Impaired type I immunity to mycobacterial infections

Cover design by Kimberly de Ruijter
Lay-out and printing by Gildeprint Drukkerijen - Enschede
ISBN: 978-94-6108-397-5

The printing of this thesis was financially supported by:
Greiner Bio-One, BD Biosciences and Life Technologies.

Impaired type I immunity to mycobacterial infections

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 20 maart 2013
klokke 16.15 uur

door

Roelof Anne de Paus

geboren te Dordrecht
in 1965

Promotie commissie

Promotor:	Prof. dr. J.T. van Dissel
Co-promotor:	Dr. E. van de Vosse
Overige Leden:	Prof. dr. T.W. Kuijpers (Academisch Medisch Centrum, Amsterdam)
	Prof. dr. T.H.M. Ottenhoff
	Prof. dr. M. Yazdanbakhsh

The research presented in this thesis was performed at the Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands.

It is better to travel a mile than to read 1609344 articles.

(naar een uitspraak van Confucius)

Voor mijn ouders

Aan Jenny en Monica

Contents

CHAPTER 1

General introduction

1. Mycobacterial diseases

Mycobacteria are associated with several human diseases. Disease-causing mycobacteria are usually classified into one of two groups, the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex and the non-tuberculous mycobacteria (NTM). The *M. tuberculosis* complex comprises a small group of mycobacteria, amongst them *M. tuberculosis* and *M. bovis*. These two mycobacteria are the cause of tuberculosis in humans. Various NTM are found to be associated with mycobacterial disease, e.g. *M. abscessus, M. avium* and *M. genavense*. Besides these two groups *M. leprae* and *M. lepromatosis* have been identified as causative agents of leprosy. The latter are not discussed within the scope of this thesis.

M. tuberculosis is a small, non-motile Gram-positive bacterium, which divides at an extremely low rate of about once every 16 to 20h. The mycobacterium, highly adapted to the human host, is transmitted between humans through aerosols. Patients with active tuberculosis cough and sneeze and can transmit the mycobacteria to individuals in their surrounding. Upon transmission of *M. tuberculosis*, primary infection will lead to clinical symptoms in less than 10% of the infected. About another 10% of the infected eradicate the mycobacteria successfully, while the majority remain with a latent infection [1]. During latency the bacteria remain dormant but may reactivate months or years later and cause active tuberculosis. An active infection of *M. tuberculosis* results in most cases in pulmonary tuberculosis. *M. tuberculosis* causes severe tissue destruction and/or necrosis in the lung, a process which is balanced by wound healing and fibrosis, resulting in the formation of scars and/or cavities with necrotic tissue. Hematological spread of the bacteria to other organs, giving rise to extrapulmonary tuberculosis disease, occurs in up to 15% of the cases [1]. Extrapulmonary tuberculous lesions are often found in peripheral lymph nodes, kidney, brain and bones, but also other organs may be affected. If not treated with antibiotics tuberculosis is a deadly disease in approximately half of the cases.

NTM infections are usually less severe than tuberculosis, because NTM infections often can be controlled and eradicated by the immune system. However, NTM can cause tuberculosis-like diseases, for example in COPD patients, and NTM may occasionally cause cutaneous diseases, lymphadenitis, soft tissue diseases or skeletal infections. The infections may remain local, may become manifest recurrently or may disseminate throughout the body. Dissemination of infection is a severe manifestation of the disease which may occur in individuals with a strongly impaired immune response. This may be due to nongenetic causes, for example the use of immunosuppressive medicines or an underlying disease (e.g. acquired immune deficiency, silicosis or diabetes). On the other hand the immunity may be impaired due to genetic defects [2-4], as for instance observed in patients with Mendelian susceptibility to mycobacterial disease (MSMD [MIM 209950]). Investigation of the causes of compromised immunity will help to make an early diagnosis and may provide guidance for

alternative or additional treatments. For example, children with an interleukin-12 receptor β1 (IL-12Rβ1) deficiency or a partial interferon-γ receptor 1 (IFN-γR1) deficiency and a NTM infection may benefit from IFN-γ treatment in addition to antibiotic therapy. The antibiotic treatment regimen depends on the NTM species involved, on the sites of infection and on underlying diseases.

The *M. bovis* Bacillus Calmette-Guérin (BCG) strain is a live attenuated strain, derived from *M. bovis*, which is used as a vaccine and protects very well against developing tuberculosis in childhood. Incidentally, immunocompromised patients, for example MSMD patients, are at high risk for developing severe BCG infections after BCG vaccination (4).

2. Immunity towards mycobacteria

The course of mycobacterial infections is dependent on the interactions of the mycobacterium and the defense system of the host. The human body is protected from invasion of pathogens by the skin and the mucosal membranes. Once pathogens, such as mycobacteria, pass this first line of defense the immune system is activated to fight the pathogens. Upon the first encounter with mycobacteria the innate arm of the immune system is rapidly activated (Figure 1). When the mycobacteria are not successfully eradicated by the innate immune system, the adaptive arm of the immune system is needed to combat the infection. In this process, granulomas may be formed to isolate infection sites from the rest of the body.

2.1. Innate immune responses to mycobacteria

Upon encounter with bacteria the immune system is activated (Figure 1). Factors of the humoral complement system are activated as well as cellular-bound pattern recognition receptors (PRR). In response bacteria are opsonized and subsequently eradicated by the phagocytic cells: the granulocytes, monocytes, macrophages and dendritic cells.

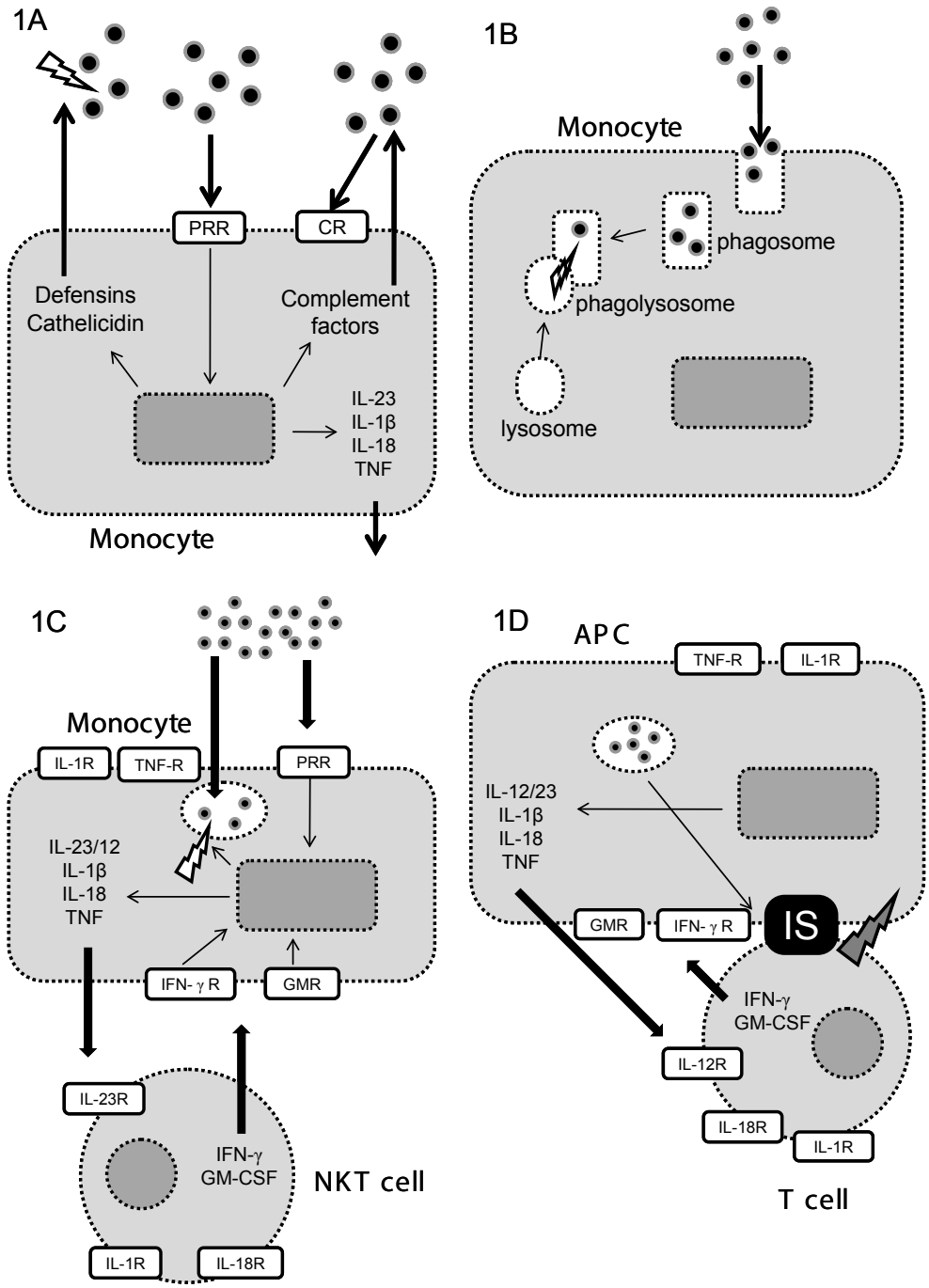
After mycobacteria break through the first line of defense the classical and alternative pathway of the complement system is activated via factors, such as C1q and C3 [5]. During the first innate immune response, the complement factor C3 is activated via the lectin pathway or the C2 pathway of the complement system [6, 7], resulting in the opsonization of mycobacteria (Figure 1A). The lectin pathway plays a pivotal role in the uptake (phagocytosis) of mycobacteria by phagocytes during the innate immune response after the first encounter with mycobacteria. This pathway of the complement system is initiated when mannose-binding lectin, present in plasma, binds to the mannose residues of mycobacterial glycoproteins. Subsequently, C3 is converted into C3a and C3b. Mycobacteria coated with C3b are opsonized after recognition of C3b by CD35 expressed on phagocytic cells. Besides CD35,

the mannose receptor expressed on macrophages and DC-SIGN expressed on dendritic cells play an important role in the uptake of mycobacteria by these phagocytic cells [8, 9]. In addition, C3b induces the formation of membrane attack complexes via the activation of C5, by cleaving C5 into C5a and active C5b. The presence of the membrane attack complexes on bacteria will lead to osmotic lysis of the bacteria. Meanwhile C3a and C5a influence B cell activation and the role of dendritic cells in T cell polarization [10]. The factors of the complement system are constitutively produced by various cell types. During an immune response the production can be enhanced by IL-1β, IFN-α, IFN-γ and endotoxins [11, 12].

Bacteria are opsonized and end up in an endosome-like vesicle, the phagosome, (Figure 1B) a process called phagocytosis. Subsequently, the phagosome fuses with a lysosome into a phagolysosome, which further matures. In the early phagosome the bacteria are exposed to reactive oxygen radicals. In the mature phagolysosome the bacteria can be killed via a lowered pH and enzymatic hydrolysis of bacterial components (Figure 1B). The phagolysosome maturation is an important step in the eradication of mycobacteria. However, mycobacteria have evolved mechanisms to evade the phagolysosomal killing mechanisms. To counterweight immune evasion, IFN-γ is an important cytokine which remarkably enhances the innate intracellular bactericidal processes in the phago- and the phagolysosome [13].

Before and during phagocytosis, the cellular part of the innate immune system is activated by membrane-bound and intracellular PRRs. PRRs, expressed by phagocytes, recognize characteristic pathogen-associated molecular patterns (PAMPs) (Table 1). Upon PRR and PAMP interaction signals within the cells will regulate the formation of an immune response. In this way, various immune responses can be initiated depending on the pathogen-specific PAMPs. Several classes of PRRs are known: Toll-like receptors (TLRs), C-type lectin receptors, nucleotide binding oligomerization domain-like receptors (NLRs) and retinoic acid inducible gene-I-like helicases (RLHs). Thus, the immune response is differentially shaped via the interactions of specific PRRs with various pathogens. For example, in the event of viral infections anti-viral responses, to stop viral replication and assembly, are initiated after interaction of typical viral products with certain PRRs. Other PRRs are stimulated by mycobacterial PAMPs (Table 1). Upon PRR interactions with mycobacterial PAMPs, the production of type I cytokines and several anti-bacterial processes are induced [7] (Figure 1A).

PRRs differentially signal via the transcription factors Nuclear factor κb (NFκb) or the interferon regulatory factors (IRFs) [6, 7, 14] in order to induce the expression of inflammatory genes, amongst them various cytokine genes. TLR1, -2, -4, -5, -6, NLRs and RLHs mediated signaling via NFκb results in the production of the proinflammatory cytokines IL-12, tumor necrosis factor (TNF) and IL-1β, while the TLR3, -7, -8, -9 and RLH signaling via IRFs results in the production of type I interferons, such as IFN-α and IFN-β.


1A
PRR
CR
Defensins
Cathelicidin
Complement
factors
IL-23
IL-1β
IL-18
TNF
Monocyte
1B
Monocyte
phagosome
phagolysosome
lysosome
1C
Monocyte
IL-1R
TNF-R
PRR
IL-23/12
IL-1β
IL-18
TNF
IFN- γ R
GMR
IL-23R
IFN-γ
GM-CSF
IL-1R
IL-18R
NKT cell
1D
APC
TNF-R
IL-1R
IL-12/23
IL-1β
IL-18
TNF
GMR
IFN- γ R
IS
IFN-γ
GM-CSF
IL-12R
IL-18R
IL-1R
T cell

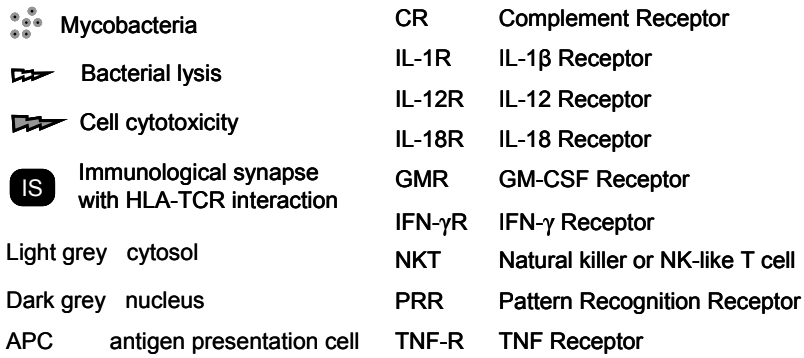


Figure 1. Cellular immune responses against mycobacteria. Upon encountering mycobacteria phagocytes, such as monocytes, are stimulated by mycobacterial-associated molecular patterns, which interact with PRRs on the cell surface of the monocytes (A). The monocyte is stimulated to produce cytokines, bactericidal defensins, cathelicidin and complement factors. The complement factors can bind to the cell surface of mycobacteria and subsequently to the complement receptors. The complement receptors mediate the phagocytosis of mycobacteria (1B). By means of phagocytosis the mycobacteria are internalized in an endosome-like particle, the phagosome. Subsequently, the phagosome fuses with the lysosome, in order to lyse the mycobacteria. Concomitantly, the monocyte is stimulated via PRRs to produce cytokines, for example IL-23. IL-23 stimulates innate NK-like T cells to produce IFN-γ and GM-CSF (1C). In turn, IFN-γ enhances the microbicidal mechanisms, type I immune responses and antigen processing and presentation by monocytes. The monocytes, stimulated by GM-CSF, mature into dendritic antigen-presenting cells, which in turn may trigger the T cells from the adaptive immune system (1D). During the cellular interaction between the antigen-presenting cell and the T cell an immunological synapse is formed, wherein the HLA with mycobacterial antigens interacts with the TCR of specific T cells. After recognition of the antigen by the TCR the T cell is stimulated. $CD8^+$ T cells employ cytotoxic functions, while $CD4^+$ T cells react by producing cytokines.

Furthermore, PRR signaling results in the expression of other inflammatory effectors and chemokines and the production of the antibacterial defensins and cathelicidin. In addition, the phagocytosis and killing of the bacteria within phagolysosomes are also regulated via PRR signaling. The latter processes can be enhanced by IFN-γ.

During the innate immune response various leukocytes are recruited to the site of infection, including, neutrophils, phagocytes, Natural Killer (NK) and NK-like T cells. Infected macrophages produce IL-8 and attract neutrophils to the infection site. Neutrophils can produce neutrophil extracellular traps (NETs). NETs are structures of chromatin with proteins that can bind and kill bacteria. These NETs are capable of trapping mycobacteria but are unable to kill them [15]. In addition, the recruited neutrophils produce anti-bacterial peptides and cooperate with monocytes and macrophages in the modulation of the immune response [16]. Other cells are attracted and various cellular interactions further strengthen the immune response. For example, both monocytes and T cells play a role in the establishment of a strong type I immune response. Monocytes, activated by mycobacterial products, produce IL-23 and IL-1β. Subsequently, NK-like T cells produce GM-CSF and IFN-γ in

response to IL-23 and IL-1β. In turn, IFN-γ strengthens the innate immune responses of monocytes to enhance anti-bacterial processes (Figure 1C) and the production of IL-12, while GM-CSF further directs the maturation of monocytes. IL-12 in turn, strongly enhances the production of IFN-γ [17]. These modulators of the innate immune response also orchestrate the development of an adaptive T cell response.

Table 1. Pattern recognition receptors (PRR) and their ligands, the pathogen-associated molecular patterns (PAMPs), which induce signals in the human phagocytes [6, 7].

Class of PRR	PRR	cellular localization	PAMPs[a]
TLRs	TLR1/2	cell surface	Triacyl lipopeptides
	TLR2	cell surface	*Phosphatidyl-myo-inositol mannoside* *Lipoarabinomannan* Hemagglutinin
	TLR3	endolysosomes	Viral ssRNA from RSV Viral dsRNA from CMV
	TLR4	cell surface	*Mannan* Lipopolysaccharide from bacteria
	TLR5	cell surface	Flagellin from flagellated bacteria
	TLR6/2	cell surface	Diacyl lipopeptides from mycoplasma
	TLR7	endolysosomes	Viral ssRNA RNA from Streptococcus
	TLR8	endolysosomes	Viral ssRNA
	TLR9	endolysosomes	Viral dsDNA from CMV CpG motifs from bacteria and virusses
C-type lectins	Mannose receptor	cell surface	*Mannose*
	DC-SIGN	cell surface	*Mannosylated lipoarabinomannan* gp120 from HIV virus
	Dectin-1	cell surface	β-glucans from fungi
	Mincle	cell surface	*Trehalose-dimycolate*
RLHs	RIG-I	cytoplasm	Viral ssRNA (negative strand) polyIC (short)
	MDA5	cytoplasm	Viral ssRNA (positive strand) polyIC (short)
NLRs	NOD1	cytoplasm	*Diaminopimelic acids*
	NOD2	cytoplasm	*Muramyl dipeptide*
	NLRP1	cytoplasm	*Muramyl dipeptide*
	NLRP3	cytoplasm	Viral and bacterial RNA β-glucan from fungi
	NLRC4	cytoplasm	Flagellin from flagellated bacteria

[a] Examples of PAMPs; mycobacterial products are given in *italics*.

2.2. Adaptive immune responses to mycobacteria

When mycobacteria manage to evade the innate immune defense, the adaptive arm of the immune system is needed to control infections in a more specific, mycobacterial-antigen restricted manner. After the first encounter with a pathogen, antigen-specific T and B cells are selected in lymphoid organs and will expand and further differentiate. The differentiated B and T cells are released from the lymphoid organs carry out their tasks at the infection sites. The generation of a primary adaptive immune response takes several days and becomes effective about 5 to 7 days after the first infection. After successful eradication of the pathogen some of these T and B-cells become memory cells. Those cells remain circulating and can be recruited and expanded more rapidly upon a second infection.

Immature B cells with membrane-bound immunoglobulins that recognize mycobacterial (non-self) antigens are positively selected in lymphoid organs. These B-cells mature further, expand and are released from the lymphoid organ. Circulating B cells are activated after recognition of specific antigens and start to produce anti-mycobacterial antibodies. These antibodies circulate in the blood and tissue fluids in the body and play a pivotal role in the humoral defense. Antibodies bound to mycobacteria help in the uptake of the pathogen by phagocytes via the classical pathway of the complement system.

Antigen specific T cells within the pool of newly generated T cells can be selected and expanded in lymphoid organs. T cells in the lymphoid tissue are instructed by dendritic cells. The foreign peptides are processed and presented within the context of HLA molecules to lymphoid $CD8^+$ and $CD4^+$ T cells [18]. Upon interaction of a dendritic cell with a T cell, carrying an αβ T-cell receptor (αβ TCR) that recognises a specific mycobacterial antigen in the context of HLA, this T cell further develops, proliferates and differentiates. The expanded T cells will be released from the lymphoid organs to carry out their tasks in the infected tissues. The differentiated $CD3^+CD8^+$ T cells employ cytotoxic functions, while $CD3^+CD4^+$ T cells further shape the immune system by producing cytokines upon recognition of the mycobacterial antigen (Figure 1D). Three main subsets of $CD4^+$ T cells are distinguished; Th0, Th1 and Th2 cells. Th0 or T helper 0 cells are naive cells which can differentiate (polarize) into typical Th1 or Th2 cells [19]. This T helper cell polarization is guided by professional antigen-presenting dendritic cells [20] or monocyte-derived dendritic cells.

Th1 or T helper 1 cells can produce IFN-γ and facilitate cell-mediated immune responses (Figure 1), for example, phagocytosis, the enhancement of cytotoxic NK and T cell responses. Th1 cells are important in the control of infections with viruses and intracellular bacteria, such as *Mycobacteria* and *Salmonellae* [21]. IL-12 is the main regulator of the polarization of naïve Th0 cells into Th1 cells. In addition, IL-12 strongly enhances the proliferation of Th1 cells and induces the production of IFN-γ, which is the main cytokine of the type I immune response, and also stimulates T-cells to produce GM-CSF and TNF. IL-12 and IFN-γ

act in concert with other type I cytokines: TNF, IL-1β and IL-18 [22, 23]. These cytokines determine the shape of the innate and adaptive immune responses against mycobacteria towards a type I immune response.

IL-4 regulates the polarization of Th0 cells into Th2 cells, while IFN-γ negatively interferes with the formation of a Th2 response [24]. This indicates that there is a certain balance between the generation of a Th1 and a Th2 response. When the Th1 immune response is impaired, relatively more T cells will be shaped into the Th2 type, as is observed in MSMD patients [25]. Thus, an impaired type I immune response correlates with a disturbed Th1/Th2 balance. Activated Th2 cells produce IL-4, together with other type 2 cytokines, such as IL-5 and IL-13. IL-4 and IL-13 inhibit the enhancing effects of IFN-γ on the bactericidal processes of macrophages [26], which further illustrates the dichotomy between type I and type II immune responses.

Th2 cells support B cells in their development and function to establish a robust antibody response. Th2 cells are important in the control of infections of extracellular pathogens, such as helminths [27]. In addition, Th2 cells are involved in several auto-immune diseases, such as SLE [28] and atopic allergic diseases [29]. Hence, factors which play a role in the Th1/Th2 balance of abovementioned diseases may also play a role in the susceptibility to or protection against to mycobacterial diseases.

Besides the Th0, Th1 and Th2 subsets, other subsets of T cells are described. For example, Th17 cells are IL-17-producing T cells, which are modulated by IL-23, IL-1β, IL-6 and/or TGF-β. These cells are not fully differentiated, but show plasticity in effector functions. To indicate the plasticity, these cells can regain the capacity to produce IFN-γ [30]. Since IL-23 is an important cytokine in the maintenance of the IL-17-producing T cells, IL-12Rβ1 deficient patients have in addition to an impaired IFN-γ-mediated immune response also an impaired IL-17-immune response [31]. A study of IL-17 deficient mice showed that IL-17 plays a major role in chemokine production and neutrophil recruitment to cutaneous infections [32]. This is confirmed by a study with IL-17 overexpression in lung epithelium of mice, which caused local chemokine production and leukocyte infiltration [33]. In humans, an impaired development of IL-17-producing T cells results in susceptibility to mucosal candida infections [34]. These studies indicate an important role of antigen-specific IL-17-producing T cells in neutrophil recruitment at mucosal sites, which is also important for lung infections with mycobacteria.

Fully differentiated $CD8^+$ effector and $CD4^+$ responder T cells may turn into memory T cells. While the selection of newly generated T cells is time-consuming, the memory T cells can be expanded and instructed rapidly after another encounter with the same pathogen. In this way the immune system responds more rapidly upon a second encounter with a mycobacterium [35] or after the reactivation of dormant Mycobacteria.

Other important classes of adaptive T cells are regulatory T-cells (Tregs) and CD1 restricted T cells. In patients with active tuberculosis higher levels of circulating Tregs were found, compared to patients with latent tuberculosis [36]. Depletion of Tregs enhances the Th1 immune responses in patients with active disease [36]. Tregs suppress IFN-γ-mediated T cell responses via the production of TGF-β and IL-10 [37]. In this way, Tregs control the function of T-cells during homeostasis and during inflammatory conditions in order to prevent uncontrolled T cell reactions resulting in unnecessary tissue damage.

CD1 restricted T cells recognize mycobacterial lipids within the context of CD1 molecules. Phagocytes can take up lipid antigens from the mycobacterial membranes and present them within CD1 molecules on the outer cell surface to special classes of T cells, for example the invariant NK-like T cells carrying an invariant TCR chain. These T cells are capable of producing IFN-γ and exhibit effector and memory functions [38] and thus are important in the control of mycobacterial infections [39].

2.3. Granuloma formation in the control of mycobacterial infections

Granulomas can be formed in the lung and other organs during *M. tuberculosis* infections, but also during NTM or BCG infections [40, 41]. A granuloma is formed by an interplay of different cell types to encapsulate infected macrophages [42]. In this way the sites of infection are walled off. Around the infected macrophages uninfected monocytes, neutrophils and lymphocytes are recruited. The infected macrophages become surrounded by other phagocytes and are enclosed by a fibrous cuff, consisting of collagens and other extracellular matrix molecules, while lymphocytes reside on this outer layer of the granuloma. In this way, mycobacteria are captured within granulomas, which are not vascularized, under hypoxic conditions. In this state the mycobacteria can remain dormant for many months or years.

Genetic immunodeficiency, HIV infection or immunosuppression due to e.g. malnutrition results in impaired granuloma formation. The $CD4^+$ T cells play a major role in the maintenance of the granuloma structure [43]. Impaired $CD4^+$ T cell functions can result in a decay of granulomatous structures [44]. Due to the loss of these structures necrosis of the infected cells occurs within the granuloma resulting in the release of mycobacteria and subsequently the infection will reactivate.

In mycobacterial diseases Th1 cytokines play a major role in orchestrating granuloma formation and homeostasis. In studies of the course of mycobacterial infections in TNF-deficient mice, it appeared that TNF is a crucial cytokine in the induction of cell recruitment and the formation of granulomas [45]. In humans, TNF blocking agents, used in the treatment of rheumatoid arthritis or Crohn's disease, can result in the resuscitation of latent *M. tuberculosis* and subsequently in severe disseminated infections [46-48]. Granuloma formation is completely absent in patients with severe disseminated NTM or BCG infections, due to a complete IFN-γR1 or IFN-γR2 deficiency. Patients with partial IFN-γR1 or complete

IL-12Rβ1 deficiency the granuloma formation is often incomplete [49]. This indicates that IFN-γ also plays an important role in granuloma formation. Besides these host factors mycobacterial factors also influence granuloma formation.

3. Cytokines in the control of type I immune responses

IL-12 and IFN-γ are the main regulatory cytokines in a strong type I immune response. IL-12, the best described cytokine of the IL-12 cytokine family, is a strong modulator of type I immune responses by NK cells and adaptive T cells. IL-23 is also a member of the IL-12 cytokine family, which is structurally and functionally closely related to IL-12 [50]. IL-23 may play a role in the activation of memory cells [51] and IL-17-producing T cells [52]. Both IL-12 and IL-23 can be produced by phagocytes and can enhance IFN-γ production and proliferation of various subsets of T-cells. IFN-γ is a pleiotropic cytokine that plays a central role in the type I immune responses important in the defense against intracellular bacteria. IFN-γ and IL-12/23 act in concert with other important type I cytokines: IL-1β, IL-18 and TNF [17, 53].

3.1. IL-12 and IL-23

IL-12 and IL-23 have similar and distinct structures and features (Figure 2). The IL-12p40 subunit is the common subunit of IL-12 and IL-23. The IL-12p35 subunit and the IL-23p19 subunits are the specific subunits of IL-12 and IL-23 respectively, which show structural similarity [54]. The cytokines bind and act on different receptor complexes (figure 2). The IL-12 and IL-23 receptors consist of a common receptor chain, IL-12Rβ1, and a specific receptor chain, the IL-12Rβ2 and the IL-23R, respectively. The IL-12R complex signals via STAT4 [55], while the IL-23R complex signals via STAT3 and STAT4 [50]. Dimeric STAT modules are transcription factors, for instance for the *IFNG* gene. IL-12, IL-23 and their receptors are expressed differentially and their receptors differ in signaling. These two cytokines display both common and distinct effects, probably due to differential binding capacities of the different STAT modules to the various STAT binding sites in promoters.

IL-23 can be produced by monocytes and dendritic cells directly upon PRR stimulation by bacterial products [17, 56, 57]. For IL-12 production IFN-γ stimulation [58, 59] and/or cellular interaction with activated T cells is needed [60]. Costimulation by CD40-CD40L [61, 62] and/or CD28-CD80/86 interactions [63] is also important for a strong induction of IL-12. Several *in vitro* studies have shown that many other factors are also involved in the regulation of IL-12 and IL-23 production. Gram-positive bacteria seem to stimulate the IL-12 production preferentially, while Gram-negative bacteria induce more IL-23 [64]. GM-CSF [17], IL-1β [65] and prostaglandin E2 [66] favor IL-23 production, while IL-4-generated

dendritic cells can produce large amounts of IL-12. The anti-inflammatory cytokine IL-10 effectively inhibits the IL-12 and IL-23 production [67]. Thus, *in vivo*, a complex interplay of immune modulators determines whether IL-12 or IL-23 or both cytokines are produced.

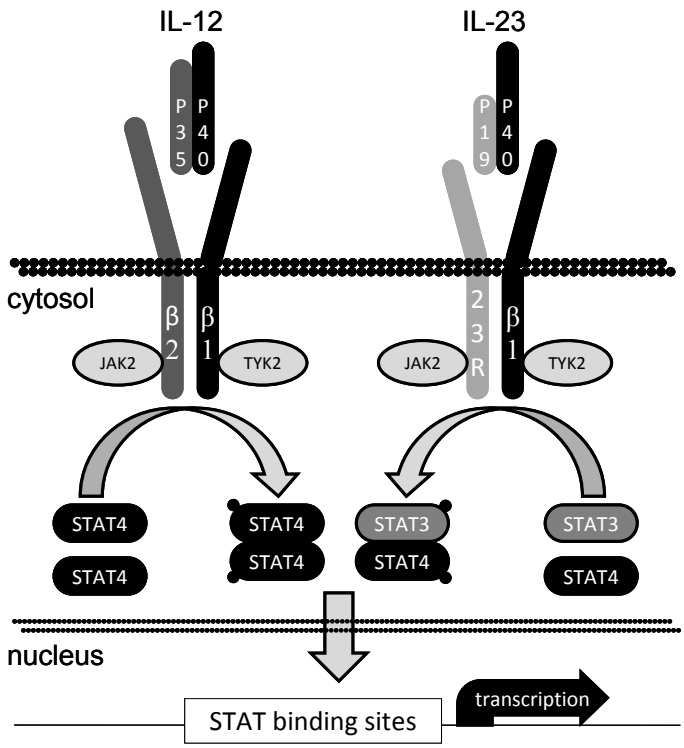


Figure 2. The IL-12 and IL-23 cytokines, their receptors and signal transduction. IL-12 and IL-23 both consist of two linked subunits. The IL-12p40 subunit is their common subunit. In IL-12 the p40 subunit is linked to the IL-12-specific p35 subunit, while in IL-23 the p40 subunit is linked to the IL-23-specific p19 subunit. Their receptors also have a common receptor chain: IL-12Rβ1. IL-12 binds to a receptor complex which contains the IL-12Rβ2 receptor chain. IL-23 binds to a receptor complex containing the IL-23R chain. After IL-12 binding to its receptor, STAT4 is phosphorylated and dimerized to form a homodimer. The STAT4 homodimer translocates to the nucleus, binds to STAT binding sites in promoters and acts as a transcription factor, for example for the IFN-γ promoter. IL-23 signals likewise, although next to STAT4, STAT3 is also phosphorylated and STAT3 and STAT4 heterodimers are formed, though STAT3 and STAT4 can each also form homodimers. [50, 54, 68]

The IL-12R consists of an IL-12Rβ1 chain and an IL-12Rβ2 chain. The IL-12Rβ1 chain is present on nearly all lymphocytes. The IL-12Rβ2 chain is present on non-stimulated NK cells and can be induced on other lymphocytes. For example, T- cells express IL-12Rβ2 after TCR triggering [69]. Subsequent stimulation of these T cells with IL-12 results in type I immune responses.

One of the main actions of IL-12 is the induction of IFN-γ production by T cells, which is synergistically enhanced by IL-18 and/or IL-1β. In addition, IL-12 enhances the cytolytic

capacity and proliferation of NK and T cells. Another important action of IL-12 is that IL-12 drives T cell polarization of the adaptive CD4+ T cells towards Th1 cells [68, 70], via the induction of the Th1-specific transcription factor T-bet [71].

The expression pattern of the IL-23R is not as well understood, because of the lack of a good antibody to detect IL-23R expression on individual cells. Thus, it is not clear which cells are responsive to IL-23. Reported IL-23 effects on certain cell types may have been induced directly or indirectly via another cell type.

Previously, IL-23 was reported to activate memory T cells and to induce IFN-γ production in humans and in mice [51, 54, 72]. Other studies with normal and IL-23p19-/- mice revealed that IL-23 plays an important role in the immune responses of IL-17-producing T cells [52]. IL-23 does not influence the IL-17 production directly, though IL-23 contributes to the survival and proliferation of IL-17-producing T cells [73], which are shaped by the cytokines IL-1β, IL-6, IL-21 and TGF-β [74]. Both IL-17 and IL-23 play an essential role in the mucosal immunity against *M. tuberculosis* [75, 76], *Klebsiella pneumoniae* [56, 77] and *Candida albicans* [78, 79]. In addition, IL-23 plays a role in the IFN-γ and IL-17-mediated immune responses, IL-23 may also regulate the production of the anti-inflammatory cytokine IL-10. Activated $CD4^+$ and $CD8^+$ T cells were also found to be able to produce IL-10 in response to IL-23 [80]. All these effects of IL-23 were found to be important in the control of infections and IL-23 plays a role in various auto-immune diseases, because IL-23R polymorphisms were found to be associated with immune diseases, such as inflammatory bowel disease [81], psoriasis [82], Graves' disease [83] and ankylosing spondylitis [84]. Taken together, it is clear that IL-23 plays a crucial role in establishment of an immune response. Still, the direct biological effects of IL-23 at the cellular level remain to be elucidated.

3.2. Interferon-γ

IFN-γ can be produced by various subsets of T cells; NK cells, iNKT cells, NK-like T cells, γδT cells and αβT cells. For instance, IL-12, IL-23 and IL-27 stimulate NK cells to produce IFN-γ, which is synergistically enhanced by IL-1β and IL-18. IFN-γ production by adaptive T cells requires TCR activation in addition to cytokine stimulation.

IFN-γ is a homodimer which signals via the IFN-γR complex (Figure 3). The IFN-γR consists of two IFN-γR1 and two IFN-γR2 chains. After IFN-γ ligation to its receptor STAT1 is phosphorylated and dimerized. STAT1 homodimers act as transcription factors on various genes, such as *CD54*, *CD64* and genes for other transcription factors, such as *IRF1* and *CIITA* [85]. Thus, IFN-γ stimulates gene transcription directly or via the induction of other transcription factors. In this way IFN-γ regulates immune responses with different kinetics. For instance, STAT1-mediated transcription occurs within minutes after IFN-γ exposure, while interferon regulatory factor 1 (IRF-1)-mediated transcription occurs with a few hours delay. Relatively late, more than 8 hours after IFN-γ stimulation, the protein inhibitor of activated STAT1 (PIAS1), a negative regulator of STAT1 mediated transcription, is produced

as a negative feedback loop [86]. PIAS1 suppresses STAT1 mediated transcription, but does not directly suppress IRF-1-induced transcription.

IFN-γ is a stable cytokine which has pleiotropic effects on various cell types. Nearly all cell types express the IFN-γR [87], though the response varies between cell types. IFN-γ alarms and activates the immune system, by means of a wide range of responses of infected and uninfected cells.

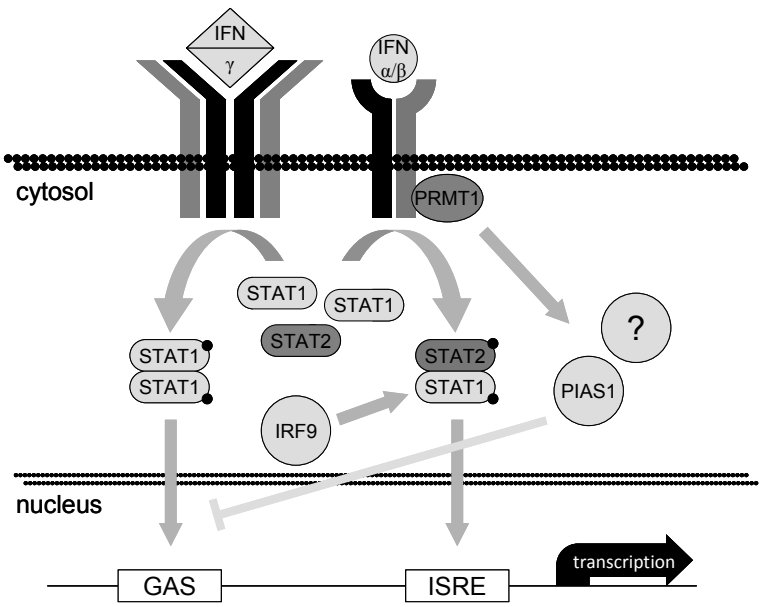


Figure 3. The signal transduction of IFN-γ and IFN-α/β. IFN-γ is a homodimer which signals via its own receptor complex, consisting of two IFN-γR1 and two IFN-γR2 chains. After ligation of IFN-γ to the IFN-γR, STAT1 is phosphorylated, dimerized and translocated to the nucleus. STAT1 homodimers bind to GAS sites and act as a transcription factor [85]. IFN-α and IFN-β are structurally related and signal via a common receptor, which consists of two chains, the IFN-αR1 en IFN-αR2. After IFN-α/β ligation to the receptor, STAT1 and STAT2 are phosphorylated and form heterodimers. Subsequently IRF-9 binds to the heterodimer to form the ISGF3 complex [88, 89]. This complex translocates to the nucleus and binds to the ISRE sites within various promoters. IFN inducible genes may have GAS sites or ISRE sites or both. IFN-α may activate PRMT1 [90], which was found to be associated with the IFN-α/β receptor (in mice). In this way, IFN-α may influence PIAS1 and other PRMT1 protein substrates [91]. PIAS1 negatively interferes with the transcription activity of STAT1 [86].

IFN-γ increases phagocytosis of pathogens via the upregulation of receptors which mediate the opsonization of bacteria, for instance, the immunoglobulin-binding receptor CD64 [92, 93] and receptors of the complement system [94, 95]. To stimulate these processes IFN-γ also enhances the synthesis of immunoglobulins [96, 97] and complement factors [98, 99], which bind to bacteria prior to opsonization.

Furthermore, several anti-bacterial processes of phagocytes are stimulated by IFN-γ. IFN-γ strengthens the magnitude of the oxidative burst within the phagosomes of phagocytes

[100] and IFN-γ stimulates the phagolysosome fusion and maturation in order to lyse the pathogen [101-103]. IFN-γ reduces the serum iron concentration [104] and the intracellular iron content of phagocytes. In this way, the bacterial growth is restrained because iron is essential for replicating intracellular bacteria [105]. IFN-γ also induces the production and secretion of anti-microbial peptides, such as the defensins [106] and cathelicidin [107], in order to lyse extracellular bacteria.

IFN-γ influences also the immune responses of various T cell subsets in several ways. The antigen presentation of various cell types is stimulated by IFN-γ, via the enhancement of the processing of bacterial antigens and upregulation of HLA Class I and Class II on various cell types [85]. In this way, the antigen-specific T cells are activated and start to proliferate. IL-12 and IFN-γ further regulate the differentiation of the activated T cells towards a Th1 type of cell and IFN-γ downregulates the formation of a Th2 response [24]. Furthermore, IFN-γ regulates the cytolytic activity of the innate NK cells [108].

The production of chemokines and cytokines by various cells is also induced by IFN-γ. The production of chemokines, such as MCP-1 and CXCL-10 can be enhanced by IFN-γ [109] as well as the TLR-ligand induced production of IL-12, IL-1β and TNF by monocytes, macrophages and dendritic cells [58, 110, 111]. These cytokines act in concert with IFN-γ to eradicate bacterial pathogens and to form granulomas [49].

Furthermore, IFN-γ competes with the anti-inflammatory cytokine IL-10 in the establishment of an effective immune response [112-114]. IFN-γ can suppress the activity of IL-10 by inhibiting STAT3 and even prime for IL-10-mediated STAT1 signaling, while IL-10 is able to reduce the IFN-γ responsiveness if IL-10 acts before IFN-γ and TLR-ligands. In this way IL-10 and IFN-γ control the extent of an immune response, in order to prevent exaggerated and/or auto-immune reactions.

3.3. The role of type I interferons in the immunity against intracellular bacteria

Type I interferons can be induced by various intracellular bacteria, such as *Legionella pneumophila* [115] and *Listeria monocytogenes* [116, 117]. Host immunity against intracellular bacteria may benefit from the production of type I IFNs, since these interferons can stimulate antibody production, proliferation of mature CD8+T cells and the function of mature dendritic cells [118]. On the other hand, both IFN-α and IFN-β were found to be able to inhibit immature immune cells. For example, the IL-12 production of antigen-presenting cells can be inhibited by IFN-α and IFN-β [119, 120]. Thus, intracellular pathogens may benefit from the induction of IFN-α/β by inhibiting the establishment of a Th1 immune response. To illustrate the latter, mice lacking the IFN-αR are resistant to *L. monocytogenes* infections [117]. *M. tuberculosis* may also result in the induction of type I interferons. The blood cells from pulmonary tuberculosis patients display a transcript signature like that of the type I interferons, while specific IFN-γ transcription is repressed [121]. After treatment

with antibiotics, during recovery, the blood cells from the patients showed a decreased type I interferon signaling and an increased IFN-γ production [122]. Direct evidence that *M. tuberculosis* is able to induce one of the type I interferons in these patients has not yet been found. In mice, virulent *M. tuberculosis* species induce the production of IFN-α and these mice show a diminished Th1 immune response [123, 124].

While IFN-γ is the sole member of the type II interferons, the type I interferons group encompasses 8 subtypes: IFN-α, -β, -δ, -ε, -ω, -κ, -τ and –ζ. All type I interferons act on the same receptor complex, albeit with different efficacy. These interferons are selectively produced by almost all cell types in response to viral infections [89]. Special PRRs, such as MDA5, TLR3, TLR8, TLR9 and RIG-I can be triggered by viral RNA or DNA [125]. Subsequently, type I interferons are produced and secreted in order to accomplish local or systemic effects. In response to type I interferons various anti-viral proteins are expressed and activated, resulting in the inhibition of viral replication and viral assembly [126].

IFN-α and the other type I interferons signal in the same way via STAT1 and STAT2 heterodimers. IRF-9 binds to this heterodimer to form the stable interferon-stimulated gene factor 3 (ISGF3) complex [88, 127]. The ISGF3 complex acts as a transcription factor on interferon-stimulated response element (ISRE) sites in the promoter of various immune genes (Figure 3), for example the promoter of *IRF1* [128]. The effect of IFN-α signaling depends on the presence of IRF-9. In IRF-9 knockout mice the signaling of IFN-α occurs via dimers of STAT1 and STAT2 instead of the ISGF3 complex. As a consequence, IFN-α stimulation results in an IFN-γ-like gene expression pattern [129].

In addition to this signaling pathway, IFN-α regulates protein arginine methyl transferase1 (PRMT1) activation in monocytes. In mice, it was found that PRMT1 associates with the IFN-αR and that IFN-α stimulation can result in PRMT1 activation [90]. PRMTs are able to methylate various kinds of proteins, amongst them PIAS1 [90]. PIAS1 is a negative regulator of STAT1 transcription. PIAS1 bound to STAT1 decreases the binding efficiency of STAT1 to certain but not all GAS sites, thereby selectively interfering with IFN-γ signaling [130]. Whether this or other PRMT pathways play a role in the immune responses of type I interferons in humans needs to be investigated.

4. Mycobacterial immune evading strategies

Mycobacterial infections may persist because of a lack of IL-12 and IFN-γ mediated immune responses. On the other hand mycobacteria have evolved mechanisms to enhance their virulence and to evade host immunity. NTM species and BCG are poorly pathogenic and the low virulence of these species wins from the host only when the host is immunodeficient or immunocompromised, due to e.g. malnutrition, HIV infection or genetic deficiencies.

Mycobacteria from the *M. tuberculosis* complex are more virulent and have additional features to evade host immune responses.

The mycobacterium has a peculiar cell wall, which is rich in mycolic acid and other lipids. Binding of antibodies to these foreign lipids results in the activation of the complement system. The mycobacteria are easily opsonized or phagocytosed, and can reside within phagocytes, thereby escaping the attack by defensins and the complement system. Various mycobacterium species have evolved mechanisms to circumvent the hostile environment of macrophages and are able to survive in the host macrophages.

Mycobacteria are taken up by phagocytes in a phagosome. In the early phagosome most bacteria are killed directly by the superoxide burst, which is mediated via the NADPH complex [131]. IFN-γ is known to strengthen the oxidative burst [132]. Pathogenic mycobacteria express superoxide dismutase, an enzyme that reduces oxygen radicals, thereby protecting the bacteria from oxidative stress [133]. Also the cell wall lipoglycans protect mycobacteria from damage by scavenging the reactive oxygen species [134].

The phagosome fuses with a lysosome into a phagolysosome, a process which is actively accomplished by tethering molecules and a fusion machinery, consisting of SNARE proteins. The bacterium can subsequently be killed within a mature phagolysosome. Within the mature phagolysosome the pH drops, whereby several proteases are activated. However, mycobacteria are able to inhibit this process. The decrease in pH in the phagolysosome after phagocytosis is restrained and the mycobacterium inhibits host factors involved in bacterial killing and phagolysosome maturation [135]. For example, mycobacteria exclude H^+ATPases from the phagosome, thereby preventing the decrease in pH and the subsequent activation of lysosomes, which only show activity at low pH. Mycobacteria also block the tethering and fusion of the phagosome with the lysosome, by inhibiting the tethering molecule EEA1 and the activation of SNARE proteins [135].

Dependent on the mycobacterial strain, the bacteria have additional evading strategies. For example, the more virulent *M. tuberculosis* confers high resistance to the killing of macrophages by reactive nitric oxide and reactive nitrogen intermediates [135]. However, *M. avium* is also able to prevent phagosome-lysosome fusion and is highly resistant to nitric oxide but is less pathogenic compared to *M. tuberculosis* [136]. Thus, the highly pathogenic *M. tuberculosis* has additional immune evading strategies. For example, virulent mycobacteria have the ESX-1 secretion system [137]. The ESX-1 system secretes ESAT-6 and CFP10 into the cytoplasm of the host cell. Secretion of these molecules leads to the lysis of membranes in the host cell and to non-apoptotic, necrotic cell death. Mycobacteria benefit from this necrosis of the infected macrophages, while apoptotic cell death of infected macrophages is more beneficial for the host [138]. After necrosis of the infected macrophage the mycobacteria easily spread and infect other monocytes or macrophages. After apoptotic cell death apoptotic vesicles containing mycobacterial antigens are released. Professional

antigen-presenting dendritic cells are able to phagocytose these vesicles and present mycobacterial antigens in HLA molecules to $CD4^+$ and $CD8^+$ T cells [139]. To evade this, the virulent *M. tuberculosis* produces a 19 kDa protein which negatively interferes with antigen presentation by HLA Class II molecules [140].

The presence and the efficiency of these immune-evading mechanisms differ between the various mycobacterial strains. In addition, host factors influence the interactions whereby the pathogen regulates the immune evasion.

5. Risk factors in the susceptibility to mycobacterial infections

Various kinds of factors enhance the susceptibility to mycobacterial diseases. The role of genetic factors in the susceptibility to tuberculosis seems less important, while genetic mutations are often the cause of the susceptibility to severe disseminated NTM or BCG infections.

Genetic deficiencies are often found in young children with severe disseminated NTM or BCG infections. Various genetic defects have been identified in several genes involved in the Th1 cytokine pathway. These patients are highly susceptible to infections with NTM species or BCG which are normally poorly pathogenic [21, 141, 142]. Some of these patients may also develop severe infections with other pathogens, such as *Salmonellae* and viruses. For example, IL-12p40 or IL-12Rβ1-deficient patients are also susceptible to *Salmonellae* infections, and signal transducer and activator of transcription 1 (STAT1)-deficient patients are also susceptible to various viral infections, while IFN-γR1 or IFN-γR2-deficient patients are relatively resistant to *Salmonellae* and viral infections [141].

Severe NTM or BCG infections are also found in patients with other genetic diseases such as chronic granulomatous disease (CGD) and severe combined immunodeficiency (SCID). For example, mutations in *CYBB* or *NCF1* are a cause of CGD. Within the phagocytes of these patients the formation of superoxide radicals is greatly reduced. This leads to the susceptibility to mycobacterial infections, but also to infections with various other bacteria and fungi. SCID patients are also highly susceptible to mycobacterial infections, amongst others, as a result of an impaired B and T cell immunity.

Several environmental and host factors that increase susceptibility to tuberculosis have been described, such as other diseases, air pollution, malnutrition, smoking, alcoholism and genetic host factors. For example, having silicosis [143], having diabetes [144] or smoking [145] increases the risk of developing tuberculosis more than twofold. Co-infection with human immunodeficiency virus (HIV) increases the risk up to 37 times [1]. Furthermore, several host genetic factors were found to be weakly associated with tuberculosis. Associations with tuberculosis were repeatedly found for polymorphisms in *SLC11A1, P2RX7, IL12B,*

IFNG, VDR, TLR1, and *TLR8* [144, 146-151]. However, tuberculosis disease usually does not follow a Mendelian inheritance pattern since the susceptibility is polygenic and multi-factorial [148].

6. Genetic deficiencies and the susceptibility to NTM and BCG infections

Among MSMD patients with NTM or BCG infections various defects were found in the genes encoding for IL-12p40, IL-12Rβ1, IFN-γR1, IFN-γR2, STAT1, Nuclear-factor-κB-essential modulator (NEMO), tyrosine kinase 2 (TYK2), Interferon-stimulated gene 15 (ISG15) and IRF-8 [141, 152, 153]. These mutations lead to a lack of Th1 immune responses.

Each of the genetic deficiencies in the genes of the Th1 cytokine pathway, as found in humans, causes a similar susceptibility in mice. Knock out (KO) mice completely deficient in IFN-γ, IFN-γR1, IL-12p40 or IL-12Rβ1 develop normally. When challenged with mycobacteria these mice develop severe infections and show a lack of Th1 immune responses [154]. In comparison with wild type mice the KO mice were more susceptible to *M. tuberculosis* infections. In addition, the KO mice show no protective immunity to *M. avium* and BCG and fail to form mature granulomas. In humans, IL-12p40 and IL-12Rβ1 deficiency lead to susceptibility to infections with poorly pathogenic *Mycobacteria* and/or *Salmonellae* and granuloma formation is impaired [21]. In patients with complete IFN-γR deficiency [4], granuloma formation is completely absent [49]. The enhanced susceptibility to infections with intracellular pathogens in humans and mice confirm that the IL-12/IFN-γ axis is essential for control of these infections and that a complete deficiency of one of the genes in the IL-12 and IFN-γ cytokine pathway is a major cause of MSMD.

STAT1, NEMO and TYK2 deficiency are not only the cause of susceptibility to mycobacterial diseases, but also lead to susceptibility to various other kinds of infections. STAT1-deficient patients are also susceptible to severe or lethal viral infections [155, 156]. This may be explained by the fact that STAT1 is not only involved in IFN-γ signaling, but also in the signaling of type I interferons. NEMO plays an essential role in the nuclear translocation of NFkB and is thus involved in the signaling of Th1 cytokines, such as IL-1, IL-18 and TNF [157]. Furthermore, NEMO plays a role in the CD40-mediated induction of IL-12 production by dendritic cells [156]. NEMO is also involved in other signaling pathways, such as TCR and B cell receptor signaling. The involvement in various immune processes explains why NEMO patients are susceptible to various bacterial, fungal and viral infections.

TYK2 deficiency was found in two unrelated patients. One patient had besides a Salmonella and a BCG infection in addition to severe viral and fungal infections as well as elevated serum IgE levels [158]. The other patient had an herpes infection and a disseminated BCG infection, but normal IgE levels [159].The susceptibility to infections with various pathogens can be explained by the fact that TYK2 not only plays a role in IL-12 signaling but also in the signaling of other cytokines, such as IL-6 and IL-10.

Recently, ISG15 and IRF-8 mutations were reported as MSMD causing mutations. ISG15 mutations were found in two unrelated patients with impaired IFN-γ immunity, who developed relatively mild mycobacterial diseases at the age of 12 and 15 year [152]. ISG15 regulates the secretion of IFN-γ by NK cells and T cells and is abundantly produced by granulocytes and induces the secretion of IFN-γ by NK cells and T cells, alone or in synergy with IL-12 [152]. The latter emphasizes the important role of the ISG15-IFN-γ axis between granulocytes and NK cells in the innate immune response against mycobacteria.

IRF-8 mutations were found in three unrelated patients who developed BCG infections after vaccination [153]. IRF-8 regulates the transcriptional response, for example the transcription of *IL12B* and *NOS2*, induced by interferons and TLR ligands [160]. A complete IRF-8 deficiency was found in one patient who presented with an absence of $CD14^+$ and CD16+ myeloid cells in the blood. Two other patients with an autosomal dominant form of the disease showed a marked loss of $CD1c^+CD11^+$ dendritic cells and their capacity to produce IL-12. This illustrates the importance of IRF-8 in the generation of myeloid and dendritic cells and their capacity to modulate a type I immune response.

6.1. Diagnosis

Severe BCG or NTM infections are rare and develop with heterogeneous symptoms. The first infections become manifest in most cases in early childhood. Rapid diagnosis of the nature of the immunodeficiency is difficult, but mandatory for immunotherapeutic interventions, to help to combat the mycobacterial infections successfully.

When a case with a severe NTM or BCG infection is presented, MSMD can be suspected. In first instance, underlying diseases such as HIV infections and chronic granulomatous disease should be excluded. The incidence of these diseases is higher and both diseases require other treatments. Whether patients with NTM or BCG infections had had severe infections with other pathogens may already give information about the nature of a defect in the immune system. Furthermore, parental consanguinity and family history may also indicate whether the disease is genetically inherited in an autosomal recessive, autosomal dominant or X-linked manner. When the patient is suspected to have MSMD, several immunological investigations are necessary to reach a clear diagnosis. Valuable information can be obtained by cellular analysis and functional assays using whole blood, PBMCs and/or cultured T cell blasts from the patient, his or her family members and unrelated controls [142].

T-cells from IL-12Rβ1 and TYK2-deficient patients show poor IFN-γ production upon TCR triggering and additional IL-12 stimulation. FACS (fluorescence activated cell sorting) analysis of the IL-12Rβ1 protein on activated T cells will give an indication of a defect in the ability to express the receptor protein on the membrane. The PBMCs from a patient with IL-12p40 deficiency show impaired IL-12p40 production upon lipopolysaccharide (LPS) stimulation. Additional tests can be done to investigate a putative mutation in TYK2. The T cells of TYK2 patients are unresponsive to IL-12, but also to IFN-α [158].

To identify an IFN-γR deficiency the monocytes of the patient can be tested for IFN-γ responsiveness. $CD14^+$ monocytes stimulated with IFN-γ upregulate the cell surface expression of CD54 and CD64 and the LPS induced IL-12p40 and TNF production. Failure in the response indicates a defect in the IFN-γR expression or IFN-γ signaling. The quantification of the IFN-γR expression level by FACS may also indicate a defect in the receptor. An absent or abnormally high expression of IFN-γR1 on the cell surface may indicate a complete defect or the inability to internalize the protein, respectively. An impaired IFN-γ responsiveness can also be due to a STAT1 deficiency. This deficiency results also in unresponsiveness to IFN-α/β and can be further characterized by functional STAT assays, for example electrophoretic mobility shift assays to study the binding of STAT modules to DNA.

Additional tests can be done to investigate a putative mutation in NEMO, ISG15 or IRF-8. The T cells of these patients are unresponsive to IL-12, but are also unable to respond to IFN-α [158]. The monocytes of putative NEMO patients can be tested for the ability to produce cytokines after stimulation of the CD40 receptor. In NEMO patients, the CD40-induced production of IL-12p40, TNF and IL-6 is markedly reduced, while the LPS-induced production of TNF and IL-1β is normal [156]. ISG15 deficiencies can be expected when whole blood leukocytes, which are stimulated with BCG or BCG with IL-12, produce reduced levels of IFN-γ. The IFN-γ production of ISG15-deficient cells can be restored with recombinant ISG15. IRF-8 deficiency can be assumed when there is a severe lack of $CD14^+$ monocytes or a selective loss of $CD1c^+CD11c^+$ monocytes.

The ultimate diagnosis can be made when the genetic basis of the defect is revealed. Next to the functional assays, the DNA or mRNA coding for the protein of interest can be amplified by (RT-)PCR in order to be sequenced for mutations. Clear null mutations can be found, but also variations with unknown impact on protein function. The impact of gene deletions or early stop codons within the coding sequence on the features and functions of the gene product is clear. The impact of a subtle genetic variation is less clear. For example, in the IL-12Rβ1, IFN-γR1 and IFN-γR2 proteins various amino substitutions, with unknown impact on protein function were found. Those variants need to be functionally characterized in order to designate them as polymorphisms or mutations, and to address the cause of the mycobacterial disease.

6.2. Management

MSMD patients with NTM or BCG infections need antibiotics and additional measures to combat the infections [2, 4]. The choice of antibiotic treatment depends on the sensitivity of the mycobacterial species to antibiotics. Antibiotic treatment is often required permanently, because of the dissemination of the mycobacteria and the hampered immunity. Vaccination of a sibling from MSMD patients with the live BCG vaccine should be avoided, until it is proven that the sibling is not affected by the same genetic deficiency. IL-12Rβ1-deficient and partial IFN-γR1-deficient patients usually respond very well to antibiotic therapy, and additional treatment with high doses of IFN-γ is possible. Complete STAT1, IFN-γR1 or IFN-γR2 deficiency usually leads to a more severe disease, for which antibiotic treatment alone may not be not successful. Full remission is not always achieved and relapse of the infection frequently occurs after withdrawal of the antibiotic therapy.

Stem cell transplantation is suggested as an optional therapy for patients with STAT1 deficiencies and patients with complete IFN-γR deficiencies. First attempts of transplantation were not always successful, only two of the eight patients experienced recovery [2]. Active infections may impede stem cell transplantation. In addition, the circulating IFN-γ in these patients may evoke graft rejection [161], whereby IFN-γ depletion is required for successful transplantation in these patients.

Additional IFN-α treatment has also been suggested as a therapeutic option for patients with IFN-γR deficiencies, since both IFN-α and IFN-γ are able to induce a broad range of interferon inducible genes. Three patients with disseminated *M. avium* infections have been treated with IFN-α [162, 163], though the benefit of this additional treatment was not clear. Type I interferons stimulate mature immune cells, but inhibit the immature immune cells [118]. For instance, IFN-α enhances the antigenic function of maturing monocyte-derived dendritic cells. In this way IFN-α may promote Th1 responses [164]. On the other hand IFN-α inhibits the IFN-γ responsiveness of primary monocytes [165]. Besides the effects on monocytes, IFN-α also has dual effects on the establishment of B and T cell responses. The isotype class switch and the antibody production of plasma cells are stimulated by type I interferons, while the development of $CD19^+$ pro-B cells is inhibited [118]. The cytotoxic effects of $CD8^+$ T cells against virus-infected cells is enhanced by IFN-α, while $CD4^+$ T cell responses and early T cell development are inhibited [118]. Thus, the outcome of IFN-α therapy is unpredictable yet, and may even be counter-effective.

Outline of the thesis

This thesis focuses on the causes of impaired type I immune responses, important in the control of non-tuberculous and tuberculous mycobacterial infections. The first part of the thesis (chapters 2-6) covers the investigations of genetic variations in the genes of key cytokine receptors in the control of type I immune responses. Studying the impact of polymorphisms and mutations in the IL-12, IL-23 and IFN-γ receptors on cytokine responses will help in the diagnosis and treatment of patients with severe NTM or BCG infections. The second part of the thesis (chapters 7 and 8) focuses on other causes of repressed IFN-γ mediated immune responses involved in the manifestation of tuberculous mycobacterial infections.

Chapter 2 is a case report of a patient with a disseminated *Mycobacterium genavense* infection. Functional tests revealed a deficiency in IL-12Rβ1. The genetic analysis showed two variations, which are putative mutations, within the transcript of *IL12RB1*: a -2C>T variation in the translational start site and a 1561C>G variation resulting in an amino acid substitution at position 521 of the protein. Using a cellular model with retroviral expression of *wild type* and mutant IL-12Rβ1, the R521G variation was found to be the cause of the disease. In the **addendum of chapter 2** a summary is given of the functional characterization of the naturally occurring amino acid substitutions within IL-12Rβ1.

In **chapter 3** the function of various IL-23R variants is investigated. Until now several MSMD causing mutations, leading to impaired IL-12 and IL-23 responses, were found in *IL12RB1,* but not in *IL12RB2* or *IL23R*. Still some IL-23R variations were found the impact of which on receptor function was unknown. An amino acid substitution Y173H in the IL-23R of a patient with pulmonary tuberculosis was reported. In **chapter 3** the influence of the Y173H amino acid substitution on the receptor function was studied using a cellular model with retroviral expression of the IL-23 receptor. Two other variations, the frequently found polymorphisms P310L and R381Q, which were previously reported to be associated with autoimmune diseases, were also under investigation. In addition, the effects of IL-12 and IL-23 were compared in this cellular model.

Little is known about the IL-23R expression patterns *in vivo* due to the lack of a good antibody against the IL-23R. In **chapter 4** the presence of a functional IL-23R on NK-like T cells was determined by studying the direct effects of IL-23 on the receptor-specific signaling. Next, a first comparison was made between the effects of IL-12 and IL-23 on these primary cells.

Chapters 5 and 6 describe the functional characterization of variations in the IFN-γR, in order to designate them as polymorphisms or mutations. To assess the nature of IFN-γR defects is important, since patients with partial and patients with complete IFN-γR deficiencies have different prognoses and treatment options. Several case reports in the literature describe

patients who are unresponsive to IFN-γ. In some of these patients single amino acid variations were found in one of the two IFN-γR genes. Whether such a variation is the cause of an IFN-γR deficiency needs to be proven. Therefore, we developed two models. **Chapter 5** evaluates the use of a cellular model to test and compare the cell surface expression and function of IFN-γR1 variants. In **chapter 6** we evaluate a model to test IFN-γR2 variants. With the use of these models it was possible to distinguish MSMD-causing mutant receptor variants from polymorphic variants.

In **chapters 7 and 8** the role of IFN-α in the type I immunity is investigated. One of the current hypotheses is that IFN-α/β aggravates the course of tuberculosis. Previously, it was found that the IFN-γ signaling, necessary to control mycobacterial infections, was impaired in the blood cells of patients with tuberculosis. IFN-α and IFN-β seem to be able to inhibit the IFN-γ mediated type I immune responses of monocytes in tuberculosis patients. In the study presented in **chapter 7** the extent of the inhibitory effects of IFN-α and IFN-β on monocytes was investigated. In addition we reveal some of the mechanisms whereby IFN-α inhibits the type I immune response.

The fact that IFN-α/β has been shown to strongly inhibit the type I immunity leads to the following hypothesis; viral infections cause the resuscitation of dormant *M. tuberculosis* and aggravate the course of tuberculosis. In **chapter 8** we tested the latter for influenza infections. Influenza viruses induce IFN-α/β in the respiratory tract and could make the host susceptible to *M. tuberculosis* infections in the lung. We studied the plasmas of tuberculosis patients and controls, which were previously collected in Jakarta in Indonesia, for the presence of antibodies against recent influenza viruses. In the group of tuberculosis patients more recent cases of influenza infections were expected.

References

[1] WHO report 2009. Global tuberculosis control - epidemiology, strategy, financing. WHO/HTM/TB/2009.411.

[2] Haverkamp MH, van Dissel JT, Holland SM. Human host genetic factors in nontuberculous mycobacterial infection: lessons from single gene disorders affecting innate and adaptive immunity and lessons from molecular defects in interferon-γ-dependent signaling. Microbes Infect 2006;8:1157-66.

[3] Wagner D, Young LS. Nontuberculous mycobacterial infections: a clinical review. Infection 2004;32:257-70.

[4] Remus N, Reichenbach J, Picard C, Rietschel C, Wood P, Lammas D et al. Impaired interferon γ-mediated immunity and susceptibility to mycobacterial infection in childhood. Pediatr Res 2001;50:8-13.

[5] van Lookeren CM, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. Cell Microbiol 2007;9:2095-102.

[6] Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. Biochem Biophys Res Commun 2009;388:621-5.

[7] Saiga H, Shimada Y, Takeda K. Innate immune effectors in mycobacterial infection. Clin Dev Immunol 2011;2011:347594.

[8] Gupta A, Kaul A, Tsolaki AG, Kishore U, Bhakta S. Mycobacterium tuberculosis: Immune evasion, latency and reactivation. Immunobiology 2011.

[9] Tailleux L, Schwartz O, Herrmann JL, Pivert E, Jackson M, Amara A et al. DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. J Exp Med 2003;197:121-7.

[10] Sacks SH. Complement fragments C3a and C5a: the salt and pepper of the immune response. Eur J Immunol 2010;40:668-70.

[11] Lappin DF, Guc D, Hill A, McShane T, Whaley K. Effect of interferon-γ on complement gene expression in different cell types. Biochem J 1992;281 (Pt 2):437-42.

[12] Colten HR, Dowton SB. Regulation of complement gene expression. Biochem Soc Symp 1986;51:37-46.

[13] Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 2004;119:753-66.

[14] O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 2007;7:353-64.

[15] Ramos-Kichik V, Mondragon-Flores R, Mondragon-Castelan M, Gonzalez-Pozos S, Muniz-Hernandez S, Rojas-Espinosa O et al. Neutrophil extracellular traps are induced by Mycobacterium tuberculosis. Tuberculosis (Edinb) 2009;89:29-37.

[16] Appelberg R. Neutrophils and intracellular pathogens: beyond phagocytosis and killing. Trends Microbiol 2007;15:87-92.

[17] van de Wetering D, de Paus RA, van Dissel JT, van de Vosse E. Salmonella induced IL-23 and IL-1β allow for IL-12 production by monocytes and Mphi1 through induction of IFN-γ in CD56 NK/NK-like T cells. PLoS One 2009;4:e8396.

[18] Watts C, Powis S. Pathways of antigen processing and presentation. Rev Immunogenet 1999;1:60-74.

[19] Del PG. The concept of type-1 and type-2 helper T cells and their cytokines in humans. Int Rev Immunol 1998;16:427-55.

[20] de Jong EC, Smits HH, Kapsenberg ML. Dendritic cell-mediated T cell polarization. Springer Semin Immunopathol 2005;26:289-307.

[21] Ottenhoff TH, Verreck FA, Lichtenauer-Kaligis EG, Hoeve MA, Sanal O, van Dissel JT. Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. Nat Genet 2002;32:97-105.

[22] Netea MG, Stuyt RJ, Kim SH, van der Meer JW, Kullberg BJ, Dinarello CA. The role of endogenous interleukin (IL)-18, IL-12, IL-1β, and tumor necrosis factor-α in the production of interferon-γ induced by Candida albicans in human whole-blood cultures. J Infect Dis 2002;185:963-70.

[23] Stuyt RJ, Kim SH, Reznikov LL, Fantuzzi G, Novick D, Rubinstein M et al. Regulation of Staphylococcus epidermidis-induced IFN-γ in whole human blood: the role of endogenous IL-18, IL-12, IL-1, and TNF. Cytokine 2003;21:65-73.

[24] Bernabei P, Allione A, Rigamonti L, Bosticardo M, Losana G, Borghi I et al. Regulation of interferon-γ receptor (IFN-γR) chains: a peculiar way to rule the life and death of human lymphocytes. Eur Cytokine Netw 2001;12:6-14.

[25] Losana G, Rigamonti L, Borghi I, Assenzio B, Ariotti S, Jouanguy E et al. Requirement for both IL-12 and IFN-γ signaling pathways in optimal IFN-γ production by human T cells. Eur J Immunol 2002;32:693-700.

[26] Harris J, Master SS, De Haro SA, Delgado M, Roberts EA, Hope JC et al. Th1-Th2 polarisation and autophagy in the control of intracellular mycobacteria by macrophages. Vet Immunol Immunopathol 2009;128:37-43.

[27] Maizels RM, Pearce EJ, Artis D, Yazdanbakhsh M, Wynn TA. Regulation of pathogenesis and immunity in helminth infections. J Exp Med 2009;206:2059-66.

[28] Mok CC, Lau CS. Pathogenesis of systemic lupus erythematosus. J Clin Pathol 2003;56:481-90.

[29] Barrett NA, Austen KF. Innate cells and T helper 2 cell immunity in airway inflammation. Immunity 2009;31:425-37.

[30] Peck A, Mellins ED. Plasticity of T-cell phenotype and function: the T helper type 17 example. Immunology 2010;129:147-53.

[31] Hoeve MA, Savage ND, de Boer T., Langenberg DM, de Waal Malefyt R, Ottenhoff TH et al. Divergent effects of IL-12 and IL-23 on the production of IL-17 by human T cells. Eur J Immunol 2006;36:661-70.

[32] Lopez KS, Dinges S, Griewank K, Iwakura Y, Udey MC, von Stebut E. IL-17 promotes progression of cutaneous leishmaniasis in susceptible mice. J Immunol 2009;182:3039-46.

[33] Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;6:1133-41.

[34] Puel A, Picard C, Cypowyj S, Lilic D, Abel L, Casanova JL. Inborn errors of mucocutaneous immunity to Candida albicans in humans: a role for IL-17 cytokines? Curr Opin Immunol 2010;22:467-74.

[35] Swain SL, Croft M, Dubey C, Haynes L, Rogers P, Zhang X et al. From naive to memory T cells. Immunol Rev 1996;150:143-67.

[36] Marin ND, Paris SC, Velez VM, Rojas CA, Rojas M, Garcia LF. Regulatory T cell frequency and modulation of IFN-γ and IL-17 in active and latent tuberculosis. Tuberculosis (Edinb) 2010;90:252-61.

[37] Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA. Anti-inflammatory and pro-inflammatory roles of TGF-β, IL-10, and IL-22 in immunity and autoimmunity. Curr Opin Pharmacol 2009;9:447-53.

[38] Cohen NR, Garg S, Brenner MB. Antigen Presentation by CD1 Lipids, T Cells, and NKT Cells in Microbial Immunity. Adv Immunol 2009;102:1-94.

[39] Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. Nat Immunol 2003;4:1230-7.

[40] Robson CD. Imaging of granulomatous lesions of the neck in children. Radiol Clin North Am 2000;38:969-77.

[41] Bartralot R, Pujol RM, Garcia-Patos V, Sitjas D, Martin-Casabona N, Coll P et al. Cutaneous infections due to nontuberculous mycobacteria: histopathological review of 28 cases. Comparative study between lesions observed in immunosuppressed patients and normal hosts. J Cutan Pathol 2000;27:124-9.

[42] Russell DG. Who puts the tubercle in tuberculosis? Nat Rev Microbiol 2007;5:39-47.

[43] Saunders BM, Frank AA, Orme IM, Cooper AM. CD4 is required for the development of a protective granulomatous response to pulmonary tuberculosis. Cell Immunol 2002;216:65-72.

[44] Diedrich CR, Mattila JT, Klein E, Janssen C, Phuah J, Sturgeon TJ et al. Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV is associated with early peripheral T cell depletion and not virus load. PLoS One 2010;5:e9611.

[45] Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J Immunol 2002;168:4620-7.

[46] Hess S, Hospach T, Nossal R, Dannecker G, Magdorf K, Uhlemann F. Life-threatening disseminated tuberculosis as a complication of TNF-α blockade in an adolescent. Eur J Pediatr 2011.

[47] Arend SM, Breedveld FC, van Dissel JT. TNF-α blockade and tuberculosis: better look before you leap. Neth J Med 2003;61:111-9.

[48] Mayordomo L, Marenco JL, Gomez-Mateos J, Rejon E. Pulmonary miliary tuberculosis in a patient with anti-TNF-α treatment. Scand J Rheumatol 2002;31:44-5.

[49] Lammas DA, De Heer E, Edgar JD, Novelli V, Ben-Smith A, Baretto R et al. Heterogeneity in the granulomatous response to mycobacterial infection in patients with defined genetic mutations in the interleukin 12-dependent interferon-γ production pathway. Int J Exp Pathol 2002;83:1-20.

[50] Parham C, Chirica M, Timans J, Vaisberg E, Travis M, Cheung J et al. A Receptor for the Heterodimeric Cytokine IL-23 Is Composed of IL-12Rβ1 and a Novel Cytokine Receptor Subunit, IL-23R. J Immunol 2002;168:5699-708.

[51] Frucht DM. IL-23: a cytokine that acts on memory T cells. Sci STKE 2002;2002:pe1.

[52] Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005;201:233-40.

[53] Ottenhoff TH, Verreck FA, Hoeve MA, van de Vosse E. Control of human host immunity to mycobacteria. Tuberculosis (Edinb) 2005;85:53-64.

[54] Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000;13:715-25.

[55] Watford WT, Hissong BD, Bream JH, Kanno Y, Muul L, O'Shea JJ. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. Immunol Rev 2004;202:139-56.

[56] Happel KI, Dubin PJ, Zheng M, Ghilardi N, Lockhart C, Quinton LJ et al. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. J Exp Med 2005;202:761-9.

[57] Schnurr M, Toy T, Shin A, Wagner M, Cebon J, Maraskovsky E. Extracellular nucleotide signaling by P2 receptors inhibits IL-12 and enhances IL-23 expression in human dendritic cells: a novel role for the cAMP pathway. Blood 2005;105:1582-9.

[58] Ma X, Chow JM, Gri G, Carra G, Gerosa F, Wolf SF et al. The interleukin 12 p40 gene promoter is primed by interferon-γ in monocytic cells. J Exp Med 1996;183:147-57.

[59] Kubin M, Chow JM, Trinchieri G. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor α, and IL-1β production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. Blood 1994;83:1847-55.

[60] Kaufmann SH, Ladel CH, Flesch IE. T cells and cytokines in intracellular bacterial infections: experiences with Mycobacterium bovis BCG. Ciba Found Symp 1995;195:123-32.

[61] Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med 1996;184:747-52.

[62] Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. Annu Rev Immunol 1998;16:111-35.

[63] Makrigiannis AP, Musgrave BL, Haeryfar SM, Hoskin DW. Interleukin-12 can replace CD28-dependent T-cell costimulation during nonspecific cytotoxic T lymphocyte induction by anti-CD3 antibody. J Leukoc Biol 2001;69:113-22.

[64] Smits HH, van Beelen AJ, Hessle C, Westland R, de Jong E, Soeteman E et al. Commensal Gram-negative bacteria prime human dendritic cells for enhanced IL-23 and IL-27 expression and enhanced Th1 development. Eur J Immunol 2004;34:1371-80.

[65] Harris KM, Fasano A, Mann DL. Cutting edge: IL-1 controls the IL-23 response induced by gliadin, the etiologic agent in celiac disease. J Immunol 2008;181:4457-60.

[66] Sheibanie AF, Tadmori I, Jing H, Vassiliou E, Ganea D. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. FASEB J 2004;18:1318-20.

[67] D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL-10) inhibits human lymphocyte interferon γ-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 1993;178:1041-8.

[68] Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev 2003;14:361-8.

[69] Wu C, Warrier RR, Wang X, Presky DH, Gately MK. Regulation of interleukin-12 receptor β1 chain expression and interleukin-12 binding by human peripheral blood mononuclear cells. Eur J Immunol 1997;27:147-54.

[70] Khader SA, Partida-Sanchez S, Bell G, Jelley-Gibbs DM, Swain S, Pearl JE et al. Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. J Exp Med 2006;203:1805-15.

[71] Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O'Shea JJ et al. Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. J Clin Immunol 2003;23:147-61.

[72] Bosticardo M, Witte I, Fieschi C, Novelli F, Casanova JL, Candotti F. Retroviral-mediated gene transfer restores IL-12 and IL-23 signaling pathways in T cells from IL-12 receptor β1-deficient patients. Mol Ther 2004;9:895-901.

[73] Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol 2007;8:345-50.

[74] Mills KH. Induction, function and regulation of IL-17-producing T cells. Eur J Immunol 2008;38:2636-49.

[75] Khader SA, Pearl JE, Sakamoto K, Gilmartin L, Bell GK, Jelley-Gibbs DM et al. IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-γ responses if IL-12p70 is available. J Immunol 2005;175:788-95.

[76] Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat Immunol 2007;8:369-77.

[77] Happel KI, Zheng M, Young E, Quinton LJ, Lockhart E, Ramsay AJ et al. Cutting Edge: Roles of Toll-Like Receptor 4 and IL-23 in IL-17 Expression in Response to Klebsiella pneumoniae Infection. J Immunol 2003;170:4432-6.

[78] Kagami S, Rizzo HL, Kurtz SE, Miller LS, Blauvelt A. IL-23 and IL-17A, but not IL-12 and IL-22, are required for optimal skin host defense against Candida albicans. J Immunol 2010;185:5453-62.

[79] Saunus JM, Wagner SA, Matias MA, Hu Y, Zaini ZM, Farah CS. Early activation of the interleukin-23-17 axis in a murine model of oropharyngeal candidiasis. Mol Oral Microbiol 2010;25:343-56.

[80] van den Eijnden S, Goriely S, de Wit D, Willems F, Goldman M. IL-23 up-regulates IL-10 and induces IL-17 synthesis by polyclonally activated naive T cells in human. Eur J Immunol 2005;35:469-75.

[81] Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 2006;314:1461-3.

[82] Capon F, Di Meglio P, Szaub J, Prescott NJ, Dunster C, Baumber L et al. Sequence variants in the genes for the interleukin-23 receptor (IL23R) and its ligand (IL12B) confer protection against psoriasis. Hum Genet 2007;122:201-6.

[83] Huber AK, Jacobson EM, Jazdzewski K, Concepcion ES, Tomer Y. Interleukin-23 receptor is a major susceptibility gene for Grave's ophthalmopathy: The IL-23/Th17 axis extends to thyroid autoimmunity. J Clin Endocrinol Metab 2008;93:1077-81.

[84] Rueda B, Orozco G, Raya E, Fernandez-Sueiro JL, Mulero J, Blanco FJ et al. The IL23R Arg381Gln non-synonymous polymorphism confers susceptibility to ankylosing spondylitis. Ann Rheum Dis 2008;67:1451-4.

[85] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. Annu Rev Immunol 1997;15:749-95.

[86] Liu B, Mink S, Wong KA, Stein N, Getman C, Dempsey PW et al. PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. Nat Immunol 2004;5:891-8.

[87] Valente G, Ozmen L, Novelli F, Geuna M, Palestro G, Forni G et al. Distribution of interferon-γ receptor in human tissues. Eur J Immunol 1992;22:2403-12.

[88] Qureshi SA, Salditt-Georgieff M, Darnell JEJr. Tyrosine-phosphorylated Stat1 and Stat2 plus a 48-kDa protein all contact DNA in forming interferon-stimulated-gene factor 3. Proc Natl Acad Sci U S A 1995;92:3829-33.

[89] Takaoka A, Yanai H. Interferon signalling network in innate defence. Cell Microbiol 2006;8:907-22.

[90] Abramovich C, Yakobson B, Chebath J, Revel M. A protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNαR1 chain in the type I interferon receptor. EMBO J 1997;16:260-6.

[91] Boisvert FM, Chenard CA, Richard S. Protein interfaces in signaling regulated by arginine methylation. Sci STKE 2005;271 re2:1-10.

[92] Becker S, Daniel EG. Antagonistic and additive effects of IL-4 and interferon-γ on human monocytes and macrophages: effects on Fc receptors, HLA-D antigens, and superoxide production. Cell Immunol 1990;129:351-62.

[93] Schiff DE, Rae J, Martin TR, Davis BH, Curnutte JT. Increased phagocyte FcγRI expression and improved Fc γ-receptor-mediated phagocytosis after in vivo recombinant human γ treatment of normal human subjects. Blood 1997;90:3187-94.

[94] Drevets DA, Leenen PJ, Campbell PA. Complement receptor type 3 mediates phagocytosis and killing of Listeria monocytogenes by a TNF-α and IFN-γ stimulated macrophage precursor hybrid. Cell Immunol 1996;169:1-6.

[95] Livingston DH, Appel SH, Sonnenfeld G, Malangoni MA. The effect of tumor necrosis factor-α and interferon-γ on neutrophil function. J Surg Res 1989;46:322-6.

[96] Leibson HJ, Gefter M, Zlotnik A, Marrack P, Kappler JW. Role of γ-interferon in antibody-producing responses. Nature 1984;309:799-801.

[97] Snapper CM, Paul WE. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 1987;236:944-7.

[98] Sacks S, Zhou W, Campbell RD, Martin J. C3 and C4 gene expression and γ-mediated regulation in human glomerular mesangial cells. Clin Exp Immunol 1993;93:411-7.

[99] Terui T, Ishii K, Ozawa M, Tabata N, Kato T, Tagami H. C3 production of cultured human epidermal keratinocytes is enhanced by IFN-γ and TNF-α through different pathways. J Invest Dermatol 1997;108:62-7.

[100] Van der Ven BC, Yates RM, Russell DG. Intraphagosomal measurement of the magnitude and duration of the oxidative burst. Traffic 2009;10:372-8.

[101] Santic M, Molmeret M, Abu KY. Maturation of the Legionella pneumophila-containing phagosome into a phagolysosome within γ interferon-activated macrophages. Infect Immun 2005;73:3166-71.

[102] Santic M, Molmeret M, Abu KY. Modulation of biogenesis of the Francisella tularensis subsp. novicida-containing phagosome in quiescent human macrophages and its maturation into a phagolysosome upon activation by IFN-γ. Cell Microbiol 2005;7:957-67.

[103] Sibley LD, Franzblau SG, Krahenbuhl JL. Intracellular fate of Mycobacterium leprae in normal and activated mouse macrophages. Infect Immun 1987;55:680-5.

[104] Feelders RA, Vreugdenhil G, Eggermont AM, Kuiper-Kramer PA, van Eijk HG, Swaak AJ. Regulation of iron metabolism in the acute-phase response: interferon γ and tumour necrosis factor α induce hypoferraemia, ferritin production and a decrease in circulating transferrin receptors in cancer patients. Eur J Clin Invest 1998;28:520-7.

[105] Nairz M, Schroll A, Sonnweber T, Weiss G. The struggle for iron - a metal at the host-pathogen interface. Cell Microbiol 2010;12:1691-702.

[106] Duits LA, Ravensbergen B, Rademaker M, Hiemstra PS, Nibbering PH. Expression of β-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells. Immunology 2002;106:517-25.

[107] Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L et al. The human antimicrobial and chemotactic peptides LL-37 and α-defensins are expressed by specific lymphocyte and monocyte populations. Blood 2000;96:3086-93.

[108] Reiter Z. Interferon-α major regulator of natural killer cell-mediated cytotoxicity. J Interferon Res 1993;13:247-57.

[109] Verreck FA, de Boer T, Langenberg DM, van der Zanden L, Ottenhoff TH. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-γ- and CD40L-mediated costimulation. J Leukoc Biol 2006;79:285-93.

[110] Hayes MP, Wang J, Norcross MA. Regulation of interleukin-12 expression in human monocytes: selective priming by Interferon-γ of lipopolysaccharide-inducible p35 and p40 genes. Blood 1995;86:646-50.

[111] Hart PH, Whitty GA, Piccoli DS, Hamilton JA. Control by IFN-γ and PGE2 of TNF α and IL-1 production by human monocytes. Immunology 1989;66:376-83.

[112] Hu X, Chakravarty SD, Ivashkiv LB. Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. Immunol Rev 2008;226:41-56.

[113] Herrero C, Hu X, Li WP, Samuels S, Sharif MN, Kotenko S et al. Reprogramming of IL-10 activity and signaling by IFN-γ. J Immunol 2003;171:5034-41.

[114] Chan LL, Cheung BK, Li JC, Lau AS. A role for STAT3 and cathepsin S in IL-10 down-regulation of IFN-γ-induced MHC class II molecule on primary human blood macrophages. J Leukoc Biol 2010;88:303-11.

[115] Opitz B, Vinzing M, van Laak V, Schmeck B, Heine G, Gunther S et al. Legionella pneumophila induces IFN-β in lung epithelial cells via IPS-1 and IRF3, which also control bacterial replication. J Biol Chem 2006;281:36173-9.

[116] O'Riordan M, Yi CH, Gonzales R, Lee KD, Portnoy DA. Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. Proc Natl Acad Sci U S A 2002;99:13861-6.

[117] Auerbuch V, Brockstedt DG, Meyer-Morse N, O'Riordan M, Portnoy DA. Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes. J Exp Med 2004;200:527-33.

[118] Seo YJ, Hahm B. Type I interferon modulates the battle of host immune system against viruses. Adv Appl Microbiol 2010;73:83-101.

[119] Nagai T, Devergne O, van Seventer GA, van Seventer JM. Interferon-β mediates opposing effects on interferon-γ-dependent Interleukin-12 p70 secretion by human monocyte-derived dendritic cells. Scand J Immunol 2007;65:107-17.

[120] Mattner J, Wandersee-Steinhauser A, Pahl A, Rollinghoff M, Majeau GR, Hochman PS et al. Protection against progressive leishmaniasis by IFN-β. J Immunol 2004;172:7574-82.

[121] Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 2010;466:973-7.

[122] Ottenhoff THM, Hari Dass R, Yang N, Zhang M, Wong HEE, Sahiratmadja E et al. Genome-wide expression profiling identifies Type I interferon response pathways in active tuberculosis. accepted for publication in PlosOne 2012;7 (9):e45839.

[123] Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-α /β. Proc Natl Acad Sci U S A 2001;98:5752-7.

[124] Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, Murray PJ et al. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res 2005;25:694-701.

[125] Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci 2008;1143:1-20.

[126] Boo KH, Yang JS. Intrinsic cellular defenses against virus infection by antiviral type I interferon. Yonsei Med J 2010;51:9-17.

[127] Ghislain JJ, Wong T, Nguyen M, Fish EN. The interferon-inducible Stat2:Stat1 heterodimer preferentially binds in vitro to a consensus element found in the promoters of a subset of interferon-stimulated genes. J Interferon Cytokine Res 2001;21:379-88.

[128] Li X, Leung S, Qureshi S, Darnell JE, Jr., Stark GR. Formation of STAT1-STAT2 heterodimers and their role in the activation of IRF-1 gene transcription by interferon-α. J Biol Chem 1996;271:5790-4.

[129] Hofer MJ, Li W, Lim SL, Campbell IL. The type I interferon-α mediates a more severe neurological disease in the absence of the canonical signaling molecule interferon regulatory factor 9. J Neurosci 2010;30:1149-57.

[130] Wesoly J, Szweykowska-Kulinska Z, Bluyssen HA. STAT activation and differential complex formation dictate selectivity of interferon responses. Acta Biochim Pol 2007;54:27-38.

[131] Vignais PV. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cell Mol Life Sci 2002;59:1428-59.

[132] Van der Ven BC, Yates RM, Russell DG. Intraphagosomal measurement of the magnitude and duration of the oxidative burst. Traffic 2009;10:372-8.

[133] Spagnolo L, Toro I, D'Orazio M, O'Neill P, Pedersen JZ, Carugo O et al. Unique features of the sodC-encoded superoxide dismutase from Mycobacterium tuberculosis, a fully functional copper-containing enzyme lacking zinc in the active site. J Biol Chem 2004;279:33447-55.

[134] Chan J, Fujiwara T, Brennan P, McNeil M, Turco SJ, Sibille JC et al. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc Natl Acad Sci U S A 1989;86:2453-7.

[135] Flynn JL, Chan J. Immune evasion by Mycobacterium tuberculosis: living with the enemy. Curr Opin Immunol 2003;15:450-5.

[136] Appelberg R. Pathogenesis of Mycobacterium avium infection: typical responses to an atypical mycobacterium? Immunol Res 2006;35:179-90.

[137] Simeone R, Bottai D, Brosch R. ESX/type VII secretion systems and their role in host-pathogen interaction. Curr Opin Microbiol 2009;12:4-10.

[138] Behar SM, Divangahi M, Remold HG. Evasion of innate immunity by Mycobacterium tuberculosis: is death an exit strategy? Nat Rev Microbiol 2010;8:668-74.

[139] Torrado E, Robinson RT, Cooper AM. Cellular response to mycobacteria: balancing protection and pathology. Trends Immunol 2011;32:66-72.

[140] Baena A, Porcelli SA. Evasion and subversion of antigen presentation by Mycobacterium tuberculosis. Tissue Antigens 2009;74:189-204.

[141] van de Vosse E, Hoeve MA, Ottenhoff TH. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. Lancet Infect Dis 2004;4:739-49.

[142] van de Vosse E, van Dissel JT, Ottenhoff TH. Genetic deficiencies of innate immune signalling in human infectious disease. Lancet Infect Dis 2009;9:688-98.

[143] Barboza CE, Winter DH, Seiscento M, Santos UP, Terra FM. Tuberculosis and silicosis: epidemiology, diagnosis and chemoprophylaxis. J Bras Pneumol 2008;34:959-66.

[144] Alisjahbana B, van Crevel R, Sahiratmadja E, den Heijer M, Maya A, Istriana E et al. Diabetes mellitus is strongly associated with tuberculosis in Indonesia. Int J Tuberc Lung Dis 2006;10:696-700.

[145] Bates MN, Khalakdina A, Pai M, Chang L, Lessa F, Smith KR. Risk of tuberculosis from exposure to tobacco smoke: a systematic review and meta-analysis. Arch Intern Med 2007;167:335-42.

[146] Davila S, Hibberd ML, Hari Dass R, Wong HE, Sahiratmadja E, Bonnard C et al. Genetic association and expression studies indicate a role of toll-like receptor 8 in pulmonary tuberculosis. PLoS Genet 2008;4:e1000218.

[147] Leandro AC, Rocha MA, Cardoso CS, Bonecini-Almeida MG. Genetic polymorphisms in vitamin D receptor, vitamin D-binding protein, Toll-like receptor 2, nitric oxide synthase 2, and interferon-γ genes and its association with susceptibility to tuberculosis. Braz J Med Biol Res 2009;42:312-22.

[148] Moller M, de Wit E, Hoal EG. Past, present and future directions in human genetic susceptibility to tuberculosis. FEMS Immunol Med Microbiol 2010;58:3-26.

[149] Sahiratmadja E, Baak-Pablo R, de Visser AW, Alisjahbana B, Adnan I, van Crevel R et al. Association of polymorphisms in IL-12/IFN-γ pathway genes with susceptibility to pulmonary tuberculosis in Indonesia. Tuberculosis (Edinb) 2007;87:303-11.

[150] Tso HW, Lau YL, Tam CM, Wong HS, Chiang AK. Associations between IL12B polymorphisms and tuberculosis in the Hong Kong Chinese population. J Infect Dis 2004;190:913-9.

[151] Meilang Q, Zhang Y, Zhang J, Zhao Y, Tian C, Huang J et al. Polymorphisms in the SLC11A1 gene and tuberculosis risk: a meta-analysis update. Int J Tuberc Lung Dis 2012;16:437-46.

[152] Bogunovic D, Byun M, Durfee LA, Abhyankar A, Sanal O, Mansouri D et al. Mycobacterial Disease and Impaired IFN-γ Immunity in Humans with Inherited ISG15 Deficiency. Science 2012;DOI: 10.1126.

[153] Hambleton S, Salem S, Bustamante J, Bigley V, Boisson-Dupuis S, Azevedo J et al. IRF8 mutations and human dendritic-cell immunodeficiency. N Engl J Med 2011;365:127-38.

[154] Dorman SE, Holland SM. Interferon-γ and interleukin-12 pathway defects and human disease. Cytokine Growth Factor Rev 2000;11:321-33.

[155] Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, Al-Jumaah S et al. Impaired response to interferon-α/β and lethal viral disease in human STAT1 deficiency. Nat Genet 2003;33:388-91.

[156] Filipe-Santos O, Bustamante J, Haverkamp MH, Vinolo E, Ku CL, Puel A et al. X-linked susceptibility to mycobacteria is caused by mutations in NEMO impairing CD40-dependent IL-12 production. J Exp Med 2006;203:1745-59.

[157] Verstrepen L, Bekaert T, Chau TL, Tavernier J, Chariot A, Beyaert R. TLR-4, IL-1R and TNF-R signaling to NF-κB: variations on a common theme. Cell Mol Life Sci 2008;65:2964-78.

[158] Minegishi Y, Saito M, Morio T, Watanabe K, Agematsu K, Tsuchiya S et al. Human tyrosine kinase 2 deficiency reveals its requisite roles in multiple cytokine signals involved in innate and acquired immunity. Immunity 2006;25:745-55.

[159] Kilic SS, Hacimustafaoglu M, Boisson-Dupuis S, Kreins AY, Grant AV, Abel L et al. A patient with tyrosine kinase 2 deficiency without hyper-IgE syndrome. J Pediatr 2012;160:1055-7.

[160] Tamura T, Yanai H, Savitsky D, Taniguchi T. The IRF family transcription factors in immunity and oncogenesis. Annu Rev Immunol 2008;26:535-84.

[161] Rottman M, Soudais C, Vogt G, Renia L, Emile JF, Decaluwe H et al. IFN-γ mediates the rejection of haematopoietic stem cells in IFN-γR1-deficient hosts. PLoS Med 2008;5:e26.

[162] Ward CM, Jyonouchi H, Kotenko SV, Smirnov SV, Patel R, Aguila H et al. Adjunctive treatment of disseminated Mycobacterium avium complex infection with interferon α-2b in a patient with complete interferon-γ receptor R1 deficiency. Eur J Pediatr 2007;166:981-5.

[163] Rapkiewicz AV, Patel SY, Holland SM, Kleiner DE. Hepatoportal venopathy due to disseminated Mycobacterium avium complex infection in a child with IFN-γ receptor 2 deficiency. Virchows Arch 2007;451:95-100.

[164] Santini SM, Lapenta C, Logozzi M, Parlato S, Spada M, Di Pucchio T et al. Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. J Exp Med 2000;191:1777-88.

[165] van de Wetering D, Van Wengen A, Savage ND, van de Vosse E, van Dissel JT. IFN-α cannot substitute lack of IFN-γ responsiveness in cells of an IFN-γR1 deficient patient. Clin Immunol 2011;138:282-90.

CHAPTER 2

Disseminated *Mycobacterium genavense* infection in a patient with a novel partial interleukin-12 receptor β1 deficiency

Judith Potjewijd[1], Roelof A. de Paus[2], Annelies van Wengen[2], Jan Damoiseaux[3], Annelies Verbon[4] and Esther van de Vosse[2].

[1]Dept. of Internal Medicine, Division of Clinical and Experimental Immunology, Maastricht University Medical Center, Maastricht, The Netherlands.
[2]Dept. of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands.
[3]Laboratory of Clinical Immunology, Maastricht University Medical Center, Maastricht, The Netherlands.
[4]Dept. of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands.

extended version of manuscript
Clinical Immunology 2012;144(2):83-86

Abstract

An adult patient with a severe disseminated *M. genavense* infection was investigated. The patient had a novel mutation in *IL12RB1*, leading to the amino acid substitution R521G. The R5212G variation was confirmed to be a mutation in a study using a cellular model, with retroviral expression of cloned IL-12Rβ1 variants in cells from a completely IL-12Rβ1 deficient patient. We demonstrate that the R521G variation results in a strong reduction of the cell surface expression of IL-12Rβ1 and a minimal response upon IL-12 stimulation. Another variation, -2C>T, was found in the Kozak consensus sequence, but this variation did not influence receptor expression in our experiments and appears to be a functional polymorphism. R521G is, next to C198R, only the second known mutation in IL-12Rβ1 leading to a partial defect, and is a cause of Mendelian susceptibility to mycobacterial disease. This case illustrates that Mendelian susceptibility to mycobacterial disease may manifest later in life and not only in childhood.

1. Introduction

Mendelian susceptibility to mycobacterial disease (MSMD, MIM #209950) is a rare disease which usually manifests in early childhood. MSMD patients often suffer from disseminated nontuberculous mycobacteria (NTM), Bacillus Calmette-Guerin (BCG) and/or Salmonella infections. Individuals with MSMD have an impaired type I immune response due to deficiencies in the type I cytokine pathway [1, 2]. MSMD causing mutations have been identified in five genes; *IL12RB1, IL12B, IFNGR1*, *IFNGR2*, and *STAT1*. While most patients suffer only from infections with *Mycobacteria* and *Salmonellae*, STAT1 deficient patients suffer in most cases from severe viral infections [3]. Complete defects in the IFN-γ receptor (IFN-γR) result in a severe manifestation of MSMD, while partial IFN-γR deficiency and complete IL-12p40 or IL-12Rβ1 deficiency usually results in a less severe disease. Until now only one IL-12Rβ1 mutation, C198R, identified in three MSMD patients [4, 5] was found to result in a partial IL-12Rβ1 deficiency [6].

Human immunity against intracellular pathogens, such as *Mycobacteria* and *Salmonellae*, is dependent on an effective cell-mediated type I immune response. Dendritic cells and macrophages can recognize these pathogens via innate pattern recognition receptors (PRRs) which interact with specific pathogen associated molecular patterns (PAMPs). Activation of PRRs by mycobacterial PAMPs induces the production of various type I cytokines cytokines, such as IL-12, IL-23, IL-1β and tumor necrosis factor (TNF).

IL-12 and IL-23 are heterodimers with IL-12p40 as a common subunit. IL-12 and IL-23 signal via their own receptor complex, respectively consisting of a specific IL-12Rβ2 and IL-23R chain and a common IL-12Rβ1 chain. Hence, IL-12p40 and IL-12Rβ1 deficiency results in both impaired IL-12 and IL-23 responses. IL-12 and IL-23 activate T cells, for instance to produce IFN-γ. IL-12 signals via STAT4 and thereby induces the IFN-γ production of various subsets of T cells [7]. IL-23 may also stimulate natural-killer-like T cells to produce IFN-γ [8]. IFN-γ acts in concert with other type I cytokines to regulate effective innate and adaptive immune responses [9] and granuloma formation [10, 11]. Defects in this IL-12/IFN-γ pathway, such as defects in IL-12Rβ1, results in a lack of the IFN-γ mediated type I immunity and impaired granuloma formation giving rise to disseminated infections with otherwise poorly pathogenic *Mycobacteria* and *Salmonellae* species.

We present a case of disseminated NTM infection in an adult, middle aged, patient with a partial IL-12Rβ1 deficiency. The clinical phenotype of this patient and the immunological investigations, in order to unravell the nature of the deficiency, are described. We found two homozygous variations within the transcript of *IL12RB1* from the patient. The variations were functionally characterized using a cellular model with retroviral transduction of *IL12RB1* constructs, from which one variation appeared to be a novel mutation, causing MSMD.

2. Patient and Methods

2.1. Case report

A 43 year old man was admitted to the hospital because of anorexia, fatigue and weight loss (27 kg) since 2 months. He had intermittent subfebrile temperatures, night sweats and a productive cough with clear sputum. The patient smoked five to ten cigarettes a day and never used (intravenous) drugs. His medical history showed congenital hearing loss and cataract at the age of 38. On physical examination a cachectic male was seen with a temperature of 38.3 ^{o}C. He had cervical lymphadenopathy and hepatomegaly, no other abnormalities were found.

Laboratory evaluation showed elevated inflammation parameters (ESR 41 mm, CRP 122 mg/L) and a pancytopenia (hemoglobin 6.3 mmol/L, leucocytes 1.8 * 10^{9} /L with a normal differentation, thrombocytes 94 * 10^{9} /L). An X-ray of the lungs showed no abnormalities. A CT scan of thorax and abdomen showed retroperitoneal, mesenterial and cervical lymphadenopathy and hepatosplenomegaly. A cervical lymphnode and bone marrow biopsy showed granulomatous infiltrations with intracellular mycobacteria. In the Ziehl Neelsen stain acid-fast rods were seen. *Mycobacterium genavense* was cultured from blood and sputum. The patient repeatedly tested negative for HIV by polymerase chain reaction (PCR).

Treatment was started with clarithromycin, ethambutol and rifampicin. After three weeks, the patient was discharged in a reasonably good physical and mental condition. A few months later he had completely recovered, and after 18 months triple antibiotic therapy was stopped. Because of the disseminated NTM infection and the absence of HIV or extensive immunosuppression, MSMD was suspected and further examination of the patient's host immunity was performed.

2.2. Generation of T cell blasts

PBMCs were isolated from the blood by Ficoll separation. Cells were cultured in IMDM supplemented 20 mM GlutaMax, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % fetal calf serum (Invitrogen). To generate T cell blasts (TCB) the PBMCs were stimulated with 800 ng/ml PHA-16 (Murex) and cultured in medium with 30 U/ml IL-2 (Chiron) in 24-wellsplates and tested in 96-wellplates (Greiner bio-one).

2.3. Detection of the IL-12Rβ1 expression

1*10^{5} PHA blasts were washed in FACS buffer; 0.2% bovine serum albumin (Roche) in phosphate buffered saline. The cells were labeled with one of the PE conjugated specific IL-12Rβ1 antibodies (clone 4D1 and 2.4E6, BD Biosciences). As controls unlabeled cells and cells labeled with a PE conjugated isotype control IgG1 (DAKO) were used. After labeling, the cells were washed twice with FACS buffer. Subsequently the cells were analyzed using a FACSCalibur and CellQuest software (BD Biosciences).

2.4. IFN-γ production assay

TCB were used 14 days after PHA stimulation. $1*10^5$ TCB were cultured in 200 µl medium and left unstimulated or stimulated with 2 µg/ml anti-CD2 (CLB-T11.1/1 and CLB-T11.2/1) and 2 µg/ml anti CD28 (CLB-CD28/1) in the presence of various concentrations of IL-12 (R&D). After 72 h the supernatants were removed and analyzed for the presence of IFN-γ by ELISA (Invitrogen).

2.5. STAT4 phosphorylation assay

To study the signal transduction $2*10^5$ cells were prestimulated overnight in 150 µl culture medium supplemented with anti CD2 and anti CD28 antibodies. Thereafter the cells were pulsed with 200, 1000 or 5000 pg/ml of IL-12. After the pulse, the cells were fixed with paraformaldehyde (Sigma) and permeabilized with methanol (Merck). Subsequently, the cells were washed with FACS buffer, blocked with 10% normal goat serum (Sanquin) and stained with a specific antibody against pY693-STAT4-alexafluor647 (BD Biosciences). Before FACS analysis the cells were washed twice.

2.6. Genetic screening

The *IL12RB1* mRNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen) from TCB-derived RNA and sequenced. To confirm the presence of the detected variations in the genomic DNA the relevant exons were amplified by PCR from the genomic DNA, isolated by the high salt method. The PCR products were sequenced by the Leiden Genome Technology Centre (LGTC in Leiden, The Netherlands). Primers and PCR conditions are available upon request.

2.7. Expression cloning of IL-12Rβ1and retroviral transduction

The coding sequences of *wild type* IL-12Rβ1 and the R521G variant were cloned, respectively from control and patient cDNA in the pGEM-T easy vector as described before using the following primers [6]; primer IL12RB1-2C-ATG: 5'-GGCTCTACGTGGATCCGATGGAGCCGCTGGTGACC-3', primer IL12RB1-2T-ATG: 5'-GGCTCTACGTGGATCTGATGGAGCCGCTGGTGACC-3' and the reverse primer IL12RB1-STOP: 5'-GGGTCCAAATGTGACTCCTGTGT-3'.The coding sequences for *wild type* IL-12Rβ1 and the R521G variant were transferred from the pGEM-Teasy vector into the bicistronic retroviral pLZRS-IRES-GFP vector with either the wild type translational start site or with the start site containing the -2C>T variation. All constructs were verified by sequencing. Helper free recombinant virus was produced and used to transduce TCB, from a patient with a Q32X mutation in *IL12RB1*, as described before [6]. GFP positive TCB were isolated by FACS sorting using the FACSAria (BD Biosciences).

2.8. Statistical analysis

The student t-test was used to determine statistical significance between two groups of measurements. Results were regarded significantly different when $p<0.05$. In order to determine the difference between the kinetics of STAT4 phosphorylation, non linear least squares regression analysis was performed with GraphPad Prism software, using 95% confidential intervals to designate significant differences.

3. Results

3.1. Phenotypic and functional evidence for an IL-12Rβ1 deficiency

Although the patient developed disease at middle-age and not in childhood, we suspected the patient has MSMD, because *M. genavense* rarely causes disseminated infections in immunocompetent individuals. Therefore we tested the blood cells from the patient for thier ability to respond to IFN-γ and IL-12. The monocytes of the patient and a control responded upon LPS and IFN-γ stimulations, with a similar induction of TNF and IL-12p40 production (data not shown). Next, we determined the IL-12Rβ1 expression and IL-12 responsiveness of the TCB from the patient. The IL-12Rβ1 expression could be detected on control TCB but not on the TCB from the patient (Figure 1). Surprisingly, the TCB from the patient showed upon IL-12 stimulation no abrogated, but a severely reduced STAT4 phosphorylation as compared to the response of the control TCB (Figure 2). Next, we examined the IFN-γ production of the activated TCB. The TCB from the patient showed upon CD2 and CD28 stimulation a strongly reduced IFN-γ production as compared to the TCB from the control (Figure 3). Addition of IL-12 enhanced the IFN-γ production by the control cells already with low IL-12 concentrations, while the IFN-γ production of the patient's TCB was only slightly enhanced after stimulation with high concentrations of IL-12 (Figure 3). Taken together the results indicate a defect in the IL-12 receptor.

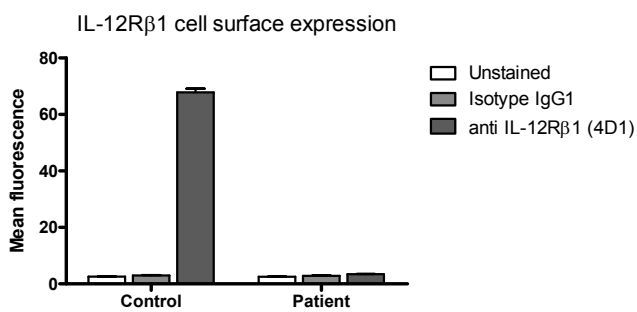


Figure 1. No detectable expression of IL-12Rβ1 on the T cells from the patient. TCB from the patient and an unrelated control were stained with an isotype antibody or an IL-12Rβ1 specific antibody (4D1). The mean fluorescence of the cells was determined by FACS. The experiment was performed in triplo. Mean+/- standard deviation is shown.

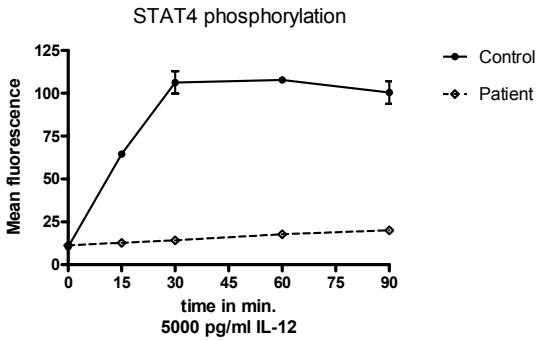


Figure 2. Minimal IL-12 induced STAT4 phosphorylation in the T cells of the patient. TCB of the patient and an unrelated control were stimulated with 5000 pg/ml IL-12. After 0, 15, 30, 60, 90 minutes the STAT4 phosphorylation was analysed by FACS using a specific antibody against tyrosine phosphorylated STAT4. The TCB from the patient cells showed a minimal but statistical significant increase in STAT4 phosphorylation.

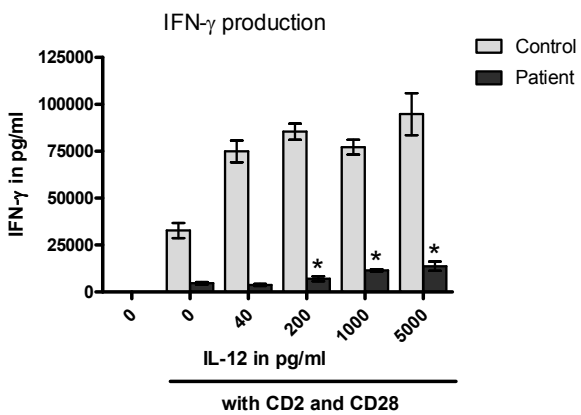


Figure 3. Strongly reduced IFN-γ production by the cells of the patient. TCB of the patient and an unrelated control were cultured without stimuli or stimulated with various concentrations of IL-12 in the presence of antibodies against CD2 and CD28. After 72h the amount of IFN-γ in the supernatant was measured by ELISA. The mean +/- standard deviation is displayed of an experiment performed in triplo. * Significantly different from the control and from the CD2 and CD28 stimulated cells (0 pg/ml IL-12), $p < 0.05$ (student t-test).

3.2. Sequence analysis of IL12RB1

To reveal the underlying genetic basis of the impaired IL-12 responsiveness, we sequenced the *IL12RB1* transcript. We identified several homozygous variations within the transcript of *IL12RB1* of the patient (data not shown). Several of these encode amino acid variations that are known not to influence IL-12Rβ1 function: R156H, Q214R, M365T, and G378R [7]. In addition, we found a -2C>T variation before the translational start-site and an r.1561C>G

variation resulting in an R521G amino acid substitution within the extracellular domain of IL-12Rβ1. Sequencing of genomic DNA from the patient revealed that both variations were homozygously present in respectively exon 1 and exon 13 of *IL12RB1* (data not shown). The r.1561C>G variation was not described before and could not be found in the genomic DNA from 95 healthy controls (data not shown).

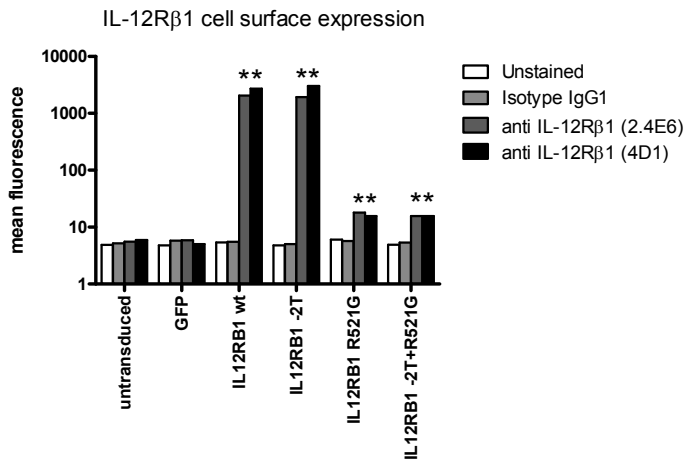


Figure 4. The influence of the -2C/T and R521G variation on the IL-12Rβ1 expression. TCB from a complete IL-12Rβ1 deficient patient were untransduced, transduced with GFP, with the wild type *IL12RB1* cDNA or with an *IL12RB1* construct carrying one or both of the genetic variations as found in the patient under study, -2C/T and R521G. The cells were labelled with a PE conjugated isotype control or an IL-12Rβ1 specific antibody (2.4E6 or 4D1). The mean fluorescence of the cells was determined by FACS. The mean +/- standard deviation is displayed of a triplo experiment. * significantly different from the untransduced and GFP controls, $p<0.05$ (student t-test).

3.3. Functional consequences of the -2C/T and the R521G variations

To establish whether the -2C>T or the R521G variation is the cause of the impaired IL-12 responsiveness we used a previously developed model system to study the functional effect of the variations in the IL-12/IL-23Rβ1 by employing a retroviral expression vector in IL-12/IL-23Rβ1 deficient cells [6]. We made four retroviral constructs carrying a GFP marker gene and the open reading frame coding for one of the four IL-12Rβ1 variants to be investigated; the wild type IL-12Rβ1, the -2C>T variant, the R521G variant and the variant with both the -2C>T and the R521G variation. TCB cells from a patient with a complete IL-12Rβ1 defect were transduced with one of these retroviral constructs and as a control TCBs were transduced with a vector carrying the GFP marker alone. GFP positive TCBs were selected by FACS sort and analyzed for IL-12Rβ1 expression. The -2C>T variation had no influence on receptor expression, as determined by FACS using two different antibodies (Figure 4).

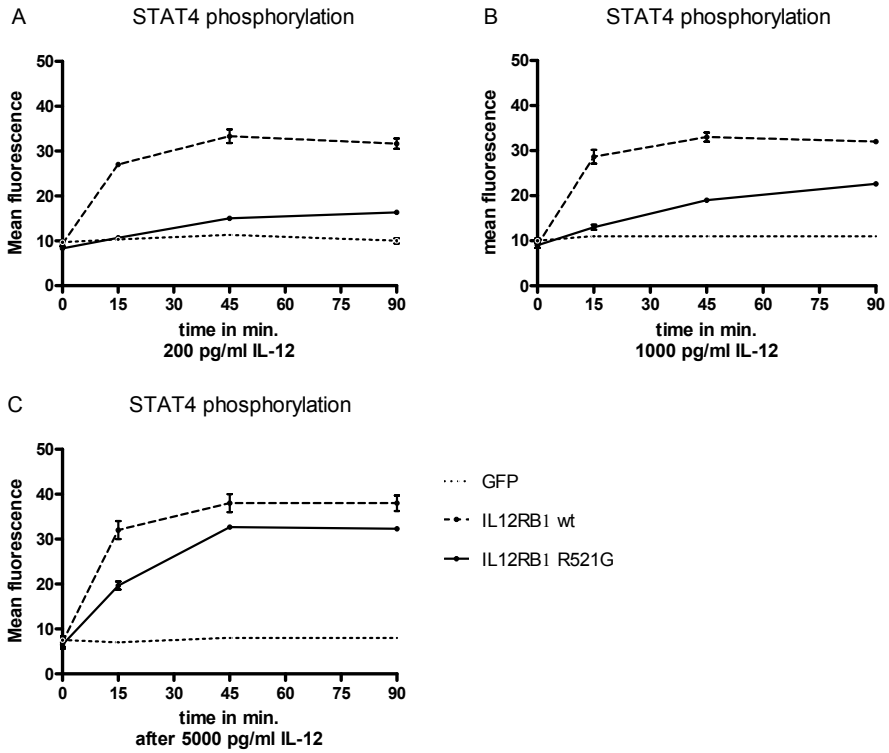


Figure 5. Influence of the R521G variation on the IL-12 signalling. TCB from a complete IL-12Rβ1 deficient patient, transduced with a GFP control vector or a vector carrying the c DNA from the *wild type* IL-12Rβ1 or the R521G variant were stimulated for 0, 15, 45, 90 minutes with 200 pg/ml (A), 1000 pg/ml (B) or 5000 pg/ml (C) IL-12. The kinetics of the IL-12 induced STAT4 phosphorytion was analysed by FACS using a specific antibody against tyrosine phosphorylated STAT4. The kinetics of STAT4 phosphorylation was significantly different from that of the GFP control and that from the wild type *IL12RB1* control at all three IL-12 concentrations tested.

The R521G variation resulted in a severely reduced IL-12Rβ1 expression at the cell surface, which was about a factor 450 lower than wild-type protein (Figure 4). Next we analyzed the effect of the R521G variation on the IL-12 induced signal transduction via STAT4. In cells transduced with the R521G variant phosphorylation of STAT4 in response to IL-12 stimulation was greatly reduced (Figure 5). And as a consequence of reduced signaling, the IL-12 induced IFN-γ production was also severely reduced by the R521G variation (Figure 6). These experiments prove that the R521G variation is a deleterious mutation.

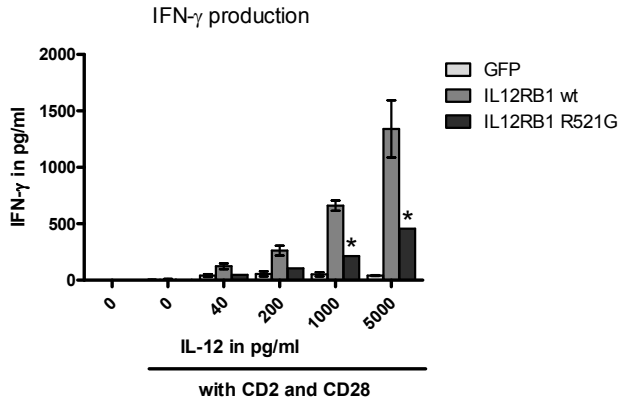


Figure 6. Influence of the R521G variation on the IL-12 induced IFN-γ production. TCB from a complete IL-12Rβ1 deficient patient, transduced with a GFP control vector or a vector carrying the cDNA from the *wild type* IL-12Rβ1 or the R521G variant were cultured in medium or stimulated with various concentrations of IL-12 in the presence of antibodies against CD2 and CD28. After 72h the IFN-γ production was measured by ELISA. The mean +/- standard deviation is displayed of a triplo experiment. * significantly different from the GFP control and the *wild type IL12RB1* construct, $p<0,05$ (student t-test).

4. Discussion

We report the case of a middle-aged patient, who developed a severe disseminated infection of *M. genavense*. The patient appeared to have a partial IL-12Rβ1 deficiency due to homozygous inheritance of the r.1561C>G mutation in *IL12RB1*, leading to the amino acid substitution R521G. As a result of this mutation no IL-12Rβ1 protein was detectable on the T cells from the patient, although the IL-12 response was not completely abrogated. This was confirmed in a cellular model using retroviral expression constructs carrying various IL-12Rβ1 variants. The patient also had another variation, -2C>T, within the *IL12RB1* gene. This variation in the Kozak consensus sequence has no influence on the cell surface expression of IL-12Rβ1 and is therefore regarded as a functional polymorphism.

The patient described in this study had a severe partial defect in IL-12Rβ1. Remarkably, the patient did not suffer from severe *Mycobacteria* nor *Salmonellae* infections in childhood and the first severe NTM infection manifested at the age of 43, although usually, infections in MSMD patients occur at a mean age of 2.4 years [12]. It has been previously reported that some siblings of IL-12Rβ1 deficient MSMD patients, who were also found to be homozygous for the IL-12Rβ1 mutation, have remained asymptomatic for many years [2]. Nine such asymptomatic individuals were described with complete IL-12Rβ1 deficiency, which were vaccinated with BCG, but did not develop BCG-itis [13]. Of two of these patients it is known

they developed mycobacterial disease later in life (before the age of 14 and 33), of most however no follow-up is known. IL-12Rβ1 deficient individuals may remain symptom free for several reasons. For example, it may be that there is a lack of exposure to NTM, in case of BCG-vaccination the vaccine in use was of poor quality, young BCG-vaccinated children may benefit form antibodies in breast milk or that some other compensatory immune mechanisms in early childhood contributes to enough resistance to NTM infections.

Recently, a survey of 141 patients from 30 countries with an IL-12Rβ1 deficiency was published [12]. In all but 2 kindreds, the patients lacked expression of the receptor on the cell surface. FACS analysis of IL-12Rβ1 expression on activated T lymphocytes from our patient was also indicative for an IL-12Rβ1 deficiency. Hence, we sequenced the transcript and parts of the *IL12RB1*gene. The patient had two unknown variations within the transcript of *IL12RB1*. The -2C>T variation, within the Kozak consensus sequence, could potentially influence the rate of translation and thereby the expression of the protein. The presence of a thymidine at 2 nucleotides before the ATG start codon could potentially decrease the rate of translation [14, 15]. However, the -2C>T substitution did not decrease the IL-12Rβ1 expression as was proven with a cellular model using a retroviral expression vector containing the open reading frame of IL-12Rβ1 and 17 bases of the Kozak sequence before the start codon. The R521G variation appeared to be a mutation, leading to a 450 times reduced cell surface expression of IL-12Rβ1 in our model system. The minimal expressed R521G protein could still elicit a marginal IL-12 response. Previously, another mutation was identified to cause a partial defect due to a C198R amino acid substitution within the cytokine binding region of the receptor [6]. The C198R mutation resulted in a severe reduction of IL-12Rβ1 expression at the cell surface, of about 250 times, as was quantified with the same cellular model as used in this study. As a consequence, the function of IL-12Rβ1 was almost completely abrogated. One of the patients, homozygous for the C198R mutation, was a child who developed BCG adenitis after vaccination with *M. bovis* BCG in the neonatal period [5]. After anti-tuberculous treatment infections recurred over a period of many years, and extra antibiotic treatment was necessary . Thus, both the C198R and the novel R521G variations are MSMD causing mutations, despite the fact that both mutations do lead to a partial functional IL-12Rβ1 protein product.

In conclusion, our study demonstrates that IL-12Rβ1 deficiency can lead to an adult onset of severe disseminated NTM infections. The patient in this study had a novel homozygous mutation, leading to the amino acid substitution R521G. The R521G variant of IL-12Rβ1 results in an almost abrogated IL-12 response, due to a severely reduced cell surface expression of IL-12Rβ1. Concomitantly, the R521G variation is considered to be a MSMD causing mutation.

References

[1] Remus N, Reichenbach J, Picard C, Rietschel C, Wood P, Lammas D et al. Impaired interferon γ-mediated immunity and susceptibility to mycobacterial infection in childhood. Pediatr Res 2001;50:8-13.

[2] van de Vosse E, Hoeve MA, Ottenhoff TH. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. Lancet Infect Dis 2004;4:739-49.

[3] Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, Al-Jumaah S et al. Impaired response to interferon-α/β and lethal viral disease in human STAT1 deficiency. Nat Genet 2003;33:388-91.

[4] de Beaucoudrey L, Puel A, Filipe-Santos O, Cobat A, Ghandil P, Chrabieh M et al. Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. J Exp Med 2008;205:1543-50.

[5] Lichtenauer-Kaligis EG, de Boer T, Verreck FA, van Voorden S, Hoeve MA, van de Vosse E et al. Severe Mycobacterium bovis BCG infections in a large series of novel IL-12 receptor β1 deficient patients and evidence for the existence of partial IL-12 receptor β1 deficiency. Eur J Immunol 2003;33:59-69.

[6] van de Vosse E, de Paus RA, van Dissel JT, Ottenhoff THM. Molecular complementation of IL-12Rβ1 deficiency reveals functional differences between IL-12Rβ1 alleles including partial IL-12Rβ1 deficiency. Hum Mol Genet 2005;14:3847-55.

[7] Watford WT, Hissong BD, Bream JH, Kanno Y, Muul L, O'Shea JJ. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. Immunol Rev 2004;202:139-56.

[8] van de Wetering D., de Paus RA, van Dissel JT, van de Vosse E. IL-23 modulates CD56+/CD3- NK Cell and CD56+/CD3+ NK-like T Cell function differentially from IL-12. Int Immunol 2008;21:145-53.

[9] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. Annu Rev Immunol 1997;15:749-95.

[10] Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J Immunol 2002;168:4620-7.

[11] Lammas DA, De Heer E, Edgar JD, Novelli V, Ben-Smith A, Baretto R et al. Heterogeneity in the granulomatous response to mycobacterial infection in patients with defined genetic mutations in the interleukin 12-dependent interferon-γ production pathway. Int J Exp Pathol 2002;83:1-20.

[12] de Beaucoudrey L, Samarina A, Bustamante J, Cobat A, Boisson-Dupuis S, Feinberg J et al. Revisiting human IL-12Rβ1 deficiency: a survey of 141 patients from 30 countries. Medicine (Baltimore) 2010;89:381-402.

[13] Fieschi C, Dupuis S, Catherinot E, Feinberg J, Bustamante J, Breiman A et al. Low penetrance, broad resistance, and favorable outcome of interleukin 12 receptor β1 deficiency: medical and immunological implications. J Exp Med 2003;197:527-35.

[14] Kozak M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 1986;44:283-92.

[15] Kozak M. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 1987;15:8125-48.

ADDENDUM TO CHAPTER 2

IL-12Rβ1 variants and Mendelian susceptibility to mycobacterial disease: an overview of polymorphisms and mutations

1. Introduction

Patients with Mendelian susceptibility to mycobacterial disease (MSMD; MIM #209950) develop severe infections with usually poorly pathogenic non-tuberculous *Mycobacteria* (NTM) or non-typhoid *Salmonellae* (NTS) [1]. The patients are susceptible to these infections due to a deficiency in one of the genes of the IL-12/IFN-γ pathway, amongst them *IL12RB1*. Various kinds of *IL12RB1* mutations have been identified in MSMD patients. For example, null mutations as a result of premature stop codons, splice mutations, base pair deletions or insertions have been identified [2]. Next to these mutations single nucleotide substitutions resulting in single amino acid substitutions, were found in the *IL12RB1* gene. Whether the latter variations are intrinsically deleterious is difficult to predict by *in silico* protein modeling only, but can be studied with the use of cellular models.

IL-12Rβ1 (Figure 1) is the common chain of the IL-12 receptor and the IL-23 receptor complex. IL-12 signals via a receptor complex, which also contains the IL-12Rβ2 chain, while IL-23 signals via a distinct receptor complex, which contains the IL-23R chain next to the IL-12Rβ1 chain. The heterodimeric cytokines IL-12 and IL-23 share a common subunit, IL-12p40, which binds to the IL-12Rβ1 receptor chain. Both IL-12 and IL-23 can be produced by monocytes, macrophages and dendritic cells, and both cytokines are able to induce IFN-γ production in certain subsets of T cells and NK cells. IFN-γ is the central cytokine in type I immune responses, important in the control of infections with intracellular pathogens. Thus, a mutation in *IL12RB1* that results in impaired type I immune responses may have as a consequence an enhanced susceptibility to atypical *Mycobacteria* and *Salmonellae* infections.

The relation of the *IL12RB1* genotype and the clinical phenotype of MSMD patients has previously been reviewed [2-4]. Still, in many case reports the impact of amino acid substitutions within *IL12RB1* on the function of the protein product was unclear. To study the influence of subtle amino acid (aa) changes, we developed a cellular model with retroviral IL-12Rβ1 expression that can be tested functionally [5]. Receptor variants were cloned and retrovirally expressed in T cells from a patient with a null mutation in *IL12RB1*. In this way, the expression and function of the various IL-12Rβ1 variants are compared within the same genetic background. The results obtained with this system are displayed in Table 1. The IL-12Rβ1 expression of the variants was analyzed using at least two different antibodies, recognizing different parts of the protein. In addition, the receptor function of the variants was tested by analyzing the IFN-γ and IL-10 production [5] and STAT4 phosphorylation (unpublished data) upon IL-12 stimulation. In this report an overview of the functional consequences of the variations in IL-12Rβ1 is presented and discussed.

2. MSMD causing mutations in IL-12Rβ1

Using our cellular model, the variants L77P, Q171P, R173P, C186S, R213W and Y367C were functionally characterized and confirmed to be deleterious mutations leading to complete IL-12Rβ1 deficiency [5] (Table 1). In the L77P variant, expression of the IL-12Rβ1 on the cell surface was severely reduced, while the other four variants were not expressed at all on the cell surface but could be detected in the intracellular compartment. In IL-12 stimulation assays, the L77P expressed on the cell surface appeared to be non-functional. Because the L77P variation is located within the cytokine binding region of IL-12Rβ1 (aa 43-237, see Figure 1), it was expected that ligation of IL-12 to its receptor was abrogated. This was confirmed by studying the binding of IL-12 to the IL-12R on cell lines, transiently transfected with *IL12RB1* constructs, and using recombinant IL-12p70 and IL-12p40 specific antibodies [2].

The cell surface expression of the C198R variant [6] and the R521G variant of IL-12Rβ1 ([7] and chapter 2 of this thesis) was also severely reduced, 250 and 450 times respectively. In contrast to the L77P variant, however, these two variants are able to respond to IL-12 stimulation, though the response to IL-12 was in both cases almost abrogated, due to the very low cell surface expression. This explains why the T cells from patients homozygous for the C198R and R521G mutation show a minimal response upon stimulation with high concentrations of IL-12 ([6, 7] and chapter 2). These rare patients, with a partial IL-12Rβ1 deficiency, and all reported patients with complete IL-12Rβ1 deficiency, suggest that the IL-12Rβ1 function has to be strongly reduced to impair the mounting of sufficient immune responses against atypical *Mycobacteria* and *Salmonellae*. A reduction of 50 % in IL-12Rβ1 expression apparently does not affect immunity to these pathogens substantially: individuals with just one mutated allele showed intermediate expression of IL-12Rβ1 and an intermediate response to IL-12 has not been associated with clinical manifestation of NTM or NTS infections (unpublished data).

In addition, it was found that some siblings from patients with NTM infections, which are also IL-12Rβ1 deficient, may remain asymptomatic for many years. This may be due to a lack of relevant exposure to NTM and NTS. On the other hand, the threshold of minimal required IL-12 and IL-23 responses to combat infections is low and other host-factors may also contribute to the susceptibility to NTM infections. Thus, sufficient residual type I immunity is present in some cases with complete IL-12Rβ1 deficiency. However, in the reported cases with severe disseminated infections and complete IL-12Rβ1 deficiency, the deficiency is evidently related to high susceptibility to disease.

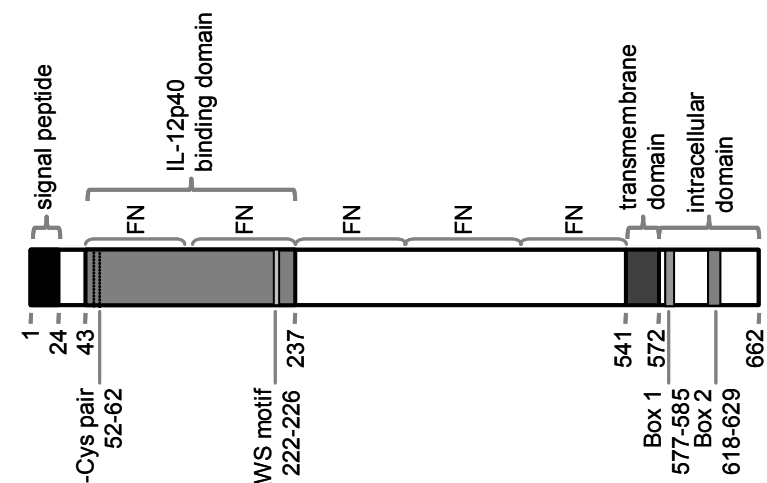


Figure 1. Schematic presentation of IL-12Rβ1. The various domains are indicated and on the left the amino acid positions of the borders of the different domains are given. The extracellular domain consists of five fibronectin type III domains (FN). The two N-terminal FN domains form the cytokine binding region with a Cys-Cys pair and a WSxWS motif, which is commonly found in cytokine receptors. The intracellular region consists of 91 amino acids and contains a box 1 cytokine receptor motif: a proline rich region involved in JAK2 binding, and a box 2 cytokine receptor motif: a stretch of hydrophobic amino acids.

Table 1. Summary of functional assays of *IL12RB1* variations on IL-12Rβ1 function.

variation[a]	receptor expression	IL-12 binding	protein function	conclusion	references
-2C>T	normal	N.A.	normal	polymorphism	[7], chapter 2
C62G	absent on cell surface [c]	N.A.	N.A.	mutation	[2]
S74R	normal	N.A.	normal	polymorphism	[5]
L77P	10 fold reduction	absent	absent	mutation	[4, 5, 15]
A91T	normal	N.A.	normal	polymorphism	unpublished
R156H	normal	N.A.	normal	polymorphism	[5]
Q171P	only intracellular	absent	absent	mutation	[4]
R173P	only intracellular	absent	absent	mutation	[4, 5, 16]
R173W	absent on cell surface [c]	absent	N.A.	mutation	[2]
R175W	absent on cell surface [c]	absent	N.A.	mutation	[2]
C186S	only intracellular	absent	absent	mutation	[4, 5]
C186Y[e]	only intracellular	N.A.	absent	putative mutation[f]	[17]
C196Y	absent on cell surface [c]	absent	N.A.	mutation	[2]
C198R	250 fold reduction	absent[d]	reduced	mutation	[5, 6]
R211P	absent on cell surface [c]	absent	N.A.	mutation	[2]
R213W	only intracellular	absent	absent	mutation	[4, 5, 18, 19]
R214Q [b]	normal	N.A.	normal	polymorphism	[5, 9]
T365M [b]	normal	N.A.	normal	polymorphism	[5, 9]
Y367C	only intracellular	absent	absent	mutation	[4, 5]
I369T	absent on cell surface [c]	absent	N.A.	mutation	[2]
R378G [b]	normal	N.A.	normal	polymorphism	[5, 9]
H438Y	normal	N.A.	normal	polymorphism	[5]
R521G	450 fold reduction	N.A.	reduced	mutation	[7], chapter 2
A525T	normal	N.A.	normal	polymorphism	[5]
G569D[f]	N.A.	N.A.	N.A.	unknown [f]	[2]
G594E	normal	N.A.	normal	polymorphism	[5]

[a] The variation -2C>T indicates a variation 2 nucleotides before the start codon. The other variations are amino acid substitutions of which the amino acid position in the protein is indicated. The variations that were proven to be mutations are indicated in bold. [b] Linked polymorphisms; two major haplotypes with the amino acids RTR or QMG respectively on the positions 214, 365 and 378. [c] Not tested for expression within the cell. [d] IL-12 binding was not detected in the assay, although the functional assay suggests that IL-12 can bind to the low amounts of the C198R present on the cell surface. [e] C186Y was found in two related patients with a disseminated coccidioidomycosis. [f] The G596D and C186Y variations are not yet evaluated in a cellular model with retroviral IL-12Rβ1 expression. N.A. not analyzed.

The functional characterization of the Q171P variant was not published before. Two patients were described to be heterozygous for the Q171P variation, while having a premature stop mutation on the other allele [4]. Both patients suffered from severe disseminated BCG infections at the age of two and three, of which one died due to the infection. The severe infections and the null mutation in one allele, suggested that the Q171P variation was also a mutation rather than a polymorphism without phenotype. Using our cellular model, the Q171P variant was confirmed to be a deleterious mutation. The Q171P variant was expressed within the cell and not at the cell surface and no residual function was detected for the Q171P variant (unpublished data).

Using a similar cellular model, not with stable retroviral expression but with transient expression, the C62G, R173W, R175W, C186S, R211P and I369T variations were also determined to be mutations, based on loss of expression and loss of IL-12 binding to the receptor [2] (Table 1). However, these variants were not tested for their ability to respond to IL-12. Thus, it remains unclear whether these mutations exhibit residual responses to high concentrations of IL-12.

3. Effects of mutations in IL-12Rβ1 on receptor expression

In a previous report the IL-12Rβ1 mutations of 141 MSMD patients were discussed [2]. Remarkably, all currently characterized mutations due to single amino acid substitutions or premature stop codons reduced the cell surface expression completely or in a few cases nearly complete (Table 1). Two of the 141 patients lacked cell surface expression of the receptor [2, 8]. These patients did not carry a single amino acid substitution in *IL-12RB1*, but had a large in frame deletion. The protein products from the latter mutation were detected on the cell surface by some but not all of the available antibodies against IL-12Rβ1. Thus, antibodies against the different parts of the extracellular domain of the receptor are useful in the diagnosis of all currently known IL-12Rβ1 deficiencies, because the lack of IL-12Rβ1 expression at the cell surface already gives a strong indication for an IL-12Rβ1 deficiency.

Single nucleotide substitutions can also lead to a premature stop codon. The variations Q32X, E67X, Y88X, Q285X, K305X, S321X, Q376X, E480X, R486X, R521X, W531X, Q542X are mutations found in MSMD patients [2]. All these mutations occurred in the extracellular part or in the transmembrane domain of the receptor, resulting in the synthesis of truncated protein products lacking the transmembrane domain (aa 541-572) and the intracellular signaling domain (aa 573-662) (Figure 1). Thus far, all amino acid substitutions and all premature stop codons resulting in severe dysfunction of the receptor were found in the extracellular part or the transmembrane region and not in the intracellular part of the receptor. This fact and the fact that all reported mutations lead to loss of receptor expression

at the cell surface or loss of IL-12 binding, suggest that perhaps the intracellular part of the receptor is dispensable for minimal required receptor function. To investigate the latter, an *IL12RB1* construct was made with a stop codon at position 578. This construct codes for a truncated IL-12Rβ1 protein with an extracellular and a transmembrane domain, but without the intracellular domain. The artificial C578X variant was expressed at the cell surface, but was unable to induce STAT4 phosphorylation and IFN-γ production upon IL-12 stimulation (unpublished data). Thus, the intracellular domain of IL-12Rβ1 is not dispensable for receptor function. Consequently, in the investigation of a putative IL-12Rβ1 deficiency of a new MSMD case, it is advisable to measure the IL-12 responsiveness of the T cells from the patient and not the IL-12Rβ1 expression and IL-12 binding only. According to current reports [2, 4], putative MSMD patients are not always screened for IL-12 responsiveness. Hence, the finding that all MSMD causing mutations in *IL12RB1* affect the IL-12Rβ1 expression and/or the IL-12 binding, may be biased. We recommend in the diagnosis of MSMD, to test for the IL-12 responsiveness.

Until now, 14 amino acid substitutions were proven to be the cause of IL-Rβ1 deficiency. As a consequence the receptor is totally or mainly expressed within the cell, and not on the cell surface. Eleven of the 14 amino acid substitutions are located within the IL-12p40 binding region, which is a 195 aa long segment of the extracellular domain of 541 aa. In contrast, the identified early stop codons are more equally distributed within the extracellular domain. Only 2 of the 12 premature stop codons were located within the IL-12p40 binding region. Taken together, this indicates that the IL-12p40 binding region is not only important for cytokine binding but that the conformation of this region is also crucial for receptor expression on the outer surface of the cellular membrane.

4. Harmless polymorphisms in IL-12Rβ1

The most common polymorphisms in IL-12Rβ1 are R214Q, T365M and R378G. These polymorphisms are tightly linked to each other [9], and hence they form the two major haplotypes RTR and QMG, which are present in several human populations in almost equal proportions. In the studies with the cellular model of retroviral IL-12Rβ1 expression the QMG variant was somewhat higher expressed and showed slightly higher responses upon IL-12 stimulation as compared to the RTR variant [5], while both variants responded similar to IL-23 stimulations [10]. This is in line with the earlier finding that the T cells from RTR homozygotes appeared to respond less well upon IL-12 stimulation [9]. In addition, it was found that the RTR haplotype is overrepresented in Japanese tuberculosis patients [9]. The latter indicates that a reduction of the Th1 immune responses may play a role in the pathogenesis of virulent tuberculous *Mycobacteria*. This is supported by recent reports that

indicate that the type I immunity in tuberculosis patients is impaired. It was found that in the blood cells from tuberculosis patients a transcript signature was found that indicated a typical type I interferon signaling which coincides with a repressed IFN-γ mediated signaling [11]. Furthermore, it was shown that the IFN-γ production of T cells in TB patients was strongly suppressed, which recovered during treatment with antibiotics [12] (R. Hari Dass, submitted for publication).

The three R214Q, T365M and R378G polymorphisms are strongly linked. The strong, but not 100% linkage suggests that other less common haplotypes exist, for example RMG, RTG, QTR or QMR. Whether one or more of these variants exist and whether they are functionally distinct from the *wild type* receptor is unknown. This would be of interest to know if such a haplotype is over- or underrepresented in a certain patient group, either with particular infections or in autoimmune disease.

The variants S74R, A91T, R156H, H438Y, A525T and G594E are abundantly expressed at the cell surface and are fully functional (Table 1). The R156H variant is present in several populations, while the S74R and the A91T variant are rare variants that were identified in MSMD patients. The H438Y, A525T and G594E variants were identified in tuberculosis patients [5]. These six amino acid substitutions are considered to be harmless polymorphisms and not disease causing deleterious mutations. For example, the A91T variation was found in a Chinese patient who initially presented with a chronic skin infection with a poorly virulent mycobacterium and eventually died from a brain infection (personal communication with Dr. Sun Dong-Jie). Using our cellular model, the A91T variant was characterized as a fully functional variant (unpublished data). Thus, this variation could not explain why the immune system of the patient had failed to control the mycobacterial infection. More extensive analyses of immune responses in the patient were unfortunately not performed.

Amino acid substitutions in the second exon of *IL12RB1* are probably harmless polymorphisms, because this exon (encoding aa 21 to aa 42) seems to be dispensable for sufficient receptor function. Investigation of exon-skipping, to sidestep the consequences of premature stop codons within the second exon, showed that a protein product without the amino acids encoded by the second exon was functional [13]. The deletion of the exon resulted in a reduction of 90% in receptor expression, while the receptor lacking exon 2 responded similar to IL-12 and IL-23 stimulations as compared to the wild type receptor [13]. The response is probably still enough for sufficient immunity against non-tuberculous *Mycobacteria* or non-typhoid *Salmonellae*. Thus far, all reported cases with complete or nearly complete IL-12Rβ1 deficiency, had more impact on receptor expression. The cases with partial IL-12Rβ1 deficiency showed more than 250 times reduction in receptor expression.

The -2C>T variation within the Kozak consensus sequence was found occasionally within the *IL12RB1* transcripts of MSMD patients (unpublished data). This variation could potentially reduce the translation rate of the transcript, because thymidine residues within the

first three bases before the ATG start codon are underrepresented in eukaryotic transcripts [14]. The -2C>T variation in the transcript of *IL12RB1* appeared to be a functional polymorphism with no impact on the translation and the cell surface expression of the receptor ([7] and chapter 2 of this thesis). This was determined with the use of our cellular model, in which the IL-12Rβ1 was retrovirally expressed, using an expression vector containing the open reading frame of *IL12RB1* and 17 bases before the ATG start codon. To ascertain that the -2C>T has no influence on the transcriptional activity of the natural transcript an *in vitro* translation assay should be performed using RNA material from T cells from patients and controls.

5. Conclusion

Recent research into the genetic nature of IL-12Rβ1 deficiencies gave insights for the diagnosis of MSMD and the role of IL-12 and IL-23 in cellular immunity. Only mutations leading to severe IL-12Rβ1 deficiency impair immunity sufficiently to enhance the susceptibility to infections with otherwise poorly pathogenic *Mycobacteria* and to *Salmonellae*. Variations in IL-12Rβ1 leading to weak or moderate changes in expression and function can be considered as harmless polymorphisms, which may be associated with subtle differences in the susceptibility to infections and the predisposition to other immune diseases.

References

[1] van de Vosse E, Hoeve MA, Ottenhoff TH. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. Lancet Infect Dis 2004;4:739-49.

[2] de Beaucoudrey L, Samarina A, Bustamante J, Cobat A, Boisson-Dupuis S, Feinberg J et al. Revisiting human IL-12Rβ1 deficiency: a survey of 141 patients from 30 countries. Medicine (Baltimore) 2010;89:381-402.

[3] van de Vosse E, Ottenhoff TH, de Paus RA, Verhard EM, de Boer T, van Dissel JT et al. Mycobacterium bovis BCG-itis and cervical lymphadenitis due to Salmonella enteritidis in a patient with complete interleukin-12/-23 receptor β1 deficiency. Infection 2010;38:128-30.

[4] Fieschi C, Dupuis S, Catherinot E, Feinberg J, Bustamante J, Breiman A et al. Low penetrance, broad resistance, and favorable outcome of interleukin 12 receptor β1 deficiency: medical and immunological implications. J Exp Med 2003;197:527-35.

[5] van de Vosse E, de Paus RA, van Dissel JT, Ottenhoff THM. Molecular complementation of IL-12Rβ1 deficiency reveals functional differences between IL-12Rβ1 alleles including partial IL-12Rβ1 deficiency. Hum Mol Genet 2005;14:3847-55.

[6] Lichtenauer-Kaligis EG, de Boer T, Verreck FA, van Voorden S, Hoeve MA, van de Vosse E et al. Severe Mycobacterium bovis BCG infections in a large series of novel IL-12 receptor β1 deficient patients and evidence for the existence of partial IL-12 receptor β1 deficiency. Eur J Immunol 2003;33:59-69.

[7] Potjewijd J, de Paus RA, Van Wengen A., Damoiseaux J, Verbon A, de Vosse EV. Disseminated Mycobacterium genavense infection in a patient with a novel partial interleukin-12/23 receptor β1 deficiency. Clin Immunol 2012;144:83-6.

[8] Fieschi C, Bosticardo M, de Beaucoudrey L, Boisson-Dupuis S, Feinberg J, Santos OF et al. A novel form of complete IL-12/IL-23 receptor β1 deficiency with cell surface-expressed nonfunctional receptors. Blood 2004;104:2095-101.

[9] Akahoshi M, Nakashima H, Miyake K, Inoue Y, Shimizu S, Tanaka Y et al. Influence of interleukin-12 receptor β1 polymorphisms on tuberculosis. Hum Genet 2003;112:237-43.

[10] de Paus RA, van de Wetering D, van Dissel JT, van de Vosse E. IL-23 and IL-12 responses in activated human T cells retrovirally transduced with IL-23 receptor variants. Mol Immunol 2008;45:3889-95.

[11] Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 2010;466:973-7.

[12] Sahiratmadja E, Alisjahbana B, de Boer T, Adnan I, Maya A, Danusantoso H et al. Dynamic changes in pro- and anti-inflammatory cytokine profiles and γ interferon receptor signaling integrity correlate with tuberculosis disease activity and response to curative treatment. Infect Immun 2007;75:820-9.

[13] van de Vosse E, Verhard EM, de Paus RA, Platenburg GJ, van Deutekom JC, Aartsma-Rus A et al. Antisense-mediated exon skipping to correct IL-12Rβ1 deficiency in T cells. Blood 2009;113:4548-55.

[14] Kozak M. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 1987;15:8125-48.

[15] Moraes-Vasconcelos D, Grumach AS, Yamaguti A, Andrade ME, Fieschi C, de Beaucoudrey L et al. Paracoccidioides brasiliensis disseminated disease in a patient with inherited deficiency in the β1 subunit of the interleukin (IL)-12/IL-23 receptor. Clin Infect Dis 2005;41:e31-e37.

[16] Aksu G, Tirpan C, Cavusoglu C, Soydan S, Altare F, Casanova JL et al. Mycobacterium fortuitum-chelonae complex infection in a child with complete interleukin-12 receptor β 1 deficiency. Pediatr Infect Dis J 2001;20:551-3.

[17] Vinh DC, Schwartz B, Hsu AP, Miranda DJ, Valdez PA, Fink D et al. Interleukin-12 receptor β1 deficiency predisposing to disseminated Coccidioidomycosis. Clin Infect Dis 2011;52:e99-e102.

[18] Sakai T, Matsuoka M, Aoki M, Nosaka K, Mitsuya H. Missense mutation of the interleukin-12 receptor β1 chain-encoding gene is associated with impaired immunity against Mycobacterium avium complex infection. Blood 2001;97:2688-94.

[19] Altare F, Ensser A, Breiman A, Reichenbach J, Baghdadi JE, Fischer A et al. Interleukin-12 receptor β1 deficiency in a patient with abdominal tuberculosis. J Infect Dis 2001;184:231-6.

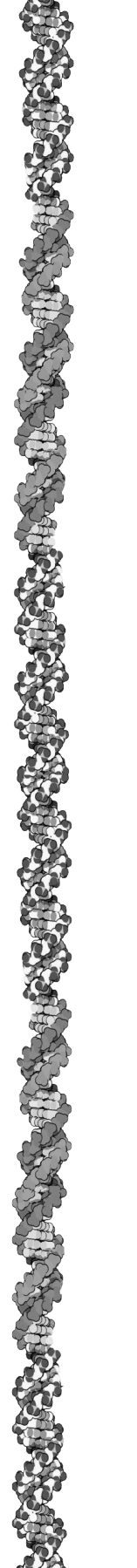
CHAPTER 3

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

Roelof A. de Paus, Diederik van de Wetering,
Jaap T. van Dissel, Esther van de Vosse.

Department of Infectious Diseases, Leiden University Medical Center,
Albinusdreef 2, 2333 ZA, Leiden, The Netherlands.

Molecular Immunology 2008;45(15):3889-95



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-12Rß1 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-12Rß1-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.

1. 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 (Hibbert *et al.*, 2003; Lucas *et al.*, 2003). IL-23 plays a role in the maintenance of immune responses by controlling T cell memory function (Frucht, 2002) and by influencing the proliferation and survival of IL-17-producing Th17 cells (Bettelli *et al.*, 2007). 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-12Rß1 chain complemented by the IL-23R and the IL-12Rß2 (Trinchieri *et al.*, 2003). IL-12Rß1 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 (Wu *et al.*, 1997). The IL-12Rß2 is only expressed on CD4+ T cells after activation (Gately *et al.*, 1998), 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 (Parham *et al.*, 2002).

The IL-12 and IL-23 receptor complexes signal via JAK2 and STAT modules to regulate gene expression (Parham *et al.*, 2002). IL-12 activates STAT4 thereby inducing IFN-γ (Watford *et al.*, 2004) and IL-10 production in various T-cell subsets (Meyaard *et al.*, 1996; Mehrotra *et al.*, 1998). IL-23 activates STAT1, STAT3, STAT4 and STAT5 and can induce IFN-γ, IL-10 and IL-17 depending on the celltype (Parham *et al.*, 2002; van den Eijnden *et al.*, 2005).

IL-23 is important in controlling mucosal host defenses (Happel *et al.*, 2005; Uhlig *et al.*, 2006) and is involved in autoimmune diseases such as inflammatory bowel diseases (IBD) (McGovern and Powrie, 2007), psoriasis (Torti and Feldman, 2007) and rheumatoid arthritis (Kim *et al.*, 2007). Patients with Mendelian susceptibility to mycobacterial disease (MSMD) due to IL-12Rß1 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 (van de Vosse *et al.*, 2004).

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 (Duerr *et al.*, 2006), psoriasis (Capon *et al.*, 2007), ankylosing spondylitis (Rueda *et al.*, 2008), and graft versus host disease after bone marrow transplantation (Elmaagacli *et al.*, 2008). The P310L allelic variant was overrepresented in patients with Grave's Disease (Huber *et al.*, 2008). 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.

2. Materials and Methods

2.1. 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 pGEMT-Easy (Promega), variations were introduced by site directed mutagenesis (Higuchi *et al.*, 1988). Three constructs were made with the variations, P310L, Y173H and R381Q. One construct designated as -Δ23R was made by introducing an early stop codon (at aa 400) and an Y397F mutation. The IL23R constructs were released from the pGEM-Teasy vector by digestion with NotI and ligated into pLZRS–IRES–GFP (Heemskerk *et al.*, 1997) or into pLZRS-IRES-ΔNGFR (Ruggieri *et al.*, 1997). 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 (Kinsella and Nolan, 1996), using a calcium-phosphate transfection kit (Invitrogen). The virus producing cells were cultured for 2–3 weeks under 2 μg/ml puromycin (Clontech) selection after which a 20 h supernatant was harvested.

2.2. 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 μg/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, $0.5*10^6$ TCB were transduced on a CH-296 coated (RetroNectin^{tm}, Takara Shuzo) 48-wells plate using 0.5 ml of virus containing supernatants as described previously (Heemskerk *et al.*, 2001). 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 (Gong *et al.*, 1994) were cultured in IMDM

supplemented with 20 mM GlutaMAX, 10% FCS, 10% Horse serum (Gibco), 100 U/ml Penicillin, 100 µg/ml Streptomycin (Invitrogen) supplemented with 100 U/ml IL-2 and 50 µM 2-mercaptoethanol (Merck) and transduced as described above.

2.3. Co-transduction of IL-12Rß1 and IL-23R into IL-12Rß1-/- TCB

TCB of a patient (TCBp1) with a null mutation in IL-12Rß1 were transduced as described above with one or two retroviral vectors. Namely the pLZRS-IRES-GFP vector in which one of the IL-12Rß1 variants (QMG or RTR) was cloned (van de Vosse *et al.*, 2005) and the pLZRS-IRES-ΔNGFR 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 ΔNGFR). 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).

2.4. 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).

2.5. Cytokine production analysis and proliferation assay

$1*10^5$ TCB were cultured in 200 µl of culture medium in 96-wellsplates (Greiner bio-one). The cells were stimulated with or without 2 µg/ml anti-CD2 (CLB-T11.1/1 and .2/1, Sanquin) and 2 µg/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 µl 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 25µl of RPMI-medium (Invitrogen) containing 0.5 µCi 3H-thymidine (PerkinElmer) was added. After 8 hours of incubation the cells were harvested and incorporated 3H 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).

2.6. STAT phosphorylation assays

To study signal transduction 10^5 TCB were pre-stimulated overnight in 150 µl culture medium with 2 µg/ml anti-CD2 and 2 µg/ml anti-CD28 in 96-wellsplates (Greiner bio-one).

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 µg/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.

3. Results

3.1. 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-12Rß1. 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. 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- 12Rß1 and IL-12Rß2 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).

3.2. 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).

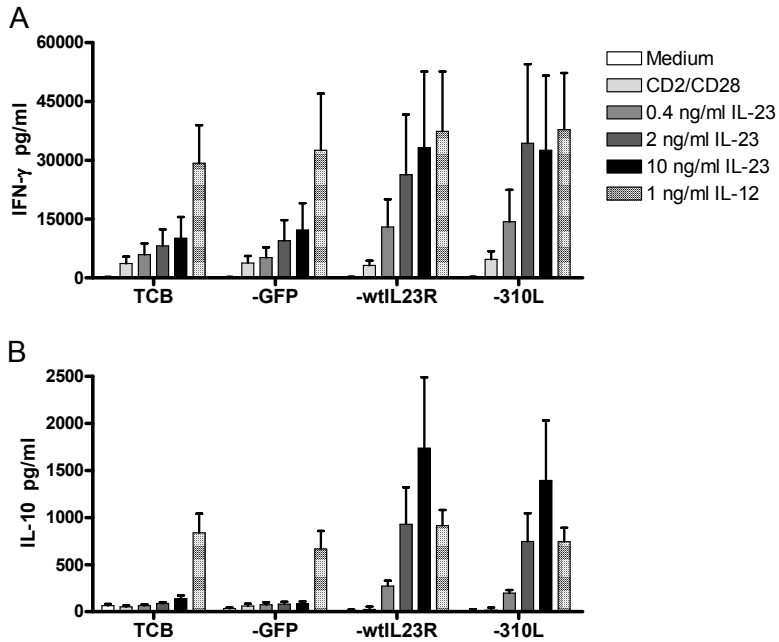


Figure 1. IL-23 induces IFN-γ and IL-10 production via retrovirally expressed IL-23R. Normal TCB, GFP transduced TCB and TCB transduced with the wtIL23R or the 310L variant were tested for their IFN-γ (A) and the IL-10 (B) production in response to anti-CD2/anti-CD28 with or without various amounts of 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.

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 (Wilson *et al.*, 2007). 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).

3.3. Kinetics of IL-23 induced STAT phosphorylation

It was previously reported that IL-23 induces STAT1, STAT3, STAT4 and STAT5 phosphorylation (Parham *et al.*, 2002). 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 (Van de Wetering, manuscript in preparation). 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).

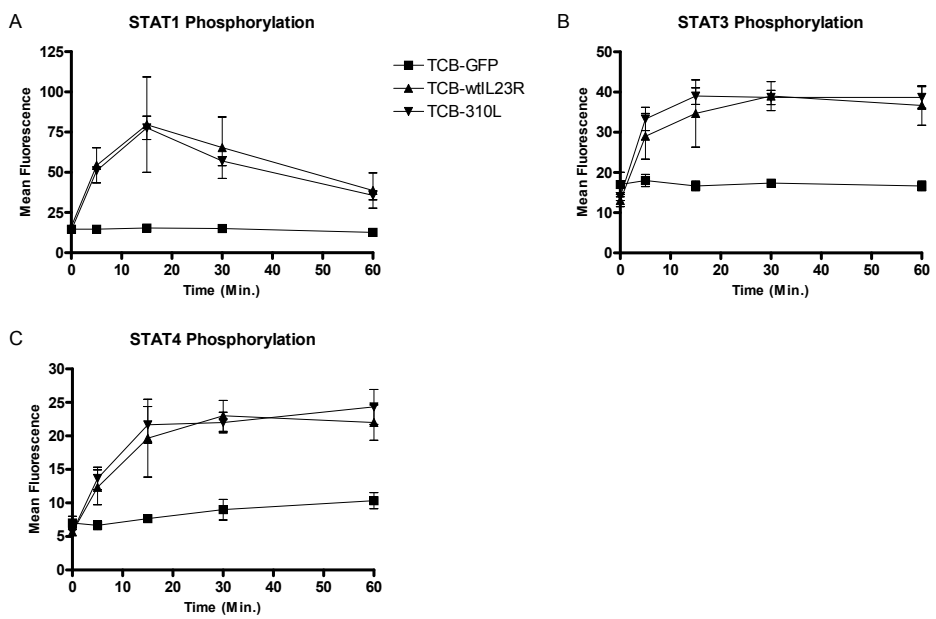


Figure 2. Kinetics of IL-23 induced STAT phosphorylation. TCB transduced with the wild-type (wtIL23R), the 310L variant or GFP alone were stimulated with 10 ng/ml IL-23 for 0, 5, 15, 30 and 60 minutes. The amount of STAT1 (A), STAT3 (B) and STAT4 (C) phosphorylation was determined by FACS using phospho-specific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

3.4. 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).

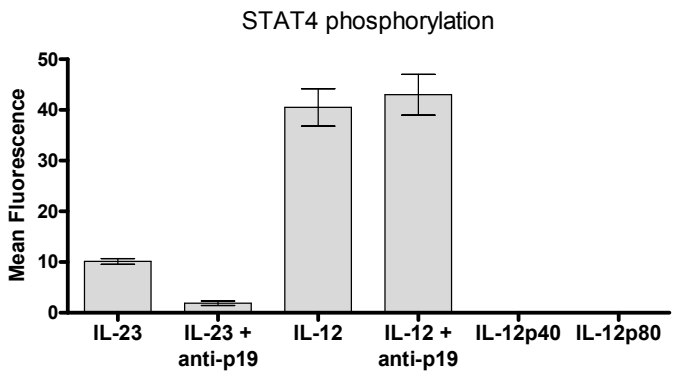


Figure 3. IL-23 but not IL-12p40 monomers or homodimers induce IL-12-like IL-23R independent STAT4 phosphorylation. Normal TCB were stimulated in the presence of anti-CD2 and anti-CD28 with 10 ng/ml IL-23, 1 ng/ml IL-12, 6.8 ng/ml IL-12p40, IL-12p80, or with IL-23 or IL-12 preincubated for 30 minutes 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.

3.5. 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-Δ23R). 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-Δ23R 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).

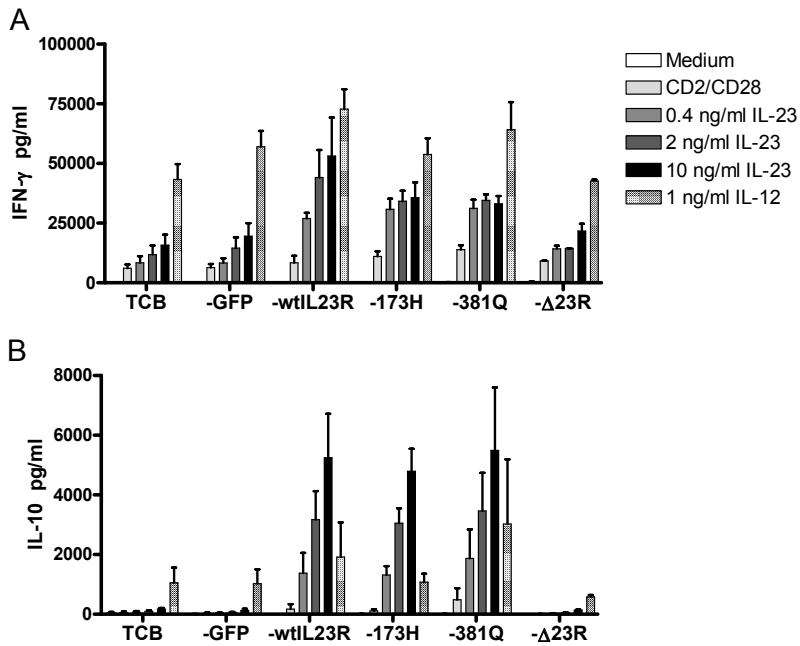


Figure 4. The IL-23R variants do not differ in IL-23 induced cytokine production. TCB transduced with the constructs of the wtIL23R, the 173H- and the 381Q-variants were compared with normal TCB, TCB-GFP and TCB-.23R. The cell-lines were tested for IFN-γ (A) and IL-10 (B) production in response to various 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.

3.6. 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 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).

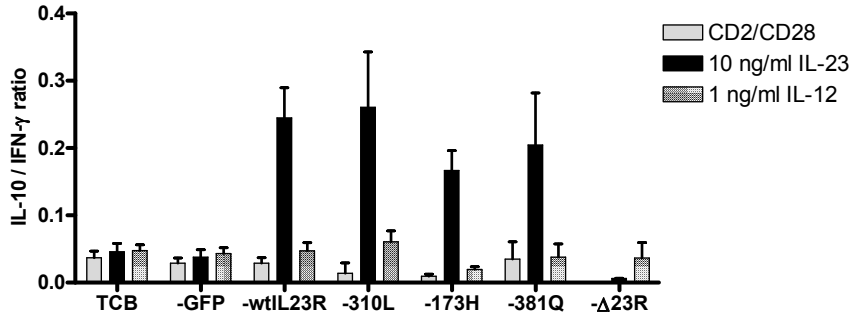


Figure 5. Influence of IL-23R expression on the IL-10/ IFN-γ ratio after IL-23 and IL-12 stimulation. The IL-10/ IFN-γ ratio was determined for each stimulation of the TCB cultures with anti-CD2/anti-CD28 alone or together with 10 ng/ml IL-23 or 1 ng/ml IL-12. Displayed are the means and standard deviations of TCB (n=13), -GFP (n=13), -wtIL23R (n=13), -310L (n=6), -173H (n=6), -381Q (n=5) and -Δ23R (n=3).

3.7. 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-Δ23R). 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).

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

IL-23 has been reported to affect proliferation and survival of T cells (Bettelli *et al.*, 2007). 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.

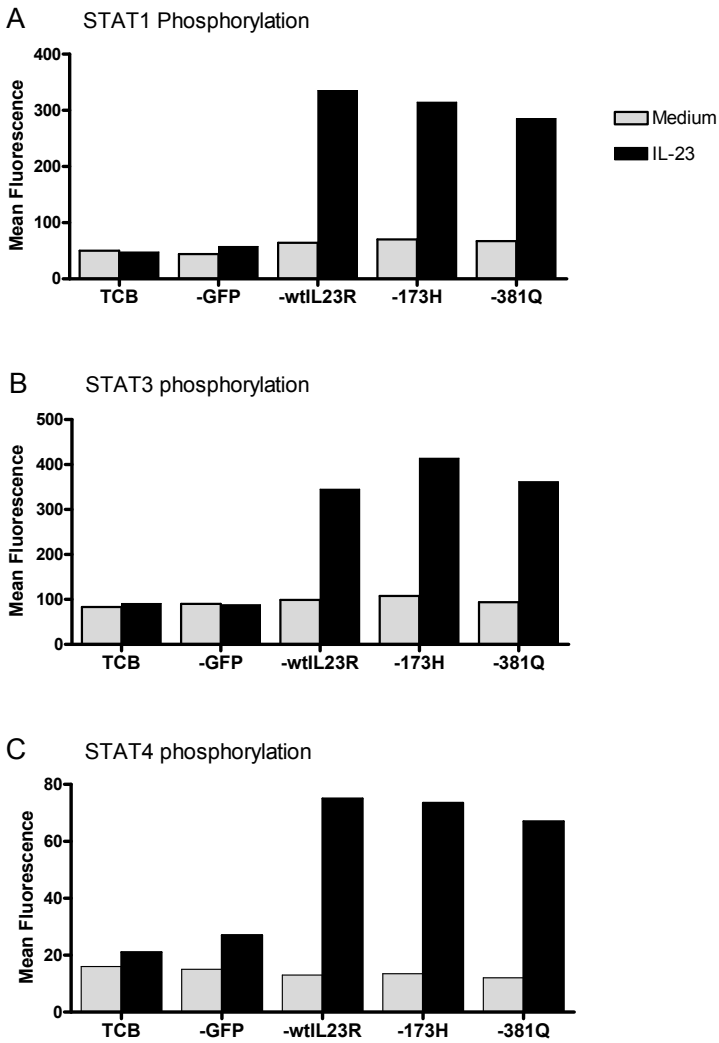


Figure 6. The IL-23R variants do not differ in IL-23 induced STAT phosphorylation. Normal TCB, TCB transduced with GFP and TCB transduced with the various IL-23R constructs (wtIL23R, 173H and 381Q) were stimulated with 10 ng/ml IL-23 for 15 (STAT1) or 30 minutes (STAT3 and STAT4). The amount of STAT1 (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.

3.9. Combined effects of IL-12Rß1 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-12Rß1 chain, therefore common IL-12Rß1 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-12Rß1 null mutation. TCBp1 cells were transduced with a retroviral vector expressing an IL-12Rß1 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-12Rß1 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.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-12Rß1 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-12Rß1 on these cells.

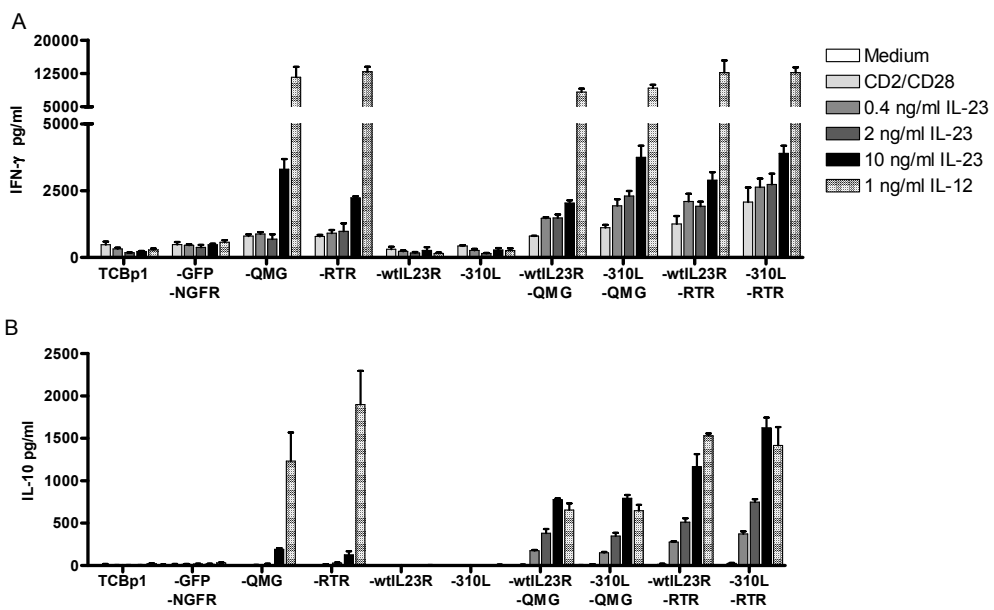


Figure 7. Influence of common IL-12Rß1 and IL-23R polymorphisms on the IL-23 response. The IL-23 responsiveness was tested on TCBp1-QMG-wtIL23R, TCBp1-QMG-310L, TCBp1-RTR-wtIL23R and TCBp1-RTR-310L and compared with the responsiveness of TCBp1 and controls (TCBp1-GFP-NGFR, -QMG, -RTR, -wtIL23R and -310L). 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.

4. 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-12Rß1 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 (Janssen *et al.*, 2002). 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 (Hill and Sarvetnick, 2002) 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 (Cummings *et al.*, 2007) and psoriasis (Capon *et al.*, 2007). The SNP designated rs11465804 for instance is strongly linked to the R381Q polymorphism (Capon *et al.*, 2007). 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-12Rß1-/- patient this IL-12-like effect of IL-23 was only observed after transduction with an IL-12Rß1 expression construct, indicating that the IL-12Rß1 chain is indeed involved in this signaling. Whether the IL-12Rß2 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-12Rß1 and not on IL-23R expression. Cells from an IL-12Rß1-/- patient were used to study the influence of various combinations of IL-12Rß1 and IL-23R polymorphisms. The QMG and RTR alleles of the IL-12Rß1 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 (Wilson *et al.*, 2007). 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-12Rß1 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.

References

Bettelli, E., Oukka,M., Kuchroo,V.K., 2007. T(H)-17 cells in the circle of immunity and autoimmunity. Nat. Immunol. 8, 345-350.

Capon, F., Di Meglio P., Szaub,J., Prescott,N.J., Dunster,C., Baumber,L., Timms,K., Gutin,A., Abkevic,V., Burden,A.D., Lanchbury,J., Barker,J.N., Trembath,R.C., Nestle,F.O., 2007. Sequence variants in the genes for the interleukin-23 receptor (IL23R) and its ligand (IL12B) confer protection against psoriasis. Hum. Genet. 122, 201-206.

Cummings, J.R., Ahmad,T., Geremia,A., Beckly,J., Cooney,R., Hancock,L., Pathan,S., Guo,C., Cardon,L.R., Jewell,D.P., 2007. Contribution of the novel inflammatory bowel disease gene IL23R to disease susceptibility and phenotype. Inflamm. Bowel. Dis. 13, 1063-1068.

Duerr, R.H., Taylor,K.D., Brant,S.R., Rioux,J.D., Silverberg,M.S., Daly,M.J., Steinhart,A.H., Abraham,C., Regueiro,M., Griffiths,A., Dassopoulos,T., Bitton,A., Yang,H., Targan,S., Datta,L.W., Kistner,E.O., Schumm,L.P., Lee,A.T., Gregersen,P.K., Barmada,M.M., Rotter,J.I., Nicolae,D.L., Cho,J.H., 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 314, 1461-1463.

Elmaagacli, A.H., Koldehoff,M., Landt,O., Beelen,D.W., 2008. Relation of an interleukin-23 receptor gene polymorphism to graft-versus-host disease after hematopoietic-cell ransplantation. Bone Marrow Transplant. 41, 821-826.

Frucht, D.M., 2002. IL-23: a cytokine that acts on memory T cells. Sci. STKE. 2002, pe1.

Gately, M.K., Renzetti,L.M., Magram,J., Stern,A.S., Adorini,L., Gubler,U., Presky,D.H., 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu. Rev. Immunol. 16, 495-521.

Gong, J.H., Maki,G., Klingemann,H.G., 1994. Characterization of a human cell line (NK- 92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia. 8, 652-658.

Happel, K.I., Dubin,P.J., Zheng,M., Ghilardi,N., Lockhart,C., Quinton,L.J., Odden,A.R.,

Shellito,J.E., Bagby,G.J., Nelson,S., Kolls,J.K., 2005. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. J. Exp. Med. 202, 761-769.

Heemskerk, M.H., Blom,B., Nolan,G., Stegmann,A.P., Bakker,A.Q., Weijer,K., Res,P.C., Spits,H., 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. J. Exp. Med. 186, 1597-1602.

Heemskerk, M.H., de Paus,R.A., Lurvink,E.G., Koning,F., Mulder,A., Willemze,R., van Rood,J.J., Falkenburg,J.H., 2001. Dual HLA class I and class II restricted recognition of alloreactive T lymphocytes mediated by a single T cell receptor complex. Proc. Natl. Acad. Sci. U. S. A. 98, 6806-6811.

Hibbert, L., Pflanz,S., de Waal Malefyt R., Kastelein,R.A., 2003. IL-27 and IFN-α signal via Stat1 and Stat3 and induce T-Bet and IL-12Rβ2 in naive T cells. J Interferon Cytokine Res. 23, 513-522.

Higuchi, R., Krummel,B., Saiki,R.K., 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. 16, 7351-7367.

Hill, N., Sarvetnick,N., 2002. Cytokines: promoters and dampeners of autoimmunity. Curr. Opin. Immunol. 14, 791-797.

Huber, A.K., Jacobson,E.M., Jazdzewski,K., Concepcion,E.S., Tomer,Y., 2008. Interleukin-23 receptor is a major susceptibility gene for Grave's ophthalmopathy: The IL-23/Th17 axis extends to thyroid autoimmunity. J Clin Endocrinol Metab 93, 1077-1081.

Janssen, R., Van Wengen,A., Verhard,E., de Boer,T., Zomerdijk,T., Ottenhoff,T.H., van Dissel,J.T., 2002. Divergent role for TNF-α in IFN-γ-induced killing of Toxoplasma gondii and Salmonella typhimurium contributes to selective susceptibility of patients with partial IFN-γ receptor 1 deficiency. J. Immunol. 169, 3900-3907.

Kim, H.R., Kim,H.S., Park,M.K., Cho,M.L., Lee,S.H., Kim,H.Y., 2007. The clinical role of IL-23p19 in patients with rheumatoid arthritis. Scand. J Rheumatol. 36, 259-264.

Kinsella, T.M., Nolan,G.P., 1996. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Hum. Gene Ther. 7, 1405-1413.

Lucas, S., Ghilardi,N., Li,J., de Sauvage,F.J., 2003. IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. Proc. Natl. Acad. Sci. U. S. A 100, 15047-15052.

McGovern, D., Powrie,F., 2007. The IL23 axis plays a key role in the pathogenesis of IBD. Gut 56, 1333-1336.

Mehrotra, P.T., Donnelly,R.P., Wong,S., Kanegane,H., Geremew,A., Mostowski,H.S., Furuke,K., Siegel,J.P., Bloom,E.T., 1998. Production of IL-10 by Human Natural Killer Cells Stimulated with IL-2 and/or IL-12. J Immunol 160, 2637-2644.

Meyaard, L., Hovenkamp,E., Otto,S.A., Miedema,F., 1996. IL-12-induced IL-10 production by human T cells as a negative feedback for IL-12-induced immune responses. J Immunol 156, 2776-2782.

Parham, C., Chirica,M., Timans,J., Vaisberg,E., Travis,M., Cheung,J., Pflanz,S., Zhang,R., Singh,K.P., Vega,F., To,W., Wagner,J., O'Farrell,A.M., McClanahan,T., Zurawski,S., Hannum,C., Gorman,D., Rennick,D.M., Kastelein,R.A., de Waal Malefyt,R., Moore,K.W., 2002. A Receptor for the Heterodimeric Cytokine IL-23 Is Composed of IL-12R.1 and a Novel Cytokine Receptor Subunit, IL-23R. J Immunol 168, 5699-5708.

Rueda, B., Orozco,G., Raya,E., Fernandez-Sueiro,J.L., Mulero,J., Blanco,F.J., Vilches,C.,

Gonzalez-Gay,M.A., Martin,J., 2008. The IL23R Arg381Gln non-synonymous polymorphism confers susceptibility to ankylosing spondylitis. Ann. Rheum. Dis. (Epub 2008, Jan. 16).

Ruggieri, L., Aiuti,A., Salomoni,M., Zappone,E., Ferrari,G., Bordignon,C., 1997. Cellsurface marking of CD(34+)-restricted phenotypes of human hematopoietic progenitor cells by retrovirus-mediated gene transfer. Hum. Gene Ther. 8, 1611-1623.

Torti, D.C., Feldman,S.R., 2007. Interleukin-12, interleukin-23, and psoriasis: current prospects. J. Am. Acad. Dermatol. 57, 1059-1068.

Trinchieri, G., Pflanz,S., Kastelein,R.A., 2003. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. Immunity. 19, 641-644.

Uhlig, H.H., McKenzie,B.S., Hue,S., Thompson,C., Joyce-Shaikh,B., Stepankova,R., Robinson,N., Buonocore,S., Tlaskalova-Hogenova,H., Cua,D.J., Powrie,F., 2006. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. Immunity. 25, 309-318.

van de Vosse, E., Hoeve,M.A., Ottenhoff,T.H., 2004. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. Lancet Infect. Dis. 4, 739-749.

van de Vosse, E., de Paus,R.A., van Dissel,J.T., Ottenhoff,T.H.M., 2005. Molecular complementation of IL-12Rβ1 deficiency reveals functional differences between IL-12Rβ1 alleles including partial IL-12Rβ1 deficiency. Hum. Mol. Genet. 14, 3847-3855.

van den Eijnden, S., Goriely,S., de Wit,D., Willems,F., Goldman,M., 2005. IL-23 upregulates IL-10 and induces IL-17 synthesis by polyclonally activated naive T cells in human. Eur. J. Immunol. 35, 469-475.

Watford, W.T., Hissong,B.D., Bream,J.H., Kanno,Y., Muul,L., O'Shea,J.J., 2004. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. Immunol. Rev. 202, 139-156.

Wilson, N.J., Boniface,K., Chan,J.R., McKenzie,B.S., Blumenschein,W.M., Mattson,J.D., Basham,B., Smith,K., Chen,T., Morel,F., Lecron,J.C., Kastelein,R.A., Cua,D.J., McClanahan,T.K., Bowman,E.P., de Waal Malefyt R., 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. Nat. Immunol. 8, 950-957.

Wu, C., Warrier,R.R., Wang,X., Presky,D.H., Gately,M.K., 1997. Regulation of interleukin-12 receptor β1 chain expression and interleukin-12 binding by human peripheral blood mononuclear cells. Eur. J. Immunol. 27, 147-154.

CHAPTER 4

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

Diederik van de Wetering, Roelof A. de Paus,
Jaap T. van Dissel, Esther van de Vosse.

Department of Infectious Diseases, Leiden University Medical Center,
Albinusdreef 2, 2333 ZA, Leiden, The Netherlands.

International Immunology 2009;21(2):145-53

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-γ (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. 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-18Rα. 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.

1. 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-γ (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 interleuk in-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-γR on the macrophages and dendritic cells to enhance their bactericidal activity and antigen presentation and increase production of IL-12 (12).

Interleukin-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-12Rβ1 chain complemented by the IL-23R and the IL-12Rβ2 respectively. While IL-12Rβ1 is constitutively expressed in naive $CD4^+$ T cells, $CD56^+$ NK-like T cells and NK cells (13;14), IL-23R and IL-12Rβ2 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 proinflammatory 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-12Rβ1 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.

2. Materials and Methods

2.1. 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).

2.2. 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 10^5 cells/well in 96-well plates 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 asses IL-18Rα 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-18Rα mAb FAB840P (R&D Systems) in combination with with FITC conjugated anti-human CD3 and Alexa 647 anti-human CD56.

2.3. Functional analysis

To determine cytokine production, overnight rested $CD56^+$ beads isolated cells were seeded 100.000 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 μl. 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. 10^6 cells were labelled in 2 ml medium containing 1 μM CFSE for 15 minutes. After labelling cells were washed twice and seeded 10^5 cells/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).

3. Results

3.1. 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).

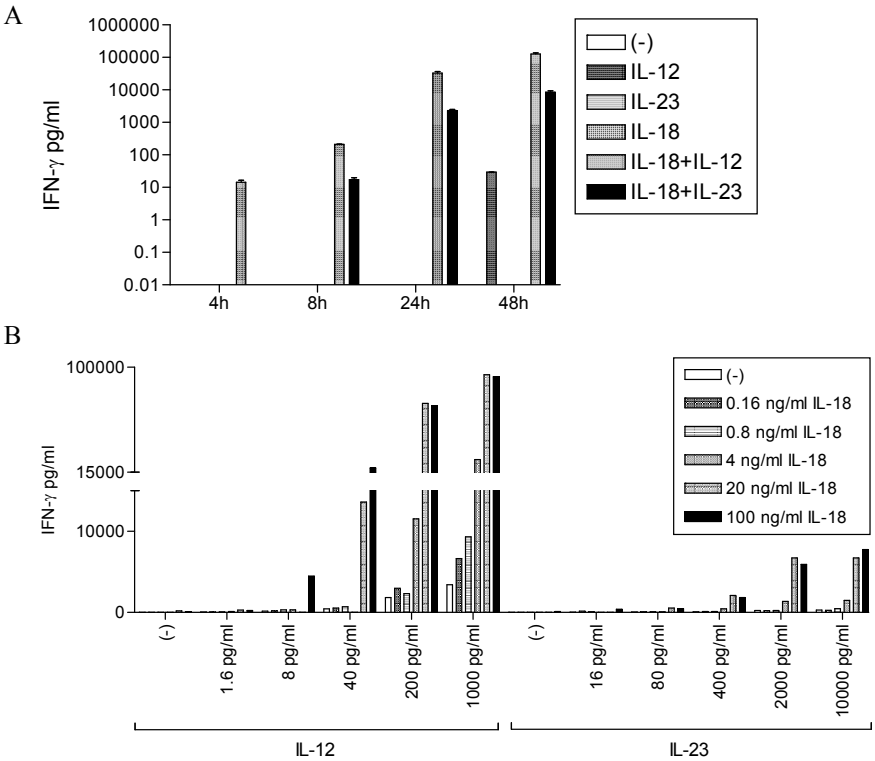


Figure 1. **IL-23, in combination with IL-18, induces IFN-γ in primary human CD56$^+$ cells.** (A) Anti-CD56 MACS bead isolated cells were rested overnight and then left unstimulated or stimulated with indicated concentrations of IL-23 or IL-12 plus or minus IL-18. Supernatants were collected 4, 8, 24 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.

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).

3.2. 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. 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).

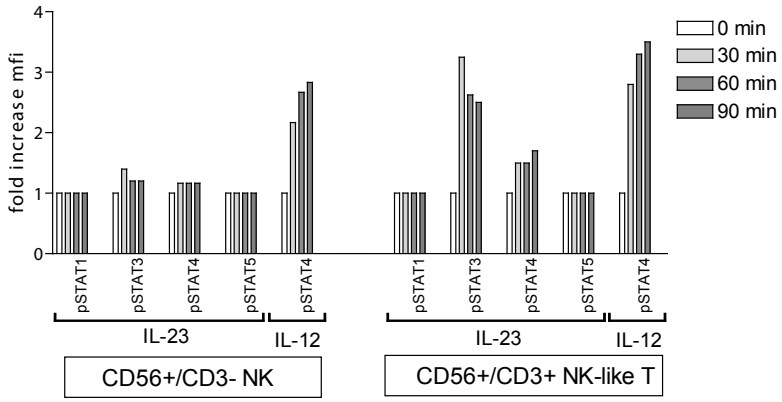


Figure 2. IL-23 induces STAT phosphorylation in CD56$^+$/CD3$^+$ NK-like T but not in CD56$^+$/CD3$^-$ NK cells. Anti-CD56 MACS bead isolated cells were rested overnight, and subsequently stimulated with 10 ng/ml IL-23 or 1 ng/ml IL-12 for indicated times. Cells were labelled with anti-human pSTAT1, 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.

3.3. 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).

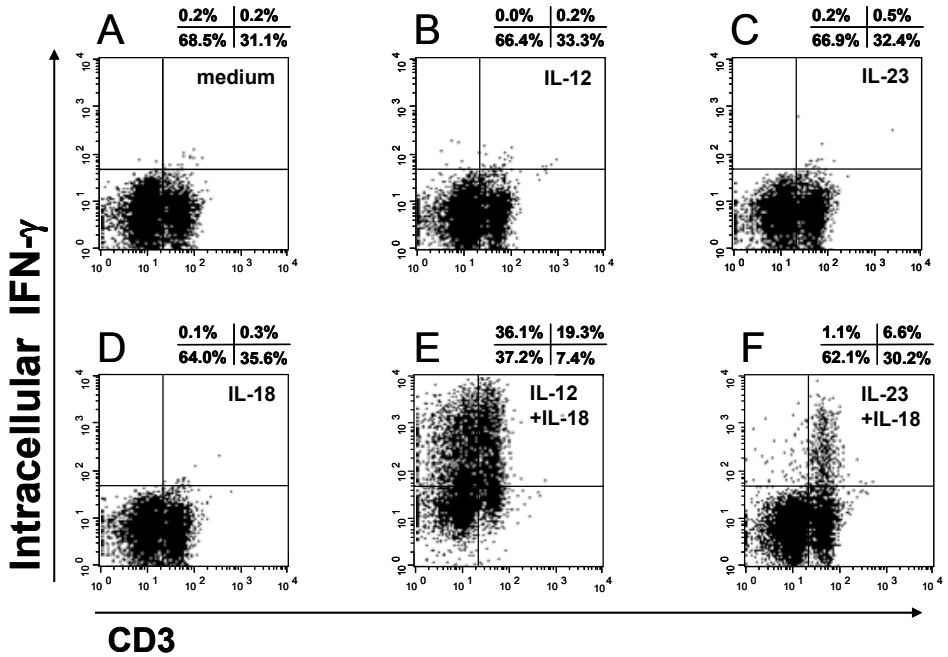


Figure 3. IL-23 in combination with IL-18 induces IFN-γ production in CD56^{+}/CD3^{+} NK-like T cell but not in CD56^{+}/CD3^{-} NK cells. Anti-CD56 MACS bead isolated cells were rested overnight and then left unstimulated (A) or stimulated with 1 ng/ml IL-12 (B), 10 ng/ml IL-23 (C), 100 ng/ml IL-18 (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.

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

	(-) % (SD)	IL-12 % (SD)	IL-23 % (SD)	IL-18 % (SD)	IL-18+IL-12 %(SD)	IL-18+IL-23 percent (SD)
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 od stimulated versus unstimulated cells. S.D., standard deviation. * indicates a p-value <0.01, ** indicates a p-value <0.02.

3.4. 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%.

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

	(-) % (SD)	**IL-12** % (SD)	**IL-23** % (SD)	**IL-18** % (SD)	**IL-18+IL-12** % (SD)	**IL-18+IL-23** % (SD)
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. S.D., standard deviation. * indicates a p-value <0.05, ** indicates a p-value <0.01

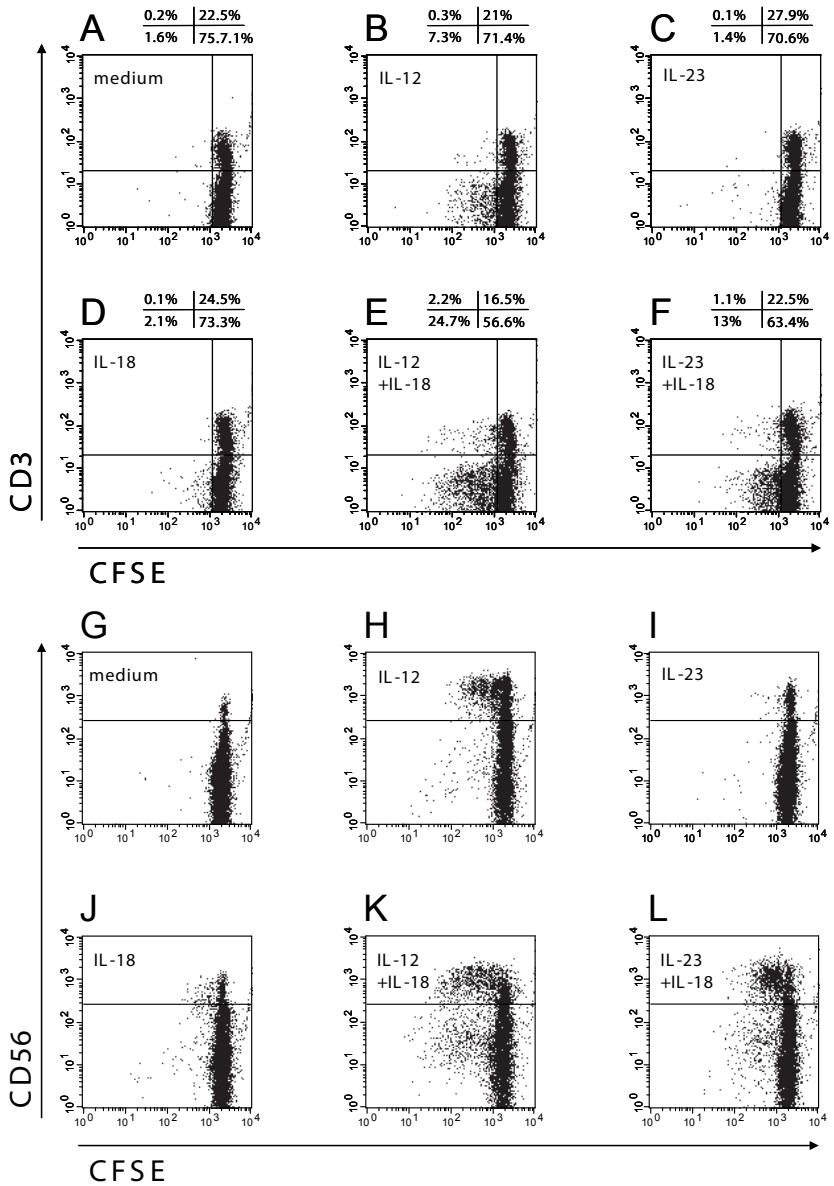


Figure 4. IL-23 in combination with IL-18 induces proliferation of CD56$^+$/CD3$^-$ NK cells and in CD56$^+$/CD3$^+$ NK-like T cells. Anti-CD56 MACS bead isolated cells were rested overnight and then CFSE labeled. These labeled cells were left unstimulated (A and G) or stimulated with 1 ng/ml IL-12 (B 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.

3.5. IL-23 enhances IL-18Rα 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-18Rα expression (23). To determine whether the synergy between IL-23 and IL-18 is similarly dependent on the up-regulation of IL-18Rα, 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-18Rα, CD3 and CD56 expression. Unstimulated NK and NK-like T cells expressed low amounts of IL-18Rα (Fig. 5A and Table 3). IL-12 enhanced IL-18Rα expression in both NK and NK-like T cells (Fig. 5B and Table 3). IL-23 up-regulated the IL-18Rα 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-18Rα only marginally in both populations (Fig. 5D and Table 3), but synergized with the effect of IL-12 on the expression of IL-18Rα 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-18Rα in NK-like T cells (Fig. 5F and Table 3). In NK cells a slight upregulation of the IL-18Rα 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-18Rα were CD56^{bright} (data not shown).

4. 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-18Rα. Secondly, IL-23 plus IL-18 induced proliferation of CD56$^+$/CD3$^-$ NK cells and CD56$^+$/CD3$^+$ NK-like T cells.

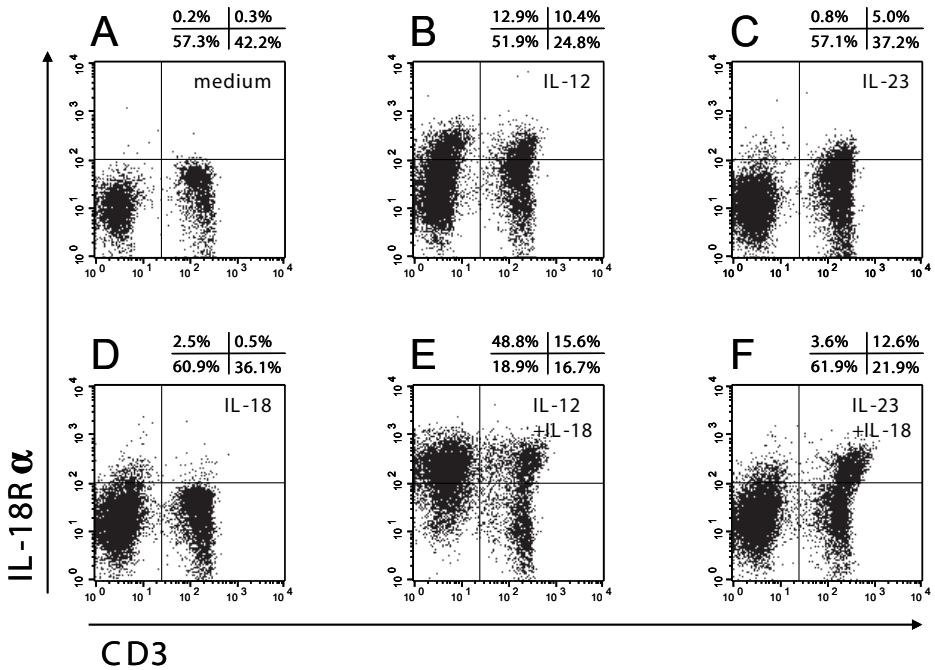


Figure 5. **IL-23 up-regulates IL-18Rα expression in CD56$^+$/CD3$^+$ NK-like T, but not in CD56$^+$/CD3$^-$ NK cells.** Anti-CD56 MACS bead isolated cells were left unstimulated (A) or stimulated with 1 ng/ml IL-12 (B), 10 ng/ml IL-23 (C), 100 ng/ml IL-18 (D), 1 ng/ml IL-12 plus 100 ng/ml IL-18 (E) 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-I8 synergizes with IL-12 and IL-23 in the up-regulation of IL-18Rα expression. One representative of experiments with cells from 6 donors.

Table 3.

	(-) % (SD)	**IL-12** % (SD)	**IL-23** % (SD)	**IL-18** % (SD)	**IL-18+IL-12** % (SD)	**IL-18+IL-23** % (SD)
CD3$^-$ NK cells upper left quadrant	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 right quadrant	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-test were performed of stimulated cells versus unstimulated cells. S.D., standard deviation. * Indicates a p-value <0.01, ** Indicates a p-value <0.02.

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-18Rα 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-18Rα expression. In $CD56^+/CD3^-$ NK cells the IL-18Rα 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-12Rβ1 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.

Acknowledgments
We would like to thank dr. Nigel Savage for carefully reading the manuscript.

References

1 Griggs, N. D. and Smith, R. A. 1994. Natural killer cell activity against uninfected and Salmonella typhimurium-infected murine fibroblast L929 cells. *Nat.Immun.* 13:42.

2 Kirby, A. C., Yrlid, U., and Wick, M. J. 2002. The innate immune response differs in primary and secondary Salmonella infection. *J.Immunol.* 169:4450.

3 Feng, C. G., Kaviratne, M., Rothfuchs, A. G., Cheever, A., Hieny, S., Young, H. A., Wynn, T. A., and Sher, A. 2006. NK cell-derived γ differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with Mycobacterium tuberculosis. *J.Immunol.* 177:7086.

4 Scharton, T. M. and Scott, P. 1993. Natural killer cells are a source of γ that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. *J.Exp.Med.* 178:567.

5 Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P., and Salazar-Mather, T. P. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu.Rev.Immunol.* 17:189-220.:189.

6 Byrne, P., McGuirk, P., Todryk, S., and Mills, K. H. 2004. Depletion of NK cells results in disseminating lethal infection with Bordetella pertussis associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. *Eur.J.Immunol.* 34:2579.

7 Martin-Fontecha, A., Thomsen, L. L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., and Sallusto, F. 2004. Induced recruitment of NK cells to lymph nodes provides γ for T(H)1 priming. *Nat.Immunol.* 5:1260.

8 van de Vosse, E. and Ottenhoff, T. H. 2006. Human host genetic factors in mycobacterial and Salmonella infection: lessons from single gene disorders in IL-12/IL-23-dependent signaling that affect innate and adaptive immunity. *Microbes.Infect.* 8:1167.

9 Verreck, F. A., de Boer, T., Langenberg, D. M., Hoeve, M. A., Kramer, M., Vaisberg, E., Kastelein, R., Kolk, A., de Waal-Malefyt, R., and Ottenhoff, T. H. 2004. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc.Natl.Acad.Sci.U.S.A* 101:4560.

10 Hunter, C. A., Timans, J., Pisacane, P., Menon, S., Cai, G., Walker, W., Aste-Amezaga, M., Chizzonite, R., Bazan, J. F., and Kastelein, R. A. 1997. Comparison of the effects of interleukin-1 α, interleukin-1 β and interferon-γ-inducing factor on the production of interferon-γ by natural killer. *Eur.J.Immunol.* 27:2787.

11 Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F., and Kastelein, R. A. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity.* 13:715.

12 van de Vosse, E., Hoeve, M. A., and Ottenhoff, T. H. 2004. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. *Lancet Infect.Dis.* 4:739.

13 Gately, M. K., Renzetti, L. M., Magram, J., Stern, A. S., Adorini, L., Gubler, U., and Presky, D. H. 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu. Rev.Immunol.* 16:495.

14 Guia, S., Cognet, C., de Beaucoudrey L., Tessmer, M. S., Jouanguy, E., Berger, C., Filipe-Santos, O., Feinberg, J., Camcioglu, Y., Levy, J., Al Jumaah, S., Al-Hajjar, S., Stephan, J. L., Fieschi, C., Abel, L., Brossay, L., Casanova, J. L., and Vivier, E. 2008. A role for interleukin-12/23 in the maturation of human natural killer and CD56+ T cells in vivo. *Blood* 111:5008.

15 Hochrein, H., O'Keeffe, M., Luft, T., Vandenabeele, S., Grumont, R. J., Maraskovsky, E., and Shortman, K. 2000. Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J.Exp.Med.* 192:823.

16 Happel, K. I., Dubin, P. J., Zheng, M., Ghilardi, N., Lockhart, C., Quinton, L. J., Odden, A. R., Shellito, J. E., Bagby, G. J., Nelson, S., and Kolls, J. K. 2005. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. *J.Exp.Med.* 202:761.

17 Aggarwal, S., Ghilardi, N., Xie, M. H., de Sauvage, F. J., and Gurney, A. L. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J.Biol.Chem.* 278:1910.

18 Vanden Eijnden, S., Goriely, S., De Wit, D., Willems, F., and Goldman, M. 2005. IL-23 up-regulates IL-10 and induces IL-17 synthesis by polyclonally activated naive T cells in human. *Eur.J.Immunol.* 35:469.

19 van de Vosse, E., de Paus, R. A., Van Dissel, J. T., and Ottenhoff, T. H. 2005. Molecular complementation of IL-12Rβ1 deficiency reveals functional differences between IL-12Rβ1 alleles including partial IL-12Rβ1 deficiency. *Hum.Mol.Genet.* 14:3847.

20 Parham, C., Chirica, M., Timans, J., Vaisberg, E., Travis, M., Cheung, J., Pflanz, S., Zhang, R., Singh, K. P., Vega, F., To, W., Wagner, J., O'Farrell, A. M., McClanahan, T., Zurawski, S., Hannum, C., Gorman, D., Rennick, D. M., Kastelein, R. A., de Waal, M. R., and Moore, K. W. 2002. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rγ1 and a novel cytokine receptor subunit, IL-23R. *J.Immunol.* 168:5699.

21 Takeda, K., Tsutsui, H., Yoshimoto, T., Adachi, O., Yoshida, N., Kishimoto, T., Okamura, H., Nakanishi, K., and Akira, S. 1998. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity.* 8:383.

22 de Paus, R. A., van de Wetering, D., Van Dissel, J. T., and van de Vosse, E. 2008. IL-23 and IL-12 responses in activated human T cells retrovirally transduced with IL-23 receptor variants. *Mol.Immunol.* 45:3889.

23 Lauwerys, B. R., Renauld, J. C., and Houssiau, F. A. 1999. Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. *Cytokine* 11:822.

24 Hayes, M. P., Wang, J., and Norcross, M. A. 1995. Regulation of interleukin-12 expression in human monocytes: selective priming by γ of lipopolysaccharide-inducible p35 and p40 genes. *Blood* 86:646.

25 Thakker, P., Leach, M. W., Kuang, W., Benoit, S. E., Leonard, J. P., and Marusic, S. 2007. IL-23 is critical in the induction but not in the effector phase of experimental autoimmune encephalomyelitis. *J.Immunol.* 178:2589.

26 MacLennan, C., Fieschi, C., Lammas, D. A., Picard, C., Dorman, S. E., Sanal, O., MacLennan, J. M., Holland, S. M., Ottenhoff, T. H., Casanova, J. L., and Kumararatne, D. S. 2004. Interleukin (IL)-12 and IL-23 are key cytokines for immunity against Salmonella in humans. *J.Infect.Dis.* 190:1755.

27 Jason, J., Buchanan, I., Archibald, L. K., Nwanyanwu, O. C., Bell, M., Green, T. A., Eick, A., Han, A., Razsi, D., Kazembe, P. N., Dobbie, H., Midathada, M., and Jarvis, W. R. 2000. Natural T, γδ, and NK cells in mycobacterial, Salmonella, and human immunodeficiency virus infections. *J.Infect.Dis.* 182:474.

28 Barcelos, W., Martins-Filho, O. A., Guimaraes, T. M., Oliveira, M. H., Spindola-de-Miranda, S., Carvalho, B. N., and Toledo, V. P. 2006. Peripheral blood mononuclear cells immunophenotyping in pulmonary tuberculosis patients before and after treatment. *Microbiol.Immunol.* 50:597.

29 Veenstra, H., Baumann, R., Carroll, N. M., Lukey, P. T., Kidd, M., Beyers, N., Bolliger, C. T., van Helden, P. D., and Walzl, G. 2006. Changes in leucocyte and lymphocyte subsets during tuberculosis treatment; prominence of CD3dimCD56+ natural killer T cells in fast treatment responders. *Clin.Exp.Immunol.* 145:252.

30 Cooper, A. M., Kipnis, A., Turner, J., Magram, J., Ferrante, J., and Orme, I. M. 2002. Mice lacking bioactive IL-12 can generate protective, antigen-specific cellular responses to mycobacterial infection only if the IL-12 p40 subunit is present. *J.Immunol.* 168:1322.

31 Lehmann, J., Bellmann, S., Werner, C., Schroder, R., Schutze, N., and Alber, G. 2001. IL-12p40-dependent agonistic effects on the development of protective innate and adaptive immunity against Salmonella enteritidis. *J.Immunol.* 167:5304.

32 Khader, S. A., Pearl, J. E., Sakamoto, K., Gilmartin, L., Bell, G. K., Jelley-Gibbs, D. M., Ghilardi, N., de Sauvage, F., and Cooper, A. M. 2005. IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-γ responses if IL-12p70 is available. *J.Immunol.* 175:788.

CHAPTER 5

Functional analysis of naturally occurring amino acid substitutions in human IFN-γR1

Diederik van de Wetering [1], Roelof A. de Paus[1],
Jaap T. van Dissel, Esther van de Vosse

[1] shared first authors
Department of Infectious Diseases, Leiden University Medical Center,
Leiden, The Netherlands

Molecular Immunology 2009;47(5):1023–1030

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-γR1 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 poorly pathogenic mycobacteria, a disorder known as Mendelian Susceptibility to Mycobacterial Disease (MSMD). More common amino acid variations in the IFN-γR1 may also influence IFN-γR function, albeit more subtle. To determine the effect of various amino acid substitutions on IFN-γR1 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-γR1 expression was determined as well as responsiveness to IFN-γ stimulation. The two newly discovered 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-γR1 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-γR1 deficiency, while V63G and I87T lead to partial IFN-γR1 deficiency.

1. 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 type1 cytokines (Ottenhoff et al., 2002). Upon encounter of these pathogens, antigen presenting cells (APC) produce cytokines such as IL-23, IL-1β, 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. and IL-18. IFN-γ in turn, binds to the IFN-γ receptor (IFN-γR), present on nearly all cell types (Valente et al., 1992). 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 (Leenen et al., 1994; Nathan and Hibbs, 1991). 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-γR is comprised of two ligand-binding IFN-γR1 chains associated with two signal-transducing IFN-γR2 chains (Boehm et al., 1997). 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-γR1, allowing for subsequent STAT1 phosphorylation (Boehm et al., 1997). 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)(Ramana et al., 2002), or indirectly via the induction of other transcription factors such as IRF1, IRF7 and CIITA (e.g. B2M and HLA) (Boehm et al., 1997). 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 (van Boxel-Dezaire and Stark, 2007). In Fig. 1 a schematic representation of the IFN-γR1 is provided.

Polymorphisms and mutations in the IFN-γR1 chain influence IFN-γ responses. For example, patients with Mendelian Susceptibility to Mycobacterial Disease (MSMD) due to IFN-γR1 deficiency, have impaired Th1-immunity and suffer from unusually severe infections caused by weakly virulent Mycobacteria (van de Vosse et al., 2004). Most recessive IFN-γR1 deficiencies result in complete loss of cellular responsiveness to IFN-γ, due to mutations that preclude the expression of IFN-γR1 on the cell surface (Pierre-Audigier et al., 1997; Rosenzweig et al., 2002; Newport et al., 1996; Jouanguy et al., 1996; Roesler et al., 1999).

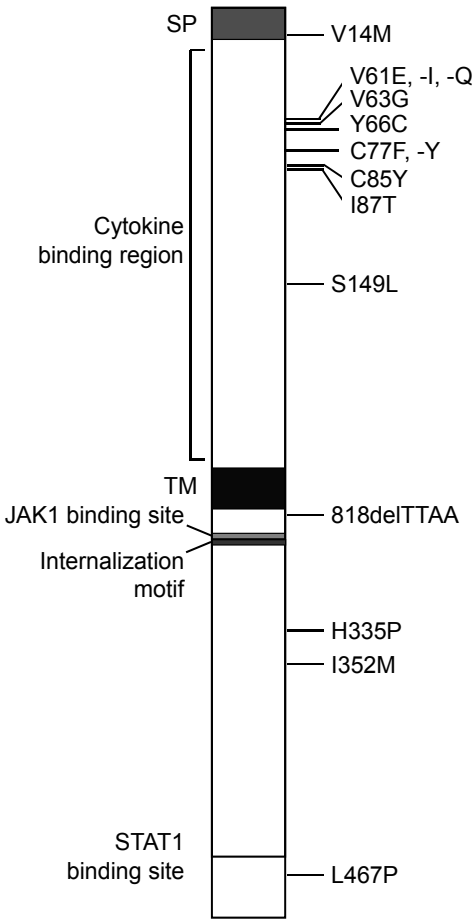


Figure 1. Schematic presentation of the IFN-γR1. On the left the various domains are indicated, on the right the variations that were analyzed are indicated. TM = Transmembrane domain, SP = signal peptide.

Another group of IFN-γR1 deficiencies is due to missense mutations which result in normal expression of IFN-γR1 at the cell surface, however, the resulting receptors show no or diminished binding of IFN-γ (Allende et al., 2001; Jouanguy et al., 2000). Dominant-negative defects such as 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 (Jouanguy et al., 1999; Glosli et al., 2008). In addition, several polymorphisms of the IFN-γR1 have been found that may have an effect on IFN-γ responses. The H335P and L467P variants of the IFN-γR1 have been associated with the production of high antibody titers against Helicobacter pylori (Thye et al., 2003) and

susceptibility to allergic disease and the production of high IgE titers (Aoki et al., 2003). 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 (Tanaka et al., 1999; Nakashima et al., 1999).

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 (Nakao et al., 2001; Tanaka et al., 1999), V61I (SNP database, 2006), H335P, L467P (Thye et al., 2003; Aoki et al., 2003), all seven reported missense mutations (V61Q (Jouanguy et al., 2000), V63G (Allende et al., 2001), Y66C (Dorman et al., 2004), C77Y (Jouanguy et al., 2000), C77F (Chantrain et al., 2006), C85Y (Noordzij et al., 2007), I87T (Jouanguy et al., 1997; Remiszewski et al., 2006)) and the 818delTTAA mutation (Jouanguy et al., 1999) on the expression and function of IFN-γR1 in the same genetic background. In addition, because the mutation at nucleotide 182 (gTA . gAA) reported by Jouanguy et al. (2000) may have been aberrantly designated as V61Q (Val . Gln), instead of V61E (Val . Glu), we analyzed both variations. For this purpose we cloned wild type *IFNGR1* and the *IFNGR1* variants (see also Fig. 1), into a retroviral expression vector and transduced the constructs into the IFN-γR1 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.

2. Materials and methods

2.1. Cloning IFN-γR1 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-' and cloned into the retroviral vector pLZRS-IRES-GFP (Heemskerk et al., 1997) after digestion with the enzymes BamHI and XhoI (Fermentas). Variations were introduced by site directed mutagenesis (Higuchi et al., 1988). 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 (de Paus et al., 2008).

2.2. Cells, culture conditions and retroviral transduction

The human IFN-γR1$^{-/-}$ cell-line SKLC-7 (Kaplan et al., 1998) 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 µg/ml Streptomycin (Gibco/Invitrogen). 0.25×10^6 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.

2.3. Analysis of IFN-γR1 expression

To detect IFN-γR1 membrane expression cells were labeled with IRγ2 (Watzka et al., 1998) and 177.10 (Novick et al., 1989) 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 γR99 antibody (Garotta et al., 1990) (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.

2.4. 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 × 105 transduced SKLC-7 or THP-1 cells in 200 µl culture medium with various amounts of IFN-γ for 20 hours in 96-wellsplates (Greiner bio-one). 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).

2.5. STAT phosphorylation assays

To study signal transduction, 2×10^5 transduced or untransduced SKLC-7 cells in 200 µl of culture medium were pulsed with various concentrations of IFN-γ (Biosource) in 96-wellsplates (Greiner bio-one). 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, pY705STAT3-PE or pY694-STAT5-PE (BD Pharmingen). Before analysis on a FACSCalibur, the cells were washed twice.

3. Results

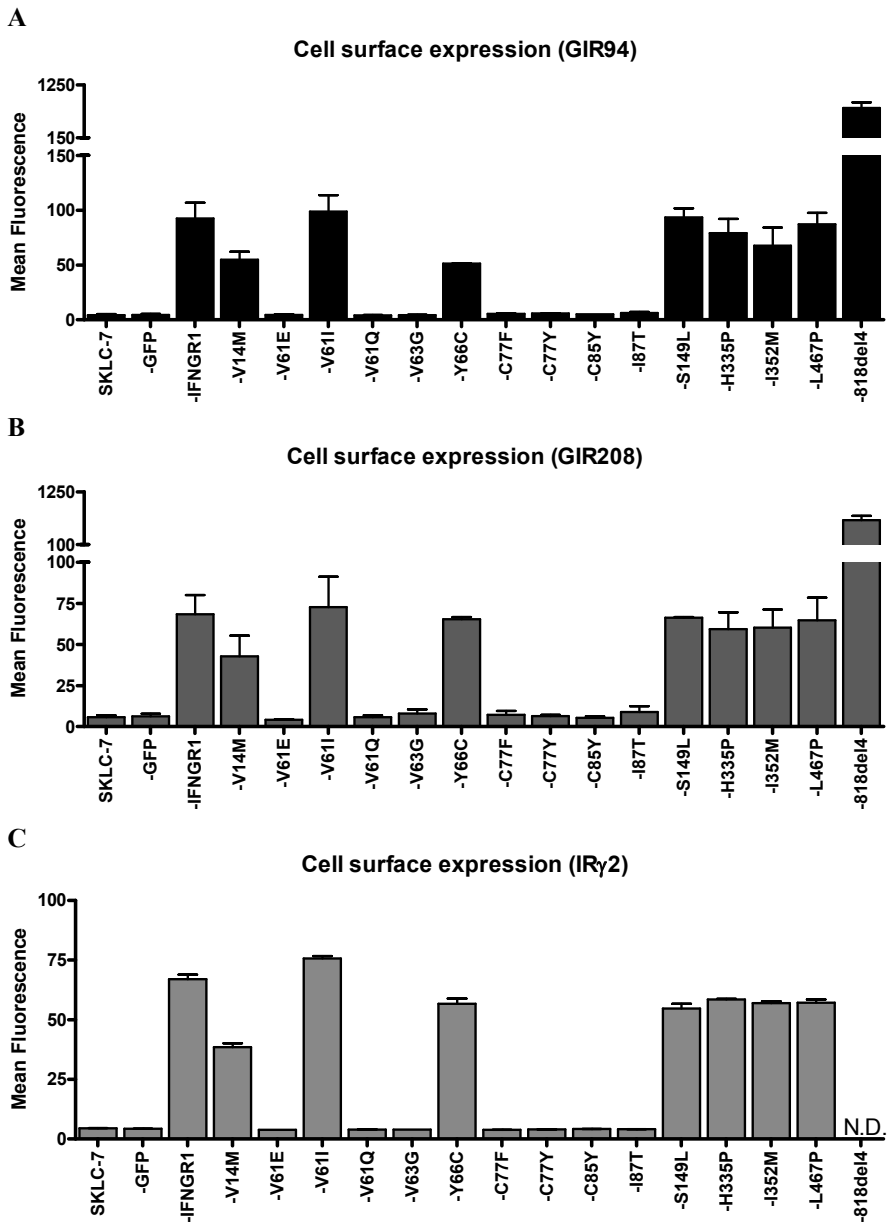
3.1. Functional transfer of the IFNGR1 in SKLC-7 cells

We cloned and expressed the wild type IFN-γR1 in the IFN-γR1 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. 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-γR1 on their cell surface, nor do they express GFP (Fig. 2A–D). After retroviral transduction of the wild-type *IFNGR1* into the SKLC-7 cells the IFN-γR1 could be detected on the cell membrane (Fig. 2A–D), whereas in cells transduced with the empty vector (GFP) it could not (Fig. 2A–D). Next we tested the responsiveness of transduced cells to IFN-γ. SKLC7 cells expressing wild type IFN-γR1 were stimulated with various concentrations of IFN-γ for 5 to 90 min before assessing STAT1) phosphorylation. STAT1 phosphorylation peaked 10–15 min 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).

3.2. Expression pattern of the IFN-γR1 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-γR1 were cloned and retrovirally transduced into the IFN-γR1-/- SKLC-7 cell line. We first determined whether the IFN-γR1 variants were expressed at the cell surface, using four different IFN-γR1 specific antibodies. Untransduced or GFP-transduced cells did not express IFN-γR1 (Fig. 2A–D). On the SKLC-7 cells transduced with wild type IFN-γR1, 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 and B).


A
Cell surface expression (GIR94)
Mean Fluorescence
1250
150
150
100
50
0
SKLC-7
-GFP
-IFNGR1
-V14M
-V61E
-V61I
-V61Q
-V63G
-Y66C
-C77F
-C77Y
-C85Y
-I87T
-S149L
-H335P
-I352M
-L467P
-818del4
B
Cell surface expression (GIR208)
Mean Fluorescence
1250
100
100
75
50
25
0
SKLC-7
-GFP
-IFNGR1
-V14M
-V61E
-V61I
-V61Q
-V63G
-Y66C
-C77F
-C77Y
-C85Y
-I87T
-S149L
-H335P
-I352M
-L467P
-818del4
C
Cell surface expression (IRγ2)
Mean Fluorescence
100
75
50
25
0
N.D.
SKLC-7
-GFP
-IFNGR1
-V14M
-V61E
-V61I
-V61Q
-V63G
-Y66C
-C77F
-C77Y
-C85Y
-I87T
-S149L
-H335P
-I352M
-L467P
-818del4

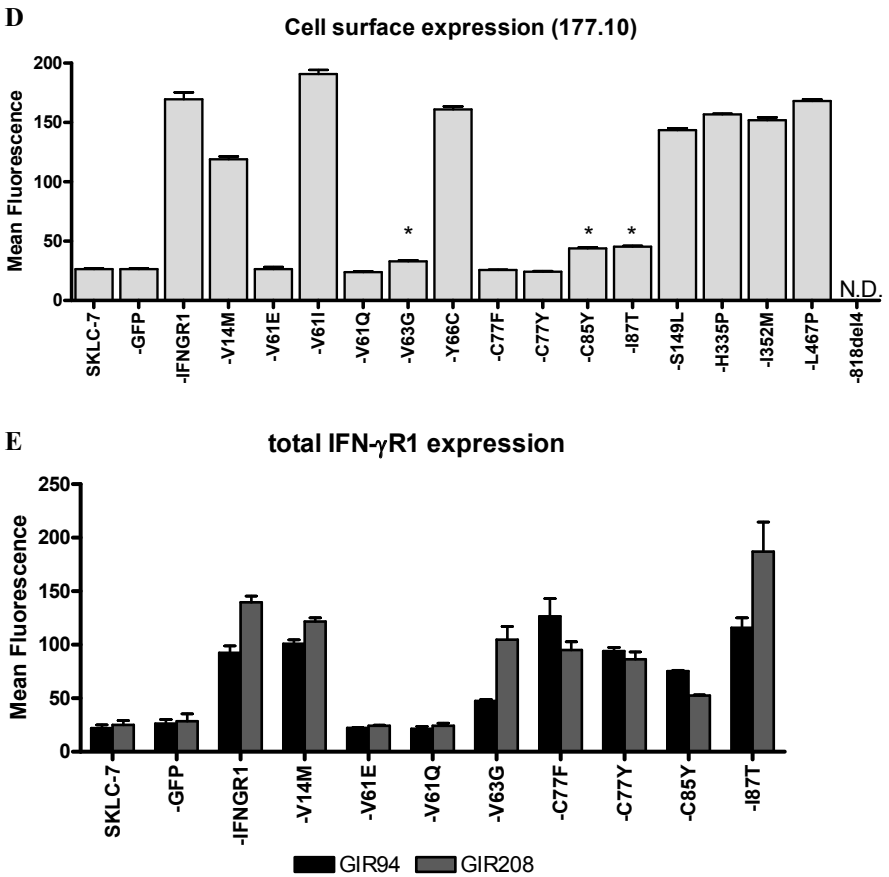


Figure 2. Extracellular and intracellular expression of the IFN-γR1 variants. SKLC-7 cells and the cells retrovirally transduced with GFP or with one of the *IFNGR1* gene variants were stained without pretreatment to detect extracellular expression (A–D or after permeabilization to detect both intracellular and extracellular expression of IFN-γR1 (E). Cells were analyzed for IFN-γR1 expression using four IFN-γR1 specific antibodies (GIR94 (A), GIR208 (B), IRγ2 (C) and 177.10 (D)). Mean ± SD fluorescence of these antibodies of 2 (IRγ2 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.

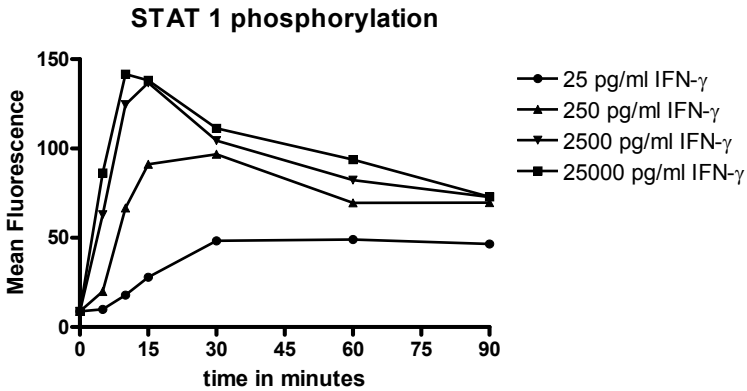


Figure 3. Kinetics of STAT1 phosporylation in response to IFN-γ. SKLC-7 cells transduced with wild type *IFNGR1* were stimulated with various concentrations of IFN-γ (25–25,000 pg/ml IFN-γ), whereafter STAT1 phosphorylation was analyzed at various time points. One representative experiment out of 2 is depicted.

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 production, 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-γR1 expression. Except for the GFP transduced cells and the V61E and V61Q variants, in all other transduced cells the IFN-γR1 could be detected intracellular, indicating protein is synthesized (Fig. 2E). The results of the expression analysis experiments are summarized in Table 1.

3.3. The effect of IFN-γR1 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 (Platanias and Fish, 1999). To test the influence of the IFN-γR1 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-γR1 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 and 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 and C). The results of the STAT1 phosphorylation experiments are summarized in Table 1.

Table 1. Summary of the expression and functional analyses of the IFN-γR1 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.

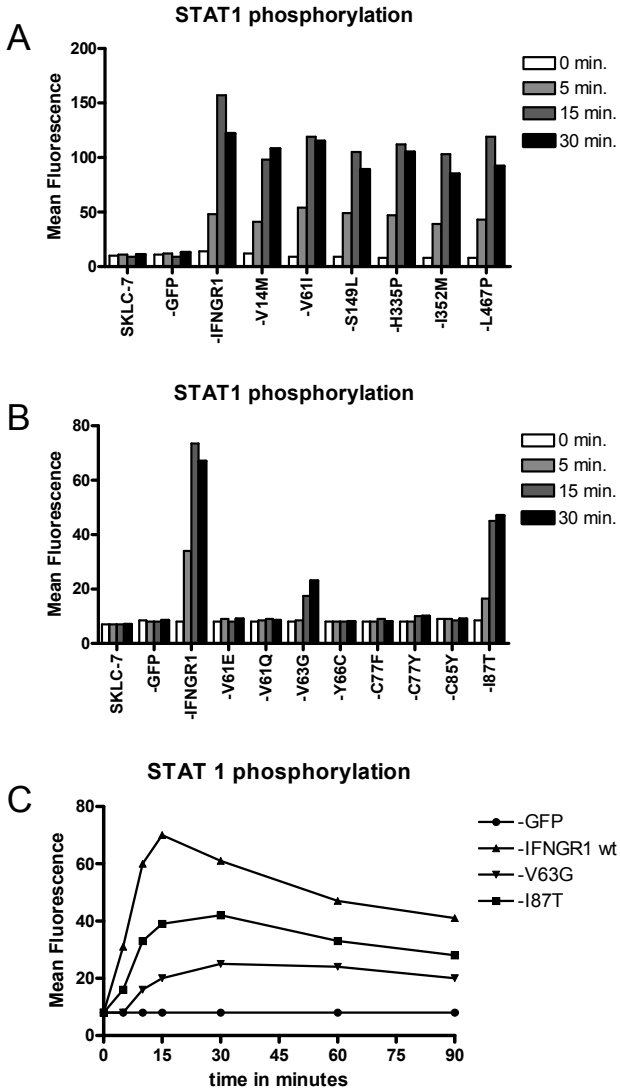


Figure 4. The influence of IFN-γR1 variations on the kinetics of STAT1 phosphorylation.
SKLC-7 cells transduced with wild type IFNGR1, GFP or with the V14M, V61I, S149L, H335P, I352M and L467P (A), V61E, V61Q, V63G, Y66C, C77F, C77Y, C85Y and I87T (B) or V63G and I87T variants (C), were stimulated for 5–90 min with 2500 pg/ml IFN-γ (A) or 250,000 pg/ml IFN-γ (B and C). The phosphorylation of STAT1 was determined by FACS, using a STAT1 phospho-specific antibody. One representative experiment out of 3 are depicted.

3.4. Influence of IFN-γR1 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 (Shang and Issekutz, 1998). CD54 functions as a costimulatory molecule on APC and other cell types, to activate CD4+ T cells and cytotoxic CD8+ T cells, respectively (van de Stolpe and van der Saag, 1996). 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 (Boehm et al., 1997; Schroder et al., 2004). We assessed the influence of the IFN-γR1 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-γR1, 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-γR1 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.

3.5. Influence of the IFN-γR1 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-γR contains two IFN-γR1 chains, in individuals heterozygous for a defective IFN-γR1 chain that is expressed on the membrane, incorporation of one defective IFN-γR1 chain in the IFN-γR 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-γR1 or a GFP vector. While expressing large amounts of Y66C IFN-γR1, 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-γR1 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-γR1 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).

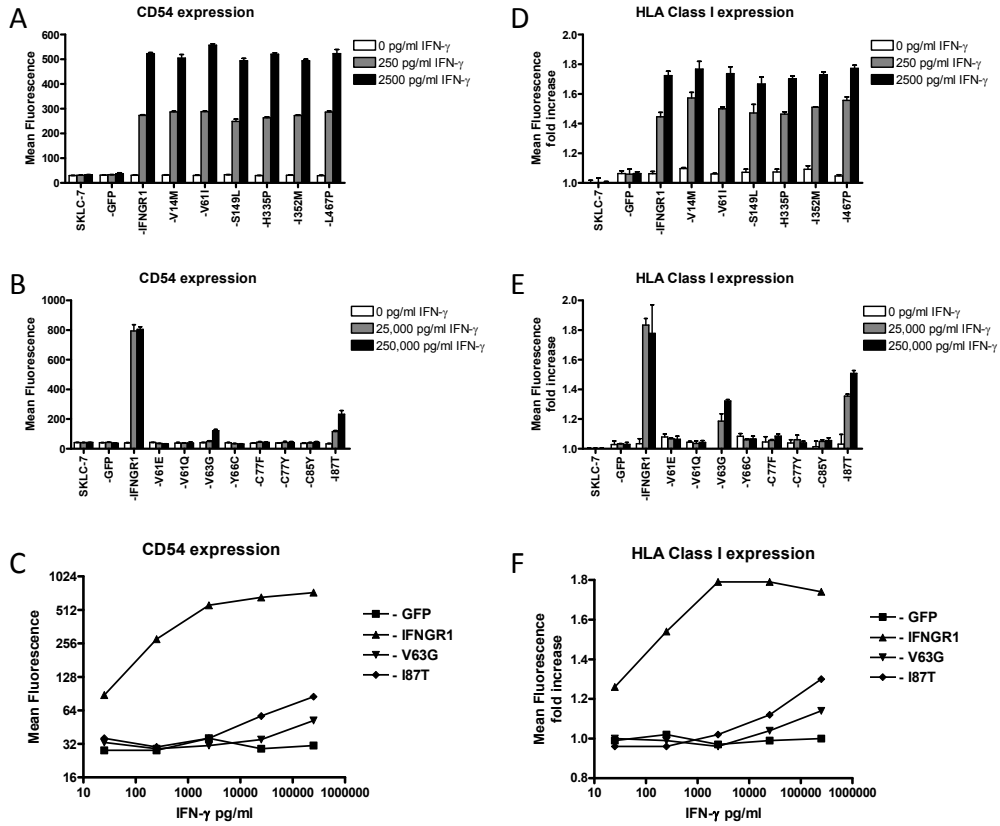


Figure 5. The effect of IFN-γR1 variations on IFN-γ induced CD54 and HLA class I upregulation. SKLC-7 cells and the cells retrovirally transduced with GFP or with one of the *IFNGR1* gene variants were stimulated for 20 h with various concentrations of IFN-γ. The CD54 expression (A–C) and the HLA Class I expression (D–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 ± SD of a triplo experiment. All variants were tested at least three times for comparison with the wild type variant.

4. Discussion

The main finding of this study is that the newly discovered IFN-γR1 variants, S149L and I352M, as well as the known V14M, V61I, H335P and L467P IFN-γR1 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 (Okada et al., 2007; Jouanguy et al., 1997).

To reach these conclusions, we retrovirally expressed the IFN-γR1 variants in the human IFN-γR1-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-γR 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 (vanBoxel-Dezaire and Stark, 2007), in our model no STAT3 or STAT5 phosphorylation could be detected, thus not allowing for detection of potential differences between the IFN-γR1 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-γR1 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-γR1. Furthermore, post-transcriptional and post-translational modifications of normal or retroviral expressed IFN-γR1 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 (Klausner and Sitia, 1990), as we have also reported for several IL-12Rβ1 mutations (van de Vosse et al., 2005). In accordance with literature, we did not observe any IFN-γ responsiveness of these two variant receptors (Chantrain et al., 2006; Jouanguy et al., 2000).

In contrast with previous publications (Jouanguy et al., 1997; Allende et al., 2001), we only detected low cell surface expression of 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. (1997) 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. (2001) that it results in complete deficiency. In line with our results with the I87T variant, Jouanguy et al. (1997) observed a response in B-cells and monocytes obtained from the patient expressing this variant when stimulated with very high doses of IFN-γ. 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-γR1 are known to be essential in the interaction between the high-affinity receptor and its ligand IFN-γ (Walter et al., 1995). Moreover, the adjacent lysine at position 64 directly interacts with IFN-γ.

In the V61Q (V61E) variant IFN-γR1, the binding of IFN-γ was reported to be abrogated (Jouanguy et al., 2000). This was determined in patient cells expressing two mutant *IFNGR1* alleles (V61Q and 652del3) (Jouanguy et al., 2000). 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. (2000) may have been the 652del3 mutation. 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 IRγ2) were raised against the extracellular domain of IFN-γR1, one (177.10) was raised against full length IFN-γR1 but is known to block IFN-γ binding, suggesting it binds to the extracellular domain of IFN-γR1 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 aminoacids, 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 (Walter et al., 1995). Although the non-functional Y66C variant was expressed on the cell surface, it did not hamper the function of the IFN-γR when this variant was co-expressed with wild type IFN-γR1. Whether or not the Y66C variant is incorporated in the IFN-γR 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-γR1 is overexpressed, as is the case in our model, subtle differences in expression and the balance between the expression of the IFN-γR1 and the IFN-γR2 within our model may not resemble the normal expression pattern. In T lymphocytes IFN-γR1 and R2 expression can individually vary from low to high. For example, the expression density of the IFN-γR2 is an important mechanism in determining the fate of T lymphocytes (Bernabei et al., 2001). Therefore differences in IFN-γR1 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-γR1 have been reported to be associated with the production of high antibody titers against H. pylori (Thye et al., 2003) and susceptibility to allergic disease and the production of high IgE titers (Aoki et al., 2003). 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-γR1. 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-γR1 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-γR1 deficiency while V63G and I87T lead to partial IFN-γR1 deficiency.

References

Allende, L.M., Lopez-Goyanes, A., Paz-Artal, E., Corell, A., Garcia-Perez, M.A., Varela, P., Scarpellini, A., Negreira, S., Palenque, E., Arnaiz-Villena, A., 2001. A point mutation in a domain of γ interferon receptor 1 provokes severe immunodeficiency. Clin. Diagn. Lab Immunol. 8, 133–137.

Aoki, M., Matsui, E., Kaneko, H., Inoue, R., Fukao, T., Watanabe, M., Teramoto, T., Kato, Z., Suzuki, K., Suzuki, Y., Kasahara, K., Kondo, N., 2003. A novel single-nucleotide substitution, Leu 467 Pro, in the interferon-γ receptor 1 gene associated with allergic diseases. Int. J. Mol. Med. 12, 185–191.

Bernabei, P., Allione, A., Rigamonti, L., Bosticardo, M., Losana, G., Borghi, I., Forni, G., Novelli, F., 2001. Regulation of interferon-γ receptor (INF-γR) chains: a peculiar way to rule the life and death of human lymphocytes. Eur. Cytokine Netw. 12, 6–14.

Boehm, U., Klamp, T., Groot, M., Howard, J.C., 1997. Cellular responses to interferon γ. Annu. Rev. Immunol. 15, 749–795.

Chantrain, C.F., Bruwier, A., Brichard, B., Largent, V., Chapgier, A., Feinberg, J., Casanova, J.L., Stalens, J.P., Vermylen, C., 2006. Successful hematopoietic stem cell transplantation in a child with active disseminated Mycobacterium fortuitum infection and interferon-γ receptor 1 deficiency. Bone Marrow Transplant. 38, 75–76.

de Paus, R.A., van de Wetering, D., Van Dissel, J.T., van de Vosse, E., 2008. IL-23 and IL-12 responses in activated human T cells retrovirally transduced with IL-23 receptor variants. Mol. Immunol. 45, 3889–3895.

Dorman, S.E., Picard, C., Lammas, D., Heyne, K., Van Dissel, J.T., Baretto, R., Rosenzweig, S.D., Newport, M., Levin, M., Roesler, J., Kumararatne, D., Casanova, J.L., Holland, S.M., 2004. Clinical features of dominant and recessive interferon γ receptor 1 deficiencies. Lancet 364, 2113–2121.

Garotta, G., Ozmen, L., Fountoulakis, M., Dembic, Z., van Loon, A.P., Stuber, D., 1990. Human interferon-γ receptor. Mapping of epitopes recognized by neutralizing antibodies using native and recombinant receptor proteins. J. Biol. Chem. 265, 6908–6915.

Glosli, H., Stray-Pedersen, A., Brun, A.C., Holtmon, L.W., Tonjum, T., Chapgier, A., Casanova, J.L., Abrahamsen, T.G., 2008. Infections due to various atypical mycobacteria in a Norwegian multiplex family with dominant interferonγ receptor deficiency. Clin. Infect. Dis. 46, e23–e27.

Heemskerk, M.H., Blom, B., Nolan, G., Stegmann, A.P., Bakker, A.Q., Weijer, K., Res, P.C., Spits, H., 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. J. Exp. Med. 186, 1597–1602.

Higuchi, R., Krummel, B., Saiki, R.K., 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. 16, 7351–7367.

Jouanguy, E., Altare, F., Lamhamedi, S., Revy, P., Emile, J.F., Newport, M., Levin, M., Blanche, S., Seboun, E., Fischer, A., Casanova, J.L., 1996. Interferon-γreceptor deficiency in an infant with fatal bacille Calmette-Guerin infection. N. Engl. J. Med. 335, 1956–1961.

Jouanguy, E., Lamhamedi-Cherradi, S., Altare, F., Fondaneche, M.C., Tuerlinckx, D., Blanche, S., Emile, J.F., Gaillard, J.L., Schreiber, R., Levin, M., Fischer, A., Hivroz, C., Casanova, J.L., 1997. Partial interferon-γ receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guerin infection and a sibling with clinical tuberculosis. J Clin. Invest. 100, 2658–2664.

Jouanguy, E., Lamhamedi-Cherradi, S., Lammas, D., Dorman, S.E., Fondaneche, M.C., Dupuis, S., Doffinger, R., Altare, F., Girdlestone, J., Emile, J.F., Ducoulombier, H., Edgar, D., Clarke, J., Oxelius, V.A., Brai, M., Novelli, V., Heyne, K., Fischer, A., Holland, S.M., Kumararatne, D.S., Schreiber, R.D., Casanova, J.L., 1999. A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. Nat. Genet. 21, 370–378.

Jouanguy, E., Dupuis, S., Pallier, A., Doffinger, R., Fondaneche, M.C., Fieschi, C., Lamhamedi-Cherradi, S., Altare, F., Emile, J.F., Lutz, P., Bordigoni, P., Cokugras, H., Akcakaya, N., Landman-Parker, J., Donnadieu, J., Camcioglu, Y., Casanova, J.L., 2000. In a novel form of IFN-γ receptor 1 deficiency, cell surface receptors fail to bind IFN-γ. J. Clin. Invest. 105, 1429–1436.

Kaplan, D.H., Shankaran, V., Dighe, A.S., Stockert, E., Aguet, M., Old, L.J., Schreiber, R.D., 1998. Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice. Proc. Natl. Acad. Sci. U.S.A. 95, 7556–7561.

Klausner, R.D., Sitia, R., 1990. Protein degradation in the endoplasmic reticulum. Cell 62, 611–614.

Leenen, P.J., Canono, B.P., Drevets, D.A., Voerman, J.S., Campbell, P.A., 1994. TNF-α and IFN-γ stimulate a macrophage precursor cell line to kill Listeria monocytogenes in a nitric oxide-independent manner. J. Immunol. 153, 5141–5147.

Nakao, F., Ihara, K., Kusuhara, K., Sasaki, Y., Kinukawa, N., Takabayashi, A., Nishima, S., Hara, T., 2001. Association of IFN-γ and IFN regulatory factor 1 polymorphisms with childhood atopic asthma. J. Allergy Clin. Immunol. 107, 499–504.

Nakashima, H., Inoue, H., Akahoshi, M., Tanaka, Y., Yamaoka, K., Ogami, E., Nagano, S., Arinobu, Y., Niiro, H., Otsuka, T., Niho, Y., 1999. The combination of polymorphisms within interferon-γ receptor 1 and receptor 2 associated with the risk of systemic lupus erythematosus. FEBS Lett. 453, 187–190.

Nathan, C.F., Hibbs Jr., J.B., 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr. Opin. Immunol. 3, 65–70.

Newport, M.J., Huxley, C.M., Huston, S., Hawrylowicz, C.M., Oostra, B.A., Williamson, R., Levin, M., 1996. A mutation in the interferon-γ-receptor gene and susceptibility to mycobacterial infection. N. Engl. J. Med. 335, 1941–1949.

Noordzij, J.G., Hartwig, N.G., Verreck, F.A., De Bruin-Versteeg, S., de Boer, T., Dissel, J.T., De Groot, R., Ottenhoff, T.H., Van Dongen, J.J., 2007. Two patients with complete defects in interferon γ receptor-dependent signaling. J. Clin. Immunol. 27, 490–496.

Novick, D., Fischer, D.G., Reiter, Z., Eshhar, Z., Rubinstein, M., 1989. Monoclonal antibodies to the human interferon-γ receptor: blocking of the biological activities of interferon-γ and purification of the receptor. J. Interferon Res. 9, 315–328.

Okada, S., Ishikawa, N., Shirao, K., Kawaguchi, H., Tsumura, M., Ohno, Y., Yasunaga, S., Ohtsubo, M., Takihara, Y., Kobayashi, M., 2007. The novel IFNGR1 mutation 774del4 produces a truncated form of interferon-γ receptor 1 and has a dominant-negative effect on interferon-γ signal transduction. J. Med. Genet. 44, 485–491.

Ottenhoff, T.H., Verreck, F.A., Lichtenauer-Kaligis, E.G., Hoeve, M.A., Sanal, O., Van Dissel, J.T., 2002. Genetics, cytokines and human infectious disease: lessons from weakly pathogenic Mycobacteria and Salmonellae. Nat. Genet. 32, 97–105.

Pierre-Audigier, C., Jouanguy, E., Lamhamedi, S., Altare, F., Rauzier, J., Vincent, V., Canioni, D., Emile, J.F., Fischer, A., Blanche, S., Gaillard, J.L., Casanova, J.L., 1997. Fatal disseminated Mycobacterium smegmatis infection in a child with inherited interferon γ receptor deficiency. Clin. Infect. Dis. 24, 982–984.

Platanias, L.C., Fish, E.N., 1999. Signaling pathways activated by interferons. Exp. Hematol. 27, 1583–1592.

Ramana, C.V., Gil, M.P., Schreiber, R.D., Stark, G.R., 2002. Stat1-dependent and independent pathways in IFN-γ-dependent signaling. Trends Immunol. 23, 96–101.

Remiszewski, P., Roszkowska-Sliz, B., Winek, J., Chapgier, A., Feinberg, J., Langfort, R., Bestry, I., Augustynowicz-Kopec, E., Ptak, J., Casanova, J.L., Rowinska-Zakrzewska, E., 2006. Disseminated Mycobacterium avium infection in a 20-year-old female with partial recessive IFNγR1 deficiency. Respiration 73, 375–378.

Roesler, J., Kofink, B., Wendisch, J., Heyden, S., Paul, D., Friedrich, W., Casanova, J.L., Leupold, W., Gahr, M., Rosen-Wolff, A., 1999. Listeria monocytogenes and recurrent mycobacterial infections in a child with complete γ-receptor (IFN-γR1) deficiency: mutational analysis and evaluation of therapeutic options. Exp. Hematol. 27, 1368–1374.

Rosenzweig, S., Dorman, S.E., Roesler, J., Palacios, J., Zelazko, M., Holland, S.M., 2002. 561del4 defines a novel small deletion hotspot in the interferon-γ receptor 1 chain. Clin. Immunol. 102, 25–27.

Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A., 2004. Interferon-γ: an overview of signals, mechanisms and functions. J. Leukoc. Biol. 75, 163–189.

Shang, X.Z., Issekutz, A.C., 1998. Contribution of CD11a/CD18, CD11b/CD18, ICAM-1 (CD54) and -2 (CD102) to human monocyte migration through endothelium and connective tissue fibroblast barriers. Eur. J. Immunol. 28, 1970–1979.

SNP database, build: 130, 2006. http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp&cmd=search&term=rs17175322.

Tanaka, Y., Nakashima, H., Hisano, C., Kohsaka, T., Nemoto, Y., Niiro, H., Otsuka, T., Otsuka, T., Imamura, T., Niho, Y., 1999. Association of the interferon-γ receptor variant (Val14Met) with systemic lupus erythematosus. Immunogenetics 49, 266–271.

Thye, T., Burchard, G.D., Nilius, M., Muller-Myhsok, B., Horstmann, R.D., 2003. Genomewide linkage analysis identifies polymorphism in the human interferon-γ receptor affecting Helicobacter pylori infection. Am. J. Hum. Genet. 72, 448–453.

Valente, G., Ozmen, L., Novelli, F., Geuna, M., Palestro, G., Forni, G., Garotta, G., 1992. Distribution of interferon-γ receptor in human tissues. Eur. J. Immunol. 22, 2403–2412.

van Boxel-Dezaire, A.H., Stark, G.R., 2007. Cell type-specific signaling in response to interferon-γ. Curr. Top. Microbiol. Immunol. 316, 119–154.

van de Stolpe, A., van der Saag, P.T., 1996. Intercellular adhesion molecule-1. J. Mol. Med. 74, 13–33.

van de Vosse, E., Hoeve, M.A., Ottenhoff, T.H., 2004. Human genetics of intracellular infectious diseases: molecular and cellular immunity against Mycobacteria and Salmonellae. Lancet Infect. Dis. 4, 739–749.

van de Vosse, E., de Paus, R.A., Van Dissel, J.T., Ottenhoff, T.H., 2005. Molecular complementation of IL-12Rβ1 deficiency reveals functional differences between IL-12Rβ1 alleles including partial IL-12Rβ1 deficiency. Hum. Mol. Genet. 14, 3847–3855.

Walter, M.R., Windsor, W.T., Nagabhushan, T.L., Lundell, D.J., Lunn, C.A., Zauodny, P.J., Narula, S.K., 1995. Crystal structure of a complex between interferon-γ and its soluble high-affinity receptor. Nature 376, 230–235.

Watzka, H., Pfizenmaier, K., Moosmayer, D., 1998. Guided selection of antibody fragments specific for human interferon γ receptor 1 from a human VH-and VL-gene repertoire. Immunotechnology 3, 279–291.

CHAPTER 6

Effect of amino acid substitutions in the human IFN-γR2 on IFN-γ responsiveness

Roelof A. de Paus[1], Sara S. Kilic[2], Jaap T. van Dissel[1], Esther van de Vosse[1]

[1]Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands.
[2] Department of Pediatric Immunology, Uludag University School of Medicine, Bursa, Turkey.

Genes and Immunity 2011;12(2):136-44.

Abstract

Patients with IFN-γR null mutations have severe infections with poorly pathogenic *Mycobacteria.* The IFN-γR complex involves two IFN-γR1 and two IFN-γR2 chains, in which several amino acid substitutions, some linked to disease and some apparently naturally occurring, have been described. We developed a model system to study functional effects of genetic variations in IFN-γR2. We retrovirally transduced wild-type IFN-γR2 and IFN-γR2 carrying presently known amino acid substitutions, in various human cell lines and next determined the IFN-γR2 expression pattern as well as IFN-γ responsiveness. We determined that the T58R, Q64R, E147K and K182E variants of IFN-γR2 are fully functional, although the Q64R variant may be expressed higher on the cell membrane. The R114C, T168N and G227R variants were identified in patients that had disseminated infections with non-tuberculous *Mycobacteria*. Of these genetic variants, T168N was confirmed to be completely non-functional, whereas the novel variant G227R, and the previously reported R114C, were partial functional. The impaired IFN-γ responsiveness of R114C and G227R is mainly due to reduced receptor function, although expression on the cell membrane is reduced as well. We conclude that the T58R, Q64R, E147K, and K182E variants are polymorphisms, while the R114C, T168N and G227R constitute mutations associated with disease.

1. Introduction

Deficiency in the interferon-γ receptor (IFN-γR) leads to susceptibility to infections with poorly pathogenic *Mycobacteria*.[1,2] Individuals with such a deficiency are among the small number of persons with "Mendelian Susceptibility to Mycobacterial Disease" (MSMD) that are prone to develop infection by low pathogenic non-tuberculous *Mycobacteria* and *Salmonellae*. Upon encounter with these bacteria, antigen presenting cells will stimulate natural killer (NK) cells and next type-1 helper T (Th1) cells to produce IFN-γ. IFN-γ is a pleiotropic cytokine which upregulates proteasome complexes, various pro-inflammatory cytokines, chemokines and cell surface molecules. At the same time, IFN-γ downregulates the production of the anti-inflammatory cytokine IL-10. By consequence, IFN-γ responses result in enhanced cytotoxicity of NK cells, stimulation of antigen presentation, maturation of B-lymphocytes, expansion of Th1 lymphocytes and induction of apoptosis in Th2 cells.[3-6]

IFN-γ mediates immune responses via the IFN-γ receptor (IFN-γR), which is comprised of two ligand-binding IFN-γR1 chains associated with two signal-transducing IFN-γR2 chains. Binding of IFN-γ to its receptor induces activation of the receptor-associated Janus kinases JAK1 and JAK2 by trans-phosphorylation,[7] which in turn phosphorylate STAT1 on its Tyrosine 701 residue.[8] Phosphorylated STAT1 dissociates from the receptor, dimerizes and translocates to the cell nucleus, where it regulates gene expression either directly (e.g. CD54, CXCL10 and CCL2)[9-11] or indirectly via the induction of transcription factors such as IRFs and CIITA (e.g. IL12B, B2M and MHC).[12,13] Although STAT1 is the main mediator of IFN-γ responses, IFN-γ has also been reported to induce STAT3 or STAT5 phosphorylation.[14] Besides the JAK2 binding site, the intracellular domain of IFN-γR2 contains a BAX inhibiting domain[15] and an internalization motif[16] (Figure 1).

Amino acid variations in the IFN-γR1 or in the R2 chain can influence IFN-γ responses. Besides null mutations some additional amino acid variations of uncertain consequence were found in the IFN-γR. Recently, we determined the impact of naturally occurring amino acid variations on the function of the IFN-γR1.[17] In the IFN-γR2 chain amino acid variations have similarly been reported in both patients as well as healthy individuals (Figure 1). For instance, an R114C variation was identified in a patient with mycobacterial disease and strongly impaired IFN-γ responses in vitro.[18] The T168N mutation results in a gain of glycosylation and complete loss of function.[19] A novel variation, G227R, was recently found in a MSMD patient (S.S. Kilic, manuscript in preparation). Variations reported in healthy individuals are T58R (population allele frequency 0~18%), Q64R (9~57%), E147K (0~2%) and K182E (0~4%) (NCBI, SNP database). Previous studies suggested an association between the Q64R polymorphism and immune related disease: for instance, the 64Q allele was described as a risk factor for systemic lupus erythematosis (SLE),[20] while in patients with multiple sclerosis the 64R allele correlated with a progressive onset of disease.[21] Furthermore, the 64Q allele

was weakly associated with low serum IgE levels.[22] The exact impact of the variations on IFN-γR2 function, with the exception of the T168N mutation, is unknown.

In this study, we analysed the impact of previously described amino acid variations in IFN-γR2 on receptor expression and function. We cloned the cDNA from wild type IFN-γR2 and its variants into a retroviral vector and transduced these into several human cell lines. In this way the variants could be compared functionally within the same genetic background. The transductants were tested for both receptor expression as well as function.

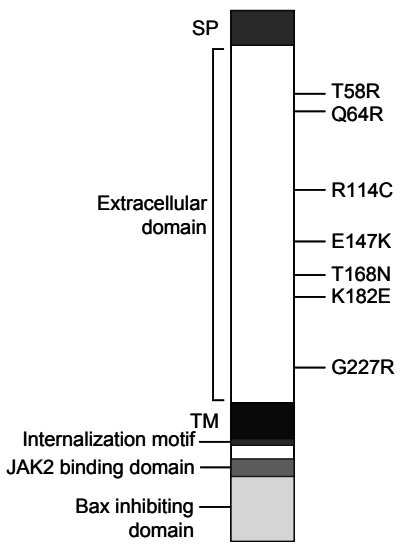


Figure 1. Schematic presentation of IFN-γR2.
On the left the various domains are indicated, on the right the amino acid variations that were analysed are indicated. TM=transmembrane domain, SP=signal peptide, C=Cysteine, E=Glutamic acid, G=Glycine, K=Lysine, N=Asparagine, Q=Glutamine, R=Arginine, T=Threonine.

2. Materials and Methods

2.1. Cloning IFNGR2 variants into a retroviral expression vector

The full-length *IFNGR2* coding sequence was PCR amplified from cDNA of a healthy control with the sense primer 5`-AATTGGATCCCGGGGCCATGCGACCGAC-3` and the anti-sense primer 5`-CCGGCTCGAGTTCAAAGCGTTTGGAGAACAT-3` and cloned into the retroviral vector pLZRS-IRES-GFP[26] as previously described.[27] Variations were introduced by site directed mutagenesis.[28] All constructs were sequence verified and were transfected in the Phoenix-A[29] packaging cell line using a calciumphosphate transfection kit (Invitrogen, Breda, The Netherlands). The virus producing cells were cultured for 2–3 weeks under 2 μg/ml puromycin (Clontech, Saint-Germain-en-Laye, France) selection after which a 20 h supernatant was harvested. *IFNGR1* constructs were previously made and described.[17]

2.2. Cell culture and retroviral transduction

Human Jurkat (T lymphoblastic cell line, ATCC TIB-152), THP-1 (myeloid cell line, ATCC TIB-202) and G1A cells[30] (fibroblasts, kind gift from George Stark) were cultured in RPMI1640 medium. B-LCL and T cell blasts (TCB) were cultured in IMDM (Lonza, Vervier, Belgium) as described before.[27] Culture media were supplemented with 10% FCS, 20 mM GlutaMax, 100 U/ml Penicillin and 100 µg/ml Streptomycin (Invitrogen). For propagation the adherent G1A cells were trypsinized in the presence of EDTA. Prior to testing, G1A cells were collected using only EDTA (Invitrogen). $0.25*10^6$ THP-1 or TCB were retrovirally transduced on a CH-296 (RetroNectin®, Takara, Saint-Germain-en-Laye, France) coated 48 wells plate by adding 1 ml of virus containing supernatant. $0.5*10^6$ B-LCL, Jurkat and G1A cells were transduced by adding 1 ml virus containing supernatant supplemented with 10 µl DOTAP (Roche, Almere, The Netherlands) to the cells. After overnight incubation the cells were washed and cultured for at least four days before analysing in further assays.

2.3. FACS analysis of IFN-γR2 expression

To detect IFN-γR2 membrane expression cells were stained with an APC conjugated polyclonal antibody BAF773 (R&D Systems, Abingdon, UK), with PE conjugated monoclonal 2HUB159, 2HUB145 (SantaCruz, Heidelberg, Germany), with monoclonal MMHGR-2 (PBL, New Brunswick, NJ- USA) and counterstained with goat-anti-mouse IgG-PE (BD biosciences, Breda, The Netherlands), or stained with biotin conjugated monoclonal MHCD119B25 (DAKO, Heverlee, Belgium) and counterstained with streptavidin-PE (BD biosciences). As a negative control the cells were stained with an IgG1 antibody. After staining, the cells were washed twice in PBS with 0.2% BSA (Sigma, Zwijndrecht, The Netherlands). All subsequent FACS measurements and analyses, using a FACSCalibur and CellQuest (BD Biosciences), were performed on cells gated for equal GFP expression. In order to detect total (intra- and extracellular) IFN-γR2 expression, cells were treated with 4% paraformaldehyde and 0.1% saponin (Sigma) prior to staining with APC conjugated BAF773.

2.4. STAT phosphorylation assay

To study signal transduction, $2*10^5$ G1A cells or TCB in 200 µl of culture medium were pulsed for 0 to 30 minutes with various concentrations of IFN-γ (Invitrogen) in 96-wellsplates (Greiner bio-one). The cells were fixated with 4% paraformaldehyde and permeabilised with 90% methanol (Merck, Amsterdam, The Netherlands). Subsequently, the cells were washed with PBS containing 0.2% BSA (Sigma), blocked with 10% normal goat serum (Sanquin, Amsterdam, The Netherlands), and stained with the phospho-specific antibody pY701-STAT1-Alexa 647, pY705-STAT3-PE or pY694-STAT5-PE (BD Biosciences). Before analysis by FACS the cells were washed twice.

2.5. IFN-γ response assays

To study the upregulation of cell surface markers $0.25*10^6$ G1A cells were stimulated in 24 Wellsplates (Greiner bio-one) for 20 h in 1 ml of culture medium with various concentrations of IFN-γ. Afterwards the cells were washed in medium and stained with PE-conjugated antibodies against CD54, CD64, HLA-DR or stained with the W6.32 antibody against HLA Class I and counterstained with GAM-PE (BD biosciences). The cells were washed twice before FACS measurements and analyses were performed on G1A cells gated for relatively low GFP expression.

Prior to cytokine production assays G1A cells were sorted by FACS for low GFP expression using a FACSAria (BD biosciences). $0.1*10^5$ G1A cells were cultured in 0.2 ml of culture medium and pulsed with various concentrations of IFN-γ (Invitrogen) using microlon plates (Greiner bio-one). After 48 hours cell free supernatants were collected and tested for the presence of CXCL10 with a specific ELISA (Invitrogen).

3. Results

3.1. Functional transfer of wild type IFNGR2 into cell lines

In order to set up a model system we cloned wild-type IFN-γR2 cDNA into the retroviral expression vector pLZRS-GFP. The use of pLZRS-GFP allows for tandem expression of IFN-γR2 and green fluorescent protein (GFP). We expressed the receptor in various human cell lines: B-LCL, THP-1, Jurkat, TCB and IFN-γR2 deficient fibroblastic G1A cells. The cell surface expression of IFN-γR2 was determined by FACS with five distinct anti-IFN-γR2 antibodies. We did not detect natural expression of IFN-γR2 on untransduced or GFP-transduced cells with any of the antibodies (data not shown). With the BAF773 antibody we detected retrovirally achieved overexpression of wild-type IFN-γR2 in all cell lines tested (Figure 2A), whereas the other four antibodies failed to detect expression of wild-type IFN-γR2 (data not shown). Cotransduction of IFN-γR1 did not influence detection of IFN-γR2 expression and vice versa (data not shown).

Next, we tested the IFN-γ responsiveness of the transduced cells by studying the intracellular signalling. The kinetics of STAT1 phosphorylation was determined by FACS. After 0 to 30 minutes of incubation with various concentrations of IFN-γ the STAT1 phosphorylation in the cells was measured using a phospho-specific antibody against pY701-STAT1. IFN-γ induced STAT1 phosphorylation in untransduced THP-1 cells and to a lesser extent in B-LCL. This indicates that, although there was no detectable natural expression of IFN-γR2 on these cells, there is a small amount of functional IFN-γR2 present. IFN-γ did not induce STAT1 phosphorylation in untransduced Jurkat, G1A and TCB cells. IFN-γR2 transduced B-LCL and Jurkat cells showed just a slight increase in the amount of

phosphorylated STAT1 in response to IFN-γ (data not shown). IFN-γR2 transduced G1A and TCB cells showed an IFN-γ-dose dependent increase in STAT1 phosphorylation upon IFN-γ stimulation (Figure 3A and 3B). The STAT1 phosphorylation in G1A (Figure 3A) and in TCB (Figure 3B) with the wild type IFN-γR2 was at maximum at 15 minutes and was strongest at the highest concentration of IFN-γ. No induction of STAT3 and STAT5 was observed in IFN-γR2 transduced B-LCL, Jurkat, TCB and G1A (data not shown).

3.2. Differential expression patterns of the IFN-γR2 variants

One novel variation (G227R), two known mutations (R114C, T168N), and two common (T58R, Q64R) and two rare variations (E147K and K182E) in IFN-γR2 were cloned and retrovirally expressed in the human cell lines: B-LCL, THP-1, Jurkat, TCB and G1A. Next, we determined the expression of the IFN-γR2 variants on the cell surface by FACS, on cells gated for equal GFP expression, with five anti-IFN-γR2 antibodies.

With the antibody BAF773 (Figure 2A) we detected cell surface expression on cells transduced with either wild-type IFN-γR2 or with the variants. Expression patterns, however, varied amongst the cell lines. For instance, the Q64R variant showed, in all experiments and in all cell lines, the highest detection signal as compared with all other variants tested. In G1A cells the expression of wild- type IFN-γR2 was similar to that of the variants T58R, R114C, E147K, T168N, K182E or G227R. By contrast, TCB cells showed a reduced expression of R114C and G227R, whereas the expression of T58R, E147K, T168N and K182E was again similar to wild-type IFN-γR2. TCB, B-LCL, THP-1 and Jurkat cells showed similar expression patterns of the IFN-γR2 variants, except that the expression of G227R on B-LCL and THP-1 varied between experiments from 29% to 123% of the expression level of wild-type IFN-γR2. Of note, the expression of R114C on one of the six TCB lines tested, TCB-5, was significantly reduced as compared to the expression of R114C on the other TCB lines ($p=0.001$, student t-test) (data not shown).

Remarkably, of the four antibodies (2HUB159, 2HUB145, MMHGR-2, MHCD119B25) that failed to detect expression of wild-type receptor, 2HUB159 was able to detect cell surface expression of R114C and G227R on Jurkat, TCB and G1A cells (Figure 2B). Just like the wild-type, the other variants could not be detected by any of these four antibodies (Figure 2B and data not shown).

Differences in detection of cell surface expression might be ascribed to an altered affinity of the antibody caused by an amino acid variation or to an actual difference in the amount of protein present on the cell membrane. Differences in amount of protein can be due to differences in protein production, in protein stability, or in misfolding followed by sequestration and degradation of the protein in the endoplasmic reticulum. To determine whether the IFN-γR2 variants are present in equal amounts within the cell but differential expressed on the external cell surface, we analysed the total (intra- and extracellular)

expression by staining TCB with BAF773 after permeabilisation of the cell membrane. In this way comparable amounts of proteins were detected for R114C, G227R, T58R, E147K, T168N, K182E and the wild type variant (Figure 2C). In contrast, the signal of Q64R was about 75% stronger as compared to the other variants.

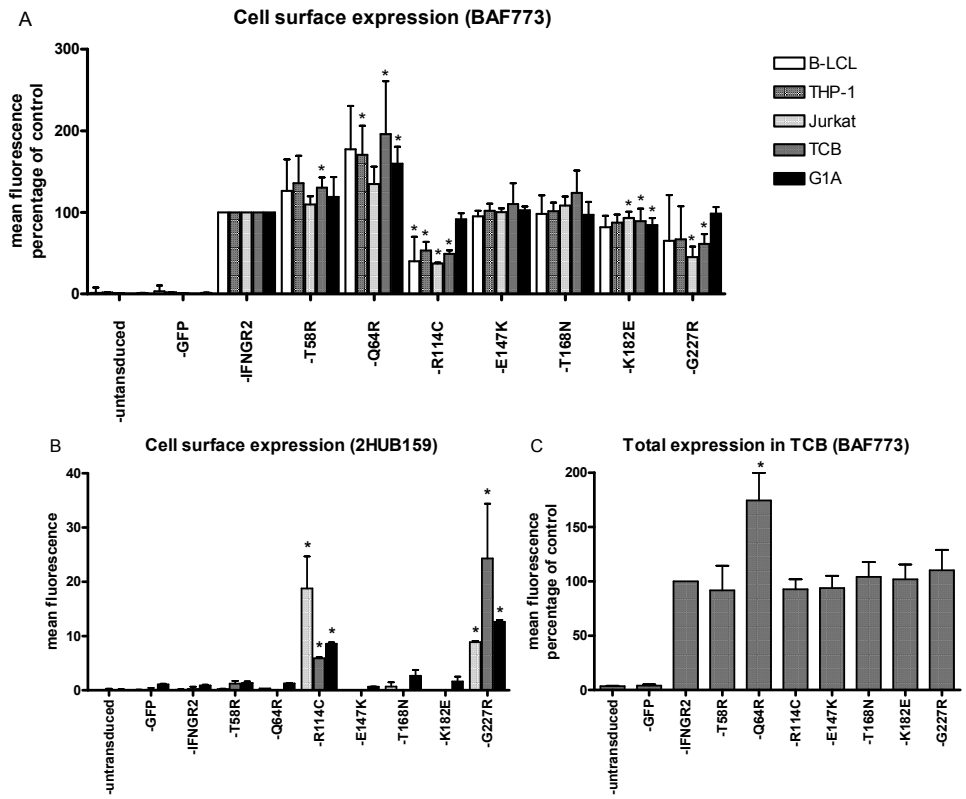


Figure 2. Extracellular and total expression of the IFN-γR2 variants.
(A) Extracellular expression of IFN-γR2 was measured on B-LCL (n=3), THP-1 (n=3), Jurkat (n=5), TCB (n=6) and G1A (n=3) cells that were untransduced, transduced with GFP alone or transduced with one of the *IFNGR2* gene variants by FACS with the antibody BAF773. **(B)** Extracellular expression of IFN-γR2 was also measured on Jurkat, TCB, G1A cells that were untransduced, transduced with GFP alone or with one of the *IFNGR2* gene variants with the 2HUB159 antibody. Experiment in triplo. **(C)** The total, intra- and extracellular, expression of IFN-γR2 in two TCB cell lines was measured after fixation and permeabilization of the cells. For all experiments the expression of IFN-γR2 was determined for the transduced cells with equal GFP expression. The mean fluorescence ± standard deviation (SD) is displayed. For A and C the mean fluorescence is depicted as percentage of the IFN-γR2 wild type variant, where the mean fluorescence of cells labelled with an isotype antibody was set at 0%. (* $p<0.05$, variant and the wild type expression were compared using a paired student t-test)..

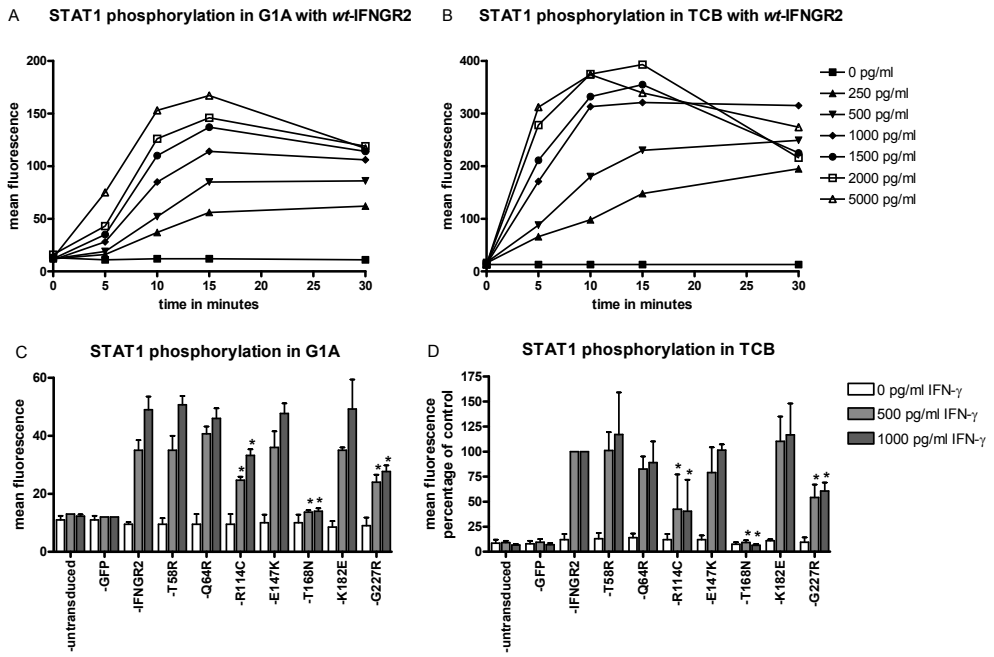


Figure 3. Kinetics of IFN-γ induced STAT1 phosphorylation and the influence of IFN-γR2 variations.
The amount of intracellular phosphorylated STAT1 was determined as a measure of IFN-γ induced signal transduction. The kinetics of STAT1 phosphorylation was first determined for **(A)** G1A and **(B)** TCB-4 cells transduced with wild-type IFN-γR2. Cells were stimulated for 0 to 30 minutes with various concentrations of IFN-γ. Next we studied the impact of the variations on IFN-γ signalling. **(C)** G1A and **(D)** TCB cells, untransduced, transduced with GFP alone or transduced with one of the IFN-γR2 variants were stimulated for 15 minutes with 500 or 1000 pg/ml IFN-γ. The amount of intracellular phosphorylated STAT1 was measured by FACS, using a phospho-specific antibody, in cells with equal GFP expression. For (C) the mean fluorescence ± SD of one representative out of two experiments is displayed. For (D) the mean fluorescence ± SD of four different TCB lines (each tested in triplo) was calculated as percentage of the IFN-γR2 wild type variant. (* $p<0.05$, variant and the wild type were compared using the student t-test).

3.3. Effect of IFN-γR2 variations on IFN-γ mediated signal transduction

Intracellular signalling via the IFN-γR complex is mediated by STAT1 molecules which are activated through tyrosine phosphorylation. We chose the TCB and G1A cells to study the functional effect of IFN-γR2 variants on STAT1 phosphorylation, because these cells are not responsive to IFN-γ, unless transduced with wild-type IFN-γR2 (Figure 3A and 3B). In untransduced and GFP transduced cells, no STAT1 phosphorylation could be detected after exposure of the cells to IFN-γ. G1A cells expressing wild-type IFN-γR2, T58R, Q64R, E147K or K182E showed similar STAT1 phosphorylation after IFN-γ exposure (Figure 3C). However, in G1A cells transduced with the T168N variant no IFN-γ induced STAT1 phosphorylation could be detected. G1A cells carrying the R114C or the G227R

variant showed diminished, intermediate levels of STAT1 phosphorylation (Figure 3C). All variants expressed in G1A cells, with the exception of T168N, displayed similar STAT1 phosphorylation plateau levels, after 60 to 90 minutes of stimulation with 500 pg/ml IFN-γ (data not shown).

The results obtained with the TCB cells (Figure 3D) did not differ from those obtained with the G1A cells. The reduction of STAT1 phosphorylation in TCB lines, transduced with R114C, varied from -34% to -98%. TCB-5 that was found to have the lowest cell surface expression of R114C (see previous section) also showed the lowest phosphorylation of STAT1.

3.4. Influence of IFN-γR2 variations on IFN-γ responses

IFN-γ stimulates, via STAT1 phosphorylation, the expression of a wide range of proteins. We tested the IFN-γ induced expression of CD54, CD64, HLA Class I and II in IFN-γR2 transduced TCB and G1A cells by FACS. Additionally, we tested the supernatants of these cells for the presence of CXCL10 by ELISA. Stimulation with IFN-γ of TCB carrying wild-type IFN-γR2 did not or, only slightly enhance, the expression of these proteins (data not shown). By contrast, G1A cells carrying the wild-type receptor showed significant upregulation of CD54, HLA Class I and CXCL10 after stimulation with 25 000 pg/ml of IFN-γ (data not shown). Subsequently, we tested the IFN-γR2 variants in G1A cells for IFN-γ-induced CD54 and HLA Class I upregulation and CXCL10 production. Untransduced and GFP transduced G1A cells did not respond to IFN-γ (Figure 4A-C). In G1A cells carrying the wild-type receptor or the T58R, Q64R, E147K or K182E variants, IFN-γ induced in a dose-dependent fashion the CD54 expression on the cell surface (Figure 4A). Cells transduced with R114C or G227R showed impaired responses; for instance in comparison to cells transduced with the wild-type receptor 8 (G227R) t o 100 times (R114C) as much IFN-γ needed to be added to the incubations to achieve the same level of CD54 expression.

Similar results were obtained when the G1A cells were analysed for HLA-Class I expression (Figure 4B) or the production of CXCL10 (Figure 4C). While induction of CD54 and HLA Class I expression could be detected after incubation of the cells with as little as 25 pg/ml IFN-γ, CXCL10 expression was induced only at concentrations of 2500 pg/ml IFN-γ or higher. G1A cells with T168N showed in four independent experiments almost a total lack of response, as a slight yet significant upregulation of cell surface expression of CD54 and HLA Class I was noted at the highest dose of IFN-γ only (Figure 4A and 4B). An overview of the results of all assays is presented in Table 1.

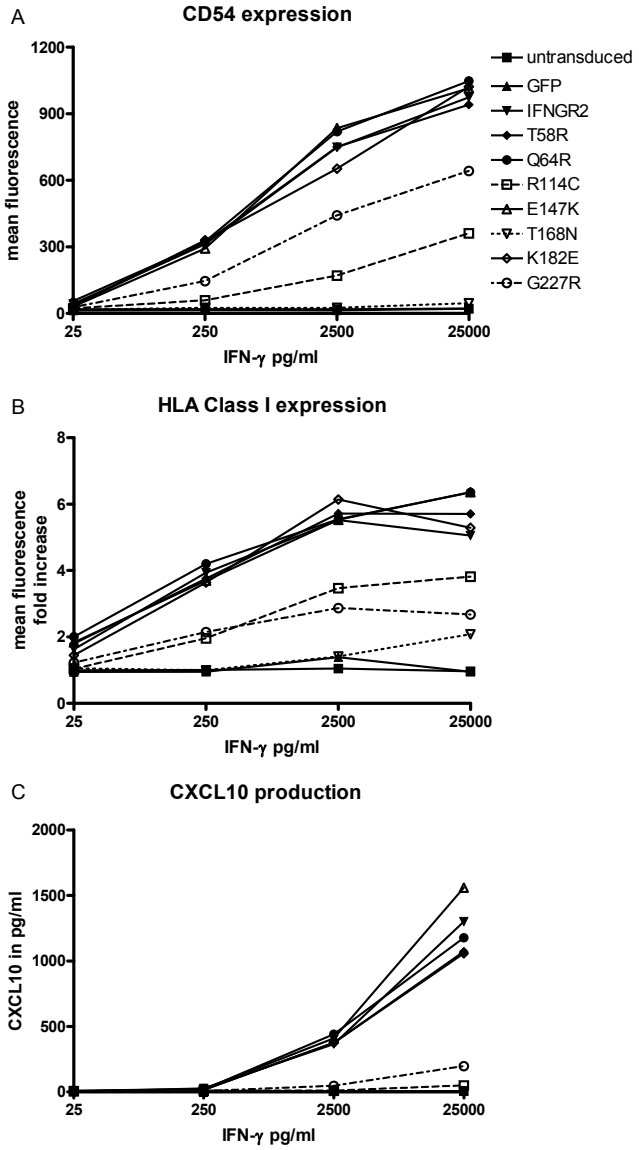


Figure 4. The effect of the IFN-γR2 variations on IFN-γ responses.
Untransduced G1A cells and G1A cells retrovirally transduced with GFP alone or with one of the IFN-γR2 variants were stimulated for 18 h (A and B) or 48 h (C) with various concentrations of IFN-γ. The CD54 expression (A) and the HLA Class I expression (B) were determined by FACS. The CXCL10 (C) production was determined by ELISA. One representative out of four (A) and one out of two (B and C) experiments are shown.

Table 1. Summary of the expression and functional analyses of the IFN-γR2 variants

Variant	Cell surface expression [a,b,c]	Total expression [a,b]	STAT1 phosphorylation [b,c]	Induction of CD54, HLA and CXCL10 [c]	Conclusion
wild-type	normal	normal	normal	normal	
T58R	slightly enhanced in TCB, normal in G1A	normal	normal	normal	polymorphism
Q64R	enhanced	enhanced	normal	normal	polymorphism
R114C	reduced in TCB, normal in G1A	normal	decreased	decreased	mutation, partial defect
E147K	normal	normal	normal	normal	polymorphism
T168N	normal	normal	not detected	nearly absent	null mutation, complete defect
K182E	normal in TCB, slightly reduced in G1A	normal	normal	normal	polymorphism
G227R	reduced in TCB, normal in G1A	normal	decreased	decreased	mutation, partial defect

[a] expression analysed with BAF773 antibody
[b] analyses with TCB
[c] analysis with G1A

3.5. No dominant negative effects of IFN-γR2 mutations

Since the IFN-γR consists of two IFN-γR1 and two R2 chains, incorporation of one defective chain in the IFN-γR could potentially have a dominant-negative effect on the receptor complex function. We determined whether the T168N, R114C or the G227R variants, which were partly or completely defective, could exert such an effect. To this end, we transduced the IFN-γ responsive cell line THP-1 with the T168N, R114C and G227R variants and as controls with wild-type IFN-γR2, wild-type IFN-γR1, a dominant negative IFN-γR1 mutant (IFN-γR1-delTTAA), or with an empty GFP vector. All THP-1 cells displayed induction of CD54 expression after stimulation with 2500 pg/ml IFN-γ. Overexpression of wild type IFN-γR1 or IFN-γR2 resulted in an upregulation of CD54 of respectively 48% and 59%, whereas overexpression of the G227R variant resulted only in 30% CD54 upregulation (Figure 5). The GFP, R114C and T168N transduced THP-1 cells were similarly responsive to IFN-γ, whereas the IFN-γR1-delTTAA transduced THP-1 cells showed a 60% decreased response (Figure 5). Thus, no dominant-negative effects of the IFN-γR2 variants were seen.

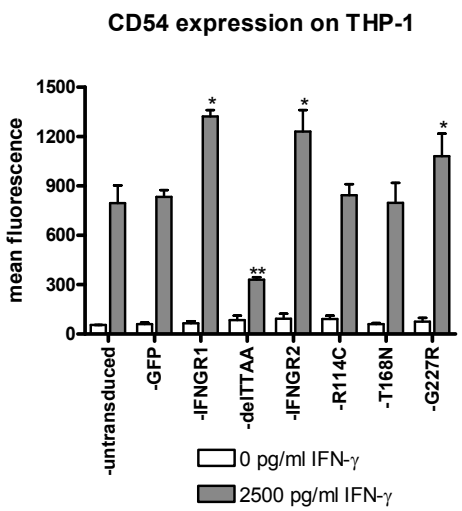


Figure 5. Influence of IFN-γR2 mutants on the function of the natural IFN-γ receptor complex. Normal and deficient IFN-γR1 and IFN-γR2 proteins were overexpressed in THP-1 cells to study their influence on the functioning of the native receptor IFN-γR complex. Untransduced THP-1 cells and THP-1 cells transduced with GFP, wild type IFN-γR2, R114C, T168N, G227R, wild type IFN-γR1 and the dominant negative delTTAA variant of IFN-γR1 were stimulated for 20 h with 2500 pg/ml IFN-γ. Hereafter the cells were analysed for CD54 expression by FACS. One representative of two experiments in triplo is shown. * significantly higher and ** significantly lower induction of CD54 expression as compared to the stimulation of THP-1 cells ($p<0.05$, student t-test).

4. Discussion

In an in vitro test model to analyse the functional impact of amino acid substitutions in IFN-γR2 we successfully characterized IFN-γR2 variants and could designate them as either innocuous polymorphisms or deleterious mutations affecting the function of the type-1 cytokine pathway to macrophage activation. We could confirm the complete loss of function of IFN-γR2 due to the T168N variation. The R114C and the G227R variations, described previously in MSMD patients, were shown to constitute partially functional receptor chains. These findings have implications for the treatment of patients homozygous for R114C or G227R mutations, since in case of mycobacterial infection, they might still benefit from IFN-γ administration in addition to antibiotic treatment.[18] Moreover, we showed that the T58R, Q64R, E147K and K182E variants do not differ in function from the wild-type receptor and can therefore be considered functional polymorphisms.

Our conclusions, summarized in Table 1, are based on the findings obtained with an in vitro model wherein cells are retrovirally transduced with the respective IFN-γR2 variants. Employing this model system, we were able to distinguish normal from defective variants. In this respect, several aspects need consideration. First, IFN-γR2 is overexpressed after transduction by the retroviral vector and overexpression might mask subtle differences in receptor function. Still, we could define the impact of mutations that resulted in an intermediate phenotype of receptor expression and reduced receptor function, as opposed to a complete, null phenotype. Naturally and overexpressed IFN-γR2 may differ also in post-transcriptional or post-translational modification, although there are no indications that such modifications in fact occur. Second, all of the commercially available antibodies failed to detect native IFN-γR2 expression on THP-1 and primary monocytes. Previous research already indicated the inability of five antibodies (three of which we employed as well) to detect cell surface expression of native IFN-γR2.[23] Apparently, the expression of native IFN-γR2 is too low to detect. In our model system, however, we successfully detected IFN-γR2, when it was overexpressed, with the BAF773 antibody but not with the other antibodies. The use of other antibodies, recognising additional, distinct epitopes, would have helped to determine whether a difference in detection is due to a difference in affinity towards the variants or due to an actual difference in the amount of protein present. However, in the system with overexpression, we observed for most variants a comparable total (i.e., intracellular and cell surface) receptor expression, suggesting the affinity is not different. Third, the IFN-γ responses in the host are not confined to the cellular responses we assayed in this report. Although this has not been reported, it cannot be excluded that other read-out systems may be more sensitive to subtle differences in IFN-γR2 at lower IFN-γ concentrations. Our model allowed us, nevertheless, to detect highly significant induction of CD54, HLA Class I and CXCL10 when using physiologically relevant concentrations of IFN-γ.[24]

The variations R114C and G227R were homozygously present in MSMD patients and could be the cause of IFN-γ deficiency, as was previously shown for the T168N variation. The T168N variation was described as a mutation resulting in a complete loss of function due to gain of glycosylation at the asparagine (N) residue.[25] We could confirm the severe impact of the T168N variant on IFN-γR2 function. When T168N was overexpressed, some upregulation of CD54 and HLA Class I occurred at the highest concentration of IFN-γ used, and this response is considered to be marginal only. For instance, the latter finding might indicate that a very small portion of the T168N variant is not fully glycosylated, since deglycosylation of the receptor can restore receptor function.[25] If the expression level of T168N is brought down to a physiological level, this effect may be absent. In all, we confirm the observation that the T168N variation results in a complete null mutation. The G227R variation, which was recently observed in a MSMD patient (S.S. Kilic, manuscript in preparation), appears to be a severe but not completely null mutation. We found that the cell surface expression of G227R on TCB was reduced, although the total expression in the cell was normal. The cell surface expression of G227R on G1A cells was normal. Both TCB and G1A transduced with G227R showed, however, a strongly reduced responsiveness to IFN-γ. Thus we conclude that G227R is a mutation leading to a partial receptor defect due to reduced functioning of the receptor and that G227R also results in a moderate loss of cell surface expression, which may depend on cell type or state of activation. The same conclusion is drawn for the R114C variation, which was first described in a patient who responded poorly to IFN-γ.[18] The IFN-γ responsiveness of the patient's cells could be restored in vitro by transfection with a vector carrying cDNA from *IFNGR2*, indicating a defect in IFN-γR2.[18] Our results further demonstrate that R114C is the cause of this defect. In our experiments R114C and G227R showed comparable reductions on IFN-γR2 expression and function.

Some differences in expression patterns of IFN-γR2 could be distinguished. The expression of both R114C and G227R was reduced in TCB, but not in G1A. We also found that the expression of G227R in B-LCL and THP-1 varied between experiments. Additionally, we found that one of the six TCB lines, TCB-5, transduced with the R114C variant showed a highly significant reduced expression as compared to the other TCB lines carrying R114C. Taken together these findings suggest that the impact of the partial mutations depends on cell type, culture state and on individual host specific factors.

The 2HUB159 antibody we used failed to detect retroviral expression of wild type IFN-γR2. 2HUB159 was raised against an unspecified fragment of the extracellular domain of the receptor and possibly recognises the receptor in a denatured, e.g. in blotting experiments, rather than natural conformation. Remarkably, we could detect cell surface expression of R114C and G227R with the 2HUB159 antibody. Therefore, the R114C and G227R variants are likely to result in an altered conformation of the receptor, as compared to wild-type IFN-γR2, and this may contribute to the reduced receptor function. Although the R114 and G227 are in two different sites of the extracellular domain, within the three dimensional

structure these could be located close together. The crystal structure of IFN-γR2 has not been elucidated yet, the putative secondary structure elements identified by Krause *et al*[7] suggest however that R114 and G227 may well be in close proximity after folding of the receptor.

We showed that the R114C, T168N and G227R mutations do not have a dominant negative effect on the function of the IFN-γ receptor complex. This is in line with the observation that heterozygous family members carrying one mutated allele are not more prone to develop *Mycobacterial* infections[18,25] and in vitro the cells, from individuals heterozygous for the G227R mutation, do not show impaired responses in immunological analyses (S.S. Kilic, manuscript in preparation).

The variants T58R, Q64R, E147K and K182E constitute fully functional polymorphisms of the IFN-γR2. Although we could detect minor differences in cell surface expression, all IFN-γR2 variants displayed a similar IFN-γ response. The cell surface as well as total cellular expression of Q64R, as determined with the BAF773 antibody, was higher in all cell lines tested as compared to the other variants. Since the expression was determined in cells gated for equal GFP expression and the *IFNGR2* and *GFP* genes are transcribed in tandem thus ensuring that equal amounts of transcripts are present, we expect that an equal amount of protein is translated of the wild-type IFN-γR2 and Q64R. An explanation may be that the Q64R variant is more stable, resulting in higher expression. Another explanation may be that the BAF773 antibody binds with higher affinity to the Q64R variant. Finally, higher expression of the Q64R variant is consistent with suggestions in the literature that this variant might be more responsive to IFN-γ. First, in agreement with the notion of a somewhat altered function, the 64R allele was overrepresented in patients with multiple sclerosis having a more progressive onset of the disease, in which Th1 immune response plays a role.[21] Second, the 64Q allele was positively associated with the occurrence of SLE, which is considered to be a Th2 disease.[20] And third, in a British population the 64Q allele showed a weak association with low IgE levels.[22] Taken together, altered cell surface expression of IFN-γR2 due to the Q64R polymorphism, may influence the strength of IFN-γ responses and by consequence the polarization of T cells towards Th1 or Th2.[6] More population association studies will be necessary to confirm an association of the common Q64R polymorphism with Th1 and Th2 mediated disease. Research on the expression pattern of IFN-γR2 variants at the cellular level urgently needs the characterization of a wider array of IFN-γR2 detecting antibodies.

We conclude that T58R, Q64R, E147K and K182E are fully functional polymorphisms of IFN-γR2. The R114C, T168N and G227R variations are deleterious mutations with T168N leading to a complete IFN-γR2 deficiency while R114C and G227R lead to a partial IFN-γR2 deficiency. The severely reduced IFN-γ responsiveness of R114C and G227R is mainly due to reduced receptor function.

References

1 Ottenhoff TH, Verreck FA, Lichtenauer-Kaligis EG, Hoeve MA, Sanal O, van Dissel JT. Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. *Nat Genet* 2002; **32**: 97-105.

2 van de Vosse E, Hoeve MA, Ottenhoff TH. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. *Lancet Infect Dis* 2004; **4**: 739-49.

3 Valente G, Ozmen L, Novelli F, Geuna M, Palestro G, Forni G, et al. Distribution of interferon-γ receptor in human tissues. *Eur J Immunol* 1992; **22**: 2403-12.

4 Hu X, Chakravarty SD, Ivashkiv LB. Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. *Immunol Rev* 2008; **226**: 41-56.

5 Saha B, Jyothi PS, Chandrasekar B, Nandi D. Gene modulation and immunoregulatory roles of interferon γ. *Cytokine* 2010; **50**: 1-14.

6 Bernabei P, Allione A, Rigamonti L, Bosticardo M, Losana G, Borghi I, et al. Regulation of interferon-γ receptor (IFN-γR) chains: a peculiar way to rule the life and death of human lymphocytes. *Eur Cytokine Netw* 2001; **12**: 6-14.

7 Krause CD, Lavnikova N, Xie J, Mei E, Mirochnitchenko OV, Jia Y, et al. Preassembly and ligand-induced restructuring of the chains of the IFN-γ receptor complex: the roles of Jak kinases, Stat1 and the receptor chains. *Cell Res* 2006; **16**: 55-69.

8 Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. *Annu Rev Immunol* 1997; **15**: 749-95.

9 Tessitore A, Pastore L, Rispoli A, Cilenti L, Toniato E, Flati V, et al. Two γ-interferon-activation sites (GAS) on the promoter of the human intercellular adhesion molecule (ICAM-1) gene are required for induction of transcription by IFN-γ. *Eur J Biochem* 1998; **258**: 968-75.

10 Valente AJ, Xie JF, Abramova MA, Wenzel UO, Abboud HE, Graves DT. A complex element regulates IFN-γ-stimulated monocyte chemoattractant protein-1 gene transcription. *J Immunol* 1998; **161**: 3719-28.

11 Clarke DL, Clifford RL, Jindarat S, Proud D, Pang L, Belvisi MG, et al. TNFα and IFNγ synergistically enhance transcriptional activation of CXCL10 in human airway smooth muscle cells via STAT-1, NF-κB and the transcriptional coactivator CREB-binding protein. *J Biol Chem* 2010.

12 van den Elsen PJ, Holling TM, Kuipers HF, van der Stoep N. Transcriptional regulation of antigen presentation. *Curr Opin Immunol* 2004; **16**: 67-75.

13 Wang IM, Contursi C, Masumi A, Ma X, Trinchieri G, Ozato K. An IFN-γ-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. *J Immunol* 2000; **165**: 271-9.

14 van Boxel-Dezaire AH, Stark GR. Cell type-specific signaling in response to interferon-γ. *Curr Top Microbiol Immunol* 2007; **316**: 119-54.

15 Gomez JA, Sun W, Gama V, Hajkova D, Yoshida T, Wu Z, et al. The C-terminus of interferon γ receptor β chain (IFNγR2) has antiapoptotic activity as a Bax inhibitor. *Cancer Biol Ther* 2009; **8**: 1771-86.

16 Regis G, Conti L, Boselli D, Novelli F. IFNγR2 trafficking tunes IFNγ-STAT1 signaling in T lymphocytes. *Trends Immunol* 2006; **27**: 96-101.

17 van de Wetering D, de Paus RA, van Dissel JT, van de Vosse E. Functional analysis of naturally occurring amino acid substitutions in human IFN-γR1. *Mol Immunol* 2010; **47**: 1023-30.

18 Doffinger R, Jouanguy E, Dupuis S, Fondaneche M-C, Stephan J-L, Emile JF, et al. Partial interferon-γ receptor signaling chain deficiency in a patient with bacille Calmette-Guerin and Mycobacterium abscessus infection. *J Infect Dis* 2000; **181**: 379-84.

19 Vogt G, Bustamante J, Chapgier A, Feinberg J, Boisson DS, Picard C, et al. Complementation of a pathogenic IFNGR2 misfolding mutation with modifiers of N-glycosylation. *J Exp Med* 2008; **205**: 1729-37.

20 Nakashima H, Inoue H, Akahoshi M, Tanaka Y, Yamaoka K, Ogami E, et al. The combination of polymorphisms within interferon-γ receptor 1 and receptor 2 associated with the risk of systemic lupus erythematosus. *FEBS Lett* 1999; **453**: 187-90.

21 Schrijver HM, Hooper-van Veen T, van Belzen MJ, Crusius JB, Pena AS, Barkhof F, et al. Polymorphisms in the genes encoding interferon-γ and interferon-γ receptors in multiple sclerosis. *Eur J Immunogenet* 2004; **31**: 133-40.

22 Gao P-S, Mao X-Q, Jouanguy E, Pallier A, Doffinger R, Tanaka Y, et al. Nonpathogenic common variants of IFNGR1 and IFNGR2 in association with total serum IgE levels. *Biochem and Biophys Res Comm* 1999; **263**: 425-9.

23 Rosenzweig SD, Dorman SE, Uzel G, Shaw S, Scurlock A, Brown MR, et al. A novel mutation in IFN-γ receptor 2 with dominant negative activity: biological consequences of homozygous and heterozygous states. *J Immunol* 2004; **173**: 4000-8.

24 Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, Modlin RL. Cytokine production at the site of disease in human tuberculosis. *Infect Immun* 1993; **61**: 3482-9.

25 Vogt G, Chapgier A, Yang K, Chuzhanova N, Feinberg J, Fieschi C, et al. Gains of glycosylation comprise an unexpectedly large group of pathogenic mutations. *Nat Genet* 2005; **37**: 692-700.

26 Heemskerk MH, Blom B, Nolan G, Stegmann AP, Bakker AQ, Weijer K, et al. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J Exp Med* 1997; **186**: 1597-602.

27 van de Vosse E, de Paus RA, van Dissel JT, Ottenhoff THM. Molecular complementation of IL-12Rβ1 deficiency reveals functional differences between IL-12Rβ1 alleles including partial IL-12Rβ1 deficiency. *Hum Mol Genet* 2005; **14**: 3847-55.

28 Higuchi R, Krummel B, Saiki RK. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res* 1988; **16**: 7351-67.

29 Kinsella TM, Nolan GP. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum Gene Ther* 1996; **7**: 1405-13.

30 Kohlhuber F, Rogers NC, Watling D, Feng J, Guschin D, Briscoe J, et al. A JAK1/JAK2 chimera can sustain α and γ interferon responses. *Mol Cell Biol* 1997; **17**: 695-706.

CHAPTER 7

Inhibition of the type I immune responses of human monocytes by IFN-α and IFN-β

Roelof A. de Paus[a], Annelies van Wengen[a], Iris Schmidt[a], Marten Visser[b], Els M. E. Verdegaal[b], Jaap T. van Dissel[a] and Esther van de Vosse[a]

[a] Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands.
[b] Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands.

Cytokine 2013; 61(2):645-55

Abstract

Interleukin-12 (IL-12), IL-23 and interferon-γ (IFN-γ) are pivotal cytokines acting in concert with tumor necrosis factor (TNF) and IL-1β to shape type I immune responses against bacterial pathogens. Recently, several groups reported that type I immunity can be inhibited by IFN-α/β. Here we show the extent of the inhibitory effects of IFN-α and IFN-β on the responsiveness of human monocytes to Toll like receptor-ligands and IFN-γ. Both IFN-α and IFN-β strongly reduced the production of IL-12p40, IL-1β and TNF and the IFN-γ induced CD54 and CD64 expression. High IFN-γ concentrations could not counterbalance the inhibitions and IFN-α still inhibited monocytes 24 h after stimulation *in vitro* as well as *in vivo* in patients undergoing IFN-α treatment. Next, we explored the mechanism of inhibition. We confirm that IFN-α/β interferes with the IFN-γR1 expression, by studying the kinetics of IFN-γR1 downregulation. However, IFN-γR1 downregulation occurred only after two hours of IFN-α/β stimulation and was transient, which can not explain the IFN-γ unresponsiveness observed directly and late after IFN-α/β stimulation. Additional experiments indeed indicate that other mechanisms are involved. IFN-α may interfere with IFN-γ-elicited phosphorylation of signal transducer and activator of transcription 1 (STAT1). IFN-α may also activate methyltransferases which in turn reduce, at least partly, the TNF and IL-1β production and CD54 expression. IFN-α also induces the protein inhibitor of activated STAT1 (PIAS1). In conclusion, IFN-α and IFN-β strongly inhibit the IFN-γ responsiveness and the production of type I cytokines of monocytes, probably via various mechanisms. Our findings indicate that IFN-α/β play a significant role in the immunopathogenesis of bacterial infections, for example *Mycobacterium tuberculosis* infection.

1. Introduction

IFN-α and IFN-β as well as IFN-γ display immunomodulatory effects to help the host to combat infections. IFN-α and IFN-β are closely related cytokines, which are important in the defence against viruses. IFN-γ is the main mediator of the type I immune response and is essential in the control of infections with intracellular pathogens, such as *Mycobacteria* and *Salmonellae* [1]. IFN-α and IFN-β also play a role in various bacterial diseases, for example tuberculosis. Patients with active infection with *Mycobacterium tuberculosis* were recently shown to have an expression profile typical of IFN-α-induced immune related genes coinciding with reduced IFN-γ signalling within their blood cells [2, 3]. This is in line with the finding that virulent *Mycobacterium tuberculosis* strains isolated from humans induced IFN-α production in mice, which correlated with decreased type I immunity [4, 5]. Thus, *Mycobacterium tuberculosis* survival in the host may benefit from enhanced IFN-α/β signalling and repressed IFN-γ signalling. In addition, virulent strains of the intracellular pathogens *Bordetella pertussis* and *Francisella tularensis* were also found to inhibit the type I immune responses of human dendritic cells, via induction of IFN-β [6, 7].

IFN-α/β and IFN-γ have both common and distinct effects on human cells [8, 9]. IFN-α/β act inhibitory on immature and stimulatory on mature antigen presenting cells, B- and T-lymphocytes [10]. Therefore IFN-α/β may favour late antibacterial responses [11], although IFN-α/β may actually dampen antibacterial responses in the early phase of infection. For example, IFN-β exposure during naïve T cell stimulation inhibits Th1 cell generation by inhibiting the IL-12 and IL-23 production of cultured human dendritic cells [12]. We recently reported that IFN-α can also reduce the IFN-γ responsiveness of human primary monocytes [13]. Yet, little is known about the precise extent and mechanisms whereby IFN-α/β inhibits type I immune responses. IFN-α/β may very well interfere with the control of infections with intracellular pathogens. The inhibitory effects of IFN-α/β may thus explain why in influenza virus infected mice, virus-induced IFN-α renders the mice highly susceptible to bacterial infections [14], and why mice that lack a functional IFN-α/β receptor are relatively resistant to infections with the intracellular pathogen *Listeria monocytogenes* [15].

Upon IFN-γ stimulation, via the IFN-γR, STAT1 is tyrosine phosphorylated, dimerizes and translocates to the nucleus. STAT1 homodimers can activate transcription of several genes via binding to IFN-γ activated sequences (GAS) in promoters [16]. IFN-α and IFN-β both signal via the IFN-α receptor complex (IFN-αR) resulting in STAT1-STAT2 heterodimers, which associate mostly with interferon regulatory factor 9 (IRF-9) to form interferon-stimulated gene factor 3 (ISGF3) complexes [17]. The transcription factor ISGF3 binds to interferon-stimulated response elements (ISREs), while STAT1-STAT2 heterodimers can also bind to certain GAS sites [9]. IFN-α/β and IFN-γ signalling may thus result in both common and distinct responses. A broad range of genes can be induced by both IFN-γ and IFN-α, albeit

with different efficacy [8]. For example, the transcription factor IRF-1 can be effectively induced by both IFN-α and IFN-γ. On the other hand, IFN-γ upregulates IRF-8 [18], CD54 and CD64 expression and enhances LPS-induced cytokine production of interleukin-1β (IL-1β), IL-12, IL-23 and TNF [13], while IFN-α on the contrary inhibits these IFN-γ effects.

Inhibition of IFN-γ responses by IFN-α/β could potentially be achieved via for instance IFN-γ receptor (IFN-γR) downregulation, prevention of STAT1 homodimer formation or activation of protein arginine methyltransferase 1 (PRMT1). PRMT1 was found to be associated with the IFN-αR1 subunit of the IFN-αR [19], which may indicate that PRMT1 is regulated by IFN-α/β [20]. Activation of PRMTs results in methylation of arginine residues of various proteins, thereby modulating their actions [21]. PRMT1 can, amongst others, methylate the protein inhibitor of activated STAT1 (PIAS1) [22]. Methylated PIAS1 binds to STAT1, thereby negatively influencing the DNA binding capacity of STAT1 homodimers to certain, but not all, GAS sites [23]. In this way PIAS1 selectively inhibits IFN-γ induced transcription of genes.

In this study, we focused first on the extent of the opposing effects of IFN-α and IFN-β on the type I immune responses of human monocytes in vitro. We investigated whether IFN-α and IFN-β display comparable opposing effects, and whether high doses of IFN-γ can overcome these effects. Second, we determined the duration of the inhibitory effects in vitro and in vivo. Third, we explored by which mechanism IFN-α can interfere with IFN-γ functions, by examining the effects of IFN-α on IFN-γR expression and STAT1 phosphorylation. Additionally, we investigated whether PRMT1 and PIAS1 could play a role in the inhibitory effects of IFN-α.

2. Materials and Methods

2.1. Monocyte isolation and culture

PBMCs were isolated from blood of healthy blood bank donors, via ficoll separation. Subsequently monocytes were isolated using CD14 MACS beads (Miltenyi) according to the manufacturer's protocol. The isolated monocytes contained less than 1.5 % CD3+ cells, as analysed by FACS using PE conjugated antibody against CD3 (BD Biosciences). Cells were cultured in 96 wellsplates in IMDM (Lonza) supplemented with 8% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 mM glutamax (Life Technologies). Cells were stimulated, as indicated in each experiment, with 1 ng/ml LPS (Sigma), 10 μg/ml of heat killed *Mycobacterium tuberculosis* (MTB) (kind gift from L. Wilson), IFN-α, IFN-β, IFN-γ (PBL) and Arginine Methyltransferase Inhibitor 1 (AMI1, Calbiochem). The IFN-α is a 1:1 mixture of the subtypes 2a and 2b. The IFN-β is of the 2b subtype. 10 Units/ml IFN-α/β equals the concentration of 94 pg/ml IFN-γ.

2.2. Patient materials and immunological screenings

Patients with cutaneous melanoma received IFN-α treatment prior to and after T cell infusions [24]. IFN-α (subtype 2a, 3 million IU Roferon, Roche) was injected subcutaneously each day. PBMCs were isolated from blood and stored in liquid nitrogen until use at two time points: once before the start of the treatment and once 2 to 4 weeks later during IFN-α treatment. Thawed PBMCs were washed three times with culture medium and directly used in further assays using 96 wellsplates (Greiner bio-one). The IFN-γR1 receptor expression and the IFN-γ induced CD54 expression were analyzed by FACS as described in section 2.3, except that the cells were also stained with FITC conjugated CD14 (BD Biosciences) , in order to assess the expression on $CD14^+$ monocytes. The LPS responsiveness was tested by stimulating $2*10^5$ PBMCs (the standard deviation of cell counting was about 5%), from patients and controls, with or without 1 ng/ml LPS in 200 µl of IMDM supplemented with 8% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mM glutamax. After 24h of incubation supernatants were harvested and analyzed for the presence of IL-12p40 by ELISA (Invitrogen). All patients gave written informed consent. The treatment protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center.

2.3. Analysis of receptor expression

$1.5*10^5$ PBMCs or $CD14^+$ monocytes were incubated with various concentrations of IFN-γ or IFN-α in 200 µl of culture medium. Cells were washed with PBS supplemented with 0.2% bovine serum albumin (BSA) (Roche). To determine CD54 and CD64 expression the cells were stained with PE labelled anti-CD54 and FITC labelled anti-CD64 (clones HA58 and 10.1, BD Biosciences). To determine the IFN-γR1 expression the cells were stained with PE labelled GIR94 (BD Biosciences). Cells were analyzed with a FACSCalibur using CellQuest software (BD Biosciences).

2.4. Measurements of cytokine production

$1.5*10^5$ $CD14^+$ monocytes were incubated with various concentrations of IFN-γ and/or IFN-α in the presence or absence of 1 ng/ml LPS. After 24h supernatants were collected and analysed for the presence of IL-12p40, IL-1β and TNF by ELISA (Invitrogen) using microlon plates (Greiner bio-one). The detection limit of the ELISAs was 150 pg/ml.

2.5. FACS analysis of STAT1 phosphorylation

$2*10^5$ $CD14^+$ monocytes were incubated for 5 to 60 minutes with various concentrations of IFN-γ and/or IFN-α in 200 µl of culture medium. In order to detect intracellular phosphorylation of STAT1, cells were fixated with 4% paraformaldehyd (Sigma) and permeabilized with 90% methanol (Merck). Subsequently, the cells were treated with 10% normal goat serum (Sanquin) and stained with Alexafluor-647 labelled mouse-anti-human-

pY701-STAT1 (clone 4a, BD Biosciences). After labelling, the cells were washed twice and analyzed by FACS using a FACSCalibur and CellQuest software (BD Biosciences).

2.6. Western blot analysis of STAT2 phosphorylation

To detect STAT2 tyrosine phosphorylation, we incubated $2*10^6$ $CD14^+$ monocytes with 100 U/ml IFN-α and/or 2.5 ng/ml IFN-γ. After incubation the cells were washed with an excess of ice-cold PBS. The cells were lysed in a radioimmune-precipitation-assay (RIPA) buffer supplemented with PMSF, protein inhibitors and sodium-orthovanadate, according to the supplier's recommendations (SantaCruz). Equal protein amounts, determined using a Bradford assay (Thermo Scientific), were run on a 10% polyacrylamidegel (Promega), and blotted on a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer) using electrophoresis and blotting apparatuses according to the manufacturer's protocols (BioRad). Membranes were blocked for 1 h in PBS supplemented with 5% milk powder (Campina). Subsequently, the membranes were incubated with mouse-anti-human-pY690-STAT2 (clone 7a, BD Biosciences) in PBS-2.5% milk powder, washed and incubated with a HRP-conjugated Fab fragment of a goat-anti-mouse antibody (SantaCruz) in PBS-2.5% milk powder. As a control the blot was subsequently incubated with a HRP-conjugated antibody against GAPDH (clone Fl335, SantaCruz). Binding was detected using an enhanced chemiluminescence kit (Thermo Scientific) and exposure to X-ray film (Fuji).

2.7. Western blot analysis of PRMT1 and PIAS1

The expression of PRMT1 and PIAS1 was analyzed by western blot as described above, with some slight modifications. Tris-buffered-saline (Roche) instead of PBS was used to incubate the PVDF membranes after blotting. Specific monoclonal rabbit-anti-human antibodies against PRMT1and PIAS1 (clone A33 and clone D33A7, Cell Signalling Technologies) and a secondary HRP-conjugated donkey-anti-rabbit antibody (sc-2077, SantaCruz) were used to determine expression. As a control GAPDH expression was subsequently analysed using a HRP-conjugated monoclonal antibody against GAPDH on the same blot.

2.8. Statistical analysis

Differences in responses were analyzed using one-way or two-way ANOVA analyses with Bonferroni adjustment, the two tailed student t-test or the paired student t-test. The statistical significance level used was $p<0.05$.

3. Results

3.1. IFN-α and IFN-β inhibit IFN-γ induced CD64 and CD54 expression

We have previously noted that IFN-α inhibited the expression of CD54 and CD64 induced by 2.5 ng/ml of IFN-γ [13]. Little is known about the dose response effects of IFN-α/β and IFN-γ, which we studied here in detail. Furthermore, we determined whether IFN-β displays similar effects and whether high concentrations of IFN-γ could overcome these inhibitory effects. $CD14^+$ monocytes were cultured for 18 h with various concentrations of IFN-γ in the presence or absence of IFN-α or IFN-β. Incubation of the $CD14^+$ monocytes with high concentrations of IFN-γ gave a 6 fold enhancement in CD64 expression (Fig. 1A and 1B). Culturing the cells with IFN-α or IFN-β alone did not affect CD64 expression. Addition of IFN-α or IFN-β together with IFN-γ gave a dose dependent reduction of the IFN-γ induced CD64 expression (Fig. 1A and 1B). IFN-α and IFN-β reduced the expression of CD64, at all IFN-γ concentrations. The IFN-γ induced enhancement of CD54 expression was reduced by IFN-α and IFN-β in a similar way (Fig. 1C and 1D). The inhibition by IFN-α and IFN-β was similar (Fig. 1E) and IFN-γ concentrations up to 250 ng/ml could not overcome the inhibitory effect of IFN-α or IFN-β (Fig 1A-D).

3.2. IFN-α and IFN-β inhibit production of IL-12p40, IL-1β and TNF

IFN-α and IFN-β are known to inhibit IL-12p40 production. We wanted to determine the extent of this inhibition and whether other cytokines are also inhibited. For the establishment of an effective type I immune response the cytokines TNF and IL-1β play a pivotal role. The production of these cytokines can be induced via TLR stimulation and is greatly enhanced by IFN-γ. Therefore, we studied the effects of IFN-α and IFN-β on the production of IL-12p40 (the common subunit of IL-12 and IL-23), IL-1β and TNF by monocytes. $CD14^+$ monocytes were stimulated with or without 1000 U/ml IFN-α or IFN-β and various concentrations of IFN-γ in the presence or absence of LPS. Incubation with one of the interferons alone did not induce cytokine production (data not shown). LPS induced the production of IL-12p40, IL-1β and TNF, which could be dose dependently enhanced by IFN-γ (Fig. 2 A, B, C). IFN-α and IFN-β strongly reduced the production of IL-12p40 induced by LPS alone or together with IFN-γ (Fig. 2A). The effect of IFN-γ on IL-1β production was also strongly reduced (Fig. 2B), while the effect of IFN-γ on TNF production was only partly reduced by IFN-α and IFN-β (Fig. 2C). Addition of high IFN-γ concentrations, up to 250 ng/ml, could not overcome the inhibitory effects of IFN-α or IFN-β on the LPS-induced IL-12p40, IL-1β and TNF production (Fig 2A, B, C). The extent of the inhibitory effects of IFN-α and IFN-β was compared in figure 2D. The inhibitory effect was significant for all three cytokines and was similar for IFN-α and IFN-β (Fig. 2D). The inhibitory effects of the IFN-α and IFN-β were not observed when LPS or IFN-γ were added 1 h prior to the addition of IFN-α (data not shown).

Besides LPS, which is a single TLR-ligand able to activate monocytes via TLR2 and TLR4, we investigated whether IFN-α and IFN-β could also inhibit the responses induced by Mycobacteria. Hereto, we stimulated $CD14^+$ monocytes with or without IFN-α or IFN-β and IFN-γ in the presence or absence of MTB. The MTB induced production of the three measured cytokines was reduced by IFN-α and IFN-β (Fig. 2E, F, G). The synergistic effect of IFN-γ on the MTB induced IL-12p40 and IL-1β production was blocked by IFN-α and IFN-β (Fig. 2E and 2F), while the TNF production was partially reduced by IFN-α and IFN-β (Fig. 2G).

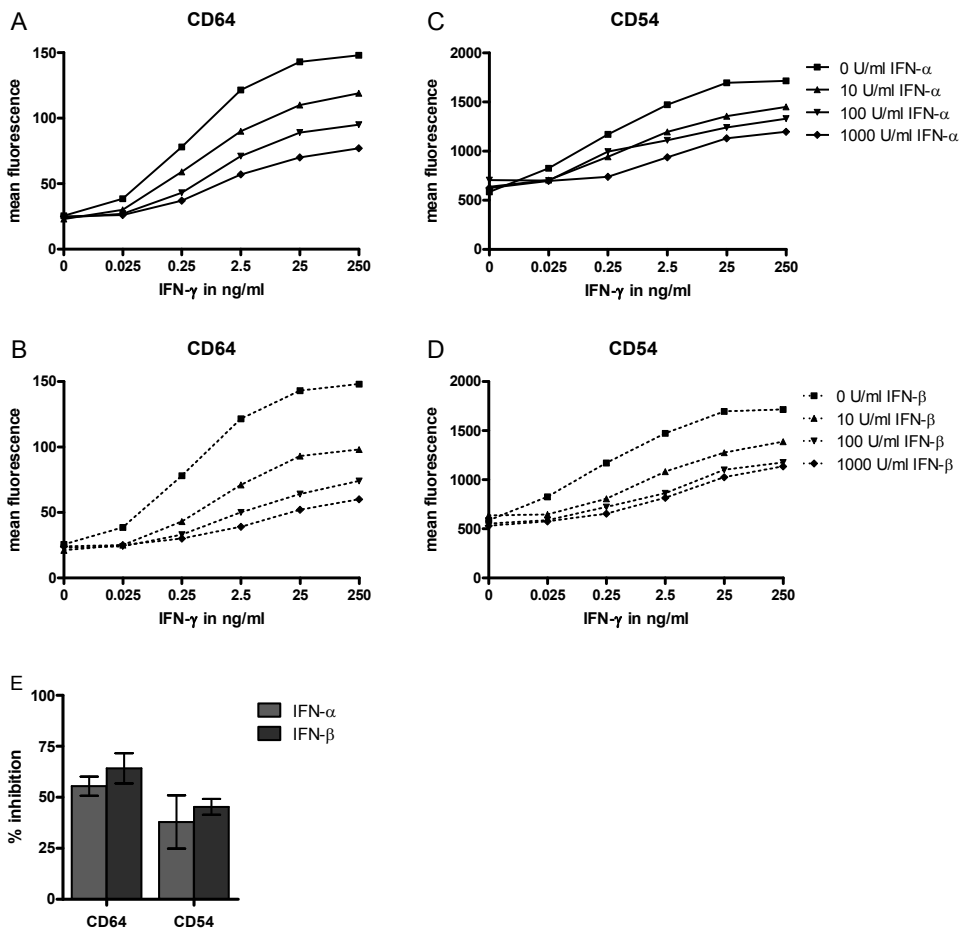


Figure 1. Inhibition of IFN-γ induced CD54 and CD64 expression by IFN-α and IFN-β. $CD14^+$ monocytes were stimulated with various concentrations of IFN-γ and IFN-α (A,B) or IFN-β (C,D). After 18 h the CD54 and CD64 expression was measured by FACS. Four donors were tested separately. One representative experiment is shown (A,B,C,D). The influence of 1000 U/ml of IFN-α on the CD64 and CD54 expression (E), induced by 250 ng/ml IFN-γ, is depicted as the percentage of inhibition by IFN-α. IFN-γ induced expression was set at 100% and expression in untreated cells was set at 0%. The mean +/- the standard deviation is displayed for four donors.

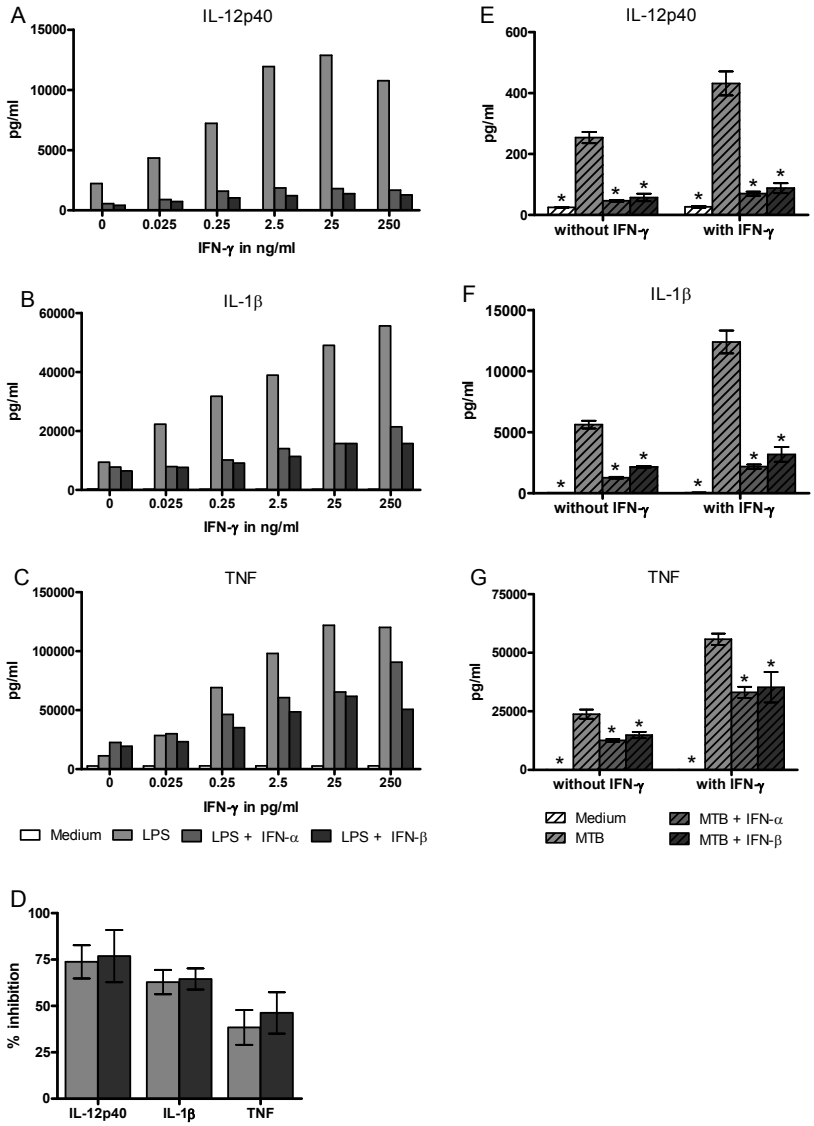


Figure 2. Inhibition of TLR-ligand and IFN-γ induced cytokine production by IFN-α and IFN-β. CD14^{+} monocytes were stimulated with 1 ng/ml LPS and various concentrations of IFN-γ with or without 1000 U/ml IFN-α or IFN-β. After 24h the production of IL-12p40 (A), IL-1β (B) and TNF (C) were determined by ELISA. Four donors were tested, one representative experiment is shown. (D) The influence of 1000 U/ml of IFN-α/β on cytokine production, induced by LPS and 250 ng/ml IFN-γ, is depicted as the percentage of inhibition by IFN-α/β. LPS/IFN-γ induced production was set at 100% and LPS induced production was set at 0%. The effect of IFN-α/β on mycobacterial stimulation (E-G) was determined by incubating CD14^{+} monocytes with 10 μg/ml MTB, with or without 2.5 ng/ml IFN-γ and with or without 1000 U/ml IFN-α or IFN-β. After 24h the production of IL-12p40 (E), IL-1β (F) and TNF (G) were determined by ELISA. Three donors were tested in triplo. One representative experiment is shown. Error bars represent the standard deviation. * $p<0.01$ (one-way ANOVA).

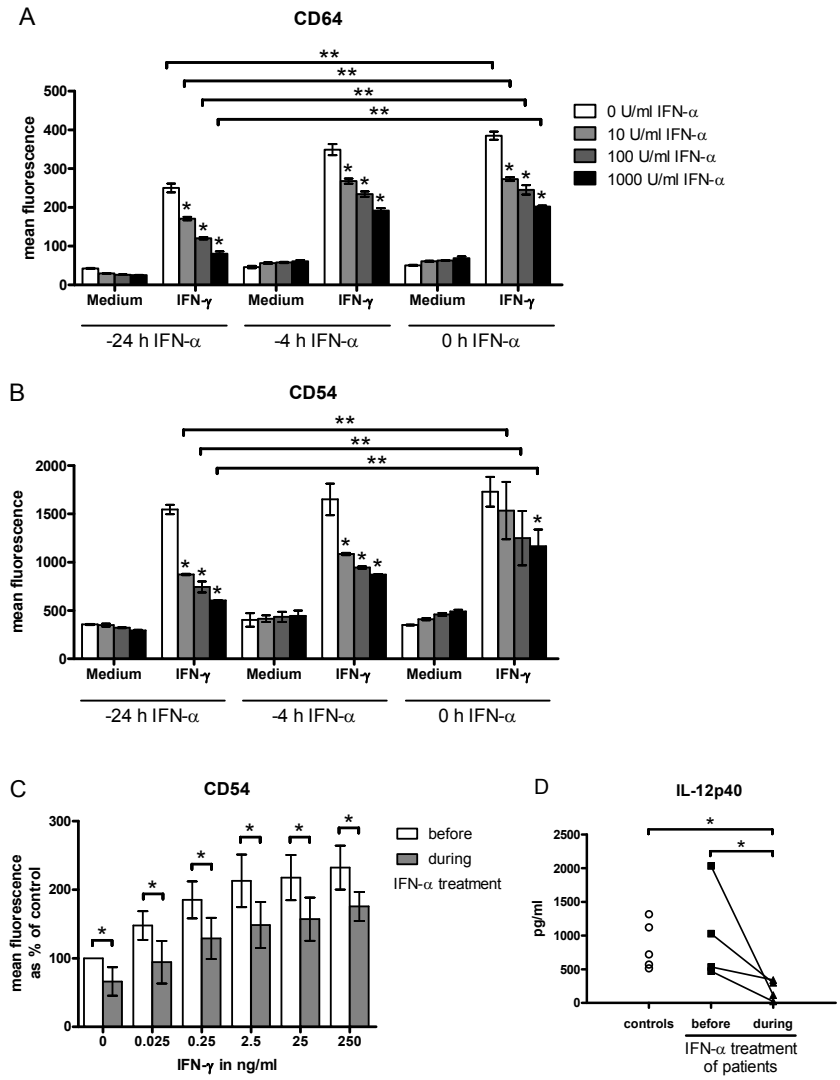


Figure 3. IFN-α elicits prolonged inhibitory effects on type I immune responses *in vitro* and *in vivo*. The duration of the inhibitory effects of IFN-α on type I immune response was studied *in vitro* (A, B). IFN-α was added to $CD14^+$ monocytes 24 h, 4 h or 0 h prior to the addition of 2.5 ng/ml IFN-γ or medium. The cells were cultured for another 18 h before expression of CD64 (A) en CD54 (B) was analyzed. The results are displayed as the mean fluorescence +/- standard deviation of an experiment performed in triplo. One representative experiment out of three is shown. * significantly different from stimulation without IFN-α and ** significantly different pairs , $p<0.05$ (two-way ANOVA). To study the inhibitory effects of IFN-α *in vivo* (C), PBMCs from melanoma patients were obtained before and during treatment with IFN-α and cultured for 18 h with various concentrations of IFN-γ. Afterwards, CD54 expression of the $CD14^+$ cells was analyzed by FACS, the relative mean fluorescence +/- standard deviation of four patients are shown (C). In addition, PBMCs from four patients and five controls were stimulated for 24 h with 10 ng/ml LPS or medium (D). IL-12p40 production in supernatants was analyzed by ELISA (D). For C * $p<0.01$ by one-way ANOVA and for D * $p<0.05$ (paired student t-test, unpaired student t-test was used for comparison with controls).

3.3. IFN-α elicits prolonged inhibition of type I immune responses in vitro and in vivo

IFN-α inhibits the IFN-γ responses when both cytokines are given together to the monocytes. It is not known how long the inhibitory effect of IFN-α lasts. We investigated whether IFN-α could still effectively inhibit type I immune responses until 24h after IFN-α stimulation. Therefore $CD14^+$ monocytes were pre-cultured in the presence of IFN-α and subsequently stimulated with IFN-γ. IFN-α inhibited the IFN-γ induced CD64 expression when given together with IFN-γ and when given 4 h prior to IFN-γ to a similar extent (Fig. 3A). When IFN-α was given 24 h prior to the addition of IFN-γ the inhibition of CD64 expression was even stronger. IFN-α also displayed strong inhibitory effects on the induction of CD54 expression when given 4 h or 24 h prior to IFN-γ (Fig. 3B).

To study whether IFN-α also inhibits monocytes *in vivo* we analyzed the PBMCs of melanoma patients before and during IFN-α treatment. Before and after at least one week of daily IFN-α injections we determined the basal and IFN-γ inducible expression of CD54 and the LPS-induced IL-12p40 production. Basal expression of CD54 was significantly reduced during IFN-α treatment (Fig. 3C). IFN-γ could not enhance the CD54 expression to maximum levels when the patients received IFN-α treatment. At all IFN-γ concentrations the CD54 expression was significantly lower during IFN-α treatment. Due to the IFN-α treatment, the capacity of the PBMCs to produce IL-12p40 upon LPS stimulation was reduced (Fig. 3D). Unstimulated PBMCs did not produce any detectable IL-12p40 (data not shown). The IFN-γR1 expression of the PBMCs, before and during IFN-α treatment, was comparable (data not shown).

3.4. Downregulation of IFN-γR1 expression by IFN-α and IFN-β

IFN-γ binding to its receptor complex, consisting of two IFN-γR1 and two IFN–γR2 chains, results in signal transduction and subsequent downregulation of the cell surface expression of IFN-γR1. Little is known about the kinetics of IFN-γR downregulation in human monocytes. We investigated the kinetics of IFN-γR1 downregulation on $CD14^+$ monocytes by IFN-γ in more detail and whether IFN-α and IFN-β could also induce IFN-γR1 downregulation, independent from IFN-γ. The cell surface expression of the IFN-γR1 receptor was, after a delay of 2 h, gradually downregulated by IFN-γ, was strongly reduced after 4 h (up to 77%), and was still remarkably reduced after 18 h of stimulation (Fig. 4A). The IFN-γR1 expression was gradually reduced at all IFN-α concentrations, also with a delay of 2 h after stimulation, and was most strongly reduced after 4 h (up to 75%). The IFN-γR1 expression was partly restored after 18 h of stimulation with IFN-α (Fig. 4A).

We next analyzed the effects of simultaneous stimulation with IFN-α and IFN-γ and whether a 4h or 24h pre-stimulation with IFN-α had an additive or synergistic effect on the IFN-γR1 downregulation induced by IFN-γ. We did not observe any additive effect of IFN-α and IFN-γ (Fig. 4B). Pre-stimulation of IFN-α did not influence the kinetics of the IFN-

γR1 downregulation by IFN-γ (Fig. 4B). IFN-β downregulated the IFN-γR1 expression in a similar manner (data not shown). As previously reported, cell surface expression of IFN-γR2 on monocytes could not be detected by FACS [25].

3.5. IFN-α signalling interferes with IFN-γ induced STAT1 phosphorylation

IFN-γ signalling occurs via the formation of STAT1 homodimers, while IFN-α signals via STAT1- STAT2 heterodimer complexes. The signalling of both interferons may influence each other through competition for STAT1. We examined the kinetics of STAT1 and STAT2 phosphorylation in $CD14^+$ monocytes after addition of IFN-α and IFN-γ alone or together. STAT1 phosphorylation was quantified by FACS (Fig. 4C). There was only a very small additive effect in STAT1 phosphorylation after stimulation for 5 or 10 minutes with 0.25 ng/ml of IFN-γ and 100 U/ml of IFN-α, although maximum STAT1 phosphorylation was not reached, as illustrated by the use of a higher concentration of IFN-γ or by longer stimulation (15 minutes). The additive effect at 5 and 10 minutes was smaller than expected of a full additive effect (which can be calculated as the sum of the response induced by IFN-α and the response induced by IFN-γ). After 5, 10 and 15 minutes of stimulation with 2.5 ng/ml IFN-γ and 100 U/ml of IFN-α, no additive effects were observed (Fig. 4C).

Because no antibodies are available to quantify tyrosine phosphorylated STAT2 by FACS, we determined the STAT2 phosphorylation by western blot analysis. No STAT2 phosphorylation was observed in unstimulated cells. As expected, IFN-γ did not induce any detectable STAT2 phosphorylation. IFN-α clearly induced STAT2 phosphorylation, which was not changed in the presence of IFN-γ (Fig. 4D).

Surprisingly, hardly any additive effects of IFN-α and IFN-γ were observed for the STAT1 phosphorylation, and IFN-α induced the same STAT2 phosphorylation in the presence or absence of IFN-γ. We argue that there may be a considerable reduction of IFN-γ induced STAT1 homodimer formation due to competition between STAT2 and STAT1 for dimer formation with STAT1. To estimate the reduction of STAT1 homodimer formation we compared the amount of STAT1 phosphorylated by IFN-γ alone with the amount of STAT1 phosphorylated by the costimulation of IFN-γ and IFN-α minus the amount of STAT1 phosphorylated by IFN-α alone. In this way, we estimate that there may be a reduction in STAT1 homodimer formation of up to 17 %, 44 % and 58 % after respectively 5, 10 and 15 minutes of stimulation. These calculated reductions are probably overestimated, because we presumed that all phosphorylated STAT1 dimerizes and because we cannot fully exclude that the IFN-α induced STAT2 phosphorylation is not slightly reduced in the presence of IFN-γ.

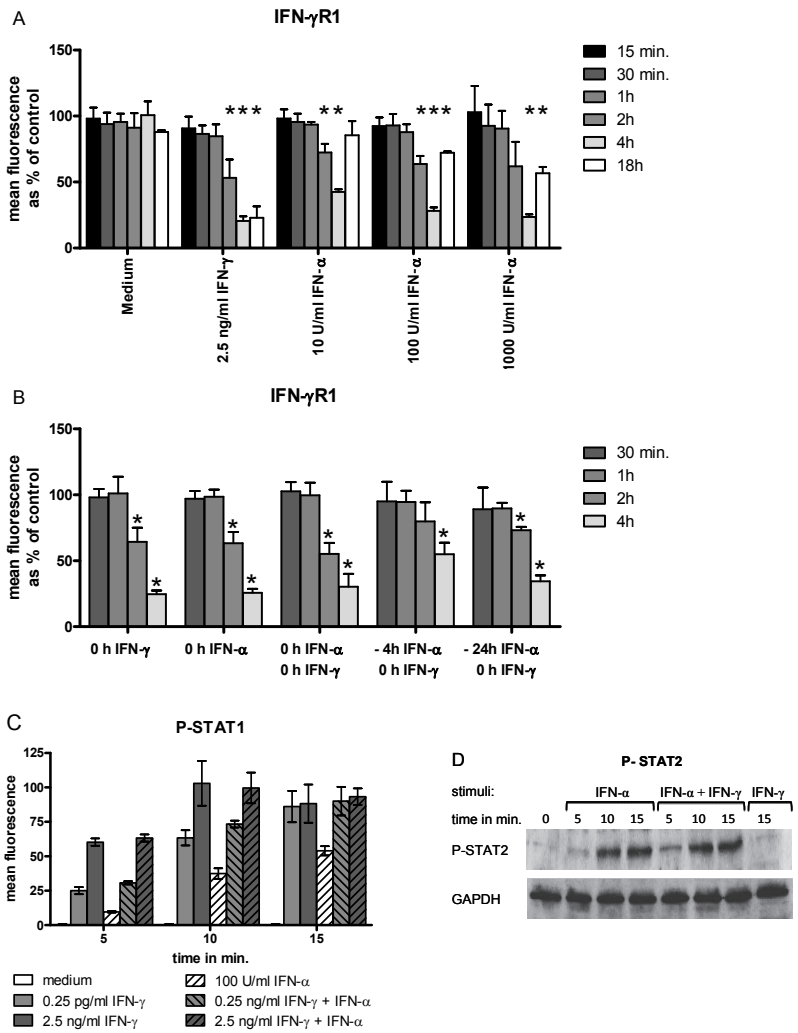


Figure 4. IFN-α downregulates IFN-γR1 expression and interferes in the IFN-γ induced signal transduction. IFN-γR1 expression on $CD14^+$ monocytes was measured by FACS after stimulation with medium, with 2.5 ng/ml IFN-γ or with various concentrations of IFN-α (A). Mean fluorescence of unlabeled monocytes was set at 0 % and mean fluorescence of labelled untreated monocytes was set at 100%. IFN-γR1 expression was calculated as percentage of the controls. The mean +/- the standard deviation of three donors is shown. * significantly different from the untreated control $p<0.05$ (two-way ANOVA). The effect of costimulation with 1000 U/ml IFN-α and the effect of prestimulation with 1000 U/ml IFN-α on the IFN-γ induced IFN-γR1 downregulation was tested in triplo for two donors (B). The measured IFN-γR1 expression at 0 h was set at 100%. The mean +/- the standard deviation is shown. * significantly different from the expression measured at 0 h $p<0.05$ (two way ANOVA). The signal transduction was determined by measuring the STAT1 and STAT2 phosphorylation (C and D). $CD14^+$ monocytes were stimulated with 0.25 ng/ml (C, D) or 2.5 ng/ml (A) IFN-γ and 100 U/ml of IFN-α (A, B). At various time points the STAT1 tyrosine phosphorylation was determined by FACS (A). The mean fluorescence +/- the standard deviation of three individual measurements is shown. The amount of tyrosine phosphorylated STAT2 and GAPDH was determined by western blot (B), one representative experiment out of three is shown.

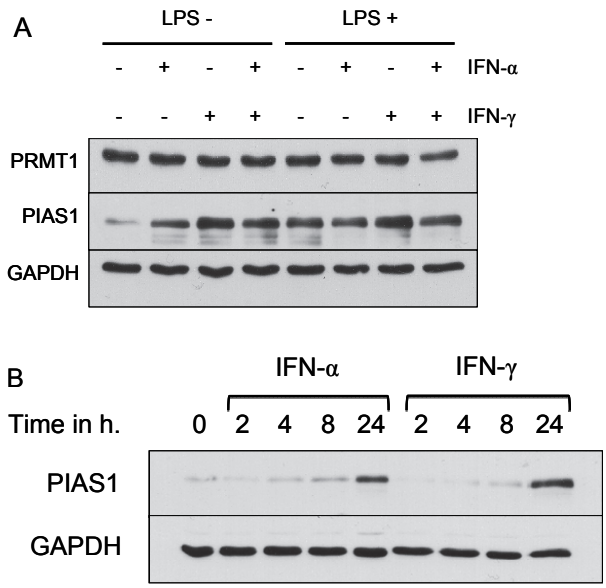


Figure 5. Basal expression of PRMT1 and interferon inducible PIAS1 expression. The expression of PRMT1, PIAS1 and GAPDH in $CD14^+$ monocytes was analyzed by western blot after 18 h of stimulation with medium, 1000 U/ml IFN-α, 25 ng/ml IFN-γ, 1 ng/ml of LPS or with combinations of the stimuli (A). The kinetics of PIAS1 expression was determined after stimulation with 1000 U/ml IFN-α or 25 ng/ml IFN-γ (B).

3.6. Monocytes show basal PRMT1 expression and inducible PIAS1 expression

IFN-α could potentially inhibit IFN-γ responses via activation of PRMT1 and/or via induction of factors that negatively influence the JAK/STAT pathway, such as PIAS1. To establish whether these factors play a role in the inhibitory effects of IFN-α we investigated the basal and inducible expression of PRMT1 and PIAS1 in monocytes. $CD14^+$ monocytes were stimulated for 18 h with combinations of IFN-α, IFN-γ and LPS. Monocytes showed basal expression of PRMT1 which was not further enhanced by the stimuli (Fig. 5A). A small amount of PIAS1 was expressed in unstimulated monocytes and could be enhanced by either IFN-α, IFN-γ or LPS alone or by combinations of these stimuli (Fig. 5A). Next, we determined the kinetics of PIAS1 upregulation by IFN-α or IFN-γ. Both interferons induced PIAS1 expression, relatively late, after more than 8 h of stimulation (Fig. 5B).

3.7. Inhibition of PRMTs reduces the inhibitory effects of IFN-α

IFN-α could potentially activate PRMT1, which in turn can influence the expression of immune genes via methylation of various regulatory proteins. With the use of a competitive PRMT inhibitor, AMI1 [26], we investigated the role of PRMTs in the inhibitory effects of IFN-α.

First, we determined the role of PRMTs in the regulation of CD54 and CD64 expression. $CD14^+$ monocytes were pre-incubated with AMI1 and cultured in the presence of IFN-γ and various concentrations of IFN-α. IFN-α reduced the IFN-γ upregulated CD54 expression (Fig. 6A). Pre-incubation with AMI1 resulted in a significantly smaller reduction (Fig. 6C). In contrast, IFN-γ induced expression of CD64 was already somewhat reduced by AMI1 itself (Fig. 6B), while AMI1 had no influence on the inhibitory effect of IFN-α on CD64 expression (Fig. 6D).

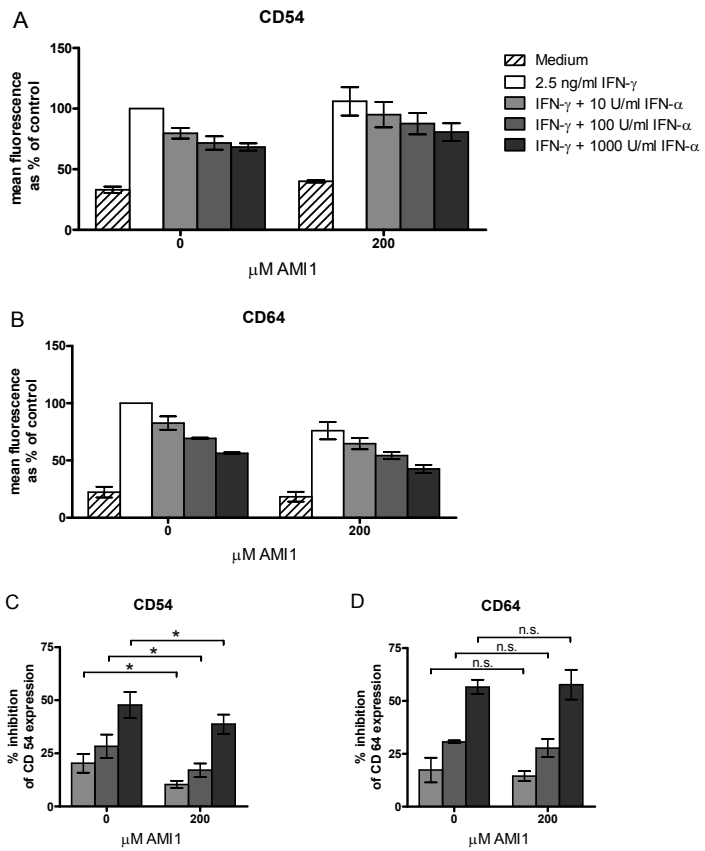


Figure 6. Inhibition of PRMTs reduces the IFN-α effect on IFN-γ induced CD54 and CD64 expression. The PRMT inhibitor AMI1 was added in a concentration of 200 μM to $CD14^+$ monocytes cultures 1 h before the addition of 2.5 ng/ml IFN-γ and various concentrations of IFN-α. The induction of CD54 (A) and CD64 (B) was measured by FACS. The fluorescence of unlabelled cells was set at 0% and the fluorescence of the IFN-γ stimulated and CD54 or CD64 labelled cells was set at 100%. The results of one donor tested in triplo is shown, error bars represent the standard deviation (A, B). To indicate significant effects of IFN-α, the results of three donors were sampled (C, D). Therefore, the inhibition of IFN-α on the IFN-γ mediated CD54 (C) and CD64 (D) expression was calculated as the percentage of inhibition: IFN-γ induced expression was set at 0% and the basal expression was set at 100%. The mean and standard deviation of three donors, each tested in triplo, is shown (C,D). $p<0.05$ (two-way ANOVA), n.s. not significant.

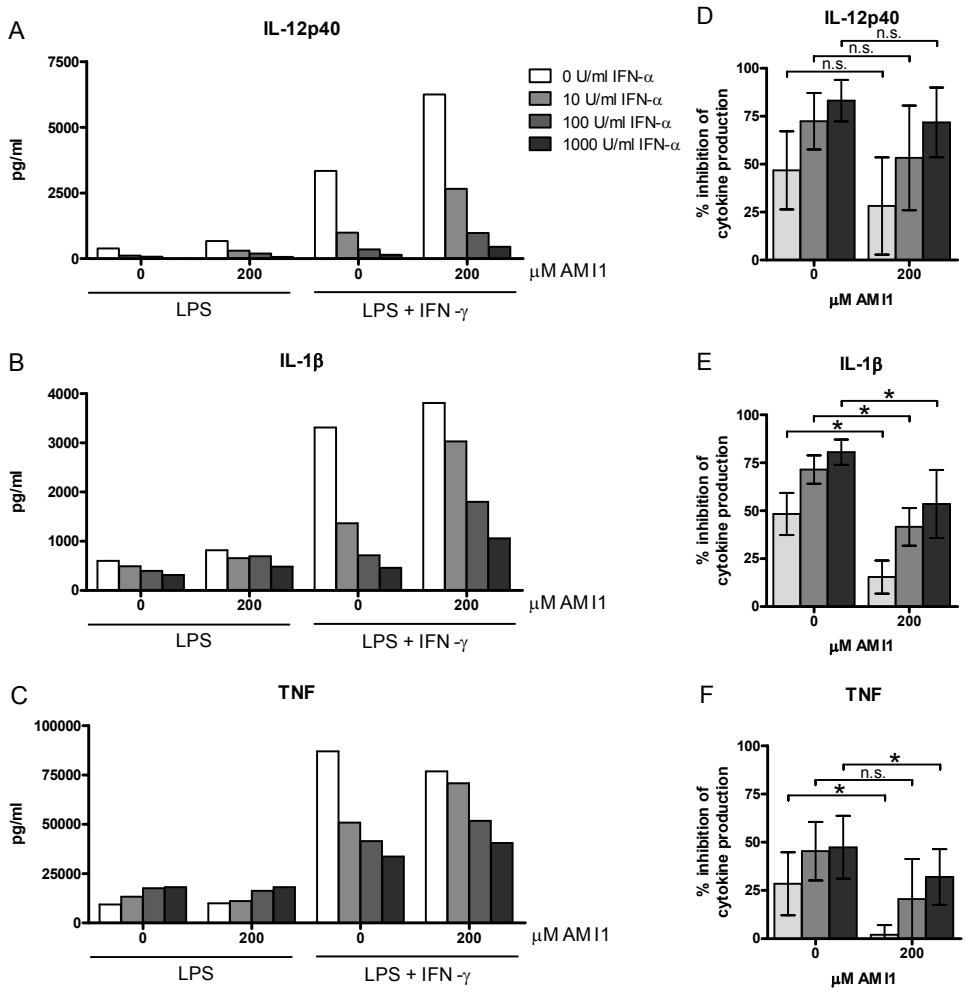


Figure 7. Inhibition of PRMTs reduces the effect of IFN-α on IFN-γ induced cytokine production. $CD14^+$ monocytes were pre-incubated for 1 h with or without 200 μM of the PRMT inhibitor AMI1. Subsequently the cells were stimulated for 24 h with 1 ng/ml LPS with or without 2.5 ng/ml IFN-γ and various concentrations of IFN-α. After 24 h the production of IL-12p40 (A, D), IL-1β (B, E) and TNF (C, F) were measured by ELISA. The results of one out of three donors are shown (A, B, C). To indicate significant effects of IFN-α, the results of three donors were sampled (D, E, F). The inhibition by IFN-α on the LPS/IFN-γ induced cytokine production was calculated as percentage of inhibition (D, E, F). The LPS/ IFN-γ induced cytokine production was set at 0% and the LPS-induced production was set at 100%. The mean and the standard deviation of three donors is shown (D, E, F). AMI1 alone did not induce cytokine production (data not shown). $p<0.05$ (two-way ANOVA), n.s. not significant.

Next, we analysed the influence of AMI1 on LPS and IFN-γ induced cytokine production. $CD14^+$ monocytes were pre-incubated with AMI1 and then stimulated with IFN-γ and various concentrations of IFN-α in the presence of LPS. AMI1 alone did not induce any cytokine production (data not shown). AMI1 pre-incubation already resulted in a 2 fold enhanced LPS/IFN-γ-induced IL-12p40 production, without IFN-α (Fig. 7A). AMI1 had little influence on the effects of IFN-α on the LPS and IFN-γ induced IL-12p40 production (Fig. 7A, D). IFN-α reduced the LPS/IFN-γ induced IL-12p40 production. Pre-incubation with AMI1 resulted in a similar reduction of IL-12p40 by IFN-α (Fig. 7D). IFN-α reduced the LPS/IFN-γ induced IL-1β and TNF production, which was for both cytokines significantly less after pre-incubation with AMI1 (Fig. 7B, C, E, F).

4. Discussion

This report demonstrates that IFN-α/β strongly inhibit the type I cytokine production and the IFN-γ responsiveness of human monocytes, probably via various mechanisms. Previously, we and others reported that exposure of monocytes to IFN-α/β leads to a reduction of IL-12p40 production and IFN-γ responsiveness [12, 13]. We now demonstrate that IFN-α and IFN-β have comparable strong inhibiting effects on human monocytes and that exposure to high concentrations of IFN-γ could not counterbalance the inhibitory effects of either IFN-α or IFN-β. Furthermore, our results indicate that IFN-α interferes with the IFN-γ responsiveness, early and late after stimulation. To explore the mechanisms of inhibition we studied the kinetics of the IFN-α induced IFN-γR1 downregulation showed that the downregulation was transient and occurred with two hours delay after IFN-α stimulation, indicating that other mechanisms are involved in the direct and late inhibitory effects. Indeed, we found some additional evidence that IFN-α directly interferes with the IFN-γ mediated signalling and that IFN-α may, at least in part, inhibit the production of IL-1β and TNF via activation of a PRMT, probably PRMT1. Furthermore, we show that IFN-α induces PIAS1, a negative regulator of STAT1 mediated transcription, which indicates that a PRMT1/PIAS1 pathway may be involved as well.

Both IFN-α and IFN-β reduced the IFN-γ induced CD64 and CD54 expression as well as the LPS and IFN-γ induced IL-12p40, IL-1β and TNF production. IFN-α and IFN-β showed the strongest inhibition on the LPS and MTB induced production of IL-12p40, the shared subunit of IL-12 and IL-23. Both IL-12 and IL-23 play an important role in the mounting of type I immune responses. IL-12 is known to stimulate IFN-γ production, Th1 polarization and expansion of T-cells subsets. IL-23 stimulates NK-like T cells to produce IFN-γ [27]. In turn, IFN-γ modulates monocytes in their type I immune functions, which can also be

antagonized by IFN-α. These inhibitory effects on monocytes occurred *in vitro* for at least 24 h after IFN-α stimulation and endured *in vivo* in IFN-α treated patients. The severe impact of IFN-α on type I immunity, the importance of IFN-γ in the control of mycobacterial diseases, the ability of virulent mycobacterial strains to induce IFN-α [5] and the finding of a typical IFN-α transcript signature in the blood cells of TB patients [2, 3] suggest an important role of IFN-α/β in the immunopathogenesis of *Mycobacterium tuberculosis* infections.

One mechanism through which IFN-α/β inhibits IFN-γ signalling may be downregulation of IFN-γR1 cell surface expression. IFN-γR downregulation by IFN-α/β was observed in mice after *Listeria monocytogenes* infection. Receptor down-regulation was found to be responsible for the IFN-γ unresponsiveness of monocytes after *Listeria* encounter [28]. In human macrophages, IFN-γR1 expression was also found to be reduced after incubation with IFN-α [29]. Our kinetic studies show that IFN-γR1 downregulation occurred only after two hours of stimulation with IFN-α and appeared to be transient. Hence, the IFN-γR1 downregulation can not explain the inhibitory effects of IFN-α we observed when IFN-α was given together with or 24 h before IFN-γ *in vitro* or when given to patients *in vivo*. Thus, our results indicate that other mechanisms are also involved to achieve direct and enduring opposing effects on the type I immune responses of monocytes.

The fact that IFN-α/β can inhibit IFN-γ mediated responses when given to monocytes simultaneous with IFN-γ indicates that these interferons can also interfere directly with IFN-γ mediated signalling. Indeed, by studying the kinetics of STAT1 and STAT2 phosphorylation we found some evidence that there is a direct negative effect of IFN-α on IFN-γ signalling. The amount of phosphorylated STAT1 induced by IFN-γ signalling is reduced in the presence of IFN-α. We reason that this reduction in STAT1 phosphorylation may result in a reduction of STAT1 homodimer formation of maximal 58% and could thereby account for a severe loss of IFN-γ responsiveness. Previous investigations with human fetal astrocytes [30] and murine macrophages [31], indicate also that IFN-β is involved in IFN-γ signalling by reducing the amount of STAT 1 homodimers bound to DNA [30] and influencing the STAT1 dephosphorylation [31]. It would be interesting to perform similar investigations with human monocytes and other phagocytes with ChIP analysis, using DNA probes coding for different GAS sites, in order to quantify the exact amounts of STAT1 homodimers, ISGF3 complexes and bioactive PIAS1 in the nucleus.

Another mechanism whereby IFN-α may oppose type I immune modulation is the activation of PRMTs. PRMT1 can associate with the intracellular part of IFN-αR1 and can be activated by IFN-α [19]. We show that PRMT1 is expressed in unstimulated human monocytes. And with the use of an inhibitor of PRMTs we revealed that only some of the inhibitory effects of IFN-α could be ascribed to a PRMT pathway. This was most prominent for the inhibition of IL-1β and TNF production, but was also observed for CD54 expression.

Further extensive investigation is needed to reveal which PRMT and which PRMT targets are involved.

PRMT1 is, for instance, able to methylate PIAS1, a negative regulator of the transcriptional activity of STAT1 [22, 23]. We show that expression of PIAS1 is induced relatively late after stimulation with IFN-γ, apparently as a negative feedback on IFN-γ signalling. IFN-α also induces PIAS1, with similar kinetics. The relatively late induction of PIAS1 may partly explain the fact that 24 h of pre-stimulation of monocytes with IFN-α results in a strong inhibition of the IFN-γ responsiveness *in vitro*, and why a lasting inhibitory effect on type I immunity could be observed in IFN-α treated patients.

Other mechanisms could also be involved. Gene transcription may be indirectly regulated by transcription factors other than STAT1 and STAT2. For example, the induction of the transcription factor IRF-8 by IFN-γ can be antagonized by IFN-α [18]. Furthermore, we speculate that there could be negative interference of ISGF3 with transcriptional activity. This may be the case with *CD64* transcription. Despite the fact that the promoter of *CD64* contains an ISRE-like element, which is a putative ISGF3 binding site, we showed that IFN-α does not induce CD64 expression. In fact, IFN-γ induced CD64 expression was inhibited by IFN-α. This suggests that the inhibitory effects of IFN-α may be due to a partial or complete block of transcriptional activity as a result of binding of ISGF3 to the promoter of *CD64*.

Taken together, IFN-α stimulation of monocytes results in a broad range of opposing effects on type I immunity, which may be established by various mechanisms. Some of these effects sustain for at least 24 h after IFN-α stimulation, *in vitro* as well as *in vivo*, indicating that IFN-α is an important negative regulator of the type I immune response. These effects may explain the enhanced susceptibility to bacterial pneumonia just after influenza infection [32]. Similarly, IFN-α/β induced by intracellular pathogens, such as *Listeria monocytogenes* [28] or virulent *Mycobacterium tuberculosis* [5] strains, can dampen the formation of an effective type I immune response. The benefit of the inhibitory effects of IFN-α for the host is not really understood. We speculate that a type I immune response during the early phase of viral infections may otherwise easily escalate in hyperinflammatory reactions.

Because IFN-α induces strong opposing effects on type I immune responses this should be taken into account in the treatment of certain patients. First, we recently reported that IFN-α treatment of IFN-γR1 deficient patients is not advisable [13]. Our present data further strengthen the advice not to use IFN-α in patients lacking Th1 immunity. Second, when considering IFN-α treatment in other types of patients, clinicians should be aware of the type I immune inhibiting effects. Several case reports of hepatitis patients who developed Tuberculosis during IFN-α treatment [33-35] indicate that perhaps patients from countries with high Tuberculosis prevalence should be screened for latent Tuberculosis prior to IFN-α treatment. Third, although cancer patients receiving IFN-α treatment to support T cell therapy may respond well to the therapy, it is possible that the inhibition of the type I

immune responses hampers the therapy to some extent. Fourth, in the development of *M. tuberculosis* infections IFN-α/β appear to play an important role [2], the source of this IFN-α/β is however still unclear. IFN-α/β may be produced by infected monocytes and/or during viral infection, allowing *M. tuberculosis* to take advantage of the situation and develop into an active infection. Thus, co-infections of *M. tuberculosis* with common types of viruses may aggravate the course of *M. tuberculosis* infections (R.A. de Paus et al, submitted for publication).

In conclusion, IFN-α and IFN-β both strongly inhibit monocytes in their capacity to produce type I cytokines and to respond to IFN-γ. The impact of IFN-α and IFN-β may indicate a major role of these interferons in the pathogenesis of infections with *M. tuberculosis* and other intracellular bacterial pathogens which are able to induce IFN-α/β. Our data provide novel insights into the mechanisms of the inhibitory effects. These effects may be achieved via various mechanisms, such as, a decreased IFN-γ signalling, a transient decrease in IFN-γR1 expression, activation of PRMTs and the induction of PIAS1. Further detailed investigations should dissect the exact contributions of each of these putative mechanisms.

References

[1] van de Vosse E, van Dissel JT, Ottenhoff TH. Genetic deficiencies of innate immune signalling in human infectious disease. Lancet Infect Dis 2009;9:688-98.

[2] Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 2010;466:973-7.

[3] Ottenhoff THM, Hari Dass R, Yang N, Zhang M, Wong HEE, Sahiratmadja E et al. Genome-wide expression profiling identifies Type I interferon response pathways in active tuberculosis. accepted for publication in PlosOne 2012;7 (9):e45839.

[4] Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-α/β. Proc Natl Acad Sci U S A 2001;98:5752-7.

[5] Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, Murray PJ et al. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res 2005;25:694-701.

[6] Spensieri F, Fedele G, Fazio C, Nasso M, Stefanelli P, Mastrantonio P et al. Bordetella pertussis inhibition of interleukin-12 (IL-12) p70 in human monocyte-derived dendritic cells blocks IL-12 p35 through adenylate cyclase toxin-dependent cyclic AMP induction. Infect Immun 2006;74:2831-8.

[7] Bauler TJ, Chase JC, Bosio CM. IFN-β Mediates Suppression of IL-12p40 in Human Dendritic Cells following Infection with Virulent Francisella tularensis. J Immunol 2011;187:1845-55.

[8] Sanda C, Weitzel P, Tsukahara T, Schaley J, Edenberg HJ, Stephens MA et al. Differential gene induction by type I and type II interferons and their combination. J Interferon Cytokine Res 2006;26:462-72.

[9] Takaoka A, Yanai H. Interferon signalling network in innate defence. Cell Microbiol 2006;8:907-22.

[10] Seo YJ, Hahm B. Type I interferon modulates the battle of host immune system against viruses. Adv Appl Microbiol 2010;73:83-101.

[11] Opitz B, Vinzing M, van Laak V, Schmeck B, Heine G, Gunther S et al. Legionella pneumophila induces IFN-β in lung epithelial cells via IPS-1 and IRF3, which also control bacterial replication. J Biol Chem 2006;281:36173-9.

[12] Nagai T, Devergne O, van Seventer GA, van Seventer JM. Interferon-b mediates opposing effects on interferon-γ-dependent Interleukin-12 p70 secretion by human monocyte-derived dendritic cells. Scand J Immunol 2007;65:107-17.

[13] van de Wetering D, Van Wengen A, Savage ND, van de Vosse E, van Dissel JT. IFN-α cannot substitute lack of IFN-γ responsiveness in cells of an IFN-γR1 deficient patient. Clin Immunol 2011;138:282-90.

[14] Shahangian A, Chow EK, Tian X, Kang JR, Ghaffari A, Liu SY et al. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. J Clin Invest 2009;119:1910-20.

[15] O'Connell RM, Saha SK, Vaidya SA, Bruhn KW, Miranda GA, Zarnegar B et al. Type I interferon production enhances susceptibility to Listeria monocytogenes infection. J Exp Med 2004;200:437-45.

[16] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. Annu Rev Immunol 1997;15:749-95.

[17] Qureshi SA, Salditt-Georgieff M, Darnell JEJr. Tyrosine-phosphorylated Stat1 and Stat2 plus a 48-kDa protein all contact DNA in forming interferon-stimulated-gene factor 3. Proc Natl Acad Sci U S A 1995;92:3829-33.

[18] Fultz MJ, Vogel SN. Analysis of the antagonist effect of IFN-α on IFN-γ-induced interferon consensus sequence binding protein messenger RNA in murine macrophages. J Inflamm 1998;48:28-39.

[19] Abramovich C, Yakobson B, Chebath J, Revel M. A protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFN-αR1 chain in the type I interferon receptor. EMBO J 1997;16:260-6.

[20] Altschuler L, Wook JO, Gurari D, Chebath J, Revel M. Involvement of receptor-bound protein methyltransferase PRMT1 in antiviral and antiproliferative effects of type I interferons. J Interferon Cytokine Res 1999;19:189-95.

[21] Lee YH, Stallcup MR. Minireview: protein arginine methylation of nonhistone proteins in transcriptional regulation. Mol Endocrinol 2009;23:425-33.

[22] Mowen KA, Tang J, Zhu W, Schurter BT, Shuai K, Herschman HR et al. Arginine methylation of STAT1 modulates IFN-α/β-induced transcription. Cell 2001;104:731-41.

[23] Liu B, Mink S, Wong KA, Stein N, Getman C, Dempsey PW et al. PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. Nat Immunol 2004;5:891-8.

[24] Verdegaal EM, Visser M, Ramwadhdoebe TH, van der Minne CE, van Steijn JA, Kapiteijn E et al. Successful treatment of metastatic melanoma by adoptive transfer of blood-derived polyclonal tumor-specific CD4+ and CD8+ T cells in combination with low-dose interferon-α. Cancer Immunol Immunother 2011;60:953-63.

[25] de Paus RA, Kilic SS, van Dissel JT, van de Vosse E. Effect of amino acid substitutions in the human IFN-γR2 on IFN-g responsiveness. Genes Immun 2011;12:136-44.

[26] Feng Y, Xie N, Wu J, Yang C, Zheng YG. Inhibitory study of protein arginine methyltransferase 1 using a fluorescent approach. Biochem Biophys Res Commun 2009;379:567-72.

[27] van de Wetering D., de Paus RA, van Dissel JT, van de Vosse E. IL-23 modulates CD56+/CD3- NK Cell and CD56+/CD3+ NK-like T Cell function differentially from IL-12. Int Immunol 2008.

[28] Rayamajhi M, Humann J, Penheiter K, Andreasen K, Lenz LL. Induction of IFN-α/β enables Listeria monocytogenes to suppress macrophage activation by IFN-γ. J Exp Med 2010;207:327-37.

[29] Liu BS, Janssen HL, Boonstra A. IL-29 and IFN-α differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFN-γ receptor expression. Blood 2011;117:2385-95.

[30] Hua LL, Kim MO, Brosnan CF, Lee SC. Modulation of astrocyte inducible nitric oxide synthase and cytokine expression by interferon-β is associated with induction and inhibition of interferon-γ-activated sequence binding activity. J Neurochem 2002;83:1120-8.

[31] Gao JJ, Filla MB, Lorsbach RB, Pace JL, Crespo A, Russell SW et al. Prolonged exposure of mouse macrophages to IFN-β suppresses transcription of the inducible nitric oxide synthase gene: altered availability of transcription factor Stat1a. Eur J Immunol 2000;30:1551-61.

[32] Ballinger MN, Standiford TJ. Postinfluenza bacterial pneumonia: host defenses gone awry. J Interferon Cytokine Res 2010;30:643-52.

[33] Farah R, Awad J. The association of interferon with the development of pulmonary tuberculosis. Int J Clin Pharmacol Ther 2007;45:598-600.

[34] Sabbatani S, Manfredi R, Marinacci G, Pavoni M, Cristoni L, Chiodo F. Reactivation of severe, acute pulmonary tuberculosis during treatment with pegylated interferon-α and ribavirin for chronic HCV hepatitis. Scand J Infect Dis 2006;38:205-8.

[35] Telesca C, Angelico M, Piccolo P, Nosotti L, Morrone A, Longhi C et al. Interferon-α treatment of hepatitis D induces tuberculosis exacerbation in an immigrant. J Infect 2007;54:e223-e226.

CHAPTER 8

The influence of influenza virus infections on the development of tuberculosis

Roelof A. de Paus[1], Reinout van Crevel[2], Ruud van Beek[3], Edhyana Sahiratmadja[4], Bachti Alisjahbana[5], Sangkot Marzuki[6], Guus F. Rimmelzwaan[3], Jaap T. van Dissel[1], Tom H.M. Ottenhoff[1] and Esther van de Vosse[1].

[1] Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands.
[2] Department of Internal Medicine, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands.
[3] Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands.
[4] Health Research Unit, Faculty of Medicine University of Padjadjaran, Bandung, Indonesia.
[5] Division of Tropical and Infectious Diseases, Internal Medicine Faculty of Medicine University of Padjadjaran, Bandung, Indonesia.
[6] Eijkman Institute for Molecular Biology, Jakarta, Indonesia.

Accepted for publication in Tuberculosis

Abstract

Recently, it was shown that interferon-γ mediated immune responses, which play a major role in the control of infection with *Mycobacterium tuberculosis* (Mtb), can be inhibited by type I interferons. Since type I interferons are abundantly induced during viral infections, we hypothesized that infections with influenza viruses might play a role in the development of active tuberculosis (TB) disease either directly after exposure to Mtb or through reactivation of latent Mtb-infection. To explore this hypothesis we investigated in a retrospective study whether newly diagnosed adult TB patients from Indonesia had had recent influenza infection. Plasma samples from TB patients and controls were assayed for antibodies against two subtypes of at that time relevant, seasonal influenza A viruses. Overall, no correlation was observed with the presence of antibodies and manifest tuberculosis. Still, antibody titers against circulating A/H3N2 influenza virus were slightly enhanced in tuberculosis patients as compared to controls, and highest in cases of advanced tuberculosis. This suggests that tuberculosis patients were recently infected with influenza, before clinical manifestation of the disease. Alternatively, the production of antibodies and susceptibility to tuberculosis may be influenced by a common confounding factor, for example the ability of patients to induce interferon-α. We conclude that in an endemic country like Indonesia, an influenza virus infection is not a major determinant for developing clinically manifest tuberculosis.

1. Introduction

Tuberculosis (TB) is a severe disease caused by *Mycobacterium tuberculosis* (Mtb). Up to 10% of the Mtb infected individuals develop active tuberculosis, while the majority of those infected develop a latent state of infection for years [1, 2]. During latency, Mtb may stay quiescent, but when the immune system fails to control the bacteria, reactivation may occur and precipitate development of active disease [3, 4], in most cases pulmonary TB disease.

Several risk factors that influence the susceptibility to TB have been described, such as host genetic factors [5], malnutrition [6], smoking [7], diabetes [8] and infection with HIV [9]. Besides these factors also the Mtb strain virulence may influence the course of TB, since virulent Mtb can inhibit the host immune system in various ways [10]. Recently, IFN-α has been described as a putative factor, which may be induced by highly virulent Mtb strains [11, 12] and can inhibit an effective IFN-γ mediated immune response [13-15]. Mouse studies revealed that during Mtb infection IFN-α is induced and that IFN-γ mediated immune responses can be impaired by IFN-α [12, 16]. In humans, a typical IFN-α/β transcript signature was found in the blood cells from TB patients [11, 17, 18]. However, it is not clear whether Mtb infection in humans leads to the production of type I interferons or whether production of type I interferons leads to TB. It is possible that in TB patients type I interferons are more abundantly induced due to infections with viruses, such as pneumotropic influenza viruses.

After influenza virus infection a period of enhanced susceptibility to bacterial infections is commonly seen in humans [19]. In mice, Toll like receptor-induced responses of alveolar macrophages to bacterial ligands remain desensitized for months after an influenza virus infection [20]. This explains why mice are highly susceptible to bacterial pneumonia for several weeks after influenza virus infection [21]. In humans, post-influenza bacterial pneumonia is a major cause of morbidity [19], with *Streptococcus pneumonia* as the main pathogen associated with post-influenza pneumonia [22]. TB is usually not diagnosed shortly after influenza infections and although some anecdotal reports suggest that the occurrence of TB was also high during influenza pandemics [23-25], a causal relationship between the epidemics of the two infectious diseases has not been investigated.

If indeed an influenza virus infection leads to the (re)-activation of a latent Mtb infection, this may remain unnoticed, because the period between (re)-activation of the bacteria and the first presentation of the clinical symptoms of TB is long. TB develops slowly, due to the slow metabolism and replication rate of Mtb. Thus it may be that within the latency period of TB, during the primary infection or after re-infection, a transient inhibitory effect on the anti-bacterial responses by an influenza virus infection influences the course of TB and leads to active disease, possibly in conjunction with other risk factors. In mice, co-infections of influenza viruses with Mtb enhanced the development of TB in the lungs [26]. In humans, it is still unclear whether influenza virus infections can influence the course of TB.

We hypothesized that influenza virus infections may promote the development of active disease after exposure to Mtb, or might play a role in the reactivation of latent Mtb-infection. To explore this hypothesis we investigated in a retrospective study whether patients with clinically-manifest TB had an influenza virus infection recently. Plasma samples collected from TB patients at time of diagnosis and from controls were screened for the presence of antibodies against influenza viruses in order to investigate a putative association between TB and influenza virus infections.

2. Patients, materials and methods

2.1. Study subjects

Patients and controls (Table 1) were recruited between March 2001 and December 2004 from the TB clinic "Perkumpulan Pemberantasan Tuberkulosis Indonesia" in Jakarta [8, 27, 28]. Patients newly diagnosed with active pulmonary TB, between the age of 15 and 70, were included. TB diagnosis was based on WHO definitions including the presence of clinical symptoms, a chest X-ray examination (CXR), microscopic detection of acid-fast bacilli in sputum and a positive culture of Mtb. Based on the CXR examinations TB patients were classified into two groups; patients with mild to moderate TB and patients with advanced TB. Patients seropositive for HIV were excluded. Community control subjects were recruited from neighboring houses and matched for age, sex and socio-economic class. Controls with a history of TB or with positive TB finding in the CXR were excluded. The control subjects underwent the same examinations as the patients, but were not tested for HIV, since the prevalence of HIV in the Indonesian population was low, as evidenced by the low prevalence of HIV amongst the TB patients in this cohort (1.8%) [29]. The influenza vaccination status of our study cohort is unknown. However, at the time of our study, vaccination against influenza viruses in Indonesia was only rarely applied and based on the low socio-economic status of our patients and matched controls these individuals are extremely unlikely to have received such vaccinations. For this study, the patients and controls were matched for the date of inclusion. Patients and matched controls were only included if the dates of inclusion were not more than 14 days apart. Written informed consent was obtained from all subjects. The study was approved by the Ethical Committee of the Medical Faculty, University of Indonesia.

Table 1. Description of the study population.

	TB patients (n=111)	Controls (n=111)	p-value
age in years (median)	18-67 (31)	17-69 (35)	0.153[a]
gender; males	72 (65%)	60 (54%)	0.132[b]
diabetes mellitus	24 (22%)	8 (8%)	<0.001[b]
individuals with BCG scar	39 (35%)	45 (41%)	0.489[b]

[a] student t-test, [b] χ^2 test.

2.2. Detection of antibodies against influenza viruses

Heparinized plasma samples were obtained from the patients and controls and stored at –80°C. Thawed plasmas were analyzed for the presence of total IgG and IgM antibodies against two subtypes of influenza A virus, a H3N2 virus (A/Moscow/10/99, vaccine strain ResVir-17) and a H1N1 virus (A/New Caledonia/20/99, vaccine strain IVR-116), using the hemagglutinating inhibition (HI) test. These strains were chosen because of their high antigenic similarity to the specific H3N2 and H1N1 viruses circulating during the sample period. For use in the HI test, the influenza virus strains were propagated in 11-day old embryonated chicken eggs. The HI test was performed in duplicate according to standard methods [30] with turkey erythrocytes and four hemagglutinating units of virus. Ferret sera raised against the test antigens were used as positive controls. All plasma samples of all study subjects were tested simultaneously, in duplicate, and were only regarded positive when both analyses gave positive results. The threshold of detection was an HI titer ≥ 10.

2.3. Statistical analysis

Data were analyzed using SPSS software. The Pearson χ^2 test and the paired samples t-test were regarded significant when $p < 0.05$.

3. Results

3.1. No correlation between the incidence of tuberculosis and the seroprevalence of antibodies against influenza viruses

To study the effect of influenza virus infections on TB we examined the plasma samples of controls and newly identified TB patients for the presence or absence of antibodies against two subtypes of influenza A virus, H1N1 and H3N2 (Table 2). The threshold of detection was an HI titer ≥10. Both influenza strains circulated in the population during the time of plasma sampling: 46% of the TB patients and 41% of the controls had antibodies against H1N1, while 82% of the TB patients and 82% of the controls had antibodies against H3N2 (Table 2). No significant differences in the number of individuals with antibodies against influenza viruses were thus observed between the control group and the group of TB cases.

Table 2. Seroprevalence of antibodies to A/H1N1 and A/H3N2 influenza viruses.

Group	H1N1 positive	H3N2 positive
TB patients (n=111)	51 (46%)	91 (82%)
controls (n=111)	46 (41%)	91 (82%)
Total (n=222)	97 (44%)	182 (82%)
χ^2 test; p-value	p=0.499	p=1.000

The threshold of detection was an HI titer of ≥ 10.

3.2. Titers of antibodies to influenza viruses correlated with the manifestation of tuberculosis
Because yearly influenza infection rates are estimated to be 5-10% [31] the high percentage of individuals with H1N1 and H3N2 antibodies indicate that many of these individuals were already exposed to these viruses in the past. Therefore, the magnitude of the antibody responses might be a better indication of recent exposure as IgG antibody levels will be boosted upon reinfection and will only wane partially. Hence, we examined the titers of antibodies against H1N1 and H3N2 influenza viruses in the TB and control group. The results are displayed in Table 3 and Figure 1. The geometric means of the titers of antibodies against the H3N2 and the H1N1 influenza viruses were higher (respectively 1.7 and 1.4 times) for the total group of pulmonary TB patients, as compared to the titers of the control group. Statistical analysis using the paired sample t test showed that only for the titers of antibodies against H3N2 this difference was significant. We next determined whether there is a correlation between the severity of the disease and the antibody titers against H3N2. The cases of advanced pulmonary TB showed a higher geometric mean titer for H3N2 (1.8 times), while the group of cases with mild and moderate TB showed a smaller increase (1.4 times) in the geometric mean titer, as compared to the matched controls.

The difference in these titers was significant between the cases with advanced TB and their matched controls but not between the cases of mild to moderate TB and their matched controls. The geometric mean titer of antibodies against H3N2 influenza viruses for the extrapulmonary TB patients was even higher (2.0 times as compared to the matched controls). However, the latter patient group was small (n=10) and the antibody titers of the cases with extrapulmonary TB were not significantly different from the titers of their matched controls.

Table 3. Number of positive cases and mean antibody titers against A/H3N2 and A/H1N1 influenza viruses for the different groups of TB patients and their matched controls.

	H3N2			H1N1		
	n	mean[a]	p-value[b]	n	mean[a]	p-value[b]
total TB patients	72	244	0.002	25	72	0.330
matched controls	72	145		25	51	
mild and moderate TB	33	211	0.152	11	52	0.995
matched controls	33	147		11	52	
advanced TB	33	262	0.021	14	93	0.222
matched controls	33	142		14	50	
only pulmonary TB	62	234	0.004	21	69	0.267
matched controls	62	143		21	45	
extrapulmonary TB	10	319	0.289	4	92	0.986
matched controls	10	157		4	94	
TB patients without DM	55	271	0.030	20	70	0.496
matched controls without DM	55	148		20	54	

[a] geometric mean.
[b] paired student t-test.

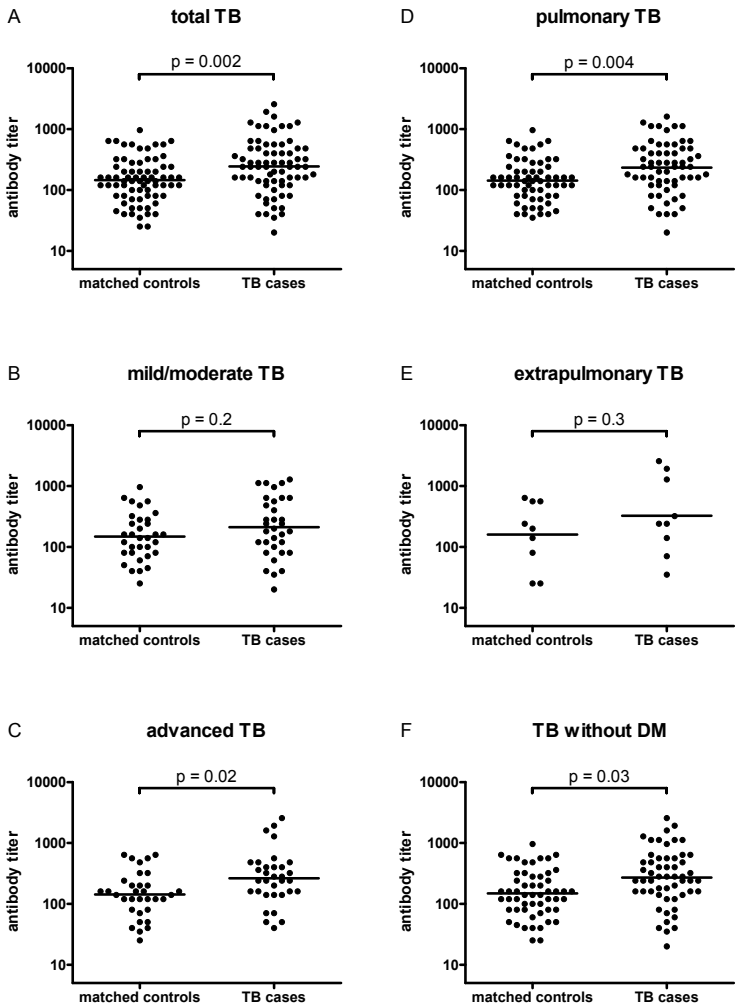


Figure 1. Antibody titer against A/H3N2 in the different groups from the Indonesian TB cohort. The antibody titers against A/H3N2 were determined for the groups of TB cases and their matched controls. The data of the TB cases and the paired controls, matched for the date of inclusion, that were both found positive for the presence of antibodies are displayed. The group of total TB cases (A) was divided into a group with mild to moderate pulmonary TB (B) and a group of cases with advanced pulmonary (C) according to CXR examinations. In a separate analysis the group of total TB cases was divided in a group of cases with only pulmonary TB (D) and a group of cases with signs of extrapulmonary TB (E). In addition, the TB cases without diabetes mellitus (DM) were compared with their matched controls without DM (F). The geometric mean of the antibody titers is indicated with a line. Cases were compared with their matched controls and statistical analysis was performed on logarithmic values of the antibody titers using the t-test for matched pairs.

3.3. The influence of diabetes mellitus on tuberculosis and the titers of antibodies to influenza viruses

In a previous study from our group, with the large cohort of patients and controls from Indonesia, Diabetes mellitus (DM) was identified as a risk factor for TB [8]. Since having DM is correlated with the incidence of TB in this study (Table 1) we investigated whether having DM could be a factor of influence on influenza virus specific antibody levels. Hence, we compared the influenza virus specific antibody titers between the TB cases and their matched controls, while excluding individuals with DM from both groups.

The group of patients with pulmonary TB and without DM showed still significantly higher amounts of antibodies against H3N2 as compared to their matched controls, (paired sample t test, Table 3 and Figure 1F). The mean titer of antibodies against H3N2 of the TB cases with DM was on the other hand significantly lower (student t test, $p=0.049$) as compared with the mean titer of the TB cases without DM (data not shown).

4. Discussion

The main finding of this report is that there is no correlation between the seroprevalence of antibodies against influenza A viruses and the development of clinically active TB in an Indonesian cohort. However, we observed an association between the level of antibody titers against influenza A/H3N2 virus and the stage of active TB lung disease. Compared to the control group the mean antibody titer of the group of TB patients was slightly enhanced, which may indicate that the TB patients were recently re-infected with an influenza virus strain, suggesting that an influenza virus infection precedes and influences the clinical manifestation of TB. However, such an epidemiological association may not be causal, and be confounded.

Previously, it was suggested that an association existed between the 20th century influenza pandemics and the incidence of TB [23-25]. The existence of this correlation can however be questioned because diagnoses in the early 20th century were based solely on clinical symptoms. In addition, an increased incidence of both diseases may also be explained by common susceptibility factors, such as malnutrition. Here, we tested the hypothesis that influenza virus infections enhance the susceptibility to develop active TB. Although we did not find an association between the number of people with antibodies against influenza viruses and the development of TB, the difference in influenza A/H3N2 virus specific antibody titers between TB cases and controls may suggest that more TB cases than controls were recently re-infected, or have been exposed more often, with these seasonal influenza viruses. A drawback of our study is the high (pre-existing) seroprevalence of the antibodies

against H3N2. Detection of antibodies against specific epitopes of the most recent influenza subtypes was unfortunately not possible, due to the low antigenic drift between the latest seasonal influenza viruses.

Within the group of TB patients the cases with extrapulmonary manifestation of the disease showed the highest mean antibody titers against A/H3N2 viruses. This association suggests that the clinical manifestation of extrapulmonary infections occurs in TB patients with stronger antibody responses after influenza virus infections. Dissemination of mycobacterial infections are more common in hosts with impaired immunity, for example in MSMD patients with impaired IFN-γ mediated immune responses [32] or in HIV patients [33]. In patients with extrapulmonary TB the plasma concentrations of IFN-γ are also reduced, as compared to patients with pulmonary TB [34]. Therefore, we speculated that viruses other than HIV, like influenza viruses, affect the Th1 immunity against mycobacteria and in this way influence the course of TB. In mice it was indeed found that influenza virus infection aggravates the course of TB [26]. It is unclear whether this is also the case in humans. Our cohort was too small to assess whether the risk of extrapulmonary manifestation is increased due to influenza virus infections.

The finding that TB patients had slightly higher plasma antibody levels to A/H3N2 influenza virus than the control group suggests a relation exists between influenza virus infections and the clinical manifestation of tuberculosis. However, the association may very well be confounded. One possible confounding factor is that a co-infection with an influenza virus in patients with TB may be more severe or lasts longer due to lung damage by Mtb, and as a result more antibodies against the virus are generated. Another possible confounding factor is that Mtb infection may result in the polyclonal activation of B cells, as was observed in mice after infection with various microorganisms [35]. Human memory B cells were also found, in response to a strong stimulus, to polyclonally produce small amounts of antibodies in the absence of specific antigens [36]. Whether such non-specific antibody production also occurs during Mtb infection and results in a detectable rise in anti-influenza antibodies is yet unknown. Since plasma of TB patients before the manifestation of the disease is unavailable we were unable to determine whether the anti-influenza antibodies rise upon development of TB. The ability to produce high amounts of IFN-α may also be a confounding factor. Some individuals are relatively strong producers of type I interferons [37] and because of this they may produce both higher amounts of antibodies against viral pathogens and may be more susceptible to developing TB. In addition, TB may be aggravated if Mtb induced type I interferons are also more abundant in these individuals, as they may impair an effective IFN-γ mediated immune response [11]. Another potential confounding factor may be diabetes mellitus (DM) since DM was previously found to be a risk factor for developing TB [8]. We investigated whether DM cases had higher antibody titers against the viruses. Based on our results, we could exclude DM as a confounding factor.

Our findings are based on a retrospective study with TB cases in Jakarta (Indonesia) in the period from March 2001 till December 2004. This is the first study which investigates a potential role for viral infections in the development of TB by determining the presence of antibodies against influenza viruses in plasmas of TB patients and matched controls. In order to determine whether the observed association between TB and higher virus-specific antibody titers indeed indicates an increased risk for TB after influenza virus infection and to exclude that confounding factors cause the increase in the antibody response to influenza viruses, a prospective study is needed. Although such a study would be difficult to set up, this should preferably investigate the influence of primary viral infections on the manifestation of TB in childhood. In such a cohort it would be possible to prove new, primary influenza virus infections.

A prospective study for adult TB is also possible, but because many adults already have antibodies against previous seasonal viruses (that may cross-react with contemporary viruses) novel infections will have to be identified by demonstrating antibody titers rises against a new seasonal strain. The current study was cross sectional and identified a high seroprevalence of antibodies against two subsubtypes (44% and 82% for H1N1 and H3N2 respectively) indicating that a large proportion of study subjects were infected during previous epidemics, as normally about 5 to 10% of the population are affected during annual influenza epidemics [31]. Studies of larger cohorts and screening for strain-specific antibodies may provide more insights into the effects of co-infections with influenza viruses on the occurrence and severity of TB.

Based on our results we conclude that influenza virus infections are not a major determinant in the development of clinically active TB in adults, either through reactivation of latent disease or directly after exposure to the bacillus, nor of more severe manifestation of the disease. The identified correlation between the titers of antibodies against influenza viruses and the manifestation of TB, is still in line with the suggestion that type I interferons may play a role in the immunopathogenesis of TB.

Funding: This study was supported partly by a grant from the Royal Netherlands Academy of Arts and Sciences (KNAW99MED01) and received supplementary support from NWO-PRIOR. The research also received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) for projects NEWTBVAC; IDEA; and ADITEC (No: 280873).

References

[1] World Health Organization: WHO report 2009. Global tuberculosis control - epidemiology, strategy, financing. website: who int/tb/publications/global_report/2009/en/index html 2009.

[2] Ottenhoff THM, Kaufmann SHE. Vaccines against tuberculosis: where are we and where do we need to go? PloS Pathogen 2012;85:e1002607.

[3] Ehlers S. Lazy, dynamic or minimally recrudescent? On the elusive nature and location of the mycobacterium responsible for latent tuberculosis. Infection 2009;37:87-95.

[4] Cardona PJ. A dynamic reinfection hypothesis of latent tuberculosis infection. Infection 2009;37:80-6.

[5] Ottenhoff TH, Verreck FA, Hoeve MA, van de Vosse E. Control of human host immunity to mycobacteria. Tuberculosis (Edinb) 2005;85:53-64.

[6] Cegielski JP, McMurray DN. The relationship between malnutrition and tuberculosis: evidence from studies in humans and experimental animals. Int J Tuberc Lung Dis 2004;8:286-98.

[7] Bates MN, Khalakdina A, Pai M, Chang L, Lessa F, Smith KR. Risk of tuberculosis from exposure to tobacco smoke: a systematic review and meta-analysis. Arch Intern Med 2007;167:335-42.

[8] Alisjahbana B, van Crevel R, Sahiratmadja E, den Heijer M, Maya A, Istriana E et al. Diabetes mellitus is strongly associated with tuberculosis in Indonesia. Int J Tuberc Lung Dis 2006;10:696-700.

[9] Aaron L, Saadoun D, Calatroni I, Launay O, Memain N, Vincent V et al. Tuberculosis in HIV-infected patients: a comprehensive review. Clin Microbiol Infect 2004;10:388-98.

[10] Ottenhoff THM. New pathways of protective and pathological host defense to mycobacteria. Trends in Immunology 2012;In press.

[11] Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 2010;466:973-7.

[12] Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, Murray PJ et al. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res 2005;25:694-701.

[13] Nagai T, Devergne O, van Seventer GA, van Seventer JM. Interferon-β mediates opposing effects on interferon-γ-dependent Interleukin-12 p70 secretion by human monocyte-derived dendritic cells. Scand J Immunol 2007;65:107-17.

[14] de Paus RA, Van Wengen A, Schmidt I, Visser M, Verdegaal EME, van Dissel JT et al. Inhibition of type I immune responses of human monocytes by interferon-α and interferon-β. Submitted 2012.

[15] van de Wetering D, Van Wengen A, Savage ND, van de Vosse E, van Dissel JT. IFN-α cannot substitute lack of IFN-γ responsiveness in cells of an IFN-γR1 deficient patient. Clin Immunol 2011;138:282-90.

[16] Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-α /β. Proc Natl Acad Sci U S A 2001;98:5752-7.

[17] Maertzdorf J, Weiner J, III, Mollenkopf HJ, Bauer T, Prasse A, Muller-Quernheim J et al. Common patterns and disease-related signatures in tuberculosis and sarcoidosis. Proc Natl Acad Sci U S A 2012;109:7853-8.

[18] Ottenhoff THM, Hari Dass R, Yang N, Zhang M, Wong HEE, Sahiratmadja E et al. Genome-wide expression profiling identifies Type I interferon response pathways in active tuberculosis. accepted for publication in PlosOne 2012;7 (9):e45839.

[19] Ballinger MN, Standiford TJ. Postinfluenza bacterial pneumonia: host defenses gone awry. J Interferon Cytokine Res 2010;30:643-52.

[20] Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, Bebien M et al. Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. J Exp Med 2008;205:323-9.

[21] McCullers JA, Rehg JE. Lethal synergism between influenza virus and Streptococcus pneumoniae: characterization of a mouse model and the role of platelet-activating factor receptor. J Infect Dis 2002;186:341-50.

[22] McNamee LA, Harmsen AG. Both influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary Streptococcus pneumoniae infection. Infect Immun 2006;74:6707-21.

[23] Housworth J, Langmuir AD. Excess mortality from epidemic influenza, 1957-1966. Am J Epidemiol 1974;100:40-8.

[24] Lofgren S, Callans A. Asian influenza and pulmonary tuberculosis. Acta Med Scand 1959;164:523-7.

[25] Noymer A. The 1918-19 influenza pandemic affected tuberculosis in the United States: reconsidering Bradshaw, Smith, and Blanchard. Biodemography Soc Biol 2008;54:125-33.

[26] Volkert M, Pierce C, Horsfall FL, Dubos RJ. The enhancing effect of concurrent infection with pneumotropic viruses on pulmonary tuberculosis in mice. J Exp Med 1947;86:203-14.

[27] Sahiratmadja E, Alisjahbana B, Buccheri S, Di Liberto D, de Boer T, Adnan I et al. Plasma granulysin levels and cellular γ production correlate with curative host responses in tuberculosis, while plasma interferon-γ levels correlate with tuberculosis disease activity in adults. Tuberculosis (Edinb) 2007;87:312-21.

[28] Sahiratmadja E, Alisjahbana B, de Boer T, Adnan I, Maya A, Danusantoso H et al. Dynamic changes in pro- and anti-inflammatory cytokine profiles and γ interferon receptor signaling integrity correlate with tuberculosis disease activity and response to curative treatment. Infect Immun 2007;75:820-9.

[29] van Crevel R, Parwati I, Sahiratmadja E, Marzuki S, Ottenhoff TH, Netea MG et al. Infection with Mycobacterium tuberculosis Beijing genotype strains is associated with polymorphisms in SLC11A1/NRAMP1 in Indonesian patients with tuberculosis. J Infect Dis 2009;200:1671-4.

[30] World Health Organization: Global Influenza Surveillance Network. Manual for the laboratory diagnosis and virological surveillance of influenza 2011. website: who int/influenza/resources/documents/manual_diagnosis_surveillance_influenza/en/index html 2011.

[31] World Health Organization. Influenza. website: www who int/biologicals/vaccines/influenza/en/ 2012.

[32] van de Vosse E, Hoeve MA, Ottenhoff TH. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. Lancet Infect Dis 2004;4:739-49.

[33] Yang Z, Kong Y, Wilson F, Foxman B, Fowler AH, Marrs CF et al. Identification of risk factors for extrapulmonary tuberculosis. Clin Infect Dis 2004;38:199-205.

[34] Hasan Z, Jamil B, Khan J, Ali R, Khan MA, Nasir N et al. Relationship between circulating levels of IFN-γ, IL-10, CXCL9 and CCL2 in pulmonary and extrapulmonary tuberculosis is dependent on disease severity. Scand J Immunol 2009;69:259-67.

[35] Montes CL, Acosta-Rodriguez EV, Merino MC, Bermejo DA, Gruppi A. Polyclonal B cell activation in infections: infectious agents' devilry or defense mechanism of the host? J Leukoc Biol 2007;82:1027-32.

[36] Lanzavecchia A, Bernasconi N, Traggiai E, Ruprecht CR, Corti D, Sallusto F. Understanding and making use of human memory B cells. Immunol Rev 2006;211:303-9.

[37] Katschinski DM, Neustock P, Kluter H, Kirchner H. Influence of various factors on interferon-α production in cultures of human leukocytes. J Interferon Res 1994;14:105-10.

CHAPTER 9

Summary and general discussion

Type I cytokine responses play an essential role in the control of mycobacterial infections. Mutations in genes involved in the type I cytokine pathway were found in patients with Mendelian susceptibility to mycobacterial disease (MSMD) [1]. These patients are highly susceptible to infections with non-tuberculous mycobacteria (NTM). NTM are usually poorly pathogenic, but can cause severe disseminated infections in MSMD patients. MSMD causing mutations have been found in genes of the type I cytokine pathway, for example in *IL12B, IL12RB1, IFNGR1* and *IFNGR2*. The role of genetic host factors in the susceptibility to virulent *Mycobacterium tuberculosis complex* strains is less evident. Likely, other host-factors, but also environmental factors and virulence factors of the mycobacterium, play an important role in the susceptibility to tuberculosis. Suppression of type I immune response by medication enhances also the susceptibility. For instance, patients treated with TNF blocking antibodies such as infliximab, effectively abrogating the effector arm of the type-I immune response including macrophage activation, and cell recruitment to and formation of granulomas, are exquisitely susceptible to mycobacterial infection. This thesis focuses on the relation between genotype and phenotype in the cause of an impaired type-I immunity, leading to the susceptibility to non-tuberculous and tuberculous mycobacterial diseases.

1. Functional consequences of amino acid substitutions in IL-12Rβ1

MSMD patients develop severe disseminated infections with NTM and sometimes non-typhoid *Salmonella,* usually in but certainly not confined to early childhood. The susceptibility of MSMD patients to these intracellular bacteria is strongly enhanced due to mutations in genes involved in the type-I cytokine pathway, for example in *IL12RB1*. Many harmless polymorphisms and MSMD causing mutations in *IL12RB1* are reported, but also variations with an unknown impact on gene and protein function. Amongst them several single nucleotide substitutions leading to amino acid substitutions are described.

Chapter 2 describes the case of an adult patient with a severe disseminated infection of the NTM *M. genavense*. Immunological screenings indicated a severe defect in IL-12Rβ1 function. Subsequent genetic screening revealed the presence of two variations, -2C>T and R521G, with unknown impact on the immunological phenotype. Using a cellular model, with retroviral expression of IL-12Rβ1 variants in cells from a complete IL-12Rβ1 deficient patient, the R521G variation was confirmed to be the mutation leading to impaired IL-12 and IL-23 responses. Remarkably, the patient developed the infection at the age of 43 and had no history of severe infections in the past. Usually, infections in MSMD patients occur in early childhood for the first time, at a mean age of 2.4 years [2], although it has also been reported that individuals, homozygous for an IL-12Rβ1 mutation, have remained asymptomatic for many years [1]. The IL-12Rβ1 deficient individuals may remain free of symptoms due to infection for several reasons. Firstly, before an infection to develop, individuals must be exposed to the pathogen. Thus, lack of exposure to NTM may play a role here, although

these low virulent pathogens are ubiquitous and hard to escape; still the level of exposure may vary according to habits and local environment. Moreover, immunological mechanisms compensating for the defect may be active that preclude a clinical manifestation for years. Later in life, subtle, innate reductions in host defense may now become manifest as the function of organs and compensatory immunological mechanisms become compromised due to aging and the cumulative effect of bad habits like smoking, drinking, or nutritional deficiencies. Consequently, clinicians should be aware that a severe disseminated NTM infection due to MSMD may manifest for the first time later in life, even at middle age.

With the use of the same cellular model, with which the R521G mutation was characterized, also other subtle variations in the *IL12RB1* gene were investigated. In the **addendum** to **chapter 2** a summary is given of the impact to immunological phenotype of all currently known variations resulting in amino acid substitutions. Several variations were confirmed to be deleterious mutations causing MSMD, while other variations were proven to be harmless polymorphisms without impact on immunological phenotype. Thus far, all known mutations in *IL12RB1* show a complete or an almost complete dysfunction of the receptor. The two mutations leading to a partial deficiency, i.e., C198R and R521G, reduced the cell surface expression of IL-12Rβ1 by 250 or 450 times, respectively. This suggests that the IL-12Rβ1 function has to be strongly reduced to impair the formation of sufficient immunity against intracellular bacteria.

Most amino acid substitutions in IL-12Rβ1 characterized as mutations, lead to absent or severely reduced cell surface expression of IL-12Rβ1. This indicates that determining the IL-12Rβ1 expression of cells from a putative MSMD patient can already give a first indication of a deleterious mutation in the receptor. Still, it is necessary to complete the genetic analysis and perform an immunological phenotypic test to determine the IL-12 responsiveness, because mutations in the intracellular part may affect receptor function but not the cell surface expression, as was proven to be the case with an artificial mutation leading to a truncated receptor which lacks the intracellular domain.

The two major haplotypes of *IL12RB1*, designated as RTR and QMG, are formed by three linked amino acid changes, known as R214Q, T365M and R378G [3]. The protein product of the QMG haplotype, the QMG variant, is slightly higher expressed and is slightly more responsive to IL-12 stimulation as compared to the RTR variant [4]. The IL-23 responses of both variants were similar (chapter 3). Thus, analyzing these functional haplotypes in genetic association studies may give information about a putative role for IL-12 in the protection or development of type-I cytokine immune diseases. Such an association was found in a study with a Japanese cohort of tuberculosis patients, in which the RTR allele appears to contribute to the susceptibility to tuberculosis [3].

2. The direct biological effects of IL-23 and variations in the IL-23R

Thus far, all reported patients with a defect in IL-12 or the IL-12R function carry a mutation in respectively IL-12p40 or IL-12Rβ1, while no mutations are found in the IL-12Rβ2 and the IL-23R chains, or in the IL-12p35 and IL-23p19 cytokine subunits of the heterodimers IL-12 and IL-23, respectively. Since IL-12p40 is also a subunit of the IL-23 cytokine and IL-12Rβ1 is the IL-12p40 -binding subunit of the IL-23R complex, this suggests that both IL-12 and IL-23 mediated immunity are impaired in enhanced susceptibility to NTM infections. Thus, only a defect in both cytokines or both receptors results in a MSMD phenotype. Probably, both cytokines complement each others actions, with subtle differences in function, e.g., in the induction of IFN-γ production by T-cells.

IL-12 is known to play a pivotal role in the type-I immune responses of various subsets of T cells. In contrast, the role of IL-23 in the establishment of a type-I immune response is less clear, because little is known about the expression pattern of the specific IL-23R chain. More insight in the role of IL-23 may give clues for novel therapeutic interventions. In **chapter 3** the effects of IL-23 on human T cell blasts (TCB), which were retrovirally transduced with the *wild type* IL-23R or a variant of the receptor, were investigated. With the use of these TCB a comparison was made between the effects of IL-12 and IL-23 within the same type of cell. Both cytokines are only able to induce effects in the TCB after TCR stimulation and differ in their elicited signal transduction via STAT modules. IL-12 signals with the use of STAT4, while IL-23 signals via its own receptor with the use of STAT1, STAT3 and STAT4. These data confirm the previous data on IL-23 signaling in the human Kit225 cell line, derived from a T cell leukemia [5]. However, in this cell line also a slight signaling via STAT5 was observed, which was not observed in the TCB cells. In primary $CD3^+$ $CD56^+$ T cells IL-23 induced only STAT3 and STAT4 phosphorylation, while STAT1 and STAT5 phosphorylation was not observed. Thus, the effects of IL-23 may depend on the type of T cell, and may depend on co-stimulations with other factors.

Remarkably, IL-23 elicited an IL-12 like response in the TCB cell lines without the retroviral expression of the IL-23R. In these cells, IL-23 elicited only STAT4 signaling, resulting in a slight IFN-γ production, which was about 50 times less as compared to IL-12 stimulation. This may indicate that IL-23 also signals via another receptor, possibly IL-12Rβ2, although with lower efficiency as compared to the IL-12 responses via the IL-12R complex.

Another interesting finding is that both IL-23 and IL-12 were shown to be able to induce the production of IFN-γ as well as the production of the anti-inflammatory cytokine IL-10, albeit with different efficiency. IL-23 induced less IFN-γ and relatively more IL-10. Probably, there is a differential binding of STAT modules on the promoters of IFN-γ and IL-10 upon IL-23 stimulation, as compared to IL-12 stimulation. These data are in line with the results of a previous report that IL-23 can enhance the IL-10 production of polyclonal stimulated naïve

T cells [6]. Apparently, both IL-12 and IL-23 have the capacity to regulate the establishment of a type-I immune response but in addition have anti-inflammatory capacity. Thus, the influence of IL-23 on the establishment of a type-I immune response is also regulated by the presence of IL-10 producing T cells. The finding that IL-23, compared to IL-12, induces less IFN-γ and relatively more IL-10 and the fact that IL-23 can be rapidly produced in the early stage of the immune response [7, 8], followed by the production of IL-12 in later stages may indicate that the type-I immune responses are gradually upregulated, e.g., 'pre-amplified' by IL-23.

In the cellular model with retroviral IL-23R expression, the function of the wild type IL-23R is compared with other IL-23R variants; Y173H, P310L and R381Q. The Y173H variant was found in a patient with intracranial abscesses and with sepsis due to a *Salmonella* infection. Although the Y173H variation was predicted to be a severe mutation, the functional characterization of the variation revealed that it does not affect the IL-23 response, and therefore is unlikely to be the cause of disease in the patient. The P310L and R381Q are common variations, which were previously found to be associated with other immune related diseases. Neither of these variations in the IL-23R had an impact on the IL-23 responses. Thus, these variations are considered to be harmless polymorphisms in the setting of NTM or *Salmonella* infections.

Recently, an antibody against the human IL-23R was generated [9]. This antibody recognizes both the wild type and the R381Q variant. The R381Q variation was reported to be associated with a decreased number of IL-23R positive cells, not with a reduced capacity of these cells to respond to IL-23 [9]. This is in line with our observation, that the R381Q variant of the receptor is fully functional. Still, it remains unclear why the R381Q variation is correlated with a decreased number of IL-23 responsive cells. It was reported that the R381Q polymorphism is strongly associated with protection against Inflammatory Bowel Disease [10]. The 381Q allele is correlated with protection against IBD. Taken together this indicates that the IL-23 responsive cells may play a role in the establishment of an auto-immune response. Thus, a reduced number of these IL-23 responsive cells may prevent the formation of a type-I immune response. It would be interesting to investigate in large cohorts whether the R381Q polymorphism is also associated with the susceptibility to tuberculosis.

After assessment of the effect of IL-23 on the TCB cell lines, we investigated the effects of IL-23 on primary cells. Because at the time of our investigations, no suitable antibody to detect the IL-23R was available, the presence of the IL-23R was determined by analyzing the IL-23 induced STAT3 phosphorylation directly after stimulation of T cell subsets with IL-23. **Chapter 4** reports the investigation of the direct biological effects of IL-23 on the type-I immune responses in cultures of $CD56^+$ cells, a mixture of $CD3^-$ $CD56^+$ NK cells and $CD3^+$ $CD56^+$ NK-like T cells. IL-23 elicited without any pre-stimulation STAT3 and STAT4 phosphorylation in NK-like T cells. Both IL-23 and IL-12 are able to induce IFN-γ

production, independent of TCR triggering, but dependent on IL-18. In contrast to IL-12, which is able to induce IFN-γ production in NK and NK-like T cells, IL-23 is only able to induce IFN-γ production in NK-like T cells, whereas both cytokines are able to induce the proliferation of NK and NK-like T cells. Since IL-23 can be rapidly produced by monocytes after stimulation with various TLR ligands [8] this indicates that IL-23 and NK-like T cells play an important role in the early phase of the immune response against pathogens, and that the production of initial, small amounts of IFN-γ boosts and amplifies the subsequent production and responses to IL-12.

Recent IL-23 research has focused on the IL-23/IL-17 axis. So far, there is a no evidence that IL-23 stimulates the IL-17 production directly, though IL-23 influences the proliferation and survival of IL-17 producing T cells [11]. In our IL-23 studies, we similarly could not detect an influence of IL-23 on production of IL-17, though we could detect other IL-23-induced responses. We showed direct effects of IL-23 on NK-like T cells, such as the IL-23 specific STAT3 phosphorylation and the IL-23 induced IFN-γ production. In contrast, IL-23 induces proliferation, but no IFN-γ production in NK cells. Taken together, this suggests that the proliferation of various subsets of T cells, such as the IL-17 producing T cells and NK cells, can be stimulated by IL-23, indirectly via NK-like T cells.

3. Functional consequences of amino acid substitutions in the IFN-γR

IFN-γ is the central effector cytokine in type-I immune responses, with pleiotropic effects on many cell types. By consequence, mutations in the IFN-γR have a major impact on the establishment of a type-I immune response. In the course of years, we and others have detected several amino acid substitutions in both chains of the IFN-γR. In order to investigate the functional consequences of these variations we developed two cellular models. Cell lines lacking IFN-γR1 or IFN-γR2 expression were retrovirally transduced with IFN-γR1 and IFN-γR2 variants, respectively. In this way the function of the receptor variants could be compared within the same genetic background.

Chapter 5 describes the functional characterization of amino acid variations in IFN-γR1, in order to make a distinction between polymorphisms and deleterious mutations. The amino acid substitutions V14M, V61I, S149L, H335P, I352M and L467P in IFN-γR1 appeared to be fully functional polymorphisms, with no major impact on receptor function. Interestingly, the S149L and I352M variations were heterozygously present in patients suspected to have MSMD, from which the blood cells showed a reduction in IFN-γ responsiveness. Since these variations were found to be functional polymorphisms, the reduced IFN-γ responsiveness of these patients cannot be explained by these variations in the receptor, but may be due to impaired signalling via STAT1 or due to other yet unknown factors. The amino acid substitutions V61E, V61Q, Y66C, C77F, C77Y and C85Y were deleterious mutations leading to complete IFN-γR1 deficiency, while the V63G and I87T mutations lead to partial IFN-γR1 deficiency.

Chapter 6 describes the evaluation of amino acid variations in IFN-γR2. The amino acid substitutions T58R, Q64R, E147K and K182E in IFN-γR2 were fully functional polymorphisms of IFN-γR2. On the other hand, the R114C, T168N and G227R variations were deleterious mutations with T168N leading to complete IFN-γR2 deficiency, while R114C and G227R lead to partial IFN-γR2 deficiency.

The partial deficiencies in IFN-γR1, due to the V63G and I87T mutations, are nearly complete deficiencies. For the V63G and I87T variants up to 10,000 times more IFN-γ was needed to obtain a similar effect on STAT1 phosphorylation of upregulation of CD64 or HLA-receptor expression, as compared with the wild type variant of IFN-γR1 [12]. This explains why the patients, homozygous for these mutations, had very severe mycobacterial infections [13, 14]. In contrast, patients heterozygous for the dominant 818delTTAA mutation in IFN-γR1, show relatively mild clinical symptoms. The 818delTTAA mutation leads to a truncated IFN-γR1 product, unable to signal, which is overexpressed at the cell surface. As a consequence, only 1-4% of the IFN-γR complexes are fully functional resulting in a dominant negative phenotype [15].

The partial deficiencies in IFN-γR2 due to the R114C [16] and the G227R mutation [17] resulted in a strong reduction of the IFN-γ responsiveness. In both cases, up to 100 times more IFN-γ was needed for the patient cells to produce similar responses as produced by the cells from healthy controls. The latter patients had, because of the strongly reduced IFN-γ responsiveness, severe disseminated NTM infections. Taken together, a deficiency in the IFN-γR2 leads to MSMD only when the receptor expression is reduced by a factor 100 or more.

The cellular models were used with success to investigate the impact of mutations and thus to reveal the nature of the IFN-γR deficiencies. However, the cellular models were less suitable to differentiate between more subtle variations with a small impact on receptor function. For example the V14M polymorphism in the IFN-γR1 showed normal function but was significantly less expressed on the cell surface. Due to the overexpression by the retroviral system, no differences in the IFN-γ responses were detected between cells expressing the wild type IFN-γR1 and the cells expressing the V14M variant. In this way, retroviral overexpression may mask subtle differences. In human subjects the V14M polymorphism was shown to correlate with an altered Th1/Th2 balance [18, 19]. This suggests that the influence of the V14M polymorphism on IFN-γR1 expression affects the IFN-γ responses in natural situations, where the IFN-γR1 expression is limited. Although it cannot be excluded that other polymorphisms linked to V14M are involved in the altered Th1/Th2 balance.

Both the V14M polymorphism in IFN-γR1 and the Q64R polymorphism in IFN-γR2 influenced the cell surface expression of the receptor chains remarkably. Consequently, these polymorphisms may have a significant impact on the Th1/Th2 balance, since the regulation of IFN-γR1 and IFN-γR2 expression and the strength of IFN-γ signaling play a decisive

role in the fate of T cells during the differentiation of Th0 cells [20, 21]. Therefore these two polymorphisms would be interesting to analyse in genetic association studies in order to investigate a putative role for IFN-γ or a lack of IFN-γ control in infection and immune related diseases. This was found to be the case for systemic lupus erythematosus [18, 19], but may also be true for infectious diseases such as tuberculosis.

4. Towards novel therapies to treat MSMD

MSMD patients with NTM infections are usually treated with antituberculous drugs, based on findings concerning the in vitro sensitivity of the mycobacterium. Unfortunately, in vitro susceptibility determinations of such pathogens is complex, and findings may not correlate with clinical response and outcome to treatment. Some patients with severe defects in IFN-γ signalling received haematopoietic stem cell transplantation (HSCT). However, HSCT is not without problems in patients with active NTM or *Salmonella* infections and also graft failures have been reported [22]. In some cases, the deficient type-I immune responses can be bypassed. For example, MSMD patients with a complete defect in IL-12p40, TYK2, IL-12Rβ1 or a partial defect in STAT1, IFN-γR1 or IFN-γR2 may benefit from treatment with high doses of IFN-γ in addition to antituberculous medication. Thus, rapid diagnosis of MSMD and investigation of the nature of the genetic defect as described in the **chapters 2, 5, 6** may help in the choice for this additional treatment. Several case reports describe the use of high doses of IFN-γ to treat MSMD patients. For example, a patient with a complete defect in IL-12Rβ1, who developed a severe disseminated *M. bovis* BCG infection, was treated with high doses of IFN-γ and showed remarkable clinical improvement without adverse effects [23]. During therapy, the mycobactericidal activity of the serum and the number of lymphocytes in the blood increased [23]. Another patient with a partial defect in IFN-γR2, who presented with a disseminated *M. abscessus* infection, showed no improvement on antituberculous therapy alone, but improved on adjunct IFN-γ therapy [16]. IFN-γ treatment is however not always successful. A report of two similar cases with disseminated *M. bovis* BCG infections, due to IL-12Rβ1 deficiency, shows successful remission after IFN-γ treatment in one case, while the other patient died despite IFN-γ treatment [16]. Still, the adjunct treatment with IFN-γ holds promise in the management of MSMD, especially in the early phase of the disease. Hence, early diagnosis may be necessary for the success of this treatment. Studying more MSMD patients in a multi-centre study would give valuable information about the effectiveness of high doses IFN-γ treatment.

Besides IFN-γ treatment, treatment with IFN-α was advocated by some and applied in a few cases. Additional IFN-α treatment was given to MSMD patients with manifestations of disseminated *M. avium* infections, although the benefit of this treatment was not clear. In the first reported case, of a patient with a complete IFN-γR1 deficiency, a reduction of the hepatosplenomegaly and lymphadenopathy was observed during treatment, although

mycobacteraemia did not ablate [24]. The latter can be explained by the fact that IFN-α in fact inhibits the type-I immune responses of monocytes [25]. The inhibitory effect of IFN-α on monocytes was further studied in detail in **chapter 7**. IFN-α was found to be a strong inhibitor of the type-I immune responses of monocytes. Thus, it could be that the residual type-I immune response in this patient was further impaired by IFN-α and that mycobacteraemia still occurred or was even aggravated. In another case report of a patient with complete IFN-γR2 deficiency, who developed a disseminated *M. avium* infection, the IFN-α therapy improved granuloma formation. Still the patient developed liver and bone lesions, and eventually succumbed to the infection [26]. This indicates that the outcome of the treatment may be counter effective and thus, the use of IFN-α in the treatment of MSMD is not adviced.

Next to the treatment with cytokines also cellular therapies, such as adoptive T cell therapies, may be possible. The investigations in **chapter 3 and 4** show that IL-23 may have an important role in the establishment of a type-I immune response, via the $CD3^+CD56^+$ NK-like T cells. This indicates that these cells, or certain subsets of this group of T cells, can potentially be used in cellular therapies. For example, CD1 restricted T cells are $CD3^+CD56^+$ and are able to produce IFN-γ and exhibit effector and memory functions [27]. Perhaps those cells can be expanded *ex vivo* in response to, e.g., mycobacterial ceramide glycolipids. Expanded CD1 T cells may than be transferred back to the patient in order to combat infections. Such therapy may be combined with gene therapy strategies. For example, the CD1 restricted T cells from IL-12Rβ1 deficient patients may be genetically modified with a vector carrying the coding sequence of *IL12RB1*. The safety and efficacy of these kinds of therapeutic strategies need to be tested in animal models, since IL-23 is also involved in various auto-immune diseases, such as inflammatory bowel disease [10]. This is further illustrated by mice studies. For example, IL-23p19-/- mice are relatively protected against the development of experimental autoimmune encephalomyelitis [28], while mice with IL-23 overexpression showed development of arthritis [29]. The effectiveness and the possible adverse auto immune effects should be evaluated in the choice of putative T cell therapies. It would be interesting to determine which IL-23 responsive T cells, amongst the $CD3^+CD56^+$ T cells have the best potentials in such cellular therapies.

5. Factors involved in impaired type I immunity in tuberculosis patients

Several studies provide evidence that the type-I immune responses of monocytes and dendritic cells can be inhibited by type-I interferons [25, 30, 31]. Recently in the blood cells of tuberculosis patients a typical type-I interferon transcript signature was found, which coincided with repressed IFN-γ signaling [32, 33]. Thus, the signaling of IFN-γ (a type II interferon) in tuberculosis patients may be repressed by type-I interferons, such as IFN-α and IFN-β.

In **chapter 7** the impact of IFN-α and IFN-β on the TLR-ligand and IFN-γ responsiveness of monocytes was investigated. The experiments showed that IFN-α and IFN-β strongly inhibit the LPS-induced IL-12p40 production, while high concentrations of IFN-γ could not counterbalance the inhibition. Furthermore, the IFN-γ responsiveness was strongly reduced. For example, for the induction of CD64 and CD54, 10 to 100 times more IFN-γ is needed in the presence of IFN-α/β to obtain similar effects as obtained in the absence of IFN-α/β. This indicates that type-I interferons may play a significant role in the repressed IFN-γ signaling, as observed in tuberculosis patients.

IFN-α inhibits the type-I immune responses of monocytes *in vitro* as well as *in vivo*, in melanoma patients who received IFN-α treatment prior to T cell therapy. The inhibition is probably achieved via various mechanisms (**chapter 7**): IFN-α may interfere directly with the IFN-γ mediated signaling via STAT1, IFN-α gradually downregulates the expression of IFN-γR1 after 2 h of stimulation, and IFN-α induces the production of the protein inhibitor of activated STAT1 (PIAS1) 8 h after stimulation. In addition, IFN-α may activate protein arginine methyltransferases (PRMTs), which influence the activity of various transcription factors and regulate via HuR antigens the stability of transcripts [34]. Further studies are needed to dissect the contributions of each of these mechanisms.

The finding that an arginine methyltransferase inhibitor was able to enhance the production of IL-12p40, IL-1β and TNF indicates that PRMTs have a certain suppressive role in the regulation of type I immune responses. Because of this, PRMTs may be putative targets for therapeutic interventions. In addition, PRMTs as well as PIAS1, which is regulated by PRMT1 [35, 36], can also be regarded as putative factors influencing the susceptibility to tuberculosis.

Our study in **chapter 7**, together with previous studies [32, 37, 38] that show impaired IFN-γ-mediated immune responses in tuberculosis patients, indicate that type-I interferons may play a role in the immunopathogenesis of tuberculosis. However, it is not clear whether type-I interferons such as IFN-α, are induced by mycobacteria in tuberculosis patients. Thus far, there is no direct evidence that *M. tuberculosis* can induce IFN-α or IFN-β in human cells. Other intracellular bacteria, for example *Bordetella pertussis* [39] and *Francisella tularensis* [30], were found to be able to induce IFN-β in human cells and may take advantage of the IFN-β induced inhibiting effects on dendritic cells. It may be the case that another type-I interferon is induced by *M. tuberculosis*, since the class of interferons consists of 13 IFN-α subtypes and 7 other types [40]. Next to the type-I interferons, type-III interferons could also be involved, since both types of interferons signal via ISGF3 modules, albeit via another receptor. These classes of interferons can be induced via special pathogen recognition receptors, such as TLR8 and RIG-I. Interestingly, the TLR8 polymorphism M1V was found to be associated with tuberculosis disease [41]. Furthermore, the TLR8 protein is present in the membrane of the phagolysosome [42], wherein the mycobacterium resides. Taken

together, there are some indirect indications that *M. tuberculosis* infections result in the induction of type-I interferons.

Type-I interferons can be abundantly produced in response to viral infections. It may be that mycobacteria take advantage of an IFN-α/β mediated repression of type-I immune responses during viral infection. This suggestion leads to the following hypothesis: viral infections promote the development of active disease either after reactivation of latent *M. tuberculosis* or after first or re-exposure to the pathogen. In **chapter 8** we tested the latter hypothesis for influenza infections. Influenza viruses may induce IFN-α/β in the respiratory tract and could locally suppress antigen presenting cells. As a consequence the mycobacterial infected host may develop active *M. tuberculosis* infection in the lung.

To explore this hypothesis we investigated, in a retrospective study, plasma of tuberculosis patients and controls. The plasma samples, previously collected in Jakarta in Indonesia [38], were tested for the presence and titer of antibodies against influenza viruses that circulated during the sampling period. If the hypothesis would be correct, in the group of tuberculosis patients more cases of recent past influenza infections were expected. However, in the case-control study the data showed no correlation between the presence of antibodies against influenza A viruses and manifest tuberculosis, which indicates that the risk of developing tuberculosis is not increased due to influenza A virus infections. Noteworthy, tuberculosis patients did have, at time of diagnosis, on average higher antibody titers against the H3N2 influenza A virus as compared to controls. In addition, we found an association between the mean antibody titer against H3N2 and the manifestation of tuberculosis. Patients with extrapulmonary manifestation of tuberculosis and patients with advanced tuberculosis at time of diagnosis had the highest antibody titers against H3N2. The association may indicate that these tuberculosis cases had had more recent influenza infections than controls. However, the association is most probably confounded by one or more factors (chapter 8). For example, the association may be explained by the fact that individuals vary greatly in their capacity to induce type-I interferons upon viral stimulation [43]. Individuals which have a high capacity to produce type-I interferons, develop more antibodies against influenza viruses and may also be more susceptible to develop tuberculosis, presuming that type-I interferons play a role in the pathogenesis of tuberculosis. Thus, it would be interesting to test the capacity of patients to produce type I interferons in relation to the susceptibility to tuberculosis and the severity of the disease. Besides these immunological hypotheses, another plausible explanation may simply be that patients developing tuberculosis and influenza infections are more sociable than others and hence more (re-)exposed to respiratory pathogens.

6. Future prospects

Until recently, the clinical management of NTM and TB infections was based on the eradication of the bacteria with antituberculous drugs. Because both the slowly replicating bacteria as well as the latently present, dormant bacteria have to be eliminated, such treatments often last for more than six months. Treatments require great compliance of the patients and are not in all cases accomplished successfully. Thus, there is an urgent need to improve current therapies and come up with additional therapeutic strategies that would shorten the antituberculous drugs regimens.

A better understanding of the interactions between the host and the mycobacterial pathogen, will generate ideas for novel therapies, tailor made and individualized for the susceptible host. More knowledge of the biology of the different mycobacterial species may yield ideas for the development of new antituberculous drugs. In addition, improvement of the immunity of the patient may also help to limit dissemination and combat the mycobacterial infection, especially in the immunocompromised host. An intrinsic lack of type-I immune responses or otherwise impaired immunity should be restored, at least partially, in order to support and strengthen the immune response of the host. Such therapy may reduce the need of extensive or prolonged medical treatments.

Currently, there are different therapeutic approaches at the various medical centers to treat disseminated NTM infections in MSMD patients. Since MSMD patients are rare, it is difficult to conduct clinical trials. Still, such trials could reveal more about the status of the infection at first presentation, the consequences of the genetic defect on the cellular immune response and the success of current therapies in relation to the genetic defect.

In order to find a genetic deficiency, the blood cells from putative MSMD patients should be screened for IL-12 and IFN-γ responsiveness. Informative data can also be directly obtained by sequencing the patient's genomic DNA or cDNA, in the search for known mutations in *IL12B*, *IL12RB1*, *TYK2*, *IFNGR1, IFNGR2* and *STAT1*. Our data, provided with the cellular models to evaluate the effects of genetic variations, will help in revealing the nature of a genetic defect and establish the genotype – immunological phenotype relationship. Rapid determination of the genetic deficiency will help guide the physician in a choice for additional treatment, e.g., supplementing deficient or inadequate levels of IFN-γ. Patients with IL-12 and IL-12R deficiency may respond to antituberculous drugs, but still may benefit from this additional treatment. So far, many patients were given extensive antituberculous medication and recovered, whereas some patients did not respond adequately and eventually died despite treatment. In some of these cases, clinicians should consider adding IFN-γ to their medical treatment, especially in IL-12p40 and IL-12Rβ1 deficient patients, and those with a partial mutation in either IFN-γR1 or IFN-γR2.

Additional treatment options for complete IFN-γR deficient patients are currently not available. Intervention with cytokines, other than IFN-γ, and cellular therapies may be possible. IL-27, which also signals via STAT1, is a putative candidate to achieve sufficient type-I immune responses against mycobacterial infections but clearly more experimental data supporting such usage in humans is needed. IL-27 may enhance the type-I immune response of monocytes and macrophages [44]. However, IL-27 is also able to signal via STAT3. For instance, regulatory T cells can be activated by IL-27, via STAT3 phosphorylation. By consequence, the cellular immune responses can be down regulated instead of stimulated. More knowledge about IL-27, in particular the factors that influence the activation of STAT1 and STAT3 by IL-27, may give clues for novel therapeutic approaches with this cytokine.

Possible therapeutic interventions with *ex vivo* treated cells should also be considered. *Ex vivo* treatment may circumvent the adverse effects of the use of cytokines *in vivo*. Therefore, it would be interesting to determine which subsets of T cells are underrepresented in the blood from MSMD patients, and to which extent the type-I immune modulating functions of these T cells are impaired. For example, NK-like T cells are underrepresented in IL-12Rβ1 deficient patients [45]. Novel therapies may be based on the gain of functions of these T cells. For example, infusion of *ex vivo* expanded and differentiated T cells may help to boost the immune responses of the patient. Because of the wide range of opportunities to modulate the type I immune responses, and the uncertainty of the outcome of such treatments, it would be helpful to explore these opportunities in animal models.

Mycobacterium tuberculosis evades host immunity via several mechanisms. Likely, an important mechanism is the blocking of the phagolysosome fusion and maturation. Next to this, virulent mycobacteria actively suppress the IFN-γ-mediated immune responses. This may be achieved via the induction of type-I interferons. Hence, it would be interesting to investigate whether virulent Mycobacteria are able to induce type I interferons in cultured human phagocytes. However, it is also possible that IFN-γ responses are inhibited by different, other factors. For instance, PRMT1 and PIAS1, which are thought to play a role in the repression of IFN-γ responses, may very well be induced and activated. Thus, these factors should be assessed in the blood cells of tuberculosis patients and if elevated, it should be determined how they are activated. Such exploratory studies will contribute greatly to the understanding of the mechanisms whereby the IFN-γ responses are repressed in tuberculosis patients and may give clues for novel immunotherapeutic interventions to treat tuberculosis.

References

[1] van de Vosse E, Hoeve MA, Ottenhoff TH. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. Lancet Infect Dis 2004;4:739-49.

[2] de Beaucoudrey L, Samarina A, Bustamante J, Cobat A, Boisson-Dupuis S, Feinberg J et al. Revisiting human IL-12Rβ1 deficiency: a survey of 141 patients from 30 countries. Medicine (Baltimore) 2010;89:381-402.

[3] Akahoshi M, Nakashima H, Miyake K, Inoue Y, Shimizu S, Tanaka Y et al. Influence of interleukin-12 receptor β1 polymorphisms on tuberculosis. Hum Genet 2003;112:237-43.

[4] van de Vosse E, de Paus RA, van Dissel JT, Ottenhoff THM. Molecular complementation of IL-12Rβ1 deficiency reveals functional differences between IL-12Rβ1 alleles including partial IL-12Rβ1 deficiency. Hum Mol Genet 2005;14:3847-55.

[5] Parham C, Chirica M, Timans J, Vaisberg E, Travis M, Cheung J et al. A Receptor for the Heterodimeric Cytokine IL-23 Is Composed of IL-12Rβ1 and a Novel Cytokine Receptor Subunit, IL-23R. J Immunol 2002;168:5699-708.

[6] van den Eijnden S, Goriely S, de Wit D, Willems F, Goldman M. IL-23 up-regulates IL-10 and induces IL-17 synthesis by polyclonally activated naive T cells in human. Eur J Immunol 2005;35:469-75.

[7] Sender LY, Gibbert K, Suezer Y, Radeke HH, Kalinke U, Waibler Z. CD40 ligand-triggered human dendritic cells mount interleukin-23 responses that are further enhanced by danger signals. Mol Immunol 2010;47:1255-61.

[8] van de Wetering D, de Paus RA, van Dissel JT, van de Vosse E. Salmonella induced IL-23 and IL-1β allow for IL-12 production by monocytes and Mphi1 through induction of IFN-γ in CD56 NK/NK-like T cells. PLoS One 2009;4:e8396.

[9] Pidasheva S, Trifari S, Phillips A, Hackney JA, Ma Y, Smith A et al. Functional studies on the IBD susceptibility gene IL23R implicate reduced receptor function in the protective genetic variant R381Q. PLoS One 2011;6:e25038.

[10] Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 2006;314:1461-3.

[11] Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol 2007;8:345-50.

[12] van de Wetering D, de Paus RA, van Dissel JT, van de Vosse E. Functional analysis of naturally occurring amino acid substitutions in human IFN-γR1. Mol Immunol 2010;47:1023-30.

[13] Allende LM, Lopez-Goyanes A, Paz-Artal E, Corell A, Garcia-Perez MA, Varela P et al. A point mutation in a domain of γ interferon receptor 1 provokes severe immunodeficiency. Clin Diagn Lab Immunol 2001;8:133-7.

[14] Jouanguy E, Lamhamedi-Cherradi S, Altare F, Fondaneche MC, Tuerlinckx D, Blanche S et al. Partial interferon-γ receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guerin infection and a sibling with clinical tuberculosis. J Clin Invest 1997;100:2658-64.

[15] Jouanguy E, Lamhamedi-Cherradi S, Lammas D, Dorman SE, Fondaneche MC, Dupuis S et al. A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. Nat Genet 1999;21:370-8.

[16] Doffinger R, Jouanguy E, Dupuis S, Fondaneche M-C, Stephan J-L, Emile JF et al. Partial interferon-γ receptor signaling chain deficiency in a patient with bacille Calmette-Guerin and Mycobacterium abscessus infection. J Infect Dis 2000;181:379-84.

[17] Kilic SS, Van Wengen A., de Paus RA, Celebi S, Meziane B, Hafizoglu D et al. Severe disseminated mycobacterial infection in a boy with a novel mutation leading to IFN-γR2 deficiency. J Infect 2012;doi 10.1016.

[18] Nakashima H, Inoue H, Akahoshi M, Tanaka Y, Yamaoka K, Ogami E et al. The combination of polymorphisms within interferon-γ receptor 1 and receptor 2 associated with the risk of systemic lupus erythematosus. FEBS Lett 1999;453:187-90.

[19] Tanaka Y, Nakashima H, Hisano C, Kohsaka T, Nemoto Y, Niiro H et al. Association of the interferon-γ receptor variant (Val14Met) with systemic lupus erythematosus. Immunogenetics 1999;49:266-71.

[20] Bernabei P, Allione A, Rigamonti L, Bosticardo M, Losana G, Borghi I et al. Regulation of interferon-γ receptor (IFN-γR) chains: a peculiar way to rule the life and death of human lymphocytes. Eur Cytokine Netw 2001;12:6-14.

[21] Bernabei P, Coccia EM, Rigamonti L, Bosticardo M, Forni G, Pestka S et al. Interferon-γ receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. J Leukoc Biol 2001;70:950-60.

[22] Rottman M, Soudais C, Vogt G, Renia L, Emile JF, Decaluwe H et al. IFN-γ mediates the rejection of haematopoietic stem cells in IFN-γR1-deficient hosts. PLoS Med 2008;5:e26.

[23] Alangari AA, Al-Zamil F, Al-Mazrou A, Al-Muhsen S, Boisson-Dupuis S, Awadallah S et al. Treatment of disseminated mycobacterial infection with high-dose IFN-γ in a patient with IL-12Rβ1 deficiency. Clin Dev Immunol 2011;2011:691956.

[24] Ward CM, Jyonouchi H, Kotenko SV, Smirnov SV, Patel R, Aguila H et al. Adjunctive treatment of disseminated Mycobacterium avium complex infection with interferon α-2b in a patient with complete interferon-γ receptor R1 deficiency. Eur J Pediatr 2007;166:981-5.

[25] van de Wetering D, Van Wengen A, Savage ND, van de Vosse E, van Dissel JT. IFN-α cannot substitute lack of IFN-γ responsiveness in cells of an IFN-γR1 deficient patient. Clin Immunol 2011;138:282-90.

[26] Rapkiewicz AV, Patel SY, Holland SM, Kleiner DE. Hepatoportal venopathy due to disseminated Mycobacterium avium complex infection in a child with IFN-γ receptor 2 deficiency. Virchows Arch 2007;451:95-100.

[27] Cohen NR, Garg S, Brenner MB. Antigen Presentation by CD1 Lipids, T Cells, and NKT Cells in Microbial Immunity. Adv Immunol 2009;102:1-94.

[28] Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 2003;421:744-8.

[29] Adamopoulos IE, Tessmer M, Chao CC, Adda S, Gorman D, Petro M et al. IL-23 is critical for induction of arthritis, osteoclast formation, and maintenance of bone mass. J Immunol 2011;187:951-9.

[30] Bauler TJ, Chase JC, Bosio CM. IFN-β Mediates Suppression of IL-12p40 in Human Dendritic Cells following Infection with Virulent Francisella tularensis. J Immunol 2011;187:1845-55.

[31] Nagai T, Devergne O, van Seventer GA, van Seventer JM. Interferon-β mediates opposing effects on interferon-γ-dependent Interleukin-12 p70 secretion by human monocyte-derived dendritic cells. Scand J Immunol 2007;65:107-17.

[32] Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 2010;466:973-7.

[33] Ottenhoff THM, Hari Dass R, Yang N, Zhang M, Wong HEE, Sahiratmadja E et al. Genome-wide expression profiling identifies Type I interferon response pathways in active tuberculosis. accepted for publication in PlosOne 2012;7 (9):e45839.

[34] Boisvert FM, Chenard CA, Richard S. Protein interfaces in signaling regulated by arginine methylation. Sci STKE 2005;271 re2:1-10.

[35] Liu B, Mink S, Wong KA, Stein N, Getman C, Dempsey PW et al. PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. Nat Immunol 2004;5:891-8.

[36] Mowen KA, Tang J, Zhu W, Schurter BT, Shuai K, Herschman HR et al. Arginine methylation of STAT1 modulates IFNα/β-induced transcription. Cell 2001;104:731-41.

[37] Sodhi A, Gong J, Silva C, Qian D, Barnes PF. Clinical correlates of γ production in patients with tuberculosis. Clin Infect Dis 1997;25:617-20.

[38] Sahiratmadja E, Alisjahbana B, de Boer T, Adnan I, Maya A, Danusantoso H et al. Dynamic changes in pro- and anti-inflammatory cytokine profiles and γ interferon receptor signaling integrity correlate with tuberculosis disease activity and response to curative treatment. Infect Immun 2007;75:820-9.

[39] Spensieri F, Fedele G, Fazio C, Nasso M, Stefanelli P, Mastrantonio P et al. Bordetella pertussis inhibition of interleukin-12 (IL-12) p70 in human monocyte-derived dendritic cells blocks IL-12 p35 through adenylate cyclase toxin-dependent cyclic AMP induction. Infect Immun 2006;74:2831-8.

[40] Takaoka A, Yanai H. Interferon signalling network in innate defence. Cell Microbiol 2006;8:907-22.

[41] Davila S, Hibberd ML, Hari Dass R, Wong HE, Sahiratmadja E, Bonnard C et al. Genetic association and expression studies indicate a role of toll-like receptor 8 in pulmonary tuberculosis. PLoS Genet 2008;4:e1000218.

[42] Cervantes JL, Dunham-Ems SM, La Vake CJ, Petzke MM, Sahay B, Sellati TJ et al. Phagosomal signaling by Borrelia burgdorferi in human monocytes involves Toll-like receptor (TLR) 2 and TLR8 cooperativity and TLR8-mediated induction of IFN-β. Proc Natl Acad Sci U S A 2011;108:3683-8.

[43] Katschinski DM, Neustock P, Kluter H, Kirchner H. Influence of various factors on interferon-α production in cultures of human leukocytes. J Interferon Res 1994;14:105-10.

[44] Yoshida H, Nakaya M, Miyazaki Y. Interleukin 27: a double-edged sword for offense and defense. J Leukoc Biol 2009;86:1295-303.

[45] Guia S, Cognet C, de Beaucoudrey L., Tessmer MS, Jouanguy E, Berger C et al. A role for interleukin-12/23 in the maturation of human natural killer and CD56+ T cells in vivo. Blood 2008;111:5008-16.

CHAPTER 10

Nederlandse samenvatting

Curriculum Vitae

Publications

Nederlandse samenvatting

Het afweersysteem, ook wel aangeduid als het immuunsysteem, is voor elk individu van cruciaal belang om infecties met micro-organismen, ziekteverwekkende microscopisch-kleine organismen, binnen de perken te houden. Veel infecties verlopen zonder dat de betreffende persoon ziek wordt. Als er ziekteverschijnselen optreden zoekt het lichaam naar een balans tussen de noodzaak micro-organismen uit de weefsels te verwijderen en het ontstaan van weefselbeschadiging. Deze complexe taak is opgedragen aan het afweersysteem. Het lichaam bestrijdt infecties met verschillende micro-organismen met verschillende afweerreacties op heel diverse wijze. Bij een afweerreactie is er vaak rechtstreeks contact tussen afweercellen, zodat signalen van de ene op de andere cel kunnen worden doorgegeven. Deze communicatie tussen de afweercellen verloopt mede door specifieke boodschappermoleculen, de zogenaamde interleukines (ILs) en interferonen (IFNs), gezamenlijk ook wel aangeduid als cytokines. Deze boodschappermoleculen binden aan specifieke oppervlaktemoleculen, receptoren genaamd, op de buitenkant van afweercellen, zoals een sleutel in een deurslot past. Deze binding initieert een reeks van signalen binnen de afweercel. De afweercel wordt daardoor geactiveerd om taken die voorgeprogrammeerd liggen in het erfelijk materiaal uit te voeren. Zo spelen het IFN-α en IFN-β een belangrijke rol in de afweer tegen virussen. Bij infecties door parasieten is een geheel ander type afweerreactie van belang, waarbij het cytokine IL-4 een regulerende rol heeft. In dit onderzoek beperk ik mij tot de afweer tegen mycobacteriën. Bij afwikkeling van een infectie met mycobacteriën is de ontwikkeling van een type I immuunreactie essentieel, waarbij de cytokines IL-12 en IFN-γ een centrale regulerende rol spelen.

Mycobacteriën komen in vele gedaanten voor. Zo zijn er virulente en minder virulente mycobacteriën. De bekendste virulente mycobacterie is ongetwijfeld *Mycobacterium tuberculosis*, de veroorzaker van de besmettelijke longziekte tuberculose. Naast deze tuberculeuze mycobacterie zijn er vele niet-tuberculeuze mycobacteriën (NTM) die normaal gesproken geen ziekte veroorzaken. Deze minder virulente NTM veroorzaken echter bij patiënten met een verstoorde afweer lokale of gedisseminеerde infecties, waarbij de bacterie zich door het lichaam verspreid. Dit laatste wordt vaak gezien bij patiënten met een erfelijk bepaalde verhoogde vatbaarheid voor infecties met mycobacteriën. De verhoogde vatbaarheid komt door een afwijking in het erfelijke materiaal (DNA) dat codeert voor een van de belangrijke componenten van een type I immuunreactie. Deze patiënten worden MSMD (Mendelian Susceptibility to Mycobacterial Disease) patiënten genoemd.

Een type I immuunreactie wordt in gang gezet nadat bepaalde afweercellen, zoals de monocyten uit het bloed, macrofagen of dendritische cellen in de weefsels, met mycobacteriën in contact komen. In de meeste gevallen zal opname van bacteriën door deze cellen ertoe leiden dat de micro-organismen snel gedood worden. Sommige mycobacteriën, kunnen

echter binnenin deze cellen overleven. Pas na activatie zijn deze afweercellen in staat de mycobacteriën in hun uitgroei te beteugelen. Bij deze activatie spelen verscheidene cytokines en andere typen afweercellen een rol. De opname van de mycobacteriën in deze afweercellen heeft afgifte van type I cytokines tot gevolg, zoals IL-23, IL-12 en IL-1β. In reactie op IL-23 en IL-12 produceren weer andere witte bloedcellen, de NK cellen en de T cellen, het IFN-γ. Deze cytokines oefenen via hun specifieke receptoren effecten uit op verschillende lichaamscellen welke er op gericht zijn de bacteriële uitgroei te bestrijden. Het is dus ook niet verwonderlijk dat afwijkingen in het genetisch materiaal dat codeert voor IL-12, de IL-12 receptor (IL-12R) en de IFN-γ receptor (IFN-γR) grote gevolgen kunnen hebben voor het bewerkstelligen van een afdoende immuunreactie tegen mycobacteriën. Anders gezegd: dergelijke genetische "afwijkingen", aangeduid als mutaties, spelen een voorname rol in een verhoogde vatbaarheid voor NTM infecties.

Het is interessant te constateren dat mutaties die zo'n essentiële rol spelen in de afweer tegen NTM geen rol lijken te spelen in de vatbaarheid voor tuberculose. Wel is in verscheidene onderzoeken aangetoond dat de IFN-γ-gemedieerde type I immuunrespons bij patiënten met actieve tuberculose onderdrukt is, terwijl reactiviteit op IFN-γ weer toeneemt tijdens een effectieve behandeling met tuberculostatica. Hoe het komt dat de type I immuunreactie onderdrukt wordt in tuberculose patiënten is nog onduidelijk.

Het eerste gedeelte van dit proefschrift (hoofdstuk 2-6) richt zich op genetische factoren die ten grondslag liggen aan een verhoogde vatbaarheid voor infecties met NTM. In het tweede gedeelte (hoofdstuk 7 en 8) worden mogelijke mechanismen onderzocht die een rol spelen bij een verminderde type I immuunrespons bij tuberculose.

Genetische factoren bij MSMD

MSMD-patiënten kunnen op jonge leeftijd ernstige NTM infecties ontwikkelen, waarbij de bacterie zich door het lichaam verspreidt en meerdere organen betrokken raken bij de infectie. Naast een verhoogde vatbaarheid voor NTM infecties zijn MSMD patiënten soms vatbaar voor ernstige infecties met veelvoorkomende *Salmonella* soorten, bacteriën die voor de meeste mensen niet of zelden ziekmakend zijn. De vatbaarheid voor infecties is bij MSMD verhoogd door afwijkingen in genen die coderen voor eiwitten die betrokken zijn bij de regulatie van de type I immuunreactie, bijvoorbeeld mutaties in de genen *IL12B*, *IL12RB1*, *IFNGR1* en *IFNGR2*. Vele MSMD-veroorzakende mutaties zijn inmiddels beschreven, maar in veel gevallen is niet onderzocht of een vastgestelde genetische variatie eigenlijk wel samengaat met een afwijkende functie van het desbetreffende eiwit. Van veel variaties in genen betrokken bij de type I immuunreactie weten we niet of het slechts polymorfismen, onschuldige DNA variaties, zijn of dat de variaties inderdaad ziekte-veroorzakende

mutaties zijn. In de studies van dit proefschrift (Hoofdstuk 2, 3, 5 en 6) is de invloed van aminozuurveranderingen in de receptoren van IL-12, IL-23 en IFN-γ op het functioneren van deze receptoren onderzocht.

Functionele consequenties van aminozuurveranderingen in IL-12Rβ1

In hoofdstuk 2 beschrijven we de ziektegeschiedenis van een patiënt met een uitzonderlijk verlopende NTM infectie. Op grond van immunologisch onderzoek werd vermoed dat de patiënt een IL-12Rβ1 deficiëntie had, waarna met genetische analyse werd vastgesteld dat de patiënt twee afzonderlijke genetische variaties in IL-12Rβ1 droeg, te weten R521G en -2C>T. Met behulp van een in vitro onderzoeksmodel werd bevestigd dat de R521G variatie de genetische verandering is die leidt tot een gedeeltelijk defecte functie van IL-12Rβ1. De R521G is in dit geval de ziekteveroorzakende mutatie, terwijl de andere variatie een onschuldig polymorfisme bleek te zijn. Opmerkelijk in dit geval was dat de patiënt pas op middelbare leeftijd (43 jaar) voor het eerst een NTM infectie ontwikkelde, terwijl de meeste MSMD patiënten met een IL-12Rβ1 defect al op jeugdige leeftijd een eerste NTM infectie doormaken. Naast deze jeugdige MSMD patiënten zijn er ook familieleden van deze patiënten beschreven bij wie genetisch onderzoek een MSMD mutatie blootlegde maar die nog nooit klachten of tekenen van een NTM infectie gehad hadden. Dit kan natuurlijk het gevolg zijn van een verminderde blootstelling aan mycobacteriën ten opzichte van de gevallen die wel NTM infecties doormaken of door een compenserend afweermechanisme. Ook leeftijd kan hierop van invloed zijn.

Met behulp van hetzelfde in vitro onderzoeksmodel zijn de functionele consequenties van meerdere aminozuurveranderingen in IL-12Rβ1 onderzocht. In het addendum bij hoofdstuk 2 staat een korte samenvatting van de invloed van alle tot dusver bekende aminozuurveranderingen op de type I immuunreactie van T cellen. Sommige variaties zijn definitief getypeerd als MSMD veroorzakende mutaties, anderen bleken onschuldige polymorfismen. Alle nu bekende MSMD mutaties leiden tot een compleet of bijna compleet defect in de expressie van IL-12Rβ1. Twee mutaties leiden tot een partieel functioneel defect, te weten C198R en R521G, en hebben tot gevolg dat het receptoreiwit sterk verminderd op T cellen aanwezig is. Deze bevindingen suggereren dat pas als de IL-12Rβ1 functie sterk verminderd is, een effectieve immuunreactie tegen NTM uitblijft.

De twee meest voorkomende varianten van IL-12Rβ1, te weten RTR en QMG, worden feitelijk gevormd door drie polymorfismen, te weten R214Q, T365M en R378G, welke als één blok overerven. De QMG variant is in hogere aantallen aanwezig op het celoppervlak van T cellen en reageert intensiever op stimulatie met IL-12 dan de RTR variant. Dit verschil maakt deze twee genvarianten zeer geschikt in genetische associatiestudies naar een rol voor IL-12 bij een bepaald ziektebeeld. Zo is bijvoorbeeld gebleken dat het dragen van een RTR variant geassocieerd is met een licht verhoogde vatbaarheid voor tuberculose ten opzichte van normale controles.

De biologische effecten van IL-23 en variaties in de IL-23R

Tot dusver hebben alle gerapporteerde MSMD patiënten met een functioneel gekarakteriseerd defect in de functie van IL-12 of het IL-12R complex, een gen mutatie in respectievelijk IL-12p40 of IL-12Rβ1. Aangezien IL-12p40 ook een onderdeel is van het eiwit IL-23, dat evenals IL-12 een heterodimeer is waarvan IL-12p40 onderdeel uitmaakt, en IL-12Rβ1 ook een keten is van het IL-23R complex, hebben deze patiënten ook een defect in de functies van IL-23 of het IL-23R complex. Het is opmerkelijk dat er geen defecten beschreven zijn in de specifieke componenten van IL-12 en IL-23 of van hun specifieke receptorketens, IL-12Rβ2 en IL-23R. Dit suggereert dat zowel de IL-12 als de IL-23-gemedieerde immuniteit verminderd moet zijn wil dit leiden tot een verhoogde vatbaarheid voor NTM infecties. Mogelijk complementeren beide cytokines elkaars effecten.

Het belang van IL-12 in de type I immuunreactie is evident. De rol van IL-23 is minder duidelijk, onder andere omdat maar weinig bekend is over welke afweercellen een IL-23R op hun celoppervlak hebben. In hoofdstuk 3 beschrijven wij het effect van IL-23 op geactiveerde T cellen, waarin tevoren op artificiële wijze de IL-23R ingebracht is. Het onderzoek toonde verschillende, maar ook overlappende, gemeenschappelijke effecten van IL-12 en IL-23 op deze cellen. IL-12 stimulatie leidt in de cel tot activatie van het eiwit STAT4, wat resulteert in een sterke productie van IFN-γ en een geringe productie van het remmende cytokine IL-10. Dit staat in contrast met de uitwerking van IL-23, dat na stimulatie van het IL-23R complex leidt tot activatie van STAT1, STAT3 en STAT4 en resulteert in een geringere IFN-γ productie dan IL-12 en relatief meer IL-10 productie. Deze bevinding en het gegeven dat IL-23 voornamelijk in de eerste fase van de immuunreactie geproduceerd wordt en IL-12 in een latere fase, is een aanwijzing voor een stapsgewijze versterking van een type I immuunreactie.

In de situatie dat er geen IL-23R tot expressie gebracht wordt kan IL-23 toch STAT4 activeren, wat resulteert in een bescheiden IFN-γ productie, veel minder dan de hoeveelheid die vrijkomt na IL-12 stimulatie. Deze bevinding suggereert dat IL-23 de afweercellen ook activeert via een andere receptor dan de IL-23R, mogelijk het IL-12 receptor complex.

In het in vitro model zijn de IL-23R varianten Y173H, P310L en R381Q wat betreft functionaliteit vergeleken met de wild type IL-23R (de meest voorkomende natuurlijke variant). Deze receptor varianten en de wild type receptor blijken vergelijkbaar te functioneren. De variaties zijn dus onschuldige polymorfismen en geen mutaties. De Y173H was gevonden in een patiënt met een Salmonella infectie en is mogelijk een mutatie met ernstige gevolgen voor de functie van IL-23R. Echter, de functionele karakterisering toont aan dat Y173H een volkomen onschuldig polymorfisme is zonder enige invloed op de IL-23 respons; het gevonden polymorfisme kan dus niet verantwoordelijk gehouden worden voor de beschreven infectie.

De R381Q variant is een polymorfisme geassocieerd met bescherming tegen de ziekte van Crohn; dit suggereert dat IL-23 mogelijk een rol speelt in auto-immuunziekten. In

een andere studie werd beschreven dat het R381Q polymorfisme is geassocieerd met een verminderde aanwezigheid van IL-23R positieve afweercellen. Bij elkaar genomen, past dit in het beeld dat IL-23 waarschijnlijk een belangrijke rol speelt in het opstarten van een type I immuunrespons.

Omdat tijdens dit onderzoek geen methode beschikbaar was om de aanwezigheid van de IL-23R op cellen aan te tonen, hebben wij onderzocht of IL-23R-geïnduceerde activatie van STAT3 te bepalen was direct na toevoeging van IL-23 aan afweercellen, als een indirecte maat voor IL-23 reactiviteit. Hoofdstuk 4 beschrijft onderzoek waaruit blijkt dat zogenaamde 'NK-achtige' T cellen reageren op IL-23. De IL-12 en IL-23-geïnduceerde IFN-γ productie door deze NK-T cellen is onafhankelijk van T-cel-receptor-activatie, maar bleek wel afhankelijk van de aanwezigheid van IL-18. Het onderzoek toonde ook aan dat IL-23 indirect de celdeling, maar niet de IFN-γ productie, van NK-cellen stimuleert. Eerder was aangetoond dat IL-23 ook de celdeling en de overleving van IL-17-producerende T cellen stimuleert. Dit wijst erop dat IL-23 stimulatie mogelijk resulteert in de productie van een factor, die essentieel is voor celdeling en overleving van NK-cellen en van T cellen.

Functionele consequenties van aminozuur veranderingen in de IFN-γR

IFN-γ is hét centrale cytokine in de type I immuunreactie met een veelheid aan effecten op meerdere celtypen. Mutaties in de genen die coderen voor elk van de twee eiwitketens van de IFN-γ receptor, IFN-γR1 of IFN-γR2, hebben grote gevolgen voor de afwikkeling van een type I immuunreactie. Door meerdere onderzoekers zijn aminozuurveranderingen beschreven in beide receptorketens. Om de functionele consequenties van deze variaties te bepalen zijn twee onderzoeksmodellen ontwikkeld, die beschreven zijn in hoofdstuk 5 en 6. In cellijnen die zelf geen IFN-γR1 of IFN-γR2 tot expressie brengen, werd met behulp van een gemodificeerd virus de genen ingebracht van één van de varianten van IFN-γR1 of IFN-γR2, of van de wild type receptoren (de meest voorkomende varianten van de receptorketens). Op deze wijze kan de functie van de receptorvarianten vergeleken worden met de wild type receptor tegen één en dezelfde genetische achtergrond. Hiermee kon een onderscheid gemaakt worden tussen onschuldige polymorfismen en ziekteveroorzakende mutaties.

De aminozuurvariaties V14M, V61I, S149L, H335P, I352M en L467P in IFN-γR1 blijken polymorfismen te zijn, welke nauwelijks invloed hebben op de receptor functie. De variaties S149L en I352M waren beschreven in patiënten van wie men vermoedde dat ze MSMD hadden, omdat de bloedcellen van deze personen vermindert reageerden op stimulatie met IFN-γ dan bloedcellen van controles. Uit ons onderzoek volgt dat dit fenotype van verminderde IFN-γ reactiviteit niet verklaard kan worden door de aanwezige variatie in IFN-γR1 en dus zeer waarschijnlijk toegeschreven moet worden aan een ander defect, bijvoorbeeld in STAT1, een eiwit dat door de IFN-γ receptor geactiveerd wordt en diverse antibacteriële processen binnen de cel in gang zet.

De aminozuurvariaties V61E, V61Q, Y66C, C77F, C77Y en C85Y konden aangemerkt worden als mutaties die tot een complete IFN-γR1 deficiëntie leiden, terwijl de V63G en I87T mutaties een partiële, bijna complete deficiëntie, veroorzaken. De functie van de laatste twee receptorvarianten bleek sterk gereduceerd te zijn, maar niet geheel afwezig. In vergelijking met de wild type receptor is bij deze varianten echter wel veel meer IFN-γ nodig om hetzelfde biologisch effect tot gevolg te hebben. Dit kan een verklaring vormen voor de bevinding dat MSMD patiënten met deze partiële mutaties ook ernstige infecties ontwikkelen.

In het IFN-γR2 gen zijn DNA variaties beschreven die de aminozuurvariaties T58R, Q64R, E147K en K182E tot gevolg hebben; allen zijn gekarakteriseerd als onschuldige polymorfismen. De beschreven variaties R114C, T168N en G227R bleken echter mutaties, waarvan R114C en G227R leiden tot een gedeeltelijk defect in de expressie van IFN-γR2. Bloedcellen van patiënten die homozygoot zijn voor een van deze mutaties, hebben inderdaad een sterk afgenomen reactie op stimulatie met IFN-γ. In vergelijking met cellen van een gezonde controle blijkt een circa 100 keer hogere concentratie van IFN-γ nodig om een vergelijkbare respons te geven. Deze gedeeltelijke, bijna complete deficiënties in IFN-γR1 en IFN-γR2 wijzen erop dat een IFN-γ deficiëntie pas tot MSMD leidt als de reactiviteit op IFN-γ sterk gereduceerd is.

Het V14M polymorfisme in IFN-γR1 en het Q64R polymorfisme in IFN-γR2 beïnvloeden de expressie van de receptorketens op de buitenkant van de afweercel aanzienlijk, maar blijken toch niet geassocieerd met MSMD. Deze polymorfismen hebben niettemin enige invloed op de ontwikkeling van een T cel reactie tot een type I of een type II immuunrespons. Daarom zijn deze twee polymorfismen interessant als markers in genetische associatiestudies om te bepalen of IFN-γ een factor is in de aanleg voor infectieziekten waartegen een type I respons gewenst is, en of IFN-γ een rol speelt in auto-immuunziekten. Ziekte-associatie met deze polymorfismen is bijvoorbeeld eerder beschreven voor systemische lupus erythematosus (SLE). Voor infectieziekten zoals longtuberculose is hiernaar nog onvoldoende onderzoek verricht.

Op zoek naar nieuwe therapieën om MSMD te behandelen

MSMD patiënten met NTM infecties moeten vaak langdurig behandeld worden met antibiotische middelen, en zelfs dan is deze therapie niet altijd succesvol. Een langdurige preventieve therapie kan mogelijk antibioticaresistentie in de hand werken. Verschillende alternatieve therapieën zijn daarom overwogen en toegepast.

Zo hebben patiënten met een ernstig IFN-γR1 defect stamceltransplantatie ondergaan. Dit bleek lang niet altijd succesvol, bijvoorbeeld omdat patiënten tijdens de transplantatie nog een actieve infectie hadden, die in de fase van ernstig gestoorde afweer na de transplantatie niet langer onder controle te houden was. Ook bleek het gebruik van donorstamcellen niet altijd succesvol, mogelijk omdat de donorcellen niet in het beenmerg “enten”.

Voor patiënten met een defect in IL-12p40 of IL-12Rβ1 of met een gedeeltelijk defect in IFN-γR1 of IFN-γR2, is een aanvullende behandeling met IFN-γ toegepast in een poging de gevolgen van de deficiëntie in de type I immuunreactie te omzeilen. Dit toont eens te meer het belang aan van een volledige karakterisering van het immunologische fenotype en de achterliggende genetische mutatie bij MSMD, waarbij de onderzoeken beschreven in hoofdstuk 2, 5 en 6 kunnen helpen bij de keuze voor een additionele behandeling.

Naast het toedienen van IFN-γ is er ook gepleit (en gekozen) voor een behandeling met IFN-α. Uit de spaarzame beschrijvingen is het succes van deze behandeling echter niet duidelijk. In een MSMD patiënt met een compleet IFN-γR1 defect nam de vergroting van lever, milt en lymfeklieren af als gevolg van de IFN-α behandeling, echter de verspreiding van de NTM via het bloed nam niet af, en de patiënt overleed uiteindelijk als gevolg van de gedisseminеerde infectie. Een mogelijke verklaring hiervoor kan zijn dat IFN-α de type I immuunreactie juist sterk blijkt te kunnen remmen (hoofdstuk 7). Het is niet uit te sluiten dat de systemische verspreiding van NTM in de beschreven patiënt mede is toe te schrijven aan de IFN-α behandeling. In een andere patiënt met een compleet IFN-γR2 defect werden infecties in de organen schijnbaar beter ingeperkt, door de vorming van weefselgranulomen tijdens IFN-α behandeling. Niettemin ontwikkelde de patiënt een niet te stuiten uitbreiding van infectiehaarden in lever en botten, en bezweek ook deze patiënt aan een gedisseminеerde NTM infectie. Mede gezien onze bevindingen beschreven in hoofdstuk 7 zou de behandeling met IFN-α dus zelfs contra-effectief kunnen zijn en ligt het niet langer voor de hand het gebruik ervan bij MSMD aan te bevelen.

Naast behandeling met cytokines zijn ook cellulaire therapieën overwogen. De onderzoeken beschreven in hoofdstuk 3 en 4 tonen aan dat IL-23 een potentiële rol speelt in de totstandkoming van een type I immuunreactie, via een subgroep van T cellen, de NK-T cellen. Het zou interessant zijn na te gaan of deze NK-T cellen gebruikt kunnen worden in een cellulaire therapie. Bijvoorbeeld T cellen die specifiek een mycobacterieel product herkennen zijn een bron van IFN-γ, het cytokine dat zij na stimulatie aanmaken. Het is in principe mogelijk deze cellen buiten het lichaam te stimuleren en te vermeerderen. Hierna kunnen de T cellen aan de patiënt teruggegeven worden, in de hoop dat de T cellen het afweersysteem helpen bij het bestrijden en afwikkelen van de mycobacteriële infectie. Eventueel zou deze vorm van therapie gecombineerd kunnen worden met gentherapie. Bijvoorbeeld, T cellen van een IL-12Rβ1 deficiënte patiënt kunnen genetisch gemodificeerd worden door het gen coderend voor IL-12Rβ1 in de cellen te brengen, waarna de receptor weer tot expressie komt aan het celoppervlak en de cellen weer kunnen reageren op IL-12. Voor het tot dergelijke behandelingen komt moet nagegaan worden welke typen T cellen het best te gebruiken zijn in deze vorm van celtherapie.

Factoren die betrokken zijn bij een verminderde type I immuniteit in tuberculose patiënten

Er zijn aanwijzingen dat de IFN-γ-gemedieerde immuniteit onderdrukt is bij patiënten met actieve tuberculose. Recentelijk is gebleken dat, naast een onderdrukte IFN-γ reactiviteit, de bloedcellen van tuberculose patiënten een cytokine profiel tonen dat past bij een IFN-α gemedieerde afweerreactie. Zoals boven beschreven kan IFN-α de type I immuunreactiviteit van monocyten remmen. Mogelijk wordt de type I immuniteit in tuberculose patiënten onderdrukt door lokaal geproduceerde interferonen, zoals IFN-α, en het daarop gelijkende IFN-β.

In hoofdstuk 7 is de invloed die IFN-α/β kunnen hebben op de vorming van een type I immuunreactie door monocyten beschreven. De experimenten tonen dat de (door bacteriële producten aangestuurde) aanmaak van IL-12p40 sterk geremd wordt door IFN-α/β, terwijl IFN-γ dit effect niet kan opheffen. Verscheidene effecten van IFN-γ werden sterk onderdrukt door IFN-α/β. Deze bevindingen zijn bewijs dat IFN-α/β een belangrijke rol kan spelen in het onderdrukken van IFN-γ reactiviteit zoals beschreven in tuberculose patiënten. De remmende effecten van IFN-α worden bewerkstelligd via meerdere mechanismen. Zo bleek IFN-α niet alleen de expressie van de receptor voor IFN-γ te kunnen verlagen, maar IFN-α beïnvloedt ook de activatie van STAT1 door IFN-γ. Daarnaast stimuleert IFN-α ook remmende factoren, zoals de eiwitremmer van STAT1 (PIAS1) en methylerende eiwitten (PRMTs). PRMTs en PIAS1 kunnen daarmee beschouwd worden als factoren die de vatbaarheid voor tuberculose negatief beïnvloeden, en zijn dus potentiële aangrijpingspunten voor immuuntherapie.

Het lijkt erop dat antivirale interferonen zoals IFN-α en IFN-β, een rol spelen in de immuunpathogenese van tuberculose, maar het is niet duidelijk of deze interferonen in de patiënt daadwerkelijk aangemaakt worden tijdens een infectie met *Mycobacterium tuberculosis*. Het is ook mogelijk dat deze interferonen door een geheel andere reden aangemaakt worden, bijvoorbeeld door een gelijktijdig optredende virus infectie. Kortom, mycobacteriën zouden voordeel kunnen hebben van het feit dat antivirale interferonen, die aangemaakt zijn tijdens een virus infectie, de type I immuunreacties tegen intracellulaire bacteriën (tijdelijk) onderdrukken. Dit leidt tot de volgende hypothese: Virale infecties bevorderen de klinische openbaring van een tuberculose. In hoofdstuk 8 is deze hypothese getoetst voor tuberculose na een recente influenza virus infectie (ofwel griep). Influenza virussen kunnen door de aanmaak van IFN-α of IFN-β in de ademhalingswegen lokaal de vorming van type I immuunreacties remmen. Mogelijk zal hierdoor de gastheer, indien besmet met *Mycobacterium tuberculosis,* eerder dan anders een klinisch-actieve longtuberculose ontwikkelen.

Om deze hypothese te testen is plasma van tuberculose patiënten en van controles onderzocht op aanwezigheid van antilichamen tegen de influenza virussen, H3N2 en H1N1.

Deze type virussen circuleerden ten tijde van het verzamelen van de plasmamonsters in de bevolking. Indien de hypothese juist is, zouden naar verwachting meer tuberculose patiënten dan controle personen antilichamen tegen deze virussen in hun plasma aantoonbaar hebben. Hoewel er geen correlatie is gevonden tussen het aantal personen met antilichamen tegen influenza en de klinische openbaring van tuberculose, werd er wel een verband gevonden tussen de concentratie van antilichamen in het bloedplasma en het hebben van klinisch actieve longtuberculose. Patiënten met tuberculose hadden ten opzichte van de gezonde controle groep een hogere concentratie van antilichamen tegen het H3N2 influenza virus in hun bloedplasma. Daarnaast hadden patiënten met extrapulmonaire tuberculose en patiënten met tuberculose in een vergevorderd stadium, de hoogste concentratie aan antilichamen. Ook de sero-immuniteit voor het H3N2 virus was hoog (rond de 82%), wat suggereert dat er meer tuberculose patiënten dan controles recentelijk opnieuw geïnfecteerd zijn met het influenza virus. Maar conclusies kunnen hieruit niet getrokken worden, want veel factoren kunnen dit verband vertroebelen. Zo kan de associatie mogelijk verklaard worden doordat individuen verschillen in de mate van IFN-α productie. Individuen die meer dan gemiddeld IFN-α produceren na een virale infectie, produceren mogelijk ook meer antilichamen tegen het virus. Dezelfde personen produceren mogelijk ook meer IFN-α tijdens een mycobacteriële infectie. En zo zijn er andere mogelijke verklaringen. Kortom, de resultaten beschreven in hoofdstuk 8 geven vooralsnog onvoldoende bewijs om de hypothese te bevestigen of ontkrachten.

Toekomstperspectieven

Tot nu toe is de behandeling van NTM infecties en tuberculose gebaseerd op het doden van de bacteriën door anti-mycobacteriële middelen. Omdat naast de al langzaam delende mycobacteriën ook de ‘slapende’ nauwelijks delende mycobacteriën gedood moeten worden, duurt een dergelijke behandeling langer dan 6 maanden, welke zeer belastend is voor de patiënt. Er is dus een sterke behoefte aan aanvullende behandelmethodes. Inzichten in de interactie tussen mycobacteriën en gastheer zullen ongetwijfeld nieuwe ideeën opleveren voor behandelmethodes. Nieuwe en krachtige tuberculostatica moeten ontwikkeld worden. Antimicrobiële middelen zijn echter niet meer dan hulpmiddelen van de afweer. De aanpak moet zich dus ook richten op ondersteuning van de afweer van de patiënt. Dit laatste kan mogelijk de tijdsduur van de behandeling met tuberculostatica bekorten.

Voor MSMD patiënten geldt dat een snelle diagnose geboden is, al was het maar omdat een deel van deze patiënten baat heeft bij een additionele behandeling met IFN-γ. Voor patiënten met een complete IFN-γR deficiëntie is momenteel geen andere behandeling mogelijk dan tuberculostatica. Mogelijk dat nieuwe inzichten in andere eiwitten die de type

I immuunrespons kunnen versterken, zoals bijvoorbeeld IL-27, hier in de toekomst uitkomst bieden.

De mogelijkheid van interventies met afweercellen, die na oogsten buiten het lichaam geselecteerd en geactiveerd worden, dient nader onderzocht te worden. Kandidaten voor een dergelijke aanpak zijn NK-achtige T cellen, welke overigens verminderd aanwezig zijn in het bloed van IL-12Rβ1-deficiënte patiënten.

Mycobacterium tuberculosis heeft verschillende mechanismen om het afweersysteem te omzeilen. Een belangrijk mechanisme is het tegenwerken van bacteriedodende mechanismen in witte bloedcellen, zoals in monocyten en weefselmacrofagen. Daarnaast onderdrukt de mycobacterie ook de IFN-γ gemedieerde immuunrespons, mogelijk door de effecten van een IFN-α gemedieerde afweerreactie. De IFN-γ respons in tuberculose patiënten kan echter ook door andere factoren geremd zijn, zoals de eerder beschreven activatie van PRMT1 en PIAS1. Het is duidelijk dat er nog veel onderzoek nodig is voordat we daadwerkelijk begrijpen waarom de ene persoon na blootstelling aan deze bacterie wel en de andere geen tuberculose ontwikkelt. Zulk onderzoek zal bijdragen aan verdere kennis over de rol van een verminderde IFN-γ-gemedieerde immuniteit bij mycobacteriële infecties en zal hopelijk nieuwe inzichten geven hoe tuberculose gerichter te behandelen.

Curriculum Vitae

Roelof Anne de Paus werd geboren op 25 juni 1965 te Dordrecht. In 1981 behaalde hij het mavo-diploma aan de gemeentelijke school Krispijn, te Dordrecht, waarna hij in 1983 het havo-diploma heeft behaald aan de gemeentelijke scholengemeenschap Zuid, te Dordrecht. Aansluitend heeft hij de opleiding tot research analist gevolgd aan het Van leeuwenhoek Instituut te Delft, welke tijdens zijn studie onderdeel werd van de Hogeschool van Rotterdam en omstreken. Tijdens zijn stage heeft hij moleculair biologisch onderzoek gedaan op de afdeling Endocrinologie van de Medische Faculteit van de Erasmus Universiteit, te Rotterdam (hoofd Prof. dr. A. Grootegoed). De opleiding tot ingenieur in de biochemie werd afgerond in 1987. Daarna heeft hij tot 1990 gewerkt als organisch chemisch analist bij de melkzuurfabriek van C.C.A. biochem (thans Purac Biochem). Vanaf januari 1990 is hij werkzaam geweest op het Laboratorium van Experimentele Hematologie (hoofd Prof. dr. J.H.F. Falkenburg) van het Leids Universitair Medisch Centrum. In Februari 2002 heeft hij binnen het Leids Universitair Medisch Centrum de overstap gemaakt naar het Laboratorium van Infectieziekten (hoofd Prof. dr. J.T. van Dissel). Het onderzoek aldaar is omgezet in een promotie onderzoek met als co-promotor Dr. E. van de Vosse.

Publications

1. Toebosch, A. M., D. M. Robertson, J. Trapman, P. Klaassen, R. A. de Paus, F. H. de Jong, and J. A. Grootegoed. 1988. Effects of FSH and IGF-I on immature rat Sertoli cells: inhibin α- and β-subunit mRNA levels and inhibin secretion. *Mol. Cell Endocrinol.* 55: 101-105.
2. Falkenburg, J. H., M. A. Harrington, R. A. de Paus, W. K. Walsh, R. Daub, J. E. Landegent, and H. E. Broxmeyer. 1991. Differential transcriptional and posttranscriptional regulation of gene expression of the colony-stimulating factors by interleukin-1 and fetal bovine serum in murine fibroblasts. *Blood* 78: 658-665.
3. de Kroon, J. F., R. A. de Paus, H. C. Kluin-Nelemans, P. M. Kluin, C. A. van Bergen, A. J. Munro, G. Hale, R. Willemze, and J. H. Falkenburg. 1996. Anti-CD45 and anti-CD52 (Campath) monoclonal antibodies effectively eliminate systematically disseminated human non-Hodgkin's lymphoma B cells in Scid mice. *Exp. Hematol.* 24: 919-926.
4. de Kroon, J. F., C. A. van Bergen, R. A. de Paus, H. C. Kluin-Nelemans, R. Willemze, and J. H. Falkenburg. 1997. Human cytotoxic $CD8^+$ T-lymphocyte clones engraft in severe combined immunodeficient (SCID) mice but show diminished function. *J. Immunother.* 20: 101-110.
5. Smit, W. M., M. Rijnbeek, C. A. van Bergen, R. A. de Paus, H. A. Vervenne, M. van de Keur, R. Willemze, and J. H. Falkenburg. 1997. Generation of dendritic cells expressing bcr-abl from CD34-positive chronic myeloid leukemia precursor cells. *Hum. Immunol.* 53: 216-223.
6. van de Corput, M. P., R. W. Dirks, R. P. van Gijlswijk, van Binnendijk E., C. M. Hattinger, R. A. de Paus, J. E. Landegent, and A. K. Raap. 1998. Sensitive mRNA detection by fluorescence in situ hybridization using horseradish peroxidase-labeled oligodeoxynucleotides and tyramide signal amplification. *J. Histochem. Cytochem.* 46: 1249-1259.
7. Vogt, M. H., R. A. de Paus, P. J. Voogt, R. Willemze, and J. H. Falkenburg. 2000. DFFRY codes for a new human male-specific minor transplantation antigen involved in bone marrow graft rejection. *Blood* 95: 1100-1105.
8. Vogt, M. H., E. Goulmy, F. M. Kloosterboer, E. Blokland, R. A. de Paus, R. Willemze, and J. H. Falkenburg. 2000. UTY gene codes for an HLA-B60-restricted human male-specific minor histocompatibility antigen involved in stem cell graft rejection: characterization of the critical polymorphic amino acid residues for T-cell recognition. *Blood* 96: 3126-3132.
9. Heemskerk, M. H., R. A. de Paus, E. G. Lurvink, F. Koning, A. Mulder, R. Willemze, J. J. van Rood, and J. H. Falkenburg. 2001. Dual HLA class I and class II restricted

recognition of alloreactive T lymphocytes mediated by a single T cell receptor complex. *Proc. Natl. Acad. Sci. U. S. A* 98: 6806-6811.

10. Noort, W. A., A. B. Kruisselbrink, P. S. in't Anker, M. Kruger, R. L. van Bezooijen, R. A. de Paus, M. H. Heemskerk, C. W. Lowik, J. H. Falkenburg, R. Willemze, and W. E. Fibbe. 2002. Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived $CD34^+$ cells in NOD/SCID mice. *Exp. Hematol.* 30: 870-878.
11. Heemskerk, M. H., M. Hoogeboom, R. A. de Paus, M. G. Kester, M. A. van der Hoorn, E. Goulmy, R. Willemze, and J. H. Falkenburg. 2003. Redirection of antileukemic reactivity of peripheral T lymphocytes using gene transfer of minor histocompatibility antigen HA-2-specific T-cell receptor complexes expressing a conserved α joining region. *Blood* 102: 3530-3540.
12. Posthuma, E. F., C. A. van Bergen, M. G. Kester, R. A. de Paus, P. A. van Veelen, A. H. de Ru, J. W. Drijfhout, E. G. Lurvink, R. Willemze, and J. H. Falkenburg. 2004. Proteosomal degradation of BCR/ABL protein can generate an HLA-A*0301-restricted peptide, but high-avidity T cells recognizing this leukemia-specific antigen were not demonstrated. *Haematologica* 89: 1062-1071.
13. van der Veken, L. T., M. Hoogeboom, R. A. de Paus, R. Willemze, J. H. Falkenburg, and M. H. Heemskerk. 2005. HLA class II restricted T-cell receptor gene transfer generates $CD4^+$ T cells with helper activity as well as cytotoxic capacity. *Gene Ther.* 12: 1686-1695.
14. van de Vosse, E., R. A. de Paus, J. T. van Dissel, and T. H. Ottenhoff. 2005. Molecular complementation of IL-12Rβ1 deficiency reveals functional differences between IL-12Rβ1 alleles including partial IL-12Rβ1 deficiency. *Hum. Mol. Genet.* 14: 3847-3855.
15. de Paus, R. A., D. van de Wetering, J. T. van Dissel, and E. van de Vosse. 2008. IL-23 and IL-12 responses in activated human T cells retrovirally transduced with IL-23 receptor variants. *Mol. Immunol.* 45: 3889-3895.
16. van Bilsen K., G. J. Driessen, R. A. de Paus, E. van de Vosse, K. van Lom, M. C. van Zelm, K. H. Lam, N. G. Hartwig, G. S. Baarsma, M. van de Burg, and P. M. van Hagen. 2008. Low level IGF-1 and common variable immune deficiency: an unusual combination. *Neth. J. Med.* 66: 368-372.
17. van de Wetering, D., R. A. de Paus, J. T. van Dissel, and E. van de Vosse. 2009. IL-23 modulates $CD56^+/CD3^-$ NK cell and $CD56^+/CD3^+$ NK-like T cell function differentially from IL-12. *Int. Immunol.* 21: 145-153.
18. van de Vosse, E., E. M. Verhard, R. A. de Paus, G. J. Platenburg, J. C. van Deutekom, A. Aartsma-Rus, and J. T. van Dissel. 2009. Antisense-mediated exon skipping to correct IL-12Rβ1 deficiency in T cells. *Blood* 113: 4548-4555.

19. van de Wetering, D., R. A. de Paus, J. T. van Dissel, and E. van de Vosse. 2009. Salmonella induced IL-23 and IL-1β allow for IL-12 production by monocytes and Mφ1 through induction of IFN-γ in CD56 NK/NK-like T cells. *PLoS. One.* 4: e8396.
20. van de Wetering, D., R. A. de Paus *(shared first author)*, J. T. van Dissel, and E. van de Vosse. 2010. Functional analysis of naturally occurring amino acid substitutions in human IFN-γR1. *Mol. Immunol.* 47: 1023-1030.
21. van de Vosse, E., T. H. Ottenhoff, R. A. de Paus, E. M. Verhard, T. de Boer, J. T. van Dissel, and T. W. Kuijpers. 2010. Mycobacterium bovis BCG-itis and cervical lymphadenitis due to Salmonella enteritidis in a patient with complete interleukin-12/-23 receptor β1 deficiency. *Infection* 38: 128-130.
22. de Paus, R. A., S. S. Kilic, J. T. van Dissel, and E. van de Vosse. 2011. Effect of amino acid substitutions in the human IFN-γR2 on IFN-γ responsiveness. *Genes Immun.* 12: 136-144.
23. Mul, D., S. Wu, R. A. de Paus, W. Oostdijk, A. C. Lankester, H. V. Duyvenvoorde, C. Ruivenkamp, M. Losekoot, M. V. Tol, F. Deluca, E. van de Vosse, and J. M. Wit. 2012. A mosaic de novo duplication of 17q21-25 is associated with growth hormone insensitivity, disturbed in vitro CD28 mediated signaling and decreased STAT5B, PI3K and NF-κB activation. *Eur. J. Endocrinol.* 116: 743-752.
24. Potjewijd, J., R. A. de Paus, van Wengen A., J. Damoiseaux, A. Verbon, and E. van de Vosse. 2012. Disseminated Mycobacterium genavense infection in a patient with a novel partial interleukin-12/23 receptor β1 deficiency. *Clin. Immunol.* 144: 83-86.
25. Kilic, S. S., A. van Wengen, R. A. de Paus, S. Celebi, B. Meziane, D. Hafizoglu, J. T. van Dissel, and E. van de Vosse. 2012. Severe disseminated mycobacterial infection in a boy with a novel mutation leading to IFN-γR2 deficiency. *J. Infect.* 65: 568-572.
26. de Paus, R. A., A. van Wengen, I. Schmidt, M. Visser, E. H. M. Verdegaal, J. T. van Dissel, and E. van de Vosse. 2013. Inhibition of the type I immune responses of human monocytes by IFN-α and IFN-β. *Cytokine, 61(2):645-55.*
27. de Paus, R. A., R. van Crevel, R. van Beek, E. Sahiratmadja, B. Alisjahbana, S. Marzuki, G. F. Rimmelzwaan, J. T. van Dissel, T. H. M. Ottenhoff, and E. van de Vosse. 2013. The influence of influenza virus infections on the development of tuberculosis. *Accepted for publication in Tuberculosis*.
28. de Paus, R.A., M. Geilenkirchen, S. van Riet, J.T. van Dissel and E. van de Vosse. 2013. Differential expression and function of human IL-12Rβ2 variants. *submitted*.