Universitat Autònoma de Barcelona

Towards a better diagnosis of mycobacterial infections IGRAs and beyond

Raquel Villar Hernández

Ph.D. Thesis

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A ella,

amante de flores y corazones

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Tuberculosis kills five thousand people every day. The social and economic impacts are devastating, including poverty, stigma and discrimination.

Dr. Tedros Adhanom Ghebreyesus, WHO General-Director.

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Summary

The genus *Mycobacterium* includes over 200 species, most of them found in the environment. This bacteria genus includes both fast-growing and slow-growing species, with the latter more frequently associated with host pathogenicity.

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB) in humans and is one of the species of the *M. tuberculosis* complex, which affects the lungs producing pulmonary TB, but can also cause extra-pulmonary TB. TB is the first cause of death worldwide due to a single infectious agent, causing approximately 1.6 million deaths per year. Additionally, 23% of the world's population is estimated to be latently infected with TB and therefore identification of these cases is key for TB control. Diagnosis of latent tuberculosis infection (LTBI) still has limitations and the existing assays are not able to differentiate between LTBI and TB, differentiate between the different stages of the TB spectrum, or predict whether an infected individual will progress to active TB. Because of this, it is of special importance to properly screen those patients that are at high risk of developing TB. This includes patients with immune-mediated inflammatory diseases (IMIDs) who are candidates for (or who are already on) immunosuppressant therapy, patients with immunosuppressive diseases, such as HIV, and children. In article 1 of this thesis, the performance of interferon (IFN)-γ release assays (IGRAs) was assessed in patients with IMIDs showing that IGRAs are a suitable tool for this purpose. Following with TB screening in patients with IMIDs, article 2 evaluates the IGRAs and IP-10 detection in patients with rheumatic diseases, with the conclusion that detecting IP-10 is comparable to detecting IFN-γ and that their combined detection could be beneficial when screening for TB infection in this type of patients because it increases the sensitivity of the test. In these two studies, the treatment regimens did not seem to affect the performance of the tests, although this could vary depending on the IMID and the specific treatment regimen. Furthermore, in article 3 the impact of biological response modifiers on respiratory tract infections in these patients is reviewed. Regarding groups at high risk of active TB progression, the use of serial QuantiFERON-TB Gold In-Tube (QFN-G-IT) testing is discussed in article 4 concluding that it should be further evaluated as high IFN-γ conversion levels may serve as indicators of active disease progression in children.

As previously mentioned, TB infection diagnostic tools need improving and one way to improve the current tests is to further stimulate the samples using additional TB specific antigens, which may intensify the immune response and even distinguish between the different phases of the TB spectrum. Following this approach, the addition of EspC, EspF and Rv2348-B to the current QFN-G-IT antigens (ESAT-6, CFP-10 and TB7.7) was evaluated (article 5). In this case, despite a mild increase in sensitivity, the addition of these antigens did not seem to improve the performance of the test under current stimulation conditions. However, when used in the absence of ESAT-6, the performance was still comparable to

that of the current stimulation mix, offering an alternative in the case that the ESAT-6 based vaccine or ESAT-6 tuberculin skin test (TST) are developed and used in the future.

Another way of improving TB infection diagnosis is by considering other approaches and methodologies that enable the detection of changes in the immune system after specific TB stimulation and not only improving those that are based solely on evaluating IFN-γ production. These approaches could be, for example, detection of other cytokines (such as IP-10, articles 1 and 7) or groups of cytokines, but also studying the different cell populations that produce these cytokines by flow cytometry. Cell markers CD27 and CCR4, amongst others, have been described as promising markers to discriminate between active TB and LTBI. In article 6, these two homing markers were evaluated on *M. tuberculosis*-specific $CD4^+$ T-cells (IFN-γ^+ and/or TNF-α^+) using flow cytometry. In agreement with previous studies, these two markers have the required characteristics to act as potential TB biomarkers to distinguish active TB from LTBI patients.

When talking about TB burden it is important to keep in mind that the numbers are underestimated due to under-reporting but also to under-diagnosis. In article 7 of this thesis, IP-10 detection in dried plasma spots (DPS) was evaluated in contacts and considered as a good approach for LTBI screening on sites where fresh plasma samples are difficult or impossible to store and transport in refrigerated conditions. This would help reduce the under-diagnosis issue.

Clinically less relevant and less studied, are the non-tuberculous mycobacteria (NTM). These bacteria are also referred to as atypical and environmental mycobacteria and are considered to be opportunistic pathogens that especially affect immunosuppressed individuals. The number of NTM species is increasing and so is the number of infections caused by them worldwide. In Europe, the incidence rates of NTM in patients with chronic pulmonary diseases (CPD) range from 0.2 to 2.9/100000 in the population. This increase has been described as a possible consequence of a decrease in TB incidence in areas with higher socioeconomic standards. Unlike TB etiological agents, isolation of NTM in respiratory samples have a different relevance depending on the species isolated and the specific guidelines that must be followed in order to classify them. However, no diagnostic method is available to distinguish whether an isolated NTM is causing a disease or it is merely colonizing. In addition, when diagnosing TB infection, NTM can cause discordant results between TB diagnostic tests (negative IGRA but positive TST despite lack of BCG vaccination) making their interpretation difficult. The last study included in this thesis (article 8) evaluates the use of glycopeptidolipids (GPLs) as specific NTM antigens for a future NTM-IGRA test. When discriminating between TB and NTM infections, stimulation with GPLs yielded a higher IFN-γ response in subjects with an NTM confirmed lymphadenopathy, those with suspicion of NTM infection (with discordant TB test results) and those with disseminated NTM infections, compared to subjects with active TB or LTBI and healthy controls. Additionally, when

considering patients with CPD and positive NTM isolate, those considered as NTM-colonized and those who had a record of a previous NTM-disease had a lower amount of IFN-γ production than those who were classified as having a disease caused by the isolated NTM. These promising results suggest that an NTM-IGRA based on stimulation with GPLs would be a very useful tool for better handling of CPD patients with NTM isolates of unclear clinical relevance and LTBI screening cases with discordant results.

Altogether, this thesis focuses on evaluating and developing different approaches beyond the current IGRAs to improve TB and NTM infection diagnosis. Adoption of improved methods would improve patient management and wellbeing and also help to decrease the TB burden.

Resumen

El género *Mycobacterium* está compuesto por más de 200 especies, la mayoría de ellas presentes en el medio ambiente. Este género de bacterias incluye especies de crecimiento rápido y crecimiento lento, siendo estas últimas las más frecuentemente asociadas con patogenicidad.

Mycobacterium tuberculosis, agente etiológico de la tuberculosis (TB) en humanos y una de las especies que componen el complejo *M. tuberculosis*, puede afectar a los pulmones, produciendo TB pulmonar, pero también puede causar TB extrapulmonar. La TB es la primera causa de muerte en todo el mundo debido a un solo agente infeccioso, causando aproximadamente 1.6 millones de muertes. Además, se estima que el 23% de la población mundial está infectada latentemente con TB y, por lo tanto, la identificación de estos casos es clave para el control de la TB. El diagnóstico de infección tuberculosa latente (ITBL) sigue teniendo limitaciones y las técnicas existentes no pueden diferenciar entre ITBL y TB, ni diferenciar entre las diferentes etapas del espectro de TB, así como tampoco predecir si un individuo infectado progresará a TB activa. Por lo tanto, es de especial importancia evaluar adecuadamente a aquellos pacientes que tienen un alto riesgo de desarrollar TB, como los pacientes con enfermedades inflamatorias inmunomediadas (IMIDs) que son candidatos para (o ya reciben) terapia inmunosupresora, pacientes con enfermedades inmunosupresoras como el VIH, y niños.

El artículo 1 de esta tesis evalúa el rendimiento de los ensayos de liberación de interferón (IFN)-γ (IGRAs) en pacientes con IMIDs mostrando que los IGRAs son una herramienta adecuada para este propósito y que los regímenes de tratamiento no parecen afectar su rendimiento, aunque esto podría variar según el tipo de IMID y el régimen terapéutico. Siguiendo con la detección de TB en pacientes con IMIDs, el artículo 2 evalúa el uso de la detección de IFN-γ y de la proteína 10 inducida por IFN-γ (IP-10) en pacientes con enfermedades reumáticas, concluyendo que la detección de IP-10 es comparable a la detección de IFN-γ, y que su detección combinada podría ser beneficiosa en el cribado de infección tuberculosa en este tipo de pacientes ya que aumenta la sensibilidad de la prueba. Tal y como se observó en el artículo 1, el tratamiento no parece afectar el rendimiento de estas pruebas. Además, el artículo 3 revisa el impacto de los modificadores de la respuesta biológica en las infecciones del tracto respiratorio en estos pacientes. Con respecto a los grupos con alto riesgo de progresión a TB activa, el artículo 4

comenta el uso seriado del QuantiFERON-TB Gold In-Tube (QFN-G-IT) concluyendo que debe evaluarse más a fondo, ya que conversiones (QFN-G-IT negativo a positivo) con altos niveles de IFN-γ podrían servir como indicadores de progresión a TB activa en niños.

Tal y como se menciona anteriormente, las herramientas de diagnóstico de infección tuberculosa necesitan ser mejoradas y una forma de hacerlo es estimulando las muestras usando otros antígenos específicos de TB que puedan intensificar la respuesta inmune e incluso distinguir entre las diferentes fases del espectro de TB. Siguiendo este enfoque, se evaluó la adición de EspC, EspF y Rv2348-B a los antígenos que se usan actualmente en el QFN-G-IT (ESAT-6, CFP-10 y TB7.7) (artículo 5). En este caso, a pesar de un ligero aumento en la sensibilidad, la adición de estos antígenos no pareció mejorar el rendimiento de las condiciones de estimulación actuales. Sin embargo, cuando se usó en ausencia de ESAT-6, el rendimiento aún era comparable al de la mezcla de estimulación que se utiliza, ofreciendo una alternativa en caso de que la vacuna o la prueba de la tuberculina (PT) basadas en ESAT-6 se desarrollen y usen en el futuro.

Otra forma de mejorar el diagnóstico de la infección tuberculosa es considerar otros enfoques y metodologías que permitan la detección de cambios en el sistema inmune después de la estimulación específica por TB y no mejorar solo aquellos que se basan únicamente en evaluar la producción de IFN-γ. Estos enfoques podrían ser, por ejemplo, la detección de otras citoquinas (como la IP-10, artículos 1 y 7) o grupos de citoquinas, pero también estudiar mediante citometría de flujo las diferentes poblaciones de células que producen estas citoquinas. Los marcadores celulares CD27 y CCR4 se han descrito, entre otros, como marcadores prometedores para discriminar entre TB activa e ITBL. Usando citometría de flujo, en el artículo 6, estos dos marcadores se evaluaron en células T $CD4^+$ específicas de *M. tuberculosis* (IFN-γ^+ y/o TNF-α^+ tras estimular con antígenos específicos de TB). De acuerdo con estudios previos, estos dos marcadores reunieron las características requeridas como posibles biomarcadores de TB, pudiendo distinguir entre pacientes con TB activa y pacientes con ITBL.

Cuando se habla de la incidencia de TB, es importante tener en cuenta que los números se subestiman debido a una subnotificación, pero también debido a un infradiagnóstico. En el artículo 7 de esta tesis, la detección de IP-10 en gotas secas de plasma se evaluó en contactos y se consideró como un buen método para la detección de ITBL en sitios en los que las muestras de plasma fresco son difíciles o imposibles de almacenar y transportar refrigeradas. Esto ayudaría a reducir el problema de infradiagnóstico.

Clínicamente menos relevantes y menos estudiadas que *M. tuberculosis* complex, son las micobacterias no tuberculosas (MNT). Estas bacterias también se conocen como micobacterias atípicas y ambientales, y se consideran patógenos oportunistas que afectan, especialmente, a personas inmunodeprimidas. El número de especies de MNT está aumentando así como el número de infecciones causadas por ellas en todo el mundo. En Europa, las tasas de incidencia de MNT en pacientes con enfermedades pulmonares

crónicas varían de 0.2 a 2.9 / 100000 habitantes. Tal aumento se ha descrito como una posible consecuencia de la disminución de la incidencia de TB en áreas con estándares socioeconómicos más altos. A diferencia de los agentes etiológicos de TB, el aislamiento de MNT en muestras respiratorias tiene una relevancia diferente dependiendo de las especies aisladas y se deben seguir pautas específicas para clasificarlas. Sin embargo, no hay un método de diagnóstico disponible para distinguir si una MNT aislada está causando una enfermedad o si simplemente está colonizando. Además, en el diagnóstico de infección tuberculosa, las MNT pueden causar resultados discordantes entre las pruebas de diagnóstico de infección tuberculosa (IGRA negativo pero PT positiva a pesar de no presentar historial de vacunación por bacille Calmette-Guérin [BCG]) dificultando su interpretación. El último estudio incluido en esta tesis (artículo 8) evalúa el uso de glicopeptidolípidos (GPLs) como antígenos específicos de MNT que podrían servir para una futura prueba IGRA-MNT. Con respecto a la discriminación entre infección por TB y MNT, la estimulación con GPLs produjo una mayor respuesta de IFN-γ en aquellos pacientes con linfadenopatía confirmada por MNT, aquellos con sospecha de infección por MNT (con resultados discordantes en las pruebas de infección tuberculosa) y aquellos con infecciones diseminadas de MNT, que en aquellos con TB activa, ITBL y controles sanos. Además, en los pacientes con enfermedades pulmonares crónicas con aislamiento positivo de MNT, aquellos considerados como colonizados por MNT y aquellos que tenían un registro de una enfermedad previa por MNT, tenían una cantidad menor de producción de IFN-γ que aquellos que fueron clasificados como enfermos por la MNT aislada. Estos resultados sugieren que un IGRA-MNT basado en la estimulación con GPLs es una herramienta útil para mejorar el manejo de pacientes con enfermedades pulmonares crónicas con aislado de MNT de relevancia clínica incierta, y en aquellos casos de cribado de ITBL con resultados discordantes.

En conjunto, esta tesis se centra en evaluar y desarrollar diferentes técnicas más allá de los IGRAs actuales para mejorar el diagnóstico de la infección por TB y MNT mejorando, por lo tanto, el manejo de los pacientes, su bienestar y, en última instancia, contribuyendo a disminuir la incidencia de TB.

Articles

Article 1

Irene Latorre, Sonia Mínguez, José-Manuel Carrascosa, Juan Naves, **Raquel Villar-Hernández**, Beatriz Muriel, Cristina Prat, Esther García-García, Irma Casas, Eugeni Domènech, Carlos Ferrándiz, Lourdes Mateo, Jose Domínguez. **Immune-mediated inflammatory diseases differently affect IGRAs' accuracy for latent tuberculosis infection diagnosis in clinical practice.** *PLoS One*, 2017. doi.org/10.1371/journal.pone.0189202

Article 2

Raquel Villar-Hernández, Irene Latorre, Sonia Mínguez, Jéssica Díaz, Esther García-García, Beatriz Muriel-Moreno, Alicia Lacoma, Cristina Prat, Alex Olivé, Morten Ruhwald, Lourdes Mateo, José Domínguez. **Use of IFN-γ and IP-10 detection in the diagnosis of latent tuberculosis infection in patients with inflammatory rheumatic diseases.** *Journal of Infection*, 2017. doi.org/10.1016/j.jinf.2017.07.004

Article 3

Alicia Lacoma, Lourdes Mateo, Ignacio Blanco, Maria J. Méndez, Carlos Rodrigo, Irene Latorre, **Raquel Villar-Hernández**, Jose Domínguez and Cristina Prat. **Impact of host genetics and biological response modifiers on respiratory tract infections.** *Frontiers in Immunology*, 2019. doi.org/10.3389/fimmu.2019.01013

Article 4

Raquel Villar-Hernández, Irene Latorre, Neus Altet, José Domínguez. **Could IFN-γ levels in serial QuantiFERON predict tuberculosis development in young children?** *Journal of Laboratory and Precision Medicine*, 2017. doi.org/10.21037/jlpm.2017.10.06

Article 5

Raquel Villar-Hernández, Thomas Blauenfeldt, Irene Latorre, Beatriz Muriel-Moreno, Esther García-García, María Luiza de Souza-Galvão, Joan Pau Millet, Josefina Sabriá, Adrián Sánchez-Montalva, Juan Ruiz-Manzano, José Pilarte, María Á. Jiménez, Carmen Centeno, Carmen Martos, Israel Molina-Pinargote, Yoel González-Díaz, Javier Santiago, Adela Cantos, Irma Casas, Rosa Guerola, Cristina Prat, José Dominguez, Morten Ruhwald. **Diagnostic benefits of adding EspC, EspF and Rv2348-B to the QuantiFERON Gold In-tube antigen combination**. Pending to submit to *Scientific Reports*, 2019

Article 6

Irene Latorre, Marco A. Fernández-Sanmartín, Beatriz Muriel-Moreno, **Raquel Villar-Hernández**, Sergi Vila, María L. de Souza-Galvão, Zoran Stojanovic, María Á. Jiménez-Fuentes, Carmen Centeno, Juan Ruiz-Manzano, Joan Pau Millet, Israel Molina-Pinargote, Yoel D. González-Díaz, Alicia Lacoma,

Lydia Luque-Chacón, Josefina Sabriá, Cristina Prat and Jose Domínguez. **Study of CD27 and CCR4 markers on specific CD4+ T-Cells as immune tools for active and latent tuberculosis management.** *Frontiers in Immunology*, 2017. doi.org/10.3389/fimmu.2018.03094

Article 7

Raquel Villar-Hernández, Irene Latorre, María Luiza de Souza-Galvão, María Á Jiménez, Juan Ruiz-Manzano, José Pilarte, Esther García-García, Beatriz Muriel-Moreno, Adela Cantos, Neus Altet, Joan Pau Millet, Yoel González-Díaz, Israel Molina-Pinargote, Cristina Prat, Morten Ruhwald & José Domínguez. **Use of IP-10 detection in dried plasma spots for latent tuberculosis infection diagnosis in contacts via mail.** *Scientific Reports*, 2019. doi.org/10.1038/s41598-019-40778-1

Article 8

Raquel Villar-Hernández, Irene Latorre, Zoran Stojanovic, Antoni Noguera-Julian, María Luiza de Souza-Galvão, María Méndez, Carlos Rodrigo, Josefina Sabriá, Carmen Martos, José Ramón Santos, Jordi Puig, Anàlia López, Aina Martínez-Planas, Antonio Soriano-Arandes, María Á Jiménez, Marisol Domínguez, Laura Minguell, Joan Pau Millet, Neus Altet, Irma Casas, Alicia Marín, Yolanda Galea, Beatriz Muriel-Moreno, Esther García-García, Miguel Pérez, Juan Ruiz Manzano, Cristina Prat, Jordi B. Torrelles and José Domínguez. **Development and testing of a new non-tuberculous mycobacteria diagnostic test using specific mycobacterial cell wall antigens.** Pending to submit to the *Lancet of Infectious Diseases*, 2019.

Annexes

Annex 1

Cell-mediated immune responses to in vivo expressed and stage specific *Mycobacterium tuberculosis* antigens in adult and adolescent latent and active tuberculosis. Study done in collaboration with Tom H.M. Ottehnhoff, Mariateresa Coppola, and Krista van Meijgaarden from the LUMC (Leiden, The Netherlands).

Annex 2

Thomas Blauenfeldt, **Raquel Villar-Hernandez**, Esther García-García, Irene Latorre, Line L. Holm, Beatriz Muriel-Moreno, María Luiza de Souza-Galvão, Joan Pau Millet, Josefina Sabriá, Adrián Sánchez-Montalva, Juan Ruiz-Manzano, Jose Pilarte, María Á. Jiménez, Carmen Centeno, Carmen Torres, Israel Molina-Pinargote, Yoel González-Díaz, Javier Santiago, Adela Cantos, Cristina Prat, José Dominguez, Morten Ruhwald. **Diagnostic accuracy of IP-10 mRNA release assay for tuberculosis.** Submitted *PLOS One*, 2019.

Abbreviations

BCG – Bacille Calmette-Guérin

CFP-10 – Culture Filtrate Protein - 10 kDa

CPD – Chronic Pulmonary Disease

DMARD – Disease-Modifying Anti-Rheumatic Drugs

DPS – Dried Plasma Spots

ELISA – Enzyme-Linked Immunosorbent Assay

ELISPOT –Enzyme-linked Immunospot Assay

ESAT-6 – Early Secretory Antigen Target – 6 kDa

GPLs – Glycopeptidolipids

HIV – Human Immunodeficiency Virus

IFN – Interferon

IGRA – Interferon Gamma Release Assay

IL – Interleukin

IMIDs – Immune-Mediated Inflammatory Diseases

IP-10 – IFN-γ Inducible protein 10

LTBI – Latent Tuberculosis Infection

MDR-TB – Multidrug-resistant TB

MFI – Median Fluorescence Intensity

mRNA – Messenger Ribonucleic Acid

NK cell – Natural Killer Cell

NTM – Non-Tuberculous Mycobacteria

PBMCs – Peripheral Blood Mononuclear Cells

PPD – Purified Protein Derivative

QFN-G-IT – QuantiFERON-TB Gold In-Tube

QFN Plus – QuantiFERON-TB Gold Plus

Rv – Region of Variance

TB – Tuberculosis

Th – T-helper (cell)

TNF – Tumor Necrosis Factor

WHO – World Health Organization

XDR-TB – Extensively Drug-Resistant TB

General Introduction

1. The world of mycobacteria

The genus *Mycobacterium* is composed by catalase-positive, non-motile, non-spore forming, rod-shaped, and acid-fast bacteria stained by Ziehl-Neelsen staining. So far, more than 200 species have been recognized as being part of this genus[1] and, most of them are found in the environment[2–6]. They are mostly aerobic, although some species are microaerophilic[7] and they are grouped in fast-growing and slow-growing species[1]. Although those from the slow-growing group are more frequently associated with host pathogenicity[8], in both groups there are species that cause pulmonary, ganglionar, disseminated and cutaneous diseases[7,9–11].

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB) in humans, and *Mycobacterium leprae*, the etiological agent of leprosy, are clinically considered the most important species. *Mycobacterium bovis* and *Mycobacterium africanum* can also cause TB in humans and, as they are genetically very similar to *M. tuberculosis*, they all belong to the *Mycobacterium tuberculosis* complex. *M. tuberculosis* complex bacilli usually affect the lungs, producing pulmonary TB, but can also affect other sites of the body, producing extrapulmonary TB.

Clinically less relevant and less studied (but increasing in incidence) are the non-tuberculous mycobacteria (NTM). These bacteria are also referred to as atypical and environmental mycobacteria and are considered opportunistic pathogens that affect, especially, immunosuppressed individuals[12]. The most frequently reported NTM species are those from the *Mycobacterium avium* complex (MAC) (*Mycobacterium avium*, *Mycobacterium chimaera* and *Mycobacterium intracellulare*, among others), *Mycobacterium abscessus, Mycobacterium chelonae,* and *Mycobacterium fortuitum*, but there are others[1,7].

2. Mycobacterial infection burden

2.1. TB burden

According to the 2018 World Health Organization (WHO) Global Tuberculosis Report[13], TB is the first cause of death worldwide due to a single infectious agent. In 2017, the estimate of TB deaths was 1.3 million considering only HIV-negative people, 300000 extra deaths if HIV-positive were taken into account. Although TB is considered mainly a poverty-related disease, it is important to state that it affects all countries and all age groups. In 2017, 90% of cases were adults (aged ≥15 years), the majority of which were male (65%), 9% were HIV-positive (72% of them in Africa) and two thirds were in eight countries: India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%). Only 3% of the cases were in the WHO European Region and another 3% in the WHO Region of the Americas.

Although TB incidence and death rates have decreased in the last years (29% less than in the year 2000 amongst HIV-negative and 44% amongst HIV-positive people) (figure 1), and many efforts are carried out to control and prevent the disease, TB still affects millions of people each year with an

estimate of 10 million new cases in 2017. In most high-income countries there were under 10 new cases per 100000 population, whereas in most of the 30 high TB burden countries the number of new cases was higher, among 150 to 400 (figure 2), and it reached 500 in a few other countries such as Mozambique, Lesotho, the Philippines and South Africa (figure 3). These estimations are affected by the under-diagnosing or under-reporting of TB as is the case of the "missing millions" in India[14,15]. It is worth mentioning that, although TB incidence rates have decreased (figure 1), given that the world's population is increasing[16], this does not necessarily mean that the total number of TB cases is decreasing but rather stayed the same or even increased.

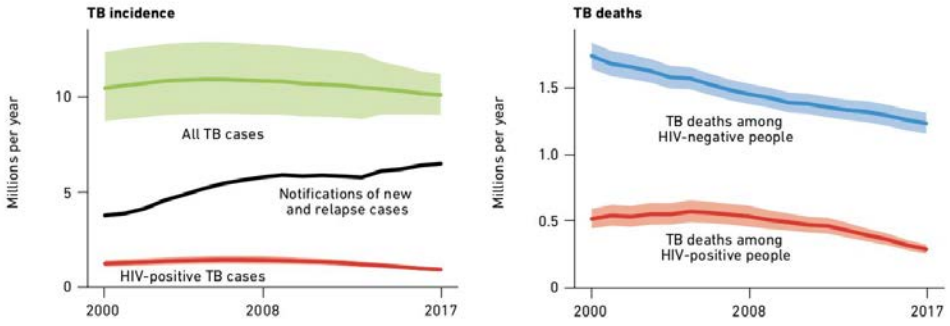

Figure 1. Global trends in the estimated number of incident TB cases and the number of TB deaths (in millions), 2000-2017. Shaded areas represent uncertainty intervals. Figure and data Source: Global Tuberculosis Report 2018[13]. WHO, 2018. © WHO 2018. All rights reserved.

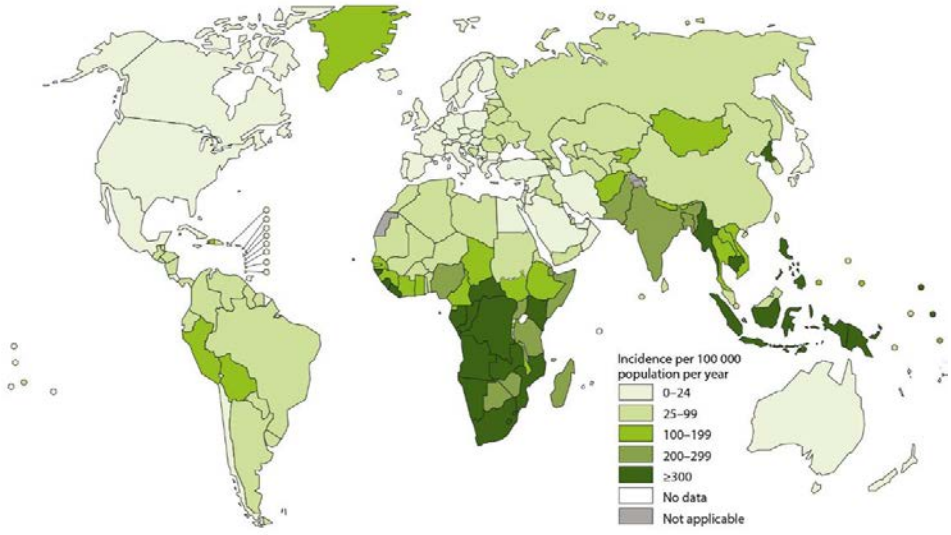

Figure 2. Estimated TB Incidence Rates in 2017. Figure and data Source: Global Tuberculosis Report 2018. WHO, 2018[13]. © WHO 2018. All rights reserved.

Apart from the TB burden public health concern *per se*, drug-resistant TB is a major threat that needs to be addressed. Briefly, according to the WHO, there are five TB drug resistance types: 1) mono-resistance: resistance to only one first-line anti-TB drug; 2) poly-resistance: resistance to more than one first-line anti-TB drug but not rifampicin nor isoniazid; 3) rifampicin resistance (RR): resistance to

rifampicin, with or without resistance to other anti-TB drugs; 4) multidrug-resistance (MDR): resistance to at least rifampicin and isoniazid; and 5) extensively drug-resistance (XDR): MDR with additional resistance to at least one fluoroquinolone and one second-line injectable agent (the two most important groups of drugs in a MDR-TB regimen). In 2017 the global estimate was 558000 new cases of RR-TB from which 82% had MDR-TB, causing 230000 deaths. Most MDR/RR-TB cases were registered in China, India, and the Russian Federation. Additionally, the reported proportion of MDR-TB cases with XDR-TB has increased from 6.2% in 2016 to 8.5% in 2017.

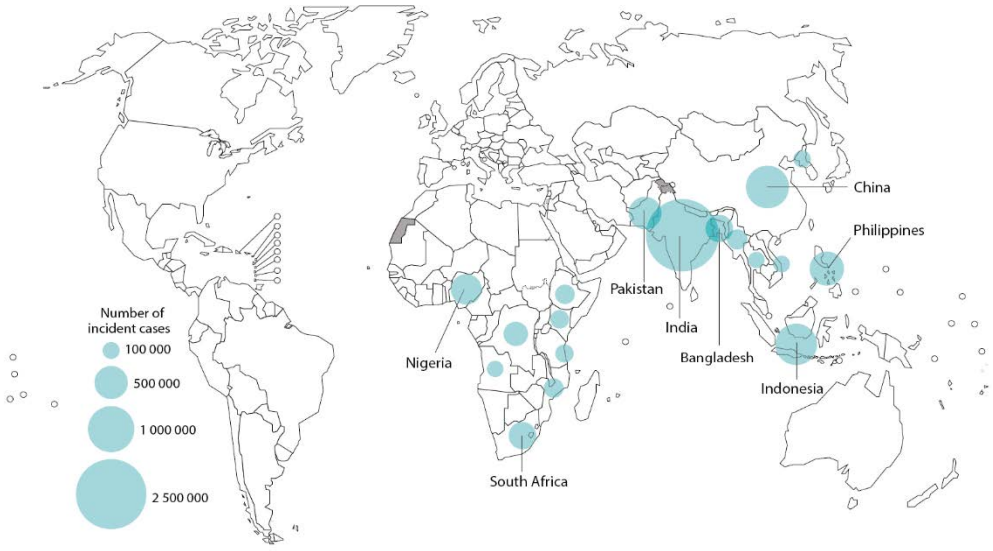

Figure 3. Estimated TB incidence in 2017, for countries with at least 100000 incident cases. Figure and data Source: Global Tuberculosis Report 2018[13]. WHO, 2018. © WHO 2018. All rights reserved.

Diagnosing active TB is based on a combination of different techniques and approaches. There are no clinical symptoms exclusive of TB, and therefore other techniques such as x-rays, microscopy, culture and molecular tests have to be used to achieve a more accurate diagnosis. However, these methods still have drawbacks. Sputum-smear microscopy, for example, is rapid, simple, and inexpensive but has a low sensitivity[17]. Mycobacterial culture, on the other hand, has the highest sensitivity, but obtaining a result requires a long time due to the slow growth of the bacteria (up to two months in solid media and up to 20 days in liquid media)[17,18]. Molecular based diagnostic tests are in constant improvement and development, and mostly aim to detect specific *M. tuberculosis* nucleic acids using polymerase chain reaction (PCR), and detect mutations related to resistance to anti-TB treatment by sequencing or nucleic acid hybridization, amongst others[19]. The GeneXpert (Cepheid, California, USA), for example, is a molecular semi-automated method that represents an important advance in TB diagnosis in the last years. It detects DNA from the bacteria in sputum samples, obtaining a result in no more than two hours, and can also detect resistance to rifampicin. Recently an optimized version of the test has been developed,

the Xpert RIF/MTB Ultra[20]. However, the need of electricity and its cost are still drawbacks and make it difficult to establish as a routinely used test, especially in low-income countries.

Furthermore, it is estimated that 23% of the world's population (1.7 billion people) are latently infected with TB (LTBI) and, therefore, at risk of developing the disease during their lifetime[21]. This LTBI population is not infectious, and only 5 to 10% will develop active TB during their lifetime (especially during the first 5 years after infection)[22]. Progression from latent to active TB depends on several factors, but the most important is the immunological status[23,24].

Prevention of new infections by *M. tuberculosis* and their progression to TB disease is critical to reduce the burden of disease and death caused by TB and to achieve the End TB Strategy targets set for 2030 and 2035. Current health interventions for TB prevention are treatment of latent TB infection (LTBI), prevention of transmission of *M. tuberculosis* through infection prevention and control, and vaccination of children with the bacille Calmette-Guérin (BCG) vaccine.

WHO guidelines published before 2018 identified four priority groups in which testing and treatment for LTBI was strongly recommended: people living with HIV (all countries), children aged under 5 years who are household contacts of bacteriologically confirmed pulmonary TB cases (all countries), people aged five years or more who are household contacts of pulmonary TB cases (upper-middle-income and high-income countries with a low incidence of TB) and clinical risk groups (upper-middle-income and high-income countries with a low incidence of TB). Updated guidelines published in 2018 includes an additional recommendation to consider testing and treatment for people aged 5 years or more who are household contacts of bacteriologically confirmed pulmonary TB cases in countries with a high incidence of TB; it also expands the recommendation for clinical risk groups to all countries.

BCG vaccination should be provided as part of national childhood immunization programmes according to a country's TB epidemiology. In 2017, 158 countries reported providing BCG vaccination as a standard part of these programmes, of which 120 reported coverage of at least 90%, up from 111 countries in 2016.

2.1. NTM infection burden

The number of NTM species is increasing and so is the number of infections caused by them worldwide[25]. This increase is not only due to improved diagnosis, recording and typing, but also due to a real increase in NTM diseases[26–32]. As NTM isolation or disease is not of compulsory notification in Europe, many cases go unrecorded and their epidemiological analyses are not entirely accurate.

Isolation of NTM in clinical samples from patients with chronic pulmonary disease (CPD) are also increasing[30,33–35]. Interestingly, such an increase has been described as a possible consequence of a decrease in TB incidence in areas with higher socioeconomic standards[36]. NTM species isolated from pulmonary samples vary between the different countries and regions worldwide, being MAC species the

predominant[37] (figure 4). Focusing on Europe, the incidence rates of NTM in patients with CPD range from 0.2 to 2.9/100000 population[38].

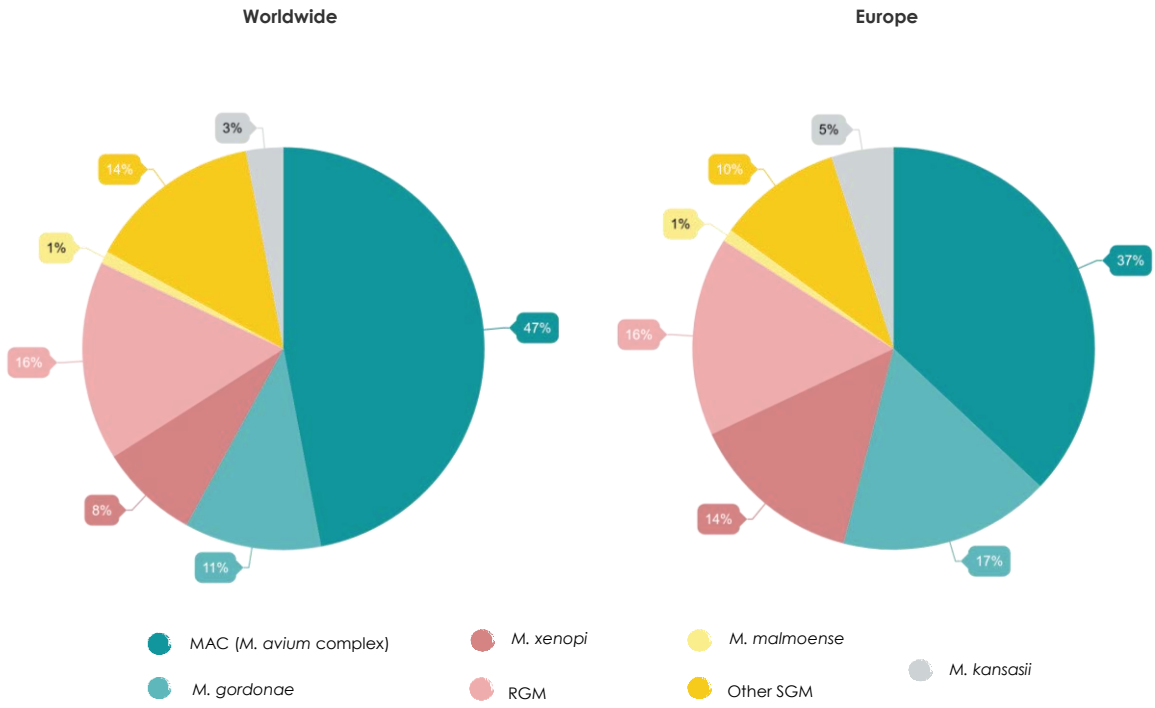

Figure 4. NTM species isolated in respiratory samples. Data source: Hoefsloot et al. 2013[37]. RGM: Rapid-growing mycobacteria; SGM: Slow-growing mycobacteria.

Unlike TB etiological agents, isolation of NTM in respiratory samples have a different relevance depending on the isolated species[39,40] (table 1) and specific guidelines, such as the Wolinsky criteria and those from the American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) (ATS/IDSA), should be followed in order to classify them[41,42].

Table 1: NTM species and their clinical relevance for pulmonary diseases[39,40]. Table source: Wassilew *et al.* 2016[38]

Clinical relevance	SGM	RGM
Predominantly pathogenic (the majority of findings are related to disease)	*M. malmoense* *M. szulgai* *M. kansasii*	
Often pathogenic (usually 30-70 % of findings are related to disease)	*M. xenopi* *M. avium*	*M. abscessus subsp. abscessus*
Mostly colonizing or contaminating (usually < 30% of findings are related to disease)	*M. simiae* *M. gordonae* *M. intracellulare**	*M. abscessus subsp. bolletii* *M. chelonae* *M. fortuitum*

* In some regions of Europe isolation of *M. intracellulare* may be more often related to disease. SGM, slowly growing mycobacteria; RGM, rapidly growing mycobacteria.

3. TB transmission and spectrum of the disease

Pulmonary TB is the contagious form of the disease. When *M. tuberculosis* bacilli are spread in aerosol droplets through the air by an ill person's cough (for example) they can be inhaled by another person close by[43,44] (figure 5).

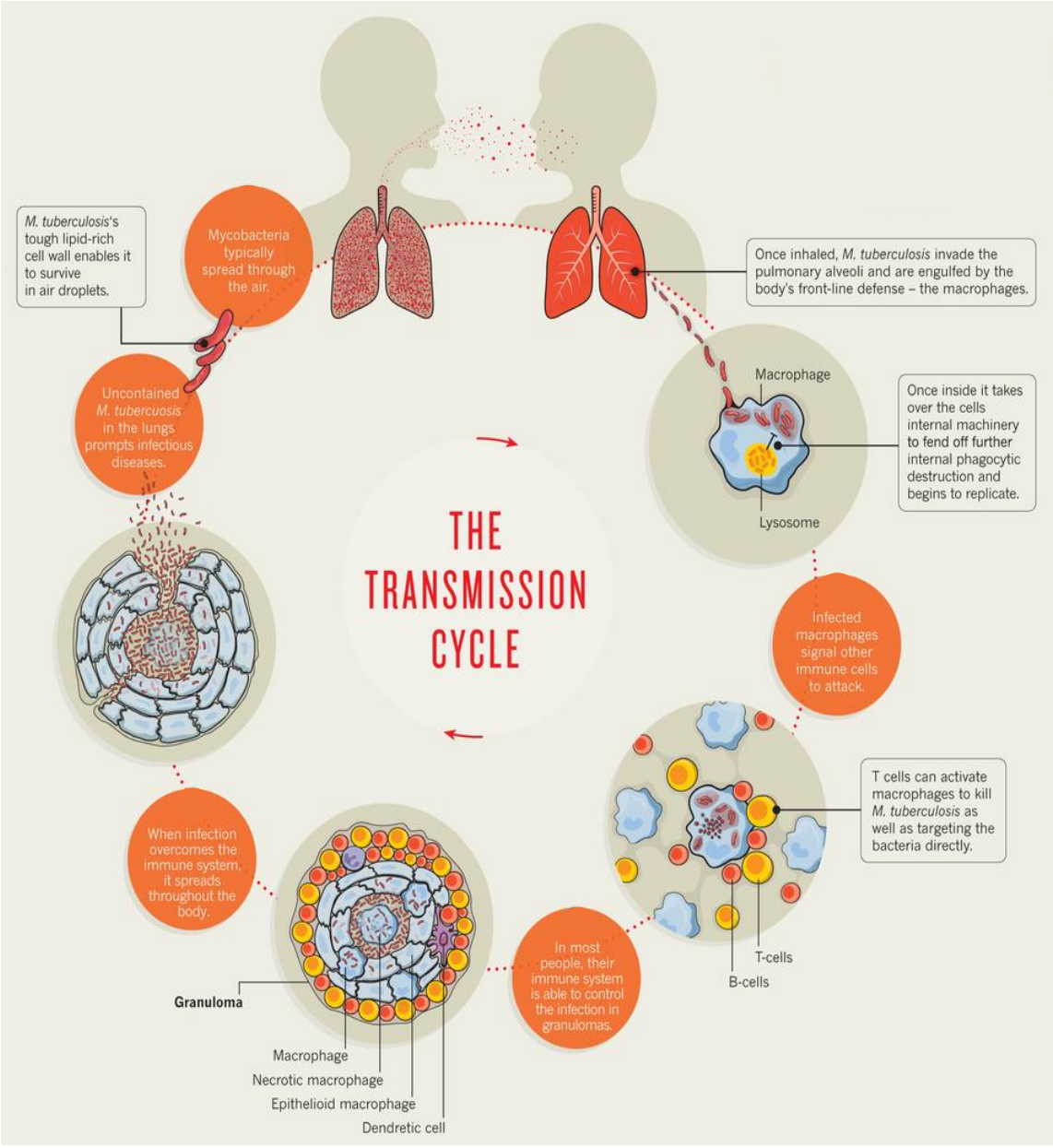

Figure 5. The TB transmission cycle. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Nature, Macmillan Publishers Limited, Paulson, T. Epidemiology: A mortal foe. Nature 502, S2 (2013) © 2013 [43]. https://doi.org/10.1038/502S2a

Briefly, when this happens, *M. tuberculosis* travels through the respiratory tract and invades the pulmonary alveoli. Here bacilli will be engulfed by the new host's macrophages where they will replicate. Infected macrophages signal other immune cells such as T-cells and B-cells, which travel to the site of infection and kill *M. tuberculosis* by activating macrophages for this purpose and/or targeting it directly.

For most people (90%)[22], their immune system is able to contain the infection in this point, forming granulomas in which the bacilli and infected macrophages are controlled. This is what is traditionally called as LTBI. However, in a 5-10% of cases[22] infection overcomes the host's immune system, spreads and replicates uncontrollably causing active TB disease. If this invasion happens in the lungs it will cause pulmonary disease and can be transmitted again (usually through air). If otherwise, it spreads and replicates in other parts of the body, it will cause extrapulmonary TB.

Traditionally this process has been considered as binary, with either an LTBI, in which the bacteria are contained and replication of the bacilli is inexistent or happens in a very low rate without causing harm to the host, or an active TB disease. However, this binary interpretation has changed in the last decade into a continuous spectrum of immunologic responses, which corresponds to different states that go from infection to active TB disease in a dynamic continuum[45–49] (figure 6).

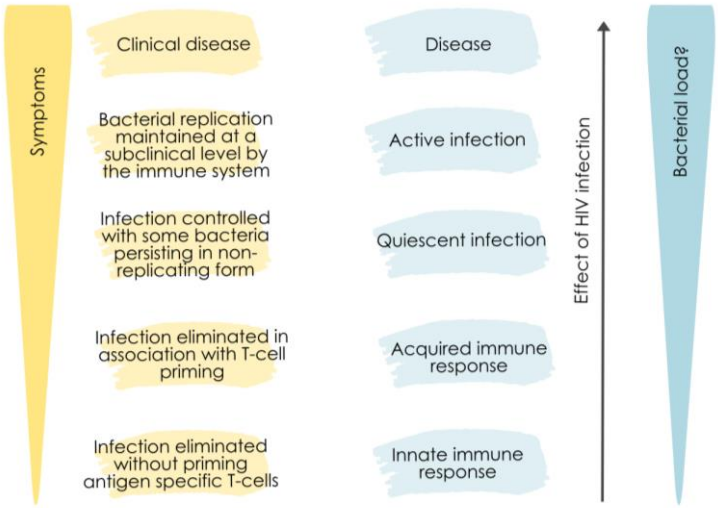

Figure 6. Tuberculosis infection as a spectrum. Figure adapted/reprinted with permission from Springer Nature Customer Service Centre GmbH: Nature Reviews Microbiology, Macmillan Publishers Limited. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Barry 3rd *et al*. © 2013[45]. https://doi.org/10.1038/nrmicro2236

Considering this dynamic continuum, Drain *et al.*, further propose five categorical states in order to better classify and define this dynamic spectrum[48]:

1. Eliminated TB infection - in which an individual exposed to *M. tuberculosis* may have immunological evidence of such infection but no longer has viable *M. tuberculosis* bacteria, either due to the host's immune system or due to anti-TB treatment.
2. LTBI - in which the exposed individual has viable *M. tuberculosis* bacteria but progression to TB is not expected.
3. Incipient TB infection - in contrast with LTBI, in this case, progression to active disease is likely to occur in the absence of treatment but still, the individual lacks clinical symptoms.

4. Subclinical TB disease - although there are still no clinical symptoms, the patient presents radiological abnormalities or microbiological evidence of active and viable *M. tuberculosis*.

5. Active TB disease - in this state the patient presents clinical symptoms with radiographic abnormalities suggestive of TB. This state corresponds to the current WHO definition of active TB disease.

In summary, following infection, *M. tuberculosis* can be eliminated, persist or cause progression to active disease. If the bacteria persists, once the latent infection is established, it can remain latent, progress to the active state, or enter a cyclic disease period in which the patient alternates between incipient and subclinical states before developing active TB (figure 7). Not every infected individual will go through all these states and if they do, the timing can be different. In addition, spontaneous recovery and *M. tuberculosis* elimination are possible at any time throughout this process. The possible outcomes of this spectrum are still poorly understood and depend on several factors from the host, the pathogen and the environment[24,50].

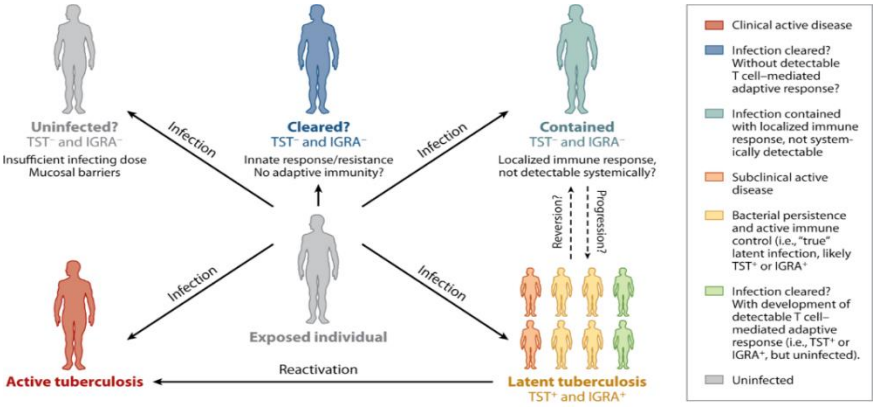

Figure 7. Heterogeneity resulting from *M. tuberculosis* infection. Reprinted with permission from Annual Reviews through Copyright Clereance Center: The Immune Response in Tuberculosis. O'Garra A, *et al*. © 2013 [55]. https://doi.org/10.1146/annurev-immunol-032712-095939

4. Immunity against TB

Immunity against TB is complex and poorly understood. To fully understand the immune processes involved in TB disease prevention and control, we have to take into account not only the genetic factors of the host but also those related to the pathogen and the environment[24,48].

Once *M. tuberculosis* is established in the lungs, alveolar macrophages phagocytose the bacilli, which are also taken up by neutrophils and dendritic cells[51–54]. The immune response triggered and the role of each of these cell types is not well understood and depends on the host and the pathogen, as well as the stage of the disease[55].

According to the central dogma, the IFN-γ produced by T-cells activates macrophages to kill intracellular bacilli. IFN-γ production by CD4+ T-cells is important for *M. tuberculosis* control; however, it is unknown to what extent more IFN-γ production increases protection, how much is needed or which cells are required. CD4+ Th1 cells are essential for protective immunity against *M. tuberculosis* by activating macrophages by producing IFN-γ and other cytokines and activating other pathways that contribute to the immune response against TB[56,57]. Other cells such as CD8+ T-cells, natural killer (NK) cells, and CD1-restricted T-cells also confer protection against *M. tuberculosis* and produce IFN-γ, however, they are not able to compensate for the lack of CD4+. Although IFN-γ is a key cytokine in TB infection, its levels seem to correlate better with bacterial burden rather than disease control[44,58] and other T-cell functions apart from IFN-γ production have been shown to also mediate protection[44,59]. Th17-type CD4+ T-cell responses, for example, have been associated with a lower risk of progression to TB[60] while CD4+ T-cells that produce interleukin-2 (IL-2) seem to correlate with bacterial burden and disease[61,62]. Additionally, a lower prevalence of NKT-cells has been associated with TB progression[63]. B-cells also seem to play an important role in the protective response against *M. tuberculosis* both by producing antibodies and production of IL-10[64–67]. Circulating antibodies against TB antigens as well as their glycosylation patterns may serve as biomarkers of subclinical TB and its control or progression to active TB disease[66,68–71]. Furthermore, dendritic cells have a fundamental role as the prime antigen-presenting cells to activate T-cell responses[72]; however, their mechanism of action is poorly understood and considered complex.

The host's immune response causes changes in *M. tuberculosis*' gene expression causing changes in the antigens produced by the bacteria and this consequently causes changes in the host's immune response[73]. Antigen Ag85B for example, is present in high amounts in the early stages of infection, while it is repressed during chronic stages[74–76]. Frequencies of T-cells that recognize lipid antigens (CD1bc-restricted T-cells) are higher during LTBI compared to TB uninfected individuals[77,78] and those of cells that recognize riboflavin biosynthetic products (MR1-restricted T-cells) are lower during active TB disease compared to LTBI[79].

Predicting TB progression is key for disease control, however, the immune mechanisms and/or biomarkers involved remain unknown and therefore there is still no method available to detect it[80]. Finding biomarkers of protection and disease control is key for vaccine development. According to Nunes-Alves *et al.*, optimal protection against TB is based on vaccines that induce changes in T-cell function rather than eliciting large numbers of T-cells that produce IFN-γ[44]. They postulate that inducing GM-CSF and IFN-γ producing CD4+ T-cells, and cytolytic CD8+ T-cells, in addition to producing IFN-γ, would protect better than vaccines which induce IFN-γ production alone[44]. Considering that *M. tuberculosis* can survive inside macrophages, vaccines that induce antibody production are not considered useful for TB protection[81].

As previously mentioned, host-related factors are not the only important players in the triggered immune response. Genetic and phenotypic variations of the pathogen (such as infection by different lineages and strains) and the diverse microenvironments created by the different infection sites produced, affect the host's immune response and therefore shape disease states and influence TB progression[82–86]. Portevin *et al.*, for example, described that strains from lineages 2, 3 and 4 (modern strains) may have adapted to cause a faster disease progression and transmission as they trigger a lower cytokine production than strains from lineages 1, 5 and 6 (ancient strains)[87]. Another example was described by Manca *et al.* in which differential monocyte activation by strains CDC1551 and HN878 showed to alter their pathogenesis by different cytokine production patterns[88]. Furthermore, proteomics, metabolomics and epigenetics, may also help define TB progression signatures[89–92]. Extrinsic factors such as cigarette smoke, malnutrition and treatment intake (especially immunomodulatory therapy) also affect such response. These variables have to be kept in mind for the development of new diagnostic tools and effective vaccines.

5. Individuals at risk of progression

As previously mentioned, a small proportion (5 to 10%) of those latently infected with TB will end developing active TB disease during their lifetime. Transition to disease depends on several factors from the host, pathogen and extrinsic[24], being the immunological status the most important[23]. Therefore, it is not surprising that the chances of developing active TB disease are much higher amongst people infected with HIV and other conditions that impact *M. tuberculosis* infection such as diabetes, undernutrition, biologic therapy or TNF-α blockers, smoking and alcohol consumption[24,93–95].

Recently, the WHO has updated and consolidated the guidelines for programmatic management of LTBI[96] where it strongly recommends LTBI testing and treatment in three priority groups: people living with HIV (regardless of age), HIV-negative household contacts with microbiologically confirmed pulmonary TB (especially children), and other HIV-negative at-risk groups such as patients initiating anti-TNF treatment.

According to the last 2018 Global Tuberculosis Report[13], the overall percentage of active TB and HIV positive patients has fallen globally since 2008. However, when considering the WHO regions separately, the only area in which decline is non-existent is the WHO European Region, where the proportion of these patients has increased, from 3% in 2008 to 13% in 2017[13].

Regarding individuals with immune-mediated inflammatory diseases (IMIDs), such as rheumatoid arthritis, psoriasis and Crohn's disease, who are candidates for disease-modifying anti-rheumatic drugs (DMARDs), biologic therapy (TNF antagonists) and corticosteroids, LTBI diagnosis is essential. Biologic agents (anti-TNF-α amongst others) are related to an increase in developing severe infections such as active TB, by newly acquired infection and/or reactivation[93,94,97–100] therefore, prescription of these therapies should go hand in hand with LTBI diagnosis, and close follow up[101–103].

Age also influences TB risk. Children under 5 years old are also considered as vulnerable individuals and therefore at high risk of developing TB after infection, progressing to active TB at a higher rate than adults[104,105].

6. TB infection diagnosis

To manage TB correctly and decrease the burden of the disease, not only prevention, early identification of active TB cases and good treatment regimens are necessary; identifying those individuals that are infected is also of great importance, as they are the ones at risk of developing the disease at any point during their lifetime. The WHO has recently published the *Latent Tuberculosis infection*: *updated and consolidated guidelines for programmatic management* in which they add the importance of a national plan for programmatic management of LTBI and includes prioritizing groups at high risk[96].

Although there is no gold standard for LTBI diagnosis, there are a few methods available.

Tuberculin skin test (TST). The classical method for infection screening is the tuberculin skin test (TST). This test is based on the intradermal injection of purified protein derivative (PPD) in the forearm of the subject using the Mantoux method. The rationale behind this test is the stimulation of T-cells with this pool of proteins. If the individual's immune system has been in contact with these antigens before, they will recognize them and therefore produce an immune response, which can be measured as an induration in the intradermal injection site 48 to 72 hours later.

Although the TST is easy to perform, not costly and therefore extensively used, it has several limitations. The performance of the TST requires a second visit of the patient in order to obtain the result, which sometimes does not happen and therefore remains undiagnosed. Additionally, some patients are not able to produce a good response to the TST due to a deficient cell-mediated immunity such as patients with chronic inflammatory diseases which are receiving anti-TNF-α agents or biologic therapy. In these cases, detection of infection is very important to prevent active TB development given their fragile immunological state[97,98]. In addition, recently, there has been a shortage of the PPD used (PPD RT23 from the Statens Serum Institut, Copenhagen, Denmark) which may jeopardize the availability of this test in the near future. Nonetheless, the main limitation of the TST is the cross-reaction with the Bacille Calmette-Guérin (BCG) vaccine strain of *Mycobacterium bovis* and non-tuberculous mycobacteria (NTM)[106] as the injected PPD is a combination of proteins purified from an extract of *M. tuberculosis*. Therefore, this extract not only contains proteins that are specific of *M. tuberculosis* but also contains proteins that are present in other mycobacteria species.

Interferon (IFN)-γ release assays (IGRAs). As an alternative to the TST, the interferon (IFN)-γ release assays (IGRAs) have emerged. Unlike the TST, IGRAs measure T-cell mediated responses to specific *M. tuberculosis* antigens (ESAT-6, CFP-10 and TB 7.7) by measuring either the amount of IFN-γ released in whole blood (QuantiFERON-TB Gold In-Tube [QFN-G-IT]; Qiagen, Düsseldorf, Germany) or the amount of peripheral blood mononuclear cells (PBMCs) that produce IFN-γ (T-SPOT.TB; Oxford

Immunotec Limited, Abingdon, UK) after an overnight stimulation (figure 8). Recently, a new QuantiFERON test has been developed: the QuantiFERON Plus (QFN-Plus)[107,108]. This version of the test includes two antigen tubes: TB1 and TB2. TB1 is equivalent to the antigen tube of the previous version which stimulates $CD4^+$ T-cells. TB2 however, contains other peptides which stimulate both $CD4^+$ and $CD8^+$ T-cells.

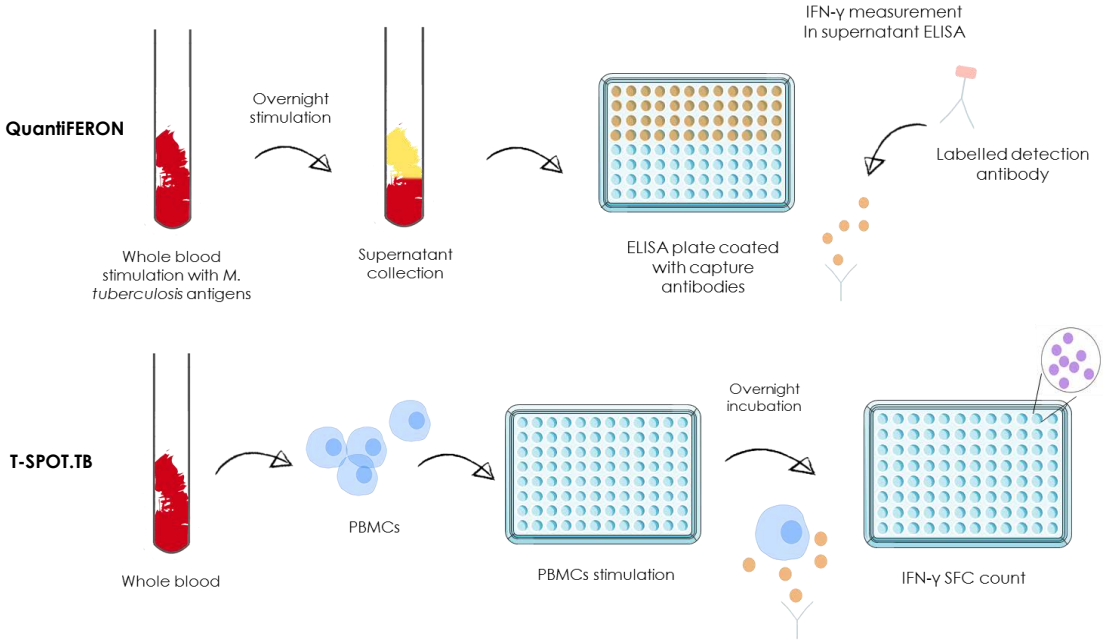

Figure 8. Graphical representation of the IGRAs. ELISA: Enzyme-Linked Immunosorbent Assay; PBMCs: peripheral blood mononuclear cells, SFCs: Spot forming cells

As stimulation of the samples in the IGRAs is done by specific *M. tuberculosis* antigens (ESAT-6, CFP-10, and TB7.7), they lack cross-reaction issues with the BCG vaccine strain and NTM, and therefore they yield a higher specificity than the TST. However, as well as the TST, these tests are based on the T-cell response, which indicates infection with *M. tuberculosis* but does not give information about the present status of such infection, progression to TB, nor the current presence of the bacilli.

As previously mentioned, the current methods available for LTBI diagnosis are limited and far from optimal. Given the importance of identifying LTBI cases, there is a clear need for further improvement of these techniques and the development of new ones.

7. Future / alternative biomarkers for TB infection

Other approaches for LTBI diagnosis are based on the detection of different cytokines and cell markers, and the search of other specific antigens to stimulate the samples with, that may be more immunogenic or trigger immunologic pathways differently in the various stages of the infection.

A well-studied example of detection of other cytokines is the case of IFN-γ inducible protein 10 (IP-10). IP-10 is a chemokine that is induced by innate and adaptive mechanisms, and is mainly expressed

by antigen-presenting cells[109]. Its expression is induced mainly by T-cell derived IFN-γ and TNF-α and, to less extent, IL-2, type II IFNs, IL-27, IL-17/IL-23, and IL-1β[110]. IP-10, unlike IFN-γ, is produced in higher levels (100-fold higher) which makes it a good candidate for assay simplification and miniaturization[111,112]. So far, the detection of IP-10 alone has not been proven to differentiate between TB infection and disease or between infection states. However, it has been described as a more robust biomarker than IFN-γ in HIV infected patients and young children, and suggested to be used in combination with the IGRAs for a better accuracy of TB diagnosis[113–117].

Apart from IP-10 and IFN-γ, other pro- and anti-inflammatory cytokines, chemokines, growth factors and cell markers (biomarkers for simplicity) have been related to the pathogenesis and control of TB infection[118–122]. These possible biomarkers have been studied to determine their usefulness for improving LTBI diagnostic tests, differentiating between LTBI infection and disease, and predicting progression to active TB[123–125]. Decrease of CD27 surface expression on *M. tuberculosis*-specific $CD4^+$ T-cells, for example, has been related to active TB disease as well as a marker of lung tissue damage during active TB[126–129]. Therefore, CD27 could serve as a biomarker to differentiate between active TB and infection, and, monitor TB treatment. Furthermore, studying HLA-DR expression in IFN-γ^+ $CD4^+$ T-cells and IFN-γ^+ TNF-α^+ $CD3^+$ cells has also proven promising to differentiate between LTBI and active TB[130].

The use of new immunogenic and *M. tuberculosis*-specific antigens for a differential sample stimulation is also in extensive study[131]. Rv1733 has shown to be very immunogenic and able to discriminate between TB uninfected individuals, LTBI and active TB patients, while Rv2389 and Rv2435n have been characterized as an LTBI biomarker[132]. In addition, responses against Rv2628 and Rv2031 may correlate with immune-mediated protection against TB[133,134]. According to the systematic review carried out by Meier *et al.* the most promising antigens for active TB and LTBI discrimination are Rv0081, Rv1733c, Rv1737c, Rv2029c, Rv2031, and Rv2628[131].

The immune response against TB is complex and triggered by multiple and variable factors. Elucidating immunologically distinct characteristics of each state of the TB infection dynamic continuum remains a challenge. In order to progress towards the WHO's "End TB Strategy"[135], we need to understand better the effect of these different variables in the immune response against TB. Only then will we be able to develop better diagnostic tools and efficient TB vaccines. Although TB infection diagnosis in high TB burden countries may not be the main concern, as most of the population may be already infected, having tools to predict active TB disease progression and effective TB vaccines could make a difference in TB management worldwide.

Justification

Tuberculosis (TB) is an infectious disease caused by bacilli from the *Mycobacterium tuberculosis* complex. Bacteria from this complex, usually affect the lungs, producing pulmonary TB, but can also cause extrapulmonary TB. According to the 2018 WHO Global Tuberculosis Report, TB is the first cause of death worldwide due to a single infectious agent.

Twenty-three per cent of the world's population is estimated to be latently infected with TB, identification of these cases is critical for TB control. However, current latent TB infection (LTBI) diagnosis faces several limitations which need to be addressed in order to improve TB control.

The traditional method used for LTBI screening, the tuberculin skin test (TST), has cross-reaction with the BCG vaccine and non-tuberculous mycobacteria (NTM) infection, and its performance is limited in patients with chronic inflammatory diseases on treatment with immunosuppressants as well as patients with other immunosuppressive diseases which affect cell response such as HIV infection. As an alternative to the TST, the interferon-gamma (IFN-γ) release assays (IGRAs) measure T-cell mediated response to specific *M. tuberculosis* antigens either by measuring the amount of IFN-γ produced (QuantiFERON) or the number of cells that produce IFN-γ (T-SPOT.TB). Due to the lack of cross-reaction issues previously mentioned, IGRAs have been extensively used. However, as happens with the TST, they do not give information on the present infection degree or stage, do not identify those cases with a higher risk of progression to active disease, nor differentiate between active disease and infection. In order to tackle these issues, several approaches have been studied, such as stimulation with different antigens, detection of cytokines other than IFN-γ, and search of other biomarkers. Recently, thanks to the increasing evidence of the performance of the IGRAs gathered through the last years and given the importance of LTBI screening in order to control the disease, the WHO updated the "LTBI guidelines for programmatic management" and included the use of IGRAs to test for LTBI worldwide. To increase the use of IGRAs in low- and middle-income countries and improve their reach in isolated rural areas, efforts in modifying the current methodology, such as simplifying sample manipulation and transport, are key.

As mentioned above, cross-reaction with NTM infections is a potential issue when it comes to TB infection diagnosis in those non-BCG vaccinated cases with a negative IGRA and a positive TST, especially in individuals at high risk of developing TB such as children. Nowadays there is no diagnostic method available to detect NTM infection, which, apart from helping in the diagnosis of these discordant cases, they could be helpful in children with lymphadenopathies caused by NTM, and in patients with chronic pulmonary diseases (CPD) such as bronchiectasis or cystic fibrosis with a positive NTM culture. In these cases, clinicians have often doubts regarding the relevance of the isolates; it is not clear whether the bacteria are colonizing or in fact causing disease. Having a diagnostic test able to differentiate between NTM disease and NTM colonization could help in the management of these patients.

In order to help solve these issues, the studies carried out for this thesis focused on different patients' cohorts including those at high risk of developing TB disease, detecting various cytokines and cell markers, and testing alternative specific antigens for sample stimulation.

The performance of IGRAs and IP-10 detection in patients with immune-mediated inflammatory diseases was tested (articles 1 and 2) and the impact of biological response modifiers on respiratory tract infections in this type of patients was reviewed (article 3). Furthermore, the use of QuantiFERON and its serial performance to predict TB disease progression in children was discussed (article 4). In order to study the use of other *M. tuberculosis* antigens as alternatives to the current IGRAs, different antigens both used individually and in combination, were evaluated in immunocompetent individuals with active TB, LTBI and TB uninfected (article 5 and annex 1). Focusing on different approaches and variations to improve the existing diagnostic methods, CD27 and CCR4 cell markers were evaluated using flow cytometry in order to differentiate active TB from LTBI (article 6). As an approach to improve sample transport and therefore increase the testing reach, IP-10 detection in dried plasma spots was evaluated after being sent by ordinary mail at room temperature (article 7). IP-10 mRNA detection was also studied as a molecular diagnostic approach for TB infection (annex 2). Regarding NTM infection diagnosis, stimulation of PBMCs with glycopeptidolipids (GPLs), specific antigens of NTM, was studied in different patient groups in order to characterize the immune response and evaluate their use in those cases in which TB diagnostic tests are discordant and also in patients with CPD with NTM isolate of unclear clinical relevance (article 8).

Although TB death rates have decreased in the last years, TB still affects millions of people each year with an estimate of 10 million new cases in 2017. TB infection diagnosis and proper handling of existing and new cases are key for keeping TB burden at a minimum. Focusing on those populations at high risk of developing the disease and reaching places where diagnosing new patients is a challenge, is of major importance. This thesis aims to go a few steps forward and help close some of the existing gaps in TB and NTM infection diagnosis.

Objectives

Individuals at high risk of developing TB:

1. Evaluate the use of IFN-γ and IP-10 detection to diagnose TB infection in individuals at high risk of developing active TB disease such as patients with immune-mediated inflammatory diseases and children (Articles 1, 2, 3 and 4).

New antigens:

2. Evaluate the use of immunogenic *M. tuberculosis* antigens in order to improve TB infection diagnosis in immunocompetent individuals (Article 5 and Annex 1).

New methodologies - improvement of the current ones:

3. Evaluate the use of CD27 and CCR4 detection in *M. tuberculosis* sensitized T-cells, by flow cytometry as a possible tool to distinguish between active TB disease and infection (Article 6).
4. Improve the technology of the current diagnostic tools to increase TB screening reach (Article 7 and Annex 2).

NTM infection:

5. Evaluate the use of GPLs for NTM infections detection to help with doubtful TB cases and aid in the handling of patients with chronic pulmonary diseases with NTM isolates of unclear clinical relevance (Article 8).

Results

Although according to the 2018 Global Tuberculosis Report[13], TB incidence worldwide is falling at about 2% per year, more needs to be done to improve disease control, management and prevention. The following studies were carried out and published in this Ph.D. thesis, with the aim of humbling aid this need, by creating new knowledge in the field of TB and NTM infection diagnostics.

These studies are grouped in four different blocks regarding their focus:

Individuals at high risk of developing TB:

Articles 1, 2, 3 and 4

New antigens:

Article 5 (and Annex 1)

New methodologies - improvement of the current ones:

Articles 6 and 7 (and Annex 2)

NTM infection:

Article 8

Article 1

Immune-mediated inflammatory diseases differently affect IGRAs' accuracy for latent tuberculosis infection diagnosis in clinical practice

Irene Latorre, Sonia Mínguez, José-Manuel Carrascosa, Juan Naves, **Raquel Villar-Hernández**, Beatriz Muriel, Cristina Prat, Esther García-García, Irma Casas, Eugeni Domènech, Carlos Ferrándiz, Lourdes Mateo, Jose Domínguez.

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Immune-mediated inflammatory diseases (IMIDs) differently affect IGRAs' performance for latent tuberculosis infection diagnosis in clinical practice

Latorre I, Mínguez S, Carrascosa JM, Naves J, Villar-Hernández R, Muriel B, Prat C, García-García E, Casas I, Domènech E, Ferrándiz C, Mateo L, Domínguez J

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Do immunosuppressants and IMIDs affect QFN-G-IT and T-SPOT.TB performance?

Materials & Methods

LTBI screening in:

- 50 patients with inflammatory rheumatic diseases
- 50 patients with psoriasis
- 30 patients with Crohn's disease
- 44 healthy individuals not immunosupressed

Results

1. Immunosuppressant > more frequent in Patients with Crohn's disease and psoriasis

2. Crohn's disease patients → ↓ Positive results; ↑ Indeterminate T-SPOT.TB results

3. IFN-γ after mitogen stimulation > in patients with psoriasis and controls

4. IFN-γ after mitogen stimulation and corticosteroids dose → Negative correlation

Conclusions

IMIDs NEGATIVELY AFFECT QFN-G-IT and T-SPOT.TB

Specially Crohn's disease

IMIDs seem to negatively affect the clinical performance of IGRAs, being Crohn's disease patients the most affected individuals due to their concomitant drug profile and impaired immune response.

IMIDs: immune mediated inflammatory diseases; QFN-G-IT: QuantiFERON-TB Gold In-Tube; TB: Tuberculosis

Raquel Villar Hernández

PLOS ONE

RESEARCH ARTICLE

Immune-mediated inflammatory diseases differently affect IGRAs' accuracy for latent tuberculosis infection diagnosis in clinical practice

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Data Availability Statement: There are ethical restrictions on sharing a de-identified data set, because of data contain potentially identifying/sensitive patient information. To share data will breach patient confidentiality. However, excerpts of the data will be available upon request to the Research Ethical Committee of the Institut d'Investigació Germans Trias i Pujol (contact email: ceic.germanstrias@gencat.cat) who will ensure that the data shared are in accordance with patient consent.

Abstract

Background

Clinical accuracy of IGRAs remains unclear on patients with immune-mediated inflammatory diseases (IMIDs). Here, we assess the impact of immunosuppressants and IMIDs on QuantiFERON-TB Gold In-Tube (QFN-G-IT) and T-SPOT.TB accuracy.

Methods

Patients with IMIDs who required latent tuberculosis infection (LTBI) screening were enrolled and classified into: (i) 50 patients with inflammatory rheumatic diseases, (ii) 50 patients with psoriasis and (iii) 30 patients with Crohn's disease. A total of 44 healthy individuals without immunosuppression were also included as controls. Tuberculin skin test (TST), T-SPOT.TB and QFN-G-IT assays were performed. IGRAs were performed following manufacturer's instructions.

Results

Immunosuppressant's intake was more frequent on patients with Crohn's disease and psoriasis. Positive IGRAs and TST results were reduced in Crohn's disease patients, whereas rate of indeterminate T-SPOT.TB results was increased in this group with respect to the other IMIDs analysed and controls. When IFN-γ response was studied, the levels of this cytokine after mitogen stimulation were significantly lower in Crohn's and inflammatory

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rheumatic diseases than in psoriasis. Interestingly, psoriatic patients were the only ones not receiving corticosteroids. Furthermore, a negative correlation was observed between the IFN-γ secreted after mitogen stimulation and corticosteroids dose.

Conclusions

IMIDs seem to negatively affect the clinical accuracy of IGRAs, being Crohn's disease patients the most affected individuals due to their concomitant drug-profile and impaired immune response.

Introduction

Patients with immune-mediated inflammatory diseases (IMIDs) have a higher risk of tuberculosis (TB) reactivation than common population because of the intake of different immunosuppressive therapies and/or their main disease itself. Tumour Necrosis Factor (TNF)-alpha (α) is a pro-inflammatory key cytokine playing an important role in the immune response against *Mycobacterium tuberculosis*. It is crucial for maintaining the granuloma formation and thus containing the infection caused by the bacilli. Therefore, ruling out latent tuberculosis infection (LTBI) and active TB is mandatory prior anti-TNF-αprescription as well as during treatment [1, 2].

Interferon (IFN)-gamma (γ) Release Assays (IGRAs) used for LTBI diagnosis detect the IFN-γ cytokine secreted by sensitized T-cells after stimulating with specific *M. tuberculosis* antigens [3]. There are two assay formats: QuantiFERON technology (QFN; Qiagen, Düsseldorf, Germany) and T-SPOT.TB (Oxford Immunotec, Abingdon, UK), both approved by the U.S. Food and Drug Administration (FDA) and the CE (for their use in Europe). Currently, these assays are useful tools for diagnosing LTBI because they are not affected by BCG-vaccination and/or non-tuberculous mycobacteria (NTM) sensitization [4–6]. In addition, IGRAs' methodology contain a positive control [stimulation with phytohaemagglutinin (PHA) as a mitogen] which detects the presence of anergy. This control is very useful in individuals with a weak immune response due to the intake of several immunosuppressant therapies or the presence of a certain underlying disease. Therefore, the inclusion of this mitogen could be particularly useful in patients with IMIDs [7]. However, clinical performance of IGRAs remains still unclear in these kind of patients. Currently, available data on the usefulness of IGRAs in patients with IMIDs is diverse due to the population heterogeneity and the variety of immunosuppressant drug-regimens analysed [8, 9]. Therefore, research in this direction should be further considered. In the present study, we have investigated the impact of different immunosuppressant therapies and IMIDs on QFN-TB Gold In-Tube (QFN-G-IT) and T-SPOT.TB assays.

Materials and methods

Study participants

A total of 130 patients and 44 healthy controls were enrolled in the study. Samples were collected from Rheumatology, Dermatology, Gastroenterology and Preventive Medicine Departments located in Hospital Universitari Germans Trias I Pujol (Badalona, Spain). The study was approved by the Ethics Committee of the Hospital Germans Trias i Pujol. All experiments were performed in accordance with the relevant guidelines and regulations. All the patients

enrolled in this study gave a written informed consent. A total of 11 mL of blood was collected from each patient.

Tuberculin skin testing

Tuberculin skin test (TST) was administered using the Mantoux method and interpreted according to Spanish Society of Pneumology Guidelines by qualified members of the research team. TST was considered positive when the induration was higher than 5mm [10]. In order to avoid TST booster effect over IGRAs, TST and blood extraction for T-SPOT.TB and QFN-G-IT were performed simultaneously [11]. The study was double-blinded: TST was interpreted without knowing IGRAs' results, and at the same time, researchers did not know the clinical data and the TST result prior to performing the tests.

IFN-γ release assays and results interpretation

T-SPOT.TB and QFN-G-IT assays were performed and interpreted following manufacturer's instructions. For T-SPOT.TB assay a total of 8 mL of blood were collected. Spot-forming cells (SFC) were counted using an automated AID ELISPOT reader (AID Systems, Strasberg, Germany). The results were also checked and validated by naked eye. The assay was considered positive when the test wells contained at least six SFC more than the negative control, and this number was at least twice the number of the negative control well. The result was considered indeterminate if both antigen-stimulated wells were negative and if the number of SFC in the positive control was less than <20; and/or if SFC counted in the negative control well was>10. For QFN-G-IT a total of 3 mL of blood was collected into three tubes (negative, TB-Antigen and positive). For this assay, a result was considered positive when the IU/mL of IFN-γ secreted was ≥0.35 for the TB-Antigen tube. A result was scored as indeterminate when the TB-antigen tube was negative and the mitogen tube presented less than 0.5 IU/mL of IFN-γ secreted (positive control); and/or if the negative control tube was higher than 8.0 UI/mL. Measurements >10IU/mL in QFN-G-IT were set as 10IU/mL according to the upper limit of the assay and manufacturer's instructions.

Statistical analysis

Differences on the assay results based on the clinical therapeutic profile and the underlying disease was assessed and analysed using the Chi-square test. Association between the IFN-γ response and corticosteroid doses was assessed using Spearman correlation coefficient. The amount of IFN-γ secreted was investigated as well in each group. In this case, comparisons among the three study groups were performed using the Kruskall-Wallis test. In addition, Mann-Whitney U test was applied for pairwise comparisons. Differences were considered statistical significant when a p-value was <0.05. All analyses were performed using the SPSS statistical software for windows (SPSS version 15.0; SPSS Inc, Chicago, IL, USA). Graphical representation is based on GraphPad Prism version 4 (GraphPad Software, Inc, San Diego, CA).

Results

Patient characteristics and concomitant drug-profile when IGRAs where performed

We enrolled a total of 130 IMID patients who required LTBI screening before starting systemic immunosuppressive treatment or during its sustained use. Patients were screened in the course of their routine examinations and had no known risk of TB exposure. They were classified into three groups: (i) 50 patients with inflammatory rheumatic diseases, (ii) 50 patients with

moderate-to-severe psoriasis and (iii) 30 patients with Crohn's disease. A total of 44 healthy individuals without immunosuppression were also included as controls. They were healthcare workers with no risk of TB exposure and recruited in the course of routine examinations.

Patient characteristics and their specific underlying disease regarding the study group is detailed in Table 1. Briefly, the proportion of patients on immunosuppressants (DMARDs or biologics) when IGRAs were performed varied significantly regarding the underlying disease ($p = 0.016$). It was higher in patients with Crohn's disease (90%; 27/30) and psoriasis (82%; 41/50) with respect to inflammatory rheumatic patients (64%; 32/50). The 75% (24/32) of rheumatic patients were receiving one DMARD and the 25% (8/32) were taking two. The 46.3% (19/41) of patients with psoriasis were receiving classic systemic treatment (cyclosporine or methotrexate) and the 53.7% (22/41) were already taking biologicals (infliximab, etanercept, adalimumab or ustekinumab). The 81.5% (22/27), 11.1% (3/27) and 7.4% (2/27) of Crohn's disease patients were treated with azathioprine, methotrexate and biologicals (infliximab) respectively. Corticosteroid therapy was frequent in patients with inflammatory rheumatic diseases (40%; 20/50) and Crohn's disease (33.3%; 10/30), while it was absent in psoriatic patients (Table 1).

IGRAs' clinical accuracy regarding IMID type

Overall, the percentage of positive results by either of the two IGRAs were 22.7% (10/44), 22% (11/50), 24% (12/50), and 13.3% (4/30) in healthy controls, patients with psoriasis, rheumatic

Table 1. Patients' characteristics, final diagnosis and present treatment regarding IMID type.

VARIABLE	Psoriasis n = 50 (%)	Inflammatory rheumatic diseases n = 50 (%)	Crohn's disease n = 30 (%)	Healthy controls n = 44 (%)
Mean age (years) ± SD	45.52±13.05	49.58±13.11	38.16±13.94	36.21±7.22
Gender				
Male	37 (74)	17 (34)	16 (53.3)	10 (22.7)
Female	13 (26)	33 (66)	14 (46.7)	34 (77.3)
Diagnosis				
Moderate-to-severe psoriasis	50 (100)	0 (0)	0 (0)	N/A
Rheumatoid arthritis	0 (0)	16 (32)	0 (0)	N/A
Ankylosing spondylitis	0 (0)	12 (24)	0 (0)	N/A
Psoriatic arthritis	0 (0)	9 (18)	0 (0)	N/A
Crohn's disease	0 (0)	0 (0)	30 (100)	N/A
Other*	0 (0)	13 (26)	0 (0)	N/A
Present corticoids				
Yes	0 (0)	20 (40)	10 (33.3)	N/A
No	50 (100)	30 (60)	20 (66.7)	N/A
Present DMARDs				
Yes	19 (38)	32 (64)	25 (83.3)	N/A
No	31 (62)	18 (36)	5 (16.7)	N/A
Present biologics				
Yes	22 (44)	0 (0)	2 (6.7)	N/A
No	28 (56)	50 (100)	28 (93.3)	N/A

SD: standard deviation

*Systemic lupus erythematosus (n = 4), SAPHO syndrome (n = 4), seronegativepolyarthritis (n = 3), undifferenciated spondiloarthropaty (n = 1) and spondylitis associated with inflammatory bowel disease (n = 1).

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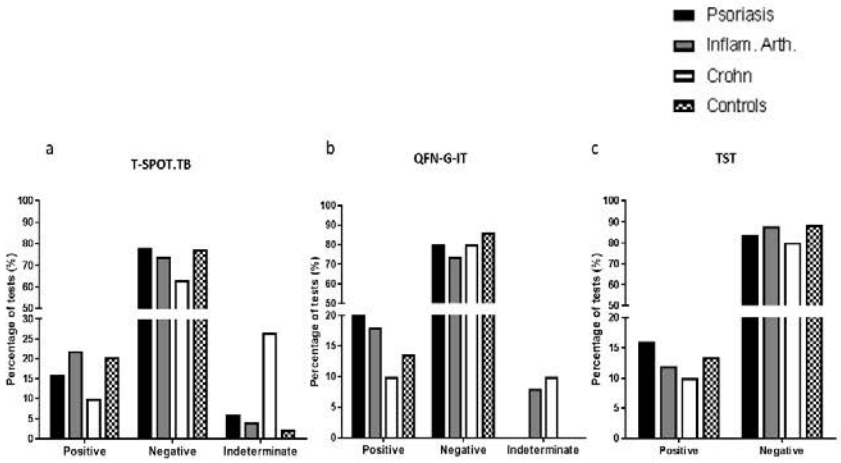

Fig 1. Percentage of positive, negative and indeterminate results. Percentages are represented for (a) T-SPOT.TB, (b) QFN-G-IT and (c) TST in patients with psoriasis (black bars), inflammatory arthritis diseases (grey bars), Crohn disease (white bars) and healthy controls (spot bars). Inflam. Arth: Inflammatory arthritis; QFN-G-IT: QuantiFERON-TB Gold In-Tube; TST: Tuberculin skin test.

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inflammatory diseases and Crohn's disease respectively. In addition, a considerable percentage of IMIDs patients with negative TST (12.7%; 14/110) were positive for one of the two IGRAs. The percentage of positive results in patients with psoriasis, inflammatory arthritis, Crohn's disease and healthy controls is represented for the three assays in Fig 1A–1C. The rate of positive results obtained by T-SPOT.TB, QFN-G-IT and TST was higher in patients with inflammatory rheumatic diseases, psoriasis and healthy controls with respect to Crohn's disease individuals (differences not significant). Indeterminate results were obtained by T-SPOT.TB and QFN-G-IT in a 10% [13/130; 10 cases due to insufficient mitogen (PHA) response and 3 due to high response in negative control) and a 5.4% (7/130; all due to insufficient PHA response)] of the cases respectively. All indeterminate results due to insufficient PHA response corresponded with negative TSTs. The percentage of indeterminate results observed by T-SPOT.TB in patients with Crohn's disease diagnosis was significantly higher than in rheumatic patients, those diagnosed with psoriasis and healthy controls ($p = 0.003$, $p = 0.009$ and $p = 0.002$ respectively). Seven out of the eight Crohn's disease patients with indeterminate results corresponded to individuals receiving azathioprine. Furthermore, three of them were receiving corticosteroid therapy. Concordances between both IGRAs were good when they were analysed globally ($\kappa = 0.817$) and based on the study groups ($\kappa = 0.849$ for rheumatic inflammatory diseases, $\kappa = 0.799$ for psoriasis and $\kappa = 0.773$ for Crohn's disease). By means of an univariate analysis, no significant differences were observed between the results obtained by either IGRAs or TST with biologics, corticoids or DMARDs received (Table 2).

Impact of immunosuppressive therapies on the IFN-γ response

The SFC and the IFN-γ response in T-SPOT.TB and QFN-G-IT was assessed in those positive responders after specific antigen stimulation, and evaluated regarding the underlying inflammatory disease. No significant differences were found between study groups ($p = 0.101$ and $p = 0.574$ respectively, data analysed with Kruskall-Wallis test). In order to further study the impact of different IMIDs on clinical assay accuracy, the amount of IFN-γ released after PHA

Table 2. Association of the immunosuppressant treatment received with TST, QFN-G-IT and T-SPOT.TB positivity by means of univariate analysis.

Risk Factors	TST; n (%)			QFN-G-IT; n (%)			T-SPOT.TB; n (%)		
	Positive	Negative	p-value	Positive	Negative	p-value	Positive	Negative	p-value
Total[a]	17	110	NA	22	101	NA	22	95	NA
Mean age (years) ± SD[b]	52.53 ±12.50	44.42 ±14.47	0.031	57.19 ±10.68	43.62 ±13.81	<0.0001	56.76 ±13.16	43.71 ±12.64	<0.0001
Gender									
Male	11 (64.7)	56 (50.9)	0.289	18 (81.8)	51 (50.5)	0.007	17 (77.3)	46 (48.4)	0.014
Female	6 (35.3)	54 (49.1)		4 (18.2)	50 (49.5)		5 (22.7)	49 (51.6)	
Present immunosuppressants (DMARDs or biologics)									
Yes	12 (70.6)	85 (77.3)	0.548[c]	17 (77.3)	78 (77.2)	0.996	15 (68.2)	76 (80.0)	0.259[c]
No	5 (29.4)	25 (22.7)		5 (22.7)	23 (22.8)		7 (31.8)	19 (20.0)	
Present DMARDs									
Yes	9 (52.9)	66 (60.0)	0.582	11 (50.0)	60 (59.4)	0.418	11 (50.0)	58 (61.1)	0.342
No	8 (47.1)	44 (40.0)		11 (50.0)	41 (40.6)		11 (50.0)	37 (38.9)	
Present biologics									
Yes	3 (17.6)	19 (17.3)	1.000[c]	6 (27.3)	18 (17.8)	0.373[c]	4 (18.2)	18 (18.9)	1.000[c]
No	14 (82.4)	91 (82.7)		16 (72.7)	83 (82.2)		18 (81.8)	77 (81.1)	
Present corticoids									
Yes	4 (23.5)	26(23.6)	1.000[c]	5 (22.7)	22(21.8)	0.923	5 (22.7)	22(23.2)	0.966
No	13 (76.5)	84(76.4)		17 (77.3)	79(78.2)		17 (77.3)	73(76.8)	

NA: non-applicable; SD: standard deviation. P vàlues less than 0.05 were considered significant.

[a]Excluding indeterminate QFN-G-IT and/or T-SPOT.TB results.

[b]Significance on age was calculated using Student's T test.

[c]Significance was calculated using Fisher's Exact test, since the 25% of expected frequency were less than 5.

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stimulation in QFN-G-IT was analysed in the overall population. Interestingly, in Crohn's and rheumatic disease patients, the IFN-γ cytokine secreted by sensitized T cells after PHA stimulation was significantly lower with respect to psoriasis and healthy controls (Fig 2; $p<0.0001$ for all comparisons). Psoriatic patients were the only ones not receiving corticosteroids. A correlation between the IFN-γ secreted after mitogen stimulation and dose (mg/day) of corticosteroid administered was performed. There was a negative moderate correlation (Fig 3; Spearman's Rho = -0.489; $p = 0.007$).

Discussion

Investigations on IGRAs in high risk individuals are still limited, and it is necessary to compare and study how IMIDs with different immunosuppressive regimen can affect *in vitro* assays' accuracy. Our research group have previously assessed in two independent studies the usefulness of IGRAs on patients with arthritic [12] and psoriatic [13] diseases, indicating that IGRAs seem to be helpful for LTBI diagnosis in such populations. In both studies we found that positive results for T-SPOT.TB/QFN-G-IT and the amount of T-cell response were not affected by clinical therapeutic profile. However, the effect of therapy on IFN-γ detection may depend on IMID type and its concomitant therapy. Here, we compare three different types of IMIDs (psoriasis, rheumatic inflammatory disease and Crohn's disease) from patients enrolled in the same study setting. Furthermore, the study shows that accuracy of T-SPOT.TB and QFN-G-IT is differentially affected regarding the IMID type and its concomitant drug-treatment. The

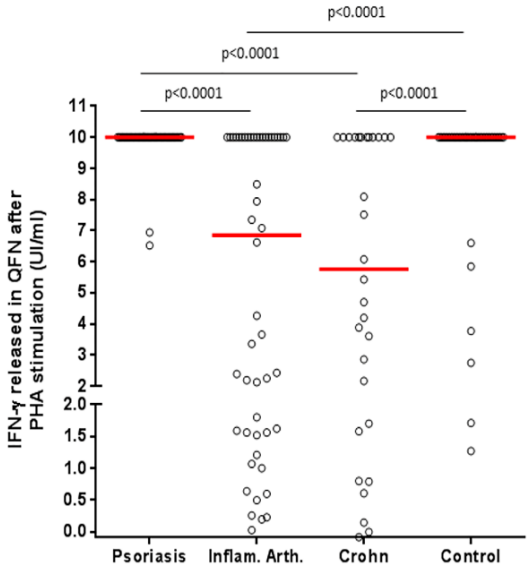

Fig 2. IFN-γ released after mitogen (PHA) overnight stimulation (Mit-Nil) in QFN-G-IT based on the study group of patients analyzed. The median cytokine levels from each group is represented by a line. Mann-Whitney U analysis was applied for pairwise comparisons. Measurements >10IU/mL were set as 10IU/mL according to the upper limit of the assay and manufacturer's instructions. Only significant differences were indicated in the figure. It was not possible to assess the IFN-γ response after PHA stimulation in T-SPOT.TB assay due to saturation in the control well (>250 spot-forming cells). Inflam. Arth: Inflammatory arthritis; PHA: Phytohaemagglutinin; QFN-G-IT: QuantiFERON-TB Gold In-Tube.

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overall number of positive results obtained here by TST and IGRAs were reduced in patients suffering Crohn's disease with respect to those with rheumatic disorders or psoriasis. Similarly, Ramos JM. et al. conducted a study using QFN-G-IT and TST assays in our region, observing that positive results were negatively affected by immunosuppressive therapy in patients with inflammatory bowel diseases. However indeterminate results obtained were low, therefore, no associations were established [14]. Low rates of positive IGRAs results on patients with inflammatory bowel diseases under azathioprine have been previously mentioned by others when they are compared with bowel disease patients under other drug-regimens or without immunosuppressive treatment [15]. Interestingly, the majority of Crohn's disease patients included in our study were under this immunosuppressant drug.

Generally, the frequency of QFN-G-IT indeterminate results has been linked to patients with chronic inflammatory diseases under glucocorticoid use [8, 16–19]. In line with this, our results reveal that number of IGRA's indeterminate results is even higher in Crohn's disease patients under azathioprine. Moreover, the induced-mitogen response was reduced in this group and also in patients with rheumatic diseases with respect to those patients with psoriasis. Intriguingly, the 33.3% of Crohn's disease patients and the 40% of individuals with rheumatic diseases were on corticosteroid therapy. Bélard E. et al. found a significant negative effect of prednisolone on IFN-γ response, indeterminate QFN-G-IT results and positive TST results. They also compared the IFN-γ response after mitogen stimulation in several therapy groups, and found that it was reduced in patients with corticosteroid treatment when they were compared with those not receiving treatment or other therapy without corticosteroid [16]. In order to study whether the immune response was influenced by corticosteroid treatment, we

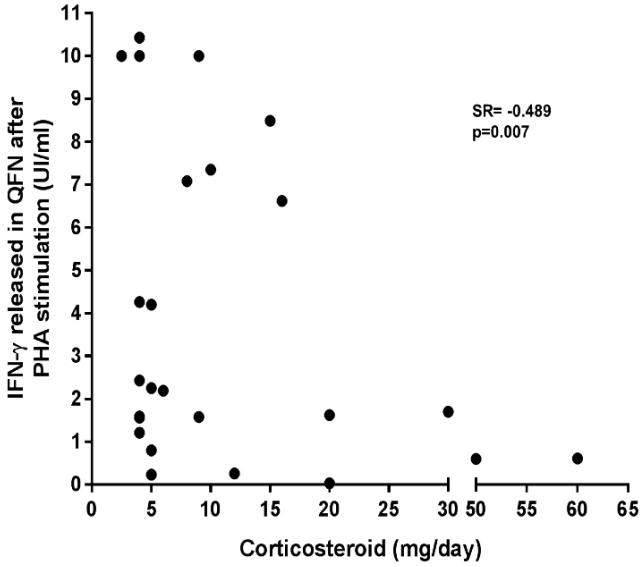

Fig 3. Correlation between the IFN-γ secreted after mitogen (PHA) stimulation and dose (mg/day) of corticosteroid administered. Association between the IFN-γ response on QFN-G-IT and corticosteroid doses was assessed using Spearman correlation coefficient. Measurements >10IU/mL were set as 10IU/mL according to the upper limit of the assay and manufacturer's instructions. It was not possible to assess the IFN-γ response after PHA stimulation in T-SPOT.TB assay due to saturation in the control well (>250 spot-forming cells). PHA: Phytohaemagglutinin; QFN-G-IT: QuantiFERON-TB Gold In-Tube; SR: Spearman's Rho.

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correlated both IFN-γ released upon PHA stimulation with dose (mg/day) of corticosteroid administered, observing that this response diminished when the dose of corticosteroids augmented. In addition, a recent study has assessed the impact of corticosteroids on QFN-G-IT suggesting that they can significantly impair IGRAs accuracy [20]. Consequently, we have to pay attention when ruling out LTBI in this specific group of patients due to false-negative TST/IGRAs and low IFN-γ cytokine secretion that can lead to indeterminate results. In this direction, some studies report that a long *in vitro* stimulation of T cells (6–9 days) can detect a memory response and may be can be used to identify LTBI individuals who resulted to have negative IGRAs. More studies need to confirm these data, however, this could be a useful tool on patients under immunosuppressive treatment who have high risk of developing TB [21–23].

Taken together, here we describe how IMIDs can negatively affect the accuracy of IGRAs, being Crohn's disease patients the most affected individuals due to their concomitant immunosuppressant drug-profile and impaired immune response. Therefore, in the absence of a gold standard, it seems prudent to diagnose LTBI using both the TST and IGRAs with the aim of detecting all possible cases in patients with IMIDs [24, 25], giving special attention to those with a poor accuracy of IGRAs and/or TST such as Crohn's disease. In addition, the use of TST in psoriasis has to be taken with caution. Previous observations indicate that psoriasis can produce a TST hyper-reactivity due to a skin over-reaction to a broad range of antigens. Therefore, it is necessary to re-evaluate a positive TST reaction in these patients because in most situations it could lead to a LTBI over-diagnosis [26].

It is still necessary to make efforts for an accurate LTBI diagnosis in IMIDs' patients. In this direction, a new improved IGRA version called QFN-Plus has appeared. This assay includes new designed peptides form *M. tuberculosis* capable to induce specific CD4 and CD8 T cells. Data about its better accuracy over classical IGRAs is still limited, and there are not yet results evidencing a better sensitivity and specificity in this population [27–30]. Another encouraging approach seems to be the study of other cytokines different from IFN-γ. In the last years, the cytokine called IFN-γ-induced protein-10 (IP-10) has emerged as a promising biomarker for LTBI diagnosis that enables to rise sensitivity in conjunction with IFN-γ [31–34]. We should focus investigations on this direction: improving LTBI diagnostics on high-risk individuals, increasing sensitivity and minimizing false-negative and indeterminate results.

In conclusion, clinical accuracy of IGRAs for LTBI diagnosis seems to be differentially affected by the IMID type. Particularly, Crohn's disease and/or its concomitant immunosuppressive drug-profile could negatively affect accuracy of T-SPOT.TB and QFN-G-IT when compared with psoriasis orinflammatory rheumatic diseases. Therefore, it is important to be prudent when diagnosing LTBI in this kind of patients due to the high frequency of indeterminate results and an attenuated IFN-γ response.

Author Contributions

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Validation: Irene Latorre, Raquel Villar-Hernández.

Visualization: Irene Latorre, Raquel Villar-Hernández, Jose Domínguez.

Writing – original draft: Irene Latorre, Jose Domínguez.

Writing – review & editing: Irene Latorre, Sonia Mínguez, José-Manuel Carrascosa, Juan Naves, Raquel Villar-Hernández, Beatriz Muriel, Cristina Prat, Esther García-García, Irma Casas, Eugeni Domènech, Carlos Ferrándiz, Lourdes Mateo, Jose Domínguez.

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Article 2

Use of IFN-γ and IP-10 detection in the diagnosis of latent tuberculosis infection in patients with inflammatory rheumatic diseases

Raquel Villar-Hernández, Irene Latorre, Sonia Mínguez, Jéssica Díaz, Esther García-García, Beatriz Muriel-Moreno, Alicia Lacoma, Cristina Prat, Alex Olivé, Morten Ruhwald, Lourdes Mateo, José Domínguez.

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USE OF IFN-γ AND IP-10 DETECTION IN THE DIAGNOSIS OF LATENT TUBERCULOSIS INFECTION IN PATIENTS WITH INFLAMMATORY RHEUMATIC DISEASES

Raquel Villar-Hernandez; Irene Latorre; Sonia Mínguez; Jéssica Díaz; Esther García-García; Beatriz Muriel-Moreno; Alicia Lacoma; Cristina Prat; Álex Olivé; Morten Ruhwald; Lourdes Mateo; Jose Dominguez

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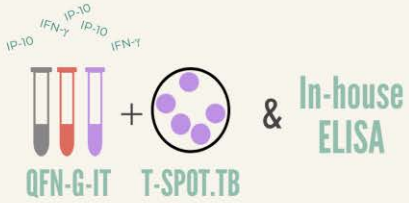
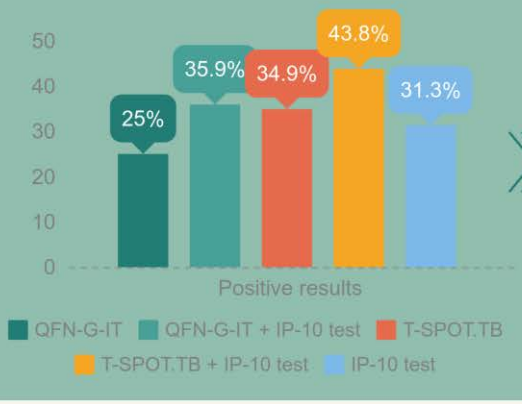
Biologic agents used by rheumatic patients increase risk of TB developement

Can we use **IP-10** detection to diagnose LTBI in rheumatic patients on different immunossuppressive therapy?

64 patients with inflammatory rheumatic disease included



Detect **IFN-γ** & **IP-10**



There is an increase of positive results when tests are combined
($p < 0.001$)

Treatment intake had no effect on in vitro test performance
($p > 0.05$)

IP-10 and **IFN-γ** detection is comparable and their combined use could increase the number of positive results in the diagnosis of LTBI in rheumatic patients

The tested assays were not influenced by rheumatoid immunosuppressive therapy in our cohort

IP-10 could be of use in the development of new and improved LTBI diagnostic tools

TB: tuberculosis; LTBI: latent tuberculosis infection; IFN-γ: Interferon gamma; IP-10: IFN-γ inducible protein 10; QFN-G-IT: QuantiFERON Gold In-Tube

Raquel Villar Hernández

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Use of IFN-γ and IP-10 detection in the diagnosis of latent tuberculosis infection in patients with inflammatory rheumatic diseases

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Tuberculosis infection;
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QuantiFERON;
T-SPOT.TB;
IGRAs

Summary *Objectives:* Biologic agents are used against rheumatic diseases, however, they increase the risk of developing severe infections and diseases such as tuberculosis. We aimed to determine the benefits of IP-10 detection to diagnose latent tuberculosis infection (LTBI) in patients with inflammatory rheumatic diseases on different immunosuppressive drug regimens, and compare these results with IFN-γ detection.
Materials and methods: We included 64 patients with inflammatory rheumatic diseases. We used QuantiFERON Gold In-Tube (QFN-G-IT) and T-SPOT.TB to detect IFN-γ production, and an in-house ELISA for IP-10 detection from the previous QFN-G-IT stimulated samples. We

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assessed the combined use of IFN-γ release assays (IGRAs) and IP-10 test, and analyzed the influence of immunotherapy on the tests performance.
Results: We obtained 34.9% positive results by T-SPOT.TB, 25.0% by QFN-G-IT and 31.3% by IP-10 test. The combined use of IGRAs and IP-10 detection increased significantly the amount of positive results ($p < 0.0001$). Treatment intake had no significant effect on *in vitro* tests ($p > 0.05$).
Conclusions: IP-10 and IFN-γ detection is comparable and their combined use could increase the number of positive results in the diagnosis of LTBI in rheumatic patients. The tested assays were not influenced by rheumatoid immunosuppressive therapy. Thus, IP-10 could be of use in the development of new and improved LTBI diagnostic tools.

Introduction

Biologic agents that target different levels of the immune response such as IL-1, IL-6, IL-12-23, CD20, and anti-tumor necrosis factor alpha agents (anti-TNF-α), among others, are often used to treat several rheumatic diseases.[1] However, although biologic agents are beneficial for a number of rheumatic diseases, anti-TNF-α agents and non-anti-TNF-α agents to a lesser extent, are related to a risk increase of developing severe infections such as active tuberculosis (TB), by newly acquired infection and/or reactivation.[2–10] Among others, TNF-α is one of the key molecules involved in granuloma formation and maintenance of *Mycobacterium tuberculosis* infection control in the host, therefore, before starting a treatment based on TNF-α inhibition and other biologic agents, it is necessary to screen for latent TB infection (LTBI) to avoid developing active TB as well as excluding current active TB in the candidate population.[11] Several guidelines have been described to address this matter.[4,12]

LTBI screening is classically based on the tuberculin skin test (TST). However, it has some limitations such as cross-reaction with the bacilli Calmette-Guérin (BCG) vaccine strain of *Mycobacterium bovis* and non-tuberculous mycobacteria (NTM).[13] Moreover, the majority of patients with chronic inflammatory diseases are already treated with immunosuppressants including corticosteroids and other disease-modifying anti-rheumatic drugs (DMARDs) prior to anti TNF-α therapy, thus, this group of patients may not be able to produce an adequate response to TST due to their deficient cell-mediated immunity.[2,3,14] Interferon (IFN)-γ release assays (IGRAs) have emerged as an alternative for TST. IGRAs measure T-cell-mediated responses after specific *M. tuberculosis* antigen stimulation either from peripheral blood lymphocytes (T-SPOT.TB; Oxford Immunotec Limited, Abingdon, UK) or whole blood (QuantiFERON-TB Gold In-Tube [QFN-G-IT]; Qiagen, Düsseldorf, Germany).

In immunosuppressed patients, especially patients with immune-mediated inflammatory diseases (IMIDs), the TST has a low sensitivity and despite the promising results, the clinical test performance of the IGRAs remains unknown.[15–17] Every study so far has described discordant results between the IGRAs and the TST.[15] Most of these discordant results could be attributed to the BCG vaccination, but also to previous sensitization to NTMs.[18]

IFN-γ inducible protein 10 (IP-10) is a chemokine mainly expressed by antigen-presenting cells, and induced by innate and adaptive mechanisms.[19] Its expression is stimulated at the transcription level by multiple signals; mainly T cell derived IFN-γ and TNF-α, but also IL-2, type II IFNs, IL-27, IL-17/IL-23 and IL-1β.[20] TNF-α alone is a weak IP-10 inducer, but a potent synergic inducer with the IFNs.[20,24] During adaptive immune response, its secretion is mainly driven by T-cell-derived IFN-γ.[19,25] IP-10 levels have been related to severity degree, infection susceptibility and to treatment effect (among others) not only in infectious diseases such as hepatitis C (HCV) and TB, but also in several autoimmune diseases, such as rheumatoid arthritis.[26–31] This suggests that IP-10 could be a promising diagnostic marker for infectious and autoimmune diseases.[25,32] Moreover, it has been reported that IP-10 levels are 100-fold higher than those of IFN-γ, therefore enabling a good option for new possibilities in assay simplification and miniaturization such as lateral flow, dried blood spots and molecular detection.[25,33] IP-10 detection is suggested to be more robust than IFN-γ in HIV-infected patients and young children, and it could be used in combination with IGRAs.[34–38] However, its role in LTBI diagnosis in rheumatic patients is still unknown. To fill in this knowledge gap, the aims of this study were to determine the utility and benefits of IP-10 detection by means of an in-house ELISA, for LTBI diagnosis in patients with inflammatory rheumatic diseases on different immunosuppressive drug-regimens, and compare these results with IFN-γ detection (T-SPOT.TB and QFN-G-IT). The impact of the immunosuppressive treatment on the immunological techniques results was also analyzed.

Material and methods

Study setting and patient recruitment

We included patients with inflammatory rheumatic diseases which attended the Department of Rheumatology of the Hospital Universitari Germans Trias i Pujol (Badalona, Spain) between January 2010 and February 2012. The study was approved by the corresponding Ethics Committee. Each patient gave written informed consent before blood sampling and a detailed questionnaire with the following variables was completed: age, gender, country of birth, treatment regimens, date and result of prior TST, TB known contact, history of prior TB, chest x-ray as well as other clinical and demographic information.

Tuberculin skin test

Tuberculin skin tests (TST) were performed following Spanish guidelines[39] and using the Mantoux method with 2-TU of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark). Briefly, 0.1 ml of PPD solution was injected intradermally in the patients' forearm by experienced personnel. The induration diameter was measured after 48—72 h. A positive TST was given when the induration was >5 mm regardless BCG vaccination status. Patients with a negative result (<5 mm induration) had the TST repeated after 7—10 days (booster effect). In this case, an induration >5 mm was considered positive as well.[40]

IFN-γ release assays (IGRAs)

In order to detect the production of IFN-γ, we used the QFN-G-IT and the T-SPOT.TB procedures. We followed the manufacturer's instructions to perform each method. Briefly, regarding the QFN-G-IT procedure, 3 ml of blood (1 ml per tube; nil, antigen [RD1 antigens cocktail: ESAT-6, CFP-10, and TB7.7] and mitogen tubes) was extracted from each patient. The nil tube reactivity was subtracted from the IFN-γ value in antigen and mitogen tubes. A result was considered positive when the amount of IFN-γ was at least 0.35 IU/ml after antigen stimulation. A result was considered indeterminate when the stimulation was negative and the value of the positive control was less than 0.5 IU/ml or if the negative control was higher than 8.0 IU/ml. For the T-SPOT.TB, 8 ml of blood from every patient was drawn into a CPT tube (Becton Dickinson Diagnostics, Franklin Lakes, NJ) for the isolation of peripheral blood mononuclear cells (PBMCs). The PBMCs isolation and the detection of IFN-γ by enzyme-linked immunospot assay (ELISPOT) after stimulation with RD1 antigens (ESAT-6 and CFP-10 separately) were done as described previously.[40] Tested wells were considered positive if they contained at least six spot forming cells (SFC) more than the negative control well and if this number was at least twice the number of the negative control well. A result was considered indeterminate if the response to both antigen panels were negative and if the number of SFCs in the control positive well was less than 20. The immune response was also considered indeterminate if the number of SFCs in the negative control was greater than 10. When considering the quantitative RD1 response, the number of SFCs was obtained counting the two stimulation wells containing ESAT-6 and CFP-10 specific antigens.

IP-10 detection by an in-house ELISA

IP-10 was retrospectively detected in the previously QFN-G-IT analyzed plasma by a non-commercial in-house ELISA.[41] Plasma samples were diluted 30 times and IP-10 levels were measured as previously described.[20] Briefly, plasma samples were diluted first in dilution buffer and then in dilution buffer with HRP-conjugated detection antibody. After a 2 h incubation in an ELISA plate pre-coated with monoclonal antibodies specific for IP-10,[20] the plate was washed, TMB substrate was added, and after a 30 min incubation the reaction was stopped using H_2SO_4, and the absorbance was read (450 nm—630 nm). The nil stimulated samples reactivity was subtracted from the IP-10 value in antigen (ESAT-6 and CFP-10) and mitogen stimulated samples. A result was considered positive when the IP-10 level was, at least, 2.3 ng/ml after antigen stimulation. A result was considered indeterminate when the stimulation was negative and the value of the positive control was below 1.5 ng/ml. These cut-off values give the highest sensitivity with minimal specificity loss and were set up using ROC curve analysis as previously described.[41]

Statistical analysis

The concordance of the tests was assessed using Cohen's kappa (κ) coefficient, excluding indeterminate results. K values below 0.40 indicate a weak agreement, values from 0.40 to 0.60 indicate an intermediate agreement and values over 0.60 indicate a strong agreement. To compare the IFN-γ and IP-10 levels released in each of the 3 QFN-G-IT test tubes, we performed a Kruskal—Wallis one-way analysis. We used Mann—Whitney *U* test for pairwise comparisons with a Bonferroni—Dunn post hoc correction which considers significant a *p*-value of 0.016. To study the proportion of positive results obtained by using a combination of IGRAs and IP-10 test, we used contingency tables and analyzed the significance of the differences by Pearson's chi-squared test. We analyzed the association between positive test results and the different variables included in this study using univariate analysis. We also used Pearson's chi-squared test analysis to calculate the differences between the results of each IGRA and IP-10 test in order to study the influence of previous and/or actual treatment with DMARD, corticosteroids and/or biologic therapy. We considered as significant *p*-values those under 0.05. Analysis and graphs were performed using SPSS statistical software (SPSS version 15.0; SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 5.00 (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Results

Study population

A total of 64 patients with inflammatory rheumatic diseases were included in this study. Demographic and clinical data is shown in Table 1. Thirty-four (53.1%) patients were taking at least one DMARD at the time of the IGRA performance. The most common DMARD used within our study group was methotrexate (MTX) (29/34; 85.3%), followed by leflunomide (15/34; 44.1%). Thirty-one (48.4%) patients were receiving corticosteroids at the moment of the IGRA performance with an average daily dose of 22.14 mg/day ± standard deviation (SD) of 88.94. Twenty-four (37.5%) patients were already receiving biologic treatment (17/24, 70.3% were anti-TNFs) at the time of the IGRA performance being adalimumab the most prescribed (5/24; 20.8%). At the moment of the IGRAs performance, 34.4% (22/64) and 12.5% (8/64) of the patients were receiving two and all three (respectively) types of therapies combined (DMARDS, corticosteroids and biologics).

Table 1 Demographic and clinical characteristics of all rheumatic patients included in the study.

Variable	Number of patients (%) in each group, $n = 64$
Age, mean (years) ± SD	52.03 ± 12.0
Gender	
Male	23 (35.9)
Female	41 (64.1)
Country of birth	
High TB incidence	9 (14.1)
Low TB incidence	55 (85.9)
Diagnosis	
Rheumatoid arthritis	27 (42.2)
Psoriatic arthritis	14 (21.9)
Ankylosing spondylitis	6 (9.4)
Spondyloarthropathies	4 (6.2)
Other[a]	13 (20.3)
Previous DMARD[b] treatment received	
Yes	47 (73.4)
No	17 (26.6)
No. of previous DMARD	
0	17 (26.6)
1	15 (23.4)
2	22 (34.4)
3	7 (10.9)
4	1 (1.6)
5	2 (3.1)
DMARD when *in vitro* tests were performed	
Yes	34 (53.1)
No	30 (46.9)
No. of DMARD when *in vitro* tests were performed	
0	30 (46.9)
1	23 (35.9)
2	10 (15.6)
3	1 (1.6)
Previous corticosteroid received	
Yes	41 (64.1)
No	23 (35.9)
Corticosteroid received when *in vitro* tests were performed	
Yes	31 (48.4)
No	33 (51.6)
Baseline corticosteroid dose (mg/day) ± SD	22.14 ± 88.94
No. of years on corticosteroid therapy mean ± SD	2.29 ± 2.85
On biologic treatment when *in vitro* tests were performed	
Anti TNF-α	17 (26.6)
Non-anti TNF-α	7 (10.9)
No	40 (62.5)
Combined therapy when *in vitro* tests were performed	
DMARD + corticosteroid	13 (20.3)
DMARD + biologic treatment	3 (4.69)
Corticosteroid + biologic treatment	6 (9.3)
Corticosteroid + biologic treatment + DMARD	8 (12.5)

[a] Idiopathic erythema nodosum (2 cases), seronegative erosive arthritis (1 case), SAPHO syndrome/psoriatic arthritis (1 case), Still disease (1 case), tuberculous arthritis of 2nd MCF (1 case), spondyloarthritis HLAB27+ (1 case), spondyloarthritis possibly psoriatic (1 case), tuberculous spondylodiscitis (1 case), oligoarthritis HLAB27 positive (1 case), seronegative oligoarthritis (1 case), systemic lupus erythematosus (1 case), Vogt-Koyanagi-Harada syndrome (1 case).
[b] DMARD disease-modifying anti-rheumatic drugs.

TST, IGRAs and IP-10 assay performance

TST was performed in 51 patients (79.7%) yielding 13 (25.5%) of positive results. Regarding the *in vitro* tests, the QFN-G-IT and the IP-10 detection was performed in all 64 patients however the T-SPOT.TB could not be performed in one patient due to the lack of enough cells in the sample. Twenty-two patients (34.9%) had a positive T-SPOT.TB result, 16 (25.0%) were positive by QFN-G-IT and 20 (31.3%) were positive by IP-10 test. The combined use of T.SPOT-TB and QFN-G-IT with IP-10 detection test increased significantly the amount of positive results ($p < 0.0001$ in both cases) to 43.8% (28/64) and 35.9% (23/64), respectively. Twelve patients with negative TST result (31.6%) were positive for at least one *in vitro* assay. We obtained 7 (10.9%) indeterminate cases overall; 5 (7.9%) by T-SPOT.TB, one (1.6%) by QFN-G-IT and 2 cases (3.1%) by IP-10 test. From the 7 indeterminate results, 5 (71.4%) were TST negative. When detecting IP-10, we obtained 2 indeterminate results (3.1%); one of them considered also indeterminate by QFN-G-IT, but negative by T-SPOT.TB and TST, and the other considered negative by both IGRAs and TST. The results of each assay on all patients included are shown in Table 2. The agreement and concordance between both IFN-γ assays was 82.5% ($\kappa = 0.603$, $p < 0.0005$). The agreement between T-SPOT.TB and QFN-G-IT assays with IP-10 test was 76.8% ($\kappa = 0.501$, $p < 0.0005$) and 83.8% ($\kappa = 0.611$, $p < 0.0005$) respectively.

By univariate analysis, we assessed the association of each variable with positive test results in order to determine possible risk factors (Table 3). There is no positive association between positive IP-10 test and treatment intake.

The amount of IFN-γ and IP-10 detected in each QFN-G-IT test tube (nil, antigen and mitogen) is significantly different ($p < 0.0001$) between tubes. The highest cytokine levels are detected in the mitogen tube and the lowest in the nil. IP-10 levels were significantly higher ($p < 0.0001$) than those of IFN-γ with a median value of 507.1 pg/ml

Table 2 Results of the IGRAs, IP-10 test and TST on all rheumatic patients included in this study.

Diagnostic test results	All rheumatic patients, $n = 64$ (%)
TST[a]	
Positive	13/51 (25.5)
Negative	38/51 (74.5)
T-SPOT.TB[b]	
Positive	22/63 (34.9)
Negative	36/63 (57.1)
Indeterminate	5/63 (7.9)
QFN-G-IT	
Positive	16/64 (25.0)
Negative	47/64 (73.4)
Indeterminate	1/64 (1.6)
IP-10 test	
Positive	20/64 (31.3)
Negative	42/64 (65.6)
Indeterminate	2/64 (3.1)

[a] TST was not performed in 13 cases when the *in* vitro tests were done.
[b] T-SPOT.TB was not possible to perform in one case due to the lack of enough cells.

(IQR = 47.78–3055) and 4.25 pg/ml (IQR = 0.0–16.38) respectively, when subtracting the nil value from the antigen tube (Fig. 1).

Impact of immunosuppressive therapies

In our patient cohort there were no significantly different effects on the *in vitro* tests results nor in the amount of cytokine secreted (Mann–Whitney U test $p > 0.05$) regarding immunosuppressive treatment intake (DMARD, corticosteroids and biologic at the time of the study) (Fig. 2, Table 4). In addition, the results were not affected by the number of DMARDs and/or corticosteroids intake previously or at the time of the study (Pearson's Chi-Squared test $p > 0.05$). The number of DMARDs and/or corticosteroids intake does not seem to have an effect upon indeterminate results. Neither the use of immunosuppressive therapy nor the amount administered affected significantly the levels of cytokine or SFCs (RD1 response) detected per each method. From the seven samples with indeterminate result, five were on DMARDs, three on corticosteroids and three on biologic treatment. Four of them had combined treatment, receiving all three types of treatment at the same time (2/5), DMARDs and biologics (1/5), and DMARDs and corticosteroids (1/5). Two out of the seven indeterminate results obtained were from patients with no treatment intake. In addition, we compared IP-10 levels in patients scored positive and negative to IGRAs, both separately and together, taking into account treatment intake. We found no significant differences of IP-10 levels among tests and treatment intake when considering positive and negative results separately, except in T-SPOT.TB positive patients receiving biologics which have a significantly lower IP-10 level than T-SPOT.TB positive patients not receiving biologics ($p < 0.02$).

Patients' follow up for active TB outcome

Each patient was periodically followed up in search of active TB symptoms, given their immunosuppressed condition. From the 64 patients included, two developed active TB: (i) one of the patients presented a negative TST and positive IGRAs/IP-10 assays. The patient had no signs and/or radiological images compatible with TB and was receiving methotrexate (10 mg), leflunomide and corticosteroids (methylprednisolone, 4 mg). After IGRAs performance, TB chemoprophylaxis was prescribed and biologic therapy was started (etanercept followed by adalimumab). Three years after the inclusion, the patient developed disseminated active TB, which led to a fatal outcome. (ii) The other patient presented negative TST, IGRAs and IP-10 assay when was included in the study. At this moment, the patient was on azathioprine and prednisolone (5 mg) intake and had no symptoms compatible with TB. Afterwards, the patient initiated biological therapy with infliximab. Four months later, the patient developed a pulmonary TB which was cured after anti-TB therapy prescription. There is no evidence of TB exposure in any of these cases.

Discussion

LTBI diagnosis in patients about to receive biologic therapy using IGRA testing has been extensively discussed.[4,5] However, there is no clear answer to the fact that each specific IMID and their concomitant treatment may affect the assay's clinical performance nor to what extent. A number of studies regarding this topic have been developed including heterogeneous study populations combining patients with different IMIDs in general with or without immunosuppressive treatment,[15,42 45] and others focus specifically on rheumatic patients.[40,46 49] In this study, we have tested the potential clinical performance of IP-10 for LTBI diagnosis in rheumatic patients, and compared it with IFN-γ responses measured by QFN-G-IT and T-SPOT.TB. To our knowledge, this is the first time that this comparison has been done in this kind of population. Our results show that the combined use of IP-10 detection in plasma and an IGRA test yields a higher number of positive results.

IP-10 detection has been largely studied in a wide number of infectious diseases such as HCV and TB, and immune diseases like rheumatoid arthritis, among others, and the different levels detected have been related to different infection/disease states and also with the possible outcomes of the disease.[26,27,29 31,50] However, in order to consider IP-10 as a biomarker of a specific disease and even specific outcome, there is a need for more longitudinal studies. Regarding TB, it is accepted that IP-10 could serve as a good biomarker of infection and, given the importance of LTBI screening in biologic therapy candidates, we consider the evaluation of IP-10 detection of great importance to improve the management of this issue.

Table 3 Association between tuberculosis risk factors and positive QFN-G-IT, T-SPOT.TB and IP-10 test results by means of univariate analysis.

Risk factors	QFN-G-IT[a]		T-SPOT.TB[a]		IP-10[a]	
	Positive *n* (%)	*p*	Positive *n* (%)	*p*	Positive *n* (%)	*p*
Gender						
Men	10 (15.9)	0.007	12 (20.7)	0.02	10 (16.1)	0.10
Women	6 (9.5)		10 (17.2)		10 (16.1)	
Age years[b]	–	0.57	–	0.09	–	0.23
Birth country						
Low prevalence	14 (22.2)	0.93	15 (25.9)	0.69[c]	16 (25.8)	0.28
High prevalence	2 (3.2)		4 (6.9)		4 (6.4)	
Rheumatic disease						
RA	9 (14.3)	0.21	12 (20.7)	0.17	8 (12.9)	0.83
Others	7 (11.1)		10 (17.2)		12 (19.3)	
Previous DMARDs						
Yes	14 (22.2)	0.13	17 (29.3)	0.67	14 (22.6)	0.75
No	2 (3.2)		5 (8.6)		6 (9.7)	
No. of previous DMARDs						
0	2 (3.2)	0.42[c]	5 (8.6)	0.18[c]	6 (9.7)	0.66[c]
1	5 (7.9)		6 (10.3)		5 (8.1)	
2	5 (7.9)		5 (8.6)		5 (8.1)	
3	3 (4.8)		4 (6.9)		2 (3.2)	
4	0 (0)		1 (1.7)		1 (1.6)	
5	1 (1.6)		1 (1.7)		1 (1.6)	
Actual DMARDs						
Yes	11 (17.5)	0.13	12 (20.7)	0.74	10 (16.1)	0.86
No	5 (7.9)		10 (17.2)		10 (16.1)	
No. of actual DMARDs						
0	5 (7.9)	0.11[c]	10 (17.2)	0.74[c]	10 (16.1)	0,77[c]
1	6 (9.5)		8 (13.8)		6 (9.7)	
2	4 (6.3)		3 (5.2)		4 (6.4)	
3	1 (1.6)		1 (1.7)		0 (0)	
Previous corticosteroids						
Yes	12 (19.0)	0.34	14 (24.1)	0.20	13 (20.9)	0.96
No	4 (6.3)		5 (8.6)		7 (11.3)	
Years on corticosteroids						
	–	0.32[c]	–	0.73[c]	–	0.33[c]
Actual corticosteroids						
Yes	9 (14.3)	0.51	12 (20.7)	0.59	10 (16.1)	0.86
No	7 (11.1)		10 (17.2)		10 (16.1)	
Actual biologics						
Yes	4 (6.3)	0.27	10 (17.2)	0.36	5 (8.1)	0.17
No	12 (19.0)		12 (20.7)		15 (24.2)	

[a] Patients with indeterminate response were excluded from the analysis.
[b] Age effect was analyzed by *t*-test.
[c] Fisher's exact test was used as more than 25% of cells had less than 5 counts.

When comparing the IGRAs performance, we obtained a moderate-high agreement rate between them (82.5% $\kappa = 0.603$) similar to what Mínguez, S et al. and Jung, YJ et al., previously described (83.01% $\kappa = 0.57$, and 78.8% $\kappa = 0.56$, respectively).[40,47] The detection of IP-10 included in our study also has a similar agreement rate between QFN-G-IT and T-SPOT.TB (83.8%, $\kappa = 0.611$; and 76.8%, $\kappa = 0.501$, respectively). When combining IGRAs with IP-10 assay, the overall number of positive results increases significantly ($p < 0.0001$). This increase when combining the two assays has previously been described in other study populations both in children and adults, therefore, the combined use of both assays would improve LTBI detection.[34,51,52] However, due to the lack of a gold standard it is not possible to know which results are false positive or false negative.

In several studies regarding IMIDs in general and rheumatic diseases in particular, TST yields fewer positive results than IGRAs, being higher when using T-SPOT.TB than QFN-G-IT.[40,43,49] In the present study, the amount of positive results we obtained by TST was also lower than by T-SPOT.TB and IP-10 detection assay but almost the same by QFN-G-IT. A substantial number of patients with negative TST result (31.6%) were positive for at least one

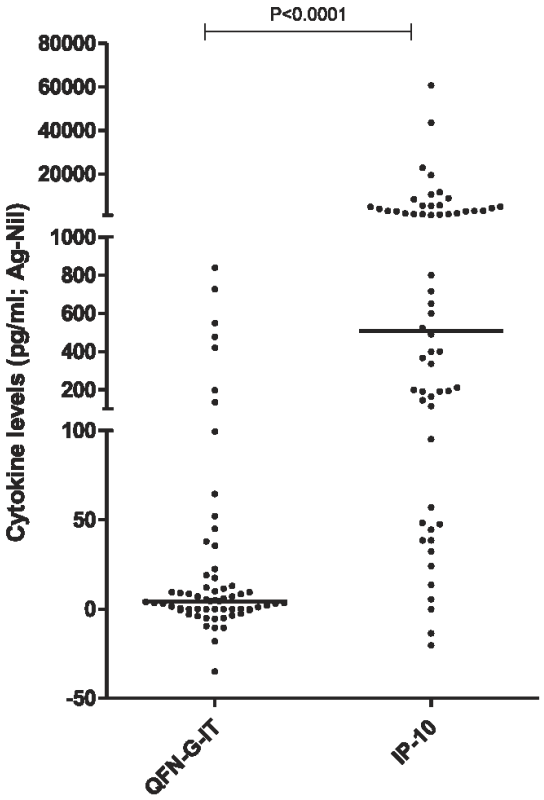

Figure 1 IFN-γ and IP-10 levels (pg/ml) in plasma after *M. tuberculosis* specific whole blood stimulation. IP-10 levels are significantly higher than the levels of IFN-γ ($p < 0.0001$) having a median value of 507.1 pg/ml and 4.3 pg/ml respectively. Statistical analysis was performed using Mann–Whitney *U* test with Dunn–Bonferroni correction. *p*-value was considered significant if <0.016.

of the *in vitro* assays, suggesting that the TST could be more influenced by immune deregulation than the *in vitro* assays. The immunosuppressive therapy effect on IGRAs results is still uncertain. According to the meta-analysis conducted by Wong SH et al.[45] in which they compare 17 studies with a total of 3197 patients with autoimmune diseases including rheumatic diseases, IGRAs results are negatively affected by immunosuppressive therapy in rheumatic patients yielding less number of positives both in QFN-G-IT and T-SPOT.TB than in patients without immunosuppressive treatment. Unlike the previous, in our study we did not find any relation between the type (immunosuppressive and not immunosuppressive therapy) nor the amount of treatment intake, and the *in vitro* technique. These findings agree with another meta-analysis conducted by Ruan, Q et al.[46] which, as in our study, only included rheumatic patients. According to this study, neither DMARDs nor steroids affected positive IGRA results. Therefore, the therapy effect on IFN-γ and IP-10 detection may depend on the kind of IMID and the kind of treatment.

The proportion of indeterminate results obtained by IGRAs varies between studies and study population. Some studies describe a higher number of indeterminate results obtained by QFN-G-IT than T-SPOT.TB,[40,42,48] whereas others describe the opposite.[43,53] Our study agrees with the latter as from the seven indeterminate results obtained, five were obtained by T-SPOT.TB, one by QFN-G-IT and two by IP-10 detection. Interestingly, five patients with negative TST had an indeterminate result for, at least, one of the *in vitro* assays. There seems to be no association between indeterminate results and immunosuppressive therapy or the negative TST outcome, however, the number of indeterminate results is too low to obtain a reliable statistic result.

Previous studies, have reported that IP-10 is secreted in higher levels than IFN-γ[32] and in this study, we have observed this same result in every stimulation condition ($p < 0.0001$). Thus, IP-10 could be of great use as a diagnostic marker for TB especially in this group of patients with a potential reduced immune response. Moreover, due to the high level production, IP-10 could also be detected in dried plasma spots.[20] As well as IP-10, there are other cytokines produced in high levels, such as TNF-α, IL-17, IL-13, IL-2, IL-1ra and MIP-1β, that are worth studying further as possible LTBI biomarkers.[32,54,55]

According to the recently published Spanish guidelines for the use of IGRAs in LTBI diagnosis,[56] LTBI screening in patients with IMID before starting biologic therapy should be done by both TST and IGRAs. However, one of the most challenging issues that remain unknown is the usefulness of IFN-γ and IP-10 detection to monitor LTBI during biologic, DMARD and/or corticosteroid therapy as well as to establish the prognostic value of progression to active TB. The booster effect of the TST makes the use of this assay limited for these purposes, but, the use of serial IGRAs testing also raises concerns about false-positive test results due to fluctuations in IFN-γ responses and conversions/reversions of QFN-G-IT over time.[57–59] These fluctuations have to be taken into account when individualizing follow-up strategies. Muñoz et al.[60] consider systemic periodic retesting of patients with negative screening results at baseline unnecessary as, in their study, no TB cases occurred after a year of anti-TNF therapy. Regarding the low progression rates in patients with positive test results, Sester et al.[44] also point out the limitation of LTBI screening in rheumatoid arthritis patients when there is no TB risk factor. In addition, M. G. Johnson et al.[12] have proposed an algorithm for monitoring patients with LTBI while on systemic biologic therapy in low-incidence TB regions that suggests an initial screening before treatment followed by annual questionnaires. Only those patients with new TB exposure will go through LTBI screening.

From our patient cohort, two patients developed active TB. One was receiving immunosuppressive therapy and TB prophylaxis in the moment of the *in vitro* assays performance and, after 3 years, developed active TB. The other patient had negative test results but after 4 months of biologic treatment it developed active TB. Regarding the first patient, it is likely that it suffered a reinfection as it only developed active TB after 3 years. In the case of the second patient, it could have been already infected in the moment of the test performance but not detected however, we

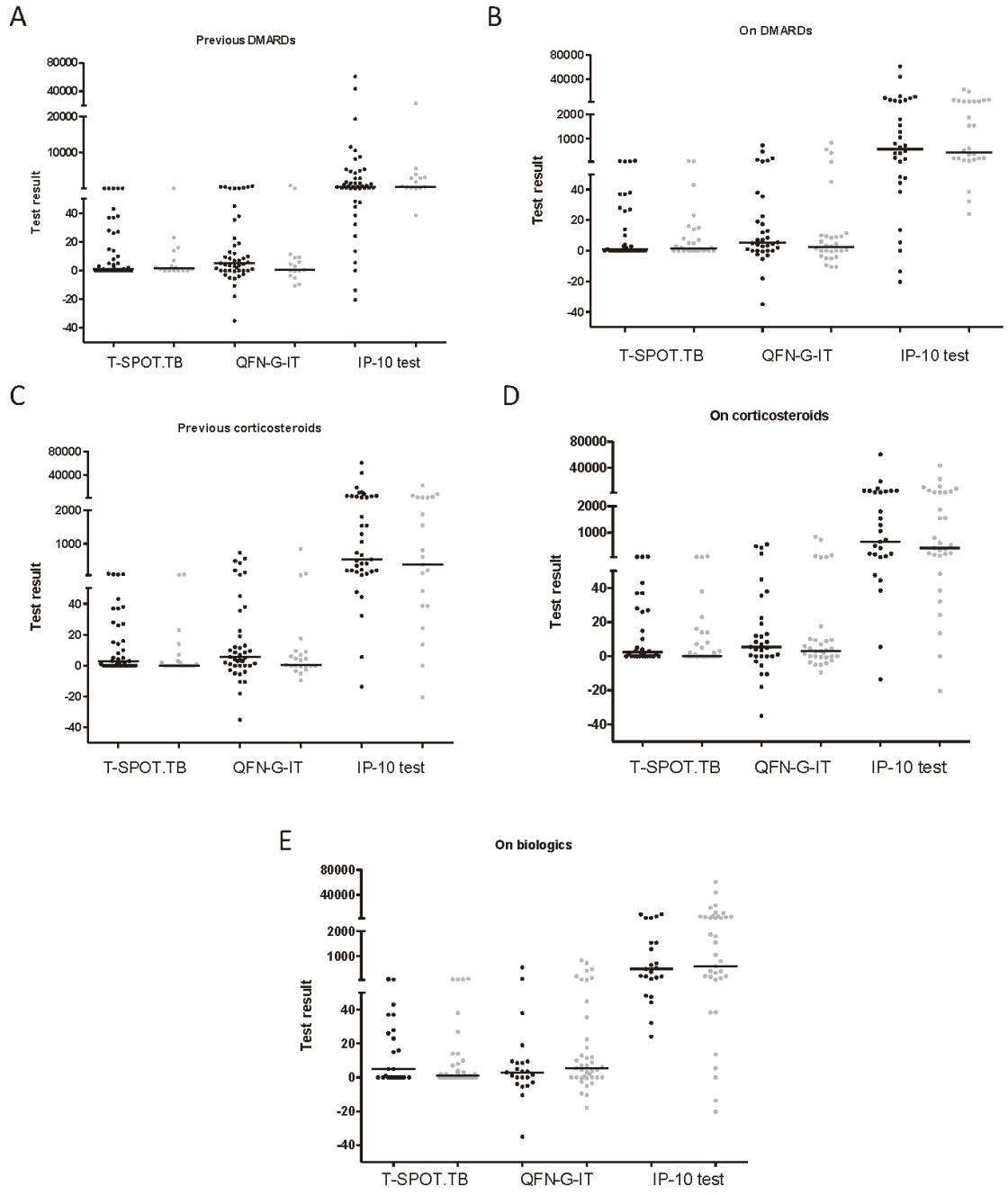

Figure 2 Test results regarding treatment intake. In each case, black dots represent patients on treatment and gray dots represent patients without treatment intake. Test results units are spots for T-SPOT.TB*, pg/ml (Ag-Nil) for QFN-G-IT and IP-10 test. A: differences regarding previous DMARDs intake. B: differences regarding DMARDs intake at the moment of the study. C: differences regarding previous corticosteroids intake. D: differences regarding corticosteroids intake at the moment of the study. E: differences regarding biologics intake at the moment of the study. No significant differences ($p \geq 0.05$). *Considering the quantitative RD1 response: spots after stimulation with ESAT-6 plus spots after stimulation with CFP-10.

Table 4 Number of positive test results according to treatment intake.

Treatment regimens	Number of positive results			
	T-SPOT.TB	QFN-G-IT	IP-10	Total
	n (%)	*n* (%)	*n* (%)	*n* (%)
Previous DMARDs intake				
Yes	17 (36.2)	14 (29.8)	14 (29.8)	47 (73.4)
No	5 (29.4)	2 (11.8)	6 (35.3)	17 (26.6)
No. of previous DMARDs intake				
1–2	11 (29.7)	10 (27.0)	10 (27.0)	37 (57.8)
3–5	6 (60.0)	4 (40.0)	4 (40.0)	10 (15.6)
Previous corticosteroids intake				
Yes	17 (41.5)	12 (29.3)	13 (31.7)	41 (64.1)
No	5 (21.7)	4 (17.4)	7 (30.4)	23 (35.9)
On DMARDs at the moment of *in vitro* test performance				
Yes	12 (35.3)	11 (32.4)	10 (29.4)	34 (53.1)
No	10 (33.3)	5 (16.7)	10 (33.3)	30 (46.9)
No. of DMARDs at the moment of *in vitro* test performance				
1–2	11 (33.3)	10 (30.3)	10 (30.3)	33 (51.6)
3	1 (100)	1 (100)	0 (0)	1 (1.6)
On corticosteroids at the moment of *in vitro* test performance				
Yes	12 (38.7)	9 (29.0)	10 (32.7)	31 (48.4)
No	10 (30.3)	7 (21.2)	10 (30.3)	33 (51.6)
On biologics at the moment of *in vitro* test performance				
Yes	10 (43.5)	4 (17.4)	5 (21.7)	23 (35.9)
No	12 (29.3)	12 (29.3)	15 (36.6)	41 (64.1)

cannot discard the possibility that the patient became infected after the screening. The study of IFN-γ and IP-10 levels in patients followed over time would be of interest in order to identify LTBI reactivations or newly acquired infections during anti-TNF-α treatment.[61] However, the risk of TB progression remains mainly during the first year of therapy and therefore retesting is not justified after a negative result.[60]

Although our overall results support the use of IP-10 in LTBI in rheumatic patients, the main limitation of our study is the treatment variability (DMARDs, corticosteroids and biologics) of the study population, which adds difficulties when grouping patients for the analysis. Moreover, the combination of IGRAs and IP-10 detection increases the number of positive results. However, due to the lack of a gold standard, we cannot evaluate if we are detecting a truly LTBI or a lasting immunity. We are aware that combination of techniques always increases the amount of positive results, increasing as well the chances of false-positives. However, a population at high risk of TB progression, such as our study cohort, could benefit from this increase of positive results if it means they can reduce this risk.

Recently, a new version of the QFN-G-IT, the QuantiFERON Plus (QFN-Plus) has been introduced.[62,63] This new test is also based on IFN-γ detection; however, it includes two antigen tubes: one contains antigens that induce a CD4 T-cells response and another contains antigens that induce both CD4 and CD8 T-cells response. Further studies need to be performed in order to assess the utility of this new version in this population.

In conclusion and according to our data, the diagnostic performance of IP-10 is comparable to that of IFN-γ. The combination of IP-10 and IFN-γ detection could increase the number of positive results in the diagnosis of LTBI in rheumatic patients. The results obtained by any of the three *in vitro* techniques previously described, are not influenced by DMARD, biologic and/or corticosteroid treatment received in this study population. Thus, the study of IP-10 could be of use in the development of new diagnostic techniques in order to improve LTBI diagnosis.

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Conflict of interest

The authors declare no competing financial interests.

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Article 3

Impact of Host Genetics and Biological Response Modifiers on Respiratory Tract Infections

Alicia Lacoma, Lourdes Mateo, Ignacio Blanco, Maria J. Méndez, Carlos Rodrigo, Irene Latorre, **Raquel Villar-Hernández**, Jose Domínguez and Cristina Prat.

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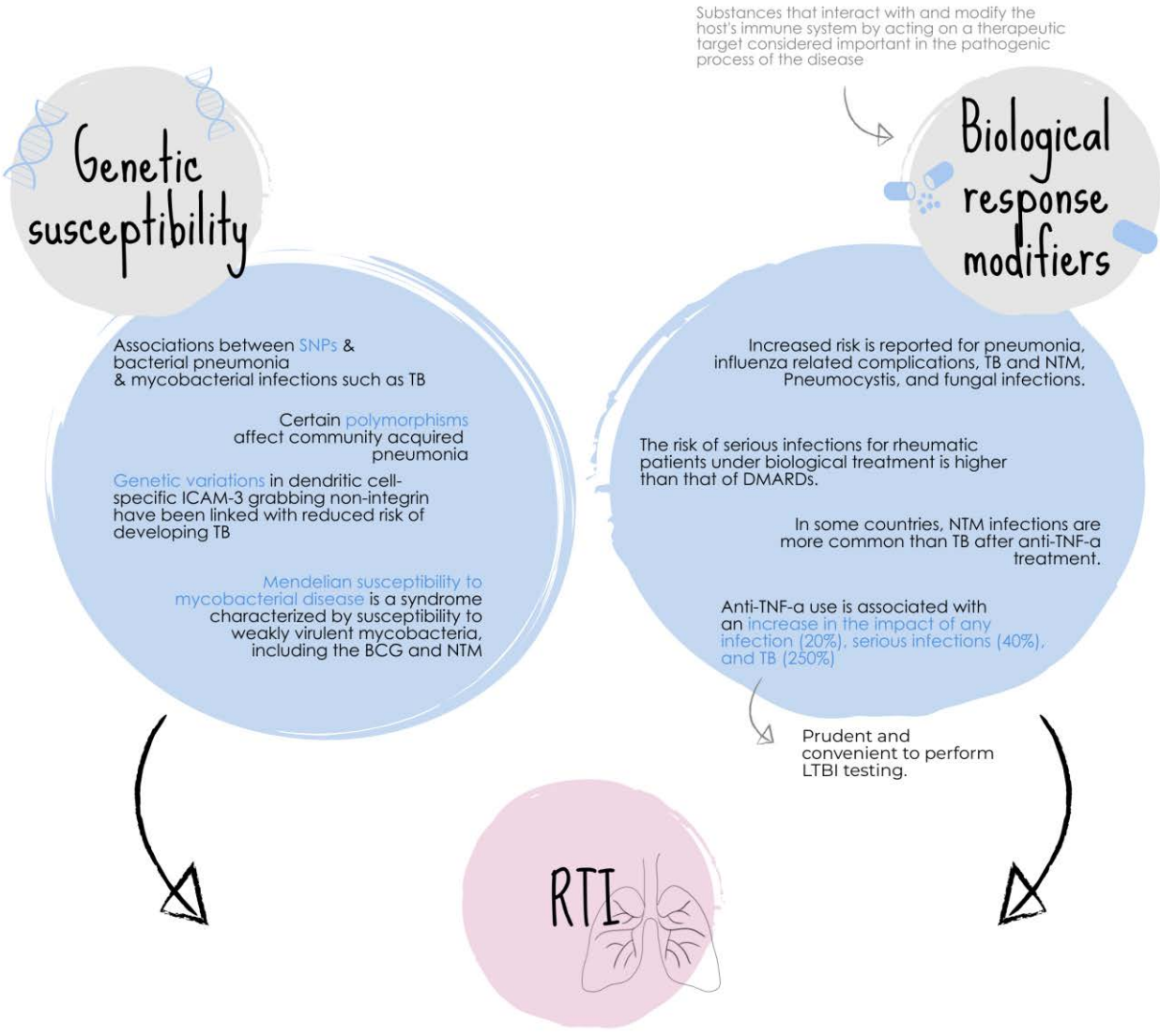

One of the most frequent causes of infections and antimicrobial prescription

&

the leading cause of death in developing countries

DMARDs: Disease-modifying anti-rheumatic drugs; LTBI: latent tuberculosis infection; NTM: non-tuberculous mycobacteria; RTI: Respiratory tract infections; SNPs: Single-nucleotide polymorphism; TB: tuberculosis

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Impact of Host Genetics and Biological Response Modifiers on Respiratory Tract Infections

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Host susceptibility to respiratory tract infections (RTI) is dependent on both genetic and acquired risk factors. Repeated bacterial and viral RTI, such as pneumonia from encapsulated microorganisms, respiratory tract infections related to respiratory syncytial virus or influenza, and even the development of bronchiectasis and asthma, are often reported as the first symptom of primary immunodeficiencies. In the same way, neutropenia is a well-known risk factor for invasive aspergillosis, as well as lymphopenia for *Pneumocystis,* and mycobacterial infections. However, in the last decades a better knowledge of immune signaling networks and the introduction of next generation sequencing have increased the number and diversity of known inborn errors of immunity. On the other hand, the use of monoclonal antibodies targeting cytokines, such as tumor necrosis factor alpha has revealed new risk groups for infections, such as tuberculosis. The use of biological response modifiers has spread to almost all medical specialties, including inflammatory diseases and neoplasia, and are being used to target different signaling networks that may mirror some of the known immune deficiencies. From a clinical perspective, the individual contribution of genetics, and/or targeted treatments, to immune dysregulation is difficult to assess. The aim of this article is to review the known and newly described mechanisms of impaired immune signaling that predispose to RTI, including new insights into host genetics and the impact of biological response modifiers, and to summarize clinical recommendations regarding vaccines and prophylactic treatments in order to prevent infections.

Keywords: immunogenetics, biological response modifiers, respiratory tract infections, primary immunodeficiencies, inborn errors

EPIDEMIOLOGY AND PATHOGENESIS OF RESPIRATORY TRACT INFECTIONS

Acute and chronic respiratory tract infections (RTI) are one of the most frequent causes of infections and antimicrobial prescription, and the leading cause of death in developing countries (1, 2). Pneumonia accounts for 1.3 million deaths annually in children <5 years of age (3). In 2017, 1.6 million people died of tuberculosis (TB). Children (aged <15 years) accounted for 15% of total deaths, higher than their share of estimated cases, suggesting poorer access to diagnosis and treatment. About 1.7 billion people, 23% of the world's population, are estimated to have a latent TB infection (4). The control of latent TB, a stage in which a person is infected with *Mycobacterium tuberculosis* plays an important role in disease control, since dormant bacilli are a reservoir of potential TB cases (5). Viral acute RTI are estimated to cause 75% of acute diseases in children, and is the main reason for hospitalization worldwide (6). The annual prevalence in an otherwise healthy child is from 3 to 10 infections (7). Early and recurrent lower RTI are linked to a higher risk to develop asthma or bronchiectasis (8–10). However, bronchiectasis secondary to recurrent and severe infections alone have declined, with an increasing proportion of patients being recognized as having underlying conditions predisposing to its development (11).

Improvements in immunization programs and the wide availability of antimicrobials, have led to optimism for most of the devastating infectious diseases. Always without forgetting that alleviation of poverty is crucial, the combination of genetic versatility and ecological opportunism of the microbial world appears to have been under-estimated (12). Some emerging pathogens, such as *Legionella*, avian influenza, and coronavirus species were described in the past decades (13). Ethnic variations in the incidence of RTI have also been reported, suggesting genetic susceptibility to disease (14). Most children, on reaching 2 years of age, have been in contact with the most common respiratory viruses, such as respiratory syncytial virus (RSV), but while some develop a mild disease, others develop severe bronchiolitis (15). Influenza viruses cause mild to moderate respiratory illness in most people, but some develop fatal infections. The virulence factors encoded by viral genes can explain seasonal or geographical differences at a population level, but are unlikely to account for inter-individual clinical variability (16). TB outcome depends on the pathogen and extrinsic elements, as well as on host factors that are still unclear (17).

As regards bacteria, focusing on those species whose normal ecological niche is the airways, therapeutic decisions are a daily clinical challenge (18). The shift from commensalism to infection is shaped by host intrinsic (genetics) and extrinsic factors (for example, diet and exposure to cigarette smoke and environmental pollution) and by bacterial features that also contribute to inter-individual variability (19). Bacteria develop adaptive mechanisms (at genetic/phenotypic level) in order to survive in a hostile environment, such as the respiratory tract (20, 21). Whether pathogen virulence generates clinical symptoms depends on how well the immune system limits its impact. Recently, changes in gut and lung microbiome composition (dysbiosis) have also been related to dysfunctional immune modulation (22).

IMMUNE RESPONSE TO RESPIRATORY TRACT INFECTIONS

Respiratory immune responses are complex, and inborn errors can be present at any level. Essential pathways can be summarized as follows: Firstly, the pathogen has to be detected by host cells. This identification relies on a set of pathogen associated molecular profiles that bind to pattern recognition receptors (PRR). PRR can be found as transmembrane, cytosolic or extracellular components. Among PRRs, it is important to mention toll-like receptors (TLR), nucleotide-binding oligomerization domain-containing (NOD) receptor, NOD-like receptors (NLR), RIG-I-Like Receptors (RLR), and receptor CD14 because of their importance during respiratory infections (23). Depending on the PRR, different intracellular signaling pathways are activated (24). Most of the signaling pathways converge on signaling hubs, such as transcription nuclear factor κβ (NF-κβ), interferon regulatory factor families (IRF3, IRF7), and mitogen-activated protein kinase, leading to the induction of gene expression encoding adhesion molecules, pro-inflammatory cytokines, chemokines, and type I interferon, among others. NLRs directly trigger inflammasome assembly and caspase-1 activation, leading to interleukin (IL)-1β and IL-18 processing (25). Type III interferons, also termed IFN-λ, have been recently identified as regulators of immunity and homeostasis in the respiratory tract (26) during infections, as well as during chronic lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD) (27). Alveolar macrophages and dendritic cells (DC) have an important role sensing microbes and thus activating lung epithelial cells and neutrophils. These are essential for the defense against bacteria, viruses, and *Aspergillus* (28, 29), as well as in the pathogenesis of acute lung injury. In a recent study, patterns of differentially expressed cellular genes shared by several respiratory pathogens were searched using transcriptomics (30). Most of the commonly up-regulated host genes were related to the innate immune response and/or apoptosis, with Toll-like, RIG-I-like, and NLR among the top 10 signalers. Some of the genes showed a high degree of interconnection and possible redundancy to respiratory viral and bacterial infections. The adaptive immune response requires the activation of antigen-specific T and B lymphocytes to trigger protective cellular and humoral responses. Most of the T lymphocyte subsets, along with B lymphocytes and DC, are essential for immune defense and/or regulation (31). In particular, the protective immunity against *M. tuberculosis* depends on $CD4^+$ T-helper1 lymphocytes that mainly secrete interferon-gamma (IFN-γ), IL-2, and tumor necrosis factor alpha (TNF-α), which leads to macrophage activation, cytokine production, and bacterial control (32). HIV-revealed T-cell lymphopenia as a well-defined risk group for *Pneumocystis jirovecii* pneumonia (PJP), but also in other situations where CD4 lymphocyte count is lower, such as renal transplant recipients (33).

GENETIC SUSCEPTIBILITY TO RESPIRATORY TRACT INFECTIONS

The study of susceptibility to lower respiratory tract infections is complex, and requires different approaches. There are three main elements playing a role: host genetic background (in relation to lung tissue functionality and immune response), pathogen virulence determinants, and environmental factors.

Early life (children under 5 years of age) is a challenging period because pulmonary tissue and the immune system are still in a maturation process while being continuously exposed to airborne antigens (34). However, the occurrence of life-threatening bacterial/viral/fungal infection in an otherwise healthy individual deserves further immunological and genetic studies (35, 36). Complications during upper RTI include sinusitis and otitis media, and in the lower airways, pneumonia, bronchitis, as well as the development of bronchiectasis, interstitial lung diseases, organizing pneumonia, and hyperreactive airway diseases (37). Indeed, genetic susceptibility for the concomitant illnesses that predispose to RTI can also play a role, including congenital defects of the airways, familial congenital bronchiectasis or tracheobronchomegaly (11). As regards impaired mucociliary clearance, cystic fibrosis is the most common autosomal recessive disorder and primary cause of bronchiectasis in the developed world. Mutations are well-defined, but its severity is influenced by genes involving inflammatory and anti-inflammatory mediators (38, 39). Other disorders include ciliopathies and disorders of humoral immunity. Alpha 1-antitrypsin is a circulating serine protease inhibitor (serpin) made in the liver that plays an important role in modulating immunity, inflammation, apoptosis, and possibly cellular senescence programs and its deficiency is considered the genetic cause of COPD, but there are other genetic factors that may affect disease activity and outcomes, even in patients without this deficiency (27).

High-throughput whole genome sequencing technologies and novel bioinformatics tools are revealing the sequence and annotation of the complete human genome, as well as genome-wide maps of polymorphic microsatellite markers and single nucleotide polymorphisms (SNP). In order to characterize genetic susceptibility, two complementary approaches can be envisaged: whole genome association studies (WGAS) for the identification of variants with high population frequency but low impact at individual level in terms of risk of infection (although SNP identification can potentially be later included in healthcare planning protocols); and mechanistic studies for identifying disease-causing mutations with deleterious effects, related to a high risk of infection at individual level, although its frequency in general population is low. Many genetic variants have been associated with complex human diseases and traits, but often confer relatively small increases in risk (40). According to a recent review, there are more than 300 primary immunodeficiency disorders (PIDs), most of them monogenic conditions with Mendelian inheritance, that are mainly associated with crucial defects in adaptive immunity (31). Innate immune responses are largely redundant, with pleiotropic nature of some gene products (31), thus most of the defects can be potentially counterbalanced. According to the literature, there is another view suggesting that while patients with broad immunodeficiencies may present with one of their many infections, the phenotype of particular inborn errors of immunity is very narrow, with susceptibility to only one specific infection (36, 41, 42). A set of inborn errors affecting "primarily" innate immunity, exercise their effect on the adaptive immune response (41). The range and nature of infections depend on several factors. The improving recognition of immune dysregulation diseases, autoinflammatory disorders, and interferonopathies leads to changes in terminology. The annual report of the authoritative International Union of Immunological Societies (43) has categorized and listed (as of February 2017) 354 inborn errors of immunity, and those with a predominant RTI phenotype have been included in **Table 1**.

Despite the limitations of molecular genetic studies in pulmonary infections, several associations have been described between SNPs and bacterial pneumonia and mycobacterial infections (14, 45). Polymorphisms affecting community-acquired pneumonia including, among others, those related to mannose-binding lectin and the IgG2 Fc gamma receptor II, and are discussed extensively elsewhere (14). The genetic contribution for the propensity to develop severe RSV infection was estimated to account for ~20% of the variance in RSV disease severity. Several studies have attempted to link candidate host SNPs to disease severity, mostly in chemokine receptors and PRRs (46, 47).

As regards TB, SNPs are frequently found in loci involving TLR-2, TNF-α, IL-12, and IFN-γ, and their corresponding receptors (45). Genetic variations in dendritic cell-specific ICAM-3 grabbing non-integrin have been linked with reduced risk of developing TB (48). Mendelian susceptibility to mycobacterial disease is a syndrome characterized by susceptibility to weakly virulent mycobacteria, including the attenuated vaccine Bacillus Calmette-Guerin (BCG) strain and non-tuberculous mycobacteria (NTM). Different gene mutations have been identified, most of which are related to IFN-γ-mediated immunity (49–51). Using exome and transcriptome sequencing, three rare loss-of-function variants have been recently characterized in theIFIH1 gene. These encode a RIG-I-like receptor involved in the sensing of viral RNA (52). The deficiency causes a primary immunodeficiency manifested in extreme susceptibility to common respiratory RNA viruses. Interestingly, human primary immunodeficiency disorders (PID) affecting T and B cells were not found to predispose to severe influenza. However, human IRF7 was shown to be essential for IFN-α/β- and IFN-λ-dependent protective immunity against primary influenza *in vivo* (53).

IMPACT OF BIOLOGICAL RESPONSE MODIFIERS IN RESPIRATORY TRACT INFECTIONS AND TUBERCULOSIS

Biological response modifiers (BRM) are substances that interact with and modify the host immune system by acting on a therapeutic target considered important in the pathogenic

TABLE 1 | Reported risk of infection and recommended prophylaxis according to functional classification of biologicals-based on ESCMID consensus document (44) and to categorization of inborn errors of immunity-based on International Union of Immunological Societies annual report (43).

	Reported risk of infection	Recommendations			
		TB screening and prophylaxis	**Pneumocystis prophylaxis**	**Pneumococcal/ capsulated bacteria vaccination**	**Influenza vaccination**
TARGETED AGENT					
Anti-tumor necrosis factor-α agents	Two to four increase in the risk of active TB compared to healthy patients and other granulomatous conditions Rate of *Legionella* 37-fold higher. Histoplasmosis and coccidioidomycosis	Yes	No	Yes	Yes
Interleukins, immunoglobulins and complement factors	IL-1 family, moderate risk of infection, IL-6 and IL-6 receptor (JAK), similar to TNF. Neutropenia in some cases C5 targeted, *Aspergillus* encapsulated bacteria, specially *Neisseria*, IL-17 upper respiratory tract infections and *Candida* IgE helminth infection, *Strongyloides*	Yes	No	Yes	Yes
Cell surface receptors/associated signaling pathways	Drug induced neutropenia Skin and soft tissue infections and sometimes pneumonia Overall risk of infection low for epidermal growth factor	Optional	No	Age appropriate	Yes
Intracellular signaling pathways	Increased overall risk of infection, cytomegalovirus and hepatitis B reactivation Difficult to distinguish from the risk of the underlying disease Cases of *Pneumocystis*, invasive fungal infection nocardiosis, mainly JAK	Yes	Yes	Age appropriate	Yes
Lymphoid cells surface antigens (CD19, CD20, CD52)	Can cause IgG hypogammaglobulinaemia and neutropenia Evidence of catheter related bacteremia, severe respiratory tract infection, hepatitis B reactivation and varicella zoster	Yes	Yes	Age appropriate	Yes
Lymphoid/Myeloid cells surface antigens (CD22, CD30, CD33, CD38, CD40, SLAMF-7, CCR4)	Similar to anti CD20	Optional	Yes	Age appropriate	Yes
Immune checkpoint inhibitors, cell adhesion inhibitors, sphingosine-1-phosphate receptor modulators and proteasome inhibitors	Associated T cell lymphopenia but no opportunistic infections reported Risk of varicella zoster virus	Yes	Yes	Yes	Yes
INBORN ERRORS OF IMMUNITY					
Severe combined immune deficiency:	Severe opportunistic disseminated infections in early childhood	Non-applicable	Yes	No	No (cohabitants)
Less severe combined immune deficiency	Some related to recurrent respiratory tract infections	Optional	Yes	Yes	Yes
Combined immune deficiencies with syndromic features e.g., Wiscot Aldrich and those altering DNA reparation	Recurrent infections	Optional	No	Yes	Yes
Humoral immune deficiencies Antibody deficiencies	Repeated respiratory tract infections (pneumonia, sinusitis, otitis, …)	Optional	No	Yes	Yes
Defects of phagocyte number or function	Fungal and bacterial infections, pulmonary abscesses, aspergillosis	No	No	Yes	Yes
Defects in intrinsic and innate immunity	Pyogenic bacterial infections	In selected cases	No (*)	Yes	Yes
Autoinflammatory diseases	No clear predisposition to infection	No	No	Yes	Yes
Complement deficiencies	Disseminated infections (meningitis/sepsis) by capsulated microorganisms and *Neisseria*	No	No (*)	Yes	Yes

**Antibiotic prophylaxis to prevent bacterial infections.*

process of the disease. Monoclonal antibodies (mAbs) are now established as therapies for malignancies, transplant rejection, several immune disorders from most organ systems, and even infectious diseases (54). Safety problems related to immunomodulation and infection have been identified in some cases (55). The use of mAb indirectly provides insights into the function of the molecule to combat particular pathogens, increasing our knowledge of the immune system (56). A recent consensus document has reviewed the groups of drugs according to the targeted site of action, the expected impact on susceptibility to infection, the evidence of risk, and the recommendation of prevention strategies. It is also important to mention the influence of previous or concomitant therapies, underlying conditions, and the accumulative exposure to the agent (44). As regards lower RTI, treatment with BRM results in an increased risk is reported for pneumonia, influenza-related complications, TB and NTM, *Pneumocystis,* and fungal infections, such as histoplasmosis, taking into account the impact of geographical variations on incidence rates (57). The knowledge obtained from experience with the prescription of BRM may be particularly valuable for the understanding of some genetic inborn errors, as the type of infections acquired as a side effect may help to identify which genetic defects favors a similar infectious phenotype. With the current knowledge and because of pleiotropic effects, it is not feasible to show how biological agents actually mimic some inborn errors of immunity, but several parallelisms can be inferred. We provide a Table containing the list of BRM according to their functional classification, and inborn errors categorized according to common infectious phenotypes (**Table 1**). Data presented are extracted from the respective consensus documents, and lists the main RTI and preventive recommendations.

Current recommendations should be focused on rheumatic diseases because of the greater experience in follow-up time (more than 15 years) and number of patients treated. Biological therapies targeting TNF-α, T cells, B cells, and various cytokines (including IL-6 and IL-1) have become essential for the treatment of rheumatic diseases [mainly rheumatoid arthritis (RA), ankylosing spondylitis, and psoriatic arthritis], as well as other immune-mediated diseases. Moreover, additional drugs with novel targets, including those that inhibit IL-12–IL-23, IL-17α, or the Janus activating kinase system have been introduced more recently. Immunomodulation offered by biological and non-biological disease-modifying therapies and prednisone contributes greatly to the increased risks of opportunistic infections (OI) (58, 59). In **Figure 1** we present the sites of action and associated risks of the most frequently prescribed BRM.

Two recent meta-analysis have calculated the relative risk of infection for rheumatic patients under biological treatment, with an odds ratio (OR) of 1.31–1.41 (60, 61). The absolute increase in the number of serious infections per 1,000 patients treated/year is six times higher than that observed with synthetic disease-modifying anti-rheumatic drugs (DMARDs). Different meta-analyses and national registries have confirmed the increase on the impact of any infections (20%), serious infections (40%), and TB (250%), associated with anti-TNF-α use (60). In addition, the risk of serious infections is highest during the first 6 months of therapy (62) (up to 4.5-fold risk), although, after 1 year this risk is no different from conventional DMARDs. Recurrent infections in RA are common. In a prospective observational cohort study, the baseline annual rate of a first serious infection was 4.6%. Additionally, 14% of this cohort experienced a recurrent episode/year during their follow-up, with the highest risk being within the first year (29%), and with respiratory infections being the most common (44% of all episodes) (63). Factors that have shown to be predictive of infection include, age, functional status, specific comorbidities (chronic renal/lung disease), corticosteroid treatment, number of previous DMARD, treatment failures, previous serious infections, and current treatment with anti-TNF-α inhibitors or non-biological DMARDs (64). Nevertheless, recent data suggest that patients having a serious infection and exposed to biological treatment have a significantly lower risk of sepsis and fatal outcome than patients treated with conventional DMARDs (62, 65). British and French national biological registries have reported OI rates of 200–270/100,000 in patients using anti-TNF-α therapies (66, 67). In particular, there is evidence of an increased risk of *M. tuberculosis*, herpes zoster, and *Listeria* infections. The overall incidence of OI is not significantly different considering drug classes; however, the rate of PJP is significantly higher in those patients using rituximab in comparison to anti-TNF-α therapy. The absolute risk of PJP is low, although corticosteroid exposure is a strong predictor. Current data do not support PJP prophylaxis for all rituximab users. However, it may be appropriate in certain high-risk individuals. Furthermore, rituximab-associated neutropenia and impaired antibody response is also well-described.

Pre-clinical and clinical evidence indicate that anti-TNF-α therapy (infliximab, adalimumab, golimumab, certolizumab pegol, and etanercept) is associated with a 2- to 4-fold increase in the risk of active tuberculosis and other granulomatous conditions. Risk seems to be lower for etanercept (68). Risk also depends on local TB prevalence: in the year 2000, Spanish investigators reported an estimated TB incidence of 1,893/100,000 person-years in patients with RA treated with infliximab (69). This rate is ∼10- to 20-fold higher than the observed rate in naïve patients. These rates have decreased dramatically since the establishment of latent tuberculosis infection (LTBI) screening prior to biological therapy (67, 70). It is essential to rule out LTBI in such individuals in order to reduce the risk of active TB reactivation. Interferon-gamma release assays (IGRAs) are useful tools for LTBI diagnosis. They are more specific than the tuberculin skin test (TST) because they do not show cross-reactivity with BCG-vaccination or NTM sensitization (71–73). Moreover, these *in-vitro* assays incorporate a mitogen control that can detect the presence of anergy, common in patients on immunosuppressive therapy (74). However, the clinical performance of IGRAs is still controversial due to the variety of concomitant immunosuppressive drug-regimens used at the time of LTBI screening, population heterogeneity, and the severity of the disease itself (75). Therefore, the clinical accuracy of IGRAs seems to be differentially affected depending on the specific type

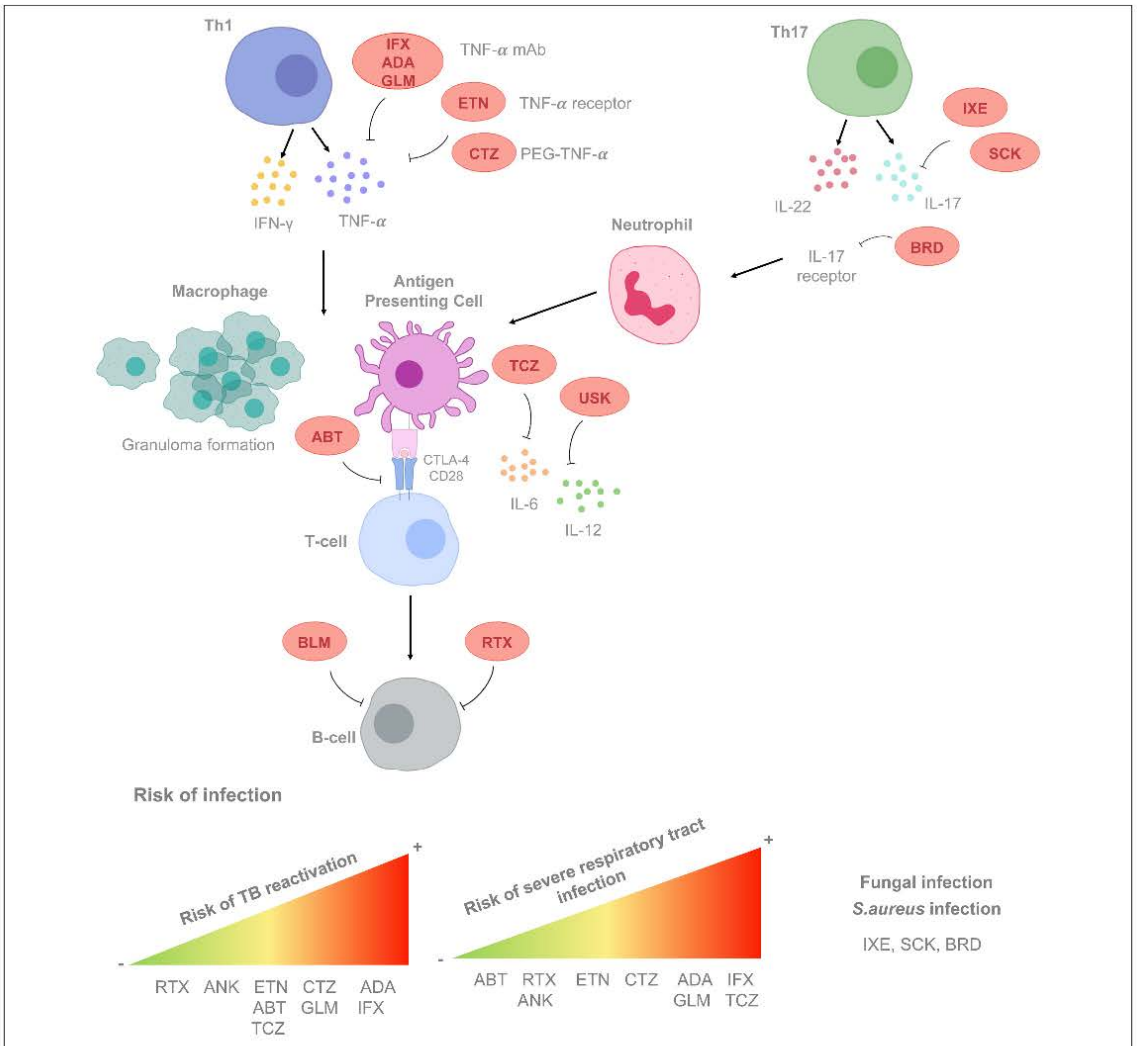

FIGURE 1 | Mode of action of biological response modifiers (BRM) according to cell type, cytokine and/or receptor targeted. Risk of developing infections according to the BRM considered is also shown. List of BRM. Anti-tumor necrosis factor-α (TNF-α) agents. ADA, adalimumab; CTZ, certolizumab; GLM, golimumab; IFX, infliximab; ETN, etanercept. Anti-interleukins, immunoglobulins, and complement factors. Anti IL-1, anakinra ANK; Anti IL-6: TCZ, tocilizumab; Anti IL-17: SCK, secukinumab; IXE, ixekizumab; BRD, brodalumab. Anti-IL12/23: USK, ustekinumab. Cell surface receptors/associated signaling pathways agents. Anti-CD28: ABT, abatacept; B-cell activating factor (BAFF): BLM, belimumab. Lymphoid cells surface antigens. Anti-CD20: RTX, rituximab. mAb, monoclonal antibody; PEG, polyethylene glycol; TB, tuberculosis; LTBI, latent tuberculosis infection.

of immune disorder. Crohn's disease and/or its concomitant drug-profile (such as azathioprine or high-dose corticosteroids) could negatively affect the clinical performance of IGRAs when compared with other immune-mediated diseases, such as psoriasis or inflammatory rheumatic diseases (76). Thus, it seems prudent and convenient to perform dual LTBI testing with TST and IGRAs (77). Patients with RA and underlying structural lung diseases are at increased risk of developing NTM infection (78), mostly *Mycobacterium avium*. In some countries, NTM infections are more common than TB after anti-TNF-α treatment. However, there are still no established recommendations as regards screening and prophylaxis (79). A baseline chest x-ray should be recommended prior to starting therapy, and in patients with chronic unexplained cough, further work-up should include chest computed tomography scans and culture of respiratory specimens.

Immunization strategies are recommended for all cases, regardless of whether the patient has PID or is receiving immunosuppressive treatment, and it is of importance to be vaccinated according to the national immunization routine schedules. For patients with anti-TNF-α treatment, pneumococcal and age-appropriate anti-viral vaccinations (i.e., influenza) should be administered (68). Immunization before and after BRM is well-established as regards inactivated vaccines, and precautions should be taken for live vaccines (57). However, even if response to vaccines is impaired in patients with PID (80), it may have an effect in patients receiving some BRM. This may be partially explained by the concept of trained immunity-based vaccines (81).

In conclusion, RTIs belong to the most common causes of infections in humans worldwide. The genetic contribution to severe RTIs may have been masked by other interventions (82). The inborn errors of innate immunity show us that the absence of a measurable immunological defect does not exclude an immunodeficiency (41). Further functional genetic studies are necessary in order to fully validate the impact of host genetics during lung infections. The knowledge obtained from experience with the prescription of BRM may be particularly valuable, as the infections acquired as a side effect may help to identify genetic defects with a similar infectious phenotype. In the meantime, recommendations based on biological rationale and clinical experience are mandatory in order to prevent re-emerging severe infections.

AUTHOR CONTRIBUTIONS

CP organized the structure and supervised the manuscript elaboration, revised literature and wrote a part of every chapter. AL revised literature, wrote the sections related to the immune response to infection and part of genetics, and edited the manuscript. IL, RV-H, and JD revised and wrote the aspects related to tuberculosis, and JD also supervised the manuscript elaboration. LM revised and wrote the section regarding the impact of biological response modifiers specially related to rheumatologic diseases. LM, AL, and RV-H prepared the figure. MM and CR revised aspects related to immunodeficiencies, and impact of BRM in children. IB revised host genetic factors. All authors revised and approved the final version of the manuscript.

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Article 4

Could IFN-γ levels in serial QuantiFERON predict tuberculosis development in young children?

Raquel Villar-Hernández, Irene Latorre, Neus Altet, José Domínguez.

Journal of Laboratory and Precision Medicine, 2017.

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Could IFN-γ levels in serial QuantiFERON predict tuberculosis development in young children?

- Editorial -

Raquel Villar-Hernández, Irene Latorre, Neus Altet, José Domínguez

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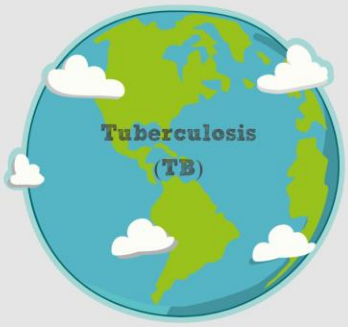

10.4 million new cases
1.8 million deaths
(in 2016)

Decrease TB burden:
Control & Management

TB screening identifying cases at a higher risk of developing active TB

IGRAs for IFN-γ detection

QFN-G-IT

and / or

T-SPOT.TB

High IFN-γ conversion levels in QFN-G-IT may indicate TB progression in children

> 4.00 IU/ml → **High probability**

High IFN-γ levels → **Less probable to revert and higher probability to progress to TB**

Recent QFN-G-IT conversion → **Higher probability to progress to TB**

IFN-γ detection is very variable so these findings should be further evaluated in order to consider a revision of the international guidelines in the use of IGRAs in children.

TB: Tuberculosis; IGRAs: Interferon-γ release assays; QFN-G-IT: QuantiFERON-TB Gold In-Tube; IU: International Units

Raquel Villar Hernández

Editorial

Could IFN-γ levels in serial QuantiFERON predict tuberculosis development in young children?

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Provenance: This is a Guest Editorial commissioned by Section Editor Maoshui Wang (Department of Lab Medicine, Shandong Provincial Chest Hospital, Jinan, China).

Comment on: Andrews JR, Nemes E, Tameris M, *et al.* Serial QuantiFERON testing and tuberculosis disease risk among young children: an observational cohort study. Lancet Respir Med 2017;5:282-90.

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Tuberculosis (TB) is an infectious disease of important global health concern. On 2016, TB caused 10.4 million new cases and 1.8 million deaths (1). Compared to adults, young children infected with *Mycobacterium tuberculosis* (*M.TB*) are at high risk of progression and have an increased risk of developing a severe TB outcome (2). TB screening recommendations in children and other risk groups are described in several international and national guidelines, as well as the recommended prophylaxis (3,4). However, in some cases, prophylaxis may turn out to be unnecessary and depending on the setting it may be impossible to access. In order to better characterize who would benefit from prophylaxis, there is a need to identify those at a higher risk of developing active TB.

Interferon gamma (IFN-γ) release assays (IGRAs) are diagnostic tests used for TB infection detection. IGRAs are based on IFN-γ detection released by T-cells after whole blood stimulation with specific *M.TB* complex antigens. Currently there are two IGRAs available: QuantiFERON Gold in-Tube (QFN-G-IT) and T-SPOT.TB, both based on an enzyme-linked immunosorbent assay. QFN-G-IT detects the amount of IFN-γ released by T-cells, whereas the T-SPOT.TB detects the number of IFN-γ producing cells. Recently, a new version of the QFN-G-IT, the QFN-Plus, has been introduced (5,6). This assay detects the IFN-γ produced by CD4 and also by CD4 and CD8 together. IGRAs have been extensively studied (7-11) and, although they have a good negative predictive value, they have a poor positive predictive value: 2.7% overall or 6.8% in high risk groups according to Diel *et al.* (9), and up to 17% according to Altet *et al.* (10), which represents an issue for prophylaxis prescription.

Several studies have assessed the amount of IFN-γ released in QFN-G-IT test at baseline, conversions and also reversions, in order to find a relation between concentrations and risk of progression, instead of focusing only on positive or negative test results (10,12-17). However, findings in this regard are conflicting and more needs to be done to assess the potential role of different IFN-γ concentrations in future TB development.

Andrews *et al.* (18) recently addressed this issue in young children studying the relation between IFN-γ in QFN-G-IT conversions and risk of TB development, and QFN-G-IT reversions. The authors included 2,512 South African young children aged 4–6 months, BCG vaccinated on the first week after birth, HIV negative, with no known TB contact and a negative QFN-G-IT (<0.35 IU/mL) at the beginning of the study. The study subjects were part of a MVA85A vaccine trial (19). However, since the vaccine did not seem to have any effect, every subject was considered in the study regardless the group they were initially divided into (placebo group, with *Candida* spp., and MVA85A vaccinated). Subjects underwent a second QFN-G-IT test 336 days after the first one, and a third one when the patient developed active TB (6–24 months after the first 336 days) or at the end of the study (October 2012). Every patient was

followed-up every 3 months in order to check for active TB exposure and symptoms.

According to IFN-γ levels, patients were divided in three groups: (I) under 0.35 IU/mL, (II) between 0.35 and 4.00 IU/mL, and (III) above 4.00 IU/mL. After the second QFN-G-IT test, 7% (172/2,512) of the patients had a positive result. From the 172 converters at day 336, 79 (46%) had IFN-γ levels between 0.35 and 4.00 IU/mL and only 2 (2.5%) patients developed active TB. A 36.6% (63/172) of the converters had IFN-γ levels above 4.00 IU/mL and 15.9% (10/63) were diagnosed with TB. When comparing with non-converters and converters at day 336 with IFN-γ values under 4.00 IU/mL, the authors found that TB incidence in converters was significantly higher when IFN-γ values were above 4.00 IU/mL. Regarding the patients with a negative QFN-G-IT test at day 336, 16 developed active TB, from which 14 were tested and QFN-G-IT positive at the diagnostic moment. Regarding reversions, 91 out of the 172 patients who had a QFN-G-IT conversion at day 336 were retested and 53 (58%) reverted to a negative result. The authors found that there were significantly more reversions in converters with IFN-γ levels below 4.00 IU/mL.

To date, many studies have been performed in the assessment of IGRAs and TB risk (7,10,20). However, studies that consider TB disease progression focusing not only on positive and negative result but also on IFN-γ levels at baseline, conversions and reversions are not abundant.

In 2002, Doherry *et al.* described a strong association between IFN-γ production and TB progression, in Ethiopian contacts after a 5-day PBMCs stimulation with ESAT-6 (13). The authors found that contacts who progressed to active TB were the ones with the highest IFN-γ levels (P<0.001). Similarly, in their contact tracing study in household contacts, del Corral *et al.* described a significant trend for TB development in high IFN-γ producers after CFP-10 stimulation (21). Pai *et al.* found that healthcare workers with tuberculin skin test (TST) and QFN-G-IT positive had high IFN-γ levels and were less likely to revert, being reversions more frequent in those individuals with IFN-γ levels close to 0.35 IU/mL and considering that conversions with high IFN-γ levels might predict TB progression (14). Moreover, it has been described that adolescents with a recent QFN-G-IT conversion have a higher risk of TB progression (17).

In a previous study, we assessed the risk of TB development based on QFN-G-IT and TST in contacts (adults and children) with both positive and negative QFN-G-IT results at baseline (10). Overall, we described a higher risk of developing active TB disease in the first 2 years after infection in those contacts with a positive QFN-G-IT test. Focusing on children, all of those who progressed to TB had, at baseline, IFN-γ levels above 0.35 IU/mL. Moreover we found that TB progression risk was higher in contacts with initial IFN-γ levels above the limit of detection (10 IU/mL).

Similarly, Diel *et al.* compared TST and QFN-G-IT in contacts (both adults and children), and evaluated progression to TB during a 4-year follow-up period (12). From the overall study population, the majority of cases with positive QFN-G-IT who developed TB had IFN-γ levels 10-fold higher than the cut-off (0.35 IU/mL), and the majority had IFN-γ levels above the limit of detection. Regarding the children cohort alone, 6 out of the 21 untreated patients with positive QFN-G-IT developed active TB during the follow up and had high IFN-γ levels: five of them with more than 4.00 IU/ml and one 3.59 IU/mL. In this study, all children with positive QFN-G-IT who did not develop active TB had responses lower than 4.5 IU/mL.

Studies developed so far show the importance of considering QFT-G-IT results not only as dichotomous (positive or negative) but also as quantitative. Results and study population vary among studies. However, overall, they seem to relate high IFN-γ levels to TB progression and low IFN-γ levels with low reversion risk. Therefore, Andrews *et al.* study has an increase value in this matter: it is a prospective study with a good follow-up period, the study population is entirely integrated by young children with negative QFN-G-IT at baseline, and the population is well described. In this study authors conclude that (I) TB incidence is significantly higher in children converters with more than 4.00 IU/mL of IFN-γ; (II) converters with IFN-γ values under 4.00 IU/mL have an increased risk of reversion. These results suggest that monitoring in young children could serve to make better decisions in prophylaxis prescription considering especially those with >4.00 IU/mL conversions.

Although these results are promising it should be taken into account that (I) even though contacts with higher IFN-γ levels are the ones who progress to active TB, there is an overlap with those positive QFN-G-IT contacts who remain healthy, and (II) although the risk of TB progression rises with high IFN-γ levels, there are also contacts who develop active TB and have low initial IFN-γ levels (10,12,22). Regarding IFN-γ levels differences in children

among latent TB infection (LTBI) and active TB groups, there is no clear association. Whittaker *et al.* (23) described a lower amount of IFN-γ in LTBI patients compared to active TB, whereas Latorre *et al.* described no significant difference between these two groups (22). In addition to these concerns, Kampmann *et al.* stated that a negative IGRA should not exclude active TB in children but rather serve to confirm it (24). Furthermore, the fact that IFN-γ levels have been described as highly variable in serial IGRA testing (25) should be taken into account and assessed in further studies in this same age cohort before changing the actual guidelines.

Altogether, high IFN-γ conversion levels could serve as indicators for TB progression in the study population, enabling better diagnosis and chemotherapy decisions. However, as the authors state, these findings should be further evaluated in order to consider a revision of the international guidelines in the use of IGRAs in children especially given the IFN-γ previously described variability.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Article 5

Diagnostic benefits of adding EspC, EspF and Rv2348-B to the QuantiFERON Gold In-tube antigen combination

Raquel Villar-Hernández, Thomas Blauenfeldt, Irene Latorre, Beatriz Muriel-Moreno, Esther García-García, María Luiza de Souza- Galvão, Joan Pau Millet, Josefina Sabriá, Adrián Sánchez-Montalva, Juan Ruiz-Manzano, José Pilarte, María Á. Jiménez, Carmen Centeno, Carmen Martos, Israel Molina-Pinargote, Yoel González-Díaz, Javier Santiago, Adela Cantos, Irma Casas, Rosa Guerola, Cristina Prat, José Dominguez, Morten Ruhwald.

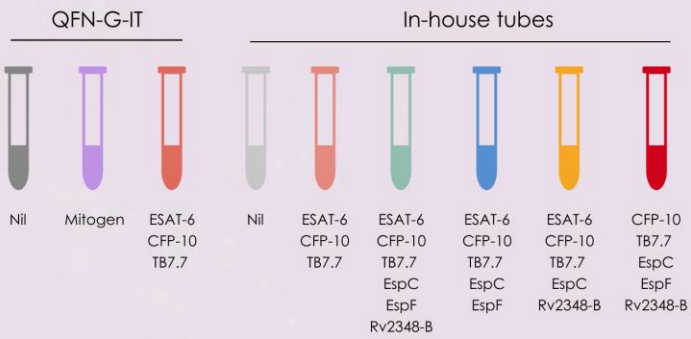
Pending to submit to *Scientific Reports*, 2019

Diagnostic benefits of adding EspC, EspF and Rv2348-B to the QuantiFERON Gold In-tube antigen combination

Raquel Villar-Hernández, Thomas Blauenfeldt, Irene Latorre, Beatriz Muriel-Moreno, Esther García-García, María Luiza de Souza- Galvão, Joan Pau Millet, Josefina Sabriá, Adrián Sánchez-Montalva, Juan Ruiz-Manzano, José Pilarte, María Á. Jiménez, Carmen Centeno, Carmen Martos, Israel Molina-Pinargote, Yoel González-Díaz, Javier Santiago, Adela Cantos, Irma Casas, Rosa Guerola, Cristina Prat, José Dominguez, Morten Ruhwald

Aim

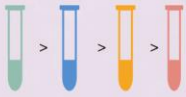
Test the immunogenicity and TB infection diagnosis capacity of new additional antigens to those present in the QFN-G-IT



Procedure

Findings

1. Very good correlation between QFN-G-IT antigens and the in-house tubes
2. In-house tubes could also differentiate between TB infected (active TB and LTBI) and not infected
   No differences between active TB and LTBI
3. More number of positives when adding EspC and EspF but less specific



4. 

It's performance is very similar to that of the QFN-G-IT

What does this mean?

Addition of these extra antigens does not seem to improve the diagnostic accuracy of the actual QFN-G-IT antigen combination; however, they could be good candidates for an ESAT-6 free IGRA in case the ESAT-6 based vaccine or TST are developed and used in the future.

IGRA: IFN-γ release assay; LTBI: Latent TB infection; QFN-G-IT: QuantiFERON-TB Gold In-Tube; TB: Tuberculosis; TST: tuberculin skin test

Raquel Villar Hernández

Diagnostic benefits of adding EspC, EspF and Rv2348-B to the QuantiFERON Gold In-tube antigen combination

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Abstract

Interferon (IFN)-γ release assays (IGRAs) are the most commonly used method to diagnose latent tuberculosis (TB) infection (LTBI). Different approaches to improve the accuracy of LTBI diagnostic tests are under study, such as detection of other cytokines and selection of other antigens to stimulate blood samples with. The aim of this study was to test the use of stimulation with a combination of additional antigens (EspC, EspF and Rv2348-B) to those present in the QuantiFERON-TB Gold In-Tube (QFN-G-IT) (ESAT-6, CFP-10 and TB-7.7) to study their immunogenicity and ability to detect *Mycobacterium tuberculosis* infection.

In this study, we included 115 subjects: 74 were active TB patients, 17 were LTBI individuals and 24 were healthy controls. Whole blood samples were collected from each study subject in the three QFN-G-IT tubes and in different in-house tubes containing different combinations of antigens EspC, EspF and Rv2348, together with ESAT-6, CFP-10 and TB7.7. After overnight incubation at 37ºC, plasma was harvested and IFN-γ levels were detected.

Correlations between IFN-γ levels detected in the QFN-G-IT and the in-house tubes were very good (Spearman Rho (r) above 0.82). After ROC curve analysis and cut-off determination, antigen combinations present in the in-house tubes were able to distinguish between healthy individuals and those with active TB and LTBI with specificities and sensitivities higher than 94.0 % and 91.0%, respectively (AUC above 0.96).

In this study, we tested the use of adding extra antigens to the QFN-G-IT stimulation condition to increase the accuracy of *M. tuberculosis* infection diagnostic assays. Addition of EspC, EspF and (to less extent) Rv2348-B, showed a mild increase in the number of positive cases in infected and active TB individuals but also in the healthy controls group. Therefore,

the addition of these antigens does not seem to improve in a great extent the accuracy of the QFN-G-IT and may even jeopardize the specificity of the assay.

Introduction

Tuberculosis (TB) remains the leading cause of death by a single infectious agent [1]. Good control, as well as proper management of the disease, are of great importance in order to decrease TB burden. For this, early diagnosis as well as a correct and efficient preventive and anti-TB treatment, are key[2]. Screening of latent TB infection (LTBI) and preventive treatment guidance generally relies on the tuberculin test (TST) performance. However, due to its cross-reaction with non-tuberculous mycobacteria and the bacilli Calmette-Guérin (BCG) vaccine, its applicability is limited[3]. To avoid cross-reaction issues, interferon (IFN)-γ release assays (IGRAs) are the most recent and commonly used alternative for TB infection detection. IGRAs are based on the stimulation with specific *Mycobacterium tuberculosis* antigens followed by the measurement of the T-cell-mediated responses from peripheral blood lymphocytes (T-SPOT.TB; Oxford Immunotec Limited, Abingdon, UK) or whole blood (QuantiFERON technology; QFN, Qiagen, Düsseldorf, Germany)[4–6]. However, neither test type (TST and IGRAs) distinguish between active and LTBI, and therefore, do not provide evidence of recent infection, or risk of progression to active TB.

In order to improve LTBI diagnosis and not only distinguish between active disease and infection but also between the different infection stages, many studies have focused on the measurement of cytokines other than IFN-γ and also on the detection of a combination of cytokines[7–9]. In addition, the search of new *M. tuberculosis* antigens and characterization of the immune response after sample stimulation has also been extensively studied[10–14]. Some of the newly described antigens have been proven to yield different responses among active TB patients, TB exposed individuals and healthy controls[12,15,16]. However promising and innovative, there are contrary results among studies and more evidence is needed to validate the use of these novel *M. tuberculosis* antigens. Recently, Ruhwald et al. evaluated a promising ESAT-6 free IGRA which yielded a comparable performance to the QFN-G-IT[17]. In this study, several antigen and peptides were tested in order to find a combination that together with CFP-10, but lacking ESAT-6, could result in a comparable specific response to the currently used combination (ESAT-6, CFP-10 and TB7.7). After whole blood stimulation and specific response analysis, the most frequently recognized antigens by active TB and LTBI individuals were ESAT-6, CFP-10 and EspC followed by Rv2348-B and EspF. As described in this study, EspC, EspF and Rv2348 have less homology with non-tuberculous mycobacteria than ESAT-6 and CFP-10 and full sequence homology with the *M. tuberculosis* complex making them suitable for a TB infection diagnosis method with no cross-reaction issue[17]. Moreover, EspC recognized cases that were not detected by CFP-10 or ESAT-6 (11% and 12%, respectively).

Following the previous findings, the aim of this study was to test the effect of adding different combinations of EspC, EspF and Rv2348-B to the ESAT-6, CFP-10 and TB7.7 present in the QFN-G-IT assay, by measuring the amount of antigen-specific IFN-γ release in whole blood, in order to improve the accuracy of current TB infection diagnostic tests.

Materials & methods

Study population and ethical statement

In this study, we included whole blood samples from (i) microbiologically confirmed TB patients by means of a positive culture and/or a positive PCR, (ii) infected individuals (LTBI) (asymptomatic and with a positive IGRA) and (iii) healthy controls (uninfected individuals with a negative IGRA).

Such samples were collected at five medical centres in Barcelona, Spain: Unitat de Tuberculosi Vall d'Hebron-Drassanes, Unidad Clínica de Tratamiento Directamente Observado "Serveis Clinics", Hospital Universitari Vall d'Hebron, Hospital Sant Joan Despí Moises Broggi, and Hospital Universitari Germans Trias i Pujol.

The study was approved by the ethical review board of the Ethics Committee of the Hospital Germans Trias i Pujol and subsequently for all the Ethics Committee of every participating health care centre (reference number CEI_PI-15-073) and by the ethical review board of the Capital Region of Copenhagen (reference number H-3-2012-008). Written consent was obtained from all study participants.

Preparation of in-house tubes

Vacutainer blood extraction tubes were prepared by the Statens Serum Institut (SSI, Copenhagen, Denmark) with different antigen cocktails for blood stimulation. Briefly, 25ul containing 10μg of the corresponding peptide mix (in-house QuantiFERON (QFN), QFN +A, QFN +B and QFN +C) together with 20IU of heparin were added in cleaned 4ml vacutainer tubes under sterile conditions. For the nil tube, 20IU of heparin alone was added. Tubes were kept at -80ºC, then freeze-dried, and vacuum for a total volume of 1ml was done. Information about the selected antigens and which antigen is added in each in-house tube are shown in table 1.

Whole blood stimulation

Whole blood was drawn into the QuantiFERON-TB Gold In-Tube (QFN-G-IT; Qiagen, Düsseldorf, Germany) and the in-house tubes following the QFN-G-IT manufacturer's instructions. Briefly, 1ml per tube was extracted from each patient. Tubes were placed in a roller for proper mixing of the antigens with the blood and afterwards, incubated overnight at 37ºC. After incubation, plasma was harvested and kept at -20ºC until IFN-γ determination.

Quantification of IFN-γ production

The IFN-γ production was measured using the QFN-G-IT ELISA kit following the manufacturer's instructions. Reactivity from both nil tubes (QFN-G-IT nil and in-house nil) was subtracted from the IFN-γ value in the corresponding QFN-G-IT and in-house tubes. In the case of the QFN-G-IT, a result was considered positive when the amount of IFN-γ was at least 0.35IU/ml after antigen stimulation. A result was considered indeterminate when the stimulation was negative and the value of the positive control was less than 0.5IU/ml or if the negative control was higher than 8.0IU/ml. Mitogen from QFN-G-IT was used as the positive control of the in-house tubes. In the case of the in-house tubes, receiver operating characteristic (ROC) curves were performed in order to obtain cut-off values for each one.

Table 1. Antigens used and in-house combinations.

	Rv number	Function	Protein length	Fraction assesed	Nil	QFN	QFN +A	QFN +B	QFN +C
ESAT-6	Rv3875	RD1, ESX1 substrate	95	1–95	-	Yes	Yes	Yes	Yes
CFP-10	Rv3874	RD1, ESX1 substrate	100	1–100	-	Yes	Yes	Yes	Yes
TB7.7	Rv2654c,p4	RD11, unknown	81	10-81	-	Yes	Yes	Yes	Yes
EspC	Rv3615c	ESX1 substrate	103	54-103	-	-	Yes	Yes	Yes
EspF	Rv3865	ESX1 associated protein	103	9-44	-	-	Yes	Yes	-
Rv2348-B	Rv2348-B	RD7 unknown	108	55-108	-	-	Yes	-	Yes

The previous antigens have the same length as described in Ruhwald et al 2017[17].

Statistics

Correlation among tested antigenic combinations was calculated using Spearman Rho correlation coefficient (r). Median and interquartile ranges (IQRs) were calculated for the levels of IFN-γ detected in each condition and differences in responses were compared using Mann-Whitney t-test. Differences among tests were considered statistically significant when p values were below 0.05. The diagnostic accuracy and cut-off values of the in-house tubes were determined using ROC curves and Youden's Index (J). Calculation of agreement among tests and the 95% confidence interval (CI) was done using Cohen's kappa (κ) coefficient. The strength of agreement was considered "perfect" when κ=1.00, "very good" when κ values were between 0.99 and 0.81, "good" when κ values were between 0.60 and 0.41, and "poor" when κ values were below 0.40. The software used for the statistical analyses and graphs were GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Study population

A total of 115 subjects were included in this study from five different health care centres in Barcelona (Spain). A 64.3% (74/115) were active TB patients, 14.8% (17/115) were LTBI individuals and 20.9%

(24/115) were healthy controls. For demographic and clinical data from the studied population see table 2.

QFN-G-IT and in-house QFN

Considering the overall study population, antigen-specific IFN-γ levels detected in the QFN-G-IT and in-house QFN tubes (both containing the same antigens: ESAT-6, CFP-10 and TB7.7) was significantly higher in the Qiagen tube than in the in-house ($p<0.05$) due to differences in the active TB group (figure 1A). Nevertheless, the correlation between both tubes was very good (Spearman $r = 0.92$) (figure 1B). In both tubes, healthy controls had a significantly lower amount of antigen-specific IFN-γ production compared to active TB and LTBI ($p<0.0001$) but no statistically significant differences were detected between LTBI and active TB samples ($p>0.05$) (figure 1C).

Table 2. Demographic and clinical data of the study population

	Overall (n=115)		
	HC (n=24)	**LTBI (n=17)**	**TB (n=74)**
Age, average (years) ± SD	37.0 ± 10.6	42.1 ± 9.9	41.1 ± 14.8
Gender (%)			
Female	20 (83.3)	4 (23.5)	21 (28.4)
Male	4 (16.7)	13 (76.5)	53 (71.6)
Country of birth (%)			
High TB burden[a]	0 (0.0)	2 (11.7)	11 (14.9)
Low TB burden	24 (100.0)	15 (88.2)	59 (79.7)
Unknown	0 (0.0)	0 (0.0)	3 (4.1)
BCG vaccination (%)	1 (4.2)	10 (58.8)	27 (36.5)
QFN-G-IT			
Positive	0 (0.0)	16 (94.1)	66 (89.2)
Negative	24 (100.0)	1 (5.9)[b]	8 (10.8)
Prophylaxis (%)			
Before starting prophylaxis	-	5 (29.4)	-
On prophylaxis	0 (0.0)	12 (70.6)	0 (0.0)
< 30 days	-	12 (100.0)	-
≥ 30 days	-	0 (0.0)	-
Average (days) ± SD in patients with prophylaxis	-	21.1 ± 7.0	-
Treatment (%)			
Before starting treatment	-	-	9 (12.2)
On treatment	0 (0.0)	0 (0.0)	65 (87.8)
< 30 days	-	-	47 (63.5)
≥ 30 days	-	-	18 (24.3)
Average (days) ± SD in patients with treatment	-	-	24.09 ± 14.9

[a]About 150 cases per 100 000 population. [b]T-SPOT.TB positive. TB: active tuberculosis patients, LTBI: latently tuberculosis infected individuals, HC: healthy controls.

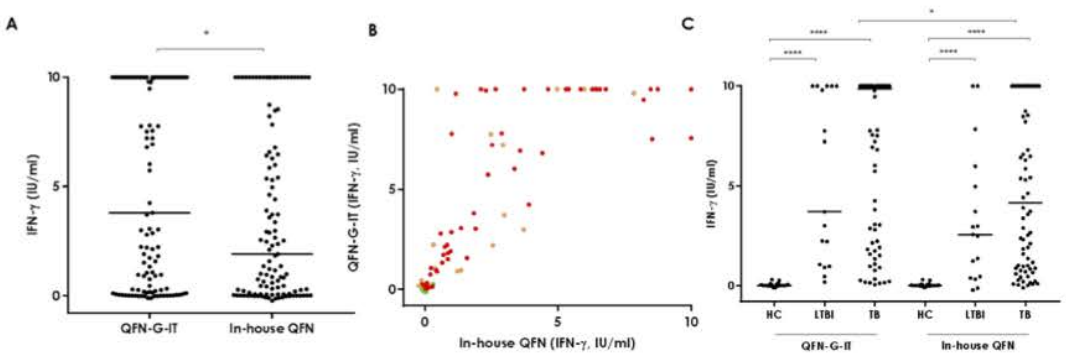

Figure 1. Antigen-specific IFN-γ release in the QFN-G-IT and in-house QFN tubes. A) Overall. Higher levels of IFN-γ were detected in the QFN-G-IT compared to the in-house QFN ($p<0.05$). Median and interquartile range (IQR) values: 3.8 IU/ml (0.21 – 10.00) for the QFN-G-IT and 1.9 IU/ml (0.07 – 7.84) for the in-house QFN. **B) Overall correlation.** Spearman r = 0.92. Green circles correspond to healthy control samples, orange to LTBI individuals and red to active tuberculosis patients. **C) Per groups.** Significantly higher levels of IFN-γ in LTBI and active TB compared to HC in both tubes ($p<0.0001$). No significant differences between IFN-γ levels in LTBI and active TB ($p>0.05$). IFN-γ levels were significantly higher in the QFN-G-IT tube than in the in-house QFN tube when considering the active TB group ($p<0.01$). HC: healthy controls, LTBI: latent tuberculosis infection, TB: active tuberculosis.

In-house QFN +A (+ EspC, EspF and Rv2348-B) and in-house QFN

In order to evaluate the benefits of adding three extra antigens (EspC, EspF and Rv2348-B) to the currently used stimulation (ESAT-6, CFP-10 and TB7.7) we compared the in-house QFN +A stimulation with the in-house QFN. Performance of the in-house QFN +A was similar to that of the in-house QFN with a strong overall correlation (Spearman r = 0.86; figure 2A) however, antigen-specific IFN-γ release was significantly higher ($p<0.05$) in the in-house QFN +A than in the in-house QFN (figure 2B). This difference between both tubes is no longer significant when excluding the three outliers from the healthy control group (data not shown). When classifying the study population in groups, healthy controls had a higher level of IFN-γ in the in-house QFN +A tube than in the in-house QFN ($p<0.05$) (figure 2C) due again to these three outliers. No significant differences were detected among the active TB and LTBI groups. Moreover, as occurred in the in-house QFN condition, IFN-γ levels in the in-house +A tube were higher in active TB patients (median [IQR] = 5.82 IU/ml [1.55 – 10.00]) and LTBI individuals (median [IQR] = 5.41 IU/ml [1.57 – 10.00]) than in healthy controls (median [IQR] = 0.02 IU/ml [0.01 – 0.08]) ($p<0.0001$) (figure 2C).

In-house QFN +B (+ EspC and EspF) and +C (+ EspC and Rv2348-B)

As described above, the addition of the three extra antigens present in the in-house QFN +A increased the number of positive results. In order to evaluate if the addition of not all three of the antigens but only two would also yield a similar response, we compared the in-house QFN +B and +C tubes with the in-house QFN +A.

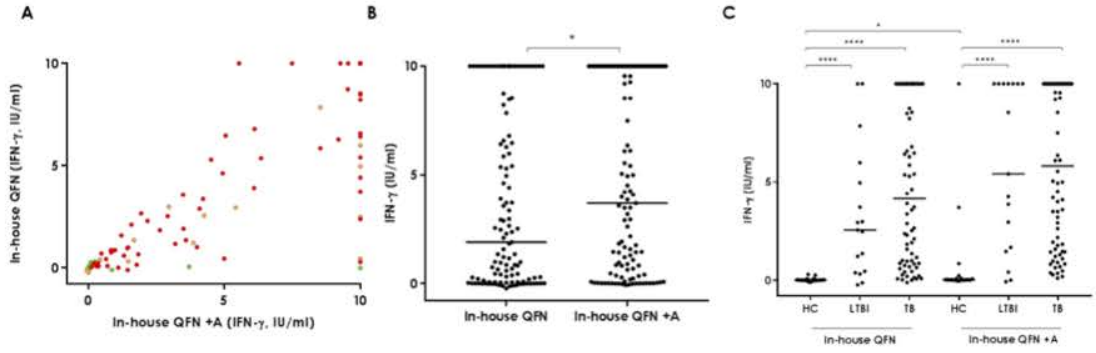

Figure 2. Antigen-specific IFN-γ release in the in-house QFN and in-house QFN +A tubes. A) Overall correlation. Spearman r = 0.86. Green circles correspond to healthy control samples, orange to LTBI individuals and red to active tuberculosis patients. **B) Overall.** IFN-γ levels were higher in the in-house QFN +A compared to the in-house QFN (p<0.05). Median and interquartile range (IQR) values: 1.9 IU/ml (0.07 – 7.84) for the in-house QFN and 3.7 IU/ml (0.41 – 10.00) for the in-house +A. **C) Per groups.** Significantly higher levels of IFN-γ in LTBI and active TB compared to HC in both tubes (p<0.0001). No significant differences between IFN-γ levels in LTBI and active TB (p>0.05). IFN-γ levels were significantly higher in the in-house +A tube than in the in-house QFN tube when considering the HC group (p<0.05). HC: healthy controls, LTBI: latent tuberculosis infection, TB: active tuberculosis.

Antigen-specific IFN-γ release in both extra in-house combinations correlated strongly with the in-house QFN +A (Spearman r = 0.95 when comparing in-house QFN +B with +A, and Spearman r = 0.87 when comparing in-house QFN +C with +A) (figure 3A and B). No significant differences were detected in the overall amount of antigen-specific IFN-γ release among tubes (figure 3C). As expected, IFN-γ levels were significantly higher in active TB patients (median $[IQR]_{\text{in-house QFN +B}}$ = 5.95 IU/ml [1.80 – 10.00], median $[IQR]_{\text{in-house QFN +C}}$ = 4.78 IU/ml [1.53 – 10.00]) and LTBI individuals (median $[IQR]_{\text{in-house QFN +B}}$ = 3.49 IU/ml [1.90 – 10.00], median $[IQR]_{\text{in-house QFN +C}}$ = 4.03 IU/ml [1.25 – 10.00]) than in healthy controls (median $[IQR]_{\text{in-house QFN +B}}$ = 0.02 IU/ml [-0.01 – 0.07], median $[IQR]_{\text{in-house QFN +C}}$ = 0.03 IU/ml [0.00 – 0.05]) (p<0.0001) (figure 3D).

ROC curves and positivity rates

ROC curve analyses were performed in order to calculate the positivity cut-off values of the in-house QFN and in-house QFN +A tubes. Those active TB and LTBI patients with a positive QFN-G-IT result were considered as positive cases and healthy controls (all of which had a negative QFN-G-IT) were considered as negative ones. The areas under the curve (AUC) were excellent, reaching 0.9863 in the case of the in-house QFN, 0.9697 in the case of the in-house QFN +A, 0.9777 in the case of the in-house +B and 0.9758 in the case of the in-house QFN +C (figure 4). Following the Youden's Index (J), the suitable cut-off value for the in-house QFN condition was 0.31IU/ml (J = 0.96) yielding a 100% sensitivity [CI: 85.75% - 100%] and 96.39% specificity [CI: 89.8% - 99.25%]. In the case of the in-house QFN +A tube, the selected cut-off was 0.29IU/ml (J = 0.89) with a 91.67% sensitivity [CI: 73% - 98.97%] and 97.59% specificity [CI: 91.57% - 99.71%]. The selected cut-off for the in-house QFN +B was 0.335IU/ml (J = 0.94) yielding a 96.0% sensitivity [CI: 79.65% - 99.9%] and 97.65% specificity [CI: 91.76% - 99.71%]. For the in-house QFN +C the cut-off was 0.345IU/ml (J = 0.96) reaching a 100% sensitivity [CI: 86.28% - 100%] and 96.43% specificity [CI: 89.92% - 99.26%]. Interestingly, using the

calculated positivity cut-offs, the overall amount of positive results obtained using the in-house QFN +A tube was higher than that obtained by the rest of the tested combinations QFN tubes (table 3). The number of positive results obtained by the in-house QFN +B and in-house QFN +C was similar to that of the in-house QFN +A, but lower when using the in-house QFN +C (table 3). Considering active TB and LTBI groups together, there was an overall of 7 more positive results using the in-house QFN +A (86/91) than using the in-house QFN (79/91). However, there are also 3 healthy controls that yielded positive results in the in-house QFN +A that were QFN-G-IT and in-house QFN negative. Compared to the in-house QFN +B and +C, the in-house QFN +A combination yielded one positive result more than the in-house QFN +B (85/91) and two more than the in-house QFN +C (84/91).

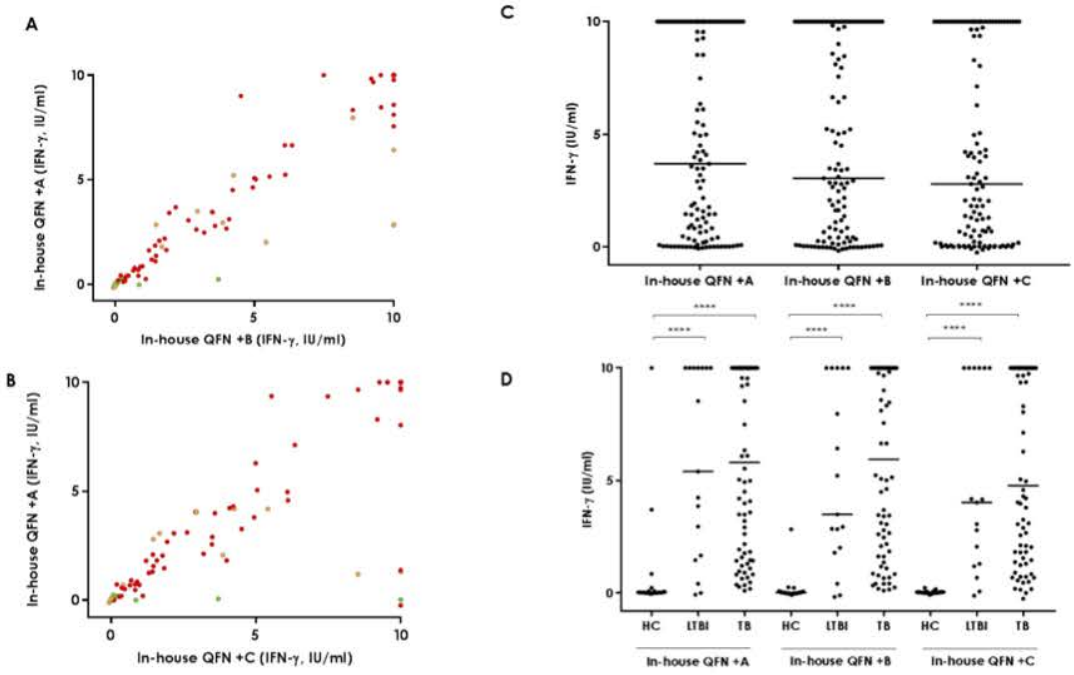

Figure 3. Antigen-specific IFN-γ release in the in-house QFN +A and in-house QFN +B and in-house QFN +C tubes. A) Correlation between in-house QFN +A and +B. Spearman r = 0.95. Green circles correspond to healthy control samples, orange to LTBI individuals and red to active tuberculosis patients. **B)** Correlation between in-house QFN +A and +C. Spearman r = 0.87. Green circles correspond to healthy control samples, orange to LTBI individuals and red to active tuberculosis patients. **C)** Overall. No significant differences overall (p>0.05). Median and interquartile range (IQR) values: 3.7 IU/ml (0.41 – 10.00) for the in-house QFN +A, 3.05 IU/ml (0.26 – 10.00) for the in-house QFN +B, and 2.79 IU/ml (0.18 – 10.00) for the in-house QFN +C tube. **D)** Per groups. Significantly higher levels of IFN-γ in LTBI and active TB compared to HC in all tubes (p<0.0001). No significant differences between IFN-γ levels in LTBI and active TB (p>0.05). No significant differences between tubes in each group (p>0.05). HC: healthy controls, LTBI: latent tuberculosis infection, TB: active tuberculosis.

The agreement between tubes was considered as very good ($\varkappa$ = 0.938; CI = 0.869 - 1.000) when comparing the commercial and the in-house QFN tube and as good when comparing the QFN-G-IT with the in-house QFN +A ($\varkappa$ = 0.750; CI = 0.613 - 0.888) and both in-house tubes ($\varkappa$ = 0.781; CI = 0.655 - 0.908). There was a very good agreement between the in-house QFN +A and the +B ($\varkappa$ = 0.881;

CI = 0.779- 0.983) and +C (κ = 0.837; CI = 0.721 - 0.953). Agreement between the in-house QFN +B and +C tubes was also very good (κ = 0.955; CI = 0.893 - 1.000).

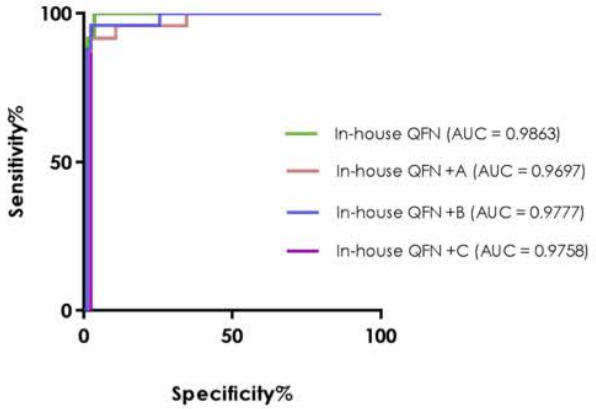

Figure 4. Receiver operating characteristic (ROC) curve analysis. Antigen-specific (antigen-nil) release of IFN-γ in the tested in-house tubes. Areas under the curve (AUCs) from each tested tube were comparable: in-house QFN AUC = 0.9863 (green line), in-house QFN +A AUC = 0.9697 (orange line), in-house QFN +B AUC = 0.9777 (blue line) and in-house QFN +C AUC = 0.9758 (purple line). Active tuberculosis patients and latently infected individuals with a QFN-G-IT positive result were considered as positive controls and QFN-G-IT negative healthy controls as negative controls.

Table 3. Test results obtained in the in-house QFN and in-house QFN +A. Cut-offs used: 0.35 IU/ml for the QFN-G-IT, 0.31 IU/ml for the in-house QFN, 0.29 IU/ml for the in-house QFN +A, 0.335 for the +B and 0.345 for the +C.

		Active TB (n=74)	LTBI (n=17)	Healthy Controls (n=24)
QFN-G-IT				
	Positive	66 (89.2)	16 (94.1)	0 (0.0)
	Negative	8 (10.8)	1 (5.9)	24 (100.0)
In-house QFN				
	Positive	64 (86.5)	15 (88.2)	0 (0.0)
	Negative	10 (13.5)	2* (11.8)	24 (100.0)
In-house QFN +A				
	Positive	71 (95.9)	15 (88.2)	3 (12.5)
	Negative	3 (4.1)	2* (11.8)	21 (87.5)
In-house QFN +B				
	Positive	70 (94.6)	15 (88.2)	1 (4.2)
	Negative	4 (5.4)	2* (11.8)	23 (95.8)
In house QFN +C				
	Positive	69 (93.2)	15 (88.2)	0 (0.0)
	Negative	5 (6.8)	2* (11.8)	24 (100.0)

*One of them had a limit QFN-G-IT positive result and the other was also negative for QFN-G-IT. TB: tuberculosis, LTBI: latently tuberculosis infected individuals.

Discussion

LTBI infection diagnosis is key for disease control and management improvement; however, the current methods remain insufficient. Therefore, many efforts are invested in the development of new diagnostic tools as well as the improvement of the current ones. Following this direction, in this study,

we evaluated the effect of adding extra specific antigens (EspC, EspF and Rv2348-B) to the ones currently used in the QFN-G-IT in order to test if their immunogenicity improved the assay's performance increasing, therefore, the accuracy of the test.

Novel *M. tuberculosis* antigens have been extensively studied with the purpose to characterize better TB infection[10,12–14]. In this study, the immune response against new combinations of antigens was evaluated by measuring the levels of IFN-γ produced after specific stimulation. The antigenic combinations here tested rendered an IFN-γ response comparable to that obtained by the QFN-G-IT (Spearman r above 0.82). Stimulation with all the tested conditions yielded significantly higher IFN-γ levels in active TB patients and LTBI individuals compared to healthy controls ($p<0.0001$). After ROC curve analysis and cut-off values selection, test results were calculated. The addition of EspC, EspF and Rv2348-B to the currently used ESAT-6, CFP-10 and TB7.7 enabled the identification of 7 more positive cases. Addition of EspC and EspF identified less positive cases than when the three extra antigens were present but yielded more positive results compared to adding EspC and Rv2349-B (2 more when considering the overall population and 1 when excluding healthy controls). These results show that the addition of EspC could increase the identification of positive cases together with EspF and Rv2348-B, being the later the antigen that less adds. However, the addition of these extra antigens could also increase the number of unspecific responses as seen in the in-house QFN+A and +B which detect as positive cases three and one healthy controls, respectively.

Taking into account the high correlation and agreement between the in-house QFN and the rest of in-house combinations, the mild increase in positive results, and the possible unspecific responses obtained in this study, the addition of EspC, EspF and Rv2348-B, does not seem to improve significantly the current QFN-G-IT assay.

Given the immunogenicity of ESAT-6, this antigen has been extensively studied as a candidate component for protective and therapeutic vaccines[18–22]. Moreover, recently the use of this *M. tuberculosis*-specific antigen has been suggested and is already on trial as the next generation skin test together with CFP-10 in several studies[23–26]. The ESAT-6 and/or CFP-10 skin tests developed, show to be safe and lack cross-reaction with the BCG vaccine and non-tuberculous mycobacteria.

Therefore, the development of an ESAT-6 free IGRA that could serve as a companion diagnostic test in case of a future ESAT-6 based vaccine has been under study[17,27]. So far, the ESAT-6 free IGRA containing CFP-10, TB7.7, EspC, EspF and Rv2348-B, has yielded promising results, showing comparable performance to the QFN-G-IT. Although done in a small cohort of patients, we consider that it is worth mentioning that during this study we also tested this same ESAT-6 free antigen combination. This condition was tested in 9 samples (5 from active TB patients, 1 from LTBI individuals and 3 from healthy controls). The amount of antigen-specific IFN-γ released in this condition was comparable to that of the in-house QFN and in-house QFN +A (1.51 IU/ml, 2.55 IU/ml and 2.73 IU/ml, respectively). Considering as responders those individuals with antigen-specific IFN-γ levels above 0.61 IU/ml (as the cut-off value recently established)[27], all healthy controls tested were negative,

the LTBI individual was positive, and all active TB patients except one (with an IFN-γ release of 0.56 IU/ml) were positive. These results are similar to those described previously by Ruhwald et al[17] with comparable specificity rates (95-98% and 99-100%, respectively) and similar sensitivity (91-100% and 89%, respectively). We are aware that the number of cases tested by the ESAT-6 free condition is very low and more cases should be added in order to arrive at a more robust conclusion. However, we consider that these results add evidence to the previous studies done in this direction and emphasize the promising perspective of using this new antigenic combination for future TB diagnosis.

In this study, the immune response generated after stimulation with the different antigen combinations was evaluated solely by the amount of IFN-γ released. However, in order to characterize better this response, it would be of great interest to study the presence of other cytokines such as interferon gamma-induced protein 10 (IP-10). IP-10 has been described as an alternative biomarker, which is expressed in concert with IFN-γ but at higher levels (100 fold)[8,28,29] and seems to have a better diagnostic performance than the IGRAs in children, HIV positive individuals and patients receiving immunosuppressant therapies[30–33]. Despite the fact that in this study IP-10 detection was not performed, considering previous studies, we expect that the results would be comparable to that of the IFN-γ and in any case, improve sensitivity. Moreover, detection of signatures comprised by a combination of cytokines instead of a single cytokine pattern could also be of use to better characterize the response to the new antigenic combinations tested in this study and perhaps enable discrimination between active and latent states.

Furthermore, TST and IGRAs have been shown to have reduced sensitivity in patients at high risk of developing TB such as children, HIV positive individuals and patients with immune-mediated inflammatory diseases (IMIDs)[34,35]. Testing the antigen combinations used in this study with a cohort of high TB risk patients would be of great interest in order to characterize their immune response and compare it with that of the actual IGRAs, in search of an improved tool for these cohorts at risk.

Much is still to be done regarding TB control and management to decrease the disease's burden. TB infection diagnosis, as well as stage characterization, are key for these tasks; however, the existing methods are not enough. In this study, we provided evidence that addition of EspC, EspF and (to less extent) Rv2384-B, to the current combination of ESAT-6, CFP-10 and TB7.7, could increase the detection of TB infected cases. Moreover, and based not only on this study but previous ones, we consider that the search for alternative stimulation combination for new TB diagnostic tests should focus on the use of an ESAT-6 free condition combined with the other specific antigens tested here. Evaluating different combination of specific immunogenic antigens such as EspC, EspF and Rv2348-B but lacking ESAT-6 and possibly CFP-10, is of great interest regarding the importance of the later in vaccine and skin test development. Moreover, evaluation of the immune response to such antigens by detection of cytokines other than IFN-γ alone should be done to evaluate their capacity not only to distinguish between TB infected and uninfected individuals, but also serve to distinguish between infection stages and disease and aid the diagnosis in high TB risk groups.

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Article 6

Study of CD27 and CCR4 Markers on Specific CD4+ T-Cells as Immune Tools for Active and Latent Tuberculosis Management

Irene Latorre, Marco A. Fernández-Sanmartín, Beatriz Muriel-Moreno, **Raquel Villar-Hernández**, Sergi Vila, Maria L. De Souza-Galvão, Zoran Stojanovic, María Á. Jiménez-Fuentes, Carmen Centeno, Juan Ruiz-Manzano, Joan Pau Millet, Israel Molina-Pinargote, Yoel D. González-Díaz, Alicia Lacoma, Lydia Luque-Chacón, Josefina Sabriá, Cristina Prat and Jose Domínguez.

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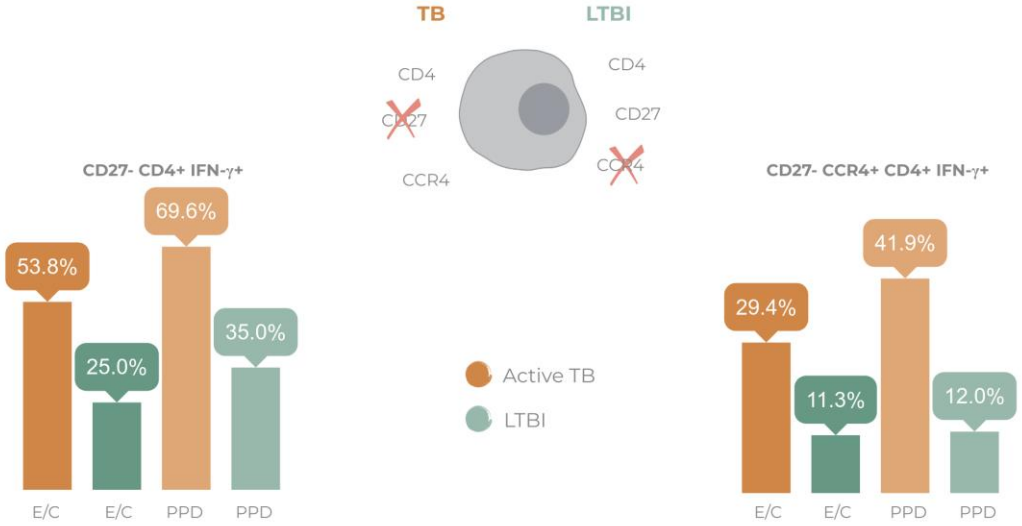
The aim of our study is focused on the assessment of several T-cell subsets to understand immune-mechanisms in TB.

This phenotypic immune characterization is based on the study of the specific immune responses of T-cells expressing CD27 and/or CCR4 homing markers.

Active TB patients have a higher amount of TB specific CD4+ CD27- and CD4+ CD27- CCR4+ cells than LTBI individuals. When using CCR4 alone, the diagnosis accuracy was not good.



Cell population differences after specific stimulation with ESAT-6 and CFP-10 (E/C) and PPD
These differences are also seen in TNF-α+ CD4+ cells

A high CD27 MFI ratio was significantly associated with active TB.

CD27 MFI ratio is the MFI of CD27 in CD4+ T-cells over the MFI of CD27 in IFN-γ+ CD4+ T-cells
This ratio allows normalization of the results avoiding subjectivity and discrepancies on CD27 positive or negative gating

CD27 and CCR4 expression could serve as a good immunodiagnostic method.

The immunological characterization of subset populations could be a promising tool for understanding the biological basis of the disease.

TB: Tuberculosis. LTBI: latent TB infection. MFI: Median fluorescence intensity

Raquel Villar Hernández

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Study of CD27 and CCR4 Markers on Specific CD4$^+$ T-Cells as Immune Tools for Active and Latent Tuberculosis Management

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The immunological characterization of different cell markers has opened the possibility of considering them as immune tools for tuberculosis (TB) management, as they could correlate with TB latency/disease status and outcome. CD4$^+$ T-cells producing IFN-γ^+ with a low expression of CD27 have been described as an active TB marker. In addition, there are unknown homing receptors related to TB, such as CCR4, which might be useful for understanding TB pathogenesis. The aim of our study is focused on the assessment of several T-cell subsets to understand immune-mechanisms in TB. This phenotypic immune characterization is based on the study of the specific immune responses of T-cells expressing CD27 and/or CCR4 homing markers. Subjects enrolled in the study were: (i) 22 adult patients with active TB, and (ii) 26 individuals with latent TB infection (LTBI). Blood samples were drawn from each patient. The expression of CD27 and/or CCR4 markers were analyzed within CD4$^+$ T-cells producing: (i) IFN-γ^+, (ii) TNF-α^+, (iii) TNF-α^+IFN-γ^+, and (iv) IFN-γ^+ and/or TNF-α^+. The percentage of CD27$^-$ within all CD4$^+$ T-cell populations analyzed was significantly higher on active TB compared to LTBI after PPD or ESAT-6/CFP-10 stimulation. As previously reported, a ratio based on the CD27 median fluorescence intensity (MFI) was also explored (MFI of CD27 in CD4$^+$ T-cells over MFI of CD27 in IFN-γ^+CD4$^+$ T-cells), being significantly increased during disease ($p < 0.0001$ after PPD or ESAT-6/CFP-10 stimulation). This ratio was also assessed on the other CD4$^+$ T-cells functional profiles after specific stimulation, being significantly associated with active TB. Highest diagnostic accuracies for active TB (AUC $\geq$ 0.91) were achieved for: (i) CD27 within IFN-γ^+TNF-α^+CD4$^+$ T-cells in response to ESAT-6/CFP-10, (ii) CD27 and CCR4 markers together within IFN-γ^+CD4$^+$

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T-cells in response to PPD, and (iii) CD27 MFI ratio performed on IFN-γ^+TNF-α^+CD4$^+$ T-cells after ESAT-6/CFP-10 stimulation. The lowest diagnostic accuracy was observed when CCR4 marker was evaluated alone (AUC $\leq$ 0.77). CD27 and CCR4 expression detection could serve as a good method for immunodiagnosis. Moreover, the immunological characterization of markers/subset populations could be a promising tool for understanding the biological basis of the disease.

Keywords: CD4 T-cells, CD27, CCR4, flow cytometry, immunity, latent tuberculosis, tuberculosis

INTRODUCTION

Given the limited knowledge on tuberculosis (TB) biomarkers, the study of different T-cell subsets, as well as *Mycobacterium tuberculosis* specific antigens and cytokines, are attractive options to follow in order to understand TB pathogenesis as well as the interplay between infection and disease (1–3). Usually, TB outcome is understood as a bimodal model between active TB and latent TB infection (LTBI). However, in the past years, infection has been associated with a dynamic and wide spectrum containing different latency phases (4). Furthermore, active TB is known to be a heterogeneous disease which comprises a wide range of manifestations and forms. The key to control the spread of TB is a rapid diagnosis in an early stage. However, active TB confirmation can be difficult and the commonly used test systems are still insufficient. Due to all these reasons, the development of alternative diagnostic methods remains a challenge for improving TB control. In this aspect, the immunological characterization of different cell markers has opened the possibility of considering them as immune tools for TB management, as they could correlate with cell differentiation, latency/disease status, and outcome (1, 5).

M. tuberculosis sensitization can be detected by tuberculin skin test (TST), classically used in LTBI diagnosis. However, this assay presents cross-reactive antigens and other proteins which could lead to false-positive results. More than a decade ago, the *in vitro* interferon (IFN)-gamma (γ) release assays (IGRAs) were introduced. These tests have been proven to be more specific and useful for LTBI diagnosis and sensitization detection than the TST. They are not affected by cross-reaction caused by BCG vaccination and/or non-tuberculous mycobacteria (NTM) infection (6–8). These techniques are based on the detection of IFN-γ released by sensitized T-cells after stimulation with specific *M. tuberculosis* antigens (ESAT-6 and CFP-10) (9). Nevertheless, both TST and IGRAs identify sensitization to the bacilli, which is translated into a detectable immune response. Therefore, they cannot discriminate between active TB and infection, nor identify those individuals with high risk of developing active TB when infected (10, 11). New biomarkers are therefore needed to improve disease immune diagnosis, providing prognostic information, assessing risk-stratification in LTBI individuals, and revealing general biological mechanisms of the pathogenesis.

CD4$^+$ T-cells with a low expression of CD27 have been described as an immune biomarker of active TB disease and lung tissue destruction. The decrease of CD27 expression indicates the existence of differentiated effector T-cells which produce cytokines upon antigen encounter. This subset phenotype (CD27$^-$CD4$^+$ or CD27^{low}CD4$^+$) is specifically increased in whole blood and at the site of infection during active disease (12–15). This is due to the process known as homing, which refers to the migration of specific cell subsets to the infected tissue. Recently, a new strategy based on CD27 marker detection has been developed for active TB diagnosis (16, 17). This assay is able to discriminate between active TB and LTBI by analyzing CD27 expression on specific *M. tuberculosis* CD4$^+$ T-cells that respond secreting IFN-γ. This new approach, assesses the ratio of the median fluorescence intensity (MFI) between CD27 on CD4$^+$ T-cells and CD27 on specific T-cells in response to PPD or ESAT-6/CFP-10 antigens. There are still other novel and potential homing markers to explore that might be useful tools for understanding TB pathogenesis and improving diagnosis. For example, the chemokine receptor CCR4, which is considered a homing marker that could be expressed on several cells of the immune system including T helper type 1 (Th1) cells. It is known that T-cells expressing CCR4 surface marker are recruited in inflammatory sites (18). Some evidence suggest that the induction of CCR4 expression is associated with the migration of CD4$^+$ T-cells into the lungs, indicating that this homing marker could play a protective role in the immunity against some respiratory pathogens (19, 20). Together, these findings open the possibility for new studies on the development of novel strategies for TB management and understanding the different mechanisms against the disease. In the present study, we focus on the assessment of several T-cell subsets in order to characterize different TB latency/disease immune-mechanisms. This immune characterization could allow the development of new strategies for TB management based on the study of the immune response of T-cells expressing CD27 and/or CCR4 markers in patients with active TB and LTBI individuals.

MATERIALS AND METHODS

Study Population and Inclusion Criteria

For this study we enrolled subjects with active TB or LTBI suspicion, who attended the four following centers located in Barcelona (Spain): Hospital Germans Trias i Pujol, Unitat de Tuberculosi Vall d'Hebron-Drassanes, Serveis Clínics-Unitat Clínica de Tractament Directament Observat de la Tuberculosi and Hospital Sant Joan Despí Moises Broggi. A total of 16 mL of blood per patient were drawn in CPT tubes (BD Biosciences, San Jose, CA, USA). Blood was directly sent to the Institut

d'Investigació Germans Trias i Pujol for peripheral blood mononuclear cells (PBMCs) isolation and cytometry testing.

Subjects enrolled in the study were classified as: (i) adult patients with active TB (pulmonary or extrapulmonary) with a positive culture and/or PCR for *M. tuberculosis*. Patients were enrolled within the first 4 weeks of starting anti-TB therapy; and (ii) individuals with LTBI enrolled during contact tracing studies or LTBI screenings. In this group, LTBI was defined based on a positive TST and/or IGRAs in the absence of clinical symptoms and radiological signs compatible with active TB. Chemoprophylaxis was prescribed in all of these subsets, being all of them enrolled during the first 4 weeks of preventive therapy.

TST was performed according the Mantoux technique using two tuberculin units of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark), and was evaluated within 48–72 h. According to the Spanish Pulmonology and Thoracic Surgery Society guidelines, a TST $\geq$ 5 mm was considered positive (21, 22). T-SPOT.TB (Oxford Immunotec, Abingdon, UK) and QuantiFERON-TB Gold In-Tube (QFN-G-IT; Qiagen, Düsseldorf, Germany) were performed and interpreted according to the manufacturer's instructions provided in the kits.

PBMCs Isolation, Preservation, and Stimulation

PBMCs were isolated using CPT tubes (BD Biosciences). Afterwards, cells were cryopreserved and stored in liquid nitrogen for later flow cytometry analyses. Cryopreserved PBMCs were thawed and rested during 2 h in a humidified incubator at 37°C with 5% CO_2 in RPMI 1,640 medium (Biowest, Nuaillé, France) containing 10% of heat-inactivated fetal calf serum (FCS) with benzonase (Sigma, St. Louis, MO, USA; final concentration 10 U/mL). PBMCs from each patient were stimulated overnight at 37°C with 5% CO_2 with the recombinant proteins ESAT-6/CFP-10 (Lionex Diagnostics and Therapeutics, Braunschweig, Germany; final concentration 2 μg/mL for each antigen) and PPD (Statens Serum Institut, Copenhagen, Denmark; final concentration 10 μg/mL). The staphylococcal enterotoxin B (SEB; Sigma; final concentration 2.5 μg/mL) was used as a positive control. A negative control without stimulation was also included. Cells were also co-stimulated with anti-CD28 and anti-CD49d monoclonal antibodies (BD Bioscience; final concentration 2 μg/mL each). After 2 h of incubation, Brefeldin A (BFA; Sigma; final concentration 3 μg/mL) was added into the culture media to inhibit the intracellular vesicular transport. Then, PBMCs were left in the incubator overnight.

Surface and Intracellular Staining

After stimulation, PBMCs were stained with the following surface antibodies: anti-CD4 BV786 (BD Bioscience; clone SK3), anti-CD3 PerCP (BioLegend, San Diego, CA, USA; clone SK7), anti-CD27 BV605 (BD Bioscience; clone L128), anti-CCR4 PE-CF594 (BD Bioscience; clone 1G1), and anti-CD8 BV510 (BD Bioscience; clone SK1). A viability marker was also used to exclude dead cells (LIVE/DEAD, Near-IR fluorescent reactive dye; Thermo Fisher, Waltham, MA, USA). For intracellular staining, PBMCs were fixed/permeabilized (IntraStain; Dako, Santa Clara, CA, USA) and then stained with anti-IFN-γ APC (BD Bioscience; clone B27) and anti-TNF-α PE-Cy7 (BD Bioscience; clone Mab11). Markers detection was performed in a BD LSRFortessa flow cytometer (BD Bioscience). A total of 100.000 alive $CD3^+$ T-cells were acquired within 2–3 h after staining.

Flow Cytometry and Data Analysis

Aggregated cells were excluded by gating on the diagonal that appears with *forward scatter* (FSC)-H and FSC-A characteristics. Then, lymphocytes were selected according to their FSC-A and *side scatter* (SSC)-A. $CD4^+$ and $CD8^+$ T-cells were gated based on alive $CD3^+$ T-cells. The gating strategy is represented in **Figure S1** in **Supplementary Material**. Specific IFN-γ and/or TNF-α secretion was analyzed on $CD4^+$ and $CD8^+$ T-cells. The expression of CD27 and/or CCR4 markers were studied within the following populations after PPD or ESAT-6/CFP-10 stimulation: (i) IFN-γ^+CD4^+ T-cells, (ii) TNF-α^+CD4^+ T-cells, and (iii) IFN-γ^+TNF-α^+CD4^+ T-cells. In addition, a boolean analysis was performed on $CD4^+$ T-cells producing IFN-$\gamma^+$and/orTNF-α^+ in order to characterize the expression of CD27 and/or CCR4 on T-cells producing any cytokine. To assess the expression of CD27 and/or CCR4 homing markers, the frequency of cytokine production after specific stimulation was defined as positive when it was twice the amount when compared to its negative control (unstimulated sample). Fluorescence Minus One (FMO) controls were included in each experiment to set up the gates. A ratio based on CD27 MFI was also calculated as suggested by Portevin et al. (16). This ratio is performed measuring the MFI of CD27 marker on $CD4^+$ T-cells over the MFI of CD27 marker on IFN-γ^+CD4^+ specific T-cells. This ratio based on CD27 MFI was also studied in the other T-cells phenotypes (TNF-α^+CD4^+; IFN-γ^+TNF-α^+CD4^+; and IFN-$\gamma^+$and/orTNF-α^+CD4^+ T-cells). Results comparing percentages of $CD27^-$ and/or $CCR4^+$ T-cells, as well as CD27 MFI ratios between groups were performed using the two-tailed Mann-Whitney *U*-test for pairwise comparisons. Differences were considered statistically significant when a *p*-value was <0.05. Correlations between the percentage of $CD27^-$ T-cells and CD27 MFI ratio were calculated using the two-tailed non-parametric Spearman test. Receiver operating characteristic (ROC) analysis and areas under the curve (AUC) were calculated in order to assess the accuracy of the different biomarkers for TB diagnosis. Flow cytometry data was analyzed using BD FACSDiva software (BD Bioscience). Graphical representation is based on GraphPad Prism version 4 (GraphPad Software, Inc, San Diego, CA).

RESULTS

Patient Characteristics

A total of 48 subjects were enrolled in the study: (i) 22 active pulmonary and extrapulmonary TB patients with *M. tuberculosis* culture confirmation, and (ii) 26 individuals with LTBI. Demographical and clinical characteristics are detailed in **Table 1**. Overall, 62.5 (30/48) were men and 37.5% (18/48) women. The mean age (years) ± standard deviation (SD) was 43.54 ± 16.06.

TABLE 1 | Demographic and clinical characteristics of the participants regarding the study group.

VARIABLES	Active TB	LTBI
Participants, *n*	22	26
Mean age, years ± SD	38.68 ± 15.35	47.65 ± 15.77
Male gender, *n*(%)	18 (81.8)	12 (46.2)
POSITIVE PCR, *n*(%)		
Positive	10 (45.4)	–
Negative	4 (18.2)	–
Unknown	8 (36.4)	–
TYPE of TB		
Pulmonary	18 (81.8)	–
Extrapulmonary [a]	4 (18.2)	–
ANTI-TB TREATMENT, *n*(%)		
Before starting treatment	3 (13.6)	–
After starting treatment (<1month)	19 (86.4)	–
Not prescribed	–	26 (100)
Mean time of anti-TB treatment, days ± SD	15.14 ± 9.77	–
CHEMOPROPHYLAXIS, *n*(%)		
Before starting treatment	–	2 (7.7)
After starting treatment (<1month)	–	24 (92.3)
Not prescribed	22 (100)	–
Mean time of chemoprophylaxis, days ± SD	–	18.6 ± 8.06
PPD CYTOMETRY RESPONDERS, *n*(%)[b]		
IFNγ^+CD4$^+$ T cells	22 (100)	26 (100)
TNF-α^+CD4$^+$ T-cells	22 (100)	26 (100)
TNF-α^+IFN-γ^+CD4$^+$ T-cells	22 (100)	26 (100)
IFN$\gamma^+$and/orTNF-α^+CD4$^+$ T-cells	22 (100)	26 (100)
ESAT-6/CFP-10 CYTOMETRY RESPONDERS, *n*(%)[b]		
IFNγ^+CD4$^+$ T cells	21 (95.5)	21 (80.8)
TNF-α^+CD4$^+$ T-cells	22 (100)	25 (96.2)
TNF-α^+IFN-γ^+CD4$^+$ T-cells	21 (95.5)	21 (80.8)
IFN$\gamma^+$and/orTNF-α^+CD4$^+$ T-cells	22 (100)	25 (96.2)

[a] *Pleural TB (n = 2), ganglionar TB (n = 1), and pericardical TB (n = 1).*

[b] *Number of individuals with a positive CD4$^+$ T-cell response to the specified antigen. The frequency of the response to any cytokine after specific stimulation was defined as positive when it was twice the amount when compared to its negative control (unstimulated sample).*

TB, tuberculosis; LTBI, latent tuberculosis infection; SD, standard deviation; PPD, purified protein derivative.

Cytokine Profile of *M. tuberculosis* Specific CD4$^+$ and CD8$^+$ T-Cell Response in Active TB and LTBI Individuals

To better define the specific *M. tuberculosis* responses in active TB and LTBI individuals, IFN-γ and/or TNF-α cytokines were measured on CD4$^+$/CD8$^+$ T-cells after PPD or ESAT-6/CFP-10 stimulation (**Figure 1A**). All individuals included in this study were responsive to SEB positive control in the cytometry assays.

Regarding CD4$^+$ T-cells functional profile after PPD stimulation, cells producing only IFN-γ were significantly lower compared to cells producing only TNF-α^+ or IFN-γ^+/TNF-α^+ simultaneously in active TB patients ($p < 0.0001$ for IFN-γ^+ vs. TNF-α^+ and $p < 0.0001$ for IFN-γ^+ vs. IFN-γ^+/TNF-α^+) and LTBI individuals ($p = 0.0001$ for IFN-γ^+ vs. IFN-γ^+/TNF-α^+). This was also observed after ESAT-6/CFP-10 stimulation in disease patients ($p < 0.0001$ for IFN-γ^+ vs. TNF-α^+ and $p = 0.035$ for IFN-γ^+ vs. IFN-γ^+/TNF-α^+) and LTBI ($p < 0.0001$ for IFN-γ^+ vs. TNF-α^+ and $p = 0.043$ for IFN-γ^+ vs. IFN-γ^+/TNF-α^+). Interestingly, cells that only produced TNF-α after PPD stimulation were significantly increased in active TB in comparison with LTBI ($p < 0.0001$). This difference was not observed after ESAT-6/CFP-10 specific stimulation (**Figure 1B**).

Regarding CD8$^+$ T-cells, specific cytokines responses were detectable in active TB patients and LTBI individuals, indicating that this T-cell population is also abundant in the immune response against *M. tuberculosis*. After PPD stimulation, cells which produced only TNF-α were predominant in active TB patients ($p = 0.009$ for TNF-α^+ vs. IFN-γ^+/TNF-α^+). This phenotype was not observed for LTBI individuals. In addition, although differences were not significant, cytokine response frequency after ESAT-6/CFP-10 stimulation was higher in active TB vs. LTBI (**Figure 1C**).

Expression of CD27 and/or CCR4 Markers Regarding the Clinical Status

The percentage of CD27$^-$ and/or CCR4$^+$ surface homing markers was studied on active TB and LTBI within the IFN-γ^+CD4$^+$ T-cells subset after *M. tuberculosis* specific stimulation (**Figure 2A**). When the expression of CD27 and CCR4 was analyzed separately, the proportion of CD27$^-$ or CCR4$^+$ within IFN-γ^+CD4$^+$ T-cells was significantly higher in active TB when compared with LTBI in response to PPD ($p < 0.0001$ for CD27$^-$ and $p = 0.006$ for CCR4$^+$) or ESAT-6/CFP-10 recombinant proteins ($p < 0.0001$ for CD27$^-$), with the exception of CCR4 marker in response to ESAT-6/CFP-10, where no statistical significance was obtained. In addition, both surface T-cell markers were analyzed together (CD27$^-$CCR4$^+$ phenotype within IFN-γ^+CD4$^+$ T-cell compartment). The proportion of CD27$^-$CCR4$^+$IFN-γ^+CD4$^+$ T-cells was significantly associated with active TB ($p < 0.0001$ after PPD or ESAT-6/CFP-10 stimulation; **Figures 2B,C**) and reduced the overlapping between the two clinical status after PPD stimulation. These findings could indicate that the loss of CD27 and the increase of CCR4 markers could be associated with *M. tuberculosis* uncontrolled replication. In our study, active TB patients were recruited within the 4 weeks of starting therapy. In order to explore if these firsts weeks after treatment initiation influenced the expression of CD27 and/or CCR4 markers, we performed a Spearman test correlation. No significant correlation was observed between days of treatment (within the 4 weeks of starting therapy) and the percentage of CD27$^-$ and/or CCR4$^+$ within IFN-γ^+CD4$^+$ T-cells in response to PPD (**Figure S2A** in **Supplementary Material**) or ESAT-6/CFP-10 (**Figure S2B** in **Supplementary Material**).

CD27 and/or CCR4 markers were further characterized on CD4$^+$ T-cells producing: (i) TNF-α^+, (ii) TNF-α^+IFN-γ^+, and (iii) IFN-γ^+ and/or TNF-α^+ (Boolean analysis) after *M. tuberculosis* specific stimulation. The proportion of CD27$^-$, CCR4$^+$, and CD27$^-$CCR4$^+$ T-cells within these three subsets was significantly higher in active TB patients compared to LTBI individuals in response to

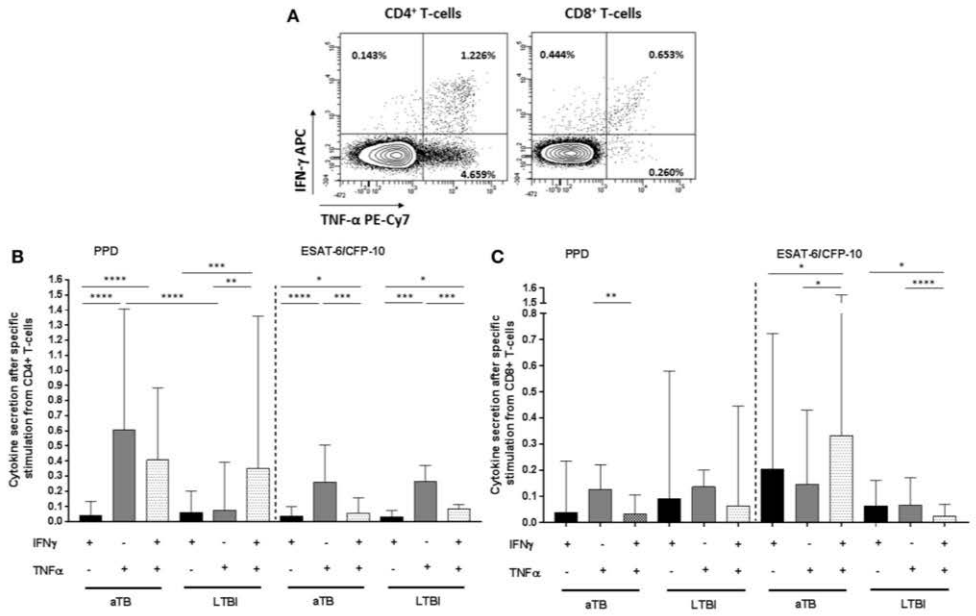

FIGURE 1 | $CD4^+/CD8^+$ T-cells specific cytokine secretion phenotype regarding active TB or LTBI. **(A)** Dot plots from one active TB representative donor showing the expression of $CD4^+$ and $CD8^+$ T-cells producing IFN-γ and/or TNF-α after PPD stimulation. The frequency of the different cytokines production profile from the total $CD4^+$ and $CD8^+$ T-cells is indicated in each dot plot. **(B)** $CD4^+$ T-cells and **(C)** $CD8^+$ T-cells cytokine secretion after PPD or ESAT6/CFP10 stimulation. Black bars represent T-cells which only produce IFN-γ cytokine. Gray bars correspond to T-cells which only produce TNF-α. Dotted bars represent T-cells producing both IFN-γ and TNF-α cytokines. Bars depict medians with interquartile ranges. Differences between conditions were calculated using the two-tailed Mann-Whitney *U*-test. Only significant differences are represented in the graphs. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. aTB, active TB; LTBI, latent tuberculosis infection.

PPD or ESAT-6/CFP-10 specific stimulation (**Figure S3** in **Supplementary Material**), with the exception of CCR4 marker in response to ESAT-6/CFP-10, where no statistical significance was obtained. Furthermore, a significant positive correlation was observed on $CD27^-$ expression between antigen-specific IFN-γ^+CD4^+ and TNF-α^+CD4^+ T-cells (**Figure S4A** in **Supplementary Material**) or TNF-α^+IFN-γ^+CD4^+ T-cells (**Figure S4B** in **Supplementary Material**).

CD27 MFI Ratio Analysis

An approach based on CD27 MFI on $CD4^+$ T-cells was assessed as suggested by Portevin et al. (16). This method consists on evaluating the ratio between CD27 MFI in $CD4^+$ T-cells and the MFI of CD27 in specific IFN-γ^+CD4^+ T-cells. Therefore, a low CD27 MFI on specific $CD4^+$ T-cells which produce IFN-γ^+ implies a high CD27 ratio (this is a consequence of an increase of the $CD27^-$IFN-γ^+CD4^+ T-cells phenotype). In this study, a high ratio was significantly associated with active TB when T-cells were stimulated with PPD (**Figure 3A**; $p < 0.0001$) or ESAT-6/CFP-10 (**Figure 3B**; $p < 0.0001$).

In order to explore whether a high CD27 MFI ratio was associated with an increase of the percentage of $CD27^-$ within IFN-γ^+CD4^+ T-cells, a Spearman test correlation was performed. A positive correlation between these two variables was observed in T-cells responding to PPD (**Figure 3C**) or ESAT-6/CFP-10 (**Figure 3D**), which is supported by a significant correlation coefficient (for PPD: Spearman's rho = 0.869, $p < 0.0001$; for ESAT-6/CFP-10: Spearman's rho = 0.892, $p < 0.0001$).

The approach based on CD27 MFI was also assessed on (i) TNF-α^+CD4^+ T-cells, (ii) IFN-γ^+TNF-α^+CD4^+ T-cells, and (iii) IFN-γ^+ and/or TNF-α^+CD4^+ T-cells after *M. tuberculosis* specific stimulation. These ratios were significantly higher in active TB patients than in LTBI individuals after PPD (**Figure S5A** in **Supplementary Material**) or ESAT-6/CFP-10 (**Figure S5B** in **Supplementary Material**) stimulation.

Diagnostic Accuracy of the Different Biomarkers

To asses TB diagnostic accuracy of the different approaches analyzed in this study, we performed a ROC curve analysis (**Table 2**). Highest AUC values (AUC > 0.90) for discriminating active TB from LTBI were achieved when evaluating: (i) CD27 within IFN-γ^+CD4^+ and IFN-γ^+TNF-α^+ $CD4^+$ T-cells in response to ESAT-6/CFP-10 [AUC (95% confidence interval, CI) 0.90 (0.79–1.01) and 0.92 (0.84–1.01) respectively], (ii) CD27 and CCR4 markers together within IFN-γ^+CD4^+, TNF-α^+CD4^+ and IFN-γ^+TNF-α^+ $CD4^+$ T-cells in response to PPD [AUC

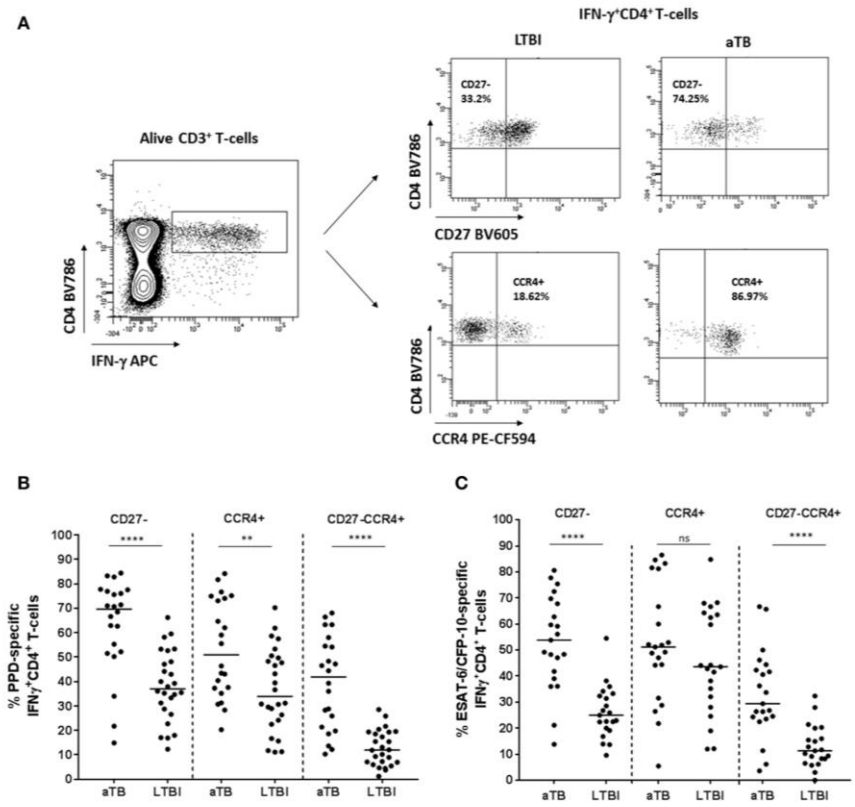

FIGURE 2 | CD27$^-$ and/or CCR4$^+$ phenotype from CD4$^+$IFN-γ^+ specific T-cells in patients with active TB and LTBI individuals. **(A)** Representative example from an active TB and a LTBI individual showing the strategy for determining CD27$^-$ or CCR4$^+$ T-cells after PPD stimulation. CD27 and CCR4 expression was analyzed within the specific IFN-γ^+CD4$^+$ T-cells population after PPD or ESAT-6/CFP-10 stimulation. A negative control without stimulation was also included in the analysis for each patient enrolled in the study. Fluorescence Minus One (FMO) controls were included in each experiment for setting up gates. **(B)** Percentage of PPD or **(C)** ESAT6/CFP10 specific CD27$^-$, CCR4$^+$, and CD27$^-$CCR4$^+$ within IFN-γ^+CD4$^+$ specific T-cells. Horizontal lines represent medians. Differences between conditions were calculated using the two-tailed Mann-Whitney *U*-test. $^{**}p < 0.01$, $^{****}p < 0.0001$. ns, non-significant. aTB, active TB; LTBI, latent tuberculosis infection.

(95% CI) 0.91 (0.83–0.99), 0.90 (0.82–0.99), and 0.90 (0.81–0.99) respectively], and (iii) CD27 MFI ratio performed on IFN-γ^+CD4$^+$ and IFN-γ^+TNF-α^+CD4$^+$ T-cells after ESAT-6/CFP-10 specific stimulation [AUC (95% CI) 0.90 (0.79–1.01) and 0.91 (0.82–1.01), respectively]. The lowest diagnostic accuracy was observed when CCR4 marker was evaluated alone (**Figure 4**).

DISCUSSION

Approaches based on the study of the host immune response have emerged as potential tools for TB management, studying the interplay between the host and *M. tuberculosis*, and discovering suitable disease biomarkers. Here we have analyzed specific immune-mechanisms based on the characterization of different T-cell subsets and the expression of surface receptors such as CD27 and/or CCR4 involved in the migration of certain lymphocytes to the disease inflammatory sites. Briefly, our results confirm previous reports on CD27 modulation in specific CD4$^+$ T-cells producing IFN-γ, which is downregulated during active disease. Furthermore, a high CD27 MFI ratio proposed as a disease biomarker by other authors (16, 17) was associated with active TB patients compared to LTBI individuals. This study adds novel information on other potential homing biomarkers such as CCR4, showing that in combination with CD27 (CD27$^-$CCR4$^+$IFN-γ^+CD4$^+$ T-cells) could be a phenotype to discriminate between disease and infection. Furthermore, we also characterized CD27 on CD4$^+$ T-cells producing TNF-α, observing that this marker has a high power of discrimination when analyzed within IFN-γ^+TNF-α^+CD4$^+$ T-cells population.

The study of cytokine profiles on specific *M. tuberculosis* T-cell responses has suggested that specific subsets may serve as disease biomarkers associated with bacterial load, treatment response or disease outcome. This data is still limited and

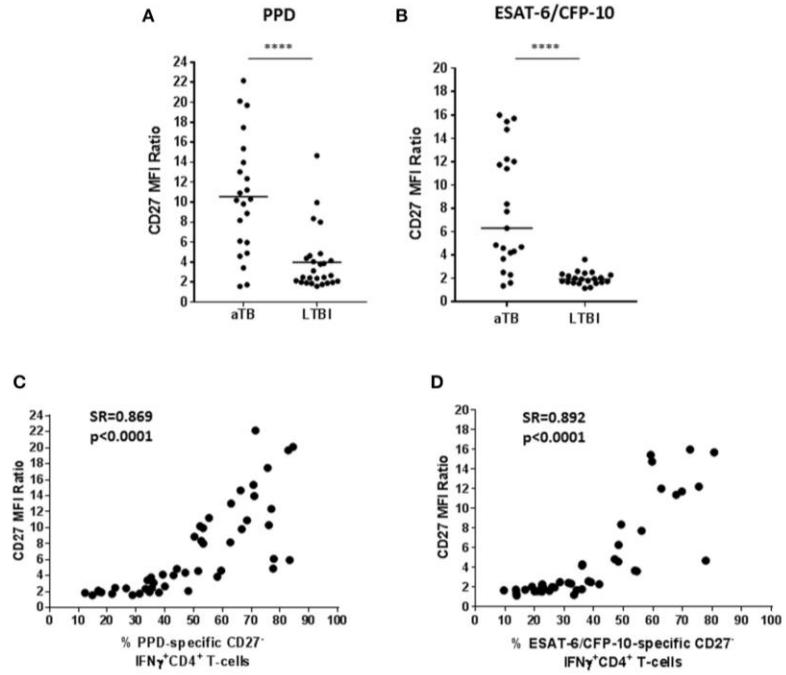

FIGURE 3 | CD27 MFI ratio according to the study group. A ratio based on CD27 MFI was calculated after specific stimulation as suggested by Portevin et al. (16). This ratio is based on the MFI of CD27 in $CD4^+$ T-cells over MFI of CD27 in IFN-γ^+CD4^+ T-cells. **(A)** CD27 MFI ratio after PPD or **(B)** ESAT-6/CFP-10 stimulation in patients with active TB and LTBI individuals. Horizontal lines represent medians. Differences between conditions were calculated using the two-tailed Mann-Whitney *U*-test. $^{****}p < 0.0001$. **(C,D)** Correlation of the percentage of $CD27^-$ marker within IFN-γ^+CD4^+ specific T-cells with the CD27 MFI ratio after PPD or ESAT-6/CFP-10 stimulation. Correlation was calculated using the two-tailed non-parametric Spearman test. aTB, active TB; LTBI, latent tuberculosis infection.

need to be further evaluated. In this context, a previous study indicated that $CD4^+$ T-cells that only produce TNF-α could be associated with active disease (23). Others have reinforced this data suggesting that PPD specific TNF-α^+ $CD4^+$ T-cells with an effector phenotype can accurately discriminate active TB from LTBI, or even recently acquired from remote LTBI (24, 25). The results we obtained on *M. tuberculosis* T-cells functional profile were also in agreement with those obtained in previous studies, as we found that $CD4^+$ T-cell only producing TNF-α in response to PPD were increased in active TB patients. While it is widely accepted that $CD4^+$ T-cells play an essential role against the bacilli's immune response, protective immunity to *M. tuberculosis* by $CD8^+$ T-cells still remains controversial. In the last years, $CD8^+$ T-cells have emerged as a possible population actively involved in the immunopathology (26–28). Our study also corroborates the importance of $CD8^+$ T-cells as TB control players, showing detectable cytokine responses in this population which tend to be higher during disease in response to ESAT-6 and CFP-10 antigens. One important challenge for TB management and diagnosis is to find specific antigens capable to elicit $CD8^+$ T-cells responses. In this context, a new generation of QFN called QFN-Plus has incorporated new peptides able to induce IFN-γ responses on $CD4^+$ and $CD8^+$ T-cells, trying to increase the accuracy of the assay and to correlate T-cell responses with antigen load or high risk of TB progression (29). However, data about its accuracy over classical IGRAs or correlation with disease state is still limited.

In this study, we confirm that the evaluation of the frequency of $CD27^-$ within functional $CD4^+$ T-cells, together with the CD27 MFI ratio, were suitable biomarkers for TB which could discriminate disease from infection with acceptable AUC values between 0.82 and 0.92 depending on the stimuli used (PPD or ESAT-6/CFP-10). The CD27 marker is a member of the TNF-receptor superfamily, expressed by lymphocytes, which is downregulated during effector differentiated T-cells able to produce cytokines (14). Thus, due to the persistent antigenic stimulation during active TB, it has been proposed as an immune biomarker of the disease (16, 17, 30, 31). The recently developed immune assay based on the detection of CD27 MFI ratio (TAM-TB assay) has also been proposed as an alternative way for measuring this receptor (16). Here, we show that the percentage of $CD27^-$ significantly correlated with CD27 MFI quantification, indicating that both immune strategies are accurate enough for TB diagnosis. However, the calculation of a ratio based on MFI allows normalization of the results avoiding subjectivity and discrepancies on CD27

positive or negative gating. In addition, our results also add new information about the CCR4 homing marker. We have observed that overexpression of CCR4 receptor within IFN-γ^+CD4$^+$ T-cells is a poor immune biomarker of disease when evaluated alone, however, when combined together with CD27$^-$IFN-γ^+CD4$^+$ T-cells, its discriminatory capacity increased (0.91 after PPD stimulation). CCR4 has been suggested as a lung homing receptor expressed on T-cells. A recent study focused on the detection of CD27 or CCR4 markers (among others) on active TB and LTBI individuals (with and without HIV infection), found that in an active TB context T-cells presented a CD27 marker downregulation. In contrast, no difference on CCR4 expression was found regarding the clinical status of the individuals (irrespective of HIV infection) when this marker was detected alone (31). In addition, other possible disease immune markers have been studied by others in order to improve TB diagnosis accuracy. For example, the expression of the activation marker HLA-DR on specific CD4$^+$ T-cells has shown a good discriminatory capacity between active TB and LTBI (30, 31). This study also adds new data on CD27 and/or CCR4 characterization within T-cells secreting IFN-γ and/or TNF-α. IFN-γ cytokine does not fully represent the response against *M. tuberculosis*, having TNF-α an important role during active TB disease. In this context, we found that CD27$^-$ and/or CCR4$^+$ expression within: (i) TNF-α^+CD4$^+$; (ii) IFN-γ^+TNF-α^+CD4$^+$ T-cells, and (iii) IFN$\gamma^+$and/orTNF-α^+CD4$^+$ T-cells, as well as CD27 MFI ratio measured in these functional populations, were increased in active TB patients in comparison with LTBI individuals. Highest discriminatory capacities were achieved when measuring CD27$^-$ or CD27 MFI ratio within IFN-γ^+TNF-α^+CD4$^+$ T-cells (AUC 0.92 and 0.91 after ESAT-6/CFP-10 stimulation). This indicates that CD4$^+$ T-cells lacking CD27 marker are able to differentiate into effector T-cells and increment their capacity to secrete IFN-γ and/or TNF-α cytokines. In addition, the study of CD27 on TNF-α producing T-cells increased the detection of positive responses by flow cytometry after ESAT-6/CFP-10 stimulation, especially in the LTBI group.

In mice it has been shown that IFN-γ^+CD4$^+$ T-cells which have CD27 receptor downregulated are accumulated preferentially in the lungs during mycobacterial infection (32). Furthermore, CD27^{low} specific CD4$^+$ T-cells are increased in lungs of patients with active TB, and percentages of this subset are higher in lung tissue than in blood. Interestingly, when this T-cell subset was detected in blood, it correlated with tissue destruction and TB severity. This correlation was not observed when the T-cell subset was detected in the lungs. The reasons of this discordance are still unclear, but could be explained by

TABLE 2 | ROC curve analysis of the different approaches.

PHENOTYPE	AUC (95% CI)	
	PPD	ESAT-6/CFP-10
CD27$^-$		
IFNγ^+CD4$^+$	0.85 (0.72–0.97)	0.90 (0.79–1.01)
TNF-α^+CD4$^+$	0.85 (0.74–0.97)	0.86 (0.75–0.97)
TNF-α^+IFN-γ^+CD4$^+$	0.88 (0.77–098)	0.92 (0.84–1.01)
IFN$\gamma^+$and/orTNF-α^+CD4$^+$	0.82 (0.69–0.95)	0.87 (0.76–0.98)
CCR4$^+$		
IFNγ^+CD4$^+$	0.73 (0.59–0.87)	0.60 (0.43–0.78)
TNF-α^+CD4$^+$	0.70 (0.55–0.85)	0.50 (0.33–0.67)
TNF-α^+IFN-γ^+CD4$^+$	0.77 (0.64–0.91)	0.65 (0.49–0.83)
IFN$\gamma^+$and/orTNF-α^+CD4$^+$	0.72 (0.58–0.87)	0.59 (0.43–0.75)
CD27$^-$CCR4$^+$		
IFNγ^+CD4$^+$	0.91 (0.83–0.99)	0.86 (0.73–0.98)
TNF-α^+CD4$^+$	0.90 (0.82–0.99)	0.84 (0.71–0.96)
TNF-α^+IFN-γ^+CD4$^+$	0.90 (0.81–0.99)	0.89 (0.77–0.99)
IFN$\gamma^+$and/orTNF-α^+CD4$^+$	0.88 (0.78–0.97)	0.84 (0.71–0.96)
MFI CD27		
IFNγ^+CD4$^+$	0.84 (0.71–0.97)	0.90 (0.79–1.01)
TNF-α^+CD4$^+$	0.84 (0.71–0.97)	0.87 (0.76–0.97)
TNF-α^+IFN-γ^+CD4$^+$	0.84 (0.71–0.97)	0.91 (0.82–1.01)
IFN$\gamma^+$and/orTNF-α^+CD4$^+$	0.84 (0.71–0.97)	0.88 (0.77–0.99)

AUC, area under the curve; CI, confidence interval; PPD, purified protein derivative.

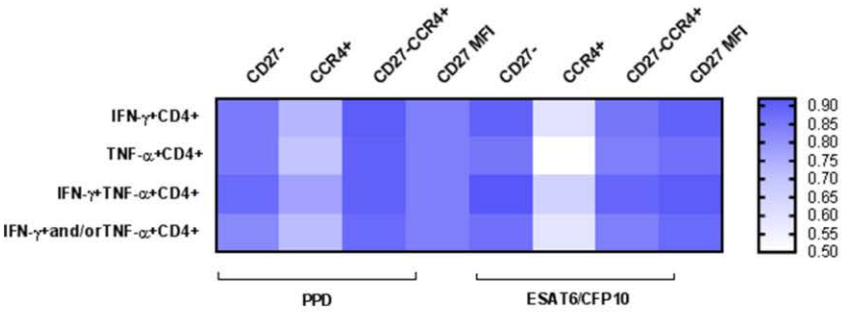

FIGURE 4 | Heatmap depicting Areas Under the Curve (AUC) values for the different approaches. A ROC curve analysis was performed to determine the diagnostic accuracy for TB diagnosis. AUC values are represented for: (i) percentage of CD27$^-$ marker, (ii) percentage of CCR4$^+$ marker, (iii) percentage of CD27$^-$CCR4$^+$ signature, and (iv) CD27 MFI ratio. Values are shown for the different CD4$^+$ T-cells functional populations analyzed after PPD or ESAT-6/CFP-10 specific stimulation. High AUC values are indicated by intensity of blue color.

its generation from different precursors. $CD27^{low}$ specific $CD4^+$ T-cells can be generated in the lymph nodes and then migrate to peripheral blood, while those located in the lungs can be generated locally from other precursors (14). In the same context, severe TB induced the upregulation of CCR4 gene (among others) in pulmonary compartments of infected rhesus monkeys (33). According to these findings, it would be interesting to study CD27 and/or CCR4 in samples from active TB patients coming from the site of infection in order to understand better the mechanisms and pathogenesis of the disease. Further studies in this direction need to be addressed.

The monitoring of anti-TB therapy efficacy is a key point for TB control. Petruccioli E. et al. showed that the expression of CD27 increases on specific T-cells in cured active TB patients after 1 year of therapy completion (17). These findings suggest that CD27 might serve as a tool for following-up active TB patients, detecting efficacy of treatment, and exploring inflammatory status. In this line, the study of a differential phenotype on T-cells expressing CD27 and/or CCR4 homing markers during the treatment follow-up of active TB patients is needed for validating these findings. Some data support that only after 2 months of TB therapy the expression of CD27 starts its modulation (14). Thus, according to these data, the detection of $CD27^-CD4^+$ T-cells during the first month of treatment on patients recruited in our study should not be altered. This hypothesis is also reinforced by our results, as no significant correlation between days of treatment (within the firsts 4 weeks of therapy) and the CD27/CCR4 expression was found.

Limitations of this study need to be addressed. First, although results obtained on CD27 are robust among different studies, it is important to uniformly validate this immune assay for routine purposes and results reproducibility, choosing common starting material (PBMCs or whole blood), same specific stimuli and fluorochromes, as well as standardizing protocols. And second, the triggering of host immune responses and disease outcome does not depend only on a single factor. Immune status of the host depends on a *troika* of multiple parameters covering host genetics, the pathogen and extrinsic elements (34). Therefore, it is necessary to study and combine all these variables together in other to translate possible host immune TB biomarkers into potential immune assays with clinical applications.

In summary, our findings on surface homing markers such as CD27 and CCR4 on *M. tuberculosis* specific $CD4^+$ T-cells gather the required features for using them as potential TB biomarkers. Therefore, it would be crucial to further evaluate these receptors in a larger cohort of patients in order to develop possible and simplified routine immune assays for TB diagnosis, assessment of therapy efficacy/relapse, and risk-stratification of LTBI individuals.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Hospital Germans Trias i Pujol (Reference number PI-17-134). All enrolled subjects gave a written informed consent for participating in this study.

AUTHOR CONTRIBUTIONS

IL and JD designed the study. IL, MF-S, and RV-H designed the experiments. BM-M, SV, RV-H, and IL performed the experiments. MDS-G, ZS, MJ-F, CC, JR-M, J-PM, IM-P, YG-D, LL-C, JS, and CP contributed with resources. IL, MF-S, and RV-H analyzed the data. IL, JD, AL, and CP supervised the study. IL and JD wrote the paper. All authors revised and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03094/full#supplementary-material

Figure S1 | Gating strategy used for $CD4^+/CD8^+$ T-cells cytokine secretion and CD27/CCR4 analysis. Aggregated cells were taken off by gating on the diagonal that appears with Forward-Scatter (height; FSC-H) vs. Forward-Scatter (area; FSC-A) dot plot. For $CD4^+$ and $CD8^+$ T-cells gating, alive $CD3^+$ T-cells were first selected. Cytokine profiles on T-cells were analyzed on $CD4^+/CD8^+$ T-cells. CD27/CCR4 expression was studied within IFN-γ^+CD4^+ T-cells gated from alive $CD3^+$ T-cells.

Figure S2 | Correlation of homing markers expression with days of treatment. Correlation of days after starting anti-TB therapy in active TB patients (within the 4 weeks of starting treatment) with **(A)** percentage of $CD27^-$ and/or $CCR4^+$ within IFN-γ^+CD4^+ T-cells in response to PPD or **(B)** ESAT-6/CFP-10 antigens. Correlation was calculated using the two-tailed non-parametric Spearman test.

Figure S3 | $CD27^-$ and/or $CCR4^+$ phenotype within functional $CD4^+$ T-cells producing IFN-γ and/or TNF-α in patients with active TB and LTBI individuals. Percentage of PPD or ESAT-6/CFP-10 specific $CD27^-$, $CCR4^+$, and $CD27^-CCR4^+$ within **(A)** TNF-α^+CD4^+ T-cells, **(B)** IFN-γ^+TNF-α^+CD4^+ T-cells,

and **(C)** IFN-γ^+ and/or TNF-α^+CD4$^+$ T-cells. Horizontal lines represent medians. Differences between conditions were calculated using the two-tailed Mann-Whitney *U*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns, non-significant; aTB, active TB; LTBI, latent tuberculosis infection.

Figure S4 | Relationship of the CD27$^-$ expression on the different antigen-specific T-cells populations analyzed. Correlation of the CD27$^-$ expression on IFN-γ^+CD4$^+$ T-cells with **(A)** TNF-α^+CD4$^+$ or **(B)** TNF-α^+IFN-γ^+ CD4$^+$ T-cells after PPD or ESAT-6/CFP-10 antigen stimulation. Correlation was calculated using the two-tailed non-parametric Spearman test.

Figure S5 | CD27 MFI ratio calculated on functional CD4$^+$ T-cells producing IFN-γ and/or TNF-α. A ratio based on CD27 MFI was calculated after specific stimulation in active TB patients and LTBI individuals. This ratio is based on the MFI of CD27 in CD4$^+$ T-cells over: (i) MFI of CD27 in TNF-α^+CD4$^+$ T-cells, (ii) MFI of CD27 in IFN-γ^+TNF-α^+CD4$^+$ T-cells, and (iii) MFI of CD27 in IFN-γ^+ and/or TNF-α^+CD4$^+$ T-cells after **(A)** PPD or **(B)** ESAT-6/CFP-10 antigen stimulation. Horizontal lines represent medians. Differences between conditions were calculated using the two-tailed Mann-Whitney *U*-test. ****$p < 0.0001$. aTB, active TB; LTBI, latent tuberculosis infection.

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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 a potential conflict of interest.

Article 7

Use of IP-10 detection in dried plasma spots for latent tuberculosis infection diagnosis in contacts via mail

Raquel Villar-Hernández, Irene Latorre, María Luiza de Souza-Galvão, María Á Jiménez, Juan Ruiz-Manzano, José Pilarte, Esther García-García, Beatriz Muriel-Moreno, Adela Cantos, Neus Altet, Joan Pau Millet, Yoel González-Díaz, Israel Molina-Pinargote, Cristina Prat, Morten Ruhwald & José Domínguez.

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Villar-Hernández R, Latorre I, De Souza-Galvao ML, Jiménez MA, Ruiz Manzano J, Pilarte J, García-García E, Muriel-Moreno B, Cantos A, Altet N, Millet JP, González-Díaz Y, Molina-Pinagorte I, Prat C, Ruhwald M, Domínguez J

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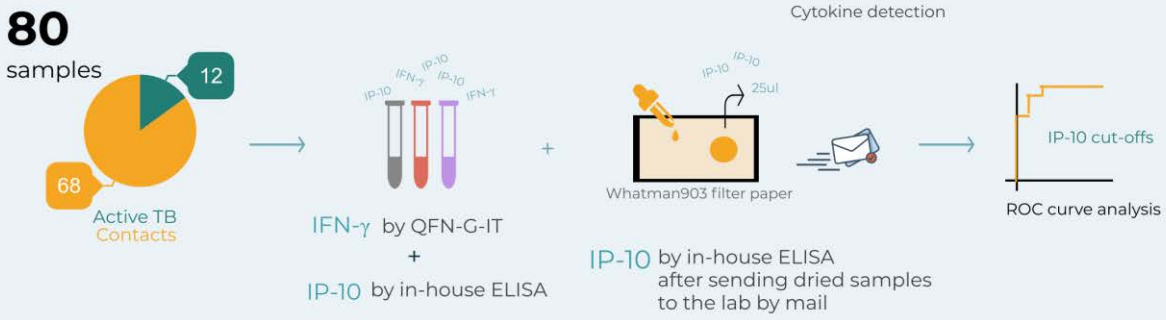
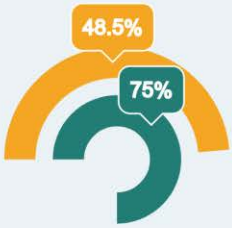
Could IP-10 detection in dried plasma be used for LTBI diagnosis?



Positive QFN-G-IT

Positive IP-10 direct plasma

Positive IP-10 dried plasma



IP-10 levels **strongly** correlate
(r=0.897)

k>0.67 agreement
among all tests

IP-10 is **stable**
in dried plasma and can be
detected

Simple and **safe**
method to **send** samples via mail
enabling LTBI diagnosis in
faraway settings.

Increasing the reach towards a better LTBI diagnosis and disease control

IFN-γ: Interferon gamma; IP-10: IFN-γ inducible protein 10; LTBI: latent tuberculosis infection; QFN-G-IT: QuantiFERON Gold In-Tube; TB: tuberculosis.

Raquel Villar Hernández

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Use of IP-10 detection in dried plasma spots for latent tuberculosis infection diagnosis in contacts via mail

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The aim of this study was to test the use of IP-10 detection in dried plasma from contact studies individuals (contacts of smear positive patients), by comparing it with IP-10 and IFN-γ detection in direct plasma, to establish IP-10 detection in DPS as a useful assay for LTBI diagnosis. Whole blood samples were collected from 80 subjects: 12 with active tuberculosis (TB), and 68 from contact studies. The amount of IFN-γ produced by sensitized T cells was determined in direct plasma by QuantiFERON Gold In-Tube test. IP-10 levels were determined in direct and dried plasma by an *in-house* ELISA. For dried plasma IP-10 determination, two 25 µl plasma drops were dried in Whatman903 filter paper and sent by mail to the laboratory. Regarding TB patients, 100.0%, 91.7% and 75.0% were positive for IFN-γ detection and IP-10 detection in direct and dried plasma, respectively. In contacts, 69.1%, 60.3% and 48.5% had positive results after IFN-γ and IP-10 in direct and dried plasma, respectively. The agreement among *in vitro* tests was substantial and IP-10 levels in direct and dried plasma were strongly correlated (r = 0.897). In conclusion, IP-10 detection in dried plasma is a simple and safe method that would help improve LTBI management.

Tuberculosis (TB) still causes high morbidity and mortality rates worldwide. The key to disease control is early diagnosis and efficient treatment regimens[1]. Latent tuberculosis infection (LTBI) screening is traditionally based on the tuberculin skin test (TST). However, this test is limited by cross-reaction with the *Mycobacterium bovis* strain present in the bacilli Calmette-Guérin (BCG) vaccine and non-tuberculous mycobacteria (NTM)[2]. Interferon (IFN)-γ release assays (IGRAs), which measure T-cell-mediated responses after specific *Mycobacterium tuberculosis* antigen stimulation either from whole blood (QuantiFERON technology; QFN, Qiagen, Düsseldorf, Germany) or peripheral blood lymphocytes (T-SPOT.TB; Oxford Immunotec Limited, Abingdon, UK)[3–5], have appeared as an alternative for the TST. Both TST and IGRAs provide indirect evidence of the presence of the bacilli by measuring cell sensitization. Despite the fact that IGRAs positive predictive value has been shown to be poor[6], their negative predictive value is excellent and correlates better with contacts degree of *M. tuberculosis* exposure that the TST[7].

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Variable	Overall patients (%) $n=80$	Active TB patients (%) $n=12$	Contacts (%) $n=68$
Age, mean (years) ± SD	29.5 ± 11.6	33.5 ± 9.2	28.8 ± 11.9
Gender			
Male	39 (48.8)	10 (83.3)	29 (42.6)
Female	41 (51.2)	2 (16.6)	39 (57.4)
Country of birth			
High TB incidence	32 (40.0)	8 (66.7)	24 (35.3)
Low TB incidence	43 (53.8)	4 (33.3)	39 (57.3)
Unknown	5 (6.2)	0 (0.0)	5 (7.4)
BCG			
Yes	28 (35.0)	2 (16.6)	26 (38.2)
No	35 (43.7)	5 (41.7)	30 (44.1)
Unknown	17 (21.3)	5 (41.7)	12 (17.6)
QFN-G-IT			
Positive	59 (73.8)	12 (100.0)	47 (69.1)
Negative	21 (26.2)	0 (0.0)	21 (30.9)

Table 1. Demographic characteristics of all patients included in the study.

In the past few years, the use of IFN-γ inducible protein 10 (IP-10) has been studied as a biomarker of LTBI. This chemokine is largely expressed by antigen-presenting cells previously induced by innate and adaptive mechanisms. During the adaptive immune response, IP-10 production is mainly driven by T-cell-derived IFN-γ[8,9]. IP-10 levels are reported to be 100-fold of those of IFN-γ, suggesting that it can be a promising diagnostic marker in TB[9,10], as well as a good option for assay simplification and miniaturization such as lateral flow, dried blood spots and molecular detection[9,11]. IP-10 detection has been proven more robust than IFN-γ in HIV-infected patients and young children[12–17].

Having procedures that make sample transportation from the extraction/collection sites to the laboratory simple and safe, and that also detect infection markers other than IFN-γ, would be of use, easing IGRA implementation, and improving LTBI control. An example of these procedures, is the dried blood and plasma drops. The use of dried blood in diagnostics was introduced for metabolic diseases in neonatal population in 1963. Since then, this technique has had different applications both with dried blood and plasma, including TB infection diagnosis and management[18–21]. Blauenfeldt *et al.* found that IP-10 mRNA isolated from dried blood spots and IP-10 isolated from dried plasma spots (DPS) had higher levels in active TB patients compared to uninfected controls[20]. Hence, the detection of IP-10 in DPS has also been described as a possible indicator of anti-TB treatment efficacy, as it declines in response to anti-TB chemotherapy[19]. Regarding LTBI diagnosis, we previously described the stability of IP-10 in DPS from active TB patients and non-infected individuals, showing that, IP-10 levels detected in DPS are stable and comparable to those detected in direct plasma[18]. However, there is little experience addressing IP-10 detection in DPS in contacts[22] yielding a low number of patients (2/60, 3.3%) with positive tests results.

Due to this knowledge gap, the main objective of this study is to test the use of IP-10 detection in dried plasma from contact studies individuals (contacts of smear positive patients), by comparing it with IP-10 and IFN-γ detection in direct plasma, to establish IP-10 detection in DPS as a useful assay for LTBI diagnosis.

Results

Study population. A total of 80 subjects were included in this study. Twelve of them (15.0%) were patients with microbiologically confirmed pulmonary active TB and 68 (85.0%) were individuals from contact tracing studies. TST was performed in 69 patients (86.3%); all of them positive, except one that was also negative for QuantiFERON Gold In-Tube (QFN-G-IT). This is due to the fact that the unit, where the samples come from, acts as a reference centre of the area and mainly receives TST positive patients from contact studies for further evaluation. Demographic and clinical data is shown in Table 1. For further analysis purposes we grouped the patients in active TB, LTBI and uninfected controls. We considered as LTBI patients those contacts with a positive QFN-G-IT result (69.1%; 47/68) and as uninfected controls those with a negative QFN-G-IT result (30.9%; 21/68). Furthermore, we also grouped contacts in three groups based on the exposure degree.

Cytokine levels detected. We compared the cytokines (IFN-γ and IP-10) levels detected (i) among each QFN-G-IT tubes (nil, antigen and mitogen), (ii) by each *in vitro* test, and (iii) in each patient group (active TB, LTBI and uninfected controls). The amount of IFN-γ and IP-10 detected in each QFN-G-IT test tube was significantly different ($p < 0.05$) between tubes being the highest amount present in the mitogen tube and the lowest in the nil, as expected (Fig. 1a–c). The amount of IP-10 detected in direct plasma was significantly higher ($p < 0.0001$) than that of DPS and IFN-γ with a median value of 4686 pg/ml (IQR = 411.2–9785), 72.4 pg/2discs (IQR = 5.6–220.4), and 113.0 pg/ml (IQR = 16.3–342.4), respectively, when subtracting the nil from the antigen stimulated tube (Fig. 1d). Moreover, IP-10 levels in direct plasma correlated well with those in DPS (Spearman correlation coefficient r = 0.897, $p < 0.0001$) (Fig. 2), meaning that both tests are comparable and that IP-10 is stable in DPS. After stimulation with specific antigens, LTBI patients had a higher production of cytokines than TB patients (not statistically significant) and uninfected controls ($p < 0.0001$) (Fig. 3a–c). After mitogen stimulation,

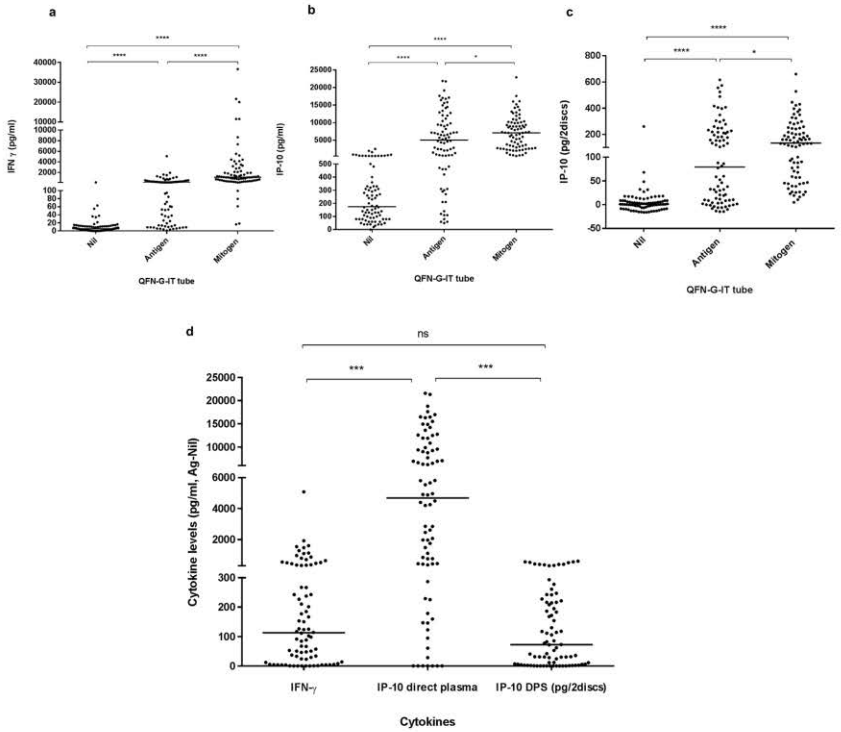

Figure 1. IFN-γ levels in plasma (**a**) and IP-10 measured both in direct plasma (**b**) and DPS (**c**) after nil, antigen and mitogen stimulation, and (**d**) comparison between IFN-γ and IP-10 levels after antigen-specific stimulation. The median cytokine levels are represented by a horizontal line. Cytokine levels in mitogen stimulated samples are higher than antigen and nil stimulated ones in every method used. The median cytokine levels are represented by a horizontal line. Differences in the cytokine levels between groups were analyzed using Mann-Whitney U test (ns = $p > 0.05$, *$p \leq 0.05$ ***$p \leq 0.0001$).

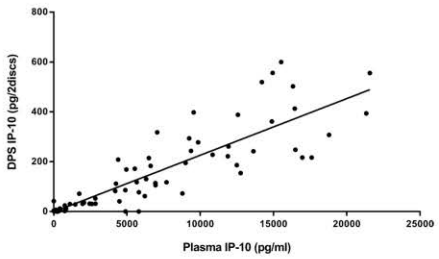

Figure 2. Correlation of IP-10 detected in dried plasma spots (DPS) and direct plasma. IP-10 detection in nil, antigen and mitogen stimulated samples. Correlation assessed using Spearman correlation coefficient ($r = 0.897$, $p < 0.0001$).

LTBI patients had higher IFN-γ and IP-10 levels than active TB patients (only significantly different ($p < 0.0001$) when looking at IFN-γ levels) and uninfected controls (not significant) (Fig. 3d–f).

ROC curve analysis. In order to establish positivity cut-offs for IP-10 detection, we performed ROC curve analysis. The areas under curves (AUC), of both IP-10 detection tests were comparable ($AUC_{direct\ plasma} = 0.908$ and $AUC_{DPS} = 0.925$). After analysing the AUCs, we chose the following cut-offs: 1.3 ng/ml (sensitivity 83.7%, 95%

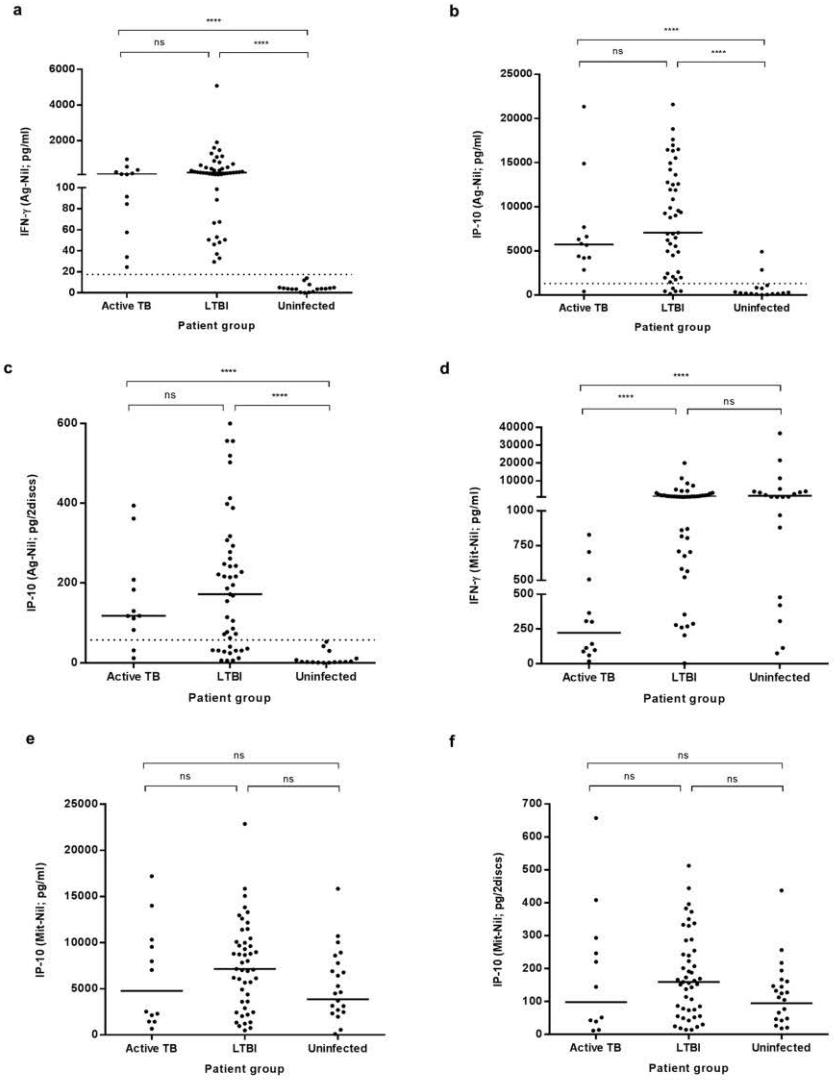

Figure 3. Cytokine levels per study group after antigen and mitogen stimulation. Specific antigen stimulation: (**a**) IFN-γ levels in active TB patients (median = 119 pg/ml; interquartile range (IQR): 64.3–331.9), LTBI patients (median = 216.5 pg/ml; IQR: 98.5–515.0) and uninfected controls (median = 3.5 pg/ml; IQR: 0.3–4.8), (**b**) IP-10 levels in direct plasma samples in active TB patients (median = 5735 pg/ml; IQR: 4213–7428), LTBI patients (median = 7060 pg/ml; IQR: 2060–12760) and uninfected controls (median = 160 pg/ml; IQR: 15–555), and (**c**) IP-10 levels in DPS in active TB patients (median = 117.9 pg/2discs; IQR: 44.1–202.0), LTBI patients (median = 172 pg/2discs; IQR: 31.8–277.8) and uninfected controls (median = 1.8 pg/2discs; IQR: −0.4–5.5). Mitogen stimulation: (**d**) IFN-γ levels in active TB patients (median = 223 pg/ml; IQR: 91.9–471.8), LTBI patients (median = 1273 pg/ml; IQR: 704.5–2079.0) and uninfected controls (median = 1161 pg/ml; IQR: 679.5–4110.0), (**e**) IP-10 levels in direct plasma samples in active TB patients (median = 4785 pg/ml; IQR: 1623–10133), LTBI patients (median = 7160 pg/ml; IQR: 3610–10080) and uninfected controls (median = 4630 pg/ml; IQR: 2600–8195), and (**f**) IP-10 levels in DPS in active TB patients (median = 97.8 pg/2discs; IQR: 19.9–281.5), LTBI patients (median = 159.3 pg/2discs; IQR: 73.0–254.7) and uninfected controls (median = 124.5 pg/2discs; IQR: 47.3–163.8). Uninfected controls are contacts with negative QFT. Statistical analysis was performed using Mann-Whitney U test (ns = $p > 0.05$, **** $p \leq 0.0001$). Dotted line indicates Ag-Nil positivity cut-off: IFN-γ = 17.5 pg/ml, IP-10 direct plasma = 1300 pg/ml, IP-10 DPS = 57.4 pg/2discs.

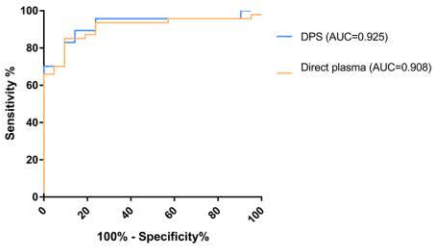

Figure 4. Receiver operating characteristic (ROC) curve analysis. Antigen-specific (ag-nil) release of IP-10 in DPS and direct plasma samples. Areas under the curve (AUCs) were comparable using both methods: DPS AUC (blue line) = 0.925 and direct plasma AUC (orange line) = 0.908. QFN-G-IT positive contacts were considered as LTBI patients and QFN-G-IT negative contacts as uninfected controls.

Patient classification	TST	QFN-G-IT	IP-10 direct plasma[a]	IP-10 DPS[b]
Active TB ($n = 12$)				
Positive	7 (100.0)	12 (100.0)	11 (91.7)	9 (75.0)
Negative	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)
Indeterminate[a]	—	0 (0.0)	0 (0.0)	3 (25.0)
Contacts ($n = 68$)				
Positive	61 (98.4)	47 (69.1)	41 (60.3)	33 (48.5)
Negative	1 (1.6)	21 (30.9)	27 (39.7)	32 (47.1)
Indeterminate[a]	—	0 (0.0)	0 (0.0)	3 (4.4)
LTBI ($n = 47$)				
Positive	41 (100.0)	47 (100.0.0)	39 (83.0)	33 (70.2)
Negative	0 (0.0)	0 (0.0)	8 (17.0)	11 (23.4)
Indeterminate[a]	—	0 (0.0)	0 (0.0)	3 (6.4)
UC ($n = 21$)				
Positive	20 (95.2)	0 (0.0)	2 (9.5)	0 (0.0)
Negative	1 (4.8)	21 (100.0)	19 (90.5)	21 (100.0)
Indeterminate[a]	—	0 (0.0)	0 (0.0)	0 (0.0)
Overall ($n = 80$)				
Positive	68 (98.6)	59 (73.8)	52 (65.0)	42 (52.5)
Negative	1 (1.4)	21 (26.2)	28 (35.0)	32 (40.0)
Indeterminate[a]	—	0 (0.0)	0 (0.0)	6 (7.5)

Table 2. Results (%) obtained with each *in vitro* technique in each group of patients. [a]Cut-offs: Ag-Nil = 1.3 ng/ml, Mit-Nil = 0.1 ng/ml. [b]Cut-offs: Ag-Nil = 57.4 pg/2discs, Mit-Nil = 17.8 pg/2discs. UC: uninfected controls.

confidence interval [CI] 69.3–93.2; specificity 94.1%, 95% CI 71.3–99.8) in direct plasma detection and 57.4 pg/2discs (sensitivity 67.4%, 95% CI 51.5–80.9; specificity 100%, 95% CI 80.5–100.0) in DPS (Fig. 4). Thus, every value above 1.3 ng/ml in direct plasma and 57.4 pg/2discs in DPS, was considered positive. As indeterminate result cut-offs, we chose: 0.1 ng/ml for the mitogen in direct plasma detection, and 17.8 pg/2discs in DPS. Therefore, results considered negative using the positivity cut-off but with cytokine production lower than 0.1 ng/ml (for direct plasma) and 17.8 pg/2discs (for DPS) after mitogen stimulation and nil subtraction, were considered indeterminate.

Test results. Active TB patients had TST performed in 58.3% (7/12) of cases obtaining 100.0% (7/7) positive results, and contacts had 91.2% (62/68) TST performed, yielding 98.4% (61/62) positive results (Table 2).

Regarding the QFN-G-IT test overall, 73.8% (59/80) of the samples had a positive result, 26.2% (21/80) had a negative result and none of the patients had an indeterminate result (Table 2). All TB patients had a positive QFN-G-IT. From the contacts with positive QFN-G-IT (69.1%, 47/68), the 41 that had the TST done were also positive for this test. From the contacts with negative QFN-G-IT (30.9%, 21/68), 4.8% (1/21) was also negative by TST, but the remaining 95.2% (20/21) were positive (11 out of these 20, 55.0%, were BCG vaccinated and the average induration size of non-BCG vaccinated was 9 mm).

Using the selected cut-offs for positive and indeterminate results, the percentages of overall positivity in direct plasma and DPS were 65.0% (52/80) and 52.5% (42/80), respectively (Table 2, Fig. 5). IP-10 detection in direct plasma yielded a 35.0% (28/80) of negative results and no indeterminate resutls. IP-10 detection in DPS yielded

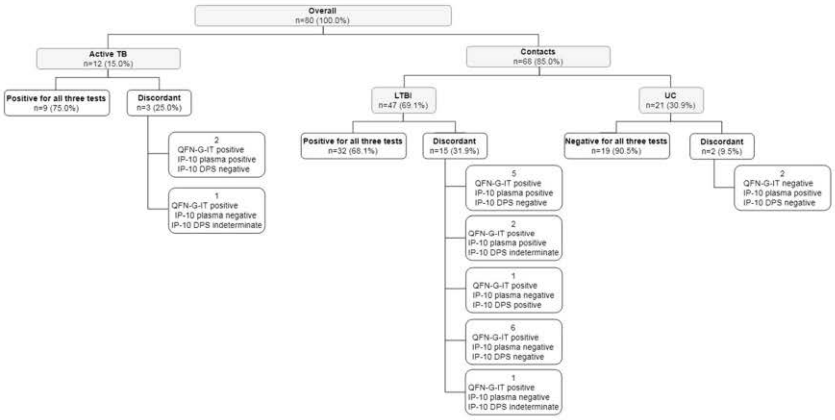

Figure 5. Flowchart of participants and test result. Graphical representation of the participants included and the results obtained for each test (QFN-G-IT, IP-10 detection in direct plasma and IP-10 detection in DPS) using the selected cut-offs. UC: uninfected controls.

a 40.0% (32/80) of negative results and a 7.5% (6/80) of indeterminate results. Not all samples from active TB patients had a positive IP-10 result: 8.3% (1/12) was negative for IP-10 detection in direct plasma but indeterminate in DPS, and other 2 (3/12, a total of 25.0%) were indeterminate when using DPS. Regarding contacts, the amount of positive and negative results obtained by IP-10 detection in direct plasma is distributed similarly to that of QFN-G-IT. IP-10 detection in DPS in contacts also yielded both positive and negative results, but also 4.4% (3/68) were indeterminate which were positive for QFN-G-IT (Table 2, Fig. 5).

A 6.2% of the contact individuals (5/80) from low prevalence countries were not BCG vaccinated but had a positive TST, a negative QFN-G-IT and IP-10 in DPS result, and 4 of them had also a negative result in IP-10 detection in direct plasma. The agreement between IP-10 detection in plasma and in DPS was strong ($\kappa = 0.77$), as that of the QFN-G-IT test and IP-10 detection in direct and DPS ($\kappa = 0.68$ in both cases). Combination of both cytokines detection in direct plasma from our total study population yielded a 76.0% (61/80) of positive results. This means a 2.5% increase in the number of positive results compared to those obtained by QFN-G-IT alone and 11.25% compared to IP-10 detection alone. Regarding contacts, cytokine detection in direct plasma yielded a 72.0% (49/68) of positive results. This is a 3.0% increase in the number of positive results compared to those obtained by QFN-G-IT alone and a 12.0% compared to IP-10 detection alone.

Contact groups. In order to study more in depth contact individuals, we grouped them regarding the contact degree: more than 6 hours (22/64, 34.4%), less than 6 hours (18/64, 28.1%) and sporadic contacts (24/64, 37.5%) (four contacts are excluded as the exposure degree is unknown) (Table 3). Although it is not statistically significant, contacts with less than 6 hours of exposure are the group with more negative results by the three *in-vitro* tests; meanwhile contacts with an exposure above 6 hours have the majority of positive results. IFN-γ in direct plasma, and IP-10 levels, both in direct and dried plasma, among the three groups were similar (no statistically significant differences among them, $p > 0.05$) but higher in more than 6-hour contacts (Fig. 6a–c). Regarding LTBI individuals (positive QFN-G-IT result) with known exposure degree, 43.2% (19/44) were more than 6-hour contacts, 22.7% (10/44) were less than 6-hour contacts and 34.1% (15/44) were sporadic contacts. As well as when considering the total contact cohort, LTBI individuals with less than 6 hours of exposure have more negative IP-10 test results, and those with more than 6 hours of exposure have more positive results (Table 3). Although IFN-γ levels in these patients are higher in less than 6-hour contacts, and IP-10 levels are higher in sporadic contacts, the differences among groups remains as not statistically significant ($p > 0.05$) (Fig. 6d–f).

Discussion

In this study we evaluated the use of IP-10 detection in DPS for LTBI diagnosis after sending the samples via mail. Although, there is a loss in positive results, IP-10 detection in filter paper could be a good alternative as the agreement between IP-10 tests remains strong. Therefore, IP-10 detection in DPS could be of use in TB infection diagnosis when long transportation is required and sample refrigeration is not possible to maintain.

IGRAs have been extensively used to measure T-cell-mediated responses after specific *M. tuberculosis* antigen stimulation. Although their positive predictive value has been characterized as poor, IGRAs have an excellent negative predictive value[6] and seem to correlate better than TST with the exposure degree to *M. tuberculosis*[7]. During the past few years IP-10 detection has been studied for TB diagnosis both in adults and children as well as immunocompromised patients[9,13,16,23–25]. Regarding the usefulness of IP-10 in children a range of sensitivity (67%-82%) and specificity (87–97%) values which depend on the enrolled cohort characteristics has been described[16,23,24]. Moreover, Ruhwald *et al.* reported an 81% sensitivity and 97% specificity when measuring IP-10 in plasma of adult active TB patients, validating the comparable use of IP-10 with IFN-γ. The overall conclusions

Exposure time	QFN-G-IT	IP-10 direct plasma	IP-10 DPS
>6 h ($n=22$)			
Positive	19 (86.4)	17 (77.3)	15 (68.2)
Negative	3 (13.6)	5 (22.7)	6 (27.3)
Indeterminate	0 (0.0)	0 (0.0)	1 (4.5)
<6 h ($n=18$)			
Positive	10 (55.6)	7 (38.9)	7 (38.9)
Negative	8 (44.4)	11 (61.1)	11 (61.1)
Indeterminate	0 (0.0)	0 (0.0)	0 (0.0)
Sporadic ($n=24$)			
Positive	15 (62.5)	14 (58.3)	10 (41.7)
Negative	9 (37.5)	10 (41.7)	12 (50.0)
Indeterminate	0 (0.0)	0 (0.0)	2 (8.3)

Table 3. Contact test results (%) per exposure degree. Four contacts had unknown exposure degree.

from the existing studies are that IP-10 detection seems to be comparable to QFT-G-IT and may add information in young children and immunocompromised patients. Despite the amount of studies on IP-10 performed, fewer assess the use of IP-10 detection in contacts. Wang *et al.* compared IFN-γ and IP-10 levels among active TB patients, household contacts (HHC) and healthy controls, finding that IFN-γ levels correlate with IP-10 and that active TB patients had significantly higher IP-10 levels than the rest followed by HHC and healthy controls[26]. When splitting the HHC into LTBI and uninfected patients, they found that active TB patients remained as the group with higher IP-10 levels but not significantly different from those of LTBI. Biraro *et al.* also compared IP-10 detection in plasma with IFN-γ in a cohort comprising active TB patients and contacts[27]. In contrast to Wang and in agreement with our findings, LTBI patients in their study had the highest production of IP-10 compared to active TB patients and uninfected contacts, and IP-10 levels in active TB patients and LTBI individuals were significantly different compared to those of uninfected contacts.

Unlike IFN-γ detection in DPS which has been considered unreliable[21], the use of IP-10 in DPS offers the possibility to diagnose TB infection when it is not possible to analyse direct plasma. Using this method, samples do not require a major processing at the clinical setting and they can be kept at room temperature unlike fresh plasma. Therefore, using DPS enables the sending of the sample easily and without damage to the laboratories, which in many cases can be far away from the sampling site. In our previous study, we described as comparable the detection of IP-10 in DPS and in direct plasma with an excellent agreement ($\kappa=0.91$) and with significantly higher levels in active TB patients compared to uninfected controls[18]. To our knowledge, this is the first time that IP-10 detection in DPS has been studied in a contact cohort with different exposure degrees and with more than 50% IP-10 positivity. Tuuminen *et al.*[22], assessed the performance of IP-10 detection in paediatric contacts, both in direct and dried plasma demonstrating the comparable performance of IFN-γ and IP-10 detection as screening tools with a 100% negative predictive value. The study participants were the two siblings of the index case and 58 classmates. The number of contacts with positive results was very low, with two positive cases at the beginning (one of them was a sibling) and three converters (one of them was the other sibling), thus, no positive predictive value could be established. Our contact cohort comprises a modestly higher amount of positive cases that are contacts form different index cases, the majority TST positive. This study increases the previous evidence that IP-10 detection in direct and dried plasma is comparable to IFN-γ detection in direct plasma in contacts[16].

Although the amount of IP-10 detected in DPS is lower than the amount detected in direct plasma, therefore causing a decrease of positive results, both IP-10 assays have a strong agreement ($\kappa=0.77$).

Following with the idea of using IP-10 detection in DPS as a potential point-of-care test, IP-10 mRNA detection in dried blood spots (DBS) has also been evaluated in TB patients for this purpose. Blauenfeldt *et al.* demonstrated that, as in IP-10 detection in DPS, IP-10 mRNA is present in significantly higher amount in active TB patients and LTBI individuals compared to uninfected controls (sensitivity 88%, specificity 96%) and that the results are comparable to those obtained in direct blood (sensitivity 85%, specificity 96%)[20].

Regarding the 5 non-BCG vaccinated contacts with negative QFN-G-IT and IP-10 test results but with a positive TST, we hypothesized, that these could be false-positive TST results due to a non-tuberculous mycobacteria sensitization[28].

The spread of TB is directly related with degree and duration of the contact exposure with the index TB case, increasing the risk of LTBI infection[29,30]. In order to check whether a closer contact with active TB patients resulted in a higher positivity rate or in the increase of specific cytokines release, contacts were stratified based on time of exposure. In our study, although not statistically significant, those contacts with a higher rate of positive results were those with more than 6 hours of exposure. Moreover, regarding cytokine levels, our results showed no significant differences among groups ($p>0.05$), therefore in our contact cohort we cannot correlate IP-10 levels with degree exposure to the index case.

Although our overall results support the use of IP-10 detection in DPS for LTBI diagnosis, this study is limited by a relatively small sample size, comprising a total of 80 subjects which may cause an imprecise assessment of the cut-offs. Moreover, there is no gold standard method for LTBI diagnosis, therefore, we cannot truly confirm whether a result is really false positive or negative. Regarding the transportation of the samples, our sending conditions did not seem to interfere in the signal strength nor in the test performance, as we have also reported

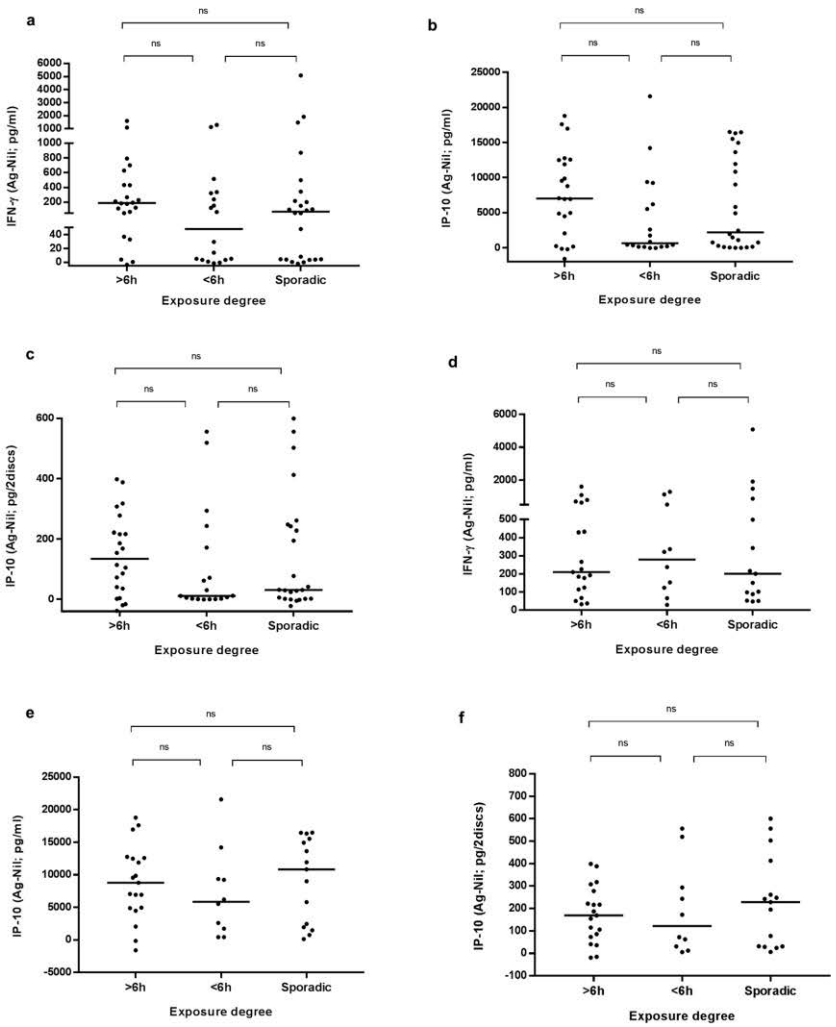

Figure 6. Cytokine levels in overall contacts (**a**–**c**) and in LTBI contacts (**d**–**f**) regarding the exposure degree. (**a**) IFN-γ detected by QFN-G-IT in direct plasma (pg/ml). Median values: 189 pg/ml, 48 pg/ml, 70.75 pg/ml in >6 h, <6 h and sporadic contatcs, respectively. (**b**) IP-10 detected in direct plasma (pg/ml). Median values: 7003 pg/ml, 640 pg/ml and 2206 pg/ml in >6 h, <6 h and sporadic contatcs, respectively. (**c**) IP-10 detected in DPS (pg/2discs). Median values: 134.5 pg/2discs, 11.6 pg/2discs and 31.45 pg/2discs in >6 h, <6 h and sporadic contatcs, respectively. (**d**) IFN-γ detected by QFN-G-IT in direct plasma (pg/ml). Median values: 210.5 pg/ml, 280 pg/ml, 201.5 pg/ml in >6 h, <6 h and sporadic contatcs, respectively. (**e**) IP-10 detected in direct plasma (pg/ml). Median values: 8780 pg/ml, 5874 pg/ml and 10841 pg/ml in >6 h, <6 h and sporadic contatcs, respectively. (**f**) IP-10 detected in DPS (pg/2discs). Median values: 168.6 pg/2discs, 122 pg/2discs and 227.8 pg/2discs in >6 h, <6 h and sporadic contatcs, respectively. Statistical analysis was performed using Mann-Whitney U test (ns = $p > 0.05$). Four contacts had unknown exposure degree (data not shown).

previously[18]. However, we cannot assure this will be the case in other settings with different transport temperature and timing.

In summary, our study has led to the following conclusions: (i) IP-10 is a useful chemokine that can be used as a biomarker for LTBI; (ii) sending dried plasma in filter paper is a simple process and enables the sending of samples for LTBI diagnosis via mail (iii) IP-10 can be detected in dried plasma by immunological methods; (iv)

IP-10 detection in DPS causes a decrease in positive results however, the agreement with IP-10 detection in direct plasma and with QFT-G-IT is high.

The clinical implication of this study is the development of a new procedure for LTBI diagnosis in contacts that simplifies the sending of previously stimulated samples from the collection site to the analysis site without sample damage using simple protocols. Having a simple and safe method to send samples through mail with no need for refrigeration enables LTBI diagnosis in faraway settings, thus setting us a step closer to improving LTBI diagnosis and disease control.

Material and Methods

Study setting and patient recruitment. In this retrospective case-control study we included whole blood samples from microbiologically confirmed active pulmonary TB patients and individuals from contact tracing studies (contacts of smear positive patients). In order to correlate the amount of cytokine detected with the time of exposure, contacts were divided into three subgroups regarding time of exposure to active TB patients: (i) more than 6 hours of exposure per day, (ii) less than 6 hours of exposure per day, and (iii) sporadic contact (not daily). Moreover, and aware of the lack of gold standard, contacts were divided into two groups regarding the QFN-G-IT result. Those with a negative test result were considered as uninfected controls and those with positive results were considered as LTBI individuals.

Such samples were collected at two medical centers in Barcelona, Spain: Unitat de Tuberculosi de Drassanes - Hospital Universitari Vall d'Hebron and Unidad Clínica de Tratamiento Directamente Observado "Serveis Clinics".

The study was approved by the Ethics Committee Hospital Universitari Germans Trias i Pujol (http://www.ceicgermanstrias.cat/). This research was performed in accordance with the relevant guidelines/regulations. Written informed consent was collected from each subject before blood sampling and a detailed questionnaire with the following variables was filled in: age, gender, country of birth, smear and culture result, tuberculin skin test result, BCG vaccination and time of exposure.

Tuberculin skin test. To perform the tuberculin skin test (TST) we followed the Spanish guidelines[31] using the Mantoux method with 2-TU of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark). Briefly, 0.1 ml of PPD solution was injected intradermally by experienced personnel in the patients' forearm[31]. The induration diameter was measured after 48–72 hours. When the induration had a diameter equal to 5 mm or above the test was considered positive regardless BCG vaccination status.

IFN-γ detection in direct plasma. To detect IFN-γ production, we used the QuantiFERON-TB Gold In-Tube (QFN-G-IT; Qiagen, Düsseldorf, Germany) procedure following manufacturer's instructions. Briefly, from each patient 3 ml of blood (1 ml per tube) were extracted. The reactivity obtained in the nil tube was subtracted from the IFN-γ value of the antigen (RD1 antigens cocktail: ESAT-6, CFP-10, and TB7.7) and mitogen tubes. An IFN-γ level equal or above 0.35 IU/ml after antigen stimulation is recorded as a positive result. If the stimulation was negative and the value of the positive control was less than 0.5 IU/ml or the negative control higher than 8.0 IU/ml the result was considered indeterminate. Throughout this study, the amount of IFN-γ will be shown in pg/ml to ease the comparison with IP-10 (1 IU/ml = 50 pg/ml, National Institute for Biological Standards and Control, [NIBSC], UK).

IP-10 detection in direct plasma. IP-10 was detected in the previously QFN-G-IT analysed plasma using a non-commercial in-house ELISA[18]. Briefly, 30 time diluted plasma samples were incubated for 2 hours in an ELISA plate pre-coated with specific IP-10 monoclonal antibodies. Plate was washed and TMB substrate was added. After incubating for 30 minutes, the reaction was stopped with H_2SO_4, and the absorbance was read (450nm-630nm)[21]. The nil stimulated sample response was subtracted from that of the antigen and mitogen stimulated samples.

IP-10 detection in dried plasma spots. At the medical centres, two plasma drops of 25 µl from each sample were dried in Whatman903 filter paper for 3–4 hours at room temperature (RT), away from direct sunlight. After 5 days at RT, the samples were sent by postal service to the lab in a plastic bag with desiccants, to avoid humidity. Once in the lab, after a 2-day transport, samples were stored at RT and IP-10 detection analyses were performed within 7 days after arrival. The cytokine detection was done using a non-commercial in-house ELISA and following the procedure previously described[21]. Briefly, each dried plasma spot (DPS) was punched by a standard office puncher of a 5 mm diameter. The two discs from each sample were placed into the same well of a 96 well plate with dilution buffer. Conjugate buffer was added and the sample was mixed. After 2 hours of incubation at RT and dark conditions, the wells were washed and the discs were knocked out. In order to measure IP-10 levels, TMB stabilized chromogen was added and after 30 min in the dark the reaction was stopped. The plates were read at 450–630 nm.

Statistical analysis. The correlation between IP-10 detection in direct plasma and dried plasma, was assessed using Spearman correlation coefficient for a stability overview approach and to determine whether IP-10 levels detected by each test were comparable. In order to study the agreement among the different tests and assess the interrater reliability, we used Cohen's kappa (κ) coefficient, excluding indeterminate results. Kappa values range from 0 (agreement that could be expected from chance) to +1 (perfect agreement)[32]. Agreement was considered strong, intermediate or weak, when kappa values were above 0.60, between 0.60 and 0.40, or below 0.40, respectively. We used Mann-Whitney U test, two tailed, to compare the amount of both cytokines released in each QFN-G-IT tube. We considered as significant p values those under 0.05.

In order to establish positivity cut-offs for both IP-10 detection tests, we performed a receiver operating characteristic (ROC) curve analysis. Due to the well-known non-specificity of the TST, we have considered QFN-G-IT

positive contacts as LTBI patients and QFN-G-IT negative contacts as uninfected controls. We chose positivity cut-offs for IP-10 detection that gave a high specificity without losing sensitivity. As indeterminate result cut-offs, we chose the lower value of mitogen after nil subtraction of those negative QFT-G-IT samples.

Analysis and graphs were performed using SPSS statistical software (SPSS version 15.0; SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 5.00 (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Data Availability

Due to participant privacy, the data collected for this study is available upon request to the corresponding author.

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Author Contributions

Study design and supervision: J.D. and M.R. Recruitment of patients: M.L.D.S.G., M.A.J., J.R.M., J.P., A.C., N.A., J.P.M., Y.G.D., I.M.P. and C.P. Acquisition of data: R.V.H., I.L., E.G.G. and B.M.M. Analysis and interpretation of data: R.V.H. and I.L. Drafting the article: R.V.H., I.L., J.D. and M.R. Revising it critically for important intellectual content and final approval of the version to be published: all authors.

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Article 8

Development and testing of a new non-tuberculous mycobacteria diagnostic test using specific mycobacterial cell wall antigens.

Raquel Villar-Hernández, Irene Latorre, Zoran Stojanovic, Antoni Noguera-Julian, María Luiza de Souza-Galvão, María Méndez, Carlos Rodrigo, Josefina Sabriá, Carmen Martos, José Ramón Santos, Jordi Puig, Anàlia López, Aina Martínez-Planas, Antonio Soriano-Arandes, María Á Jiménez, Marisol Domínguez, Laura Minguell, Joan Pau Millet, Neus Altet, Irma Casas, Alicia Marín, Yolanda Galea, Beatriz Muriel-Moreno, Esther García-García, Miguel Pérez, Juan Ruiz Manzano, Cristina Prat, Jordi B. Torrelles and José Domínguez.

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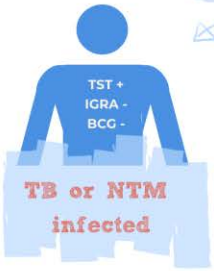
Why diagnosing NTM infections is so important?



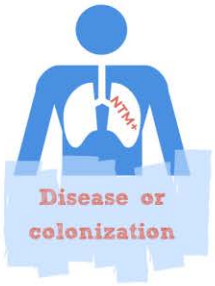
To discard tuberculosis (TB) infection when test results are discordant

&

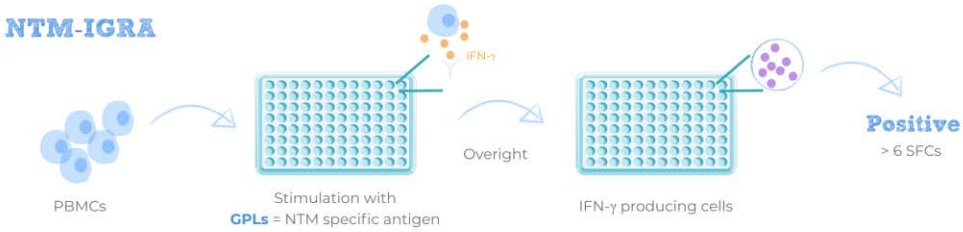
To help handling patients with CPD that have NTM isolations of unclear clinical relevance

&

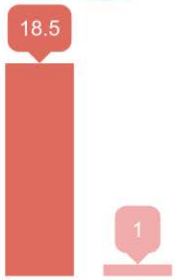
NTM infections are increasing in frequency



How can we do it?

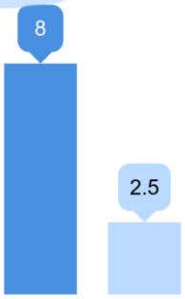


What do we get?



TST+, IGRA-, BCG- patients have a significantly higher number of SFCs after stimulation with GPLs than TB infected individuals

CPD patients with NTM causing disease have significantly higher number of SFCs after stimulation with GPLs than those with colonizing NTM or previous NTM disease



Bars represent median values

NTM-IGRA could be a useful diagnostic tool to improve NTM diagnosis. It would guide proper clinical decisions, avoid unnecessary treatment and improve patient's handling.

CPD: Chronic pulmonary disease; GPLs: Glycopeptidolipids; IGRA: Interferon-γ release assay; NTM: Non-tuberculous mycobacteria; PBMCs: Peripheral Blood Mononuclear Cells; QFN-G-IT: QuantiFERON-TB Gold In-Tube; TB: Tuberculosis; SFCs: Spot forming cells; TST: Tuberculin Skin Test.

Raquel Villar Hernández

Development and testing of a new non-tuberculous mycobacteria diagnostic test using specific mycobacterial cell wall antigens

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ABSTRACT

Diagnosing non-tuberculous mycobacteria (NTM) infection is important to discard tuberculosis (TB) infection when test results are discordant and could be useful to handle patients with chronic pulmonary diseases (CPD) with NTM isolations of unclear clinical relevance. This study describes an immunological NTM-Interferon (IFN)-γ Release Assay (IGRA) that we have developed using specific NTM antigens to detect NTM sensitization.

We enrolled 332 patients: 90 latently TB infected (LTBI), 69 active TB, 52 NTM culture positive (18 lymphadenopathies, 31 pulmonary infectious diseases, and 3 disseminated infections), 13 NTM infection suspicion (positive TST, negative IGRA and no BCG vaccination record), 31 lymphadenopathies of unknown etiology, 3 lymphadenopathies not caused by NTM, 52 HIV positive, and 22 uninfected controls with no TB exposure record. Peripheral blood mononuclear cells were isolated from whole blood samples. Cells were then stimulated overnight with NTM specific antigens. Detection of IFN-γ producing cells was evaluated by ELISPOT.

NTM positive and suspicion groups had a significantly higher count of INF-γ-producing cells after NTM antigen stimulation. Using ROC curve analysis, a positivity cut-off of 6 spots was determined. The NTM suspicion group, NTM positive and non-culture confirmed lymphadenopathies groups, had a higher number of positive results than LTBI, active TB and HIV positive patients (76.9%, 69.2%, and 37.5%, compared to 26.7%, 17.4%, and 23.1%, respectively). Additionally, CPD patients with NTM isolates considered as causing disease

yielded a higher number of positive results than those considered as colonized (84.6% and 33.3%, respectively). Uninfected individuals and those with lymphadenopathies not caused by NTM all yielded negative results.

The NTM-IGRA described in this study could serve as an NTM infection diagnosis test capable of distinguishing NTM infection from LTBI. Furthermore, it could enlighten the clinical relevance of NTM isolates in clinical samples from patients with CPD. Altogether, the NTM-IGRA would improve the handling of the patient and avoid unnecessary treatment. Ongoing studies are focused on characterizing the immune response against these antigens.

INTRODUCTION

Non-tuberculous mycobacteria (NTM) are a group of over 200 ubiquitous environmental species[1,2]. NTM are commonly considered as clinically less important[3] than other bacteria from the *Mycobacterium* genus such as *Mycobacterium tuberculosis complex* species and *Mycobacterium leprae* and therefore less frequently reported to public health authorities. However, in some cases, NTM infections are associated with respiratory diseases, lymphadenitis and disseminated infections in immunocompromised patients, among others[4,5]. In the past years, NTM infections have increased and not solely due to improved diagnosis, recording, and typing, but also due to a real increase in diseases caused by NTM[6–12].

Isolations of NTM in clinical samples from patients with chronic pulmonary diseases (CPD) are also increasing[10,13–15]. Recently, Shah et al. described a two-fold increased incidence of *Mycobacterium avium* complex (MAC) pulmonary infections from the year 2007 to 2012[14] and Russell et al. also described an increase of *Mycobacterium avium* during the years 2000 and 2010[15]. The clinical relevance of NTM isolation in CPD patients is not fully understood nor possible to determine. In these cases, NTM isolation could be the cause of disease and therefore anti-NTM treatment would be established accordingly, or the isolated NTM could be a mere colonizer. Thus, in these situations in which the role of the NTM isolates is unclear, having a method that can detect the NTM infection and distinguish disease from colonization, would help the patient's handling and, therefore, increase the chances of recovery.

NTM are also a source of confusion in latent tuberculosis (TB) infection (LTBI) diagnosis due to cross-reaction with the tuberculin skin test (TST). The purified protein derivative (PPD) used in the TST includes a combination of *M. tuberculosis* antigens that are also shared by the Bacillus Calmette-Guérin (BCG, TB vaccine available) and NTM species. The development of the interferon (IFN)-γ release assay (IGRA) which use specific *M. tuberculosis* antigens (ESAT-6, CFP-10, and TB7.7) to stimulate T-cells have enabled the detection of TB infection by detection of IFN-γ production by the sensitized T-cells. However, there are cases in which the TST is positive, the IGRA is negative and there is no record of BCG vaccination[16,17]. In these cases, especially in children, who are at high risk of developing active TB, preventive chemotherapy is recommended in order to prevent progression to TB.

However, such results are also consistent with NTM infection and therefore anti-TB treatment and follow up may not be necessary in these cases[18,19]. Although IGRAs are specific for TB and have been shown to discriminate between NTM and TB infections[19] in some cases with diagnostic discrepancies there is a need to increase the certainty that infection is in fact caused by NTM and not TB before considering no to establish anti-TB therapy.

Glycopeptidolipids (GPLs) are a type of glycolipids produced by a wide range of NTM such as *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium smegmatis*, and species from the MAC such as *M. avium* and *Mycobacterium intracellulare*[20–23], but not by species from the *M. tuberculosis* complex. These NTM, synthesize GPLs with a common lipopeptide core but depending on the species their glycosylation, methylation, and acetylation patterns will be different[21,22,24,25]. Various functions have been attributed to GPLs such as colony morphology, bacteria physiology, host immune response, motility, biofilm formation and virulence[22,26–29].

In this study, we describe the use of GPLs for PBMCs stimulation using an Enzyme-Linked Immunospot Assay (ELISPOT) based NTM-IGRA that we have developed. The aims of this study were to evaluate the use of the developed NTM-IGRA to detect specifically NTM infections and evaluate its applicability in patients with CPD for the determination of the NTM isolate's clinical relevance.

MATERIALS & METHODS

Study design

Patient recruitment and sample collection was carried out prospectively at 8 health care centres in Barcelona, Spain: Germans Trias i Pujol University Hospital, Unitat de Tuberculosi Vall d'Hebron-Drassanes, Serveis Clínics TB Directly Observed Treatment Unit, Sant Joan Despí Moises Broggi Hospital, Sant Joan de Déu Barcelona Children's Hospital, Mar Hospital, Arnau de Vilanova University Hospital, and Granollers Hospital.

Ethical approval for this study was provided by the Ethics Committee of the Germans Trias i Pujol University Hospital (http://www.ceicgermanstrias.cat/) (reference number: PI 17-120) and by the other centres involved. Together with the collected samples, we obtained a written informed consent from each patient or their legal representatives, and a detailed questionnaire indicating, demographic and clinical data, such as TST results, BCG vaccination status, details of any contact with an active TB patient, culture results, history of prior or current TB, LTBI and other co-morbidities, among others.

Study groups

A varied group of patients was included in this study. The subgroups in which they were classified and the criteria used were:

1) Active TB patients with a microbiological confirmation - PCR and/or culture positive for *M. tuberculosis,*
2) LTBI screening/contact studies:
    i) IGRA positive, and
    ii) NTM infection suspicion group - NTM infection suspicion due to a positive TST but negative IGRA and no BCG vaccination.
3) Patients with CPD and an NTM isolation
    i) diseased patients, and
    ii) colonized patients.
4) Immunosuppressed patients with a disseminated disease caused by NTM.
5) Patients with lymphadenopathies:
    i) with an NTM isolation,
    ii) of unknown aetiology, and
    iii) not caused by NTM.
6) HIV positive patients, coming from the HIV unit from Hospital Germans Trias i Pujol.
7) Healthy controls - uninfected immunocompetent controls with no known exposure to TB or other mycobacteria.

NTM microbiological diagnosis in patients with CPD

In the case of CPD patients, X-ray and culture of respiratory samples were performed. The clinical relevance of NTM isolates in order to classify the patient as diseased or colonized, was performed following the American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) 2007 guidelines (ATS/IDSA)[3]. Briefly, clinical relevant NTM isolates are characterized by: (i) compatible chest radiograph or computerized tomography scan, (ii) compatible clinical symptoms and exclusion of other similar diseases such as TB, and (iii) at least two positive separate expectorated sputum samples or a positive culture from at least one bronchial wash or lavage or NTM isolation from a sterile site[3].

Purification of NTM specific antigens

Glycopeptidolypids (GPLs) purified from the cell wall of *M. avium* serovar 4 were used as NTM specific antigens.

M. avium complex colonies were scraped from the surface of Middlebrook 7H11 agar plates containing oleic acid-albumin-dextrose-catalase (OADC) enrichment, and were transferred aseptically to sterile screw-capped glass culture tubes containing 5 ml of methanol (CH_3OH) followed by the addition of 10 ml of chloroform ($CHCl_3$) for a final ratio of $CHCl_3$–CH_3OH (2:1 v/v) and incubated at 37°C overnight. After this incubation period, samples were centrifuged for 5 min at 11,500 x g at 4°C to obtain supernatants (organic extractions containing total lipids). Then, total lipids were treated with mild alkali (an equal 20 volume of 0.2 N sodium hydroxide (NaOH) in CH_3OH at 37 °C for 35 min) followed by neutralization using 100 microliters of glacial acetic acid (2 to 3 drops, check pH to be 7.0). The alkali-treated lipids were further partitioned between $CHCl_3$–CH_3OH (2:1 v/v) and H_2O at a ratio of 1:1 (v/v) to remove 25 generated sodium acetate salts. This 'washing' step was repeated twice. The final chloroform layer containing the total GPLs fraction was transferred to a pre-weight tube and dried down under nitrogen to avoid lipid oxidation. Dried samples were kept at -20°C under nitrogen until further use. This allows for obtaining total GPLs.

Sample type and PBMCs isolation

Whole blood samples were collected from all study subjects in vacutainer glass mononuclear cell preparation tubes (CPT, Becton Dickinson Diagnostics) for the subsequent peripheral blood mononuclear cells (PBMCs) isolation for future stimulation. Briefly, blood collected in CPTs was centrifuged at 1600g for 30 minutes at room temperature. PBMCs layer was harvested and washed twice with RPMI 1640 medium with L-Glutamine (Biowest, France) supplemented with 10% fetal bovine serum (FBS, Biowest, France). Washed cells were resuspended in AIM-V medium (Gibco, Life Technologies Ltd. Fountain Dr, Inchinnan, UK), for a final concentration of 2'5 $x10^6$ cells/ml.

TST

Following the Spanish guidelines[30], 2-TU of PPD (Statens Serum Institut, Copenhagen, Denmark) were injected intradermally in the patient's forearm by experienced personnel. Induration was read after 48-72 hours. Induration diameters equal or above 5mm were considered as positive.

T-SPOT.TB

Stimulation of PBMCs with specific TB antigens, ESAT-6 and CFP-10, was done using the T-SPOT.TB test (Oxford Immunotec Limited, Abingdon, UK) following the manufacturer's instructions. Briefly, 50ul of the negative control (medium), the positive control and the antigenic TB panels A and B, were added to the corresponding wells. Afterwards, 100ul of cell suspension containing 250.000 cells were added into the previous wells (figure 1). The plates were incubated overnight at 37ªC in a 5% CO_2 atmosphere after which, wells were washed and the conjugate and substrate were added in order to detect the spot forming cells (SFCs). Interpretation of the results was done following the manufacturer's

instructions. As positive results, we considered 8 SFCs or more in panel A and/or B than in the negative control well and at least as twice the number. If the highest number of SFCs in either panel was 5, 6 or 7 more than in the negative control, these samples were considered as borderline. An indeterminate result was given when (i) the response to both antigen panels were negative and the number of SFCs after positive control stimulation was less than 20 or (ii) there were more than 10 SFCs in the negative control well. To calculate the number of SFCs in the case of RD1 response, we counted the response obtained in both panels. Result readout was done using an AID ELISPOT reader (AID GmbH, Strassberg, Germany).

NTM-IGRA

For PBMCs stimulation with the purified NTM specific antigens, the same ELISPOT based technique was used. Throughout this article, we will refer to this variation as the NTM-IGRA test. Cells (100ul containing 250000 cells) were added into an extra well in which 2.5ul of GPLs at 1mg/ml were added, mixing thoroughly (figure 1). This GPL volume was established after evaluating the addition of different amounts of the antigen for cell stimulation; 2.5ul was the minimum amount that did not cause a decrease in the response and did not affect cell viability. The plates were incubated and processed as described above for the T-SPOT.TB. For result interpretation in the case of NTM specific antigen stimulation, we first determined the cut-off value using Receiver Operating Characteristic (ROC) curve analysis and then followed the same criteria as for panel A and B. In those wells in which the amount of SFCs was too high, we considered the quantitative result as saturated and in order to plot the results, we used 100 SFCs as the standard value. Spare PBMCs were kept in a cryopreservation media and stored in liquid nitrogen for future use.

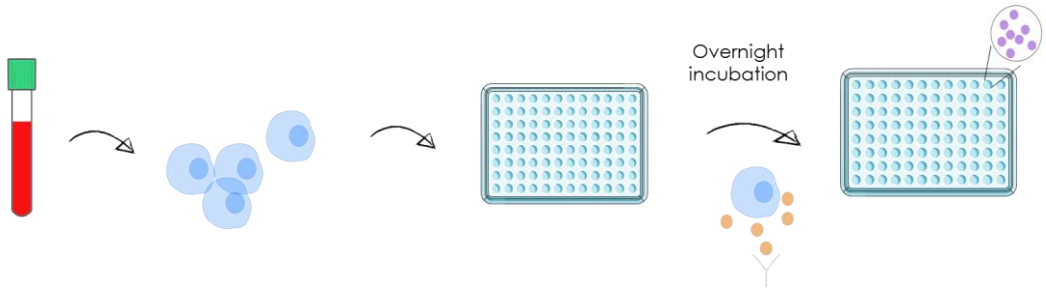

Figure 1. Diagram of the T-SPOT.TB and NTM-IGRA procedures. PBMCs are isolated from whole blood and stimulated overnight with TB and GPLs in an IFN-γ antibody coated plate. Each well contains 250.000 cells in 100µl of medium and the antigens or controls tested.

Statistical analysis

Statistical analyses and graphical representations of the study results were performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Mann-Whitney U-test was used for unpaired comparison between groups considering as statistically significant a p-value below 0.05.

ROC curve analyses were performed and the area under the curve (AUC) was calculated in order to determine the cut-off values of the GPLs stimulation. Negative controls were those uninfected individuals with a 0-1 SFC, and the positive cases were those with a positive NTM culture. Youden's index (=Sensitivity -[1-Specificity]) was used to determine the best cut-off value.

RESULTS

Study population

We received whole blood samples from 332 patients. A 15.7% (52/332) of the included patients had a positive NTM culture: 34.6% (18/52) of them had lymphadenopathies, 59.6% (31/52) a CPD and 5.8% (3/52) a disseminated infection caused by *M. chimaera*. A 20.8% (69/332) of samples were from active TB patients, 27.1% (90/332) were LTBI, 3.9% (13/332) were characterized as patients with discordant results between TST (positive) and IGRA (negative), 9.3% (31/332) of patients had lymphadenopathies of unknown aetiology, 0.9% (3/332) were lymphadenopathies not caused by NTM, 15.7% (52/332) were HIV positive and 6.6% (22/332) were healthy controls.

Demographic and clinical data are shown in table 1.

NTM-IGRA

ROC curve analysis

After ROC curve analysis, a positive value was considered when there were 6 SFCs or more (AUC = 0.8977, Youden's index = 0.82) to obtain an 82.0% sensitivity and a 100.0% specificity, (CI: 68.56% to 91.42% and 84.56% to 100%, respectively) (figure 2).

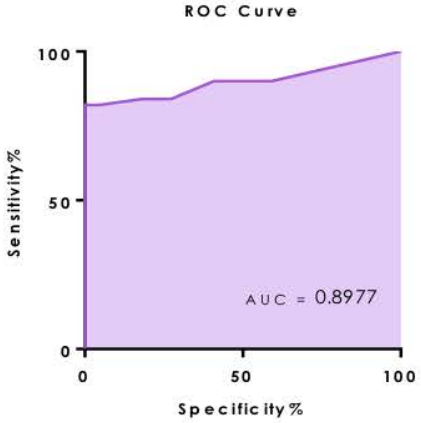

Figure 2. Receiver operating characteristic (ROC) curve analysis. AUC = area under the curve.

Table 1. Demographic and clinical data of the study subjects

Variable		n (%)	
Overall (n=332)			
Age, mean (years) ± SD		37.0 ± 23.1	
Gender			
	Male	196 (59.0)	
	Female	136 (41.0)	
Active TB (n=69)			
Age, mean (years) ± SD		44.5 ± 19.1	
Gender			
	Male	43 (62.3)	
	Female	26 (37.7)	
Country of birth			
	High TB burden[a]	18 (26.1)	
	Low TB burden	51 (73.9)	
Anti-TB treatment (%)			
	Before starting treatment	13 (18.8)	
	On treatment	56 (81.2))	
	< 30 days		37 (66.1)
	≥ 30 days		19 (33.9)
	Average (days) ± SD in patients with treatment	50.1 ± 79.7	
LTBI screening (n=103)			
LTBI (TST & IGRA +) (n=90)			
Age, mean (years) ± SD		39.7 ± 17.0	
Gender			
	Male	49 (54.4)	
	Female	41 (45.5)	
Country of birth			
	High TB burden[a]	11 (12.2)	
	Low TB burden	79 (87.8)	
Anti-TB prophylaxis (%)			
	Before starting prophylaxis	17 (18.9)	
	On prophylaxis	73 (81.1)	
	< 30 days		56 (76.7)
	≥ 30 days		17 (23.3)
	Average (days) ± SD in patients with prophylaxis	33.4 ± 31.6	
NTM suspicion (TST +, IGRA-, BCG-) (n=13)			
Age, mean (years) ± SD		17.9 ± 14.2	
Gender			
	Male	7 (53.8)	
	Female	6 (46.2)	
Country of birth			
	High TB burden[a]	0 (0.0)	
	Low TB burden	12 (92.3))	
	Unknown	1 (7.7))	
Anti-TB prophylaxis (%)			
	Before starting prophylaxis	9 (69.2)	
	On prophylaxis	4 (30.8)	
	< 30 days		2 (50.0)
	≥ 30 days		2 (50.0)
	Average (days) ± SD in patients with prophylaxis	50.8 ± 35.7	
CPD NTM+ (n=31)			
NTM disease (n=13)			
Age, mean (years) ± SD		67.2 ± 13.0	
Gender			
	Male	7 (53.8)	
	Female	6 (46.2)	
Anti-NTM treatment (%)			
	Before starting treatment	8 (61.5)	
	On treatment	5 (38.5)	
	< 30 days		2 (40.0)
	≥ 30 days		3 (60.0)
	Average (days) ± SD in patients with treatment	55.8 ± 40.3	
NTM colonization (n=6)			
Age, mean (years) ± SD		76.5 ± 10.5	
Gender			
	Male	3 (50.0)	
	Female	3 (50.0)	

NTM disease or colonization (n=6)*			
Age, mean (years) ± SD		49.2 ± 31.3	
Gender			
	Male	3 (50.0)	
	Female	3 (50.0)	
Anti-NTM treatment (%)			
	Before starting treatment	4 (66.7)	
	On treatment	2 (33.3)	
	< 30 days		0 (0.0)
	≥ 30 days		2 (100.0)
	Average (days) ± SD in patients with treatment	328.5 ± 236.9	
Previous NTM disease (n=6)			
Age, mean (years) ± SD		47.7 ± 14.6	
Gender			
	Male	4 (66.7)	
	Female	2 (33.3)	
Anti-NTM treatment (%)			
	On treatment	2 (33.3)	
	Average (days) ± SD in patients with treatment	429.5 ± 85.6	
	Ended treatment	4 (66.7)	
Disseminated NTM (n=3)			
Age, mean (years) ± SD		56.7 ± 16.0	
Gender			
	Male	3 (100.0)	
	Female	0 (0.0)	
Anti-NTM treatment (%)			
	Before starting treatment	0 (0.0)	
	On treatment	3 (100.0)	
	< 30 days		0 (0.0)
	≥ 30 days		3 (100.0)
Lymphadenopathies (n=52)			
NTM+ (n=18)			
Age, mean (years) ± SD		6.7 ± 14.6	
Gender			
	Male	8 (44.4)	
	Female	10 (55.6)	
Anti-NTM treatment (%)			
	Before starting treatment	12 (66.7)	
	On treatment	6 (33.3)	
	< 30 days		2 (33.3)
	≥ 30 days		2 (33.3)
	Unknown		2 (33.3)
	Average (days) ± SD in patients with treatment	43.8 ± 50.1	
Lymphadenopathies of unknown aetiology (n=31)			
Age, mean (years) ± SD		4.5 ± 3.4	
Gender			
	Male	12 (38.7)	
	Female	19 (61.3)	
Lymphadenopathies not caused by NTM (n=3)			
Age, mean (years) ± SD		6.5 ± 5.3	
Gender			
	Male	2 (66.7)	
	Female	1 (33.3)	
HIV positive (n=52)			
Age, mean (years) ± SD		47.3 ± 13.6	
Gender			
	Male	44 (84.6)	
	Female	8 (15.4)	
CD4⁺ counts (cells/ul) Average ± SD		695.6 ± 321.8	
Viral load <40 c/ml		39 (75.0)	
Healthy controls (n=22)			
Age, mean (years) ± SD		25.6 ± 17.1	
Gender			
	Male	11 (50.0)	
	Female	11 (50.0)	

ᵃAbout 150 cases per 100 000 population. TB: active tuberculosis patients, LTBI: latently tuberculosis infected individuals, NTM: non-tuberculous mycobacteria *This group of patients are cases in which the NTM role was not clear. These unclear cases did not meet all ATS/IDSA criteria but clinically were considered as the cause of the disease and treated.

LTBI screening

In order to evaluate the use of GPLs as a tool to enlighten the diagnosis of patients with negative IGRA but positive TST despite no BCG vaccination record (suspicion NTM infection group), we compared the results obtained in this group with those from the LTBI and HC samples. As shown in figure 3, those samples from the suspicion of NTM infection group had a statistically significantly higher response to GPL stimulation than LTBI and healthy control ones ($p<0.001$ and $p<0.0001$, respectively). There were no significant differences between the response obtained in the LTBI and healthy controls.

HIV positive patients

Samples from HIV positive patients had a significantly lower IFN-γ release in response to GPLs than those patients that were suspected to have an NTM infection ($p<0.0001$) (figure 3). No significant differences were observed when compared to the LTBI and healthy controls groups. All HIV positive patients had $CD4^+$ counts above 200 cells/μl, except two, which had 164 and 172 cells/μl.

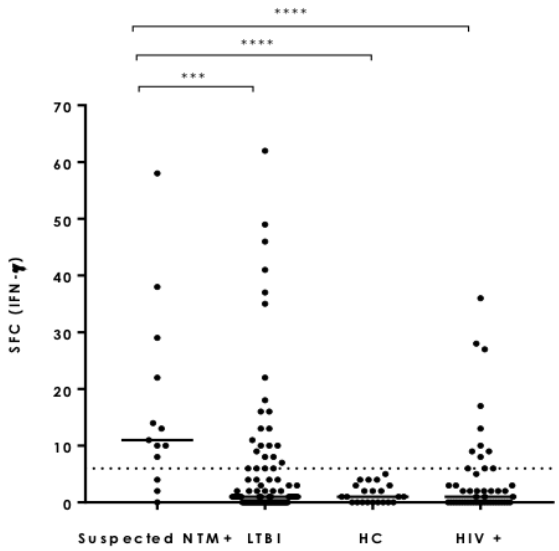

Figure 3. Spot forming cells (SFC) obtained after stimulation with GPLs for LTBI differential diagnosis and NTM infection diagnosis in HIV positive patients. Median (IQR) values in SFCs of each group were: 18.5 (5.5 – 51.5) in NTM positive samples, 11 (6 – 25.5) in samples from the suspected NTM infected group, 1 (0 – 6) in LTBI, 1 (0 – 3) in HC, and 1 (0 - 5.5) in HIV positive patients. Results refer to the SFCs in the GPL stimulation condition after response subtraction of the negative control. Mann Whitney test was performed to compare between groups: *** = $p<0.001$; **** = $p<0.0001$. HC: healthy controls, IQR: interquartile range. Two LTBI and three HIV positive samples are not shown due to their indeterminate result. Dotted line indicates the 6 SFC positivity cut-off.

Lymphadenopathies

Regarding samples from the 52 patients with lymphadenopathies, those with a positive NTM culture (34.6%, 18/52) had a significantly higher response to GPL stimulation than those of unknown etiology that had a TST result below 5mm (28.8%, 15/52) ($p<0.001$) and those not caused by NTM ($p<0.05$)

(figure 3). No statistically significant differences were found when comparing NTM positive lymphadenopathies with those of unknown aetiology that had a TST result above 5mm (30.8%, 16/52) (figure 4). From the 18 cases of lymphadenopathies that were positive for NTM, 14 (77.8%) had a MAC isolate, 3 (16.7%) were *Mycobacterium malmoense* and one (5.6%) had both a MAC isolate and *Mycobacterium chimaera*.

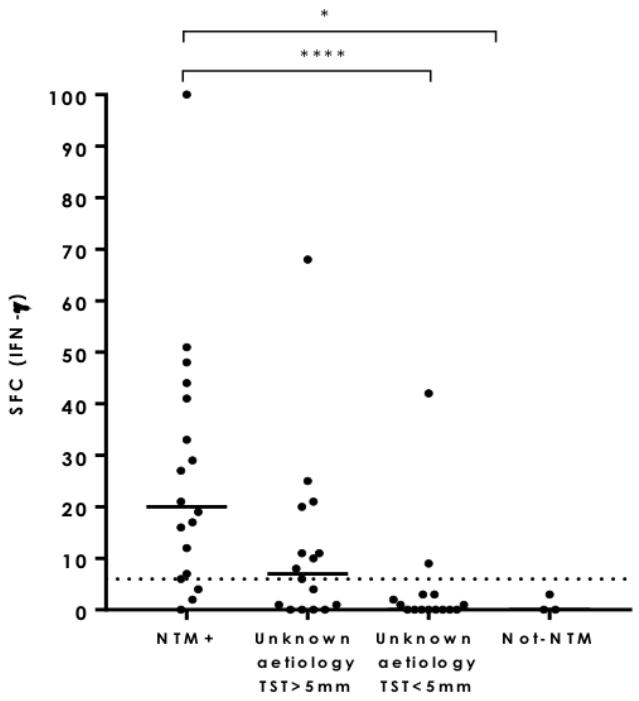

Figure 4. Response to stimulation with GPLs in samples from patients with lymphadenopathies. Median (IQR) values in SFCs for each group were: 20 (6.75 – 41.75) for positive NTM lymphadenopathies, 7 (0.25 – 17.75) for lymphadenopathies of unknown aetiology with a TST result >5mm, 0 (0 - 3) for lymphadenopathies of unknown aetiology with a TST result <5mm and 0 SFCs (0 – 3) for lymphadenopathies not caused by NTM. Mann Whitney test was performed to compare between groups: * = p<0.05; *** = p<0.001. Results refer to the SFCs in the GPL stimulation condition after response subtraction of the negative control. IQR: interquartile range. Dotted line indicates the 6 SFC cut-off.

Disseminated infections

Among the overall NTM positive group (52 cases), three patients (5.8%) had a disseminated infection by *M. chimaera*. In these cases, stimulation with GPLs yielded over 100 SFCs. This response was significantly higher to that of the NTM suspicion group and the rest of NTM positive samples (lymphadenopathies and CPD) ($p<0.01$).

Patients with CPD and NTM isolates

From the overall NTM positive samples, 59.6% (31/52) were from patients with CPD. Seven patients had more than one CPD. Thirteen samples (40.9%) were from patients with bronchiectasis, 4 (12.9%)

from patients with cystic fibrosis, 7 (22.6%) with chronic obstructive pulmonary disease (COPD), 6 (19.4%) with asthma and 7 (22.6%) had a past active TB. The responses against GPLs were significantly higher in samples from patients with bronchiectasis than COPD ($p<0.05$) and higher in samples from cystic fibrosis patients compared to COPD ($p<0.01$), asthma ($p<0.01$) and past active TB ($p<0.05$) (figure 5A). Altogether, samples from patients with CPD with positive NTM isolate yielded a statistically significant higher response to GPLs than those of active TB patients ($p<0.001$).

Considering the challenging task of knowing whether NTM isolations in these kinds of patients are in fact of clinical relevance (are they the cause of disease or mere colonizers), we separated them further into different groups. This clinical classification was done following the ATS/IDSA criteria and the interpretation by the clinicians in charge of each case. In 41.9% (13/31) of the CPD patients, the isolated strain was considered as the cause of disease; 19.4% (6/31) were classified as having NTM disease in the past but had already finished their anti-NTM treatment regimen; 19.4% (6/31) were considered as colonizers; and 19.4% (6/31) were cases in which the NTM role was not clear. These unclear cases did not meet all ATS/IDSA criteria but clinically were considered as the cause of the disease and treated. The response to GPLs in samples from CPD patients in which the isolated NTM was considered as the cause the disease, was significantly higher to that of the ones considered as colonizers ($p<0.05$), active TB ($p<0.0001$) and healthy controls ($p<0.0001$) (figure 5B). Samples from the unclear group (disease or colonization) had a significantly higher response to GPLs than those of active TB patients ($p<0.05$). Interestingly, no significant differences were observed among the colonization, NTM disease in the past, active TB and healthy control groups (figure 5B).

The NTM species isolated in CPD patients' samples were mainly from the MAC (67.7%, 21/31) followed by *M. abscessus* (22.6%, 7/31) which was in 4 cases co-isolated with MAC. All cases considered as unclear (disease or colonization) had a MAC isolate. The majority of cases classified as previous NTM disease had a MAC isolate (83.3%, 5/6). The same happened in the group in which the NTM was considered as causing disease in which 61.5% of the cases (8/13) had a MAC isolate. None of the patients considered as NTM-colonized had an *M. abscessus* isolate. Other species were also isolated such as *Mycobacterium fortuitum* (in two cases considered as colonized), *Mycobacterium xenopi* (in one case considered as previous NTM disease), *Mycobacterium celatum* (in one case considered as colonized) and *M. chimaera* (in two cases, one considered as causing disease and the other as colonizing).

Positive results

The positive results obtained per group and subgroups of samples are detailed in table 2. Considering the CPD cases with NTM isolates, the majority of positive results were obtained in the group of patients where it was considered that the isolated NTM was causing disease (84.6%), while those considered as colonized and previously infected had the lowest amount of positive results (33.3% in each case). Regarding NTM positive lymphadenopathies, the majority of positive results for the NTM-IGRA was

obtained in those with a TST above 5 mm (56.3%). Regarding those cases with suspicion of being NTM infected, the majority had a positive NTM-IGRA results (76.9%). Importantly, none of the healthy controls nor patients with lymphadenopathies not caused by NTM had a positive result.

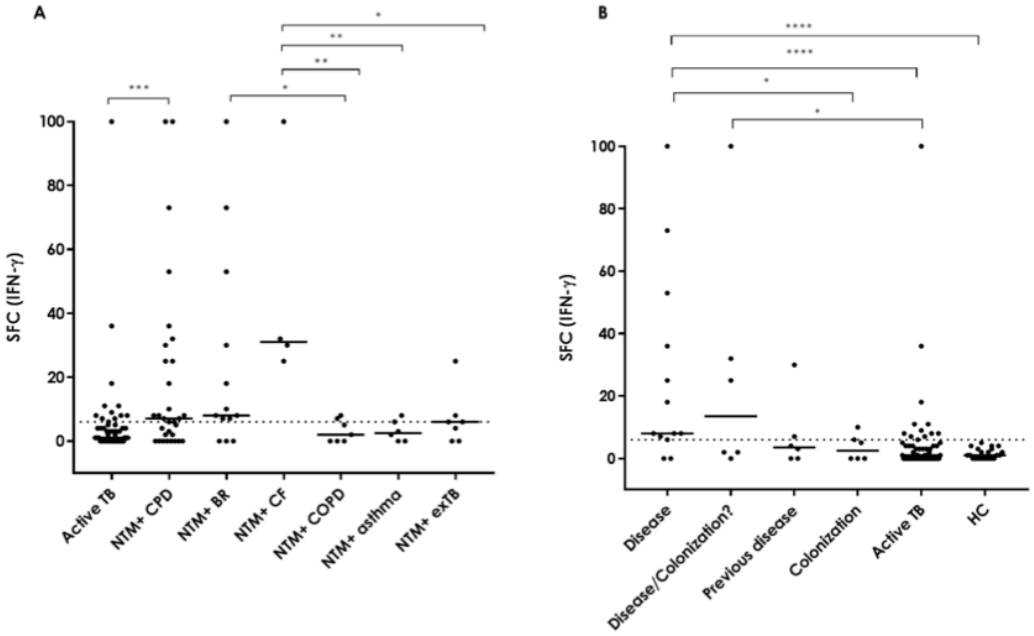

Figure 5. Response to GPL stimulation in samples from NTM positive CPD patients and active TB. A. *Regarding the type of CPD and compared to active TB.* Median values and IQR for each group were: 7 SFC (0 – 25) for the overall CPD cases, 8 SFC (3.5 – 41.5) for the bronchiectasis cases, 31 SFC (26.3 – 83) for the cystic fibrosis cases, 2 SFC (0 – 7) for the COPD cases, 2.5 SFC (0 – 6.5) for the asthma case, 6 SFC (0 – 8) for the previous active TB cases and 1 SFC (0 – 4) for active TB. There are 6 patients that are present in more than one group because they had more than one CPD. **B.** *Regarding NTM role as disease or colonization*. Median (IQR) values in SFCs for each group were: 8 SFC (6.5 – 44.5) in disease, 13.5 SFC (1.5 – 49) in the unclear disease/colonization group, 3.5 SFC (0 – 12.75) in previous disease, 2.5 SFC (0 – 7) in colonization, 1 SFC (0 – 3) in HC and 1 SFC (0 – 4) for active TB. Mann Whitney test was performed to compare between groups: *=p<0.05, **=p<0.01, ***=p<0.001 and ****=p<0.0001. Results refer to the SFCs in the GPL stimulation condition after response subtraction of the negative control. CPD: chronic pulmonary disease, BR: bronchiectasis, CF: cystic fibrosis, COPD: chronic obstructive pulmonary disease, exTB: previous/past active TB, HC: healthy controls, IQR: interquartile range. Dotted line indicates the 6 SFC positivity cut-off.

DISCUSSION

NTM cause a wide range of infections such as lymphadenopathies in children, disseminated infections in immunosuppressed individuals and pulmonary infections, which are not simple to detect nor handle. Distinguishing between TB and NTM infection in cases in which TB diagnostic tests are discordant due to the TST cross-reaction issues and establishing the clinical significance of NTM isolations in CPD patients (causing disease or colonizing), is key to avoid unnecessary treatment, and

therefore reduce antibiotic resistance, extra-costs, and side effects, and ensure a proper handling of the patients. Despite this great need, there is no available method that can enable such differentiation.

This study describes an NTM- IGRA that we have developed using specific NTM antigens, the GPLs, to detect NTM infection. To our knowledge, this is the first time that stimulation with GPLs and measurement of the immune response to them, has been used for NTM infection detection. Similarly to this study, in 2010 Latorre et al evaluated the use of *M. avium* sensitins for stimulation of PBMCs and detection of NTM infection in children with discordant TST and T-SPOT.TB results[31]. The results obtained were promising, determining that some positive TST could be in fact negative and that those cases could correspond to NTM infected children. However, there were cross-reaction issues and therefore, the use of more specific antigens (such as the GPLs) was necessary[32,33].

Table 2. Number of positive results (%) obtained per group after GPL stimulation. Cut-off used was 6 SFC.

Sample group	Positive results (%)	
NTM + (n=52)[a]	36****** (69.2)	
Lymphadenopathies (n=18)	15** (83.3)	
CPD (n=31)	18****(58.1)	
Diseased (n=13)		11** (84.6)
Previous NTM disease (n=6)		2*(33.3)
Colonized (n=6)		2*(33.3)
Unclear NTM role (disease or colonization) (n=6)		3 (50.0)
Disseminated infections (by *M. chimaera*) **(n=3)**	3 (100.0)	
Lymphadenopathies of unknown aetiology (n=31)	11* (35.5)	
TST<5mm (n=15)	2 (13.3)	
TST>5mm (n=16)	9* (56.3)	
Lymphadenopathies not NTM (n=3)	0 (0.0)	
NTM infection suspicion (n=13)	10 (76.9)	
LTBI (n=90) (2 indeterminate results)	24***** (26.7)	
Active TB (n=69) (1 indeterminate result)	12*** (17.4)	
HIV positive (n=52) (3 indeterminate results)	12*** (23.1)	
HC (n=22)	0 (0.0)	

[a]*M. avium, M. intracellulare, M. abscessus, M. chimaera, M. fortuitum, M. malmoense, M. xenopi*. The number of * indicates the number of positive results with a limit value (6 or 7 SFC).

When discriminating between TB and NTM infections, the higher response to GPLs in the NTM positive suspicion group compared to LTBI and healthy controls indicates that GPLs trigger a specific immune response in those patients infected with NTM. This would mean that (using the calculated cut-off of 6 SFC) 10 out of the 13 cases (76.9%) that had discordant LTBI diagnostic tests could be the result of NTM infection. Although there are LTBI cases that have a response to GPL above 6 SFC, their QFN-G-IT and/or T-SPOT.TB is also positive and it is possible that some of these patients are co-infected with NTM.

Considering HIV patients, 23.1% had a positive NTM-IGRA. As all patients, except two, had $CD4^+$ counts above 200, we do not consider the test to be hampered by a weak immune response. Given the health risk condition of this group of patients, determining whether they are co-infected by NTM is important in order to manage them properly, especially when suffering a decrease in CD4+ counts, the moment in which NTM could worsen the state of the patient.

Identification of NTM infection in children with lymphadenopathies is very important in order to differentiate between TB from NTM infections, and treat or not treat accordingly. Considering the high amount of positive NTM-IGRA results obtained in the NTM positive lymphadenopathy group, those lymphadenopathies of unknown aetiology with a positive NTM-IGRA could be due to NTM. Interestingly, those cases with lymphadenopathies of unknown aetiology with a TST induration above 5mm had a higher response compared to those with a TST induration below 5mm (not statistically significant).

As previously mentioned in the case of NTM infections, being able to distinguish between disease and colonization is of extreme importance in patients with CPD. In order to do this, the clinicians follow the American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) 2007 guidelines (ATS/IDSA)[3], which are based on clinical features, radiographic findings, and microbiological studies, and therefore not completely precise and often difficult to interpret. There is another set of criteria that have been suggested for the same purpose. According to Wolinsky, clinically relevant NTM isolations can differ from those that are not by (i) having a moderate or high bacillary growth, (ii) being isolated in several occasions, (iii) being isolated from sterile body sites (such as lung tissue), (iv) the isolated species, and (v) the patient's risk factors such as CPD[34]. These criteria have the same interpretation issues as the ATS/IDSA guidelines.

In this study samples from CPD patients were analyzed with the NTM-IGRA as a possible test that could help in the diagnosis of these patients and therefore aid their handling when making treatment decisions. So far, the results obtained are promising. Those subjects that were considered as having a disease by NTM yielded an 84.6% (11/13) of positive results while those considered as colonized or previously infected yielded 33.3% (2/6) of positive results each. In the case of the unclear CPD group (disease or colonization), given the high response obtained in CPD patients considered as having an NTM disease compared to the low response in those considered as NTM-colonized, we could argue that the three cases that have a positive NTM-IGRA in this group, may be, in fact, cases with disease caused by NTM.

The rationale behind the different response obtained in CPD patients with NTM-disease and NTM-colonization is not clear. In fact, the host's immune response to NTM infection, in general, remains poorly understood[35,36]. The clinical outcome of NTM infections, as occurs with other infections, is dependent not only on the pathogen (pathogenicity, bacterial load) nor the host (susceptibility, local

defence, immunity, risk factors) but on the interactions of both as well as environmental factors[37–39]. We hypothesize that when NTM are colonizing, there is low or no interaction with the systemic immune system and/or that the recognition by the immune system may be located intracellularly or on the basolateral surface and therefore, in colonizing-NTM the interaction with the immune system is low or inexistent while in infective-NTM causing disease, such interaction takes place or is stronger. This would also explain the very high response (over 100SFC) obtained in the cases with disseminated infections included in this study. The fact that the response against GPLs is attenuated in those CPD cases that have completed anti-NTM treatment, could mean that a lower bacillary load induces a lower immune response and could serve for treatment monitoring and patient recovery. More studies with a higher number of CPD patients per group, and accompanied by the study of cellular markers and cytokines, are needed in order to better evaluate this new diagnostic test and characterize the immune response related to disease and colonization.

As GPLs are not present in *M. tuberculosis* complex species and are specific for NTM, they have been previously described as candidates for the development of new serodiagnostic methods to detect MAC infection and differentiate them from TB infection[40–44]. However, they are based on the detection of antibodies against GPLs instead of stimulation based on these antigens and focused on infections caused by *M. avium* complex[40–44]. Matsunaga et al., for example, described the potential use of anti-GPL antibodies detection to distinguish between MAC infections and TB infection using sera from MAC infected guinea pigs[44]. In 2013, Shu et al. described a method to detect also MAC infection in lung disease patients by detecting serum immunoglobulin A (IgA) antibody against MAC-GPL[43]. These studies are promising however, none has been successfully applied into the clinical practice and in the case of CPD, there is no information about their ability to differentiate between disease and colonization. Additionally, the NTM-IGRA here described yielded positive results in cases with NTM isolates of various species and not only from MAC.

The main limitation of this study is the overlapping response present in patients with NTM isolate (both in lymphadenopathies and those considered as CPD with NTM causing disease) and those suspected of having NTM infection after LTBI screening (negative IGRA and positive TST despite no BCG vaccination). Differentiating between NTM causing disease and colonizing NTM seems to be possible in patients with CPD. However, in the suspicion NTM group, we would expect a different range of response that could be specific for sensitization. It is possible that different kind of patients (CPD, lymphadenopathies or asymptomatic) and different sites of infection (lung, lymph nodes or mere exposure) trigger different immune responses and therefore, further analysis using flow cytometry are needed in order to characterize the immune response in each case. This would give more information on whether memory cells, for example, are involved in order to explain sensitization after exposure and how this memory does not seem to have a role in CPD patients with previous disease caused by NTM and how treatment may affect such responses. Another limitation is that the classification of CPD

patients as NTM colonized or patients with disease caused by NTM are based on the ATS/IDSA guidelines, which, as mentioned previously, are difficult to interpret and not precise. This complicates the proper classification of patients and can cause mistakes when interpreting the results obtained by the NTM-IGRA.

The increasing frequency of NTM infections is a reality. A simple method to detect NTM infection and differentiate it from LTBI, and detect NTM colonization in CPD patients, is necessary to guide proper clinical decisions, avoid unnecessary treatment and improve the handling of patients. In this study, we test the use of GPLs for sample stimulation as a method to detect NTM infections and we consider that this newly developed NTM-IGRA could be a useful diagnostic tool to improve NTM diagnosis.

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CONFLICT OF INTEREST

Raquel Villar-Hernández, Irene Latorre, Jordi B Torrelles and José Domínguez are registered as inventors on a patent filed by Institut d'Investigació Germans Trias i Pujol, CIBER and Ohio State University, disclosing the use of GPLs for NTM infection diagnosis.

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Discussion

TB is the first cause of death worldwide due to a single infectious agent[13] and therefore disease management and control is an urgent need.

The studies included in this thesis are centered on the improvement of TB infection diagnosis by focusing on population at high risk of developing TB (such as patients with IMIDs and children) (articles 1-4), exploring new biomarkers for future assays (such as antigens and cytokines) (article 5), testing new methodologies (articles 6, 7 and annex 1), and developing a new diagnostic tool to detect NTM infection (article 8).

LTBI screening in individuals at high risk of developing active TB disease is highly necessary but evidence of the performance of current assays is still limited. The immune response in this type of patients could induce false-negative results due to not only their condition and the disease itself but also their treatment. Regarding patients with IMIDs, (articles 1 and 2), IGRAs and IP-10 detection seem to be a helpful LTBI diagnostic tool. The number of positive results obtained and the amount of T-cell response were not affected by clinical therapeutic profile, although this may depend on the type of IMID and the specific treatment. Individuals who suffer from Crohn's disease, for example, have a reduced number of positive TST and IGRAs results, as well as an increase of indeterminate results, than those with rheumatic diseases and psoriasis (article 1). This has also been previously described in other studies[136–142] suggesting that special care should be taken for these kinds of individuals, as a negative IGRA or TST result may be in fact false. To improve these assays and avoid false-negative and indeterminate results, other approaches such as longer in vitro stimulation, the recently introduced QFN Plus, or detection of other cytokines such as IP-10, could be of use[107,109,116,143–146] (article 2). IP-10 detection for LTBI diagnosis has been extensively studied in the past years and its usefulness in individuals at high risk of developing active TB disease, such as children and HIV infected patients, has been proven[113–117]. As discussed in article 2, detection of IP-10 in combination with the IGRA tests increases the number of positive results in rheumatic patients and these results were not affected by the treatment regimen. Although there is no certainty about whether these positive results are not false-positives (due to a lack of gold standard), detecting all the possible cases in this kind of at-risk population is of major importance especially when they are about to start biologic therapy.

In these populations at high risk, apart from diagnosing LTBI, indicators of TB progression would be of great use. However, up to now, no specific biomarker has been used for this purpose. The IGRAs despite the fact that they have a good negative predictive value, their positive predictive value is poor[147,148]. Changes in the amount of IFN-γ detected over time has been the subject of many studies regarding TB progression[58,148–153] however, no clear conclusion has been established as the IFN-γ fluctuation patterns do not seem to reflect specific stages. Recently a study focused on children, has however suggested that high IFN-γ conversion levels, and not only conversions and reversions *per se*, could serve as indicators for progression to disease, enabling better diagnosis and chemotherapy

decisions[154] (article 4). These findings should be further evaluated in order to consider a revision of the international guidelines on the use of IGRAs in children especially given the variability of IFN-γ levels over time.

Apart from new combinations of cytokine detection, sample stimulation with other *M. tuberculosis* antigens that could trigger the immune response differently, is of interest. In article 5 the addition of EspC, EspD, and Rv2348-B to the current QFN-G-IT antigen combination (ESAT-6, CFP-10 and TB7.7), was assessed. Their addition increased the number of positive results (specially EspC) compared to the QFN-G-IT, however, this increase in sensitivity was at the cost of specificity decrease as several uninfected individuals yielded positive results. Given their immunogenicity, these antigens have been studied together with CFP-10 and TB7.7 but in the absence of ESAT-6 in order to be used as a companion LTBI diagnostic tool in the case that the ESAT-6 vaccine is approved[155,156]. Apart from the antigens tested in this study, there are more promising antigens that could serve to identify not only LTBI cases and differentiate them from active TB but also characterize different stages of the TB dynamic spectrum[131–134,157]. Additionally, the evaluation of the immune response to these antigens is key not only for diagnostic purposes but also in the search on new TB vaccines[157–159].

The study of different cytokine patterns and use of new antigens should be further studied by characterizing the immune response at the cell level in search of specific cell subsets that produce certain cytokines and are stimulated by certain antigens. This approach could also give more information regarding the differentiation between active TB disease and mere infection, which current methods are incapable of doing. In article 7, surface receptors CD27 and CCR4 were studied in CD4+ T-cells showing that by evaluating the frequency of these two markers alone or in combination in specific *M. tuberculosis* CD4+ T-cells (IFN-γ^+ and/or TNF-α^+) discrimination between active TB disease and infection could be assessed. Active TB patients had a higher CD27 MFI ratio and their specific *M. tuberculosis* CD4+ T-cells CD27- and/or CCR4+ phenotype was also increased compared to LTBI individuals.

Another approach to step forward in the improvement of TB control is increasing the diagnosis reach. The difficult access to certain areas (especially rural areas) and long-distance from basic healthcare centres to specialized laboratories make it difficult or impossible to properly store and transport the samples (refrigerated or frozen plasma, for example) and therefore infected cases are missed. IP-10 detection in dried plasma on filter paper has been proven as a good alternative to fresh plasma in active TB patients[1] but until now, no studies included contacts. Despite a mild loss of positive results, the agreement between IP-10 detection in fresh plasma and in DPS is strong (article 6). Given the high production of IP-10, its stability in DPS, and the extensively studied good performance of IP-10 detection, this approach could serve in isolated and poor areas to increase TB infection diagnosis. This alteration to the traditional assay will make the sample suitable to be stored and transported without

refrigeration thus, enabling the identification of infected cases that otherwise would have remained undetected.

As mentioned several times throughout this thesis, proper LTBI diagnosis is key for TB control. Characterizing those BCG unvaccinated cases that have a positive TST and a negative IGRA and knowing whether they are infected by NTM or TB is important to avoid misleading TB infection estimations and unnecessary treatment, especially in children. In article 8, GPLs are described as good candidates for NTM infection diagnosis as they are specific of this type of bacteria. The evaluated assay is an IGRA test based on the ELISPOT and therefore quantifies the amount of cells that produce IFN-γ after PBMCs stimulation with the antigens overnight. In active TB and LTBI cases, the majority are negative (81.2% and 71.1%, respectively) and although there are some that are positive this probably means that they are simultaneously TB and NTM infected. Those BCG unvaccinated cases with a positive TST and negative IGRA, have also a significantly higher amount of cells producing IFN-γ than those with active TB and LTBI. Regarding the group of patients with lymphadenopathies, the majority of samples from patients with a positive NTM lymphadenopathy were positive for the NTM-IGRA (75%), suggesting that those lymphadenopathies of unknown aetiology with a positive NTM-IGRA could be also due to NTM; especially, those with a positive TST above 5mm.

Being able to detect NTM infections has also a very important role in patients suffering from CPD in which there is a positive NTM culture but the clinical relevance is unclear. Knowing whether that NTM is the causative agent of the infection or otherwise it is just colonizing is critical for the handling of the patient given that the treatment regimens will be different. Using GPLs to stimulate PBMCs from patients with CPD classified as NTM diseased or NTM colonized enables the differentiation between them, being higher the number of cells producing IFN-γ in those considered as NTM diseased. These are preliminary data that are being currently evaluated in a larger cohort and also by flow cytometry in order to understand better the role of each cell type in this process. In the meantime, we hypothesize that in an NTM colonization, interactions between bacteria and the systemic immune system are inexistent or very low, whilst when NTM are causing disease, such interactions do take place or are stronger.

These studies have some limitations which are subjected to be improved in further studies. The absence of a gold standard diagnostic test will undoubtedly be conditioning the results and conclusions obtained. However, this is the only available way to compare the new approaches with the ones currently in use. In the case of the articles 1 and 2, the variability on treatment regimens (DMARDs, corticosteroids and biologics) of the studied population added some difficulty when grouping the patients. For further studies, a wider number of patients with less therapy variability should be included. Regarding the study of IP-10 detection in DPS (article 7), the sample size is relatively small, which may compromise the assessment of the cut-offs. Although the transportation conditions used for this study did not interfere with the test results, it is unknown how other transport temperatures and timings may

affect such results. The main limitation of the NTM-IGRA study (article 8) relies on the classification of CPD patients in infection and colonization groups. As previously mentioned, this classification has been done following the ATS/IDSA guidelines and therefore some samples could be wrongly classified or not classified at all. To finish, a very important limitation and area of improvement of these studies is that when assessing host immunity we should keep in consideration that this response depends on a combination of factors that come from not only the host's genetics but also the pathogen and extrinsic factors[24]. It is very important to study all these factors together in order to fully understand the results obtained and be able to give more accurate conclusions about possible biomarkers, future diagnostic assays, and, possibly, specific individualized patient management.

By finding new specific *M. tuberculosis* antigens, studying new cytokine profiles and cell marker combinations, we get closer to understanding the complex TB immune response. Discovering biomarkers associated with bacterial load, treatment response or disease outcome will allow us to not only discriminate between disease and infection but also characterize the dynamic spectrum and predict those individuals that will progress to active disease.

Much is still to be done to win the fight against TB. Multisectoral efforts are needed to close the existing gaps in TB prevention, diagnosis and treatment. As stated in the WHO Report 2018, these efforts should not only address intensified research but also social and economic determinants and consequences of this disease, focusing on its elimination in low TB burden countries, especially in vulnerable groups in which the risk of infection and disease is high. The studies described in this thesis have increased the knowledge in the diagnostic field both of *M. tuberculosis*, as prioritized by the WHO, and NTM infections, getting closer to the necessary gap closing in this fields.

Conclusions

Individuals at high risk of developing TB

IGRAs are suitable for LTBI screening in patients with IMIDs about to start biologic treatment.

IP-10 detection is comparable to that of IFN-γ.

IP-10 detection in combination with IGRAs increases sensitivity of LTBI screening in patients with rheumatic diseases and therefore its inclusion in the current tests should be considered, especially in individuals at high risk of developing TB.

In the presented studies, treatment regimens (DMARDs, corticosteroids and/or biologics) did not affect the assessed in-vitro test results (QFN-G-IT, T-SPOT.TB and IP-10 detection). However, this could change regarding IMID and therapy type.

Considering the extensive amount of studies on different approaches to predict TB progression and given the importance of this matter in TB control, serial QFN-G-IT testing should be further evaluated for this purpose as high IFN-γ conversion levels may serve as indicators for progression to disease in children.

New antigens

Addition of EspC, EspF and Rv2348-B to the current stimulation combination used in the IGRAs (ESAT-6, CFP-10 and TB7.7) increases sensitivity although some combinations may affect the test's specificity.

Given their immunogenicity, EspC, EspF and Rv2348 are suitable for an ESAT-6 free diagnostic test in combination with CFP-10 and TB7.7 in case of a future ESAT-6 based TB vaccine or TST.

New methodologies - improvement of the current ones

Homing markers such as CD27 and CCR4 on *M. tuberculosis*-specific CD4+ T-cells gather the required features as potential TB biomarkers.

IP-10 detection in DPS is a good approach for LTBI screening in contacts for sites in which fresh plasma samples are difficult or impossible to store and transport refrigerated.

NTM infection

PBMCs stimulation with GPLs enables the detection of NTM infections.

Individuals with a confirmed NTM lymphadenopathy have a higher response against GPLs than those not caused by NTM.

Response to GPLs is higher in patients with TST positive and IGRA negative results than in patients infected with TB, suggesting that the majority of these patients are in fact NTM infected.

Patients with CPD that have an NTM isolation considered as producing disease have a higher response to GPLs than those considered as colonizers. This means that the developed NTM-IGRA could aid in the handling of patients with CPD that have an NTM isolation of unclear clinical relevance.

Altogether, the developed NTM-IGRA would avoid unnecessary treatment regimens and improve patient's handling.

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- Infographics and some of the figures present in this Ph.D. thesis have been created using Piktochart.

Annexes

Annex 1

Cell-mediated immune responses to in vivo expressed and stage specific *Mycobacterium tuberculosis* antigens in adult and adolescent latent and active tuberculosis.

Study done in collaboration with Tom H.M. Ottenhoff, Mariateresa Coppola and Krista van Meijgaarden from the Leiden University Medical Center (LUMC) in Leiden, The Netherlands

Cell-mediated immune responses to in vivo expressed and stage specific *Mycobacterium tuberculosis* antigens in adult and adolescent latent and active tuberculosis.

ABSTRACT

A quarter of the global population is estimated to be latently infected by *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB). TB remains the global leading cause of death of a single pathogen ranking among the top 10 causes of overall mortality. Current immune diagnostic tests cannot discriminate between latent, active and past TB, nor predict progression of latent infection to active disease. The only TB vaccine in use, Bacillus Calmette-Guérin (BCG), does not adequately prevent pulmonary TB in adolescents and adults, thus allowing continued aerosol-mediated transmission. Several *Mtb* proteins have been proposed as targets for new TB-diagnostic tests or -vaccines. However, the expression of these antigens during *Mtb* infection in the human lung has remained virtually unstudied. Moreover, most studies have relied on IFN-γ as main immunological read-out but we previously reported, in two small Northern European LTBI cohorts, that many *Mtb* antigens elicit other cytokine responses, including in the absence of concomitant IFN-γ production (Coppola *et al,* Sci Rep 2016).

Here, we (i) extend and validate the recognition of novel *Mtb* antigens, including *in-vivo* expressed *Mtb* (IVE-TB), latency and Rpf stage specific antigens, in an independent Southern European cohort, (ii) and demonstrate their immunogenicity in both adults and adolescents with either LTBI or TB. Furthermore (iii) we provide independent validation that these responses are marked by multiple cytokines, in addition to or even in the absence of IFN-γ immune responses. Interestingly, except for TNF-α, the magnitude of those responses appeared to be more pronounced in latent *Mtb* infected subjects. Finally, (iv) using unbiased analyses of high dimensional single cell data, 17 clusters of antigen specific TNF-α+ cells could be captured. T-cell clusters with a CD8+TEMRA or CD8+CD4+ phenotypes defined by the expression of multiple intracellular markers were found to be prominent in adult LTBI, while CD14+TNFa+ myeloid like clusters were most prominent in adolescent LTBI.

Our findings validate and extend the importance of evaluating immune responses using broader sets of parameters than IFN-γ alone, at an early stage of *Mtb* antigen discovery. Our results also provide proof of concept on how unbiased cell subset analysis by advanced multiparametric technologies and analysis platforms can identify less-defined cell subsets participating in the immune response against *Mtb*.

INTRODUCTION

Tuberculosis (TB) kills more than 1.6 million persons per year and is the most life-threatening disease caused by a single infectious agent, *Mycobacterium tuberculosis* (*Mtb*) (1). Although one fourth of the global population is estimated to be latently infected (2), none of the current immunodiagnostics can neither discriminate between present and past *Mtb* infection nor predict risk of possible TB progression (3, 4). Currently, the only licensed vaccine, Bacillus Calmette-Guérin (BCG), is insufficient to prevent active pulmonary TB, which propagates aerosol *Mtb* transmission in adults and adolescents (5). To boost or replace BCG, multiple TB vaccine candidates have been proposed and are being evaluated at different preclinical or clinical trial stages (6). Nevertheless, to date, only one subunit vaccine candidate, M72/AS01E, induced significant protection as it reduced the number of active TB cases in a 2 year follow-up cohort of latently *Mtb* infected (LTBI) adults (7). Although based on relatively low numbers of prevented cases as yet, the outcome of this phase IIb trial supports the value of *Mtb* antigen-based subunit TB vaccine development.

Ideally, *Mtb* antigens selected as targets of new TB vaccines should be expressed during active *Mtb* lung infection and efficiently trigger immune effector cells capable of controlling or clearing the infection without inflicting major tissue damage. Recently, we identified a new class of *Mtb* antigens, named IVE-TB, encoded by *Mtb* genes highly and consistently expressed in the lung of susceptible (C3HeB/FeJ) as well as resistant (C57BL/6J) mice following aerosol *Mtb* (Erdman) challenge (8, 9). Besides their high expression in *Mtb* infected lung, these IVE-TB proteins constitute an attractive group of candidate antigens for multiple other reasons: (i) they are conserved among 219 *Mtb* clinical isolates and thus cover a wide array of *Mtb* strains; (ii) they share high homology with BCG and other pathogenic mycobacteria, including *M. leprae*; (iii) they contain a large number of epitopes predicted to bind to HLA-Ia and HLA-II alleles (coverage of 85% of the human population); (iv) they are well recognized by immune blood cells from *Mtb* exposed subjects (as shown in 37 *Mtb* exposed individuals); and (v) they elicit immune cells that are producing multiple cytokines besides IFN-γ, which is known to be necessary but not sufficient in conferring protection against TB (9).

INF-γ has been used as the main readout to study other *Mtb* antigens than IVE-TB, such as latency antigens (DosR regulon encoded antigens, HBHA) and resuscitation-promoting factors (Rpfs) (10). Latency antigens are thought to be mostly expressed during latent stages of *Mtb* infection (11), while Rpfs proteins functionally appear to be required in the transition from a dormant into an active replicating state of *Mtb* (12). In support of this, several *Mtb* stage specific antigens were recognized more strongly by IFN-γ producing cells from LTBI than from TB patients and therefore have been proposed as novel *Mtb* antigen specific tools to differentiate latent *Mtb* infection from active TB disease (13-17). Additionally, although to only a limited extent, the recognition of some of these *Mtb* stage specific antigens has been assessed by immune parameters other than IFN-γ, including IL-12, IP-10, IL-10, TNF-α (17-22).

To date, the most extensively characterized cellular subsets participating in the response to *Mtb* antigens have been mono- or poly-functional CD4+ T cells producing IFN-γ, TNF-α, and/or IL-2 (23). However, it remains understudied which other cell subsets recognizing *Mtb* antigens may be involved in the overall response. Recent evidence for example has highlighted a role for NK cells in protective immunity to TB. In this study, we first validated the recognition of several recently defined *Mtb* antigens by multicomponent cytokine signatures in an independent cohort of LTBI and TB patients. The magnitude of those responses was stronger in latent *Mtb* infected subjects. Additionally, the use of high dimensional single cell data analysis revealed numerous clusters of antigen specific TNF-α+ cells, uncovering immunological heterogeneity in cellular subsets responding to *Mtb* antigens in different LTBI and TB age groups.

MATERIALS AND METHODS

Study setting and patients recruitment. In this study, whole blood samples were collected from 20 adults (age range=27-51) and 15 adolescents (age range=13-17) with pulmonary active TB (n=18) or latent *Mtb* infection (LTBI) (n=17) (Table 1). Active TB patients were determined by a compatible X-ray, positive *Mtb* culture and/or positive PCR. In adolescents the TB diagnosis was also supported by a positive TST and a known TB contact. LTBI cases were defined by a positive TST and/or QuantiFERON-TB Gold In tube (QFN-G-IT) test. Donors were recruited from four centers located in Barcelona, Spain: Germans Trias i Pujol University Hospital, Unitat de Tuberculosi Vall d'Hebron-Drassanes, Serveis Clínics TB Directly Observed Treatment Unit, Vall d'Hebron University Hospital, Sant Joan Despí Moises Broggi Hospital and Sant Joan de Déu Barcelona Children's Hospital. The study was approved by the Ethics Committee of the Hospital Universitari Germans Trias i Pujol (reference CEIC: PI-15-073) (http://www.ceicgermanstrias.cat/), also approved by the Ethics Committee of every participating centre that recruited cases, and performed following the relevant guidelines and regulations. For each sample a written informed consent was collected together with a detailed questionnaire about clinical and demographic data of the study participant.

Recombinant proteins. A total of 59 *Mtb* recombinant proteins, previously identified by different *Mtb* antigen discovery approaches (10), were tested in this study (Table 2). As described previously (9), *Mtb* genes were amplified by PCR from genomic H37Rv DNA and cloned by Gateway technology (Invitrogen, Carlsbad, CA, USA) in a bacterial expression vector containing, overexpressed in *Escherichia coli* (*E. coli*) BL21 (DE3) and purified. Gel electrophoresis and western blotting with an anti-His Ab (Invitrogen) and an anti-*E. coli* polyclonal antibody (a kind gift of Statens Serum Institute, Copenhagen, Denmark) were used to check the size and purity of the recombinant proteins. Rv0287-Rv0288, Rv2346c-Rv2347, and Rv3614-Rv3615 were produced as fusion proteins to mirror the pairwise

dependent secretion pathway followed by T7S systems. All recombinant proteins here included were previously tested to exclude protein-nonspecific T-cell stimulation and cellular toxicity (9).

Table 1. Demographic and clinical characteristics of all patients included in the study

	Overall (n=35)		Adults (n=20)		Adolescents (n=15)	
	TB (n=18)	LTBI (n=17)	TB (n=12)	LTBI (n=8)	TB (n=6)	LTBI (n=9)
Age, average (years) ± SD	32.7 ± 14.1	26.8 ± 13.5	41.1 ± 8.6	40 ± 6.1	15.8 ± 1.2	15.0 ± 1.0
Gender (%)						
Female	5 (27.8)	10 (58.8)	2 (16.7)	4 (50.0)	3 (50.0)	6 (66.7)
Male	13 (72.2)	7 (41.2)	10 (83.3)	4 (50.0)	3 (50.0)	3 (33.3)
Country of birth (%)						
High TB burden[a]	2 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)
Low TB burden	16 (88.9)	17 (100.0)	12 (100.0)	8 (100.0)	4 (66.7)	9 (100.0)
BCG vaccination (%)	11 (61.1)	5 (29.4)	7 (58.3)	5 (62.5)	4 (66.7)	0 (0.0)
Known TB contact (%)	4 (22.2)	14 (82.3)	2 (16.7)	5 (62.5)	2 (33.3)	9 (100.0)
TST >15mm (%)						
≥15mm (%)	5 (27.8)	10 (58.8)	1 (8.3)	5 (62.5)	4 (66.7)	5 (55.6)
<15mm (%)	5 (27.8)	5 (29.4)	3 (25.0)	1 (12.5)	2 (33.3)	4 (44.4)
Unknown/not tested	8 (44.4)	2 (11.8)	8 (66.7)	2 (25.0)	0 (0.0)	0 (0.0)
QFN-G-IT						
Positive	17 (94.4)	16 (94.1)	11 (91.7)	7 (87.5)	6 (100.0)	9 (100.0)
Negative	1 (5.6)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)
Unknown/not tested	0 (0.0)	1 (5.9)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)
Prophylaxis (%)						
< 30 days	-	14 (82.3)	-	6 (75.0)	-	0 (0.0)
> 30 days	-	1 (5.9)	-	1 (12.5)	-	8 (88.9)
Average (days) ± SD	-	22.9 ± 7.1	-	27.4 ± 8.2	-	19.0 ± 2.5
No prophylaxis	-	2 (11.8)	-	1 (12.5)	-	1 (11.1)
Anti-TB treatment (%)						
< 30 days	6 (33.3)	-	4 (33.3)	-	2 (33.3)	-
> 30 days	10 (55.6)	-	6 (50.0)	-	4 (66.7)	-
Average (days) ± SD	40.2 ± 17.0	-	37.6 ± 18.0	-	48.8 ± 33.2	-
No treatment	2 (11.1)	-	2 (16.7)	-	0 (0.0)	-

[a] About 150 incident cases per 100 000 population
Definitions of abbreviations: BCG = bacillus Calmette-Guérin; LTBI = latent tuberculosis infection; QFN-G-IT = QuantiFERON Gold In-Tube; SD = standard deviation; TB = tuberculosis; TST = tuberculin skin test.

Table 2. Tested antigens

List	Rv number	Function	Category
1	**Rv0066**		IVE-TB
2	Rv0287/Rv0288	EsxG/EsxH	IVE-TB
3	**Rv0383c**	Possible conserved secreted protein	IVE-TB
4	**Rv0423c**	ThiC	IVE-TB
5	Rv0440	GroEL2	IVE-TB
6	**Rv0467**	icl1	IVE-TB

7	**Rv0468**	FadB2	IVE-TB
8	**Rv0470c**	PcaA	IVE-TB
9	**Rv0501**	GalE2	IVE-TB
10	**Rv0640**	RplK	IVE-TB
11	**Rv0642c**	MmaA4	IVE-TB
12	**Rv0645**	MmaA1	IVE-TB
13	**Rv0824c**	Acyl-desaturase DesA1	IVE-TB
14	**Rv0826**	Conserved hypothetical protein	IVE-TB
15	**Rv0991**	Conserved serine rich protein	IVE-TB
16	**Rv1038c**	EsxJ	IVE-TB
17	**Rv1131**	PrpC	IVE-TB
18	**Rv1221**	SigE	IVE-TB
19	**Rv1284**	Beta-carbonic anhydrase	IVE-TB
20	**Rv1390**	RpoZ	IVE-TB
21	**Rv1479**	MoxR1	IVE-TB
22	**Rv1738**	Conserved hypothetical protein	IVE-TB/latency antigen
23	**Rv1791**	PE19	IVE-TB
24	**Rv1792**	EsxM	IVE-TB
25	**Rv1846**	BlaI	IVE-TB
26	**Rv1872**	IldD2	IVE-TB
27	**Rv1980c**	Mpt64	IVE-TB
28	**Rv2007**	FdxA	IVE-TB
29	**Rv2031**	HspX	IVE-TB
30	**Rv2215**	DlaT	IVE-TB
31	**Rv2245**	KasA	IVE-TB
32	**Rv2346c/ Rv2347c**	EsxO/ EsxP	IVE-TB
33	**Rv2382**	Polyketide synthetase mbtC	IVE-TB
34	**Rv2431c**	PE25	IVE-TB
35	**Rv2461**	ClpP1	IVE-TB
36	**Rv2626**	Hrp1	IVE-TB/latency antigen
37	**Rv2657c**	PhiRv2 prophage protein	IVE-TB
38	**Rv2710**	SigB	IVE-TB
39	**Rv2873**	Mpt83	IVE-TB
40	**Rv2941**	28	IVE-TB
41	**Rv3048c**	R1F protein	IVE-TB
42	**Rv3052**	FadB4	IVE-TB
43	**Rv3407**	VapB47	IVE-TB
44	**Rv3462**	IF-1 infA	IVE-TB
45	**Rv3583c**	Possible transcription factor	IVE-TB
46	**Rv3614/3615**	/EspC	IVE-TB
47	**Rv3615**	EspC	IVE-TB
48	**Rv3616***	EspA	IVE-TB
49	**Rv3846**	SodA	IVE-TB
50	**Rv3865***	EspF	IVE-TB
51	**Rv3874/Rv3875**	CFP10ESAT6	IVE-TB
52	Rv0867c	RpfA	Rpf
53	Rv1009	RpfB	Rpf
54	Rv1733c	Rv1733c	latency antigen
55	Rv2032	acg	latency antigen
56	Rv2034	Rv2034	IVE-TB
57	Rv2389c	RpfD	Rpf
58	Rv2450c	RpfE	Rpf
59	Rv3353c	Rv3353c	IVE-TB

Resuscitation-promoting factors (Rpf). Immunodominant. *patent

Diluted whole blood assay. Within 3 hours of collection, heparinized venous blood was diluted 1:10 in AIM-V medium (Invitrogen, Breda, The Netherlands). Samples were incubated (450 µl/well) in 48-well plates at 37°C, 5% CO_2, with 50 µl antigen solution (final concentration of 10 µg/ml). After 6 days, 200 µl of the supernatants were collected from each well and frozen in aliquots at −20°C until further analysis.

Multiple cytokine array and analysis of diluted whole blood supernatant. As it has been recommended by a cross-laboratory evaluation of multiplex bead assays, in this study we used one multiplex kit from the same manufacturer (24). The R&D TM premixed Multi-analyte kit (Cat #: 1415903) was used to measure the concentrations of eight analytes (IL-13, IL-22, IL-17A, IFN-γ, induced protein 10 [IP-10 (CXCL10)], IL-10, GM-CSF and TNF-α) in diluted whole blood culture supernatants according to manufacturer's instructions. To rule out batch effects, for each multiplex run samples with different disease status and age were always mixed. Data were acquired using Luminex 200 (Luminex Corp., Austin, TX) and analysed using Bio-Plex Manager software 6.0 (Bio-Rad Laboratories), as previously described (9). The median background values of unstimulated samples were: 14 pg/ml (GM-CSF, LTBI=14 pg/ml and TB=14 pg/ml), 47 pg/ml (IFN-γ, LTBI=47 pg/ml and TB=47 pg/ml), 3 pg/ml (IL-10, LTBI=3 pg/ml and TB=4 pg/ml), 5 pg/ml (IL-17A, LTBI=4 pg/ml and TB=5 pg/ml), 10 pg/ml (IL-22, LTBI=12 pg/ml and TB=9 pg/ml), 17 pg/ml (IP-10, LTBI=10 pg/ml and TB=20 pg/ml) and 3pg/ml (TNF-α, LTBI=3 pg/ml and TB=4 pg/ml). Values outside the upper (ULOQ) or lower (LLOQ) limits of quantification were set as the values of the analyte detection limits. Due to the high LLOQ (median=177 pg/ml), the values detected for IL-13 did not fall in the linear part of the standard curve and therefore were not further analysed.

IL-32 ELISA. To assess the IL-32 alpha concentration in supernatants, the DuoSet ELISA (R&D Systems, Catalog #: DY3040-05) was used according to the manufacturer's instructions. Samples were diluted 2-fold in reagent diluent (R&D Systems, Catalog #: DY995). The optical density (O.D.) was acquired by Varioskan Flash (Thermo Electron Corporation) using the SkanIt software version 2.4.1. Data were linearized by plotting the log of the human IL-32 concentration (pg/ml) versus the log of the O.D. and the fit line was determined by regression analysis using GraphPad Prism (version 7). The concentration read from the standard curve was multiplied by the dilution factor of 2. The median background value of unstimulated samples was 2 pg/ml (LTBI=2 pg/ml and TB=2 pg/ml).

Blood processing and Whole Blood Intracellular Staining (WB-ICS) assay. As previously described (25), venous blood was collected from 15 study participants in sodium heparin tubes and, within 60 min (26), 1 ml of blood was transferred into Sarstedt tubes containing either AIM-V medium, a pool of *Mtb* antigens (Rv1131, Rv2461 and Rv3616c), or PPD, in the presence of co-stimulants (anti-CD28 and anti-CD49d, each at 1-µg/ml, BD Biosciences, Eerembodegem, Belgium). The IVE-TB

proteins were tested at a final concentration of 10 μg/ml while PPD (Statens Serum Institut, Copenhagen, Denmark) was used at a final concentration of 5-μg/ml.

After 3h of incubation in a water-bath set at 37°C, Brefeldin A (3- μg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands) and Monensin (1:1000; BD Biosciences) were added. Samples were then transferred back to the water-bath for 12 extra hours, after which the water-bath switched-off automatically. Nine hours after the water-bath was switched-off, EDTA was added (2mM final concentration) and blood incubated for 15 min at room temperature in order to detach adherent cells. Erythrocytes were lysed and white blood cells fixed with FACS lysing solution (BD Biosciences). The fixed cells were pelleted and cryopreserved in 1ml of FCS with 10% dimethyl sulfoxide (DMSO).

Flow cytometry reagents. Fixed whole-blood samples were thawed in a water bath at 37°C for 2min and stained with a 14-colour FACS panel in permeabilization solution (Fix&Perm cell permeabilization kit, An Der Gurb BioResearch GMBH, Susteren, The Netherlands). The 14-colour FACS panel included: CD3-PE-TexasRed (clone S4.1) (TermoFischer), CD4-Pacific Blue (clone RPA-T4), CD28 PerCP-Cy5.5 (clone L293), CD8-PECy5 (clone RPA-T8), CD14-HorizonV500 (clone RPA-T8), IFN-γ-AlexaFluor700 (clone B27) (all BD Biosciences), CD45RA-Brilliant Violet 650 (clone HI100), CD27-Brilliant Violet 605 (clone O323), TNF-α-APC-Cy7 (clone MAb11), IL-10-PE-Cy7 (clone JES3-9D7), IL-13-PE (clone JES10-5A2) (all BioLegend), IL-17-A-FITC (clone eBio17B7), IL-22-PerCP-eFluor710 (clone 22 URTI) (all eBioscience), IL-32-allo-phycocyanin (APC) (clone 373821) (R&D). Samples were acquired on a BD LSRFortessa using FACSDiva as software (version 6.2, BD Bioscences) with compensated parameters.

Data analysis. Significant differences (p-value < 0.05) between cytokine levels in stimulated and unstimulated samples were evaluated by Mann-Whitney U test corrected for multiple comparisons (FDR, Benjamini-Hochberg test correction). The R package "phenotypicForest" was used to construct the polar histogram (27). After dividing by the background-values, i.e. AIM-V medium, for each response in each donor. Differences between cytokine levels in LTBI donors and TB patients were assessed by Mann-Whitney U test (p-value < 0.05) and $\log_2$ median fold-changes ($\log_2$LTBI/TB > 1 or <-1). Results are reported only for *Mtb* antigens that induced increased cytokine levels compared to the background-values.

Flow cytometry data were analysedd using FlowJo v10. Results from single-stained and unstained mouse/rat κ beads were used to calculate compensations. Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters. After debris exclusion, TNF-α+ cells were gated and FCS files of stimulated samples (antigen pool and PPD) were exported. FACS data were transformed using hyperbolic arcsin with a cofactor of 150 directly within Cytosplore^{+HSNE} (28). Then, for each donor and condition the gated events were randomly down-sampled to the lowest amount of TNF-α+ cells

found (n=153), to ensure equal representation of all samples. Next, a HSNE analysis was performed on a total of 3366 TNF-α+ cells after defining the markers used for the similarity computation (CD45, CD3, CD4, CD28, CD8, CD14, IFN-γ, CD27, IL-10, IL-13, IL-17A, IL-22, IL-32). We used the standard parameters for the hierarchy construction; number of random walks for landmark selection: N = 100, random walk length: L = 15, number of random walks for influence computation: N = 15, number of scale = 2. For any clustering that occurred the GMS grid size was set to S = 256 (28). The iterations chosen of the HSNE analysis were 1000. We clustered the data with a kernel size sigma of 30 on the overview level without manual modifications. Cell clusters were inspected using the integrated heatmap visualization. The median abundance (i.e. median cell frequency) of the cells composing each cluster was calculated per each group of donors and conditions (heatmap generated in Morpheus: https://software.broadinstitute.org/morpheus).

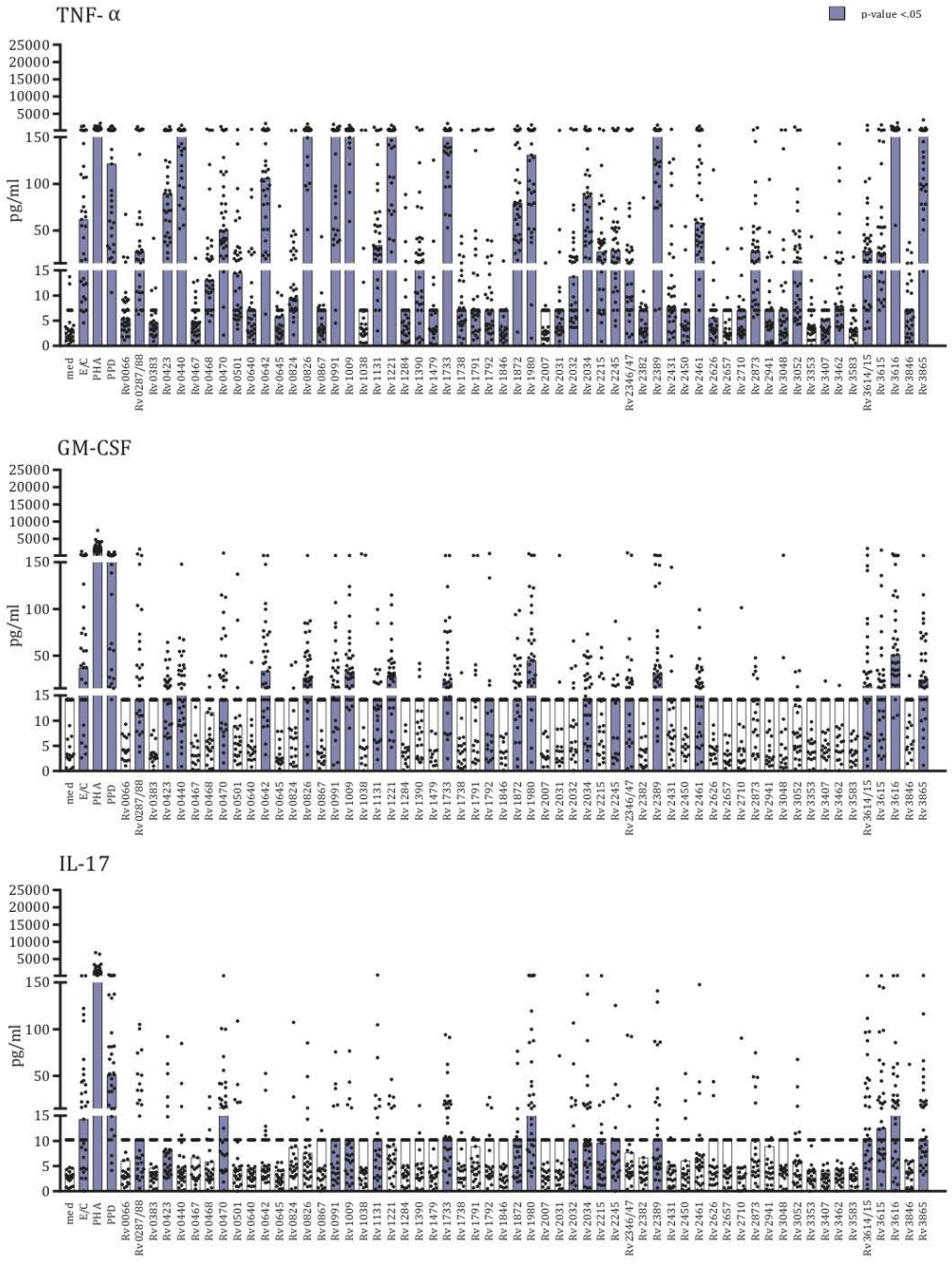
RESULTS

Several IVE-TB *Mtb* antigens, as well as *Mtb* DosR-regulon and Rpf antigens, are recognized by multiple-cytokine-producing blood cells of *Mtb* exposed subjects.

We recently described a new class of *Mtb* antigens, which we termed IVE-TB antigens and that were recognized by blood cells of *Mtb* exposed subjects not only by eliciting canonical IFN-γ but also alternative cytokines including GM-CSF, IP-10, IL-13, TNF-α and IL-17 (9). To formally and independently validate these findings, which were all derived from two small Northern European LTBI cohorts (n=37), we tested those antigens in a new Southern European cohort of 35 *Mtb* exposed individuals which included both LTBI and (for the first time also) TB patients (Table 1). In addition to IVE-TB proteins, we also included two known latency antigens (Rv1733c and Rv2032) and four resuscitation-promoting factors (Rv0867c, Rv1009, Rv2389c and Rv2450c), accumulating to a total of 59 *Mtb* proteins (Table 2). When measuring multicomponent cytokine signatures in the supernatants of diluted whole blood (incubated for 6 days with single or fusion *Mtb* antigens), we found secretion of five cytokines to be significantly increased in stimulated vs. unstimulated samples, namely TNF-α, GM-CSF, IFN-γ, IP-10 and IL-17A (Mann-Whitney U test with FDR multiple test correction) (Figure 1A). In agreement with our previous findings, not all antigens induced the same signature and number of cytokines (Figure 1A). However, most of the antigens studied predominantly induced TNF-α production. Interestingly, out of 59 *Mtb* proteins, 30 were significantly recognized by cells producing two to five cytokines not always including IFN-γ (Figure 1B).

Together these results extend, and most importantly, validate our previous findings in a completely independent cohort of donors, by demonstrating multi- rather than single- component cytokine signatures in the response to IVE-TB antigens, and for the first time also to DosR and Rpf stage-specific *Mtb* antigens. Importantly, we confirm immune recognition of *Mtb* antigens in the absence of detectable IFN-γ production in a significant number of cases (Figure 1B).

A


TNF- α
p-value <.05
GM-CSF
IL-17
pg/ml
25000
20000
15000
10000
5000
150
100
50
15
10
5
0
med
E/C
PHA
PPD
Rv0066
Rv0287/88
Rv0383
Rv0423
Rv0440
Rv0467
Rv0468
Rv0470
Rv0501
Rv0640
Rv0642
Rv0645
Rv0824
Rv0826
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Rv1284
Rv1390
Rv1479
Rv1733
Rv1738
Rv1791
Rv1792
Rv1846
Rv1872
Rv1980
Rv2007
Rv2031
Rv2032
Rv2034
Rv2215
Rv2245
Rv2346/47
Rv2382
Rv2389
Rv2431
Rv2450
Rv2461
Rv2626
Rv2657
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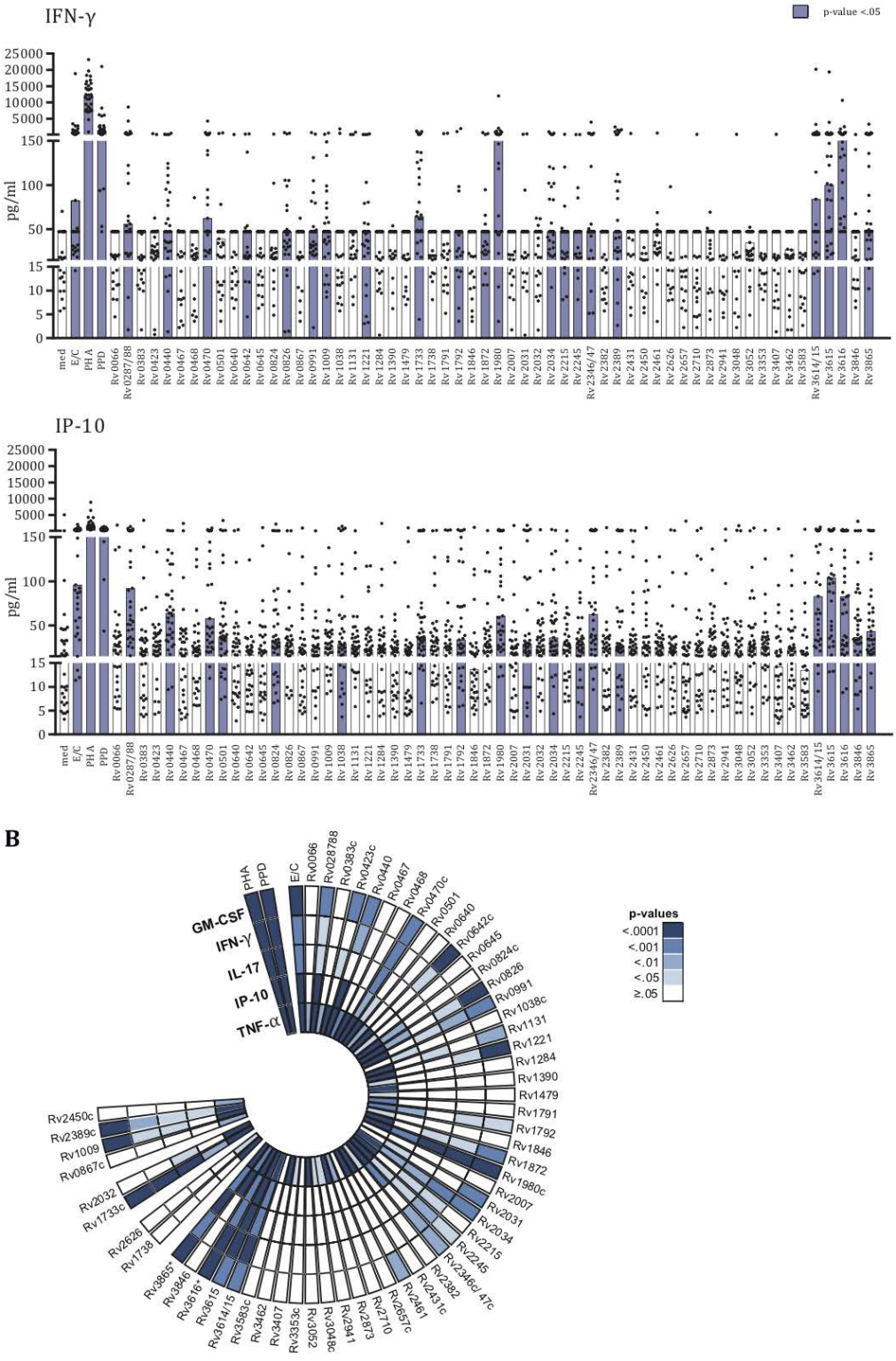


Figure 1. IVE-TB, as well as DosR and Rpf antigens, are recognized by multiple-cytokine-producing blood cells of *Mtb* exposed subjects. The levels of eight cytokines were measured in diluted whole blood supernatants from a cohort of *Mtb* exposed individuals (*n*=35) after 6 days stimulation with either IVE-TB, DosR, Rpf (10 µg/ml), control antigens ESAT6/CFP10 (E/C) (10 µg/ml), PPD (5 µg/ml), or the positive control, mitogen PHA (2 µg/ml). The statistical significance of the differences between cytokine levels in stimulated and unstimulated samples was evaluated by Mann-Whitney U test with FDR multiple test correction for IL-13, IL-22, IL-17A, IFN-γ, IP-10, IL-10, GM-CSF, IL-32 and TNF-α. (**A**) Results are shown only for TNF-α, GM-CSF, IP-10, IFN-γ and IL-17A for which significant differences were found among single *Mtb* antigens and unstimulated samples. Each dot represents a donor. Bars indicate medians. Purple bars indicate statistically significant increases in responses compared to the unstimulated samples with a p-value <0.05 (Mann-Whitney U test with FDR multiple test correction).

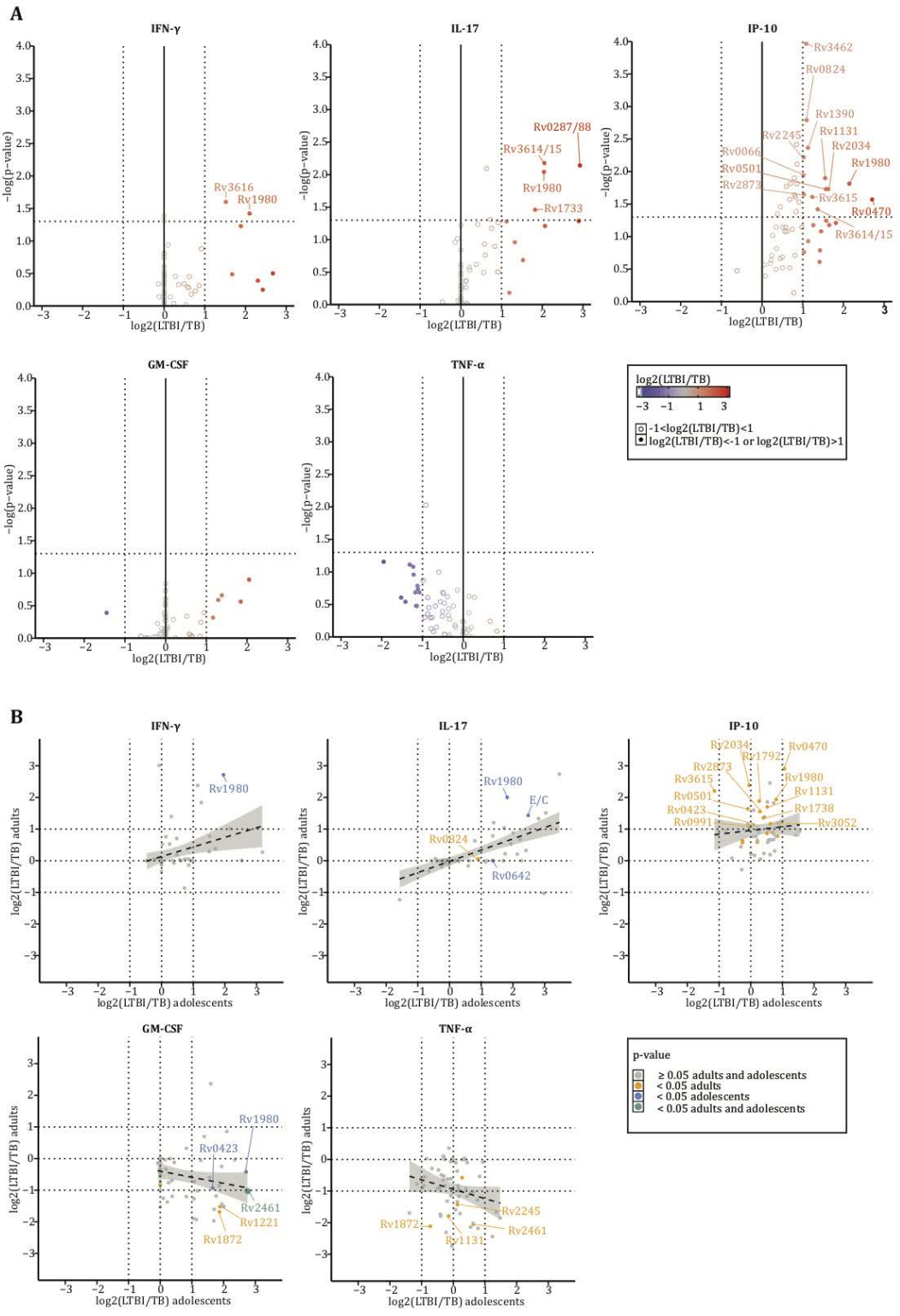
(**B**) The polar histogram displays the different p-values found among single *Mtb* antigens (or control) and unstimulated samples. Stimuli are ordered clockwise as follows: controls, IVE-TB antigens, IVE-TB/latency antigens, latency antigens and resuscitation-promoting factors. The different classes of antigens are separated by interruptions of the histogram.

LTBI and TB patients' blood cells secrete different amounts of cytokines in response to IVE-TB and stage-specific *Mtb* antigens.

Next, we assessed whether the *in vitro* cytokine profiles in response to the selected 59 *Mtb* antigens (Table 2) were similar in case of stimulated blood cells from LTBI subjects (n=17) or active (n=18) *Mtb* infection (Table 1). This comparison was performed for five cytokines, TNF-α, GM-CSF, IFN-γ, IP-10 and IL-17A, that had been found to be significantly increased in stimulated samples (Figure 1). Within each donor, prior to analysis, the concentrations detected in response to every antigen were normalized to the background-values, i.e. divided by the medium concentration, and $\log_2$ transformed. Cytokine differences were assessed by Mann-Whitney U test (significant for p-values < 0.05) and by $\log_2$ median fold-changes ($\log_2$FC(LTBI/TB) > 1 or < -1). Results described below are focusing only on the *Mtb* antigens that induced significantly different responses as confirmed by both p-values and $\log_2$ median fold-changes (Figure 2).

Of all 59 *Mtb* antigens, 16 were preferentially recognized by blood cells of LTBI donors (Rv0066, Rv0287/88, Rv0470, Rv0501, Rv0824, Rv1131, Rv1390, Rv1733, Rv1980, Rv2034, Rv2245, Rv2873, Rv3462, Rv3615, Rv3616 and Rv3614/15) (Figure 2A). Except for TNF-α, the cytokines detected in response to *Mtb* antigen stimulation were consistently found to be higher in blood samples from LTBI (Figure 2A). Interestingly, this was not identical upon subgroup analyses comparing adults (TB n=8; LTBI n=12) and adolescents (TB n=9; LTBI n=6) separately. Although IFN-γ and IL-17A were abundantly secreted by blood cells of LTBI donors independent of age (multiple R-squared derived by linear models: 0.1037 and 0.4192, for IFN-γ and IL-17), differences between LTBI and TB were less marked in adolescents for IP-10 (multiple R-squared derived by linear models: 0.009575); significantly higher in adult TB for TNF-α (multiple R-squared derived by linear models: 0.04714) and higher in adolescents for GM-CSF (multiple R-squared derived by linear models: 0.05153) (Figure 2B). Interestingly, these contrasting responses were consistent in response to stimulation with a wide array of specific antigens (Figure 2B), lending further validity to these observations.

Collectively, these data suggest that blood cells from LTBI and TB patients produce qualitatively and quantitatively different cytokines in response to stimulation with specific *Mtb* antigens. The data also suggest that these responses can differ by age groups.


A
IFN-γ
IL-17
IP-10
GM-CSF
TNF-α
−log(p−value)
log2(LTBI/TB)
Rv3616
Rv1980
Rv0287/88
Rv3614/15
Rv1733
Rv3462
Rv0824
Rv1390
Rv1131
Rv2034
Rv2245
Rv0066
Rv0501
Rv2873
Rv3615
Rv0470
log2(LTBI/TB)
-1<log2(LTBI/TB)<1
log2(LTBI/TB)<-1 or log2(LTBI/TB)>1
B
IFN-γ
IL-17
IP-10
GM-CSF
TNF-α
log2(LTBI/TB) adults
log2(LTBI/TB) adolescents
Rv1980
E/C
Rv0824
Rv0642
Rv2034
Rv1792
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Rv0501
Rv1738
Rv0423
Rv0991
Rv3052
Rv2461
Rv1221
Rv1872
Rv2245
Rv1131
p-value
≥ 0.05 adults and adolescents
< 0.05 adults
< 0.05 adolescents
< 0.05 adults and adolescents

Figure 2. LTBI and TB patients blood cells secrete different amount of cytokines in response to IVE-TB and stage specific antigens. The levels of nine cytokines were measured in diluted whole blood supernatants after 6 days stimulation with either IVE-TB, DosR, Rpf antigens (10 µg/ml), ESAT6/CFP10 (E/C) (10 µg/ml), PPD (5 µg/ml) or PHA (2 µg/ml). The cytokine responses were compared between LTBI donors (n=17) and TB patients (n=18). This comparison is displayed for five cytokines, TNF-a, GM-CSF, IFN-γ, IP-10 and IL-17A, which were found to be significantly increased in stimulated samples compared to unstimulated (Figure 1). Within each donor, prior to further analysis, the pg/ml values detected in response to every antigen were normalized to the background-values, i.e. divided by the medium, and then log_2 transformed. Results are reported only for *Mtb* antigens that induced increased cytokine levels compared to the background-values. (**A**) Cytokine differences were assessed by Mann-Whitney U test (significant for p-values < 0.05) and by log_2 median fold-changes (log2FC(LTBI/TB) > 1 or <-1). P-values and log_2 median fold-changes are reported on the y and x-axes, respectively. Thresholds for p-values and log_2 median fold-changes are indicated by dotted lines intersecting the axes (x:-1 and 1; y: 1.30103). Closed circles define when the log2FC(LTBI/TB) is >1 (red) or <-1 (blue). Open circles define when the log2FC(LTBI/TB) is >-1 and <1(grey). **(B)**Differences between LTBI and TB patients were analysed separately among adults (TB n=8; LTBI n=12) and adolescents (TB n=9; LTBI n=6). X and y-axes indicate the log_2 median fold-changes in adolescents and adults, respectively. The color-coded dots indicate p-values: ≥ 0.05 in adults and adolescents (grey), <0.05 in adults (orange), <0.05 in adolescents (blue) and <0.05 in adults and adolescents (green). Both in (A) and in (B), the Rv numbers are coupled to antigens recognised differently by LTBI and TB according to both p-values and log_2 median fold-changes.

Quantitative and qualitative differences in specific TNF-α+ responding cellular subsets between LTBI and TB patients.

To further characterize the functional blood cells involved in the recognition of *Mtb* antigens in a phenotype unbiased manner, we focused on TNF-α+ cells, since TNF-α was the most abundant cytokine found in the supernatant of diluted whole blood in response to *Mtb* antigen stimulation (Figure 1). To address this question in the same samples as used in the cytokine analyses, we performed intracellular cytokine staining on whole blood (ICS-WB) as previously described by others (25, 26, 29). Due to restriction in blood collection, the ICS-WB could be performed only on a limited number of donors (n=11). Samples were left unstimulated or stimulated for 21 hours with a pool of three *Mtb* antigens (Rv1131, Rv2461 and Rv3616c), that had been selected prior to commencing the study on the basis of previous results (9), or PPD, in the presence of co-stimulants (anti-CD28 and anti-CD49d). Interestingly, two antigens of the pool (Rv1131 and Rv2461) were among those recognised differently by LTBI and TB patients of different age groups in the cytokine screening (Figure 2B). After staining cryopreserved fixed blood cells with a 14-colour FACS panel, all TNF-α+ cells were enumerated in each sample (Figure 3A). Already after 12 hours of stimulation, TB patients had larger numbers of TNF-α+ cells (quantified by log_2FC(LTBI/TB)) in response to PPD compared to LTBI, after background correction (Figure 3B). The same trend was also observed in response to the *Mtb* antigen pool but only in the adult group, thus mirroring the amount of TNF-α protein secreted in the 6-day supernatants (Figure 2B).

We then selected and imported the TNF-α+ cells from all stimulated conditions (both the antigen pool and PPD) into Cytosplore, and analysed all data using hierarchical stochastic neighbour embedding (HSNE) (28). HSNE landmarks revealed a clear heterogeneity in the memory cell compartment, distinguishing central memory phenotype like subsets (CD45RA-CD27+CD28+) from effector (CD45RA+CD27+/-CD28+/-) and terminally differentiated effector (CD45RA+CD27-CD28-)

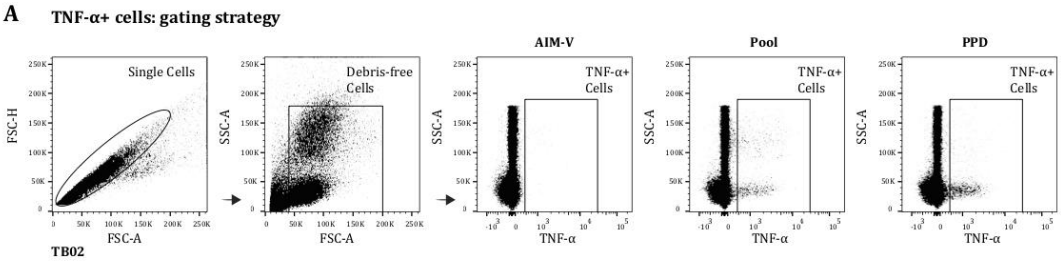
memory like subsets (Figure 3C). By applying a Gaussian mean-shift clustering to the flow cytometry data, 17 distinct cell clusters were defined by unique combinations of expressed markers. Hierarchical clustering of the heat map revealed two major groups, corresponding to the absence or presence of CD45RA. First, cells lacking CD45RA clustered in 3 main subpopulations: either co-expressing either CD14 but not CD3 (cluster 3, number of cells in the cluster (N) =310), or co-expressing CD3 and CD4 (cluster 4, N=352), or expressing IL-17 (cluster 12, N=235). A fourth CD45RA negative cluster, mostly characterized by high IL-32 and IFN-γ expression was also identified but since it was defined based on a low number of cells (cluster 17, N=22), it is not further described here, awaiting further confirmation. The second major group was characterized by a progressively higher expression of CD45RA, combined with differential expression of CD28 and CD27 molecules, co-expressing either CD4 (cluster 6, N=219; cluster 15, N=125 and cluster 4, N =352) or CD8 (cluster 8, N=92; cluster 9, N=93 and cluster 14, N=87) or both (cluster 2, N=196 and cluster 10, N =335) and several intracellular markers including IL-32, IL-17A, IFN-γ and IL-22 but not IL-10 and IL-13 (Figure 3D). The phenotype of three-cell clusters expressing IL-17 could not be fully captured and better defined by the markers included in our analysis (cluster 11, N=454; cluster 12, N=235 and cluster 13, N=88). Two cell clusters were only defined by different expression of CD45RA, CD27 and CD28 (cluster 16, N=87; cluster 7, N=302).

The proportion of cells (median relative abundance), derived from LTBI and TB, which contributed to the above cell clusters was different between adolescents and adults (Figure 3D). More specifically, in our analysis TNF-α+ CD8+ (CD3+CD4-) cells with a poly-functional (IL-22, IFN- and IL-32) terminal effector memory-like phenotype were mainly found in LTBI adults after *Mtb* antigen pool stimulation (clusters 8, 9 and 14, Figure 3D). On the contrary, TNF-α+ cells co-expressing CD14 (clusters 1, cells number=301; cluster 3 and cluster 5, cell numbers=68; Figure 3D) mostly originated from LTBI adolescents and were almost absent in samples from LTBI adults. Observed mostly in adolescent LTBI and TB patients were two TNF-α+ CD4 central memory/effector like clusters moderately co-expressing IFN-γ (clusters 4 and 6; Figure 3D). One cluster of TNF-α+ T cells co-expressing CD4 and CD8 and IFN-γ (cluster 10) was found to be more abundant in adults LTBI than in TB patients in response to PPD.

Despite the limited number of donors, these results support the presence of higher numbers of TNF-α+ cells in the blood of TB patients compared to LTBI in response to specific *Mtb* antigens (IVE-TB antigen pool or PPD) except for adolescent LTBI. In addition to these quantitative differences, our high dimensional analysis defined phenotypically and functionally different TNF-α+ cells in the blood of LTBI and TB patients enriching our knowledge of the existence of less defined, *Mtb* antigen-specific cell subsets.

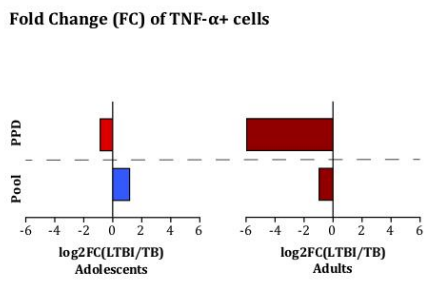
Collectively, our findings confirm and independently validate the recognition of recently described *Mtb* antigens in an independent South European cohort of LTBI and extend this also to TB patients. We further confirmed that these responses are defined by multiple cytokines besides IFN-γ and show

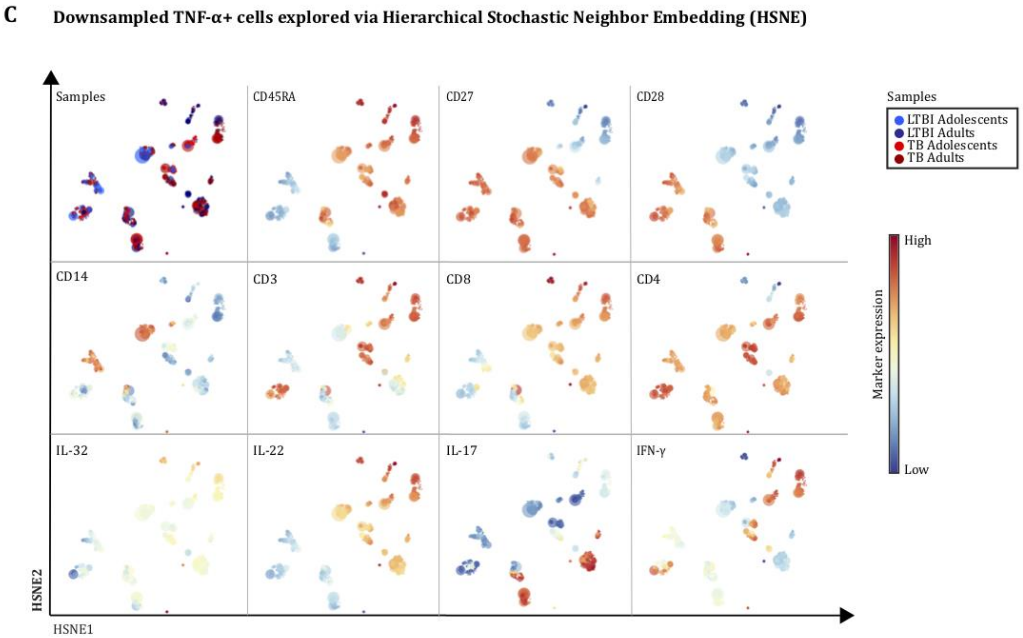
this to be the case both in adults and adolescents. Of note, except for TNF-α+, the magnitude of those responses was more pronounced during latent *Mtb* infection. Additionally, through unbiased analyses of high dimensional single-cell data, 17 clusters of antigen-specific TNF-α+ cells were defined revealing the presence of as yet poorly defined but phenotypically and functionally different *Mtb* antigen responsive TNF-α+ cells in LTBI and TB patients of different age groups.



Donors and absolute number of TNF-α+ cells

Code	Donor	Age	Number of TNF-α+ cells		
			AIM-V	Pool	PPD
TB02	LTBI	Adolescent	187	4002	2861
TB05	LTBI	Adolescent	192	1918	587
TB06	TB	Adolescent	68	1046	1675
TB41	TB	Adolescent	78	219	508
TB39	LTBI	Adult	134	181	703
TB72	LTBI	Adult	289	1023	1152
TB73	LTBI	Adult	138	214	1984
TB42	TB	Adult	86	289	689
TB43	TB	Adult	182	663	3834
TB45	TB	Adult	74	388	6123
TB47	TB	Adult	68	153	535





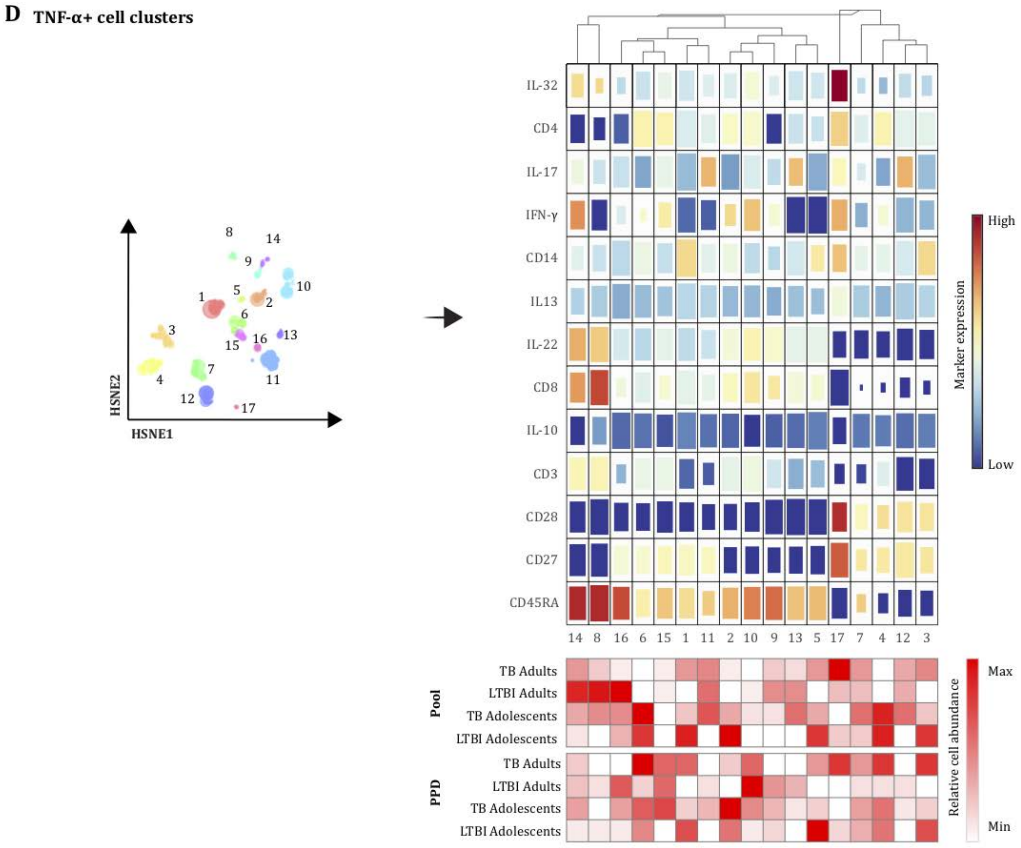


Figure 3. Quantitative and qualitative differences are present in specific TNF-a+ cell subsets between LTBI and TB patients. Intracellular cytokine staining was performed on whole blood (ICS-WB) of a limited number of *Mtb* exposed individuals recruited in this study (n=15). Samples were left unstimulated or stimulated for 12 hours with a pool of three *Mtb* antigens (Rv1131, Rv2461 and Rv3616c) or PPD, in the presence of (T-cell) co-stimulants (anti-CD28 and anti-CD49d). After staining cryopreserved fixed blood cells with an extensive 14-colour FACS panel, TNF-a+ cells were quantified in each sample. (**A**) The gating strategy is shown for one sample (TB02) to illustrate the selection of single debris-free TNF-a+ cells in unstimulated (AIM-V) and stimulated (antigen pool or PPD) sample. In the left upper table, the number of TNF-a+ cells is indicated for each donor. (**B**)The difference in the absolute number of TNF-a+ cells between LTBI and TB was defined by log_2 median fold change (log2FC(LTBI/TB)) and calculated separately in adolescents and adults. Blue or red bars represent log_2FC(LTBI/TB) > 0.5 or <-0.5, respectively. (**C**) For each of the 11 donors, TNF-a+ cells responding to the antigen pool and PPD were used as input in Cytosplore. Input cells were randomly downsampled to 153 events. A total of 3366 TNF-a+ cells was then explored via hierarchical stochastic neighbour embedding (HSNE). In the upper left HSNE plot, blue and red dots indicate cells isolated from LTBI or TB patients (colours tonality distinguishes adolescents from adults). In the other 11HSNE plots the coloured dots indicate the expression range of 11 singular cellular markers on the cells analysed (high expression = red, medium expression=yellow, low expression=blue). (**D**) By applying a Gaussian mean-shift clustering to the FACS data, 17 distinct TNF-a+ cell clusters (displayed in the HSNE plot) were defined by a unique combination of expressed markers as shown by the heatmap (upper right). Differences in the size of coloured squares indicate the contribution of each marker in defining the specific cell subset, while colours indicate the expression of cellular markers (high = red, blue = low). The lower right heatmap shows the proportion of cells composing each cluster per type of donors (age grouped LTBI and TB) and stimuli (PPD and antigen Pool). Numbers in the last row refer to the number of the cluster.

DISCUSSION

Mtb antigens selected as targets for TB diagnostic tests or TB vaccines need to be able to activate immune cells in *Mtb* exposed individuals (10). This has classically been studied, at least in humans, by measuring *Mtb* antigen-induced IFN-γ production in *in vitro* assays. While useful, sensitive and robust, clearly many other molecules are secreted by immune cells, often in the absence of IFN-γ. Our previous work showed that these molecules include GM-CSF, IP-10, IL-13, TNF-α and IL-17 (9). In the current study, we confirm and validate these findings in an independent cohort across different age groups and phases of *Mtb* infection (TB vs LTBI). These molecules all participate in the immune response against *Mtb* antigens, including several IVE-TB and stage-specific antigens. Next to quantitative differences in the cytokine production between LTBI and TB patients after *Mtb* antigen stimulation, interestingly, high dimensional unbiased single-cell data analysis defined multiple new, yet ill-defined clusters of TNF-α+ cells and also suggested distinct abundancy in cell subsets in LTBI and TB across different age groups in response to *Mtb* antigen stimulation.

Although IL-12/IFN-γ axis deficiency results in susceptibility to unusual mycobacterial infections (30, 31), the role of IFN-γ in TB resistance and immunopathology, especially in the lung, is not undisputed (4, 32, 33). In spite of this, most *Mtb* proteins have been proposed as candidate antigens almost exclusively on the basis of their recognition by IFN-γ producing cells obtained from latently infected individuals (LTBI) and TB patients (10, 17). Despite the fact that several *Mtb* proteins triggered a different type of cytokines and chemokines, including IL-12, IP-10, IL-10 and TNF-α, these broader immune responses have been examined almost only for candidates that had already been selected by prior IFN-γ screening approaches (17-22, 34). Here, in a new cohort of *Mtb* exposed individuals, we corroborated our previous finding on the added value of TNF-α, GM-CSF, IP-10 and IL-17A in the screening of putative *Mtb* antigens using an entirely non-IFN-γ centric approach (Figure 1).

As for IFN-γ, there is no unequivocal evidence defining IL-17A, GM-CSF and IP-10 as essential to controlling latent *Mtb* infection (35-39) or active TB disease (40-43). TNF-α, generally associated with tissue damage (34, 44), is also considered a key factor in TB granuloma integrity and protective immunity (45). In our cohort, the concentrations of GM-CSF, IL-17A, IFN-γ and IP-10 were higher in the stimulated blood cell supernatants from LTBI compared to TB patients (Figure 2A). If LTBI control *Mtb* infection better than TB patients (46), those *Mtb* antigens more strongly recognised by blood cells of LTBI correlate with, and potentially could contribute to the host immune response containing *Mtb*. This rationale has led to the identification of several TB vaccine candidates whose efficacy has been proven in animal models (47-49), but not yet tested in humans. It, therefore, remains to be seen whether there is a difference in efficacy between TB vaccine candidates based on antigens preferentially recognized by LTBI vs. TB patients (6, 7, 50-52).

Despite the fact that most of the cytokines in response to *Mtb* antigens were found more abundantly in LTBI, as expected by previous reports (34, 44) we found TNF-α responses to be higher in TB patients (Figure 2A). Interestingly, when analysing adults and adolescents separately, this difference was more pronounced in the adult group (Figure 2B). This different trend in cytokine secretion between the two age groups was also found for GM-CSF (Figure 2B). Adolescents and adults represent the priority target population for TB vaccination (53). However, the immune response of adolescents is not often analysed as a separate entity but merged with that found in either children (40, 54) or adults (29). The need for carefully considering different age groups is supported by the M72/AS01E efficacy trial results, which showed by far that the highest vaccine efficacy in preventing active TB among LTBI was in individuals of 25 years of age or younger (7). This observed age-effect on the M72/AS01E vaccine efficacy has been explained by possibly different timing of primary *Mtb* infection occurrence. Based on this reasoning, younger LTBI due to a more-recent primary *Mtb* infection would be less likely to have the infection under immune control and therefore could benefit more from boosting immune responses by the administration of M72/AS01E (7). In our cohort, IFN-γ producing cells were not compromised by active TB disease or age (or time of *Mtb* exposure), since blood cells of all groups were similarly effective in secreting IFN-γ in response to antigens present in the QuantiFERON-TB Gold (ESAT-6/CFP-10/TB-7.7(p4)) (Table 1) and to other specific *Mtb* antigens (Figure 1). However, such impairment could not be excluded for IFN-γ-independent immune mechanisms, such as for GM-CSF and TNF-α responses.

Finding age-related differences among TB and LTBI might suggest that either the abundance, the functionality or the phenotype of GM-CSF and TNF-α producing cells are heterogeneous in relation to age. To start addressing this issue we focused first on TNF-α+ cells since the levels of TNF-α were the highest detectable ones in the supernatants of dilute whole blood in response to antigen stimulation. Although the number of donors was limited (n=11), we found that TNF-α+ cells stimulated with three *Mtb* antigens (Rv1131, Rv2461, Rv3616c) or PPD were more abundant in TB patients than LTBI in adults (Figure 3A). An opposite trend was seen in adolescent LTBI samples when stimulated with the pool of *Mtb* antigens (Figure 3B). Of note, this pool contained two antigens (Rv1131 and Rv2461) recognised differently by LTBI and TB patients of different age groups in the cytokine screening (Figure 2B). Therefore, one could speculate that part of the differences found in the secreted cytokine profiles reflected the number of TNF-α+ cells recognizing the pool of *Mtb* antigens (Figure 3A).

More interestingly, by analysing high dimensional single-cell data (28), we observed additional differences in the phenotype and functionality of TNF-α+ cells between age groups. TNF-α+ cells co-expressing CD14 (Cluster 1, 5 and 3; Figure 3D) were mainly found in adolescent LTBIs after stimulation with the *Mtb* antigen pool or PPD while almost absent in adult LTBIs. This is interesting since the major source of TNF in human blood seems to be a subset of pro-inflammatory non-classical monocytes, which have recently been associated with recent *Mtb* exposure (38, 55).

In contrast, from the cells from adult LTBIs TNF-α+ cellular subsets were found to be CD8+ cells with terminal effector memory (TEMRA) like phenotype co-expressing IL-22, IL-32 and IFN-γ after *Mtb* antigen pool stimulation (Clusters 8, 9 and 14; Figure 3D). TNF-α+ CD8+ TEMRA cells have been already described in adult LTBI and suggested to play a role in antimicrobial activity against TB reactivation (56, 57). In one of these studies, it was elegantly demonstrated that anti-TNF therapy correlated with lower numbers of TNF-α+ CD8+ TEMRA cells and decreased anti-mycobacterial activity, that was reverted by the addition of TNF-α+ CD8+ TEMRA cells (56). Thus, it would be interesting to assess whether there is a link between the "protection-associated" TNF-α+ CD8+ TEMRA subset previously described and the ones found in our cohort. One subset co-expressing CD4 and CD8 with IFN-γ (cluster 10) was also mainly formed by cells originating from LTBI adults. Recently, a new subset of CD4 CD8 double-positive T cells able to produce cytokines and cytolytic markers has been identified in the blood, airways and lung granulomas of *Mtb* infected cynomolgus macaques. Additional studies would be required to clarify the role of those cells in the context of active and latent *Mtb* infection. Larger cohorts studied by advanced multiparametric technologies might help to resolve the cell heterogeneity, especially present in LTBI, found in our as well as in previous studies (58). High-dimensional cytometry analyses have been already harnessed to explore cells from differently exposed *Mtb* adolescents uncovering new cell subsets, including those expressing CD16+ and other populations defined as (NK) cells, CD27−CD8+ αβ T cells and B cells (59) and ILC3.

Despite the relatively low number of subjects included, our cell subset unbiased analysis reveals the presence of heterogeneous TNF-α+ cell subsets associated with responses to *Mtb* antigens and PPD. These responses could reflect the spectrum of *Mtb* infection and *Mtb* exposure, but their identity and exact function need further elucidation (60, 61).

In conclusion, our data validate our previous antigen discovery approach, support the value of assessing broader immune responses than IFN-γ alone at an early stage of *Mtb* antigen discovery, and show how in-depth unbiased profiling of functional cells recognising *Mtb* antigens can identify a yet ill-defined cell subset participating in the immune response against TB.

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Annex 2

Diagnostic accuracy of IP-10 mRNA release assay for tuberculosis

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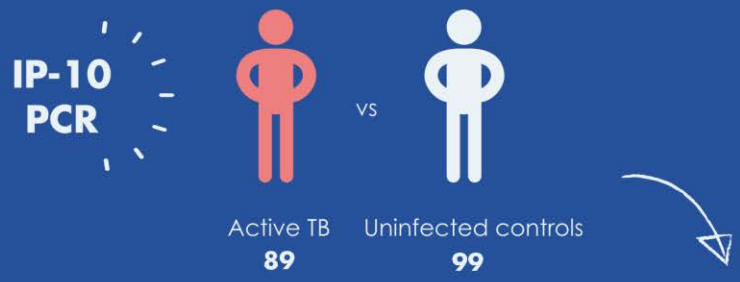

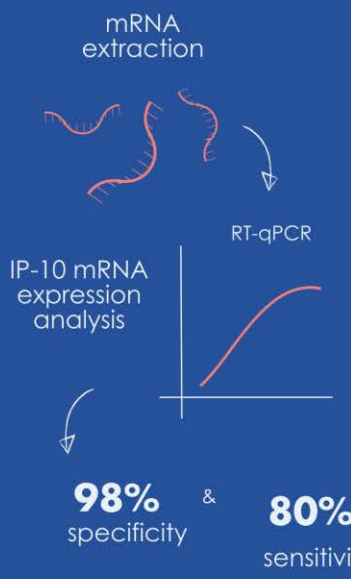

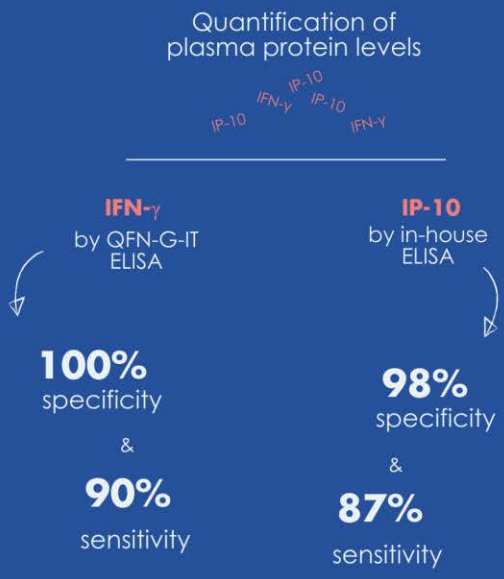

IP-10 mRNA detection assay is very robust molecular technique

As a diagnostic tool this assay would gain from further optimization especially on the kinetics of IP-10 mRNA expression

IFN-γ: Interferon gamma; IP-10: IFN-γ inducible protein 10; QFN-G-IT: QuantiFERON Gold In-Tube; RT-qPCR: Quantitative reverse transcription polymerase chain reaction; TB: tuberculosis.

Raquel Villar Hernández

Diagnostic accuracy of IP-10 mRNA release assay for tuberculosis

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Abstract

The current gold standard test for latent TB infection (LTBI) is the ELISA-based IFN-γ release assays (IGRAs). Although highly specific, IGRAs have a relatively high false-negative rate of approximately 20%. A more sensitive assay is needed. IP-10 is an alternative biomarker with a 100 fold high expression level compared to IFN-γ, allowing for different analysis platforms including molecular detection. The PCR technique is already an integrated tool in most TB laboratories and thus an obvious platform to turn to. In this case-control study, we investigated the diagnostic sensitivity and specificity of a molecular assay detecting IP-10 mRNA expression following antigen stimulation of a blood sample.

We included 89 TB patients and 99 healthy controls. Blood was drawn in QuantiFERON-TB (QFT) tubes. Eight hours post-stimulation IP-10 mRNA expression was analyzed and 20 hours post-stimulation IP-10 and IFN-γ protein plasma levels were analyzed using an in-house IP-10 ELISA and the official QFT ELISA, respectively.

The IP-10 mRNA assay provided a high specificity (98%) and sensitivity (80%) and AUC=0.97, however, the QFT assay provided a higher overall diagnostic potential with specificity (100%) and sensitivity (90%) and AUC=0.99. The IP-10 protein assay performed on par with the QFT assay with specificity (98%) and sensitivity (87%) and AUC=0.98.

We have provided proof of a very robust molecular assay detecting IP-10 mRNA expression. As a diagnostic tool, this assay would gain from further optimization especially on the kinetics of IP-10 mRNA expression.

Introduction

The 2016 United Nations Millennium Development goals boldly aim for a TB free world by 2035. Similar optimistic targets were recently proposed by the World Health Organization in the End TB strategy [1]. With 10.4 million new cases of TB annually, a surge in antibiotic resistance and an estimated 2-3 billion people already infected with *Mycobacterium tuberculosis* (*M.tb*) - reaching these optimistic goals will require innovation in treatment, vaccines and diagnostics as well as better use of the currently

available tools [2,3]. One such approach launched by the WHO was a policy change with a renewed focus on preventive treatment of latent *M.tb* infection in low income and high burden countries. Rational use of preventive treatment is an effective strategy to prevent progression to active TB in people at risk, however, the currently available tools have several limitations and new initiatives are needed [2].

Currently, preventive treatment is guided by tuberculin skin test (TST) and Interferon Gamma Release Assay (IGRA) testing. TSTs are in-vivo diagnostic tests measuring a delayed-type hypersensitivity response in the skin 48-72 hours after intradermal injection of antigens specific for mycobacteria. A major limitation of TSTs is cross-reactivity to the BCG vaccine and environmental mycobacteria rendering the test unspecific in many cases [4,5]. The IGRAs are in-vitro diagnostic alternatives to TSTs. These tests are based on stimulation of peripheral T-cells found in the blood with *M.tb* specific antigens and do not deliver false-positive results after BCG vaccination or exposure to most environmental mycobacteria [6–8].

However, a limitation to the use of IGRAs in low and middle-income countries is their dependence on trained laboratory personnel and specialized equipment for analysis and interpretation of the result. In terms of diagnostic performance, both IGRA and TST have reduced diagnostic sensitivity in young children and people with immunosuppression e.g. due to HIV infection, corticosteroid treatment and other immunosuppressant drugs, although IGRAs perform superior to TST in immunosuppressed individuals [5,9].

Interferon gamma-induced protein 10 (IP-10) is an alternative biomarker to IFN-γ in the IGRA test format [10]. IP-10 is expressed in concert with IFN-γ but at 100 fold higher levels [11,12]. The relative abundance of IP-10 allows for innovation in detection methods including lateral flow, dried blood spots and molecular methods not available for IFN-γ [12,13]. In addition, IP-10 seems to improve diagnostic performance by improving the detection of infection in children, HIV infected and rheumatic patients receiving immunosuppressant therapies [14–19].

We recently established proof-of-concept for a rapid molecular detection platform for IP-10 mRNA in the IGRA test format [20]. The aim of this study is to investigate the diagnostic sensitivity and specificity of this method in a case-control study conducted in a clinical routine lab.

Materials and Methods

Ethical statement

The study was approved by the ethical review board of the Ethics Committee of the Hospital German Trias i Pujol and subsequently for all the Ethics Committee of all the health care centres participating (reference number CEI_PI-15-073) and by the ethical review board of the Capital Region of Copenhagen (reference number H-3-2012-008). Written consent was obtained from all patients and controls enrolled in this study.

Study population

For this study, we enrolled 95 suspected tuberculosis patients through 5 different health care centres in Barcelona, Spain. Tuberculosis was confirmed by standard culture and/or PCR analysis in all but five participants, who were clinically diagnosed and had a relevant response to treatment. We further enrolled 100 healthy controls with no known exposure to *M.tb* at Statens Serum Institut in Denmark.

Whole blood stimulation and sample preparation

Whole blood was drawn in the Nil, TB Ag and Mitogen QuantiFERON-TB Gold tubes (QFT, Qiagen, Hilden, Germany) according to manufacturer's protocol. As the enrolment of the TB patients was performed at 5 different locations, the samples were transported to the main laboratory in a portable 37°C incubator. After arrival at the main laboratory, the tubes were transferred to a stationary 37°C incubator.

Enrolment of healthy controls was performed at the site of the laboratory so samples were placed directly in a stationary 37°C incubator. Whole blood incubated for 20 hours before plasma isolation. Plasma was stored at -20°C before analysis.

RNA extraction from whole blood

After 8 hours of incubation, 250µl whole blood from each tube was removed and total RNA was isolated using HighPure RNA isolation kit (Roche, Schlieren, Switzerland) according to manufacturers' protocol. In brief whole blood cells were lysed and total RNA were column purified. DNase step was included to limit contamination from DNA. Total RNA was eluted in 50 µl elution buffer and stored at -80°C. Due to the very small extraction of blood volume, it was not possible to do a contamination assessment (RNA/DNA) or a purity analysis.

Multiplex RT-qPCR

RT-qPCR was performed with the extracted RNA as template using primers and hydrolysis probes specific for target gene IP-10 (NM_001565.3) with β-Actin (NM_001101.3) as reference gene using the HawkZ05 Fast one-step RT-PCR kit (Roche Custom Biotech, Mannheim, Germany) as per manufacturer's protocol. In brief, 4 µl total RNA was used as template in a total reaction volume of 20 µl. Reaction mix contained a final Manganese Acetate concentration of 1.5 mM. The primer and probe sequences and concentrations are given: IP-10 forward: 5'-TGT CCA CGT GTT GAG ATC ATT G-3', IP-10 reverse: 5'-GGC CTT CGA TTC TGG ATT CA-3', 0.3 mM, 75 bp. IP-10 probe: FAM-5'-TAC AAT GAA AAA GAA GGG TGA GAA-39-MGB, 0.2 mM. β-actin forward: 5'-AGC CTC GCC TTT GCC GA-3', β-actin reverse: 5'-CTG GTG CCT GGG GCG-3', 0.5 mM, 174 bp, β-actin probe: HEX-5'-CCG CCG CCC GTC CAC ACC CGC C-3'-BHQ-1, 0.05 mM.

The RT -qPCR parameters for all targets were 5 minutes at 55°C, 5 minutes at 60°C and 5 minutes at 65°C for the reverse transcription step followed by 45 cycles of 10 seconds at 94°C and 40 seconds at

56°C. IP-10 and β-actin were analysed in multiplex and average Cq values were based on duplicate measurements. All samples were analysed on a Roche LightCycler 96 with default software (Roche, Basel, Switzerland). Primer and probe concentration and temperature optimization were performed on a Roche LightCycler 96 (Roche, Basel, Switzerland) as previously described[20]. The mRNA fold change was calculated using the ΔΔCq equation. All samples were measured on the same machine at one site to limit technical variability.

IFN-γ and IP-10 protein detection

IP-10 protein levels were determined in plasma samples using an in-house IP-10 ELISA assay in a 30 times dilution as described previously[17]. IFN-γ levels were determined using the QuantiFERON-TB ELISA (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Statistics

Differences in responses were compared using Mann-Whitney t-test, diagnostic accuracy using Receiver operating characteristic (ROC) curves using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Study population

We included 95 patients with TB in Spain and 100 healthy controls in Denmark. Six patient samples were excluded from the final analysis: 4 due to technical issues with the PCR (unexplained difference in reference gene expression), 1 patient was included twice (only the first sample was included) and finally 1 patient was not considered to have TB as a final diagnosis. One control sample was excluded due to technical issues with the PCR. This rendering a final cohort of 89 TB patients and 99 controls (table 1).

Five patients considered TB cases were negative in both, culture and/or PCR, and 1 had a positive culture for *M. kansasii*. This last donor was kept in the final analysis as M.*tb*. and M. *kansasii* both contain ESAT-6 and CFP-10, the two major antigens in the QFT test.

We found no difference in age between the two groups, however, the patient group was more ethnically diverse and included more males (72%) compared to the controls. Among the patients, four (5%) were HIV seropositive and three (3%) were receiving immunosuppressive treatment or having other immune-suppressive co-morbidities. In both groups, approximately half the enrolled participants were BCG vaccinated. Of the 89 TB patients, 81 (90%) tested QFT positive, whereas all controls tested negative.

IFN-γ and IP-10 protein biomarker levels

Antigen-specific IFN-γ and IP-10 protein levels were significantly higher in TB patients compared with healthy controls (figure 1). Mitogen specific release was similar for IP-10 between cases and

controls. This comparison was not possible for QFT as most participants had IFN-γ release at levels overshooting the upper limit of the assay (10IU/ml) (table 2 and fig S1A-B). Background levels (Nil) were significantly higher in TB patients than in controls for both IFN-γ and IP-10 (table 2 and fig S1D-E).

Table 1. Demographic and clinical data of the study participants.

		TB patients	Controls
Participants, n		89	99
Age, (years)		40.6 ± 14.4	41.8 ± 10.7
Males, n		65 (72%)	30 (30%)
Ethnicity, n (%)			
	European	46 (52%)	95 (96%)
	African	16 (18%)	0 (0%)
	Asian	15 (17%)	4 (4%)
	South America	9 (10%)	0 (0%)
	Not announced	3 (3%)	0 (0%)
PCR/culture-positive, n (%)			
	Positive	83* (93%)	0 (0%)
	Negative	5 (6%)	0 (0%)
	Not done	1 (1%)	99 (100%)
In treatment, n (%)			
	Yes	77 (87%)	0 (0%)
	No	12 (13%)	99 (100%)
	Not announced	-	0 (0%)
Mean time of treatment (days)		22,6	-
Previous TB infection, n (%)			
	Yes	12 (13%)	0 (0%)
	No	64 (72%)	99 (100%)
	Unknown	13 (15%)	0 (0%)
Previous TB treatment, n (%)			
	Yes	8 (9%)	0 (0%)
	No	54 (61%)	95 (96%)
	Unknown	27 (30%)	4 (4%)
BCG vaccinated, n (%)			
	Yes	35 (39%)	45 (45%)
	No	27 (30%)	42 (42%)
	Unknown	27 (30%)	12 (12%)
HIV infected, n (%)			
	Yes	4 (5%)	0 (0%)
	No	84 (93%)	96 (97%)
	Unknown	1 (1%)	3 (3%)
Other immunosuppressions, n (%)			
	Yes	3 (3%)	-
	No	86 (97%)	-
	Unknown	0 (0%)	-
QFT, n (%)			
	Positive	80 (90%)	0 (0%)
	Negative	9 (10%)	99 (100%)
	Indeterminate	0 (0%)	0 (0%)
Close contact w TB patient, n (%)			
	Yes	8 (9%)	5 (5%)
	No	73 (82%)	88 (89%)
	Unknown	8 (9%)	6 (6%)

*Considering at least 1 of the tests positive.

IP-10 mRNA expression

IP-10 mRNA expression levels were determined in an aliquot of the same sample used for protein detection isolated after 8 hours incubation. As with the protein-based assays, we found a significant difference between TB patients and healthy controls (figure 2 and table 2).

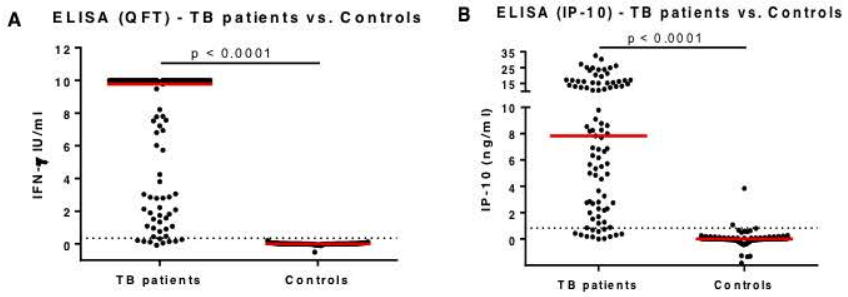

Figure 1. IFN-γ and IP-10 plasma release in TB patients and healthy controls. Whole blood samples from 89 TB patients and 99 healthy controls was stimulated 20 hours in QFT tubes and IFN-γ and IP-10 plasma levels were measured. **A)** IFN-γ release was determined using official QFT ELISA. Dotted line represents cut-off (0.35IU/ml). **B)** IP-10 plasma release was determined using an in-house IP-10 ELISA. Dotted line represents calculated cut-off (0.81pg/ml). Data represented are background subtracted (Ag-Nil) and median values are indicated in red. Statistics was a Mann-Whitney test.

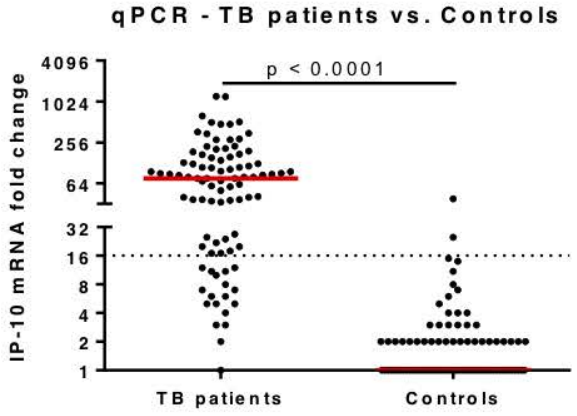

Figure 2. IP-10 mRNA expression in TB patients and healthy controls. Whole blood samples from 89 TB patients and 99 healthy controls was stimulated 8 hours in QFT tubes and total RNA was extracted. IP-10 mRNA expression was evaluated using our in-house RT-qPCR. Dotted line represents calculated cut-off (16 fold change). Median values are indicated in red. Statistics was a Mann-Whitney test.

Technical performance qPCR

The repeatability of the qPCR assay was very high with SD < 0.5 in more than 97% of duplicate measurements, which fell within the linear range of the IP-10 and ACTB assays (table S1 and figure S2A-B). The reproducibility of the assay is also very high; comparable average PCR efficiency for target

gene (IP-10) and reference gene (β-actin) at 108% ± 4% and 99% ± 5% and with a calibration curve r^2 of 1.00 and 0.99 across 42 individual runs of the 5-point standard curve (table S1 and figure S2C-D).

Table 2. Median (interquartile range) and p values obtained by each test.

	TB patients	Controls	p value
Subjects n	89	99	
ELISA (QFT)			
Nil*	0.19 (0.15-0.31)	0.03 (0.02-0.08)	<0.0001
TB antigen#	9.77 (2.08-10.00)	0.00 (-0.01-0.01)	<0.0001
Mitogen#	10.00 (10.00-10.00)	10.00 (10.00-10.00)	0.022
ELISA (IP-10)			
Nil*	0.32 (0.16-0.70)	0.12 (0.04-0.26)	<0.0001
TB antigen#	7.83 (2.69-15.20)	0.00 (-0.04-0.06)	<0.0001
Mitogen#	9.71 (5.67-16.70)	9.65 (7.78-12.96)	0.646
qPCR (IP-10)			
TB antigen	76 (20-153)	1.25 (0.76-1.95)	<0.0001
Mitogen	91 (37-230)	54 (22.09-97.68)	0.005

ELISA (QFT) data are presented as median concentration in IU/ml (interquartile range), ELISA (IP-10) data are presented as median concentration in pg/ml (Interquartile range) and qPCR (IP-10) are presented as median fold change (interquartile range). *: Unstimulated; #: QFT TB-antigen/Mitogen tube stimulated subtracted Nil.

Correlation between IFN-γ and IP-10 and IP-10 protein and mRNA

The correlation between the IFN-γ and IP-10 protein levels in the TB patients was good (spearman r= 0.80). After omission on the control group (with almost all data points centred around 0 positively biasing the correlation) the correlation remained unaltered (r = 0.80 (figure 3A)). The correlation between IP-10 mRNA and protein was poor in the patients (r = 0.43) but better when including the controls (r = 0.79) (figure 3B).

Diagnostic performance of IP-10 assays

Diagnostic performance of the two protein assays was evaluated using Receiver Operating Characteristics (ROC) curve analysis (figure 4). Cut-offs for IP-10 assays were determined at the level which rendered the test not more than 2% less specific than QFT. The IP-10 protein assay rendered an AUC at 0.98 and suggested a cut-off at 0.83ng/ml with 97/99 (98%) specificity and 78/89 (87%) sensitivity. The cut off for the mRNA based IP-10 assay was best at 16 fold upregulation, with an AUC at 0.97, rendering a 97/99 (98%) specificity and 72/89 (80%) sensitivity (table 3 and figure 4). The concordance of the two protein assays was very good with only 5/89 (6%) and 2/99 (2%) discordance for TB patients and controls respectively (table 4). The concordance between the QFT assays and the IP-10 mRNA assay was lower than the protein assays with 15/89 (17%) and 2/99 (2%) samples

discordant for TB patients and controls, respectively. Finally, the discordance between the two IP-10 assays was also lower than the two ELISA assays with 14/89 (16%) and 4/99 (4%) of the samples being discordant in TB patients and controls, respectively (table 4). None of the assays had indeterminate results.

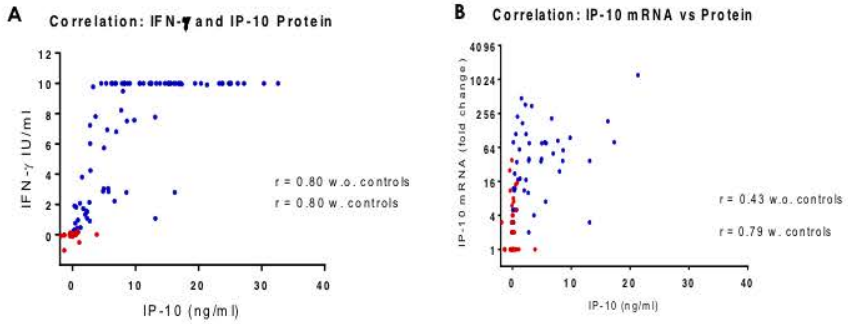

Figure 3. Correlation analysis between IFN-γ and IP-10 protein and IP-10 protein and mRNA. **A)** Correlation between plasma IFN-γ and IP-10 protein levels. TB patients and healthy controls are plotted together (n=146). Due to the maximum limit of 10 IU/ml in the IFN-γ ELISA, all samples at this level (n=42) have been omitted from the analysis however they are not omitted from the graph. **B)** Correlation between plasma IP-10 protein and mRNA expression. TB patients and healthy controls are plotted together (n=188). Red dots are healthy controls, blue dots are TB patients. Spearman correlation analysis was performed with and without healthy controls to disclose any bias of the healthy control samples.

Table 3. Results obtained by each test.

	ELISA (QFT)	ELISA (IP-10)	qPCR (IP-10)
Cut-off	0.35IU/ml	0.83pg/ml	16 fold change
TB patients (n=90)			
Positive	80/89 (90%)	77/89 (87%)	71/89 (80%)
Negative	9/89 (10%)	12/89 (13%)	18/89 (20%)
Indeterminate	0/89 (0%)	0/89 (0%)	-
Controls (n=99)			
Positive	0/99 (0%)	2/99 (2%)	2/99 (2%)
Negative	99/99 (100%)	97/99 (98%)	97/99 (98%)
Indeterminate	0/99 (0%)	0/99 (0%)	-

AUC (ELISA: QFT): 0.99, AUC (ELISA:IP-10): 0.98, AUC (qPCR:IP-10): 0.97. Indeterminate for ELISA: IP-10 is set to Mit-Nil < 0.5ng/ml. Indeterminate for qPCR: IP-10 is not applied due to assay setup.

Discussion

In this study, we evaluated the diagnostic potential of a molecular IP-10 release assay for detection of infection with *M.tb* in a case-control study. The molecular assay was compared to an IP-10 protein-based IGRA and the QuantiFERON-TB. We found that, at the protein level, the QFT and the IP-10 release assays performed comparably, however, the sensitivity of the molecular assay was significantly lower compared to QFT.

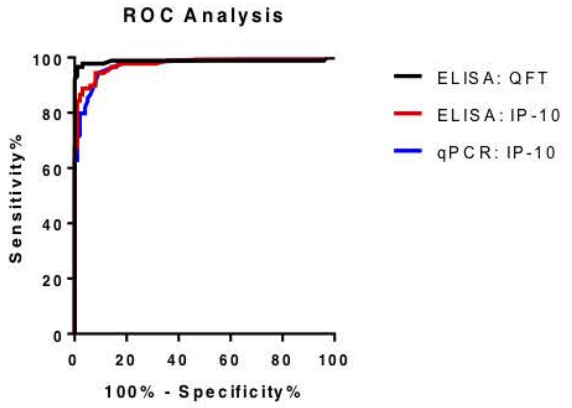

Figure 4. ROC curve analysis comparing the three assays; QFT (ELISA), IP-10 (ELISA), IP-10 (RT-qPCR). The analysis included all 89 TB patient and all 99 healthy control samples. The area under the curve (AUC) reflects the diagnostic potential of the individual assays. AUC for QFT (ELISA) 0.99 (95% CI 0.97-1.01), AUC for IP-10 (ELISA) 0.98 (95% CI 0.96-1.00) and AUC for IP-10 (RT-qPCR) 0.97 (95% CI 0.95-0.99).

IP-10 is emerging as a more versatile and potentially superior immunodiagnostic marker compared to IFN-γ as demonstrated in multiple studies using various assay platforms including ELISA, lateral flow, dried blood spots and molecular technology [11,12,17,21]. In the IGRA test format, IP-10 is expressed in concert with IFN-γ when the specific T cells recognize *M.tb* specific peptides presented on the MHC molecules on the surface of the antigen-presenting cells (APCs). The mechanisms triggering IP-10 release from the APCs involve cell surface receptor interactions at the immunological synapsis and detection of cytokines (IFN-γ, TNF and others) from the adjacent T cells [12]. The reason for the difference in magnitude of release probably stems from the fact that IFN-γ exerts its role as signal molecule in the immunological synapsis between T cell and APC, whereas IP-10 is a chemoattractant meant for long-distance signalling in the tissue by creating an IP-10 gradient for T cells to migrate along.

As expected from the immunological interdependence of IP-10 and IFN-γ, we found highly correlated release at the protein level, also confirming findings in other studies[17]. However, we were surprised to see the lower agreement and inferior diagnostic performance with the molecular assay in the TB patients. By isolating mRNA in an aliquot of the exact same blood sample at 8 hours during the 20 hour incubation period, we had designed the study to limit between-sample variability. However, given the fact that we were able to detect IP-10 at the protein level after completion of the incubation in the exact same sample, it seems likely we mistimed the mRNA sampling at the 8 hour time point.

In a previous study we have shown that after stimulation with TB antigenic peptides, the mRNA expression is a relatively rapid event with expression undetectable in the first couple of hours. This is followed by an exponential increase to often several hundred fold upregulation with a subsequent decline over the following hours. However the expression peak time is not uniform but is highly dependent on the individual and the stimulant. We previously found that individual variability peaks at 6, 8 and 10

hours post stimulation[20]. Others have investigated the kinetics of mRNA expression of both IFN-γ and IP-10 upon stimulation with M.tb antigens with various conclusions. Some found that IFN-γ mRNA peaks around 6 hours post stimulation followed by a rapid degradation[20,22] while others found no major difference in mRNA expression levels between 8 and 18 hours post stimulation[23,24]. Kim et al analyzed the IP-10 mRNA expression level in cell pellet 24 hours post stimulation with *M.tb* antigens and found a very high median response from active TB patients[25]. However, given that IP-10 protein was upregulated in several of the samples with low mRNA signal, it seems likely that the main cause of the molecular platform underperforming in this present study is the 8 hour time point selected for mRNA isolation.

Table 4. Specificity and sensitivity obtained by each test.

qPCR (IP-10) vs. ELISA (QFT)				
Sensitivity	**N=89**		**qPCR: IP-10**	
			Positive	**Negative**
	ELISA: QFT	**Positive**	68	12
		Negative	3	6
Specificity	**N=99**		**qPCR: IP-10**	
			Positive	**Negative**
	ELISA: QFT	**Positive**	0	0
		Negative	2	97
ELISA (IP-10) vs. ELISA (QFT)				
Sensitivity	**N=89**		**ELISA: IP-10**	
			Positive	**Negative**
	ELISA: QFT	**Positive**	76	4
		Negative	1	8
Specificity	**N=99**		**ELISA: IP-10**	
			Positive	**Negative**
	ELISA: QFT	**Positive**	0	0
		Negative	2	97
ELISA (IP-10) vs. qPCR (IP-10)				
Sensitivity	**N=89**		**qPCR: IP-10**	
			Positive	**Negative**
	ELISA: IP-10	**Positive**	67	10
		Negative	4	8
Specificity	**N=99**		**qPCR: IP-10**	
			Positive	**Negative**
	ELISA: IP-10	**Positive**	0	2
		Negative	2	95

This illustrates a major challenge in mRNA based diagnostic tests. In contrast to protein-based cytokine release assays where protein accumulates in the plasma, mRNA increase is rapid and transient. Inhibition of the degradation of mRNA with measurement of an accumulated amount of mRNA would significantly improve the correlation between the protein and the mRNA levels and subsequently also lead to an overall higher diagnostic performance of the assay. However, the kinetics of the IP-10 mRNA release also suggests an opportunity to study whether mRNA expression responsiveness is associated with ongoing immune activation and risk of developing disease which could be explored for superior predictive performance.

Intra- and inter-assay variability of this molecular assay has been described previously, and this paper supports the high technical performance of the PCR now in a much larger cohort. The average PCR efficiency was >92% for both the target IP-10 and the reference gene β-actin. Furthermore, less than 2.5% of all measurements within the linear dynamic range had a standard deviation >0.5 Cq values (Fig S2 + table S1). The technical performance of the assay validates the assay as suitable as a diagnostic tool. Adding to this, the platform itself is good for automation and is already integrated in most TB labs used as a diagnostic tool for active TB.

In conclusion, we compared a molecular IP-10 release assay to QFT and protein-based IP-10 release assay and found a comparable performance of the protein-based assays, but a slightly lower sensitivity for the molecular assay. We suggest that the molecular platform underperforms, due to suboptimal timing of mRNA sampling. A more detailed description of the mRNA expression kinetics is needed for further assay development. Overall, we found a high technical performance of the assay making it suitable as a diagnostic tool. The rapid technological advances in hand-held and field-friendly PCR machinery and microfluidics suggests that faster, cheaper and importantly simpler test platforms could be developed for molecular detection of LTBI.

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Supplementary data

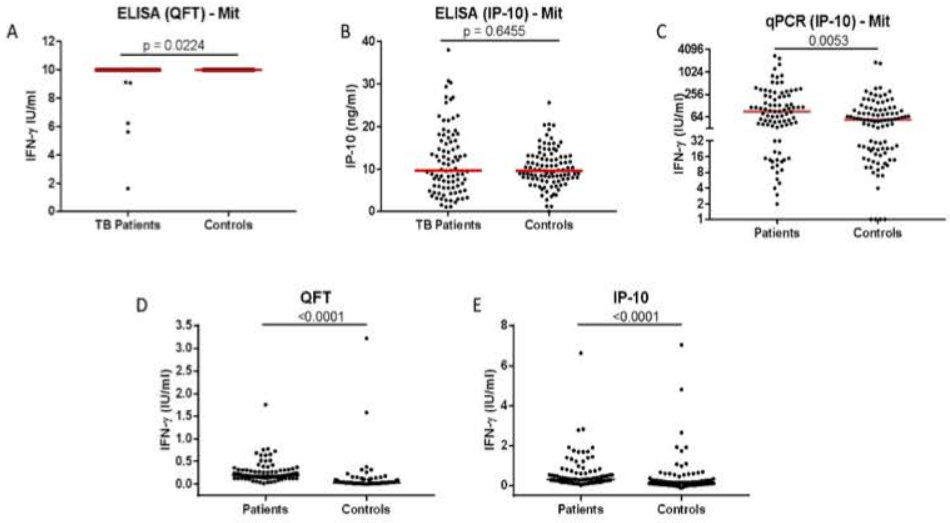

Figure S1. Cytokine levels detected per method and patients group.

Table S1. qPCR technical performance.

qPCR technical performance		
	IP-10 (target)	**β-actin (Reference)**
Measurements (n)	42	42
Linear dynamic range	22.70-35.23	19.75-32.84
Reproducibility		
Lower LOD (SD)	22.70 (± 0.18)	19.75 (± 0.33)
Upper LOD (SD)	35.23 (± 0.32)	32.84 (± 0.50)
Average PCR efficiency (SD)	1,08 (0.04)	0,99 (0.05)
Y-intercept	19.87 ± 0.3654	16.83± 0.7339
Slope	-3.139 ± 0.1102	-3.355 ± 0.2213
r^2 calibration curve	1.00	0.99
Repeatability		
Measurements (n)	567	567
SD<0.5 n/n_{total} (%)	13 (2.3%)	4 (0.7%)

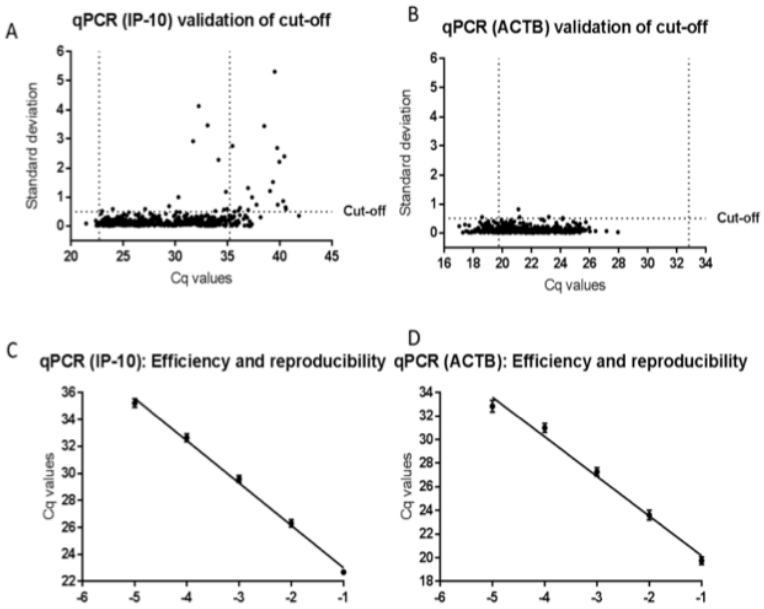

Figure S2. qPCR technical performance graphs.

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About Raquel Villar Hernández

I was born on January 1988 in Toronto (Canada) and in 1994 moved to Tenerife, Canary Islands (Spain) where I attended primary and secondary school. In September 2000, I started my bachelor's degree in Biology at La Laguna University in Tenerife. During the academic year 2009-2010, I collaborated in the Microbiology Department of La Laguna University under the supervision and mentorship of Dr. Laila Moujir-Moujir, testing a previously purified natural compound against HeLa cells. The last year of her bachelor's degree (2011-2012), I moved to Wroclaw (Poland) to continue my studies at Wroclaw University. During this period, I worked at three different labs: Parasitology, Microbiology, and Plant Biology. At the Parasitology lab, I was under the supervision of Dr. Agnieszka Perec-Matysiak, looking for *Cryptosporidium* and *Giardia spp* in rodent stool samples and hemoparasites in rodent spleen samples. At the Microbiology lab, I was supervised by Dr. Zuzanna Drulis-Kawa, involved in the study of the activity of bacteriophages obtained from water and soil against *Pseudomonas aeruginosa*. At the Instytut Biologii Roslin Plant Biology lab, I was supervised by Dr. Malgorzata Janicka-Russak and mentored by Dr. Anna Wdowikowsk and Dr. Agata Bogusz, learning different plant biotechnology techniques, such as genetic engineering. In June 2012, I graduated in Biology specializing in both Health and Molecular Biology by La Laguna University.

In September 2012, I moved to Barcelona (Spain) to start my master studies in Advanced Microbiology at the University of Barcelona. Throughout the master I worked at the Microbiology Department of the University Hospital Germans Trias i Pujol under the mentorship of Dr. Cristina Prat and Dr. José A. Domínguez. During this time, I learned about respiratory samples processing, molecular biology diagnostics, and cell culture techniques, amongst others. In June 2014, I obtained the master's degree after defending my thesis entitled "*Haemophilus influenzae* after pneumococcal vaccination".

From June until December 2014, I obtained a research contract by CIBER Respiratory Diseases, to work under the supervision of Dr. Cristina Prat and Dr. Alicia Lacoma on the development of alveolar macrophages culture to study the intracellular life of *Staphylococcus aureus*, and evaluating the use of a natural compound (seconeolitsin) against *S. aureus*. From October to December 2014, I did a research stay at the Bacterial Genetics Unit at the National Center for Microbiology, ISCIII, in Madrid (Spain), mentored by Dr. Adela González de la Campa. During this period, I tested the activity of seconeolitsin on DNA topoisomerase I of *S. aureus*, by previously cloning the topoisomerase I encoding gene into an *Escherichia coli* expression vector, overproducing the enzyme and then purifying it.

In January 2015, I started my Ph.D. studies at the Genetics and Microbiology Department from Universitat Autònoma de Barcelona, under the mentorship of Dr. José Domínguez, Dr. Cristina Prat, and Dr. Irene Latorre. During this time I worked at the Institute for Health Science Research Germans

Trias i Pujol (IGTP) (Barcelona, Spain) studying the host-pathogen interaction in mycobacterial infections in order to improve their diagnosis by looking at different biomarkers and stimulating biological samples and cells with different antigens. During this period, I was involved in several international and national projects related mostly with tuberculosis infection diagnosis. The results of the work done are presented in this Ph.D. thesis and available in other publications. I spent 4 months at Leiden University Medical Center (LUMC) (Leiden, The Netherlands), under the mentorship of Prof. Dr. Tom H. M. Ottenhoff, studying the immunogenicity of newly selected *Mycobacterium tuberculosis* in vivo expressed (NETB) antigens. Because of the involvement in other international projects, I also did a research stay at the Statens Serum Institut (SSI), (Copenhagen, Denmark), under the supervision of Dr. Morten Ruhwald, analyzing samples from the IP-RAs project in which I was already collaborating actively from Barcelona; and another one at the TB Clinics in Odessa (Ukraine), under the supervision of Dr. Olena Rzhepishevska, working with TB patients' samples. Furthermore, and as a result of my research on non-tuberculous mycobacteria (NTM) infections, I am registered as one of the inventors on a patent filed by the IGTP, CIBER and Ohio State University, disclosing the use of GPLs for NTM infection diagnosis.

Apart from being focused on research in itself, I am fascinated by the idea of combining science and art to reach and engage society. Throughout my Ph.D. studies, I have spent part of my spare time developing skills in research communication. Because of this, I created and currently manage my research group's Twitter account (@Oneandahalf_Lab), and made videos and infographics explaining what we do in more engaging and understandable terms. In addition, I developed, designed, and currently manage the website, Instagram, and Twitter accounts of the INNOVA4TB International Consortium (www.innova4tb.com and @innova4tb). Following with these interests, during 2018, I was involved in the PERFORM project where I was trained in responsible research and innovation focusing on how to maximize the local impact of research. In addition, during two months, I took part in the participatory research process of this project, developing communication and science education activities based on performing arts with students from the Institut Consell de Cent, Barcelona. In June 2018, I participated in the final conference of the PERFORM project that took place at the UNESCO headquarters in Paris.

As to what concerns *life after the Ph.D. thesis defense*, I am very interested in further characterizing the immune response against mycobacterial infections, taking into account not only the host and the pathogen but also external factors. Additionally, I wish to continue exploring the combination of different media to explain what I do in more understandable terms. In fact, together with Barcelona's Public Health Agency, I am currently elaborating a photojournalism project proposal about TB control. By visually showing the management of the disease, this project aims to create awareness about tuberculosis while helping decrease stigma and discrimination.

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