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

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## Summary and General Discussion

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

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

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

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

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

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

**Genetic variations in the Th1 pathway.**

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

Mantoux skin reactivity only); progression to clinical disease after infection; clinical characteristics and course of the disease, and different outcome measures like spontaneous recovery, progressive disease or death. Therefore, case-control studies that 'simply' compare 'patients with clinical tuberculosis' to those without signs of disease, without taking into account specific disease characteristics and determination who became infected but escaped disease, often lack realism.
Clearly, clinical expression of disease and severity of immunopathology depends on the cross-talk between *M. tuberculosis*, with its specific virulence characteristics and invasiveness, and a host immune response comprising innate as well as adaptive elements the activity of many of which is genetically preset. Thus, variability between individuals in clinical outcome results at least in part from variability in the genes that control the host defense processes. However, the influence of host genetic factors as weighed against environmental factors on an individual's susceptibility is a matter of debate. Genetic factors can be of decisive importance in the extreme susceptibility of rare, selected cases and in **chapter 3** and **4** we analyzed the functional consequences of genetic variability in two components central to type-1 cytokine pathway, the IL23R and IFN-γR1; in case of malfunctioning of these receptors, patients are highly prone to infection by intracellular bacterial pathogens like mycobacteria and salmonella but the consequence of various genetic variations in the respective genes is not yet clear.

Polymorphisms in the IL-23R chain may influence the cellular response to IL-23. Two polymorphisms occur at relatively high frequency in the population and were investigated first. The polymorphism P310L occurs at a frequency of 2–30% and the R381Q polymorphism at a frequency of 0–17%, somewhat depending on the population background. In population studies, the R381Q allele appears to confer protection against several inflammatory states like inflammatory bowel disease, psoriasis, ankylosing spondylitis, and graft versus host disease after bone marrow transplantation. The P310L allelic variant was reported to be overrepresented in patients with Grave's disease. Indeed, indirect evidence for a role of the type-1 cytokine pathway in the immunopathogenesis of inflammatory diseases is provided by the effectiveness of treatment with anti-TNF antibodies like Infliximab in many of these disease states. In view of these associations with diseases, it was suggested that the R381Q and P310L variants of the IL-23R might be functionally distinct. In **chapter 3** we show that there are no functional differences between these two variants in the IL-23-induced STAT phosphorylation, IFN-γ induction, or T cell proliferation. Furthermore, we tested a newly identified Y173H variant of the IL-23R, which also did not show functional differences when compared to the wild type receptor. Combined with the IL-12Rβ1, IL-23R forms the functional IL-23 receptor complex. Signal transduction of IL-23 is dependent on either chain, indicating that polymorphisms of both chains may influence IL-23 responsiveness. We therefore tested whether common IL-12Rβ1 haplotypes (QMG and RTR) differentially influenced the IL-23 response. Again, no major functional differences were detected in the IL-23 responses between various combinations of IL-12Rβ1 and IL-23R chains. These results indicate that the

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

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

In **chapter 4** we compared the effect of two newly discovered IFN-γR1 variations, identified in patients with mycobacterial infections (S149L, I352M), four known polymorphisms (V14M,V61I, H335P, L467P), all seven reported missense mutations (V61Q, V63G, Y66C, C77Y, C77F, C85Y, I87T) and the 818delTTAA mutation on the expression and function of IFN-γR1 in the same genetic background. 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. Expression on the cell surface of V14M is reduced which may result in slightly reduced IFN-γ responses, when IFN-γR1 gene transcription *in vivo* is limited to natural numbers of the receptors. This polymorphism may influence susceptibility to infections or predisposition to autoimmune disease such as systemic lupus erythematosus. The other variants are deleterious mutations with V61E, V61Q, Y66C, C77F, C77Y and C85Y leading to complete IFN-γR1 deficiency, while V63G and I87T lead to partial IFN-γR1 deficiency.

Complete IFN-γR1 deficiency is characterized by severe infections with environmental mycobacteria or *M. bovis* BCG and patients with these deficiencies usually present within the first 5 years of life. In case of active infection, patients with partial IFN-γR deficiencies can benefit from treatment with IFN-γ. By contrast, patients with complete IFN-γR1 deficiencies are not able to respond to IFN-γ and thus will not benefit from treatment with recombinant IFN-γ. In **chapter 5** we evaluated whether IFN-α could compensate for the absence of IFN-γ effects in cells obtained from an IFN-γR1 deficient patient. IFN-α and IFN-γ activate common signaling pathways and the induced

genes and biological activities they induce overlap. Like IFN-γ, IFN-α induces STAT1 phosphorylation in its target cells. Therefore, treatment with exogenous IFN-α might (partly) compensate for the absent effect of IFN-γ in patients with a deficiency of the IFN-γR. However, IFN-α could not compensate for the abrogated IFN-γ effects; despite the fact that IFN-α induced STAT1 phosphorylation in cells of an IFN-γR1 deficient patient, IFN-α did not upregulate CD64 and CD54 expression, both considered markers of IFN-γ activation signaling. Furthermore, IFN-α did not prime for enhanced cytokine production in response to LPS. In control cells, IFN-α even antagonized the IFN-γ induced upregulation of CD64 and the priming effect of IFN-γ on LPS induced cytokine production. In patients suffering complete IFN-γR deficiencies this is not likely to be a problem; however, in patients with partial deficiencies IFN-α may abrogate the rest response to IFN-γ.

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

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

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

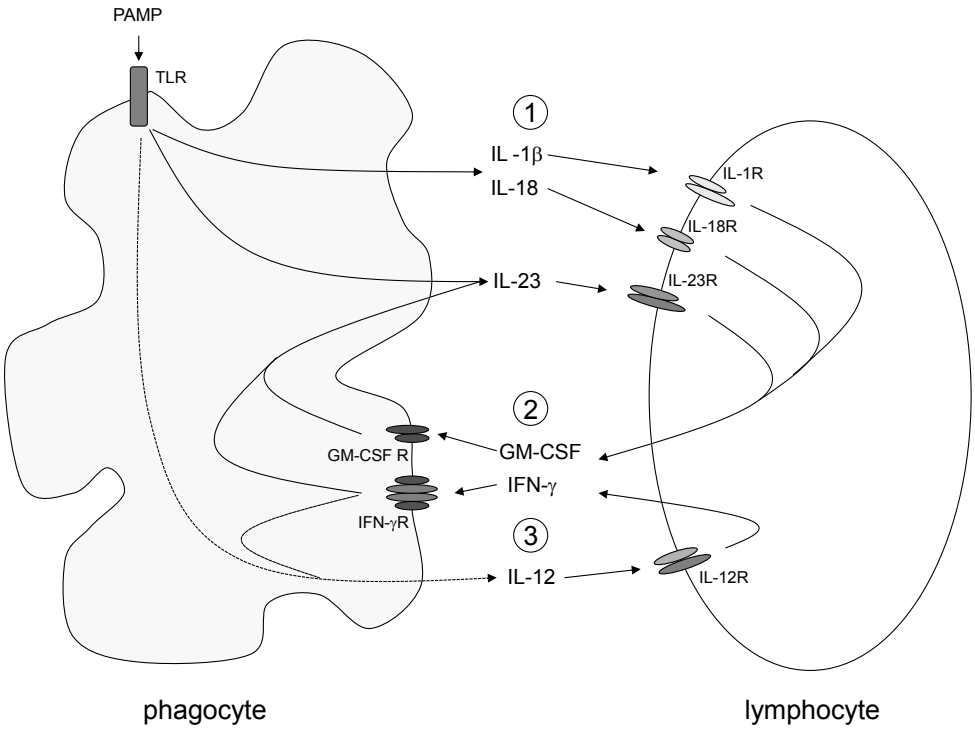


**Figure 1. Initiation of a Th1 response.** (1) In response to pathogen associated molecular patterns (PAMPs) phagocytes like monocytes and macrophages start to produce IL-23, IL-1β and IL-18, but no IL-12. (2) IL-23, in combination with IL-1β and IL-18, induces IFN-γ and GM-CSF production in lymphocytes.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

**Concluding remarks**

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

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

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