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

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Edited by John J. O'Shea, NIH, Bethesda, MD, and accepted by Editorial Board Member Tadatsugu Taniguchi December 10, 2018 (received for review October 3, 2018)

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.

This article is a PNAS Direct Submission. J.J.O. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1816698116/-/DCSupplemental.

Published online January 16, 2019.

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.

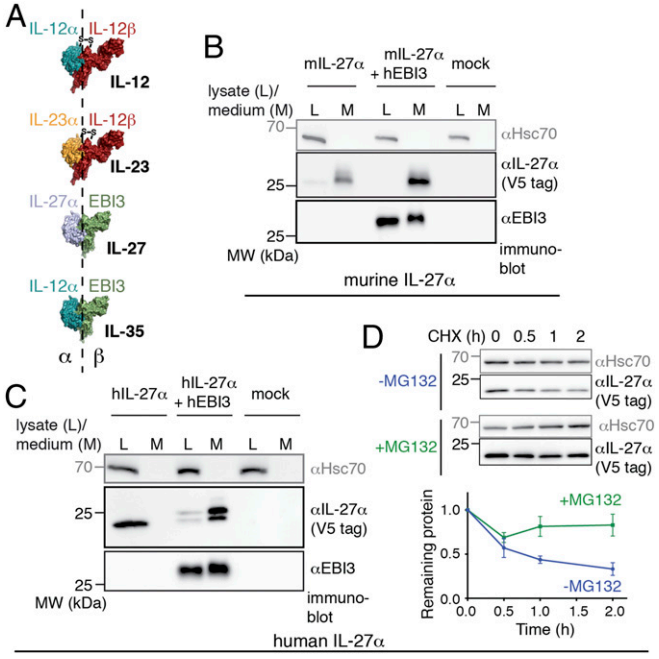

Fig. 1. Murine IL-27α is secreted, whereas its human ortholog is degraded via ERAD. (*A*) Schematic of the IL-12 family. Each family member is an αβ heterodimer. IL-12 and IL-23 are covalent dimers, connected by a disulfide bond (S-S), whereas IL-27 and IL-35 are noncovalent dimers. (*B*) Mouse IL-27α (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).

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

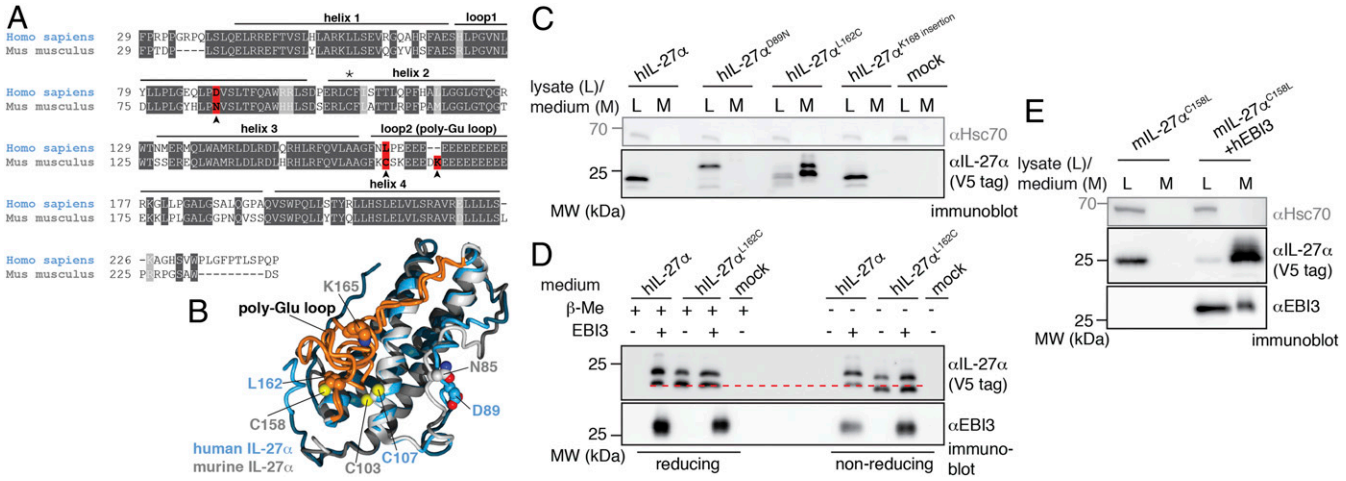

Fig. 2. A single amino acid switch regulates IL-27α secretion. (*A*) Potentially folding-relevant differences in the amino acid sequence of hIL-27α and mIL-27α (without ER import sequences). Identical residues are shaded in dark gray, homologous residues in light gray, and differences with a potential 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

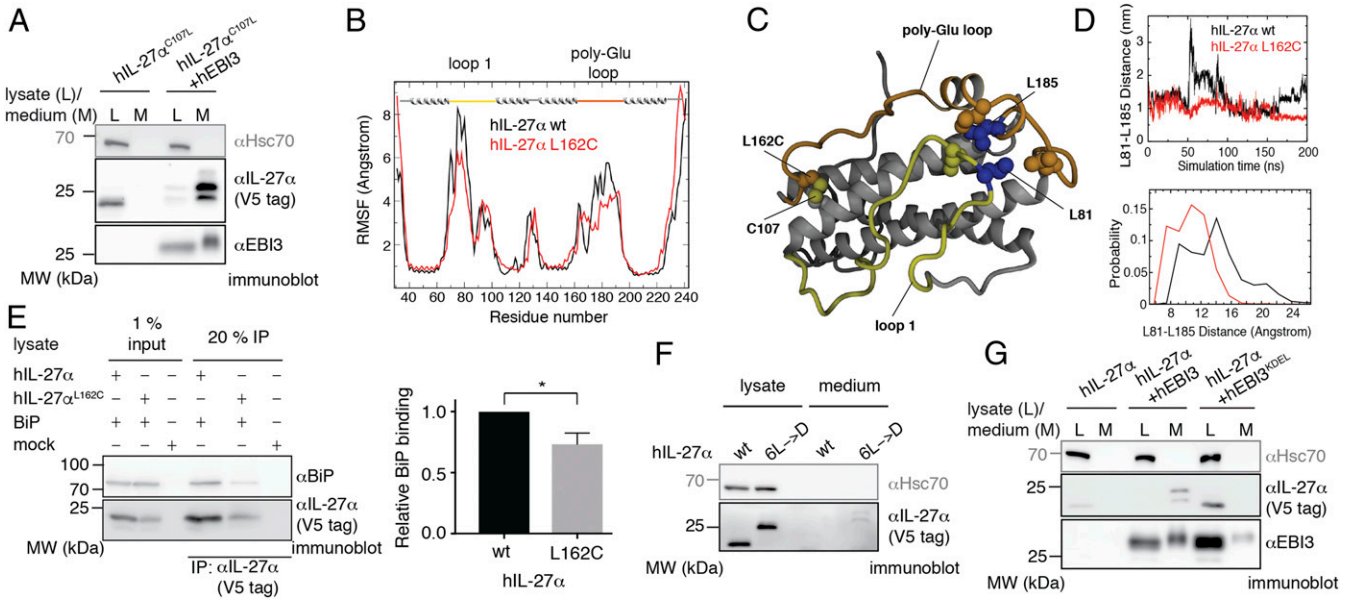

Fig. 3. Molecular determinants of IL-27α retention versus secretion. (*A*) The single free Cys of hIL-27α does not lead to its ER retention: hIL-27α^{C107L} is retained in the cell in isolation and secreted upon coexpression of EBI3. (*B*) Molecular dynamics simulations reveal locally reduced flexibility 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

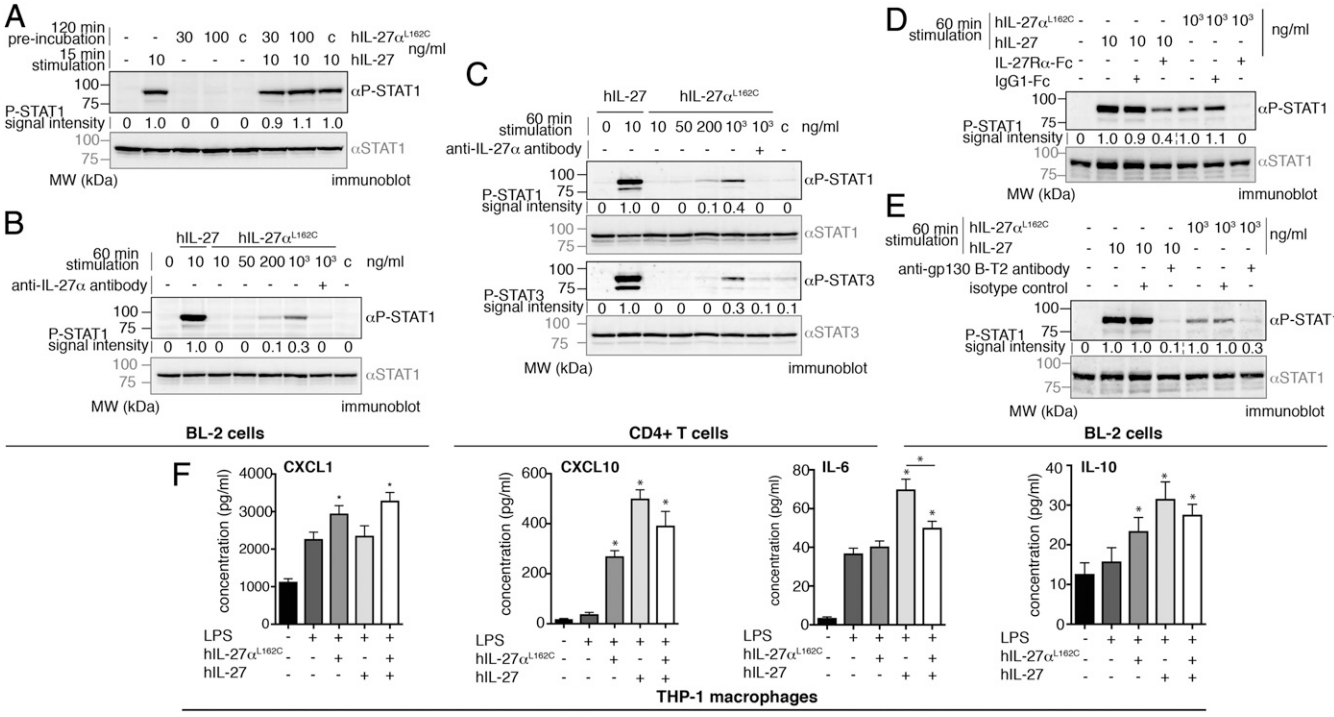

Fig. 4. Human IL-27α^{L162C} is biologically active. (*A*) hIL-27α^{L162C} does not antagonize IL-27–induced STAT1 phosphorylation. BL-2 cells were preincubated with hIL-27α^{L162C} and then stimulated with hIL-27 (c, control supernatant of nontransfected cells). (*B*) Human IL-27α^{L162C} induces STAT1 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.

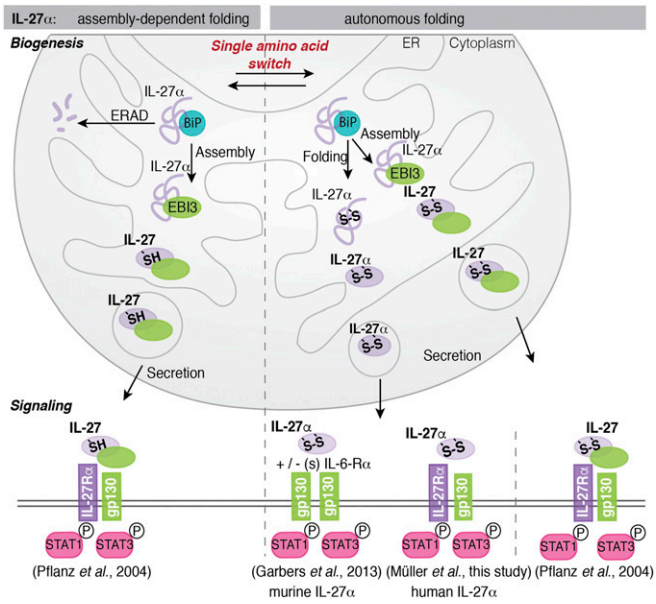

Fig. 5. A single amino acid protein folding switch underlies differences in the IL-27 system. The absence or presence of a disulfide bond-forming Cys pair defines if IL-27α depends on EBI3 interaction for folding and secretion or if it can be secreted autonomously, inducing downstream signaling. Signaling-competent 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*.

ACKNOWLEDGMENTS. We thank Julia Behnke and Johannes Buchner [Technical University of Munich (TUM)] and Linda Hendershot (St. Jude Children's Research Hospital) for helpful comments on the manuscript; Linda Hendershot for kindly providing the BiP expression plasmid and BiP antiserum; Diakho Kebe (INSERM) for the isolation of peripheral blood mononuclear cells; Jordane Divoux and Martina Lubrano di Ricco (Benoit Salomon's laboratory, Centre d'Immunologie et des Maladies Infectieuses-Paris) for providing mouse cells; and Anna Miesl (TUM) for excellent experimental support. S.I.M. was supported by a PhD scholarship from the German Academic Scholarship Foundation. M.J.F. is a Rudolf Mössbauer Tenure Track Professor and was funded through the Marie Curie COFUND program and the TUM Institute for Advanced Study, which is funded by the German Excellence Initiative and the European Union Seventh Framework Program under Grant Agreement 291763. O.D. was supported by grants from the Fondation ARC pour la Recherche sur le Cancer and the Ligue Nationale contre le Cancer (Comité de Paris) and J.E.-v.B., by grants from the German Research Foundation (DFG) (ES 471/2-1) and by the Helmholtz Association (VH-NG-1331). This work was performed in the framework of the DFG SFB1035, Projects B02 and B11.

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