Pathognomonic effects of human tuberculosis on host immune response in an endemic population: impact on T-cell functions and M. *tuberculosis* infection diagnosis

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List of Publications

Paper I

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Paper II

Harling, K., Adankwah, E., Güler, A., Afum-Adjei Awuah, A., Adu-Amoah, L., Mayatepek, E., Owusu-Dabo, E., Nausch, N. & Jacobsen, M. Constitutive STAT3 phosphorylation and IL-6/IL-10 co-expression are associated with impaired T-cell function in tuberculosis patients. *Cellular and Molecular Immunology.* 2018;16(3):275-87.

Paper III

Adankwah, E., Guler, A., Mayatepek, E., Phillips, R.O., Nausch, N, & Jacobsen, M. CD27 expression of T-cells discriminates IGRA-negative TB patients from healthy contacts in Ghana. *Microbes & Infection* 2020;22(1): 65-68.

Paper IV

Lundtoft, C., Afum-Adjei Awuah, A., Rimpler, J., Harling, K., Nausch, N., Kohns, M., Adankwah, E., Lang, F., Olbrich, L., Mayatepek, E., Owusu-Dabo, E. & Jacobsen, M. Aberrant plasma IL-7 and soluble IL-7 receptor levels indicate impaired T-cell response to IL-7 in human tuberculosis. *PLoS pathogens.* 2017;13(6):e1006425.

Publications not included in this thesis

Güler, A., Lopez Venegas, M., **Adankwah, E**., Mayatepek, E., Nausch, N. & Jacobsen, M. Suppressor of cytokine signalling 3 is crucial for interleukin-7 receptor re-expression after T-cell activation and interleukin-7 dependent proliferation. *European Journal of Immunology***.** 2020;50(2):234-244

Adankwah, E., Nausch, N., Franken, K.L.M.C., Ottenhoff, T.H.M., Mayatepek, E., Phillips, R.O. & Marc Jacobsen (Preliminary). Alternative cytokines and Mycobacterium tuberculosis dormancy-associated antigens improve immune diagnostic tests for human tuberculosis.

Summary

Mycobacterium (M.) *tuberculosis* (*Mtb*) is a globally prevalent pathogen that threatens humans since ancient time. Exposure to *Mtb* infection leads to a spectrum of outcomes including an asymptomatic state, known as latent tuberculosis (LTBI) and an active, symptomatic infectious state, tuberculosis (TB).

T-cells and canonical T helper type 1 (TH1) cytokines, including the key effector Interferon-γ (IFN-γ), centrally contribute to protection against *Mtb* and impaired T-cell response can lead to disease progression. However, *Mtb* immunodiagnostic IFN-γ release assays (IGRA), like the QuantiFERON, are unable to discriminate LTBIs from active TB suggesting more complex mechanisms involved in immune protection against TB. The underlying hypothesis of this thesis was that TB pathognomonic features affect T-cell mediated protection because of impaired T-cell response to mitogen and limited sensitivity of the IGRA tests in highly endemic populations had been shown. In an initial approach, the capacity of altered *in-vitro* assay conditions and alternative *Mtb* antigen usage to improve sensitivity of immune tests was investigated. Finally, the capacity of alternative *Mtb* antigens (i.e. dormancy antigens) were analyzed to unravel differences of immune responses between TB patients and LTBI.

Included in this thesis, are four independent studies from TB patients and potentially latently *Mtb* infected healthy (LTBI) contacts recruited from Ghana, West Africa. Using standard IGRA test (QuantiFERON), we confirmed own previous studies of assays' suboptimal performance in detection of *Mtb* infection in both study groups. To explore factors that might contribute to limited sensitivity and/or improve IGRA test, we initially, analyzed the serum cytokine milieu from TB patients and healthy LTBI contacts. IL6 and IL-10 were significantly higher in both serum and QuantiFERON supernatants of TB patients as compared to healthy LTBI contacts. High IL6/IL-10 expression in TB patients was accompanied by constitutive STAT3 phosphorylation and high SOCS3 levels, indicating aberrant functional cytokine signaling effects on T-cells from TB patients. Pathognomonic TB effects on T-cell biology were also identified by T-cell immunophenotyping showing low CD27 and IL-7R expression. Finally, possibilities to circumvent impaired T-cell functions on *Mtb*-specific immune tests were assessed by alternative assay conditions and *Mtb*-specific latency antigen usage. A two-hit *in-vitro* assay improved detection of *Mtb* infection also in IGRA non responders from both TB patients and healthy LTBI contacts. Interestingly, *Mtb* latency antigen specific T-cell responses were highly capable of discriminating healthy LTBI contacts from active TB patients. Thus, optimized assay conditions have the potential to overcome pathognomonic TB effects and thereby improving the sensitivity for detection of *Mtb* infection in an endemic population.

Together, these findings offer crucial insights into potential pathognomonic features/markers which characterize impaired T-cell function in tuberculosis and introduce a novel assay with the capacity to improve *Mtb* infection detection in an endemic population.

List of Abbreviations

BCG	Baccille Calmette and Guerin
DosR	Dormany survival regulon
ESAT-6	Early secretory antigen target-6
CFP-10	Culture filtrate protein-10
IGRA	Interferon-γ release assay
IL-7R	Interleukin-7 receptor
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-7	Interleukin -7
JAK	Janus Kinase
LTBI	Latent tuberculosis infection
MHC	Major histocompatibility complex
Mtb	Mycobacterium *tuberculosis*
MTBC	Mycobacterium *tuberculosis* complex
RD1	Region of difference 1
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
TH_1	T-helper type 1 cells
TH_2	T-helper type 2
TH_{17}	T-helper 17 cells
TST	Tuberculin skin test

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1.0. Introduction

1.1. **Biology of Mycobacterium *tuberculosis***

Human tuberculosis (TB) is a chronic infectious disease caused by bacteria of the *Mycobacterium Tuberculosis Complex (MTBC)* (Pai et al., 2016). MTBC species are responsible for most pulmonary and extrapulmonary TB cases. The complex includes Mycobacterium *tuberculosis* (*Mtb*), the most common causative agent of TB in humans and M. *africanum* that causes human TB only in limited geographic regions in Africa (de Jong et al., 2010; Pai et al., 2016). Besides M. *cannetti* which is presumed environmental from origin, other members of the MTBC like M. *bovis*, M. *caprae*, M. *microti* are known to cause TB in wild and domesticated animals (Koeck et al., 2011). However, M. *bovis* and M. *cannetti* may also sporadically cause TB in humans (Davidson et al., 2017). These strains are distributed across the world and they account for most of reported TB cases (Coscolla and Gagneux, 2014). *Mtb* is a slowing growing bacterium with a replication time of approximately 20 hours. The rate of growth accounts for the chronicity of infection, increase difficulty in microbial diagnosis and requirement of prolonged drug treatment for TB patients (Kaufmann, 2001). It has a peculiar mycobacterial cell wall structure that enables survival in host phagocytes contributing to virulence (Kaufmann, 2001). In addition, the main virulence factors for most pathogens is through the use of protein secretion systems and in *Mtb,* it has five type 7 secretion systems known as (ESX1-5) (Abdallah et al., 2007). Region of difference 1 (RD1) in the *Mtb* genome contains genes that encode for ESX1 secretion system and this has been well-characterized (Abdallah et al., 2007). Contextually, the ESX1 is absent in attenuated Baccille Calmette and Guerin (BCG) and non-tuberculosis mycobacteria strains (Abdallah et al., 2007). *Mtb* maintains persistence and virulence by using this as a secretion system to translocate from phagosome to the cytosol of infected macrophages (Ernst, 2012). The ESX1 secrete among other proteins early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10). These two antigens are highly immunogenic and this forms the basis for their use in interferon gamma release assay (IGRA) tests for *Mtb* infection diagnosis (Delogu et al., 2013; Ernst, 2012). Importantly, since BCG lack ESX1 and cannot express ESAT-6 and CFP-10, IGRA are most useful in the diagnosis of *Mtb* infection in individual previously vaccinated with BCG (Abdallah et al., 2007; Pai et al., 2016).

Further, *Mtb* also utilizes another unique set of so-called latency-associated proteins that are regulated by the dormancy survival regulon (DosR) in response to hypoxia, low-dose nitric oxide and carbon monoxide (Barry et al., 2009). These conditions are encountered by *Mtb* during its long-term persistence in immunocompetent hosts. DosR can control the expression of more than 40 latency-associated genes (Voskuil et al., 2003; Meier et al., 2018). These genes provide *Mtb* with a niche to survive and overcome immune surveillance in a state of dormancy for a long time until it reverts to its proliferating state (Ulrichs & Kaufmann, 2006).

1.2. Epidemiology of human tuberculosis

Mtb is known to affect about 2 billion people of the world's population, making it the most efficient human pathogen to plaque mankind (Kaufmann, 2001; Yong et al., 2019). It remains one of the topmost killers from an infectious disease with 10 million infected cases and approximately 1.6 million deaths recorded in 2017, translating to 4000 deaths per day (WHO, 2018; Yong et al., 2019). Although, TB is a major treat globally, its impact on developing countries in Africa is alarming. It accounts for more than 4 million of active TB cases with the increase rates being driven by the HIV epidemic (Kaufmann & Parida, 2008). TB-related deaths of about 650,000 are reported every year with one-third of these deaths occurring in HIV co-infected individuals (Kaufmann & Parida, 2008). The treat of TB in sub-Saharan Africa is exacerbated due to inefficiency of current diagnostic tools, for example, the most common diagnostic test for TB in these regions is sputum smear microscopy which detects acid-fast *Mtb* bacilli. However, its detects less than half of individuals with active TB disease (Yong et al., 2019). Also, current drug regimen for treatment of tuberculosis need a combination of 3 to 4 drugs for over a period of 6 to 9 months. This is logistically inefficient and leads to poor compliance which contribute to the rise in multidrug-resistant cases (Kaufmann & Parida, 2008; Pai et al., 2016). Moreover, BCG - the only licensed vaccine for TB, is only effective against disseminated TB in children and cannot prevent active pulmonary TB in adolescents and adults (Yong et al., 2019). To overcome this treat, an improved vaccine and complete coverage of drugs supplied for TB treatment alongside better diagnostic tools are needed.

1.3. Pathophysiology: Latent infection vs. active tuberculosis

Mtb infection can affect different organs of the body but most frequently leads to a pulmonary TB manifestation. Extrapulmonary TB may affect any part of the body and accounts for up to 15-20 % of TB cases.50-60% HIV-coinfected cases are usually extrapulmonary (Blankley et al., 2016). *Mtb* is transmitted through aerosol droplets containing few bacilli exhaled by individuals with active pulmonary tuberculosis. Upon entry into the lung, the bacilli are engulfed by alveolar macrophages, the dominant cell type infected by *Mtb* (Pai et al., 2016). Immune cells, particularly T-cells, are activated in draining lymph nodes. The interplay between alveolar macrophages and sensitized T-cells results in bacterial containment through the formation of granulomatous lesions. These consist of an aggregation mature macrophage in a fibrotic matrix, neutrophils, DC and different T cell population in the lung (Figure 1). Bacterial containment by immune surveillance is known as a state of latency (LTBI) and can persistent for probably lifelong. When this containment fails (due to changes in host immunity or be overwhelmed), active disease can occur (Figure 1). LTBIs do not show any clinical symptoms and their diagnosis depend solely on immunodiagnostic test (Pai et al., 2006). About 90% of the infected remain LTBI. Increase risk of active TB in LTBIs occurs within the first two years and approximately 10% will develop active TB in their lifetime (Kaufmann, 2001; Nieuwenhuizen & Kaufmann, 2018; Prezzemolo et al., 2014). In LTBI, progression to disease is usually not the result of primary infection but a reactivation of existing foci. Primary infection translates to disease more frequently in immunologically naïve and immunosuppressed individuals (Hunter, 2018; Kaufmann, 2001). It is now becoming increasingly clear that the state of latency involves a range of clinical outcomes and it is characterized by the degree of pathogen replication, inflammation and host immune resistance (Cadena et al., 2017; Ling &Flynn, 2012). Active TB disease on the other hand, is markedly diverse and differs according to bacterial load and replication, degree of pathology, immune activation and inflammation, area of infected lung and rate of disease progression (Cadena et al., 2017). Thus, active interaction of immune players in fight against this intracellular pathogen play a crucial role as they can lead to these *Mtb* infection outcomes; total eradication of the bacteria, containment in granulomatous lesions, primary active disease and reactivation of latent infection. Overall, *Mtb* infection outcome is not just a simple two-way arm including TB and LTBI but represent a complex continuous spectrum that can differ based on host

immune activity and pathogen factors. Therefore, stage and status of *Mtb* infection must be accurately diagnosed to ensure effectiveness of treatment regimens and diagnostic tools.

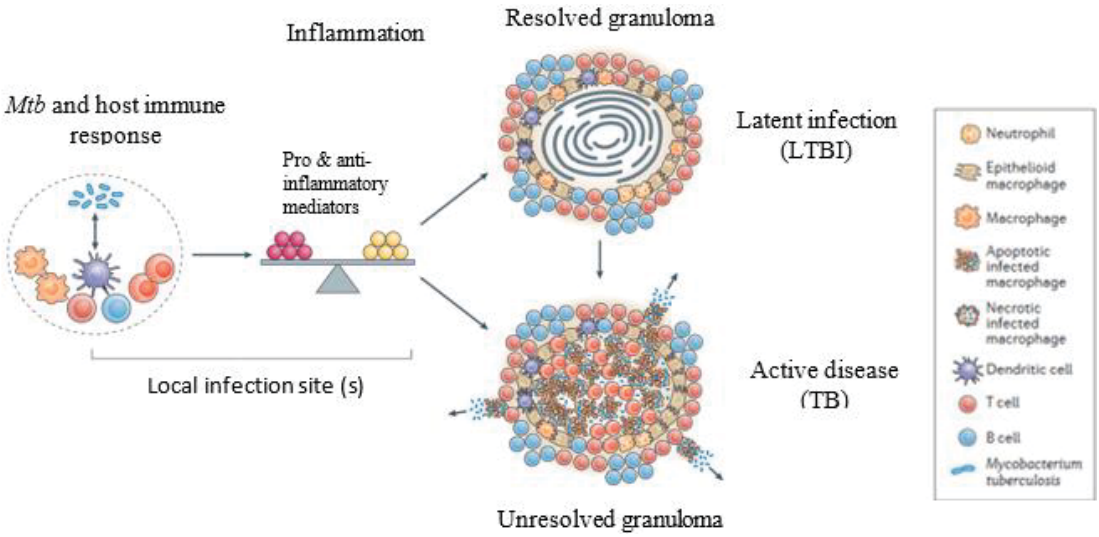

Figure 1: Interplay of host immune cells in *Mtb* infection. Figure adapted from Cadena et al., 2017 and reprinted by permission Springer Nature: Nature reviews.

1.4. The central role of T-cells for immune surveillance against *Mtb* infection

The outcome of *Mtb* infection is known to largely rely on the type and the extent of host immune response to mycobacteria. Host immune surveillance is essential as it can induce protective immune cues against *Mtb* infection (Pai et al., 2016). After *Mtb* infection, numerous innate and adaptive effector pathways are induced. However, the adaptive immune response especially T-cells are critical for the control of infection whereas the role of B-cells is not clear (Prezzemolo et al., 2014). Additionally, the intracellular location of the bacteria shields it from the influence of antibody-mediated effects and thus, the involvement of T-cell subsets become paramount for control (Ernst, 2012; Kaufmann, 2002). The initiation of the adaptive immune response to *Mtb* is a delayed CD4+ T-cells driven type IV hypersensitivity response that becomes detectable in about 3-8 weeks after infection (Urdahl et al., 2011). This delayed response is exemplified in antigen-induced reactivity response in majority of *Mtb* infected individuals to the administration of *Mtb* purified protein derivative (PPD) or tuberculin skin test (TST), in which visible reaction are detected 24-72 hours (Jasenosky et al., 2015). Importantly, the generation of memory T-cell after antigen exposure is essential for *Mtb* control. Memory T-cells that remain after infection is cleared, proliferate and elicit strong effector mediated function on subsequent exposure. Consequently, the quality of memory response make a significant difference between protective response against *Mtb* infection and disease progression (Prezzemolo et al., 2014).

The protective role of T-cells in TB immunity only began to be understood through studies using mice models, in which prevention of infection was based on the absence of T-cells subsets (Cooper et al., 1997; Pai et al., 2016). Subsequently, the role of T-cell immunity in the control of *Mtb* infection was described in humans and non-human primates (Cadena et al., 2017; Jasenosky et al., 2015; Pai et al., 2016). Mycobacterial-host interactions induce a dynamic immune response that usually requires the participation of both conventional and unconventional T cells (Kaufmann, 2001). However, the major effector cell types involve CD4+ T cells and a supporting role for CD8+ T cells (Jasenosky et al., 2015). Resident *Mtb* inside host phagosome secretes antigens that are processed by MHC class II antigen processing machinery which are present on monocytes, dendritic cells and macrophages (Urdahl et al., 2011). Antigens present on MHC II are recognized by CD4+ T cells, typically helper T cell

(TH_1) resulting in their activation. These *Mtb*-specific CD4+ T cells become potent producers of IFN-γ, that is, involved in activating macrophages, induction of phagocytosis, phagosome maturation, and production of reactive nitrogen intermediates. These effector functions restrict intracellular bacteria growth and promote *in-vivo* killing (Kaufmann, 2001; Urdahl et al., 2011). During acute infection, specific cytokines like IL-12, IL-18 and other co-stimulatory molecules are released and they promote the differentiation of CD4+ T cells to T helper type 1 (TH_1) cells (Lyadova & Panteleev, 2015). These induced cytokines milieu and activated T-cell sub-populations are essential in fine-tuning of *Mtb*-specific immune response (Kaufmann, 2001; Prezzemolo et al., 2014). It has been shown that genetic defects in IL-12 or IFN-γ increase susceptibility to TB disease progression (Jasenosky et al., 2015; Rook et al., 2005). Additionally, HIV-infected patients with low CD4+ T cells count are highly susceptible to reactivation of latent infection and active disease (Lyadova & Panteleev, 2015). In view of this, *Mtb* specific TH_1 and its canonical cytokine, IFN-γ are now well-established markers that are prerequisite for protection against TB. However, it must be noted that frequencies of *Mtb*-specific IFN-γ producing T cells or IFN-γ levels do not correlate to protection and this has been demonstrated by several studies in mice and humans (Majlessi et al., 2006; Mittrücker et al., 2007; Sakai et al., 2016). Although IFN-γ is the influential factor in *Mtb* infection control, other cytokines, in particular TNF-α also play a role. The importance of TNF-α came to light when rheumatoid arthritis patients showed increased risk of reactivation of tuberculosis after anti-TNF-α therapy (Kaufmann, 2002; Urdahl et al., 2011).

CD8+T cells activation are restricted to MHC class-I antigen presention and *Mtb* has been shown to induce this class presentation that also contribute to host protection (Brighenti & Andersson, 2010; Bruns et al., 2009). CD8+ T cells like CD4+T cells also produce IFN-γ but their main role is associated with target-mediated killing. This involves the direct killing of *Mtb* through the secretion of granulysins and perforins, where antigen-specific killing is achieved through the induction of apoptosis (Prezzemolo et al., 2014). Other T cells subset are involved in the control or suppression of *Mtb*-specific immune response. For example, T helper type 2 (TH_2) and regulatory T cells can counter-regulate the effects of TH_1 cells leading to impaired TH_1 mediated immunity against TB (Lyadova & Panteleev, 2015). In recent times, IL-17 producing T helper 17 (TH_{17}) cells have been described to play a protective role against TB by mediating proinflammatory response during the early stages of infection (Jasenosky et

al., 2015; Prezzemolo et al., 2014). Together, *Mtb* infection induce a broad range of immune response. Better understanding of host immune players in the fight against *Mtb* infection is crucial, in order to inform the development of effective TB vaccines, and to monitor disease progression and treatment efficacy and the implementation of interventional strategies for TB control.

1.5. Pathognomonic effects of Tuberculosis on Host immune response

Cellular immunity especially T-cells mediated immune response against *Mtb* is crucial for protection but unrestricted response can result in damage of host tissues promoting disease severity (Prezzemolo et al., 2014). To maintain balance, host cells utilize different mechanisms including molecular restraints using cytokines and growths factors, initiating regulatory function of lymphoid subpopulation and upregulation of effector molecules (Rottenberg & Carow, 2014). Active TB is characterize by diverse inflammatory response associated with dysregulation of inflammatory cytokines and effector pathways that can affect T- cell function in the blood and site of infection (Pai et al., 2016; Rottenberg & Carow, 2014). Several cytokines involved in this immune process signal via receptor complexes that active Janus kinases (JAK) and signal transducers and activators of transcription (STATs) pathway and they play important role in protective immune response against active TB (Rottenberg & Carow, 2014). It is now only becoming clear that aberrant regulation of cytokines in TB disease in particular, IL-6, IL-10 and IL-7, have the potential to reveal underlying disease mechanisms that may indicate impaired role of T-cells in TB pathology.

1.5.1. The role of IL-10 in immunity to tuberculosis

IL-10 is a central anti-inflammatory cytokine originally described as a TH_2 related but in recent times other IL-10 related cell types like type 1 regulatory T-cells and TH_{17} effector cells have also been identified (Lyadova & Panteleev, 2015). It is produced by many hematopoietic cells and it plays a key role in maintaining optimal immune response and balance. In TB, it has inhibitory effects on the functions on DCs and macrophage that are required for uptake, control and initiation of cellular immunity against *Mtb* (P. S. Redford et al., 2011). IL-10 has been associated with immunosuppressive functions that results in susceptibility to TB disease in humans and mice models (Rottenberg & Carow, 2014). It is known to antagonize IL-12 release

and IFN-γ mediated killing of infected *Mtb* macrophages through downregulation of antigen presentation by DCs and impairment of the production of reactive oxygen and nitrogen intermediates (Abdalla et al., 2016; P. S. Redford et al., 2011). In *Mtb* infected mice, IL-10 has been shown to impair the migration of T-cells to the site of infection, the lungs, limiting the capacity of TH_1-mediated response (Redford et al., 2010). In humans, immunosuppressive effects of IL-10 on immune response against *Mtb* was first described in immunocompetent active TB patients which showed anergy to TST skin test. T-cells from these patients, upon antigen stimulation, produced high levels of IL-10 that inhibited T-cell proliferation. Conversely, blocking IL-10 restored proliferation of these cells (Boussiotis et al., 2000). O'Leary and co-authors also demonstrated that IL10 impairs phagosome maturation in infected macrophages that promoted *Mtb* growth and survival with partial dependency on STAT3 induction (O'Leary et al., 2011). Further, several other studies have shown significant levels IL-10 in the lung and serum of tuberculosis patients with potential effect on T-cell function (Abdalla et al., 2016; Chowdhury et al., 2014). Collectively, these studies show that IL-10 has immunosuppressive effects on immune response and, therefore, may contribute to TB pathology.

1.5.2. IL-6, effect on T-cells subsets and M. *tuberculosis*

IL-6 is a multifunctional cytokine that is involved in the regulation of acute-phase response, differentiation of B cells and T cells activation (Hunter & Jones, 2015). IL-6 serum levels are low (1-5pg/ml) in physiological conditions but their levels get rapidly elevated in disease settings (Hunter & Jones, 2015). Previous studies showed that IL-6 is lymphocyte stimulating factor and lack of IL-6 signaling leads to impaired innate and adaptive immune response to viral and bacterial infections (Hoge et al., 2013; Longhi et al., 2008). Thus, a role of IL-6 has been implicated in many infectious and auto-immune diseases (Hunter & Jones, 2015). IL-6 induces functional activities via STAT3 signaling and genetic mutation of this transcription factor have been associated with impaired IL-6 activity and recurrent infection (Freeman & Holland, 2010). Also, it has a polarizing effect on distinct populations of T helper cells like IL-17 producing TH_{17} cells and drives their expansion in humans and mice disease models (Dileepan et al., 2011; Lyadova & Panteleev, 2015; Nowell et al., 2009). While IL-6 is known to dampen immunosuppressive effect on T- cell function by inhibiting regulatory T-cells , it

can also promote IL-10 production by T-cells, counteracting this effect (Hunter & Jones, 2015; Singh & Goyal, 2013). Further, IL-6 functions synergistically with other cytokines like TNF-α and IL-1β to promote pro-inflammatory response (Singh & Goyal, 2013). Importantly, the protective role of IL-6 *Mtb* infection outcomes has been described (Singh & Goyal, 2013). It known to drive TH_1-mediated protective immune responses against *Mtb* infection (Nowell et al., 2009). However, its paradoxical role in promoting intracellular growth of mycobacterial species and the dampening of IFN-γ response in *Mtb*-infected macrophages has been reported (Leal et al., 2001). Collectively, IL-6 has a broad effect on immune response in several diseases setting but its influence and impact on TB pathology is still under investigation.

1.5.3. Role of IL-7 in tuberculosis

IL-7 regulate T-cell homeostasis by promoting cellular survival and proliferation (Lundtoft,et al., 2017; Lawson et al., 2015). Its role in the modulation of T-cell mediated antigen-specific response has also been described (Lawson et al., 2015). IL-7 mediated effects on T-cells depends on membrane bound IL-7Rα receptor (IL-7Rα) expression. This receptor is highly expressed on conventional T cell subsets including naive and memory with the exception T regulatory cells (Heninger et al., 2012; MacKall et al., 2011). IL-7 signaling requires the recruitment of IL-7Rα and common-γ chain that activates JAK/STAT pathway via the phosphorylation of STAT5 (MacKall et al., 2011). Compared to other cell types that express IL-7Rα, TH_1 cells showed improve cellular response after IL-7 stimulation (Arbelaez et al., 2015). Earlier studies in animal models showed the potential role of IL-7 in T-cell immunity against tuberculosis, as adoptive transfer of *Mtb*-specific immune cells after IL-7 treatment showed decrease *Mtb* load and prolonged survival of mice (Maeurer et al., 2000). Similarly, co-administration of IL-7 and IL-15 promoted survival and enhanced BCG vaccination efficacy against *Mtb* (Singh et al., 2010). Additionally, it was shown that TB infected non-human primates had elevated levels of lL-7 and soluble IL-7Rα in their lung tissue, indicating an underlying role for IL-7 in TB pathology (Rane et al., 2011). Interestingly, the role of IL-7 in the improvement of sensitivity standard IFN-γ release assay in children and immunocompromise patients has also be demonstrated. It was shown that co-incubation with IL-7 improved cytokine release in response to acute *Mtb* antigens resulting in increased sensitivity of assay (Feske et al., 2008). Other studies identified that in chronic viral infections,

especially in AIDS, impaired IL-7 regulation affects T-cell function (Benito et al., 2008; Tanaskovic et al., 2014). For example, immune reconstitution in AIDS patients during anti-viral therapy is affected by impaired T-cell response to IL-7 (Tanaskovic et al., 2014). Furthermore, our own study described impaired IL-7 mediated T cell function as a feature of TB disease (Lundtoft et al., 2017). Indeed, IL-7 is important in TB disease but its regulatory role in *Mtb* infection remains largely unexplored.

1.6. *Mtb* infection diagnosis: prospects and challenges diagnostic tools

Fast and accurate diagnostic methods are important for the control of tuberculosis. Diagnosis of tuberculosis for the past years has been based on bacterial cultures and a triage of clinical symptoms, chest x-rays and sputum smear analysis (Wallis et al., 2010). However, analysis of sputum smears for *Mtb* detects less than half of active tuberculosis cases while as a negative chest x-ray do not exclude pulmonary TB especially in children (Qiu et al., 2019; Yong et al., 2019). Although *Mtb* bacterial culture is the most accurate tool for diagnosis, it is time consuming, specimen quality dependent and limited to active TB (Qiu et al., 2019). The introduction of molecular-based technique for *Mtb* DNA amplification i.e. GeneXpert MTB/RIF represent a great achievement in TB diagnosis. This diagnostic tool has two major advantages; it detects both *Mtb* infection and rifampicin resistant *Mtb* in approximately 2 hours and its user-friendly set-up requires minimum training of laboratory personnel. Nonetheless, its usage is limited to active TB diagnosis with other inherent limitations including; inability to differentiate live and dead mycobacteria and lower sensitivity in the diagnosis of paucibacillary pulmonary and extra-pulmonary TB in children (Yong et al., 2019). Importantly, T-cell immune responses to *Mtb* antigens can be evaluated with better consistency as indicators of *Mtb* infection, in contrast to antibody-mediated responses (Pinto et al., 2011; Yong et al., 2019). Additionally, T-cell based immune tests especially those performed on blood are attractive options as they meet the target product profile for point-of-care testing as recommended by World Health organization (World Health Organization, 2014).

1.6.1. Interferon-γ release assay (IGRA)

Despite the fact that different diagnostic tools exist for active TB, diagnosis of latent infection on the other hand relies heavily on the use of T-cell based immune tests (i.e. IGRA). IGRA

test utilizes the recall of IFN-γ production by T-cells sensitized with *Mtb* antigens i.e. Early Secreted Antigenic Target-6 (ESAT-6) and Culture Filtrate Protein-10 (CFP-10) to indicate evidence of previous exposure in absence of clinical symptoms (Kwon et al., 2015). Two commercialized IGRA-based formats, TST.SPOTB and QuantiFERON, are commonly used (Kwon et al., 2015; Pinto et al., 2011). In addition to these IGRA tests, TST a century old diagnostic test is still in used especially in some endemic countries (Pinto et al., 2011). It measures local response to *Mtb* antigens i.e. purified protein derivative after intradermal administration. However, its specificity is greatly reduced in BCG-vaccinated individuals and immunocompromised individual (Pinto et al., 2011; Yong et al., 2019). IGRA tests were introduced to overcome this limitation of TST. However, previous studies have indicated limited sensitivity especially in children and highly TB endemic population (Lundtoft et al., 2017; Nausch et al., 2017). Further, a negative or positive IGRA test result cannot conclusively rule out or confirm *Mtb* infection (Rangaka et al., 2012). In high burden countries, false negatives associated with IGRA tests have been described (De Visser et al., 2015; Masood et al., 2020; Rangaka et al., 2012). Thus, improved understanding and evaluation of factors associated with suboptimal performance of IGRA are required. Previous studies have indicated different potential etiological factors that may account for inconclusive IGRA test results. These included; age, immunodeficiency, extra- pulmonary TB, genetic variability and the immunomodulatory action of regulatory T cells (Cho et al., 2012; De Visser et al., 2015; Kwon et al., 2015). These factors have the potential to increase the number of missed *Mtb* infected cases which can perpetuate the spread of the disease. Moreover, factors that clearly elucidate why *Mtb* specific adaptive immune response remains undetectable at the time of diagnosis for TB or LTBIs particularly in endemic populations remains unclear.

1.6.2. T-cell activation markers

TST and IGRA tests, as diagnostic tools, are not able to discriminate LTBIs from active TB. In view of this, host biomarkers that have the potential to diagnose and classify LTBIs and TB patients are needed. Cellular phenotypic markers of T cells associated with T-cell activation

and antigenic dependent differentiation have been demonstrated to show promise as candidate biomarkers (Lyadova & Panteleev, 2015). Among these, CD27 a T-cell maturation marker and a co-stimulatory receptor, has been best characterized (Ahmed et al., 2018; Yong et al., 2019). Naïve T-cells show high CD27 expression which is downregulated at the effector cell level (Lyadova & Panteleev, 2015). Loss of CD27 expression in *Mtb* infected murine model indicates TB activity with enhanced lung migratory properties and are potent in IFN-γ production (Lyadova et al., 2004). In humans, low CD27 expression on CD4+ T-cells has also been shown to be associated with TB disease (Adekambi et al., 2012; Streitz et al., 2007). Additionally, T cells with this phenotype have been linked with degree of *Mtb* replication and might be a useful indicator for sub clinical *Mtb* infection (Schuetz et al., 2011). Further, the examination of CD27 expressing IFN-γ T cells subsets have been described by several studies to distinctively classify LTBIs from active TB patients (Adekambi et al., 2012; Ahmed et al., 2019; Lyadova & Panteleev, 2015; Portevin et al., 2014). Interestingly, Portevin and colleagues adapted T cell activation marker-tuberculosis (TAM-TB) approach, where they showed utility of CD27 expression was able to achieve 83.3% sensitivity and 96.8% specificity in children with culture confirmed TB cases (Portevin et al., 2014).

Similarly, diagnostic utility of other markers like CD38; an immune activation marker and Ki67; proliferation marker, for *Mtb* infection have been shown (Ahmed et al., 2019; Mupfumi et al., 2020). Ahmed and co-authors, showed the discriminatory potential of CD38/CD27, HLADR and Ki67 expression in active TB and LTBIs (Ahmed et al., 2018). Increase proportions of $CD38{+}CD27_{low}$ T cells and $CD38_{low}CD27_{high}$ were associated with active TB or LTBI respectively. Additionally, the expression of CD38, HLA-DR and Ki67 were shown to be stable markers that could predict time of sputum conversion and serve as surrogates for monitoring treatment efficacy and disease progression (Ahmed et al., 2018, 2019; Riou et al., 2014). Collectively, T-cells immune effector markers may prove to be reliable biomarkers for TB but robust validation and evaluation of their suitability for use especially in an endemic population is required.

1.6.3. *Mtb* latency antigen in immune-based tests

The nature of acute antigens used i.e secretory antigens, ESAT-6 and CFP-10 may be a compelling reason for limited sensitivity of IGRA tests in *Mtb* infection diagnosis. These

antigens are expressed early in acute tuberculosis and may partially reflect the dynamic spectrum of host immune response (Coppola & Ottenhoff, 2018). Hence, the use of alternative *Mtb*-stage specific antigens is required. Non-active stage antigens of *Mtb* is the so-called latency antigens. These are regulated by a set of *Mtb* genes that resides in the dormancy survival regulon (DosR) and play a role in adaptation of *Mtb* during dormancy (Barry et al., 2009; Meier et al., 2018). DosR antigens show moderate immunogenicity in humans (Schuck et al., 2009). Interestingly, adapted BCG with adjuvant of latency associated antigens conferred improved protection against TB than conventional BCG in murine model (Nieuwenhuizen & Kaufmann, 2018; Reece et al., 2011). Others also showed that latency proteins expressed at different stage of *Mtb* infection elicits strong TH_1-type immune response (Rakshit et al., 2017). Notably, several studies have described the potential of latency-related antigens to differentiate LTBIs from active TB disease (Meier et al., 2018; Rakshit et al., 2017; Schuck et al., 2009). In particular, Rakshit and co-authors, demonstrated that latent *Mtb* infection as compared to active TB, was significantly associated with latency-associated specific central memory T cells, polyfunctional and regulatory TH_{17} T cells. Further, others also described differential cytokines induced by latency-associated antigens as useful candidates (Arroyo et al., 2018; Chegou et al., 2012; Kassa et al., 2012). Thus, measuring host T-cell immune response against latency antigens may serve as important biomarker for latent infection. Together, the incorporation of latency antigens in current diagnostic tool may become useful indicators not only for *Mtb* infection but also in monitoring of TB disease progression.

2.0. Research Objectives

In the four research articles included in this dissertation, we aimed to identify potential mechanisms underlying immune failure in *Mtb* infection leading to TB disease progression. In this regard we compared healthy LTBI contacts with TB patients to identify pathognomonic features and potentially biomarkers of immune protection in LTBI. Importantly, pathognomonic features may also affect sensitivity of immune-based test for *Mtb* infection. In

view of this, possibilities to circumvent *Mtb*-mediated effects on immune-based tests were also investigated.

These were included objectives:

- identification of T-cell phenotype differences and serum markers of TB patients and healthy LTBI contacts.
- characterization of mechanisms underlying impaired T-cell functions in TB patients through the identification components of T-cell effector and cytokine expression signaling pathways.
- optimization of immune tests for diagnosis of *Mtb* infection.
- characterization of antigen specific differences between healthy LTBI contacts and TB patients.
- evaluate host biomarkers that robustly discriminate TB patients from healthy LTBI contacts.

3.0. Paper I: Two-Hit in vitro T-Cell stimulation detects Mycobacterium *tuberculosis* infection in QuantiFERON Negative tuberculosis patients and healthy Contacts from Ghana

Adankwah, E., Lundtoft, C., Güler, A., Franken, K.L.M.C., Ottenhoff, T.H.M., Mayatepek, E., Owusu-Dabo, E., Phillips, R.O., Nausch, N. & Jacobsen, M. Two-Hit in vitro T-Cell stimulation

detects Mycobacterium *tuberculosis* infection in QuantiFERON Negative tuberculosis patients and healthy Contacts from Ghana. *Frontiers in Immunology*. 2019; 10:1518

Contribution to this publication: 90%

- Patients recruitment
- FACS analysis
- ELISA
- *Ex-vivo & In-vitro* assays
- Data analysis
- Writing of the manuscript

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ORIGINAL RESEARCH
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Two-Hit *in vitro* T-Cell Stimulation Detects *Mycobacterium tuberculosis* Infection in QuantiFERON Negative Tuberculosis Patients and Healthy Contacts From Ghana

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IFN-γ release assays [e.g., QuantiFERON (QFT)] are widely used for diagnosis of *Mycobacterium tuberculosis* (*Mtb*) infection. T-cell responses against QFT antigens ESAT6 and CFP10 are highly *Mtb* specific but previous studies indicated suboptimal assay sensitivity. Especially for potentially infected healthy contacts (HCs) of tuberculosis patients, alternative antigen usage and more sensitive tests may contribute to improved detection of latent *Mtb* infection. In a pilot case-control study of tuberculosis patients ($n = 22$) and HCs ($n = 20$) from Ghana, we performed multifaceted *in vitro* assays to identify optimal assay conditions. This included a two-hit stimulation assay, which is based on initial and second re-stimulation with the same antigen on d6 and intracellular IFN-γ analysis, to compare T-cell responses against ESAT6/CFP10 (E6/C10) and selected latency antigens (i.e. Rv2628, Rv1733, Rv2031, Rv3407) of *Mtb*. Considerable subgroups of tuberculosis patients (64%) and HCs (75%) had negative or indeterminate QFT results partially accompanied by moderate PHA induced responses and high IFN-γ background values. Intracellular IFN-γ analysis of E6/C10 specific $CD4^+$ T-cell subpopulations and evaluation of responder frequencies had only moderate effects on assay sensitivity. However, two-hit *in vitro* stimulation significantly enhanced E6/C10 specific IFN-γ positive T-cell proportions especially in QFT non-responders, and in both study groups. *Mtb* latency antigen-specific T cells against Rv1733 and Rv2628 were especially detected in HCs after two-hit stimulation and T-cell responses against Rv2628 were highly capable to discriminate tuberculosis patients and HCs. Two-hit *in vitro* stimulation may improve moderate sensitivity of short term IFN-γ based assays, like QFT, to detect *Mtb* infection. Latency stage-specific antigens added significantly to detection of *Mtb* infection in HCs and tuberculosis patients with negative QFT test results.

Keywords: tuberculosis, LTBI, IGRA, *Mycobacterium tuberculosis* latency antigens, ESAT6, CFP10

INTRODUCTION

Tuberculosis is a chronic infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). The pathogen is transmitted via aerosols from tuberculosis patients with cavernous disease to close contacts (HCs), who have a high risk of becoming *Mtb* infected. A minor subset of *Mtb* infected HCs will develop active tuberculosis whereas the majority will control the pathogen by immune surveillance and remain latently *Mtb* infected (LTBI) (1). IFN-γ producing $CD4^{+}T_{helper}$ cells are central for protection of LTBI against progression to active disease. T_{helper} cells induce a delayed type hypersensitivity reaction against mycobacterial antigens (i.e., *Mtb* purified protein derivative; PPD) and this recall immune response forms the basis of the tuberculin skin test (TST) for detection of previous *Mtb* infection. The TST test has been used for more than a century to diagnose *Mtb* infection. In several regions where tuberculosis is endemic the TST is still applied but replacement by more specific immunological *in vitro* tests (i.e., IFN-γ release assays, IGRAs) is ongoing (2).

Immunological tests are essential for diagnosis of *Mtb* infection since direct detection of *Mtb* in affected body fluids is only possible for a subgroup of tuberculosis patients and generally not for LTBI. Identification of LTBI within HCs, however, is crucial especially for individuals with high risk of tuberculosis disease progression (e.g., young children) (3). IGRAs, like the QuantiFERON® Gold Plus test (QFT) are based on IFN-γ measurement after *in vitro* stimulation of whole blood with selected *Mtb* antigens (i.e., Early Secretory Antigenic Target (ESAT)-6 and Culture Filtrate Protein (CFP)-10). IGRAs can be assessed faster (i.e., after 16h) with TST comparable sensitivity but higher specificity especially in BCG vaccinated individuals (4, 5). Own previous studies showed that QFT sensitivity varies markedly between children with tuberculosis from different regions, being high in children with tuberculosis from Germany and alarmingly low in children from Ghana (6, 7). The usage of few selected antigens may contribute to population-dependent differences in QFT sensitivity due to differential MHC or exposure background. In addition, QFT antigens are predominantly expressed during active stage of *Mtb* and may only partially reflect host immune response against *Mtb* at different stages (i.e., dormancy, reactivation, resuscitation) (8). The term "latency antigens" of *Mtb* has been widely used for non-active stage antigens and is adopted here.

Several studies investigated T-cell response against *Mtb* latency in study groups of tuberculosis patients and controls [reviewed in (9)]. These studies were either using whole blood (10–13) or purified peripheral blood mononuclear cells (PBMC) (10, 14–16) in short-term (i.e., 16–24 h) (11, 13–15) or long-term (i.e., 5–7d) (11, 12, 14, 16, 17) assays measuring IFN-γ in the supernatant (10–17) or intracellularly (10, 11, 13). Only few studies compared different assays and found marked differences of individual donors between short- and long-term assays (18–21). We previously demonstrated that long-term culture (i.e., 7 days) including a second stimulation with the respective antigen on d6 (two-hit) and intracellular IFN-γ analysis enhanced the sensitivity for detection of T-cell responses against latency antigens (19). To investigate the immunogenicity of latency antigens, the vast majority of studies were based on QFT positive individuals, especially for LTBI. However, this excludes significant subgroups of both tuberculosis patients and LTBI, that may well show false-negative QFT results. Especially for HCs, early identification of *Mtb* infection is crucial to avoid spread of tuberculosis in endemic countries like Ghana.

Latency antigen specific T-cell responses may especially be important as a biomarker for LTBI, where *Mtb* dormancy contributes to pathogen survival (22). These T cells are, therefore, not only potential indicators of *Mtb* infection but may contribute to immune surveillance crucial to avoid *Mtb* reactivation and disease progression.

This pilot study aims at improving *in vitro* culture conditions to detect T-cell responses against QFT antigens ESAT6 and CFP10 (E6/C10) and selected latency antigens (i.e., Rv2628, Rv1733, Rv2031, Rv3407) in well-characterized tuberculosis patients and HC cohorts. A special focus was on tuberculosis patients with negative (or indeterminate) QFT results. For these we evaluated the capacity of alternative assays and antigens for detection of *Mtb* infection. Furthermore, the efficacy of latency antigen-specific T-cell based tests to classify tuberculosis patients and HCs was investigated.

MATERIALS AND METHODS

Study Cohort

We recruited tuberculosis patients ($n = 22$) and HCs ($n = 20$) from August to October 2018 at the Presbyterian Hospital in Agogo/Ghana. An independent cohort used for classification analyses [i.e., tuberculosis patients ($n = 4$) and HCs ($n = 13$)] was recruited between January and February 2016 at the Komfo Anokye Teaching Hospital, Kumasi/Ghana.

Diagnosis for active tuberculosis was based on patient history, clinical evaluation, chest X-ray, and sputum smear test. GeneXpert (Cepheid, USA) analyses were done for the main tuberculosis study group. 15 out 22 tuberculosis patients were GeneXpert-positive. GeneXpert negative tuberculosis patients had chest X-ray and clinical symptoms (i.e., blood coughing, weight loss) that were strongly suggestive for tuberculosis. 4 of 7 GeneXpert negative tuberculosis patients had positive QFT results. Sputum culture is not routinely performed. All tuberculosis patients of the second "classification" cohort were sputum smear positive for Mtb and no GeneXpert was performed for those. All tuberculosis patients were included prior to initiation of treatment and chemotherapy has been initiated immediately thereafter. The vast majority of tuberculosis patients had pulmonary disease manifestation besides two children, who had lymph node tuberculosis. HIV infection was excluded for all participants.

HCs had no symptoms of tuberculosis but were close relatives living in the same household with indexed tuberculosis patients according to self-report and direct observation. Study group characteristics are shown in **Table 1**.

Ethical Statement

The present study received approval from the Committee on Human Research, Publication and Ethics (CHRPE/AP/023/18;

TABLE 1 | Study cohort characteristics.

	Group	*n*	Median age (range, y)	Gender (m/f)	Tested conducted		
					QFT	X-ray	Sputum smear (microscopy or GeneXpert)
Study cohort	TB	22	37 (3–74)	18/4	√	√	√ (15 GeneXpert positive*)
	HC	20	33.5 (5–61)	12/8	√	nd	nd
Test cohort	TB	4	61(13–73)	3/1	√	√	√ (all microscopy positive)
	HC	13	36 (20–76)	8/5	√	nd	nd

*Y, years; m, male; f, female, n, numbers, nd, not done; TB, tuberculosis patients; HCs, healthy controls; *Tests included GeneXpert analyses; four of seven GeneXpert negative TB patients were QFT positive.*

CHRPE/221/14) at the School of Medical Sciences (SMS) at the Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, Ghana. All study subjects gave written informed consent prior to recruitment and for children written informed consent was provided by their parents or legal guardians.

QFT and QFT$_{in\text{-}vitro}$ Assays

Whole blood was taken in a single venepuncture into heparin tubes and 0.8 ml were added to each of the four QFT tubes (i.e., Nil, TB1, TB2, and PHA; Qiagen). The tubes were immediately incubated at 37°C for about 20 h. For the QFT$_{in\text{-}vitro}$ assay, 100 µl whole blood was cultured in 100 µl RPMI supplemented with, Penicillin/Streptomycin (100 U/ml) and L-glutamine (2 mM) using a 96 U-bottom plate (Greiner). Samples were stimulated with recombinant ESAT6/CFP10 fusion protein (E6/C10: 2 µg/ml), purified protein derivative of *Mtb* (PPD: 10 µg/ml; Statens Serum Institute), phytohemagglutinin (PHA: 10 µg/ml; Sigma-Aldrich) or left unstimulated for 20 h at 37°C and 5% CO_2. The term QFT$_{in\text{-}vitro}$ has been introduced since the results serve as reference between QFT and ICS-based *in vitro* assays (see below). In contrast to QFT, the QFT$_{in\text{-}vitro}$ uses recombinant proteins (not optimized peptide mixtures) and blood dilution. However, for comparison of PPD stimulation, as well as PBMC and intracellular cytokine staining (ICS), this QFT comparable assay was needed.

Supernatants from both assays were harvested and stored at −80°C until further analysis.

Measurement of IFN-γ Concentrations in the Supernatant

Supernatants from QFT and QFT$_{in\text{-}vitro}$ assay were thawed and IFN-γ concentrations were measured by ELISA (R&D) according to manufacturer's instruction. All samples were analyzed in duplicate and measured using an Infinite M200 ELISA reader (Tecan). Concentrations were calculated from respective standard curves by applying 4-parametric logistic regression. IFN-γ concentration of non-stimulated controls were subtracted from *Mtb* antigen specific and PHA induced IFN-γ to retrieve Δ values. Values below 1 pg/ml were set to 1 pg/ml. A previously described algorithm for the interpretation of IFN-γ values based on manufacturer's criteria was used for comparison of QFT (**Table S1**). These criteria have been adjusted for evaluation of QFT$_{in\text{-}vitro}$ results (**Table S2**) (6). QFT includes two tubes (TB1, TB2 according to manufacturer's nomenclature) containing *Mtb* antigens optimized for $CD4^+$ and $CD8^+$ T-cell response, respectively. Since both tubes showed similar results (data not shown) only TB1 (termed Ag1 in this manuscript) has been included for comparisons.

Indeterminate test results from QFT assay can have different explanations (e.g., NIL > 400 pg, see **Table S1**) and the underlying cause for indeterminate tests cannot be deduced from single parameters. For this reason, we did not include evaluation results as different symbol colors in **Figure 1A** but depict effects of high NIL values in combination with PHA response on indeterminate QFT results in **Figure 1B**.

Short-Term Intracellular IFN-γ Assays: ICS$_{Blood}$ and ICS$_{PBMC}$

Peripheral blood and PBMCs were used concomitantly. PBMCs were isolated from heparinized whole blood according manufacturer's instruction using density centrifugation (Ficoll, Biochrom). PBMCs (1.2×10^5) were cultured in RPMI (200 µl) and heparinized whole blood (100 µl) was diluted 1:1 in RPMI. Co-stimulatory antibodies were applied to optimize detection of *Mtb* specific T cells in this assay (23). All wells received co-stimulatory antibodies αhuman CD49d (clone 9F10) (1 µg/ml) and αhuman CD28 (clone CD28.2) (1 µg/ml). PPD (10 µg/ml), E6/C10 (2 µg/ml), or PHA (10 µg/ml) were added to the indicated samples. PBMC samples were also stimulated with *Mtb* latency antigens Rv2628 and Rv3407 (each 2 µg/ml) and were generally supplemented with human recombinant IL-7 (5 ng/ml). Samples were incubated for 2 h prior to addition of Brefeldin A (2.5 µg/ml; Sigma Aldrich). Subsequently, samples were incubated for about 20 h at 37°C and 5% CO_2. Afterwards, erythrocytes were lysed (RBC lysis buffer; Sigma Aldrich) for the whole blood assay. Samples were then fixed, permeabilized and stained with the following panel of αhuman antibodies: CD3 APC (clone UCTH1), CD4 PerCP/Cy5.5 (clone RPA-T4), IFN-γ PE (clone B27) and TNF-α FITC (clone Mab 11) (all Biolegend). Cells were acquired using BDAccuri C6 flow cytometer (BD Biosciences). The median number of $CD4^+$ T cells analyzed was 6142 (range: 1,120–20,671) for tuberculosis patients and 11,097 (range: 3,375–48,661) for HCs. The data were analyzed by FlowJo software (Version 10, FlowJo LLC). A representative example for the gating procedure is depicted in **Figure S1**. A threshold of

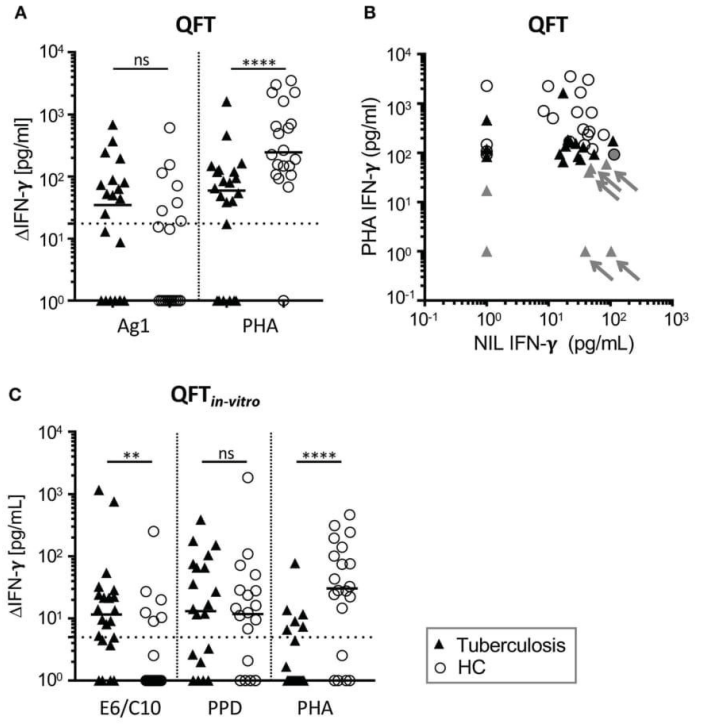

FIGURE 1 | High proportions of QFT and $QFT_{in\text{-}vitro}$ negative (or indeterminate) results from tuberculosis patients and healthy contacts (HCs). IFN-γ concentrations in supernatants of QFT **(A,B)** or the $QFT_{in\text{-}vitro}$ **(C)** whole blood overnight culture shown for tuberculosis patients (TB) ($n = 22$) and HCs ($n = 20$). Symbols indicate mean values of duplicates for individual tuberculosis patients (black triangles) and HCs (open circles). **(A,C)** Results for antigen-specific stimuli [Ag1 for QFT; ESAT6/CFP10 recombinant fusion protein (E6/C10), purified protein derivative of *Mtb* (PPD)] and PHA were subtracted for IFN-γ measured in the unstimulated sample (ΔIFN-γ). Significant differences are indicated by asterisks. Nominal *p*-values for the Mann-Whitney *U*-test (two-tailed) were calculated and shown as $^{**}p < 0.01$ and $^{****}p < 0.0001$. ns: not significant. **(B)** NIL (x-axis) and PHA (y-axis) induced IFN-γ concentrations of QFT supernatants are shown for TB patients and HCs. Individuals with indeterminate QFT results are indicated by gray color. Arrows indicate donors evaluated as indeterminate because of high IFN-γ NIL values and weak PHA response. PHA, phytohemaglutinin; NIL, Non-stimulated sample.

0.03 % IFN-γ producing $CD4^+$ T cells (three times higher than the assumed detection limit of 0.01% for flow cytometry) was set for classifying an individual donor as a "responder."

Two-Hit Assay

The two-hit assay has been performed [as described previously (19)] with minor modifications. In brief, 10^5 PBMCs were cultured in 200 µl RPMI supplemented with Penicillin/Streptomycin (100 U/ml), L-glutamine (2 mM), 10 mM HEPES buffer (all Gibco), 7.5% human serum (Sigma Aldrich) and 5 ng/ml of recombinant IL-7 (Sigma-Aldrich) in 96-U bottom plate. Cells were stimulated or left unstimulated with two *Mtb* antigens PPD (1µg/ml), ESAT6/CFP10 fusion protein (E6/C10; 1 µg/ml) and four latency antigens (i.e., Rv1733, Rv2628, Rv2031, and Rv3407; 1µg/ml). E6/C10 as well as the *Mtb* latency antigens are recombinant proteins produced in the laboratory of K. Franken and have been thoroughly used in previous studies (18, 19, 24). Samples were then incubated for 6 days at 37°C and 5% CO_2. On the sixth day, 100 µl of culture supernatants were discarded and samples were re-stimulated with the respective *Mtb* antigens (same concentrations as on d1) and Brefeldin A (3.75 µg/ml) (Sigma Aldrich) in reconstituted in fresh medium for 20 h. Afterwards, cells were then fixed, permeabilized and stained with the following panel of antibodies: CD3 APC (clone UCTH1), CD4 PerCP/Cy5.5 (clone RPA-T4), IFNγ PE (clone B27) and TNF-α FITC (clone Mab 11) (all BioLegend). Cells were measured as described above.

Statistical Analyses

Statistical analysis was performed using GraphPad prism Software v7 (Graphpad Software). A non-parametric Mann Witney U and Wilcoxon matched-pairs rank test were applied and indicated in the according figure legends. The Spearman Rank test was used to determine significant correlations between E6/C10 specific $CD4^+$ T-cell proportions and participant age for all donors and both study groups separately. Correlation

coefficients (r) and nominal p-values are given. Receiver Operating Characteristic (ROC) curve analysis was performed to evaluate the diagnostic performance of *Mtb* and latency antigens in the discrimination of active tuberculosis from HCs. Ternary plots were generated to determine qualitative difference of T-cell responses against *Mtb* E6/C10 and latency antigens using Grapher Software (Golden Software, LLC). Significance was considered at a p-value of ≤ 0.05.

RESULTS

Low Sensitivity of the QFT in Tuberculosis Patients and HCs

We performed QFT of tuberculosis patients ($n = 22$) and healthy contacts ($n = 20$) from Agogo in Ghana. Eight patients with tuberculosis (36%) and five contacts (25%) had positive QFT results (**Table 2**) according to manufacturer's criteria (**Table S1**). Notably, the majority of tuberculosis patients and contacts were negative or indeterminate (**Table 2**). Indeterminate results were more frequent in tuberculosis patients (32%) as compared to healthy contacts (5%) (**Table 2**). Generally impaired T-cell response of tuberculosis patients has been described and we found lower PHA-induced IFN-γ of tuberculosis patients (**Figure 1A**). In addition, differences in IFN-γ background (NIL) levels may affect result interpretation since NIL-subtracted Δ values are used for QFT evaluation (**Table S1**). The majority of donors had detectable IFN-γ values in the NIL sample and, in combination with impaired T-cell response of tuberculosis patients to PHA (**Figures 1A,B**), NIL IFN-γ_{high} background caused indeterminate QFT results in a subgroup of tuberculosis patients (**Figure 1B**). To investigate possible effects of NIL IFN-γ_{high} background on positive QFT results, we compared proportions of QFT positive individuals between tuberculosis patient and HC subgroups (i.e., NIL IFN-γ_{high} vs. NIL IFN-γ_{low}). Comparable proportions of NIL IFN-γ_{high} individuals between QFT positive and negative/indeterminate individuals did not indicate NIL IFN-γ effects on positive test results (**Figure S2**). We concluded that QFT diagnosed *Mtb* infection only for a minority of tuberculosis patients and HCs. In addition, impaired T-cell response in tuberculosis patients in combination with high NIL_{IFN}-γ levels hampered interpretation of QFT results.

A $QFT_{in\text{-}vitro}$ Assay Confirmed Marginal Sensitivity of QFT to Detect *Mtb* Infection

Since GeneXpert analyses confirmed *Mtb* infection for the majority of tuberculosis cases (including six of seven patients with indeterminate QFT results), we concluded that QFT results were at least partially false negative. Suboptimal assay conditions may lead to low assay sensitivity as a possible reason for negative test results. To analyse this, we established an *in vitro* assay based on recombinant *Mtb* antigen-specific *in vitro* culture and IFN-γ measure. Initially, we applied QFT comparable conditions and measured IFN-γ concentrations in the supernatant of E6/C10, PPD, and PHA stimulated T cells ($QFT_{in\text{-}vitro}$). IFN-γ concentrations were generally lower in the $QFT_{in\text{-}vitro}$ assay as compared to the QFT (**Figure 1**) and E6/C10 specific IFN-γ concentrations in HCs were significantly lower as compared to tuberculosis patients (**Figure 1C**; $p = 0.004$). To facilitate direct comparison of both assays, we adjusted the threshold of positive results for the $QFT_{in\text{-}vitro}$ assay (according to PHA induced median IFN-γ; **Figure 1C**) and classified tuberculosis patients and contacts as positive, indeterminate, or negative in both assays (**Table S2**). Proportions were largely comparable between the assays and, like for the QFT, high numbers of tuberculosis patients and HCs were indeterminate or negative in the $QFT_{in\text{-}vitro}$ assay (**Table 2**).

Moderately Increased PPD-Specific $CD4^+$ T-Cell Proportions Detected by $ICS_{in\text{-}vitro}$

To exclude possible effects of bystander IFN-γ production and/or differential IFN-γ serum levels that may affect QFT and $QFT_{in\text{-}vitro}$, we next performed intracellular IFN-γ measurements in *Mtb* specific T cells in whole blood (ICS_{Blood}) as well as purified PBMCs (ICS_{PBMCs}). Proportions of $CD4^+$ IFN-γ positive T cells largely reflected results from QFT/$QFT_{in\text{-}vitro}$ showing significantly higher responses for PHA in HCs as compared to tuberculosis patients for ICS_{Blood} and ICS_{PBMCs} (**Figures 2A,B**). To compare the obtained results between different assays, we set a threshold of 0.03% IFN-γ positive $CD4^+$ T cells to classify "responders" to respective stimuli (**Figures 2A,B**; **Table 3**). Similar proportions of E6/C10 responders were found for tuberculosis patients in different assays [$QFT_{in\text{-}vitro}$: 9 (41%); ICS_{Blood}: 9 (41%); ICS_{PBMC}: 11 (50%)] (**Table 3**). For HCs, the proportions of responders were higher in both ICS assays [ICS_{Blood}: 11 (55%); ICS_{PBMC}: 8 (40%)] as compared to the $QFT_{in\text{-}vitro}$ [4 (20%)]. PPD responders were generally more frequent than E6/C10 responders (**Table 3**) and ICS assays detected higher proportions of PPD responders (77 to 86%) as compared to $QFT_{in\text{-}vitro}$ (45 %) (**Table 3**). These results indicated that additional *Mtb* antigens may improve sensitivity and that all examined short-term assays showed suboptimal sensitivity to detect *Mtb* infection using E6/C10 antigen.

Two-Hit Stimulation Improves Detection of E6/C10 and Latency Antigen-Specific T Cells

Previously, we demonstrated that seven days of *in vitro* culture including two-hit stimulation enhanced sensitivity for detection of IFN-γ expressing T cells and enabled identification of T-cell response against *Mtb* latency antigens (19). Importantly, this "two-hit" assay did not prime *Mtb* specific T-cell response in the absence of previous *Mtb* infection (19). Two-hit stimulation was performed with PPD, E6/C10, and selected *Mtb* latency antigens (i.e., Rv2628, Rv1733, Rv2031, Rv3407) of tuberculosis patients and HCs. Tuberculosis patients and HCs had comparable proportions of PPD- or E6/C10- specific $CD4^+$ T cells (**Figure 2C**). Classification of responders (>0.03% IFN-γ^+ $CD4^+$ T cells) revealed that the vast majority of tuberculosis patients and HCs responded to E6/C10 in the two-hit assay (tuberculosis patients: 18 (82%); HCs 16 (80%); **Figure 2C**). For

TABLE 2 | QFT and $QFT_{in\text{-}vitro}$ evaluation of test results.

	QFT			$QFT_{in\text{-}vitro}$		
	Positive *n* (%)	**Indeterminate *n* (%)**	**Negative *n* (%)**	**Positive *n* (%)**	**Indeterminate *n* (%)**	**Negative *n* (%)**
TB ($n = 22$)	8 (36%)	7 (32%)	7 (32%)	9 (41%)	9 (41%)	4 (18%)
HC ($n = 20$)	5 (25%)	1 (5%)	14 (70%)	4 (20%)	4 (20%)	12 (60%)

n, number.

PPD, all HCs and 21 (96%) of the tuberculosis patients showed a positive response in the two-hit assay (**Figure 2C**).

For latency antigens, two-hit stimulation with Rv2628 and Rv1733 induced IFN-γ producing T cells in the majority of individuals, whereas smaller subgroups had specific T cells for Rv2031 and Rv3407 (**Figure 2D**). Notably, and in contrast to E6/C10, Rv2628, Rv1733, and Rv3407 specific T cells were significantly more frequent in HCs as compared to tuberculosis patients (Rv2628: $p < 0.0001$; Rv1733: $p = 0.0003$; Rv3407: $p = 0.039$; **Figure 2D**). Classification of responders revealed that all HCs responded to Rv1733 and 18 (90%) of HCs to Rv2628 (**Figure 2D**). In contrast, only 11 (50%) and 7 (32%) of tuberculosis patients were responders to Rv1733 or Rv2628, respectively (**Figure 2D**).

To determine whether *Mtb* latency antigens could also be used to detect *Mtb* infection in short-term assays, we selected two candidates (i.e., Rv2628, Rv3407) to perform additional ICS_{PBMC} assays. IFN-γ^+ $CD4^+$ T cells were detectable for both latency antigens (**Figure S3**) and proportions were comparable to E6/C10 in the same assay (**Figure 2B**; **Table 3**). As for E6/C10, however, considerable proportions of tuberculosis patients ($n = 11$, 50%) and HCs ($n = 10$, 50%) were negative for Rv2628 specific T-cell responses and even less individuals had detectable T-cell responses against Rv3407 after short-term stimulation (**Figure S3**). In addition, there was no significant difference between tuberculosis patients and HCs (**Figure S3**). We concluded that short-term stimulation was also suboptimal for *Mtb* latency antigens Rv2628 and Rv3407 and focused on the two-hit assay for further analyses.

Since individuals from both study groups varied markedly in age at recruitment, we determined possible age-dependent effects on two-hit results. No correlation was seen between age and two-hit E6/C10 responses (**Figure S4A**). In addition, exclusion of children/adolescents (below 18 years) from analyses had no detectable effects on two-hit results (**Figure S4B**). Therefore, age-dependent effects on T-cell response in two-hit assays were not found.

$QFT_{in\text{-}vitro}$ Negative Tuberculosis Patients and LTBI Are Predominantly Promoted in the Two-Hit Assay

False negative (or indeterminate) QFT results of tuberculosis patients are a major obstacle in the diagnosis of active tuberculosis especially if direct proof of *Mtb* infection is not possible. Furthermore, negative QFT results preclude identification of LTBI within HCs. To address the question whether E6/C10 results from the two-hit assay could be used for detection of *Mtb* infection in QFT negative individuals, we compared short-term ICS_{PBMC} with two-hit results (**Figure 2E**) and evaluated the median fold change (**Table 4**) for the study cohorts. A significant increase of E6/C10 specific T-cell proportions was found in the long-term two-hit assay as compared to the short-term ICS_{PBMC} assay for both study groups (tuberculosis patients: $p < 0.0001$; HCs: $p = 0.0003$; **Figure 2E**). Notably, ICS_{PBMC} non-responders within both study groups showed significantly enhanced E6/C10 specific T-cell responses (tuberculosis: median fold-change 33.7; HCs: median fold-change 42.8; **Table 4**) whereas E6/C10 ICS_{PBMC} responders were hardly affected by two-hit stimulation (tuberculosis: median fold-change: 1.2; HCs: median fold-change 1.3) (**Table 4**). In accordance, 10 of 13 (77 %) of the ICS_{PBMC} negative tuberculosis patients and 12 of 16 (75 %) of the ICS_{PBMC} negative HCs were classified as responders in the two-hit assay.

Short-Term Assay Non-responders Are Detected by Two-Hit Rv2628/Rv1733 Stimulation

To evaluate the capacity of Rv2628 and Rv1733 to detect *Mtb* infection in E6/C10 non-responders, we compared ICS_{PBMCs} values with the two-hit results for Rv2628 and Rv1733 between the study groups. Tuberculosis patients showed heterogeneous responses against both latency antigens compared to E6/C10 whereas HCs had markedly higher T-cell proportions against Rv1733 and Rv2628 (**Figure 2F**). Notably, ICS_{PBMCs} non-responders from both study groups showed a significantly stronger T-cell response against Rv1733 and Rv2628 in the two-hit assay as compared to $ICS_{in\text{-}vitro}$ responders (tuberculosis patients, Rv2628 $p = 0.03$, Rv1733 $p = 0.02$; HCs, Rv2628 $p = 0.008$, Rv1733 $p = 0.002$) (**Table 4**). Four tuberculosis patients and three HCs were non-responders in both, E6/C10 specific ICS_{PBMCs} and two-hit, assays. Inclusion of Rv1733 two-hit results confirmed *Mtb* infection in all E6/C10 non-responder HCs of this subgroup and two of four tuberculosis patients. Therefore, inclusion of latency antigen specific T-cell responses in the two-hit assay may increase sensitivity for detection of *Mtb* infection.

Mtb Latency Antigens Distinguish LTBIs From Tuberculosis Patients

Discrimination of tuberculosis patients from HCs (especially LTBIs) has important implications e.g., for intervention

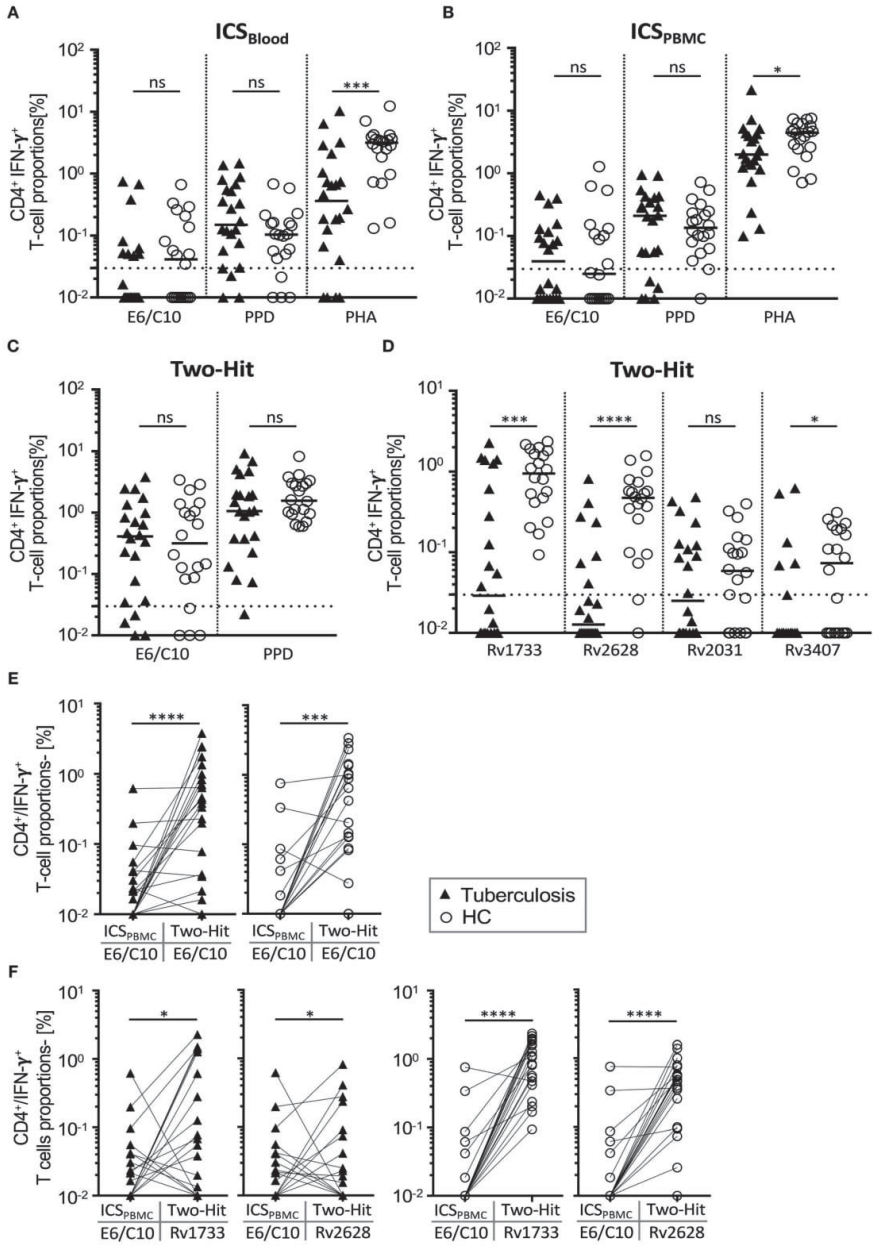

FIGURE 2 | IFN-γ positive CD4$^+$ T-cell proportions specific for E6/C10 and latency Mtb antigens after short-term and two-hit *in vitro* culture. IFN-γ positive CD4$^+$ T cells of TB patients and HCs were measured by intracellular flow cytometry after stimulation short-term **(A,B)**, long-term culture (two-hit) **(C,D)** or comparison between both **(E,F)**. E6/C10, PPD, or PHA were used in short-term whole blood **(A)** or purified PBMCs culture **(B)**. E6/C10, PPD, and latency antigens (i.e., E6/C10, Rv1733, Rv2628, Rv2031, or Rv3407) were used in long-term two-hit stimulation **C,D**). Symbols indicate mean values of duplicates for individual tuberculosis patients (black triangles) and HCs (open circles). **(E,F)** Paired comparison of short-termE6/C10 specific CD4$^+$ T-cell proportions with long-term two-hit stimulation for E6/C10 **(E)** and Rv1733, Rv2628 **(F)** in TB patients (left graphs) and HCs (right graphs). Significant differences are indicated by asterisks. Nominal *p*-values for the Mann-Whitney *U*-test (two-tailed) were calculated and shown as $^*p < 0.05$; $^{***}p < 0.001$ and $^{****}p < 0.0001$. ns, not significant.

TABLE 3 | $QFT_{in\text{-}vitro}$ comparison with intracellular cytokine-based assays.

	E6/C10 responder			PPD responder		
	$QFT_{in\text{-}vitro}$ *n* (%)	ICS_{Blood} *n* (%)	ICS_{PBMC} *n* (%)	$QFT_{in\text{-}vitro}$ *n* (%)	ICS_{Blood} *n* (%)	ICS_{PBMC} *n* (%)
TB (n=22)	9 (41%)	9 (41%)	11 (50%)	10 (45%)	19 (86%)	17 (77%)
HCs (n=20)	4 (20%)	11 (55%)	8 (40%)	9 (45%)	16 (80%)	18 (82%)

ICS, intracellular cytokine analysis; PPD, purified protein derivatives of Mtb.

TABLE 4 | Two-hit results for categorized strong and weak E6/C10 responders.

	TB		HCs	
E6/C10, d1:	**Low**	**High**	**Low**	**High**
E6/C10, fold-change median (range)	33.7 (0.4–381.1)	1.2 (0.8–31.6)	42.8 (1.0–341.2)	1.3 (0.32–10.6)
Rv1733, fold-change median (range)	5.5 (0.5–149.5)	0.4 (0.02–14.71)	106.8 (9.4–237.4)	3.3 (0.6–19.4)
Rv2628, fold-change median (range)	1.5 (0.5–41.0)	0.33 (0.02–8.5)	46.1 (1.0–139.5)	1.5 (1.0–11.7)

R, range; fc, fold-change.

strategies. Therefore, we next determined the capacities of Rv2628 and Rv1733 to classify tuberculosis patients and LTBIs. ROC analyses showed robust classification efficacy for Rv2628 (AUC: 0.88, $p < 0.0001$) and Rv1733 (AUC: 0.81, $p = 0.0006$) (**Figure 3A**). To validate classification results we included an independent test cohort of tuberculosis patients ($n = 4$) and HCs ($n = 13$). ROC analyses verified the discrimination capacity of Rv2628 (AUC:0.98, $p = 0.0046$) whereas Rv1733 (AUC:0.67, $p = 0.30$) was less effective in the test cohort (**Figure 3B**). In conclusion, T-cell responses against Rv2628 from the two-hit assay effectively discriminated HCs from tuberculosis patients.

Mtb Antigen Specificity Pattern Differ Between QFT-Negative LTBIs and Tuberculosis Patients

Finally, we characterized qualitative differences of T-cell responses against E6/C10, Rv2628, and Rv1733 for QFT low (or indeterminate) individuals from both study groups in the two-hit assay. The sum of individual T-cell proportions specific for any of the three antigens was set to one and relative contribution of individual antigens is calculated. None of the individuals had a dominant (more than 50 % of IFN-γ positive T cells) Rv2628 specific T-cell response (open triangle region). HCs predominantly had either a Rv1733 dominant response (blue triangle region) or no dominance (center) (**Figure 4**, upper graph). Interestingly, tuberculosis patients showed two main phenotypes (**Figure 4**, lower graph). The majority had an E6/C10 dominant T-cell response (red triangle) but a considerable subgroup of QFT negative tuberculosis patients, 5 of 14 (36 %), had a Rv1733 dominant T-cell response (blue triangle). We concluded that *Mtb* latency antigens and especially Rv1733 may improve detection of *Mtb* infection in HCs and tuberculosis patients without detectable E6/C10 specific T cells.

DISCUSSION

In this pilot study we demonstrate the capacity of a two-hit long-term *in vitro* assay to improve detection of *Mtb* infection of tuberculosis patients and HCs from Ghana. QFT results were negative or indeterminate for the majority of tuberculosis patients and healthy contacts. This outcome confirmed own previous studies that showed low sensitivity of QFT tests for detection of *Mtb* infection in children with tuberculosis from Ghana (6). In contrast, a parallel study in children with tuberculosis and LTBI from Germany showed almost optimal sensitivity of the QFT (7). This suggested an effect occurring in a tuberculosis high-endemic country like Ghana and raised the question if low sensitivity was specific for children and/or acute tuberculosis. Here, we show that low QFT sensitivity is also found in adults from Ghana and that both, tuberculosis patients and HCs, were affected. For tuberculosis patients, impaired T-cell responses to PHA stimulation was seen as an additional effect that increased the frequency of indeterminate test results in combination with high IFN-γ background. Generally impaired T-cell functions has been described and this may contribute to low QFT sensitivity for *Mtb* specific T-cells (25).

In the present study, we established a QFT comparable *in vitro* assay ($QFT_{in\text{-}vitro}$) to characterize mechanisms involved suboptimal QFT sensitivity. It turned out that intracellular IFN-γ measurements [after including costimulatory antibodies (23)] and purification of PBMCs only marginally improved sensitivity for *Mtb* specific T cells for healthy contacts. However, intracellular IFN-γ measurements in purified PBMCs largely reversed impaired T-cell response to PHA in tuberculosis patients, indicating that serum factors at least partially accounted for impaired PHA induced T-cell response. A possible explanation would be high levels of inflammatory and regulatory cytokines found in serum of patients with acute tuberculosis (26, 27). Recently, we showed

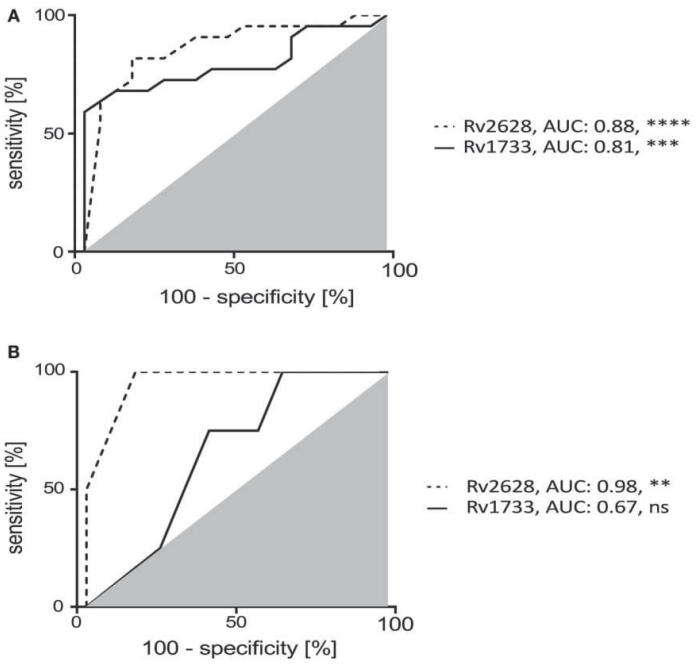

FIGURE 3 | Two-hit assay induced Mtb latency antigen specific IFN-γ positive $CD4^+$ T cells discriminate TB patients and HCs. Receiver Operator Characteristic (ROC) analysis for classification of TB patients and HCs using Rv1733 and Rv2628 specific IFN-γ positive $CD4^+$ T cells of TB patients and HCs after two-hit stimulation. Area Under Curve (AUC) values of ROC analysis for the study groups **(A)** and an independent test cohort **(B)** [TB patients ($n = 4$) and HCs ($n = 13$)]. Deduced AUC-values and nominal p-values are shown as $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. ns: not significant.

that high serum IL-10 and IL-6 levels were accompanied by constitutive STAT3 phosphorylation and SOCS3 expression (27). Especially SOCS3 correlated negatively with T-cell IFN-γ production and may therefore contribute to low PHA response (27).

Intracellular IFN-γ measurements also improved detection of *Mtb* PPD-specific T cells as compared to the $QFT_{in\text{-}vitro}$ assay. This suggested that inclusion of additional antigens could improve the sensitivity and that short-term assay conditions may be suboptimal for detection of *Mtb* infection in Ghana. Several studies indicated higher sensitivity of long-term (i.e., five to seven days) *in vitro* stimulation using latency *Mtb* antigens (20, 21). These antigens have been well characterized as immunodominant in different *Mtb* exposed populations across the world (28–32).

Previously, we demonstrated that the two-hit assay detected T-cell responses against latency *Mtb* antigens in LTBIs as well as in a minor subgroup of tuberculosis patients but not in IGRA-negative controls (19). We did not include controls without a known recent history of *M. tuberculosis* contact in the present study since identification of non-*M. tuberculosis* infected controls is difficult in a country with high tuberculosis prevalence like Ghana. In addition, we demonstrated that the QFT test fails to detect T-cell responses in confirmed *Mtb* infected patients and, hence, QFT negative results do not reliably exclude *Mtb* infection in Ghana. Like for most Sub-Saharan countries, children are BCG vaccinated at birth and this limits the significance of the TST. Against this background, large cohorts are needed to evaluate differences between potential LTBI (IGRA-positive or negative) and IGRA-negative donors (with an unknown history of *Mtb* infection) with sufficient statistical power. This was not possible as part of this pilot study but will be performed as part of a follow-up study. We concluded from the present study that QFT tests do not reliably exclude or confirm *Mtb* infection in Ghana and that more sensitive assays are needed to diagnose *Mtb* infection. The two-hit assay is a candidate for an immune test with higher sensitivity but needs to be evaluated by future studies.

In the present study, two-hit stimulation with the same respective antigen markedly enhanced T-cell response against E6/C10 in both study groups. Notably, tuberculosis and HCs without T-cell response in short-term assays benefited most from the re-stimulation with E6/C10. Previous studies predominantly focused on IGRA positive tuberculosis patients and healthy control cohorts to determine *Mtb* latency antigen specific T-cell responses (9). QFT/IGRA negative (or indeterminate) individuals were excluded in the majority of these studies, to avoid inclusion of misdiagnosed tuberculosis patients and

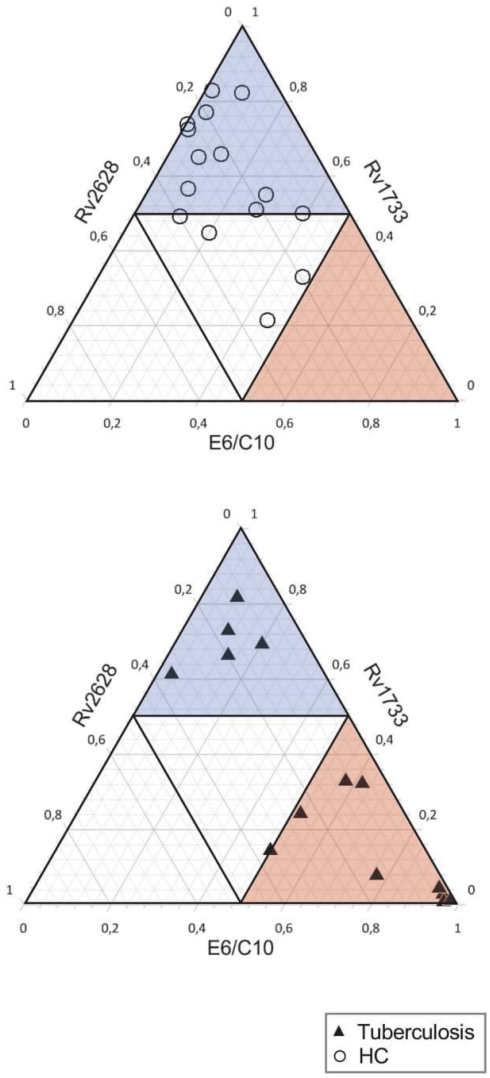

FIGURE 4 | E6/C10 and Rv1733 dominant T-cell recognition pattern from the two-hit assay indicate two distinct subgroups in QFT negative (or indeterminate) tuberculosis patients. Ternary plots depict relative T-cell proportions specific for E6/C10, Rv1733, and Rv2628 of HCs (upper graph) and tuberculosis patients (lower graph) with low (indeterminate) QFT response. The sum of all IFN-γ positive T cells after two-hit stimulation with one of these antigens is set to 1. Dominant results (defined as > 50% positive T cell) are indicated as open triangle for Rv2628, blue transparent triangle for Rv1733, and red transparent triangle for E6/C10. Each symbol depicts results for an individual tuberculosis patient (black triangle) or HC (open circle).

non-*Mtb* infected HCs. But by implication, these studies excluded tuberculosis patients and HCs that were false negative in QFT/IGRA. Since previous studies clearly demonstrated that also IFN-γ negative $CD4^+$ T cells producing alternative cytokines contribute to immunity against *Mtb* (6, 30), we hypothesize that negative QFT/IGRA results do not preclude Mtb infection.

Immune-based assays with high sensitivity are of paramount importance to allow early interventions strategies especially in *Mtb* infected HCs with high risk to develop active tuberculosis (e.g., young children, immune compromised patients). For young children, BCG effects on *Mtb* antigen specific immune responses are generally possible but previous studies did not see effects of BCG vaccination on T-cell responses against latency antigens (33, 34).

To our knowledge this is the first study to show that long-term *in vitro* culture with two-hit stimulation may improve detection of *Mtb* infection in QFT negative (or indeterminate) tuberculosis patients and HCs. The current study design did not allow to directly prove of *Mtb* infection for HCs. However, we used strict inclusion criteria for HCs to ensure tight and long-term contact to a known index patient, this way strongly increasing the likelyhood of being *Mtb* infected. Therefore, we assume that at least the majority of HCs are LTBI as indicated by the two-hit assay. Future studies -including follow-up of HCs and identification of tuberculosis progressors- will address the question if differences in the response against E6/C10 in two hit assays may contribute to diagnosis of *Mtb* infection in HCs.

For HCs, future studies will determine if high sensitivity of two-hit responses (100% in our cohort) is accompanied by high specificity to detect LTBI within HCs. These results may contribute to the decision about early prevention therapy in highly tuberculosis susceptible individuals.

Several studies have addressed the question if *Mtb* latency antigens can be used as T-cell targets of immune assays in *Mtb* infection [reviewed in (9)].We selected the most promising candidates from those and own previous studies (19). Rv2628 and Rv1733 were identified by others (12, 14–16) and also the capacity to discriminate tuberculosis patients from LTBI has been described for Rv2628 (15, 18). Our results confirm these studies and render Rv2628 and Rv1733 proteins most promising candidates for *Mtb* immune assays. T-cell responses were less frequently found for Rv2031 and Rv3407, although differences between the study groups for Rv3407 were detected. In general, the overlap of promising latency antigen candidates found in previous studies was moderate (9). This may be due to differences in the genetic background of the study populations examined and different assay types used. Application of a small group of latency antigens covering this heterogeneity and optimization of assay conditions for individual antigens could circumvent this problem. In addition, a group of antigen candidates can include antigens which are more generally recognized (e.g., Rv1733, E6/C10) as well as those with a higher capacity to discriminate (e.g., Rv2628).

Future studies will need to address the question whether immune response against latency antigens, Rv2628 and Rv1733, can help to predict tuberculosis disease progression

in recently *Mtb* infected HCs. This way immune correlates of tuberculosis risk vs. reduced risk can be identified with implications for treatment intervention and vaccine design strategies. This long-term stimulation and the requirement of sophisticated flow cytometry measurement restricts the applicability of this assay for clinical routine. Therefore, this test may only be performed in well-equipped research laboratories accessible for few hospitals in Africa like in Kumasi/Ghana. Especially, potentially *Mtb* infected individuals with high risk of tuberculosis disease progression (i.e., young children, HIV co-infected individuals, patients treated with anti-TNFα immune modulatory drugs) may benefit from this test.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

The present study received approval from the Committee on Human Research, Publication and Ethics (CHRPE/AP/023/18; CHRPE/221/14) at the School of Medical Sciences (SMS) at the Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, Ghana. All study subjects gave written informed consent prior to recruitment and for children written informed consent was provided by their parents or legal guardians.

AUTHOR CONTRIBUTIONS

EA, CL, and AG performed the experiments and contributed to analyses. EM, EO-D, RP, NN, and MJ designed the study. EA, EO-D, and RP recruited patients and contacts. KF and TO provided antigens and expertise. NN, RP, and MJ supervised the study. EA and MJ analyzed the data and wrote 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.2019.01518/full#supplementary-material

REFERENCES

1. Kaufmann SH. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol.* (2001) 1:20–30. doi: 10.1038/35095558
2. Lalvani A. Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. *Chest.* (2007) 131:1898–906. doi: 10.1378/chest.06-2471
3. Marais BJ, Donald PR, Gie RP, Schaaf HS, Beyers N. Diversity of disease in childhood pulmonary tuberculosis. *Ann Trop Paediatr.* (2005) 25:79–86. doi: 10.1179/146532805X45665
4. Pai M, Riley LW, Colford JM Jr. Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. *Lancet Infect Dis.* (2004) 4:761–76. doi: 10.1016/S1473-3099(04)01206-X
5. Auguste P, Tsertsvadze A, Pink J, Court R, McCarthy N, Sutcliffe P, et al. Comparing interferon-gamma release assays with tuberculin skin test for identifying latent tuberculosis infection that progresses to active tuberculosis: systematic review and meta-analysis. *BMC Infect Dis.* (2017) 17:200. doi: 10.1186/s12879-017-2301-4
6. Lundtoft C, Awuah AA, Nausch N, Enimil A, Mayatepek E, Owusu-Dabo E, et al. Alternative Quantiferon cytokines for diagnosis of children with active tuberculosis and HIV co-infection in Ghana. *Med Microbiol Immunol.* (2017) 206:259–65. doi: 10.1007/s00430-017-0501-6
7. Nausch N, Lundtoft C, Schulz G, Henckel H, Mayatepek E, Fleischer B, et al. Multiple cytokines for the detection of Mycobacterium tuberculosis infection in children with tuberculosis. *Int J Tuberculosis Lung Dis.* (2017) 21:270–7. doi: 10.5588/ijtld.16.0351
8. Coppola M, Ottenhoff TH. Genome wide approaches discover novel Mycobacterium tuberculosis antigens as correlates of infection, disease, immunity and targets for vaccination. *Semin Immunol.* (2018) 39:88–101. doi: 10.1016/j.smim.2018.07.001
9. Meier NR, Jacobsen M, Ottenhoff THM, Ritz N. A systematic review on novel mycobacterium tuberculosis antigens and their discriminatory potential for the diagnosis of latent and active tuberculosis. *Front Immunol.* (2018) 9:2476. doi: 10.3389/fimmu.2018.02476
10. Govender L, Abel B, Hughes EJ, Scriba TJ, Kagina BM, de Kock M, et al. Higher human CD4 T cell response to novel Mycobacterium tuberculosis latency associated antigens Rv2660 and Rv2659 in latent infection compared with tuberculosis disease. *Vaccine.* (2010) 29:51–7. doi: 10.1016/j.vaccine.2010.10.022
11. Delogu G, Chiacchio T, Vanini V, Butera O, Cuzzi G, Bua A, et al. Methylated HBHA produced in M. smegmatis discriminates between active and non-active tuberculosis disease among RD1-responders. *PLoS ONE.* (2011) 6:e18315. doi: 10.1371/journal.pone.0018315
12. Chegou NN, Black GF, Loxton AG, Stanley K, Essone PN, Klein MR, et al. Potential of novel *Mycobacterium tuberculosis* infection phase-dependent antigens in the diagnosis of TB disease in a high burden setting. *BMC Infect Dis.* (2012) 12:10. doi: 10.1186/1471-2334-12-10
13. Chiacchio T, Delogu G, Vanini V, Cuzzi G, De Maio F, Pinnetti C, et al. Immune characterization of the HBHA-specific response in Mycobacterium tuberculosis-infected patients with or without HIV infection. *PLoS ONE.* (2017) 12:e0183846. doi: 10.1371/journal.pone.0183846
14. Chen J, Su X, Zhang Y, Wang S, Shao L, Wu J, et al. Novel recombinant RD2- and RD11-encoded Mycobacterium tuberculosis antigens are potential candidates for diagnosis of tuberculosis infections in BCG-vaccinated individuals. *Microbes Infect.* (2009) 11:876–85. doi: 10.1016/j.micinf.2009.05.008

3.1. Paper II: Constitutive STAT3 phosphorylation and IL-6/IL-10 co-expression are associated with impaired T-cell function in tuberculosis patients

Harling, K., Adankwah, E., Güler, A., Afum-Adjei Awuah, A., Adu-Amoah, L., Mayatepek, E., Owusu-Dabo, E., Nausch, N. & Jacobsen, M. Constitutive STAT3 phosphorylation and IL-6/IL-10 co-expression are associated with impaired T-cell function in tuberculosis patients. *Cellular and Molecular Immunology.* 2018;16(3):275-87

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- ELISA
- CBA
- Data analysis
- Writing of the manuscript

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ARTICLE

Constitutive STAT3 phosphorylation and IL-6/IL-10 co-expression are associated with impaired T-cell function in tuberculosis patients

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T-cells critically contribute to protection against *Mycobacterium tuberculosis* infection, and impaired T-cell responses can lead to disease progression. Pro-inflammatory and immunosuppressive cytokines affect T-cells, and fine-tuned regulation of cytokine signaling via the Jak/STAT signaling pathways is crucial for appropriate T-cell function. Constitutive STAT3 phosphorylation as a consequence of aberrant cytokine signaling has been described to occur in pathognomonic T-cell responses in inflammatory and autoimmune diseases. We characterized blood samples from tuberculosis patients ($n = 28$) and healthy contacts ($n = 28$) from Ghana for *M. tuberculosis*-specific T-cell responses, constitutive cytokine production, and SOCS3 and pSTAT3 expression. Lentiviral modulation of primary CD4$^+$ T-cells was performed to determine the effects of SOCS3 on T-cell functions. T-cells from tuberculosis patients expressed higher levels of IL-10 and IL-6 and lower levels of T helper type (T_H)17 cytokines after *M. tuberculosis*-specific stimulation compared to healthy contacts. In addition, tuberculosis patients had higher IL-10 and IL-6 levels in the supernatants of non-stimulated immune cells and plasma samples compared to healthy contacts. Notably, aberrant cytokine expression was accompanied by high constitutive pSTAT3 levels and SOCS3 expression in T-cells. Multivariate analysis identified an IL-6/IL-10 co-expression-based principal component in tuberculosis patients that correlated with high pSTAT3 levels. SOCS3 contributed to a regulatory component, and tuberculosis patients with high SOCS3 expression showed decreased T_H1 cytokine expression and impaired IL-2-induced STAT5 phosphorylation. SOCS3 over-expression in primary CD4$^+$ T-cells confirmed the SOCS3 inhibitory function on IL-2-induced STAT5 phosphorylation. We conclude that constitutive pSTAT3 and high SOCS3 expression are influential factors that indicate impaired T-cell functions in tuberculosis patients.
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Keywords: tuberculosis; Interleukin-6; Interleukin-10; STAT3; SOCS3

INTRODUCTION

Tuberculosis is a chronic infectious disease caused by *M. tuberculosis*. *M. tuberculosis* is transmitted via aerosols exhaled by tuberculosis patients and eventually inhaled by close contacts. Immune surveillance protects the vast majority of infected individuals, and only a minority of *M. tuberculosis*-infected individuals develops tuberculosis disease. T-cells are crucial for protection against *M. tuberculosis*, and impaired T_H1 immunity is specifically associated with an increased risk of developing tuberculosis disease.[1] The key T_H1 cytokine IFN-γ produced by *M. tuberculosis*-specific T-cells is a well-established marker for detection of *M. tuberculosis* infection. However, quantification of IFN-γ expression of T-cells from *M. tuberculosis*-infected healthy donors is neither sufficient to predict the risk of developing tuberculosis nor reliable as a biomarker indicating acute disease.[2] Additional candidate biomarkers have been identified that may indicate disease mechanisms and T-cell failure to protect against tuberculosis more adequately.[2] Several studies have reported higher IL-6 expression by T-cells and increased IL-6 serum concentrations in tuberculosis

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patients.[3–8] Most of these studies concomitantly detected higher IL-10 serum concentrations in tuberculosis patients.[3–7] Both IL-6 and IL-10 are produced by T-cells (as well as by other immune cell subsets) and directly affect T-cell functions via receptor signaling.[9] IL-6 is a mediator of inflammation, and its crucial role in inflammatory and auto-immune diseases is well-established.[10] Modulation of IL-6 availability and signaling of immune cells has been proven to be an efficient therapeutic approach, such as for rheumatoid arthritis.[11] During T-cell polarization, IL-6 is of central importance for the generation of IL-17-producing T_H17 cells, which are major immune drivers of inflammation.[12] Furthermore, IL-6 inhibits the generation of regulatory T-cells, the main immunosuppressive T-cell population.[13] IL-10 is a central effector molecule of immune regulation and characterizes type 1 regulatory T-cells in particular.[14] Originally described as a T_H2-related cytokine, IL-10 plays an important role in balancing immunity and limiting inflammatory responses. Consistent with this role, IL-10-deficient mice develop spontaneous inflammatory bowel disease.[15] Many sources of IL-10 have been described, including T_H1 and T_H17 effector cells, which can produce IL-10 in an IL-6- or IL-27-dependent manner.[16] In chronic viral infections, IL-10 expression by T_H1 cells has been associated with antigen persistence and self-limiting immune responses.[17,18]

IL-6 and IL-10 signal via cellular receptor complexes that lack inherent signaling activity. Therefore, recruitment of subsidiary cofactors is required, and signal transduction function is mainly exerted by Jak/STAT pathway molecules for IL-6 and IL-10 receptors.[19] Both IL-6 and IL-10 receptors specifically interact with Jak1, leading to recruitment, phosphorylation, and dimerization of the transcription factor STAT3.[19] Similar transcription factor usage by both cytokines led to the obvious question of how IL-6 and IL-10 promote opposite cellular functions. Differences between immune cell subpopulations, including distinct IL-6/IL-10 receptor expression, may partially explain this phenomenon.[9,20] Another important difference between IL-6 and IL-10 receptor signaling is exerted at the level of feedback regulation. IL-6 and IL-10 both induce expression of the inhibitory molecule SOCS3; however, whereas SOCS3 inhibits IL-6 signaling (by interacting with the gp130 receptor chain[21]), IL-10 receptor signaling is not affected by SOCS3 expression.[22] Consistently, IL-10 induces prolonged STAT3 phosphorylation compared to IL-6.[23] SOCS3 plays a role in multiple processes affecting T-cell functions.[24] In addition to regulation of T-cell polarization, SOCS3 also inhibits T-cell receptor activation and proliferation.[25,26] These SOCS3 effects are exerted via inhibition of the T-cell receptor, the CD28 co-receptor, and IL-2R signaling.[25–28] SOCS3 is strongly implicated in host immune responses against *M. tuberculosis* infection.[29] Previous studies have also reported increased levels of SOCS3 RNA in T-cells from tuberculosis patients,[8,30,31] and effects of SOCS3 in resistance against tuberculosis have been identified in animal models.[20]

The goal of this study was to elucidate possible roles of aberrant cytokine expression and cytokine receptor signaling events on T-cell functions during tuberculosis pathogenesis in humans. To this end, we determined T-cell and plasma cytokine production levels *in vitro*, as well as STAT phosphorylation and SOCS3 protein expression levels in T-cells from tuberculosis patients and healthy contacts. Principal component analysis was performed to identify underlying complex profiles that distinguished tuberculosis patients and healthy contacts. Functional T-cell assays and modulation of SOCS3 expression were performed to elucidate impaired effector T-cell functions in human tuberculosis patients.

Table 1 Study group characteristics

	TB	*Contacts*
Number of Persons (N)	19	23
Median age, years (range)	39.5 (20–77)[a]	40.0 (21–65)
Mean age, years	40.7	39.8
Male/Female (% male)	13/6 (68)	9/14 (39)

[a]one value missing.

MATERIALS AND METHODS

Donor recruitment

We recruited adult tuberculosis patients ($n=28$) and exposed household contacts without symptoms of tuberculosis (hereafter referred to as 'healthy contacts' throughout the manuscript) ($n=28$) in an observational hospital-based study. Tuberculosis patients ($n=5$) and healthy contacts ($n=2$) with known HIV infection, as well as individuals with incomplete experimental data sets (i.e., tuberculosis patients ($n=4$); healthy contacts ($n=3$)), were excluded from this study. The study group characteristics of the ultimately included tuberculosis patients ($n=19$) and healthy contacts ($n=23$) are shown in Table 1. Tuberculosis patients were recruited at the Komfo Anokye Teaching Hospital (KATH), the Kwame Nkrumah University of Science and Technology (KNUST) Hospital, the Suntreso Government Hospital (SGH), and the Manhyia Hospital, all in Ghana, between 2015 and 2016. Healthy contacts had lived in the same household as tuberculosis index patients for over four months prior to diagnosis. Diagnosis of tuberculosis was based on patient history, chest X-ray, and sputum smear test. Chemotherapy according to the Ghanaian guidelines was initiated immediately after blood samples (approximately 10 ml) were taken. Individual donor samples from these study groups have been included for functional T-cell assays and analyses of IL-6 plasma levels published previously.[31]

Ethical statement

The study was approved by the Committee on Human Research, Publication and Ethics (CHRPE/221/14, CHPRE/AP/328/15) at the School of Medical Sciences (SMS) at the Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, Ghana. All study participants provided written informed consent.

Whole blood *in vitro* cultures for measurement of supernatant cytokines

Heparinized whole blood (100 µl) diluted 1:1 in RPMI 1640 medium supplemented with L-Glutamine (2 mM) and Penicillin/Streptomycin (50 U/ml)) was cultured for 72 h in 96-well U-bottom plates at 37 °C and 5% CO_2. Cells were stimulated with *M. tuberculosis* purified protein derivative (PPD) (10 µg/ml, Statens Serum Institute, Copenhagen, Denmark) or left unstimulated (medium only). After the culture period, plates were centrifuged, and supernatants were harvested and immediately frozen at −80 °C. Samples were then simultaneously thawed and measured using a flow cytometry-based LEGENDplex kit (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, culture supernatants (12.5 µl) were diluted 1:2 in assay buffer and incubated for 2 h at room temperature with specific antibody-labelled beads for cytokine analyses. Streptavidin-PE was added, and samples were incubated for an additional 30 min. Samples were then washed and analyzed with a BD LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA). Concentrations of each cytokine were determined by LEGENDplex Data Analysis Software v7 (VigeneTech, Carlisle, MA, USA) according to the manufacturer's instructions.

PBMC *in vitro* cultures for measurement of phosphorylated STAT molecules

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by density centrifugation (Ficoll, Biochrom, Berlin, Germany) according to the manufacturer's instructions. Anti-human CD4 antibodies (AlexaFluor488-labelled, clone RPTA-4, BioLegend) were added to each sample, which were then incubated for 15 min at 37 °C and 5% CO_2 with pre-warmed (37 °C) X-vivo15 medium (100 µl, Lonza, Basel, Switzerland) supplemented with L-Glutamine (2 mM), and Penicillin/Streptomycin (50 U/ml) with or without human recombinant IL-2 (25 U/ml) or IL-6 (25 ng/ml) (both from BioLegend). Then, cells were fixed using True-Nuclear Transcription Factor buffer (BioLegend) and permeabilized with methanol as described previously.[31] Samples were then centrifuged, washed in PBS containing FCS (10%) and stained for pSTAT3 (APC-conjugated, clone LUVNKLA, eBioscience, Waltham, MA, USA) or pSTAT5 Y694 (PE-conjugated, clone SRBCZX, eBioscience). Sample measurement was performed on a BD Accuri C6 flow cytometer (BD Biosciences). Flow cytometry-based STAT phosphorylation analyses were performed to characterize pSTAT levels in T-cell subpopulations. Similar approaches for quantification of constitutive STAT3 phosphorylation have been published previously.[32,33] Alternative methods (e.g., Western blot) were not used because of the restricted donor sample volumes.

Measurement of plasma cytokine and soluble IL-6R levels

Blood plasma was harvested from EDTA BD vacutainer tubes (BD Biosciences) according to manufacturer's instructions and immediately stored at −80 °C. After simultaneous thawing, the levels of a subset of cytokines, including IL-10, IL-21, IL-23, IL-27 and soluble IL-6R, were measured by ELISA Ready-SET-Go! (eBioscience) and Human sIL-6R INSTANT ELISA kits (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. All samples were analyzed in duplicate. Samples were measured using an Infinite M200 ELISA reader (Tecan, Männedorf, Switzerland). Concentrations were calculated from respective standard curves by applying 4-parametric logistic regression. Samples outside the detection range were set to the corresponding lower or upper range value.

SOCS3 analysis in $CD4^+$ T-cells in whole blood

Whole blood was stained with PE-conjugated anti-human CD4 (RPTA-4; BioLegend) and FITC-conjugated anti-human CD45RA (clone HI100; BioLegend) for 30 min on ice in the dark. After incubation, red blood cell lysis was performed (Red Blood Cell lysis buffer, Roche, Basel, Switzerland) according to the manufacturer's instructions, followed by washing with PBS supplemented with 10% FCS (Sigma Aldrich, St Louis, MO, USA). PBMCs were then fixed with Fixation Buffer (BioLegend) and permeabilized using permeabilization/washing buffer (BioLegend). Subsequently, permeabilized cells were stained with an anti-human SOCS3 antibody (Abcam, Cambridge, UK) conjugated with Dy650 (DyLight™ 650 microscale antibody labelling kit; ThermoFisher) and analyzed using a BD Accuri C6 flow cytometer (BD Biosciences). For data analysis, we used FlowJo software (Version 10, FlowJo LLC, Ashland, OR, USA).

Whole blood *in vitro* stimulation for measurement of T-cell activation and intracellular cytokine levels

Heparinized blood was diluted (1:1) in RPMI 1640 supplemented with L-glutamine (2 mM) and Penicillin/Streptomycin (50 U/ml). Cells were then stimulated with PPD (10 µg/ml) or left unstimulated at 37 °C, 5% CO_2 for 16 h. After 2.5 h, Brefeldin A (Sigma Aldrich) was added at a concentration of 3.75 µg/ml. Thereafter, erythrocytes were lysed (Red Blood Cell Lysis Buffer, Roche) according to the manufacturer's instructions. Then, cells were fixed and permeabilized (BioLegend) and stained with an antibody against CD4 (AlexaFluor488-labelled, clone RPTA-4, BioLegend), IFN-γ (PE, clone 25723.11, BD Biosciences) or IL-2 (PerCP-Cy5.5, clone MQ1-17H12) and CD40L/CD154 (APC, clone 24.31, BioLegend). Cells were measured using a BD Accuri C6 flow cytometer (BD Biosciences) and analyzed with FlowJo software Version 10, FlowJo LLC). To avoid bias for PPD-specific cytokine production and T-cell activation, spontaneous cytokine expression was subtracted from PPD-induced cytokine production. Limited fluorescence channels restricted our analyses to four markers in Kumasi/Ghana. Therefore, we included the CD4 T-cell marker in addition to effector cytokine and activation markers and omitted the often-used T-cell marker CD3 as previous studies have shown a predominant $CD3^+/CD4^+$ phenotype of cytokine-producing T cells. In addition, cytokine-expressing $CD3^-CD4^+$ cells were rarely detected by the applied *in vitro* assay.[34,35]

Lentiviral transduction of SOCS3 and small hairpin RNA targeting SOCS3

Lentiviral transduction using the Lentiviral Gene Ontology (LeGO) assay was performed as described previously.[36] Briefly,

enriched CD4$^+$ T-cells from healthy donors were activated by *in vitro* culture (X-vivo15 medium as described above) with anti-CD3/CD28-coated beads (0.2 µl, ThermoFisher) and IL-7 (10 ng/ml) for two days. Thereafter, T-cells were transduced using supernatants containing viral particles carrying SOCS3 cDNA,[36] anti-SOCS3 shRNA (TRCN0000057075,

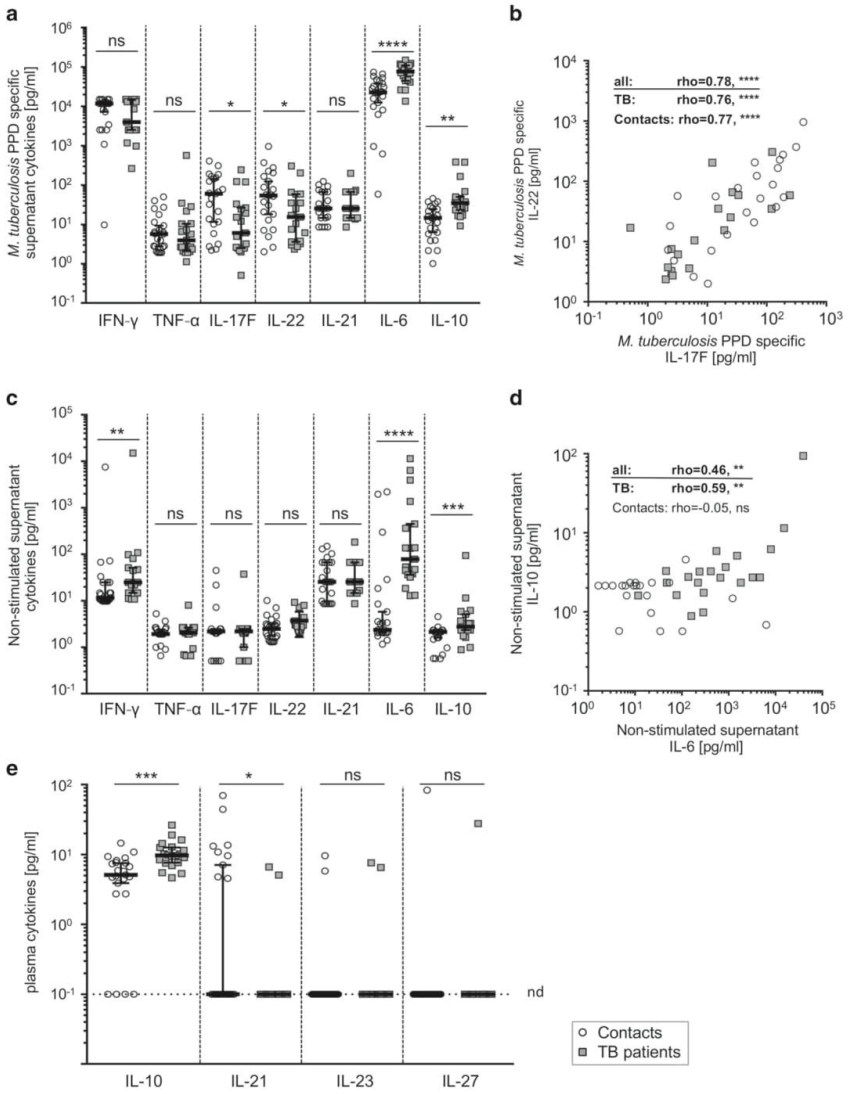


Figure 1 *M. tuberculosis* PPD-specific and constitutive cytokine expression and plasma cytokine concentrations from tuberculosis patients and healthy contacts. Cytokine concentrations of IFN-γ, TNF-α, IL-17F, IL-22, IL-21, IL-6, and IL-10 of supernatants from diluted whole blood after 72 h *in vitro* culture with (**a** and **b**) or without (**c** and **d**) *M. tuberculosis* PPD are depicted. Association of differentially expressed cytokines are shown in (**b**) for PPD-specific IL-17F and IL-22, and (**d**) for constitutive IL-6 and IL-10 values. (**e**) Plasma concentrations of IL-10, IL-21, IL-23, IL-27 are shown for healthy contacts and tuberculosis patients. The dotted line indicates the detection limit for the respective cytokines. Symbols placed on this line indicate values below the detection limit. All samples were measured in duplicate, and mean values are indicated as open circles for healthy contacts and grey squares for tuberculosis patients. Study group medians and percentiles (25, 75) are shown. Significant differences are indicated by asterisks. Nominal *P*-values for the Mann-Whitney *U*-test (two-tailed) were calculated and shown as * for $P<0.05$, ** for $P<0.01$, *** for $P<0.001$, and **** for $P<0.0001$. The Spearman Rank test was used to determine significant correlations for all donors and both study groups separately. Correlation coefficients (rho) and nominal *P*-values are given. ns: not significant, nd: not detectable.

Sigma-Aldrich), a vector control, and eBFP for detection of transduced T-cells by flow cytometry. After transduction, cells were washed and incubated for four days. Then, flow cytometry analyses for SOCS3 expression were performed as described previously.[36] Briefly, cells were incubated with Fixable Viability Dye eFluor 780 (eBioscience) at room temperature for 15 min prior to fixation and permeabilization. Cells were stained with PE-Cy7-conjugated anti-human CD4 (OKT4, BioLegend) and an anti-human SOCS3 antibody (Abcam) conjugated with Dy650 (DyLight™ 650 microscale antibody labelling kit; ThermoFisher) for 30 min on ice in the dark and analyzed using a BD LSRFortessa flow cytometer (BD Biosciences). For quantitative analyses, mean fluorescence intensities were compared between vector control, shRNA, and SOCS3 cDNA-treated cells. However, absolute differences could not be directly deduced from these analyses because of background cellular fluorescence.[36] Therefore, these results confirm differential expression, but the exact level of SOCS3 differences could not be deduced.

pSTAT5 analysis after IL-2 stimulation was performed three days after transduction as described above, with the following modifications: after methanol fixation, samples were stored at − 20 °C for up to one week in methanol. An anti-human CD4 antibody (PE-Cy7, clone RPTA-4, BioLegend) was stained along with the pSTAT5 antibody (PE-conjugated, clone SRBCZX, eBioscience) and analyzed using a BD LSRFortessa flow cytometer (BD Biosciences).

Statistical analyses

Statistical analyses were performed using SPSS software version 24 (IBM Corp., Armonk, NY, USA) and GraphPad prism version 7 (GraphPad Software, La Jolla, CA, USA). The respective tests used are indicated in the Figure legends.

To reduce the complexity of measured immunological parameters into a smaller number of variables, a principal component-based multivariate analysis (PCA) was performed as described previously.[37] Briefly, variables were log10-transformed to factor in the skewness of data (e.g., non-normal distributions and low proportions of positive producers for several cytokines) and to minimize the effects of outlier values. PCA was performed using SPSS v24. Only principal components (PC) accounting for a sufficient amount of variance (i.e., an eigenvalue >2, chosen based on the scree plot curve) were considered, and regression factor scores were extracted for subsequent analysis. Within a PC, variables with a loading factor ≥ 0.5 or ≤ -0.5 were considered to be reflected by the PCA.[37]

RESULTS

Lower *M. tuberculosis*-specific IL-17/IL-22 and higher IL-6/IL-10 expression in tuberculosis patients

M. tuberculosis PPD-specific cytokine expression was compared between tuberculosis patients and healthy contacts. High IFN-γ concentrations were detected in supernatants of PPD stimulated whole blood samples, but there was no significant difference between the study groups (Figure 1a). TNF-α concentrations were generally low, with several values below the limit of detection. T_H17 cytokine levels, e.g., IL-17F and IL-22, were lower in tuberculosis patients compared to healthy contacts (IL-17F: $P=0.015$, IL-22: $P=0.038$, respectively) (Figure 1a). As expected, IL-17F and IL-22 expression correlated strongly in individual donors from both study groups (for all donors: $r=0.78$, $P<0.0001$; Figure 1b). Notably, IL-6 and IL-10 levels were higher in tuberculosis patients after PPD-specific stimulation compared to healthy contacts (Figure 1a). In summary, PPD-induced cytokines showed lower T_H17 responses and higher IL-6/IL-10 production, whereas T_H1 responses were similar between tuberculosis patients and healthy contacts.

Higher spontaneous IL-6 and IL-10 expression levels in tuberculosis patients

Marked variability was observed for cytokine concentrations of non-stimulated whole blood cultures from individual donors. Hence, we next compared cytokine levels in non-stimulated supernatants in the study groups. TNF-α, IL-17, and IL-22 concentrations were generally low and comparable between the study groups. In contrast, IL-10, IL-6, and IFN-γ concentrations were higher in tuberculosis patients compared to healthy contacts (for IL-6, $P<0.0001$; for IL-10, $P=0.006$; and for IFN-γ, $P=0.007$; Figure 1c). For tuberculosis patients, IL-6 and IL-10 levels correlated positively ($r=0.59$, $P=0.0019$; Figure 1d), whereas no correlation was found for healthy contacts ($r=-0.05$, $P=0.83$; Figure 1d). Since spontaneous cytokine expression may affect the interpretation of PPD-specific cytokines, we calculated ΔPPD values for individual donors (Supplementary Figure 1). Similar results, including higher IL-6/IL-10 and comparable IFN-γ concentrations, were found compared to non-subtracted PPD comparisons (Supplementary Figure 1). Therefore, tuberculosis patients had higher *M. tuberculosis*-specific IL-6/IL-10 concentrations, as well as higher spontaneous IL-6, IL-10, and IFN-γ expression in cultured whole blood. We speculated that spontaneous *in vitro* IL-6/IL-10 expression could be due to aberrantly high plasma concentrations of causative cytokines.

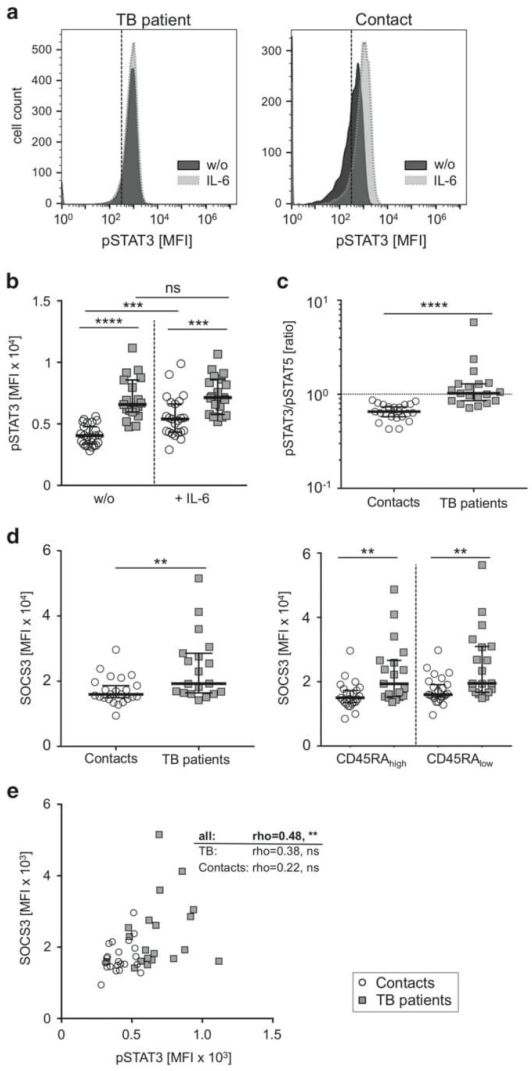
Higher IL-6 and IL-10 plasma levels may trigger constitutive T-cell cytokine expression in tuberculosis patients

Several cytokines have been reported to induce IL-6 and IL-10 expression in T-cells. To identify possible triggers of spontaneous IL-6 and IL-10 expression, we evaluated several plasma cytokine candidates (i.e., IL-6, IL-10, IL-21 IL-23, IL-27). IL-23 and IL-27 concentrations were below the detection limit for the vast majority of donors, and no differences were detected between the study groups (Figure 1e). IL-21 was detected in a minority of donors, primarily in healthy contacts ($P=0.015$; Figure 1e). In contrast, IL-10 levels were significantly higher in tuberculosis patients compared to healthy contacts ($P=0.0002$; Figure 1e). The IL-6 plasma levels from these study groups have already been published, with significantly higher IL-6 levels in tuberculosis patients.[31] Since IL-6 signaling partly depends on the presence of the soluble IL-6 receptor (sIL-6R),

we also determined sIL-6R plasma concentrations; however, we found no differences between the study groups (Supplementary Figure 2). These findings raised the question of whether IL-6/IL-10-dependent receptor signaling processes are affected in tuberculosis patients.

Ex vivo aberrantly high STAT3 phosphorylation and impaired IL-6 responses of T-cells from tuberculosis patients

IL-6 and IL-10 signal transduction is largely dependent on STAT3.[19] Therefore, we analyzed STAT3 phosphorylation in T-cells from tuberculosis patients and healthy contacts by intracellular flow cytometry analysis. Representative histograms for a tuberculosis patient and a healthy contact are shown in Figure 2a. In the absence of *in vitro* re-stimulation, we found significantly higher pSTAT3 levels in $CD4^+$ T-cells of tuberculosis patients compared to healthy contacts ($P<0.0001$; Figure 2b). Whereas the addition of IL-6 led to increased pSTAT3 levels in healthy contacts ($P=0.0007$; Figure 2b), no significant IL-6-induced STAT3 phosphorylation was detected in tuberculosis patients ($P=0.51$; Figure 2b). However, pSTAT3 levels in the presence of IL-6 were higher in tuberculosis patients as compared to healthy contacts ($P=0.0003$; Figure 2b). To avoid a possible bias from serial mean fluorescence analyses, we included non-stimulated pSTAT5 levels as an internal control for individual donor pSTAT3 measures. No differences for pSTAT5 expression were detected between tuberculosis patients and healthy contacts (Supplementary Figure 3). However, comparison of individual donor pSTAT3/pSTAT5 ratios confirmed higher constitutive pSTAT3 levels in tuberculosis patients ($P<0.0001$; Figure 2c). Since IL-6-induced STAT3 phosphorylation is inhibited by SOCS3, we next evaluated SOCS3 protein expression in T-cells.



Higher SOCS3 expression in $CD4^+$ T-cells from tuberculosis patients correlates with pSTAT3 levels

Previously, we and others described increased SOCS3 mRNA expression levels in T-cells from acute tuberculosis patients.[8,30,38] Here, we determined SOCS3 protein expression levels by intracellular flow cytometry to characterize $CD4^+$ T-cells and additional subpopulations. $CD4^+$ T-cells of tuberculosis patients had higher SOCS3 protein expression compared to healthy contacts ($P=0.006$; Figure 2d, left graph). Similarly, we found higher SOCS3 levels in naïve ($CD45RA_{high}$) and memory ($CD45RA_{low}$) $CD4^+$ T-cells in tuberculosis patients compared to healthy contacts (Figure 2d, right graph).

Figure 2 Constitutive and IL-6-induced STAT3 phosphorylation and SOCS3 expression in $CD4^+$ T-cells from tuberculosis patients and healthy contacts. (**a**–**c**) Phosphorylation of STAT3 with or without IL-6 *in vitro* stimulation and (**d**) SOCS3 expression of $CD4^+$ T-cells from tuberculosis patients and healthy contacts. (**a**) Representative histograms indicating non-stimulated (w/o) and IL-6-induced pSTAT3 expression of samples from a tuberculosis patient (left graph) and a healthy contact (right graph) are shown. Dotted lines indicate similar mean fluorescence intensities (MFI) of non-stained control samples for both respective study groups. (**b**) Study group comparisons of non-stimulated (w/o) and IL-6-induced pSTAT3 levels are shown. (**c**) The ratios between non-stimulated pSTAT3 and pSTAT5 values calculated for each individual donor. (**d**) SOCS3 expression for all $CD4^+$ T-cells (left graph) as well as for $CD45RA_{high}$ and $CD45RA_{low}$ (right graph) $CD4^+$ T-cell subpopulations are shown. Tuberculosis patients are represented by grey squares, healthy contacts are depicted as open circles. Study group medians and percentiles (25, 75) are given. Significant differences are indicated by asterisks. Nominal *P*-values for the Mann-Whitney *U*-test (two-tailed) were calculated and shown as * for $P<0.05$, ** for $P<0.01$, *** for $P<0.001$, and **** for $P<0.0001$. ns: not significant. (**e**) Correlation plots for constitutive pSTAT3 and SOCS3 expression are shown for $CD4^+$ T-cells. The Spearman Rank test was used to determine significant correlations for all donors and both study groups separately. Correlation coefficients (rho) and nominal *P*-values are given.

Since pSTAT3 induces SOCS3 transcription, we next determined the association between SOCS3 and STAT3 expression in T-cells. A significant correlation (for all donors; rho = 0.48, $P = 0.001$) was detected for $CD4^+$ T-cells (Figure 2e) and was also found for $CD4^+$ T-cell subpopulations (Supplementary Figure 4). Therefore, constitutive STAT3 phosphorylation is accompanied by higher SOCS3 protein levels in tuberculosis patients.

SOCS3 is negatively correlated with T-cell activation and IFN-γ expression

SOCS3 has previously been shown to regulate T-cell receptor activation.[25–28] In addition, we and others have reported SOCS3 inhibitory effects on T_H1 cells.[36,39,40] We therefore measured the expression of T-cell activation marker CD40L in combination with intracellular IFN-γ and IL-2 after PPD-specific *in vitro* stimulation (Figure 3a). Tuberculosis patients and healthy contacts had similar proportions of *M. tuberculosis* PPD-induced CD40L-positive T-cells co-expressing IL-2 (Figure 3b). The results in CD40L/IFN-γ co-expressing T-cells were comparable to CD40L/IL-2 and have been published previously.[31] Notably, CD40L/IFN-γ- and CD40L/IL-2-positive T-cell proportions were negatively correlated with SOCS3 expression exclusively in tuberculosis patients (for CD40L/IFN-γ: rho = −0.59, $P = 0.007$; for CD40L/IL-2: rho = −0.64, $P = 0.004$; Figure 3c)), whereas no correlation was detected for healthy contacts or for all donors collectively (for CD40L/IFN-γ: rho = −0.09, $P = 0.54$; for CD40L/IL-2: rho = −0.16, $P = 0.29$; Figure 3c). Negative correlations between SOCS3 and T-cell activation/cytokine expression were also found for $CD45RA_{high}$ and for $CD45RA_{low}$ T-cell subpopulations (Supplementary Figure 5). This suggested potential inhibitory effects of high SOCS3 levels on activated T_H1 cytokine-expressing $CD4^+$ T-cells from tuberculosis patients.

High SOCS3 expression in $CD4^+$ T-cells from tuberculosis patients inhibits IL-2-induced STAT5 phosphorylation

The negative correlation of T-cell cytokine expression with high SOCS3 expression in tuberculosis patients may be due to reported SOCS3 inhibitory effects on IL-2-dependent T-cell proliferation.[26,36] Therefore, we measured levels of STAT5 phosphorylation, which is the main mediator of IL-2 receptor signaling. After stimulation with IL-2, we found lower pSTAT5 levels in $CD4^+$ T-cells from tuberculosis patients compared to healthy contacts ($P = 0.031$; Figure 4a). A moderate but significant correlation of lower pSTAT5 levels with higher SOCS3 expression was detected for individual donors (rho = −0.31, $P = 0.046$; Figure 4b), which was mainly due to a tendency towards negative correlation in tuberculosis patients (rho = −0.38, $P = 0.1$; Figure 4b). To characterize SOCS3-mediated effects on IL-2 receptor signaling, we modulated SOCS3 expression in $CD4^+$ T-cells by lentiviral transduction. Representative graphs of eBFP expression are shown in Figure 4c. Flow cytometry mean fluorescence analyses confirmed ectopic SOCS3 over-expression ($P = 0.021$) and shRNA-mediated inhibition of SOCS3 expression ($P = 0.004$) in $CD4^+$ T-cells (Figure 4d). Analysis of IL-2-induced STAT5 phosphorylation confirmed decreased pSTAT5 levels in $CD4^+$ T-cells over-expressing SOCS3 (mean: 0.12, equivalent to an 88% reduction compared to vector control, $P = 0.0004$; Figure 4e), whereas shRNAs targeting SOCS3 had only minor effects on SOCS3 after short-term IL-2 stimulation ($P = 0.8$; Figure 4e). These results suggested that high SOCS3 levels in T-cells from tuberculosis patients impaired IL-2 receptor signaling.

Multivariate analyses identified a principal component of T-cells correlating with constitutive pSTAT3 levels that further classifies tuberculosis patients

Multiple factors determined in this study may contribute to failed immunity in human tuberculosis. To decipher the complex associated immunological factors and to identify patterns, we performed principal component multivariate analysis. We included data from supernatants of non-stimulated and PPD-stimulated whole blood, plasma cytokines, and $CD4^+$ T-cells with PPD-specific intracellular cytokines and SOCS3 expression, as described above. Overall, four principal components (PC) were extracted based on an eigenvalue > 2, which together accounted for 56.9% of the total variance (Table 2). Within each PC, variables with a loading factor of > 0.5 were considered strong influencing factors. PC-1 accounted for the largest amount of the total data variance (20.5%) and was primarily characterized by constitutive and PPD-induced T_H17-related cytokines (e.g., IL-17F and IL-22) from supernatants, as well as activated T-cell populations expressing IFN-γ or IL-2. PC-2 (accounting for 15.7% of the total data variance) was characterized by SOCS3 expression in T-cells, plasma IL-6 and IL-10 concentrations, constitutive IL-6/IL-10 production and PPD-induced IL-6/IL-10 expression (Table 2). PC-3 was characterized by IL-21 expression (in the presence or absence of PPD), and PC-4 included only constitutive IL-17F expression (negatively associated). To determine the relationship of PCs to constitutive pSTAT3 levels, we correlated the regression factor scores of each PC with pSTAT3 levels. Whereas PC-1 was not correlated with pSTAT3 (rho = −0.01, $P = 0.95$; Figure 5a, left graph), a strong positive correlation of PC-2 (rho = 0.75, $P < 0.0001$) with constitutive pSTAT3 levels (Figure 5a, right graph) was observed. Neither PC-3 nor PC-4 significantly correlated with pSTAT3 levels. Furthermore, regression scores were compared between TB patients and healthy contacts. Notably, the median regression score of PC-1 was comparable between tuberculosis patients and healthy contacts (Figure 5b, left graph), whereas the regression score for PC-2 was significantly different between tuberculosis patients and healthy contacts (Figure 5b, right graph). We concluded that constitutive pSTAT3 expression is associated with simultaneously elevated levels of SOCS3, spontaneous IL-6/IL-10 expression, and PPD-specific IL-6/IL-10 expression in $CD4^+$ T-cells in tuberculosis patients.

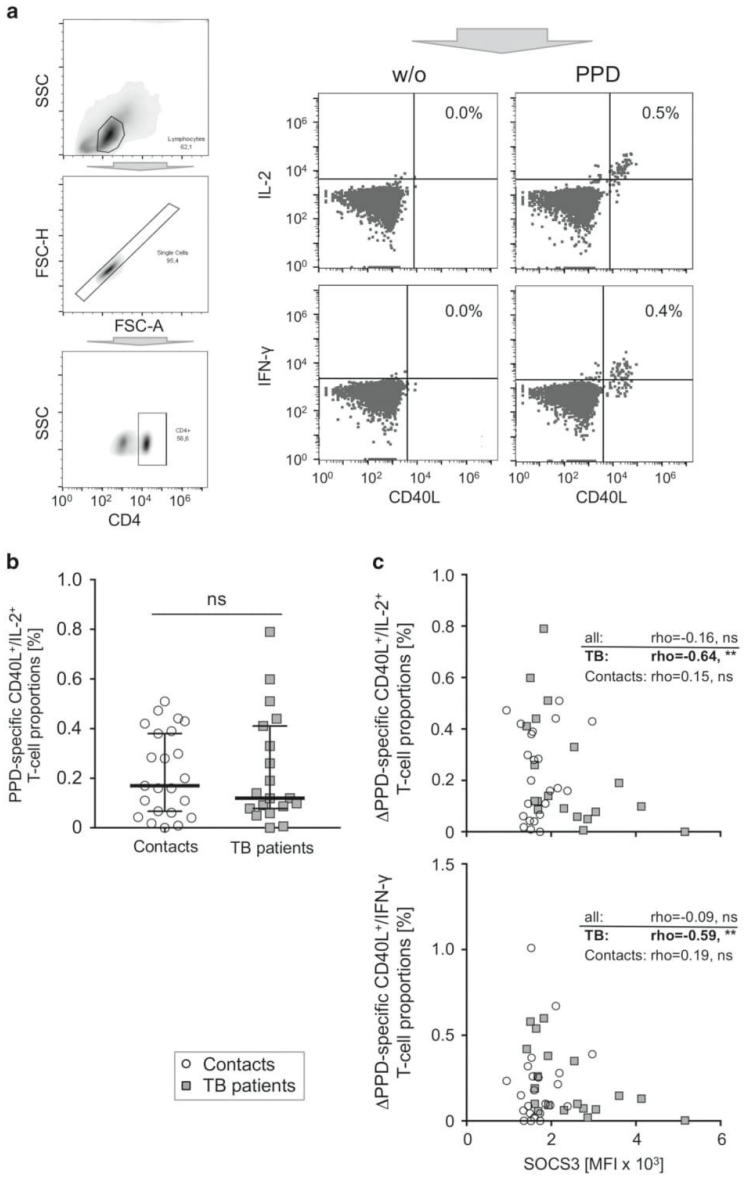


Figure 3 *M. tuberculosis*-specific activation and intracellular cytokine expression in association with SOCS3 expression in both study groups. (**a**) Representative graphs depicting the gating procedure for intracellular cytokine analysis are shown. (**b**) Proportions of CD40L/IL-2-positive CD4$^+$ T-cells are shown for tuberculosis patients (grey squares) and healthy contacts (open circles). (**c**) Correlation plots between SOCS3 expression and CD40L/IL-2 (upper graph) as well as CD40L/IFN-γ (lower graph) are shown for CD4$^+$ T-cells. The Spearman Rank test was used to determine significant correlations for all donors and both study groups separately. Correlation coefficients (rho) and nominal *P*-values are given. ns: not significant.

DISCUSSION

This study investigated the role of cytokines and their associated T-cell functions in human tuberculosis patients. To our knowledge, this is the first report identifying constitutive STAT3 phosphorylation in association with high SOCS3 protein expression as a feature of CD4$^+$ T-cells in tuberculosis patients. Spontaneous *in vitro* and *M. tuberculosis*-specific IL-6 and IL-10 expression, as well as higher IL-6/IL-10 plasma levels,

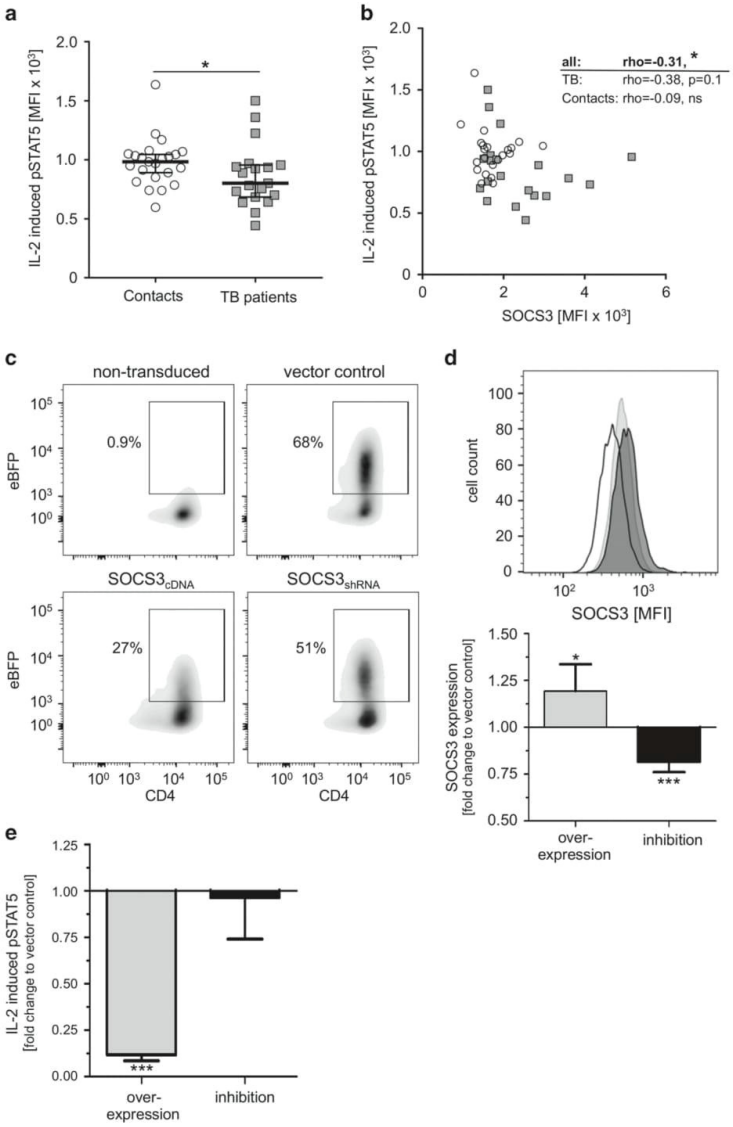


Figure 4 IL-2-induced pSTAT5 levels in T-cells from both study groups, and effects of lentiviral SOCS3 modulation on IL-2-induced pSTAT5. (**a** and **b**) IL-2-induced STAT5 phosphorylation of $CD4^+$ T-cells is shown for tuberculosis patients (grey squares) and healthy contacts (open circles). (**b**) Correlation of IL-2-induced pSTAT5 with SOCS3 expression. The Spearman Rank test was used to determine significant correlations for all donors and both study groups separately. Correlation coefficients (rho) and nominal *P*-values are given. (**c**) Representative flow cytometry histogram indicating fluorescence (eBFP) expression of non-transduced, vector control-, SOCS3cDNA-, and SOCS3shRNA-transduced $CD4^+$ T-cells are shown. (**d**) Flow cytometry-based quantification of $CD4^+$ T-cells with SOCS3cDNA (dark grey) and SOCS3shRNA (black line, open) compared to vector control (bright grey). The results from seven experiments are shown. (**e**) STAT5 phosphorylation in $CD4^+$ T-cells from three experiments with increased or reduced SOCS3 expression. Nominal *P*-values for the paired *t* Test (two-tailed) were shown. MFI, mean fluorecence intensity.

likely cause aberrant cytokine signaling events in tuberculosis patients. Multivariate principal component analysis revealed that spontaneously produced IL-6/IL-10, PPD-specific IL-6/IL-10 production, and SOCS3 expression in $CD4^+$ T-cells clustered together in a single component that strongly correlated with high constitutive pSTAT3 levels. High SOCS3 levels correlated with

Table 2 Principal Component analyses

	Parameter	*Principal component*			
		1	*2*	*3*	*4*
Inhibitory	SOCS3 on CD4	−0.065	**0.674**	−0.152	0.146
Plasma cytokines	plasma IL-6	−0.046	**0.758**	0.203	0.122
	plasma IL-6R	**0.565**	0.041	0.211	−0.250
	plasma IL-10	0.226	**0.537**	0.044	0.014
	plasma IL-21	0.377	−0.260	−0.176	0.186
	plasma IL-23	0.489	−0.018	−0.068	−0.115
	plasma IL-27	−0.303	−0.206	−0.276	−0.259
Restim cytokines	IL-6 unstim	**0.514**	**0.595**	−0.056	0.137
	IL-6 PPD	0.396	**0.543**	0.153	0.483
	IL-10 unstim	0.393	**0.528**	−0.228	−0.379
	IL-10 PPD	0.499	**0.602**	0.148	0.015
	IFNγ unstim	**0.574**	0.129	−0.132	−0.518
	IFNγ PPD	0.380	−0.124	−0.338	0.344
	TNFα unstim	0.123	−0.341	**0.694**	−0.148
	TNFα PPD	**0.571**	−0.191	0.088	0.163
	IL-17f unstim	**0.571**	−0.211	0.054	**−0.596**
	IL-17f PPD	**0.620**	−0.418	−0.383	−0.020
	IL-21 unstim	0.330	−0.200	**0.847**	0.111
	IL-21 PPD	0.250	−0.161	**0.886**	0.079
	IL-22 unstim	**0.528**	0.245	−0.023	−0.468
	IL-22 PPD	**0.587**	−0.464	−0.250	0.056
ICS cytokines	PPD_CD40L^{hi}IFNγ^{hi}	**0.597**	−0.160	−0.194	**0.505**
	PPD_CD40L^{hi}IL2^{hi}	**0.601**	−0.336	−0.133	0.402
Eigenvalue[a]	4.714	3.602	2.715	2.06	
Amount of variance (%)		20.496	15.661	11.803	8.957
Cumulative variance (%)		20.496	36.157	47.96	56.917

[a]extracted if eigenvalue >2. Variables with a loading factor ≥0.5 or ≤−0.5 are indicated in bold.

weak T_H1 responses in tuberculosis patients, likely due to inhibited IL-2 signaling and T-cell proliferation.

Constitutive STAT3 phosphorylation has been described for malignant diseases, and several functions during tumorigenesis have been attributed to STAT3 activation.[41] In T-cell lymphoma, aberrantly high pSTAT3 levels are accompanied by high SOCS3 expression and contribute to tumor survival.[42,43] High pSTAT3 levels have also been described for inflammatory diseases.[32,33,44–46] Lovato *et al.* described constitutive STAT3 phosphorylation of intestinal and peripheral blood T-cells from patients with Crohn's disease.[44] Additional studies have further confirmed the important role of STAT3 in Crohn's disease,[46–48] and identified the IL-6/STAT3 cytokine signaling pathway as a therapeutic target against chronic inflammation and cancer development in inflammatory bowel disease.[49] More recently, constitutive STAT3 phosphorylation was described in T-cells from rheumatoid arthritis patients.[32,33,45] These studies reported a dependence between pSTAT3 levels—mainly in $CD4^+$ T-cells and monocytes—and high IL-6 plasma concentrations in rheumatoid arthritis patients.[32,33,45] The central role of IL-6 in constitutive STAT3 phosphorylation in rheumatoid arthritis was strengthened by transcriptomic and functional T-cell analyses.[45] Furthermore, the levels of other potential causative cytokines of pSTAT3 induction (i.e., IL-10, IL-21, IL-23, and IL-27) were not increased in plasma samples from rheumatoid arthritis patients.[32,33,45,50] Of note, whereas constitutive STAT3 phosphorylation in inflammatory and autoimmune disease studies could be increased by the addition of IL-6 *in vitro*, pSTAT3 levels in $CD4^+$ T cells from tuberculosis patients were not increased in the presence of exogenous IL-6, and IL-6-induced pSTAT3 expression in healthy contacts did not reach the levels observed in tuberculosis patients. This indicates aberrantly high pSTAT3 expression and potentially impaired IL-6-mediated T-cell responses in tuberculosis patients. We strengthened the finding of increased constitutive pSTAT3 expression by including concomitant measures of pSTAT5 levels for individual donors. Both study groups had similar median pSTAT5 levels and, more importantly, individual donor pSTAT3/pSTAT5 ratios confirmed differential

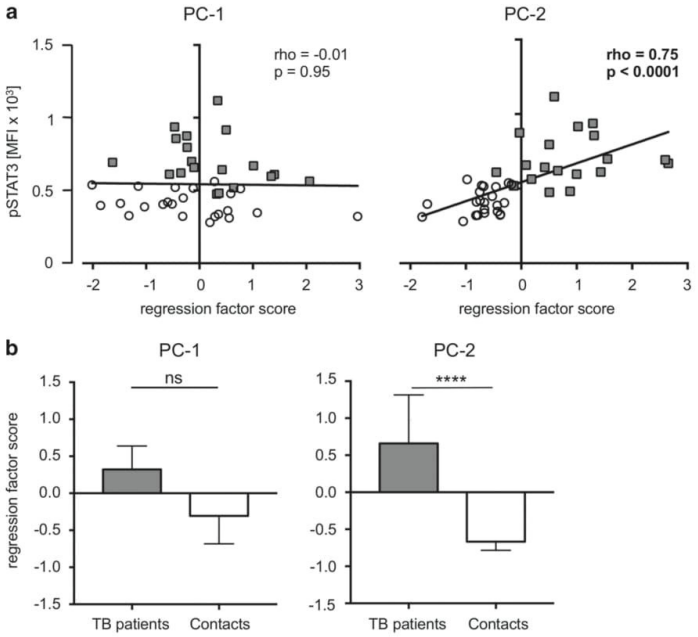


Figure 5 Multivariate analyses of influential factors and correlation with constitutive pSTAT3 in tuberculosis patients and healthy contacts. (**a**) Correlation of principal component (PC)-1 (left graph) and PC-2 (right graph) with constitutive pSTAT3 levels. The Spearman Rank test was used to determine significant correlations for all donors and both study groups separately. Correlation coefficients (rho) and nominal *P*-values are given. (**b**) Regression scores for PC-1 (left graph) and PC-2 (right graph) are compared between tuberculosis patients and healthy contacts. Data are presented as medians, and nominal *P*-values for the Mann-Whitney *U*-test (two-tailed) are shown.

pSTAT3 levels and excluded possible effects of asynchronous flow cytometry measurements.

IL-6 is a well-known key factor in the development of T_H17 cells, which are centrally involved in the development of inflammatory diseases.[12] We detected lower proportions of IL-17F- and IL-22-producing T_H17 cells in tuberculosis patients, which was consistent with previous studies.[6,51] Different roles of STAT3 activation between inflammatory diseases and chronic infectious tuberculosis may reflect the complexity of STAT3 induction and regulation in different cell populations.[29] The important role of STAT3 and its regulator, SOCS3, in the immune response against tuberculosis is well-established.[24] Several studies have demonstrated the increased expression of SOCS3 mRNA expression in T-cells and PBMCs during tuberculosis infection.[8,30,31,36,38] Analysis of protein expression by flow cytometry enabled us to characterize SOCS3 expression in several T-cell subpopulations in the present study. We identified increased SOCS3 protein expression in naïve ($CD45RA_{high}$) and memory ($CD45RA_{low}$) $CD4^+$ T-cells from tuberculosis patients. We also detected a correlation between constitutive STAT3 phosphorylation and SOCS3 expression in different T-cell subpopulations. In contrast to previous studies concerning pSTAT3/SOCS3 expression in inflammatory diseases, we detected higher IL-10 plasma levels, as well as higher spontaneous IL-10 production, in PBMCs from tuberculosis patients compared to healthy contacts. Higher IL-10 plasma concentrations have been described for tuberculosis patients,[3–7] and spontaneous IL-10 production in non-activated PBMCs *in vitro* from tuberculosis patients has also been reported previously.[4,5] Higher IL-10 expression was accompanied by higher IL-6 plasma levels in the present and previous studies.[3–7] In addition, *M. tuberculosis*-infected macrophages were shown to have increased pSTAT3 levels. In macrophages, ESAT-6-induced IL-6 or IL-10 expression have been identified as possible causes.[52,53] In addition to spontaneous IL-10 and IL-6 production, T-cells from tuberculosis patients were strongly activated in the presence of *M. tuberculosis* PPD to produce IL-10 and IL-6. These results suggested a dominant immunosuppressive T-cell phenotype in tuberculosis patients characterized by IL-10-positive *M. tuberculosis*-specific T-cells. Type 1 regulatory T-cells, as well as IL-10-producing effector T-cells, may account for the phenomenon. Induction of IL-10 expression in effector T-cells is well-established in chronic viral infections[17,54] and has also been described in tuberculosis.[55–58] In general, IL-10 co-expression in effector T-cells is hypothesized to play a role in down-regulating immune responses after pathogen eradication to minimize harmful side effects of immune responses.[59,60] Interestingly, the induction of IL-10 in effector T-cells is dependent on IL-6 (or IL-27)-mediated STAT3 activation.[16]

These seemingly opposed functions of STAT3 are at least partially explained by its regulator, SOCS3.[61] Whereas transient SOCS3 expression has been shown to be important for inflammatory T-cell and monocyte functions, the absence of SOCS3—and also permanent SOCS3 expression—was found to promote immunosuppressive or modulatory immune responses.[61] This is reflected in IL-6 and IL-10 functions, as well as in their receptors. IL-6 induces SOCS3 via STAT3 phosphorylation, and IL-6 receptor signaling is suppressed by SOCS3 binding to the gp130 chain.[61] In contrast, IL-10 induces SOCS3 expression, but no inhibitory functions of SOCS3 on IL-10R signaling have been described.[22] Against this background, we hypothesized that the concomitant IL-6 and IL-10 expression measured in T-cells and plasma of tuberculosis patients promotes IL-10-mediated immune suppression and not the inflammatory arms of immune responses. Consistently, we found a correlation between high SOCS3 expression and low T_H1 responses in tuberculosis patients and contacts. Although T_H1 responses were comparable between tuberculosis patients and contacts, we conclude that T_H1 responses may be stronger during acute tuberculosis in the absence of SOCS3. SOCS3 was found to suppress T-cell activation and proliferation via inhibition of T-cell co-stimulation by IL-2.[36] To determine the role of SOCS3 in this context we performed lentiviral modulation of SOCS3 expression and detected inhibition of IL-2-mediated STAT5 phosphorylation when over-expressing SOCS3.

Overall, our data confirm previous findings of higher IL-10/IL-6 cytokine levels in tuberculosis patients and add novel data concerning constitutive pSTAT3/SOCS3 expression, which likely drives an immunosuppressive/anti-proliferation T-cell response in tuberculosis patients. This impaired T-cell function may contribute to the inefficient immune response in active tuberculosis disease that renders long-term multi-drug chemotherapy necessary. Against the background of ongoing immunomodulatory treatment trials for chronic infectious diseases, this study identifies novel candidate biomarkers for evaluating the efficacy of T-cell recovery.

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1 Cooper AM, Flynn JL. The protective immune response to Mycobacterium tuberculosis. *Curr Opin Immunol* 1995; **7**: 512–516.
2 Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. *Nature reviews Immunology* 2011; **11**: 343–354.
3 Chowdhury IH, Ahmed AM, Choudhuri S, Sen A, Hazra A, Pal NK *et al.* Alteration of serum inflammatory cytokines in active pulmonary tuberculosis following anti-tuberculosis drug therapy. *Molecular immunology* 2014; **62**: 159–168.
4 Masood KI, Rottenberg ME, Salahuddin N, Irfan M, Rao N, Carow B *et al.* Expression of M. tuberculosis-induced suppressor of cytokine signaling (SOCS) 1, SOCS3, FoxP3 and secretion of IL-6 associates with differing clinical severity of tuberculosis. *BMC infectious diseases* 2013; **13**: 13.
5 Masood KI, Rottenberg ME, Carow B, Rao N, Ashraf M, Hussain R *et al.* SOCS1 gene expression is increased in severe pulmonary tuberculosis. *Scand J Immunol* 2012; **76**: 398–404.
6 Chen X, Zhang M, Liao M, Graner MW, Wu C, Yang Q *et al.* Reduced Th17 response in patients with tuberculosis correlates with IL-6R expression on CD4+ T Cells. *Am J Respir Crit Care Med.* 2010; **181**: 734–742.
7 Verbon A, Juffermans N, Van Deventer SJ, Speelman P, Van Deutekom H, Van Der Poll T. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clin Exp Immunol* 1999; **115**: 110–113.
8 Ashenafi S, Aderaye G, Bekele A, Zewdie M, Aseffa G, Hoang AT *et al.* Progression of clinical tuberculosis is associated with a Th2 immune response signature in combination with elevated levels of SOCS3. *Clin Immunol* 2014; **151**: 84–99.
9 Jones LL, Alli R, Li B, Geiger TL. Differential T Cell Cytokine Receptivity and Not Signal Quality Distinguishes IL-6 and IL-10 Signaling during Th17 Differentiation. *J Immunol* 2016; **196**: 2973–2985.
10 Hirano T, Kishimoto T. Interleukin-6: possible implications in human diseases. *Ric Clin Lab* 1989; **19**: 1–10.
11 Tanaka T, Narazaki M, Kishimoto T. Interleukin (IL-6) Immunotherapy. *Cold Spring Harb Perspect Biol* 2017.
12 Iwakura Y, Ishigame H. The IL-23/IL-17 axis in inflammation. *J Clin Invest* 2006; **116**: 1218–1222.
13 Romagnani S. Regulation of the T cell response. *Clin Exp Allergy.* 2006; **36**: 1357–1366.
14 Wu K, Bi Y, Sun K, Wang C. IL-10-producing type 1 regulatory T cells and allergy. *Cell Mol Immunol* 2007; **4**: 269–275.
15 Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993; **75**: 263–274.
16 Stumhofer JS, Silver JS, Laurence A, Porrett PM, Harris TH, Turka LA *et al.* Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* 2007; **8**: 1363–1371.
17 Parish IA, Marshall HD, Staron MM, Lang PA, Brustle A, Chen JH *et al.* Chronic viral infection promotes sustained Th1-derived immunoregulatory IL-10 via BLIMP-1. *J Clin Invest* 2014; **124**: 3455–3468.
18 Brooks DG, Teyton L, Oldstone MB, McGavern DB. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. *J Virol.* 2005; **79**: 10514–10527.
19 Murray PJ. The JAK-STAT signaling pathway: input and output integration. *J Immunol* 2007; **178**: 2623–2629.
20 Carow B, Reuschl AK, Gavier-Widen D, Jenkins BJ, Ernst M, Yoshimura A *et al.* Critical and independent role for SOCS3 in either myeloid or T cells in resistance to Mycobacterium tuberculosis. *PLoS pathogens* 2013; **9**: e1003442.
21 Lang R, Pauleau AL, Parganas E, Takahashi Y, Mages J, Ihle JN *et al.* SOCS3 regulates the plasticity of gp130 signaling. *Nat Immunol* 2003; **4**: 546–550.
22 Niemand C, Nimmesgern A, Haan S, Fischer P, Schaper F, Rossaint R *et al.* Activation of STAT3 by IL-6 and IL-10 in primary human macrophages is differentially modulated by suppressor of cytokine signaling 3. *J Immunol* 2003; **170**: 3263–3272.
23 Braun DA, Fribourg M, Sealfon SC. Cytokine response is determined by duration of receptor and signal transducers and activators of transcription 3 (STAT3) activation. *J Biol Chem* 2013; **288**: 2986–2993.
24 Carow B, Rottenberg ME. SOCS3, a Major Regulator of Infection and Inflammation. *Frontiers in Immunology* 2014; **5**: 58.
25 Banerjee A, Banks AS, Nawijn MC, Chen XP, Rothman PB. Cutting edge: Suppressor of cytokine signaling 3 inhibits activation of NFATp. *J Immunol* 2002; **168**: 4277–4281.
26 Yu CR, Mahdi RM, Ebong S, Vistica BP, Gery I, Egwuagu CE. Suppressor of cytokine signaling 3 regulates proliferation and activation of T-helper cells. *J Biol Chem* 2003; **278**: 29752–29759.

27 Cohney SJ, Sanden D, Cacalano NA, Yoshimura A, Mui A, Migone TS *et al*. SOCS-3 is tyrosine phosphorylated in response to interleukin-2 and suppresses STAT5 phosphorylation and lymphocyte proliferation. *Molecular and Cellular Biology* 1999; **19**: 4980–4988.
28 Matsumoto A, Seki Y, Watanabe R, Hayashi K, Johnston JA, Harada Y *et al*. A role of suppressor of cytokine signaling 3 (SOCS3/CIS3/SSI3) in CD28-mediated interleukin 2 production. *The Journal of experimental medicine* 2003; **197**: 425–436.
29 Rottenberg ME, Carow B. SOCS3 and STAT3, major controllers of the outcome of infection with Mycobacterium tuberculosis. *Semin Immunol* 2014; **26**: 518–532.
30 Jacobsen M, Repsilber D, Kleinsteuber K, Gutschmidt A, Schommer-Leitner S, Black G *et al*. Suppressor of cytokine signaling-3 is affected in T-cells from tuberculosisTB patients. *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2011; **17**: 1323–1331.
31 Lundtoft C, Afum-Adjei Awuah A, Rimpler J, Harling K, Nausch N, Kohns M *et al*. Aberrant plasma IL-7 and soluble IL-7 receptor levels indicate impaired T-cell response to IL-7 in human tuberculosis. *PLoS pathogens* 2017; **13**: e1006425.
32 Isomaki P, Junttila I, Vidqvist KL, Korpela M, Silvennoinen O. The activity of JAK-STAT pathways in rheumatoid arthritis: constitutive activation of STAT3 correlates with interleukin 6 levels. *Rheumatology (Oxford)* 2015; **54**: 1103–1113.
33 Kuuliala K, Kuuliala A, Koivuniemi R, Oksanen S, Hamalainen M, Moilanen E *et al*. Constitutive STAT3 Phosphorylation in Circulating CD4+ T Lymphocytes Associates with Disease Activity and Treatment Response in Recent-Onset Rheumatoid Arthritis. *PLoS One* 2015; **10**: e0137385.
34 Mueller H, Detjen AK, Schuck SD, Gutschmidt A, Wahn U, Magdorf K *et al*. Mycobacterium tuberculosis-specific CD4+, IFNgamma+, and TNFalpha+ multifunctional memory T cells coexpress GM-CSF. *Cytokine* 2008; **43**: 143–148.
35 Schuck SD, Mueller H, Kunitz F, Neher A, Hoffmann H, Franken KL *et al*. Identification of T-cell antigens specific for latent mycobacterium tuberculosis infection. *PLoS One* 2009; **4**: e5590.
36 Kleinsteuber K, Heesch K, Schattling S, Sander-Juelch C, Mock U, Riecken K *et al*. SOCS3 promotes interleukin-17 expression of human T cells. *Blood* 2012; **120**: 4374–4382.
37 Sokal RR, RF. *Biometry: the principles and practice of statistics in biological research*. New York: W.H. Freeman & Co Ltd, 1981.
38 Mistry R, Cliff JM, Clayton CL, Beyers N, Mohamed YS, Wilson PA *et al*. Gene-expression patterns in whole blood identify subjects at risk for recurrent tuberculosis. *The Journal of infectious diseases* 2007; **195**: 357–365.
39 Croker BA, Krebs DL, Zhang JG, Wormald S, Willson TA, Stanley EG *et al*. SOCS3 negatively regulates IL-6 signaling in vivo. *Nat Immunol* 2003; **4**: 540–545.
40 Egwuagu CE, Yu CR, Zhang M, Mahdi RM, Kim SJ, Gery I. Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. *J Immunol* 2002; **168**: 3181–3187.
41 Yuan J, Zhang F, Niu R. Multiple regulation pathways and pivotal biological functions of STAT3 in cancer. *Sci Rep* 2015; **5**: 17663.
42 Brender C, Lovato P, Sommer VH, Woetmann A, Mathiesen AM, Geisler C *et al*. Constitutive SOCS-3 expression protects T-cell lymphoma against growth inhibition by IFNalpha. *Leukemia* 2005; **19**: 209–213.
43 Brender C, Nielsen M, Kaltoft K, Mikkelsen G, Zhang Q, Wasik M *et al*. STAT3-mediated constitutive expression of SOCS-3 in cutaneous T-cell lymphoma. *Blood* 2001; **97**: 1056–1062.
44 Lovato P, Brender C, Agnholt J, Kelsen J, Kaltoft K, Svejgaard A *et al*. Constitutive STAT3 activation in intestinal T cells from patients with Crohn's disease. *J Biol Chem* 2003; **278**: 16777–16781.
45 Anderson AE, Pratt AG, Sedhom MA, Doran JP, Routledge C, Hargreaves B *et al*. IL-6-driven STAT signalling in circulating CD4+ lymphocytes is a marker for early anticitrullinated peptide antibody-negative rheumatoid arthritis. *Ann Rheum Dis* 2016; **75**: 466–473.
46 Mudter J, Weigmann B, Bartsch B, Kiesslich R, Strand D, Galle PR *et al*. Activation pattern of signal transducers and activators of transcription (STAT) factors in inflammatory bowel diseases. *Am J Gastroenterol* 2005; **100**: 64–72.
47 Nieminen JK, Niemi M, Sipponen T, Salo HM, Klemetti P, Farkkila M *et al*. Dendritic cells from Crohn's disease patients show aberrant STAT1 and STAT3 signaling. *PLoS One* 2013; **8**: e70738.
48 Li C, Iness A, Yoon J, Grider JR, Murthy KS, Kellum JM *et al*. Noncanonical STAT3 activation regulates excess TGF-beta1 and collagen I expression in muscle of stricturing Crohn's disease. *J Immunol* 2015; **194**: 3422–3431.
49 Atreya R, Neurath MF. Signaling molecules: the pathogenic role of the IL-6/STAT-3 trans signaling pathway in intestinal inflammation and in colonic cancer. *Curr Drug Targets* 2008; **9**: 369–374.
50 Pratt AG, Swan DC, Richardson S, Wilson G, Hilkens CM, Young DA *et al*. A CD4 T cell gene signature for early rheumatoid arthritis implicates interleukin 6-mediated STAT3 signalling, particularly in anti-citrullinated peptide antibody-negative disease. *Ann Rheum Dis* 2012; **71**: 1374–1381.
51 Bandaru A, Devalraju KP, Paidipally P, Dhiman R, Venkatasubramanian S, Barnes PF *et al*. Phosphorylated STAT3 and PD-1 regulate IL-17 production and IL-23 receptor expression in Mycobacterium tuberculosis infection. *European Journal of Immunology* 2014; **44**: 2013–2024.
52 Jung BG, Wang X, Yi N, Ma J, Turner J, Samten B. Early Secreted Antigenic Target of 6-kDa of Mycobacterium tuberculosis Stimulates IL-6 Production by Macrophages through Activation of STAT3. *Sci Rep* 2017; **7**: 40984.
53 Queval CJ, Song OR, Deboosere N, Delorme V, Debrie AS, Iantomasi R *et al*. STAT3 Represses Nitric Oxide Synthesis in Human Macrophages upon Mycobacterium tuberculosis Infection. *Sci Rep* 2016; **6**: 29297.
54 Rojas JM, Avia M, Martin V, Sevilla N. IL-10: A Multifunctional Cytokine in Viral Infections. J. *Immunol Res*. 2017; **2017**: 6104054.
55 Gerosa F, Nisii C, Righetti S, Micciolo R, Marchesini M, Cazzadori A *et al*. CD4(+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients. *Clin Immunol* 1999; **92**: 224–234.
56 Cyktor JC, Carruthers B, Beamer GL, Turner J. Clonal expansions of CD8+ T cells with IL-10 secreting capacity occur during chronic Mycobacterium tuberculosis infection. *PLoS One* 2013; **8**: e58612.
57 Boussiotis VA, Tsai EY, Yunis EJ, Thim S, Delgado JC, Dascher CC *et al*. IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. *J Clin Invest* 2000; **105**: 1317–1325.
58 Pinheiro RO, de Oliveira EB, Dos Santos G, Sperandio da Silva GM, de Andrade Silva BJ, Teles RM *et al*. Different immunosuppressive mechanisms in multi-drug-resistant tuberculosis and non-tuberculous mycobacteria patients. *Clin Exp Immunol* 2013; **171**: 210–219.
59 McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T *et al*. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* 2007; **8**: 1390–1397.
60 Haringer B, Lozza L, Steckel B, Geginat J. Identification and characterization of IL-10/IFN-gamma-producing effector-like T cells with regulatory function in human blood. *The Journal of experimental medicine* 2009; **206**: 1009–1017.
61 Yasukawa H, Ohishi M, Mori H, Murakami M, Chinen T, Aki D *et al*. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat Immunol* 2003; **4**: 551–556.

Supplementary Information for this article can be found on the *Cellular & Molecular Immunology* website (http://www.nature.com/cmi)

3.2. Paper III: CD27 expression of T-cells discriminates IGRA-negative TB patients from healthy contacts in Ghana.

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- Data analysis
- Writing of the manuscript

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Short communication

CD27 expression of T-cells discriminates IGRA-negative TB patients from healthy contacts in Ghana

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ABSTRACT

IFN-γ release assays (IGRAs) have suboptimal sensitivity for detection of *Mycobacterium tuberculosis* (*Mtb*) infection and cannot discriminate between tuberculosis (TB) patients and healthy -potentially *Mtb* infected- contacts (HCs). In a case-control study, we determined T-cell phenotypes of IGRAs in TB patients (n = 20) and HCs (n = 20) from Ghana. CD27 expression of T-cells was significantly lower in TB patients as compared to HCs independent from *Mtb*-specificity. CD27 expression discriminated both study groups - including TB patients with low or indeterminate IGRA results - effectively. We conclude that CD27 is a promising biomarker for diagnosis of TB patients with inconclusive IGRA results.

IFN-γ release assays (IGRAs) are widely used for detection of T-cell responses against *Mtb* infection. IGRAs are based on short-term *in vitro* stimulation with *Mtb*-specific antigens (mainly Early Secreted Antigen Target (ESAT)-6 and Culture Filtrate Protein (CFP)-10) and subsequent measure of IFN-γ in the supernatant. IGRAs are highly specific but fail to detect *Mtb* infection in subgroups of TB patients from Sub-Saharan Africa [1,2]. In addition, discrimination between active TB disease and healthy -potentially *Mtb* infected- contacts (HCs) is not possible on the basis of IGRA results [3,4].

Previous studies demonstrated the capacity of T-cell phenotyping in combination with IGRA antigen *in vitro* stimulation to distinguish between active TB disease and HCs [5,6]. These studies found higher *Mtb*-specific T-cell proportions with low CD27 expression in TB patients [6]. CD27 is a co-stimulatory receptor expressed on naïve and memory T-cells. During maturation of effector T-cells, CD27 expression decreases progressively in accordance with effector T-cell independency of co-receptor stimulation. In the present study, we determined CD27 expression on T-cells stimulated with IGRA antigens, purified protein derivative (PPD) of *Mtb*, mitogen, and without stimulation in study groups of TB patients and HCs.

1. Material and methods

1.1. Study cohort

This study recruited TB patients (n = 20) (median age 37.5, range 27–74; male/female: 18/2) and HCs (n = 20) (median age 33.5, range 5–61; male/female: 12/8) in 2018 at the Agogo Presbyterian Hospital in Ghana. HCs showed no symptoms of TB but were close relatives living in the same household with TB patients. Diagnosis for TB was based on clinical evaluation, chest X-ray, patient history, and GeneXpert (Cepheid) results. TB patients were included prior to treatment initiation. All study participants were tested using the QuantiFERON (Qiagen) IGRA. Manufacturer's criteria were used for evaluation of QuantiFERON results. The majority of TB patients (n = 13; 65%) had negative (n = 7) or indeterminate (n = 6) IGRA results but GeneXpert results confirmed *Mtb* infection in the vast majority (10 of 13; 77%) of TB patients. GeneXpert negative tuberculosis patients had chest X-ray and clinical symptoms (i.e. blood coughing, weight loss) that were strongly suggestive for TB. All patients diagnosed with TB responded well to treatment. As for TB patients, the vast majority of household contacts had negative

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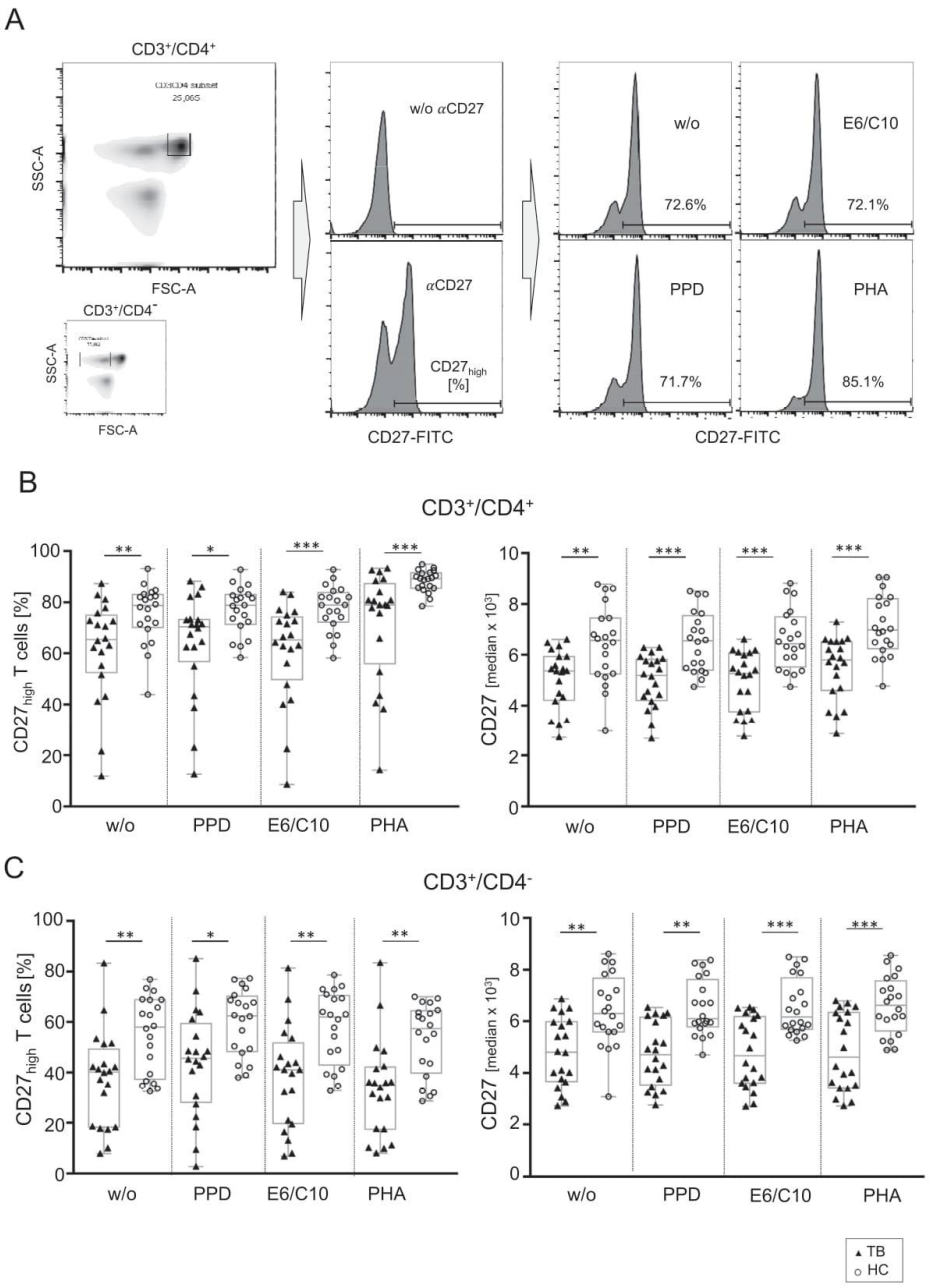

Fig. 1. Lower CD27 expression of $CD4^+$ and $CD4^-$ T-cells from TB patients as compared to HCs. CD27 expression of T-cells of TB patients and HCs after overnight PBMC culture with *Mtb* antigens (i.e. PPD, E6/C10), the mitogen PHA, and without stimulation were determined using a non-CD27 stained control sample as depicted in (A). (**B, C**) Proportions of $CD27_{high}$ T-cells (left graphs) and median CD27 protein expression of $CD27_{high}$ T-cells (right graphs) are shown for $CD3^+/CD4^+$ (**B**) and $CD3^+/CD4^-$ (**C**) T-cells. TB patients are indicated by black triangles and HCs as open circles. Each symbol depicts the mean of duplicates from an individual donor. Nominal p-values for the Mann–Whitney–*U*-test (two-tailed) are given. E6/C10: ESAT-6/CFP-10.

(70%) or indeterminate (5%) QuantiFERON results. The present study received approval from the Committee on Human Research, Publication and Ethics (CHRPE/AP/023/18; CHRPE/221/14) at the Kwame Nkrumah University of Science and Technology in Kumasi. All study subjects gave written informed consent. For children written informed consent was provided by their parents.

1.2. T-cell phenotyping and intracellular IFN-γ measure by flow cytometry

PBMCs were isolated from heparinized blood by density centrifugation (Ficoll, Biochrom) and 1.2×10^5 cells were cultured with co-stimulatory antibodies α-human CD49d (9F10) (1 μg/ml) and CD28 (CD28.2) (1 μg/ml) as described [5]. PPD (10 μg/ml; Statens Serum Institute), ESAT-6/CFP-10 (2 μg/ml; provided by T. Ottenhoff, Leiden University Medical Center), or PHA (1 μg/ml; Sigma–Aldrich) were used for stimulation (20 h, 37°C, 5% CO_2) in the presence of Brefeldin A (2.5 μg/ml; Sigma–Aldrich). The following antibodies were used: αhuman CD3-APC (UCTH1), CD4-PerCP/Cy5.5 (RPA-T4), CD27-FITC (O323), and IFN-γ-PE (B27) (all BioLegend). Cells were measured using BDAccuri C6 (BD Biosciences) and data were analyzed by FlowJo software (BD Biosciences). Samples not stained with CD27 antibodies were used to set the threshold for CD27 positive T-cells. A representative example for the gating procedure is depicted in Supplementary Figure 1.

1.3. Statistical analyses

Statistical analyses were performed using GraphPad prism Software v7 (Graphpad Software). The non-parametric Mann–Whitney-*U*-test was applied. Receiver Operating Characteristic (ROC) curve analysis was performed to evaluate the sensitivity and specificity of CD27 expression for discrimination of TB patients (as well as the TB patient's subgroup with negative or indeterminate QFT results) from HCs. Significance was considered below a p-value of 0.05.

2. Results and discussion

We analyzed CD27 expression of $CD4^+$ T-cells from TB patients and HCs cultured with different stimuli. A threshold was set to define $CD27_{high}$ T-cells using an all-minus-one antibody control (Fig. 1A) and proportions were compared between TB patients and HCs. We found significantly lower proportions of $CD4^+/CD27_{high}$ T-cells in TB patients as compared to HCs (Fig. 1B, left graph). Significant differences were detected in samples stimulated with *Mtb*-specific antigens (ESAT-6/CFP-10: $p = 0.0007$; PPD: $p = 0.02$), PHA ($p = 0.0006$), and without stimulation ($p = 0.009$) (Fig. 1B, left graph). Since CD27 differences could indicate changes in the proportions of $CD4^+$ T-cell subsets and/or impaired CD27 expression on individual T-cells, we next compared CD27 protein expression levels on $CD27_{high}$ T-cells. Samples from TB patients had generally

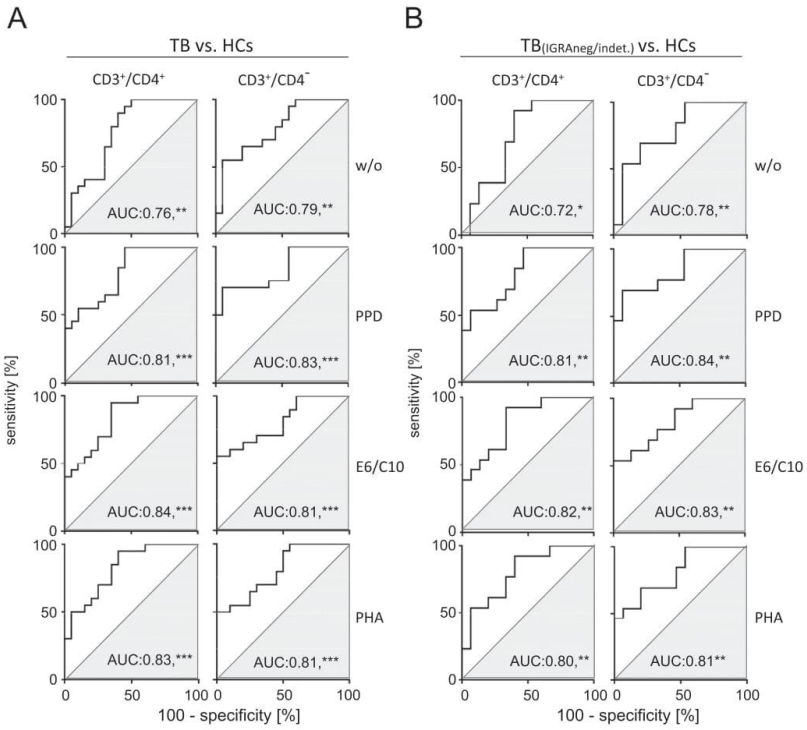

Fig. 2. CD27 expression discriminates between TB patients and HCs. Receiver Operator Characteristic (ROC) analysis for classification of TB patients and HCs using CD27 median expression of stimulated $CD4^+$ and $CD4^-$ T-cells of TB patients and HCs. Area Under Curve (AUC) values for discrimination of (**A**) TB patients from HCs as well as (**B**) QFT negative/indeterminate TB patients and HCs are given. Nominal p-values are shown as * for p<0.05, ** for p<0.01, and *** for p<0.001.

lower median CD27 levels on $CD4^+/CD27_{high}$ T-cells as compared to HCs (Fig. 1B, right graph). These results indicated impaired CD27 expression on $CD4^+$ T-cells as the potential cause for higher proportions of $CD27_{low}$ T-cells of TB patients. To determine if other T-cell subpopulations showed also differences, we next analyzed CD4 negative T-cells for CD27 expression. Similar differences between study groups were detected for CD4 negative T-cells (Fig. 1C) indicating generally decreased CD27 expression of T-cells from TB patients.

CD27 differences were found without re-stimulation and with PHA suggesting mechanisms independent of *Mtb* antigen specificity. Therefore, we next determined CD27 expression on IFN-γ^+/$CD4^+$ T-cells (Supplementary Fig. 1A). PPD and ESAT6/CFP10 specific $CD27_{low}$ IFN-γ^+ T-cells were more frequent in TB patients as compared to HCs whereas PHA-activated T-cells showed no significant differences (Supplementary Fig. 1B). This indicated a retained $CD27_{low}$ phenotype of *Mtb*-specific T-cells when re-stimulated *in vitro*. This finding was in accordance with previous studies demonstrating lower CD27 expression as a feature of *Mtb*-specific $CD4^+$ T-cells in active TB [5–8]. Our results confirmed lower CD27 expression of T-cells from TB patients but suggested pathognomonic mechanisms independent from *Mtb* specificity. A possible explanation for general (i.e. antigen-specificity independent) effects on the T-cell phenotype could be aberrant serum cytokine levels, which we previously described for TB patients [9]. Future studies are needed to address this important point and to determine if decreased CD27 expression influences T-cell functions in TB patients.

Furthermore, we investigated if differential CD27 expression of T-cells can be used to discriminate TB patients from HCs. ROC analyses showed strong capacity of CD27 expression level of T-cells to distinguish TB patients from HCs. Similar AUC values and significant discrimination was detected for *Mtb* antigens, PHA, and without stimulation (Fig. 2A). A significant subgroup of TB patients in our study group was IGRA-negative or -indeterminate (n = 13; 65%) although *Mtb* infection was confirmed for the vast majority of cases (see Methods). Since CD27 differences were seen also in the absence of a *Mtb*-specific stimulation, we assumed that $CD27_{low}$ expression of T-cells could contribute to the diagnosis for this TB patients' subgroup. ROC analyses efficiently discriminated IGRA-negative and -indeterminate TB patients from HCs (Fig. 2B). These results showed that increased proportions of $CD27_{low}$ T-cells characterize patients with active TB and can contribute to TB diagnosis especially in the context of negative or inconclusive IGRA results that are frequent in Sub-Saharan Africa [1,2]. However, interindividual heterogeneity of TB patients/healthy contact responses rendered the efficacy of CD27 expression alone limited for discrimination. These results may reflect heterogeneity of TB pathogenesis e.g. due to confounding environmental and immune genetic factors. Additional studies may therefore analyze the capacity of a group of candidates, including CD27 expression, in combination with learning algorithms (e.g. using support vector machines, linear discriminant analyses, classification/regression trees) for discrimination as it has been described for TB [10].

Since *in vitro* cultured PBMCs without stimulation showed CD27 differences as well, we speculate that *ex vivo* analyzed T-cells from TB patients would also present with lower CD27 expression. Future studies will address this point and the question if functional differences of $CD27_{low}$ T-cells affect TB disease progression.

Conflict of interest

The authors declare to have no conflict of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2019.07.003.

References

[1] Adetifa IM, Lugos MD, Hammond A, Jeffries D, Donkor S, Adegbola RA, et al. Comparison of two interferon gamma release assays in the diagnosis of Mycobacterium tuberculosis infection and disease in the Gambia. BMC Infect Dis 2007;7:122.
[2] Lundtoft C, Awuah AA, Nausch N, Enimil A, Mayatepek E, Owusu-Dabo E, et al. Alternative Quantiferon cytokines for diagnosis of children with active tuberculosis and HIV co-infection in Ghana. Med Microbiol Immunol 2017;206:259–65.
[3] Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma interferon release assays for detection of Mycobacterium tuberculosis infection. Clin Microbiol Rev 2014;27:3–20.
[4] Lu P, Chen X, Zhu LM, Yang HT. Interferon-Gamma release assays for the diagnosis of tuberculosis: a systematic review and meta-analysis. Lung 2016;194:447–58.
[5] Portevin D, Moukambi F, Clowes P, Bauer A, Chachage M, Ntinginya NE, et al. Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study. Lancet Infect Dis 2014;14:931–8.
[6] Streitz M, Tesfa L, Yildirim V, Yahyazadeh A, Ulrichs T, Lenkei R, et al. Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. PLoS One 2007;2:e735.
[7] Jiang J, Wang X, Wang X, Cao Z, Liu Y, Dong M, et al. Reduced CD27 expression on antigen-specific CD4+ T cells correlates with persistent active tuberculosis. J Clin Immunol 2010;30:566–73.
[8] Latorre I, Fernandez-Sanmartin MA, Muriel-Moreno B, Villar-Hernandez R, Vila S, Souza-Galvao ML, et al. Study of CD27 and CCR4 markers on specific CD4(+) T-cells as immune tools for active and latent tuberculosis management. Front Immunol 2018;9:3094.
[9] Harling K, Adankwah E, Guler A, Afum-Adjei Awuah A, Adu-Amoah L, Mayatepek E, et al. Constitutive STAT3 phosphorylation and IL-6/IL-10 co-expression are associated with impaired T-cell function in tuberculosis patients. Cell Mol Immunol 2019 Mar;16(3):275–87.
[10] Jacobsen M, Repsilber D, Gutschmidt A, Neher A, Feldmann K, Mollenkopf HJ, et al. Candidate biomarkers for discrimination between infection and disease caused by Mycobacterium tuberculosis. J Mol Med (Berl) 2007;85:613–21.

3.3. Paper IV: Aberrant plasma IL-7 and soluble IL-7 receptor levels indicate impaired T cell response to IL-7 in human tuberculosis

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Contribution to this publication: 20%

- ELISA
- Data analysis
- Writing of the manuscript

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RESEARCH ARTICLE

Aberrant plasma IL-7 and soluble IL-7 receptor levels indicate impaired T-cell response to IL-7 in human tuberculosis

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Abstract

T-cell proliferation and generation of protective memory during chronic infections depend on Interleukin-7 (IL-7) availability and receptivity. Regulation of IL-7 receptor (IL-7R) expression and signalling are key for IL-7-modulated T-cell functions. Aberrant expression of soluble (s) and membrane-associated (m) IL-7R molecules is associated with development of autoimmunity and immune failure in acquired immune deficiency syndrome (AIDS) patients. Here we investigated the role of IL-7/IL-7R on T-cell immunity in human tuberculosis. We performed two independent case-control studies comparing tuberculosis patients and healthy contacts. This was combined with follow-up examinations for a subgroup of tuberculosis patients under therapy and recovery. Blood plasma and T cells were characterised for IL-7/sIL-7R and mIL-7R expression, respectively. IL-7-dependent T-cell functions were determined by analysing STAT5 phosphorylation, antigen-specific cytokine release and by analysing markers of T-cell exhaustion and inflammation. Tuberculosis patients had lower soluble IL-7R ($p < 0.001$) and higher IL-7 ($p < 0.001$) plasma concentrations as compared to healthy contacts. Both markers were largely independent and aberrant expression normalised during therapy and recovery. Furthermore, tuberculosis patients had lower levels of mIL-7R in T cells caused by post-transcriptional mechanisms. Functional *in vitro* tests indicated diminished IL-7-induced STAT5 phosphorylation and impaired IL-7-promoted cytokine release of *Mycobacterium tuberculosis*-specific $CD4^+$ T cells from tuberculosis patients. Finally, we determined T-cell exhaustion markers PD-1 and SOCS3 and detected increased SOCS3 expression during therapy. Only moderate correlation of PD-1 and SOCS3 with IL-7 expression was observed. We conclude that diminished soluble IL-7R and increased IL-7 plasma concentrations, as well as decreased membrane-associated IL-7R expression in T cells, reflect impaired T-cell sensitivity to IL-7 in tuberculosis patients. These findings show similarities to pathognomonic features of impaired T-cell functions and immune failure described in AIDS patients.

decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Author summary

IL-7 is important for the development and homeostasis of T cells and promotes antigen-specific T-cell responses. Aberrant expression of plasma IL-7 and soluble IL-7R are found in autoimmune diseases and chronic viral infections. In AIDS patients—especially those who fail to reconstitute T-cell numbers during therapy—impaired IL-7-promoted T-cell functions indicated T-cell exhaustion/senescence. In order to evaluate the potential impact of IL-7 on tuberculosis, we characterised various parameters involved in the IL-7-response of tuberculosis patients and healthy contacts. Despite IL-7 being available at higher plasma levels among tuberculosis patients, the T-cell response to IL-7 was impaired when compared to healthy contacts. Soluble IL-7R levels were aberrantly low in plasma during acute tuberculosis but did not account for impaired IL-7 usage. Chronic inflammation in tuberculosis patients—reflected by increased IL-6 plasma levels—did not account for dysfunctional T-cell responses and analysed T-cell exhaustion markers were only moderately correlated. Our findings demonstrate that availability of IL-7 alone is not sufficient to promote protective T-cell immunity against tuberculosis. We describe aberrant IL-7/soluble IL-7R expression and impaired IL-7-mediated T-cell functions in tuberculosis patients with similarities and differences to described IL-7 dysregulation seen in patients with AIDS.

Introduction

T cells are crucial for protection against *Mycobacterium (M.) tuberculosis* infection but biomarkers that characterise T-cell failure and progression towards tuberculosis disease are not available [1]. $CD4^+$ T cells are key to anti-mycobacterial immune protection [2] and $CD4^+$ T-cell deficiency, e.g. of AIDS patients, results in increased susceptibility against tuberculosis [3–5]. There is growing evidence that impaired $CD4^+$ T-cell functions play a role in tuberculosis [6]. Recent studies identified T-cell exhaustion as a feature of tuberculosis [7, 8]. T-cell exhaustion impairs immunity against chronic viral infections and harms memory T-cell potential [9]. IL-7 is central for generation of memory T cells and was shown to revert T-cell exhaustion in chronic viral infections [10]. Notably, IL-7 induced T-cell memory was hampered in the presence of persistent antigen and inflammation as seen for chronic viral infections [11]. In AIDS patients, failure of immune reconstitution is accompanied by a dysfunctional T-cell response that showed features of senescence and exhaustion [12–14]. Recently, persistent inflammation characterised e.g. by increased IL-6 serum concentrations from AIDS patients were found to correlate with T-cell exhaustion/senescence and impaired T-cell response to IL-7 [14, 15]. High IL-7 plasma levels as well as decreased membrane-associated (m)IL-7R expression of T cells were found in AIDS patients with immune failure [16, 17]. Concomitantly impaired T-cell response to IL-7 was detected in immune failure patients [13–15, 18–20].

The regulation of IL-7R expression is central for control of IL-7-mediated effects on T cells [21]. On IL-7 binding, the mIL-7R assembles as a heterodimer (comprising the IL-7Rα (CD127) and the common γ-chain (CD132)) and induces signalling cascades mainly via the Jak/STAT pathway. Jak1 and Jak3 are involved in IL-7R signalling, and STAT5 gets phosphorylated and initiates multiple transcription events [22]. As part of IL-7 signalling, the mIL-7R is rapidly internalised, becomes partly degraded or recycles to the cell surface [23]. Regulation of IL-7R expression is also controlled on the transcriptional level and IL-7 and other cytokines were shown to suppress IL-7R mRNA expression [24]. Alternative splicing of the *IL7RA* gene generates a soluble IL-7R (sIL-7R) variant [25]. The sIL-7R variant binds IL-7 although with

lower affinity as compared to the mIL-7R heterodimer and is present in blood plasma at high molar excess relative to IL-7 [26]. The exact role of the sIL-7R for IL-7 metabolism remains elusive. Competitive inhibition of IL-7 uptake as well as IL-7 reservoir functions have been described [26–28]. Differential sIL-7R plasma concentrations are found in immune pathologies, e.g. autoimmune diseases [26, 29, 30] and AIDS [28, 31]. In addition, a functional *IL7RA* polymorphism (rs6897932) that interferes with IL-7R alternative splicing and thereby leads to reduced sIL-7R levels in plasma was found to be associated with autoimmune diseases [32, 33] and to affect immune reconstitution in AIDS patients [34–36].

Initial results indicating a role of IL-7 during T-cell immunity against tuberculosis were derived from animal models. Increased IL-7 and soluble IL-7R expression in pulmonary tissue of primates with tuberculosis was found, indicating a possible role of IL-7 metabolism in tuberculosis pathogenesis [37, 38]. Furthermore IL-7 was shown to promote survival and to improve BCG vaccination efficacy in *M. tuberculosis*-infected mice [39, 40]. However, a comprehensive understanding of the possible role of IL-7 or IL-7R functions in human tuberculosis has not yet been developed.

This present study aimed to elucidate a possible role of IL-7 modulated T-cell responses in human tuberculosis. We determined sIL-7R and IL-7 plasma concentrations and mIL-7R expression of T cells from tuberculosis patients—before, during, and after chemotherapy—and compared these to healthy contacts. Since results resembled pattern seen in AIDS patients with impaired T-cell response to IL-7, we then performed functional T-cell assays in a second set of tuberculosis patients and healthy contacts to determine IL-7-mediated signalling and promoted cytokine release on *M. tuberculosis*-specific T-cell activation. Finally, mRNA expression of exhaustion markers was compared in $CD4^+$ T cells between the cohorts to evaluate a possible causative role of T-cell exhaustion for impaired IL-7 response in tuberculosis.

Results

Decreased sIL-7R plasma concentrations in acute tuberculosis patients

Aberrant sIL-7R plasma levels indicate pathologic T-cell immunity in autoimmune, inflammatory, and chronic viral diseases. Hence, we determined sIL-7R plasma concentrations in individuals infected with *M. tuberculosis*. Patients with active tuberculosis (n = 57) and healthy contacts (n = 151) were included. Tuberculosis patients had significantly lower sIL-7R concentrations as compared to healthy contacts ($p < 0.001$) (Fig 1a). Since study groups differed in gender distributions (tuberculosis: 30% females; contacts: 56% females; Table 1), we compared sIL-7R between male and female subgroups. Female patients with tuberculosis showed moderately lower sIL-7R concentrations as compared to male patients, whereas no differences were detected for healthy contacts (S1 Fig). Therefore, differences in plasma sIL-7R were not due to gender differences. Next we determined the influence of anti-tuberculosis therapy and recovery on plasma sIL-7R in tuberculosis patients (i.e. 2 months and 6 months after therapy onset). Analyses revealed significantly increased sIL-7R plasma levels after 2 months ($p = 0.03$) and after recovery ($p = 0.009$) (Fig 1b). sIL-7R plasma concentrations of recovered tuberculosis patients were comparable to healthy contacts (Fig 1b). To determine if changes in sIL-7R under therapy were dependent on sIL-7R concentrations prior to treatment, we compared initial sIL-7R concentrations with changes of sIL-7R expression between 0 and 6 months. Absolute differences and ratios were calculated. Absolute differences (month 6 –month 0) showed only moderate negative correlation with initial sIL-7R levels (rho = -0.26; $p = 0.13$) (S2a Fig), but changes of ratios (month 6 / month 0) were strongly associated with sIL-7R levels prior to treatment (rho = -0.61, $p < 0.001$) (S2a Fig). Therefore, especially tuberculosis patients with

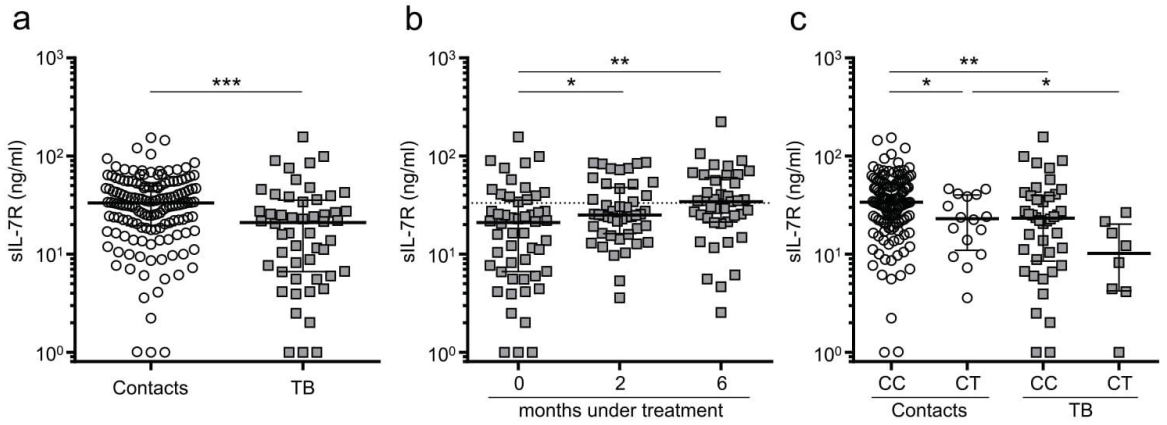

Fig 1. Plasma sIL-7R level in healthy contacts and tuberculosis during chemotherapy. (a) Concentrations of sIL-7R in plasma from tuberculosis patients (n = 52) and healthy contacts (n = 149) determined by cytometric bead array. **(b)** sIL-7R plasma concentration prior to (0 months, n = 52), during (2 months, n = 46) and after (6 months, n = 41) treatment of tuberculosis patients. Median sIL-7R plasma concentration of healthy contacts is indicated with a dotted line. **(c)** Plasma sIL-7R levels stratified for the *IL7RA* exon 6 single nucleotide polymorphism rs6897932C>T for healthy contacts (n = 142) and tuberculosis patients (n = 50). Median and interquartile range is depicted, and each symbol indicates mean values of duplicates from each individual donor. Exact Mann-Whitney U test was used for comparison of groups, while paired data was evaluated by Wilcoxon Signed-Rank test. Nominal p-values are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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low sIL-7R concentrations prior to treatment showed increased sIL-7R levels after recovery and a relative gain of sIL-7R plasma concentration was detected.

The IL7RA functional polymorphism rs6897932 contributed to differential sIL-7R plasma levels

A functional single nucleotide polymorphism (SNP, rs6897932C>T) in exon 6 of the *IL7RA* gene interferes with splicing and impairs sIL-7R expression [32]. Therefore, we determined

Table 1. Patient characteristics.

Cohort 1	**Healthy Contacts**	**TB**
Number of participants	151	57
Age (y)	31 [18–68]	33 [18–71]
Gender		
Female	84 (56%)	17 (30%)
Male	67 (44%)	40 (70%)
BCG vaccination		
Yes	90 (60%)	28 (49%)
No	50 (33%)	28 (49%)
No information	11 (7%)	1 (2%)
Cohort 2		
Number of participants	24	22
Age (y)	41 [21–65]	39 [15–72]
Gender		
Female	14 (58%)	15 (68%)
Male	10 (42%)	7 (32%)

Median [range] or number (proportion) is shown.

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the rs6897932 minor T allele (rs6897932T) frequency in tuberculosis patients and healthy contacts. Tuberculosis patients had a marginally higher MAF proportion (7.3%) as compared to healthy contacts (5.6%). No homozygous rs6897932T/T carriers were identified in the study groups. As expected, lower levels of plasma sIL-7R were detected for rs6897932C/T healthy contacts as compared to rs6897932C/C wild type healthy contacts ($p = 0.02$), and the same tendency was seen for the tuberculosis patients ($p = 0.06$) (Fig 1c). However, stratification for SNP genotypes confirmed lower plasma sIL-7R among tuberculosis patients when compared to healthy contacts ($p < 0.001$). We concluded that increased frequencies of IL-7R rs6897932T alleles in tuberculosis patients contributed to differential sIL-7R levels but did not account for lower sIL-7R plasma concentrations of tuberculosis patients.

Increased IL-7 plasma concentrations in tuberculosis patients but no correlation with sIL-7R

We hypothesised that differential sIL-7R plasma levels would affect IL-7 consumption. Consequently we next determined IL-7 plasma concentrations in tuberculosis patients and healthy contacts. Tuberculosis patients showed significantly increased IL-7 concentrations prior to therapy as compared to healthy contacts ($p < 0.001$) (Fig 2a). IL-7 concentrations decreased under therapy and recovery (0 vs. 6 months, $p < 0.001$) and reached levels comparable to healthy contacts (Fig 2b). Higher initial IL-7 levels were associated with stronger decrease rates until month 6 (rho = -0.58, $p < 0.001$; S2b Fig). Notably, and in contrast to sIL-7R results, also absolute differences between month 0 and 6 correlated strongly with IL-7 levels prior to therapy (rho = -0.79, $p < 0.001$; S2b Fig). This indicated different mechanisms involved in IL-7 and sIL-7R regulation during tuberculosis. In accordance, no dependency was detected between IL-7 and sIL-7R plasma concentrations for tuberculosis patients or healthy contacts (Fig 2c).

These results suggested that IL-7 and sIL-7R could be useful as biomarkers for diagnosis of tuberculosis patients. Comparison of tuberculosis patients and healthy contacts revealed moderate discrimination capacity for both sIL-7R (AUC = 0.67) and IL-7 (AUC = 0.73) using Receiver Operating Characteristic (ROC) analysis (Fig 2d). Independency of IL-7 and sIL-7R plasma levels (Fig 2c) prompted us to calculate the combined efficacy of both markers using Random Forest analysis (for details see Methods section). Correct prediction of tuberculosis patients and healthy contacts was achieved for 73% of all donors, and IL-7 was about two times more influential on prediction than sIL-7R. These results indicated that IL-7 and sIL-7R plasma concentrations were largely independent and may contribute to tuberculosis diagnosis.

Increased proportions of mIL-7R$_{low}$ CD4$^+$ and CD8$^+$ T cells in tuberculosis patients

Increased IL-7 plasma concentrations are likely caused by decreased T-cell consumption of IL-7. Low T-cell numbers or impaired T-cell receptivity of IL-7 may account for this. Hence we compared mIL-7R protein expression for subgroups of tuberculosis patients and healthy contacts by flow cytometry. We detected lower mean mIL-7R expression for CD8$^+$ T cells ($p = 0.02$) and a tendency for CD4$^+$ T cells ($p = 0.05$) (Fig 3a). Analysis of mIL-7R on T-cell subpopulations revealed increased proportions of mIL-7R$_{low}$ CD4$^+$ ($p = 0.006$) and CD8$^+$ T cells ($p = 0.02$) from tuberculosis patients as compared to healthy contacts (Fig 3b). To confirm these observations, we performed mIL-7R analysis in a second independent cohort study including additionally recruited tuberculosis patients (n = 22) and healthy contacts (n = 24). Due to restriction in the number of flow cytometry parameters, CD4$^+$ and CD4$^-$ T cells were analysed for mIL-7R protein expression. Tuberculosis patients showed significantly decreased

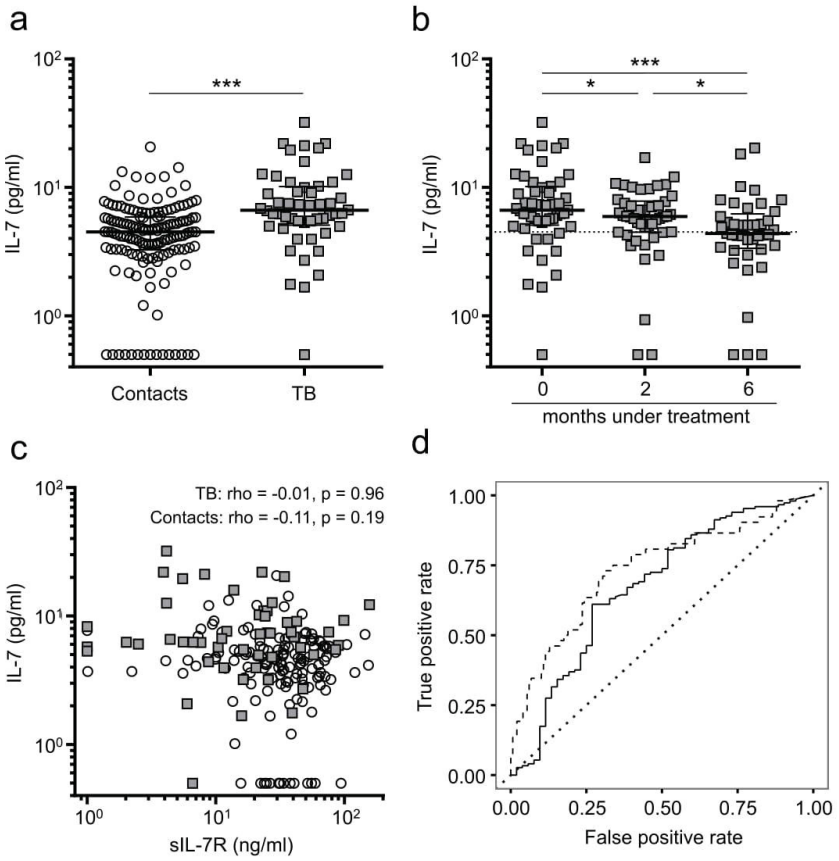

Fig 2. Plasma IL-7 concentration of tuberculosis patients and healthy contacts. (a) Comparison of IL-7 plasma concentrations between tuberculosis patients (n = 52) and healthy contacts (n = 148). **(b)** Plasma IL-7 concentrations prior to (0 months, n = 52), during (2 months, n = 46), and after (6 months, n = 41) treatment of tuberculosis patients. Median IL-7 plasma level of healthy contacts is represented with a dotted line. **(c)** Spearman correlation between plasma IL-7 and sIL-7R for tuberculosis patients prior to treatment (squares), and healthy contacts (circles). **(d)** Receiver operating characteristic (ROC) curve indicates sensitivity and specificity of plasma sIL-7R (solid line) and plasma IL-7 (dashed line) to discriminate between patients with tuberculosis and healthy contacts. The line of no discrimination is indicated as a dotted line. Median and interquartile range is depicted, and each symbol indicates mean values of duplicates from each individual donor. Exact Mann-Whitney U test was used for comparison of groups, whereas paired data was evaluated by Wilcoxon Signed-Rank test. Nominal p-values are indicated as: * $p < 0.05$, *** $p < 0.001$.

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mIL-7R expression for both $CD4^+$ ($p = 0.01$) and $CD4^-$ ($p = 0.006$) T cells (S4 Fig). This confirmed initial results and led us to the conclusion that impaired mIL-7R expression of T cells resulted in increased proportions of mIL-7R_{low} $CD4^+$ and $CD8^+$ T cells in tuberculosis patients.

Differential mIL-7R expression may be affected by plasma IL-7 and sIL-7R levels. We determined correlation between these parameters to identify possible interactions. A tendency of positive correlation between mIL-7R expression and sIL-7R plasma (rho = 0.42, p = 0.06) was

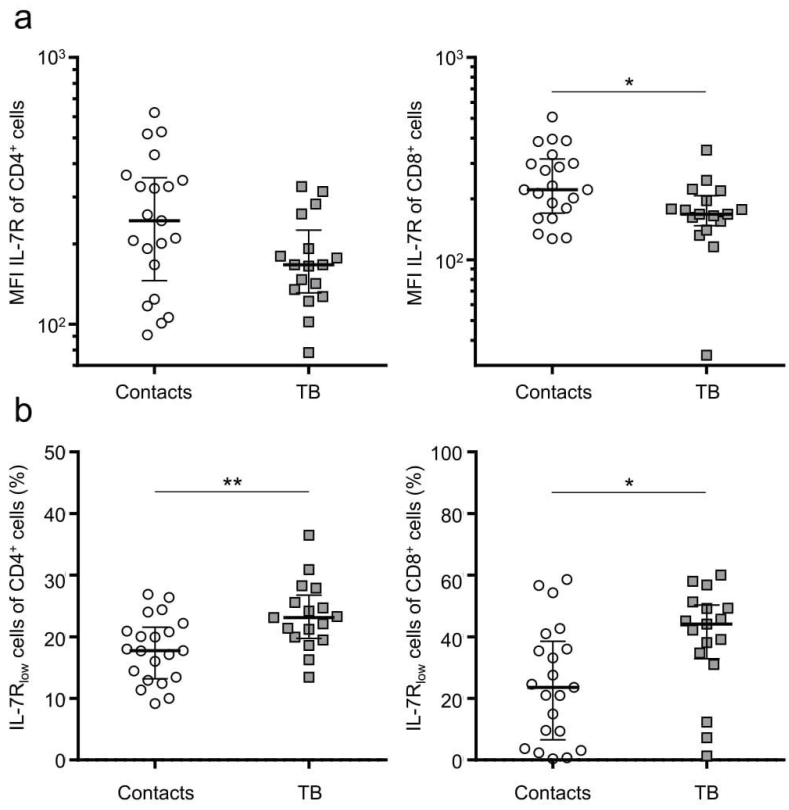

Fig 3. Membrane-associated IL-7R expression on CD4⁺ and CD8⁺ T cells. Membrane-associated IL-7R expression of CD4⁺ (left graphs) and CD8⁺ (right graphs) T cells from tuberculosis patients (n = 17) and healthy contacts (n = 21) analysed by flow cytometry. **(a)** Mean fluorescence intensity (MFI) analyses of IL-7R. **(b)** Proportions of IL-7R$_{low}$-expressing CD4⁺ and CD8⁺ T cells from tuberculosis patients and healthy contacts. Median and interquartile range is depicted, and each symbol indicates mean values of duplicates from each individual donor. Exact Mann-Whitney U test used for comparison of groups. Nominal p-values are indicated as * $p < 0.05$, ** $p < 0.01$.

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found only in the group of contacts, whereas mIL-7R and IL-7 showed a marginal negative correlation (rho = -0.38, p = 0.10) (S1 Table) in this study group. No correlation between any parameters was found for tuberculosis patients (S1 Table).

Similar IL-7R isoform mRNA expression of CD4⁺ T cells from tuberculosis patients and healthy contacts

High IL-7 plasma levels and low mIL-7R expression of T cells have previously been described for HIV/AIDS patients [16, 17, 41, 42]. In AIDS patients these differences are accompanied with mIL-7R regulatory dysfunctions [43]. Therefore we questioned whether aberrant expression of IL-7R variants in tuberculosis patients is caused by differential regulation on the transcriptional or post-transcriptional level. Hence, we analysed IL-7R mRNA transcripts of purified CD4⁺ T cells from tuberculosis patients and healthy contacts. Three IL-7R transcripts

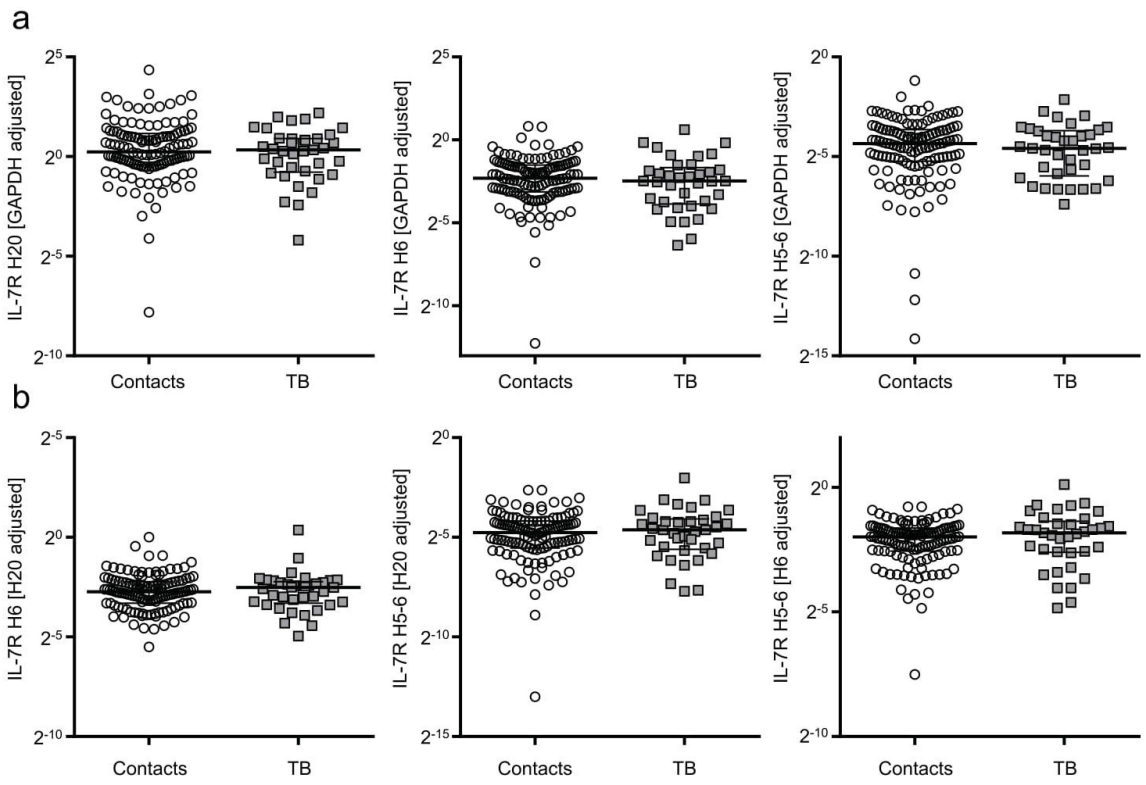

Fig 4. mRNA expression of IL-7R variants of CD4⁺ T cells from tuberculosis patients and healthy contacts. The expression of full length IL-7R (H20), and IL-7R lacking exon 6 (H6) or exon 5–6 (H5-6) was evaluated using mRNA isolated from $CD4^+$ T cells. **(a)** Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or **(b)** IL-7R variants were applied as references. Cycle threshold differences ($2^{-\Delta Ct}$) are shown for tuberculosis patients (n = 37) and healthy contacts (n = 120). Median and interquartile range is depicted, and each symbol indicates mean values of duplicates from each individual donor. Exact Mann-Whitney U test used for comparison of groups.

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coding for the mIL-7R (all 8 exons included; H20) and a sIL-7R (H6 and H5-6; for details see Methods section [25]) were measured. None of the IL-7R variants were differentially expressed on the mRNA level of $CD4^+$ T cells between tuberculosis patients and healthy contacts (Fig 4a). Also relative expression of sIL-7R vs. mIL-7R transcripts was similar between study groups (Fig 4b). These results indicated that differential IL-7R mRNA expression is not the cause for aberrant sIL-7R and mIL-7R expression in tuberculosis patients and render causative post-transcriptional mechanisms likely.

Impaired IL-7-induced STAT5 phosphorylation and IL-7-promoted T-cell cytokine release in tuberculosis patients

Impaired IL-7 signalling has been associated with diminished IL-$7R_{low}$ expression of T cells from AIDS patients, but different mechanisms about the role of STAT5 were described [18, 44, 45]. To evaluate the effect of IL-7 signalling, we recruited a second cohort of tuberculosis patients (n = 22) or healthy contacts (n = 24) (Table 1). A lower surface level of mIL-7R on T

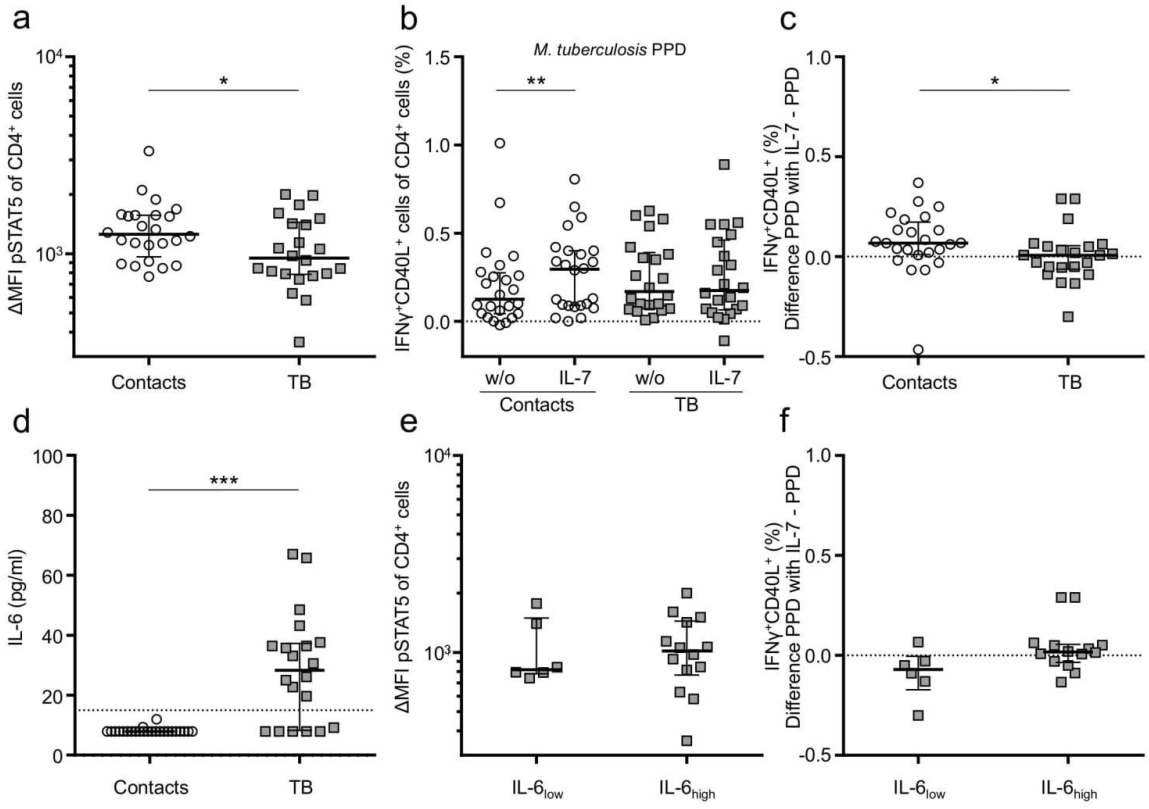

Fig 5. IL-7 response of CD4⁺ T cells from tuberculosis patients and healthy contacts. (a) IL-7-induced (10 ng/ml) STAT5 phosphorylation of CD4⁺ T cells from tuberculosis patients (n = 22) and healthy contacts (n = 24) measured by flow cytometry. The STAT5 phosphorylation level of non-stimulated cells has been subtracted of all values. **(b)** IFNγ/CD40L-expressing CD4⁺ T cells after PPD re-stimulation in the presence or absence of IL-7 detected by flow cytometry. Non-stimulated values with or without IL-7 have been subtracted. **(c)** Induction of IFNγ/CD40L-expressing CD4⁺ T cells by IL-7 and PPD stimulation. Absolute differences as compared to PPD alone are shown. **(d)** Plasma IL-6 levels of tuberculosis patients (n = 20) and healthy contacts (n = 24). An arbitrary threshold indicated by a dotted line was set to define tuberculosis patients with high ($IL\text{-}6_{high}$) and low ($IL\text{-}6_{low}$) concentrations of plasma IL-6. **(e)** STAT5 phosphorylation, and IL-7-induced PPD response **(f)** for tuberculosis patients with high or low plasma IL-6 level, as defined in (d). Median and interquartile range is depicted, and exact Mann-Whitney U test was applied for comparison of groups, whereas paired data was evaluated by Wilcoxon Signed-Rank test. Nominal p-values are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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cells from tuberculosis patients was confirmed in this cohort (S4 Fig). Next, we measured IL-7-induced STAT5 phosphorylation and detected decreased phosphorylated STAT5 in CD4⁺ T cells from tuberculosis patients as compared to healthy contacts ($p = 0.04$) (Fig 5a). Since IL-7 was shown to enhance specific T-cell cytokine release [46], we determined intracellular cytokines after *M. tuberculosis* antigen (PPD) *in vitro* stimulation in the presence or absence of IL-7. No differences were detected for PPD-specific T cells co-expressing IFNγ and CD40L when comparing tuberculosis patients and healthy contacts (Fig 5b). However, co-stimulation with IL-7 induced increased proportions IFNγ-producing T cells solely in the study group of healthy contacts ($p = 0.003$), but not in tuberculosis patients ($p = 0.94$) (Fig 5b). Next, IL-7-specific effects were quantified by calculating the difference of PPD induced T cells with or without IL-7 for each individual (Fig 5c). We found a significantly stronger effect of IL-7 on

cytokine release in healthy contacts as compared to tuberculosis patients ($p = 0.02$). These results suggested impaired T-cell responses to IL-7 in patients with tuberculosis.

Increased IL-6 plasma concentrations in tuberculosis patients were not associated with impaired T-cell responses to IL-7

Chronic inflammation and increased IL-6 serum concentrations were found in AIDS patients with impaired T-cell immunity to IL-7 [14, 15]. One study found a direct inhibitory effect of IL-6 on IL-7-mediated T-cell functions [15]. Since increased IL-6 plasma levels were described in tuberculosis previously [47], we measured plasma IL-6 levels and detected increased IL-6 concentrations in tuberculosis patients as compared to healthy contacts ($p < 0.001$) (Fig 5d). The distribution of IL-6 plasma concentrations indicated two subgroups of tuberculosis patients. Hence we set an arbitrary threshold (15 pg/ml) and compared IL-6_{high} and IL-6_{low} tuberculosis patients for IL-7-promoted T-cell responses. No significant differences in IL-7-induced STAT5 phosphorylation or IL-7 co-stimulated IFNγ/CD40L expression was found between the two IL-6_{high} and IL-6_{low} subgroups of tuberculosis patients (Fig 5e and 5f). Therefore differential IL-6 serum levels were not associated with impaired IL-7-promoted T-cell responses in tuberculosis patients.

Exhaustion markers PD-1 and SOCS3 were not associated with IL-7-impaired T-cell response in tuberculosis patients

Programmed cell death (PD)-1, a marker of T-cell exhaustion and senescence was recently found to be expressed on T cells with impaired response to IL-7 [14]. We determined PD-1 mRNA expression of purified $CD4^+$ T cells and found similar PD-1 expression among healthy contacts and tuberculosis patients prior to therapy (Fig 6a). Under therapy, a decrease of PD-1 expression was found for tuberculosis patients ($p = 0.007$) followed by an increase until recovery ($p < 0.001$). PD-1 levels in recovered tuberculosis patients were even higher as compared to healthy contacts ($p = 0.04$). We found a moderate but significant positive correlation of PD-1 ($rho = 0.22$, $p = 0.005$) with IL-7 (Fig 6b). Previously, we identified SOCS3 as a marker of $CD4^+$ T cells in tuberculosis [48], and others described SOCS3 as a central regulator of T-cell exhaustion and target of IL-7 in chronic viral infections [10]. Therefore we determined SOCS3 mRNA expression of $CD4^+$ T cells. Marginal increased SOCS3 expression was detected in tuberculosis patients prior to therapy ($p < 0.16$), and significantly increased SOCS3 levels were detected at two months under therapy ($p < 0.001$) and after six months ($p = 0.04$) as compared to healthy contacts (Fig 6c). As for PD-1, a moderate positive correlation between SOCS3 expression and IL-7 concentrations was found ($rho = 0.22$, $p = 0.005$) (Fig 6d). We concluded that expression of T-cell exhaustion marker SOCS3 was increased in tuberculosis patients during therapy but was only moderately associated with aberrant IL-7 plasma concentrations. These observations indicated similarities and differences of aberrant IL-7 pathway features in tuberculosis patients as compared to AIDS patients.

Discussion

In the presented study, we identified alterations in the IL-7 pathway and impaired T-cell response to IL-7 co-stimulation in tuberculosis patients.

First, we detected higher IL-7 plasma concentrations in tuberculosis patients that decreased during therapy and recovery. Lymphopenia may cause high IL-7 plasma levels [49, 50] and few reports indicated a role of lymphopenia in tuberculosis [51–53], but this has not been verified by others [54]. We did not determine lymphocyte counts in the present study and cannot

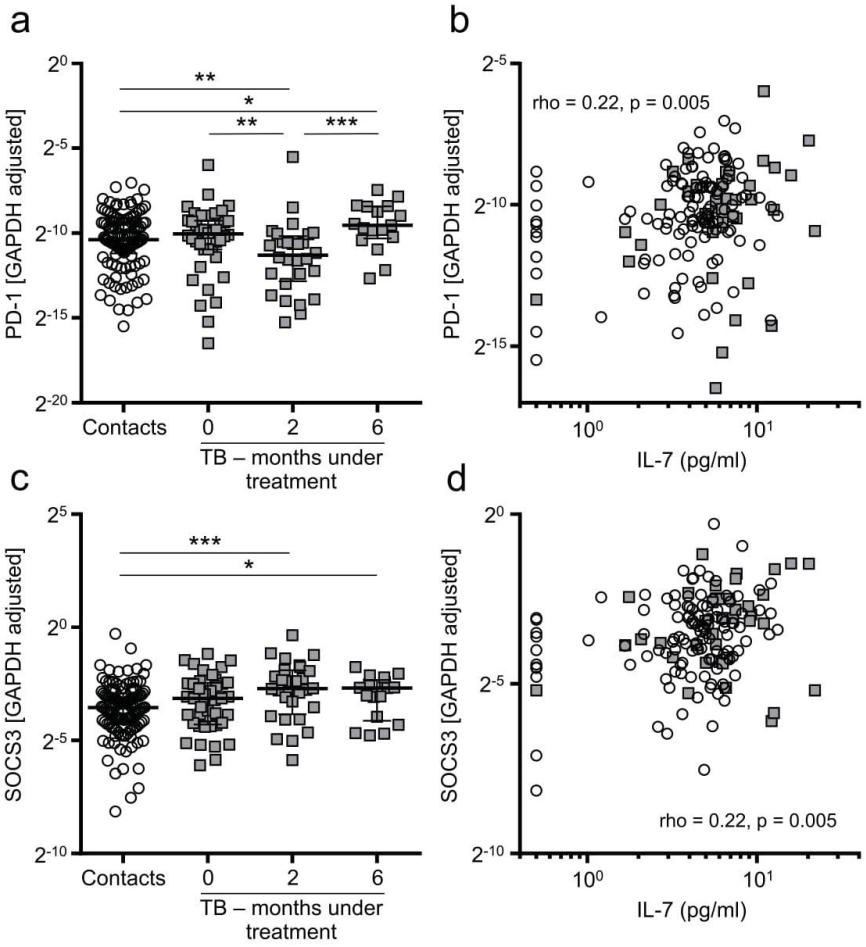

Fig 6. PD-1 and SOCS3 mRNA expression of CD4+ T cells from tuberculosis patients and healthy contacts. The expression of PD-1 **(a)** and SOCS3 **(c)** was determined for mRNA isolated from $CD4^+$ T cells, using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference. Cycle threshold differences ($2^{-\Delta Ct}$) are shown for healthy contacts [n = 117 (PD-1), n = 119 (SOCS3)], and for tuberculosis patients prior to (0 months, n = 40), during (2 months, n = 28), and after (6 months, n = 17) treatment. Median and interquartile range is depicted. Spearman correlation between plasma IL-7 and **(b)** PD-1 or **(d)** SOCS3 for healthy contacts (circles) or tuberculosis patients (squares) prior to treatment. Each symbol indicates mean values of duplicates from each individual donor. Due to a low overlap between tuberculosis patients, exact Mann-Whitney U test used for comparison of all groups. Nominal p-values are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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prove or refute lymphopenia as a possible cause for high IL-7 levels. However, there is evidence that IL-7 serum concentrations are affected only at very low $CD4^+$ T-cell numbers in AIDS patients [41, 55], and these levels are far below lymphopenia described in tuberculosis [49, 50]. Another possible explanation for higher IL-7 plasma concentrations is impaired receptivity/consumption of IL-7 by T cells [21]. Our investigations provide evidence for reduced mIL-7R expression and impaired IL-7 co-stimulatory effects on T cells from tuberculosis patients.

Strong evidence for impaired IL-7 regulation and T-cell function was found for chronic viral infections, especially AIDS [56]. In AIDS patients increased IL-7 plasma levels and decreased mIL-7R expression of T cells were described [17, 42, 43, 57–59]. Furthermore, impaired T-cell response to IL-7 in AIDS patients was shown to affect immune reconstitution during anti-retroviral therapy [13, 60, 61]. In order to determine possible dependencies between mIL-7R expression on T cells and IL-7/sIL-7R plasma concentrations, we performed correlation analyses. For contacts there was a tendency of positive correlation between mIL-7R and sIL-7 levels, whereas IL-7 plasma levels showed a marginal negative correlation with mIL-7R expression. Given the described regulatory influence of IL-7/sIL-7R on mIL-7R expression [21], we speculate that IL-7 and sIL-7R plasma level alterations caused by tuberculosis disrupted this dependency that indicates the homeostatic balance in healthy individuals. The low number of samples included for mIL-7R analyses restricted the validity of these results. In addition, analyses of mIL-7R during disease course and after recovery are needed to confirm this thesis.

Several mechanisms and T-cell phenotypes were described to play a role in impaired IL-7 functions of AIDS patients. Chronic inflammation and increased serum concentrations of IL-6 were found in HIV/AIDS [14, 15], and functional *in vitro* assays indicated inhibitory effects of the pro-inflammatory cytokines IL-6 and IL-1β on IL-7-mediated signal transduction [15]. Higher IL-6 plasma concentrations were described for tuberculosis [47, 62], and we confirmed higher IL-6 plasma concentrations in a subgroup of tuberculosis patients in the present study. However, we did not detect IL-7 response differences between IL-6_{high} and IL-6_{low} subgroups among tuberculosis patients (Fig 5e and 5f). Hence there was no indication for an association between IL-6 plasma concentrations and impaired IL-7 T-cell response of tuberculosis patients.

T-cell exhaustion was found in AIDS patients [12, 13, 60, 63–65] and was associated with decreased IL-7R expression [60, 64] and impaired IL-7 response [13]. Initial studies indicated a role of T-cell exhaustion in tuberculosis animal models [7, 8]. Our results on SOCS3 and PD-1 expression did not support a major role of T-cell exhaustion in human tuberculosis and this is in accordance with a previous study [66]. These differences might at least partly be due to the fact that exhaustion is poorly defined for $CD4^+$ T cells in contrast to $CD8^+$ T cells [67]. Therefore, other marker molecules may be indicative for exhaustion in $CD4^+$ T cells. We were not able to study the phenotype of $CD8^+$ T cells in detail in the present study but decreased mIL-7R expression (Fig 3a) may indicate exhaustion of $CD8^+$ T cells in tuberculosis patients.

Impaired mIL-7R signalling was described for T cells from AIDS patients [13, 14, 18, 68]. We detected lower STAT5 phosphorylation and showed also impaired IL-7 promoted cytokine release in T cells from tuberculosis patients. The capacity of IL-7 to promote IFNγ-expressing T cells for detection of *M. tuberculosis* infection has been shown before [46]. Here we provide first evidence that IL-7 mediated increased sensitivity of T cells to stimulation (e.g. by decreasing the T-cell receptor activation threshold [69]) was impaired in tuberculosis patients. One may therefore speculate that impaired IL-7 response not only hampered generation of effective memory but also effector T-cell response against acute tuberculosis. This raised the question if impaired T-cell response to IL-7 can be interpreted as a feature of T-cell anergy. Anergy is defined as unresponsiveness of T cells to their cognate antigen and anergy against PPD—measured by tuberculin skin test—has been described for tuberculosis patients before [70]. We did not detect differences in the PPD response of $CD4^+$ T cells between tuberculosis patients and healthy contacts in the present study (Fig 5b). However, we would speculate that impaired T-cell responses to IL-7 contributed to the phenomenon of diminished tuberculin reactivity in tuberculosis patients as this *in vivo* test would be better reflected by IL-7-supplemented PPD stimulation in our *in vitro* assay. Since IL-7 effects on T-cell function include a decreased T-cell receptor activation threshold [69], impaired mIL-

7R signaling may contribute to diminished T-cell receptor signaling characteristic for T-cell anergy [71]. Therefore impaired mIL-7R signaling may contribute to tuberculin skin test anergy described for tuberculosis patients but additional studies are needed to further clarify the exact role of IL-7.

We also detected lower sIL-7R plasma concentrations in tuberculosis patients and normalisation during therapy and recovery. sIL-7R levels were previously shown to affect IL-7-availability for T cells, but the role of aberrant sIL-7R levels in immune pathologies is a matter of controversy [26–28]. Crawley et al. detected increased sIL-7R concentrations in plasma samples from AIDS patients and described sIL-7R-Fc chimera-mediated inhibition of IL-7 bioactivity [28]. They hypothesised that increased sIL-7R concentrations limited availability of IL-7 for T cells [28]. In contrast, Rose et al. found decreased sIL-7R plasma concentrations in HIV/AIDS patients as compared to controls [31]. sIL-7R plasma concentrations of this study were similar to the present study and 5 to 10 times lower for both study groups as compared to the study published by Crawley et al. [28]. Recently, Lundstrom et al. proposed an alternative model of IL-7 storage provided by the sIL-7R [26]. They demonstrated that sIL-7R even potentiates the bioactivity of IL-7 by forming a reservoir of accessible IL-7 [26]. In accordance, high sIL-7R as well as IL-7 plasma concentrations were associated with multiple sclerosis, and sIL-7R had potentiating effects on exacerbation of experimental autoimmune encephalomyelitis [26]. From this, they concluded that increased plasma concentrations of sIL-7R supported generation of autoimmunity by promoting IL-7-dependent T cells [26]. Since IL-7 serum levels are predominantly regulated by T-cell consumption [21], both restriction and reservoir hypotheses suggest dependency of IL-7 on sIL-7R levels. In the present study, we did not detect a correlation between IL-7 and sIL-7R plasma levels in tuberculosis patients or healthy contacts, although both factors were affected during tuberculosis pathogenesis. It is therefore tempting to speculate that sIL-7R has either no regulatory activities on IL-7, or that additional factors influence sIL-7R and/or IL-7 serum levels. In accordance, the proposed regulatory function of sIL-7R on IL-7 has been questioned by others [72].

We evaluated the utility of IL-7 and sIL-7R plasma concentrations as biomarkers for diagnosis of active tuberculosis using ROC curve and Random Forest-based statistics. Both markers showed moderate classification capacity and the combined efficacy of both markers revealed correct prediction for 73% of all donors. Since normalization of low sIL-7R and high IL-7 plasma concentrations during recovery from tuberculosis was found, these parameters may qualify as biomarker candidates for successful tuberculosis chemotherapy. This study was not designed to evaluate markers for the efficacy of tuberculosis therapy but future studies may address this important question.

Immunomodulatory therapies of tuberculosis gained increasing interest during recent years to complement antibiotic therapy that is periled e.g. by multi-drug resistant mycobacteria [73]. IL-7 is a promising candidate for immunotherapies and is already applied in clinical trials against chronic viral infections [74, 75]. However, the mechanisms underlying impaired IL-7 signalling pathways during chronic infections may antagonise IL-7-based novel therapy strategies. Our study contributed to the characterisation of impaired IL-7 T-cell response that may indeed counteract IL-7 treatment in tuberculosis.

We provide initial evidence that IL-7-availability is not critical during tuberculosis. Instead, T-cell functions in response to IL-7 are impaired, and therefore approaches targeting T-cell abnormalities—causative for reduced IL-7 response—may be helpful. Since IL-7 availability is a crucial factor for the development of memory T-cell induction [76], such an approach might also aim at improving protection against recurrent *M. tuberculosis* infection and disease.

Methods

Study design and samples

In this hospital-based observational study, we recruited adult tuberculosis patients (n = 57; Table 1) and exposed but healthy household contacts (healthy contacts) (n = 151). Tuberculosis patients were recruited at the Komfo Anokye Teaching Hospital (KATH), the Kumasi South Hospital (KSH), and the Kwame Nkrumah University of Science and Technology (KNUST) Hospital, Ghana, in 2011–2012. Diagnosis of tuberculosis was based on patient history, chest X-ray, and sputum smear test. For sputum smear negative cases, laboratory confirmation by *M. tuberculosis* sputum culture was performed. Tuberculosis patients with a known history of HIV infection were excluded from this study. Chemotherapy according to the Ghanaian guidelines was initiated immediately after the first blood sample was taken. For the patient study group, peripheral heparinised blood was taken consecutively (i.e. prior to treatment, under treatment (at 2 months), and after recovery (at 6 months)). Only a subgroup of tuberculosis patients (n = 36) completed the study procedure. Twenty-one tuberculosis patients were not included at all time points, including nine patients included only prior to treatment; six patients prior to treatment and under treatment; two patients prior to treatment and after recovery, and four patients during treatment and after recovery. Healthy tuberculosis patient contacts (short: healthy contacts) were recruited at the homes of tuberculosis index cases and showed no clinical symptoms of tuberculosis. A subgroup of healthy contacts (n = 19) and tuberculosis patients (n = 32) was tested for *M. tuberculosis* PPD-specific immune response before and showed significant IFNγ expression [77]. We took heparinised blood (up to 30 ml) from each donor. Not all samples were included for all experiments, and the respective numbers of samples included are given in the figure legends. A second cohort of tuberculosis patients (n = 22) and healthy contacts (n = 24) were recruited in the period of October 2015 to March 2016. HIV-positive individuals were excluded from the analysis (First Response HIV 1–2.0 Card Test, Premier Medical Corporation).

Ethics statement

All study participants were adults who gave written informed consent. All participants were free to drop out at any time of the study. The studies were approved by the Committee on Human Research, Publication and Ethics (CHRPE) at the School of Medical Sciences (SMS) at the Kwame Nkrumah University of Science and technology (KNUST) in Kumasi, Ghana.

Measurement of sIL-7R concentrations using cytometric bead assay

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised whole blood (diluted 1:1 in PBS) by density centrifugation (Ficoll, Biochrom) according to manufacturer's instructions. PBMCs were cryopreserved in DMSO/FCS (each 10%) containing medium. The plasma layer (diluted 1:1 in PBS) were collected and frozen at -80˚C until processing. Diluted plasma samples were thawed in parallel and analysed for sIL-7R expression. Quantification of sIL-7R was performed according to the protocol of Faucher et al. [78] with minor modifications. In brief, we applied cytometric bead array (CBA) (Bead A4, BD Biosciences). Conjugation of beads with polyclonal goat anti-human CD127 (IL-7Rα) antibody (R&D Systems, AF306) was done according to manufacturer's instructions. Biotinylated mouse anti-human CD127 (clone HIL-7R-M21, BD Biosciences) was used as detection antibody. Samples were incubated with labelled beads in PBS for 1 hour at room temperature and then the detection antibody (5 μl) was added for overnight incubation in the fridge. Afterwards, Streptavidin-PE (1 μl) (Southern Biotech) was added and incubated for 30 min at room

temperature. Finally the beads were washed twice in PBS. For analyses, the bead pellets were resuspended in 80 μl PBS and analysed using a BD LSRFortessa flow cytometer (BD Biosciences) and the FCS Express 4 (De Novo Software) software. For absolute quantification, the assay was calibrated with dilutions of rhIL-7R alpha-Fc chimera (R&D Systems). sIL-7R concentrations were calculated using the non-linear regression tool of GraphPad Prism 6 (Graphpad Software Inc.). Possible effects of IL-7 on sIL-7R measure were excluded by Faucher et al. [78].

Measurement of plasma IL-6 and IL-7

IL-6 and IL-7 was determined in duplicate for diluted plasma samples using Human IL-6 ELISA Ready-SET-Go! (eBioscience) and Human IL-7 Quantikine HS ELISA kit (R&D Systems), respectively, according to manufacturer's instructions. Samples were measured using the Infinite M200 ELISA reader (Tecan). Concentrations were calculated from the respective standard curves by applying 4-parametric logistic regression. Samples outside the detection range were set to the corresponding lower or upper range value.

Real-Time PCR of IL-7R variants and T-cell exhaustion markers

$CD4^+$ cells were isolated from freshly isolated PBMCs (1.5 x 10^7 cells) using anti-human CD4 magnetic particles (BD Biosciences) according to manufacturer's recommendations. Cell purity was evaluated by flow cytometry and was generally higher than 95%. miRNA was isolated from at least 5 x 10^6 enriched $CD4^+$ cells using mirVanaTM miRNA Isolation Kit (Life Technologies) following manufacturer's instructions. cDNA was generated by Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific), while RT-PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen) for full-length IL-7R (H20: forward 5'-AATAATAGCTCAGGGGAGATGG-3', reverse 5'-ATGACCAACAGAGCGACAGAG-3'), IL-7R lacking exon 6 (H6: forward 5'-GATCAATAATAGCTCAGGATTAAGC-3', reverse 5'-AAGATGTTCCAGAGTCTTCTTATG-3'), and IL-7R lacking exon 5–6 (H5-6: forward 5'-ATGAAAACAAATGGACGGATTAAGC-3', reverse 5'-AAGATGTTCCAGAGTCTTCTTATG-3'), PD-1 (forward 5'-CTCAGGGTGACAGAGAGAAG-3', reverse 5'-GACACCAACCACCAGGGTTT-3'), SOCS3 (forward 5'-GACCAGCGCCACTTCTTCAC-3', reverse 5'-CTGGATGCGCAGGTTCTTG-3') using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping control gene (forward 5'-CACCATCTTCCAGGAGCGAG-3', reverse 5'-GACTCCACGACGTACTCAGC-3'). The reaction with a final volume of 25 μl was run 2 min. at 50˚C, 10 min. at 95˚C, 45 cycles of 15 s at 95˚C, 30 s at 53˚C and 30 s at 72˚C, followed by a melt curve sequence of 15 s at 95˚C, 60 s at 60˚C with a slow gradient to 95˚C and finally 15 s at 60˚C. Data from duplicate reactions was evaluated using the $2^{-\Delta Ct}$ method. A 7500 Real-Time PCR machine (Applied Biosystems) was used for quantitative PCR analyses.

Genotyping of IL7RA single nucleotide polymorphism

DNA was isolated from PBMCs using QIAamp DNA Mini Mini kit (Qiagen) followed by rs6897932C>T genotyping using a predesigned TaqMan SNP Genotyping Assay (Applied Biosystems) following manufacturer's instructions.

Staining of PBMCs

Frozen PBMCs were thawed and washed with RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-Glutamine, 10 mM HEPES, and 50 U/ml Penicillin-Streptomycin (all from Thermo Fisher). Cells were stained with Viability Dye eFluor 780 (eBioscience) and

antibodies against CD3 (PE-labelled, clone HIT3a, BD Biosciences), CD4 (BrilliantViolet510-labelled, clone OKT4, BioLegend), CD8 (PerCP-Cy5.5-labelled, clone HIT8a, BioLegend), CD25 (PE-Cy7-labelled, clone 2A3, BD Biosciences) and CD127 (AlexaFluor647-labelled, clone HIL-7R-M21, BD Biosciences). After cell wash, PBMCs were fixed with Fixation Buffer (BioLegend) and subsequently analysed using a BD LSRFortessa flow cytometer (BD Biosciences). Gating procedures are depicted in S3 Fig. For detection of mIL-7R in the second independent cohort of tuberculosis patients and healthy contacts we used the CD127 antibody clone A019D5 (BioLegend). Comparison of both antibody clones revealed similar T-cell binding pattern as well as percentages of mIL-7R_{high} and mIL-7R_{low} T cells.

STAT5 phosphorylation by IL-7

Freshly isolated PBMCs were stained for CD4 (AlexaFluor488, clone RPTA-4, BioLegend) followed by addition of 100 μl pre-warmed X-VIVO 15 medium (Lonza) added 50 U/ml Penicillin-Streptomycin with or without human recombinant IL-7. The concentration of IL-7 was titrated prior to the study and a concentration of 10 ng/ml was sufficient to induce pSTAT5 in 94% of the T cells (S5 Fig). Higher IL-7 concentrations (25 or 50 ng/ml) did not further increase STAT5 phosphorylation (S5 Fig). Therefore we cultured the samples with and w/o 10 ng/ml of recombinant human IL-7 in this study. After 15 min incubation at 37˚C, 5% CO_2, cells were fixed for 15 min. with 100 μl 1x True-Nuclear Transcription Factor buffer (BioLegend). Subsequently, cells were permeabilised with 100% methanol, washed in PBS/10% FCS and stained for p-STAT5 Y694 (PE, clone SRBCZX, eBioscience). Analysis was performed on a BD Accuri C6 flow cytometer. Gating procedure is shown in S5 Fig.

Ex vivo stimulation of whole blood

Heparinised blood was diluted 1:2 in RPMI 1640 supplemented with 2 mM L-Glutamine and 50 U/ml Penicillin-Streptomycin in a 96-well U bottom plate. Cells were stimulated with 10 μg/ml PPD (Statens Serum Institute) and/or 10 ng/ml recombinant human IL-7 (BioLegend), or left unstimulated. After 2.5 hours of stimulation at 37˚C, 5% CO_2, Brefeldin A (Sigma Aldrich) was added at a concentration of 3.75 μg/ml followed by 16 hours of incubation. Erythrocytes were subsequently lysed in two rounds by resuspending pelleted cells in 100 μl RBC Lysis Buffer (Roche) followed by 10 min incubation at room temperature. Next, cells were fixed and permabilised (BioLegend) and stained with antibody against CD4 (AlexaFluor488, clone RPTA-4, BioLegend), IFNγ (PE, clone 25723.11, BD Biosciences) and CD154 (APC, clone 24.31, BioLegend). Cells were analysed using a BD Accuri C6 flow cytometer (BD Biosciences). Gating procedure is shown S6 Fig.

Statistical analysis

Statistical analyses were performed using R version 3.3.0, applying Exact Mann-Whitney U test from the package *coin* for comparison between groups and Wilcoxon signed-rank test for evaluation of repeated measurements. Spearman correlation was used to evaluate association between continuous variables, while Receiver Operating Characteristic (ROC) was performed using the package *ROCR*. Random forest analysis was performed with the package *ranger*, applying 10^5 random trees and adjusting the importance measure by permutation. Plots were generated in R and GraphPad Prism version 6.07.

Supporting information

S1 Table. Correlation between mIL-7R expression on T cells and plasma IL-7 or sIL-7R concentrations.
(EPS)

S1 Fig. Gender differences in plasma sIL-7R levels. Plasma concentrations of sIL-7R from TB contacts (n = 149) and tuberculosis patients prior to (0 months, n = 52), during (2 months, n = 46) and after (6 months, n = 41) treatment was determined by cytometric bead array. Exact Mann-Whitney U test used for comparison of gender differences.
(PDF)

S2 Fig. Changes in plasma sIL-7R and plasma IL-7 during chemotherapy. Absolute (left panel) and relative (right panel) differences of a) plasma sIL-7R and b) plasma IL-7 level after (6 months) and prior treatment for tuberculosis. Concentration of sIL-7R in plasma from TB patients was determined by cytometric bead array, while plasma IL-7 level was determined by ELISA (n = 36). p-values for Spearman correlation are shown, while linear regression lines are shown for guidance.
(PDF)

S3 Fig. Gating strategy for IL-7R$_{low}$, and IL-7R MFI of CD4$^+$ and CD8$^+$ cells. Proportions (%) of cells within the individual gates are indicated.
(PDF)

S4 Fig. Surface level of IL-7R on CD3$^+$CD4$^+$ and CD3$^+$CD4$^-$ cells. Heparinised blood from TB patients (n = 22) and contacts to TB patients (n = 24) was lysed (RBC Lysis Buffer, Roche) and leukocytes were stained for CD3 APC (clone UCHT1, BD Biosciences), CD4 AlexaFluor 488 (clone RPTA-4, Biolegend) and IL-7R (CD127) PE-Cy7 (clone A019D5, Biolegend). Cells were analysed on a BD Accuri C6 Flow Cytometer (BD Biosciences). Mean Fluoresence Intensity (MFI) of IL-7R is shown for (a) CD3$^+$CD4$^+$ and (b) CD3$^+$CD4$^-$ cells. Exact Mann-Whitney U test is used for comparison of groups.
(PDF)

S5 Fig. STAT5 phosphorylation of CD4$^+$ cells after IL-7 stimulation. (a) Gating strategy for STAT5 phosphorylation (pSTAT5) on CD4$^+$ cells stimulated with (solid line) or without (shaded) 10 ng/ml IL-7 for 15 min. Proportions (%) of cells in the individual gates are indicated, and mean fluorescence intensity (MFI) is shown for the two stimulations. (b) Titration of IL-7. PBMCs stimulated as in (a) with various concentrations of IL-7 shown for CD4$^+$ cells.
(PDF)

S6 Fig. Gating strategy for IFNγ$^+$CD40L$^+$ cells. Gating strategy for IFNγ$^+$CD40L$^+$ cells of CD4$^+$ cells after overnight stimulation of whole blood with PPD. Proportions (%) of cells in the individual gates are indicated.
(PDF)

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References

1. Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. Nature reviews Immunology. 2011; 11(5):343–54. Epub 2011/04/09. https://doi.org/10.1038/nri2960 PMID: 21475309.
2. Green AM, Difazio R, Flynn JL. IFN-gamma from CD4 T cells is essential for host survival and enhances CD8 T cell function during Mycobacterium tuberculosis infection. J Immunol. 2013; 190(1):270–7. https://doi.org/10.4049/jimmunol.1200061 PMID: 23233724;
3. Casanova JL, Abel L. Genetic dissection of immunity to mycobacteria: the human model. Annu Rev Immunol. 2002; 20:581–620. https://doi.org/10.1146/annurev.immunol.20.081501.125851 PMID: 11861613.
4. Scanga CA, Mohan VP, Yu K, Joseph H, Tanaka K, Chan J, et al. Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. The Journal of experimental medicine. 2000; 192(3):347–58.
5. Geldmacher C, Zumla A, Hoelscher M. Interaction between HIV and Mycobacterium tuberculosis: HIV-1-induced CD4 T-cell depletion and the development of active tuberculosis. Curr Opin HIV AIDS. 2012; 7(3):268–75. https://doi.org/10.1097/COH.0b013e3283524e32 PMID: 22495739.
6. Behar SM, Carpenter SM, Booty MG, Barber DL, Jayaraman P. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. Seminars in immunology. 2014; 26(6):559–77. https://doi.org/10.1016/j.smim.2014.09.003 PMID: 25311810;
7. Henao-Tamayo M, Irwin SM, Shang S, Ordway D, Orme IM. T lymphocyte surface expression of exhaustion markers as biomarkers of the efficacy of chemotherapy for tuberculosis. Tuberculosis (Edinb). 2011; 91(4):308–13. Epub 2011/05/03. https://doi.org/10.1016/j.tube.2011.04.001 PMID: 21530406;
8. Jayaraman P, Jacques MK, Zhu C, Steblenko KM, Stowell BL, Madi A, et al. TIM3 Mediates T Cell Exhaustion during Mycobacterium tuberculosis Infection. PLoS pathogens. 2016; 12(3):e1005490. https://doi.org/10.1371/journal.ppat.1005490 PMID: 26967901;
9. Angelosanto JM, Blackburn SD, Crawford A, Wherry EJ. Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. Journal of virology. 2012; 86(15):8161–70. Epub 2012/05/25. https://doi.org/10.1128/JVI.00889-12 PMID: 22623779;
10. Pellegrini M, Calzascia T, Toe JG, Preston SP, Lin AE, Elford AR, et al. IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology. Cell. 2011; 144(4):601–13. Epub 2011/02/08. https://doi.org/10.1016/j.cell.2011.01.011 PMID: 21295337.
11. Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proceedings of the National Academy of Sciences of the

United States of America. 2004; 101(45):16004–9. Epub 2004/10/27. https://doi.org/10.1073/pnas.0407192101 PMID: 15505208;

12. Grabmeier-Pfistershammer K, Steinberger P, Rieger A, Leitner J, Kohrgruber N. Identification of PD-1 as a unique marker for failing immune reconstitution in HIV-1-infected patients on treatment. J Acquir Immune Defic Syndr. 2011; 56(2):118–24. https://doi.org/10.1097/QAI.0b013e3181fbab9f PMID: 20980914.

13. Tanaskovic S, Fernandez S, Price P, French MA. Interleukin-7 signalling defects in naive CD4+ T cells of HIV patients with CD4+ T-cell deficiency on antiretroviral therapy are associated with T-cell activation and senescence. AIDS. 2014; 28(6):821–30. https://doi.org/10.1097/QAD.0000000000000213 PMID: 24499954.

14. Shive CL, Clagett B, McCausland MR, Mudd JC, Funderburg NT, Freeman ML, et al. Inflammation perturbs the IL-7 axis, promoting senescence and exhaustion that broadly characterize immune failure in treated HIV infection. J Acquir Immune Defic Syndr. 2015. https://doi.org/10.1097/QAI.0000000000000913 PMID: 26627102.

15. Shive CL, Mudd JC, Funderburg NT, Sieg SF, Kyi B, Bazdar DA, et al. Inflammatory cytokines drive CD4+ T-cell cycling and impaired responsiveness to interleukin 7: implications for immune failure in HIV disease. The Journal of infectious diseases. 2014; 210(4):619–29. https://doi.org/10.1093/infdis/jiu125 PMID: 24585897;

16. Rethi B, Fluur C, Atlas A, Krzyzowska M, Mowafi F, Grutzmeier S, et al. Loss of IL-7Ralpha is associated with CD4 T-cell depletion, high interleukin-7 levels and CD28 down-regulation in HIV infected patients. AIDS. 2005; 19(18):2077–86. PMID: 16284456.

17. Sasson SC, Zaunders JJ, Zanetti G, King EM, Merlin KM, Smith DE, et al. Increased plasma interleukin-7 level correlates with decreased CD127 and Increased CD132 extracellular expression on T cell subsets in patients with HIV-1 infection. The Journal of infectious diseases. 2006; 193(4):505–14. https://doi.org/10.1086/499309 PMID: 16425129.

18. Bazdar DA, Kalinowska M, Sieg SF. Interleukin-7 receptor signaling is deficient in CD4+ T cells from HIV-infected persons and is inversely associated with aging. The Journal of infectious diseases. 2009; 199(7):1019–28. https://doi.org/10.1086/597210 PMID: 19239367;

19. Fernandez S, Tanaskovic S, Helbig K, Rajasuriar R, Kramski M, Murray JM, et al. CD4+ T-cell deficiency in HIV patients responding to antiretroviral therapy is associated with increased expression of interferon-stimulated genes in CD4+ T cells. The Journal of infectious diseases. 2011; 204(12):1927–35. https://doi.org/10.1093/infdis/jir659 PMID: 22006994.

20. Cha L, de Jong E, French MA, Fernandez S. IFN-alpha exerts opposing effects on activation-induced and IL-7-induced proliferation of T cells that may impair homeostatic maintenance of CD4+ T cell numbers in treated HIV infection. J Immunol. 2014; 193(5):2178–86. https://doi.org/10.4049/jimmunol.1302536 PMID: 25063872.

21. Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. Nature reviews Immunology. 2007; 7(2):144–54. Epub 2007/01/30. https://doi.org/10.1038/nri2023 PMID: 17259970.

22. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. Nature reviews Immunology. 2009; 9(7):480–90. https://doi.org/10.1038/nri2580 PMID: 19543225;

23. Henriques CM, Rino J, Nibbs RJ, Graham GJ, Barata JT. IL-7 induces rapid clathrin-mediated internalization and JAK3-dependent degradation of IL-7Ralpha in T cells. Blood. 2010; 115(16):3269–77. https://doi.org/10.1182/blood-2009-10-246876 PMID: 20190194.

24. Park JH, Yu Q, Erman B, Appelbaum JS, Montoya-Durango D, Grimes HL, et al. Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. Immunity. 2004; 21(2):289–302. https://doi.org/10.1016/j.immuni.2004.07.016 PMID: 15308108.

25. Rane L, Vudattu N, Bourcier K, Graniar E, Hillert J, Seyfert V, et al. Alternative splicing of interleukin-7 (IL-7) and interleukin-7 receptor alpha (IL-7Ralpha) in peripheral blood from patients with multiple sclerosis (MS). Journal of neuroimmunology. 2010; 222(1–2):82–6. Epub 2010/03/17. https://doi.org/10.1016/j.jneuroim.2010.02.014 PMID: 20226540.

26. Lundstrom W, Highfill S, Walsh ST, Beq S, Morse E, Kockum I, et al. Soluble IL7Ralpha potentiates IL-7 bioactivity and promotes autoimmunity. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(19):E1761–70. Epub 2013/04/24. https://doi.org/10.1073/pnas.1222303110 PMID: 23610432;

27. Jager J, Schulze C, Rosner S, Martin R. IL7RA haplotype-associated alterations in cellular immune function and gene expression patterns in multiple sclerosis. Genes and immunity. 2013; 14(7):453–61. Epub 2013/08/30. https://doi.org/10.1038/gene.2013.40 PMID: 23985573.

28. Crawley AM, Faucher S, Angel JB. Soluble IL-7R alpha (sCD127) inhibits IL-7 activity and is increased in HIV infection. J Immunol. 2010; 184(9):4679–87. Epub 2010/03/23. https://doi.org/10.4049/jimmunol.0903758 PMID: 20304824.

29. Badot V, Luijten RK, van Roon JA, Depresseux G, Aydin S, Van den Eynde BJ, et al. Serum soluble interleukin 7 receptor is strongly associated with lupus nephritis in patients with systemic lupus erythematosus. Annals of the rheumatic diseases. 2013; 72(3):453–6. Epub 2012/12/25. https://doi.org/10.1136/annrheumdis-2012-202364 PMID: 23264357.

30. Monti P, Brigatti C, Krasmann M, Ziegler AG, Bonifacio E. Concentration and activity of the soluble form of the interleukin-7 receptor alpha in type 1 diabetes identifies an interplay between hyperglycemia and immune function. Diabetes. 2013; 62(7):2500–8. Epub 2013/03/05. https://doi.org/10.2337/db12-1726 PMID: 23454692;

31. Rose T, Lambotte O, Pallier C, Delfraissy JF, Colle JH. Identification and biochemical characterization of human plasma soluble IL-7R: lower concentrations in HIV-1-infected patients. J Immunol. 2009; 182 (12):7389–97. Epub 2009/06/06. https://doi.org/10.4049/jimmunol.0900190 PMID: 19494261.

32. Gregory SG, Schmidt S, Seth P, Oksenberg JR, Hart J, Prokop A, et al. Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. Nature genetics. 2007; 39 (9):1083–91. Epub 2007/07/31. https://doi.org/10.1038/ng2103 PMID: 17660817.

33. Todd JA, Walker NM, Cooper JD, Smyth DJ, Downes K, Plagnol V, et al. Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. Nature genetics. 2007; 39 (7):857–64. Epub 2007/06/08. https://doi.org/10.1038/ng2068 PMID: 17554260;

34. Rajasuriar R, Booth DR, Gouillou M, Spelman T, James I, Solomon A, et al. The role of SNPs in the alpha-chain of the IL-7R gene in CD4+ T-cell recovery in HIV-infected African patients receiving suppressive cART. Genes and immunity. 2012; 13(1):83–93. https://doi.org/10.1038/gene.2011.65 PMID: 21938017.

35. Hartling HJ, Thorner LW, Erikstrup C, Harritshoj LH, Kronborg G, Pedersen C, et al. Polymorphism in interleukin-7 receptor alpha gene is associated with faster CD4(+) T-cell recovery after initiation of combination antiretroviral therapy. AIDS. 2014; 28(12):1739–48.

36. Guzman-Fulgencio M, Berenguer J, Jimenez-Sousa MA, Micheloud D, Garcia-Alvarez M, Bellon JM, et al. IL7RA polymorphisms predict the CD4+ recovery in HIV patients on cART. Eur J Clin Invest. 2015; 45(11):1192–9. https://doi.org/10.1111/eci.12539 PMID: 26402121.

37. Vudattu NK, Magalhaes I, Hoehn H, Pan D, Maeurer MJ. Expression analysis and functional activity of interleukin-7 splice variants. Genes and immunity. 2009; 10(2):132–40. Epub 2008/12/19. https://doi.org/10.1038/gene.2008.90 PMID: 19092841.

38. Rane L, Rahman S, Magalhaes I, Ahmed R, Spangberg M, Kondova I, et al. Increased (6 exon) interleukin-7 production after M. tuberculosis infection and soluble interleukin-7 receptor expression in lung tissue. Genes and immunity. 2011; 12(7):513–22. Epub 2011/06/17. https://doi.org/10.1038/gene.2011.29 PMID: 21677672.

39. Maeurer MJ, Trinder P, Hommel G, Walter W, Freitag K, Atkins D, et al. Interleukin-7 or interleukin-15 enhances survival of Mycobacterium tuberculosis-infected mice. Infection and immunity. 2000; 68 (5):2962–70. Epub 2000/04/18. PMID: 10768995;

40. Singh V, Gowthaman U, Jain S, Parihar P, Banskar S, Gupta P, et al. Coadministration of interleukins 7 and 15 with bacille Calmette-Guerin mounts enduring T cell memory response against Mycobacterium tuberculosis. The Journal of infectious diseases. 2010; 202(3):480–9. Epub 2010/06/24. https://doi.org/10.1086/653827 PMID: 20569158.

41. Napolitano LA, Grant RM, Deeks SG, Schmidt D, De Rosa SC, Herzenberg LA, et al. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nature medicine. 2001; 7(1):73–9. https://doi.org/10.1038/83381 PMID: 11135619.

42. Paiardini M, Cervasi B, Albrecht H, Muthukumar A, Dunham R, Gordon S, et al. Loss of CD127 expression defines an expansion of effector CD8+ T cells in HIV-infected individuals. J Immunol. 2005; 174 (5):2900–9. PMID: 15728501.

43. Colle JH, Moreau JL, Fontanet A, Lambotte O, Joussemet M, Jacod S, et al. Regulatory dysfunction of the interleukin-7 receptor in CD4 and CD8 lymphocytes from HIV-infected patients—effects of antiretroviral therapy. J Acquir Immune Defic Syndr. 2006; 42(3):277–85. https://doi.org/10.1097/01.qai.0000214823.11034.4e PMID: 16810123.

44. Juffroy O, Bugault F, Lambotte O, Landires I, Viard JP, Niel L, et al. Dual mechanism of impairment of interleukin-7 (IL-7) responses in human immunodeficiency virus infection: decreased IL-7 binding and abnormal activation of the JAK/STAT5 pathway. Journal of virology. 2010; 84(1):96–108. https://doi.org/10.1128/JVI.01475-09 PMID: 19864382;

45. Landires I, Bugault F, Lambotte O, de Truchis P, Slama L, Danckaert A, et al. HIV infection perturbs interleukin-7 signaling at the step of STAT5 nuclear relocalization. AIDS. 2011; 25(15):1843–53. Epub 2011/07/01. https://doi.org/10.1097/QAD.0b013e32834a3678 PMID: 21716071.

46. Feske M, Nudelman RJ, Medina M, Lew J, Singh M, Couturier J, et al. Enhancement of human antigen-specific memory T-cell responses by interleukin-7 may improve accuracy in diagnosing tuberculosis. Clinical and vaccine immunology: CVI. 2008; 15(10):1616–22. Epub 2008/08/30. https://doi.org/10.1128/CVI.00185-08 PMID: 18753334;

47. Chowdhury IH, Ahmed AM, Choudhuri S, Sen A, Hazra A, Pal NK, et al. Alteration of serum inflammatory cytokines in active pulmonary tuberculosis following anti-tuberculosis drug therapy. Molecular immunology. 2014; 62(1):159–68. https://doi.org/10.1016/j.molimm.2014.06.002 PMID: 25019566.

48. Jacobsen M, Repsilber D, Kleinsteuber K, Gutschmidt A, Schommer-Leitner S, Black G, et al. Suppressor of cytokine signaling-3 is affected in T-cells from tuberculosisTB patients. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2011; 17(9):1323–31. https://doi.org/10.1111/j.1469-0691.2010.03326.x PMID: 20673263.

49. Ponchel F, Cuthbert RJ, Goeb V. IL-7 and lymphopenia. Clin Chim Acta. 2011; 412(1–2):7–16. https://doi.org/10.1016/j.cca.2010.09.002 PMID: 20850425

50. Puronen CE, Thompson WL, Imamichi H, Beq S, Hodge JN, Rehm C, et al. Decreased interleukin 7 responsiveness of T lymphocytes in patients with idiopathic CD4 lymphopenia. The Journal of infectious diseases. 2012; 205(9):1382–90. https://doi.org/10.1093/infdis/jis219 PMID: 22454463;

51. Zaharatos GJ, Behr MA, Libman MD. Profound T-lymphocytopenia and cryptococcemia in a human immunodeficiency virus-seronegative patient with disseminated tuberculosis. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2001; 33(11):E125–8. https://doi.org/10.1086/324086 PMID: 11692315.

52. Beck JS, Potts RC, Kardjito T, Grange JM. T4 lymphopenia in patients with active pulmonary tuberculosis. Clin Exp Immunol. 1985; 60(1):49–54. PMID: 3874015;

53. Al-Aska A, Al-Anazi AR, Al-Subaei SS, Al-Hedaithy MA, Barry MA, Somily AM, et al. CD4+ T-lymphopenia in HIV negative tuberculous patients at King Khalid University Hospital in Riyadh, Saudi Arabia. Eur J Med Res. 2011; 16(6):285–8. PMID: 21810564; https://doi.org/10.1186/2047-783X-16-6-285

54. Gambon-Deza F, Pacheco Carracedo M, Cerda Mota T, Montes Santiago J. Lymphocyte populations during tuberculosis infection: V beta repertoires. Infection and immunity. 1995; 63(4):1235–40. PMID: 7890378;

55. Fry TJ, Connick E, Falloon J, Lederman MM, Liewehr DJ, Spritzler J, et al. A potential role for interleukin-7 in T-cell homeostasis. Blood. 2001; 97(10):2983–90. PMID: 11342421.

56. Crawley AM, Angel JB. The influence of HIV on CD127 expression and its potential implications for IL-7 therapy. Seminars in immunology. 2012; 24(3):231–40. Epub 2012/03/17. https://doi.org/10.1016/j.smim.2012.02.006 PMID: 22421574.

57. Shalekoff S, Tiemessen CT. Circulating levels of stromal cell-derived factor 1alpha and interleukin 7 in HIV type 1 infection and pulmonary tuberculosis are reciprocally related to CXCR4 expression on peripheral blood leukocytes. AIDS Res Hum Retroviruses. 2003; 19(6):461–8. https://doi.org/10.1089/088922203766774504 PMID: 12882655.

58. Boutboul F, Puthier D, Appay V, Pelle O, Ait-Mohand H, Combadiere B, et al. Modulation of interleukin-7 receptor expression characterizes differentiation of CD8 T cells specific for HIV, EBV and CMV. AIDS. 2005; 19(17):1981–6. PMID: 16260904.

59. Koesters SA, Alimonti JB, Wachihi C, Matu L, Anzala O, Kimani J, et al. IL-7Ralpha expression on CD4 + T lymphocytes decreases with HIV disease progression and inversely correlates with immune activation. European journal of immunology. 2006; 36(2):336–44. https://doi.org/10.1002/eji.200535111 PMID: 16421946.

60. Benito JM, Lopez M, Lozano S, Gonzalez-Lahoz J, Soriano V. Down-regulation of interleukin-7 receptor (CD127) in HIV infection is associated with T cell activation and is a main factor influencing restoration of CD4(+) cells after antiretroviral therapy. The Journal of infectious diseases. 2008; 198(10):1466–73.

61. Rajasuriar R, Booth D, Solomon A, Chua K, Spelman T, Gouillou M, et al. Biological determinants of immune reconstitution in HIV-infected patients receiving antiretroviral therapy: the role of interleukin 7 and interleukin 7 receptor alpha and microbial translocation. The Journal of infectious diseases. 2010; 202(8):1254–64. https://doi.org/10.1086/656369 PMID: 20812848.

62. Chandrashekara S, Anupama KR, Sambarey A, Chandra N. High IL-6 and low IL-15 levels mark the presence of TB infection: A preliminary study. Cytokine. 2016; 81:57–62. https://doi.org/10.1016/j.cyto.2016.02.003 PMID: 26878649.

63. Fernandez S, Price P, McKinnon EJ, Nolan RC, French MA. Low CD4+ T-cell counts in HIV patients receiving effective antiretroviral therapy are associated with CD4+ T-cell activation and senescence but

not with lower effector memory T-cell function. Clin Immunol. 2006; 120(2):163–70. https://doi.org/10.1016/j.clim.2006.04.570 PMID: 16765088.

64. Kiazyk SA, Fowke KR. Loss of CD127 expression links immune activation and CD4(+) T cell loss in HIV infection. Trends Microbiol. 2008; 16(12):567–73.

65. Petrovas C, Chaon B, Ambrozak DR, Price DA, Melenhorst JJ, Hill BJ, et al. Differential association of programmed death-1 and CD57 with ex vivo survival of CD8+ T cells in HIV infection. J Immunol. 2009; 183(2):1120–32. https://doi.org/10.4049/jimmunol.0900182 PMID: 19564339;

66. Hassan SS, Akram M, King EC, Dockrell HM, Cliff JM. PD-1, PD-L1 and PD-L2 Gene Expression on T-Cells and Natural Killer Cells Declines in Conjunction with a Reduction in PD-1 Protein during the Intensive Phase of Tuberculosis Treatment. PloS one. 2015; 10(9):e0137646. https://doi.org/10.1371/journal.pone.0137646 PMID: 26359860;

67. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nature reviews Immunology. 2015; 15(8):486–99. https://doi.org/10.1038/nri3862 PMID: 26205583.

68. Nguyen TP, Shukla S, Asaad R, Freeman ML, Lederman MM, Harding CV, et al. Responsiveness to IL-7 but not to IFN-alpha is diminished in CD4+ T cells from treated HIV infected patients who experience poor CD4+ T-cell recovery. AIDS. 2016; 30(13):2033–42. https://doi.org/10.1097/QAD.0000000000001161 PMID: 27191978;

69. Pellegrini M, Calzascia T, Elford AR, Shahinian A, Lin AE, Dissanayake D, et al. Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies. Nature medicine. 2009; 15(5):528–36. Epub 2009/04/28. https://doi.org/10.1038/nm.1953 PMID: 19396174.

70. Delgado JC, Tsai EY, Thim S, Baena A, Boussiotis VA, Reynes JM, et al. Antigen-specific and persistent tuberculin anergy in a cohort of pulmonary tuberculosis patients from rural Cambodia. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99(11):7576–81. Epub 2002/05/29. https://doi.org/10.1073/pnas.062056099 PMID: 12032325;

71. Chappert P, Schwartz RH. Induction of T cell anergy: integration of environmental cues and infectious tolerance. Current opinion in immunology. 2010; 22(5):552–9. https://doi.org/10.1016/j.coi.2010.08.005 PMID: 20869863;

72. Blom-Potar MC, Bugault F, Lambotte O, Delfraissy JF, Theze J. Soluble IL-7Ralpha (sCD127) and measurement of IL-7 in the plasma of HIV patients. J Acquir Immune Defic Syndr. 2009; 51(1):104–5. https://doi.org/10.1097/QAI.0b013e31819d8a20 PMID: 19390329.

73. Uhlin M, Andersson J, Zumla A, Maeurer M. Adjunct immunotherapies for tuberculosis. The Journal of infectious diseases. 2012; 205 Suppl 2:S325–34. Epub 2012/03/30. https://doi.org/10.1093/infdis/jis197 PMID: 22457298.

74. Toe JG, Pellegrini M, Mak TW. Promoting immunity during chronic infection-The therapeutic potential of common gamma-chain cytokines. Molecular immunology. 2013; 56(1–2):38–47. Epub 2013/05/21. https://doi.org/10.1016/j.molimm.2013.04.008 PMID: 23685259.

75. Thiebaut R, Jarne A, Routy JP, Sereti I, Fischl M, Ive P, et al. Repeated Cycles of Recombinant Human Interleukin-7 in HIV-Infected Patients with low CD4 T cell Reconstitution on Antiretroviral Therapy: Results of two Phase II Multicentre Studies. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2016. https://doi.org/10.1093/cid/ciw065 PMID: 26908786.

76. van Leeuwen EM, Sprent J, Surh CD. Generation and maintenance of memory CD4(+) T Cells. Current opinion in immunology. 2009; 21(2):167–72. Epub 2009/03/14.

77. Afum-Adjei Awuah A, Ueberberg B, Owusu-Dabo E, Frempong M, Jacobsen M. Dynamics of T-cell IFN-gamma and miR-29a expression during active pulmonary tuberculosis. International immunology. 2014; 26(10):579–82. https://doi.org/10.1093/intimm/dxu068 PMID: 24981709.

78. Faucher S, Crawley AM, Decker W, Sherring A, Bogdanovic D, Ding T, et al. Development of a quantitative bead capture assay for soluble IL-7 receptor alpha in human plasma. PloS one. 2009; 4(8):e6690. Epub 2009/08/20. https://doi.org/10.1371/journal.pone.0006690 PMID: 19690616;

4.0. Discussion

4.1. IGRA tests and *Mtb* infection diagnosis in high TB burden population

Study subjects were mostly adults recruited in high TB burden regions in Ghana, West Africa. Commercialized QuantiFERON® Gold Plus test (QFT) was used to confirm *Mtb* infection or evidence of exposure, and we showed that majority of confirmed TB and healthy contacts were either negative or had inconclusive results (Adankwah et al., 2019). Low sensitivity of QFT in *Mtb* infection detection confirmed our own previous findings in Ghanaian children with tuberculosis (Lundtoft et al., 2017; Nausch et al., 2017). Interestingly, a parallel study in TB and LTBI children from Germany showed higher sensitivity for QFT in *Mtb* infection detection (Nausch et al., 2017). In support, a meta-analysis of IGRA tests in *Mtb* infection diagnosis, showed that QFT sensitivity was 0.79 in high income countries but only 0.57 in low-income countries (Sollai et al., 2014). Specifically, we showed that lower QFT sensitivity was not only found in children but affected adults with latent *Mtb* infection and acute tuberculosis from Ghana (Adankwah et al., 2019). The use of IGRA test as evidence of *Mtb* infection exposure is apparently better and well-defined in low endemic settings in contrast to high endemic areas (Pai et al., 2006). The reason for this phenomenon still remains unclear. However, several factors including low mitogen response, impaired T-cell response, genetic variation and tropical infections have been suggested (Pai et al., 2006; Pinto et al., 2011). Indeed, we identified high IFN-γ background and impaired T-cell response to mitogen as possible confounding factors for inconclusive QFT results in TB patients (Adankwah et al., 2019). To further characterize underlying causes for inconclusive QFT results, we analyzed the effects of modified short termed (i.e 16-24hrs) *in-vitro* conditions. However, sensitivity for *Mtb* infection detection was only marginally improved. Importantly, none of these conditions were robust to distinguish between active and latent infection (Adankwah et al., 2019). It is likely that aberrant serum levels of pro and anti-inflammatory cytokines found in TB patients may account for assays' suboptimal performance (Chowdhury et al., 2014). In line with this, our serum cytokine measures identified potential immune modulating factors in tuberculosis patients (Harling et al., 2018). Additionally, it has been suggested that limited sensitivity might be due to the use of peripheral blood, as number of potent IFN-γ producing effector T-cells are low in the blood and that the use of purified immune cells might offer a better sensitivity (Lange et al., 2009). Conversely, we showed that short term assays using blood or purified immune cells and only acute *Mtb* antigens may not be sufficient in improving sensitivity for *Mtb* infection detection, but better

sensitivity may be associated with validated long-term assays that incorporate the usage of alternative *Mtb* antigens (Adankwah et al., 2019).

4.2. Effects of pathognomonic markers on T-cell functions with potential impact on IGRA tests

Several risk factors has been described to be associated with limited sensitivity or inconclusive test results of IGRAs including immunodeficiency, age, lymphocytopenia and malignancy (Kwon et al., 2015). These are linked with low cell -mediated host immune response. Indeed, IGRAs are T-cell based assays and host factors that characterize impaired T-cell function can potentially lead to limited sensitivity.

4.2.1. IL6/IL-10 concentrations as potential causes for impaired T-cell function

We found higher concentrations of IL-6/IL-10 concomitantly expressed in cell culture supernatants after *Mtb* specific stimulation and in plasma of TB patients as compared to healthy contacts (Harling et al., 2018). This was consistent with other studies where higher expression of IL-6 by T-cells and high levels of IL-6/IL-10 levels in serum of tuberculosis patients were shown (Chowdhury et al., 2014; Joshi et al., 2015). Indeed, how these two cytokines with opposing cellular functions (i.e. pro & anti-inflammatory), are preferentially induced in TB patients warrant further investigation. However, it is possible for *Mtb* to exploits the mechanistic interaction of IL6/IL-10 to favour its survival and persistence. To evaluate this further, we hypothesized that since IL6/IL-10 are regulated by transcriptional and regulatory elements, aberrant levels are associated with perturbed cytokine signaling components with implicating effects on T-cell function. Interestingly, we demonstrated that high levels of IL6/IL-10 in TB patients were associated with aberrant cytokine signaling function involving constitutive pSTAT3 and high protein expression of SOCS3, a negative regulator of STAT3 mediated signaling (Harling et al., 2018). This is supported by findings from cancers, autoimmune and inflammatory diseases, where constitutive pSTAT3 expression is associated with severity of disease (Sugimoto, 2008; Yuan et al., 2015). Additionally, increase SOCS3 mRNA expression levels in T-cells during *Mtb* infection with potential influence on T-cell function has been shown (Ashenafi et al., 2014; Jacobsen et al., 2011). Accordingly, we showed that high SOCS3 protein expression levels in both naive and memory T cell subsets and this was associated with impaired T cell function in study cohorts (Harling et al., 2018). Although Carow

and colleagues earlier described a dispensable role of SOCS3 expression in impaired T cell response in *Mtb* infection, we showed that high SOCS3 levels in CD4+ T-cells rather correlated with impaired TH_1 cytokine response in TB patients and healthy contacts (Carow et al., 2013; Harling et al., 2018). In accordance with previously described (Jacobsen et al., 2011; Matsumoto et al., 2003), we identified that SOCS3 might have suppressive effects on T- cell activation through the inhibition of IL-2 co-stimulation (Harling et al., 2018). Overall, aberrant IL-6/ IL-10 and constitutive expression of pSTAT3/SOCS3 promote immune suppressive effects on T-cell function in TB disease.

4.2.2. Down regulation of CD27 and IL-7R

Antigen dependent activation of T-cells results in stage-specific (early, late and terminal) differentiated effector cells. These stages up or down-regulate the expression of specific immune markers on T-cell cells (Lyadova & Panteleev, 2015). Phenotypic and functional characterization of immune markers especially, CD27 expression, involved in these differentiation processes have been implicated TB disease. We demonstrate that proportions and median expression of CD27 were significantly lower in TB patients than healthy contacts (Adankwah et al., 2020). This finding is consistent with previous studies where loss of CD27 expression were found in TB patients and this was associated with bacterial load and TB disease (Jiang et al., 2010; Lyadova & Panteleev, 2015). Notably, Rakshit and co-authors also identified that effector memory (i.e CD45RA-$CD27_{low}$) subsets were significantly increased in TB patients, possibly because antigen persistence linked with high bacterial load can lead to increased effector memory responses (Rakshit et al., 2017). Interestingly, several studies had described the loss of CD27 expression on CD4 T cells as an *Mtb*-specific feature in TB patients, by excluding its expression on non-*Mtb* specific T cells (Ahmed et al., 2018; Latorre et al., 2019; Portevin et al., 2014; Streitz et al., 2007). In contrast, we demonstrate that $CD27_{low}$ expression as a feature of non-*Mtb* specific CD4 T cells in TB patients (Adankwah et al., 2020). Importantly, our findings indicate that aberrant low CD27 expression in TB patients is likely not a consequence of antigen-specific stimulation. Rather other mechanisms e.g. a pathognomonic cytokine profile or possible bystander effects are causative. However, whether loss of CD27 is a feature of impaired T-cell function will be required further evaluation.

IL-7 plays a crucial role in T-cell homeostasis and its dysregulation during infection leads to impaired T-cell function (Lundtoft et al., 2017). We showed that high plasma levels of IL-7 in TB patients as compared to healthy contacts (Lundtoft et al., 2017) This could be due to decreased T-cell consumption, impaired T-cell receptivity or low T-cell count as indicated by previous studies where high plasma levels of IL-7 are associated with low T cell numbers or lymphopenia (Napolitano et al., 2001; Ponchel et al., 2011). Importantly, evaluation of membrane bound IL-7R on CD4+T cells showed that TB patients had significantly increased proportions of mIL-7R_{low} CD4+ and CD8+ T cells, suggesting that impaired mIL-7R expression is a contributory factor to increase percentage of mIL7R_{low} T cells in tuberculosis patients (Lundtoft et al., 2017). These findings are similar in chronic viral infections like HIV where impaired IL-7 signaling has been described (Benito et al., 2008; Tanaskovic et al., 2014). IL-7 mediate its functions via receptor signaling through activation of STAT5 through a phosphorylation process. We show that low mIL-7R expression was associated with low pSTAT5 levels in TB patients, suggesting an impaired T-cell function in response to IL-7 (Lundtoft et al., 2017).

Interestingly, a recent report suggests a modulation effect of CD27 signaling on IL-7Ra expression on T -cells and function (Dong et al., 2019). Dong and co-authors, showed that CD27 co-stimulation enhanced IL-7R expression and this contributed to long-term memory generation and survival CD8+Tcells (Dong et al., 2019).Whilst CD27 mediated effect on IL-7R in tuberculosis remains to be explored, these findings demonstrate that loss of CD27 expression in TB patients may significantly contribute to aberrant T-cell phenotype. Indeed, lower CD27 expression and impaired IL-7 signaling in TB patients from high burden population characterizes a pathognomonic state with significant impact T-cell function in TB pathology.

4.3. *Mtb* infection diagnosis: Potential of novel Two-Hit assay and usage of latency antigens

Control of *Mtb* infection which usually results in latent infection, is mediated by specific cell-mediated immune response. This state of latency is associated with non-active state of the pathogen and antigens expressed in these conditions are candidate indicators for latent infection and reactivation. These antigens have been described to induce differential response in active TB and latent infection (Meier et al., 2018; Rakshit et al., 2017). Already, we identified that short term assays are suboptimal in the detection of *Mtb* infection and previous studies indicated relative higher sensitivity of long term assays (i.e 5 to 7 days) (Cehovin et al., 2007; Schuck et al., 2009; Serra-Vidal et al., 2014). Therefore, we performed a novel long term two-hit assay described previously by Schuck et al (2009), which is based the detection of *Mtb*-specific T-cell responses after two rounds of *in-vitro* re stimulation with the same antigen followed by intracellular cytokine measure. Included in this assay are both *Mtb* secretory antigens (i.e. ESAT-6/CFP10) and selected latency antigens. In contrast to the short-term assays, where majority of both study cohorts did not show detectable T-cell responses, the two-hit assay markedly improved T-cell response to secretory antigen in both study cohorts. A notable observation of our study is non-responders (i.e IGRA negative/indeterminate) as indicated by short term assay in both study subjects showed improved detectable T-cell response after restimulation with secretory antigens (Adankwah et al., 2019). This is of great relevance as most studies exclude non-responders to avoid including misdiagnosed *Mtb* infected patients (Latorre et al., 2019; Rakshit et al., 2017). It must be noted that, since IFN-γ-negative CD4+T cells may produce alternative cytokines in T-cell immunity against TB, it is possible that negative IGRA results does not exclude *Mtb* infection.

Importantly, we identified that our selected latency antigen candidates in particular, Rv2628 and Rv1733 also showed enhanced the detection of IGRA non-responders after two-hit latency-antigen specific stimulation. Additionally, two-hit assay showed high sensitivity for the detection of *Mtb* infection in almost 100% of healthy contacts (Adankwah et al., 2019). However, further studies will be needed to verify if high sensitivity to detect LTBI in our study bears the brunt of specificity. Although, poly functional *Mtb* and latency antigen specific T-cells have been reported as promising candidates by previous studies, these were not evaluated in this study.

Importantly, we are the first to comprehensibly demonstrate the potential of this long-term two-hit assay in enhancing sensitivity for detection of *Mtb* infection in IGRA non-responders in both TB patients and healthy contacts.

4.4. Potential host candidate biomarkers for the discrimination latent infection from TB disease

Global control of TB disease can be achieved by the identification of host immune biomarkers that can differentiate active TB from latent infection. These biomarkers could serve as guidelines for monitoring TB treatment efficacy, vaccine-induced immunity and disease progression (Kaufmann & Parida, 2008; Yong et al., 2019). We identified CD27 expression and latency antigens i.e Rv1733 and Rv2628 as promising candidates. By analyzing differential CD27 expression, we demonstrated it had a strong capacity to distinctively classify TB patients from healthy contacts independent of different stimuli conditions (Adankwah et al., 2020). Our findings corroborated with several studies where CD27 expression differentiated TB patients from latent infection (Ahmed et al., 2018, 2019; Latorre et al., 2019). Interestingly, CD27 expression efficiently discriminated among subgroup of study cohorts that were IGRA non-responders (Adankwah et al., 2020). Importantly, CD27 expression also classified TB from healthy contacts with antigen stimulation and it might be useful tool in TB diagnosis especially among IGRA non-responders. However, it must be noted that heterogeneity among study cohort limited the efficacy for the use of CD27 expression only for discrimination purposes. Further studies that validate CD27 in combination of other host candidates may have improved diagnostic potential. On the other hand, we showed two-hit assay induce strong T-cell response against secretory antigens but this did not discriminate between TB and healthy contacts. Conversely, latency-associated antigens induced strong T-cell response that distinctively classified healthy contacts from active TB patients. Our ROC analyses confirmed *Mtb*-specific IFN-γ response against latency antigens (i.e Rv1733 and Rv2628) showed robust capacity to discriminate healthy contacts from TB patients with area under the curve (AUC) 0.81 and 0.88 respectively (Adankwah et al., 2020). Our findings are in support with previous studies who also identified Rv628 and Rv733 as promising host candidates for *Mtb* infection detection (Chegou et al., 2012; Chen et al., 2009; Rakshit et al., 2017).

It is worthwhile to note, very few successes have been achieved in search of TB biomarkers with current rise in a number of biomarker studies. This is largely due to heterogeneity of *Mtb*-specific

immune response that may exist in human populations. Moreover, immune response may be influence by confounding immune genetic factors, HIV co-infection and other environmental factors. Therefore, rigorous validation of these candidate host biomarkers in both endemic and low endemic areas are needed before they can be rolled out as *Mtb* diagnostic tools.

4.5. Outlook

We described here that immunomodulatory factors including aberrant serum levels of IL-6/IL-10/ IL-7, high pSTA3/SOCS3 protein expression and low CD27/IL-7R expression in TB patients described a pathognomonic state that is associated with impaired T-cell function. In addition, these factors may be associated with limited sensitivity of IGRA tests in highly endemic regions. In view of this, interpretation of IGRA tests results in endemic regions must be done in the context of a full evaluation of these immunomodulatory factors. Our two-hit assay showed promise in circumventing the effects of these factors, as it improves sensitivity for *Mtb* infection detection. Additionally, we identified CD27 and selected latency antigens (i.e Rv1733 and Rv2628) as potential diagnostic tools for discriminating LTBIs from active TB disease. However, a holistic multi-center, large and longitudinal studies are required to validate the utility and applicability of these tools in TB endemic setting.

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6.0. Statutory declaration

I declare under oath that I have compiled my dissertation independently and without any undue assistance by third parties under consideration of the 'Principles for the Safeguarding of Good Scientific Practice at Heinrich Heine University Düsseldorf'.

The dissertation has not been presented to other faculties, nor do I have any record of previous successful or unsuccessful attempts to obtain a doctorate.

Ernest Adankwah

Name

E.A 25/06/2020

Date/Signature

7.0. REFERENCES

Abdalla, A. E., Lambert, N., Duan, X., & Xie, J. (2016). Interleukin-10 family and tuberculosis: An old story renewed. *International Journal of Biological Sciences*, *12*(6), 710–717. https://doi.org/10.7150/ijbs.13881

Abdallah, A. M., Gey van Pittius, N. C., DiGiuseppe Champion, P. A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C. M. J. E., Appelmelk, B. J., & Bitter, W. (2007). Type VII secretion - Mycobacteria show the way. *Nature Reviews Microbiology*, *5*(11), 883–891. https://doi.org/10.1038/nrmicro1773

Adankwah, E., Güler, A., Mayatepek, E., Phillips, R. O., Nausch, N., & Jacobsen, M. (2020). CD27 expression of T-cells discriminates IGRA-negative TB patients from healthy contacts in Ghana. *Microbes and Infection*, *22*(1), 65–68. https://doi.org/10.1016/j.micinf.2019.07.003

Adankwah, E., Lundtoft, C., Güler, A., Franken, K. L. M. C., Ottenhoff, T. H. M., Mayatepek, E., Owusu-Dabo, E., Phillips, R. O., Nausch, N., & Jacobsen, M. (2019). Two-Hit in vitro T-Cell stimulation detects mycobacterium tuberculosis infection in QuantiFERON negative tuberculosis patients and healthy contacts from Ghana. *Frontiers in Immunology*, *10*(JUL), 1–12. https://doi.org/10.3389/fimmu.2019.01518

Adekambi, T., Ibegbu, C. C., Kalokhe, A. S., Yu, T., Ray, S. M., & Rengarajan, J. (2012). Distinct effector memory CD4 + T cell signatures in latent Mycobacterium tuberculosis infection, BCG vaccination and clinically resolved tuberculosis. *PLoS ONE*, *7*(4). https://doi.org/10.1371/journal.pone.0036046

Ahmed, M. I. M., Ntinginya, N. E., Kibiki, G., Mtafya, B. A., Semvua, H., Mpagama, S., Mtabho, C., Saathoff, E., Held, K., Loose, R., Kroidl, I., Chachage, M., von Both, U., Haule, A., Mekota, A. M., Boeree, M. J., Gillespie, S. H., Hoelscher, M., Heinrich, N., & Geldmacher, C. (2018). Phenotypic Changes on Mycobacterium Tuberculosis-Specific CD4 T Cells as Surrogate Markers for Tuberculosis Treatment Efficacy. *Frontiers in Immunology*, *9*(September), 2247. https://doi.org/10.3389/fimmu.2018.02247

Ahmed, M. I. M., Ziegler, C., Held, K., Dubinski, I., Ley-Zaporozhan, J., Geldmacher, C., & von Both, U. (2019). The TAM-TB assay-A promising TB immune-diagnostic test with a potential for treatment monitoring. *Frontiers in Pediatrics*, *7*(FEB), 1–7. https://doi.org/10.3389/fped.2019.00027

Arbelaez, C. A., Glatigny, S., Duhen, R., Eberl, G., Oukka, M., & Bettelli, E. (2015). IL-7/IL-7 Receptor Signaling Differentially Affects Effector CD4 + T Cell Subsets Involved in Experimental Autoimmune Encephalomyelitis . *The Journal of Immunology*, *195*(5), 1974–1983. https://doi.org/10.4049/jimmunol.1403135

Arroyo, L., Marín, D., Franken, K. L. M. C., Ottenhoff, T. H. M., & Barrera, L. F. (2018). Potential of DosR and Rpf antigens from Mycobacterium tuberculosis to discriminate between latent and active tuberculosis in a tuberculosis endemic population of Medellin Colombia. *BMC Infectious Diseases*, *18*(1), 1–9. https://doi.org/10.1186/s12879-017-2929-0

Ashenafi, S., Aderaye, G., Bekele, A., Zewdie, M., Aseffa, G., Hoang, A. T. N., Carow, B., Habtamu, M., Wijkander, M., Rottenberg, M., Aseffa, A., Andersson, J., Svensson, M., & Brighenti, S. (2014). Progression of clinical tuberculosis is associated with a Th2 immune response signature in combination with elevated levels of SOCS3. *Clinical Immunology*, *151*(2), 84–99. https://doi.org/10.1016/j.clim.2014.01.010

Barry, C. E., Boshoff, H. I., Dartois, V., Dick, T., Ehrt, S., Flynn, J. A., Schnappinger, D., Wilkinson, R. J., & Young, D. (2009). The spectrum of latent tuberculosis: Rethinking the biology and intervention strategies. *Nature Reviews Microbiology*, *7*(12), 845–855. https://doi.org/10.1038/nrmicro2236

Benito, J. M., López, M., Lozano, S., González-Lahoz, J., & Soriano, V. (2008). Down-Regulation of Interleukin-7 Receptor (CD127) in HIV Infection Is Associated with T Cell Activation and Is a Main Factor Influencing Restoration of CD4 + Cells after Antiretroviral Therapy . *The Journal of Infectious Diseases*, *198*(10), 1466–1473. https://doi.org/10.1086/592716

Blankley, S., Graham, C. M., Turner, J., Berry, M. P. R., Bloom, C. I., Xu, Z., Pascual, V., Banchereau, J., Chaussabel, D., Breen, R., Santis, G., Blankenship, D. M., Lipman, M., & O'Garra, A. (2016). The transcriptional signature of active tuberculosis reflects symptom status in extra-pulmonary and pulmonary tuberculosis. *PLoS ONE*, *11*(10), 1–14. https://doi.org/10.1371/journal.pone.0162220

Boussiotis, V. A., Tsai, E. Y., Yunis, E. J., Thim, S., Delgado, J. C., Dascher, C. C., Berezovskaya, A., Roussec, D., Reynes, J. M., & Goldfeld, A. E. (2000). IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. *Journal of Clinical Investigation*, *105*(9), 1317–1325. https://doi.org/10.1172/JCI9918

Brighenti, S., & Andersson, J. (2010). Induction and regulation of CD8+ cytolytic T cells in human tuberculosis and HIV infection. *Biochemical and Biophysical Research Communications*, *396*(1), 50–57. https://doi.org/10.1016/j.bbrc.2010.02.141

Bruns, H., Meinken, C., Schauenberg, Philipp Härter, G., Kern, P., Modlin, R. L., Antoni, C., & Stenger, S. (2009). Anti-TNF immunotherapy reduces CD8 + T cell–mediated antimicrobial activity against. *The Journal of Clinical Investigation*, *119*(5), 1167–1177. https://doi.org/10.1172/JCI38482.ated

Cadena, A. M., Fortune, S. M., & Flynn, J. L. (2017). Heterogeneity in tuberculosis. *Nature Reviews Immunology*, *17*(11), 691–702. https://doi.org/10.1038/nri.2017.69

Carow, B., Reuschl, A. K., Gavier-Widén, D., Jenkins, B. J., Ernst, M., Yoshimura, A., Chambers, B. J., & Rottenberg, M. E. (2013). Critical and Independent Role for SOCS3 in Either Myeloid or T Cells in Resistance to Mycobacterium tuberculosis. *PLoS Pathogens*, *9*(7). https://doi.org/10.1371/journal.ppat.1003442

Cehovin, A., Cliff, J. M., Hill, P. C., Brookes, R. H., & Dockrell, H. M. (2007). Extended culture enhances sensitivity of a gamma interferon assay for latent Mycobacterium tuberculosis infection. *Clinical and Vaccine Immunology*, *14*(6), 796–798. https://doi.org/10.1128/CVI.00093-07

Chegou, N. N., Black, G. F., Loxton, A. G., Stanley, K., Essone, P. N., Klein, M. R., Parida, S. K., Kaufmann, S. H. E., Doherty, T. M., Friggen, A. H., Franken, K. L., Ottenhoff, T. H., & Walzl, G. (2012). Potential of novel Mycobacterium tuberculosis infection phase-dependent antigens in the diagnosis of TB disease in a high burden setting. *BMC Infectious Diseases*, *12*. https://doi.org/10.1186/1471-2334-12-10

Chen, J., Su, X., Zhang, Y., Wang, S., Shao, L., Wu, J., Wang, F., Zhang, S., Wang, J., Weng, X., Wang, H., & Zhang, W. (2009). Novel recombinant RD2- and RD11-encoded Mycobacterium tuberculosis antigens are potential candidates for diagnosis of tuberculosis infections in BCG-vaccinated individuals. *Microbes and Infection*, *11*(10–11), 876–885. https://doi.org/10.1016/j.micinf.2009.05.008

Cho, K., Cho, E., Kwon, S., Im, S., Sohn, I., Song, S., Kim, H., & Kim, S. (2012). Factors associated with indeterminate and false negative results of quantiFERON-TB gold in-tube test in active tuberculosis. *Tuberculosis and Respiratory Diseases*, *72*(5), 416–425. https://doi.org/10.4046/trd.2012.72.5.416

Chowdhury, I. H., Ahmed, A. M., Choudhuri, S., Sen, A., Hazra, A., Pal, N. K., Bhattacharya, B., & Bahar, B. (2014). Alteration of serum inflammatory cytokines in active pulmonary tuberculosis following anti-tuberculosis drug therapy. *Molecular Immunology*, *62*(1), 159–168. https://doi.org/10.1016/j.molimm.2014.06.002

Cooper, B. A. M., Magram, J., Ferrante, J., & Orme, I. M. (1997). *Interleukin 12 (IL-12) Is Crucial to the Development of with Mycobacterium tuberculosis*. *186*(1).

Coppola, M., & Ottenhoff, T. H. (2018). Genome wide approaches discover novel Mycobacterium tuberculosis antigens as correlates of infection, disease, immunity and targets for vaccination. *Seminars in Immunology*, *39*(July), 88–101. https://doi.org/10.1016/j.smim.2018.07.001

Coscolla and Gagneux. (2014). Consequences of genomic diversity. *Semin Immunol*, *23*(1), 1–7. https://doi.org/10.1038/jid.2014.371

Davidson, J. A., Loutet, M. G., Connor, C. O., Kearns, C., Smith, R. M. M., Lalor, M. K., Thomas, H. L., Abubakar, I., & Zenner, D. (2017). Epidemiology of Mycobacterium bovis Disease in. *Emerging Infectious Diseases*, *23*(3), 377–386.

de Jong, B. C., Antonio, M., & Gagneux, S. (2010). Mycobacterium africanum-review of an important cause of human tuberculosis in West Africa. *PLoS Neglected Tropical Diseases*, *4*(9). https://doi.org/10.1371/journal.pntd.0000744

De Visser, V., Sotgiu, G., Lange, C., Aabye, M. G., Bakker, M., Bartalesi, F., Brat, K., Chee, C. B. E., Dheda, K., Dominguez, J., Eyuboglu, F., Ghanem, M., Goletti, D., Dilektasli, A. G., Guglielmetti, L., Koh, W. J., Latorre, I., Losi, M., Polanova, M., … Bossink, A. (2015). False-negative interferon-γ release assay results in active tuberculosis: A TBNET study. *European Respiratory Journal*, *45*(1), 279–283. https://doi.org/10.1183/09031936.00120214

Delogu, G., Sali, M., & Fadda, G. (2013). The biology of mycobacterium tuberculosis infection. *Mediterranean Journal of Hematology and Infectious Diseases*, *5*(1). https://doi.org/10.4084/mjhid.2013.070

Dileepan, T., Linehan, J. L., Moon, J. J., Pepper, M., Jenkins, M. K., & Cleary, P. P. (2011). Robust antigen specific th17 t cell response to group a streptococcus is dependent on il-6 and intranasal route of infection. *PLoS Pathogens*, *7*(9). https://doi.org/10.1371/journal.ppat.1002252

Dong, H., Buckner, A., Prince, J., & Bullock, T. (2019). Frontline Science: Late CD27 stimulation promotes IL-7Rα transcriptional re-expression and memory T cell qualities in effector CD8+ T cells. *Journal of Leukocyte Biology*, *106*(5), 1007–1019. https://doi.org/10.1002/JLB.1HI0219-064R

Ernst, J. D. (2012). The immunological life cycle of tuberculosis. *Nature Reviews Immunology*, *12*(8), 581–591. https://doi.org/10.1038/nri3259

Feske, M., Nudelman, R. J., Medina, M., Lew, J., Singh, M., Couturier, J., Graviss, E. A., & Lewis, D. E. (2008). Enhancement of human antigen-specific memory T-cell responses by interleukin-7 may improve accuracy in diagnosing tuberculosis. *Clinical and Vaccine Immunology*, *15*(10), 1616–1622. https://doi.org/10.1128/CVI.00185-08

Freeman, A. F., & Holland, S. M. (2010). Clinical manifestations of hyper IgE syndromes. *Disease Markers*, *29*(3–4), 123–130. https://doi.org/10.3233/DMA-2010-0734

Harling, K., Adankwah, E., Güler, A., Afum-Adjei Awuah, A., Adu-Amoah, L., Mayatepek, E., Owusu-Dabo, E., Nausch, N., & Jacobsen, M. (2018). Constitutive STAT3 phosphorylation and IL-6/IL-10 co-expression are associated with impaired T-cell function in tuberculosis patients. *Cellular and Molecular Immunology*, *16*(3), 275–287. https://doi.org/10.1038/cmi.2018.5

Heninger, A.-K., Theil, A., Wilhelm, C., Petzold, C., Huebel, N., Kretschmer, K., Bonifacio, E., & Monti, P. (2012). IL-7 Abrogates Suppressive Activity of Human CD4 + CD25 + FOXP3 + Regulatory T Cells and Allows Expansion of Alloreactive and Autoreactive T Cells . *The Journal of Immunology*, *189*(12), 5649–5658. https://doi.org/10.4049/jimmunol.1201286

Hoge, J., Yan, I., Jänner, N., Schumacher, V., Chalaris, A., Steinmetz, O. M., Engel, D. R., Scheller, J., Rose-John, S., & Mittrücker, H.-W. (2013). IL-6 Controls the Innate Immune Response against Listeria monocytogenes via Classical IL-6 Signaling . *The Journal of Immunology*, *190*(2), 703–711. https://doi.org/10.4049/jimmunol.1201044

Hunter, C. A., & Jones, S. A. (2015). IL-6 as a keystone cytokine in health and disease. *Nature Immunology*, *16*(5), 448–457. https://doi.org/10.1038/ni.3153

Hunter, R. L. (2018). The pathogenesis of tuberculosis: The early infiltrate of post-primary (adult pulmonary) tuberculosis: A distinct disease entity. *Frontiers in Immunology*, *9*(SEP), 1–9. https://doi.org/10.3389/fimmu.2018.02108

Jacobsen, M., Repsilber, D., Kleinsteuber, K., Gutschmidt, A., Schommer-Leitner, S., Black, G., Walzl, G., & Kaufmann, S. H. E. (2011). Suppressor of cytokine signaling-3 is affected in T-cells from tuberculosis TB patients. *Clinical Microbiology and Infection*, *17*(9), 1323–1331. https://doi.org/10.1111/j.1469-0691.2010.03326.x

Jasenosky, L. D., Scriba, T. J., Hanekom, W. A., & Goldfeld, A. E. (2015). T cells and adaptive immunity to Mycobacterium tuberculosis in humans. *Immunological Reviews*, *264*(1), 74–87. https://doi.org/10.1111/imr.12274

Jiang, J., Wang, X., Wang, X., Cao, Z., Liu, Y., Dong, M., Tong, A., & Cheng, X. (2010). Reduced CD27 expression on antigen-specific CD4+ T cells correlates with persistent active tuberculosis. *Journal of Clinical Immunology*, *30*(4), 566–573. https://doi.org/10.1007/s10875-010-9418-1

Joshi, L., Ponnana, M., Sivangala, R., Chelluri, L. K., Nallari, P., Penmetsa, S., Valluri, V., & Gaddam, S. (2015). Evaluation of TNF-α, il-10 and il-6 cytokine production and their correlation with genotype variants amongst tuberculosis patients and their household contacts. *PLoS ONE*, *10*(9), 1–15. https://doi.org/10.1371/journal.pone.0137727

Kassa, D., Ran, L., Geberemeskel, W., Tebeje, M., Alemu, A., Selase, A., Tegbaru, B., Franken, K. L. M. C., Friggen, A. H., Van Meijgaarden, K. E., Ottenhoff, T. H. M., Wolday, D., Messele, T., & Van Baarle, D. (2012). Analysis of immune responses against a wide range of Mycobacterium tuberculosis antigens in patients with active pulmonary tuberculosis. *Clinical and Vaccine Immunology*, *19*(12), 1907–1915. https://doi.org/10.1128/CVI.00482-12

Kaufmann, S. H.E. (2002). Protection against tuberculosis: Cytokines, T cells, and macrophages. *Annals of the Rheumatic Diseases*, *61*(SUPPL. 2), 54–58. https://doi.org/10.1136/ard.61.suppl_2.ii54

Kaufmann, Stefan H.E., & Parida, S. K. (2008). Tuberculosis in Africa: Learning from Pathogenesis for Biomarker Identification. *Cell Host and Microbe*, *4*(3), 219–228. https://doi.org/10.1016/j.chom.2008.08.002

Kaufmann, Stefan H E. (2001). How can immunology contributed to TB control. *Nature Reviews Immunology*, *1*(October), 20–30.

Koeck, J. L., Fabre, M., Simon, F., Daffé, M., Garnotel, É., Matan, A. B., Gérôme, P., Bernatas, J. J., Buisson, Y., & Pourcel, C. (2011). Clinical characteristics of the smooth tubercle bacilli "Mycobacterium canettii" infection suggest the existence of an environmental reservoir. *Clinical Microbiology and Infection*, *17*(7), 1013–1019. https://doi.org/10.1111/j.1469-0691.2010.03347.x

Kwon, Y. S., Kim, Y. H., Jeon, K., Jeong, B. H., Ryu, Y. J., Choi, J. C., Kim, H. C., & Koh, W. J. (2015). Factors that predict negative results of QuantiFERON-TB Gold In-Tube Test in patients with culture-confirmed tuberculosis: A multicenter retrospective cohort study. *PLoS ONE*, *10*(6), 1–9. https://doi.org/10.1371/journal.pone.0129792

Lange, C., Pai, M., Drobniewski, F., & Migliori, G. B. (2009). Interferon-γ release assays for the diagnosis of active tuberculosis: Sensible or silly? *European Respiratory Journal*, *33*(6), 1250–1253. https://doi.org/10.1183/09031936.00019709

Latorre, I., Fernández-Sanmartín, M. A., Muriel-Moreno, B., Villar-Hernández, R., Vila, S., De Souza-Galvão, M. L., Stojanovic, Z., Jiménez-Fuentes, M. A., Centeno, C., Ruiz-Manzano, J., Millet, J. P., Molina-Pinargote, I., González-Díaz, Y. D., Lacoma, A., Luque-Chacón, L., Sabriá, J., Prat, C., & Domínguez, J. (2019). Study of CD27 and CCR4 markers on specific CD4+ T-cells as immune tools for active and latent tuberculosis management. *Frontiers in Immunology*, *10*(JAN), 1–11. https://doi.org/10.3389/fimmu.2018.03094

Lawson, B. R., Gonzalez-Quintial, R., Eleftheriadis, T., Farrar, M. A., Miller, S. D., Sauer, K., McGavern, D. B., Kono, D. H., Baccala, R., & Theofilopoulos, A. N. (2015). Interleukin-7 is required for CD4+ T cell activation and autoimmune neuroinflammation. *Clinical Immunology*, *161*(2), 260–269. https://doi.org/10.1016/j.clim.2015.08.007

Leal, I. S., Flórido, M., Andersen, P., & Appelberg, R. (2001). Interleukin-6 regulates the phenotype of the immune response to a tuberculosis subunit vaccine. *Immunology*, *103*(3), 375–381. https://doi.org/10.1046/j.1365-2567.2001.01244.x

Longhi, M. P., Wright, K., Lauder, S. N., Nowell, M. A., Jones, G. W., Godkin, A. J., Jones, S. A., & Gallimore, A. M. (2008). Interleukin-6 is crucial for recall of influenza-specific memory CD4 + T cells. *PLoS Pathogens*, *4*(2), 2–9. https://doi.org/10.1371/journal.ppat.1000006

Lundtoft, et al. (2017). Aberrant plasma IL-7 and soluble IL-7 receptor levels indicate impaired T-cell response to IL-7 in human tuberculosis. *PLoS Pathogens*, *13*(6), 1–22. https://doi.org/10.1371/journal.ppat.1006425

Lundtoft, C., Awuah, A. A. A., Nausch, N., Enimil, A., Mayatepek, E., Owusu-Dabo, E., & Jacobsen, M. (2017). Alternative Quantiferon cytokines for diagnosis of children with active tuberculosis and HIV co-infection in Ghana. *Medical Microbiology and Immunology*, *206*(3), 259–265. https://doi.org/10.1007/s00430-017-0501-6

Lyadova, I. V., & Panteleev, A. V. (2015). Th1 and Th17 Cells in Tuberculosis: Protection, Pathology, and Biomarkers. *Mediators of Inflammation*, *2015*. https://doi.org/10.1155/2015/854507

Lyadova, Irina V., Oberdorf, S., Kapina, M. A., Apt, A. S., Swain, S. L., & Sayles, P. C. (2004). CD4 T cells producing IFN-γ in the lungs of mice challenged with mycobacteria express a CD27-negative phenotype. *Clinical and Experimental Immunology*, *138*(1), 21–29. https://doi.org/10.1111/j.1365-2249.2004.02573.x

MacKall, C. L., Fry, T. J., & Gress, R. E. (2011). Harnessing the biology of IL-7 for therapeutic application. *Nature Reviews Immunology*, *11*(5), 330–342. https://doi.org/10.1038/nri2970

Maeurer, M. J., Trinder, P., Hommel, G., Walter, W., Freitag, K., Atkins, D., & Störkel, S. (2000). Interleukin-7 or interleukin-15 enhances survival of Mycobacterium tuberculosis-infected mice. *Infection and Immunity*, *68*(5), 2962–2970. https://doi.org/10.1128/IAI.68.5.2962-2970.2000

Majlessi, L., Simsova, M., Jarvis, Z., Brodin, P., Rojas, M. J., Bauche, C., Nouzé, C., Ladant, D., Cole, S. T., Sebo, P., & Leclerc, C. (2006). An increase in antimycobacterial Th1-cell responses by prime-boost protocols of immunization does not enhance protection against tuberculosis. *Infection and Immunity*, *74*(4), 2128–2137. https://doi.org/10.1128/IAI.74.4.2128-2137.2006

Masood, K. I., Jamil, B., Akber, A., Hassan, M., Islam, M., & Hasan, Z. (2020). Testing for Mycobacterium tuberculosis infection using the QuantiFERON-TB GOLD assay in patients with

comorbid conditions in a tertiary care endemic setting. *Tropical Diseases, Travel Medicine and Vaccines*, *6*, 3. https://doi.org/10.1186/s40794-020-0102-z

Matsumoto, A., Seki, Y. ichi, Watanabe, R., Hayashi, K., Johnston, J. A., Harada, Y., Abe, R., Yoshimura, A., & Kubo, M. (2003). A role of suppressor of cytokine signaling 3 (SOCS3/CIS3/SSI3) in CD28-mediated interleukin 2 production. *Journal of Experimental Medicine*, *197*(4), 425–436. https://doi.org/10.1084/jem.20020939

Meier, N. R., Jacobsen, M., Ottenhoff, T. H. M., & Ritz, N. (2018). A systematic review on novel mycobacterium tuberculosisantigens and their discriminatory potential for the diagnosis of latent and active tuberculosis. *Frontiers in Immunology*, *9*(NOV). https://doi.org/10.3389/fimmu.2018.02476

Mittrücker, H. W., Steinhoff, U., Köhler, A., Krause, M., Lazar, D., Mex, P., Miekley, D., & Kaufmann, S. H. E. (2007). Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(30), 12434–12439. https://doi.org/10.1073/pnas.0703510104

Mupfumi, L., Mpande, C. A. M., Reid, T., Moyo, S., Shin, S. S., Zetola, N., Mogashoa, T., Musonda, R. M., Kasvosve, I., Scriba, T. J., Nemes, E., & Gaseitsiwe, S. (2020). Immune phenotype and functionality of Mtb-specific T-cells in HIV/TB co-infected patients on antiretroviral treatment. *Pathogens*, *9*(3), 1–19. https://doi.org/10.3390/pathogens9030180

Napolitano, L. A., Grant, R. M., Deeks, S. G., Schmidt, D., De Rosa, S. C., Herzenberg, L. A., Herndier, B. G., Andersson, J., & Mccune, J. M. (2001). Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: Implications for T-cell homeostasis. *Nature Medicine*, *7*(1), 73–79. https://doi.org/10.1038/83381

Nausch, N., Lundtoft, C., Schulz, G., Henckel, H., Mayatepek, E., Fleischer, B., Marx, F. M., & Jacobsen, M. (2017). Multiple cytokines for the detection of Mycobacterium tuberculosis infection in children with tuberculosis. *International Journal of Tuberculosis and Lung Disease*, *21*(3), 270–277. https://doi.org/10.5588/ijtld.16.0351

Nieuwenhuizen, N. E., & Kaufmann, S. H. E. (2018). Next-generation vaccines based on Bacille Calmette-Guérin. *Frontiers in Immunology*, *9*(FEB). https://doi.org/10.3389/fimmu.2018.00121

Nowell, M. A., Williams, A. S., Carty, S. A., Scheller, J., Hayes, A. J., Jones, G. W., Richards, P. J., Slinn, S., Ernst, M., Jenkins, B. J., Topley, N., Rose-John, S., & Jones, S. A. (2009). Therapeutic Targeting of IL-6 Trans Signaling Counteracts STAT3 Control of Experimental Inflammatory Arthritis . *The Journal of Immunology*, *182*(1), 613–622. https://doi.org/10.4049/jimmunol.182.1.613

O'Leary, S., O'Sullivan, M. P., & Keane, J. (2011). IL-10 blocks phagosome maturation in Mycobacterium tuberculosis-infected human macrophages. *American Journal of Respiratory Cell and Molecular Biology*, *45*(1), 172–180. https://doi.org/10.1165/rcmb.2010-0319OC

P. Ling, J. Flynn. (2012). Understanding Latent Tuberculosis: A Moving Target. *Journal of Immunology*, *185*(1), 15–22. https://doi.org/10.4049/jimmunol.0903856.Understanding

Pai, M., Behr, M. A., Dowdy, D., Dheda, K., Divangahi, M., Boehme, C. C., Ginsberg, A., Swaminathan, S., Spigelman, M., Getahun, H., Menzies, D., & Raviglione, M. (2016). Tuberculosis. *Nature Reviews Disease Primers*, *2*. https://doi.org/10.1038/nrdp.2016.76

Pai, M., Kalantri, S., & Dheda, K. (2006). New tools and emerging technologies for the diagnosis of tuberculosis: Part I. Latent tuberculosis. *Expert Review of Molecular Diagnostics*, *6*(3), 413–422. https://doi.org/10.1586/14737159.6.3.413

Pinto, L. M., Grenier, J., Schumacher, S. G., Denkinger, C. M., Steingart, K. R., & Pai, M. (2011).

Immunodiagnosis of tuberculosis: State of the art. *Medical Principles and Practice*, *21*(1), 4–13. https://doi.org/10.1159/000331583

Ponchel, F., Cuthbert, R. J., & Goëb, V. (2011). IL-7 and lymphopenia. *Clinica Chimica Acta*, *412*(1–2), 7–16. https://doi.org/10.1016/j.cca.2010.09.002

Portevin, D., Moukambi, F., Clowes, P., Bauer, A., Chachage, M., Ntinginya, N. E., Mfinanga, E., Said, K., Haraka, F., Rachow, A., Saathoff, E., Mpina, M., Jugheli, L., Lwilla, F., Marais, B. J., Hoelscher, M., Daubenberger, C., Reither, K., & Geldmacher, C. (2014). Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: A prospective proof-of-concept study. *The Lancet Infectious Diseases*, *14*(10), 931–938. https://doi.org/10.1016/S1473-3099(14)70884-9

Prezzemolo, T., Guggino, G., La Manna, M. P., Di Liberto, D. Di, Dieli, F., & Caccamo, N. (2014). Functional signatures of human CD4 and CD8 T cell responses to Mycobacterium tuberculosis. *Frontiers in Immunology*, *5*(APR), 1–13. https://doi.org/10.3389/fimmu.2014.00180

Qiu, X., Tang, Y., Zou, R., Zeng, Y., Yue, Y., Li, W., Qu, Y., & Mu, D. (2019). Diagnostic accuracy of interferon-gamma-induced protein 10 for differentiating active tuberculosis from latent tuberculosis: A meta-analysis. *Scientific Reports*, *9*(1), 1–10. https://doi.org/10.1038/s41598-019-47923-w

Rakshit, S., Adiga, V., Nayak, S., Sahoo, P. N., Sharma, P. K., Van Meijgaarden, K. E., Uk, A. J., Dhar, C., Souza, G. D., Finak, G., De Rosa, S. C., Ottenhoff, T. H. M., & Vyakarnam, A. (2017). Circulating Mycobacterium tuberculosis DosR latency antigen-specific, polyfunctional, regulatory IL10+ Th17 CD4 T-cells differentiate latent from active tuberculosis. *Scientific Reports*, *7*(1), 1–15. https://doi.org/10.1038/s41598-017-10773-5

Rane, L., Rahman, S., Magalhaes, I., Ahmed, R., Spångberg, M., Kondova, I., Verreck, F., Andersson, J., Brighenti, S., & Maeurer, M. J. (2011). Increased (6 exon) interleukin-7 production after M. tuberculosis infection and soluble interleukin-7 receptor expression in lung tissue. *Genes and Immunity*, *12*(7), 513–522. https://doi.org/10.1038/gene.2011.29

Rangaka, M. X., Wilkinson, K. A., Glynn, J. R., Ling, D., Menzies, D., Mwansa-Kambafwile, J., Fielding, K., Wilkinson, R. J., & Pai, M. (2012). Predictive value of interferon-γ release assays for incident active tuberculosis: A systematic review and meta-analysis. *The Lancet Infectious Diseases*, *12*(1), 45–55. https://doi.org/10.1016/S1473-3099(11)70210-9

Redford, P. S., Murray, P. J., & O'Garra, A. (2011). The role of IL-10 in immune regulation during M. tuberculosis infection. *Mucosal Immunology*, *4*(3), 261–270. https://doi.org/10.1038/mi.2011.7

Redford, Paul S., Boonstra, A., Read, S., Pitt, J., Graham, C., Stavropoulos, E., Bancroft, G. J., & O'Garra, A. (2010). Enhanced protection to Mycobacterium tuberculosis infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. *European Journal of Immunology*, *40*(8), 2200–2210. https://doi.org/10.1002/eji.201040433

Reece, S. T., Nasser-Eddine, A., Dietrich, J., Stein, M., Zedler, U., Schommer-Leitner, S., Ottenhoff, T. H. M., Andersen, P., & Kaufmann, S. H. E. (2011). Improved long-term protection against Mycobacterium tuberculosis Beijing/W in mice after intra-dermal inoculation of recombinant BCG expressing latency associated antigens. *Vaccine*, *29*(47), 8740–8744. https://doi.org/10.1016/j.vaccine.2011.07.144

Riou, C., Gray, C. M., Lugongolo, M., Gwala, T., Kiravu, A., Deniso, P., Stewart-Isherwood, L., Omar, S. V., Grobusch, M. P., Coetzee, G., Conradie, F., Ismail, N., Kaplan, G., & Fallows, D. (2014). A subset of circulating blood mycobacteria-specific CD4 T cells can predict the time to Mycobacterium tuberculosis sputum culture conversion. *PLoS ONE*, *9*(7).

https://doi.org/10.1371/journal.pone.0102178

Rook, G. A. W., Dheda, K., & Zumla, A. (2005). Immune responses to tuberculosis in developing countries: Implications for new vaccines. *Nature Reviews Immunology*, *5*(8), 661–667. https://doi.org/10.1038/nri1666

Rottenberg, M. E., & Carow, B. (2014). SOCS3 and STAT3, major controllers of the outcome of infection with Mycobacterium tuberculosis. *Seminars in Immunology*, *26*(6), 518–532. https://doi.org/10.1016/j.smim.2014.10.004

Sakai, S., Kauffman, K. D., Sallin, M. A., Sharpe, A. H., Young, H. A., Ganusov, V. V., & Barber, D. L. (2016). CD4 T Cell-Derived IFN-γ Plays a Minimal Role in Control of Pulmonary Mycobacterium tuberculosis Infection and Must Be Actively Repressed by PD-1 to Prevent Lethal Disease. *PLoS Pathogens*, *12*(5), 1–22. https://doi.org/10.1371/journal.ppat.1005667

Schuck, S. D., Mueller, H., Kunitz, F., Neher, A., Hoffmann, H., Franken, K. L. C. M., Repsilber, D., Ottenhoff, T. H. M., Kaufmann, S. H. E., & Jacobsen, M. (2009). Identification of T-cell antigens specific for latent mycobacterium tuberculosis infection. *PLoS ONE*, *4*(5), 1–10. https://doi.org/10.1371/journal.pone.0005590

Schuetz, A., Haule, A., Reither, K., Ngwenyama, N., Rachow, A., Meyerhans, A., Maboko, L., Koup, R. A., Hoelscher, M., & Geldmacher, C. (2011). Monitoring CD27 expression to evaluate mycobacterium tuberculosis activity in HIV-1 infected individuals in vivo. *PLoS ONE*, *6*(11), 2–7. https://doi.org/10.1371/journal.pone.0027284

Serra-Vidal, M., Latorre, I., Franken, K., Díaz, J., de Souza-Galvão, M., Casas, I., Maldonado, J., Milà, C., Solsona, J., Jimenez-Fuentes, M. A., Altet, N., Lacoma, A., Ruiz-Manzano, J., Ausina, V., Prat, C., Ottenhoff, T. H. M., & Domínguez, J. (2014). Immunogenicity of 60 novel latency-related antigens of Mycobacterium tuberculosis. *Frontiers in Microbiology*, *5*(SEP), 1–13. https://doi.org/10.3389/fmicb.2014.00517

Singh, P. P., & Goyal, A. (2013). Interleukin-6: A potent biomarker of mycobacterial infection. *SpringerPlus*, *2*(1), 2–9. https://doi.org/10.1186/2193-1801-2-686

Singh, V., Gowthaman, U., Jain, S., Parihar, P., Banskar, S., Gupta, P., Gupta, U. D., & Agrewala, J. N. (2010). Coadministration of Interleukins 7 and 15 with Bacille Calmette-Guérin Mounts Enduring T Cell Memory Response against Mycobacterium tuberculosis . *The Journal of Infectious Diseases*, *202*(3), 480–489. https://doi.org/10.1086/653827

Sollai, S., Galli, L., de Martino, M., & Chiappini, E. (2014). Systematic review and meta-analysis on the utility of Interferon-gamma release assays for the diagnosis of Mycobacterium tuberculosis infection in children: A 2013 update. *BMC Infectious Diseases*, *14*(Suppl 1), 1–11. https://doi.org/10.1186/1471-2334-14-S1-S6

Streitz, M., Tesfa, L., Yildirim, V., Yahyazadeh, A., Ulrichs, T., Lenkei, R., Quassem, A., Liebetrau, G., Nomura, L., Maecker, H., Volk, H. D., & Kern, F. (2007). Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. *PLoS ONE*, *2*(8). https://doi.org/10.1371/journal.pone.0000735

Sugimoto, K. (2008). Role of STAT3 in inflammatory bowel disease. *World Journal of Gastroenterology*, *14*(33), 5110–5114. https://doi.org/10.3748/wjg.14.5110

Tanaskovic, S., Fernandez, S., Price, P., & French, M. A. (2014). Interleukin-7 signalling defects in naive CD4+ T cells of HIV patients with CD4+ T-cell deficiency on antiretroviral therapy are associated with T-cell activation and senescence. *Aids*, *28*(6), 821–830.

https://doi.org/10.1097/QAD.0000000000000213

Ulrichs, T., & Kaufmann, S. H. E. (2006). New insights into the function of granulomas in human tuberculosis. *Journal of Pathology*, *208*(2), 261–269. https://doi.org/10.1002/path.1906

Urdahl, K. B., Shafiani, S., & Ernst, J. D. (2011). Initiation and regulation of T-cell responses in tuberculosis. *Mucosal Immunology*, *4*(3), 288–293. https://doi.org/10.1038/mi.2011.10

Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R., & Schoolnik, G. K. (2003). Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *Journal of Experimental Medicine*, *198*(5), 705–713. https://doi.org/10.1084/jem.20030205

Wallis, R. S., Pai, M., Menzies, D., Doherty, T. M., Walzl, G., Perkins, M. D., & Zumla, A. (2010). Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. *The Lancet*, *375*(9729), 1920–1937. https://doi.org/10.1016/S0140-6736(10)60359-5

WHO. (2018). Global Tuberculosis Report 2018. Geneva: World Health Organization; 2018. In *WHO Publication*. https://doi.org/WHO/HTM/TB/2017.23

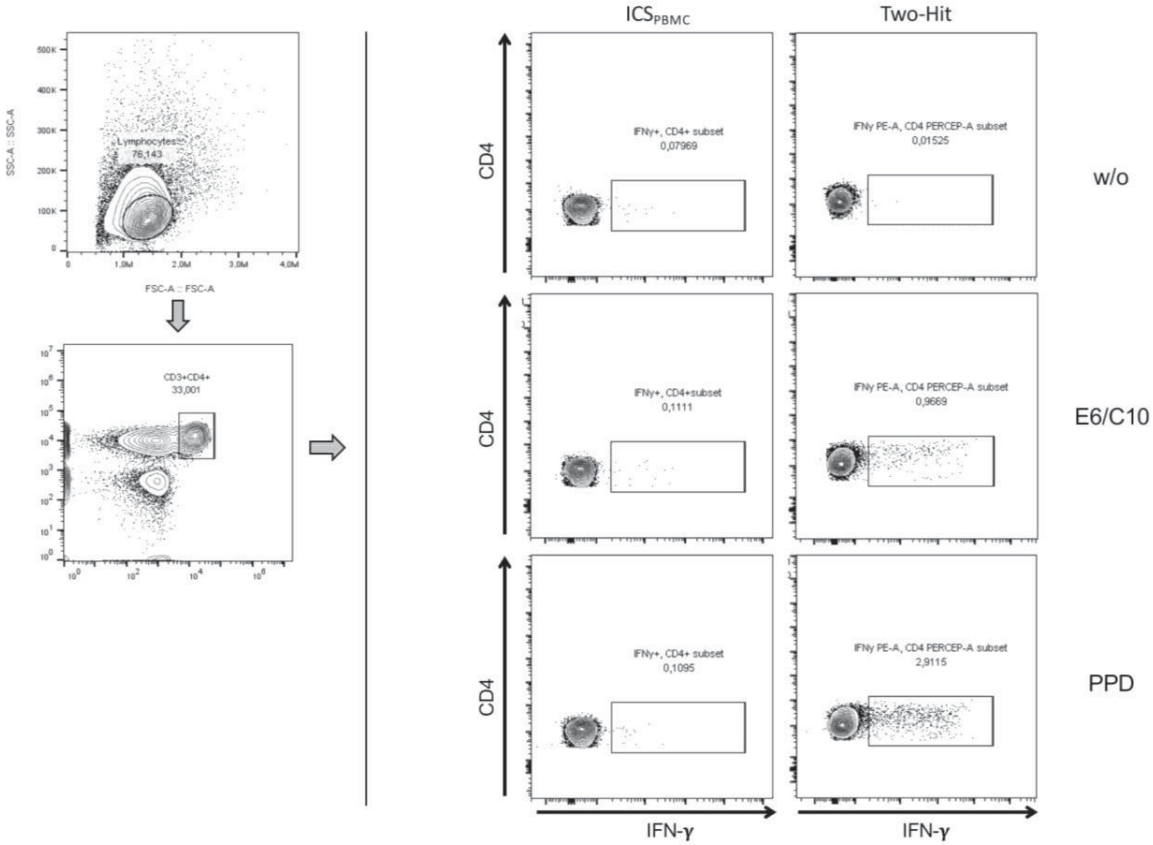
World Health Organization. (2014). *High-priority target product profiles for new tuberculosis diagnostics: report of a consensus meeting*. *April*, 1–96.

Yong, Y. K., Tan, H. Y., Saeidi, A., Wong, W. F., Vignesh, R., Velu, V., Eri, R., Larsson, M., & Shankar, E. M. (2019). Immune Biomarkers for Diagnosis and Treatment Monitoring of Tuberculosis: Current Developments and Future Prospects. *Frontiers in Microbiology*, *10*(December). https://doi.org/10.3389/fmicb.2019.02789

Yuan, J., Zhang, F., & Niu, R. (2015). Multiple regulation pathways and pivotal biological functions of STAT3 in cancer. *Scientific Reports*, *5*, 1–10. https://doi.org/10.1038/srep17663

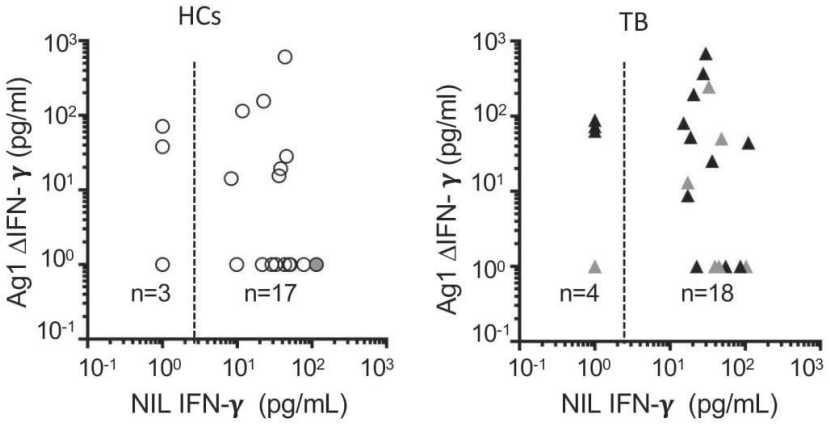
Supplementary Information: Paper I

Supplementary Figure 1



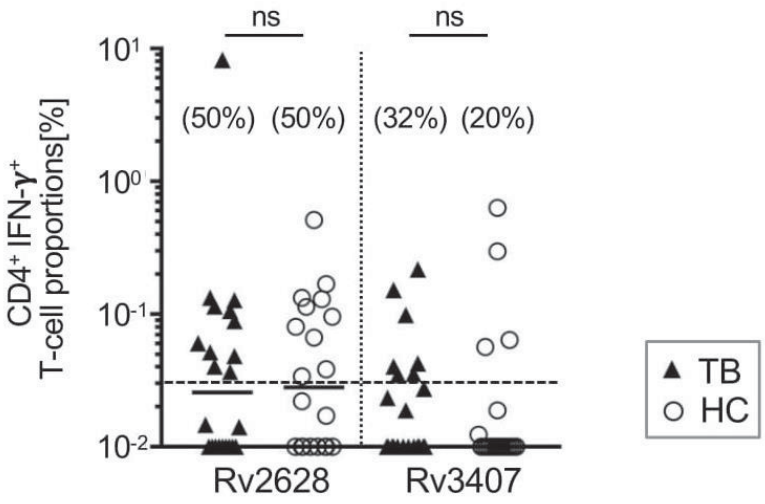
A representative data set depicting analysis procedure of flow cytometry data for intracellular cytokine measurement of ICS$_{PBMC}$ and the two-hit assay for an individual donor is shown.

Supplementary Figure 2



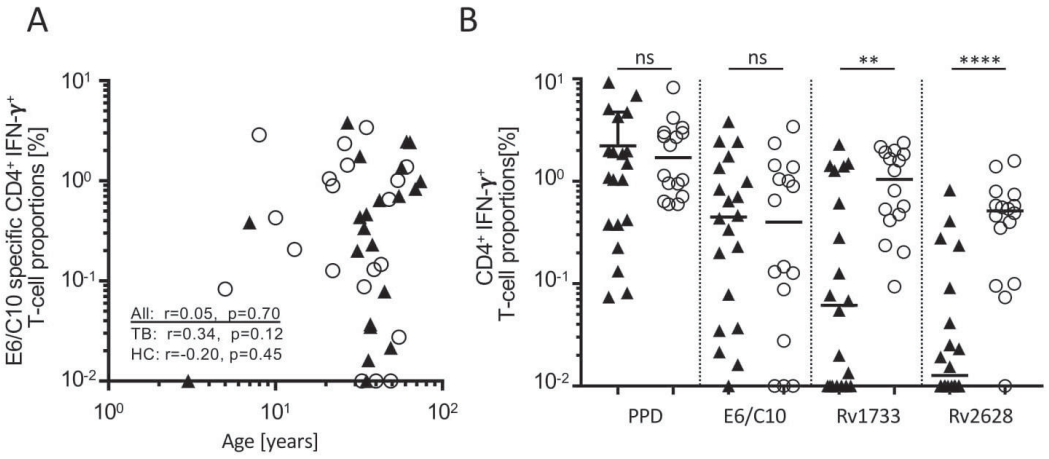
NIL (x-axis) and Ag1 (y-axis) induced IFN-γ concentrations of QFT supernatants are shown for tuberculosis patients (TB) patients and healthy contacts (HCs). Donors with indeterminate QFT are indicated by grey color. Numbers (n) of donors with low and high NIL IFN-γ values are given for both study groups

Supplementary Figure 3



IFN-γ positive CD4+ T-cell proportions specific for latency *Mtb* antigens Rv2628 and Rv3407 after short-term *in vitro* stimulation. Symbols indicate mean values of duplicates for individual tuberculosis patients (black triangles) and HCs (open circles). Proportions of responders (according to the threshold of 0.03%) are indicated in brackets. Mann-Whitney *U*-test (two-tailed) indicated no significant differences between the study groups. ns: not significant.

Supplementary Figure 4



IFN-γ positive CD4+ T-cell proportions specific for *Mtb* antigens after two-hit stimulation. Symbols indicate mean values of duplicates for individual tuberculosis patients (black triangles) and HCs (open circles). (A) E6/C10 specific T-cell proportions (y-axis) and donor age (x-axis) are depicted and association was determined using the Spearman Rank test for all donors and both study groups separately. Correlation coefficients (r) and nominal p-values are given. (B) CD4+ T-cell proportions after two-Hit stimulation with PPD, E6/C10, Rv2628, and Rv3407 are depicted for study subgroups of adult (>18y) donors. Nominal p-values for the Mann-Whitney *U*-test (two-tailed) were calculated and shown as ** for p<0.01 and **** for p<0.0001.

Supplementary Table 1: Evaluation criteria for QFT.

Interpretation	ΔTB1 specific antigen response[1] (pg/ml)[2]	Nil control (pg/ml)[2]	ΔMitogen control[1] (pg/ml)[2]
Positive	≥ 17.5 (and ≥ 25% of Nil)	≤ 400	any
Negative	< 17.5 OR ≥ 17.5 and < 25% of Nil	≤ 400	≥ 25
Indeterminate	< 17.5 OR ≥ 17.5 and < 25% of Nil	≤ 400	< 25
	any	> 400	any

[1]Corrected for Nil response. [2]IFN-γ concentration converted by 1 IU/ml = 50 pg/ml. The table was adopted from manufacturers' instructions.

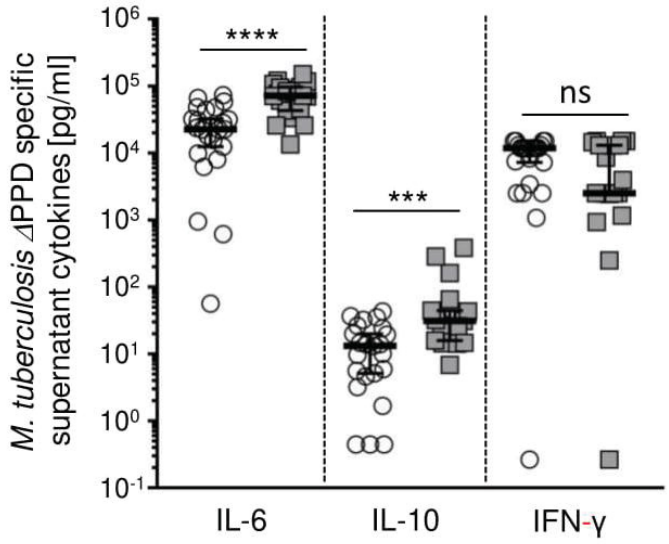
Supplementary Table 2: Evaluation criteria for $QFT_{in\text{-}vitro}$.

Interpretation	ΔE6C10 specific response[1] (pg/ml)	ΔMitogen control[1] (pg/ml)
Positive	≥ 1.5 (and ≥ 25% of Unstim.)	any
Negative	< 1.5 OR ≥ 1.5 and < 25% of Nil	≥ 5
Indeterminate	< 1.5 OR ≥ 1.5 and < 25% of Nil	< 5
	any	any

[1]Corrected for unstimulated response

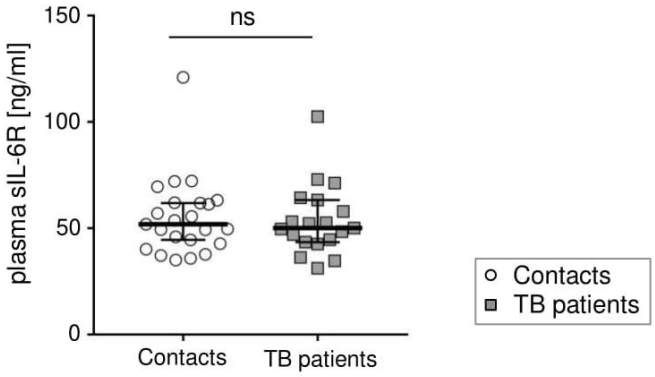
Supplementary Information: Paper II

Sup. Fig. 1



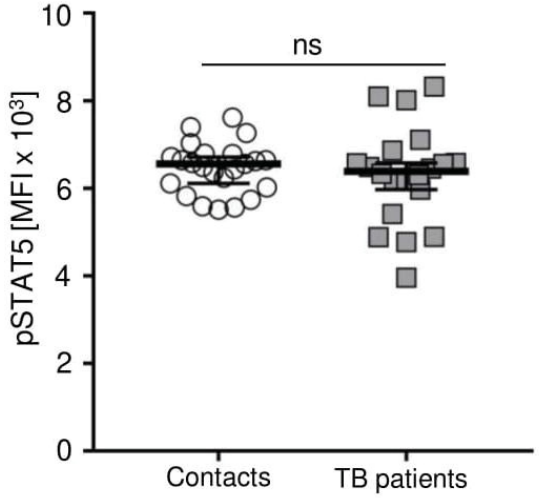
IL-6, IL10, and IFN-γ concentrations calculated as ΔPPD values. Concentrations of non-stimulated samples were subtracted from respective *M. tuberculosis* PPD induced concentrations determined in supernatants of diluted blood after 72 h *in vitro* culture are depicted. All samples were measured as duplicates and mean values are indicated as open circles for healthy contacts and grey squares for tuberculosis patients. Study group medians and percentiles (25, 75) are shown. Significant differences are indicated by asterisks. Nominal p-values for the Mann-Whitney *U*-test (two-tailed) were calculated and shown as *** for $p < 0.001$, and **** for $p < 0.0001$. ns: not significant.

Sup. Fig. 2



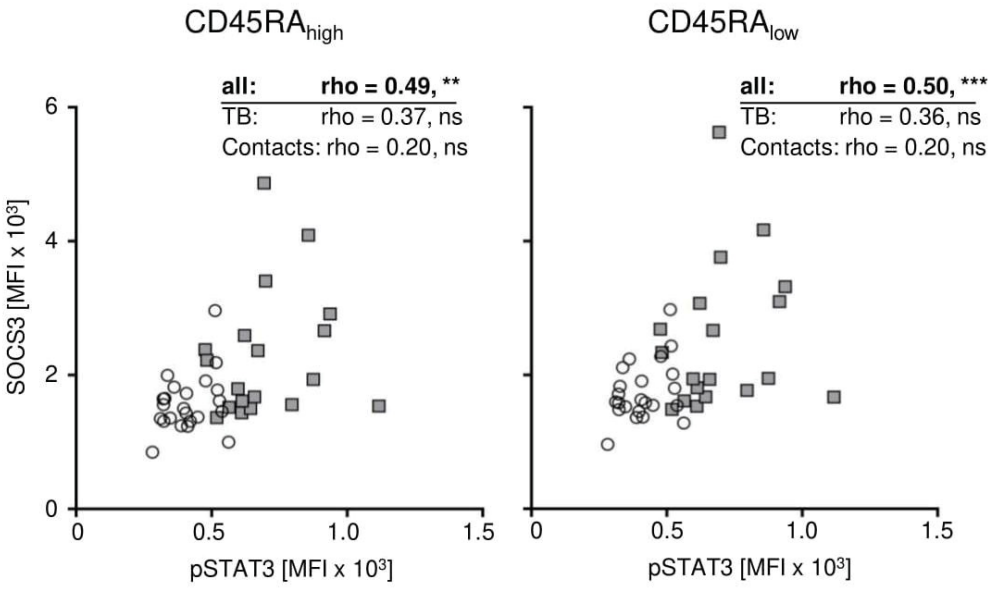
Soluble IL-6 receptor (sIL-6R) plasma concentrations. sIL-6R plasma concentrations are shown for healthy contacts (open circles) and tuberculosis patients (grey squares). ns: not significant.

Sup. Fig. 3



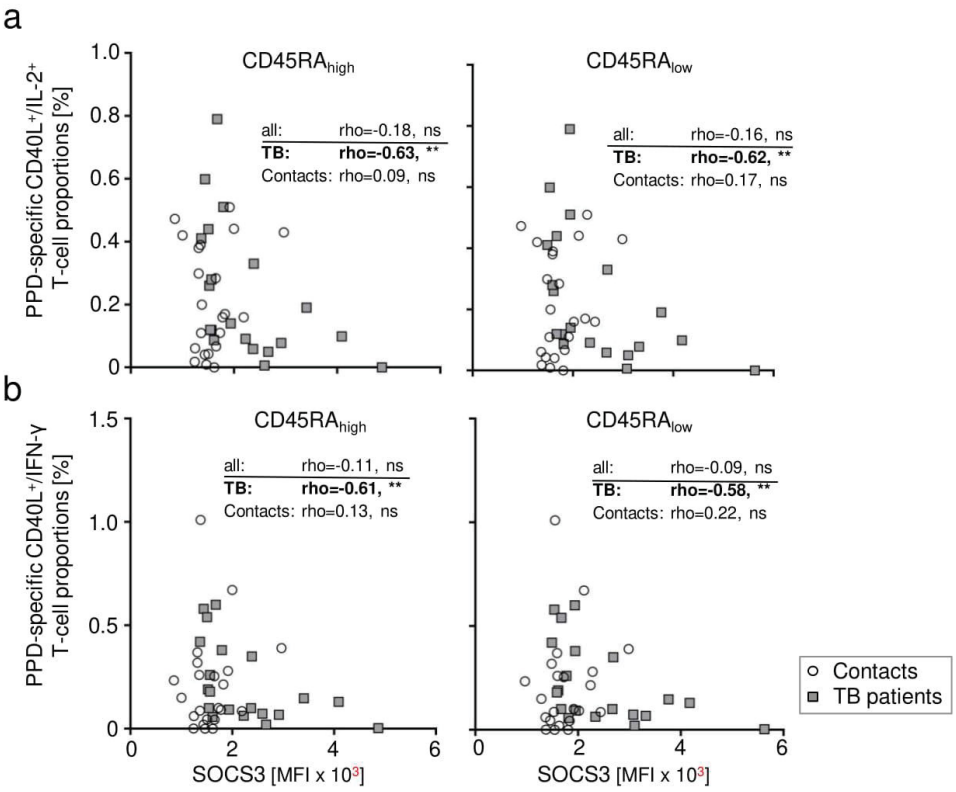
STAT5 phosphorylation of CD4⁺ T cell without *in vitro* re-stimulation. Individual values are shown for tuberculosis patients (grey squares) and healthy contacts (open circles). All samples were measured as duplicates and mean values are indicated. Nominal p-values for the Mann-Whitney U-test (two-tailed) were calculated and shown as **** for $p < 0.0001$. ns: not significant.

Sup. Fig. 4



Correlation plots for constitutive pSTAT3 and SOCS3 expression of T-cell subpopulations. Individual values are shown for CD45RA$_{high}$ (left graph) and CD45RA$_{low}$ (right graph) CD4$^+$ T-cell subpopulations. The Spearman Rank test was used to determine significant correlations for all donors and both study groups separately. Correlation coefficients (rho) are given and p-values are shown as ** for $p < 0.01$ and *** for $p < 0.001$. ns: not significant.

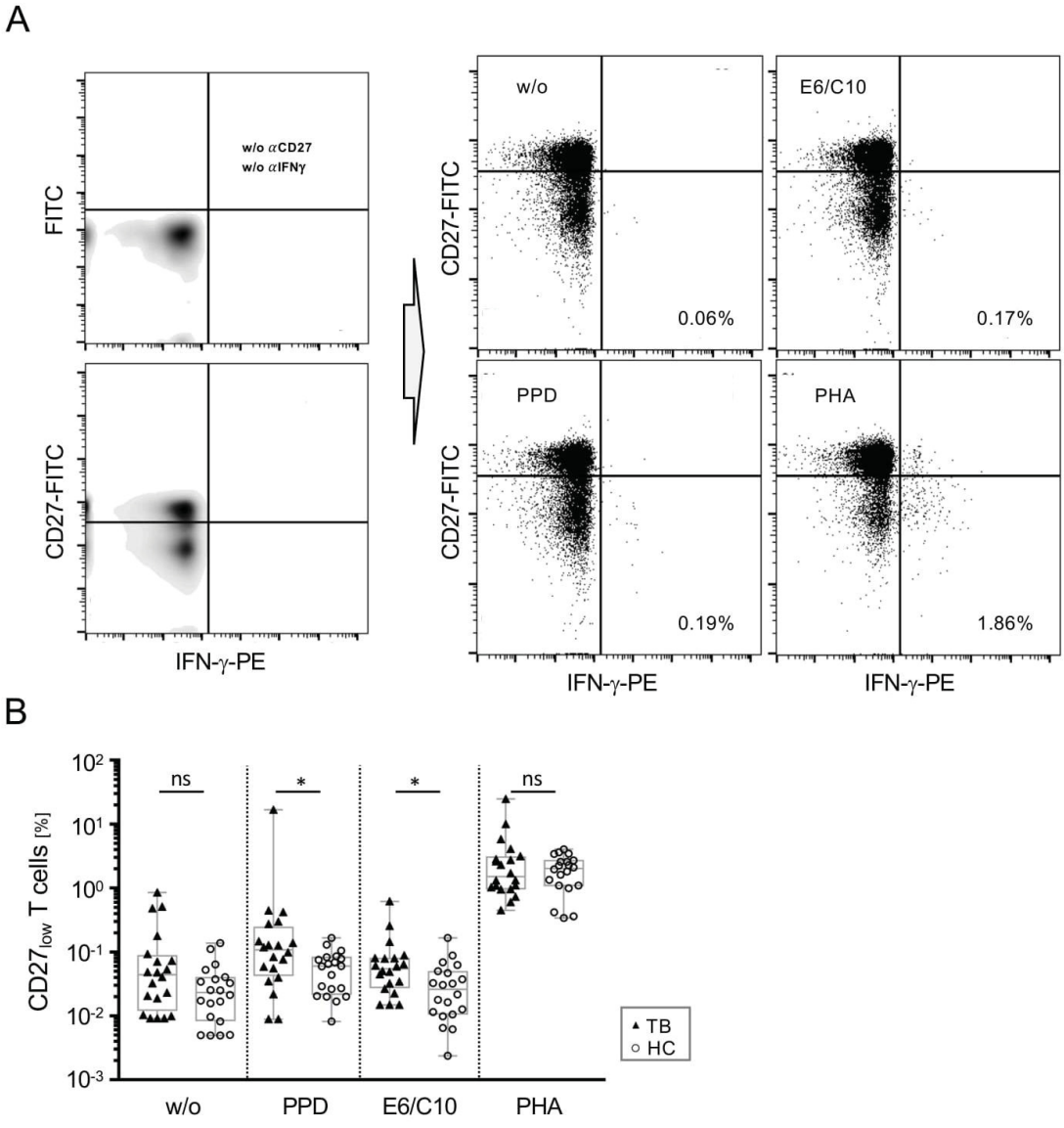
Sup. Fig. 5



Correlation plots for SOCS3 expression in CD4$^+$ T-cell subpopulations. Individual values are shown for CD40L/IL-2 **(a)** and CD40L/IFN-γ **(b)** for CD4$^+$ CD45RA$_{high}$ (left graphs) and CD45RA$_{low}$ (right graphs) T-cell subpopulations from tuberculosis patients (grey squares) and healthy contacts (open circles). All samples were measured as duplicates and mean values are indicated. The Spearman Rank test was used to determine significant correlations for all donors and both study groups separately. Correlation coefficients (rho) are given. Nominal p-values are shown as ** for $p < 0.01$. ns: not significant.

Supplementary Information: Paper III

Supplementary Figure 1



CD27 expression of CD4⁺/IFN-γ⁺ T-cells of TB patients and HCs was measured after overnight PBMC culture with *Mtb* antigens (i.e. PPD, E6_C10), the mitogen PHA, and without stimulation. (**A**) Schematic depiction of flow cytometric analysis of $CD27_{low}$ T-cell proportions is shown. (**B**) $CD27_{low}$ IFN-γ⁺/CD4⁺ T-cell proportions after *in vitro* culture with different stimuli are shown. TB patients are indicated by black triangles and HCs as open circles. Each symbol depicts the mean of duplicates from an individual donor. Nominal p-values for the Mann-Whitney *U*-test (two-tailed) are shown as * for $p<0.05$. ns: not significant.