Technische Universität München
Fakultät für Chemie Arbeitsgruppe für
Zelluläre Proteinbiochemie

Folding and quality control of the interleukin 12 family cytokines interleukin 27 and interleukin 35

Stephanie Irene Müller

Vollständiger Ausdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender:		Prof. Dr. Bernd Reif
Prüfende der Dissertation:	1.	Prof. Dr. Matthias J. Feige
	2.	Prof. Dr. Johannes Buchner
	3.	Prof. Dr. Martin Zacharias

Die Dissertation wurde am 09.05.2019 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 15.07.2019 angenommen.

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Verteidige dein Recht zu denken.
Denken und sich zu irren ist besser als nicht zu denken.
−*Hypatia von Alexandria zugeschrieben*

Abstract

Interleukins (ILs) are small secretory proteins that mediate communication between immune cells and are crucial for the immune system to exert its central task of balancing infectious immunity and self-tolerance. The IL-12 family is particularly interesting due to its combinatorial setup, which consists of three α subunits pairing with two β subunits and additional single signaling-competent subunits. Besides chain sharing promiscuity, the assembly-dependent folding of subunits is another characteristic feature of IL-12 cytokines, which is structurally and mechanistically not understood thus far. Despite their structural similarities, IL-12 family members perform distinct biological functions with biomedical relevance: IL-12 and IL-23 act mostly pro-inflammatory, IL-27 immunomodulatory and IL-35 acts anti-inflammatory. The aim of this PhD thesis was thus to study cellular IL-27 and IL-35 biosynthesis to gain fundamental insights into basic protein folding mechanisms and to ultimately use this knowledge to rationally change subunit folding in order to engineer novel immuno-functions. This thesis will in the following focus on IL-27.

IL-27 is built of the α subunit IL-27α and the β subunit EBI3. Interestingly, in mice, IL-27α folds autonomously and performs immuno-protective functions on its own. Human IL-27α, in contrast, is dependent on assembly with EBI3 for folding and secretion and therefore IL-27α is not present in humans. Combining mutational analyses with computational molecular dynamics simulations, a single amino acid folding switch was identified to be responsible for the differences in mouse and human. Presence or absence of a single cysteine residue determines if IL-27α can form an intramolecular disulfide bond resulting in restricted loop dynamics and the formation of a hydrophobic cluster within the protein, stabilizing the protein-fold. Binding by the chaperone BiP otherwise retains human IL-27α within the cell, where it is targeted for degradation by the proteasome. The human IL-27α^{L162C} mutant, however, was not only secretion- but also signaling-competent on human immune cells inducing STAT1 and STAT3 activation. Moreover, it was able to modulate IL-27 induced cytokine secretion by macrophages and had additional distinct functions on its own, with an overall immunomodulatory profile. A folding switch in IL-27α thus yielded a molecular phenocopy of hIL-27α for future more human-like mouse models and a novel immune modulating molecule with potential applications in infectious diseases like sepsis. A broad interspecies analysis of the amino acid sequence of IL-27α coupled with cell biological experiments revealed that intramolecular disulfide bond formation conferring folding autonomy to IL-27α is an evolutionary conserved feature. Subsequently, a mutagenesis-guided molecular docking provided structural insights into α β

heterodimerization of IL-27 which will be paramount for engineering novel variants of IL-27, *e.g.* for structure resolution. Finally, a common construction principle of IL-27 across species was uncovered, where one secretion-incompetent subunit always pairs with one being dependent on assembly with its partner subunit thereby evolutionary safeguarding a modular, flexible cytokine repertoire in an organism.

In toto, this PhD thesis illustrates how protein folding in the ER can shape the cytokine repertoire and thus immunoregulation of an organism and how we can use our knowledge about these processes to design novel immunotherapeutic agents.

Zusammenfassung

Interleukine (IL) sind kleine sekretorische Proteine, die Immunzellkommunikation vermitteln. Für das Immunsystem sind sie essentiell, da sie die Balance zwischen Infektionsimmunität und der Immuntoleranz gegenüber körpereigenen Strukturen gewährleisten. Die IL-12 Familie ist wegen ihres kombinatorischen Aufbaus, der aus drei α und zwei β Untereinheiten besteht, besonders interessant. Die assemblierungsabhängige Faltung der Untereinheiten ist ein weiteres charakteristisches Merkmal, welches, wie die Promiskuität der Proteinkettenkombination, weder strukturell noch mechanistisch verstanden ist. Trotz ihrer strukturellen Gemeinsamkeiten, haben IL-12 Zytokine sehr unterschiedliche Funktionen und sind dadurch biomedizinisch besonders relevant. IL-12 und IL-23 agieren pro-inflammatorisch, IL-27 immunomodulatorisch und IL-35 immunsuppressiv. Das Ziel dieser Doktorarbeit war folglich, fundamentale Einblicke in grundlegende Proteinfaltungsmechanismen durch die Untersuchung der zellulären IL-27 und IL-35 Biosynthesen zu erlangen. Dieses Wissen sollte schließlich dazu verwendet werden, die Faltung von IL-12 Untereinheiten rationell zu verändern, um neue immunologische Funktionen zu kreieren. Diese Arbeit wird sich im Folgenden auf IL-27 konzentrieren.

IL-27 besteht aus der α Untereinheit IL-27α und der β Untereinheit EBI3. In Mäusen kann sich IL-27α eigenständig falten und hat immun-protektive Funktionen, während die IL-27α-Faltung im Menschen abhängig ist von EBI3. IL-27α ist folglich nicht im menschlichen Körper vorhanden. Durch die Kombination von Mutagenese und computergestützten Simulation von Molekulardynamiken, wurde eine einzelne Aminosäure identifiziert, die für die Faltungsunterschiede in Mensch und Maus verantwortlich sind. Die An- oder Abwesenheit eines einzelnen Cysteinrestes (Cys) entscheidet, ob IL-27α eine intramolekulare Disulfidbrücke ausbilden kann, welche die Dynamik zweier großer Proteinschleifen einschränkt und dadurch die Ausbildung eines faltungsstabilisierenden hydrophoben Clusters ermöglicht. In Abwesenheit des Cys, wurde humanes IL-27α durch Bindung an das Chaperon BiP in der Zelle zurückgehalten und schließlich vom Proteasom abgebaut. Die humane IL-27α^{L162C} Mutante konnte hingegen eigenständig sekretiert werden und löste Signaltransduktion durch STAT1 und STAT3 Aktivierung in humanen Immunzellen aus. Sie war außerdem fähig IL-27 induzierte Zytokinausschüttung in Makrophagen zu modulieren und zeigte zusätzlich IL-27 unabhängige immunomodulatorische Funktionen. Ein konformationeller Schalter ermöglichte folglich das Design einer molekularen humanen IL-27α Phenokopie zur zukünftigen Generation von Mausmodellen, welche dem Menschen ähnlicher sind, sowie eines neuartigen Immunsystem-

modulierenden Molekül mit potentiellen Anwendungsmöglichkeiten in Infektionskrankheiten, wie der Sepsis. Eine umfassende Interspeziesanalyse der IL-27α Aminosäuresequenz kombiniert mit zellbiologischen Experimenten offenbarte, dass die intramolekulare Disulfidbrückenausbildung ein evolutionär konservierter Mechanismus ist, der IL-27α Faltungsautonomie verleiht. Anschließend wurde ein durch Mutagenese angeleitetes molekulares Docking durchgeführt, welches strukturelle Einblicke in die α β Heterodimerisierung von IL-27 gewährte. Diese sind essentiell für die Generation neuer IL-27 Varianten, beispielsweise zu Strukturlösungszwecken. Schließlich wurde ein grundsätzliches und evolutionär konserviertes Konstruktionsprinzip von IL-27 aufgedeckt, welches in der Kombination einer sekretionskompetenten Untereinheit mit einer sekretionsinkompetenten besteht. Die Faltung von letzterer ist dabei abhängig von der Assemblierung mit einer Partneruntereinheit. Dies gewährleistet Organismen die Konservierung eines modularen, flexiblen Zytokinrepertoires.

In toto verdeutlicht diese Doktorarbeit wie Proteinfaltung im ER das Zytokinrepertoire einer Spezies und somit die Immunoregulation eines Organismus beeinflussen kann und wie wir dieses Wissen nutzen können, um neue Immuntherapeutika zu designen.

Parts of this thesis have been published in peer-reviewed journals as listed below:

Müller SI, Friedl A, Aschenbrenner I, Esser-von Bieren J, Zacharias M, Devergne O[2], Feige MJ[2]

A folding switch regulates interleukin 27 biogenesis and secretion of its α-subunit as a cytokine.

Proceedings of the National Academy of Sciences of the United States of America, Jan 2019, 116 (5) 1585-1590; DOI: 10.1073/pnas.1816698116

Müller SI[1], Aschenbrenner I[1], Zacharias M[2], Feige MJ[2]

An interspecies analysis reveals molecular construction principles of interleukin 27.

Accepted manuscript, in press at the Journal of Molecular Biology, 2019, DOI: 10.1016/j.jmb.2019.04.032

Parts of this thesis have been published in patents as listed below:

Technische Universität München, 2018.

Secretion-competent muteins of the human IL-27 alpha-subunit.

Inventors: Matthias J Feige and Stephanie Müller. 08.11.2018 Filing date: 04.05.2018. PCT/EP2018/061561

Research topics that are not covered in this thesis:

Intrinsic instability of interleukin 35 explains its pleiotropic effects and receptor repertoire.

Karen Hildenbrand, Susanne Meier, Stephanie I. Müller[2], Matthias J. Feige[2]

Manuscript in preparation

[1] these authors contributed equally

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SFB1035 – Control of proteinfunction by conformational switches

3rd – 6th May 2018, San Servolo, Italy

Flashtalk and poster presentation:

A single amino acid switch regulating interleukin 27α folding.

Cellular protein biochemistry lab retreat

14th-17th September 2017, Regen, Germany

oral presentation:

Interleukin 12 family cytokines as model substrates for protein folding and quality control.

PhD & PostDoc Retreat

SFB1035 – Control of proteinfunction by conformational switches

3rd – 5th April 2017 Spitzingsee, Germany

oral presentation:

Structural mechanisms and cellular regulation of assembly-induced protein folding.

27th Faltertage on "Protein Folding, Dynamics and Stability"

21st – 23rd October 2016, Halle an der Saale, Germany

poster presentation:

A single residue toggles an interleukin between autonomous and assembly-induced folding in the ER, affecting an organism's cytokine repertoire.

Application for membership in the

SFB1035 – Control of proteinfunction by conformational switches

11th February 2016, Garching, Germany

poster presentation:

Conformational switches upon IL-27 and IL-35 assembly.

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1 Introduction

Mammalian cells produce billions of proteins, which perform important functions throughout the body. A correct three-dimensional protein structure is the prerequisite for performing them accurately. To guarantee homeostasis and ultimately the survival of an organism, it is thus of utmost importance that proteins are controlled for their folding and assembly. While main players of this fundamental process have been identified in the past decades, a detailed understanding of the underlying mechanisms is starting to emerge only recently.[1] Still fundamental questions remain. How does a cell distinguish slowly folding proteins from ultimately misfolded proteins? Which structural features are recognized by the quality control (QC) machinery? How is protein assembly regulated in the presence of multiple possible assembly partners? A rewarding model system to study protein folding and QC *in vivo* is the unique heterodimeric interleukin (IL) 12 family. Its four members are built of only three α and two β subunits, which show assembly-dependent folding. As secretory proteins, ILs interact with the cellular environment and therefore have direct immunological and biomedical impacts. Using an interdisciplinary approach that combines computational, biochemical and cellular methods, we aim at better understanding folding, assembly and QC of secretory proteins in order to enable targeted interventions in the biogenesis of IL-12 family members and to develop ultimately approaches for rational engineering of novel immunomodulatory proteins with therapeutic potential.

1.1 The protein folding problem

How does a protein obtain its three-dimensional native structure in face of the many possible confirmations of its amino acid sequence? In 2005, Science listed this question, also known as the protein folding problem, among the 125 biggest scientific questions of the next 25 years.[2] Proteins are built out of a combination of 20 different amino acids - their primary sequence, which illustrates the immense diversity of possible protein molecules ($20^{n = \#\ of\ residues}$). Depending on the physicochemical properties of each amino acid, the primary sequence forms secondary and tertiary structures that define the protein's native state. Folding processes take up to milliseconds, sometimes minutes, depending on the protein.[1] Yet random sampling of all possible conformations of a 100-residue protein with potentially 3 conformations per residue would take billions of years.[3] The so called Levinthal paradox was postulated in 1968 and is still relevant today as we are not yet able to predict the folding pathway or native structure of a protein reliably and solely from its primary sequence. The large gap between deposited PDB

structures (~150 000) and total UniProt entries (~1.2 millions having evidence on the transcript level) illustrates the potential that *de novo* protein structure prediction would have for fundamental biological and biomedical research.[4,5]

Since Christian Anfinsen's discovery that the amino acid primary sequence encodes all necessary information for a protein to fold spontaneously *in vitro* and that its native state is the conformation with the lowest Gibbs free energy (ΔG), our understanding of protein folding has developed further.[6,7] Today's perception of protein folding is a stochastic search of trial and error in a funnel-shaped protein folding landscape of free energy (Fig. 1).[8] Many unfolded states move on different routes - representing different folding pathways - down the funnel towards its bottom: the global energy minimum, where the energetically most favorable native state is located. Amino acids shape the landscape of the funnel and evolution has resulted in the fast and efficient folding of proteins.[9] The folding process *in vitro* is driven by the collapse of hydrophobic residues into a buried core to avoid contact with hydrophilic water molecules.[10] This effect restricts the search of possible conformations and contributes to efficient folding.

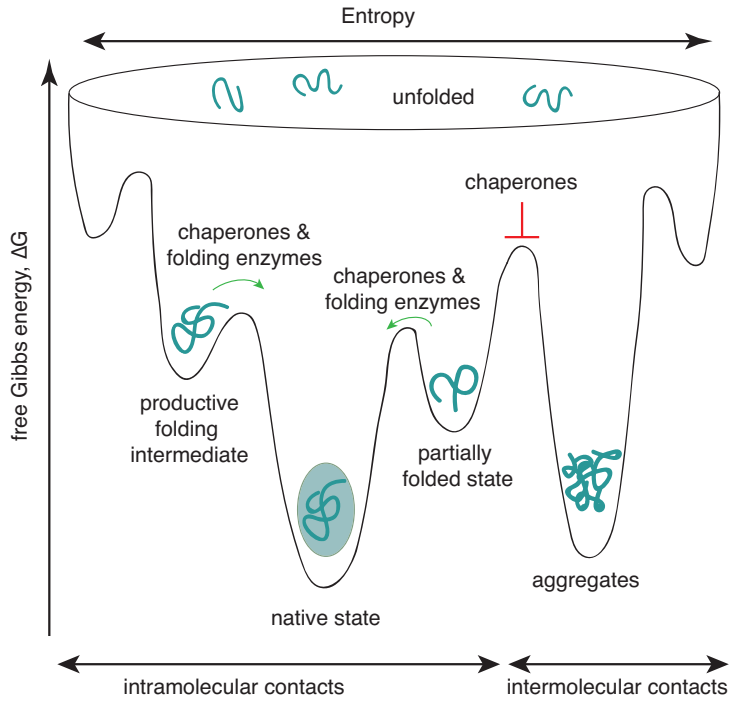

Fig. 1 The free energy landscape of a protein folding funnel. Proteins can take different routes towards their native state, characterized by low ΔG and small entropy. Chaperones and folding enzymes help kinetically trapped folding state to reach their correct structure and inhibit aggregate formation. Adapted from[15].

We also learnt that large proteins (>100 amino acids) have more complex folding mechanism and might require chaperones and folding enzymes, which prevent unfavorable side reactions and catalyze slow folding steps; factors the typical *in vitro* environment does not provide.[11] In the test tube, these proteins need longer to fold and often do not reach their native state at all.[12] In contrast to the rather smooth folding landscape of smaller proteins that follow a simple two state folding pathway, the energy folding landscape of larger proteins is rugged, meaning that kinetic barriers of local energy minima have to be passed in order for the protein to reach its native conformation (Fig.1).[13,14] These minima can represent either productive folding intermediates with native like structures or kinetically trapped partially folded states, caused by *e.g.* unspecific backbone and/or side chain interactions. Other side reactions are due to the highly crowded cellular environment with protein concentrations reaching up to 300 mg/mL.[15] Folding intermediates exposing hydrophobic sequences are thus prone to aggregation. To enable a productive folding pathway in face of possible side reactions, cells have developed a QC system consisting of chaperones that keep proteins in conformationally dynamic states needed for folding by a mechanism of kinetic partitioning.[14] Chaperones rebind non-native states to prevent unspecific interactions as well as aggregation until the protein is either correctly folded or sent to degradation. Given the vectorial process of protein synthesis *in vivo* (from the N- to the C-terminus of the protein), the necessity of the evolution of a buffering system becomes obvious. As ΔG values of protein folding are small, proteins need continuous monitoring by chaperone networks to maintain cellular proteostasis.[14,16]

While studying protein folding *in vitro* is imperative for high resolution insights and has, in combination with computer simulations, enlightened our understanding of folding pathways, cellular *in vivo* studies are paramount to complement our understanding of physiological protein folding and assembly mechanisms. 14 years after Science's 125 years anniversaries edition, we have advanced considerably along the route towards solving the protein folding problem. Understanding and being able to pointedly change the folding and QC of secretory proteins are another means to contribute to deciphering the physical folding code and pathways of protein folding.

1.2 Interleukins: Secretory proteins mediating immune cell communication

For the immune system, the folding, assembly and QC of secretory proteins are crucial to maintain organism homeostasis. Immunglobulin (Ig) G antibody heavy chain secretion, for example, is coupled to association with light chains by a chaperone-mediated mechanism guaranteeing that only fully functional antibody molecules consisting of two light and two heavy

chains are released from the cell.[17,18] This QC mechanism prevents the secretion of unassembled heavy chains that would be able to elicit unspecific immune responses *via* their Fc fragment and because of the incomplete specificity-promoting antigen-binding domain. Rare heavy chain diseases are an example for the possible, and severe consequences of the lack of this important QC step.[18] The folding and QC of interleukins (ILs) is similarly important for an organism due to the central role ILs play in the immune system. As small secretory proteins belonging to the class of cytokines, ILs are produced by cells of the innate and adaptive immune system and act as pro- and anti-inflammatory messenger molecules between these two layers. After secretion, ILs bind to receptor chains on secreting cells (autocrine signaling) or on target cells (paracrine signaling) of the innate and adaptive immune system. Upon binding, these receptor chains dimerize and elicit intracellular signaling with varying immunological effector outputs (Fig. 2).

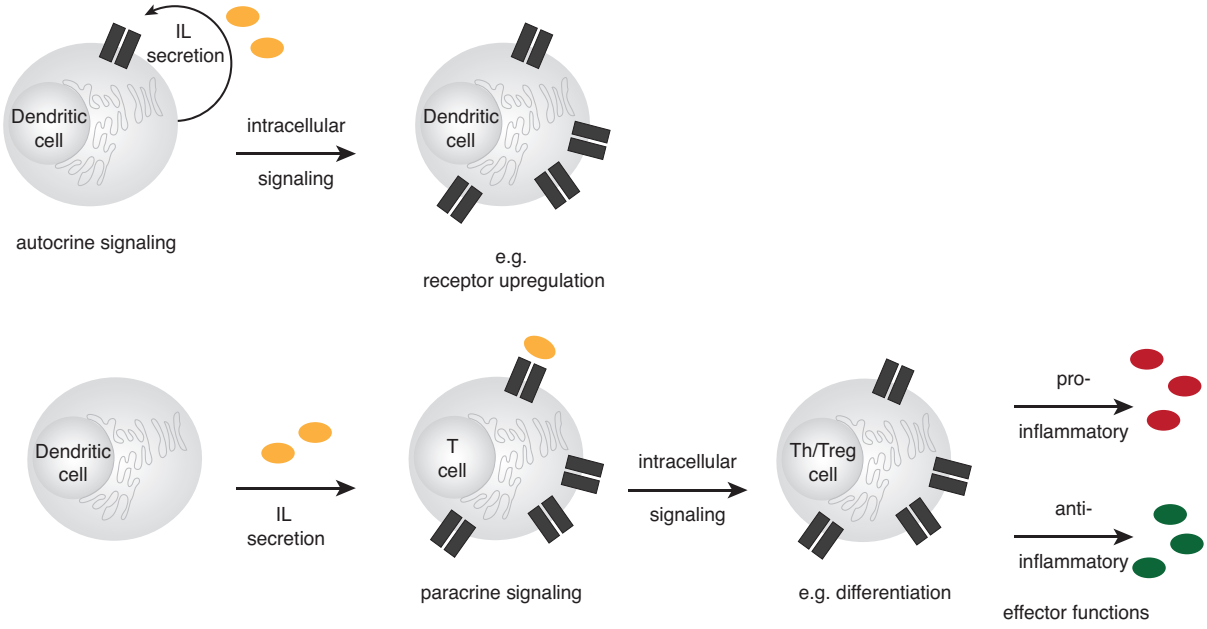

Fig. 2 Autocrine and paracrine signaling modes of interleukins (ILs). ILs are secreted by immune cells and can either bind to receptors on secreting cells inducing intracellular signaling leading to *e.g.* receptor upregulation, which is called autocrine signaling. Or they can bind to recpetors expressed by different immune cells, then called paracrine signaling. T cell proliferation and differentiation to specialized T cell subsets that can then act pro- or anti-inflammatory is a common pathway of this signaling mode.

The functioning of the immune system is based on its ability to distinguish self from non-self enabling it to protect the body from pathogens, while maintaining homeostasis.[19] Cells of the innate immune system, like macrophages, dendritic cells or neutrophils, express receptors that recognize common pathogen associated molecular patterns (PAMPs).[20] Upon PAMP binding the innate host response is elicited. It leads to a fast pathogen control by phagocytosis and activation of cells and humoral components of this unspecific, yet rapid part of the immune system. In

addition, it induces a pathogen specific and long lasting defense program, the adaptive immune response. Naïve B and T cells proliferate and differentiate into diverse B and T cell subsets with antigen-specific potent effector functions including antibody production, helper, cytotoxic and memory function. Long lasting infectious immunity is provided by the generation of memory B and T cells. Upon infection with the same pathogen, the body is thus able to elicit an efficient and specialized response much faster. The role of regulatory B and T cells in concert with suppressive cytokines is to restore and maintain immune homeostasis. In case of misregulation, chronic inflammatory diseases, autoimmunity and cancer can be the consequence.

All immunological processes are mediated by receptor interaction and cytokine signaling between immune cells, illustrating the important role of interleukins in the immune system. Nonetheless, the therapeutic potential of the ~40 known ILs in humans is largely unexploited.[21] Being able to rationally engineer the outcome of immunomodulatory decisions might open up novel avenues for immunotherapies. The investigation of folding, assembly and QC of the IL-12 family is in this regard especially interesting as the four members epitomize the concept of immune balance in just one single IL-family.

1.3 The IL-12 family - A unique model system to study protein folding, assembly and QC in the endoplasmic reticulum

The folding of IL-12 family members takes place in the endoplasmic reticulum (ER), where they enter the secretory pathway and obtain their proper structure. The IL-12 family is an especially interesting structural model system with immediate biomedical relevance: It is the only strictly heterodimeric cytokine family and the four members are built of only five different subunits (Fig. 3a). Nonetheless, they perform distinct, sometimes even opposing immunological functions.

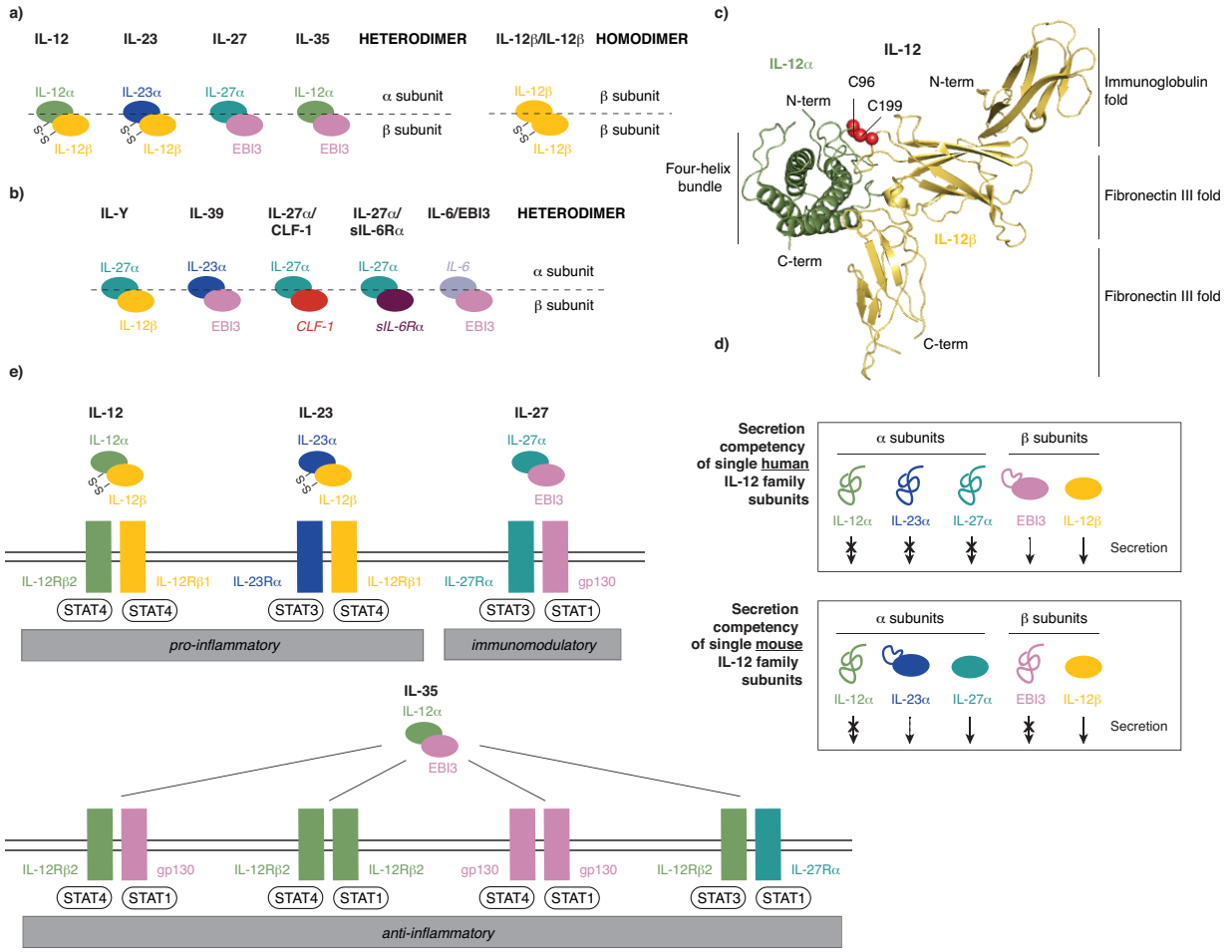

Fig. 3 Overview of the IL-12 cytokine family. a) Chain-sharing promiscuity of the IL-12 family members IL-12, IL-23, IL-27 and IL-35. They are all built of one α and one β subunit, which are shared between the members. IL-12α (green) is part of IL-12 and IL-35, IL-12β (yellow) part of IL-12 and IL-23 and EBI3 (pink) part of IL-27 and IL-35. Homodimerformation of the β subunit IL-12β has also been reported. IL-12, IL-23 and the IL-12β homodimer are disulfide-linked. b) Uncoventional subunit pairings have been reported for IL-12 family subunits. IL-Y has been only reported as synthetic protein, whereas the other pairings have physiological relevance. c) Crystal structure of IL-12 showing the typical protein folds of IL-12 family subunits: α subunits (IL-12α, green) are four-helix bundle proteins, whereas the β subunits are formed by an N-terminal Ig-domain and two fibronectin III-like domains in the case of IL-12β (yellow) and two fibronectin III-like domains only in the case of EBI3 (pink). The intermolecular disulfide bond of IL-12 is represented in red CPK. d) Secretion competency of IL-12 family members differs in human and mouse. While the α subunits are not secretion competent in humans, the β subunits are secreted in isolation. In mice, IL-12α and EBI3 are retained in the cell, whereas IL-23α, Il-27α and IL-12β are secretion competent. e) Receptor and signaling complexes of IL-12, IL-23, IL-27 and IL-35. Chain-sharing extends to the receptor level of the IL-12 family. IL-12, IL-23 and IL-27 signal through heterodimeric receptor chains. For IL-35 an unconventional receptor repertoire has been reported consisting of IL-12Rβ2 and gp130 heterodimers, as well as homodimers of each receptor chains. The combination of IL-12Rβ2 and IL-27Rα has been exclusively described for B cells. IL-12 cytokine binding to receptor chains induces dimerization and STAT activation by phosphorylation *via* the Jak pathway (not depicted here). Effector functions and STAT proteins that are essential for these functions are depicted for each receptor pair.

1.3.1 Members of the heterodimeric IL-12 family

Three α subunits (IL-12α/p35; IL-23α/p19; IL-27α/p28) and two β subunits (IL-12β/p40; Epstein-Barr virus-induced gene 3, EBI3) pair to form IL-12 (IL-12α/p35, IL-12β/p40), IL-23 (IL-23α/p19, IL-12β/p40), IL-27 (IL-27α/p28, EBI3) and IL-35 (IL-12α/p35, EBI3) (Fig. 3a).[22-28] More combinations, namely IL-Y (IL-27α/p28, IL-12β/p40) and IL-39 (IL-23α/p19, EBI3) have been reported but need further evidence to prove their physiological relevance in humans.[29-33] Intriguingly, cross interaction of IL-12 family subunits with members of the homologous IL-6 family has also been reported: IL-27α can pair with cytokine-like factor 1 (CLF), while EBI3 is able to interact with IL-6 highlighting that chain sharing promiscuity might be a feature that is shared across families (Fig. 3b).[34,35] Reports of IL-12β acting as an extracellular adapter for other proteins support this notion.[36,37]

1.3.2 Structures of IL-12 family members

The IL-12 family α subunits share homology with IL-6 family cytokines, which have a four-helix bundle structure. Likewise, β subunits are homologous to the soluble receptor chains of the IL-6 family, and have a N-terminal immunoglobulin (Ig)-like fold and two fibronectin III-like domains. EBI3 is predicted to lack the Ig domain.[38] Structures of the disulfide-linked IL-12 and IL-23 have been crystallized and confirm the predicted topologies for α and β subunits (Fig. 3c).[39,40] Their subunit interaction is mediated by a charged residue interaction between a conserved Arg residue in the α subunits and an Asp residue in a pocket formed by IL-12β. The hydrophobic inner and a polar outer shell of the pocket result in a charge and hydrogen-bonding network representing the interlocking topography of IL-12 and IL-23. Resolving the structure of the non-covalently linked and therefore less stable IL-27 and IL-35 cytokines, in contrast, is an ongoing challenge in IL-12 family research.[41]

1.3.3 Assembly-dependent secretion, a common feature of IL-12 family cytokines

Similar to other immunologically relevant secretory or membrane proteins, like antibodies or T cell receptors, the exit of IL-12 family members from the ER is dependent on protein assembly.[17,42] In humans, α subunits are only able to fold, if their cognate β subunit is co-expressed in the same cell. They are secreted solely as heterodimers.[23,24,28] β subunits, in contrast, do not underlie this QC step to the same extent in humans, with IL-12β being readily, and EBI3 weakly secreted when expressed alone in a cell.[25,27,28] Nonetheless, increased EBI3 secretion can be observed upon presence of a corresponding α subunit suggesting mutually induced conformational changes and stabilization between the subunits.[27] Interestingly, secretion-incompetency and -competency of IL-27α and EBI3, respectively, is not a conserved

feature between species, as in mice, IL-27α is secreted in isolation, while EBI3 is retained (Fig. 3d).[24] Accordingly, there is evidence for immunological function of the individual subunits IL-27α, EBI3 and IL-12β.[43-45] Weak secretion of IL-23α in mice has also been reported, however, without any functional implications.[23] The underlying mechanisms of assembly-dependent folding and secretion are not understood for all family members. For IL-12α, it has been shown that misfolding and degradation occurs in absence of IL-12β and that assembly with IL-12β rescues IL-12α from this fate by inducing its folding.[46] Open questions yet remain*, e.g.*: Which structural determinants does the ERQC machinery recognize to mediate secretion *versus* retention of proteins? And how is the specific subunit assembly realized on a molecular level, while chain-sharing promiscuity is realized at the same time?

1.3.4 Composition of IL-12 family receptor complexes

Interestingly, each IL-12 family subunit seems depend on a distinct receptor chain for signaling, extending the chain-sharing feature to the receptor level (Fig. 3e). IL-12 binds to IL-12Rβ2 and IL-12Rβ1 receptor chains, while IL-23 engages IL-23Rα and IL-12Rβ1.[47,48] IL-27 signals *via* IL-27Rα, also known as WSX-1 or TCCR and the common IL-6 family receptor chain gp130.[24,49] A soluble IL-27Rα has been reported to bind IL-27, inhibiting binding of the cytokine to the membrane bound receptor complex and thereby antagonizing IL-27 signaling.[50] For IL-35, in contrast, unconventional signaling *via* multiple receptor combinations has been reported which lead to different effector functions and consist of IL-12Rβ2 and gp130 or homodimers of each receptor chain as well as a combination of IL-27Rα and IL-12Rβ2 on B cells.[51,52] Dependent on the receptor chain, different Jak kinases transmit the signal to IL and receptor-specific STAT proteins (Fig. 3e).[41,53]

1.3.5 Functional diversity within the IL-12 family

Despite sharing similar receptor chains, IL-12 family cytokines span a broad range of biological functions from pro-inflammatory and immunomodulatory to anti-inflammatory (Fig. 3e). The family's founding member IL-12 was discovered in 1989 and is produced by antigen presenting cells, *e.g.* dendritic cells and macrophages, after contact with pathogens.[22,54,55] As a pro-inflammatory cytokine, it activates the differentiation of Th1 cells and can induce interferon-γ (IFNγ) secretion by natural killer (NK) cells illustrating its roles in cell-mediated immunity, respectively.[22] IL-23, discovered in 2000, has also pro-inflammatory functions, and is secreted by activated dendritic cells and macrophages upon infection.[23] It is essential for Th17 development and IL-17 expression in these cells.[56] In 2002, IL-27 joined the ranks of the IL-12 family.[24] It performs immunomodulatory roles by inhibiting Th17 cell development and inducing

a Tr1 cell population secreting the anti-inflammatory IL-10.[57-59] In combination with IL-12, IL-27 can also have pro-inflammatory functions, like the induction of IFNγ production by NK cells.[24] It is secreted mainly by monocytes and activated dendritic cells after direct or cell-mediated pathogen contact.[60-62] IL-35 is the youngest member of the family as its biological function was first reported in 2007.[26] It is located at the other end of the functional spectrum: Being secreted by regulatory B and T cell populations as well as tolerogenic dendritic cells, it performs anti-inflammatory functions, *e.g.* by suppressing T cell differentiation.[26,52,63-65] How can these different functionalities be realized when functionally opposing IL-12 members engage similar receptor chains? There is evidence that signaling specificity is regulated on the level of STAT proteins.[41] Homo- and heterodimers might be able to dictate different gene activation patterns and therefore different immunoregulatory outcomes.

From a systems biological view, the IL-12 family and their receptors constitute a set of immunological modules that can, depending on their combination, elicit various immune responses. Understanding the underlying concepts and mechanisms of how these modules are combined *in vivo* allows us to intervene in this process by strengthening or inhibiting a known combination, or even creating a completely new one. Studying folding, assembly and quality control of IL-12 family members promises thus not only fascinating insights into basic cellular processes and immunoregulation. It offers also the prospects of novel immunotherapies as IL-12 family cytokines are implicated in the etiology of many human diseases; reflected by their ability to control the development of many different T and B cell subsets. IL-12 and IL-23 are for example involved in autoimmunity, IL-27 harbors potential as anti-tumor agent and IL-35 limits tumor-immunity.[66] Due to their immunoregulatory and –suppressive functions, IL-27 and IL-35 are two especially interesting candidates for the treatment of inflammatory diseases, autoimmune reactions and cancer. Structurally, however, they are the least understood members of the IL-12 family making the molecular mechanisms of their biosynthesis particularly worth studying.

1.4 Interleukin 27, an immunomodulatory interleukin

IL-27 consists of the α subunit IL-27α and the β subunit EBI3 (Fig. 3a). Identified as an Epstein-Barr virus regulated gene in infected B cells, EBI3 was first described in 1996 as an IL-12β related protein.[25] In 2002, a computational search for homologues of the IL-6 family members established IL-27α as an interacting partner of EBI3.[24]

1.4.1 A short introduction into the complex immunobiology of IL-27

The same study reported IL-27 to induce, in concert with IL-12, the proliferation of naïve T cells and their differentiation into Th1 cells that secrete IFNγ, which characterized IL-27 initially to be pro-inflammatory. In the past decades, though, increasing evidence emerged that IL-27 is actually an immunomodulatory cytokine crucial for preventing immune pathology by limiting the development of multiple T cell populations.[67,68] The common understanding of IL-27 to date is that it plays dual roles in immunity by preventing hyperreactivity and promoting certain pro-inflammatory functions. IL-27 is a negative regulator of Th2 responses and inhibits Th17 development as well as production of the pro-inflammatory IL-17.[69-71] Moreover, it contributes to the development of the $Foxp3^-$ regulatory T cell population Tr1, which produces IL-10 that is important for dampening immune reactions as an anti-inflammatory IL.[57-59,71] These functions are involved in IL-27 limiting experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis.[67] In addition, IL-27 holds pro-inflammatory properties like the stimulation of growth and survival of CD8+ T, which seem to be crucial for tumor regression in cancer.[24,71,72] However, IL-27 has been also reported to induce the expression of the inhibitory receptors PD-L1 and PD-1 in immune cells, which plays a central role in the evasion of tumor cells from the immune system.[73-75] Lastly, there are studies suggesting an involvement of IL-27 in limiting $Foxp3^+$ regulatory (reg) T cell populations (Treg) and in controlling B cell responses leading to immune system activation.[71,76,77] The challenge in studying IL-12 family immunobiology and in particular in studying the dual functionality of IL-27 is to dissect direct from indirect effects. The intricacy of the immune network makes this difficult as well as the fact that subunits and receptor chains are shared within and between the IL-12 and IL-6 families. Protective effects in a number of disease conditions including *e.g.* multiple sclerosis, colitis and cancer, however, emphasize the great potential IL-27 holds as immunotherapeutic agent.[67] More studies with better model systems and methods will be thus critical for constituting a comprehensive picture of how the pleiotropic effects of IL-27 shape immunity and disease. Studying the biosynthesis of IL-27 on a molecular level holds great potential for a development along these lines and will hopefully contribute to the use of IL-27 in the treatment of cancer, autoimmunity and inflammatory diseases.

1.4.2 Is murine IL-27α a potential novel cytokine with immuno-functions in humans?

The intriguing fact that secretion competency of IL-27 subunits behaves reciprocal in mice to how it does in humans adds another layer of complexity to IL-27 immunobiology. In mice, IL-27α can be readily secreted without EBI3, while EBI3 is dependent on IL-27α for

secretion.[24] Accordingly, there have been studies reporting biological functions for IL-27α , also known as IL-30, in mice. Similarly to IL-12β homodimers, antagonizing IL-12 signaling, murine IL-27α (mIL-27α) as a monomer has been described to inhibit IL-27 and gp130-mediated signaling.[78,79] In 2013, however, a study reported mIL-27α to instead act agonistic through recruitment of gp130 homodimers and IL-6Rα or *via trans*-signaling with soluble IL-6Rα, as well as through signaling without any β chain at high concentrations.[43] The authors suggested that the bacterially produced mIL-27α by Stumhofer *et al.* was not correctly folded and therefore not able to induce signaling.[79] In fact, a study in 2018 took advantage of the commercially available recombinant mIL-27α from mammalian cells and demonstrated its activity on human monocytes through sIL-6Rα interaction and gp130-mediated signaling.[80] Functional studies of mIL-27α indicate it exerting pro- and anti-inflammatory roles comparable to the dichotomy of IL-27 signaling, however not always acting along the same lines as IL-27 does.[78,80-82] When co-administrated with IL-27 it is *e.g.* able to modulate IL-27 induced effects.[78] Nonetheless, there is appealing evidence that IL-27α might be a promising route for a number of conditions as it has been shown to limit anti-allogeneic immune responses, plays a protective role in liver injury as well as macrophage-mediated inflammation, alleviates experimental sepsis and induces inflammatory responses in human monocytes, while abrogating anti-tumor responses.[78,80-83] Moreover, in 2009, secretion of IL-27α by murine activated dendritic cells as a complex with CLF has been reported.[34] Pro- and anti-inflammatory effects like IFNγ production after NK cell activation and inhibition of CD4+ T cell proliferation and induction of IL-10 secretion by CD4+ T cells, respectively, have been attributed to the IL-27α/CLF complex. IL-27α was shown to engage IL-6Rα, in addition to gp130, for increased signal transduction *via* STAT1 and STAT3. Crabé *et al.* hypothesized that CLF interacts with IL-27α through the receptor-binding site of IL-27α, as CLF does with its cognate partner CLC.[84] This assumption would explain the engagement of the non-signaling IL-6Rα comparable to the recruitment of the non-signaling CNTFRα receptor chain to the cytokine binding domain of CLC. Nonetheless, IL-27α/CLF seemed to be able to transmit signals without IL-6Rα, albeit less efficiently and through STAT3 induction only. While STAT induction was verified to be dependent on gp130 and IL-6Rα, IL-27Rα involvement could not be studied due to the lack of a neutralizing antibody that was described only in 2014.[50] The recruitment of a tripartite receptor consisting of IL-27Rα, gp130 and IL-6Rα was thus proposed as possible model as IL-27α/CLF has been shown to bind to IL-27Rα. IL-27Rα knock down was not tested. *In toto*, the transferability of these studies to the human context remains to be determined. Since human (h) IL-27α is not secretion competent on its own, one would expect it to be absent in the human cytokine profile. Since it has been

proposed that CLF is not needed for IL-27α signaling, IL-27α-secretion induction by CLF could be a possible scenario for the human context. However, it has not been addressed to date, if the IL-27α/CLF complex is produced in humans and which functional impact it might have. Ultimately, it needs to be investigated if mIL-27α can induce similar effects in humans as it does in mice. Petes and coworkers provided the first evidence that this might be the case putting IL-27α to the forefront of novel immunotherapeutics.[80]

1.4.3 Is EBI3 a signaling- competent member of the human cytokine repertoire?

In contrast to hIL-27α, hEBI3 is secreted by human cells. Many studies aimed at identifying EBI3-related functions by using $EBI3^{-/-}$ KO mouse strains. Since mice lack EBI3 due to its secretion incompetence in isolation, these studies, however, provide only information about IL-27 and IL-35 dependent effects. Research providing insights about IL-27 and IL-35 independent EBI3 effects instead is rare: In LPS stimulated human monocytes, EBI3 mRNA levels were maintained above background levels 72 h post stimulation, while the expression of IL-12α, IL-27α and IL-12β dropped back to background levels after 24 h already.[85] The long lasting expression kinetics of EBI3 imply that it might play a role in down-regulation of the immune system after infection in order to maintain immune homeostasis. Moreover, it is highly expressed throughout human pregnancy in placental throphoblasts and secreted in large amounts from human first trimester and term placental explant cultures suggesting its involvement in the formation of fetal-maternal tolerance.[25,44] There is also evidence that hEBI3 might be involved in limiting anti-tumor responses.[86-88] Moreover, a study by Dambuza and coworkers showed that recombinant (r) mEBI3 inhibited mouse CD4+ T cell proliferation comparable to rIL-35 and induced its own expression within CD19+ B cells.[89] Although these observations propose that EBI3 has immunosuppressive effects, it has been reported to have pro-inflammatory functions when expressed in combination with IL-6. Chehboun *et al.* showed that murine and human EBI3 can bind IL-6, thereby acting as soluble receptor chain comparable to sIL-6Rα, and induce gp130 mediated IL-6 *trans*-signaling, eliciting IL-6 related pro-inflammatory effects on murine and human cells.[35] While research to date has proven that EBI3 is *per se* signaling-competent, more studies using rEBI3 or a more human-like mouse model with secretion-incompetent IL-27α and secretion-competent EBI3 are needed in order to decipher the role of EBI3 in the immune system in more detail.

Structural insights into folding and QC of IL-27 and its subunits will lay the groundwork to better understand their immunobiology and are the prerequisite for influencing an organisms cytokine repertoire. However, the molecular mechanisms of folding, QC and subsequent

secretion of IL-27 and its subunits are not know so far. Also how assembly *versus* single subunit secretion is regulated, *e.g.* which cellular factors are implicated in secretion and retention is an open question. Deciphering these mechanisms will be key for the development of tailor-made immunotherapies urgently needed in face of the pleiotropic functions of IL-12 family members.

1.4.4 The IL-27 interface

Since the structure of IL-27 has not been solved yet, homology modeling and mutagenesis-guided molecular docking of subunits is a common approach to study the IL-27 interface. Taking into consideration homologues of the IL-6 and IL-12 families, Rousseau *et al.* identified Trp97 in IL-27α and Phe97 as well as Asp210 in EBI3 to be critical residues for subunit interaction in IL-27.[38] Molecular docking predicted the interface to mimic site 1 interaction of the IL-6 family homologue CNTFRα and its ligands and to consist of hydrophobic and polar areas. IL-27α^{W97} and EBI3^{F97} are part of a cluster mediating the hydrophobic interaction, while there are oppositely charged surfaces in IL-27α (Arg55, Arg67, Arg219) and EBI3 (Glu124, Glu159, Asp210) predicted to contribute to subunit interaction. Another study choosing this approach, albeit working with mEBI3, confirmed EBI3 residues Phe97 and Asp210, which are conserved in mice, to be involved in IL-27 subunit interaction, while the corresponding residue to Glu159 being Asp157 in mice did not interrupt heterodimer formation. Furthermore, mEBI3 residues Phe159 and Lys161 were reported to interrupt subunit interaction in mIL-27 and are conserved in hEBI3.[90] Now, almost 10 years later, computational state-of-the-art techniques combined with evolutionary investigations might reveal novel structural insights into α β heterodimerization in IL-27.

1.4.5 The biosynthesis of human IL-27

Whereas the interface of IL-27 has been studied to a certain extent, the mechanisms of cellular folding, assembly and QC of its subunits are completely unknown. It has been described that human IL-27α is retained in cells when expressed in isolation, while human EBI3 is secreted alone.[24] Co-expression leads to subunit interaction, releases IL-27α from cellular retention and increases EBI3 secretion. The subunits are secreted as non-covalently linked heterodimers. Although IL-27α has a free Cys, it does not form an intermolecular disulfide bond with EBI3 as compared to IL-12 and IL-23. EBI3 contains four Cys residues in its N-terminal domain, which are predicted to form intramolecular disulfide bridges and it does not contain the intermolecular disulfide bridge forming Cys of IL-12β.[27,91] IL-27α has an intriguing and for type I cytokines unique 13 amino acid long Glu-stretch that has been reported to provide IL-27 with hydroxyapatite binding properties able to localize it to bone structures *in vivo.*[92] Moreover, there

is evidence that it might be involved in EBI3 interaction, as a ΔE_{13} IL-27α mutant did not co-immunoprecipitate with EBI3 and a ΔE_{13} IL-27 fusion protein did not bind the IL-27 receptor anymore.[38] While IL-27α is not predicted to have any *N*- or *O*-glycosylation it does appear as multiple bands on Western blots indicative of different *O*-glycosylation species.[24] EBI3 is predicted to have two *N*-glycosylation sites what is supported *inter alia* by its binding to the lectin chaperone Calnexin.[25,27] Many questions regarding IL-27 biosynthesis, however, remain open. Why is IL-27α not secreted in isolation? What happens to IL-27α when EBI3 is not expressed? Which conformational changes does EBI3 induce in IL-27α that enable secretion, and how does IL-27α increase EBI3 secretion upon assembly? Which structural features of EBI3 induce folding of the two α subunits EBI3 is interacting with, IL-12α and IL-27α, respectively? Is there any competition between α subunits upon assembly with EBI3 that might contribute to assembly regulation? And finally, which factors chaperone folding and regulate specific subunit assembly, *i.e.* why do antigen-presenting cells that produce IL-12 and IL-27 do not secrete IL-35?

Investigating the molecular mechanisms of IL-27α and EBI3 folding, assembly and QC will provide valuable insights into IL-12 family biosynthesis and enable us to rationally change IL folding thereby shaping the cytokine repertoire of a species.

1.5 The immunosuppressive interleukin 35

IL-35 is the only IL-12 family cytokine that has been thus far associated with strictly immunosuppressive roles. It is built of the α subunit IL-12α and the β subunit EBI3. Although their interaction has been reported already in 1997, the biological function of the heterodimer was described only a decade later.[26,27] Nonetheless, the identification of IL-12α and EBI3 expression in human placental throphoblasts in 1997 already pointed towards possible immunosuppressive functions of IL-35 and suggested a role in fetal-maternal tolerance.[27]

1.5.1 Does IL-35 exist as a stable cytokine?

Signaling of IL-35 is exceptional from the other IL-12 family cytokines in that it has been shown to use multiple receptor complexes to transmit different effector responses. Heterodimers consisting of IL-12Rβ2 and gp130 can inhibit T cell proliferation and induce the development of regulatory T cells that secrete IL-35 (iTreg35).[51] In contrast, IL-35 signaling through homodimers of IL-12Rβ2 and gp130, respectively, limits only T cell proliferation and cannot induce the iTr35 population. IL-27Rα and IL-12Rβ2 on B cells have been reported to transmit IL-35 mediated induction of regulatory B cells secreting IL-10 (iBreg10) and IL-35 (iBreg35).[52]

The unconventional receptor repertoire of IL-35 and the fact that the subunits are not covalently linked prompted the hypothesis that IL-35 does not exist as a stable cytokine but rather as a mixture of heterodimers and homodimers or possibly even as homodimers exclusively. In 2014, rmIL-12α and rmEBI3 generated in insect cell culture were shown for the first time to be signaling competent in isolation, as they inhibited lymphocyte proliferation.[52] In 2017, rmIL-12α monomers and homodimers were shown, furthermore, to recapitulate anti-inflammatory functions of IL-35 like the induction of i10- and i35-Breg cells or the inhibition of Th1 and Th17 responses.[89,93] While this finding qualifies IL-12α generally as novel immune signaling molecule with the potential application in autoimmune therapy, the activity of rmIL-12α on human cells remains yet to be established. Finally, the biological relevance of extracellularly existing IL-12α monomers and homodimers in a human setting needs further investigations as Dambuza *et al.* examined murine cell lysate only.

1.5.2 The immunobiology of IL-35

With its immunosuppressive functions, IL-35 is an important regulator of immune homeostasis. It limits the proliferation of effector T cells like Th17 populations by cell cycle arrest and leads to the expansion of IL-10 secreting regulatory T and B cells.[26,52,64,94-97] In addition, IL-35 is able to transmit this tolerance promoting state to other cells by inducing naïve T cells and activated B cells to differentiate into exclusively IL-35 secreting iTr35^{Foxp3-} and i35-Breg cells.[52,65] Besides conferring infectious tolerance, IL-35 is able to limit anti-tumor immunity and therefore represents a cytokine with tremendous biomedical relevance in allergic and autoimmune diseases as well as cancer.[65,98] Its physiological functions have been associated with limiting autoimmune diseases and other pathologies, *e.g.* EAE, EA uveitis, collagen induced arthritis, allergic airway inflammation, colitis, diabetes, graft versus host disease, viral myocarditis and atherosclerosis.[52,64,94-96,99-102] Autologous i35-Breg and iTr35 therapy might be a promising approach for these conditions.[52,103] Whereas in cancer, Treg derived IL-35 can lead to tumor progression and qualifies IL-35 as an important anti-cancer target.[65]

1.5.3 The biosynthesis of IL-35

As for IL-27, a major challenge in using IL-35 as a therapeutic agent or target is its missing structure. An extensive mutational screen was not able to identify any residues interrupting IL-35 formation suggesting that the subunit interaction might be mediated by many weak interactions.[90] However, it was shown that the interface differs from IL-12 and IL-27, as residues interrupting formation of these cytokines had no influence on IL-35. Similar to the other IL-12 family members, IL-35 subunits induce and facilitate their mutual secretion.[27] IL-12α has

been shown to misfold and form non-native redox species, mainly homodimers, in isolation.[46] It is degraded by the ER associated degradation (ERAD) machinery with a half-life of ~1.6 h. EBI3 seems to prevent misfolding of IL-12α by inducing its folding and secretion as heterodimer. How this is realized on a molecular level is not known. Studying the structural mechanisms of IL-35 folding and QC in the ER will enable novel engineering approaches that might ultimately be used for the generation of targeted immunotherapies.

1.6 The ER, a dedicated protein folding organelle

The IL-12 family belongs to one third of the mammalian proteome, which is produced in the secretory pathway ensuring correct folding, maturation, assembly and transport of proteins destined for the cell membrane or the extracellular space.[104,105] A central role is played by the ER where most ILs gain their functional structure and are assembled, and misfolded proteins are routed for degradation *via* a stringent QC system, maintaining proteome homeostasis.

The journey of most secretory proteins begins with the cytosolic signal recognition particle binding to the hydrophobic, amino-terminal signal sequence of nascent polypeptides and targeting ribosome-nascent chain complexes to the ER.[106,107] After binding to its ER associated receptor complex, it directs the unfolded polypeptide to the Sec61 translocon, an aqueous channel, which imports the nascent chain co-translationally.[108] Once imported, the signal sequence is cleaved by the signal peptidase complex.[109] A large variety of folding helpers and enzymes, abundantly present in the ER, aids and safeguards protein folding and assembly in the ER, which, due to its oxidizing environment and specific ion composition, resembles the extracellular space.[1,110] Chaperones interact with exposed hydrophobic amino acid residues of incompletely folded proteins, thus preventing aggregation in the highly crowded ER and providing their clients with opportunities to reach their native conformation.[111] Moreover, the binding of immature proteins constitutes a crucial ERQC mechanism in which hydrophobic stretches serve as ER retention signals ensuring that only correctly folded proteins leave the ER. ER-resident chaperones comprise the Hsp70 family members BiP[112] and Grp170, accessory Hsp40 DnaJ-like co-chaperones of BiP (ERdjs1-7 in the ER)[113,114], the Hsp90 homologue Grp94 and lectin chaperones, *i.e.* Calnexin (CNX), Calreticulin (CRT).[115-117] BiP binds promiscuously to proteins exposing hydrophobic parts *via* its C-terminal substrate-binding domain (SBD) and is not only implicated in protein translocation and folding but also in targeting misfolded proteins for degradation, stress regulation *via* the unfolded protein response (UPR) and in regulating Ca^{2+} homeostasis, which is crucial for a lot of folding factors.[114,118-120] Client binding and release by

BiP are regulated by an ATPase cycle mediated through the N-terminal nucleotide-binding domain (NBD) of BiP (Fig. 4).

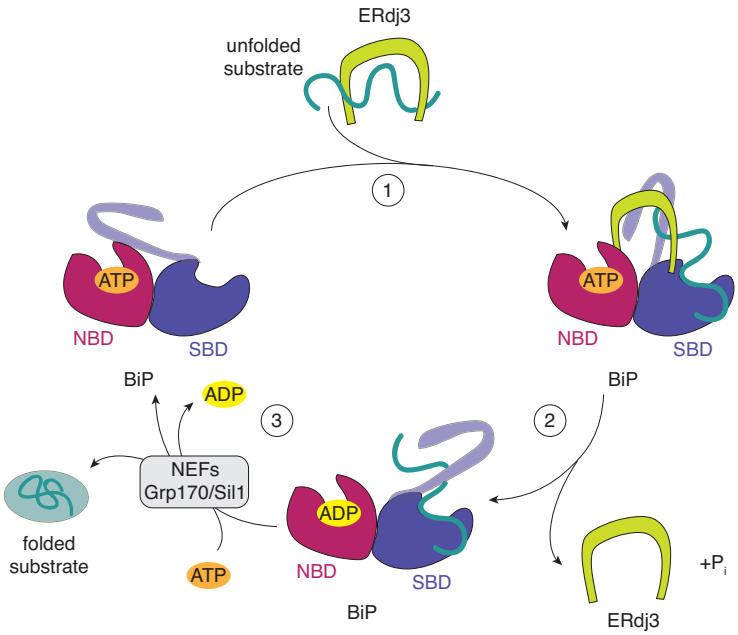

Fig. 4 The ATPase cycle of BiP. Co-chaperones like ERdj3 recruit client substrates to the substrate binding domain (SBD, pink) of BiP (1) and stimulate ATP hydrolysis by BiP's nucleotide binding domain (NBD, dark violet) upon which ERdj proteins dissociate from the BiP client complex. BiP undergoes a conformational change, closing its lid domain (light violet) (2). The nucleotide exchange factors (NEFs) Grp170 and Sil1 facilitate ADP exchange against ATP and thereby substrate release through induction of opening the BiP lid (3). Adapted from[121].

Binding of ATP to the NDB induces conformational changes in BiP, enabling it to bind substrate *via* its SBD. ERdj proteins help with substrate recruitment and can stimulate ATP hydrolysis through their J domain, which interacts with BiP. The nucleotide exchange factors Sil1 and Grp170 then assist in exchanging ADP against ATP, which releases the substrate and makes BiP available for a new binding round.[121] Grp170 has additionally been reported to bind unfolded proteins and act as a chaperone itself, through a different mechanism than BiP.[122] Besides helping with client recruitment, ERdj co-chaperones have roles in preventing nascent chains from entering into the ER in the absence of BiP (ERdj1), can help in protein folding (ERdj3) and are implicated in ERAD (ERdj4 and 5). ERdj5, for example, recruits misfolded proteins to BiP and reduces them prior to retrotranslocation to the cytosol for degradation.[123] Moreover, it has been reported to interact with EDEM, a protein involved in ERAD of glycoproteins.[123] The functions of ERdj2 and 7 are not clear up to date. Although Grp94 is one of the most abundant chaperones in the ER and ubiquitously expressed, only few clients have been identified for it and substrate recognition motifs are unknown.[124] Besides its role as a folding helper, it has been reported to interact with the lectin chaperone OS-9, which recognizes

terminally misfolded proteins, and therefore has been suggested to be implicated in ERAD.[125,126] Peptidyl-prolyl isomerases (PPIases) and protein disulfide isomerases (PDIs) with their recycling oxidoreductases (ER oxidoreductin 1 (Ero1) α and β) are abundant enzymes in the ER, which catalyze rate-limiting protein folding processes and introduce post-translational modifications.[127-133] PPlases catalyze *cis-trans*-isomerization of the peptide bond preceding Pro residues and include cyclophilins, FK-binding proteins and parvulins, latter acting on folded substrates.[120] Without their action, the folding of proteins would be markedly slowed down, due the high activation energy of this process.[134] Approximately 20 PDI family member have been identified in the ER.[129] PDIs act co- and post-translationally on folding proteins and can form, isomerize and reduce disulfide bonds by the active CXXC center in their thioredoxin-like (Trx) domains.[135] The formation of disulfide bonds starts co-translationally, post-translational reshuffling until the correct native conformation is achieved is common however.[136] The first discovered and best-characterized member, PDI, additionally acts as a chaperone by binding hydrophobic patches in unfolded proteins.[137] ERp57, another PDI family member, in contrast, binds to the lectin chaperones CNX and CRT and although its exact function has not been unraveled yet, the lectin interaction suggests its involvement in glycoprotein folding.[138-140] There is evidence that interaction with chaperones is a common mechanism for targeting PDIs to their substrates.[141,142] PDIs are recycled by the ER oxidoreductases Ero1 α and β.[132,133] Besides disulfide bridge formation, *N*-glycosylation of Asn-X-Ser/Thr (X≠Pro) sequons is very common among the ER-specific modifications. It plays an important role in folding and QC of proteins as it increases solubility and can be recognized by lectin chaperones. Moreover, *N*-glycans on proteins have been associated with many biological functions ranging from conferring stability to meditating protein-protein interactions.[143] *N*-glycosylation occurs co-translationally *via* the Sec61-associated oligosaccharyl transferase (OST) complex, which adds the sugar core Glc3Man9GlcNAc2 consisting of 14 residues to Asn residue of the consensus sequence.[144-146] Subsequent Glucose trimming by glucosidase I and II qualifies proteins for recognition by the lectin chaperones membrane-bound CNX and the soluble CRT.[147-150] After glucosidase II has cleaved off the last glucose residue resulting in Man9GlcNAc2, CNX and CRT are unable to rebind a protein, which then is susceptible for binding to UDP-glucose:glycoprotein glycosyltransferase (UGT).[151,152] UGT is able to reshuffle not yet fully folded protein-intermediates into the CNX/CRT cycle by specifically recognizing and re-glucosylating incompletely folded proteins.[151] Intermediates that travel too long through this CNX/CRT cycle without gaining their native state are recognized by ER mannosidase I and EDEM (ER

degradation enhancing alpha mannosidase like) as molecular clocks and targeted to ERAD. During ERAD, misfolded proteins are retrotranslocated back from the ER to the cytosol, polyubiquitinated and degraded by the proteasome.[110,153] Under stress conditions, ER homeostasis may fail and un- and misfolded proteins can accumulate and overwhelm the ER chaperone machinery. A feedback loop from the ER/Golgi exists that can address such an overload: the unfolded protein response (UPR). The stress sensor proteins IRE1, PERK and ATF6 mediate an up-regulation of the ER folding machinery[154], an ER expansion and a transient inhibition of the synthesis of most proteins.[155] If stress persists, the UPR can even lead to apoptosis.[156] After folding, assembly and concomitant release from ER chaperones, proteins are transported to the Golgi where *N*-glycan maturation and processing as well as *O*-glycosylation takes place.[157] Although, *O*-glycosylation can be initiated in the ER depending on the first sugar molecule that is attached to the oxygen in Ser or Thr (*e.g. O*-mannosylation), actual biosynthesis takes place post-translationally in the Golgi.[158] In contrast to *N*-glycosylated proteins, which arrive decorated with the core glycan Man9GlcNAc2 in the Golgi and are then further modified, *O*-glycosylation is more heterogeneous as different sugar moieties can be attached to Ser and Thr.[159,160] Moreover, *O*-glycosylation does not have an as clearly defined consensus sequence as *N*-glycosylation does. *O*-glycans are implicated in a variety of biological functions.[161] Single *O*-mannose-residues, *e.g.,* have been associated with promoting folding when present near the N-terminus, while the Notch pathway is a prime example for the role of *O*-glycosylations in signaling.[162,163] Little is known about Golgi-based QC processes. ERp44, another member of the PDI family, is an example for Golgi QC as it cycles between the Golgi and the ER recognizing free Cys of unassembled proteins, like IgM molecules, and transporting them back to the ER for proper folding or degradation.[164] Moreover, Quiescin-sulfhydryl oxidase (QSOX) enzymes have been reported to catalyze disulfide rearrangements downstream of the ER. QSOX are mainly localized to the Golgi but can also be secreted by certain cell types.[165]

The three main principles of ER early secretory pathway QC is thus recognition of exposed hydrophobic polypeptides and reactive thiol groups, and glycan-monitoring. While the key players of the ER folding machinery and the major ER stress response mechanisms, UPR and ERAD, have been identified and characterized in recent years, detailed insights into the molecular mechanisms of folding and quality control in the ER, which is a prerequisite for targeted interventions, is still missing. For the IL-12 family, folding in the secretory pathway has hardly been understood and raises many intriguing questions some of which this PhD thesis aims

to answer. A combined cell biological and computational approach is likely to provide important insights into this complex issue.

1.7 Aims and scope of this PhD thesis

The overall aim of this PhD thesis was to uncover structural and cellular mechanisms, which define if a protein is folding-competent on its own or depends on assembly with a folded matrix protein to gain its native structure. As a model system we used the heterodimeric IL-12 family cytokines IL-27 and IL-35, small secretory proteins, which both are built up of a folding-incompetent α subunit and an autonomously folding β subunit, IL-27α/EBI3 and IL-12α/EBI3, respectively. In order for the α subunits to fold and be secreted, they need to assemble with their cognate β subunit, while EBI3 can be secreted on its own.[24,27] Interestingly, the folding competence of IL-27 subunits in mice is the exact opposite with IL-27α being secretion-competent and EBI3 dependent on the α subunit for folding.[24] The molecular basis of these folding differences is unknown.

One main goal of this PhD thesis was thus to uncover why hIL-27α is retained in cells whereas mIL-27α is secreted. Which structural features and cellular folding factors determine if a protein is secreted or not and can we change this using protein engineering? For a number of disease conditions, beneficial effects of mIL-27α have been reported. Are these effects relevant for humans if the protein is not present in the human body? Moreover, the question arises if the composite nature of IL-27 in humans *versus* mice is an evolutionary conserved construction principle and if yes, can a broader interspecies analysis provide us with fundamental insights into IL-27 subunit folding? A better understanding of IL-27 biosynthesis would ultimately contribute to the development of novel IL-related immunotherapies.

IL-35 is particularly enigmatic as a cytokine, both in terms of subunit interaction and signal transduction. While comprehensive mutational analyses failed to define the interface of IL-35, its actual existence as a heterodimer is also under debate due to the unconventional receptor repertoire it has been shown to signal through.[51,90] Nonetheless, it harbors great therapeutical potential being an immunosuppressive IL. The second main goal of this PhD thesis was thus to learn more about the IL-35 interface by studying the cellular folding and assembly of its subunits in order to ultimately generate a disulfide bridged IL-35 as well as the single subunits IL-12α and EBI3. These three proteins should then be tested for their individual signaling modalities using a receptor chain coupled NanoBRET system.[166] This project was started during the PhD but is not part of the contents of this PhD thesis.

Studying the folding, assembly and quality control of the IL-27 and IL-35 with cell-biological as well as computational methods, enables fundamental insights into the mechanisms of cellular protein folding. These can be used for rationally changing or improving the folding of IL-27 and IL-35 subunits and heterodimers and hopefully contribute to ultimately resolving the structure of IL-27 and IL-35. All of which is paramount for the development of novel immunotherapies.

1.8 Overview of Methods

Constructs for mammalian expression. Interleukin cDNAs were cloned into a vector for mammalian expression. Where indicated, epitope tags were C-terminally introduced *via* a $(GS)_4$-linker. Mutants were generated by site-directed mutagenesis. All constructs were sequenced for verification.

Cell culture and transient transfections. HEK293T, THP1, BL-2 and Expi293 cells were grown and cultured as recommended by the cell line provider. Transient transfections were carried out in poly D-lysine coated dishes using GeneCellin (BioCellChallenge) according to the manufacturer's instructions.

Secretion, redox and cycloheximide chase experiments. Secretion experiments were used to determine if a protein was correctly folded and thus released from ER retention. Redox tests were conducted to investigate the formation of disulfide bridges and cycloheximide chase assays allowed us to study newly synthesized proteins only, as opposed to steady state conditions. Deglycosylation experiments were carried out to examine the *N*- and *O*-glycosylation state of proteins.

Immunoprecipitation experiments and immunoblots. Immunoprecipitations were used to investigate protein-protein interactions. Immunoblots were used to prove the presence of a certain protein in a sample *via* antibody boding and chemiluminescence detection.

Recombinant protein production. Recombinant proteins for cytokine assays and for a reference were expressed in Expi293 cells and *E.coli,* respectively.

Quantification and statistics. Where indicated, Western blots and multiplex assays were quantified and statistically analyzed. All experiments were performed at least three times.

Sequence and structural analyses. DNA sequence alignments were performed to investigate sequence conservation between proteins of different species. Homology modeling of proteins was performed when no structure was available. Molecular dynamics simulations were

undertaken to simulate physical movement of modeled protein structures. Molecular docking was carried out to investigate protein subunit interaction interfaces.

Cytokine assays. Different cell lines and isolated primary cells were used to study STAT activation, *i.e.* phosphorylation, upon cytokine incubation. The analysis of cytokine secretion from a macrophage cell line was performed to monitor downstream biological effects of STAT signaling.

Methodological details can be found in the Materials and Methods section of the included papers.

2 Results

2.1 A folding switch regulates interleukin 27 biogenesis and secretion of its α-subunit as a cytokine.

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Published by Stephanie I. Müller, Antonie Friedl, Isabel Aschenbrenner, Julia Esser-von Bieren, Martin Zacharias, Odile Devergne[2] and Matthias J. Feige[2]

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2.1.1 Summary

The heterodimeric IL-12 family of cytokines is an intriguing example for the complexity of the immune system, with the four members IL-12, IL-23, IL-27 and IL-35, consisting of only five building blocks (IL-12α, IL-23α, IL-27α, EBI3, IL-12β) and additional single subunits performing immunological functions on their own. IL-27 is an immunomodulatory cytokine built of the non-covalently linked subunits IL-27α and EBI3. In mice IL-27 function is complemented by IL-27α, while in humans this protein is retained in cells and not secreted. In this study, IL-27 biogenesis was investigated structurally and mechanistically to identify what determines subunit retention *versus* secretion and subsequent biological functionalities.

Human (h) IL-27α in isolation was not secreted and instead sent to ERAD in absence of EBI3, while mouse (m) IL-27α was secreted and performs immunomodulatory roles on its own. As for mIL-27α protective roles in a number of pathologies have been reported, understanding its autonomous folding might be beneficial for therapies in humans. The comparison of the amino acid sequences of human and murine IL-27α revealed that mIL-27α has two Cys residues, while hIL-27α has only a single Cys. Introducing this Cys at the corresponding position in the human protein rendered hIL-27α^{L162C} secretion competent and intramolecular disulfide formation was shown. Moreover, substitution of Cys by Leu in the mouse protein rendered mIL-27α^{C158L} dependent on assembly with EBI3 for secretion. A single amino acid switch thus changed IL-27α folding from autonomous to being assembly-dependent. In order to understand why hIL-27α is retained in cells, contribution of its single free Cys residue as well as a long poly-Glu loop was tested but turned out to not induce retention of hIL-27α in the ER. A molecular dynamics simulation comparing hIL-27α^{WT} and hIL-27α^{L162C} revealed the disulfide bond to reduce

dynamics of the two large loops 1 and 2 in hIL-27α and the burial of hydrophobic residues as an interacting cluster between loops 1 and 2 and helices 2 and 4. The ER chaperone BiP plays a key role in binding hydrophobic residues in incompletely folded proteins and was thus tested for interaction with hIL-27α^{WT} and hIL-27α^{L162C}. Indeed, hIL-27α^{L162C} showed decreased BiP binding. Moreover, mutation of a potential BiP binding site in hIL-27α^{WT} decreased BiP interaction even more and partial autonomous secretion of the hIL-27α$^{6L\rightarrow D}$ mutant was visible arguing for BiP retaining hIL-27α^{WT} in the ER. In order to test if hIL-27α^{L162C} had immunological functions, as has been reported for mIL-27α, its signaling competence on human BL-2 cells expressing the IL-27 receptor complex was tested. While for mIL-27α agonistic and antagonistic roles are still debated, IL-27 signal inhibition on STAT levels was not observed when co-incubating cells with IL-27 and an excess of hIL-27α^{L162C}. It was shown that signal transduction was, indeed, mediated by the IL-27 receptor subunits IL-27Rα and gp130. In addition, the effects of hIL-27α^{L162C} induced signaling were tested with LPS (non-) stimulated THP1-derived macrophages. hIL-27α^{L162C} was able to modulate IL-27 induced CXCL10, L-6 and IL-10 secretion in LPS stimulated cells, while in cells not treated with LPS it could also induce effects distinct from IL-27. hIL-27α^{L162C} is thus a signaling-competent molecule able to induce downstream immunological effects.

Using an interdisciplinary approach, this study dissected the folding of IL-27α structurally and mechanistically. By a single amino acid switch folding and secretion of IL-27α can now be changed from being assembly-dependent to autonomous and *vice versa*. This resulted in a molecular phenocopy of hIL-27α for a more human-like mouse model and a novel human immunomodulator. This study illustrates thus how protein folding and ERQC can shape immuno-functions in a species.

2.1.2 Contribution of the PhD candidate

Stephanie I. Müller, Odile Devergne, and Matthias J. Feige designed research. Stephanie I. Müller, Antonie Friedl, Isabel Aschenbrenner, Martin Zacharias, and Odile Devergne performed research. Stephanie I. Müller, Antonie Friedl, Isabel Aschenbrenner, Julia Esser-von Bieren, Martin Zacharias, Odile Devergne, and Matthias J. Feige analyzed data. Stephanie I. Müller, Julia Esser-von Bieren, Odile Devergne, and Matthias J. Feige wrote the paper.

PNAS

A folding switch regulates interleukin 27 biogenesis and secretion of its α-subunit as a cytokine

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A common design principle of heteromeric signaling proteins is the use of shared subunits. This allows encoding of complex messages while maintaining evolutionary flexibility. How cells regulate and control assembly of such composite signaling proteins remains an important open question. An example of particular complexity and biological relevance is the interleukin 12 (IL-12) family. Four functionally distinct αβ heterodimers are assembled from only five subunits to regulate immune cell function and development. In addition, some subunits act as independent signaling molecules. Here we unveil key molecular mechanisms governing IL-27 biogenesis, an IL-12 family member that limits infections and autoimmunity. In mice, the IL-27α subunit is secreted as a cytokine, whereas in humans only heterodimeric IL-27 is present. Surprisingly, we find that differences in a single amino acid determine if IL-27α can be secreted autonomously, acting as a signaling molecule, or if it depends on heterodimerization for secretion. By combining computer simulations with biochemical experiments, we dissect the underlying structural determinants: a protein folding switch coupled to disulfide bond formation regulates chaperone-mediated retention versus secretion. Using these insights, we rationally change folding and assembly control for this protein. This provides the basis for a more human-like IL-27 system in mice and establishes a secretion-competent human IL-27α that signals on its own and can regulate immune cell function. Taken together, our data reveal a close link between protein folding and immunoregulation. Insights into the underlying mechanisms can be used to engineer immune modulators.

protein folding | protein assembly | protein quality control | interleukins | immune engineering

A central task of any immune system is the balanced regulation of pro- and antiinflammatory responses, which allows rapid eradication of threats while protecting the host (1). Interleukin 12 (IL-12) cytokines, namely IL-12, IL-23, IL-27, and IL-35 (2), epitomize this concept of balanced immune regulation within a single family. Although each family member is functionally diverse, IL-12 and IL-23 have mostly been associated with proinflammatory functions, whereas IL-35 performs immune-suppressive roles (2, 3). IL-27 is multifaceted with immunomodulatory pro- and antiinflammatory functions, acting on different types of T cells (4). It can limit autoimmune reactions but is also crucial in fighting infections as well as regulating cancer development (5–7).

This broad range of biological functions exerted by IL-12 cytokines goes hand in hand with a unique structural complexity. Each IL-12 family member is a heterodimer composed of a four-helical bundle α-subunit (IL-12α/p35, IL-23α/p19, or IL-27α/p28) and of a β-subunit composed of two fibronectin domains (IL-27β/EBI3) or one immunoglobulin and two fibronectin domains (IL-12β/p40) (8, 9). Despite their distinct roles in regulating immune responses, all known heterodimeric IL-12 family members are made up of only these three α- and two β-subunits. The regulatory capacity of the IL-12 family, but also its assembly complexity, is further increased by the secretion and biological activity of some isolated α- and β-subunits (10, 11). A prominent example is IL-27α/p28. Murine IL-27α, also designated as IL-30, is secreted in isolation (12) and performs immunoregulatory roles (13–16). In contrast, no autonomous secretion of human IL-27α has been reported yet. The molecular basis for this difference has remained unclear, but it is likely to have a profound impact on immune system function, since in mice IL-27 and its α-subunit both strongly influence inflammatory diseases (4, 17), where significant differences between mouse and man exist (18).

Insights into IL-12 family biogenesis, which could potentially explain this difference, are very limited so far (19, 20). It has been shown that all human α-subunits are retained in cells in isolation and depend on assembly with their cognate β-subunit to be secreted (12, 21–23), but the underlying mechanisms remain incompletely understood. In this study, we thus investigated IL-27 biosynthesis to delineate how folding and assembly regulate secretion of isolated subunits versus heterodimeric molecules with their distinct biological functions. Using an interdisciplinary approach, we structurally and mechanistically dissect key reactions in IL-27α biosynthesis. Building on our analyses, we rationally change IL-27α secretion and thus provide the basis for a more human-like IL-27 system in mice as well as a human IL-27α subunit that acts as an immune modulator.

Significance

Interleukins are small secreted proteins that drive immune cell communication. Understanding how cells produce interleukins is thus key for decoding and modulating immune responses. Our study elucidates the molecular mechanisms of interleukin 27 (IL-27) biosynthesis, a key cytokine in control of autoimmunity and infections. IL-27 is composed of two subunits, α and β. In humans, these have to assemble to form bioactive IL-27, whereas in mice, IL-27α can be secreted alone, modulating immune reactions and reducing sepsis-related mortality. Strikingly, differences in a single amino acid regulate IL-27α secretion. Using our molecular insights, we engineer a more human-like IL-27 system in mice and design a secretion-competent and functional human IL-27α subunit. This may provide an approach toward treating inflammatory diseases.

Author contributions: S.I.M., O.D., and M.J.F. designed research; S.I.M., A.F., I.A., M.Z., and O.D. performed research; S.I.M., A.F., I.A., J.E.-v.B., M.Z., O.D., and M.J.F. analyzed data; and S.I.M., J.E.-v.B., O.D., and M.J.F. wrote the paper.

Conflict of interest statement: A patent describing variants of IL-27α has been submitted.

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Results

Murine IL-27α Is Secreted from Cells, Whereas Its Human Ortholog Is Degraded. As a member of the heterodimeric IL-12 family, IL-27 is composed of an α- and a β-subunit (IL-27α/p28 and EBI3, respectively, Fig. 1*A*), which assemble noncovalently to form heterodimeric IL-27 (12). Once secreted, IL-27 signals via the heterodimeric IL-27Rα/gp130 receptor (24). In addition, in mice IL-27α/p28 is secreted in isolation and performs biological functions (13–16). In agreement with this notion and the first study on IL-27 (12) we found murine IL-27α (mIL-27α) to be secreted from transfected cells (Fig. 1*B*). Human EBI3 (hEBI3) further increased secretion of mIL-27α (Fig. 1*B*) and both proteins interacted in coimmunoprecipitation experiments (*SI Appendix*, Fig. S1*A*), arguing for a conserved IL-27 interface. In contrast to mIL-27α, human IL-27α (hIL-27α) was retained in cells if expressed in isolation and its β-subunit, EBI3, induced its secretion (Fig. 1*C*) (12). Upon coexpression of EBI3, hIL-27α populated two major species with reduced electrophoretic mobility (Fig. 1*C*), which we could attribute to hIL-27α becoming O-glycosylated (*SI Appendix*, Fig. S1 *B–D*). O-glycosylation occurs in the Golgi, and thus hIL-27α appears to be retained in the endoplasmic reticulum (ER) in the absence of EBI3 and only traverses the Golgi in its presence. To assess the fate of ER-retained hIL-27α, we performed cycloheximide chase assays in the absence and presence of the proteasome inhibitor MG132. Human IL-27α was rapidly degraded with a half-life of only ∼0.5 h and stabilized by MG132, showing that isolated hIL-27α is a substrate of ER-associated degradation (ERAD) (Fig. 1*D*).

A Single Amino Acid Switch Regulates IL-27α Secretion. The difference in secretion between human and murine IL-27α warrants further investigation, since murine IL-27α has immunomodulatory functions (13–16) and exerts protective roles in sepsis, graft-versus-host disease, liver fibrosis, and autoimmunity (15, 17, 25, 26). These functions will be absent in the human system due to the lack of hIL-27α secretion. Our data show that hIL-27α is retained and becomes degraded by the ER quality-control machinery. Together, this argues that hIL-27α is recognized as incompletely structured by the cell and that analyzing the structure of IL-27α may hold a clue to its secretion and immunological functions. To identify differences between the human and murine IL-27α subunits, we thus modeled their structures and performed a sequence and structure alignment (Fig. 2 *A* and *B*). Despite approximately 75% sequence identity, three major differences exist in the murine protein in comparison with its human ortholog: (*i*) an N-glycosylation site; (*ii*) a second cysteine, which both could affect structure formation and protein maturation in the endoplasmic reticulum (27, 28); and (*iii*) a lysine that disrupts a poly-Glu stretch in IL-27α. To assess the effect of these differences, we individually introduced corresponding mutations into hIL-27α (D89N, L162C, or K168 insertion) and monitored secretion of the three mutants. Neither hIL-27α^{D89N} nor hIL-27α$^{K168\ insertion}$ were secreted (Fig. 2*C*). In striking contrast, hIL-27α^{L162C} was very efficiently secreted, including bona fide O-glycosylation (Fig. 2*C* and *SI Appendix*, Fig. S2*A*). Our structural modeling suggested that disulfide bond formation may underlie this behavior: in mIL-27α, its two cysteines are close enough to form a disulfide bond, which would stabilize the protein. In the human protein, L162 was predicted to be exactly at the position of the second cysteine (Fig. 2*B*). An SDS-PAGE analysis confirmed this hypothesis: a clear mobility shift, an indication of disulfide bond formation (29), existed for hIL-27α^{L162C} under reducing versus nonreducing conditions (Fig. 2*D*). To further verify this assumption, we tested an expanded set of hIL-27α mutants. Based on our structural modeling, N161C, P163C, and L181C could possibly also allow for the formation of a disulfide bond in hIL-27α (*SI Appendix*, Fig. S2*B*). In complete agreement with our hypothesis, all additional mutants for which we observed secretion, namely hIL-27α^{N161C} and hIL-27α^{P163C}, also formed a disulfide bond (*SI Appendix*, Fig. S2 *C* and *D*). To assess evolutionary conservation of this principle, we analyzed disulfide bond formation and secretion competency for IL-27α derived from three important model organisms: Chinese hamster, pig, and macaque monkey. For Chinese hamster IL-27α, disulfide bond formation and secretion were observed. In contrast, no disulfide bond formation and thus cellular retention and only EBI3-induced secretion were observed for pig and macaque monkey (*SI Appendix*, Fig. S2 *E* and *F*), indicating evolutionary conservation.

Our data reveal that the secretion of human IL-27α and thus potential immune regulation by this subunit depend on a single cysteine residue. Based on this observation, we queried if we could rationally switch the secretion behavior of murine IL-27α, making it dependent on EBI3. Indeed, when we replaced the second cysteine in mIL-27α by a leucine (C158L), mIL-27α^{C158L} was now retained in cells in isolation. And just like for its human counterpart, its secretion was induced by EBI3 (Fig. 2*E*). By a single point mutation, we could thus change an autonomous protein folding reaction into an assembly-dependent one and generate a molecular phenocopy of human IL-27α for the mouse protein.

Molecular Determinants of IL-27α Retention Versus Secretion. To further understand molecular determinants of IL-27α retention versus secretion and thus the biogenesis of IL-27 as a key cytokine, we performed a comprehensive mutational analysis, molecular dynamics (MD) simulations and queried chaperone binding of hIL-27α. Recognition of unpaired cysteines constitutes an important step in secretory protein quality control (30), and one free cysteine is present in human IL-27α. We thus first mutated the free cysteine of hIL-27α (C107, see Fig. 2*B*) to leucine and monitored its secretion. However, like the wild-type protein (hIL-27α^{wt}), hIL-27α^{C107L} was secreted only upon coexpression of EBI3

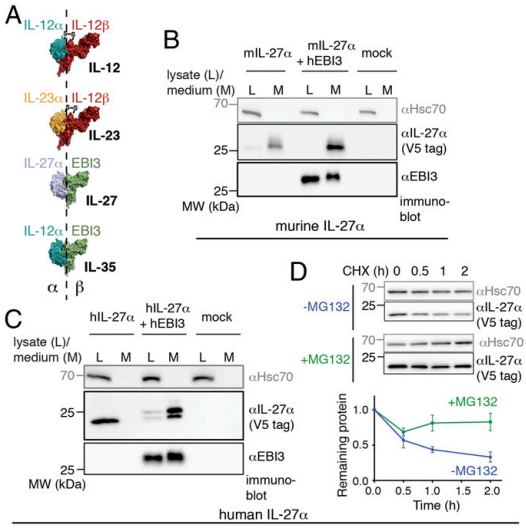

Fig. 1. Murine IL-27α is secreted, whereas its human ortholog is degraded via ERAD. (*A*) Schematic of the IL-12 family. Each family member is an αβ heterodimer. IL-12 and IL-23 are covalent dimers, connected by a disulfide bond (S-S), whereas IL-27 and IL-35 are noncovalent dimers. (*B*) Mouse IL-27α (mIL-27α) is secreted in isolation into the medium of transfected 293T cells, and human EBI3 (hEBI3) further increases its secretion. (*C*) Human IL-27α (hIL-27α) is retained in cells in isolation and depends on coexpression of its β-subunit EBI3 for secretion. (*D*) Isolated hIL-27α is degraded by ERAD. CHX, cycloheximide. Where indicated, the proteasome was inhibited with MG132 ($n = 4 \pm$ SEM).

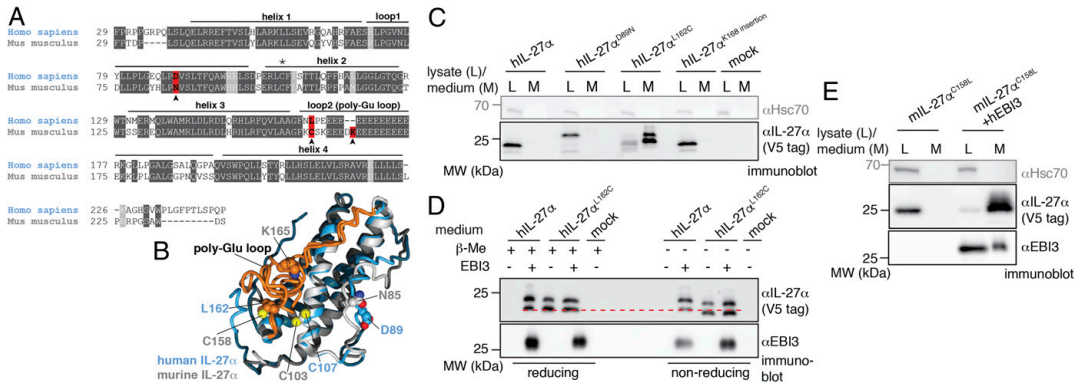

Fig. 2. A single amino acid switch regulates IL-27α secretion. (*A*) Potentially folding-relevant differences in the amino acid sequence of hIL-27α and mIL-27α (without ER import sequences). Identical residues are shaded in dark gray, homologous residues in light gray, and differences with a potential impact on protein structure formation in red (arrowheads). The conserved Cys residue is marked with an asterisk. (*B*) Superposition of hIL-27α (blue) and mIL-27α (gray) structural models. Potentially folding-relevant residues (*A*) are shown in a CPK representation; the poly-Glu loop is highlighted in orange. (*C*) Substitution of Leu162 with Cys (L162C) in hIL-27α allows for its secretion in isolation. (*D*) Secreted hIL-27α^{L162C} forms a disulfide bond. Where indicated, samples were treated with β-mercaptoethanol to reduce disulfide bonds. A dashed line highlights mobility differences. (*E*) Substitution of Cys158 with Leu (C158L) in mIL-27α leads to its cellular retention and dependency on EBI3 coexpression for secretion.

(Fig. 3*A*). Next, we focused on the characteristic poly-Glu stretch of IL-27α (31). It was predicted to be unstructured (Fig. 2*B*) and may thus be involved in retention. Loop deletion as well as replacement mutants of hIL-27α, however, behaved like the wild-type protein (*SI Appendix*, Fig. S3*A*). This indicates that other features cause ER retention. To obtain insights into possible underlying principles, we performed MD simulations on hIL-27α^{wt} and hIL-27α^{L162C} with a disulfide bond formed. The presence of the disulfide bond reduced the dynamics of two large loops within hIL-27α^{L162C}: of the poly-Glu loop, whose N terminus is fixed by the disulfide bond (Fig. 2*B*), and additionally of the loop connecting helices 1 and 2 in hIL-27α (Fig. 3 *B* and *C*). Together, this led to more persistent interactions of hydrophobic residues between this loop, the poly-Glu loop, and in helices 2 and 4 of

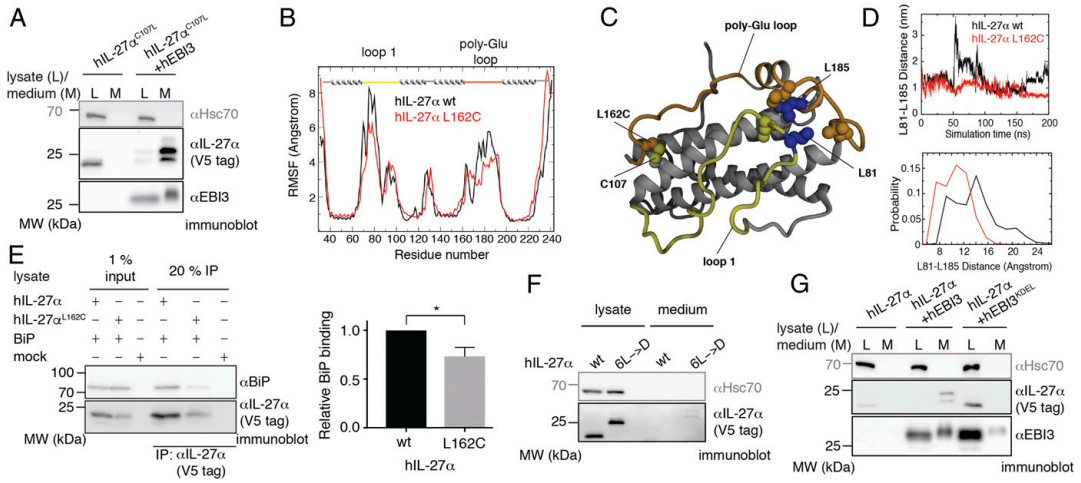

Fig. 3. Molecular determinants of IL-27α retention versus secretion. (*A*) The single free Cys of hIL-27α does not lead to its ER retention: hIL-27α^{C107L} is retained in the cell in isolation and secreted upon coexpression of EBI3. (*B*) Molecular dynamics simulations reveal locally reduced flexibility of hIL-27α^{L162C} in loop1 and the poly-Glu loop. The root mean square fluctuation (rmsf) values for hIL-27α^{wt} and hIL-27α^{L162C} (with its disulfide bond formed) are overlaid. (*C*) Regions with reduced flexibility in hIL-27α^{L162C} with its disulfide bond formed compared with hIL-27α^{wt} are highlighted in the modeled structure of hIL-27α (*B*). A cluster of hydrophobic Leu residues including L81 and L185 is shown in a CPK representation. (*D*) The distance of L81 and L185 over time, derived from MD simulations, as well as a distribution analysis of their distances for hIL-27α^{wt} versus hIL-27α^{L162C} are shown. L81 and L185 were selected as a proxy for hydrophobic cluster formation. (*E*) The chaperone BiP binds significantly better to hIL-27α^{wt} than to hIL-27α^{L162C}. Coimmunoprecipitation data are shown on the *Left* and their quantification on the *Right* ($n = 4 \pm$ SEM; $*P < 0.05$). (*F*) Replacement of six Leu residues in loop1/poly-Glu loop of hIL-27α by Asp (hIL-27α$^{6L\rightarrow D}$), including L81 and L185 (*C* and *D*), leads to partial secretion of hIL-27α. (*G*) Human EBI3 with an ER retention sequence (hEBI3^{KDEL}) does not induce hIL-27α secretion.

hIL-27α in silico (Fig. 3 *C* and *D*). Thus, a conformational switch coupled to disulfide bond formation led to burial of hydrophobic amino acids in IL-27α. Stretches of hydrophobic amino acids are the signature recognition motif for the key ER chaperone BiP (heavy chain binding protein) (32). Indeed, BiP strongly bound to hIL-27α^{wt}, whereas binding to hIL-27α^{L162C} was significantly reduced (Fig. 3*E*), in very good agreement with our hypothesis derived from the MD simulations. To lend further experimental support to this idea, we replaced a subset of six Leu residues in loop1 and the poly-Glu loop of hIL-27α by Asp residues to reduce BiP binding (hIL-27α$^{6L\rightarrow D}$) (33). Consistent with our hypothesis, the hIL-27α$^{6L\rightarrow D}$ mutant showed reduced BiP binding (*SI Appendix*, Fig. S3*B*) and was partially secreted (Fig. 3*F*).

Our data revealed a folding switch to underlie hIL-27α retention versus secretion. Furthermore, IL-27 is a noncovalent heterodimer (Fig. 1*A*). We thus wondered if EBI3 was only needed to induce correct folding of hIL-27α, to allow for its presence as a cytokine in the organism, or if stable heterodimerization was a prerequisite for secretion. To decide between these possibilities, we used a human EBI3 construct with a C-terminal KDEL ER retention sequence (hEBI3^{KDEL}), which would not be secreted any more but should still induce hIL-27α folding. This hEBI3^{KDEL} construct did not induce hIL-27α secretion (Fig. 3*G*). Thus, human IL-27 needs to be secreted as a heterodimer, further corroborating the absence of free IL-27α in humans.

Human IL-27α Is a Functional Immune Signaling Molecule. The hIL-27α^{L162C} mutant provided us with secretion-competent human IL-27α. For the corresponding mouse cytokine, it is still debated if it performs agonistic or antagonistic roles (13–17). To assess the biological effects of hIL-27α^{L162C}, we produced the protein in mammalian cells (*SI Appendix*, Fig. S4 *A*–*C*) and assessed its effect on human cell lines and primary cells, beginning with the lymphoma BL-2 cell line. BL-2 cells express a functional IL-27 receptor on their surface as shown by STAT1 phosphorylation in response to IL-27 stimulation (34). In these cells, hIL-27α^{L162C} did not inhibit IL-27–induced STAT1 phosphorylation even at a several hundredfold excess (Fig. 4*A* and *SI Appendix*, Fig. S5*A*). Instead, hIL-27α^{L162C} induced STAT1 phosphorylation in BL-2 cells with the effect being approximately 700-fold weaker and slightly slower than that observed for IL-27 as determined from quantifying the phospho-STAT1 signals (Fig. 4*B* and *SI Appendix*, Fig. S5 *B* and *C*). These findings in human cells are in agreement with the agonistic functions for mIL-27α suggested in the literature (14) and confirmed by our experiments (*SI Appendix*, Fig. S5 *D* and *E*). Since at lower levels mIL-27α can potentially induce STAT1 phosphorylation via IL-6R/gp130 receptors (14), which are not present on BL-2 cells (*SI Appendix*, Fig. S5*F*), we next assessed the effects of hIL-27α^{L162C} on primary human CD4$^+$ T cells. Despite the presence of IL-6R (*SI Appendix*, Fig. S5*G*), similar levels of hIL-27α^{L162C} as observed for BL-2 cells were needed to induce signaling in human CD4$^+$ T cells (Fig. 4*C*). Furthermore, hIL-27α^{L162C} did not inhibit IL-6 signaling in primary human CD4$^+$ T cells (*SI Appendix*, Fig. S5*H*). Based on these findings, we next analyzed which receptors mediate hIL-27α^{L162C} signaling in BL-2 cells. A soluble IL-27Rα as an antagonist of IL-27Rα–mediated signaling (34) as well as anti-gp130

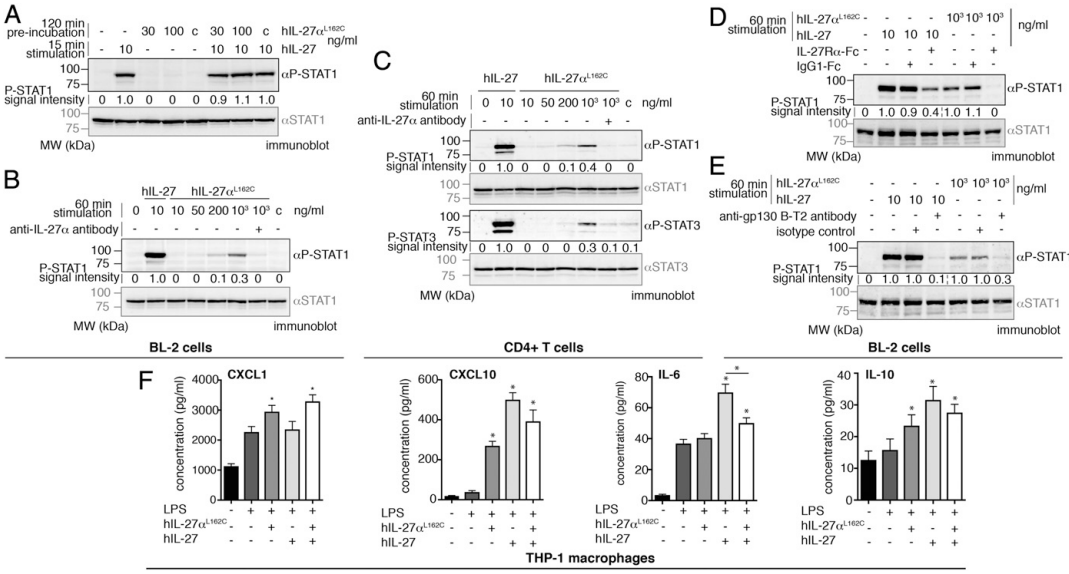

Fig. 4. Human IL-27α^{L162C} is biologically active. (*A*) hIL-27α^{L162C} does not antagonize IL-27–induced STAT1 phosphorylation. BL-2 cells were preincubated with hIL-27α^{L162C} and then stimulated with hIL-27 (c, control supernatant of nontransfected cells). (*B*) Human IL-27α^{L162C} induces STAT1 phosphorylation. BL-2 cells were incubated with the indicated concentrations of hIL-27 or hIL-27α^{L162C}. Effects can be inhibited by an anti–IL-27 antibody. (*C*) hIL-27α^{L162C} induces STAT1 and STAT3 phosphorylation in human CD4$^+$ T cells. CD4$^+$ T cells were incubated with the indicated concentrations of hIL-27 or hIL-27α^{L162C} with or without an anti–hIL-27 antibody (10 μg/mL) as indicated. (*D*) hIL-27α^{L162C} signals via IL-27Rα. BL-2 cells were stimulated with hIL-27α^{L162C} or hIL-27 for 60 min in the presence of an ∼1.5-fold molar excess of soluble IL-27Rα (IL-27Rα-Fc) or control fusion protein (IgG1-Fc). (*E*) hIL-27α^{L162C} signals via gp130. BL-2 cells were treated as described in *D* using an anti-gp130 antibody or the corresponding isotype control (each at 10 μg/mL). (*A*–*E*) P-STAT signals were quantified and normalized for IL-27–induced levels. (*F*) hIL-27α^{L162C} increases the secretion of CXCL1, CXCL10, and IL-10, and modulates IL-27–induced IL-6 secretion from LPS-stimulated THP-1 macrophages (multiplex assays, $n = 11 \pm$ SEM from three independent biological replicates, $^*P < 0.003$, ANOVA). THP-1 macrophages were stimulated for 2 h with 0.5 μg/mL hIL-27α^{L162C} and/or 10 ng/mL hIL-27. Subsequently, 1 μg/mL LPS was added for an additional 4 h in the presence of hIL-27α^{L162C} and/or hIL-27.

antibodies both decreased signaling induced by hIL-27α^{L162C}, similar to IL-27 signaling (Fig. 4 *D* and *E*). This argues that both proteins signal via IL-27Rα and gp130.

Our data show that hIL-27α^{L162C} is a signaling-competent immune protein obtained by a single point mutation. To further assess biological consequences of IL-27α–induced signaling in human immune cells, we focused on THP-1 macrophages, since we found IL-27α to also be active on primary human monocytes (*SI Appendix*, Fig. S6*A*). To provide a comprehensive picture of hIL-27α^{L162C} effects, THP-1 cells differentiated into macrophages were stimulated with LPS either in the absence or presence of human IL-27, IL-27α^{L162C}, or a combination of both proteins. THP-1 cytokine secretion was assessed by multiplex assays. In these experiments, hIL-27α^{L162C} increased the secretion of the chemokines CXCL-1 and CXCL-10 that are involved in immune cell recruitment and host defense (Fig. 4*F*). In addition, hIL-27α^{L162C} modulated the LPS-driven secretion of pro- and antiinflammatory cytokines (IL-10 and IL-1β) and reduced IL-27–triggered IL-6 release, corroborating its regulatory activity on immune cells (Fig. 4*F* and *SI Appendix*, Fig. S6*B*). For THP-1 macrophages not treated with LPS, we observed similar trends but also differences for certain cytokines, e.g., for CXCL2, which was induced by hIL-27α^{L162C} (*SI Appendix*, Fig. S7).

Thus, in some cases hIL-27α^{L162C} modulated IL-27 functions (e.g., IL-6 in the presence of LPS), whereas in others, e.g., for CXCL-1 and CXCL-2 in the absence of LPS, hIL-27α^{L162C} had distinct effects from IL-27. By engineering a protein folding reaction, we could thus generate secretion-competent human IL-27α that acts as a functional immune signaling protein.

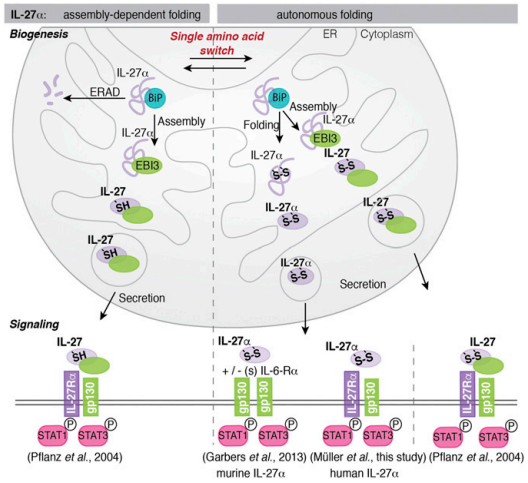

Fig. 5. A single amino acid protein folding switch underlies differences in the IL-27 system. The absence or presence of a disulfide bond-forming Cys pair defines if IL-27α depends on EBI3 interaction for folding and secretion or if it can be secreted autonomously, inducing downstream signaling. Signaling-competent IL-27 species are shown in bold. Information on receptors were taken from refs. 14 and 24 and this study.

Discussion

Our study reveals that differences in a single cysteine residue toggle IL-27α between being secretion competent in isolation or depending on heterodimerization with EBI3 as a prerequisite to leave the cell (Fig. 5). A combined computational and biochemical approach revealed that disulfide bond formation is coupled to shielding hydrophobic residues in IL-27α, which are otherwise recognized by BiP, lead to IL-27α ER retention, and ultimately its degradation. This disulfide-regulated folding switch solves the longstanding question about differences in IL-27α secretion in mouse and man: if no disulfide bond can form in IL-27α, it depends on assembly with EBI3 to obtain its correct structure and leave the cell. If a disulfide bond can form, IL-27α can be secreted and act as an immune modulator. This allows an organism's cytokine repertoire to evolve by just changing a single residue in IL-27α that decisively influences protein folding. Interestingly, we find that polymorphisms in human IL-27α can affect regions we identified to be important for its assembly-induced folding (*SI Appendix*, Fig. S8). This may be relevant, e.g., in the context of tumor immunity, where IL-27 has recently been reported to be prominently involved (35). Although all human IL-12 family α-subunits depend on assembly with their β-subunits for secretion (12, 21–23), different molecular mechanisms seem to underlie secretion control. This is exemplified by IL-12α, which, unlike IL-27α, shows pronounced misfolding in the absence of IL-12β (19). These differences can potentially be explained by evolutionary relationships as, e.g., IL-27α is particularly closely related structurally to human ciliary neurotrophic factor (CNTF) (36), which does not follow a typical signal sequence-dependent secretion pathway.

Assembly-induced quality-control processes are a common theme for proteins of the immune system: antibodies (37, 38), the αβ T cell receptor (39–41), as well as interleukins (19, 20, 42) rely on this. IL-27α adds a very important aspect to this principle: in this case the individual components of heterodimeric IL-27 have independent functions in the immune system. Regulated and controlled protein assembly processes are thus not only used to safeguard proper biosynthesis of immune signaling molecules, but also define which of the possible signaling molecules are ultimately secreted and in which ratios. Modulating protein folding and assembly in the ER thus provides opportunities for regulating immune signaling.

Insights into the underlying mechanisms contribute to our understanding of secretory protein biogenesis as well as immune system function, but also provide approaches for rational interventions: introducing a point mutation into one of the cysteines in mouse IL-27α renders it dependent on EBI3 for secretion. This aids in establishing a molecular phenocopy of the human IL-27 system for future studies and may also reveal functions of IL-27 versus IL-27α. The biological roles of IL-27 are still incompletely understood (4–6) and insights are mostly gained from mouse models. In mice, however, deletion of EBI3 will indeed ablate IL-27, but not free IL-27α. In fact, removing its interaction partner EBI3 even increases levels of free IL-27α with its independent functions in mice (16). Analogously, deleting IL-27α will ablate IL-27 but also IL-27α functions in mice. Our study suggests that this can potentially be circumvented by introducing a single point mutation into one of the cysteines in mouse IL-27α, thus rendering it dependent on EBI3 for secretion, as in humans. We furthermore show that our findings are evolutionarily conserved in different species, thus also informing studies in other model organisms.

Vice versa, by a single point mutation we could obtain secretion-competent human IL-27α with biological activity on immune cells. IL-27 signaling is an attractive therapeutic target and different approaches have already been developed to alter IL-27 function. Soluble IL-27 receptor subunits may provide one way to block IL-27 function (34, 43). Alternatively, structural modeling on IL-27 has been used to design IL-27 mutants deficient in receptor activation, thus acting as IL-27 antagonists (36). Secretion-competent IL-27α, however, goes beyond these approaches: it acts as an immune signaling molecule itself. We find IL-27α^{L162C} to modulate the production of multiple cytokines by human monocytic cells, including neutrophil chemoattractants (CXCL-1 and CXCL-10) and antiinflammatory IL-10. This is of particular relevance since these mediators may contribute to the protective roles of

IL-27α in murine models of sepsis (17, 44, 45) or graft-versus-host disease (46). No good treatment options are currently available for these conditions in human patients. Building on a mechanistic analysis of protein folding in the ER, our study now establishes a secretion-competent and functional human IL-27α, which may serve as the basis for treatment options.

Materials and Methods

Constructs and Transient Transfections. Interleukin cDNAs were cloned into the pSVL vector (Amersham) for transient transfections of HEK293T cells. Secretion, redox, and cycloheximide chase experiments were performed as described in *SI Appendix*.

Recombinant Protein Production. IL-27α^{L162C} was expressed in Expi293 cells. Details can be found in *SI Appendix*.

Sequence and Structural Analyses. Multiple DNA sequence alignments were performed using Clustal Omega (47). iTasser (48) was used for homology modeling. Structural alignments and molecular dynamics simulations were performed as described in *SI Appendix*.

Cytokine Assays. STAT experiments were performed using the human Burkitt lymphoma BL-2 cell line, human primary CD4$^+$ T cells, or human primary monocytes. For multiplex assays, cytokine secretion from THP-1 cells was analyzed as detailed in *SI Appendix*.

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[03/19]

2.2 An interspecies analysis reveals molecular construction principles of interleukin 27.

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2.2.1 Summary

IL-27 is built of the α subunit IL-27α and the β subunit EBI3. The formation of an intramolecular disulfide bond qualifies IL-27α for autonomous folding in mice, while human IL-27α is dependent on assembly with EBI3 for folding. In this study the evolutionary relevance of disulfide bridge formation for autonomous folding of IL-27α as well as the construction principle of IL-27 across species was investigated. The combination of mutagenesis and computational methods aimed furthermore at gaining more structural insights into IL-27 heterodimer formation, of which no structure has been resolved thus far.

In order to determine if intramolecular disulfide bond formation is an evolutionary conserved feature, a multiple amino acid alignment of IL-27α from 15 species was performed. In fact, five IL-27α classes could be defined differing in the number and localization of Cys residues. Homology models of representatives of each class predicted the proximity Cys residues for forming a disulfide bond. To confirm these predictions, IL-27α of one representative from each class was tested experimentally for secretion competency as well as disulfide bond formation in isolation. Indeed, IL-27α with none or a single Cys was secretion-incompetent, while IL-27α with two or more Cys was secretion-competent. Secretion of IL-27α from all species was induced and/or increased by human EBI3 arguing for a conserved interface between species, which was also confirmed *in silico*. As no structure is available for IL-27, a mutagenesis-guided molecular docking combined with molecular dynamics simulations was used to gain a deeper structural understanding of subunit interaction. The refined interface of IL-27 was experimentally validated and revealed that IL-27α is orientated differently to its β subunit than the α subunits are to their β subunits in the crystal structures of covalently linked IL-12 and IL-23. Subsequently, the composite nature of IL-27 consisting of pairing a secretion-incompetent with a secretion-competent subunit was studied across species using secretion tests.

It turned out that this is a common construction principle of IL-27. Analysis of the assembly state of secreted EBI3 revealed that it is secreted as a homodimer.

In summary, this study revealed that intramolecular disulfide bond formation is an evolutionary conserved mechanism to provide secretion autonomy to IL-27α. Pairing of an autonomously folding subunit with one being dependent on assembly for folding is the common design principle of IL-27, which expands the cytokine repertoire in a given species by conserving one secretion and signaling competent subunit in addition to the IL-27 heterodimer. These observations in combination with a refined IL-27 molecular docking provide the groundwork for engineering IL-27α, EBI3 and IL-27 mimics and a disulfide linked IL-27 crucial for the challenging structure resolution of this cytokine.

2.2.2 Contribution of the PhD candidate

Matthias J. Feige and Stephanie I. Müller conceived the experimental part of the study. Martin Zacharias conceived the docking part of the study. All experiments were performed by Stephanie I. Müller and Isabel Aschenbrenner. Martin Zacharias performed docking simulations. Stephanie I. Müller, Isabel Aschenbrenner, Martin Zacharias and Matthias J. Feige analyzed data and wrote the paper.

2.2.3 Manuscript

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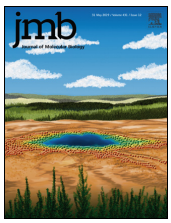

An Interspecies Analysis Reveals Molecular Construction Principles of Interleukin 27

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Abstract

Interleukin 27 (IL-27) is a cytokine that regulates inflammatory responses. It is composed of an α subunit (IL-27α) and a β subunit (EBI3), which together form heterodimeric IL-27. Despite this general principle, IL-27 from different species shows distinct characteristics: Human IL-27α is not secreted autonomously while EBI3 is. In mice, the subunits show a reciprocal behavior. The molecular basis and the evolutionary conservation of these differences have remained unclear. They are biologically important, however, since secreted IL-27 subunits can act as cytokines on their own.

Here, we show that formation of a single disulfide bond is an evolutionary conserved trait, which determines secretion-competency of IL-27α. Furthermore, combining cell-biological with computational approaches, we provide detailed structural insights into IL-27 heterodimerization and find that it relies on a conserved interface. Lastly, our study reveals a hitherto unknown construction principle of IL-27: one secretion-competent subunit generally pairs with one that depends on the other to induce its secretion.

Taken together, these findings significantly extend our understanding of IL-27 biogenesis as a key cytokine and highlight how protein assembly can influence immunoregulation.

Introduction

Secreted proteins like cytokines, extracellular enzymes or antibodies are produced in the endoplasmic reticulum (ER) and released by cells to interact with their environment. In multicellular organisms, most physiological functions essentially depend on secreted proteins. Interleukins (ILs), as a prime example, mediate immune cell communication. They maintain organism homeostasis by activating or suppressing defense mechanisms. It is thus of utmost importance that secreted ILs possess their correct structure, which defines their biological activity. A dedicated quality control (QC) system in the ER, composed of chaperones and folding enzymes [1,2], ensures that ILs and other secretory proteins fold and assemble correctly before being released into the extracellular space.

For interleukin 27 (IL-27), a member of the heterodimeric IL-12 cytokine family [3,4], cellular QC is not only a means to prevent secretion of aberrant proteins, but also a way to modulate immune reactions: IL-27 consists of the α subunit IL-27α and the β subunit Epstein–Barr virus induced gene 3 (EBI3) [5]. IL-27 is secreted by antigen-presenting cells and signals *via* a heterodimeric receptor composed of IL-27Rα and gp130 to regulate immune functions, mainly by controlling T cell differentiation [4–9]. In mice, IL-27α is stabilized by a single disulfide bond and therefore can pass ERQC and be secreted from cells without its partner subunit EBI3 to act within the murine cytokine repertoire [5,10–13]. In humans, however, IL-27α is incompletely folded in isolation and depends on EBI3 to leave the cell [5,13]. Interestingly, EBI3 behaves reciprocally to IL-27α in these two species: human

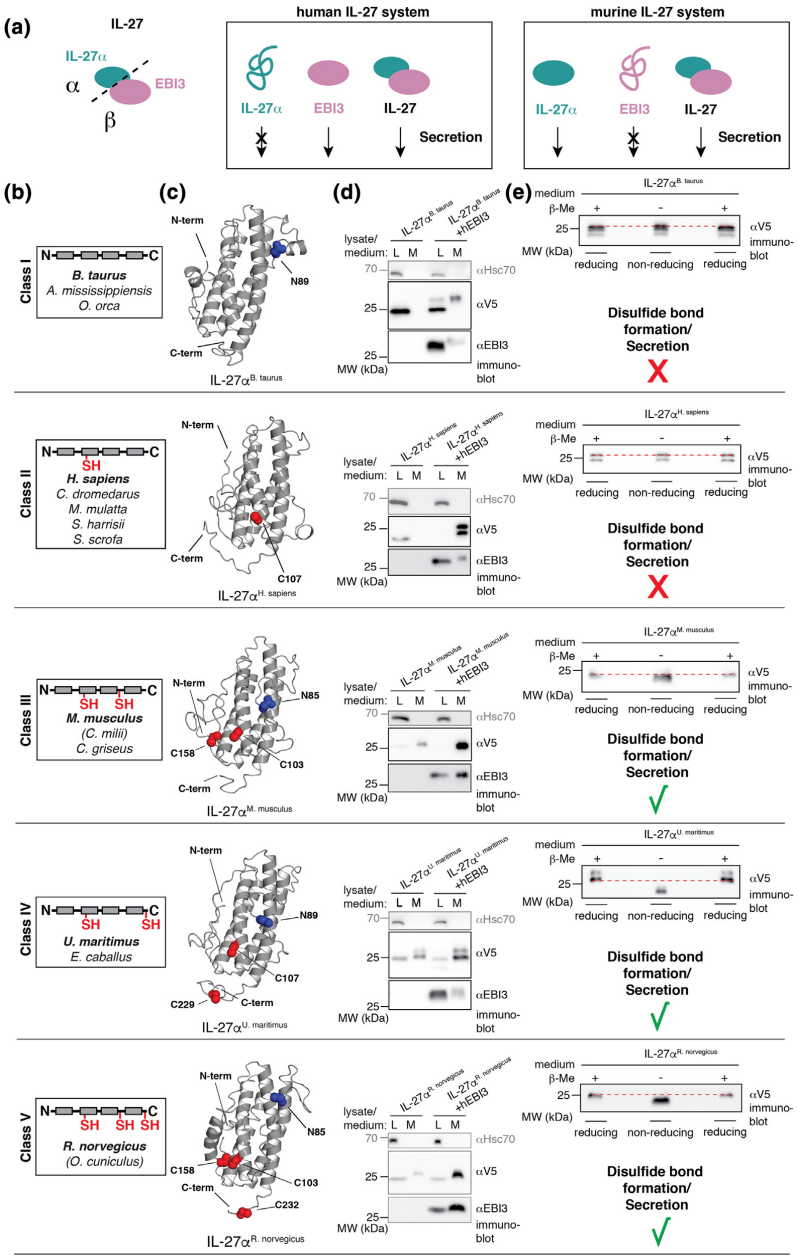

Fig. 1 (*legend on next page*)

EBI3 can be secreted in absence of human IL-27α [5] and has been proposed to be an immunomodulator of maternal tolerance during pregnancy [14]. Murine EBI3, in contrast, is retained in cells and cannot be secreted without its α subunit [5,15]. The molecular mechanisms underlying EBI3 retention *versus* secretion remain unclear. Furthermore, although immunological functions of IL-27 have been well characterized [8,9], its structure remains unresolved. While IL-27α is predicted to have the typical 4-helix bundle structure of type I cytokines [16], EBI3 is homologous to soluble members of the class I cytokine receptor family like IL-6Rα, consisting of two Fibronectin III domains connected by a hinge region [17].

To provide insights into general construction principles of the key cytokine IL-27, further define its structural setup and immunoregulatory capabilities, we pursued an approach combining evolutionary analyses with structural investigations. We define basic criteria of IL-27α and EBI3 secretion and show how the IL-27 system has evolved to maintain immune balance in various organisms.

Results

Disulfide bond formation is an evolutionary conserved determinant for IL-27α secretion

In all species examined, IL-27 is a heterodimeric cytokine composed of IL-27α and EBI3 (Fig. 1a). Its assembly control, however, varies in different organisms: Human IL-27α (hIL-27α) is retained in the cell and can only be secreted upon co-expression of its β subunit EBI3 [5]. Murine IL-27α (mIL-27α), in contrast, is secretion-competent on its own and acts as a cytokine [5,10–12] (Fig. 1a). Very recently, it has been shown that the folding- and secretion-competency of human and murine IL-27α can be changed by introducing or eliminating a single disulfide-bond forming Cys residue, respectively [13]. In order to reveal if disulfide bond formation is a more general, evolutionary conserved trait that defines autonomous IL-27α secretion, we performed a multiple sequence alignment of IL-27α from 15 different species, laying emphasis on the number and location of Cys in the sequences (Supplementary Fig. S1a). This alignment allowed us to group IL-27α into five different classes. These either contain no Cys (class I), a single Cys (class II), two Cys with the second Cys being located centrally (class III) or towards the C-terminus of the protein (class IV), or three Cys (class V) (Fig. 1b and Supplementary Fig. S1a). To structurally understand how the Cys residues are arranged within the proteins, we generated homology models for the various α subunits (Fig. 1c and Supplementary Fig. S1b). The modeled structures illustrate that, whenever present, the first Cys is always located in the second α-helix of IL-27α. The second Cys is located either in a characteristic poly-Glu loop of IL-27α [18] (class III and V) or towards the C-terminus of the protein (class IV), where also the third Cys in class V is located (Fig. 1b and c).

Based on this *in silico* analysis, we proceeded to test two hypotheses concerning IL-27α experimentally. First, that predicted proximity of two Cys in the modeled structure is sufficient for disulfide bond formation to occur. And second, that disulfide bond formation correlates with autonomous secretion of IL-27α. Towards this end, we analyzed the secretion behavior and disulfide bond formation of representatives from each class. In complete agreement with our hypotheses, we found that whenever none or a single Cys was present, IL-27α was dependent on assembly with EBI3 for secretion (classes I and II, Fig. 1b and d and Supplementary Fig. S1b and c). In agreement with this data, IL-27α of classes I and II did not form a disulfide bond (Fig. 1e and Supplementary Fig. S1d). The presence of two or more Cys, however, always

Fig. 1. Folding- and secretion-competency of IL-27α depend on disulfide bond formation. (a) Schematic of the heterodimeric IL-27, consisting of the non-covalently linked subunits IL-27α and EBI3. In humans (left box), IL-27α is retained in cells in isolation and depends on co-expression of EBI3, whereas in mice (right box), IL-27α is secretion-competent and is needed to induce EBI3 secretion. (b) Classification of IL-27α was performed according to the number and location of Cys residues. Species belonging to each class are listed – with the representative species selected for experiments in bold. The location of Cys (red) in helix 2 (box, gray), loop 3 (line, black) and/or the C-terminus (C) are depicted within a schematic structure of IL-27α. Parenthesized species share the same number of Cys but slightly differ in Cys location compared to the class consensus. (c) Homology models of IL-27α subunits from representative species of classes I-V. Within the 4-helix bundle structure, Cys residues (red) and predicted *N*-glycosylation sites (blue) are shown in a CPK representation. (d) Secretion-competency of IL-27α varies between classes. IL-27α of class I (no Cys) or class II (single Cys) is retained in cells in isolation (L), whereas co-expression with human EBI3 (hEBI3) induces its secretion (M). With two or three Cys (classes III, IV and V), IL-27α is secretion-competent even in absence of the β subunit. 2% L/M were applied to the gel and blotted with the indicated antibodies. Hsc70 served as a loading control. (e) Disulfide bond formation as a prerequisite for folding- and secretion-competency of IL-27α. Secretion-competent IL-27α (classes III-V) forms a disulfide bond. Secreted α subunits were analyzed by non-reducing SDS-PAGE and blotted with anti-V5 antibody. 1–2% medium (M) were applied to the gel and blotted with the indicated antibodies. Where indicated (+), samples were treated with β-mercaptoethanol (β-Me) to reduce disulfide bonds. To highlight mobility differences, dashed lines are shown. *N*-glycosylated IL-27α from *B. taurus*, *M. musculus* and *R. norvegicus* were deglycosylated with PNGase F prior to SDS-PAGE analysis. (d, e) L, lysate. M, medium. MW, molecular weight.

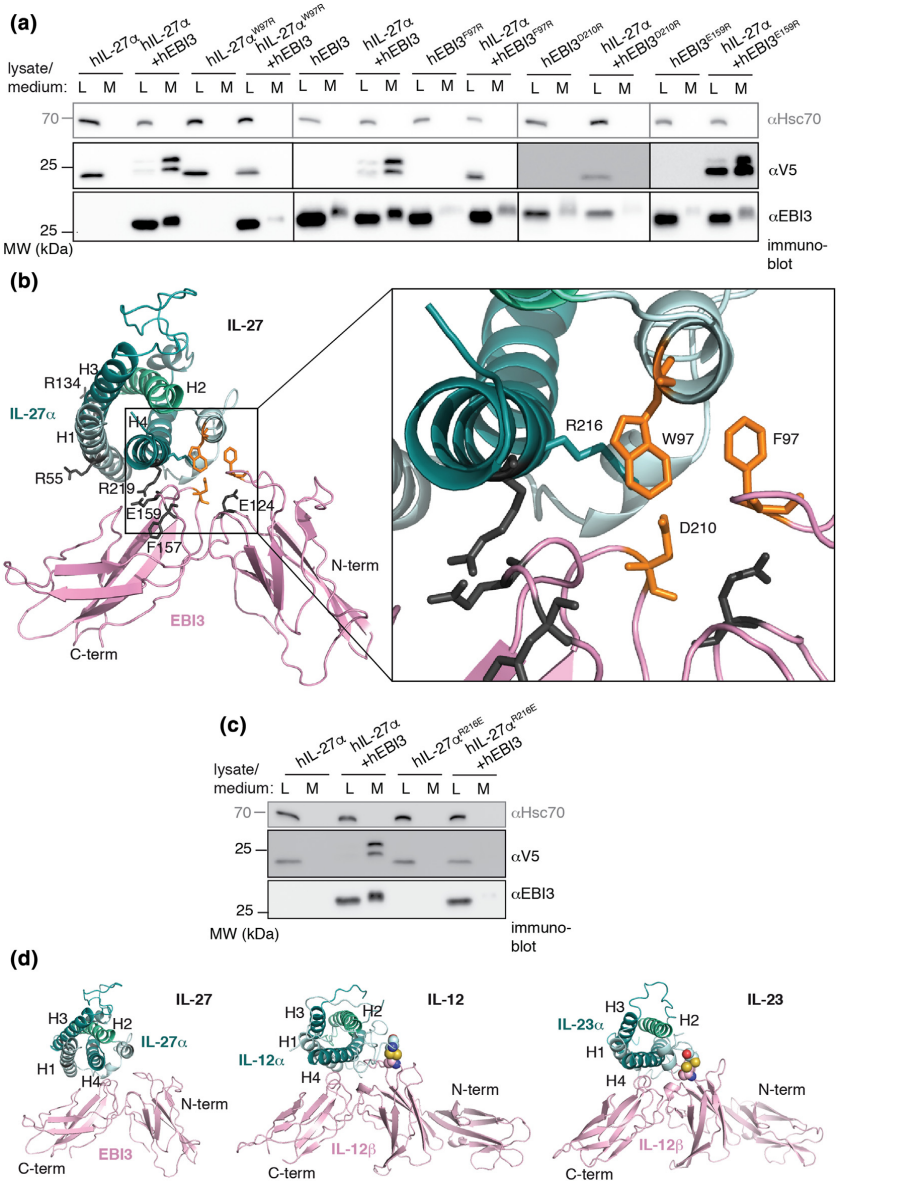

Fig. 2. A structural analysis of IL-27 heterodimerization. (a) hIL-27α^{W97R}, hEBI3^{F97R} and hEBI3^{D210R} but not hEBI3^{E159R} disrupt subunit interactions in IL-27. EBI3^{D210R} contained a C-terminal FLAG-tag. (b) Molecular docking of human IL-27 shows hIL-27α^{W97}, hEBI3^{F97} and hEBI3^{D210} to be located within the interface of the human α and β subunit. Helices 1–4 (H1–4) in IL-27α are indicated. (c) The hIL-27α^{R216E} mutation disrupts IL-27 formation. (d) Comparison of docked IL-27 with IL-12 and IL-23 crystal structures. Disulfide bonds in IL-12 and IL-23 are shown in a CPK representation. Helices 1–4 (H1–4) in the α subunits are indicated. (a, c) L, lysate. M, medium. MW, molecular weight. 2% L/M were applied to the gel and blotted with the indicated antibodies. Hsc70 served as a loading control.

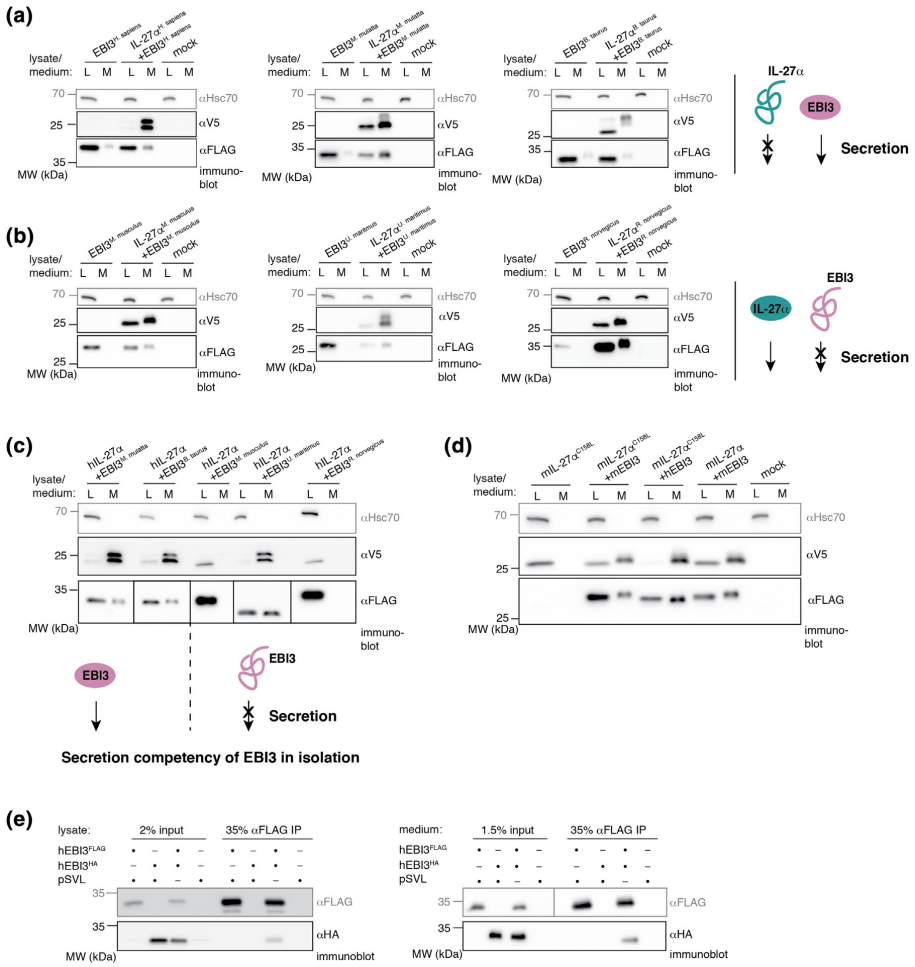

Fig. 3. An evolutionary conserved molecular construction principle of IL-27. (a,b) In all species tested, secretion-competent EBI3 subunits pair with secretion-incompetent IL-27α subunits to form IL-27 and *vice versa*. (a) EBI3 is secretion-competent (M) in isolation whenever its corresponding IL-27α is secretion-incompetent alone. (b) EBI3 from species with secretion-competent IL-27α is secretion-incompetent and thus retained in cells in isolation (L). Darker exposures are shown for EBI3 in Supplementary Fig. S3. (c) Secretion-incompetent hIL-27α was co-expressed with EBI3 from different species, being itself either secretion-competent or -incompetent as indicated. Secretion-incompetent EBI3 proteins were not able to induce hIL-27α secretion except for polar bear EBI3. (d) The secretion-incompetent mIL-27α^{C158L} mutant and mEBI3 mutually induce their secretion. (e) EBI3 forms homodimers. 35% lysate or medium of cells transfected with FLAG-tagged EBI3/mock and/or HA-tagged EBI3 was immunoprecipitated with anti-FLAG antibody, applied together with input samples to the gel and immunoblotted with the indicated antibodies (MW, molecular weight). (a-d) L, lysate. M, medium. MW, molecular weight. 2% L/M were applied to the gel and blotted with the indicated antibodies. Hsc70 served as a loading control.

rendered IL-27α secretion-competent in isolation (classes III, IV and V in Fig. 1b and d). Autonomous secretion of IL-27α was always concurrent with disulfide bond formation (Fig. 1e). In agreement with the structural models, a larger number of residues between Cys connected by an intramolecular disulfide bond resulted in a larger shift of IL-27α under non-reducing conditions on SDS-PAGE gels (*e.g.* mouse *versus* polar bear IL-27α in Fig. 1e). Furthermore, whenever *N*-linked glycosylation sites were predicted

(Fig. 1c and Supplementary Fig. S1a and b), modification of these sites upon secretion could be detected (Fig. 1d and Supplementary Fig. S1c and e). Remarkably, human EBI3 (hEBI3) was able to induce or increase secretion of IL-27α from all species tested (Fig. 1d and Supplementary Fig. S1c). Taken together, our data show that disulfide bond formation is an evolutionary conserved determinant for autonomous secretion and thus potential biological functions of IL-27α. Furthermore, our findings point towards a conserved mode of IL-27 heterodimerization that we next investigated further.

A structural analysis of the IL-27 interface reveals evolutionary conservation

Despite its important immunological functions, no experimentally determined structure is available for IL-27 yet. Thus, to provide further structural insights into IL-27 heterodimerization, we decided to use a mutational approach combined with docking and molecular dynamics (MD) simulations. A broad set of mutations was selected based on i) a previous structural analysis of IL-27 heterodimerization [16], and ii) the homology of IL-27 to CNTF, IL-12 and IL-23 [16,19–22]. This approach led to nine possible interface residues (Fig. 2a and b and Supplementary Fig. S2a), which we tested experimentally by single point mutations. To investigate the effect of the different mutations, we analyzed hEBI3-induced secretion of hIL-27α. Secretion of hIL-27α can be assessed *via O*-glycosylation, which occurs during Golgi passage: while hIL-27α populates a single species only detectable in the lysate (L, Fig. 2a), co-expression of hEBI3 induces secretion into the medium concomitant with the formation of different *O*-glycosylated species (M, Fig. 2a) [13]. Using this assay, we found that in agreement with previous data [16], hIL27a^{W97R}, hEBI3^{F97R} and hEBI3^{D210R} disrupted hIL-27α secretion (Fig. 2a). The hEBI3^{E159R} mutant, in contrast to what has been reported previously [16], did not inhibit hIL-27α secretion in our experiments (Fig. 2a). This is in agreement with studies on mouse EBI3 [23] and may be due to the different assays used: induction of hIL-27α secretion by EBI3 would also report on weak interactions, as opposed to co-immunoprecipitations [16]. All other five residues tested did not inhibit EBI3-induced secretion of hIL-27α (Supplementary Fig. S2b-d). Together, this provided us with a comprehensive set of disruptive and non-disruptive single point mutants, which we used to guide a docking approach combined with MD refinement simulations (Fig. 2b). In the obtained model, Trp97 of hIL-27α is located at the center of the interface and binds at the hinge region between the two domains of hEBI3. It contacts Phe97 and, in addition, several other residues in hEBI3 such as Leu96, Thr209 and Asp210. The model predicts a salt bridge contact between Arg216 in hIL-27α and Asp210 in hEBI3 as well as a stacking contact between Phe94 (hIL-27α) and Phe97 (hEBI3) that further stabilizes binding. In order to test our model experimentally, we substituted Arg216 in hIL-27α with a negatively charged Glu residue, which would interrupt the predicted interaction between hIL-27α Arg216 and hEBI3 Asp210 (Fig. 2b). In complete agreement with our docking, the mutant hIL-27α^{R216E} disrupted IL-27 formation (Fig. 2c). The model was also compatible with all residues found to be of lesser or no importance for binding on both partners (Supplementary Fig. S2a-d) since these residues are not at the interface, with the exception of Arg219 in hIL-27α. However, the guanidinium group of Arg219 does not form any specific contact, *e.g.* a salt bridge with the hEBI3 partner, compatible with the finding that substitution of this residue did not interfere with complex formation (Supplementary Fig. S2c, right panel). Importantly, all the IL-27α residues that form the predicted interface (defined as residues in contact with residues of the hEBI3 partner, *i.e.* a side chain atom-atom distance of <5 Å) are highly conserved among the IL-27α molecules from different species (Supplementary Fig. S2e). This is consistent with our finding that human EBI3 could induce secretion of IL-27α from all tested species (Fig. 1d). Interestingly, despite this fact, some differences appeared to exist in the molecular details of interactions: co-expression of hEBI3^{F97R} could not induce secretion of IL-27α from Tasmanian devil and monkey, like observed for human IL-27α (Fig. 2a and Supplementary Fig. S2f). IL-27α from cow and pig, however, were able to leave the cell in the presence of this mutant (Supplementary Fig. S2f). A comparison of IL-27α residues from these species, predicted to be located at the interface with hEBI3, did not indicate any difference (Supplementary Fig. S2e). Hence, the structural model suggests that the origin of the different behavior of IL-27α from Tasmanian devil and monkey *versus* cow and pig is not associated with residue differences at the subunit interface. It might thus be possible that hEBI3^{F97R} has generally lower affinity to (any) IL-27α and this weak subunit association is sufficient for IL-27α from pig and cow to fold correctly and assemble, whereas it is insufficient in case of IL-27α from Tasmanian devil and monkey.

Similar to the known structures of IL-12 [19] and IL-23 [20], helix 4 (H4) of hIL-27α contributed most to the interface with hEBI3 (Fig. 2d). Interestingly, despite this overall similarity, the arrangement of the hIL-27α subunit relative to the β subunit was slightly rotated and translated in the IL-27 model – which positions the residues found critical for IL-27 formation at the interface – compared to the corresponding placement in the IL-12 and IL-23 complexes (Fig. 2d). A global arrangement like in IL-12 and IL-23 would shift the critical Trp97 in IL-27α away from the interface. Note, that both in the IL-12 and IL-23 complex the arrangement of α and

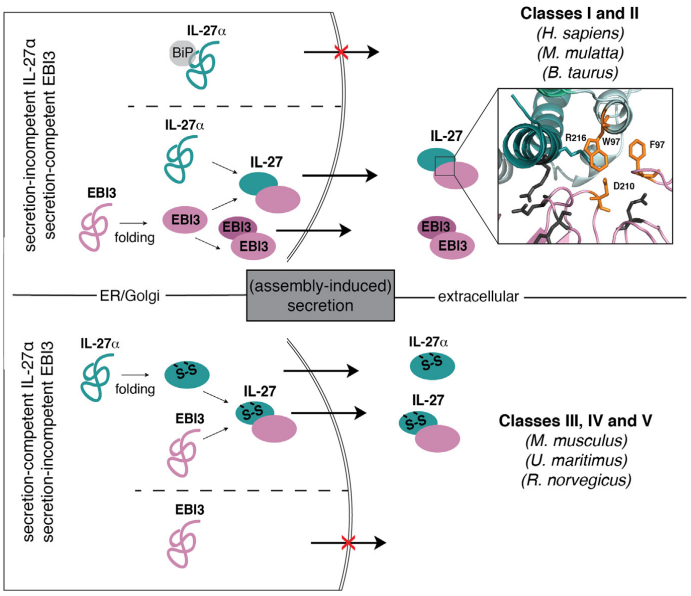

Fig. 4. A model for IL-27 biogenesis. Secretion-incompetent IL-27α (classes I and II) pairs with secretion-competent EBI3 and *vice versa* (classes III, IV and V). Species from which IL-27 was investigated in this study are shown in brackets. Disulfide bond formation (S-S) determines folding and secretion-competency of IL-27α subunits. IL-27α is retained in the cell by BiP binding if no disulfide bond is present [13]. EBI3 can form homodimers (shown for human EBI3 in this study). The inset highlights the heterodimerization interface of human IL-27 as defined in this study.

β partners is stabilized by an intermolecular disulfide bond (Fig. 2d) that is absent in IL-27 and may contribute to the slightly shifted arrangement. Taken together, our approach provides an improved structural model for IL-27 that is compatible with our comprehensive mutational analysis and reveals molecular details of αβ heterodimerization modes within the IL-12 family.

Reciprocally induced secretion of its constituent subunits is an evolutionary conserved trait of IL-27

Reminiscent of the IL-27α subunit, the IL-27β subunit EBI3 shows a different secretion behavior depending on the species. In humans, EBI3 can be secreted without co-expressing its corresponding IL-27α subunit, albeit weakly ([24] and Fig. 3a). In contrast, in mice, IL-27α is needed to induce EBI3 secretion ([5,15] and Fig. 3b). Interestingly, in both species, one secretion-competent subunit thus pairs with one secretion-incompetent subunit to form IL-27, only differing in the allocation of these traits to α or β subunit (depicted by the scheme in Fig. 3a and b on the right). Intrigued by this observation, we investigated if this construction principle was evolutionary conserved by testing the secretion-competency of the β subunit from species, where the α subunit is either secretion-incompetent (monkey and cow) or secretion-competent (polar bear and rat). Strikingly, EBI3 from monkey and cow were secreted into the medium without co-expression of IL-27α (Fig. 3a and Supplementary Fig. S3). Moreover, EBI3 from polar bear and rat were retained in cells in isolation and only secreted into the medium upon co-expression of their secretion-competent α subunits (Fig. 3b and Supplementary Fig. S3). Our data thus suggest that combination of a secretion-competent with a secretion-incompetent subunit is a common construction principle of IL-27. In the light of this finding, we wondered if only IL-27 subunits that co-evolved with assembly-dependent partner subunits were able to induce their secretion. To test this idea, we co-expressed human IL-27α with EBI3 from five different species and analyzed if the subunits were retained in or secreted by the cells (Fig. 3c). In complete agreement with this idea, hIL-27α was well-secreted upon co-expression of secretion-competent monkey and cow EBI3, but was retained in the cell when co-expressed with secretion-incompetent EBI3 from mouse and rat (Fig. 3c). The only exception from this rule was EBI3 from polar bear, which induced hIL-27α secretion comparable to secretion-competent EBI3 species. This pointed towards the interesting possibility that two secretion-incompetent subunits could in principle pair to become secreted together. To further test this, we examined if mEBI3 could induce secretion of the previously described mutant mIL-27α^{C158L}. This mutant is rendered secretion-incompetent in isolation by replacing Cys158 by Leu, removing its disulfide bond [13]. Indeed, co-expression with mEBI3 led to secretion of both subunits, mEBI3 and mIL-27α^{C158L} (Fig. 3d), constituting another

example of two secretion-incompetent subunits that can become secreted together. This finding furthermore underscores that mIL-27α^{C158L} together with mEBI3 recapitulates the behavior of the human system in regard to IL-27α, which qualifies it as a tool to further analyze the IL-27α and IL-27 biology in mouse models.

Lastly, we were wondering in which assembly state EBI3 would be secreted. This is of relevance, since generally two receptor chains are needed to initiate signaling by IL-12 family members [3]. Co-immunoprecipitation experiments of differentially tagged human EBI3 revealed the formation of homodimers in cells as well as in the medium (Fig. 3e), showing that EBI3 can be secreted as a homodimer. In this context it is noteworthy, that the shared IL-12/IL-23 β subunit (IL-12β) can also be secreted autonomously, including formation of a homodimer, and performs immunoregulatory functions [25,26].

Summary

In conclusion, our study reveals that disulfide bond formation is an evolutionary conserved determinant of IL-27α secretion and its potential role as a cytokine (Fig. 4). Furthermore, by an in-depth mutational and computational analysis, we were able to provide detailed insights into the molecular architecture of the IL-27 interface, which will also help in future engineering approaches. And lastly, our study reveals a hitherto unappreciated but intriguing setup of the IL-27 system, where one secretion-competent subunit pairs with one, that needs to assemble in order to be secreted. Our data show that this is not rooted in the biophysics of IL-27 subunit assembly, since we also could generate pairs of two assembly-incompetent subunits that mutually promoted their secretion. This suggests that the combinatorial principle realized for IL-27 has evolved for a functional benefit rather than due to structural requirements, *e.g.* to maintain a functional and balanced immune system. Using different protein assembly states with distinct functionalities appears to be a common design principle in immune systems to extend the signaling repertoire of cytokines and maintain immune homeostasis [12,13,25,27–29].

Materials and methods

Constructs for mammalian expression

For mammalian expression, all interleukin cDNAs were cloned into the pSVL vector (Amersham BioSciences). Human interleukin cDNAs (hIL-27α and hEBI3) were obtained from OriGene and amino acid sequences correspond to the UniProt accession numbers Q8NEV9 and Q14213, respectively. For a species comparison of IL-27α, sequences corresponding to GenBank identifiers NP_001158125.1 (*Bos taurus*), EHH31550.1 (*Macaca mulatta*), NP_663611.1 (*Mus musculus*), XP_344963.5 (*Rattus norvegicus*), XP_012398754.1 (*Sarcophilus harrisii*) and XP_008683452.1 (*Ursus maritimus*) were synthesized by GeneArt (Thermo Fisher Scientific) with optimized codon-usage for human expression. A $(GS)_4$-linker followed by a V5-tag was introduced at the C-terminus of the different IL-27α constructs. For a species comparison of EBI3, sequences corresponding to GenBank identifiers AAI49503.1 (*Bos taurus*), XP_014977995.1 (*Macaca mulatta*), NP_001102891.1 (*Rattus norvegicus*) and XP_008709480.1 (*Ursus maritimus*) were synthesized by GeneArt with optimized codon-usage for human expression (Thermo Fisher Scientific). Mouse EBI3 cDNA, according to GenBank identifier NM_015766.2 (*Mus musculus*), was obtained from OriGene. Where indicated, EBI3 genes were C-terminally tagged with a $(GS)_4$-linker followed by a FLAG- or an HA-tag. Mutants were generated by site-directed mutagenesis. All constructs were sequenced.

Cell culture and transient transfections

Human embryonic kidney (HEK) 293 T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-Ala-L-Gln (AQmedia, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS; Biochrom or Gibco, Thermo Fisher Scientific) at 37 °C and 5% CO_2. The medium was complemented with a 1% (v/v) antibiotic-antimycotic solution (25 µg/ml amphotericin B, 10 mg/ml streptomycin and 10,000 units of penicillin; Sigma-Aldrich) (complete DMEM). Transient transfections were carried out in in either poly D-lysine coated p35 or p60 dishes (BioCoat, Corning) or p60 dishes (Corning BioCoat) using GeneCellin (BioCellChallenge) according to the manufacturer's instructions. Equal amounts of constructs or empty vector were transfected with a total DNA amount of 2 µg (p35) or 4 µg (p60).

Secretion and redox experiments

For secretion and redox-status experiments by immunoblotting, cells were transfected for 8 h, washed twice with phosphate buffered saline (PBS; Sigma-Aldrich) and then supplemented with fresh medium for another 16 h. To analyze secreted proteins, the medium was centrifuged for 5 min, 300 *g*, 4 °C. Subsequently, the supernatant was transferred into a new reaction tube and supplemented with 0.1 volumes of 500 mM Tris/HCl, pH 7.5, 1.5 M NaCl, complemented with 10x Roche complete Protease Inhibitor w/o EDTA (Roche Diagnostics). Prior to lysis, cells were

washed twice in ice-cold PBS. Cell lysis was carried out in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1.0% Nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1% SDS, 1x Roche complete Protease Inhibitor w/o EDTA; Roche Diagnostics) on ice. Both lysate and medium samples were centrifuged for 15 min, 20,000 g, 4 °C. *N*-glycosylated IL-27α from *B. taurus*, *S. harrissii, M. musculus* and *R. norvegicus* were deglycosylated with PNGase F (SERVA) under non-reducing conditions according to the manufacturer's instructions prior to running redox gels to improve visibility of disulfide-induced down-shifts of proteins on SDS-polyacrylamide gel electrophoresis (PAGE) gels. Endo H (New England Biolabs) deglycosylation experiments were carried out according to the protocol of the manufacturer. Samples were supplemented with 0.2 volumes of 5x Laemmli buffer (0.3125 M Tris/HCl pH 6.8, 10% SDS, 50% glycerol, bromphenol blue) containing either 10% (v/v) β-mercaptoethanol (β-Me) for reducing SDS-PAGE or 100 mM *N*-Ethylmaleimide (NEM) for non-reducing SDS-PAGE.

Immunoprecipitation experiments

For co-immunoprecipitation (co-IP) experiments of FLAG-tagged proteins, cells were washed twice with ice-cold PBS. Cell lysis was carried out in Triton buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1x Roche complete Protease Inhibitor w/o EDTA; Roche Diagnostics) on ice. Lysates were cleared by centrifugation at 20,000 *g*, 15 min, 4 °C. ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich, A2220) was used for immunoprecipitation according to the manufacturer's protocol using Triton buffer for washes. Proteins were eluted by adding 2x Laemmli buffer (supplemented with 4% (v/v) β-Me) and boiling at 95 °C for 5 min. For immunoprecipitations of secreted proteins, the medium was treated as described for the analysis of secreted protein and subsequently treated like the lysate, using ANTI-FLAG M2 Affinity Gel.

Immunoblotting

For immunoblots, samples were run on 12% SDS-PAGE gels or 8–16% gradient gels (Bio-Rad, for redox experiments of IL-27α *M. musculus* and *R. norvegicus*) and transferred to polyvinylidene difluoride (PVDF) membranes by blotting overnight (o/n) at 30 V and 4 °C. Thereafter, membranes were blocked for at least 3 h with Tris-buffered saline (TBS) containing skim milk powder and Tween-20 (M-TBST; 25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5% *w/v* skim milk powder, 0.05% v/v Tween-20) or gelatin buffer (0.1% gelatin, 15 mM Tris/HCl, pH 7.5, 130 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.002% NaN_3) for Hsc70 immunoblots. Binding of the primary antibody was carried out o/n at 4 °C with anti-Hsc70 (Santa Cruz, sc-7298, 1:1000) in gelatin buffer, anti-V5 (Abcam, ab27671, 1:1000), anti-FLAG (Sigma-Aldrich, F7425, 1:1000) and anti-HA (BioLegend, 902,302, 1:1000) antibodies in M-TBST. Anti-EBI3 (1:20 in PBS) antiserum has been described previously [14]. Species-specific HRP-conjugated secondary antibodies (Santa Cruz Biotechnology; 1:10,000 in M-TBST) were used. Immunoblots were detected using Amersham ECL prime (GE Healthcare) and a Fusion Pulse 6 imager (Vilber Lourmat).

Sequence analysis, homology modeling and structural analyses

Multiple DNA sequence alignments were performed using Clustal Omega [30] and depicted with BoxShade server (EMBnet). Homology models of isolated IL-27α subunits were generated using iTasser [38]. For generating starting structures for protein–protein docking we followed the homology modeling procedure of Rousseau *et al.* [16]. It is based on a structure-based alignment of IL-27α and hEBI3 sequences to the IL-6 family of cytokines. Structural models of hIL-27α and hEBI3 were generated using the program Modeller [31] and are based on the alignment with known ciliary neurotrophic factor (CNTF) cytokine (pdb: 1cnt) and IL-6 (pdb: 1p9m) structures. For structure modeling the alignments as published by Rousseau *et al.* [16] were used. Generated models were energy minimized (5000 steps) using the Amber18 package [32]) to remove any residual sterical overlap. Docking searches were performed using the protein–protein docking program ATTRACT [33,34]. In ATTRACT, partner proteins are represented by a reduced protein model with four centers per residue [35] to allow rapid energy minimization of docked complexes. The initial search was restricted to starting placements in the vicinity of ~ 20 Å from the Trp97 residue on the hIL-27α side and to ~ 20 Å from Phe97, Asp210 on the hEBI3 partner protein. Substitution of any of these three residues was found experimentally to disrupt hIL-27α secretion induced by hEBI3. Hence, it is reasonable to assume that these residues are located at the protein–protein interface. Initial docking geometries were energy minimized and favorable docking solutions were screened for geometries with residues hIL-27α Trp97 and hEBI3 Phe97, Asp210 located at the interface and residues substitutions not affecting binding outside of the interface. The most favorable binding geometry was refined at atomic resolution after superimposing the atomistic model structures onto the docked model complex. Refinement was performed by energy minimization (5000 steps) followed by atomistic MD simulation (5 ns using the Amber18 package [32]) and first using an implicit (generalized Born) solvation model [36] for 5 ns at 300 K. A center of mass distance restraint between protein partners was included to avoid dissociation during the refinement step because of possible sterical overlap in the starting structure. For further relaxation of sterical strain the structure was

solvated in explicit TIP3P water [37] and equilibrated for another 10 ns at 300 K at constant pressure (1 bar) after heating up the system over a time frame of 1 ns. The resulting structure fulfilled the experimental restraints such that all residues critical for binding are at the interface and those substitution positions that were found experimentally not to affect binding are all located >6 Å (mostly >10 Å) from the binding interface. An exception is Arg219 in hIL-27α that is located at the rim of the protein–protein interface (Fig. 2b). However, the side chain of Arg219 is not forming specific (*e.g.* salt bridge) contacts to the hEBI3 partner. In the final model hIL-27α Trp97 is in contact with several aromatic and non-polar interface residues of hEBI3 (including hEBI3 Phe97). The Asp210 in hEBI3 forms a salt bridge with Arg216 of hIL-27α. Visualization and structural alignments and further structural analyses were performed using Yasara Structure (www.yasara.org) and PyMOL (PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

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Author contributions

MJF and SIM conceived the experimental part of the study, MZ conceived the docking part. All experiments were performed by SIM and IA. MZ performed docking simulations. All authors analyzed data and wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2019.04.032.

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BACK CLOSE WINDOW

3 Summary and outlook

The research presented within this thesis provides a comprehensive understanding of the molecular and cellular mechanisms of IL-27 biogenesis throughout evolution.

First, the structural basis of secretion incompetency *versus* competency of the IL-27α subunit was uncovered; a long-standing puzzle that finally has been solved. A single amino acid switch determines if IL-27α can form an intramolecular disulfide bond and consequently fold autonomously or if it is folding-dependent on its β subunit EBI3. Intramolecular disulfide bond formation in

IL-27α leads to reduced dynamics of two large loops and the formation of a hydrophobic cluster stabilizing the overall fold of the protein and qualifies it for secretion from the cell in absence of EBI3. Without the stabilizing disulfide bond, BiP binds to the exposed hydrophobic residues otherwise covered by cluster formation. The presence or absence of a single Cys residue at position 162 thus decides if human (h) IL-27α is routed for degradation and BiP binding or folded and secreted. This process can however not only be influenced by intramolecular disulfide bond formation but also by assembly with EBI3. It remains to be explored how EBI3 can induce conformational changes within hIL-27α and if these resemble the structural transformations prompted by the amino acid folding switch. An important step towards answering this question will be the resolution of the IL-27 structure.

Second, it was shown that hIL-27α^{L162C} is not only secretion- but also signaling-competent on human immune cells. This was determined by STAT activation in a number of cell lines and primary cells as well as by its ability to induce macrophages to secrete a certain subset of pro- and anti-inflammatory cytokines. This study thus showed for the first time that human IL-27α has immunological functions and added hIL-27α^{L162C} as a novel cytokine to the human cytokine repertoire. In mice, where IL-27α is endogenously present due to its inherent secretion competency, *inter alia*, a protective effect in infections has been shown.[81-83] Especially in the case of sepsis, where hyperreactivity of the immune system paired with subsequent immune paralysis often leads to organ damage, which can be fatal, the immunomodulatory functions of IL-27α^{L126C} might be of great therapeutic benefit.[81,167] Of note, human and murine (m) IL-27α differ in their glycosylation state, while hIL-27α consists of two *O*-glycosylated species, mIL-27α is *N*-glycosylated.[24] Possible functional roles of the different glycosylation species, especially in terms of *O*-glycosylation, which is characteristically heterogeneous, should be investigated in the future. To validate the therapeutic potential of hIL-27α^{L162C}, the protein

should be produced by a mammalian expression system so that glycosylation patterns can be reproduced, a purification strategy has to be established as well as first functional tests in a humanized sepsis mouse model have to be undertaken. If the therapeutic potential has been successfully established, hIL-27α^{L162C} would be a very promising candidate molecule for sepsis therapy and clinical studies. A patent describing hIL-27α^{L162C} has been submitted.[168]

This study showed that hIL-27α^{L162C} did not antagonize IL-27 signaling by receptor blockage but instead induced signaling *via* IL-27Rα and gp130. In contrast, Garbers *et al.* showed that mIL-27α can bind to gp130 homodimers *via* soluble or membrane bound IL-6Rα interaction, and this without any IL-6Rα help at high mIL-27α concentrations. However, that study excluded IL-27Rα involvement only at low mIL-27α concentrations, its involvement at high mIL-27α concentrations was not examined. There is thus no common consensus up to date about which receptor chains are recruited by IL-27α. In fact, Crabé *et al.* even proposed that IL-27α engages a tripartite receptor complex consisting of IL-27Rα, gp130 and IL-6Rα. An analogous receptor repertoire has been reported for the neuroprotective, mitochondrial 1-helix peptide Humanin signaling *via* IL-27Rα, gp130 and CNTF-Rα.[169] It would be hence of general interest to determine the complete receptor repertoire IL-27α is able to use. The implementation of methods that enable the investigation of protein interaction in life cells would be of great interest to answer this fundamental biological question.[166] It could be used to investigate which receptor chain combinations hIL-27α^{L162C} can recruit as dimers and if there is the requirement for a third receptor chain to induce receptor complex formation. This approach could then be complemented by STAT phosphorylation assays with immune cells expressing all three receptor chains to decipher the involvement of a third receptor chain in more detail.

This first part of the thesis explained the molecular basis of the longstanding observation that mIL-27α can be secreted without EBI3, while hIL-27α cannot. Most importantly, it contributed a novel human cytokine, the folding- and signaling-competent hIL-27α^{L162C}, to the ranks of possible immunotherapeutic agents and provides a more human-like IL-27α in mice, namely the secretion-incompetent mIL-27α^{C158L}.

In a broad interspecies analysis, this PhD thesis further investigated the evolutionary relevance of disulfide formation as an ERQC mechanism for IL-27α and the conservation of the construction principle of IL-27 being the pairing of one secretion-incompetent with one secretion-competent subunit. By comparing the amino acid sequence of IL-27α from 15 species, five classes could be defined, characterized by the number and position of Cys residues within these proteins. The analysis of the folding and secretion behavior of representatives from each

class revealed that the presence of two or more Cys residues in IL-27α consistently qualified it for autonomous folding, while one or none Cys led to assembly-dependent folding. This result raised the question if pairing of a secretion-competent with a secretion-incompetent subunit is a fundamental construction principle conserved in evolution. Indeed, the analysis of secretion competency of EBI3 from the investigated species upon co-expression with their cognate species α subunit revealed this composite nature to be common throughout evolution. Whenever the α subunit of IL-27 was secretion-incompetent, the cognate β subunit was secretion-competent and *vice versa*. When investigating if co-evolution with a secretion-incompetent subunit was the prerequisite for being able to induce the folding of a partner subunit, it was found that even though this being mostly the case, it was not mandatory. The combination of two secretion incompetent subunits led in two cases to the secretion of IL-27 as a heterodimer. These subunits were thus able to induce folding in each other, although none of them carried the trait to fold autonomously. This is indicative of IL-27 evolution being function-driven rather than by structural requirements. The composite nature of IL-27 throughout species can thus be considered as convergent evolution where the immune system of different species has developed divergent mechanisms to maintain a similar modular flexibility. Interestingly, hEBI3 was able to chaperone the secretion of all tested assembly-dependent IL-27α species arguing for a conserved IL-27 interface between species. This was further illustrated by computational modeling and docking of secretion-incompetent IL-27α subunits from different species with hEBI3, which showed that key interacting residues were conserved within the interface of these subunits.

Subsequently, the human IL-27 interface was refined by a mutagenesis-guided molecular docking. The result highlights that the same helix is involved in interaction with the β subunit in IL-27α as it is in IL-12α and IL-23α. However, the α subunit of IL-27 orientates differently towards its β subunit than do the α subunits of IL-12 and IL-23, which are connected to their β subunit *via* a disulfide bond. This results in the positioning of residues critical for IL-27 subunit interaction into the interface. Four residues that were identified by Rousseau *et al.* to lie within the IL-27 interface did not interrupt subunit interaction in our assay (*i.e.* IL-27α Arg55, Arg219 and EBI3 Glu124 and Glu159), whereas we could experimentally validate our newly generated docking model.[38] Moreover, also the mEBI3 Phe159 residue that was reported by the Vignali lab to interrupt mIL-27 formation did not interfere with subunit interaction in hIL-27 pointing towards certain differences in the hIL-27 interface as compared to mouse.[90] This refined, experimentally validated molecular docking broadens our understanding of IL-27 subunit

interaction and will contribute to the design of a more stable covalently linked IL-27 heterodimer.

The observed homodimer formation of secreted hEBI3 suggests that it can bind to receptor dimers as has been shown for IL-12β.[170,171] This finding is also relevant for the investigation of the unconventional receptor repertoire of IL-35 and might hint to the fact that IL-35 actually consists of a mixture of subunit homo- and heterodimers as well as eventually monomers.

While the ability of IL-27α to fold autonomously has been associated with the presence of an intramolecular disulfide bond, the molecular basis for EBI3 retention *versus* secretion remains obscure. Homodimer formation would be a possible QC mechanism that retains subunits not able to form homodimers and exposing hydrophobic stretches within the cell. This retention could be BiP mediated. Analysis of homodimer formation and BiP binding did however not reveal conclusive differences (data not shown). Human and murine EBI3 were both able to form homodimers. Moreover, they bound similarly well to BiP, which usually is a good indicator of the folding state of proteins.[172] Moreover, strong binding by the lectin chaperone system usually points towards proteins not being correctly folded. Intriguingly, the secretion-competent hEBI3 showed significantly stronger CRT binding than non-secreted mEBI3. Although this result might also be indicative of hEBI3 folding better and mEBI3 being more directly routed to ERAD with molecules not binding CRT at all, this topic needs further investigation. A comprehensive analysis of chaperone interaction with human *versus* murine EBI3 would be an interesting approach that might provide clues about how to change mEBI3 folding to being autonomous. This could be realized by *e.g.* direct sampling of possibly EBI3-interacting, ER resident chaperones, generally expressed by mammalian cells, through co-IP.[173] Grp94, for example, is a chaperone abundantly present in the ER for which only few substrates have been identified and which has been reported to interact with the lectin OS-9 together in ERAD.[124,126] Moreover, fibronectin III domains, of which EBI3 consists, have been reported to contain unpaired, free Cys residues, making the probing of mEBI3 with members of the PDI attractive.[165] Another possible approach would be affinity enrichment mass spectrometry of endogenously expressed chaperones interacting with EBI3.[174] Once interacting chaperones have been identified, their expression or binding sites could be altered to investigate their impact on EBI retention.[175] This could yield insights that might be used to influence biosynthesis of EBI3. Being able to rationally change folding of mIL-27α and mEBI3 would enable the design of a more human-like mouse model. This would be of paramount relevance for deciphering the respective effects of IL-27α, EBI3 and the IL-27 heterodimer in more detail.

Taken together this second part of the PhD thesis provided insights into basic molecular construction principles of IL-27 and laid the groundwork for the design of a disulfide-linked and therefore more stable IL-27 molecule. A covalently linked IL-27 will facilitate structure resolution noticeably. Additionally, it will contribute to the development of IL-27 as a therapeutic agent as low protein stability is a major limitation in using ILs as biopharmaceuticals.[176]

The structural insights this PhD thesis provided will also be of value for developing targeted, non-toxic therapies for IL-27. The following example of IL-12 shows how important these are. In 1995, clinical trials with IL-12 as an anti-cancer drug were stopped after the death of two patients. Constantly high IFNγ levels led to the severe toxicity of IL-12.[177] Targeted administration is especially important in the context of the IL-12 family, which shares subunits and receptor chains not only within the family, but also with the IL-6 family.

Fusion of cytokines to antibodies or antibody fragments would be an appropriate approach to target cytokines to restricted cell types thereby preventing undesirable side effects. There are some promising cancer drug candidates in preclinical and even clinical phases being tested at the moment among them two IL-12 immunocytokines in Phase I.[178] While no toxicity of IL-27 has been reported in mouse studies, its overexpression is associated with severe defects in blood cell formation and loss of Treg populations.[71,179,180] Studies using targeted administration of IL-27, *e.g.* adenovirus expressing IL-27 delivered to inflamed tissues in mice with collagen-induced arthritis, which is the animal model of autoimmune rheumatoid arthritis, reported, in contrast, beneficial effects, qualifying IL-27 as an interesting immunocytokine candidate.[181]

Another compelling approach for providing target specificity is to control receptor binding of ILs. Very recently, an IL-2/IL-15 mimic was designed *de novo* by a computational approach.[182] While receptor binding sites to IL-2Rβ and γ_c were maintained in the design, binding sites to IL-2Rα and IL-15Rα were not included as IL-2Rα deficient animal models showed a significant decrease in toxicity when rIL-2 was administered. A completely new topology was added around these binding regions. The resulting IL-2Rβ and γ_c selective, highly stable and non-immunogenic IL-2/IL-15 neoleukines prevented undesired effects on non-target cells and had in general better pharmaceutical properties than other mutants reducing IL-2Rα interaction.[183,184] A similar approach could be selected to design IL-27α and EBI3 molecules without binding sites for sIL-6Rα and IL-6, respectively, in order to prevent IL-12/IL-6 family cross reactivity. High EBI3 serum levels during the last months of pregnancy prompted, for example, the suggestion of its implication in the reported role of IL-6 in preterm delivery.[35,44,185]

Abrogating IL-6 interaction for EBI3, as well as sIL-6Rα interaction for IL-27α, could prove beneficial for promoting their immunosuppressive over their pro-inflammatory functions. The Humanin, a mitochondrial secretory peptide, could be an interesting starting point for the design of IL-12 family neoleukins, as it consists of only 24 amino acid residues in a single helix and is able to signal through a receptor complex of IL-27Rα, gp130 and CNTF-Rα.[169] Since crystal structures of IL-receptor complexes are, however, mandatory as starting point for this *de novo* approach and none is available for IL-12 family members, this will need further work before being applicable to the IL-12 family.

A different approach for influencing receptor binding is to introduce mutations into receptor binding sites that enable binding but no signaling or directed evolution of super-binder mutations increasing the potency of IL signaling. This has been done *e.g.* for IL-27 where Trp197 in IL-27α has been substituted with Ala which led to a non-signaling IL-27 mutant.[38] Super-binder engineering is also a common approach with *e.g.* hyper-IL6 consisting of IL-6 fused to sIL-6Rα or super-2, a version of IL-2 that has been evolved by mutation.[184,186] However, all of these variants harbor still the problem of off-target effects and thus toxicity, making newer approaches of immunocytokines and neoleukines more favorable.

Given the combinatorial organization of IL-12 family cytokines, the quest for novel functionalities is ongoing. The fact that IL-27α and EBI3 can pair with members of the IL-6 family, suggests that pairings between the remaining members of these families are also possible and hold the potential of therapeutic benefit. Revealing more IL-12/Il-6 family cross subunit interactions *in vivo* would also raise the question if IL-12 and IL-6 family members actually form one big IL-family. The pairing of cytokines naturally not forming a complex has however also been reported, *e.g.* colony stimulating factors and ILs or chemokines, called GIFTs.[187] Their administration had beneficial effects in autoimmune diseases and cancer.[188,189] These so called fusokines might be a potential route for IL-12/IL-6 family cytokines as well.

Since also immuno-receptor engineering has seen an intriguing development thanks to the combination of synthetic biology and genetic engineering, synthetic cytokine-receptor systems can be used to generate controllable cellular responses. Anti-GFP/mCherry nanobodies have been, for example, extracellularly fused to the IL-12 family IL-23R to generate a synthetic receptor, which can induce IL-23 signaling by GFP/mCherry binding.[190] This is of special interest in the context of the recently FDA approved chimeric antigen receptor (CAR) T cells for immunotherapy.[191] CAR T cells express receptors against antigens expressed by a specific type of cancer, which sets a hallmark strategy for developing specific immunotherapy regimens,

although severe side effects are still common. In combination with synthetic cytokine signaling systems, these T cells have the potential to be engineered to produce certain cytokines upon non-natural ligand binding which would inhibit background effects.[187]

The growing number of engineered cytokines under pre- and clinical examination illustrates the trend towards IL engineering.[176] Our completely new approach of engineering immune signaling molecules with novel functionalities by investigating basic cellular folding and QC mechanisms of IL-12 family cytokines adds a powerful method to the field of IL-engineering.

In toto, this PhD thesis provides a comprehensive picture of how cellular protein folding and QC in the ER can influence the functioning of the immune system. It illustrates how we can influence the immune system by changing these processes rationally. Hopefully, this knowledge will contribute to novel routes that will ultimately lead to non-toxic and effective immunotherapies against infectious diseases, autoimmune reactions and cancer.

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6 Appendix

6.1 Supplemental information for "A folding switch regulates interleukin 27 biogenesis and secretion of its α-subunit as a cytokine"

SI Appendix for:

A folding switch regulates interleukin 27 biogenesis and secretion of its α-subunit as a cytokine

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Supplementary Materials and Methods

Constructs for mammalian expression. Interleukin cDNAs were obtained from Origene (human IL-27α and human EBI3) or GeneArt (murine IL-27α) and cloned into the pSVL vector (Amersham) for mammalian expression. hIL-27α, mIL-27α and hEBI3 amino acid sequences correspond to the UniProt accession numbers Q8NEV9, Q8K3I6, and Q14213, respectively. For a species comparison of IL-27α, sequences corresponding to genbank identifiers EHH31550.1 (*Macaca mulatta*), NP_001007521.1 (*Sus scrofa*) and XP_003514905.1 (*Cricetulus griseus*) were synthesized by Geneart (Thermo Fisher Scientific) with optimized codon-usage for human expression. Where indicated, a V5-tag (preceded by a $(GS)_4$-linker) was introduced at the C-terminus of the different IL-27α constructs. Mutants were generated by site-directed mutagenesis. Constructs for BiP expression have been described previously (1). All constructs were sequenced.

Cell culture and transient transfections. HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing L-Ala-L-Gln (AQmedia, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Biochrom) at 37 °C and 5% CO_2. The medium was complemented with a 1% (v/v) antibiotic-antimycotic solution (25 µg/ml amphotericin B, 10 mg/ml streptomycin, and 10,000 units of penicillin; Sigma-Aldrich). Transient transfections were carried out for 24 h in either p35 (Becton Dickinson) or p60 (Corning BioCoat) poly D-lysine coated dishes using GeneCellin (BioCellChallenge) according to the manufacturer's instructions. Equal amounts of constructs or empty vector

were transfected with a total DNA amount of 2-3 μg (p35), 4-6 μg (p60). For BiP interaction studies, a 3:1 ratio of α subunit over chaperone DNA was used.

Secretion, redox and cycloheximide chase experiments. For secretion and redox-status experiments by immunoblotting, cells were transfected for 8 h, washed twice with PBS and then supplemented with 0.5 ml (p35) or 2 ml (p60) fresh medium for additional 16 h. For cycloheximide (CHX) (Sigma-Aldrich) chase experiments, cells were transfected for 24 h and subsequently treated with 50 μg/ml CHX for the indicated time periods before cell lysis. Where indicated, cells were pre-treated with 10 μM MG132 (Sigma-Aldrich) for 3 h before additional supplementation with CHX. Prior to lysis, cells were washed twice in ice cold PBS. Cell lysis was carried out in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1.0% Nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1% SDS, 1x Roche complete Protease Inhibitor w/o EDTA; Roche Diagnostics). To analyze secreted proteins, the medium was centrifuged for 5 min, 300 g, 4 °C. Subsequently, samples were supplemented with 0.1 volumes of 500 mM Tris/HCl, pH 7.5, 1.5 M NaCl and protease inhibitor and centrifuged for 15 min, 20,000 g, 4 °C. Samples were supplemented with 0.2 volumes of 5x Laemmli containing either β-mercaptoethanol for reducing SDS-PAGE or 100 mM NEM for non-reducing SDS-PAGE. EndoH / PNGaseF / O-glycosidase (New England Biolabs) deglycosylation experiments were carried out according to the protocols of the manufacturer.

Immunoblots and immunoprecipitation experiments. For immunoblots, samples were run on 12% SDS-PAGE gels (15% for redox experiments), transferred to PVDF membranes and blotted with anti-Hsc70 (Santa Cruz, sc-1059 or sc-7298, 1:1,000 in gelatin buffer (0.1% gelatin, 15 mM Tris/HCl, pH 7.5, 130 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.002% NaN_3)), anti-V5 (Thermo Fisher Scientific, MA5-15253, 1:500 or Abcam, ab27671, 1:1,000), or anti-hIL-27 antibodies (R&D Systems, AF2526, 1:200) in TBS, 0.05% Tween, 5% milk. Anti-EBI3 and anti-BiP antisera have been described previously (2, 3). Species-specific HRP-conjugated secondary antibodies (in TBS, 0.05% Tween, 5% milk or gelatin buffer) were used (Santa Cruz). Blots were detected using Amersham ECL prime (GE Healthcare) and a Fusion Pulse 6 imager (Vilber Lourmat). Immunoprecipitations of V5-tagged proteins were performed using the same antibodies as for immunoblotting. For co-immunoprecipitation of V5-tagged proteins, cells were washed with PBS, lysed with NP40 lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% NP40, 0.5% DOC, 1x Roche complete Protease Inhibitor w/o EDTA; Roche Diagnostics) and centrifugation-cleared lysates (15 min, 20,000 g, 4 °C) were incubated rotating o/n at 4 °C with 2 μg target-specific antibody. After rotation with 25 μl Protein A/G agarose (Santa Cruz) for 1 h at 4 °C, beads were washed three times with NP40-wash buffer (50 mM Tris/HCl pH 7.5, 400 mM NaCl, 0.5% NP40, 0.5% DOC) and proteins were eluted by boiling in 2x Laemmli buffer containing β-mercaptoethanol for reducing SDS-PAGE. For the co-immunoprecipitation of BiP, NP40 lysis buffer was supplemented with 10 U/ml apyrase (Sigma Aldrich) and 20 mM NEM. For immunoprecipitations of secreted proteins, the medium was treated as described for the analysis of secreted protein,

precleared for 1 h with 30 μl Protein A/G agarose (Santa Cruz) and subsequently treated like the lysate, using 2-2.5 μg of antibody.

Recombinant protein production. Human IL-27α cDNA optimized for expression in *E. coli* (without ER-import sequence) was obtained from GeneArt and cloned into the pET21a vector (Merck Millipore) with an N-terminal hexa-Histidine-tag and TEV protease cleavage site after the tag. The L162C mutation was introduced by site-directed mutagenesis. Proteins were expressed as inclusion bodies in selective LB medium. The culture was induced at OD_{600}=0.6 with 1 mM IPTG and harvested after another 4 h by centrifugation (5,000 rpm, 15 min, 4 °C). To isolate inclusion bodies, cells were lysed by sonication on ice in 100 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, SigmaFAST protease inhibitor and subsequently spun down (20,000 g, 20 min, 4 °C). The cell pellet was resuspended, washed twice with 100 mM Tris/HCl, pH 7.5, 500 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, and finally once with 100 mM Tris/HCl, pH 7.5, 100 mM NaCl. Inclusion bodies were solubilized in 50 mM sodium phosphate, pH 7.5, 250 mM NaCl, 6 M GdmCl and 10 mM β-mercaptoethanol at 4 °C. After o/n solubilization, the solution was cleared by centrifugation (20,000 g, 20 min, 20 °C). The supernatant was diluted with 1 volume of 50 mM sodium phosphate, pH 7.5, 250 mM NaCl, 5 M GdmCl and applied to Ni-Sepharose HP column (GE Healthcare). Bound protein was washed with 50 mM sodium phosphate, pH 7.5, 250 mM NaCl, 5 M GdmCl, 30 mM imidazole, 1 mM DTT and eluted with 50 mM sodium phosphate, pH 3.5, 250 mM NaCl, 5 M GdmCl and 1 mM DTT. Eluted protein was further purified and buffer exchanged into 50 mM MES pH 6.0, 6 M

urea, 1 mM EDTA by size exclusion chromatography using a HiPrep 16/60 Sephacryl S-400 HR column (GE Healthcare). Protein concentrations were determined spectrophotometrically using A_{280nm}. Human IL-27α^{L162C} cDNA optimized for expression in *H. sapiens* was obtained from GeneArt (Thermo Fisher Scientific) and cloned into the pHEK293 Ultra Expression Vector I (TaKaRa Clontech) for mammalian cell expression. Protein expression was carried out with the Expi293 expression system according to the manufacturer's protocol (Thermo Fisher Scientific). 48 h post-transfection, the medium was harvested by centrifugation (300 g, 15 min, 4 °C), concentrated to 2.5 μg/ml using Amicon Ultra-15, PLBC Ultracel-PL membrane, 3 kDa (Sigma-Aldrich) and used for immunological assays. hIL-27$\alpha^{L162C}$$His_6$ purified from inclusion bodies in *E. coli* was used as a reference to obtain a standard curve with linear fit for quantification of hIL-27α^{L162C} in Expi293 supernatants using immunoblot signals.

Sequence analysis and homology modeling. Multiple DNA sequence alignments were performed using Clustal Omega (4) and depicted with boxshade. iTasser (5) was used for homology modeling of human and murine IL-27α structures. Structural alignments and models of point mutants (which were also energy minimized) were generated using Yasara Structure (www.yasara.org).

Molecular dynamics simulations. Comparative Molecular Dynamics (MD) simulations were performed starting from the human wt hIL-27α and hIL-27α^{L162C} model structures, respectively, including a disulfide bond between L162C and C107 in the case of hIL-

27α^{L162C}. All MD simulations and the analysis of root-mean square deviation (RMSD) and fluctuations (RMSF) were performed using the Amber14 package (6) and the parm14SB force field (7). Proteins were first solvated in octahedral boxes including explicit Na^+ and Cl^- ions (~0.15 M) and explicit (TIP3P) water molecules (8) keeping a minimum distance of 10 Å between any protein atom and the box boundary. The simulation systems were first energy minimized (5,000 steps) followed by heating up to 300 K in steps of 100 K with position restraints on all non-hydrogen atoms of the proteins. Subsequently, positional restraints were gradually removed from an initial 12 kcal•mol^{-1}•Å^{-2} to 0.5 kcal•mol^{-1}•Å^{-2} within 0.5 ns followed by a 1 ns unrestrained equilibration phase at 310 K. All production simulations (200 ns) were performed at a temperature of 310 K and a pressure of 1 bar.

IL-27 cytokine assays. STAT experiments were performed using the human Burkitt lymphoma BL-2 cell line, human primary CD4+ T cells purified by negative magnetic cell isolation (purity >97%) or human primary monocytes purified by positive selection using CD14 beads (purity >95%) (Miltenyi Biotech) from PBMCs of adult donors (Etablissement Français du Sang, Paris, France). In some cases, total mouse spleen cells (C57BL/6) or purified murine splenic CD4+ T cells (obtained by electronic cell sorting using a FACSAria, BD Biosciences) were used. Murine IL-27 was obtained from eBioscience and murine IL-27α (produced in mammalian cells) from R&D Systems. BL-2 cells were starved overnight in serum-free RPMI-1640 medium before use. Cells were incubated in 48- or 24-well plates (1 to 2 x10^6 cells/well) in RPMI-1640 medium supplemented with

0.5 % BSA, for different times and with various concentrations of hIL-27α^{L162C} Expi293 supernatant or non-transfected control Expi293 supernatant. As a specificity control, an anti-IL-27 antibody (R&D Systems, AF2526; final concentration: 10 μg/mL) was added into the culture. In some cases, sIL-27R-Fc (R&D Systems) in parallel to control IgG1-Fc protein (Enzo Life Sciences), or neutralizing gp130 antibodies (B-T2, mouse IgG1, Diaclone) in parallel to control isotype antibodies (IgG1, Sigma) were used. In most competition experiments, cells were first pre-incubated for 2 h with Expi293 expressed hIL-27α^{L162C} or control Expi293 supernatant before activation with IL-27 (R&D Systems) or IL-6 (Immuno Tools) for 15 min. Alternatively, cells were stimulated with Expi293 expressed hIL-27α^{L162C} or control Expi293 supernatant and IL-27 simultaneously. The reaction was stopped by diluting cells in ice-cold PBS buffer and lysis in NP40 lysis buffer supplemented with protease and phosphatase inhibitors. Phosphorylated and total STAT proteins were detected by immunoblots using rabbit or mouse antibodies from Cell Signaling Technology (P-STAT1, #9167; P-STAT3, #9145; STAT1, #9172, STAT3, #9139).

Macrophage assays. THP-1 cells were grown in RPMI-1640 medium containing L-Ala-L-Gln (AQmedia, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Biochrom) at 37 °C and 5% CO_2. The medium was complemented with a 1% (v/v) antibiotic-antimycotic solution (25 μg/ml amphotericin B, 10 mg/ml streptomycin, and 10,000 units of penicillin; Sigma-Aldrich) (complete RPMI-1640). THP-1 cultures were kept below a density of $1x10^6$ cells/ml, seeded at a density of $2x10^5$ cells/ml in 0.5 ml and

differentiated to macrophages by continuous 48 h incubation with 25 nM PMA (9). Subsequently, the medium was exchanged against PMA-free complete RPMI-1640. After 24 h, the cells were pre-incubated for 2 h with 500 ng/ml of Expi293 expressed hIL-27α^{L162C}; 10 ng/ml hIL-27 (R&D systems, 219-IL-005); 500 ng/ml hIL-27α^{L162C} pre-mixed with 10 ng/ml hIL-27; control medium and then stimulated for another 4 h with 1 μg/ml LPS (Sigma-Aldrich, L4516) in the presence of hIL-27α^{L162C}, hIL-27, hIL-27α^{L162C} and hIL-27 or control medium. Cell supernatants were harvested by centrifugation (300 g, 15 min, 4 °C). Cytokine levels in THP-1 macrophage cell supernatants were determined using multiplex cytokine assays (Magnetic Luminex Assay, R&D Systems) for Eotaxin (CCL11), GROα (CXCL1), GROβ (CXCL2), IL-1β, IL-6, IL-10, IL-12 p70, IL-18, IL-33, IP-10 (CXCL10), ITAC-1 (CXCL11), MIG (CXCL9), RANTES (CCL5), TARC (CCL17) and TNFα according to the manufacturer's instructions on a Bio Plex 200 System (Bio-Rad). Duplicates of each biological replicate were measured and cytokine concentrations were determined using standard curves with five-parameters logistic fits.

Quantification and statistics. Western blots were quantified using the Bio-1D software (Vilber Lourmat). For CHX chase assays, signals were normalized to t=0 h. Western blot signals of co-immunoprecipitated BiP were normalized for BiP expression levels by dividing by the respective BiP input signals. BiP binding was determined by dividing the normalized BiP signal by the signal of immunoprecipitated α subunit (Fig. 3) or input signals of the α subunit (SI *Appendix*, Fig. S3). For the quantification of P-STAT immunoblots, western blot signals of non-treated cells were subtracted from signals of

treated cells, with negative values set to 0 and normalized to the IL-27 or IL-27α^{L162C} signal, as indicated. Molecular weights of 25 kDa (IL-27α) or 50 kDa (IL-27 heterodimer) were used for fold-calculations of induced signaling. Statistical analyses were performed using GraphPad Prism, version 6.0 (GraphPad Software). Where indicated, data were analyzed with two-tailed, unpaired Student's *t*-tests. Differences were considered statistically significant when $p<0.05$. For multiplex assays, significance was determined by ANOVA with Holm-Sidak's multiple comparison test, followed by Bonferroni correction and p-values <0.003 were considered statistically significant. Where no statistical data are shown, all experiments were performed at least three times, and one representative experiment was selected.

Supplementary References

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Supplementary Figures S1-S8

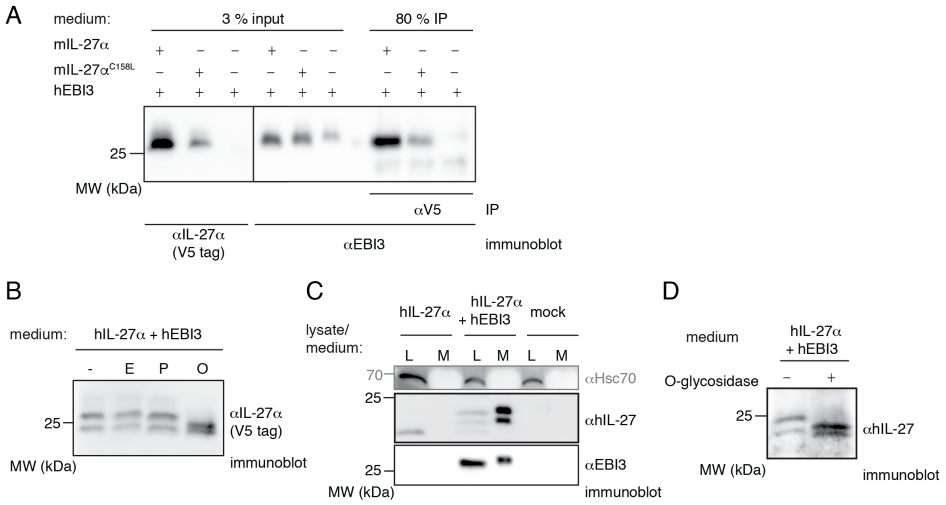

Fig. S1. Defining the glycosylation state and EBI3 interaction of IL-27α. (*A*) Secreted murine IL-27α and IL-27α^{C158L} both interact with human EBI3. 3% medium of cells transfected with V5-tagged mIL-27α, mIL-27α^{C158L} and/or hEBI3 as indicated were immunoblotted against V5 and EBI3 (inputs). 80% medium of cells transfected with mIL-27α, mIL-27α^{C158L} and/or hEBI3 were immunoprecipitated with α-V5 antibody (IP) and immunoblotted against EBI3. (*B*) O-glycosylation gives rise to different species of secreted V5-tagged hIL-27α. 1.6% or 3.2% (in case of EndoH) culture medium of cells co-transfected with hIL-27α and hEBI3 was treated (+) or not (-) with the N-glycosidases EndoH (E), PNGaseF (P) or an O-glycosidase (O) as indicated, and subsequently immunoblotted against V5. (*C*) Untagged hIL-27α depends on co-expression of its β subunit EBI3 for secretion. hIL-27α was transfected alone or co-expressed with the human IL-27β subunit EBI3 (hEBI3) in 293T cells as indicated (mock: empty pSVL vector).

2% lysate (L) or culture medium (M) were immunoblotted against Hsc70 as loading control, hIL-27α or hEBI3. Note that untagged hIL-27α as well as V5-tagged hIL-27α each populate three species (compare to Fig. 1*C* and SI Appendix, Fig. S1*B*), but these are shifted up for the V5-tagged hIL-27α since the linker+tag introduce an additional O-glycosylation site. (*D*) O-glycosylation gives rise to different species of secreted untagged hIL-27α. 1.2% medium of cells co-transfected with hIL-27α and hEBI3 was treated (+) or not (-) with O-glycosidase, and subsequently immunoblotted against hIL-27α.

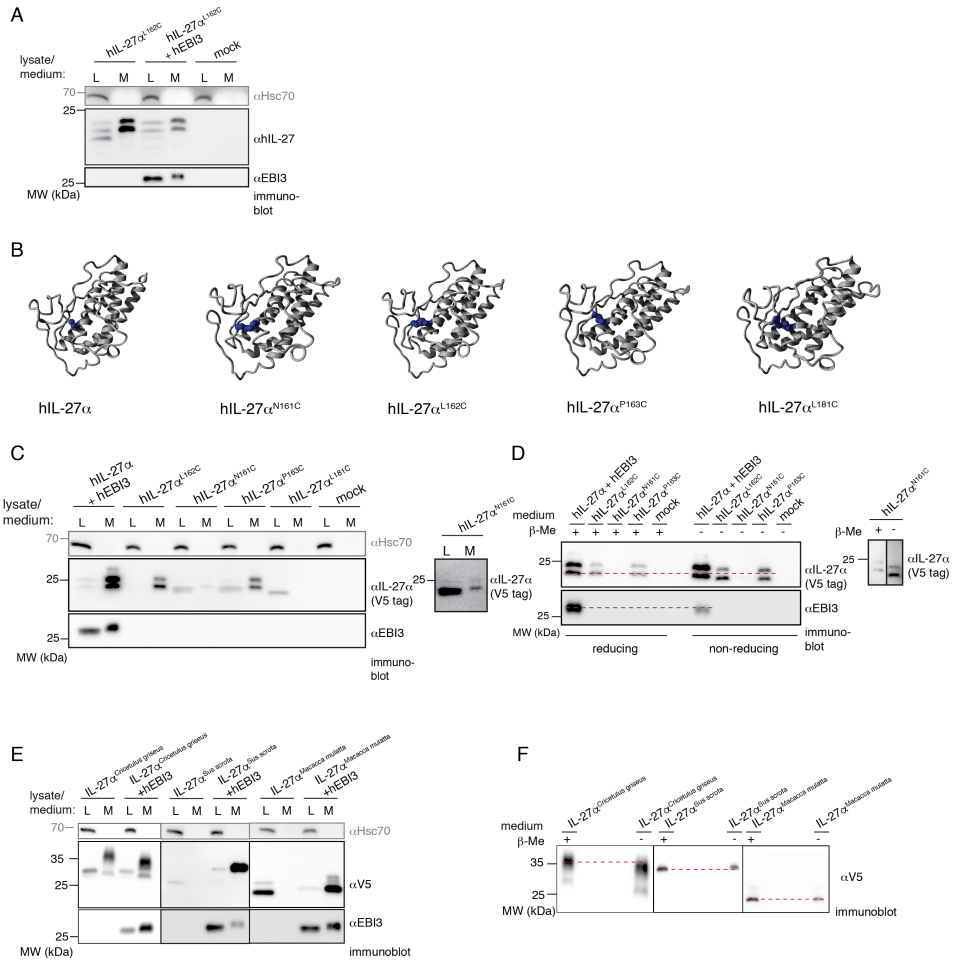

Fig. S2. Disulfide bond formation leads to secretion competency of IL-27α. (*A*) Untagged hIL-27α^{L162C} is secretion-competent in isolation. hIL-27α^{L162C} was transfected alone or co-expressed with hEBI3 in 293T cells as indicated (mock: empty pSVL vector). 2% lysate (L) or medium (M) were immunoblotted against Hsc70 as loading control, hIL-27α or hEBI3. (*B*) Homology models of other possible cysteine mutations rendering hIL-27α

secretion competent. The structural model of hIL-27α was generated by iTasser. Mutants (hIL-27α^{N161C}, hIL-27α^{L162C}, hIL-27α^{P163C} and hIL-27α^{L181C}) were generated with Yasara structure. A disulfide bond was introduced between the two cysteines *in silico* and structures were subsequently energy minimized using Yasara structure. Cysteines are highlighted in a blue CPK representation. (*C*) In addition to hIL-27α^{L162C}, hIL-27α^{N161C} and hIL-27α^{P163C} are secretion-competent in isolation. hIL-27α and hEBI3, hIL-27α^{N161C}, hIL-27α^{L162C}, hIL-27α^{P163C}, hIL-27α^{L181C} or empty pSVL vector (mock) were transfected in 293T cells and 2% medium (M) were immunoblotted against Hsc70, V5 and hEBI3. An image with increased contrast is shown on the side for hIL-27α^{N161C}. (*D*) In addition to hIL-27α^{L162C}, hIL-27α^{N161C} and hIL-27α^{P163C} form a disulfide bond. Secreted hIL-27α, hIL-27α^{N161C}, hIL-27α^{L162C} and hIL-27α^{P163C} were analyzed by non-reducing SDS-PAGE. 2% medium of cells transfected as depicted was immunoblotted against V5 and EBI3. Where indicated (+) samples were treated with β-mercaptoethanol. Dashed lines are shown to highlight mobility differences between reduced/non-reduced samples. An image with increased contrast is shown on the side for hIL-27α^{N161C}. (*E*) IL-27α from Chinese hamster (*Criteculus griseus*) is secretion competent, while IL-27α from pig (*Sus scrofa*) and monkey (*Macaca mulatta*) is not. IL-27α from the different species was either expressed in isolation or in the presence of human EBI3. 2% of lysate (L) or medium (M) of cells transfected with the indicated constructs was immunoblotted against Hsc70, V5 and EBI3. Glycosylation gives rise to the different mobilities observed for the IL-27α subunits derived from the different species. Due to the different expression levels of IL-27α from different species, the blot was separated into sections with optimal exposure

each. (*F*) Secretion competency of IL-27α correlates with disulfide bridge formation in different species. Secreted IL-27α from Chinese hamster, pig and monkey were analyzed by non-reducing SDS-PAGE. 2% medium of cells transfected as depicted was immunoblotted against V5 and EBI3. Where indicated, samples were treated with β-mercaptoethanol. Dashed lines are shown to highlight mobility differences between reduced/non-reduced samples.

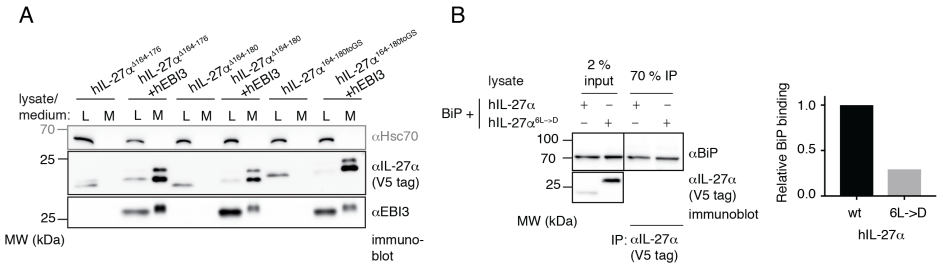

Fig. S3. Influence of the poly-glutamate loop on retention of hIL-27α and BiP binding of hIL-27α mutants**.** (*A*) The flexible poly-Glu loop does not cause ER retention of hIL-27α. Deletion mutants hIL-27α$^{\Delta 164\text{-}176}$ and hIL-27α$^{\Delta 164\text{-}180}$ as well as a replacement mutant hIL-27α$^{164\text{-}180toGS}$ (Glu residues replaced by a Gly-Ser sequence) are retained in the cell in isolation, whereas co-expression of EBI3 induces their secretion. hIL-27α$^{\Delta 164\text{-}176}$, hIL-27α$^{\Delta 164\text{-}180}$ or hIL-27α$^{164\text{-}180toGS}$ and EBI3 were co-transfected in cells as indicated and 2% of lysate (L) or medium (M) was immunoblotted against Hsc70, V5 and EBI3. (*B*) Lower BiP binding of hIL-27α$^{6L\rightarrow D}$ in comparison to hIL-27α^{wt}. 70% lysate of cells transfected with hIL-27α^{wt} or hIL-27α$^{6L\rightarrow D}$ and hamster BiP were immunoprecipitated with anti-V5 antibody and immunoblotted against BiP. Relative BiP binding was calculated using the signals of V5-inputs and co-immunoprecipitated BiP (normalized for BiP expression levels).

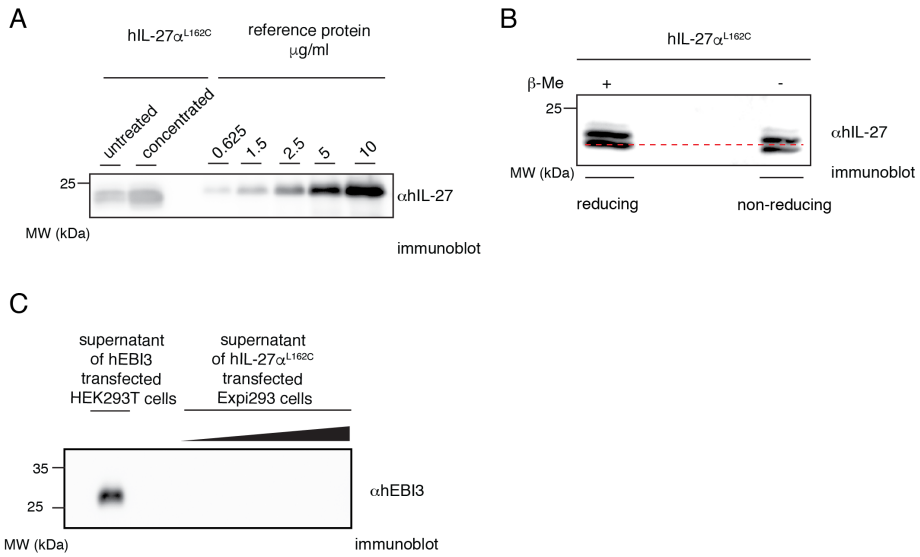

Fig. S4. Production of hIL-27α^{L162C} in mammalian cells. (*A*) Concentration determination of Expi293-secreted hIL-27α^{L162C} using hIL-27α^{L156C}His$_6$ from bacteria as a reference. Same volumes of cell supernatants of hIL-27α^{L162C} expressing Expi293 cells and hIL-27α^{L156C}His$_6$ standards with different concentrations were loaded onto SDS-PAGE gels and immunoblotted against IL-27α. The Expi293-expressed hIL-27α^{L162C} concentration was determined by comparing its immunoblot signal to a linear fit for the signals of the bacterially expressed hIL-27α^{L156C}His$_6$ standards. Where indicated, samples were further concentrated by ultrafiltration. (*B*) Expi293 expressed hIL-27α^{L162C} forms a disulfide bond. 0.03% cell supernatant of hIL-27α^{L162C}-transfected Expi293 cells was analyzed on SDS-PAGE gel under reducing and non-reducing conditions and immunoblotted against hIL-27α. Where indicated (+), samples were treated with β-mercaptoethanol. Dashed lines

are shown to highlight mobility differences between reduced/non-reduced samples. (*C*) Expi293 cells do not endogenously secrete hEBI3. 2% medium of HEK293T cells transfected with hIL-27α^{L162C} and hEBI3 as well as increasing amounts (0.005% to 0.04%) of cell supernatant of hIL-27α^{L162C}-transfected Expi293 cells were immunoblotted against hEBI3.

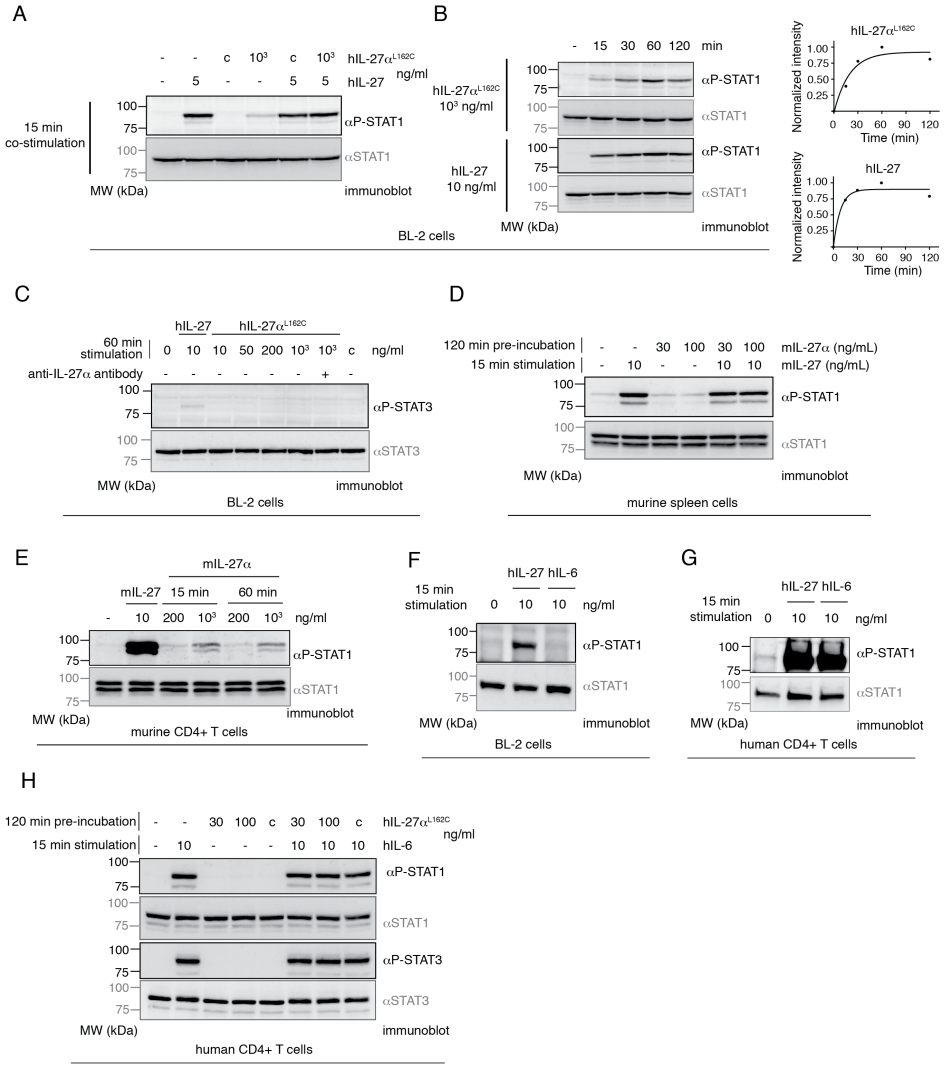

Fig. S5. Analysis of hIL-27α^{L162C} induced effects on immune cells. (*A*) hIL-27α^{L162C} does not inhibit IL-27 signaling upon co-incubation with hIL-27. BL-2 cells were co-stimulated with hIL-27α^{L162C} and hIL-27 for 15 minutes and STAT1 activation was assessed by

immunoblotting against phosphorylated STAT1 (c: control, cell supernatant of non-transfected cells). (*B*) hIL-27α^{L162C} shows slightly slower STAT1 phosphorylation kinetics than observed for hIL-27. BL-2 cells were incubated with hIL-27α^{L162C} or hIL-27 for increasing periods of time (the exposure time for hIL-27α^{L162C} was longer). Quantifications of the phosphorylated STAT1 intensities are shown on the right with a single exponential fit. (*C*) hIL-27α^{L162C} does not induce STAT3 phosphorylation in BL-2 cells. (*D*) Murine IL-27 signaling is not blocked by murine IL-27α. Murine spleen cells were pre-incubated with or without mIL-27α for 2 hours and subsequently stimulated with mIL-27 for 15 minutes. STAT1 phosphorylation was assessed by immunoblotting. (*E*) mIL-27α induces STAT1 signaling. Murine CD4+ T cells were incubated with or without mIL-27α with the indicated concentration for the indicated time periods. STAT1 phosphorylation was assessed by immunoblotting. (*F*) Stimulation of BL-2 cells with IL-6 reveals lack of an IL-6 receptor. (*G*) Human CD4+ T cells express a functional IL-6 receptor. ((*F*), (*G*): BL-2 or CD4+ T cells were incubated either with hIL-27 or hIL-6 and STAT1 phosphorylation was assayed by immunoblotting.) (*H*) hIL-6 signaling is not inhibited by hIL-27α^{L162C}. Human CD4+ T cells were pre-incubated with or without control medium/hIL-27α^{L162C} for 2 hours and then stimulated for 15 min with hIL-6. Concentrations were as indicated. STAT1 and STAT3 activation were assessed by immunoblotting against phosphorylated STAT1 or STAT3, respectively (c: control, cell supernatant of non-transfected cells).

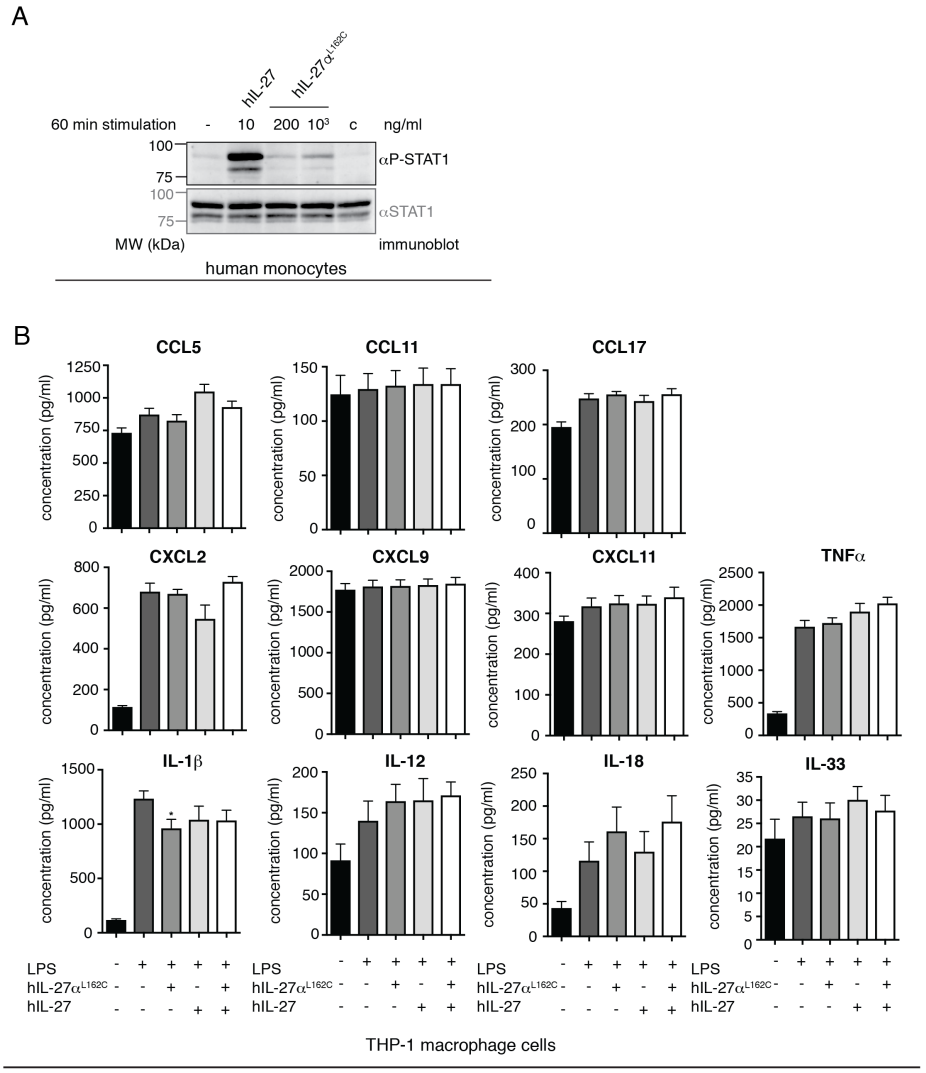

Fig. S6. Analysis of hIL-27α^{L162C} induced effects on primary human monocytes and LPS-stimulated THP-1 cells. (*A*) hIL-27α^{L162C} induces STAT1 phosphorylation in primary human monocytes. Human monocytes were incubated for 60 min with the indicated

concentrations of hIL-27, hIL-27α^{L162C} or control supernatant (c) and STAT1 phosphorylation was assessed by immunoblotting. (*B*) hIL-27α^{L162C} suppresses the secretion of IL-1β but has no significant effect on the secretion of CCL5, CCL11, CCL17, CXCL2, CXCL9, CXCL11, TNFα, IL-12, IL-18 or IL33 from LPS-stimulated THP-1 macrophages (N=11±SEM, *p<0.003, ANOVA). THP-1 macrophages were stimulated for 2 hours with 0.5 μg/ml hIL-27α^{L162C} or/and 10 ng/ml hIL-27. Subsequently, 1 μg/ml LPS was added for an additional incubation time of 4 hours in the presence of hIL-27α^{L162C} or/and hIL-27. THP-1 macrophage supernatants were analyzed with cytokine multiplex assays.

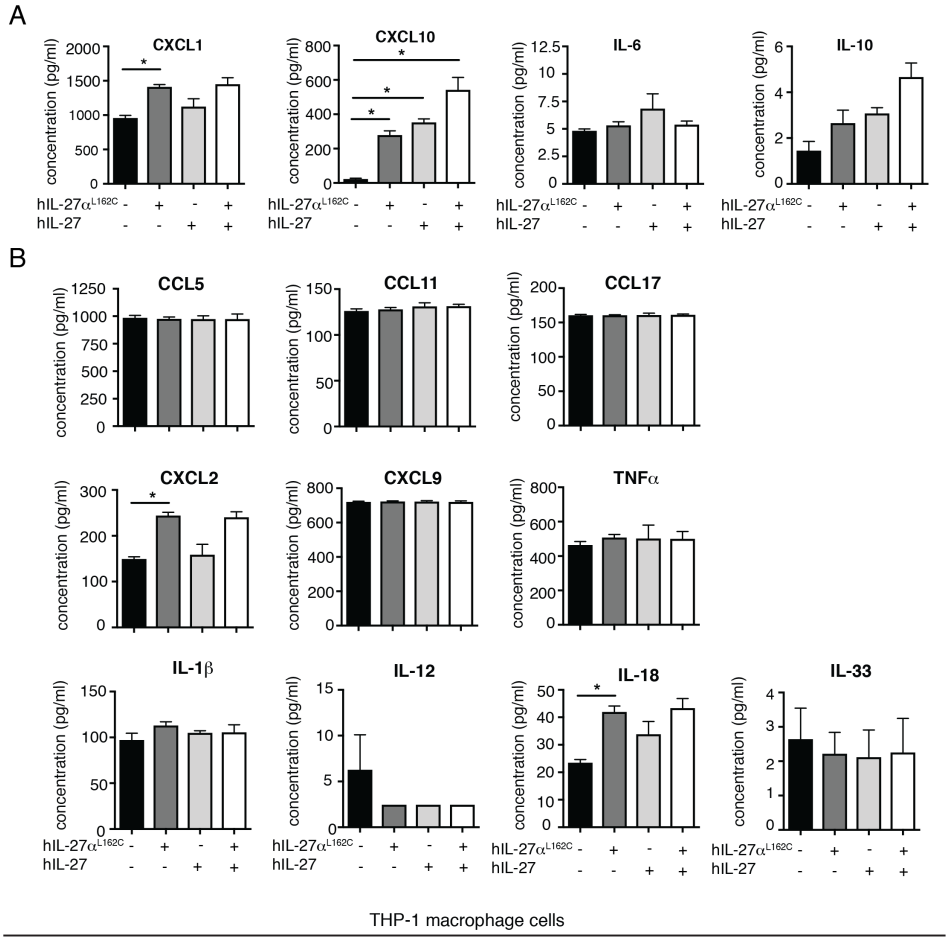

Fig. S7. Analysis of hIL-27α^{L162C} induced effects on non-LPS-stimulated THP-1 cells. THP-1 macrophages were stimulated for 6 hours with 0.5 μg/ml hIL-27α^{L162C} or/and 10 ng/ml hIL-27. THP-1 macrophage supernatants were analyzed with cytokine multiplex assays (N=4±SEM, *p<0.003, ANOVA). (*A*, *B*): sorted as shown in the main Figure 4, Figure S6.

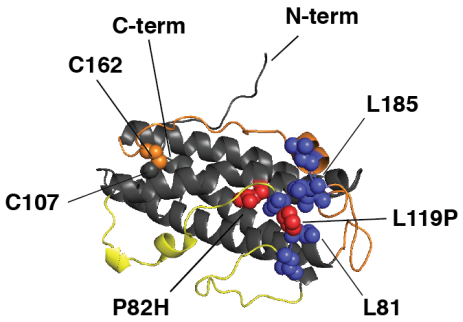

Fig. S8. Polymorphisms in human IL-27α affect potentially folding-relevant sites. Reported polymorphisms for hIL-27α^{wt} that lie in the hydrophobic cluster of hIL-27α^{L162C} and may perturb folding of hIL-27^{wt}. Structural model of hIL-27α^{L162C} with potentially folding relevant polymorphisms shown in a red CPK representation. Residues of the hydrophobic cluster are shown in blue. The poly-Glu loop and loop1 are highlighted in orange and yellow, respectively.

6.2 Supplemental information for "An interspecies analysis reveals construction principles of interleukin 27"

An Interspecies Analysis Reveals Molecular Construction Principles of Interleukin 27

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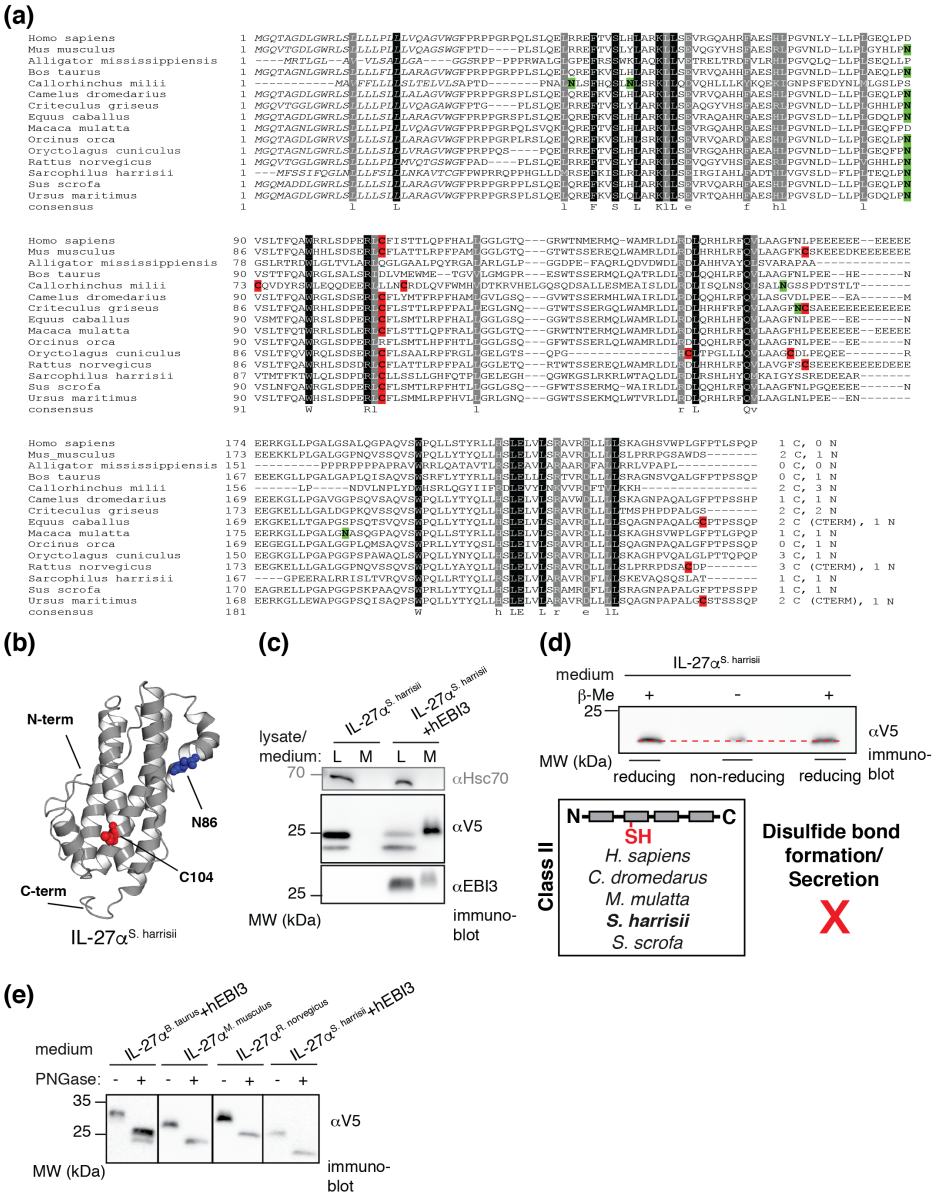
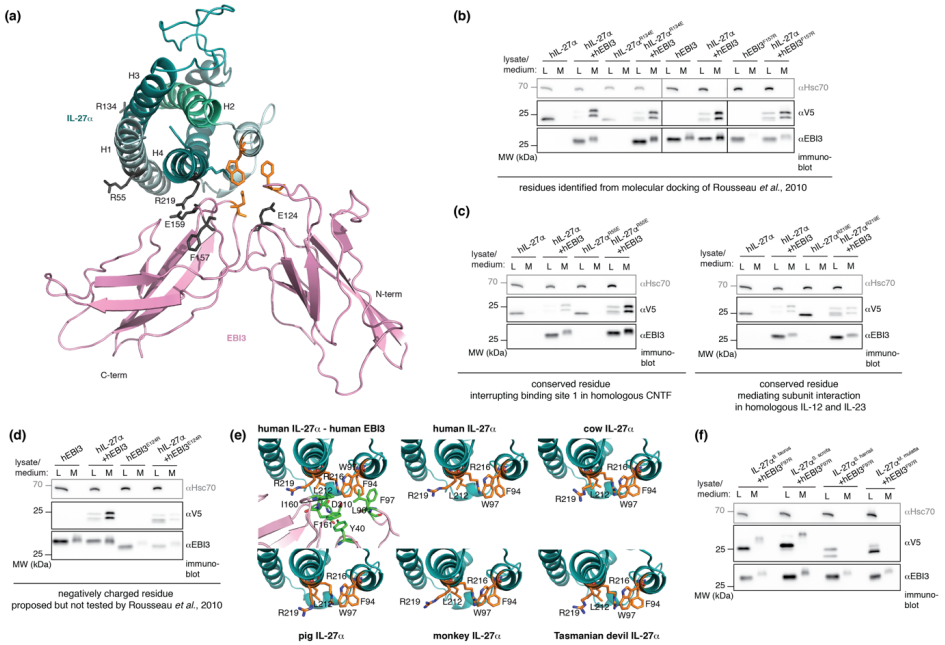
Supplementary Material



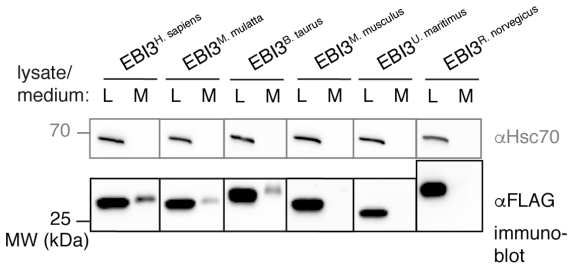
Supplementary Fig. S1 Species comparison as a basis for an IL-27α classification based on number and location of Cys residues. (a) An IL-27α sequence alignment shows potentially folding-relevant differences in the amino acid sequences from 15 different species as cause of distinct secretion behavior. Identical residues are shaded in black, homologous amino acids in gray, *N*-glycosylation sites in green and Cys

residues in red. ER import sequences are shown in italic. Numbers of Cys residues and predicted *N*-glycosylation sites (N) are listed at the end of each sequence. (b) Homology model of *Sarcophilus harrisii* IL-27α as a further representative of class II. (c) *S. harrisii* IL-27α is not secretion-competent in isolation (L, lysate) but becomes secreted upon co-expression of hEBI3 (M, medium). 2% L/M was applied to the gel and blotted with the indicated antibodies. Hsc70 served as a loading control. (d) Absence of disulfide bond formation due to a single Cys residue within *S. harrissii* IL-27α. 2% deglycosylated medium was analyzed by non-reducing SDS-PAGE like in Fig. 1c. No mobility shift was visible, indicating absence of a disulfide bond (class II). (e) Deglycosylation control of PNGase F-treated IL-27α species. For improved mobility shift visualization on redox blots (classes III-V), *N*-glycosylated IL-27α medium samples of the depicted species were previously deglycosylated with PNGase F (indicated with (+)). 2% medium was applied to the gel and blotted with anti-V5-antibody. (c-e), MW, molecular weight.



Supplementary Fig. S2 Test of possible interface residues for disruption of human IL-27 subunit interaction. (a) Molecular docking of IL-27 showing residues identified as non-essential for subunit interactions in gray. (b) Secretion behavior of IL-27 subunit mutants identified by a molecular docking from Rousseau *et al.* [16]. All mutants shown did not disrupt IL-27 formation, as hIL-27α mutants became secreted upon co-expression of hEBI3. Likewise, shown hEBI3 mutants did not prevent hIL-27α secretion. (c) hIL-27α^{R55E} (Arg25 in CNTF, which disrupts formation of CNTF/CNTFRα heterodimers [22]) is secreted into the medium upon co-expression of hEBI3. The same applies to hIL-27α^{R219E} (Arg189 in IL-12α and Arg159 in IL-23α, which disrupt formation of IL-12 and IL-23 heterodimers, respectively [16]). (d) Mutation of hEBI3 Glu124, a residue proposed but not tested by Rousseau *et al.* [16], did not prevent secretion of hIL-27α into the medium. (e) Interface residues of hIL-27α-hEBI3, hIL-27α and IL-27α from cow, pig, monkey and Tasmanian devil. (f) hEBI3^{F97R} shows differences in promoting secretion of IL-27α subunits from the species shown. (b-d, f)

L, lysate. M, medium. MW, molecular weight. 2% L/M were applied to the gel and blotted with the indicated antibodies. Hsc70 served as a loading control.



Supplementary Fig. S3 Overexposure of immunoblots for EBI3 secretion. Depending on the species, EBI3 was secreted (M, medium) or retained in cells in isolation (L, lysate). 2% L/M were applied to the gel and blotted with anti-FLAG antibody. Hsc70 served as a loading control. MW, molecular weight.