Genes and Immunity (2011) 12, 136–144

www.nature.com/gene

ORIGINAL ARTICLE

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

RA de Paus[1], SS Kilic[2], JT van Dissel[1] and E van de Vosse[1]
[1]Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands and [2]Department of Pediatric Immunology, Uludag University School of Medicine, Bursa, Turkey

*Patients with interferon-γ receptor (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, whereas the R114C, T168N and G227R constitute mutations associated with disease.*
Genes and Immunity (2011) **12,** 136–144; doi:10.1038/gene.2010.74; published online 20 January 2011

**Keywords:** *IFNGR2; interferon-γ; immunodeficiency; mutation; mycobacterium; polymorphism*

## 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*. On encounter with these bacteria, antigen-presenting cells will stimulate natural killer cells and next type-1 helper T (Th1) cells to produce IFN-γ. IFN-γ is a pleiotropic cytokine that 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 interleukin-10. By consequence, IFN-γ responses result in enhanced cytotoxicity of natural killer 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 transphosphorylation,[7] which in turn phosphorylate STAT1 on its tyrosine 701 residue.[8] Phosphorylated STAT1 dissociates from the receptor, dimerises and translocates to the cell nucleus, where it regulates gene expression either directly (for example, *CD54*, *CXCL10* and *CCL2*)[9–11] or indirectly via the induction of transcription factors such as IRFs and CIITA (for example, *IL12B*, *B2M* and major histocompatibility complex).[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 internalisation 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 effect 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 (SS Kilic,

Correspondence: Dr E van de Vosse, Department of Infectious Diseases, Leiden University Medical Center, Albinusdreef 2, Leiden 2333 ZA, The Netherlands.
E-mail: E.van_de_Vosse@lumc.nl
Received 5 October 2010; revised and accepted 17 November 2010; published online 20 January 2011

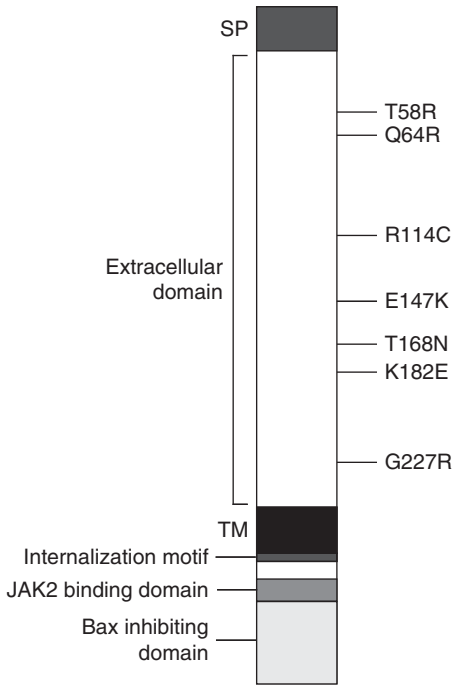


**Figure 1** Schematic presentation of IFN-γR2. On the left the various domains are indicated, on the right the amino acid variations that were analysed are indicated. TM, transmembrane domain; SP, signal peptide; C, cysteine; E, glutamic acid; G, glycine; K, lysine; N, asparagine; Q, glutamine; R, arginine T, threonine.

manuscript in preparation). Variations reported in healthy individuals are T58R (population allele frequency 0–18%), Q64R (9–57%), E147 K (0–2%) and K182E (0–4%; National Center for Biotechnology Information, single-nucleotide polymorphism 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,[20] whereas 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 immunoglobulin E levels.[22] The exact effect of the variations on IFN-γR2 function, with the exception of the T168N mutation, is unknown.

In this study, we analysed the effect of previously described amino acid variations in IFN-γR2 on receptor expression and function. We cloned the complementary DNA 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.

## Results

*Functional transfer of wild- type IFN-γR2 into cell lines*

To set up a model system, we cloned a wild-type IFN-γR2 complementary DNA into the retroviral expression vector pLZRS-green fluorescent protein (GFP). The use of pLZRS-GFP allows for tandem expression of IFN-γR2 and GFP. We expressed the receptor in various human cell lines: B-LCL, THP-1, Jurkat, T-cell blasts (TCB) and IFN-γR2-deficient fibroblastic G1A cells. The cell surface expression of IFN-γR2 was determined by fluorescence-activated cell sorting (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 the 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 0–30 min 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 on IFN-γ stimulation (Figures 3a and b). The STAT1 phosphorylation in G1A (Figure 3a) and in TCB (Figure 3b) with the wild-type IFN-γR2 was at maximum at 15 min 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).

*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

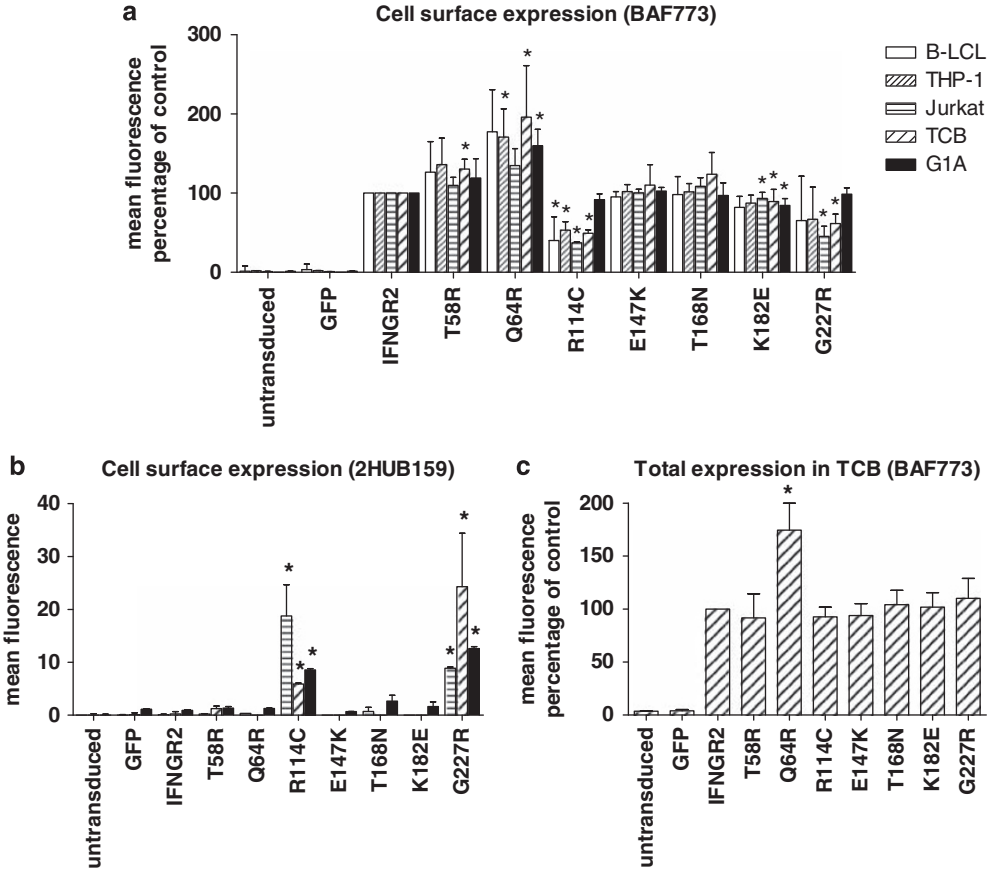


**Figure 2** Extracellular and total expression of the IFN-γR2 variants. (**a**) Extracellular expression of IFN-γR2 was measured on B-LCL ($n=3$), THP-1 ($n=3$), Jurkat ($n=5$), TCB ($n=6$) and G1A ($n=3$) cells that were untransduced, transduced with GFP alone or transduced with one of the *IFNGR2* 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 permeabilisation of the cells. For all experiments the expression of IFN-γR2 was determined for the transduced cells with equal GFP expression. The mean fluorescence ± s.d. is displayed. For **a** and **c,** the mean fluorescence is depicted as percentage of the IFN-γR2 wild-type variant, in which 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).

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 with 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 and 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). Similar to the wild type, 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 the amount of protein can be caused by 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 with the other variants.

### *Effect of IFN-γR2 variations on IFN-γ-mediated signal transduction*

Intracellular signalling via the IFN-γR complex is mediated by STAT1 molecules that are activated through tyrosine phosphorylation. We chose 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 (Figures 3a and b). 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

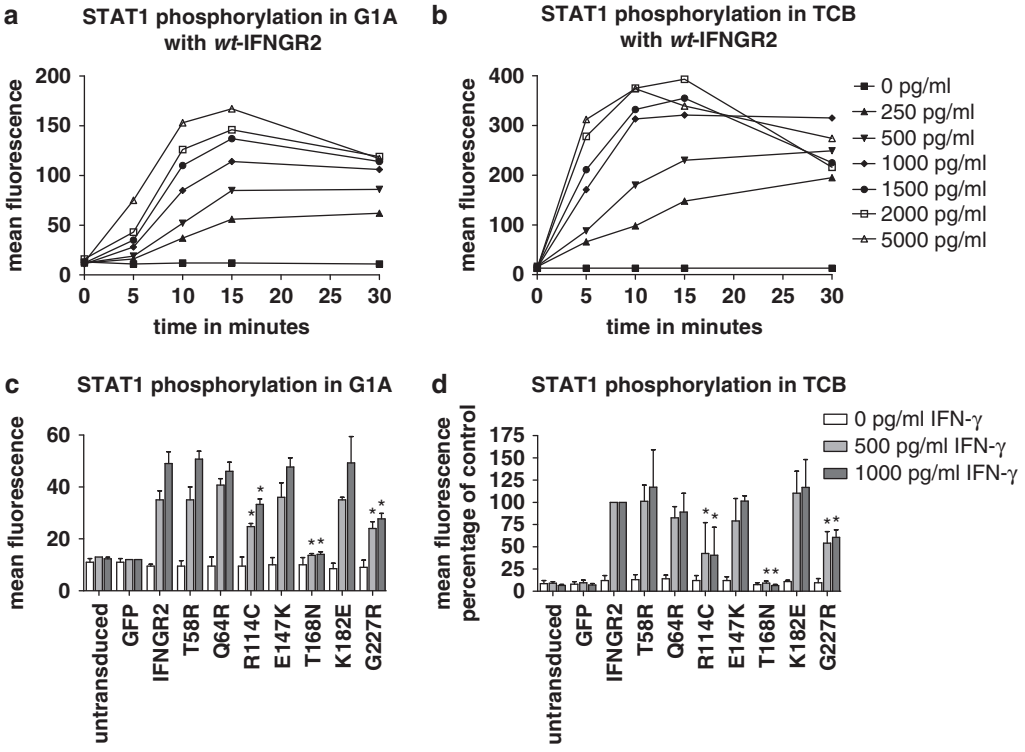


**Figure 3** Kinetics of IFN-γ induced STAT1 phosphorylation and the influence of IFN-γR2 variations. The amount of intracellular phosphorylated STAT1 was determined as a measure of IFN-γ-induced signal transduction. The kinetics of STAT1 phosphorylation was first determined for (**a**) G1A and (**b**) TCB-4 cells transduced with wild-type IFN-γR2. Cells were stimulated for 0–30 min with various concentrations of IFN-γ. Next, we studied the effect 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 min with 500 or 1000 pg ml$^{-1}$ 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 ± s.d. of one representative out of two experiments is displayed. For (**d**) the mean fluorescence ± s.d. 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).

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 60–90 min of stimulation with 500 pg ml$^{-1}$ 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.

*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, human leukocyte antigen (HLA) class I and II in IFN-γR2-transduced TCB and G1A cells by FACS. In addition, we tested the supernatants of these cells for the presence of CXCL10 by enzyme-linked immunosorbant assay. 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$^{-1}$ 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 (Figures 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 manner the CD54 expression on the cell surface (Figure 4a). Cells transduced with R114C or G227R showed impaired responses; for instance, in comparison with cells transduced with the wild-type receptor 8 (G227R) to 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). Although the induction of CD54 and HLA class I expression could be detected after incubation of the cells with as little as 25 pg ml$^{-1}$ IFN-γ, CXCL10 expression was induced only at concentrations of 2500 pg ml$^{-1}$ 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 (Figures 4a and b). An overview of the results of all assays is presented in Table 1.

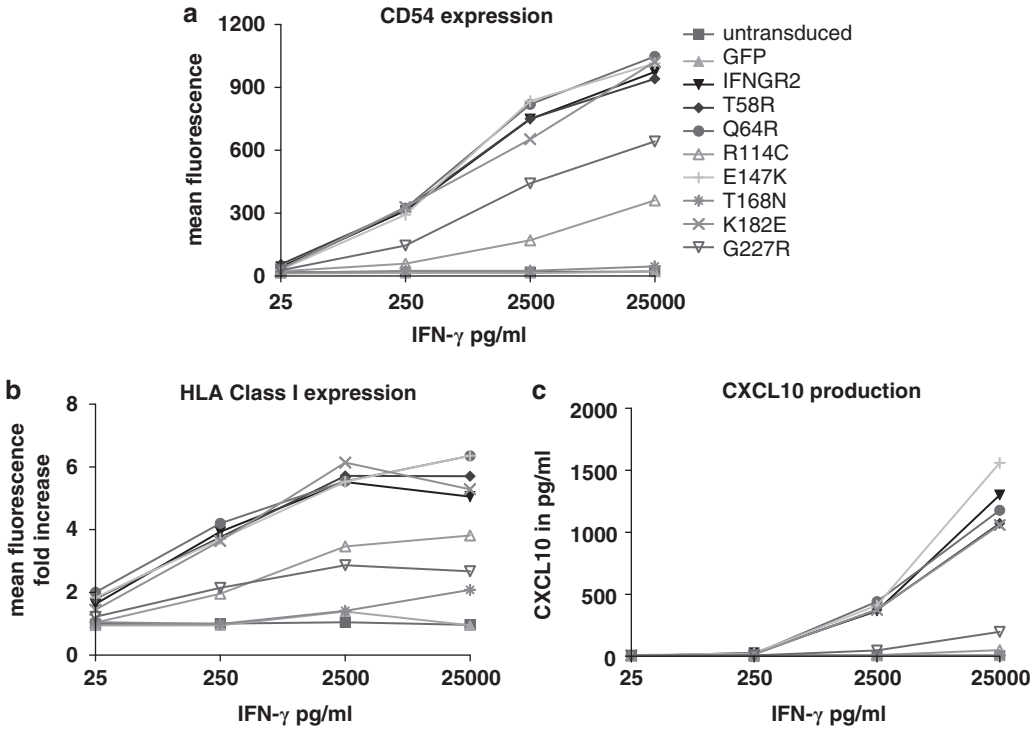


**Figure 4** The effect of the IFN-γR2 variations on IFN-γ responses. Untransduced G1A cells and G1A cells retrovirally transduced with GFP alone or with one of the IFN-γR2 variants were stimulated for 18 h (**a**, **b**) or 48 h (**c**) with various concentrations of IFN-γ. The CD54 expression (**a**) 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**, **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 |

Abbreviations: HLA, human leukocyte antigen; IFN-γR2, interferon-γ receptor; TCB, T-cell blasts.
[a]Expression analysed with BAF773 antibody.
[b]Analyses with TCB.
[c]Analysis with G1A.

*No dominant-negative effects of IFN-γR2 mutations*

As 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 T168N, R114C or 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$^{-1}$ 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

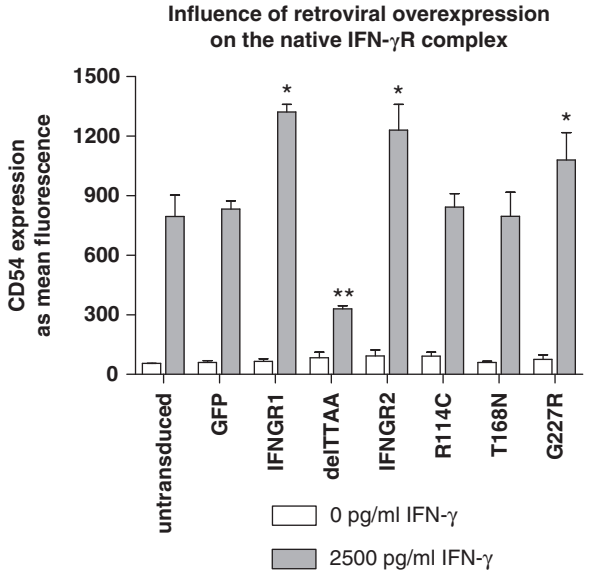


**Figure 5** Influence of IFN-γR2 mutants on the function of the natural IFN-γ receptor complex. Normal and deficient IFN-γR1 and IFN-γR2 proteins were overexpressed in THP-1 cells to study their influence on the functioning of the native receptor IFN-γR complex. Untransduced THP-1 cells and THP-1 cells 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$^{-1}$ IFN-γ. Hereafter, the cells were analysed for CD54 expression by FACS. One representative of two experiments in triplo is shown. * Indicates significantly higher and ** indicates significantly lower induction of CD54 expression as compared with the stimulation of THP-1 cells ($P < 0.05$, student *t*-test).

60% decreased response (Figure 5). Thus, no dominant-negative effects of the IFN-γR2 variants were seen.

## Discussion

In an *in vitro* test model to analyse the functional effect of amino acid substitutions in IFN-γR2, we successfully characterised 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, as 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, summarised 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. Using 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 effect 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 used 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 caused by a difference in affinity towards the variants or caused by an actual difference in the amount of protein present. However, in the system with overexpression, we observed for most variants a comparable total (that is, 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 readout 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-γR deficiency, as was previously shown for the T168N variation. The T168N variation was described as a mutation resulting in a complete loss of function because of the gain of glycosylation at the asparagine residue.[25] We could confirm the severe effect 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, as 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 (SS Kilic, manuscript in preparation), seems 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 because of the 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 complementary DNA 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. In addition, we found that one of the six TCB lines, TCB-5, transduced with the R114C variant showed a highly significant reduced expression as compared with the other TCB lines carrying R114C. Taken together, these findings suggest that the effect 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. The 2HUB159 was raised against an unspecified fragment of the extracellular domain of the receptor and possibly recognises the receptor in a denatured, for example in blotting experiments, rather than natural conformation. Remarkably, we could detect cell surface expression of R114C and G227R with the 2HUB159 antibody. Therefore, R114C and G227R variants are likely to result in an altered conformation of the receptor, as compared with wild-type IFN-γR2, and this may contribute to the reduced receptor function. Although 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 (SS 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 showed 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 with the other variants. As 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 has a role.[21] Second, the 64Q allele was positively associated with the occurrence of systemic lupus erythematosis, which is considered to be a Th2 disease.[20] And third, in a British population the 64Q allele showed a weak association with low immunoglobulin E 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 polarisation 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 characterisation 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, whereas 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.

## Materials and methods

*Cloning* IFNGR2 *variants into a retroviral expression vector*
The full-length *IFNGR2* coding sequence was PCR amplified from complementary DNA of a healthy control with the sense primer 5′-AATTGGATCCCGGGGCCATG CGACCGAC-3′ and the anti-sense primer 5′-CCGGCTC GAGTTCAAAGCGTTTGGAGAACAT-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 calcium phosphate transfection kit (Invitrogen, Breda, The Netherlands). The virus producing cells were cultured for 2–3 weeks under 2 μg ml$^{-1}$ puromycin (Clontech, Saint-Germain-en-Laye, France) selection after which a 20 h supernatant was harvested. *IFNGR1* constructs were previously made and described.[17]

*Cell culture and retroviral transduction*
Human Jurkat (T lymphoblastic cell line, ATCC TIB-152, ATCC, Manassas, VA, USA), 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 TCB were cultured in IMDM (Lonza, Vervier, Belgium) as described before.[27] Culture media were supplemented with 10% fetal calf serum, 20 mM GlutaMax, 100 U ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin (Invitrogen). For propagation, the adherent G1A cells were trypsinised in the presence of ethylenediaminetetraacetic acid. Before testing, G1A cells were collected using only ethylenediaminetetraacetic acid (Invitrogen). In all, $0.25 \times 10^6$ 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. In all, $0.5 \times 10^6$ 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 4 days before analysing in further assays.

*FACS analysis of IFN-γR2 expression*
To detect IFN-γR2 membrane expression, cells were stained with an allophycocyanin-conjugated polyclonal antibody BAF773 (R&D Systems, Abingdon, UK), with phycoerythrin (PE)-conjugated monoclonal 2HUB159, 2HUB145 (SantaCruz, Heidelberg, Germany), with monoclonal MMHGR-2 (PBL, New Brunswick, NJ, USA) and counterstained with goat-anti-mouse immunoglobulin G-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 immunoglobulin G1 antibody. After staining, the cells were washed twice in phosphate-buffered saline with 0.2% bovine serum albumin (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. To detect total (intra- and extracellular) IFN-γR2 expression, cells were treated with 4% paraformaldehyde and 0.1% saponin (Sigma) before staining with APC conjugated BAF773.

*STAT phosphorylation assay*
To study signal transduction, $2 \times 10^5$ G1A cells or TCB in 200 μl of culture medium were pulsed for 0–30 min with various concentrations of IFN-γ (Invitrogen). The cells were fixated with 4% paraformaldehyde and permeabilised with 90% methanol (Merck, Amsterdam, The Netherlands). Subsequently, the cells were washed with phosphate-buffered saline containing 0.2% bovine serum albumin (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.

*IFN-γ response assays*
To study the upregulation of cell surface markers, $0.25 \times 10^6$ G1A cells were stimulated 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 and 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.

Before cytokine production assays, G1A cells were sorted by FACS for low GFP expression using a FACSAria (BD biosciences). In all, $0.1 \times 10^5$ G1A cells were cultured in 0.2 ml of culture medium and pulsed with various concentrations of IFN-γ (Invitrogen). After 48 h, cell free supernatants were collected and tested for the presence of CXCL10 with a specific enzyme-linked immunosorbent assay (Invitrogen).

## Conflict of interest

The authors declare no conflict of interest.

## 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**: 739–749.
3 Valente G, Ozmen L, Novelli F, Geuna M, Palestro G, Forni G *et al*. Distribution of interferon-gamma receptor in human tissues. *Eur J Immunol* 1992; **22**: 2403–2412.
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 gamma. *Cytokine* 2010; **50**: 1–14.
6 Bernabei P, Allione A, Rigamonti L, Bosticardo M, Losana G, Borghi I *et al*. Regulation of interferon-gamma receptor (IFN-gammaR) 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-gamma 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-gamma. *Annu Rev Immunol* 1997; **15**: 749–795.
9 Tessitore A, Pastore L, Rispoli A, Cilenti L, Toniato E, Flati V *et al*. Two gamma-interferon-activation sites (GAS) on the promoter of the human intercellular adhesion molecule (ICAM-1) gene are required for induction of transcription by IFN-gamma. *Eur J Biochem* 1998; **258**: 968–975.
10 Valente AJ, Xie JF, Abramova MA, Wenzel UO, Abboud HE, Graves DT. A complex element regulates IFN-gamma-stimulated monocyte chemoattractant protein-1 gene transcription. *J Immunol* 1998; **161**: 3719–3728.
11 Clarke DL, Clifford RL, Jindarat S, Proud D, Pang L, Belvisi MG *et al*. TNFalpha and IFNgamma synergistically enhance transcriptional activation of CXCL10 in human airway smooth muscle cells via STAT-1, NF-kappaB and the transcriptional coactivator CREB-binding protein. *J Biol Chem* 2010; **285**: 29101–29110.
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-gamma-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. *J Immunol* 2000; **165**: 271–279.
14 van Boxel-Dezaire AH, Stark GR. Cell type-specific signaling in response to interferon-gamma. *Curr Top Microbiol Immunol* 2007; **316**: 119–154.
15 Gomez JA, Sun W, Gama V, Hajkova D, Yoshida T, Wu Z *et al*. The C-terminus of interferon gamma receptor beta chain (IFNgammaR2) has antiapoptotic activity as a Bax inhibitor. *Cancer Biol Ther* 2009; **8**: 1771–1786.

16 Regis G, Conti L, Boselli D, Novelli F. IFNgammaR2 trafficking tunes IFNgamma-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-gammaR1. *Mol Immunol* 2010; **47**: 1023–1030.
18 Doffinger R, Jouanguy E, Dupuis S, Fondaneche M-C, Stephan J-L, Emile JF *et al*. Partial interferon-gamma receptor signaling chain deficiency in a patient with bacille Calmette-Guerin and Mycobacterium abscessus infection. *J Infect Dis* 2000; **181**: 379–384.
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**: 1729–1737.
20 Nakashima H, Inoue H, Akahoshi M, Tanaka Y, Yamaoka K, Ogami E *et al*. The combination of polymorphisms within interferon-gamma receptor 1 and receptor 2 associated with the risk of systemic lupus erythematosus. *FEBS Lett* 1999; **453**: 187–190.
21 Schrijver HM, Hooper-van Veen T, van Belzen MJ, Crusius JB, Pena AS, Barkhof F *et al*. Polymorphisms in the genes encoding interferon-gamma and interferon-gamma receptors in multiple sclerosis. *Eur J Immunogenet* 2004; **31**: 133–140.
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**: 425–429.
23 Rosenzweig SD, Dorman SE, Uzel G, Shaw S, Scurlock A, Brown MR *et al*. A novel mutation in IFN-gamma receptor 2 with dominant negative activity: biological consequences of homozygous and heterozygous states. *J Immunol* 2004; **173**: 4000–4008.
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**: 3482–3489.
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**: 1597–1602.
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**: 3847–3855.
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**: 7351–7367.
29 Kinsella TM, Nolan GP. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum Gene Ther* 1996; **7**: 1405–1413.
30 Kohlhuber F, Rogers NC, Watling D, Feng J, Guschin D, Briscoe J *et al*. A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. *Mol Cell Biol* 1997; **17**: 695–706.