HHS Public Access

Author manuscript

Crit Rev Clin Lab Sci. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

Crit Rev Clin Lab Sci. 2018 May ; 55(3): 184–204. doi:10.1080/10408363.2018.1444580.

Laboratory evaluation of the IFN-γ circuit for the molecular diagnosis of Mendelian susceptibility to mycobacterial disease

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Abstract

The integrity of the interferon (IFN)-γ circuit is necessary to mount an effective immune response to intra-macrophagic pathogens, especially *Mycobacteria*. Inherited monogenic defects in this circuit that disrupt the production of, or response to, IFN-γ underlie a primary immunodeficiency known as Mendelian susceptibility to mycobacterial disease (MSMD). Otherwise healthy patients

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Disclosure statements:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

display a selective susceptibility to clinical disease caused by poorly-virulent mycobacteria such as BCG (bacille Calmette-Guérin) vaccines and environmental mycobacteria, and more rarely by other intra-macrophagic pathogens, particularly *Salmonella* and *M. tuberculosis*. There is high genetic and allelic heterogeneity, with 19 genetic etiologies due to mutations in 10 genes that account for only about half of the patients reported. An efficient laboratory diagnostic approach to suspected MSMD patients is important, because it enables the establishment of specific therapeutic measures that will improve the patient's prognosis and quality of life. Moreover, it is essential to offer genetic counseling to affected families. Herein, we review the various genetic and immunological diagnostic approaches that can be used in concert to reach a molecular and cellular diagnosis in patients with MSMD.

Keywords

Mycobacteria; intracellular pathogens; interferon gamma; primary immunodeficiency; diagnosis; MSMD

Introduction

Mendelian susceptibility to mycobacterial disease (MSMD) is a primary immunodeficiency (PID) characterized by a selective predisposition in otherwise healthy individuals to disease when infected by bacille Calmette-Guérin (BCG) vaccines or environmental mycobacteria [1,2]. It is included in the PID classification by the IUIS (International Union of Immunology Societies) in the VIth group of defects in Intrinsic and Innate immunity [3]. Immunity to mycobacteria relies on the IFN-γ (interferon) circuit (Figure 1), as shown by the study of mice both *in vitro* and *in vivo,* and by the study of humans with MSMD. Pattern recognition receptors are important sensors of mycobacteria after infection; however, their role in generating a protective response is apparently redundant [4,5]. After bacilli/us phagocytosis, antigen-presenting cells (APC), including macrophages, are activated and produce tumor necrosis factor (TNF)-α, interferon-stimulated gene (ISG) 15, and interleukin (IL)-12p70, which induce T helper (Th) cells to produce IFN-γ and differentiate into Th1 cells. This creates a positive loop between the T cell and the APC, which enhances the former's microbicidal capacity through production of oxygen reactive species (ROS) [6–10].

IL-12 (IL-12p70) is a heterodimer composed of a p40 subunit (in common with IL-23) and a p35 subunit that bind IL-12Rβ1 and IL-12Rβ2, respectively, activating both natural killer (NK) and Th cells [11]. Janus-associated kinase 2 (JAK2) binds to IL-12Rβ2 subunit and tyrosine kinase 2 (TYK2) to IL-12Rβ1 subunit. After IL-12p70 binds to the IL-12 receptor (IL-12Rβ1-IL-12Rβ2 dimer), TYK2 and JAK2 come closer and JAKs are trans-phosphorylated, thereby phosphorylating the receptor chains. Signal transducer and activator of transcription 4 (STAT4) binds to phosphorylated IL-12Rβ2, becomes auto-phosphorylated, and dimerizes. Then, STAT4 homodimers translocate to the nucleus, where they bind to the *IFNG* promoter, inducing its transcription [12]. In parallel, secreted free ISG15 from APCs also promotes IFN-γ production by T cells and CD3$^-$CD56$^+$ NK cells, which are considered the key ISG15-responder leukocytes [7,13]. Thus, ISG15 and IL-12p70 act synergistically to induce IFN-γ production.

IFN-γ response in APCs, especially in macrophages, is mediated by its binding to IFN-γ receptor (IFN-γR) 1 and IFN-γR2, followed by internalization and signalization via the receptor complex. After IFN-γ binding, the two subunits of the receptor, as well as JAK1 (bonded to IFN-γR1) and JAK2 (bonded to IFN-γR2) come closer. JAK1 and JAK2 then cross-phosphorylate and phosphorylate IFN-γR2, creating a docking site for STAT1. After binding, STAT1 is activated by phosphorylation of tyrosine 701 and dimerizes, forming γ-activated factor (GAF) and translocating to the nucleus where it binds to γ-interferon-activated site (GAS) of ISG, promoting its expression [14,15]. MSMD is caused by monogenic defects in different steps of this circuit (Figure 1), which impair the production of, or the response to, IFN-γ, thereby disrupting protective immunity to mycobacterial infection.

Although the first clinical description of MSMD was published in 1951 [16], it was not until 1996 that the first genetic etiology of MSMD, autosomal recessive (AR) IFN-γR1 deficiency, was described in an infant with fatal BCG infection [17,18]. Afterwards, defects in other genes encoding proteins involved in IFN-γ immunity have been discovered, affecting both IFN-γ production (*IL12RB1* [19–21], *IL12B* [22,23], *ISG15* [7,24], *NEMO* [25], *IRF8* [26], and *TYK2* [27]), and cellular responses to IFN-γ (*IFNGR1* [17,19,28–31], *IFNGR2* [32,33], *STAT1* [34–36], *IRF8* [26] and *CYBB* [25,37,38]). There are currently 19 different genetic etiologies of MSMD that involve the impact of the mutation (null or hypomorphic), the mode of transmission in the family (dominant or recessive), the expression of the mutant allele (absent or detectable), or the function affected by the mutation (one domain or another, in the case of a detectable protein); the most common defect is IL-12Rβ1 deficiency, and the second most common, IFN-γR1 deficiency [2,27,39]. The number of genetic etiologies is likely to increase in the coming years. With so many forms, the clinical boundaries of MSMD syndrome and of each genetic etiology are not yet fully defined; the disease spectrum ranges from the complete forms of IFN-γR deficiencies in the most severe cases of MSMD, with an outcome that leads to death if hematopoietic stem cell transplantation (HSCT) is not performed [17,40], to other defects (for example, IL-12Rβ1 or IL-12p40 deficiencies), in which patients can be treated with exogenous human recombinant IFN-γ (hrIFN-γ) in addition to antibiotics [20,21]. For this reason, accurate genetic diagnosis, and the distinction between complete and partial defects, as well as the careful description of the immunological signs, are of the utmost importance to ensure the best possible management of MSMD patients.

Published immunological approaches for the molecular and cellular diagnosis of MSMD are diverse. Some are complex and results, even among healthy controls, can be highly variable. [19,21,23,30,39]. Nevertheless, they are necessary, since they facilitate targeted gene sequencing and the prediction of effectiveness of adjuvant therapies such as exogenous hrIFN-γ. Our main aim is to summarize the current warning signs of MSMD, as well as the functional and genetic approaches available for the study of the IFN-γ circuit, both in clinical practice and in research, and their limitations, in order to guide physicians and immunologists in the diagnosis of MSMD.

Infectious spectrum of MSMD

Patients affected with MSMD are otherwise usually healthy and can present a wide range of severity of the disease, from local and recurrent to disseminated and lethal. The severity of the disease depends on the type of underlying defect (complete or partial). Clinical disease is usually caused by environmental mycobacteria (EM), and BCG after infant vaccination, which is the most common, and sometimes the only, infectious event [2,41]. Some patients are also susceptible to *Mycobacterium tuberculosis* [42]. Different etiologies of MSMD, especially IL-12Rβ1 [2,42,43], IFN-γR1[2,29], STAT1[2,34], and IL-12p40 [2,23] deficiencies, were found in patients with severe tuberculosis (TB) (disseminated/extrapulmonary or recurrent TB). There are currently 23 reported patients with tuberculosis due to inborn errors of IFN-γ, 13 of whom are IL-12Rβ1-deficient; these include six who did not suffer from any other mycobacterial disease (BCG, EM)[2,42,43]. Interestingly, MSMD underlying *Mycobacterium tuberculosis* infection restricted to the lung has been described not only in IL-12Rβ1 deficiency, but also in IFN-γR1 deficiency [30,44,45]. Besides mycobacteria, there is a wide range of causative organisms of disease that includes *Salmonella*, fungi (especially *Candida*), other intra-macrophagic bacteria, and parasites (*Leishmania, Toxoplasma* [46]). MSMD usually, but not always, manifests in childhood [2].

Interestingly, specific clinical manifestations have been associated with specific gene defects: the correlation of pathogens and/or clinical forms with all described genetic etiologies of MSMD was nicely reviewed by Bustamante et al. [2]. Briefly, patients with IFN-γ production defects caused by mutations in *IL12RB1* and *IL12B* (encoding IL-12Rβ1 and IL-12p40, respectively) commonly suffer from disease caused by *Salmonella* (recurrent or not) and, to a lesser extent, by *Candida*. Patients with IFN-γ production defects do not usually present with viral infections. Regarding IFN-γ response defects, the presence of multifocal osteomyelitis should raise the suspicion of a partial autosomal dominant (AD) IFN-γR1, partial AR, or AD STAT1 loss of function (LOF) [36,47–53]. Patients with complete deficiency in IFN-γR1 and IFN-γR2, abolishing IFN-γ response, are more prone to viral diseases such as cytomegalovirus, respiratory syncytial virus and varicella-Zoster virus, among others [2].

Laboratory testing

Who should be tested?

Children or adults without any other hemato-immunological conditions who develop recurrent or severe/disseminated mycobacterial infectious disease caused by BCG, EM, *M. tuberculosis,* or *Salmonella* alone or in combination with other intracellular pathogens or viruses should be tested. Specific warning signs of MSMD are presented in Table 1.

Defects in the IFN-γ circuit are not the only PID predisposing to mycobacterial disease [3,42]. Before performing specific MSMD tests, severe combined immunodeficiency, combined immunodeficiency and chronic granulomatous disease must be ruled out [54], because they are more common than MSMD and they confer susceptibility to various infectious diseases including mycobacteria.

Other less common PIDs confer susceptibility to various infectious diseases including mycobacteria and should also be ruled out in parallel with MSMD testing: 1) X-linked NF-kB deficiency: anhidrotic ectodermal dysplasia with immunodeficiency (XR-EDA-ID) syndrome. Patients suffering XR-EDA-ID are susceptible to a wide range of pathogens (pyogenic bacteria, viruses) including mycobacteria. Immunologically, these patients present altered NK cell mediated cytotoxicity and TNF-α production after Toll-like receptor (TLR)-4 lipopolysaccharide (LPS) stimulation [2,55–57]; 2) GATA2 deficiency, particularly in otherwise healthy adults with disseminated EM infections [58,59]. Patients with GATA2 deficiency show susceptibility to viral infections and mycobacteria and usually present with severe circulating monocytopenia (78% of patients), and B (86% of patients) and NK (82% of patients) [58,59] lymphopenias. Characteristically, patients with GATA2 deficiency show specific loss of the $CD56^{bright}$ subset [60]. Due to these characteristic myeloid and lymphoid cytopenias, consideration of GATA2 deficiency as a genetic etiology of MSMD is currently open to debate, because MSMD-causing defects occur in otherwise healthy subjects without other significant immune abnormalities except for the defect in the IFN-γ circuit; 3) severe innate PID, predisposing to mycobacteria and viruses (AR STAT1, AR STAT2, AR JAK1, and AR interferon regulatory factor 8 (IRF8) deficiencies [26,61–68]) or mycobacteria and fungi (AR RAR related orphan receptor C (RORC) deficiency [69]).

Beyond PID, other causative conditions such as immunosuppressive drug exposure, including anti-TNFα antibodies, azathioprine, cyclophosphamide, mycophenolate, and cyclosporine, need to be ruled out [70,71]. In addition, long-term potent oral steroids can lead to secondary mycobacterial infection [72]. Also, acquired immunodeficiency by HIV infection [42,71,73] and malignancies such as hairy cell leukemia need to be tested for [74–77]. Finally, patients who have neutralizing anti-IFN-γ autoantibodies can develop MSMD-like clinical manifestations; they are included in group IX of the IUIS classification, which is called PID phenocopies [3]. Patients with neutralizing anti-IFN-γ autoantibodies have impaired IFN-γ production and STAT1 phosphorylation in the presence of autologous serum that is rescued after lavage. This phenomenon has been mostly, but not exclusively, observed in adult Asian populations [78–81].

Baseline IFN-γ in plasma

Detection of baseline IFN-γ in plasma by enzyme-linked immunosorbent assay (ELISA) is a simple technique that can help to rapidly identify patients with complete IFN-γR deficiency [2,82,83]. These patients present with increased levels of IFN-γ in plasma; patients with partial recessive forms of IFN-γR deficiency present with detectable levels of IFN-γ while it is undetectable in other MSMD forms and in healthy controls [30,33,40,82–84]. The threshold to consider a patient with a complete defect as a candidate for HSCT was defined as 2 standard deviations above the mean level in patients with partial AR IFN-γR1 defects (>80 pg/mL), while observed levels in complete IFN-γR deficiency were 150 1700 pg/mL [82,83]. Several years later [82], Sologuren et al. published a case series of partial AR IFN-γR1 defects showing a range of baseline IFN-γ of 51 222 pg/mL, with an outlier of 925 pg/mL [30]. They suggested that the very high concentration of baseline IFN-γ observed in the outlier could reflect an acute mycobacterial disease. Thus, the infectious state of the patient needs to be considered, as baseline IFN-γ plasma levels may vary in acute infection

compared with the convalescent phase [30], making it possible that levels may overlap in partial AR IFN-γR1 or IFN-γR2 deficiency in rare cases [30,33,83]. Therefore, if possible, baseline IFN-γ should be measured at least one month after resolution of acute infection. In any case, no IFN-γ is usually detected in the plasma of healthy individuals [82,83]. Plasma samples need to be diluted at least 1:2 to avoid interference from other proteins such as fibrinogen.

To optimize the ELISA technique, IFN-γ measurements on patients' plasma samples should be batched. Then the cost of an individual determination of IFN-γ can range from 1–12€, depending on the kit used. The selection of the ELISA kit will also determine the hands-on time required (3 h to approximately 6–8 h), depending on whether or not an overnight sensitization step is required. Optimization can lead to an increased response time (turn-around time) when returning the results to the clinician if the number of patients is low.

Cytokine production

The gold standard for the study of IFN-γ circuit integrity, cytokine production, was developed by Feinberg et al. [19]. This assay is based in the measurement of IL-12p40, IL-12p70 and IFN-γ after stimulation of whole blood or peripheral blood mononuclear cells (PBMCs). Stimulation conditions comprise live BCG stimulation at a multiplicity of infection of 20 BCG/leukocyte with or without hrIL-12p70 (20 ng/mL), or hrIFN-γ (5000 IU/mL) co-stimulation for 18 h (for IL-12 measurement) or 48 h (for IFN-γ and IL-12 measurements).

For healthcare practices and laboratories subject to ISO 15189 European regulations, the use of BCG as a stimulus impedes the standardization of the protocol. An alternative that avoids the use of BCG is the use of mitogens as follows: phytohemagglutinin (PHA; 1%) [85] or LPS (from *Salmonella minesotta*; 100 ng/mL) in combination with hrIL-12p70 or hrIFN-γ (10^2, 10^3 and 10^4 IU/mL) [30]. The output of both BCG and mitogen whole blood or PBMC stimulation is similar (measurement of IL-12p40, IL-12p70 and IFN-γ). Detection of the cytokines produced may be performed with ELISA or multiplex assays by means of flow cytometry (Luminex Technology (Luminex, Austin, TX, USA) or cytometric bead array systems [19,30,40]. As the interval between blood extraction and performance of testing reduces the cytokine production [21], it is important to take this into account when analyzing the results in samples that are assayed 24 h after the blood extraction.

Results obtained from the cytokine production assay will help to distinguish between IFN-γ response defects and IFN-γ production defects. Complete forms of IFN-γR1, IFN-γR2, IL-12Rβ1 or IL-12p40 can be detected with this approach; however, some genetic etiologies of MSMD, such as CYBB or AD IRF8 deficiency, will show normal responses to this stimulation [26,37,86]. IFN-γ production defects are characterized by the absence or low production of IFN-γ after BCG stimulation. If there is no recovery of IFN-γ after hrIL-12p70 co-stimulation, IL-12Rβ1 deficiency should be studied first [21], followed by ISG15 or TYK2 deficiencies [7,27]. Patients with IL-12p40 deficiency produce low or very low levels of IFN-γ in response to BCG stimulation, which can be rescued, at least partially, with exogenous hrIL-12p70. In complete IFN-γ response defects (complete IFN-γR1 and IFN-γR2 deficiencies), there is no response to hrIFN-γ in terms of IL-12 production

[19,29,85,87–92]. On the other hand, in partial IFN-γ response defects (partial AR IFN-γR1/IFN-γR2 and partial AD STAT1 LOF deficiency), the response to hrIFN-γ is impaired in a dose-dependent manner, but not abolished [28–30,34,36,45,93–98].

In nuclear factor-kappa B essential modulator (NEMO)-deficient patients, IL-12 production is normal after BCG stimulation but impaired after PHA/CD3 PBMC stimulation [38,99].

It is difficult to establish cut-off values for diagnosis, because published cases cannot always be compared due to differences in the techniques used for cytokine production determination. There have been attempts to study cohorts of IL-12Rβ1 and IL-12p40 patients [19,23,100] functionally: i) in IL-12Rβ1 deficient patients, IL-12p70 production was normal but IFN-γ production was low or null after BCG (4 – 726 pg/mL) and did not increase after IL-12p70 co-stimulation [19,21]; ii) IL-12p40 deficient patients showed no IL-12p70 production and a decreased IFN-γ production that in most cases was undetectable or below 100 pg/mL; only one patient showed IFN-γ production of 1000 pg/mL [19,23]. Patients with complete deficiency of IFN-γR1 or IFN-γR2 showed normal production of IFN-γ but failed to induce IL-12p70 after BCG or BCG + IFN-γ. Expected results of cytokine production in the different genetic forms of the IFN-γ circuit are summarized in Tables 2 and 3.

The main advantage of this technique is that it is the test that most closely assesses the patients' real immune function. However, it also has limitations: by itself, it only clearly detects complete defects, while partial defect identification can be difficult. To date, the specification of cut-off values to define disease for routine healthcare practice has not been possible. Although it has limitations, cytokine detection after whole blood/PBMC culture is a powerful option with room for improvement.

The culture itself takes 48 h, but the hands-on time is limited, and depends on if it is performed in whole blood (30–45 min) or in PBMCs (120–165 min). Depending on the concentration and the source of the stimuli used, the costs may vary. In this technique, an economic limitation may be the acquisition of the stimuli for the first time, because some are expensive, but they can be used for many tests. For the detection of secreted cytokines, the most economic option is to perform ELISA for IFN-γ and for IL-12p70, with an estimated cost from around 6–7€ to 80–100€ per individual[a], but it will vary depending on the duplicates run, the assay conditions and the chosen kit. This technique requires the same hands-on time as IFN-γ baseline detection, including the possibility that optimization of the technique by batching of patients can lead to increased response times[b].

[a]Approximate costs of the different techniques are calculated for each sample processed to which the cost of healthy (normal) control/s sample/s must be added; only reagent-derived costs are included. It is important to take into account that prices are approximate and that they may vary depending on the supplier/country or type of kit used. Moreover, when evaluating the costs of implementing these techniques, other costs need to be considered, such as sample preservation, including frozen PBMCs and plasma, DNA extraction and preservation, and general materials such as phosphate buffer saline, plastic materials and culture media. Because all laboratories may not have a flow cytometer, they may need to use flow cytometry facilities, which likely charge the users for the use of the cytometers and for technical assistance. This is an important variable to consider in all flow cytometry techniques as it may significantly increase the final cost.

[b]Hands-on time is an estimation of the time needed to perform the technique; however, the response time (turn-around time) can vary depending on different factors including i) the need to batch patient samples, ii) the number of patient samples, and iii) the time required for analysis (from receipt of specimen to reporting the result).

Cytometric detection of extracellular receptors

IFN-γR1/IFN-γR2 expression—IFN-γR detection by flow cytometry is a fast technique for the detection of complete forms of AR IFN-γR1 and AR IFN-γR2 deficiency with absent protein expression in the membrane of monocytes; it can be performed in both WB and PBMCs (Figure 2). However, different mutations in *IFNGR1* and *IFNGR2* can lead to distinct patterns of expression (Table 1). In partial AR IFN-γR1 defects, there is usually a weak expression of the receptor [2,30], and partial AD IFN-γR1 deficiency leads to increased protein expression due to mutations in the recycling motif [2,31]. In case of expression of the receptor, its detection could be affected by the antibody used: for example, IFN-γR1 in cells of patients with the C77Y complete AR IFN-γR1 defect would be detected with the gR99 clone but not with the gR38 clone [28]. Similarly, there are some AR IFN-γR2 defects with protein expression, and partial AD/AR defects show low but detectable IFN-γR2 in the membrane of monocytes [2,33,92]. The currently-available antibodies for the evaluation of IFN-γR2 expression are not optimal.

We estimate that cytometric evaluation of IFN-γR1 should allow the identification of approximately 80% of complete AR IFN-γR1 deficiencies; however, normal expression of IFN-γR does not exclude a deficiency. In such cases of expression of normal receptors but suspected MSMD, other techniques that evaluate cellular responses to IFN-γ, such as IL-12p70 production, STAT1 phosphorylation in response to increasing doses of IFN-γ or IFN-γ binding studies, should be used.

Flow cytometry staining for the usual number of samples (1–2 patients and a control) takes about 90 min of hands-on time[b]. The cost of antibodies is around 8€ per individual[a], but as for all techniques, it can vary depending on the laboratory provider and region.

IL-12Rβ1 expression—IL-12Rβ1 deficiency is the most common genetic form of MSMD [2]. IL-12Rβ1 expression detection with flow cytometry is performed in PBMCs after 72 h of stimulation with PHA [21] (Figure 2). As stated for IFN-γR, not all IL-12Rβ1 described defects have an absence of IL-12Rβ1 in the membrane of activated lymphocytes (Table 3) [21,101]. Only two mutations lead to a detectable but nonfunctional expression of IL-12Rβ1 protein in the membrane; one is a large deletion (700 + 362_1619-944del) in *IL12Rβ1* [102], and the other is caused by an N-terminal signal peptide stop-gain homozygous mutation [103]. Cytometric determination of IL-12Rβ1 expression is a powerful and easy-to-perform technique that allows the detection of more than 99% of the described mutations. In the absence of the protein in the membrane of activated lymphocytes, genetic studies of *IL12Rβ1* need to be performed, but its presence does not rule out a defect. In such cases, an evaluation of cellular responses to IL-12 is needed.

From receipt of the blood to the acquisition of results, this technique takes 4 days, with a hands-on time of approximately 2 h and 15 min (90 min on day 1 for the PBMCs isolation and stimulation, and approximately 45 min for the staining and acquisition in the cytometer on day 3[b]. The estimated antibody cost is around 10€ per individual[a].

IFN-γ binding studies—Because some defects in IFN-γR do not affect their membrane expression, IFN-γ binding studies can help to evaluate their functionality. These techniques

may be performed with radiolabeled ^{125}IFN-γ or by flow cytometry [30–32]. For flow cytometry, PBMCs are first incubated with hrIFN-γ for 30 min, and then washed and incubated for 20 min with an anti-IFN-γ antibody. If the anti-IFN-γ antibody is fluorescence-labeled, cells can be directly acquired with a flow cytometer [32]; otherwise, further steps are needed [30]. With this technique, membrane-expressing IFN-γR1 defects can be easily detected. IFN-γ-binding assays with flow cytometry do not yield consistent results with Epstein-Barr virus-transformed B cells (EBV-B cells), and when using PBMCs, gating on monocytes is required [30]. Some MSMD etiologies (AD IFN-γR1 deficiency) will escape this detection [31].

This technique, performed in PBMCs, includes four incubation steps. From receipt of the blood sample to acquisition of results in the cytometer, the technique can be performed in approximately 5 h for a patient sample, a health control and a negative control (medium), and the cost of consumables is approximately 34€[a]. However, this technique was specifically developed to analyze whether a particular mutation in the IFN-γR1 confers a partial recessive or a complete recessive deficiency, and increasing doses of IFN-γ (1–10,000 IU/mL) are required for this analysis; in such a case, the cost and the hours of work may increase to 120€[a] and 6–6.5 h[b], respectively. To our knowledge, only three patients and three healthy controls have been evaluated so far. Therefore, it is particularly difficult to provide sensitivity and reference ranges for this non-radioactive and flow cytometry-based technique. In addition, the antibodies used, and the model of the flow cytometer and its configuration may significantly affect the results. In our hands, mean fluorescence intensity (MFI, binding of the anti-IFN-γ antibody to monocytes) increases 4- to17-fold in cells incubated with as low as 1 IU IFN-γ/mL compared to cells incubated with medium alone. No or a very low MFI is observed in cells from patients with partial AR IFN-γR1 deficiency at the same concentrations of IFN-γ. At high IFN-γ concentrations, binding (MFI) is similar to or only slightly diminished in cells from patients with partial AR IFN-γR1 deficiency compared to cells from healthy controls.

Cytometric detection of phosphorylated STAT molecules

STAT proteins play a crucial role in cytokine signaling. They bind to activated extracellular receptors, and then phosphorylate, dimerize, and translocate to the nucleus to bind to specific DNA regions and activate gene transcription [14,15,104]. The two most relevant STAT molecules implicated in the IFN-γ circuit are STAT1, which is activated after IFN-γ/IFN-α stimulation, and STAT4, which is activated after IL-12p70 stimulation [105]. Flow cytometric determination of STAT1 phosphorylation can be performed in both whole blood and isolated PBMCs, while STAT4 phosphorylation in response to IL-12p70 needs to be performed in activated lymphocytes. First, cells are stimulated with different cytokine concentrations for 15–30 min. Then cells are fixed and permeabilized with special buffers that maintain the phosphorylation state of the cell, are stained with anti-phosphorylated STAT antibodies in conjunction with the extracellular antibodies of choice, and are acquired with a flow cytometer [34,36,102,106] (Figure 2). It should be stressed that when working with anti-STAT antibodies, proper negative controls are mandatory, and, if possible, the results should be corroborated by other techniques such as western blot, to avoid artifacts.

Of note, STAT4 phosphorylation evaluation is limited by the lack of a proper antibody to detect total STAT4.

STAT1 phosphorylation—STAT1 phosphorylation is a useful technique to test the response to IFN-γ, as *IFNGR* mutations may or may not lead to abolished receptor expression on the surface of monocytes [2,28,30,31,33,92]. Complete defects in *IFNGR1* and *IFNGR2* genes lead to abolished STAT1 phosphorylation in response to hrIFN-γ and normal phosphorylation in response to hrIFN-α, respectively [32,88,92,107], while partial defects lead to impaired, but not abolished, STAT1 phosphorylation in a dose-dependent manner, with normal responses at high doses [30,33,92,94,95,107–109]. When stimulating cells for STAT1 phosphorylation analysis, the IFN-γ dosage is a key factor to consider; a range from 10–10^5 IU/mL of hrIFN-γ or hrIFN-α is used, with 10^3 IU/mL and 10^5 IU/mL being the most common concentrations [30,31,34,35,62,93,96,107].

It is not only mutations affecting STAT1 phosphorylation that cause loss of function. Although Tyr701 phosphorylation is the first step for STAT1 function, mutations on other STAT1 domains implicated in later events can also impair its function. AD *STAT1* LOF mutations in the tail segment domain or SH2 domain (with the exception of the M654K mutation [106]) lead to impaired STAT1 phosphorylation in response to hrIFN-γ but not hrIFN-α [35,36,93]. In contrast, mutations in the DNA-binding domain can lead to both normal (E320Q and Q463H mutations [34]) and altered (E157K and G250E mutations [110]) STAT1 phosphorylation. Impaired or abolished phosphorylation to both hrIFN-γ and hrIFN-α suggests a STAT1 deficiency (which is considered a combined immunodeficiency if it is AR or an MSMD if it is AD), while normal phosphorylation does not exclude it. STAT1 phosphorylation after low-dose IFN-γ stimulation will detect almost all IFN-γR defects and approximately 70% of STAT1 defects. Furthermore, it has been recently reported that patients with AD *STAT1* gain of function mutations, who usually develop chronic mucocutaneous candidiasis, can also develop mycobacterial infectious disease [110].

STAT1 phosphorylation determination is a very informative technique that can be performed in approximately 4 h[b], depending on the number of tubes to be processed, with consumable costs of about 40€ per individual[a] tested.

STAT4 phosphorylation—STAT4 is an essential part of the downstream signaling cascade that occurs after IL-12 stimulation. After IL-12 binding to the IL-12 receptor, IL-12Rβ1 binds TYK2 and IL-12Rβ2 associates with JAK2, which initiates trans-phosphorylation of the receptors, creating docking sites for STAT4. At these sites, STAT4 is phosphorylated at tyrosine 693, dimerizes, and undergoes nuclear translocation where it binds to its target DNA sequences [12]. The STAT4 phosphorylation cytometric assay needs to be performed in stimulated PBMCs cultured with IL-2 and then stimulated with hrIL-12p70; the whole assay takes approximately 1 week [102,111]. Abolished STAT4 phosphorylation in response to rhIL-12p70 has been observed in both IL-12Rβ1-[101,102] and TYK2-deficient patients [27]. Of interest, STAT4 phosphorylation after IFN-α is normal in IL-12Rβ1-deficient patients [102] and impaired in TYK2 deficient patients [27]. However, STAT4 phosphorylation results must be interpreted with caution due to the lack of a proper STAT4 antibody to assess total STAT4 in the cell with flow cytometry. Bi-allelic

mutations in STAT4 have not been described to date. For this reason, STAT4 is meant to help only in the diagnosis of other forms of MSMD.

As explained above, a pre-stimulation step is needed for the detection of phosphorylated STAT4 in response to IL-12p70. For this reason, the technique takes 7 d, with a hands-on time of approximately 90 min on day 1 for PBMC isolation and pre-stimulation, 15 min on day 4 for change of medium, and approximately 4 h for stimulation, staining and acquisition in the flow cytometer[b], with a cost of approximately 30€ per individual[a].

Detection of anti-IFN-γ autoantibodies

Another form of MSMD-like susceptibility to mycobacteria is due to the presence of neutralizing anti-IFN-γ autoantibodies in the blood of affected patients. Although it is not strictly an MSMD-diagnosis technique, we have included it in this review because this condition is a phenocopy of MSMD and should be included in the differential diagnosis of MSMD, especially, but not exclusively, in adults of Asian descent. The most direct approach for detecting IFN-γ autoantibodies is by using an ELISA system and by observing IFN-γ level recovery after the addition of exogenous IFN-γ to patient serum. In both situations, the level of autoantibodies can be titrated by performing an ELISA against anti-IFN-γ-antibodies with different serum dilutions or by increasing the concentration of exogenous IFN-γ in the recovery strategy [78–80,112–114]. In addition, it has been shown recently that undetectable or very low IFN-γ production in the QuantiFERON-TB Gold In-tube assay (Quiagen, Hilden, Germany) is a warning sign for the presence of anti-IFN-γ antibodies[81]. If performed with ELISA, the cost[a] and the time[b] needed to perform the test may be similar to those required for the detection of baseline IFN-γ levels in plasma.

Particular considerations in some MSMD

Some genetic defects of MSMD present with characteristic immunological features. For example, in partial AD IRF8 deficiency, there is a loss of $CD11c^{+}CD1c^{+}$ blood myeloid dendritic cells [26]. MSMD patients with CYBB deficiencies present an abolished respiratory burst in monocyte-derived macrophages in response to purified protein derivative (PPD) or BCG, and in EBV-B cells. However, this oxidative burst defect cannot be detected in a routine dihydrorhodamine test, since monocytes, neutrophils, and monocyte-derived dendritic cells have normal responses [37,115]. Although these are not common tests for MSMD diagnosis, it is important to have these special features in mind in suggestive patients.

Interpretation of results

Cytokine production is the gold standard in the diagnostic pursuit of an inborn error of IFN-γ underlying MSMD. Complete defects often lead to abolished production of, or response to, IFN-γ (Tables 2 and 3). However, although it has not been possible to establish broad cut-off values, it may be possible to have in-house healthy-control range values. In the case of blood samples that have to be shipped, samples from a healthy control are required. As there is great variability in this control group, the lower 10^{th} percentile of the control cohort may define a weak response. Cytokine production data is robust for complete deficiencies but may show limitations in partial defects.

Cytometric determination of receptor presence can be a fast, easy tool to detect complete forms of IFN-γR1, IFN-γR2, and IL-12Rβ1 deficiencies, as their absence confirms the defect. However, the presence of these receptors does not exclude an underlying defect. Functionally, normal phosphorylation of STAT1 in response to IFN-γ rules out complete defects of *IFNGR1* and *IFNGR2*, and virtually all partial defects. Partial *IFNGR1* and *IFNGR2* may present STAT1-phosphorylation but only at high concentrations of hrIFN-γ. Abolished STAT1 phosphorylation in response to both hrIFN-γ and hrIFN-α is a sign of AR STAT1 defect. If phosphorylation is abolished only after hrIFN-γ stimulation, IFN-γR deficiency must be suspected; in contrast, if it is abolished or impaired after hrIFN-α stimulation, the *TYK2* gene may be studied. Abolished STAT4 phosphorylation after IL-12p70 stimulation suggests a defect in IL-12p70 receptor or in TYK2 (Figure 3). The presence or absence of IL-12Rβ1 and STAT1 phosphorylation after hrIFN-α will help to differentiate between these two defects.

Genetic approaches

For a full diagnosis and genetic counseling, genetic studies are needed. Sanger sequencing is a good option if functional tests have identified specific candidate genes. Otherwise, next generation sequencing (NGS) will be less time-consuming and may cost less [116]. For healthcare practice, gene panels with known genes are the option with the best cost-efficiency ratio. However, a great proportion of patients with clinical signs suggestive of MSMD do not show mutations in the known disease-causing genes [2]. In such cases, whole exome sequencing (WES) or whole genome sequencing (WGS) may be required. WGS may reveal mutations in non-coding regulatory regions that would be undetectable by WES, but WGS is more expensive and difficult to interpret than WES. It is important to emphasize that new mutations require further functional confirmation. Different strategies are proposed in order to study the deleterious effects of specific mutations [110]. Recommended guidelines for considering single-patient mutations to be disease-causing have been recently published [117]. An increasingly-used approach for the evaluation of PID (including MSMD) is to start with NGS either with a gene panel or with WES/WGS and then to perform functional tests to confirm the mutations found. Genetic filiation of patients (achievement of a genetic diagnosis) is of utmost importance, as it will condition treatment of the current or future infection and/or prophylaxis. For WES, the cost would be around 500€ but it depends on the coverage. However, analysis of WES studies requires specialized staff. Prices for genetic studies, especially for NGS, are changing rapidly with the development of new technologies and the expansion of their use.

Other useful tests in research

Intracellular detection of IFN-γ producing T-cell blasts can be measured after activation with rhIL-12 in T-cell blasts cultured with PHA; PMA/ionomycin can be used as a positive control for the assay. T-cell blasts are fixed and permeabilized for subsequent intracellular staining with anti-human monoclonal IFN-γ or isotype-matched negative control. Some patients with defects in IFN-γ production (such as IL-12Rβ1 deficiency) may present with normal or only slightly diminished values so that its diagnostic value is limited, particularly in the absence of the analysis of IFN-γ production with PHA or BCG in culture supernatants.

Because samples of primary cells from patients are not infinitely available, some tests have been adapted to the use of patient-derived cell lines. EBV-B cells, herpes virus saimiri-transformed T cells (T-saimiri cells), and immortalized SV-40 fibroblasts are the most common cell lines used [27,30,31,33,107,109,118]. Additional commonly-used cells and techniques are summarized in Table 4. This group of tests requires a laboratory with experience in the field of MSMD and is usually performed in a research laboratory.

Defects in IFN-γ response are caused by diverse genetic etiologies. Assessment of the effect of the different mutations in the response to IFN-γ is crucial to determine treatment and patient management; for this reason, techniques other than STAT1 phosphorylation may be needed. For example, expression of activation markers such as HLA-DR and CD64 after stimulation with different hrIFN-γ concentrations may be used to determine response to IFN-γ, both in primary cells and in transformed SV40-fibroblasts and EBV-B cells [45,97,107], as not all defects in STAT1 lead to altered phosphorylation in response to hrIFN-γ. To prove that a mutation in *STAT1* with normal phosphorylation is pathogenic, other tests showing defective response to IFN-γ are needed. The electrophoretic mobility shift assay (EMSA) is useful to detect forms of IFN-γR and AD STAT1 LOF deficiencies with the presence of phosphorylation, as it reveals STAT1 translocation and DNA binding. In addition, it can help to identify AD STAT1 gain of function deficiencies [35,93]. In the same line, it is possible to study induction of GAS in response to IFN-γ. Specifically, expression of CXCL9 and CXCL10, among others, or the activation of GAS elements by luciferase detection [30,34,62,93,106,110] can help to determine the effect of specific mutations. These approaches are usually used in research rather than in healthcare practice.

Other advanced research techniques are used for the characterization of new mutations in newly-discovered genes or mutations causing MSMD. These methods for the confirmation of a pathogenic effect of new mutations are beyond the scope of this review, and they have been carefully reviewed elsewhere [117]. Briefly, transfection of different cell lines with wild-type or mutated genes may be useful for the evaluation of a mutated allele in terms of protein expression and function. It is possible to perform expression assays, as disease-causing variations commonly have altered expression. Also, transfection of a wild-type copy of the mutated gene into patient cells (for example, in EBV-B or T-saimiri cells) that restores protein function can reveal a possible loss-of-function mutation [119]. Furthermore, new techniques such as CRISPR/Cas9 open up the possibility of reversing the mutation in patient cells or mutating control cells, especially in the event that no cells are available from the patient, to confirm that the phenotype that is observed in the patient is due to the mutation.

Discussion

Tuberculosis was thought by many to be a hereditary disease until the discovery of the characteristic bacterium by Koch in 1882 [120]. It was not until the middle of the 20th century that infections after BCG vaccination were understood to be related to inborn errors of immunity [16], and only in 1996 did Jouanguy [17] and Newport [18] et al. show for the first time that inheritable single monogenic defects in the IFN-γ circuit conferred susceptibility to mycobacterial infection rather than to a broad range of pathogens.

These findings boosted the concept of atypical PID in which monogenic defects confer selective susceptibility to specific pathogens [121,122]. Twenty years and ten disease-causing genes later, MSMD diagnosis is still a clinical challenge. In the present review, we provide an overview of the different assays available for the study of suspected defects in the IFN-γ circuit that can be performed in diagnostic and research laboratories. Table 5 summarizes their advantages and disadvantages.

Some issues in MSMD diagnosis need to be resolved. First, there is a need for awareness about MSMD, so that physicians taking care of children or adults can suspect this disorder. Knowledge of the specific warning signs is of utmost importance, as well as knowledge of other conditions that can lead to susceptibility to mycobacterial diseases and that must be included in the differential diagnosis: patients, especially children, with BCG-itis or BCG-osis, EM infections, or severe tuberculosis, alone or in combination with other intracellular infections, are to be suspected of having MSMD. Global frequency of MSMD has been estimated to be at least 1/50,000, although it was previously thought to be rare.

Second, there is a need to facilitate the diagnosis of MSMD, once suspected. Indeed, the detection of the genetic defect is necessary to offer the patient the best treatment options and genetic counseling, and therefore to decrease mortality. This is exemplified by complete deficiency of IFN-γR where the only curative treatment attempted is HSCT; most other forms of MSMD will benefit from prolonged antibiotics to which exogenous hrIFN-γ therapy can be added - even partial defects of IFN-γR respond to exogenous hrIFN-γ therapy [2,29,123]. When a member of a family is diagnosed with MSMD, BCG vaccination in family members should be avoided until a genetic defect has been ruled out. It is also important to consider that some MSMD etiologies have incomplete penetrance, meaning that not all the individuals presenting with the mutation will present the clinical phenotype [2,20,22]. For example, in IL-12Rβ1 deficiency, it is estimated that 21% of the individuals with MSMD genotype do not show the phenotype at 20 years [2,20]. Genetic counseling in these patients is thus challenging.

Functional tests for MSMD diagnosis are also challenging: in this review we have described a broad array of available tests; however, some of these techniques are limited by the timing and the requirement of qualified staff, making the full diagnosis of MSMD usually only possible in specialized immunology laboratories. Genetic approaches are gaining ground and could overcome these limitations. Nevertheless, genetic results usually need a functional confirmation of the identified mutation.

Conclusions

We describe the currently-available techniques to study patients with suspected MSMD defects in diagnostic and research laboratories. MSMD should be considered in patients with significant infection (severe, disseminated, or recurrent) after BCG vaccination and infection by mycobacteria, particularly EM, especially in combination with *Salmonella*, *Candida* or virus. When suspected, acquired causes of immunodeficiency, T cell defects and chronic granulomatous disease first need to be ruled out. Then, MSMD-specific evaluation should be started. The tests performed, and their order, may depend on laboratory facilities, technical

staff, and clinical orientation. Genetic studies may be performed after functional studies have suggested a specific defect or may be performed upfront and be followed by functional confirmation. Given the number of different genetic etiologies causing MSMD, NGS technologies may be especially suitable to help in the identification of new disease-causing genes, because almost 60% of patients with suspected MSMD today have no identified genetic cause.

Acknowledgments

Funding All phases of this work were supported by the Plan Nacional de I+D+I and co-financed by the ISCIII – Subdirección General de Evaluación y Fomento de la Investigación Sanitaria – and the Fondo Europeo de Desarrollo Regional (FEDER) under the grants PI12/01990 and PI15/01094 to LA and PI13/00676 to MJ, PI13/01456 and PI16/00759 to CRG. The Laboratory of Human Genetics of Infectious Diseases is supported by grants from the National Institute of Allergy and Infectious Diseases (under grants 5R01AI089970 and 5R37AI095983), the National Center for Research Resources and the National Center for Advancing Sciences of the National Institutes of Health (grant numbers 8UL1TR000043 for J.L.C.), The Rockefeller University, the St. Giles Foundation, Institut National de la Santé et de la Recherche Médicale (INSERM), Paris Descartes University, the Integrative Biology of Emerging Infectious Diseases Laboratory of Excellence (ANR-10-LABX-62-IBEID), and the French National Research Agency (ANR) under the “Investments for the Future” program (grant number ANR-10-IAHU-01), ANR-GENMSMD (for J.B.), SRC2017 (support of Clinical research, Fondation du Souffle et du Fonds de Recherche en Santé Respiratoire, for J.B.). RMB was supported by the European Molecular Biology Organization (EMBO). COQ was supported by ANR-14-CE15-006-01.

Abbreviations

AD	autosomal dominant
APC	antigen presenting-cell
AR	autosomal recessive
BCG	bacille Calmette-Guérin
EBV-B cells	Epstein-Barr virus-transformed B cells
ELISA	enzyme-linked immunosorbent assay
EM	environmental mycobacteria
EMSA	electrophoretic mobility shift assay
GAF	γ-activated factor
GAS	γ interferon-activated site
hrIFN-γ	human recombinant IFN-γ
HSCT	hematopoietic stem cell transplantation
IFN	interferon
IFN-γR	IFN-γ receptor
IL	interleukin
IRF	interferon regulatory factor

ISG	interferon-stimulated gene
IUIS	International Union of Immunology Societies
JAK2	Janus-associated kinase 2
LOF	loss of function
LPS	lipopolysaccharide
MFI	mean fluorescence intensity
MSMD	Mendelian susceptibility to mycobacterial disease
NEMO	nuclear factor-kappa B essential modulator
NGS	next generation sequencing
NK	natural killer
PBMCs	peripheral blood mononuclear cells
PHA	phytohemagglutinin
PID	primary immunodeficiency
PPD	purified protein derivative
RORC	RAR related orphan receptor C
ROS	reactive oxygen species
STAT	signal transducer and activator of transcription
TB	tuberculosis
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
T-saimiri cells	herpes virus saimiri-transformed T cells
TYK2	tyrosine kinase 2
WB	whole blood
WES	whole exome sequencing
WGS	whole genome sequencing
XR-EDA-ID	anhidrotic ectodermal dysplasia with immunodeficiency

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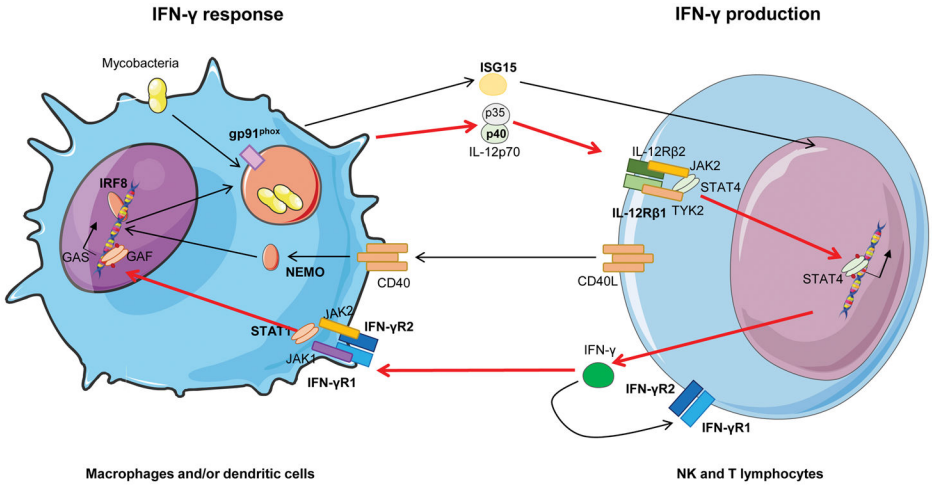

Figure 1. IFN-γ circuit

Summary of molecules implicated in the IFN-γ circuit. Molecules represented with bold characters are known to cause of MSMD. GAS: γ-interferon activated site; GAF: γ-activated factor.

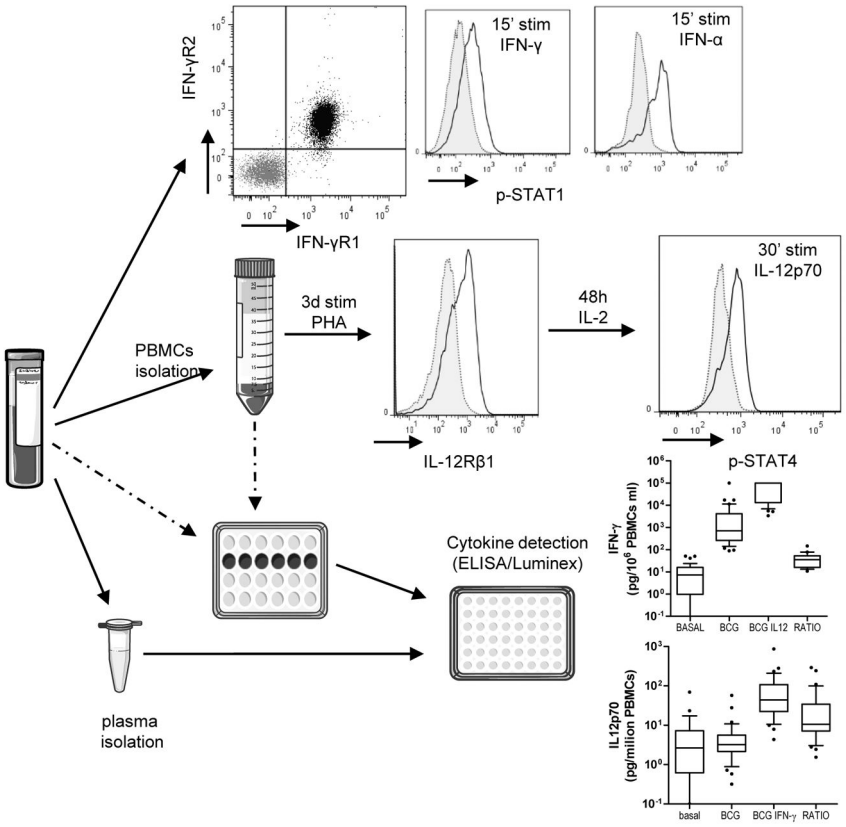

Figure 2. Diagram of the laboratory analysis of MSMD defects with examples

IFN-γR and STAT1 phosphorylation detection is performed in whole blood assay. IL-12Rβ2 detection is performed in PBMCs after 72 h stimulation with PHA. STAT4 phosphorylation detection is performed in PBMCs after 72 h stimulation with PHA and at least 48 h of culture in the presence of IL-2 or PHA + IL-2. An example of a healthy control is shown for each technique. Cytokine production is detected after 18 h of culture (for IL-12p70) and 48 h of culture (for IFN-γ and IL-12p70) in the gold standard procedure, BCG with or without IFN-γ or IL-12p70 co-stimulation. Control cohort is shown.

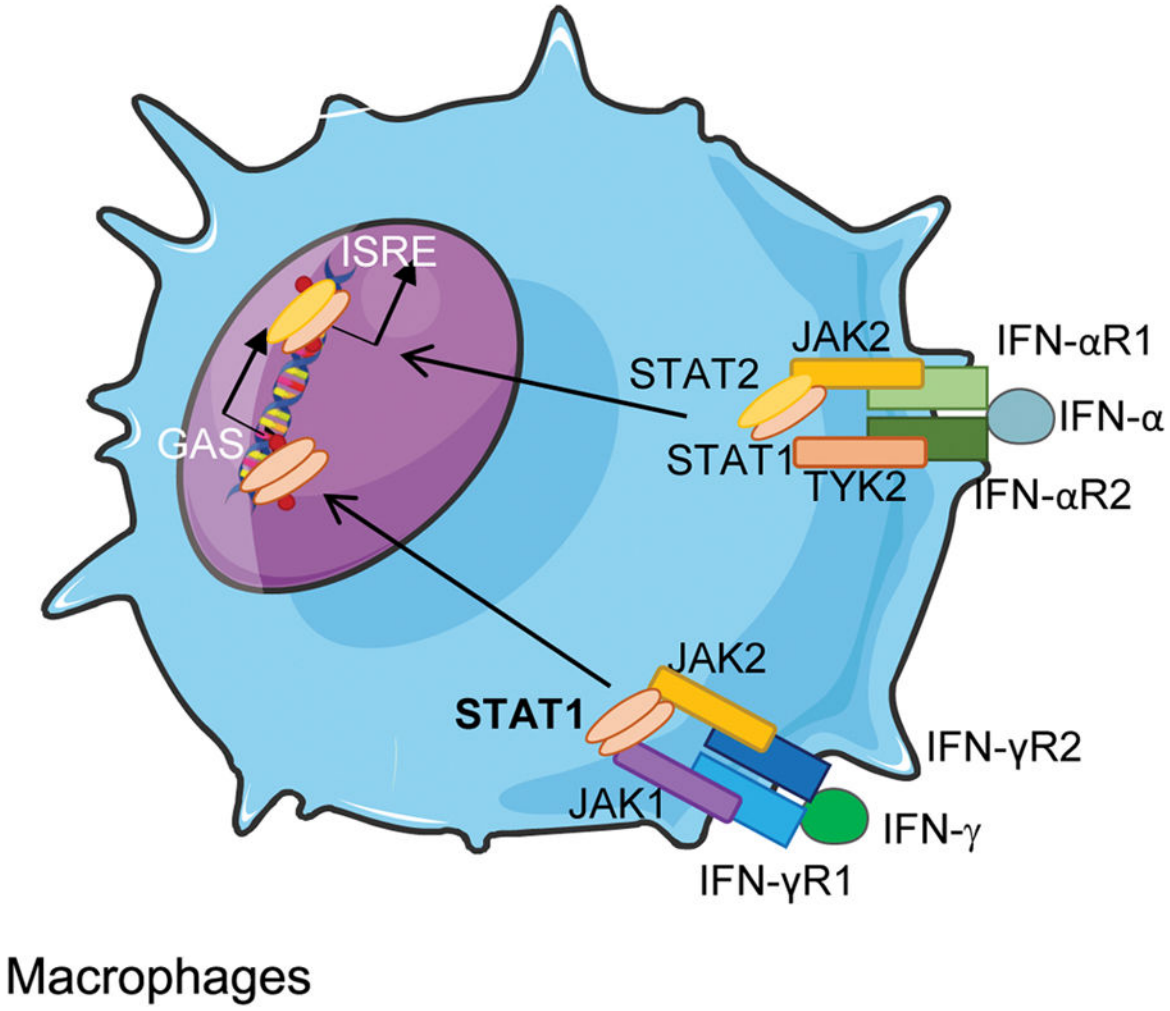

Figure 3. IFN-α and IFN-γ signaling

GAS: γ-interferon activated site; ISRE: interferon-sensitive response element.

Table 1

MSMD warning signs.

Sign	Description
Age at presentation	Usually in childhood, also in adolescence and adulthood
General state	Otherwise healthy individuals
Infectious spectrum	Invasive or recurrent infections by: Mycobacteria: BCG infection (*Mycobacterium bovis* vaccine strain) Environmental mycobacteria (*M. chelonae, M. fortuitum, M. mageritense, M. peregrinum, M. smegmatis, M. scrofulaceum*...) *Mycobacterium tuberculosis* Intramacrophagic bacteria (alone or in combination with mycobacteria): *Salmonella* spp. *Listeria monocytogenes*/*Nocardia* spp./*Klebsiella* spp. Fungi (in combination with mycobacteria) *Candida* spp. *Histoplasma capsulatum/Paracoccides brasilensis/coccicoides* spp. Parasites (alone or in combination with mycobacteria, rare): *Leishmania* spp. *Toxoplasma gondii* Virus (in combination with mycobacteria, rare) Cytomegalovirus, human herpes virus 8, parainfluenza virus type 3, respiratory syncytial virus and varicella zoster virus.
Other	Family history of invasive or recurrent mycobacterial infection Undetectable or very low IFN-γ production in Interferon-Gamma Release Assays (IGRAs) (i. e. QuantiFERON-TB Gold In-Tube)

Table 2

Diagnostic tests in IFN-γ response defects.

Gene	Inheritance	Defect complete/ partial (C/P)	Microbial stimulation (BCG)		Mitogen stimulation		Baseline IFN-γ	Cytometric determination			
			IFN-γ production	IL-12 production	IFN-γ production	IL-12 production		IFN-γ R1	IFN-γ R2	IFN-γ binding	STAT1-phosphorylation in response to IFN-γ
IFNGR1	AR	C	Normal	Does not increase with IFN-γ costimulation	Normal in response to PHA (WB, PBMCs)	Normal or reduced with LPS, no response to IFN-γ (WB, PBMCs)	Very high	Present	NP	NP	Abolished[1]
	AR	C	Normal	Does not increase with IFN-γ costimulation	Normal (WB); low in response to PHA, rescued with IL-12p70 (PBMCs)	No response to IFN-γ	Very high	Absent	NP	NP	Abolished
	AD	P	Normal	Increase with IFN-γ costimulation	Normal in response to CD2/ CD28 in T cells; low IFN-γ secretion in T cells after IL-12 stimulation	Impaired but not abolished response to LPS + IFN-γ (WB, isolated monocytes)	Normal	Increased	NP	NP	Impaired but not abolished [2]
	AR	P	Normal	Does only increase with high IFN-γ costimulation	NP	Low response to low/ medium doses of IFN-γ	High	Present	NP	Impaired, dosage dependent	Impaired but not abolished
IFNGR2	AR	C	Normal	Does not increase with IFN-γ costimulation	NP	NP	High	Present	Present	NP	Abolished
	AR	C	Normal	Does not increase with IFN-γ costimulation	Low after PHA stimulation; increased to normal levels with IL-12 (PBMCs)	NP	High	Present	Absent	NP	Abolished
	AR	P	Normal (WB)	Impaired increase with IFN-γ costimulation	NP	NP	Increased or not	Present (measured in PBMCs)	Low levels	NP	NP
	AD	P	NP	NP	NP	NP	NP	NP	NP	NP	Normal in monocytes, T cells and monocyte derived-macrophages
STAT1	AD	P	Normal increase with IL-12 costimulation	Impaired increase with IFN-γ costimulation	NP	NP	NP	NP	NP	NP	Normal
	AD	P	Normal increase with IL-12 costimulation	Impaired increase with IFN-γ costimulation	NP	NP	NP	NP	NP	NP	Impaired[3]

AR: autosomal recessive; AD: autosomal dominant; NP: not published; PBMC: peripheral mononuclear cells; WB: whole blood.

[1] Abolished response: no phosphorylation of STAT1 after IFN-γ stimulation

[2] Impaired response: impaired phosphorylation of STAT1 after IFN-γ stimulation; phosphorylation is present only after high-dose IFN-γ (>10^5 IU/ml)

[3] Abolished or impaired STAT1 phosphorylation in response to IFN-γ may be due to mutations in the coiled-coil domain and in the DNA-binding domain. Some patients have impaired STAT1-p and impaired binding to DNA.

Table 3

Diagnostic tests in IFN-γ production defects.

Gene	Inheritance	Defect complete / partial (C/P)	Microbial stimulation (BCG)		Mitogen stimulation		Cytometric determination					
			IFN-γ production	IL-12 production	IFN-γ production	IL-12 production	Baseline IFN-γ	IL-12Rγ 1 presence	STAT4 phosphorylation in response to IL-12p70	IFN-γ R1 presence	IFN-γ R2 presence	STAT1 phosphorylation in response to IFN-γ
IL12B	AR	C	Impaired but not abolished, increases after IL-12p70 costimulation (lower than controls) (WB); impaired and restored after IL-12p70 costimulation (PBMCs)	Abolished IL-12p40, severely impaired IL-12p70 in WB and in PBMCs	Severely impaired/ increases after IL-12p70 costimulation (PHA, PBMCs and WB)	Abolished (SAC stimulation, PBMCs), abolished (CD40L, PBMCs)	Not detectable	NP	NP	Normal	NP	NP
IL12Rβ 1	AR	C	Low, does not increase after IL-12p70 costimulation	Normal	NP	NP	Not detectable	Present (normal or impaired)	Abolished	Normal	Normal	NP
	AR	C	Low, does not increase after IL-12p70 costimulation	Normal	Abolished or severely impaired (PHA, PHA +IL-12; CD3+CD28; CD2+CD28, PBMCs)	NP	Not detectable	Abolished	Abolished	Normal	Normal	Normal in response to IFN-γ
IRF8	AD	p	Normal after BCG stimulation (also PPD, in PBMCs, WB)	Normal after BCG (WB)	Normal with PHA (WB)	1/3 production after R848 stimulation (PBMCs)	NP	NP	NP	NP	NP	NP
ISG15	AR	C	No IFN-γ in response to BCG, partially recovers with IL-12 costimulation (recovers with ISG15 addition, resembles IL-12p40 def)	Normal	NP	NP	NP	Normal	NP	NP	NP	NP
CYBB	XR	C	Normal	Normal	NP	NP	NP	NP	NP	NP	NP	NP
NEMO	XR	P	NP	Normal	Low levels after PHA/ anti-CD3	NP	NP	NP	NP	NP	NP	NP
TYK2	AR	P	Impaired IFN-γ production, but not abolished, does not recover with IL-12 costimulation	Normal	NP	NP	NP	NP[1]	NP[1]	NP[1]	NP[1]	NP[1]

AR: autosomal recessive; AD: autosomal dominant; XR: X-linked; NP: not published; PBMC: peripheral mononuclear cells; PHA: phytohemagglutinin, WB: whole blood

[1]Results tested in EBV-B cells are included in Table 4

Table 4

Other techniques described to diagnose or confirm different genetic entities.

Gene	Inheritance	EBV-B cells	Fibroblast (SV40)	Other tests
IFNGR1	AR complete	Abolished GAF DNA binding in response to IFN-γ (EMSA); Abolished IFN-γ binding to IFN-γ receptors (with radiolabeled IFN-γ)	Abolished HLA-II expression response to IFN-γ	No increase of TNF-α secretion after LPS + IFN-γ stimulation in comparison with LPS alone (PBMCs)
	AR complete	Abolished GAF DNA binding in response to IFN-γ; Abolished IFN-γ binding to IFN-γ receptors	Abolished HLA-II expression response to IFN-γ	Lack of CD64 upregulating capacity after IFN-γ stimulation (monocytes). Abolished STAT1 DNA binding in primary cells
	AD	Impaired but not abolished GAF DNA binding in response to IFN-γ, normal IFN-γ binding; Normal levels of *IFNGR1* mRNA, increased IFN-γR1 expression	Impaired HLA-II expression in response to IFN-γ at low doses, almost normal at high doses	Impaired but not abolished CD64 upregulation after low/medium IFN-γ stimulation (monocytes); Low STAT1 binding to DNA (PMN); STAT1p after IFN-γ stimulation can be detected. Low LPS+IFN-γ/LPS ratio of TNF-α secretion (PBMCs). Impaired but not abolished STAT1 migration to the nucleus in primary cells.
	AR partial	Impaired but not abolished GAF DNA binding in response to IFN-γ, impaired IFN-γ binding; STAT1 translocation after IFN-γ stimulation is impaired but not abolished	NP	CD64 upregulation in response to IFN-γ detectable but low; TNF-α secretion after LPS+IFN-γ impaired but not abolished. Impaired but not abolished STAT1 migration to the nucleus in primary cells.
IFNGR2	AR complete	Abolished GAF DNA binding in response to IFN-γ	NP	Abolished upregulation of HLA-DR after IFN-γ treatment
	AR complete	Abolished GAF DNA binding in response to IFN-γ, abolished STAT1p in response to IFN-γ	Abolished GAF DNA binding and no STAT1p in response to IFN-γ	Normal production of TNF-α after PHA/LPS stimulation, no increase with IFN-γ (PBMCs); Abolished upregulation of HLA-DR after IFN-γ stimulation
	AR	IFN-γ R2 detection, impaired but not abolished GAF DNA binding and GAF dependent genes (*CXCL9, CXCL10, IRF8*) induction in response to IFN-γ; impaired nuclear translocation. Normal IFN-γ R1 expression, normal affinity and number of binding sites for IFN-γ	No induction of HLA-II expression in response to IFN-γ; impaired but not abolished GAF DNA binding; IFN-γ R2 retained in the ER; normal response to IFN-γ after kifunensine treatment	Decreased STAT1p and *CD4, HLA, CXCL10* induction after IFN-γ stimulation in diverse cell types with overexpression of the mutants.
	AD partial	Impaired STAT1-p; impaired but not abolished GAF DNA binding and GAF dependent genes induction in response to IFN-γ	NP	NP
STAT1	AD partial	Normal/Impaired STAT1p, impaired GAS (but normal ISRE) and DNA binding in response to IFN-γ	Normal/impaired nuclear translocation	Impaired STAT1 DNA binding in primary cells
	AD partial	Impaired GAS in response to IFN-γ and IFN-α (normal ISRE in response to IFN-α) and impaired DNA binding.	Impaired nuclear translocation	Severely impaired TNF-α production after IFN-γ stimulation in isolated CD14+ monocytes or PBMCs. Impaired STAT1p in U3A cells in heterozygosis, abolished in homozygosis. Impaired STAT1 DNA binding in primary cells.
IL-12B	AR	No IL-12p40/p70 production after PDBu stimulation, normal TNF-α levels	NP	Lower than normal frequency of CD3+IL-17A+ cells ex vivo, but T cells responded to IL-23 by producing IL-17

Gene	Inheritance	EBV-B cells	Fibroblast (SV40)	Other tests
IL12Rβ1	AR complete	Present IL-12Rβ1 expression	NP	NP
	AR complete	Abolished IL-12Rβ1 expression	NP	IL-17-producing T cells *ex vivo*, T-cell blasts do not express IL-17 in response to IL-23. Normal IL-12Rβ2 expression. Reduced circulating memory Tfh and memory B cells, lower avidity of tetanus toxoid-specific serum antibodies
IRF8	AD partial	Normal IRF8 expression with low DNA binding in the IL-12B promoter;	NP	Loss of $CD11c^{+}CD1c^{+}$ blood myeloid DCs
ISG15	AR complete	Absence of ISG15, normal response to IFN-α	Absence of ISG15	Impaired but not abolished IFN-γ production by T and NK cells; Normal TNF-α production in response to BCG
CYBB	XR partial	Normal O^{-}, H2O2 production after PMA stimulation on neutrophils, monocytes	NP	Abolished respiratory burst in monocyte derived-macrophages in response to PPD, BCG, IFN-γ, normal in monocytes, neutrophils and monocyte derived-dendritic cells
NEMO	XR partial	Normal NEMO expression	Normal NEMO expression	Normal NEMO expression in monocytes and T cells. PHA stimulation in T cell/Monocytes co-culture system: low production IFN-γ, IL-12p40, IL-12p70, Lower IL10 production after TNF-α stimulation (WB);
TYK2	AR complete	Normal STAT1p and GAS in response to IFN-γ; diminished cell surface expression of IL-10R2, and IL-12Rβ1, normal IFN-γR1 & IFN-γR2. Abolished IL-23 response (STAT3-p, IFN-γ production), abolished pSTAT3, impaired production of ISG15 in response to IFN-γ; impaired pSTAT3 and EMSA after IL-10 stimulation, normal pSTAT3 and EMSA after IL-6/IL-21/IL-27 stimulation.	Impaired pSTAT1 and EMSA (GAS and ISRE) in response to IFN-γ; impaired STAT3p after IL-10 stimulation, normal STAT3p after IL-6 stimulation	Impaired response to IL-10 after LPS and TNF-α stimulation of PBMCs; normal IL17+ cells in PBMCs after PMA/Iono stimulation; impaired IL17A and F production in naive T cells. In HVS transformed T cells: Abolished STAT4 phosphorylation, GAS and IFN-γ production in response to IL-12p70, abolished pSTAT1 in response to IFN-α

AR: autosomal recessive; AD: autosomal dominant; XR: X-linked; NP: not published; GAF: γ activated factor, GAS: γ interferon-activated site; ISRE: interferon-sensitive response element; PDBu: phorbol 12,13-dibutyrate; STAT1p: STAT1 phosphorylation.

Table 5

Strengths and limitations of the different techniques for MSMD diagnosis.

Procedure	Test	Strengths	Limitations	MSMD defect detected (published)
Culture and cytokine determination	Basal IFN-γ in plasma	Easy and cheap to perform, when established, high levels of IFN-γ are indicative of IFN-γ receptor defect	Differences between kits, normalization of IFN-γ levels after acute phase of the infection	AR IFN-γ receptors defects
	WB culture with BCG stimulation	Most similar condition to reality	Partial defects can be occult, some MSMD defects have normal results; big variability in healthy controls, use of BCG difficult a lot ISO regulations acceptance Needs to be performed in fresh blood (max. 48h after extraction)	AR IFN-γ receptors, IL-12Rβ1, IL-12p40, ISG15/TYK2 defects
	PBMCs culture with BCG stimulation	Can be performed in cryopreserved cells	Partial defects can be occult, some MSMD defects have normal results; big variability in healthy controls, use of BCG difficult a lot ISO regulations acceptance	
	WB culture with mitogen stimulation	NO use of BCG	Mycobacterial-specific immunity is not tested Needs to be performed in fresh blood (max. 48h after extraction)	AR IFN-γR1, IL-12Rβ1, IL-12p40 defects NEMO deficiency performed in PBMCs
	PBMCs culture with mitogen stimulation	NO use of BCG. Can be performed in cryopreserved cells	Mycobacterial-specific immunity is not tested	
Cytometry (in primary cells)	IFN-γ binding	Useful to detect defects in IFN-γR1 when it is expressed in the membrane	There are some defects that bind IFN-γ but have no functional IFN-γR1	IFN-γR1 defects
	IFN-γR1 determination	Easy to perform, rapid diagnosis of some forms of IFN-γR1deficiency.	Some forms of IFN-γR1 deficiency present a non-functional form of IFN-γR1 in the membrane, so presence of the receptor does not exclude the defect.	IFN-γR1 defects
	IFN-γR2 determination	Easy to perform, rapid diagnosis of some forms of *IFNGR2* deficiency	Some forms of *IFNGR2* present a non-functional form of IFN-γR2 in the membrane, so presence of the receptor does not exclude the defect.	IFN-γR2 defects
	STAT1 phosphorylation determination	Rapid test that evaluates the function of both IFN-γR1/IFN-γR2 and can detect some forms of STAT1 deficiency	Partial forms of IFN-γ R1/2 defects can be occult if only high levels of IFN-γ are used, some STAT1 defects present normal STAT1 phosphorylation after IFN-γ stimulation	IFN-γR1/2 defects, STAT1, TYK2 (help)
	IL-12Rβ1 determination	Rapid/easy test; all but one mutation in *IL12Rβ1* present a lack of IL-12Rβ1 in the membrane.	There is a form of IL-12Rβ1 defect that present IL-12Rβ1 in the membrane of act lymphocytes, so it's present does not exclude the defect	IL-12Rβ1 defects
	STAT4 determination	Evaluates both IL-12Rβ1 and Tyk2 function.	Long technique; no STAT4 antibody to detect total STAT4.	IL-12Rβ1, TYK2 defects

Procedure	Test	Strengths	Limitations	MSMD defect detected (published)
Genetics	Sanger	When the number of candidate genes is small, it can be easy, rapid and cheap. Easy to analyze and interpret	Slow and expensive if there is not a clear orientation of the defect. Not useful for the discovery of new genes.	All known defects
	Next generation sequencing	Indicated when there is no clear candidate gene, study of all known defects at once	Expensive, difficult to analyze and interpret results due to the huge amount of generated data. Especially with whole genome sequencing, gene panels are more affordable and easy to interpret.	All known defects, discovery of new genes or new manifestations of known genes
		Useful for new genes discovery; detection of mutations in non-coding/regulatory regions with whole genome sequencing (not whole exome sequencing)		

PBMC: peripheral mononuclear cells; WB: whole blood.

Crit Rev Clin Lab Sci. Author manuscript; available in PMC 2019 May 01.