CHAPTER 5

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

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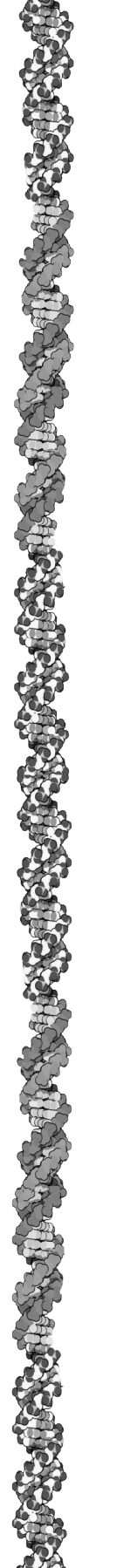


Abstract

IFN-γ plays an essential role in the IL-12/IL-23/ IFN-γ pathway that is required for the defense against intracellular pathogens. In the IFN-γR1 several amino acid substitutions have been reported that abrogate IFN-γ signaling. These substitutions can lead to a null phenotype and enhanced susceptibility to infection by poorly pathogenic mycobacteria, a disorder known as Mendelian Susceptibility to Mycobacterial Disease (MSMD). More common amino acid variations in the IFN-γR1 may also influence IFN-γR function, albeit more subtle. To determine the effect of various amino acid substitutions on IFN-γR1 expression and function we cloned two newly identified amino acid substitutions (S149L, I352M), four common variations (V14M, V61I, H335P, L467P), seven reported missense mutations (V61Q, V63G, Y66C, C77Y, C77F, C85Y, I87T) and the 818delTTAA mutation in a retroviral expression vector. IFN-γR1 expression was determined as well as responsiveness to IFN-γ stimulation. The two newly discovered variants, and the four common polymorphisms could be detected on the cell surface, however, the V14M, H335P and I352M variants were significantly lower expressed at the cell membrane, compared to the wild type receptor. Despite the variance in cell surface expression, these IFN-γR1 variants did not affect function. In contrast to literature, in our model the expression of the V63G variant was severely reduced and its function was severely impaired but not completely abrogated. In addition, we confirmed the severely reduced function of the I87T mutant receptor, the completely abrogated expression and function of the V61E, V61Q, C77F, C77Y and the C85Y mutations, as well as the overexpression pattern of the 818delTTAA mutant receptor. The Y66C mutation was expressed at the cell surface, it was however, not functional. We conclude that the V14M, V61I, S149L, H335P, I352M and L467P are functional polymorphisms. The other variants are deleterious mutations with V61E, V61Q, Y66C, C77F, C77Y and C85Y leading to complete IFN-γR1 deficiency, while V63G and I87T lead to partial IFN-γR1 deficiency.

1. Introduction

Interferon-γ (IFN-γ) is a pleiotropic cytokine playing a central role in type-1 immunity. Human host immunity against intracellular pathogens such as Mycobacteria and Salmonellae depends on an effective cell-mediated immune response mediated by type1 cytokines (Ottenhoff et al., 2002). Upon encounter of these pathogens, antigen presenting cells (APC) produce cytokines such as IL-23, IL-1β, IL-18, IL-12 and TNF via the stimulation of pattern recognition receptors, including Toll-like receptors. Type-1 helper T (Th1) cells and natural killer (NK) cells express receptors for these cytokines and produce IFN-γ in response to IL-12 or IL-23 which can be enhanced by IL-1. and IL-18. IFN-γ in turn, binds to the IFN-γ receptor (IFN-γR), present on nearly all cell types (Valente et al., 1992). APCs, including monocytes, macrophages and DCs, are activated by IFN-γ to produce increased levels of IL-12 and to enhance both antigen presentation and bactericidal activity (Leenen et al., 1994; Nathan and Hibbs, 1991). In addition IFN-γ can, depending on cell type, block proliferation, induce apoptosis and enhance expression of cell surface molecules such as HLA Class I and II, CD54 and CD64.

The IFN-γR is comprised of two ligand-binding IFN-γR1 chains associated with two signal-transducing IFN-γR2 chains (Boehm et al., 1997). Binding of IFN-γ to its receptor induces receptor oligomerization and activation of the receptor-associated Janus kinases JAK1 and JAK2 by trans-phosphorylation. The JAKs phosphorylate the tyrosine 440 that is part of the STAT1 docking site in the intracellular domain of the IFN-γR1, allowing for subsequent STAT1 phosphorylation (Boehm et al., 1997). Phosphorylated STAT1 dissociates from the receptor, dimerizes and translocates to the nucleus, where it regulates the expression of IFN-γ responsive genes directly (e.g. CD54)(Ramana et al., 2002), or indirectly via the induction of other transcription factors such as IRF1, IRF7 and CIITA (e.g. B2M and HLA) (Boehm et al., 1997). Although STAT1 is the main mediator of IFN-γ responses, IFN-γ has also been reported to induce STAT3 or STAT5 phosphorylation in a few other cell types (van Boxel-Dezaire and Stark, 2007). In Fig. 1 a schematic representation of the IFN-γR1 is provided.

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

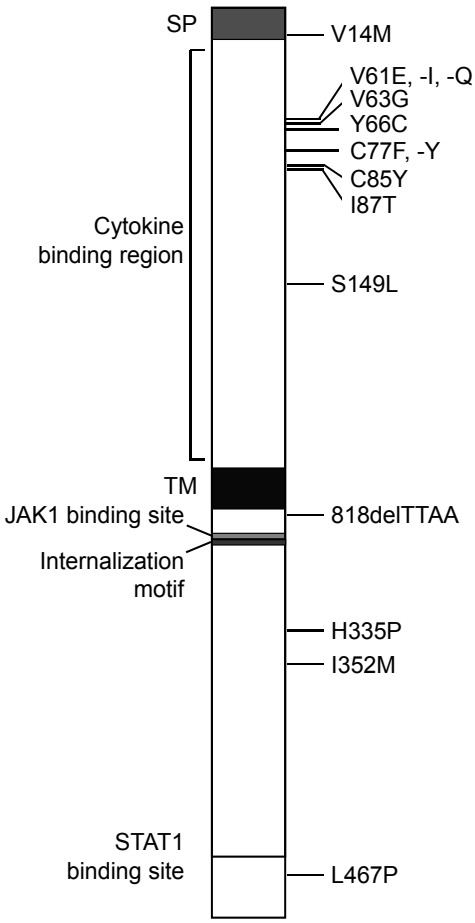


Figure 1. Schematic presentation of the IFN-γR1. On the left the various domains are indicated, on the right the variations that were analyzed are indicated. TM = Transmembrane domain, SP = signal peptide.

Another group of IFN-γR1 deficiencies is due to missense mutations which result in normal expression of IFN-γR1 at the cell surface, however, the resulting receptors show no or diminished binding of IFN-γ (Allende et al., 2001; Jouanguy et al., 2000). Dominant-negative defects such as the 818delTTAA mutation in the intracellular domain of the receptor result in milder forms of MSMD. The 818delTTAA mutation product lacks the JAK1 and STAT docking site as well as the receptor recycling domain which leads to accumulation of aberrant receptor complexes on the cell surface (Jouanguy et al., 1999; Glosli et al., 2008). In addition, several polymorphisms of the IFN-γR1 have been found that may have an effect on IFN-γ responses. The H335P and L467P variants of the IFN-γR1 have been associated with the production of high antibody titers against Helicobacter pylori (Thye et al., 2003) and

susceptibility to allergic disease and the production of high IgE titers (Aoki et al., 2003). The frequency of the V14M allele in SLE patients is significantly higher than that of the healthy control population and the presence of a V14M allele correlated with an altered Th1/Th2 balance in favor of Th2 (Tanaka et al., 1999; Nakashima et al., 1999).

In this report we compare the effect of two novel variations, identified in patients suffering mycobacterial infections (S149L, I352M unpublished data), four known polymorphisms (V14M (Nakao et al., 2001; Tanaka et al., 1999), V61I (SNP database, 2006), H335P, L467P (Thye et al., 2003; Aoki et al., 2003), all seven reported missense mutations (V61Q (Jouanguy et al., 2000), V63G (Allende et al., 2001), Y66C (Dorman et al., 2004), C77Y (Jouanguy et al., 2000), C77F (Chantrain et al., 2006), C85Y (Noordzij et al., 2007), I87T (Jouanguy et al., 1997; Remiszewski et al., 2006)) and the 818delTTAA mutation (Jouanguy et al., 1999) on the expression and function of IFN-γR1 in the same genetic background. In addition, because the mutation at nucleotide 182 (gTA . gAA) reported by Jouanguy et al. (2000) may have been aberrantly designated as V61Q (Val . Gln), instead of V61E (Val . Glu), we analyzed both variations. For this purpose we cloned wild type *IFNGR1* and the *IFNGR1* variants (see also Fig. 1), into a retroviral expression vector and transduced the constructs into the IFN-γR1 deficient cell line SKLC-7. We analyzed the signal transduction, the regulation of CD54, CD64, HLA-DR and HLA class I expression and the cytokine production in response to IFN-γ.

2. Materials and methods

2.1. Cloning IFN-γR1 variants into a retroviral expression vector

The full-length *IFNGR1* coding sequence was PCR amplified from cDNA of a healthy control with the sense primer 5'-AATTGGATCCGGTAGCAGCATGGCTCTCCT-3' and the anti-sense primer 5'-AAGGCTCGAGTCATGAAAATTCTTTGGAATCT-' and cloned into the retroviral vector pLZRS-IRES-GFP (Heemskerk et al., 1997) after digestion with the enzymes BamHI and XhoI (Fermentas). Variations were introduced by site directed mutagenesis (Higuchi et al., 1988). All constructs were sequence verified and were transfected in the Phoenix-A packaging cell line using calciumphosphate (Invitrogen). Supernatants with retroviral particles carrying the expression construct were generated as described before (de Paus et al., 2008).

2.2. Cells, culture conditions and retroviral transduction

The human IFN-γR1$^{-/-}$ cell-line SKLC-7 (Kaplan et al., 1998) and the human monocytic cell-line THP-1 (ATCC TIB-202) were cultured in RPMI1640 medium supplemented with

10% FCS, 20 mM GlutaMax, 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco/Invitrogen). 0.25×10^6 cells were retrovirally transduced by overnight incubation on a CH-296 (RetroNectinTM, Takara Shuzo) coated 48 wells plate in the presence of 1 ml of virus containing supernatant. Cells were washed and cultured for at least four days before analysis in further assays. All subsequent FACS measurements were performed on cells gated for equal GFP expression.

2.3. Analysis of IFN-γR1 expression

To detect IFN-γR1 membrane expression cells were labeled with IRγ2 (Watzka et al., 1998) and 177.10 (Novick et al., 1989) antibodies (kindly provided by Heiner Böttinger and Daniela Novick respectively) and PE conjugated monoclonal antibodies GIR94, GIR208, and as an isotype control IgG1 (BD Biosciences). After labeling with the γR99 antibody (Garotta et al., 1990) (kindly provided by Francesco Novelli) the cells were counterstained with goat-anti-mouse-PE (BD Biosciences). 1×10^5 cells were stained in PBS supplemented with 0.2% BSA (Fraction V, Sigma) and washed twice before analysis on a FACSCalibur (BD Biosciences). In order to detect intracellular expression 1×10^5 cells were fixated with 4% paraformaldehyde (Sigma) and permeabilized with 4% saponin (Sigma–Aldrich) before staining with a directly labeled antibody.

2.4. Detection of cell surface markers

Regulation of CD54, HLA Class I, CD64 and HLA-DR expression in response to IFN-γ was determined by stimulation of 2 × 105 transduced SKLC-7 or THP-1 cells in 200 µl culture medium with various amounts of IFN-γ for 20 hours in 96-wellsplates (Greiner bio-one). Subsequently, the cells were washed and stained with either PE conjugated anti-CD54, the HLA Class-I antibody W6.32 and counterstained with goat-anti-mouse-PE, PE conjugated anti-CD64 or with PE conjugated anti-HLA-DR (BD Biosciences). After staining the cells were washed twice and analyzed on a FACSCalibur (BD Biosciences).

2.5. STAT phosphorylation assays

To study signal transduction, 2×10^5 transduced or untransduced SKLC-7 cells in 200 µl of culture medium were pulsed with various concentrations of IFN-γ (Biosource) in 96-wellsplates (Greiner bio-one). The cells were fixated with 4% paraformaldehyde and permeabilized with 90% methanol (Merck). Subsequently, the cells were washed with PBS, 0.2% BSA, blocked with normal goat serum (Sanquin), and stained with the phospho-specific antibodies pY701-STAT1-Alexa 647, pY705STAT3-PE or pY694-STAT5-PE (BD Pharmingen). Before analysis on a FACSCalibur, the cells were washed twice.

3. Results

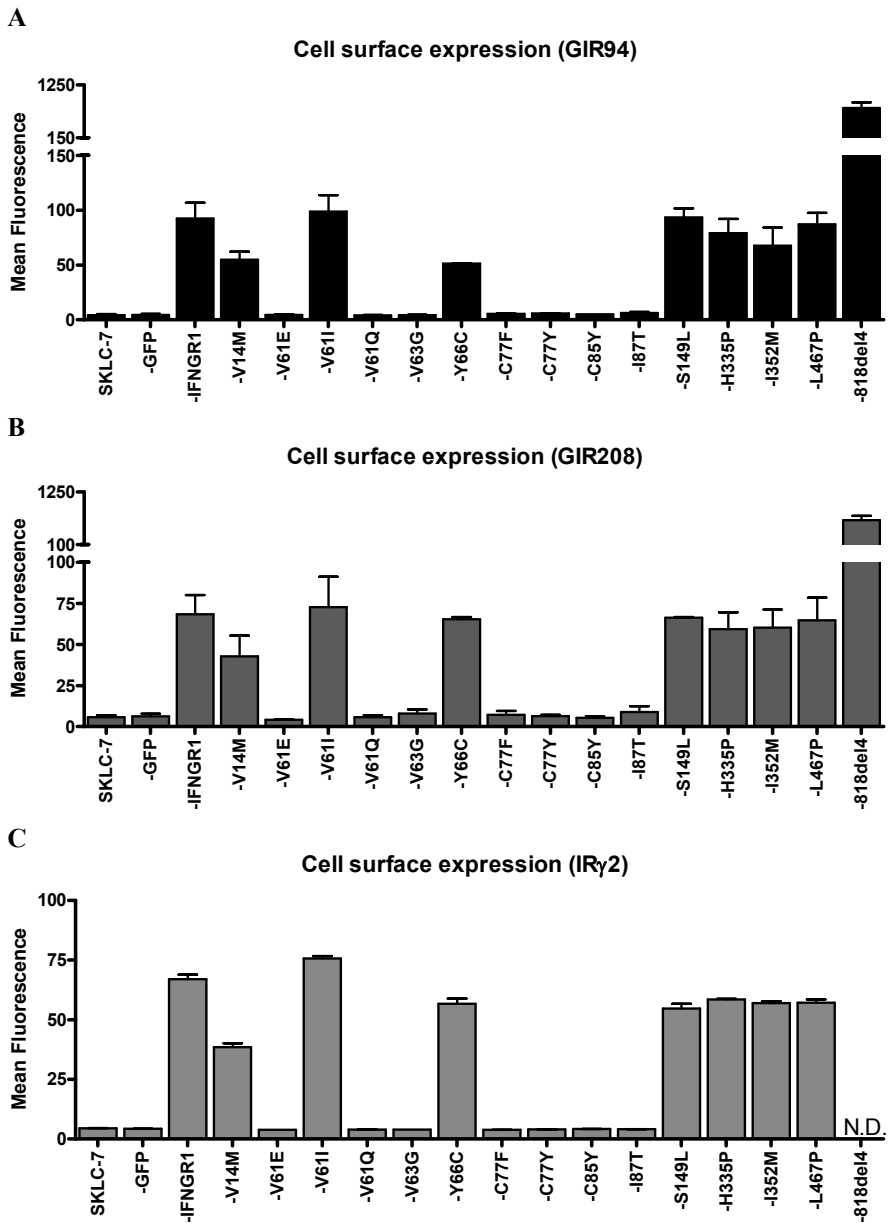
3.1. Functional transfer of the IFNGR1 in SKLC-7 cells

We cloned and expressed the wild type IFN-γR1 in the IFN-γR1 deficient cell line SKLC-7. The use of the retroviral expression vector pLZRS ensures transcription and expression of the *IFNGR1* and green fluorescent protein (GFP) genes in tandem and allows for selection of transduced cells by fluorescence-activated cell sorting (FACS) for the GFP signal. Transduction efficiency of SKLC-7 cells was typically between 20 and 40%. The transduction efficiency depends on target cell type and its proliferation rate. SKLC-7 cells divide slowly and are therefore more difficult to transduce than other cell types. With the same viral supernatants we obtained transduction efficiencies of up to 80% in Jurkat or K562 cells.

SKLC-7 cells do not express the IFN-γR1 on their cell surface, nor do they express GFP (Fig. 2A–D). After retroviral transduction of the wild-type *IFNGR1* into the SKLC-7 cells the IFN-γR1 could be detected on the cell membrane (Fig. 2A–D), whereas in cells transduced with the empty vector (GFP) it could not (Fig. 2A–D). Next we tested the responsiveness of transduced cells to IFN-γ. SKLC7 cells expressing wild type IFN-γR1 were stimulated with various concentrations of IFN-γ for 5 to 90 min before assessing STAT1) phosphorylation. STAT1 phosphorylation peaked 10–15 min after stimulation with high doses of IFN-γ, whereas with lower doses of IFN-γ STAT1 phosphorylation peaked later (Fig. 3). In addition, the induction of STAT1 phosphorylation was dose dependent and the highest IFN-γ concentrations induced the largest amounts of STAT1 phosphorylation (Fig. 3). No induction of STAT3 or STAT5 phosphorylation was observed in response to IFN-γ at any time point (data not shown).

3.2. Expression pattern of the IFN-γR1 variants

Four known polymorphisms (V14M, V61I, H335P, L467P), the known mutations (V61E, V61Q, V63G, Y66C, C77Y, C77F, C77Y, C85Y, I87T, and 818delTTAA) and two novel amino acid substitutions (S149L and I352M) in IFN-γR1 were cloned and retrovirally transduced into the IFN-γR1-/- SKLC-7 cell line. We first determined whether the IFN-γR1 variants were expressed at the cell surface, using four different IFN-γR1 specific antibodies. Untransduced or GFP-transduced cells did not express IFN-γR1 (Fig. 2A–D). On the SKLC-7 cells transduced with wild type IFN-γR1, the receptor was detected on the cell surface (Fig. 2A–D). The V61I, Y66C, S149L and L467P variants could be detected on the cell surface in similar amounts as the wild type receptor while the H335P and I352M variants, showed a small (13% and 18% respectively) reduction of expression that was significant with three out of four antibodies. The V14M variant showed significant reduced expression (42%) compared to the wild type construct with all four antibodies. As expected, the 818delTTAA mutant lacking the receptor recycling-domain was expressed ten times higher on the cell membrane than the wild type construct (Fig. 2A and B).


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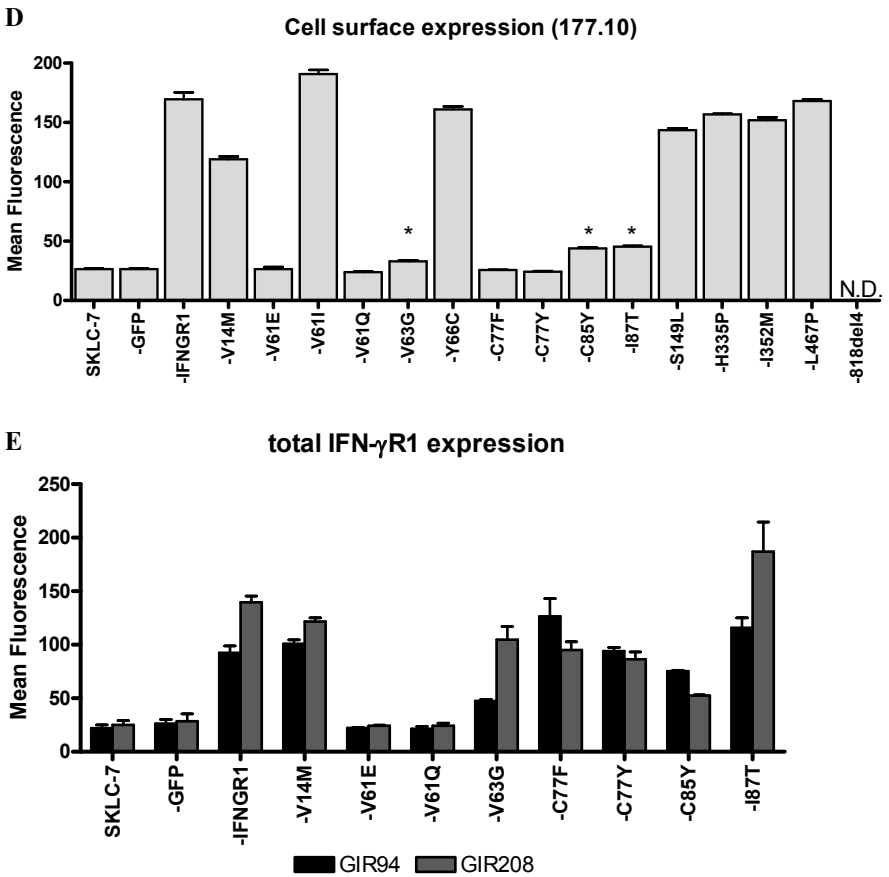

Figure 2. Extracellular and intracellular expression of the IFN-γR1 variants. SKLC-7 cells and the cells retrovirally transduced with GFP or with one of the *IFNGR1* gene variants were stained without pretreatment to detect extracellular expression (A–D or after permeabilization to detect both intracellular and extracellular expression of IFN-γR1 (E). Cells were analyzed for IFN-γR1 expression using four IFN-γR1 specific antibodies (GIR94 (A), GIR208 (B), IRγ2 (C) and 177.10 (D)). Mean ± SD fluorescence of these antibodies of 2 (IRγ2 and 177.10) or 3 (GIR94 and GIR208) experiments in triplo is depicted. N.D.: Not determined. * $p < 0.004$ higher expression compared to GFP construct.

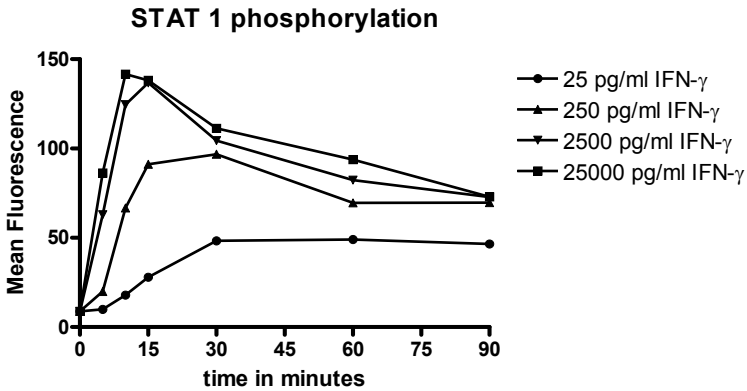

Figure 3. Kinetics of STAT1 phosporylation in response to IFN-γ. SKLC-7 cells transduced with wild type *IFNGR1* were stimulated with various concentrations of IFN-γ (25–25,000 pg/ml IFN-γ), whereafter STAT1 phosphorylation was analyzed at various time points. One representative experiment out of 2 is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

In contrast, membrane expression of the V61E, V61Q, C77F and C77Y variants could not be detected (Fig. 2A–D). A small amount of cell-surface expression of the V63G, C85Y and I87T variants was detected by one antibody (Fig. 2D).

Lack of cell surface expression can be due to a lack of protein production, or due to misfolded proteins being sequestered in the endoplasmic reticulum (ER) or due to a defect in trafficking of the receptor to the cell membrane. To determine whether the variants that were undetectable at the cell surface were synthesized, we stained the cells for intracellular IFN-γR1 expression. Except for the GFP transduced cells and the V61E and V61Q variants, in all other transduced cells the IFN-γR1 could be detected intracellular, indicating protein is synthesized (Fig. 2E). The results of the expression analysis experiments are summarized in Table 1.

3.3. The effect of IFN-γR1 variations on IFN-γ induced STAT1 phosphorylation

STAT1 plays a critical role in the IFN-γ signal transduction and the IFN-γ induced host defence against infections (Platanias and Fish, 1999). To test the influence of the IFN-γR1 alleles on IFN-γ signal transduction, STAT1 phosphorylation was assessed. The kinetics of STAT1 phosphorylation in response to IFN-γ was determined in cells transduced with each of the variants. In cells expressing the wild type IFN-γR1 or the V14M, S149L, H335P, I352M, and L467P variants, comparable STAT1 phosphorylation was observed (Fig. 4A). In cells transduced with the V63G or the I87T variant, STAT1 phosphorylation was detected, however, it was significantly reduced compared to cells transduced with the wild type receptor (Fig. 4B and C). In cells transduced with the V61E, V61Q, Y66C, C77F, C77Y and C85Y variants, no STAT1 phosphorylation could be observed in response to IFN-γ (Fig. 4B and C). The results of the STAT1 phosphorylation experiments are summarized in Table 1.

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

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

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

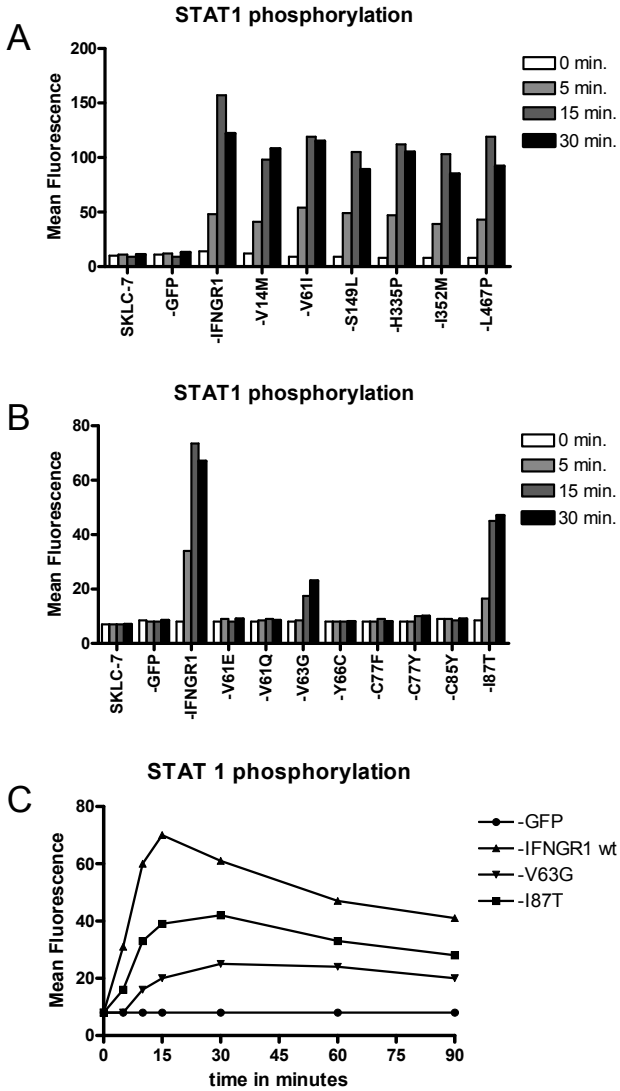

Figure 4. The influence of IFN-γR1 variations on the kinetics of STAT1 phosphorylation.
SKLC-7 cells transduced with wild type IFNGR1, GFP or with the V14M, V61I, S149L, H335P, I352M and L467P (A), V61E, V61Q, V63G, Y66C, C77F, C77Y, C85Y and I87T (B) or V63G and I87T variants (C), were stimulated for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

3.4. Influence of IFN-γR1 variations on CD54 and HLA Class I regulation by IFN-γ

IFN-γ stimulates the expression of many cell surface markers,including CD54 and HLA Class I molecules. CD54 (also known as ICAM-1) is involved in the binding of cells to

endothelia and transmigration into tissues (Shang and Issekutz, 1998). CD54 functions as a costimulatory molecule on APC and other cell types, to activate CD4+ T cells and cytotoxic CD8+ T cells, respectively (van de Stolpe and van der Saag, 1996). IFN-γ increases antigen processing and the expression of the HLA class I molecules, thereby increasing antigen presentation and promoting the induction of cell-mediated immunity (Boehm et al., 1997; Schroder et al., 2004). We assessed the influence of the IFN-γR1 variants on the IFN-γ induced expression of CD54 and HLA Class I molecules. IFN-γ upregulated the expression of CD54 in SKLC-7 cells expressing the wild type IFN-γR1, but not in GFP-transduced or in untransduced SKLC-7 cells (Fig. 5A). In cells expressing the V14M, V61I, S149L, H335P, I352M or L467P, similar upregulation of CD54 expression was observed as in cells expressing the wild type receptor (Fig. 5A). In cells expressing the V63G or the I87T variants, CD54 upregulation was seen when cells were stimulated with high concentrations of IFN-γ, however, this upregulation was less compared to cells expressing the wild type receptor (Fig. 5B and C). No regulation of CD54 by IFN-γ was observed in cells expressing the V61E, V61Q, Y66C, C77F, or C77Y receptor variants (Fig. 5B). Similar results for all IFN-γR1 variants were observed for IFN-γ induced HLA Class I cell surface expression (Fig. 5D–F). The results of the expression analysis experiments are summarized in Table 1.

The expression of two other cell surface markers that can be regulated by IFN-γ, CD64 and HLA-DR, was not altered by IFN-γ stimulation in the SKLC7 cell line with or without *IFNGR1* construct (data not shown). Therefore expression of these two markers was not analyzed in the full panel of *IFNGR1* variations.

3.5. Influence of the IFN-γR1 Y66C variation on the function of the wild type receptor

The Y66C variation was expressed on the cell surface but appeared to be non-functional. Since the IFN-γR contains two IFN-γR1 chains, in individuals heterozygous for a defective IFN-γR1 chain that is expressed on the membrane, incorporation of one defective IFN-γR1 chain in the IFN-γR complex could potentially have a dominant-negative effect. To determine whether the Y66C variation exerts such an effect we transduced the IFN-γ responsive cell line THP-1 with the Y66C variation and as controls with the wild type IFN-γR1 or a GFP vector. While expressing large amounts of Y66C IFN-γR1, Y66C THP-1 transductants showed a dose dependent induction of CD54 after stimulation with IFN-γ, which was comparable to the dose response curve of untransduced THP-1 cells (data not shown). Transduction of the wild type receptor led to a higher total expression of IFN-γR1 at the cell surface, as well as a stronger upregulation of CD54 in response to IFN-γ (data not shown). In THP-1 cells transduced with the 818delTTAA IFN-γR1 variant, total receptor cell surface expression was increased, while the CD54 upregulation in response to IFN-γ was severely reduced, confirming the dominant negative effect of this variant (data not shown).

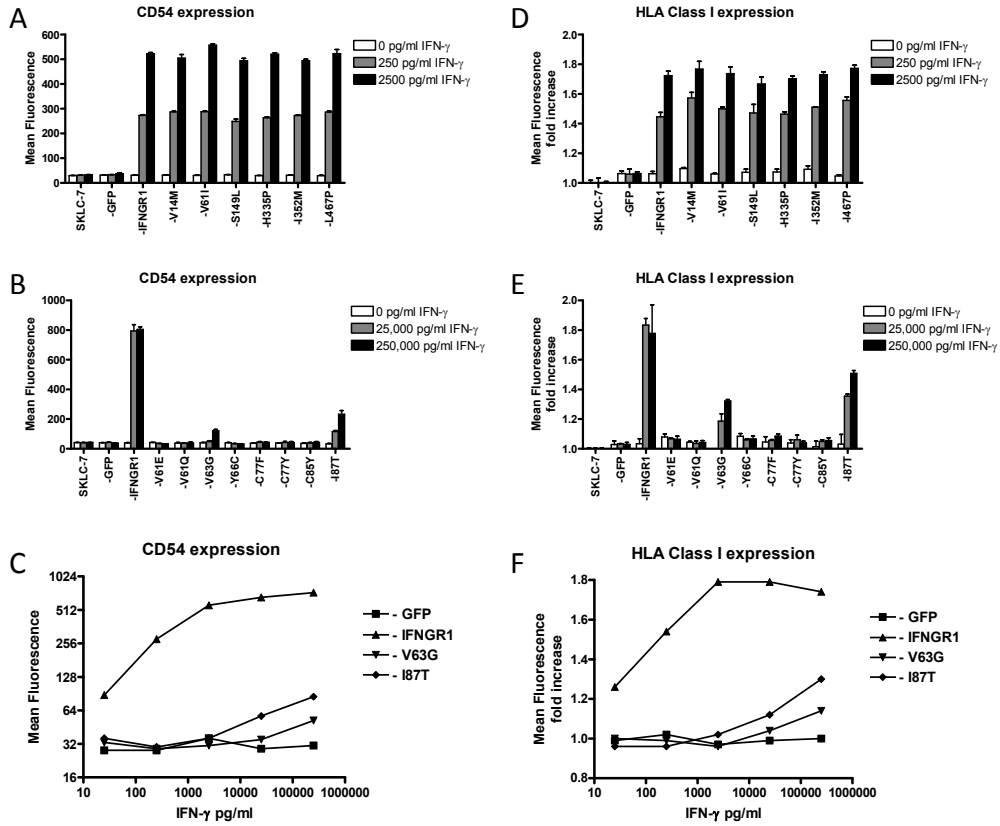

Figure 5. The effect of IFN-γR1 variations on IFN-γ induced CD54 and HLA class I upregulation. SKLC-7 cells and the cells retrovirally transduced with GFP or with one of the *IFNGR1* gene variants were stimulated for 20 h with various concentrations of IFN-γ. The CD54 expression (A–C) and the HLA Class I expression (D–F) was determined by FACS. CD54 expression is depicted as mean fluorescence, HLA Class I expression is depicted as fold increase compared to the mean of untransduced cells. Displayed is the mean ± SD of a triplo experiment. All variants were tested at least three times for comparison with the wild type variant.

4. Discussion

The main finding of this study is that the newly discovered IFN-γR1 variants, S149L and I352M, as well as the known V14M, V61I, H335P and L467P IFN-γR1 variants do not functionally differ from the wild type receptor and are therefore considered polymorphisms. In contrast to literature, we showed a severely reduced expression of the V63G receptor variant, and found that its function was severely reduced although not completely abrogated. In addition, we confirmed the severely reduced function of the I87T mutant receptor, the

completely abrogated function of the V61E, V61Q, Y66C, C77F, C77Y and C85Y variants, as well as the overexpression patterns of the 818delTTAA mutant receptor. Thus far the only mutant proteins that have been characterised after transduction into cells were the 818delTTAA (into HEK293 cells) and the I87T (into fibroblasts) variants (Okada et al., 2007; Jouanguy et al., 1997).

To reach these conclusions, we retrovirally expressed the IFN-γR1 variants in the human IFN-γR1-deficient SKLC-7 cell line and analyzed its functional activity. The conclusions are summarized in Table 1. With the in vitro model used, we were able to distinguish between functional and non-functional variants and were also able to detect the partial responsiveness of the V63G and I87T variants. However, the following points should be considered. First, while the IFN-γR is expressed by all nucleated cells in the human body, the cells used in this study are not representative for all IFN-γ responsive cells. For example, in a few cell-types STAT3 and STAT5 phosphorylation in response to IFN-γ can be observed (vanBoxel-Dezaire and Stark, 2007), in our model no STAT3 or STAT5 phosphorylation could be detected, thus not allowing for detection of potential differences between the IFN-γR1 variants in signaling through these molecules. Second, the concentrations of stimuli chosen may not fully resemble physiologically relevant conditions. Though we tested a range of IFN-γ concentrations as commonly found in physiological relevant situations, the present approach cannot exclude subtle differences in the lowest range of ligand binding. Third, the overexpression of the IFN-γR1 by the retroviral system could mask differences in effects due to alterations in transcript or protein stability. We were nevertheless able to detect accumulation on the membrane of the 818delTTAA mutant, a significant lower expression of some of the polymorphisms, as well as the extremely low expression of partial mutations compared to the wild type IFN-γR1. Furthermore, post-transcriptional and post-translational modifications of normal or retroviral expressed IFN-γR1 may differ, although thus far no findings suggest that such modifications occur. Fourth, the IFN-γ induced STAT1 phosphorylation and subsequent induction of CD54 and HLA class I molecules are not the only responses induced. Subtle differences between the different variants may be missed due to the choice of read-out system.

The C77F and C77Y receptor variants could not be detected on the cell surface, however, these variants could be detected intracellularly. Lack of cell surface expression can be due to a lack of protein expression or due to a defect in trafficking of the receptor to the cell membrane. Alternatively, the protein quality control system in the endoplasmic reticulum (ER) could prevent transport of mutant, misfolded, or incorrectly complexed proteins, and target these for degradation (Klausner and Sitia, 1990), as we have also reported for several IL-12Rβ1 mutations (van de Vosse et al., 2005). In accordance with literature, we did not observe any IFN-γ responsiveness of these two variant receptors (Chantrain et al., 2006; Jouanguy et al., 2000).

In contrast with previous publications (Jouanguy et al., 1997; Allende et al., 2001), we only detected low cell surface expression of the V63G and I87T receptor variants. With one out of the four antibodies used, low cell surface expression of the V63G, C85Y and I87T variants could be detected. In addition, we were able to detect STAT1 phosphorylation as well as a slight upregulation of CD54 and HLA Class I in the V63G and I87T variants in response to more than 10,000 times higher IFN-γ concentrations than needed to induce IFN-γ responses in cells expressing the wild type receptor. This confirms the observation by Jouanguy et al. (1997) that I87T is a partial deficiency and identifies V63G as a partial deficiency as well in contrast to the earlier report by Allende et al. (2001) that it results in complete deficiency. In line with our results with the I87T variant, Jouanguy et al. (1997) observed a response in B-cells and monocytes obtained from the patient expressing this variant when stimulated with very high doses of IFN-γ. The diminished response of the I87T variant to IFN-γ may be largely due to the low cell-surface expression. The diminished response observed of the V63G variant can be due to either the severely diminished cell-surface expression or to disrupted binding of IFN-γ to the receptor since the neighboring amino acids (64 and 66–68) of the IFN-γR1 are known to be essential in the interaction between the high-affinity receptor and its ligand IFN-γ (Walter et al., 1995). Moreover, the adjacent lysine at position 64 directly interacts with IFN-γ.

In the V61Q (V61E) variant IFN-γR1, the binding of IFN-γ was reported to be abrogated (Jouanguy et al., 2000). This was determined in patient cells expressing two mutant *IFNGR1* alleles (V61Q and 652del3) (Jouanguy et al., 2000). We tested both the V61Q and V61E variants. Interestingly, in our experiments, with four independently cloned and sequence-verified V61Q and V61E constructs, neither variant was detected on the cell surface nor was the protein detectable intracellular. The fact that these mutants could not be detected on the cell surface with four different antibodies suggests that the variant detected on the cell surface by Jouanguy et al. (2000) may have been the 652del3 mutation. The failure to detect protein expression may be caused by a changed protein conformation, leading to the disappearance of epitopes recognized by the antibodies. Three of the antibodies (GIR-94, GIR-208 and IRγ2) were raised against the extracellular domain of IFN-γR1, one (177.10) was raised against full length IFN-γR1 but is known to block IFN-γ binding, suggesting it binds to the extracellular domain of IFN-γR1 as well. Of none of these antibodies the exact binding site is known.

The V61I variant is an amino acid substitution at the same position as the V61Q and V61E variants that was however normally expressed on the cell surface and showed a normal STAT1 phosphorylation in response to IFN-γ· Both valine (V) and isoleucine (I) are hydrophobic aminoacids, V to I substitutions are conserved substitutions while substitutions of valine to amino acids glutamine (Q) or glutamic acid (E) substitutions are not. The Y66C variant could be detected on the cell surface, however, no STAT1 phosphorylation, nor CD54

or HLA class I induction was observed in response to IFN-γ. This finding is in accordance with the fact that phenylalanine residue on position 66 is essential for IFN-γ binding (Walter et al., 1995). Although the non-functional Y66C variant was expressed on the cell surface, it did not hamper the function of the IFN-γR when this variant was co-expressed with wild type IFN-γR1. Whether or not the Y66C variant is incorporated in the IFN-γR complex is not known.

We show that the variants V14M, V61I, H335P, L467P, S149L and 352M were detectable on the cell surface and were fully functional and not different in receptor function from the wild type receptor. Despite the fact that cell surface expression of the V14M variant was nearly halved, we were not able to detect a functional difference compared to the wild type receptor. The V14M variation is within the signal peptide, thereby likely influencing transport of the receptor to the cell surface. When IFN-γR1 is overexpressed, as is the case in our model, subtle differences in expression and the balance between the expression of the IFN-γR1 and the IFN-γR2 within our model may not resemble the normal expression pattern. In T lymphocytes IFN-γR1 and R2 expression can individually vary from low to high. For example, the expression density of the IFN-γR2 is an important mechanism in determining the fate of T lymphocytes (Bernabei et al., 2001). Therefore differences in IFN-γR1 expression levels as seen with the V14M variant, which did not result in differences in our read outs, may nonetheless influence signaling in natural conditions.

The H335P and L467P variants of the IFN-γR1 have been reported to be associated with the production of high antibody titers against H. pylori (Thye et al., 2003) and susceptibility to allergic disease and the production of high IgE titers (Aoki et al., 2003). The identification of associations between an allele and an observed clinical outcome does not necessarily mean that the allele itself conveys a functional difference. The functional difference may be due to a variation that is merely linked to the polymorphism under study. We can however not exclude more subtle functional differences of the studied alleles, since we used overexpression constructs of the IFN-γR1. In addition, the observed associations may be due to alterations in signaling pathways other than the ones we explored in this study.

When a variation affects the function of a protein it is considered a mutation, when it does not affect protein function it is considered a polymorphism. We therefore conclude that the V14M, V61I, S149L, H335P, I352M and L467P are functional polymorphisms. Expression on the cell surface of V14M is reduced which may result in slightly reduced IFN-γ responses, when IFN-γR1 gene transcription is in vivo limited to natural amounts. This polymorphism may influence susceptibility to infections or predisposition to SLE. The other variants are deleterious mutations with V61E, V61Q, Y66C, C77F, C77Y and C85Y leading to complete IFN-γR1 deficiency while V63G and I87T lead to partial IFN-γR1 deficiency.

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