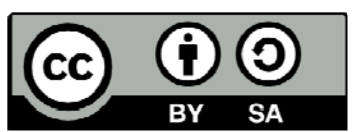
Primary and secondary immunodeficiencies of the IL-12/IFN-γ axis

Ana Esteve-Solé



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"Primary and secondary immunodeficiencies of the IL-12/IFN-γ axis"

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Yaya, avi, abuelo, abuela: gracias.

Failure is a very important part of success,
if you don’t fail, it means you are not risking anything
or you are repeating yourself
Marina Abramovic

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Abbreviations

Term	Abbreviation
Acquired immune deficiency syndrome	AIDS
Antigen-presenting cells	APC
Autosomal dominant	AD
Autosomal recessive	AR
B regulatory cells	Breg
Bacillus-Calmette Guérin	BCG
Chronic granulomatous disease	CGD
Common variable immunodeficiency	CVID
Constant fraction	Fc
Cord blood of healthy neonates	hUCB
Co-stimulation ratio	Co-SR
Crohn's disease	CD
Death domains	DD
Dendritic cells	DC
Environmental mycobacteria	EM
Epstein-Barr virus	EBV
Extensive-drug resistant	XDR
Food and Drug Administration	FDA
Gamma interferon-activated site	GAS
Gamma-activated factor	GAF
Granzyme B	GZMB
Hematopoietic stem cell transplantation	HSCT
Human immunodeficiency virus	HIV
Human recombinant IFN-γ	hr-IFN-γ
IFN-γ receptor	IFN-γR
Immunoglobulin	Ig
Indoleamine 2,3-dioxygenase	IDO
Inducible nitrogen oxide synthase	iNOS
Inflammatory bowel disease	IBD
Interferon	IFN
Interferon regulatory factor 8	IRF8
Interferon-stimulated genes	ISG
Interleukin	IL
International Union of Immunological Societies	IUIS
Janus associated kinase	JAK
Lipopolysaccharide	LPS
Loss of function	LOF
Mendelian susceptibility to mycobacterial disease	MSMD
Monoclonal antibody	mAb
Multi-drug resistant	MDR
Mycobacterium tuberculosis	Mtb
Next generation sequencing	NGS
Nitric oxygen	NO
Natural killer	NK
Non-related healthy controls	NR-HC
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Nuclear factor-kappa B essential modulator	NEMO
Peripheral blood mononuclear cells	PBMCs
Peripheral blood of healthy adults	hAPB
Phytohemagglutinin	PHA
Primary immune deficiencies	PID
Purified protein derivative	PPD
RAR related orphan receptor C	RORC
Reactive oxygen intermediates	ROI
Related healthy controls	R-HC
Rifampicin resistant	RR
Severe Combined immunodeficiency	SCID
Signal Transducer Activator of Transcription	STAT
T helper cells	Th
T regulatory cells	Treg
TNF Receptor	TNFR
TNF superfamily	TNFSF
Toll-like receptors	TLR
Tuberculosis	TB
Tumor growth factor-β	TGF-β
Tumor necrosis factor	TNF
Tyrosine kinase 2	TYK2
Ulcerative colitis	UC
Visceral leishmaniasis	VL
Whole exome sequencing	WES
Whole genome sequencing	WGS
World Health Organization	WHO

INTRODUCTION

1. Immunodeficiencies

The immune system is a tightly controlled and extremely complex system with the ability to distinguish between dangerous and non-dangerous elements, self and non-self, in order to mount effector responses. Its main effector responses are against pathogens, but the immune system is also a principal actor in a myriad of different processes in health and disease, including cancer, pregnancy establishment and tissue healing, amongst others. There are two major components of the immune system that are closely inter-related: the innate branch and the adaptive branch. The innate immune system is a non-specific defense system: by recognizing danger signals through Toll-like receptors (TLR), amongst other receptors, it produces a powerful inflammatory response that prevents or limits the development of infections by virtually all potentially pathogenic microorganisms. The epithelium, neutrophils, macrophages, monocytes and dendritic cells (DC) are the major actors of the innate immune system. Depending on the species, the innate immune system may be the only system to deal with infections, such as in invertebrates; however, in vertebrates, the innate immune system is the first step. Following this first response, it will enhance antigen-specific responses characteristic of the adaptive immune system, both by attracting cells of the adaptive immune system (mainly lymphocytes) and by presenting them the processed antigens to induce their activation. Adaptive responses are effector responses of antibody secreting B cells and cytotoxic or helper T cells, which will then contribute to the generation of memory, that will allow a rapid secondary immune adaptive response when the same antigen is reencountered [1,2].

Defects in the function of the immune system, congenital or acquired, lead to disease: inborn or genetic errors of the immune system can induce immunodeficiency. In the other hand, secondary/acquired defects of the immune can also lead to immunodeficiency, including treatments such as cancer chemotherapy or biological drugs targeting the immune system and pathogens such as human immunodeficiency virus. Clinical spectrum of immunodeficient individuals is broad since it depends on the nature of the immunodeficiency, from susceptibility to single pathogens to susceptibility to a wide range of microorganisms, with or without associated malignancy and immune dysregulation.

1.1.Primary immunodeficiency

Primary immunodeficiencies (PID) are caused by inborn errors of the immune system generating susceptibility to infections, autoimmunity and malignancies. Most of PID are caused by monogenic defects with classical Mendelian inheritance: autosomal recessive (AR), dominant (AD) or X-linked. Recently, with the use of next generation sequencing technologies (NGS), multi-gene or somatic causes of PID are being increasingly described [3]. Study of PIDs is an expanding field: new PID-causing genes or new phenotypes of known genes are constantly being reported (**Figure 1**). Currently there are 357 known genes described to be causative of PID (collected by the International Union of Immunological Societies (IUIS) in its biannual classification of PIDs)[4].

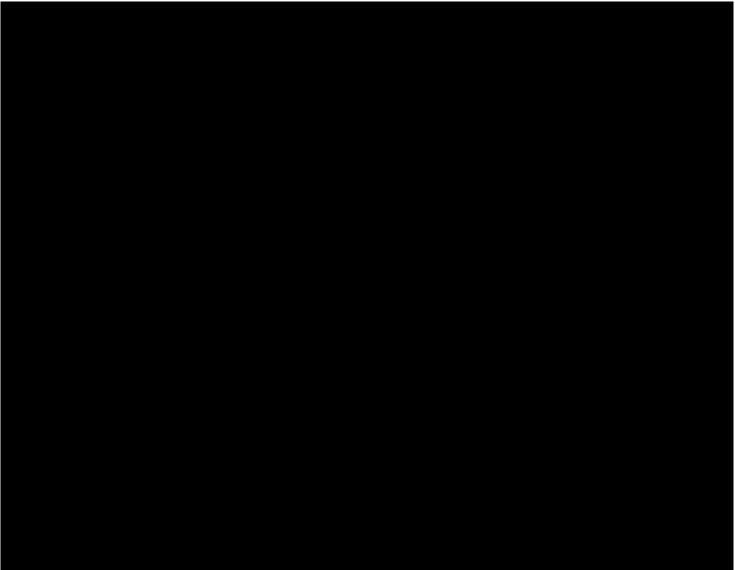

Figure 1 Number of inborn errors of immunity described by the International Union of Immune Societies since 1979. Adapted from Picard C et al, JCI, 2018 [4]

Although individually rare, incidence of PID as a group is thought to range between 1:2,000 and 1:10,000 live births [5]. In countries with high consanguinity rates (such as the Middle-East where approximately a 65% of unions are consanguineous), the incidence of AR PIDs is obviously higher [6]. PID are chronic multiorgan disorders with a high burden of disease, that can be minimized if specific treatment is started early in the course of the disease, such as antibiotic prophylaxis, immunoglobulin replacement therapy, hematopoietic stem cell transplantation (HSCT) or gene therapy. Early diagnosis and early treatment have, therefore, a huge impact on patient's prognosis.

The concept of a PID has dramatically changed over the last years. Classically, PIDs were characterized by chronic or recurrent life-threatening infections starting during childhood, with a susceptibility to a broad range of infections. Severe Combined Immunodeficiency (SCID), Chronic Granulomatous Disease (CGD) or Common Variable Immunodeficiency (CVID) are hallmarks of classical or "typical" PIDs. However, recent investigations in the last 10 years have described a new type of PID, named "atypical" PID. This atypical PID can confer susceptibility to a narrow range of pathogens (even just one) or manifest with autoimmune events without infections, starting even in adulthood. Prognosis of "typical" PID patients is usually poor, as patients worsen with age without treatment; instead, some atypical PID etiologies improve with age, implying that the adaptive immunity can "learn" to overcome the infections. Also, typical or conventional PID usually have an inherited recessive familial phenotype with complete penetrance, while new PID are often AD, can be spontaneous, have incomplete penetrance or can be originated from somatic mutations **(Table 1)** [7].

Table 1. Clinical differences between classical and atypical primary immunodeficiencies (PID)

	Classical PID	**“Atypical” PID**
Age of onset	Infancy - childhood	Infancy – childhood to young adults
Clinical phenotype	Recurrent infections Wide range of pathogens Failure to thrive	Low number of infections Low number of pathogens Otherwise healthy children
Prognosis	High mortality Worsens with age	High mortality Improves after adolescence
Genetics	Monogenic (AD, AR, XL)	Monogenic (AD, AR, XL) Somatic mutations Incomplete penetrance
Examples	Severe Combined immunodeficiency Congenital Neutropenia Chronic granulomatous disease	Complement defects Toll-like Receptors defects IFN-γ and IFN-α defects

The discovery of atypical PIDs has greatly contributed to our understanding of the function of the immune system. Single gene mutations conferring susceptibility to individual pathogens in otherwise healthy patients are considered by some to be “experiments of nature”: their study has helped understand how the immune system battles with different types of pathogens, though the identification of specific and non-redundant pathways responsible for the host-immunity to particular pathogens [8,9]: some examples are the interleukin (IL)-12/interferon (IFN)-γ pathway for anti-mycobacterial immunity or TLR3 pathway in central nervous system immunity against herpesvirus [10]. The discovery of this type of genetic infectious susceptibility gave support to the development of the genetic theory of infectious disease.

1.1.1.PID classification

The immune system is a broad and highly specialized network. Defects in different genes or gene-pathways give rise to different clinical manifestations. To try to simplify the broad and diverse forms of PID; they have been categorized based on the function of the immune system most represented. Given the continuous discovery of new etiologies, the IUIS revises the PID classification every 2 years since 1970; the last update from August 30, 2017 [4] includes 357 genes classified in 9 categories:

1. Immunodeficiencies affecting cellular and humoral immunity,
2. Combined immunodeficiencies with associated or syndromic features
3. Predominantly antibody deficiencies
4. Diseases of immune dysregulation
5. Congenital defects of phagocyte number or function
6. Defects in intrinsic and innate immunity
7. Autoinflammatory disorders
8. Complement deficiencies
9. Phenocopies of inborn errors of immunity

PID phenocopies are a new category of disorders, which are caused by two main mechanisms: (a) somatic mutations (mutations only present in specific cell types as opposed to germline mutations) and (b) auto-antibodies (majorly against cytokines) mimicking primary immune deficiencies. One example of this last group is the adult onset immunodeficiency mimicking Mendelian Susceptibility to Mycobacterial Disease (MSMD; OMIM 209950) caused by the presence of anti-IFN-γ autoantibodies[4,11].

In any case, PIDs are still underdiagnosed diseases: although awareness of PID among clinicians is increasing, there is still a proportion of patients which are not being diagnosed, mostly because of the lack of awareness in some geographic areas and in some medical specialties, but also because of the technical and economic difficulties to perform genetic and functional tests to diagnose a PID in a given patient. Also, the rapid advance of the discovery of new phenotypes is broadening the spectrum of PID. Therefore, training of clinicians and laboratory personnel is of outmost importance to better diagnose PID patients and, therefore, to decrease morbi-mortality of the disease.

1.2.Secondary immunodeficiency

Defects in the function of the immune system causing immunodeficiency can be secondary or acquired, reversible or not. Although the most known form of acquired immunodeficiency is the Acquired Immune Deficiency Syndrome (AIDS), caused by a non-controlled Human Immunodeficiency Virus (HIV) infection, severe malnutrition is currently the most prevalent form of secondary immunodeficiency in the world. Secondary immunodeficiencies occur by many different causes:

1. Severe malnutrition: caused by lack of protein intake or extreme protein loss [12]. Other forms of malnutrition such as severe micronutrient deficiencies can also lead to some form of immunodeficiency [13].
2. Viral infections: HIV infection, if untreated, leads to a state of immunodeficiency in which opportunistic infections and malignancy can be lethal. Besides, other infections such as measles virus, cytomegalovirus or influenza virus may induce transitory states of immunodeficiency [12].
3. Malignancies: both by the disease itself (i.e. chronic lymphocytic leukemia and multiple myeloma) and/or because of treatment's secondary effects [12,14].
4. Iatrogenic: Immunosuppressive or immunomodulatory drug treatment, including biologic drugs targeting immune-system molecules and splenectomy (patients whose spleen has been removed are more susceptible to *Streptococcus pneumoniae* and have higher probability of developing sepsis).
5. Age: prematurely born neonates and old age people have altered immune responses leading to infection susceptibility.
6. Metabolic diseases: such as diabetes mellitus in which phagocytosis, chemotaxis and proliferation to mitogens may be impaired.
7. Defects of the epithelial barrier: by trauma, surgery or burn.
8. Environmental causes such as ultraviolet light, ionizing radiation, high altitude, chronic hypoxia and space flights [12].

1.2.1. Secondary immunodeficiency caused by biologic drugs targeting immune-system molecules

The use of therapeutic antibodies targeting immune system molecules has dramatically changed the treatment of different diseases such as inflammatory bowel disease (IBD), rheumatic disease, cancer (both hematological and solid tumors), allergic and infectious diseases. Antibodies are secreted by B cells and are antigen-specific; they have a constant region (Fc) that defines the isotype, and a variable region that confers its specificity. The first therapeutic use of antibodies was approximately in 1890, with the use of serum from immunized animals for the treatment of some infections [15,16]. However, after the discovery of antibiotics by Fleming in 1928, being a cheaper and safer treatment, this practice was abandoned [15]. Afterwards, since 1976, when the first monoclonal antibody (mAb) was synthetized [17], the possibility of producing specific antibodies for therapeutic purposes was launched [18]. After this initial discovery, the use of murine mAbs for human therapy had disappointing results due to human anti-murine antibodies responses. However, the development of chimeric (constant fraction being from human origin and variant regions being murine sequences), humanized (all but the complementarity determining region being from human origin) and totally human antibodies overcame this limitation and paved the way for their safe and effective use in humans [19–21]. Although the first murine mAb for treatment was the anti-CD3 muronomab (OKT3) for transplant rejection therapy [22], it was not until 1997 when the first mAb for treatment of malignancy (anti-CD20 rituximab) was approved by the Food and Drug Administration (FDA) [23]. Nowadays, more than 60 antibodies have been approved for their use in humans [18,24].

Recently, it has been hypothesized that biologic drugs targeting immune system molecules can lead to phenotypes resembling PID, depending on the inhibited or modulated pathway by the mAb [25,26]. This theory is supported by the identification of PID phenotypes caused by naturally occurring auto-antibodies in the context of an autoimmune disorder: for example, anti-IL-17 auto-antibodies presence mimics chronic mucocutaneous candidiasis caused by congenital defects of IL-17A, anti-IFN-γ autoantibodies generates the same phenotype observed in patients with mutations in IFN-γ receptors, anti-IL-6 resembles Signal Transducer Activator of Transcription 3 (STAT3) deficiency and anti-granulocyte and monocyte colony stimulating factor causes pulmonary alveolar proteinosis as *CSF2RA* deficiency [27–29]. These forms of PID are named PID phenocopies, as mentioned in chapter 1.1.1.

Although the observed phenotype after mAb therapy can resemble its corresponding PID, there are factors to be considered. PID defects are present in the individual since birth (even before), whereas treatment with mAbs is usually used in adults, whose immune system is completely developed. Besides, germline mutations leading to immunodeficiencies are present in all cell types and the effect of a certain mAb can be restricted to some cell types or tissues. For these reasons, clinical outcome in mAb therapy is usually a milder version of the phenotype observed in PID. However, there are some confounding factors including disease stage, immune status, duration of treatment, concomitant medication, age, ethnicity and environmental exposure to infectious agents, amongst others, that modify the phenotype and can lead to more severe phenotypes that the corresponding PID [25,26].

Currently, there are biologic treatments targeting both molecules of the adaptive and innate immune system, mainly for the treatment of autoimmune or inflammatory diseases, cancer and during transplantation [26]. The use of anti-CD20 rituximab to deplete B cells for a prolonged

time (B cell lymphoma, autoimmune disorders, graft rejection), with small intervals or at high doses, can produce hypogammaglobulinemia, resembling primary antibody deficiencies [30,31]. Antibodies targeting molecules of the IL-12/23 – IFN-γ pathway, which are being recently used, including antibodies against IL-12p40, CD40, IFN-γ [26] and Janus associated kinase (JAK) inhibitors [32], can produce an increased susceptibility to mycobacterial and viral infections [32–35]. Secondary immunodeficiencies related to the use of anti-Tumor Necrosis Factor (TNF)-α antibodies and their relationship with mycobacterial infections will be extensively developed in the 4th section of the introduction.

2. Primary immunodeficiencies as a paradigm of the genetic infectious disease theory

Since infection has been, and already is, the principal cause of death in the world, the understanding of *how* and *why* have been major goals. There are four major theories trying to explain the basis for the development of infectious diseases: the genetic and immunological theories of infectious disease, which rely on host factors, and the ecological and microbiological theories, which rely on environmental factors. These theories are complementary and overlapping, and have been developing in parallel with the scientific knowledge advance [36].

Microbiological or germ theory arose in the late XIXth century after the works performed mainly by Pasteur and Koch. It stated that infectious diseases are caused by microorganism's infection, and that these microorganisms are as well sufficient and necessary for the development of the disease. Pasteur's [37] and other's observations [38,39] were not sufficient for the general acceptance of this theory [40–43]. It was not until the observation of tuberculous bacilli made by Koch [44], that germ theory was accepted, giving rise to the Koch-Henle postulates: "(1) The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease. (2) It [the microorganism] occurs in no other disease as a fortuitous and nonpathogenic parasite. (3) After being fully isolated from the body and repeatedly grown in pure culture, it can induce the disease anew" [45]. However, the microbiological theory encountered problems with the variability observed in infectious disease [36]. (**Figure 2**)

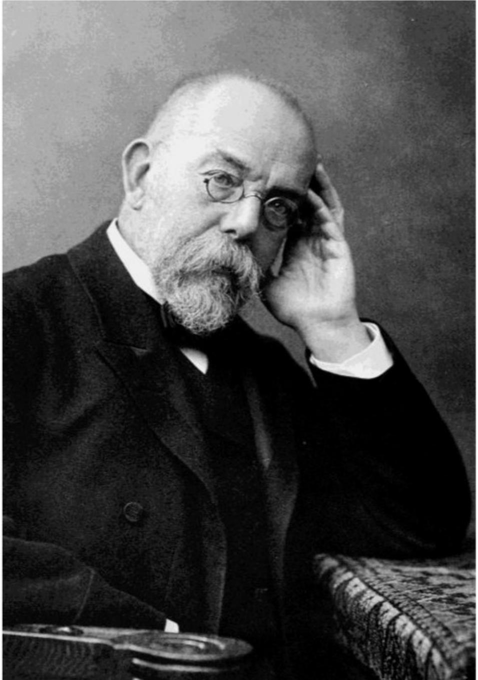

Figure 2. Studio portrait of Louis Pasteur (1822-1895) by Félix Nadar (before 1895), left panel; and photo engraving of Robert Koch (1843-1910) by Wilhelm Fechner (1900), right panel. Under creative common licence

Ecological theory of infectious disease was strongly supported and developed by Dr. Dubos during the 1950's. It questioned the statement of germ theory defending that pathogens were not sufficient for the establishment of infectious disease; it proposed that "pathogens are the agent of infection but the instigator of the disease is an unrelated disturbance of the host" and "whether the man lives in equilibrium with microbes or became the victim depends upon the circumstances which he encounters them" [46]. Those "circumstances" include climatological variants (cold, humidity, …[47]), metabolic state (malnutrition [48], uncontrolled diabetes [49],…) or co-infections with other pathogens [50]. The ecological theory of infectious disease does not invalidate the other theories but encompass them by considering that extrinsic factors modulate the relation between the pathogen and the host.

The immunological theory appeared trying to explain the differences between individuals upon an infection with microorganisms. It started with Pasteur being again a ground-breaker with the use of attenuated microbes for the protection from severe infections [51,52]. With the use of vaccines and serotherapy [15,16], the common thought was that variability in the response to normally lethal infections was due to individuals' attenuated, in terms of quantity or virulence, previous exposure [36]. However, it was after the discovery of the antigen specificity of antibodies by Paul Ehlrich [53,54] that this theory gained significance. With this fact, the experience gained by the adaptive immune system seemed to determine the host response to pathogens. This theory explained the variability observed after infection reactivation but failed to explain the great variability after primary infections. The discovery of inapparent infections, by Charles Nicolle, in which pathogens were present in asymptomatic individuals that were able to transmit the disease [55] was not easily explained by the immunological theory of infections.

After these theories and their limitations, genetic theory appeared, stating that the host's genetic background determined the outcome of a microorganism infection, either at population [36,56] or clinical levels [36,57]. Further studies gave support to these theories including, i) the differential mice susceptibility to infections depending on the studied strain [58–63], ii) tween studies in tuberculosis [64], iii) the observation of the protection to malaria infection by the presence of a relatively common allele causing sickle cell trait at a population level (showing genetic control of a disease-course) [65,66] and the description for the first time of a primary immunodeficiency, Bruton's disease [67]. Initially the two currents of genetic theory (population- and patient-based) were growing separately.

2.1. The unified genetic theory of infectious disease

Until middle nineties, despite the convincement that genetic background played a major role in infection outcomes, the division between the population- and clinical-based approaches continued to exist. Population-level studies showed association between a common background including many genes to one single infection while PIDs were the example of the opposite (i.e. one defective gene conferring susceptibility to many infections, named "one gene, multiple infections"). It was not until the description of mutations conferring susceptibility to a unique type of pathogens ("one gene, one infection"), named "atypical" primary immunodeficiencies (described in the first section), then the two currents of genetic theory unified. The first entities in which single gene mutations were found to confer susceptibility to one type of pathogen were infections by *Neisseria* in complement deficient patients [68], patients with mutations in *SH2D1A*

causing X-linked lymphoproliferation after Epstein-Barr virus (EBV) infection [69,70] and the first genetic descriptions of MSMD by mutations in *IFNGR1* [71,72].

After these discoveries, the unified theory of genetic diseases was finally established. It stated that "**life-threatening childhood infections are due to single-gene inborn errors of immunity in the course of primary infections**, […] symptomatic reactivation and secondary infections in young adults may result from the impact of a major locus, whereas in older adults the cause may be more polygenic" [36], first proposed by Jean-Laurent Casanova and Laurent Abel in 2007 [73] and nicely reviewed afterwards [7,36,73,74].

The unified genetic theory of infectious disease is "a reconciliation" with the old thoughts of hereditable infections that were discredited after the advent of the germ theory, trying to explain why not all individuals respond similarly to infections. Of note, until the discovery of Koch bacilli in 1882, tuberculosis (TB) was thought to be a hereditary disease [64,75]; more than a century after it has been shown that genetic background is of outmost importance for clinical development of tuberculosis [76–79] and, what is more, that in some cases severe infections by *Mycobacterium tuberculosis* (*Mtb*), especially during childhood, are due to PID caused by single-gene defects in T cell function, oxidative burst [78,80] or IFN-γ responses [81–95]. **In conclusion, since 2010, the genetic theory of infectious disease states that any more than usual severe primoinfection in pediatric patients might underlie an inborn error of immunity or PID.**

2.2. Mendelian susceptibility to mycobacterial disease

Some years after the initiation of broad vaccination with Bacille-Calmette Guerin (BCG), infectious secondary effects including both localized (BCGitis)[96–102] or disseminated (BCGosis) [103–108] were reported over the world, some ending in the death of the patient [105,106,108]. After the observation of familial pattern of susceptibility to develop infections [105], in the middle sixties, BCGosis after vaccination was associated with HIV infection [109–111] and PID [110–114], mainly T cell defects (SCID) and CGD [106,110–115] or were labeled as "idiopathic" if the patient was otherwise healthy and without apparent immune alterations [110,111]. However, these immune deficiencies did not explain all the phenotypes observed. In fact, BCGitis and/or BCGosis cases were reported in otherwise healthy individuals without immunological defects detectable with the ordinary tools [108,116]. The case reported by Heyne et al of two siblings with generalized BCG infection and enteric salmonellosis in 1978 was the first time in which BCG susceptibility was linked to the innate system: "No known immunodeficiency disease could be correlated with this familial syndrome of increased susceptibility to intracellular vital microbes. A defective function of the patient's macrophage system is suspected" [116].

Jean Laurent Casanova *et al* theorized that those "idiopathic" infections after BCG vaccination respond to an heritable immunodeficiency [110,111] and Levin *et al* were also for the run of the locus of susceptibility to mycobacterial disease [117]. Later in the same year, the two groups simultaneously reported on the same issue of the New England Journal of Medicine mutations in the *IFNGR1* gene as the first genetic etiology of Mendelian Susceptibility to Mycobacterial Disease, known with the acronym MSMD [71,72].

MSMD is a PID characterized by a selective predisposition in otherwise healthy individuals to disease after exposure to BCG vaccines or environmental mycobacteria [95,118]. It is included in the VIth group of defects in Intrinsic and Innate immunity in the PID classification by the IUIS

[4,11] . It is one of the most studied “atypical” PID. MSMD is caused by inborn errors impairing the final killing of intra-macrophagic pathogens. Immunity to these pathogens, whose major representative are mycobacteria, relies on the IFN-γ circuit or IL-12/IFN-γ axis [95,118], which is deeply reviewed in section 3.1.

Currently 10 causal genes have been described, whether affecting IFN-γ production, such as *IL12RB1* [81,119,120], *IL12B* [89,121], *ISG15* [90,122], *NEMO* [123], *IRF8* [124], and *TYK2* [125], or cellular responses to it, such as *IFNGR1* [72,91,93,119,126,127], *IFNGR2* [128,129], *STAT1* [94,130,131], *IRF8* [124] and *CYBB* [92,123,132]. There are currently 19 different genetic etiologies of MSMD deficiency involving either 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) [95,125]. This number is likely to increase in the coming years. With so many forms of MSMD, the clinical boundaries of the syndrome and each genetic etiology are not yet fully defined.

2.2.1.Clinical spectrum of Mendelian susceptibility to mycobacterial disease

Patients affected with MSMD are usually otherwise healthy and can present a wide range of severity of the disease, from local and recurrent to disseminated and lethal. Severity of the disease depends on the type of underlying defect (complete or partial). MSMD usually, but not always, manifests in childhood [95]. Besides, one of the main issues in MSMD is the incomplete penetrance observed in IL-12Rβ1 deficiency and partial IFN-γ receptor (IFN-γR) deficiencies mainly [81,91,95]. This incomplete penetrance opens the door for the study of modifying factors explaining the different susceptibility of individuals with the same mutation but different clinical outcomes. Clinical phenotype and general features of the different MSMD etiologies was thoroughly reviewed by Bustamante et al in 2014 [95]; an updated summary is showed in the following subsections.

Clinical manifestations of MSMD comprise atypical mycobacteria infection, being BCG after infant vaccination the most common [95,133] or environmental mycobacteria (EM). Some patients are also susceptible to *Mtb* [78]. Besides mycobacteria, there is a wide range of disease-causing organisms that includes other intra-macrophagic infections such as salmonella or listeria, fungi (especially *Candida spp.*), parasites as *Leishmania* [134] or virus in combination with mycobacteria as cytomegalovirus (**Table 2**).

Table 2. Current MSMD warning signs.

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 (i. e. QuantiFERON-TB Gold In-Tube)

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 broadly reviewed by Bustamante et al [95]. 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 S*almonella* (recurrent or not) and, although to a lesser extent, from *Candida*. Some patients with severe TB have been diagnosed with IL-12Rβ1 defects [78,82]. These patients do not usually present viral infections. In parallel, the presence of multifocal osteomyelitis should raise the suspicion of a partial AD IFN-γR1, partial AR, or STAT1 AD loss of function (LOF) [131,135–141]. Patients with complete deficiency in IFN-γR1 and IFN-γR2, abolishing IFN-γ response, are more prone to viral disease such as cytomegalovirus, respiratory syncytial virus and varicella virus Zoster, among others [95]. Treatment of patients with MSMD ranges from HSCT for the most severe forms [72,142], to exogenous human recombinant IFN-γ (hr-IFN-γ), in addition to antibiotics, for the milder forms.

2.2.1.1. IL-12Rβ1 defects

AR defects in *IL12RB1* gene are the most common cause of MSMD, they were first reported 1998 [143,144]. IL-12Rβ1 is a common chain of two cytokine receptors, when combined with IL-12Rβ2 it binds IL-12p70 and when combined with IL-23R binds IL-23. It is expressed on activated T cells and NK cells [145]. More than 200 patients with IL-12Rβ1 deficiency have been reported [95,146–155] with complete loss-of-function of mutations including nonsense, missense, splice site mutations, small and large deletions, insertions and duplications (www.LOVD.nl/IL12RB1). With exception of two described mutations, all mutations give rise to the absence of protein expression [95,155,156].

The clinical phenotype was initially thought to be mild [157], but it has been showed to be very variable, ranging from asymptomatic condition in adult individuals to death at early ages [81,95]. Atypical mycobacterial infections are the most common, especially after BCG vaccination; recurrence or EM disease after BCG vaccination are rare, suggesting altered primary anti-mycobacterial responses, but not secondary. Another clinical typical feature, observed in more than one third of IL-12Rβ1 deficient patients, is invasive salmonellosis. Besides, mucocutaneous *Candida* infection is observed in one in four patients approximately [95,151]. Other bacterial (e.

g. *Klebsiella*), fungal (e. g. *Histoplasma* spp.) and parasitic (e. g. leishmaniasis) infections have been sporadically reported; malignancy is described in just one patient [95,150]. IL-12Rβ1 defect is one of the etiologies with the most incomplete clinical penetrance, being 0.64 at 5 years and 0.79 at 20 years [81,95].

2.2.1.2. IL-12p40 defects

Mutations in *IL12B* were discovered the same year than in *IL12RB1* [95,121], being the first discovered inherited cytokine defect. As happens with *IL12RB1*, encoding for the common chain of IL-12 and IL-23 receptors, *IL12B* encodes for IL-12p40, a subunit of both IL-12 and IL-23. More than 50 patients have been identified, all from 5 countries: India, Iran, Pakistan, Saudi Arabia and Tunisia. All patients have complete LOF defects that cause the absence of the protein. Clinically, IL-12Rβ1 and IL-12p40 deficiency are phenocopies, with the same type of infections described. There is also incomplete penetrance, that reached 50% at one year old [89,95].

2.2.1.3. IFN-γR1 deficiency

IFN-γR1 is the chain of the IFN-γ receptor responsible of IFN-γ binding. AR null mutations in *IFNGR1* were the first genetic MSMD etiology to be identified [71,72,95]. Different mutations lead to different patterns of receptor's expression and levels of clinical affectation [91,95]. Complete defects of IFN-γR1 cause severe disease with infections characterized by early-onset (before three years-old), with commonly disseminated and life-threatening infections by BCG and EM and, also, *Mtb* infections have been observed. Besides mycobacterial infection, viral infections have been described, 3 patients have been reported with malignancies [95,158,159] and hemophagocytic lymphocytosis have also been observed [95,160]. Partial IFN-γR1 deficiency (both AR and AD) patients develop a milder clinical phenotype, with osteomyelitis as a typical disease presentation [91,95]. Infection with *Mtb* has also been observed in one patient; infections with other intracellular bacteria, viruses and parasites were sporadically observed [93,95].

2.2.1.4. IFN-γR2 deficiency

IFN-γR2 is the subunit of the IFN-γ responsible of transmitting the signal, first described in 1998 [95,161]. Similar to IFN-γR1 defects, there are AR complete defects with presence or not of the protein and AR or AD partial defects with protein expression. Complete defects with protein expression are related to glycosylation events, as the one caused by T182N mutation, in which the defective response to IFN-γ is caused by the creation a novel N-glycosylation site. IFN-γR2 deficiency is less common than IFN-γR1 deficiency. Clinical presentation is similar to AR complete defects of IFN-γR1, with early onset life-threatening infections and other infections although BCG is infrequently involved [95]. Partial AR IFN-γR2 is also rare and related with infections caused by BCG, other EM; besides 2 of the 6 described patients developed osteomyelitis. There is only one mutation causing an AD partial disease and there have been described cases of haploinsufficiency with milder clinical phenotypes and a very low clinical penetrance [95].

2.2.1.5. AD STAT1 deficiency

STAT1, along with JAK family members 1 and 2, mediates the response to type I and II IFN [95,162]. AR complete forms of STAT1 deficiency lead to life-threating viral and mycobacterial infections with abolished responses to both IFN-γ and IFN-α; patients with partial AR STAT1 deficiency have a milder phenotype that also comprises mycobacterial and viral susceptibility [95,130]. On the other hand, AD STAT1 mutations were first described in 2001 and have a

dominant negative effect only in IFN-γ signaling and give susceptibility to mycobacterial disease; because of that AD STAT1 mutations are considered MSMD-causing. Effects are partial thus explaining the milder clinical phenotype, with mycobacterial infections caused majorly by BCG and *Mycobacterium avium*. Similar to partial defects of IFN-γ receptors, multifocal osteomyelitis is often seen. Like in the other cases of partial defects, clinical penetrance is incomplete [95,130,131,162].

2.2.1.6. AD Interferon Regulatory Factor 8 deficiency

Interferon Regulatory Factor (IRF)8 is one of the nine members of the IRF family of transcription factors, involved in the regulation of the expression of IFN stimulated genes (ISG). Concretely, IRF8 is expressed in macrophages and DCs and is important for myeloid cell development. AR mutations in IRF8 lead to a life-threatening disease only described in one patient [95], with disseminated BCG disease, oral candidiasis and severe respiratory infections that needed antibiotic and antifungal therapy in combination with HSCT. There is another described patient with a compound heterozygosis mutations with recurrent viral infection, granuloproliferation, and intracerebral calcification [163] On the other hand, the two described patients with AD partial defects showed BCGosis as the unique infectious disease, for that reason, this type of defects were considered to be MSMD-causing. Absent myeloid dendritic cells CD11c^{+}, potent producers of IL-12, are characteristic of this defect [95,124,163–165].

2.2.1.7. ISG15 deficiency

ISG15 is an interferon-induced ubiquitin-like protein that modifies its substrate in a process that resembles ubiquitination. It is secreted by different proteins and synergistically with IL-12 induces strongly IFN-γ production by lymphocytes. AR mutations in ISG15 were first reported in 2012 and lead to BCG disease and intracranial calcifications with or without epileptic seizures and an increase type I IFN immunity and autoinflammation [90,95,166].

2.2.1.8. X-linked recessive Nuclear Factor-kappa B Essential Modulator deficiency

Nuclear Factor-kappa B Essential Modulator (NEMO) is encoded by the *IKBKG* gene and is a regulatory subunit of the inhibitor of NF-κB kinase. Null mutations totally abolish NF-κB activation dependent of NEMO and cause X-linked dominant *incontinentia pigmenti* in females, males being not viable. Hypomorphic mutations that impair but not abolish NF-κB activation cause X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency syndrome in males, conferring susceptibility to pyogenic bacteria, mycobacteria and viruses, being pneumococcal disease the classical warning signs [27,95]. There are two specific mutations in *IKBKG* that cause exclusively MSMD (no viral or pyogenic infections), interfering with the CD40-NEMO-NF-κB signaling pathway, important for anti-mycobacterial immunity. Affected patients developed disseminated infection mostly from *Mycobacterium avium*; besides, *Mtb* and *Haemophilus influenzae* type b were detected at least in one patient [95,167].

2.2.1.9. X-linked CYBB deficiency

CYBB encodes for gp91^{phox}, an essential protein of the nicotinamide adenine dinucleotide phosphate oxidase complex, expressed majorly in phagocytes. Mutations in *CYBB* give rise to the most common form of CGD, these patients have recurrent and life-threatening infections by bacteria (*Staphylococcus*) and fungi (*Aspergillus*) and can also present with BCG disease after vaccination. There is another described form of mutation that only affects monocytes,

macrophages and B cells, thus causing MSMD with BCG disease and tuberculosis, without recurrences [92,95,123,168].

2.2.1.10. TYK2 deficiency

There is a form of TYK2 deficiency that leads to viral, fungal and mycobacterial infections with hyperIgE syndrome, consisting in atopy, high levels of circulating IgE and recurrent mucocutaneus staphylococcal infections; because of this complex clinical course, it is not considered a form of MSMD [169]. However, in 2015 a form of TYK2 deficiency with viral and mycobacterial infections without hyperIgE syndrome was classified as a genetic etiology of MSMD. There are only 7 patients described, who presented with impaired responses to IL-12 and type I IFNs and suffered mainly from mycobacterial infections, both after BCG vaccination and TB as well as cutaneous viral infections [125].

2.2.2. MSMD diagnostic procedures

Children or adults without any other haemato-immunological conditions, HIV infection or immunosuppressive drug exposure (including anti-TNF-α) [78,80,170–172] who develop recurrent or severe/disseminated mycobacterial infectious disease caused by BCG, EM, *Mtb,* or *Salmonella* alone or in combination with other intracellular pathogens or viruses should be suspected of having MSMD. MSMD diagnosis comprises complex functional tests that need to be performed in specialized immunology laboratories. We have extensively reviewed the reported methodology used for MSMD diagnosis [173], included in Annex 1.

Briefly, evaluation of cytokine production is the gold standard for study of IFN-γ circuit integrity, and was developed by Feinberg *et al* [119]. This assay is based in the measurement of IL-12p40, IL-12p70 and IFN-γ after whole blood or peripheral blood mononuclear cells (PBMCs) stimulation. Stimulation conditions comprise live BCG stimulation leukocyte with or without hrIL-12p70 or hr-IFN-γ co-stimulation for 18 h (for IL-12 measurement) or 48 h (for IFN-γ and IL-12 measurements). Although powerful, this technique has several limitations: (1) the intrinsic variability observed yet in healthy controls; that hampers interpretation of results (2) if fresh whole blood is used, it should be performed during the first 48h after extraction and (3) the use of BCG stimulation can be limiting in diagnostic laboratories following ISO 15189 regulations. In an attempt to solve limitations different strategies have been developed, including the performance of the test in cryopreserved cells to eliminate time-from-extraction limitation and the use of phytohemagglutinin (PHA) and lipopolysaccharide (LPS) as stimuli to avoid the use of BCG.

Quantitation of IFN-γ levels in plasma is a fast an easy technique for detection of IFN-γR deficiencies, since high levels of this cytokine are a sign for these defects, especially of complete defects [174]. Cytometric evaluation of the presence of the different receptors of the axis (IFN-γR1, IFN-γR2 and IL-12Rβ1) is also a very useful tool to detect defects in these proteins leading to altered or absent protein expression [81,91,95]. However, receptors’ presence does not exclude a defect, since there are forms (especially in IFN-γR) in which non-functional proteins are expressed [81,91,95,126,127,129,175,176]. Finally, cytometric evaluation of receptors’ downstream signaling after specific stimulation (STAT1 phosphorylation after IFN-γ and IFN-α stimulation [128,130,131,175,177–180] and STAT4 phosphorylation after IL-12 stimulation

[156,176]) can help detect these defects and STAT1 defects with affected phosphorylation [130,131,179,180] (**Figure 3**).

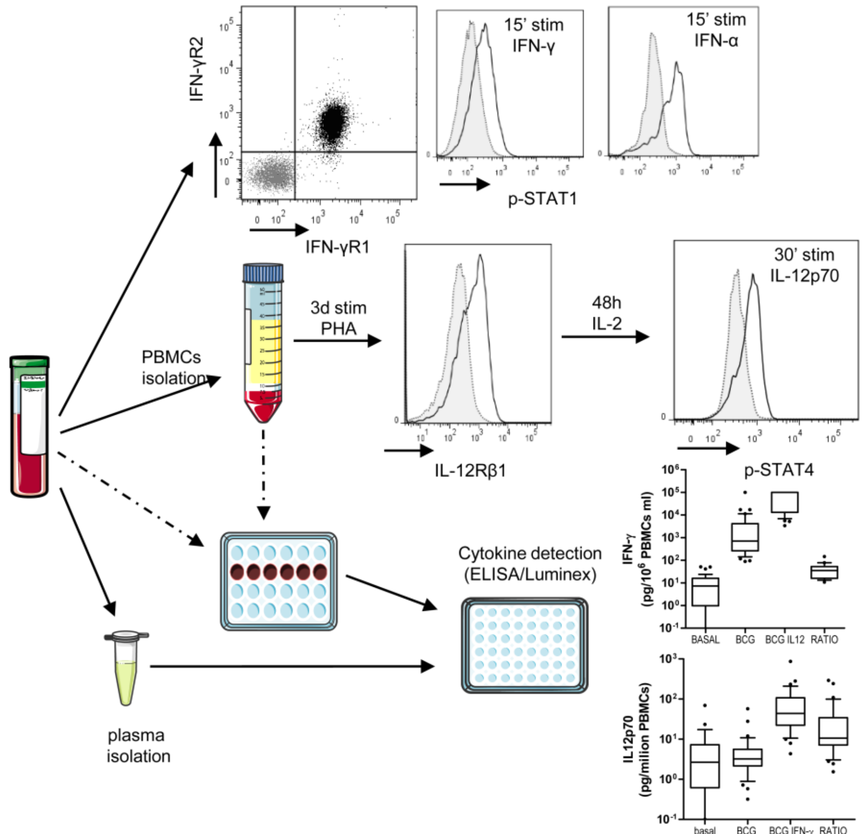

Figure 3. Diagram of the laboratory analysis of MSMD defects with examples. IFN-γ receptors and STAT1 phosphorylation detection is performed in whole blood assay. IL-12Rβ2 detection is performed in PBMCs after 72h stimulation with PHA. STAT4 phosphorylation detection is performed in PBMCs after 72h stimulation with PHA and at least 48h of culture in presence of IL-2 or PHA + IL-2. A healthy control representative for each technique is shown. Cytokine production is detected after 18h (for IL-12p70 in some cases) and 48h (for IFN-γ specially) culture with, in the gold standard, BCG with or without IFN-γ or IL-12p70 co-stimulation. Control cohort is shown. From Esteve-Solé et al, Critical Reviews in Clinical Laboratory Sciences, 2018 [173].

Genetic diagnosis of MSMD is of outmost importance, since it will condition the treatment of the patient and will allow familial genetic counseling. When functional defects suggest a specific defect Sanger sequencing is the option of choice. NGS technology, both in the form of gene panels or whole exome sequencing (WES), can be also useful to screen for all genetic etiologies simultaneously. In a more research-like setting, whole genome sequencing (WGS) is used to detect new disease-causing variations in non-protein coding regions of the genome.

In summary, MSMD is a paradigmatic PID of the genetic theory if infectious diseases, caused by a primary or inborn defect in the function of the IL-12/ IFN-γ axis, mainly manifested by a high susceptibility to intra-macrophagic infections. Many aspects of MSMD syndrome remain still unresolved, and are current areas of research, such as the identification of other infectious susceptibilities in MSMD patients, new genetic variants, and better approaches for its diagnosis and treatment.

3. Immunity to intra-macrophagic infections

Macrophages and neutrophils are the first line of defense against invading microorganisms. Some pathogens have evolved to overcome this first barrier and live and replicate inside these cells. Microorganisms with intracellular, concretely intra-macrophagic, lifestyle are both eukaryotic, as *Candida spp*¸ *Cryptococcus neoformans* [181,182] or *Leishmania spp* [183] among others) and prokaryotic (*Mycobacterium spp* [184], *Salmonella spp* [185], *Yersinia pestis* [181], among others). *Mtb* and *Leishmania spp* are two of the most relevant pathogens within intra-macrophagic infectious agents, causing disease with global relevance.

Common strategies between pathogens living inside macrophages include the capacity to inhibit phagosome maturation and fusion with lysosome or the inhibition of apoptosis in favor of necrosis. Crosstalk between adaptive and innate cells of the immune system is then crucial to destroy these microorganisms or form the granuloma that will contain it. Principal players are the macrophage itself, followed by its activation through the action of specific cytokines, such as IFN-γ and TNF-α.

3.1. The importance of IL-12/IFN-γ axis

Although different systems are involved in defense to intra-macrophagic pathogens, the IL-12/IFN-γ pathway plays a key/non-redundant role. After bacilli/us phagocytosis, pattern recognition receptors are important sensors of mycobacteria, starting a protective response; however, their role in generating this response is apparently redundant [186,187]. Antigen-presenting cells (APC) are then activated and produce TNF-α, ISG15 and IL-12p70. Cytokine secretion by APC promote IFN-γ production by Th cells and differentiation into Th1 cells. This creates a positive loop between the T cell and the APC, which enhances the former's microbicidal capacity through reactive oxygen intermediates (ROI) production [90,188–191]. **(Figure 4)**,

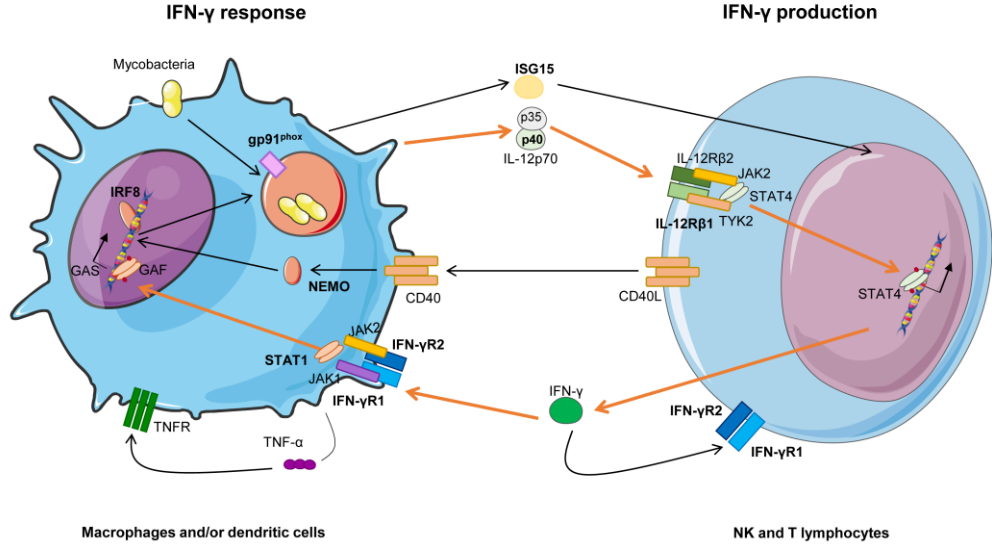

Figure 4. **IL-12/IFN-γ axis.** After intracellular microorganism phagocytosis, macrophages and dendritic cells get activated and secrete IL-12p70 and ISG15. These cytokines activate T lymphocytes and NK cells. Activated T lymphocytes secrete IFN-γ that will further activate the macrophage and promote IL-12p70 and TNF-α secretion as well as impulse the oxidative burst. Besides, CD40L-CD40

interaction will also promote an increase in IL-12p70 production by the NEMO pathway. The creation of this positive loop is necessary to clear the intracellular infection. Known MSMD defects are marked in bold. Red circles in transcription factors STAT1 and STAT4 represent phosphorylation.

The JAK – STAT axis is common for different cytokine signaling, which is conserved though evolution from lower organisms such as the *Dictyostelium* to mammals [192,193]. The JAK family receives its name by analogy to the two-sided god Janus because of the presence of two domains: the kinase domain and a domain with high similarity to kinases (pseudo-kinase domain) [194]. It has four members in mammals (JAK1, JAK2, JAK3 and TYK2). These are non-receptor tyrosine kinases and they share the same structure: a FERM domain mediating the interaction with upstream receptors and promoting kinase function, a SH-2 like domain that also mediates the interaction with upstream receptors, and a pseudokinase domain that limits kinase response and the kinase domain [193]. STAT proteins are classical transcription factors that directly interact with DNA. There are seven STAT proteins described in mammals (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6). Like JAKs, STAT proteins share a common structure: an N-terminal domain involved in protein-protein interactions, a coiled-coil domain also involved in protein-protein interactions and nuclear localization, the DNA-binding domain that directly interacts with DNA and has nuclear import-export signals, a linker domain that promotes transcriptional activity, a transactivation domain containing the tyrosine residues that can be phosphorylated and the C-terminal domain with serine residues that can also be phosphorylated [192,193,195].

Multiple cytokines use a specific combination of JAKs and STATs that lead to different cellular responses following the same mechanism of action. Different cytokines and JAK-STAT combinations are showed in **Figure 5**. Despite after each cytokine ligation a ‘preferred’ STAT/STATs are activated, there is still “promiscuity” and other STATs may be also activated. Ligand binding to cytokine receptors induces an aggregation of the associated JAKs allowing their transactivation. Activated JAKs phosphorylate tyrosine residues in the receptor, thus creating SH2-binding domains that serve as docking sites for STAT molecules. Then, STATs become phosphorylated in a tyrosine residue in the C-terminal end, permitting their homo- or heterodimerization and translocation to the nucleus, where they will bind to response elements of cytokine-dependent genes. STATs transcription factor activities include promoter binding and activation or repression; besides, they also have activity in distal binding events in enhancers, epigenetic hotspots and non-coding loci. What is more, it has been observed that STATs colocalize and work together with other transcription factors. Although less studied, there are other non-canonical pathways of signaling of the JAK-STAT axis that do not include tyrosine phosphorylation or kinase activities [9,192,193,195,196]. Both IFN-γ and IL-12p70, the two major cytokines for intracellular pathogen immunity, signal through the JAK-STAT axis, while TNF-α, also crucial for intracellular pathogen immunity, signals using a different downstream cascade that will be further revised in section 4.

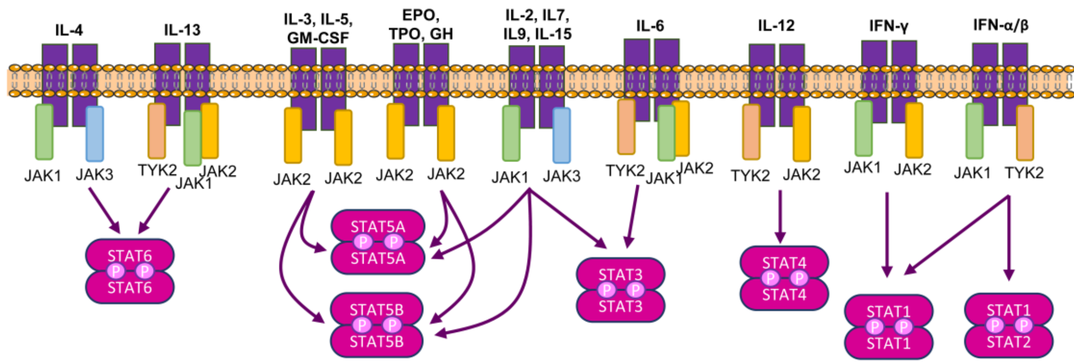

Figure 5. Cytokines and JAK-STAT pathway. Preferred JAK-STAT combinations for different cytokines and molecules. GM-CSF: granulocyte and monocytes colony stimulation factor, EPO: erythropoietin, TPO: thrombopoietin, GH: growth hormone. Adapted from O'Shea *et al* [195]

IL-12p70 is a heterodimer composed of a p40 subunit, shared with IL-23, and a p35 subunit. IL-12p70 is recognized with different affinities for its receptors, IL-12Rβ1 and IL-12Rβ2. They both belong to the gp130 subgroup of the cytokine receptor superfamily, with a high percentage of homology. When presenting as mono-dimers or oligomers, the receptors alone have low affinity for IL-12p70 ($K_d \approx 3 - 6nM$), becoming a high-affinity receptor ($K_d \approx 55pM$) when they heterodimerize. IL-12Rβ1 binds IL-12p40 and is associated with JAK2; on the other hand, IL-12Rβ2 binds IL-12p35 and is associated with TYK2. 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 their receptor chains; STAT4 binds to phosphorylated IL-12Rβ2, becomes auto-phosphorylated into tyrosine 693, and homodimerizes. Then, STAT4 homodimers translocate to the nucleus, where they bind to the *IFNG* promoter, inducing its transcription [152,197–199]. 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 [90,200]. Thus, ISG15 and IL-12p70 act synergistically to induce IFN-γ production activating both NK and Th cells [201].

IFN-γ response in APCs, especially in macrophages, is mediated by its binding to IFN-γR1 and IFN-γR2. IFN-γR are members of the class II family of cytokine receptors. Differently to what is observed in the two chains of the IL-12 receptor, the two chains of the IFN-γR do not share a significative amount of homology. IFN-γR1 is associated with JAK1 by the box1 motif (${}_{266}$LPKS${}_{269}$) and has a high affinity for IFN-γ homodimers and has the binding motif for STAT1. On the other hand, IFN-γR2 is associated with JAK2 by the box 2 motif (${}_{263}$PPSIPLQIEEYL${}_{274}$) and has stabilization and signal transmission functions. After IFN-γ homodimer binding to two IFN-γR1 chains, two IFN-γR2 chains are recruited, bringing JAK1 and JAK2 closer. JAK1 and JAK2 then cross-phosphorylate and phosphorylate IFN-γR1, creating a docking site for STAT1 (${}_{440}$YDKPH${}_{444}$) by its SH2 domain. After binding, STAT1 is activated by phosphorylation of tyrosine 701 causing its dimerization, STAT1 forms the γ-activated factor (GAF) that translocates to the nucleus where it binds to γ-interferon-activated site (GAS) of ISGs, promoting their expression. This binding site has been identified in the regulatory regions of more than 200 genes. STAT1 activation causes, among other responses, an increase of the TNF-α and IL-12 secretion, thus creating a positive loop [9,202–205]. MSMD is caused by monogenic defects in different points of this circuit **(Figure 4)**, impairing the

production of (or the response to) IFN-γ, disrupting protective immunity to mycobacterial infection.

3.2.Tuberculosis

TB is the clinical manifestation of *Mtb* infection. It can be localized in the lungs (pulmonary TB) or in other parts of the organism (extra-pulmonary TB). It is a global problem, with more than 10 million affected people per year. Nowadays, it is the ninth cause of death globally and the first cause of death from a single infectious agent, with approximately 1.6 million deaths in 2016. Besides, HIV coinfection is one of the biggest problems of TB, representing 29% of the total TB deaths of HIV-coinfected individuals. The World Health Organization (WHO) has TB burden reduction as one of its general objectives, with a global TB program since 1995 [206].

Although TB is a global problem, TB burden mostly occurs in low or middle-income countries (incidence ranging from 10 per 100,000 population in high-income countries to 500 per 100,000 population in countries such as Democratic People's Republic of Korea, Lesotho, Mozambique, the Philippines and South Africa). Most of the incident cases in 2016 occurred in South-East Asia (45%), Africa (25%) and Western Pacific (17%). Almost a 20% of the incident cases (2 million people) of TB are attributable to malnutrition. The vast majority of TB-related mortality occurs in the African and South-East Asian region **(Figure 6)**, despite mortality has decreased a 37% since 2000 [206]. There is currently no effective vaccine protective for pulmonary TB, but 12 TB vaccines are being tested in clinical trials [206].

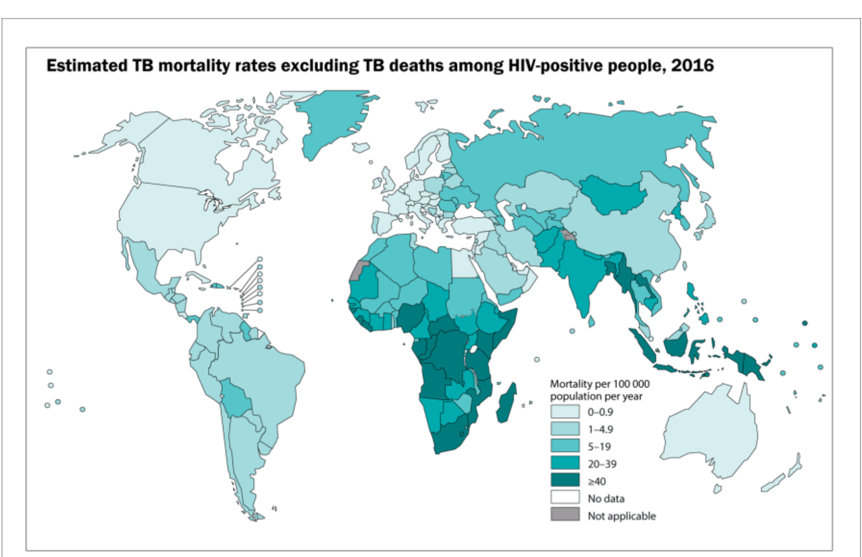

Figure 6. Estimated tuberculosis and mortality rates in 2016. Data from World Health Organization Global Tuberculosis Report, 2017.

Mtb causes disease only in a fraction of the infected individuals. After exposure to *Mtb* almost a 90% of individuals get infected, of those, 5% develop primary tuberculosis within two years of infection. Primary TB usually is mostly observed during childhood **(Figure 7)**, during which can have an extrapulmonary location. The rest of infected individuals who do not develop disease are considered to have latent TB infection. Of these, only 5 to 10% will develop active pulmonary TB as a reactivation; this type of disease is typically observed in adult patients years after the initial infection **(Figure 8)** [76,78]. As stated in section 2, because of that and because of the familial

association in the development of the disease and associated mortality, before the description of the Koch bacilli, tuberculosis was thought to be a hereditary disease.

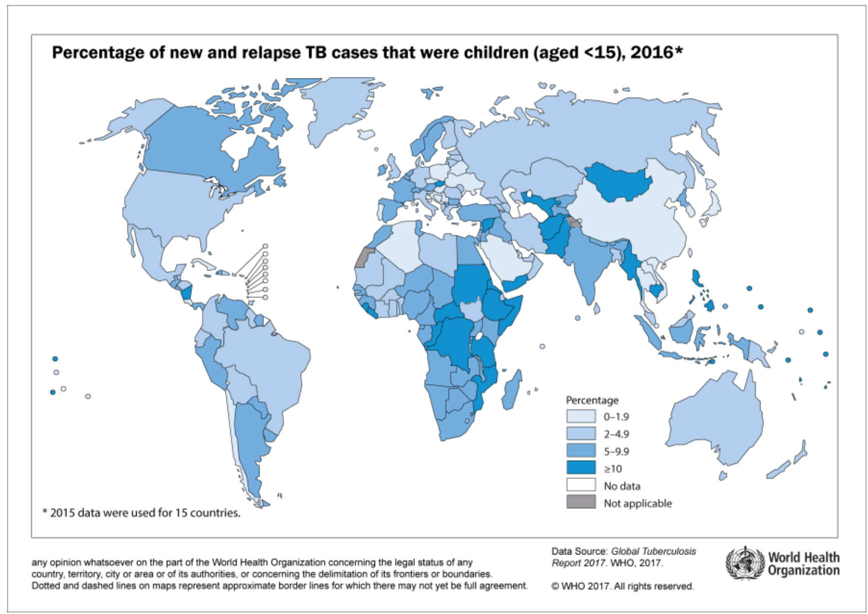

Figure 7. **Epidemiology of pediatric tuberculosis**. Data from World Health Organization Global Tuberculosis Report, 2017.

Reports from the pre-therapy era have been clue for the description of the infection's natural history. Since TB is an airborne disease, when bacilli reach a terminal airway, the alveolar macrophages phagocyte it. If the bacilli resist phagocytizing, it multiplies inside the macrophage until necrosis occurs. After necrosis, bacilli are released to the extracellular milieu and are re-phagocyted. Virulent mycobacteria have developed mechanisms to avoid destruction after phagocytosis, implying the need of an adaptive immune response to stop progression [207]. The first localized pneumonic inflammatory focus is called the parenchymal focus (Ghon sign): both the Ghon focus and the infected lymph nodes are called the primary complex [208,209]. Primary progression is variable and is commonly seen in children. Reactivation of latent tuberculosis infection is seen after local or systemic immunosuppression, although it is also seen in immunocompetent hosts. Reactivation usually takes place in the upper lobes and a process of liquification occurs, favoring the extracellular growth of the bacilli and increasing the inflammatory response, resulting in alveolar damage and cavity formation in the lung [207,210]. Pulmonary TB is characterized by persistent cough, weight loss or failure to thrive, fatigue and reduced playfulness in children [208].

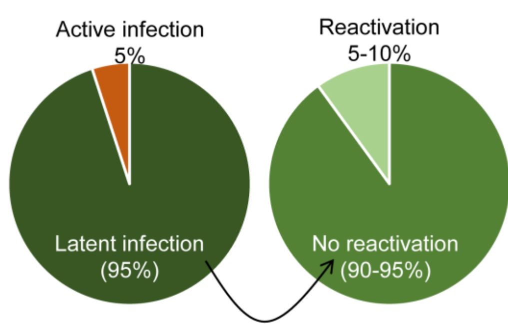

Figure 8. A schematic of the natural history of human infection by *Mycobacterium tuberculosis*. About 5% of infected individuals develop clinical TB within 2 years of infection; this 'primary' TB is particularly common in children, and could be associated with extrapulmonary disease. The remaining persons infected with *Mycobacterium tuberculosis* develop latent TB infection. Only a minority of subjects with latent TB infection (approx. 5–10%) develop clinical TB during their lifetime,

typically owing to reactivation of the original infection Adapted from Abel et al, *Philos Trans R Soc Lond B Biol Sci*, 2014 [76]

Pediatric TB represents almost a 7 % of new cases notified in 2016. Most of TB-related deaths in children occur during the first 4 years of life [206]. A clear majority of progressing children do so within the first 12 months after exposure, especially very young children. Clinically, lymph node disease is a common form of primary TB, cervical lymphadenitis is also common and if left untreated, leads to a prolonged and relapsing course. Progression of lymph node disease in the thoracic area may be dangerous if penetrating other anatomical structures, giving rise to pneumonia, unilateral diaphragmatic palsy and chylothorax formation. On the other hand, dissemination of the disease and tuberculous meningitis are life-threatening forms of primary TB, especially seen in young and immunocompromised children [209]. Extrapulmonary TB can affect any organ of the body, thus presenting with a variety of clinical manifestations. It specially spreads to the spleen, bone, kidney and cerebral cortex. Symptoms of miliary disease include prolonged pyrexia, lassitude, anorexia and weight loss [208].

Diagnosis of *Mtb* infection is still a challenge, especially in low-income countries. Different diagnostic techniques have been developed for the detection of *Mtb*, both in active (direct tests) or latent (indirect tests) infection [211]. For the detection of active infection three approaches have been made: (1) sputum smear microscopy, (2) culture based methods and (3) molecular tests [206,211]. These tests are performed with a variety of body fluids or tissues from the patient. (1) Sputum smear microscopy was developed more than 100 years ago and is the most widely used test [211,212]; it has a high specificity but moderate sensitivity. It is popular because it is inexpensive and rapid. (2) Culture is the most sensitive method and the gold standard for TB diagnosis, as it can be partly automatized or performed by microscopic evaluation in low-income countries. This technique can be coupled to drug sensitivity tests. However, performance of these tests in low-income countries can be conditioned by staff and water/electricity supply limitations. In children, positive cultures are less frequently observed than in adults in pulmonary TB due to children's difficulty to generate sputum. (3) Molecular detection of *Mtb* has changed TB diagnosis. Many nucleic acid amplification tests have been developed with a good sensitivity and specificity in pulmonary TB patients, which decrease in non-respiratory samples. The GeneXpert MTB/RIF assay is a closed system that performs real-time PCR and can give results in less than 2 hours; sensitivity is almost as good as culture (also in extrapulmonary tuberculosis) with good specificity. The use of this technique is endorsed by WHO and its use has increased the proportion of TB patients with bacterial confirmation [211,212]. Again, application of this test in low-income countries is difficult because of economical and supply limitations.

Indirect tests are useful to detect individuals with latent TB infection. The most commonly used test because of its reduced costs is the tuberculin skin test (Mantoux test). Purified protein derivative (PPD) consists of a mixture of proteins found in *Mtb* but also in BCG. PPD is injected intracutaneously, when an intradermal infiltration of 5mm in children (or immunocompromised hosts) or 10 mm in adults is observed, the test is considered positive. This test has a low sensitivity and specificity, especially because of the cross-reactivity with BCG exposure. IFN-γ release assays detect the production of IFN-γ after the stimulation of patient's cells with *Mtb* complex specific antigens, absent in BCG. These tests have better sensitivity and specificity than tuberculin skin tests but are suboptimal for the diagnosis of TB in immunocompromised individuals [211].

Although advances had been made during the last years, more studies are needed to improve the diagnosis of latent TB infection.

Since the development of the first effective treatment in 1940, more than 15 drugs have been developed for the treatment of *Mtb*. For drug-susceptible cases the treatment lasts 6 months, with four first-line drugs: isoniazid, rifampicin, ethambutol and pyrazinamide (just 40 $/individual) and 85% of individuals respond to this regimen. If resistances are found, the treatment is longer (20 months) and has increased costs (2000-5000 $/individual), also with increased toxicities. Currently there is a tendency towards a reduction in the treatment length, lasting 9 to 12 months if there is no resistance to second line drugs, reducing the costs to approximately 1000 $). Resistance is a real problem: rifampicin resistance (RR) is growing: 41% of all new reported cases in 2016 were resistant, and an increase of 10% from 2015 has been observed [206]. There are three grades of TB resistance: rifampicin resistant, multi-drug resistant (MDR, isoniazid and rifampicin resistant) and extensive-drug resistant (XDR, MDR + at least one fluoroquinolone and a second-line injectable agent). Nowadays combination of phenotypic and genotyping testing methods to evaluate resistance to these drugs are available [206,211,212].

3.2.1.Immunity to tuberculosis

Immunity to tuberculosis is complex and incompletely characterized, however it is clear that macrophages, $CD4^+$ T-cells, IL-12, IFN-γ and TNF-α play a critical role. Current knowledge on tuberculosis immunity has been extensively reviewed. Briefly, on entrance, *Mtb* bacilli are phagocyted by macrophages and neutrophils thanks to their antigen uptake machinery [213–215]. The role of neutrophils in mycobacterial infection is up for debate, since it is not clear if their action is beneficial or detrimental for the host [216]. Macrophages are innate cells specialized in the engulfment and destroy of pathogens [217]; one of the virulence factors of *Mtb* is its ability to inhibit the phagosome maturation and its fusion with the lysosome, thus preventing its destruction [213–215]. Besides, dendritic cells get activated both by direct mycobacterial infection and by bystander activation to act as APCs; however, the mechanisms are not totally clear [213–215,218]. Activated/infected macrophages and dendritic cells migrate to the lymph node, where they will produce IL-12. This production of IL-12, besides other molecules, prime Th cells to develop into Th1 IFN-γ producing $CD4^+$ T-cells, which will migrate to the lung to perform their action [213–215,218].

IFN-γ-mediated activation is a key factor for *Mtb* growth restraining. The main action of IFN-γ, in synergy with TNF-α, is the activation of the infected macrophage, thus promoting the maturation of the phagosome and autophagy to induce mycobacterial destruction. TNF-α is important for the construction and maintenance of granuloma and for inflammation. Besides, it induces the inducible nitrogen oxide synthase (iNOS) production and activity, resulting in the production of ROI, thus helping in the final killing of the mycobacteria. Nonetheless, in most cases the macrophage is not completely capable of destroying the mycobacteria [213–215]. Stimulated macrophages that differentiate into multinucleated giant cells are the central mass of the granulomas, along with other infected macrophages, epithelioid cells, foamy macrophages and neutrophils. T (specially $CD4^+$ cells) and B cells surround this inner mass and are covered by a fibrotic capsule. The center of the granuloma contains an hypoxic and hostile environment for the pathogen's growth, hence limiting the infection and leading to “dormant bacilli” [213–215,219].

Of note, if there is a real dormancy or a dynamic equilibrium of infection-reinfection due to recirculation of the bacilli, is nowadays up for debate [207,220].

Although IFN-γ producing CD4$^+$ cells are the main actors of adaptive immunity to *Mtb*, there are other adaptive cells implied. CD8$^+$ cells also produce IFN-γ and secrete molecules (perforin and granulysin) that can directly lyse the mycobacteria. Besides, as the outer membrane of *Mtb* is covered with glycolipids, atypical CD1$^+$ T cells have an important role by producing IFN-γ and are thought to possibly be one of the first barriers of defense in the lung after macrophage activation [213–215,221]. Gamma-delta T cells recognize phospholigands (antigens containing phosphates), including prenylpyrophosphates that are abundant in mycobacteria. After stimulation, these cells produce IFN-γ and express granule-dependent mycobactericidal activity [213–215,222]. Although the role of B cells has not been thoroughly studied, they are also thought to play a role in *Mtb* pathogenesis. B cells could promote protection against *Mtb* by antibody production, antigen presentation and cytokine production [223].

There is a dynamic balance between the bacilli and the host, related to a balance between regulatory and effector immune responses. Besides the vigorous immune response displayed after *Mtb* infection, the activation of the inhibitory machinery is important to avoid hazardous immune responses. An excessive response could lead to destruction of the pulmonary tissue. The anti-inflammatory machinery (T regulatory (Treg) cells, IL10 production, exhaustion…) inhibits the effector response, but also protects and heals the host tissue from the immune response. When the balance is broken, the disease appears. If the crosstalk between the innate and adaptive cells is not optimal, the bacilli can scape and invade other organs, leading to extrapulmonary TB [213–215,224].

3.2.2.Mtb infection in the immunocompromised host

There are some acquired conditions predisposing to tuberculosis. Malnutrition is a potent immunosuppressive agent, considered responsible of 20% of the new cases of TB during 2016. Also is HIV coinfection, since 10% of the new cases of TB occur among HIV infected individuals, having an increased mortality [206]. HIV infection, if untreated, causes a progressive degradation of Th cells, thus increasing the risk of clinical TB. HIV infected individuals usually manifest extrapulmonary forms of tuberculosis. Besides, immunosuppressive treatments, malignancies such as leukemia, organ solid or bone marrow transplantation, severe aplastic anemia or malignant infantile osteoporosis can also cause increased risk for TB development [78]. Biological treatments, such as anti-TNF-α drugs, can also be causative of a *Mtb* reactivation, such so, that all patients need to be tested and treated for *Mtb* infection prior treatment [225,226].

The importance of the host-genetic background was dramatically illustrated during the Lübeck accident in Germany, where 251 newborns were administered a BCG vaccine contaminated with *Mtb*. From those infected infants, 72 died, 135 had a spontaneous recovery and 44 had no clinical symptoms [78,227]. Non immune-related inherited conditions affecting the lungs have been described to confer TB susceptibility such as primary ciliary dyskinesia [78,228], pulmonary alveolar proteinosis [78,229] or cystic fibrosis [78,230]. Besides, during the last years, pediatric extrapulmonary TB has been linked to PIDs including CGD [78,80], T-cell deficiencies including SCID and combined immunodeficiencies [28], autosomal GATA2 deficiency [78], NF-κB

deficiencies causing anhidrotic dysplasia with immunodeficiency [78,231], X-linked recessive CD40L deficiency [78,232] and autosomal recessive TYK2 deficiency [78,125].

Classical clinical spectrum of MSMD mostly include non-tubercular mycobacteria, but can also confer increased susceptibility to TB infection. Two systematic studies had been performed in order to unravel MSMD defects in *Mtb* infected individuals, the first showing a 4% (2 out of 50) of TB patients with IL-12Rβ1 deficiency in an area of high consanguinity [82]. The second, performing genetic MSMD studies of children and adults with severe *Mtb* infection in an area of low consanguinity, revealed no defects [233]. Despite the lack of more systematic studies, different etiologies of MSMD, were found in patients with severe TB, especially IL-12Rβ1 deficiency [78,95,234], followed by IFN-γR1 [93,95], STAT1 [94,95], and IL-12p40 deficiency [89,95]. There are currently 26 patients reported with severe TB due to inborn errors of IFN-γ, 13 of whom are IL-12Rβ1-deficient, including six who did not suffer from any other mycobacterial disease (BCG, EM) [78,95,154,234]. It has been estimated that around a 45% of the patients with severe TB are carriers of single gene inborn errors [78,235] although this theory has not been demonstrated.

3.3.Leishmaniasis

Leishmaniasis is a tropical, sub-tropical and Mediterranean basin vector-borne disease caused by distinct species of parasites from the genus *Leishmania* [236,237]. It's the second cause of death and the fourth cause of disease by tropical infections in the world. Infections are endemic in more than 98 countries (including Spain), and it is considered one of the most important neglected diseases. Due to the suboptimal casuistic studies in endemic areas, there is not an exact number of infected patients, but there is an estimated annual incidence of 0.9 to 1.6 million cases of leishmaniasis worldwide [236]. *Leishmania* species are obligated intra-macrophagic protozoan parasites in their mammalian cell hosts, living extracellularly in their vector, the female sand-fly [236,238,239]. (**Figure 9**)

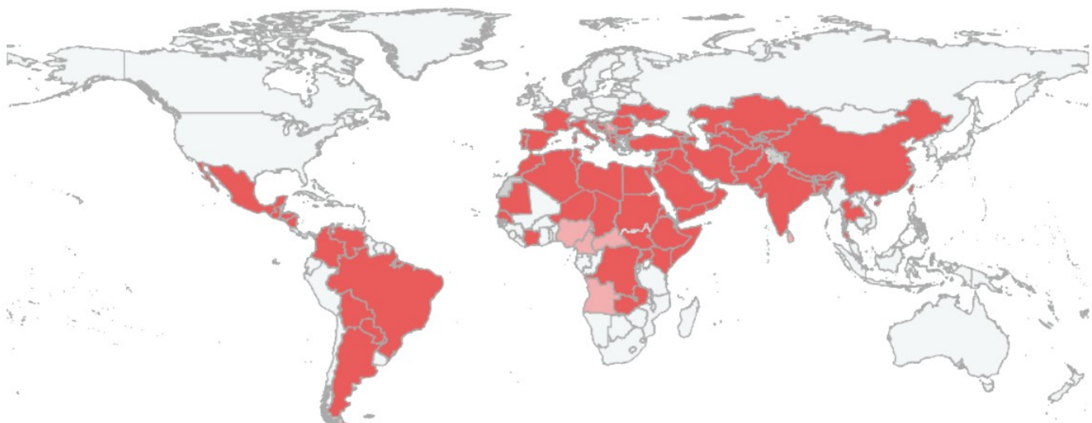

Figure 9. Map of countries with endemic visceral leishmaniasis. Countries with endemic visceral leishmaniasis are displayed in red and countries with past reported cases of visceral leishmaniasis are displayed in pink. Map from (http://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html), accessed on 2018/03/22.

There are two major genus of sand flies that are vectors of this pathogen: *Phlebotomus* in the Old World and *Lutzomyia* in the New World [236,237]. Sand flies are smaller than mosquitoes, silent and bite from dusk to dawn. They do not have a strong power in their fly, since they cannot fly against a wind current, and do not fly at high heights. For this reason, they often byte in the non-protected skin of the legs, arms and face. In the Mediterranean region they are active in the warm summer months and tend to bite the same host more than one time, increasing the chance of transmission. One of the most successful forms of leishmaniasis prevention is the control of sand fly's population with insecticides, bed nets… [236,238].

Leishmania parasites vary their shape/form during the different stages of their life cycle **(Figure 10)**, represented by the promastigote morphology in the sand-fly and the amastigote form in the mammalian host. The same modules compose both forms of the parasite: the flagellum, the basal body, the mitochondrial kinetoplast unit (a mass of concatenated mitochondrial DNA and a Golgi) and the flagellar pocket neck unit. During the amastigote phase, the parasite is smaller and more spherical; the flagellum is short and immotile, and is thought to have sensory functions. On the other hand, the promastigote has an elongated ovoid cell form, and the flagellum is long and motile, providing propulsive force. On entrance, the promastigote is engulfed by the macrophage and transitions to a amastigote phase, then divides by binary fusion until macrophage's lysis, and is then prepared to infect other cells; after the bite of another sand-fly, the amastigote is ingested with the blood and transforms into the promastigote form in the intestine of the sand-fly [239].

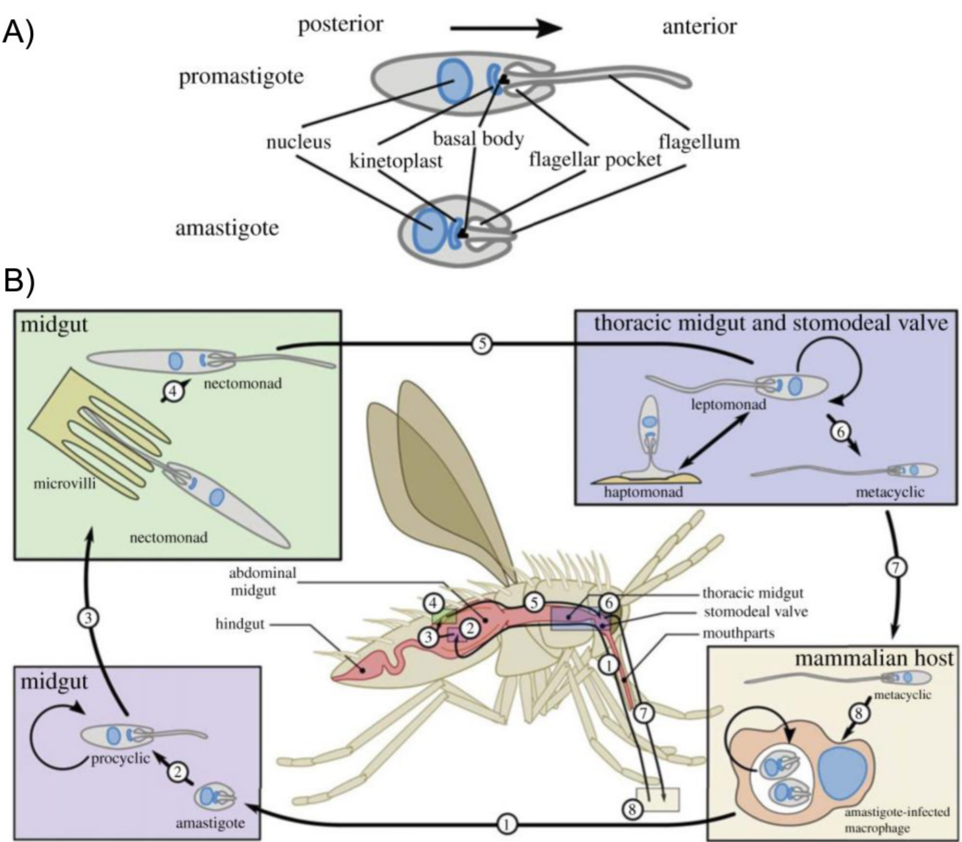

Figure 10 Schematic of promastigote and amastigote morphologies and the *Leishmania* life cycle with different cell types highlighted. A) Promastigote and amastigote morphologies aligned along the posterior anterior axis with key structures in the cells indicated. B) Cartoon of the current

understanding of the Leishmania life cycle. A sand fly takes a blood meal from an infected mammalian host and ingests a macrophage containing Leishmania amastigotes. Once in the sand fly amastigotes differentiate into promastigotes. After several phases in the sand-fly, metacyclic promastigotes are ready to infect mammalian hosts, where promastigotes will be phagocyted and differentiate to amastigotes. A circular arrow indicates proliferative stages. From Sunter et al, *Open Biology,* 2017 [239].

Leishmanial infection leads to a broad range of clinical manifestations of the infection, from subclinical signs to lethal infection. The major forms of leishmaniasis are i) cutaneous leishmaniasis, ii) diffuse cutaneous leishmaniasis, iii) muco-cutaneous leishmaniasis and iv) visceral leishmaniasis (VL). Although boundaries are not clearly set, there are different species of *Leishmania* considered dermotropic or viscerotropic. Also, there is differentiation between New World and Old World species. The New World's are considered to be more aggressive. Although there are many zones in which *Leishmania* is endemic, over 90% of the cases of cutaneous leishmaniasis occur in Afghanistan, Iran, Saudi Arabia, Syria, Alegria, Tunisia, Brazil and Peru and 90% of visceral leishmaniasis occur in Bangladesh, India, Nepal, Ethiopia, Kenya, Sudan and the northeast of Brazil [236].

The main species causing leishmaniasis in the Mediterranean basin is *Leishmania infantum,* known to be causative agent of both cutaneous and visceral disease. In Spain it is transmitted both by *Phlebotomus perniciosus*, present in almost all the peninsula, and *Phlebotomus ariasi*, especially present in the Mediterranean basin and the North-East area of Spain **(Figure 11)** [240,241]. Three patterns of infection have been described in the area: i) sporadic infections due to endemicity, being the dog the reservoir [240,241]; ii) epidemic flares, with the recent example of the leishmaniasis outbreak recently occurred in Madrid Community with the hare as reservoir [240–242] and iii) infection of immunocompromised individuals [241,243]. People living in rural and peri-urban areas are the most prone to develop disease [240,241,244]. The rising of temperatures due to climate change has facilitated vector arrival to northern or higher zones that used to be vector-free, thus increasing the probability to contract the infection [244].

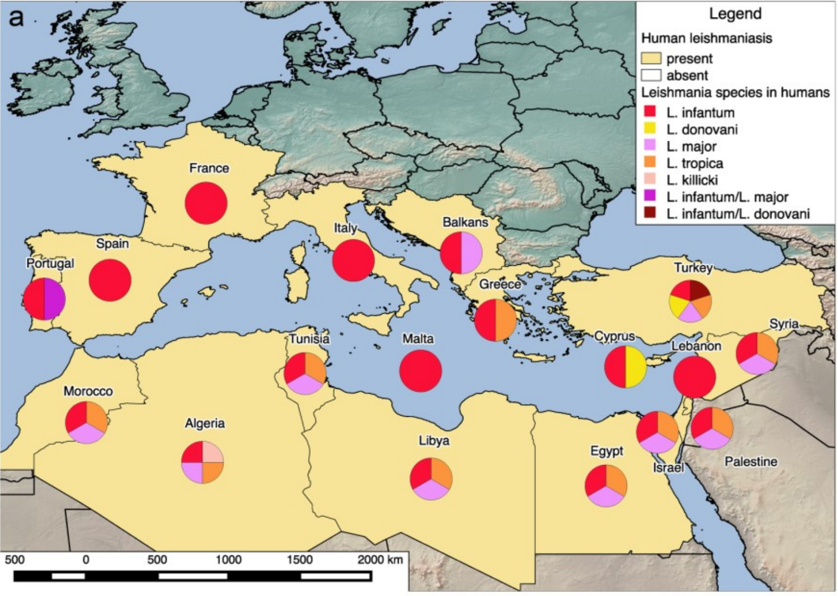

Figure 11. Distribution of *Leishmania spp.* in the Mediterranean region. Autochthonous human leishmaniasis in the Mediterranean area. Countries where human leishmaniasis was diagnosed by serological tests and/or Leishmania isolation and/or PCR method are depicted in yellow. Diagrams show Leishmania species identified and/or isolated in human cases. Adapted from Moriconi et al, *PLoS Negl Trop Dis*, 2017[245].

Microbiological confirmation of *Leishmania* infection is of outmost importance, especially in visceral leishmaniasis. Detection of the parasite is performed by direct observation or by PCR methods. Direct observation of the amastigotes can be performed in slit skin smears for cutaneous leishmaniasis or in medullar or splenic aspirates for VL. Direct observation has a moderate sensitivity. PCR targets conserved sequences in the *Leishmania* kinetoplasts or the ribosomal RNA gene; it can be performed in different tissue samples or blood, and is especially useful in cases of low parasite load, such as in patients with HIV co-infection. Besides, anti-leishmanial antibody detection or leishmanin skin test may be useful but does not distinguish between active or past infection [236,238]. Culture isolation of parasites is also performed but has a low efficacy and is expensive and time consuming [236].

VL is also known as Kala-Azar, and is the result of the dissemination through the reticuloendothelial system of infected macrophages. Its clinical signs include fevers, weight loss, anorexia, weakness, pallor, cough, diarrhea, epistaxis, hepatosplenomegaly, lymphadenopathy and growth retardation in children. Patients can develop normocytic anemia, thrombocytopenia, neutropenia, transaminitis, hypoalbuminemia and hypergammaglobulinemia. Within the immunocompetent hosts, visceral leishmaniasis occurs especially during infancy, from 1 to 4 years. Once started, and without treatment, visceral leishmaniasis lead to death from infection, severe anemia or hemorrhage within two years [238,246,247].

Treatment for visceral leishmaniasis, as well as other forms of leishmaniasis, has high toxicity, high costs, lack of efficacy in some circumstances and lack of access and/or emerging drug resistance. Amphotericin B deoxycholate is very effective against visceral leishmaniasis, leading to more than 90% rate of curation. However, it is difficult to tolerate as it can lead to fevers, rigors, nephrotoxicity, hypokalemia and anemia. In high-income countries, first line treatment for VL is liposomal amphotericin B, which has a shorter regimen, decreased toxicity and more than 95% cure rate. However, it is very expensive, making it unaffordable for the zones with higher burden of disease. Miltefosine is the unique oral treatment with efficacy against all types of leishmaniasis; it also has a 95% cure rate in VL. The adverse events are vomiting, nausea, motion sickness, headache, diarrhea and creatinine elevation, but is an alternative to amphotericin B [238,246,247].

3.3.1.Immunity to visceral leishmaniasis

Leishmania infection is asymptomatic in most individuals; however, it can give rise to cutaneous or visceral disease. The outcome of a *Leishmania* infection depends on three major factors: the parasite itself, the vector and the host immune response and genetic background. During the last decade, efforts have been made to unravel immunologic responses that confer resistance or susceptibility to clinical leishmaniasis; however, it is still a neglected disease and more research must be performed to better understand, treat and prevent *Leishmania* infection. A large amount of the knowledge on immunity to *Leishmania* has been developed in murine

models, but they have a different natural history of infection: even susceptible mice develop a self-limited visceral disease, in contrast to life-threatening disease in dogs and humans [248].Different strains of mice have different degrees of susceptibility to *Leishmania* infection, with B6 as a commonly used resistant strain and BALB/c used as a susceptible one, indicating the importance of the genetic background on the clinical outcome of *Leishmania* infection [249].

After a blood-meal the sand-fly release the promastigotes into the dermis, where the complement system kills most of the parasites by lysis. *Leishmania* parasites can be phagocyted by macrophages, neutrophils and dendritic cells. Upon macrophage phagocytizing, the parasites transform into amastigotes. Immune response following phagocytosis is genetic background-dependent. In resistant mice, infected dendritic cells produce the Th-1 polarizing cytokine IL-12, enhancing IFN-γ production by T and NK cells thus activating macrophages to produce nitric oxygen (NO) and ROI which will lead to parasite destruction. In susceptible strains, IL-4 is produced thus driving Th-2 like responses that in combination with IL-10 and tumor growth factor (TGF)-β production by Treg cells, deactivate infected macrophages, inhibiting NO and ROS production [249–251].

The role of neutrophils in *Leishmania* pathogenesis also depends on the genetic background: in susceptible mice, they are considered as Trojan horses delivering parasites after apoptosis to macrophages and promoting Th2 immunity, while in resistant mice they induce TNF-α production by macrophages [250,251]. Some *Leishmania* strains can inhibit oxidative burst and avoid the targeting to the lysosome in monocytes [250]. Macrophage responses after infection are also different in susceptible and resistant mice strains. While in susceptible mice, infection activates FasL-dependent apoptosis of resident macrophages, in resistant mice it induces a cellular stress with ROI production and activation of stress activated protein kinases [251]. *Leishmania spp.* have developed tools to inhibit immunologic responses: although macrophages are the last step on the destruction of the parasite, they have altered IL-12 production after *Leishmania* infection [250,251]. One of the mechanisms is the inhibition of JAK1/JAK2/STAT1 signaling in the infected macrophage [250].

Normal human serum has an important anti-leishmanial activity, killing most promastigotes at the time of infection, it has been shown that when cultured with human serum, 85—90% promastigotes are lysed in less than three minutes [252]. However, complement-dependent lysis on entrance is not sufficient for the clearance of the parasite in most individuals. Patients with VL present with altered responses during infection. While healthy infected individuals can respond to stimulation with leishmanial agent, PBMCs from patients with active infection do not produce IFN-γ. However, response to leishmanial agent is restored after the infection is cleared [248,253]. Differently to what is observed in murine studies, the dichotomy between Th1 association with resistance and Th2 with susceptibility is blurrier in humans. Indeed, elevated levels of IFN-γ have been found in the spleen and bone marrow during infection accompanied with increased IL-10 production [248,253]. Other mechanisms of susceptibility in humans include helminth infections that can alter the Th1/Th2 balance. Besides, TNF-α production has a role in granuloma formation and maintenance to contain *Leishmania* parasites[253].

3.3.2.Leishmania in the immunocompromised host

Leishmaniasis is thought to occur both in immunocompetent and immunocompromised hosts; however, immunocompromised hosts are more vulnerable to leishmaniasis and tend to have a more severe disease. Acquired forms of immunosuppression such as HIV infection, malignancy, immunosuppressive treatment (including biologic drugs specially anti-TNF-α antibodies [254,255]) and malnutrition are the most common forms of susceptibility to *Leishmania* infection; to date, infection resulting from a PID is rare [243]. Nonetheless, patients with PID causing leishmaniasis have been described: one patient with Ataxia-Telangiectasia [256], one patient with CVID and Evan's syndrome, 3 patients with hypogammaglobulinemia [257] and 2 patients with CGD [258]. Recently, five cases of Leishmania infections in patients with mutations in MSMD-causing genes have been described, both alone [134,259] or in combination with other infections [82,89,260]. Patients had mutations in *IL12RB1* [82,134,259,260] and *IL12B* [134]. Despite the few cases of PID-related leishmaniasis reported, more entities of single-gene mutations causing severe leishmaniasis will probably be discovered during the following years.

4. Anti-TNF-α biologic treatments

4.1.TNF-α

The effects of TNF-α stimulation are a double edge sword, with beneficial consequences when tightly regulated and potential destruction when dysregulated. TNF-α was first described by its anti-tumor properties, however it is a major player in immune system regulation, organogenesis and development, especially in lymphoid organ architecture and in defense against pathogens, including destruction of infected cells and granuloma formation, tissue regeneration and resolution of inflammation with functions in cell survival, proliferation, differentiation and apoptosis.[261–263]. On the other hand, it can also promote (1) tumorigenesis by the expression of invasion and metastasis related genes and inhibition of apoptosis and (2) autoimmunity and chronic inflammation, atherogenesis, hyper-nociception and tissue destruction [262,263]. TNF-α is a member of the TNF superfamily (TNFSF), members of this family have multiple receptors that differentiate into two groups depending on the presence or absence of death domains (DD) in their structure. The first group, with presence of DD, lead to the activation of the caspase cascade upon TNF-α ligation and, usually, apoptosis; on the other hand, the second group signals through adaptor proteins and their activation leads to activation of transcription factors associated with cell survival. For this reason, the activity of these proteins is strictly bonded to the balance between pro- and antiapoptotic signals on the cell [261,262,264].

Since its first description in 1975 [265], TNF-α is one of the most studied cytokines [261] and its signaling and function have been extensively reviewed [261,262,264,266]. TNF-α is a homo-trimeric protein, first expressed as a transmembrane molecule, then cleaved by the TNF-α converting enzyme, ADAM17 metalloprotease, into a soluble extracellular protein; both forms are bioactive. It can be produced by macrophages, T, B, natural killer (NK), mast endothelial cells, fibroblasts and neurons [261–263,267,268]. The trimeric glycoproteins TNF Receptor (TNFR) 1 and TNFR2 are its two receptors [267]. TNFR1 is virtually constitutively expressed in all cells but erythrocytes, and preferentially binds soluble TNF-α; TNFR1 contains a DD and its activation is majorly related to inflammation and tissue degradation. TNFR2 does not contain DD and gets activated by, preferentially, the transmembrane form of TNF-α; its expression is tightly regulated and is present mostly in immune and endothelial cells. It is associated with homeostasis, by regulating cell survival and tissue regeneration [261–264,268]. Regulation of TNF-α and its receptors expression is clue for the development of fine-tuned cell type-specific responses [261,263].

TNF-α ligation to TNFR2 promotes cell survival while TNFR1 activation can lead to both cell death or survival [261]. Signaling upon TNF-α ligation to TNFR1 and TNFR2 leads to NF-κB activation with different consequences. The regulation of TNFR1/TNFR2 expression and signaling is not completely understood: since immune cells commonly co-express TNFR1 and TNFR2, it is difficult to predict the cell fate after TNF-α stimulation. After stimulation, both receptors recruit adaptor molecules and occurring phosphorylation/ubiquitination processes determine the outcome of TNF-α ligation [261,262,264]. The ligation of transmembrane TNF-α can lead to reverse-signaling, giving rise to its immunomodulatory response. Immune complexes formed after anti-TNF-α biologic drug therapy can mimic this regulatory mechanism [267].

After TNF-α binding to TNFR1 both cell survival or death can occur. Differently to what is observed after FAS – FAS-ligand interaction, TNF-α binding to TNFR1 leads to a complex apoptotic

cascade with many components. It is thought that RIPK1, a protein downstream TNFR1 activation, is a central molecular switch that, depending on its ubiquitylation state, mediates TNFR1 signaling to cell survival or apoptosis. When RIPK1 is polyubiquitylated, TNF-α ligation to TNFR1 leads to NF-κB activation and nuclear translocation, where it will promote the transcription of genes involved in cell survival [261,262,264]. On the contrary, if it is not polyubiquitylated, there is no NK-κB activation and the balance falls into the apoptotic signals and cell death via caspase 8 activation. Besides, TNFR1 activation can also lead to necrosis, a pro-inflammatory form of cell death when RIPK1 is not ubiquitylated and the caspases are inactivated **(Figure 12)** [261,263]. Of note, pathways leading to TNF-α production by different cells are highly redundant, including different pattern recognition receptors and signaling pathways, thus showing the importance of this cytokine [269].

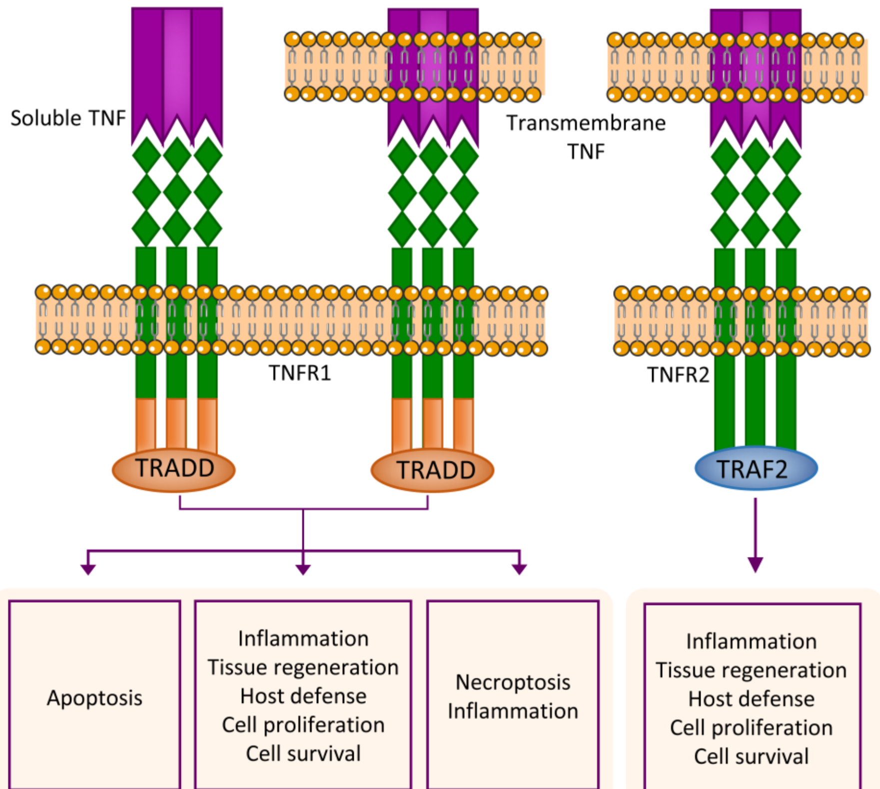

Figure 12. Different bioactivities downstream of TNF receptors. TNF receptor 1 (TNFR1) signaling is activated by both soluble and transmembrane TNF-α. TNFR1 bears a death domain that recruits the adaptor protein TNFR1-associated death domain protein (TRADD). Ligation of TNFR1 by soluble TNF-α or transmembrane TNF-α leads to different outcomes: i) inflammation, tissue degeneration, cell survival and proliferation, and orchestrates the immune defense against pathogens; ii) apoptosis; iii) necroptosis and inflammation. TNFR2 is proposed to be fully activated primarily by transmembrane TNF-α, in the context of cell-to-cell interactions. TNFR2 recruits TNFR-associated factor 2 (TRAF2) via its TRAF domain and mediates primarily homeostatic bioactivities including tissue regeneration, cell proliferation and cell survival. This pathway can also initiate inflammatory effects and host defense against pathogens. Adapted from Kalliolas *et al*, Nat Rev Rheumat, 2016 [263]

4.2.Anti-TNF-α treatment

The use of anti-TNF-α drugs in IBD represented the first FDA-approved indication for an anti-TNF-α biological treatment in 1998 [270]. The success of this therapy led to an increase in the research and commercialization of biologic drugs. Nowadays anti-TNF-α biologics are within the first sales positions drugs in the world [271,272]. Global spent on anti-TNF-α drugs is beyond $US 25 billion per year. This enormous quantity of money derives from the particularly high cost of anti-TNF-α treatment per patient/year, which ranges from $US 25,000 to 30,000. Because of this price, not all people eligible for anti-TNF-α therapy can be treated. Hopefully, this situation will be rescued in the near future with the appearance of biosimilars. Biosimilars are to biologic treatments what generic drugs are to classical small molecules. 'Remsima' is the first biosimilar for Infliximab that was approved for its use in Europe [271].

Currently, there are 5 FDA-approved anti-TNF-α biologic drugs: Infliximab (Remicade, 1998), Adalimumab (Humira, 2003), Golimumab (Simponi, 2009), Etanercept (Enbrel, 2000) and Certolizumab pegol (Cimzia, 2009) [271–274]. The first approved indication for all drugs was Rheumatic Arthritis, except for adalimumab (approved for Crohn's Disease). The approved indications include rheumatoid arthritis, ankylosing spondylitis, psoriasis and psoriatic arthritis, IBD (ulcerative colitis (UC) and Crohn disease (CD)) and hidradenitis suppurativa [263,272]. Besides, it is used in several off-label indications such as Behcet's disease and uveitis [272]. Anti-TNF-α drugs may be used both in adults and in pediatric patients, especially after failure of classic therapy [272]. This treatment is contraindicated in patients with uncontrolled infections. While vaccination against pneumococcus, influenza and human papillomavirus is recommended, live vaccines should be avoided until 3 months after discontinuation of therapy [273,275].

Infliximab is a chimeric antibody with a 25% of murine-derived amino-acids, consisting in a murine Fab region and a human constant fraction. Adalimumab was the first totally humanized antibody approved by the FDA. It is a recombinant human immunoglobulin (Ig)G1 antibody produced using phage-display technology. Golimumab is a human IgG1κ mAb, created in engineered mice immunized with human TNF-α; it has increased stability, affinity and neutralization capacity and it was not until 2013 that golimumab was approved for IBD treatment. Certolizumab pegol is a humanized anti-TNF-α, consisting on murine-derived amino-acids inserted into a human Fab region linked to pegol to increase half-life of the drug and improve solubility. Etanercept is a soluble recombinant TNFR2 protein, fused to IgG1 constant fraction, thus preventing the union of TNF-α to the receptor; it is the unique anti-TNF-α approved drug not used for IBD treatment **(Figure 13)** [268,272–274].

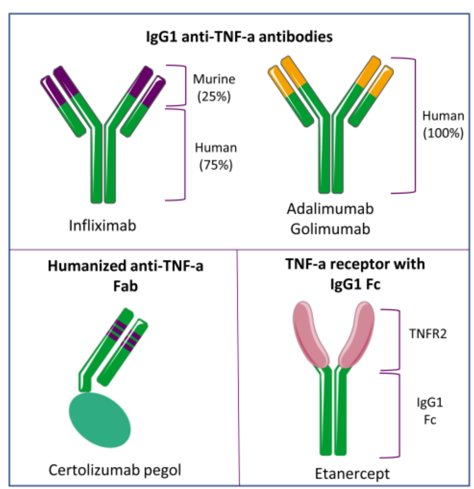

Figure 13. Approved anti-TNF-α biologic drugs. Fc: constant fraction; TNFR2: TNF

Anti-TNF-α drugs comprise different mechanisms of action. The most obvious mechanism is the neutralization of soluble TNF-α in blood, and also the binding to transmembrane TNF-α (inhibiting the interaction with its receptor). The binding of transmembrane TNF-α to anti-TNF-α drugs

with IgG1 constant region (thus excluding certolizumab pegol) leads to 3 different mechanisms of inhibition: (1) antibody-dependent cellular cytotoxicity, (2) complement dependent cytotoxicity, which occurs by the classical pathway and (3) reverse signaling, meaning the capacity to induce intracellular signals in the cell that express transmembrane TNF-α, leading to regulation of cytokine production and caspase-dependent FAS-independent apoptosis (this last mechanism is currently on debate) [268,272–274].

As expected, anti-TNF-α biologics are not free of adverse events. Infliximab, because of the need of intravenous administration and its chimeric nature, has a 10% frequency of acute and delayed infusion reactions including anaphylaxis, which have been described with the 5 drugs. Infections are a serious adverse effect of TNF-α inhibitors, being TB the major problem. It has been proved that exposure to more than one immunomodulatory or immunosuppressive treatment dramatically increases the risk of severe infection. There is no agreement in the statement that anti-TNF-α treatment can lead to malignancy, especially regarding immune-cell related cancer. Although an increased risk has been observed in some studies, it was related to the use of concomitant azathioprine treatment, which already caused similar effects in monotherapy. Patient monitoring is of outmost importance. Infections should be closely monitored by urinalysis, complete blood count, acute-phase reactants, creatinine and electrolytes measurement. Besides, liver enzyme levels should be also checked regularly as well as the apparition of cancer; dermatologic careful follow-up is highly recommended, since both melanoma and non-melanoma skin cancer has been observed [261,270,272,273,275].

4.3. Acquired susceptibility to mycobacteria and leishmaniasis after anti-TNF-a treatment

The knowledge on the importance of TNF-α role in mycobacterial, specially *Mtb,* and *Leishmania* infections *,* has dramatically increased with the anti-TNF-α use [213,269,276,277]. Patients exposed to anti-TNF-α showed higher frequency of TB, histoplasmosis, cryptococcosis, coccidomycosis, listeriosis, aspergillosis [213,225,277,278] and leishmaniasis [255,279]. The increased susceptibility to TB is one of the most relevant adverse effect of anti-TNF-α antibodies. It has been described that these treatments increase at least 5 times the risk of TB infection after drug exposure [213]. The risk of TB is mainly due to reactivation of latent tuberculosis infection, leading to disseminated forms; TNF-α is thought to be important for the containment of the dormant mycobacteria [225,226,269,276]. As opposed to the common pulmonary tuberculosis in adults, the forms seen after anti-TNF-α are specially severe, usually extrapulmonary [269,276]. Besides, the withdrawal of anti-TNF-α treatment in patients with latent tuberculosis infection can lead to a state of increased inflammation due to the immune reconstitution syndrome. For this reason, prior to anti-TNF-α treatment it is mandatory to perform a TB screening and, if needed, antibiotic therapy to avoid TB reactivation [225,269,276–278,280].

Low levels of TNF-α are associated with fatal TB progression. It plays a non-redundant role in preventing primary progression and control of the reactivation of latent TB [213,269]. Of note, virulent strains have mechanisms to inhibit TNF-α production while non-pathogenic mycobacteria lead to increased TNF-α production [269]. After mycobacterial encounter, macrophages and dendritic cells are the major source of TNF-α, especially when co-stimulated with IFN-γ [269]. Together with IFN-γ, TNF-α stimulates macrophages to produce reactive nitrogen and oxygen

intermediates as well as killing peptides [276]. Normal levels of TNF-α release by mononuclear cells lead to recruitment of T cells to the site of infection, activation cytotoxic T cells, promotion of monocyte maturation [276] and apoptosis in infected macrophages [269]

TNF-α production is highly modulated by cytokines, enzymes and lipid mediators to avoid unwanted responses. Dysregulation of TNF-α production in the context of a mycobacterial infection leads to increased disease. TNF-α has a major role in granuloma formation and maintenance [213,269,276,277]; for example, animal models have shown that low levels of TNF-α lead to disorganized granulomas, with impaired activation of macrophages and presence of increased numbers of bacilli; on the other hand, increased levels of TNF-α, although with well-defined granulomas, can lead to the hyperactivation of infected macrophages, necrosis and extracellular replication after the release of the bacilli causing hyperinflammation and tissue destruction in the host [269]. Of note, the effect on the granuloma structure in humans is not totally understood yet. Thus, the balance between TNF-α production and other immunomodulatory molecules as IL-10 is of outmost importance to prevent excessive harm.

Of interest, this increased infectious risk is observed with monoclonal antibodies and not with soluble molecules anti-TNF-α [213,277]. Differences observed between types of biologic drugs against TNF-α can be explained by two major reasons. Frist, because monoclonal antibodies as infliximab and adalimumab can form immune complexes with the complement system and provoke the lysis of the cell [277] and second, because of the differential inhibition of TNFR1 – TNFR2. Second, TNFR1 has a major role in the outcome of mycobacterial infection, infliximab and adalimumab monoclonal antibodies block both TNFR1 and TNFR2, while etanercept totally inhibits TNFR2 signaling but only blocks partially TNFR1 [276]. While whole blood treated with infliximab showed reduced CD69 early activation marker expression, reduced IFN-γ production and a tendency towards an increased intracellular viability of the mycobacteria inside the macrophage; treatment with etanercept did not cause any of these effects [277].

Although there are not known PID causing specifically a deficiency in TNF-α, it plays a key role in the IL-12/IFN-γ axis by synergistically acting with IFN-γ and macrophage activation for intra-macrophagic pathogen destruction **(Figure 14)**. Infections observed in adult patients treated with anti-TNF-α mAbs resemble those observed in patients with MSMD [26].

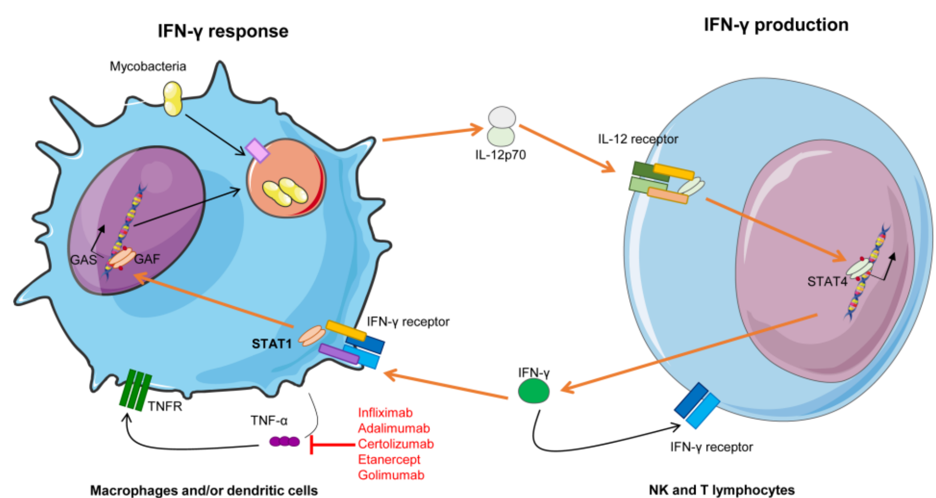

Figure 14. **Impact of anti-TNF-α blocking on the IL-12/IFN-γ axis.** Simplified representation of the IL-12/IFN-γ axis and the impact of blocking the synergistic effect of TNF-α. Drugs used for TNF-α inhibition are displayed in red.

4.4.Anti-TNF-α drugs in inflammatory bowel disease

IBD is an advancing problem in westernized countries and is characterized by chronic inflammation of the intestine and/or the colon. UC and CD are the main representatives of IBD. The prevalence of IBD in Europe is 505 and 322 per 100,000 for UC and CD, respectively [281]. IBD has serious implications for the patient, with high morbidity and a decrease in quality of life [275].UC is characterized by a relapsing inflammation of the colonic mucosa and is normally diagnosed after the presence of bloody diarrhea. Disease severity can range from mild inflammation to severe fulminant disease, dependent on the affected colonic extent. When the disease is not controlled by drugs, surgery (including proctocolectomy) can be needed; besides, at the long term, UC is related with increased risk of colorectal cancer [282]. CD is a more complex relapsing disease affecting the gastrointestinal tract but including also extraintestinal manifestations such as osteoporosis or pyoderma gangrenosum. Besides, it is associated with other immune disorders such as psoriasis or multiple sclerosis. CD usually presents with abdominal pain, fever, bowel obstruction, diarrhea that can be bloody and/or contain mucus. If not controlled, CD can become an invalidating disease; a big fraction of CD patients will need surgery and, almost half of them, more than once [275,283]. Anti-TNF-α mAb have revolutionized IBD treatment [275,281,284–286], by decreasing the rate of hospitalization due to complications or need for surgery [275].

Microbiota and immune system relationship is tightly regulated. The intestinal mucosa is covered by commensal bacteria; consequently, the interaction between the patrolling immune system and the environmental stimuli needs to be strictly regulated to prevent intestinal inflammation [275]. IBD is caused by a destructive loop between a non-controlled immune response against normal intestinal flora and epithelial barrier disruption after TNF-α over-production [261,264,275]. UC has been associated with the disruption of the barrier function while CD with a dysfunction on the microbe sensing [275]. Overexpression of TNF-α is related to hyper-inflammation and tissue damage [267], concretely in the disruption of the epithelial barrier by both the rearrangement of adhesion proteins and the induction of intestinal cell apoptosis after an increased iNOS expression [264,268]. After this disruption, luminal antigens can penetrate and, then, promote inflammation [264]. TNF-α presence in serum directly correlates with disease severity in IBD patients, giving conceptual rationale for anti-TNF-α treatment.

Anti-TNF-α drugs are normally used after failure of conventional treatments. Conventional treatments include budesonide, 5-aminosalycilic acid, topical or oral glucocorticoids, antibiotics, immunomodulatory molecules such as azathioprine, mercaptopurine or methotrexate (only for CD patients). Among biological therapies, infliximab and adalimumab are the most common used anti-TNF-α mAbs; however, certolizumab pegol and golimumab have also been recently approved for their use in IBD therapy. Placebo-controlled trials have proven that infliximab, adalimumab and certolizumab pegol are efficacious in the treatment of moderate-to-severe CD, promoting mucosal healing and the sparing or stop of glucocorticoid treatment. Randomized trials for UC proved adalimumab, infliximab and golimumab useful in patients resistant to conventional

therapy [273,275]. The effect of these drugs in IBD pathogenesis has been proven by different clinical trials, showing clinical response, reduction of acute inflammatory molecules and mucosal healing [268].

The mechanism of action of anti-TNF-α drugs in IBD is complex. It includes apoptosis of lamina propria T cells and the induction of M-2 type wound-healing macrophages. Lamina propria T cell apoptosis occurs as fast as 24h after administration of infliximab, adalimumab or certolizumab (not yet studied in golimumab) and continued after 4 weeks of treatment, thus stopping or reducing inflammation. $CD206^{+}$ M-2 type macrophage generation is dependent on the Fc binding activity of monocytes; for that reason, only full antibodies can exert this action. The appearance of $CD206^{+}$ macrophages is related with good outcome in infliximab treated patients. The production of these macrophages is increased when biological drugs are used in combination with azathioprine. Besides, it has been shown that anti-TNF-α treatment modifies transcription factor and gene expression profiles in intestinal cells, as they respond differently to other stimuli that would trigger the inflammatory response [274].

Anti-TNF-α therapies in IBD start with an induction phase followed by a stable phase. Infliximab is typically administered intravenously at a dose of 5 mg per kilogram of body weight; induction phase consists of three doses at weeks 0, 2 and 6, followed by a subsequent treatment every 6 to 8 weeks., Adalimumab, certolizumab pegol and golimumab are administered subcutaneously, and the administration can be performed in-house by the trained patient. For adalimumab, the first dose is administered at 160 mg, the second at 80 mg and then at 40 mg every two weeks [272,274,275,287]; for Certolizumab pegol the recommended dose is of 400 mg, first at weeks 0, 2 and 4, and then after every 4 weeks [272,275,288]. Golimumab is administered at a dose of 200 mg the first time, then 100 mg at week 2, followed by 100 mg administration every four weeks [272,275,289].

Although anti-TNF-α treatments have greatly improved the prognosis of IBD patients, there are still patients that do not respond to treatment. Approximately from 10 to 40% of CD patients and a 50% of UC patients do not have clinical responses to anti-TNF-α treatments. If persistent inflammation is observed, dose or frequency of administration of anti-TNF-α can be increased or the anti-TNF-α drug can be changed [272,273,275]. Treatment with infliximab and azathioprine has increased clinical responses, compared with the two treatments alone. Also the occurrence of anti-infliximab antibodies is reduced [275]. Levels of anti-TNF-α and presence of anti-drug antibodies should be monitored to prevent relapses of the disease, as persistently detectable drug levels correlate with favorable responses. Therapeutic dosage for adalimumab is >4 µg/ml and for infliximab >3 mg/ml. Undetectable drug levels may correspond with the apparition of anti-drug antibodies, as their presence detection calls for medical actions regarding treatment [290].

4.5.Anti-TNF-α drugs during pregnancy

There is increasing interest in the use of anti-TNF-α drugs during pregnancy in women with IBD. Indeed, IBD debut occurs frequently in women during childbearing age (78% of UC and 51% of CD patients debuted between 20 and 29 years, with a female predominance) [281,286]. Nevertheless, pregnancy is not associated with IBD improvement, as opposed to other

inflammatory diseases such as rheumatoid arthritis [291–295]. Active IBD can lead to increased pre-term deliveries and spontaneous abortion (up to 31% in active UC patients), and increased probability of low birth weight [291–294,296]. These risks have resulted in an increased frequency of elected childless woman within the IBD group [286]. To increase safety in IBD pregnant woman, sustained remission of IBD is mandatory, often only achievable with anti-TNF-α treatment.

Anti-TNF-α mAb cross placenta mostly from week 28 of gestation to delivery [297]; they are included in category B (no apparent risk) of FDA-classification for pregnancy risk. However, drug level's safety in newborns and the full consequences of such exposure in newborn's immune system development are unclear. Several recent studies in extensive cohorts (PIANO (n=426), OTIS registry (n=74) and CRIB study (n=31)) of infants exposed to anti-TNF-α drugs during, at least, the second and third trimester of pregnancy, showed that both anti-TNF-α adalimumab and infliximab were detectable in infants from treated mothers for 12 months after birth, while certolizumab was barely detectable. Infants showed no apparent major clinical consequences [298–301]. Nevertheless, a fatal case of disseminated BCG disease after vaccination was reported in an infant whose mother had been treated with high doses of infliximab during pregnancy [302]. It is recommended to delay immunization with live vaccines until 12 months or after confirmation of negative drug levels [300,303].

TNF-α plays a pivotal role in fetal development. Both in regulation of necroptosis and fate-programming during the embryonic state [304–307] and, afterwards, in B cell development. TNF-α knock out mice present abnormal B cell structures; they lack splenic B cell follicles, organized follicular dendritic cell networks and germinal centers. These alterations lead to a decreased humoral response and increased infection risk [308,309]. Nonetheless, mice exposed to anti-TNF-α mAb during gestation did not show any abnormal B cell structures. However, B cell development occurs 3 weeks after birth in mice. Instead, in humans, B cell development occurs during the third trimester of pregnancy and through 8 weeks after delivery [310–312]. A study on the impact of the exposure to golimumab during pregnancy in macaques revealed no effect in B or T cell frequency, nor in humoral responses, or in lymphoid organ formation, but maturation status of the immune system was not assessed [313].

5. The immune system in the neonate

Upon labor, the fetus must transition from the semi-allogenic but sterile uterus of the mother, to a world full of antigens. Most of these antigens will be harmless but some will be harmful and dangerous, and the development of different strategies to overcome these different challenges is of dramatic importance. Just after birth, especially after vaginal delivery, the mucosa of the infant is colonized by commensal microbiota. Thus, the avoidance of excessive responses after this massive first contact with microorganisms is critical, as well as to reduce the risk of alloimmune reactions during late pregnancy; to do so, the immune system has developed different strategies to manage this situation. The neonate's immune system plays a principal role in this special and delicate moment. Study of the neonate's immune system is still on development, and current knowledge has been thoroughly reviewed [314–318]; concretely, Kollmann *et al* have recently elegantly reviewed immune defense against infections in newborns [314]

Neonatal infection is responsible for approximately 700.000 newborns' deaths per year. Transition from the last stages of pregnancy, labor and the first encounters of the newborn with the real-world, claims for rapid changes and adaptation of the immune system: from cell autonomous and innate immunity to the adaptive immune system. Although the neonate's immune system can fight pathogen infections, as a result of the need of tolerance, there is an increased risk for severe infections [314]. For this reason, immunization through maternal IgG during late pregnancy is critical. IgG transfer can confer around three months of broad protection for a variety of infectious diseases including measles, mumps, rubella and varicella. Altered IgG transfer, observed in very low weigh and preterm infants, results in an increased risk of lethal neonatal infection. Newborns have an increased risk of intracellular pathogens infections requiring Th1 responses, especially *Listeria monocytogenes*, *Salmonella spp*. and *Mycobacterium* infections [314–316].

External factors, such as maternal condition during pregnancy and microbiota acquisition, will shape the development of the neonate's immune system. Maternal conditions include infections during the last trimester of pregnancy, highlighting parasitic infections, maternal nutritional imbalance and inflammatory conditions such as autoimmune or inflammatory disease. Microbiota acquisition during delivery and lactation will shape long-term immunological responses, being essential for optimal immune development. Full gut colonization lasts 12 to 18 months, and a symbiosis relation is established between the microbiota and the host. This colonization has been related with tolerance acquisition and innate training to mount effective immune responses upon pathogen encounter. Pharmacological treatments, such as antibiotics, can cause dysbiosis during early age, which is associated with increased risk of immune-mediated diseases or allergy in the newborn [314–317].

The immune system of the newborn has different regulatory mechanisms to promote tolerance. Hypoxia during labor can cause tissue damage enhancing inflammation, so the strong immune bias towards resolution of inflammation and healing is very important. In fact, vaginal or cesarean delivery can affect leukocyte populations and plasma concentration of cytokines [319]. Upon TLR-mediated activation, APCs promote Treg cell differentiation; besides, non-inherited maternal antigens challenge also results in $CD4^+$ T-cell differentiation into Treg cells. Along with Treg cells, myeloid-derived suppressor cells and erythroid suppressor cells are present in cord

blood and regulate $CD4^+$, $CD8^+$ and NK cell activity. Besides, high adenosine levels in blood after inflammatory events and hypoxic states contribute to the generation of an increased presence of cyclic adenosine monophosphate. The presence of these purines promotes a tolerogenic state of the immune system through the impairment of MyD88 pathway activation, thus reducing TLR-mediated Th1 polarizing cytokine induction and neutrophil activation. Besides, altered TLR activation in neonates partially responds to a different micro-RNA profile, including increased levels of the inhibitory micro RNA146a [314–317].

Besides the above-mentioned inflammatory responses' regulation, the neonate has other compensatory non-inflammatory weapons to protect against germ insult. Newborn's skin and mucosa produce high amounts of antimicrobial proteins and peptides. What is more, at birth the skin of the neonate is covered with the vernix, consisting of a waxy-coating full of microbicidal peptides to compensate the reduced toughness of the skin. Innate regulation of the immune system comprises 1) decreased complement system function; 2) decreased neutrophil quantity and functions, including respiratory burst; 3) mononuclear presenting cells (monocytes, macrophages and dendritic cells) have decreased IL-1β, TNF-α and IL-12p70 production, along with normal IL-23 and IL-6 production; 4) decreased IFN-γ response in certain conditions; 5) IRF3 has decreased DNA binding capacity; 6) monocytes produce increased levels of IL-10 and cyclic adenosine monophosphate; 7) conventional DC number is decreased and cells are more immature and produced less inflammatory cytokines and 8) NK cells express higher levels of the inhibitory receptor NKG2A and lower levels of the activating receptors NKG2C, LIR-1 and KIR [314–318].

In the neonate, $CD4^+$ T-helper cells are biased towards Th2-like immunity, with a more anti-inflammatory profile. There is low IFN-γ production, in concordance with decreased IL-12p70 production by mononuclear antigen presenting cells. However, in response to some insults such as BCG vaccination, the newborn can develop adult-like Th1 responses and the Th2 bias can be reverted after this BCG training. This bias is related with epigenetic modifications, as regulatory regions of the Th2 locus are hypomethylated and IFN-γ and other Th1 associated molecules promoter sites are hypermethylated. Besides, Th17 cell levels are low because of reduced transcription of *RORC* transcription factor. There is also impaired T cell signaling by the T cell receptor resulting in decreased transcription of CD40L, IL-2 and IFN-γ genes. B cells are mostly naïve with a poor repertoire and diminished B-cell receptor activity, resulting in decreased antigen response. For this reason, not all vaccines are successful when given at birth, as observed with oral polio, measles and rubella vaccination [314–317].

Neonatal B-cells have been less studied. B cell development and maturation is a complex and regulated process. In peripheral blood we can encounter different B cell subsets that include naïve, transitional, marginal zone like B-cells (expressing IgM, IgD and CD27 in their membrane [320,321]), mature B cells and plasmablasts [320,322]. B cells have been thought as mere "antibody-factories" during years; nowadays it is known that they have different functions including cytokine production and regulation of T cell responses. Activation status of B cells has been studied and is tightly modulated. CD22 is a B cell-restricted molecule that down-regulates the signal between CD19 and the BCR [323–325], the lack of this regulatory molecule results in an increase in B10 cells in mice [326]. Some few published studies on B cells in the neonate have associated B cells with the Th2 bias: asthmatic mothers of infants with early-allergy had an increase in transitional B cells in the late-pregnancy period, suggesting that these cells could have a role in the Th1/Th2 bias observed in neonates [327]. Murine studies have shown that $CD5^+$ B

cells of newborn mice also contribute to the decrease in IL-12p70 production [328]. On the other hand, it is known that infusion of stem cells from cord blood rather than adult bone marrow enables transplantation in patients with increased donor-recipient HLA-mismatch [329], and one of the possible mechanisms explaining this augmented allogenic tolerance is the B cell-mediated regulation trough B regulatory cells (Breg). [330] The frequency of Breg cells in the neonate can predict the severity of acute bronchiolitis disease after respiratory syncytial virus, thus showing how neonatal Breg cells can modulate microbial pathogenesis [331].

5.1.Breg cells

Avoidance of unwanted immune responses through peripheral tolerance involves several regulatory/suppressor molecules and cell types. Breg cells are a rare B cell subpopulation with this regulatory/suppressor function. Although B cells [332] and, more concretely, IL-10 production by B cells [333] have been shown to be important in inflammation in mice, it was not until 2010 by the hand of Blair *et al* that the first description of the phenotype and function of a human Breg cell subset was published [334]. Since then, several markers have been described for the detection and sorting of these cells, although there is not yet a consensus. Breg cells represent fewer than a 10% of total B-cells in circulation; their regulatory activity is mostly but not uniquely performed through IL-10 production; nevertheless, less than 20% of cells from the different described subsets are IL-10 producers after stimulation [335,336].

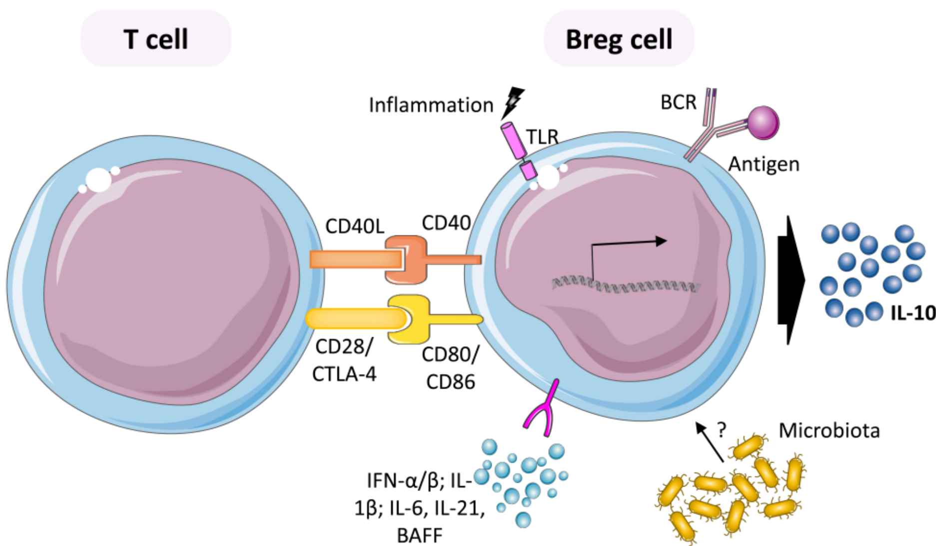

Figure 15. Induction of Bregs. IL-10–producing Bregs that express regulatory genes are generated as a consequence of immune activation. The signals that induce the differentiation of Bregs include inflammatory stimuli (via TLRs), costimulatory signals (CD40, CD80, CD86), microbiota, and cytokines (IFN-α/β, IL-1β, IL-6, IL-21, and BAFF). Adapted from Mauri 2017[335]

There are two theories regarding Breg development: the first states that B cells are a specific lineage with a specific transcription factor that controls the suppressive nature of the cells; the second suggests that B cells can take on a regulatory phenotype after certain stimuli to suppress inflammation. Inflammation is a potent trigger of Breg cell development and differentiation, Breg cells need combination of different molecules to get activated, including TLRs, CD40, the B cell receptor, CD80, CD86 and cytokines **(Figure 15)** [335,336]..

With that, there are 8 different described types of Breg cells in humans [335–338]:

1. Immature B-cells, phenotypically described as $CD24^{hi}CD38^{hi}$. These cells perform their action on CD4 and CD8 T-cells, plasmacitoid DCs and invariant NKT cells by IL-10 secretion, PD-L1, CD80, CD86 and CD1d ligation [334,339–342].
2. B10 cells, described as $CD24^{hi}CD27^{hi}$, produce IL-10 and regulate monocytes and effector CD4 T cells [343].
3. Granzyme B^{+} (GZMB) cells, described as $CD38^{+}CD1d^{+}IgM^{+}CD147^{+}$ exert their regulatory function on CD4 T-cells by IL10, indoleamine 2,3-dioxygenase (IDO) and GZMB [344].
4. Br1 cells are defined as $CD25^{hi}CD71^{hi}CD73^{low}$ B cells and they produce IL-10 and allergen-specific IgG4, thus suppressing allergen-specific CD4 cells, thus maintaining allergen tolerance [345].
5. Plasmablasts, defined as $CD27^{int}CD38^{hi}$; some of them can produce IL-10 but their target cell type is not known yet [346].
6. B-cells expressing CD39 and CD73 that regulate CD4 and CD8 T cells by adenosine formation, thus reducing inflammation by ATP [347].
7. Induced Breg cells, without known phenotypic markers produce TGF-ß and IDO to suppress CD4 T cells. These cells are developed after T-cell CTLA-4 interaction [348].
8. Cells expressing TIM1 inhibit CD8 and CD4 T cells by producing IL-10 [349].

Main functions of Breg cells include inhibition of Th1 cells activation, Th17 differentiation and promotion and maintenance of the Treg cell population [335–337]. The major suppressive mechanism for Breg cell function is IL-10 secretion. IL-10 is a suppressor cytokine that can inhibit chemokine and pro-inflammatory cytokine production, thus inhibiting the effector mechanisms of the immune system; Breg cells are known IL-10 producers and IL-10 blockade partially inhibits their regulatory function. Although IL-10 is a key player in Breg inhibition of inflammation, other mechanisms have been described, including TGF-β (specially for the differentiation of tolerogenic DCs) and IDO production, cell to cell contact by CD80/86 interaction with T-cells, PD-L1 inhibition of T follicular helper cells and CD73-dependent adenosine production [334,336,338,339,343,350–352] .

The most studied subset of Breg cells is defined by $CD24^{hi}$ and $CD38^{hi}$ expression in B-cells [334,339,353]. Phenotypically, these cells also express IgM, IgD, CD5, CD10 and CD1d [334], resembling transitional B cells [354]. Breg-cells are mainly defined by its regulatory function: Mauri *et al.* demonstrated that the $CD19^{+}CD24^{hi}CD38^{hi}$ subset is enriched in IL-10 production and can inhibit IFN-γ production [334,355] and block Th1 and Th17 differentiation while maintaining Treg cell population [339]. Their implication in human immune-related diseases has mostly been studied in autoimmune and allergic diseases [356–365], persistent infections such as HIV [340], HBV [366] and *Mtb* [367], cancer [368–371], transplantation [355,372–375] and, as demonstrated recently, pregnancy [327,356,376–380].

Viruses, bacteria, helminths [381] and parasites [382] can induce B cells with regulatory functions. Rapidly after infection, *Salmonella* and *Listeria* induce the apparition of IL-10 producing cells in a TLR/MyD88 dependent fashion in mice. Some helminth-derived molecules can directly promote IL-10 stimulation in murine B cells. These B cells can suppress immune responses towards

allergens, studies in humans have also shown this bystander regulatory functions after helminthic infections. The lack of helminth infections in westernized countries has been proposed as one of the reasons that can explain the increased incidence of allergy and autoimmunity. Breg role in viral infection has been more studied because of their role in HIV infection and in chronic HBV infection [381]. Immature Breg cells inhibit IFN-γ production by $CD8^+$ T-cells after HBV virus infection [335,366]. Besides, $CD24^{hi}CD38^{hi}$ IL-10 producing cell frequency directly correlates with HIV virus load [383]; what is more, after in vitro Breg depletion, $CD8^+$ T-cell effector function was restored and HIV infected $CD4^+$ cells were cleared *in vitro* [340].

Breg cells are considered to promote a stable tolerant immune profile in the local microenvironment. Recently, Guzman-Genuino *et al*, reviewed how the previous knowledge of the role of Breg cells in autoimmunity and transplantation (promoting tolerance) and cancer (promoting tumor growth) could help in the understanding of the role of Breg cells in the establishment and maintenance of pregnancy, where a semi-allogenic mass of cells grows inside the women [379]. Immunological changes are needed to avoid allogenic reactions that could lead to miscarriage. Body conditions to allow conception and implantation, as well as changes needed to allow embryonic and fetal growth, are regulated by pregnancy hormones. Therefore, it is logic to think about human gonadotropic hormone, estrogen and progesterone as regulators or promoters of these changes.

Similar to what happens in the neonate, after conception, the mother needs to modify its immune system in order to tolerate the semi-allogenic embryo. Maternal-fetal tolerance is achieved through different mechanisms that include an increase of Treg cells, IL-10 and TGF-β expression [384], increase of the inhibitory molecule PD-L1 in the trophoblastic tissue, reduction of APCs and of mononuclear phagocytes and, as reported recently, an increase of Breg-cells [380,385,386]. Pregnancy hormones modify immune responses after conception, including Breg cells. It is known that estrogen can modify decidual immune cells by turning DCs and NK cells into tolerogenic cells; besides it can regulate T cell activities including the expansion of Treg cells [379]. It also has a role in B cells, as it is known to increase, in mice, $CD1d^{high}CD5^+$ IL-10 producing Breg cell subset and PD-L1 expression in B cells, thus protecting mice from experimental autoimmune encephalomyelitis [379,380,387,388]. Progesterone promotes Th2-like immune responses with a reduction in pro-inflammatory cytokines and increased IL-10 production that is associated with a B cell expansion. Human gonadotropic hormone from pregnant women sera increase IL-10 production by B-cells **(Figure 16)** [376,380].

First associations of Breg cells with pregnancy success were performed in mice. In pregnant mice, the increase in $CD5^+CD1d^+$ Breg cells is necessary to avoid immunological abortion. In fact, the transfer of Breg cells to abort-prone mice promotes fetal-maternal tolerance by leading to a Treg cell increase and by maintaining DCs in an immature state [377]. In humans, it was observed that women treated with rituximab, a B cell depleting antibody, during pregnancy presented a higher rate of first-trimester pregnancy loss[389], thus suggesting a principal role of B-cells in pregnancy. Later, $CD24^{hi}CD38^{hi}$ [334] and $CD24^{hi}CD27^+$ [343] have been recently used for the study of Bregs in pregnant women [378,380]. $CD24^{hi}CD27^{hi}$ cells increase in the first trimester of pregnancy but, as observed with mice Breg cells, this increase does not occur in patients with spontaneous abortion. What is more, almost 95% of $CD24^{hi}CD27^{hi}$ B cells express the receptor for human gonadotropic hormone [376]. These data highlight the importance of B-cells, specifically

Breg, in the mother's achievement of immune tolerance during the first steps of pregnancy. The importance of this subset of cells in the fetus and the newborn is still yet to be determined.

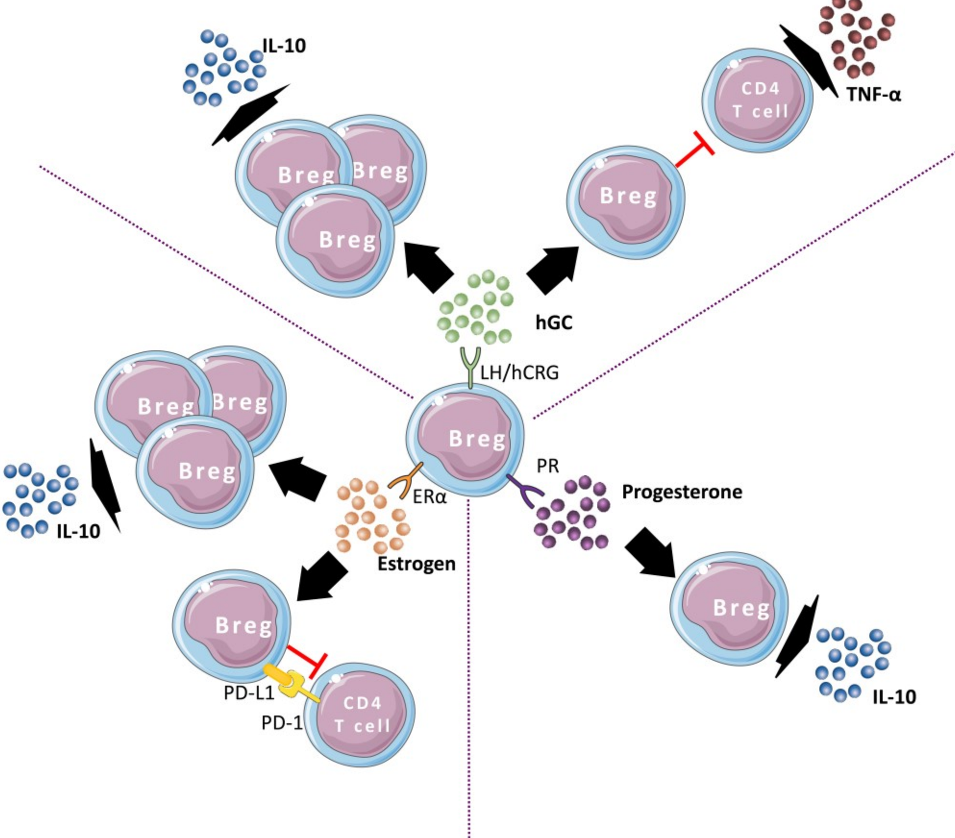

Figure 16. Schematic representation depicting the influence of female sex hormones in the activation and subsequent function of regulatory B cells (Bregs). (A) Estradiol (E2), acting through estradiol receptor alpha (ERα), induces the proliferation as well as the production of IL-10 by regulatory B cells (Breg). In addition, E2 mediates the control of T cells' activation by B cells throughout a mechanism involving PDL1/PD1 pathway. (B) Human chorionic gonadotropin (hCG) was proposed to induce proliferation of regulatory B cells. Besides, this hormone induces the production of IL-10 by B cells that in turn inhibit the production of TNF-α by T cells. (C) Progesterone (P4) induces the production of the potent anti-inflammatory and Breg hallmark cytokine IL-10 by B cells. Adapted from Muzzio 2014 [380]

HYPOTHESIS and OBJECTIVES

1. Hypothesis

This thesis is framed in the concept that the IL-12/IFN-γ axis is fundamental for the control of intracellular infections. A dysfunction in this axis, inherited or acquired, can be observed in specific clinical situations not yet explored, concretely:

We hypothesize that patients with extrapulmonary *Mycobacterium tuberculosis* infection or visceral leishmaniasis have a **primary dysfunction** of the IL-12/IFN-γ axis and that the study of these patients will lead to detection of new cases of MSMD, in which specific treatment approaches could be applied and that exposure to anti-TNF-α antibodies during whole pregnancy in children born to mothers with inflammatory bowel disease affects the normal development of the neonatal immune system, conferring a **secondary immunodeficiency**, which includes a dysfunction of the IL-12/IFN-γ axis.

2. Objetives

The general objective of this project is to better understand the state of IL-12/IFN-γ axis defects both by inborn errors of immunity and secondary to anti-TNF-α exposure during pregnancy.

The specific objectives are:

Objective 1. To elucidate if patients with intra-macrophagic infections (visceral leishmaniasis or severe/tuberculosis) have defects on the IL-12/IFN-γ axis.

Objective 2. To define the effect of whole-pregnancy exposure to anti-TNF-α antibodies used in inflammatory bowel disease in the exposed infant immune system.

Objective 2.1. To determine if IL-12/IFN-γ axis is affected in the exposed infants

Objective 2.2. To characterize different lymphoid populations in the exposed infants

Objective 3. To establish lymphoid populations normality values in cord blood, including Breg cells and IL-12/IFN-γ axis

RESULTS

Chapter 1. Study of IL-12/IFN-γ axis in patients with severe/extrapulmonary tuberculosis and visceral leishmaniasis

We have studied the IL-12/IFN-γ axis in patients with severe TB or VL. For the recruitment of patients, we established different collaborations with autonomic, state and international centers, including Hospital Sant Joan de Déu and Hospital Vall d'Hebron in Barcelona, Hospital Joan XXIII in Tarragona, Hospital General in Granollers, Hospital La Fe in Valencia, Hospital Gregorio Marañón and 12 de Octubre in Madrid, Hospital Son Espases in Mallorca and Instituto Nacional del Niño in Lima in Perú. Thanks to this collaboration, we have been able to recruit 25 patients with TB and 23 with VL. However, one of the patients was removed from the TB cohort because, although first diagnosed as suffering from TB, he was misdiagnosed and indeed suffered from complicated BCGosis. We have also included in the study 41 healthy controls, of whom 23 were related to the patients (R-HC, mostly parents) and 18 were non-related to the patients (NR-HC).

Epidemiologically, patients with TB presented a broader ethnic variation while most of VL where from Caucasian origin. Of interest, VL patients were younger at age of infection (1.58 years old, 0.25 to 8 years old in VL patients and 3 years old, 0.47 to 16 years old in TB patients, median, range). However, these differences disappeared when comparing the age at test (7.42 years old, 0.42 to 12.54 years old in VL patients, 7.28 years old, 0.62 to 12.54 years old in TB patients, median, range). Only two families declared consanguinity. In TB patients, location of the infection was majorly disseminated (33% of total), with (36.4% of disseminated infections) or without (63.6% of disseminated infections) meningitis, adenopathic (21% of total) or meningitis (17% of total). All patients showed normal lymphocytic subsets, proliferation response to mitogens and oxidative burst responses.

Gold-standard technique for the study of the integrity of IL-12/IFN-γ axis, used for MSMD diagnosis, is the detection of secreted cytokines after a whole blood culture in presence of BCG, with or without hrIL-12p70 or hr-IFN-γ in order to evaluate anti-mycobacterial response *ex vivo* [119,173] (see section 2.2.2). This technique is especially useful for the detection of complete defects of the IL-12/IFN-γ axis. However, there is a big variability in the observed responses both in healthy controls and patients [81,89,119] that hinders the identification of partial defects. Besides of the culture, we have studied IFN-γR1, IFN-γR2 presence in monocytes, IL-12Rβ1 presence in activated lymphocytes and STAT1 phosphorylation in response to IFN-γ in monocytes, without observing any alterations.

High variability observed in cytokine production was observed in both NR- and R-HC. However, after the performance of co-stimulation ratio (Co-SR, cytokine production with 'BCG+Co-stimulus'/BCG alone), there was a reduction of the coefficient of variation in NR-HC that was not observed in R-HC. Also, the pattern of produced cytokines was different between the two groups. R-HC, when compared to NR-HC, had a decreased production of IFN-γ and increased production of IL-6, IL-10 and TNF-α after BCG stimulation. Besides, there was a reduced Co-SR of IL-6 and TNF-α after hr-IL-12p70 co-stimulation and a reduced Co-SR of TNF-α and IL-12p70 after hr-IFN-γ co-stimulation.

Concordantly with the cytometric studies results **we did not find any complete defects of the IL-12/INF-g axis in patients with extrapulmonary TB or VL infections**. However, we observed different cytokine production patterns. Patients with TB showed an increased IL-10 and TNF-α

secretion and a diminished IL-1RA secretion after BCG stimulation when compared with NR-HC (this difference was not observed in R-HC). Of note, response to IFN-γ in terms of IL-12p70, IL-1β and TNF-α production was diminished. VL patients presented diminished IFN-γ production after BCG stimulation compared to all the other groups studied, which was rescued to normal levels after IL-12p70 co-stimulation. **Altogether, these data showed that studied TB patients had an impaired response to IFN-γ while studied VL patients had an impaired production of IFN-γ.**

Detailed methods, results and figures are shown in "*Altered IFN-γ circuit in extrapulmonary tuberculosis and visceral leishmaniasis patients and related controls*" manuscript.

The case of the patient that was removed from the TB cohort was of special interest. She was a Peruvian 6 years old girl diagnosed with a recurrent, severe and disseminated TB. Her case was remitted to us for the study of the integrity of the IL-12/IFN-γ axis. We observed no expression of IL-12Rβ1 in the membrane of activated lymphocytes. Further studies confirmed this functionally, as there was no STAT4 phosphorylation in response to IL-12p70 in activated lymphocytes and the production of IFN-γ after BCG stimulation was low and non-rescued after IL-12p70 co-stimulation. Genetic studies confirmed a mutation in *IL12RB1* gene (p. (Arg211*; c.631C>T). Thus, being the first Peruvian patient with a genetic confirmation of MSMD disease. To receive the appropriate treatment, consisting in hr-IFN-γ, she was transferred to the National Institute of Health in the United States of America. Once there, further microbiological studies revealed that the patient had been initially misdiagnosed and that BCG-derived *Mycobacterium bovis* infection was the infectious agent. Anti-mycobacterial treatment was then optimized and supplemented with hr-IFN-γ. The patient is now stable and without active infection. This case demonstrates the importance of PID suspicion amongst clinicians. BCGosis is a much rarer infectious event compared with TB; however, its presence is virtually always associated with immunodeficiency states, inherited or acquired.

Detailed methods, results and figures are shown in "*Severe BCGosis misdiagnosed as multidrug-resistant tuberculosis in an IL-12Rβ1-deficient Peruvian girl*" manuscript.

1.1. Manuscript "Altered IFN-γ circuit in extrapulmonary tuberculosis and visceral leishmaniasis patients and related controls"

Title page:

Altered IFN-γ circuit in extrapulmonary tuberculosis and visceral leishmaniasis patients and related controls.

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Key words:

IFN gamma, mycobacterium tuberculosis, visceral leishmaniasis, immunodeficiency, infection

Abbreviated title: (<55 characters)

Altered IFN-γ circuit in MTB and VL patients and families.

Running title: (<44 characters)

Altered IFN-γ circuit in MTB and VL patients.

Abstract:

Background: Genetic defects in IL-12/IFN-γ axis confer susceptibility to intracellular pathogens in otherwise healthy children, especially but not restricted to non-tuberculous mycobacteria. The main aim of this work is to understand the status of the IL-12/IFN-γ axis in patients with/severe extrapulmonary tuberculosis (TB) or visceral leishmaniasis (VL) and unravel if this susceptibility is due to complete defects in the pathway.

Methods: A retrospective and prospective cohort study of the IL-12/IFN-γ axis of 23 patients with VL and 24 patients with TB compared to controls (n=41, 18 non-related controls and 23 related controls). After discarding other PIDs, whole blood culture in the presence of BCG with or without IL-12p70 or IFN-γ co-stimulation was performed to analyze cytokine secretion.

Results: Related and non-related controls presented differential cytokine production patterns. Although we did not detect any complete defect, patients with VL or severe TB showed an altered IL-12/IFN-γ axis with reduced IFN-γ production and response, respectively.

Conclusions: TB and VL infections in our media are not warning signs for complete defects of the IL-12/IFN-γ axis. However, the altered cytokine production after mycobacterial challenge reveals the need for thorough genetic studies searching for partial or somatic defects as well as epigenetic changes.

Introduction

Genetic theory of infectious tries to explain why not all individuals respond the same way to infections. It states that susceptibility to severe primary infections in the pediatric age can be explained by single-gene mutations in immune-related genes. This concept has been stated and nicely reviewed by Casanova and Abel[1–4]. Primary immune deficiencies (PID) are the paradigm of single-gene mutations conferring susceptibility to infectious disease. During many years, PIDs were thought to be extremely rare and to confer susceptibility to a wide range of pathogens. However, in the last years, “atypical” PIDs have shown how single-gene errors can lead to susceptibility to one or a narrow range of pathogens, and that there can be only one infection during lifetime which, by the way, can be lethal[1–4].

Mendelian Susceptibility to Mycobacterial Disease (MSMD) is one of the better known “atypical” PID. It is caused by inborn errors impairing the final killing of intra-macrophagic pathogens. Immunity to these pathogens, whose major representative are mycobacteria, relies on the IFN-γ circuit[5,6]. Briefly, after mycobacteria (or other intra-macrophagic pathogens) are engulfed by dendritic cells or macrophages, these cells produce IL-12p70, thus activating T helper (Th) cells

and enhancing IFN-γ production. IFN-γ makes macrophages produce more IL-12p70; creating a positive loop; besides, it activates oxidative burst leading to the killing of intra-macrophagic pathogens[7–11]. Currently, at least ten MSMD-causing genes (*IFNGR1*, *IFNGR2*, *STAT1*, *IRF8*, *CYBB*, *IL12B*, *IL12RB1*, *IKBKG*, *ISG15*[6] and *TYK2*[12]) have been described, giving place to 19 different genetic etiologies.

MSMD has not yet defined its clinical boundaries, but is characterized by infections by non-pathogenic mycobacteria (including BCG and environmental mycobacteria) alone or in combination with *Salmonella* or *Candida*. Infection by other intra-macrophagic pathogens has also been observed[6]; including severe forms of *Mycobacterium tuberculosis* (*Mtb*) infections and visceral leishmaniasis (VL) (alone[13,14] or in combination with other infections[6]). *Leishmania infantum* is endemic in Spain, especially in the Mediterranean basin; giving rise to VL in childhood[15–18].

Before the suspect of MSMD in patients with severe disease caused by *Mtb* or VL, other forms of PID need to be discarded, such as chronic granulomatous disease[19,20], T-cell deficiencies, NF-κB deficiencies, CD40L deficiency and AR TYK2 deficiency[21]. Different etiologies of MSMD, especially IL-12Rβ1 deficiency[22], followed by IFN-γR1, STAT1, and IL-12p40 deficiency, were found in patients with severe tuberculosis (TB). There are currently 26 reported patients with severe TB due to inborn errors of IFN-γ, 13 of whom are IL-12Rβ1-deficient, including six who did not suffer from any other mycobacterial disease[21–23]. Recently, five cases of *Leishmania* infections in patients with mutations in MSMD-causing genes have been described, both alone[13,14] or in combination with other infection[24–26]; patients had mutations in *IL12RB1*[13,14,24,26] and *IL12B*[13].

The main aim of this work was to reveal whether patients with extrapulmonary *Mtb* or visceral *Leishmania spp.* infections had defects in the common disease-causing described genes in MSMD or have defects in mycobacterial response. We have observed no complete defects in the studied patients but a distinct cytokine secretion pattern in related healthy controls, extrapulmonary TB and VL patients showing a decreased response after mycobacterial challenge, rising the need to perform thorough studies at genetic and epigenetic level.

Materials and methods:

Study design

This is a transversal study. We recruited patients both retrospective and prospectively. Inclusion criteria consisted of pediatric patients with extrapulmonary *Mycobacterium tuberculosis complex* infection, all of them were pyrazinamide sensitive to exclude *Mycobacterium bovis* infection, or VL confirmed by direct observation or positive PCR. Patients with other PID, concomitant treatment with immunosuppressive drugs or other conditions predisposing to infections were excluded. All samples were obtained at least 1 month after infection's resolution.

For ethical reasons, controls were not paired by age. However, a cohort of anonymous non-related healthy controls (NR-HC) was recruited along with related (mostly parents) healthy controls (R-HC) cohort.

This study was carried out in accordance with the recommendations of Ley General de Sanidad (25/4/1986) Art. 10., with written informed consent from parents of pediatric patients and from healthy controls. The protocol was approved by the ethics committee of the Hospital Sant Joan de Déu (Number of the *Comité Ético de Investigaciones Clínicas*: PIC-50-12).

Whole blood culture and cytokine detection

Venous blood was collected into heparinized tubes, and diluted 1:2 in complete medium (RPMI (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA), 1 μg/ml penicillin and 1 μg/ml streptomycin (Invitrogen, Grand Island, NY, USA) and incubated at 37ºC in a 5% CO2 humidified incubator for 18h and 48h. Activation conditions: medium alone, live BCG (*Mycobacterium bovis* BCG, Pasteur sub-strain, kindly provided by Dr. Julià González at the Microbiology Department at Hospital Clínic de Barcelona) at a multiplicity of infection of 20 BCG per leukocyte, BCG plus human recombinant IL-12p70 (hrIL-12p70, 20ng/ml, Milteny Biotec, Germany), BCG plus hr-IFN-γ (5,000 IU/mL) as described elsewhere[20,27].

IFN-γ detection was performed by enzyme-linked immunosorbent assay (eBiosciences, San Diego, CA, USA) at 48h culture point and IFN-γ, IL-10, IL-12p70, IL-6, IL-1RA, IL-1β and TNF-α secretion determination was assessed by Luminex (Millipore, Billerica, MA, USA) at 18h culture point following the manufacturer's instructions.

Besides the production of cytokines to mycobacteria itself, an important part of the mycobacterial response is the cross-talk from T cells and macrophages by responding to cytokines. Here, we have evaluated BCG co-stimulation with hrIL-12p70 or IFN-γ by studying the capacity of the system to respond to two key cytokines (IL-12p70 and IFN-γ) co-stimulation, as a co-stimulation ratio (Co-SR; i. e. IFN-γ costimulation ratio = 'IFN-γ production after BCG + IL-12p70 stimulation' ⁄ IFN-

γ production after BCG stimulation'). Methods for cytometric determination of IFNGR1, IFNGR2, IL-12Rβ1 and STAT1 phosphorylation by flow cytometry are explained in supplementary methods.

Statistical analysis

Data distribution and shape were studied with coefficient of variation calculation (standard deviation/mean) and asymmetry tests. Asymmetry tests revealed that data did not follow a normal distribution (data not shown). Accordingly, non-parametric tests were performed. First, Krustal-Wallis test was performed to study if differences observed between groups were statistically significant. If statistically-significant differences were found, U from Mann-Whitney test was performed to study the differences between the groups by pairs. Statistical significance was set for p values lower than 0.05.

To assess correlation between variables, Spearman test was performed. If $p<0.05$ variables were considered to be associated. Besides, correlation coefficient determined the strength of the association. When correlation coefficients were lower than 0.5 association was considered weak association, if between 0.5 and 0.8 moderate association and higher than 0.8 strong association.

In favor of the understanding and legibility of the paper, median and standard deviation values are showed in **Supplementary Table 1 and 2** instead of inserted in the text. Statistical analysis was performed with SPSS v22 (IBM corporation, Armonk, NY, USA) and graphical representation was performed both with SPSS and GraphPad 7 (GraphPad Software, La Jolla, CA, USA).

Results

Cohort description

We included 24 patients with extrapulmonary TB infection, 23 patients with VL and 41 healthy controls (18 non-related and 23 related to the patients, usually parents). There was a bias to female patients in VL (65,2% female *vs*. 34.8% male). Patients with TB infection presented with a variety of ethnicities while VL patients were mostly Caucasian (Table 1). Of note, only 2 families reported consanguinity. Patients with VL were younger at principal infection than TB patients (1.58 years old (yo); range 0.25 to 8 yo in VL *vs.* 3 yo; range 0.47 to 16 yo in TB; p:0.002). However, this difference disappeared when comparing the age when the test was performed (7.42 yo; range 0.42 to 12.54 yo for VL *vs.* 7.28 yo; range 0.62 to 18 yo in TB; p: 0.496). Table 1. TB infections were mostly disseminated (33%), with (12%) or without meningitis (21%), adenopathic (21%) or meningitis (17%) (Figure 1).

Immunophenotype, proliferation in response to mitogens and oxidative burst response studies discarded more common PID associated with the infections (data not shown). Besides, patients showed no alterations after cytometric study of IFN-γ receptors 1-2 expression in the membrane of monocytes and IL-12Rβ1 expression in the membrane of PHA-activated lymphocytes and STAT1 phosphorylation in response to IFN-γ (data not shown). Thus, discarding common causes of complete defects giving rise to susceptibility to mycobacterial disease.

Mycobacterial responses present a big interindividual variability in healthy controls

To assess mycobacterial response, we measured cytokine secretion in the supernatants of a whole blood culture with live BCG. We have measured IFN-γ, IL-12p70, IL-1β, IL-1RA, IL-6, IL10 and TNF-α after 18h and IFN-γ after 48h culture. In almost all cytokines assayed, coefficient of variation (CV) of cytokine production after BCG stimulation was >100, thus showing a high variability both in R-HC and NR-HC Variability of Co-SR was reduced significantly in NR-HC compared to cytokine production after BCG stimulation (p=0.014). This did not happen in R-HC, in fact, we observed increased CV in R-HC compared to NR-HC, although it did not reach statistical significance (p=0.086). (Figure 2, Supplementary table 1).

R-HC showed an altered cytokine secretion pattern

Comparison between NR- and R-HC revealed differences in cytokine production. R-HC produced less IFN-γ (p=0.013) and more IL-10 (p=0.002) after 18h post-stimulation than NR-HC while presenting with increased IL-6 (p=0.028), and TNF-α (p=0.044) and a trend towards an increased IL-1β production (p=0.07). After IL-12p70 co-stimulation, R-HC presented a reduced Co-SR in IL-6 (0.003) and TNF-α (0.01), and a trend towards a diminished Co-SR in IL-1β (p=0.068). On the other hand, after IFN-γ co-stimulation, they presented a reduced Co-SR in TNF-α (p=0.028). Also, we observed a trend towards a diminished Co-SR in IL-12p70 (p=0.066) and IL-1β (p=0.076). Altogether, this data shows an altered response after mycobacterial challenge in R-HC (Figure 3, Supplementary Table 2).

TB and VL patients have altered cytokine producing patterns compared with NR-HC.

BCG stimulation in TB patients led to increased production of IL-10 (p=0.01) and TNF-α (0.019) and a trend towards increased IL-6 (p=0.077) after BCG stimulation when compared with NR-HC. Besides, we observed a diminished IL-1RA production compared to both R-HC (p=0.03) and NR-HC (p=0.014). As observed in R-HC, TB patients presented a decreased IL-12p70 Co-SR after IFN-γ co-stimulation (p=0.008). These patients also presented a decreased IL-1β and TNF-α Co-SR both to IL-12p70 (IL-1β, p=0.016; TNF-α, p=0.006) and to IFN-γ (IL-1β, p=0.016; TNF-α, p=0.021). We can then state that TB patients presented a decreased response to IFN-γ.

IFN-γ decreased production after BCG stimulation was a distinctive feature in VL patients. IFN-γ production was reduced in VL patients in comparison with all the other groups studied both at 18h (TB, p<0.001; R-HC, p<0.001; NR-HC, p<0.001) and 48h (TB, p=0.001; R-HC, p<0.008; NR-HC, p<0.001) post-culture. IL-10 production was also reduced in comparison to NR-HC (p=0.022). VL patients presented differences with R-HC: a diminished IL-1β (p=0.0.49) and IL-6 (p=0.047). IFN-γ Co-SR after IL-12p70 addition was superior in VL patients than in the other groups studied (MTB, p=0.003; R-HC, p=0.015; NR-HC, p=0.005). Besides, there was a trend towards a decreased IL-1β Co-SR ratio after IFN-γ co-stimulation (p=0.064).

The ratio between inflammatory and anti-inflammatory cytokines conditions the final response. As we have observed differential changes in IL-10 and IFN-γ production, we wanted to compare the ratio between these cytokines; of note, IFN-γ/IL-10 ratio after BCG stimulation was lower in all studied groups when compared with NR-HC, and was especially low in VL patients (Figure 4).

Neither age at infection nor age are strongly associated with cytokine secretion.

As the range of age at which both the test is performed (0.42 to 18 yo) and the age of primary infection (0.25 to 16 yo) was large we wanted to test if there was any association of the different cytokine production with age. After correlation test was performed, we found no association between the age of the test and cytokine production or fold-change after co-stimulation in VL patients. In TB patients, however, we observed a weak association between age at infection and IL-1RA production (r: 0.454, p=0.026). Besides, after IL-12 co-stimulation we observed a weak inverse association in IL-10 Co-SR (r: -0.483, p=0.017) and moderate in TNF-α Co-SR (r: -0.704, p<0.001).

Discussion

Development of severe intra-macrophagic infections in otherwise healthy children without any acquired predisposition implies the suspicion of inborn errors of immunity. Since disease-causing mutations have been found in MSMD-related genes in patients with severe extrapulmonary TB[26,28] and VL we considered that the study of the function of the IL-12/IFN-γ axis in these patients needed to be studied. To our knowledge, this is the first time that the function of the IL-12/IFN-γ axis has been systematically studied in patients with severe/extrapulmonary TB and VL. After functional evaluation of the pathway with described methods[20], we did not find any classical complete defect on the IFN-γ circuit. Nonetheless, both groups showed altered cytokine secretion patterns: a defect in IFN-γ production in VL patients and of IFN-γ response in TB patients.

The gold-standard technique for MSMD diagnosis consists in cytokine secretion determination after whole blood stimulation with live BCG in the presence/absence of IL-12 or IFN-γ co-

stimulation[20,27]. However, cytokine secretion detection techniques have intrinsic associated variability; cytokine production is strongly influenced by concurrent or past infections[29], time of blood drawn[30,31], stress[32,33], seasonality[34] or the point in the menstrual cycle in women[35]. High variability in healthy controls have been already observed[25,36,27] but has not been quantified. Quantification of the CV revealed a high variability in healthy controls, especially on R-HC. There are two main types of functional impairment of the IL-12/IFN-γ axis, IFN-γ production defects and IFN-γ response defects. We speculate that the increased variability in response to IFN-γ and IL-12p70 observed in R-HC may respond to the differential affectation of the pathway observed in the two groups of patients, carried by their parents.

Patients with VL had a defective response after mycobacterial challenge. Although we have not detected any complete defect, VL patients had a defect in IFN-γ production in response to BCG, rescued after IL-12p70 co-stimulation. Since IFN-γ production is required for parasite killing in the macrophage, an altered production may result in defective killing[37–39]. Of note, during active infection, PBMC's of infected individuals have an altered IFN-γ production that is restored upon clearance of the infection[40,41]. Since in all patients the study was performed, at least, one month after resolution of the infection, the inhibitory capacity of *Leishmania* parasites should not interfere with the test. Innate immune training theory states that at birth innate immunity is immature and acquires Th-1-like characteristics after antigenic exposition due to epigenetic changes[42–45]. On the other hand, BCG vaccination is shown to have Th-1 driving capacities, and it has been shown to confer cross-protection to other pathogens by increasing IFN-γ and TNF-α production after specific epigenetic changes[46–48]. The low IFN-γ production after mycobacterial challenge might respond to both inborn errors of immunity or, also, to epigenetic changes derived from the exposure to *Leishmania* at young ages, alone or in combination.

Severe/extrapulmonary TB patients present with altered cytokine production after BCG stimulation and reduced response to IFN-γ. IL-1β, along with TNF-α, contributes to granuloma formation and maintenance and restriction of intracellular growth in macrophages[49–54]. TNF-α is necessary for the control of both non-virulent, as BCG, and virulent species as *Mtb* of mycobacteria, IL-1β is only necessary for the control of virulent mycobacteria[49]. Decreased IL-1RA (reflect of IL-1β production) might reflect defects on granuloma formation, thus promoting dissemination of the disease to other organs that may not be rescued with an increased production of TNF-α. Impaired response to IFN-γ may be somehow explained by the increase of IL-6 production after mycobacterial challenge, as IL-6 has been shown to inhibit IFN-γ responses in mycobacterial infection[55]. Of note, a similar pattern of cytokine production and response has also been observed in the R-HC group.

There are two other studies screening for MSMD-related defects in *Mycobacterium tuberculosis* infections, one searching for mutations in *IL12RB1* (2/50 deficient patients) in endemic and highly consanguineous regions[26] and the other searching for common mutations in 114 adults with severe tuberculosis and for common or new mutations in 10 children with extrapulmonary mutations in *IL12RB1, IFNGR1, IFNGR2* and *STAT1* genes without encountering any mutations in a region with low consanguinity rates[28]. With these and our results, we hypothesize that severe/extrapulmonary TB in patients without consanguinity is not a warning sign for known MSMD-related complete defects. However, studies searching for hypo-morphic or somatic mutations as well as epigenetic defects are needed to better characterize and treat these patients.

Although this study has several strengths, it also has some limitations: 1) the variability observed in healthy controls difficult the interpretation of patient's results, 2) the size of the cohort and 3) the impossibility to compare the results to healthy controls paired by age. However, we have observed coherent and robust results showing statistically significant differences between the studied groups. Altogether, these data suggest that severe/extrapulmonary TB and VL patients have a familial altered mycobacterial response. More research needs to be performed to discover new mechanisms conferring this susceptibility. The use of high throughput technologies may facilitate the study of epigenetic changes, somatic mutations or complex mechanisms of susceptibility. Besides, it is necessary to develop tools to diminish this variability in order to maximize the diagnostic value of this technique.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures:

Figure 1. ***Mycobacterium tuberculosis*** **infection localization in TB patients.** (n=24)

Figure 2. Coefficient of variation after mycobacterial challenge. Coefficient of variation (CV) is calculated as "standard deviation/mean". CV for different conditions is represented as a heat map; blue indicates the minimum values, red the maximum, and yellow median values. NR-HC, n=18; R-HC, n=23. NR-HC: non-related healthy control; R-HC: related healthy control; CV: coefficient of variation

Figure 3. Differential cytokine secretion after mycobacterial challenge. Cytokine production after BCG stimulation, and co-stimulation ratio is represented as a heat map. Co-stimulation ratio (Co-SR) is calculated as "BCG + co-stimulation (IFN-γ or IL-12p70) condition /BCG condition". Blue indicates the minimum values, red the maximum, and yellow median value for cytokine secretion after BCG stimulation and next to one for co-stimulation ratio. NR-HC, n=18; R-HC, n=23; VL patients, n=23; MTB patients, n=24. NR-HC: non-related healthy control; R-HC: related healthy control; VL: visceral leishmaniasis; TB: tuberculosis.

Figure 4. IFN-γ/IL-10 ratio. Ratio of IFN-γ/IL-10 after 18h of culture with BCG. NR-HC, n=18; R-HC, n=23; VL patients, n=23; MTB patients, n=24. NR-HC: non-related healthy control; R-HC: related healthy control; VL: visceral leishmaniasis; MTB: *Mycobacterium tuberculosis*.

Table 1. Cohort characterization

		TB n=24	VL n=23
Gender	Female	54.2% (13)	65.2% (15)
	Male	45.8% (11)	34.8% (8)
Ethnicity	Caucasian	29.2% (7)	95.7% (22)
	North-African	16.7% (4)	3.3% (1)
	Sud-African	8.3% (2)	
	Arabic	20.8% (5)	
	Latin-American	16.7% (4)	
	Asiatic	8.3% (2)	
Age at infection[1]	Median	3	1.58
Years old	Range	0.47 to 16	0.25 to 8
Age at test[2]	Median	7.28	7.42
Years old	Range	0.62 to 18	0.42 to 12.54

[1]p=0.002; [2]p=0.496

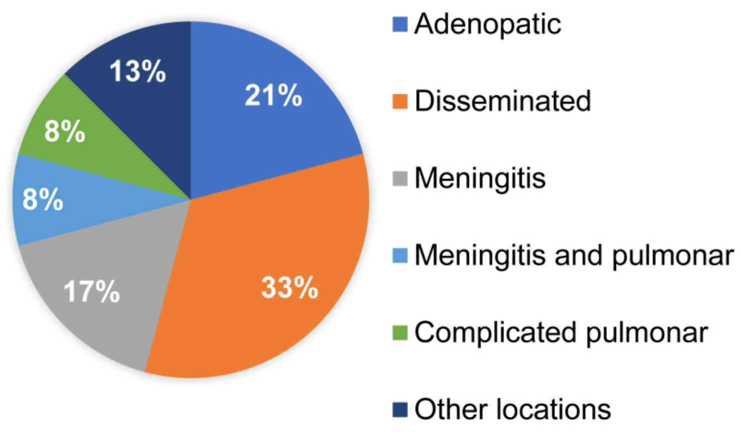

Figure 1

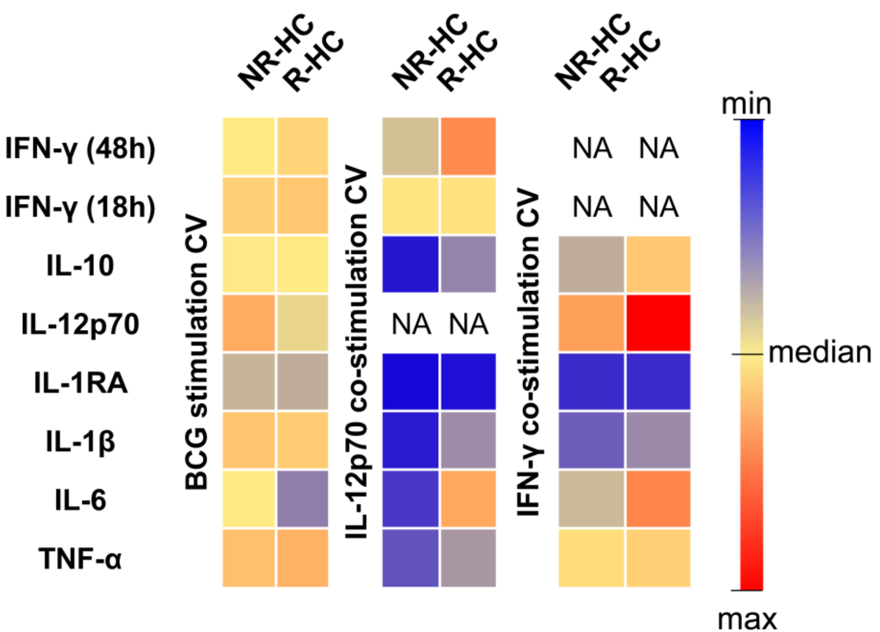

Figure 2

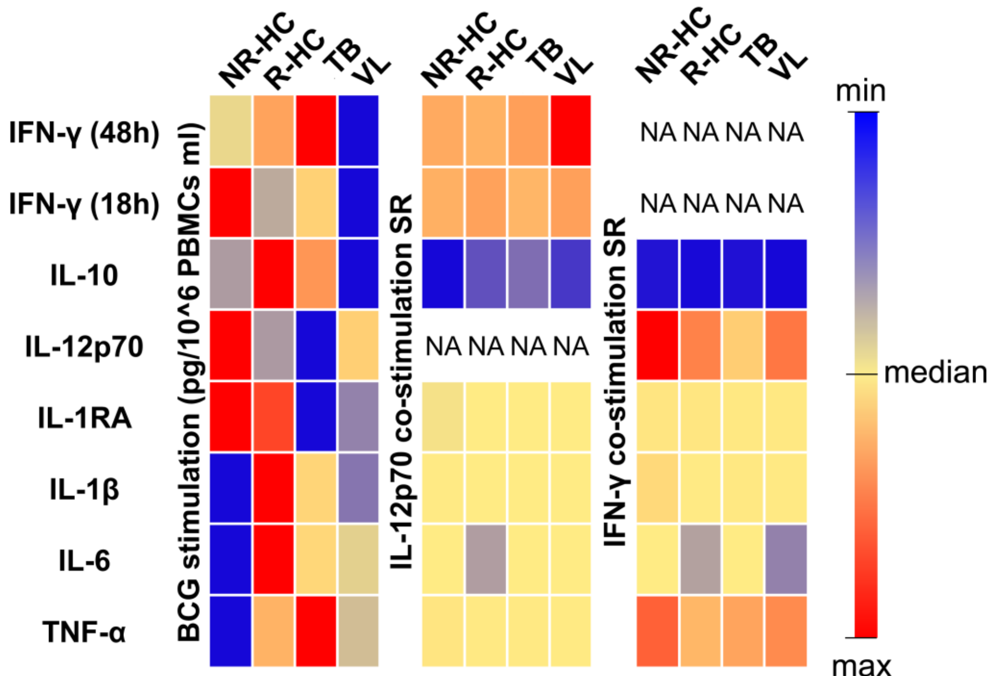

Figure 3

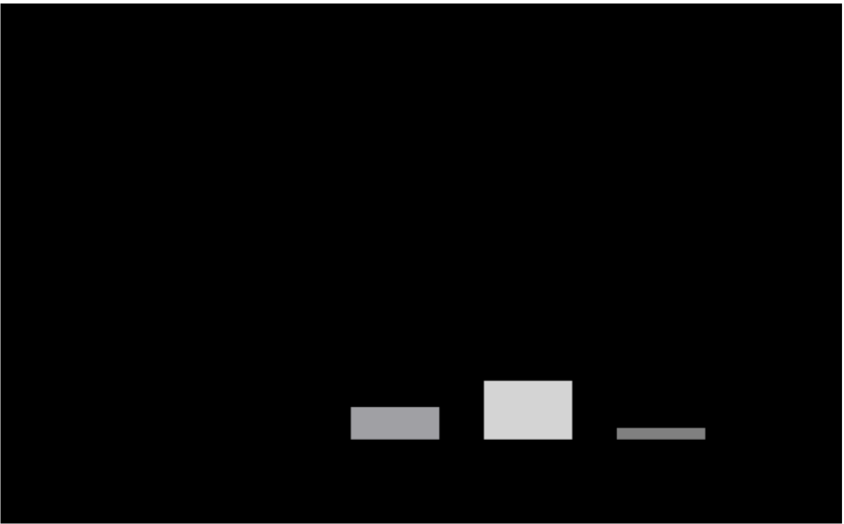

Figure 4

Supplementary methods

Flow cytometry tests

Cytometric determination of IFN-γR1 and IFN-γR2 presence and STAT1 phosphorylation in response to IFN-γ were performed in whole blood. For IFN-γR1 (GIR-94, BD Bioscience, San Jose, CA, USA) and IFN-γR2 (polyclonal, R&D, Minneapolis, MN, USA). 50ul of whole blood were blocked with goat serum, then stained with specific antibodies, lysed (BD lysing solution, BD Bioscience) and washed twice with phosphate buffer saline (PBS, Roche, Spain, EU). For STAT1 phosphorylation detection (clone 4a, BD Bioscience), 50 µl of whole blood were stimulated with $5 \cdot 10^3$ IU/ml of IFN-γ (Imukin, Boehringer Ingelheim, Spain) for 15 minutes prior to intracellular staining following BD PhosFlow protocol.

IL-12Rβ1 presence and was performed in density gradient isolated peripheral blood mononuclear cells. Cells were stimulated 72h with phytohemagglutinin (5ug/ml, Sigma-Aldrich, St. Louis, MO, USA) previously to IL-12Rβ1 (clone 2.4E6, BD Bioscience) detection.

Cells were acquired with a Canto II cytometer in the next two hours after staining. Data was analyzed with FlowJo 7.3 software (TreeStar, Inc., Ashland, OR, USA).

Supplementary table 1. Coefficient of variation of the different parameters.

	NR-HC	R-HC	TB	VL
	BCG stimulation CV			
IFN-γ (48h)	110.25	127.48	151.86	325.04
IFN-γ (18h)	129.81	136.32	126.96	169.87
IL-10	109.10	111.09	72.80	90.82
IL-12p70	156.48	102.05	165.37	200.59
IL-1RA	89.68	86.74	107.10	107.90
IL-1β	138.59	133.54	461.24	110.68
IL-6	111.39	69.65	85.73	91.79
TNF-α	141.55	152.19	285.46	141.98
	IL-12p70 co-stimulation CV			
IFN-γ (48h)	93.59	179.66	93.76	136.23
IFN-γ (18h)	114.40	116.47	123.96	161.51
IL-10	31.55	71.95	42.40	55.95
IL-1RA	26.01	29.25	22.90	32.17
IL-1β	33.08	74.55	198.81	56.91
IL-6	44.34	157.93	33.45	96.51
TNF-α	53.56	78.77	77.86	62.22
	IFN-γ co-stimulation CV			
IL-10	86.84	135.47	73.62	78.30
IL-12p70	164.54	279.87	305.34	168.85
IL-1RA	39.49	39.11	47.25	58.44
IL-1β	57.55	74.23	405.89	108.46
IL-6	91.38	183.75	180.35	118.03
TNF-α	120.38	129.48	129.24	125.93

CV: Coefficient of variation; NR-HC: non-related healthy controls, R-HC: related healthy controls; TB: *Mycobacterium tuberculosis* patients; VL: visceral leishmaniasis patients.

Supplementary table 2. Median, standard deviation and statistical significance values for the levels of different cytokines and the stimulation ratio of co-stimulation conditions.

Supplementary table 2. Median, standard deviation and statistical significance values for the levels of different cytokines and the stimulation ratio of co-stimulation conditions.

	BCG stimulation (pg/10^6 PBMCs ml)													
	NR-HC		R-HC		TB		VL		NR-HC vs TB	R-HC vs TB	NR-HC vs VL	R-HC vs VL	NR-HC vs R-HC	TB vs VL
	Median	SD	Median	SD	Median	SD	Median	SD	p					
IFN-γ (48h)	1585.79	3209.01	1839.61	9037.44	2121.31	8261.14	254.24	5400.70	0.446	0.848	0.000***	0.000***	0.618	0.000***
IFN-γ (18h)	100.79	283.98	27.83	104.54	42.80	131.61	8.99	43.86	0.099	0.328	0.000***	0.008**	0.013*	0.001**
IL-10	146.94	256.77	225.93	341.16	189.01	166.93	107.56	141.24	0.010*	1.000	0.022*	0.316	0.002**	0.441
IL-12p70	3.87	13.66	1.79	2.12	1.38	5.06	2.24	12.86	0.559	0.831	0.293	0.089	0.511	0.081
IL-1RA	158.25	220.47	147.85	168.14	67.78	133.63	97.36	162.32	0.014*	0.030*	0.088	0.144	0.599	0.287
IL-1β	458.01	1249.52	1041.95	2577.60	593.92	190056.79	501.32	872.32	0.263	0.360	0.495	0.049*	0.070#	0.431
IL-6	1626.47	3417.20	3777.04	3066.14	2651.18	3763.87	2435.12	2710.55	0.077#	0.542	0.599	0.047*	0.028*	0.185
TNF-α	145.00	563.98	334.65	1487.95	433.31	6616.17	272.12	701.33	0.019*	0.826	0.306	0.180	0.044*	0.125
IFN-γ/IL-10	1.075	0.7711	0.18	0.484	0.315	0.9169	0.07	0.7117	0.043*	0.328	0.000***	0.06	0.0009***	0.016*
	IL-12p70 co-stimulation SR													
	NR-HC		R-HC		TB		VL		NR-HC vs TB	R-HC vs TB	NR-HC vs VL	R-HC vs VL	NR-HC vs R-HC	TB vs VL
	Median	SD	Median	SD	Median	SD	Median	SD	p					
IFN-γ (48h)	14.97	25.02	13.28	115.54	17.42	23.26	51.29	121.17	0.629	0.966	0.005**	0.015*	0.655	0.003**
IFN-γ (18h)	13.66	37.64	16.76	34.42	12.40	37.25	17.08	69.54	0.879	0.456	0.937	0.904	0.618	0.934
IL-10	0.65	0.22	0.76	0.61	0.81	0.36	0.72	0.43	0.170	0.377	0.803	0.843	0.723	0.989
IL-1RA	0.99	0.28	1.06	0.32	1.11	0.24	1.03	0.32	0.694	0.932	0.990	0.652	0.703	0.885
IL-1β	1.36	0.52	1.11	1.07	1.09	4.66	1.02	0.69	0.016*	0.932	0.043*	0.709	0.068*	0.225
IL-6	1.19	0.64	0.88	2.29	1.00	0.34	1.00	1.26	0.110	0.062*	0.062*	0.605	0.003**	0.514
TNF-α	2.41	1.46	1.45	1.41	1.48	1.33	1.42	1.09	0.006**	0.992	0.005**	0.860	0.010**	0.219

	IFN-γ co-stimulation SR													
	NR-HC		R-HC		TB		VL		NR-HC vs TB	R-HC vs TB	NR-HC vs VL	R-HC vs VL	NR-HC vs R-HC	TB vs VL
	Median	SD	Median	SD	Median	SD	Median	SD	p					
IL-10	0.12	0.12	0.08	0.25	0.11	0.10	0.07	0.07	0.770	0.701	0.211	0.183	0.990	0.159
IL-12p70	14.10	83.79	6.90	145.93	2.69	94.91	7.51	35.51	0.008**	0.450	0.109	0.575	0.066	0.680
IL-1RA	1.34	0.61	1.33	0.56	1.22	0.65	1.21	0.86	0.274	0.617	0.415	0.913	0.572	0.726
IL-1β	2.05	1.28	1.06	1.23	1.22	41.60	1.13	2.16	0.016*	0.924	0.064	0.886	0.076	0.327
IL-6	1.00	1.39	0.70	2.42	1.00	2.24	0.57	1.35	0.377	0.600	0.318	0.991	0.202	0.721
TNF-α	8.69	21.07	3.93	12.95	4.97	10.25	6.37	15.39	0.021*	0.898	0.259	0.339	0.028*	0.923

NR-HC: non related healthy cotrols, R-HC: related healthy controls; TB: Severe/extrapulmonary tuberculosis patients; VL: visceral leishmaniosis patients. SD: standard deviation. *: $p<0.05$; **: $p<0.01$; *** $p<0.001$

1.2.Manuscript "Severe BCGosis misdiagnosed as multidrug-resistant tuberculosis in an IL-12Rβ1-deficient Peruvian girl"

Title page:

Severe BCGosis misdiagnosed as multidrug-resistant tuberculosis in an IL-12Rβ1-deficient Peruvian girl.

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BCGosis masquerading as MDR-TB in IL-12Rβ1 deficiency

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Abstract

We report the first Peruvian patient with IL-12Rβ1 deficiency. The patient developed a severe recurrent systemic mycobacterial disease, initially misdiagnosed as tuberculosis. The identification of the IL-12Rβ1 deficiency led to microbiological reevaluation of the patient, confirming BCG vaccine-related infection. Treatment was then adjusted, eventually with good response. Identification of the underlying primary immunodeficiency led to specific and successful treatment.

Key words: BCGosis; IFN-γ; IL-12Rβ1deficiency; Mendelian susceptibility to mycobacterial diseases (MSMD); *Mycobacterium tuberculosis*.

Introduction

Mendelian susceptibility to mycobacterial disease (MSMD, OMIM 209950) syndrome is a rare primary immunodeficiency (PID) caused by monogenic inborn errors of interferon (IFN)-γ and IL-12 mediated immunity, with at least ten causal genes known to date[1,2]. MSMD was classically defined as conferring susceptibility to poorly pathogenic mycobacteria, including attenuated *Mycobacterium bovis bacillus* Calmette-Guérin (BCG) vaccine and environmental mycobacteria, being IL-12Rβ1 deficiency the most common genetic etiology. Recently, it has been shown that patients with MSMD can also develop severe disease from *Mycobacterium tuberculosis* (*Mtb*)[2]. Differentiating BCG from *Mtb* infection, both included in *Mtb complex* (MTC), is difficult [3] but important since these conditions are treated differently[1]. We report and discuss here the first Peruvian patient diagnosed with IL-12Rβ1 deficiency, suffering from a severe recurrent BCGosis initially misdiagnosed as multidrug-resistant *Mtb* infection.

Methods

Mycobacterial identification within MTC was performed directly on DNA extracted from an acid-fast bacillus smear positive stool sample by a polymerase chain reaction (PCR) assay targeting the mycobacterial secA1 gene[4]. For species identification a second PCR was performed[3]: Touchdown PCR amplification with a first step of 98°C (30s), 20 cycles of 98°C (10s), annealing starting at 65°C (20s; decreasing 0.6°C/cycle), and extension at 72°C (30s) followed by a second step of 20 cycles of 98°C (10s), 53°C (20s), 72°C (30s). PCR products were sequenced on ABI3130 Genetic Analyzer (Applied Biosystems, Waltham, MA) and their identity was confirmed by comparison with NCBI GenBank database, using BLAST.

IFN-γ production was evaluated in heparinized-venous blood after stimulation with live BCG (Pasteur sub-strain) at a multiplicity of infection of 20 BCG/leukocyte and BCG plus human recombinant IL-12p70 (hrIL-12p70, 20ng/ml, Milteny Biotec, Germany) during 48h as previously described [5,6]. IFN-γ detection in the culture's supernatant was performed with enzyme linked immunosorbent assay (eBiosciences, San Diego,

CA, USA) following the manufacturer's instructions. Cytometric determination of IL-12Rβ1 presence and STAT4 phosphorylation in response to hrIL-12p70 (STAT4p) was performed in peripheral blood mononuclear cells as described elsewhere [6]. *IL12RB1* exons and flanking regions were amplified by PCR in genomic DNA and sequenced with 3730xl DNA Analyzer apparatus (Applied Biosystems).
This study was carried out in accordance with the recommendations of Spanish Ley General de Sanidad (25/4/1986), and parental written informed consent was obtained. The protocol was approved by the local ethics committee (PIC-50-12).

Results

The patient was a 6-year-old Peruvian (Cuzco region) girl from non-consanguineous parents, with no relevant family history. She had received routine Peruvian vaccinations, including BCG at birth. At 10 months of age, she was diagnosed with axillary *Mtb* lymphadenitis, ipsilateral to the site of BCG vaccination (fine needle aspiration with positive bacilloscopy and positive culture to MTC), with negative tuberculin skin test. Anti-tuberculosis treatment was started (see **Figure 1** for a detailed chronogram of clinical evolution and treatment). Three months later, an enlargement of the lymphadenitis was observed needing surgical excision. At that time, a culture-based phenotypic drug susceptibility testing (DST) from the initial sample yielded resistance to pyrazinamide. She was switched to a directly observed treatment with an empiric regimen[7] and clinical signs of infection resolved. Four months after finishing the treatment, she presented an early relapse, with an ipsilateral pectoral abscess (positive bacilloscopy and MTC culture). The strain was tested for drug resistance by Genotype® MTBDR PLUS (rifampicin and isoniazid) and resistance to rifampicin was identified. After 10 months on a new regimen, the patient was admitted with fevers and weight loss because of a disseminated mycobacterial infection, including a massive thoracic wall abscess that was debrided, with positive bacilloscopies in urine and feces. Simultaneously, mucocutaneous candidiasis (vaginal, oral and esophageal) was observed. She had an initial good response to a new empiric treatment for multi-drug resistant tuberculosis, but was then readmitted with intestinal semi-occlusion due to mesenteric adenopathies' growth, persistent cough, weight loss, a psoas abscess, and Pott's disease (T12 and L1 vertebrae), with acid-fast bacilli present in feces and gastric aspirates. Abdominal computed tomography showed signs of ileitis and portal hypertension. Extended resistance to Etoposide was confirmed by DST causing a switch to an empiric therapy for extensively drug-resistant tuberculosis regimen. A primary immunodeficiency (PID) was then suspected, and she was transferred to a reference center in Peru, for an immunologic diagnostic workup. Lymphocyte populations, lymphocyte proliferation to mitogen stimulation and phagocyte oxidative response were within the normal limits for patient's age, ruling out T-cell deficiencies and chronic granulomatous disease (data not shown). Both IL-12Rβ1 expression and STAT4 phosphorylation in response to hrIL-12p70 were absent **(**see **Figure 2a**). After 48h culture with BCG, IFN-γ levels were low (30.46pg/ml) without recovering after hrIL-12p70 addition (43pg/ml); these results were consistent with IL-12Rβ1 deficiency (**Figure 2b**). A homozygous mutation p. (Arg211*; c.631C>T) was found in *IL12RB1 g*ene, creating a premature stop codon in exon 7 (**Figure 2c)**. This mutation was found in heterozygosis in her parents; her 5-year-old brother was found to have the same homozygous mutation but no symptoms despite BCG vaccination. The patient was transferred to the National Institute of Health Clinical Center (United States of

America) for hr-IFN-γ treatment and further evaluation. Simultaneously, she had developed a severe protein-losing enteropathy with lymphopenia and hypogammaglobulinemia.
Microbiological studies performed from stool samples identified MTC[4], speciated as *M. bovis*[3], assumed to be from the vaccine strain due to its initial location next to the BCG administration site, timing of the initial disease in infancy and the early demonstration of pyrazinamide resistance. Identification of *M. bovis BCG* was later confirmed on grown isolate by the US Centers for Disease Control and Prevention. hr-IFN-γ was initiated at a dose of 25mcg/m^2 subcutaneously three times weekly, and titrated up to approximately 150mcg/m^2 subcutaneously three times weekly over several months, without complete clinical improvement. Antimicrobial regimen was switched, and in five months, stool cultures cleared, lymphocyte count normalized and albumin improved, consistent with the improvement of her protein-losing enteropathy. The patient was discharged on anti-mycobacterial therapy but hr-IFN-γ was interrupted. She has remained clinically stable, except for a diarrhea from Cryptosporidium and a *Candida parapsilosis* central line infection, both of which were successfully treated. She was maintained on clotrimazole troches for oral candidiasis.

Discussion

We describe a patient with a mutation in *IL12RB1* gene causing MSMD whose infection was misdiagnosed as tuberculosis being indeed a BCG-derived infection. This mutation has already been reported in a Turkish patient with salmonellosis, mycobacterial (not BCG-related) and fungal infections[8]. Warning signs for IL-12Rβ1 deficiency include adverse events after BCG vaccination, invasive BCG infection, invasive or recurrent salmonellosis, or a combination of severe mycobacterial infection and candidiasis, although other intra-macrophagic infections can occur[1]. There is an unexplained variation in infection susceptibility[1], since not all mutation-carriers develop infections despite abnormal functional studies, as for the sibling of our index case. This unexplained variation in infectious susceptibility in IL-12Rβ1 deficiency is currently a new line of research for the identification of modifying factors.

PID suspicion in the context of a mycobacterial infection is of outmost importance. Disseminated *M. bovis* BCG infection (BCGosis) is very uncommon (estimated at 1 : 10,000–1 : 1,000,000)[9] and almost only occurs in patients with AIDS, severe combined immunodeficiency, chronic granulomatous disease and MSMD. The confirmation of an MSMD-causing mutation has therapeutic implications: exogenous hr-IFN-γ can improve the outcome of patients with deficient production of, or partial response to IFN-γ; it is useless in complete IFN-γ response defects[1]. In IL-12Rβ1 deficiency, treatment is aimed at extensive and prolonged antimycobacterial treatments followed by secondary prophylaxis, and exogenous hr-IFN-γ is typically recommended[1,8,10], with doses ranging from 50 mcg/m^2 up to 200 mcg/m^2 subcutaneously three times weekly over several months[1,8,10].

Inborn errors of IFN-γ should be considered in patients with severe mycobacterial infections worldwide. As MDR-TB is an emerging problem in Peru[7], the patient's lack of clinical response was first ascribed to drug-resistant Mtb, thus delaying the suspicion of other less virulent mycobacteria in the context of an underlying

immunodeficiency. A similar case of disseminated tuberculosis from Colombia, finally diagnosed with IL-12Rβ1 deficiency has recently been published[10]. Both cases developed severe gut inflammation, causing protein-losing enteropathy, hypoalbuminemia, and antimicrobial malabsorption, needing IV administration. Both *M. bovis* BCG and *Mtb* must be considered in the differential, as different therapeutic approaches will be needed.

M. bovis is closely related to *Mtb* within the MTC[3] and the BCG vaccine is derived from a virulent strain of *M. bovis*[9]. BCG is difficult to differentiate from other strains such as *M. bovis* and other members of the *MTC* by conventional methods as mycobacterial culture. All BCG vaccine strains are intrinsically resistant to pyrazinamide, which is uncommon in naïve patients with *M.tb*[3], and the study of specific regions by PCR is needed[3]; unfortunately, these studies are not widely available. Besides, IFN-γ release diagnostic assays, which are specific for virulent MTC species such as *Mtb* and *M. bovis*, avoiding cross-reactivity with *M. bovis BCG*[11], are less reliable in patients with inborn errors of IFN-γ mediated immunity, such as MSMD.

Conclusions

This case highlights the need to consider PID (especially defects in the IFN-γ circuit) in patients with severe mycobacterial infection, and/or major adverse events after BCG vaccination[1] and how BCGosis can mimic disseminated tuberculosis and delay appropriate diagnostic and therapeutic management. The consideration of these concepts in the healthcare practice can help to reduce morbi-mortality of possible immunodeficient patients.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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Figure Legends

Figure 1. Chronogram of clinical evolution and treatment. Isoniazid (H); rifampicin (R); pyrazinamide (Z); ethambutol (E); amikacin (Am); levofloxacin (Lfx); ethionamide (Eto); moxifloxacin (Mfx); capreomycin (Cm); cycloserine (Cs); paraamino salicylic acid (PAS); ciprofloxacin (Cfx); linezolid (Lnz); Imipenem/Cilastatin (Ipm/Cln); amoxicillin/clavulanate (Amx/Clv); thioridazine (Tio); multidrug resistant tuberculosis (MDR-TB); extensively multidrug resistant tuberculosis (XDR-TB).

Figure 2. **Immunological and genetic studies.** A) IFN-γ production after no stimulation, BCG stimulation, BCG + hrIL-12p70 stimulation, ratio between BCG+hrIL-12p70/BCG stimulations. B) Cytometric determination of IL-12Rβ1 and STAT4 phosphorylation after hrIL-12p70 stimulation in PHA-stimulated lymphocytes. Solid line represents stimulated condition and dashed line represents basal condition) Electropherograms for the mutation site in a healthy control (WT/WT), and in the father, mother and patient.

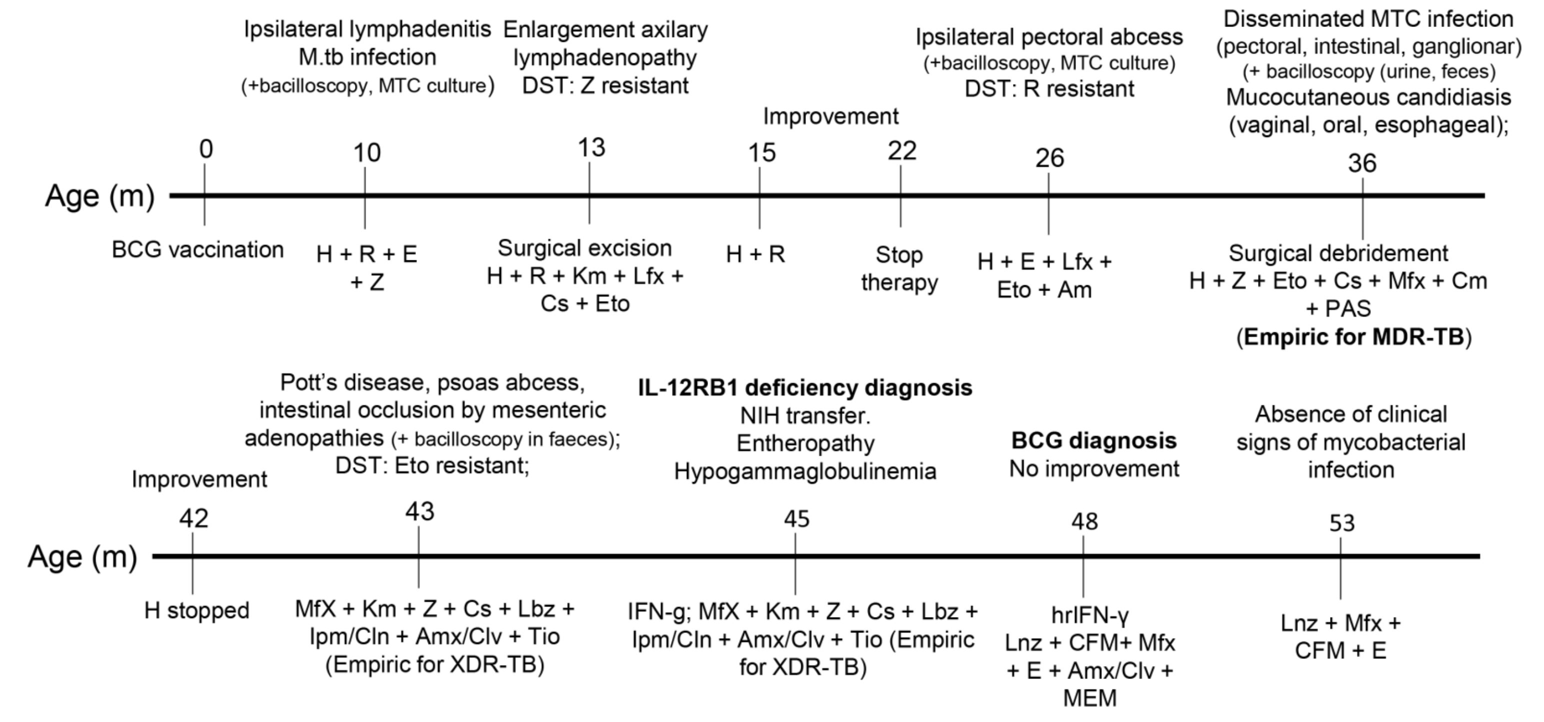

Figure 1

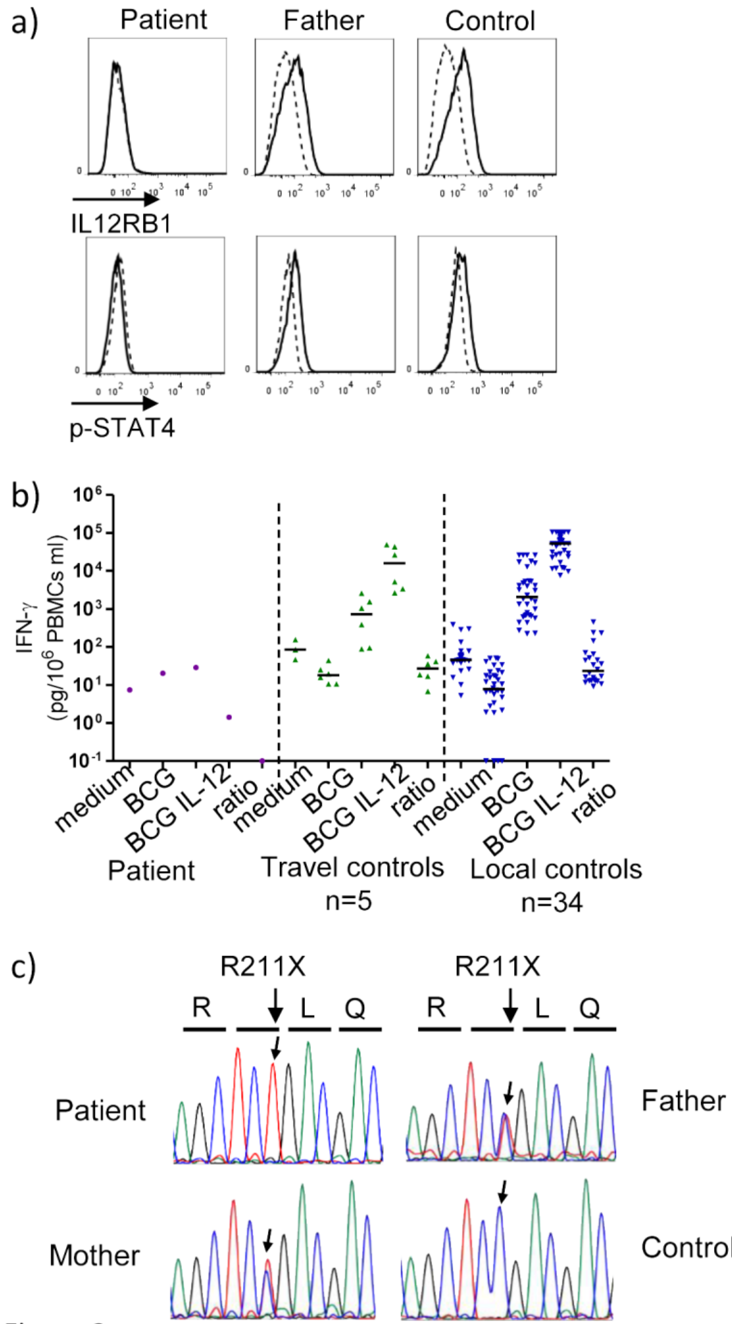

Figure 2

Chapter 2. Study of the impact of whole-pregnancy exposure to anti-TNF-α on the development of the exposed infant's immune system.

For the study of the effect of the exposure to anti-TNF-α during pregnancy 7 patients (out of the 8 patients fulfilling inclusion criteria) enrolled the study. From these, 2 stopped analytical follow up after birth, 4 completed the study until 12 months and 1 until 18 months. The size of the cohort was limited by the fact that, at the time of project conception, the IBD Unit of Hospital Clinic de Barcelona was the only place in Spain where anti-TNF-α was maintained during whole-pregnancy in patients with IBD.

During the follow up of the anti-TNF-α drug levels during the first 12 months in exposed infants', clearance of the drug corresponded with the expected immunoglobulin's half-life and the drug was detectable until 6 months after birth. We did not observe changes in the main lymphocytic and leukocytic populations. However, at birth, T and B subpopulations showed a more immature phenotype in exposed infants compared to non-exposed infants. After one year, T and B population were within reference range defined per age. This initial immaturity had no consequences in immunoglobulin production or vaccine responses (against tetanus, diphtheria and pneumococcus). We also observed a diminished frequency of Treg cells, that inversely correlated with the through level of anti-TNF-α in the mother during the third trimester of pregnancy (r: -0.9; p: 0.03). Treg cell frequency also inversely correlated with T cell proliferation after the challenge with a weak T cell stimulus (r: -0.68; p:0.01), increased in exposed infants (p: 0.01). On the other hand, $CD24^{hi}CD38^{hi}$ Breg cells were increased (p: 0.0007).

We evaluated anti-mycobacterial response by studying surface activation markers and IL-12/IFN-γ axis by cytokine secretion in whole blood cultures in the presence (non-washed condition) and absence (washed condition) of autologous sera. Exposure to anti-TNF-α during pregnancy reduced the response after mycobacterial challenge. We observed a diminished stimulation ratios (SR) in the early activation marker CD69 (p: 0.004) expression and in HLA-DR (p: 0.003), which were partially rescued after drug removal. Without stimulation, washed samples from exposed infants produced higher amounts of IL-17 compared with the non-washed condition (*p*: 0.03) and with non-exposed infants (*p*: 0.02). On the other hand, after BCG stimulation, IL-17 production in exposed infants was reduced. Also, at birth, TNF-α induction was reduced in the exposed-infant (*p*: 0.0002), being only partially recovered after drug removal (*p*: 0.005) although still being reduced (*p*: 0.02) in comparison to non-exposed infants. Despite not reaching statistical significance, IL-1β SR was reduced and IL-6 and IL-1RA SR were increased. Along with the decrease of anti-TNF-α drug presence (3 to 12 months after birth), IFN-γ, IL-12p70, IL-1β, and TNF-α production increased while IL-6 production was stable.

Detailed methods, results and figures are shown in "*Esteve et al, Immunological Changes in Blood of Newborns Exposed to Anti-TNF-α during Pregnancy. Frontiers in Immunology. 2017*" manuscript.

2.1.Manuscript "Immunological Changes in Blood of Newborns Exposed to Anti-TNF-α during pregnancy".

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Immunological Changes in Blood of Newborns Exposed to Anti-TNF-α during Pregnancy

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Background: Although anti-TNF-α monoclonal antibodies are considered safe during pregnancy, there are no studies on the development of the exposed-infant immune system. The objective was to study for the first time the impact of throughout pregnancy exposure to anti-TNF-α has an impact in the development of the infant's immune system, especially B cells and the IL-12/IFN-γ pathway.

Methods: Prospective study of infants born to mothers with inflammatory bowel disease treated throughout pregnancy with anti-TNF-α (adalimumab/infliximab). Infants were monitored both clinically and immunologically at birth and at 3, 6, 12, and 18 months.

Results: We included seven patients and eight healthy controls. Exposed infants had detectable levels of anti-TNF-α until 6 months of age; they presented a more immature B- and helper T-phenotype that normalized within 12 months, with normal immunoglobulin production and vaccine responses. A decreased Treg cell frequency at birth that inversely correlated with mother's peripartum anti-TNF-α levels was observed. Also, a decreased response after mycobacterial challenge was noted. Clinically, no serious infections occurred during follow-up. Four of seven had atopia.

Conclusion: This study reveals changes in the immune system of infants exposed during pregnancy to anti-TNF-α. We hypothesize that a Treg decrease might facilitate hypersensitivity and that defects in IL-12/IFN-γ pathway might place the infant at risk of intracellular infections. Pediatricians should be aware of these changes. Although new studies are needed to confirm these results, our findings are especially relevant in view of a likely increase in the use of these drugs during pregnancy in the coming years.

Keywords: adalimumab, inflammatory bowel disease, infliximab, monoclonal antibodies, pregnancy, prenatal exposure

INTRODUCTION

Anti-TNF-α monoclonal antibodies (mAb) have revolutionized inflammatory bowel disease (IBD) treatment (1–4). IBD onset is frequently observed in women during childbearing age (3, 4); nevertheless, pregnancy is not associated with IBD improvement (5–9). Active IBD can lead to increased pre-term deliveries and spontaneous abortion, and sustained remission of IBD is often only achievable with anti-TNF-α treatment. Anti-TNF-α mAb cross placenta mostly from week 28 of gestation to delivery (10); they are included in category B (no apparent risk) of FDA-classification for pregnancy risk. However, drug level's safety in newborns and the full consequences of such exposure in newborn's immune system development are unclear.

Several recent studies in extensive cohorts [PIANO ($n = 426$), OTIS registry ($n = 74$), and CRIB study ($n = 31$)] of infants exposed to anti-TNF-α drugs during, at least, the second and third trimester of pregnancy showed that both anti-TNF-α adalimumab (ADA) and infliximab (IFX) are detectable in infants from treated mothers for 12 months after birth while certolizumab was barely detectable. Infants showed no apparent major clinical consequences (11–14). Nevertheless, a fatal case of disseminated Bacillus Calmette–Guérin (BCG) disease after vaccination was reported in an infant whose mother had been treated with high doses of IFX during pregnancy (15). It is recommended to delay immunization with live vaccines until 12 months or after confirmation of negative drug levels (13, 16).

It has been hypothesized that biologic drugs targeting immune-system molecules can lead to phenotypes resembling primary immune deficiencies (PID) related to the inhibited or modulated pathway (17). Although there are not known PID caused specifically by a deficiency in TNF-α, it plays a key role in the IL-12/IFN-γ pathway, responsible for the response to intracellular pathogens. Infections observed in adult patients treated with anti-TNF-α mAbs resemble those observed in patients with Mendelian Susceptibility to Mycobacterial Disease (OMIM 209950) (18), caused by monogenic errors in the IL-12/IFN-γ pathway. Severe or recurrent infections by atypical/non-pathogenic mycobacteria and other intra-macrophagic infections, including salmonellosis are characteristic in patients with Mendelian Susceptibility to Mycobacterial Disease and in patients treated with anti-TNF-α (19). Anti-TNF-α treatment alters the IL-12/IFN-γ pathway by inhibiting IL-12p70 production in response to CD40L stimulation (18). Besides, it is thought that it plays a pivotal role in B cell development: TNF-α knockout mice present abnormal B cell structures. These alterations lead to a decreased humoral responses and increased infection risk (20).

The in-depth study of the effect of prenatal anti-TNF-α exposure on a developing immune system remains a relevant clinical question. Therefore, this was the aim of our study: our hypothesis was that throughout exposure during pregnancy to anti-TNF-α affects the development of the infant's immune system, especially B-cells (20, 21) and the IL-12/IFN-γ pathway (18). To our knowledge, this is the first study to shed light on how prenatal anti-TNF-α influences the human immune system development.

MATERIALS AND METHODS

Patients Included

We conducted a prospective study (January 2014–January 2016) of infants born to mothers with IBD who received anti-TNF-α mAb (ADA or IFX) throughout during pregnancy, including the third trimester. Infants undergoing immunosuppressive treatment or with an immunodeficiency were excluded. All IBD patients included in the study were recruited from the IBD Unit of Hospital Clinic de Barcelona (HCB), and underwent close monitoring of pregnancy and childbirth. At the time of the initiation of our study, the IBD Unit of HCB was the only institution in our region maintaining this treatment during throughout pregnancy and all patients but one fulfilling inclusion criteria were enrolled in the study.

This study was carried out in accordance with the recommendations of Ley General de Sanidad (25/4/1986) Art. 10, with written informed consent from all subjects. The protocol was approved by the ethics committee of the Hospital Sant Joan de Déu (Comité Ético de Investigaciones Clínicas number PIC-50-12). All patients included in the study signed the informed consent, complying with current legislation.

We included seven exposed-infants and eight healthy controls. Exposed-infants were monitored both clinically and immunologically at birth and at 3, 6, 12, and 18 months. On the delivery day, 20 ml of heparinized blood was extracted from umbilical cord blood (seven exposed-infants and eight controls). From 3 months, 6 ml heparinized blood and 2.5 ml sera without anticoagulant factor were obtained from all exposed-infants but 2, due to the mother's refusing subsequent blood draws after an unsuccessful first attempt.

Immune and Clinical Follow-up

Immune parameter study included: quantification of anti-TNF-α blood levels, basic T/B/NK immunophenotype, T-cell and B-cell subphenotypes with evaluation of regulatory cells (Treg and Breg), lymphocyte proliferation to mitogens, and IFN-γ/IL-12 pathway (Tables S1 and S2 in Supplementary Material). Common procedures as immunophenotyping and cell proliferation are detailed in the supplementary methods. To facilitate reading and interpretation, populations appear in the text with a given name, while markers definitions are presented in **Table 1**.

Study of IL-12/IFN-γ Pathway in Response to Mycobacterial Stimulus

For the study of IL-12/IFN-γ pathway, we performed a whole blood culture (22). Heparinized blood was diluted 1:2 in complete medium [RPMI (Gibco, Grand Island, New York, NY, USA)] supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA), 1 μg/

TABLE 1 | Lymphocyte subpopulation cell markers.

Name	Cell marker
T lymphocytes	CD45$^+$CD3$^+$CD19$^-$
B lymphocytes	CD45$^+$CD3$^-$CD19$^+$
NK cells	CD45$^+$CD3$^-$CD16CD56$^+$
CD4 T cells	CD45$^+$CD3$^+$CD4$^+$
CD8 T cells	CD45$^+$CD3$^+$CD8$^+$
Double-negative T cells	CD45$^+$CD3$^+$CD4$^-$CD8$^-$
TCRγδ T cells	CD3$^+$TCRγδ$^+$
TCRαβ T cells	CD3$^+$TCRαβ$^+$
Naive T cells	CD3$^+$CD45RA$^+$CD45RO$^-$
Cytotoxic naive T cells	CD3$^+$CD45RA$^+$CD45RO$^-$CD8$^+$
Helper naive T cells	CD3$^+$CD45RA$^+$CD45RO$^-$CD8$^-$
Memory T cells	CD3$^+$CD45RO$^+$
Cytotoxic memory T cells	CD3$^+$CD45RO$^+$CD8$^+$
Helper memory T cells	CD3$^+$CD45RO$^+$CD8$^-$
Late-memory T cells	CD3$^+$CD45RA$^-$CD45RO$^+$
Cytotoxic late-memory T cells	CD3$^+$CD45RA$^-$CD45RO$^+$CD8$^+$
Helper late-memory T cells	CD3$^+$CD45RA$^-$CD45RO$^+$CD8$^-$
Early-memory T cells	CD3$^+$CD45RA$^+$CD45RO$^+$
Cytotoxic early-memory T cells	CD3$^+$CD45RA$^+$CD45RO$^+$CD8$^+$
Helper early-memory T cells	CD3$^+$CD45RA$^+$CD45RO$^+$CD8$^-$
T regulatory cells	CD45$^+$CD3$^+$CD4$^+$CD25^{hi}CD127^{low}FoxP3$^+$
IgD$^+$IgM$^+$ B cells	CD19$^+$IgD$^+$IgM$^+$
Marginal zone B cells	CD19$^+$IgD$^+$IgM$^+$CD27$^+$
Naive B cells	CD19$^+$IgD$^+$CD27$^-$
Transitional B cells	CD19$^+$IgM$^+$CD38^{hi}
B regulatory cells	CD19$^+$CD24^{hi}CD38^{hi}
IgD$^+$IgM$^-$ B cells	CD19$^+$IgD$^+$IgM$^-$
Switched B cells	CD19$^+$IgD$^-$IgM$^-$
Switched memory B cells	CD19$^+$IgD$^-$IgM$^-$CD27$^+$
IgM only B cells	CD19$^+$IgD$^-$IgM$^+$
Activated B cells	CD19$^+$CD38^{low}CD21^{low}
Plasmablasts	CD19$^+$IgM^{low}CD38^{hi}

ml penicillin, and 1 μg/ml streptomycin (Invitrogen, Grand Island, New York, NY, USA) and incubated at 37°C in a 5% CO_2 humidified incubator for 48 h. To assess the effect of anti-TNF-α mAb on the function of IL-12/IFN-γ pathway, the same whole blood culture was performed in two ways: using blood from exposed infants without washing and using the blood washed with PBS three times to eliminate anti-TNF-α drug. After the washing, complete medium (RPMI with 10% FBS and penicillin/streptomycin) was added to restore blood to the same volume as before the washing with PBS. In this way, we maintained cellular amounts of cells and cell concentration in the two conditions assayed. Activation conditions: medium alone, live BCG (*M. bovis* BCG, Pasteur substrain) at a multiplicity of infection of 20 BCG per leukocyte, BCG plus human recombinant IL-12p70 (hrIL-12p70, 20 ng/ml, Miltenyi Biotec, Germany), BCG plus hrIFN-γ (5,000 IU/ml; Imukin, Boehringer Ingelheim, Germany) as described elsewhere (22). We analyzed activation markers after 48 h of culture by flow cytometry.

Cytokine production determination was assessed by Luminex (Millipore, Billerica, MA, USA) at 48-h culture point following the manufacturer's instructions. Briefly, supernatants were incubated for 2 h with corresponding anti-cytokine magnetic beads, and then washed with 1× washing buffer and stained with detection antibodies (provided) for 1 h. Strepatividin-PE was then added for 30 more minutes. During all incubation steps, the plate was agitated at 650 rpm. After washing, plate was agitated for 15 min at 650 rpm and read in the xMAP Luminex reader (Waltham, MA, USA). IL-17 detection was assessed by ELISA (Invitrogen, Carlsbad, CA, USA) at 48 h culture point following manufacturer's instructions.

Statistical Analysis

As data did not follow a Gaussian distribution, unpaired *t*-test was performed to compare different cell populations between exposed and non-exposed infants. Significance of correlation between populations/drug levels was studied with Spearman test for non-parametric populations.

Statistical significance of functional studies was performed with two-way ANOVA test and Bonferroni post-test. For the comparison between the results obtained with autologous sera and washed condition, two-way ANOVA for repeated measures was used. In heat-map representation, blue corresponds with the minimum, red with the maximum, and yellow with one for each parameter.

Statistical analysis and graphical representation of the data were performed with Prism5 software (GraphPad, La Jolla, CA, USA) and Microsoft Excel (2010). Detailed results (mean ± SE of the mean and *p*) for immune phenotype are detailed in **Tables 2** and **3**.

RESULTS

All Studied Mothers Presented Therapeutic Levels of Anti-TNF-α

Seven moderate-to-severe IBD pregnant patients (mean 34 years old, range 27–36) with long-lasting IBD (mean 9 years since diagnosis, range 2.5–11) were treated with anti-TNF-α for a period longer than 6-months prior to pregnancy. Six patients suffered from Crohn's disease (CD) and one from extended ulcerative colitis. Five patients were treated with ADA, and two with IFX. All mothers presented supra-therapeutic levels of anti-TNF-α (ADA >4 μg/ml; IFX >3 μg/ml) before or during pregnancy. Three patients were treated with other immunosuppressive drugs—2 with azathioprine (AZA) and one with prednisolone—and two of them had active IBD during pregnancy (Table S3 in Supplementary Material).

For all patients, the interval between the last dose of drug and the delivery was ≤7 days (range: 3–7 days). The delivery was programmed only in two patients; the five remaining mothers gave birth by caesarian section (1) or vaginal (4) delivery, with one preterm infant (35 weeks in one mother with active disease); while the remaining 4-cases delivered at 39 weeks on average (range 37–41). All newborns were normal weight for gestational age, Apgar 9/10, and did not present any malformations. Five mothers breastfed.

Levels of Anti-TNF-α in the Infant

Exposed-infants had positive anti-TNF-α drug levels in cord blood (mean 11.42 μg/ml, range: 5.87–42.52 μg/ml; **Figure 1A**);

with especially high levels in one IFX-exposed infant whose mother received the drug 4 days before delivery. There was a direct correlation between the trough level of ADA in the mother and the cord blood level ($r = 0.9$ $p = 0.04$); this correlation was not determinable in IFX patients due to the low number of samples (**Figure 1B**). The ratio between the mother and cord blood drug's trough level was close to 1 (mean: 0.99) for ADA and higher for IFX (mean: 3.25).

Drug level clearance in the infant followed a one-phase decay with a mean half-life of 29.6 days (range 23.93–35.53 days), slightly longer than the described half-life of immunoglobulin (Ig) G (23). Drug levels were detectable until 6 months in all infants studied (**Figure 1C**; Table S4 in Supplementary Material).

Mild Changes in Leukocyte and Lymphocyte Population in Exposed-Infants

There were no statistical differences in cord blood counts in red blood cells (Figure S1 in Supplementary Material), total leukocytes, lymphocytes, neutrophils, and basophils between exposed-infants and healthy controls (Figure S2 in Supplementary Material). However, although due to sample-size limitations comparisons between ADA- and IFX-exposed infants should be approached with caution. We have observed that, compared with healthy controls, IFX-exposed infants had lower monocyte count in cord blood (0.44 vs 1.16×10^3 cells/μl blood) and that ADA-exposed infants presented increased eosinophil counts in

TABLE 2 | Leukocyte populations in umbilical cord blood of exposed and non-exposed infants.

		Exposed (n =6)		Non-exp (n = 8)		p	ADA-exposed (n = 4)			IFX-exposed (n = 2)		
		Mean	SEM	Mean	SEM		Mean	SEM	p	Mean	SEM	p
Absolute numbers (10^3 cells/μl blood)	Leukocytes	16.06	3.12	13.12	1.00	0.28	20.15	3.65	0.15	12.36	6.15	>0.9999
	Lymphocytes	7.18	1.47	5.39	0.41	0.08	9.31	1.47	0.02	5.27	2.93	>0.9999
	Neutrophils	7.29	1.83	6.18	0.74	0.85	8.54	2.81	0.9	6.48	3.42	0.84
	Monocytes	1.04	0.24	1.16	0.10	0.94	1.28	0.26	0.15	0.44	0.04	0.04
	Basophils	0.11	0.02	0.39	0.11	0.09	0.09	0.03	0.07	NA	NA	NA
	Eosinophils	0.44	0.21	0.08	0.02	0.07	0.63	0.55	0.004	NA	NA	NA
Absolute numbers (cells/μl blood)	T lymphocytes	5593.00	1006.00	3479.00	256.70	0.04*	6298	1045	0.004*	4184	2376	1
	B lymphocytes	959.20	303.80	978.60	166.30	0.85	1112	420.4	0.57	653.6	428.8	0.71
	NK cells	1100.00	304.00	796.40	217.80	0.66	1552	164.8	0.11	196.8	4.92	0.18
	CD4 T cells	3935.00	779.40	2442.00	169.80	0.14	4470	854.2	0.49	2864	1736	1
	CD8 T cells	1523.00	294.70	925.10	101.00	0.14	1736	343.5	0.07	1097	567.9	0.89
	Double-negative T cells	182.80	45.22	70.90	10.66	0.01*	217.6	59.69	0.008*	113.4	45.37	0.09
% of T cells	TCRγδ T cells	3.57	0.79	2.11	0.28	0.28	3.12	0.67	0.26	2.39	0.38	0.67
	TCRαβ T cells	94.84	0.80	94.26	0.45	0.15	95.18	0.78	0.57	96.15	0.25	0.07
	Naive T cells	62.72	6.82	46.90	2.32	0.13	54.88	7.226	0.47	78.4	3.9	0.07
	Cytotoxic naive T cells	15.11	1.22	16.20	1.24	0.82	13.25	0.4575	0.25	18.82	0.625	0.28
	Helper naive T cells	47.62	6.08	30.73	2.33	0.13	41.64	7.293	0.47	59.59	4.525	0.07
	Memory T cells	34.68	7.98	52.65	2.30	0.13	44.9	7.391	0.48	14.25	0.35	0.07
	Cytotoxic memory T cells	11.74	4.11	9.77	1.17	0.7	11.74	4.108	0.61	5.675	0.305	0.07
	Helper memory T cells	22.95	5.393	42.9	1.784	0.009*	22.95	5.393	0.04*	8.575	0.655	0.07
	Late-memory T cells	9.07	1.39	8.05	0.80	0.81	8.948	2.156	0.91	9.31	0.89	0.43
	Cytotoxic late-memory T cells	2.55	0.70	0.12	0.03	0.002*	2.21	1.046	0.01*	3.225	0.145	0.07
	Helper late-memory T cells	6.52	1.05	7.92	0.78	0.13	6.738	1.625	0.25	6.085	0.745	0.28
	Early-memory T cells	25.64	8.53	44.87	2.09	0.18	35.73	8.952	0.61	5.46	1.02	0.07
	Cytotoxic early-memory T cells	9.22	3.85	9.69	1.15	0.69	12.47	5.132	0.61	2.715	0.705	0.07
	Helper early memory T cells	16.42	5.71	35.18	1.90	0.03*	23.26	5.901	0.11	2.745	0.315	0.07
% of CD4 T cells	T regulatory cells	0.24	0.04	0.5	0.10	0.04	0.22	0.037	0.049	0.29	0.11	0.4
% of B cells	IgD⁺IgM⁺ B cells	91.97	2.38	92.03	1.30	0.46	90.13	3.20	0.920	95.65	1.85	0.29
	Marginal zone B cells	20.16	9.02	40.74	2.46	0.03*	29.58	40.74	0.190	1.33	0.45	0.04*
	Naive B cells	72.21	10.87	47.70	3.49	0.045*	60.77	12.82	0.270	95.11	1.42	0.04*
	Transitional B cells	59.75	10.78	53.89	4.88	0.44	50.43	13.66	0.920	78.40	10.00	0.09
	B regulatory cells	49.31	1.42	34.39	2.49	0.0007*	47.62	1.31	0.004*	53.55	0.45	0.04*
	IgD⁺IgM⁻ B cells	3.36	2.13	3.64	1.65	0.72	4.35	3.18	0.780	1.37	1.15	0.89
	Switched B cells	2.84	1.00	3.90	1.30	0.045*	3.59	1.35	0.130	1.32	0.79	0.17
	Switched memory B cells	2.26	0.82	2.57	0.58	0.35	2.76	1.16	0.630	1.28	0.78	0.4
	IgM only B cells	1.81	0.16	0.43	0.09	0.002*	1.90	0.24	0.010*	1.64	0.10	0.04*
	Activated B cells	1.23	0.73	0.05	0.03	0.003*	1.74	1.03	0.010*	0.21	0.12	0.09
	Plasmablasts	1.33	0.32	1.39	0.28	0.94	1.81	0.16	0.500	0.7	0.41	0.27

ADA, adalimumab; IFX, infliximab.
**indicates statistically significant p-values.*

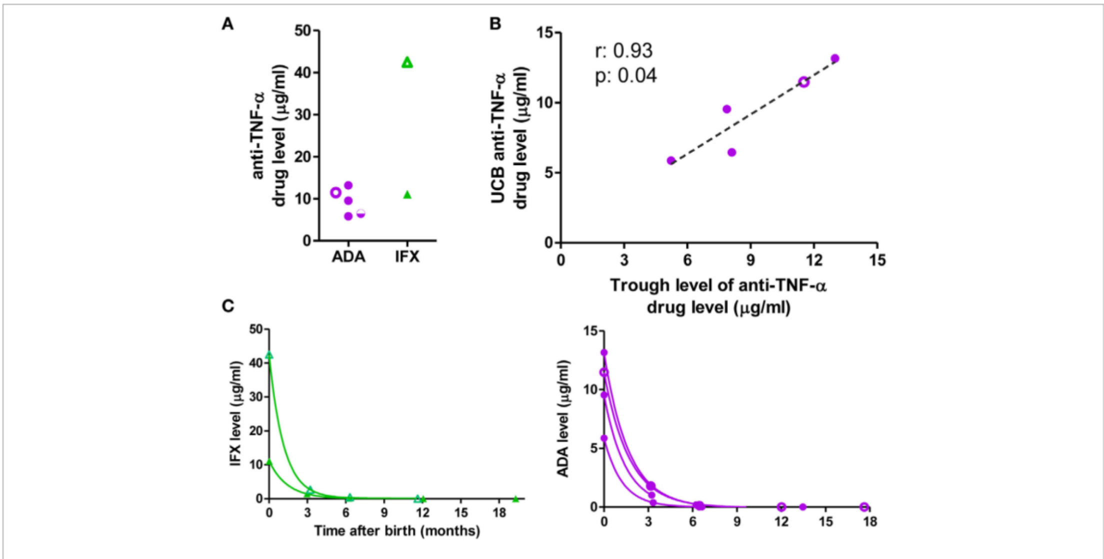

FIGURE 1 | Anti-TNF-α drug levels. **(A)** Anti-TNF-α levels in cord blood. **(B)** Correlation between mother's adalimumab (ADA) trough level during the third trimester of pregnancy and level in cord blood. **(C)** Clearance of the anti-TNF-α monoclonal antibodies. Purple circles: ADA-exposed; green triangles: infliximab (IFX)-exposed, empty symbols: co-exposure with azathioprine; half-empty symbols: co-exposure with prednisone. ADA-exposed infants, $n = 5$; IFX-exposed infants, $n = 2$.

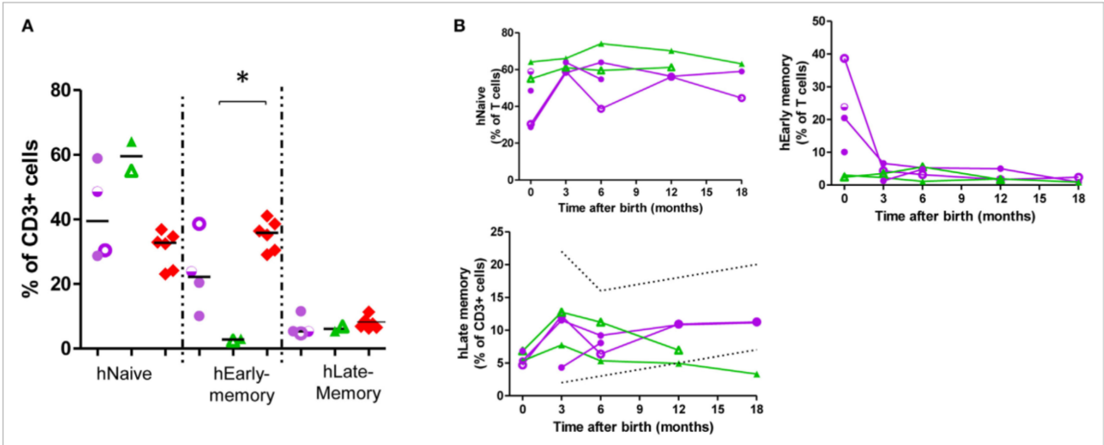

FIGURE 2 | Helper CD45RO⁺CD45RA⁺ T-cells are at a low frequency in exposed infants cord blood. T-cell subpopulation frequency at birth **(A)** and at follow-up **(B)**. Dotted lines, described reference ranges; purple circles, adalimumab (ADA)-exposed; green triangles, infliximab (IFX)-exposed; red diamonds, cord blood healthy controls; empty symbols, azathioprine co-exposure; half-empty symbols, prednisone co-exposure. ADA-exposed infants, $n = 4$; IFX-exposed infants, $n = 2$.

cord blood (0.63 vs 0.08 × 10^3 cells/µl blood; $p = 0.004$) (Figure S2 in Supplementary Material; **Table 2**).

Absolute number of T-cells was increased in cord blood of ADA-exposed-infants (6.294 vs 3.479 cells/µl of blood; $p = 0.004$), which fell within normal ranges at 12 months. No differences were observed in other lymphocyte subpopulations (Figures S2 and S3 in Supplementary Material). One ADA-treated patient, also treated with AZA, presented very low numbers of B-cells (0.54% of lymphocytes and 32 cells/µl of blood) (this effect was not observed in the other AZA-treated infant).

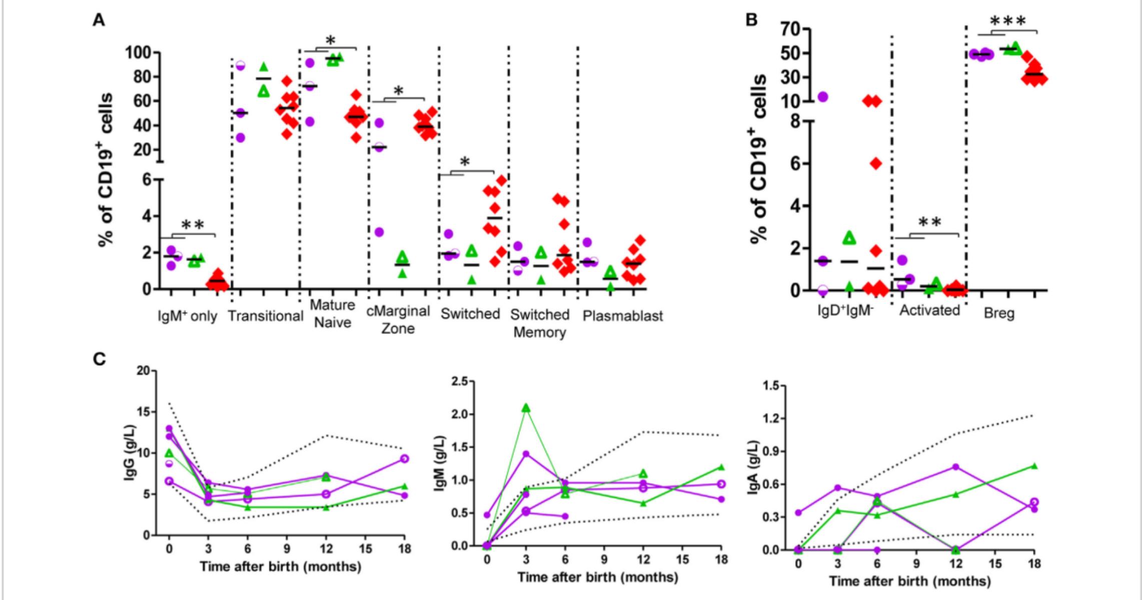

FIGURE 3 | B-cells present higher levels of naïve markers and normal levels of immunoglobulins (Igs) in exposed infants. **(A,B)** B-cell subpopulation frequencies at birth. adalimumab (ADA)-exposed infants, $n = 3$; infliximab (IFX)-exposed infants, $n = 2$. **(C)** Ig production follow-up. ADA-exposed infants, $n = 4$; IFX-exposed infants, $n = 2$. Dotted lines, described reference ranges; purple circles, ADA-exposed; green triangles, IFX-exposed; red diamonds, cord blood healthy controls; empty symbols, azathioprine co-exposure; half-empty symbols: prednisone co-exposure.

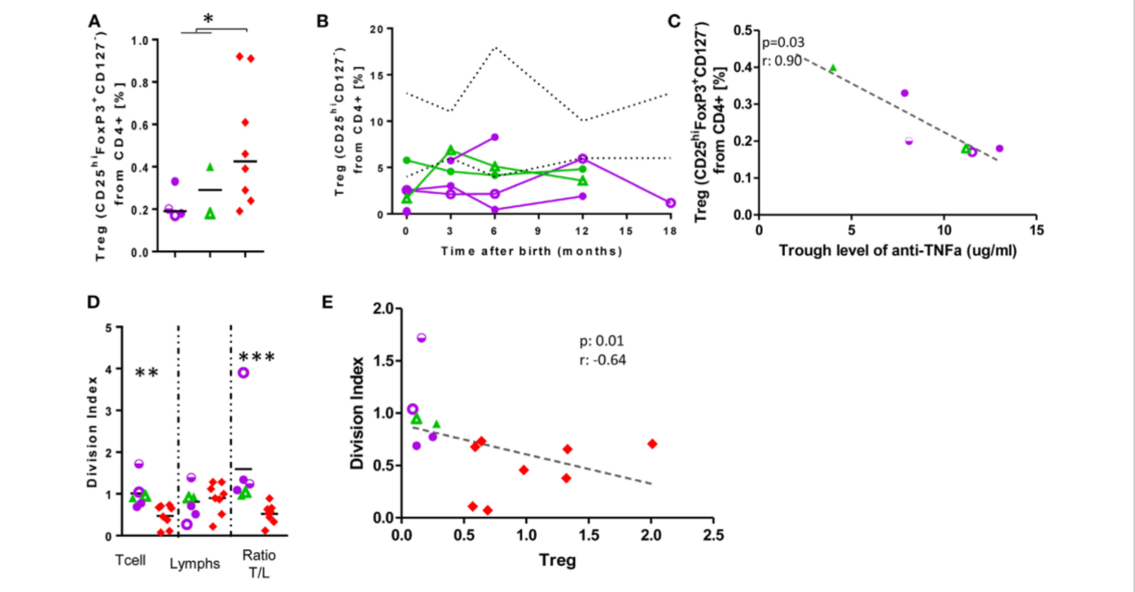

FIGURE 4 | Low Treg levels at birth are not rescued and correlate with increased T-cell proliferation. Treg frequency at birth **(A)** and follow-up **(B)**. **(C)** Correlation between Treg frequency and mother's anti-TNF-α trough level. **(D)** Division Index after 7-days' stimulation with ConA. **(E)** Correlation between Treg frequency and T-cell division index. Dotted lines, described reference ranges; purple circles, adalimumab (ADA)-exposed; green triangles, infliximab (IFX)-exposed; red diamonds, cord blood healthy controls; empty symbols, azathioprine co-exposure; half-empty symbols: prednisone co-exposure. ADA-exposed infants, $n = 4$; IFX-exposed infants, $n = 2$.

At 12 months of age, all exposed infants showed normal-for-age levels of leukocyte and lymphocyte subsets; neutrophils and NK were at the lower limit of the normal ranges (24) (Figure S2 in Supplementary Material; **Table 2**).

Differences in T- and B-Lymphocyte Maturation Status in Exposed-Infants

In the T-cell compartment (**Figure 2**; Figure S4 in Supplementary Material; **Table 2**), there was a tendency toward an increase in the naïve population in exposed infants (62.72 vs 46.9% of T-cells, $p = 0.13$) due to T helper (Th)-subset. Furthermore, CD45RO$^+$ (memory) population frequency was decreased among Th-cells (22.95 vs 42.9% of T-cells; $p = 0.009$) due to a decrease in Th-early-memory-cells (16.42 vs 35.18% of CD3$^+$; $p = 0.03$) while there were no significant differences in late-memory T-cells. By contrast, there was an increase in the T cytotoxic Tc-memory-cells (2.55 vs 0.12% of T-cells; $p = 0.002$). At 12 months of age, CD45RO$^+$ T-cells, both Th and Tc, were in range (25).

In the B-cell compartment (**Figure 3**; Figure S5 in Supplementary Material; **Table 2**), naïve B-cell population

TABLE 3 | Activation markers' expression and cytokine production after 48 h whole blood culture with different stimulations.

			Ratio stim/basal			Ratio w_exp/exp			
Variable		**Stimulus**	**Non-exp**	**Exp**	**w_exp**	**UCB**	**3 months**	**6 months**	**12 months**
ACTIVATION MARKERS EXPRESSION									
CD71	Freq%	Bacillus Calmette–Guérin (BCG)	2.36	1.416	1.162	2.307	3.434	4.004	3.788
		BCG IL-12	2.379	1.768	1.434	2.227	2.768	3.826	3.368
		BCG IFN-γ	3.018	1.682	1.252	1.883	3.204	4.394	3.51
			Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**			
		p	0.06	0.06	0.17	0.13			
	MFI	BCG	1.154	1.238	1.116	3.443	2.164	2.016	1.93
		BCG IL-12	1.183	1.168	1.218	3.487	2.034	1.926	1.75
		BCG IFN-γ	1.266	1.28	1.032	2.733	2.006	2.026	1.69
			Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**			
		p	0.91	0.71	0.27	0.003			
CD69	Freq%	BCG	2.306	2.052	1.904	1.14	1.434	1.018	2.11
		BCG IL-12	4.359	2.664	3.778	1.103	1.412	1.09	2.143
		BCG IFN-γ	4.829	2.69	3.424	2.653	1.48	1.15	1.938
			Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**			
		p	0.13	0.13	0.09	0.15			
	MFI	BCG	5.478	2.106	1.838	1.513	1.526	0.918	1.02
		BCG IL-12	11.34	2.57	4.38	1.647	1.674	1.044	1.438
		BCG IFN-γ	12.22	2.464	3.232	1.887	1.66	1.038	1.008
			Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**			
		p	0.001*	0.004*	0.02*	0.009*			
HLA-DR	Freq%	BCG	1.123	1.854	1.674	1.053	1.08	0.938	0.9725
		BCG IL-12	1.231	2.18	1.854	1.03	1.038	0.94	1.018
		BCG IFN-γ	1.443	2.016	1.61	0.9867	1.03	0.976	0.955
			Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**			
		p	0.21	0.1	0.26	0.58			
	MFI	BCG	2.024	1.166	1.926	2.673	1.368	0.962	1.318
		BCG IL-12	2.38	1.19	2.198	2.093	1.164	1.046	1.165
		BCG IFN-γ	2.564	1.482	2.404	2.99	1.338	1.204	1.213
			Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**			
		p	0.003*	0.05	0.1	0.009*			

(Continued)

TABLE 3 | Continued

Variable		Ratio stim/basal				Ratio w_exp/exp			
Variable		**Stimulus**	**Non-exp**	**Exp**	**w_exp**	**UCB**	**3 months**	**6 months**	**12 months**
CYTOKINE SECRETION									
IFN-γ	BCG	282.1	12.68	85.44	15.97	3.378	0.946	1.195	
	BCG IL-12	2994	2835	6680	10.4	1.616	7.866	0.9375	
		Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**				
	p	0.87	0.22	0.18	0.13				
IL-10	BCG	775.6	510	611.5	0.4733	0.376	0.442	0.5175	
	BCG IL-12	841.5	447.4	523.9	0.4567	0.202	0.366	0.35	
	BCG IFN-γ	120.6	60.8	22.45	0.6933	0.304	0.302	0.295	
		Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**				
	p	0.42	0.5	0.74	0.61				
IL-12p70	BCG	0.3638	2.132	0.032	0.54	0.2	0.2	0.25	
	BCG IFN-γ	38.23	73.18	88.62	4.78	2.87	1.308	3.07	
		Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**				
	p	0.48	0.44	0.5	0.5				
IL1-RA	BCG	42.72	144.4	69.55	1.157	1.272	1.69	1.83	
	BCG IL-12	39.82	140.4	69.27	1.197	1.07	1.45	1.385	
	BCG IFN-γ	47.04	144.8	70.63	0.93	1.086	0.968	1.435	
		Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**				
	p	0.07	0.32	0.07	0.12				
IL-1β	BCG	4223	2176	2069	0.94	0.626	1.284	0.6775	
	BCG IL-12	4361	2232	2099	1.063	0.838	1.76	0.7725	
	BCG IFN-γ	5264	2180	2139	0.8967	0.738	0.948	0.5525	
		Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**				
	p	0.06	0.06	0.39	0.05				
IL-6	BCG	953.3	2676	3551	0.7233	4.172	0.882	0.785	
	BCG IL-12	1783	2703	3538	0.61	2.124	1.114	0.9425	
	BCG IFN-γ	1002	3176	3571	5.92	0.704	0.614	0.6575	
		Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**				
	p	0.06	0.03*	0.52	0.25				
TNF-α	BCG	621.8	42.86	243.7	20.91	18.15	6.604	1.06	
	BCG IL-12	638	44.09	165.8	24.34	51.46	9.276	1.23	
	BCG IFN-γ	818.8	87.72	509.1	34.44	40.06	3.528	0.85	
		Non-exp vs exp	**Non-exp vs w_exp**	**Exp vs w_exp**	***p* of the effect of time in the changes observed**				
	p	0.0002*	0.02*	0.005	0.02				

Stim, stimulation; non-exp, healthy neonate; exp, infants exposed to anti-TNF-α during pregnancy, blood cultured with autologous sera; w_exp, infants exposed to anti-TNF-α during pregnancy, blood cultured after PBS washing; MFI, mean fluorescence intensity; freq, frequency of lymphocytes.
Age expressed in months.
**indicates statistically significant p-values.*

frequency was greater in exposed infants (72.21 vs 47.70% of B cells, $p = 0.045$) and switched B-cell population was smaller (2.84 vs 3.90% of B-cells, $p = 0.045$). Circulating marginal zone B-cells were decreased (20.16 vs 40.74% of B cells; $p = 0.03$), and activated B-cells (1.23 vs 0.05% of B cells; $p = 0.003$) and IgM-only B-cell-frequency (1.81 vs 0.43% of B-cells, $p = 0.002$) were increased. Although at some time-points, some B-cell subsets showed values outside the range, at 12–18 months all values were within age-matched ranges (23, 24, 26, 27). In all exposed-infants, IgG, IgA, and IgM production were since birth in range; at 3 months there was an increase in IgM with respect to reference ranges (**Figure 3C**). IgG-responses to vaccines (tetanus, diphtheria, and pneumococcus) were normal (**Table 3**).

Differences in Treg- and Breg Cells in Exposed Infants

In cord blood of anti-TNF-α-exposed-infants, Treg (**Figure 4**) were diminished (0.24 vs 0.5% of $CD4^+$-cells; $p = 0.04$). An inverse correlation between mother's anti-TNF-α trough level and Treg frequency was observed ($r = -0.9$; $p = 0.03$); at follow-up, almost all Treg values were below the lower reference limit (24). Treg frequency inversely correlated with

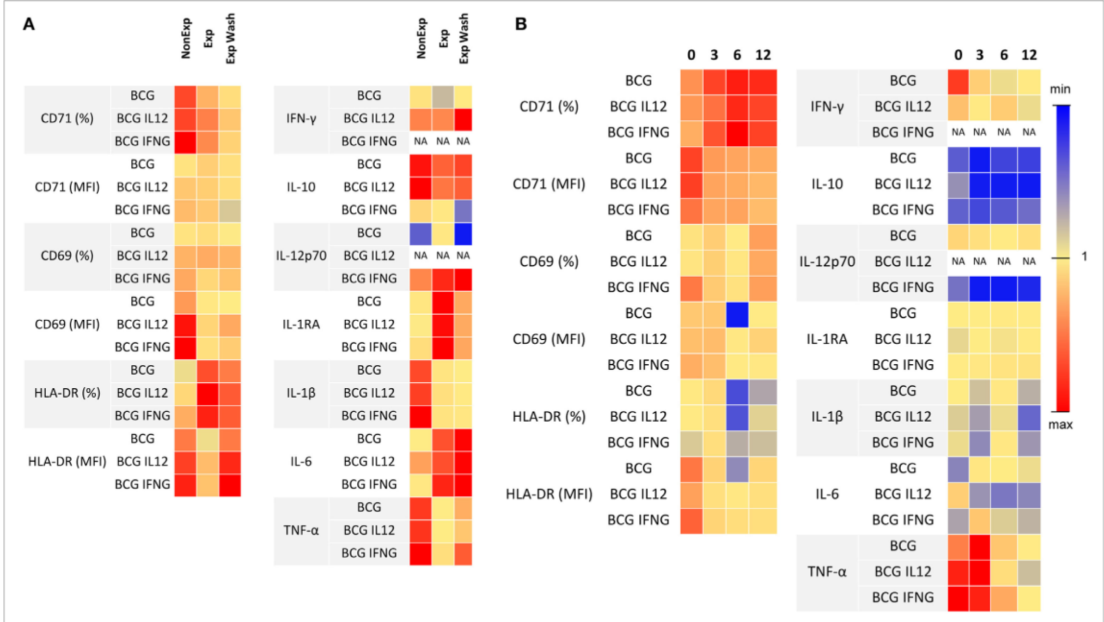

FIGURE 5 | Effects of anti-TNF-α exposure in the IL-12/IFN-γ pathway. **(A)** Stimulation ratio of lymphocyte activation markers expression (measured with flow cytometry) and secreted cytokines (measured with luminex) is represented as a heat map. Stimulation ratio is calculated as "stimulated culture condition/baseline condition." **(B)** Ratio of lymphocyte activation markers expression and secreted cytokines after Bacillus Calmette–Guérin (BCG) culture using washed blood and non-washed blood during follow-up is represented as a heat map. Blue indicates the minimum values, red the maximum, and yellow next to one for each parameter. Adalimumab-exposed infants, $n = 4$; infliximab-exposed infants, $n = 2$.

T-cell proliferation to ConA (a weak T-cell mitogen; $r = -0.64$, $p = 0.01$; **Figure 4**). Ratio of division indexes between T-cells/total lymphocytes was significantly statistically higher in exposed infants than controls (1.6 vs 0.52; $p = 0.01$; **Figure 4**), while there were no differences when strong T-cell mitogens were used (Figure S6 in Supplementary Material). Inversely to the decrease in Treg, we observed in cord blood an increase in Breg (49.31 vs 34.39% of B-cells, $p = 0.0007$; **Figure 4**). We have observed similar amounts of IL-10 production in exposed-infant's B cells as in cells from non-exposed infants. We also observed that Breg cell frequency positively correlated with the frequency of IL-10⁺ B cells (Figure S8 in Supplementary Material).

Deficient Mycobactericidal Response in Exposed-Infants

We evaluated anti-mycobacterial response by studying surface activation markers and cytokine secretion after whole blood cultures in the presence (non-washed condition) and absence (washed condition) of autologous sera.

Exposure to anti-TNF-α during pregnancy reduced the response after mycobacterial challenge (**Figure 5A**; **Table 3**). Exposed-infants presented at birth a lower stimulation ratio (SR, stimulated condition/basal condition) of CD69 ($p = 0.004$) and HLA-DR ($p = 0.003$) MFI (expression per cell). CD69 expression was partially recovered after drug removal ($p = 0.02$) although still reduced ($p = 0.004$); drug removal had a mild effect on HLA-DR SR reduction ($p = 0.05$). There was a tendency toward a reduction of $CD71^+$ frequency SR, with ($p = 0.06$) or without (0.06) drug presence.

At birth, TNF-α induction was reduced in the exposed-infant ($p = 0.0002$), being partially recovered after drug removal ($p = 0.005$) although still reduced ($p = 0.02$). Although without statistical significance, IL-1ß secretion was reduced ($p = 0.06$), and IL-6 ($p = 0.06$) and IL-1RA ($p = 0.07$) SR were increased. IL-1ß was not recovered after washing, IL-1RA SR was rescued and IL-6 secretion increased even more ($p = 0.03$). Without stimulation, washed samples from exposed infants produced higher amounts of IL-17 (measured by ELISA) compared with the non-washed condition ($p = 0.03$) and with non-exposed infants ($p = 0.02$). On the other hand, after BCG stimulation, IL-17 production in exposed infants was reduced, as none of the exposed infants produced any detectable IL-17 but one; however, differences did not reach statistical significance. Differences between non-washed and washed samples from exposed infants were maintained ($p = 0.03$) but disappeared when compared with non-exposed infants ($p = 0.95$) (Figure S9 in Supplementary

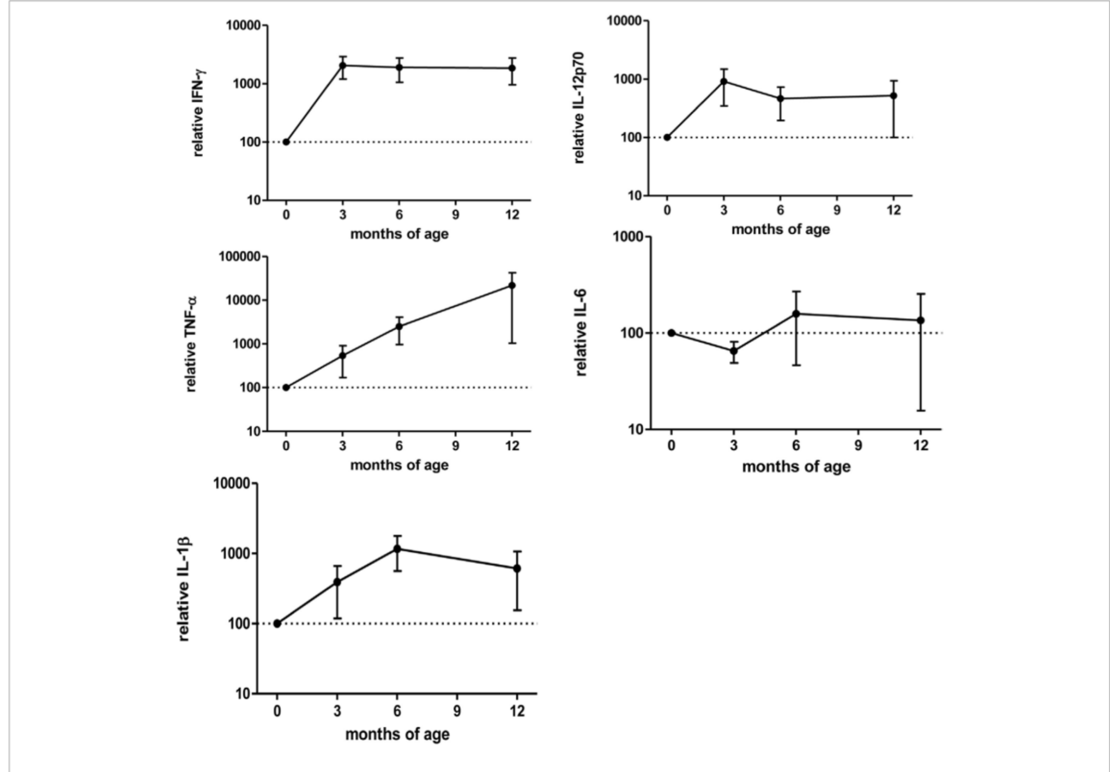

FIGURE 6 | Cytokine secretion in the first months of age related to birth in exposed infants. Cytokine secretion measured by Luminex after 48 h culture with Bacillus Calmette–Guérin. Values are relative to "birth" (0) time point. Points represent mean and bars SE of the mean. Adalimumab-exposed infants, *n* = 3; infliximab-exposed infants, *n* = 1 Tables.

Material). Altogether, these data suggest that immune system activation upon mycobacterial challenge may be compromised.

After anti-TNF-α clearing (3–12 months after birth), IFN-γ, IL-12p70, IL-1β, and TNF-α production increased while IL-6 production was stable (**Figure 6**) and the differences between washed and non-washed blood of exposed infants decreased (overall effect of time on the ratio between washed and non-washed blood, $p = 0.006$). This effect was observed in CD69 ($p = 0.009$) and HLA-DR ($p = 0.0009$) MFI, $CD71^+$ frequency ($p = 0.003$), and TNF-α secretion ($p = 0.02$) (**Figure 5B**).

Clinical Manifestations

All children showed normal growth and neurological development. Although one child suffered from recurrent infections from 6 to 12 months of age, no other exposed-infants manifested any significant infections, despite normal microbial exposure (attendance to daycare or siblings below 5 years old). Although our recommendation was to avoid rotavirus vaccination, it was used in four exposed-infants with no adverse effects. Atopic dermatitis was observed in four children (two of them without family history), and food allergy was diagnosed in one of them (**Table 4**).

DISCUSSION

In accordance with published data (11, 13), anti-TNF-α mAbs were detectable until 6 months post-partum. Exceptionally, anti-TNF-α mAbs were detectable at 12 months in one exposed-infant. Our results on the child/mother ratio level show some differences from those of a recent publication: mean ratio of 0.99 for ADA and 3.25 for IFX vs reported ratios of 1.21 and 1.97, respectively (13). These differences may be justified because we measured mother's trough levels during pregnancy instead of levels at birth; also, all patients included received the treatment only 1 week before delivery, while in Julsgaard et al. patients received the last dose from 0 to 25 weeks before delivery. Of interest, we observed a greater ratio with IFX than ADA, attributable to the larger inter-dose interval of IFX than ADA (4–8 folds), and all patients received the last dose a week prior to birth. These discrepancies in the "transferred dosage" of anti-TNF-α may explain why effects observed seemed to be stronger in IFX-exposed infants. Our recommendation would be to try to separate as much as possible from birth the administration of anti-TNF-α in the mother to reduce the drug level in the newborn. In this sense, there are other studies recommending ADA and IFX discontinuation

TABLE 4 | Clinical evolution of exposed infants.

ID	Gender	Treatment	Blood follow-up	General information	Family history	Siblings	Infections	Other clinical symptoms	Vaccinations
1	Male	ADA + Pred	No	Normal development, 2w BF, daycare from 9 m	Father with atopic dermatitis and allergic conjunctivitis	Yes. Exposed to ADA and Pred. No significant infections, or atopy	No severe infections. 2 upper respiratory tract infections, 2 gastroenteritis	Atopic dermatitis	Yes, including Prevenar, varicella and MMR
2	Male	ADA + AZA	18 m	Normal development, 3 m BF, daycare from 12 m	Father with psoriasis and allergic rhinitis	NO	No severe infections. 3 gastroenteritis, 3 upper respiratory tract infections, 1 conjunctivitis, 1 bronchitis, and 2 otitis.	At 2y, auto-limited tics	Yes, including varicella, MMR, and RotaTeq® without adverse effects. Urticarial reaction, facial edema, and fever after Prevenar
3	Male	ADA	18 m	Normal development, 5 m BF, daycare from 5 m	Mother with atopic dermatitis and allergic rhinitis	NO	No severe infections. 2 upper respiratory tract infections, 5 recurrent bronchitis, 4 otitis media, and 1 conjunctivitis. Most infections between 6 and 12 months.	Atopic dermatitis and egg allergy	Yes, including *p* Prevenar, varicella, MMR and RotaTeq® without adverse effects.
4	Male	IFX	18 m	Normal development, BF continues at 2y, no daycare	NO	Yes. Exposed to mesalazine. No infections but atopy	No severe infections. 3 bronchitis, thrush that responded to treatment, 1 tonsillitis with otitis media without complications, and 3 upper respiratory tract infections without complications.	Atopic dermatitis	Yes, including Prevenar, varicella, MMR, and RotaTeq® without adverse effects
5	Female	IFX + ADA	12 m	Normal development, no BF, no daycare	NO	NO	No severe infections. 1 otitis media; 1 upper respiratory tract infection complicated with bronchitis; and 2 urinary tract infections without pyelonephritis.	Atopic dermatitis	Yes, including Prevenar and MMR without adverse effects
6	Female	ADA	3 m	Normal development, BF continues at 12 m, daycare from 7 m	NO	NO	No severe infections. 4 otitis media. Mouth-hand-feed infection	NO	Yes, including Prevenar and MMR without adverse effects
7	Male	ADA	12 m	Normal development, no BF, no daycare	NO	Yes, exposed to ADA without significant infections or atopy	No severe infections. 2 upper respiratory tract infections, not complicated	NO	Yes, including Prevenar, MMR and RotaTeq® without adverse effects

ADA, adalimumab; AZA, azathioprine; IFX, infliximab; Pred, prednisone; w, week; m, month; y, year; BF, breastfeeding.

after week 20 of pregnancy to try to reduce drug levels in the newborn (28–30).

In some patients, it has been seen that anti-TNF-α exposure during pregnancy can lead to neutropenia (28). Neutrophils from four children exposed to IFX during pregnancy (including third trimester, without other immunosuppressants) were decreased when measured "a few days" or 15 days after birth, with levels below $<0.5 \times 10^9$ ANC/L in 3 and 1.1×10^9 ANC/L, and increased 3 months after birth; infectious skin lesions occurred during neutropenia. In our study, three exposed infants had normal levels of neutrophils at birth in cord blood, two had values below the reference range for the age ($4.3–11.4 \times 10^9$ ANC/L): 2.78 (exposed to ADA + steroids) and 3.06×10^9 ANC/L (exposed to IFX + AZA) and one had values in the limit of the reference range: 4.73×10^9 ANC/L (exposed to ADA + AZA). We observed a decrease of neutrophil levels below the reference ranges at 3 months of age ($2.2–6.3 \times 10^9$ ANC/L): one infant with severe neutropenia (0.25×10^9 ANC/L; exposed to ADA) and two with moderate neutropenia (1.35 and 1.14×10^9 ANC/L, exposed to ADA + AZA and ADA, respectively). None of them presented infectious skin lesions. Differences in the results may be due to differences in the drug infusion pattern. In the four cases described, the infusion of the last IFX dose was, at least, 8 weeks' prepartum. Instead, patients included in our study received the last dose of either IFX or ADA from 3 to 7 days' pre-partum. Also, other factors (such as prematurity and presence of positive neutrophil-specific CD16 autoantibodies) differentiate our cohort from the cases published by GuiddirT et al. However, we agree that neutrophil count should be routinely performed in infants exposed to anti-TNF-α drugs during pregnancy, especially in the event of an infection.

We have observed normal numbers of B cells at birth, although with a more immature phenotype. It is known that TNF-α knock out mice presented abnormal B cell structures. They lack splenic B cell follicles, organized follicular dendritic cell networks and germinal centers. These alterations lead to a decreased humoral response and increased infection risk (20, 21). Nonetheless, mice exposed to anti-TNF-α mAb during gestation did not show any abnormal B cell structures. This difference might be ascribed to the fact that in mice, B cell development occurs 3 weeks after birth. Instead, in humans B cell development occurs during the third trimester of pregnancy and through 8 weeks after delivery (29–31). A study on the impact of the exposure to golimumab during pregnancy in macaques revealed no effect in B or T cell frequency, nor in humoral responses, or in lymphoid organ formation, but maturation status of B cells was not assessed (32). Data from animal models along with our study reinforce the theory that TNF-α plays a role in B cell development and maturation in humans.

Based on empirical experience [adverse event to BCG vaccine (15) and theoretical knowledge (33)], the use of all live vaccines is delayed from 6 to 12 months of age in infants exposed to ADA or IFX during the late second and third trimester of pregnancy (13, 15). Here, we provide objective data to ponder this statement: at birth, exposed infants showed more immature B- and T-cell subsets. However, we observed a normal T-cell proliferation to mitogens, as well as T- and B-cell numbers and maturation, Ig production, and inactivated vaccine responses, accomplishing the criteria for attenuated vaccination in patients with cellular immunodeficiency (34). One infant presented B-cell lymphopenia at birth after ADA + AZA exposure; it is known that AZA exposure during pregnancy can lead to B cell lymphopenia at birth (35). Also, none of the four infants who received rotavirus-inactivated vaccine presented adverse events.

Immune system dysregulation needs to be considered: four of seven of our children presented atopy in the first year (two of them without family history), and all ADA-exposed infants had increased eosinophil counts in cord blood. Exposed infants showed an altered T- and B-regulatory compartment. There was an increased Breg frequency, a population having an anti-inflammatory role in cord blood (36). By contrast, we can speculate that a decreased Treg cell frequency correlating with increased T-cell response to weak stimulus may be a sign of a more responsive immune system, which might be related to the atopy in these patients. However, we cannot rule out the possibility that this may be influenced by the mother's disease (37). A decrease on Treg cell population has also been observed in infants born to mothers that had received a kidney transplant and were exposed to immunosuppressive drugs during pregnancy. However, in this case, Treg cell numbers were rescued with age (38). As Treg did not increase over time, clinical evolution of exposed infants should be specifically followed-up, with special attention to allergic, inflammatory, and autoimmune events. More studies with larger cohorts are needed to confirm these results.

We have observed a diminished frequency of Treg cells described as $CD4^+CD25^{hi}CD127^{low}FoxP3^+$ T cells in all exposed infants compared to healthy controls. Interestingly, there are some publications showing an increase in Treg cells in responder patients after anti-TNF-α treatment (39–41). These differences may be explained by the possibility that (1) It is described that the cells that increase in adult are not natural ($CD62L^+$) but induced ($CD62L^-$) Treg cells (40). As induced Treg cells are differentiated upon an antigenic insult (42), in the umbilical cord blood, the majority of Treg cells would be expected to be natural Treg as they express high levels of CD62L (43) and (2) the effect of anti-TNF-α drugs on the development of induced Tregs in exposed infants is difficult to assess since as early as at 3 months the amount of anti-TNF-a in blood had significantly decreased. It has been noted that the functional capacity of Tregs after anti-TNF-a treatment is increased. Although it would be interesting to study the inhibitory capacity of Treg cells in exposed infants, we cannot test this in samples from our cohort due to limitations related to cell number requirements.

We have shown that drug exposure decreases the response after a mycobacterial challenge at birth, which did not totally recover after drug cleaning. In adults, anti-cytokine biological treatments are thought to cause an immune-deficiency-like phenotype (17). This should also be applied to infants who, besides, intrinsically present a Th2-biased response (44). It has been observed that there is a decreased production of IL-12 but not of IL-6 after anti-TNF-α therapy in adults (45). Also, that there is a decrease in IFN-γ-producing CD8 T cells and in Th1/Th17 subset with an increase in IFN-γ-producing NK cells (46). Results obtained after BCG stimulation do not correspond with those published; we have observed no significant differences in

IL-12p70 production in comparison with non-exposed infants and an increased production of IL-6. However, from our results and others (36, 44, 47–50), it would seem that the immune system of patients with inflammatory diseases and neonates show differentiable characteristics. Also, presence of anti-TNF-a mAbs reduced IL-17 production after BCG stimulation that was rescued after whole-blood washing. The advent of biosimilars will broaden the use of biological treatments in developing countries, some of which have endemic tuberculosis or BCG vaccination soon after birth. Until more investigations are performed, BCG vaccination must be absolutely avoided in exposed infants until recovery of antimycobacterial function is verified or at least until 12 months of age. *In vitro* functional studies would be relevant for this purpose.

Although this study has several strengths, including the thorough immune system analysis, it also has some limitations: our cohort of exposed infants is small, and a broader group would probably provide more robust information. Nevertheless, all observations were consistent from sample to sample. Our study control group included infants born to healthy mothers, since no IBD pregnant women with moderate-to-severe disease were without anti-TNF-α treatment; thus, we have not been able to evaluate the effect of IBD itself. Finally, immunological follow-up of healthy controls was not performed for ethical reasons.

This study is the first thorough evaluation of the impact of prenatal anti-TNF-α on the immune system development of exposed-infants. Although we observed specific changes, infants were not clinically compromised. Our results aim at generating consciousness of the need to further study and follow-up on exposed-infants. The pediatrician should be informed of the mother's mAb treatment during pregnancy, because of the impact on vaccine recommendations, especially with regards to BCG due to the observed mycobacterial-deficient response.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ley General de Sanidad (25/4/1986) Art. 10, with written informed consent from all subjects. The protocol was approved by the ethics committee of the Hospital Sant Joan de Déu (Comité Ético de Investigaciones Clínicas number PIC-50-12). All patients included in the study signed the informed consent, complying with current legislation.

AUTHOR CONTRIBUTIONS

AE-S performed immunological studies, carried out the analyses, drafted the initial manuscript, and approved the final manuscript as submitted. IT performed the clinical management of pregnant women, critically reviewed the manuscript, and approved the final manuscript as submitted. AD-M performed clinical follow-up of exposed infants, critically reviewed the manuscript, and approved the final manuscript as submitted. ER and MG performed the clinical management of IBD patients, critically reviewed the manuscript, and approved the final manuscript as submitted. MT, NM, and EG performed anti-TNF-α monitoring, critically reviewed the manuscript, and approved the final manuscript as submitted. JY coordinated anti-TNF-α monitoring, analyzed anti-TNF-α monitoring results, critically reviewed the manuscript, and approved the final manuscript as submitted. AP and MJ conceptualized and designed the study, critically reviewed the manuscript, and approved the final manuscript as submitted. LA performed clinical follow-up of exposed infants, conceptualized and designed the study, critically reviewed the manuscript, and approved the final manuscript as submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01123/full#supplementary-material.

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Supplementary Material

Immunological changes in blood of newborns exposed to anti-TNF-α during pregnancy.

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Supplementary Data

Supplementary methods

Anti-TNF-a monitoring:

Levels of anti-TNF-α monoclonal antibody (adalimumab or infliximab) were measured in the serum/plasma of the exposed infants and their mothers with Promonitor ® kits (Movaco, Grifols, Spain) using the Triturus Immunoassay System (Grifols Movaco, Spain). Levels of immunoglobulin A, G, and M were determined by nephelometry (Siemens, Germany).

Immunophenotyping:

Immune-phenotype cell staining was performed in heparinized whole blood: for surface staining, 50µL of whole blood was incubated for 15 min at room temperature (RT) with the appropriate monoclonal antibodies (BD Bioscience, San Jose, CA, USA). To lyse erythrocytes and fix cells, stained cells were incubated with 2 ml of BD lysing solution 1x (BD Bioscience) for 15 min at RT. Cells were then washed two times with FACS buffer [phosphate buffered saline (PBS) with 5% fetal calf serum, 0.5% bovine serum albumin, and 0.07% NaN_3] and samples were acquired using FACSCanto-II (BD Bioscience) cytometer. A minimum of events were acquired for the different populations: 20,000 T cells for T cells subpopulations, 10,000 B cells for B cells subpopulations, and 10,000 lymphocytes for stimulation markers detection. The antibodies used (Ab) are presented in Supplementary Table 2. Gating strategy was previously described (1).

Treg intracellular staining was performed with Treg Detection Kit (CD4/CD25/FoxP3) (Milteny Biotec, Germany) following the manufacturer's instructions. Briefly, after surface staining performed as described above, cells were fixed with 500 µl of Fixation Buffer for 30 min at 4ºC. Cells were washed two times with FACS buffer and after that incubated with Perm Buffer. To block non-

specific Ab binding, cells were incubated with 20 µl of Perm Buffer and 5 µl of FcR Blocking Reagent for 5 min at RT. Cells were then stained with FoxP3-APC Ab for 30 min at 4ºC. Finally, cells were then washed with FACS buffer and acquired by the cytometer.

To study the level of Treg cells in the described reference range, we determined the correlation between the gold standard phenotype for Treg ($CD3^{+}CD4^{+}CD25^{hi}CD127^{+}FoxP3^{+}$) and the one in which the reference values are calculated ($CD3^{+}CD4^{+}CD25^{hi}CD127^{+}$). We found a good correlation between the two populations (r: 0.67; p: 0.0009), **Supplementary Figure 6.**

Intracellular cytokine staining

For IL-10 production detection, as previously reported (18), cells were maturated 48h with CD40L and LPS and stimulated during the last 5h with LPS, PMA and ionomycin in the presence of Brefaldin A (BFA; 10µl/ml, Sigma-Aldrich, St. Louis, MO, USA). Cells were washed with FACS Buffer and incubated with mAb for anti-human surface molecules for 15 min RT, cells were then permeabilized and then incubated for 30 min, RT, dark with anti-IL-10 Ab. Cells were then washed with FACS buffer and acquired by the cytometer.

Proliferation analysis

Peripheral blood mononuclear cells (PBMCs) from hUCB and healthy controls were isolated by Ficoll-Hipaque (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation of heparinized blood. Cells were subsequently washed three times with PBS 1x (Roche Diagnostics, Barcelona, Spain) and cultured with complete medium [RPMI (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA), 1 µg/ml penicillin, and 1µg/ml streptomycin (Invitrogen, Grand Island, NY, USA)]. Viable cells were counted using a hemocytometer in an inverted microscope.

$1.5\cdot10^{6}$ PBMC/ml was labeled with 5µM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Invitrogen, Grand Island, NY, USA) for 10 min at 37ºC; then 5mL of cold PBS supplemented with 10% FCS was added for 5 min at 4ºC and washed twice with PBS + 2% FCS before stimulation. CFSE-stained PBMCs (10^{6} PBMCs/ml) were stimulated with phytohemaglutinin A (5µg/mL; PHA, Sigma, St. Louis, MO, USA), pokeweed mitogen (2 µg/mL; PWM; Sigma, St. Louis, MO, USA) and Concavalin A (2 µg/mL; ConA, Sigma, St. Louis, MO, USA) or medium only in a 96-well plate for 7 days at 37ºC in a humidified incubator, with 5% CO2.

We analyzed division index and proliferation index calculated with the FlowJo 7.3 software. Division index is the average number of cell divisions per cell in the original population and it includes the entire population, whereas proliferation index refers to the total number of divisions per proliferating cell as it only takes into account the cells that underwent at least one division(2).

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Supplementary Figures and Tables

Supplementary Table 1. Follow-up of anti-TNF-α exposed infants.

Clinical follow-up (months)					
	Birth	**3m**	**6m**	**12m**	**18m**
Neonatologist and immunologist visit	✓				
Immunologist visit		✓	✓	✓	✓
Immunological follow-up (months)					
	Birth	**3m**	**6m**	**12m**	**18m**
Sample	UCB	PB	PB	PB	PB
Drug levels	✓	✓	✓	✓	✓
Whole blood count	✓	✓	✓	✓	✓
T/B/NK immune-phenotype	✓	✓	✓	✓	✓
T/B sub-phenotype	✓	✓	✓	✓	
T/B regulatory cells detection	✓	✓	✓	✓	
Mitogen proliferation	✓	✓	✓	✓	
IL-12/IFN-γ study	✓	✓	✓	✓	
anti-tetanus Ab					✓
anti-diphtheria Ab					✓
anti-rubella Ab					✓
anti-pneumococci Ab					✓

UCB: umbilical cord blood; PB: peripheral blood.

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Supplementary Figures and Tables

Supplementary Table 2. Antibody panels for umbilical cord and peripheral blood phenotyping.

Panel	Antibody - Fluorochrome					
T-B-NK phenotype	CD3[1] APCCy7 SK7	CD4[2] FITC VIT4	CD8[1] PerCPCy5.5 RPA-T8	CD16/CD56[1] PE B73/MY31	CD19[1] PECy7 HIB19	CD45[1] APC 2D1
T-subphenotype	CD3[1] APCCy7 SK7	CD8[1] PerCPCy5.5 RPA-T8	CD45RA[1] PECy7 L48	CD45RO[1] APC UCHL1	TCRab[1] FITC WT31	TCRgd[1] PE 11F2
B-subphenotype	CD19[1] PECy7 SJ25C1	CD21[1] FITC B-ly4	CD38[1] APC HIT2	IgD[1] PE IA6-2	IgM[1] PerCPCy5.5 SA-DA4	CD27[3] PECy7 1A4CD27
T regulatory cells	CD3[3] AlexaFluor750 UCHT1	CD4[2] FITC VIT4	CD25[2] PE 4E3	CD127[1] PECy7 HIL-7R-M221		
B regulatory cells	CD19[1] PECy7 SJ25C1	CD24[1] PerCPCy5.5 ML5	CD38[1] APC HIT2			
Activation markers	CD11b[1] PECy7 ICRF44	CD69[1] APCCy7 FN50	CD71[1] APC M-A712	HLA-DR[1] PerCPCy5.5 G46-6		

[1]BD Biosciences; [2]Milteny Biotech; [3]Beckman coulter (Brea, CA, USA);

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Supplementary Figures and Tables

Supplementary Table 3. Mother's clinical data, treatment details, and delivery conditions.

Clinical data	
Age	34 (range 27-36y)
IBD	6 Crohn 1 ulcerative colitis
IBD duration (years)	9 (range 2.5-11 years)
Severity of disease	Crohn: 4 ileal (3 resections); 2 colonic localizations Extensive ulcerative colitis
Active disease during pregnancy	5 remissions 2 activity (1-3 Tm and 1st Tm)
Other co-morbidities	1 epilepsy (treated with levetiracetam + CMZ) + erythema nodosum 1 hypothyroidism
Treatment details	
Anti-TNF-a dosage during pregnancy	5 Adalimumab: 40 mg/2 weeks (4) 40 mg/1 weeks (1) 2 Infliximab 5 mg/kg/8 weeks 10 mg/kg/6-8 weeks
>6 month anti-TNF-a before delivery?	7/7
Anti-TNF-a levels during pregnancy	Measured 12 days before delivery (4-30 days) Adalimumab: 4/5: > 4µg/ml (5.2 - >12 µg/ml) Infliximab: 2/2: > 3 µg/ml (4.1 to 12.4 µg/ml)
anti-TNF-a mAb	7/7 negative
Other immunosuppressive treatment during pregnancy	3/7 Prednisone 20-40 mg/day (1) Azathioprine 125-150 mg/day (2)
Pregnancy and labor	
Other risk factors	1 smoker
Pregnancy complications	2 mild pre-eclampsia
Delivery	4 normal 3 caesarean sections (1 urgent, 2 elective due to previous caesarian section)
Gestational age	1 premature 35 weeks (IBD maternal activity) 6 on term: 39 weeks (37-41 weeks)
Apgar 1/5	9/10 all
Low weight for gestational age or malformations	No
Post-partum IBD reactivation	2/7

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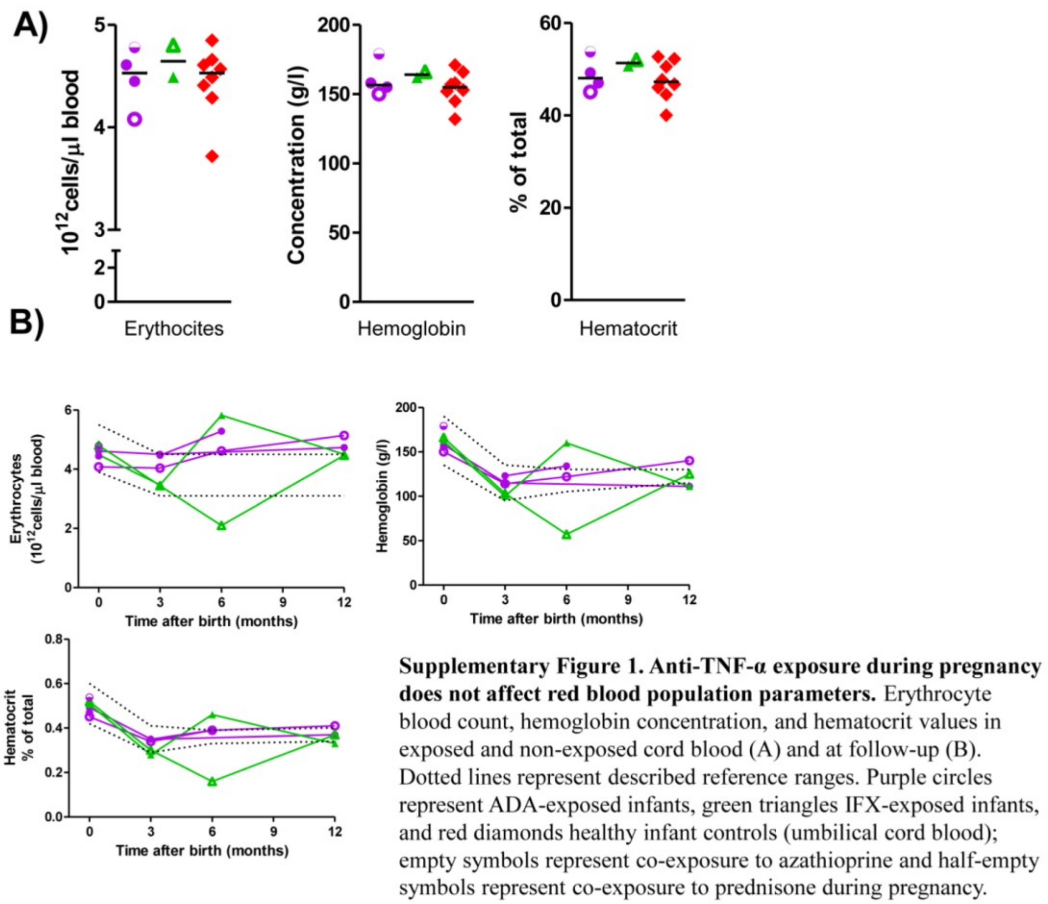
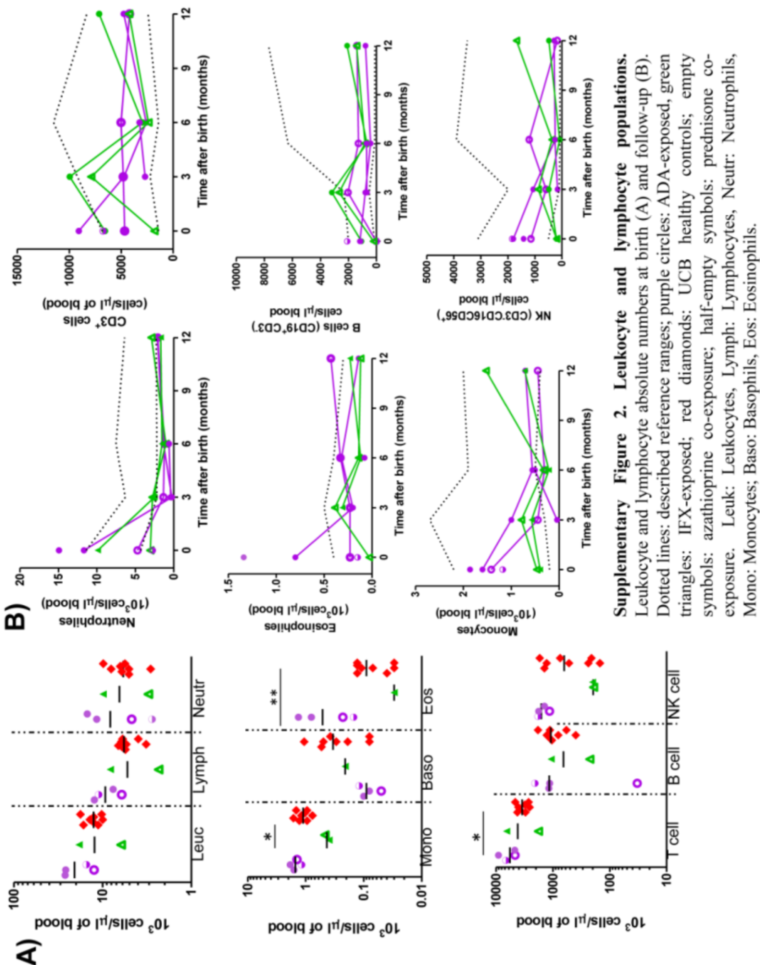
Supplementary Figures and Tables

Supplementary Table 4. Anti-TNF-α drug levels. ADA: adalimumab, IFX: infliximab

	Drug	Trough drug level (µg/ml) Mother	Drug level (µg/ml) Cord blood	Days from last mother's anti-TNF-α dose to labor	Months with detectable drug in blood
1	ADA	--	6.46	--	--
2	ADA	>12	11.48	7	12
3	ADA	>12	13.168	5	6
6	ADA	7.8	--	7	--
7	ADA	5,2	5.87	3 0	--
4	IFX	4.1	11.11	7	6
5	IFX	12.4	42.52	4 0	6

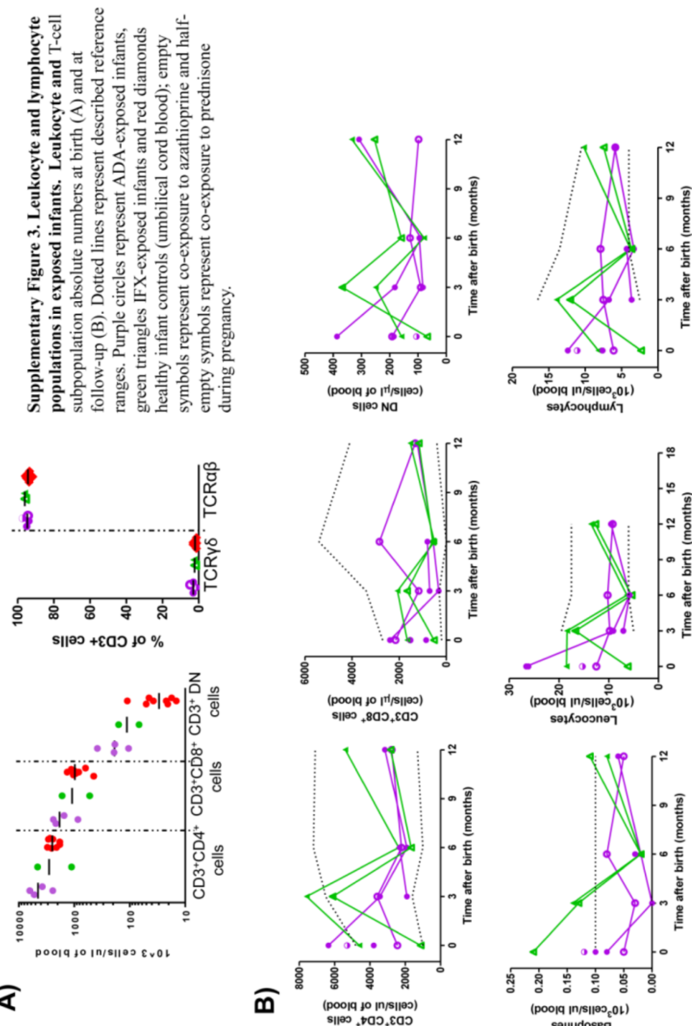


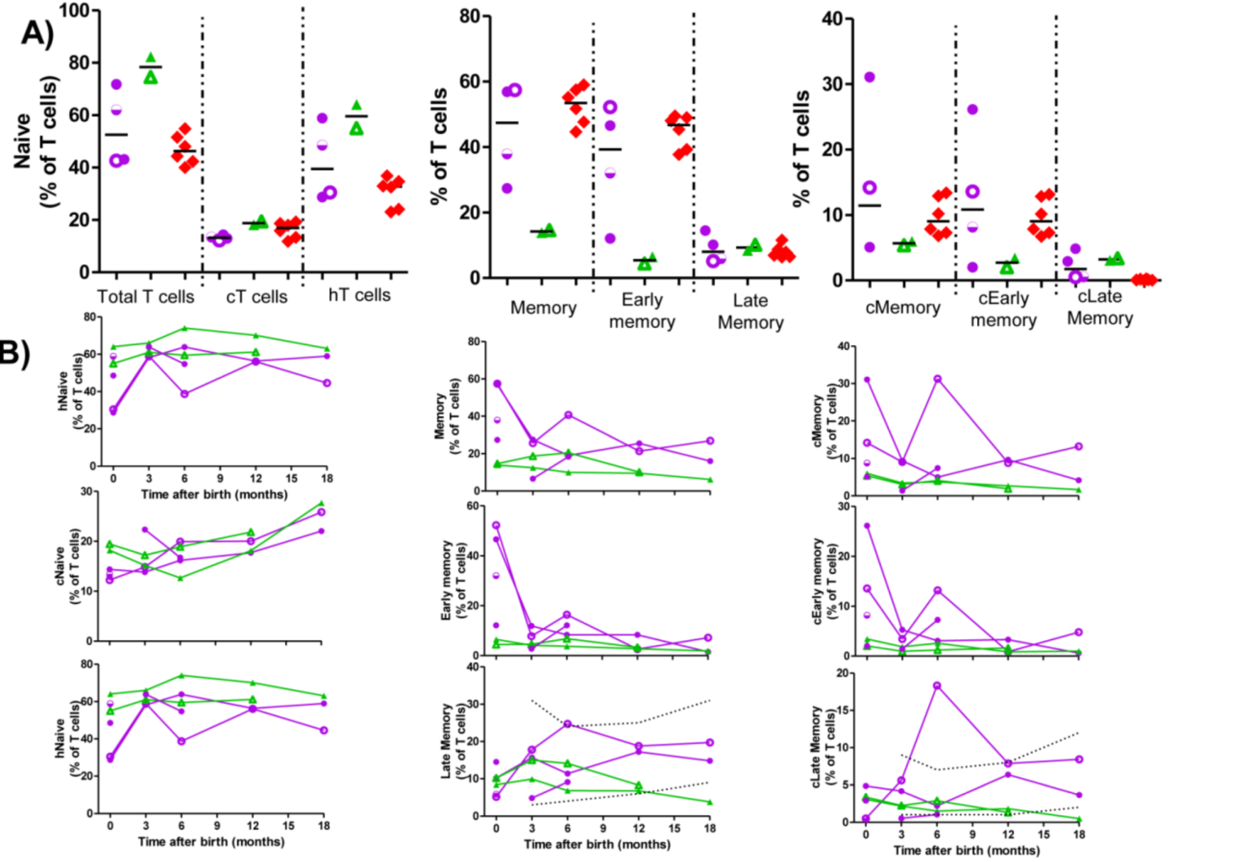
Supplementary Figure 1. Anti-TNF-α exposure during pregnancy does not affect red blood population parameters. Erythrocyte blood count, hemoglobin concentration, and hematocrit values in exposed and non-exposed cord blood (A) and at follow-up (B). Dotted lines represent described reference ranges. Purple circles represent ADA-exposed infants, green triangles IFX-exposed infants, and red diamonds healthy infant controls (umbilical cord blood); empty symbols represent co-exposure to azathioprine and half-empty symbols represent co-exposure to prednisone during pregnancy.



Supplementary Figure 2. Leukocyte and lymphocyte populations. Leukocyte and lymphocyte absolute numbers at birth (A) and follow-up (B). Dotted lines: described reference ranges; purple circles: ADA-exposed, green triangles: IFX-exposed; red diamonds: UCB healthy controls; empty symbols: azathioprine co-exposure; half-empty symbols: prednisone co-exposure. Leuk: Leukocytes, Lymph: Lymphocytes, Neutr: Neutrophils, Mono: Monocytes; Baso: Basophils, Eos: Eosinophils.

Supplementary Figure 3. Leukocyte and lymphocyte populations in exposed infants. Leukocyte and T-cell subpopulation absolute numbers at birth (A) and at follow-up (B). Dotted lines represent described reference ranges. Purple circles represent ADA-exposed infants, green triangles IFX-exposed infants and red diamonds healthy infant controls (umbilical cord blood); empty symbols represent co-exposure to azathioprine and half-empty symbols represent co-exposure to prednisone during pregnancy.





Supplementary Figure 4. Maturation state of T cells based on CD45RA/CD45RO expression. T cell subpopulation frequency at birth (A) and at follow-up (B). Dotted lines represent described reference ranges. Purple circles represent ADA-exposed infants, green triangles IFX-exposed infants, and red diamonds healthy infant controls (umbilical cord blood); empty symbols represent co-exposure to azathioprine and half-empty symbols represent co-exposure to prednisone during pregnancy. cT cells: cytotoxic T cells, h T cells: helper T cells.

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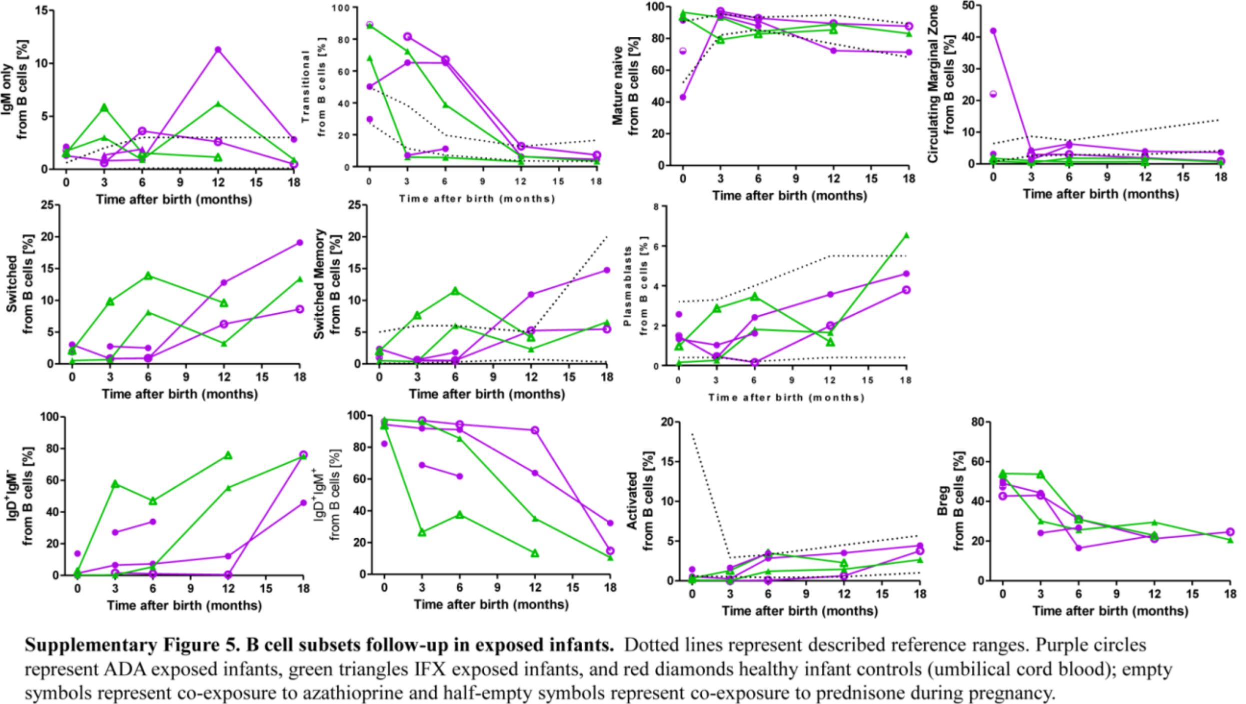
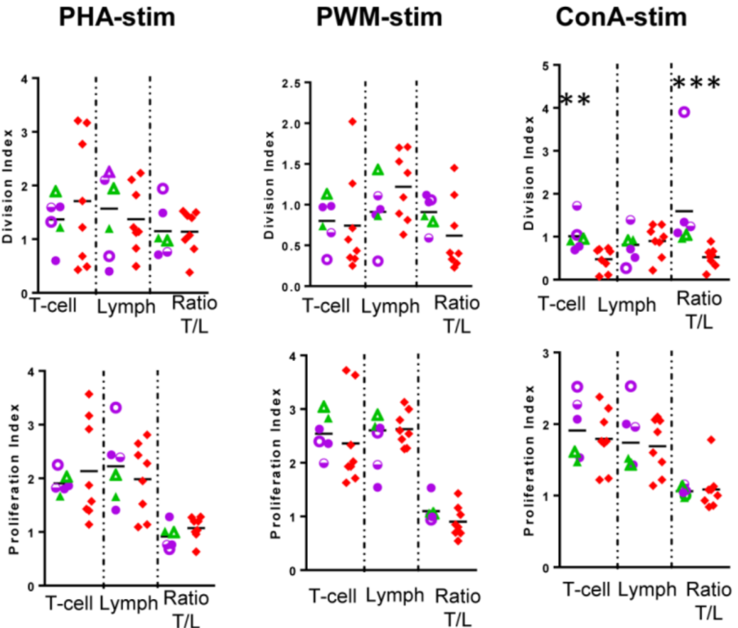
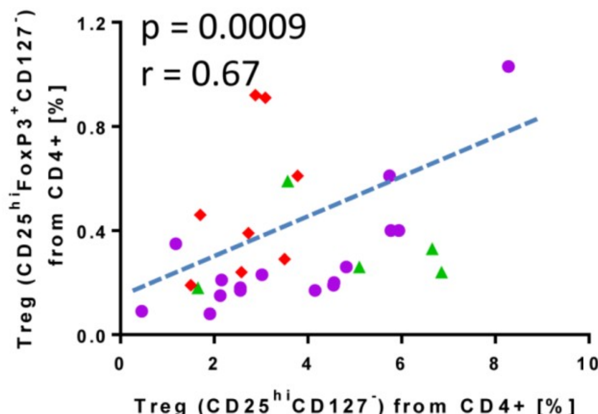


Supplementary Figure 5. B cell subsets follow-up in exposed infants. Dotted lines represent described reference ranges. Purple circles represent ADA exposed infants, green triangles IFX exposed infants, and red diamonds healthy infant controls (umbilical cord blood); empty symbols represent co-exposure to azathioprine and half-empty symbols represent co-exposure to prednisone during pregnancy.

Supplementary Material

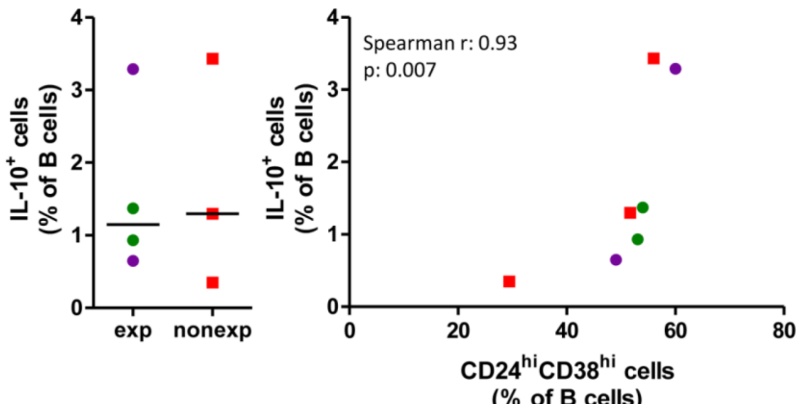


Supplementary Figure 6. Mitogen proliferation at birth and at follow-up in exposed infants. Division and proliferation index after PHA, PWM, and ConA stimulation during 7 days at birth. Purple circles represent ADA exposed infants, green triangles IFX exposed infants, and red diamonds healthy infant controls (umbilical cord blood); empty symbols represent co-exposure to azathioprine and half-empty symbols represent co-exposure to prednisone during pregnancy.

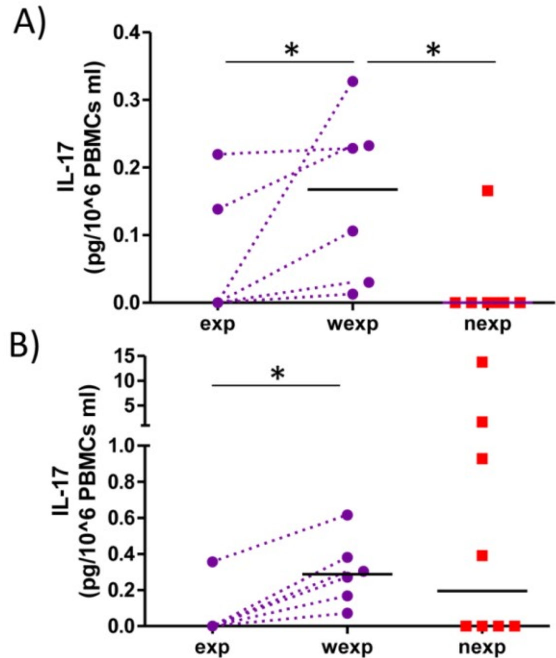


Supplementary Figure 7. Correlation between gating strategy with and without FoxP3 expression. Correlation between the two Treg gating strategies. Purple circles represent ADA exposed infants, green triangles IFX exposed infants, and red diamonds healthy infant controls (umbilical cord blood).

Supplementary Material



Supplementary Figure 8. B-cell production of IL-10 correlates with CD24hiCD38hi B cell frequency. A) IL-10 production after 48h with CD40L+LPS stimulation and 5h restimulation with LPS, PMA and Ionomicin in the presence of BFA. (% of IL-10^+ cells = % of IL-10^+ B cells in stimulated condition - % of IL-10^+ B cells in non-stimulation condition). B) Correlation between IL10^+ B cell frequency and CD38^{hi}CD34^{hi} B cells. Purple circles represent ADA exposed infants, green circles IFX exposed infants, and red squares healthy infant controls (umbilical cord blood). Exposed infants n=4, non-exposed infants n=3



Supplementary Figure 9. IL-17 production is increased after removal of autologous sera. IL-17 was measured by ELISA in SN of whole-blood cultures from cord blood from exposed infants (with and without PBS washing) and non-exposed infants at basal condition (A) and after 48h BCG stimulation (B). Bar represent median production of IL-17. Exposed infants n=6; non-exposed infants: n=8.

Chapter 3. Study of Breg cells and IL-12/IFN-γ axis in healthy neonates

Since TNF-α is a key factor for B cell development, we decided to perform a sub-study evaluating Breg cells in healthy neonates, which, by the time of the study, had not been described. Also, it would provide us with normality values for the evaluation of the impact of anti- TNF-α drugs. Breg cells (defined as $CD19^{+}CD24^{hi}CD38^{hi}$) are tolerance promoters in the adult immune system. They are capable of inhibiting IFN-γ and IL-17 production by T cells and have a significant role in different situations, including pregnancy. We hypothesized that Breg might have a role in the achievement of intrauterine tolerance expanded to the first moments after birth.

We studied $CD19^{+}CD24^{hi}CD38^{hi}$ in cord blood on healthy neonates (hUCB) and in peripheral blood of healthy adults (hAPB). Frequency of Breg cell was increased in hUCB in comparison with hAPB (34.39% vs. 2.49%; p: 0.0002), especially in the circulating marginal zone B cells, where the difference was bigger (60.8% vs. 4.94%). hUCB Breg cells were capable of producing IL-10 and to inhibit IFN-γ production after co-culture with T cells (1.63 T cells alone, 0.95 Tcell:Breg, stimulation ratio, SR; p: 0.004) and IL-4 (1.66 T cells alone vs. 0.86 Tcell:Breg SR; p: 0.02), which was not observed when co-cultured with noBreg ($CD19^{+}CD24^{low}CD38^{low}$) cells. hUCB Breg cells presented a $IgM^{hi}IgD^{hi}CD5^{+}CD10^{+}CD27^{-}$ phenotype, similar to that of adult Breg cells but with some differences. There was an increment of the quantity of IgM per cell and a decreased expression of CD27, CD22 and CD73 markers. Our work has characterized the frequency, phenotype and function of hUCB Breg.

Detailed methods, results and figures are shown in "*Esteve et al, Characterization of the Highly Prevalent Regulatory CD24(hi)CD38(hi) B-Cell Population in Human Cord Blood. Front Immunol. 2017*" manuscript.

Besides Breg cell characterization, we also studied the state of the IL-12/IFN-γ axis in healthy neonates. Overall response to mycobacterial challenge was reduced in neonates: hUCB cells produced significantly less IFN-γ (p: 0.001) and IL-6 (p: 0.005) upon BCG stimulation, as well as displayed lower frequency (p: 0.014) and levels (p: 0.051) of CD69. Besides, IFN-γ production after BCG stimulation inversely correlated with Breg cell frequency (spearman correlation test, R^2: 0.618, p: 0.043). After the study of the general anti-mycobacterial response we studied the integrity of the system in terms of response to hr-IL-12p70 and hr-IFN-γ co-stimulation. We observed a decreased Co-SR after hrIL-12p70 co-stimulation in TNF-α production (p: 0.022), however, we observed a tendency towards an increased IFN-γ Co-SR (p: 0.073). We have not observed any significant differences in cytokine production in Co-SR after hr-IFN-γ addition but an increased CO-SR in HLA-DR positive cells frequency (p: 0.026).

Detailed methods, results and figures are shown in "*Breg cells in umbilical cord blood: two sides of the same coin*" manuscript.

3.1.Manuscript "Characterization of the Highly Prevalent Regulatory CD24^{hi}CD38^{hi} B-cell Population in Human Cord Blood"

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Characterization of the Highly Prevalent Regulatory CD24^{hi}CD38^{hi} B-Cell Population in Human Cord Blood

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The newborn's immune system must transition from a sterile haploidentical uterus to the world full of antigens. Regulatory B-cells (Breg; broadly defined as CD19$^+$CD24^{hi}CD38^{hi}) are tolerance promoters in the adult immune system. They can inhibit IFN-γ and IL-17 production by T-cells and are essential in different conditions, including pregnancy. Breg have still not been well characterized in umbilical cord blood, where we hypothesize that they are pivotal in the achievement of tolerance. We studied CD19$^+$CD24^{hi}CD38^{hi} Breg in healthy umbilical cord blood (hUCB) compared to healthy peripheral adult blood (hAPB). Total numbers of Breg were increased in hUCB compared to hAPB (34.39 vs. 9.49%; $p = 0.0002$), especially in the marginal zone-like B-cell subset, in which the most marked difference could be observed between hUCB and hAPB (60.80 vs. 4.94%; $p = 0.1$). CD24^{hi}CD38^{hi} subset in hUCB produced IL-10 and inhibited T-cell IFN-γ [1.63 vs. 0.95 stimulation ratio (SR); $p = 0.004$] and IL-4 (1.63 vs. 1.44 SR; $p = 0.39$) production. Phenotypically, hUCB Breg cells presented IgM^{hi}IgD^{hi}CD5$^+$CD10$^+$CD27$^-$ markers, similar to those described in hAPB Breg cells, but they showed increased IgM concentration and decreased expression of CD22 and CD73 markers. Our work characterized the frequency, phenotype, and function of Breg in hUCB, which may contribute to understanding of immune tolerance during pregnancy, paving the way to a new approach to immune-related diseases in the fetus and the newborn.

Keywords: regulatory B-cells, newborn, tolerance, B-lymphocytes, umbilical cord

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INTRODUCTION

Avoidance of unwanted immune responses through peripheral tolerance involves several regulatory/suppressor molecules and cell types. Regulatory B (Breg)-cells are a rare B-cell subpopulation with this regulatory/suppressor function. Several markers have been described for the detection and sorting of these cells. This is nicely reviewed by Rosser and Mauri (1). Breg cells are known

Abbreviations: BFA, brefeldin A; Breg, B regulatory cell; FCS, fetal calf serum; hAPB, human adult peripheral blood; hUCB, human umbilical cord blood; ICS, intracellular cytokine staining; NoBreg, non-regulatory B-cell; PIM, PMA–ionomycin–monensin; RT, room temperature; SR, stimulation ratio; Th, T helper cell; Treg, T regulatory cell.

to perform their suppressive action by IL-10/TGF-β production, cell-to-cell contact by CD80/86 interaction with T-cells, and CD73-dependent adenosine production (1–6). $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ have been used recently for the study of Bregs in pregnant women (7).

The most studied subset of Breg cells is defined by $CD24^{hi}$ and $CD38^{hi}$ expression in B-cells (2, 6, 8). Phenotypically, these cells also express IgM, IgD, CD5, CD10, and CD1d (2), resembling transitional B-cells (9). Breg cells are mainly defined by their regulatory function: Mauri et al. demonstrated that the $CD19^{+}CD24^{hi}CD38^{hi}$ subset is enriched in IL-10 production and can inhibit IFN-γ production (2, 10) and block T helper-cell (Th)1 and Th17 differentiation while maintaining T regulatory (Treg) cell population (6). Their implication in human immune-related diseases has been studied mostly in autoimmune (11) and allergic diseases (12, 13), persistent infections such as HIV (14), HBV (15), and *Mycobacterium tuberculosis* (16), cancer (17), transplantation and, as demonstrated recently, pregnancy (7, 12, 18–20).

Regulatory B-cells are an important player in the achievement of the tolerogenic immune state of the mother to its haploidentical fetus during pregnancy. Maternal–fetal tolerance is achieved through different mechanisms such as an increase of Treg cells, expression of CD274 (PD-L1) in the trophoblastic tissue, and an increase of Breg cells (21, 22). Early pregnancy factor enhances Treg-cell production and IL-10 and TGF-β expression in splenocytes from female mice (23). In pregnant mice, the increase in Breg is necessary to avoid immunological abortion. In fact, the transfer of Breg cells to abortion-prone mice leads to a Treg-cell increase and maintains dendritic cells in an immature state, promoting fetal–maternal tolerance (19). In humans, B-cells increase IL-10 production in response to human gonadotropic hormone from pregnant woman serum (18). Also, there is an increase of Breg during the first trimester of pregnancy that does not occur in women with spontaneous abortion (18). Moreover, women treated with rituximab, a B-cell-depleting antibody, during pregnancy presented a higher rate of first-trimester pregnancy loss (24). The role of B cells during pregnancy changes in its various stages. A decrease in $CD24^{hi}CD38^{hi}$ B cells in the third trimester of pregnancy has been described recently (7), as lower levels of IL-10 in pregnant women (25). Furthermore, there are lower BAFF levels in pregnant women suffering from preeclampsia in comparison with healthy ones; BAFF levels are higher in healthy umbilical cord blood (hUCB) than in the pregnant mother at the time of delivery (26). These data highlight the importance of B-cells, specifically Breg, in the mother's achievement of immune tolerance during the first stages of pregnancy.

B-cell development and maturation is a complex and regulated process. In peripheral blood, we can encounter different B cell subsets that include naïve, transitional, marginal zone-like B-cells [expressing IgM, IgD, and CD27 in their membrane (27, 28)], mature B-cells, and plasmablasts (27, 29).

B-cells have been thought to be mere antibody factories for years, but it is now known that they have different functions that include cytokine production and regulation of T-cell responses. Activation status of B-cells has been studied. CD25 expression in B-cells is related with better antigen presentation, more proliferation, and an increased response to IL-2 (30). Another B-cell activation marker is CD71, the transferrin receptor. CD71 regulates the iron uptake of activated B-cells (31). Activation of B-cells is tightly modulated. CD22 is a B-cell-restricted molecule that downregulates the signal between CD19 and the BCR (32–34). The lack of this regulatory molecule provokes an increase in B10 cells in mice (35).

Along with an important anti-infection role, the immune system of the fetus must also tolerate its haploidentical mother as well as harmless antigens after delivery. To reduce the risk of alloimmune reactions between mother and fetus, APCs from the newborn selectively impair production of Th1-related cytokines (36). Although vaginal or cesarean delivery can affect leukocyte populations and plasma concentration of some cytokines (37), hUCB T-cells presented lower IFN-γ production after mitogen stimulation independently of the way of delivery (38). This regulation is partially explained by impaired IL-12 production caused by a defect in nucleosome remodeling and the repression of IL-12p35 at the chromatin level. Also, murine $CD5^{+}$ B-cells in neonates have been described as contributing to the reduced production of IL-12 by APCs through IL-10 production in response to TLR9 stimulation (39). Recently, it was described how asthmatic mothers of infants with early allergy had an increase in transitional B-cells in the late-pregnancy period, suggesting that these cells may play a role in the Th1/Th2 bias observed in neonates (20). Furthermore, it is known that infusion of stem cells from hUCB rather than adult bone marrow enables transplantation in patients with increased donor–recipient HLA mismatch (40). Recently, it was shown that B-cell-mediated regulation was one of the possible mechanisms explaining this augmented allogenic tolerance (41).

Human Breg cells in umbilical cord blood and their implications in the achievement of tolerance are not fully characterized. This work is an attempt to further characterize the Breg cell population in cord blood of healthy neonates. We observed an increased number of $CD19^{+}CD24^{hi}CD38^{hi}$ B-cells, especially among marginal zone-like B-cells, phenotypically comparable to adult Breg but with particular differences and functionally inhibiting IFN-γ and IL-4 production.

MATERIALS AND METHODS

Blood Extraction

Eight healthy pregnant women with uneventful pregnancies and eight anonymous adult healthy controls [three males and five non-pregnant females; median age, 25.5 years (range, 21–50 years)] were enrolled in this study over a period of 2 months. hUCB from the enrolled pregnant women was drawn at the time of delivery. A total volume of 20 ml was drawn from each subject or from the hUCB into lithium-heparinized and EDTA-treated tubes.

This study was carried out in accordance with the recommendations of *Ley General de Sanidad (25/4/1986) Art. 10* with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committee of the Hospital Sant Joan de Déu (number of the *Comité Ético de Investigaciones* Clínicas: PIC-50-12).

Peripheral Blood Mononuclear Cells (PBMCs) Isolation, Sorting, and Culture

Peripheral blood mononuclear cells from hUCB and healthy controls were isolated with Ficoll-Hipaque (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation of heparinized blood. Cells were subsequently washed three times with PBS 1× (Roche Diagnostics, Barcelona, Spain) and cultured with complete medium [RPMI (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA), 1 µg/ml penicillin, and 1 µg/ml streptomycin (Invitrogen, Grand Island, NY, USA)]. Viable cells were counted using a hemocytometer in an inverted microscope.

We explored the regulatory function of hUCB B-cells by studying $CD3^-CD19^+CD24^{hi}CD38^{hi}$ cell subset because although it is a heterogeneous population, most Breg cells lie in this compartment. For Th1/Th2/Th17 evaluation, coculture assays were performed in sorted PBMCs isolated from hUCB: hUCB PBMCs were sorted using the FACS-ARIA II sorter (BD Bioscience, San Jose, CA, USA). Cell populations were defined as Breg: $CD3^-CD19^+CD24^{hi}CD38^{hi}$, NoBreg: $CD3^-CD19^+CD24^{int}CD38^{int}$ (2), and T-cells: $CD3^+CD19^-CD25^-$. These hUCB sorted cells were cocultured in a CD3 pretreated plate (24 h, 4°C, 0.5 µg/ml, clone 33-2A3 developed in HCB by Dr. R. Vilella) in three different conditions: T-cells only, T-cells and Breg at 1:1 ratio, and T-cells and NoBreg cells at 1:1 ratio. After 72 h of coculture, cells were stimulated for 5 h with PMA–ionomycin–monensin [PIM; PMA 50 ng/ml (Sigma-Aldrich, St. Louis, MO, USA) + ionomycin 1 µl/ml (Sigma-Aldrich, St. Louis, MO, USA) in the presence of monensin (GolgiStop from Th1/Th2/Th17 phenotyping kit, 4 µl for 6 ml of culture)] or left with monensin alone to stop cytokine secretion in the Golgi compartment. Cells were stained for flow cytometry.

For IL-10 detection in the supernatant of B-cell cultures, we performed positive magnetic separation of $CD19^+$ cells (>98% purity). After separation, 400,000 B-cells were cultured in a 96-well plate with or without CD40L (1 µg/ml) and CpG-B (1 µg/ml). After 48 h, 100 µl of supernatant were removed and analyzed by Luminex following manufacturer's instructions. Cells were cultured for 5 more hours in the presence of brefeldin A (BFA) alone or LPS/PMA/ionomycin and BFA. Intracellular cytokine staining (ICS) for IL-10 detection was then performed.

Flow Cytometry

For the immunophenotype, cell staining was performed in 50 µl EDTA whole blood. For surface staining, whole blood was incubated for 15 min at room temperature (RT) with the surface antigen antibodies at proper concentration. To lyse and fix cells, cells were incubated with 2 ml of BD FACS lysing solution 1× (BD bioscience, San Jose, CA, USA) for 15 min at RT. Cells were then washed two times with FACS buffer (phosphate-buffered saline with 5% FCS, 0.5% BSA, and 0.07% NaN_3) and acquired using a FACSCanto-II (BD Bioscience) cytometer.

T regulatory intracellular staining was performed with Treg Detection Kit (CD4/CD25/FoxP3) (Milteny Biotec, Germany) following the manufacturer's instructions. Briefly, surface staining was performed as described above, and then cells were fixed with 500 µl of fixation buffer for 30 min at 4°C. Cells were washed two times with FACS buffer and a third time with Perm Buffer. To perform the blocking, cells were incubated with 20 µl of Perm Buffer and 5 µl of FcR Blocking Reagent for 5 min at RT. Cells were then stained with FoxP3-APC antibody for 30 min at 4°C. Finally, cells were washed with FACS buffer and acquired with the cytometer.

For detection of IL-10 production, as previously reported (21), cells were matured 48 h with CD40L (1 µg/ml; Insight genomics, Falls Church, VA, USA) and ODN-2006 Cp (USA), PMA (50 ng/mL), and ionomycin (1 µl/ml) in the presence of BFA (10 µl/ml, Sigma-Aldrich, St. Louis, MO, USA). For detection of spontaneous IL-10 production, cells were incubated for 5 h in the presence of BFA to stop cytokine secretion *via* Golgi transport and then washed with FACS Buffer and incubated with mAb anti-human surface molecules for 15 min at RT; cells were then permeabilized following "Th1, Th2, Th17 phenotyping kit®" (BD Bioscience, San Jose, CA, USA) protocol described below and incubated for 30 min at RT, dark with anti-IL-10 Ab. Cells were then washed with FACS buffer and acquired with the cytometer.

Intracellular Cytokine Staining for IFN-γ, IL-4, and IL-17 was performed following the "Th1, Th2, Th17 phenotyping kit®" (BD Bioscience, San Jose, CA, USA) protocol. Briefly, cells were first washed with FACS buffer and then fixed incubating with 600 µl of Cytofix buffer for 10 min at RT and washed twice with FACS buffer. To permeabilize them, the membrane cells were incubated with 600 µl of Perm/Wash for 15 min at RT and then incubated for 45 min with the corresponding surface and intracellular antibodies. Cells were then washed with FACS buffer and acquired with the cytometer.

Panels of direct fluorochrome-conjugated anti-human antibodies were used with the following antibodies: CD3-APCCy7, CD3-AlexaFluor750, CD4-FITC, CD4-PerCPCy5.5, CD5-APC, CD8-PerCPCy5.5, CD10-APCH7, CD16-PE, CD19-PECy7, CD19-APCCy7, CD19-BV510, CD21-FITC, CD21-PECy5.5, CD22 BV421, CD24-PerCPCy5.5, CD25-PE, CD25-APCH7, CD27-PECy7, CD38-APC, CD38-PECy7, CD45-APC, CD45-PECy7, CD56-PE, CD71-APC, CD73-BV421, CD86-FITC, CD127-PECy7, IFN-γ-FITC, IgD-PE, IgD-FITC, IgM-FITC, IgM-BV421, IL-4-APC, IL-10-PE, IL-17-PE, PD-L1-APC, TCRab-FITC, and TCRgd-PE. See **Table 1** for panel and antibodies details.

Cells were acquired on a FACS CANTO II flow cytometer (BD Bioscience, San Jose, CA, USA) within the next 2 h after cell staining. Data analysis was performed using FlowJo 7.3 software (TreeStar, Inc., Ashland, OR, USA). Absolute number of lymphocytes was determined with a hematological cell counter.

Statistical Analysis

Statistical analysis was performed using Prism 6 software (GraphPad, La Jolla, CA, USA) and Microsoft Excel (2010). As the cohort did not show normal distribution, we performed Mann–Whitney test for all conditions. Differences between values were considered statistically significant when $p < 0.05$.

TABLE 1 | Antibody panels used for umbilical cord and adult peripheral blood phenotyping.

Panel	Antibody specificity–fluorochrome-Clone					
T–B–NK phenotype	CD3[a] APCCy7 SK7	CD4[b] FITC VIT4	CD8[a] PerCPCy5.5 RPA-T8	CD16/CD56 PE B73/MY31	CD19[a] PECy7 HIB19	CD45[a] APC 2D1
T-subphenotype	CD3[a] APCCy7 SK7	CD8[a] PerCPCy5.5 RPA-T8	CD45RA[a] PECy7 L48	CD45RO[a] APC UCHL1	TCRab[a] FITC WT31	TCRgd[a] PE 11F2
B-subphenotype-1	CD19[a] PECy7 SJ25C1	CD21[a] PECy5 B-ly4	CD38[a] APC HIT2	IgD[a] PE IA6-2	IgM[c] FITC SA-DA4	
B-subphenotype-2	CD19[a] PECy7 SJ25C1	CD27[c] PECy7 1A4CD27	CD38[a] APC HIT2	IgD[a] PE IA6-2	IgM[c] FITC SA-DA4	
Regulatory T-cells	CD3[c] AlexaFluor750 UCHT1	CD4[b] FITC VIT4	CD25[b] PE 4E3	CD127[a] PECy7 HIL-7R-M221		
Regulatory B-cells	CD19[a] PECy7 SJ25C1	CD24[a] PerCPCy5.5 ML5	CD38[a] APC HIT2			
Breg sorting	CD3[c] FITC UCHT1	CD19[a] PECy7 HIB19	CD25[b] PE 4E3	CD24[a] PerCPCy5.5 ML5	CD38[a] APC HIT2	
IL-10 intracellular cytokine staining	CD19[a] PECy7 HIB19	IL-10[a] PE JES3-9D7				
Th1/tTh2/Th17 phenotyping kit	CD3[a] APCCy7 SK7	CD4[a] PerCPCy5.5 SK3	IFNg[a] FITC B27	IL4[a] APC MP4-25D2	IL17[a] PE N49-653	
Breg panel 1	CD19[a] APCCy7 SJ25C1	CD24[a] PerCPCy5.5 ML5	CD38[a] APC HIT2	CD27[c] PECy7 1A4CD27	IgM[a] BV421 G20-127	IgD[c] FITC IADB6
Breg panel 2	CD19[a] BV510 SJ25C1	CD24[a] PerCPCy5.5 ML5	CD38[a] PECy7 HB7	CD25[a] APCH7 M-A251	CD73[a] BV421 AD2	CD71[d] APC HI166
Breg panel 3	CD19[a] BV510 SJ25C1	CD24[a] PerCPCy5.5 ML5	CD38[a] PECy7 HB7	CD21[a] FITC B-Ly4	CD5[c] APC BL1a	CD10[e] APCH7 HI10a
Breg panel 4	CD19[a] APCCy7 SJ25C1	CD24[a] PerCPCy5.5 ML5	CD38[a] PECy7 HB7	PD-L1[e] APC 29E-2A3	CD22[e] BV421 S-HCL-1	CD86[d] FITC BU63

[a]*BD Biosciences.*
[b]*Milteny Biotech.*
[c]*Beckman Coulter (Brea, CA, USA).*
[d]*Immunotools (Friesoythe, Germany).*
[e]*Biolegend (San Diego, CA, USA).*

RESULTS

Description of Reference Values of Leukocyte and Lymphocyte Subsets in hUCB

We first analyzed monocyte, neutrophil, and lymphocyte subset frequency. For lymphocyte subset study, we based our panels on and used the reference values of clinical guide references (27, 42–44). Values observed were within-reported reference values (27, 42, 43, 45, 46). We encountered, as expected, significant differences between healthy adult peripheral blood [healthy peripheral adult blood (hAPB), $n = 5$] and hUCB ($n = 6$). Specifically, we observed more naïve T-cell compartment ($CD45RA^{+}CD45RO^{-}$, 46.9 vs. 24.18% of T-cells, $p = 0.004$). B-cells were also enriched in the more immature subsets. There was an increase in IgD/IgM double-positive B-cells (92 vs. 62.28% of B-cells, $p = 0.002$), marginal zone-like B-cells ($CD19^{+}IgM^{+}Ig$

D^+CD27^+; 40.74 vs. 17.29% of B-cells, $p = 0.002$), naïve B-cells ($CD19^+IgD^+CD27^-$; 47.7 vs. 17.4% of B-cells; $p = 0.0002$), and transitional B-cells ($CD19^+CD38^+IgM^{hi}$; 54 vs. 18.78% of B-cells, $p = 0.006$) (**Table 1**; **Table 2**; Figure S1 in Supplementary Material).

Breg Cells Are Present at Higher Frequency in hUCB than in hAPB

There was an increased frequency of Breg cells among total $CD19^+CD24^{hi}CD38^{hi}$ B-cells in hUCB (34.39% ± 2.49 of B-cells in hUCB, 9.49 ± 1.27% of B-cells in hAPB; mean ± SD; $p = 0.0002$) (**Figure 1**). After evaluating the presence of Breg cells among total B-cells, we studied Breg cells in different B-cell subsets (**Figures 2A,B**) in both hUCB and hAPB. Studied B-cell subsets were defined as (i) $CD19^+IgM^+IgD^+$, (ii) $CD19^+IgM^-IgD^+$, (iii) naïve cells ($CD19^+IgD^+CD27^-$), (iv) marginal zone-like cells ($CD19^+IgD^+CD27^+$), and (v) class-switched cells ($CD19^+IgM^-IgD^-$). In both hUCB and hAPB, $CD19^+CD24^{hi}CD38^{hi}$ Breg cells were present in all the above-described B-cell subsets. Nevertheless, the marginal zone-like B-cell subset was the most enriched in Breg and showed the most marked difference between hUCB and hAPB (60.8 vs. 4.94%; $p = 0.1$), compared to the other subsets: naïve subset (30.58 vs. 3.26%; $p = 0.1$), IgD^+IgM^+ B-cells (30.76 vs. 3.39%; $p = 0.057$), IgD^+IgM^- B-cells (6.02 vs. 0.51% of B-cells; $p = 0.057$), and class-switched B-cells (10.8 vs. 0.49% of B-cells; $p = 0.1$) (**Figure 2C**).

TABLE 2 | Evaluation of different leukocyte and lymphocyte populations and subpopulations in hUCB vs hAPB.

Population	hUCB		hAPB		p
	Mean	SEM	Mean	SEM	
% of leukocytes					
Monocytes	7.54	1.39	5.77	1.25	0.02
Lymphocytes	29.63	7.19	22.42	4.69	0.11
Neutrophils	41.31	9.47	52.28	8.19	0.04
% of lymphocytes					
T lymphs	65.96	4.33	74.88	1.79	0.22
B lymphs	17.81	2.03	13.76	1.36	0.23
NK cells	13.80	3.20	9.47	2.19	0.41
NKT-cells	1.62	0.23	6.16	1.15	0.002
$CD3^+CD4^+$	46.70	3.47	45.54	2.50	0.62
$CD3^+CD8^+$	17.21	1.44	23.56	1.79	0.03
$CD3^+CD4/8^-$	1.30	0.15	5.11	1.13	0.01
% of T-cells					
$TCR_{\alpha\beta}$	93.85	0.4822	89.3	1.202	0.009
$TCR_{\gamma\delta}$	1.90	0.3044	7.32	1.976	0.03
$CD45RA^+CD45RO^-$	46.9	2.321	24.18	5.006	0.004
$CD8^+CD45RA^+CD45RO^-$	16.2	1.236	10.71	2.866	0.25
$CD8^-CD45RA^+CD45RO^-$	30.73	2.333	13.45	2.866	0.009
$CD45RO^+$	52.65	2.301	75.72	5.035	0.004
$CD8^+CD45RO^+$	9.765	1.174	19.14	2.185	0.004
$CD8^-CD45RO^+$	42.9	1.784	56.51	4.081	0.004
$CD45RA^+CD45RO^+$	44.87	2.091	37.94	5.966	0.66
$CD8^+CD45RA^+CD45RO^+$	10	1.147	17.79	1.95	0.004
$CD8^-CD45RA^+CD45RO^+$	35.18	1.895	20.1	4.82	0.02
$CD45RA^-CD45RO^-$	8.048	0.7985	37.78	2.205	0.004
$CD8^+CD45RA^-CD45RO^-$	0.1217	0.03497	1.35	0.3404	0.004
$CD8^-CD45RA^-CD45RO^-$	7.923	0.7755	36.41	2.145	0.004
Treg cells	0.52	0.13	0.23	0.08	0.13
% of B-cells					
IgD^+IgM^+	92	1.304	62.28	4.617	0.002
Marginal zone-like B-cells	40.74	2.464	17.29	3.669	0.002
Naïve B-cells	47.7	3.487	4.046	0.7584	0.002
Transitional B-cells	54	4.88	18.78	2.761	0.002
IgD^+IgM^-	3.638	1.649	14	2.875	0.006
IgD^-IgM^-	3.903	0.5775	1.639	0.3791	0.003
Switched memory B-cells	2.574	0.5774	63.8	5.043	0.002
IgD^-IgM^+	0.4338	0.08744	3.603	0.7951	0.0003
$CD38^{low}CD21^{low}$ B-cells	0.0475	0.02541	2.377	0.5152	0.0003
Plasmablasts	1.391	0.2774	3.92	0.8957	0.02

hUCB, human umbilical cord blood; hAPB, human adult peripheral blood; p, significance value; marginal zone-like B-cells, $CD19^+IgM^+IgD^+CD27^+$; switched memory B-cells, $CD19^+IgM^-IgD^-CD27^+$; naïve B-cells, $CD19^+IgD^+CD27^-$; transitional B-cells, $CD19^+IgM^{hi}CD38^{hi}$; plasmablasts, $CD19^+IgM^+CD38^{low}$.

IL-10 Production at Baseline Is Increased in hUCB Compared with hAPB, in Both Breg and Non-Breg Compartments

Human Breg cannot be defined solely based on a phenotype composed of conventional B-cell markers. Analyses of Breg functionality are necessary to confirm the regulatory function of this population. The capacity to produce IL-10 is an important functional item that defines $CD19^+CD24^{hi}CD38^{hi}$ Breg cells (2–4), although it is not unique to this cell type (47, 48). We observed

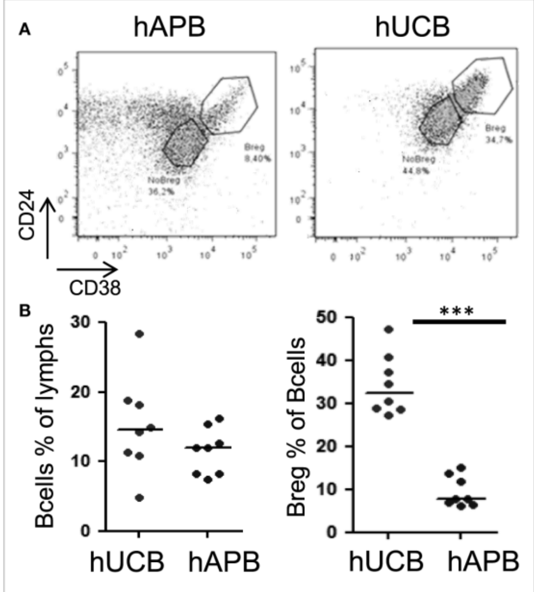

FIGURE 1 | hUCB is enriched in Breg population when compared with hAPB. (A) Breg gating strategy and representative example of hAPB/hUCB Breg and NoBreg cells. **(B)** Frequency of B-cells and Breg cells among B-cells in hAPB ($n = 8$) and hUCB ($n = 8$). hUCB, human umbilical cord blood; hAPB, human adult peripheral blood; Breg, B regulatory cell; NoBreg, non-regulatory B-cell. ***$p < 0.001$.

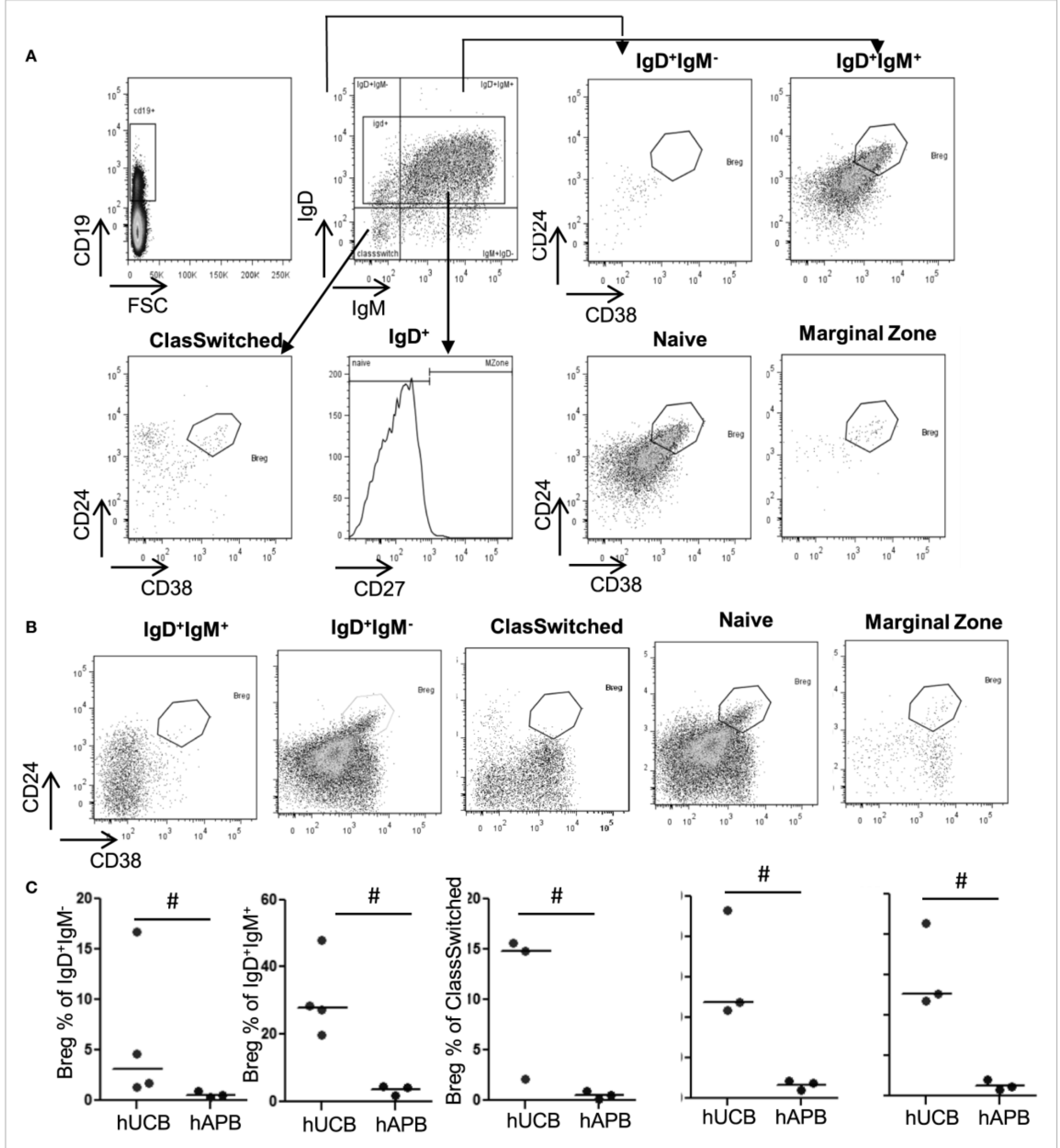

FIGURE 2 | **Breg frequency is significantly higher in hUCB than in hAPB among marginal zone-like, naïve, and IgD⁺IgM⁺ cells.** **(A)** Gating strategy and representative hUCB example. **(B)** Representative hAPB example. **(C)** Breg percentage in different B-cell populations in hUCB ($n = 3$) and hAPB ($n = 4$). hUCB, human umbilical cord blood; hAPB, human adult peripheral blood; Breg, regulatory B-cell; NoBreg, non-regulatory B-cell; MZ, marginal zone-like. $^{*}p < 0.05$.

that hUCB B-cells produced spontaneously (by incubating cells for 5 h in the presence of BFA) higher levels of IL-10 than hAPB, but this difference was no longer observed after stimulation (**Figure 3**; Figure S3 in Supplementary Material). When we analyzed the different B-cell subsets, we observed that both Breg and NoBreg [defined as $CD19^{+}CD24^{int}CD38^{int}$ (2)] from hUCB showed a tendency toward a spontaneously higher IL-10 production than hAPB (3.93 vs. 2.29%; $p = 0.2$ for B-cells; 5.54 vs. 1.92%;

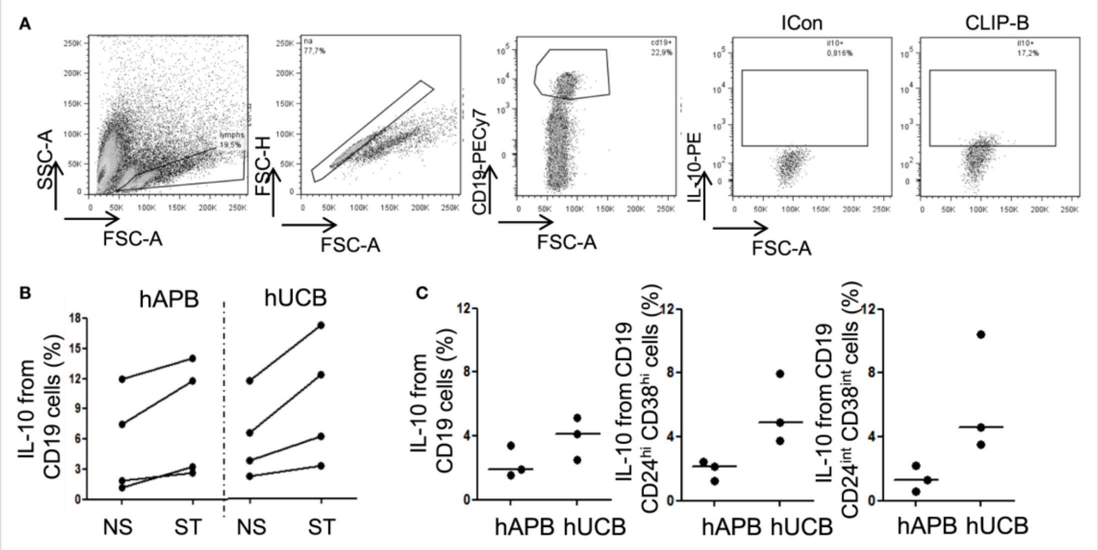

FIGURE 3 | **Breg cells from hUCB can produce IL-10 after stimulation and spontaneously. (A)** Intracellular IL-10 staining in one representative hUCB example in CD40L + CPG maturation + CLIP-B stimulation condition. **(B)** IL-10 intracellular staining at 48 h of maturation with CD40L plus GPG followed by non-stimulation or stimulation with CLIP-B in hUCB ($n = 4$) and healthy peripheral adult blood (hAPB) ($n = 4$). **(C)** B, Breg, and NoBreg cells spontaneous production of IL-10 after 5 h incubation in the presence of BFA B in hUCB ($n = 3$) and hAPB ($n = 3$). CLIP-B, CpG, LPS, PMA, and ionomycin after 5 h in the presence of BFA; BFA, brefeldin A; hAPB, human adult peripheral blood; hUCB, human umbilical cord blood; Breg, regulatory B-cells; NoBreg, non-regulatory B-cell. $^{**}p < 0.01$.

$p = 0.1$ for Breg and 6.15 vs. 1.35%, $p = 0.1$ for NoBreg; **Figure 3**; Table SII in Supplementary Material), suggesting an important role for IL-10 from B-cells in hUCB.

hUCB Breg Inhibit IFN-γ and IL-4 Production by T-Cells When Cocultured

To corroborate the suppressive capacity of phenotypically described hUCB Breg, we performed T:Breg and T:NoBreg coculture assays in hUCB sorted PBMCs. We analyzed IFN-γ, IL-4, and IL-17 production by T-cells after 72 h coculture in the presence of plate-bound CD3 with or without hUCB Breg (as defined by $CD19^+CD24^{hi}CD38^{hi}$) or NoBreg cells (as defined by $CD19^+CD24^{int}CD38^{int}$), with PMA–ionomycin in the presence of monensin during the previous 5 h. We observed a statistically significant inhibition of IFN-γ stimulation ratio (SR) by T-cells when cocultured with Breg cells [1.63 vs. 0.95 SR; $p = 0.004$]. This was not observed when cocultured with NoBreg cells (1.63 vs. 1.44 SR; $p = 0.39$). For IL-4, we observed a significant decrease in the SR in the T-cell-Breg coculture condition in comparison with T-cells alone (1.66 vs. 0.86 SR; $p = 0.02$) and T-cells:NoBreg (0.86 vs. 1.79 SR; $p = 0.009$). We cannot state the effect of Breg cells in the IL-17 production as we cannot clearly detect its production after PIM stimulation (**Figure 4**; Table SIII in Supplementary Material). These data demonstrated that hUCB Breg cells play a regulatory function by inhibiting IFN-γ production by T-cells when cocultured.

hUCB Breg Show Phenotypical Differences with hAPB-Described Breg

In concordance with the reported phenotype (2), hAPB Breg were $IgM^{hi}IgD^{hi}CD5^+CD10^+CD27^{low}$. hUCB Breg presented a similar phenotype but with some differences: hUCB Breg showed more IgM per cell [mean fluorescence intensity (MFI); 13,380 vs. 7,716; $p = 0.057$] and a diminished frequency of $CD27^+$ cells (0.65 vs. 6.11%; $p = 0.057$). hUCB Breg were then $IgM^{hi+}IgD^{hi}CD5^+CD10^+CD27^-$. This difference in IgM and CD27 expression was extendable to, but less marked in, NoBreg cell compartment. Concretely, NoBreg cells in hUCB presented a $IgM^+IgD^+CD5^-CD10^-CD27^{low}$ phenotype, which compared to the adult had higher MFI of IgM (4,953 vs. 2,706; $p = 0.057$), an increased CD5 frequency (26.01 vs. 6.59%; $p = 0.057$) and MFI (202.5 vs. 39.88; $p = 0.057$), and a diminished frequency of $CD27^+$ cells (0.92 vs. 5.34%; $p = 0.1$) (**Figure 5**; Table SIV and Figure S2 in Supplementary Material).

hUCB Breg Present Lower Membrane Expression of CD22 than hAPB Breg

In addition to the previously described markers, we studied others related to the activation and maturation state of B-cells. For each marker, we compared hUCB Breg cells with NoBreg cells and with their adult equivalents. For the activation status of Breg cells, we studied CD22, CD25, CD71, CD86, and PD-L1 markers. In hUCB, we observed an increase in frequency and

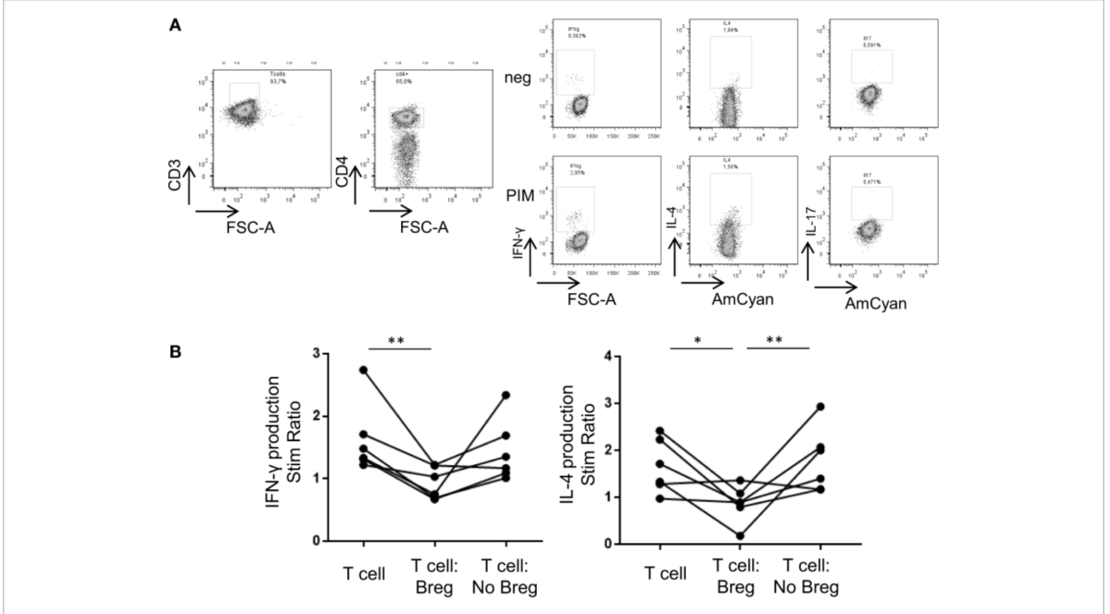

FIGURE 4 | **Breg cells from hUCB inhibit IFN-γ and IL-4 production by T-cells when cocultured. (A)** Intracellular cytokine staining in one representative hUCB example of a T-cell-only culture with PIM stimulation. **(B)** hUCB Breg and NoBreg effect on T-cell SR (stimulated/basal) after 72 h coculture plus 5 h stimulation with PIM at 1:1 ratio (T-cells alone, T-cells:Breg, T-cells:NoBreg) in a CD3-coated plate ($n = 6$). SR, stimulation ratio; PIM, PMA and ionomycin in the presence of monensin; hAPB, human adult peripheral blood; hUCB, human umbilical cord blood; Breg, Regulatory B cells; NoBreg, non-regulatory B-cells. $^{**}p < 0.005$.

a decrease in MFI of CD25 in Breg cells when compared with NoBreg cells (4.37 vs. 2.78%; $p = 0.03$ and MFI: 7.46 vs. 54.33; $p = 0.03$). We observed no differences in CD71, CD22, CD86, and PD-L1 expression between hUCB Breg and NoBreg cells. Interestingly, there were differences between Breg from hUCB and hAPB: there was a decreased CD22 expression in hUCB (MFI: 800.9 vs. 1,293; $p = 0.057$) and a lower CD86 frequency (0.58 vs. 5.8%; p: 0.057). These differences did not reach statistical significance possibly due to the high variability observed in adults (**Figure 5**; Table SIV in Supplementary Material; Figure S2 in Supplementary Material). This variability may account for the different antigenic stimulations occurring during the lifespan of a person and the divergence of this experience increasing over the years.

CD73 Ectonucleotidase Seems Not to Be a Mechanism of Regulation in hUCB

CD73 is an ectonucleotidase that produces adenosine-inhibiting T-cell responses in an IL-10-independent way that has been related with regulatory functions of B-cells (5). To explore the presence of an IL-10-independent suppression mechanism in hUCB, we studied CD73 expression in the membrane of Breg and NoBreg cells in both hUCB and hAPB. There was a lower CD73 expression frequency in Breg cells compared with NoBregs, in both hUCB (13.45 vs. 50.83%; $p = 0.03$) and hAPB (37.98 vs. 85.7%; $p = 0.1$). Of note, the frequency observed in hUCB Breg was lower than hAPB ($p = 0.057$). This fact suggests that the IL-10-independent suppression by $CD19^{+}CD24^{hi}CD38^{hi}$ Breg cells is not related to CD73 expression (**Figure 5**; Table SIV in Supplementary Material; Figure S2 in Supplementary Material).

DISCUSSION

This study presents the functional and phenotypical characterization of the increased Breg cell subset in hUCB defined as $CD19^{+}CD24^{hi}CD38^{hi}$ cells. Bregs were present with greater frequency in hUCB and were most markedly observed in marginal zone-like B-cells. At a functional level, hUCB B-cells (including both Breg and NoBreg) produced higher levels of IL-10 spontaneously. hUCB Bregs demonstrated their regulatory capacity by differentially inhibiting IFN-γ and IL-4 production by T-cells when cocultured. Phenotypically, hUCB Bregs presented a similar phenotype to that described in hAPB with an increased quantity of IgM per cell and a diminished frequency of CD27 and with less CD22 in their membrane. Finally, hUCB Bregs also presented lower expression levels of the IL-10-independent inhibitor CD73. To our knowledge, this is the second attempt to characterize the population of $CD19^{+}CD24^{hi}CD38^{hi}$ cells in human cord blood. The first attempt was recently reported (41), but the phenotype of hUCB Breg was not fully characterized.

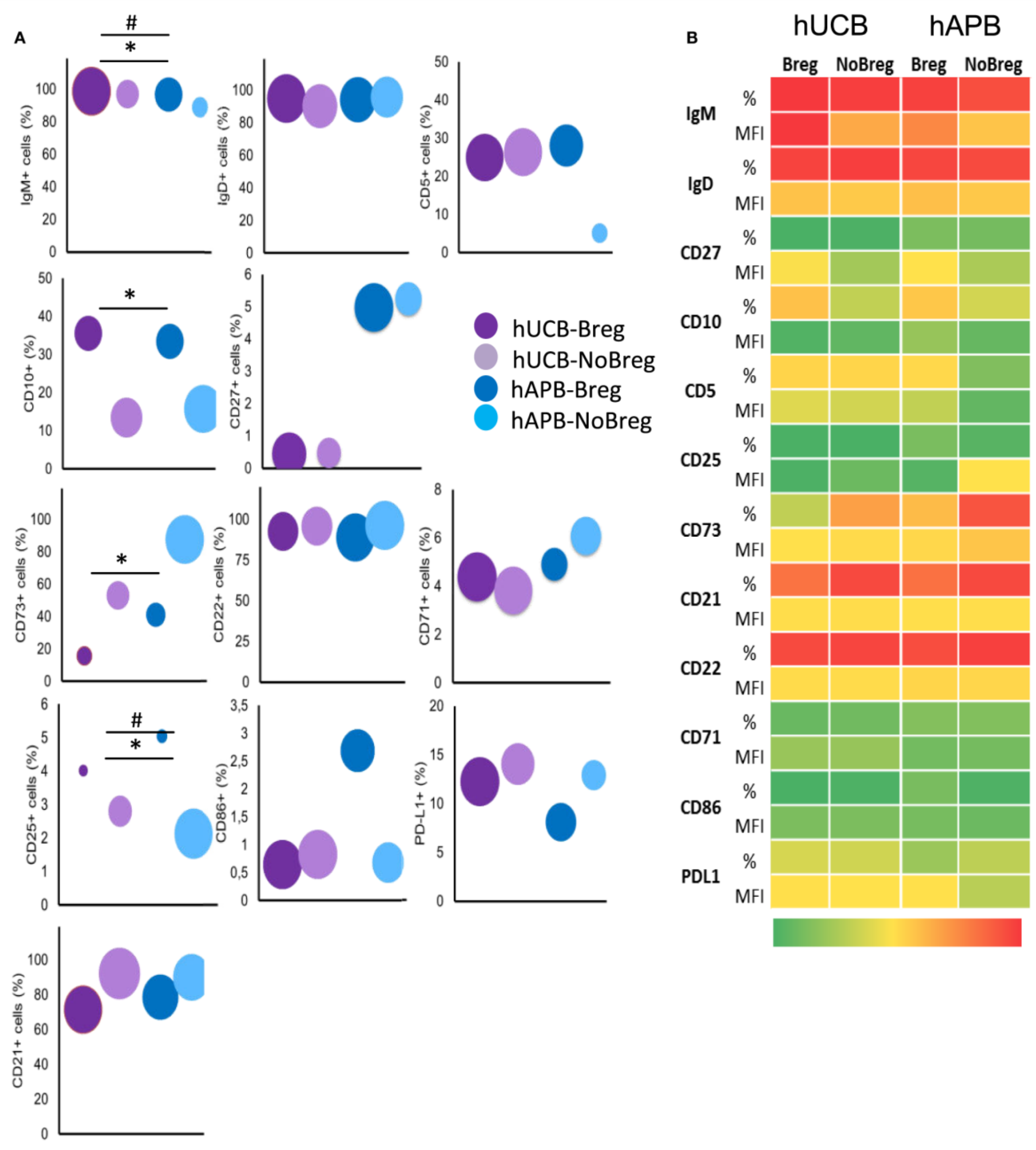

FIGURE 5 | **hUCB Breg cell-defining phenotype corresponds with that previously described for Bregs. (A)** Frequency of known Breg cell markers and others among Breg and NoBreg cells. Y axis value corresponds to % of positive cells, and area of the circle corresponds to the MFI of the whole population. **(B)** Heatmap of % and MFI of the different T-cell markers in Bregs and NoBregs from hAPB (n = 3) and hUCB (n = 4), with green being the lowest values and red the highest. hUCB, human umbilical cord blood; hAPB, human adult peripheral blood; Breg, B regulatory cell; NoBreg, non-regulatory B-cell; MFI, mean fluorescence intensity. * and # denote MFI and frequency statically significant differences, respectively. $^{*/\#}p < 0.05$, $^{**/\#\#}p < 0.01$, and $^{***/\#\#\#}p < 0.001$.

Healthy umbilical cord blood marginal zone-like B-cells presented the greatest difference in proportion of Breg cells when compared with adults. Circulating marginal zone B-cells are representative of splenic marginal zone B-cells, having a prediversified immunoglobulin repertoire and initiating T-cell-independent responses through TLRs as activation signals. Their responses are mainly directed against encapsulated bacteria, including commensal microbiota (49–51). Marginal zone B-cells also play a role in normal pregnancy development (52), and it is known that antiencapsulated bacteria responses are lower in infants and young children (36). As TLRs activate Bregs (2, 3), the greater proportion of marginal zone-like B-cells observed might indicate increased cooperation in regulatory function after encapsulated bacterial stimulation. Also, we observed lower levels of CD22 in the membrane of hUCB Bregs, suggesting a lower activation threshold (32), which could be of importance in the rapid abrogation of unwanted responses to commensal bacteria. During the first contacts with the extrauterine environment and the adoption of microbiota, we might hypothesize the increased proportion of Breg cells among marginal zone B-cells to be one of the mechanisms by which the neonatal immune system protects itself from an exacerbated response to the new range of antigens it is exposed to.

We observed that all B-cells, Breg, and NoBreg, from hUCB spontaneously produced more IL-10 than their adult counterparts, but there were no significant differences between Breg and NoBreg hUCB cells. It has previously been observed that $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD24^{int}CD38^{in}$ T-cells from hUCB are capable of producing similar amounts of IL-10 after stimulation (41). Unlike other recently published studies (41), we observed a significant inhibition of IFN-γ production only when T-cells and Bregs were cocultured. We speculate that this discrepancy could be due to differences in the experimental design. We performed a coculture with either Breg or NoBreg with $CD3^{+}CD25^{-}$ T-cells for 72 h in the presence of plate-bound CD3 and, following that, a restimulation for ICS. Sarvaria et al. cocultured $CD4^{+}$ cells with either Breg or NoBreg cells, stimulated them with anti-CD3/CD28 for 96 h, and then analyzed the concentration of inflammatory cytokines in the supernatant (41).

The mechanism by which hUCB Breg cells perform their regulatory function is unclear. We observed a differential regulatory activity of Breg and NoBreg cells spontaneously producing similar amounts of IL-10. It is known that Breg cells can carry out part of their functions *via* IL-10-independent mechanisms, such as IL-35 and TGF-β secretion and cell-to cell-contact (1, 6). Immune regulation by hUCB B-cells seems to be partially mediated by (i) IL-10 production, as seen in IL-10-blockade experiments and (ii) cell-to-cell direct contact (mediated by CD80/CD86), but independently of TGF-β (41). The mechanisms by which hAPB Bregs perform their suppressive activity have been further studied (5–7, 53), but more investigation is needed to understand whether there are other mechanisms in hUCB Breg cell regulation of T-cells.

We observed an increased quantity of IgM per cell in hUCB Breg cells compared to NoBreg cells and hAPB Breg cells, which could have implications in the mechanism of action of these Breg cells. Naturally occurring IgM antileukocyte autoantibodies (IgM-ALA) are antibodies with suppressor capacity that can inhibit T-cell activation and chemotaxis (54, 55). IgM-ALA antibodies are present at birth (55), and they inhibit pro-inflammatory cells from producing IFN-γ and IL-17 in response to alloantigens in mice (54). Altogether, we speculate that this could be a new Breg IL-10-independent mechanism for their regulatory function that could be of special importance during pregnancy and the postnatal period. More research should be done to evaluate whether these are true regulatory mechanisms of hUCB Breg function.

Healthy umbilical cord blood Breg cells presented diminished CD22 expression, suggesting a lower activation threshold. CD22 modulates the BCR signal and prevents the hyperactivation of B-cells upon stimulation (32–34). CD22 knock-out mice presented increased circulating IgM and with more IL-10-competent T-cells (35) and Breg cells $CD1d^{hi}CD5^{+}$ (56) in the spleen. These B-cells have the capacity to inhibit the IgG response to non-self and self-antigens (35). Also, *in vivo* treatment of mice with anti-CD22 mAb depletes spleen B10 and marginal zone cells in mice (57). We hypothesize that the lower level of CD22 in hUCB Breg is one of the explanations for the increased frequency of Breg among marginal zone-like B-cells. Although there is a Breg cell subset characterized by high expression of CD25 in the membrane of B-cells (53), we did observe a decrease in the presence of this receptor. $CD25^{hi}$ Bregs described by Kessel et al. also presented high levels of CD86 and CD27 (53), suggesting that these two Breg cell groups have a convergent function but are not the same subsets.

Regulatory B cell population can be of importance in the Th1/Th2 bias observed in neonates (39). The neonate immune system has the enormous challenge of modulating the migration from the sterile uterus to the real world (36). During pregnancy, the fetus tolerates the mother's allogenicity, dealing after birth with a huge range of antigens and microorganisms, pathogenic or not (36, 39). We hypothesized that, as $CD5^{+}$ murine B-cells contribute to the reduced production of IL-12 by APCs (58), increased hUCB Breg contributes to the limited Th1 response observed in neonates inhibiting IFN-γ production. It has been observed that B-cells recovered from patients after stem cell transplantation without GvHD had increased levels of IL-10-producing B-cells, suggesting the inhibitory role of these cells (41). Also, $CD24^{hi}CD38^{hi}$ B-cells have been studied during pregnancy, and it has been shown that they correlate with Treg cell numbers during pregnancy and that they increase postpartum (7, 12). There was an increase in transitional B-cells ($IgM^{hi}CD38^{hi}$) in asthmatic pregnant women whose progeny developed allergy signs before 6 months of age (20). It would be of interest to confirm whether these cells express high levels of CD24, thus producing one more clue to the role of Breg cells in the Th1/Th2 bias observed in neonates.

Availability of hUCB samples is the major limitation of our study. However, it should be mentioned that in spite of the small number of samples, the same findings were consistently found in each of the samples analyzed, which strengthens the results.

Finally, we show that Breg cells were not only present in hUCB but also present at an increased frequency compared with hAPB, especially among marginal zone-like B-cells, and that they have the capacity to produce more IL-10 already at baseline while

developing their regulatory function by inhibiting T-cell IFN-γ and IL-4 production, among others, thereby likely contributing to the limited Th1 response observed in neonates at delivery. The phenotypical differences observed between hUCB and hAPB Bregs lead us to construct hypotheses about the mechanisms of tolerance achievement in the fetus. Understanding of how immune tolerance to the newborn's haploidentical mother and to harmless antigens is achieved is of the utmost importance to further advance knowledge and treatment of immune-related diseases in the fetus and the newborn. However, more investigation with neonatal hUBC Breg cells is required to decipher their regulatory mechanisms and their role in special pregnancy conditions such as chronic autoimmune disease, HIV infection, and premature delivery.

AUTHOR CONTRIBUTIONS

AE-S performed the experiments, designed the study, and wrote the paper; IT and AD-M performed the experiments and revised the manuscript; and AP-M, JY, MJ, and LA designed the experiments and wrote and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00201/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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Supplementary Material

Characterization of the highly prevalent regulatory CD24hiCD38hi B cell population in human cord blood

Ana Esteve-Solé[1.2], Irene Teixidó [3], Angela Deyà-Martínez [1.2], Jordi Yagüe [4], Ana M Plaza-Martín [1], Manel Juan [2.4#*], Laia Alsina [1.2#]
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Supplementary Figures and Tables

Supplementary Tables

Breg. % of	UCB			APB			p
	Mean	±	std	mean	±	std	
Marginal Zone	60.8	±	12.28	4.94	±	1.46	0.1
Naive	30.58	±	7.95	3.26	±	0.65	0.1
IgD+IgM+	30.76	±	5.999	3.39	±	0.84	0.057
IgD+IgM-	6.016	±	3.625	0.514	±	0.166	0.057
Class Switched	10.8	±	4.363	0.49	±	0.23	0.1

Supplementary Table I. Breg cell frequency among different B cell populations

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Supplementary Figures and Tables

Supplementary Tables

	48h CD40L+CpG					
	5h BFA			5h CLIP-B		
	mean	±	std	mean	±	std
UCB	6.06	±	2.08	9.73	±	3.13
APB	5.63	±	2.54	7.92	±	2.9

	UCB			APB			p
	mean	±	std	mean	±	std	
Bcell	3.93	±	1.32	2.287	±	0.97	0.2
Breg	5.54	±	2.15	1.92	±	0.63	0.1
NoBreg	6.16	±	3.72	1.35	±	0.79	0.1

Supplementary Table II. IL10 production after maturation ± CLIP-B stimulation (CpG LPS Ionomycine PMA + BFA) and spontaneously after 5h incubation in the presence of BFA

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Supplementary Figures and Tables

1.1 Supplementary Tables

	Tcell			Tcell:Breg			Tcell:NoBreg			Tcell	T cell	Breg
	mean	±	std	mean	±	std	mean	±	std	Breg	NoBreg	NoBreg
IFNg	1.63	±	0.23	0.93	±	0.1	1.44	±	0.21	**0.004**	0.39	0.09
IL4	1.66	±	0.23	0.86	±	0.16	1.9	±	0.28	0.02	0.9	0.009

Supplementary table III. Stimulation Ratio of T cells when co-cultured with Bregs, Cells were co-cultured during 72h in the presence of plate-bound CD3 and stimulated 5h with PMA and Ionomycine in the presence of monensin.

Supplementary Material

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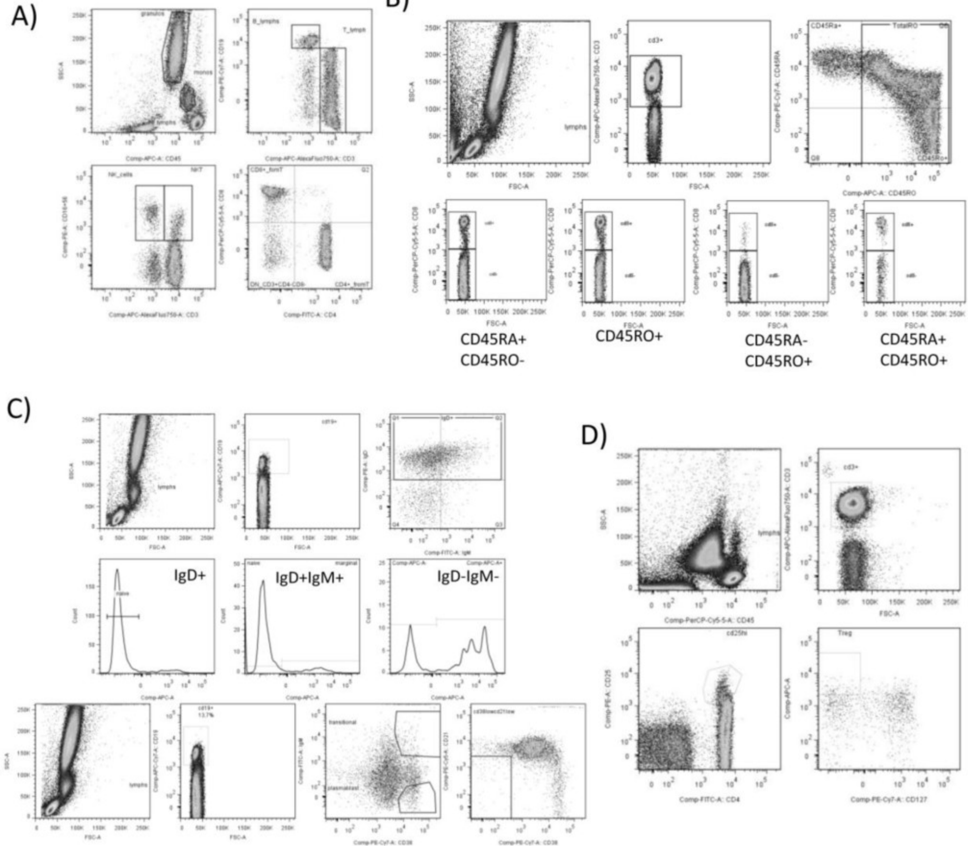
Supplementary Figures and Tables

Supplementary Tables

		UCB			APB			UCB vs APC	
		Breg mean ± std	NoBreg mean ± std	p	Breg mean ± std	NoBreg mean ± std	p	Breg	NoBreg
IgM	%	98.6 ± 0.194	95.99 ± 1.23	0.029	94.83 ± 2.2	88.58 ± 0.45	0.100	0.057	0.057
	MFI	14380 ± 1758	4953 ± 318.4	0.029	7716 ± 1278	2706 ± 255.8	0.100	0.057	0.057
IgD	%	93.78 ± 1.43	95.84 ± 0.17	0.340	92.5 ± 2.85	90.33 ± 0.4	0.700	>0.9999	0.057
	MFI	2918 ± 326.8	2297 ± 105.5	0.114	3139 ± 139.9	2434 ± 187	0.100	0.400	0.630
CD27	%	0.652 ± 0.21	0.9207 ± 0.45	>0.9999	6.108 ± 1.209	5.338 ± 0.53	>0.9999	0.100	0.100
	MFI	263.8 ± 35.35	134.3 ± 18.34	0.100	301 ± 1.26	147 ± 5.57	0.100	0.700	0.700
CD10	%	35.48 ± 6.73	13.66 ± 1.49	0.030	32.65 ± 0.93	15.51 ± 2.07	0.100	>0.9999	0.630
	MFI	15.8 ± 15.7	37.16 ± 4.06	0.340	25 ± 0.95	45.35 ± 5.21	0.700	0.620	0.230
CD5	%	26.43 ± 2.88	26.01 ± 3.91	0.690	24.74 ± 7.84	6.59 ± 1.89	0.200	0.860	0.057
	MFI	223.4 ± 20.75	202.5 ± 22.49	0.880	179.1 ± 22.04	39.88 ± 7.88	0.100	0.200	0.057
CD25	%	4.373 ± 0.416	2.776 ± 0.129	0.030	5.822 ± 0.85	2.142 ± 0.34	0.100	0.220	0.220
	MFI	7.46 ± 0.93	54.33 ± 11.1	0.030	20.66 ± 11.37	276.2 ± 182.6	0.100	0.230	0.230
CD73	%	13.45 ± 2.92	50.83 ± 10.79	0.029	37.98 ± 8.23	85.7 ± 2.49	0.100	0.057	0.057
	MFI	431.4 ± 77.81	918 ± 226.8	0.200	1185 ± 515.2	2623 ± 342.7	0.200	0.057	0.057
CD21	%	71.43 ± 7.31	91.03 ± 2.79	0.057	72.3 ± 6.6	90.78 ± 2.03	0.100	0.860	0.860
	MFI	631.5 ± 85.22	770.8 ± 87.99	0.490	584.3 ± 90.23	624.2 ± 71.47	0.700	0.230	0.400
CD22	%	90.53 ± 4.43	92.96 ± 3.69	0.690	89.08 ± 0.28	95.17 ± 1.39	0.100	0.400	0.860
	MFI	800.9 ± 44.77	809.4 ± 36.15	>0.9999	1293 ± 109.3	1226 ± 50.23	>0.9999	0.057	0.057
CD71	%	4.058 ± 1.06	4.85 ± 1.59	0.890	6.955 ± 2.804	6.72 ± 2.222	>0.9999	0.860	0.630
	MFI	120.3 ± 30.34	117.1 ± 29.8	>0.9999	63.95 ± 18.95	66.32 ± 10.14	>0.9999	0.230	0.400
CD86	%	0.58 ± 0.1	1.02 ± 0.23	0.110	5.8 ± 3.87	1.22 ± 0.59	0.200	0.057	0.630
	MFI	77.28 ± 9.84	77.98 ± 9.909	>0.9999	69.42 ± 8.89	53.22 ± 4.73	0.200	0.630	0.230
PDL1	%	15.89 ± 7.056	15.1 ± 6.49	0.890	9.5 ± 2.07	13.12 ± 1.289	0.400	>0.9999	>0.9999
	MFI	439.4 ± 19.41	309.3 ± 62.12	0.200	385.8 ± 174.8	166.8 ± 3.87	0.400	0.630	0.057



Supplementary Figure 1. Lymphocyte subsets characterization. Gating strategy of one hAPB representative example of A) T-B-NK phenotyping, B) T-cell sub-phenotyping, C) B-cell sub-phenotyping and D) Treg-cell detection.

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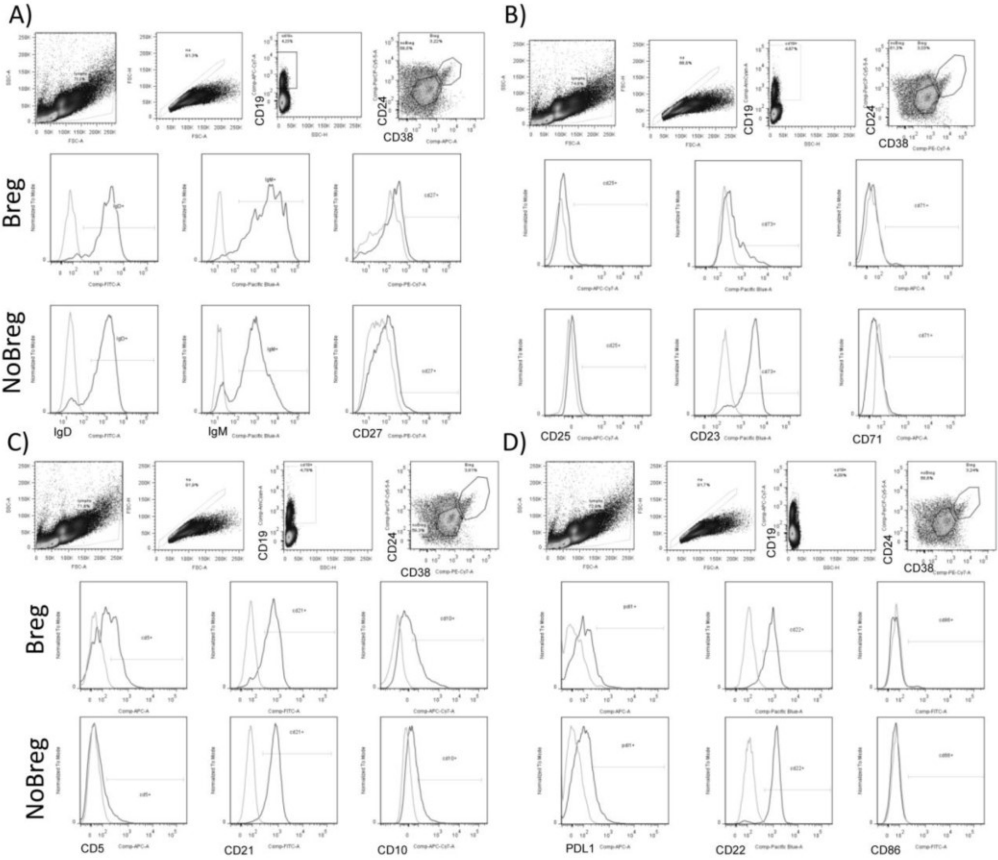
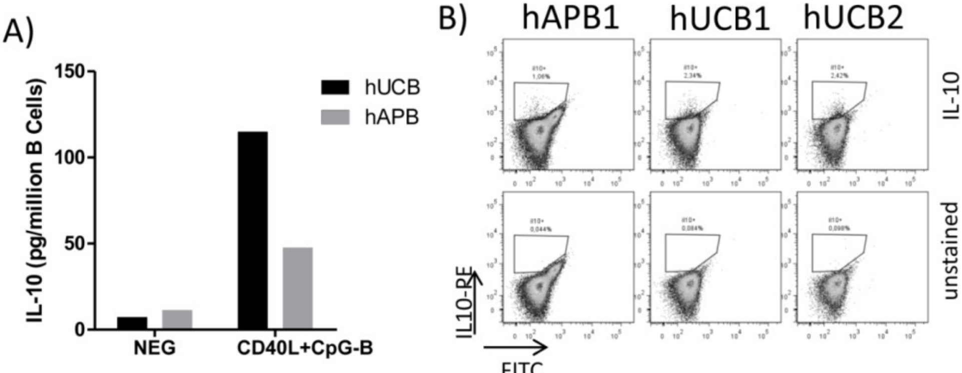


Supplementary Figure 2. Breg phenotyping. Gating strategy and one hAPB representative example of A) Panel 1, B) Panel 2, C) Panel 3 and D) Panel 4.



Supplementary figure 3. Consistency between IL-10 detection in the supernatant of cultured B cells and by ICS. A) IL-10 levels in the supernatant (height of the bar represents the mean of the values, n=2 for hAPB and for hUCB); B) IL-10 production in the CD40L/CpG-B – LPS/PMA/ionomycin condition (n=1 for hAPB and n=2 for hUCB). hUCB: human Umbilicar Cord Blood, hAPB: human Adult Peripheral Blood.

3.2. Manuscript: “Breg cells in umbilical cord blood: two sides of the same coin”

Title page

Breg cells in umbilical cord blood: two sides of the same coin

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Key words:
Neonate, cord blood, BCG, mycobacteria, Interferon-gamma, cytokines, B regulatory

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Abbreviations
APB: adult peripheral blood.
BCG: Bacille Calmette-Guerin
Breg: B regulatory cells
hr: human recombinant
Co-SR: co-stimulation ratio
UCB: umbilical cord blood
GvHD: Graft versus Host Disease

Abstract:
Immunological responses in neonates are specifically shaped for managing the transition between the allogenic sterile uterus to the real world full of antigens. We have compared antimycobacterial responses in neonate's cord blood compared to adult's peripheral blood, observing a diminished response to Bacille Calmette-Guerin challenge, with a decreased CD69 expression stimulation ratio (SR) and a decreased IL-6 and IFN-γ production SR with a normal response to IFN-γ and IL-12p70. B regulatory (Breg) cells are known to be increased in cord blood, as part of the tolerogenic mechanisms to maintain pregnancy, here we show for the first time that IFN-γ SR production after mycobacterial challenge inversely correlated with Breg cell frequency. This association may have implications for the indication of cord blood transplantation in conditions with constitutively increased IFN-γ production such as in IFNGR1 deficiency, and in understanding increased susceptibility to intracellular infections after cord blood transplantation.

Introduction

Umbilical cord blood (UCB) has been considered a safer source of hematopoietic progenitors for stem cell transplantation. Its superior immune plasticity of UCB grafts allows a less stringent HLA matching, increasing the probabilities to find a compatible donor. Still, infection is a major problem in cord blood stem cell recipients, especially during the first 100 days post-transplantation, including bacterial and fungal infections[1–3]. This has been ascribed to the lower number of infused cells, the more immature immune system and to the observed transitory neutropenia[1,4]; however, the mechanisms of increased susceptibility to infections have still not been fully studied.

UCB recipients can present with severe disseminated forms of *Mycobacterium tuberculosis* infection[5] that resemble those observed in patients with Mendelian Susceptibility to Mycobacterial Disease (MSMD)[6]. MSMD is a primary immunodeficiency with a clinical spectrum including severe disease after poorly pathogenic mycobacteria, including attenuated Mycobacterium bovis bacillus *Calmette-Guérin* (BCG) vaccine and environmental mycobacteria, severe disseminated *Mycobacterium tuberculosis*, infection by *Salmonella* species and fungal infections[6,7]. It is caused by inborn errors of the IFN-γ circuit that impair IFN-γ production or response to IFN-γ[6,7]. Thus, the correct production and/or response to IFN-γ after the exposure to mycobacteria are crucial in the ability to control the infection.

The neonate's immune system must effectively manage the transition from the sterile uterus to the real world full of antigens. Just after birth, especially after vaginal delivery, the mucosa of the infant is colonized by commensal microbiota. To avoid excessive responses to this colonization, the immune system of the neonate is modulated: for example, antigen presenting cells from the newborn selectively impair production of T helper-cell 1-related cytokines such as IFN-γ[8,9]. This regulation is partially explained by impaired IL-12 production caused by a defect in nucleosome remodeling and the repression of IL-12p35 at the chromatin level. Besides, B regulatory (Breg) cells, which have the capacity to suppress IFN-γ production by T cells, are increased in UCB[10,11] and have been shown to be pivotal in the establishment of pregnancy[12,13].

The main aim of this study was to evaluate anti-mycobacterial responses in UCB compared to adult peripheral blood (APB). We have observed diminished responses to mycobacterial challenge with a diminished IFN-γ production. Interestingly, this observation inversely correlated with Breg cell frequency. This allows us to hypothesize that the benefit of Breg in tolerance may have as a counterpoint that there is a reduced IFN-γ production in response to mycobacteria.

Results and discussion

Overall response to mycobacterial challenge was reduced in neonates. Cytokine production and cytokine markers expression were measured in neonate's UCB and APB cultured with live BCG with or without exogenous rhIL-12p70 or hr-IFN-γ during 48h. Compared to adults, neonates showed no statistically significant changes in IL-10, IL-12p70, IL-1RA, IL-1β and TNF-α stimulation ratio. Instead, they showed a diminished IFN-γ (p: 0.001) and IL-6 (p: 0.005) stimulation ratio. Stimulated lymphocytes displayed also lower frequency (p:0.014) and levels (p: 0.051) of CD69 (**Figure 1A, Table 1**). Different from what we observed, it has been published that IL-6 production in UCB can be higher than in APB in basal conditions and after TLR stimulation[8,14]; these differences may be explained by the strong Th-1 polarizing capacity of BCG[15].

Currently, there is still a lack of consensus for the phenotypic definition of Breg cells[16–20]. However, $CD19^{+}CD24^{hi}CD38^{hi}$-defined Breg cells have been studied by us and others in neonate's cord blood, demonstrating the capacity to suppress IFN-γ production by T cells[10,11]. Given that neonatal Breg cells are increased in UCB and block IFN-γ production by T cells[10,11] we hypothesized that Breg cells are, in part, responsible of the decreased IFN-γ production after BCG stimulation observed in neonates. After performing linear regression tests, we observed statistical association between frequency of Breg cells and IFN-γ stimulation ratio after BCG stimulation (spearman correlation test, R^2: 0.618, p: 0.043, **Figure 2**), thus suggesting that Breg cells may be, at least in part, responsible of the diminished antimycobacterial response observed in UCB.

The IL-12/IFN-γ axis is responsible for anti-mycobacterial responses and is impaired in susceptible patients with inborn errors in this axis, also known as Mendelian Susceptibility to Mycobacterial Disease[7,21,22]. We tested the integrity of the axis by analyzing the effect of the co-stimulation with hrIL-12p70 and hr-IFN-γ. We observed a decreased co-stimulation ratio (Co-SR) in TNF-α production after hr-IL-12p70 co-stimulation (p: 0.022). However, we observed a tendency towards an increased IFN-γ Co-SR (p: 0.073), showing that the axis can be, at least, partially rescued by the addition of hr-IL-12p70. We did not observe any significant differences in cytokine production in Co-SR after hr-IFN-γ addition, but an increased Co-SR in HLA-DR positive cells frequency (p: 0.026; **Figure 1B**). Altogether these data show that responses to proinflammatory cytokines were preserved in neonates' cord blood.

High IFN-γ levels are associated with graft versus host disease (GvHD) development[23,24]. Consistently with the lower rates of GvHD observed in cord-blood transplanted patients[25], Breg cells are increased in UCB[10,11] and are found to be reduced in cord blood transplanted patients that developed GvHD[10]. On the other hand, UCB transplantation is also associated with increased morbi-mortality due to infections[25]. IFN-γ plays a major role in immunity to infections, especially in tuberculosis[5]: thus, after showing for the first time a correlation between Breg cell frequency with a diminished IFN-γ production after BCG stimulation, we hypothesize that Breg cells might play a double edge sword role in cord blood transplantation.

This work presents some limitations, including a big variability in the response to mycobacteria with respect to cytokine production, which has already been described[26–28] and observed by our group (unpublished results), along with the size of the studied cohort. However, we have observed coherent, consistent and robust results showing statistically significant differences between the two groups studied.

Concluding remarks

In summary, we have observed that neonate's immune system has a diminished response to mycobacterial challenge, concretely diminished IFN-γ production, which is partially determined by increased Breg cells frequency in UCB. This association deserves further investigation and it

might have implications in clinical practice, especially in UCB stem cell transplantation. For example, in IFN-γR1 deficiency, where basal levels of IFN-γ in blood are increased[7,29] and bone marrow transplantation has been difficult to perform with success[30], UCB transplantation might help to reduce IFN-γ levels in blood, and improve engraftment.

Materials and methods

This study was carried out in accordance with the recommendations of Ley General de Sanidad (25/4/1986) Art. 10, with written informed consent from parents. The protocol was approved by the ethics committee of the Hospital Sant Joan de Déu (Comité Ético de Investigaciones Clínicas number PIC-50-12). All healthy controls and parents of the included infants in the study signed the informed consent, complying with current legislation. Eight umbilical cord blood samples from uneventful pregnancies from healthy mothers were included; on the delivery day, 20mL of heparinized blood was extracted from umbilical cord blood. 20ml of venous blood from 6 adult healthy controls was extracted into heparinized blood-collection tubes.

We evaluated the function of the IL-12/IFN-γ axis based on a whole blood culture [27,28]: heparinized blood was diluted 1:2 in complete medium (RPMI (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA), 1 µg/ml penicillin, and 1 µg/ml streptomycin (Invitrogen, Grand Island, NY, USA) and incubated at 37ºC in a 5% CO_2 humidified incubator for 48h. Activation conditions: medium alone, live BCG (Pasteur sub-strain) at a multiplicity of infection of 20 BCG per leukocyte, BCG plus human recombinant IL-12p70 (hrIL-12p70, 20ng/ml, Milteny Biotec, Germany), BCG plus hr-IFN-γ (5,000 IU/mL; Imukin, Boehringer Ingelheim, Germany) as described elsewhere[27]. Cytokine production determination was assessed by Luminex (Millipore, Billerica, MA, USA) at 48h culture point following the manufacturer's instructions. Breg cells (CD19-SJ25C1, CD25-ML5, CD38-HIT2[11]) and activation marker expression (CD69-FN50, CD71-M-A712 and HLA-DR G46-6[31]) were evaluated by flow cytometry. All antibodies are from BD Bioscience, San Jose, CA, USA.

Data did not follow normal distribution; thus Mann-Whitney test was performed. Statistical association studies were performed with linear regression studies and spearman correlation test. Statistical analysis and graphical representation of the data were performed with SPSS 22 (IBM, Armonk, NY, USA) and Microsoft Excel (2016). In favor of the understanding and legibility of the paper, detailed results (median, standard deviation and p values) for cytokine production and activation marker expression are shown in **Table 1.**

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure legends

Figure 1. Cytokine and activation markers stimulation ratio. Cytokine (A) and activation markers (B) stimulation ratio is represented as a heatmap where blue indicates the minimum values, red the maximum, and yellow next to 1 for each parameter. SR: stimulation ratio, Co-SR: costimulation ratio. UCB: umbilical cord blood, APB: adult peripheral blood. APB, n=6, UCB, n=7.

Figure 2. Correlation between Breg frequency and IFN-γ SR. SR: stimulation ratio. R2 calculated with spearman correlation test. APB, n=6, UCB, n=7

Tables and figures

Table 1. Cytokine production and activation markers expression stimulation ratios in lymphocytes. SR: stimulation ratio, Co-SR: co-stimulation ratio APB: adult peripheral blood, UCB: umbilical cord blood.

		IFN-γ	IL-10	IL-12p70	IL-1RA	IL-1β	IL-6	TNF-α	CD71	CD69		HLA-DR	
									%	%	MFI	%	%
SR (BCG/basal)													
APB	**Median**	440.15	349.82	0.60	26.01	2235.41	4506.58	544.11	3.66	7.32	17.44	3.66	7,32
	STD	883.14	191.93	0.75	170.30	1552.16	5151.57	564.65	4.06	3.23	9.13	4.06	3,23
UCB	**Median**	2.89	608.50	0.82	38.64	538.13	514.52	140.77	1.75	1.93	4.57	1.75	1,93
	STD	14.08	1101.39	0.37	25.42	4188.00	699.90	657.04	2.08	1.25	5.48	2.08	1,25
	p	0,001**	0.534	0.445	0.534	0.731	0.005**	0.731		0.101	0.014*	0.051#	0.101
Co-SR (BCG + hrIL-12p70/BCG)													
APB	**Median**	11.72	0.70	NA	1.04	1.61	1.00	2.58	1.19	1.53	1.96	1.19	1,53
	STD	26.94	0.22	NA	0.28	0.63	0.49	1.48	0.29	0.36	0.96	0.29	0,36
UCB	**Median**	79.14	0.77	NA	0.93	1.00	1.27	1.04	1.01	1.84	2.41	1.01	1,84
	STD	507.20	0.70	NA	0.24	1.03	1.04	0.99	0.20	0.74	1.10	0.20	0,74
	p	0,073#	0.366	NA	0.534	0.945	0.366	0.022*		0.366	0.366	0.945	0.366
Co-SR (BCG + hrIFN-γ/BCG)													
APB	**Median**	NA	0.07	356.71	0.91	1.54	1.00	4.60	1.32	1.37	2.06	1.32	1,37
	STD	NA	0.08	349.70	0.50	2.07	0.56	2.07	0.29	0.49	0.47	0.29	0,49
UCB	**Median**	NA	0.16	12.33	1.12	1.22	1.23	1.59	1.21	2.36	2.27	1.21	2,36
	STD	NA	0.43	243.41	0.40	10.18	0.34	7.63	0.81	0.72	1.46	0.81	0,72
	p	NA	0.295	0.181	0.731	0.534	0.181	0.628		0.366	0.073#	0.366	0.366

* p<0.05, ** p<0,01; #: statistical trend

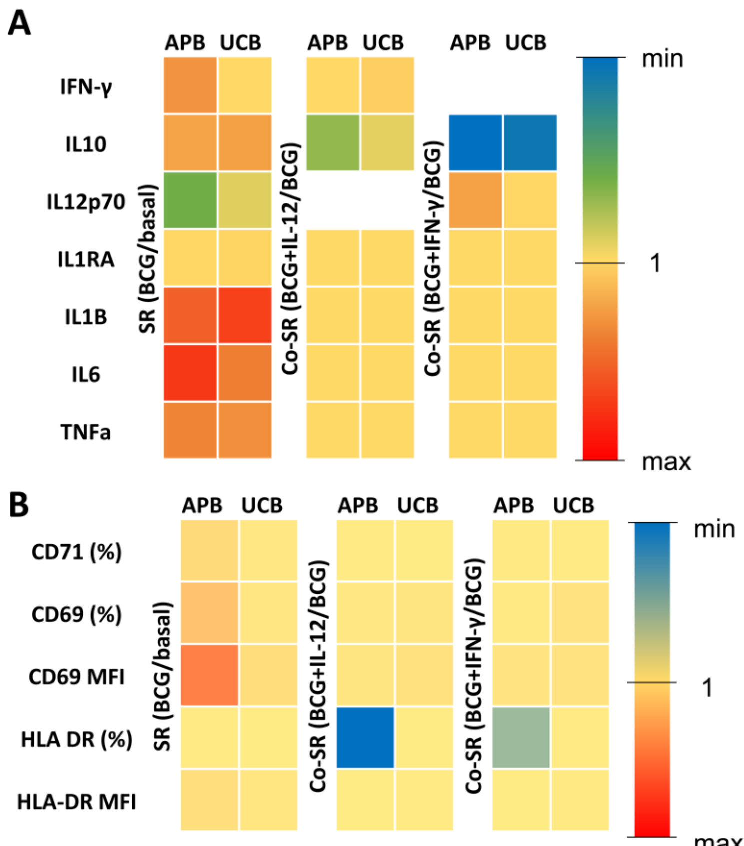

Figure 1

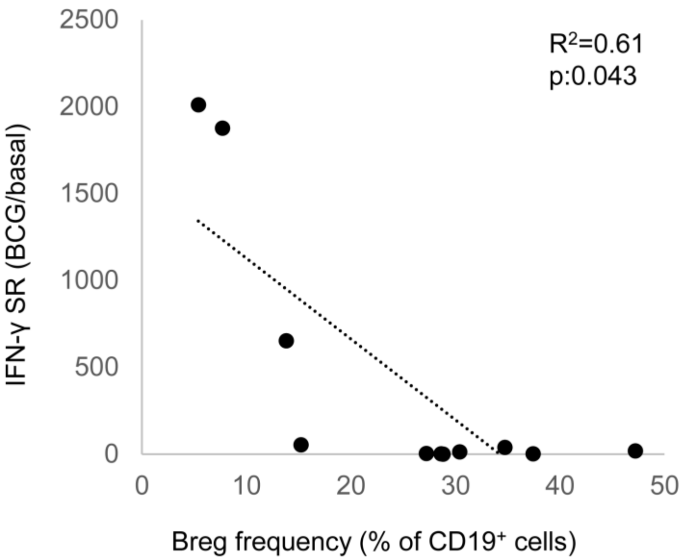

Figure 3

DISCUSSION

The center of this thesis is the analysis of the IL-12/IFN-γ axis in health and disease (primary and secondary immunodeficiencies). We wanted to explore the function of this pathway in two different settings: first in the context of children with severe intracellular infectious diseases, concretely VL and severe/extrapulmonary TB. These infections during childhood have sporadically been associated with inborn errors of the IL-12/IFN-γ axis, but this axis has never been systematically evaluated in big cohorts. Second, in infants prenatally exposed to anti-TNF-α monoclonal antibodies, since the death of one exposed child due to BCG vaccination [302] suggested that exposure to this drug during pregnancy impaired the infant's immune system, conferring a secondary immunodeficiency, being the IL-12/IFN-γ axis most probably involved.

For the study of the impact of anti-TNF-α drugs on the immune system of exposed infants, we had to first establish normality values for the different immune populations as well as for the analysis of IL-12/IFN-γ axis. On the light of recent studies highlighting the role of Breg cells in different conditions of health and disease, including in pregnancy, and the role of TNF-α in B cell development, we included in this evaluation, the assessment (quantitatively and qualitatively) of $CD38^{hi}CD24^{hi}$ B cell-subset (Bregs) since there were no previous published studies on the status of this subset in newborns.

3. Strengths and limitations of our studies

1.1.Cohort size

Samples used for the present thesis represent both strength and a limitation. Fortunately, in our area, severe/extrapulmonary TB and VL diseases in pediatric population are not common. Thus, we tried to collaborate with as much hospitals as possible. Despite this multicentric approach the size of the cohort remains small. We included 23 pediatric patients with VL, 24 pediatric patients with severe/extrapulmonary TB and 41 controls in which we performed an immunophenotype, oxidative burst test, proliferative assays and testing of the IL-12/IFN-γ axis. The performance of these tests on fresh whole blood was a big effort of coordination between clinicians and the laboratory. Two previous studies have been reported searching for mutations in MSMD-causing genes in TB patients [82,233] but this is, to our knowledge, the first systematic and thorough evaluation of the state of the IL-12/IFN-γ axis function searching for inborn defects of immunity in both sets of patients.

Our work on the normality in cord blood has also been marked by the low number of samples: cord blood samples were not easily achievable and, besides, for functional studies high numbers of cells were needed. With no doubt, the cohort of infants born to mothers with IBD for the study of the effects of the exposure to anti-TNF-α drugs during whole-pregnancy represented the higher challenge. As stated in the results section, the IBD unit in Hospital Clinic de Barcelona was unique in our area that maintained the treatment during whole pregnancy in severe cases of IBD at the time of the study. Between January 2014 and January 2016, we were able to recruit 7 patients fulfilling inclusion criteria of the 8 eligible patients in Catalonia. Although results need to be interpreted on the light of this limitation, we could obtain robust and statistically significant results. Due to the high value of the studied cohorts, we strongly believe that the results obtained are of special interest.

1.2.Control groups

The choice of control groups is of outmost importance for further interpretation of results. This project has been performed in pediatric cohorts, increasing the difficulty to recruit an optimal control group. Unfortunately, recruitment of age-paired pediatric healthy controls was not possible due to ethical limitations. We have therefore compared the cohort of children with VL or severe/extrapulmonary TB with healthy adults. Age-paired comparison would have been of especial interest in patients below 2 years old, where Th1-like immunity has been shown to be physiologically diminished [314–318]. It is of special interest that, from our knowledge, description of IL-12/IFN-γ axis in the context of a mycobacterial challenge during infancy and childhood have never been assayed. We might also speculate that variability in young children could be lower than in adults due to decreased immunological experience conditioning the immune response.

For the study of anti-TNF-α exposed infants, the control group for the birth time-point consisted of infants born to healthy mothers. The perfect control group would have been a group of infants born to mothers with moderate-to-severe IBD without immunomodulatory treatment (or treatment without transplacental pass-through). However, virtually all pregnant women with moderate-to-severe IBD were treated with anti-TNF-α or another immunomodulatory drug. Future perspectives need to dissociate the effects of the treatment itself and the changes due to

maternal condition. However, this does not hamper the traslationability of our study in IBD-pregnant women, the original clinical scope, as they can mostly be reassured based on our results.

The availability of cord blood samples after the recruitment of a control group of infants born to healthy mothers was a unique opportunity. On one hand, it allowed us to generate our normality values and, on the other hand, to study Breg cells in this special state of immunity. The study of Breg cells in human neonates was of great novelty as, at the time of the start of this study, there were no previous reports. Unfortunately, another group working on cord blood stem transplantation published for the first time the presence of $CD38^{hi}CD24^{hi}$ B cells with regulatory functions in human cord blood, just 9 months before (Sarvaria *et al* [330]). Their study was more focused on the description of the functional properties of neonatal cord blood and their implication in cord blood transplantation. We then pursued this work by relating Breg cells and IFN-γ production after mycobacterial challenge: we have showed an association between infant's reduced IFN-γ production after microbial challenge and increased Breg cell frequency.

1.3.Variability in cytokine production

Whole blood culture stimulation with live BCG is (although no-perfect) one of the best simulations of what happens in "real-life" after an intra-macrophagic infection in humans, being the gold-standard technique for MSMD diagnosis [81,89,119,173]. The classical read-out of this technique is the detection of the different secreted cytokines after stimulation. Cytokine production is strongly influenced by a broad variety of factors including different concurrent or past infections [390], time of blood drawn [391,392], stress [393,394], seasonality [395] or the time-point in the menstrual cycle in women [396]. High variability, already seen in adult healthy controls, is characteristic of this technique [81,89,119]. This intrinsic variability does not influence the detection of complete defects, as we have shown in the cytokine production pattern of the IL-12Rβ1 deficient patient we diagnosed. In any case we hypothesize that this variability could hamper the detection of partial defects, a problem that would need to be solved.

Reduction of variability in this technique in healthy controls could improve its diagnostic power. One method would be the use of Co-SR, in which we study the capacity of the system to respond to IFN-γ or IL-12p70. We observed that variability is reduced, but only in non-related controls but is maintained in related ones. This suggests that the use of Co-SR of non-related controls may be useful for diagnostic procedures as well as for the study of this pathway in the research context. Complex data analysis performed with the aid of bioinformatic tools could be an option to better differentiate between normal responses, partial functional defects and complete defects.

4. Study of the IL-12/IFN-γ axis in extrapulmonary severe *Mycobacterium tuberculosis* and visceral leishmaniasis

Severe/extrapulmonary TB and VL are two severe diseases with global impact. The development of severe disease by these pathogens is increased in low-income countries, where pediatric patients have more possibilities to have acquired predisposition because of malnutrition or HIV infections. Patients included in this study did not have any of these acquired predisposition factors, nor were treated with immunosuppressive or immunomodulatory drugs. The reason why these children developed the severe infectious disease, while other community members did not, is an important question to improve prevention and treatment strategies. To evaluate the genetic theory of infectious diseases (severe infections in childhood respond to monogenic inborn errors of immunity), we studied first well-described PIDs (SCID by performing a general immunophenotype and a proliferation assay and CGD by performing an oxidative burst assay). None of the studied patients presented a phenotype compatible with these diseases. After discarding them, we evaluated MSMD.

Although VL and severe/extrapulmonary TB are not within the classical warning signs for MSMD, patients with *Leishmania spp.* or *Mtb* infections have been described to have inborn errors in described MSMD-causing genes [78,95,134,234,259]. Therefore, we hypothesized that pediatric patients that had suffered from severe/extrapulmonary TB or VL lacking other factors of predisposition/susceptibility could have congenital susceptibility to these diseases, and that this susceptibility could be explained by inborn errors of the IL-12/IFN-γ axis. To rapidly detect complete defects of the pathway we performed cytometric analysis of the IFN-γR1, IFN-γR2 and IL-12Rβ1 receptors presence as well as functional assays to determine STAT1 phosphorylation in response to IFN-γ. We did not found alterations in cytometric determinations in the studied patients, thus lowering the possibilities to detect an MSMD-causing defect.

The gold standard technique to detect defects in the IL-12/IFN-γ axis consists in the measure of cytokine secretion after whole blood stimulation with BCG [119,173]. We did not find any pattern of cytokine production that could be associated with a complete defect in the integrity of the IL-12/IFN-γ axis. There are two other studies screening for MSMD-related defects in *Mtb* infections, one searching for mutations in *IL12RB1* (2/50 deficient patients) in endemic and highly consanguineous regions [82] and the other searching for i) common mutations in *IL12RB1, IFNGR1, IFNGR2* and *STAT1* in 114 adults with severe tuberculosis and ii) common or new mutations in children with extrapulmonary TB in an area of low consanguinity rates, not encountering any mutations [233]. In our studied cohort consanguinity was also a rare event. With that, we suggest that complete forms of MSMD may not to be suspected at first in patients with severe/extrapulmonary TB or VL in our area. Nevertheless, we found suggestive and clear differences in cytokine production profiles between healthy controls and patients, pointing out that still, the axis is partially impaired, which needs further studies.

Both related and non-related controls presented high variability after BCG-stimulation, shown by coefficients of variation higher than 100. MSMD defects are divided in two main categories: ones causing defective production to IFN-γ and others causing defective response to IFN-γ. Defective production of IFN-γ is usually caused by IL-12 response or production defects. For this reason, the combination of cytokine production levels after BCG stimulation with the response to IL-12 or IFN-γ co-stimulation is the readout that provides the most accurate

information on the integrity of the pathway. We performed Co-SR ratios with the intention of reduce the variability in healthy controls. This was true for NR-HC but not for related controls, where variability did not decrease. We speculate that the increased variability of Co-SR in response to IFN-γ and IL-12p70 observed in R-HC may be ascribed to the different defects of the pathway observed in patients, since they both carry the same genetic background.

Studied patients with VL or severe/extrapulmonary TB had differentially altered cytokine secretion patterns. While VL patients had decreased IFN-γ production after mycobacterial challenge, rescued with IL-12p70 co-stimulation, extrapulmonary TB patients had an impaired response to IFN-γ. Production and response to IFN-γ is required for parasite/mycobacteria killing in the macrophage [213–215,249–251]. Of note, during active VL infection, PBMC's of infected individuals have an altered IFN-γ production that is restored upon clearance of the infection [248,253]. Since in all patients the study was performed at least one month after resolution of the infection, the inhibitory capacity of *Leishmania* parasites should not have interfered with the test. Besides, epigenetic changes responsible for the transition to a more Th-1 like immunity [397–400] occur in an accelerated way after BCG immunization [318,401,402]. With that, we speculate that the exposure to *Leishmania* at young ages may interfere with these epigenetic changes or, alternatively, that susceptible patients have inborn errors in genes implicated on IFN-γ production.

Besides of the defective IFN-γ response, extrapulmonary TB patients showed an altered cytokine production pattern after BCG stimulation. This group produced decreased amounts of IL-1RA and increased levels of TNF-α and IL-10. TNF-α and IL-1ß are both contributors of the granuloma formation and maintenance as well as restriction of intracellular growth in macrophages [213,269,276,277,403,404]. TNF-α is necessary for the control of both non-virulent (i. e. BCG), and virulent species (i. e. *Mtb*) of mycobacteria; on the other hand IL-1β is only necessary for the control of virulent mycobacteria [403]. Decreased IL-1RA (a reflect of IL-1β production) might reflect defects on granuloma formation, thus promoting dissemination of the disease to other organs that may not be rescued with an increased production of TNF-α. Although we did not find any complete defect, this altered cytokine production and response to IFN-γ suggest the need of more studies searching for hypomorphic or somatic mutations as well as epigenetic defects to better characterize and treat these patients.

Altogether, these data strongly suggest that severe/extrapulmonary TB and VL patients have a familial altered anti-mycobacterial response, that involves the IL-12/IFN-γ axis, still undefined. The use of high throughput technologies may facilitate the study of epigenetic changes, somatic mutations or complex mechanisms of susceptibility. Decreased frequency of MSMD in our media could be explained by the low rates of consanguinity, reducing the chances of recessive diseases. Besides, detection of MSMD may be also decreased since BCG infection after vaccination is the main sign of MSMD, and BCG vaccination is no longer used in our country. The warning signs of MSMD in our media are still to be defined, and also training of physicians on the need to evaluate immunity in children with severe infections.

Differentiation from a BCG-vaccination related disease from *Mtb* infection have major implications in diagnosis procedure and treatment. The described patient with disseminated vaccine-related BCGosis was misdiagnosed as resistant extrapulmonary. Since MDR-TB is an emerging problem in Peru, the patient's lack of clinical response was first ascribed to drug-

resistant *Mtb*. BCG and *Mtb* differentiation is not easy because *Mycobacterium bovis* (the virulent strain from where BCG derives [405]) is closely related to *Mtb* within the *Mycobacterium tuberculosis complex* [406]. Mycobacterial culture from patient's sterile liquids or tissues (the gold standard diagnostic technique), does not differentiate between these two microbes. Resistance studies may help, since all BCG vaccine strains are intrinsically resistant to pyrazinamide, which is uncommon in naïve patients with *Mtb* [406]. Only PCR studies and IFN-γ release assays specific for virulent strains of the *Mycobacterium tuberculosis complex* are capable of clearly differentiate between *Mycobacterium bovis* and *Mtb*, but they are not widely available[406,407].

Besides the primary microbiologic misdiagnosis, immunologic and genetic diagnosis of the underlying immune defect (IL-12Rβ1 deficiency in our case), allowed for a specific management and improved survival: in our case, the patient was transferred to the National Institute of Health (United States of America) for specific treatment with extensive and prolonged antimycobacterial drugs followed by secondary prophylaxis, in combination with exogenous hr-IFN-γ [95,154,408]. After hr-IFN-γ treatment was started, the patient started to respond and get cured of her infectious disease. This case highlights the need to consider PID (especially defects in the IL-12/IFN-γ axis) in patients with severe mycobacterial infection, and/or major adverse events after BCG vaccination [95] and how BCGosis can mimic disseminated TB and delay appropriate diagnostic and therapeutic management. The consideration of these concepts in the healthcare practice can help to reduce morbi-mortality of possible immunodeficient patients.

5. MSMD diagnosis: from research to clinical practice

Set up and performance of specific functional techniques for MSMD diagnosis is not trivial. Besides of the technical limitations, interpretation of the results can also be challenging; therefore, presence of qualified staff is required. Especially in low-income countries, the needed infrastructures and equipment's could be also limiting, as there is a need to work in sterile conditions with high cost equipment and consumables, specifically in relation to flow cytometry or some cytokine detection techniques. MSMD diagnosis is usually only possible in specialized immunology laboratories. Functional techniques for MSMD diagnosis were set up to study patients with severe/extrapulmonary TB and VL in our laboratory, deriving in the implementation of MSMD diagnosis in the clinical practice in the Immunology Service of the *Hospital Clinic de Barcelona* from the Functional Unit of Clinical Immunology Sant Joan de Déu-Clínic, leading to the publication of a manuscript reviewing current laboratory tests for MSMD diagnosis [173].

In fact, currently there is a broad array of available tests for MSMD diagnosis, each one with different strengths and limitations [173]. However, considering price, working hours and complexity in the interpretation of results, we propose a step-by-step algorithm **(Figure 17)** for MSMD diagnosis in the routine practice after clinical suspicion: 1) discard other more frequent intra-macrophagic-susceptibility-conferring PIDs, 2) performance of cytometric determination of the presence of IL-12Rβ1, IFN-γR1, IFN-γR2 and, when possible, of STAT1 phosphorylation in response to IFN-γ, 3) genetic evaluation depending if in step 2 there is a clear candidate (targeted Sanger sequencing) or not (NGS with a panel or WES) and 4) functional confirmation of the genetic result, which normally needs to be performed in the context of a research laboratory. Nevertheless, only genetics with a functional confirmation of the identified mutation will lead to a real diagnosis. Thus, functional studies will always be necessary.

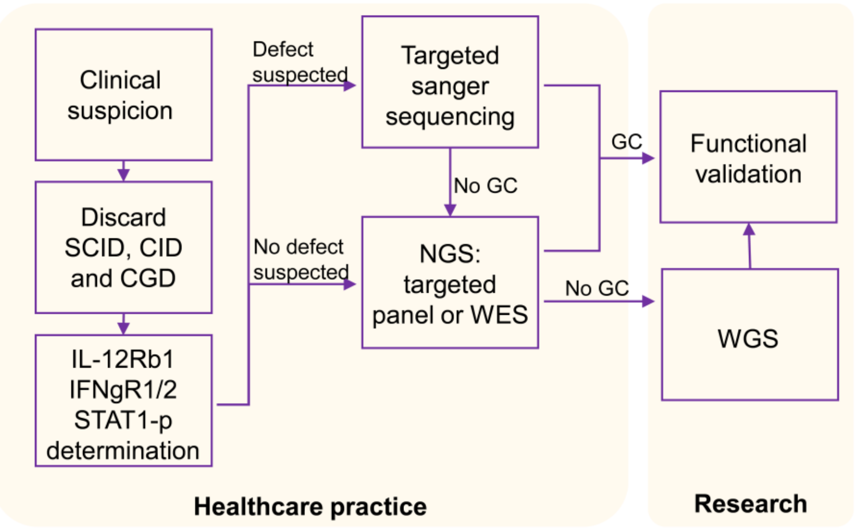

Figure 17. Step-by-step diagnosis algorithm for MSMD diagnosis. SCID: severe combined immunodeficiency, CID: combined immunodeficiency, CGD: chronic granulomatous. GC: genetic confirmation, NGS: next generation sequencing, WES: whole exome sequencing; WGS: whole genome sequencing; GC: genetic confirmation.

More than thirty years have passed since the first genetic diagnosis of MSMD [71,72] and there are, still, many issues in its diagnosis which need to be resolved [173]. First, there is a need

for awareness regarding MSMD, so that physicians taking care of children or adults, can suspect these disorders. The knowledge of the specific warning signs is of outmost importance. Patients, especially children, with BCGitis or BCGosis or EM infections, alone or in combination with other intracellular infections, should be suspected of having a PID, especially MSMD. Global frequency of MSMD has been estimated to be around 1/50,000, although it had been previously thought to be more infrequent.

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 option and genetic counseling, and, therefore, decrease mortality, as the final genetic diagnostic will condition the treatment of the infectious disease in patients with MSMD [81,89,93,95,409]. For this reason, we are now setting up a panel with all the known disease-causing genes that would serve to both better study the cohorts described here and as a diagnostic tool in the clinical practice for patients with clinical and/or functional suspicion. Unfortunately, although during the last years big efforts have been made to discover new MSMD-causing mutations, there is still a great proportion of MSMD patients (almost 50%) without a definitive genetic diagnostic. Further studies for the discovery of new genes should imply WES or WGS and functional validations.

6. Prenatal exposure to anti-TNF-α drugs impacts on the immune system maturation in exposed infants

Anti-TNF-α treatment is a new opportunity to female patients with IBD to have a non-complicated pregnancy. Still, a big group of families decide not to have children when the woman suffers from IBD because of the fear of reactivation of the disease or the effect that the disease itself or the treatments might have on the infant. TNF-α treatment is, sometimes, the only way to control the disease in patients with moderate-to-severe IBD. For this reason, the assessment of the effects of the exposure to this drug during whole pregnancy on the infant is an imperative for the management of these patients. Although other studies concerning safety and general effects on the immune response of exposed infants exist, we aimed at thoroughly studying the effect of anti-TNF-α exposure in the developing immune system, especially focused in the study of mycobacterial response.

The transfer of anti-TNF-α to the fetus during third trimester of pregnancy leads to drug exposure in the exposed neonate until approximately 6 months. In accordance with published data [298,300], we could detect TNF-α inhibitors in the blood of the exposed infants until 6 months of age. The observed level on the infant was related to the though level of the mother and the time-to-delivery of the last injection. It has been shown that exposed-infants had increased levels compared with their mothers [300]. Our results were slightly different to those published: for adalimumab exposure reported child/mother mean ratio was 1.21 while ours was 0.99; on the other hand, for infliximab reported ratio was 1.97 and ours was 3.25. Experimental design differences may explain the differences observed. First, we measured mother's trough level during pregnancy instead of birth's levels. Besides, all patients included in this study received the last dose of treatment the last week before delivery while in the previous study [300] there was a huge range of time from the last dose to delivery, from 0 to 25 weeks.

Although due to sample size limitations we could not make comparisons between adalimumab and infliximab exposed infants, it seemed that the effects of infliximab on the immune system of the exposed infant were stronger than in adalimumab exposed ones. This possible different affectation could be explained by the larger inter-dose interval of infliximab than adalimumab (4-8 folds), being the dose higher in infliximab patients. Our recommendation would be to try to separate as much as possible from birth the administration of the last dose of anti-TNF-α in the mother, to reduce the drug level in the newborn. There are also studies recommending ADA and IFX discontinuation after week 20 of pregnancy in order to try to reduce drug levels in the newborn [410–412]; however, in some circumstances the risk of a disease reactivation prevent the adoption of these strategies.

Neutropenia can be an associated complication of anti-TNF-α exposure during pregnancy. Neutropenia in infliximab-exposed infants after approximately 15 days after birth had been previously reported: 3 patients presented with severe and 1 with moderate neutropenia that improved after 3 months [413]. In our studied cohort, at birth none of the included infants had severe neutropenia, although 2 infants presented with levels below the reference range for the age. On the other hand, we observed one case of severe and two cases of moderate neutropenia in 3-months old exposed infants. Reported exposed infants had also infectious skin lesions that occurred during the neutropenic period [413] while none of the included infants presented infectious skin lesions. Differences in the results may be due to differences in the drug

infusion pattern. In the 4 cases described, the infusion of the last infliximab dose was, at least, 8 weeks' pre-partum. Instead, patients included in our study received the last dose of either infliximab or adalimumab from 3 to 7 days pre-partum. After these observations, we conclude that neutrophil count should be routinely performed in infants exposed to anti-TNF-α drugs during pregnancy, especially in the event of an infection.

Exposure to anti-TNF-α drugs affects immune system maturation. For the first time, we evaluated the maturation status of B and T cell compartments. In concordance to what had been previously observed in anti-TNF-α exposed non-human primates [313], there was no impairment of total numbers of B and T cells in all exposed infants but in one that had B-cell lymphopenia at birth after adalimumab and azathioprine exposure (azathioprine exposure during pregnancy can lead to B cell lymphopenia at birth [414]). Although maturation is impaired at birth, after 12 months values of B and T cell subpopulations were within the normality values for age, and patients produced normal amounts of immunoglobulins with normal responses to vaccines. Except for one infant with recurrent non-severe infections from 6 to 12 months, no clearly increased susceptibility to infections was observed in exposed infants, possibly ascribed to the transferred immunoglobulins from the mother in late-pregnancy.

Immune dysregulation should be considered and monitored in anti-TNF-α exposed infants. 4 of out the 7 studied children presented atopy in the first year (2 of them without family history), and all ADA-exposed infants had increased eosinophil counts in cord blood. We have observed a diminished frequency of Treg cells in all exposed infants compared to healthy controls. Interestingly, there are some publications showing an increase in Treg cells in responder adult patients after anti-TNF-α treatment [415–417]. However, cells that increase in adult are not natural ($CD62L^+$) but induced ($CD62L^-$) Treg cells [416]. As induced Treg cells are differentiated upon an antigenic insult [418], in the umbilical cord blood the majority of Treg cells would be expected to be natural Treg (also suggested by the fact that they express high levels of CD62L)[419]. The decreased Treg cell frequency correlated with increased T-cell response to weak stimulus, thus suggesting higher reactivity of T cells. However, we cannot rule out the possibility that this may be influenced by the mother's disease [420]. On the other hand, there was an increased immature Breg frequency. Regulatory populations in TNF-α inhibitors exposed infants need to be further studied in larger cohorts to confirm these results. Besides, clinical evolution of exposed infants should be specifically followed-up, with special attention to allergic, inflammatory, and autoimmune events.

We have shown that drug exposure decreases the response to mycobacterial challenge at birth, which is not totally rescued after drug cleaning and increased with age. Comparison between the effect of anti-TNF-α inhibitors exposure in the immunity of adults with inflammatory disease with respect to the exposure of infants during late pregnancy and early infancy, is difficult because both cases are special situations of immunity [318,421–425]. In adults, it has been observed that there is a decreased production of IL-12 (but not of IL-6) after anti-TNF-α therapy [426] as well as a decrease in IFN-γ-producing $CD8^+$ T-cells and in the Th1/Th17 subset with an increase in IFN-γ-producing NK cells [427]. Our results obtained after BCG stimulation do not totally correspond with those published: we observed no significant differences in IL-12p70 production in comparison with non-exposed infants and an increased production of IL-6. Also, presence of anti-TNF-α mAbs reduced IL-17 production after BCG stimulation that was rescued

after whole-blood washing. On the other hand, the reduced CD69 expression has been reported previously in exposed patients' T cells [277].

Based on empirical experience, including the death of an infliximab exposed infant after BCG vaccination [302] and theoretical knowledge [428], the use of all live vaccines was delayed from 6 to 12 months of age in infants exposed to adalimumab or infliximab during the late second and third trimester of pregnancy [298,303]. With this work, we provide objective data to ponder this statement: at birth, exposed infants showed more immature B- and T-cell-subsets. However, we observed a normal T-cell proliferation to mitogens, as well as T- and B-cell numbers and maturation, immunoglobulin production and inactivated vaccine responses, accomplishing the criteria for attenuated vaccination in patients with cellular immunodeficiency [429]. Also, none of the 4 infants who received rotavirus-inactivated vaccine presented adverse events. After these results, we speculate that vaccine policy in those infants could be revised, highlighting the avoidance of BCG vaccination in anti-TNF-α exposed neonates.

The advent of new biosimilars will broaden the use of biological treatments in developing countries, some of which have endemic TB or vaccination programs including BCG immunization soon after birth. Until more investigations are performed, BCG vaccination must be absolutely avoided in exposed infants until recovery of antimycobacterial function is verified or at least until 12 months of age. *In vitro* functional studies would be relevant for this purpose. This study is the first thorough evaluation of the impact of prenatal anti-TNF-α on the immune system development of exposed-infants. Although we observed specific changes, infants were not clinically compromised. Our results aim at generating consciousness of the need to further study and follow-up on exposed-infants. Pediatricians should be informed of the mother's mAb treatment during pregnancy, because of the impact on vaccine recommendations, especially with regards to BCG due to the observed mycobacterial-deficient response **(Figure 18)**.

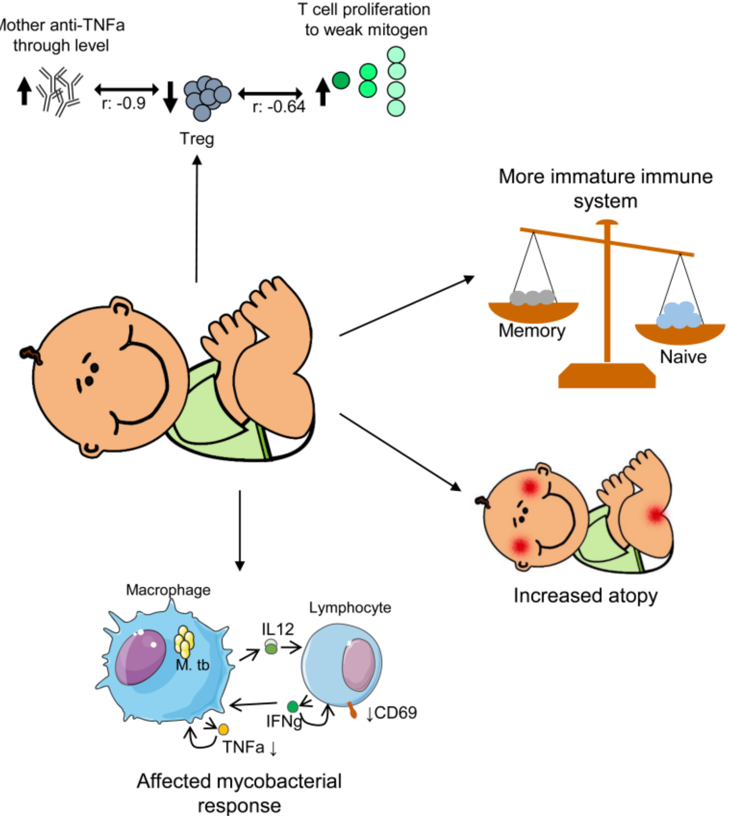

Figure 18. Graphical representation of the effects of anti-TNF-α exposure during pregnancy

7. Breg cells can play a major role in neonate immunity

The immune system has developed mechanisms to accommodate the especially important period before, during and after birth: there is a breaking transition from the allogenic but sterile uterus to the 'outside-maternal' world, where the newborn will be massively colonized by commensal microbiota and exposed to a myriad of different non-hazardous antigens, while will also be attacked by pathogens. When we try to define reference values in newborns, we detected a surprisingly increased frequency of CD24^{hi}CD38^{hi} B cells in cord blood; this B-subpopulation showed regulatory capacities with a phenotype similar to that of adult CD24^{hi}CD38^{hi} cells. Of note, Breg cell frequency was associated with IFN-γ production after whole blood BCG-stimulation. Neonatal response to BCG was, therefore, reduced, showing lower levels of IFN-γ and IL-6 as well as diminished CD69 response upon stimulation. However, responses to IL-12 and IFN-γ co-stimulation were preserved.

Increased frequency of CD24^{hi}CD38^{hi} B cells in neonates could be due to the immaturity of the system, being these cells 'only' transitional B-cells. We made functional studies to check if, neonatal CD24^{hi}CD38^{hi} B cells had regulatory functions. We observed a capacity to produce IL-10 upon stimulation and a suppressive activity on IFN-γ and IL-4 production by T cells after co-culture that did not occur after CD24^{int}CD38^{int} co-culture. Besides, they have a similar phenotype compared with adult CD24^{hi}CD38^{hi} Breg cells, with an increased IgM and a decreased CD27 expression. Since CD27 is a memory marker, the decrease can be explained by their very young age [334]. Our results are in consonance with the studies performed with adult CD24^{hi}CD38^{hi} Breg cells, named 'immature Breg cells' with proven regulatory functions [334,335,339].

Interestingly, without any stimulation, both Breg and noBreg cells produced more IL-10 than their adult counterparts, in consonance to what was observed in the other study on neonatal Breg cells [330]. After IL-10 blockade experiments, the authors concluded that although IL-10 is in part responsible of their suppressive functions, it is not the only mechanism involved. However, differently to our observation, they observed inhibition of the production of inflammatory cytokines by both CD24^{hi}CD38^{hi} and CD24^{int}CD38^{int} [330]. We speculate that discrepancy in the results could be explained by differences in the experimental design. While we co-cultured either Breg or noBreg cells with CD3^{+}CD25^{-} T cells for 72h in the presence of plate-bound CD3 and a re-stimulation step with PMA and Ionomycin before intracellular cytokine staining; Sarvaria *et al* co-cultured CD4^{+} cells with either Breg or NoBreg cells, stimulated them with anti-CD3/CD28 for 96h, and then analyzed the concentration of inflammatory cytokines in the supernatant [330]. Although we were both analyzing CD4^{+} cytokine production; in our setting, cells were in contact with other types of T-cells (as CD8^{+} cells), while in the other study there were only CD4^{+} cells. With that, we can speculate that Breg cells affect differentially to different subsets of T cells.

It is known that Breg cells can carry out part of their functions via IL-10-independent mechanisms. Immune regulation by cord blood B cells seems to be partially mediated by i) IL-10 production and ii) cell-to-cell direct contact (mediated by CD80/CD86), but independently of TGF-β, as has also been observed in adults [330,335]. Other mechanisms for Breg cell inhibitory functions have been proposed, including the production of adenosine by CD73. After studying this mechanism in neonatal CD24^{hi}CD38^{hi} Breg cells we conclude that adenosine production by CD73 seems not to be a mechanism of action for these cells, as CD73 expression is decreased in neonatal Breg cells. On the other hand, we speculate that the increased quantity of IgM per cell in neonatal Breg cells compared both no NoBreg cells and adult Breg cells could be a new mechanism of action

for their inhibitory function. Naturally occurring IgM Anti-Leukocyte Autoantibodies (IgM-ALA) are antibodies with suppressor capacity that can inhibit T cell activation and chemotaxis [430,431]. IgM-ALA antibodies are present at birth [431], and they inhibit proinflammatory cells from producing IFN-γ and IL-17 in response to alloantigens in mice [430]. More research should be done to evaluate whether if this is a true regulatory mechanism of neonatal Breg function.

Neonatal marginal zone-like B cells presented the greatest difference in proportion of Breg-cells when compared with adults. Circulating marginal zone B cells are representative of splenic marginal zone B cells; these cells are characterized for having a pre-diversified Ig repertoire and initiating T-cell independent responses through TLRs as activation signals. Their responses are mainly directed against encapsulated bacteria, including commensal microbiota [321,432,433] and play a role in normal pregnancy development [434]. As TLRs activate Bregs [334,343], the greater proportion of marginal zone-like B cells observed might indicate increased regulatory responses after encapsulated bacterial stimulation, thus explaining lower responses to encapsulated bacteria in infants [318]. Besides, we observed lower levels of CD22; this surface molecule modulates the BCR signal and prevents the hyperactivation of B cells upon stimulation [323–325]. Besides, in CD22 knock-out mice, increased circulating IgM was detected and more Breg-cells were observed in the spleen [326,435]. These B cells had the capacity to inhibit the IgG response to estrange and self-antigens [326]. Overall, it suggested a lower activation threshold of these neonatal Breg cells. This could be of importance in the rapid abrogation of unwanted responses to commensal bacteria. We might hypothesize that, during the first contacts with the extra-uterine environment and the adoption of microbiota, the increased proportion of Breg-cells among marginal zone B cells could be one of the mechanisms by which the neonatal immune system protects itself from an exacerbated response to the new range of antigens encountered.

Breg increased levels in umbilical cord blood have been related to lower rates of graft versus host disease in cord-blood transplanted patients [330,436]. Besides, patients developing GvHD present a decreased frequency of Breg cells [330]. On the other hand, cord-blood transplantation has also been associated with an increased morbi-mortality due to infections [436]. We hypothesized that, as $CD5^+$ murine B-cells contribute to the reduced production of IL-12 by APCs [437], increased hUCB Breg contributes to the limited Th1 response observed in neonates inhibiting IFN-γ production. We evaluated the state of the IL-12/IFN-γ axis in neonates using the gold standard technique used to detect inborn errors of this pathway [119,173] and observed decreased response to mycobacterial challenge consisting in decreased IFN-γ and IL-6 secretion as well as diminished expression of CD69 early activation marker. BCG infection has been proven as one of the stimulus capable of inducing IL-12p70 by neonatal lymphocytes [398], concordantly, there were no significant differences in IL-12p70.

We propose that neonates maintain the IL-12/IFN-γ axis integrity after observing normal responses to IL-12p70 and IFN-γ co-stimulation; what is more, IFN-γ production ratio after IL-12p70 co-stimulation showed a tendency to be higher, as well as increased $HLA\text{-}DR^+$ T-cell frequency after IFN-γ co-stimulation. BCG vaccination has been shown to confer protection to other non-related diseases as well as to reduce total infant mortality due to infections. This protection has been proposed to be due to epigenetic modifications in innate cells, what is known as innate training. After these results, we hypothesize that although IFN-γ production is low after the first 48h post-infection, the effect of the secreted IFN-γ together with IL-12p70 might lead to the Th1 differentiation of the cells, thus leading to a new balance between Th1 and Th2 cells.

Breg cell frequency is inversely associated with IFN-γ secretion after whole blood BCG stimulation. It is known that after BCG challenge in neonates, the major providers of IFN-γ are NK cells instead of T cells [438]; therefore, we hypothesize that Breg cells could be, at least in part, responsible of this decreased T cell response. Since high IFN-γ levels are associated with graft versus host disease development [439,440], we consider that this association altogether with the increased Breg cell frequency in neonates, deserves further investigation and may have implications in clinical practice, especially in stem cell transplantation from cord blood. For example, in IFN-γR1 deficiency, where basal levels of IFN-γ in blood are increased [95,174], stem cell transplantation has been difficult to perform with success [142]. We propose that UCB transplantation in those patients could help to reduce IFN-γ levels in blood, thus helping the engraftment.

In summary, we have observed that neonate's immune system has a diminished response to mycobacterial challenge with a diminished IFN-γ production, possibly partially determined by Breg cell frequency. More research on neonatal Breg cell subset is required in order to decipher their regulatory mechanisms and their role in special pregnancy conditions such as chronic autoimmune disease, HIV infection, premature delivery, in cord blood transplantation and in neonatal infections.

8. Implications and prospects

The work presented here is the result of a translational project; therefore, it has direct implications for clinical practice. The consecution of the different objectives has raised more questions than were answered.

None of the studied patients with severe/extrapulmonary TB or VL had a complete MSMD defect. However, the two groups of patients had altered function of the IL-12/IFN-γ axis, known to be essential for immunity against both *Leishmania* and mycobacterial infections. We strongly believe that the increased susceptibility of these patients to develop these severe diseases might be hereditary (at least in part), because their parents showed also an altered IL-12/IFN-γ axis function. Although it will be mandatory to study the presence of mutations in the known MDMS-causing genes, currently approximately half of the patients with clinical signs of MSMD have not a defined genetic diagnosis. For this reason, we believe that WES or WGS will expand the understanding of the basis of the infectious susceptibility of these patients. Besides, since only partial defects of the IFN-γ/IL-12 axis were identified, we hypothesize that other genetic factors could be occurring in susceptible patients, such as epigenetic variations or somatic mutations affecting both IFN-γ/IL-12 axis and the macrophage function.

Due to intrinsic variability in cytokine secretion, which is the basis for the gold standard technique for MSMD diagnosis, this approach hampers the diagnosis of partials defects. We believe that this assay has still options for improvement: we propose the performance of multiparametric assays in order to integrate the different read-outs of the technique to decrease variability and/or to find other forms of analyzing results in order to better differentiate between a normal and a pathogenic response. This type of studies can only be performed with the use of advanced bioinformatic technology; for this reason, collaboration with bioinformatic experts is critical.

Because of the methods established, we have developed a clinical pipeline for the management of patients with suspicion of MSMD in our area. We are currently a reference laboratory for the study of suspected MSMD defects. I would like to highlight the diagnosis of the IL-12Rβ1 deficiency of a Peruvian patient, despite it is probably not the most relevant scientific contribution of this thesis. In fact, our diagnosis allowed the transfer of the patient to the National Institute of Health in the United States of America, where she was properly treated and microbiologically diagnosed. As a biologist, it is uncommon to observe direct clinical consequences of an experimental research.

The results obtained with the study of the impact of whole-pregnancy anti-TNF-α treatment on the immune system of infants born to IBD patients arose interest in other medical areas where anti-TNF-α treatment is used, such as rheumatology (in fact, most of studies on the effect of anti-TNF-α have been performed in rheumatic patients); like in IBD, a great proportion of patients with rheumatic disease are women in childbearing age. To our knowledge, our study was first of its kind, by thoroughly studying the immune system of the exposed baby. For this reason, and in collaboration with the group for the study of the effect of biologic drugs administered during pregnancy, there is on ongoing project to study the influence of different treatments with TNF-α blockers in pregnant patients with inflammatory rheumatic diseases.

The decreased response to mycobacterial challenge of exposed infants has implications on their clinical management. The fact that antimycobacterial immunity was affected by the

exposure to anti-TNF-α during pregnancy was first suspected after the death of an exposed infant after BCG vaccination [302]; however mechanisms leading to this susceptibility were not further studied. After the results obtained, we energetically stress that BCG vaccination must not be administered to anti-TNF-exposed infants, as well as other attenuated vaccines for intra-macrophagic pathogens (oral typhoid vaccine) [441–443]. Therefore, a global study of the capacity of exposed neonates to respond to pathogens (especially of intracellular lifestyle) is still to be performed.

The study of regulatory subsets, especially Treg cells, in exposed infants is a new area to be developed: we observed a significant reduction on Treg cell frequency in exposed infants that did not recover after 12 months of follow-up. We aim at following this population at one more time-point around 3 years-old, as well as better studying in the new project all the different Treg subpopulations in exposed infants (including their functionality). Due to this altered Treg cell subset and the fact that 4 out of 7 patients presented atopy, the follow-up of clinical signs of allergy, inflammation or autoimmunity in this cohort is of special interest. Besides, to study the effect to anti-TNF-α exposure on the microbiota seems also to be potentially relevant: it is known that the composition of the microbiota is of outmost importance for the development of the immune system. Besides, different maternal conditions determine the composition of the microbiota in the infant. Therefore, we speculate that exposed infants might show differences in the microbiota that can lead to different maturation of the immune system.

The study of the safety of anti-TNF-α drugs can change the course of some pregnancy-related diseases. Amongst them, obstetric antiphospholipid syndrome (a known cause for reduced fertility in women) where anti-phospholipid-related thrombosis in the placenta partially explains the increased frequency of miscarriage in affected women, has been recently linked to inflammation, especially related to TNF-α production [444,445]. The use of anti-TNF-α drugs to overcome infertility has been successfully used before [446], and their use in patients with antiphospholipid syndrome is being now proposed [444,445]. The study of the effect of TNF-α on the exposed infant is an imperative especially when using this drug for fertility problems. We believe that our work increases the safety of TNF-α drugs during pregnancy and improves the knowledge of the management of the exposed infant. Nevertheless, similar studies in different conditions and with bigger cohorts are obviously needed.

On a more basic-research area, together with Sarvaria *et al* [330] we were the first to describe the population of Breg cells in neonates, thus opening the door for new research and possible treatments. We propose three lines of research on neonatal Breg cells:

1. To study of new mechanisms of action, such as the possible presence of IgM Anti-Leukocyte Antibodies in Breg, that could explain the increased levels of IgM per cell in neonatal Breg cells.
2. To define the role of Breg in different conditions, such as neonatal infection, autoimmunity, inflammation as well as the effect of maternal conditions on the Breg cell population or the evolution of this subset during the first years of life.
3. To characterize the role of Breg cells in cord blood transplantation, as beneficial actors to achieve tolerance or as a detrimental factor in infection susceptibility.

CONCLUSIONS

1. In our area, we did not detect an underlying primary immunodeficiency in pediatric patients with visceral leishmaniasis or severe/extrapulmonary tuberculosis, including T cell defects, chronic granulomatous disease and complete defects of IL-12/IFN-γ axis or MSMD.
2. Nevertheless, IL-12/IFN-γ axis was partially impaired in pediatric patients with visceral leishmaniasis and severe/extrapulmonary tuberculosis, suggesting an intrinsic susceptibility to intra-macrophagic infections. Definition of the concrete mechanism of susceptibility needs new approaches including next generation sequencing and epigenetic studies.
3. The early diagnosis of an MSMD, based on the development of specific warning signs and a diagnostic methodology of MSMD, is of outmost importance, since immunological and genetical confirmation of an MSMD will condition patient's management.
4. Prenatal exposure to anti-TNF-α drugs for the treatment of inflammatory bowel disease in the pregnant woman can be considered safe, with no significant clinical events.
5. Nevertheless, changes in the infant's immune system development are observed; especially regarding the IL-12/IFN-γ axis, which is partially defective, possibly increasing their susceptibility to mycobacteria. Therefore, BCG vaccination must be avoided at birth.
6. Infants exposed to TNF-α inhibitors during pregnancy born to mothers with inflammatory bowel disease should be followed-up on the long term for the advent of allergic, autoimmune or inflammatory events.
7. B regulatory cell subset is expanded in healthy neonates' cord blood, with a confirmed regulatory function. This observation might have a pivotal role in explaining the particularities of both neonatal response to infections and microbiota, and cord blood transplantation.

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ANNEXES

1. Laboratory evaluation of the IFN-γ circuit for the molecular diagnosis of Mendelian Susceptibility to Mycobacterial Disease.

Critical Reviews in Clinical Laboratory Sciences

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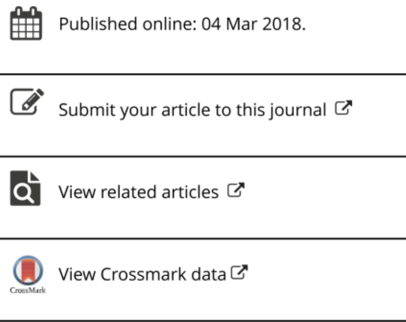

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Annex 1

CRITICAL REVIEWS IN CLINICAL LABORATORY SCIENCES, 2018
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REVIEW ARTICLE

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 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.

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

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; JAK: Janus-associated kinase; 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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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 (EM) [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 (APCs), 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 reactive oxygen 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 (JAK2)-2 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 ISGs, promoting their 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.

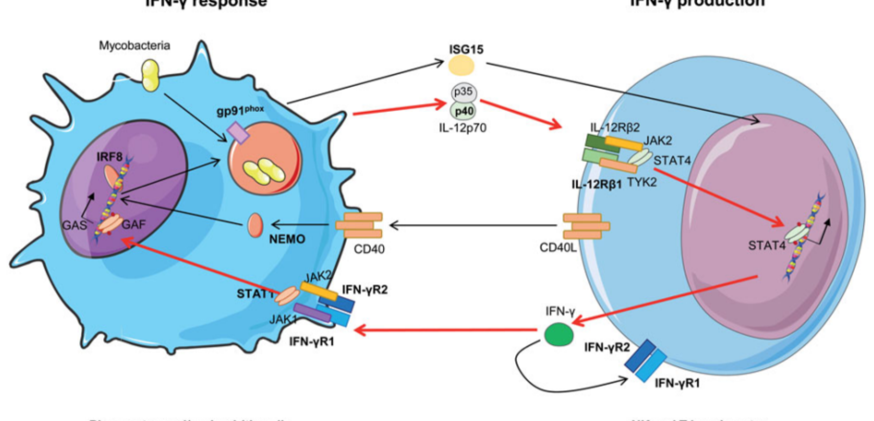

Figure 1. IFN-γ circuit. Summary of molecules implicated in the IFN-γ circuit. Molecules represented with bold characters are known to cause of MSMD. GAS: γ-interferon activated site; GAF: γ-activated factor.

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 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 (e.g. IL-12Rβ1 or IL-12p40 deficiencies), in which patients can be treated with exogenous human recombinant IFN-γ (hrIFN-γ) in addition to antibiotics [2,21]. 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 with 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 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 TB 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 intramacrophagic 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 also 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

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*, etc.)
	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)

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 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-TNF-α 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 two 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[1] to 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)[2], 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 on 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) 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 cutoff 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

Table 2. Diagnostic tests in IFN-γ response 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	IFN-γR1	IFN-γR2	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[a]
	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[b]
	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[c]

AR: autosomal recessive; AD: autosomal dominant; NP: not published; PBMC: peripheral mononuclear cells; WB: whole blood.
[a]Abolished response: no phosphorylation of STAT1 after IFN-γ stimulation.
[b]Impaired response: impaired phosphorylation of STAT1 after IFN-γ stimulation; phosphorylation is present only after high-dose IFN-γ ($>10^5$ IU/mL).
[c]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.

			Microbial stimulation (BCG)		Mitogen stimulation		Cytometric determination					
Gene	Inheritance	Defect complete/ partial (C/P)	IFN-γ production	IL-12 production	IFN-γ production	IL-12 production	Baseline IFN-γ	IL-12Rβ1 presence	STAT4 phosphorylation in response to IL-12p70	IFN-γ R1 presence	IFN-γR2 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
IL12RB1	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[a]	NP[a]	NP[a]	NP[a]	NP[a]

AR: autosomal recessive; AD: autosomal dominant; XR: X-linked; NP: not published; PBMC: peripheral mononuclear cells; PHA: phytohemagglutinin, WB: whole blood.
[a]Results tested in EBV-B cells are included in Table 4.

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 cutoff 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,[1] 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.[2]

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 2). 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 and 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.[2] The cost of antibodies is around 8€ per individual,[1] 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 of the described IL-12Rβ1 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 *IL12RB1* [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 *IL12RB1* 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 four 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).[2] The estimated antibody cost is around 10€ per individual.[1]

IFN-γ binding studies

As some defects in IFN-γR do not affect their membrane expression, IFN-γ binding studies can help to evaluate

Annex 1

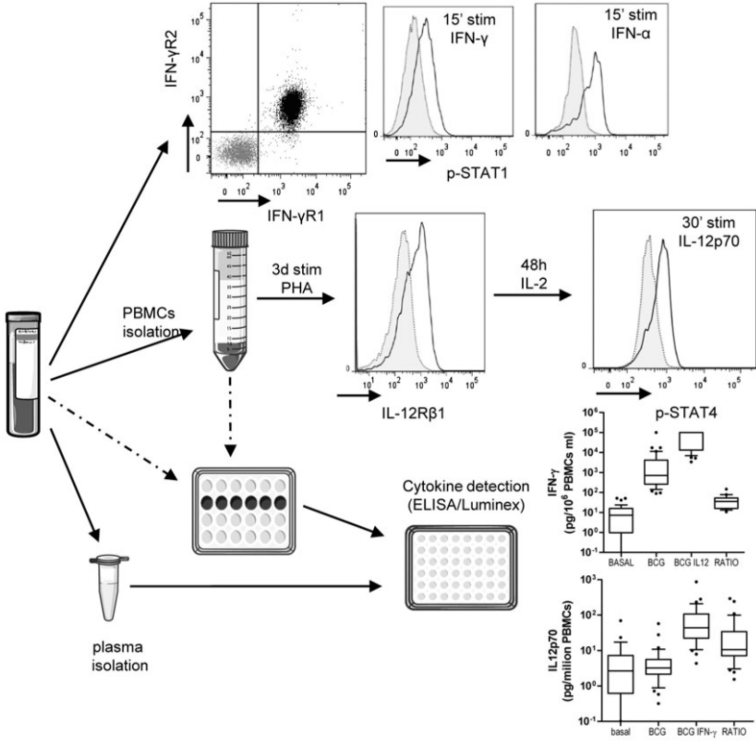

Figure 2. Diagram of the laboratory analysis of MSMD defects with examples. IFN-γR and STAT1 phosphorylation detection is performed in whole blood assay. IL-12Rβ2 detection is performed in PBMCs after 72 h stimulation with PHA. STAT4 phosphorylation detection is performed in PBMCs after 72 h stimulation 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.

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 healthy control and a negative control (medium), and the cost of consumables is approximately 34€.[1] 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€[1] and 6–6.5 h[2], 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- to 17-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 [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 to 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 LOF. 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), 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,[2] depending on the number of tubes to be processed, with consumable costs of about 40€ per individual[1] 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

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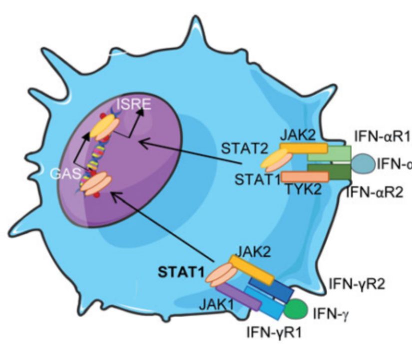

Figure 3. IFN-α and IFN-γ signaling. GAS: γ-interferon activated site; ISRE: interferon-sensitive response element.

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), whole genome sequencing (WGS) or copy number variation 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 hrIL-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.

As 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].

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 radio-labeled 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

(*continued*)

Table 4. Continued

Gene	Inheritance	EBV-B cells	Fibroblast (SV40)	Other tests
IL12RB1	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^-, H_2O_2 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 and 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.

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 (e.g. in EBV-B, T-saimiri cells or SV-40 fibroblasts) 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 twentieth century that infections after BCG vaccination were understood to be related to inborn errors of immunity [16], and only in 1996 did Jouanguy et al. [17] and Newport et al. [18] 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

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. 48 h 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. 48 h 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-γR1 deficiency	Some forms of IFN-γR1 deficiency present a nonfunctional 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 nonfunctional 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 two mutations in *IL12RB1* present a lack of IL-12Rβ1 in the membrane	There are two forms of IL-12Rβ1 defect that present IL-12Rβ1 in the membrane of activated lymphocytes, so its presence does not exclude the defect	IL-12Rβ1 defects
	STAT4 phosphorylation determination	Evaluates both IL-12Rβ1 and Tyk2 function	Long technique; no STAT4 antibody to detect total STAT4	IL-12Rβ1, TYK2 defects
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 Useful for new genes discovery; detection of mutations in non-coding/regulatory regions with whole genome sequencing (not whole exome sequencing)	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

PBMC: peripheral mononuclear cells; WB: whole blood.

10 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 TB, alone or in combination with other intracellular infections, are to be suspected of having MSMD. The 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.

Notes

1. 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. As 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.
2. 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).

Disclosure statement

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

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2. Resumen de la tesis

2.1.Introducción:

2.1.1.Inmunodeficiencias

El sistema inmunitario es un sistema altamente complejo y controlado con capacidad para discernir entre lo propio y lo extraño, entre lo peligroso y lo inocuo para poder responder de manera efectiva. Su función principal es la defensa ante patógenos, pero también tiene un papel importante en otros procesos como el cáncer, el establecimiento y mantenimiento del embarazo y la regeneración tisular entre otros. Defectos en la función del sistema inmunitario, ya sean congénitos o adquiridos, pueden ser causantes de inmunodeficiencia [1,2]. Las inmunodeficiencias primarias (IDP) son enfermedades consideradas raras causadas por defectos congénitos del sistema inmunitario. Las IDP "clásicas" son aquellas en que la mutación en un gen genera susceptibilidad a un amplio rango de patógenos, con un inicio temprano y mal pronóstico, como en la inmunodeficiencia combinada severa. Por otro lado, la descripción de IDP que confieren susceptibilidad a un rango de patógenos más estrecho, que pueden aparecer desde edades tempranas hasta la edad adulta y que tienen un pronóstico variable generó el concepto de "IDP atípicas" [3]; uno de los ejemplos más estudiados es la susceptibilidad mendeliana a las micobacterias (*Mendelian Susceptibility to Mycobacterial Disease*, MSMD; OMIM 209950) [4,5].

Las inmunodeficiencias secundarias son defectos adquiridos del sistema inmunitario que pueden ser reversibles o no. Pese a que el síndrome de inmunodeficiencia adquirida causado por el virus de la inmunodeficiencia humana es la causa de inmunodeficiencia secundaria más conocida y estudiada, la malnutrición es la causa más común [6]. Por otro lado, se ha postulado que los pacientes tratados con fármacos biológicos dirigidos a moléculas del sistema inmunitario pueden presentar estados similares a esos pacientes con inmunodeficiencias primarias [7]. Las infecciones que se observan en los pacientes tratados con anticuerpos monoclonales (AcMos) anti-TNF-α recuerdan a aquellas observadas en pacientes afectos de MSMD [7], como son las infecciones atípicas, diseminadas y miliares por micobacterias [8].

2.1.2.MSMD como paradigma de la teoría genética de las infecciones

El estudio del "por qué" y "cómo" de las enfermedades infecciosas está en el centro de numerosas investigaciones. Existen 4 teorías principales que tratan de explicarlo, centrándose o bien en el ambiente (teoría microbiológica y teoría ecológica) o en el huésped (teoría inmunológica y teoría genética). Estas teorías se han desarrollado en paralelo con el avance del conocimiento y son complementarias. La teoría microbiológica de las infecciones estableció que los gérmenes eran necesarios y suficientes para el desarrollo de enfermedad. La variabilidad observada en las enfermedades infecciosas hizo cuestionar esta teoría. Por su parte, la teoría ecológica propuso que los microorganismos son necesarios para que se desarrolle la enfermedad, pero que es el entorno (estado nutricional, coinfecciones, …) lo que va a determinar que se desarrolle o no la enfermedad. Más adelante, tras la observación de las respuestas a microorganismos atenuados apareció la teoría inmunológica de las infecciones, que justificaba la variabilidad observada por la previa exposición a patógenos atenuados, por ser

menos virulentos o en menor número. Esta teoría explicaba la variabilidad tras reactivaciones o reinfecciones, pero no en las infecciones primarias. Por último, la teoría genética de las infecciones propone que el *background* genético del individuo determina la respuesta a los microorganismos, concretamente postula que “las infecciones primarias que amenazan la vida en la infancia responden a defectos heredados en un único gen mientras que las reactivaciones e infecciones secundarias en adultos jóvenes son consecuencia de defectos en locus principales y en adultos mayores son debidas a causas poligénicas”. En conclusión, siguiendo la teoría genética de las infecciones, cualquier infección primaria severa en la infancia puede deberse a defectos heredados en el sistema inmunitario, es decir, a una IDP [10].

La MSMD es una IDP de las consideradas “atípicas”, causada por defectos genéticos en la vía IL-12/IFN-γ; confiere predisposición a infecciones causadas principalmente por micobacterias poco virulentas, como son las micobacterias no tuberculosas y la cepa vacunal BCG (bacilos vivos atenuados de una cepa de *Mycobacterium bovis*), pero también por *Mycobacterium tuberculosis* [4,5,11]; más raramente, se han reportado también infecciones graves por otras bacterias intracelulares, hongos y parásitos (como la leishmaniasis [12,13]) que debutan habitualmente en la infancia [4]. Se considera hoy en día una enfermedad de base mendeliana (formas autosómicas recesivas, dominantes o ligadas al cromosoma X), en la cual se conocen diez defectos genéticos: *IFNGR1*, *IFNGR2*, *STAT1*, *IRF8*, *CYBB*, *IL12B*, *IL12RB1*, *NEMO*, *ISG15* [4] y *TYK2* [14]. Dichos defectos genéticos tienen en común un fallo en la inmunidad mediada por IFN-γ, ya sea completo o parcial según si la mutación es deletérea o hipomórfica, respectivamente.

La gravedad de las manifestaciones clínicas en los pacientes con MSMD depende en gran parte del genotipo: los defectos completos de IL-12 y su receptor y los defectos parciales del receptor de IFN-γ habitualmente predisponen a infecciones menos graves en comparación con los defectos completos del receptor de IFN-γ que predisponen a infecciones devastadoras a edades tempranas. Dentro del mismo genotipo, existe igualmente gran variabilidad en la expresividad clínica: pacientes con el mismo defecto genético pueden tener formas clínicas con severidad dispar, como ha podido objetivarse en amplias series de pacientes con los defectos de IL-12Rβ1 [15] y de IFN-γR1 [16], en los que aparecen infecciones recurrentes junto a asintomáticas, incluso dentro de la misma familia. Ello sugiere que mecanismos patogénicos adicionales, potencialmente determinados por factores ambientales, pueden determinar la presentación de la enfermedad, y la forma en que ésta progresa [4,5].

El estudio de posibles defectos causantes de MSMD se debe realizar en niños o adultos que desarrollen infecciones severas/diseminadas por micobacterias o *Salmonella* sin otras alteraciones hemato-inmunológicas, infección por el virus de la inmunodeficiencia humana o exposición a fármacos inmunosupresores (incluyendo los fármacos biológicos anti-TNF-α). El diagnóstico de la MSMD se basa en la realización de pruebas funcionales sobre la vía IL-12/IFN-γ, en concreto, la medición de la producción de IFN-γ e IL-12 tras estimulación de sangre total con BCG (+/- IL-12 e IFN-γ), junto a la medición por citometría de flujo de la expresión del receptor de IL-12 (IL-12Rβ1) y de IFN-γ (IFN-γR1 e IFN-γR2), así como la fosforilación de STAT1 en respuesta a IFN-γ, seguido del estudio genético dirigido [4,17–19]. Con el desarrollo de las nuevas tecnologías para el estudio genético, el algoritmo diagnóstico ha variado en algunos centros, de manera que la realización de estudios de secuenciación masiva es el primer paso para luego confirmar los resultados mediante pruebas funcionales. El abordaje terapéutico de

los pacientes depende del defecto genético: por ejemplo, en los defectos completos que afecten al receptor de IFN-γ, la administración de IFN-γ exógeno no será eficaz y serán candidatos a trasplante de progenitores hematopoyéticos [20]; en cambio, el resto de formas de MSMD se benefician del tratamiento con IFN-γ exógeno. La identificación de los pacientes afectos de MSMD, con la consiguiente indicación de un tratamiento específico marcarán el pronóstico de los pacientes.

2.1.3.Inmunidad a infecciones intramacrofágicas

Dado que los macrófagos forman parte de la primera línea de defensa contra los patógenos, las infecciones por microorganismos intramacrofágicos representan un reto para el sistema inmunitario. Comúnmente, estos patógenos han desarrollado estrategias para evadir los mecanismos innatos para su destrucción, por lo que la cooperación entre el sistema inmunitario adaptativo y el innato es imprescindible. Esta colaboración se da principalmente a través de la vía de IL-12/IFN-γ. En resumen, tras la fagocitosis de la micobacteria o patógeno intramacrofágico se activa la célula fagocítica, que producirá IL-12p70 e ISG15. Estas citocinas son detectadas por las células T y *natural killer* que, a su vez, se van a activar y a producir IFN-γ causando la sobre activación de las células fagocíticas induciendo la producción de TNF-α, IL-12p70 y la activación de la respuesta oxidativa que permitirá la destrucción de los patógenos. De esta manera se crea un círculo de retroalimentación positiva que es necesario para el control de la infección [21–25].

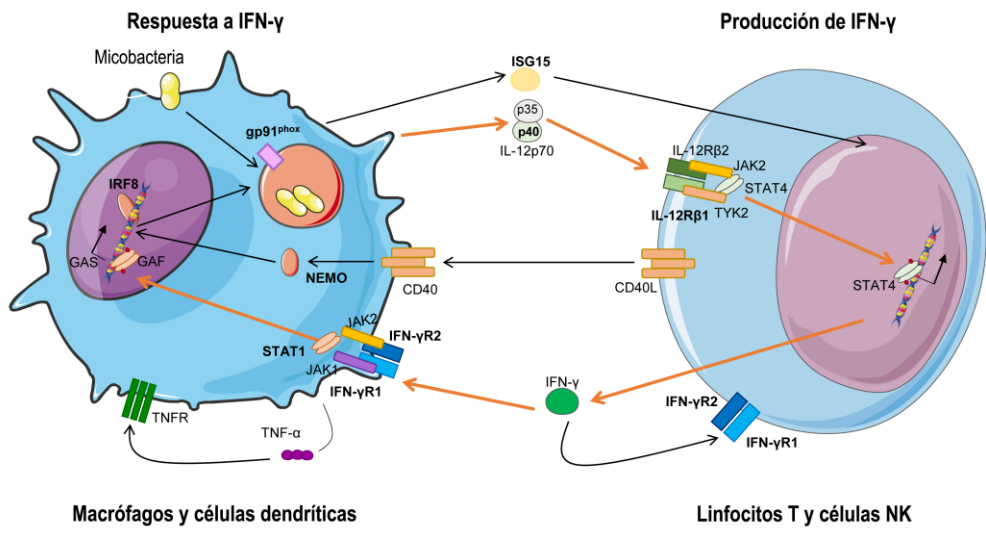

Figura 1. Esquema de la vía de IL-12/IFN-γ en respuesta a micobacterias

El mecanismo de señalización principal de IL-12 e IFN-γ se basa en eje *Janus associated kinase* (JAK) – *signal transducers and activation of transcription* (STAT). Este eje es usado por diversas citocinas que comparten el mecanismo de acción. Existen 4 miembros de la familia de JAK (JAK1, JAK2, JAK2 y TYK2) y 7 miembros de la familia de STAT (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6). Las moléculas JAK se encuentran asociadas con las cadenas de los receptores de citocinas. Tras la unión de la citocina con su receptor hay una agregación del receptor (o de sus cadenas) que hace que las moléculas de JAK se agreguen también. Esta

proximidad física activa a sus dominios quinasa, de manera que se da fosforilación de los receptores y fosforilación de las JAKs. Estas fosforilaciones van a crear sitios de unión para los STATs que, a su vez, serán también fosforilados y dimerizarán formando homo o heterodímeros para migrar al núcleo y, allí, realizar su función como factores de transcripción [26,27]. La señalización de IFN-γ se da tras la agregación de sus dos receptores, IFN-γR1 e IFN-γR2, que se encuentran asociados a JAK1 y JAK2 respectivamente; similarmente, la IL-12 se une a su receptor formado por las cadenas IL-12Rβ1 e IL-12Rβ2 que se encuentran asociadas a TYK2 y JAK2. La agregación de los receptores y la fosforilación de los adaptadores JAK creará un lugar de unión para STAT1 en el caso del IFN-γ y para STAT4 en el caso de la IL-12p70 [28–33]

La tuberculosis es la manifestación clínica de la infección por *Mycobacterium tuberculosis*. Esta enfermedad es un problema global y una de las primeras causas de muerte en el mundo, sobre todo en países empobrecidos; de hecho, un 20% de los nuevos casos se pueden atribuir a la malnutrición [34]. La inmunidad frente a la tuberculosis depende principalmente en la vía de IL-12/IFN-γ con un gran papel del TNF-α. La respuesta inmunitaria es compleja y lleva normalmente al control de la infección, pero muy raramente da lugar a una inmunidad “esterilizante” [35–37]. Pese a su potencial patogénico, solo aproximadamente un 5% de los individuos infectados por *Mycobacterium tuberculosis* va a desarrollar tuberculosis primaria (con formas extrapulmonares, principalmente en la infancia) y, del resto, un 5% va a desarrollar tuberculosis secundaria (forma pulmonar “clásica” en adultos) [11,38]. A parte de la malnutrición o la co-infección con el virus de la inmunodeficiencia humana, existen IDPs que confieren susceptibilidad aumentada a este patógeno como son la inmunodeficiencia combinada severa, la enfermedad granulomatosa crónica y los defectos autosómicos dominantes de GATA2 entre otros. Además, se han descrito 26 pacientes con mutaciones en genes de la vía IL-12/IFN-γ causantes de MSMD [4,11,39–43].

La leishmaniasis es una enfermedad parasitaria que se da en las zonas tropicales, subtropicales y en el área del Mediterráneo, siendo endémica en España. La organización mundial de la salud la ha catalogado como una enfermedad “descuidada”, afectando principalmente en zonas en situación de pobreza. Se transmite tras la picada de flebótomos hembra y tiene un ciclo de vida complejo con forma de promastigota en el insecto y de amastigota en el huésped humano [44–46]. Hay cuatro formas clínicas de leishmaniasis, la cutánea, la cutánea difusa, la muco-cutánea y la leishmaniasis visceral. La leishmaniasis visceral, también conocida como Kala-Azar, es una forma grave de la infección por *Leishmania spp* que, sin tratamiento, es letal. La respuesta inmunitaria a *Leishmania* es, como en el caso de la tuberculosis, dependiente de la vía de IL-12/IFN-γ para conseguir la activación del macrófago y, así, la destrucción del patógeno. Dada la casuística de la enfermedad, se han descrito sobre todo causas secundarias de susceptibilidad a leishmaniasis; además, se han descrito 12 pacientes con IDP asociada a leishmaniasis, de los cuales 5 tenían mutaciones en genes causantes de MSMD [12,42,47–49]

2.1.4.Tratamiento con fármacos biológicos anti-TNF-α

La enfermedad inflamatoria intestinal (EII), mayoritariamente representada por la enfermedad de Crohn y la colitis ulcerosa, es potencialmente invalidante y se da mayoritariamente en mujeres en edad fértil. Al contrario que otras enfermedades inflamatorias, la EII no remite durante el embarazo; de hecho, puede empeorar, siendo la reactivación de la

enfermedad un riesgo tanto para el feto en desarrollo como para la madre. El uso de fármacos biológicos ha proporcionado un gran avance en el tratamiento de los pacientes afectos. En concreto, en la EII se emplean fármacos anti-TNF-α (infliximab, adalimumab golimumab y certolizumab pegol) [50–53], una molécula que participa en diversos procesos, incluyendo la inflamación, la inmunidad frente patógenos intracelulares y el desarrollo del sistema inmunitario durante la gestación [54].

Por su rol en la inmunidad frente a patógenos intracelulares, los pacientes adultos tratados con fármacos biológicos anti-TNF-α tienen un riesgo infeccioso aumentado frente a estos patógenos, incluyendo la leishmaniasis [55,56] y, como elemento principal, la tuberculosis [8,57]. En el caso de la tuberculosis, se observa la reactivación de infecciones latentes con formas extrapulmonares que recuerdan a las infecciones observadas en niños con MSMD; este hecho hace que antes de administrar un tratamiento biológico anti-TNF-α se deba contemplar la posibilidad de la presencia de tuberculosis latente, que deberá ser tratada antes de empezar el tratamiento biológico [58,59]. El TNF-α es una molécula clave para la inmunidad frente a tuberculosis, tanto en su acción sinérgica con el IFN-γ para la estimulación de la respuesta oxidativa en los macrófagos como el reclutamiento de células T, la activación de las células T citotóxicas y la promoción de la maduración del monocito, siendo crucial para la formación y el mantenimiento del granuloma [35,58,59].

Los AcMos anti-TNF-α más usados hasta el momento en EII, adalimumab e infliximab, son AcMos IgG que atraviesan la barrera placentaria sobre todo a partir de la semana 30 de gestación, existiendo niveles detectables en el suero del niño hasta 6-7 meses después del nacimiento [60]. En los casos en que las pacientes con EII no pueden suspender el tratamiento durante la gestación, se ha observado que estos fármacos no tienen un efecto teratogénico. Sin embargo, no se conoce el alcance de los efectos sobre el sistema inmunitario en desarrollo para poder determinar el riesgo infeccioso real del lactante y la necesidad de tomar medidas específicas al respecto [61]. Por ello, existe gran controversia sobre la necesidad o no de interrumpir dicho tratamiento en caso de embarazo [62,63]. Los efectos a largo plazo de la exposición a anti-TNF-α del sistema inmunológico en desarrollo son todavía desconocidos [54,64]. En base a datos limitados provenientes de casos reportados, los bebés que nacen con niveles detectables de anticuerpos anti-TNF-α no parecen tener un mayor riesgo de infecciones en su primer año de vida y tienen una respuesta normal a las vacunas inactivadas [61]. Sin embargo, un caso mortal de infección diseminada por BCG fue reportada en un niño cuya madre había sido tratada con infliximab durante el embarazo y que recibió la vacuna BCG a los 3 meses de edad [65].

2.1.5.El sistema inmunitario en el neonato

El sistema inmunitario tiene un papel crucial en la transición del feto entre el útero materno, estéril y alogénico, y el mundo exterior. Al nacer, especialmente tras el parto vaginal, el neonato es masivamente colonizado por diferentes microorganismos. La mayoría de éstos serán no-patogénicos, pero algunos podrán ser dañinos. Por esta razón, es necesario un balance entre la respuesta antimicrobiana hacia microorganismos patogénicos y la tolerancia para los nuevos antígenos inocuos. Los mecanismos de control del sistema inmunitario neonatal incluyen una tendencia hacia la tolerancia y la baja inflamación, incluyendo la producción de péptidos

antimicrobianos no inflamatorios, la transferencia transplacentaria de anticuerpos maternos, un sesgo de la inmunidad adaptativa hacia células T colaboradoras tipo 2 a expensas de las células T colaboradoras tipo 1, baja respuesta a receptores tipo toll, entre otros mecanismos. Con esto, las infecciones intramacrofágicas en edad neonatal son especialmente peligrosas, destacando especialmente los casos de *Listeria monocytogenes*, *Salmonella spp*. y *Mycobacterium spp.* [66–70].

El establecimiento de la tolerancia periférica necesita diferentes estrategias reguladoras o supresoras. En los últimos años se ha ido desvelando el papel regulador de las células B en humanos [71,72]. Las células B reguladoras en humanos no tienen un fenotipo único definido, si no que se han descrito diferentes poblaciones con actividad reguladora, siendo las células $CD24^{hi}CD38^{hi}$ las más estudiadas, también conocidas como células B reguladoras inmaduras. Estas células hacen su función reguladora mediante la producción de IL-10 y el contacto directo entre células principalmente; de esta manera, pueden bloquear la diferenciación de las células Th1 y Th17 y promover la diferenciación de las células T reguladoras [73–76]. Se ha visto la importancia de estas células en diferentes situaciones como las infecciones crónicas, la autoinmunidad y, recientemente, sobre el embarazo. Se ha observado que, tanto en modelos murinos como en humanos, estas células están reguladas por las hormonas del embarazo y que son claves para el establecimiento y el mantenimiento del embarazo [77].

2.2.Hipótesis y objetivos:

Esta tesis se enmarca en el concepto que la vía IL-12/IFN-γ es fundamental para el control de las infecciones intracelulares. Con esto, disfunciones, heredadas o adquiridas, en este eje pueden ser detectadas en condiciones clínicas no exploradas en la actualidad. Concretamente, hipotetizamos que pacientes con infecciones severas/extrapulmonares por *Mycobacterium tuberculosis* o con leishmaniasis visceral tienen una disfunción primaria de la vía de IL-12/IFN-γ, de manera que el estudio de estos pacientes va a llevar a la detección de nuevos casos de MSMD, en los que se podrá realizar un manejo específico. Además, que la exposición a anticuerpos anti-TNF-α durante el embarazo afecta al desarrollo del sistema inmunitario, pudiendo generar una inmunodeficiencia secundaria a esta exposición que incluiría la disfunción del eje de IL-12/IFN-γ.

Con esto, el objetivo principal de esta tesis doctoral: entender los defectos de la vía IL-12-IFN-γ causados por defectos heredados y secundarios a la exposición a anti-TNF-α durante el embarazo. Del objetivo general se derivan los siguientes objetivos específicos:

Objetivo 1. Elucidar si los pacientes con infecciones intramacrofágicas severas como la tuberculosis severa/extrapulmonar o la leishmaniasis visceral tienen defectos en la vía IL-12/IFN-

Objetivo 2. Definir el efecto del tratamiento de la madre con enfermedad inflamatoria intestinal con anti-TNF-α durante el embarazo en el desarrollo del sistema inmune del niño expuesto.

Objetivo 2.1. Determinar si la vía IL-12/IFN-γ se encuentra afectada en los niños expuestos a anti-TNF-α

Objetivo 2.2. Caracterizar las diferentes poblaciones linfocitarias en los niños expuestos.

Objetivo 3: Establecer valores de normalidad para las diferentes poblaciones linfocitarias en sangre de cordón umbilical, especialmente en el subgrupo de células B reguladoras y la integridad del eje IL-12/IFN-γ

2.3. Resultados

2.3.1.Estudio del eje IL-12/IFN-γ en pacientes con tuberculosis severa/extrapulmonar y leishmaniasis visceral

Para el reclutamiento de pacientes hemos establecido colaboraciones con diferentes centros autonómicos, estatales e internacionales incluyendo el Hospital Sant Joan de Déu y el Hospital Vall d'Hebron en Barcelona, el Hospital Joan XXIII (Tarragona), el Hospital General (Granollers), el Hospital La Fe (Valencia), el Hospital Gregorio Marañón y 12 de Octubre en Madrid, el Hospital Son Espases (Mallorca) y el Instituto Nacional del Niño (Lima, Perú). Hemos recogido 25 pacientes con infección por *Mycobacterium tuberculosis* y 23 con leishmaniasis visceral, así como 41 controles sanos, de los cuales 23 están relacionados con los pacientes (mayoritariamente padres) y 18 no están relacionados. Uno de los pacientes de la cohorte de tuberculosis tuvo que ser excluido del estudio dado que primeramente se catalogó como una infección por *Mtb* para luego confirmarse que se trataba de una infección por BCG.

Los pacientes con infección por *Mycobacterium tuberculosis* presentaron una mayor variación étnica mientras que los pacientes con leishmaniasis visceral eran mayoritariamente de origen caucásico. Cabe destacar que los pacientes con leishmaniasis visceral eran más pequeños en el momento de la infección (mediana: 1,58 años, rango: 0.25 a 8 años en leishmaniasis visceral y mediana: 3 años, rango: 0,47 a 16 años en *Mycobacterium tuberculosis*). Por otro lado, estas diferencias desaparecieron cuando se compara la edad en el momento del estudio (mediana: 7,42 años, rango: 0,42 a 12,54 años en leishmaniasis visceral y mediana: 7,28 años, rango: 0,62 a 18 años en *Mycobacterium tuberculosis*). En los pacientes con infección extrapulmonar por *Mycobacterium tuberculosis*, la localización de la infección fue mayoritariamente diseminada (33%), con (12%) o sin (21%) meningitis, adenopática (21%) o meníngea (17%). Dos de las familias reportaron consanguineidad. Se descartó la presencia de inmunodeficiencia combinada severa y enfermedad granulomatosa crónica mediante el estudio de las poblaciones celulares, proliferación en respuesta a mitógenos y respuesta oxidativa. También resultaron sin alteraciones evidentes las pruebas citométricas específicas para la MSMD que incluyeron la detección de IFN-γR1 e IFN-γR2 en monocitos y de IL-12Rβ1 en linfocitos activados, así como la fosforilación de STAT1 en respuesta a IFN-γ.

La técnica *gold-standard* para el diagnóstico de pacientes con MSMD es la detección de la producción de citocinas tras el cultivo de sangre entera con BCG con o sin co-estimulación con IL-12p70 o IFN-γ exógeno. Esta técnica permite observar cómo es la respuesta a micobacterias en el paciente ex-vivo, siendo muy útil para la detección de defectos completos. Sin embargo, existe una gran variabilidad en controles sanos que dificulta la detección de defectos parciales. Observamos esta gran variabilidad en la producción de citocinas tanto en los controles sanos relacionados como no-relacionados con los pacientes. Cabe destacar que cuando se realizaron

ratios de co-estimulación (producción de citocinas con "BCG + co-estímulo" / "BCG") se observó una disminución del coeficiente de variación en los controles sanos no-relacionados que no se observó en la cohorte de controles relacionados.

Se observó un patrón de producción de citocinas diferente entre los dos grupos de controles, habiendo una menor producción de IFN-γ y mayor producción de IL-6, IL-10 y TNF-α en los controles relacionados en los que, además, observamos una disminución de la ratio de co-estimulación de IL-6, y TNF-α en respuesta a IL-12 y de IL-12p70 en respuesta a IFN-γ. En los pacientes con infección por *Mycobacterium tuberculosis* o leishmaniasis visceral no detectamos defectos completos en la vía de IL-12/IFN-γ, pero si detectamos una función alterada de esta vía. Los pacientes con infecciones por *Mycobacterium tuberculosis* destacaron por una respuesta a IFN-γ alterada, acompañada de un aumento de la producción de IL-10 y TNF-α tras la estimulación con BCG. Por el otro lado, los pacientes con infecciones por *Leishmania* se caracterizaron por la baja producción de IFN-γ, en respuesta a BCG. Cabe destacar que todos los grupos presentaron una ratio de IFN-γ/IL-10 en respuesta a BCG disminuida en relación con los controles no relacionados, especialmente evidente en los pacientes con leishmaniasis visceral. Con estos datos, concluimos que tanto los pacientes con tuberculosis severa/extrapulmonar como los pacientes con leishmaniasis visceral tienen alterada la vía de IL-12/IFN-γ.

El caso sacado de la cohorte de tuberculosis por un error en el diagnóstico microbiológico es de especial interés. Se trató de una niña de 6 años peruana diagnosticada con tuberculosis recurrente, severa y diseminada. Con esta evolución se decidió estudiar la integridad de la vía IL-12/IFN-γ, de manera que se nos remitió el caso. Al realizar las pruebas funcionales y genéticas diagnosticamos un defecto completo de IL-12Rβ1 caracterizado por la ausencia de la expresión de IL-12Rβ1, así como de fosforilación de STAT-4 tras la estimulación con IL-12p70. La producción de IFN-γ tras la estimulación con BCG fue muy reducida y no se rescató con la co-estimulación con IL-12p70. Los estudios genéticos confirmaron una mutación en *IL12RB1* en p. (Arg211*; c.631C>T), siendo la primera paciente con diagnóstico genético de MSMD en Perú. Al tiempo del diagnóstico la paciente empeoró y fue trasladada al *National Institute of Health* en Estados Unidos para su tratamiento con IFN-γ exógeno. Una vez allí los estudios microbiológicos confirmaron que había habido un error en el diagnóstico inicial, siendo la infección derivada de la vacunación por BCG recibida en la infancia. Con esto se optimizó el tratamiento micobactericida acompañado de IFN-γ exógeno. La paciente se encuentra ahora estable y sin clínica infecciosa.

2.3.2.Estudio del efecto de la exposición a fármacos anti-TNF-α en el desarrollo del sistema inmunitario del niño expuesto.

Para el estudio del efecto de los fármacos anti-TNF-α administrado a mujeres con EII embrazadas sobre el desarrollo del sistema inmunitario del niño expuesto se enrolaron 7 pacientes de las 8 pacientes que cumplieron los criterios de inclusión. De éstas, 2 dejaron el seguimiento analítico después del nacimiento, 4 lo han completado hasta los 12 meses y 1 hasta los 18 meses. El tamaño de la cohorte de debe a que, en el momento de realización del estudio, solo la unidad de EII del Hospital Clínic de Barcelona mantenía el tratamiento durante todo el embarazo.

Hemos encontrado niveles de fármaco detectables en todos los pacientes al nacimiento (media 11,42 µg/ml, rango: 5,87 – 42,52 µg/ml), siendo el *clearance* de éstos el propio que corresponde a la vida media esperada de las inmunoglobulinas. Pudimos detectar fármaco hasta los 6 meses. No observamos cambios en las principales poblaciones leucocitarias y linfocitarias. De todas maneras, al nacimiento encontramos un sistema inmunitario inmaduro (subpoblaciones linfocitarias B y T) comparado con controles sanos no expuestos a los fármacos. Al año de vida las subpoblaciones linfocitarias B y T se encontraron dentro de los rangos definidos para la edad. La inmadurez inicial no tiene consecuencias a nivel de producción de inmunoglobulinas o de respuesta a vacunas. Por otro lado, observamos una disminución de las células T reguladoras que correlacionó de manera inversa con el nivel valle de fármaco anti-TNF-α de la madre durante el embarazo. Este nivel de células T reguladoras también correlacionó inversamente con la proliferación de las células T a un estímulo débil. De esta manera, a más alto es el nivel de fármaco valle de la madre durante el embarazo, menos células T reguladoras se encuentran en el neonato y mayor es la proliferación de sus células T a un estímulo débil.

La respuesta a micobacterias fue menor en los recién nacidos expuestos al fármaco durante el embarazo, con una reducción de la expresión de la molécula de activación temprana CD69 y de HLA-DR, recuperadas parcialmente después de lavar el fármaco de la sangre para el cultivo. Además, la ratio de estimulación (RE) para IL-17 y TNF-α se encontró disminuida, así como para la producción de IL-1β, aunque no alcanzó significancia estadística. De igual manera la RE de IL-6 y de IL-1RA se encontró aumentada sin obtener significancia estadística. Observamos que, con el paso del tiempo, y coincidiendo con la desaparición del fármaco en sangre existió un aumento en la producción de IFN-γ, IL-12p70 y TNF-α, mientras que la producción de IL-6 se mantuvo estable.

Clínicamente, ninguno de los niños sufrió infecciones severas, solo uno de los niños expuestos (en este caso a adalimumab + azatioprina) tuvo un aumento de las infecciones entre los 6 y los 12 meses. 4 de los 7 niños expuestos desarrollaron dermatitis atópica. Con todo, podemos concluir que la exposición a fármacos anti-TNF-α durante el embarazo no tiene consecuencias clínicas severas durante el primer año de vida, pero que es necesario ampliar el número de niños expuestos con estudios inmunológicos y ampliar su seguimiento clínico para otros defectos. Es importante sobre todo el control de estos pacientes en países endémicos para micobacterias y evitar la su vacunación con BCG, al menos en los primeros meses de vida.

2.3.3.Estudio de las células B reguladoras y la vía IL-12/IFN-γ en neonatos sanos

Dado que el TNF-α es un factor clave para el desarrollo de las células B, decidimos incluir el estudio de las células B reguladoras que, en el momento de realización del estudio, no se había descrito en el neonato. Estudiamos las células B reguladoras definidas como $CD19^{+}CD24^{hi}CD38^{hi}$ en sangre de cordón umbilical de neonatos sanos (hUCB) y en sangre periférica de individuos adultos sanos (hAPB). La frecuencia de células Breg estaba incrementada en hUCB en comparación con hAPB (34,39% vs. 9,49%; p=0,0002), especialmente en la subpoblación B de células de la zona marginal circulantes, donde se observó la mayor diferencia entre hUCB y hAPB (60,80% vs. 4,94%). Las células B reguladoras en hUCB fueron capaces de producir IL-10 e inhibir la producción de IFN-γ (1,63 vs. 0,95 RE p=0.004) e IL-4 (1,66 vs. 0,86 RE; p=0,02) por las células T. Las células Breg de hUCB presentaron el siguiente fenotipo:

$IgM^{hi}IgD^{hi}CD5^{+}CD10^{+}CD27^{-}$, siendo similar al descrito en hAPB con algunas diferencias. Se encontró un aumento de la concentración por célula de IgM y un descenso de la expresión de los marcadores CD22 y CD73. Nuestro trabajo ha caracterizado la frecuencia, fenotipo y función de las células Breg en hUCB, lo que puede contribuir en el conocimiento de la tolerancia inmunitaria durante el embarazo, abriendo una puerta a nuevos acercamientos a las enfermedades inmuno-mediadas en el feto y el recién nacido.

Además de las células B reguladoras estudiamos el estado de la vía de IL-12/IFN-γ en neonatos. Observamos que, en general, la respuesta tras el estímulo con BCG estaba disminuida, concretamente observamos una menor producción de IFN-γ ($p=0.001$) y de IL-6 ($p=0.005$), así como una menos frecuencia de células $CD69^{+}$ y un nivel de CD69 por célula más bajo. Por otro lado, la respuesta a IL-12 e IFN-γ no se encontraba disminuida. Al estudiar la relación entre la producción de citocinas y las células B reguladoras descubrimos una asociación entre la frecuencia de células B reguladoras y el nivel de producción de IFN-γ tras la estimulación con BCG.

2.4.Discusión

Este trabajo aborda el estudio de la integridad de la vía IL-12/IFN-γ en dos situaciones de posible inmunodeficiencia: por un lado, pacientes con sospecha de inmunodeficiencia primaria por el tipo y gravedad de las infecciones que padecen y, por el otro, el estudio de una posible inmunodeficiencia secundaria en el neonato, derivada de la exposición a un fármaco biológico anti-TNF-α durante el embarazo. Para desentrañar el efecto de la exposición a fármacos anti-TNF-α durante el embarazo era necesario establecer valores de normalidad en un grupo control, especialmente respecto a la vía IL-12/IFN-γ. Además, dado el papel del TNF-α en el desarrollo de las células B y del papel de las células B reguladoras en salud y enfermedad, decidimos incluir su estudio en el neonato.

2.4.1.Fortalezas y limitaciones:

Diferentes fortalezas y limitaciones condicionan la interpretación de este trabajo, incluyendo el tamaño de las cohortes, los grupos controles y la variación intrínseca de la producción de citocinas. Dadas las características de las cohortes estudiadas, el tamaño muestral es reducido en todos los casos, especialmente en el estudio del efecto de los fármacos anti-TNF-α en niños de madres con EII. Aunque los resultados deben ser interpretados teniendo en cuenta esta limitación, valoramos que provienen de cohortes altamente valiosas y son robustos.

Por otro lado, el grupo control ideal pareado por edad no se pudo reclutar debido a limitaciones éticas. Por tanto, en el estudio de pacientes pediátricos con tuberculosis severa/extrapulmonar o leishmaniasis visceral, éstos se compararon con controles adultos. Para el estudio del efecto de los inhibidores del TNF-α en el neonato expuesto se compararon los valores obtenidos en sangre de cordón con una cohorte de neonatos nacidos de madres sanas, dado que virtualmente todas las madres con EII moderada a severa reciben tratamientos inmunomoduladores que nos impedirían evaluar el efecto del fármaco separadamente de la patología materna. Al no poder disponer de una cohorte control pediátrica, los resultados del estudio evolutivo se compararon con los valores de normalidad publicados o de manera relativa

en la evolución cuando no existían valores de normalidad establecidos. Cabe destacar la valía de la cohorte control de sangre de cordón de madres sanas, que nos permitió establecer valores de normalidad y, además, el estudio de la población de células B reguladoras que, en el momento del estudio no se había explorado en neonatos. Desafortunadamente, otro grupo trabajando en trasplante de progenitores hematopoyéticos de sangre de cordón publicó por primera vez la presencia de la población $CD24^{hi}CD38^{hi}$ en sangre de cordón umbilical 9 meses antes [78].

El cultivo sangre entera del paciente con BCG, en presencia o no de co-estímulos, es una de las mejores simulaciones de lo que ocurre en el paciente después de una infección intramacrofágica. Es considerado *gold standard* para el diagnóstico de la MSMD aunque presenta una gran variabilidad interindividual como demuestran los datos de los controles sanos, y como también han descrito otros grupos [15,18,42]. Esta variabilidad hace que mediante esta técnica sea complejo distinguir los defectos parciales de los controles sanos. Mediante el uso de ratios de co-estimulación se puede reducir esta variabilidad en controles sanos no relacionados con los pacientes. Hemos observado que esta disminución de la variabilidad no se observa en controles sanos relacionados con los pacientes. Creemos que al estar relacionados con un grupo niños con posibles defectos parciales de respuesta o producción de IFN-γ, la variabilidad se mantiene aun realizando ratios de respuesta a los co-estímulos, reforzando la hipótesis de que existe un componente familiar/genético en la susceptibilidad a dichas infecciones.

2.4.2.Estudio de la vía de IL-12/IFN-γ en pacientes con tuberculosis severa/extrapulmonar y leishmaniasis visceral

Siguiendo la teoría genética de las infecciones, que postula que infecciones severas en la infancia, en pacientes sin predisposición adquirida, se deben a defectos heredados en un único gen [79], hemos estudiado dos cohortes de pacientes con infecciones no típicas en la MSMD: pacientes con infección extrapulmonar por *Mycobacterium tuberculosis* y pacientes con leishmaniasis visceral como única infección. Estas infecciones son más graves de lo común en niños con un estado de salud general normal, sugiriendo un defecto en el control de estos patógenos.

Ya en controles sanos relacionados con los pacientes observamos un patrón de producción de citocinas alterado, sugiriendo una afectación de la vía. Los pacientes con infección tuberculosa presentaron una producción de IFN-γ comparable a los controles sanos pero una respuesta a éste disminuida, incluyendo la producción de IL-12p70, TNF-α e IL-1β. Por otro lado, la producción de IFN-γ estaba disminuida en los pacientes con infección por leishmaniasis visceral comparado con los demás grupos estudiados. Se conoce que durante la infección por *Leishmania*, las células de los pacientes tienen una menor capacidad de producción de IFN-γ que se recuera tras la desaparición de la infección [80,81], por lo que dado que todas las muestras se procesaron al menos un mes después de la infección no debería afectar a los resultados. Por otro lado, la teoría de la inmunidad innata entrenada sostiene que, al nacer, la inmunidad innata es inmadura y que va adquiriendo la capacidad para generar respuestas inflamatorias con el tiempo y la exposición a antígenos debido a cambios principalmente epigenéticos [82–84], con esto, podría ser posible que la infección con leishmaniasis en edades muy tempranas previniera estas modificaciones epigenéticas o que fuera debido a errores heredados. El estudio genético en busca de defectos parciales o somáticos en estos pacientes, así como una aproximación

epigenética, podría dilucidar el origen de la susceptibilidad de estos pacientes y, así, optimizar su tratamiento y manejo.

Hasta el momento no hay publicado ningún trabajo en que se estudie la integridad de la vía IL-12/IFN-γ en busca de defectos en pacientes con leishmaniasis visceral; sin embargo, hay un estudio describiendo que 2 de 50 niños estudiados presentaron mutaciones en *IL-12RB1*en el pacientes con infecciones severas por *Mycobacterium tuberculosis* en una zona endémica de tuberculosis con alta consanguinidad [48]*;* por otro lado, otro estudio de genes causantes de MSMD en pacientes adultos y pediátricos con infecciones severas por *Mtb* realizado en una zona de baja consanguinidad no detectó ninguna mutación causante [85]. En el estudio presentado no hemos detectado ningún paciente con un defecto completo, lo que sugiere que defectos completos en genes conocidos causantes de MSMD no deberían ser sospechados a priori en pacientes con estas infecciones en zonas de baja consanguineidad; de todas maneras, es necesario seguir estudiando la alteración observada en la integridad de la vía de IL-12/IFN-γ.

La baja frecuencia de pacientes diagnosticados de MSMD en nuestro medio se puede deber a la baja consanguinidad presente y, también, al hecho que no exista la vacunación con BCG, el signo de alarma principal de esta entidad. El desarrollo de enfermedad infecciosa tras la vacunación con BCG es muy raro [86] y es signo de inmunodeficiencia [4], ya sea adquirida o congénita. Al derivar de *Mycobacterium bovis*, la diferenciación entre una infección por *Mycobacterium tuberculosis* y BCG es compleja y puede llevar a confusión en áreas endémicas para *Mycobacterium tuberculosis* [86–88]. La correcta identificación del elemento infeccioso, así como la sospecha de inmunodeficiencia es básica para el correcto manejo del paciente. En el caso de la paciente diagnosticada de una deficiencia de IL-12Rβ1 no se sospechó la presencia de una inmunodeficiencia hasta la diseminación y recurrencia de la mal diagnosticada infección por *Mycobacterium tuberculosis*. Si la sospecha de inmunodeficiencia se hubiera realizado desde el principio, se hubiera podido aplicar el tratamiento adecuado y, posiblemente, haber reducido la severidad de la enfermedad.

2.4.3.El diagnóstico de la MSMD, de la investigación a la práctica clínica

El diagnóstico de la MSMD es complejo y se realiza en laboratorios de inmunología especializada. Gracias al desarrollo de la metodología necesaria para el estudio de la vía de IL-12/IFN-γ en pacientes con leishmaniasis visceral o tuberculosis severa/extrapulmonar hemos podido establecer la metodología necesaria para el diagnóstico de MSMD. Concretamente proponemos un algoritmo diagnóstico que incluye, tras la sospecha diagnóstica: 1) descartar otras inmunodeficiencias que, además de otras características, causan susceptibilidad a micobacterias, 2) determinar mediante citometría de flujo la presencia de los receptores de IFN-γ y el receptor de IL-12R así como la fosforilación de STAT1 en respuesta a IFN-γ para descartar de manera rápida los defectos completos de la vía más frecuentes, 3) estudios genéticos, o bien enfocados tras las pruebas citométricas o mediante secuenciación masiva (con el uso de paneles o secuenciación del exoma) y 4) si se ha encontrado una posible mutación causante de enfermedad se deberá realizar la validación funcional que suele ser realizada en el contexto de un laboratorio de investigación. El diagnóstico final de MSMD puede reducir la morbi-mortalidad de esta inmunodeficiencia, ya que condiciona el tratamiento y ayuda en el manejo paciente.

2.4.4.La exposición prenatal a fármacos anti-TNF-α tiene un impacto en la maduración del sistema inmunitario de los niños expuestos

Existe una proporción elevada de familias que escogen no tener hijos entre las pacientes con EII, principalmente por el miedo a la reactivación de la enfermedad o al efecto de los fármacos en el niño. En los niños expuestos a anti-TNF-α prenatalmente observamos que, como se había publicado anteriormente [89,90], existía presencia del fármaco en sangre detectable hasta, aproximadamente, los seis meses de vida. La exposición al fármaco afectó a la maduración del sistema inmunitario durante el embarazo, normalizándose a los 12 meses de vida, incluyendo una respuesta vacunal adecuada respecto a la edad. A los tres meses de edad, 3 de los 5 niños expuestos que seguían realizando seguimiento analítico desarrollaron neutropenia. Previamente se había asociado el uso de fármacos anti-TNF-α durante el embarazo con neutropenia severa en el neonato expuesto al nacimiento [91], por este motivo. Aunque en la cohorte incluida en el estudio ninguno de los niños desarrolló las infecciones cutáneas observadas previamente, creemos que el conteo de neutrófilos debería incluirse en el seguimiento de esta población.

Cabe destacar que observamos un descenso de células T reguladoras que no se recupera a los 12 meses. Se ha descrito en algunos trabajos que la exposición a fármacos anti-TNF-α aumenta la frecuencia de células T reguladoras en pacientes respondedores [92–94]. Una posible explicación de estas diferencias es que las células que aumentan en los pacientes adultos parecen ser células T reguladoras inducidas [93]. Dado que las células T reguladoras inducidas se diferencian tras un insulto antigénico, en el cordón umbilical esperaríamos que la mayoría de las células reguladoras sean naturales [95]. Además, el efecto del fármaco anti-TNF-α en el desarrollo de las células Treg inducidas es difícil de cuantificar dado que el nivel de fármaco presente en sangre disminuye de manera significativa a los 3 meses de edad. Es destacable en este contexto que observamos que 4 de los 7 niños estudiados presentaron atopia. Por estas razones creemos aconsejable realizar un seguimiento clínico de estos niños para valorar la aparición de alergias, atopia u otros eventos relacionados.

La exposición a fármacos anti-TNF-α durante todo el embarazo produce una disminución transitoria de la respuesta a micobacterias. En adultos, los tratamientos con fármacos biológicos son considerados causantes de fenotipos tipo inmunodeficiencia [96]; esto se puede aplicar también a los neonatos que, además, tienen una respuesta sesgada hacia las células T colaboradoras tipo 2 [97]. En adultos con enfermedad inflamatoria, estos fármacos producen disminución de la producción de IL-12 [98], así como un descenso en las células T CD8 productoras de IFN-γ y en las subpoblaciones Th1/Th17 [99]. Los resultados obtenidos en neonatos no corresponden totalmente con los observados en adultos, presumiblemente explicable por el hecho de que el sistema inmunitario del neonato y el del adulto con enfermedad inflamatoria son diferentes [70,97,100–103]. La respuesta disminuida a micobacterias al nacimiento es de especial importancia en el contexto de la vacunación con BCG.

Recientemente han aparecido en el mercado los biosimilares anti-TNF-α, de manera que se va a poder ampliar el tratamiento con este tipo de fármacos biológicos en países con menos recursos económicos, donde la vacunación con BCG está regulada justo al nacer. Hasta que se realicen más investigaciones, la vacunación con BCG está totalmente contraindicada en estos niños al menos hasta los 12 meses. Por otro lado, la muerte del neonato expuesto a anti-TNF-α

durante el embarazo y vacunado con BCG [104], así como la práctica en pacientes con EII tratados con fármacos inmunomoduladores [105], llevaron a la decisión de evitar las vacunas vivas en estos niños hasta, al menos, los 12 meses [89,106]. Tras observar que no hubo reacciones adversas tras la administración de la vacuna del rotavirus en 4 pacientes, la correcta maduración de las poblaciones linfocitarias, la proliferación en respuesta a mitógenos y la respuesta a vacunas, cumpliendo los criterios para poder administrar vacunas atenuadas en pacientes con inmunodeficiencia celular [107], especulamos que la política de vacunación de los niños expuestos a fármacos anti-TNF-α durante el embarazo podría ser revisada, siempre teniendo en cuenta la evitación de la vacunación con BCG u otras vacunas con microorganismos de vida intramacrofágica atenuados.

2.4.5. Las células B reguladoras pueden jugar un papel principal en la inmunidad neonatal

El sistema inmunitario se ha adaptado para mediar la transición entre el útero materno, estéril y alogénico, y el mundo exterior donde será masivamente colonizado por microorganismos y atacado por patógenos. En sangre de cordón de neonatos sanos, las células con fenotipo $CD19^{+}CD24^{hi}CD38^{hi}$ se encuentran en una mayor frecuencia y son capaces de producir IL-10 así como de inhibir la producción de citocinas inflamatorias como el IFN-γ con un fenotipo similar al de las células B reguladoras en el adulto. Cabe destacar que encontramos una asociación entre el nivel de células B reguladoras y la producción de IFN-γ tras la estimulación con BCG, estando ésta disminuida en los neonatos.

La inhibición de la producción de IFN-γ por las células B reguladoras no se da con las células “no B reguladoras” (subpoblación definida con los marcadores $CD24^{int}CD38^{int}$). En el otro estudio publicado estudiando las células B reguladoras en sangre de cordón ratificaron que eran capaces de inhibir la producción de IFN-γ por las células T, pero que la subpoblación de células no B reguladoras también era capaz de hacerlo [78], estas diferencias no son irreconciliables y se pueden deber a diferentes aproximaciones experimentales. La proporción de células B reguladoras entre la población de células circulantes de la zona marginal llama la atención. Cabe destacar que las células de la zona marginal se caracterizan por ser de rápida respuesta tras el insulto con bacterias encapsuladas, incluyendo la microbiota comensal [108–110]. El aumento de células con función reguladora dentro de esta población, así como la disminución de la molécula CD22, que podría resultar en una reducción del umbral de activación, podría ser uno de los mecanismos mediante los cuales el sistema inmunitario del neonato previene una respuesta inflamatoria exagerada en respuesta al gran rango de antígenos encontrados.

Además, observamos un aumento de la cantidad de IgM por célula en las células B reguladoras en cordón umbilical, que especulamos podría estar implicado en el mecanismo de acción de éstas. Los anticuerpos IgM anti-leucocitarios son definidos como anticuerpos “naturales”, que tienen capacidad de inhibir la activación de las células T y su quimiotaxis [111,112]. Estos anticuerpos se encuentran presentes al nacimiento [112] y son capaces de inhibir la producción de IFN-γ e IL-17 por las células T en respuesta a aloantígenos en ratón [111]. Especulamos que este puede ser un nuevo mecanismo independiente de IL-10 por el cual las células B reguladoras neonatales serían capaces de ejercer su función reguladora.

Las células B podrían ser un arma de doble filo en el trasplante de progenitores hematopoyéticos de sangre de cordón, por un lado, parece que favorecen la tolerancia [78] y, por otro lado, tras la observación de una respuesta disminuida al insulto con BCG en neonatos

y la correlación de la producción de IFN-γ con la frecuencia de células B reguladoras, hipotetizamos que también podrían estar implicadas en la aumentada susceptibilidad a infecciones observada en los pacientes trasplantados con sangre de cordón [113]. Esta asociación merece una investigación más exhaustiva para determinar el papel de estas células en el trasplante de células de cordón, así como para estudiar el papel de estas células en situaciones especiales del embarazo.

2.4.6.Implicaciones del trabajo y expectativas de futuro

El trabajo presentado es el resultado de un proyecto traslacional, por lo que los resultados obtenidos tienen implicaciones en la práctica clínica. La consecución de los diferentes objetivos ha generado más preguntas de las que ha resuelto.

Ninguno de los pacientes con algún episodio de leishmaniasis visceral o tuberculosis severa/extrapulmonar presentó defectos completos con las pruebas funcionales disponibles. De todas maneras, estos pacientes presentaron una alteración de la vía de IL-12/IFN-γ, por lo que se deberían estudiar, al menos, los genes conocidos con mutaciones causantes de MSMD. De todas maneras, existe un gran número de pacientes con cuadros compatibles con MSMD, sin defecto genético conocido aún tras la realización de estudios de secuenciación masiva, incluyendo el exoma [4]. Es probable que genes candidatos y vías cruciales para la inmunidad micobacterias aún no se hayan identificado; así como la existencia de otros mecanismos causantes de enfermedad todavía no explorados como las mutaciones somáticas o las variaciones epigenéticas. Complementariamente al estudio de mecanismos de enfermedad alternativos, la técnica *gold standard* actual tiene todavía rango de mejora, sobre todo en la variabilidad existente en los resultados obtenidos en controles sanos, el análisis de los resultados mediante técnicas bioinformáticas avanzadas podría ayudar a reducir esta variabilidad y, así, facilitar la interpretación de los resultados.

En el efecto de la exposición con fármacos anti-TNF-α sobre el sistema inmunitario del niño expuesto destaca el efecto sobre la frecuencia de células T reguladoras. Por esto, estudiar en profundidad los cambios que se producen en estas células, así como su funcionalidad sería de gran ayuda para poder entender los efectos y las consecuencias del uso de estos fármacos sobre éstas. En la misma línea, dado que existe una respuesta alterada a micobacterias que no se recupera totalmente tras limpiar el fármaco de la sangre para el cultivo, sería muy interesante estudiar la posibilidad que se produzcan cambios epigenéticos por la exposición al fármaco durante la fase final del desarrollo intra-uterino del sistema inmunitario que expliquen esta reducida respuesta a micobacterias. Dada la importancia de las condiciones maternas en el desarrollo del sistema inmunitario, creemos que es recomendable estudiar los efectos del fármaco en las diferentes enfermedades en que se quiera implementar su uso durante esta etapa.

Finalmente, la observación de una población expandida de células B reguladoras en sangre de cordón abre la puerta a ahondar en el estudio de estas células en diferentes situaciones, incluyendo la posibilidad de estudiar un nuevo mecanismo de acción mediante la producción de anticuerpos IgM anti-leucocitarios, el estudio de las células B reguladoras en diferentes condiciones como las infecciones neonatales y su importancia en el trasplante de células hematopoyéticas cordón umbilical, evaluando en qué casos podría ser beneficioso y en cuales podría ser perjudicial.

2.5.Conclusiones

1. No detectamos ningún caso de inmunodeficiencia primaria en los pacientes estudiados con leishmaniasis visceral o tuberculosis severa/extrapulmonar en nuestra área, incluyendo defectos T, enfermedad granulomatosa crónica y defectos completos de la vía IL-12/IFN-γ o MSMD.
2. Sin embargo, el eje IL-12/IFN-γ estaba parcialmente alterado en pacientes pediátricos con leishmaniasis visceral y con tuberculosis severa/extrapulmonar, sugiriendo una susceptibilidad intrínseca a infecciones intramacrofágicas. Para la definición de los mecanismos concretos de susceptibilidad son necesarias nuevas estrategias de estudio, incluyendo el uso de técnicas de secuenciación masiva y estudios epigenéticos.
3. El diagnóstico temprano de la MSMD, basado en el desarrollo de signos de alarma específicos y de la metodología diagnóstica de MSMD, es primordial ya que la confirmación genética e inmunológica de MSMD va a condicionar el tratamiento del paciente.
4. La exposición prenatal a anti-TNF-α, usado para el tratamiento de mujeres embarazadas con enfermedad inflamatoria intestinal, puede considerarse segura, sin eventos clínicos significantes
5. Sin embargo, cambios en el sistema inmunitario del neonato expuesto se han observado, especialmente teniendo en cuenta el eje IL-12/IFN-γ que se encuentra parcialmente defectuoso, posiblemente aumentando la susceptibilidad a micobacterias. Por este motivo, la vacunación con BCG debe ser evitada al nacimiento
6. Los niños expuestos a anti-TNF-α, usado para el tratamiento de mujeres embarazadas con enfermedad inflamatoria intestinal, deberían ser seguidos a largo término para controlar la aparición de eventos alérgicos, autoinmunes o autoinflamatorios.
7. Las células B reguladoras se encuentran expandidas en sangre de cordón de neonatos sanos, con una función regulatoria confirmada. Esta observación podría tener un rol importante explicando las particularidades tanto de la respuesta neonatal a infecciones y microbiota como del trasplante con sangre de cordón.

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