REVIEW ARTICLE

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Inherited Disorders of the Interleukin-12-Interferon Gamma Axis: Mendelian Predisposition to Mycobacterial Disease in Man

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INTRODUCTION

Over the past 50 years there have been a number of reported cases of severe disseminated infection with weakly virulent mycobacteria in individuals without recognised predisposing immunodeficiency. Mycobacterial species included various environmental mycobacteria (EM), and several *M. bovis* BCG vaccine substrains. High rates of affected siblings and parental consanguinity suggested the existence of a novel primary immunodeficiency syndrome, subsequently named Mendelian susceptibility to mycobacterial disease (MSMD, MIM 209950). Molecular investigation of these families has identified mutations in five genes in the interleukin-12-dependent interferon-gamma axis highlighting the importance of this pathway in human immunity to mycobacteria. There remain a number of patients for whom a genetic aetiology has yet to be identified, suggesting that mutations in other genes await discovery.

Sporadic cases of disseminated EM infection in the absence of recognised immunodeficiency are well described (1,2). Familial disseminated EM infection was first reported in 1964-three members of the same Danish family had fatal disseminated *M. avium complex* (MAC) (3). Uchiyama identified 2 siblings with *M. avium* infection (4). More recently, Holland et al reported 3 male members of one family with disseminated EM infection (5,6,7).

Idiopathic disseminated BCG infection following vaccination was first reported in 1951 (8). A sporadic case born to consanguinous parents was described in 1973 (9), and families with more than one affected member were first reported at around the same time (10,11). In 1976, That patients with inherited susceptibility to mycobacterial infections may also be at increases risk of Salmonella infection was first highlighted in a report by Heyne who described a brother and sister from Germany who both developed generalized infection after neonatal BCG vaccination (12). The boy later developed Salmonella enteritis and osteomyelitis. In Prague, a 3 -year- old boy who had been vaccinated with BCG at the age of 3 days developed disseminated Salmonella and BCG infection resulting in his death 3 years later (13). A first cousin of this child also had disseminated BCG infection (14).

The first family in which the molecular basis of increased susceptibility to EM was elucidated was described in 1995 (15). Four children from the same village in Malta all developed disseminated EM infection. Two were brothers related to a third child as fourth cousins, whilst the fourth child was not knowingly

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related to the others. The parents of the brothers were second cousins and both related to both parents of the fourth cousin. Each child was infected with a different mycobacterial species (*M. chelonae*, *M. fortuitum* and two different stains of MAC), suggesting an innate defect in host immunity was responsible. However, extensive immunological investigation failed to identify any known defect predisposing to such infections. Patients had defective upregulation of monocyte function in response to endotoxin and IFN-γ (15) and defective antigen presentation (16). The high degree of consanguinity within the Maltese family suggested the children were homozygous for a rare recessive mutation inherited from a common ancestor. A whole genome search for homozygosity in three of the affected children mapped the gene to the region of chromosome 6q containing the gene encoding the IFN-γ receptor ligand binding chain (IFN-*R1) of the IFN-γ receptor complex (17). A mutation in the coding region of this gene (*IFNGR1*), which resulted in complete absence of IFN-γR1_expression at the cell surface, was subsequently identified as the cause of the defect (17).

Meanwhile, a survey conducted in parallel to the work described above found that of 108 cases of disseminated infection following BCG vaccination reported since 1951, 50% were idiopathic (18). A retrospective study of all cases of disseminated BCG infection following vaccination in France between 1974 and 1994 revealed that of 32 children identified, 16 had no recognised predisposing immunodeficiency (19). Among a total of 60 children worldwide with idiopathic disseminated BCG infection for whom information was available, four pairs of siblings and one pair of first cousins were identified, and parental consanguinity was noted in 24 families. Clinical and histopathological features in a Tunisian child with disseminated BCG infection, born to consanguineous parents, were remarkably similar to those of the Maltese children with EM infections. A series of candidate genes involved in anti-mycobacterial immunity in the mouse model of BCG infection were tested by homozygosity mapping. The segregation of markers within *IFNGR1* suggested linkage and a frameshift mutation resulting in the absence of IFNγR1 was identified (20).

Although mutations in *IFNGR1* were subsequently identified in other cases of MSMD, there were a number of patients in whom mutations within *IFNGR1* were not detected. Investigation of other candidate genes within the IFN-γ pathway led to the identification of mutations in four other genes (table 1), all of which are involved in the IL-12 dependent IFN-γ mediated immunity reviewed in (21,22,23).

Table 1. Genes involved in defective macrophage activation

Gene	Gene product	Chromosomal location	MIM number
IFNGR1	Interferon-γ receptor ligand binding chain	6q23-q2s4	107470
IFNGR2	Interferon-γ receptor signal transducing chain	21q22.1-22.2	145659
IL-12RB1	Interleukin-12 receptor beta-1 subunit	19p13.1	601604
IL-12B	Interleukin-12 p40 subunit	5q31.1-33.1	161561
STAT1	Signal transducer and activator of transcription 1	2q32.2-32.3	600555

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

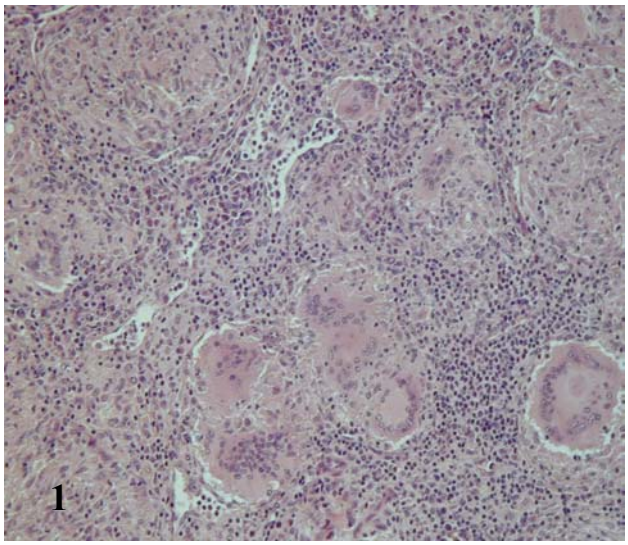
The central feature of MSMD is infection with weakly pathogenic mycobacteria. In keeping with the genetic heterogeneity there is a clinical spectrum of MSMD. At one end of the spectrum mutations in *IFNGR1 or IFNGR2* which result in a lack of functional protein at the cell surface (complete receptor deficiency) have a very poor prognosis with the development of disseminated infection in early childhood and progressively fatal disease (17,20,24). At the other end, screening of family members has identified individuals who carry mutations involving *IFNGR1*, STAT1, IL-12B and *IL-12RB1* who have not developed infection with either mycobacteria or salmonella (25,26,27,28). Other *IFNGR1* and *IFNGR2* mutations resulting in the _expression of an abnormal protein causing partial receptor deficiency are associated with milder phenotypes and response to IFN-γ treatment (27,29,30). Similarly, mutations in the genes encoding the IL-12 p40 subunit (*IL-12B*) or the IL-12 receptor *1 subunit (*IL-12RB1*) resulting in complete deficiency of either protein result in a less severe phenotype and good response to

antimicrobial and IFN-γ treatment. The signal transducer and activator of transcription 1 (*STAT1*) mutation reported is phenotypically similar to partial IFN-γR deficiency (31).

A striking feature of MSMD is the specific susceptibility to poorly pathogenic mycobacterial species. Various mycobacteria species have been isolated including slow-growing species, such as *M. kansasii*, *M. avium* and *M. szulgai* and rapid-growing species, such as *M. smegmatis*, *M. abscessus, M. chelonei*, *M. fortuitum,* and *M. peregrinum. M. smegmatis* (32) and *M. peregrinum* (Koscielnak *et al*., submitted) had not previously been documented as causes of disseminated EM disease. The more virulent *M. tuberculosis* has been implicated in or isolated from individuals with IFN-γR1, IL-12p40 and IL-12Rβ1 deficiency (25,28,30). The mycobacterial species identified correlate with the genetic defect; for example, rapid-growing mycobacterial species are mostly observed in children with complete IFN-γR1 or IFN-γR2 deficiency (Table 2).

Septicaemia and disseminated infection occurred in about a quarter of reported cases, more commonly in association with IL-12p40 and IL-12R*1 deficiency. Other pathogens isolated from MSMD cases include *Listeria monocytogenes* (33), *Histoplasma capsulatum* (27) and *Norcardia asteroides* (28). Fungal and bacterial pathogens such as candida and staphylococci have not caused infection, despite the presence of indwelling intravenous catheters in many patients. Increased susceptibility to viral infections, particularly with herpes viruses, has been noted in some patients in whom MSMD has been shown to be due to IFN-γR1 deficiency (34,35,36). However, this is not universal, and most other patients have had classical childhood viral infections without problems. Mutation of *STAT1* has not resulted in increased susceptibility to viral infection despite the role of STAT1 in both IFN-γ and IFN-α mediated immunity. Patients with MSMD due to complete IFN-γR1 deficiency may present in childhood with a characteristic syndrome of chronic fever, weight loss, lymphadenopathy, hepatosplenomegaly and evidence of disseminated infection which may involve bone, skin, soft tissues, lung and meninges. The clinical presentation appears to vary according the genetic defect involved. For example, dominant partial IFN-γR1 deficiency is almost always associated with osteomyelitis (27,37,38) while lymphadenopathy is a very common feature of IL-12p40 or IL-12R*1 deficiency [Altare, 1998 #1065; de Jong, 1998 #1066; (25,28,37,39,40). The clinical features of each genetic defect remain to be carefully described (Dorman and Picard in progress). The age of onset varies according to the gene involved, the type of mutation and whether the affected individual received BCG vaccination at birth or acquired EM infection via natural routes.

Correlation between clinical phenotype and histopathological findings has been observed (41). Two distinct histological types have been documented which appear to be associated with distinct clinical phenotypes (figure 1,2).



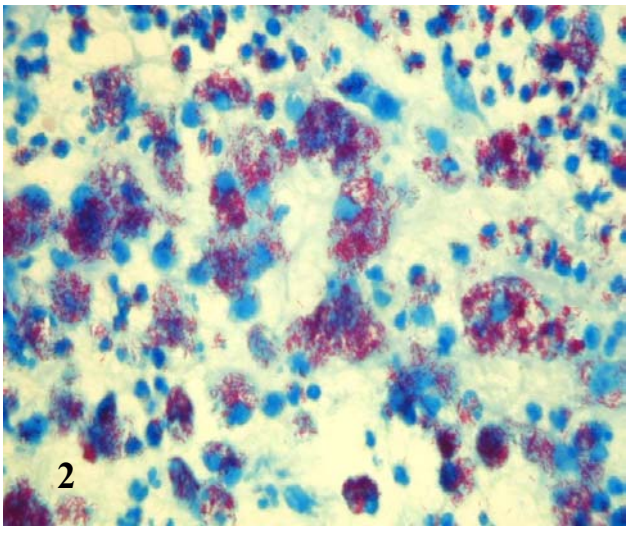


Figure 1,2. Granulomata type 1 and type 2

Table 2. Clinical features of inherited disorders of the interleukin-12-interferon gamma axis indicating pathogens isolated and organs involved.

Disorder	Infection	RES	Bone	CNS	GIS	RS	Skin	References
CIFN-γR1	MAC	+	+	+		+		[Newport, 1996 #480; Jouanguy, 1996 #663; Pierre-Audigier,
	M. fortuitum	+	+					1997 #907; Vesterhus, 1998 #1363; Holland, 1998 #1093; Altare,
	M. chelonae	+	+					1998 #1088; Roesler, 1999 #1340; Allende, 2001 #1399;
	BCG	+	+	+				Koscielnak, 2002 #1425; Cunningham, 2000 #1330; Jouanguy,
	M. smegmatis	+	+	+				2000 #1337 Rosenzweig, 2002 #1430]
	M. kansasii	+	+				+	
	M. szulgai	+					+	
	Salmonella	+						
	L. monocytogenes	+		+	+	+		
CIFN-γR2	M. fortuitum	+				+		[Dorman, 1998 #1092]
	MAC	+				+		
AR pIFN-γR1	BCG	+					+	[Jouanguy, 1997 #1067]
	Salmonella	+						
AR pIFN-γR2	BCG	+					+	[Doffinger, 2000 #1270]
	M. abcessus	+					+	
AD pIFN-γR1	M. tuberculosis	+				+		[Jouanguy, 1999 #1086; Villela, 2001 #1392]
	MAC	+	+			+	+	
	BCG	+	+			+	+	
	M. kansasii	+	+					
	Salmonella							
	H. capsulatum							
clL-12p40	BCG	+					+	[Altare, 1998 #1118; Picard, 2002 #1426]
	M. tuberculosis	+						
	Salmonella spp	+						
	Norcardia asteroides				+	+		
clL-1R*1	BCG	+						[Altare, 1998 #1065; de Jong, 1998 #1066; Sakai, 2001 #1401;
	MAC	+				+		Asku, 2001 #1393; Altare, 2001 #1398]
	M. fortuitum-	+			+	+		
	chelonae	+			+			
	M. tuberculosis	+			+			
	Salmonella							
STAT1	BCG	+						[Dupuis, 2001 #1394]
	MAC	+						

Abbreviations:

clFN-γR1 complete interferon-gamma 1 deficiency
clFN-γR2 complete interferon-gamma 2 deficiency
AR plFN-γR1 autosomal recessive partial interferon-gamma 1 deficiency
AR plFN-γR2 autosomal recessive partial interferon-gamma 2 deficiency
AD plFN-γR1 autosomal dominant partial interferon-gamma 1 deficiency
clL-12p40 complete interleukin-12 p40 deficiency
clL-12*1 complete interleukin-12 receptor *1 deficiency
STAT1 partial signal transducer and activator of transcription 1 deficiency
RES Reticuloendothelial system
CNS Central nervous system
GIS Gastrointestinal system
RS Respiratory system
BCG bacille Calmette-Guerin
MAC Mycobacterium avium complex

Approximately half of the patients with disseminated BCG infections had tuberculoid (typeI) granulomata with well-defined epithelioid and giant cells surrounded by lymphocytes and fibrosis containing only occasional acid-fast bacilli. The remaining patients had lepromatous-like (type II) lesions with poorly formed granulomata containing large numbers of acid fast bacilli. Patients with type I granulomata had a good prognosis but virtually all the children with poor granuloma formation (type II) type II died. EM granulomata tend to be poorly formed irrespective of the clinical outcome and underlying genetic defect.

LABORATORY FINDINGS

Chronic infection leads to normochromic, normocytic anemia, and raised inflammatory markers. Immune function in patients with MSMD has been extensively investigated in patients with MSMD in an attempt to identify a known immunodeficiency, and is in general remarkably normal. CD4 T helper cells are often normal but may be low secondary to chronic infection. Levels of serum immunoglobulin isotypes, including IgG subclasses, are normal or elevated, and antigen-specific antibody titers are normal. T cell proliferation in vitro in response to various mitogens and recall antigens are also normal. Polymorphonuclear cells are normal in terms of morphology, CD18 _expression, chemotaxis, and respiratory burst. Delayed type hypersensitivity testing in vivo and blastogenesis in vitro to PPD are normal in patients with complete IFN-γR1 and IL-12R*1 deficiency, indicating that IL-12 and IFN-γ are not required for DTH or blastogenesis to mycobacterial antigens.

MOLECULAR BASIS OF THE DISEASE

Mutations in 5 genes of the IL-12/IFN-γ axis causing increased susceptibility to mycobacteria have been identified to date (summarized in figure 3).

This pathway is central to the immune response to intracellular pathogens such as mycobacteria and the functions of these genes.

IFNGR1

Mutations in this gene were the first to be identified as the cause of MSMD (17,20). Subsequent investigation of patients with increased susceptibility to poorly pathogenic mycobacteria has led to the identification of at least 15 null recessive mutations in this gene (32,33,34,42,43,44,45,46); Koscielnak, et al., 2002; 47). The identification of families in which mycobacterial infections occurred in more than one generation suggested dominant mutations might also exist (27). Investigation of 18 individuals from 12 kindreds led to the identification of a small deletion hotspot within *IFNGR1*. A 4 base pair deletion at nucleotide position 818 (818del4) was identified in 11 of the unrelated kindreds and the 12th family had a single nucleotide deletion (T) in this position (818delT). The 818del4 mutation leads to a premature stop codon at position 827-829 within the intracellular domain of the receptor. The receptor is expressed on the cell surface but the mutant receptor lacks the three motifs required for intracellular signaling (the JAK1 and STAT1 binding sites, and the tyrosine phosphorylation site). It also lacks a recycling motif so the truncated receptor accumulates on the cell surface and interferes with signaling by the normal receptor encoded by the normal copy of *IFNGR1*. Thus the mutant allele has a dominant effect (in comparison to the recessive form of IFN-γR1 deficiency, where parents are healthy carriers of the mutations). Subsequently, other *IFNGR1* mutations resulting in a dominantly inherited phenotype have been identified (35,37,38). A second small deletion hotspot was recently identified in *IFNGR1*, in this case with a recessive phenotype (46). In summary, a range of mutations including frameshift, insertion, deletion, nonsense, missense and splice mutations have been identified in *IFNGR1*.

All recessive mutations identified to date occur in the part of the gene encoding the extracellular domain of the receptor chain, the majority of which result in complete lack of receptor _expression. Two of the recessive mutations allow _expression of a poorly-functioning protein leading to partial deficiency (30). Partial receptor deficiency may also result from dominant mutations leading to a receptor deprived of its intra-cytoplasmic segment.

IFNGR2

Complete deficiency of IFNγR2 was found in a child with disseminated *M. fortuitum* and MAC infections in whom cell surface _expression of IFN-γR1 and *IFNGR1* sequence was normal (24). Sequence analysis of *IFNGR2* led to the identification of a 2 base pair deletion (277del2), which in turn led to a premature stop codon. The truncated protein lacked both the transmembrane and intracellular (signalling) domains and was not expressed at the cell surface. Both parents, though unrelated, carried this mutation for which the child was homozygous. Recently an Iranian child with complete IFN-γR2 deficiency suffering from disseminated BCG infection has been detected. (Mansoori D, Casanova JL, et al., unpublished).

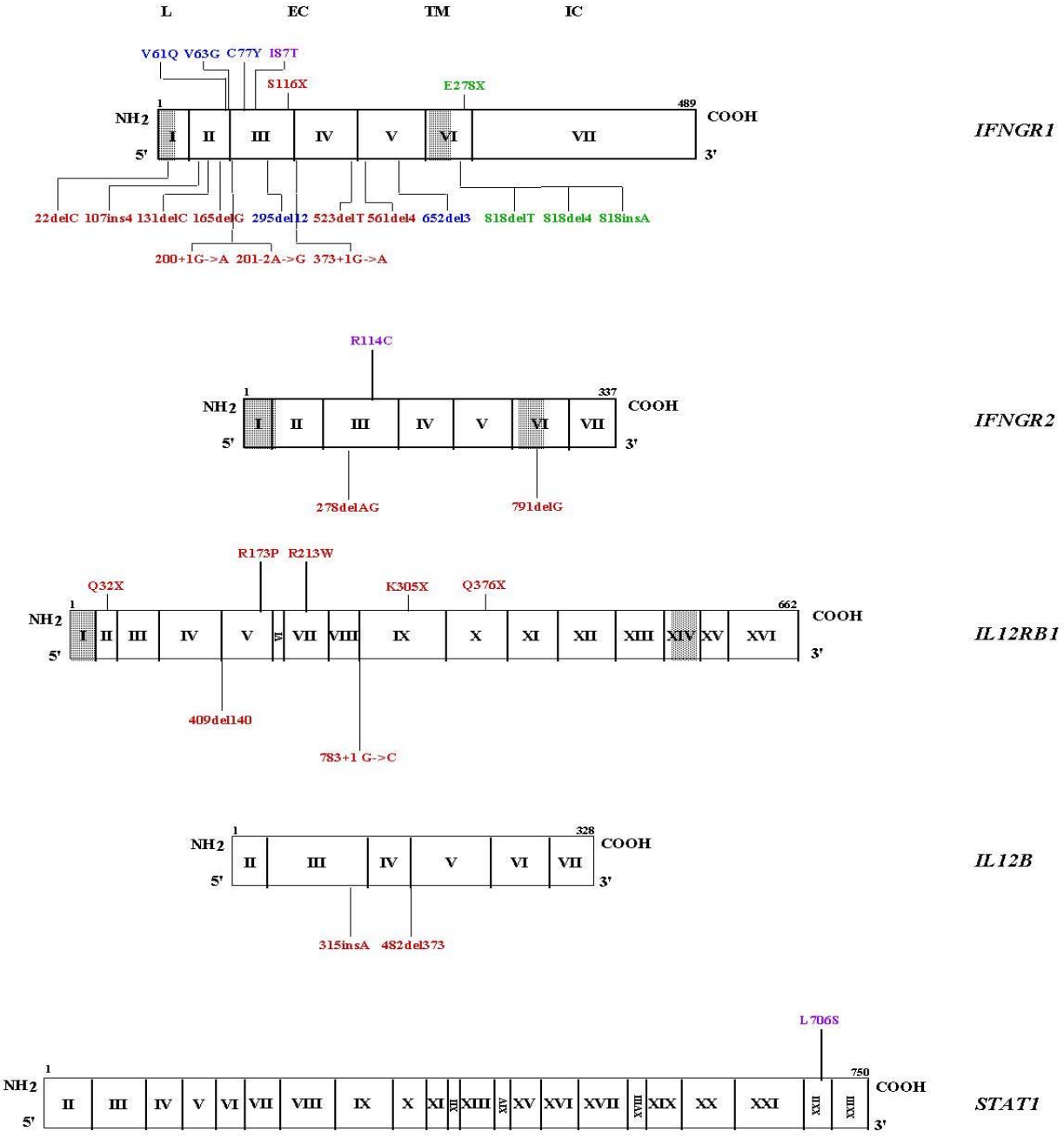


Figure 3. Mutations identified to date in the 5 MSMD genes. The gene-coding regions are indicated with vertical bars separating the exons, designated by roman numerals. Mutations in red (nonsense and splice mutations and frameshift insertions and deletions; recessive) cause complete deficiency with no detectable protein _expression at the cell surface. Mutations in blue (missense mutations and in-frame deletions; recessive) cause complete deficiency with detectable surface protein _expression. Mutations in purple (recessive) and green (dominant) cause partial deficiency.

A case of partial IFN-γR2 deficiency has also been described (29). A child born to related Portuguese parents developed disseminated infection following BCG vaccination. At the age of 16 she developed *M. abscessus* infection. A point mutation was identified in the *IFNGR2* sequence: the patient was homozygous for the mutation while both parents were heterozygous. This mutation results in an amino acid substitution at position 141 (arginine->cysteine) within the extracellular domain. The mutant IFN-γR2 is expressed normally on the cell surface but presumably the affinity between IFN-γR1 and IFN-γR2 is impaired.

IL-12B

Complete IL-12 p40 subunit deficiency leading to MSMD and Salmonella infections has also been described. The first case was born to consanguineous Pakistani parents and was immunised with BCG at birth (40). Sequencing of *IL-12B* revealed a deletion involving two coding exons. The parents and a healthy sibling were carriers of this mutation: the affected child was homozygous. Eleven other patients from 5 other families have recently been identified (28). One child had only salmonellosis. All other patients had mycobacterial disease, BCGosis in 10 children and *M. chelonei* in one child. Four children with BCGosis also had salmonellosis, one had tuberculosis, and one has nocardiosis. Five children died but all survivors are well off all treatment. Interestingly, one kindred from India had the same large deletion previously reported in the Pakistani kindred. A founder effect was documented and dated to approximately 29 generations ago (95% CI 9-115) and 700 years ago (95% CI 216-2,760) using a novel mutation dating method (Abel L. et al. submitted). The other four kindreds originated from the Arabic peninsula and were all found to carry the same IL-12B frameshift insertion. A founder effect was again documented, and dated to 47 generations ago (95% CI 22-110) and 1,100 years ago (95% CI 528-2,640). The fact that all patients with IL-12p40 deficiency identified to date have *IL-12B* mutations resulting from a founder effect, one in the Indian subcontinent and another in the Arabic peninsula, is consistent with the rarity of IL-12p40 deficiency among patients with MSMD. It is the first example of a founder effect among Mendelian mycobacterial susceptibility genes. IL-12B (IL-12 p40) has recently been shown to be to be a component of IL23. Thus IL-12B deficiency probably results in IL23 deficiency: however owing to the lack of human IL23 specific antibodies, this cannot be ascertained as yet.

IL-12RB1

Mutations in *IL-12RB1* which encodes the β1 subunit of the IL-12 receptor have been identified in seven patients from eight different families with EM infection to date (25,48, 49,50,51,52). Five of these patients also had Salmonella infections. A total of 8 mutations have been identified to date, including nonsense, splice and frameshift mutations which lead to premature termination of translation in the extra cellular domain. This abrogates cell surface _expression resulting in complete IL-12Rβ1 deficiency. Two missense mutations, also resulting in a lack of receptor _expression at the cell surface, were recently validated by gene transfer studies (25,51). Only recessive, loss-of-function mutations have been identified in *IL-12RB1* to date. Recently, a series of 31 patients from 23 kindred with IL-12Rb1 deficiency has been described (Fieschi et al. in preparation). Most patients had BCG/NTM disease, often with salmonellosis, but several were found to suffer from salmonellosis only and some from tuberculosis only. A significant fraction of patients were strictly asymptomatic. The recent observation that IL-12Rβ1 also serves as a subunit in the IL23 receptor suggests that *IL-12RB1* mutations prevent IL23 activation, but this has not been experimentally tested as yet.

STAT1

The identification of two unrelated families presenting with MSMD in the absence of mutation in any of the above genes led recently to the discovery of the fifth MSMD gene. A 33 year old woman with a history of disseminated BCG infection following childhood vaccination, and a 10 year old girl with disseminated *M. avium* infection were found to carry a de novo mutation in the coding region of *STAT1* (26). Both were heterozygous for a single T-C nucleotide change at position 2116 results in L706S in the COOH terminal region. The abnormal protein exerts a dominant negative effect on the normal protein in terms of STAT-1 dimer (also known as gamma activating factor, GAF) activation, but not in terms of STAT-1/STAT-2/p48 trimer (also known as interferon-stimulated gamma factor 3, ISGF3) activation. The STAT1 mutation is loss-of-function for the two

cellular phenotypes (it impairs phosphorylation of tyrosine 701) but dominant for one (GAF activation) and recessive for another (ISGF3 activation) in heterozygous cells stimulated with either type of IFN. It is to our knowledge the first reported mutation in a human gene to be dominant and recessive for two cellular phenotypes. Vulnerability to mycobacteria and resistance to viruses in the patients thus imply that GAF mediates anti-mycobacterial IFN-γ activity, whereas the anti-viral effects of IFNs are either STAT-1 independent or ISGF3 dependent. This novel disorder proves that IL-12-induced IFN-γ mediated immunity against mycobacteria is both STAT1 and GAF dependent.

FUNCTIONAL ASPECTS OF THE PROTEINS

The receptor for IFN-γ consists of two subunits: IFN-γR1, the ligand binding chain (previously known as the α chain) and IFN-γR2, the signal transducing chain (previously known as the β chain or accessory factor-1) (53). As the ligand binding chains interact with IFN-γ homodimers, they dimerise and become associated with two signal transduction chains. This leads to the activation of specific members of two protein families – the Janus kinases (JAK) and the signal transducers and activators of transcription (STAT). JAK1 and JAK2 phosphorylate key tyrosine residues on the ligand binding chains. This leads to the recruitment and activation of STAT1 which translocates to the nucleus as a phosphorylated homodimer (GAF) to activate a wide range of IFN-γ responsive genes. After signaling, the receptor complex is internalised and dissociates. The IFN-γR1 chain is then recycled to the cell surface. IFN-γR1 is expressed constitutively at moderate levels on the surface of all nucleated cells. IFNγR2 is also constitutively expressed a low levels but _expression is regulated by external stimuli, including IFN-γ itself (54).

Interleukin-12 is a heterodimeric cytokine comprised of two subunits, p40 and p35, which together form the biologically active p70 molecule. IL-12 is produced by activated antigen presenting cells such as dendritic cells and macrophages in response to a number of micro-organisms and microbial products, including lipopolysaccharide, lipoarabinomannan and bacterial superantigens (55). It can also be secreted upon stimulation by T cells in a CD154-CD40 dependent and IFN-γ dependent manner. It promotes cell-mediated immunity to intracellular pathogens by inducing the production of IFN-γ by both T and NK cells. The IL-12 receptor complex, expressed on activated T and NK cells, is comprised of two subunits known as the β1 and β2 subunits. Binding of IL-12 to the heterodimeric IL-12 receptor complex induces the phosphorylation of tyrosine kinase 2 (Tyk2) and JAK2, and subsequent activation of STAT4 which dimerises and translocates to the nucleus to activate IL-12 inducible genes. Figure 3 shows the cytokine interactions between the macrophage and T or NK cell in the context of mycobacterial infection, illustrating the interaction between IL-12 and IFN-γ.

MUTATION ANALYSIS

Mutations in *IFNGR1*, *IFNGR2*, *STAT1*, *IL-12B* and *IL-12RB1* can be detected by SSCP and/or sequencing. Primers to amplify and sequence all exons and flanking intron regions have been published and are available upon request.

STRATEGIES FOR DIAGNOSIS

Clinical diagnosis

Inherited defects of IL-12-IFN-γ axis may be considered in the differential diagnosis of all patients presenting with severe infection (including disseminated and recurrent diseases) with intracellular microorganisms, particularly when the organism is considered to be non-pathogenic in the "immunocompetent" individual. However, these defects should be sought aggressively in patients with severe nontuberculous mycobacterial or salmonella infections. Furthermore, a high index of suspicion is warranted in patients presenting with chronic fever, wasting, hepatosplenomegaly lymphadenopathy and anemia in whom a pathogen is not isolated, as cultures may be persistently negative (15,32). Diagnosis may also be cofounded by the lack of usually diagnostic granulomata, in which microbes may or may not be visible. An initial diagnosis of histiocytosis X has occasionally been made, hence MSMD should be considered in chemotherapy-resistant children with a tentative diagnosis of histiocytosis without formal histological criteria (56). In many individuals, MSMD becomes apparent following BCG

vaccination – vaccination history is therefore essential. The high incidence of parental consanguinity and affected relatives stresses the important of the family history. Specific attention must be directed at possible parental relatedness. Defects in the IL-12/IFN-γ axis should not only be sought in patients with disseminated or recurrent BCG/EM disease but also should be considered in patients with acute local BCG/EM, severe tuberculosis, severe histoplasmosis, listeriosis, and severe viral infections. Despite the progress outlined in this chapter, our understanding of the molecular basis of MSMD is still in its relative infancy and it is likely that there are many aspects of the disease that are yet to be unravelled. It is therefore prudent to consider the disorder in those diagnostic conundrums in which infectious, malignant or inflammatory diagnoses are entertained.

In vitro diagnosis

Circulating IFN-γ levels

Protein _expression (FACS, ELISA)

Functional studies

DNA analysis

1) Measurement of circulating IFN-γ in either plasma or serum is a simple means to differentiate patients with complete IFN-γR deficiency from those with other MSMD mutations (57). These children have high levels of plasma IFN-γ whereas IFN-γ is low or undetectable in plasma taken from healthy controls, or MSMD patients with IL-12p40 or IL-12Rβ1 receptor deficiency or partial IFNγR1 or 2 deficiency. This is thought to be due to sustained production of IFNγ in the most severe form of MSMD and/or the requirement for an intact IFNγR for ligation and removal of IFN-γ from the circulation. This observation provides a simple diagnostic assay for individuals presenting with severe BCG/EM disease. However, it should be kept in mind that elevated plasma or local (e.g., pleural tuberculosis) IFN-γ levels may also be seen in more normal hosts with tuberculosis.

2) The IFN-γR is expressed ubiquitously on all nucleated cells, while the IL-12 receptor is found only on NK and T cells. Analysis of EBV-B cells, SV-40 fibroblasts, or PBMC . IFN? R1 cell surface _expression by FACS is a simple means to assess the presence or absence of these receptors, as was demonstrated for the first reported cases of complete IFNγR1 deficiency [Newport, 1996 #480; (20). Mutations causing the dominant form of IFNγR1 deficiency abrogate the receptor recycling motif leading to high levels of cell surface IFN-γR1 _expression (up to 10-fold), which is easily detectable by FACS staining (27,37,38). Normal _expression of IFNγR1, as detected by FACS, even using blocking antibodies, does not exclude partial or even complete IFNγR1 deficiency resulting from mutations which result in the surface _expression of an abnormal protein (30,45). Antibodies that recognize the low levels of IFN-γR2 present on resting cells are not yet adapted to routine laboratory use. Antibodies that recognize IL-12Rβ1 work well on PHA blasts, and allow a diagnosis of IL-12Rβ1 deficiency. To date, all *IL-12RB1* mutations identified cause a loss of _expression of the encoded chain. Staining of *H. saimiri*-transformed T cell lines also works well: this is not the case for other cell lines such as EBV-B and SV40-transformed fibroblasts. Secreted IL-12p40 and p70 can be detected by ELISA in the supernatant of blood cells stimulated by BCG, or in the supernatant of EBV-B cells stimulated with pDBU. To date, all *IL-12B* mutations identified have been associated with a lack of detectable IL-12p40 and IL-12p70. However, prolonged stimulation of IL-12R*1 deficinet T cell lines appears to rescue some degree of IL-12 responsiveness (52).

3) Expression of IFN-γR or IL-12R does not imply function, and therefore other in vitro assays are required to identify which component(s) of the IL-12/IFN-γ pathway are defective. On binding to its receptor, IFN-γ induces pleiotropic effects including the upregulation of MHC class II _expression and TNFα production by monocytes. These effects are mediated by the binding of phosphorylated STAT1 to gamma activating sequences (GAS) in gamma responsive genes. Thus IFN-γR deficiency may be diagnosed functionally by studying in vitro responses to IFN-γ. A simple whole blood assay was used to demonstrate defective responses to IFN-γ in the Maltese kindred (15) and this technique was modified to study PBMC responses in a patient with partial IFN-γR1 deficiency (30). MHC class II _expression is easily studied by flow cytometry (43), while phosphorylation and nuclear translocation of STAT1 in response to IFN-γ can be assessed using electrophoretic mobility shift assays (53,26), or more simply by flow cytometry, using a STAT1 specific monoclonal antibody (58). Cellular responses to

IFN-γ should be tested at low and high concentrations of IFN-γ to differentiate between partial and complete receptor deficiency. In vitro studies in patients with IL-12 p40 deficiency show defective IFN-γ production by PBMC or whole blood following stimulation with BCG. This defect was restored by the addition of recombinant IL-12 to the culture medium. IL-12Rβ1 deficient patients also have diminished mitogen-induced IFN-γ production, but IL-12 p70 production in response to LPS, tuberculin or mycobacteria is normal. A flow cytometric assay which detects phosphorylated STAT4 has also been developed (59). Notably, children with complete IFN-γR deficiency have a low in vitro production of IFN-γ, due to impaired production of IL-12 (44).

4) With the exception of the dominant *IFNGR1* hotspot mutations and the two *IL-12B* (Il-12 p40) mutations (founder effects), every MSMD family has a unique mutation. It is therefore not cost effective to set up mutation screening assays looking for known mutations. A combination of in vitro phenotyping (_expression and functional studies), and direct gene sequencing is recommended. Once the causative mutation has been established in a family, other family members can be screened directly for the mutation. Accurate molecular diagnosis by biochemical, functional, and genetic studies is of the utmost importance for predicting clinical outcome and guiding the treatment of patients.

GENETIC COUNSELING AND PRENATAL DIAGNOSIS

Defects in the IL-12/IFN-γ pathway may be inherited either as dominant or recessive disorders depending on the mutation. All mutations reported in *IFNGR2*, *IL-12RB1* and *IL-12B* are recessive: many patients are homozygous for recessive mutations, reflecting the high frequency of parental consanguinuity within this group of patients. *IFNGR1* mutations were initially identified as homozygous recessively inherited, but dominant mutations have subsequently been identified as well. Compound heterozygotes have also been identified. The *STAT1* mutation identified in three individuals to date is dominant. Finally, X linked recessive inheritance has been suggested in one kindred, though the molecular basis of increased susceptibility to EM in this family has yet to be established (5,6,7).

Given the heterogeneity of this syndrome, coupled with its rarity, carrier detection/screening is not currently feasible. In one family with recessive IFN-γR1 deficiency, heterozygous carriers had an intermediate cellular phenotype in vitro (15,17) although this may have been dependent on the assay used. To date, there is no clinical phenotype associated with heterozygosity for any of the recessive alleles. Once the molecular basis is known within a family it is simplest to screen other members by looking directly at their DNA. Counseling within families where the mutation is known is straightforward in terms of the risk of inheriting a 'susceptible' genotype (25% risk of an affected child if recessive, 50% risk of an affected child if dominant inheritance). However, any discussion must also take into account the following:

a) The clinical phenotype depends on the gene affected and whether the mutation leads to complete or partial protein deficiency

b) The development of disease is dependent on pathogen exposure

c) There are individuals with less severe mutations involving either *IFNGR1* or *IL-12RB1* who have inherited a susceptible genotype but have not developed disease. Presumably they have some residual antimycobacterial immune function. To date, there are no known patients with complete IFNγR1 or IFNγR2 deficiency who have not been affected.

Complete IFNγR1 or IFNγR2 deficiency is the most severe phenotype and is frequently lethal despite antibiotics. BCG vaccination must be withheld from potentially affected children until IFNγR status is clarified. Bone marrow transplantation has proved very difficult, and less successful than would be anticipated (see below) perhaps because transplantation has typically been attempted after disseminated mycobacterial disease has occurred.

Once a molecular diagnosis has been established, prenatal diagnosis can be offered to affected families with severe disease, i.e. complete IFNγR deficiency. The role of prenatal diagnosis for other mutations is less obvious as the phenotype is less severe, disease is preventable and many individuals carrying mutations are disease free.

TREATMENT AND PROGNOSIS

The treatment of defects in the IL-12/IFN-γ axis should be tailored to the individual patient according to their mutation, the clinical pattern of disease and the pathogens involved (60). Established infection should be treated with appropriate antimicrobial drugs as determined by the genus and species. Thus microbiological isolation and characterization of the causative pathogen at an early stage is desirable. The role for in vitro susceptibilities in directing treatment of EM is still unproven and poorly defined. EM are notoriously resistant to a number of antimicrobials. Cytokine therapy has helped clear mycobacterial infection in patients with full or partial function of the IFN-γ receptor (7,61). Patients with IL-12B (IL-12p40) or IL-12R*1 deficiency, or partial IFN-γR deficiency respond well to IFN-γ treatment. However, intestinal/mesenteric/splenic infections can be resistant to antibiotics and IFN-γ. Splenectomy was helpful in two children with splenic sequestration (IFN-γ induced in one child); on occasion abdominal lymph node resection may be indicated (62) (Casanova unpublished). Overall, patients with partial IFN-γR/STAT1 deficiency or complete IL-12R*1 deficiency can achieve prolonged clinical remission after antibiotics and IFN-γ are discontinued. Relapses may occur years after the initial episode. Treatment with antibiotics and IFN-γ should be prolonged, even after clinical remission is obtained.

In contrast, children with complete IFN-γR deficiency achieve full clinical remission less often and mycobacterial infections often relapse weeks to months after antibiotics are discontinued. Therefore, successful antibiotic therapy should not be discontinued. Due to lack of specific receptors, IFN-γ therapy is not indicated. The role for other cytokines such as IFN-*, GM-CSF or IL-12 is undefined. The only curative treatment available for patients with complete IFN-γR deficiency is bone marrow transplantation (BMT). An international survey of 10 patients who underwent BMT is currently in progress (33). Preliminary results indicate that BMT in patients with complete IFN-γR deficiency is associated with an unexpectedly high level of post-BMT morbidity and mortality. The only child who received an HLA-haplo-identical transplant rejected the graft. Of nine unrelated patients who received an HLA-identical intrafamilial graft, despite an initial full engraftment in all cases, the graft was secondarily rejected in six children, of whom four have died. Therefore, there appears to be a selective advantage of IFN-γR-deficient over WT hemopoietic progenitors in IFN-γR-deficient children. This makes gene therapy for bone marrow IFN-γR deficiency challenging, as a selective advantage of transduced cells is absolutely required.

Prevention of infection is desirable, although many pathogens to which these individuals are susceptible are ubiquitous in the environment. BCG should be avoided and mycobacterial infection (both primary and secondary) may be prevented by the use of a macrolide such as clarithromycin or azithromycin. In patients with mild MSMD, prophylactic antibiotics are not absolutely required, as infectious episodes are relatively infrequent and can be controlled by IFN-γ and antibiotics if treated promptly. However, physicians and patients should weigh carefully the risks and benefits of recurrence of infection, especially if it recurs in bone, as is the case often with the dominant form of IFN-γR1 deficiency. In these patients recurrence of infection can have serious consequences, despite curative therapy.

In patients with complete IFN-γR deficiency, antibiotics should be continued indefinitely) after therapy of acute infections. There is considerable diversity of pathogenic EM (particularly rapidly-growing species), making absolute recommendations difficult. However, most EM are susceptible to macrolides, and these should be strongly considered for long-term prophylaxis regardless of cure of other acute infections. Immunosupression such as corticosteroids should be avoided as a rule, particularly in children with complete IFN-γR deficiency, although in some circumstances they may be helpful. Children with MSMD should be treated on an individual basis, and treatment undertaken in close collaboration with a center specialized in the care of such patients.

Animal models

The study of gene disrupted mice has greatly enhanced our understanding of the IL-12/IFN-γ pathway. Although not completely concordant, the phenotypic similarities between these animal models and patients with mutations in this axis are striking (63). Mice lacking *Ifngr1* are highly susceptible to BCG infection, with poorly defined granuloma formation and death

(64). Mice lacking *Ifn-γ* also fail to control BCG, *M. avium* or *M. tuberculosis* growth (65,66). More recently, *Ifngr2* knockout mice were shown to have defective Ifn-γ production and susceptiblity to *L. monocytogenes* infection (67). *IL-12b* (il-12 p40) knockout mice are more susceptible to *M. tuberculosis* infection than normal mice, leading to higher bacterial loads and disseminated disease (68). Granulomata were poorly formed and multibacillary.. *IL-12rb1* knockout mice have defective Ifn-γ responses to mitogens and LPS (69). Disruption of *Ifng* in mice also leads to lethal infection with an attenuated strain of *S. typhimurium* whereas wild type mice clear infection within four weeks (70,71). However, comparisons between mouse and man are limited in several ways:-. most of the infections in MSMD patients are naturally occurring while those in mice are experimental, often administered intravenously, and the strain and dose of pathogen is controlled. There are certain infections, such as *Toxoplasma gondii* and *Cryptococcus neoformans,* to which IFN/IL-12 knockout mice have increased susceptibility which have not been observed in humans (72,73). This may reflect lack of exposure, experimental design, or the fact that knockout mice are generated in highly inbred strains. Genetic variation at other immunity modifying loci is low in inbred mice whereas humans are outbred, even in the setting of consanguinity. Experimental infections in mice probably highlight even minor effects of the IL-12/IFN-γ axis. Alternatively, mice and humans may be divergent in their handling of some of these non-mycobacterial infections.

CONCLUDING REMARKS AND FUTURE CHALLENGES

Mutations in 5 genes involved in the IL-12/IFN-γ axis have been associated with the syndrome of MSMD, which encompasses a range of clinical phenotypes. The severity of the clinical phenotype primarily depends on the gene involved and the specific mutation. IFN-γ-mediated immunity appears to be a genetically controlled quantitative trait that determines the outcome of mycobacterial invasion (31). IFN-γ immunity to mycobacteria is dependent on IL-12 stimulation, and mediated by STAT1 and its homodimeric complex GAF. These defects are most pronounced with respect to mycobacteria and to a lesser extent salmonella and viruses. (74). The investigation of more patients is necessary to broaden our knowledge of these genotype-phenotype correlations. Clinically, molecular diagnosis guides rational treatment based on pathophysiology.

Are there other MSMD genes?

There remain patients with the clinical syndrome of MSMD who do not have mutations in *IFNGR1*, *IFNGR2*, *STAT1, IL-12B*, or *IL-12RB1* (approximately 50% at our centres, Holland, Levin and Casanova, unpublished). Characterization of the molecular defects in these patients will identify other MSMD genes, and contribute further to our understanding of human mycobacterial immunity. Relevant genes upstream of IL-12 and downstream of STAT1 are expected to expand and define the limits of the IL-12/IFN-γ axis, especially the inducer and effector mechanisms of immunity to mycobacteria.

Definition of the clinical boundaries of MSMD

The genetic defects of the IL-12/IFN-γ axis were found by investigating patients with disseminated, often lethal, BCG/EM disease. Subsequently, it was found that some affected individuals have recurrent local disease, while others are asymptomatic. International surveys are currently underway to define the clinical features of each inherited disorder, based on the clinical history of the patients identified. The question arises whether patients with unexplained local BCG/EM disease may suffer from these or related genetic defects. For example, EM pneumonitis in the elderly, and EM adenitis in childhood, are currently unexplained. Patients with various forms of BCG/EM disease thus need to be explored for the IL-12/IFN-γ axis, in order to define the clinical frontiers of each genetic defect.

What is the role of MSMD genes in susceptibility to tuberculosis and leprosy?

It is estimated that approximately 2 billion individuals world-wide are infected with *M. tuberculosis* (75). The World Health Organization estimates there were 8 million new cases of tuberculosis (TB) and 1.9 million deaths from the disease in 1998. The fact that only 10% of individuals infected with *M. tuberculosis* go on to develop clinical disease suggests that exposure to virulent mycobacteria alone is not sufficient and that the host immune response is an important determinant of susceptibility (or resistance) to disease (76). Several studies

demonstrate a role for host genetic factors as determinants of susceptibility to TB (74). However, the identification of specific genes involved in susceptibility to infectious diseases in outbred human populations is difficult. Complex interactions between the pathogen, which also has a genome, the environment and host factors determine whether an individual is resistant or susceptible to disease. It is likely that a number of genes are involved, but it is not known exactly how many, nor how they interact. Population based studies have reported associations between candidate genes and TB but the effects have been modest and the functional relevance of these findings is yet to be established (77,78,79,80,81).

There is a spectrum of disease within the MSMD syndrome ranging from severe disease which is fatal in early childhood (complete IFN-γR deficiency) to moderate disease in individuals with partial IFN-γR1deficiency (30). The IL-12/IL-12Rβ1 mutations have a less severe clinical course. Mutation in *IL-12RB1* and *IL-12B* have been identified recently as a susceptibility factor for the development of abdominal *M. tuberculosis* infection (25) and tuberculous adenitis (28). Partial deficiency of either IL-12B or IL-12R*1 would be expected to have a less severe phenotype than complete deficiency, and to predispose to only more virulent pathogens (82). More subtle polymorphisms in the MSMD genes identified so far could result in impaired _expression of a normal protein or normal _expression of a slightly altered protein. It is also likely that mutations or polymorphisms in other genes involved in mycobacterial immunity, which have a different role and may cause a different immune defect. Such individuals may retain immunity to organisms of low virulence while remaining susceptible to more virulent species.

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