Partial recessive IFN-γR1 deficiency: genetic, immunological and clinical features of 14 patients from 11 kindreds

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We report a series of 14 patients from 11 kindreds with recessive partial (RP)-interferon (IFN)-γR1 deficiency. The I87T mutation was found in nine homozygous patients from Chile, Portugal and Poland, and the V63G mutation

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was found in five homozygous patients from the Canary Islands. Founder effects accounted for the recurrence of both mutations. The most recent common ancestors of the patients with the I87T and V63G mutations probably lived 1600 (875–2950) and 500 (200–1275) years ago, respectively. The two alleles confer phenotypes that are similar but differ in terms of IFN-γR1 levels and residual response to IFN-γ. The patients suffered from bacillus Calmette-Guérin-osis (n = 6), environmental mycobacteriosis (n = 6) or tuberculosis (n = 1). One patient did not suffer from mycobacterial infections but had disseminated salmonellosis, which was also present in two other patients. Age at onset of the first environmental mycobacterial disease differed widely between patients, with a mean value of 11.25 ± 9.13 years. Thirteen patients survived until the age of 14.82 ± 11.2 years, and one patient died at the age of 7 years, 9 days after the diagnosis of long-term Mycobacterium avium infection and the initiation of antimycobacterial treatment. Up to 10 patients are currently free of infection with no prophylaxis. The clinical heterogeneity of the 14 patients was not clearly related to either IFNGR1 genotype or the resulting cellular phenotype. RP-IFN-γR1 deficiency is, thus, more common than initially thought and should be considered in both children and adults with mild or severe mycobacterial diseases.

INTRODUCTION

Mendelian susceptibility to mycobacterial disease (MSMD) is a rare primary immunodeficiency (1,2). Patients with MSMD present an apparently selective and inherited predisposition to mycobacterial diseases, suffering from severe clinical disease caused by weakly virulent mycobacterial species, such as bacillus Calmette-Guérin (BCG) vaccines and non-tuberculous, environmental mycobacteria (EM) (2–4). The patients are also susceptible to Mycobacterium tuberculosis (4,5). Other infections are rare, with the exception of extra-intestinal salmonellosis, which has been documented in less than half the patients (2–4,6). In the last 13 years, MSMD-causing germline mutations in five autosomal (IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1) and one X-linked gene (NEMO) have been reported (2–4). Most of the affected gene products are immunologically related, as they are involved in IL-12-dependent and interferon (IFN)-γ-mediated immunity. Disorders of IL12B, IL12RB1 and NEMO result in impairment of the secretion of IL-12-dependent IFN-γ and IL-23-dependent IL-17 (2–4,7). Disorders of IFNGR1, IFNGR2 and STAT1 impair cellular responses to IFN-γ (2–4). The high level of allelic heterogeneity accounts for the definition of up to 13 different genetic disorders causing MSMD (2–4). Two related disorders, complete and partial recessive forms of signal transducer and activator of transcription 1 (STAT-1) deficiency, also impair IFN-α/β and IFN-α responses, thus conferring a broader susceptibility to mycobacteria and viruses (8–10). Other mutations in NEMO are also associated with a broader infectious phenotype (11).

The first genetic etiology of MSMD was described in 1996, with null mutations in IFNGR1 (12,13). Three other molecular forms of IFN-γR1 deficiency have since been described (2–4). Autosomal recessive complete IFN-γR1 (RC-IFN-γR1) deficiency is the result of mutations abolishing the response to IFN-γ (4,14). Most patients present null mutations, due to the presence of stop codons upstream from the exon encoding the transmembrane domain, preventing the production of IFN-γR1 (12,15–18). In-frame deletions and missense mutations in the segment encoding the extracellular domain of IFN-γR1 have been reported in four patients with RC-IFN-γR1 deficiency. The cells of these patients produced IFN-γR1 molecules that were unable to bind IFN-γ, resulting in a complete loss of responsiveness to IFN-γ (18). One patient with a mutation in the initiation codon of the IFNGR1 gene and residual IFN-γ signaling due to weak IFN-γR1 expression presented an immunological and clinical form of the disease almost as severe as that of patients with RC-IFN-γR1 deficiency (19). The most common form of IFN-γR1 deficiency, the dominant partial (DP) type (54 patients from 35 kindreds), results from heterozygous mutations in the cytoplasmic segment of IFNGR1, giving rise to truncated molecules that accumulate at the cell surface (4). These molecules bind IFN-γ but cannot transduce signals; they therefore have a dominant-negative effect (4,14). RC-IFN-γR1 deficiency confers a predisposition to severe and often fatal mycobacterial infection, mostly at an early age, whereas autosomal dominant partial IFN-γR1 (DP-IFN-γR1) deficiency is less severe, several patients with this deficiency having reached or having been diagnosed in adulthood (14).

Not all patients with IFN-γR1 deficiency present with these forms. In particular, the I87T mutation was shown 10 years ago to lead to an autosomal recessive form of partial (RP)-IFN-γR1 deficiency in two patients from a Portuguese kindred (20). The cells of these patients expressed the receptor at the cell surface, and displayed weak, but not completely abolished IFN-γ-mediated signaling. The mechanism by which the I87T mutation exerts its deleterious effect remained unclear. This disorder was thought to be restricted to this single family, until recently, when a patient from Poland was reported to be homozygous for the same mutation (21). We report here six new patients homozygous for the I87T mutation, from five unrelated families of Portuguese and Chilean descent. We also report four unrelated Spanish kindreds with five patients homozygous for the V63G mutation. We demonstrate that homozygosity for the V63G allele, previously identified as potentially responsible for the RC-IFN-γR1 deficiency in another kindred (22), actually confers a RP form of defect. We show that both the V63G and I87T mutations result from a founder effect and provide age estimates for the most recent common ancestor (MRCA) of each mutation. Finally, we compare the immunological data and clinical features of the 14 patients with
RESULTS

**IFNGR1 genotype and familial segregation**

We investigated 14 patients with mycobacterial disease from 11 unrelated families (Table 1; Fig. 1). The sequencing of two different polymerase chain reaction (PCR) products generated from the coding regions of the IFNGR1 mRNA revealed that patients I.1, I.2, II.1, III.1 and IV.1 were homozygous for a T → G nucleotide substitution at position 188 (exon 2), leading to the conservative replacement of a valine residue by a glycine residue at position 63 (V63G) in the extracellular domain of the IFN-γR1 molecule. Homozygosity for the mutation was confirmed by genome analysis. The parents of the five patients, one brother of patients I.1 and I.2, one brother of patient III.1 and one brother of patient IV.1 were heterozygous for the V63G allele and the wild-type allele and are all healthy. The V63G mutation was not found in 128 unrelated healthy individuals from the population of Gran Canaria. The V63G mutation is, therefore, not an irrelevant polymorphism. It segregates with the clinical phenotype as an autosomal recessive trait.

Direct sequencing of IFNGR1 exons and flanking intron regions showed that patients V.1, VI.1, VII.1, VIII.1, IX.1 and IX.2 were homozygous for a nucleotide substitution at position 260 (T → C, exon 3), leading to the non-conservative replacement of an isoleucine residue by a threonine residue at position 87 (I87T). The I87T mutation causes a recessive hypomorphic lesion in IFNGR1 previously identified in only three known patients with RP-IFN-γR1 deficiency (patients X.1, X.2 and XI.1 in this study) (20,21). Residues 63 and 87 are highly conserved in mammals, being identical in 14 of the 18 species considered. Any changes observed are always conservative, with these residues being replaced by another branched-chain amino acid (V, L or I) in all but one case (Fig. 1).

**Founder effect analysis**

An analysis of the SNP Array 6.0 data showed that patients carrying the I87T mutation had a common homozygous haplotype around IFNGR1, encompassing 2.8 Mb (corresponding to 968 SNPs) for patients VI.1 and X.1 (Fig. 2). A similar pattern was observed for the V63G mutant, with a longest shared haplotype of 4.7 Mb (corresponding to 1540 SNPs) for the three patients analyzed (Fig. 2). The ESTIAGE method estimated the age of the MRCA at 20 generations [95% CI (8–51)] for the V63G mutation and 64 generations [95% CI (35–118)] for the I87T mutation. Assuming a generation time of 25 years, the MRCA of the patients with the V63G and I87T mutations lived 500 (200–1275) and 1600 (875–2950) years ago, respectively. Unlike the patients with the V63G mutation, all of whom came from Gran Canaria and had homozygous haplotypes of similar lengths around IFNGR1, the patients with the I87T mutation came from different countries and had shared haplotypes of different lengths. An analysis of these haplotypes by subgroup for the patients with the I87T mutation showed that the most closely related patients were the two Portuguese patients, with a MRCA estimated to have lived 475 (150–1725) years ago. The next most closely related patients were the two Chileans, who were estimated to have a MRCA with the two mainland Portuguese patients 1000 (475–2175) years ago, followed by the patient from the Azores, with a MRCA 1300 (675–2600) years ago, and finally the Polish patient, who was the most distantly related (age of MRCA estimated at 1600 years when considering all the patients as mentioned above).

Studies based on autosomal markers in the current population of Gran Canaria highlight a major European influence, mostly from the Iberian peninsula (23). However, due to a strong sexually asymmetric bias favoring mating between European men and indigenous women of north-west African Berber origin, there is an autochthonous contribution, mostly from maternal lineages (24,25). We tried to determine the most probable origin of the families carrying the V63G mutation, by analyzing haplotype variation for the uniparentally inherited Y chromosome and mitochondrial DNA (mtDNA) (Supplementary Material, Tables S1 and S2). MtDNAs from the patients and their parents belonged to haplogroups of probable pre-Hispanic origin, although haplogroups H (patients I.1, I.2 and their mother) and J (patients II.1 and III.1 and their mothers, and the father of patient IV.1—no samples from patient IV.1 were available) are also observed in Europeans. Similarly, we cannot rule out the possibility of a sub-Saharan origin, through the slave trade, for haplogroups L1b (mother and brother of patient IV.1) and L3e4 (father of patients I.1 and I.2). The Y chromosomes of the male patients and their fathers were mostly of European origin. In contrast, haplogroup E1b1b, of possible pre-Hispanic origin, was found in the father of patient IV.1 (haplogroup J for mtDNA). However, as haplogroup E1b1b is present at low frequency on the Iberian Peninsula, we cannot rule out the possibility of inheritance from Iberian colonizers.

**IFN-γR1 expression and IFN-γ binding**

Monocytes from patients bearing the I87T allele have been shown to express IFN-γR1 molecules on their surface (20). We found that monocytes from patients bearing the V63G allele showed only weak specific fluorescence with four anti-IFN-γR1 mouse monoclonal antibodies (mAbs) (GIR94, GIR208, BB1E2 and 92101), whereas fluorescence was normal or nearly normal with the fifth mAb (MMHG1-1) (Fig. 3). We then compared IFN-γR1 levels on Epstein Barr virus-transformed B (EBV-B) cells from patients homozygous for the V63G and I87T mutations (Fig. 4). EBV-B cells from V63G homozygous patients were stained slightly less strongly with the five mAbs than most I87T homozygous EBV-B cells, but these differences were not statistically significant. However, staining was variable in cells from patients with the same mutation and in healthy controls, particularly for the MMHG1 mAb (Supplementary Material, Table S3). No IFN-γR1 expression was observed with any of the five mAbs in EBV-B cells from a patient with RC-IFN-γR1 deficiency, suggesting that IFN-γR1 labeling was specific.
### Table 1. Infections and organisms isolated from the IFN-γ R1 deficient patients

<table>
<thead>
<tr>
<th>Patient I.I (Spain)</th>
<th>Patient I.2 (Spain)</th>
<th>Patient II.1 (Spain)</th>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td><strong>Episode</strong></td>
<td><strong>Age</strong></td>
</tr>
<tr>
<td>5 days</td>
<td>Urinary infection and omphalitis</td>
<td>2 years</td>
</tr>
<tr>
<td>5 years 6 months</td>
<td>Varicella; unremarkable course</td>
<td>3 years</td>
</tr>
<tr>
<td>5 years 9 months</td>
<td>Aseptic meningitis(^b)</td>
<td>4 years</td>
</tr>
<tr>
<td>16 years</td>
<td>Mediastinal conglomerate; pericardectomy</td>
<td>6 years</td>
</tr>
<tr>
<td>16 years 6 months</td>
<td>Osteomyelitis, retroperiosteal lymphadenopathies. Acid-fast bacilli(^d)</td>
<td>21 years</td>
</tr>
<tr>
<td>27 years</td>
<td>Healthy. No prophylaxis</td>
<td>22 years</td>
</tr>
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<td></td>
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<tr>
<th>Patient III.1 (Spain)</th>
<th>Patient IV.1 (Spain)</th>
<th>Patient V.1 (Chile)</th>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td><strong>Episode</strong></td>
<td><strong>Age</strong></td>
</tr>
<tr>
<td>22 months</td>
<td>Pain in the left ankle</td>
<td>23 months</td>
</tr>
<tr>
<td>33 months</td>
<td>Multifocal osteomyelitis, <em>Mycobacterium avium</em></td>
<td>41 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 years 11 months, 7 years 1 month</td>
</tr>
<tr>
<td>6 years 3 months</td>
<td>Healthy. No prophylaxis</td>
<td>7 years 2 months</td>
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<th>Patient VI.1 (Portugal)</th>
<th>Patient VII.1 (Chile)</th>
<th>Patient VIII.1 (Chile)</th>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td><strong>Episode</strong></td>
<td><strong>Age</strong></td>
</tr>
<tr>
<td>2 days</td>
<td>BCG vaccination</td>
<td>2 days</td>
</tr>
<tr>
<td>14 weeks</td>
<td>Adenopathy. Hepatomegaly. BCGosis</td>
<td>7 months</td>
</tr>
<tr>
<td>5 years</td>
<td>On anti-mycobacterial therapy</td>
<td>5 years 6 months</td>
</tr>
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<tr>
<th>Patient IX.1 (Portugal-Azores)</th>
<th>Patient IX.2 (Portugal—Azores)</th>
<th>Patient X.1 (Portugal)</th>
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<tr>
<td><strong>Age</strong></td>
<td><strong>Episode</strong></td>
<td><strong>Age</strong></td>
</tr>
<tr>
<td>2 days</td>
<td>BCG vaccination</td>
<td>2 months</td>
</tr>
<tr>
<td>2 months</td>
<td>BCGitis</td>
<td></td>
</tr>
<tr>
<td>7 years</td>
<td>Pneumonia, controlled with ampicillin(^b)</td>
<td>12 years</td>
</tr>
<tr>
<td>8 years 3 months</td>
<td>Encephalitis. <em>Toxoplasma gondii</em></td>
<td>8 years 11 months</td>
</tr>
<tr>
<td>8 years 11 months</td>
<td>On anti-toxoplasma therapy</td>
<td></td>
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<tr>
<th>Patient X.2 (Portugal)</th>
<th>Patient XI.1 (Poland)</th>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td><strong>Episode</strong></td>
</tr>
<tr>
<td>3 years</td>
<td>Clinical tuberculose(^b)</td>
</tr>
<tr>
<td>6 years</td>
<td>Pneumonitis. <em>Mycoplasma pneumoniae</em></td>
</tr>
<tr>
<td>22 years</td>
<td>Healthy. No prophylaxis</td>
</tr>
</tbody>
</table>

-Anti-A60, IgG antibodies against mycobacterial antigen 60; BCG, Bacillus Calmette-Guérin.
-\(^{a}\)Unlike patients with other primary immunodeficiencies and IFN-γ-deficient mice (52–55), the patient spontaneously recovered from the disease without specific drug treatment and no recurrences were observed.
-\(^{b}\)Unidentified microorganism.
-\(^{c}\)Histological examination (Ziehl-Neelsen staining).
We investigated the functional relevance of the V63G mutation, by first analyzing the binding of IFN-γ to the surface-expressed V63G IFN-γR1 molecules in a novel fluorescent IFN-γ-binding assay (Fig. 5). Monocytes from patients I.1, I.2 and III.1 displayed lower levels of specific binding of IFN-γ at low concentrations of the cytokine (1 IU IFN-γ/ml) than control cells: mean fluorescence intensity (MFI) was 12.3 ± 9.7 in cells from the patients versus 114.9 ± 45.7 in cells from the controls (P = 0.019; the values given are MFI after incubation with rhIFN-γ minus MFI after incubation with medium alone ± standard deviation). However, these differences were less marked at medium (10^3 IU IFN-γ/ml; MFI 55.1 ± 24.7 for the patients’ cells versus 144.5 ± 60.2 for the control cells; P = 0.076) and high IFN-γ concentrations (10^5IU IFN-γ/ml; MFI 110.7 ± 47.1 for the patients’ cells versus 190.5 ± 135.9 for the control cells; P = 0.39).

EBV-B cell lines homozygous for I87T have been shown to be capable of signal transduction at medium and high IFN-γ concentrations, consistent with an ability to bind IFN-γ (20). Thus, these results suggest that patients homozygous for either the I87T or the V63G IFNGR1 allele displayed IFN-γR1 deficiency with the detectable expression of IFN-γR1 molecules on the surface of their cells. The deficiency observed thus resulted from the presence of too few receptors on the surface of the cell, low levels of IFN-γ binding to the receptors present or both.

Consistent with the expression of abnormal IFN-γR1 molecules, plasma IFN-γ concentrations were high in our patients (51 pg/ml in I.1, 70 pg/ml in I.2, 51 pg/ml in II.1, 129 pg/ml in III.1, 40 pg/ml in V.1, 915 pg/ml in VI.1, 222 pg/ml in VII.1, 89 pg/ml in IX.1, 55 pg/ml in IX.2 and 48 pg/ml in XI.1), whereas no IFN-γ was detected in the plasma of healthy individuals. Analysis of a plasma sample from patient III.1 7 months after diagnosis showed plasma IFN-γ concentration to be 76 pg/ml. However, plasma IFN-γ concentration was excessively high in patient IV.1, in the 3 days preceding his death (post-mortem analysis): 15253, 25972 and 17360 pg/ml 1, 2 and 3 days before death, respectively. Tumor necrosis factor-α (TNF-α), IL-1β and IL-2 were undetectable or present at very low concentrations in the plasma of this patient, but high levels of IL-6 (846 pg/ml), IL-8 (927 pg/ml) and IL-10 (108.4 pg/ml) were also detected. The very high concentrations of IFN-γ observed in two patients (V.1 and VII.1) probably reflect the fact that the plasma samples from these patients were obtained during the course of mycobacterial disease. Patient IV.1 was the only other patient from whom plasma samples were collected during mycobacterial disease. The IFN-γ levels observed in patient IV.1 may reflect a high mycobacterial burden due to late diagnosis of the infection.

No differences in serum IFN-γ concentrations were observed between patients bearing the V63G and the I87T alleles, when patient IV.1 was excluded from the comparison.

These results contrast with the very high plasma IFN-γ concentrations observed in patients with RC-IFN-γR1 deficiency (249 ± 101 pg/ml). This cytokine was undetectable in the plasma of patients with the DP-IFN-γR1 deficiency and in healthy individuals (26). We previously showed that these high IFN-γ concentrations result from high levels of IFN-γ production and/or impaired clearance of this cytokine in the mouse model (27). Our results reflect the impairment, but not the abolition, of IFN-γ binding and clearance from the blood in patients with RP-IFN-γR1 deficiency. Plasma IFN-γ concentration appears to be a good indicator of the...
activity of severe infections, and of mycobacterial diseases in particular, in patients with RP-IFN-\(\gamma\)R1 deficiency, as in patients with RC-IFN-\(\gamma\)R1 deficiency (unpublished data).

STAT-1 phosphorylation and DNA binding in cells from the patients

Like the I87T allele (20), the V63G allele may be a hypomorphic mutation. We analyzed the phosphorylation of STAT-1 (pY-701), and found that monocytes from patients bearing the V63G allele showed no detectable STAT-1 phosphorylation following incubation with 10 IU IFN-\(\gamma\)/ml, whereas a significant increase in pY-701 was observed in control cells (Fig. 6). At higher IFN-\(\gamma\) concentrations (10\(^2\) and 10\(^3\) IU/ml), an increase in STAT-1 phosphorylation was observed in V63G cells and, at the highest concentrations of IFN-\(\gamma\), phosphorylation levels were similar to those achieved after stimulation with IFN-\(\alpha\). Nevertheless, STAT-1 phosphorylation levels remained slightly lower than those in cells from healthy individuals or healthy carriers of the V63G allele (Fig. 6).

STAT-1 translocation to the nucleus in response to low concentrations of IFN-\(\gamma\) (10 IU IFN-\(\gamma\)/ml) has been shown to be abolished in EBV-B cells from patients with the I87T mutation. However, a response to intermediate (10\(^2\) IU/ml) and high (10\(^3\) IU/ml) concentrations of IFN-\(\gamma\) was observed, with no detectable plateau (20,28). We compared the IFN-\(\gamma\)R1-mediated signaling of the V63G and I87T molecules, by quantifying the nuclear translocation and DNA-binding activity of STAT-1 by enzyme-linked immunosorbent assay (ELISA) (Fig. 7). EBV-B cells from three V63G homozygotes were less likely to have STAT-1 present in the nucleus (9.33 ± 10.8% of control samples, range 2.9–21.8%), than cells from five I87T homozygotes (31.16 ± 27.8% of control samples, range 12.18–80%) after stimulation with 10\(^3\) IU IFN-\(\gamma\)/ml, although these differences were not significant. At higher concentrations of IFN-\(\gamma\) (10\(^5\) IU/ml) similar, but slightly stronger responses were observed (50.9 ± 15.1%, range 33.49–60.47% that observed with the control cell line for V63G cells versus 46.2 ± 21.47%, range 23.3–79.36%, for I87T cells). EBV-B cells from a patient with DP-IFN-\(\gamma\)R1 deficiency responded less strongly than V63G
and I87T cells, particularly at high IFN-γ concentrations. No response was observed in cells from a patient with RC-IFN-γR1 deficiency. All cell lines showed a similar response to IFN-α (data not shown).

**Distal events of IFN-γ-mediated signaling**

The ratio of IL-12p40 production in response to BCG plus IFN-γ to that in response to BCG alone was much lower in cultures of cells from our patients than in cells from healthy controls (Fig. 8). Monocytes from patients homozygous for the I87T allele have been shown to display an upregulation of CD64 in response to high concentrations of IFN-γ (10^4 IU/ml) (20). CD64 upregulation was detectable, but weaker in V63G homozygous monocytes than in monocytes from healthy controls stimulated with high (10^4 IU/ml) concentrations of IFN-γ, in which the response to low (10 IU/ml) concentrations was poor or entirely absent (Fig. 8). Like peripheral blood mononuclear cells (PBMCs) from healthy individuals, PBMCs from I87T patients were found to respond to high, but not to low concentrations of IFN-γ in terms of TNF-α production in response to lipopolysaccharide (LPS) plus IFN-γ (data not shown) (20).

We then determined, in whole-blood cultures, the ratio of IL-12p70 production in response to LPS plus various concentrations of IFN-γ to IL-12p70 production in response to LPS alone. Like cells from I87T homozygous patients, cells from V63G homozygous patients responded to high, but not to low concentrations of IFN-γ (Fig. 8), and similar results were also obtained for the ratio of TNF-α production (data not shown). However, unlike I87T cells (20), V63G cells responded significantly less strongly to high IFN-γ concentrations than cells from healthy controls. No IFN-inducible protein 10 (IP-10) production was observed in whole-blood cultures from patients with the V63G mutation stimulated with low to medium IFN-γ concentrations (10–10^3 IU/ml), by contrast to the results obtained for cultured cells from healthy individuals (Fig. 8). However, at very high IFN-γ concentrations (5 x 10^5 IU/ml), the increase in IP-10 production by cells from the patients was similar to that observed in cells from healthy controls. Similar results were obtained for monokine induced by gamma-interferon (MIG) production (data not shown). Thus, the four V63G homozygous patients described here, such as I87T homozygous patients, clearly suffer from RP-IFN-γR1 deficiency.

**Mycobacterial infections**

An analysis of the 14 patients with RP-IFN-γR1 deficiency showed that the onset of the first EM disease occurred at ages of 15.75 years (I.1), 22.25 years (I.2), 4.17 years (II.1), 1.83 years (III.1) and 20 years (X.1), with the first onset of
tuberculosis at the age of 3 years (X.2). Clinical data suggested a possible age at onset of EM disease of 2 to 3 years in patient IV.1. BCG vaccination was the cause of disseminated BCG disease in six patients, although one patient had been vaccinated in infancy without complications (patient XI.1). *Mycobacterium abscessus*, a rapidly growing species, caused disseminated infection in patient II.1; infection with this rapidly growing species has been reported before in patients with IFN-γR2 deficiency (29,30). We cannot exclude the possibility of a causal effect of immunosuppression with IV methylprednisolone and IV hydrocortisone in the infection of patient II.1, and immunosuppressive treatment (corticosteroids and cyclosporin A, see the Supplementary patients section) may also have contributed to the dissemination of *M. avium* infection in patient XI.1.

The mean number of organs infected in patients with EM disease was $1.83 \pm 1.33$ ($n = 6$), or $1.71 \pm 1.25$ if the patient with clinical tuberculosis (X.2) was taken into account ($n = 7$). Five of these six patients suffered from osteomyelitis, and four (patients I.2, III.1, IV.1 and XI.1) had no other apparent site of mycobacterial disease at the onset of disease. After 9 months of osteomyelitis, patient XI.1 also developed brain and lung infiltration immediately after the introduction of immunosuppressive treatment. The mean number of organs infected in patients with BCG disease was $2.66 \pm 1.51$ ($n = 6$); only two of these six patients developed osteomyelitis.

![Figure 4](image-url). IFN-γR1 expression on EBV-B cells from patients homozygous for the V63G and I87T mutations. EBV-B cells from three V63G-homozygous patients (I.1, I.2 and II.1), five I87T-homozygous patients (V.1, VI.1, X.1, X.2 and XI.1), two healthy controls (positive control, C1+ and C2+) and a patient with complete IFN-γR1 deficiency and no expression of IFN-γR1 described in a previous study (negative control, C−/−) were stained with five IFN-γR1-specific mAbs (solid lines) and isotypic control primary antibodies (dashed lines).

![Figure 5](image-url). Cells from IFNGR1-V63G-homozygous patients show impaired but detectable IFN-γ binding. (A) Representative experiment showing the binding of anti-IFN-γ antibody to monocytes from patient I.1 and a healthy control (C+) when cells were incubated with medium alone (dashed line), 1 IU IFN-γ/ml (thin line) and $10^3$ IU IFN-γ/ml (thick line). (B) Data are the MFI of anti-IFN-γ antibody binding to monocytes from patients I.1, I.2 and III.1 (closed symbols) and three healthy controls (open symbols). Each symbol represents the value for an individual patient or healthy control.
Non-mycobacterial infections

Non-typhoidal systemic salmonellosis was observed in three patients. Other serious infections, caused by Shigella sonnei, Haemophilus influenzae, Legionella spp., Mycoplasma pneumoniae and Klebsiella spp., or pneumonia of suspected bacterial etiology were recorded in six patients (Table 1). The V63G homozygous patient reported in a previous study (22) also had pneumonia due to K. pneumoniae. Patient IX.1 suffered from Toxoplasma encephalitis (Gonçalo-Marques et al., manuscript in preparation). Viral infections (aseptic meningitis, molluscum contagiosum and bronchiolitis due to respiratory syncytial virus) were also observed in three patients (Table 1). Severe viral infections with clinical signs have been reported in a few IFN-γR1-deficient patients (14,31,32). In contrast, patients I.1 and I.2, and at least seven previously reported patients (14,31), suffered an uneventful course of chicken-pox. Some patients presented several different bacterial and viral infections, making it difficult to establish a causal relationship.

Outcome

Thirteen of our 14 patients survived until last follow-up at 14.82 ± 11.2 years. Ten patients were healthy, without prophylaxis, at the ages of 3, 6, 8 years and 2 months, 8 years and 6 months, 14, 22, 27, 28, 29 and 31 years (Table 1); one patient subsequently developed Toxoplasma encephalitis at the age of 8 years and 3 months. Three patients are healthy (VI.1) or recovering (VII.1 and VIII.1) on antimycobacterial therapy at the ages of 5 years, 5 1/2 years and 26 months, respectively. In patient VII.1, lung and cerebral lesions caused by multiresistant BCG still developed while the patient was on antimycobacterial therapy, although his clinical status improved while on a multiple-drug mycobacterial treatment regimen supplemented with IFN-γ and IFN-α. Patient IV.1 died at the age of 7 years, 9 days after the diagnosis of a previously undetected long-term M. avium infection and the initiation of antimycobacterial treatment.

DISCUSSION

We report here a series of 14 patients with RP-IFN-γR1 deficiency resulting from mutations in the region encoding the extracellular domain of IFN-γR1. The I87T mutation was found in six kindreds from Portugal, Poland and Chile, and the V63G mutation was found in four unrelated kindreds from Gran Canaria (Spain).

A founder effect was found for both the I87T and V63G mutations. Simulation studies have shown that the ESTIAGE method provides excellent age estimates for the MRCA from small number of individuals, and the precision of this method has been greatly improved by the advent of high-resolution genotyping (33). The MRCA of the I87T and V63G patients date back to the colonization of the Canary Islands by Europeans, mostly from Spain (late 15th and 16th centuries). This, together with the known major genetic contribution of Spain.
to the population of the islands (24,25), strongly suggests that this mutation arrived with a settler from the Iberian Peninsula, probably from Spain. However, the Canary Islands archipelago was probably first populated by settlers of north-west African Berber origin, in the first millennium BC, and an aboriginal substrate is also observed in the current population of the Canary Islands (24,25). Indeed, analyses of uniparentally inherited markers reveal a strong sexually asymmetric event, with mating between European men and indigenous women favored (24,25). The likely European origin of the Y chromosome in our male patients is consistent with our hypothesis of a mutation brought by a Spanish male settler in the 15th or 16th century.

With the exception of the recently reported mutation in the first initiation codon of the IFNGR1 gene, which leads to almost complete IFN-γR1 deficiency that is difficult to distinguish from complete deficiency at the cellular and clinical levels (10), I87T is the mutation that first defined and best illustrates RP-IFN-γR1 deficiency (20). Homozygosity for the V63G allele was previously reported in another patient, who was thought to suffer from RC-IFNγR1 deficiency (2–4,22). Our analysis of early and late events in IFN-γ-induced activation shows that the V63G allele is actually hypomorphic, conferring RP-IFN-γR1 deficiency. The V63G allele was also recently reported to be a hypomorphic variant in experiments comparing the IFN-γ responses of an IFN-γR1-deficient lung adenocarcinoma cell line transduced with several IFNGR1 alleles obtained by site-directed mutagenesis (34). However, this previous study suggested that the V63G mutation, such as the I87T mutation, results in much more severe impairment of IFN-γR1 expression and IFN-γR1-mediated activation than observed in the analysis of PBMC and EBV-B cell lines from our patients. These differences were particularly evident in experiments with intermediate and high concentrations of IFN-γ.

 Assessments of STAT-1 translocation to the nucleus in IFN-γ-stimulated EBV-B cells showed that cells from our patients displayed a mild response to intermediate IFN-γ concentrations and a normal response to high concentrations, with no detectable plateau. Lower levels of STAT-1 translocation to the nucleus were observed in V63G EBV-B cells when both cell lines were stimulated with intermediate IFN-γ concentrations, although this difference was not significant, but no differences were observed at high concentrations of IFN-γ. However, consistent with the previous result (28), EBV-B cells from a patient with DP-IFN-γR1 deficiency were shown to respond poorly to intermediate and high concentrations of IFN-γ, with a plateau at intermediate concentrations. Our results show that the V63G and I87T mutations lead to a less severe cellular phenotype, as demonstrated by the level of STAT-1 translocation to the nucleus, than mutations leading to DP-IFN-γR1 deficiency.

The IFN-γR1 is a type II cytokine receptor with extracellular regions consisting of two immunoglobulin-like domains. Each of these domains forms a β sandwich consisting of a three-β-strand and a four-β-strand antiparallel β-sheet (35–37). The net result of the V63G and I87T mutations contrasts with that of the other five in-frame mutations affecting the extracellular domain (C77Y, C77F, V61Q, 295del12 and 652del3), which result in cell surface-expressed dysfunctional molecules that are unable to bind IFN-γ, and RC-IFN-γR1 deficiency (4,18). The non-polar, aliphatic amino acids valine and isoleucine are overrepresented in β-sheets. However, the factors stabilizing β-strands and the rules governing β-sheet formation are poorly understood (38). Further studies are required to elucidate the observed differences.
conferring these mutations in terms of ligand-binding and signal transduction.

Clinically, the patients with RP-IFN-γR1 deficiency reported had a much less severe clinical phenotype than patients with RC-IFN-γR1 deficiency (3,4,14). The first EM disease occurred later in patients with RP-IFN-γR1 deficiency (mean age at onset: 11.25 ± 9.13 years) than in patients with RC deficiency (3.1 ± 2.5 years), but age at onset was similar in patients with RP-IFN-γR1 deficiency and in those with DP deficiency (13.4 ± 14.3 years). The mean number of organs affected in patients with RC and DP deficiencies with *M. avium* disease was 4.1 ± 0.8 and 2.0 ± 1.1, respectively. If we take into account non-BCG mycobacterioses, a mean of 1.71 ± 1.25 organs were affected in the seven RP-IFN-γR1 patients concerned. Five of these seven patients presented osteomyelitis, which was the only presentation in four of the patients, at least at the time of diagnosis. The patients with the V63G mutation described in a previous study also had osteomyelitis due to *M. avium* and *M. szulgay* as the only presentation (20). Mycobacterium avium osteomyelitis, with no other apparent site of mycobacterial disease, was observed in about a third of all patients with DP deficiency, but not in patients with RC-IFN-γR1 deficiency (14). BCG vaccination was the cause of curable, disseminated BCG disease in five RP-IFN-γR1-deficient patients, although one patient had been vaccinated in infancy without complications. Similarly, the penetrance of vaccine-associated BCG disease in patients with DP deficiency is high, but not complete, whereas all BCG-vaccinated patients with RC-IFN-γR1 deficiency developed BCG disease. Patients with RC-IFN-γR1 deficiency have chronic mycobacterial disease that does not resolve with treatment with relapses following the discontinuation of antibiotics. Bone marrow transplantation is the only effective curative treatment (3,4,39,40). Like patients with DP-IFN-γR1 deficiency, patients with RP-IFN-γR1 deficiency had mycobacterial disease that resolved rapidly with adequate treatment. Even in the absence of prophylaxis, recurrent or multiple mycobacterial infection was observed in only one patient with multidrug-resistant *M. abscessus* (II.1), who received a short course of antimycobacterial treatment because of its toxicity. This suggests that infection with mycobacteria, including the BCG vaccine strain, prevented the subsequent development of EM infections. However, a high rate of mycobacterial relapses was observed in patients with DP-IFN-γR1 deficiency (3,4), and late-onset infection with EM is common in patients with DP-IFN-γR1 deficiency presenting curable BCG infection (14). This suggests that the clinical phenotype of RP-IFN-γR1 deficiency may be even milder than that of DP-IFN-γR1 deficiency. However, additional patients with V63G and I87T mutations must be diagnosed and studied for firm conclusions to be drawn. Our findings highlight the close correlation between IFNGRI genotype, the cellular phenotype of IFN-γ responsiveness and clinical phenotype.

Only 4 of the 22 patients with RC-IFN-γR1 deficiency described in previous studies reached the age of 12 years, whereas only two deaths have occurred among the 38 reported patients with DP-IFN-γR1 deficiency (4,14). Thirteen of our 14 patients with RP-IFN-γR1 deficiency are alive. Ten of our patients were healthy, without prophylaxis, at the ages of 3, 6, 8 years and 2 months, 8 years and 6 months, 14, 22, 27, 28, 29 and 31 years, after a follow-up of 11.15 ± 7.49 years since the onset of the disease. One of these patients developed encephalitis due to *Toxoplasma gondii* at the age of 8 years and 3 months (Gonçalo-Marcos et al., manuscript in preparation). This patient is now 8 years and 11 months old and is recovering on anti-Toxoplasma treatment. Three patients are healthy (VI.1) or recovering (VII.1 and VIII.1) on anti-mycobacterial therapy with (VII.1 and VIII.1) or without (VI.1) IFN-γ at the ages of 5 years, 5½ years and 26 months. The clinical phenotype of 1 patient (VII.1) was much more severe than those of the other 10 living patients, although his clinical status improved on multidrug anti-mycobacterial treatment supplemented with IFN-γ and IFN-α. One patient with RP-IFN-γR1 deficiency (IV.1) died from multiorgan dysfunction syndrome at the age of 7 years, 4 days after the diagnosis of *M. avium* infection. However *M. avium* disease probably began when the patient was between 2 and 3½ years old. The V63G homozygous patient described in a previous study is healthy at the age of 17 years, with no prophylaxis. However, a brother of this patient died at the age of 10 years from virulent *M. bovis* menigitis (caused by a strain other than BCG) (22). This patient’s history, and that of patient X.2, suggests that, like patients with IL-12Rβ1 deficiency (41), patients with RP-IFN-γR1 deficiency may present with bona fide tuberculosis.

In conclusion, this first series of patients shows that RP-IFN-γR1 deficiency is not limited to the single kindred published in 1997 (20). After excluding patients with mycobacterial cervical lymphadenitis, mycobacterial infection secondary to cystic fibrosis and catheter-related infections, four of the five children from Gran Canaria (838 397 inhabitants in 2009) diagnosed with disseminated EM infections in the 1997–2009 period suffered from RP-IFN-γR1 deficiency, and one child had IL-12Rβ1 deficiency (22,41,42) (this report). In addition, two adult patients with disseminated mycobacterial infection (probably caused by EM, patients I.1 and I.2) were also diagnosed with RP-IFN-γR1 deficiency in the same period. Taken together, our results indicate that RP-IFN-γR1 is much more common than initially thought. Moreover, although the I87T and V63G mutations result from two founder events at the IFNGRI locus, conferring similar, but not identical, cellular phenotypes of IFN-γ responsiveness, the clinical phenotype of the patients varies considerably, even among individuals homozygous for the same mutation. Several factors may be involved in this clinical heterogeneity, including differences in environmental exposure, type of microorganism, the virulence and antibiostic resistance of different strains, and even delays in diagnosis and the adequacy of antibiotic treatment. In addition, host variability for other genes may also influence the immune response. RP-IFN-γR1 deficiency should therefore be considered in both children and adults with mild or severe mycobacterial diseases.

**PATIENTS AND METHODS**

We investigated 14 patients from 11 unrelated kindreds. The clinical features of the patients are shown in Table 1 and the Supplementary patients section. Patients X.1, X.2 and XI.1 have been described elsewhere (20,21). Informed consent was obtained from each patient or the patient’s family. The protocol was approved by the local ethics committees of the various institutions involved.
Sequencing of the IFNGRI gene

RNA was isolated from phytohemagglutinin-activated T-cell blasts grown in suspension, with the RNAeasy Mini Kit (Qiagen), used according to the manufacturer’s instructions. Reverse transcriptase (RT)–PCR was performed with the First Strand complementary DNA (cDNA) Synthesis Kit for RT–PCR (Roche). The resulting cDNA was amplified with specific oligonucleotides, as previously reported (22). The PCR products were subjected to agarose gel electrophoresis, excised from the gel and purified with the QiAquick Gel Extraction Kit (Qiagen). The purified amplification products were sequenced by dideoxy-nucleotide termination with BigDye terminator kit v3.0 and the PCR primers (Applied Biosystems), in an ABI Prism 3100 apparatus (Applied Biosystems). Primers and conditions are available upon request. Genomic DNA was isolated from whole blood, according to a standard phenol-chloroform procedure, and the IFNGR1 exons and flanking introns were amplified and sequenced, as reported elsewhere (18,20). Screening for the V63G mutation in the population of Gran Canaria was carried out by PCR amplification for refractory mutations. Primers and conditions are available upon request.

Founder effect analysis

Founder effect analyses were carried out in a subset of 6 [two Chilean, two Portuguese from mainland Portugal and one Azorean (Portuguese nationality), one Polish] apparently unrelated patients with the I87T mutation and three apparently unrelated patients from Gran Canaria with the V63G mutation. Genotypes were obtained for >900 000 SNPs from the Affymetrix Genome-Wide Human SNP Array 6.0. SNPs with 100% call rates were scanned for continuous stretches of homozygosity up- and downstream from the IFNGR1 locus on chromosome 6. Pairwise comparisons within each mutation group revealed the limits of the longest shared haplotype and the positions of subsequent recombination breakpoints. The likelihood-based ESTIAGE method (33) was used to estimate the age of the MRCA for each mutation, from the observed shared haplotypes and the recombination rates and haplotype frequencies obtained in the HapMap Project (43).

Analyses of Y chromosome and mtDNA haplotypes

Hypervariable sequences I and II of mtDNA were sequenced with the primer pairs and PCR conditions used in a previous study (24,44). Sequences were sorted into haplogroups according to the most recent classification (45). For the analysis of Y chromosome haplotypes, we amplified 16 Y-chromosomal short tandem repeat (STR) loci with the Applied Biosystems Y-filer™ PCR Amplification kit (www.appliedbiosystems.com). STR alleles were named according to current recommendations (46–48).

IFN-γR1 detection with antibodies

Non-specific binding to monocytes was minimized by the prior incubation of ethylenediaminetetraacetic acid (EDTA)-treated whole blood samples with purified mouse IgG1, IgG2a and IgG2b isotype controls (BD Biosciences). After 15 min of incubation, the samples were washed twice and stained with phycoerythrin (PE)-labeled mAbs specific for human IFN-γR1 92101 (R&D Systems), GIR-94 (BD Biosciences), GIR-208 (BD Biosciences) and MMHGR-1 (Caltag Laboratories), the human IFN-γR1-specific fluororescein isothiocyanate (FITC)-labeled mAb BB1E2 (Cytognos) or isotype-matched negative controls (BD Biosciences). Erythrocytes were lysed with Lysing Solution (BD Biosciences), and washed twice. White blood cells were then resuspended in phosphate-buffered saline (PBS) and analyzed by flow cytometry. Monocytes were gated on the basis of forward/side light scattering and positive staining with an allotrophocyanin (APC)-labeled mAb directed against human CD14 (BD Biosciences). EBV-B cells were stained by incubation with purified mouse IgG1, IgG2a and IgG2b isotype controls, followed by washing and incubation with PE-labeled 92101, GIR-94 and GIR-208 mAbs, FITC-labeled BB1E2 mAb or isotype-matched negative controls. Purified MMHGR-1 (Biomedical Laboratories) or an isotype-matched negative control was also used for the staining of EBV-B cells. After incubation with these antibodies, cells were washed twice and incubated with biotinylated goat anti-mouse antibody (Caltag Laboratories) and streptavidin-PE (Caltag Laboratories). EBV-B cells and gated monocytes were analyzed on a FACSCalibur flow cytometer, with CellQuest Pro software (BD Biosciences).

IFN-γ binding assay

PBMCs were obtained from EDTA-treated whole blood by the standard density gradient centrifugation procedure, in Ficoll Hypaque. PBMCs were prepared at a density of 5 × 10⁶ cells/ml in RPMI 1640 (Bio-Whittaker) and 200 μl of cell suspension was cultured with medium alone or with variable concentrations of recombinant human IFN-γ (rHIIFN-γ, R&D Systems) for 20 min at 4°C. The samples were washed once with 0.1% sodium azide in PBS, and 0.5 mg/ml purified mouse anti-human IFN-γ monoclonal antibody (clone 4S.B3; BD Biosciences) or an isotypic control (purified mouse IgG1, BD Biosciences) was added and incubated with the samples for 30 min at 4°C. Samples were washed twice in calcium- and magnesium-free PBS (Euroclone), and incubated with goat anti-mouse IgG1 biotin conjugate (Caltag Laboratories) for 30 min at 4°C. Finally, samples were washed twice with calcium- and magnesium-free PBS and incubated for 30 min with APC-conjugated anti-human CD14-antibody and PE-conjugated streptavidin. The samples were analyzed with a FACS-Calibur cytometer and CellQuest Pro software.

Phosphorylation of STAT-1 in monocytes in response to IFN-γ

PBMCs were obtained from EDTA-treated whole blood by density gradient centrifugation. STAT-1 phosphorylation was assessed as previously described (49). As a positive control, cells were incubated with high concentrations of IFN-α. Intracellular labeling was carried out with an Alexa Fluor 488-labeled anti-human STAT-1-pY701 (BD Biosciences) mAb or with the relevant isotypic control. STAT-1 phosphorylation at STAT-1-pY701 was assessed on monocytes.
gated on the basis of forward/side light scattering and positive staining with an APC-conjugated antibody against CD14 in a FACS Calibur flow cytometer, with CellQuest Pro software. Antibody binding to monocytes is expressed as MFI.

**Nuclear translocation of STAT-1**

EBV-B cells were stimulated for 30 min with $10^3$ and $10^5$ IU rhIFN-γ/ml or with $10^5$ IU IFN-α/ml. We quantified the translocation of STAT-1 homodimers to the nucleus and their binding to DNA with the TransAM STAT family kit (Active Motif Europe), used according to the manufacturer’s protocol. Briefly, nuclear extracts from rhIFN-γ-stimulated EBV-B cell lines were incubated in the wells of ELISA plates coated with oligonucleotides containing a STAT consensus binding site. The wells were then washed and incubated with anti-STAT-1 antibodies specific for an epitope present only on DNA-bound-activated STAT-1, washed again and incubated with a horseradish peroxidase-conjugated secondary antibody. The wells were then washed and incubated with anti-STAT-1 antibodies specific for an epitope present only on DNA-bound-activated STAT-1, washed again and incubated with a horseradish peroxidase-conjugated secondary antibody. The plates were washed again, a developing solution was added and absorbance was measured at 450 nm.

**Late responses to IFN-γ in blood cells**

Late events in IFN-γ-mediated stimulation were studied in heparin-treated whole-blood cultures. We analyzed the production of IL-12p40 in response to IFN-γ by incubating whole blood with BCG, with or without rhIFN-γ, as previously described (50).

For analysis of the induction of CD64 expression in response to IFN-γ, whole blood was diluted 1:4 in RPMI 1640 and incubated with medium alone or with various concentrations of rhIFN-γ for 24 h at 37°C and under an atmosphere containing 5% CO₂. The erythrocytes were lysed and the cell surface expression of CD64 on blood monocytes was assessed with an FITC-labeled anti-human CD64 mAb (BD Biosciences), by flow cytometry with CellQuest Pro software. Monocytes were gated on the basis of forward/side light scattering and positive staining for CD14 with an APC-CD14 mAb. The production of IL-12p70 and TNF-α in response to rhIFN-γ was assessed in whole blood cultures diluted 1:2 in RPMI 1640 and left unstimulated or stimulated with 100 ng/ml LPS from *S. enteritidis* (Sigma), alone or in combination with various concentrations ($10^2$–$10^5$ IU/ml) of rhIFN-γ. Supernatants were collected after 24 h of incubation at 37°C under an atmosphere containing 5% CO₂. The production of (IP-10, CXCL10) and (MIG, CXCL9) was assessed in whole blood diluted 1:2 with RPMI 1640 incubated with medium alone or with various concentrations (1–5 × 10³ IU/ml) of rhIFN-γ at 37°C, under an atmosphere containing 5% CO₂. Supernatants were collected after 24 h.

**Determination of cytokine levels**

Levels of IL-12p40 in cultures and of IFN-γ in plasma were determined by ELISA, as previously reported (26,50). The levels of TNF-α, IL-12p70, IP-10 and MIG in cultures were determined with a flow cytometry-based bead array system (BD Biosciences), according to the manufacturer’s protocols.

**Authors’ Contributions**


**Supplementary Material**

Supplementary Material is available at HMG online.

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