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

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## Chapter 3

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

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## Abstract

Interleukin-23 (IL-23) is a regulator of cellular immune responses involved in controlling infections and autoimmune diseases. Effects of IL-23 on T cells are mediated via a receptor complex consisting of an IL-12R $\beta$ 1 and a specific IL-23R chain. The R381Q and P310L variants of the IL-23R were recently reported to be associated with autoimmune diseases, suggesting they have an effect on IL-23R function. To investigate this matter, these variants and a newly identified variant, Y173H, were retrovirally transduced into human T cell blasts and functionally characterized by measuring the IL-23-induced signal transduction pathway (i.e., STAT1, STAT3 and STAT4 phosphorylation), and IFN- $\gamma$  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- $\gamma$  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- $\gamma$  inducer. In addition, IL-23 also exerted a minor IL-12-like effect by inducing IL-23R-independent, IL-12R $\beta$ 1-dependent STAT4 phosphorylation and IFN- $\gamma$  production. In conclusion, the reported association between R381Q and P310L variants of the IL-23R and autoimmune diseases does not depend on differences in functional activity between wild-type and R381Q and P310L variants of the IL-23R.

## Introduction

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

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

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

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

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

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

### Materials and Methods

#### *Cloning IL-23R variants into a retroviral expression vector*

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

#### *Cells, culture conditions and retroviral transduction*

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors by Ficoll separation and cultured in IMDM supplemented with 20 mM GlutaMAX, 10% FCS, 100 U/ml Penicillin, 100  $\mu$ g/ml Streptomycin (Invitrogen) supplemented with 30 U/ml IL-2 (Proleucin, Chiron). T-cell blasts (TCB) were generated by stimulating PBMCs with 800 ng/ml phytohemagglutinin (PHA) (Murex). On day 2, after PHA-stimulation,  $0.5 \times 10^6$  TCB were transduced on a CH-296 coated (RetroNectin<sup>TM</sup>, Takara Shuzo) 48-wells plate using 0.5 ml of virus containing supernatants as described previously (28). On day 10, cells were sorted on GFP or NGFR signal by fluorescence activated cell sorting (FACS) and restimulated in the presence of 800 ng/ml PHA and irradiated allogeneic PBMCs (pool from two donors) and irradiated B-LCL in a cellular ratio of 2:10:1 respectively. After each restimulation, cells were allowed to expand for at least 14 days. The cells were washed three times before each functional assay. NK-92 cells (29) were cultured in IMDM supplemented with 20 mM GlutaMAX, 10% FCS, 10% Horse serum (Gibco), 100 U/ml Penicillin, 100  $\mu$ g/ml Streptomycin (Invitrogen) supplemented with 100 U/ml IL-2 and 50  $\mu$ M 2-mercaptoethanol (Merck) and transduced as described above.

*Co-transduction of IL-12R $\beta$ 1 and IL-23R into IL-12R $\beta$ 1<sup>-/-</sup> TCB*

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

*FACS analysis*

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

*Cytokine production analysis and proliferation assay*

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

*STAT phosphorylation assays*

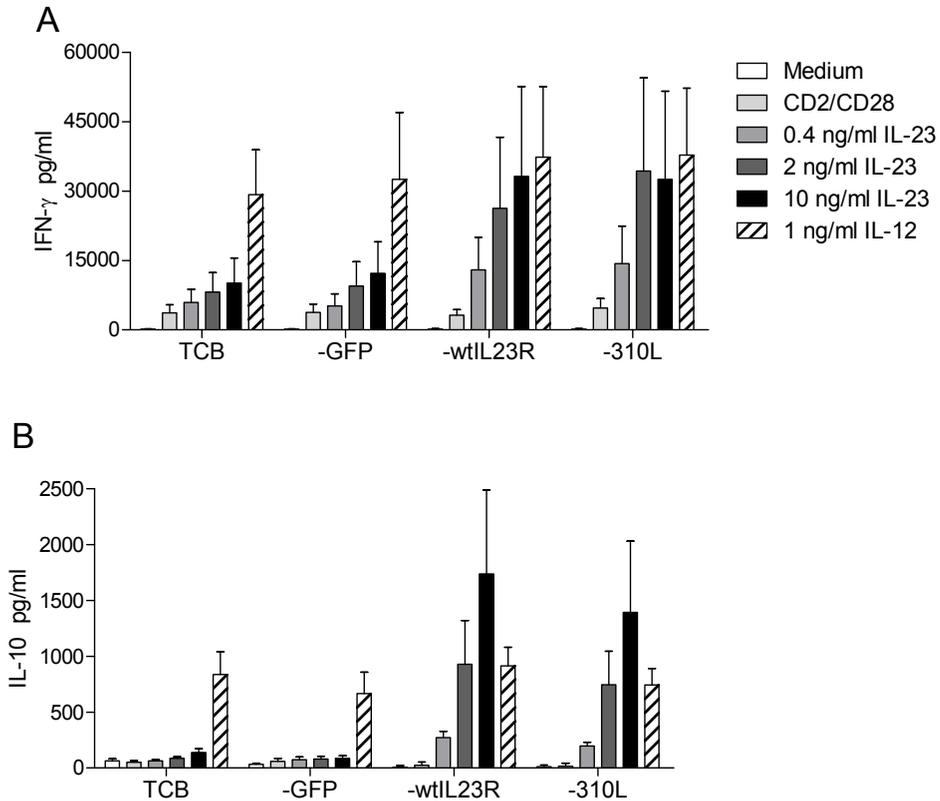
To study signal transduction  $10^5$  TCB were pre-stimulated overnight in 150  $\mu$ l culture medium with 2  $\mu$ g/ml anti-CD2 and 2  $\mu$ g/ml anti-CD28. Thereafter the cells were pulsed with 10 ng/ml IL-23, 1 ng/ml IL-12, 6.8 ng/ml IL-12p40 (Peprotech) or 6.8 ng/ml IL-12p80 (Peprotech). For use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

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

## Results

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

We used a retroviral expression system to study the impact of various IL-23R polymorphisms on the function of the IL-23 receptor in normal control PHA stimulated TCB that express IL-12Rβ1. The retroviral expression vector, pLZRS, ensures transcription and expression of the *IL23R* gene and green fluorescent protein (*GFP*) genes in tandem and allows for selection of transduced cells by FACS for the GFP signal. Transduction efficiency was typical between 5 and 20%; after FACS sorting 96-99% (98% average) of the cells were GFP positive.



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

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

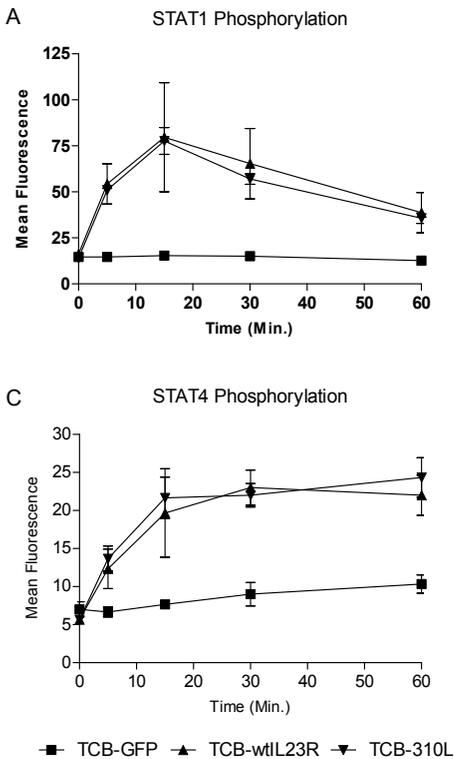
#### *Detection of IL-23R expression on the cell membrane*

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

#### *Kinetics of IL-23 induced STAT phosphorylation*

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

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

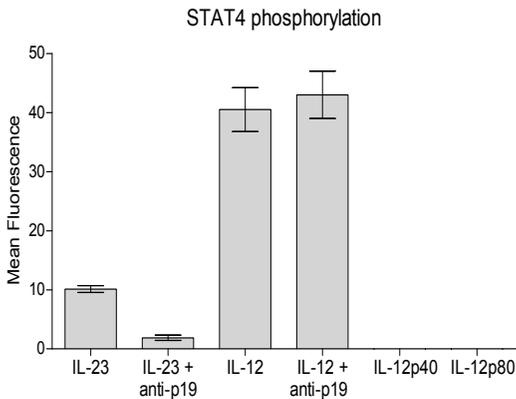


**Figure 2. Kinetics of IL-23 induced STAT phosphorylation.** TCB transduced with the wild-type (wtIL23R), the 310L variant or GFP alone were stimulated with 10 ng/ml IL-23 for 0, 5, 15, 30 and 60 minutes. The amount of STAT1 (A), STAT3 (B) and STAT4 (C) phosphorylation was determined by FACS using phospho-specific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

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

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

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



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

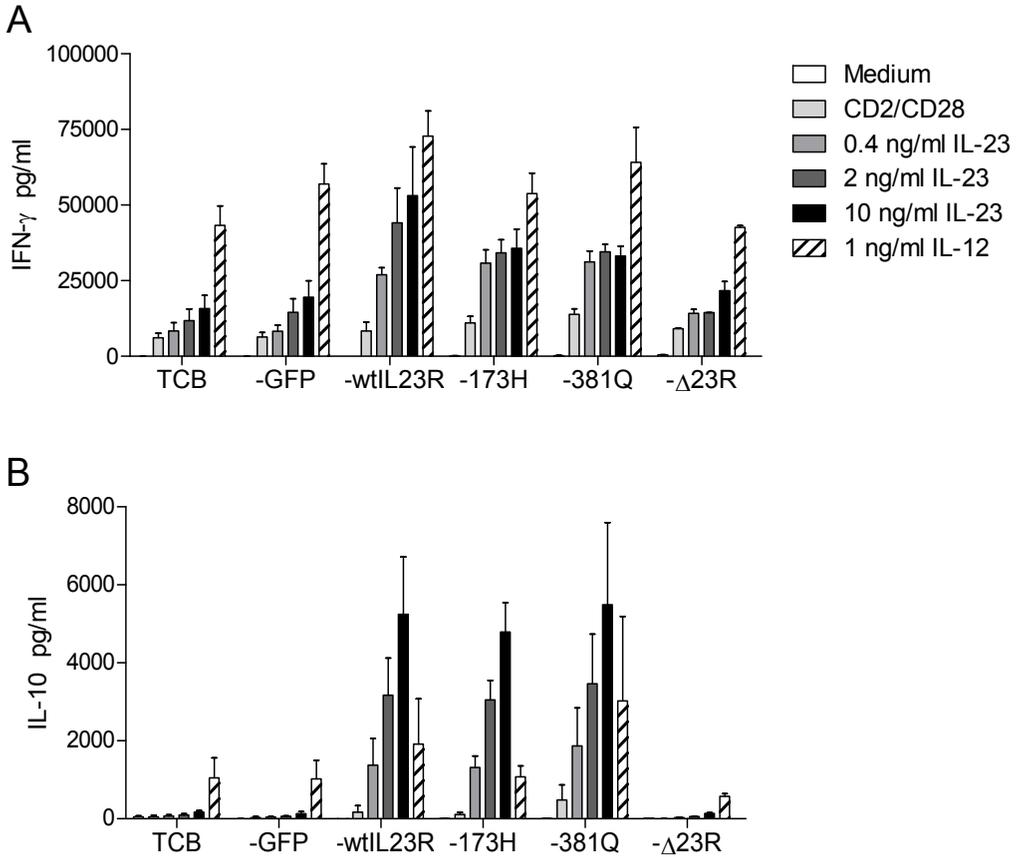
#### *Effect of variations in the IL-23R on IL-23 induced IFN- $\gamma$ and IL-10 production*

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- $\Delta$ 23R). TCB and TCB-GFP cultures were used as controls. IFN- $\gamma$  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- $\gamma$  production by TCB-wtIL23R, TCB-173H and TCB-381Q cultures were detected. Although it appeared that IFN- $\gamma$  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- $\gamma$  production in the TCB- $\Delta$ 23R culture, comparable with other cells lacking the IL-23R: the control TCB and the TCB-GFP cultures (Fig. 4A). Comparable large amounts of IL-10 were induced by IL-23 in TCB-wtIL23R, TCB-173H and TCB-381Q (Fig. 4B).

#### *IL-23 enhances IL-10 production relatively more than IFN- $\gamma$ production*

We demonstrated that IL-23, similar to IL-12, is a potent inducer of both IFN- $\gamma$  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- $\gamma$  production were calculated (Fig. 5). All TCB cultures stimulated with anti-CD2 and anti-CD28 produced around twenty times less IL-10 than IFN- $\gamma$  (ratio  $\sim$  0.05), a similar ratio was observed



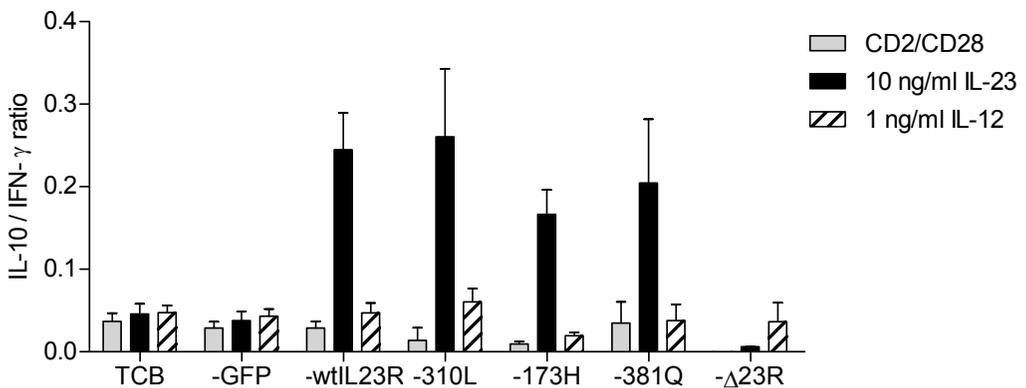
**Figure 4. The IL-23R variants do not differ in IL-23 induced cytokine production.** TCB transduced with the constructs of the wtIL23R, the 173H- and the 381Q-variants were compared with normal TCB, TCB-GFP and TCB- $\Delta$ 23R. The cell-lines were tested for IFN- $\gamma$  (A) and IL-10 (B) production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

in response to IL-12. Addition of IL-23 to TCB lacking a functional IL-23R (TCB, TCB-GFP and TCB- $\Delta$ 23R) 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- $\gamma$  ratio by a factor five. No major differences were observed between the IL-23R alleles (Fig. 5).

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

Although no difference was detected in IFN- $\gamma$  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

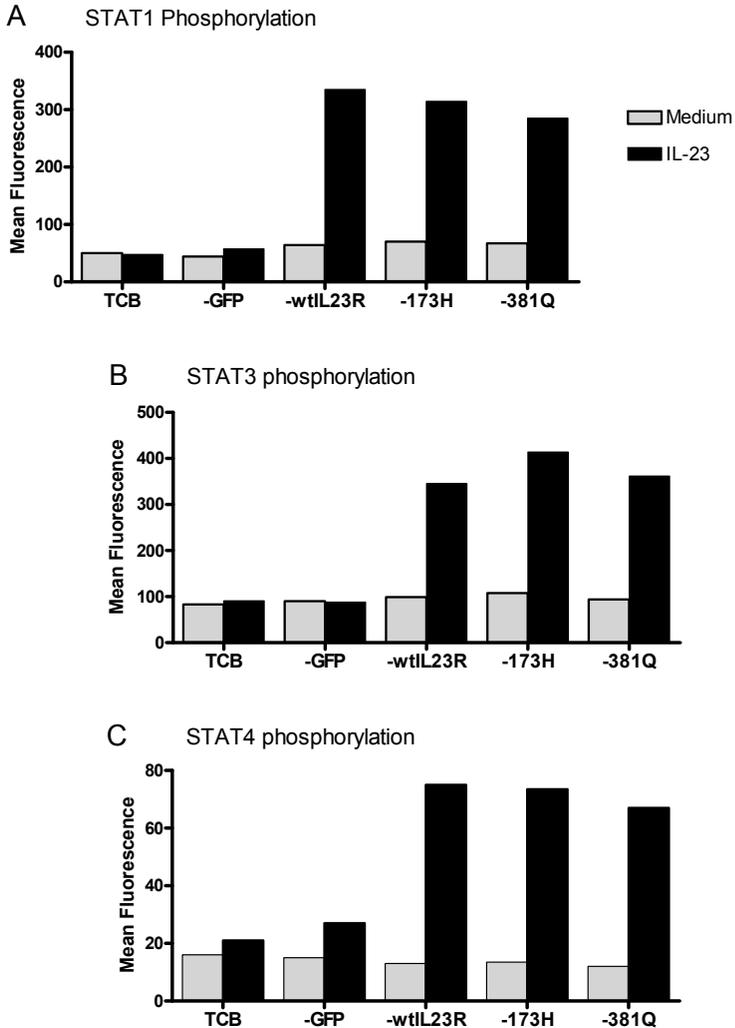
with the controls (TCB-wtIL23R, TCB, TCB-GFP and TCB- $\Delta$ 23R). Cells were pre-stimulated with anti-CD2 and anti-CD28 followed by a short incubation with or without IL-23. STAT1, STAT3 and STAT4 phosphorylation were analyzed by FACS using phospho-specific antibodies. STAT 1 and STAT3 phosphorylation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



**Figure 5. Influence of IL-23R expression on the IL-10/ IFN- $\gamma$  ratio after IL-23 and IL-12 stimulation.** The IL-10/ IFN- $\gamma$  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 - $\Delta$ 23R (n=3).

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

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



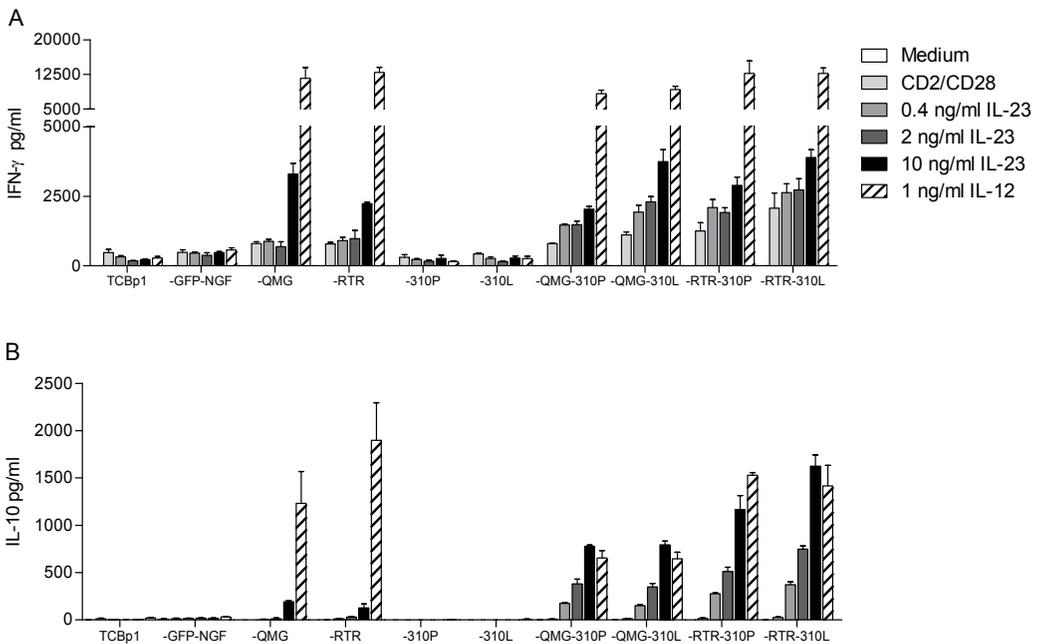
**Figure 6. The IL-23R variants do not differ in IL-23 induced STAT phosphorylation.** Normal TCB, TCB transduced with GFP and TCB transduced with the various IL-23R constructs (wtIL23R, 173H and 381Q) were stimulated with 10 ng/ml IL-23 for 15 (STAT1) or 30 minutes (STAT3 and STAT4). The amount of STAT1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

#### *Combined effects of IL-12R $\beta$ 1 and IL-23R variations on IL-23 driven responses*

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

null mutation. TCBp1 cells were transduced with a retroviral vector expressing an IL-12R $\beta$ 1 allele in tandem with GFP, or transduced with a retroviral vector expressing an IL-23R allele in tandem with a truncated NGF-R as marker, or co-transduced with both. We selected the cells for expression of the markers and examined the cytokine release in response to IL-12 and IL-23. TCBp1 transduced with the IL-12R $\beta$ 1 alleles QMG or RTR produced IFN- $\gamma$  (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- $\gamma$ , 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 $\beta$ 1 and IL-23R chains.

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



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

## Discussion

The main finding of the present study is that the natural variants of the human IL-23R, P310L, Y173H and R381Q do not differ in receptor transfer function from the wild-type allele. This conclusion is based on analysis of IL-23R downstream intracellular signaling pathways (STAT1, STAT3 and STAT4 phosphorylation) following ligand binding, as well as the IFN- $\gamma$  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- $\gamma$ -to-IL-10 production that is distinct from that induced by IL-12. Finally, IL-23 exhibited a minor IL-12-like effect by inducing STAT4 phosphorylation dependent on IL-12R $\beta$ 1 but independent of IL-23R expression.

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

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

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

We demonstrated that IL-23 via its receptor induced STAT1, STAT3 and STAT4 phosphorylation, but not STAT5 phosphorylation whereas IL-12 could only induce STAT4 phosphorylation. Both cytokines can induce IFN- $\gamma$  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- $\gamma$ . The effect could be blocked by a specific antibody against IL-23p19, while IL-12p40 or IL-12p80 could not induce this IL-12 like effect, indicating that IL-23 itself and not IL-23 byproducts mediate this effect. These findings demonstrate that IL-23 is also able to signal through another receptor besides the IL-23 receptor, albeit with a much lower efficiency. Because this signal transduction, similar to IL-12 signaling, only involves STAT4 phosphorylation, an obvious candidate would be the IL-12 receptor. Indeed, in T cells from an IL-12R $\beta$ 1<sup>-/-</sup> patient this IL-12-like effect of IL-23 was only observed after transduction with an IL-12R $\beta$ 1 expression construct, indicating that the IL-12R $\beta$ 1 chain is indeed involved in this signaling. Whether the IL-12R $\beta$ 2 chain or another receptor chain is involved and to which extent IL-23 can achieve this IL-12-like effect *in vivo* remains to be investigated. Based on our findings we conclude that IL-23 exerts an IL-12-like effect that depends on IL-12R $\beta$ 1 and not on IL-23R expression.

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

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

used in a study to describe the cytokine profile of IL-23R positive CD45RO<sup>+</sup> T cells (31). The authors did not test the IL-23 responsiveness of the 'IL-23R positive' cells, which might have proven whether or not these cells indeed expressed a functional IL-23R. We were also able to stain this subset of human CD45RO<sup>+</sup> 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-12Rβ1 but independent of IL-23R expression. The P310L, Y173H and the R381Q variations in the IL-23R are fully functional and do not show differences in IL-23 responsiveness.

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