Influence of glycosylation on IL-12 family cytokine biogenesis and function

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Abstract

The interleukin 12 (IL-12) family of cytokines regulates T cell functions and is key for the orchestration of immune responses. Each heterodimeric IL-12 family member is a glycoprotein. However, the impact of glycosylation on biogenesis and function of the different family members has remained incompletely defined.

Here, we identify glycosylation sites within human IL-12 family subunits that become modified upon secretion. Building on these insights, we show that glycosylation is dispensable for secretion of human IL-12 family cytokines except for IL-35. Furthermore, our data show that glycosylation differentially influences IL-12 family cytokine functionality, with IL-27 being most strongly affected.

Taken together, our study provides a comprehensive analysis of how glycosylation affects biogenesis and function of a key human cytokine family and provides the basis for selectively modulating their secretion *via* targeting glycosylation.

Keywords

interleukins / protein glycosylation / protein assembly / protein secretion / immune signaling

1. Introduction

Interleukins (ILs) are secreted proteins that regulate immune cell function. Among the more than 60 ILs known to date (Akdis et al., 2016), the interleukin 12 (IL-12) family is functionally and structurally particularly complex (Tait Wojno et al., 2019; Vignali and Kuchroo, 2012). Each of its family members is a heterodimer composed of an α- and a β-subunit with extensive subunit sharing occurring in the family: IL-12 consists of IL-12α and IL-12β (Gubler et al., 1991; Kobayashi et al., 1989; Stern et al., 1990; Wolf et al., 1991), the latter is shared with IL-23 where IL-12β pairs with IL-23α (Oppmann et al., 2000). Likewise, EBI3 is the β-subunit for IL-27, where it pairs with IL-27α (Pflanz et al., 2002), but also for IL-35, where it assembles with IL-12α (Collison et al., 2007; Devergne et al., 1997). This structural complexity goes hand in hand with the broad functional spectrum of the IL-12 family. All family members are produced by antigen presenting cells and regulate T cell functions, thus connecting innate and adaptive immunity, except for IL-35, which is produced by regulatory T cells (Tait Wojno et al., 2019; Vignali and Kuchroo, 2012). IL-12 family cytokines span a broad range of pro-inflammatory, immuno-regulatory and anti-inflammatory functions and are involved in diseases from infection *via* autoimmunity to cancer (Croxford et al., 2012; Sawant et al., 2015; Teng et al., 2015; Trinchieri et al., 2003; Yoshida and Hunter, 2015). The structural complexity and functional repertoire of the family are extended even further by the fact that also isolated IL-12 family subunits can be secreted and act as regulatory molecules in the immune system (Dambuza et al., 2017; Espigol-Frigole et al., 2016; Garbers et al., 2013; Gately et al., 1996; Lee et al., 2015; Ling et al., 1995; Muller et al., 2019b; Stumhofer et al., 2010).

Like other secreted mammalian proteins, IL-12 family cytokines are produced in the endoplasmic reticulum (ER). There, they also obtain their native structure and assemble into heterodimeric complexes before being transported further along the secretory pathway

towards the cell surface for secretion. Most secreted proteins acquire post-translational modifications in the ER, with disulfide bond formation between cysteines and glycosylation of Asn residues being the most prevalent ones (Braakman and Bulleid, 2011). IL-12 family cytokines are no exception to this rule: human IL-12α, IL-12β, and EBI3 are *N*-glycosylated and each subunit except IL-27α contains at least one intramolecular disulfide bond. Additionally, IL-12 and IL-23 are disulfide-linked heterodimers (Carra et al., 2000; Devergne et al., 1996; Lupardus and Garcia, 2008; Oppmann et al., 2000; Pflanz et al., 2002; Podlaski et al., 1992; Yoon et al., 2000). For all human IL-12 family α-subunits it was shown that oxidative folding is a key step in their biogenesis and functional roles have been delineated for intra- and intermolecular IL-12 family cytokine disulfide bonds (Jones et al., 2012; Meier et al., 2019; Muller et al., 2019a; Muller et al., 2019b; Reitberger et al., 2017; Yoon et al., 2000). In contrast, the role of glycosylation in IL-12 family biogenesis and function remains incompletely defined. Several studies indicate that glycosylation of IL-12, on its α- and on its β-subunit, is dispensable for IL-12 secretion and function *per se* – but modulates IL-12 activity (Aparicio-Siegmund et al., 2014; Carra et al., 2000; Ha et al., 2002; Podlaski et al., 1992). No comprehensive analyses are available for IL-23, IL-27 or IL-35 yet in this regard. In this study, we thus systematically investigated the effect of *N*- and *O*-glycosylation, the latter occurring in the Golgi, on IL-12 family cytokine biogenesis and function.

2. Materials and Methods

2.1 Constructs. Human interleukin cDNAs (Origene) were cloned into the pSVL vector (Amersham BioSciences) for mammalian expression. Mutants were generated by site-directed mutagenesis. All constructs were sequenced.

2.2 Sequence analysis, structural modeling and structural analyses. *N*-glycosylation sites were predicted by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and *O*-glycosylation sites were assessed by the NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) (Steentoft et al., 2013). Both servers evaluate the potential of glycosylation by using a threshold. Sequence alignments were performed with Clustal Omega (Sievers et al., 2011). Structures were taken from the PDB database (3D87, 3HMX) and missing loops were modeled using Yasara Structure (www.yasara.org) with a subsequent steepest descent energy minimization. The homology-modeled structure of IL-27 was used (Muller et al., 2019a). Structures were depicted with PyMOL (PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

2.3 Cell culture and transient transfections. Human embryonic kidney (HEK) 293T 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; Gibco, ThermoFisher) and 1% (v/v) antibiotic-antimycotic solution (25 μg/ml amphotericin B, 10 mg/ml streptomycin, and 10,000 units of penicillin; Sigma-Aldrich) at 37 °C and 5% CO_2. Transient transfections were carried out in poly D-lysine coated p35 dishes (VWR), or uncoated p60 dishes (VWR) for the functionality assays, using GeneCellin (Eurobio) according to the manufacturer's protocol. A total DNA amount of 2 μg (p35) or 4 μg (p60) was used. The α-subunit DNA was co-transfected with the β-subunit DNA or empty pSVL

vector in equal amounts (IL-27), in a ratio of 1:2 (IL-23) or 2:1 (IL-12, IL-35) for secretion and de-glycosylation experiments. BL-2 cells were cultured in RPMI-1640 medium with L-Gln and sodium bicarbonate (Sigma-Aldrich), supplemented with 20% (v/v) heat-inactivated FBS (Gibco, ThermoFisher) and 1% (v/v) antibiotic-antimycotic solution (25 μg/ml amphotericin B, 10 mg/ml streptomycin, and 10,000 units of penicillin; Sigma-Aldrich) at 37 °C and 5% CO_2.

2.4 Immunoblotting experiments. For secretion and de-glycosylation experiments, cells were transfected for 8 h and then supplemented with fresh medium for another 16 h. To analyze secreted proteins, the medium was centrifuged (5 min, 300xg, 4 °C), transferred to a new reaction tube and supplemented with 0.1 volumes 500 mM Tris/HCl, pH 7.5, 1.5 M NaCl, complemented with 10x Roche complete Protease Inhibitor w/o EDTA (Roche Diagnostics). Cells were lysed after washing twice with ice-cold PBS. Cell lysis was carried out in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40 substitute, 0.5% DOC, 0.1% SDS, 1x Roche complete Protease Inhibitor w/o EDTA; Roche Diagnostics) on ice. Both lysate and medium samples were centrifuged (15 min, 15,000xg, 4 °C). Samples were de-glycosylated with PNGase F (SERVA) or a mix of *O*-Glycosidase and α2-2,6,8 Neuraminidase (New England Biolabs) according to the manufacturer's protocol. For SDS-PAGE, 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 10% (v/v) β-mercaptoethanol (β-Me). For immunoblots, samples were run on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes by blotting overnight (o/n) at 30 V (4 °C). After blocking the membrane with Tris-buffered saline (25 mM Tris/HCl, pH 7.5, 150 mM NaCl; TBS) containing 5% (w/v) skim milk powder and 0.05% (v/v) Tween-20 (M-TBST), binding of the primary antibody was carried out o/n at 4 °C with anti-Hsc70 (Santa

Cruz, sc-7298, 1:1,000), anti-IL-12β (abcam ab133752, 1:500), anti-IL-12α (abcam ab133751, 1:500), anti-IL-23α (BioLegend 511202, 1:500), anti-IL-27α (R&D Systems, Bio-Techne, 1:200) in M-TBST containing 0.002% NaN_3 or anti-EBI3 antiserum (Devergne et al., 2001) (1:20) in PBS. Species-specific HRP-conjugated secondary antibodies (Santa Cruz Biotechnology; 1:10,000 in M-TBST or 1:5,000 for IL-23α in M-TBST) were used to detect the proteins. Amersham ECL prime (GE Healthcare) and a Fusion Pulse 6 imager (Vilber Lourmat) were used for detection.

2.5 Functionality assays. The **IL-12 activity assay** was performed following a previously published protocol (Reitberger et al., 2017). CD14-negative PMBCs were thawed and resuspended in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS (GE Healthcare) and 100 μg/ml streptomycin, 1 μg/ml gentamicin, 100 units/ml penicillin, and 2 mM L-glutamine (Thermo Fisher Scientific). Cells were seeded at a density of 5 x 10^5 cells/ml and stimulated with the supernatants of transfected HEK293T cells expressing the IL-12 constructs for 24 h at 37 °C and 5% CO_2. After harvesting (2 min, 1,000xg, 4 °C), cells were washed once with PBS prior to lysis in RLT buffer (Qiagen) supplemented with 1% β-Me. Total RNA was isolated (QuickRNA™ MicroPrep, Zymo Research) and cDNA (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific) was synthesized following the instructions of the manufacturer's protocol. Real-time PCR was performed using a ViiA 7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) and the FastStart Universal SYBR Green Master Mix (Roche). Transcript levels were normalized to actin (ACTB forward, 5'-GGATGCAGAAGGAGATCACT-3'; ACTB reverse, 5'-CGATCCACACGGAGTACTTG-3'; IFNγ forward, 5'-TCAGCCATCACTTGGATGAG-3'; IFNγ reverse, 5'-CGAGATGACTTCGAAAAGCTG-3').

For the **IL-23 activity test**, CD14-negative PBMCs were thawed in RPMI-1640 (Thermo Fisher Scientific) and 300,000 cells were resuspended in 100 µl RPMI-1640 supplemented with 5% human serum (Sigma Aldrich), 1% non-essential amino acids, 100 µg/ml streptomycin, 100 units/ml penicillin, 1 mM sodium pyruvate, and 2 mM L-glutamine (Thermo Fisher Scientific). The cells were stimulated with HEK293T supernatants containing 100 ng/ml secreted IL-23 constructs, previously quantified *via* immunoblotting with the help of recombinant IL-23 (R&D), and 10 µg/ml Phytohemagglutinin-L (Sigma Aldrich) for 72 h at 37 °C and 5% CO_2. After harvesting (2 min, 1,000xg, 4 °C), supernatants were analyzed for IL-17 secretion using human IL-17 DuoSet ELISA (R&D Systems), according to the manufacturer's protocol.

For IL-12 and IL-23, a **receptor activation assay** was performed using IL-12 or IL-23 iLite® reporter cells, respectively (Svar Life Science AB) according to the supplier's instructions. The cells were stimulated with HEK293T supernatants containing 10 ng/ml secreted IL-12 or IL-23 constructs, previously quantified *via* immunoblotting by comparing immunoblot signals to those of recombinant IL-12 or IL-23 with known concentrations (R&D Systems). The firefly and renilla luminescence signals were detected *via* the Dual-Glo Luciferase Assay System (Promega) in a multimode microplate reader (CLARIOstar Plus, BMG LABTECH).

To determine **IL-27 activity** dependent on its glycosylation pattern, BL-2 cells were stimulated with IL-27 protein, derived from transiently transfected HEK293T, secreted into the medium. Protein amounts of the IL-27 variants used in this functionality assay were determined by quantification *via* immunoblotting with anti-IL-27α antibody (R&D Systems, Bio-Techne, 1:200) relative to the wild-type protein signal prior to stimulation. BL-2 cells were starved o/n in serum-free RPMI-1640 and seeded into uncoated 48-well plates (Sigma-Aldrich) resuspended in RPMI-1640 supplemented with 0.5% (w/v) bovine serum albumin (BSA; Sigma A3294) at a cell number of 1 x 10^6 cells/well. Subsequently, cells were

stimulated for one hour with IL-27 protein or control supernatant (mock) and the reaction was stopped by adding ice-cold PBS. Cells were transferred to reaction tubes, centrifuged (5 min, 300xg, 4 °C) and lysed with NP-40 lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% NP40, 0.5% DOC) supplemented with 1x Roche complete Protease Inhibitor w/o EDTA (Roche Diagnostics) and 1x Phosphatase Inhibitor (SERVA). The supernatant (5 min, 20,000xg, 4 °C centrifugation) was complemented with 0.2 volumes 5x Laemmli buffer containing 10% (v/v) β-Me and loaded on 12% SDS-PAGE gels. After blotting o/n, membranes were washed with TBS, blocked with TBS containing 5% (w/v) skim milk powder and 0.1% (v/v) Tween-20 for one hour, washed again with TBS with 0.1% Tween-20 and incubated in the primary antibody (α-STAT1 or α-STAT1-P, Cell Signaling Technology, 1:1,000 in TBS with 5% (w/v) BSA, 0.1% Tween-20) o/n. Anti-rabbit HRP-conjugated antibody (Santa Cruz Biotechnology; 1:10,000 in 5% (w/v) skim milk powder and 0.1% (v/v) Tween-20 in TBS) was used for subsequent detection.

2.6 Quantification and statistics. Immunoblots were quantified using the Bio-1D software (Vilber Lourmat). Statistical analyses were performed using Prism (GraphPad Software). Differences were considered statistically significant when $p<0.05$. Where no statistical data are shown, all experiments were performed at least two times, with one representative experiment selected.

3. Results

3.1 Defining IL-12 family subunit glycosylation sites

Glycosylation often is a major determinant of protein biogenesis and function. To develop a comprehensive understanding of the role of glycosylation within the IL-12 family, we first aimed at defining glycosylation sites within each constituent subunit. In this study, all analyses and experiments were performed on the human proteins. Based on a sequence analysis (Fig. S1a), multiple *N*-glycosylation sites are expected to be present within both IL-12 subunits (IL-12α, IL-12β) as well as in EBI3, the β-subunit of IL-27 and IL-35 (Fig. 1a). For both β-subunits, the Asn residues predicted to be *N*-glycosylated are located within the two Fibronectin type-III (FnIII) domains, whereas the immunoglobulin (Ig) domain of IL-12β lacks glycosylation sites. The remaining subunits of the heterodimeric IL-12 family, IL-23α and IL-27α, are not predicted to be *N*-glycosylated (Figs. 1a and S1a).

Hence, at least one subunit of every IL-12 family cytokine is predicted to be *N*-glycosylated (Fig. 1a). To assess these predictions, we first investigated the overall glycosylation status of each IL-12 family subunit by enzymatic assays. Subsequently, if glycosylation was detectable, we identified modified residues by mutational analyses (Fig. 1b). In these experiments, αβ pairs were always co-expressed and secreted cytokines were analyzed. Using PNGase F, which removes *N*-linked glycans, electrophoretic mobility shifts were observed for IL-12α, IL-12β, and EBI3 but not for IL-23α and IL-27α (Fig. 1b), verifying overall predictions of *N*-glycosylation (Figs. 1a and S1a). Likewise, *O*-Glycosidase was used to detect *O*-glycosylation of each IL-12 family subunit. Of note, secreted wild-type IL-12α migrates differently when enzymatically digested with the *O*-Glycosidase mix. However, this mix also contains α2-2,6,8 Neuraminidase, which cleaves terminal sialic acid residues from glycosylation moieties. IL-12α has previously been shown to be modified with sialic acid (Carra et al., 2000), which is consistent with this observation. In agreement with this notion,

the mutant IL-12α^{N93,107Q} lacking *N*-glycosylation sites did not show any different migration upon this enzymatic de-glycosylation treatment any more (Fig. 1b). The shift in molecular weight for wild-type IL-12α can thus most likely be attributed to the enzymatic removal of terminal sialic acid residues from the complex *N*-linked sugar moiety, which arises from glycoprotein processing along the secretory pathway (Bohm et al., 2015; Zhang et al., 2019). In contrast, IL-27α actually was *O*-glycosylated (Fig. 1a and b), in agreement with previous studies (Muller et al., 2019a; Muller et al., 2019b; Pflanz et al., 2002).

Using mutational analyses, we next proceeded to identify individual glycosylation sites within each modified subunit. For IL-12α, mutation of two Asn residues (N93, N107), both predicted to be *N*-glycosylation sites (Fig. S1a), to Gln resulted in a protein with the same electrophoretic migration behavior as the wild-type counterpart after PNGase F treatment (Fig. 1b). Furthermore, treating this mutant with PNGase F did not lead to any further changes in electrophoretic mobility, verifying that mutation of N93 and N107 was sufficient to completely abolish IL-12α *N*-glycosylation (Fig. 1b).

Using mutated variants of IL-12β (IL-12β^{N125,135,222,303Q}) and EBI3 (EBI3^{N55,105Q}) that lacked all possible glycosylation sites, we could verify *N*-glycosylation of these two proteins (Fig. 1a and b). In EBI3^{N55,105Q}, only the two predicted sites were mutated (Fig. S1a), confirming these (Fig. 1b). In case of IL-12β, two Asn residues were predicted to be *N*-glycosylated (N135 and N222, Fig. S1a) but of those only N222 could be experimentally validated (Fig. S1b). Since secreted IL-12β^{N222Q} still showed glycosylation, we analyzed further possible *N*-glycosylation sites that were below the threshold for glycosylation-prediction (Fig. S1b). This approach revealed that also N125 and N303 were glycosylated to a certain extent in IL-12β.

To identify *O*-glycosylated Ser/Thr residues within human IL-27α, we made use of the fact that murine IL-27α is not *O*-glycosylated (Muller et al., 2019a; Muller et al., 2019b;

Pflanz et al., 2002) despite approximately 75% sequence conservation. A sequence alignment of the murine and human IL-27 α-subunits showed only few Ser and Thr residues that were present in the human but not the murine protein (Fig. S2a). Focusing on surface-exposed residues among those (Fig. S2b), mutational analyses of respective residues in a secretion-competent human IL-27α variant (Muller et al., 2019b) identified two C-terminal *O*-glycosylated residues, T238 and S240 (Fig. S2c). Mutation of these residues to Ala completely abolished *O*-glycosylation in human IL-27α (Figs. 1b and S2c).

Taken together, we could confirm predicted and identify new glycosylation sites of human IL-12 family cytokine subunits. Although modifications of subunits vary within the IL-12 family, each heterodimer was modified (Fig. 1a and b). Next, we thus aimed at defining how glycosylation affected heterodimerization and secretion of each IL-12 family cytokine.

3.2 Glycosylation is essential for IL-35 secretion, but not for secretion of other IL-12 family members

When expressed in isolation, all human IL-12 family α-subunits are retained in the cell. Only the presence of a corresponding β-subunit leads to assembly-induced secretion of the heterodimeric cytokine (Devergne et al., 1997; Gubler et al., 1991; Oppmann et al., 2000; Pflanz et al., 2002). Conversely, IL-12β is readily secreted in isolation, whereas EBI3 shows only inefficient secretion (Devergne et al., 1996; Ling et al., 1995). Thus, heterodimerization is a prerequisite for secretion of most of the IL-12 subunits to occur or increase. This suggests that glycosylation, which is often coupled to ER folding and quality control processes, may impact IL-12 family cytokine secretion. To assess the effect of subunit glycosylation on the secretion of single subunits as well as heterodimer formation, we investigated the secretion behavior of each IL-12 family member.

For IL-12 and IL-23, even the complete absence of glycosylation did not result in pronounced effects on secretion and thus heterodimerization (Fig. 2a and b). IL-12β^{N125,135,222,303Q} (IL-12β$^{\Delta N}$) behaved comparable to the wild-type protein with regard to its secretion levels and was still able to induce secretion of IL-12α, its non-glycosylated variant IL-12α^{N93,107Q} (IL-12α$^{\Delta N}$) (Fig. 2a) and the naturally non-glycosylated IL-23α (Fig. 2b).

In contrast to IL-12β, glycosylation of EBI3 turned out to be essential for its secretion as EBI3^{N55,105Q} (EBI3$^{\Delta N}$) was not secreted in isolation anymore (Fig. 2c and d). Wild-type EBI3 induced the secretion of both IL-27α and IL-27α^{T238,S240A} (IL-27α$^{\Delta O}$), lacking *O*-glycosylation. Interestingly, although retained itself, EBI3$^{\Delta N}$ also induced secretion of both these IL-27α variants and was co-secreted (Fig. 2c). Thus, IL-27 subunit mutants lacking glycosylation are still able to interact and enhance their mutual secretion even when secretion-incompetent in isolation.

Although sharing the same β-subunit as IL-27, secretion of IL-35 was strongly affected by missing glycosylation (Fig. 2d). Mutation of either of its subunits, IL-12α or EBI3, was sufficient to block or severely reduce secretion of the other subunit in this heterodimeric cytokine. Furthermore, co-expression of non-glycosylated IL-12α$^{\Delta N}$ and EBI3$^{\Delta N}$ showed no co-secretion into the medium at all (Fig. 2d). The fact that IL-12α$^{\Delta N}$ reduced secretion of wild-type EBI3, which is secretion-competent on its own, implies that heterodimerization still occurred for this pair leading to retention of the heterodimeric cytokine by ER quality control. Based on these findings, we examined the effect of the individual glycosylation sites of each IL-35 subunit. IL-12α single mutants N93Q and N107Q were secreted upon co-expression of EBI3, but to a lesser extent than wild-type IL-12α (Fig. 2e). The two individual EBI3 mutants N55Q and N105Q were secreted in isolation, but to decreased levels, which was not further enhanced upon IL-12α co-expression. Especially glycosylation at N105 seemed to be critical for efficient secretion of EBI3 (Fig. 2e).

In summary, the absence of glycosylation affects the secretion of IL-12 family cytokines in a surprisingly different manner. IL-12 and IL-23 seem to be secreted independently of their glycosylation status, whereas deleting glycosylation in IL-27 subunits led to a decreased secretion. Most pronounced effects were observed for IL-35, where absence of glycosylation caused an almost complete and dominant retention in the cell.

3.3 Lack of glycosylation does not compromise IL-12- or IL-23-mediated responses, but reduces IL-27 signaling

Our data indicate that for the IL-12 family members IL-12, IL-23, and IL-27 glycosylation was dispensable for heterodimer formation and secretion (Fig. 2). For these three cytokines, we thus investigated the impact of glycosylation on their biological functions by comparison of the different glycosylation mutants with their wild-type counterparts.

A major physiological activity of IL-12 is the induction of IFNγ expression (Chan et al., 1991). We therefore assessed the IL-12-induced IFNγ production in human peripheral blood mononuclear cells (PBMCs) by qPCR, using wild-type IL-12 and its glycosylation mutants (Figs. 3a and S3a). All IL-12 glycosylation variants induced IFNγ gene expression (Fig. 3a). Mutation of *N*-glycosylation sites in either the α- or β-subunit (IL12$\alpha^{\Delta N}$ + IL-12β, IL12α + IL-12$\beta^{\Delta N}$) did not significantly change the level in gene expression compared to wild-type IL-12. Surprisingly, non-glycosylated IL-12 (IL12$\alpha^{\Delta N}$ + IL-12$\beta^{\Delta N}$) showed an increase of IFNγ-induction in PBMCs (Fig. 3a), whereas a slight decrease in receptor activation of IL-12 iLite® reporter cells compared to wild-type heterodimer was observed (Fig. S4a).

Next, we examined the dependency of IL-23 signaling on cytokine glycosylation by measuring IL-23-induced IL-17 production in PBMCs (Langrish et al., 2005). Since IL-23α has no glycosylation sites (Fig. 1), only IL-17 production after stimulation with wild-type IL-23 and the mutant heterodimer consisting of IL-23α and IL-12$\beta^{\Delta N}$ were assessed using

ELISA (Figs. 3b and S3b). In these experiments, no significant change in IL-17 secretion was observed for wild-type IL-23α in comparison to the non-glycosylated variant (Fig. 3b). Stimulation of IL-23 iLite® reporter cells with the IL-23 glycosylation mutant (IL-23α + IL-12$\beta^{\Delta N}$) showed a slightly reduced receptor activation (Fig. S4b).

Lastly, we assessed the impact of glycosylation on IL-27 activity. Toward this end, we used the lymphoma BL-2 cell line which, in response to IL-27 stimulation, shows induction of STAT1 phosphorylation (Dietrich et al., 2014). Quantification of the phospho-STAT1 signals *via* immunoblotting confirmed signaling-competency for all IL-27 glycosylation variants in BL-2 cells (Figs. 3c and S3c). The heterodimer composed of IL-27$\alpha^{\Delta O}$ and wild-type EBI3 showed no significant change in activity compared to IL-27. In contrast, the activity of the complex of wild-type IL-27α with EBI3$^{\Delta N}$ as well as of the non-glycosylated IL-27 heterodimer (IL-27$\alpha^{\Delta O}$ + EBI3$^{\Delta N}$) was significantly decreased in comparison to IL-27 wild type (Fig. 3c).

Taken together, glycosylation in the interleukin-12 family does not seem to be essential for cytokine signaling. However, not only the secretion but also the biological functions of IL-12 family cytokines seem to be affected by the absence of glycosylation to a different extent: IL-27 signaling was reduced when EBI3 lacked glycosylation, whereas IL-12 and IL-23 function were less dependent of their glycosylation status, but may be modulated (Ha et al., 2002).

4. Discussion

In this study we provide a comprehensive analysis of how glycosylation influences human IL-12 family cytokine biogenesis and function. This extends previous studies on the impact of disulfide bond formation within IL-12 family cytokines (Jalah et al., 2013; Meier et al., 2019; Muller et al., 2019a; Muller et al., 2019b; Reitberger et al., 2017; Yoon et al., 2000) by insights into the second major post-translational modification occurring in the ER. We could verify that all IL-12 family subunits except IL-23α are glycosylated and identified new glycosylation sites. Moreover, our study reveals that loss of glycosylation affected secretion, heterodimer formation and biological activity of the IL-12 family members to different extents (Fig. 4).

A general principle for the human IL-12 family is assembly-induced folding of the α-subunit by a suitable β-subunit and subsequent secretion of the heterodimeric cytokine (Devergne et al., 1997; Gubler et al., 1991; Jalah et al., 2013; Meier et al., 2019; Muller et al., 2019b; Oppmann et al., 2000; Pflanz et al., 2002; Reitberger et al., 2017). It can thus be expected that whenever glycosylation is a prerequisite for proper β-subunit folding and assembly with its cognate α-subunit, effects on cytokine secretion should be dominant. In complete agreement with this idea, non-glycosylated and consequently cell-retained EBI3 abrogated secretion of IL-35 and significantly reduced IL-27 secretion. Interestingly, although diminished, mutant IL-27 subunits (IL-27α and EBI3) lacking glycosylation were still able to interact and induce their mutual secretion even when being secretion-incompetent in isolation. This possibility of pairing of two secretion-incompetent subunits to become secreted together has previously also been observed in the context of oxidative subunit folding for IL-27 (Muller et al., 2019a). It suggests that IL-27 subunits are (partially) folded even when lacking glycosylation but still expose features that do not allow them to pass ER quality control. Among the four human IL-12 family cytokines investigated, our

study reveals IL-35 formation to be most strongly dependent on glycosylation. This may explain failed attempts of IL-35 reconstitution using a recombinant non-glycosylated IL-12α subunit purified from bacteria (Aparicio-Siegmund et al., 2014).

In contrast to EBI3, IL-12β without glycosylation behaved comparable to its wild-type counterpart and still formed IL-12 and IL-23. Thus, heterodimers containing IL-12β are in general less affected than those containing EBI3 by their extent of glycosylation. Of note, IL-12 and IL-23 contain intermolecular disulfide bridges (Lupardus and Garcia, 2008; Yoon et al., 2000), which may facilitate heterodimer secretion even without other stabilizing factors like sugar moieties. In agreement with this notion, IL-12 and IL-23 were still functional with regard to IFNγ or IL-17 induction in human lymphocytes, respectively, and revealed receptor activation capabilities in reporter cell lines. In contrast, we observed impaired functionality for IL-27, when its β-subunit lacked *N*-glycosylation.

IL-12 family cytokines are attractive therapeutic targets and potential biopharmaceuticals (Muller et al., 2019b; Tait Wojno et al., 2019; Teng et al., 2015; Vignali and Kuchroo, 2012; Yan et al., 2016; Yeku and Brentjens, 2016). Our study assesses the impact of glycosylation on IL-12 family cytokine secretion and functionality by a limited number of tests and therefore builds the basis for further pharmaceutical investigations. Although we observed functionality for non-glycosylated IL-12 family cytokines, it should be considered that the biological activity of the tested cytokines may vary dependent on their glycosylation patterns. Furthermore, glycosylation serves not only as a checkpoint for trafficking along the secretory pathway but also influences protein characteristics such as solubility, stability, and biodistribution within the human body, as already investigated for an antibody-p40 fusion protein (Bootz et al., 2016). On the other hand, since our study reveals IL-12, IL-23, and IL-27 to be secreted and functional even when completely lacking glycosylation, modifying glycosylation patterns may also open new doors towards rationally

modifying IL-12 family cytokine functionality, as also exemplified by G-CSF (Chamorey et al., 2002).

Finally, it is noteworthy that deleting glycosylation sites abolished IL-35 formation yet was compatible with formation of functional IL-12 and IL-27, which each share one subunit with IL-35. In the light of the chain sharing promiscuity within the IL-12 family this is relevant, since a simple knockout of single IL-12 family subunits generally affects more than one of the heterodimeric family members. Mutating glycosylation sites may thus be a viable way to selectively delete individual IL-12 family members from an organism's cytokine repertoire. Our findings concerning glycosylation-dependent secretion of IL-35 could also be of interest with regard to the immunosuppressive role of this cytokine (Vignali and Kuchroo, 2012; Xue et al., 2019), e.g. in cancer forms that are difficult to treat (Mirlekar et al., 2018; Pylayeva-Gupta et al., 2016) where IL-35 subunit glycosylation may be worth investigating since its plays a pivotal role in IL-35 formation as our study shows.

5. Conclusions

In this study, we characterized the human IL-12 family in regard of their glycosylation profile. We were able to verify that at least one subunit of all IL-12 family members is glycosylated and furthermore identified new glycosylation sites. Our data indicate that loss of glycosylation does not severely affect IL-12 and IL-23 secretion, heterodimer formation, and biological activity. In contrast, non-glycosylated IL-27 shows partially impaired biogenesis and reduced signaling, whereas missing glycosylation of IL-35 led to complete retention in the cell. These findings extend our insights into this key cytokine family and provide a possibility to selectively remove individual IL-12 cytokines from an organism's cytokine repertoire.

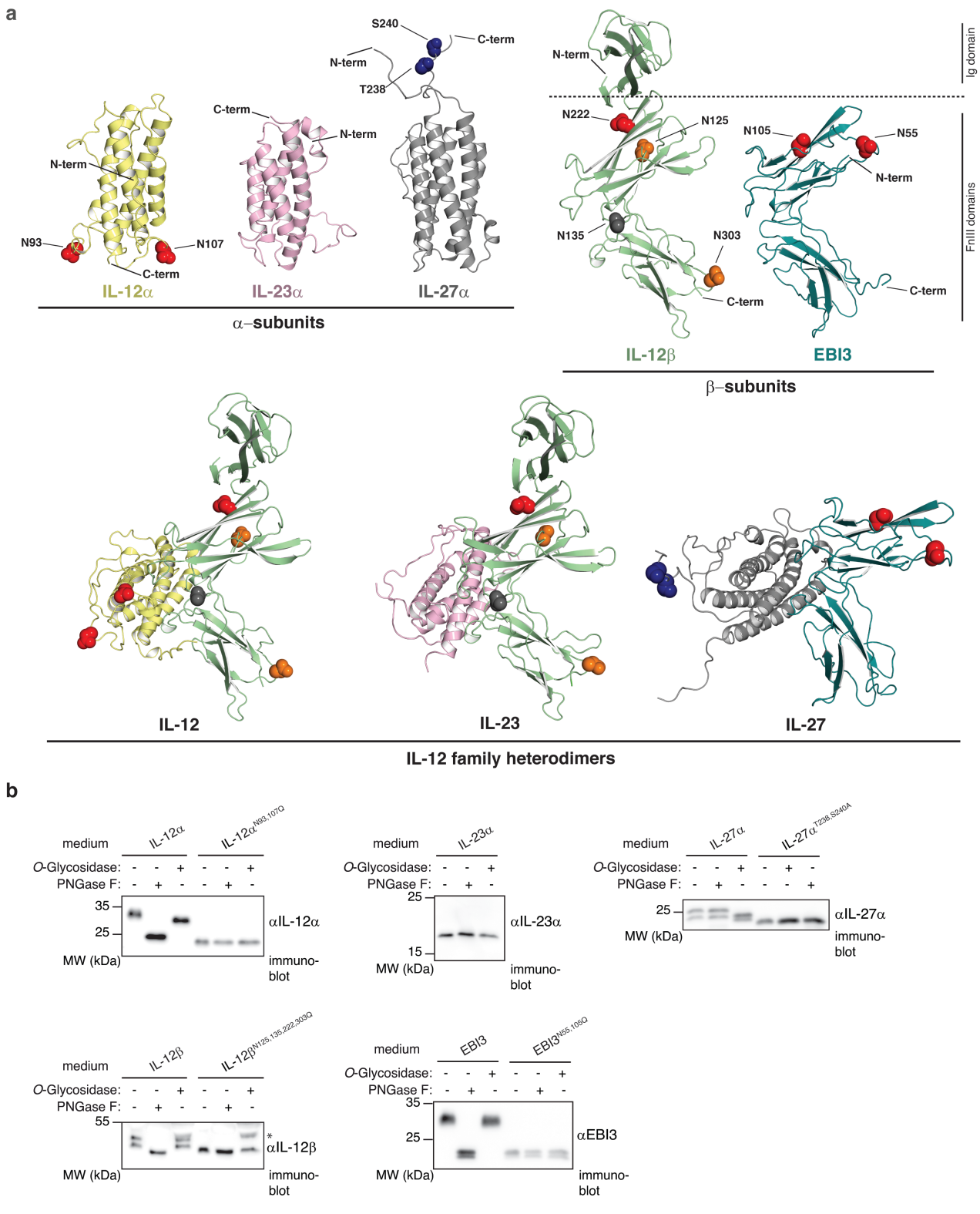

Fig. 1. Interleukin 12 family cytokines differ in their glycosylation patterns. (a) Top: Structural overview and glycosylation sites of the five shared subunits of the heterodimeric IL-12 family. Within the structures of the 4-helix bundle α-subunits IL-12α (PDB: 3HMX), IL-23α (PDB: 3D87), and the homology model of IL-27α (Muller et al., 2019a), glycosylation sites are

labeled and shown in a CPK representation. The β-subunits IL-12β (PDB: 3HMX) and EBI3 (homology model, (Muller et al., 2019a)) possess predicted *N*-glycosylation sites in the Fibronectin III (FnIII) domains, but not in the immunoglobulin (Ig) domain of IL-12β. Experimentally verified *N*-glycosylation sites are shown in red, *O*-glycosylation sites in blue. For IL-12β, the residue N135 (gray) was predicted as *N*-glycosylation site but could not be experimentally verified. Instead, two other *N*-glycosylation sites (N125, N303) that were below the threshold for prediction, were experimentally identified as glycosylation-modified and are shown in orange. Bottom: The heterodimers IL-12 (PDB: 3HMX) and IL-23 (PDB: 3D87) share IL-12β (green), whereas the β-subunit EBI3 (cyan) is part of IL-27 (homology model, (Muller et al., 2019a)) and IL-35 (no structural model available). (b) Verification of the predicted glycosylation sites of IL-12 family subunits by enzymatic treatment (PNGase F or *O*-Glycosidase) and mutagenesis of the respective sites. IL-12α is *N*-glycosylated at N93 and N107, whereas IL-23α is not glycosylated. IL-27α is *O*-glycosylated at two residues (T238, S240). Both β-chains, IL-12β and EBI3, are *N*-glycosylated at multiple sites (IL-12β: N125, N222, N303; EBI3: N55, N105). For EBI3, a double band pattern arose only after enzymatic digest and thus seems to be an artefact related to the enzymatic treatment. α- and β-subunits were co-expressed and treated with PNGase F or *O*-Glycosidase/Neuraminidase. 1.8% medium, or 3.6% medium in case of IL-12β, was applied to the gel and blotted with antibodies against the respective subunits. Electrophoretic mobility shifts indicate de-glycosylation. The asterisk indicates non-specific detection of the Neuraminidase. MW, molecular weight.

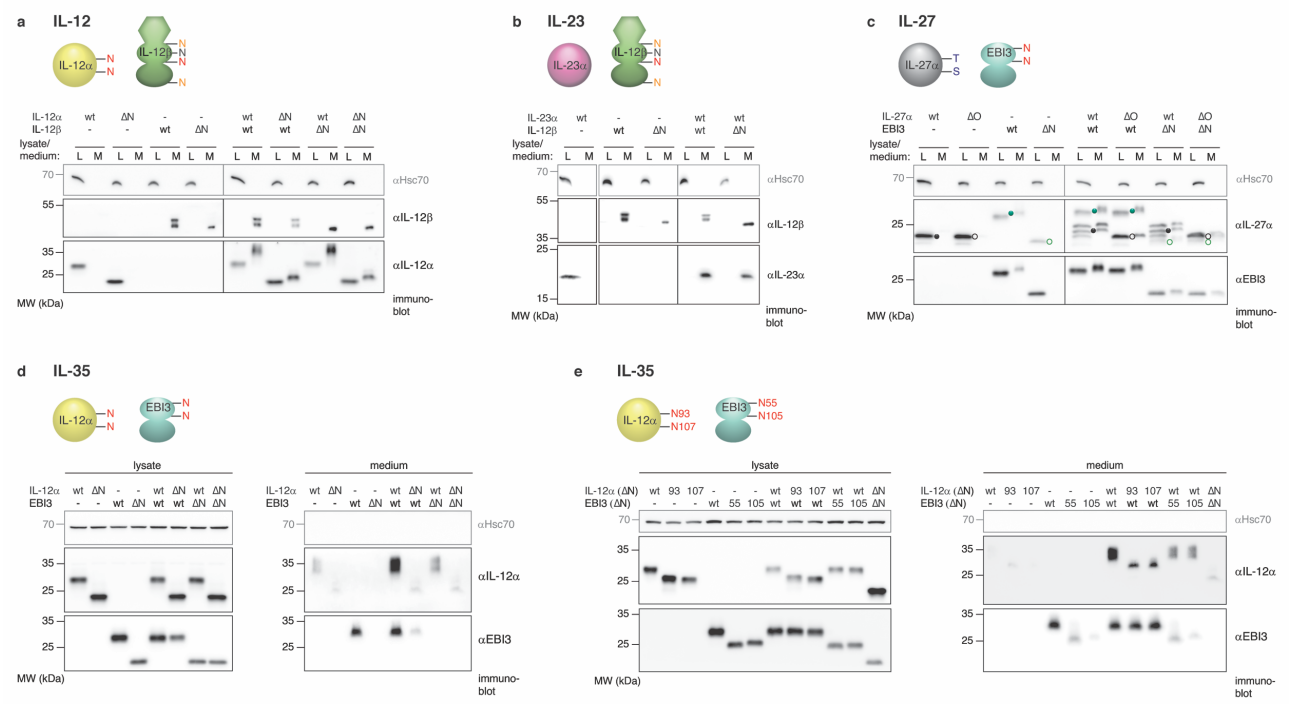

Fig. 2. Impact of glycosylation on IL-12 family cytokine secretion. (a-d) All human wild-type α-subunits are secretion-incompetent in isolation (IL-12α, IL-23α, IL-27α) and retained in cells (L). Their pairing with secretion-competent wild-type β-subunits (IL-12β, EBI3) to form the heterodimeric ILs induces secretion into cell media (M). (a) For IL-12, glycosylation mutants do neither affect the formation of the heterodimer nor its secretion behavior. (b) IL-23 heterodimer formation is not affected by lacking glycosylation of IL-12β and is secreted similarly to the wild type. (c) For IL-27, the α-subunit lacking *O*-glycosylation (open gray circle) behaves analogous to the wild type (filled gray circle), whereas the mutant EBI3 without *N*-glycosylation (open cyan circle) is retained in cells in isolation in contrast to wild-type EBI3 (filled cyan circle). Co-transfection of both subunits results in heterodimer formation as both non-glycosylated cytokine subunits become secreted. The used αIL-27α antibody detects also the EBI3 subunit. (d) For IL-35, both IL-12α$^{\Delta N}$ and EBI3$^{\Delta N}$ are not secreted in isolation and heterodimer formation is severely impaired. (e) Secretion behavior and heterodimer formation of IL-35 are affected by the individual *N*-glycosylation sites of IL-12α and EBI3. IL-12α^{N93Q} and IL-12α^{N107Q} are only secreted to a much lower extent upon

co-expression with EBI3, compared to the wild type. Similarly, the individual EBI3 glycosylation mutants are mostly retained in isolation and only induce reduced secretion levels of IL-12α. (d-e) To facilitate an analysis regarding low IL-35 secretion levels, lysate and medium samples were analyzed on separate blots. (a-e) ΔN and ΔO indicate subunits where *N*-glycosylation sites are mutated to Gln or *O*-glycosylation sites are mutated to Ala, respectively. In the schematic models, circles represent the 4-helix bundle α-subunits, hexamers stand for the Ig domains and ellipses for the FnIII domains of the β-subunits. L, lysate. M, medium. MW, molecular weight. 2% L/M (in (b): 4% L/M, for IL-23α transfected alone 6.4% L/M, in (d-e): 6% M) were applied to the gel and blotted with antibodies against the respective subunits. Hsc70 served as a loading control.

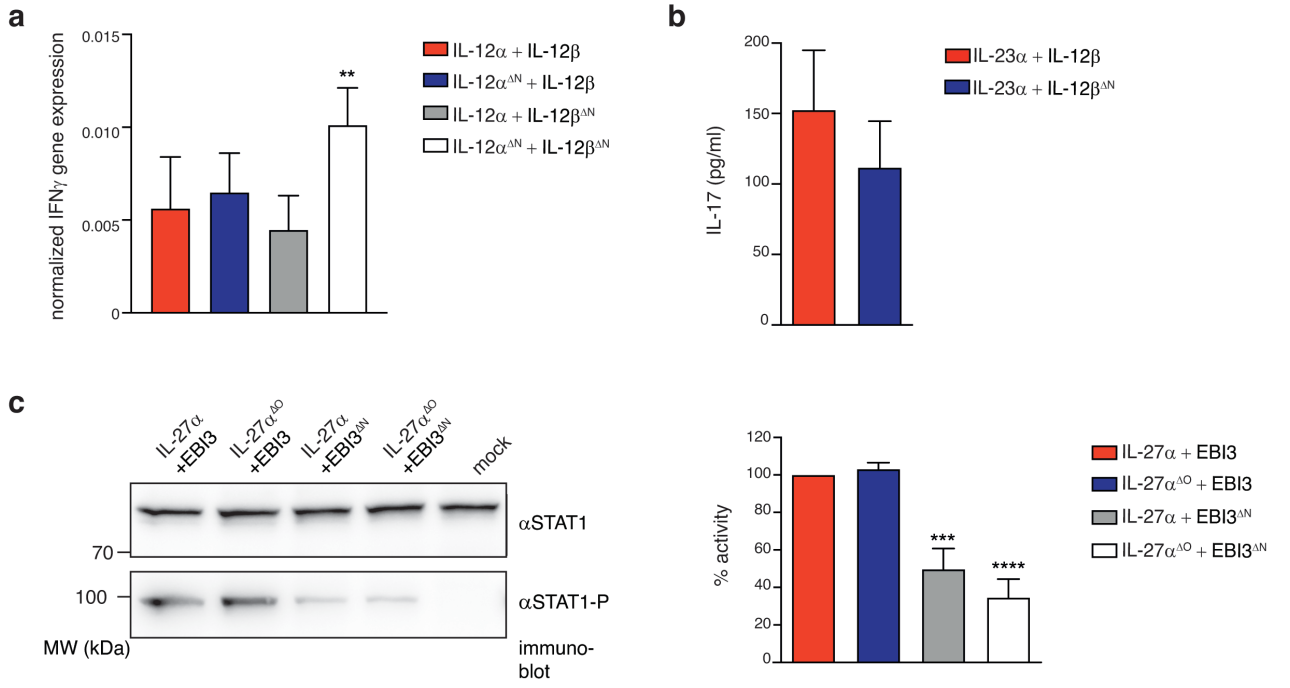

Fig. 3. Glycosylation influences the functionality of IL-12 family cytokines differently. (a) Glycosylation states of IL-12α and IL-12β influence biological functionality of heterodimeric IL-12. Stimulation of human peripheral blood mononuclear cells (PBMCs) with the indicated IL-12 glycosylation-variants show IFNγ induction by all analyzed heterodimers measured by qPCR. Data were normalized to secretion levels (Fig. S3a) and transcription levels were normalized to actin. The IL-12 heterodimer lacking glycosylation in both subunits shows

significantly enhanced gene expression levels compared to the wild-type proteins. Data are presented as mean ± SEM, PBMCs were from n=9 donors. Statistical significance was determined by the Friedman test (more than 2 groups). (b) IL-23 function is not significantly affected by lacking glycosylation. PBMCs were stimulated with previously quantified supernatants of HEK293T cells co-transfected with IL-23α and IL-12β variants (Fig. S3b). IL-17 production measured in the supernatant *via* ELISA, was not significantly changed for the glycosylation mutant. Data are shown as mean ± SEM, PBMCs were from n=6 donors. Statistical significance was calculated using a two-tailed paired t-test. (c) Functionality of IL-27 depends on the glycosylation states of EBI3. Stimulation of human BL-2 cells with differently glycosylated IL-27, using previously quantified HEK293T supernatants co-transfected with IL-27α and EBI3 (Fig. S3c), shows significantly reduced cytokine signaling for $EBI3^{\Delta N}$ in complex with IL-27α or $IL\text{-}27\alpha^{\Delta O}$. Levels of STAT1 phosphorylation (α-STAT1-P) were quantified and indicate receptor activation by heterodimeric IL-27. αSTAT1-immunoblot signals serve as loading control. Activity levels were determined from at least four independent experiments (shown ± SEM). Signals were normalized to the wild-type signal which was set to 100% activity. Statistical significance was calculated using a two-way ANOVA. (a-c) **p<0.01, ***p<0.001, and ****p<0.0001 indicate statistical significance. Mock, empty vector transfection. MW, molecular weight.

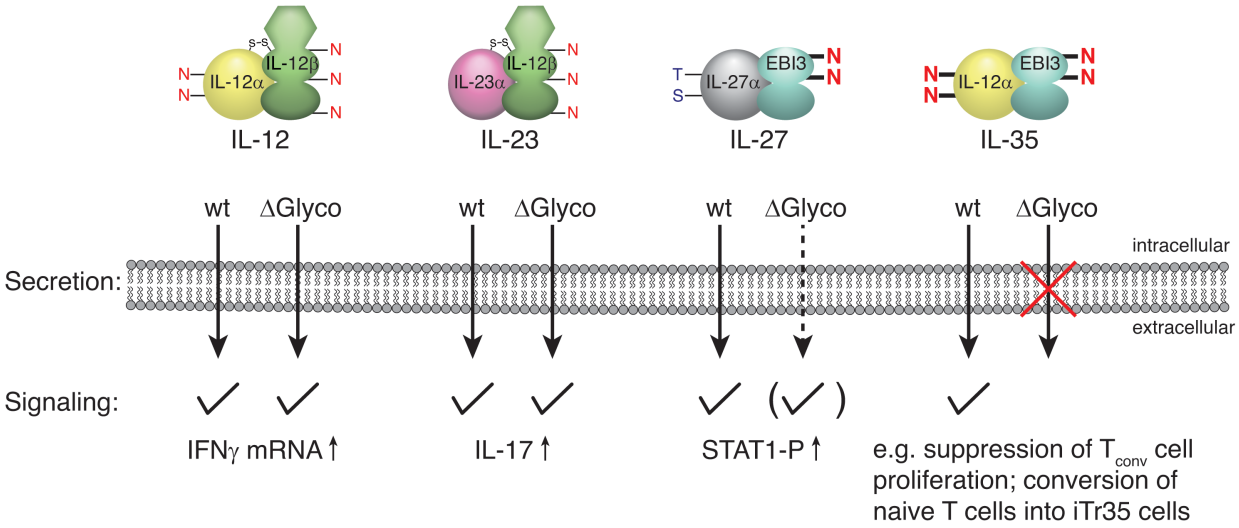

Fig. 4. The impact of glycosylation on human IL-12 family biogenesis and function. All heterodimeric wild-type (wt) IL-12 family cytokines are secreted and show biological function. IL-35 wild-type signaling was previously described (Collison et al., 2012; Collison et al., 2007). Both, IL-12 and IL-23 were secreted irrespective of their glycosylation status and also their biological functions (IFNγ gene expression and IL-17 production, respectively) were not compromised by lacking glycosylation. In contrast, lacking glycosylation of IL-27 led to a decreased secretion and signaling (phosphorylation of STAT1). Non-glycosylated IL-35 was almost completely retained in the cell. Red letters indicate *N*-glycosylated sites, blue letters *O*-glycosylated sites. Bold letters indicate glycosylation sites critical for secretion and functionality.

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Author contributions

MJF conceived the study. Experiments were performed by SB, KH, IA, and SIM. SB, KH, IA, SIM, JEvB, and MJF analyzed data. SB, KH, IA, JEvB, and MJF wrote the paper.

Conflict of interest

A patent on IL-27 glycosylation mutants has been submitted.

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