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Regulation of the Th1 immune response : the role of IL-23 and the influence of genetic variations

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Regulation of the Th1 immune response:
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Regulation of the Th1 immune response: the role of IL-23 and the influence of genetic variations

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Introduction

The immune system

The human immune system is crucial to our survival since among other functions it protects the integrity of the tissues against pathogens invading from our environment and helps limit damage exerted by our microbial adversaries. The intact skin and the mucosal membranes in the gut and lung provide a first physical barrier that effectively prevent pathogens such as bacteria from invading the human body. In case this first line of defence is breached, the pathogen is confronted by the immune system. The human immune system can be divided into an innate and an adaptive arm.

Innate immune system

The first line of defence against invading pathogens consists of the innate immune system. The innate immune response has evolved to recognize a broad spectrum of pathogens and defends the host from infection by pathogens in an apparent non-specific manner. Moreover, this part of the immune system provides immediate defense against infection (1). The system is composed of both humoral and cellular mechanisms. The complement system is key component of the humoral mechanism (2), whereas the cellular arm consists of many cell types, including granulocytes, monocytes, macrophages and natural killer (NK) cells. In contrast to the adaptive immune system, exposure of the innate immune system to a pathogen does not induce immunological memory, i.e., the ability to react more rapid and specific upon a second contact with the pathogen.

Phagocytosis of invading pathogens represents an early and crucial event in host defense. Phagocytic cells include granulocytes, monocytes, tissue macrophages and dendritic cells. Phagocytosis starts with the binding of a pathogen to cell surface receptors, and is followed by the uptake of the pathogen into a vesicle designated phagosome (3). Next, the phagosome fuses with lysosomes and subsequently matures into a phagolysosome (3). Pathogens contained within a phagolysosome are destroyed by lowered pH, enzymatic hydrolysis, and radical attack (3,4).

Recognition of pathogens is dependent on the binding of pathogen-derived structures to germline-encoded pathogen recognition receptors (PRRs) expressed by phagocytes (5,6). PRRs bind pathogen-associated molecular patterns (PAMPs) which are conserved between classes of pathogens (7,8). PRRs are involved in phagocytosis of pathogens and in the activation of pro-inflammatory pathways and recruitment of inflammatory cells (7). Phagocytes express membrane-bound as well as cytosolic PRRs. Of membrane-bound PRRs, perhaps the best known are the Toll-like receptors (TLRs). The TLR family forms a class of PRRs consisting of at least 10 members that each recognize a different classe of pathogen-derived patterns (9). Apart from TLRs, membrane-bound C-type lectins and the cytosolic nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid inducible gene (RIG)-I-like helicases (RLHs) play important roles in triggering innate immune responses (10-12). Members of the NLR family form the central

components of inflammasomes and act as intracellular sensors that detect cytosolic microbial components (10). The inflammasome is a multiprotein complex involved in activating caspase-1 and -5, leading to the processing and secretion of the pro-inflammatory cytokines IL-1 β and IL-18. In addition, some of the PRRs are secreted into the bloodstream. Secreted PRRs include the C-type lectin mannan-binding lectin (MBL), collectins and ficolins (12). These PRRs bind to a wide range of bacteria, viruses, fungi and protozoa and serve to opsonise pathogens, help to activate complement and allow for enhanced recognition by phagocytic cells (12).

The early phase of an inflammatory response is characterised by an alteration of the local vascular permeability and influx of inflammatory cells. Upon recognition of pathogens via PRRs, cells of the innate immune system produce a wide range of inflammatory mediators such as cytokines, chemokines and interferons (IFN). These mediators orchestrate the development of the adaptive immune response.

Adaptive immune system

When pathogens successfully evade the innate immune system, a second line of protection is provided by the specific or adaptive immune system. The adaptive immune system provides a stronger and pathogen specific immune response and induces an immunological memory that enables it to react more promptly and specifically upon a second encounter with the same pathogen. Like the innate immune system, the adaptive immune system consists of a humoral and a cellular part. B cells and T cells are the major types of lymphocytes of the specific immune system (13). B cells are involved in the humoral part, whereas T cells are involved mainly in the cellular part, though intracellular signalling by soluble factors, interleukins or cytokines, plays a role in both arms.

B cells produce specific antibodies that specifically recognize and neutralize pathogens. Naïve B cells express a unique membrane bound B cell receptor (BCR). Ligation of the BCR on these cells by pathogen-derived structures induces the proliferation and differentiation of the cells into antibody secreting plasma cells (14). Generally, B cells require help from activated T helper cells to mature into fully activated plasma cells (15).

Two major subtypes of T cells can be distinguished: CD4⁺ T helper cells and CD8⁺ cytotoxic T cells, subtypes that play a distinct function. T cells express a membrane bound T cell receptor (TCR), which can recognize antigens bound to the major histocompatibility complex (MHC). There are two different classes of MHC molecules of importance here: MHC class I and MHC class II. MHC class I molecules are expressed on all cells and present intracellular-derived antigens to CD8⁺ cytotoxic T cells, whereas MHC class II molecules are expressed only on professional antigen presenting cells (APC) like dendritic cells (DC) and macrophages, and present antigens of an extracellular origin to CD4⁺ T cells. CD4⁺ T helper cells help regulate the immune response, for instance to intracellular bacterial pathogens like *Salmonellae* and *Mycobacteria*, whereas CD8⁺ cytotoxic T cells eliminate mainly virally-infected cells.

T helper cell immune responses: Th1, Th2 and Th17

An effective and specific immune response against pathogens that invade the human body is dependent on the differentiation of T lymphocytes into effector cells. Specific immune responses are driven by the emergence of pathogen-specific effector CD4⁺ T-cell subsets: the Th1 cells, which primarily produce IFN- γ ; Th2 cells, which produce IL-4, IL-5, IL-10 and IL-13 (16); and the recently discovered Th17 cells, producing IL-17.

Differentiation of naïve CD4⁺ T cells into effector CD4⁺ T cells is dependent on the type of pathogen and is guided by cells of the innate immune system. Th1 cells control intracellular pathogens, including viruses and bacteria, and are involved in autoimmune diseases (17). For example, host defense against intracellular bacterial pathogens such as *Salmonellae* and *Mycobacteria* is dependent on the type-1 IL-12/IL-23/IFN- γ pathway and deficiencies of components of this pathway render patients highly susceptible to infections with these pathogens (18). The Th2 cells are important in defence against helminths. In addition, the Th2 cell is thought to be the central effector cell in the pathogenesis of atopic allergic diseases (19). For example, IL-4 and IL-13 elicit antigen-specific IgE production in B cells, essential for the immediate hypersensitivity response, leading to mast cell degranulation and the subsequent release of histamine (20). The exact function of Th17 cells still has to be established. However, this T helper cell subset is thought to play an important role in the defense against bacterial, fungal, and viral infections at mucosal surfaces (21). In addition, Th17 cells are thought to be crucial for the development of various auto-immune diseases.

The activation and differentiation of naïve T helper cells is thought to be dependent on three signals (22). The first signal results from ligation of T-cell receptors (TCRs) by pathogen-derived peptides, presented by MHC class II molecules on the cell surface of APCs. This signal determines the antigen-specificity of the response. The second signal is provided by the co-stimulatory molecules CD80 and CD86 expressed by the APC, which bind and activate CD28 on the T cell. TCR triggering in the absence of this co-stimulatory signal leads to the induction of anergic T helper cells and might lead to tolerance. The third signal is the polarizing signal and depends on the cytokines activating the T cell. IL-12 and IL-4 are seen as the major polarizing cytokines for naïve T helper cells to differentiate into Th1 and Th2 cells, respectively (23,24). Both signals 2 and 3 are thought to be dependent on the binding of specific microbial products or endogenous danger signals on receptors expressed by APCs and the resultant cytokine production (22,25).

Cytokines

Innate and adaptive immunity are not just sequential mechanisms, but rather are interwoven and regulating each other in many reciprocal pathways. For instance, the innate immune system provides the cells of the adaptive system with information via the secretion of soluble mediators and

cell-cell contact. One of these soluble mediators is IFN- γ . IFN- γ is critical to many immune responses and regulates many processes.

Interferon- γ

Effective defence against different types of pathogens require different types of immune responses to be elicited. IFN- γ plays a central role in the type-1 immune response and is critical to innate and adaptive immunity against intracellular bacteria and parasites. IFN- γ and IFN- γ R-deficient mice develop normally, however, these mice display selective defects in the immune response against pathogens, including *Salmonellae* and *Mycobacteria* (26-31). Humans with IFN- γ R1 or IFN- γ R2 deficiency show a clinical presentation similar to that observed in the mouse models; the patients have impaired Th1-immunity and suffer from unusually severe infections caused by weakly virulent *Mycobacteria* (32). On the other side, in both IFN- γ transgenic mice and in SOCS1-deficient mice, lacking negative feedback of the IFN- γ signal transduction pathway, fatal multi-system inflammation is observed (33,34).

Interferon- γ production

At early stages of infection, IFN- γ secretion by NK cells, NK-like T cells, NKT cells, $\gamma\delta$ T cells and CD8⁺ T cells is crucial, whereas later on, T lymphocytes become the major source of IFN- γ when the adaptive immune response has evolved (35-39). IFN- γ produced in the early phases of an infection by the innate immune system skews the adaptive immune response towards a T helper 1 (Th1) phenotype (40-42). IFN- γ production is controlled by cytokines secreted by antigen presenting cells (APC). The IL-12 cytokine family (e.g. IL-12, IL-23 and IL-27) is thought to be of major importance in the induction of IFN- γ production. IL-18 and IL-1 β are known to synergise with IL-12 and IL-23 in inducing IFN- γ production in various cell types (43,44). Together, these cytokines serve in the coupling of the innate and adaptive immune response (45).

Interferon- γ functions

IFN- γ is produced by Th1 cells, skews the immune response further towards a Th1 phenotype and inhibits Th2 cell populations. After binding of IFN- γ to the IFN- γ R complex on its target cells, many effects are induced, including the following:

- 1) IFN- γ enhances the synthesis of components of the complement cascade and the acute phase response, thereby enhancing the humoral arm of innate immunity (46-49).
- 2) The availability of sufficient iron is essential for the growth of many pathogens. IFN- γ limits the availability of iron by reducing serum iron and increasing ferritin levels, thereby inhibiting growth of many extracellular pathogens (50). To inhibit the growth of intracellular

pathogens within infected macrophages, IFN- γ reduces the uptake of iron and increases iron efflux (51).

3) IFN- γ contributes to macrophage activation by increasing phagocytosis (52-54). IFN- γ increases the expression of the high-affinity IgG receptor, Fc γ RI (CD64) on the granulocyte, monocyte and macrophage cell surface. This receptor binds IgG, allowing for enhanced phagocytosis, binding of immune-complexes, and antibody-dependent cellular cytotoxicity (55,56).

4) IFN- γ induces antimicrobial effector function. After phagocytosis of a pathogen, IFN- γ enhances phagosome-lysosome fusion in infected macrophages and speeds up the maturation and acidification of the phagolysosome (57-60). IFN- γ enhances the expression of proteins involved in the subsequent destruction of pathogens in the phagolysosome, *including* immunity-related GTPases (IRG) (61), natural resistance-associated macrophage protein (NRAMP1) and proteins involved in the oxidative burst (62-64). The membrane-associated complex NADPH mediates the formation of superoxide anions (O_2^-). IFN- γ in synergy with TNF, enhances the transcription of genes encoding gp91-phox, p47-phox and p67-phox, all components of the NADPH complex, thereby promoting formation of this complex and the production of O_2^- anions and hydrogen peroxide (65). Furthermore, IFN- γ enhances the production of nitric oxide (NO), an unstable radical gas. IFN- γ maximises the expression of inducible nitric oxide synthase (iNOS), an enzyme that catalyzes the oxidation of one of the guanidino nitrogens of L-arginine to nitric oxide (NO) (52).

5) In addition, IFN- γ enhances antigen processing and the expression of the MHC class I and II molecules, thereby increasing antigen presentation and promoting the induction of cell-mediated immunity (66,67). The IFN- γ induced up-regulation of class I MHC increases the potential for cytotoxic T cells to recognize infected cells. On the other hand, via the up-regulation of class II MHC molecules, IFN- γ promotes the activation of CD4⁺ T helper cells.

6) Immunoglobulines are important for the opsonisation and neutralisation of many pathogens. IFN- γ stimulates immunoglobulin (Ig) production and matures the antibody response by the induction of class switching in B cells (68).

7) IFN- γ primes cells for more rapid and heightened production of pro-inflammatory cytokines upon TLR stimulation (69-71). For example, in the course of an immune response, the IFN- γ produced by innate immune cells provides a strong positive feedback loop to enhance IL-12, IL-23 and TNF production by monocytes and macrophages (69-71), thereby enhancing Th1 immunity.

Interferon- γ signal transduction

The IFN- γ R is comprised of two ligand-binding IFN- γ R1 chains associated with two signal-transducing IFN- γ R2 chains (Fig. 1) (66). Binding of IFN- γ as a non-covalently linked homodimer to its receptor induces receptor oligomerization and activation of the receptor-associated Janus kinases (JAK)1 and JAK2 by trans-phosphorylation. JAK1 and JAK2 associate with the IFN- γ R1 and IFN- γ R2, respectively. JAK1 phosphorylates the functionally critical tyrosine residue on position 440 (Y440) in the intracellular domain of each IFN- γ R1 chain. The Y440 serves as the docking site for signal transducer and activator of transcription (STAT)1. After docking to the Y440, STAT1 is phosphorylated

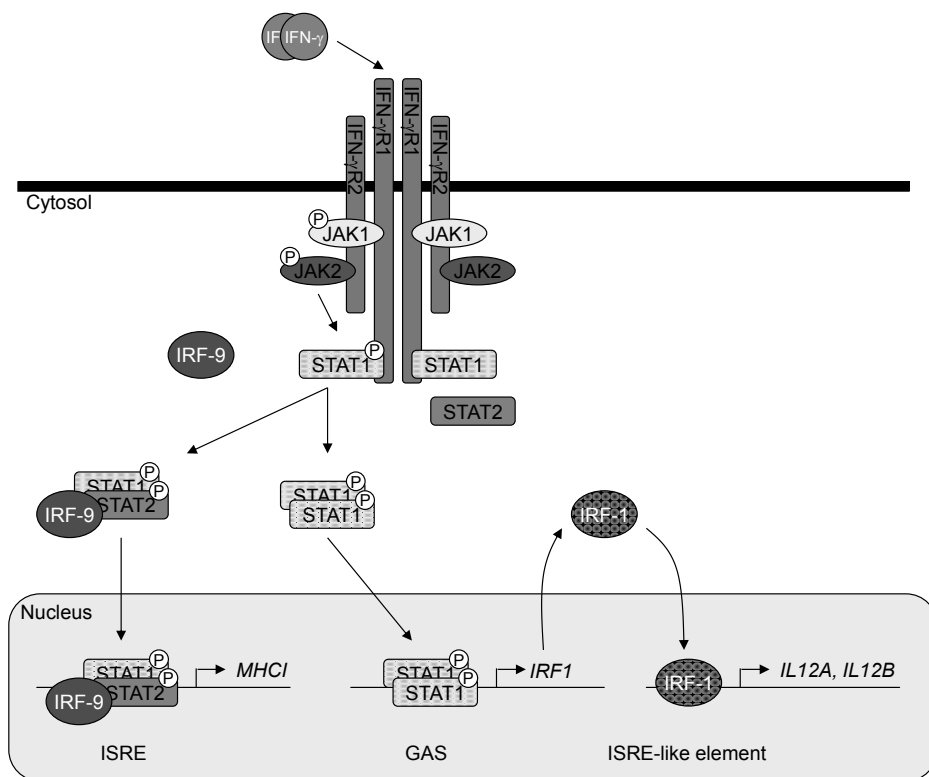


Figure 1. IFN- γ signal-transduction. IFN- γ oligomerization of the IFN- γ -receptor subunits IFN- γ R1 and IFN- γ R2 leads to the phosphorylation and activation of JAK1, JAK2 and the tyrosine 440 of the IFN- γ R1. Subsequently STAT1 docks to the phosphorylated IFN- γ R1 to become phosphorylated itself. Phosphorylated STAT1 dimerizes and translocates to the nucleus where it binds to GAS-elements. Among the primary response genes are a number of transcription factors including IRF-1. IRF-1 plays a role in regulating the expression of secondary response genes through promoters with IRSE-like motifs. To a lesser extent, IFN- γ signalling also results in the formation of STAT1:STAT1:IRF-9 and STAT1:STAT2:IRF-9 (ISGF3) complexes

on its tyrosine 701 (Y701) and serine 727 (S727) residues (66). Phosphorylation of the Y701 is essential for subsequent dimerisation of STAT1 molecules, while the phosphorylation of the S727 is

needed for full transcriptional activity. After being phosphorylated, STAT1 dissociates from the receptor, dimerizes and translocates to the nucleus, where it directly regulates the expression of IFN- γ responsive genes (e.g. *ICAM1*) (72), or indirectly activates genes, via the induction of transcription factors such as interferon regulatory factor (*IRF1*, *IRF8* and *CIITA* (e.g. *IL12B*, *IL12A*, *B2M* and *HLA*) (73-76). Apart from STAT1 homodimers, IFN- γ induces, although to a lesser extent, STAT1/STAT2/IRF-9 complexes (also known as IFN-stimulated gene factor 3 (ISGF3)). ISGF3 induces target genes containing an interferon-stimulated response element (ISRE) element. Via the induction of these transcription factors, IFN- γ regulates a wide range of distinct cellular programs via the activation of multiple genes.

The IL-12 cytokine family

Cytokines can be classified based on size, gene organization, sequence homology and structural motifs, such as the common four-helix bundle present in type I cytokines (77). Type I cytokines regulate development, differentiation, and activation of immune cells and cells of the inflammatory system (77). The cytokines IL-12, IL-23, IL-27 and IL-35 are members of the IL-12 cytokine subfamily, which is part of the type I cytokine superfamily. A specific feature of the members of the IL-12 cytokine family is that they are formed by heterodimerization of specific subunits. IL-12p40 can be secreted as a monomer or as a disulfide-linked heterodimer, linked to IL-12p35 or IL-23p19 (78). When IL-12p40 is bound to IL-12p35, it forms IL-12. While the IL-12p40 is homologous to the α -chain of the soluble IL-6 receptor, the IL-12p35 subunit is structurally related to the type I cytokines. The complex of IL-23p19 and IL-12p40 forms IL-23. IL-23p19 was identified on the basis of its homology with IL-6 and the IL-12p35 chain (78). The discovery of IL-23 led to a re-evaluation of the function of IL-12 in experiments based on the neutralization or deletion of IL-12p40. In mice, IL-12p40 can form a homodimer named IL-12p80, in humans however, IL-12p80 is not reported (79,80).

Similar to IL-12 and IL-23, IL-27 is a heterodimeric cytokine consisting of Epstein-Barr virus-induced molecule 3 (EBI3), an IL-12p40 homologue, noncovalently bound to p28, an IL-12p35 homologue (81). EBI3 has also been reported to bind to IL-12p35, forming IL-35 (82,83). The major cellular source of IL-12, IL-23 and IL-27 are APCs. By contrast, murine IL-35 is produced by regulatory T cells (83). Human regulatory T cells do not constitutively express IL-35 (84). However, human rhinovirus stimulated DCs induce IL-35 production in FOXP3 negative regulatory T cells (85). The IL-12 cytokine family members are thought to be crucial in the development and maintenance of a Th1 response and in mice deficiency in either IL-23 or IL-12 significantly compromises the host's ability to eliminate pathogens (18,86).

Regulation of IL-12 and IL-23 production

IL-12 and IL-23 share the common IL-12p40 subunit. For the production of IL-12, both IL-12p40 and IL-12p35 must be co-expressed, whereas for the expression of IL-23 IL-12p40 and IL-23p19 must be co-expressed. The expression of the p40, p35 and IL-23p19 genes is independently regulated. IL-12p40 is produced in large excess over the IL-12 and IL-23 hetero-dimers; a role of this excess IL-12p40 remains unknown. Prior to secretion, the various subunits are assembled to form the different hetero-dimeric cytokines. After dimerisation, N-linked glycosylation of IL-12p35 is a required step in the secretion of IL-12 (87).

A variety of stimuli can induce IL-12 and IL-23 production. For example, monocytes, macrophages and dendritic cells (DCs) produce IL-23 following exposure to PAMPs that act through TLRs (88). In contrast to IL-23, an extra stimulus such as IFN- γ is needed for the induction of IL-12 (40,89). In line, in whole blood assays with bloodcells obtained from patients with complete IFN- γ R deficiency, no IL-12 production can be detected in response to *M. bovis* BCG infection *in vitro* (90). IFN- γ primes for IL-12 production and enhances IL-23 production in human monocytes and macrophages (71,89,91). In contrast, IFN- γ is reported to inhibit IL-23 production in human DCs (89), indicating a dual role for IFN- γ in the regulation of IL-23 production, depending on the cell type.

The balance between IL-12 and IL-23 production is dependent on the specific TLR being stimulated. In human DCs TLR2 ligands increase IL-23, but at the same time inhibit IL-12 production (91). In addition, activation of human myeloid DCs by TLR4 ligands, like lipopolysaccharide (LPS), leads to the expression of p19, p35 and p40 the building blocks of both IL-23 and IL-12. TLR2 activation by peptidoglycan (PGN), however, induces high levels of IL-23, but not of p35 expression or IL-12 synthesis (92,93). Not all TLR2 agonists are equally good IL-23 inducers: for instance, PGN is a more potent IL-23 inducer than PAM₃CSK₄ (94), which can be explained by the fact that PGN not only stimulates IL-23 production via TLR2 but also via NOD2 stimulation by the intracellular PGN metabolite muramyl dipeptide (MDP) (93). These data indicate that synergy between TLR2 and NOD2 agonists favour the induction of IL-23. In addition, several combinations of TLR agonists induce both IL-12 and IL-23. TLR3 and TLR4 ligands synergize with TLR8 ligands in the induction of both IL-12 and IL-23 (88,95,96). Other PRRs influence the balance between IL-12 and IL-23 production. The dectin-1 agonist β -glucan curdlan induces IL-23 synthesis by DCs without inducing IL-12 production (97), indicating that signalling through C-type lectins also favours IL-23 production.

Commensal gram-negative bacteria as well as gram-positive bacteria are able to induce p19 expression in DCs. Intact gram-positive bacteria, however, preferentially stimulate production of IL-12 over IL-23 (98). Gram-negative bacteria induce more IL-23 compared to gram-positive bacteria, indicating that a component of gram-negative bacteria synergizes with PGN, a cell-wall component of both gram-negative and gram-positive bacteria, in the induction of IL-23.

A variety of cytokines is known to modulate IL-12 and IL-23 production. Harris et al. showed that IL-1 β induces IL-23 production in human monocytes in response to gliadin (99). GM-

CSF primes monocytes for enhanced IL-23 production in response to a variety of TLR agonists (88). Moreover, macrophages generated in the presence of GM-CSF (type 1 macrophages) produce IL-23 in response to various stimulations, whereas macrophages generated under pressure of M-CSF (type 2 macrophages) do not (89). Furthermore, GM-CSF is known to amplify the IL-23 inducing activity of prostaglandin E₂ (PGE₂) in a synergistic manner (100). In combination with GM-CSF, IL-4 is used for the in vitro generation of DCs and known to enhance IL-12 production (101,102). IL-4 downregulates IL-23p19 mRNA, IL-12p40 mRNA and IL-23 protein expression in virally infected macrophages (103) and inhibits TLR agonist induced IL-23 production in DCs (104). These results suggest that IL-4 can shift the balance between IL-23 and IL-12 production towards IL-12. In contrast to IL-4, PGE₂ *inhibits* IL-12 production, but stimulates IL-23 expression in LPS-treated DC in mice (100,105,106), thereby inducing a shift in the IL-23/IL-12 balance in favour of IL-23. The effect of PGE₂ is dependent on the induction of cAMP (107). The nucleotide adenosine triphosphate (ATP) also influences the balance between IL-12 and IL-23 in DCs, favouring a shift towards IL-23 (107). IL-10 is a potent negative regulator of many pro-inflammatory cytokines, including IL-12 and IL-23. IL-10^{-/-} mice produce more IL-23 in response to LPS, compared to wild-type mice (108). Both IL-12 and IL-23 can induce IL-10 production (109-111), which may provide a negative feedback signal. Finally, IL-12 and IL-23 seem to cross-regulate each other. IL-23 deficient mice produce higher levels of IL-12 in a colitis model and IL-23 suppresses IL-12 production in murine DCs upon TLR stimulation (112), suggesting an inhibiting role of IL-23 on IL-12 synthesis.

Activated T cells regulate IL-12 and IL-23 production by cell-cell contact, via a mechanism in which CD40L expressed on activated T cells after activation binds CD40 on APCs. Apart from IFN- γ , CD40-CD40L interactions seem to be crucial for the induction of IL-12. CD40 or CD40L deficient mice fail to produce IL-12 in response to *Leishmania* infection (113). In monocyte-derived DCs, both IL-12 and IL-23 production are upregulated after CD40 ligation (78,98,104,114-117). Furthermore, ligation of CD40L by CD40 enhances the IFN- γ inducing capacities of IL-12 on T cells (118).

Summarizing, regulation of IL-12 and IL-23 production during infection is dependent on a complex network of mediators; the TLRs stimulated by the infective agent, the cytokines induced and contact dependent T-cell regulation. Importantly, IL-12 production is dependent on IFN- γ and CD40-CD40L interactions, whereas these stimulations are not needed for the induction of IL-23. Moreover, IL-23 production is preceding IL-12 production during infections (89,119,120).

Biological functions of IL-12 and IL-23: induction of IFN- γ and the Th1 (and Th17) response

Generally, IL-12 is thought to be the primary trigger that initiates Th1 differentiation and IFN- γ production (Fig. 2). As mentioned earlier, paradoxically, for the induction of IL-12 production, the Th1 cytokine IFN- γ is needed (40). Moreover, Abdi et al. showed that Th1 polarization is critically dependent on IFN- γ . This means that for the initiation of Th1 differentiation, IFN- γ is needed to

complement this “third signal”. Naïve T cells and APCs are unable to produce this cytokine, indicating that other cells producing IFN- γ are needed for the initiation of Th1 differentiation. As mentioned, the primary sources of IFN- γ include effector cells of the innate immune system like NK cells, NKT cells, NK-like T cells and $\gamma\delta$ T cells, therefore these cells may provide this third signal (41,121,122). NK and NKT cells constitutively express IFN- γ mRNA, allowing rapid induction and secretion of IFN- γ (123). Moreover, NK derived IFN- γ is known to push differentiation of T helper cells into the Th1 subclass (41,42). However, the stimuli leading to the initial IFN- γ production in these cells are not well characterized.

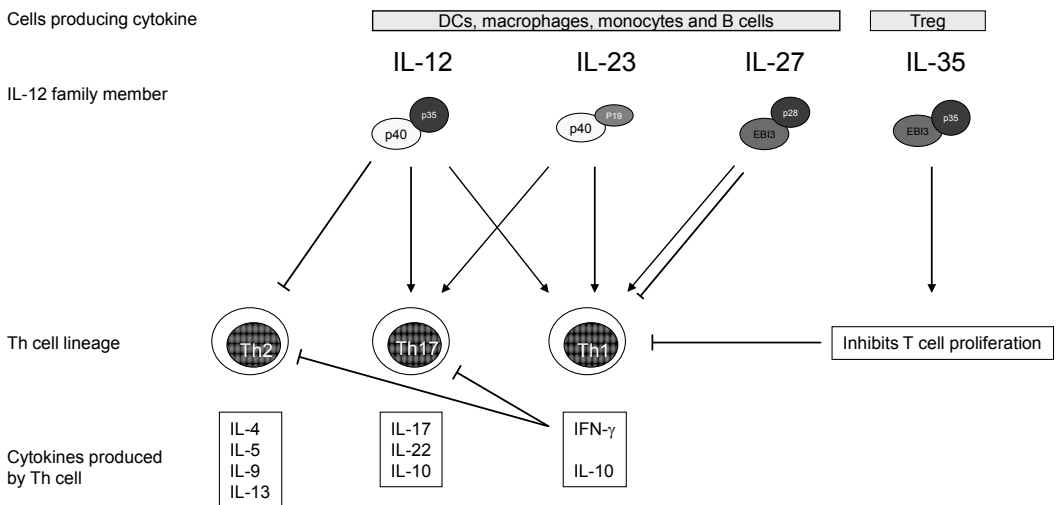


Figure 2: IL-12 family members and T helper cell subsets. All IL-12 family members are composed of two subunits. IL-12, IL-23 and IL-27 are produced by dendritic cells, macrophages, monocytes and B cells. IL-12 promotes Th1 cell differentiation. IL-23 can induce both IFN- γ and IL-17 and is involved in both Th1 and Th17 cell differentiation. IL-27 promotes Th1-cell differentiation, an effect that is most prominent in the absence of IL-12, whereas at later stages of the immune response IL27 inhibits Th1 cells. IFN- γ inhibits both Th2 and Th17 cells. In contrast to the other IL-12 family members IL-35 is produced by regulatory T cells and inhibits proliferation of all T cell lines.

IL-23 is produced early in infections, without the need of IFN- γ . Moreover, this cytokine is capable of inducing IFN- γ production (43,78,88,103,124). IL-23 is among the first cytokines secreted by APCs in response to stimulation with PAMPs such as LPS (89). Thus, early APC-derived IL-23 may be an initial trigger of IFN- γ production. Experimental data in favour of this hypothesis comes from the observation that mice deficient in the p40 subunit of IL-12/23 are far more impaired in their ability to generate Th1 responses to pathogens as *Salmonellae* and *Mycobacteria* than are mice lacking the p35 subunit of IL-12 (125-128), suggesting a role for IL-23 in the induction of IFN- γ production. Also the fact that IL-23 induces the expression of substance P (129), a peptide reported to enhance IFN- γ

production by Th1 cells, in macrophages points to a role of IL-23 in the initiation of IFN- γ production (130).

Other cytokines, including the IL-12 family member IL-27, are reported to regulate Th1 differentiation early in infection, independently of IL-12 (131-133) (Fig. 2). Although several lines of evidence indicate that IL-23 and IL-27 have a role in the induction of IFN- γ , it should be noted that none of these cytokines is indispensable for the induction of a Th1 response (134-136). This suggests that several factors, including IL-23, IL-27 can induce IFN- γ production, without the need of all these factors being produced. Apart from IL-23 and IL-27, IL-1 β and IL-18, both members of the IL-1 family of cytokines, are likely to be involved in the induction of innate IFN- γ . Both IL-1 β and IL-18 are released soon after the first contact between phagocytes and pathogens and both these cytokines promote IFN- γ production by NK cells and T cells (44,137). Moreover, IL-18 is essential in the innate immune activation of CD4 T cells in *Salmonella*-infected mice (137).

Concluding, IL-12 is likely to be involved in the maintenance of an ongoing Th1 response, while other cytokines like IL-23 are involved in the initiation of a Th1 immune response.

IL-12 and IL-23 receptor

Structure and expression

IL-23 and IL-12 signal through a common IL-12R β 1 chain complemented by the IL-23R and the IL-12R β 2, respectively (Fig. 3). While IL-12R β 1 is constitutively expressed in activated CD4⁺ T cells, NK cells and NK-like T cells (17,138), IL-23R and IL-12R β 2 expression are critical for the ability to respond to IL-23 and IL-12, respectively. To generate a high affinity binding site for IL-12, both the IL-12R β 1 and IL-12R β 2 chains need to be co-expressed (139). The IL-12R β 2 expression is highly regulated on T cells. Resting T cells do not express IL-12R β 1 or IL-12R β 2 receptors, but these subunits are induced upon T-cell activation, and receptor expression correlates with IL-12 responsiveness (140). Furthermore, the IL-12R β 2 is expressed on Th1 cells, but not on Th2 cells (141). In Th1 cells, the expression of the IL-12R β 2 chain is upregulated by IL-12 itself, IFN- γ , IL-18 and IL-27 (124,142).

The IL-23R protein is very similar to IL-12R β 2, in particular with respect to the two cytokine binding domains and the immunoglobulin domain. IL-23R expression patterns are not well defined, because a specific antibody for FACS staining is not available (109). Expression of the *IL23R* mRNA has been reported in a broad range of cells, including memory T cells, NK-like T cells, Th1, Th2 as well in Th17 clones, $\gamma\delta$ T cells, NKT cells, NK cells, DCs and in activated macrophages (43,78,143-148). Apart from lymphocytes, also microglia, a type of glial cell that acts as the first and main form of active immune defense in the central nervous system, are reported to express *IL23R* mRNA and to respond to IL-23 (149). The Th1 associated transcription factor T-bet is involved in the induction of both the *IL-23R* mRNA and the IL-12R β 2 (150).

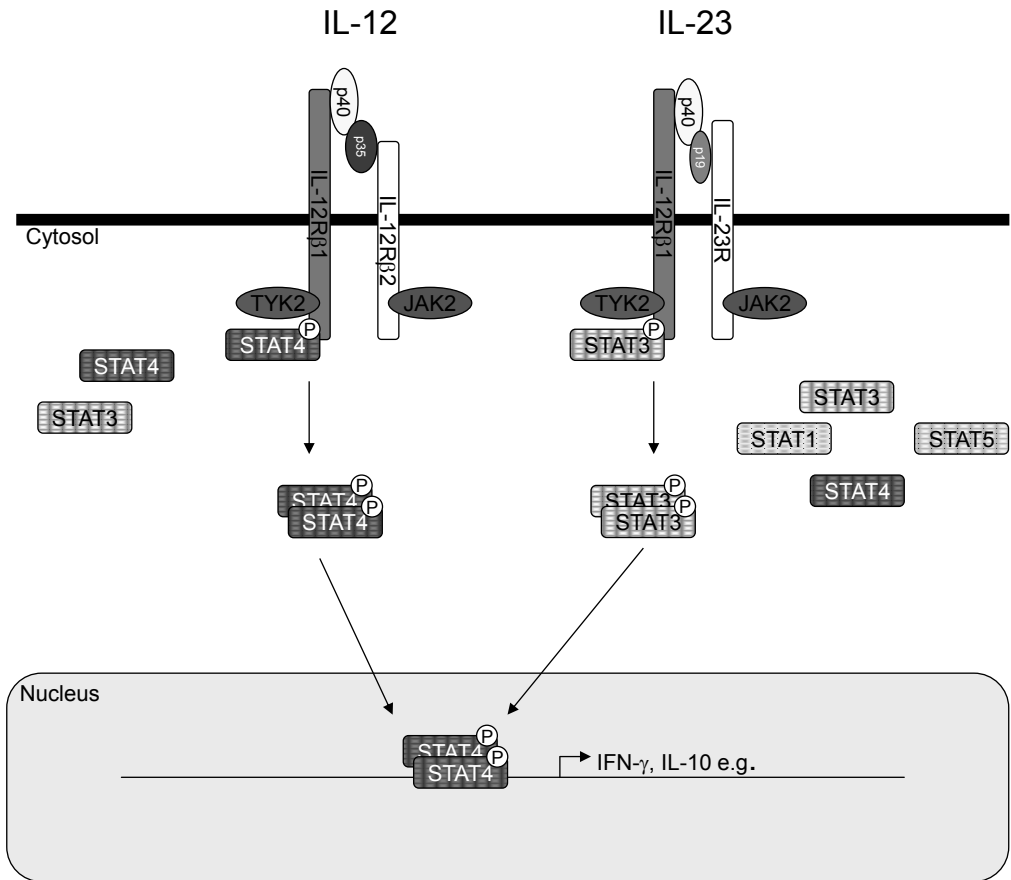


Figure 3. IL-12 and IL-23 signal transduction. IL-12 and IL-23 activate the JAK/STAT pathway. IL-12 is composed of IL-12p40 and p35 subunits that bind to IL-12Rβ1 and β2, respectively. Upon binding of IL-12 to its receptor, TYK2 and JAK2 associate with the IL-12Rβ1 and β2, respectively, followed by JAK transphosphorylation and subsequent phosphorylation of the receptor chains by activated JAKs. Subsequently, the IL-12Rβ2 is phosphorylated and serves as a docking site for STAT molecules. STAT4 binds to the receptor chain and is phosphorylated. Next STAT4 homodimers are formed and translocate to the nucleus where they bind to STAT-binding elements, like in the IFN-γ promoter, and regulate transcription. IL-23 is a heterodimeric cytokine composed of p40 and p19 that bind to the IL-12Rβ1 and the IL-23 receptor (IL-23R), respectively. Like IL-12, IL-23 stimulation induces JAK2 and TYK2 phosphorylation. Subsequently, mainly STAT3 is phosphorylated.

Signal transduction

The IL-23R associates with JAK2, whereas the IL-12Rβ1 associates with TYK2 (Fig. 3) (144). Upon binding of IL-23 to the IL-23 receptor complex, JAK2 and TYK2 are phosphorylated. Subsequently, STAT1, STAT3, STAT4 and STAT5 can dock to the receptor and are reported to be phosphorylated, however, the most prominent STAT induced by IL-23 is STAT3 (43,109,144,151). The tyrosine residues of the IL-23R responsible for STAT recruitment have not yet been established. The human

IL-23R contains seven tyrosine residues, three of which represent putative Src Homology 2(SH2)-binding sites which may be involved in binding the SH2-sites of STAT molecules (144,152).

Similar to the IL-23R, the IL-12R β 2 associates with JAK2, which is phosphorylated upon binding of IL-12 to the IL-12 receptor complex. IL-12R β 1 bound TYK2 is also phosphorylated after binding of IL-12 to the receptor complex. During IL-12 signal transduction, STAT4 docks to the tyrosine 800 of the IL-12R β 2 chain and this tyrosine is critical for IL-12 signal transduction (153). In contrast to IL-23, IL-12 mainly induces STAT4 phosphorylation.

After phosphorylation of STAT molecules in response to either IL-12 or IL-23, the phosphorylated STAT molecules dimerize to form homo- and heterodimers and subsequently translocate to the nucleus to regulate their target genes. STAT3 is critical in IL-23-dependent induction of IL-17 (151,154-156). Experiments elucidating the role of STAT4 in the development of Th17 cells are contradictory (154,157,158). IL-23 and IL-12 are both inducers of IFN- γ and IL-10 (78,109). STAT4 is essential for IL-12 induced IFN- γ (159). STAT4 is likely to be important in IL-23 induced IFN- γ , however, this is not yet elucidated.

Mendelian Susceptibility to Mycobacterial Disease.

Patients have been described with unusually severe infections caused by otherwise poorly pathogenic *Mycobacteria* and *Salmonellae*, a condition also known as Mendelian Susceptibility to Mycobacterial Disease (MSMD [MIM 209950 [OMIM]]) (18). MSMD is a heterogeneous disorder that can be caused by mutations in genes involved in the Th1 - IL-12/-23/IFN- γ - cytokine signalling cascade. In these patients, genetic defects have been identified in the genes encoding IL-12p40, IL-12R β 1, TYK2, IFN- γ R1, IFN- γ R2, and STAT1 proteins (32).

Deficiencies in the IL-12 and IL-23 pathways

IL-12p40

In total 20 cases of IL-12p40 deficiency have been reported and five different mutations in the *IL12B* gene have been found (160). All known *IL12B* mutations are recessive and result in complete IL-12p40 deficiency with a lack of detectable IL-12p40 secretion by cells of the patients (161,162). These patients lack active IL-12 and IL-23. As a result, these patients produce severely reduced amounts of IFN- γ *in vitro* (162).

In three children (one Pakistani and two Indian), a large homozygous deletion causing a frameshift in the *IL12B* gene has been identified, resulting in a protein of 184 amino acids including only 139 out of the 328 original amino acids in the N-terminal region and 45 novel amino acids in the C-terminal region (161-163). This protein cannot be detected and the stability is unknown. Interestingly, Pulickal et al report one female sibling homozygous for this mutation who was not clinically affected, suggesting a multifactorial basis for the observed phenotype in the clinically

affected sibling. Four kindreds from Saudi Arabia were found to have a small insertion (316insA) causing a frameshift, resulting in a short protein lacking the amino acids essential for dimerization with IL-12p35 (161). Small frameshift deletions (528del2 and 297del8) resulting in a premature stop-codon were detected in four patients (age between 3 months-28 years) who presented with *Bacillus Calmette-Guérin* (BCG)-itis (164,165). Interestingly, all patients with IL-12p40 deficiencies who were BCG vaccinated developed BCG-itis (161,162,164,165). In about half of the cases, salmonellosis was reported (161,162).

IL-12R β 1

Since IL-12R β 1 is an essential component of IL-12R as well as IL-23R, deficiencies of this receptor chain will affect both IL-12R and IL-23R dependent signalling. Over eighty individuals have now been reported with complete IL-12R β 1 deficiency (165-177), resulting in deficient IFN- γ production. IL-12R β 1 deficiency is the most frequent known genetic cause of MSMD. Mycobacterial infection and salmonellosis are the infectious diseases most frequent found in these patients.

Most reported IL-12R β 1 mutant alleles cause recessive complete IL-12R β 1 deficiency. In most cases IL-12R β 1 expression is abrogated due to a diverse array of mutations, including nonsense (167), missense (169-171,173,178-180), and splice mutations (165,169,175,181), microinsertions (182), microdeletions (165,169), microduplications (182) and large deletions (167,174,183,184). Fieschi et al. reported a patient with a large in-frame deletion in the *IL12RB1*, resulting in the surface expression of nonfunctional IL-12R β 1 (183). This same deletion was found in an unrelated 6 year old boy (184). In all IL-12R β 1-deficient patients both IL-12 and IL-23 responses are abrogated and IL-12 and IL-23-dependent production of IFN- γ is severely impaired (90,183,185). One patient with a partial IL-12R β 1 deficiency has been reported (169). In this patient, IL-12 response was diminished, but not abrogated.

Several siblings of IL-12R β 1 deficient patients have been identified that were homozygous for the same *IL12RB1* mutation as the index patient, however, they remained asymptomatic with regard to unusual infections with intracellular bacteria (172,173). The clinical penetrance of IL-12R β 1 deficiency is estimated between 66 (10-15) and 91% (49 of 54) (32,186), suggesting more factors play a role in the increased susceptibility to mycobacterial and salmonella infections in these patients. Patients with a deficiency of the IL-12p40 subunit or the IL-12R β 1 have defects in both the IL-12 and IL-23 cascade, however, the relative roles of IL-12 and IL-23 are not known.

Infections with BCG in patients with IL-12p40 deficiency or IL-12R β 1 deficiency apparently protect against subsequent illness due to nontuberculous environmental *Mycobacteria*, but not against disease due to *Salmonellae*. Unlike salmonellosis, recurrent mycobacterial infections are rare in these patients (173). Moreover, patients with a defect in the IL-12R β 1 are reported who controlled BCG after receiving vaccinations, indicating that in some patients IL-12 and IL-23 are redundant for mounting an effective immune response against BCG (187).

TYK2

TYK2 is involved in multiple signalling pathways, including IL-23 and IL-12 (173).

Minegishi et al. identified a homozygous TYK2 mutation in a patient who had been clinically diagnosed with hyper-IgE syndrome (188). This patient suffered a BCG lymphadenitis and a non-typhi *Salmonella* infection. Furthermore, this patient showed unusual susceptibility to various microorganisms including virus and fungi (188). Apart from IL-23 and IL-12, the IL-6, IL-10 and type I interferon pathways were impaired in cells from this patient, demonstrating that TYK2 plays obligatory roles in multiple cytokine signals involved in innate and adaptive immunity. These data suggest that TYK2 deficiency is characterised by a broad spectrum of clinical features, including both autosomal recessive hyper-IgE syndrome (AR-HIES) and MSMD.

Deficiencies in the IFN- γ signalling pathway

IFN- γ R deficiencies selectively predispose to severe infections due to poorly pathogenic mycobacteria, but by contrast with IL-12/IL-23 system defects, only rarely leads to non-typhoid *Salmonellae* infection. IFN- γ R deficiency is a heterogeneous disorder with different clinical, genetic, immunological, and histopathological types. In addition, various STAT1 deficiencies have been described, all impairing IFN- γ signal transduction.

IFN- γ R1

Patients with partial and complete IFN- γ R1 deficiencies have been described. All patients with autosomal recessive complete IFN- γ R1 deficiency present with symptoms of BCG-itis or environmental mycobacteria infection within the first 5 years of life (189). Complete IFN- γ R1 deficiency is characterised by a complete lack of functional IFN- γ R1. This lack of functional IFN- γ R1 can be due to mutations that preclude expression of IFN- γ R1 on the cell surface (190-194). Mutations leading to the abrogation of cell surface expression identified to date, include nonsense and splice mutations and frameshift deletions and insertions, all resulting in a premature stop codon upstream from the segment encoding the transmembrane domain. In several patients, missense mutations or a small deletion in the region encoding the ligand binding domain of the IFN- γ R1 have been identified. The resulting mutant receptors (C77Y, V61Q, 295del12 and 652del3) show no binding of IFN- γ (195).

In contrast to complete deficiency, partial IFN- γ R1 deficiency is associated with milder course of infections and patients with partial IFN- γ R1 deficiency may remain asymptomatic for much longer periods of time, possibly even until late adulthood (189). Partial IFN- γ R1 deficiencies are mainly due to truncations in the cytoplasmic domain of the receptor. In exon 6, a small deletion hotspot is found around nucleotide 818, favouring mispairing events during replication (196-198). A frameshift due to the deletion of four nucleotides (818del4) leads to a premature stop codon. The

truncated receptor lacks the signalling domains as well as the receptor recycling domain, resulting in accumulation of non-functional IFN- γ R1 proteins that are thought to exert a dominant-negative effect over normal IFN- γ R1 molecules encoded by the wild-type allele. In a patient suffering recurrent *Mycobacterium avium* osteomyelitis, a mutation at nucleotide 832 (designated E278X) was identified (199). This mutation causes a premature stop codon and results in truncated IFN- γ R1 lacking the recycling domain, leading to accumulation of the non-functional receptor on the cell membrane. This mutant receptor, like the 818del4 mutant receptor, exerts a dominant-negative effect. In a Japanese girl suffering BCG lymphadenitis after BCG vaccination, another dominant negative mutation (774del4) was detected (200). Like the 818del4, this mutation produces a truncated form of the IFN- γ R1 which lacks the recycling domain, resulting in overexpression of the mutant receptor and a dominant-negative effect on IFN- γ signalling. Storgaard et al. report a patient with a nucleotide deletion at position 794, resulting in an overexpressed negative dominant receptor as well (201). Two children of healthy heterozygous parents have been reported with a homozygous variant (I87T) IFN- γ R1, affecting the extracellular part of the receptor (202). When homozygous, this mutation leads to a severely reduced, but not completely abrogated, response to IFN- γ . In addition, in one patient a missense mutation (V63G) in the region encoding the ligand binding domain of the IFN- γ R1 has been identified, severely impairing cellular response to IFN- γ (203).

IFN- γ R2

IFN- γ R2 deficiency is a rare form of MSMD. Mutations can lead to partial and complete IFN- γ R2 deficiencies. Four mutations leading to complete IFN- γ R2 deficiencies have been reported. In a child with disseminated *Mycobacterium fortuitum* and *M. avium* complex infections, a two-base pair homozygous recessive frameshift deletion has been found in the *IFNGR2* coding region (204). This deletion results in a premature stop in the extracellular region and the abrogation of IFN- γ responsiveness. The second form of complete IFN- γ R1 deficiency was detected in three children from two families. In these children a T168N missense mutation was detected in the IFN- γ R2. This missense mutation resulted in a receptor with an extra consensus site for N-glycosylation, resulting in a extra polysaccharide chain being added to the IFN- γ R2. The addition of this polysaccharide chain resulted in the expression of a non-functional IFN- γ R2 on the cell surface (205). In another patient, an in-frame microinsertion in the *IFNGR2* was discovered, leading to a receptor of abnormally high molecular weight and most of the protein remained intracellular (206). The IFN- γ R2 expressed on the cell surface was non-functional. In two siblings, a deletion in the IFN- γ R2 transmembrane domain (791delG) causing a frameshift and premature stop codon 8 triplets downstream was found (207). This deletion in the transmembrane domain disables golgi processing and subsequent cell surface expression. The two siblings were homozygous for this mutation and

their cells were not responsive to IFN- γ . Their heterozygous parents were healthy and showed a diminished IFN- γ responsiveness.

One partial IFN- γ R2 deficiency has been reported so far. In a person with a history of recurrent infections with BCG and *Mycobacterium abscessus*, a homozygous nucleotide substitution in *IFNGR2* was detected (208). This nucleotide substitution resulted in an amino acid substitution (A114C) in the extracellular region of the encoded receptor. The resulting receptor was expressed on the cell surface. Cellular responses to IFN- γ however, were impaired but not abolished, indicating a partial deficiency.

STAT1

Signal transducer and activator of transcription 1 (STAT1) is critical for cellular responses to type I (IFN- α/β) and type II (IFN- γ) IFNs (67). In 2001, the first human germline mutation of STAT1 was found and associated with susceptibility to mycobacterial disease (209). In cells from a heterozygous patient, a missense mutation (L706S) resulted in impaired STAT1 Tyr-701 phosphorylation and affects the IFN- γ activation factor (GAF) mediated IFN- γ response, but not the interferon-stimulated gene factor 3 (ISGF3) mediated IFN- γ response, in a dominant negative manner (209). Two siblings homozygous for the STAT1 missense mutation P696S, displayed severely impaired but not abolished responses to IFN- γ , IFN- α/β , IFN- λ 1 and IL-27 (210). The mutation impaired splicing of the mRNA. The misspliced forms lacking exon 23 were not translated into a mature protein and residual full-length mRNA resulted in low but detectable normal functional STAT1 protein. The affected patients suffered severe but curable intracellular bacterial and viral infection. Two recessive complete mutations of the STAT1-binding domain have been described as well (211). The resulting STAT molecules are normally phosphorylated but the nuclear-translocated STAT1-dimers do not bind correctly to IFN- γ activation site (GAS) elements. The patients having these mutations were highly susceptible to mycobacterial infections. Both patients died of overwhelming viral infections (212), indicating both the type I and type II interferon pathways were affected by these mutations. In a patient suffering disseminated BCG infection, abrogation of STAT1 expression was reported (213). Complete STAT1 deficiency resulted from a frameshift due to a homozygous insertion (1928insA), causing a premature stop codon and thereby abrogation of STAT1 expression. The lack of STAT1 resulted in unresponsiveness of cells to IFN- α and IFN- γ . This patient died after bone marrow transplantation, from a fulminant EBV infection.

IFN- γ

No patients have been described with genetic IFN- γ deficiency. Despite the fact that IFN- γ deficient mice develop normally (29), in humans IFN- γ deficiency may be incompatible with life. However, acquired IFN- γ deficiencies secondary to neutralizing auto-antibodies have been described in over ten patients (214-217). These patients were susceptible primarily to mycobacterial infections.

Treatment options

For all patients, early diagnosis of infection and appropriate antibiotic therapy, based on the sensitivity of the (myco-) bacterial species identified, is crucial. Patients with IL-12p40, IL-12R β 1, and partial IFN- γ R defects usually respond well to antibiotic treatment.

Patients with mutations in the IL-12R β 1 or IL-12p40 lack the effects of IL-12 and IL-23 and therefore produce little IFN- γ in response to infections. Apart from the appropriate antibiotics, these patients can benefit from treatment with recombinant IFN- γ (163,184,218,219). Studies of IFN- γ therapy in patients with partial IFN- γ R1 or partial IFN- γ R2 deficiencies have not been performed. However, patients suffering partial IFN- γ R deficiency can benefit from treatment with high dose recombinant IFN- γ (189). By contrast, patients with complete IFN- γ R1, complete IFN- γ R2 or STAT1 deficiencies are not able to respond to IFN- γ and thus will not benefit from treatment with recombinant IFN- γ . Moreover, these patients are less responsive to antimicrobial treatment (220). Hematopoietic stem cell transplantation (HSCT) is theoretically a curative treatment for these patients, however, a poor outcome is reported in most of the patients who received HSCT (221). To prevent infections in these patients, prophylactic life-long anti-microbial therapy is indicated. In contrast to patients with complete IFN- γ R or complete STAT1 deficiency, mycobacterial infection in patients with other deficiencies usually can be controlled and the need for prophylactic treatment is controversial. Patients doing well without prophylaxis have been reported (208,222). In addition, in patients with defects in the IL-12p40 subunit or in the IL-12R β 1 chain the use of prophylactic treatment after the clearance of the first infection, to prevent the recurrence of mycobacterial infections is unclear, while in these patients new episodes of mycobacterial infections generally do not develop (172). However, recurrent infections with *Salmonellae* are common.

In two patients, with complete IFN- γ R1 and complete IFN- γ R2 deficiency respectively, suffering from disseminated infection with *Mycobacterium avium* complex, treatment with IFN- α as additional therapy has been described (223,224), however, the effect of this treatment remains unclear. Hematopoietic stem cell transplantation is the only available curative treatment of complete IFN- γ R and STAT1 deficiencies, however, the overall success rate of stem cell transplantation is low (221).

Outline of Thesis

Part 1: The role of IL-23 in inducing IFN- γ production and in the initiation of a Th1 response.

IL-12 is thought to be the classical IFN- γ inducing cytokine. However, for APCs to produce IL-12 in response to PAMPs like LPS, IFN- γ itself is needed as an additional stimulus. This indicates that another factor is needed to drive early IFN- γ production, allowing for IL-12 production and subsequent amplification of IFN- γ production. IL-23 and IL-18 are among the first cytokines secreted by APC in response LPS, without the need of IFN- γ . We hypothesized that early APC-derived IL-23 may be an initial trigger of IFN- γ production in NK and NK-like T cells and in **Chapter 1**, we evaluated the IFN- γ inducing capacities of IL-23 and IL-18 on CD56⁺ NK and NK-like T cells.

IL-23 is produced by APC like monocytes and macrophages. In **Chapter 2**, the effect of a variety of TLR agonists, as well as infection with live *Salmonella*, on the production of IL-23, IL-18 and IL-1 β by monocytes and macrophages were tested. Next, the role of these cytokines in the induction of IFN- γ was elucidated. Finally, we evaluated the role of IL-23 induced IFN- γ in the priming for subsequent IL-12 production by monocytes.

Part 2: Genetic variations in the type-1 cytokine pathway.

The effects of IL-23 on its target cells are mediated via a receptor complex consisting of an IL-12R β 1 and a specific IL-23R chain. The R381Q and P310L variants of the IL-23R have been reported to be associated with autoimmune diseases, suggesting they have an effect on IL-23R function. In **chapter 3**, these variants and a newly discovered IL-23R variant, namely Y173H, were functional characterized.

In the IFN- γ R1, one of the IFN- γ receptor chains, several amino acid substitutions have been reported that abrogate IFN- γ signalling. These substitutions lead to enhanced susceptibility to infection with weakly pathogenic mycobacteria and salmonellae, as described above. More common amino acid variations in the IFN- γ R1 may also have an effect on IFN- γ R function, albeit more subtle. In **chapter 4** we describe two new variants of the IFN- γ R1, namely S149L and 352M. To compare the effect of the variations at a molecular level, we cloned the IFN- γ R1 and the newly discovered *IFNGR1* variants, as well as four known polymorphisms (V14M, V61I, H335P, L467P, all six reported missense mutations (V61Q, V63G, C85Y, C77Y, C77F, I87T) and the 818del4 mutant. For all these variants, we analyzed the signal transduction, the regulation of CD54, CD64, HLA-DR and HLA class I expression and the cytokine production in response to IFN- γ .

Part 3: Treatment options for a genetic deficiency in the type-1 cytokine pathway.

Patients with complete deficiency of the IFN- γ R1 are not responsive to IFN- γ and therefore adding IFN- γ to traditional treatment of infections with antibiotics is not an option in these patients. A severe clinical course is seen in IFN- γ R deficient patients and these patients often succumb to mycobacterial infections very early in life. In **chapter 5** we evaluated the ability of recombinant IFN- γ to compensate for the absence of IFN- γ effects in cells obtained from an IFN- γ R1 deficient patient.

In the final section the results of the previous chapters will be summarized and discussed.

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Chapter 1

IL-23 modulates CD56⁺/CD3⁻ Natural Killer Cell and CD56⁺/CD3⁺ Natural Killer-like T Cell function differentially from IL-12.

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Abstract

Natural killer (NK) and natural killer-like T (NK-like T) cells play an essential role in linking innate and adaptive immunity, through their ability to secrete interferon gamma (IFN- γ). The exact trigger initiating production of IFN- γ is uncertain. Antigen presenting cell (APC)-derived interleukin-12 (IL-12) is thought to be the classical IFN- γ inducing cytokine, but requires an additional stimulus such as IFN- γ itself. Interleukin (IL)-23 and IL-18 are among the first cytokines secreted by APC in response to binding of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS). Thus, early APC-derived IL-23 may be an initial trigger of IFN- γ production in NK and NK-like T cells. Herein, we characterized the effect of IL-23 on IFN- γ secretion by NK and NK-like T cells. Our findings show that IL-23 and IL-18 synergistically elicit IFN- γ production in NK-like T cells but not in NK cells. In contrast, IL-12 together with IL-18 induced secretion of IFN- γ in both populations. The observed synergy between IL-23 and IL-18 in NK-like T cells coincided with IL-23 mediated up-regulation of IL-18R α . Furthermore, IL-23 up-regulated CD56 expression in NK-like T cells and, together with IL-18, induced proliferation of NK and NK-like T cells. We postulate a role for APC-derived IL-23 in the activation of NK and NK-like T cell early in infection and in shaping Th1-differentiation, via induction of IFN- γ , which provides the additional stimulus needed for APC to subsequently produce IL-12.

Introduction

Innate immunity plays an essential role in the defense against pathogens. NK cells constitute an important arm of the innate immune system and several murine models have shown NK cell-derived cytokines to be critical in the early response against intracellular pathogens such as *Salmonella typhimurium* (1,2) and *Mycobacterium tuberculosis* (3). Early in infection, NK cells are thought to be the primary source of interferon gamma (IFN- γ) (4-6), shaping the acquired immune response through differentiation of T helper (Th) cells to the Th1 subclass (6,7). At a later stage of infection, Th1 cells become the predominant source of IFN- γ .

Host defense against intracellular bacterial pathogens such as *Salmonellae* and *Mycobacteria* is dependent on the type-1 cytokine pathway (8). Generally thought, this pathway is initiated by bacterial stimulation of pattern recognition receptors on APC, resulting in the production of interleukin-23 (IL-23), IL-12 and IL-18 (9). IL-12 and IL-18 subsequently induce IFN- γ production in NK cells and Th1 cells by binding to their respective receptors (10), while IL-23 is known to induce IFN- γ production in naïve T cells and in memory T cells (11). IFN- γ in turn binds to the IFN- γ R on the macrophages and dendritic cells to enhance their bactericidal activity and antigen presentation and increase production of IL-12 (12).

IL-23 and IL-12 are heterogenic cytokines composed of a shared IL-12p40 subunit bound to an IL-23p19 or IL-12p35 subunit, respectively. IL-23 and IL-12 signal through a common IL-12R β 1 chain complemented by the IL-23R and the IL-12R β 2 respectively. While IL-12R β 1 is constitutively expressed in naïve CD4⁺ T cells, CD56⁺ NK-like T cells and NK cells (13,14), IL-23R and IL-12R β 2 expression are critical for the ability to respond to IL-23 and IL-12, respectively.

The role of IL-23 in shaping the immune response is poorly defined. IL-23 is produced by APC in response to PAMPs like LPS. Moreover, IL-23 rather than IL-12 is the first type 1 cytokine released by activated pro-inflammatory macrophages (9). To induce production of IL-12 by monocytes and macrophages, IFN- γ signaling is required in addition to a PAMP (9). In dendritic cells both IFN- γ and IL-4 can enhance PAMP induced IL-12 production (15). Since IL-23 is expressed early in infections (16) and capable of inducing IFN- γ (17-19), we hypothesized that IL-23 may serve as a factor important in initiating early Th1 differentiation by inducing the extra signal needed, IFN- γ , for APC to produce IL-12 in response to pathogens or PAMPs. In order to achieve this, IL-23 needs to target cells of the innate immune system and induce IFN- γ production in these cells. NK and NK-like T cells may be candidate cells providing IFN- γ as they are part of the innate immune system and both known to express IL-12R β 1 mRNA (14,20). Moreover, NK cells are reported to express *IL23R* mRNA (20). To verify this hypothesis, we tested the ability of IL-23 to induce activation and IFN- γ secretion in primary human CD56⁺/CD3⁻ NK and CD56⁺/CD3⁺ NK-like T cells.

Methods

Cells and culture conditions

CD56⁺ cells were isolated from buffy coats from healthy donors (Sanquin, Leiden, The Netherlands) by Ficoll-Amidotrizoate density gradient centrifugation and subsequent selection using anti-CD56 MACS beads (Miltenyi Biotech, Utrecht, The Netherlands). For the proliferation assay, CD56⁺ bead isolated cells were labeled with PE-labeled anti-human CD3 (BD PharMingen, Amsterdam, The Netherlands) and CD3⁻ cells were sorted with a FACSVantage SE (BD Biosciences, Amsterdam, The Netherlands). Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Bio-Whittaker) supplemented with 20 mM GlutaMAX (Gibco/Invitrogen, Breda, The Netherlands), 10% FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin (Gibco/Invitrogen, Breda, The Netherlands).

FACS analysis

To assess STAT phosphorylation by FACS analysis, overnight-rested CD56⁺ bead isolated cells were stimulated with recombinant human 10 ng/ml IL-23 or 1 ng/ml IL-12 for times indicated. Cells were fixed using 4% formaldehyde and permeabilised with 90% methanol. Cells then were labeled directly with anti-phosphorylated STAT1 (pY701)-Alexa 647, anti-phosphorylated STAT3 (pY705)-PE or STAT3 (pY705)-Alexa 647, anti-phosphorylated STAT4 (pY693)-Alexa 647 or anti-phosphorylated STAT5 (pY694)-PE (BD PharMingen). CD56⁺ magnetic bead isolated cells were stained in combination with anti-human CD3 and anti-human CD56.

For intracellular staining for IFN- γ , CD56⁺ -isolated cells were seeded 10⁵ cells per well in 96-well plates (Greiner Bio-One) and stimulated with IL-23 (R&D Systems, Abingdon, United Kingdom), IL-12 (R&D Systems), IL-18 (MBL, Woburn, USA) or a combination of these cytokines for 48 hours. The last 6 hours of stimulation BD GolgiPlug (BD PharMingen) was added (final concentration 1:1000). Cells were fixed in 4% paraformaldehyde (Sigma, Zwijndrecht, The Netherlands) and permeabilised in 90% methanol. Cells were stained with Alexa 647-labeled anti-human IFN- γ in combination with PE-labeled anti-human CD56 and FITC-labeled anti-human CD3 (BD PharMingen). To assess IL-18R α expression, overnight rested CD56⁺ beads isolated cells were stimulated for 2 days with IL-23 10 ng/ml, IL-12 1 ng/ml, IL-18 100 ng/ml or a combination of these cytokines. Cells were directly labeled with PE-conjugated mouse-anti-human IL-18R α mAb FAB840P (R&D Systems) in combination with FITC-conjugated anti-human CD3 and Alexa 647 anti-human CD56.

Functional analysis

To determine cytokine production, overnight rested CD56⁺ beads isolated cells were seeded 10⁵ cells per well and stimulated for 48h with IL-23, IL-12, IL-18 or a combination of these cytokines in a

96-well plate in a final volume of 200 μ l. Concentrations are indicated in the figures. The concentration of IFN- γ , IL-10 and IL-17 in the supernatants was determined by cytokine-specific ELISAs (Biosource, Etten-Leur, The Netherlands). For proliferation assays in CD56⁺-isolated cells, cells were carboxyfluorescein (CFSE) (Celltrace, Invitrogen, Breda, The Netherlands) labelled. 10⁶ cells were labelled in 2 ml medium containing 1 μ M CFSE for 15 minutes. After labelling cells were washed twice and seeded 10⁵ cells per well in 96 well plate (Costar, Badhoevedorp, The Netherlands). Cells were stimulated with 10 ng/ml IL-23, 1 ng/ml IL-12, 100 ng/ml IL-18 or a combination of these cytokines. Four days after stimulation, cells were directly labelled with PE-labelled anti-human CD3 and Alexa 647-labelled anti-human CD56 and analyzed on a FACS Calibur (BD Bioscience).

Results

IL-23 synergizes with IL-18 in inducing IFN- γ production in primary human CD56⁺ cells.

IL-23 is one of the first cytokines produced by APC in response to PAMPs, while NK and NK-like T cells may be the first innate immune system cells to respond to IL-23. Therefore, we determined whether IL-23 was able to induce IFN- γ production in CD56⁺/CD3⁻ NK and CD56⁺/CD3⁺ NK-like T cells. As IL-18 has been reported to stimulate Th1 responses synergistically with IL-12 by enhancing NK activity and IFN- γ production (21), we also tested the effect of IL-18 in combination with IL-23 on IFN- γ production. Isolated human CD56⁺ cells were analyzed by FACS for CD56 and CD3 expression. More than 90% of the cells were CD56⁺ and of these, 30-70% were CD3⁺. To determine the kinetics of the IFN- γ production, CD56⁺ cells were stimulated for 4, 8, 24 and 48 hours with IL-12 or IL-23 with or without IL-18. IL-23 or IL-18 alone did not induce IFN- γ production at any time point (Fig. 1A and table 1). IL-12 alone induced IFN- γ production 48 hours after stimulation. Four hours after stimulation, small amounts of IFN- γ were detected in supernatants from cells stimulated with IL-12 plus IL-18 (Fig. 1A). IL-23 plus IL-18 induced IFN- γ with slower kinetics and IFN- γ was not detected until 8 hours of stimulation. Highest amounts of IFN- γ were detected after 48 hours of stimulation, in response to IL-12 plus IL-18 or IL-23 plus IL-18 (Fig. 1A).

To determine the response to various concentrations of IL-23 and IL-12 in combination with various concentrations of IL-18, CD56⁺ cells were stimulated for 48 hours. Again, IL-23 or IL-18 alone did not induce IFN- γ production in CD56⁺ cells (Fig. 1B). However, when IL-23 was combined with IL-18, we observed synergistic effects on IFN- γ secretion (Fig. 1A and 1B). IL-12 alone induced minimal IFN- γ production. As expected, a synergistic effect on IFN- γ production was observed when IL-12 and IL-18 were combined (Fig. 1A and 1B). Both IL-23 and IL-12 showed strongest synergy with the highest concentrations of IL-18 (Fig. 1B). Furthermore, as IL-15 is known to enhance IL-12 induced IFN- γ production, we tested IL-15 in combination with IL-23. IL-15 synergized with IL-23 in

inducing IFN- γ production by CD56⁺ cells; however, this synergy was not as strong as the synergy observed between IL-18 and IL-23 (data not shown). We also stimulated CD56⁺ cells with IL-18 in combination with IL-12 plus IL-23. IL-23 slightly inhibited the effect of IL-12 (data not shown). As IL-23 is reported to play a role in the induction of IL-17, we tested IL-17 production in these supernatants as well. However, no IL-17 was detected (data not shown). In antiCD2/antiCD28 activated CD56⁺ cells, IL-23 inhibited the production of the Th2 cytokines IL-4 and IL-13 (data not shown).

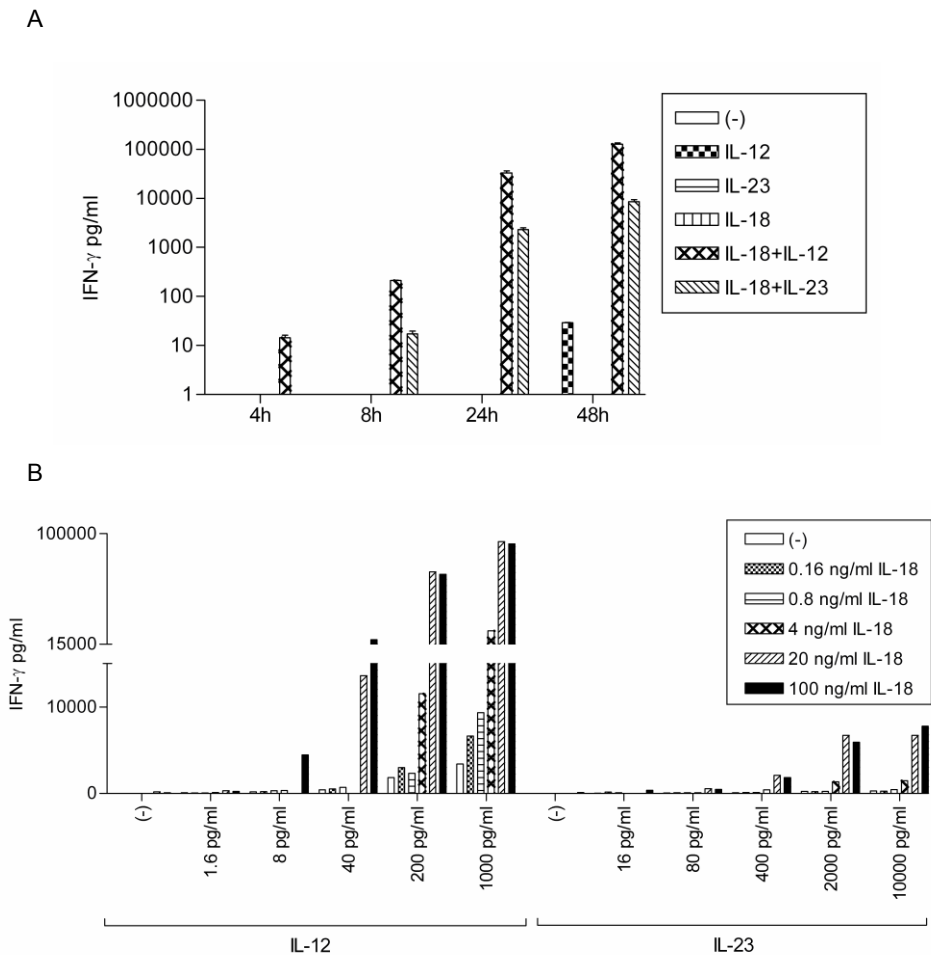


Figure 1. IL-23, in combination with IL-18, induces IFN- γ in primary human CD56⁺ cells. **A** Anti-CD56 MACS bead isolated cells were rested overnight and then left unstimulated or stimulated with indicated concentrations of IL-23 or IL-12 plus or minus IL-18. Supernatants were collected 4, 8, 24 and 48 hours after stimulation and IFN- γ concentration was measured by ELISA. Both IL-23 and IL-12 synergize with IL-18 in the induction of IFN- γ . **B** Overnight rested CD56⁺ cells were left unstimulated or stimulated with indicated concentrations of IL-23 or IL-12 in combination with various concentrations of IL-18 for 48 hours. IFN- γ concentrations were determined by ELISA. One representative of experiments with cells from 3 donors.

IL-23 induces STAT phosphorylation in CD56⁺/CD3⁺ NK-like T, but not in CD56⁺/CD3⁻ NK cells.

IL-23 has been reported to induce STAT1, STAT3, STAT4 and STAT5 tyrosine phosphorylation in the human T cell line Kit225 (20). Primary human CD56⁺ can be divided into CD56⁺/CD3⁻ NK cells and CD56⁺/CD3⁺ NK-like T cells. To test in which of these CD56⁺ populations IL-23 induces STAT phosphorylation, we stimulated CD56⁺ cells for 30-90 minutes with IL-23 and determined STAT phosphorylation by FACS analysis, using antibodies specifically directed against phosphorylated STAT1, STAT3, STAT4 or STAT5, in combination with anti-CD3 and anti-CD56. IL-23 induced both STAT3 and STAT4 phosphorylation in CD56⁺/CD3⁺ NK-like T cells, but not in CD56⁺/CD3⁻ NK cells (Fig. 2). We did not observe any STAT1 or STAT5 phosphorylation in response to IL-23 (Fig. 2). These results suggest a direct effect of IL-23 on CD56⁺/CD3⁺ NK-like T cells.

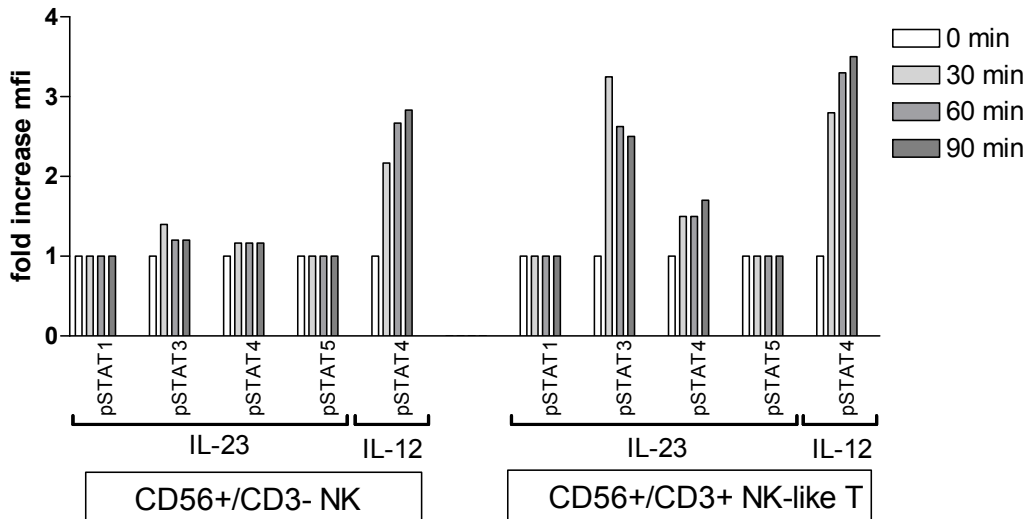


Figure 2. IL-23 induces STAT phosphorylation in CD56⁺/CD3⁺ NK-like T but not in CD56⁺/CD3⁻ NK cells. Anti-CD56 MACS bead isolated cells were rested overnight, and subsequently stimulated with 10 ng/ml IL-23 or 1 ng/ml IL-12 for indicated times. Cells were labelled with anti-human pSTAT1, pSTAT3 or pSTAT4-Alexa 647 in combination with anti-human CD3-FITC and anti-human CD56-PE and analyzed by FACS. Graph shows fold increase of the mean fluorescence intensity (mfi) as compared to medium stimulated cells. IL-23 induces phosphorylation of STAT3 and STAT4, but not STAT1 and STAT5, in CD56⁺/CD3⁺ NK-like T cells, but not in CD56⁺/CD3⁻ NK cells. IL-12 induces STAT4 phosphorylation in both populations. One representative of experiments with cells from 3 donors.

For control purposes, cells were also stimulated with IL-12 and IL-2. IL-12 induced STAT4 phosphorylation in both NK and NK-like T cells (Fig. 2). IL-2 induced STAT5 phosphorylation in both CD3⁺ and CD3⁻ populations (data not shown). The fact that only CD3⁺ NK-like T-cells and not in CD3⁻ NK cells are responsive to IL-23 may indicate that only NK-like T cells express the IL-23R. To

date, no antibody is available for the detection of the IL-23R on the cell membrane (22). Instead, we analyzed *IL23R* mRNA expression in both CD3⁺ NK-like T and in CD3⁻ NK cells by real time PCR and observed a 5-6 fold higher expression of *IL23R* mRNA in CD3⁺ NK-like T cells, compared to CD3⁻ NK cells (data not shown).

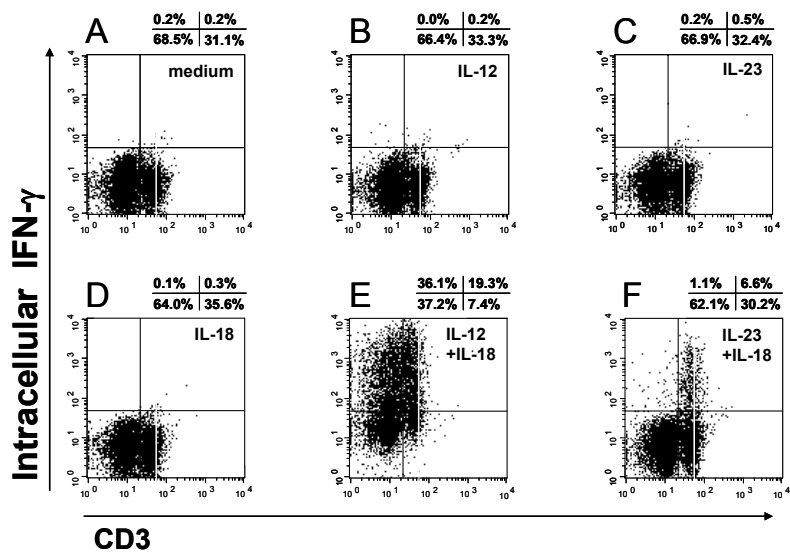


Figure 3. IL-23 in combination with IL-18 induces IFN- γ production in CD56⁺/CD3⁺ NK-like T cell but not in CD56⁺/CD3⁻ NK cells. Anti-CD56 MACS bead isolated cells were rested overnight and then left unstimulated (A) or stimulated with 1 ng/ml IL-12 (B), 10 ng/ml IL-23 (C), 100 ng/ml IL-18 (D), 100 ng/ml IL-18 plus 1 ng/ml IL-12 (E), 100 ng/ml IL-18 plus 10 ng/ml IL-23 (F), for 48 hours. Cells were fixed and permeabilised and labeled with anti-human CD3-PE, anti-human CD56-FITC and anti-human IFN- γ -Alexa 647. Unstimulated cells and cells stimulated with IL-12, IL-23 or IL-18 do not produce IFN- γ . IL-12 plus IL-18 induce IFN- γ production in CD3⁻ NK and CD3⁺ NK-like T cells. IL-23 in concert with IL-18 induced IFN- γ production in CD3⁺ NK-like T cells, but not in CD3⁻ NK cells. One representative of experiments with cells obtained from 6 donors.

IL-23, in concert with IL-18, induces IFN- γ in CD56⁺/CD3⁺ NK-like T, but not in CD56⁺/CD3⁻ NK cells. In the STAT phosphorylation assay we observed a specific effect of IL-23 on CD56⁺/CD3⁺ NK-like T cells. We next wanted to determine whether these IL-23 responsive CD56⁺/CD3⁺ NK-like T cells were responsible for the observed IFN- γ production by CD56⁺ cells. CD56⁺ cells were stimulated with IL-23, IL-12, IL-18, combinations of these cytokines, or left unstimulated two days. Intracellular IFN- γ production, as well as CD56 and CD3 expression were assessed by FACS, to compare IFN- γ production by CD56⁺/CD3⁻ NK cells and CD56⁺/CD3⁺ NK-like T cells. Unstimulated cells did not produce IFN- γ (Fig. 3A and Table 1). In response to IL-12 or IL-23 or IL-18 alone no IFN- γ production could be detected (Fig. 3B-D and Table 1). IL-12 in combination with IL-18 induced IFN- γ production in both NK cells and NK-like T cells (Fig. 3E and Table 1). In contrast, after stimulation with IL-23 in combination with IL-18, NK-like T cells produced IFN- γ , whereas NK cells did not (Fig.

3F and Table 1). Moreover, IL-23 plus IL-18 induced IFN- γ production only in CD56^{bright} NK-like T cells, whereas IL-12 plus IL-18 induced IFN- γ production in CD56^{bright} and CD56^{dim} cells (data not shown).

Table 1. Percentages of IFN- γ positive NK and NK-like T cells from 6 donors

	medium	IL-12	IL-23	IL-18	IL-18+IL-12	IL-18+IL-23
IFN- γ ⁺ ve NK cells	0.24 (0.22)	0.55 (0.57)	0.16 (0.13)	0.23 (0.22)	33.6 (11.8)*	2.05 (1.38)
IFN- γ ⁺ ve NK-like T cells	0.53 (0.38)	1.06 (0.88)	0.56 (0.30)	0.70 (0.53)	26.57 (9.11)*	10.76 (3.56)**

Isolated CD56 cells were stimulated and analysed exactly as in Figure 3. Average percentages and standard deviation (SD) of IFN- γ positive cells are shown of 6 donors. Two-tailed paired t-tests were performed of stimulated versus unstimulated cells. * indicates a p-value <0.01, ** indicates a p-value <0.02.

IL-23 in combination with IL-18 induces proliferation of CD56⁺/CD3⁻ NK and CD56⁺/CD3⁺ NK-like T cells

Because the above observed expansion of the CD56^{bright} CD3⁻ NK cells could be due to upregulation of CD56, to enhanced survival, or to proliferation of CD56^{bright} cells we analyzed proliferation in response to cytokines. CD56⁺ cells were CFSE labeled and stimulated with IL-23, IL-12, IL-18, combinations of these cytokines, or left unstimulated.

Proliferation was measured by FACS six days after stimulation. Cells were anti-CD56 and anti-CD3 labeled. Unstimulated cells did not proliferate (Fig. 4A and G and Table 2). IL-12 induced proliferation of CD56⁺/CD3⁻ NK cells (Fig. 4B and table 2), predominantly of CD56^{bright} cells (Fig. 4H and Table 2). In contrast to IL-12, IL-23 alone did not induce proliferation of NK or NK-like T cells (Fig. 4C and I and Table 2). IL-18 alone induced proliferation of a small population of CD56⁺/CD3⁻ NK cells (Fig. 4D and Table 2), these cells were mostly CD56^{bright} (Fig. 4J). IL-12 in combination with IL-18 (Fig. 4E and Table 2) induced proliferation of CD56⁺/CD3⁻ NK cells and, to a lesser extent, of CD56⁺/CD3⁺ NK-like T cells. Both CD56^{bright} and CD56^{dim} cells proliferated in response to IL-12 plus IL-18 (Fig 4K). IL-23 in combination with IL-18 induced proliferation of CD56⁺/CD3⁻ NK cells (Fig. 4F and Table 2). Both IL-12 and IL-23 in combination with IL-18 induced proliferation of CD56⁺/CD3⁺ NK-like T cells, but less compared to CD56⁺/CD3⁻ NK cells (Fig. 4E and F).

Because in these experiments CD56⁺/CD3⁻ NK and CD56⁺/CD3⁺ NK-like T cells are co-cultured, the effects observed in CD3⁻ NK cells could have been induced directly in CD3⁻ NK cells or induced indirectly via IL-23 activated CD3⁺ NK-like T cells. However, when CD3⁺ NK-like T cells were depleted from the CD56⁺ cells using FACS sort, proliferation in response to IL-23 plus IL-18 was still observed in the CD56⁺/CD3⁻ NK cells (data not shown). The purity of the sorted CD56⁺/CD3⁻ NK cells was >99%.

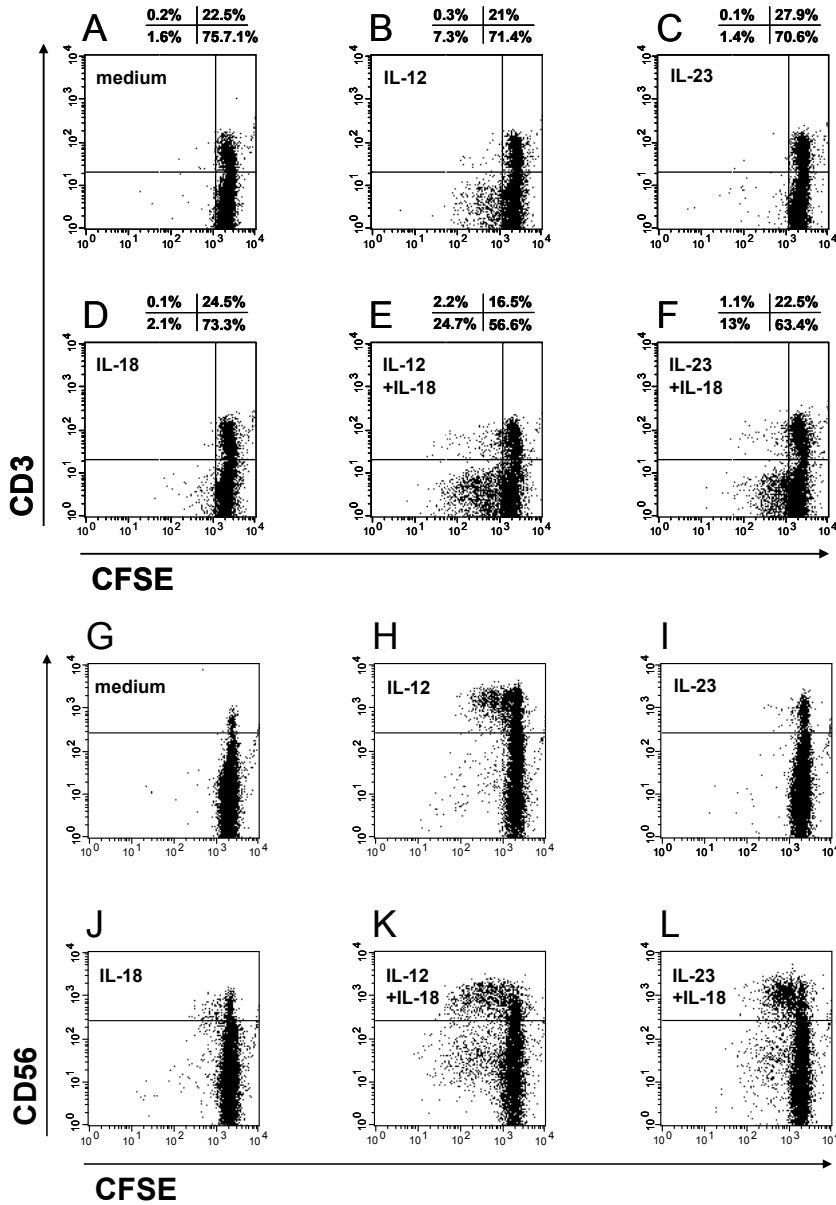


Figure 4. IL-23 in combination with IL-18 induces proliferation of CD56⁺/CD3⁺ NK cells and in CD56⁺/CD3⁺ NK-like T cells. Anti-CD56 MACS bead isolated cells were rested overnight and then CFSE labeled. These labeled cells were left unstimulated (A and G) or stimulated with 1 ng/ml IL-12 (B and H), 10 ng/ml IL-23 (C and I), 100 ng/ml IL-18 (D and J), 100 ng/ml IL-18 plus 1 ng/ml IL-12 (E and K), 100 ng/ml IL-18 plus 10 ng/ml IL-23 (F and L). Six days after stimulation cells were labeled with anti-human CD3-PE and CD56 alexa 647 and analyzed by FACS. IL-23 and IL-18 alone do not induce or induce only little proliferation. IL-23 and IL-18 together induce proliferation of CD3⁺ NK cells. IL-12 alone induces proliferation of CD3⁺ NK cells. IL-18 enhances the proliferative effect of IL-12. CD3⁺ NK-like T cells proliferate less compared to CD3⁺ NK cells in response to all stimuli. Experiments were performed with cells obtained from 6 donors. Representative graphs from one donor are shown.

Table 2. Percentages of proliferating NK and NK-like T cells from 6 donors

	(-)	IL-12	IL-23	IL-18	IL-18+IL-12	IL-18+IL-23
Proliferating NK cells	1.53 (0.32)	7.08 (2.38)*	1.39 (0.43)	2.54 (0.83)	22.17 (2.04)*	9.47 (3.76)*
Proliferating NK-like T cells	0.28 (0.09)	0.99 (0.74)*	1.16 (2.40)	0.29 (0.27)	3.83 (1.53)**	2.16 (0.69)*

Isolated CD56⁺ cells were stimulated and analysed exactly as in Figure 4. Average percentages of proliferating NK and NK-like T cells obtained from 6 different donors. Two-tailed paired t-tests were performed of stimulated cells versus unstimulated cells. * indicates a p-value <0.05, ** indicates a p-value <0.01

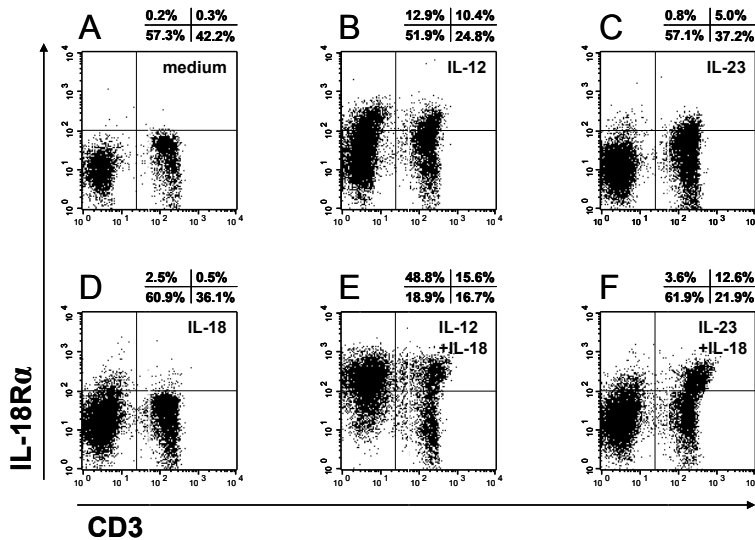


Figure 5. IL-23 up-regulates IL-18Rα expression in CD56⁺/CD3⁺ NK-like T, but not in CD56⁺/CD3⁻ NK cells. Anti-CD56 MACS bead isolated cells were left unstimulated (A) or stimulated with 1 ng/ml IL-12 (B), 10 ng/ml IL-23 (C), 100 ng/ml IL-18 (D), 1 ng/ml IL-12 plus 100 ng/ml IL-18 (E) or 10 ng/ml IL-23 plus 100 ng/ml IL-18 (F). 48 hours after stimulation cells were labelled with anti-human CD56-Alexa 647, anti-human CD3-FITC and anti-human IL-18Rα-PE and analyzed by FACS. Unstimulated CD3⁺ NK-like T cells express slightly more IL-18Rα as compared to CD3⁻ NK cells. IL-12 up-regulates IL-18Rα in NK and NK-like T cells. IL-23 up-regulates IL-18Rα expression mainly in NK-like T cells. IL-18 alone slightly enhances IL-18Rα in NK cells. IL-18 synergizes with IL-12 and IL-23 in the up-regulation of IL-18Rα expression. One representative of experiments with cells from 6 donors.

IL-23 enhances IL-18R α expression in CD56⁺/CD3⁺ NK-like T but not in CD56⁺/CD3⁻ NK cells.

We have shown that IL-23 induces IFN- γ production in CD56⁺/CD3⁺ NK-like T cells in synergy with IL-18. We have also shown that IL-23 in synergy with IL-18 induced enhanced CD56 expression in NK-like T cells and that IL-23 plus IL-18 induced proliferation in CD56⁺/CD3⁻ NK cells. The mechanism underlying the synergy between IL-18 and IL-12 involves IL-12 induced IL-18R α expression (23). To determine whether the synergy between IL-23 and IL-18 is similarly dependent on the up-regulation of IL-18R α , CD56 isolated cells were stimulated for two days with IL-23, IL-12, IL-18, IL-23 plus IL-18 or IL-12 plus IL-18. Cells were then analyzed for IL-18R α , CD3 and CD56 expression. Unstimulated NK and NK-like T cells expressed low amounts of IL-18R α (Fig. 5A and Table 3). IL-12 enhanced IL-18R α expression in both NK and NK-like T cells (Fig. 5B and Table 3). IL-23 up-regulated the IL-18R α expression in NK-like T cells, but only marginally in NK cells (Fig. 5C and Table 3). IL-18 alone enhanced the expression of the IL-18R α only marginally in both populations (Fig. 5D and Table 3), but synergized with the effect of IL-12 on the expression of IL-18R α in both NK and NK-like T cells (Fig. 5E and Table 3). IL-18 synergized with IL-23 in the up-regulation of the IL-18R α in NK-like T cells (Fig. 5F and Table 3). In NK cells a slight upregulation of the IL-18R α was observed in response to IL-23 plus IL-18 (Fig. 5F and Table 3). Regardless of the stimulation, cells with high expression of the IL-18R α were CD56^{bright} (data not shown).

Table 3. IL-18R α expression in NK and NK-like T cells from 6 donors

	(-)	IL-12	IL-23	IL-18	IL-18+IL-12	IL-18+IL-23
CD3 ⁻ NK cells upper half	0.19 (0.08)	10.30 (5.48)**	0.85 (0.48)**	1.11 (1.60)	34.99 (21.24)*	4.17 (1.23)
CD3 ⁺ NK-like T cells upper half	0.36 (0.26)	11.84 (10.4)	3.98 (4.85)	0.65 (0.49)	50.98 (24.12)*	36.34 (2.92)*

Isolated CD56 cells were stimulated and analysed exactly as in Figure 5. Average percentages and standard deviation of data obtained from 6 different donors are shown. Two-tailed paired t-tests were performed of stimulated cells versus unstimulated cells. * indicates a p-value <0.01, ** indicates a p-value <0.02.

Discussion

The main finding of this study is that IL-23, an APC-derived cytokine, in combination with IL-18, another cytokine elicited in APCs early after binding of pathogen-associated molecular patterns, can elicit the production of IFN- γ by NK-like T cells as well as proliferation and activation of human NK and NK-like T cells. We hypothesize that in this way, APCs become primed to subsequently produce large amounts of IL-12 and thus amplify the production of IFN- γ . This

conclusion is supported by the following findings. Firstly, IL-23 and IL-18 synergistically increase IFN- γ production in CD56⁺/CD3⁺ NK-like T cells. This synergy between IL-23 and IL-18 in NK-like T cells coincided with IL-23 mediated up-regulation of IL-18R α . Secondly, IL-23 plus IL-18 induced proliferation of CD56⁺/CD3⁻ NK cells and CD56⁺/CD3⁺ NK-like T cells.

We observed a strong synergy between IL-23 and IL-18 to induce cytokine production and induce cell proliferation. In this respect, the combination of IL-23 and IL-18 on NK and NK-like T cells differed from that induced by IL-12 in combination with IL-18: IL-23 plus IL-18 induced IFN- γ production in NK-like T cells only, whereas IL-12 plus IL-18 induced IFN- γ in both NK as well as NK-like T cells. Previously it has been described that IFN- γ production by NK-like T cells can be triggered by IL-12/IL-18 (14) stimulation or TCR stimulation. We show that in NK-like T cells, IL-23 plus IL-18 can also induce IFN- γ production, in absence of TCR ligation. In CD56⁺/CD3⁻ NK cells and to a lesser extent in CD56⁺/CD3⁺ NK-like T cells, IL-23 and IL-18 synergized in eliciting cell proliferation.

In CD56⁺/CD3⁺ NK-like T cells IL-18R α expression was up-regulated by IL-23. The synergy between IL-23 and IL-18 in inducing IFN- γ production in these cells is likely to be dependent on this upregulation of IL-18R α expression. In CD56⁺/CD3⁻ NK cells the IL-18R α expression was not enhanced by IL-23, suggesting that in these cells the synergistic effect of IL-23 and IL-18 on proliferation is achieved via another mechanism. The proliferation observed in NK cells could be indirectly induced via NK-like T cells. However, IL-23 in combination with IL-18 also induced proliferation in FACS sorted CD56⁺/CD3⁻ NK cells that were cultured without CD56⁺/CD3⁺ NK-like T cells (>99% pure). This result suggests a direct effect of these cytokines on NK cells.

Upon contact with pathogens APCs are unable to release IL-12 in sufficient amounts to recruit and activate T-helper cells and thus initiate a cellular immune response. For substantial IL-12 production by APC, in addition to PAMPs or pathogens, an extra stimulus such as IFN- γ is needed (9,24). IL-23 on the other hand is produced by APC in response to PAMPs or pathogens without the need of an additional stimulus (9). Because IL-23 and IL-18 are both released soon after first contact between phagocytes and pathogens, we hypothesize that, *in vivo*, IL-23 plus IL-18 triggers release of IFN- γ by NK-like T cells and that this IFN- γ could provide APCs with the necessary priming to subsequently produce IL-12. The IFN- γ induced by IL-23 plus IL-18 might thus be important to initiate Th1 immunity at early stages of infection. In line with this hypothesis, IL-23 is shown to be critical for the induction of Ag-specific Th1 development in an experimental autoimmune encephalomyelitis mouse model (25). Moreover, IL-23 inhibited the induction of the Th2 cytokines IL-4 and IL-13 in antiCD2/antiCD28 activated CD56⁺ cells. Taken together, the synergy of IL-18 with IL-23 is likely important in initiating Th1 differentiation early in infections, whereas the synergy between IL-18 and IL-12 may be important in further Th1 response in subsequent stages of infection. Host defense against intracellular bacterial pathogens such as

Salmonellae and *Mycobacteria* depends on the IL-23/IL-12/IFN- γ cytokine pathway (12,26). However, the cells that produce IL-23/IL-12 and IFN- γ at different stages of infection in human are not well defined. For instance, the role of CD56⁺ NK-like T cells in *Mycobacteria* and *Salmonellae* infections has not been studied widely. These cells are likely important because of the following observations. First, the number of peripheral blood CD56⁺ T-cells are increased during these infections in humans (27,28). Second, high numbers of CD56⁺ NK-like T cells at diagnosis of pulmonary tuberculosis correlated significantly with negative sputum culture after 8 weeks of treatment (29). Third, in the presence of macrophages infected with live *M. bovis* BCG or *S. typhimurium*, CD56⁺ NK-like T cells, but not CD56⁻ T cells, produce IFN- γ in the absence of TCR stimulation (14). Fourth, in patients with unusual susceptibility to *Mycobacteria* and *Salmonellae* infections due to deficiency of IL-12R β 1 or IL-12p40, the number of CD56⁺ NK-like T-cells is drastically reduced (14). The reduced numbers of NK-like T cells in these last patients indicate that IL-12 and IL-23 are also needed for the differentiation and/or maintenance of these cells. In addition to these findings, we have shown that IL-23, in combination with IL-18, is able to drive IFN- γ production in CD56⁺ NK-like T cells, in the absence of IL-12 and TCR ligation. This finding indicates that IL-23 could be important in driving IFN- γ production in these cells in early stages of infection, before sufficient IL-12 is produced to drive IFN- γ production. Consistent with this hypothesis, p40^{-/-} mice, lacking both IL-12 and IL-23, infected with *S. enteritidis* or *M. tuberculosis* produce lower levels of IFN- γ than p35^{-/-} mice, lacking only IL-12 (30,31). Moreover, p35^{-/-} mice infected with *S. enteritidis* show higher survival rates or longer survival times than p40^{-/-} mice (31). In mycobacterial infection, IL-23 provides protection in the absence of IL-12 (32). Together, this points to a role for IL-23 in protection, independent of IL-12. The relative roles of either IL-12 or IL-23 in driving the IFN- γ response early in infection have not been addressed yet. Monitoring the kinetics of IFN- γ in response to infections with *Salmonellae* and *Mycobacteria* in p19^{-/-} and p35^{-/-} mice could provide information about the contribution of IL-23 and IL-12 to the induction of IFN- γ .

Taken together, these observations indicate that IL-23 has different effects on NK cells as compared with NK-like T cells. The effects of IL-23 in combination with IL-18 on NK and NK-like T cells differ from the effects induced by IL-12 in combination with IL-18. Moreover, we showed that IL-23, in synergy with IL-18, activates NK-like T cells. This activation was independent of IL-12 and independent of TCR ligation. In conclusion, IL-23 may have an important role in activating NK and NK-like T cells and the initiation of the (Th1) immune response early in an infection.

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Chapter 2

Salmonella Induced IL-23 and IL-1 β Allow for IL-12 Production by Monocytes and M ϕ 1 through Induction of IFN- γ in CD56⁺ NK/NK-like T cells

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Abstract

Background The type-1 cytokine pathway plays a pivotal role in immunity against intracellular bacterial pathogens such as *Salmonellae* and *Mycobacteria*. Bacterial stimulation of pattern recognition receptors on monocytes, macrophages and dendritic cells initiates this pathway, and results in the production of cytokines that activate lymphocytes to produce interferon (IFN)- γ . Interleukin (IL)-12 and IL-23 are thought to be the key cytokines required for initiating a type-1 cytokine immune response to *Mycobacteria* and *Salmonellae*. The relative contribution of IL-23 and IL-12 to this process is uncertain.

Methodology/Principal Findings We show that various TLR agonists induce the production of IL-23 but not IL-12 in freshly isolated human monocytes and cultured human macrophages. In addition, type 1 pro-inflammatory macrophages (M ϕ 1) differentiated in the presence of GM-CSF and infected with live *Salmonella* produce IL-23, IL-1 β and IL-18, but not IL-12. Supernatants of *Salmonella*-infected M ϕ 1 contained more IL-18 and IL-1 β as compared with supernatants of M ϕ 1 stimulated with isolated TLR agonists, and induced IFN- γ production in human CD56⁺ cells in an IL-23 and IL-1 β -dependent but IL-12-independent manner. In addition, IL-23 together with IL-18 or IL-1 β led to the production of GM-CSF in CD56⁺ cells. Both IFN- γ and GM-CSF enhanced IL-23 production by monocytes in response to TLR agonists, as well as induced IL-12 production.

Conclusions/Significance The findings implicate a positive feedback loop in which IL-23 can enhance its release via induction of IFN- γ and GM-CSF. The IL-23 induced cytokines allow for the subsequent production of IL-12 and amplify the IFN- γ production in the type-1 cytokine pathway.

Introduction

Immunity against intracellular bacterial pathogens such as *Salmonellae* and *Mycobacteria* depends on the type-1 cytokine pathway (1). This pathway is initiated by bacterial stimulation of pattern recognition receptors on monocytes and macrophages, resulting in the production of cytokines that activate lymphocytes and induce IFN- γ production. The IFN- γ in turn activates monocytes and macrophages, to enhance bactericidal effector mechanisms and to further pro-inflammatory cytokine production. Thus, the type-1 cytokine pathway critically depends on the cross-talk between monocytes/macrophages and lymphocytes. Abdi *et al.* state that IL-12p70 cannot be the primary trigger that initiates Th1 T-cell responses, as it is not produced in response to bacterial stimulation when costimulation in the form of activated T cells or IFN- γ are absent (2). In addition, in whole blood assays with blood obtained from patients with complete IFN- γ R deficiency, no IL-12p70 production can be detected in response to *M. bovis* BCG infection *in vitro* (3). How the type-1 pathway is initiated, therefore, has remained uncertain.

Interleukin-23 (IL-23) is a cytokine which is produced early in the immune response (4). Monocytes as well as type 1 macrophages (M ϕ 1) produce IL-23 in response to the binding of pathogens and pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), to Toll-like receptors (TLRs) (5,6). In contrast to IL-23, for the production of IL-12 in response to PAMPs an additional stimulus such as IFN- γ is required (5). IL-23 is known to induce IFN- γ production in naïve T cells, in memory T cells (7) and in NK-like T cells (8), thereby potentially providing the necessary, additional stimulus to induce IL-12 production. Next, IL-12 and IL-18 enhance IFN- γ production in NK, NK-like T cells and Th1 cells by binding to their respective receptors (9). Though IFN- γ is not required for the induction of IL-23, the precise role of IFN- γ in the regulation of IL-23 is not well established.

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) is a cytokine produced by NK cells and T cells in response to a variety of stimuli, for instance IL-15 and IL-18 (10). GM-CSF activates monocytes and enhances their bactericidal activity (11-13). Moreover, monocytes pre-stimulated with GM-CSF secrete increased quantities of tumor necrosis factor (TNF) and IL-1 β when stimulated with LPS (14,15). Furthermore, GM-CSF induces differentiation of human monocytes into M ϕ 1, a macrophage type that is capable of producing IL-23 (5).

In this study we addressed the roles of the cytokines IL-23, IL-1 β , IL-18, GM-CSF and IFN- γ in the crosstalk between NK/NK-like T cells and monocytes/ macrophages in the early activation of the type-1 cytokine pathway. To this end we determined which TLR agonists induce IL-23 production in human monocytes and macrophages and assessed the roles of GM-CSF and IFN- γ in the regulation of IL-23 production by human monocytes in response to TLR agonists. Furthermore, we explored the role of *Salmonella*-induced IL-23, IL-1 β and IL-18 in the induction of

IFN- γ in primary human NK/NK-like T cells, and tested the capacities of IL-23, IL-1 β and IL-18 to induce GM-CSF and IFN- γ in human NK and NK-like T cells.

Materials and Methods

Cells and culture conditions

Human CD14⁺ cells and CD56⁺ cells were isolated from buffy coats from healthy donors (Sanquin) by Ficoll-Amidotrizoate density gradient centrifugation and subsequent selection with anti-CD14 MACS beads or anti-CD56 MACS beads (Miltenyi Biotech).

CD14⁺ cells were cultured in RPMI-1630 medium, supplemented with 20 mM GlutaMAX (Gibco/Invitrogen), 10% FCS, 100 U/ml Penicillin, 100 μ g/ml Streptomycin (Gibco/Invitrogen). CD56⁺ cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Bio-Whittaker) supplemented with 20 mM GlutaMAX, 10% FCS, 100 U/ml Penicillin, 100 μ g/ml Streptomycin. To generate M ϕ 1, CD14⁺ cells were cultured for 6 days in RPMI 1640 with 10% FCS and 5 ng/ml GM-CSF (Biosource) in 75 cm² or 175 cm² cell culture flasks (Greiner Bio-One).

Cytokine induction and measurement

To determine cytokine production by monocytes, CD14⁺ beads isolated cells were seeded in a 96-well plate at $1 \cdot 10^5$ cells per well and cultured for 24 h in the presence or absence of the following TLR agonists: 100 ng/ml *S. minnesota* LPS (Sigma), 200 ng/ml recombinant flagellin (tlrl-flic, InvivoGen), 1 μ g/ml Pam3CSK4 (tlrl-pms, InvivoGen), 100 μ g/ml Zymosan A (Sigma), 1 μ g/ml CL075 (tlrl-c75, InvivoGen) or 1 μ g/ml CL087 (tlrl-c87, InvivoGen) in a final volume of 200 μ l. Supernatants were taken and IL-23 (eBioscience), IL-1 β (Biosource) IL-18 (MBL) and IL-12p70 (Sanquin) concentrations were determined by ELISA. For experiments with M ϕ 1, cells were harvested with trypsin-EDTA, washed with PBS and seeded at $3.3 \cdot 10^5$ per well in 24-well or $1 \cdot 10^6$ in 12-well culture plates (Corning Life Sciences) and allowed to adhere. Subsequently M ϕ 1 were stimulated with TLR agonists, infected with group B *Salmonella* at a 10:1 multiplicity of infection as described below, or left unstimulated. Twenty-four hours after stimulation, supernatants were collected and cytokine production was determined by ELISA. To test the effect of GM-CSF and IFN- γ pre-stimulation, $1 \cdot 10^5$ CD14⁺ monocytes were cultured for 16 hours with 50-5000 pg/ml GM-CSF, 50-5000 pg/ml IFN- γ (Biosource) or left unstimulated. Subsequently cells were stimulated with 100 ng/ml LPS plus 1 μ g/ml CL075 for 24 hours, or left unstimulated. After stimulation cell free supernatants were collected and IL-23 production was determined by ELISA (eBioscience).

Supernatant transfer experiments

To generate supernatants from *Salmonella* stimulated M ϕ 1, $1 \cdot 10^6$ cells were seeded in a 12-well plate and infected with group B *Salmonella* at a 10:1 multiplicity of infection. To promote the uptake of bacteria, the bacteria were spun onto the macrophages by centrifugation at $300 \times g$ for 5 min and the cells were allowed to internalize the bacteria for 30 min at 37°C, 5% CO₂. Cells were washed 3 times with PBS after which medium containing 50 μ g/ml gentamicin (Sigma) was added to kill extracellular *Salmonellae*. After 30 minutes, medium was replaced with 2 ml fresh medium containing 10 μ g/ml gentamicin. Twenty-four hours after infection, cell free supernatants were collected and used to stimulate anti-CD56 beads isolated cells. Overnight rested CD56⁺ cells were seeded $1 \cdot 10^5$ cells per well in a 96-well plate and stimulated for 48 hr with 100 μ l of conditioned M ϕ 1 medium, with or without 100 ng/ml IL-18 (MBL), in a final volume of 200 μ l. After 48 hours of culture, cell free supernatants were collected and IFN- γ production was measured by cytokine specific ELISA (Biosource). For cytokine neutralization assays, macrophage supernatants were pre-incubated with 10 μ g/ml anti-IL-12p40 (C8.6, eBiosciences) or 10 μ g/ml anti-IL-18 (MBL) or 5 μ g/ml anti IL-1 β (BD Biosciences) for 30 minutes at 37°C.

To generate supernatants of CD56⁺ cells, $1 \cdot 10^6$ cells were stimulated with 10 ng/ml IL-23 plus 10 ng/ml IL-1 β , or left unstimulated, for 24 hours. Cell free supernatants were collected and used to stimulate CD14⁺ monocytes. CD14⁺ cells were seeded $1 \cdot 10^5$ cells per well in a 96-well plate and stimulated for 24 hr with 100 ng/ml LPS, in combination with 100 μ l supernatant of CD56⁺ cells, 2.5 ng/ml IFN- γ (Biosource) or medium in a final volume of 200 μ l. To neutralize IFN- γ , supernatants from stimulated CD56⁺ cells were pre-incubated with 2 μ g/ml anti-IFN- γ (Genzyme) for 30 minutes at 37°C. Supernatants were collected and IL-12p70 was measured by cytokine specific ELISA (BD Bioscience).

IFN- γ and GM-CSF production

To determine GM-CSF and IFN- γ production in NK and NK-like T cells, overnight-rested CD56⁺ beads-isolated cells were seeded $1 \cdot 10^5$ cells per well in a 96-well plate and stimulated for 24 hr with various concentrations of IL-23, alone or in combination with various concentrations of IL-18 (MBL) or IL-1 β (Biosource) in a final volume of 200 μ l. Concentrations of IFN- γ and GM-CSF in the cell free supernatants were determined by cytokine-specific ELISA (Biosource).

For intracellular staining of IFN- γ , CD56⁺ beads-isolated cells were seeded 10^5 cells/well in 96-well plates and stimulated with IL-23 (R&D Systems), IL-18 (MBL), IL-1 β (Biosource) or a combination of these cytokines for 48 hours. The last 5 hours of stimulation BD GolgiPlug (BD PharMingen) was added (final concentration 1:1000). Cells were fixed in 4% paraformaldehyde (Sigma) and permeabilised in 90% methanol. Cells were stained with Alexa 647-labeled anti-human IFN- γ in

combination with PE-labeled anti-human CD56 and FITC-labeled anti-human CD3 (BD PharMingen).

Results

Monocytes and M ϕ 1 produce IL-23, but not IL-12, in response to various TLR agonists.

LPS is known to induce IL-23, but not IL-12, production in M ϕ 1 (5). To determine whether various TLR agonists are capable of inducing IL-23 or IL-12 production in monocytes and M ϕ 1, we first tested these capacities in freshly isolated human monocytes. Unstimulated monocytes did not produce IL-23, whereas Zymosan A (agonist for TLR2/6) induced minimal IL-23 production, LPS (TLR4) and CL075 (TLR8/7) each induced small amounts of IL-23, while LPS and CL075 synergized in the induction of IL-23 production in monocytes (Fig. 1A). IL-23 production was also observed in response to LPS plus flagellin (TLR5) but the amount released was similar to that induced by LPS alone. Of note, the amounts of IL-23 produced by CD14⁺ monocytes varied somewhat between different donors, the relative amount released upon the various TLR stimulations was however similar (data not shown). In none of the supernatants IL-12p70 was detected, given a detection limit of the IL-12p70 ELISA of 3 pg/ml (data not shown).

Next, we compared the amount of IL-23 and IL-12 produced by monocytes to those by cultured M ϕ 1. Upon incubation with each of the TLR agonists M ϕ 1 produced IL-23 (Fig. 1B). LPS synergized with both CL075 and flagellin in the induction of IL-23. As compared with freshly isolated monocytes at identical cell number and incubation conditions, M ϕ 1 produced markedly more IL-23 in response to the TLR agonists. Again, no IL-12p70 production was detected in response to any of the stimuli (data not shown). To verify that the M ϕ 1 used in these experiments are capable of producing IL-12p70 they were stimulated with LPS in combination with IFN- γ . Large amounts of IL-12p70 were detected in the supernatants (data not shown).

*Infection of M ϕ 1 with live *Salmonella* induces IL-23, but not IL-12, production.*

To determine whether not only PAMPs such as the TLR agonists, but also live pathogens induce IL-23 rather than IL-12 production in M ϕ 1, we infected cultured M ϕ 1 with live *Salmonella* and assessed cytokine release in the culture supernatants. M ϕ 1 exposed to and containing ingested *Salmonellae* produced IL-23 whereas M ϕ 1 left unstimulated did not (Fig. 1C). Of note, *Salmonella* infected M ϕ 1 produced markedly more IL-23 as compared with M ϕ 1 stimulated with large amounts of LPS (Fig 1C). Similar to stimulation with TLR agonists, we did not detect any IL-12p70 in the supernatants of infected M ϕ 1 (data not shown). Moreover, in a related project, micro-array analysis of M ϕ 1 infected with live *Salmonella* for 1, 2, 4, 8 and 24 hours revealed that infection did not induce

IL12A (IL-12p35) transcription, whereas *IL12B* (IL-12p40) and *IL23A* (IL-23p19) transcription were both upregulated in response to *Salmonella* (16).

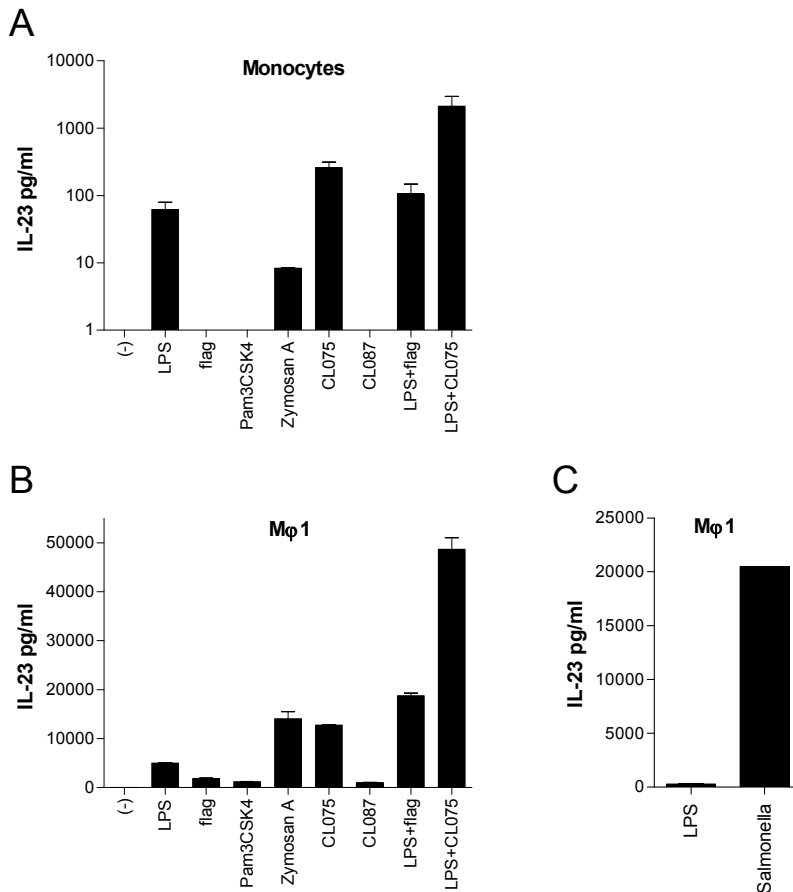


Figure 1. TLR stimuli and *Salmonella* infection induce IL-23 production in human monocytes and Mφ1.

$1 \cdot 10^5$ overnight rested anti-CD14 beads-isolated monocytes (A) or $5 \cdot 10^4$ Mφ1 (B) were stimulated with 100 ng/ml LPS, 200 ng/ml flagellin (flag), 1 μ g/ml Pam3CSK4, 100 μ g/ml Zymosan A, 1 μ g/ml CL075, 1 μ g/ml CL087, 100 ng/ml LPS plus 200 ng/ml flagellin, LPS 100 ng/ml plus 1 μ g/ml CL075 for 24 hours, or left unstimulated. (C) Mφ1 were infected with live *Salmonella* with a multiplicity of infection (MOI) of 10, left uninfected or were stimulated with 100 ng/ml LPS. Supernatants were collected after 24 hours and IL-23 protein production was assessed by ELISA. Data are means $\pm$ standard deviation (SD) of triplicates in one representative experiment of five (A and B) or means of duplicates in one representative experiment of three (C).

TLR stimuli and infection with live Salmonella induce IL-18 and IL-1 β production.

We have shown previously that for IL-23 to induce IFN- γ production in NK-like T cells, an additional stimulus such as IL-18 is required (8). Similar to IL-18, IL-1 β can costimulate for IFN- γ production (17,18). To determine whether the production of IL-23 occurs in concert with that of IL-18 or IL-1 β ,

we assayed the production of these cytokines by M ϕ 1 in response to LPS or infection with *Salmonella*. Unstimulated cells produced minimal IL-18, but no IL-1 β . Although IL-18 and IL-1 β production in response to LPS and infection varied markedly between donors; M ϕ 1 of all donors produced both IL-18 and IL-1 β in response to these stimulations (Fig. 2A and B). *Salmonella*-infected M ϕ 1 produced markedly more IL-18 and IL-1 β as compared with cells stimulated with LPS (Fig. 2A and B).

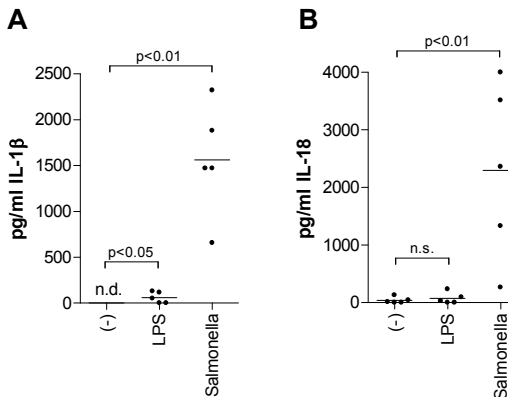


Figure 2. *Salmonella* infection induces IL-18 and IL-1 β production in M ϕ 1. $3.3 \cdot 10^5$ M ϕ 1 of five different donors were left unstimulated, stimulated with LPS or infected with *Salmonella* with a MOI of 10. Supernatants were collected after 24 hours and IL-18 (A) and IL-1 β (B) production was measured by ELISA. Each dot represents one donor. The mean is indicated by a horizontal line. A paired two-tailed student's t-test was used for statistical analysis. n.d. = not detected.

IL-23 and IL-1 β synergize in the induction of IFN- γ in CD56 $^+$ cells.

Infection of M ϕ 1 with live *Salmonella* resulted in the production of IL-23, IL-18 and IL-1 β , IL-23 is reported to induce IFN- γ production in NK, NK-like T cells and in T cells, in synergy with IL-18 (7,8,19). As mentioned before, IL-1 β is known to enhance IL-12 induced IFN- γ production (17,18). Therefore, we tested whether perhaps IL-23 in combination with IL-1 β could also induce IFN- γ production in CD56 $^+$ (NK and NK-like T) cells. Each cytokine alone was not able to induce IFN- γ production (Fig. 3). However, when IL-23 and IL-1 β were combined, they synergized in inducing IFN- γ production (Fig. 3). Isolated CD56 $^+$ cells consist of CD56 $^+$ /CD3 $^-$ NK cells and CD56 $^+$ /CD3 $^+$ NK-like T cells. We next determined which of these two populations were responsible for the observed IFN- γ production by IL-23 plus IL-1 β stimulated CD56 $^+$ cells. Because sorting CD3 $^+$ cells with an anti-CD3 antibody induces undesired activation of the cells, we used intracellular staining for IFN- γ in combination with CD3/CD56 labeling. We arbitrarily designated a population positive for IFN- γ when more than 1% of the cells stained IFN- γ positive. CD56 $^+$ cells obtained from eight donors were stimulated with IL-23, IL-1 β , IL-18, IL-23 plus IL-1 β , IL-23 plus IL-18, or left unstimulated for 48 hours. No significant IFN- γ production was observed in unstimulated cells or cells stimulated with either IL-23, IL-1 β or IL-18 (Fig 4). Surprisingly, the cells producing IFN- γ in response to IL-23 plus IL-1 β or IL-23 plus IL-18 varied between donors. In response to IL-23 plus IL-1 β in half of the donors

IFN- γ production was observed in both CD56⁺/CD3⁻ NK cells and CD56⁺/CD3⁺ NK-like T cells, whereas in the other half IFN- γ production was only observed in CD56⁺/CD3⁻ NK-like T cells (Table 1). In response to IL-23 plus IL-18 IFN- γ production was observed in both populations in seven donors, while in one donor only CD56⁺CD3⁻ cells produced IFN- γ (Table 1). On average, significant IFN- γ production was observed in response to IL-23 plus IL-1 β or IL-18 in NK cells, while in NK-like T cells only IL-23 plus IL-1 β resulted in significant IFN- γ production (Fig 4).

Since IL-23 in combination with IL-1 β is known to promote the production of IL-17 in memory T cells (20), we also analyzed IL-17 production by CD56⁺ cells in response to IL-23 and IL-1 β . However, in none of the supernatants IL-17 was detected (data not shown).

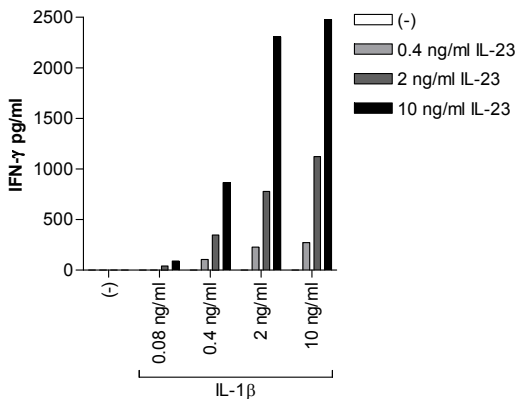


Figure 3. IL-23, in combination with IL-1 β , induces IFN- γ production in primary human CD56⁺ cells. Anti-CD56 MACS bead isolated cells were rested overnight and then left unstimulated or stimulated with indicated concentrations of IL-23 in combination with various concentrations of IL-1 β . Supernatants were collected 24 hours after stimulation and IFN- γ concentration was measured by ELISA. IL-23 and IL-1 β synergize in the induction of IFN- γ . One representative experiment of three is shown.

Supernatants of Salmonella infected M ϕ 1 induce IFN- γ production in primary CD56⁺ cells.

So far we found that IL-23, IL-18 and IL-1 β , but not IL-12p70, are present in supernatants of *Salmonella* infected M ϕ 1. We showed that IL-23 can induce IFN- γ production in CD56⁺ when IL-1 β is present. Previously, we have shown that CD56⁺ cells produce IFN- γ when stimulated with IL-23 plus IL-18, in the absence of IL-12 (8). Based on these data we expected that supernatants from *Salmonella* infected M ϕ 1 would be able to induce IFN- γ production in CD56⁺ cells. Supernatants of *Salmonella* infected M ϕ 1 were indeed able to induce IFN- γ production in primary CD56⁺ cells (Fig. 5A). Neutralization of LPS in these supernatants did not alter their ability to induce IFN- γ production (data not shown).

Since IL-18 is induced in small amounts only, even after infection by *Salmonella*, we determined whether IL-18 was a limiting factor in the induction of IFN- γ in CD56⁺ cells by supernatants of M ϕ 1 infected with *Salmonella*. The addition of recombinant IL-18 to the supernatants increased the IFN- γ production by these CD56⁺ cells (Fig. 5A).

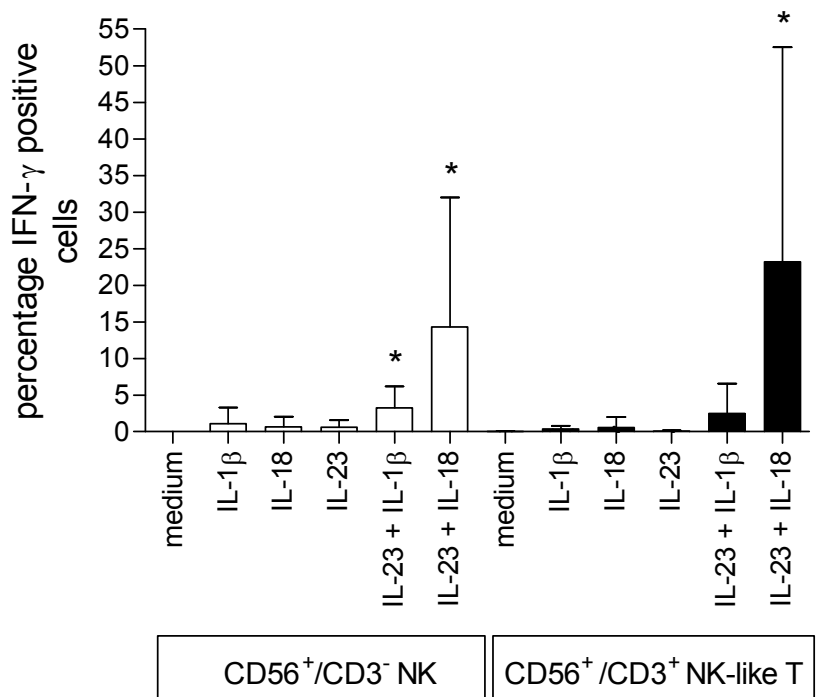


Figure 4. IL-23 in combination with IL-1 β or IL-18 induces IFN- γ production in both CD56⁺/CD3⁻ NK cells and CD56⁺/CD3⁺ NK-like T cells. Anti-CD56 MACS bead isolated cells were rested overnight and then left unstimulated or stimulated with 10 ng/ml IL-1 β , 100 ng/ml IL-18, 10 ng/ml IL-23, 10 ng/ml IL-1 β plus 10 ng/ml IL-23 or 100 ng/ml IL-18 plus 10 ng/ml IL-23, for 48 hours. Cells were fixed, permeabilised and labeled with anti-human CD3-PE, anti-human CD56-FITC and anti-human IFN- γ -Alexa 647. Unstimulated cells and cells stimulated with a single cytokine do not produce significant amounts of IFN- γ . CD3⁻ NK cells stimulated with IL-1 β or IL-18 in combination with IL-23 induce significant IFN- γ production, while in CD3⁺ NK-like T cells only IL-23 plus IL-18 induces significant IFN- γ production. Data are means $\pm$ SD of experiments with cells obtained from 8 donors. Two-tailed paired t-tests were performed of stimulated cells versus unstimulated cells. * indicates a p-value <0.05.

IL-23 and IL-1 β , but not IL-18, in supernatants are critical for the induction of IFN- γ production in CD56⁺ cells.

To verify the role of IL-23 in the induction of IFN- γ by supernatants of *Salmonella* infected M ϕ 1, we neutralized IL-23 using an antibody binding the p40 subunit of IL-23. (Note that although the p40 antibody can also neutralize IL-12, we have shown above that no IL-12 was present in these supernatants). When IL-23 was thus neutralized, IFN- γ production was effectively abrogated (Fig. 5B), even when exogenous IL-18 was added (data not shown).

Similarly, to specify the role of IL-18 and IL-1 β in the supernatants of *Salmonella*-infected M ϕ 1 in the induction of IFN- γ production, we used a specific antibody to neutralize these interleukins. Pilot experiments had confirmed that the IL-18 antibody could neutralize a large amount

of recombinant IL-18 (4 ng/ml) effectively (data not shown). Although exogenous IL-18 enhanced the M ϕ 1 supernatant-induced IFN- γ production by CD56⁺ cells, neutralization of IL-18 in this supernatant did not affect the production of IFN- γ significantly (Fig. 5B), suggesting IL-18 is not crucial in this respect. In contrast, neutralization of IL-1 β reduced the capacity of the supernatants to induce IFN- γ production significantly (Fig. 5B).

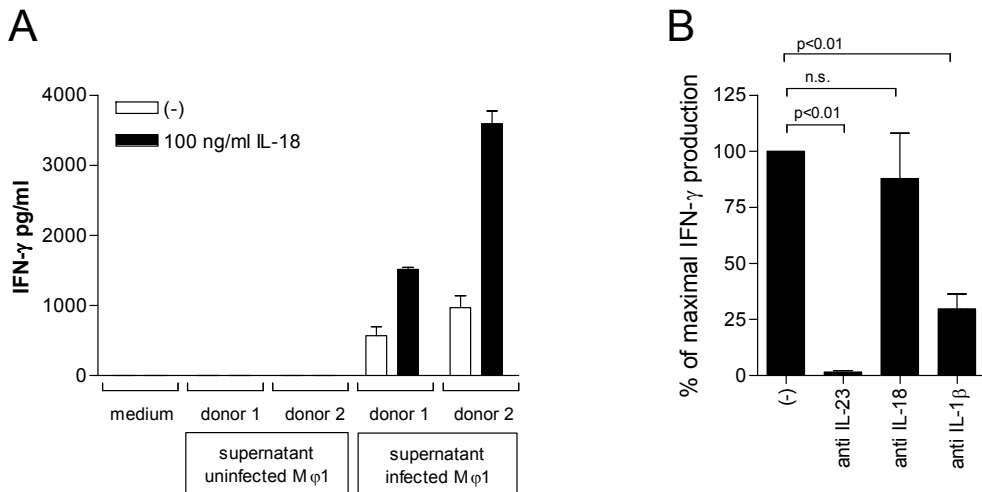


Figure 5. Supernatants of *Salmonella*-infected M ϕ 1 induce IFN- γ production in primary human CD56⁺ cells. (A) IFN- γ production by CD56⁺ NK/NK-like T cells from two donors. Cells were cultured for 48 hours in the presence of supernatants obtained from uninfected or *Salmonella* infected M ϕ 1 (from two donors) with or without additional exogenous recombinant IL-18. As a control, medium only was used. Data are means $\pm$ SD of triplicates in one representative experiment of three. (B) IFN- γ production in CD56⁺ cells induced by supernatants of *Salmonella*-infected M ϕ 1 is blocked by neutralizing IL-12p40 or IL-1 β , but not by neutralizing IL-18. CD56⁺ NK/NK-like T cells were cultured for 48 hours in the presence or absence of supernatant obtained from M ϕ 1 cells infected with *Salmonella*, in the presence or absence of an IL-12p40, IL-18 or IL-1 β neutralizing antibody. IFN- γ concentration was determined by ELISA. IFN- γ production induced by supernatants of *Salmonella* infected M ϕ 1 is set at 100%. A paired two-tailed student's t-test was used for statistical analysis. Data are means $\pm$ SEM of experiments with cells obtained from 10 different donors.

IL-23, in synergy with IL-18 or IL-1 β , induces GM-CSF production in CD56⁺ NK/NK-like T cells.

IL-18 is known to induce GM-CSF production in NK cells in combination with various cytokines (10). To determine whether IL-18 or IL-1 β , combined with IL-23 can induce GM-CSF production in human CD56⁺ NK/NK-like T cells, these cells were stimulated with IL-18, IL-1 β or IL-23 and combinations of these cytokines, or left unstimulated for 24 hours. Unstimulated cells and cells stimulated with IL-23 alone did not produce any GM-CSF (Fig. 6A). In contrast, IL-18 alone induced a small amount of GM-CSF, when high concentrations were used. In combination with IL-23 on the other hand, IL-18 induced considerable amounts of GM-CSF in CD56⁺ cells (Fig. 6A). CD56⁺ cells stimulated with high

concentrations of IL-1 β produced GM-CSF, whereas cells stimulated with lower concentrations of IL-1 β did not. IL-23 synergized with IL-1 β in inducing GM-CSF (Fig 6B). For both IL-18 and IL-1 β , strongest synergy was observed at highest concentrations.

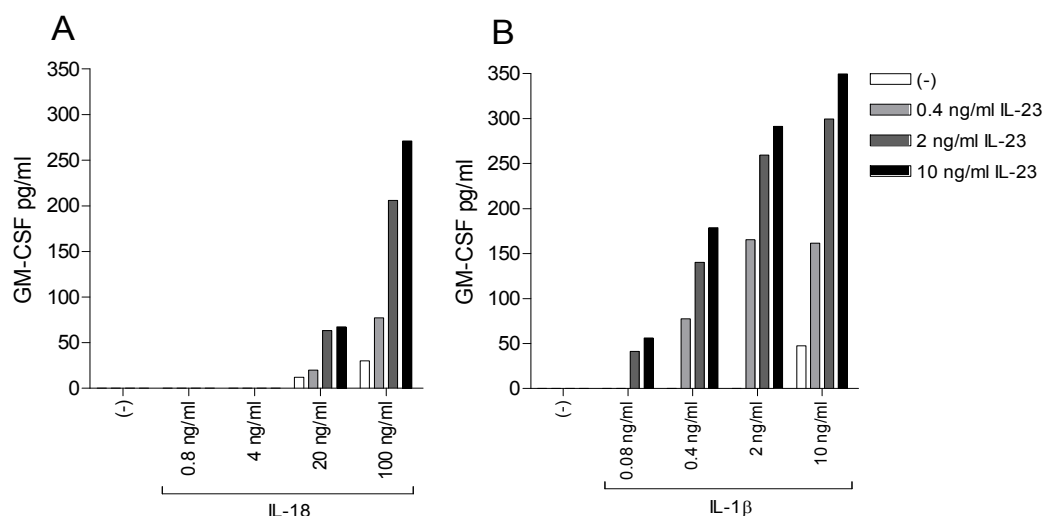


Figure 6. IL-23 in synergy with IL-18 or IL-1 β induces GM-CSF in CD56 $^{+}$ cells. $1 \cdot 10^5$ anti-CD56 beads isolated cells were rested for 24 hours and then left unstimulated or stimulated with indicated concentrations of IL-23 in combination with various concentrations of IL-18 (A) or IL-1 β (B). IL-18 or a combination of these cytokines. Supernatants were collected 24 hours after stimulation and GM-CSF protein production was measured by ELISA. One representative experiment of three is shown.

GM-CSF and IFN- γ prime monocytes for enhanced IL-23 production.

M ϕ 1 generated by culturing CD14 $^{+}$ monocytes for six days in the presence of GM-CSF are strong producers of IL-23 compared with freshly isolated CD14 $^{+}$ monocytes ((5) and above). To investigate whether GM-CSF or IFN- γ (known to enhance the expression of the IL-23 subunit IL-12p40 (21)) could prime monocytes directly for enhanced IL-23 production in response to stimulation with heat killed *Salmonella*, we pre-stimulated CD14 $^{+}$ cells for 16 hours with GM-CSF or IFN- γ and assessed IL-23 production. Stimulation of monocytes with GM-CSF or IFN- γ alone did not induce IL-23 production (Fig. 7A and 7B). However, sixteen hours of pre-stimulation of monocytes with as little as 50 pg/ml GM-CSF enhanced IL-23 production in response to heat killed *Salmonella* (Fig. 7A), with a dose dependent priming effect (Fig. 7A). Similar to GM-CSF, pre-stimulation of monocytes with IFN- γ also enhanced IL-23 production in response to heat killed *Salmonella*, again in a dose dependent manner (Fig. 7B).

Table 1 Percentages of IFN- γ producing CD56⁺ cells after stimulation with cytokines.

Donor	Fraction	Stimulation					
		-			IL-23		
		-	IL-1 β	IL-18	-	IL-1 β	IL-18
1	CD3 ⁻	0,2	0,8	0,2	0,4	3,0	6,7
	CD3 ⁺	0,0	0,1	0,0	0,1	0,2	0,7
2	CD3 ⁻	0,1	0,3	0,2	0,3	1,9	4,4
	CD3 ⁺	0,1	0,1	0,1	0,2	0,6	4,8
3	CD3 ⁻	0,1	0,1	0,0	0,2	1,5	7,5
	CD3 ⁺	0,1	1,2	0,0	0,1	11,6	35,9
4	CD3 ⁻	0,1	0,3	0,0	0,1	3,2	53,4
	CD3 ⁺	0,0	0,4	0,0	0,0	1,2	84,1
5	CD3 ⁻	0,2	6,1	3,9	2,8	9,7	15,8
	CD3 ⁺	0,1	0,6	3,9	0,3	2,3	19,5
6	CD3 ⁻	0,0	0,1	0,1	0,1	1,2	3,4
	CD3 ⁺	0,1	0,0	0,0	0,0	0,5	10,0
7	CD3 ⁻	0,1	0,1	0,2	0,5	2,4	9,0
	CD3 ⁺	0,1	0,2	0,0	0,1	1,0	7,5
8	CD3 ⁻	0,1	0,3	0,0	1,0	4,7	17,8
	CD3 ⁺	0,0	0,0	0,0	0,0	0,1	2,7

Percentages of IFN- γ producing CD56⁺/CD3⁻ NK cells and CD56⁺/CD3⁺ NK-like T cells for each of eight donors after stimulation of CD56⁺ cells with the indicated cytokines for 48h.

IL-23 plus IL-1 β stimulated CD56⁺ cells prime monocytes for IL-12p70 production.

To produce IL-12p70 in response to LPS, IFN- γ is needed as an extra stimulus. CD56⁺ cells produce IFN- γ in response to IL-23 plus IL-1 β , therefore we wanted to know whether supernatants of IL-23/IL-1 β stimulated CD56⁺ cells could prime monocytes for IL-12p70 production in response to LPS. Monocytes were stimulated with LPS, plus or minus supernatants of IL23/IL-1 β stimulated CD56⁺ cells or, as a positive control, IFN- γ . Monocytes stimulated with LPS alone did not produce IL-12p70, whereas cells stimulated with LPS in combination with IFN- γ or supernatants of IL-23 plus IL-1 β stimulated CD56⁺ cells both produced IL-12p70 (Fig. 8A). To assess the role of IFN- γ present in the supernatants of IL-23 plus IL-1 β stimulated CD56⁺ cells in the priming effect on IL-12p70 production by monocytes, we used an IFN- γ neutralizing antibody. Neutralization of IFN- γ greatly diminished the priming effect of these supernatants on LPS induced IL-12p70 production by monocytes (Fig. 8B). Of note, IL-23 plus IL-1 β did not directly prime monocytes to produce IL-12p70 in response to LPS (data not shown).

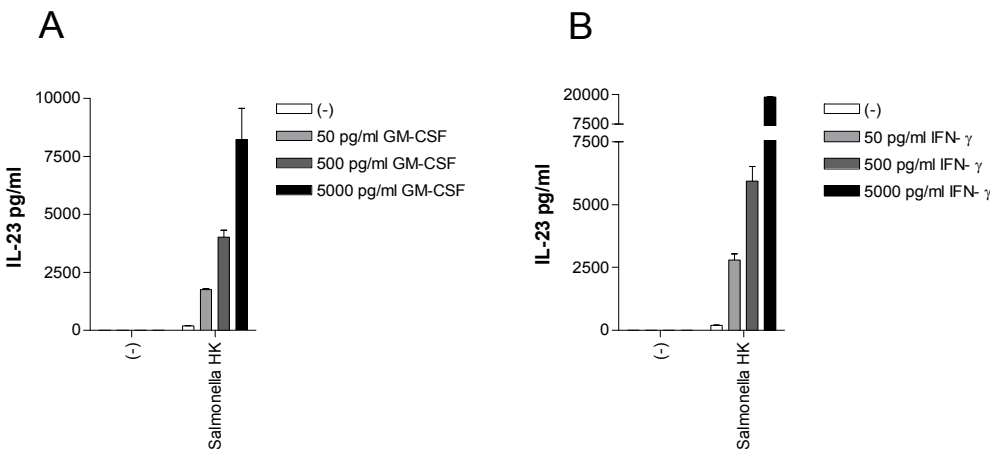


Figure 7. GM-CSF and IFN- γ prime monocytes for enhanced IL-23 production. $1 \cdot 10^5$ CD14 $^{+}$ monocytes were prestimulated with GM-CSF (A) or IFN- γ (B) for 16 hours in concentrations indicated. Subsequently cells were stimulated with heat killed Salmonella (moi=10) or left unstimulated for 24 hours. Supernatants were taken and IL-23 production was measured by ELISA. Data are means $\pm$ SD of triplicates in one representative experiment of five.

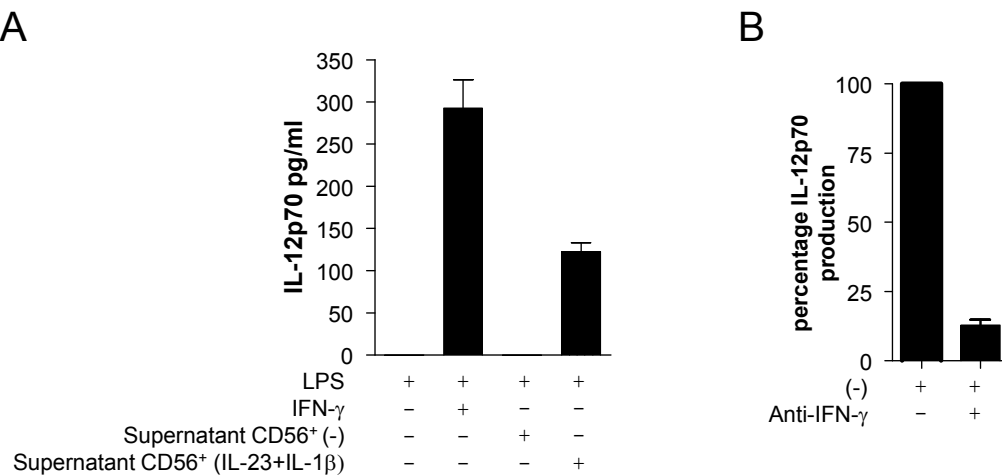


Figure 8. IFN- γ in supernatants of IL-23 plus IL-1 β stimulated CD56 $^{+}$ cells primes monocytes for IL-12p70 production in response to LPS. $1 \cdot 10^5$ CD14 $^{+}$ monocytes were stimulated with 100 ng/ml LPS in combination with 2.5 ng/ml IFN- γ , supernatants of unstimulated CD56 $^{+}$ cells, supernatants of IL-23 plus IL-1 β stimulated CD56 $^{+}$ cells, or medium alone for 24 hours (A). $1 \cdot 10^5$ CD14 $^{+}$ monocytes were stimulated with 100 ng/ml LPS in combination supernatants of IL-23 plus IL-1 β stimulated CD56 $^{+}$ cells, plus or minus 2 μ g/ml anti-IFN- γ for 24 hours (B). Supernatants were taken and IL-12p70 production was measured by ELISA. Data are means $\pm$ SD of triplicates from one representative experiment of three.

Discussion

In this study we demonstrate that human monocytes and cultured pro-inflammatory macrophages (i.e., M ϕ 1) both produce IL-23, IL-18 and IL-1 β in response to various TLR agonists, during co-incubation and upon ingestion of live *Salmonella*. Subsequently, in response to IL-23 in combination with IL-18 or IL-1 β , CD56 $^{+}$ cells produce IFN- γ and GM-CSF. These cytokines in turn enhance IL-23 production by monocytes and macrophages in response to TLR agonists, implying a positive feedback loop. Furthermore, supernatants of *Salmonella* infected M ϕ 1 induce IFN- γ production in CD56 $^{+}$ cells and this induction of IFN- γ was critically dependent on IL-23 and IL-1 β . Finally, supernatants from IL-23 plus IL-1 β stimulated CD56 $^{+}$ cells allowed monocytes to produce IL-12p70 in response to LPS.

The assumption of an IL-23 positive feedback loop is based on the following observations: **(1)** We demonstrated that monocytes as well as M ϕ 1 are able to produce IL-23 in response to various TLR agonists. In addition, monocytes and M ϕ 1 produced small amounts of IL-18 and IL-1 β in response to individual TLR agonists, while cells infected with live *Salmonella* produced large amounts of IL-18, IL-1 β and IL-23. In line, Verreck *et al.* showed that M ϕ 1 produce IL-23 and IL-1 β in response to LPS or mycobacterial sonicate (22). **(2)** Guia *et al.* reported that in the presence of macrophages infected with *Salmonella* in vitro, CD56 $^{+}$ NK-like T cells, but not CD56 $^{-}$ T cells produce IFN- γ in absence of TCR stimulation (23). Here we show that IL-23 and IL-1 β in these supernatants of *Salmonella* infected M ϕ 1 were critical components for the induction of IFN- γ production in the CD56 $^{+}$ NK/NK-like T cells, because neutralization of these interleukins resulted in abrogation or a strong reduction of IFN- γ production, respectively. **(3)** Recombinant IL-18 was able to enhance the induction of IFN- γ in CD56 $^{+}$ in response to supernatants of *Salmonella* infected M ϕ 1. However, neutralization of IL-18 did not significantly reduce the IFN- γ production. These results support that IL-23 and IL-1 β , but not IL-18, in the supernatant of *Salmonella* infected M ϕ 1 are critical for the induction of IFN- γ in CD56 $^{+}$ cells. Moreover, in CD56 $^{+}$ cells stimulated with IL-23 no synergy is seen with IL-18 when concentrations lower than 20 ng/ml IL-18 are used (8), indicating that the IL-18 concentration induced by *Salmonella* (which was ~1-4 ng/ml) may be too low to synergize with IL-23 in inducing IFN- γ . In addition, *Salmonella* infection is known to induce TNF production in macrophages and TNF has been reported to enhance IFN- γ production (18,24). Therefore, in our experiments, TNF in the supernatants of infected M ϕ 1 may also enhance IFN- γ production. However, we did not determine the role of TNF in this respect. **(4)** IFN- γ and GM-CSF both enhanced IL-23 production in monocytes in response to various stimulations. **(5)** Moreover, both cytokines could be induced by IL-23, in combination with IL-18 or IL-1 β , in CD56 $^{+}$ cells. Depending on the concentrations used, IL-23 plus

IL-18 or IL-1 β induced more than 200 pg/ml GM-CSF, a concentration which we have shown to be sufficient to prime CD14⁺ monocytes for enhanced IL-23 production.

Together these observations confirm the presence of a positive feedback loop in which IL-23 can enhance its own expression via the induction of IFN- γ and GM-CSF. In line with our observations, IFN- γ was recently reported to enhance *Francisella tularensis* induced IL-23 in human monocytes (25). IFN- γ allows for the transcription of IL-12p35 in response to LPS (26) and for the induction of IL-12 in response to a PAMP an additional stimulus such as IFN- γ is needed (5). Herein we show that IL-23 together with IL-1 β present in supernatants of *Salmonella* infected M ϕ 1 can provide this costimulus by the induction of IFN- γ in NK-like T cells, thereby allowing subsequent IL-12p70 production in response to *Salmonella* and the initiation of a Th1 response. In contrast, in human DCs IFN- γ can inhibit IL-23p19 mRNA transcription (27), emphasizing the complexity of the regulation of this cytokine.

In this study we report on a positive feedback of IL-23 production, but negative feedback mechanisms may exist as well. For instance, IL-4 and IL-10 are both reported to inhibit the induction of IL-23 and may serve as a negative feedback (28). IL-4 may be important in balancing IL-23 and IL-12 production as IL-4 can enhance IL-12p70 production (29-31). It would be interesting to explore the effects of these and other potential negative regulators of IL-23.

To reach the conclusion on a positive feedback loop in which IL-23 enhances its own expression via the induction of IFN- γ and GM-CSF, the following pitfalls of this study need to be considered. Firstly, in supernatants of M ϕ 1 infected with live *Salmonella*, we neutralized IL-23 using an antibody which is able to neutralize IL-12p70 as well. Though we did not detect IL-12p70 and IL-12p35 mRNA expression was not induced by infection with *Salmonella*, one should bear in mind that the detection limit of the IL-12p70 ELISA used was 3 pg/ml and that we can not exclude that the effect observed after neutralizing the IL-23 is due to the neutralization of small, undetectable amounts of IL-12p70. Secondly, the concentrations of the TLR agonists used may exceed physiological relevant conditions. For example, the concentrations of *Salmonella* LPS added to the cells used in this study are likely to differ from LPS concentration when M ϕ 1 are being infected with live *Salmonella*. During sepsis 5-10 pg/ml LPS can be detected in the blood (32). Thirdly, between donors we observed interindividual differences in cytokine production in response to the stimulations used. This may reflect differences in responsiveness to stimulation and differences in capacity to produce cytokines between cells obtained from different donors. We observed for example remarkable interindividual differences in the subset of IFN- γ producing CD56⁺ cells in response to IL-23 in combination with IL-1 β or IL-18.

In addition to the elucidation of the positive feed-back loop of IL-23 expression we observed a synergy between LPS (TLR4) and flagellin (TLR5) and between LPS and CL075 (TLR8/7) with respect to the induction of IL-23 production in monocytes and M ϕ 1. During an infection, synergy

between TLRs is likely of importance because pathogens express multiple TLR agonists. LPS and flagellin for instance, which in synergy induce large quantities of IL-23 in M ϕ 1, are both expressed by *Salmonella*. Synergy between LPS (TLR4) and R848, another TLR8/7 agonist, has been observed in the induction of IL-12 and IL-23 in human DCs (33). Both TLR8 and TLR4 are implicated in the resistance against *M. tuberculosis* (34,35), suggesting the synergy we observed between the TLR4 and the TLR8/7 agonists may be relevant in mycobacterial infections.

In conclusion, we have shown that various TLR agonists and infection with *Salmonella* can induce IL-23, IL-18 and IL-1 β , but not IL-12, production in monocytes and M ϕ 1. Furthermore, our findings indicate that a positive feedback loop exists in which IL-23 can enhance its own production via the induction of IFN- γ and GM-CSF, which both prime monocytes for enhanced IL-23 production. Last, IL-23, in combination with IL-1 β , could prime monocytes to produce IL-12p70 in response to LPS, via the activation of CD56⁺ cells, thereby amplifying the type-1 cytokine pathway to IFN- γ production.

IL-23 plus IL1 β induced IFN- γ production in CD56⁺ cells, which consist of CD56⁺/CD3⁻ NK cells and CD56⁺CD3⁺ NK-like T cells. In half of the donors IFN- γ production was observed in both NK cells and NK-like T cells, whereas in the other half IFN- γ production was observed only in the NK cells. Possible explanations for these interindividual differences may be previous (recent) exposure to different pathogens or genetic differences between donors; these may be elucidated when individual donors are assayed at regular intervals over a longer time period to determine whether or not the individual cytokine production profiles are constant.

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Chapter 3

IL-23 and IL-12 responses in activated human T cells retrovirally transduced with IL-23 receptor variants

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Abstract

Interleukin-23 (IL-23) is a regulator of cellular immune responses involved in controlling infections and autoimmune diseases. Effects of IL-23 on T cells are mediated via a receptor complex consisting of an IL-12R β 1 and a specific IL-23R chain. The R381Q and P310L variants of the IL-23R were recently reported to be associated with autoimmune diseases, suggesting they have an effect on IL-23R function. To investigate this matter, these variants and a newly identified variant, Y173H, were retrovirally transduced into human T cell blasts and functionally characterized by measuring the IL-23-induced signal transduction pathway (i.e., STAT1, STAT3 and STAT4 phosphorylation), and IFN- γ and IL-10 production. No differences were detected between the genetic variants and wild-type in the function of the IL-23R-chain. Furthermore, while comparing IFN- γ and IL-10 production in response to IL-23 and IL-12, we found IL-23 to be a more potent IL-10 inducer, and IL-12 a more potent IFN- γ inducer. In addition, IL-23 also exerted a minor IL-12-like effect by inducing IL-23R-independent, IL-12R β 1-dependent STAT4 phosphorylation and IFN- γ production. In conclusion, the reported association between R381Q and P310L variants of the IL-23R and autoimmune diseases does not depend on differences in functional activity between wild-type and R381Q and P310L variants of the IL-23R.

Introduction

Interleukin-23 (IL-23) is a member of the IL-12 family of cytokines which plays an essential role in the cellular immune response. IL-12 directs Th1 polarization and induces IFN- γ release by CD4⁺ T-cells in concert with IL-27 or IFN- α (1,2). IL-23 plays a role in the maintenance of immune responses by controlling T cell memory function (3) and by influencing the proliferation and survival of IL-17-producing Th17 cells (4). Furthermore, IL-23 can shape Th1-immunity via CD3⁺CD56⁺ T cells, through the production of IFN- γ early in the immune response (Van de Wetering, manuscript in preparation).

IL-23 and IL-12 are heterogenic cytokines composed of a shared IL-12p40 subunit bound to an IL-23p19 or IL-12p35 subunit, respectively. IL-23 and IL-12 signal through a common IL-12R β 1 chain complemented by the IL-23R and the IL-12R β 2 (5). IL-12R β 1 is expressed on lymphocytes and can be upregulated via activation and costimulation of the T-cell and by the cytokines IL-2, IL-7 and IL-15 (6). The IL-12R β 2 is only expressed on CD4⁺ T cells after activation (7), whereas the membrane expression patterns of the IL-23R chain are still undefined. IL-23R transcripts are however found in bone marrow and in various T-cell subsets (8).

The IL-12 and IL-23 receptor complexes signal via JAK2 and STAT modules to regulate gene expression (8). IL-12 activates STAT4 thereby inducing IFN- γ (9) and IL-10 production in various T-cell subsets (10,11). IL-23 activates STAT1, STAT3, STAT4 and STAT5 and can induce IFN- γ , IL-10 and IL-17 depending on the celltype (8,12).

IL-23 is important in controlling mucosal host defenses (13,14) and is involved in autoimmune diseases such as inflammatory bowel diseases (IBD) (15), psoriasis (16) and rheumatoid arthritis (17). Patients with Mendelian susceptibility to mycobacterial disease (MSMD) due to IL-12R β 1 or IL-12p40 deficiency lack both IL-12 and IL-23 mediated signaling, have impaired Th1 immunity and suffer from severe recurrent infections with poorly virulent *Salmonella* or *Mycobacterium* species (18).

Polymorphisms in the IL-23R chain may influence IL-23 responses. The polymorphism P310L occurs at a frequency of 2-30 % and the R381Q polymorphism at a frequency of 0-17 % depending on the population. The R381Q allele confers protection against IBD (19), psoriasis (20), ankylosing spondylitis (21), and graft versus host disease after bone marrow transplantation (22). The P310L allelic variant was overrepresented in patients with Grave's Disease (23). In view of these associations with diseases, it was suggested that the R381Q and P310L variants of the IL-23R may be functionally different. To investigate this matter we functionally characterized the IL-23R allelic variants P310L, R381Q and Y173H (a newly identified allele), as well as an IL-23R lacking the intracellular domain.

We cloned the IL-23R variants into a retroviral expression vector and transduced them into T cell blasts (TCB). IL-23 and IL-12 responsiveness in signal transduction and cytokine production by the TCB were compared.

Materials and Methods

Cloning IL-23R variants into a retroviral expression vector

Full-length *IL23R* coding sequence was PCR amplified using cDNA from a healthy control. The PCR product of the wild-type allele (wtIL23R) was first cloned into pGEM-T-Easy (Promega), variations were introduced by site directed mutagenesis (24). Three constructs were made with the variations, P310L, Y173H and R381Q. One construct designated as Δ 23R was made by introducing an early stop codon (at aa 400) and an Y397F mutation. The *IL23R* constructs were released from the pGEM-T-easy vector by digestion with *NofI* and ligated into pLZRS-IRES-GFP (25) or into pLZRS-IRES- Δ NGFR (26). As negative controls vectors without an *IL23R* insert were used. All constructs were verified by sequencing. Helper-free recombinant retrovirus was produced after introducing the constructs into a 293T-based amphotropic retroviral packaging cell line, Phoenix (27), using a calcium-phosphate transfection kit (Invitrogen). The virus producing cells were cultured for 2–3 weeks in 175 cm² cell culture flasks (Greiner Bio-One) under 2 μ g/ml puromycin (Clontech) selection after which a 20 h supernatant was harvested.

Cells, culture conditions and retroviral transduction

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors by Ficoll separation and cultured in IMDM supplemented with 20 mM GlutaMAX, 10% FCS, 100 U/ml Penicillin, 100 μ g/ml Streptomycin (Invitrogen) supplemented with 30 U/ml IL-2 (Proleucin, Chiron). T-cell blasts (TCB) were generated by stimulating PBMCs with 800 ng/ml phytohemagglutinin (PHA) (Murex). On day 2, after PHA-stimulation, 0.5 x10⁶ TCB were transduced on a CH-296 coated (RetroNectinTM, Takara Shuzo) 48-wells plate using 0.5 ml of virus containing supernatants as described previously (28). On day 10, cells were sorted on GFP or NGFR signal by fluorescence activated cell sorting (FACS) and restimulated in the presence of 800 ng/ml PHA and irradiated allogeneic PBMCs (pool from two donors) and irradiated B-LCL in a cellular ratio of 2:10:1 respectively. After each restimulation, cells were allowed to expand for at least 14 days. The cells were washed three times before each functional assay. NK-92 cells (29) were cultured in IMDM supplemented with 20 mM GlutaMAX, 10% FCS, 10% Horse serum (Gibco), 100 U/ml Penicillin, 100 μ g/ml Streptomycin (Invitrogen) supplemented with 100 U/ml IL-2 and 50 μ M 2-mercaptoethanol (Merck) and transduced as described above.

Co-transduction of IL-12R β 1 and IL-23R into IL-12R β 1^{-/-} TCB

TCB of a patient (TCBp1) with a null mutation in IL-12R β 1 were transduced as described above with one or two retroviral vectors. Namely the pLZRS-IRES-GFP vector in which one of the IL-12R β 1 variants (QMG or RTR) was cloned (30) and the pLZRS-IRES- Δ NGFR vector in which the wild-type IL23R (wtIL23R) or the P310L variant was cloned. As controls TCBp1 cells were transduced with empty vectors (GFP and Δ NGFR). Cells were selected for comparable GFP and NGF-R expression by FACS sorting after staining the cells with a PE conjugated antibody against the NGF-R (BD biosciences).

FACS analysis

To detect IL-23R expression two commercial IL-23R antibodies, the biotinylated BAF1400 polyclonal and the PE conjugated FAB14001P monoclonal antibody (R&D Systems) and two IL-23R antibodies raised in-house in rabbits against peptides (FLB2, aa 343-352 and FLJ2, aa 62-75) were used. Cells were blocked with 10% normal mouse serum or normal goat serum in PBS, 0.2% BSA (Fraction V, Sigma) and washed before labeling with antibody. The cells were washed three times and where necessary counterstained with streptavidine-PE (BD) or goat-anti-rabbit-PE (SouthernBiotech), washed again and analyzed on a FACScalibur (BD biosciences).

Cytokine production analysis and proliferation assay

1×10^5 TCB were cultured in 200 μ l of culture medium in a 96 wells format. The cells were stimulated with or without 2 μ g/ml anti-CD2 (CLB-T11.1/1 and .2/1, Sanquin) and 2 μ g/ml anti-CD28 (CLB-CD28/1, Sanquin) in the presence of various amounts of IL-23 or IL-12 (R&D). After two days 150 μ l supernatant of each well was removed. The concentrations of IFN- γ , IL-10, TNF and IL-17 were determined by cytokine-specific ELISAs (Biosource). To the remaining cells 25 μ l of RPMI-medium (Invitrogen) containing 0.5 μ Ci ^{3} H-thymidine (PerkinElmer) was added. After 8 hours of incubation the cells were harvested and incorporated ^{3} H was determined using a liquid scintillation counter (Wallac). Results were calculated as a stimulation index (ratio mean cpm of the test sample/mean cpm of the medium).

STAT phosphorylation assays

To study signal transduction 10^5 TCB were pre-stimulated overnight in 150 μ l culture medium with 2 μ g/ml anti-CD2 and 2 μ g/ml anti-CD28. Thereafter the cells were pulsed with 10 ng/ml IL-23, 1 ng/ml IL-12, 6.8 ng/ml IL-12p40 (Peprotech) or 6.8 ng/ml IL-12p80 (Peprotech). For use in blocking experiments IL-23 and IL-12 were preincubated for 30 minutes with 5 μ g/ml anti-IL-23p19 (R&D). The cells were fixed with paraformaldehyde and permeabilized with methanol. Then the cells were washed with PBS, 0.2% BSA, blocked with normal goat serum, and stained with the phospho-

specific antibodies pY701-STAT1-alexa 647, pY705-STAT3- PE, pY693-STAT4-alexa 647 or pY694-STAT5-PE (BD Pharmingen). Before analyzing by FACS the cells were washed twice.

Results

Retroviral transduction of IL-23R alleles results in functional IL-23 receptor complexes

We used a retroviral expression system to study the impact of various IL-23R polymorphisms on the function of the IL-23 receptor in normal control PHA stimulated TCB that express IL-12R β 1. The retroviral expression vector, pLZRS, ensures transcription and expression of the *IL23R* gene and green fluorescent protein (*GFP*) genes in tandem and allows for selection of transduced cells by FACS for the GFP signal. Transduction efficiency was typical between 5 and 20%; after FACS sorting 96-99% (98% average) of the cells were GFP positive.

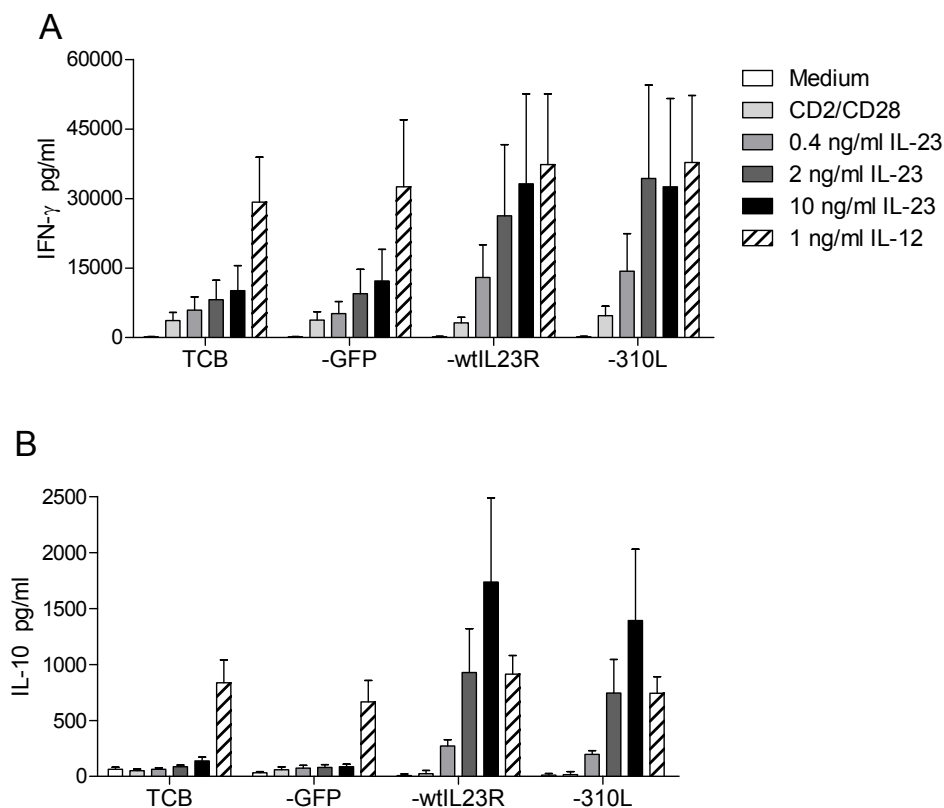


Figure 1. IL-23 induces IFN- γ and IL-10 production via retrovirally expressed IL-23R. Normal TCB, GFP transduced TCB and TCB transduced with the wtIL23R or the 310L variant were tested for their IFN- γ (A) and the IL-10 (B) production in response to anti-CD2/anti-CD28 with or without various amounts of IL-23 or as a control to 1 ng/ml IL-12. The graph represents the average data of four donors, one donor was tested twice, the error bars indicate the standard deviation.

The IL-23 responses of sorted TCB cultures transduced with the wild-type and the P310L IL-23R variant (TCB-wtIL23R and TCB-310L) were compared with GFP transduced (TCB-GFP) and untransduced (TCB) cultures. In the absence of anti-CD2 and anti-CD28 neither IL-23 nor IL-12 induced any effect (data not shown). Therefore all subsequent cultures were stimulated with cytokines in the presence of anti-CD2 and anti-CD28. IL-12 induced IFN- γ and IL-10 production in all cultures, indicating that IL-12R β 1 and IL-12R β 2 are present on all cells (Fig. 1A and 1B). In response to IL-23 stimulation TCB-wtIL23R and TCB-310L produced large amounts of IFN- γ (Fig. 1A) and IL-10 (Fig. 1B). TCB and TCB-GFP produced a small amount of IFN- γ (Fig. 1A) but no IL-10 (Fig. 1B) in response to increasing doses of IL-23. Tumor necrosis factor (TNF) or IL-17 production was not detectable in any of the cultures (data not shown).

Detection of IL-23R expression on the cell membrane

To detect IL-23R expression on the membrane FACS analysis was performed with two commercially available IL-23R antibodies (BAF1400 and FAB14001P; R&D systems) as well as two antibodies (FLB2 and FLJ2) raised in-house in rabbits against two IL-23R peptides. The FLB2 and FLJ2 antibodies specifically detect the immunizing peptides in an ELISA, in dilutions up to a ten-thousand fold (data not shown). TCB, TCB-GFP and TCB-IL23R cells were stained with these four IL-23R antibodies and identical staining patterns were detected (data not shown) even though we have shown in the paragraph above that a functional IL-23R is only present on the TCB-IL23R cells. Similar results were obtained with IL-23 responsive, NK-92 cells transduced with the IL-23R construct and with IL-23 unresponsive, untransduced NK-92 cells (Van de Wetering, unpublished data). The antibody BAF1400 was recently used in a study to select a subset of CD45RO⁺ T cells that were however not analyzed for IL-23 responsiveness (31). We were able to stain the same subset of human CD45RO⁺ T cells with the BAF1400 antibody (data not shown). Moreover, no staining with the BAF1400 was observed of IL-23 responsive primary CD3⁺CD56⁺ T cells from various donors (Van de Wetering, unpublished data).

Kinetics of IL-23 induced STAT phosphorylation

It was previously reported that IL-23 induces STAT1, STAT3, STAT4 and STAT5 phosphorylation (8). We have shown previously that IL-23 induces phosphorylation of STAT3 and STAT4 but not STAT1 or STAT5 in human primary CD3⁺CD56⁺ T cells (32). We also found that phosphorylation of STAT1, STAT3 and STAT4 but not STAT5 in an IL-23R transduced NK-92 cell line (unpublished data). To assay IL-23 induced STAT phosphorylation kinetics in T cells, TCB containing IL-23R constructs (TCB-wtIL23R, TCB-310L) and TCB-GFP were pre-stimulated with anti-CD2 and anti-CD28 and subsequently stimulated for 5 to 60 minutes with IL-23 or with IL-12. STAT1, STAT3, STAT4 and STAT5 phosphorylations were determined by intracellular FACS. IL-23 induced STAT1,

STAT3 and STAT4 phosphorylation in TCB-wtIL23R and TCB-310L (Fig. 2A-C). STAT1 phosphorylation diminished after 15 minutes, whereas STAT3 and STAT4 phosphorylation persisted. IL-12 induced STAT4 but no STAT1 or STAT3 phosphorylation in all cultures examined (data not shown). TCB-GFP cultures stimulated with 10 ng/ml IL-23 showed a small amount of STAT4 (Fig. 2C), but no STAT1 or STAT3 phosphorylation (Fig. 2A and 2B). STAT5 was strongly phosphorylated in cells pre-stimulated with anti-CD2 and anti-CD28, upon stimulation with IL-23 or IL-12 no additional phosphorylation could be detected (data not shown).

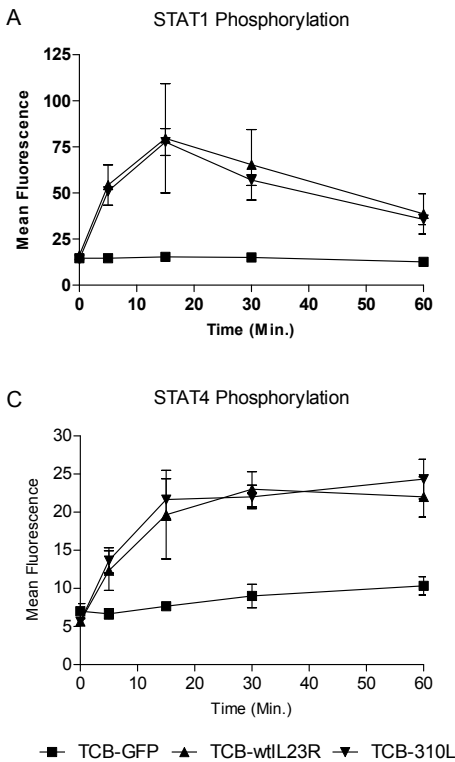


Figure 2. Kinetics of IL-23 induced STAT phosphorylation. TCB transduced with the wild-type (wtIL23R), the 310L variant or GFP alone were stimulated with 10 ng/ml IL-23 for 0, 5, 15, 30 and 60 minutes. The amount of STAT1 (A), STAT3 (B) and STAT4 (C) phosphorylation was determined by FACS using phospho-specific antibodies. The graph displays the mean fluorescence signal of three different donors, the error bars indicate the standard deviation.

IL-23 also exhibits a minor IL-12-like effect independent of IL-23R expression

We observed that IL-23 induced in normal, untransduced TCB some STAT4 but no STAT1 and STAT3 phosphorylation, which resulted in the production of small amounts of IFN- γ . This effect may be induced by IL-23 itself or by potential IL-23-byproducts such as IL-12p40 monomers or IL-12p40 dimers (IL-12p80). Therefore we determined whether a specific antibody against the IL-23p19 subunit could block the STAT4 phosphorylation and whether IL-12p40 or IL-12p80 alone could induce STAT4 phosphorylation. In normal, untransduced TCB prestimulated with anti-CD2 and anti-CD28 an anti-IL23p19 antibody was able to block 82% of the IL-23 induced STAT4 phosphorylation,

while it did not block IL-12 induced STAT4 phosphorylation (Fig. 3). IL-12p40 or IL-12p80 did not induce STAT4 phosphorylation (Fig. 3).

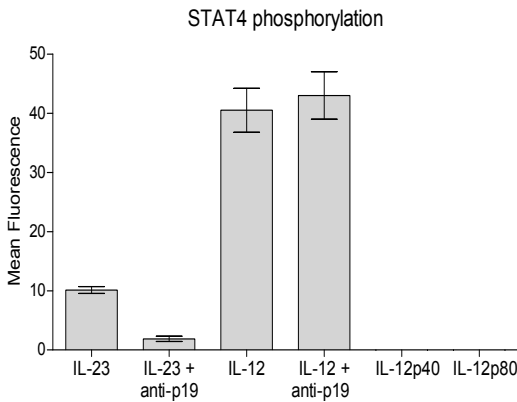


Figure 3. IL-23 but not IL-12p40 monomers or homodimers induce IL-12-like IL-23R independent STAT4 phosphorylation. Normal TCB were stimulated in the presence of anti-CD2 and anti-CD28 with 10 ng/ml IL-23, 1 ng/ml IL-12, 6.8 ng/ml IL-12p40, IL-12p80, or with IL-23 or IL-12 preincubated for 30 minutes with anti-IL-23p19. STAT4 phosphorylation was determined by FACS using a phospho-specific antibody. Displayed are the mean minus the mean of the anti-CD2 / anti-CD28 stimulation and the standard deviations of eight measurements from two donors.

Effect of variations in the IL-23R on IL-23 induced IFN- γ and IL-10 production

Variations in the IL-23R may influence IL-23 mediated responses. To investigate this we transduced TCB cultures with two IL-23R variants (TCB-173H and TCB-381Q). These two variants were compared with TCB-wtIL23R and with TCB transduced with a truncated form of the receptor (TCB- Δ 23R). TCB and TCB-GFP cultures were used as controls. IFN- γ and IL-10 production were determined after two days of culture in the presence of anti-CD2/anti-CD28 with or without IL-23 or IL-12 (Fig. 4).

No major differences in the IL-23 induced IFN- γ production by TCB-wtIL23R, TCB-173H and TCB-381Q cultures were detected. Although it appeared that IFN- γ production in response to IL-23 stimulation of TCB-173H and TCB-381Q, as compared to the TCB-wtIL23R, reached a plateau at lower IL-23 concentrations (Fig. 4A), these differences were not significant. IL-23 induced low amounts of IFN- γ production in the TCB- Δ 23R culture, comparable with other cells lacking the IL-23R: the control TCB and the TCB-GFP cultures (Fig. 4A). Comparable large amounts of IL-10 were induced by IL-23 in TCB-wtIL23R, TCB-173H and TCB-381Q (Fig. 4B).

IL-23 enhances IL-10 production relatively more than IFN- γ production

We demonstrated that IL-23, similar to IL-12, is a potent inducer of both IFN- γ and IL-10. To compare the induction capacity of IL-23 to the induction capacity of IL-12 the ratios of IL-10 and IFN- γ production were calculated (Fig. 5). All TCB cultures stimulated with anti-CD2 and anti-CD28 produced around twenty times less IL-10 than IFN- γ (ratio ~ 0.05), a similar ratio was observed

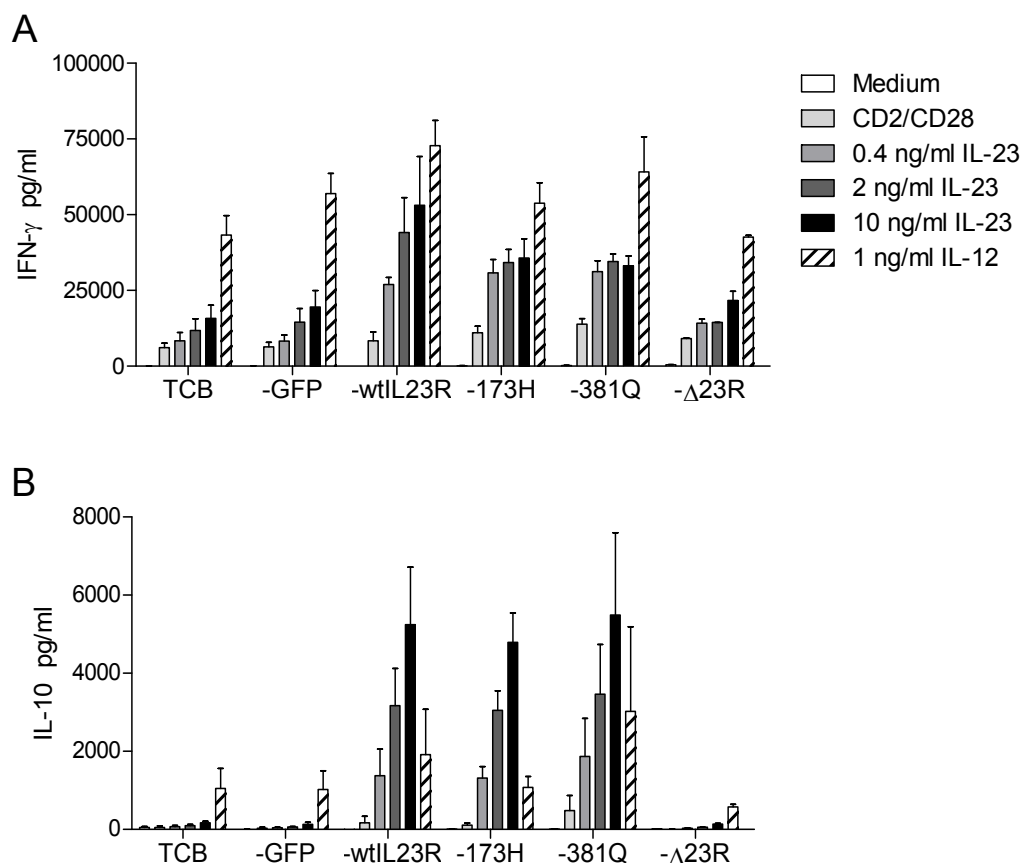


Figure 4. The IL-23R variants do not differ in IL-23 induced cytokine production. TCB transduced with the constructs of the wtIL23R, the 173H- and the 381Q-variants were compared with normal TCB, TCB-GFP and TCB-Δ23R. The cell-lines were tested for IFN- γ (A) and IL-10 (B) production in response to various amounts of IL-23 or as a control to 1 ng/ml IL-12 in the presence of CD2/CD28. Displayed are the means and the standard deviation of two experiments with three donors each

in response to IL-12. Addition of IL-23 to TCB lacking a functional IL-23R (TCB, TCB-GFP and TCB-Δ23R) had no effect on the ratio, whereas addition of IL-23 to TCB with a functional IL-23R (TCB-wtIL23R, TCB-310L, TCB-173H and TCB-381Q) increased the IL-10/ IFN- γ ratio by a factor five. No major differences were observed between the IL-23R alleles (Fig. 5).

Effect of variations in the IL-23R on signal transduction

Although no difference was detected in IFN- γ or IL-10 production, a difference may exist in the production of an as yet unknown factor. To analyze putative effects of IL-23R variants we assayed STAT phosphorylation in the TCB cultures transduced with the variants R381Q and Y173H together

with the controls (TCB-wtIL23R, TCB, TCB-GFP and TCB- Δ 23R). Cells were pre-stimulated with anti-CD2 and anti-CD28 followed by a short incubation with or without IL-23. STAT1, STAT3 and STAT4 phosphorylation were analyzed by FACS using phospho-specific antibodies. STAT 1 and STAT3 phosphorylation was induced by IL-23 in all TCB cells transduced with an IL-23R variant (Fig. 6A and 6B). Truncation of the intracellular domain of the IL-23R completely abolished STAT1 and STAT3 phosphorylation (data not shown). IL-23 induced STAT4 phosphorylation was high in all the cells transduced with IL-23R variants (Fig. 6C). As observed before, IL-23 also induced a low amount of STAT4 phosphorylation due to the IL-12-like effect of IL-23 in cells lacking a functional IL-23R (control TCB, TCB-GFP) (Fig. 6C).

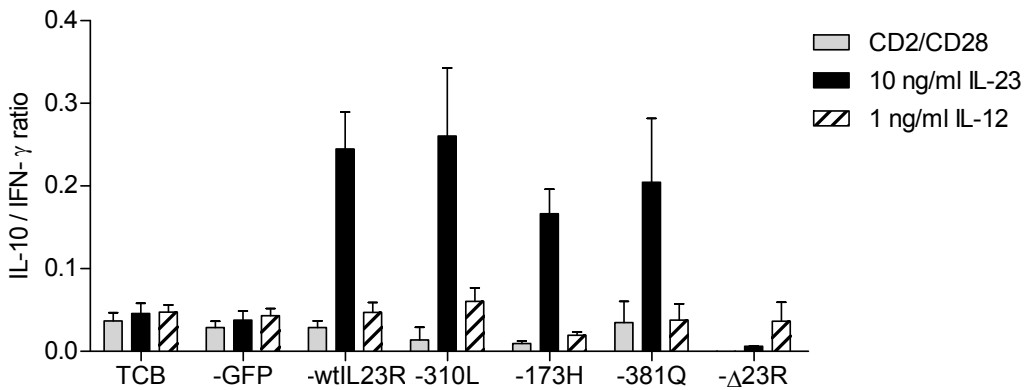


Figure 5. Influence of IL-23R expression on the IL-10/ IFN- γ ratio after IL-23 and IL-12 stimulation. The IL-10/ IFN- γ ratio was determined for each stimulation of the TCB cultures with anti-CD2/anti-CD28 alone or together with 10 ng/ml IL-23 or 1 ng/ml IL-12. Displayed are the means and standard deviations of TCB (n=13), -GFP (n=13), -wtIL23R (n=13), -310L (n=6), -173H (n=6), -381Q (n=5) and - Δ 23R (n=3).

Effect of IL-23R variants on IL-23 induced proliferation

IL-23 has been reported to affect proliferation and survival of T cells (4). We tested the IL-23 and IL-12 induced proliferation using a tritium thymidine incorporation assay of TCB, TCB-GFP and TCB transduced with one of the natural variants of the IL-23R. IL-12 had no effect on the proliferation. TCB and TCB-GFP had no increased tritium incorporation upon IL-23 stimulation (data not shown). Cells carrying an IL-23R variant incorporated 1.2 to 1.5 times more tritium upon IL-23 and anti-CD2/anti-CD28 stimulation compared to anti-CD2/anti-CD28 stimulation alone (data not shown). No significant differences were observed between the proliferations induced via the IL-23R variants.

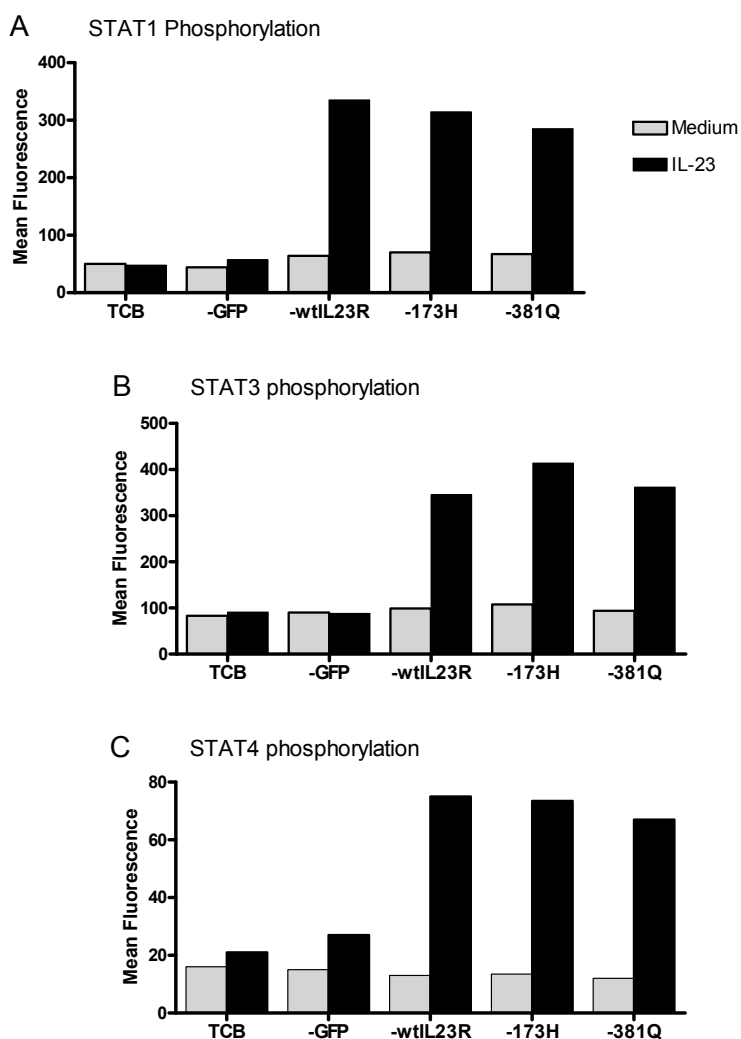


Figure 6. The IL-23R variants do not differ in IL-23 induced STAT phosphorylation. Normal TCB, TCB transduced with GFP and TCB transduced with the various IL-23R constructs (wtIL23R, 173H and 381Q) were stimulated with 10 ng/ml IL-23 for 15 (STAT1) or 30 minutes (STAT3 and STAT4). The amount of STAT1 (A), STAT3 (B) and STAT4 (C) phosphorylation was determined by FACS using phospho-specific antibodies. Displayed is the mean fluorescence signal of one representative of three experiments.

Combined effects of IL-12R β 1 and IL-23R variations on IL-23 driven responses

The IL-23 receptor does not only consist of the IL-23R chain but also of the IL-12R β 1 chain, therefore common IL-12R β 1 haplotypes (QMG and RTR) may differentially influence the IL-23 response. We designed a model system using TCB from a patient (TCBp1) carrying an IL-12R β 1

null mutation. TCBp1 cells were transduced with a retroviral vector expressing an IL-12R β 1 allele in tandem with GFP, or transduced with a retroviral vector expressing an IL-23R allele in tandem with a truncated NGF-R as marker, or co-transduced with both. We selected the cells for expression of the markers and examined the cytokine release in response to IL-12 and IL-23. TCBp1 transduced with the IL-12R β 1 alleles QMG or RTR produced IFN- γ (Fig. 7A) and IL-10 (Fig. 7B) in response to IL-12. The IL-12 induced IL-10 production was reduced for the QMG allele when the IL-23R was co-expressed. IL-23 induced low amounts of IFN- γ , but relatively more IL-10 in cultures of TCBp1 co-transduced with both receptor subunits. No major differences were detected in the IL-23 responses between the TCBp1 carrying various combinations of IL-12R β 1 and IL-23R chains.

As observed before, IL-23 induced IFN- γ (Fig. 7A) and small amounts of IL-10 (Fig. 7B) in the IL-12R β 1 expressing cultures (TCBp1-QMG, TCBp1-RTR) but not in the TCBp1, TCBp1-wtIL23R or TCBp1-310L cultures. This IL-12-like effect of IL-23 on the IFN- γ production by TCBp1-QMG and TCBp1-RTR was relatively high as compared with the effect of IL-23 on the TCBp1 expressing both receptor chains, probably due to overexpression of the IL-12R β 1 on these cells.

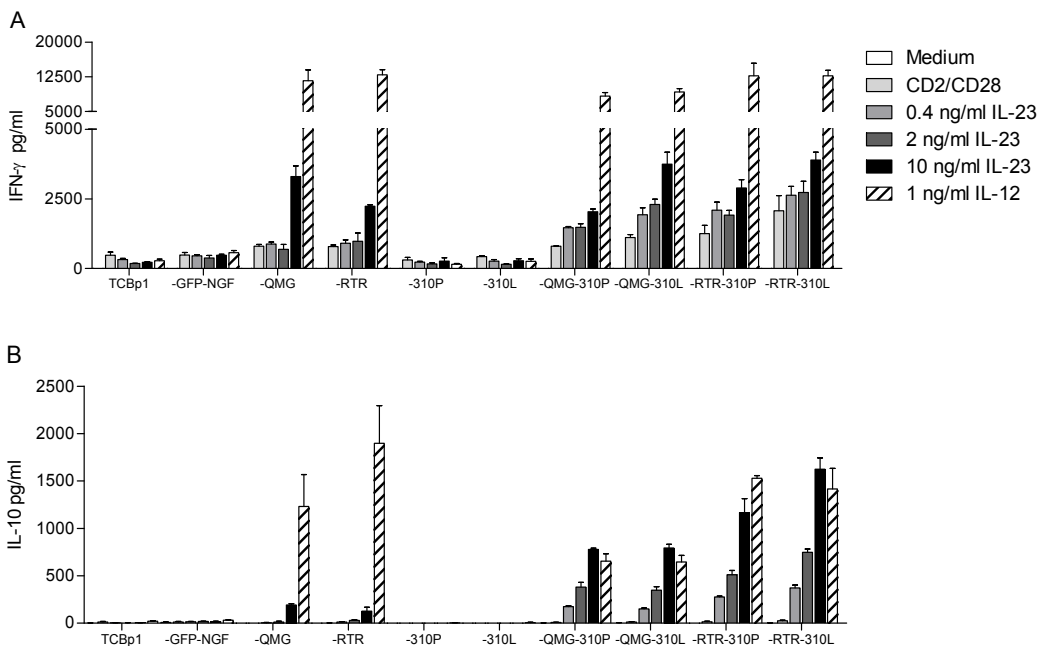


Figure 7. Influence of common IL-12R β 1 and IL-23R polymorphisms on the IL-23 response. The IL-23 responsiveness was tested on TCBp1-QMG-wtIL23R, TCBp1-QMG-310L, TCBp1-RTR-wtIL23R and TCBp1-RTR-310L and compared with the responsiveness of TCBp1 and controls (TCBp1-GFP-NGFR, -QMG, -RTR, -wtIL23R and -310L). The cells were stimulated with or without various concentrations of IL-23 or IL-12 in the presence of anti-CD2/anti-CD28. IFN- γ (A) and IL-10 (B) production were measured by ELISA. Displayed are the means and the standard deviations of a triplicate experiment. One representative out of three experiments is shown.

Discussion

The main finding of the present study is that the natural variants of the human IL-23R, P310L, Y173H and R381Q do not differ in receptor transfer function from the wild-type allele. This conclusion is based on analysis of IL-23R downstream intracellular signaling pathways (STAT1, STAT3 and STAT4 phosphorylation) following ligand binding, as well as the IFN- γ and IL-10 production of human T cells retrovirally transduced with the IL-23R variants. In recent population studies, two of the IL-23R variants were linked to increased incidence of autoimmune disease. The present findings show that such an association cannot readily be explained by differences in the function of the IL-23R variants after binding of their natural ligand. Moreover, we found that IL-23 stimulation results in a ratio of IFN- γ -to-IL-10 production that is distinct from that induced by IL-12. Finally, IL-23 exhibited a minor IL-12-like effect by inducing STAT4 phosphorylation dependent on IL-12R β 1 but independent of IL-23R expression.

To reach the conclusion that the IL-23R variants do not differ in receptor function, we retrovirally expressed the IL-23R in human T-cells and analyzed its functional activity. In this respect, the following points should be considered. First, although there is consensus in the literature that T-cells are the relevant effector cells of IL-23 mediated signaling in humans and thus provide a relevant model to study these effects, the TCB used in this study may not fully resemble the T-cell subsets that are normally IL-23 responsive, even though TCB appear to have all the factors to enable STAT signaling and IFN- γ production up to biological active concentrations (33). Second, the concentrations of stimuli chosen may not resemble physiological relevant conditions. The present approach cannot exclude small subtle differences in the lowest range of ligand binding. However we failed to detect differences within the range of IL-23 stimulation (0.4 to 10 ng/ml) which resulted in reproducible cytokine production. Third, the STAT1, STAT3 and STAT4 phosphorylation and subsequent IFN- γ and IL-10 production may not be the only important responses. However, both cytokines are important mediators in controlling autoimmunity (34) and other read-out functions of IL-23R ligand binding are presently unknown. Fourth, the overexpression of the IL-23R by the retroviral system could mask differences in effects due to alterations in transcript or protein stability. Furthermore, post-transcriptional and post-translational modifications of normal or retroviral expressed IL-23R may differ, but thus far no findings suggest that such modifications occur.

We show that the three variants P310L, Y173H and R381Q, were fully functional and not different in receptor function from the wild-type form. Several associations of the R381Q and P310L alleles with immune related diseases have been identified. Identifying associations between a specific allele and protection from a disease does not necessarily mean that the allele itself conveys a functional difference. The functional difference may be due to a variation that is merely linked to the single nucleotide polymorphism (SNP) under study. Indeed, several other SNPs in the IL-23R

besides the R381Q polymorphism also correlated with decreased susceptibility for IBD (35) and psoriasis (20). The SNP designated rs11465804 for instance is strongly linked to the R381Q polymorphism (20). These or other SNPs in the IL-23R may be responsible for the observed associations with immune related diseases. We can however not exclude functional differences of the studied alleles due to an effect of these variants on the level of expression of the receptor, since we used overexpression constructs of the IL-23R.

We demonstrated that IL-23 via its receptor induced STAT1, STAT3 and STAT4 phosphorylation, but not STAT5 phosphorylation whereas IL-12 could only induce STAT4 phosphorylation. Both cytokines can induce IFN- γ and IL-10, although IL-23 is a more potent inducer of IL-10 in this system. In this way, IL-23 and IL-12 have a different impact on the balance of pro- and anti-inflammatory immune responses. Furthermore IL-23 enhanced the proliferation of IL-23R transduced human T-cells slightly, whereas IL-12 did not.

We discovered that in addition to the signaling through the IL-23 receptor, IL-23 also exhibits an IL-12-like effect. This effect is independent of IL-23R expression and results through phosphorylation of small amounts of STAT4 in the production of small amounts of IFN- γ . The effect could be blocked by a specific antibody against IL-23p19, while IL-12p40 or IL-12p80 could not induce this IL-12 like effect, indicating that IL-23 itself and not IL-23 byproducts mediate this effect. These findings demonstrate that IL-23 is also able to signal through another receptor besides the IL-23 receptor, albeit with a much lower efficiency. Because this signal transduction, similar to IL-12 signaling, only involves STAT4 phosphorylation, an obvious candidate would be the IL-12 receptor. Indeed, in T cells from an IL-12R β 1^{-/-} patient this IL-12-like effect of IL-23 was only observed after transduction with an IL-12R β 1 expression construct, indicating that the IL-12R β 1 chain is indeed involved in this signaling. Whether the IL-12R β 2 chain or another receptor chain is involved and to which extent IL-23 can achieve this IL-12-like effect *in vivo* remains to be investigated. Based on our findings we conclude that IL-23 exerts an IL-12-like effect that depends on IL-12R β 1 and not on IL-23R expression.

Cells from an IL-12R β 1^{-/-} patient were used to study the influence of various combinations of IL-12R β 1 and IL-23R polymorphisms. The QMG and RTR alleles of the IL-12R β 1 in the IL-23 receptor complex were comparable in IL-23 responsiveness. When the IL-23R was co-expressed with the QMG allele the IL-12 responsiveness was decreased. This effect was however seen with TCB of one specific patient, in a model of overexpression of both receptor chains, while normal TCB did not show decreased responsiveness for IL-12 when transduced with the IL-23R.

We have provided ample evidence that we could express functional IL-23R in human T cells using a retroviral expression system. Despite the presence of functional IL-23R in these T cells, membrane expression of IL-23R could not be detected by FACS using four different antibodies, suggesting that perhaps the secondary structure of the IL-23R protein hampers detection with peptide-raised antibodies. One of the commercially available antibodies (BAF1400) was previously

used in a study to describe the cytokine profile of IL-23R positive CD45RO⁺ T cells (31). The authors did not test the IL-23 responsiveness of the 'IL-23R positive' cells, which might have proven whether or not these cells indeed expressed a functional IL-23R. We were also able to stain this subset of human CD45RO⁺ T cells with the BAF1400 antibody. We were however unable to obtain specific staining of various IL-23 responsive cells that expressed IL-23R naturally or retrovirally with this or three other antibodies. Based on our results we conclude that none of the available 'IL-23R antibodies' is specific for the IL-23R. Generation of new antibodies directed against a larger part of the IL-23R or the full-length protein may solve this problem in the future.

We conclude that IL-23 can induce STAT1, STAT3 and STAT4 phosphorylation via the IL-23R, but that IL-23 also exhibits IL-12-like effects, via STAT4 phosphorylation, dependent on IL-12R β 1 but independent of IL-23R expression. The P310L, Y173H and the R381Q variations in the IL-23R are fully functional and do not show differences in IL-23 responsiveness.

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Chapter 4

Functional analysis of naturally occurring amino acid substitutions in human IFN- γ R1.

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Abstract

IFN- γ plays an essential role in the IL-12/IL-23/IFN- γ pathway that is required for the defense against intracellular pathogens. In the IFN- γ R1 several amino acid substitutions have been reported that abrogate IFN- γ signaling. These substitutions can lead to a null phenotype and enhanced susceptibility to infection by low pathogenic mycobacteria and salmonellae, a disorder known as Mendelian Susceptibility to Mycobacterial Diseases (MSMD). More common amino acid variations in the IFN- γ R1 may influence IFN- γ R function, albeit more subtle. To determine the effect of various amino acid substitutions on IFN- γ R1 expression and function we cloned two newly identified amino acid substitutions (S149L, I352M), four common variations (V14M, V61I, H335P, L467P), seven reported missense mutations (V61Q, V63G, Y66C, C77Y, C77F, C85Y, I87T) and the 818delTTAA mutation in a retroviral expression vector. IFN- γ R1 expression was determined as well as responsiveness to IFN- γ stimulation. The two newly discovered IFN- γ R1 variants, and the four common polymorphisms could be detected on the cell surface, however, the V14M, H335P and I352M variants were significantly lower expressed at the cell membrane, compared to the wild type receptor. Despite the variance in cell surface expression, these IFN- γ R1 variants did not affect function. In contrast to literature, in our model the expression of the V63G variant was severely reduced and its function was severely impaired but not completely abrogated. In addition, we confirmed the severely reduced function of the I87T mutant receptor, the completely abrogated expression and function of the V61E, V61Q, C77F, C77Y and the C85Y mutations, as well as the overexpression pattern of the 818delTTAA mutant receptor. The Y66C mutation was expressed at the cell surface, it was however, not functional. We conclude that the V14M, V61I, S149L, H335P, I352M and L467P are functional polymorphisms. The other variants are deleterious mutations with V61E, V61Q, Y66C, C77F, C77Y and C85Y leading to complete IFN- γ R1 deficiency, while V63G and I87T lead to partial IFN- γ R1 deficiency.

Introduction

Interferon- γ (IFN- γ) is a pleiotropic cytokine playing a central role in type-1 immunity. Human host immunity against intracellular pathogens such as *Mycobacteria* and *Salmonellae* depends on an effective cell-mediated immune response mediated by type-1 cytokines (1). Upon encounter of these pathogens, antigen presenting cells (APC) produce cytokines such as IL-23, IL-1 β , IL-18, IL-12 and TNF via the stimulation of pattern recognition receptors, including Toll-like receptors. Type-1 helper T (Th1) cells and natural killer (NK) cells express receptors for these cytokines and produce IFN- γ in response to IL-12 or IL-23 which can be enhanced by IL-1 β and IL-18. IFN- γ , in turn, binds to the IFN- γ receptor (IFN- γ R), present on nearly all cell types (2). APCs, including monocytes, macrophages and DCs, are activated by IFN- γ to produce increased levels of IL-12 and to enhance both antigen presentation and bactericidal activity (3,4). In addition IFN- γ can, depending on cell type, block proliferation, induce apoptosis and enhance expression of cell surface molecules such as HLA Class I and II, CD54 and CD64.

The IFN- γ R is comprised of two ligand-binding IFN- γ R1 chains associated with two signal-transducing IFN- γ R2 chains (5). Binding of IFN- γ to its receptor induces receptor oligomerization and activation of the receptor-associated Janus kinases JAK1 and JAK2 by trans-phosphorylation. The JAKs phosphorylate the tyrosine 440 that is part of the STAT1 docking site in the intracellular domain of the IFN- γ R1, allowing for subsequent STAT1 phosphorylation (5). Phosphorylated STAT1 dissociates from the receptor, dimerizes and translocates to the nucleus, where it regulates the expression of IFN- γ responsive genes directly (e.g. *CD54*) (6), or indirectly via the induction of other transcription factors such as IRF1, IRF7 and CIITA (e.g. *B2M* and *HLA*) (5). Although STAT1 is the main mediator of IFN- γ responses, IFN- γ has also been reported to induce STAT3 or STAT5 phosphorylation in a few other cell types (7). In Figure 1, a schematic representation of the IFN- γ R1 is provided.

Polymorphisms and mutations in the IFN- γ R1 chain influence IFN- γ responses. For example, patients with Mendelian Susceptibility to Mycobacterial Disease (MSMD) due to IFN- γ R1 deficiency, have impaired Th1-immunity and suffer from unusually severe infections caused by weakly virulent *Mycobacteria* and *Salmonellae* (8). Most recessive IFN- γ R1 deficiencies result in complete loss of cellular responsiveness to IFN- γ , due to mutations that preclude the expression of IFN- γ R1 on the cell surface (9-13). Another group of IFN- γ R1 deficiencies is due to missense mutations which result in normal expression of IFN- γ R1 at the cell surface, however, the resulting receptors show no or diminished binding of IFN- γ (14,15). Dominant-negative defects like the 818delTTAA mutation in the intracellular domain of the receptor result in milder forms of MSMD. The 818delTTAA mutation product lacks the JAK1 and STAT docking site as well as the receptor

recycling domain which leads to accumulation of aberrant receptor complexes on the cell surface (16,17). In addition, several polymorphisms of the IFN- γ R1 have been found that may have an effect on IFN- γ responses. The H335P and L467P variants of the IFN- γ R1 have been associated with the production of high antibody titers against *H. pylori* (18) and susceptibility to allergic disease and the production of high IgE titers (19). The frequency of the V14M allele in SLE patients is significantly higher than that of the healthy control population and the presence of a V14M allele correlated with an altered Th1/Th2 balance in favor of Th2 (20,21).

In this report we compare the effect of two novel variations, identified in patients suffering mycobacterial infections (S149L, I352M, unpublished data), four known polymorphisms (V14M (20,22), V61I (23), H335P, L467P (18,19), all seven reported missense mutations (V61Q (15), V63G (14), Y66C (24), C77Y (15), C77F (25), C85Y (26), I87T (27,28)) and the 818delTTAA mutation (16) on the expression and function of IFN- γ R1 in the same genetic background. In addition, because the mutation at nucleotide 182 (gTA $\rightarrow$ gAA) reported by Jouanguy et al. may have been aberrantly designated as V61Q (Val $\rightarrow$ Gln) (15), instead of V61E (Val $\rightarrow$ Glu), we analyzed both variations. For this purpose we cloned wild type *IFNGR1* and the *IFNGR1* variants (see also Figure 1), into a retroviral expression vector and transduced the constructs into the IFN- γ R1 deficient cell line SKLC-7. We analyzed the signal transduction, the regulation of CD54, CD64, HLA-DR and HLA class I expression and the cytokine production in response to IFN- γ .

Materials and Methods

Cloning IFNGR1 variants into a retroviral expression vector

The full-length *IFNGR1* coding sequence was PCR amplified from cDNA of a healthy control with the sense primer 5'-AATTGGATCCGGTAGCAGCATGGCTCTCCT-3' and the anti-sense primer 5'-AAGGCTCGAGTCATGAAAATTCTTTGGAATCT-3' and cloned into the retroviral vector pLZRS-IRES-GFP (29) after digestion with the enzymes *Bam*HI and *Xho*I (Fermentas). Variations were introduced by site directed mutagenesis (30). All constructs were sequence verified and were transfected in the Phoenix-A packaging cell line using calciumphosphate (Invitrogen). Supernatants with retroviral particles carrying the expression construct were generated as described before (31).

Cells, culture conditions and retroviral transduction

The human IFNGR1^{-/-} cell-line SKLC-7 (32) and the human monocytic cell-line THP-1 (ATCC TIB-202) were cultured in RPMI1640 medium supplemented with 10% FCS, 20 mM GlutaMax, 100 U/ml Penicillin and 100 μ g/ml Streptomycin (Gibco/Invitrogen). 0.25 $\times$ 10⁶ cells were retrovirally transduced by overnight incubation on a CH-296 (RetroNectintm, Takara Shuzo) coated 48 wells plate in the presence of 1 ml of virus containing supernatant. Cells were washed and cultured for at

least four days before analysis in further assays. All subsequent FACS measurements were performed on cells gated for equal GFP expression.

Analysis of IFN- γ R1 expression

To detect IFN- γ R1 membrane expression cells were labeled with IR γ 2 (33) and 177.10 (34) antibodies (kindly provided by Heiner Böttinger and Daniela Novick respectively) and PE conjugated monoclonal antibodies GIR94, GIR208, and as an isotype control IgG1 (BD biosciences). After labeling with the γ R99 antibody (35) (kindly provided by Francesco Novelli) the cells were counterstained with goat-anti-mouse-PE (BD Biosciences). 1×10^5 cells were stained in PBS supplemented with 0.2% BSA (Fraction V, Sigma) and washed twice before analysis on a FACSCalibur (BD Biosciences). In order to detect intracellular expression 1×10^5 cells were fixated with 4% paraformaldehyde (Sigma) and permeabilized with 4% saponin (Sigma-Aldrich) before staining with a directly labeled antibody.

Detection of cell surface markers

Regulation of CD54, HLA Class I, CD64 and HLA-DR expression in response to IFN- γ was determined by stimulation of 2×10^5 transduced SKLC-7 or THP-1 cells in 200 μ l culture medium with various amounts of IFN- γ for 20 hours. Subsequently, the cells were washed and stained with either PE conjugated anti-CD54, the HLA Class-I antibody W6.32 and counterstained with goat-anti-mouse-PE, PE conjugated anti-CD64 or with PE conjugated anti-HLA-DR (BD Biosciences). After staining the cells were washed twice and analyzed on a FACSCalibur (BD Biosciences).

STAT phosphorylation assays

To study signal transduction, 2×10^5 transduced or untransduced SKLC-7 cells in 200 μ l of culture medium were pulsed with various concentrations of IFN- γ (Biosource). The cells were fixated with 4% paraformaldehyde and permeabilized with 90% methanol (Merck). Subsequently, the cells were washed with PBS, 0.2% BSA, blocked with normal goat serum (Sanquin), and stained with the phospho-specific antibodies pY701-STAT1-Alexa 647, pY705-STAT3-PE or pY694-STAT5-PE (BD Pharmingen). Before analysis on a FACSCalibur, the cells were washed twice.

Analysis of cytokine production

To study cytokine production, 2×10^5 transduced or untransduced SKLC-7 cells in 200 μ l of culture medium were pulsed with various concentrations of IFN- γ (Biosource). Twenty-four hours cell free supernatants were collected and cytokine production was checked by a Elisa: IL-12p40, TNF (Biosource), CXCL-10 and MCP-1 (Biorad).

Results

Functional transfer of the *IFNGR1* in SKLC-7 cells

We cloned and expressed the wild type IFN- γ R1 in the IFN- γ R1 deficient cell line SKLC-7. The use of the retroviral expression vector pLZRS ensures transcription and expression of the *IFNGR1* and green fluorescent protein (*GFP*) genes in tandem and allows for selection of transduced cells by fluorescence-activated cell sorting (FACS) for the GFP signal. Transduction efficiency of SKLC-7 cells was typically between 20 and 40%. The transduction efficiency depends on target cell type and its proliferation rate. SKLC-7 cells divide slowly and are therefore more difficult to transduce than other cell types.

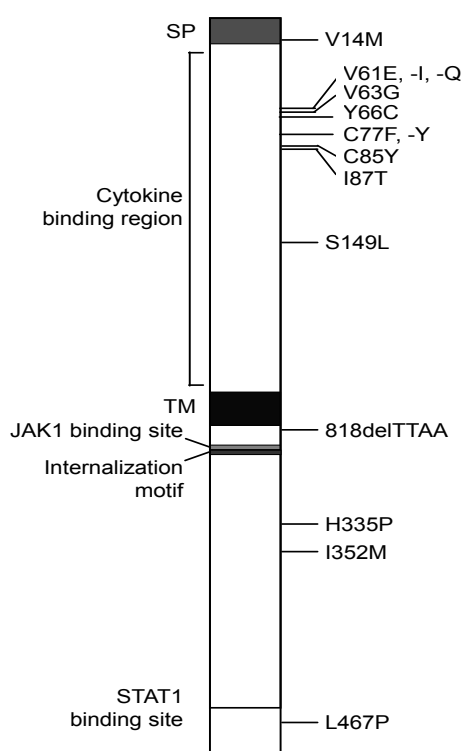


Figure 1. Schematic presentation of the IFN- γ R1. On the left the various domains are indicated, on the right the variations that were analyzed are indicated. TM=transmembrane domain, SP=signal peptide.

With the same viral supernatants we obtained transduction efficiencies of up to 80% in Jurkat or K562 cells. SKLC-7 cells do not express the IFN- γ R1 on their cell surface, nor do they express GFP (Fig 2 A-D). After retroviral transduction of the wild-type *IFNGR1* into the SKLC-7 cells the IFN- γ R1 could be detected on the cell membrane (Fig 2 A-D), whereas in cells transduced with the empty vector (GFP) it could not (Fig. 2 A-D).

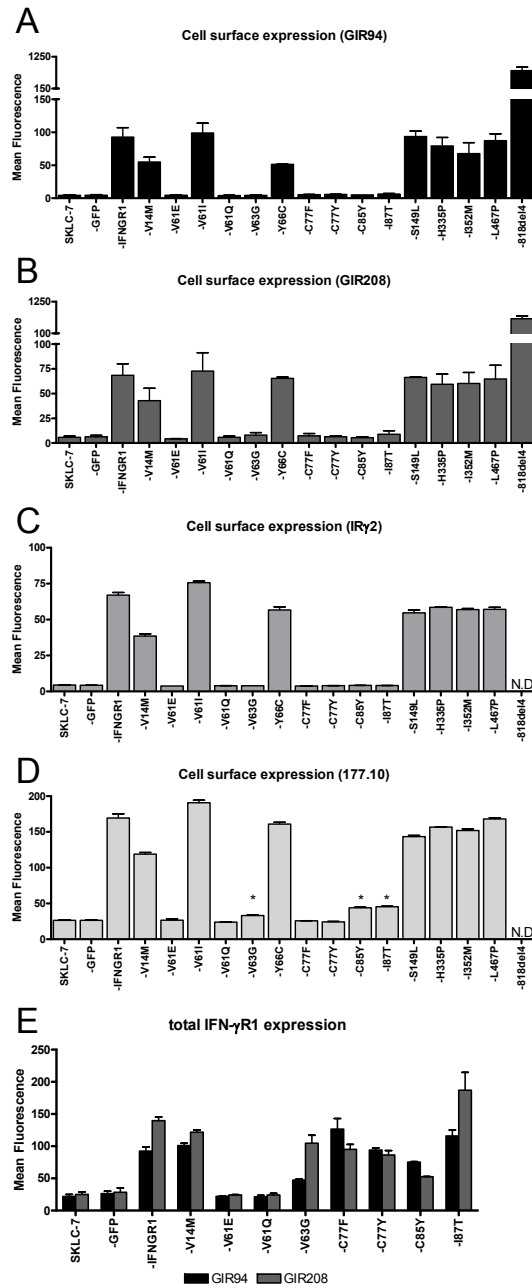


Figure 2. Extracellular and intracellular expression of the IFN- γ R1 variants. SKLC-7 cells and the cells retrovirally transduced with GFP or with one of the *IFNGR1* gene variants were stained without pretreatment to detect extracellular expression (A-D) or after permeabilization to detect both intracellular and extracellular expression of IFN- γ R1 (E). Cells were analyzed for IFN- γ R1 expression using four IFN- γ R1 specific antibodies (GIR94 (A), GIR208 (B), IR γ 2 (C) and 177.10 (D)). Mean $\pm$ SD fluorescence of these antibodies of 2 (IR γ 2 and 177.10) or 3 (GIR94 and GIR208) experiments in triplo is depicted. N.D. = Not Determined. * = $p < 0.004$ higher expression compared to GFP construct.

Next we tested the responsiveness of transduced cells to IFN- γ . SKLC7 cells expressing wild type IFN- γ R1 were stimulated with various concentrations of IFN- γ for 5 to 90 minutes before assessing STAT1 phosphorylation. STAT1 phosphorylation peaked 10-15 minutes after stimulation with high doses of IFN- γ , whereas with lower doses of IFN- γ STAT1 phosphorylation peaked later (Fig. 3). In addition, the induction of STAT1 phosphorylation was dose dependent and the highest IFN- γ concentrations induced the largest amounts of STAT1 phosphorylation (Fig 3). No induction of STAT3 or STAT5 phosphorylation was observed in response to IFN- γ at any time point (data not shown).

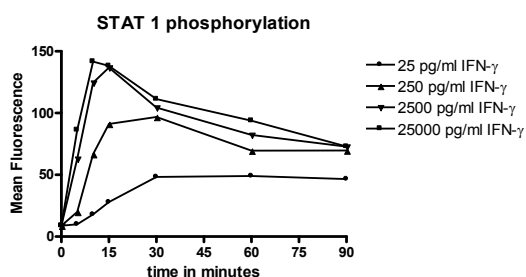


Figure 3. Kinetics of STAT1 phosphorylation in response to IFN- γ . SKLC-7 cells transduced with wild type IFNGR1 were stimulated with various concentrations of IFN- γ (25-25000 pg/ml IFN- γ), whereafter STAT1 phosphorylation was analyzed at various time points. One representative experiment out of 2 is depicted.

Expression pattern of the IFN- γ R1 variants

Four known polymorphisms (V14M, V61I, H335P, L467P), the known mutations (V61E, V61Q, V63G, Y66C, C77Y, C77F, C77Y, C85Y, I87T, and 818delTTAA) and two novel amino acid substitutions (S149L and I352M) in IFN- γ R1 were cloned and retrovirally transduced into the IFN- γ R1^{-/-} SKLC-7 cell line. We first determined whether the IFN- γ R1 variants were expressed at the cell surface, using four different IFN- γ R1 specific antibodies. Untransduced or GFP-transduced cells did not express IFN- γ R1 (Fig 2A-D). On the SKLC-7 cells transduced with wild type IFN- γ R1, the receptor was detected on the cell surface (Fig. 2A-D). The V61I, Y66C, S149L and L467P variants could be detected on the cell surface in similar amounts as the wild type receptor while the H335P and I352M variants, showed a small (13% and 18% respectively) reduction of expression that was significant with three out of four antibodies. The V14M variant showed significant reduced expression (42%) compared to the wild type construct with all four antibodies. As expected, the 818delTTAA mutant lacking the receptor recycling-domain was expressed ten times higher on the cell membrane than the wild type construct (Fig 2A-B). In contrast, membrane expression of the V61E, V61Q, C77F and C77Y variants could not be detected (Fig. 2A-D). A small amount of cell-surface expression of the V63G, C85Y and I87T variants was detected by one antibody (Fig. 2D).

Lack of cell surface expression can be due to a lack of protein expression, or due to misfolded proteins being sequestered in the endoplasmic reticulum (ER) or due to a defect in trafficking of the receptor to the cell membrane. To determine whether the variants that were

undetectable at the cell surface were synthesized, we stained the cells for intracellular IFN- γ R1 expression. Except for the *GFP* transduced cells and the V61E and V61Q variants, in all other transduced cells the IFN- γ R1 could be detected intracellular, indicating protein is synthesized (Fig. 2E). The results of the expression analysis experiments are summarized in Table 1.

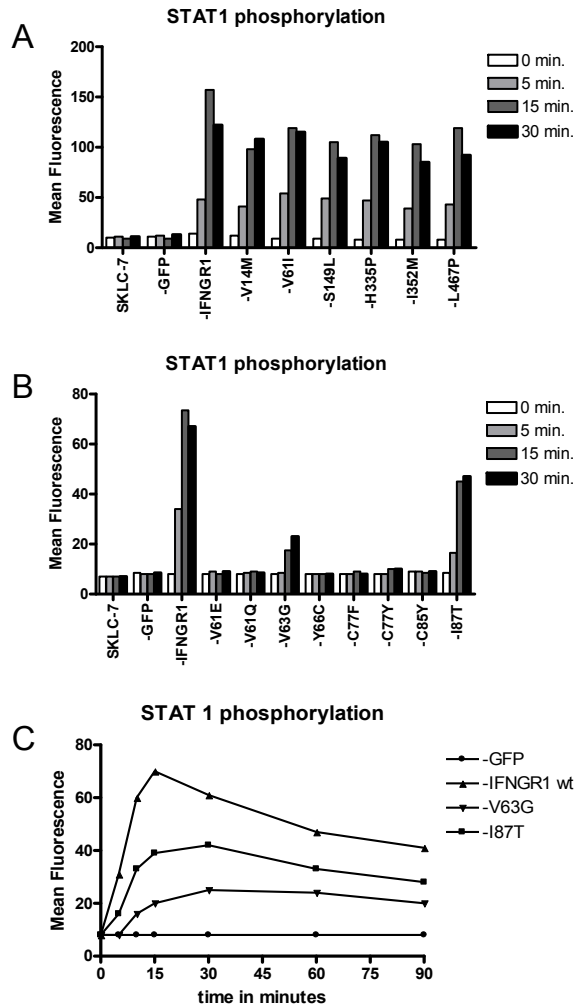


Figure 4. The influence of IFN- γ R1 variations on the kinetics of STAT1 phosphorylation. SKLC-7 cells transduced with wild type IFNGR1, GFP or with the V14M, V61I, S149L, H335P, I352M and L467P (A), V61Q, V63G, C77F, C77Y, C85Y and I87T (B) or V63G and I87T variants (C), were stimulated for 5 to 90 minutes with 2500 pg/ml IFN- γ (A), or 250,000 pg/ml IFN- γ (B-C). The phosphorylation of STAT1 was determined by FACS, using a STAT1 phospho-specific antibody. One representative experiment out of 3 are depicted.

The effect of IFN- γ R1 variations on IFN- γ induced STAT1 phosphorylation

STAT1 plays a critical role in the IFN- γ signal transduction and the IFN- γ induced host defence against infections (36). To test the influence of the IFN- γ R1 alleles on IFN- γ signal transduction, STAT1 phosphorylation was assessed. The kinetics of STAT1 phosphorylation in response to IFN- γ was determined in cells transduced with each of the variants. In cells expressing the wild type IFN- γ R1 or the V14M, S149L, H335P, I352M, and L467P variants, comparable STAT1 phosphorylation was observed (Fig. 4A). In cells transduced with the V63G or the I87T variant, STAT1 phosphorylation was detected, however, it was significantly reduced compared to cells transduced with the wild type receptor (Fig. 4B-C). In cells transduced with the V61E, V61Q, Y66C, C77F, C77Y and C85Y variants, no STAT1 phosphorylation could be observed in response to IFN- γ (Fig 4B-C). The results of the expression analysis experiments are summarized in Table 1.

Influence of IFN- γ R1 variations on CD54 and HLA Class I regulation by IFN- γ

IFN- γ stimulates the expression of many cell surface markers, including CD54 and HLA Class I molecules. CD54 (also known as ICAM-1) is involved in the binding of cells to endothelia and transmigration into tissues (37). CD54 functions as a costimulatory molecule on APC and other cell types, to activate CD4⁺ T cells and cytotoxic CD8⁺ T cells, respectively (38). IFN- γ increases antigen processing and the expression of the HLA class I molecules, thereby increasing antigen presentation and promoting the induction of cell-mediated immunity (5,39). We assessed the influence of the IFN- γ R1 variants on the IFN- γ induced expression of CD54 and HLA Class I molecules. IFN- γ upregulated the expression of CD54 in SKLC-7 cells expressing the wild type IFN- γ R1, but not in GFP- transduced or in untransduced SKLC-7 cells (Fig. 5A). In cells expressing the V14M, V61I, S149L, H335P, I352M or L467P, similar upregulation of CD54 expression was observed as in cells expressing the wild type receptor (Fig 5A). In cells expressing the V63G or the I87T variants, CD54 upregulation was seen when cells were stimulated with high concentrations of IFN- γ , however, this upregulation was less compared to cells expressing the wild type receptor (Fig 5B and C). No regulation of CD54 by IFN- γ was observed in cells expressing the V61E, V61Q, Y66C, C77F, or C77Y receptor variants (Fig 5B). Similar results for all IFN- γ R1 variants were observed for IFN- γ induced HLA Class I cell surface expression (Fig. 5D-F). The results of the expression analysis experiments are summarized in Table 1. The expression of two other cell surface markers that can be regulated by IFN- γ , CD64 and HLA-DR, was not altered by IFN- γ stimulation in the SKLC7 cell line with or without *IFNGR1* construct (data not shown). Therefore expression of these two markers was not analyzed in the full panel of *IFNGR1* variations.

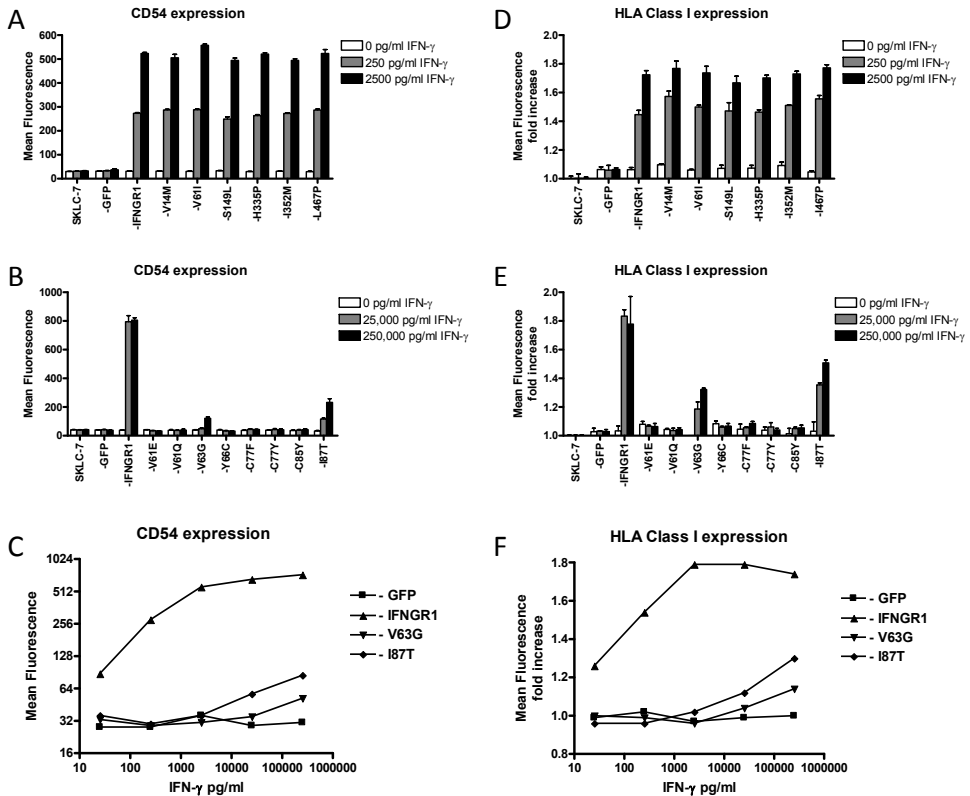


Figure 5. The effect of IFN- γ R1 variations on IFN- γ induced CD54 and HLA class I upregulation. SKLC-7 cells and the cells retrovirally transduced with GFP or with one of the *IFNGR1* gene variants were stimulated for 20h with various concentrations of IFN- γ . The CD54 expression (A, B and C) and the HLA Class I expression (D, E and F) was determined by FACS. CD54 expression is depicted as mean fluorescence, HLA Class I expression is depicted as fold increase compared to the mean of untransduced cells. Displayed is the mean $\pm$ SD of a triplo experiment. All variants were tested at least three times for comparison with the wild type variant.

Influence of the IFN- γ R1 Y66C variation on the function of the wild type receptor

The Y66C variation was expressed on the cell surface but appeared to be non-functional. Since the IFN- γ R contains two IFN- γ R1 chains, in individuals heterozygous for a defective IFN- γ R1 chain that is expressed on the membrane, incorporation of one defective IFN- γ R1 chain in the IFN- γ R complex could potentially have a dominant-negative effect. To determine whether the Y66C variation exerts such an effect we transduced the IFN- γ responsive cell line THP-1 with the Y66C variation and as controls with the wild type IFN- γ R1 or a GFP vector. While expressing large amounts of Y66C IFN- γ R1, Y66C THP-1 transductants showed a dose dependent induction of CD54 after stimulation with IFN- γ , which was comparable to the dose response curve of untransduced THP-1 cells (data not shown). Transduction of the wild type receptor led to a higher total expression of IFN- γ R1 at the cell

surface, as well as a stronger upregulation of CD54 in response to IFN- γ (data not shown). In THP-1 cells transduced with the 818delTTAA IFN- γ R1 variant, total receptor cell surface expression was increased, while the CD54 upregulation in response to IFN- γ was severely reduced, confirming the dominant negative effect of this variant (data not shown).

Discussion

The main finding of this study is that the newly discovered I IFN- γ R1 variants, S149L and I352M, as well as the known V14M, V61I, H335P and L467P IFN- γ R1 variants do not functionally differ from the wild type receptor and are therefore considered polymorphisms. In contrast to literature, we showed a severely reduced expression of the V63G receptor variant, and found that its function was severely reduced although not completely abrogated. In addition, we confirmed the severely reduced function of the I87T mutant receptor, the completely abrogated function of the V61E, V61Q, Y66C, C77F, C77Y and C85Y variants, as well as the overexpression patterns of the 818delTTAA mutant receptor. Thus far the only mutant proteins that have been characterised after transduction into cells were the 818delTTAA (into HEK293 cells) and the I87T (into fibroblasts) variants (27,40).

To reach these conclusions, we retrovirally expressed the IFN- γ R1 variants in the human IFN- γ R1-deficient SKLC-7 cell line and analyzed its functional activity. The conclusions are summarized in Table 1. With the *in vitro* model used, we were able to distinguish between functional and non-functional variants and were also able to detect the partial responsiveness of the V63G and I87T variants. However, the following points should be considered. First, while the IFN- γ R is expressed by all nucleated cells in the human body, the cells used in this study are not representative for *all* IFN- γ responsive cells. For example, in a few cell-types STAT3 and STAT5 phosphorylation in response to IFN- γ can be observed (7), in our model no STAT3 or STAT5 phosphorylation could be detected, thus not allowing for detection of potential differences between the IFN- γ R1 variants in signaling through these molecules. Second, the concentrations of stimuli chosen may not fully resemble physiologically relevant conditions. Though we tested a range of IFN- γ concentrations as commonly found in physiological relevant situations, the present approach cannot exclude subtle differences in the lowest range of ligand binding. Third, the overexpression of the IFN- γ R1 by the retroviral system could mask differences in effects due to alterations in transcript or protein stability. We were nevertheless able to detect accumulation on the membrane of the 818delTTAA mutant, a significant lower expression of some of the polymorphisms, as well as the extremely low expression of partial mutations compared to the wild type IFN- γ R1. Furthermore, post-transcriptional and post-translational modifications of normal or retroviral expressed IFN- γ R1 may differ, although thus far no findings suggest that such modifications occur. Fourth, the IFN- γ induced

STAT1 phosphorylation and subsequent induction of CD54 and HLA class I molecules are not the only responses induced. Subtle differences between the different variants may be missed due to the choice of read-out system.

The C77F and C77Y receptor variants could not be detected on the cell surface, however, these variants could be detected intracellularly. Lack of cell surface expression can be due to a lack of protein expression or due to a defect in trafficking of the receptor to the cell membrane. Alternatively, the protein quality control system in the endoplasmic reticulum (ER) could prevent transport of mutant, misfolded, or incorrectly complexed proteins, and target these for degradation (41), as we have also reported for several IL-12R β 1 mutations (42). In accordance with literature, we did not observe any IFN- γ responsiveness of these two variant receptors (15,25).

In contrast with previous publications (14,27), we only detected low cell surface expression of these the V63G and I87T receptor variants. With one out of the four antibodies used, low cell surface expression of the V63G, C85Y and I87T variants could be detected. In addition, we were able to detect STAT1 phosphorylation as well as a slight upregulation of CD54 and HLA Class I in the V63G and I87T variants in response to more than 10,000 times higher IFN- γ concentrations than needed to induce IFN- γ responses in cells expressing the wild type receptor. This confirms the observation by Jouanguy et al. (27) that I87T is a partial deficiency and identifies V63G as a partial deficiency as well in contrast to the earlier report by Allende et al. that it results in complete deficiency(14). In line with our results with the I87T variant, Jouanguy et al. observed a response in B-cells and monocytes obtained from the patient expressing this variant when stimulated with very high doses of IFN- γ (27). The diminished response of the I87T variant to IFN- γ may be largely due to the low cell-surface expression. The diminished response observed of the V63G variant can be due to either the severely diminished cell-surface expression or to disrupted binding of IFN- γ to the receptor since the neighboring amino acids (64 and 66-68) of the IFN- γ R1 are known to be essential in the interaction between the high-affinity receptor and its ligand I IFN- γ (43). Moreover, the adjacent lysine at position 64 directly interacts with IFN- γ .

In the V61Q (V61E) variant IFN- γ R1, the binding of IFN- γ was reported to be abrogated (15). This was determined in patient cells expressing two mutant *IFNGR1* alleles (V61Q and 652del3) (15). We tested both the V61Q and V61E variants. Interestingly, in our experiments, with four independently cloned and sequence-verified V61Q and V61E constructs, neither variant was detected on the cell surface nor was the protein detectable intracellular. The fact that these mutants could not be detected on the cell surface with four different antibodies suggests that the variant detected on the cell surface by Jouanguy et al. may have been the 652del3 mutation (15). The failure to detect protein expression may be caused by a changed protein conformation, leading to the disappearance of epitopes recognized by the antibodies. Three of the antibodies (GIR-94, GIR-208 and IR γ 2) were raised against the extracellular domain of IFN- γ R1, one (177.10) was raised

against full length IFN- γ R1 but is known to block IFN- γ binding, suggesting it binds to the extracellular domain of IFN- γ R1 as well. Of none of these antibodies the exact binding site is known.

The V61I variant is an amino acid substitution at the same position as the V61Q and V61E variants that was however normally expressed on the cell surface and showed a normal STAT1 phosphorylation in response to IFN- γ . Both valine (V) and isoleucine (I) are hydrophobic amino acids, V to I substitutions are conserved substitutions while substitutions of valine to amino acids glutamine (Q) or glutamic acid (E) substitutions are not.

The Y66C variant could be detected on the cell surface, however, no STAT1 phosphorylation, nor CD54 or HLA class I induction was observed in response to IFN- γ . This finding is in accordance with the fact that phenylalanine residue on position 66 is essential for IFN- γ binding (43). Although the non-functional Y66C variant was expressed on the cell surface, it did not hamper the function of the IFN- γ R when this variant was co-expressed with wild type IFN- γ R1. Whether or not the Y66C variant is incorporated in the IFN- γ R complex is not known.

We show that the variants V14M, V61I, H335P, L467P, S149L and 352M were detectable on the cell surface and were fully functional and not different in receptor function from the wild type receptor. Despite the fact that cell surface expression of the V14M variant was nearly halved, we were not able to detect a functional difference compared to the wild type receptor. The V14M variation is within the signal peptide, thereby likely influencing transport of the receptor to the cell surface. When IFN- γ R1 is overexpressed, as is the case in our model, subtle differences in expression and the balance between the expression of the IFN- γ R1 and the IFN- γ R2 within our model may not resemble the normal expression pattern. In T lymphocytes IFN- γ R1 and R2 expression can individually vary from low to high. For example, the expression density of the IFN- γ R2 is an important mechanism in determining the fate of T lymphocytes (44). Therefore differences in IFN- γ R1 expression levels as seen with the V14M variant, which did not result in differences in our read outs, may nonetheless influence signaling in natural conditions.

The H335P and L467P variants of the IFN- γ R1 have been reported to be associated with the production of high antibody titers against *H. pylori* (18) and susceptibility to allergic disease and the production of high IgE titers (19). The identification of associations between an allele and an observed clinical outcome does not necessarily mean that the allele itself conveys a functional difference. The functional difference may be due to a variation that is merely linked to the polymorphism under study. We can however not exclude more subtle functional differences of the studied alleles, since we used overexpression constructs of the IFN- γ R1. In addition, the observed associations may be due to alterations in signaling pathways other than the ones we explored in this study.

When a variation affects the function of a protein it is considered a mutation, when it does not affect protein function it is considered a polymorphism. We therefore conclude that the V14M,

V61I, S149L, H335P, I352M and L467P are functional polymorphisms. Expression on the cell surface of V14M is reduced which may result in slightly reduced IFN- γ responses, when IFN- γ R1 gene transcription is *in vivo* limited to natural amounts. This polymorphism may influence susceptibility to infections or predisposition to SLE. The other variants are deleterious mutations with V61E, V61Q, Y66C, C77F, C77Y and C85Y leading to complete IFN- γ R1 deficiency while V63G and I87T lead to partial IFN- γ R1 deficiency.

Table 1 Summary of the expression and functional analyses of the IFN- γ R1 variants

variant	cell surface expression ^a	total expression ^d	STAT1 phosphorylation	CD54 upregulation	HLA class I upregulation	conclusion
wild type	+	+	+	+	+	
V14M	42% reduced	+	+	+	+	polymorphism
V61E	absent	absent	absent	absent	absent	mutation
V61I	+	+	+	+	+	polymorphism
V61Q	absent	absent	absent	absent	absent	mutation
V63G	severely reduced ^b	+	severely reduced	severely reduced	severely reduced	mutation ^e
Y66C	+	+	absent	absent	absent	mutation
C77F	absent	+	absent	absent	absent	mutation
C77Y	absent	+	absent	absent	absent	mutation
C85Y	severely reduced ^b	+	absent	absent	absent	mutation
I87T	severely reduced ^b	+	severely reduced	severely reduced	severely reduced	mutation ^e
S149L	+	+	+	+	+	polymorphism
H335P	13% reduced ^c	+	+	+	+	polymorphism
I352M	18% reduced ^c	+	+	+	+	polymorphism
L467P	+	+	+	+	+	polymorphism

^a determined with four different antibodies. ^b low expression was detectable with one out of four antibodies. ^c observed with three out of four antibodies. ^d determined with two different antibodies. ^e mutation leading to partial deficiency.

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Chapter 5

IFN- α can not substitute lack of IFN- γ responsiveness in peripheral blood mononuclear cells of an IFN- γ R deficient patient

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Submitted

Abstract

Background. Patients with complete IFN- γ R deficiency are unable to respond to the macrophage-activating cytokine IFN- γ , have impaired Th1-immunity and recurrent, severe infections with weakly virulent *Mycobacteria*. Since IFN- α and IFN- γ share signalling pathways and both induce STAT1 phosphorylation, treatment with IFN- α was proposed as possibly beneficial intervention in complete IFN- γ R deficiency.

Methods. We stimulated peripheral blood mononuclear cells from healthy controls and from a patient lacking IFN- γ R1 with IFN- α and IFN- γ to establish whether IFN- α would substitute for IFN- γ activating effects. Mycobactericidal activity of pro-inflammatory macrophages was assessed after incubation with IFN- α and IFN- γ .

Results. IFN- α induced STAT1 phosphorylation in monocytes of the IFN- γ R1 deficient patient, but did not prime the cells for IL-12p70, IL-12p40, IL-23 or TNF production in response to LPS. In cells of healthy controls, IFN- α inhibited the priming effect of IFN- γ on LPS-induced pro-inflammatory cytokine release; IFN- γ but not IFN- α induced mycobacterial killing of *M. smegmatis* in cultured human macrophages.

Conclusions. No evidence was found to support the use of IFN- α in IFN- γ R-deficient patients as intervention against mycobacterial infection, on the contrary, the findings suggest that treatment of individuals with IFN- α may adversely affect host defence against mycobacterial infections.

Introduction

Infections with intracellular pathogens such as *Mycobacteria* are generally controlled effectively by the cell-mediated immune response (1). Upon encounter of these pathogens, antigen presenting cells (APC) produce cytokines such as IL-23, IL-12, IL-1 β , IL-18 and TNF (2). IL-23 and IL-12, in combination with IL-18 or IL-1 β , in turn, induce IFN- γ production by NK, NK-like T and Th1 cells (3,4). IFN- γ subsequently, in synergy with TNF, enhances antimicrobial activity of macrophages (5), and enhances IL-23 and IL-12 production (4,6).

Patients with unusual, persistent and severe infections caused by otherwise poorly pathogenic *Mycobacteria*, may have a condition known as Mendelian Susceptibility to Mycobacterial Disease (MSMD). MSMD is a heterogeneous disorder that can be caused by mutations in the *IL12B*, *IL12RB1*, *IFNGR1*, *IFNGR2* and *STAT1* genes that are involved in the IL-12/-23/IFN- γ cytokine signalling cascade (7). Due to these defects, no effective immune response is generated in response to mycobacterial infection. Patients with IL-12p40 or IL-12R β 1 deficiency are unable to produce or respond to IL-12 and IL-23. Since IL-12 and IL-23 signalling is imperative for IFN- γ production, these patients do not produce sufficient IFN- γ to control infections. These patients benefit from treatment with recombinant IFN- γ in addition to antibiotics (8). Patients with partial IFN- γ R deficiency benefit from treatment with high dose recombinant IFN- γ (9). A more severe clinical course is seen in complete IFN- γ R1 and IFN- γ R2 deficient patients whereby these individuals often succumb to mycobacterial infections very early in life (7). Patients with complete IFN- γ R1 or IFN- γ R2 deficiencies are unable to respond to IFN- γ and thus will not benefit from treatment with recombinant IFN- γ . The only currently available curative treatment of complete IFN- γ R deficiency is hematopoietic stem cell transplantation; however, the overall success rate of stem cell transplantation in this setting is low.

In two patients, with complete IFN- γ R1 and complete IFN- γ R2 deficiency respectively, suffering from disseminated infection with *Mycobacterium avium* complex, treatment with IFN- α as additional therapy has been described (10,11). The rationale behind this therapy being that IFN- α and IFN- γ activate common signalling pathways and the induced genes and biological effects partly overlap. Through the activation of overlapping effects of IFN- α and IFN- γ , treatment with exogenous IFN- α is thought to (partly) compensate for the absence of IFN- γ signalling in patients deficient in IFN- γ R1 or IFN- γ R2. The IFN- α receptor is composed of IFN- α R1 and IFN- α R2 subunits, associated with Janus kinases (JAKs) TYK2 and JAK1. In response to IFN- α , these JAKs are activated and subsequently phosphorylate signal transducer and activator of transcription (STAT)1 and STAT2 (12). STAT1 and STAT2 form heterodimers, which associate with interferon regulatory factor-9 (IRF-9) to form a STAT1/STAT2/IRF-9 complex (also known as IFN-stimulated gene factor 3 (ISGF3))

(13). This complex migrates to the nucleus and regulates primary response genes by binding specific DNA response elements. IFN- γ signals via the IFN- γ R1 and IFN- γ R2. Upon binding of IFN- γ , receptor-associated JAK1 and JAK2 are phosphorylated, which in mature monocytes and macrophages leads to phosphorylation of STAT1 (5). STAT1 dimerizes and translocates to the nucleus to regulate primary response gene transcription (5). STAT1 can, in theory, also form homodimers in response to IFN- α (14). IFN- α signalling is not affected by deficiencies of the IFN- γ R1 or the IFN- γ R2.

The potential effect of IFN- α treatment in patients with complete IFN- γ R deficiency has not been investigated at the cellular level. We therefore determined whether IFN- α can (partly) compensate for absence of IFN- γ signalling in cells obtained from an IFN- γ R1 deficient patient.

Materials and Methods

Cells, culture conditions and infection protocol

Human CD14⁺ cells and PBMCs were isolated from healthy donor buffy coats (Sanquin) or from venipuncture from healthy consenting volunteers, or an IFN- γ R1 deficient patient (P2 in (15)), by Ficoll-Amidotrizoate density gradient centrifugation and subsequent selection with anti-CD14 MACS beads (Miltenyi Biotech). CD14⁺ cells were cultured in RPMI-1630 medium, supplemented with 20 mM GlutaMAX (Gibco/ Invitrogen), 10% FCS, 100 U/ml Penicillin, 100 μ g/ml Streptomycin (Gibco/Invitrogen). To generate pro-inflammatory macrophages (M ϕ 1), CD14⁺ cells were cultured for 6 days in 75 cm² or 175 cm² cell culture flasks (Greiner Bio-One), in the presence of 5 ng/ml GM-CSF (Biosource) as previously described (2).

For assessment of in vitro mycobactericidal activity as described in (16), cells were seeded at $3 \cdot 10^5$ in 24 well plates, left to adhere then treated with IFN- α (IFN- α 2b (Intron[®]A)/ IFN- α 2a (Roferon[®]A)) and/or IFN- γ (Immukine[®], Boehringer) or H-89 (Sigma Aldrich) overnight prior to infecting with log phase growth *M. smegmatis*::GFP at an MOI of 1. Extracellular mycobacteria were eliminated by low dose gentamycin treatment (5 μ g/ml) for the duration of the experiment. All cells were subsequently collected, lysed, plated out on 7H9 agar plates and levels of fluorescence assayed in a Mithras LB940 platereader using GFP excitation and emission filter sets after 24 hrs of expansion.

FACS analysis

To assess STAT1 phosphorylation, PBMC were stimulated with recombinant human 2.5 ng/ml IFN- γ (Biosource) or 1000 U/ml IFN- α for 15 minutes, or left unstimulated. Cells were fixed using 4% paraformaldehyde and permeabilised with 90% methanol, then labeled with anti-phospho-STAT1 (pY701)-PE antibody (BD Bioscience).

To assess CD64 regulation in response to IFN- α/γ , PBMC were seeded $5 \cdot 10^5$ per well in hydron coated 24-well plates (Corning Life Sciences). Cells were stimulated overnight with 2.5 ng/ml IFN- γ and/or 1000 U/ml IFN- α , or left unstimulated. Cells were stained with anti-CD64-FITC, anti-CD54-PE and anti-CD14-PE antibodies (BD Biosciences). Samples were acquired and analyzed on a FACS Calibur/CellQuest Pro (BD Biosciences).

Cytokine induction and measurement

CD14⁺-bead-isolated monocytes were seeded in 96-well plates at $1 \cdot 10^5$ cells per well and cultured for 24 h in the presence or absence of 100 ng/ml LPS (Sigma), with and without various concentrations of IFN- α and/or 2.5 ng/ml IFN- γ . Cell free supernatants were collected and TNF, IL-1 β , IL-10, IL-12p40 (Biosource), IL-12p70 (BD bioscience) and IL-23 (eBioscience) concentrations were determined by ELISA.

Results

IFN- α , but not IFN- γ induces STAT1 phosphorylation in PBMCs from an IFN- γ R1 deficient patient.

STAT1 phosphorylation is known to be induced by both IFN- α and IFN- γ (17). STAT1 can therefore be an important factor in inducing potential overlapping effects of these two interferons. To compare IFN- α and IFN- γ induced STAT1 phosphorylation, PBMCs were stimulated with IFN- α , IFN- γ or both interferons and STAT1 phosphorylation in CD14⁺ monocytes was assessed by FACS.

In monocytes obtained from healthy donors, STAT1 phosphorylation was observed in response to IFN- α , to IFN- γ as well as to IFN- α plus IFN- γ (Fig. 1A). Of note, the response to IFN- α plus IFN- γ was not higher than the response to either interferon alone (Fig. 1A). In monocytes derived from an IFN- γ R1 deficient patient, STAT1 phosphorylation was induced in the presence of IFN- α but not in response to IFN- γ (Fig. 1B).

IFN- α does not substitute IFN- γ mediated upregulation of CD64 and CD54 cell-surface expression.

IFN- γ induces an increase in the cell surface expression of the high affinity IgG receptor, Fc γ RI (CD64) in monocytes and macrophages, enhancing phagocytosis, binding of immune-complexes, and antibody-dependent cellular cytotoxicity (18). This upregulation is mediated by IFN- γ -induced STAT1 phosphorylation and subsequent binding to the CD64 promoter, resulting in increased transcription and translation of the gene (14). Likewise, IFN- γ enhances the expression of CD54 (ICAM-1) (19). CD54 is involved in the adhesion to endothelial cells and the transmigration into tissues (20), and functions as a costimulatory molecule on APC and other cell types, to activate CD4⁺ T cells and cytotoxic CD8⁺ T cells, respectively (21).

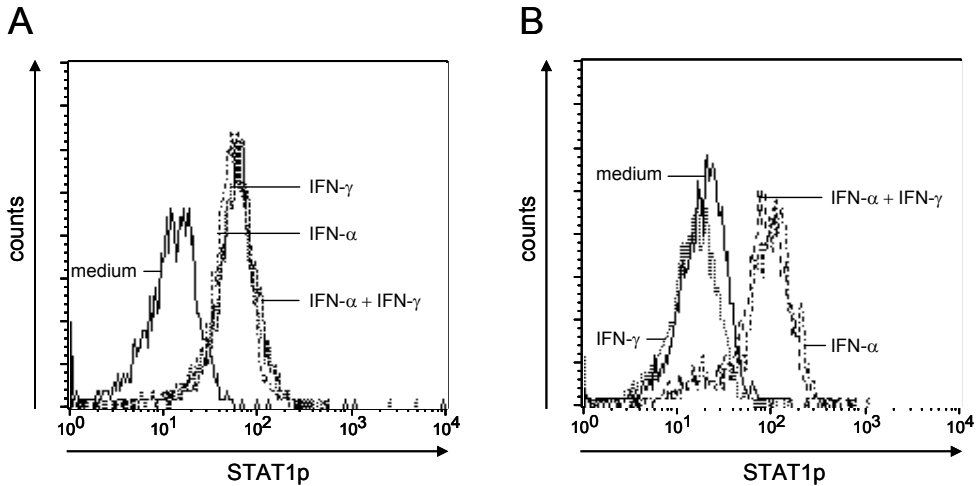


Figure 1. IFN- α induces STAT1 phosphorylation in monocytes from an IFN- γ R1 deficient patient. Healthy control (A) or patient (B) derived PBMC were stimulated with 1000 U/ml IFN- α , 2.5 ng/ml IFN- γ , 1000 U/ml IFN- α plus 2.5 ng/ml IFN- γ , or left unstimulated for 15 minutes. Cells were labelled with anti-human pSTAT1-Alexa 647 and CD64-FITC antibodies and analyzed by FACS. Histograms shown are gated on CD64 positive monocytes. In monocytes from a healthy control, IFN- α , IFN- γ and IFN- α plus IFN- γ induced STAT1 phosphorylation. In monocytes from the patient IFN- α and IFN- α plus IFN- γ induced STAT1 phosphorylation, whereas stimulation with IFN- γ alone did not.

To determine whether the IFN- α -induced STAT1 phosphorylation could also upregulate CD64 and CD54 expression, PBMCs were stimulated with IFN- α , IFN- γ , or both these interferons, and analyzed by FACS. In monocytes from healthy controls, IFN- γ enhanced the expression of CD64, whereas IFN- α did not (Fig. 2A). By contrast, in patient's monocytes lacking IFN- γ R1, neither IFN- α nor IFN- γ affected CD64 expression (Fig 2B). Moreover, upon stimulation of monocytes of healthy controls by both IFN- α plus IFN- γ , IFN- α reversed IFN- γ -dependent upregulation of CD64 (Fig. 2A). In experiments using a CD54 read-out, similar observations were made (data not shown).

IFN- α does not synergize with LPS to induce pro-inflammatory cytokines.

Cytokines such as IL-12, IL-23, TNF and IL-1 β play an important role in controlling mycobacterial infections (22,23). During an immune response, IFN- γ produced by innate immune cells provides a strong positive feedback loop to enhance secretion of IL-12, IL-23, TNF and IL-1 β by monocytes and macrophages in response to Toll-like receptor (TLR) stimuli such as lipopolysaccharide (LPS) (4-6). To compare effects of IFN- α with the effects of IFN- γ on the LPS-induced cytokine production, monocytes were stimulated with LPS in combination with IFN- α or IFN- γ and supernatants assayed for cytokine secretion.

IFN- γ enhanced the IL-12p40, IL-12p70, IL-23, TNF and IL-1 β production induced by LPS in monocytes from healthy donors (Fig. 3A-E). By contrast, IFN- α did not prime for enhance cytokine production in response to LPS in monocytes from healthy controls (Fig 3A-E). In cells obtained from an IFN- γ R1 deficient patient, IFN- α could not substitute for IFN- γ as no priming effect was observed (Fig 3F-K). Moreover, IFN- α decreased the LPS-induced IL-12p40 and IL-23 production in cells obtained from the patient (Fig 3F and 3H), as well as in control cells (Fig 3A and 3C).

No significant effect of IFN- α was manifest on LPS-induced TNF or IL-1 β production. Of note, patient-derived monocytes secreted about a four-fold higher amount of IL-12p40 in the absence of interferons as compared to control monocytes.

Opposing effects of IFN- α and IFN- γ on LPS-induced IL-12p40 and IL-23 production observed above, prompted us to assess the effects of adding both interferons, together, to stimulate pro-inflammatory cytokine release by monocytes from healthy controls. When monocytes were stimulated with LPS in combination with both interferons, IFN- α inhibited the priming effect of IFN- γ : IL-12p40, IL-12p70, IL-23, TNF and IL-1 β production were reduced compared to cells stimulated with LPS and IFN- γ (Fig. 3A-E). This inhibiting effect of IFN- α occurred in a dose-dependent fashion. When IFN- α was added 1 hour after stimulation with IFN- γ and LPS, an inhibitory effect of IFN- α was still observed, although less markedly than when both interferons were added at the same time (data not shown).

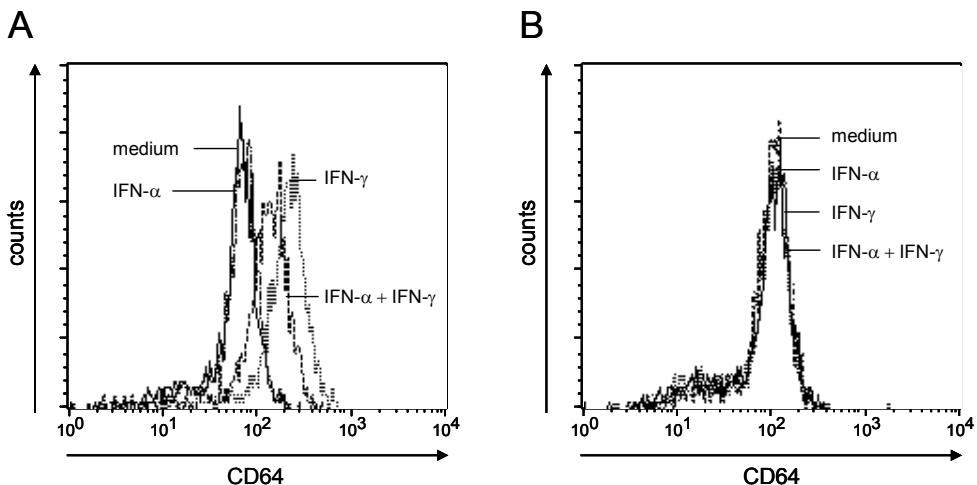


Figure 2. IFN- α does not upregulate CD64 expression. (A) PBMC obtained from a healthy control were incubated with 2.5 ng/ml IFN- γ , 1000 U/ml IFN- α or 2.5 ng/ml plus 1000 U/ml IFN- α for 24 hrs CD14⁺ monocytes were gated and analysed for CD64 expression. IFN- γ upregulated CD64 expression, whereas IFN- α had no effect on CD64 cell surface expression; IFN- α inhibited the IFN- γ induced CD64 upregulation. (B) IFN- γ R1 deficient PBMC were stimulated with IFN- γ or IFN- α , or both IFN- γ and IFN- α for 24 hours and CD64 expression on CD14⁺ monocytes was assessed by FACS. Neither IFN- γ , nor IFN- α or IFN- γ plus IFN- α upregulated CD64 expression

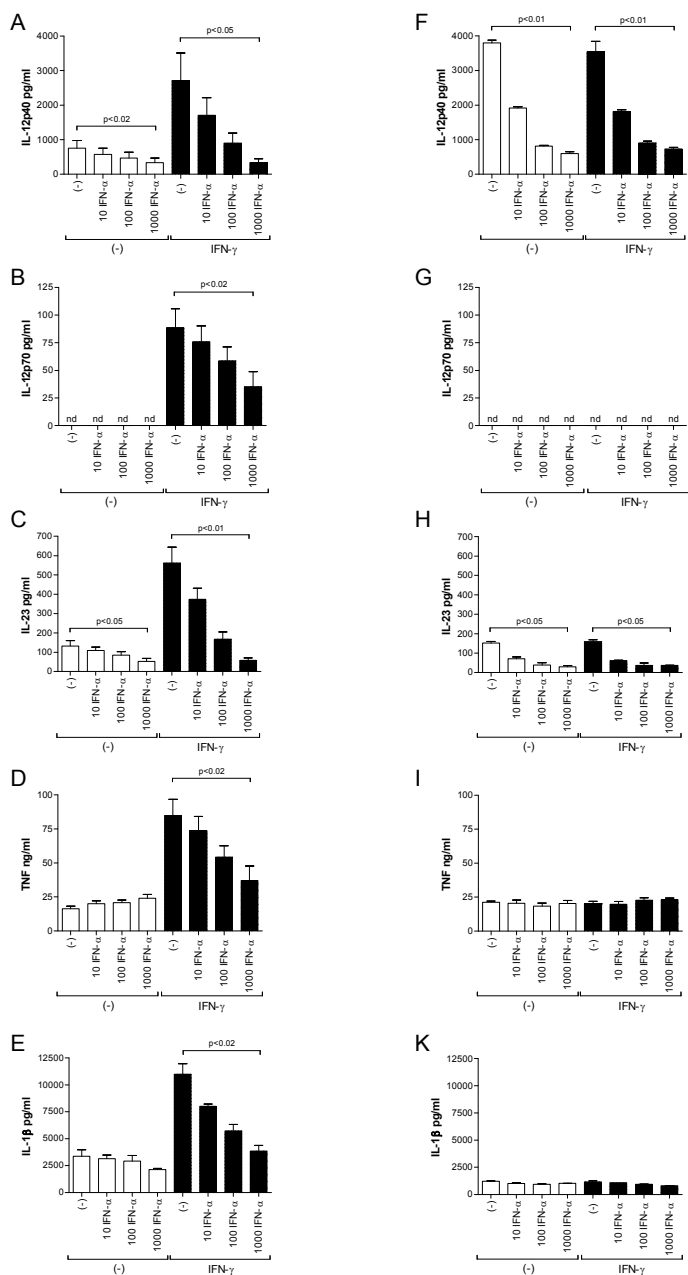


Figure 3. IFN-α does not prime monocytes for enhanced pro-inflammatory cytokine production in response to LPS. Control (A-E) and patient (F-K) derived monocytes were stimulated with 100 ng/ml LPS plus or minus indicated concentrations (in units/ml) IFN-α and/or 2.5 ng/ml IFN-γ for 24 hours. IL-12p40 (A and F), IL-12p70 (B and G), IL-23 (C and H), TNF (D and I) and IL-1β (E and K) concentrations were determined by ELISA. Depicted are average protein concentrations of 4 control donors (A-E) and average protein concentrations of triplicates of an experiment with patient derived cells (F-K). SD is indicated by the bars. nd=not detectable. For statistical analysis, two-tailed (paired) t-tests were performed.

Both IFN- γ and IFN- α inhibit LPS induced IL-10 production.

Monocytes primed with IFN- γ in a dose-dependent manner release less anti-inflammatory cytokine IL-10 in response to LPS (24), thereby favouring a pro-inflammatory response. In the IFN- γ R1-deficient patient the inhibiting effect of IFN- γ on IL-10 production is absent, which may result in relative overproduction of IL-10.

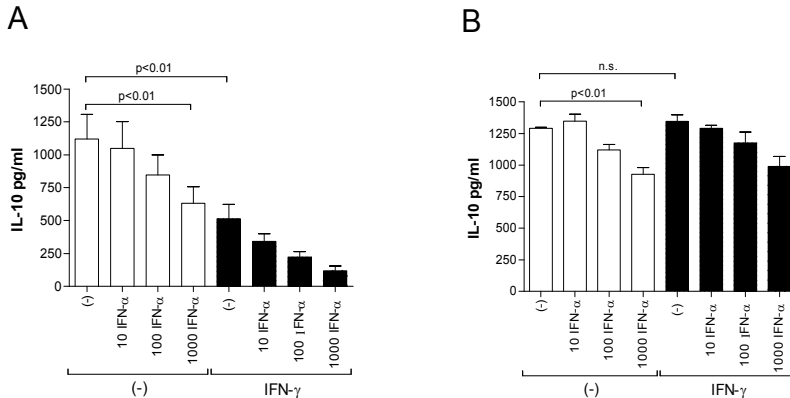


Figure 4. IFN- α inhibits LPS induced IL-10 production. Control (A) and patient (B) derived monocytes were stimulated with 100 ng/ml LPS plus or minus indicated concentrations (U/ml) IFN- α and/or 2.5 ng/ml IFN- γ for 24 hours. IL-10 concentration in supernatants was determined by ELISA. Depicted are average protein concentrations of 4 control donors (A) and average protein concentrations of triplicates of an experiment with patient derived cells (B). SD is indicated by the bars. For statistical analysis, two-tailed (paired) t-tests were performed. n.s. = not significant.

We tested whether IFN- α could substitute for absence of an IFN- γ effect in IFN- γ R1 deficient cells to reduce IL-10 production in response to LPS. Patient's monocytes were stimulated with LPS in the presence or absence of IFN- γ and/or IFN- α . In cells from healthy donors, IFN- γ and IFN- α each inhibited LPS-induced production of IL-10 while the strongest reduction of IL-10 production was observed in cells incubated with both interferons (Fig 4A). IL-10 production by IFN- γ R1 deficient monocytes in response to LPS was slightly, but significantly, inhibited by IFN- α but - as expected - not by IFN- γ . No additive effect of adding IFN- γ to IFN- α was observed (Fig. 4B) Similar inhibiting effects of IFN- γ and IFN- α on IL-10 production were observed in five experiments with LPS-stimulated whole blood of healthy controls; in a whole blood assay with patient-derived blood, no effect of IFN- γ was seen, while IFN- α inhibited LPS induced IL-10 production (data not shown).

*IFN- γ but not IFN- α induces killing of intracellular *M.smegmatis* in human macrophages.*

To determine whether IFN- α could induce killing of intracellular pathogens in macrophages, as does IFN- γ we tested the effect of both interferons on the intracellular survival of *Mycobacterium smegmatis*. Pro-inflammatory macrophages isolated and differentiated from healthy donors were

pre-stimulated with IFN- α , IFN- γ or both interferons and subsequently infected with *M. smegmatis*-GFP. As a control, infected macrophages were treated with H-89, a kinase inhibitor known to inhibit intracellular growth of mycobacteria (25). The intracellular load of *M. smegmatis* was assayed by fluorimetry. As expected, H-89 severely reduced intracellular growth of *M. smegmatis* (Fig 5). IFN- γ treatment dose-dependently induced mycobactericidal activity in macrophages whereas IFN- α showed no such activity. Additionally, upon treatment with both interferons IFN- α acted to reverse mycobactericidal effects of IFN- γ (Fig. 5).

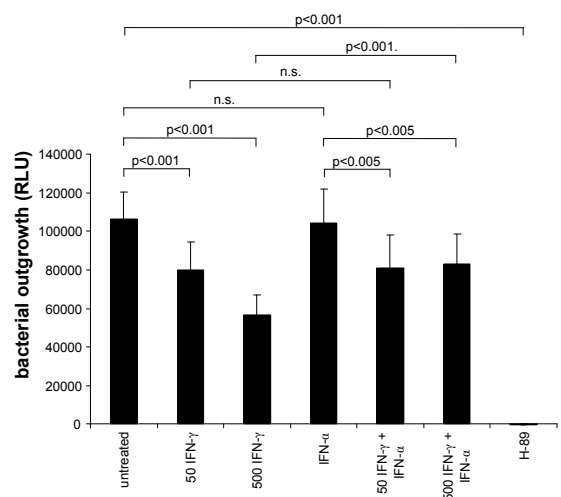


Figure 5. IFN- γ but not IFN- α , induces bactericidal activity to *M. smegmatis* in infected macrophages. Cultured pro-inflammatory macrophages were stimulated with IFN- α (1000 U/ml), IFN- γ (50 or 500 U/ml), IFN- α plus IFN- γ , H-89 (10 μ M) or left unstimulated for 16 hours prior to infection with *M. smegmatis*-GFP at a MOI of 1. Intracellular growth of *M. smegmatis*-GFP was assessed by fluorimetry 24hrs post infection. Data shown is average of 13 data points on duplicate experiments. For statistical analysis, non-parametric Mann-Whitney tests were performed. n.s. = not significant.

Discussion

The main observation in the present study is that IFN- α lacks the ability to compensate or substitute for an absence of the IFN- γ -activating effect in monocytes and macrophages of a patient with complete IFN- γ R1 deficiency. This lack of an activating effect of IFN- α was demonstrated in respect to the LPS-induced pro-inflammatory cytokine release by these cells, the upregulation of CD64 and CD54 cell surface expression, as well as engagement of bactericidal processes, all considered markers of IFN- γ activation signalling. Moreover, in cells from healthy controls we observed a negative effect on LPS induced IL-12p40 and IL-23 production and cell surface marker upregulation upon adding IFN- α to IFN- γ -stimulated cells. Thus, although we confirm that IFN- α just like IFN-

γ was capable of inducing STAT1 phosphorylation, our findings do not support the use of IFN- α in patients lacking the ability to respond to IFN- γ .

Ward et al. describe adjunct treatment of IFN- α in a patient with complete IFN- γ R1 deficiency suffering from disseminated infection with *Mycobacterium avium* complex (MAC) resistant to multiple anti-mycobacterial agents (10). Although the patient continued to be mycobacteremic, they observed reduced hepatosplenomegaly and lymphadenopathy which they credited to the additional IFN- α treatment. In addition, Rapkiewicz et al. describe an IFN- γ R2 deficient child with disseminated MAC who was treated with antimycobacterials in combination with IFN- α , GM-CSF or IFN- γ (11). The authors observed a better defined granulomatous inflammation and clinical improvement after he received IFN- α in combination with GM-CSF, although, the contribution of IFN- α to the observed effects of combined therapy is difficult to establish; this patient subsequently died during a second episode of IFN- α treatment (11). The authors of both reports hypothesized that the beneficial effects of IFN- α could be attributed to its activation of STAT1 and downstream signalling pathways shared by IFN- α and IFN- γ (10,11).

In line with our observations, IFN- α was previously found to negatively regulate IL-12 as well as the resulting IFN- γ production in mice, *in vitro* and *in vivo* (26-28). For instance, hypervirulent strains of *M. tuberculosis* isolates were shown in mouse models to induce elevated levels of IFN- α mRNA with a concordant depression in Th1 response (28). Additionally, neutralization of IFN- α/β in similar mouse models led to a significant increase in survival of animals and a corresponding increase in IL-12p40 mRNA in the lungs (29). Therefore, in IFN- γ R1 deficient patients, inhibition of IL-12 and IL-23 production by IFN- α administration may reduce the (IFN- γ independent) induced protective effects of these two cytokines. In mice, IL-23 is needed for the induction of IL-17 producing *M. tuberculosis* specific T cells in the lung and for the recall response of these cells after infection with *M. tuberculosis* (30). The Th17 recall response precedes the Th1 recall response and in the absence of IL-23 both these recall responses are diminished, resulting in a diminished clearance of *M. tuberculosis* from the lungs (30). These observations suggest that the inhibition of IL-23 production by IFN- α may have a negative effect on bacterial clearance.

IFN- α also negated the bactericidal activity of IFN- γ . In line with these observations, type I interferons are reported to enhance the intracellular replication of *M. bovis* in human monocytes and macrophages (31).

Several case reports described the appearance of mycobacterial infections in patients receiving IFN- α treatment; however, also large numbers of patients are treated with IFN- α apparently without acquiring mycobacterial infections (32-34). Administration of aerosolized IFN- α in an uncontrolled setting appeared to have a slight beneficial effect in patients suffering pulmonary tuberculosis, but randomized controlled trials have not been done (35). In five patients with

advanced intractable multidrug-resistant pulmonary tuberculosis who were treated with IFN- α no positive effect was observed (36).

Despite the fact that both IFN- α and IFN- γ cause STAT1 phosphorylation, the effects of these interferons differed considerably. One explanation for the observed differences is that phosphorylated STAT1 is incorporated in different complexes; mainly STAT1 homodimers after IFN- γ stimulation versus mainly STAT1/STAT2/IRF-9 complexes (ISGF3) after IFN- α stimulation. Both complexes can bind and activate promoters of interferon-responsive genes: STAT1 homodimers mainly activate promoters that contain gamma interferon activation site (GAS) elements, while ISGF3 mainly activates promoters that contain interferon-stimulated responsive elements (ISRE). The inhibitory effect of IFN- α on IFN- γ priming of cells may be explained by the competition for signalling molecules such as STAT1 when both stimulations are present at the same time. It would therefore be interesting to determine the balance between STAT1/STAT1 homodimers and STAT1/STAT2 after stimulation of cells with either IFN- γ or IFN- α .

Enhanced production of pro-inflammatory cytokines such as IL-12, IL-23 and TNF and IL-1 β in response to IFN- γ is absent in IFN- γ R1 deficient patients, thus leading to low levels of these cytokines during infection. Pro-inflammatory cytokines are required to mount an effective protective immune response against mycobacterial infection (23) and patients receiving anti-TNF treatment are more susceptible to mycobacterial infections (37). Adjunct treatment with cytokines such as IL-12, IL-23, TNF and IL-1 β may have beneficial effects on the eradication of mycobacterial infection.

Puzzlingly, monocytes derived from the IFN- γ R1 deficient patient produced higher amounts of IL-12p40 subunit in response to LPS compared to control derived monocytes. The reason for this is unclear. It can be hypothesized that due to a lack of IFN- γ stimulation of the IFN- γ R1 deficient monocytes these cells may have developed a higher IL-12p40 production in response to LPS, either due to the lack of an - as yet unknown - negative feedback of IFN- γ on IL-12p40 production, or as an active compensation mechanism. However, no IL-12p70 production by patient derived cells was observed and the IL-23 production in response to LPS by patient derived monocytes was comparable to that of control cells, suggesting the higher IL-12p40 production does not amount to higher production of a functional cytokine. The IL-12p40 homodimer that is reportedly produced in mice (38) and that could potentially be formed by the IL-12p40 molecules has not been detected in human cells (39).

Taken together, our study did not find support for the proposed positive effect of IFN- α treatment in mycobacterial infections while inhibitory effects of IFN- α on pro-inflammatory cytokine production and inhibition of bactericidal activity may even be counter-productive. Without evidence backing its merit, we feel one should restrain from using IFN- α as additional treatment in IFN- γ R deficient patients suffering from mycobacterial infection.

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Summary and General Discussion

Host defense against intracellular bacterial pathogens such as *Salmonellae* and *Mycobacteria* critically depends on the integrity of the type-1 cytokine pathway to macrophage activation. Generally spoken, this pathway is initiated by bacterial binding to pattern recognition receptors on APC, resulting in the production of interleukin-12 (IL-12), IL-18 and IL-23. IL-12 and IL-18 subsequently induce IFN- γ production in NK cells and Th1 cells by binding to their respective receptors, while IL-23 is known to induce IFN- γ production in naïve T cells and in memory T cells. IFN- γ in turn binds to the IFN- γ R on macrophages and dendritic cells to enhance their bactericidal activity and antigen processing and presentation, respectively, and increase production of IL-12. Macrophage's bactericidal activity is enhanced by IFN- γ stimulated release of TNF that acts on the macrophage in an autocrine fashion. Moreover, these mediators orchestrate macrophages and lymphocytes to form tissue granulomas into which the intracellular bacterial pathogens are restrained and separated from the normal tissue.

Until recently, it was thought that IL-12, produced by APCs like monocytes, macrophages and DCs upon stimulation by bacterial pathogens or lipopolysaccharide (LPS), acts as the cytokine initiating Th1 differentiation, thereby bridging the innate and adaptive immune responses. However, for the production of IL-12 in response to pathogens, the Th1 cytokine IFN- γ is needed. IFN- γ knock-out mice barely produce IL-12 in response to LPS unless costimulated by exogenous IFN- γ , underscoring that the prior presence of IFN- γ is necessary for substantial production of IL-12. This indicates that IL-12 has no role in initiating Th1 differentiation, but that its function is to sustain an ongoing Th1 response once it has been initiated by other means. We hypothesized that IL-23 might be an important factor in the initiation of Th1 differentiation, as IL-23 rather than IL-12 is the first type 1 cytokine released by activated pro-inflammatory macrophages. In **chapter 2** we show that monocytes and macrophages produce IL-23, but not IL-12, in response to a variety of TLR agonists, as well as live *Salmonella*. In combination with IL-18 or IL-1 β , IL-23 induces IFN- γ production in CD56⁺ NK-like T cells and CD56⁺ NK cells (**chapter 1 and 2**), even in the absence of T cell receptor (TCR stimulation). Like IL-23, IL-1 β and IL-18 are produced by monocytes and macrophages in response to infection with *Salmonella* in vitro (**chapter 2**). These data indicate that IL-23, IL-1 β and IL-18 are produced early in infection and that these cytokines can induce IFN- γ production without the need of a specific immune response (e.g. as evidenced by need for TCR stimulation). Previously it was described that IFN- γ production by NK-like T cells can be triggered by either IL-12/IL-18 stimulation or TCR stimulation. In addition, we now showed that supernatants of *Salmonella* infected macrophages induced IFN- γ production in an IL-23 and IL-1 β dependent manner in CD56⁺ NK and NK-like T cells (**chapter 3**). Together, these results indicate that cytokines which are produced early in an immune response can induce the Th1 cytokine IFN- γ , independently of IL-12. IFN- γ is essential

for IL-12 production in response to stimulations such as LPS, further amplifying the release of IFN- γ . In addition, we showed that the IFN- γ containing supernatants of IL-23/IL-18 activated CD56⁺ cells prime monocytes for LPS induced IL-12 production.

Apart from IFN- γ , IL-23 induced GM-CSF production in CD56⁺ cells, when combined with IL-18 or IL-1 β . GM-CSF is used to generate type 1 macrophages in vitro, capable of producing large amounts of IL-23. We tested the effects of IFN- γ and GM-CSF on the production of IL-23 (**chapter 3**). Both cytokines enhanced IL-23 production, implying a positive feedback loop, in which IL-23 can enhance its own production via the induction of IFN- γ and GM-CSF. In addition, IFN- γ and GM-CSF can be induced by IL-23 and both these factors are capable of facilitating IL-12 production in response to pathogens.

The role of IL-23 and its downstream cytokines has recently been investigated in a variety of infection models using IL-23p19 knockout mice. Most of the IL-23 research focuses on the role of this cytokine in the development of a Th17 response. Our results demonstrate a role for IL-23 in development of a Th1 response as well. Likely, both these types of immune responses are regulated by IL-23, suggesting an intricate link between Th17 and type-1 cytokine pathways.

CD56⁺/CD3⁺ NK-like T cells are likely to play a protective role against mycobacterial infection. In **chapter 1**, we describe that IL-23 modulates the function of these NK-like T cells. CD56⁺/CD3⁺ T cells can be divided in CD4⁺ and CD8⁺ cells; however, we did not investigate which of these populations responded to IL-23. It would be interesting to determine the IL-23 responsive populations and their exact functions. For example, in both CD4⁺ and CD8⁺ T cells, CD56 expression is associated with cytotoxic T cell function. In future studies, cytotoxicity assays could address whether IL-23 influences the cytotoxic capacity of these cells.

Genetic variations in the Th1 pathway.

The development of a protective immune response to a pathogen depends on the crosstalk between pathogen and host, and to a large extent is determined by their genetic make-up. As far as the host reaction to mycobacterial and salmonella infections is concerned, polymorphisms and mutations in genes encoding the components of the Th1 pathway are expected to influence the generation of an adequate protective response. In this respect, however, little is known in detail about the complex relationship of clinical phenotype (e.g., chance of developing disease after exposure, severity and characteristics of disease) and host genotype. Often, analyses of the genotype-phenotype relationship in infections are performed in population studies, examining the occurrence of particular genetic variations in a case-control setting. It must be realized, however, that many processes complicate such analyses, e.g., unknown size of inoculums, possibility of multiple exposures, influence of co-morbidity, concurrent treatments, etc. Furthermore, in mycobacterial infections for instance, a distinction must be made between infection after exposure (e.g., evidenced by change in

Mantoux skin reactivity only); progression to clinical disease after infection; clinical characteristics and course of the disease, and different outcome measures like spontaneous recovery, progressive disease or death. Therefore, case-control studies that 'simply' compare 'patients with clinical tuberculosis' to those without signs of disease, without taking into account specific disease characteristics and determination who became infected but escaped disease, often lack realism.

Clearly, clinical expression of disease and severity of immunopathology depends on the cross-talk between *M. tuberculosis*, with its specific virulence characteristics and invasiveness, and a host immune response comprising innate as well as adaptive elements the activity of many of which is genetically preset. Thus, variability between individuals in clinical outcome results at least in part from variability in the genes that control the host defense processes. However, the influence of host genetic factors as weighed against environmental factors on an individual's susceptibility is a matter of debate. Genetic factors can be of decisive importance in the extreme susceptibility of rare, selected cases and in **chapter 3** and **4** we analyzed the functional consequences of genetic variability in two components central to type-1 cytokine pathway, the IL23R and IFN- γ R1; in case of malfunctioning of these receptors, patients are highly prone to infection by intracellular bacterial pathogens like mycobacteria and salmonella but the consequence of various genetic variations in the respective genes is not yet clear.

Polymorphisms in the IL-23R chain may influence the cellular response to IL-23. Two polymorphisms occur at relatively high frequency in the population and were investigated first. The polymorphism P310L occurs at a frequency of 2–30% and the R381Q polymorphism at a frequency of 0–17%, somewhat depending on the population background. In population studies, the R381Q allele appears to confer protection against several inflammatory states like inflammatory bowel disease, psoriasis, ankylosing spondylitis, and graft versus host disease after bone marrow transplantation. The P310L allelic variant was reported to be overrepresented in patients with Grave's disease. Indeed, indirect evidence for a role of the type-1 cytokine pathway in the immunopathogenesis of inflammatory diseases is provided by the effectiveness of treatment with anti-TNF antibodies like Infliximab in many of these disease states. In view of these associations with diseases, it was suggested that the R381Q and P310L variants of the IL-23R might be functionally distinct. In **chapter 3** we show that there are no functional differences between these two variants in the IL-23-induced STAT phosphorylation, IFN- γ induction, or T cell proliferation. Furthermore, we tested a newly identified Y173H variant of the IL-23R, which also did not show functional differences when compared to the wild type receptor. Combined with the IL-12R β 1, IL-23R forms the functional IL-23 receptor complex. Signal transduction of IL-23 is dependent on either chain, indicating that polymorphisms of both chains may influence IL-23 responsiveness. We therefore tested whether common IL-12R β 1 haplotypes (QMG and RTR) differentially influenced the IL-23 response. Again, no major functional differences were detected in the IL-23 responses between various combinations of IL-12R β 1 and IL-23R chains. These results indicate that the

association reported in the literature concerning the IL-23R haplotypes and protection or increased susceptibility to disease can not readily be explained by differences in the function of the IL-23R variants after binding of their natural ligand. One explanation for this is that the observed haplotypes may be due to variations that are merely linked to the single nucleotide polymorphism (SNP) studied. The role of the IL-23R variants in infections is unknown.

Alternatively, the readout of the test system we used may be too limited to detect subtle, functional differences between the IL-23R variants. The exact mechanisms of signal transduction of the IL-23R are not precisely known. Better knowledge of the signal transduction mechanisms may provide the information needed to generate a more complete and sensitive readout system. For example, the human IL-23R cytoplasmic domain contains seven tyrosine residues, six of which are conserved in murine IL-23R. It would be interesting to determine whether these tyrosines can be phosphorylated upon stimulation and if so, what their role in the signal transduction of IL-23 is. In addition, since no specific antibody is available to stain for IL-23R expression, we were not able to test for the influence of the IL-23R polymorphisms on cell surface expression. The development of a specific IL-23R antibody would provide researchers the ability to study the regulation and the expression patterns of this receptor in more detail.

In **chapter 4** we compared the effect of two newly discovered IFN- γ R1 variations, identified in patients with mycobacterial infections (S149L, I352M), four known polymorphisms (V14M, V61I, H335P, L467P), all seven reported missense mutations (V61Q, V63G, Y66C, C77Y, C77F, C85Y, I87T) and the 818delTTAA mutation on the expression and function of IFN- γ R1 in the same genetic background. The newly discovered IFN- γ R1 variants, S149L and I352M, as well as the known V14M, V61I, H335P and L467P IFN- γ R1 variants do not functionally differ from the wild type receptor. Expression on the cell surface of V14M is reduced which may result in slightly reduced IFN- γ responses, when IFN- γ R1 gene transcription *in vivo* is limited to natural numbers of the receptors. This polymorphism may influence susceptibility to infections or predisposition to autoimmune disease such as systemic lupus erythematosus. The other variants are deleterious mutations with V61E, V61Q, Y66C, C77F, C77Y and C85Y leading to complete IFN- γ R1 deficiency, while V63G and I87T lead to partial IFN- γ R1 deficiency.

Complete IFN- γ R1 deficiency is characterized by severe infections with environmental mycobacteria or *M. bovis* BCG and patients with these deficiencies usually present within the first 5 years of life. In case of active infection, patients with partial IFN- γ R deficiencies can benefit from treatment with IFN- γ . By contrast, patients with complete IFN- γ R1 deficiencies are not able to respond to IFN- γ and thus will not benefit from treatment with recombinant IFN- γ . In **chapter 5** we evaluated whether IFN- α could compensate for the absence of IFN- γ effects in cells obtained from an IFN- γ R1 deficient patient. IFN- α and IFN- γ activate common signaling pathways and the induced

genes and biological activities they induce overlap. Like IFN- γ , IFN- α induces STAT1 phosphorylation in its target cells. Therefore, treatment with exogenous IFN- α might (partly) compensate for the absent effect of IFN- γ in patients with a deficiency of the IFN- γ R. However, IFN- α could not compensate for the abrogated IFN- γ effects; despite the fact that IFN- α induced STAT1 phosphorylation in cells of an IFN- γ R1 deficient patient, IFN- α did not upregulate CD64 and CD54 expression, both considered markers of IFN- γ activation signaling. Furthermore, IFN- α did not prime for enhanced cytokine production in response to LPS. In control cells, IFN- α even antagonized the IFN- γ induced upregulation of CD64 and the priming effect of IFN- γ on LPS induced cytokine production. In patients suffering complete IFN- γ R deficiencies this is not likely to be a problem; however, in patients with partial deficiencies IFN- α may abrogate the rest response to IFN- γ .

IFN- α is used to treat various diseases, including hepatitis B and C infection, hairy cell leukaemia and multiple myeloma. One patient has been reported in whom reactivation of severe, acute pulmonary tuberculosis was seen during treatment with IFN- α for chronic HCV hepatitis. In addition, in a clinical trial of 34 hairy cell leukaemia patients treated with IFN- α in combination with deoxycoformycin, one case of *M. avium* infection was reported. Despite the case reports describing the appearance of mycobacterial infections in patients receiving IFN- α treatment, many patients are treated with IFN- α apparently without acquiring mycobacterial infections. In addition, IFN- α has been used in the treatment of mycobacterial infections in non-MSMD patients. Administration of aerosolized IFN- α in an uncontrolled setting appeared to have slight beneficial effects in patients suffering pulmonary tuberculosis, but randomized controlled trials have not been done. In another study of five patients with advanced intractable multidrug-resistant pulmonary tuberculosis treated with IFN- α no positive effect was observed. Concluding, the use of IFN- α does not seem to be a risk factor to acquire mycobacterial disease, however, on the other hand, IFN- α does not seem to have an abeneficial effect in the treatment of mycobacterial disease.

Despite the fact that we mainly observed anti-inflammatory effects of IFN- α on these cells *in vitro*, it is difficult to establish the net effect of the additional treatment with IFN- α in IFN- γ R deficient patients suffering infections *in vivo*. Therefore, it would be interesting to test the effect of various cytokines, including IFN- α , *in vivo*. A randomized trial in which mycobacterial infections in IFN- γ R deficient patients would be treated with IFN- α would provide most information, however, the number of patients is too low to set up a study with enough statistical power. Firstly, however, the effect of recombinant IFN- α treatment on the mycobacterial clearance and survival of IFN- γ R deficient mice infected with mycobacteria should be investigated by the effects of IFN- α on the mycobacterial clearance and survival of infected mice.

In conclusion, too little is known about the safety and the net effects of IFN- γ when used as an adjuvant treatment of (mycobacterial) infections in IFN- γ R deficient patients. Therefore one should be careful in the use of IFN- α in the treatment of infections in these patients.

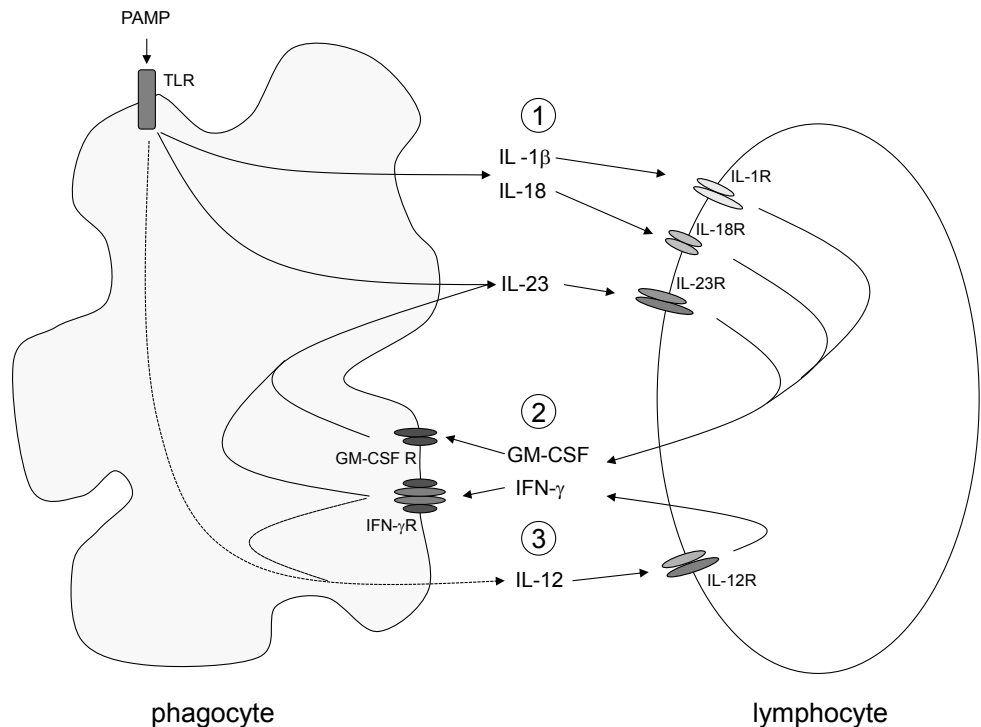


Figure 1. Initiation of a Th1 response. (1) In response to pathogen associated molecular patterns (PAMPs) phagocytes like monocytes and macrophages start to produce IL-23, IL-1 β and IL-18, but no IL-12. (2) IL-23, in combination with IL-1 β and IL-18, induces IFN- γ and GM-CSF production in lymphocytes. (3) This lymphocyte derived IFN- γ , in combination with PAMPs, allow for subsequent IL-12 production by monocyctic cells. IFN- γ and GM-CSF serve in a positive feedback loop: both cytokines enhance PAMP induced IL-23 production.

Concluding remarks

This thesis describes the role of IL-23 in the Th1 immune response. Generally, IL-12 is thought to be the primary trigger that initiates Th1 differentiation and IFN- γ production. However, for the induction of IL-12 production, the Th1 cytokine IFN- γ is needed. In this thesis we show that IL-23 can induce initial IFN- γ production in CD56⁺ cells, thereby providing the stimulus needed for subsequent IL-12 production by APCs in response to PRRs. This means that IL-23 may be important in initiating a Th1 immune response. Despite the fact that IL-23 is able to induce IFN- γ production in various cells, IL-23 probably is not indispensable to mount a Th1 response. Other cytokines induced early in infection, like IL-27, are reported to induce IFN- γ production as well. The cytokines involved in the

initiation of the Th1 response still need to be explored further. For example, the development of T helper cell responses in IL-23p19/IL-27 double knockout mice in response to infectious agents could provide additional insight in the role of these two cytokines in the induction of a Th1 response.

Concluding, we propose a model in which IL-12 is involved in the maintenance of an ongoing Th1 response, while other cytokines including IL-23 are involved in the initiation of a Th1 immune response (fig. 1)

Nederlandse samenvatting

Het immuunsysteem

Het immuunsysteem is essentieel voor de mens om te overleven. Het immuunsysteem verdedigt het lichaam tegen pathogenen zoals virussen en bacteriën die het lichaam binnen dringen. Tevens beschermt het ons tegen de ontwikkeling van kanker door ontspoorde cellen van het eigen lichaam op te ruimen. De huid en de mucosale membranen, zoals in de darm, voorkomen dat ziekteverwekkers het lichaam binnendringen. In het geval deze eerste verdedigingslijn wordt doorbroken, dan zal ons immuunsysteem proberen de ziekteverwekker uit te schakelen. Het immuunsysteem kan worden onderverdeeld in een aspecifiek (aangeboren) en een adaptief specifiek (verworven) deel. De specifieke afweer heeft dagen tot weken nodig om een volledig effect te hebben. De aspecifieke afweer daarentegen werkt direct, maar is minder effectief.

De aspecifieke afweer.

De eerste verdedigingslijn tegen ziekteverwekkers die het lichaam zijn binnengedrongen wordt gevormd door het aspecifieke immuunsysteem. Het aspecifieke immuunsysteem heeft zich ontwikkeld om zo snel mogelijk een enorm breed scala aan pathogenen te kunnen herkennen. Het systeem bestaat uit zowel een humoraal deel, als een cellulair deel. Het humorale deel van de afweer bestaat uit eiwitten die zich in lichaamsvloeistoffen bevinden, zoals bijvoorbeeld antilichamen. Het complement systeem is een belangrijk onderdeel van de humorale arm. Het complement systeem bestaat uit een reeks eiwitten die in het bloed na activatie een cascade vormen en aanleiding geven tot lysis van pathogenen. Daarnaast leidt activatie van het complement tot het aantrekken van ontstekingscellen. Fagocytose (= het internaliseren van pathogenen door cellen) is een van de eerste stappen in een immuun reactie. Fagocyten zoals monocyten en macrofagen brengen receptoren tot expressie op het cel oppervlak waarmee ze ziekteverwekkers kunnen herkennen. De aspecifieke afweer werkt met receptoren die verschillende structuren die in vele pathogenen voorkomen herkennen. Binding van ziekteverwekkers aan de receptoren leidt tot internalisatie en uiteindelijk tot de destructie van de ziekteverwekker. Tegelijkertijd geeft de fagocyt informatie aan andere cellen van het immuunsysteem over de ziekteverwekker.

De specifieke afweer.

Als ziekteverwekkers niet kunnen worden uitgeschakeld door het aspecifieke deel van de afweer, dan komt het specifieke deel van de afweer in actie. Dit deel van het afweer systeem zorgt voor een sterkere en specifiekere respons. Net als het aspecifieke immuunsysteem bestaat het specifieke deel van het immuunsysteem uit een cellulaire en een humorale compartiment. B-cellen en T-cellen zijn de voornaamste witte bloedcellen die deel uitmaken van het specifiek afweer systeem. B-cellen produceren specifieke antilichamen die ziekteverwekkers kunnen binden en daarmee de uitschakeling van deze ziekteverwekkers kunnen bevorderen. Antilichamen zelf maken deel uit van

het humorale deel van de specifieke afweer. T-cellen hebben een belangrijke rol in het uitschakelen van geïnfecteerde cellen. Tevens reguleren deze cellen de immuun respons.

De aspecifieke en de specifieke immuun respons zijn niet simpelweg opeenvolgende afweerreacties, maar twee met elkaar verweven mechanismen die elkaar reguleren en beïnvloeden. Cellen van het aspecifieke immuunsysteem bijvoorbeeld, voorzien cellen van het specifieke immuunsysteem van informatie over de ziekteverwekker middels oplosbare signaalstoffen en cel-cel contact. Een van deze signaalstoffen is interferon gamma (IFN- γ).

Interferon gamma.

Het immuunsysteem heeft verschillende repertoires om verschillende pathogenen te bestrijden.

IFN- γ speelt een centrale rol in de aspecifieke en in de specifieke immuun respons tegen een verscheidenheid aan pathogenen. Een immuun respons welke wordt gedomineerd door IFN- γ wordt een type-1 ofwel een Th1 respons genoemd. IFN- γ geproduceerd door Th1 cellen heeft vele verschillende effecten op meerdere celtypen. IFN- γ zet macrofagen bijvoorbeeld aan tot het uitschakelen van pathogenen die zij hebben gefagocyteerd. Tevens zorgt het ervoor dat macrofagen delen van het pathogeen beter presenteren op hun oppervlak, zodat o.a. T cellen hierdoor weer beter kunnen worden geïnformeerd over het pathogeen wat het lichaam is binnengedrongen. De productie van IFN- γ wordt door meerdere factoren gereguleerd. IL-12 wordt gezien als het belangrijkste IFN- γ inducerende cytokine gezien, echter, voor de productie van IL-12 zelf is IFN- γ nodig. Het is niet goed bekend hoe vroeg in een immuunrespons de IFN- γ productie wordt gereguleerd.

Het 2^e en 3^e hoofdstuk beschrijven hoe monocyt en macrofagen IL-23 en geen IL-12 produceren na infectie met *Salmonella* of na stimulatie met verschillende pathogene structuren. Naast IL-23 produceren monocyt en macrofagen IL-1 β en IL-18. Het is bekend dat IL-12, in combinatie met IL-18 of IL-1 β , de productie van IFN- γ kan induceren. In het 3^e hoofdstuk wordt beschreven hoe IL-23, in combinatie met IL-1 β en/of IL-18, de productie van IFN- γ induceert in CD56⁺ NK-achtige T cellen en in NK cellen, zonder dat daarbij specifieke stimulatie van de T cel receptor nodig is. Deze resultaten laten zien dat de door infectie met *Salmonella* geïnduceerde cytokines IL-23, IL-1 β en IL-18 samen de productie van IFN- γ kunnen induceren, zonder dat daarvoor IL-12 nodig is. Het door deze combinatie van cytokines geïnduceerde IFN- γ maakt vervolgens de productie van IL-12 mogelijk. Ook zorgde stimulatie van CD56⁺ cellen met IL-23 in combinatie met IL-1 β of IL-18, voor de productie van GM-CSF. Van GM-CSF is bekend dat het differentiatie van monocyt in IL-23 producerende pro-inflammatoire macrofagen stimuleert. In hoofdstuk 3 wordt getoond dat zowel IFN- γ , als GM-CSF, de productie van IL-23 door monocyt in respons op verschillende stimulaties

versterkt. Dit impliceert dat er sprake is van een zogenaamde positieve terugkoppeling, waarin IL-23 zijn eigen productie kan versterken via de inductie van IFN- γ en GM-CSF.

Genetische variaties.

In het 4^e en 5^e hoofdstuk wordt beschreven hoe genetische varianten van de IL-23R en de IFN- γ R1 de respons op respectievelijk IL-23 en IFN- γ beïnvloeden.

In hoofdstuk 4 worden verschillende varianten van de IL-23R functioneel met elkaar vergeleken. Samen met de IL-12R β 1 keten, vormt de IL-23R het IL-23 receptorcomplex. Van de R381Q en de P310L varianten is beschreven dat deze geassocieerd zijn met auto-immuun ziekten. Dit suggereert dat deze varianten functioneel verschillen van de *wild-type* receptor. Om de verschillende receptor varianten functioneel te vergelijken met de *wild-type* receptor hebben wij deze varianten en een nieuw ontdekte variant, namelijk de Y173H, gekloneerd en tot expressie gebracht in T-cel blasten. Echter, in T-cel blasten konden wij geen verschillen in signaaltransductie aantonen. Ook vonden wij geen significante verschillen in de door IL-23 geïnduceerde cytokine productie. Wij concluderen dan ook dat de beschreven associatie tussen de R381Q en P310L varianten van de IL-23R en auto-immuun ziekten niet wordt bepaald door functionele verschillen van deze receptor varianten. De door ons nieuw beschreven variant Y173H verschilde functioneel niet van de *wild-type* receptor

Hoofdstuk 5 toont het effect van genetische variaties van de IFN- γ R1 op de signaaltransductie en cellulaire effecten van IFN- γ . Tegen eenzelfde genetische achtergrond hebben wij twee nieuw ontdekte IFN- γ R1 variaties, ontdekt in patiënten met mycobacteriële infecties, vier bekende polymorfismen (V14M, V61I, H335P, L467P), en alle zeven gerapporteerde missense mutaties (V61Q, V63G, Y66C, C77Y, C77F, C85Y, I87T) en een mutant met een deletie (818delTTAA), in functie met elkaar en de *wild-type* receptor vergeleken. De twee nieuw ontdekte mutanten, S149L en I352M, evenals de V14M, V61I, H335P en L467P verschilden in functionaliteit niet van de *wild-type* receptor. De V14M expressie op het cel oppervlak was significant minder. De andere varianten zijn mutanten waarvan V61E, V61Q, Y66C, C77F, C77Y en C85Y resulteren in complete IFN- γ R1 deficiëntie, en V63G en I87T leiden tot een partiële IFN- γ R1 deficiëntie.

Complete IFN- γ R deficiëntie wordt gekarakteriseerd door ernstige infecties met mycobacteriën of *M. bovis* BCG (vaccinatiestam). Patiënten met een partiële IFN- γ R deficiëntie kunnen baat hebben bij behandeling met recombinant IFN- γ tijdens infecties. Patiënten die een complete IFN- γ R deficiëntie hebben, hebben geen baat bij behandeling met recombinant IFN- γ , simpelweg omdat de receptor niet functioneert. IFN- α vertoont qua signaal transductie veel overeenkomsten met IFN- γ ; beiden induceren de fosforylatie van het signaaltransductiemolecuul STAT1. Vanwege deze overlap zou IFN- α mogelijk (deels) kunnen compenseren voor de afwezige effecten van IFN- γ in patiënten met een complete IFN- γ R deficiëntie. In verschillende artikelen wordt gesuggereerd dat tijdens mycobacteriële infecties het geven van IFN- α aan patiënten die een defect

van de IFN- γ R hebben, een gunstig effect zou hebben. In hoofdstuk 6 is gekeken of IFN- α kan compenseren voor de afwezige effecten van IFN- γ in cellen van een patiënt met een complete IFN- γ R1 deficiëntie. Ondanks het feit dat IFN- α STAT1 fosforylatie induceerde in cellen van de patiënt, zorgde stimulatie van cellen met IFN- α niet voor de opregulatie van de membraangebonden activatie markers CD54 en CD64, waarvoor IFN- γ normaal zorgt. Ook kon IFN- α niet substitueren voor de afwezigheid van een IFN- γ effect in het versterken van de productie van bepaalde cytokine in respons op het bacteriële lipopolysaccharide (LPS). In cellen van gezonde donoren inhibeerde IFN- α zelfs effecten van IFN- γ . Concluderend kan gesteld worden dat er *in vitro* geen aanwijzingen zijn dat het geven van IFN- α tijdens infecties aan patiënten met een complete IFN- γ R deficiëntie nuttig is.

Conclusies

Dit proefschrift beschrijft de rol van IL-23 in de Th1 immuun respons. In het algemeen wordt aangenomen dat IL-12 de primaire stimulus is die leidt tot de inductie van de Th1 respons. Echter, voor de inductie van IL-12 productie is het Th1 cytokine IFN- γ nodig. In dit proefschrift laten we zien dat voor de inductie van IL-23 geen IFN- γ nodig is en dat IL-23 zelf wel IFN- γ productie kan induceren. Door het induceren van IFN- γ productie, maakt IL-23 vervolgens de productie van IL-12 mogelijk. Hoewel IL-23 de productie van IFN- γ kan induceren, is uit muizenstudies reeds gebleken dat IL-23 niet strikt noodzakelijk is voor de inductie van een Th1 respons. Van andere cytokinen die vroeg in de immuun respons worden geproduceerd, zoals IL-27, is bekend dat zij ook de productie van IFN- γ kunnen aanzetten. De primaire prikkel en de exacte cytokinen die leiden tot de vorming van een Th1 respons zijn nog niet opgehelderd. Concluderend suggereren wij een model, waarin IL-12 nodig is voor de versterking en de continuering van een Th1 respons, terwijl andere cytokinen, waaronder IL-23, nodig zijn voor de inductie van een Th1 respons.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 9 maart 1977 te Naarden. Het VWO werd doorlopen op het Praedinius gymnasium te Groningen, waar in 1995 het diploma werd behaald. In September 1995 werd begonnen met de studie Medische Biologie aan de Universiteit van Amsterdam. Een jaar later werd tevens begonnen aan de studie Geneeskunde aan dezelfde universiteit. In het kader van de studie Medische Biologie werd een onderzoeksstage gedaan op de afdeling celbiologie en histologie van het Academisch Medisch Centrum Amsterdam over de regulatie van de interleukine-12 receptor $\beta 2$ promotor, onder leiding van Prof. Dr. M.L. Kapsenberg. Tevens werd in Webuye (Kenia) een onderzoek verricht naar de resistentie van de malaria parasiet tegen Amodiaquine en Sulphadoxin-Pyrimethamin. Dit onderzoek werd verricht voor de African Medical and Research Foundation (AMREF), onder supervisie van Prof. dr. P.A. Kager (afdeling tropische geneeskunde, afdeling infectie ziekten, Academisch Medisch Centrum Amsterdam). In 2002 werden zowel het doctoraaldioma Medische Biologie, als het doctoraal diploma Geneeskunde behaald. Het arts-examen volgde op 24 maart 2004, na o.a. een tropen co-schap in het St. Theresa's Mission Hospital te Ibenga, Zambia. In augustus 2004 werd een aanstelling verkregen als Onderzoeker in Opleiding (OIO) op de afdeling Infectieziekten van het Leids Universitair Medisch Centrum. Tijdens deze aanstelling is promotieonderzoek verricht onder begeleiding van Prof. dr. J.T. van Dissel en dr. E. van de Vosse. In 2009 heeft de auteur van dit proefschrift een jaar als assistent niet in opleiding tot specialist op de afdeling interne geneeskunde van het Medisch Centrum Alkmaar gewerkt. In februari 2010 werd begonnen met de opleiding Interne Geneeskunde in het Universitair Medisch Centrum Groningen (opleider prof. dr. R.O.B. Gans).

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submitted