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

Dissertation

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German summary

Infektionen mit *Mycobacterium tuberculosis (M. tuberculosis)* stellen weiterhin eine große Herausforderung für die Gesundheit der Weltbevölkerung dar. T-Zellen spielen eine zentrale Rolle in der Immunantwort auf *M. tuberculosis*. Bei der Feinregulation ihrer Funktion ist das komplexe Zusammenspiel von Zytokinen entscheidend für die Ausbildung von T-Zell-Phänotyp und Effektorfunktionen. Bei Tuberkuloseinfektionen (TB) konnte eine aberrante Expression der jeweils pro- und antiinflammatorischen Interleukine (IL)- 6 und 10 gezeigt werden. Beide Zytokine aktivieren *signal transducer and activator of transcription (STAT)3* und induzieren die Expression des Feedback-Inhibitors *suppressor of cytokine signaling (SOCS)3* in T-Zellen. SOCS3 hemmt als pleiotroper Inhibitor sowohl Zytokin-Signale als auch die Aktivierung von T-Zellen. Um herauszufinden, ob das Zytokinmilieu bei Tuberkulosepatienten die T-Zellantwort beeinflusst, charakterisierten wir Blutproben von TB-Patienten und gesunden Kontrollspendern mittels FACS-Analyse, ELISA und Cytometric Bead Assay. Wir untersuchten die spontanen und *M. tuberculosis*-spezifischen Zytokinexpressionsprofile, die Expression von SOCS3 sowie die Zytokin-vermittelte Aktivierung von pSTAT3 und pSTAT5 in T-Zellen. Darüber hinaus verglichen wir die *M. tuberculosis*-spezifische T-Zell-Aktivierung anhand von Aktivierungsmarkern und Effektorzytokinproduktion. Zusätzlich führten wir lentivirale Transduktion von CD4+-Zellen durch, um den Effekt von SOCS3-Überexpression auf T-Zellfunktionen zu erforschen.

In unseren Studien zeichneten sich Blutproben von TB-Patienten im Vergleich zu gesunden Kontrollspendern sowohl im Plasma als auch in unstimulierter - und *M. tuberculosis*-spezifischer Kultur durch höhere Konzentrationen von IL-6 und IL-10 aus. Außerdem konnten wir bei Tuberkulosepatienten eine geringere *M. tuberculosis*-spezifische Produktion der Effektorzytokine IL-17F und IL-22 nachweisen.

Weiterhin fanden wir heraus, dass CD4+ T-Zellen von Tuberkulosepatienten im Gegensatz zu gesunden Kontrollspendern durch konstitutive pSTAT3-Aktivierung charakterisiert sind, die – anders als bei Kontrollspendern – nicht auf zusätzliche Stimulation mit IL-6 anspricht. Wir fanden zudem eine erhöhte Expression von SOCS3 in TB-Patienten. Die Expression von SOCS3 war mit einer reduzierten T-Zellaktivierung in TB-Patienten und einer reduzierten IL-2-vermittelten STAT5-Phosphorylierung bei allen Probanden assoziiert. Der inhibitorische Effekt von SOCS3 auf die IL-2-vermittelte Aktivierung von pSTAT5 konnte durch lentivirale Überexpression von SOCS3 in primären CD4+ T-Zellen bestätig werden.

Eine *Principal Component Analysis* konnte eine IL-6-, IL-10- und SOCS3-basierte *Principal Component* identifizieren, die mit hoher pSTAT3-Aktivierung korreliert. Daraus schlussfolgern wir, dass die aberrant gesteigerte Expression von IL-6 und IL-10 sowie die erhöhte Expression von pSTAT3 und SOCS3 zur gestörten T-Zellfunktion bei Tuberkuloseinfektionen beiträgt.

English Summary

Infections with *Mycobacterium tuberculosis (M. tuberculosis)* continue to pose a major challenge to global health. In the immune response to *M. tuberculosis*, T-cells play a crucial role. Their function is intricately regulated, and cytokines centrally contribute to the complex interplay determining T-cell phenotype and effector functions. In *M. tuberculosis* infections *(TB)*, pro- and anti-inflammatory *interleukins (IL) -6* and *-10* have been shown to be aberrantly expressed. Both cytokines activate *signal transducer and activator of transcription (STAT)3* and induce feedback inhibitor *suppressor of cytokine signaling (SOCS)3* in T-cells. SOCS3 is a pleiotropic inhibitor of both cytokine signaling and T-cell activation. In order to find out whether the cytokine milieu affects T-cell activation in tuberculosis patients, we characterized blood samples from TB patients and healthy controls using FACS analysis, ELISA and Cytometric Bead Assay. We investigated spontaneous and *M. tuberculosis*-specific cytokine expression profiles, expression of SOCS3 and cytokine-mediated activation of pSTAT3 and pSTAT5 in T-cells. Furthermore, we compared *M. tuberculosis*-specific T-cell activation assessed by activation markers and effector cytokine production. In addition, we lentivirally transduced CD4+ T-cells to overexpress SOCS3 in order investigate the effect of SOCS3 on T cell functions.

In our studies, blood samples from TB patients were characterized by higher concentrations of IL-6 and IL-10 in both plasma and unstimulated - and *M. tuberculosis*-specific culture compared to healthy contacts. We also demonstrated lower *M. tuberculosis*-specific production of the effector cytokines IL-17F and IL-22 in TB patients. Furthermore, we found constitutively activated pSTAT3 in CD4+ T-cells in tuberculosis patients (but not healthy controls), which could not be further induced by stimulation with IL-6. We also found higher expression of SOCS3 in tuberculosis patients as compared to controls. High expression of SOCS3 was associated with reduced T-cell activation responses in TB patients and reduced IL-2-induced phosphorylation of STAT5 in all donors. Likewise, lentiviral overexpression of SOCS3 in primary CD4+ T-cells confirmed the inhibitory effect on IL-2-mediated phosphorylation of STAT5.

Principal component analysis identified an IL-6, IL-10-, SOCS3 co-expression-based principal component in tuberculosis patients that correlated with high pSTAT3 levels. We conclude that aberrant high levels of IL-6 and IL-10 in combination with elevated levels of pSTAT3 and SOCS3 contribute to the impaired T-cell function in tuberculosis disease.

List of abbreviations

APC	Antigen-presenting cell
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette Guérin
CBA	Cytometric Bead Assay
CD	Cluster of differentiation
CD	Crohn's disease
CFP 10	Culture filter protein 10
CFR	Case fatality ratio
DC	Dendritic cells
DNA	Desoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secreted antigenic target 6
FACS	Fluorescence activated cell sorting
GM-CSF	Granulocyte macrophage colony stimulating factor
gp130	glycoprotein 130
HIV	Human immunodeficiency virus
HTLV	Human T cell lymphotropic virus
IFN-γ	Interferon-γ
IGRA	Interferon- γ Release Assay
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
KIR	Kinase inhibitory region
LAG-3	Lymphocyte activation gene 3
LPS	Lipopolysaccharide
LTBI	Latently *M. tuberculosis*-infected individuals
M. tuberculosis	Mycobacterium tuberculosis
MDT	Multidrug therapy
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NK	Natural killer cells
NO	Nitric oxide
NTM	Non-tuberculous mycobacteria
PBMC	Peripheral blood mononuclear cells
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PPD	Purified protein derivate
pSTAT	phosphorylated signaling transducer and activator of transcription
QFT	QuantiFERON-TB Gold In-Tube
R	Receptor
RA	Rheumatoid arthritis
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SH2	Src homology
SHP	SH2-containing phosphatase
shRNA	small hairpin RNA
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TB	Tuberculosis disease
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper cell
TNFα	Tumor necrosis factor α
Tr1	Type I regulatory T-cells
Treg	Regulatory T cell
TST	Tuberculin skin test
WHO	World Health Organization

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1. Introduction

1.1. Tuberculosis epidemiology and global challenges

Tuberculosis disease *(TB)* caused by Mycobacterium tuberculosis *(M. tuberculosis)* remains one of the major challenges in global health. In 2016, the year of our study, global incidence was estimated by the World Health Organisation *(WHO)* Global Tuberculosis Report 2017 to range around 10.4 million cases per year[1]. Incidence and prevalence vary greatly among different regions and countries, with the majority of cases still being reported in the WHO South-East Asia region and the African region [1, 2]. Of the cases reported in 2016, 10% of diseased individuals were found to be co-infected with human immunodeficiency virus *(HIV)*, with the highest rate of co-infections diagnosed in the WHO African region[1]. Over the past years, the rate of HIV co-infections in incident cases has been declining to 6,7%[2].

In 2016, TB mortality was estimated to be 1.3 million per year among HIV-negative people, while an additional 374 000 deaths were attributed to *M. tuberculosis* infections in HIV-positive individuals[1]. While the number of deaths from TB in HIV-negative people has risen slightly to 1.4 million in 2021, with 187 000 individuals, less people died from TB/HIV-co-infections[2]. Tuberculosis is therefore currently still among the leading causes of death worldwide, although as TB incidence, TB mortality varies greatly among the different regions[1, 2]. It is believed that TB mortality could be significantly lower, as global treatment success rate was 83% in 2016[1] and has risen to 86% in 2021[2]. This is supported by the fact that in 2016, the estimated case fatality ratio *(CFR)*, thus the number of deaths per incident cases, varied from less than 5% in some countries, to more than 20% in most of the WHO African region countries[1]. Thus, facilitating early diagnosis and treatment can be considered the key to reducing the case fatality ratio[1]. From 2000 to 2021, antimycobacterial treatment and antimycobacterial treatment combined with antiretroviral therapy were estimated to have prevented a total of 74 million deaths[1]. Of note, due to COVID-19 pandemic, access to both TB diagnosis and treatment has deteriorated, slowing down or even reversing progress made in the wake of the WHO End TB strategy[2].

Despite ongoing research and innovative diagnostic tools and technologies, to date, timely diagnosis of infection with *M. tuberculosis* remains out of reach for most patients, predominantly from high-burden low-ressource countries[3]. As numerous as diagnostic procedures for detecting TB are their limitations. Microscopy of sputum, bronchial secretion or bronchoalveolar lavage *(BAL)* allows for identification of infectious individuals. However, it does not distinguish between *M. tuberculosis* and non-tuberculous mycobacteria *(NTM)* and sensitivity varies widely between 20-80%[4]. Culture is currently the gold standard for detecting *M. tuberculosis* and identifying drug resistences. Unfortunately, culture generally

takes between one to three weeks but in some cases up to eight to twelve weeks are recommended[4]. Chest X-Ray may be used as a screening method in prevalence studies or else to contribute to clinical diagnosis when bacteriological testing is negative. However, chest X-Ray has poor specificity[5]. The diagnostic screening assays relying on pathogen-specific Interferon *(IFN)*-γ production discussed below are not suitable for use in high-prevalence countries because they do not discriminate between latently infected individuals *(LTBI)* and active cases[6]. Rather, their use is recommended in low-burden countries for screening for LTBI among contacts of tuberculosis patients[4]. Furthermore, sensitivity and specificity are compromised in vulnerable populations. These include children[7], pregnant women[8] as well as HIV-positive patients[8, 9]. Of note, a particularly low sensitivity of Interferon-γ release assays *(IGRA)* for both children[10] and adults[11] has been demonstrated in sub-saharan Ghana, where our study took place. In an effort to close the diagnostic gap, new devices such as the GeneXPert® MTB/RIF assay which offer automated polymerase chain reaction *(PCR)* assays and which detect anti-tuberculosis drug resistance have been rolled out and endorsed by the WHO[3]. However, because of high cost, this procedure is not available to most people in poorly-ressourced communities[3]. Also, to date, international efforts have failed to provide a test that facilitates identification of LTBI at risk for disease progression or relapse after treatment[3, 6]. Ongoing investigations are focussing on a combination of candidate biomarkers[10, 12, 13] and analysis of transcriptional biosignatures [14, 15]. Because first-line and second-line anti-tuberculous therapy require a several month long multidrug therapy *(MDT)* regimen with potentially severe adverse effects[4], a better understanding of the factors leading to disease activation, relapse after treatment or immune reconstitution will help to define therapy initiation and endpoints. This will help save ressources and limit exposure of patients to potentially harmful side-effects of antimycobacterial therapy.

1.2. Pathogenesis of Mycobacterium tuberculosis infections

M. tuberculosis is an intracellular pathogen that is usually transmitted via aerosol exhaled by an actively infected individual and inhaled by individuals in the proximity[16]. The bacteria typically become ingested by professional antigen-presenting cells *(APC)* such as dendritic cells *(DC)* and, predominantly, macrophages. Infected macrophages mount an initial microbicidal response against *M. tuberculosis* using reactive nitrogen intermediates *(RNI)* and reactive oxygen intermediates *(ROI)*[17]. Only a minority of around 10% of infected individuals develops primary progressive infections following exposure to *M. tuberculosis*, while in the vast majority, mycobacteria persist within the macrophages[16, 17]. Consequently, further immune cells are recruited to the site of infection, including T-cells, natural killer cells *(NK)* and neutrophils[17]. In the long-term control of *M. tuberculosis*, adaptive T-cell immunity is thought to be central to preventing disease progression. DC

priming of $CD4^+$ and $CD8^+$ T lymphocytes takes place in regional draining lymph nodes from where activated T-cells migrate to the granulomatous lesions and stimulate antibacterial macrophage activity[16, 17]. In immune-competent individuals, granulomas are formed consisting of macrophages differentiated into epithelioid cells surrounded by a lymphocyte cuff[18]. Inside these granulomas, mycobacteria are contained and isolated from functional organ tissue and mycobacterial growth is restricted[16, 17]. Confined mycobacteria are considered to undergo transcriptional and metabolic changes to go into dormant state characterizing latency[19]. However, latently infected patients remain at risk for reactivation of *M. tuberculosis* infection, so called post-primary tuberculosis, throughout their lives[19]. While it is generally accepted that treatment with immunosuppressive drugs such as tumor necrosis factor *(TNF)*α blockers[20] and glucocorticoids[21] or HIV co-infections[22] predispose latently *M. tuberculosis*-infected individuals for disease progression, a large body of research is currently aiming to elucidate further mechanisms leading to reactivation of *M. tuberculosis* infection.

1.3. Protective T-cell immunity in Tuberculosis

Much attention has been paid to the interaction between T helper cells *(T_h)* type 1 and infected macrophages. CD4+ T_h1 cells sustain macrophage activation and, like macrophages themselves, produce T_h1 signature cytokine IFN-γ, a cytokine that is considered crucial for mycobacterial control within the macrophages [23]. In immune-competent individuals, it has been shown that IFN-γ expressed by activated T-cells stimulates production of antimycobacterial agent nitric oxide by inducing expression of macrophage inducible nitric oxide synthase *(iNOS)*[24]. Further mechanisms contribute to mycobacterial growth restriction, including production of TNFα, a cytokine required for granuloma formation, RNI synthesis[18] as well as IFN-γ-induced phagosome maturation and acidification in the macrophage[25].

In accordance, mice incapable of expressing functional IFN-γ show increased susceptibility to developing systemic *M. tuberculosis* infections, leading to decreased survival[26] and higher bacterial loads [24, 27]. Likewise, in humans, a range of mutations in the genes encoding the IFN-γ receptor or IFN-γ-inducing cytokine interleukin *(IL)*-12 and its receptor IL-12R have been described to lead to increased susceptibility to infections with mycobacteria. Patients with impaired IFN-γ-mediated immunity present a variety of clinical symptoms ranging from lethal disseminated disease to recurrent local infections with low-virulence NTM following vaccination with attenuated Bacille Calmette Guérin *(BCG)* strain [28, 29]. While initially the protective role of IFN-γ was widely ascribed to its ability to elicit an antimicrobial response in macrophages, accumulating evidence shows that IFN-γ also has pivotal immune-modulatory functions, limiting pathology[26] and morbidity[30].

Accordingly, multiple studies in human tuberculosis have shown a reduced antigen-specific IFN-γ response in active TB [31-35] that rises during treatment[31, 32, 34]. However, others have also found similar *M. tuberculosis*-specific production of IFN-γ between TB patients and contacts, with no detectable change during treatment[36, 37]. Another study even reported increased IFN-γ responses in TB when compared to tuberculin skin test *(TST)*-negative, but not TST-positive controls[12].

M. tuberculosis-specific production of IFN-γ is currently the basis for diagnostics in screening efforts for latent tuberculosis. IGRAs, such as the QuantiFERON-TB Gold In-Tube *(QFT)* or the T-SPOT.TB use *M. tuberculosis*-specific antigens such as early secreted antigenic target 6 *(ESAT-6)* and culture filter protein 10 *(CFP-10)* to elicit an *M. tuberculosis*-specific T cell activation[9]. Quantification of either IFN-γ *(QFT)* or number of IFN-γ-producing T-cells *(T-SPOT.TB)* is then used to identify patients that have previously been exposed to *M. tuberculosis*[7, 9]. Likewise, the TST (or Mendel-Mantoux skin test) utilizes T_h1-mediated delayed type I hypersensitity for diagnostic purposes by assessing skin induration after intradermal injection of *M. tuberculosis* antigens purified protein derivate *(PPD)*[38]. Unfortunately, using the protective T_h1 response as a screening marker leaves vulnerable populations at risk for being tested falsely negative[6-9, 39]. Furthermore, as mentioned above, in case of a positive result, no distinction can be made between active TB and LTBI[6]. Consequently, IGRAs also fail at identifying individuals at risk for progression to TB disease[9].

A variety of studies has also focussed on the role of another T helper cell subset, the T_h17 population, in *M. tuberculosis* infection. T_h17 cells mediate pro-inflammatory effects, they are implicated in epithelial and mucosal immunity and in immune responses to both extracellular pathogens as well as fungal infections[40]. They produce inflammatory cytokines IL-17A-F[40] and IL-22[41] as well as granulocyte-macrophage colony stimulating factor *(GM-CSF)* and granulocyte attracting chemokines, thereby inducing granulopoiesis and myeloid tissue infiltration[40, 41]. Unrestrained T_h17 activation leads to hyperinflammation and has been described to play a role in chronic inflammatory and autoimmune diseases[40, 41].

In humans, some have described higher expression of IL-17 in TB patients as compared to healthy BCG-vaccinated controls[35]. Others, on the other hand, postulate a protective role against TB disease activation. Chen and colleagues showed a reduced proportion of T_h17 cells in TB patients as compared to healthy controls. Furthermore, they found T_h17 responses to be even lower in severe TB complicated by tuberculous meningitis[42].

Murine studies, however, point to a more differential role in *M. tuberculosis* infections. Two groups have shown that IFN-γ signaling in BCG- and *M. tuberculosis*-infected mice inhibits production of T_h17 signature cytokine IL-17 [30, 43]. Consequently, high IL-17 production increased pathology and morbidity in IFN-γ knockout mice. Interestingly, although levels of mycobacteria were not significantly different, the inflammatory response in mice deficient in

IFN-γ signaling was higher, and survival was decreased[30]. Likewise, a higher frequency of neutrophil granulocytes was reported at the site of infection when IFN-γ signaling was disrupted by receptor or cytokine deficiency[43]. This complex interplay has prompted research into the benefit of T_h17 immunity in generating vaccination response. In a murine ESAT-6 vaccination model, IL-17 production at primary exposure preceded IFN-γ production, and both were present in recall responses[44]. Others showed that in mucosal antimycobacterial vaccination, knockout of IL-17, but not IFN-γ leads to an increase in lung bacterial burden and reduced macrophage activation [45]. Another study by Gopal and colleagues reports a differential requirement for T_h17 immunity in protection against *M. tuberculosis* infections, with IL-17 being crucial for protection against high-virulence strains, but dispensable in infection with attenuated lab strains[46].

Given the complex role of T_h17 immunity in TB, evaluation of the T_h17 response as a surrogate marker for immune status has been encouraged[47] and in recent years, antigen-specific production of IL-17 has often been used as a surrogate marker for a protective T-cell immune response [48, 49]. Consistently, screening for LTBI is recommended before initiating treatment of autoimmune diseases with inhibitors of IL-17 signaling[50].

1.4. The role of cytokines IL-6 and IL-10 in T cell activation and polarization

Sufficient and on-target effector T-cell responses are dependent on both T-cell receptor activation and T-cell polarization. Early polarizing events determine the differentiation into different T helper subsets such as effector cells T helper type 1, type 2 or type 17 or regulatory T-cells (T_{reg})[51]. Among those polarizing events is the exposure to cytokines such as IL-6, a pro-inflammatory cytokine which has repeatedly been shown to be elevated during active tuberculosis disease[42, 52, 53]. A prospective study by Chowdhury and colleagues followed up on patients and was able to report that IL-6 levels decreased over time during treatment and normalized to, or approached, levels of IL-6 found in healthy subjects. Interestingly, at the moment of diagnosis, they were able to report a positive correlation between plasma levels of IL-6 and radiological severity scoring and bacterial load[53].

Skewing of naïve T-cells toward a T_h17 phenotype in mice is dependent on IL-6 signaling in combination with transforming growth factor β *(TGF-β)* signaling[54, 55]. In humans, there is ongoing controversy regarding the requirement of TGF-β for generation of T_h17 cells [56]. Although IL-6 is commonly regarded as the cytokine tipping the scale from a regulatory to an inflammatory phenotype, IL-21 and IL-23 have also been described to be capable of inducing T_h17 responses in the presence of TGF-β and IL-1b *in vitro*[57]. However, regardless of polarization driving cytokine, it has been demonstrated that T_h17 generation depends on the activation of shared signal transducer and activator of transcription *(STAT)* 3[58, 59]. STAT3-mediated polarization towards a T_h17 lineage can be inhibited by suppressor of cytokine

signaling (SOCS) 3[55, 60], which will be discussed below. In the absence of IL-6, TGF-β signaling alone drives T-cell polarization towards the immunosuppressive T_{reg} T-cell population[54]. Chen et al reported a diminished T_h17 response in TB patients and found low T_h17 frequencies to correlate with decreased levels of the IL-6R, suggesting that in spite of high IL-6 levels in TB, cytokine signaling pathways may be affected[42].

Following primary activation by APCs, naïve T-cells are induced to proliferate and gain antigen specificity[61]. Activation of T-cells involves binding of the T-cell receptor *(TCR)* to its cognate antigen, while cell-to-cell interactions, such as binding of T-cell co-receptor CD28 to its ligands expressed on APCs (e.g. CD80 and CD86), provide important co-stimulatory signals[61, 62]. Activation and proliferation of T-cells to TCR stimulation must be carefully balanced to allow for pathogen clearance while restraining pathogenic immune responses. T-cell proliferation following antigen-specific stimulation is dependant on secretion of IL-2[63], a cytokine that signals through activation of STAT5[64, 65]. On the other hand, potent negative regulation of T-cell activation is mediated by IL-10[66], a cytokine secreted by a variety of immune cells which attenuates inflammatory responses[67]. Chronic exposure to IL-10 during TCR activation confers antigen-specific T-cell anergy, a state of tolerance with decreased activation and proliferation in response to antigeneic stimulation[66]. IL-10 deficiency leads to tissue damage and is implicated in autoimmune diseases[67] and allergy[68] as well as excess pathology during infection clearance[69-71]. Conversely, high expression of IL-10 is a feature of persistant viral infections[72-74] and some viruses even encode viral homologues of IL-10 as a mechanism of immune modulation to attenuate antiviral responses[75].

In murine models of *M. tuberculosis* infections, overexpression of IL-10 was associated with increased bacterial loads, reduced numbers of CD4+ T-cells and decreased antigen specific IFN-γ production in animal lungs[76]. In addition, exogenously administered IL-10 was shown to suppress expression of iNOS and nitrix oxide (NO) in infected macrophages[77]. Conversely, IL-10 deficiency was associated with reduced bacterial loads and faster pathogen elimination rate, along with augmented recruitment of T-cells and macrophages to the granulomas, where their presence resulted in a higher production of iNOS[78].

Consequently, many studies have investigated the role of IL-10 in active *M. tuberculosis* disease in humans, consistently reporting IL-10 levels to be increased compared to LTBI cases[53, 79, 80]. Culture experiments with peripheral blood mononuclear cells *(PBMC)* from TB patients have furthermore demonstrated an increase in proliferation when PPD stimulation was supplemented with anti-IL-10 antibody[31].

1.5. Signaling and regulation of IL-6 and IL-10 by SOCS3

Because IL-10 and IL-6 mediate distinct, even opposing functions, their relationship deserves special attention. IL-6 signal can be delivered either via membrane-bound receptor expressed on a number of cells such as immune cells, neurons and hepatocytes among others, or via soluble IL-6 receptor (*mIL-6R and sIL-6R*, respectively) [81]. Binding of IL-6 to sIL-6R or mIL-6R alone does not lead to activation as the IL-6R lacks an inherent intracellular signaling domain. Instead, upon binding of IL-6, the IL-6/IL-6R receptor complex associates with homodimers of glycoprotein *(gp)*130, a glycoprotein ubiquitously expressed and shared with other cytokines that form the gp130 cytokine family [81]. This association of the IL-6R to gp130 leads to recruitment and activation of Janus kinase (JAK) 1[82]. IL-10, on the other hand, signals via binding to the membrane-bound IL-10R1 subunit that associates with stably expressed IL-10R2 subunit to activate constitutively associated Janus kinases JAK1 and Tyk2, respectively[67].

Curiously, activation of JAK family members by both cytokines leads to phosphorylation and activation of the signaling molecule STAT3, which then homodimerizes and translocates into the nucleus to initiate target gene transcription [82, 83]. Utilization of the same transcription factor raises the obvious question how opposed cellular functions can be mediated in response to cytokine exposure, a conundrum that is still subject to extensive research. It is established, however, that some distinction is achieved by differential subjection to feedback inhibition. Both IL-6 and IL-10 share the ability to induce SOCS3 [82, 83], a potent member of the SOCS family whose transcription is driven by activated phosphorylated STAT *(pSTAT)* 3[84, 85]. However, only IL-6, but not IL-10 signaling is targeted by SOCS3 inhibition[83, 86].

SOCS3 exerts its inhibition of cytokine signaling via different mechanisms. It has been shown that SOCS3 contains a kinase inhibitory region *(KIR)* that, upon binding of SOCS3 to JAK, blocks access of substrates to the JAK binding groove and provides residues that serve as pseudosubstrates for JAK phosphorylation. Beside the KIR, SOCS3 also has a Src homology 2 *(SH2)*-domain which is known to bind to gp130, thereby probably enhancing binding affinity. Additional inhibition is exerted by the C-terminal domain called SOCS box, a structure that induces ubiquitinization of molecules bound to the SH2-domain for proteasomal degradation [87].

1.6. The role of SOCS3 in T-cell-mediated immunity

Although exerting major functions as a negative feedback inhibitor, SOCS3 does not only act on cytokine signaling. An inhibitory role for SOCS3 has been shown for both TCR activation[88] and CD28-mediated co-stimulation of TCR activation[89]. Accordingly,

overexpression of SOCS3 suppresses T-cell proliferation[90] and IL-2 production[91] while knockdown of SOCS3 leads to an increase of TCR-induced T-cell proliferation[92].

Many studies have investigated the role of SOCS3 and STAT3 in different immune cell types in infections with *M. tuberculosis* and many propose a role for these mediators in the course of disease[93]. A mouse study reported significantly increased susceptibility to *M. tuberculosis* infections when SOCS3-deficient T-cells were created, resulting in increased bacterial loads, higher pathology as well as decreased survival[94], suggesting that expression of SOCS3 may play a pivotal role in *M. tuberculosis* infections. In humans, several studies have found elevated levels of SOCS3 mRNA during tuberculosis disease in both sputum[80], BAL fluid[95] and blood[15, 96].

1.7. Ethic vote

The study was approved by the local Committee on Human Research, Publications and Ethics (CHRPE/AP/221/14 and CHRPE/AP/328/15) at the School of Medical Sciences (SMS) at Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi/Ghana.

1.8. Research objectives

CD4+ T-cell functions are crucial in host immune defense in *Mycobacterium tuberculosis* infections[19, 22]. The nature of T-cell immunity greatly depends on T-cell polarization and their capacity to respond to antigeneic stimuli, both of which are centrally regulated by cytokines[51, 61, 66, 97]. Many studies have therefore focused on the characterization of the *M. tuberculosis*-specific T-cell response [12, 32-37, 42, 98], yielding heterogenous results[99]. At the same time, a differential role of various cytokines has been described during active tuberculosis disease compared to healthy controls[42, 52, 53, 79, 80]. We used a case-control study design in order to characterize the TB immune response between infected index cases and healthy contacts in Ghana.

The objectives of our study included:

- To characterize the plasma cytokine milieu and identify possible differences between TB patients and healthy contacts. We therefore determined plasma concentrations of IL-6, IL-10, IL-21, IL-23 and IL-27 in both study groups.
- To compare CD4+ T-cell responses to *M. tuberculosis*-specific antigen stimulation using classical markers of T-cell activation such as IFN-γ, IL-2 and CD40L.
- To characterize the PPD-specific T-cell response for additional cytokine signatures in both TB patients and healthy contacts. To this end, we performed whole blood stimulation and analyzed supernatants for expression of IFN-γ, TNFα, IL-6, IL-10, IL-17F and IL-22.

- To find out whether previously described increased cytokine concentrations are accompanied by concomitant changes in downstream signaling. We therefore performed stimulation of PBMCs from both study groups with IL-6 and IL-2 and analyzed subsequent phosphorylation of STAT3 and STAT5, respectively. In addition, we quantified intracellular expression of SOCS3 protein in total CD4+ T-cells as well as in $CD45RA_{high}$ and $CD45RA_{low}$ subsets.

2. **Constitutive STAT3 phosphorylation and IL-6/IL-10 co-expression are associated with impaired T-cell function in tuberculosis patients, Kirstin Harling, Ernest Adankwah, Alptekin Güler, Anthony Afum-Awuah Adjei, Louis Adu-Amoah, Ertan Mayatepek, Ellis Owusu-Dabo, Norman Nausch, Marc Jacobsen. 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, 275–287 (2019)**

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3. Discussion

3.1. Results summary

The role of CD4+ T-cells during active pulmonary tuberculosis is a matter of ongoing investigation. We performed functional T-cell analysis and found TB patients as well as contacts to be characterized by similar activation to PPD-specific stimulation as assessed by intracellular expression of CD40L, IL-2 and IFN-γ. At the same time, analysis of cytokine production in unstimulated whole blood culture supernatant revealed increased levels of regulatory and pro-inflammatory cytokines IL-10 and IL-6 in TB patients compared to healthy contacts. TB patients furthermore exhibited higher levels of unstimulated IFN-γ production, but no difference was observed for unstimulated expression of TNFα, IL-17F, IL-22 or IL-21. Upon stimulation of whole blood culture with PPD, robust expression of IL-6 and IL-10 were induced, and cytokine levels were still higher in TB patients compared to healthy contacts, whereas PPD-induced levels of IFN-γ where similar between the cohorts. Furthermore, we found reduced PPD-specific levels of IL-17F and IL-22 in TB patients as compared to contacts.

We further looked at phosphorylation of STAT3 and STAT5 in CD4+ T-cells. To our knowledge, this is the first time that constitutive phosphorylation of STAT3 is reported in a TB cohort. Furthermore, we describe diminished inducibility of JAK-STAT signaling pathways, which are centrally involved in both activation and polarization of T-cells. TB patients showed no inducibility of constitutive STAT3 phosphorylation beyond baseline upon stimulation with IL-6 and this distinguished them from healthy controls. Analysis of STAT5 phosphorylation revealed no constitutive activation but here, too, reduced phosphorylation in response to IL-2 stimulation was found in TB patients compared to healthy contacts.

In addition, we corroborate data from previous studies that report elevated expression of negative regulator SOCS3 in T-cells from TB patients[15, 80, 95, 96]. Of note, while differential SOCS3 expression has previously been described on the mRNA level, we provide evidence that increased SOCS3 mRNA expression in TB patients is mirrored on the protein level. Furthermore, SOCS3 expression levels correlated positively with activation levels of constitutive pSTAT3 in all donors, but negatively with IL-2-induced pSTAT5 activation in TB patients only. We confirmed the inhibitory effect of SOCS3 on STAT5 phosphorylation by creating CD4+ T-cells lentivirally transduced to overexpress SOCS3. T-cells overexpressing SOCS3 displayed a significant reduction of IL-2-induced STAT5 phosphorylation compared to vector control.

We also found a significant negative correlation for SOCS3 expression levels with co-expression of T_h1 activation markers CD40L with either IL-2 or IFN-γ in TB patients only,

suggesting that SOCS3 may exert inhibitory effects on T_h1 cell activation in active pulmonary tuberculosis disease.

To unravel patterns underlying the complex immune response in tuberculosis disease, we performed multivariate principal component analysis *(PCA).* PCA identifies factors accounting for variation in multi-dimensional data sets and allows for extraction of correlation patterns. We thus identified a principal component (PC) accounting for 15.7% of data variance which consisted of SOCS3 levels, unstimulated and PPD-specific IL-6/IL-10 expression as well as IL-6/IL-10 plasma levels. The regression factor score for this PC differed significantly between TB patients and healthy contacts. Furthermore, the regression factor score of this PC was significantly positively correlated with constitutive pSTAT3 levels. This highlights the association of differential cytokine expression and expression of SOCS3 with newly described constitutive phosphorylation of STAT3 in human *M. tuberculosis* infections[100].

3.2. Constitutive activation of pSTAT3 - a feature in other pathologies

We determined phosphorylation of STAT3 in unstimulated CD4+ T-cells from TB patients and healthy contacts by flow cytometry. Intriguingly, in TB patients, there was a high level of unstimulated, constitutive pSTAT3 while controls did not display any unstimulated activation. We then stimulated PBMCs with IL-6 and found that healthy contacts responded to the stimulus with a robust phosphorylation of STAT3 that was significantly higher than that seen in unstimulated samples, while at the same time not reaching the level of constitutive STAT3 phosphorylation in TB patients. Noteworthy, phosphorylation of STAT3 could not be further increased by addition of IL-6 in active TB cases. This raises the question of what factors drive constitutive STAT3 activation and how cytokine-stimulated activation of STAT3 is inhibited in TB patients.

In a past study, Jacobsen and colleagues investigated gene expression patterns in tuberculosis patients and analyzed mRNA expression of STAT3. They found no significant difference in STAT3 gene expression levels between TB patients and LTBI controls[15]. This indicates that in TB patients, increased levels of activated pSTAT3 are likely not due to increased availability due to differential gene expression of STAT3.

Constitutive phosphorylation of STAT3 molecules has been described in numerous diseases, and extensive efforts have aimed to elucidate the underlying pathomechanism. Among the conditions for which constitutive pSTAT3 activation has been identified are autoimmune [101, 102] and inflammatory diseases[103] as well as oncological pathologies[104-106].

Constitutive phosphorylation of STAT3 in CD4+ lymphocytes was found in rheumatoid arthritis *(RA)* patients, and this correlated with increased levels of serum IL-6[101, 102, 107]. Both disease modifying antirheumatic drug treatment in general and treatment with IL-6

receptor antagonist Tocilizumab led to normalization of pSTAT3 levels[102]. Of note, unlike in our TB plasma samples, IL-10 and other cytokines known to induce phosphorylation of STAT3 were not elevated[107]. It is further interesting to note that although inducibility was reduced in RA patients that had constitutive pSTAT3 activation, stimulation of IL-6 led to increased activation of pSTAT3 beyond constitutive levels[102, 107]. In contrast to the reduced response to IL-6, stimulation of RA patient T-cells showed regular STAT3 phosphorylation beyond constitutive levels in response to IL-10[107]. We, however, found that constitutively high pSTAT3 in the TB cohort could not be further induced by stimulation with IL-6. As we did not perform IL-10 stimulation assays on TB and healthy contact PBMCs, we can not report whether CD4+ T-cells in TB patients remain sensitive to IL-10. Intriguingly, further parallels in the pathogenesis of TB and RA have been highlighted by gene signature analysis in both pathologies. Anderson and colleagues reported a signature of STAT3 gene targets SOCS3, PIM-1 and Bcl-3 which discriminated RA and non-RA patients[108]. Notably, all three genes had previously been described to be differentially expressed between TB patients and LTBIs[15].

Another inflammatory condition for which constitutive phosphorylation of STAT3 has been reported is Crohn's disease *(CD)*. In a study by Lovato and colleages, constitutively phosphorylated STAT3 was found in CD4+ mucosal and peripheral T-cells in 5 out of 7 patients with Crohn's disease. Cytokine stimulation assays with IL-10 revealed a preserved phosphorylation response to high doses of this cytokine in both patients and healthy volunteers. However, no stimulation with IL-6 was performed. Of note, in a macrophage model engineered to express constitutively active pSTAT3, inducibility was higher in response to IL-10 stimulation when compared to IL-6 stimulation[109]. As mentioned above, we did not perform cytokine stimulation with IL-10 and therefore can not infer whether the preserved inducibility of constitutive pSTAT3 above baseline is a feature differentially regulated in inflammatory and infectious diseases or whether inducibility of pSTAT3 activation depends on the cytokine chosen for stimulation.

In CD patients, co-culture experiments revealed that constitutive STAT3 phosphorylation was not the result of paracrine cytokine effects. In fact, neither was exposition to CD patients' T-cells sufficient to induce constitutive pSTAT3 activation in healthy volunteers, nor did repeated washing or starvation of CD patient T-cells have an abrogative effect on constitutive pSTAT3 activation. This led the authors to conclude that in Crohn's disease, constitutive pSTAT3 is not due to exogenous factors such as cytokines[103]. We, on the other hand, found elevated plasma levels of possible causative cytokines IL-6 and IL-10 in TB patients and PCA analysis confirmed the association with constitutive phosphorylation of STAT3. However, because we cannot exclude that other factors contribute to aberrant pSTAT3 activation, it is therefore worthwhile to look into other modes of inducing constitutive pSTAT3.

Constitutively active pSTAT3 has been described repeatedly in a number of oncological conditions[84, 105, 106] and constitutive pSTAT3 expression has been shown to be sufficient to induce cell transformation[110]. This has lead to the classification of STAT3 as an oncogene[110]. In Sézary syndrom, a cutaneous T-cell lymphoma, and in human myeloma cells, for example, constitutive pSTAT3 activation coincides with activation of JAKs and expression of anti-apoptotic proteins[104, 105]. In myeloproliferative neoplasms, activating JAK2 point mutation V617F is frequently detected[111] and has recently been reported to result in constitutive phosphorylation of predominantly STAT3[106]. Beside activating mutations in JAKs, changes both upstream and downstream have been proposed to account for this pathomechanism [112, 113].

The many possible modes of induction of constitutive pSTAT3 expression are also highly suggestive of possible targets for adjuvants in anti-tuberculous MDT. Although our data points towards a role of elevated cytokine levels in constitutive pSTAT3 activation in TB, these characteristics might be accompanied by concomitant changes of expression and activation of signaling molecules and their inhibitors. A thorough understanding of the underlying factors leading to constitutive pSTAT3 activation is therefore essential and further investigations are required to elucidate the undoubtedly complex mechanism.

We report increased levels of protein expression for STAT3 inhibitor SOCS3 in CD4+ T-cells from TB patients as compared to healthy contacts. Indeed, we found a positive correlation for pSTAT3 activation and expression of SOCS3, indicating that high SOCS3 might be driven by increased phosphorylation of STAT3. Past studies that investigated other pathologies characterized by constitutive phosphorylation of STAT3 also showed a concomitant increase in SOCS3 expression. These include oncological[84] as well as inflammatory conditions[103, 108].

3.3. Potential roles for SOCS3-inducing cytokines IL-6 and IL-10 in constitutive activion of pSTAT3

Several studies have addressed the dynamics of STAT3 activation and have reported that pSTAT3 activation is prolonged when induced by IL-10 as compared to IL-6[83, 86, 114]. SOCS3 expression patterns were found to correlate with IL-6 signal termination[55], but not to have any effect on IL-10 induced pSTAT3 activation[86, 114]. Consistently, down-regulation of SOCS3 by other cytokines[55], selective SOCS3 knock-down[92] or creation of a point mutation in the SOCS3-binding site of gp130[115] have been reported to prolong IL-6 stimulated activation of pSTAT3[55, 92, 115].

Both IL-6 and IL-10 are known to induce transcription and expression of SOCS3[83] and SOCS3 mRNA levels have been reported to be higher in TB patients compared to controls[15, 80, 95]. We therefore analyzed SOCS3 protein expression levels in both naïve ($CD45RA_{high}$) and memory ($CD45RA_{low}$) CD4+ T-cells. We found increased SOCS3 protein

expression in all CD4+ T-cells from TB patients compared to healthy contacts. Interestingly, a higher expression of SOCS3 in samples from TB patients was also present in both $CD45RA_{high}$ and $CD45RA_{low}$ subsets. If we consider that SOCS3 has been described to have profound effects on T-cell activation and proliferation[88, 89, 116, 117], this finding is suggestive of an immunosuppressive effect not just in memory T-cells, but also in non-committed naïve T-cells.

We observed that in TB patient samples with constitutively active pSTAT3, IL-6 stimulation was incapable of inducing a phosphorylation of STAT3 above baseline. This suggests that inhibition of IL-6 signaling by simultaneously elevated SOCS3 remains intact in TB patients, making it interesting to speculate that IL-10 might induce constitutive pSTAT3 activation. But, as we found both high IL-6 and IL-10 levels, we cannot conclude which of these cytokines drives constitutive pSTAT3 activation. In theory, we might consider the possibility that SOCS3-inhibition of IL-6 signaling may be impaired in TB patients. Brender and colleages sequenced SOCS3 cDNA to investigate whether deficiency of feedback inhibition contributed to constitutive activation of pSTAT3 in cutaneous T-cell lymphoma, but found no aberration[116]. To our knowledge, to date, no naturally occurring mutants of SOCS3 have been described that result in insufficient inhibtion of JAK-STAT signaling. Unlike in TB CD4+ T-cells, incubation with IL-6 did cause STAT3 activation above unstimulated levels in healthy contacts, highlighting the fact that differential cytokine receptivity distinguishes TB patients from LTBI controls.

Responsiveness to IL-6 may also be determined by availability of either mIL-6R and sIL-6R[81]. Chen and colleagues have already reported diminished expression of IL-6R on CD4+ T-cells from TB patients compared to LTBI and healthy donors[42]. However, in a process termed trans-signaling, IL-6 may associate with sIL-6R and then bind to ubiquitously expressed gp130, initiating IL-6 signaling even in cells that lack the mIL-6R[81]. We thus considered the possibility that reduced sIL-6R levels may, at least partially, explain diminished responsiveness to IL-6. But, when we determined plasma levels of sIL-6R in TB patients and healthy contacts, we found no significant difference between the cohorts.

Future studies are required to investigate the inducibility of STAT3 phosphorylation in CD4+ T-cells from TB patients. For instance, incubation assays of PBMCs from healthy donors with plasma from TB patients and subsequent analysis of pSTAT3 and SOCS3 expression might help to strengthen our hypothesis that constitutive phosphorylation and reduced inducibility of pSTAT3 is caused by cytokines rather than cell-inherent changes. To further investigate this, it may be worthwhile to perform culture experiments in the presence of neutralizing anti-IL-6- or anti-IL-10-antibodies to elucidate the role of IL-10 and IL-6 in induction of constitutive pSTAT3 and its implications for pSTAT3 gene targets like SOCS3.

3.4. Constitutive pSTAT3 activation may convey immunosuppressive effects

SOCS3 effects alone are not sufficient to explain how IL-6 and IL-10 induce opposed cellular responses albeit predominantly using the same transcription factor. A study by Jones et al reported pSTAT3 signal peak and duration were greatly dependant on the level of IL-6R and IL-10Rα expression characterizing T-cell subsets, and retrogenic expression of IL-10Rα on CD4+ T-cells resulted in mimickry of IL-6 signaling. Interestingly, IL-10 could replace IL-6 under both T_h1 and T_h17 conditions, leading to reduced IFN-γ expression and increased production of T_h17 related cytokines and transcription factors, respectively[118]. In support of the hypothesis that receptor availability determines the cellular response, Braun and colleagues modified IL-10 receptor signaling by adding an anti-IL-10R antibody to IL-10-stimulated DC cultures, thereby terminating IL-10 signal. They found that early abrogation of IL-10 signaling resulted in an IL-6-like transcriptional response. In addition, their microarray analysis revealed that early STAT3 signal, whether triggered by IL-6 or IL-10, leads to similar transcriptional programmes and differential regulation of target genes does not occur until later time points of STAT3 activation[114].

Conversely, Yasukawa and colleagues showed that IL-6 can exert immunosuppressive functions if SOCS3 negative feedback inhibition is abrogated. Using selective knockdown or point mutations of SOCS3 in macrophages, they were able to show that disinhibition of IL-6 signaling was sufficient to imitate the anti-inflammatory effects of IL-10 on Lipopolysaccharide (LPS)-exposed cells and this corresponded with a sustained pSTAT3 activation[119]. Of note, IL-10-driven immunosuppressive effects can not only be mimicked by prolonging IL-6 signal, but also by generating constitutively active pSTAT3. Williams and colleagues created a macrophage mutant containing a constitutively active pSTAT3 termed STAT3C that spontaneously dimerizes and exerts transcriptional functions. They found that in STAT3C-macrophage cultures exposed to LPS, transcription of pro-inflammatory cytokines was equally diminished as in wild-type macrophages supplemented with IL-10[109].

Together, these studies implicate that signal duration - whether mediated by receptor expression, negative feedback inhibitor or mimickry of constitutive signaling - is a relevant factor in determining the effect of activated shared signaling molecule pSTAT3. It is tempting to speculate that constitutive phosphorylation of STAT3 may confer an immunosuppressive dysfunctional T-cell phenotype in active *M. tuberculosis* infections. At the same time, and in the light of non-inducibility of pSTAT3 above baseline by IL-6, the effect of IL-6 may be lost over the constitutive activation of its transcription factor. As IL-6 is a major driver of T_h17 differentiation, this may be reflected by the reduced T_h17 responses we and others[42] have observed. It is therefore conceivable that, despite the presence of high levels of both IL-6

and TGF-β[53], polarization towards a potentially protective T_h17 lineage may be compromised due to the impaired transduction of IL-6 signal.

3.5. *M. tuberculosis*-specific cytokine production in whole blood culture

We aimed to further characterize *M. tuberculosis*-specific T-cell responses and determined cytokine concentrations in unstimulated and PPD-stimulated whole blood culture supernatants. TB patients showed higher levels of IFN-γ in unstimulated supernatants compared to healthy contacts. Interestingly, PPD-specific IFN-γ production (although higher than in the unstimulated supernatant) did not differ between the cohorts. This is in agreement with previous studies conducted in West African cohorts[12, 36]. We further detected low and comparable levels of TNFα in both stimulated and unstimulated samples.

In addition to T_h1 effector cytokines, we analyzed effector cytokines IL-17F and IL-22, which are mainly produced by T_h17 cells, a cell line whose generation is driven by activated pSTAT3[58, 120]. We found no differences between unstimulated samples in the two cohorts. We did, however, find antigen-specific production of both cytokines in active TB cases to be diminished compared to contacts, who mounted a robust response to PPD. Decreased expression of IL-17 and IL-22 in tuberculosis patients has previously been reported[42, 121, 122]. Furthermore, Xu and colleagues reported that levels of IL-17 decreased after sputum smear conversion during antimycobacterial treatment compared to baseline levels[123]. However, they analyzed plasma levels and did not look at antigen-specific IL-17 production capacity which indicates that the decrease in T_h17 cytokines may reflect the reduced exposure to mycobacterial antigens rather than the T-cell effector status. Another study identified the potential of antigen-specific IL-17 (in combination with TNFα and IL-12p40) in long-term culture as a biomarker for distinction between active TB and latent *M. tuberculosis* infection[12]. Likewise, recent studies have reported an increase in antigen-specific expression of IL-22 during TB treatment[124]. Together, our findings and the findings of others highlight the critical role of T_h17 cytokines in the course of an infection with *M. tuberculosis*. Once more, against the background of differential expression of T_h17 cytokines at different stages of infection, a thorough understanding of aberrant activation of pSTAT3 – the T_h17-differentiation-driving signal transducer – appears mandatory.

We further determined the supernatant concentration of proinflammatory cytokine IL-6 and, as with IL-10 levels, found significantly higher levels in the TB cohort compared to the healthy contact group. Upon PPD stimulation, these IL-6 levels were greatly augmented, while the difference between actively infected individuals and healthy contacts was maintained. IL-6 has been consistently shown to be elevated in TB in several studies[53, 79, 125] and this has previously been reported to be accompanied by increased IL-10 levels[53]. Noteworthy, since our study, another study in a Ghanaian cohort has confirmed that *M. tuberculosis*-specific IL-6 production is higher in blood samples from TB patients compared

to contacts[13]. In fact, quantification of IL-6 following ESAT-6/CFP-10-stimulation has even been shown to be superior to IFN-γ quantification when it comes to detection of *M. tuberculosis* infection[13]. Likewise, Vivekanandan and colleagues showed that IL-6 plasma levels, too, have a potent capacity to discriminate between TB patients and contacts[126]. Interestingly, they also found increased IL-6 plasma levels in sputum smear positive patients compared to paucibacillary patients and contacts which indicated that IL-6 production may reflect antigen burden. Furthermore, they found that IL-6 levels declined faster in individuals that responded rapidly to antituberculous MDT compared to slow treatment responders, suggesting that IL-6 may be of use for monitoring treatment success[126]. Together, these recent findings confirm and highlight the clinical relevance of the increased spontaneous and *M. tuberculosis*-specific IL-6 levels that we were able to detect.

3.6. Low T-cell responses in both TB patients and contacts

We further found higher unstimulated expression of IL-10 as well as *M. tuberculosis*-specific production of IL-10 in TB patients as compared to healthy contacts. Although innate immune cells such as macrophages and DCs might contribute to IL-10 production in our whole blood culture, high levels of IL-10 and especially PPD-specific inducibility of IL-10 suggest the presence of an *M. tuberculosis*-specific T-cell line. Different T-cell subsets may account for high IL-10 production, like regulatory T-cells, anergic T-cells or even effector T-cells.

As early as 1999, Gerosa et al identified a CD4+ T cell population co-expressing IFN-γ and IL-10 in BAL fluid. Of note, the frequency of CD4+ cells co-expressing these two cytokines was higher in TB patients as compared to controls[127]. In other infections with intracellular pathogens, such as Leishmania or Toxoplasma gondii, this phenomenon has been further characterized in both mice[71, 128] and humans[129]. In these studies, IL-10 was reported to be co-produced by T_h1 effector cells expressing IFN-γ[128]. These findings prompted the hypothesis that in a strong inflammatory environment of chronic infection, T helper cells are programmed to co-produce IL-10 as part of an autocrine feedback mechanism which may then limit excess inflammation but contribute to pathogen persistence[128, 130]. Elevated IL-6 levels that we report both in plasma and whole blood culture from TB patients likely contribute to such an inflammatory milieu in tuberculosis disease and IL-10 production from T-cells has been described to be stimulated by IL-6[131]. It is therefore tempting to hypothesize that there might be a causative relationship between the two cytokines, with high IL-6 levels sustaining IL-10 production from T-cells. In line with this hypothesis, we found a positive correlation between IL-6 and IL-10 levels in unstimulated whole blood culture.

Another potential source of PPD-specific IL-10 production are inducible-, or Type I regulatory *(Tr1)* T-cells, a CD4+CD25- cell line that emerges following chronic exposure to

IL-10 during antigeneic TCR stimulation[97, 132]. Tr1 cells are distinguished by a low proliferative capacity and low IL-2 expression, but moderate IFN-γ - and high IL-10 production[66]. In co-incubation assays, Tr1 and Tr1-like cells furthermore inhibit the proliferation of responder T helper cells, predominantly by secreting inhibitory cytokines IL-10 and TGF-β[97, 133]. Tr1 cells are potent negative regulators of T cell-mediated immune responses with adoptive transfer of Tr1 cells or Tr1-like cells being sufficient to prevent colitis in mice[132, 134] or to inhibit Th2-mediated airway hyperreactivity[135].

In 2000, Boussiotis and colleagues investigated PPD- and *M. tuberculosis*-specific IFN-γ and IL-10 responses as well as TCR activation and proliferatory responses. They compared cytokine expression and activation between patients that had an intact delayed type I hypersensitivity reaction to TST testing to those that were anergic to TST testing. They found IL-10 in anergic donors to be expressed at high levels both constitutively as well as after stimulation with PPD, while IFN-γ production could not be induced. Contrarily, non-anergic patients showed inducible IL-10 production without unstimulated constitutive expression, accompanied by a robust IFN-γ response following PPD stimulation. Because in anergic patients, treatment with anti-IL-10 neutralizing antibody increased proliferation to PPD and non-specific mitogens, it was concluded that a T-cell population with a Tr1 phenotype must be present in anergic donors.

In line with previous reports of elevated serum levels of IL-10[53, 79, 80], we found higher IL-10 levels in TB patients compared with controls as well as higher PPD-induced IL-10 expression. Plasma- and culture supernatant cytokine analysis does neither enable us to determine whether the IL-10 detected is produced by T-cells co-expressing IFN-γ nor whether T-cells with either an Tr1 or effector T-cell phenotype contribute to its production. Due to limited fluorescence channels in Kumasi/Ghana, initially, on-site analysis of intracellular cytokine expression was limited to CD40L, IFN-γ and IL-2 but PPD-cultured whole blood was frozen for future analysis. Unfortunately, after transfer to Germany, the permeabilized cell samples were not viable for analysis.

T-cell receptor stimulation in the presence of IL-10 may result in T-cell anergy characterized by decreased proliferation and production of effector cytokines[66]. T-cell anergy is a state that is used to achieve tolerance to either self-, or allogenous antigens, thereby providing a powerful checkpoint against autoimmunity and hyperinflammation. Beside TCR activation accompanied by exposure to IL-10, TCR engagement in the absence of co-stimulation may also lead to the induction of anergic T-cells[136]. Functionally, anergy is characterized by a reduced production of effector cytokines such as IFN-γ, TNFα and IL-2 following adequate TCR restimulation[137], a cytokine profile which is also described as a defining feature of exhausted T-cells [138]. In line with the hypothesis of a suppressive immune cell population and dysfunctional T-cells, we report low activation to PPD-specific stimulation in TB patients characterized by low percentages of CD4+ T-cells co-expressing

high levels of CD40L and either proliferative cytokine IL-2 (CD40L/IL-2) or effector cytokine IFN-γ (CD40L/IFN-γ)[139]. Past studies have reported similarly reduced cytokine expression profiles and this was attributed to an exhausted T-cell phenotype[140]. However, because anergic and exhausted T-cells also have overlapping inhibitory receptor expression [137], it may be rash to attribute impaired cytokine expression and activation in TB disease exclusively to either T-cell exhaustion or anergy. Further studies are required to characterize the composition of T-cell subsets contributing to IL-10-production. Beside quantification of unstimulated and PPD-specific intracellular IL-10 and IFN-γ expression, newly identified Tr1 surface markers co-expressed CD49b and lymphocyte activation gene 3 *(LAG-3)* may help to address this question[141]. In order to elucidate the role of exhausted or anergic T-cells, further studies are warranted to first reliably distinguish between these cell types and then ultimately unravel their relative contributions in the pathogenesis of active TB.

Meanwhile, it is worth noting that we found no significant difference in activation response as assessed by CD40L/IFN-γ[139] and CD40L/IL-2 proportions of CD4+ T-cells between TB patients and contacts. Interestingly, a study by Day and colleagues also investigated intracellular IL-2 expression in CD4+ T-cells following whole blood stimulation with PPD. They stratified TB patients into sputum smear positive, and sputum smear negative TB patients to reflect higher and lower mycobacterial load, respectively. They found intracellular IL-2 expression to be reduced in smear positive donors as compared to both smear negative TB patients and LTBI contacts[142]. It is interesting to speculate that subgroup analysis might reveal differences in cytokine expression between our cohorts as well. As we do not have any data on the sputum smear status of our TB donors, we cannot verify this hypothesis. However, a very recent study conducted in a Ghanaian cohort aimed to characterize functional T-cell immunity between TB patients, stratified into smear-positive and paucibacillary subgroups, and contacts. They found levels of both PPD-specific and PHA-stimulated IFN-γ in smear positive, but not paucibacillary patients to be significantly lower compared to contacts[124]. These findings advocate a role for *M. tuberculosis* antigen burden in influencing the magnitude of T-cell responses and offer an explanation for seemingly equally low T-cell responses between TB patients and contacts.

Another possible explanation for low numbers of CD40L/IFN-γ and CD40L/IL-2 CD4+ T-cells in healthy contacts may be that we performed stimulation with PPD, a cocktail of hundreds of potentially antigenic proteins[143]. Several studies have demonstrated that, while T-cells from healthy controls only respond poorly or moderately to PPD stimulation, stimulation with *M. tuberculosis* so-called “latency antigens” results in a higher rate of CD4+ T cell activation[49]. This observation has also been confirmed in another Ghanaian cohort by our group[11]. The perceived hyporesponsiveness in healthy controls may therefore reflect differential antigen exposure at different stages of disease. Indeed, accumulating evidence suggests that evaluation of mitogen-induced cytokine responses may reflect T-cell

immune status more accurately than *M. tuberculosis* antigen-specific stimulation[11, 124, 144]. In 2018, a study by Feng et al. demonstrated that IFN-γ responses to mitogen PHA but not antigen-specific responses at diagnosis are associated with both treatment response and on-treatment mortality[144].

In our study, low antigen-specific T-cell activation is accompanied by aberrantly high IL-6 and IL-10 plasma levels as well as higher levels of unstimulated - and PPD-specific IL-6 and IL-10 in TB patients compared to contacts. Follow-up studies in Ghanaian cohorts from the same region have further investigated the relationship of IL-6 with T-cell activation in TB. Vivekanandan and colleagues reported that plasma IL-6 levels negatively correlated with PHA-induced IFN-γ production in TB patients[124]. Noteworthy, they also demonstrated that levels of PHA-induced IFN-γ rise signifcantly during treatment[124] while plasma levels of IL-6 decline[126]. Another study aiming to elucidate the factors leading to reduced QFT sensitivity in TB patients and contacts in Ghana performed PHA stimulation in both whole blood and PBMC culture. They found a reduced PHA-induced IFN-γ response in whole blood culture compared to PBMC culture, concluding that soluble factors such as cytokines might impair T-cell function[11]. Together, these findings strengthen our hypothesis that T-cell immunity in TB patients is critically regulated by aberrant cytokine expression.

3.7. A role of SOCS3 in T cell activation in tuberculosis patients

Meanwhile, we provide evidence that expression of SOCS3 is affected during active TB. This is in line with previous studies[15, 80, 95, 96]. Interestingly, although these studies consistently show increased levels of SOCS3 mRNA in TB as compared to LTBI, Jacobsen and colleagues showed that SOCS3 mRNA levels in non-infected healthy donors are higher than that seen in LTBI and not significantly different from those detected in TB samples[15]. These findings make it interesting to speculate that downregulation of SOCS3 during infection with *M. tuberculosis* may enhance the protective T-cell response, thereby contributing to maintaining infection latent.

Higher expression levels of SOCS3, although possibly a consequence of consitutive pSTAT3 activation sustained by increased IL-6 or IL-10 levels, may lead to interaction and inhibition with other signaling pathways targeted by SOCS molecules beside the STAT pathway. Because SOCS3 has been reported to inhibit antigen-specific TCR activation[88, 89, 91], we correlated SOCS3 expression levels in CD4+, CD4+CD45RA$_{high}$ and CD4+CD45RA$_{low}$ cells with PPD-specific T-cell activation. Curiously, we found expression of SOCS3 in all subsets to be correlated negatively with T-cell activation and cytokine production only in the TB-, but not the contact cohort. This indicates that SOCS3 may contribute to impaired T-cell activation in active tuberculosis.

Several mechanisms may account for or contribute to an immune-suppressive effect of SOCS3 in TB patients. Firstly, targets for SOCS3 have been identified among the complex

signaling pathway of the T-cell receptor in several in vitro studies. Secondly, increased production of SOCS3 is likely to have profound effects on cytokine signaling.

As for TCR activation, Yu and colleagues have shown that upon T-cell receptor stimulation, SOCS3 is transiently downregulated, and this downregulation has been proposed to facilitate activation[88]. Therefore, it is possible that increased expression of SOCS3 interferes with T-cell activation. Vice versa, and in line with the above-mentioned findings by Jacobsen et al, lower levels of SOCS3 in healthy contacts might reflect a protective downregulation required for adequate T-cell immunity. In our PCA analysis, SOCS3 levels were found to contribute to a Principal Component that included high levels of IL-6 and IL-10 in plasma, unstimulated-, and PPD-stimulated whole blood culture and this PC correlated positively with pSTAT3 activation. It is therefore tempting to speculate that in active TB, due to high expression of IL-6 and IL-10 and consecutive high activation of pSTAT3, a protective downregulation of SOCS3 required for full T-cell activation is inhibited.

To add to the complexity of the relationship between SOCS3, IL-2 and TCR activation, an *in vitro* study showed that that overexpression of SOCS3 suppresses the IL-2 production usually induced by TCR activation via interaction with TCR downstream signaling molecules [91]. Matsumoto and colleagues reported that SOCS3-inhibited TCR-induced IL-2 production by directly targeting co-stimulatory molecule CD28.[89]. If we recall that TCR stimulation in the absence of co-stimulation may lead to T-cell anergy [136], the ability of SOCS3 to interfere with co-stimulation pathways is particularly intriguing. Although the exact mechanisms by which SOCS3 affects T cell activation in chronic infectious diseases remains unclear, our data suggest that SOCS3 may affect T-cell activation in TB patients *in vivo*.

After T-cell activation, proliferation of antigen-specific T-cells is essential for an effective immune response. Kleinsteuber et al have shown that ectopic expression of SOCS3 leads to reduced proliferation of CD4+ T-cells *in vitro*[90]. T-cell proliferation, in turn, has been shown to be facilitated via production of IL-2[63] and IL-2 signaling via phosphorylation of STAT5 has been identified as a target of SOCS3[117]. We therefore measured activation of pSTAT5 following IL-2 stimulation in TB patients and healthy contacts and correlated IL-2-induced phosphorylation of STAT5 with levels of SOCS3 expression. A moderate negative correlation indicated that in TB patients, IL-2 signaling may be affected by increased inhibition of SOCS3. In additional experiments, we performed lentiviral transduction of CD4+ T-cells to generate SOCS3-overexpressing cells. IL-2 stimulation in these cells confirmed lower activation of pSTAT5, whereas inhibition with SOCS3 shRNA had negligible effects on STAT5 phosphorylation levels. This strengthened our hypothesis that in TB patients, IL-2 signaling may be modulated by SOCS3.

The implications of the suppressive effect of SOCS3 on IL-2 signaling appear even more intriguing when we look at recent work on the role of IL-2 in exhausted or anergic T-cells. Past studies had already suggested that IL-2 has the potential to reverse T-cell anergy[136].

Recently, Liu and colleagues were able to show that persistent stimulation of mice with *M. tuberculosis* antigens leads to T-cell dysfunction when compared to transient antigeneic stimulation. Curiously, IL-2 treatment of mice exposed to persistent antigen stimulation was able to reverse the dysfunctional immune response that these animals displayed upon challenge with antigen or BCG infection[145]. The potential of IL-2 to boost the immune response has also prompted research into the therapeutic effects of recombinant IL-2. A meta-analysis by Zhang et al reviewed four studies in which anti-tuberculous MDT was supplemented with recombinant human IL-2. They concluded that adjuvant recombinant IL-2 treatment improves sputum smear and culture conversion rate[146]. The potential of IL-2 to restore a robust immune response in previously dysfunctional T-cells as well as its potential to contribute to treatment success highlights the importance of an in-depth understanding of mechanisms that interfere with IL-2 signaling. We thus added evidence that in *acute M. tuberculosis* infections, high levels of SOCS3 – likely as consequence of an aberrant cytokine milieu - may play a role in compromising T-cell effector functions by affecting IL-2/pSTAT5-mediated T-cell proliferation.

4. Outlook

In the present study, we describe for the first time that constitutive phosphorylation of STAT3 and its reduced inducibility in response to cytokine stimulation in CD4+ T-cells is a feature that characterizes acute TB infections and distinguishes them from healthy contacts. We furthermore confirmed previously described concomitantly increased levels of IL-6 and IL-10 in both serum and unstimulated-, as well as PPD-specific culture which is likely to account for high expression of SOCS3 in TB patients. Because PPD-specific activation and cytokine production of CD4+ T-cells were low and negatively correlated with expression of SOCS3 levels in TB patients, but not healthy controls, we explored the potential of SOCS3 to interfere with IL-2-mediated T-cell proliferation. As IL-2-induced phosphorylation levels of STAT5 correlated negatively with SOCS3 levels in TB patients, we used lentivirally transduced T-cells overexpressing SOCS3 to confirm the potential of SOCS3 to inhibit STAT5-mediated IL-2 signaling. Multivariate principal component analysis strenghtened our hypothesis that IL-6- and IL-10 levels, constitutive phosphorylation of STAT3 and high levels of SOCS3 in T-cells contribute to the dysfunctional immune response in *M. tuberculosis* infections.

Future studies may address multiple questions arising from our data. First, it would be interesting to confirm that constitutively active pSTAT3 is indeed caused by soluble extracellular molecules, i.e. increased levels of IL-6/IL-10. Co-culture experiments might further investigate whether exposure to such an altered cytokine milieu is sufficient for generating the dysfunctional T-cell immunity we and others[11, 13, 124, 144] have observed. If confirmed, specifically targeting either the cytokine or a mediator of downstream signaling may present an attractive adjuvant in MDT. In addition, further research is required to identify whether a regulatory, anergic -, exhausted -, or even effector T-cell phenotype prevails in the secretion of pSTAT3-inducing cytokine IL-10. Identification of a potentially dominant T-cell population would offer additional immune therapy targets that could supplement MDT. And, because IL-17 levels and antigen-specific IL-17 production have been described as a characteristic feature in TB patients, further experiments should aim to elucidate whether constitutive activation of T_h17-polarization-driving signal transducer pSTAT3 interferes with a potentially protective arm of the complex immune response to infection with *M. tuberculosis*.

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