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Molecular principles of interleukin 12 family cytokine biogenesis and their exceptions for interleukin 35

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Stay hungry. Stay foolish.

Steve Jobs

To my parents

Parts of this thesis have been published in peer-reviewed journals

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

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Biogenesis and engineering of interleukin 12 family cytokines

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Trends in Biochemical Sciences, *in press*

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Annual SFB meeting

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Oral and poster presentation

Research topics that are not covered in this thesis

Glycosaminoglycans bind human IL-27 and regulate its activity

Marie-Charlotte Cavé, Solène Maillard, Karen Hildenbrand, Claire Mamelonet, Matthias J. Feige, Odile Devergne

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Abstract

Interleukins (ILs) are small, secreted proteins that are crucial for cell-cell communication and facilitate a precise regulation of the immune response. Cytokines of the IL-12 family are structurally unique due to their strictly heterodimeric architecture, with each heterodimer being composed of an α and a β subunit. Combinations of only three α and two β subunits result in the formation of the four established members IL-12, IL-23, IL-27, and IL-35, which is enabled by extensive subunit sharing. In order to achieve correct folding that permits their secretion, all human α subunits depend on assembly with their designated β subunit. Despite sharing many structural hallmarks, IL-12 family cytokines act in a broad range of divergent and even opposing biological pathways, ranging from pro- to anti-inflammatory reactions. Recent years have provided a better understanding of how cells specifically assemble and control the various IL-12 family members as well as structural insights into their receptor engagement. Only a comprehensive understanding of the underlying molecular mechanisms can open the door to translate these insights into new approaches for immunotherapy, for which IL-12 family cytokines are uniquely qualified.

In this thesis, we investigated general principles of IL-12 family members with regard to their glycosylation pattern and in a further project focused our research on the immunosuppressive IL-35 by a thorough investigation of its unique secretion mode and the resulting biological effects.

Protein glycosylation is a fundamental and highly regulated process which has significant effects on protein folding, conformation, distribution, stability, and activity. Here, we identified glycosylation sites within all human IL-12 family subunits that become modified upon secretion. Building on these insights, we showed that glycosylation is dispensable for secretion of human IL-12 family cytokines except for IL-35, which is strictly dependent on its glycosylation. Furthermore, our data reveal that glycosylation differentially impacts 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 the secretion of its members via targeting glycosylation.

IL-35 is among the most recently described and by far least understood member of the established IL-12 family. Our detailed investigation of this cytokine revealed that although IL-35 is commonly described as a strict heterodimer, its subunits IL-12α and EBI3 can also be secreted in an unassembled form. It therefore appears that IL-35 is in fact a compound cytokine, consisting of heterodimers and soluble subunits. By purifying

human IL-12α and EBI3 from mammalian cells, we were able to assess the biological function of both subunits in primary human immune cells and attribute them with immuno-suppressive features. This exciting discovery manifests the outstanding position of IL-35 in the IL-12 family and is key to understand its pleiotropic immunological effects. By considering IL-12α and EBI3 as natural extensions of the human cytokine repertoire, our results may pave the way for the development of therapeutic approaches in the treatment of severe immune diseases including asthma, chronic infections, and cancer.

Zusammenfassung

Interleukine (IL) sind kleine sekretorische Proteine, die eine entscheidende Rolle in der Zell-Zell-Kommunikation und der präzisen Regulation des Immunsystems spielen. Die Zytokine der IL-12 Familie sind aufgrund ihres streng heterodimeren Aufbaus strukturell einzigartig, wobei jedes Heterodimer aus einer α und einer β Untereinheit besteht. Nur drei α und zwei β Untereinheiten werden benötigt um die vier etablierten Mitglieder IL-12, IL-23, IL-27 und IL-35 zu bilden, was durch eine intensive gemeinsame Nutzung der Untereinheiten ermöglicht wird. Desweitern sind alle humanen α Untereinheiten auf die Assemblierung mit ihrer entsprechenden β Untereinheit angewiesen, um eine korrekte Faltung zu erreichen und sekretiert zu werden. Trotz der vielen strukturellen Gemeinsamkeiten ist das biologische Wirkspektrum der IL-12 Familie überraschend breit und ihre Mitglieder sind an pro- bis hin zu antiinflammatorischen Reaktionen beteiligt. In den letzten Jahren hat sich unser Wissen wie Zellen die verschiedenen Mitglieder der IL-12 Familie spezifisch zusammensetzen und kontrollieren sowie durch strukturelle Einblicke in ihre Rezeptorbindung signifikant erweitert. Dies sind entscheidende Fortschritte, denn nur aus einem umfassenden Verständnis der zugrundeliegenden molekularen Mechanismen können neue Ansätze für die Immuntherapie abgeleitet werden.

In dieser Arbeit wurden allgemeine Prinzipien der IL-12 Familie im Hinblick auf ihr Glykosylierungsmuster und in einem zweiten Forschungsschwerpunkt der einzigartige Sekretionsmodus und die daraus resultierenden biologischen Folgen für IL-35 untersucht.

Die Glykosylierung von Proteinen ist ein grundlegender und stark regulierter Prozess, welcher erhebliche Auswirkungen auf die Faltung, Konformation, Verteilung, Stabilität und Aktivität von Proteinen hat. Auch die Mitglieder der humanen IL-12 Familie werden vor ihrer Sekretion entsprechend modifiziert, wobei wir Glykosylierungsstellen in allen Zytokinen im Detail definieren konnten. Prinzipiell wiesen unsere Daten darauf hin, dass diese Modifizierung für die Sekretion der IL-12 Familienmitglieder entbehrlich ist, mit Ausnahme von IL-35, welches nur bei vollständiger Glykosylierung sekretiert werden kann. Darüber hinaus zeigten unsere Ergebnisse, dass die Funktionalität der IL-12 Familien Zytokine auf unterschiedliche Weise durch die Abwesenheit von Glykosylierungen beeinflusst wird, wobei der stärkste Effekt für IL-27 beobachtet wurde. Somit bietet unsere Studie eine umfassende Analyse zur Auswirkung von Glykosylierung auf die Biogenese und Funktion einer wichtigen menschlichen Zytokinfamilie und liefert darüber hinaus die Grundlage für eine gezielte Modulation einzelner Mitglieder durch Anpassung ihrer Glykosylierung.

IL-35 ist das jüngste und bei weitem am wenigsten erforschte Mitglied der etablierten IL-12 Familie. Obwohl IL-35 generell als striktes Heterodimer beschrieben wird, ergab unsere detaillierte Untersuchung dieses Zytokins, dass seine Untereinheiten IL-12α und EBI3 auch in nicht assemblierter Form sekretiert werden. Somit ist IL-35 aller Voraussicht nach ein komplexes Zytokin, welches aus Heterodimeren und löslichen Untereinheiten besteht. Durch Reinigung von rekombinanten, humanen IL-12α und EBI3 aus Säugetierzellen konnten wir biologische Effekte beider Untereinheiten in primären menschlichen Immunzellen untersuchen und ihnen immunsuppressive Eigenschaften zuordnen. Diese faszinierende Entdeckung bestätigt die herausragende Stellung von IL-35 innerhalb der IL-12 Familie und ist der Schlüssel zum Verständnis seines pleiotropen immunologischen Wirkspektrums. Unter Berücksichtigung von IL-12α und EBI3 als natürliche Erweiterung des menschlichen Zytokinrepertoires, können unsere Ergebnisse einen spannenden Weg eröffnen, der zur Entwicklung von therapeutischen Ansätzen in der Behandlung von schweren Immunkrankheiten wie Asthma, chronischen Infektionen und Krebs führt.

Abbreviations

ALP	autophagosomal-lysosomal pathway
Amp	ampicillin
APC	antigen-presenting cell
β-Me	β-mercaptoethanol
BiP	immunoglobulin heavy-chain binding protein
Breg	regulatory B cell
BRET	bioluminescence resonance energy transfer
CD	circular dichroism
CNX	calnexin
CRT	calreticulin
CSF	colony-stimulating factor
DAMP	damage associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
DNA	desoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental Autoimmune Uveitis
EBI3	Epstein-Barr virus induced gene 3
ECL	enhanced chemiluminescence reagent
EDEM	ER degradation-enhancing alpha-mannosidase-like protein
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERAD	ER-associated degradation
Ero1	ER oxidoreductin 1
EV	extracellular vesicle
FBS	fetal bovine serum
Fn	fibronectin
Foxp3	forkhead box P3 transcription factor
GSH	reduced glutathione
GSSG	oxidized glutathione
HC	heavy chain
HCD	heavy chain disease
HEK	human embryonic kidney
HSF1	heat shock factor 1
HSP	heat shock protein
HRP	horseradish peroxidase
Ig	immunoglobulin
IFN	interferon
IL	interleukin
IP	immunoprecipitation
Jak	Janus kinase
kDa	kilodalton
LC	light chain
LPS	lipopolysaccharide
MBL	mannose-binding lectin
MHC	major histocompatibility complex
MTBST	skim milk/Tris(hydroxymethyl)aminomethane buffered saline with Tween20
MW	molecular weight
NBD	nucleotide-binding domain
NEF	nucleotide exchange factor
NMR	nuclear magnetic resonance
o/n	overnight
OST	oligosaccharyl transferase

PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PDI	protein disulfide isomerase
PPIase	peptidylprolyl isomerase
PRR	pattern recognition receptor
PVDF	polyvinylidene difluoride
QC	quality control
RIPA	radio immunoprecipitation assay
RT	room temperature
SBD	substrate-binding domain
SDS	sodium dodecyl sulfate
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SP	signal peptide
SPC	signal peptidase complex
SRP	signal recognition particle
STAT	signal transducer and activator of transcription
TGF	transforming growth factor
TLR	toll-like receptors
TNF	tumor necrosis factor
TRAP	translocon-associated protein complex
Treg	regulatory T cell
UGGT	UDP-glucose:glycoprotein glucosyltransferase
UPS	ubiquitin-proteasome system
w/o	without
wt	wildtype

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1 Introduction

1.1 The world of proteins

Within living systems, proteins are by far the most versatile macromolecules and play crucial functions in virtually every biological process. It is therefore fundamental to keep the proteome in a balanced and functional state, which is summarized by the term *proteostasis.* This portmanteau combines the two words protein and homeostasis and describes the dynamic regulation of concentration, conformation, binding interaction, and location of proteins[1]. In human cells, around 20% of the proteome are involved in this task and referred to as the proteostasis network[2]. This considerable amount highlights the effort and resources the cell invests in a well-balanced protein homeostasis. Key mediators of this crucial task are molecular chaperones by their assistance in client folding, assembly, targeting, transport, and degradation, amongst others. The processes they are involved in can be roughly subdivided into three main categories, protein synthesis and folding, maintenance of conformational stability, and protein degradation, which will be briefly discussed in the following.

Protein synthesis and folding

Proteins are generally assembled from 20 proteinogenic amino acids by forming covalent peptide bonds between the single residues. Newly synthesized proteins need to acquire their native conformation, since in most cases the function of a protein is a direct consequence of its three-dimensional structure[3]. The loss of structure does not only result in loss of function, but in several cases also leads to severe diseases, like Alzheimer's or Parkinson's disease. Almost 60 years ago, Anfinsen et al. discovered in an *in vitro* experiment that the primary amino acid sequence contains the entire information needed for a protein to fold into its native state[4]. Therefore, the native structure of a protein seems to be the thermodynamically most stable conformation[5]. Due to the large number of degrees of freedom in an unfolded polypeptide chain, a protein with 101 amino acids can theoretically explore 5×10^{47} different conformations. Even with very fast sampling rates of 10^{13} 1/s it would still take 10^{27} years to adopt all configurations in a trial-and-error approach[6]. This contradicts the general observation that most proteins gain their native structure within milliseconds to minutes and is summarized by Levinthal's paradox[7]. By considering both – kinetics and thermodynamics of protein folding – our current understanding of the folding process can be depicted by a complex funnel-shaped energy landscape in which a protein can navigate by several routes towards its native state (Figure 1.1). Some of those pathways lead to local energy minima however, trapping the protein in an intermediate state, which

could finally result in the formation of aggregates. To convert proteins from an intermediate towards their native state, chaperones are required to assist protein folding *in vivo*[8].

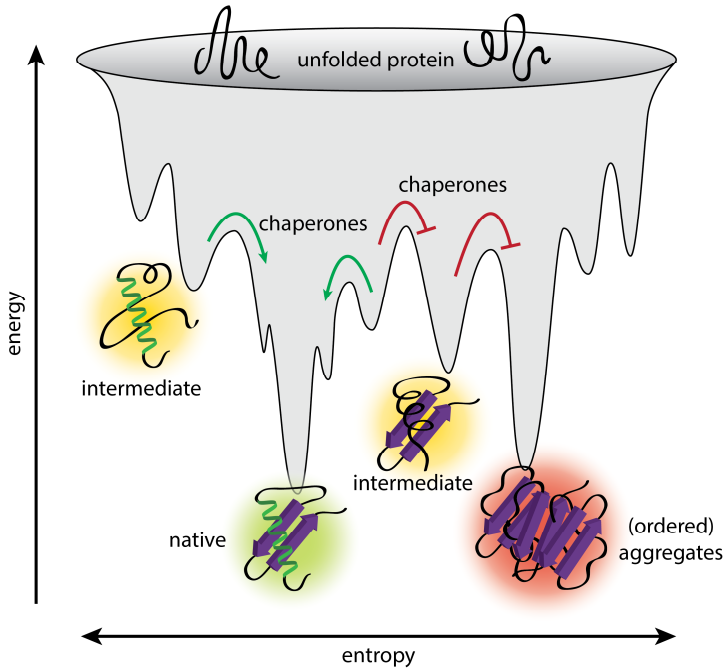

Figure 1.1: Energy landscape of the protein folding funnel. Unfolded proteins possess both high entropy and high free energy. As the protein starts to fold and descends the funnel, free energy is reduced and entropy decreases, according to the restriction of number of possible conformational states. Local minima along the way to a native state (green) trap the protein in folding intermediates (yellow) or potentially toxic (ordered) aggregates (red) resulting from intermolecular contacts. *In vivo*, molecular chaperones assist protein folding and promote reaching the native state by preventing folding intermediates and formation of aggregates.

For decades, insights into protein structure were only possible via laboratory experiments like X-ray crystallography and nuclear magnetic resonance (NMR), which are often complex as well as time consuming, and therefore less than 10% of the human proteome is covered by experimental structures[9]. Although all the information for protein folding is comprised in the amino acid sequence, computational predictions of their three-dimensional structure remained the Holy Grail of structural biology. A recent breakthrough was achieved in 2020 by release of Deepmind's open source artificial intelligence program AlphaFold[10]. By predicting highly accurate protein structures it has already revolutionized the field of structural biology in short time and will be the driver for further progress, like RoseTTA fold from David Baker's Lab, which generates accurate models of multi-protein complexes[11].

Maintenance of conformational stability

After reaching their native state, proteins do not rigidly keep this conformation, but functional or environmental effects lead to structural dynamics in the lifetime of a protein.

The presence of heavy metals, reactive oxygen species, increased temperature or redox stress can provoke structural alterations that might lead to incorrect protein interactions and finally accumulation[12]. Considering the high energy requirements of *de novo* synthesis, it is efficient for the cell to avoid erroneous proteins by maintaining their conformational stability. A dedicated quality control system based on molecular chaperones performs this task, which acts in multiple cellular processes including assistance of folding and assembly or prevention of aggregation of its clients. Based on common characteristics, chaperones are classified into different families, with small heat shock proteins (sHSPs), HSP60, HSP70, and HSP90 being the most widely studied ones[13]. Molecular chaperones usually recognize hydrophobic, unstructured regions in their clients and assist the folding process in an either ATP-dependent or -independent way. The name HSP originates due to their first description by Ritossa, who observed chromosomal “puffs” after heat exposure in *Drosophila* larvae[14]. The so-called heat shock response is a prominent stress response in the cytosol, which is controlled by several transcription factors, including heat shock factor 1 (HSF1). Under normal conditions, HSF1 is in a complex with HSP90 and HSP70. Increasing amounts of unfolded proteins lead to the recruitment of these chaperones, thereby releasing HSF1, enabling its trimerization followed by localization into the nucleus and induction of heat shock related gene transcription[15]. Once the concentration of non-native proteins diminishes, HSC70 and HSC90 have the capacity to bind to HSF1 again, and the system returns to a balanced state.

Protein degradation

Protein degradation is a crucial process for the regulation of protein concentration and disposal of terminally misfolded proteins, which are a serious threat to the cell. Moreover, the synthesis of proteins is rather inefficient, resulting in the estimation that up to 30% of newly synthesized polypeptides are targeted for immediate degradation[16]. Two major proteolytic pathways are responsible for regulated protein degradation, which both require the support of chaperones to identify their substrates and keeping them in a degradation-competent state[17]. The ubiquitin-proteasome system (UPS) requires ATP and depends on a hierarchical organized set of enzymes, which transfer the degradation signal ubiquitin to their clients, thereby targeting them to the proteasome. The autophagosomal-lysosomal pathway (ALP) recognizes large, destructive cellular clusters like protein aggregates or even whole organelles. By enveloping its target and fusion with the lysosome, the biological material is enzymatically degraded[18].

1.2 Protein folding in the endoplasmic reticulum

Approximately one third of all proteins is destined for the cell membrane or the extracellular space and traverses the endoplasmic reticulum (ER), the central hub for protein biogenesis of the secretory pathway[19]. The ER is the largest organelle in the cell consisting of a continuous membrane network that forms tubules and sheets[20]. It quite likely originated from invagination of the cell membrane, as its luminal conditions resemble those of the extracellular space, therefore allowing protein maturation in an environment which already simulates their final destination[21]. Major differences between ER and cytosol are manifested in the Ca^{2+} concentration and the redox potential. With Ca^{2+} concentrations ranging from 100-800 µM, the ER exceeds the concentration of the cytosol by three to four orders of magnitude[22,23]. As many ER resident enzymes require Ca^{2+} ions for their native structure, the concentration must be held at a high level. This gradient is maintained by the restricted membrane permeability and active calcium pumping by sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs)[24]. Furthermore, the ER enables oxidative protein folding due to the presence of many disulfide-generating enzymes in an optimized redox environment. The redox potential is generally mediated by the principal thiol redox couple glutathione (GSH) and glutathione disulfide (GSSG)[25]. Compared to the cytosol, the GSH to GSSG ratio is significantly lower in the ER with a roughly estimated ratio of 1:1-3:1[26]. GSH is involved in reduction and activation of the ER oxidoreductin (Ero) 1, a flavoenzyme that constitutes, together with protein disulfide isomerases (PDIs), the major pathway of protein disulfide bond formation[27,28]. Whether the GSH/GSSG constitutes the ultimate redox couple of the ER, or if other mechanisms exist, is part of ongoing discussions[29,30].

The process of co-translational ER import begins with a protein carrying an amino-terminal signal peptide (SP), characterized by hydrophobic residues. During the early stage of protein expression, the SP emerges from the ribosomal exit tunnel and is recognized by the signal recognition particle (SRP), which targets the ribosome-nascent chain complex to the ER. Binding to the SRP receptor results in conformational changes of the heterotrimeric Sec61 complex, a highly conserved, channel-forming pore in the ER membrane[31]. The translocation of precursor proteins into the ER lumen or membrane sometimes requires additional factors for channel opening of the Sec61 translocon, such as the translocon-associated protein complex (TRAP) or the Hsp70 molecular chaperone immunoglobulin heavy-chain binding protein (BiP)[32]. Furthermore, BiP can directly bind to the precursor polypeptide, assists efficient translocation, and inhibits backtrack into the translocon[33]. With its arrival in the ER, the translation of the protein is continued and it is introduced to the crowded environment of the ER, which includes a selected set of

ER resident factors that assist the folding and add modifications to their clients. One of the first modifications to occur is the cleavage of the SP, which is performed by the signal peptidase complex (SPC), an intramembrane-cleaving protease complex in the ER. Another important modification is the addition of sugar moieties to the protein. Due to their size and biophysical properties, glycosylations have a significant impact on the protein. The oligosaccharyltransferase (OST) complex transfers $Glc_3Man_9GlcNAc_2$ units to the asparagine of the target protein in an NXS/T sequence, where X can be any amino acid residue except proline[34]. As N-glycosylation is a co-translational process, the folding of the protein is already affected by the presence of the large sugar moiety at an early state. Once secreted, a major effect of protein glycosylation is an increase in solubility and contribution to stability[35]. In addition to glycosylation, most proteins that pass the secretory pathway contain disulfides. Their introduction provides thermodynamic stability to the protein by reducing the entropy of the unfolded state and enthalpically stabilizing the folded confirmation[36–38]. The catalysis of oxidization and reduction of cysteine residues is performed by PDIs, a family of ER resident oxidoreductases. Structurally, most PDIs are characterized by four thioredoxin-like domains, two of which contain a CXXC active-site motif. In its oxidized state, the PDI can form a transient mixed disulfide with its client and by its reduction, the bond is subsequently transferred to the client. Ero1α and Ero1β are required for recharging the PDI's active site by transferring the electrons to their cofactor FAD and finally to molecular oxygen[39]. Another important class of ER resident enzymes are the so called peptidyl-prolyl isomerases (PPIases), which catalyze the *cis-trans* isomerization of the peptide bonds preceding prolines[40]. *Cis* peptide bonds are characteristic features of turns in the secondary structure. Since spontaneous isomerization is a rate limiting step in the folding process of a protein, PPIases are required to facilitate this transition.

The ER also harbors a large number of chaperones that are crucial to prevent protein aggregation in its crowded environment. One of the most abundant proteins in the ER is the HSP70 family member BiP, which binds exclusively to hydrophobic regions[41]. Its N-terminal nucleotide-binding domain (NBD) is followed by a linker, connecting it to the substrate-binding domain (SBD) with a helical lid. In the ATP bound state, the NBD is in close proximity to the SBD, which is characterized by an open conformation of the lid and allows for low-affinity substrate binding. The substrate can be recruited to BiP by co-chaperones of the Hsp40 family, the ERdjs, which additionally catalyze the intrinsic ATPase activity of BiP. Upon ATP hydrolysis, NBD and SBD become undocked, which induces closure of the lid. The substrate is trapped in a high-affinity state and the peptide exchange is much slower than in the ATP bound form. In a last step, nucleotide exchange

factors (NEFs) release ADP, which enables ATP binding, substrate release, and the start of another ATPase cycle of BiP[42,43].

Another major chaperone family of the ER are the lectins, which generally interact with glycosylated, incompletely folded proteins. Therefore, the attachment of glycans as described previously can additionally serve as a ticket to another quality control pathway termed the calnexin/calreticulin (CNX/CRT) cycle[44]. After trimming of two of the three glucose residues, the nascent glycoprotein can interact with CNX or CRT. While CNX is anchored to the ER membrane due to its transmembrane domain, CRT is a soluble protein. Additional folding factors like the PDI ERp57 are recruited by CNX/CRT to aid in folding of the client and subsequently trimming off the final glucosidase releases the protein from the lectin cycle[45]. However, if the protein is still unfolded, the UDP-glucose:glycoprotein glucosyltransferase (UGGT) reverses this reaction by glucosylation of the substrate and reshuffles the protein into the CNX/CRT cycle, granting the protein precious time to attain a folded conformation. If too many cycles are required, ER mannosidase (ERMan) I and ER degradation-enhancing alpha-mannosidase-like proteins (EDEMs) remove mannose residues, thereby excluding the protein from re-entering the CNX/CRT cycle and targeting it for degradation via ER associated degradation (ERAD) by transfer to the cytosolic UPS[46,47].

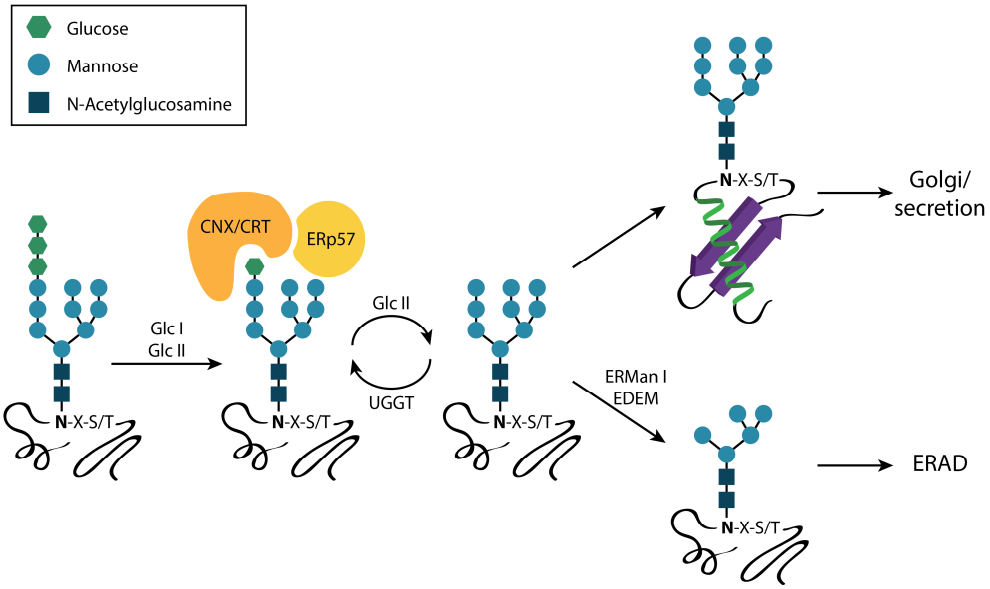

Figure 1.2: The calnexin/calreticulin (CNX/CRT) cycle in glycoprotein folding. Newly synthesized proteins are co-translationally equipped with the oligosaccharide $Glc_3Man_9GlcNAc_2$ at the asparagine (N) residue of the consensus sequence N-X-S/T by the oligosaccharyltransferase (OST) complex. Glucosidase (Glc) I and II sequentially trim the two distal glucose residues. The resulting $GlcMan_9GlcNAc_2$ intermediate is recognized by membrane bound CNX or its soluble counterpart CRT, which together with protein disulfide isomerases (PDIs, e. g. ERp57) assist protein folding by their lectin activity. Glc II trims the final glucose residue and if correctly folded, the protein is released for transport to the Golgi complex. The folding sensor UDP-glucose:glycoprotein glucosyltransferase (UGGT) adds a glucose residue to folding intermediates, thereby allowing re-entering into the CNX/CRT cycle. Permanently misfolded proteins are recognized by ER mannosidase (ERMan) I and ER degradation-enhancing alpha-mannosidase-like proteins (EDEMs), which trim further mannose residues, prevent re-entering into the cycle, and target the protein to degradation.

1.2.1 Assembly of protein complexes

The term *molecular chaperone* was originally introduced by Laskey et al. to describe a protein that enabled the assembly of histone proteins and DNA into an oligomeric structure[48]. Although the term nowadays usually confines to protein folding, the original context highlights a further and major function of chaperones. Protein complexes are key organizational units of the proteome and indispensable for proper cell function. Their interface, however, is often hydrophobic and, when unassembled, requires shielding to prevent aberrant interactions[49]. In the cytosol, assembly appears to be dependent on a co- rather than a post-translational process, evading decreased concentrations of the assembly-competent subunits after release from the ribosome[50]. This is opposed to the ER, where oligomerization generally occurs after the subunits have acquired near-native structures[51]. In many cases, unassembled monomers are retained by assembly factors and can only be released after binding of the actual partner, which competes for the same binding site. A well-studied example is given by Immunoglobulin G (IgG) antibodies, which are composed of two light and two heavy chains. Two or four Ig domains, characterized by a compact β-sheet structure, are required to build the light or heavy chain, respectively. Usually, unassembled heavy chains are tightly prevented from secretion, as their antigen-binding domain is incomplete and unspecific immune responses might occur by cellular recognition of their Fc region. The heavy chain disease (HCD) is a striking demonstration about the impact of erroneous retention of unassembled heavy chains[52]. On a molecular level, an intrinsically disordered domain of the heavy chain is recognized by BiP, which binds to its client and prevents its secretion. Only after assembly with the light chain, BiP is released and allows for complete folding of the heavy chain and formation of an interchain disulfide bond between light and heavy chain, which finally allows the covalent complex to be secreted[53]. Another prominent example for assembly-induced folding is the T cell receptor (TCR), a protein complex found on the surface of T cells. The formation of the central αβTCR heterodimer is a prerequisite to release the α chain from ER retention, which is not correctly folded when unassambled[54]. Only upon interaction with the β chain, the α chain obtains its native structure, thereby preventing further processing of unassembled chains along the secretory pathway and efficient quality control of this important immune receptor.

1.3 Our immune system and its language

Our immune system is strikingly efficient and reliable in its enduring competition with pathogens. Basically, it can be described as a vast communication network of lymphoid organs, cells, and chemical signals distributed in blood and tissue throughout the human body, which regulates normal growth and development of the organism while protecting

against disease. Fulfilling this task requires a fine-tuned system that is able to discriminate self from non-self. However, the immune system is also a double-edged sword, in that over- or underreaction can lead to severe diseases including autoimmune deficiencies or cancer. Key for the effectiveness of the human immune system is its highly interconnected multi-layered structure, which can be categorized into two main lines of defense: the innate and the adaptive immunity. Figure 1.3 summarizes the main components of the innate and adaptive immunity, which will be shortly described in the following.

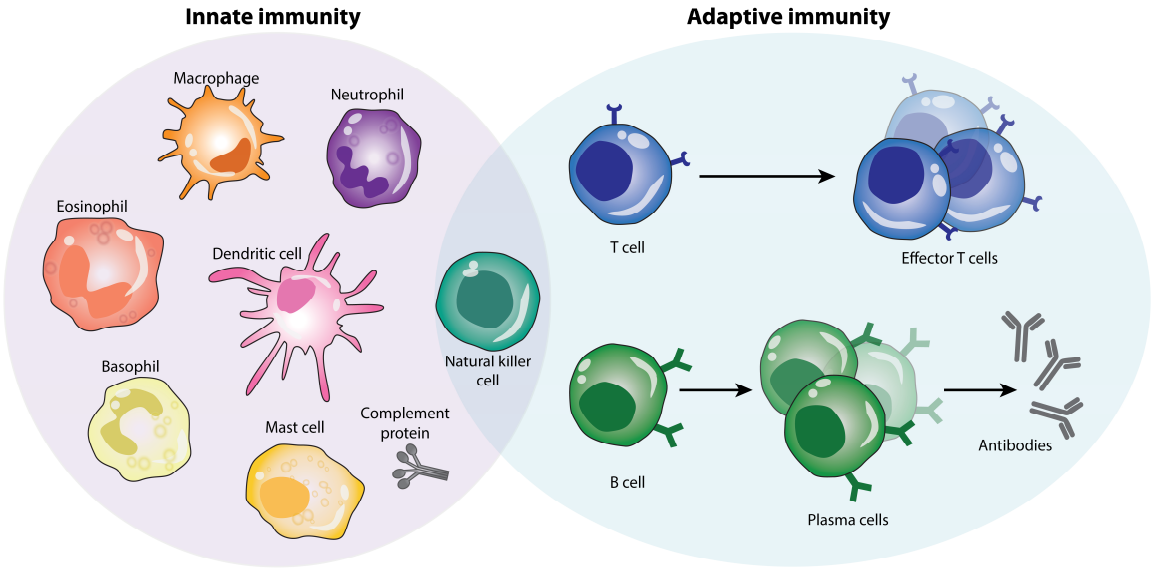

Figure 1.3: Components of the immune system. Cells of the innate immune system and the complement cascade provide rapid but poorly specific immune responses. The later onset adaptive immunity includes B cell-mediated humoral immunity by production of antigen-specific antibodies and T cell-mediated cellular immunity.

1.3.1 Innate immune system

The innate immune system owes its name to the fact that its main constituents are encoded in the host's germline. It builds the first barrier against invading pathogens and is rapid but unspecific. Moreover, innate immunity can be subcategorized into further types of defensive barriers: anatomic, physiologic, endocytic and phagocytic, and inflammatory[55]. Although it is referred to as nonspecific, recognition of pathogens is possible due to conserved pathogen associated molecular patterns (PAMPs), including lipopolysaccharides (LPS). Molecules released by damaged host cells (DAMPs) can also be recognized, allowing the innate immune system to furthermore react on infected cells. Identification of PAMPs or DAMPs occurs via a specialized set of pattern recognition receptors (PRRs), which can be either soluble, including the mannose-binding lectin (MBL), or on the surface of different immune cells, like the toll-like receptors (TLR). Signaling via PRRs activates different pro-inflammatory and microbicidal responses,

such as phagocytosis, that finally lead to the rapid elimination or containment of infectious drivers[56]. Another critical effector mechanism is the so-called complement system, which consists of constitutively expressed serum glycoproteins that act in a biochemical cascade to identify and opsonize bacteria and other pathogens[57]. The cellular components of the innate immune response consist of many different cell types, such as phagocytes (macrophages and neutrophils), dendritic cells, natural killer cells, mast cells, basophils, and eosinophils (Figure 1.3). One of the first cell classes that respond to initial infections are macrophages and neutrophils. Macrophages originate from blood monocytes, which differentiate into distinct macrophage populations depending on the tissues they are migrating into. The heterogeneity amongst those different populations is reflected in their morphology and production levels of distinct cytokines and demonstrates their required level of specialization[58]. Alveolar macrophages, for example, are the most abundant type of immune cells in the lung and are specialized in initiation and control of the immunity in response to respiratory pathogens[59]. Both macrophages and neutrophils are able to phagocytose harmful organisms by engulfing them via pseudopodia, thereby mediating pathogen clearing. Further signaling pathways are initiated by their release of cytokines, and macrophages are additionally able to display antigens via their major histocompatibility complex (MHC) class II, thereby recruiting cells of the adaptive immune system.

1.3.2 Adaptive immune system

While the innate immune system provides an effective early defense, it is in many cases inefficient, and the elimination of escaped pathogens requires the second branch of immunity. The adaptive immune system is antigen-specific, therefore extremely precise, and a hallmark of higher animals[60]. It creates an immunological memory, which facilitates a more rapid response after recurring antigen exposure. However, this high specificity comes at the cost of speed, resulting in a lag time between exposure and response that can last from days to weeks[61]. Only if both systems work hand in hand, the host is able to combat infection while minimizing collateral damage.

Key players of the adaptive immune response are T and B cells, which express antigen-specific receptors on their membrane (Figure 1.3). For their activation, cells of the adaptive immune response require the help of so-called antigen presenting cells (APCs). After contact with the antigen, APCs, including macrophages, dendritic cells, and B cells, present fragments of this peptide via their MHC class I or II molecules on their surface. T cells can recognize the peptide-MHC complex, which leads to their activation and differentiation.

B cells develop in the bone marrow and are characterized by a unique B cell receptor consisting of a membrane bound Ig molecule and a signal transduction moiety[62]. Binding to the antigen occurs directly and results in proliferation and differentiation into antibody-secreting plasma cells or memory B cells. While the first group is rather short-lived and concentrates on the immediate secretion of large amounts of soluble antibodies, memory B cells have life spans ranging from several months to a lifetime[63]. A small population of B cells exhibits immunosuppressive functions and is referred to as regulatory B cells (Bregs). Together with regulatory T cells (Tregs) they dampen harmful immune responses by the production of inhibitory cytokines.

T cells mature in the thymus from common lymphoid progenitors and each of these cells is characterized by an antigen receptor of a single specificity[64]. Binding to the complementary antigen induces the differentiation into two major subtypes, marked by the cluster of differentiation (CD): cytotoxic T cells ($CD8^+$) or T-helper cells (Th, $CD4^+$). Cytotoxic T cells are activated via interaction with MHC class I molecules and are generally involved in the elimination of infected cells or tumor cells that express appropriate antigens[55]. They are directly toxic to their target cells by inducing apoptosis, either by releasing granzymes or binding of target-cell death receptors[65]. Th cells bind to MHC class II molecules and secrete a distinct selection of cytokines, which shape the immune reaction of other cells. They are functionally subdivided into Th1, Th2, Th17, Th9, and Treg cells, depending on their cytokine expression pattern[62]. Tregs are crucial mediators of immune homeostasis by their ability to dampen excessive immune responses. Generally, they can be grouped into natural Tregs (nTregs) and induced Tregs (iTregs)[66]. The first subset is generated in the thymus and depends on the expression of the forkhead box P3 transcription factor (Foxp3) for its development. On the other hand, iTreg cells differentiate from naïve $CD4^+$ T cells in extrathymic tissues and the expression of Foxp3 seems to be variable in this subtype[64]. Due to their development in the periphery, they appear to be able to recognize both self- and foreign antigens[67]. Both classes of Tregs depend on the anti-inflammatory cytokines IL-10, TGF-β, and IL-35 to mediate their suppressive function[68–70].

1.3.3 Interleukins mediate immune cell communication

As previously illustrated, immune protection is orchestrated by a myriad of cell types with dedicated, highly specialized roles. This division of labor is only possible due to the existence of an efficient and reliable communication based on soluble signals, the so-called cytokines. This inhomogeneous group of small, secreted proteins bind to their specific receptors in an autocrine and paracrine fashion, and downstream signaling is in most cases mediated by the Janus kinase (Jak) family of molecules that subsequently

phosphorylate the signal transducers and activators of transcription (STAT) family[64]. Cytokines are further subdivided into chemokines, interferons (IFNs), colony stimulating factors (CSFs), tumor necrosis factors (TNFs), and interleukins (ILs)[71].

ILs are a structurally diverse class of secreted proteins with more than 60 known members to date[72]. They are important players in immune cell communication and facilitate a wide variety of functions, including cell growth, differentiation, and activation. Despite low sequence homology, different attempts were made to classify ILs into subfamilies resulting in a partially confusing nomenclature[73]. Depending on literature, ILs are currently categorized into nine families based on structural similarities and receptor engagement[74]. Overlaps exists, as demonstrated by the IL-6/IL-12 family[75], the latter of which will be described in more detail in the following.

1.4 The Interleukin 12 family

First identified in 1989, IL-12 was the founding cytokine of a whole family[76]. It was originally described as “cytotoxic lymphocyte maturation factor” and “natural killer cell stimulatory factor”, based on its observed functions[76,77]. With the discovery of IL-23[78], IL-27[79], and IL-35[80] the family was extended and even today there are discussions about further existing members[81–83]. By their ability to bridge the innate and adaptive immune system, IL-12 family cytokines play a pivotal role in shaping our immune response. Severe pathologies, including cancer and immune-mediated inflammatory diseases, relate to impaired IL-12 family signaling and highlight its importance for a balanced immune system. Accordingly, IL-12 family cytokines are already targeted by antibodies in the clinics and currently investigated as promising approaches in immunotherapy. In the following, the IL-12 family will be described in more detail with emphasis on structure, function, and assembly.

1.4.1 Similar in structure yet distinct in function

The IL-12 family is unique amongst all ILs due to its strictly heterodimeric character, which is reflected by its evolutionary history: each family member consists of an α and a β subunit. The α subunits comprise a four-helical bundle structure typical for many human cytokines, including IL-2 and IL-4[84]. The four α-helices are arranged in an ‘up-up-down-down’ fashion, which originates from the anti-parallel orientation of two consecutive pairs of helices first described for porcine growth hormone[85]. The secondary structure of the β subunits is defined by a β-sheet architecture, similar to cell-surface type I and II cytokine receptors and comprises fibronectin type III and immunoglobulin domains (Figure 1.4A). And indeed, heterodimeric IL-12 cytokines have likely evolved from ‘classical’ cytokines and their receptors, the latter of which have become soluble

components of the cytokines themselves during evolution[86]. This is reminiscent of proteolytic shedding of cell-surface cytokine receptors, where shed receptor subunits pair with their cytokine partner to act as one signaling entity[87]. Each IL-12 cytokine consists of one α (IL-12α, IL-23α, IL-27α) and one β (IL-12β, Epstein-Barr virus-induced gene 3 (EBI3)) subunit. Extensive subunit sharing allows the formation of the four distinct heterodimers IL-12 (IL-12α:IL-12β), IL-23 (IL-23α:IL-12β), IL-27 (IL-27α:EBI3), and IL-35 (IL-12α:EBI3) (Figure 1.4B). This combinatorial complexity also applies to IL-12 family receptors, which are generally also heterodimers, formed by five different shared chains (Figure 1.4C). Binding of the corresponding IL induces receptor chain heterodimerization thereby activating Jak/STAT signaling pathways.

Subunit sharing on the level of cytokines and receptors may suggest closely related functions of IL-12 family cytokines, but this notion is misleading. IL-12 is a key immune-activating cytokine that is secreted from APCs and regulates pro-inflammatory functions of T cells, e. g. by inducing Th1 cells and $CD8^+$ cytotoxic T cells that are key for inflammatory reactions in fighting infections but also tumors[88]. IL-23 is the most pro-inflammatory IL-12 family member and plays a key role in inducing and maintaining Th17 cell populations that secrete the strongly pro-inflammatory cytokine IL-17[78]. IL-27 is an immune-modulatory cytokine, which on the one hand is able to promote Th1 differentiation and on the other hand suppresses pro-inflammatory Th17 cells and induces anti-inflammatory IL-10 producing Treg1 cells[79]. IL-35 is the only strictly immune-inhibitory family member. It suppresses conventional T cells and induces their conversion into suppressive Treg cells[89]. With the distinct and even opposing immune functions of its members, the IL-12 family holds a key role in balancing our immune system.

In addition to the four established IL-12 family members, new intra-family subunit pairings termed IL-Y and IL-39 have been reported, yet their natural existence is debated[82,90] (Figure 1.4D). In addition to the heterodimeric family members, isolated IL-12 family subunits can also perform immune-regulatory functions. Unpaired forms of IL-12β, IL-27α and EBI3 are secreted from certain cell populations and in certain species, and free IL-12β and EBI3 are often secreted in excess over IL-12, IL-23 and IL-27 heterodimers[79,80,91] (Figure 1.4D). Another exception of the rule is the unusual receptor repertoire of IL-35[92] (Figure 1.4D), which will be discussed in a later chapter. Together, this establishes a highly complex molecular network that underlies IL-12 family cytokine functions. Since IL-12 family members have very potent immune-regulatory functions, their secretion thus needs to be tightly regulated.

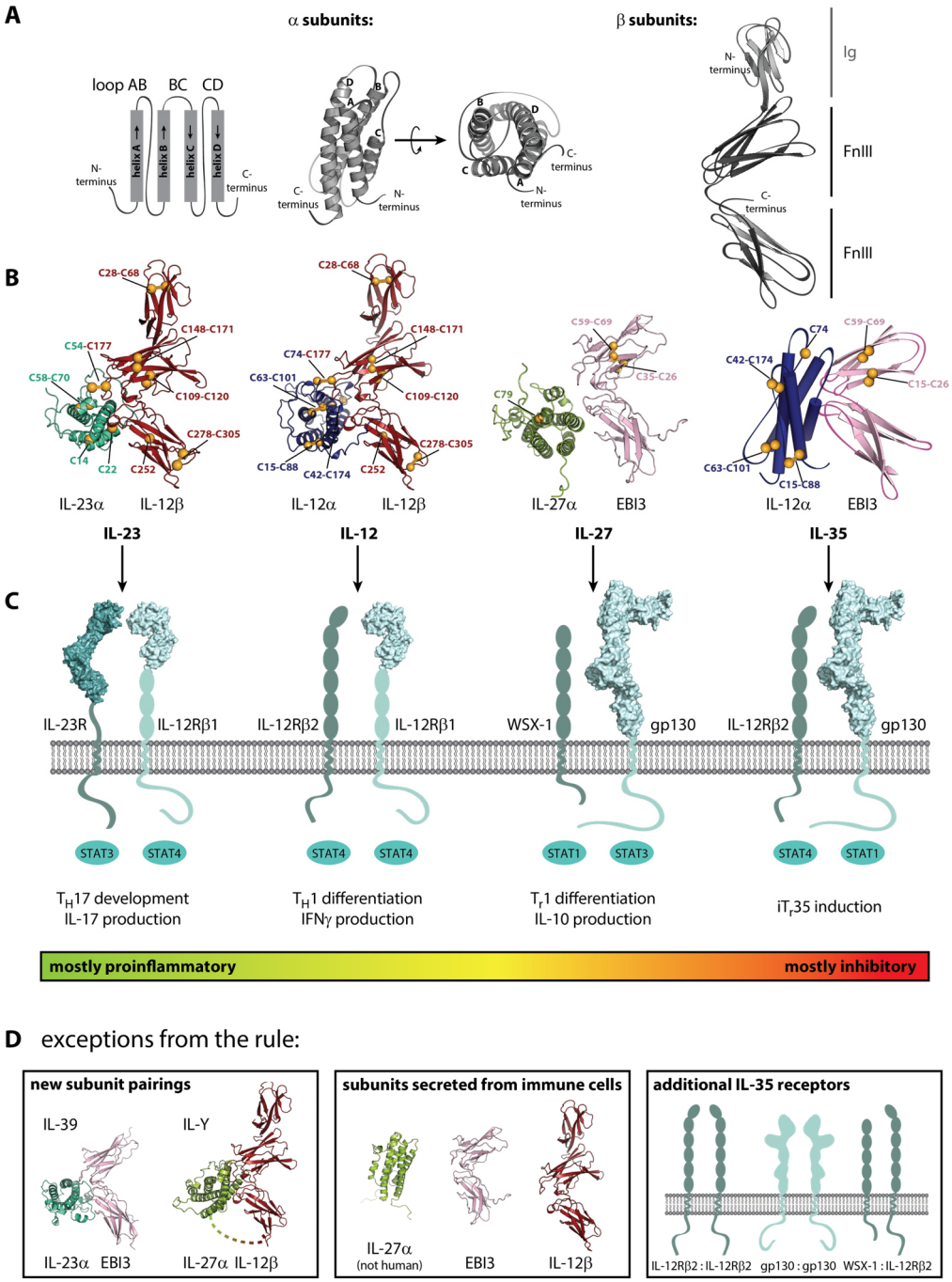

Figure 1.4: Setup of the human IL-12 family. A) Schematics of IL-12 family cytokine subunits. α subunits possess a four-helical bundle structure with an 'up-up-down-down' topology (arrows) of helix A to helix D connected via loops. β subunits contain two fibronectin type III (FnIII) domains and in case of IL-12β an additional N-terminal immunoglobulin (Ig)-like domain. B) The four IL-12 family members are made up of five different subunits: IL-23α, IL-12α, IL-27α, IL-12β, and EBI3. One α subunit pairs with one β subunit to form the respective heterodimeric cytokine IL-23, IL-12, IL-27, or IL-35. Crystal structures of IL-23 (PDB: 3D87) and IL-12 (PDB: 3HMX), a modeled structure of IL-27[93] and a hypothetical model for IL-35 are depicted. Position of free cysteines and disulfide bonds are indicated as orange spheres, numbering of residues corresponds to processed proteins. C) IL-12 family receptors pair to form functional receptors and are shared between ILs. High resolution structures for IL-23R (PDB: 5MZV), gp130 (PDB: 3L5H), and IL-12Rβ1 (PDB: 6WDP) are shown in surface representation, unresolved structures are depicted as schematic. Divergent immunological functions are mediated via different Janus kinase (Jak1, Jak2, Tyk2)-Signal Transducers and Activators of Transcription (STAT) pathways[94,95]. D) Left: New IL-12 family members IL-39 and IL-Y (covalently linked) are depicted as AlphaFold models. Middle: Single subunits IL-27α, EBI3, and IL-12β were reported to be secreted independent of their partner subunits. Right: In murine T and B cells, further IL-35 receptor pairings in addition to IL-12Rβ2/gp130 (see (C)) have been reported.

1.4.2 Detailed insights into IL-12 family cytokine assembly

Once translated, IL-12 family cytokines need to assemble to form bioactive αβ heterodimers. This assembly occurs in the ER, under the control of intricate mechanisms and machineries that support IL-12 cytokine formation. This is highly relevant due to the shared subunits IL-12 family cytokines use and the autonomous functions some unpaired subunits possess. At the same time, it provides another layer of control for immune system functions whose basis we are just beginning to understand. Biogenesis in the ER will be described in the following for each of the four established IL-12 family members.

Biogenesis of IL-12

Whereas IL-12α secretion depends on its pairing with a partner subunit, IL-12β can also be secreted in isolation, as a monomer and homodimer with likely autonomous and distinct immunological functions[96,97]. Other modes of IL-12α secretion and IL-12 formation are discussed, but their mechanistic basis remains to be defined[98]. Since IL-12α is expressed in many cell types, the dependency on a partner subunit generally allows regulation of IL-12 secretion. Recent work has revealed the molecular basis of this behavior (Figure 1.5A). If unpaired, IL-12α fails to acquire its native structure, forms non-native disulfide bonds and various molecular species including homodimers and oligomers. Due to this misfolding in isolation, unpaired IL-12α is recognized and retained in the cell by the ER chaperone machinery, including members of the PDI family[99–101]. In the light of this finding, it is interesting to note that IL-12α has three intramolecular disulfide bonds (Figure 1.4B), two of which are dispensable for assembly-induced folding and formation of IL-12[99]. This may argue that cysteine:PDI interactions play a role in regulating the secretion of IL-12 family members. The same applies to the disulfide bond that covalently stabilizes the interaction between IL-12α and IL-12β: it is dispensable for secretion of the heterodimer but may stabilize the heterodimer against dissociation once secreted[102]. If no pairing with IL-12β occurs, IL-12α will ultimately be degraded via ER-associated degradation, which involves the ERAD factor HERP and potentially also the PDI ERdj5 to reduce wrong disulfide bonds[99,103,104]. In contrast, if pairing with IL-12β occurs, IL-12α correctly folds and is efficiently secreted as heterodimeric IL-12[97,99]. Since the IL-12 cytokine family subunits are shared and structural changes occur upon heterodimer formation, the dimerization interface is of special interest. The interlocking topography of IL-12α and IL-12β is mediated by several charged residues via the helical face of the A and D helices and the AB-loop of IL-12α packing against loops 1 and 3 from domain 2 and loops 5 and 6 from domain 3 of IL-12β. Characteristic is the central

Arg189 residue of IL-12α helix D reaching into a deep arginine-binding pocket of IL-12β with Asp290 at its base to form an Arg-Asp salt bridge. Hydrogen bonds and hydrophobic interactions line the pocket, completing the interface[102] (Figure 1.5A). Considering this complex interface, it is noteworthy that, whereas isolated IL-12α has a strong tendency to misfold, no misfolding is observed in model cell lines upon co-expression of IL-12β[99]. This may argue for a substantial amount of structure also in unpaired IL-12α, where parts of the dimerization interface outlined above may be present. Such species could potentially form a reservoir for assembly with IL-12β, once expression of IL-12β begins, allowing for rapid pro-inflammatory responses.

Biogenesis of IL-23

Although IL-12 and IL-23 share the same β subunit, the interactions between IL-23α and IL-12β are somewhat different compared to IL-12. In comparison to IL-12α, the IL-23α subunit is tilted and rotated within the heterodimeric structure. As a consequence, IL-23α Arg159 of helix D forms the critical salt bridge to Asp290 of the IL-12β arginine-binding pocket and is surrounded by different hydrophobic contacts and polar interactions at the interface periphery than for IL-12. Furthermore, compared to the largely disordered AB-loop in IL-12α, IL-12β forms several more contacts with the AB-loop of IL-23α near its interchain disulfide bond linkage[105] (Figure 1.5B). IL-12β thus engages in at least two cytokines. Although the nature of the interactions that constitute the interface, i.e., charged interactions lined with hydrophobic and peripheral polar interactions, is conserved in IL-12 and IL-23, most of the interface is structurally distinct and unique. Like IL-12α, IL-23α depends on assembly with IL-12β for secretion and forms multiple redox species via non-native disulfide bonds in its absence[78,99,106]. Interestingly, like IL-12α, IL-23α populates dimeric species in the absence of IL-12β[99,106]. Assembly with IL-12β induces correct folding of the IL-23α subunit, a process into which structural insights could be obtained recently (Figure 1.5B). In the absence of IL-12β, IL-23α shows overall high flexibility and the first of its four helices (helix A) appears to be mostly unstructured. Upon assembly with IL-12β, helix A becomes structured and the remainder of IL-23α is stabilized[106]. These structural changes are coupled to an intricate chaperone recognition mechanism: helix A contains two free cysteines and binding sites for BiP. While unpaired, the sites are free to engage PDIs, including ERp44 as well as BiP[106]. This confers ER retention of unassembled IL-23α. Once assembled with IL-12β, due to the folding of helix A, the chaperone binding sites are buried within the native structure, allowing release from the ER. The single internal disulfide bond within IL-23α is needed for IL-23 secretion, whereas, like for IL-12 the covalent disulfide linkage of IL-23α with IL-12β is

dispensable for subunit interaction and secretion[106]. IL-23 has thus developed an intricate mechanism that allows the ER chaperone machinery to oversee its assembly, and which likely plays an active role in tuning it. In the light of this complex assembly control, it is noteworthy that biological functions for unpaired IL-23α have also been described[107]. It will be important to reveal how IL-23α in this case obtains a biologically active structure.

Biogenesis of IL-27

IL-27α shows close structural homology to the aforementioned α subunits with the exception of a unique glutamic acid stretch (poly-Glu loop) in the CD loop[108]. In isolation, human IL-27α is not secreted but requires interaction with EBI3 for assembly-induced secretion as part of the heterodimeric IL-27 complex[79]. The underlying mechanisms were recently revealed (Figure 1.5C). Structural dynamics of the poly-Glu loop and exposed hydrophobic residues causes BiP binding to IL-27α, which results in ER retention and rapid degradation of the unassembled IL-27α subunit via ERAD[109]. Only upon co-expression of EBI3, IL-27α is stabilized and hydrophobic regions are buried within the helical structure, which releases it from chaperone-mediated retention[109]. Once assembled, the heterodimeric complex further traverses through the Golgi where human IL-27α becomes *O*-glycosylated before secretion[79,110]. Unlike IL-12α and IL-23α, human IL-27α has only one single cysteine residue located in helix B. It therefore lacks an internal disulfide bond[109]. Within the IL-27 interface the conserved and solvent-exposed Trp97 of IL-27α interacts with Phe97 of EBI3. This aromatic interaction is further supported by salt bridges between the rather positively charged surface of IL-27α (e. g. Arg216) and the negatively charged surface of EBI3 (e. g. Glu159, Asp210)[93,111] (Figure 1.5C). Although an IL-27 crystal structure is yet not available, critical interface residues could be verified via mutation and the slightly rotated conformation of α and β subunits compared to IL-12 and IL-23 could be confirmed by molecular docking[93], further extending the structural setups found for IL-12 cytokine heterodimerization.

Biogenesis of IL-35

In contrast to IL-12, IL-23, and IL-27, for which detailed structural insights into the heterodimerization interface are available, the IL-35 interface remains completely undefined (Figure 2D). Furthermore, IL-35 is largely unaffected by amino acid substitutions that do affect other IL-12 family members[112]. IL-35 thus seems to have a distinct pairing interface compared to all other IL-12 family members. In agreement with this notion, bacterially produced IL-12α could form IL-12 but not IL-35, which may be due to the lack of glycosylation in bacterially produced proteins, as recent data suggest

glycosylation to be important for IL-35 formation[110,113]. Together, these findings indicate that complex formation of IL-12α and EBI3 might be weaker and that it clearly differs from the other family members. Despite these differences and analogous to IL-12β, EBI3 is required to release IL-12α from ER retention and for subsequent secretion of the heterodimeric complex[80] (Figure 1.5D). In contrast to IL-12, IL-35 secretion is by far less efficient, which might be due to the inefficient secretion of EBI3 itself compared to IL-12β and likely due to differences in complex formation[80,112]. Furthermore, EBI3 not only induces secretion of IL-12α but is itself more efficiently secreted in the presence of IL-12α, although the effect is less pronounced. This mutual secretion of an α and β subunit can further be observed for IL-27 arguing that, for human IL-27 and IL-35, both the α and the β subunit are labile in isolation and need each other for stabilization and efficient export. In agreement with this, the lectin chaperone CNX associates with EBI3[114]. Normally, CNX mostly acts on membrane-associated proteins[115]. It is thus interesting to note that, although lacking a membrane anchor, EBI3 has been described to localize to the cell surface[114], potentially also in a complex with IL-23Rα[116] and that co-localization of IL-12α and EBI3 on the surface of tolerogen-specific regulatory T cells has been reported[117]. In agreement with a somewhat special role of EBI3 in the IL-12 family, EBI3 itself seems to have chaperone-like functions also on other proteins. By binding of EBI3 to CNX and IL-23Rα in a peptide-dependent manner, IL-23Rα expression is augmented, and degradation is reduced[116]. Even though CNX is necessary for this EBI3-mediated folding, and further data to back up these ideas are needed, EBI3 may thus perform intracellular roles independent of the cytokines to which it contributes.

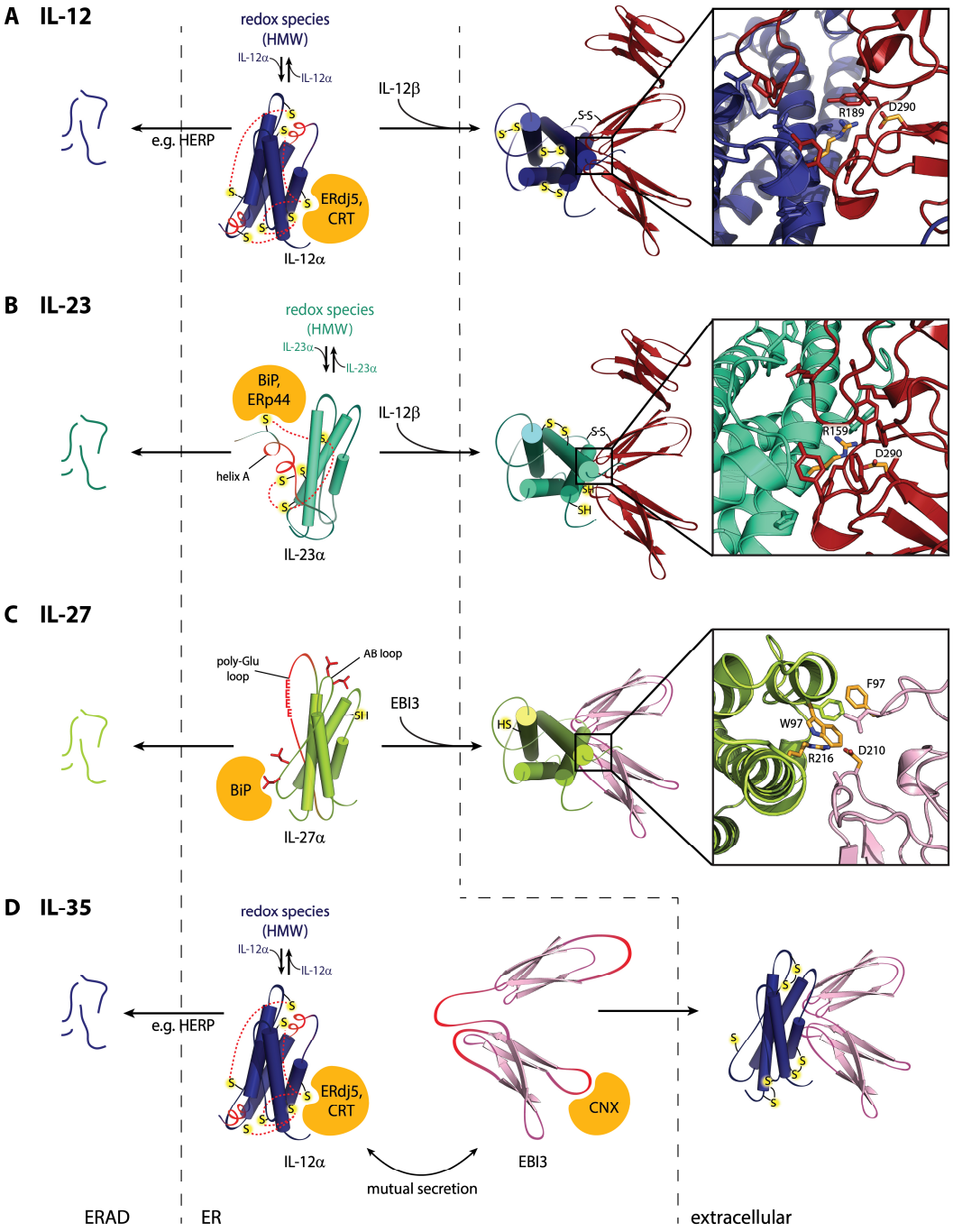

Figure 1.5: Molecular mechanisms of human interleukin (IL)-12 cytokine biogenesis. Critical elements (highlighted in red) and molecular chaperones involved in IL-12 family ER quality control are depicted for each member. Successful biogenesis of heterodimeric ILs leads to secretion (extracellular), otherwise subunits are degraded via ER-associated degradation (ERAD). Interfaces are shown as magnified images (boxes) (IL-12, PDB: 3HMX; IL-23, PDB: 3D87; IL-27 model[93]). Critical interface residues are labelled and marked in orange. Residues forming salt-bridges or hydrophobic interactions in the direct surrounding of the core are shown as sticks. Details on the subunit pairings: (A) In isolation, IL-12α forms non-native disulfide bonds (red dashed lines), thus misfolds and forms redox species of high molecular weight (HMW), including dimers. Upon pairing with IL-12β, the correctly folded, disulfide-linked IL-12 is secreted. The arginine-binding pocket with its charged interaction is highlighted in the interface structure. (B) Helix A of IL-23α is unstructured, highly flexible and erroneous disulfide-linked redox species of IL-23α are formed in absence of IL-12β. The interface of the covalently linked IL-23 heterodimer with its charged interaction is magnified. (C) Hydrophobic residues of IL-27α are exposed in the absence of EBI3 due to the flexibility of the AB and poly-Glu loops[109]. Subunit interactions of the non-covalently linked IL-27 are mediated via aromatic and charged residues. (D) For IL-35, IL-12α and EBI3 mutually enhance their secretion. IL-12α in isolation behaves like described in (A). EBI3 is bound by the chaperone calnexin (CNX). For IL-35, depicted as hypothetical model, neither its structure nor the interface is known.

Biogenesis of IL-12 family cytokines: general principles

For human IL-12 family cytokines, a clear principle has emerged in recent years to underlie their biogenesis: α subunits are incompletely structured in isolation and hardly, if at all, secreted by themselves. Instead, isolated α subunits are targeted for degradation. In contrast, human β subunits are secretion-competent on their own, induce structure formation in their designate partner α subunit and thus regulate trafficking and export of the heterodimeric cytokine. Interestingly, in other species this behavior is reversed, e. g. mouse IL-27α is readily secreted, but mouse EBI3 depends on IL-27α co-expression for secretion[79,93]. Notwithstanding these differences, we generally have one secretion-competent and one assembly-dependent subunit. This principle is of great biological relevance: the secretion competent IL-12 family subunits, be it the α or the β subunit, often perform autonomous immune-modulatory functions. This argues that this behavior has likely evolved due to functional needs of e. g. maintaining balanced immune reactions. A further general principle that emerges from recent studies on IL-12 family cytokines is that, quite unusual for secreted proteins, they have several disulfide bonds that are dispensable for secretion. These cysteines may thus be present to engage cellular chaperone machineries to regulate and control IL-12 family cytokine assembly, which is crucial in the light of distinct biological functions but subunit sharing.

1.5 IL-35 – stranger in the family?

In 1996 Devergne et al. discovered a novel gene encoding soluble receptor with homology to ciliary neurotrophic factor receptor (CNTFR) (30%) and IL-12β (27%)[114]. This protein, which lacks a membrane anchoring motif and was initially found to be secreted by Epstein-Barr virus (EBV)-transformed lymphocytes, was designated Epstein-Barr-virus-induced gene 3 (EBI3) and gave rise to further expansion of the IL-12 family. Only one year later, the same group demonstrated non-covalent binding of EBI3 to IL-12α, the first hint of a new, IL-12 related cytokine[80]. Immunological properties were not yet identified, although high levels of both subunits in human placental trophoblasts already suggested a possible immunosuppressive role[118]. For another decade, the physiological relevance of the cytokine remained elusive until Collison et al. found high expression levels of IL-12α and EBI3 in a distinct set of Tregs[89]. In their study they were able to assign functions to this newly established cytokine and named it IL-35. After the discovery of IL-23 (in 2000[78]) and IL-27 (in 2002[79]), IL-35 was the latest addition to the IL-12 family.

1.5.1 Composition of IL-35 and its unusual receptor repertoire

The heterodimeric IL-35 is built by non-covalent interaction of IL-12α and EBI3, which are both shared subunits of other IL-12 family members (IL-12, IL-27). While experimental IL-12 and IL-23 structures are known and computational, experimentally-verified models of IL-27 exist, further supported by recent insights into its receptor-binding[119], IL-35 remained elusive to detailed insights into its architecture. Since all α and β subunits share high structural homology, it would appear that the mode of interaction within the IL-12 family follows a conserved mechanism. Indeed, structural studies concerning the rest of the family propose a common principle of interaction with residues of helices A and D from the α subunit interacting with the hinge region between the two FnIII domains of the β subunit[93,102,105,111]. These so-called Site 1 interactions can be further found in the IL-6 family of cytokines, where the helical cytokine interacts with its primary receptor (e. g. IL-6 and IL-6Rα)[120]. To shed light on the IL-35 heterodimerization mode, Jones et al. followed this path by an extensive mutational screen in 2012[112]. By mutating residues within the conserved interface, they were able to affect the complex formation of IL-12 and IL-27 but no such effect could be observed in case of IL-35. Though complex formation between mouse IL-12α and human EBI3 (and vice versa) indicate at least an interspecies conserved interface[112], their results indicate that the character of interaction for IL-35 substantially differs from the other family members. It should be noted however, that the experiments in this study were performed on cell lysates only. Since only secreted IL-35 is fully post-translationally modified, this might affect its complex formation as well. In agreement with this, recombinant IL-12α purified from bacteria was indeed not able to form a complex with EBI3, in contrast to IL-12β[113]. In the same study, linked constructs of essential members of the IL-6 and IL-12 cytokine families were generated, which were all secretion competent and biologically active with only one exception: Hyper-IL-35 remained secretion incompetent, regardless of using different cell types or linker lengths[113]. Collectively, IL-35 assembly differs in several aspects from the other family members, and it is questionable whether only weak interactions are the source of this fundamentally different behavior of IL-35. One possible explanation could be the existence of additional factors that are required for effective subunit interaction and secretion but have yet been unexplored. Another hypothesis was provided by Sullivan et al., who observed IL-12α and EBI3 co-localization on the surface of tolerogen-specific Tregs[117]. In their study they postulate an alternative and atypical mode for IL-35 signaling, in which both subunits are present on the surface of extracellular vesicles (EVs) in association with the tetraspanin CD81[117]. While their results provide an explanation for the difficulty in producing a stable,

soluble, bioactive cytokine, it remains to be investigated whether this mode of secretion is limited to specific cell types and if only a minor fraction of secreted IL-35 is associated to EVs.

Chain sharing is a common principle within the IL-12 family and furthermore extends to their receptors and downstream signaling components. Considering that IL-35 shares subunits with IL-12 and IL-27, it was reasonable to assume that also receptor chains of both cytokines are utilized. Indeed, a heterodimer consisting of IL-12Rβ2 and gp130 could be identified on T cells as the main IL-35 receptor[92]. Surprisingly, even in the absence of one chain, immunological effects were still detectable and IL-35 was consequently reported to signal via a receptor triad consisting of IL-12Rβ2 homodimers, gp130 homodimers, and IL-12Rβ2:gp130 heterodimers, though the latter is required for its maximal suppressive capacity[92]. Only a few years later, Wang et al. further expanded this receptor repertoire of IL-35, by identification of a novel heterodimeric receptor comprising IL-12Rβ2 and IL-27Rα on the surface of B cells[121]. Since B cells additionally express gp130 the discovery of this completely new receptor combination was quite unexpected. Downstream signaling in T cells revealed that IL-35 induced the formation of a STAT1:STAT4 heterodimer, which induces a positive feedback loop by binding the *Il12a* and *EBI3* promoters, thereby inducing their gene transcription[92]. Furthermore, signaling via IL-12Rβ2 homodimers resulted in STAT1 and engagement of gp130 homodimers in STAT4 phosphorylation, respectively[92]. Although gp130 signaling via homo- and heterodimers is described for other proteins[122–124], it comes with a surprise that in case of IL-35, STAT3 phosphorylation was obsolete, which has not been reported for another cytokine that signals via gp130[75]. By a receptor shuffling approach, Floss and colleagues reconstituted all described IL-35 receptors but detected a more canonical signaling via STAT1 and STAT3[125]. However, the genetic lack of STAT4 in Ba/F3 cells confines the global impact of their observations. Likewise, IL-35 signaling in B cells was also mediated via STAT1 and STAT3 activation and the authors of the same study detected STAT3 phosphorylation in T cells, as opposed to Collison et al.[121]. This is further supported by Liu et al., who confirmed that trophoblast derived IL-35 activates STAT1 and STAT3 phosphorylation in human T cells but STAT4 phosphorylation was absent[126]. Moreover, in metastatic tumor cells, macrophage secreted IL-35 revealed activation of STAT6 in an IL-12Rβ2 dependent way[127]. This multi layered complexity indicates that IL-35 might utilize different receptors and signaling components depending on cell type, source, and species. Despite minor inconsistencies, it can be noted that IL-35 signals via multiple receptors that are constructed by IL-12Rβ2, gp130, and IL-27Rα and a selection of different STAT molecules, including STAT1, STAT3, STAT4, and STAT6.

How one cytokine is able to signal via this apparent receptor complexity is currently ill-defined and further research is necessary to establish the physiological relevance of all receptor combinations.

1.5.2 The complex immunobiology of IL-35 and its role in disease

Within the IL-12 family, IL-35 holds an outstanding position as its immunobiology contrasts with the other family members. While IL-12, IL-23, and IL-27 are reported to be primarily produced by APCs, IL-35 was initially found to be secreted by Foxp3+ Treg cells only[105]. With the steadily increasing number of IL-35 based studies, further IL-35 producing cell types were identified, such as Breg cells[121,128], DCs[129,130], and macrophages[127]. Additionally, IL-35 secretion was also detected by non-immune cells like tumor cells[131], or placental trophoblasts[126], suggesting an even broader impact of this cytokine.

Not only the producer cells are remarkably variable, but also the IL-35 mediated effects are considerably distinct from those of its siblings. IL-35 is the only IL-12 family member with exclusively inhibitory functions and was initially found to cause primary immunosuppression of effector T cell responses[89]. Moreover, its inhibitory role is not only mediated by suppressing the proliferation of effector T cells but also by their conversion into IL-35 producing Foxp3$^-$ cells, a process called infectious tolerance[70]. This novel iT$_R$35 population which was generated by stimulation of naïve T cells with IL-35, did not require other suppressive cytokines like IL-10 or TGF-β for its conversion, but IL-35 secretion was necessary for its maximal suppressive activity[70]. The inhibitory impact of IL-35 is not only restricted to CD4$^+$ Tregs but further includes a CD8$^+$ tumor specific Treg line in prostate cancer patients[132]. Moreover, Treg derived IL-35 has been demonstrated to suppress Th17 responses by inhibiting the differentiation of CD4$^+$ T cells into Th17 effector cells[133]. Besides T cells, IL-35 was also shown to act on other cell populations, including B cells, for which the conversion into IL-35$^+$ Breg cells has been reported[121,128], Macrophages[134], and DCs[135].

With its high suppressive capacity, IL-35 is associated with the development of a variety of diseases. Generally, increased IL-35 levels are often connected with immune escape and are therefore present in different cancers[136]. In acute myeloid leukemia, patients revealed significantly higher IL-35 plasma levels than the healthy donor control group, and there was a positive correlation between the severity of the disease and IL-35 expression levels[137]. Furthermore, murine models of melanoma and colorectal carcinoma indicate increased IL-35 production by tumor-infiltrating Treg cells[70]. On the other hand, impaired IL-35 signaling stands in direct relation to different autoimmune

diseases. Recent studies revealed that the IL-35 plasma concentration in patients with psoriasis, a chronic, inflammatory skin disease, is significantly lower than in healthy individuals[134,138]. A similar coherence of decreased IL-35 levels is reported for asthma and chronic obstructive pulmonary disease[139], atherosclerosis and coronary artery disease[140], and multiple sclerosis[141].

The broad impact of IL-35 on different immune-related diseases indicates its crucial role in a balanced and healthy immune system. With an increasing number of studies, the incorporation of IL-35 based therapeutic strategies in the near future might hold a great clinical potential. Gene therapy, injection of rIL-35, transfer of iT_R35 cells, or blocking antibodies are possible applications that are currently studied in murine disease models and first encouraging results are reported. In cancer, promising achievements were reported among others by Turnis et al[136]. In their study they investigated the effect of IL-35 blockade in murine models of human melanoma, colon adenocarcinoma, and metastatic tumors and were indeed able to detect limited tumor growth and enhanced T cell memory responses. By intraperitoneal injection of rIL-35, Niedbala and coworkers demonstrated a significant reduction in the incidence and intensity of a murine model of collagen-induced arthritis, and Wirtz et al. used gene therapy of a single-chain IL-35 construct to treat inflammatory bowel disease in mice resulting in decreased symptoms of colitis and a reduction of inflammatory markers[133,142]. Another desirable feature of IL-35 is its capability to induce a potent inhibitory iTreg cell population. In immunotherapy there is a substantial interest in *ex vivo* generated iTreg cells and their potential in antigen-specific regulatory populations, however current approaches are limited due to complex generation protocols and short half-lives[143,144]. In their study, members of the Vignali lab were however able to convert T cells into iT_R35 cells *in vitro* with highly potent and stable characteristics *in vivo*[70].

While the increasing number of therapeutical approaches imply a bright future for IL-35 in the clinics, discernible limitations and contradictions in the current literature are reasons to proceed with caution. The immunobiology of IL-35 still seems to be ill-defined, as there are additional studies suggesting anti-tumor capacities in colon cancer and hepatocellular carcinoma, thereby disagreeing with the exclusive inhibitory role of IL-35[145,146]. It should be further mentioned that in order to study IL-35, knockout models come with the complexity of IL-12 family subunit and receptor sharing, and research based on knockout cells requires very careful interpretation to dissect the observed effects. So far, successful purification of recombinant mouse or human IL-35 has not been described and is one of the major hindrances in the field. The questionable activity and purity of currently available IL-35 versions should be re-evaluated and thus the linked

publications. Another significant limitation is the lack of high-quality antibodies against IL-35. Recent data reveal serious drawbacks in previous published studies based on an allegedly IL-35 specific antibody despite its demonstrated unspecificity against IL-35[147]. However, although accurate assessment of IL-35 reagents and controls is inevitable, the overall trend indicates that increased IL-35 serum levels correlate with a state of immune escape in different tumors, while reduced levels seem to be associated with chronic infections and autoimmunity.

1.5.3 IL-12α and EBI3: Roles beyond IL-35?

IL-12 family cytokines are strictly heterodimeric, yet, as always, there are exceptions to the rule. A prominent example is IL-12β, which is secreted independently of a partnering subunit and is considered to signal both through receptor competition in an antagonistic way[148], as well as in a direct agonistic fashion[149]. It is therefore conceivable that the principle of independent subunits also extends to other members of the IL-12 family. The second β subunit, EBI3, can likewise be secreted in absence of IL-12α or IL-27α, although its secretion is comparatively inefficient[79,80,150]. To investigate the effect of unassembled EBI3, Chehboun et al. used recombinant human or mouse EBI3 purified from bacteria and demonstrated complex formation of rEBI3 with IL-6, thereby inducing IL-6 *trans*-signaling via gp130[151]. In their study, EBI3 was able to promote the pro-inflammatory signaling of IL-6. However, high concentrations were required and the lack of glycosylation might impair with natural EBI3 functions[110]. On the other hand, EBI3 promoted anti-inflammatory effects were observed in NK cells, although the authors could not distinguish between IL-35 or EBI3 derived signaling[152]. Furthermore, Dambuza et al. used insect cell purified mouse EBI3 in a murine model of experimental autoimmune uveitis (EAU) and demonstrated inhibition of $CD4^+$ proliferation by EBI3 to a similar degree as IL-35[153]. In the same study, equally purified mouse IL-12α showed a similar behavior and was additionally able to induce expansion of Bregs *in vivo* dependent on IL-12Rβ2*[153]*. Experimental autoimmune encephalomyelitis (EAE) mice showed reduced symptoms after IL-12α administration connected with a reduced number Th17 cells, investigated by the same group[154].

Thus, while immunological effects have been demonstrated for IL-12α and EBI3 that are in some cases even overlapping with IL-35 mediated effects, it is still unclear, whether the physiological concentration in a human setting is sufficient to promote signaling. Especially in case of IL-12α, which is believed to be secreted in an assembled complex only, a physiologically relevant role seemed unlikely. Since EBI3 lacks the interchain cysteine homologous to IL-12β, the IL-35 complex might be less stable and subunit

dissociation in principle possible, which could provide a source of IL-12α. Structural insights into assembly and secretion of IL-35 and its subunits are thus urgently required for a better understanding of their immunobiology and are a prerequisite to shed light on the complex signaling of IL-35.

1.6 Aims of this work

This thesis aims to address two different research objectives, which both focus on the underlying molecular and cellular principles that allow for biogenesis and signaling of IL-12 family cytokines.

IL-35 shares structural hallmarks of the IL-12 family, yet its biological function, its receptor repertoire, its structure, and the cells that produce IL-35 contrast sharply with the characteristics of other family members (IL-12, IL-23, and IL-27). Thus, a main goal of this work was to extend our knowledge of this cytokine. One important question that has remained unanswered is how one single cytokine can signal via multiple receptors and trigger such diverse immunological effects. We therefore aimed to investigate human IL-35 assembly and secretion in detail on a cellular level. By comparison with IL-12 and IL-27, which both share one subunit with IL-35, characteristic features of IL-35 should be revealed. Furthermore, to circumvent functional studies with heterogenous protein solutions, we intended to purify human IL-35 components from mammalian cells and test them in a next step under controlled *in vitro* conditions in immunologically relevant primary cell models.

Given the immunological relevance of the IL-12 family and its increasing relevance in immunotherapy, its glycosylation pattern – as one major structural modification – has however remained ill-defined. In the second goal of this thesis, we intended to uncover common and distinct principles of protein glycosylation for each member of the IL-12 family. A particular focus on cytokine assembly and secretion should reveal if the absence of glycosylation affects the formation of heterodimers and subsequently their assembly induced secretion. Moreover, we aimed to analyze the functionality of glycosylation-deficient mutants in relevant reporter cells to assess the impact of glycosylation for IL-12, IL-23, IL-27, and IL-35 comparatively.

2 Interleukin 35 subunits are anti-inflammatory cytokines

2.1 Introduction

Interleukins are key signaling molecules of our immune system and are classified into families based on structural similarities[73]. The Interleukin (IL) 12 family consists of four established members (IL-12, IL-23, IL-27, IL-35) and is assigned to one family due to its strictly heterodimeric character, a unique feature amongst the more than 60 ILs known to date[72]. Each member comprises an α subunit with a cytokine-characteristic four-helix bundle fold (IL-12α, IL-23α, IL-27α) and a β subunit composed of two fibronectin (Fn) III domains (EBI3) with an optional immunoglobulin (Ig) domain (IL-12β). Structurally, heterodimerization within the IL-12 family resembles binding of IL-6 family cytokines, which share the four-helix bundle fold, to class I cytokine receptor chains, which comprise fibronectin and Ig domains[102,105,155], implying their evolution from interleukin:receptor pairs[86]. Only five subunits are required to build the four distinct IL-12 heterodimers, which is enabled by extensive subunit sharing (IL-12: IL-12α/IL-12β, IL-23: IL-23α/IL-12β, IL-27: IL-27α/EBI3, IL-35: IL-12α/EBI3). Analogously, this combinatorial complexity extends to the IL-12 family receptors, which are also heterodimers formed by five different chains (IL-12Rβ1, IL-12Rβ2, IL-23R, IL-27Rα, gp130). Binding of the corresponding interleukin induces receptor chain heterodimerization thereby activating Jak STAT signaling pathways[94].

Despite sharing many structural hallmarks and even subunits, the effects of the four family members are remarkably diverse and even opposing. The mostly pro-inflammatory cytokines IL-12 and IL-23 are drivers of inflammation via Th1 differentiation and Th17 development, respectively[156]. IL-27 performs immunomodulatory roles on the one hand by promoting Th1 differentiation and on the other hand by suppressing pro-inflammatory Th17 cells as well as induction of anti-inflammatory IL-10 producing Tregs[157]. Conversely, IL-35 is the only strictly inhibitory family member, which is reflected by its ability to suppress conventional T cells and converting them into immunosuppressive Tregs[158]. Besides the four established IL-12 family members, reports suggest the existence of further subunit combinations[81,83,90] and some subunits are even functional by themselves[109,159–161].

Despite its many important biological functions, IL-35 remains the most incompletely characterized member of the IL-12 family. In contrast to its siblings, IL-35 is predominantly produced by Tregs and Bregs, to a lesser extent by DCs and placental trophoblasts, and is mainly responsible for the immunosuppressive capabilities of these different cell types[158]. Due to its inhibitory character, IL-35 plays a pivotal role in keeping

immune reactions in check, but when signaling is impaired IL-35 is also associated with a broad range of different immune-related diseases. In cancer, IL-35 is able to induce tumor growth by enhancing myeloid cell accumulation and promoting angiogenesis[162]. High serum levels of IL-35 can be found in melanoma[70,162], breast[163,164], pancreatic[165–167], and lung[168] cancer. On the other side, a variety of autoimmune, inflammatory, and allergic diseases are linked to reduced circulating IL-35 levels, underlining its importance in maintaining self-tolerance[169,170]. While the biological functions of IL-35 are under intensive investigation, only little is known about its structure and receptor repertoire. Experimental structures of isolated IL-12[102] and IL-23[105] and experimentally validated structural models for IL-27[93,111] reveal a conserved mode of interaction for cytokines of the IL-12 family. In contrast, even comprehensive studies based on this model have failed to identify the heterodimerization interface of IL-35[112], which is thus clearly distinct from the other family members. Furthermore, recent studies point towards independent functions of IL-35 subunits, but molecular insights remain limited[151–154]. Lastly, IL-35 signaling does not occur via a single heterodimeric receptor, as described in case of the other IL-12 family cytokines, but four possible receptor chain combinations (IL-12Rβ2:gp130, IL-12Rβ2:IL-12Rβ2, gp130:gp130, IL-12Rβ2:IL-27Rα) are currently reported as the receptor repertoire for IL-35 signaling[92,121]. Although the biological functions of IL-35 qualify it as a highly attractive therapeutic molecule and target, our biochemical lack of knowledge on IL-35 currently limits further progress in the field.

In this study, we investigated the mode of secretion for IL-35 and found that its two subunits IL-12α and EBI3 can either be secreted in assembled or unassembled forms. Instead of a strictly heterodimeric protein – the hallmark of IL-12 family cytokines – IL-35 appears to be a compound cytokine. We thus established procedures to recombinantly produce the human IL-12α and EBI3 subunits from mammalian cells and investigated their effects on human PBMCs and an HDM induced airway inflammation model of human monocyte derived macrophages. Stimulation with our recombinant proteins resulted in the downregulation of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNFα. Surprisingly, while our recombinant subunits were able to reconstitute functional IL-12 and IL-27, they showed no sign of interaction to form IL-35, therefore further manifesting their independent character and the exceptional position of IL-35 in the IL-12 family. In summary, the results obtained in this study point towards a new mode of IL-35 secretion, which allows not only the heterodimeric IL-35 but also its subunits IL-12α and EBI3 to act as independent cytokines.

2.2 Results

2.2.1 IL-35 subunits mutually promote their secretion

Since its discovery and functional characterization, IL-35 has remained a structurally ill-defined cytokine. This has hindered progress in understanding this potent immunosuppressive molecule, but also in potential application or targeting it in the clinics. IL-35 consists of IL-12α and EBI3, subunits that are shared with IL-12 or IL-27, respectively (Figure 2.1). While the subunits of IL-12 are connected by an intermolecular disulfide bridge, IL-27 and IL-35 are non-covalent heterodimers.

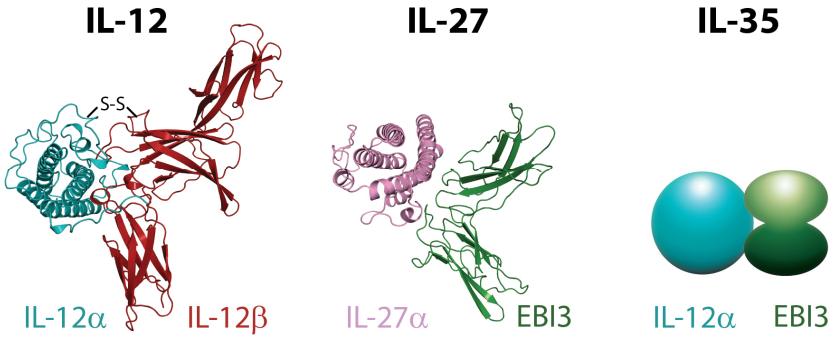

Figure 2.1: IL-35 shares subunits with IL-12 and IL-27. IL-35 (no structural model available) shares its α-subunit IL-12α with IL-12 (PDB: 3HMX), and its β-subunit EBI3 with IL-27 (experimentally verified homology model[93]).

All human IL-12 family α subunits are retained in cells in isolation and depend on interaction with their cognate β subunit to be secreted. Human IL-35 is no exception to the rule and in accordance with the first report on IL-35, we observed a titratable direct correlation between EBI3 expression and IL-12α secretion and *vice versa*, although the effect on EBI3 secretion is less pronounced (Figure 2.2A and B).

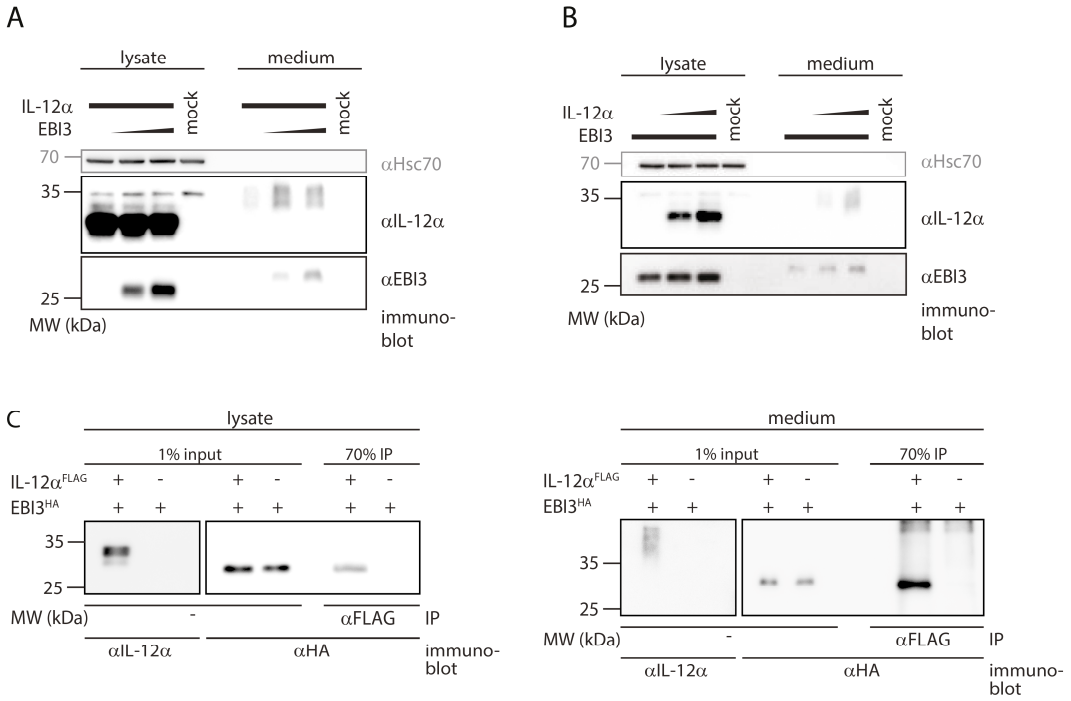

Figure 2.2: IL-12α and EBI3 mutually promote their secretion and form IL-35. To assess mutually induced secretion of IL-12α and EBI3, constant DNA amounts of IL-12α were co-transfected with increasing DNA amounts of EBI3 (A), or vice versa (B). C) Co-immunoprecipitation of HA-tagged EBI3 with FLAG-tagged IL-12α in cell lysates and in the medium verifies assembly for these two proteins. Constructs are expressed in HEK293T cells. One representative immunoblot is shown.

In agreement with this finding, we detected interaction of IL-12α and EBI3 in human cells and after their secretion into the medium (Figure 2.2C). The observed molecular weight shift upon secretion (Figure 2.2) can be attributed to modification of the glycans when traversing the Golgi after release from ER retention. Thus, at a first glance, IL-35 shares key features with other IL-12 family members: assembly-induced secretion of the subunits and their interaction in co-immunoprecipitation experiments. Recent data, however, point towards independent biological functions of IL-12α[153,154] and EBI3[150,151] as well as the possibility that IL-12α may assemble with IL-12β outside cells to form IL-12[98]. Particularly in case of IL-12α it is unclear, how the subunit can be secreted in an unassembled form, since only marginal amounts are detected when expressed without its cognate partner (Figure 2.2A). This prompted us to a more thorough investigation of IL-12α and EBI3 secretion upon co-expression. Surprisingly, in contrast to other IL-12 family members our analyses revealed a fundamentally different behavior of IL-35: When either IL-12α or EBI3 were equipped with a C-terminal KDEL sequence (IL-12α^{KDEL} or EBI3^{KDEL}, respectively), these subunits were retained in cells (Figure 2.3A and B). This was expected as a C-terminal KDEL sequence generally leads to ER retention of the respective protein due recognition and retrieval from post-ER compartments[171]. However

and quite unexpectedly, EBI3^{KDEL} could still induce the secretion of IL-12α, and IL-12α^{KDEL} also still increased secretion of EBI3 (Figure 2.3A and B).

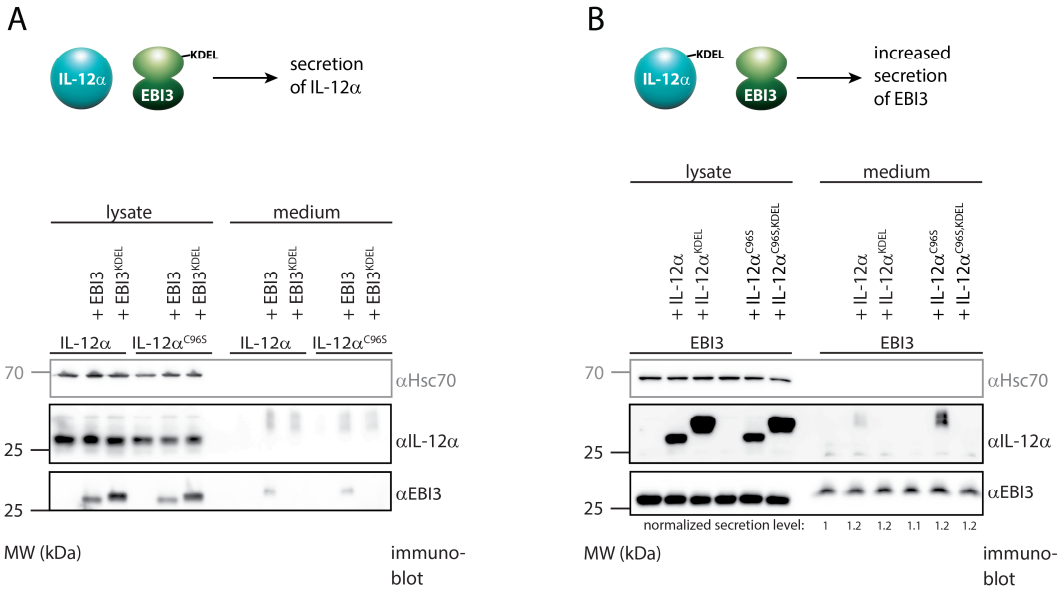

Figure 2.3 IL-35 subunits can be secreted independently when one subunit is ER retained. A) EBI3 induces the secretion of IL-12α even when it is retained in the ER (via a C-terminal KDEL sequence). EBI3-induced secretion is observed for wild-type IL-12α and a variant lacking the cysteine that forms an interchain disulfide bond in IL-12 (C96S). B) The same as in A), only that IL-12α was furnished with a KDEL ER-retention sequence and secretion of EBI3 was monitored. EBI3 secretion was slightly increased by co-expression with both IL-12α and the C96S variant. Quantifications of EBI3 secretion are shown below the blot. Constructs are expressed in HEK293T cells. One representative immunoblot is shown.

No difference in the secretion pattern was observed when we used a cysteine mutant of IL-12α that lacks the free cysteine in IL-35 (IL-12α^{C96S}). These results indicate that IL-35 subunits depend on each other to mutually induce their secretion but are not necessarily secreted as a heterodimer. This behavior is unique for IL-35 and in contrast to all other known pairings within the IL-12 family that share subunits with IL-35. For IL-12, IL-12β^{KDEL} did not induce the secretion of IL-12α, even after deletion of interchain-disulfide bond forming cysteines which are dispensable for IL-12 secretion[99], and led to co-retention of IL-12α instead (Figure 2.4A). Analogously, the same pattern could be observed for IL-27, where IL-27α^{KDEL} did not increase the secretion of EBI3 but instead also co-retained it (Figure 2.4B). *Vice versa*, it has been recently demonstrated that EBI3^{KDEL} does not permit secretion of unassembled IL-27α but instead leads to co-retention of IL-27α in cells[93]. Once they assembled, IL-12 and IL-27 seem to be stable heterodimers, even in the absence of an intermolecular disulfide bridge in case of IL-12.

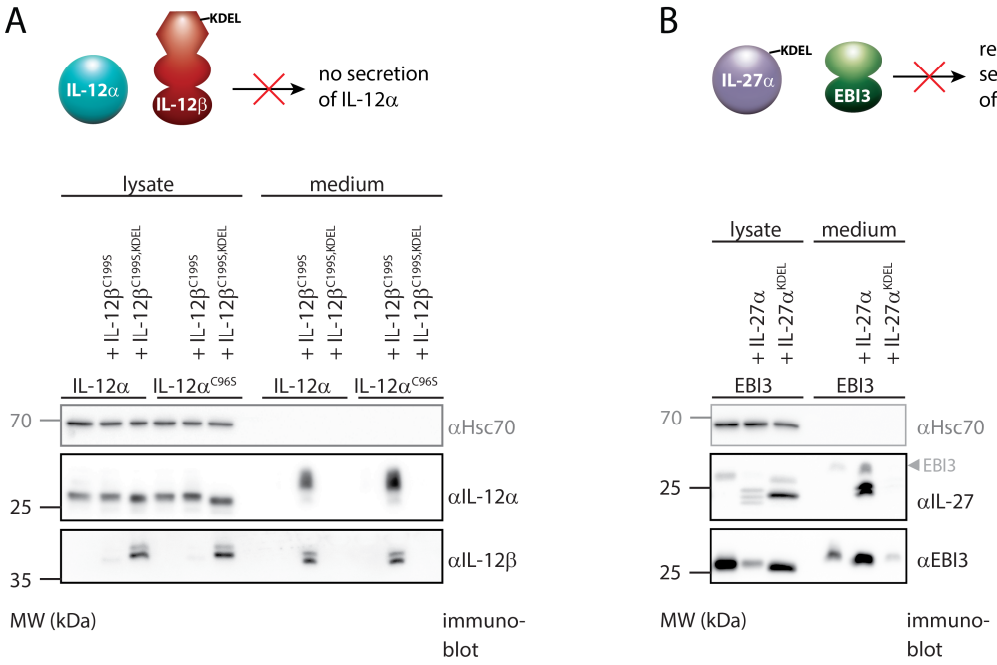

Figure 2.4: IL-12 and IL-27 subunits cannot be secreted independently when one subunit is ER retained. A) A similar analysis for IL-12 reveals that IL-12β with an ER retention sequence does not induce secretion of free IL-12α, but instead co-retains it in the cell. The same is observed for the combination of IL-12α^{C96S} with IL-12β^{C199S}, with both proteins lacking the cysteines that form the interchain disulfide bond in IL-12. B) Co-expression of wildtype EBI3 and IL-27α leads to the secretion of IL-27. When IL-27α was ER-retained (IL-27α^{KDEL}), EBI3 secretion was reduced, accordingly. Constructs are expressed in HEK293T cells. One representative immunoblot is shown.

Together, our data reveal a strikingly different behavior of IL-35 in comparison to other IL-12 family members and suggest that cells expressing IL-12α and EBI3 may in fact secrete IL-35 as a compound cytokine, including the heterodimer but also unassembled subunits. We therefore searched for the existence of free IL-12α and EBI3 in the medium of cells expressing both IL-35 subunits without utilizing an ER-retention sequence. And indeed, after pulldown of EBI3 we were able to detect both unassembled EBI3 and IL-12α in the supernatant of co-expressing cells (Figure 2.5). Comparing IL-12α+EBI3^{HA} to IL-12α and IL-12α+EBI3^{HA,KDEL}, the band intensities for IL-12α+EBI3^{HA} after the final IL-12α-IP suggest that a significant part of IL-12α is unassembled, since EBI3 was not co-immunoprecipitated. The levels of unassembled IL-12α seem to be much higher when co-transfected with EBI3, suggesting that EBI3 is required for secretion but not always part of the complex. Furthermore, EBI3 could be found in the input II but was absent after IL-12α-IP, indicating free EBI3, as well. Taken together, our data indicate that cells expressing IL-12α and EBI3 secrete assembled IL-35, but also free IL-12α and EBI3.

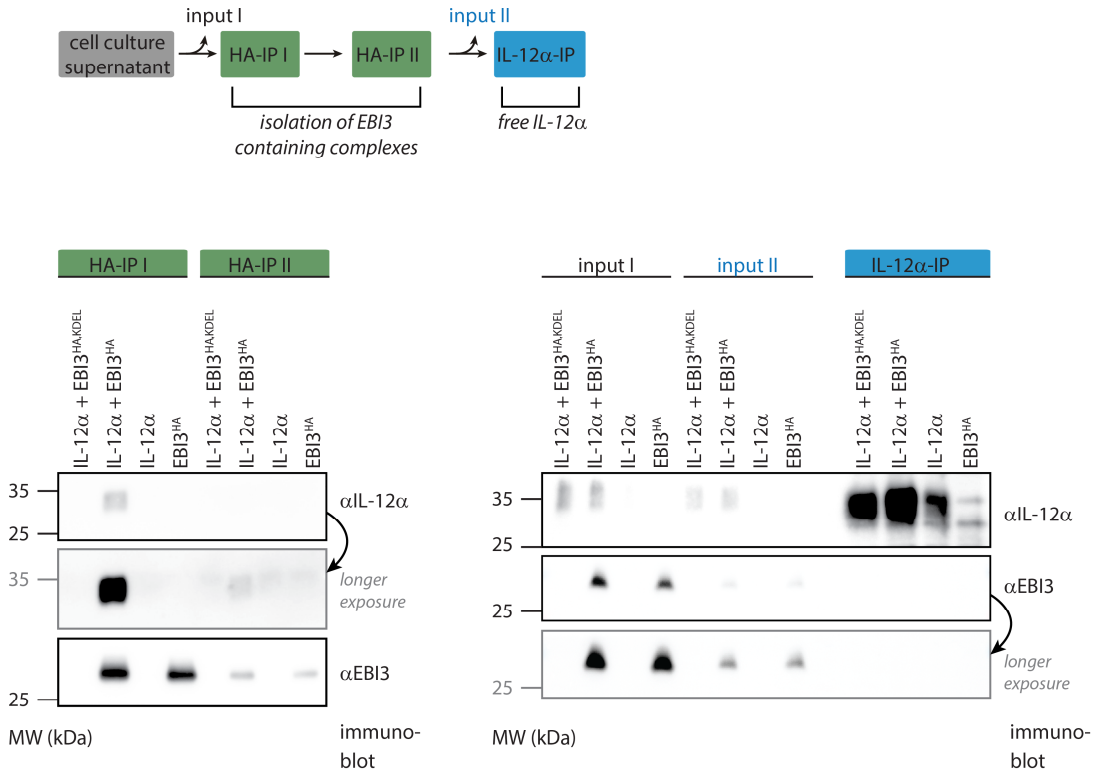

Figure 2.5: Unassembled secreted subunits can be detected in supernatants of IL-12α EBI3 co-transfected cells. Free IL-12α can be detected in medium samples after pulldown of EBI3. IL-12α and EBI3 are co-transfected and cell supernatants are applied to two consecutive HA-IPs to isolate EBI3 containing complexes. IL-12α is also co-immunoprecipitated as can be seen in the IL-12α blot after HA-IP I and II indicating pulldown of IL-35. The final IL-12α-IP reveals remaining IL-12α in the medium that is not interacting with EBI3. Band intensities imply a significant higher amount of free IL-12α when co-expressed with EBI3 (or $EBI3^{KDEL}$) than when transfected in isolation of EBI3. Constructs are expressed in HEK293T cells. One representative immunoblot is shown.

2.2.2 IL-12α and EBI3 are stable cytokines in isolation

The existence of secreted unassembled but stable IL-12α and EBI3 potentially qualifies them as independent cytokines secreted from IL-35 producing cells. To further support our hypothesis of stable cytokines, we aimed to purify human IL-12α and human EBI3. Both subunits are glycosylated and contain disulfide bonds, requiring for production in mammalian cells and purification from their supernatants. IL-12α, however, is not secreted in isolation (Figure 2.2A) but instead degraded[99] and EBI3 is only barely secreted in isolation (Figure 2.2B). Moreover, even upon co-expression only small amounts of secreted protein are detected (Figure 2.2), which has precluded purification of IL-35 thus far, also in our hands (data not shown). We thus devised strategies that allowed for the production of IL-12α and EBI3 in sufficient quantities from mammalian cells by utilizing the chain sharing of IL-12 family members. IL-12, which also comprises IL-12α (Figure 2.1), is secreted efficiently and we therefore developed a protocol to purify IL-12 lacking the intermolecular disulfide bridge in mammalian cells and subsequently

separate IL-12α^{C96S} from IL-12β^{C199S} (Figure 2.6A). Analogously, we purified IL-27 and finally separated EBI3 from IL-27α^{L162C}, a secretion competent mutant that was previously described in our laboratory[109], (Figure 2.6B) and obtained in both cases milligram-quantities of glycosylated pure protein.

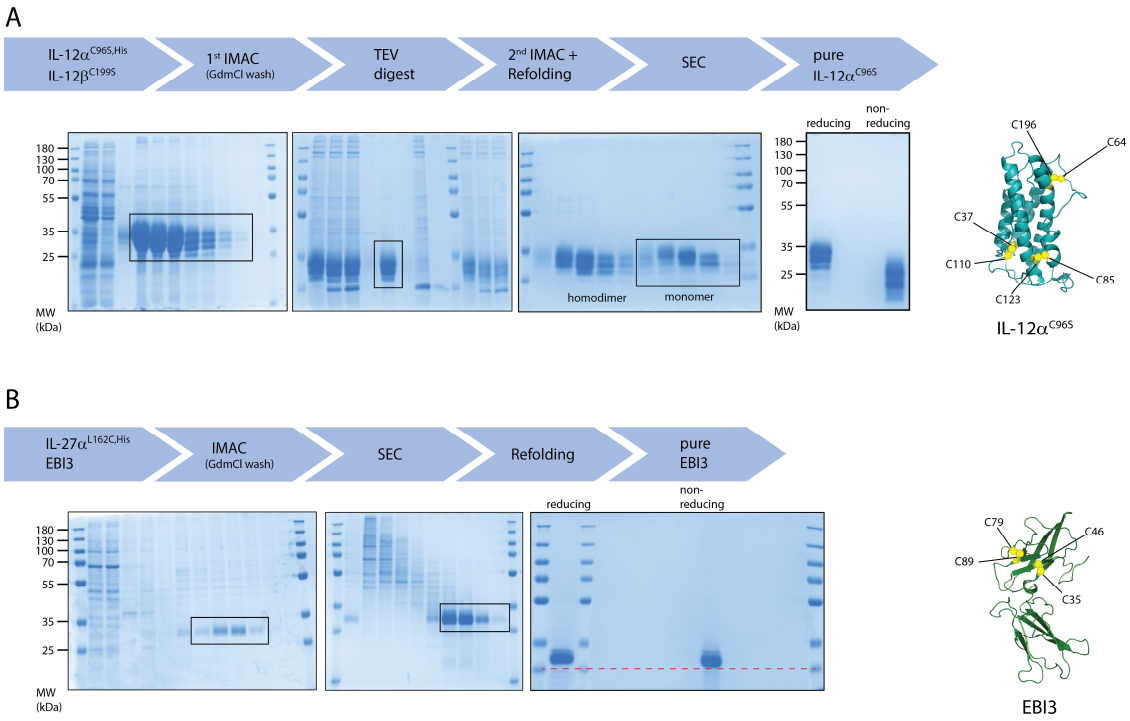

Figure 2.6: Recombinant IL-12α^{C96S} and EBI3 are pure proteins. A) Detailed purification strategy with the corresponding Coomassie stained SDS-gels of IL-12α^{C96S} and B) EBI3. Pooled fractions are shown in rectangles. Structural models indicate positions of cysteines. For IL-12α^{C96S}, the molecular weight shift under non-reducing conditions indicates formation of internal disulfide bridges (A). In case of EBI3, the minor shift in molecular weight is a result of disulfide bridge formation between cysteines that are only separated by 9 or 10 residues (B).

Since our purification strategies require washing with chaotropic agents and subsequent refolding, we used far-UV circular dichroism spectroscopy to verify the α-helical structure of IL-12α^{C96S} and the predominantly β-sheet comprising structure of EBI3, in agreement with available structural models[93] (Figure 2.7A and B). To support the idea of stable, soluble subunits, we used analytical ultracentrifugation. This approach revealed a predominantly monomeric state of IL-12α^{C96S} and EBI3 in solution (Figure 2.7C and D). Additionally, we found both proteins to be relatively stable with melting temperatures of 47±0.2 °C (IL-12α^{C96S}) and 50±0.2 °C (EBI3), respectively (Figure 2.7E and F).

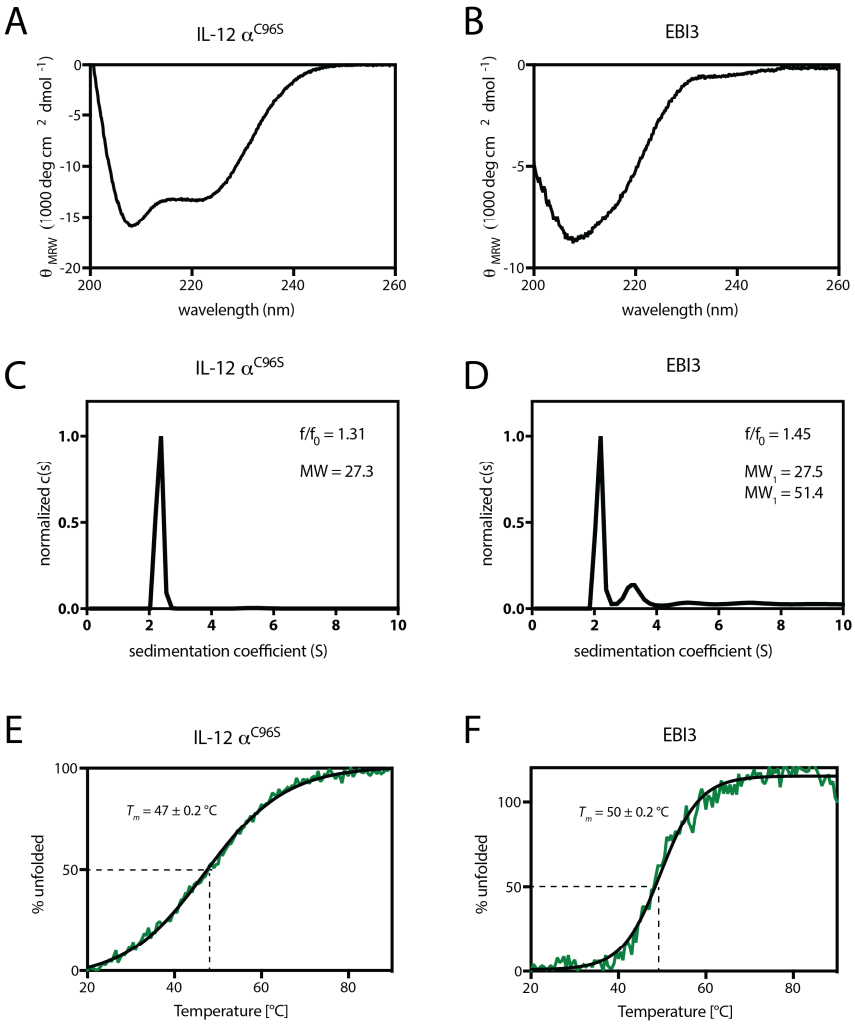

Figure 2.7: IL-12α^{C96S} and EBI3 are stable, soluble proteins. Far-UV CD spectrum of IL-12α^{C96S} (A) and EBI3 (B) reveal the signature of α-helical and predominantly β-sheet containing proteins, respectively. Analytical ultracentrifugation experiments of IL-12α^{C96S} (C) and EBI3 (D) confirm a mostly monomeric state of both proteins, with frictional ratios of 1.31 (IL-12α^{C96S}) and 1.45 (EBI3) and calculated molecular weights of 27.5 kDa (IL-12α^{C96S}) and 27.5 kDa (EBI3). For EBI3 a smaller second peak indicates the existence of a homodimeric species with a calculated molecular weight of 51.4 kDa. IL-12α^{C96S} unfolds cooperatively with a melting temperature of 47 ± 0.2 °C (E) and EBI3 with a melting temperature of 50 ± 0.2 °C (F).

We could also demonstrate via co-immunoprecipitation (Co-IP), that IL-12α^{C96S} produced in this manner remained assembly-competent with IL-12β^{C199S} (Figure 2.8A). The reconstituted IL-12 was able to induce receptor heterodimerization (Figure 2.8B) and STAT4 phosphorylation in NK-92 cells (Figure 2.8C). In contrast, signaling via receptor binding or STAT phosphorylation did not occur when cells were stimulated with IL-12α^{C96S} or IL-12β^{C199S}, respectively, supporting that our assays are sensitive for a functional IL-12 heterodimer. The same holds true for our recombinant EBI3, which dimerized with murine IL-27α to reconstitute IL-27 (Figure 2.8D) and was functional in a NanoBRET assay to demonstrate receptor heterodimerization (Figure 2.8E) and STAT1 phosphorylation in BL-2 cells (Figure 2.8F). Again, EBI3 or mIL-27α alone were not able

to initiate IL-27 signaling in our assays. Together, this provides strong evidence that our protocol yielded properly structured proteins that are stable in solution and can form functional heterodimeric cytokines.

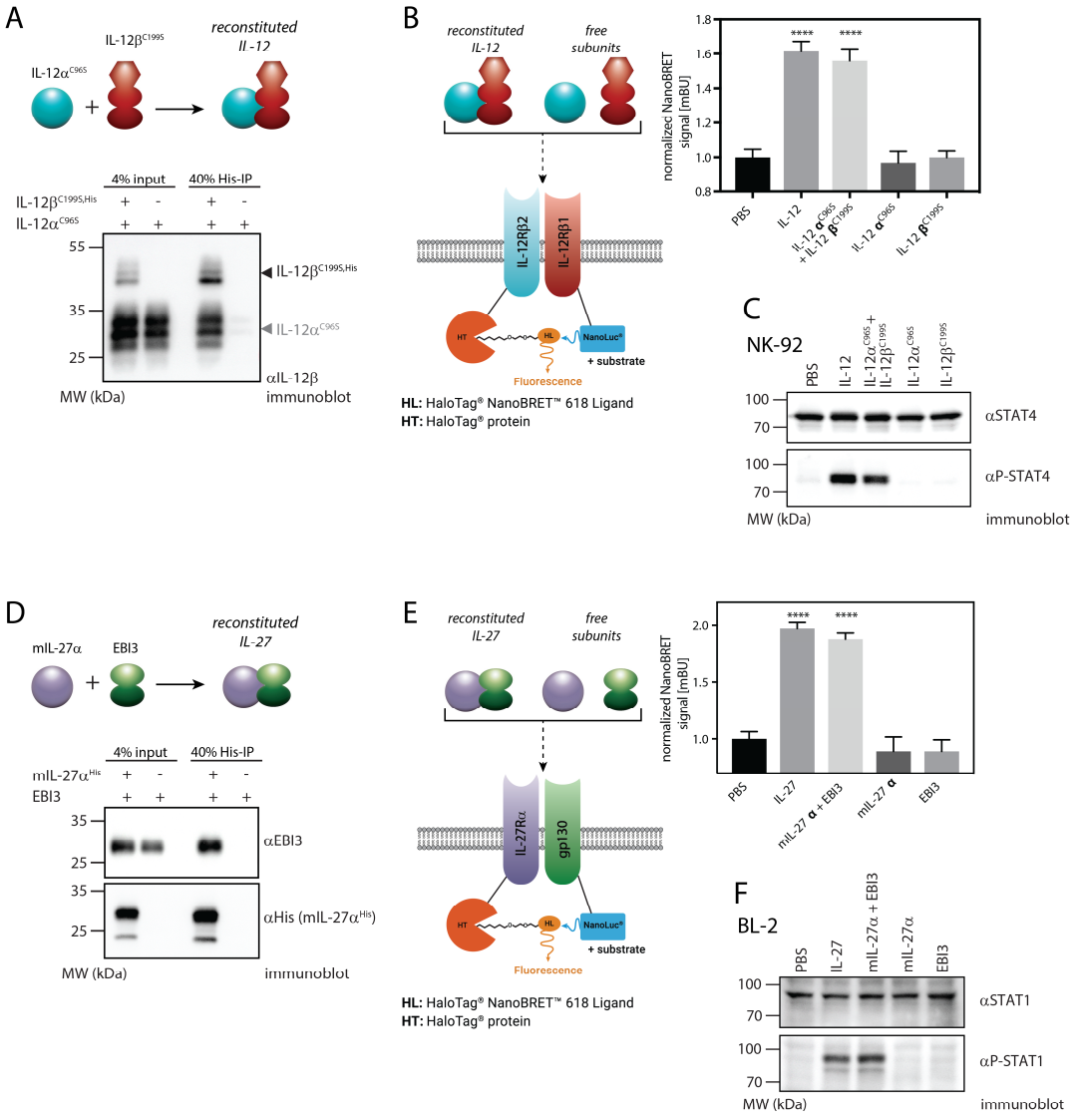

Figure 2.8: Recombinant human IL-12α^{C96S} and EBI3 interact with their partner subunits IL-12β and IL-27α to form functional IL-12 and IL-27. A) IL-12α^{C96S} and IL-12β^{C199S} were incubated, and their interaction was verified via co-immunoprecipitation. B) Reconstituted IL-12 was able to induce receptor heterodimerization monitored by NanoBRET signal. C) Both, IL-12 and its reconstituted analog were able to induce STAT4 phosphorylation in NK-92 cells. D) Murine IL-27α (mIL-27α) and EBI3 were incubated, and subsequent co-immunoprecipitation revealed complex formation. Receptor binding (E) and downstream signaling by STAT1 phosphorylation in BL-2 cells (F) of reconstituted IL-27 were indistinguishable from IL-27 and indicate a biologically active heterodimer.

It came thus as a great surprise, that although IL-12α^{C96S} and EBI3 were correctly structured, both proteins did not assemble with each other to reconstitute IL-35 *in vitro*. The lack of interaction was demonstrated by Co-IP, analytical ultracentrifugation, and hydrogen-deuterium exchange mass spectrometry experiments (Figure 2.9A-C). In

summary, these detailed biophysical and functional analyses revealed that IL-12α^{C96S} and EBI3 can be purified as stable, correctly folded proteins but do not reconstitute IL-35 *in vitro*.

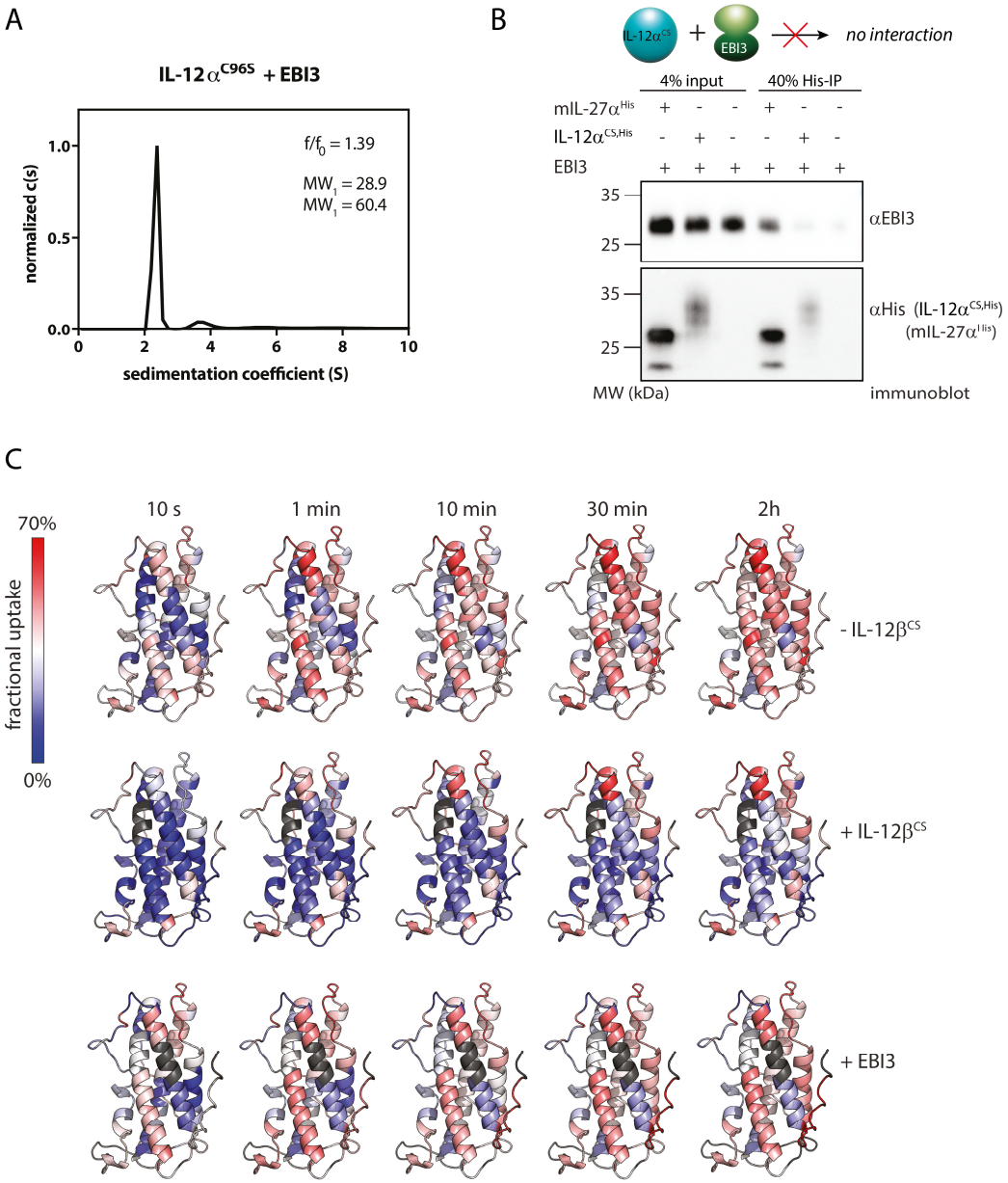

Figure 2.9: Recombinant IL-12α^{C96S} and EBI3 do not interact to form IL-35. A) Analytical ultracentrifugation experiment after incubation of IL-12α^{C96S} and EBI3 reveals mostly monomeric proteins and does not indicate the formation of a heterodimeric complex (compare also with Figure 2.7C and D). B) IL-12$\alpha^{C96S,His}$ and EBI3 do not co-immunoprecipitate in contrast to EBI3 and mIL-27α^{His}. C) Hydrogen/deuterium exchange experiments reveal IL-12α^{C96S} stabilization through complex formation with IL-12β^{C199S}. In contrast, incubation with EBI3 does not result in a stabilized conformation of IL-12α^{C96S}. IL-12α^{C96S} is colored according to the fractional uptake of H/DX measurements. Blue color indicates a low (less flexible and potentially shielded regions) and red colors a high (flexible and solvent accessible regions) fractional uptake. Gray: no sequence coverage in H/DX measurements.

Although sharing key signatures of the IL-12 family, we were thus not able to reconstitute IL-35 under the same conditions that we applied for the reconstitution of IL-12 and IL-27.

It is therefore likely, that IL-35 complex formation significantly differs from all other family members. This is also supported by mutational studies in our group (data not shown) and Jones et al.[112], where a large number of mutations based on different approaches (e. g. structural homology within the IL-12 family and computational docking) showed no effect on IL-35 heterodimer disruption, in contrast to other family members. Recent data suggest a novel secretion mode for IL-35: The authors of this study propose an infectious tolerance mechanism in which $CD81^+$ extracellular vesicles (EV), which also present IL-12α and EBI3 on their surfaces, are released from Treg cells and perform their inhibitory capacity by either primary or secondary suppression[117]. To test this hypothesis, we used the same Co-IP protocol as previously applied (Figure 2.2C) to detect whether CD81 is associated with the IL-35 complex. Although we were able to detect CD81 in the cell lysate, it was not present in the pulldown fraction and completely absent from medium samples (Figure 2.10). This indicates, that in our experiments IL-35 was not associated with CD81. To exclude that the detection of weak assembly was impaired due to the experimental setup, we integrated a chemical crosslinker in our Co-IP protocol to capture transient interactions. In line with our previous result, mass spectrometry of the DSSO-crosslinked interactome of secreted IL-35 also indicated lack of CD81, as well as CD39 or CD73, which were also mentioned by Sullivan et al. as putative IL-35 coated EV markers[117] (data not shown). Since HEK293 cells principally secrete EVs[172], a cellular effect could be excluded and we were not able to support the idea of IL-35 decorated EVs.

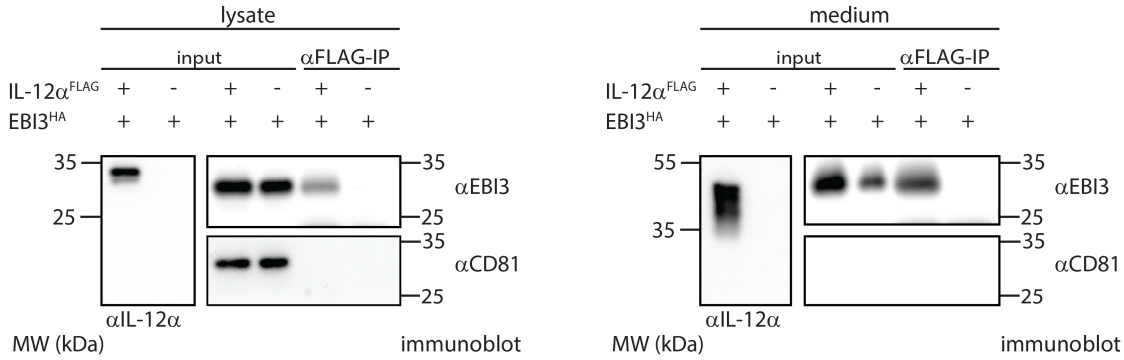

Figure 2.10: CD81 is not associated with IL-35. CD81 is expressed in HEK293T cells and can be detected in the input of lysate fractions. Co-immunoprecipitation experiments indicate complex formation between IL-12α and EBI3 but CD81 is not part of the complex. Medium samples do not contain CD81. Constructs are expressed in HEK293T cells. One representative immunoblot is shown.

2.2.3 IL-12α and EBI3 act as anti-inflammatory cytokines

The fact that IL-35 could not be reconstituted *in vitro* can be seen as a further indication of our hypothesis that IL-12α and EBI3 are, once secreted, independent, stable cytokines. In agreement with our finding that both subunits can be secreted individually and therefore occur naturally, detailed functional studies of IL-12α and EBI3 are urgently demanded. IL-35 is described as an immunosuppressive cytokine that affects signaling in various cell types[158]. Since our data show secretion of unassembled IL-12α and EBI3 from cells producing both IL-35 subunits, we wondered if immunological effects could also be exerted by these subunits as recent studies indicate[151,153,154]. Pure human IL-12α^{C96S} and EBI3 derived from mammalian cells have not been investigated in this regard but were now available to us. Thus, for investigation of immunological effects of IL-12α^{C96S} and EBI3 we used primary human peripheral blood mononuclear cells (PBMCs). PBMCs, which are composed of a heterogenous population of blood cells, including dendritic cells, monocytes, and lymphocytes allow us to study effects on cells of both, the innate and adaptive immune system. To assess anti-inflammatory effects, we treated PBMCs with 100 ng/ml LPS to induce an inflammatory response and assessed potential immunomodulatory capacities of IL-12α^{C96S} and EBI3 (Figure 2.11A). In agreement with a globally anti-inflammatory role, we detected reduced levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNFα both on mRNA and on protein levels in PBMCs after treatment with IL-12α^{C96S} or EBI3 upon LPS challenge, although in several cases not significantly (Figure 2.11B and C).

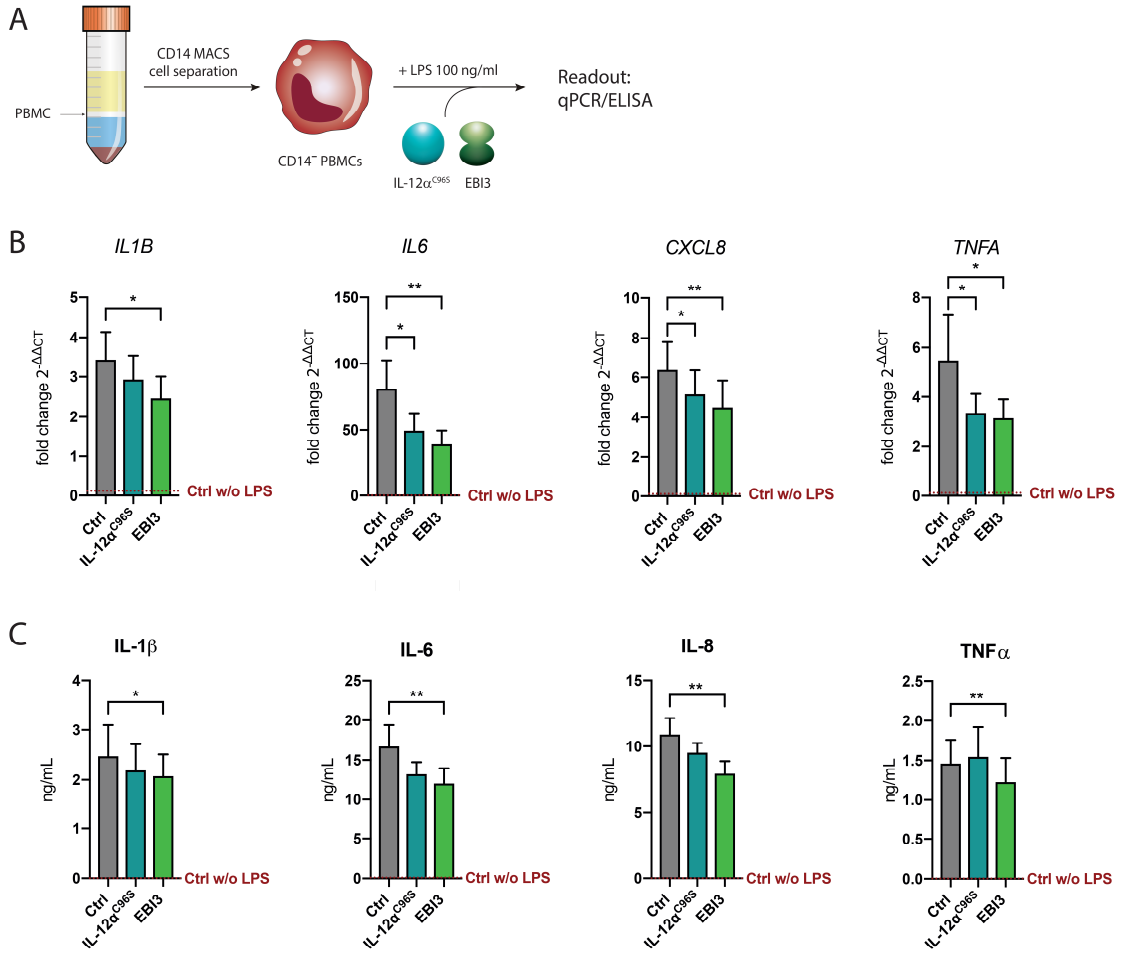

Figure 2.11: IL-12α^{C96S} and EBI3 act as immunosuppressors in LPS stimulated PBMCs. A) CD14$^-$ PBMCs were isolated from human blood and stimulated with LPS (100 µg/ml) and IL-12α^{C96S} or EBI3 (10 ng/ml). B) Gene expression of *IL1B*, *IL6*, *CXCL8*, and *TNFA* (qPCR) from LPS stimulated PBMCs after cytokine stimulation (n = 6 to 10). C) Concentration of secreted IL-1β, IL-6, IL-8, and TNFα (ELISA) in supernatants from LPS stimulated human PBMCs after cytokine stimulation (n = 10). Data are presented as means + SEM. Statistical significance was determined by Friedmann test. *P < 0.05; **P < 0.01.

Our results indicate potential immunosuppressive capacities for both IL-35 subunits in the presence of a pro-inflammatory stimulus. As mainly pro-inflammatory cytokines of the monocyte/macrophage compartment were affected by both subunits, we decided to concentrate further on this cell subset. Thus, to test the effects of IL-12α^{C96S} and EBI3 in a disease-relevant setting involving monocytes, we isolated CD14$^+$ monocytes from PBMCs and differentiated them into alveolar like macrophages by stimulation with GM-CSF and TGF-β[173] (Figure 2.12A). Alveolar macrophages are the most abundant leukocyte population in the lung and are described as essential players in the development of allergic airway diseases such as asthma[59], where IL-35 (and thus possibly IL-12α or EBI3) play important roles[139,174,175]. To investigate potential effects of IL-12α and EBI3 in this model we treated the differentiated alveolar like monocyte-derived macrophages with 10 µg/ml house dust mite extract (HDM), one of the most

frequent allergens to cause allergic airway inflammation. In this activated state, we stimulated the cells with either IL-12α^{C96S} or EBI3 (Figure 2.12A). This led to a significant downregulation of gene transcription and translation of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNFα, like in PBMCs (Figure 2.12B and C). Hence, IL-12α^{C96S} and EBI3 act as immunosuppressors in a state of active immune-inflammation and this function was also present in an *in vitro* model of allergic airway inflammation.

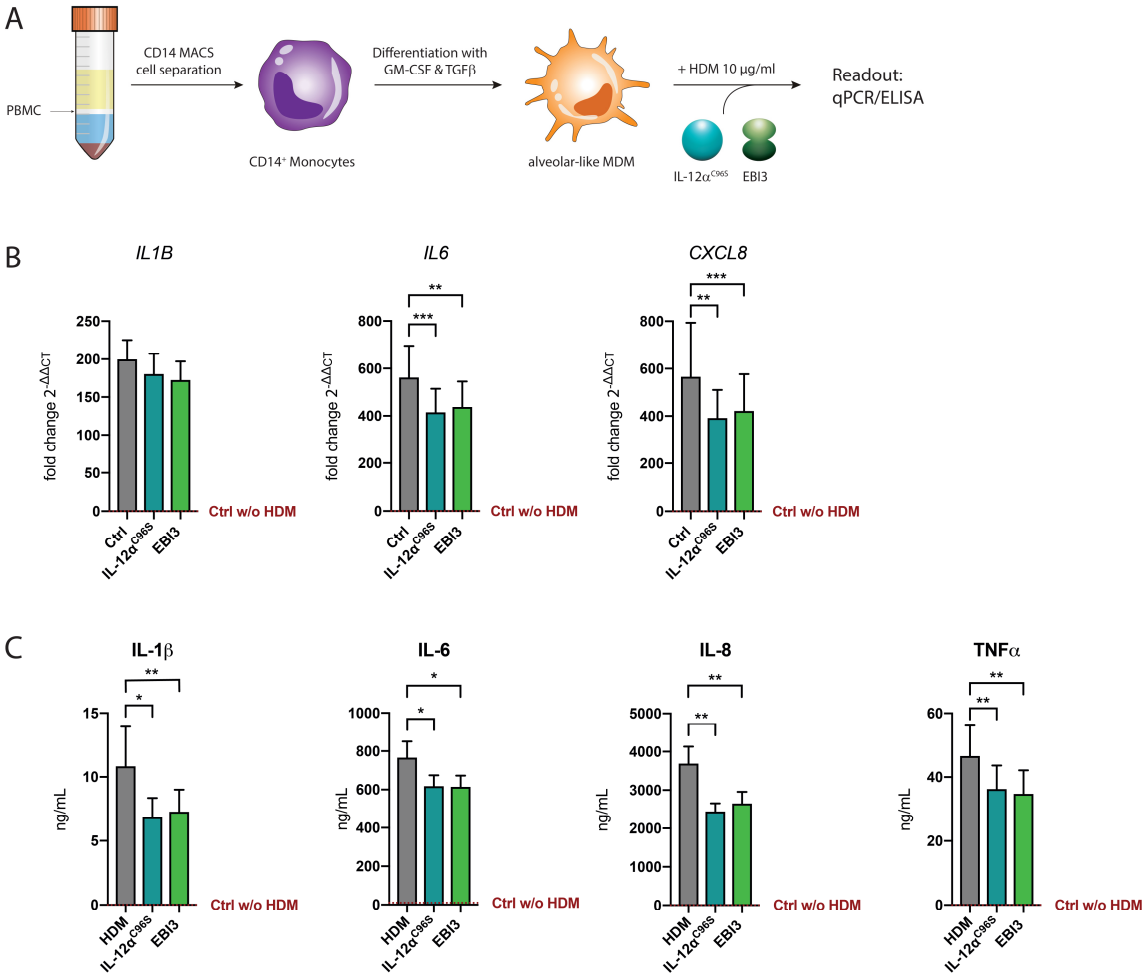

Figure 2.12: IL-12α^{C96S} and EBI3 act as immunosuppressors in HDM stimulated MDMs. A) CD14^{+} monocytes were isolated from human blood, differentiated into alveolar-like MDMs and stimulated with HDM extract (10 µg/ml) and IL-12α^{C96S} or EBI3 (10 ng/ml). B) Gene expression of *IL1B*, *IL6*, and *CXCL8* (qPCR) from HDM stimulated MDMs after cytokine stimulation (n = 13). C) Concentration of secreted IL-1β, IL-6, IL-8, and TNFα (ELISA) in supernatants from HDM stimulated human MDMs after cytokine stimulation (n = 10 to 17). Data are presented as means + SEM. Statistical significance was determined by Friedmann test. *P < 0.05; **P < 0.01; ***P < 0.001.

2.3 Discussion

IL-12 family members are defined as strictly heterodimeric cytokines, each comprising an α subunit that depends on assembly with a β subunit to obtain its native structure and escape ER associated degradation. Consequently, the heterodimeric complex can be secreted and function as an immune-active molecule.

Surprisingly, our study revealed that assembly and secretion of IL-35 substantially differs from all other IL-12 family cytokines. We were able to demonstrate that the IL-35 subunits IL-12α and EBI3 can either be secreted in an assembled or unassembled form. It thus seems, that complex formation between both subunits is of a rather weak nature and allows the subunits to dissociate once they have assisted each other's folding. Low complex stability is supported by additional evidence, as reduced secretion levels in comparison to IL-12 and IL-27, which both share one subunit of IL-35, point towards inefficient assembly induced folding[113,120]. Moreover, mutational studies for IL-35 failed to identify critical hotspot residues, analogously to IL-12 and IL-27, indicating that multiple but weak interactions build the foundation of the IL-35 interface[112]. In this context, it might be possible that IL-12α and EBI3 interact only transiently during their folding process and dissociate after complete maturation. This principle of a transient complex would provide a reasonable explanation for the lack of interaction of our recombinant subunits as well as their stability as independent soluble proteins. A similar observation was communicated by Aparicio-Siegmund and colleagues, who demonstrated IL-12 reconstitution with recombinant IL-12α purified from bacteria as well as IL-27 reconstitution by using EBI3-conditioned supernatant but failed to rebuild IL-35 with the same building blocks[113]. At this point we cannot exclude that IL-35 – in contrast to all other IL-12 family cytokines – requires additional, yet unidentified factors for efficient subunit interaction that were not included in our assays. However, the apparent instability of IL-35 might as well be attributed as a biologically relevant mechanism to prevent systemic immunosuppressive effects and reducing its bioavailability to the local tissue environment. Recent data for IL-6 signaling, consider sIL-6R and sgp130 as a systemic buffer by their ability to transiently bind IL-6, which might lead to an increased half-life and enables classical- and tans-signaling[176,177]. Given the structural homology between IL-12 and IL-6 family cytokines this concept of transient interaction to regulate signaling might as well be expanded on IL-35.

While all human α subunits of the IL-12 family are retained in the ER, the human β subunits can be secreted in absence of a partner subunit. IL-12β is readily secreted as a homodimer and secretion levels of this homodimeric form exceed heterodimeric IL-12

in certain conditions by a 10-20-fold excess[178,179]. For EBI3 no such data are available, yet its secretion generally appears to be inefficient[93,114]. Intracellular EBI3 however, might not simply be degraded but has been shown to exert a further cellular function in augmenting IL-23Rα expression by collaborating with calnexin, presumably through enhancing its chaperone activity[116]. Detailed molecular insights into the cellular mechanisms are however missing and identification of further target molecules is necessary to manifest if EBI3 has a general role in assisting the process of intracellular chaperoning. Besides a cellular role, there are numerous studies that report autonomous immunological effects for secreted EBI3[151,152], supported by data indicating high secretion levels of EBI3 in certain cells and conditions[118,180] and even in the absence of potential interaction partners[150]. Likewise, recent data provide examples for immunological properties of IL-12α[153,154]. However, no insights are provided how IL-12α can be secreted to act as an independent cytokine, since it is dependent on a β subunit to escape from ER retention. Only two proteins are reported to perform this task and in case of IL-12β the formation of a covalent interchain disulfide bridge excludes subsequent subunit dissociation. In our study we were able to demonstrate that EBI3 is required as a folding matrix for IL-12α, assisting its secretion, but low complex stability allows for subunit dissociation. We are thus able to provide a model for the secretion of both proteins and their endogenous existence as immune-active cytokines in an unassembled form. Moreover, by producing recombinant IL-12α^{C96S} and EBI3 from mammalian cells, we could demonstrate their stability as unassembled subunits, which is a prerequisite for their immunological relevance.

In accordance with previous reports, we were able to attribute immunosuppressive capabilities to the IL-12α^{C96S} and EBI3 subunits. Although significance was not achieved in each analysis for both subunits, there is a clear trend of an overall dampening effect in immune-activated primary cells. In LPS activated PBMCs, the most pronounced effects could be detected for reduction of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNFα. To investigate our proteins in a disease-relevant setting, we treated monocyte derived macrophages (MDM) with a house dust mite (HDM) extract. HDM represents a major source of indoor allergens and is strongly associated with the development of asthma[181]. Macrophages, as part of the innate immune system, are among the first and most abundant cells in the lung to encounter allergens and contribute to chronic airway inflammation like asthma through the production of pro-inflammatory cytokines[182,183]. In this model of strong immune-activation, standard concentrations (10 ng/ml) of IL-12α^{C96S} and EBI3 were sufficient to detect a significant decline of the

pro-inflammatory cytokines IL-6, IL-8, IL-1β, and TNFα. Thus, our *in vitro* data propose beneficial anti-inflammatory properties for both recombinant IL-35 subunits.

In summary, our study reveals that IL-35, in contrast to all other IL-12 family members, is not secreted as a strict heterodimer, but assembly-induced folding and transient interactions enable the secretion of unassembled subunits. Based on our discovery that IL-35 is in fact a compound of heterodimers and unassembled IL-12α and EBI3, this might, for the first time, provide a reasonable explanation for the pleiotropic signaling pathways and effects that are observed for IL-35. This is further supported by autonomous immune-suppressive properties of both subunits, which we were able to detect in primary immune cells, using recombinant human IL-12α^{C96S} and EBI3 for stimulation. While immunological effects should be re-evaluated in further cell models our data indicate a potential application of IL-12α^{C96S} and EBI3 for the treatment of inflammatory diseases and provide a basis for the development of specific antibodies against these subunits.

2.4 Material and methods

Constructs

Human interleukin cDNAs were obtained from Origene (Rockville) and subsequently cloned into the pSVL vector (Amersham Biosciences). Amino acid sequences of IL-12α, IL-12β, IL-27α, and EBI3 correspond to the UniProt accession numbers P29459, P29460, Q8NEV9, and Q14213, respectively. Where indicated, constructs where C-terminally tagged with an HA or FLAG epitope-tag, separated by a $(GS)_2$- or $(GS)_4$-linker, or equipped with a KDEL-sequence, separated by a $(GS)_3$-linker. Mutants were generated by site-directed mutagenesis. For mammalian protein purification IL-12α^{C96S} (C-terminal TEV cleavage site followed by a GG-linker and His-tag), IL-12β^{C199S} (untagged), and IL-27α^{L162C} (C-terminal HRV-3C Protease cleavage site followed by a $(GG)_2$-linker and His-tag) were cloned into the pcDNA3.4 vector (Gibco) and EBI3 (untagged) into the pHEK293 ultra expression vector 1 (TAKARA). All constructs were sequenced.

Cell culture and transient transfections

Mammalian cell experiments were performed in HEK293T cells, which were cultivated in Dulbecco's Modified Eagle Medium (DMEM) containing L-alanyl-L-glutamine (AQmedia, Sigma-Aldrich) at 37 °C and 5% CO_2. The medium was supplemented with 10% (v/v) FBS (Gibco) and 1% (v/v) antibiotic-antimycotic solution (25 µg/ml amphotericin B, 10 mg/ml streptomycin, 10,000 units of penicillin; Sigma-Aldrich).

Transient transfections were performed in poly-D-lysine coated 6-wells (Corning) by using GeneCellin (BioCellChallenge) according to manufacturer's protocol. The total transfected DNA amount was 2 µg with a DNA ratio α subunit to β subunit of 2:1 in case of IL-35 and IL-27 and 1:1 in case IL-12. For subunit titration experiments either 1 µg constant α subunit or 1 µg constant β subunit were transfected, while increasing the corresponding variable subunit. If only one subunit was transfected, empty pSVL vector was co-transfetced to maintain a total amount of 2 µg DNA.

Secretion experiments

Cells were transfected for 8 h, washed twice with PBS (Sigma-Aldrich) and cultivated in 0.5 ml fresh DMEM for another 16 h. To analyze secreted proteins, the medium was centrifuged for 5 min at 300 g and 4 °C. The supernatant was transferred into a new reaction tube, 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) and again centrifuged for 15 min at 20,000 g and 4 °C. For cell lysis, cells

were washed twice with ice-cold PBS and subsequently 0.5 ml of 1x RIPA lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS) supplemented with 1x protease inhibitor were added to each well. After 20 min, cells were scraped off and centrifuged for 15 min at 20,000 g and 4 °C. For further analysis, 0.2 volumes of 5x Laemmli buffer supplemented with 10% β-mercaptoethanol (β-Me) were added to the samples, which were then heated at 95 °C for 5 min.

Immunoblots and Co-immunoprecipitation (Co-IP) experiments

Protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% SDS-PAGE gels at 100 V for 2 h and subsequently transferred to polyvinylidene difluoride (PVDF) membranes by blotting overnight (o/n) at 30 V and 4 °C. Thereafter, membranes were blocked for 3 h at RT with Tris buffered saline containing skim milk powder and Tween-20 (MTBST; 25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5% w/v skim milk powder, 0.05% v/v Tween-20). Binding of the primary antibody was carried out o/n at 4 °C with anti-IL-12α (Abcam, ab133751; 1:500 in MTBST), anti-IL-12β (Abcam, ab133752; 1:500 in MTBST), anti-EBI3 antisera (generously provided by O. Devergne; 1:20 in PBS buffer), anti-IL-27 (R&D Systems, AF2526; 1:200 in MTBST), anti-HA tag (BioLegend, 902301; 1:1000 in MTBST), anti-Hsc70 (Santa Cruz Biotechnology, sc-1059; 1:1000 in MTBST), anti-CD81 (Proteintech 66866, 1:1000 in MTBST) or anti-HIS tag (Proteintech, HRP-66005; 1:1000 in MTBST). After washing, membranes were incubated for 1 h in species-specific HRP-conjugated secondary antibodies (Santa Cruz Biotechnology; 1:10,000 in MTBST). Immunoblots were detected with Amersham ECL prime (GE Healthcare/Cytiva) and a Fusion Pulse 6 imager (Vilber Lourmat).

For co-immunoprecipitation experiments, cells were lysed after PBS-washing in 0.5 ml Triton lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1x protease inhibitor) and cleared by centrifugation at 20,000 g for 15 min and 4 °C. To analyze secreted proteins, the medium was treated as described above, then pre-cleared by the addition of 30 µl Protein A/G agarose (Santa Cruz) and rotation for 1 h at 4 °C. Subsequently, all samples were incubated with 25 µl target-specific magnetic beads (anti-FLAG M2 (Sigma-Aldrich, M8823) or anti-HA (Thermo Fisher Scientific, 88837)) while rotating for 2 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). By adding 2x Laemmli buffer containing 4% β-Me and heating for 5 min at 95 °C, proteins were eluted and then separated by SDS-PAGE.

For co-immunoprecipitation experiments with purified proteins 1 µg IL-12$\alpha^{C96S,His}$ and 1 µg IL-12β^{C199S} (previously purified in our lab), 1 µg EBI3 and 1 µg mIL-27α (R&D

Systems 7430-ML-010), or 1 µg IL-12α^{C96S,His} and 1 µg EBI3 were mixed in a final volume of 200 µl (PBS) and incubated for 1 h at RT. 30 µl NiNTA beads (Sigma-Aldrich) were added to the solution and incubated while rotating for 2 h at 4 °C. Beads were washed with PBS containing 20 mM imidazole and elution was performed by addition of PBS supplemented with 500 mM imidazole and incubation for 10 min at 4 °C. For further analysis by SDS-PAGE, 0.2 volumes of 5x Laemmli buffer supplemented with 10% β-mercaptoethanol (β-Me) were added to the supernatants, which were then heated at 95 °C for 5 min.

Mammalian protein production and purification

For expression of IL-12$\alpha^{C96S,His}$ ExpiCHO cells (Gibco) were co-transfected with IL-12β^{C199S} in a DNA ratio of 1:1 according to manufacturer's protocol (high titer). After protein expression for 7 days, the medium was centrifuged (5,000 g, 30 min, 4 °C) and applied to a HisTrap HP column (GE Healthcare/Cytiva). A guanidimium chloride gradient (final 2.5 M GdnCl) was performed to separate IL-12β^{C199S} from IL-12α^{C96S}, subsequent washing with PBS on column was applied to refold IL-12α^{C96S}. Elution was performed in PBS supplemented with 500 mM imidazole and the His-tag was cleaved by addition of TEV protease (TEV: IL-12α^{C96S} 1:10 (w/w)) o/n at 4 °C and removed by a second HisTrap HP column in PBS. Final purification was performed using a HiLoad 26/600 Superdex 200 pg column (GE Healthcare/Cytiva) in PBS. EBI3 was co-transfected with IL27$\alpha^{L162C,His}$ into Expi293F (Gibco) cells with a DNA ratio of 1:1 according to manufacturer's protocol. After 2 days the medium was centrifuged and applied to a HisTrap column. To separate the complex a guanidinium chloride wash gradient (final 6 M GdnCl) was performed to elute EBI3 from the column, which was further purified by a HiPrep 16/60 Sephacryl S-200 HR with PBS and 3M GdnCl. EBI3 containing fractions were pooled, concentrated and stepwise dialysed against PBS.

Far-UV circular dichroism (CD) spectroscopy

Measurements were performed on a J-1500 CD spectrometer (Jasco) using a 10 µM protein solution in PBS in a quartz cuvette with 1 mm pathlength. Spectra were recorderd from 200-260 nm at 20 °C, temperature transitions from 20-90 °C at 222 nm for IL-12α^{C96S}, and 218 nm for EBI3 with a heating rate of 30 °C/h.

Hydrogen deuterium exchange mass spectroscopy

Hydrogen/deuterium exchange (HDX) measurements were performed on a ACQUITY UPLC M-class system equipped with automated HDX technology (Waters). HDX kinetics was recorderd for biological triplicates tracking data points at 0 s, 10 s, 1 min, 10 min,

30 min, and 2 h. At each data point, 3 µl of a 20 µM protein solution were diluted automatically 1:20 with PBS buffer (pH 7.4) containing 99.9% D_2O or the H_2O-containing reference buffer. The exchange was stopped by addition of 1:1 quenching buffer (200 mM Na_2HPO_4, 200 mM NaH_2PO_4, pH 2.3, containing 3 M GdmCl and 200 mM TCEP) at 1 °C. Proteolytic on-column digestion was performed using an immobilized Waters Enzymate BEH Pepsin Column (2.1 × 30 mm) at 20 °C. The resulting peptides were separated by reverse phase chromatography at 0 °C using a Waters Acquity UPLC C18 1.7 µm Vangard 2.1 × 5 mm trapping-column and a Waters Aquity UPLC BEH C18 1.7 µm 1 × 100 mm separation column by an H_2O to acetonitrile gradient with both eluents containing 0.1% formic acid (v/v). Eluted peptides were analyzed using an in-line Synapt G2-S QTOF HDMS mass spectrometer (Waters). UPLC was performed in protonated solvents (0.1% formic acid), to allow deuterium replacement with hydrogen from side chains and N-/C-termini that exchange faster than backbone amide linkages. Duplicates of all samples were measured. The use of an automated system ensures identical sample handling. Therefore, deuterium levels were not corrected for back exchange and are reported as relative levels. MS data were collected over an m/z range of 100–2000. Glu-fibrino peptide B (Waters) was used to ensure mass accuracy and peptides were identified by MSE ramping the collision energy automatically from 20–50 V. Data analysis was performed with PLGS (version 3.0.3) and DynamX (version 3.0) software packages (Waters).

Analytical Ultracentrifugation

Sedimentation velocity analytical ultracentrifugation (SV-AUC) experiments were performed on a Beckman Coulter Optima™ AUC analytical ultracentrifuge (Beckman Coulter) equipped with absorbance optics. For each sample, 350 µl of 10 µM IL-12α^{C96S} and EBI3 or the incubated proteins in PBS, pH 7.4 were loaded into a standard 12 mm double-sector epon-filled centerpiece, covered with quartz windows, alongside with 450 µl of the reference buffer solution. Samples were centrifuged at 42,000 rpm using an An-50 Ti rotor at 20 °C (with an initial test run at 3,000 rpm). Radial absorbance scans were acquired continuously at 230 nm with a radial step size of 0.001 cm. The obtained sedimentation velocity profiles were analyzed using SEDFIT software with a non-model based continuous Svedberg distribution method (c(s)), with time (TI) and radial (RI) invariant noise[184]. The density (ρ), viscosity (η) and partial specific volume ($\bar{v}$) of the potassium phosphate buffer used for data analysis was calculated with SEDNTERP[185].

NanoBRET Assay

Receptor chains were cloned into the pHTC HaloTag® CMV-neo Vector (IL-12Rβ2, gp130) or the pNLF1-C [CMV/Hygro] Vector (IL-12Rβ2, IL-27Rα) (Promega). COS7 cells were cultivated under the same conditions as described for HEK293T cells and transient transfections were performed in uncoated 6-well plates using GeneCellin according to manufacturer's protocol. In total, 2 µg DNA were transfected per well with a ratio of 100:1 HT:NL. After 16 h, transfected cells were detached via accutase (Sigma-Aldrich), divided into two pools and 1 ul HaloTag® NanoLuc® 618 Ligand (Promega) or DMSO as a control per ml cells were added. 2 x 10^4 cells were seeded into white bottom 96-wells and incubated for another 20 h at 37 °C. On the next day, reconstituted proteins were incubated for 1 h at RT and cytokines were added 30 min at a final concentration of 10 nM before measurement on a platereader (BMG Labtech CLARIOstar®). Mean milliBRET units (mBU) are calculated by deviding the acceptor emission by the donor emission and multiplication with 1,000. To determine the mean NanoBRET® ratio, the no-acceptor control mean is subtracted from the experimental mean. Samples are measured in technical triplicates and one representative result from at least biological triplicates is shown.

STAT Assays

BL-2 cells were cultivated in RPMI-1640 (ATCC modification, Thermo Fisher Scientific) supplemented with 20% heat inactivated FBS (Gibco) and 1% (v/v) antibiotic-antimycotic solution (25 µg/ml amphotericin B, 10 mg/ml streptomycin, 10,000 units of penicillin; Sigma-Aldrich). Cells were starved o/n in RPMI-1640 without FBS or antibiotic-antimycotic solution and 1 x 10^6 cells in RPMI-1640 + 0.5% (w/v) bovine serum albumin (Sigma-Aldrich) were then seeded into 48-wells and incubated for 15 min at 37 °C. Reconstituted proteins were incubated for 1 h at RT and BL-2 cells were stimulated with cytokines for 1 h at a final concentration of 10 ng/ml. 600 µl ice-cold PBS + 0.05% NaN_3 were added to each well to stop the reaction and the cells were transferred into a reaction tube, which was then centrifuged at 300 g for 5 min at 4 °C. Supernatant was discarded and cells were lysed by addition of 100 µl NP40 buffer supplemented with 1x protease inhibitor and phosphatase inhibitor (Serva) and incubated for 20 min, 4 °C while rotating. After centrifugation for 5 min at 20,000 g, and 4 °C, Laemmli buffer + β-Me was added to the supernatant, boiled for 5 min and further analyzed via immunoblot.

NK-92 cells were cultivated at 37 °C, 5% CO_2 in Minimum Essential Medium (MEM) α (Sigma-Aldrich, I-7508), containing 2.2 g/L $NaHCO_3$. The medium was further supplemented with 0.2 mM Myo-inositol (Sigma-Aldrich, I-7508), 0.1 mM 2-

mercaptoethanol, 0.02 mM folic acid (Sigma-Aldrich, F-8758), 12.5% FBS (Gibco), 12.5% horse serum (Thermo Fisher Scientific, 16050122), and 100 U/ml freshly added IL-2 (Peprotech, 200-02). NK-92 cells were starved o/n in medium without serum or IL-2. 1 x 10^6 cells were seeded into 48-wells and incubated at 37 °C for 15 min before stimulation with cytokines (reconstituted proteins were previously incubated for 1 h at RT) for 30 min at a final concentration of 10 ng/ml. Cells were harvested on ice, transferred into a reaction tube and centrifuged at 300 g for 5 min at 4 °C. The cell pellet was reconstituted in 100 µl RIPA lysis buffer supplemented with 1x protease and phosphatase inhibitor and lysis was performed for 20min at 4 °C while rotating. Cell debris was removed by centrifugation at 20,000 g, 5 min at 4 °C and the supernatant was supplemented with Laemmli buffer + β-Me, heated for 5 min at 95 °C and further analyzed via immunoblot.

PBMCs and Monocyte-derived macrophage culture

Peripheral blood mononuclear cells (PBMCs) of healthy individuals were isolated. The CD14-negative fraction was directly resuspended in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% FBS (BioSell), 1 µg/ml Gentamycin (Thermo Fisher Scientific), 100 U/ml Penicillin-Streptomycin (Thermo Fisher Scientific) and 2 mM L-Glutamine (Thermo Fisher Scientific). 1 x 10^6 cells were seeded into 24-wells and stimulated for 24 h with 100 ng/ml LPS (Invivogen) and either with 10 ng/ml EBI3 or 10 ng/ml IL-12α^{C96S}. The $CD14^+$ fraction was used to generate monocyte-derived macrophages (MDM) as previously reported[186,187]. 0.5 x 10^6 cells/ml were cultured in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% FBS (BioSell), 1 µg/ml Gentamycin (Thermo Fisher Scientific), 100 U/ml Penicillin-Streptomycin (Thermo Fisher Scientific), 2 mM L-Glutamine (Thermo Fisher Scientific), 10 ng/mL human GM-CSF (Miltenyi) and 2 ng/ml human TGFβ (Miltenyi) for six days at 37 °C with 5% CO_2 to differentate the cells into alveolar-like macrophages[173]. After 6 days incubation MDMs were harvested, 1-2 x 10^5 cells were seeded into 96-wells and stimulated for 24 h 10 µg/ml house dust mite extract (HDM) (Citeq Biologics) and either with 10 ng/ml EBI3 or 10 ng/ml IL-12α^{C96S}. During harvest, supernatants were stored at -70 °C until further cytokine analysis and cells were lysed in RLT buffer (Qiagen) supplemented with 1% β-Me and stored at -70 °C until RNA isolation.

Cytokine analysis (ELISA)

PMBC and MDM supernatants were analyzed for IL-6, IL-1β and IL-8 secretion using the human ELISA Sets (BD Biosciences, 555220, 557953, 555244). IFNγ and TNF secretion

were analyzed in supernatants using the human DuoSet ELISA (R&D Systems, DY285B, DY210). All ELISAs were performed according to the manufacturer's instructions.

RNA isolation

RNA was extracted using a spin-column kit according to the manufacturer's instruc- tions (Zymo Research) and transcribed into DNA using the HighCapacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystems) or submitted for total RNA sequencing.

RT qPCR

10 ng cDNA were used as a template and primers were mixed with FastStart Universal SYBR Green Master Mix (Roche). Fluorescence was measured on a ViiA7TM Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The expression levels were normalized to the house-keeping genes GAPDH (for MDM), ACTB and 18S (for PBMCs). Relative gene expression was calculated as 2ΔCT (ΔCT = CT (Housekeeper) - CT(Gene)).

Primer sequences used for qPCR:

Human Primer	Forward sequence	Reverse sequence
ACTB	GGATGCAGAAGGAGATCACT	CGATCCACACGGAGTACTTG
CXCL8	GAAGTTTTTGAAGAGGGCTGAGA	TGCTTGAAGTTTCACTGGCAT
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC
IL1B	AGAAGTACCTGAGCTCGCCA	CTGGAAGGAGCACTTCATCTGT
IL6	ACATGTGTGAAAGCAGCAAAG	GGCAAGTCTCCTCATTGAATCC
IFNg	TCAGCCATCACTTGGATGAG	CGAGATGACTTCGAAAAGCTG
IL17A	CCATCCCCAGTTGATTGGAA	CTCAGCAGCAGTAGCAGTGACA
TNF	CCCATGTTGTAGCAAACCCTC	TATCTCTCAGCTCCACGCCA
CXCL6	ACGCTGAGAGTAAACCCCAA	TTCTTCAGGGAGGCTACCAC
CCL1	TCCATCTGCTCCAATGAGGG	ATCCAACTGTGTCCAAGGCG
FASN	CTTCAAGGAGCAAGGCGTGA	ACTGGTACAACGAGCGGATG
TNFRSF6B	GAAACACCCACCTACCCCTG	GCTGCACAAAGGTGCCTG
CCL17	AGGGAGCCATTCCCCTTAGA	GCACAGTTACAAAAACGATGGC
MRC1	CGATCCGACCCTTCCTTGAC	TGTCTCCGCTTCATGCCATT

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. For immunological data, Friedmann test was used and $p<0.05$ was considered statistically significant. Details of statistical tests and sample sizes are provided in the figures.

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

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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.

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3.1 Introduction

Interleukins (ILs) are secreted proteins that regulate immune cell function. Among the more than 60 ILs known to date[72], the interleukin 12 (IL-12) family is functionally and structurally particularly complex[94,156]. Each of its family members is a heterodimer composed of an α- and a β-subunit. Only five different subunits form the building blocks of the four heterodimeric cytokines, which is enabled due to extensive subunit sharing occurring in the family: IL-12 consists of IL-12α and IL-12β[76,77,88,96], the latter is shared with IL-23 where IL-12β pairs with IL-23α[78]. Likewise, EBI3 is the β-subunit for IL-27, where it pairs with IL-27α[79], but also for IL-35, where it assembles with IL-12α[80,89]. 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 predominantly produced by regulatory T cells[94,156]. Although sharing structural hallmarks, subunits, and even receptors, 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[157,188–191]. 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[107,109,153,159,192–195].

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[50]. 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[78,79,102,105,114,196,197]. 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[93,99,102,106,109,112]. 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[113,196–198]. 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.

3.2 Results

3.2.1 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 (Figure 3.1a), 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 (Figure 3.1b). 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 (Figure 3.1). Hence, at least one subunit of every IL-12 family cytokine is predicted to be *N*-glycosylated (Figure 3.1). 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 (Figure 3.2). 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 (Figure 3.2a), verifying overall predictions of *N*-glycosylation (Figure 3.1). 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[196], 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 anymore (Figure 3.2a). 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[199,200]. In contrast, IL-27α actually was *O*-glycosylated, in agreement with previous studies[79,93,109].

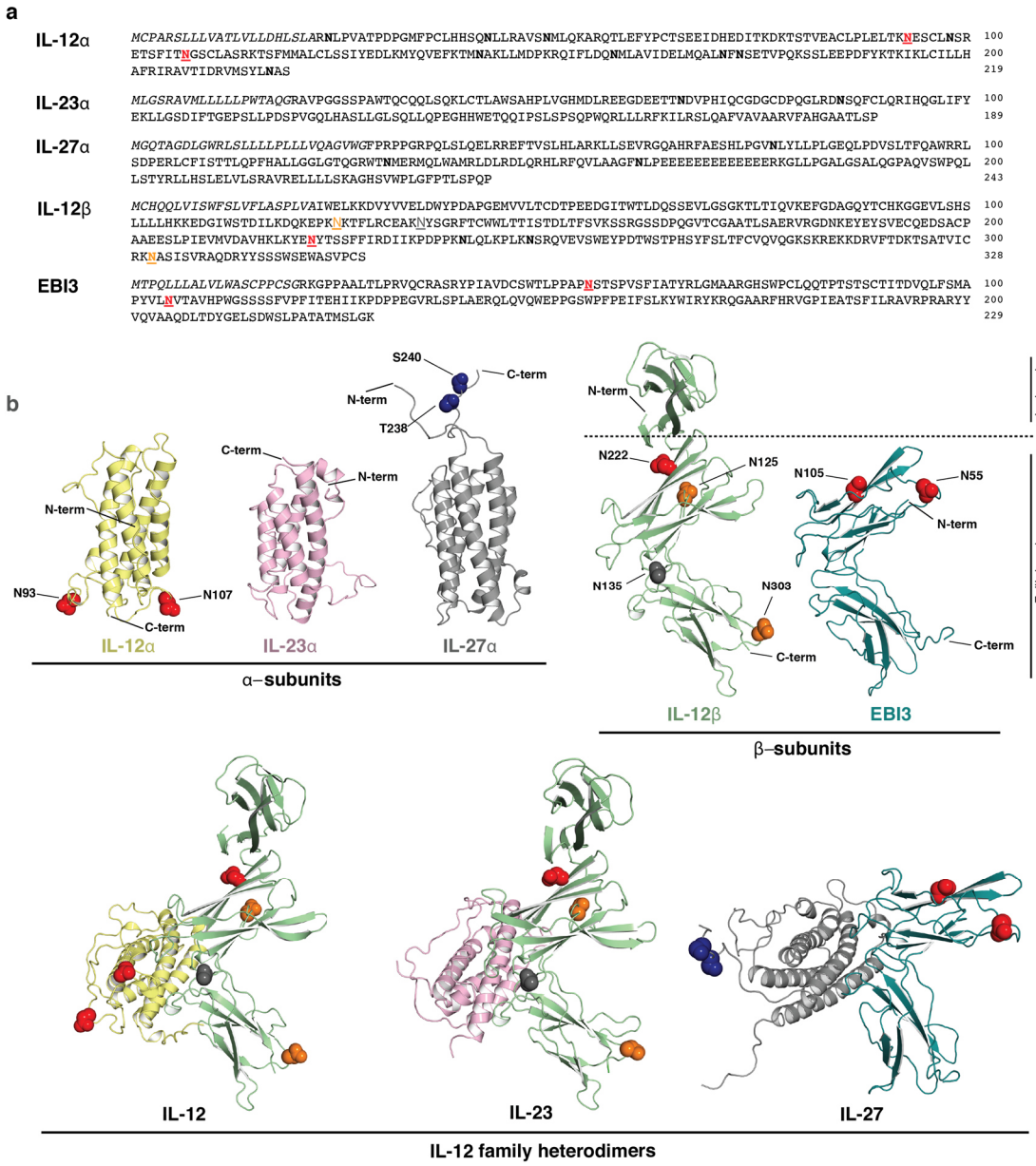

Figure 3.1: Interleukin 12 family cytokines differ in their glycosylation patterns. (a) Sequences of IL-12 family cytokine subunits show potential N-glycosylation sites. All Asn residues are highlighted in bold, predicted and experimentally verified N-glycosylation sites in red. For IL-12β, a predicted but not experimentally verified N-glycosylation site (N135) is shown in gray and not predicted (below the threshold) but experimentally detected N-glycosylation sites (N125, N303) are colored in orange. All relevant and mutated Asn residues are underlined. ER import sequences are shown in italic. (b) 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).

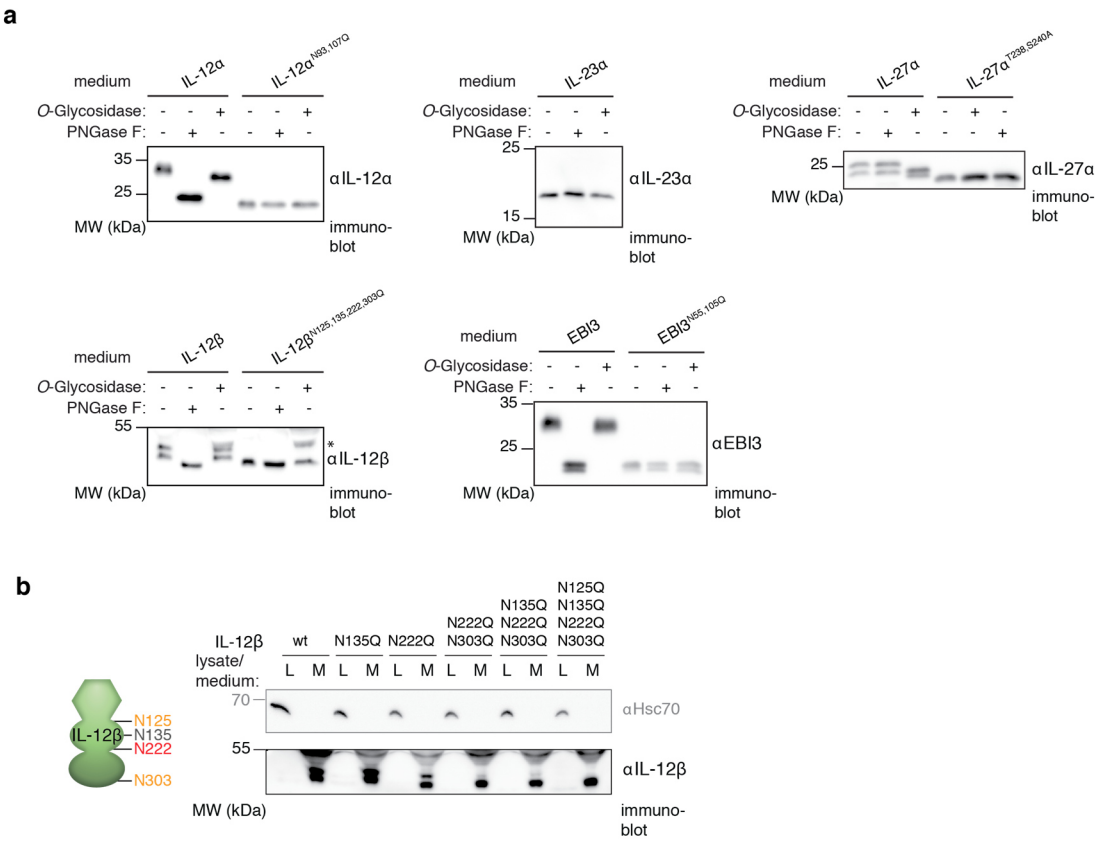

Figure 3.2: Verification of the predicted glycosylation sites of IL-12 family subunits. (a) Enzymatic treatment (PNGase F or *O*-Glycosidase) and mutagenesis of the respective sites. IL-12α is *N*-glycosylated at N93 and N107, whereas IL-23α is not glycosylated. IL-27α is *O*-glycosylated at two residues (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. (b) Sequential mutation of Asn residues in IL-12β enables identification of *N*-glycosylation sites (N125, N222, N303) *via* electrophoretic mobility shifts. The schematic shows the Ig domain (hexamer) and FnIII domains (ellipses) of the IL-12β subunit. L, lysate. M, medium. MW, molecular weight. 4% L/M were applied to the gel and blotted with antibodies against IL-12β. Hsc70 served as a loading control.

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 (Figure 3.1b), to Gln resulted in a protein with the same electrophoretic migration behavior as the wild-type counterpart after PNGase F treatment (Figure 3.2a). 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 (Figure 3.2a).

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 (Figure 3.2a). In EBI3^{N55,105Q}, only the two predicted sites were mutated (Figure 3.1a), confirming these (Figure 3.2a). In case of IL-12β, two Asn residues were predicted

to be *N*-glycosylated (N135 and N222, Figure 3.1a) but of those only N222 could be experimentally validated (Figure 3.2b). Since secreted IL-12β^{N222Q} still showed glycosylation, we analyzed further possible *N*-glycosylation sites that were below the threshold for glycosylation-prediction (Figure 3.2b). 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[79,93,109] 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 (Figure 3.3a). Focusing on surface-exposed residues among those (Figure 3.3b), mutational analyses of respective residues in a secretion-competent human IL-27α variant[93] identified two C-terminal *O*-glycosylated residues, T238 and S240 (Figure 3.3c). Mutation of these residues to Ala completely abolished *O*-glycosylation in human IL-27α (Figure 3.3).

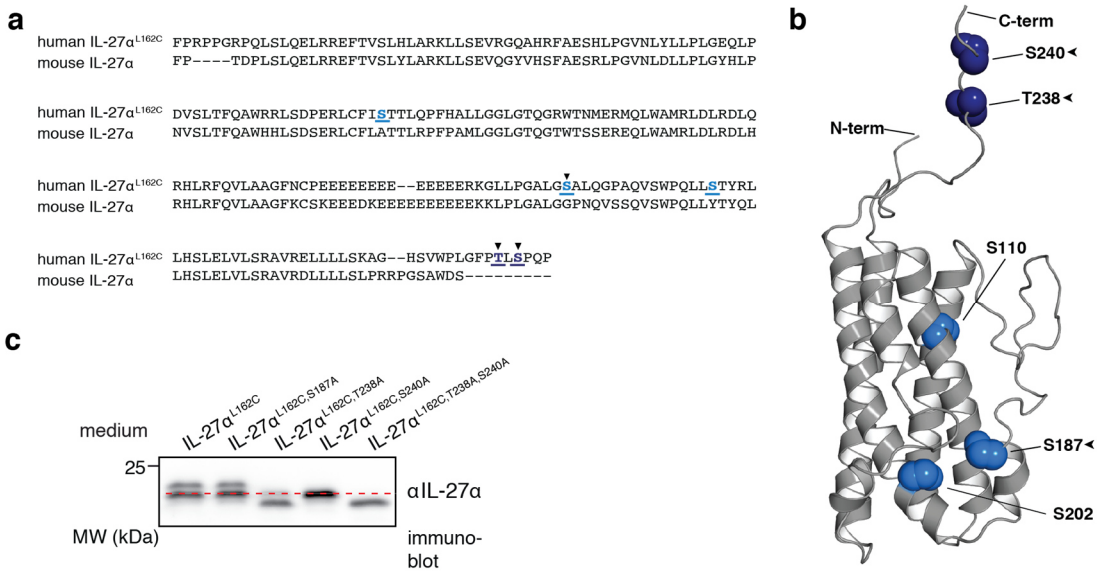

Figure 3.3: C-terminal *O*-glycosylation sites of IL-27α were determined by sequence alignment, structural assessment and verification by mutation. (a) Sequence alignment of human, *O*-glycosylated IL-27α^{L162C} [109], with murine, non-glycosylated IL-27α and (b) homology model of human IL-27α [93] show Thr and Ser residues (blue) only existing in the human protein and not in murine IL-27α. Of these, surface-exposed residues are highlighted with an arrowhead and experimentally confirmed *O*-glycosylation sites are colored in dark blue. Predicted *O*-glycosylation sites are underlined. (c) Mutation of potential *O*-glycosylation sites in human IL-27α^{L162C} identifies T238 and S240 as *O*-glycosylated residues. Electrophoretic shifts indicate lacking *O*-glycosylation due to residue exchange to Ala. Mutation of both T238 and S240 results in only one protein species which shifts to a lower molecular weight than other mutants due to completely missing *O*-glycosylation (as also verified by absence of any shift upon treatment with *O*-Glycosidase, see Fig. 1b). MW, molecular weight. 2% medium was applied to the gel and blotted with antibodies against IL-27α.

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 (Figure 3.1b). Next, we thus aimed at defining how glycosylation affected heterodimerization and secretion of each IL-12 family cytokine.

3.2.2 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[78–80,96]. Conversely, IL-12β is readily secreted in isolation, whereas EBI3 shows only inefficient secretion[114,195]. 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 (Figure 3.4a 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$^{\Delta N}$) (Figure 3.4a) and the naturally non-glycosylated IL-23α (Figure 3.4b).

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 (Figure 3.4c 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 (Figure 3.4c). 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 (Figure 3.4d). 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 (Figure 3.4d). 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.

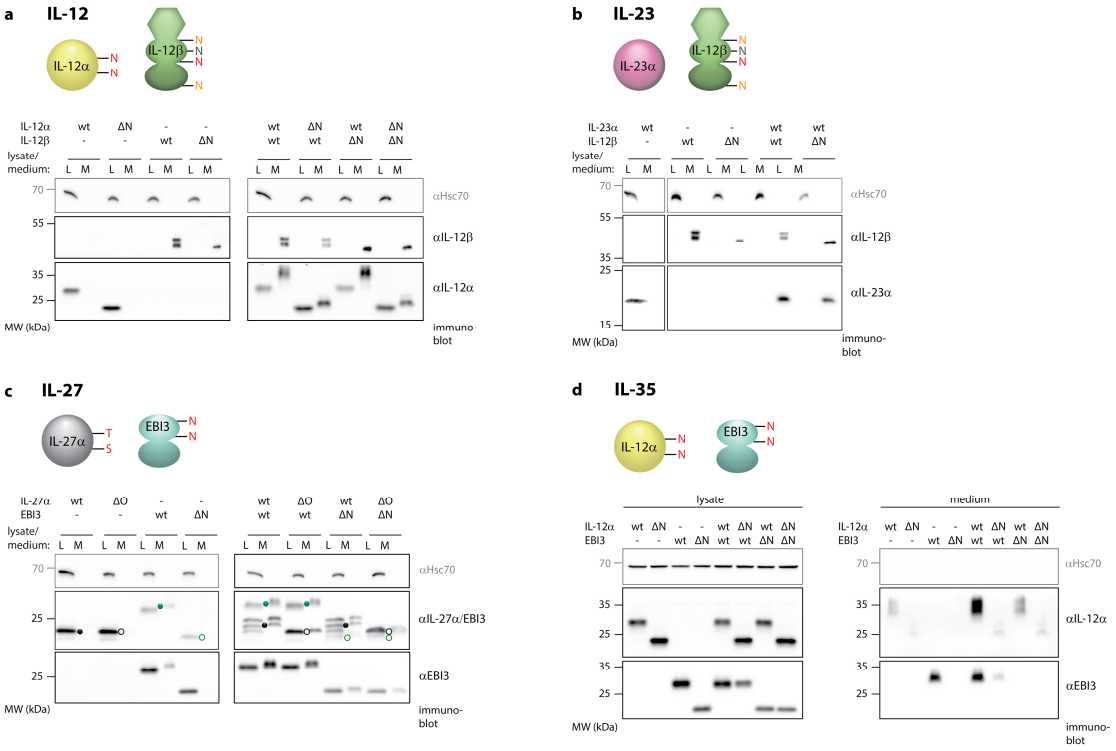

Figure 3.4: Impact of glycosylation on IL-12 family cytokine secretion. (a-d) All human wild-type α-subunits are secretion-incompetent in isolation (IL-12α, IL-23α, IL-27α) and retained in cells (L). Their pairing with secretion-competent wild-type β-subunits (IL-12β, EBI3) to form the heterodimeric 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. To facilitate an analysis regarding low IL-35 secretion levels, lysate and medium samples were analyzed on separate blots. (a-d) 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): 6% M) were applied to the gel and blotted with antibodies against the respective subunits. Hsc70 served as a loading 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 (Figure 3.4e). 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 (Figure 3.4e).

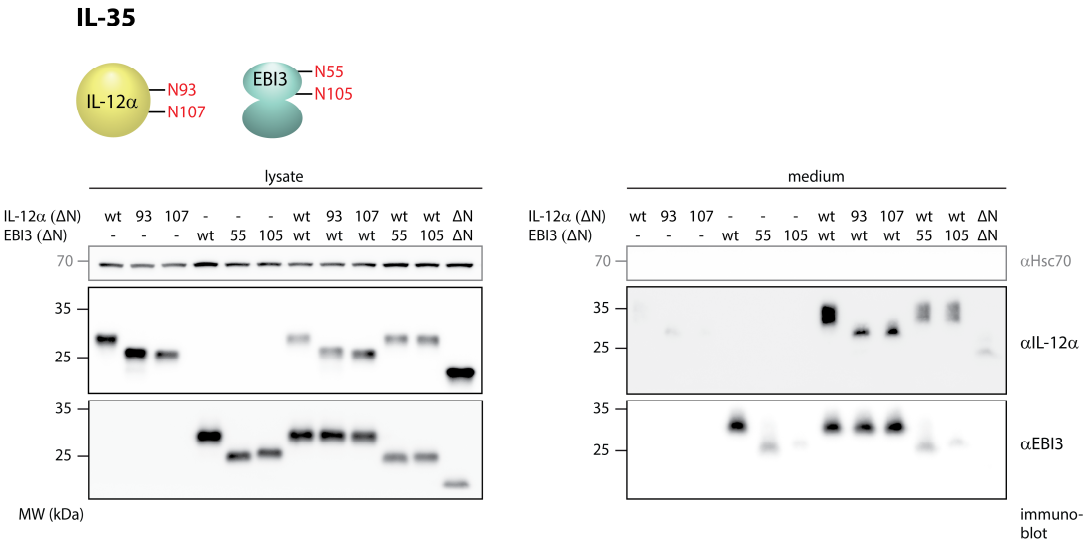

Figure 3.5: Secretion behavior and heterodimer formation of IL-35 are affected by the individual *N*-glycosylation sites of IL-12α and EBI3. IL-12α^{N93Q} and IL-12α^{N107Q} are only secreted to a much lower extent upon co-expression with EBI3, compared to the wild type. Similarly, the individual EBI3 glycosylation mutants are mostly retained in isolation and only induce reduced secretion levels of IL-12α. To facilitate an analysis regarding low IL-35 secretion levels, lysate and medium samples were analyzed on separate blots. ΔN indicates subunits where *N*-glycosylation sites are mutated to Gln. In the schematic models, circles represent the 4-helix bundle α-subunits ellipses for the FnIII domains of the β-subunits. L, lysate. M, medium. MW, molecular weight. 2% L and 6% M were applied to the gel and blotted with antibodies against the respective subunits. Hsc70 served as a loading control.

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.

3.2.3 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 (Figure 3.4). 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[201]. 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 (Figure 3.6a and b). All IL-12 glycosylation variants induced IFNγ gene expression (Figure 3.6a). Mutation of *N*-glycosylation sites in either the α- or β-subunit (IL-12α$^{\Delta N}$ + IL-12β, IL-12α + IL-12β$^{\Delta N}$) did not significantly change the level in gene expression compared to wild-

type IL-12. Surprisingly, non-glycosylated IL-12 (IL-12$\alpha^{\Delta N}$ + IL-12$\beta^{\Delta N}$) showed an increase of IFNγ-induction in PBMCs (Figure 3.6), whereas a slight decrease in receptor activation of IL-12 iLite® reporter cells compared to wild-type heterodimer was observed (Figure 3.6c).

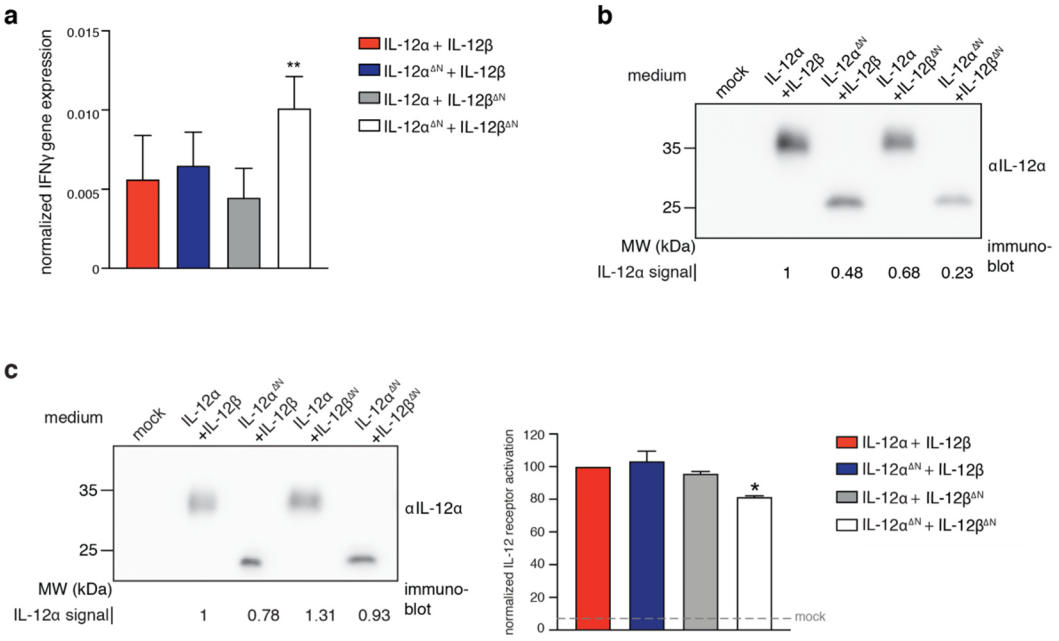

Figure 3.6: Glycosylation states of IL-12α and IL-12β influence biological functionality of heterodimeric IL-12. (a) Stimulation of human peripheral blood mononuclear cells (PBMCs) with the indicated IL-12 glycosylation-variants show IFNγ induction by all analyzed heterodimers measured by qPCR. Data were normalized to secretion levels (b) 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) Secretion of IL-12 was quantified via immunoblotting of medium samples from transiently transfected HEK293T cells relative to the wild type. (c) Secretion of IL-12 was quantified *via* immunoblotting of medium samples from transiently transfected HEK293T cells relative to the wild type, which was additionally quantified in comparison to recombinant IL-12 (data not shown). Induction of IL-12 signaling by the indicated glycosylation mutants (10 ng/ml final concentration each) was assessed with a receptor activation assay (iLite®). Receptor activation was measured in duplicates (shown ± SEM) and normalized to the wild-type signal, which was set to 100%. Statistical significance was calculated using one-way ANOVA. *p<0.05, indicates statistical significance.

Next, we examined the dependency of IL-23 signaling on cytokine glycosylation by measuring IL-23-induced IL-17 production in PBMCs[202]. Since IL-23α has no glycosylation sites (Figure 3.1), 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 (Figure 3.7a and b). In these experiments, no significant change in IL-17 secretion was observed for wild-type IL-23α in comparison to the non-glycosylated variant (Figure 3.7a). 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 (Figure 3.7c).

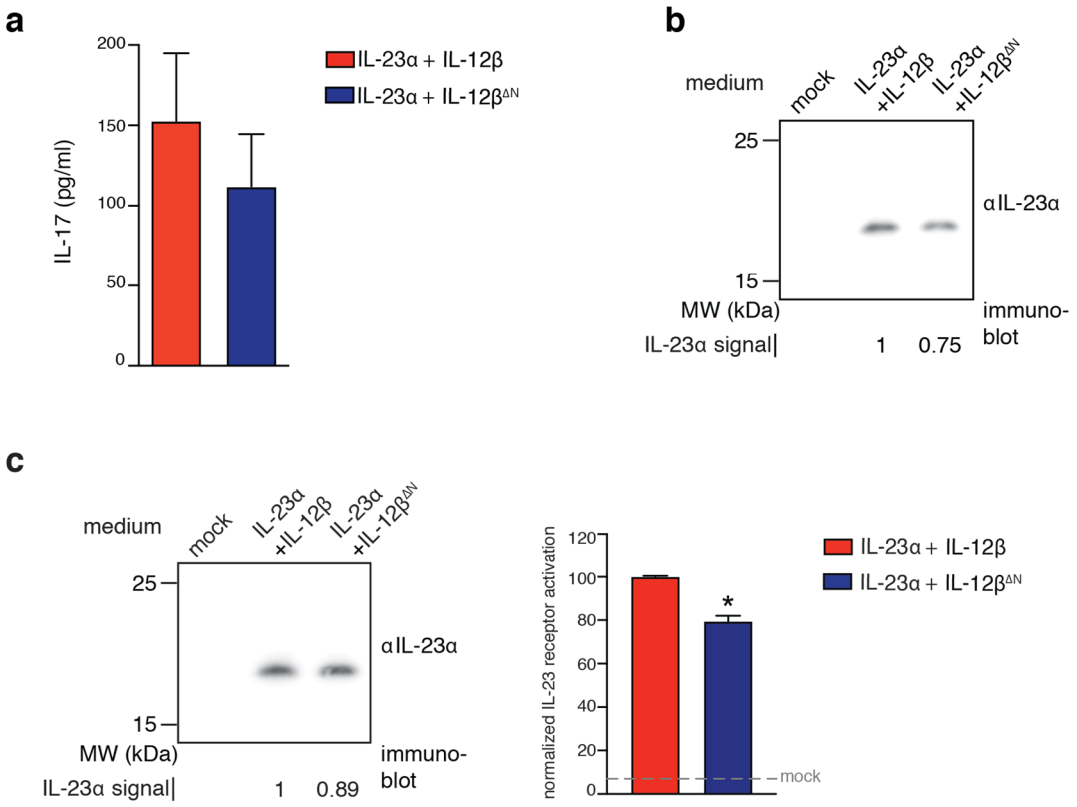

Figure 3.7: IL-23 function is not significantly affected by lacking glycosylation. (a) PBMCs were stimulated with quantified supernatants of HEK293T cells co-transfected with IL-23α and IL-12β variants (b). IL-17 production measured in the supernatant *via* ELISA, was not significantly changed for the 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. (b) Secretion of IL-23 was quantified via immunoblotting of medium samples from transiently transfected HEK293T cells relative to the wild type. IL-23 wild type was additionally quantified in comparison to recombinant IL-23 (data not shown). (c) Secretion of IL-23 was quantified *via* immunoblotting of medium samples from transiently transfected HEK293T cells relative to the wild type, which was additionally quantified in comparison to recombinant IL-12/IL-23 (data not shown). Induction of IL-23 signaling by the indicated glycosylation mutants (10 ng/ml final concentration each) was assessed with a receptor activation assay (iLite®). Receptor activation was measured in duplicates (shown ± SEM) and normalized to the wild-type signal, which was set to 100%. Statistical significance was calculated using one-way ANOVA. *p<0.05, indicates statistical significance.

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[203]. Quantification of the phospho-STAT1 signals *via* immunoblotting confirmed signaling-competency for all IL-27 glycosylation variants in BL-2 cells (Figure 3.8a and b). 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 (Figure 3.8).

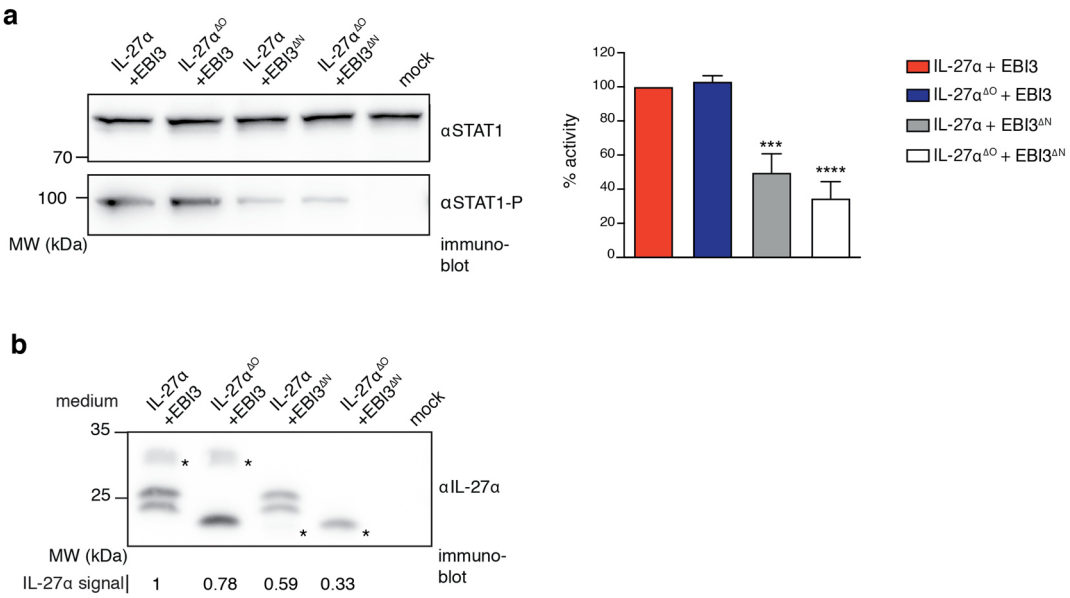

Figure 3.8: Functionality of IL-27 depends on the glycosylation states of EBI3. (a) Stimulation of human BL-2 cells with differently glycosylated IL-27, using previously quantified HEK293T supernatants co-transfected with IL-27α and EBI3 (b), shows significantly reduced cytokine signaling for EBI3$^{\Delta N}$ in complex with IL-27α or IL-27α$^{\Delta O}$. 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. (b) Secretion of IL-27 was quantified *via* immunoblotting of medium samples from transiently transfected HEK293T cells relative to the wild type. The asterisks indicate EBI3 detected by the αIL-27α antibody.

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[198].

3.3 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[93,97,99,102,106,109] 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 (Figure 3.9).

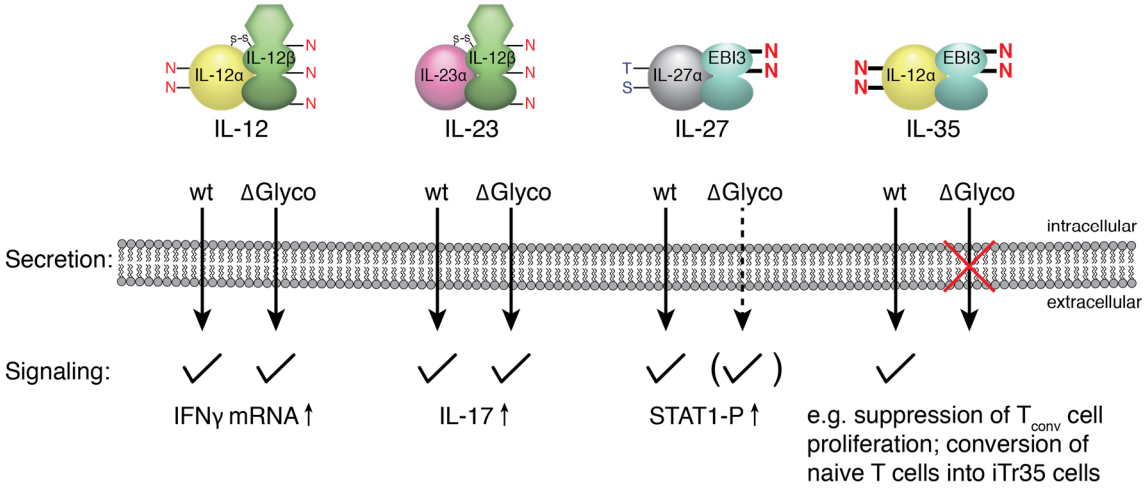

Figure 3.9: The impact of glycosylation on human IL-12 family biogenesis and function. All heterodimeric wild-type (wt) IL-12 family cytokines are secreted and show biological function. IL-35 wild-type signaling was previously described[89,92]. Both, IL-12 and IL-23 were secreted irrespective of their 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.

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 complex[78–80,93,96,97,99,106,109]. 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[93]. 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[113].

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[102,105], 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[94,109,156,190,204,205]. 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 in detail for an antibody-p40 fusion protein[206]. On the other hand, since our study reveals that IL-12, IL-23, and IL-27 can be secreted and remain 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[207].

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[94,208], e.g. in cancer forms that are difficult to treat[209,210] where IL-35 subunit glycosylation may be worth investigating since its plays a pivotal role in IL-35 formation as our study shows.

3.4 Experimental part

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.

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/)[211]. Both servers evaluate the potential of glycosylation by using a threshold. Sequence alignments were performed with Clustal Omega[212]. 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[93]. Structures were depicted with PyMOL (PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

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.

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[118] (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.

Functionality assays

The **IL-12 activity assay** was performed following a previously published protocol[99]. 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.

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.

4 Conclusion and outlook

This PhD thesis covers two distinct research projects, both contributing to our knowledge of the IL-12 family in general and on IL-35 in particular.

In the first project, we focused on IL-35, the latest addition and by far least understood member of the IL-12 family. While there are numerous reports describing its immunological functions, we aimed to obtain a better understanding of the early processes in its biogenesis. By a thorough and comparative investigation of its secretion pattern we were able to demonstrate that the two IL-35 subunits, IL-12α and EBI3, can be secreted either in assembled or unassembled forms. In contrast to all other IL-12 family members, IL-35 appears thus to be a compound cytokine rather than a strict heterodimer. Recombinant production of the human subunits from mammalian cells resulted in stable soluble proteins and furthermore allowed us to investigate their immunological effects in human PBMCs and an HDM-induced airway inflammation model of human monocyte derived alveolar like macrophages. In this context, we were able to detect downregulation of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNFα after stimulation with our subunits.

Our new model for IL-35 secretion elucidates for the first time how physiologically relevant amounts of unpaired IL-12α can be found outside the cell. In isolation, IL-12α fails to acquire its native structure and instead forms erroneous disulfide bridges and high molecular weight species, including homodimers. Members of the PDI family recognize unfolded IL-12α, which leads to its ER retention and finally to degradation[99]. This study shows that EBI3 can assist the folding process of IL-12α thereby promoting its secretion. Detailed structural insights into the resulting conformational changes are however missing. An important step towards answering this and many other questions will be the resolution of the assembled IL-35 structure.

To achieve this significant milestone, a purification strategy for functional IL-35 has to be established. In the light of our finding of weak complex stability, protein engineering is required for the design of a stabilized IL-35 variant. One common and well-established approach would be the production of a cytokine fusion protein[78,122,213], which is already utilized in case of IL-35 by research groups[89,133] and companies. Detailed insights into purification protocols and biophysical characterization of the final product are however not provided and it should be further considered that a linker between both subunits does not necessarily guarantee for stable interaction. Especially in the case of IL-35, IL-12α and EBI3 might still co-exist rather than interact. Conversely, by taking the same route we found different linked L-12α-EBI3 constructs to be secretion-incompetent (data not

shown), therefore most likely unfolded and not completely glycosylated, which is also in agreement with other reports[113]. A more nature-inspired approach for the generation of a stable complex is the introduction of an interchain disulfide bond, analogously to IL-12 and IL-23. To identify appropriate residues that enable covalent complex formation, detailed insights into the IL-35 interface are urgently required but currently not available[112]. Several interface mutants based on different structural models were tested in our laboratory during this thesis but we were not able to identify critical interface residues nor to introduce an intermolecular disulfide bond yet (data not shown). Due to the recent breakthrough in protein structure prediction, refined model and docking approaches are now available and might open new doors to tackle this long-standing puzzle.

A stabilized and functional IL-35 complex would bring a substantial progress to the field and facilitate re-evaluation of many of its biological aspects. It would further allow to dissect the enigmatic receptor repertoire of IL-35, which is unique in the IL-12 family. With the establishment of our receptor reporter system, we have now a powerful tool in our hands to monitor cytokine binding, yet the lack of positive controls hindered our investigation for IL-35 so far. Up to this point, we can only speculate if the unconventional receptor pluralism is in fact a result of our hypothesis of secreted unassembled subunits. To identify the receptor chains that are engaged by recombinant IL-12α^{C96S} and EBI3, it might be reasonable to envisage cooperation and make use of reporter assays which are already established in other laboratories[92,121].

Although details in receptor engagement and downstream signaling are still obscure, we successfully developed purification protocols to produce stable and authentic human IL-35 subunits from mammalian cells in the milligram-scale, which were immune-active cytokines. By stimulation of HDM extract treated human MDMs we investigated a concrete disease model of allergic airway inflammation and were able to observe beneficial effects of IL-12α^{C96S} and EBI3 *in vitro*. Since IL-35 is under current focus as a potential treatment in allergic diseases[174,214,215], our subunits might bring valuable progress to this field. As a next step, it is key to broaden our understanding of the immunological scopes of IL-12α^{C96S} and EBI3 and incorporate other cell types and disease models in our study. Especially T cells are attractive candidates, due to their global effector function in our immune system and in particular due to their major role in IL-35 signaling.

One puzzle that still remains to be solved, is the lack of interaction of our recombinant subunits to reconstitute IL-35. It might support our theory of low complex stability and unassembled subunits, manifest their independent character and consequently the

exceptional position of IL-35 in the IL-12 family. In this regard, apparent instability of IL-35 might be a crucial physiological mechanism to confine its strong immunosuppressive capacity to the local tissue environment. We can however not exclude that important factors are missing in our *in vitro* experiments, which are crucial for IL-35 complex formation. This question will be addressed by interaction proteomics of cell secreted IL-35, which will ultimately reveal if additional factors are part of the complex and is currently under way. At the same time, differences in the posttranslational modification pattern will be analyzed via mass spectrometry and compared for IL-35, IL-12, IL-27, IL-12α, and EBI3. By this thorough investigation we will be able to exclude, that structural variances prevent interaction of recombinant IL-12α^{C96S} and EBI3. Of note, in the course of our purification, we observed two peaks for IL-12α^{C96S}, matching the molecular weight of a monomeric and a homodimeric species, which was further confirmed by non-reducing SDS-PAGE for wildtype IL-12α and the data from Dambuza et al.[153] This opens the possibility that IL-35 is not a 1:1 complex, but EBI3 may in fact interact with a covalent IL-12α-homodimer. We will in the future elucidate this by integrating the dimer fraction of wildtype IL-12α into our assays and re-evaluate reconstitution.

In our immunological assays, we decided to stimulate cells with the monomeric fraction of IL-12α^{C96S} as non-reducing SDS-PAGE indicated it as the clearer defined species. We cannot completely rule out, that IL-12α homodimers have a slightly distinct immunological signature. However, similar to complex formation with IL-12β, homodimer formation should not be completely abrogated by mutation of the free cysteine but only render it less stable. Therefore, we do not expect a substantially different outcome for our functional studies, which is in accordance with Dambuza et al. who received comparable results by including both IL-12α monomers and homodimers in their assays, suggesting that they can be used interchangeably[153]. It should be further noted, that EBI3 is also able to form homodimers[93] and our analytical ultracentrifugation data reveal a small homodimeric fraction, which could hypothetically allow complex formation between IL-12α and EBI3 homodimers.

In summary, our data indicate that assembly-induced folding and transient complex formation extends our cytokine repertoire by the unassembled subunits IL-12α and EBI3. It remains open in which ratio the heterodimer vs. the individual subunits exist and if certain conditions or triggers are able to shift this balance. On a broader scale, these insights could ultimately lead to novel therapeutic approaches in the treatment of severe immune diseases including asthma, chronic inflammation, and cancer.

In the second part of this work, we characterized the human IL-12 family in regard to 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. It thus appears, that whenever a cytokine comprises EBI3 as a β subunit its stability is significantly more affected by loss of glycosylation. This is impressively demonstrated by comparison of IL-12 and IL-35, which share the same α subunit. Furthermore, our data highlight that in contrast to IL-12β, EBI3 is secreted inefficiently indicating partially disordered regions. It thus seems that loss of glycosylation further leads to the destabilization of EBI3. While this unstable character might be of physiological relevance, molecular dynamics simulations combined with protein engineering is a desirable approach to produce a stabilized EBI3 mutein. This could help to further investigate the effect of glycosylation on its structure and ultimately route to novel therapeutical approaches. In summary, the here presented 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.

5 Bibliography

1. Balch, W. E., Morimoto, R. I., Dillin, A. & Kelly, J. W. Adapting proteostasis for disease intervention. *Science (New York, N.Y.)* **319,** 916–919 (2008).
2. Klaips, C. L., Jayaraj, G. G. & Hartl, F. U. Pathways of cellular proteostasis in aging and disease. *The Journal of cell biology* **217,** 51–63 (2018).
3. Orengo, C. A., Todd, A. E. & Thornton, J. M. From protein structure to function. *Current Opinion in Structural Biology* **9,** 374–382 (1999).
4. HABER, E. & Anfinsen, C. B. Side-chain interactions governing the pairing of half-cystine residues in ribonuclease. *The Journal of biological chemistry* **237,** 1839–1844 (1962).
5. Anfinsen, C. B. Principles that govern the folding of protein chains. *Science (New York, N.Y.)* **181,** 223–230 (1973).
6. Dinner, A. R., Šali, A., Smith, L. J., Dobson, C. M. & Karplus, M. Understanding protein folding via free-energy surfaces from theory and experiment. *Trends in biochemical sciences* **25,** 331–339 (2000).
7. Levinthal, C. Are there pathways for protein folding? *J. Chim. Phys.* **65,** 44–45 (1968).
8. Dill, K. A., Ozkan, S. B., Shell, M. S. & Weikl, T. R. The protein folding problem. *Annual review of biophysics* **37,** 289–316 (2008).
9. SWISS-MODEL. Homo sapiens (human), https://swissmodel.expasy.org/repository/species/9606 (2021).
10. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A. W., Kavukcuoglu, K., Kohli, P. & Hassabis, D. Highly accurate protein structure prediction with AlphaFold. *Nature* **596,** 583–589 (2021).
11. Baek, M., DiMaio, F., Anishchenko, I., Dauparas, J., Ovchinnikov, S., Lee, G. R., Wang, J., Cong, Q., Kinch, L. N., Schaeffer, R. D., Millán, C., Park, H., Adams, C., Glassman, C. R., DeGiovanni, A., Pereira, J. H., Rodrigues, A. V., van Dijk, A. A., Ebrecht, A. C., Opperman, D. J., Sagmeister, T., Buhlheller, C., Pavkov-Keller, T., Rathinaswamy, M. K., Dalwadi, U., Yip, C. K., Burke, J. E., Garcia, K. C., Grishin, N. V., Adams, P. D., Read, R. J. & Baker, D. Accurate prediction of protein structures and interactions using a three-track neural network. *Science (New York, N.Y.)* **373,** 871–876 (2021).
12. Hartl, F. U., Bracher, A. & Hayer-Hartl, M. Molecular chaperones in protein folding and proteostasis. *Nature* **475,** 324–332 (2011).
13. Hipp, M. S., Kasturi, P. & Hartl, F. U. The proteostasis network and its decline in ageing. *Nature reviews. Molecular cell biology* **20,** 421–435 (2019).
14. Ritossa, F. Discovery of the heat shock response. *Cell Stress Chaper* **1,** 97 (1996).
15. Zou, J., Guo, Y., Guettouche, T., Smith, D. F. & Voellmy, R. Repression of Heat Shock Transcription Factor HSF1 Activation by HSP90 (HSP90 Complex) that Forms a Stress-Sensitive Complex with HSF1. *Cell* **94,** 471–480 (1998).
16. Schubert, U., Antón, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W. & Bennink, J. R. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404,** 770–774 (2000).

17. Dikic, I. Proteasomal and Autophagic Degradation Systems. *Annual review of biochemistry* **86,** 193–224 (2017).

18. Mauvezin, C., Nagy, P., Juhász, G. & Neufeld, T. P. Autophagosome-lysosome fusion is independent of V-ATPase-mediated acidification. *Nature communications* **6,** 7007 (2015).

19. Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., et al. Initial sequencing and analysis of the human genome. *Nature* **409,** 860–921 (2001).

20. Csala, M., Bánhegyi, G. & Benedetti, A. Endoplasmic reticulum. A metabolic compartment. *FEBS letters* **580,** 2160–2165 (2006).

21. Dallner, G., Siekevitz, P. & Palade, G. E. Biogenesis of endoplasmic reticulum membranes. I. Structural and chemical differentiation in developing rat hepatocyte. *The Journal of cell biology* **30,** 73–96 (1966).

22. Clapham, D. E. Calcium signaling. *Cell* **131,** 1047–1058 (2007).

23. Samtleben, S., Jaepel, J., Fecher, C., Andreska, T., Rehberg, M. & Blum, R. Direct imaging of ER calcium with targeted-esterase induced dye loading (TED). *Journal of visualized experiments : JoVE,* e50317 (2013).

24. Burk, S. E., Lytton, J., MacLennan, D. H. & Shull, G. E. cDNA cloning, functional expression, and mRNA tissue distribution of a third organellar Ca2+ pump. *The Journal of biological chemistry* **264,** 18561–18568 (1989).

25. Ellgaard, L. Catalysis of disulphide bond formation in the endoplasmic reticulum. *Biochemical Society transactions* **32,** 663–667 (2004).

26. Appenzeller-Herzog, C. Glutathione- and non-glutathione-based oxidant control in the endoplasmic reticulum. *Journal of cell science* **124,** 847–855 (2011).

27. Sevier, C. S. & Kaiser, C. A. Ero1 and redox homeostasis in the endoplasmic reticulum. *Biochimica et biophysica acta* **1783,** 549–556 (2008).

28. Csala, M., Kereszturi, É., Mandl, J. & Bánhegyi, G. The endoplasmic reticulum as the extracellular space inside the cell. Role in protein folding and glycosylation. *Antioxidants & redox signaling* **16,** 1100–1108 (2012).

29. Tsunoda, S., Avezov, E., Zyryanova, A., Konno, T., Mendes-Silva, L., Pinho Melo, E., Harding, H. P. & Ron, D. Intact protein folding in the glutathione-depleted endoplasmic reticulum implicates alternative protein thiol reductants. *eLife* **3,** e03421 (2014).

30. Poet, G. J., Oka, O. B., van Lith, M., Cao, Z., Robinson, P. J., Pringle, M. A., Arnér, E. S. & Bulleid, N. J. Cytosolic thioredoxin reductase 1 is required for correct disulfide formation in the ER. *The EMBO journal* **36,** 693–702 (2017).

31. Lang, S., Pfeffer, S., Lee, P.-H., Cavalié, A., Helms, V., Förster, F. & Zimmermann, R. An Update on Sec61 Channel Functions, Mechanisms, and Related Diseases. *Frontiers in physiology* **8,** 887 (2017).

32. Fons, R. D., Bogert, B. A. & Hegde, R. S. Substrate-specific function of the translocon-associated protein complex during translocation across the ER membrane. *The Journal of cell biology* **160,** 529–539 (2003).

33. Matlack, K. E., Misselwitz, B., Plath, K. & Rapoport, T. A. BiP Acts as a Molecular Ratchet during Posttranslational Transport of Prepro-α Factor across the ER Membrane. *Cell* **97,** 553–564 (1999).

34. Nilsson, I. M. & Heijne, G. von. Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *The Journal of biological chemistry* **268,** 5798–5801 (1993).

35. Shental-Bechor, D. & Levy, Y. Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 8256–8261 (2008).

36. Betz, S. F. Disulfide bonds and the stability of globular proteins. *Protein science : a publication of the Protein Society* **2,** 1551–1558 (1993).

37. Doig, A. J. & Williams, D. H. Is the hydrophobic effect stabilizing or destabilizing in proteins? *Journal of molecular biology* **217,** 389–398 (1991).

38. Feige, M. J. & Hendershot, L. M. Disulfide bonds in ER protein folding and homeostasis. *Current opinion in cell biology* **23,** 167–175 (2011).

39. Frand, A. R. & Kaiser, C. A. Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. *Molecular cell* **4,** 469–477 (1999).

40. Fanghänel, J. & Fischer, G. Insights into the catalytic mechanism of peptidyl prolyl cis/trans isomerases. *Frontiers in bioscience : a journal and virtual library* **9,** 3453–3478 (2004).

41. Hellman, R., Vanhove, M., Lejeune, A., Stevens, F. J. & Hendershot, L. M. The in vivo association of BiP with newly synthesized proteins is dependent on the rate and stability of folding and not simply on the presence of sequences that can bind to BiP. *The Journal of cell biology* **144,** 21–30 (1999).

42. Pobre, K. F. R., Poet, G. J. & Hendershot, L. M. The endoplasmic reticulum (ER) chaperone BiP is a master regulator of ER functions: Getting by with a little help from ERdj friends. *The Journal of biological chemistry* **294,** 2098–2108 (2019).

43. Behnke, J., Feige, M. J. & Hendershot, L. M. BiP and its nucleotide exchange factors Grp170 and Sil1: mechanisms of action and biological functions. *Journal of molecular biology* **427,** 1589–1608 (2015).

44. Hammond, C., Braakman, I. & Helenius, A. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 913–917 (1994).

45. Ellgaard, L. & Frickel, E.-M. Calnexin, Calreticulin, and ERp57: Teammates in Glycoprotein Folding. *CBB* **39,** 223–248 (2003).

46. Vembar, S. S. & Brodsky, J. L. One step at a time. Endoplasmic reticulum-associated degradation. *Nature reviews. Molecular cell biology* **9,** 944–957 (2008).

47. Guerriero, C. J. & Brodsky, J. L. The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. *Physiological reviews* **92,** 537–576 (2012).

48. Laskey, R. A., Honda, B. M., Mills, A. D. & Finch, J. T. Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature* **275,** 416–420 (1978).

49. Christis, C., Lubsen, N. H. & Braakman, I. Protein folding includes oligomerization - examples from the endoplasmic reticulum and cytosol. *The FEBS journal* **275,** 4700–4727 (2008).

50. Braakman, I. & Bulleid, N. J. Protein folding and modification in the mammalian endoplasmic reticulum. *Annual review of biochemistry* **80,** 71–99 (2011).

51. Hurtley, S. M. & Helenius, A. Protein oligomerization in the endoplasmic reticulum. *Annual review of cell biology* **5,** 277–307 (1989).

52. Galian, A., Lecestre, M.-J., Scotto, J., Bognel, C., Matuchansky, C. & Rambaud, J.-C. Pathological study of alpha-chain disease, with special emphasis on evolution. *Cancer* **39,** 2081–2101 (1977).

53. Feige, M. J., Groscurth, S., Marcinowski, M., Shimizu, Y., Kessler, H., Hendershot, L. M. & Buchner, J. An unfolded CH1 domain controls the assembly and secretion of IgG antibodies. *Molecular cell* **34,** 569–579 (2009).

54. Feige, M. J., Behnke, J., Mittag, T. & Hendershot, L. M. Dimerization-dependent folding underlies assembly control of the clonotypic αβT cell receptor chains. *The Journal of biological chemistry* **290,** 26821–26831 (2015).

55. Marshall, J. S., Warrington, R., Watson, W. & Kim, H. L. An introduction to immunology and immunopathology. *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology* **14,** 49 (2018).

56. Amarante-Mendes, G. P., Adjemian, S., Branco, L. M., Zanetti, L. C., Weinlich, R. & Bortoluci, K. R. Pattern Recognition Receptors and the Host Cell Death Molecular Machinery. *Frontiers in immunology* **9,** 2379 (2018).

57. Carroll, M. C. The complement system in regulation of adaptive immunity. *Nature immunology* **5,** 981–986 (2004).

58. Italiani, P. & Boraschi, D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. *Frontiers in immunology* **5,** 514 (2014).

59. Joshi, N., Walter, J. M. & Misharin, A. V. Alveolar Macrophages. *Cellular immunology* **330,** 86–90 (2018).

60. Parkin, J. & Cohen, B. An overview of the immune system. *The Lancet* **357,** 1777–1789 (2001).

61. Palm, N. W. & Medzhitov, R. Not so fast: adaptive suppression of innate immunity. *Nature medicine* **13,** 1142–1144 (2007).

62. Bonilla, F. A. & Oettgen, H. C. Adaptive immunity. *The Journal of allergy and clinical immunology* **125,** S33-40 (2010).

63. Khodadadi, L., Cheng, Q., Radbruch, A. & Hiepe, F. The Maintenance of Memory Plasma Cells. *Frontiers in immunology* **10,** 721 (2019).

64. Chaplin, D. D. Overview of the immune response. *The Journal of allergy and clinical immunology* **125,** S3-23 (2010).

65. Smith, N. C., Rise, M. L. & Christian, S. L. A Comparison of the Innate and Adaptive Immune Systems in Cartilaginous Fish, Ray-Finned Fish, and Lobe-Finned Fish. *Frontiers in immunology* **10,** 2292 (2019).

66. Beissert, S., Schwarz, A. & Schwarz, T. Regulatory T cells. *The Journal of investigative dermatology* **126,** 15–24 (2006).

67. Ciurkiewicz, M., Herder, V. & Beineke, A. Beneficial and Detrimental Effects of Regulatory T Cells in Neurotropic Virus Infections. *International journal of molecular sciences* **21** (2020).

68. Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T. & Nomura, T. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunological reviews* **212,** 8–27 (2006).

69. Curotto de Lafaille, M. A. & Lafaille, J. J. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* **30,** 626–635 (2009).

70. Collison, L. W., Chaturvedi, V., Henderson, A. L., Giacomin, P. R., Guy, C., Bankoti, J., Finkelstein, D., Forbes, K., Workman, C. J., Brown, S. A., Rehg, J. E., Jones, M. L., Ni, H.-T., Artis, D., Turk, M. J. & Vignali, D. A. A. IL-35-mediated induction of a potent regulatory T cell population. *Nature immunology* **11,** 1093–1101 (2010).

71. Turner, M. D., Nedjai, B., Hurst, T. & Pennington, D. J. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et biophysica acta* **1843,** 2563–2582 (2014).

72. Akdis, M., Aab, A., Altunbulakli, C., Azkur, K., Costa, R. A., Crameri, R., Duan, S., Eiwegger, T., Eljaszewicz, A., Ferstl, R., Frei, R., Garbani, M., Globinska, A., Hess, L., Huitema, C., Kubo, T., Komlosi, Z., Konieczna, P., Kovacs, N., Kucuksezer, U. C., Meyer, N., Morita, H., Olzhausen, J., O'Mahony, L., Pezer, M., Prati, M., Rebane, A., Rhyner, C., Rinaldi, A., Sokolowska, M., Stanic, B., Sugita, K., Treis, A., van de Veen, W., Wanke, K., Wawrzyniak, M., Wawrzyniak, P., Wirz, O. F., Zakzuk, J. S. & Akdis, C. A. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α. Receptors, functions, and roles in diseases. *The Journal of allergy and clinical immunology* **138,** 984–1010 (2016).

73. Brocker, C., Thompson, D., Matsumoto, A., Nebert, D. W. & Vasiliou, V. Evolutionary divergence and functions of the human interleukin (IL) gene family. *Hum Genomics* **5,** 30 (2010).

74. Briukhovetska, D., Dörr, J., Endres, S., Libby, P., Dinarello, C. A. & Kobold, S. Interleukins in cancer: from biology to therapy. *Nature reviews. Cancer* **21,** 481–499 (2021).

75. Garbers, C., Hermanns, H. M., Schaper, F., Müller-Newen, G., Grötzinger, J., Rose-John, S. & Scheller, J. Plasticity and cross-talk of interleukin 6-type cytokines. *Cytokine & growth factor reviews* **23,** 85–97 (2012).

76. Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chan, S., Loudon, R., Sherman, F., Perussia, B. & Trinchieri, G. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *The Journal of experimental medicine* **170,** 827–845 (1989).

77. Stern, A. S., Podlaski, F. J., Hulmes, J. D., Pan, Y. C., Quinn, P. M., Wolitzky, A. G., Familletti, P. C., Stremlo, D. L., Truitt, T. & Chizzonite, R. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proceedings of the National Academy of Sciences of the United States of America* **87,** 6808–6812 (1990).

78. Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y.-J., Abrams, J. S., Moore, K. W., Rennick, D., Waal-Malefyt, R. de, Hannum, C., Bazan, J. & Kastelein, R. A. Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12. *Immunity* **13,** 715–725 (2000).

79. Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W. M., Mattson, J. D., Wagner, J. L., To, W., Zurawski, S., McClanahan, T. K., Gorman, D. M., Bazan, J. F., Waal Malefyt, R. de, Rennick, D. & Kastelein, R. A. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells. *Immunity* **16,** 779–790 (2002).

80. Devergne O, Birkenbach M, Kieff E. Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. *Immunology,* 12041–12046 (1997).

81. Wang, X., Wei, Y., Xiao, H., Liu, X., Zhang, Y., Han, G., Chen, G., Hou, C., Ma, N., Shen, B., Li, Y., Egwuagu, C. E. & Wang, R. A novel IL-23p19/Ebi3 (IL-39) cytokine mediates inflammation in Lupus-like mice. *European journal of immunology* **46,** 1343–1350 (2016).

82. Wang, R.-X., Yu, C.-R., Mahdi, R. M. & Egwuagu, C. E. Novel IL27p28/IL12p40 cytokine suppressed experimental autoimmune uveitis by inhibiting autoreactive Th1/Th17 cells and promoting expansion of regulatory T cells. *The Journal of biological chemistry* **287,** 36012–36021 (2012).

83. Lee, S.-Y., Moon, S.-J., Moon, Y.-M., Seo, H.-B., Ryu, J.-G., Lee, A. R., Lee, C. R., Kim, D.-S., Her, Y.-M., Choi, J. W., Kwok, S.-K., Park, S.-H. & Cho, M.-L. A novel cytokine consisting of the p40 and EBI3 subunits suppresses experimental autoimmune arthritis via reciprocal regulation of Th17 and Treg cells. *Cellular & molecular immunology* **19,** 79–91 (2022).

84. Leonard, W. J., Lin, J.-X. & O'Shea, J. J. The γc Family of Cytokines: Basic Biology to Therapeutic Ramifications. *Immunity* **50,** 832–850 (2019).

85. Abdel-Meguid, S. S., Shieh, H. S., Smith, W. W., Dayringer, H. E., Violand, B. N. & Bentle, L. A. Three-dimensional structure of a genetically engineered variant of porcine growth hormone. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 6434–6437 (1987).

86. Boulay, J.-L., O'Shea, J. J. & Paul, W. E. Molecular Phylogeny within Type I Cytokines and Their Cognate Receptors. *Immunity* **19,** 159–163 (2003).

87. Lokau, J., Agthe, M., Flynn, C. M. & Garbers, C. Proteolytic control of Interleukin-11 and Interleukin-6 biology. *Biochimica et biophysica acta. Molecular cell research* **1864,** 2105–2117 (2017).

88. Wolf, S. F., Temple, P. A., Kobayashi, M., Young, D., Dicig, M., Lowe, L., Dzialo, R., Fitz, L., Ferenz, C. & Hewick, R. M. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *Journal of immunology (Baltimore, Md. : 1950)* **146,** 3074–3081 (1991).

89. Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., Cross, R., Sehy, D., Blumberg, R. S. & Vignali, D. A. A. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **450,** 566–569 (2007).

90. Bridgewood, C., Alase, A., Watad, A., Wittmann, M., Cuthbert, R. & McGonagle, D. The IL-23p19/EBI3 heterodimeric cytokine termed IL-39 remains a theoretical cytokine in man. *Inflammation research : official journal of the European Histamine Research Society … [et al.]* **68,** 423–426 (2019).

91. Coulomb-L'Herminé, A., Larousserie, F., Pflanz, S., Bardel, E., Kastelein, R. A. & Devergne, O. Expression of interleukin-27 by human trophoblast cells. *Placenta* **28,** 1133–1140 (2007).

92. Collison, L. W., Delgoffe, G. M., Guy, C. S., Vignali, K. M., Chaturvedi, V., Fairweather, D., Satoskar, A. R., Garcia, K. C., Hunter, C. A., Drake, C. G., Murray, P. J. & Vignali, D. A. A. The composition and signaling of the IL-35 receptor are unconventional. *Nature immunology* **13,** 290–299 (2012).

93. Müller, S. I., Aschenbrenner, I., Zacharias, M. & Feige, M. J. An Interspecies Analysis Reveals Molecular Construction Principles of Interleukin 27. *Journal of molecular biology* **431,** 2383–2393 (2019).

94. Vignali, D. A. A. & Kuchroo, V. K. IL-12 family cytokines. Immunological playmakers. *Nature immunology* **13,** 722–728 (2012).

95. Han, L., Chen, Z., Yu, K., Yan, J., Li, T., Ba, X., Lin, W., Huang, Y., Shen, P., Huang, Y., Qin, K., Geng, Y., Liu, Y., Wang, Y. & Tu, S. Interleukin 27 Signaling in Rheumatoid Arthritis Patients: Good or Evil? *Frontiers in immunology* **12,** 787252 (2021).

96. Gubler, U., Chua, A. O., Schoenhaut, D. S., Dwyer, C. M., McComas, W., Motyka, R., Nabavi, N., Wolitzky, A. G., Quinn, P. M., Familletti, P. C. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 4143–4147 (1991).

97. Jalah, R., Rosati, M., Ganneru, B., Pilkington, G. R., Valentin, A., Kulkarni, V., Bergamaschi, C., Chowdhury, B., Zhang, G.-M., Beach, R. K., Alicea, C., Broderick, K. E., Sardesai, N. Y., Pavlakis, G. N. & Felber, B. K. The p40 subunit of interleukin (IL)-12 promotes stabilization and export of the p35 subunit. Implications for improved IL-12 cytokine production. *The Journal of biological chemistry* **288,** 6763–6776 (2013).

98. Gerber, A. N., Abdi, K. & Singh, N. J. The subunits of IL-12, originating from two distinct cells, can functionally synergize to protect against pathogen dissemination in vivo. *Cell reports* **37,** 109816 (2021).

99. Reitberger, S., Haimerl, P., Aschenbrenner, I., Esser-von Bieren, J. & Feige, M. J. Assembly-induced folding regulates interleukin 12 biogenesis and secretion. *The Journal of biological chemistry* **292,** 8073–8081 (2017).

100. Alloza, I., Martens, E., Hawthorne, S. & Vandenbroeck, K. Cross-linking approach to affinity capture of protein complexes from chaotrope-solubilized cell lysates. *Analytical biochemistry* **324,** 137–142 (2004).

101. Alloza, I., Baxter, A., Chen, Q., Matthiesen, R. & Vandenbroeck, K. Celecoxib inhibits interleukin-12 alphabeta and beta2 folding and secretion by a novel COX2-independent mechanism involving chaperones of the endoplasmic reticulum. *Molecular pharmacology* **69,** 1579–1587 (2006).

102. Yoon, C., Johnston, S. C., Tang, J., Stahl, M., Tobin, J. F. & Somers, W. S. Charged residues dominate a unique interlocking topography in the heterodimeric cytokine interleukin-12. *The EMBO journal* **19,** 3530–3541 (2000).

103. Mideksa, Y. G., Fottner, M., Braus, S., Weiß, C. A. M., Nguyen, T.-A., Meier, S., Lang, K. & Feige, M. J. Site-Specific Protein Labeling with Fluorophores as a Tool To Monitor Protein Turnover. *Chembiochem : a European journal of chemical biology* **21,** 1861–1867 (2020).

104. McLaughlin, M., Alloza, I., Quoc, H. P., Scott, C. J., Hirabayashi, Y. & Vandenbroeck, K. Inhibition of secretion of interleukin (IL)-12/IL-23 family cytokines by 4-trifluoromethyl-celecoxib is coupled to degradation via the endoplasmic reticulum stress protein HERP. *The Journal of biological chemistry* **285,** 6960–6969 (2010).

105. Lupardus, P. J. & Garcia, K. C. The structure of interleukin-23 reveals the molecular basis of p40 subunit sharing with interleukin-12. *Journal of molecular biology* **382,** 931–941 (2008).

106. Meier, S., Bohnacker, S., Klose, C. J., Lopez, A., Choe, C. A., Schmid, P. W. N., Bloemeke, N., Rührnößl, F., Haslbeck, M., Bieren, J. E., Sattler, M., Huang, P.-S. & Feige, M. J. The molecular basis of chaperone-mediated interleukin 23 assembly control. *Nature communications* **10,** 4121 (2019).

107. Espígol-Frigolé, G., Planas-Rigol, E., Ohnuki, H., Salvucci, O., Kwak, H., Ravichandran, S., Luke, B., Cid, M. C. & Tosato, G. Identification of IL-23p19 as an endothelial proinflammatory peptide that promotes gp130-STAT3 signaling. *Science signaling* **9,** ra28 (2016).

108. Tormo, A. J., Beaupré, L. A., Elson, G., Crabé, S. & Gauchat, J.-F. A polyglutamic acid motif confers IL-27 hydroxyapatite and bone-binding properties. *Journal of immunology (Baltimore, Md. : 1950)* **190,** 2931–2937 (2013).

109. Müller, S. I., Friedl, A., Aschenbrenner, I., Esser-von Bieren, J., Zacharias, M., Devergne, O. & Feige, M. J. 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* **116,** 1585–1590 (2019).

110. Bohnacker, S., Hildenbrand, K., Aschenbrenner, I., Müller, S. I., Bieren, J. E. & Feige, M. J. Influence of glycosylation on IL-12 family cytokine biogenesis and function. *Molecular immunology* **126,** 120–128 (2020).

111. Rousseau, F., Basset, L., Froger, J., Dinguirard, N., Chevalier, S. & Gascan, H. IL-27 structural analysis demonstrates similarities with ciliary neurotrophic factor (CNTF) and leads to the identification of antagonistic variants. *Proceedings of the National Academy of Sciences of the United States of America* **107,** 19420–19425 (2010).

112. Jones, L. L., Chaturvedi, V., Uyttenhove, C., van Snick, J. & Vignali, D. A. A. Distinct subunit pairing criteria within the heterodimeric IL-12 cytokine family. *Molecular immunology* **51,** 234–244 (2012).

113. Aparicio-Siegmund, S., Moll, J. M., Lokau, J., Grusdat, M., Schröder, J., Plöhn, S., Rose-John, S., Grötzinger, J., Lang, P. A., Scheller, J. & Garbers, C. Recombinant p35 from bacteria can form Interleukin (IL-)12, but Not IL-35. *PloS one* **9,** e107990 (2014).

114. Devergne, O., Hummel, M., Koeppen, H., Le Beau, M. M., Nathanson, E. C., Kieff, E. & Birkenbach, M. A novel interleukin-12 p40-related protein induced by latent Epstein-Barr virus infection in B lymphocytes. *Journal of Virology* **70,** 1143–1153 (1996).

115. Hebert, D. N. & Molinari, M. Flagging and docking: dual roles for N-glycans in protein quality control and cellular proteostasis. *Trends in biochemical sciences* **37,** 404–410 (2012).

116. Mizoguchi, I., Ohashi, M., Hasegawa, H., Chiba, Y., Orii, N., Inoue, S., Kawana, C., Xu, M., Sudo, K., Fujita, K., Kuroda, M., Hashimoto, S.-I., Matsushima, K. & Yoshimoto, T. EBV-induced gene 3 augments IL-23Rα protein expression through a chaperone calnexin. *The Journal of clinical investigation* **130,** 6124–6140 (2020).

117. Sullivan, J. A., Tomita, Y., Jankowska-Gan, E., Lema, D. A., Arvedson, M. P., Nair, A., Bracamonte-Baran, W., Zhou, Y., Meyer, K. K., Zhong, W., Sawant, D. V., Szymczak-Workman, A. L., Zhang, Q., Workman, C. J., Hong, S., Vignali, D. A. A. & Burlingham, W. J. Treg-Cell-Derived IL-35-Coated Extracellular Vesicles Promote Infectious Tolerance. *Cell reports* **30,** 1039-1051.e5 (2020).

118. Devergne, O., Coulomb-L'Herminé, A., Capel, F., Moussa, M. & Capron, F. Expression of Epstein-Barr Virus-Induced Gene 3, an Interleukin-12 p40-Related Molecule, throughout Human Pregnancy. *The American Journal of Pathology* **159,** 1763–1776 (2001).

119. Caveney, N. A., Glassman, C. R., Jude, K. M., Tsutsumi, N. & Garcia, K. C. *Structure of the IL-27 quaternary receptor signaling complex* (2022).

120. Jones, L. L. & Vignali, D. A. A. Molecular interactions within the IL-6/IL-12 cytokine/receptor superfamily. *Immunologic research* **51,** 5–14 (2011).

121. Wang, R.-X., Yu, C.-R., Dambuza, I. M., Mahdi, R. M., Dolinska, M. B., Sergeev, Y. V., Wingfield, P. T., Kim, S.-H. & Egwuagu, C. E. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nature medicine* **20,** 633–641 (2014).

122. Pflanz, S., Hibbert, L., Mattson, J., Rosales, R., Vaisberg, E., Bazan, J. F., Phillips, J. H., McClanahan, T. K., Waal Malefyt, R. de & Kastelein, R. A. WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. *Journal of immunology (Baltimore, Md. : 1950)* **172,** 2225–2231 (2004).

123. Pennica, D., Shaw, K. J., Swanson, T. A., Moore, M. W., Shelton, D. L., Zioncheck, K. A., Rosenthal, A., Taga, T., Paoni, N. F. & Wood, W. I. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *The Journal of biological chemistry* **270,** 10915–10922 (1995).

124. Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T. & Kishimoto, T. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* **58,** 573–581 (1989).

125. Floss, D. M., Schönberg, M., Franke, M., Horstmeier, F. C., Engelowski, E., Schneider, A., Rosenfeldt, E. M. & Scheller, J. IL-6/IL-12 Cytokine Receptor Shuffling of Extra- and Intracellular Domains Reveals Canonical STAT Activation via Synthetic IL-35 and IL-39 Signaling. *Scientific reports* **7,** 15172 (2017).

126. Liu, J., Hao, S., Chen, X., Zhao, H., Du, L., Ren, H., Wang, C. & Mao, H. Human placental trophoblast cells contribute to maternal-fetal tolerance through expressing IL-35 and mediating iTR35 conversion. *Nature communications* **10,** 4601 (2019).

127. Lee, C.-C., Lin, J.-C., Hwang, W.-L., Kuo, Y.-J., Chen, H.-K., Tai, S.-K., Lin, C.-C. & Yang, M.-H. Macrophage-secreted interleukin-35 regulates cancer cell plasticity to facilitate metastatic colonization. *Nature communications* **9,** 3763 (2018).

128. Shen, P., Roch, T., Lampropoulou, V., O'Connor, R. A., Stervbo, U., Hilgenberg, E., Ries, S., van Dang, D., Jaimes, Y., Daridon, C., Li, R., Jouneau, L., Boudinot, P., Wilantri, S., Sakwa, I., Miyazaki, Y., Leech, M. D., McPherson, R. C., Wirtz, S., Neurath, M., Hoehlig, K., Meinl, E., Grützkau, A., Grün, J. R., Horn, K., Kühl, A. A., Dörner, T., Bar-Or, A., Kaufmann, S. H. E., Anderton, S. M. & Fillatreau, S. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature* **507,** 366–370 (2014).

129. Dixon, K. O., van der Kooij, S. W., Vignali, D. A. A. & van Kooten, C. Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members. *European journal of immunology* **45,** 1736–1747 (2015).

130. Seyerl, M., Kirchberger, S., Majdic, O., Seipelt, J., Jindra, C., Schrauf, C. & Stöckl, J. Human rhinoviruses induce IL-35-producing Treg via induction of B7-H1 (CD274) and sialoadhesin (CD169) on DC. *European journal of immunology* **40,** 321–329 (2010).

131. Hao, S., Chen, X., Wang, F., Shao, Q., Liu, J., Zhao, H., Yuan, C., Ren, H. & Mao, H. Breast cancer cell-derived IL-35 promotes tumor progression via induction of IL-35-producing induced regulatory T cells. *Carcinogenesis* **39,** 1488–1496 (2018).

132. Olson, B. M., Jankowska-Gan, E., Becker, J. T., Vignali, D. A. A., Burlingham, W. J. & McNeel, D. G. Human prostate tumor antigen-specific CD8+ regulatory T

cells are inhibited by CTLA-4 or IL-35 blockade. *Journal of immunology (Baltimore, Md. : 1950)* **189,** 5590–5601 (2012).

133. Niedbala, W., Wei, X.-Q., Cai, B., Hueber, A. J., Leung, B. P., McInnes, I. B. & Liew, F. Y. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *European journal of immunology* **37,** 3021–3029 (2007).

134. Zhang, J., Lin, Y., Li, C., Zhang, X., Cheng, L., Dai, L., Wang, Y., Wang, F., Shi, G., Li, Y., Yang, Q., Cui, X., Liu, Y., Wang, H., Zhang, S., Yang, Y., Xiang, R., Li, J., Yu, D., Wei, Y. & Deng, H. IL-35 Decelerates the Inflammatory Process by Regulating Inflammatory Cytokine Secretion and M1/M2 Macrophage Ratio in Psoriasis. *Journal of immunology (Baltimore, Md. : 1950)* **197,** 2131–2144 (2016).

135. Liu, X., Sun, Y., Zheng, Y., Zhang, M., Jin, X., Kang, K., Wang, Y., Li, S., Zhang, H., Zhao, Q., Zhang, S., Wu, J. & Yu, B. Administration of Interleukin-35-Conditioned Autologous Tolerogenic Dendritic Cells Prolong Allograft Survival After Heart Transplantation. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* **49,** 1180–1196 (2018).

136. Turnis, M. E., Sawant, D. V., Szymczak-Workman, A. L., Andrews, L. P., Delgoffe, G. M., Yano, H., Beres, A. J., Vogel, P., Workman, C. J. & Vignali, D. A. A. Interleukin-35 Limits Anti-Tumor Immunity. *Immunity* **44,** 316–329 (2016).

137. Wu, H., Li, P., Shao, N., Ma, J., Ji, M., Sun, X., Ma, D. & Ji, C. Aberrant expression of Treg-associated cytokine IL-35 along with IL-10 and TGF-β in acute myeloid leukemia. *Oncology letters* **3,** 1119–1123 (2012).

138. Li, T., Gu, M., Liu, P., Liu, Y., Guo, J., Zhang, W., Qian, C. & Deng, A. Clinical Significance of Decreased Interleukin-35 Expression in Patients with Psoriasis. *Microbiology and immunology* (2018).

139. Chen, C., Deng, Y., Chen, H., Wu, X., Cheng, S., Xu, Y., Xiong, W. & Xie, J. Decreased concentration of IL-35 in plasma of patients with asthma and COPD. *Asian Pacific journal of allergy and immunology* **32,** 211–217 (2014).

140. Lin, Y., Huang, Y., Lu, Z., Luo, C., shi, Y., Zeng, Q., Cao, Y., Liu, L., Wang, X. & Ji, Q. Decreased plasma IL-35 levels are related to the left ventricular ejection fraction in coronary artery diseases. *PloS one* **7,** e52490 (2012).

141. Jafarzadeh, A., Jamali, M., Mahdavi, R., Ebrahimi, H. A., Hajghani, H., Khosravimashizi, A., Nemati, M., Najafipour, H., Sheikhi, A., Mohammadi, M. M. & Daneshvar, H. Circulating levels of interleukin-35 in patients with multiple sclerosis: evaluation of the influences of FOXP3 gene polymorphism and treatment program. *Journal of molecular neuroscience : MN* **55,** 891–897 (2015).

142. Wirtz, S., Billmeier, U., Mchedlidze, T., Blumberg, R. S. & Neurath, M. F. Interleukin-35 mediates mucosal immune responses that protect against T-cell-dependent colitis. *Gastroenterology* **141,** 1875–1886 (2011).

143. Verbsky, J. W. Therapeutic use of T regulatory cells. *Current opinion in rheumatology* **19,** 252–258 (2007).

144. Horwitz, D. A., Zheng, S. G., Gray, J. D., Wang, J. H., Ohtsuka, K. & Yamagiwa, S. Regulatory T cells generated ex vivo as an approach for the therapy of autoimmune disease. *Seminars in immunology* **16,** 135–143 (2004).

145. Long, J., Guo, H., Cui, S., Zhang, H., Liu, X., Li, D., Han, Z., Xi, L., Kou, W., Xu, J., Li, T.-S. & Ding, Y. IL-35 expression in hepatocellular carcinoma cells is associated with tumor progression. *Oncotarget* **7,** 45678–45686 (2016).

146. Zhang, J., Zhang, Y., Wang, Q., Li, C., Deng, H., Si, C. & Xiong, H. Interleukin-35 in immune-related diseases. Protection or destruction. *Immunology* **157,** 13–20 (2019).

147. Larousserie, F., Kebe, D., Huynh, T., Audebourg, A., Tamburini, J., Terris, B. & Devergne, O. Evidence for IL-35 Expression in Diffuse Large B-Cell Lymphoma and Impact on the Patient's Prognosis. *Frontiers in oncology* **9,** 563 (2019).

148. Mattner, F., Fischer, S., Guckes, S., Jin, S., Kaulen, H., Schmitt, E., Rüde, E. & Germann, T. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. *European journal of immunology* **23,** 2202–2208 (1993).

149. Abdi, K. IL-12: the role of p40 versus p75. *Scandinavian journal of immunology* **56,** 1–11 (2002).

150. Cassatella, M. A., Gardiman, E., Arruda-Silva, F., Bianchetto-Aguilera, F., Gasperini, S., Bugatti, M., Vermi, W., Larousserie, F., Devergne, O. & Tamassia, N. Human neutrophils activated by TLR8 agonists, with or without IFNγ, synthesize and release EBI3, but not IL-12, IL-27, IL-35, or IL-39. *Journal of leukocyte biology* **108,** 1515–1526 (2020).

151. Chehboun, S., Labrecque-Carbonneau, J., Pasquin, S., Meliani, Y., Meddah, B., Ferlin, W., Sharma, M., Tormo, A., Masson, J.-F. & Gauchat, J.-F. Epstein-Barr virus-induced gene 3 (EBI3) can mediate IL-6 trans-signaling. *The Journal of biological chemistry* **292,** 6644–6656 (2017).

152. Jensen, H., Chen, S.-Y., Folkersen, L., Nolan, G. P. & Lanier, L. L. EBI3 regulates the NK cell response to mouse cytomegalovirus infection. *Proceedings of the National Academy of Sciences of the United States of America* **114,** 1625–1630 (2017).

153. Dambuza, I. M., He, C., Choi, J. K., Yu, C.-R., Wang, R., Mattapallil, M. J., Wingfield, P. T., Caspi, R. R. & Egwuagu, C. E. IL-12p35 induces expansion of IL-10 and IL-35-expressing regulatory B cells and ameliorates autoimmune disease. *Nature communications* **8,** 719 (2017).

154. Choi, J. K., Dambuza, I. M., He, C., Yu, C.-R., Uche, A. N., Mattapallil, M. J., Caspi, R. R. & Egwuagu, C. E. IL-12p35 Inhibits Neuroinflammation and Ameliorates Autoimmune Encephalomyelitis. *Frontiers in immunology* **8,** 1258 (2017).

155. Glassman, C. R., Mathiharan, Y. K., Jude, K. M., Su, L., Panova, O., Lupardus, P. J., Spangler, J. B., Ely, L. K., Thomas, C., Skiniotis, G. & Garcia, K. C. Structural basis for IL-12 and IL-23 receptor sharing reveals a gateway for shaping actions on T versus NK cells. *Cell* **184,** 983-999.e24 (2021).

156. Tait Wojno, E. D., Hunter, C. A. & Stumhofer, J. S. The Immunobiology of the Interleukin-12 Family: Room for Discovery. *Immunity* **50,** 851–870 (2019).

157. Yoshida, H. & Hunter, C. A. The immunobiology of interleukin-27. *Annual review of immunology* **33,** 417–443 (2015).

158. Ye, C., Yano, H., Workman, C. J. & Vignali, D. A. A. Interleukin-35: Structure, Function and Its Impact on Immune-Related Diseases. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **41,** 391–406 (2021).

159. Stumhofer, J. S., Tait, E. D., Quinn, W. J., Hosken, N., Spudy, B., Goenka, R., Fielding, C. A., O'Hara, A. C., Chen, Y., Jones, M. L., Saris, C. J. M., Rose-John, S., Cua, D. J., Jones, S. A., Elloso, M. M., Grötzinger, J., Cancro, M. P., Levin, S. D. & Hunter, C. A. A role for IL-27p28 as an antagonist of gp130-mediated signaling. *Nature immunology* **11,** 1119–1126 (2010).

160. Jana, M., Dasgupta, S., Pal, U. & Pahan, K. IL-12 p40 homodimer, the so-called biologically inactive molecule, induces nitric oxide synthase in microglia via IL-12R beta 1. *Glia* **57,** 1553–1565 (2009).

161. Mondal, S., Kundu, M., Jana, M., Roy, A., Rangasamy, S. B., Modi, K. K., Wallace, J., Albalawi, Y. A., Balabanov, R. & Pahan, K. IL-12 p40 monomer is different from other IL-12 family members to selectively inhibit IL-12Rβ1 internalization and suppress EAE. *Proceedings of the National Academy of Sciences of the United States of America* **117,** 21557–21567 (2020).

162. Wang, Z., Liu, J.-Q., Liu, Z., Shen, R., Zhang, G., Xu, J., Basu, S., Feng, Y. & Bai, X.-F. Tumor-derived IL-35 promotes tumor growth by enhancing myeloid cell accumulation and angiogenesis. *Journal of immunology (Baltimore, Md. : 1950)* **190,** 2415–2423 (2013).

163. Zhao, Z., Chen, X., Hao, S., Jia, R., Wang, N., Chen, S., Li, M., Wang, C. & Mao, H. Increased interleukin-35 expression in tumor-infiltrating lymphocytes correlates with poor prognosis in patients with breast cancer. *Cytokine* **89,** 76–81 (2017).

164. Chen, G., Liang, Y., Guan, X., Chen, H., Liu, Q., Lin, B., Chen, C., Huang, M., Chen, J., Wu, W., Liang, Y., Zhou, K. & Zeng, J. Circulating low IL-23: IL-35 cytokine ratio promotes progression associated with poor prognosisin breast cancer. *American Journal of Translational Research* **8,** 2255–2264 (2016).

165. Jin, P., Ren, H., Sun, W., Xin, W., Zhang, H. & Hao, J. Circulating IL-35 in pancreatic ductal adenocarcinoma patients. *Human immunology* **75,** 29–33 (2014).

166. Nicholl, M. B., Ledgewood, C. L., Chen, X., Bai, Q., Qin, C., Cook, K. M., Herrick, E. J., Diaz-Arias, A., Moore, B. J. & Fang, Y. IL-35 promotes pancreas cancer growth through enhancement of proliferation and inhibition of apoptosis: evidence for a role as an autocrine growth factor. *Cytokine* **70,** 126–133 (2014).

167. Huang, C., Li, N., Li, Z., Chang, A., Chen, Y., Zhao, T., Li, Y., Wang, X., Zhang, W., Wang, Z., Luo, L., Shi, J., Yang, S., Ren, H. & Hao, J. Tumour-derived Interleukin 35 promotes pancreatic ductal adenocarcinoma cell extravasation and metastasis by inducing ICAM1 expression. *Nature communications* **8,** 14035 (2017).

168. Heim, L., Kachler, K., Siegmund, R., Trufa, D. I., Mittler, S., Geppert, C.-I., Friedrich, J., Rieker, R. J., Sirbu, H. & Finotto, S. Increased expression of the immunosuppressive interleukin-35 in patients with non-small cell lung cancer. *British journal of cancer* **120,** 903–912 (2019).

169. Ning, X., Jian, Z. & Wang, W. Low Serum Levels of Interleukin 35 in Patients with Rheumatoid Arthritis. *The Tohoku journal of experimental medicine* **237,** 77–82 (2015).

170. Li, W., Gao, R., Xin, T. & Gao, P. Different expression levels of interleukin-35 in asthma phenotypes. *Respiratory research* **21,** 89 (2020).

171. Munro, S. & Pelham, H. R. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48,** 899–907 (1987).

172. Lee, S.-S., Won, J.-H., Lim, G. J., Han, J., Lee, J. Y., Cho, K.-O. & Bae, Y.-K. A novel population of extracellular vesicles smaller than exosomes promotes cell proliferation. *Cell communication and signaling : CCS* **17,** 95 (2019).

173. Yu, X., Buttgereit, A., Lelios, I., Utz, S. G., Cansever, D., Becher, B. & Greter, M. The Cytokine TGF-β Promotes the Development and Homeostasis of Alveolar Macrophages. *Immunity* **47,** 903-912.e4 (2017).

174. Dong, J., Wong, C. K., Cai, Z., Jiao, D., Chu, M. & Lam, C. W. K. Amelioration of allergic airway inflammation in mice by regulatory IL-35 through dampening inflammatory dendritic cells. *Allergy* **70,** 921–932 (2015).

175. Wing, R., Gjelsvik, A., Nocera, M. & McQuaid, E. L. Association between adverse childhood experiences in the home and pediatric asthma. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* **114,** 379–384 (2015).

176. Baran, P., Hansen, S., Waetzig, G. H., Akbarzadeh, M., Lamertz, L., Huber, H. J., Ahmadian, M. R., Moll, J. M. & Scheller, J. The balance of interleukin (IL)-6, IL-6·soluble IL-6 receptor (sIL-6R), and IL-6·sIL-6R·sgp130 complexes allows simultaneous classic and trans-signaling. *The Journal of biological chemistry* **293,** 6762–6775 (2018).

177. Rose-John, S. The Soluble Interleukin 6 Receptor: Advanced Therapeutic Options in Inflammation. *Clinical pharmacology and therapeutics* **102,** 591–598 (2017).

178. D'Andrea, A., Rengaraju, M., Valiante, N. M., Chehimi, J., Kubin, M., Aste, M., Chan, S. H., Kobayashi, M., Young, D. & Nickbarg, E. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *The Journal of experimental medicine* **176,** 1387–1398 (1992).

179. Cooper, A. M. & Khader, S. A. IL-12p40: an inherently agonistic cytokine. *Trends in immunology* **28,** 33–38 (2007).

180. Gonin, J., Larousserie, F., Bastard, C., Picquenot, J.-M., Couturier, J., Radford-Weiss, I., Dietrich, C., Brousse, N., Vacher-Lavenu, M.-C. & Devergne, O. Epstein-Barr virus-induced gene 3 (EBI3): a novel diagnosis marker in Burkitt lymphoma and diffuse large B-cell lymphoma. *PloS one* **6,** e24617 (2011).

181. Calderón, M. A., Linneberg, A., Kleine-Tebbe, J., Blay, F. de, Hernandez Fernandez de Rojas, D., Virchow, J. C. & Demoly, P. Respiratory allergy caused by house dust mites: What do we really know? *The Journal of allergy and clinical immunology* **136,** 38–48 (2015).

182. van der Veen, T. A., Groot, L. E. S. de & Melgert, B. N. The different faces of the macrophage in asthma. *Current opinion in pulmonary medicine* **26,** 62–68 (2020).

183. Draijer, C., Robbe, P., Boorsma, C. E., Hylkema, M. N. & Melgert, B. N. Characterization of macrophage phenotypes in three murine models of house-dust-mite-induced asthma. *Mediators of inflammation* **2013,** 632049 (2013).

184. Dam, J., Velikovsky, C. A., Mariuzza, R. A., Urbanke, C. & Schuck, P. Sedimentation velocity analysis of heterogeneous protein-protein interactions: Lamm equation modeling and sedimentation coefficient distributions c(s). *Biophysical journal* **89,** 619–634 (2005).

185. Harding, S. E., Arthur Jennison Rowe, and J. C. Horton. (ed.). *Analytical ultracentrifugation in biochemistry and polymer science* (Cambridge [England]: Royal Society of Chemistry, 1992).

186. Dietz, K., Los Reyes Jiménez, M. de, Gollwitzer, E. S., Chaker, A. M., Zissler, U. M., Rådmark, O. P., Baarsma, H. A., Königshoff, M., Schmidt-Weber, C. B., Marsland, B. J. & Esser-von Bieren, J. Age dictates a steroid-resistant cascade of Wnt5a, transglutaminase 2, and leukotrienes in inflamed airways. *The Journal of allergy and clinical immunology* **139,** 1343-1354.e6 (2017).

187. Esser-von Bieren, J., Mosconi, I., Guiet, R., Piersgilli, A., Volpe, B., Chen, F., Gause, W. C., Seitz, A., Verbeek, J. S. & Harris, N. L. Antibodies trap tissue

migrating helminth larvae and prevent tissue damage by driving IL-4Rα-independent alternative differentiation of macrophages. *PLoS pathogens* **9,** e1003771 (2013).

188. Croxford, A. L., Mair, F. & Becher, B. IL-23. One cytokine in control of autoimmunity. *European journal of immunology* **42,** 2263–2273 (2012).

189. Sawant, D. V., Hamilton, K. & Vignali, D. A. A. Interleukin-35. Expanding Its Job Profile. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **35,** 499–512 (2015).

190. Teng, M. W. L., Bowman, E. P., McElwee, J. J., Smyth, M. J., Casanova, J.-L., Cooper, A. M. & Cua, D. J. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. *Nature medicine* **21,** 719–729 (2015).

191. Trinchieri, G., Pflanz, S. & Kastelein, R. A. The IL-12 Family of Heterodimeric Cytokines. *Immunity* **19,** 641–644 (2003).

192. Garbers, C., Spudy, B., Aparicio-Siegmund, S., Waetzig, G. H., Sommer, J., Hölscher, C., Rose-John, S., Grötzinger, J., Lorenzen, I. & Scheller, J. An interleukin-6 receptor-dependent molecular switch mediates signal transduction of the IL-27 cytokine subunit p28 (IL-30) via a gp130 protein receptor homodimer. *The Journal of biological chemistry* **288,** 4346–4354 (2013).

193. Gately, M. K., Carvajal, D. M., Connaughton, S. E., Gillessen, S., Warrier, R. R., Kolinsky, K. D., Wilkinson, V. L., Dwyer, C. M., Higgins, G. F., Podlaski, F. J., Faherty, D. A., Familletti, P. C., Stern, A. S. & Presky, D. H. Interleukin-12 antagonist activity of mouse interleukin-12 p40 homodimer in vitro and in vivo. *Annals of the New York Academy of Sciences* **795,** 1–12 (1996).

194. Lee, S.-Y., Jung, Y. O., Kim, D.-J., Kang, C.-M., Moon, Y.-M., Heo, Y.-J., Oh, H.-J., Park, S.-J., Yang, S.-H., Kwok, S. K., Ju, J.-H., Park, S.-H., Sung, Y. C., Kim, H.-Y. & Cho, M.-L. IL-12p40 Homodimer Ameliorates Experimental Autoimmune Arthritis. *Journal of immunology (Baltimore, Md. : 1950)* **195,** 3001–3010 (2015).

195. Ling, P., Gately, M. K., Gubler, U., Stern, A. S., Lin, P., Hollfelder, K., Su, C., Pan, Y. C. & Hakimi, J. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *Journal of immunology (Baltimore, Md. : 1950)* **154,** 116–127 (1995).

196. Carra, G., Gerosa, F. & Trinchieri, G. Biosynthesis and posttranslational regulation of human IL-12. *Journal of immunology (Baltimore, Md. : 1950)* **164,** 4752–4761 (2000).

197. Podlaski, F. J., Nanduri, V. B., Hulmes, J. D., Pan, Y.-C. E., Levin, W., Danho, W., Chizzonite, R., Gately, M. K. & Stern, A. S. Molecular characterization of interleukin 12. *Archives of Biochemistry and Biophysics* **294,** 230–237 (1992).

198. Ha, S. J., Chang, J., Song, M. K., Suh, Y. S., Jin, H. T., Lee, C. H., Nam, G. H., Choi, G., Choi, K. Y., Lee, S. H., Kim, W. B. & Sung, Y. C. Engineering N-glycosylation mutations in IL-12 enhances sustained cytotoxic T lymphocyte responses for DNA immunization. *Nature biotechnology* **20,** 381–386 (2002).

199. Böhm, E., Seyfried, B. K., Dockal, M., Graninger, M., Hasslacher, M., Neurath, M., Konetschny, C., Matthiessen, P., Mitterer, A. & Scheiflinger, F. Differences in N-glycosylation of recombinant human coagulation factor VII derived from BHK, CHO, and HEK293 cells. *BMC biotechnology* **15,** 87 (2015).

200. Zhang, Z., Shah, B. & Richardson, J. Impact of Fc N-glycan sialylation on IgG structure. *mAbs* **11,** 1381–1390 (2019).

201. Chan, S. H., Perussia, B., Gupta, J. W., Kobayashi, M., Pospísil, M., Young, H. A., Wolf, S. F., Young, D., Clark, S. C. & Trinchieri, G. Induction of interferon gamma production by natural killer cell stimulatory factor. Characterization of the responder cells and synergy with other inducers. *The Journal of experimental medicine* **173,** 869–879 (1991).

202. Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A. & Cua, D. J. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *The Journal of experimental medicine* **201,** 233–240 (2005).

203. Dietrich, C., Candon, S., Ruemmele, F. M. & Devergne, O. A soluble form of IL-27Rα is a natural IL-27 antagonist. *Journal of immunology (Baltimore, Md. : 1950)* **192,** 5382–5389 (2014).

204. Yan, J., Mitra, A., Hu, J., Cutrera, J. J., Xia, X., Doetschman, T., Gagea, M., Mishra, L. & Li, S. Interleukin-30 (IL27p28) alleviates experimental sepsis by modulating cytokine profile in NKT cells. *Journal of hepatology* **64,** 1128–1136 (2016).

205. Yeku, O. O. & Brentjens, R. J. Armored CAR T-cells: utilizing cytokines and pro-inflammatory ligands to enhance CAR T-cell anti-tumour efficacy. *Biochemical Society transactions* **44,** 412–418 (2016).

206. Bootz, F., Venetz, D., Ziffels, B. & Neri, D. Different tissue distribution properties for glycosylation variants of fusion proteins containing the p40 subunit of murine interleukin-12. *Protein engineering, design & selection : PEDS* **29,** 445–455 (2016).

207. Chamorey, A.-L., Magné, N., Pivot, X. & Milano, G. Impact of glycosylation on the effect of cytokines. A special focus on oncology. *European cytokine network* **13,** 154–160 (2002).

208. Xue, W., Yan, D. & Kan, Q. Interleukin-35 as an Emerging Player in Tumor Microenvironment. *Journal of Cancer* **10,** 2074–2082 (2019).

209. Mirlekar, B., Michaud, D., Searcy, R., Greene, K. & Pylayeva-Gupta, Y. IL35 Hinders Endogenous Antitumor T-cell Immunity and Responsiveness to Immunotherapy in Pancreatic Cancer. *Cancer immunology research* **6,** 1014–1024 (2018).

210. Pylayeva-Gupta, Y., Das, S., Handler, J. S., Hajdu, C. H., Coffre, M., Koralov, S. B. & Bar-Sagi, D. IL35-Producing B Cells Promote the Development of Pancreatic Neoplasia. *Cancer discovery* **6,** 247–255 (2016).

211. Steentoft, C., Vakhrushev, S. Y., Joshi, H. J., Kong, Y., Vester-Christensen, M. B., Schjoldager, K. T.-B. G., Lavrsen, K., Dabelsteen, S., Pedersen, N. B., Marcos-Silva, L., Gupta, R., Bennett, E. P., Mandel, U., Brunak, S., Wandall, H. H., Levery, S. B. & Clausen, H. Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. *The EMBO journal* **32,** 1478–1488 (2013).

212. Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D. & Higgins, D. G. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* **7,** 539 (2011).

213. Fischer, M., Goldschmitt, J., Peschel, C., Brakenhoff, J. P., Kallen, K. J., Wollmer, A., Grötzinger, J. & Rose-John, S. I. A bioactive designer cytokine for human hematopoietic progenitor cell expansion. *Nature biotechnology* **15,** 142–145 (1997).

214. Gao, P., Su, Z., Lv, X. & Zhang, J. Interluekin-35 in Asthma and Its Potential as an Effective Therapeutic Agent. *Mediators of inflammation* **2017,** 5931865 (2017).

215. Huang, C.-H., Loo, E. X.-L., Kuo, I.-C., Soh, G. H., Goh, D. L.-M., Lee, B. W. & Chua, K. Y. Airway inflammation and IgE production induced by dust mite allergen-specific memory/effector Th2 cell line can be effectively attenuated by IL-35. *Journal of immunology (Baltimore, Md. : 1950)* **187,** 462–471 (2011).

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Declaration

I hereby declare that this thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in preparation of this thesis other than those indicated in the thesis itself.

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Karen Hildenbrand