Immune cells and soluble immune markers in different stages of tuberculosis

Potential biomarkers for diagnosis and treatment efficacy

Ida Wergeland

Thesis for the degree of philosophiae doctor (PhD)
at the University of Bergen

2017

Date of defence: March 30th

Year: 2017

Title: Immune cells and soluble immune markers in different stages of tuberculosis

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Author: Ida Wergeland

Print: AiT Bjerch AS / University of Bergen

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Scientific environment

The present work was conducted at the Department of Clinical Science, Faculty of Medicine and Dentistry, University of Bergen (UoB).

The first paper of this thesis and the education part of the PhD program were carried out as a part of the Medical student research program at the Faculty of Medicine and Dentistry, UoB, in the period from 2004-2010. After graduation and completion of the internship period, the work was continued from 2012-2017, then as a full-time PhD-student. The studies constituting the second and fourth paper were conducted in collaboration with researchers at the Arctic University of Norway, Oslo University hospital and the University of Oslo.

Acknowledgements

I would like to thank my main supervisor, Anne Ma Dyrhol-Riise. With her knowledge and enthusiasm she inspired me to start working with TB research in 2004 when I was a medical student, and encouraged me to continue the work as a PhD student eight years later. Thank you for invaluable supervision throughout these years.

My co-supervisor Tehmina Mustafa, your genuine interest in the challenges of TB will always inspire me, and your contagious enthusiasm encourages me to continuing TB research. Thank you for your helpfulness and supervision whenever needed.

I wish to express my gratitude to all my co-authors, especially to Kristian Tonby for the collaboration on the last paper of this thesis and to Jörg Assmuss, Statistician at Center for clinical research, HUH, for your advices and patience when teaching me statistical methods.

I would like to thank Steinar Sørnes at the Research laboratory, University of Bergen, for your advice and help. Your technical expertise in the lab has been invaluable.

To my fellow PhD students at the office: Thanks for our innumerable everyday discussions!

To my friends: Thanks for all enjoyable distraction throughout the years!

I would also like to thank my parents, Heidrun and Einar, for your interest in my work and your care for all of us. To my parents-in-law, Wenche and Fred, thanks for all your help and care. To my brother Stig and Gro Janne, you have both showed me that a PhD project includes considerable frustration, but that hard work and patience will pay off.

Last but not least, I wish to thank my dearest husband Tony and my children Vemund and Sondre, you are my life!

List of abbreviations

ALCAM	Activated leukocyte cell adhesion molecule
ART	Antiretroviral therapy
BCG	Bacillus Calmette-Guérin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFP-10	Culture filtrate protein 10
COX	Cyclooxygenase
CRP	C-reactive protein
CT	Computed tomography
DC	Dendritic cell
DOT	Directly observed treatment
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
ESAT-6	Early secretory antigenic target 6
ESX-1	Early secreted antigen 6 secretion system 1
FasL	Fas Ligand
FMO	Fluorescence-minus-one
FOXP3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony stimulating factor

HDAC	Histone deacetylase
HDT	Host directed therapy
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen-D related
HUH	Haukeland University Hospital
ICS	Intracellular cytokine staining
IGRA	Interferon gamma release assay
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IFN-γR	Interferon gamma receptor
IL	Interleukin
IP-10	Interferon gamma inducible protein 10
IL-1ra	Interleukin-1 receptor antagonist
LAM	Lipoarabinomannan
LDL	Lower detection limit
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MCP	Macrophage chemoattractant protein
mDCs	Myeloid dendritic cells
MDR	Multidrug resistant

MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon gamma
MIP	Macrophage inflammatory protein
Mtb	*Mycobacterium tuberculosis*
mTOR	Mammalian target of rapamycin
NTM	Non-tuberculous mycobacteria
OPG	Osteoprotegerin
OUS	Oslo University Hospital
pDCs	Plasmacytoid dendritic cells
PBMCs	Peripheral blood mononuclear cells
PDE4	Phosphodiesterase isozyme 4
PDGF-BB	Platelet-derived growth factor –BB
PET	Positron emission tomography
PGE2	Prostaglandin E2
PPARγ	Peroxisome proliferator- activated receptor-gamma
PPR	Pattern recognition receptor
PPD	Purified protein derivative
PTX3	Pentraxin 3
QFT	QuantiFERON-TB Gold

RANTES	Regulated on activation, normal T cell expressed and secreted
REK	Regional Committees for Ethics in Medical Research
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
sFRP3	Secreted frizzled-related protein 3
SCC	Sputum culture conversion
TARC	Thymus and activation regulated chemokine
sTNFr	Soluble tumour necrosis factor receptor
TB	Tuberculosis
TBAg-Nil	Background corrected tuberculosis antigen stimulated
TCR	T cell receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cells
TST	Tuberculin skin test
UDL	Upper detection limit
UoB	University of Bergen
WHO	World Health Organization
XDR	Extensively drug resistant

Abstract

Tuberculosis (TB) is a major global health problem, especially in the developing world. In order to end the TB epidemic, reliable and rapid diagnostic tools that can identify and discriminate between latent and active TB are required. In addition, the emergence of multi- and extensively drug resistant strains of *Mycobacterium tuberculosis* (*Mtb*) highlights the need of improved treatment regimens and tools for monitoring the effect of treatment. It has been suggested that adjunct treatment that targets the host response to infection has the potential to facilitate eradication of *Mtb*, reduce tissue inflammation and shorten the treatment duration.

The main aim of this thesis was to characterise immune cells and soluble immune markers in different stages of TB infection with focus on identifying potential biomarkers that may improve TB diagnostics and monitoring of treatment efficacy. The secondary aim was to explore the *in vitro* effects of the potential adjunct treatment option cyclooxygenase (COX)-inhibition on *Mtb* specific T cell responses.

Peripheral blood mononuclear cells, plasma and supernatants from the QuantiFERON-TB Gold (QFT) test were obtained from individuals with active and latent TB before and during TB treatment and from QFT-negative controls. T cell subsets were studied by flow cytometry and potential immune modulating effects of the COX-inhibitor indomethacin on *Mtb* specific T cell responses were examined *in vitro*. Multiple soluble markers were measured in plasma and QFT supernatants by multiplex and enzyme immunoassays.

In paper I, we found that the level of regulatory T cells (Treg) was higher in both the active and latent TB group compared with controls. The results of paper IV indicate that the COX-inhibitor indomethacin may be used to modulate the immune response in active TB by reducing the number of *Mtb* specific Treg.

In paper II, we report that the plasma level of interferon gamma inducible protein 10 (IP-10), although not specific for TB, may differentiate between active and latent TB

irrespective of human immunodeficiency virus (HIV) infection and may also be used to monitor the effect of treatment.

In paper III, we did not find any marker with potential to differentiate between active and latent TB infection when *Mtb* specific marker levels were analysed in QFT supernatants. However, *Mtb* specific interleukin (IL)-1ra, IL-2 and IP-10 levels distinguished individuals with borderline QFT test results from QFT negative controls and these markers may improve the differentiation between latent TB and non-TB infected individuals.

In conclusion, the results support that Treg may be a target for adjunct host directed therapy in TB, and that *Mtb* specific Treg can be reduced by COX-inhibitors which are well known drugs approved for other clinical conditions. Potential biomarkers for TB diagnosis and treatment efficacy have been identified. However, further studies are needed to examine whether it is possible to establish sufficient sensitive and specific test cut-offs for use in clinical practice.

List of publications

I. Wergeland I, Assmuss J, Dyrhol-Riise AM. T regulatory cells and immune activation in *Mycobacterium tuberculosis* infection and the effect of preventive therapy. Scand J Immunol. 2011 Mar;73(3):234-42.

II. Wergeland I, Pullar N, Assmuss J, Ueland T, Tonby K, Feruglio S, Kvale D, Damås JK, Aukrust P, Mollnes TE, Dyrhol-Riise AM. IP-10 differentiates between active and latent tuberculosis irrespective of HIV status and declines during therapy. J Infect. 2015 Apr;70(4):381-91.

III. Wergeland I, Assmuss J, Dyrhol-Riise AM. Cytokine patterns in tuberculosis infection; IL-1ra, IL-2 and IP-10 differentiate borderline QuantiFERON-TB samples from uninfected controls. PLoS One. 2016 Sep 29;11(9):e0163848.

IV. Tonby K, Wergeland I, Lieske NV, Kvale D, Tasken K, Dyrhol-Riise AM. The COX-inhibitor indomethacin reduces Th1 effector and T regulatory cells *in vitro* in *Mycobacterium tuberculosis* infection. BMC Infect Dis. 2016 Oct 24;16(1):599.

1. Introduction

Tuberculosis (TB) is an infection caused by *Mycobacterium tuberculosis* (*Mtb)* and is together with human immunodeficiency virus (HIV) and malaria among the leading causes of death from infectious diseases worldwide [1]. There is currently no optimal vaccine available. Strategies for TB control aim to reduce transmission by identification and treatment of infectious cases as well as offering preventive therapy to individuals with latent TB. An "End TB strategy" including intensified research and innovation has been developed by the World Health Organization (WHO) and aims to reduce the TB incidence rate by 90% within 2035 [2].

1.1 The tuberculosis epidemic

The WHO estimates that one third of the world's population has latent TB and in 2015 there were an estimated 10.4 million new cases of active TB and 1.8 million TB deaths [1]. 1.2 million of the cases and 0.4 million of the deaths were in HIV-positive individuals. There are great geographical variations both in the incidence of TB (figure 1) and in the prevalence of HIV in new TB cases. The majority of the cases, 61%, occurred in the South-East Asia and Western Pacific regions. The African region with 26% of the cases had the most severe burden relative to population and in addition had the highest proportion of HIV-positive TB cases.

The TB incidence has fallen by an average of 1.5% per year since 2000 and the WHO's Millennium development goal of halting and reversing the TB epidemic by 2015 has been met in all six WHO regions. However, the decline needs to accelerate to reach the milestone of the end TB strategy and the emergence of multi- and extensively drug resistant (MDR and XDR) strains of *Mtb* has aggravated the TB epidemic. In 2015, an estimated 3.9% of new TB cases had MDR-TB, and 9.5% of these were XDR-TB. India, China and the Russian Federation accounted for 45% of

the MDR-TB cases, and the proportion of new TB cases with MDR-TB varies from 0-6 % in most countries to >20% in areas of the former Soviet Union.

In Norway, TB was a high-endemic disease at the end of the 18th century. As a result of improvements of living conditions and the availability of anti-tuberculous drugs from the 1940s, the incidence declined and was at the lowest level in 1996 with 201 reported cases [3]. The last decade, the incidence of active TB cases has been 300-400 per year [3]. TB cases among Norwegian-born individuals are now rare and variations in the incidence are associated with the number of immigrants from high-endemic countries. A nationwide study of MDR-TB in Norway from 1995-2014 found that MDR-TB is rare in Norway and is predominantly seen in immigrants from the Horn of Africa and countries of the former Soviet Union [4].

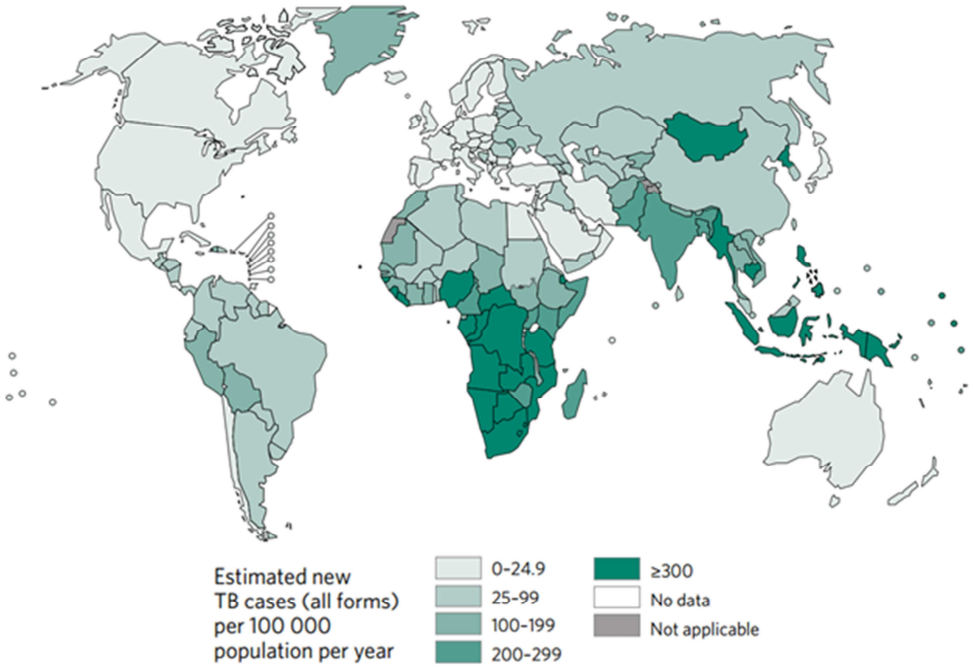


Figure 1. Estimated TB incidence rates, 2015. Adapted from WHO global tuberculosis report 2016 [1].

1.2 The clinical course of tuberculosis

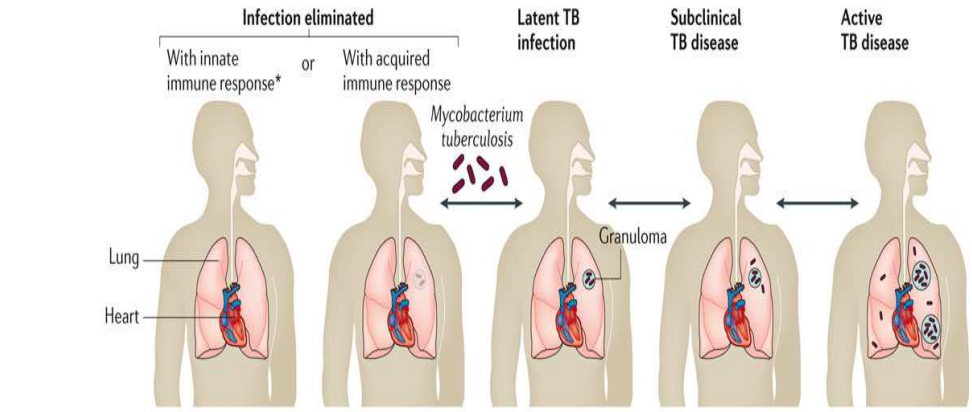
TB infection is acquired by inhalation of aerosol droplets expelled from individuals with active pulmonary TB. Primary TB infection is usually asymptomatic, and in most individuals the immune response is sufficient to constrain the infection. However, the primary infection foci may harbour viable bacteria in a latent state for years until reactivation and progression to active disease.

In exposed individuals, *Mtb* infection can be detected by development of an antigen-specific T cell response. Latent TB is defined by WHO as "a state of persistent immune response to stimulation by *Mtb* antigens without clinically manifested active TB" [5]. The lifetime risk of developing active TB from latent TB is estimated to 5-15% [1], and most commonly, reactivation occurs within a few years after the initial infection [6]. Predisposing factors like HIV coinfection, anti-tumour necrosis factor (TNF) treatment, diabetes, malnutrition and other immunosuppressive conditions increase the risk of reactivation considerably [7]. It is estimated that the risk of developing active TB is 26 times greater in HIV-positive individuals compared to HIV-negative individuals, and TB is the leading cause of death among HIV-positive individuals [8].

Pulmonary TB is the most common manifestation of active disease. Typically there is a gradual onset of symptoms including fever, cough, malaise, anorexia and weight loss. Extrapulmonary TB constituted about 15% of the 6.1 million TB cases notified in 2015 [1], and is more frequent in HIV co-infected individuals [9]. Organs typically involved are the lymph nodes and pleura, but any organ system can be affected. Miliary TB is a condition with widespread dissemination of *Mtb* giving millet-like lesions in the involved organs.

Traditionally, TB infection has been categorised either as latent or active, where latent TB constitutes an asymptomatic condition with containment of inactive bacteria and active TB constitutes active replication of bacteria giving clinical disease. However, neither of the diagnostic tests available for latent TB infection provides any direct evidence of the presence of viable bacteria and while progressing

from latent to active disease, patients often undergo asymptomatic stages with only radiological or bacteriological manifestations indicating active disease [10,11]. It is suggested that the responses to TB infection is better understood as a continuous spectrum with sterilizing immunity and fulminant active disease at the extremes (figure 2) [6,8]. In this model, the old concept of latent TB includes individuals with sterilizing immunity as well as individuals with active replicating bacteria at a subclinical level. The model is supported by results from imaging studies using positron emission tomography (PET) and computed tomography (CT) showing that individuals with latent TB display a range of findings which in part correspond to observations in patients with active TB [6].



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

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Figure 2. The spectrum of TB-from *Mtb* infection to active (pulmonary) disease. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviwes Disease primers, Pai M.[8]. Copyright 2016.

1.3 Immune responses against *Mycobacterium tuberculosis*

Complex interactions between *Mtb* and the host including both innate and adaptive immune responses contribute to the defence against *Mtb*. In active TB, the immune response limits mycobacterial replication and dissemination, but also causes formation of granulomas, tissue necrosis and cavitation. The balance between the protective and pathological effects of the immune response determines the outcome of the infection.

1.3.1 Innate immune responses

The innate immune response is the first line of defence against *Mtb* and is important in promoting the generation of adaptive immune responses. However, innate immune cells are also manipulated by *Mtb* to serve as niches for bacterial replication and to delay priming of adaptive immune cells [12].

After inhalation, *Mtb* reaches the alveolar space where it interacts with dendritic cells (DCs) [13,14], macrophages and pulmonary epithelial cells [15,16]. The macrophage is the primary host cell of *Mtb* which enters through receptor-mediated phagocytosis. Complement and mannose receptors on the surface of the macrophage are the main receptor groups involved in phagocytosis [17]. The macrophages also express several other pattern-recognition receptors, including Toll-like and C-type lectin receptors, that recognise *Mtb* components and induces the expression of cytokines and chemokines that are essential for eliciting the adaptive immune response [18]. To be able to persist and survive within the macrophages, *Mtb* has evolved multiple strategies to evade their antimicrobial mechanisms [19].

DCs have an important role in initiating the adaptive immune response by priming of naïve lymphocytes [20], and are also involved in the induction and expansion of regulatory T cells (Treg) [21]. Migration of DCs to the regional lymph nodes is critical in the immune response against *Mtb* [22], and infection of DCs by *Mtb* leads to upregulation of the antigen presenting molecules major histocompatibility complex

(MHC)-1 and MHC-2 in addition to co-stimulatory molecules [23,24]. However, it has also been reported that *Mtb* impairs the function of DCs by decreasing the ability of infected DCs to activate T cells and delaying their migration to the lymph nodes [22,25]. Blood DCs are categorised into myeloid (mDCs) and plasmacytoid DCs (pDCs) based on differences in phenotype and function [20]. mDCs secrete IL-12 and induce Th1 immune responses whereas pDCs secrete interferon alpha (IFN-α) and induce Th2 responses [26]. It has been found that the absolute numbers of both DC subsets are decreased in patients with active TB compared to controls and that the numbers are restored following successful anti-tuberculous therapy [27].

Monocytes are found in the circulation and during infection and inflammation they traffic to inflamed tissues were they differentiate and supply the tissues with macrophages and dendritic cells [28,29]. It has been demonstrated that infected monocyte derived DCs are essential for the transport of *Mtb* to the local lymph node [30], and humans with a certain genotype of the macrophage chemoattractant protein (MCP)-1 promoter gene, which are important in monocyte trafficking, have increased susceptibility to TB infection [31]. Increased susceptibility has also been associated with the ratio of monocytes to lymphocytes in blood [32]. Monocytes can be divided into subsets based on cell surface markers and it has been demonstrated that cluster of differentiation (CD)16+ monocytes are expanded in patients with active TB and correlates with disease severity [33]. As CD16+ monocytes are refractory to DC differentiation [34], less resistant to *Mtb* intracellular growth and less prone to migrate than CD16- monocytes, the expansion in CD16+ monocytes may promote microbial resilience [35]. However, the relative importance of monocyte subsets in human TB remains to be defined [25].

1.3.2 Adaptive immune responses

Since *Mtb* is an intracellular pathogen the adaptive immune response is predominantly dependent on cell-mediated immunity. In humans, the adaptive immune response to *Mtb* is detectable approximately 3-8 weeks after exposure [36].

CD4+ T cells

The CD4+ T cell subset is essential for protection against *Mtb* which is clearly demonstrated by the increased risk of development of active TB in HIV-positive individuals [37,38]. The increased risk is observable as early as HIV seroconversion and further increases as CD4+ T cell levels decrease, while immune reconstitution by antiretroviral therapy (ART) reduces the risk [39].

The CD4+ T cell response is mainly a Th1 response characterised by expression of the cytokines interferon gamma (IFN-γ) and interleukin (IL)-2. IFN-γ plays a central role in activating macrophages and their antimicrobial functions, and humans with defects in the IFN-γ and IFN-γ-receptor genes are highly susceptible to *Mtb* infection [40]. IL-2 promotes the expansion and differentiation of T cells into effector and memory cells [41]. In addition, *Mtb* induces T cell expression of TNF-α which act synergistically together with IFN-γ to stimulate production of nitric oxide and other reactive nitrogen intermediates by macrophages [42].

Regulatory T cells

Treg are a subset of T cells that may impair immune responses necessary for adequate control of infection, but also limit excessive inflammation causing tissue damage [43]. The mechanisms of Treg mediated immune suppression is incompletely understood, but includes secretion of inhibitory cytokines and cell contact dependent mechanisms [44]. Patients with active TB have reduced purified protein derivative (PPD) stimulated production of IFN-γ compared with tuberculin skin test (TST) positive healthy individuals [45], and it has been suggested that Treg contribute to the suppression of the Th1 immune responses [46]. Increased numbers of Treg have been observed in active TB infection, both at the sites of infection and in blood [46–51], and there seems to be a correlation between the severity of the infection and the number of Treg [51–53]. Several studies have reported a decline in the frequency of

Treg during TB treatment [54–56], whereas others have found sustained [47] or initially increased levels [57].

Studies of Treg in mouse models of TB have shown that *Mtb* specific Treg expand early during infection, delay the onset of adaptive immunity and are eliminated after the initial phase of infection [58,59]. Although Treg are considered to have a negative effect during the initial stage of infection, it is not known to which degree Treg are detrimental versus beneficial during active TB disease [60]. Treg cell depletion studies in mice has shown that efficient depletion of Forkhead box P3 (FOXP3)+ Treg cells decrease the bacterial burden in *Mtb*-infected lungs, but also lead to robust autoimmune activation [61]. Studies using other strategies for Treg depletion based on the marker CD25 have shown contradictory results. Quinn *et al* reported no impact on bacterial load when Treg numbers were reduced using anti-CD25 antibodies [62], whereas Kursar *et al* found a lower bacterial load when Treg levels was reduced in a model using adoptive transfer of CD25+ T cells into T cell deficient mice [63]. The contradictory results may be due to the limitations of CD25 as a Treg marker (Treg markers are discussed in section 4.2.4).

A review have discussed the potential of Treg as a target for immunotherapeutic strategies to improve T cell responses and vaccine efficacy [64]. It has been shown that depletion of CD4+ Treg significantly improves the protective capacity of the Bacillus Calmette-Guérin (BCG) vaccine [65] and it is suggested that vaccine designing methodologies must consider suppression of different Treg for enhancing vaccine efficacy as well as search for *Mtb* antigen epitopes that will selectively suppress Treg. Immunotherapeutic strategies targeting Treg may also improve favourable host responses during *Mtb* infection. However, extreme caution is emphasized when considering Treg manipulation because of their dual role in the immune response [64].

CD8+ T cells

A requirement for CD8+ T cells in the immune response against *Mtb* has not been proven in humans, but animal model data support a non-redundant and complex role for CD8+ T cells [66]. In humans there is no equivalent condition with loss of CD8+ T cells as the loss of CD4+ T cells in HIV-positive individuals, and information on the contribution of CD8+ T cells in TB infection is therefore mainly based on mouse models. Studies of knockout mice have shown that classically restricted CD8+ T cells are necessary for control of infection [67,68], and depletion of CD8+ T cells in the chronic stages of infection in mice leads to a substantial increase in bacterial burden [69]. *Mtb* specific CD8+ T cells have been detected in humans with active TB and in healthy contacts [70]. These cells are able to secrete IFN-γ in response to stimulation with *Mtb*-infected targets and have cytolytic activity [71,72]. Rozot *et al* found that *Mtb* specific CD8+ T cells responses were detected in 60% of patients with active TB compared with only 15% of subjects with latent TB infection [73] and a combination of both CD4+ and CD8+ T cell responses may improve the diagnostic tools of active TB [74].

B cells and humoral immunity

It is generally thought that B cells and humoral immunity play an important role in host defence against extracellular pathogens, whereas control of intracellular pathogens relies on cell-mediated immunity [75]. Since *Mtb* is an intracellular pathogen, the role of B cells and humoral immunity in TB infection has been controversial and is less defined than the role of T cells [76]. However, there is increasing evidence that B cells and antibodies has a significant impact on the immune response against *Mtb* by both classical and non-classical mechanisms of antibody action and interaction of B cells with other immune cells [76].

1.3.3 Immune correlates of protection against *Mycobacterium tuberculosis*

It is not clear what constitutes a protective immune response against *Mtb* infection. The lack of an immune correlate of protection hampers vaccine development as empirically determining whether a vaccine reduces the number of active TB cases is a daunting task.

Despite being essential in the immune response against *Mtb*, CD4+ T cell production of IFN-γ does not correlate with protection [77]. Polyfunctional (IFN-γ+TNF-α+IL-2+) T cells are considered to be superior effectors compared with single producing T cells [78], and have been associated with control of chronic viral infections [79–81]. Studies of the cytokine profiles of *Mtb* specific CD4+ T cells by flow-cytometry have shown that TNF-α single producing cells dominates in active TB whereas polyfunctional (IFN-γ+TNF-α+IL-2+) T cells characterise latent TB [82,83]. However, other studies have found contradictory results with increased levels of polyfunctional cells in active compared with latent TB [84,85], and an immune correlate study in BCG vaccinated infants found no correlation between the frequency and cytokine profile of *Mtb* specific T cells with protection against TB [86]. Further, a recent study reported that the frequency of activated human leukocyte antigen-D related (HLA-DR)+CD4+ T cells is associated with increased TB disease risk in BCG vaccinated infants and in *Mtb* infected adolescents, and that BCG specific IFN-γ producing T cells were a correlate of protection [87]. Due to the contradictory results of single markers, a review has suggested that a broad characterisation of immune mediators and cell types, including mechanisms that appear to have minor roles, is needed to define protective immunity and that an effective vaccine might need to engage multiple immune mechanisms [77].

1.4 Diagnosis

In 2015, only 56% of the estimated incident active TB cases were notified [1]. This is explained by under-reporting of diagnosed TB cases and under-diagnosis. The main reasons for under-diagnosis is poor access to health care facilities, the often unspecific clinical presentation of the infection and the lack of an affordable, simple and accurate point-of-care diagnostic test. TB diagnosis is especially difficult in HIV co-infected individuals as HIV changes the presentation of active TB disease and affects the performance of the available diagnostic tools [8].

1.4.1 Diagnosis of latent tuberculosis

There is no gold standard for diagnosing latent TB. The tests available are based on detection of an acquired immune response upon rechallenge with antigen and do not give any direct information about the presence of viable bacteria. For nearly a century, the TST was the only tool available for detection of latent TB. The TST measures the induration caused by a delayed type hypersensitivity response to PPD injected intradermally. Limitations of the TST include poor sensitivity in immunocompromised individuals and poor specificity because PPD contains antigens also present in the BCG vaccine and non-tuberculous mycobacteria (NTM). Efforts have been made to develop new more specific skin test by replacing PPD with more specific antigens, but higher specificity might compromise sensitivity [88].

Blood based *in vitro* assays that measure IFN-γ released by T cells in response to stimulation with *Mtb* antigens (interferon gamma release assays (IGRAs)) was developed the past decade. Commercially available IGRAs include the QuantiFERON-TB Gold (QFT) and the T-SPOT.TB (figure 3). In the QFT assay whole blood is drawn into three QFT tubes containing TB antigen, mitogen-positive control and a negative control respectively. After incubation, an enzyme-linked immunosorbent assay (ELISA) is conducted to quantify the amount of IFN-γ produced in the antigen tubes compared with the control tubes. The test is considered

positive if the IFN-γ response to TB antigens is above the cut-off after subtracting the background IFN-γ response in the negative control. The T-SPOT.TB is an enzyme-linked immunospot (ELISPOT) assay based on separated peripheral blood mononuclear cells (PBMCs). After incubation with TB antigens and a negative and positive control, the result is reported as the number of IFN-γ-producing T- cells, and as for the QFT assay, the background in the negative control is subtracted from the result of the TB antigen stimulated cells.

The IGRAs are more specific than the TST because the TB antigens used (ESAT-6 and CFP-10, and in QFT also TB7.7(p4)) are not shared with the BCG vaccine strain or most NTM. As for the TST, the sensitivity in HIV-positive individuals and in children is suboptimal [89,90]. Further, the use of IGRAs in resource limited settings is limited by high costs and need of laboratory facilities. QFT-Plus is a next generation IGRA launched in 2015 which include an additional TB antigen tube [88]. The second TB antigen tube contains peptides designed to elicit a response from CD8+ T cells and the aim is to increase the sensitivity of the test.

In contrast to the TST, the IGRAs can be repeated without any sensitisation or boosting which make them more applicable for repeated screening. However, studies of IGRAs for serial TB screening of health care workers [91,92] and in HIV infection [93,94] have shown that the interpretation of IGRA results is complicated by relative high rates of conversions and reversions and within subject variability. Subjects with QFT baseline results around the diagnostic cut off (0.35 IU/mL) are more likely to have inconsistent results on serial testing and introduction of a borderline zone from 0.20-0.70 IU mL when screening health care workers have been suggested [91].

Neither TST nor IGRAs can distinguish between active and latent TB. They also have limited prognostic value as most TST and IGRA-positive individuals will not progress to active TB [95]. New biomarkers are required to distinguish between the different stages of infection and to guide the use of preventive therapy to the subgroup that most likely will benefit from it.

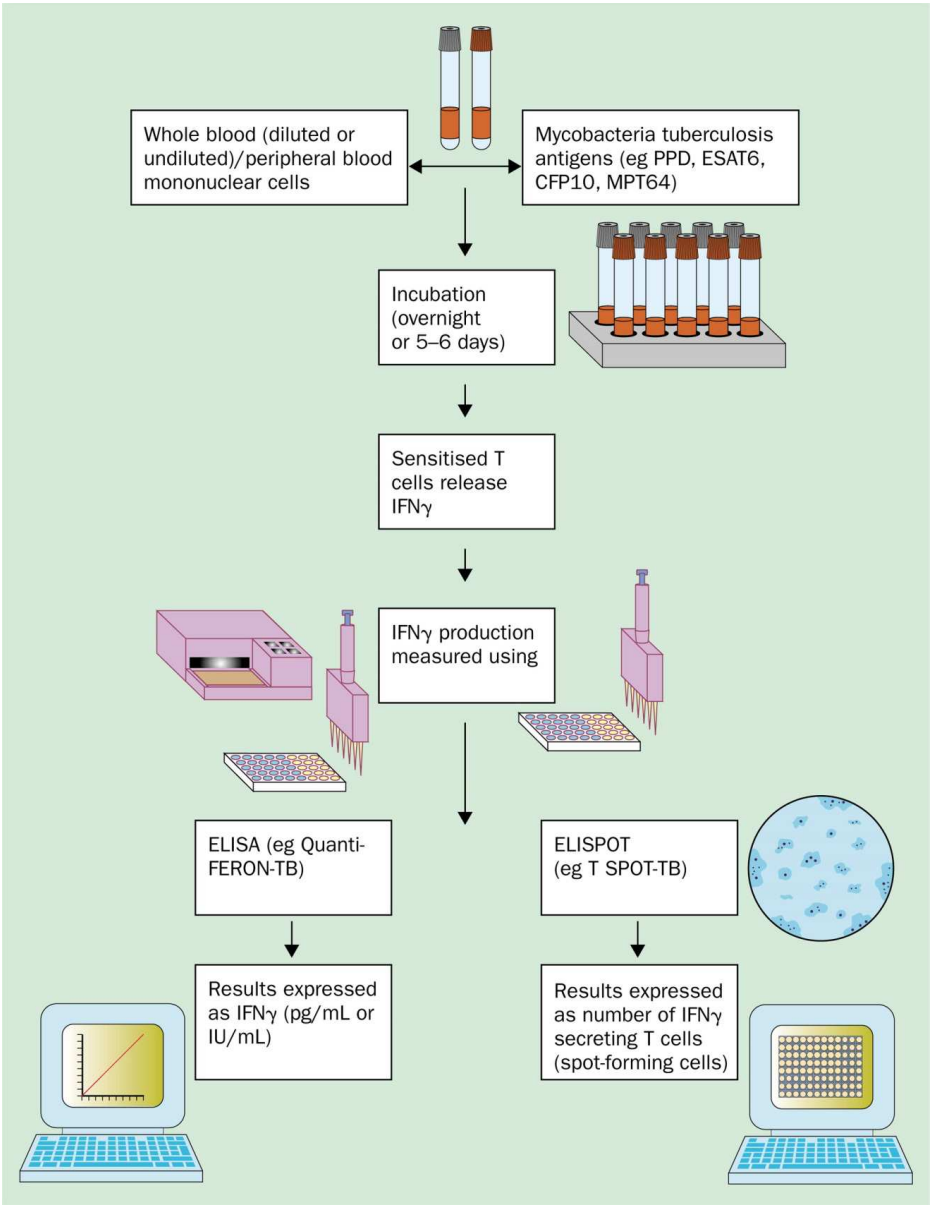

Figure 3. The test procedures of the QFT and T-SPOT.TB assays. Reprinted from The Lancet Infectious Diseases, Vol 12, Pai et al, Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review, pp 761-76 [96], Copyright (2004), with permission from Elsevier.

1.4.2 Diagnosis of active tuberculosis

Diagnosis of active TB traditionally relies on smear microscopy and culture of *Mtb* from clinical samples. Despite poor sensitivity and inability to distinguish between *Mtb* and NTM, microscopy is still widely used and is the mainstay of active TB diagnosis in resource limited settings. The sensitivity ranges from 20-80% and is lowest in HIV-positive individuals and in children [97].

Culture is the gold standard for diagnosing active TB. *Mtb* is growing slowly and detection of growth using traditional solid media requires incubation for 3-8 weeks. Automated liquid culture systems have decreased the detection time to 1-3 weeks and have higher sensitivity, but the contamination rates are higher, they are expensive and require considerable laboratory facilities [98].

In 2010 WHO recommended the use of a recently developed nucleic acid amplification-based test, the Xpert MTB/RIF, for detection of pulmonary TB. In 2013, a policy update widened the recommended use to also include diagnosis of paediatric TB and for selected extrapulmonary specimens [99]. The Xpert MTB/RIF enables rapid and simultaneous detection of *Mtb* and rifampicin resistance and clinical trials have shown that use of the Xpert/MTB/RIF assay increases the numbers of patients identified compared with smear microscopy and decreases the time to initiation of treatment [100]. However, the assay is expensive, and no significant impact on patient morbidity and mortality has been reported [100,101]. As with smear microscopy, the sensitivity is lower in HIV-positive individuals and children, and for extrapulmonary samples the sensitivity varies with sample type [102]. In addition to the Xpert MTB/RIF, two other nucleic acid amplification-based tests have been endorsed by WHO for detection of drug resistance, the GenoType MTBDRplus and the NTM+MDRTB tests [103]. These tests detect resistance against both isoniazid and rifampicin, but are only validated for testing of smear positive specimens or isolates of *Mtb* [102,103].

1.5 Treatment

The first antibiotic with activity against *Mtb*, Streptomycin, was discovered in 1944. However, it soon became evident that monotherapy lead to development of drug resistance. The following two decades several other antituberculous drugs with different mechanisms of action were discovered including isoniazid, pyrazinamide, rifampicin and ethambutol, and it was shown that combination therapy reduced the risk of drug resistance. The standard drug regimens currently recommended for drug susceptible TB still relies on the drugs discovered in the 1950s and 1960s. Efforts have been made to develop new anti-tuberculous drugs, and in 2012-2014 two new drugs were approved for treatment of MDR-TB when an otherwise effective regimen is not available [104].

1.5.1 Treatment of latent tuberculosis

Preventive treatment of individuals with latent TB infection reduces the risk of developing active TB [105]. There are several recommended regimens including six or nine months isoniazid alone, three-four months isoniazid plus rifampicine, three-month weekly rifapentine plus isoniazid and three-four months rifampicin alone [5,106]. Benefit of treatment must be balanced against risk of drug-related side-effects, and testing and preventive therapy is therefore offered to individuals with the highest risk of progression to active disease. In high and middle-income countries, WHO recommends systematic testing and treatment of latent TB in contacts of pulmonary TB cases, individuals with HIV coinfection, patients starting anti-TNF treatment, patients receiving dialysis, patients preparing for organ transplantation and patients with silicosis [5]. In resource limited settings, testing and treatment of latent TB is recommended for HIV-positive individuals and children < five years of age who are close contacts of people with pulmonary TB [107].

1.5.2 Treatment of active tuberculosis

The standard treatment regimen for new TB patients with drug susceptible TB currently consist of a two month intensive phase with rifampicin, isoniazid, pyrazinamide and ethambutol, and a four month continuation phase with rifampicin and isoniazid [106,108]. To ensure patient compliance and prevent development of drug resistance, the treatment is given as directly observed treatment (DOT).

MDR-TB is defined as TB caused by *Mtb* strains resistant to at least rifampicin and isoniazid. XDR-TB in addition involves resistance to any of the fluoroquinolones and to at least one of the three second line drugs amikacin, capreomycin and kanamycin. MDR- and XDR-TB cases require extended treatment regimens with combinations of drugs that increase the risk of serious side effects. The two new anti-tuberculous drugs, bedaquiline and delamanid are promising for MDR-TB treatment. However, access in high burden countries is limited and there are unresolved safety concerns with bedaquiline [109]. Globally, the treatment success rate for new TB cases treated in 2014 were 83%, whereas the corresponding rates for MDR- and XDR-TB cases were only 52% and 28% respectively [1].

1.5.3. Host directed therapy

The low treatment success rates for MDR- and XDR- TB highlight the need of improved treatment strategies. Adjunct treatment that targets the host response to infection (host directed therapies (HDTs)) have been suggested to may facilitate eradication of *Mtb*, shorten treatment duration and reduce permanent lung injury by augmenting cellular antimicrobial mechanisms and reducing excessive inflammation [110,111]. The range of potential targets and candidate HDTs is broad (figure 4) and includes several well-known drugs approved for other clinical indications.

Cyclooxygenase (COX)-2 inhibitors reduce the production of prostaglandin E2 (PGE2) which has a key role in the generation of the inflammatory response. In addition to proinflammatory properties, PGE2 is involved in suppression of T cell

functions [112–114] and both monocytes and adaptive Treg seem to supress T cell immune responses by a COX-2-PGE2 dependent mechanism [115,116].

In mouse models of TB it has been shown that treatment with COX-2 inhibitors enhances Th1 cytokines and reduces inflammation and bacillary loads [117–120]. However, the effect of COX-inhibition is phase dependent as a beneficial effect of PGE2 has been reported in the early, but not late phase of TB infection [119,121]. Clinical trials in HIV-positive patients have shown that COX-2 inhibitors improve T cell mediated immune responses [122–124]. The effect of COX inhibition in human TB has not been published, but studies are ongoing (ClinicalTrials.gov Identifier: NCT02503839 and NCT02781909).

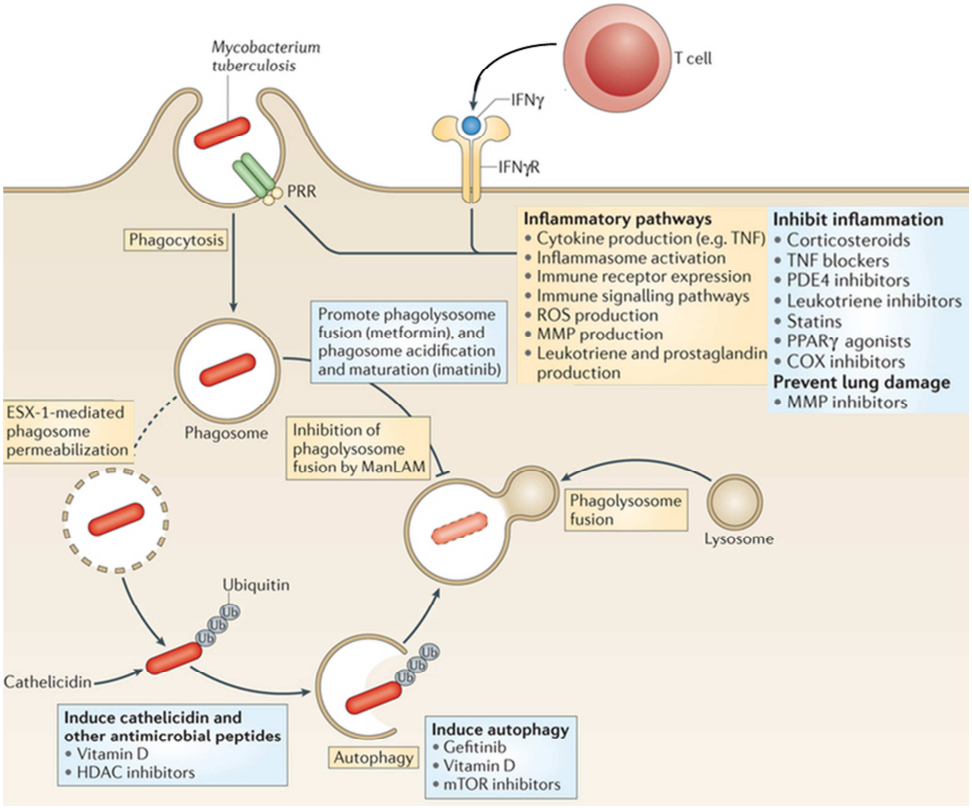

Figure 4. Potential targets of host-directed therapy against *Mtb*. Yellow boxes indicate pathological processes. Blue boxes indicate points of intervention by host-directed therapies. Reprinted by permission from Macmillan Publishers Ltd: Nature reviews Immunology, Wallis R.S. [110], copyright (2015).

1.5.4. Monitoring treatment efficacy

In pulmonary TB cases, sputum culture conversion (SCC) status at the end of the two month intensive phase of treatment is the most well established predictor of non-relapsing cure [125]. The two-month SCC status is also associated with treatment success in MDR-TB patients, but the association is substantially stronger for six-month SCC [126]. As culture is not always available in high burden settings and, especially on solid media, takes several weeks to give a result, SCC status has not been widely used clinically [127].

WHO currently recommends smear microscopy at completion of the intensive phase of treatment to identify individuals at risk of poor treatment outcome [108]. Sputum smear microscopy status is a poor predictor with respect to which patients will relapse, but are used to trigger further patient assessment and additional sputum monitoring.

Several studies have investigated the potential of IGRAs for monitoring effect of treatment. A review found no uniform pattern in IGRA conversion and reversion rates at the end of treatment for active and latent TB, and concluded that IGRAs are unlikely to be useful for monitoring effect of TB treatment [128]. In most of the studies reviewed, the majority of the IGRA results remained positive at the end of treatment. In a subsequent longitudinal study of HIV-positive individuals with latent TB, the QFT reversion rate was 23% for individuals receiving preventive therapy and 44% in individuals with untreated latent TB, supporting the unreliability of QFT for treatment monitoring [94].

Nucleic acid amplification based tests, like the Xpert MTB/RIF are not suitable for monitoring treatment because they detect DNA from both viable and non-viable bacteria [129]. However, it has been suggested that pre-treatment of samples with propidium monoazid allows selective amplification of DNA from viable *Mtb* [130].

New accurate and rapid tools for monitoring of treatment efficacy would be a major advance as it would simplify TB drug treatment trials and prevent inadequate treatment that further may lead to transmission from subsequent reactivation.

1.6 Potential biomarkers for tuberculosis diagnosis and treatment efficacy

A biomarker can be defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention [131]. In TB, biomarkers are needed for multiple purposes including diagnosis of the different stages of infection, identification of individuals with high risk of progression to active disease, monitoring of treatment efficacy and evaluation of the protective efficacy of vaccines [132]. The ideal diagnostic TB test would be an affordable point-of-care test providing accurate diagnosis of active TB in both HIV-negative and HIV-positive individuals with pulmonary and extrapulmonary TB and detection of drug resistance to the first-line anti-tuberculous drugs [132].

Biomarkers in infectious diseases can be either host- or pathogen derived. In TB, an example of a pathogen derived biomarker is lipoarabionmannan (LAM) which is a component of the outer wall of *Mtb* that is released from metabolically active or degrading cells and is detectable in urine. In contrast to other diagnostic tools, the sensitivity of urine LAM for active TB detection is increased in HIV co-infected individuals compared to HIV-negative individuals and further increases with lower CD4 counts [133]. A point-of-care lateral flow dipstick for urinary LAM detection is commercially available (LF-LAM) and may be used to assist in the diagnosis of TB in HIV-positive adults with low CD4 counts [134].

Host response derived biomarkers for TB diagnosis and treatment efficacy have been sought using technologies like transcriptomics, proteomics and multiplex bead assays. The blood transcriptional signatures and proteomic profiles for active TB reported are heterogeneous and currently there are no diagnostic assays based on these techniques available [135]. Biomarkers have also been searched for among the multiple cytokines and chemokines expressed as a part of the immune response against *Mtb* (figure 5) [136,137]. Both unstimulated plasma and *in vitro Mtb* specific stimulated levels of various markers have been investigated in the different stages of

TB infection in a number of explorative studies. A review by Chegou *et al* summarizes that although several new potential cytokine biomarkers to detect *Mtb* specific immunity have been identified, there is no clear pattern of markers able to differentiate between active and latent TB infection [136]. Similarly, a review concludes that although several cytokines have potential to monitor TB treatment, no single cytokine or combinations of cytokines have been shown to provide a sufficiently robust correlate of treatment success [137]. The diverse results emphasize the need of larger confirmatory studies to validate the diagnostic potential of the suggested biomarkers. There is also lack of studies including HIV co-infected individuals in whom the current diagnostic tools have the greatest limitations.

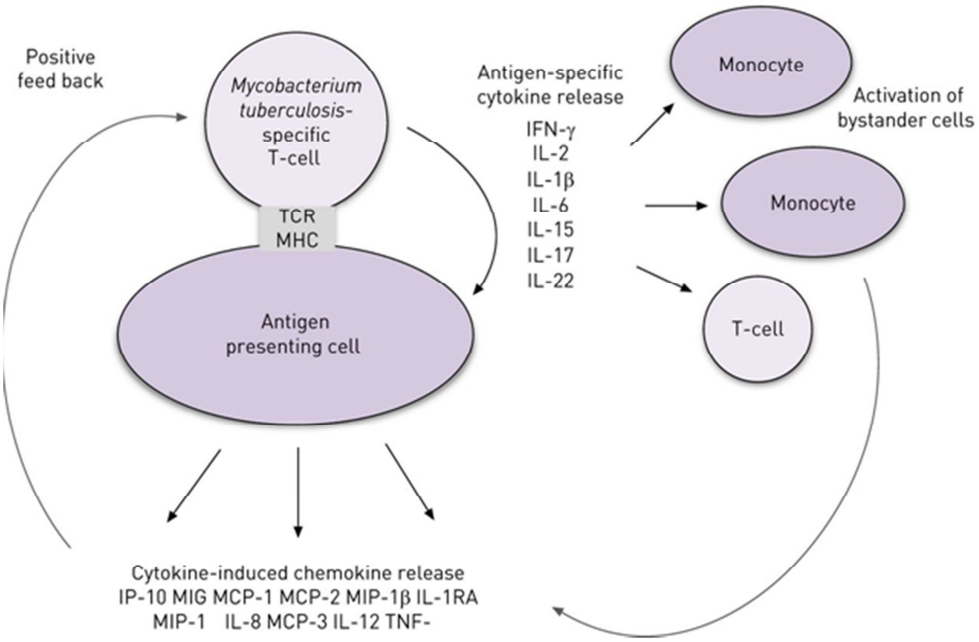

Figure 5. Overview of key cells and cytokines involved in the immune response towards *Mtb* specific antigens in immunodiagnostic tests. Reprinted by permission from European respiratory society. Chegou NN, European Respiratory journal, 2014 [136].

Interferon gamma inducible protein 10 (IP-10) is one of the most studied candidate biomarkers in TB. It is a chemokine secreted by antigen presenting cells and can be induced at high levels as part of the adaptive immune response. Secretion is driven by multiple cytokines, but mainly by IFN-γ and TNF-α [136]. It has been found that plasma levels of IP-10 are elevated in patients with active TB [138–140], as well as in patients with other inflammatory conditions like bacteremia [141], infection with HIV [142,143] and hepatitis C virus [144]. Several studies have reported a decrease in plasma levels of IP-10 in response to TB treatment of HIV-uninfected active TB cases [138,140,145–147], whereas there are conflicting results in HIV co-infected cases [146,147]. As a readout marker in *Mtb* specific immunoassays, IP-10 has comparable diagnostic accuracy to IFN-γ [148]. The relative high levels of IP-10 secreted compared with other biomarkers have made it possible to develop simplified detection methods based on dried blood/plasma spots which performs comparably to the QFT [149–151].

TNF-α plays a central role in the immune response against *Mtb* which is demonstrated by the increased risk of reactivation of latent TB in patients receiving anti-TNF treatment [152]. Soluble TNF receptors (sTNFr) act as TNF antagonists by competing with the cell membrane receptors for cytokine binding, and elevated plasma levels of both sTNFr1 and sTNFr2 have been found in patient with active TB [153].

Pentraxin 3 (PTX3) and C-reactive protein (CRP) belong to the pentraxin family and are involved in the acute phase reaction to inflammation. It has been shown that mycobacterial LAM induces PTX3 production by human monocytes [154]. Further, Azzuri *et al* found that plasma levels of PTX3 are increased in patients with active TB, decreases during successful treatment and increases in patients with treatment failure [138]. CRP is produced in the liver and is extensively used as a marker of the extent of inflammation. As for PTX3, CRP levels decrease during successful TB treatment [155,156].

The Th1 cytokine IL-2 is mainly produced by antigen-activated T cells but at a lower magnitude compared with IFN-γ. TB antigen specific stimulated levels of IL-2 have comparable diagnostic accuracy for active TB as IFN-γ and IP-10 [136], and a meta-analysis concludes that IL-2 is also a valid marker for diagnosing latent TB and combined with IFN-γ may increase sensitivity [157].

IL-1 receptor antagonist (IL-1ra) is a competitive inhibitor of the proinflammatory cytokines IL-1α and IL-1β and is secreted by various cells including monocytes, macrophages and neutrophils [158]. Juffermans *et al* showed that serum levels of IL-1ra are increased in patients with active TB compared with contacts and controls and declined during treatment [153]. Further, studies have shown that when detected in QFT supernatants, IL-1ra differentiate TB infected individuals from controls [159,160] and may also differentiate active from latent TB infection [159].

2. Aims

The main aim of this thesis was to characterise immune cells and soluble immune markers in different stages of TB infection with focus on identifying potential biomarkers that may improve TB diagnostics and monitoring of treatment efficacy. The secondary aim was to explore the in vitro effects of COX-inhibition on *Mtb* specific T cell responses.

The specific aims were:

- To examine levels of Treg, activated CD4+ and CD8+ T cells and DC subsets in blood from individuals with active TB, latent TB and QFT-negative controls (paper I).

- To examine the potential of soluble immune markers detected in plasma to differentiate between the stages of TB infection in patients with and without HIV coinfection, and to study the effect of TB treatment on levels of these markers (paper II).

- To examine the potential of soluble immune markers detected in QFT supernatants to differentiate between the stages of TB infection, and to compare the pattern of markers in subjects with a QFT test result in the borderline zone with those with higher values and with QFT negative controls (paper III).

- To analyse COX-2 expression in monocytes from patients with latent and active TB, and to explore the *in vitro* effects of the COX–inhibitor indomethacin on *Mtb* specific T cell responses (paper IV).

3. Summary of papers

3.1 Paper I: T Regulatory cells and immune activation in *Mycobacterium tuberculosis* infection and the effect of preventive therapy

The levels of Treg, DC subsets and activated CD4+ and CD8+ T cells in blood from patients with active TB, latent TB before and after three months of preventive therapy and from QFT-negative controls were examined by flow cytometry.

The level of Treg, identified as CD25+CD127-, was significantly higher in both the active and latent TB group compared with the controls. Further, the active TB group had the highest median level of CD25+FOXP3+ Treg although there were no significant differences between any of the groups. Increased T cell activation of both CD4+ and CD8+ T cells, represented by higher proportion of HLA-DR+CD38+ cells, was found in the active TB group and there was a significant positive correlation between the level of activated CD4+ T cells and both Treg subsets. There were no significant differences in the proportion of mDCs or pDCs among the study groups and no correlation between DC and Treg subsets. The level of CD25+FOXP3+Treg significantly increased in the latent TB group after preventive TB therapy, whereas no significant changes were observed in the expression of activation markers or DC subsets.

3.2 Paper II: IP-10 differentiates between active and latent tuberculosis irrespective of HIV status and declines during therapy

In this study we searched for plasma biomarkers with potential to differentiate between the stages of TB infection in HIV-negative and HIV-positive individuals, and examined changes in biomarker levels during TB treatment of HIV-negative patients with active TB by ELISA and multiplex bead assays.

Of the 38 markers examined, IP-10 and sTNFr2 were the only markers that significantly differentiated active TB from both latent TB and QFT negative controls irrespective of HIV status. The level of IP-10 declined gradually and significantly in response to TB treatment of HIV-uninfected active TB cases, whereas the level of sTNFr2 fluctuated.

The diagnostic accuracy of IP-10 was investigated by receiver operating characteristics (ROC) curve analyses. In HIV-infected individuals IP-10 discriminated active from latent TB with 100% sensitivity and specificity, whereas in HIV-uninfected individuals the sensitivity and specificity were 71% and 82%, respectively.

3.3 Paper III: Cytokine patterns in tuberculosis infection; IL-1ra, IL-2 and IP-10 differentiate borderline QuantiFERON-TB samples from uninfected controls

Using a multiplex bead assay, we searched for biomarkers in QFT supernatants with potential to differentiate between the various stages of TB infection and examined the pattern of markers in subjects with QFT test result in the borderline zone. In addition, we examined changes in marker levels after preventive TB therapy.

The unstimulated (Nil) level of IL-1β, IL-1ra, IL-9 and IL-17a were significantly lower in the active TB compared with the latent TB group. In contrast, the background corrected TB antigen stimulated (TBAg-Nil) levels of none of the 27 markers analysed were able to differentiate between these groups.

The TBAg-Nil level of seven markers was significantly higher in both the active TB and latent TB group than in QFT negative controls. However, only IL-1ra, IL-2 and IP-10 also differentiated the QFT borderline group from the controls. Using cut-offs determined by ROC curve analyses, the majority of the subjects were classified in accordance with the QFT test by all these three markers. There were no significant changes in the Nil or TBAg-Nil levels of any of the markers with diagnostic potential after preventive TB treatment of the latent TB group.

3.4 Paper IV: The COX- inhibitor indomethacin reduces Th1 effector and T regulatory cells *in vitro* in *Mycobacterium tuberculosis* infection

We studied COX-2 expression in monocytes from patients with latent and active TB and explored the *in vitro* effects of the COX-inhibitor indomethacin on *Mtb* specific T cell responses and regulation.

Although not statistically significant, unstimulated monocytes from patients with active TB tended to express higher levels of COX-2 compared to individuals with latent TB. Monocytes from both the latent and active TB group significantly upregulated COX-2 expression after *in vitro* lipopolysaccharide (LPS) stimulation.

In response to ESAT-6 and Ag85 stimulation there was a significant increase in FOXP3+CD25++ Treg and proliferating and cytokine (IL-2, TNF-α, IFN-γ) producing T cells. Indomethacin significantly reduced the fraction of FOXP3+CD25++ Treg, but also the fraction of total IL-2 producing and total TNF-α producing CD4+ T cells as well as the proliferative capacity of T cells in *Mtb* antigen stimulated samples.

4. Methodological considerations

4.1 Study design and participants

The studies which constitute this thesis are comparative retrospective studies including comparisons of patients with active TB, individuals with latent TB and TB negative controls. In addition, the studies of paper I, II and III include longitudinal data on selected study groups, and paper IV an exploratory cross-sectional *in vitro* study of patients with active TB. An overview of the study designs, number of participants, methods and factors investigated are given in table 1.

Table 1. Overview of study design, number of study participants, methods and factors investigated

Paper	Study design	Study participants (sample size)			Methods	Factors investigated
		Active TB	Latent TB	Controls		
I	1) Comparative retrospective, 2) Longitudinal data for the latent TB group	20	20	28	Flow cytometry of PBMCs	Regulatory T cells, activated CD4+ and CD8+ T cells, dendritic cells
II	1) Comparative retrospective 2) Longitudinal data for the HIV-negative active TB group	65	34	65	Multiplex bead assay, Enzyme immunoassays	Levels of 38 different markers in plasma
III	1) Comparative retrospective 2) Longitudinal data for the latent TB group	18	48	16	Multiplex bead assay	Levels of 27 different markers in supernatants of the QFT test
IV	1) Comparative retrospective 2) Exploratory in vitro study of the active TB group	33	9	-	Flow cytometry of PBMCs	1) Cox-2 expression on monocytes 2) Effects of indomethacin on *Mtb* specific Treg and T cell cytokine responses and proliferation

In paper I, III and IV, all the study participants were HIV-negative. In paper II, the study participants were further classified according to HIV status. An overview of the sample size in each subgroup in paper II is given in table 2.

Table 2. Overview of the sample size of the study groups in paper II

Active TB n=65		Latent TB QFT-positive n=34		QFT-negative controls n=65	
HIV+ n=6	HIV- n=59	HIV+ n=23	HIV- n=11	HIV+ n=52	HIV- n=13

4.1.1. Inclusion of study participants

The study participants in paper I and III were recruited at Haukeland University Hospital (HUH) in the period 2006-2007. Individuals referred to the TB outpatient clinic for medical evaluation of latent or active TB based on a positive TST and/or suspected exposure to TB were included as part of a study of the performance of IGRA in clinical practise [161]. In addition, patients diagnosed with active TB were recruited from the inpatient ward, and QFT and TST negative controls were recruited from age-matched employees at the hospital with no known exposure to TB.

The study population in paper II comprised participants included from clinical studies at different hospitals in Norway. In addition to the participants recruited at HUH described above, HIV-positive individuals were included from a national IGRA multicentre study in the period 2009-2010 [162]. HIV-negative patients with active TB followed longitudinally during 24 weeks of TB treatment were included from a clinical study at Oslo university hospital (OUS) in the period 2009-2012 [57].

The study population in paper IV comprised participants recruited at HUH described above and active TB patients recruited at OUS in the period 2010-2014.

4.1.2 Definition of study groups

1. Active TB.

Active TB cases were defined by the presence of *Mtb* detected by culture, or by the presence of clinical and radiological or histopathological signs of active disease.

2. Latent TB.

Subjects with a positive QFT, no signs of active TB based on X-ray, sputum examination and clinical evaluation and no previous active TB were defined as having latent TB.

3. Controls

Subjects with a negative QFT, no signs of active TB and no previous active TB served as TB negative controls.

4.1.3. Limitations of study design

In general, the sample size in the studies comprising this thesis is low. This is mainly due to the low TB prevalence in Norway and logistical difficulties in inclusion of patients and sample processing. The limitations in study groups and sample size for each paper are specified below.

Paper I

Due to logistical difficulties at HUH, we were not able to collect blood samples from the active TB group at the end of therapy or to perform longitudinal blood sampling

from QFT-negative subjects. We were therefore not able to investigate the effect of treatment on Treg, DC and activated CD4+ and CD8+ in the active TB group or to evaluate longitudinal variation in these factors in the QFT negative control group.

Paper II

There was a low prevalence of active TB in the HIV-infected population from whom the study participants were recruited [162]. The sample size of HIV-infected patients with active TB in the study is therefore especially low, and there is also a lack of longitudinal plasma samples from this group during treatment. Further, as plasma IP-10 is an unspecific marker for TB, the study should ideally have included both an HIV–positive and HIV–negative group with infections and inflammatory diseases other than TB.

Paper III

Repeated testing of subjects with QFT test result in the borderline zone was not performed and we were therefore not able to examine the variability of potential biomarkers in longitudinal samples.

Paper IV

Due to a limited number of samples and PBMCs available from the latent TB group, the effects of indomethacin on *Mtb* specific Treg and T cell cytokine production and proliferation was only studied in patients with active TB where we believed the effects would be most pronounced and relevant.

4.2 Laboratory assays

4.2.1 Processing and storage of samples

Blood samples were drawn from the study participants at defined time-points and PBMCs were cryopreserved to enable simultaneous batch analysis of multiple samples. It has been shown that cryopreserved cells can be stored for at least 12 years with no tendency of cell loss or decrease in viability over time [163], and that the functionality of CD4+ and CD8+ T cells are unaffected by cryopreservation in cytokine ELISPOT assays [164]. However, it has also been reported that cryopreservation can cause changes in the frequencies and function of T cell subpopulations [165,166].

The quality of cryopreserved PBMCs is highly dependent on optimal cryopreservation and thawing methods and a number of factors including time until processing and storage and thawing temperatures affect the results [167]. In general, PBMCs with viability ≥70% are considered suitable for functional analyses [168]. Despite the use of standardized cryopreservation and thawing protocols some of the samples in our study had low viability after thawing and these were excluded from further analyses.

Plasma and excess of supernatants from the QFT test were frozen and stored at -80°C until analysis. Long term storage can cause degradation of cytokines even at -80°C [169]. In our study, the majority of the cytokines analysed in both plasma and QFT supernatants had levels detectable above the lower detection limit of the Multiplex bead assays.

4.2.2 Flow cytometry

Flow cytometry has the capability of measuring several parameters of thousands of single cells per second which allows detailed analysis of cell surface and intracellular markers. The basic principles of flow cytometry are shown in figure 6. Cells are

stained with fluorochrome conjugated antibodies to the markers of interest and pass through a laser beam one by one. Forward and side scattered light and emitted light from the fluorochromes passes through a system of filters and mirrors to route specific wavelengths to designated optical detectors. Flow cytometry is widely used to study cell subsets and antigen specific T cell responses. However, several aspects of the protocols used need careful consideration to ensure reliable assays.

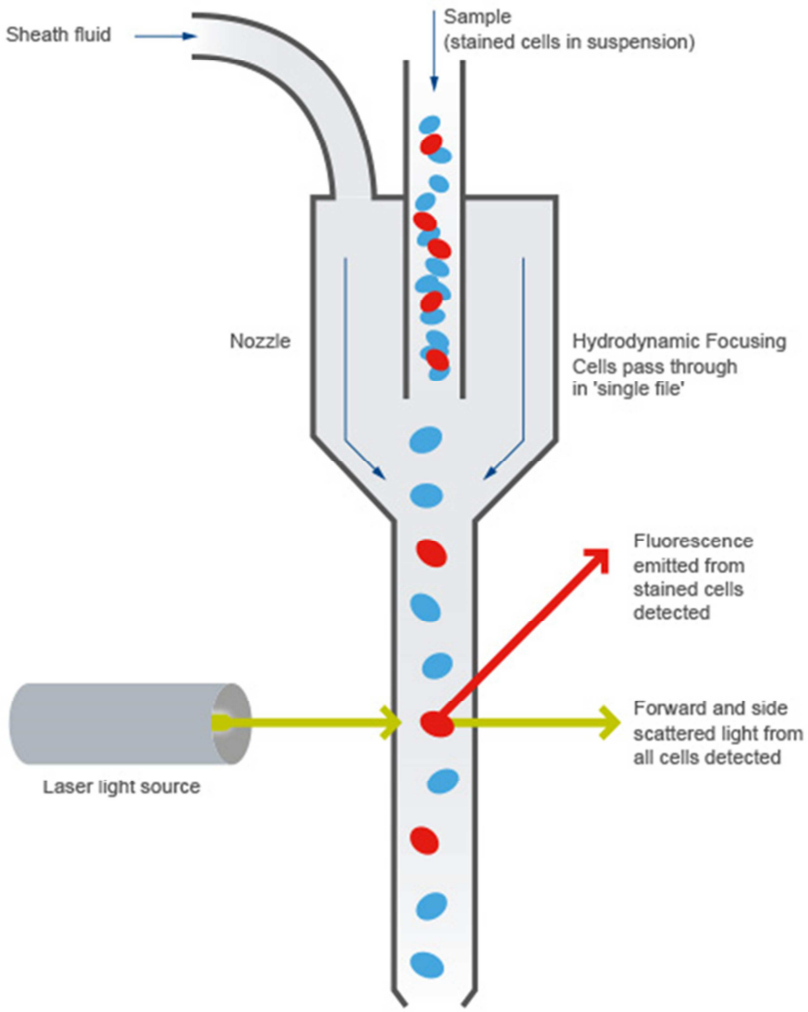

Figure 6. The basic principles of flow cytometry. Reprinted by permission from abcam [170].

Multicolour detection

Advances in technology and availability of fluorochrome conjugated antibodies have increased the number of parameters possible to detect, but with increasing number of markers also the number of fluorochromes with overlapping spectra increases. Spillover occurs when fluorescence emission from a fluorochrome is detected in a detector designed to measure the signal from another fluorochrome. This lead to false signals and compensation must be performed to correct multiparameter flow cytometric data for spectral overlap [171]. Still, it's important to avoid spillover from bright cell population into detectors requiring high resolution sensitivity [172]. In our flow cytometry assays, the maximum number of fluorochromes used was eight and the assays were optimized according to Mahnke *et al* [173]. Bright fluorochromes were chosen for weak antigens, and antibody conjugates were titrated to determine optimal concentrations. Compensation beads or PBMCs were used to prepare single stained compensation controls.

Gating and defining regions

Analysis of flow cytometry data involves gating on the cells of interest (figure 7). Differences in gating may be the largest contributor to variability in flow cytometry data [174]. Some markers, as CD3, CD4 and CD8, give discrete positive and negative populations that are easily discriminated from each other. However, for several markers such as CD25 and FOXP3, cell populations display a more continuous distribution of staining intensity making the definition of regions more complicated. Strategies applied to guide definition of regions include use of fluorescence-minus-one (FMO) and isotype controls or setting the cut-off based on another cell population within the sample having similar surface characteristics as the target population, but which do not express the marker of interest.

Most flow cytometry data are described as the percentage of cells positive for a particular marker or set of markers. For markers having a continuous distribution it

may be more appropriate to report the median fluorescence intensity (MFI) of the cell population. In paper IV we report both the percentage of FOXP3+CD25++ cells and the FOXP3 MFI.

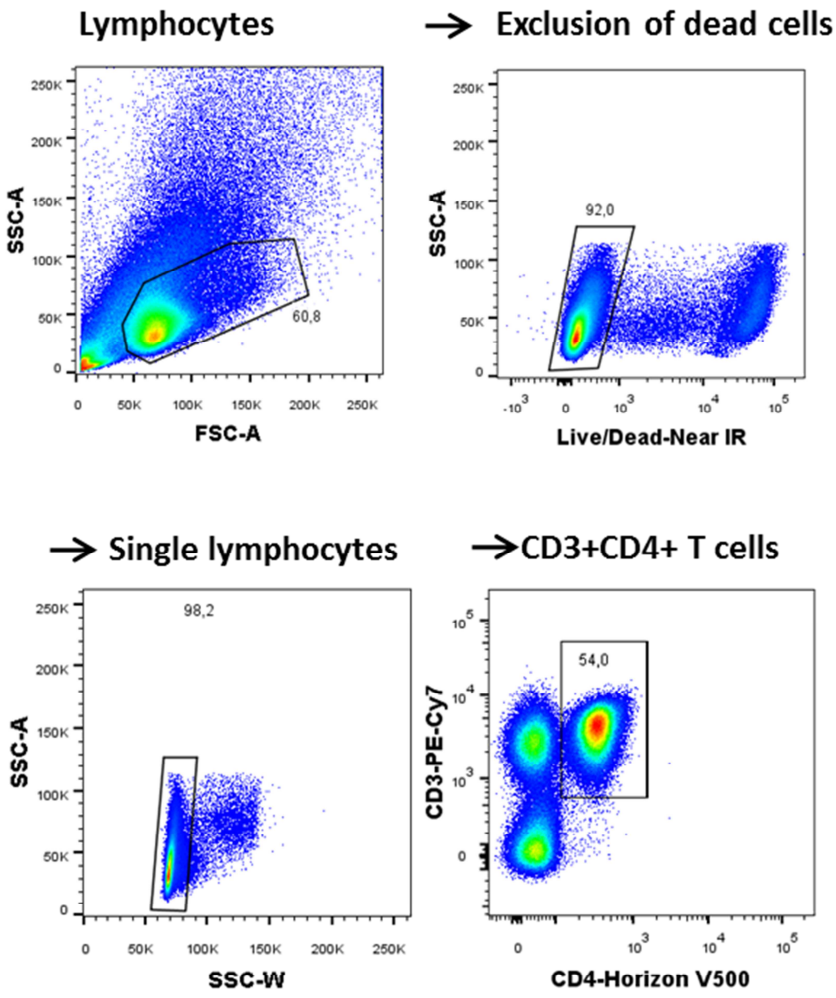

Figure 7. Example of flow cytometry data analysis and gating.

Number of events

When small subpopulations are of interest, the total number of cells acquired must include sufficient events of the population of interest. The number of cells acquired influences the precision of the analysis [175], and the number required to achieve a given precision can be determined by simple calculation [176]. We acquired at least 10.000 CD4+ and CD8+ T cells for the intracellular analyses of cytokines and FOXP3 expression in T cells and minimum 1.000 monocytes for the analyses of COX-2 expression in monocytes.

Viability staining

Dead cells often show high levels of nonspecific binding, and exclusion of dead cells is particularly important when analysing rare populations. In paper IV, a fixable viability stain was included in all analyses. In paper I, no viability stain was included due to the analysis being performed on a flow cytometer allowing only five colour detection. The flow cytometry analyses in paper I did not include any stimulation of cells or detection of rare events, and occupying one of the detectors by a viability stain was therefore not prioritized. Dead cells were to some extent excluded by forward and side scatter gating. Still, optimally a viability stain should have been included.

Intracellular cytokine staining assays

Intracellular cytokine staining (ICS) assays involves stimulation and culturing of cells in the presence of a protein secretion inhibitor followed by surface staining, fixation, permeabilization, intracellular staining and flow cytometry analysis. Published methods for optimization of intracellular cytokine assays [172,177] were used as guidelines for design of our protocols.

ICS is commonly used to study antigen specific T cell responses. However, variations between studies in the protocols used often make the results difficult to compare. In addition to the challenges of multicolour detection and gating, the duration of antigen stimulation has a significant impact on the T cell responses detected [178,179]. The recommended stimulation period for detection of IL-2, TNF-α and IFN-γ is 6-12 hours [172,177,180], whereas the expression of FOXP3 has been found to peak after 36h stimulation [181]. Han Q. *et al* showed that initial cytokine secretion from T cells are predominantly monofunctional and the results indicated that simultaneous release of IL-2, TNF-α and IFN-γ is a short-lived state [182].

T cell responses to antigen stimulation are commonly presented as background subtracted, i.e. responses in the unstimulated control are subtracted from the response in the antigen stimulated samples. In paper IV, the main objective was to study the effect of indomethacin on *Mtb* specific T cell responses. To better delineate the effect of indomethacin on stimulated versus unstimulated PBMCs, the results were all shown without subtracting background values. Gates for intracellular cytokines was set based on the unstimulated sample and boolean gating strategy used to define single-, double- and triple cytokine positive cells.

Carboxyfluorescein diacetate succinimidyl ester proliferation assay

In paper IV, T cell proliferation was assessed by a carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay. CFSE is an intracellular fluorescent dye which is equally distributed to the daughter cells following cell division [183]. Cell surface and intracellular markers can be measured in the same assay allowing analyses of proliferation of specific lymphocyte subsets. The cut-off for proliferating cells was set to the peak of the second generation of $CFSE^{dim}$ CD4+ or CD8+ T cells.

4.2.3 Markers of T cells, activation, apoptosis, dendritic cells and monocytes

An overview of the markers of T cells, Treg, activation, apoptosis, DCs and monocytes used in paper I and IV is given in table 3. CD3 in combination with CD4 and CD8 are used as key markers to identify T helper and T cytotoxic cells respectively. CD3 is expressed by T cells only, whereas CD4 and CD8 also are expressed by other immune cells.

In paper I, T cell activation was assessed by expression of CD38, HLA-DR and CD28 and apoptosis by expression of CD95. CD38, HLA-DR and CD95 are upregulated following T cell activation whereas CD28 is transiently down regulated [184–187]. DCs were characterized by a Lineage 1 (CD3, CD14, CD16, CD19, CD20 and CD56) negative HLA-DR+ phenotype and further classified as CD11c+ mDCs or CD123+ pDCs which differ in origin and function [20,26].

In paper IV, monocytes were identified using the markers CD14, CD16 and HLA-DR according to the gating strategy described by Abeles *et al* [188], and COX-2 expression analysed. Th1 type T cell responses were evaluated by intracellular staining of IFN-γ, IL-2 and TNF-α.

Table 3. Markers of T cells, activation, apoptosis, DCs and monocytes used in flow cytometry assays in paper I and/or IV [189].

Marker	Expressed by	Function	Marker of
CD3	T cells	T cell signal transduction	T cells
CD4	T helper cells, monocytes, macrophages, granulocytes	Binds MHC II, involved in T cell activation	T helper cells
CD8	T cytotoxic cells, Natural killer cells	Binds MHC I, involved in T cell activation	T cytotoxic cells
CD38	T cells, B cells, DCs, natural killer cells, monocytes, macrophages	Involved in calcium signalling and cell adhesion	Activation
HLA-DR	Professional antigen presenting cells	MHC II, antigen presentation	Activation, monocytes

Table 3 continued.

Marker	Expressed by	Function	Marker of
CD28	T cells	Co-stimulatory receptor, binds CD80 and CD86	Activation
CD95	T cells, B cells, natural killer cells, monocytes, macrophages, granulocytes	Involved in apoptosis	Apoptosis
CD11c	T cells, B cells, DCs, natural killer cells, macrophages, monocytes, granulocytes	Involved in cell adhesion	Myeloid DC
CD123	DCs, granulocytes	Interleukin 3 receptor, involved in cell growth and differentiation	Plasmacytoid DC
Lineage 1	-	Mix of CD3, CD14, CD16, CD19, CD20 and CD56	DCs (Lineage 1 negative)
CD14	Monocytes, macrophages, granulocytes	Co-receptor for LPS	Monocytes
CD16	T cells, DCs, natural killer cell, monocytes, macrophages, granulocytes	Fc receptor	Monocytes
FOXP3	T cells	Transcription factor	Treg
CD25	T cells, B cells, natural killer cells, monocytes, macrophages	Interleukin-2 receptor	Treg
CD127	T cells, monocytes, macrophages	Interleukin-7 receptor α	Treg
CD45RA	T cells, B cells, DCs, natural killer cells, monocytes, macrophages	Involved in regulation of cell growth and differentiation	Naive T cells
COX-2	Monocytes, fibroblasts, endothelial cells	Enzyme involved in synthesis of prostaglandins	Monocyte activation
IL-2	T cells, natural killer cells, natural killer T cells, DCs	Involved in proliferation and differentiation of T cells	Th1 T cell response
IFN-γ	T cells, natural killer cells, natural killer T cells	Activation of macrophages	Th1 T cell response
TNF-α	Monocytes, macrophages, T cells, B cells	Pro-inflammatory	Th1 T cell response

4.2.4 Regulatory T cell markers

Treg were first identified by expression of CD25 and the cells with high expression of CD25 were shown to have the greatest suppressive function [190]. However, CD25 is also expressed by activated non-regulatory T cells [191], and the continuous distribution of staining intensity make the definition of the CD25 expression level required to define the Treg population difficult. The transcription factor FOXP3 has been identified as a more specific marker for Treg being crucial for their development and function [192], but may also be up-regulated following activation [193,194] and since FOXP3 is an intracellular marker, the necessity of fixation/permeabilization protocols precludes isolation of viable cells. An additional marker, CD127, which allows isolation of viable Treg for assessment of functional capacity, has also been introduced [195]. As a substantial fraction of CD25+CD127- Treg do not express FOXP3 and a small proportion of CD25+FOXP3+ cells retain high expression of CD127 [196,197], it has been suggested that these markers do not represent the same population of Treg [197]. Further, gating of a clear CD25+CD127- population has been considered more difficult than gating of CD25+FOXP3+ T cells [197,198]. In 2015, an international workshop group proposed that CD3, CD4, CD25, CD127 and FOXP3 are the minimally required markers to define human Treg [199]. Further, it was proposed to use CD3+CD4- cells to define the limits of the CD25 gate as this strategy results in objective CD25+ gating rather than subjective gating on $CD25^{high}$/CD25++ cells.

In paper I, frequencies of both CD25+FOXP3+ and CD25+CD127- Treg were examined whereas in paper IV only CD25++FOXP3+ Treg were studied. The methods used to set the limit for the CD25 and the FOXP3 gate differed between the two papers. In paper I, the FOXP3 gate was set based on the CD4 negative population, and the CD25 gate to include all CD25 positive cells based on isotype controls. In paper IV, the FOXP3 gate was set visually as the positive and negative population was clearly separated, and the CD25 gate was set to include only the CD25high (CD25++) cells. Due to limited numbers of PBMCs available we were not

able to perform sorting of Tregs for further assessment of their immunosuppressive capacity.

4.2.5 Multiplex bead assays

Multiplex bead assays are based on flow cytometry and enable simultaneous measurement of multiple analytes in a small sample volume. The basic principles of multiplex beads assays are shown in figure 8. Distinctly coloured bead sets are created by the use of two fluorochromes at distinct ratios, and antibodies to a specific analyte is attached to a set of beads with the same colour. A second antibody to the analyte is conjugated to a reporter fluorochrome.

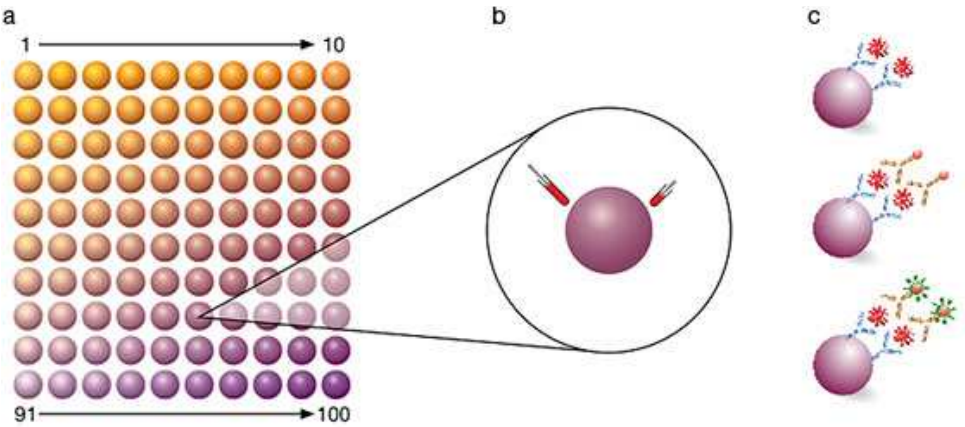

Figure 8. The basic principles of multiplex bead assays. Reprinted with permission from Bio-Rad [200].

In paper II and III, a multiplex cytokine assay was used to detect levels of 27 different markers in plasma and QFT supernatants, respectively. The 27 markers included in the assay are listed in table 4. When analysing markers with a wide range of concentrations simultaneously it can be a problem to determine optimal dilutions allowing detection of all analytes within the range of the assay. In paper III, we experienced that some of the markers analysed had concentrations above the upper

detection limit (UDL) of the multiplex assay despite fourfold dilution of the samples. On the other hand, the level of one of the markers was below the lower detection level (LDL). In paper II nine of the markers were below the LDL (Table 4).

Table 4. The 27 markers included in the Bio-Plex Human Cytokine 27-plex Panel

Marker	Abbreviation	Out of detection range	
		Paper II	Paper III
Interleukin-1β	IL-1β	<LDL	
Interleukin-1 receptor antagonist	Il-1ra		
Interleukin-2	IL-2	<LDL	
Interleukin-4	IL-4	<LDL	
Interleukin-5	IL-5	<LDL	<LDL
Interleukin-6	IL-6		
Interleukin-7	IL-7		
Interleukin-8/CXCL8	IL-8		>UDL
Interleukin-9	IL-9	<LDL	
Interleukin-10	IL-10	<LDL	
Interleukin-12(p70)	IL-12(p70)		
Interleukin-13	IL-13	<LDL	
Interleukin-15	IL-15	<LDL	
Interleukin-17	IL-17		
Basic fibroblast growth factor	bFGF		
Granulocyte colony stimulating factor	G-CSF		
Granulocyte macrophage colony stimulating factor	GM-CSF	<LDL	
Interferon-γ	IFN-γ		
Eotaxin	Eotaxin		
Interferon-γ induced protein-10	IP-10		>UDL*
Macrophage chemoattractant protein 1	MCP-1		>UDL*
Macrophage inflammatory protein 1α	MIP-1α		
Macrophage inflammatory protein 1β	MIP-1β		>UDL
Regulated on activation, normal T cell expressed and secreted	RANTES		>UDL
Tumour necrosis factor α	TNF-α		
Platelet-derived growth factor -BB	PDGF-BB		
Vascular endothelial growth factor	VEGF		

UDL=upper detection limit. LDL=lower detection limit. *Levels of IP-10 and MCP-1 were above UDL only in the TB antigen stimulated samples

4.2.6. Enzyme immunoassays

ELISA are in contrast to multiplex bead assays only able to detect the level of a single analyte in each assay and requires a higher sample volume per analyte measured [201]. ELISA is a plate-based technique in which the wells are coated with an antibody to the analyte of interest. The analyte is detected by a second antibody linked to an enzyme that generates a coloured product when chromogenic substrate is added. The intensity of the colour is proportional to the amount of analyte captured in the well, and the result can be assessed by a plate reader.

In paper II, ELISA was used to detect plasma levels of CXCL16, PTX3, sTNFr2, Fas Ligand (FasL) thymus and activation regulated chemokine (TARC)/CCL17, osteoprotegerin (OPG), activated leukocyte cell adhesion molecule (ALCAM), IL-23, secreted frizzled-related protein 3 (sFRP3), CRP and MD-2.

4.3 Statistical analyses

Nonparametric statistic tests were used in all four papers. Mann-Whitney U test was used to detect pairwise differences between groups, whereas Wilcoxon sign rank test was used to compare related samples. Correlations were investigated using Spearman's rank correlation coefficient. In paper II, we applied a binary logistic regression model to evaluate whether the markers examined were able to differentiate between the study groups when adjusted for HIV-status, age and sex. In paper II and III, ROC curve analyses were performed on selected markers to determine optimal cut-off levels for differentiation between the study groups.

There are two main limitations in the statistical analyses performed. Firstly, the sample sizes in paper I-IV are relatively small which increases the risk of type II errors, i.e. incorrectly retaining a false null hypothesis. Secondly, multiple testing increases the risk of type I errors, i.e. incorrect rejection of the null hypothesis, and was an issue in all studies. There is no gold standard or consensus on how to handle multiple comparisons. The Bonferroni adjustment of significance level is developed for independent tests, and is too conservative when tests are dependent. In paper I-III we performed preliminary analyses of the correlation between the variables investigated. In general, we found that many of the variables were highly correlated leading to dependent tests and the significance level used were therefore adjusted more moderately than by Bonferroni. Also in paper IV we examined partially highly correlated variables. However, in this paper we chose to show the statistical analysis in a simple manner without adjusting the significance level.

4.4 Ethical considerations

Written informed consent was obtained from all participants. The studies were approved by the respective Regional Committees for Ethics in Medical Research (REK-Vest, REK-Nord and REK-Sør-øst). Plasma, QFT supernatants and PBMCs were stored in approved biobanks at Department of medicine, HUH and OUS ("Research Biobank Infectious diseases").

5. General discussion

In this project we have characterised immune cells and soluble immune markers in different stages of TB and explored the *in vitro* effects of immune modulation by cox-inhibition on *Mtb* specific T cell responses. The results improve the understanding of the immune mechanisms involved in the spectrum of TB infection, and potential candidate biomarkers for TB diagnosis and monitoring of treatment efficacy have been identified.

5.1. T cell and monocyte activation in the different stages of tuberculosis

In concordance with previous studies [202,203], we found increased levels of activated T cells in the active TB group compared with controls. Further monocytes from patients with active TB tended to express higher levels of COX-2 compared to patients with latent TB. The latent TB group showed a large variation in the levels of activated T cells, overlapping with values found both in the active and the QFT negative control group. Although monocytes from the majority of individuals with latent TB expressed very low levels of COX-2, two out of nine had monocytes with considerable elevated COX-2 expression. One may speculate that the variation in immune cell activation in the latent TB group is associated with the suggested understanding of TB infection as a spectrum of responses where latent TB includes individuals with sterilizing immunity as well as individuals with controlled infection or active replicating bacteria at a subclinical level [6]. Our data thereby may indicate that immune activation gradually increases throughout the various stages of TB infection corresponding to the level of bacterial burden. This is supported by a study of Sullivan *et al* of HIV-positive individuals showing gradually elevated T cell activation in individuals with latent and active TB compared with TB negative controls [204]. We found no differences in the proportion of DC subsets among the study groups suggesting that the balance between mDCs and pDCs are maintained although absolute numbers may be decreased in patients with active TB [27].

5.2. The role of regulatory T cells in the different stages of tuberculosis

The role of Treg in the different stages of TB infection has not been clarified. In paper I, we found an increased level of CD25+CD127- Treg in both the active and latent TB group compared with controls. However, when Treg were characterized as CD25+FOXP3+, we found no significant differences between any of the groups although the median level was higher in the active TB group. Previous studies have reported significantly higher levels of CD25+FOXP3+ T cells or FOXP3 mRNA expression in active TB compared with both uninfected controls [46,50,51] and individuals with latent TB [48,49]. In contrast, Chiacchio *et al* found comparable levels of CD25highFOXP3+ T cells in active TB cases and healthy controls, and also no significant difference in CD25highCD127- T cells between these groups [205]. The variation in results may, to some extent, be explained by differences in gating strategies and that CD127- and FOXP3+ characterize partially different populations of Treg [196,197].

Information on Treg levels or FOXP3 expression in latent TB infection compared with healthy controls has been scarce and only represented by a study showing higher FOXP3 mRNA expression in response to PPD in TST positive vs TST negative individuals [206]. However, a subsequent study by Herzman *et al* has found similar frequencies of CD25+CD127- Treg in blood from individuals with latent TB and healthy controls, but increased levels in bronchoalveolar lavage from latent TB individuals [207]. Recently, Serrano *et al* reported no differences in various Treg subset in blood between QFT positive and QFT negative individuals, except from a significantly higher level of CD39+CD127- cells in QFT+ individuals [208].

When assessing changes in Treg levels in response to preventive therapy, we found a significant increase in CD25+FOXP3+ Treg, whereas there were no significant changes in the level of CD25+CD127- Treg. The significance of the increase in CD25+FOXP3+ Treg is not clear. Several studies have reported a decline in the frequency of Treg during TB treatment of active TB cases [54–56], whereas others

have found sustained [47] or initially increased levels [57]. Increased Treg levels have also been found in persons with previously treated extrapulmonary TB [209]. It has been suggested that this may be explained by redistribution of redundant Treg to peripheral blood from local sites of infection in response to reduced *Mtb* load and inflammation during TB therapy [57].

Incongruent with the presumed suppressive effect of Treg, we observed a significant positive correlation between the level of activated CD4+ T cells and both CD25+FOXP3+ and CD25+CD127- Treg. The immunosuppressive function of the Treg identified in paper I was not assessed, whereas others have found that depletion of $CD4^{+}CD25^{high}$ cells from PBMCs from patients with TB results in increased production of IFN-γ upon TB antigen stimulation [46–48]. A positive correlation between T cell activation and Treg levels have also been found in persons with previous active TB [209] and in HIV-positive fast progressors [210].

Taken together, the results indicate that Treg may have a role in both latent and active TB infection and is still present at the end of preventive therapy. However, there is variation in the results depending on the markers used for Treg characterisation.

5.3 The potential of regulatory T cells as target for immune modulation by COX-inhibitors

During infections, Treg they may be beneficial by limiting excessive inflammation causing tissue damage, while on the other hand, may impair immune responses necessary for adequate control of infection [43]. With focus on the negative effects of Treg, it has been suggested that they may be a target for host directed therapy [211].

It has been shown that PGE2 induces FOXP3 expression in CD4+CD25- T cells [116,212] and that this upregulation of FOXP3 and the suppressive effect of Treg are reversed by COX-inhibitors [116]. Several studies of *Mtb* infected mice have shown that treatment with COX-2 inhibitors enhances Th1 cytokines and reduces

inflammation and bacillary loads [117–120]. However a beneficial effect of PGE2 has been reported in the early phase of infection [119,121], indicating that the timing of adjunctive therapy with COX inhibitors is critical [213].

In paper IV we hypothesized that the COX-inhibitor indomethacin would reduce Treg levels and thereby result in enhanced T cell cytokine responses and proliferation. Accordingly, we found that indomethacin significantly down-regulated the fraction of *Mtb* specific FOXP3+ T regulatory cells. In contrast, there was an unexpected concomitant decrease in *Mtb* induced T cell TNF-α and IL-2 production and T cell proliferation. This may be due to the direct effects of indomethacin on pathways other than COX/PGE2, e.g. the intracellular NF-κB pathway [214]. NF-κB is a transcription factor regulating genes involved the inflammatory response [215], and it has been reported that COX-inhibitors inhibit NF-κB activation in cell culture [216]. In our study COX-inhibitors thus may have a stronger inhibitory effect on Th1 effector cells than the presumed beneficial effect following reduced Treg numbers. As in paper I, a major limitation of the study is the lack of assays assessing the suppressive capacity of the Treg.

There is no clear definition of what constitutes protective cytokine responses in TB infection. Our data showed that indomethacin had a most distinct effect on the CD4+TNF-α+ T cell subset. TNF-α is essential for control of TB infection [152,217], but excessive production contributes to immune mediated pathology [218]. Thus, the effect of adjunctive therapy decreasing the cytokine response may be beneficial in TB patients with chronic infection and a high level of inflammation, whereas other patients may need an increased inflammatory response [219].

5.4 Biomarkers for tuberculosis diagnosis

In order to end the TB epidemic, reliable and rapid diagnostic tools that can identify and discriminate between latent and active TB are required. As the diagnostic use of

plasma levels of markers is generally limited by lack of specificity for TB infection we searched for alternative biomarkers in both QFT supernatants (paper III) and plasma (paper II).

We showed that the plasma level of IP-10 and sTNFr2 significantly differentiates between active and latent TB infection irrespective of HIV-status. Our results are in agreement with previous studies showing elevated plasma levels of IP-10 in HIV-negative active TB cases compared with controls [138–140], and Juffermans *et al* showing elevated sTNFr2 levels in active TB cases [153]. We reported that IP-10 had 100% sensitivity and specificity for differentiation between active and latent TB in HIV co-infected individuals, whereas the results for HIV-negative individuals were less optimal with a sensitivity and specificity of 71% and 82%, respectively. The sample size, especially of HIV-positive active TB cases (n=6), in our study was small. A recent study by Sullivan *et al* which included a higher simple size also found higher plasma levels of IP-10 in HIV co-infected active TB cases compared with individuals with latent TB [204]. However, there was a noticeable overlap in IP-10 levels in the two groups, indicating that the diagnostic accuracy of IP-10 may be less optimal in HIV co-infected individuals than found in our study.

One of the main limitations of plasma levels of IP-10 is the lack of specificity for TB infection. In addition to in HIV infection [142,143], elevated levels have also been found in patients with bacteremia [141] and infection with hepatitis C virus [144]. In our study, two of the HIV-positive patients in the QFT negative control group had AIDS defining infections other than TB and had plasma levels of IP-10 that were above the median level of the HIV-infected active TB group. In addition, Clifford *et al* found no significant difference in serum IP-10 concentrations between patients with active TB and sick controls with lower respiratory tract infections caused by pathogens other than *Mtb* [220].

None potential biomarkers for differentiation between active and latent TB infection were identified when analysing background corrected TB antigen stimulated cytokine levels in QFT supernatants. However, the unstimulated Nil level of IL-1β, IL-1ra, IL-

9 and IL-17a were significantly lower in the active TB compared with the latent TB group. Several studies of biomarkers in QFT supernatants have previously been performed and unstimulated or stimulated levels of various markers have been found to differentiate between active and latent TB infection [159,221–224]. Still, the results show substantial variation and a review concludes that no clear pattern of candidate biomarkers have been identified [136]. Our findings add to the heterogeneous pattern of results. Whereas we and Chegou *et al* [223] found lower Nil levels of IL-1ra in active compared latent TB, two other studies have found higher levels in active compared with latent TB infection [224,225]. Also for the Nil levels of IL-1β and IL-17a higher and not lower levels have been reported in active TB compared with latent TB infection [224]. Opposed to our results in plasma, the level of IP-10 in the Nil QFT supernatant were not able to differentiate between active and latent TB infection. Other studies also show contradictory results. In a study of children, Chegou *et al* on reported that Nil levels of IP-10 differentiates between active and latent TB [223], whereas no differences were found in a study of adults [222].

In high and middle-income countries, WHO recommends systematic testing and treatment of latent TB in high risk individuals and IGRAs have been increasingly used for this purpose the last decade. However, the interpretation of IGRA results is complicated by relative high rates of conversions and reversions, and subjects with QFT baseline results around the diagnostic cut off (0.35 IU/mL) are more likely to have inconsistent results on serial testing [91–94]. When investigating the levels of markers in subjects with QFT test results in the borderline zone, we found that background corrected TB antigen stimulated levels of IP-10, IL-1ra and IL-2 significantly differentiated this group from QFT negative controls. As there is no gold standard for diagnosing latent TB, the diagnostic accuracy of alternative markers is difficult to assess. The IL-1ra and IP-10 levels in the QFT borderline group were not significantly different from neither the QFT high nor the active TB group, supporting true TB infection in the majority of the subjects in the QFT borderline group. Further

studies are needed to examine the variability of IL-1ra, IL-2 and IP-10 in serial testing.

5.5 Biomarkers for tuberculosis treatment efficacy

Accurate and rapid tools for monitoring of treatment efficacy would be a major advance as it would simplify TB drug treatment trials and prevent inadequate treatment. In accordance with others, we found a decrease in plasma levels of IP-10 during treatment of HIV negative active TB cases [138,140,145–147]. Azzuri *et al* reported that plasma levels of IP-10 increased in household contacts during progression to active TB and during relapse of TB in patients who previously had completed TB treatment [138]. Further, Hong *et al* showed that active TB cases with moderate to high risk of relapse decline less in IP-10 during treatment compared with low risk patients [145]. Taken together, these results support the use of IP-10 as a biomarker for monitoring treatment efficacy.

In contrast to IP-10, which showed a uniform decrease over time during treatment, the levels of sTNFr2 fluctuated, which limits its potential as a marker for monitoring treatment efficacy.

Markers to confirm successful therapy are also needed for latent TB infection as microbiological methods and IGRAs are unhelpful [128]. We examined changes in markers in QFT supernatants after preventive therapy of 15 individuals with latent TB. However, we did not identify any potential markers for the efficacy of preventive therapy. Background corrected TB antigen stimulated levels of IL-2 and IP-10, in addition to IFN-γ, remained significantly higher than in the QFT negative control group, indicating that the *Mtb* specific immune responses is maintained after treatment.

6. Conclusions

- There seems to be an increased level of T cell and monocyte activation in active TB, whereas large variation in the level of activated immune cells in the latent TB group supports the suggested understanding of TB infection as a continuous spectrum of disease ranging from true latency to subclinical and fulminant active disease.
- Treg cells may be involved in the immune response in both latent and active TB infection and our *in vitro* data indicate that indomethacin may modulate immune responses in active TB by reducing the fraction of *Mtb* specific Treg.
- The role of the observed indomethacin induced reduction of *Mtb* specific T cell cytokine production and proliferation is not clear and needs further evaluation in human models.
- The plasma level of IP-10 has potential to serve as a biomarker for monitoring treatment efficacy of active TB cases.
- Although not specific for TB, plasma level of IP-10 may give information about the stage of TB infection in both HIV-positive and HIV-negative individuals. However, it is questionable whether it is possible to establish a sufficient sensitive and specific test cut-off for use in clinical practice.
- TB antigen stimulated levels of IL-1ra, IL-2 and IP-10 differentiate individuals with borderline QFT values from controls and may improve differentiation between latent TB and non-TB infected individuals. However, inconsistency was seen and further studies are needed to determine proper cut-offs and the variability of these markers in serial testing.

7. Future perspectives

Early diagnosis and treatment of all people with TB are included in the key components of the WHO's end TB strategy. To reach the aim of a 90% reduction in TB incidence by 2035, intensified research and innovation are needed to improve the diagnostic tools and TB treatment.

The results of paper I and IV together with other studies of the role of Treg and COX/PGE2 in TB infection forms the basis for future animal studies and human clinical trials exploring the potential of COX-inhibitors as adjunct host directed therapy in TB disease. An ongoing clinical trial (ClinicalTrials.gov Identifier: NCT02503839) aims to study the immune effects and safety of the COX-2 inhibitor etoricoxib given to patients with active TB together with standard TB treatment. Further, another clinical trial estimating the potential efficacy and safety of using adjunctive ibuprofen for the treatment of XDR-TB have recently been registered (ClinicalTrials.gov Identifier:NCT02781909).

We have identified potential biomarkers that may improve TB diagnostics and monitoring of treatment efficacy. The number of participants in our studies was small, but the results are still relevant for choosing candidate markers for evaluation in larger studies. Future studies of IP-10 for monitoring treatment of efficacy should include a sufficiently large number of both drug sensitive and MDR, and pulmonary and extrapulmonary TB cases to examine whether there are any differences in IP-10 kinetics during treatment between these groups, and whether treatment failure are reflected by IP-10 levels. Larger studies are also needed to determine whether it is possible to establish sufficient sensitive and specific test cut-offs for the potential diagnostic biomarkers identified and to investigate the variability of these markers in serial testing.

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doi: 10.1111/j.1365-3083.2010.02496.x

T Regulatory Cells and Immune Activation in *Mycobacterium tuberculosis* Infection and the Effect of Preventive Therapy

I. Wergeland*, J. Aßmus† & A. M. Dyrhol-Riise*‡

*Institute of Medicine, University of Bergen, Bergen, Norway; †Centre for Clinical Research, Haukeland University Hospital, Bergen, Norway; and ‡Department of Medicine, Haukeland University Hospital, Bergen, Norway

Received 27 May 2010; Accepted 12 November 2010

Correspondence to: A. M. Dyrhol-Riise, Department of Medicine, Haukeland University Hospital, Jonas Lies vei 65, N-5021 Bergen, Norway. E-mail: anne.riise@medisin.uib.no

Abstract

Mycobacterium tuberculosis (TB) often causes persistent infection and many immune cell subsets and regulatory mechanisms may operate throughout the various stages of infection. We have studied dendritic cell (DC) subsets, regulatory T cells (Treg) and the expression of activation and apoptosis markers on $CD4^+$ and $CD8^+$ T cells in blood from patients with active TB (n = 20), subjects with positive QuantiFERON-TB GOLD (QFT) test (LTBI, latent TB infection) (n = 20) before and after 3 months of preventive anti-tuberculous therapy and from QFT-negative controls (n = 28). The frequency of $CD4^+CD25^+CD127^-$ Treg was highest in the group with active TB (P = 0.001), but also increased in the LTBI group (P = 0.006) compared to controls. The highest level of activated T cells, defined as $CD38^+$HLA-DR^+ cells, was found in the active TB group, for the $CD4^+$ T cell subset positively correlated to the level of $CD25^+CD127^-$ Treg ($P < 0.001$, r = 0.4268). After 3 months of preventive therapy, there was an increase in the fraction of $foxp3^+$ Treg, but no differences in markers of activation or apoptosis. In conclusion, there seems to be an increased level of immune activation and Treg in both latent and active TB infection that is only modestly influenced by preventive therapy.

Introduction

Mycobacterium tuberculosis (TB) infection is a major global health problem, especially in the developing world. In 2008, there were an estimated 8.9–9.9 million incident cases and approximately 2 million deaths from TB [1]. In addition, it is estimated that one-third of the world's population is infected by TB. If the immunological balance between host and pathogen is disturbed, reactivation of latent TB infection (LTBI) and development of active disease may occur. Globally, the human immunodeficiency virus (HIV) is the most dominant risk factor for reactivation of LTBI as well as contracting primary TB infection.

The cellular immune system plays a pivotal role in the immune defense against TB, and there is a critical balance between anti-TB T cell responses and immune-mediated pathology. TB induces a state of immune activation in the infected host, and an increased expression of activation markers on T cells in blood from patients with active TB has been described [2, 3]. T regulatory cells (Treg) are $CD4^+$ T cells involved in regulation of self-tolerance, autoimmunity and suppression of immune responses during infections [4, 5]. Treg cells were first recognized as $CD4^+$ $CD25^+$ T cells, but expression of the intracellular marker forkhead box p3 (foxp3) and low cell-surface expression of the IL-7 receptor α-chain (CD127) have been suggested as more accurate markers [6–8]. However, recent studies have questioned whether these markers represent different populations of Treg [9]. Patients with active TB seem to have higher levels of $CD4^+CD25^{high+}foxp3^+$ Treg cells in blood when compared to both subjects with LTBI and uninfected controls [10–12]. It has been shown that Treg depress T cell-mediated immune responses to protective TB antigens during active TB disease [11]. The level of Treg seems to decrease after 1 month of anti-tuberculous therapy [13].

Dendritic cells (DCs), professional antigen-presenting cells, initiate adaptive immune responses and stimulate induction and expansion of Treg [14]. Studies have shown that DCs serve an important role in the initiation and control of immune responses to TB [15]. Two DC subsets have been characterized in blood based on differences in phenotype markers and function; myeloid

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dendritic cell (mDC) and plasmacytoid dendritic cell (pDC) [16]. Decreased numbers of both DC subsets have been found in patients with active TB when compared to controls as well as increased pDC levels following successful anti-tuberculous therapy [17].

There is no accurate diagnostic gold standard for LTBI. However, the interferon-gamma release assays (IGRA), commercially available as the QuantiFERON-TB GOLD (QFT) and T-SPOT.TB tests, are more specific in the diagnosis of LTBI than the tuberculin skin test (TST) because they are unaffected by *Bacille Calmette Guérin* (BCG) vaccination and most infections with atypical mycobacteria. A meta-analysis including studies using microbiologically confirmed active TB and healthy low-risk individuals to assess sensitivity and specificity, respectively, conclude that the QFT test offers a overall sensitivity of 70–78% and a specificity of 96–99% when also immune suppressed individuals are included [18].

Little is known about the distribution and role of the various T cell and DC subsets in QFT-positive patients and the effects of preventive anti-tuberculous therapy. Thus, in this study, we have examined DC and Treg subsets and the expression of activation and apoptosis markers in $CD4^+$ and $CD8^+$ T cells from patients with active TB infection, subjects with positive QFT test before and after 3 months of preventive therapy and compared to QFT-negative controls to describe immune regulation in various stages of TB infection.

Methods

Study participants. Individuals referred to the TB outpatient clinic at Haukeland University Hospital, Bergen, Norway, for medical evaluation of latent or active TB disease based on a positive TST and/or suspected exposure of TB and patients diagnosed with active TB admitted to the inpatient ward were included in the study during the period of 2006–2007. The QFT-negative control group was also recruited from age-matched employees at the hospital with no known exposure to TB. There were no known HIV positives among the participants although they were not routinely tested as part of the clinical evaluation.

The TST was performed in the primary health care system according to standard procedures with 2 IU purified protein derivative RT 23 (2 TU) (Statens Serum Institute, Copenhagen, Denmark) and read after 72 h. According to national guidelines, an induration of ≥6 mm is considered a positive test [19]. The TST was performed between one and 3 months prior to inclusion. Overall, a total of 481 persons were referred to the TB outpatient clinic for QFT testing and examination of possible TB infection [20]. Thoracic X-ray and clinical examination were performed and an induced sputum sample was obtained for acid fast staining and culture.

Table 1 Characteristics of the study participants.

	Active TB (n = 20)	Latent TB (QFT^+) (n = 20)	Controls (QFT^-) (n = 28)
Age, mean (range)	35 (18–82)	33 (15–65)	33 (14–59)
Males/females	13/7	8/12	8/20
BCG vaccinated	13	15	26
Origin from TB endemic country	16	14	2
TST, mean (range) in mm	16 (10–20)	16 (9–23)	15 (6–23)*

BCG, *Bacille Calmette Guérin*; TB, tuberculosis; TST, tuberculin skin test.

*Mean and range for the twenty controls that were TST positive (≥6 mm). In addition, eight controls were TST negative (<6 mm).

Blood samples for further flow cytometry analyses were collected from randomly selected and approving individuals. The study subjects were classified into three groups; (1) Active TB (n = 20), (2) QFT-positive LTBI (n = 20) and (3) QFT-negative controls (n = 28). The ages, gender, BCG vaccination status, TST result and origin are described in Table 1.

In the active TB group, 16 patients had pulmonary TB and four had extrapulmonary TB. There was positive TB culture in 18 patients, whereas in two patients, diagnosis was based on histopathological findings in biopsies. The patients diagnosed with active TB were given standard anti-tuberculous treatment according to national guidelines [19]. Subjects with no signs of active TB based on X-ray, sputum examination and clinical evaluation and with a positive QFT test were defined as LTBI and offered preventive anti-tuberculous therapy with isoniazid and rifampicin for 3 months. The decision to treat was made by the clinician and the QFT test was known at the time of decision. Blood samples for flow cytometry analyses were obtained before start of any anti-tuberculous therapy, and for the LTBI group also at the end of therapy. Seventeen were followed with repetitive blood sampling at the end of therapy, whereas three were lost to follow up. 13/20 were still QFT positive, 4/20 had turned negative whereas in 3/20 no QFT test was performed. Because of logistic difficulties, we were not able to collect blood samples from the active TB group at the end of therapy or to perform longitudinal blood sampling from QFT-negative subjects not starting preventive therapy.

Written informed consent was obtained from all participants. The study was approved by the Regional Committee for Ethics in Medical Research (REK) in Bergen, Norway.

QuantiFERON-TB GOLD in-tube assay. The assay was performed according to the manufacturer's instructions (Cellestis International Pty Ltd., Chadstone, Vic., Australia). One ml of whole blood was added to each of the three QFT tubes containing TB antigen (ESAT-6, CFP-10 and TB 7.7 [p4]), mitogen-positive control

[phytohemagglutinin (PHA)] and a negative control, respectively. The tubes were incubated at 37 °C for 16–24 h, centrifuged and plasma removed. The amount of interferon-gamma (IFN-γ) in plasma was quantified by enzyme-linked immunosorbent assay (ELISA). The QFT Analysis Software (Cellestis International Pty Ltd) was used to analyse raw data (optical density values) and calculate results. The level of IFN-γ was corrected for background by subtracting the IU/ml value obtained for the respective negative control. The cut-off value for positive test was ≥0.35 IU/ml.

Flow cytometry analyses. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood using density gradient centrifugation (Lymphoprep™, Fresenius Kabi Norge AS, Halden, Norway), cryopreserved in 10% dimethyl sulfoxide (DMSO)/90% foetal calf serum (FCS) and stored in liquid nitrogen before analysis. Cryovials were thawed, washed and resuspended in RPMI media with 10% FCS to a final concentration of $4{\cdot}10^6$cells/ml. PBMC (50 μl) were incubated for 30 min in the dark with directly conjugated monoclonal antibodies (mAbs) to cell-surface markers of T lymphocytes; CD3-Phycoerythrin-Cyanin 7 (PC7) (clone UCHT1), CD4-PC7 [clone SFCI12T4D11 (T4)], CD8-Phycoerythrin-Cyanin 5 (PC5) (clone SFCI21Thy2D3) (Beckman Coulter (BC), Brea, CA, USA) and CD4-fluorscein isothiocynate (FITC) (clone SK3) (Becton Dickinson Biosciences (BDB), San Jose, CA, USA), activation; CD38-phycoerythrin (PE) (clone HB7), HLA-DR-FITC (clone L243), CD28-PE (clone L293) (BDB), CD28-energy couplet dye (ECD) (clone CD28.2) (BC), apoptosis; CD95-FITC (clone DX2) (BDB), regulatory T lymphocytes; CD25-ECD (clone B1.49.9) (BC), CD25-FITC (clone B1.49.9) (Immunotech-BC), CD127-FITC (clone eBioRDR5) (eBioscience, San Diego, CA, USA) and DC; HLA-DR- Peridinin-chlorophyll-protein complex (PerCP)-clone L243 (G46-6), Lineage 1 (CD3, CD14, CD16, CD19, CD20 and CD56)-FITC, CD11c-PE (clone S-HCL-3), CD123-PE (clone 9F5) (BDB). Anti-human foxp3-PE (clone PCH101) staining set (eBioscience) was

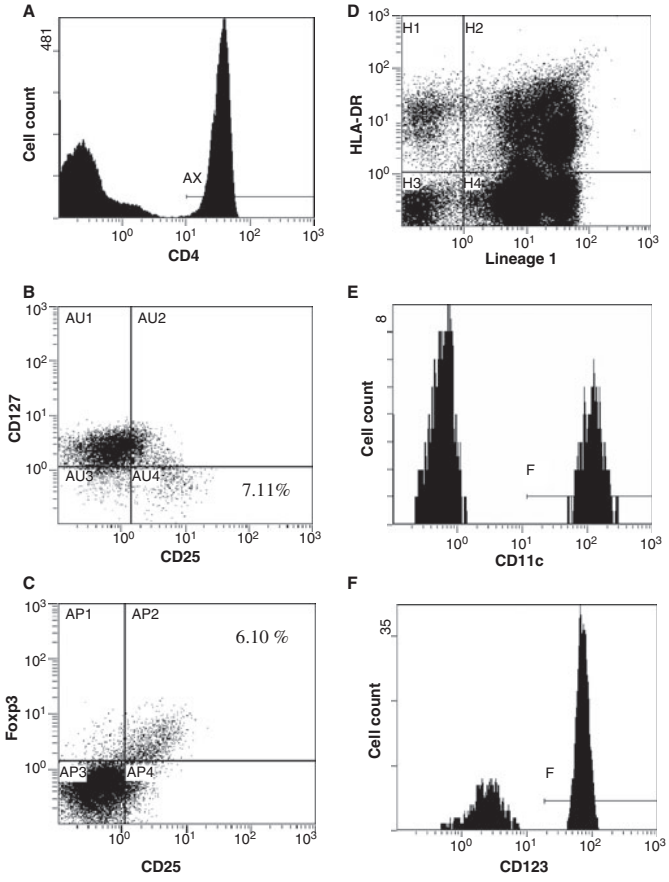

Figure 1 Flow cytometry analysis of peripheral blood mononuclear cells. (A)–(C) show the Treg subsets identified by gating from (A) $CD4^+$ T cells to (B) $CD25^+CD127^-$ $CD4^+$ T cells (quadrant 'AU4') and (C) $CD25^+foxp3^+$ $CD4^+$ T cells (quadrant 'AP2'), respectively. (D)–(F) show the dendritic cell subsets identified by gating from (D) $HLA\text{-}DR^+$ and Lineage (CD3, CD14, CD16, CD19, CD20 and CD56) negative PBMC (quadrant H1) to (E) and (F) for analysis of CD11c- and CD123-positive cells, respectively.

used for intracellular staining of foxp3. The cells were analysed on a Beckman Coulter Cytomics FC 500 MPL flow cytometry equipped with argon and diode laser for five-colour detection. Analyses were performed using MXP version 2.0 (Beckman Coulter, Inc., Brea, CA, USA) flow cytometry software. A gate was set on the lymphocytes according to forward and side scatter properties. Statistical regions were set according to isotype controls. For foxp3, the statistical marker was set at the upper cut-off for the CD4-negative population following the manufacturer's instruction. Treg subsets were defined as $CD25^+$/$foxp3^+$ or $CD25^+$/$CD127^-$ $CD4^+$ T cells (Fig. 1A–C). DC was analysed for the expression of CD11c and CD123 by gating from $HLA\text{-}DR^+$ Lineage (CD3, CD14, CD16, CD19, CD20 and CD56)-negative cells (Fig. 1D–F).

Statistical analyses. In a preliminary step, we investigated the data by using histograms and QQ plots for all cell subsets, and computing the Spearman correlations between all pairs of cell subsets. This was carried out for the entire data set and for each patient group. Spearman correlations were chosen because of their wider range of detectable relations. Investigating these 12 cell subsets leads to 66 tests, i.e. we have to take into account multiple effects. Because these tests are not independent, the Bonferroni level is too conservative. Thus, we used a significance level of 0.01.

The research question contains two different types of comparisons. Comparing the different groups (controls, LTBI and active TB), we used a two-step test procedure. First, we used a Kruskal–Wallis test to detect differences in cell subsets fractions between the groups. In the second step, we selected the cell subsets where the Kruskal–Wallis test detected a significant difference and tested the groups pairwise using a Wilcoxon test to decide where the differences detected by the Kruskal–Wallis test were located. In both cases, we used the Bonferroni significance level, i.e. 0.0042 for Kruskal–Wallis test (12 tests) and 0.0167 for the Wilcoxon test (three tests for each cell subset).

Comparing the pre/post-therapy measurements for the QFT^+ patients, we used a signed rank test, again with a Bonferroni level of 0.0042. In all investigated cases, we used non-parametric tests because the preliminary analysis indicated a non-Gaussian distribution at least for some of the variables. All statistical analyses were performed using Matlab R2010a.

Results

$CD4^+$ $CD25^+$ $CD127^-$ T regulatory cells increase in latent and active TB

There were no significant differences in the percentage of $CD4^+$ or $CD8^+$ T cells between any of the groups. Because Treg can be characterized by various immune markers possibly characterizing different Treg populations, we analysed both $CD4^+$ $CD25^+foxp3^+$ T cells (Fig. 2A) and $CD4^+$ $CD25^+CD127^-$ T cells (Fig. 2B). Both the active TB ($P = 0.001$) and the LTBI ($P = 0.006$) groups demonstrated significantly higher levels of $CD127^-$ Treg compared to the control group, whereas there was no significant difference between the LTBI and the active TB groups. Likewise, the highest level of $foxp3^+$ Treg was found in the active TB group, but for this Treg subset, there were no significant differences between any of the groups.

T cell activation increase in active, but not in latent TB

T cell activation was evaluated by the expression of the activation markers CD38, HLA-DR, the co-stimulatory molecule CD28 and the apoptosis marker CD95 (Fas receptor) on $CD4^+$ and $CD8^+$ T cells. For both the $CD4^+$ and the $CD8^+$ T cell subsets, the fraction of $HLA\text{-}DR^+CD38^+$ cells was higher in the active TB group compared to both the LTBI ($P < 0.01$) and the control ($P < 0.001$) groups (Fig. 3A,B). Likewise, the expression of CD28 on $CD8^+$ T cells was significantly lower in the active TB group compared with both the LTBI

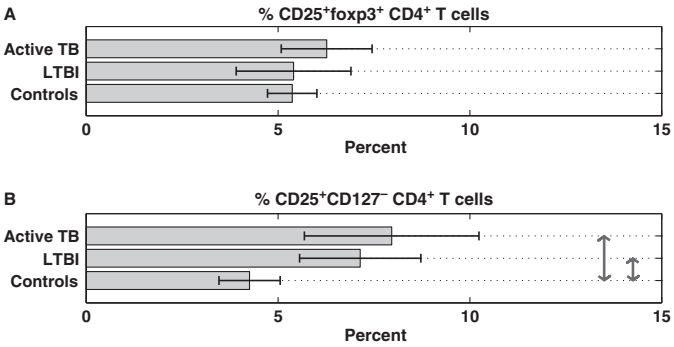

Figure 2 T regulatory cells. The fraction of $CD4^+$ T cells expressing (A) $CD25^+$ $foxp3^+$ and (B) $CD25^+$ $CD127^-$ in active tuberculosis (TB) ($n = 20$), latent TB infection before treatment (LTBI) ($n = 20$) and controls ($n = 28$). The bars represent the means with 95% symmetric confidence intervals. Double arrows show significant differences between the respective groups ($P < 0.0167$).

(P = 0.014) and control (P = 0.0001) groups, but no significant differences were found for the $CD4^+$ T cells (Fig. 3C,D). We found no significant differences in the expression of CD95 between any of the groups in any of the T cell subsets (Fig. 3E,F).

The relationship between T regulatory cells and T cell activation in TB

The possible association between the various T cell subsets was studied. When all groups were analysed together, there was a significant positive correlation between $CD127^-$ Treg and activated $CD4^+HLA\text{-}DR^+CD38^+$ T cells (P < 0.001, r = 0.4268) (Fig. 4A). This was also found for the $foxp3^+$ Treg although at a lower level of significance (P = 0.0113, r = 0.2689) (Fig. 4B). However, when the analyses were performed for each study group separately, the correlation between $CD127^-$ Treg and activated $CD4^+HLA\text{-}DR^+CD38^+$ T cells was maintained only in the control group. Further, the $foxp3^+$ Treg subset correlated positively with the expression of CD95 on both $CD4^+$ and $CD8^+$ T cells (P < 0.001, r = 0.4461 and r = 0.4325, respectively) (Fig. 4C,D), but again when the analyses were performed for each study group separately, the only correlation that remained was between $foxp3^+$ Treg and $CD95^+$ $CD4^+$ T cells in the

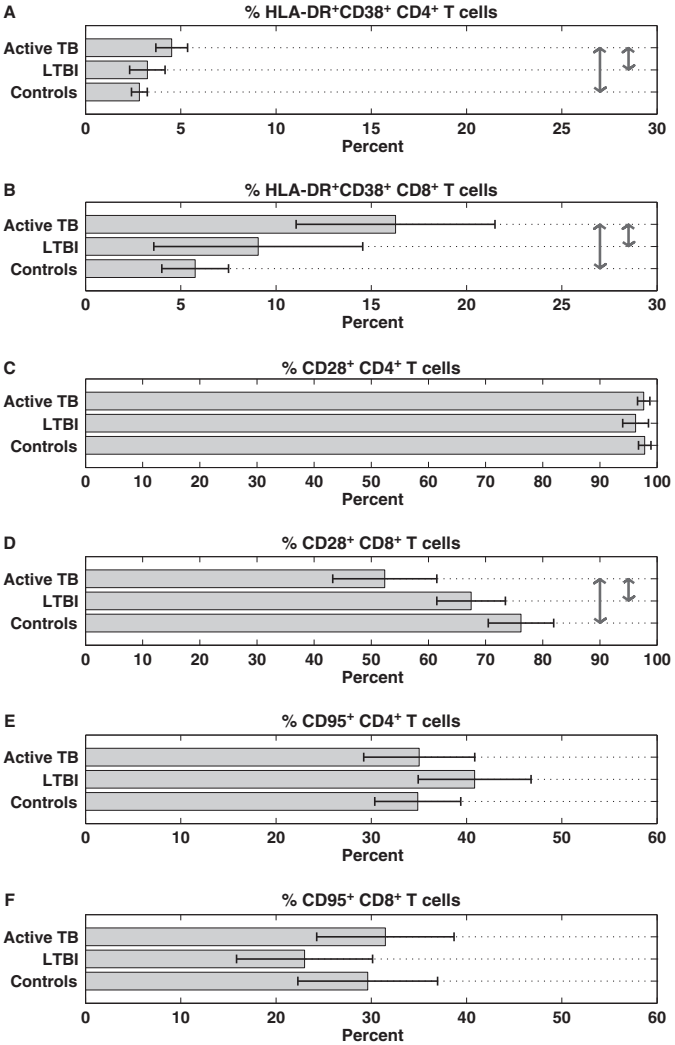

Figure 3 T cell activation and apoptosis. The fraction of $CD4^+$ and $CD8^+$ T cells co-expressing the activation markers HLA-DR and CD38 (A and B), the co-stimulatory molecule CD28 (C and D) and the apoptosis marker CD95 (E and F) in active tuberculosis (TB) (n = 20), latent TB infection before treatment (LTBI) (n = 20) and controls (n = 28). The bars represent the means with 95% symmetric confidence intervals. Double arrows show significant differences between the respective groups (P < 0.0167).

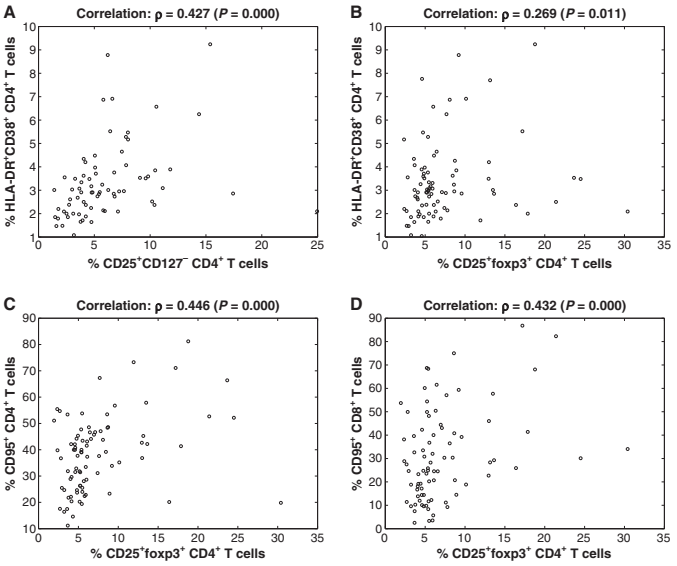

Figure 4 Correlations between T cell subsets. Scatter plots illustrating the correlation structure for different T cell subsets when all study groups were analysed together. % HLA-DR$^+$ CD38$^+$ CD4$^+$ T cells versus (A) % CD25$^+$CD127-CD4$^+$ T cell and (B) % CD25$^+$foxp3$^+$CD4$^+$ T cells. % CD25$^+$foxp3$^+$ CD4$^+$ T cells versus (C) % CD95$^+$CD4$^+$ T cells and (D) % CD95$^+$CD8$^+$ T cells. All given correlations are Spearman correlations and the *P*-values are for the test for vanishing correlation.

control group. No overall correlation was found between CD127$^-$ and foxp3$^+$ Treg except in the QFT-negative control group (P = 0.0014, r = 0.5735).

Myeloid and plasmacytoid dendritic cells in TB

Dendritic cells were phenotyped as CD11c$^+$ mDC or CD123$^+$ pDC. We found no significant difference in the proportions of mDC or pDC among PBMC between any of the groups (Fig. 5).

Effects of preventive therapy on T cell activation and regulation in latent TB

The percentage of foxp3$^+$ Treg increased in the QFT$^+$ group after preventive anti-TB treatment to a level significantly higher than that found before initiation of therapy (P = 0.0008), whereas no statistical significant difference was observed for the CD127$^-$ Treg subset (Fig. 6). We found no significant changes in the expression of activation or apoptosis markers on CD4$^+$ or CD8$^+$ T cells or in the fractions of the DC subsets.

Because of a low number of subjects converting to QFT negative after treatment (4/20), we could not perform statistical analyses of possible differences between converters and subjects who remained QFT positive (13/20). However, there seems to be a trend towards increased expression of HLA-DR and CD38 on CD8$^+$ T cells in subjects who remained QFT positive indicating persistent immune activation. The subjects converting to QFT negative contributed predominantly to the increase in foxp3$^+$ Treg seen after therapy (data not shown).

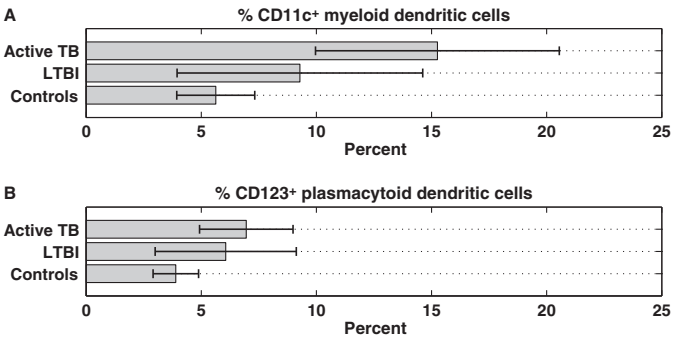

Figure 5 Myeloid and plasmacytoid dendritic cells. The fraction of CD11$^+$ myeloid dendritic cells (A) and CD123$^+$ plasmacytoid dendritic (B) cells of peripheral blood mononuclear cells from active tuberculosis (TB) (n = 20), latent TB infection before treatment (LTBI) (n = 20) and controls (n = 28). The bars represent the means with 95% symmetric confidence intervals.

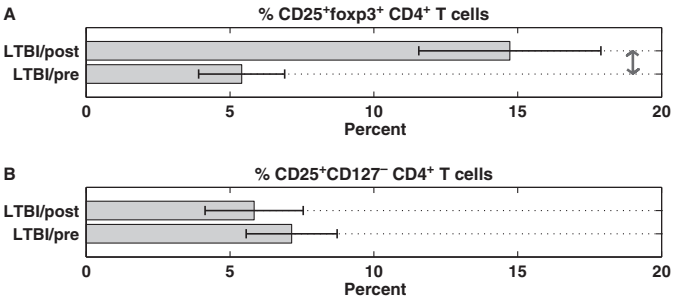

Figure 6 T regulatory subsets before and after preventive anti-tuberculous treatment. The fraction of $CD25^+foxp3^+$ (A) and $CD25^+CD127^-$ Treg (B) in LTBI before (LTBI/pre) and after (LTBI/post) 3 months of prophylactic anti-tuberculous treatment. The bars represent the means with 95% symmetric confidence intervals. Double arrows show significant differences between the respective groups ($P < 0.0167$).

Discussion

The role of the various T cell and DC subsets in TB infection and their contribution to immunopathogenesis in disease progression has not been clarified. We found that the level of blood Treg, identified as $CD4^+CD25^+CD127^-$ T cells, was higher in both the active TB and the LTBI groups compared to QFT-negative controls. In contrast, increased T cell activation was predominately found in the active TB group. The proportions of mDC and pDC subsets were comparable between the study groups. After 3 months of preventive anti-tuberculous therapy, there was an increase in the fraction of $foxp3^+$ Treg in patients with LTBI , but we observed no differences in the expression of activation or apoptosis markers on T cells.

Increased levels of T cell activation have been described in patients with active pulmonary TB and are even more pronounced in HIV/TB co-infected patients [2, 3]. Consistent with these studies, we found an increased expression of the activation markers CD38 and HLA-DR and a corresponding lower expression of the co-stimulatory molecule CD28 on $CD8^+$ T cells from patients with active TB. The level of $CD4^+$ T cell activation was also increased in patients with active TB. Although large variations among the subjects in the LTBI group were seen, our data indicate that immune activation gradually increases throughout the various stages of TB infection corresponding to the level of bacterial burden.

There have been few studies of Treg in patients with LTBI [21]. High levels of circulating Treg have previously been found in patients with active TB [10–12], but our data demonstrate that CD127-negative Treg are elevated already from the latent stage of infection. Studies have shown that $CD4^+CD25^{high+}foxp3^+$ Treg cells are elevated in active TB compared with both uninfected controls [10] and subjects with LTBI [11, 12]. In another study, the level of Treg in patients with active TB decreased after 1 month of anti-tuberculous therapy [13]. In a TB case contact study, the level of foxp3 mRNA was lower in the TB ELISPOT-positive contacts compared to the TB ELISPOT-negative contacts and both groups had lower levels than that found in patients with active TB [22]. In contrast, when we used CD25 and foxp3 as markers of Treg, we found no significant differences between the study groups.

It has been suggested that $CD127^-$ Treg and $foxp3^+$ Treg possibly represent different populations [9]. In our study, a correlation between these two Treg subsets was found only in the control group. In a study of HIV infection, the positive correlation between $foxp3^+CD127^-$ and $CD25^+CD127^-$ $CD4^+$ T cells found in healthy HIV-negative subjects was not present in the early chronic stage of HIV infection [23]. Together these data indicate that different Treg may contribute in various stages of chronic infections. It has been shown that depletion of $CD4^+CD25^{high}$ and $CD4^+CD25^+foxp3^+$ cells from PBMCs from patients with TB, results in increased production of IFN-γ upon TB stimulation [10, 11, 24], indicating that there is an inverse correlation between Treg and immune activation. In contrast, although the immunosuppressive function of Treg was not characterized in our study, we found a positive correlation between the fractions of Treg and activated $CD4^+$ T cells.

DC can initiate immune responses and stimulate induction and expansion of Treg [14]. Absolute numbers of DC have been shown to decrease in patients with TB compared to healthy controls [17]. Still, although the numbers of pDC and mDC were not estimated, in our study, we did not find any differences in the fraction of DC subsets among the various groups or any correlation between DC and Treg subsets.

Altogether, these data suggest that different Treg subsets may have different capability to regulate immune activation and that modulation may be induced by different signals in the various stages of TB infection. As we found gradually higher fractions of $CD127^-$ Treg throughout the various stages of TB infection correlating to immune activation, a possible theory is that higher bacterial burden and inflammation stimulate to increased levels of Treg to balance between anti-TB T cell responses and immune-mediated pathology. In support of

this, in a study of macaques, there were increased frequencies of Treg cells in blood as the animals developed disease [25]. An alternative explanation may be that Treg inhibit protective Th1 responses facilitating mycobacterial replication and act as a causative factor in the progression to active disease [12]. We found an increase in foxp3$^+$ Treg after preventive anti-TB treatment. Our very limited data demonstrate that this was most dominant in patients converting to QFT negative and with reduced CD8$^+$ T cell activation after treatment, possibly indicating that expansion of this Treg subset contributes to suppression or eradication of TB.

Apoptosis of TB reactive T cells may account for the depression of TB-induced T cell responses seen in active TB, but data are conflicting [3, 26]. CD95 (Fas receptor), which upon ligation with Fas ligand induces an apoptotic death signal, was expressed by a higher proportion of CD8$^+$ T cells and a lower proportion of CD4$^+$ T cells in patients with pulmonary TB [3]. To our knowledge, no such studies have been performed in patients with LTBI. In our study, we could not demonstrate any differences in the expression of CD95 on T cells in the various study groups, but there was a positive correlation between foxp3$^+$ Treg and the expression of CD95 on both CD4$^+$ and CD8 T cells.

In this study, patients with no signs of active TB based on X-ray and clinical evaluation, and with a positive QFT test, were assumed having LTBI. The QFT test is more specific in the diagnosis of LTBI than the TST and at a 90% certainty threshold LTBI is best diagnosed by the QFT test in immunocompetent persons [27]. The TST-positive/QFT-negative subjects in our study consisted predominately of ethnic Norwegians with little risk of TB infection [20]. They are probably not TB infected and believed to have false-positive TST because of previous BCG vaccination.

There are some limitations of our study. First, immune responses specific to TB were not evaluated. Our findings may therefore be influenced by immune activation mediated by other stimuli than TB. However, both the LTBI and the control groups were all healthy at inclusion. Second, we had no samples available for analyses from the active TB group after therapy, where, due to higher bacterial burden, we would expect larger effects on T cell subsets in response to treatment. Third, as often carried out in such studies because of logistics, flow cytometry analyses were performed on cryopreserved PBMCs possibly affecting the results. To minimize this problem, the lymphocyte gate was set according to forward and side scatter properties excluding dead cells. Finally, we have studied cells from peripheral blood rather than from the disease compartment itself. Studies were clinical samples from disease sites have been compared with time matched blood samples indicate that results from peripheral blood give an attenuated picture of events at the disease site [10, 24]. In macaques studies, it has also been shown that right after infection the frequency of Treg cells in peripheral blood rapidly decreased whereas they increased in the airways [25]. Still, we believe our results are valid because we have demonstrated differences between the TB groups and controls that could be explained by biological mechanisms.

In conclusion, there seems to be an increased level of immune activation including interactions of different Treg subsets in active and LTBI still present at the end of preventive therapy. The results indicate that different Treg subsets may have different functions and that the degree of bacterial burden and immune activation is associated with the level of CD127$^-$ Treg in patients with TB infection.

Acknowledgment

We want to thank Steinar Sørnes, Institute of Medicine, University of Bergen, Norway for technical support and the staff at the Department of Pulmonary medicine, Haukeland University Hospital for help recruiting patients. The study was supported by L Meltzers Høyskolefond.

Authors Contributions

Ida Wergeland has performed the experiments and participated in interpretation of data and preparation of the manuscript. Jörg Aßmus has performed the statistical analyses. Anne Ma Dyrhol-Riise has designed the study, participated in interpretation of data and preparation of the manuscript.

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Journal of Infection (2015) 70, 381–391

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IP-10 differentiates between active and latent tuberculosis irrespective of HIV status and declines during therapy

I. Wergeland [a], N. Pullar [b,c], J. Assmus [d], T. Ueland [e,f], K. Tonby [e], S. Feruglio [e], D. Kvale [e,g], J.K. Damås [h,i], P. Aukrust [e,f,j], T.E. Mollnes [e,h,k], A.M. Dyrhol-Riise [a,e,g,*]

[a] Department of Clinical Science, University of Bergen, N-5021 Bergen, Norway
[b] Department of Internal Medicine, Section for Infectious Diseases, University Hospital of Northern Norway, N-9038 Tromsø, Norway
[c] Department of Clinical Medicine, University of Tromsø, N-9037 Tromsø, Norway
[d] Center for Clinical Research, Haukeland University Hospital, N-5020 Bergen, Norway
[e] Institute of Clinical Medicine and K.G. Jebsen IRC, University of Oslo, N-0424 Oslo, Norway
[f] Research Institute of Internal Medicine, Oslo University Hospital, N-0424 Oslo, Norway
[g] Department of Infectious Diseases, Oslo University Hospital, N-0424 Oslo, Norway
[h] Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim, Norway
[i] Department of Infectious Diseases, St Olav's Hospital, Trondheim, Norway
[j] Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, N-0424 Oslo, Norway
[k] Research Laboratory, Nordland Hospital, Bodø, and Faculty of Health Sciences, K.G. Jebsen TREC, University of Tromsø, Tromsø, Norway

Accepted 17 December 2014
Available online 15 January 2015

KEYWORDS
Cytokines;
Biomarker;
IP-10;
sTNFr2;
Tuberculosis;
HIV;
Therapy

Summary *Objectives:* Biomarkers for diagnosis and therapy efficacy in tuberculosis (TB) are requested. We have studied biomarkers that may differentiate between active and latent TB infection (LTBI), the influence of HIV infection and changes during anti-TB chemotherapy.
Methods: Thirty-eight plasma cytokines, assessed by multiplex and enzyme immunoassays, were analyzed in patients with active TB before and during 24 weeks of anti-TB chemotherapy (n = 65), from individuals with LTBI (n = 34) and from QuantiFERON-TB (QFT) negative controls (n = 65). The study participants were grouped according to HIV status.

* Corresponding author. Department of Infectious Diseases, Oslo University Hospital, PO Box 4956, Nydalen, NO-0424 Oslo, Norway. Tel.: +47 92857261; fax: +47 22119181.
E-mail address: a.m.d.riise@medisin.uio.no (A.M. Dyrhol-Riise).

http://dx.doi.org/10.1016/j.jinf.2014.12.019

Results: Plasma levels of the CXC chemokine IP-10 and soluble TNF receptor type 2 (sTNFr2) significantly differentiated active TB from the LTBI group, irrespective of HIV status. In the HIV-infected group the sensitivity and specificity was 100% for IP-10 with a cut-off of 2547 pg/mL. Plasma IP-10 declined gradually during anti-TB chemotherapy (12—24 weeks, $p = 0.002$) to a level comparable to LTBI and QFT negative control groups. sTNFr2 fluctuated throughout therapy, but was decreased after 12—24 weeks ($p = 0.006$).
Conclusions: IP-10 distinguished with high accuracy active TB from LTBI irrespective of HIV infection and declined during anti-TB chemotherapy. Plasma IP-10 may serve as a diagnostic biomarker to differentiate between the stages of TB infection and for monitoring therapy efficacy.

Introduction

Tuberculosis (TB) is a major global health problem, especially in the developing world. The World Health Organization estimates that one third of the world's population has latent TB infection (LTBI), and that there were 9.0 million incident active TB cases and 1.5 million deaths caused by TB in 2013.[1] Human immunodeficiency virus (HIV) infection is globally the major risk factor contributing to the resurgence of TB,[2] and the emergence of multi- and extensively drug resistant (MDR and XDR) strains of *Mycobacterium tuberculosis* (*Mtb*) have further aggravated the situation.[1]

Strategies for TB control aim to reduce transmission by identification and treatment of infectious cases as well as to reduce development of active disease by offering preventive therapy to individuals with LTBI. However, there is shortage of reliable and rapid diagnostic tools that can identify and discriminate between latent and active TB, and predict therapy responses.[3,4] The currently available tests for diagnosing LTBI are the century old tuberculin skin test (TST) and the more recent interferon gamma (IFN-γ)-release assays (IGRAs). The IGRAs offer better specificity compared to the TST, but both tests have suboptimal sensitivity in HIV co-infected individuals, and cannot distinguish between the various stages of TB infection or be used for evaluation of treatment efficacy.[3,5] Sputum culture conversion within the first 2 months of anti-TB chemotherapy could serve as a predictor of a favorable outcome,[6,7] but may also fail as predictive marker of cure in individual patients,[8,9] and are not applicable in extra-pulmonary and culture negative TB infection. Further, the need of alternative tools for monitoring treatment efficacy and for identifying patients at high risk of relapse is emphasized by the challenges of MDR- and XDR-TB cases, which require long lasting treatment with potentially inefficient and toxic drugs. Tools for monitoring treatment are also essential for accelerating drug development.

Alternative biomarkers have been sought to improve immune diagnosis of TB and monitoring of treatment efficacy. Various single or combinations of markers have been suggested to differentiate between latent and active TB infection.[10—15] However, a recent review concludes that there is no clear pattern of markers able to differentiate between the various TB infection stages.[16] Tests based on IFN-γ -inducible Protein 10 (IP-10)/CXCL10, one of the most studied surrogate biomarkers, perform comparably to the QuantiFERON-TB (QFT) in most patient groups,[17] but may increase the diagnostic accuracy of TB infection in both HIV-infected individuals[18—20] and children.[21—24] It has also been suggested that IP-10 can be used to monitor treatment efficacy,[14,25—29] but longitudinal studies performed on diverse patient populations are limited.

The aims of this study were to examine the potential of 38 selected cytokines, including interleukins, chemokines and growth factors to differentiate between the stages of TB infection as well examine the influence of HIV co-infection and the changes in these markers during anti-TB chemotherapy. Plasma IP-10 appeared to be the most consistent of the biomarkers studied, as it was the only marker that significantly differentiated active TB from both the LTBI and QFT negative control groups irrespective of HIV status and also gradually and significantly declined during anti-TB chemotherapy.

Materials and methods

Study participants and sample collection

The study population was included from clinical studies performed at three different hospitals in Norway in the period 2006—2012; Haukeland University Hospital,[30,31] University Hospital of Northern Norway[31] and Oslo University Hospital.[32] The study participants were recruited from patients diagnosed with active TB and from individuals referred to the hospitals for medical evaluation of LTBI based on a positive TST and/or exposure of TB.[30,32] HIV-infected individuals were recruited from outpatient infectious disease clinics.[31] QFT negative/HIV-uninfected individuals from age-matched employees with no known exposure to TB served as controls.

Thoracic X-ray and clinical examination were performed in all participants and an induced sputum sample and/or biopsy was obtained for acid fast staining and culture by BACTEC 960 MGIT liquid culture media (BD) or Lövenstein Jensen solid media. The patients diagnosed with active TB were given standard anti-TB chemotherapy according to national guidelines.[33] Subjects with a positive QFT, but no clinical signs or symptoms of active TB and no prior TB that could explain QFT positivity, were defined as LTBI.

Blood samples were obtained before start of any anti-TB chemotherapy and drawn into EDTA tubes. Plasma was

harvested after centrifugation, snap-frozen and stored at −80 °C until analysis. Plasma samples from patients with active TB were also obtained at 2–4 weeks, 6–12 weeks and 12–24 weeks after the start of anti-TB chemotherapy.

Written informed consent was obtained from all participants. The study was approved by the respective Regional Committees for Ethics in Medical Research (REK-Vest, REK-Nord and REK-Sør-øst).

QuantiFERON-TB Gold In-tube assay

The QuantiFERON TB-Gold In-tube® assay was performed according to the manufacturer's instructions (Cellestis Ltd, Qiagen, Chadstone, VIC, Australia) at the respective hospitals. The cut-off value for positive test was $\geq$0.35 IU/ml.

Multiplex cytokine analysis

Plasma samples were analyzed using a multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA) containing assays for interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (bFGF), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), IFN-γ, eotaxin/CCL11, IP-10/CXCL10, macrophage chemotattractant protein 1 (MCP-1)/CCL2, macrophage inflammatory protein 1 alpha (MIP-1α)/CCL3, MIP-1β/CCL4, regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5, tumor necrosis factor (TNF), platelet-derived growth factor -BB (PDGF-BB) and vascular endothelial growth factor (VEGF).

The samples were analyzed on a Multiplex Analyser using Bio-Plex Manager 6.0 (Bio-Rad Laboratories) according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation were <12% for all analytes. Nine of the markers evaluated (IL-1β, IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-15 and GM-CSF) were below the lower detection level (LDL) or detected at very low levels in all samples (>80% below the LDL of the assay), and were therefore excluded from further statistical analysis. The remaining occasional values (<20%) below the LDL were replaced by a defined common value below the LDL (0.001), which allowed non-parametric statistical analysis.

Enzyme immunoassays (EIA)

Plasma levels of CXCL16, pentraxin 3 (PTX3), soluble TNF receptor 2 (sTNFr2), Fas Ligand (FasL), thymus and activation regulated chemokine (TARC)/CCL17), osteoprotegerin (OPG), activated leukocyte cell adhesion molecule (ALCAM), IL-23, secreted frizzled-related protein 3 (sFRP3) and C-reactive protein (CRP) were measured by EIAs (R&D Systems, Stillwater, MN). Intra-and inter-assay coefficients of variation were <10% for all except FasL (<15%). MD-2 was analyzed by an in-house ELISA as previously described.[34] Briefly, MD-2 was captured on immune plates coated with a TLR-4-Fc fusion protein and detected with digoxygenin labelled MD-2 mAb 5D7 or digoxigenin labelled IIC1 and anti-digoxygenin-HRP (Roche). The MD-2 standard was purchased from R&D Systems, Stillwater, MN.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistics 21, Matlab 7.10. or GraphPad Prism 6. We initially analyzed the HIV-infected and HIV-uninfected TB groups separately using Mann–Whitney U-test to detect pairwise differences between the study subgroups at baseline. In addition, we applied a binary logistic regression model to evaluate whether each of the markers was able to differentiate between active, LTBI and QFT negative controls pairwise, when adjusted for age, sex and HIV-status.

Mann–Whitney U-test was used to compare HIV-uninfected MDR-*Mtb* vs. drug sensitive *Mtb*, and extrapulmonary vs. pulmonary active TB cases, at time points where the sample size in each of these groups were $n \geq 5$. Wilcoxon signed rank test was used to evaluate changes in the levels of markers in patients with active TB from baseline to each follow up time point during anti-TB chemotherapy. Spearman's correlations were used to detect relationships between the CD4 counts and selected markers in the HIV-infected group.

The general significance level was set to 0.05. Taking into account effects of multiple testing, the Bonferroni adjustment would be too conservative due to the dependence between the markers. Thus, we decided to use a marginal significance level of 0.005 (corresponding to Bonferroni adjustment for 10 tests), and to describe results with p-values equal to or above 0.005 as significant and less than 0.05 ($0.005 \leq p < 0.05$) as tendencies.

Receiver operator characteristic (ROC) curve analyses were performed for the most promising marker differentiating between active and LTBI based on the results of the aforementioned tests. The optimal cut-off levels were defined by the minimum Euclidian distance to maximal specificity and sensitivity.

Results

Study participant characteristics

A total of 164 study participants had plasma samples available for analysis and these were classified into three groups; 1) Active TB (n = 65), 2) QFT-positive LTBI (n = 34) and 3) QFT negative controls (n = 65). The patients were further grouped according to HIV status. The clinical characteristics of the study participants are summarized in Table 1. Six (9.2%) of the patients with active TB, 23 (67.6%) of the individuals with LTBI and 52 (80.0%) of the QFT negative controls were HIV-infected. A total of 51 (63%) of the HIV patients were treated with antiretroviral therapy at inclusion in the study. Two of the QFT negative HIV-infected controls had acquired immune deficiency syndrome (AIDS)-defining infections (*Herpes simplex virus*, *Mycobacterium avium complex*, *Varicella zoster virus*), with high HIV viral load and CD4 counts below 100, but with no clinical signs of TB. AIDS-defining infections were not seen in the LTBI group.

The active TB diagnosis was based on positive *Mtb* culture in 60 of the patients, whereas in five patients the

Table 1 Characteristics of the study participants.

	Active TB (n = 65)		LTBI (QFT-positive, n = 34)		QFT-negative controls (n = 65)	
	HIV-infected (n = 6)	HIV-uninfected (n = 59)	HIV-infected (n = 23)	HIV-uninfected (n = 11)	HIV-infected (n = 52)	HIV-uninfected (n = 13[a])
Median age (range)	46 (38–64)	30 (16–91)	38 (21–48)	44 (24–61)	37 (19–68)	36 (26–70)
Sex: males/females	4/2	29/30	13/10	4/7	24/28	5/8
Origin: TB high/low endemic country	5/1	52/7	23/0	8/3	34/18	1/12
Median CD4 count (cells/μL, range)	286 (50–425)	ND	475 (110–1870)	ND	394 (3–1270)	ND
HIV patients on antiretroviral therapy (%)	4 (67)	ND	9 (39)	ND	38 (73)	ND
TB localization: pulm./ extrapulm./disseminated	6/0/0	39/18/2				
MDR-TB/mono-resistant TB[b]	1/1	10/4				
Culture: positive/negative or not performed	5/1	55/4				

[a] 5 QFT-negative/HIV-uninfected controls were included in the Multiplex bead assay. 13 QFT-negative/HIV-uninfected controls were included in the ELISA assays. ND: Not determined/relevant.
[b] Monoresistance against isoniazid, streptomycin or pyrazinamide.

diagnosis was based on clinical evaluation and characteristic histopathological or radiological findings. There were 11 MDR-TB cases and two patients with disseminated TB disease.

The patients with active TB had plasma samples obtained at 2–4 weeks (n = 24), at 6–12 weeks (n = 24) and at 12–24 weeks (n = 20). Only two HIV-infected patients with active TB had available plasma samples during treatment, and these were therefore excluded from the statistical analyses. All the patients with plasma samples available during treatment were successfully treated, except one patient who developed concomitant serious disease with intestinal perforation and septicaemia leading to death after 8 weeks of therapy. Thus, this patient was excluded from the follow-up analyses.

Impact of HIV infection on cytokine levels in various stages of TB infection

We found significant higher levels of IP-10 and eotaxin ($p < 0.005$) and a tendency of higher levels of FasL and sTNFr2 ($0.005 \leq p < 0.05$) in HIV co-infected compared with HIV-uninfected active TB cases (Figs. 1 and 2). In contrast, there was no significant impact of HIV infection on the levels of any of the markers neither in subjects with LTBI nor in QFT negative controls, although a tendency of increased IP-10 and CXCL16 levels ($0.005 \leq p < 0.05$) were seen in the HIV-infected compared to the HIV-uninfected LTBI group. Among QFT negative controls a tendency of decreased level of MIP-1α, MIP-1β, sFRP3 ($0.005 \leq p < 0.05$) and increased level of OPG ($0.005 \leq p < 0.05$) were observed in HIV-infected compared with HIV-uninfected individuals.

We further analyzed whether there were any correlations between the CD4 count and the level of IP-10 and eotaxin, respectively, in the HIV-infected individuals. We found a significant negative correlation between the CD4 count and the level of IP-10 ($p < 0.005$, $r = -0.349$) (Fig. 3), but not with eotaxin ($p = 0.098$, $r = -0.185$).

Cytokines differentiating between the various stages of TB infection

Since we observed a significant impact of HIV co-infection on the pattern of biomarkers in patients with active TB we analyzed the HIV-infected and HIV-uninfected TB groups separately.

In HIV-infected individuals there were significantly higher levels of IP-10 and sTNFr2 in the active TB group compared with both the LTBI group and QFT negative controls ($p < 0.005$, Fig. 2), and there was a tendency of PTX3, eotaxin and MCP-1 differentiating between active and LTBI ($0.005 \leq p < 0.05$). In contrast, in HIV-uninfected individuals, PTX3 was the only marker that significantly differentiated between active and LTBI ($p < 0.005$, Fig. 2), but in addition IP-10, IL-8, VEGF, MD-2 and sFRP3 showed a tendency of differentiating ($0.005 \leq p < 0.05$). However, several markers significantly distinguished (IL-8, PDGF-BB, TARC and CRP, $p < 0.005$) or tended to distinguish (sFRP3, IL-7, eotaxin, OPG and CXCL16, $0.005 \leq p < 0.05$) active TB from QFT negative controls.

IP-10 and sTNFr2 also differentiated significantly ($p < 0.005$) between active TB and both LTBI and QFT negative controls when a binary logistic regression model adjusting for HIV status, age and gender was applied. In addition, in this model, PTX3 and CRP significantly differentiated between active TB and QFT negative controls ($p < 0.005$), and tended to differentiate between active TB and LTBI ($0.005 \leq p < 0.05$).

The diagnostic accuracy of IP-10 in differentiating between active TB, LTBI and the QFT negative control group was investigated by ROC curve analyses (Table 2). In HIV-infected individuals a cut-off level of 2547 pg/mL

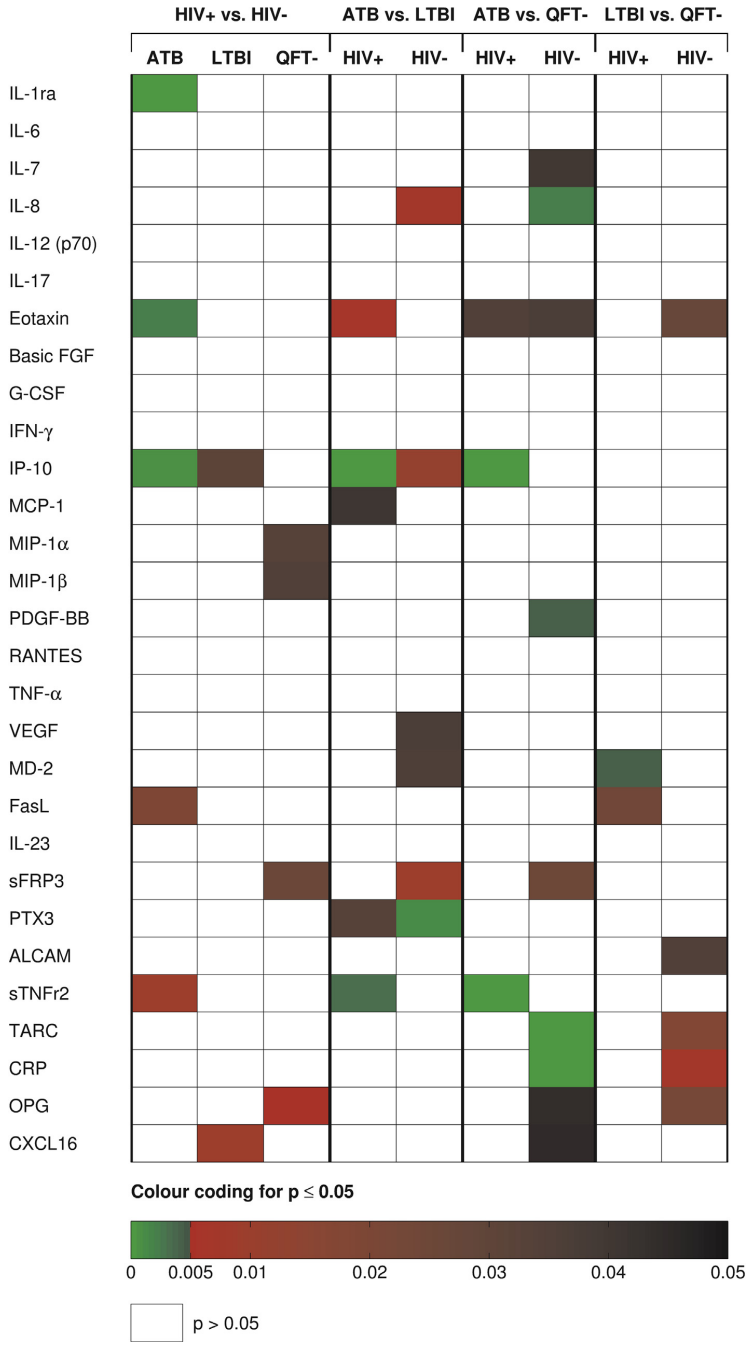

Figure 1 Comparisons of cytokine levels between patients with active TB, latent TB and QFT-negative controls and between HIV-infected and HIV-uninfected patients (Mann—Whitney U test). Significant p-values ($p < 0.005$) are shown as a colour scale in green, and p-values described as tendencies ($0.005 \leq p < 0.05$) are shown as a colour scale ranging from red to black. ATB: active TB infection. LTBI: Latent TB infection. QFT-: QuantiFERON-TB negative controls.

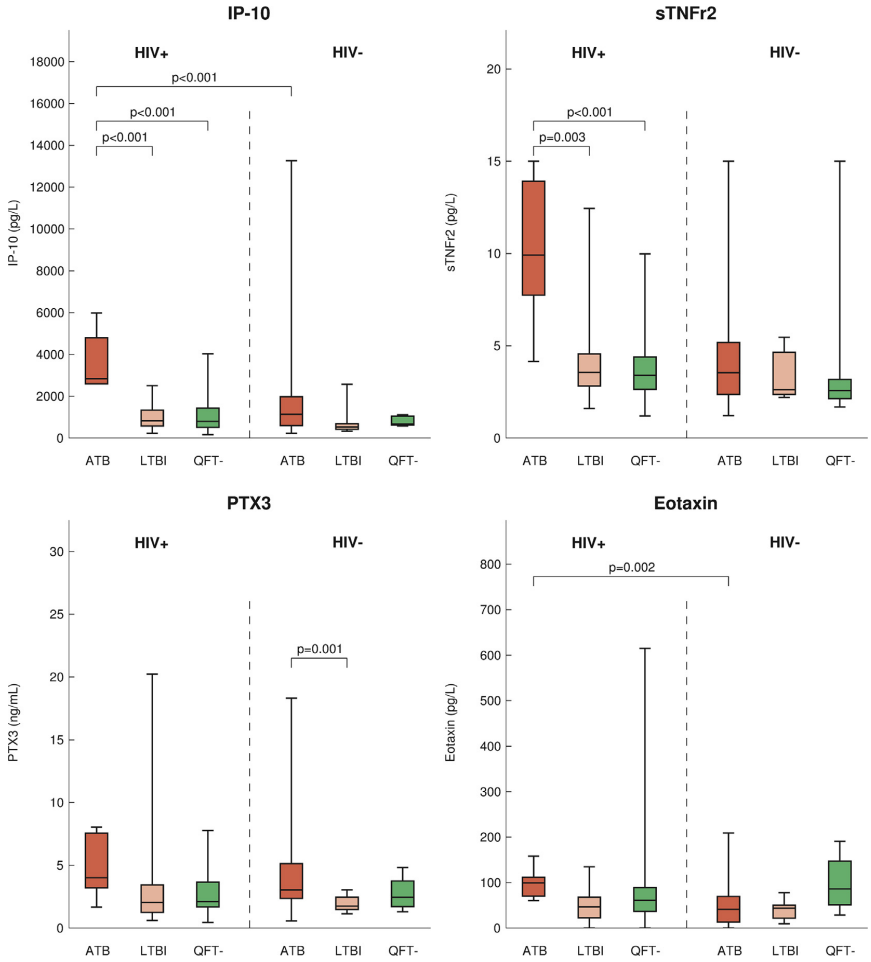

Figure 2 Plasma levels of IP-10, sTNFr2, PTX3 and eotaxin in HIV-infected and HIV- uninfected patients with active TB, latent TB and in QFT negative controls. The boxes show the median and interquartile range, and the whiskers show minimum and maximum values. Statistical analysis was performed using the Mann—Whitney U test. Brackets represent statistically significant differences ($p < 0.005$).

discriminated active TB from LTBI with 100% sensitivity and specificity, whereas in HIV-uninfected individuals the optimal cut-off level was 689 pg/mL, and the sensitivities and specificities were 71% and 82%, respectively.

Changes in cytokine levels during anti-TB chemotherapy

In HIV-uninfected patients with active TB, we analyzed the levels of the various markers at different time-points throughout therapy. The level of IP-10 decreased gradually during treatment. There was a tendency of decrease observed already after 6—12 weeks ($p = 0.022$, Fig. 4). However, the decrease was significant first after 12—24 weeks ($p = 0.002$), and the level was then comparable to that seen both among the LTBI and in the QFT negative control group. By using the optimal cut-off level of 674 pg/mL for differentiation between active TB and non-TB in HIV-uninfected patients, 43/59 (73%) of the active TB patients scored positive for plasma IP-10 at baseline, whereas 9/20 (45%) were positive at week 12—24. Only one patient with extrapulmonary TB scored negative at baseline and became positive during treatment. However, this patient had IP-10 levels close to cut-off both at baseline and during therapy. sTNFr2 tended to decrease to the level of LTBI cases and QFT negative controls after 12—24 weeks of treatment ($p = 0.006$), but in contrast to IP-10, which showed a uniform decrease over time, the level of sTNFr2 fluctuated (Fig. 4).

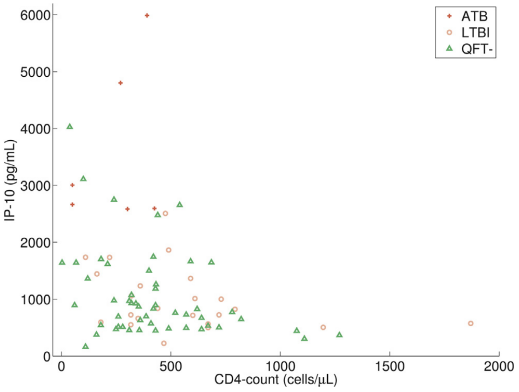

Figure 3 Negative correlation between CD4 counts and plasma IP-10 levels in HIV-infection. Spearman's correlations were used to detect relationships between the CD4 counts and selected markers in the HIV-infected group. There was a significant negative correlation between the CD4 count (cells/μL) and the plasma level of IP-10 (pg/mL) in HIV infected individuals ($p < 0.005$, $r = -0.349$). ATB: active TB infection. LTBI: Latent TB infection. QFT-: QuantiFERON-TB negative controls.

Several of the other markers that significantly differentiated between active TB and the LTBI and/or the QFT negative control groups at baseline also declined throughout therapy. PDGF-BB and TARC were significantly decreased already after 2–4 weeks ($p = 0.002$ and $p = 0.004$ respectively, Fig. 4), and there was a tendency of decrease in CRP and IL-8, which reached the significance level at week 12–24 ($p = 0.005$, Fig. 4). PTX3, that significantly differentiated between active and LTBI at baseline, did not decline during treatment. In contrast, a tendency of increasing concentration of PTX3 was found after 6–12 weeks of treatment ($p = 0.042$). However, when changes in biomarker levels were analyzed in the *Mtb* sensitive group separately, we found that the level of PTX3 tended to decrease at week 6–12 ($p = 0.013$), whereas there was an increase in PTX3 for the majority of the MDR TB patients in the same period. For the other markers analyzed we found no distinct differences in response patterns when studying the drug sensitive *Mtb* cases separately versus all active TB cases taken together.

There were also several markers which did not discriminate significantly between the various study groups at baseline, but where a significant decrease in concentrations were observed in active TB cases during treatment; RANTES (2–4 weeks), IL-1ra, IL-6, IL-12(p70), bFGF, IFN-γ, MIP-1α, TNF and VEGF (6–12 weeks), and G-CSF, MD-2 and IL-7 (12–24 weeks), $p < 0.005$, Fig. 4).

Discussion

In this study we have examined the potential of plasma cytokines to differentiate between active and LTBI in HIV-infected and HIV-uninfected individuals and longitudinal changes in these markers during anti-TB chemotherapy. For the first time we document that IP-10 and sTNFr2 can differentiate active TB cases from both individuals with LTBI and QFT negative controls irrespective of HIV status, age and gender. Moreover, we found that the level of IP-10 in HIV-uninfected active TB cases decreased gradually to the same level as LTBI and QFT negative controls after 12–24 weeks of anti-TB chemotherapy.

IP-10 is a chemokine produced by antigen presenting cells mainly in response to IFN-γ and TNF. It has been shown that plasma levels of IP-10 are increased in bacteremia[35] and infections with hepatitis C virus,[36] HIV[37,38] and TB,[14,25] and appear to correlate with the extent of inflammation. In our study, the level of IP-10 was significantly higher in HIV co-infected active TB cases compared with HIV-uninfected cases. This is in concordance with a study by Juffermans et al.,[39] but in contrast, Mihret et al.[14,27] and Riou et al.[28] found no significant differences in the level of IP-10 with respect to HIV-infection in patients with active TB before anti-TB chemotherapy. We found a significant negative correlation between the CD4 count and the level of IP-10 in HIV-infected individuals supporting studies of HIV-infected individuals without TB co-infection.[37,40] The discordant results could therefore be due to differences in CD4 counts between the HIV-infected groups in the various studies.

IP-10 circulates at much higher levels compared to IFN-γ and has been extensively studied as an alternative to IFN-γ for immunodiagnosis of TB.[16,17] In agreement with our results, it has been shown that plasma levels of IP-10 are elevated in active TB cases compared with household contacts.[14,25,41] Our study is a valuable contribution to the literature since HIV-infected subgroups are included both in the active TB, the LTBI and the QFT negative groups,

Table 2 ROC analyses with sensitivity and specificity of plasma IP-10 for tuberculosis in HIV-infected and HIV-uninfected patients.

		Cut-off (pg/mL)	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	p-value
HIV infected	ATB vs. LTBI	2547	100 (54.1–100)	100 (85.2–100)	1.00 (1.00–1.00)	<0.001
	ATB vs. QFT negative	2532	100 (54.1–100)	92.3 (81.5–97.9)	0.96 (0.91–1.00)	<0.001
HIV uninfected	ATB vs. LTBI	689	71.2 (57.9–82.2)	81.8 (48.2–97.7)	0.74 (0.59–0.89)	0.012
	ATB vs. QFT negative	674	72.9 (59.7–83.6)	60.0 (14.7–94.7)	0.66 (0.52–0.80)	0.235

ATB: Active TB infection. LTBI: Latent TB infection. QFT: QuantiFERON-TB. AUC: Area under the ROC curve. 95% CI: 95% confidence interval.

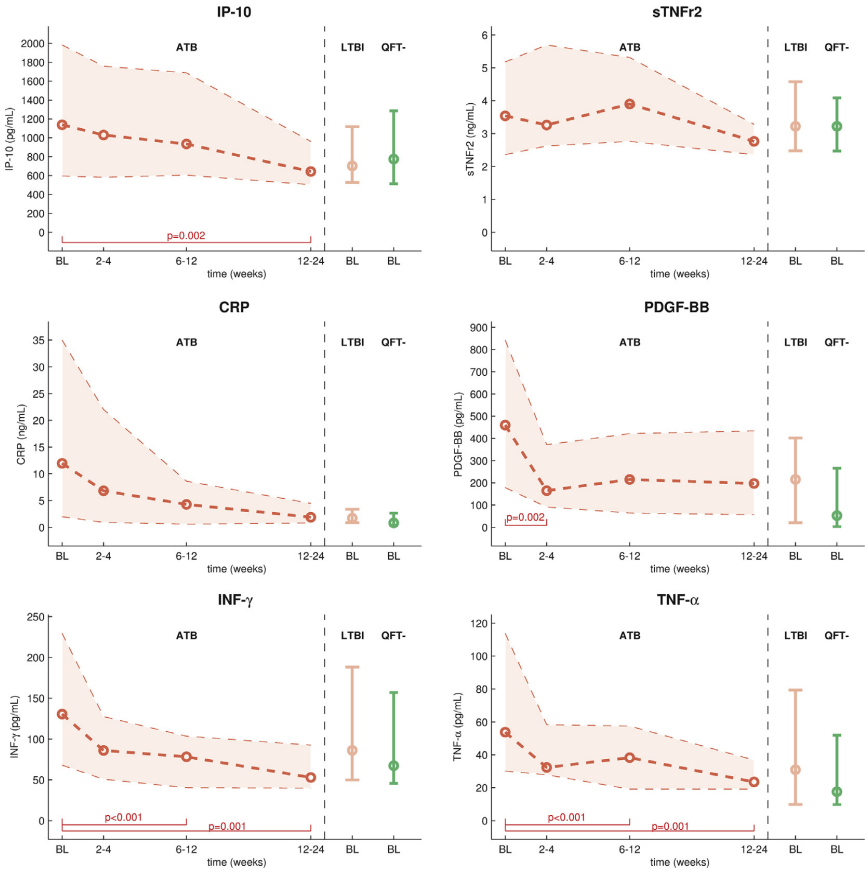

Figure 4 Plasma levels of selected soluble markers during anti-TB chemotherapy; IP-10, sTNFr2, CRP, PDGF-BB, IFN-γ and TNF-α in HIV-uninfected patients with active TB before and after 2—4, 6—12 and 12—24 weeks compared to the HIV-uninfected LTBI and QFT-negative groups. Changes in the plasma level of markers from baseline to each follow up time point during treatment were analyzed using Wilcoxon matched pairs signed rank test. Brackets represent statistically significant differences ($p < 0.005$). ATB: active TB infection. LTBI: Latent TB infection. QFT-: QuantiFERON-TB negative controls. BL: baseline.

and we demonstrate that the plasma level of IP-10 distinguish active TB from LTBI and QFT negative controls irrespective of HIV co-infection. The ROC curve analyses show that plasma IP-10 with a cut of 2547 pg/mL clearly differentiates between active TB and LTBI in HIV-infected individuals with 100% sensitivity and specificity. In HIV-uninfected individuals the diagnostic accuracy was less optimal and the cut-off level was 689 pg/mL which is notably higher than reported by Hong et al. (119.5 pg/mL).[41] This discrepancy is most likely explained by differences in methods of detection and variations in study populations, and emphasizes the importance of determining validated cut-offs before IP-10 can be used in clinical practice.

Our results support previous studies demonstrating a decrease in plasma level of IP-10 upon anti-TB chemotherapy of HIV-uninfected active TB cases,[14,25—28] whereas there are conflicting results in HIV co-infected cases. Riou et al. reported that IP-10 decreased irrespective of HIV infection,[28] but Mihret et al. documented a decline only for HIV-uninfected cases.[27] In a study by Azzuri et al., IP-10 increased in household contacts during progression to active TB and during relapse of TB in patients who previously had completed anti-TB chemotherapy.[25] Patients with active TB with moderate to high risk of relapse decline less in IP-10 during treatment compared with low risk patients.[26] It has also been suggested that serial determinations of serum IP-10 can be used to identify individuals with rheumatoid arthritis at high risk of developing active TB during TNF inhibitor treatment.[42] Further, plasma level of IP-10 correlates with the sputum smear acid fast bacilli grade[26,43] and the degree of lung involvement.[25] Although there was a tendency of decline already at 6—12 weeks, we found defined by our definition of significance level, a decrease in the level of IP-10 first after 12—24 weeks of treatment. We report a reduction in the fraction of active

TB patients with positive plasma IP-10 from 73% at baseline to 45% during anti-TB chemotherapy in line with a previous report.[26] Other markers, including PDGF-BB, TARC and IFN-γ showed an earlier significant decrease in response to therapy compared with IP-10, but these markers were not able to differentiate between the various stages of TB infection.

IP-10 measured directly in plasma is unspecific for TB, but IP-10 has also been studied as a potential readout biomarker in *in vitro Mtb* specific immunoassays. A review by Ruhwald et al. concludes that IP-10 release assays perform comparably to the QFT-test in most patient groups, but may improve the diagnostic accuracy in children and in HIV-infected individuals with low CD4 counts.[17] However, the *Mtb* specific immunoassays does not seem to be able to differentiate between active and LTBI,[3,17] and IGRAs cannot be used for monitoring treatment.[5] Hong et al. found that IP-10 secretion in response to QFT antigens decreased during anti-TB chemotherapy,[26] whereas Kabeer et al. could not confirm this, but rather detected decrease in response to RD1-selected peptides.[29]

The relatively high level of plasma IP-10 compared with other biomarkers, allows for simplification of analyzes. Methods for quantification of IP-10 in dried blood and plasma spots (DBS/DPS) have been developed, facilitating point-of-care tests and transport of samples at ambient temperatures.[44–46] DPS/DBS-based IP-10 release assays offer comparable diagnostic accuracy to the QFT.[44,47] IP-10, in contrast to IFN-γ and TNF-α, could also be detected in urine from TB patients and pulmonary TB patients have significantly higher urine IP-10 levels than healthy controls.[48] Moreover, the urine IP-10 level in cured TB patients were comparable to that found in healthy controls.[48] Urine samples have several advantaged including non-invasive collection, low cost and no need of special equipment or personnel. Still, care most be taken when detecting IP-10 in urine, as high urine IP-10 levels are also associated with chronic hepatitis C virus infection.[49] Thus, further studies are needed to determine whether urine IP-10 is a reliable alternative to plasma IP-10.

sTNFr2 can act as a TNF antagonist by competing with the cell membrane receptors for cytokine binding. It is the biologically active component of the TNF inhibitor etanercept, which, among other TNF inhibitors, has been associated with increased risk of reactivation of latent TB infection.[50] Elevated levels of TNF have been found in patients with active TB compared with household contacts and healthy controls.[51] Further, Jufferman et al. found that both sTNFr1 and sTNFr2 are increased in active TB cases and decline during treatment.[52] In our study, although finally declined, the level fluctuated during treatment. Riou et al. show that the level of TNF also fluctuates during treatment.[28] We found a gradual decrease in the level of TNF, which was significant after 6–12 weeks of therapy. However, since there were no significant differences in TNF level between any of our study groups at baseline this questions the usefulness as biomarker for therapy efficacy. Finally, if sTNFr2 may serve as a reliable marker of TNF activity is also unknown.

The pentraxin family, including CRP and PTX3, is involved in the acute phase reaction to inflammation.[53] PTX3 was the only marker that significantly differentiated between active and LTBI when HIV-uninfected individuals were studied separately, and also showed a tendency of differentiating between these groups in HIV-infected individuals. Azzuri et al. found that the plasma level of PTX3 was higher in patients with active TB compared with healthy household controls, decreased with successful treatment and increased in patients with treatment failure.[25] When patients with MDR-TB and drug sensitive *Mtb* were analyzed as one group we found a tendency of increase in the level of PTX3 during treatment. However, when analyzed separately, PTX3 tended to decrease in the drug sensitive *Mtb* group whereas there was an initial increase, followed by a decline after 24–48 months in MDR TB patients (data not shown). This may indicate that there is a delayed response to treatment in MDR compared with drug sensitive *Mtb*. CRP, which is extensively used as a marker of inflammation, declined steadily, but only near significantly, during treatment.

The main limitations of our study are the low sample size of HIV-infected patients with active TB and the lack of longitudinal follow up plasma samples from this group during treatment. This was included in the design of the original study,[31] but there was a low prevalence of active TB in the HIV-infected population, and only two HIV-infected patients had plasma samples obtained during treatment. Also, the changes in markers during treatment of active TB were assessed together regardless of drug resistant or sensitive *Mtb*, although the treatment differed in composition of antibiotics and length. We found indications that the kinetics of some of the biomarkers may differ between MDR and drug sensitive *Mtb*. Still, the majority of patients had *Mtb* sensitive strains, received standard anti-TB chemotherapy and all patients responded to treatment. Finally, since plasma IP-10 is unspecific for TB, our study should also have included patients with diagnoses other than TB, including AIDS-defining infections, to optimally validate the accuracy of the method. The two HIV patients in the QFT negative control group with other opportunistic infections clearly demonstrate this since they had plasma IP-10 levels above the median level of the HIV-infected active TB group. Still, our study was not designed to fully validate IP-10, but we recommend awareness of infections other than TB in the interpretation of plasma levels of IP-10, particularly in patients with HIV-confection.

In conclusion, IP-10 appears to be the most consistent of the biomarkers studied as it was the only marker that significantly differentiated active TB from both LTBI and QFT negative controls irrespective of HIV status, and also significantly declined during anti-TB chemotherapy of HIV-uninfected active TB cases. Although not specific for TB, plasma level of IP-10 may give information about the stage of infection, and may also be used to monitor the effect of treatment, but further studies are needed to validate and standardize IP-10 assays.

Acknowledgements

We wish to thank the staff at the research laboratories at the Dep. of Infectious Diseases, Oslo University Hospital and Haukeland University Hospital for contributing to inclusion of patients and processing of samples, especially Mette

Sannes, Helene Gjelsås and Christel Gill Haanshuus. The study was funded by grants from the Norwegian Extra-Foundation for Health and Rehabilitation (2008-2-0159), The North Norway Regional Health Authority (SFP890-09) and the "Blakstad and Maarschalk Tuberkulosefond". The funding sources were not involved in design of the study, interpitation of data or in writing of the manuscript.

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Erratum

In the paper "Wergeland I, Pullar N, Assmuss J, Ueland T, Tonby K, Feruglio S, Kvale D, Damås JK, Aukrust P, Mollnes TE, Dyrhol-Riise AM. IP-10 differentiates between active and latent tuberculosis irrespective of HIV status and declines during therapy. J Infect. 2015 Apr;70(4):381-91." there are errors in figure 1 and 2.

In Fig 1, it is incorrectly shown that there is a significant difference in the level of IL-1ra between HIV+ vs HIV- individuals, illustrated by a green box in the upper left corner of the figure. In the attached Fig 1 this is corrected to show that there is no significant difference and that the p-value is above 0.05, illustrated by a white coloured box.

In Fig 2, the unit on the y-axis of the graphs showing IP-10, sTNFr2 and Eotaxin is wrong. In the attached Fig 2 the unit is corrected to pg/mL for IP-10 and Eotaxin and to ng/mL for sTNFr2.

We apologize for the errors. The erratum has been sent to the Journal of Infection, but is not yet published.

The corrected figures are as follows:

Figure 1

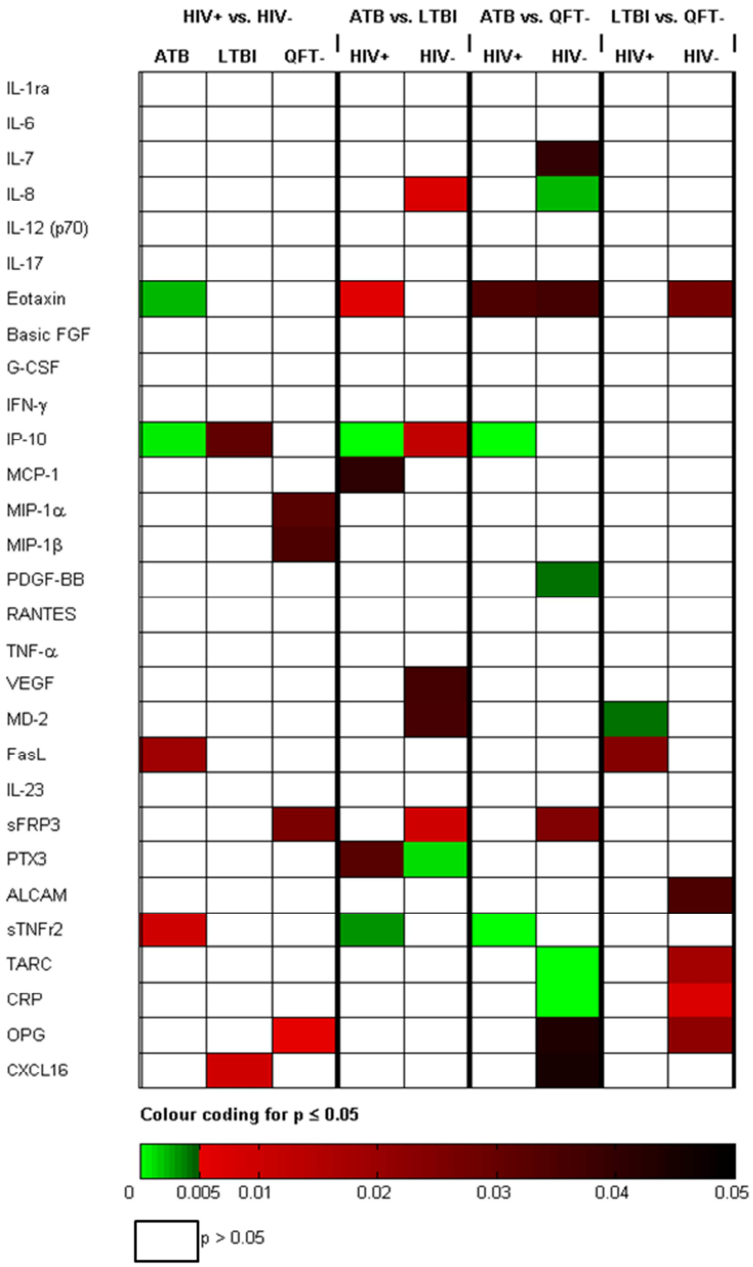
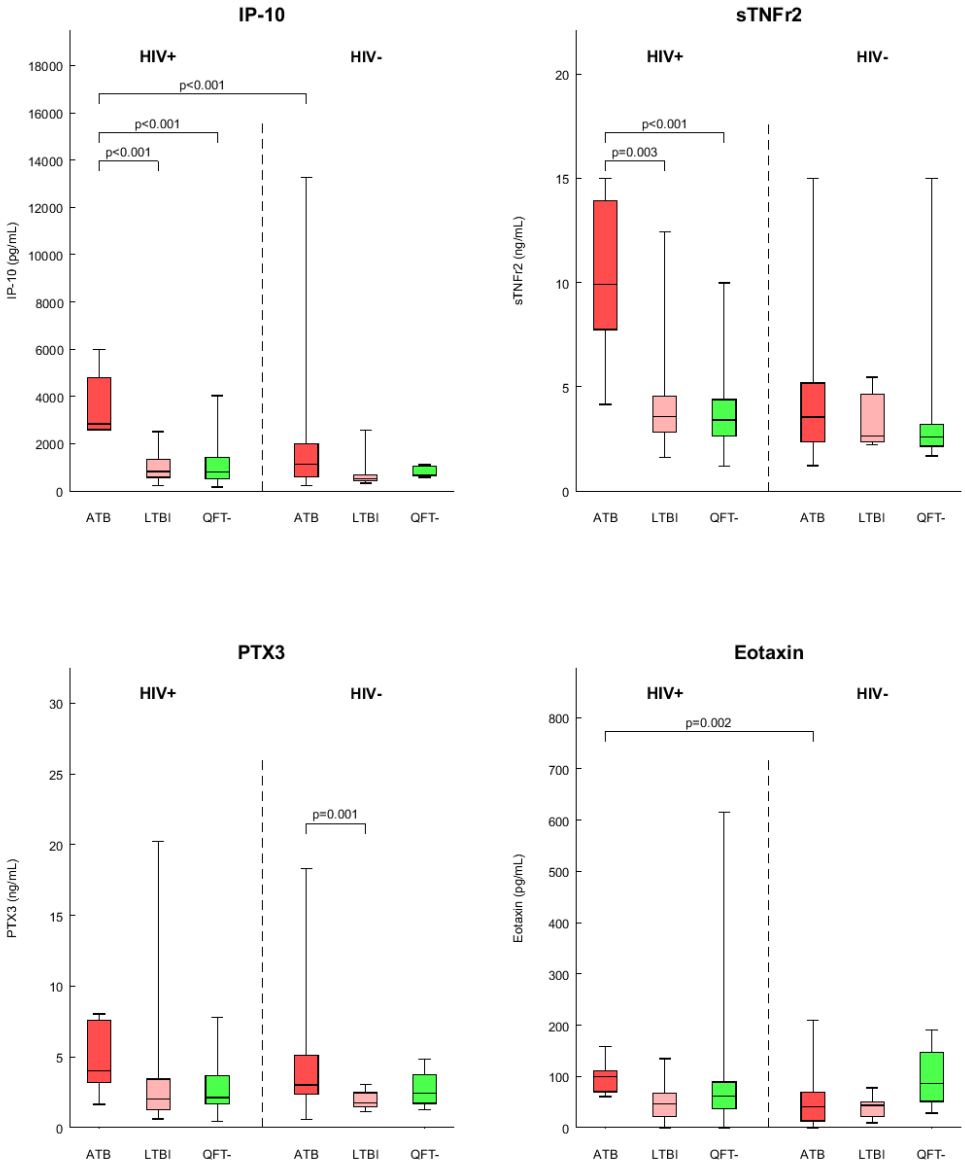

HIV+ vs. HIV-
ATB vs. LTBI
ATB vs. QFT-
LTBI vs. QFT-
ATB
LTBI
QFT-
HIV+
HIV-
HIV+
HIV-
HIV+
HIV-
IL-1ra
IL-6
IL-7
IL-8
IL-12 (p70)
IL-17
Eotaxin
Basic FGF
G-CSF
IFN-γ
IP-10
MCP-1
MIP-1α
MIP-1β
PDGF-BB
RANTES
TNF-α
VEGF
MD-2
FasL
IL-23
sFRP3
PTX3
ALCAM
sTNFr2
TARC
CRP
OPG
CXCL16
Colour coding for p ≤ 0.05
0
0.005
0.01
0.02
0.03
0.04
0.05
p > 0.05

Figure 2


IP-10
HIV+
HIV-
p<0.001
p<0.001
p<0.001
IP-10 (pg/mL)
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
ATB
LTBI
QFT-
ATB
LTBI
QFT-
sTNFr2
HIV+
HIV-
p<0.001
p=0.003
sTNFr2 (ng/mL)
0
5
10
15
20
ATB
LTBI
QFT-
ATB
LTBI
QFT-
PTX3
HIV+
HIV-
p=0.001
PTX3 (ng/mL)
0
5
10
15
20
25
30
ATB
LTBI
QFT-
ATB
LTBI
QFT-
Eotaxin
HIV+
HIV-
p=0.002
Eotaxin (pg/mL)
0
100
200
300
400
500
600
700
800
ATB
LTBI
QFT-
ATB
LTBI
QFT-

III

PLOS ONE

RESEARCH ARTICLE

Cytokine Patterns in Tuberculosis Infection; IL-1ra, IL-2 and IP-10 Differentiate Borderline QuantiFERON-TB Samples from Uninfected Controls

Ida Wergeland[1], Jörg Assmus[2], Anne Ma Dyrhol-Riise[1,3,4]*

1 Department of Clinical Science, Faculty of Medicine and Dentistry, University of Bergen, N-5020 Bergen, Norway, **2** Center for Clinical Research, Haukeland University Hospital, N-5020 Bergen, Norway, **3** Department of Infectious Diseases, Oslo University Hospital, Oslo, Norway, **4** Institute of Clinical Medicine and K.G. Jebsen IRC, Faculty of Medicine, University of Oslo, Oslo, Norway

* a.m.d.riise@medisin.uio.no

OPEN ACCESS

Citation: Wergeland I, Assmus J, Dyrhol-Riise AM (2016) Cytokine Patterns in Tuberculosis Infection; IL-1ra, IL-2 and IP-10 Differentiate Borderline QuantiFERON-TB Samples from Uninfected Controls. PLoS ONE 11(9): e0163848. doi:10.1371/journal.pone.0163848

Editor: Richard van Zyl-Smit, University of Cape Town Lung Institute, SOUTH AFRICA

Received: November 20, 2015

Accepted: August 30, 2016

Published: September 29, 2016

Data Availability Statement: All relevant data are within the paper.

Funding: This study was funded by the University of Bergen. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

Abstract

Background

Interferon gamma release assays (IGRAs) do not discriminate between active tuberculosis (TB) and latent TB infection (LTBI), which limit their use in TB endemic areas. Subjects with QuantiFERON-TB (QFT) results around the diagnostic cut-off more likely show inconsistent results on serial testing which makes the interpretation of the assay difficult. We have studied potential biomarkers in patients with various stages of TB infection and with borderline QFT tests compared to those with higher values.

Methods

27 soluble biomarkers were analysed in QFT supernatants from patients with active TB (n = 18), individuals with LTBI (n = 48) and from QFT negative controls (n = 16) by the Multiplex bead assay. The LTBI group was classified into two groups according to QFT IFN-γ levels; QFT borderline (0.35–0.70 IU/mL, n = 11) or QFT high (>0.70 IU/mL, n = 36).

Results

The levels of IL-1ra, IL-2, IL-13, IL-15, IFN-γ, IP-10 and MCP-1 in background corrected TB antigen stimulated supernatants (TBAg-Nil) significantly distinguished both active TB and LTBI QFT high groups from the QFT negative controls ($p \leq 0.004$). In addition, IL-1ra, IL-2 and IP-10 significantly differentiated the QFT borderline group from the controls ($p \leq 0.001$). Still, in the QFT borderline group the IL-1ra and IP-10 levels were not significant different from neither the QFT high nor the active TB group, whereas the IL-2 levels were lower ($p \leq 0.003$). The level of IP-10 showed the best separation between the QFT borderline group and the QFT negative controls (AUC 0.92) and offered 100% sensitivity for active TB.

Conclusion

IL-1ra, IL-2 and IP-10 differentiate QFT borderline samples from uninfected controls and the majority of QFT borderline subjects were classified as LTBI by these markers. Still, inconsistency was seen, and further studies are needed to examine the performance of alternative markers before concluded if they could be used as diagnostics tools.

Introduction

Screening for latent tuberculosis infection (LTBI) has traditionally been performed by the tuberculin skin test (TST), but during the last years interferon gamma (IFN-γ) release assays (IGRAs) have been increasingly used for this purpose. IGRAs offer better specificity than the TST, but have suboptimal sensitivity in children and human immunodeficiency virus (HIV) co-infected individuals, and the tests cannot distinguish between active tuberculosis (TB) and LTBI [1]. This limits their use in high TB incidence areas where the burden of LTBI and HIV co-infection is high. In addition, studies of IGRAs for serial TB screening of health care workers (HCW) [2,3] and in HIV infection [4,5] have shown that the interpretation of IGRA results is complicated by relative high rates of conversions and reversions and within subject variability. Subjects with QuantiFERON-TB (QFT) baseline results around the diagnostic cut off are more likely to have inconsistent results on serial testing, and a systematic review suggests introduction of a borderline zone for QFT when screening HCW [2]. It has also been shown that there is substantial variability in QFT response when the test is repeated in the same patient sample [6]. As strategies for TB control and elimination include preventive therapy to individuals with LTBI to reduce the risk for development of active disease, there is a need of more robust and reliable diagnostic tools which can differentiate TB infected from non-infected individuals.

Several studies aiming to identify alternative biomarkers for use in TB diagnostics have been conducted. IFN-γ inducible protein 10 (IP-10) is the most studied soluble biomarker, and a review by Ruhwald *et al* concludes that whereas IP-10 based tests perform comparably to QFT in most patient groups, it seems to be more robust in children and HIV-infected individuals [7]. However, IP-10 has not been studied for use in serial testing. Further, although several markers or combination of markers have been suggested that may differentiate between the various stages of TB infection [8–12], no obvious candidate has been identified [13].

The aims of this study were to examine the potential of 27 different soluble markers detected in QFT supernatants to differentiate between the various stages of TB infection and to compare the pattern of markers in subjects with QFT test results in the borderline zone with those with higher values as well as with QFT negative controls. We show that the background corrected TB antigen stimulated levels (TBAg-Nil) of seven soluble markers (IL-1ra, IL-2, IL-13, IL-15, IFN-γ, IP-10 and macrophage chemoattractant protein 1 (MCP-1)) distinguished both the active TB and LTBI groups from the QFT negative controls. The level of IL-1ra, IL-2 and IP-10 also differentiated the QFT borderline group from the controls, supporting true TB infection in the majority of these patients.

Material and Methods

Study participants

Persons referred to the TB clinic at Haukeland University Hospital, Bergen, Norway for QFT testing and medical evaluation of LTBI based on known exposure of TB or origin from a high

TB endemic country with a concomitant positive TST and patients diagnosed with active TB admitted to the inpatient ward, were included in a study of the performance of IGRA in clinical practice [14].

The diagnosis of active TB was based on a positive *Mycobacterium tuberculosis (Mtb)* culture or on clinical and radiological findings. Subjects with no signs of active TB based on X-ray, sputum or biopsy examination and clinical evaluation, and with a positive QFT test were defined as LTBI and offered preventive anti-TB chemotherapy with isoniazid and rifampicin for 3 months. A repetitive QFT test was performed during the first year after preventive therapy was ended. None of the study participants were HIV infected. The LTBI group were further classified according to QFT values 0.35–0.70 IU/mL (QFT borderline) or $>$ 0.70 IU/mL (QFT high).

Written informed consent was obtained from all participants. The study was approved by the Regional Committee for Ethics in Medical Research (REK-Vest, 3.2005.823), Norway.

QuantiFERON-TB GOLD in-tube assay

The assay was performed according to the manufacturer's instructions (Cellestis Ltd, Qiagen, Chadstone, VIC, Australia). One ml of whole blood was added to each of the three QFT tubes containing TB antigen (ESAT-6, CFP-10 and TB 7.7), mitogen-positive control (phytohemagglutinin (PHA)) and a negative control, respectively. The tubes were incubated at 37°C for 16–24 h, centrifuged and the supernatant removed. The amount of IFN-γ in the supernatant was quantified by enzyme-linked immunosorbent assay (ELISA). The level of IFN-γ in the TB antigen (TBAg) tube was corrected for background by subtracting the IU/ml value obtained for the respective negative control tube (Nil). The cut-off value for positive test (TBAg-Nil) was $\geq$ 0.35 IU/ml. The excess of supernatants from the QFT tubes were frozen and stored at -80°C until further analysis.

Multiplex cytokine analysis

Levels of biomarkers in QFT supernatants were measured using Bio-Plex Pro Human Cytokine Group 27-Plex Panel (Bio-Rad Laboratories Inc., Hercules, CA) on a Luminex 100 platform according to the manufacturer's instructions. Levels of interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (basic FGF), eotaxin, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), IFN-γ, IP-10, MCP-1, macrophage inflammatory protein 1 alpha (MIP-1α), MIP-1β, platelet-derived growth factor -BB (PDGF-BB), regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) were analysed in supernatants both from the unstimulated (Nil) and the TB antigen stimulated QFT tubes (TBAg). Supernatants from the mitogen stimulated QFT tubes were not analysed. The supernatants were diluted 1:4 in Bio-Plex sample diluent, and levels obtained were therefore multiplied by four to correct for the dilution. STarStation v.3 software was used for data analysis.

For three of the 27 markers analysed (IL-8, MIP-1β and RANTES), a considerable proportion of the study subjects in both the active TB and LTBI groups had both TBAg and Nil levels that were above the upper detection limit (UDL) of the assay despite dilutions. Thus, these TBAg-Nil results were excluded from all statistical analyses. This pattern was also found for IP-10 and MCP-1, but only for the TBAg samples. Thus, the TBAg-Nil results of IP-10 and MCP-1 were not used in statistical analyses when the active TB and LTBI groups were compared, whereas when comparing the two TB infection groups with the controls the TBAg levels

above the UDL were replaced by the highest value of the standard curve for the respective marker to allow non-parametric statistical analysis. Thus, when comparing the level of some markers both within the TB infection groups and with the controls, significant differences may not be detected because the actual median levels in the active TB and LTBI group may be higher than we were able to detect within the range of the assay. Also occasional values (<15% of the total data set) above the UDL for some of the other markers (IL-1β, IL-6 and MIP-1α), were replaced by the highest value of the standard curve. The level of IL-5 was below the lower detection level (LDL) of the assay in >75% of all samples, and were therefore excluded from all statistical analysis. For the other markers, the occasional values (<10% of the total data set) below the LDL were replaced by a defined common value below the LDL (0.001), which allowed non-parametric statistical analysis.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistics 21 and GraphPad Prism 6. The unstimulated level (Nil) and the background corrected TB stimulated level (TBAg-Nil) of each marker were included as separate variables in the statistical analyses.

Mann-Whitney U test was used to detect differences between the study groups. Wilcoxon matched pairs signed rank test was used to detect differences in the level of markers before and after prophylactic anti-TB chemotherapy. The general significance level was set to 0.05. In a preliminary step, the Spearman correlations between all pairs of biomarkers were calculated, showing a high proportion of correlated variables. Taking into account multiple testing effects the Bonferroni adjustment would be too conservative due to the dependence between the markers. Thus, we decided to use a marginal level of 0.005 (corresponding to Bonferroni adjustment for 10 tests).

Receiver operator characteristic (ROC) curve analyses were performed for the markers which, by the aforementioned tests, significantly differentiated between the LTBI borderline group and the QFT negative group. The optimal cut-off levels were defined by the minimum Euclidian distance to maximal specificity and sensitivity.

Results

Study participants

A total of 82 study participants had stored QFT supernatants available for Multiplex analysis, and these were classified into three groups; 1) active TB (n = 18), 2) QFT positive LTBI (n = 48), and 3) QFT negative controls (n = 16). Eleven of the QFT positive LTBI individuals were further classified as QFT borderline, 36 as QFT high and in one IFN-γ level was not determined. Fifteen individuals with LTBI had available repetitive samples obtained during the first year after preventive therapy. The clinical characteristics of the study participants are summarized in Table 1.

The diagnosis of active TB was based on a positive *Mtb* culture in 16 of the patients, whereas in two patients the diagnosis was based on clinical and radiological findings. Fourteen patients had pulmonary TB and four extrapulmonary TB, and there were no multi- or extensively drug-resistant TB cases.

Cytokine patterns in latent and active TB infection

Twenty-seven different cytokines, including chemokines and markers of inflammation, were analysed in QFT supernatants and compared in patients with various stages of TB infection and with QFT negative controls. We first examined if there were any differences between

Table 1. Clinical characteristics of the study participants.

	Active TB, (n = 18)	LTBI QFT high (n = 36)*	LTBI QFT borderline (n = 11)*	QFT negative (n = 16)
Age; median (range)	32 (18–62)	40 (13–67)	40 (25–53)	47 (16–68)
Sex; males/females	6/12	13/23	4/7	8/8
Origin; TB high/low endemic country	16/2	22/14	9/2	4/12

LTBI QFT borderline = 0.35–0.70 IU/ml. LTBI QFT high >0.70 IU/mL.

*In total, n = 48 QFT positive individuals with LTBI were included in the study, but for one them the exact value of the QFT-test was not known. This subject was therefore excluded from the subgroup analyses.

doi:10.1371/journal.pone.0163848.t001

patients with active TB and LTBI. For the markers within the range of the assay, we found no significant differences in TBAg-Nil levels between the active and the LTBI group. In contrast the Nil levels of IL-1β, IL-1ra, IL-9 and IL-17a were significantly lower in the active TB group compared with the LTBI group ($p \leq 0.004$, Fig 1).

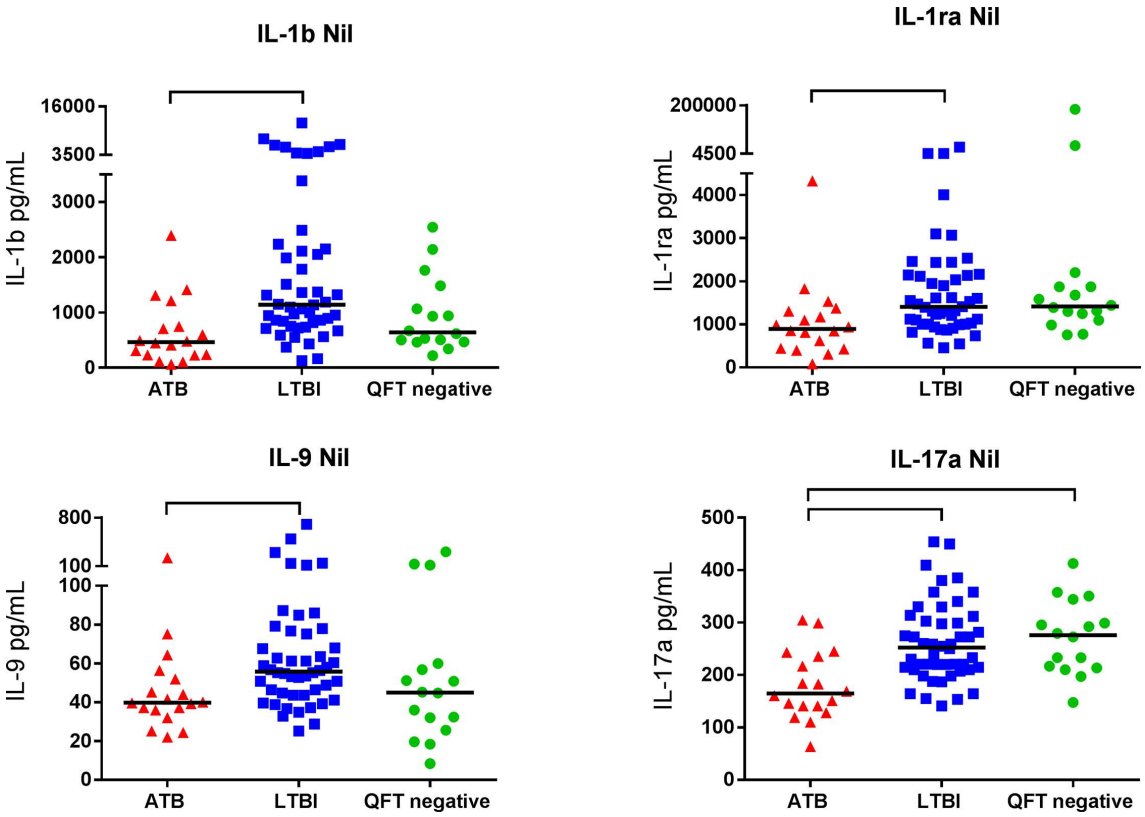


Fig 1. Markers differentiating between active and latent TB infection. Nil levels (pg/mL) of IL-1β, IL-1ra, IL-9 and IL-17a in patients with active TB (ATB), latent TB infection (LTBI) and in QFT negative controls (QFT negative). The horizontal lines show the median values. Mann-Whitney U test was used for comparison between groups. Brackets represents statistically significant differences ($p \leq 0.004$).

doi:10.1371/journal.pone.0163848.g001

We then analysed if any marker could distinguish between TB infection and QFT negative controls. We found that the TBAg-Nil levels of seven of the markers; IL-1ra, IL-2, IL-13, IL-15, IFN-γ, IP-10 and MCP-1, were significantly higher in both the active TB and LTBI group than in QFT negative controls ($p \leq 0.004$) (Fig 2).

In the unstimulated supernatants, the Nil level of IL-15, eotaxin and basic FGF were significantly lower ($p < 0.0002$) and the Nil level of RANTES significantly higher ($p = 0.0003$) both in the active TB and LTBI groups compared with the QFT negative controls. The median Nil levels of several other markers (IL-2, IL-4, IL-13, IL-17a and IFN-γ) were also lower in the active TB ($p \leq 0.003$) group than in controls (data not shown).

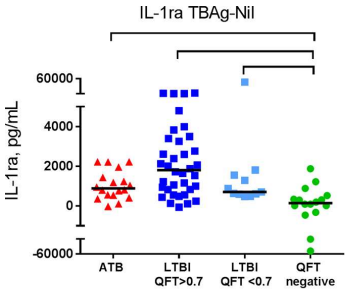
Fifteen of the study participants in the LTBI group (13/15 QFT high) had available supernatants from QFT tests performed during the first year after prophylactic anti-TB chemotherapy. There were no significant changes in the Nil or TBAg-Nil levels for any of the markers in response to treatment, except from a decline in the Nil level of PDGF-BB ($p = 0.002$). Also the TBAg-Nil level of IL-2, IFN-γ and IP-10 remained significantly higher than that seen in the QFT negative controls indicating a maintained response to TB antigens (data not shown).

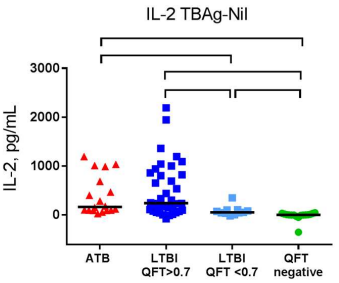
Cytokine patterns in subjects with borderline zone QFT responses. Finally, we investigated if LTBI patients with borderline zone QFT IFN-γ values in the range 0.35–0.70 IU/ml, close to cut-off for the QFT test, demonstrated a different cytokine pattern than LTBI patients with higher QFT IFN-γ values > 0.70 IU/ml. The TBAg-Nil levels of IL-1ra, IL-2, IL-13, IL-15, IFN-γ, IP-10, MCP-1 and IL-17a were all significantly higher in the QFT high group than in the QFT negative controls ($p \leq 0.004$, Fig 2).

However, only the levels of IL-1ra, IL-2 and IP-10 significantly differentiated the QFT borderline group from the QFT negative controls ($p \leq 0.001$) (Fig 2). Whereas the IL-1ra and IP-10 levels in the QFT borderline group were not significant different from neither the QFT high nor the active TB group, the IL-2 level was significantly lower in the QFT borderline group ($p \leq 0.003$). In the Nil supernatants, only IL-15 and basic FGF significantly differentiated the QFT borderline group (lower levels) from the QFT negative controls ($p \leq 0.0008$), whereas for the QFT high LTBI group a similar pattern as seen for the overall LTBI group was found (data not shown).

The diagnostic accuracy of the TBAg-Nil levels of IL-1ra, IL-2 and IP-10 in differentiating between the QFT borderline group and the QFT negative group were investigated by ROC curve analyses. The AUC, p-value, optimal cut-off levels, sensitivity and specificity for the respective markers are given in Table 2. The TBAg-Nil level of IP-10 had the highest AUC (0.92).

Based on the cut-off levels found by the ROC curve analyses, four subjects classified as LTBI by the QFT test, two in the QFT borderline group and two in the QFT high group, were classified as not TB infected by IP-10, and two subjects classified as negative by the QFT test were classified as LTBI (Table 3). IP-10 had a 100% sensitivity for active TB. IL-1ra and IL-2 classified four of the subjects in the QFT high group as not TB infected, and IL-2 also classified two subjects in the QFT borderline group as not infected. In the QFT negative control group, four and one subjects, respectively, were classified as LTBI by IL-1ra and IL-2. In total, 18/81 of the study subjects were classified in discordance with the QFT test by one or several of the three alternative markers. For eleven subjects only one of the markers showed a discordant result, whereas for seven subjects two or all three of the alternative markers were discordant with the QFT classification. In the QFT high group, four of the seven subjects that were classified in discordance with the QFT test by one or several of the three alternative markers, had low QFT test values between 0.70–1.0 IU/mL.


IL-1ra TBAg-Nil
IL-1ra, pg/mL
60000
4000
2000
0
-60000
ATB
LTBI QFT>0.7
LTBI QFT <0.7
QFT negative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IL-2, pg/mL
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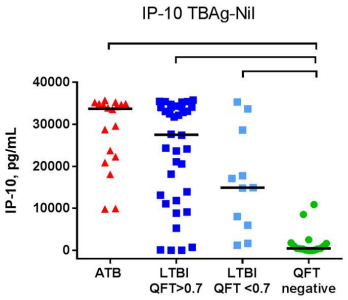
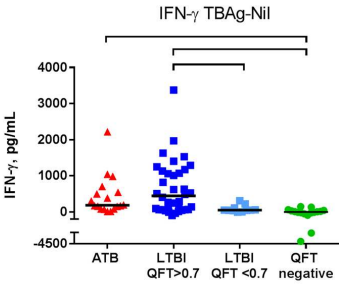
2000
1000
0
ATB
LTBI QFT>0.7
LTBI QFT <0.7
QFT negative


IP-10 TBAg-Nil
IP-10, pg/mL
40000
30000
20000
10000
0
ATB
LTBI QFT>0.7
LTBI QFT <0.7
QFT negative


IFN-γ TBAg-Nil
IFN-γ, pg/mL
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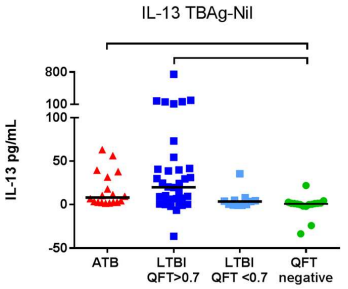
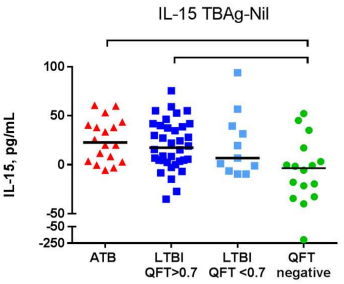
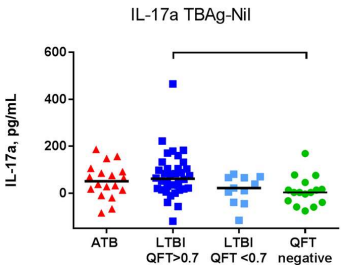
3000
2000
1000
0
-4500
ATB
LTBI QFT>0.7
LTBI QFT <0.7
QFT negative


IL-13 TBAg-Nil
IL-13 pg/mL
800
100
100
50
0
-50
ATB
LTBI QFT>0.7
LTBI QFT <0.7
QFT negative


IL-15 TBAg-Nil
IL-15, pg/mL
100
50
0
-50
-50
-250
ATB
LTBI QFT>0.7
LTBI QFT <0.7
QFT negative


IL-17a TBAg-Nil
IL-17a, pg/mL
600
400
200
0
ATB
LTBI QFT>0.7
LTBI QFT <0.7
QFT negative

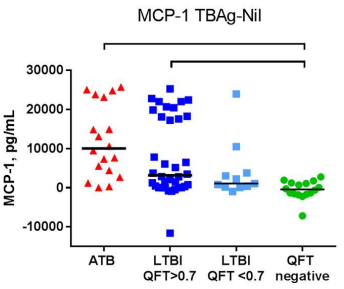

MCP-1 TBAg-Nil
MCP-1, pg/mL
30000
20000
10000
0
-10000
ATB
LTBI QFT>0.7
LTBI QFT <0.7
QFT negative

Fig 2. Markers differentiating TB infection from QFT negative controls. TB antigen stimulated background corrected (TBAg-Nil) levels of IL-1ra, IL-2, IP-10, IFN-γ, IL-13, IL-15, IL-17a and MCP-1 in patients with active TB (ATB), latent TB infected subjects with QFT result >0.70 IU/mL (LTBI QFT>0.7), subjects with QFT result in the borderline zone 0.35–0.70 IU/mL (LTBI QFT<0.7) and in QFT negative controls (QFT negative). The horizontal lines show the median values. Mann-Whitney U test was used for comparison between groups. The TBAg-Nil level of IP-10 and MCP-1 were excluded from statistical analyses when the LTBI and ATB groups were compared because high proportions of study subjects in both groups had levels above the UDL of the assay. Brackets represents statistically significant differences (p<0.005).

doi:10.1371/journal.pone.0163848.g002

Discussion

We have examined the potential of several soluble chemokines and cytokines measured in QFT supernatants to diagnose and differentiate between the various stages of TB infection and uninfected controls as well as the pattern of markers in subjects with QFT test result in the borderline zone of 0.35–0.70 IU/mL. We found that the background corrected TB antigen stimulated levels of seven markers (IL-1ra, IL-2, IL-13, IL-15, IFN-γ, IP-10 and MCP-1) distinguished both the active TB and LTBI group from the QFT negative controls. IL-1ra, IL-2 and IP-10 also differentiated the QFT borderline group from the controls, and these three markers classified the majority of the study participants in the QFT borderline group in accordance with the QFT test.

Previous studies of alternative biomarkers from analyses of QFT supernatants [8–11] have found that unstimulated or TB antigen stimulated levels of various markers (EGF, MIP-1β, TGF-α, IL-1α, sCD40L, VEGF, IFN-α2, IL-1ra, IP-10, IL-2, IL-15 and MCP-1) may have potential for differentiating between active TB and LTBI. However, a review by Chegou *et al* concludes that there is no clear pattern of markers that are able to differentiate between the various stages of TB infection [13]. In support of this we also did not find any marker that was able to differentiate between active TB and LTBI when the background corrected TB antigen stimulated levels were analysed. However, in the unstimulated Nil supernatants, the levels of IL-1b, IL-1ra, IL-9 and IL17a were significantly lower in the active TB group compared with the LTBI group. This confirms studies that report that unstimulated levels of IL-1ra differentiate between active and latent TB in both children and adults [10,12].

In serial TB screening, relative high rates of conversions and reversions of QFT tests, especially around the diagnostic cut off, complicate the interpretation of IGRA results [2,3, 14]. A large variation in both these rates has been reported from different studies of HCW in TB low endemic countries (reversion rates 22–71% and conversion rates 1–14%) [2]. This was also seen in a cohort of health care students from a high TB incidence setting in which the variation could not be explained by occupational exposure [15]. Likewise, discordant IGRA results have been found in repeated testing of HIV-infected individuals living in TB low-endemic countries [4,5]. Pullar *et al* reported QFT reversions rates in HIV patients with untreated LTBI and in those receiving preventive TB therapy of 44% and 23%, respectively, during two years of follow-up as well as a conversion rate of 7% in the TB negative control group despite no known new TB exposure [4]. Finally, Metacalfe *et al* show that there is substantial variability in QFT responses when the test is repeated in the same patient sample [6]. In studies performed in TB

Table 2. ROC curve analyses for differentiation between the QFT borderline group and QFT negative controls.

Marker	Cut-off (pg/mL)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	p-value
IL-1ra (TBAg-Nil)	409	100 (72–100)	75(48–93)	0.852 (0.701–1.000)	0.002
IL-2 (TBAg-Nil)	37	82 (48–98)	94 (50–100)	0.875 (0.720–1.000)	0.001
IP-10 (TBAg-Nil)	4235	82 (48–98)	88 (62–98)	0.920 (0.821–1.000)	<0.001

doi:10.1371/journal.pone.0163848.t002

Table 3. Number of subjects classified as active TB and LTBI by IL-1ra, IL-2 and IP-10.

Marker	Active TB (n = 18)	LTBI QFT high (n = 36)	LTBI QFT borderline (n = 11)	QFT negative (n = 16)
IL-1ra	14	32	11	4
Il-2	17	32	9	1
IP-10	18	32*	9	2

*Two subjects in the LTBI QFT high group had an inconclusive IP-10 TBAg-Nil value because both the TBAg and the Nil value were out of range of the assay.

doi:10.1371/journal.pone.0163848.t003

low-endemic countries, the relative high conversion rates have been considered a result of false positive conversions. Thus, a borderline zone for QFT from 0.20–0.70 IU/mL has been suggested in the screening of HCW [2]. Altogether, these data highlights the need for additional markers to better discriminate between truly TB infected and uninfected individuals in order to identify those that should be offered preventive TB therapy.

In this study, the background corrected TB antigen stimulated levels of three markers, IP-10, IL-1ra and IL-2, differentiated the QFT borderline group from the QFT negative controls. ROC curve analyses for differentiation between these two groups showed that IP-10 had the highest AUC (0.92) and a sensitivity and specificity of 82 and 88% respectively, whereas IL-1ra had better sensitivity (100%) and IL-2 better specificity (94%). Based on the cut off levels found by the ROC curve analyses, IP-10, IL-1ra and IL-2 classified most of the study subjects as TB infected or not-TB infected in accordance with the QFT test. However, a few subjects in the active TB group were wrongly classified as not-TB infected by IL-2 and IL-1ra whereas some of the QFT negative subjects were classified as TB infected by the study markers. IP-10 had 100% sensitivity for active TB and classified two subjects in the QFT borderline zone as not TB. It is of interest that four of seven subjects in the QFT high group classified as not-TB by one or several of the three aforementioned markers had rather low QFT test results in the range of 0.70–1.0 IU/mL. Altogether this may indicate that a broader QFT borderline zone is more appropriate or at least that a positive QFT test in the lower range of the assay must be interpreted with caution. Still, the cut-offs from these limited ROC analyses can not at this stage be used as diagnostic tools in clinical practice as the data need to be validated in larger prospective studies.

IL-1ra, IL-2 and IP-10 have previously been suggested as diagnostic biomarkers for TB infection and IP-10 is the most extensively studied [7,13]. Although IP-10 is mainly secreted by antigen presenting cells (APCs), secretion is initiated by T cell recognition of specific peptides presented by APCs, and mainly driven by T cell derived IFN-γ. In accordance with our results, Ruhwald *et al* found that TB stimulated levels of IL-1-ra were significantly higher in patients with active TB compared with unexposed controls [16]. Frahm *et al* found the same pattern when comparing TB infected (active and LTBI combined) with uninfected subjects, and also a tendency of lower levels in LTBI versus active TB infection [11]. IP-10 and IL-2 offered comparable or even better sensitivity than IFN-γ for detection of patients with active TB [17–19] on support of our data. It has also been reported that the performance of IL-2 is similar to QFT in TB exposed individuals [19,20]. Whereas IP-10 has been equal to QFT in some studies [21,22] others have identified a higher number of infected contacts defined by IP-10 [19,20]. In our study, IL-1ra, IL-2 and IP-10 classified four, one and two, respectively, of the 16 QFT negative subjects as LTBI. To our knowledge, no studies of the variability of the alternative biomarkers in serial testing have been performed.

Previous studies show that unstimulated plasma levels of IP-10 distinguish between active and latent TB cases/household contacts [23,24]. We have also recently demonstrated that plasma IP-10 distinguished active TB from LTBI irrespective of HIV-infection and declined

during anti-TB chemotherapy [25]. In support of our data, Chegou *et al* also found that the IP-10 Nil, but not TBAg-Nil level, in QFT supernatants distinguished between active TB and LTBI in children [10]. However, in the present study and a previous study in adults [9], neither the Nil nor the TBAg-Nil level of IP-10 in QFT supernatants were able to differentiate between these stages of infection. This discrepancy could be due to differences in the handling of the samples, the method of analysis and in the studied population.

There are some limitations to our study. Firstly, as there is no gold standard for diagnosing LTBI, the diagnostic accuracy of alternative markers is difficult to assess. In this study the diagnosis of LTBI was based on information of TB exposure, clinical examination excluding active TB and the QFT test result. Secondly, repeated testing of subjects with QFT test result in the borderline zone was not performed and we were therefore not able to examine the variability of IL-1ra, IL-2 and IP-10 in longitudinal samples. Thirdly, for some of the markers analysed, a considerable proportion of the study subjects in both the active TB and LTBI had TB stimulated and Nil levels that were above the upper detection level of the assay despite dilutions of the samples. Differences in these markers between the TB infection groups could therefore not be evaluated. When comparing the levels of these markers in TB groups with the controls, significant differences may not be detected because levels that were above the upper detection level were replaced by the highest value of the standard curve. The actual median levels in the active and LTBI group therefore may be higher than we were able to detect. Still, significant data presented in this study were all based on valid parameters. Finally, due to a small sample size increasing the risk of type 2 errors the data needs to be confirmed in larger prospective studies to determine whether the markers have reliable predictive capacity.

Conclusions

Analysis of unstimulated levels of IL-1b, IL-1ra, IL-9 and IL-17a in QFT supernatants may help to discriminate active TB from LTBI whereas the respective TB antigen stimulated levels do no separate between the groups. Still, TB antigen stimulated IL-1ra, IL-2 and IP-10 levels differentiate the QFT borderline group from controls, supporting true TB infection in the majority of these patients. However, inconsistency was seen and further studies are needed to examine proper cut-offs and the variability of these markers in serial testing.

Acknowledgments

We would like to thank Gerd Gran and Christel Gill Haanshuus at Haukeland University Hospital for help in recruiting study participants, Karl Brokstad, University of Bergen for technical support and Tehmina Mustafa, University of Bergen for help with clinical data. The study was funded by the University of Bergen.

Author Contributions

Conceived and designed the experiments: AMDR IW.

Performed the experiments: IW.

Analyzed the data: IW AMDR JA.

Contributed reagents/materials/analysis tools: AMDR.

Wrote the paper: IW AMDR JA.

Recruited patients and provided clinical data and patient material: AMDR.

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IV

Tonby et al. BMC Infectious Diseases (2016) 16:599
DOI 10.1186/s12879-016-1938-8

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

The COX- inhibitor indomethacin reduces Th1 effector and T regulatory cells in vitro in Mycobacterium tuberculosis infection

Kristian Tonby[1,2*], Ida Wergeland[3], Nora V. Lieske[4], Dag Kvale[1,2,5], Kjetil Tasken[2,4,5,6] and Anne M. Dyrhol-Riise[1,2,3,5]

Abstract

Background: Tuberculosis (TB) causes a major burden on global health with long and cumbersome TB treatment regimens. Host-directed immune modulating therapies have been suggested as adjunctive treatment to TB antibiotics. Upregulated cyclooxygenase-2 (COX-2)-prostaglandin E2 (PGE2) signaling pathway may cause a dysfunctional immune response that favors survival and replication of *Mycobacterium tuberculosis (Mtb)*.

Methods: Blood samples were obtained from patients with latent TB ($n = 9$) and active TB ($n = 33$) before initiation of anti-TB chemotherapy. COX-2 expression in monocytes and ESAT-6 and Ag85 specific T cell cytokine responses (TNF-α, IFN-γ, IL-2), proliferation (carboxyfluorescein succinimidyl ester staining) and regulation (FOXP3+ T regulatory cells) were analysed by flow cytometry and the in vitro effects of the COX-1/2 inhibitor indomethacin were measured.

Results: We demonstrate that indomethacin significantly down-regulates the fraction of *Mtb* specific FOXP3+ T regulatory cells (ESAT-6; $p = 0.004$ and Ag85; $p < 0.001$) with a concomitant reduction of *Mtb* specific cytokine responses and T cell proliferation in active TB. Although active TB tend to have higher levels, there are no significant differences in COX-2 expression between unstimulated monocytes from patients with active TB compared to latent infection. Monocytes in both TB groups respond with a significant upregulation of COX-2 after in vitro stimulation.

Conclusions: Taken together, our in vitro data indicate a modulation of the Th1 effector and T regulatory cells in *Mtb* infection in response to the COX-1/2 inhibitor indomethacin. The potential role as adjunctive host-directed therapy in TB disease should be further evaluated in both animal studies and in human clinical trials.

Keywords: Tuberculosis, COX-inhibitors, Tregs, Regulatory T cells, Host-directed therapy, Monocytes, Cytokines

Background

Infection with *Mycobacterium tuberculosis (Mtb)* causes a major burden on global health with 9 million people suffering from tuberculosis (TB) disease and with 1.5 million deaths every year [1]. The current TB treatment strategies consist of long lasting multiple drug regimens with risk of serious side-effects and development of multi-drug resistant TB (MDR-TB). Host-directed therapies (HDTs) in conjunction with standard anti-TB drug regimens may reduce the duration of therapy, achieve better treatment outcomes, lower the risk of developing further drug resistance and decrease the chances of relapse or reinfection [2, 3].

In chronic infections such as TB, immune-mediated tissue injury may become more detrimental than the pathogen itself and the immune system have evolved mechanisms to balance pro and anti-inflammatory signals [4]. FOXP3+ T regulatory cells (Tregs) are involved in the regulation of inflammatory processes and exert immunosuppressive functions by cell contact-dependent suppression of CD4+ T cells and by secretion of inhibitory cytokines and soluble factors [5, 6]. Tregs may dampen protective immunity facilitating pathogen multiplication and dissemination [7] and may also limit vaccine immunogenicity [8]. Thus, targeting of Tregs may have potential as host directed adjunctive therapies [9].

Prostaglandin E2 (PGE2) is generated by the constitutive cyclooxygenase 1 (COX-1) and the inducible cyclooxygenase 2 (COX-2) enzymes and is regarded as a key mediator of immunopathology with immune regulatory effects in chronic

* Correspondence: kristian.tonby@medisin.uio.no
[1]Institute of Clinical Medicine, University of Oslo, Oslo, Norway
[2]Department of Infectious Diseases, Oslo University Hospital, Oslo, Norway
Full list of author information is available at the end of the article

infections [10, 11]. Macrophages and monocytes up-regulate COX-2 enzymes in response to inflammatory signals and are thereby major producers of PGE2 and other eicosanoids [12]. Monocytes as well as adaptive Tregs seem to inhibit effector T cell functions and suppress T cell immune responses by a COX-2-PGE2-dependent mechanism [13–15]. It has been shown that highly expressed COX-2 in malignant tissue is associated with poor prognosis and outcome in cancer disease [16, 17]. However, no data exists on COX-2 expression of immune cells in human TB disease.

Standard TB antibiotics are directed against the pathogen, but various host directed immune therapies, including reduction of PGE2 production by COX- inhibitors (COX-i) has potential to become part of a treatment strategy for resistant or clinically complicated TB cases or as part of a TB vaccination scheme [18–20]. Accordingly, studies of TB animal models have shown that targeting PGE2 with COX-i have significant impact on the immune responses and outcome of disease [21–25]. Based on these data, it is suggested that a human intervention study with anti-inflammatory drugs given in combination with anti-TB chemotherapy should be performed [24]. Clinical trials in HIV infected patients have also shown that COX-i improve T cell mediated immune responses [26–28].

Indomethacin is a widely used non-steroidal anti-inflammatory drug (NSAID) of the methylated indole class with analgesic and antipyretic properties exerting its pharmacological effects by inhibiting the synthesis of prostaglandins via the arachidonic acid pathway [29]. Indomethacin inhibits both COX-1 and COX-2 with greater selectivity for COX-1 [30] and due to water soluble characteristics the compound is practical to use in in vitro studies. Indomethacin has previously been shown to increase the bactericidal activity of *Mtb* infected macrophages [31] and to improve T cell proliferative responses in HIV-infected patients [28], but to our knowledge the effect of indomethacin on T cell responses has not been studied in TB infection.

The objective of this study was to analyze COX-2 expression in monocytes from patients with latent and active TB and to explore the in vitro effects of the COX-i indomethacin on *Mtb*-specific T cell responses and regulation. We show that indomethacin down-regulates *Mtb* antigen stimulated Tregs, antigen induced cytokine responses, in particular TNF-α+ cells, and T cell proliferation. Our data suggest a potential role for COX-i in modulation of immune responses in TB infection. However, the functional consequences of our data need to be further evaluated and the potential for COX-i as HDT in TB infection should be explored in both animal studies and in human clinical trials.

Methods

Study participants

Ten patients with active TB disease (ATB) and nine patients with latent TB (LTB) were recruited from Haukeland University hospital, Bergen, Norway (cohort A). Additional, 23 patients with active TB disease were recruited from Oslo University hospital, Oslo, Norway (cohort B). Table 1 summarizes demographic and clinical patient characteristics. All patients were HIV uninfected. The diagnosis of active TB was based on a positive *Mtb* culture or on clinical and radiological findings. Subjects with a positive QuantiFERON®-TB test and with no signs of active TB based on X-ray, sputum or biopsy examination and clinical evaluation were defined as LTB.

Sample processing

Blood samples were obtained before initiation of anti-TB chemotherapy. Peripheral blood mononuclear cells (PBMCs) from participants recruited in cohort A were isolated using density gradient centrifugation (Lymphoprep™, Fresenius Kabi Norge AS, Halden, Norway), cryopreserved in 10 % DMSO/90 % fetal bovine serum (FBS, GIBCO, Life technologies) and stored in liquid nitrogen until analysis. PBMCs from participants recruited in cohort B were isolated in cell preparation tubes (CPT) (Becton Dickinson, BD, New Jersey, USA) with sodium heparin and cryopreserved in 90 % fetal calf serum (FCS, Sigma, Missouri, USA)/10 % DMSO and stored at −145 °C until analysis. Participants recruited in cohort A were only included in the monocyte assay and participants recruited in cohort B were only included in the Treg, proliferation and intracellular cytokine assays. Therefore, all assays only included PBMCs isolated by either of the methods. Figure 1 gives an overview of the number of samples used in the different assays in the study.

Table 1 Patient characteristics

	Cohort A		Cohort B
	ATB ($n = 10$)	LTB ($n = 9$)	ATB ($n = 23$)
Age (median years, range)	28 (16–72)	43 (26–67)	29 (20–60)
Female (%)	3 (30)	4 (44)	9 (39)
Origin (%)			
Africa	4 (40)	4 (44)	10 (43)
Asia	2 (20)	2 (22)	10 (43)
Europe	4 (40)	3 (33)	3 (14)
Localisation (%)			
Pulmonary	10 (100)	-	17 (74)
Extrapulmonary[a]	-	-	6 (26)
ESR[b] (median mm/hour, range)	42 (21–77)	16 (7–23)	21 (1–103)

Characteristics of patients included at Haukeland University hospital, Bergen, Norway (cohort A) and Oslo University hospital, Oslo, Norway (cohort B)
ATB active TB, *LTB* latent TB
[a]glandular, pericardial, pleural
[b]Erythrocyte Sedimentation Rate

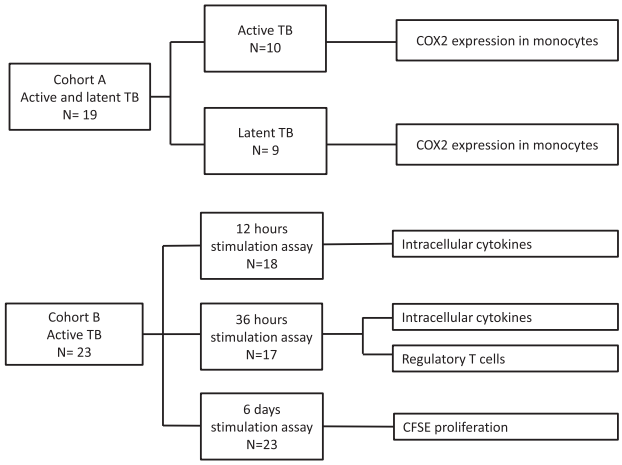

Fig. 1 Flowchart of patient samples. Patients were included at two study locations. Cohort A (latent TB and active TB) denotes patients included at Haukeland University Hospital, whilst cohort B denotes patients included at Oslo University Hospital. The figure also shows the distribution of patient samples in the different assays performed in the study

Detection of COX-2 in monocytes

Cryovials from active and latent TB patients were thawed, washed and resuspended in RPMI-1640 media (Sigma-Aldrich) with 10 % FBS. PBMCs with viability <70 % were excluded from further analyses. After resting 6 h, PBMCs were allocated to a monocyte assay for 12 h stimulation with Lipopolysaccharide (LPS) (10 ng/mL, Sigma-Aldrich) or unstimulated RPMI media. The cells were stained with Live/Dead discriminator, followed by cell surface staining of CD3, CD4, CD14, CD16 and HLA-DR and intracellular staining of COX-2. The following live/dead discriminator stain and directly conjugated monoclonal antibodies were used in the monocyte assay: Live/Dead Fixable Near-IR Dead Cell Stain kit (Life technologies), anti-CD3-PE-Cy7, anti-CD4-Horizon V500, anti-CD14-APC, anti-CD16-PE, anti-HLA-DR-BV421 (all from BD Bioscience) and anti-COX-2 (Cayman Chemicals, Ann Arbor, MI, USA). Human FOXP3 buffer set (BD bioscience) was used for fixation and permeabilisation.

T regulatory cells and intracellular cytokine assays

PBMC's (0.75 -1 × 10^6 cells/well) from patients with untreated active TB were rested 6 h before stimulation with *Mtb* derived 6 kDa early secretory antigenic target (ESAT-6, 2 ug/ml) and Antigen 85 (Ag85, 2 ug/ml) complex (both 15mer overlapping peptide, Genscript, HK limited; >85 % purity). The samples were stimulated with or without the COX-i indomethacin (25 μM, Sigma Aldrich). Staphylococcal enterotoxin B (SEB) (1 ug/ml, Sigma-Aldrich) was used as positive control and serum-free medium (AIM V; Gibco Invitrogen, Carlsbad, CA, USA) with 0.1 % highly purified human albumin as negative control. Due to limited number of cells indomethacin was not added to the SEB stimulated samples. Only PBMCs with viability >80 % were included. Samples with COX-i were pre-treated with indomethacin 2 h before stimulation and then incubated for 12 and 36 h. In the 12 h assay, Brefeldin A (BFA), final concentration 10 ug/ml (BD Bioscience) was added at time of stimulation, whilst in the 36 h assay BFA was added for the last 10 h to avoid prolonged incubation with potential toxic effects of BFA [32]. In both the 12 and 36 h assay, the cells were washed and stained with live/dead discriminator in azide-free and serum/protein-free PBS followed by CD4, CD3 and CD25 surface staining. The samples were washed and fixed/permeabilized with the FOXP3 staining kit (BD Biosciences) according to the manufacturer's instructions. Subsequently, cells were stained for intracellular TNF-α, IFN-γ, IL-2 and FOXP3. The following reagents were used in the 12 and 36 h stimulation assays for detection of intracellular cytokines and Tregs: anti-CD4- APC- H7, anti-CD3-PerCP-Cy5.5, anti-CD25-BV605, anti-IL-2-PE, anti-IFN-γ- PE-Cy7, anti-TNF-α- APC, anti-FOXP3- AF 488 (all from BD Bioscience) and Fixable Viability Dye eFluor® 450 (eBioscience, San Diego, USA).

CFSE proliferation assay

PBMCs (5 × 10^5cells/well) from active TB patients were thawed and rested 6 h before labeling with Carboxyfluorescein succinimidyl ester (CFSE) according to manufacturer's procedure. In the COX-i treated samples, indomethacin was added 2 h prior to stimulation with ESAT-6 and Ag85. The samples were incubated for 6 days and then washed

and stained for surface markers and viability staining. Fluorochromes used in the 6 days proliferation assay; anti-CD3-V450, anti-CD4- APC H7, anti-CD45RA -BV 605, anti-HLA DR- APC, anti-CD25-PE, anti-CD127- PeCy7 and 7AAD- PerCP (all from BD Bioscience) and CellTrace™ CFSE Cell Proliferation (Life technologies).

Flow cytometry analyses

Flow cytometric acquisition was performed on a BD FACS Canto II and a BD LSR Fortessa flow cytometer. At least 10.000 CD4+ and CD8+ T cells were analyzed for the intracellular analyses of cytokines and FOXP3 expression in T cells and minimum 1.000 monocytes were required for the analyses of COX-2 expression in monocytes. FlowJo version 10 (TreeStar Inc, Ashland, OR, USA) was used for data analyses. Dead cells were excluded from the lymphocyte and monocyte populations before applying the different gating strategies. In the cytokine analyses, frequencies (percentage of parent population) of *Mtb* antigen stimulated cytokine producing T cells were calculated. Total IFN-γ+, IL-2+ or TNF-α+ describe all CD4+ or CD8+ cells positive for the cytokine measured, while Boolean gating strategy was used to create cytokine combinations defined as: polyfunctional (IFN-γ+IL-2+TNF-α+), double positive (IFN-γ+IL-2+ or IL-2+TNF-α+or IFN-γ+TNF-α+) and single positive (IFN-γ+ or IL-2+ or TNF-α+) producing CD4+ and CD8+ T cells. Tregs were defined as FOXP3+CD25++CD4+ or as FOXP3+ CD45RA-CD4+ T cells [33]. In the T cell proliferation assay, cut-off for proliferating cells was set to the peak of the 2nd generation of $CFSE^{dim}$ CD4+ or CD8+ T cells. Frequencies (percentage of parent population) of *Mtb* antigen-stimulated cytokine-producing T cells, Tregs and proliferation T cells are all shown without subtracting background values (unstimulated controls) to better delineate the effect of indomethacin on stimulated versus unstimulated samples. Monocytes were defined as "true monocytes" after exclusion of CD3+ and CD16+HLA-DR- cells [34] and COX-2 expression (percentage of parent population) by true monocytes was analysed. The statistical region in the monocyte population was set by use of COX-2 human blocking peptide (Cayman chemicals) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed by SPSS statistics 22 (IBM) and Statistica v 7.0 (Statsoft, Tulsa, OK, USA). Non-parametrical statistical methods were applied. For group-wise comparison Mann-Whitney U test was applied and for dependent variables the two-tailed Wilcoxon matched pair test. A significance level of 0.05 was used. All values are presented as median and interquartile range [IQR]. Graphical presentations were made using Prism V5.04 and V6 software (GraphPad, San Diego, USA).

Results

COX 2 expression in monocytes in active and latent tuberculosis

We first analyzed COX-2 levels in monocytes from patients with latent or active TB (gating strategy, Fig. 2a). Although not significant, unstimulated monocytes from patients with active TB tended to express higher levels of COX-2 compared to patients with latent TB (Fig. 2b). Still, monocytes from both TB groups were able to significantly up-regulate COX-2 expression after 12 h in vitro LPS stimulation (Fig. 2b).

Indomethacin reduces up-regulation of *Mtb* antigen induced FOXP3+CD25++ Tregs

We then assessed the in vitro effects of the COX-i indomethacin on Tregs from patients with active TB disease prior to initiation of anti-TB chemotherapy (Fig. 3). FOXP3+CD25++ Tregs were analyzed after 36-h TB antigen stimulation, shown to be the optimal time for analyzing FOXP3 changes in Tregs [35]. We observed a significant up-regulation both in the fraction and the median fluorescence intensity (MFI) of FOXP3+CD25++ Tregs in the ESAT-6 and Ag85 stimulated CD4+ T cells with a significant reduction in the samples treated with indomethacin both for the fraction of FOXP3+CD25++ Tregs (ESAT-6; $p = 0.004$ and Ag85; $p < 0.001$) (Fig. 3a) and FOXP3 MFI (unstim; $p = 0.024$ and Ag85; $p = 0.023$) (Fig. 3b).

Indomethacin reduces *Mtb* antigen induced T cell cytokine production

To assess the effect of indomethacin on *Mtb* specific CD4+ T cells, we stimulated cells with *Mtb* peptides with or without addition of indomethacin and measured intracellular cytokine production after 12 and 36 h. There were significant up-regulation of CD4+ T cell subsets producing both IL-2, TNF-α and IFN-γ, in ESAT-6 stimulated cells already after 12 h and in Ag85 stimulated samples also after 36 h (Fig. 4). Indomethacin reduced the fraction of total IL-2 producing cells after 12 h (ESAT-6; $p = 0.032$) (Fig. 4a) and the fraction of total TNF-α producing cells after 36 h stimulation (ESAT-6; $p = 0.002$ and Ag85; $p = 0.026$) (Fig. 4b), whereas no significant changes were seen for the IFN-γ producing cells.

Single, double and polyfunctional CD4+ T cells were also determined. After 12 h of stimulation there was no clear pattern in response to indomethacin (Fig. 5a). The TNF-α+IFN-γ+CD4+ cells demonstrated a slight increase (ESAT-6; $p = 0.023$), whilst single TNF-α+ cells decreased (Ag85; $p = 0.043$). However, after 36 h stimulation, we observed a more distinct pattern in cells treated with indomethacin with down-regulation of the following cytokine producing CD4+ T cell subsets; IFN-γ+IL-2+TNF-α+T cells (Ag85; $p = 0.030$), IL-2+TNF-α+(ESAT-

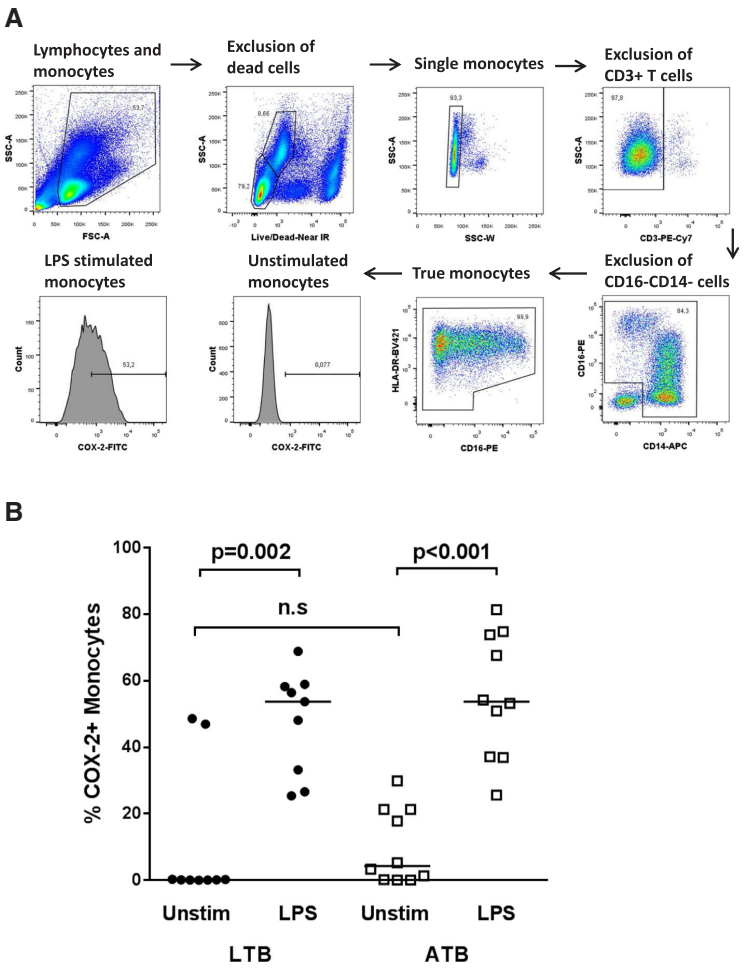

Fig. 2 COX-2 expression in monocytes. **a** Gating strategy for identification of true monocytes [34]. **b** Comparison of COX-2 expression in unstimulated true monocytes in PMBCs from patients with latent TB (LTB, *circles*) and active TB (ATB, *squares*). PBMCs from patients with LTB and ATB were left unstimulated (Unstim) or stimulated for 12 h with LPS. P- values were calculated by Wilcoxon matched pairs test for paired samples and Mann-Whitney U test for group wise comparison (n.s = non-significant). *Horizontal lines* in **b** represent median values

6; $p = 0.046$ and Ag85; $p = 0.007$) and single-TNF-α+ cells (ESAT-6; $p = 0.011$ and Ag85; $p = 0.031$) (Fig. 5b).

CD8+ T cells also significantly up-regulated their cytokine production upon *Mtb* antigen stimulation, but the effect of indomethacin was less pronounced. Overall there was a decrease in cytokine production, but only significant for total IFN-γ+ and polyfunctional IFN-γ-+IL-2+TNF-α+CD8+ T cells after 36 h stimulation (ESAT-6; $p = 0.039$ and $p = 0.017$, respectively) (data not shown).

Indomethacin modulates *Mtb* antigen induced T cell proliferation

The proliferative capacity of *Mtb* peptide stimulated CD4+ and CD8+ T cells was assessed by CFSE staining in a 6 days stimulation assay. There was a significant increase in both proliferating CD4+ (ESAT-6 and Ag85; $p < 0.001$) and CD8+ T cells (ESAT-6 and Ag85 $p < 0.001$) in stimulated compared to unstimulated cells (Fig. 6). A significant, but modest, decrease of proliferating CD4+ T cells was observed in the indomethacin treated ESAT-

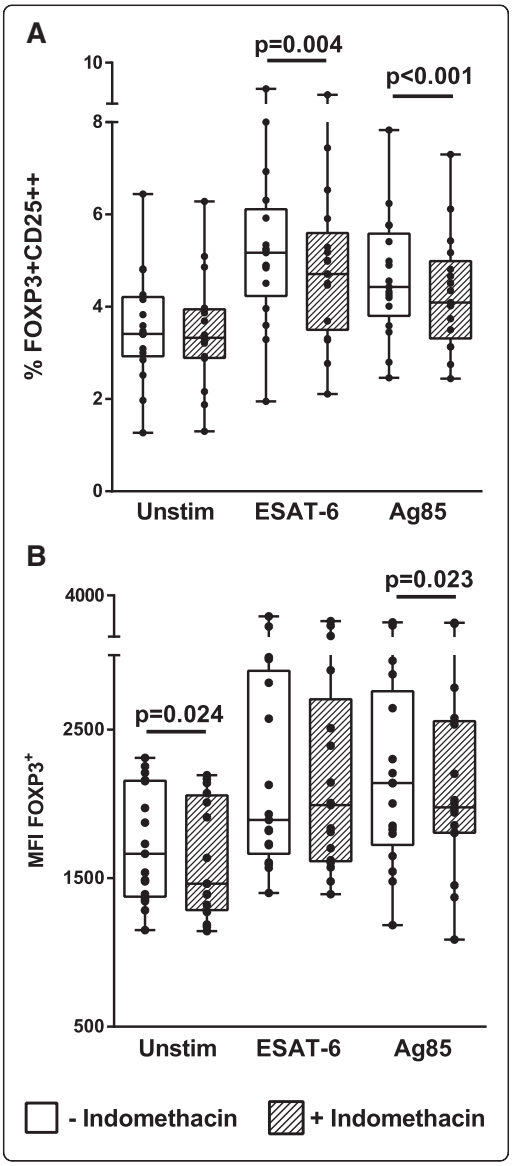

Fig. 3 Effect of indomethacin on TB antigen induced FOXP3+CD25++ CD4+ Tregs. PBMCs were obtained from patients with active TB ($n = 17$) prior to treatment and left unstimulated (unstim) or stimulated for 36 h with ESAT-6 or Ag85 without (open boxes) or with (hatched boxes) addition of indomethacin. The figures show percentages of FOXP3 +CD25++ (**a**) and FOXP3 median fluorescence intensity (MFI) (**b**) in the CD4+ cells. P- values were calculated by Wilcoxon matched pairs test. Plots are shown as *box plots* together with individual data points with median, IQR and minimum/maximum values. Significant changes between stimulated samples with and without indomethacin are denoted with *p*-values in figure. Levels of FOXP3+CD25++ CD4+ T cells and FOXP3 MFI were significantly upregulated upon stimulation with both E6 and Ag85 ($p < 0.01$, values not shown in figure)

6 stimulated samples ($p = 0.008$) (Fig. 6a), whilst no significant changes were seen in the Ag85 stimulated samples. For the CD8+ subsets, indomethacin likewise reduced proliferation of Ag85 stimulated cells ($p = 0.002$) (Fig. 6b). Cell surface activation markers were also analyzed after 6 days stimulation and showed a decrease in the fraction of activated CD25+HLA-DR+ (ESAT-6; $p = 0.011$ and Ag85; $p = 0.044$) CD4+T cell subsets in response to indomethacin treatment (data not shown).

Discussion

Host-directed immune modulating therapies have been suggested as adjunct treatment in combination with standard anti-TB antibiotics. Anti- inflammatory drugs such as corticosteroids are already in use as adjunctive therapy in TB meningitis with reduction of both morbidity and mortality [36]. Non-steroid Anti- inflammatory drugs (NSAIDS) are already in use to relieve symptoms in non-severe cases of paradoxical TB-IRIS [37] and there are anecdotal reports of use of NSAIDS in complicated cases of TB disease, however such practice is poorly documented in literature. In this study we explored the in vitro effects of the COX-i indomethacin on immune cells obtained from patients with active TB disease prior to initiation of anti-TB chemotherapy. We demonstrate for the first time that indomethacin significantly down-regulates the fraction of *Mtb* antigen induced Tregs as well as T cell proliferation and TNF-α cytokine production.

First, we explored COX-2 expression in monocytes, the major source for PGE2 production in a small cohort of active and latent TB infection. Monocytes from patients with active TB exhibit functional and phenotypical alterations compared with healthy controls [38, 39] that could be restored by TB treatment [40]. Different species of mycobacteria have been suggested to induce expression of COX-2 through different signaling pathways [41]. To our knowledge, there are no previous studies comparing COX-2 expression in latent versus active TB in humans. Interestingly, we found a tendency of higher spontaneous expression of COX-2 in the unstimulated

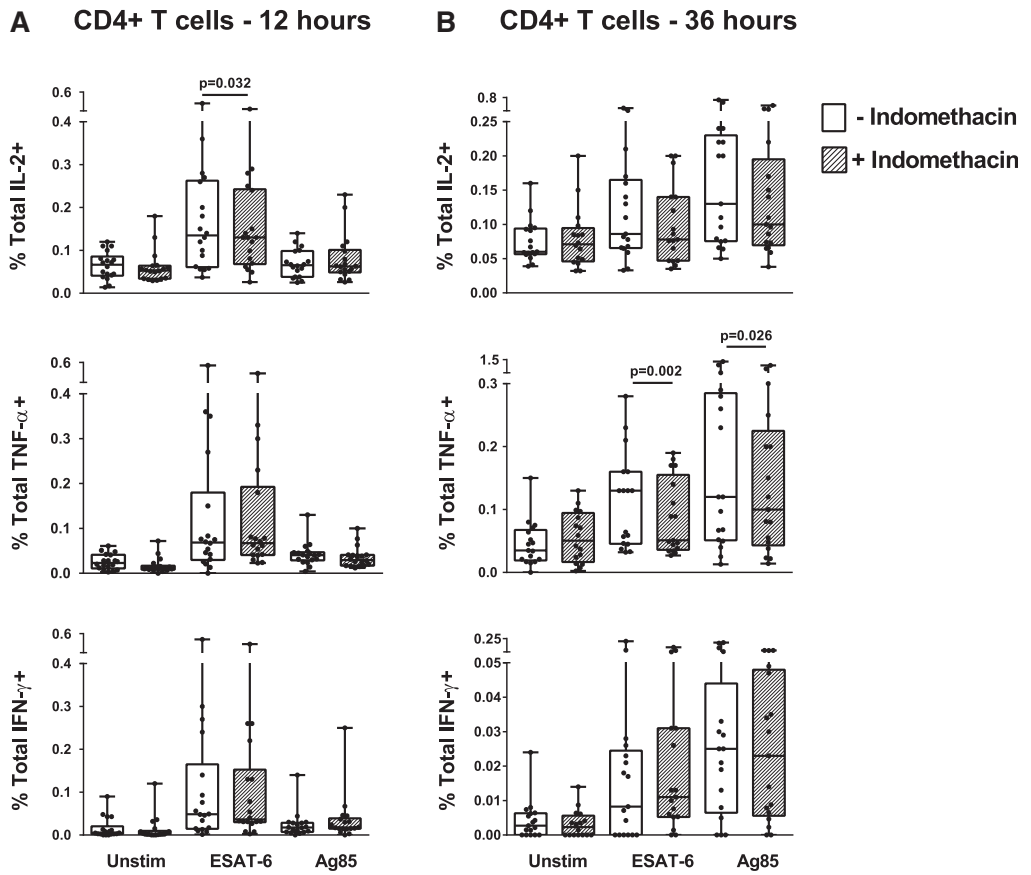

Fig. 4 Effect of indomethacin on TB antigen induced intracellular cytokines in CD4+ T cells. Total IFN-y+, IL-2+ and TNF-α+ T cell responses in unstimulated (Unstim), ESAT-6 and Ag85 stimulated PBMCs without (*open boxes*) and with (*hatched boxes*) indomethacin after 12 h (**a**, $n = 18$) and 36 h (**b**, $n = 17$) stimulation. PBMCs were obtained from patients with active TB prior to treatment. *P*- values calculated by Wilcoxon matched pairs test. Plots are shown as *box plots* with individual data points with median, IQR and minimum/maximum values. Significant changes between stimulated samples with and without indomethacin are denoted with p-values in figure

active TB compared to the latent TB samples, possibly indicating an ongoing activation of the COX-2-PGE2 pathway in patients with active disease. However, as shown by our data, also in otherwise healthy patients with latent TB high levels of COX-2 could be expressed. Thus, COX-2 levels in monocytes at different stages of TB infection and any implication for disease progression needs to be further studied in larger cohorts.

The main finding in our study was the reduction of antigen induced Tregs in the indomethacin treated samples. Of note, indomethacin also reduced the levels of FOXP3 MFI in the unstimulated samples, although to a lesser extent than seen in the stimulated samples. This possibly reflects an up-regulated COX expression and increased PGE2 production in vivo in patients with active TB. There are several reports of increased levels of Tregs in TB disease [42–44], but the role of these immune regulatory cells during chronic TB infection is unclear [45]. Still, experience from animal models has proven detrimental effects of Tregs during the initial stage of infection [46]. Treg mediated suppression of effector T cells occurs through different mechanisms [47] and Tregs may inhibit T cell effector functions in a COX-2-PGE2 dependent manner [14]. We find support for the use of COX-i in targeting Tregs from studies showing that PGE2 induces FOXP3 gene expression and Treg function in human CD4+ T cells [48]. Both the up-regulation of FOXP3 in Tregs and their suppressive effect on effector T cells have been shown to

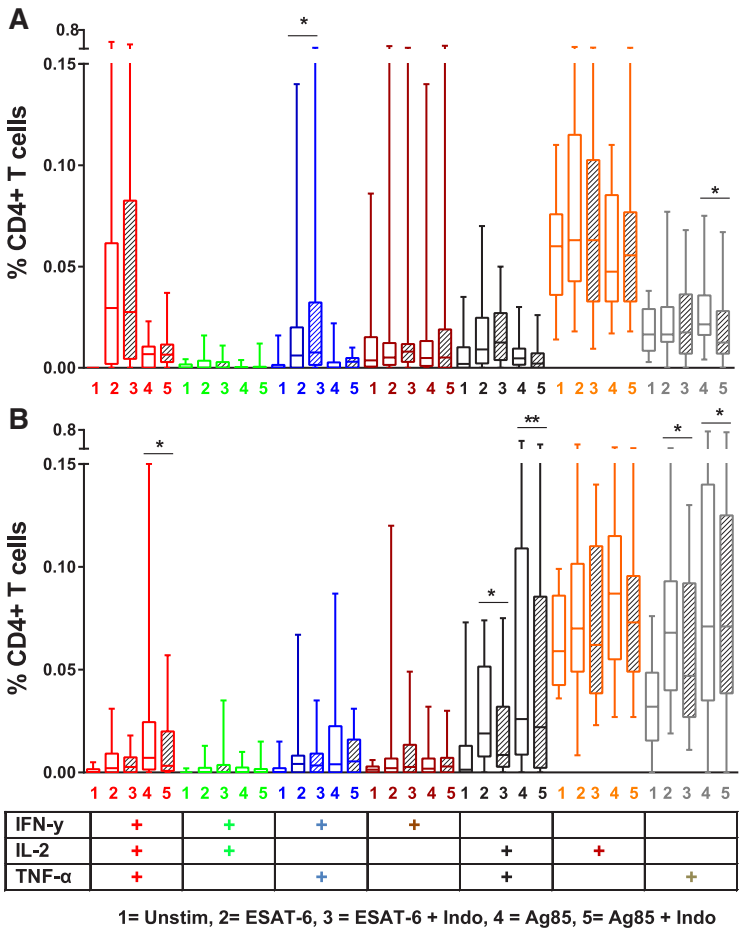

Fig. 5 *Mtb* antigen induced single, duo and polyfunctional cytokine producing CD4+ T cells. PBMCs were obtained from patients with active TB prior to treatment and left unstimulated or stimulated with ESAT- 6 or Ag85 without (*open boxes*) or with indomethacin (*hatched boxes*) after 12 h (**a**) and 36 h (**b**) stimulation ($n = 18$ and $n = 17$ respectively). Boolean gating strategy was used to create cytokine combinations of single-producing, duo-producing and polyfunctional T cells. *P*- values were calculated by Wilcoxon matched pairs test ($*p < 0.05$, $**p < 0.01$). Plots are shown as *box plots* with median, IQR and minimum/maximum values

be reversed by COX- i [14]. To our knowledge there are no published data of immune therapy aiming to modulate Tregs in human TB disease. There is however an ongoing phase I/II clinical trial on COX-2-i in active TB where Tregs will be studied (ClinicalTrials.gov Identifier: NCT02503839). An additional clinical trial aims to reduce the risk of developing active TB by oral supplementation of a probiotic containing heat-killed environmental mycobacteria (ClinicalTrials.gov Identifier: NCT02076139). Preclinical animal models to this study have shown that the mycobacteria delay progression to active TB in mice by inducing PPD specific Treg populations [49]. Nevertheless, we claim that our observed reduction of FOXP3 in indomethacin treated Tregs may be beneficial to the host, shifting the balance towards a more effective immune response in long-lasting and advanced TB disease.

Animal models also support PGE2 as a significant factor in the pathogenesis of a dysfunctional hyperactivated immune system in TB, concluding with beneficial effects of COX-i with suppressed PGE2 concentrations; reductions in pulmonary inflammation and bacillary load, reversal to a Th1 cell profile and improved survival of TB infected mice

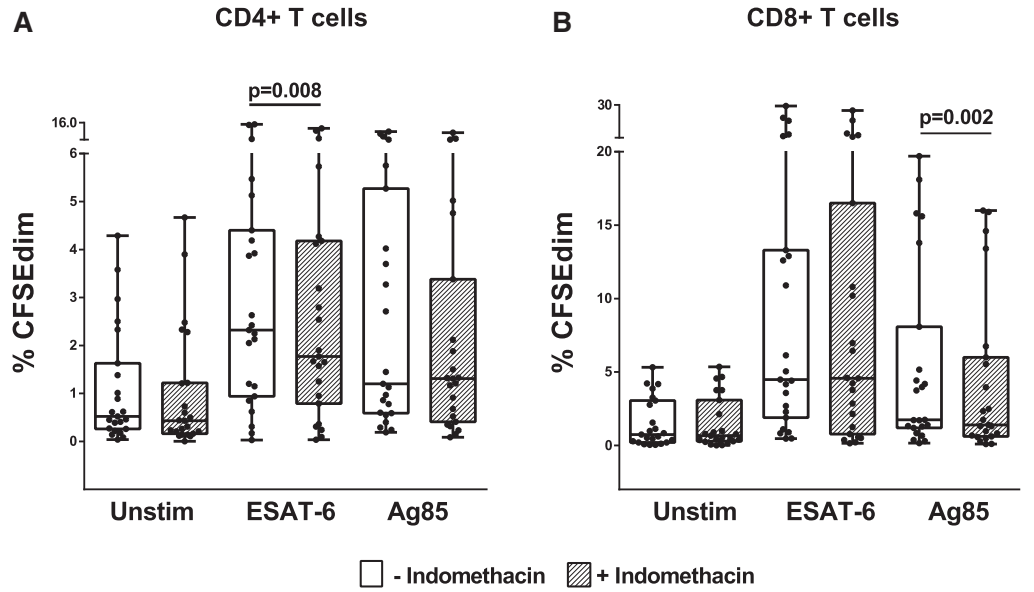

Fig. 6 Effect of Indomethacin on CD4+ and CD8+ T cell proliferation. Proliferative responses measured by percentages of $CFSE^{dim}$ in unstimulated, ESAT-6 and Ag85 stimulated PBMCs without (*open boxes*) and with (*hatched boxes*) indomethacin after 6 days stimulation. PBMCs were obtained from patients with active TB prior to treatment. **a** CD4+ T cells ($n = 23$). **b** CD8+ T cells ($n = 23$). *P*- values were calculated by Wilcoxon matched pairs test. Plots are shown as *box plots* with individual data points with median, IQR and minimum/maximum values. Significant changes between stimulated samples with and without indomethacin are denoted with *p*-values in figure. %CFSEdim cells were significantly upregulated upon stimulation with both E6 and Ag85 ($p < 0.001$, values not shown in figure)

[21–24]. In contrast, one study reports reductions in morbidity and mortality by augmenting PGE2 levels in *Mtb*-infected mice [25]. The conflicting reports may be due to differences in time since TB infection, emphasizing the need to be aware of differential effects of PGE2 in recently versus chronically TB infected mice.

Both CD4+ and CD8+ cytokine producing T cells play a major role in protection and immunity against TB disease [50], but contrasting results have made it difficult to define protective TB immunity based on cytokine responses [51–53]. In previous clinical trials, we have shown that HIV infected patients on antiretroviral therapy experience an improved immune response with concomitant treatment with COX2-i [26–28]. In the present study, the COX-i indomethacin modestly reduced the *Mtb* antigen induced CD4+ and CD8+ cytokine responses as well as the proliferative capacity of the T cells. Our data show that in particular the CD4+TNF-α+ cell subsets were significantly reduced when treated with indomethacin, but still with detectable levels. Experience from animal models show that TNF-α is important for control of TB [54, 55] and treatment with TNF-α blockers have been associated with the progression from latent to active TB in humans [56, 57]. TNF-α is essential for maintenance and formation of the granuloma, but excess TNF-α production also contributes to increased inflammation and pathology in TB [58]. Thus, one of the purposes of immune modulating therapy may be to decrease pathology related to excess TNF-α production while maintaining adequate TNF-α levels necessary for containment of *Mtb* [59]. Accordingly, the decreased levels of TNF-α producing CD4+ T cells observed in our study may constitute a potential beneficial response in a setting with chronic untreated TB disease.

The observed reduction of cytokines and proliferative capacity of T cells may be due to the direct effects of indomethacin inhibiting activation of the intracellular NF-κB pathway [60]. The NF-κB pathway is a key mediator of genes involved in the control of cellular proliferation and apoptosis [61]. Inhibition of the NF-κB pathway may be involved in the anti-inflammatory as well as the growth inhibitory properties of certain COX-i [62, 63] and COX-i have also been reported to inhibit NF-κB activation in cell culture [64]. In TB, reports have shown that TNF-α-induced NF-κB signaling pathway is central to the *Mtb*-specific immune response, and regulation of intracellular NF-κB signaling dynamics may be a key to control TB infection [65]. Thus, in summation, one must consider that COX-i may exert a relatively stronger

inhibitory effect on the Th1 effector cells than the anticipated beneficial indirect effect following reduced Treg numbers.

There are limitations to our study. First, the relatively small number of patients gives reduced power in the statistical calculations and increases the risk for Type II statistical errors. Second, multiple testing increases the risk of type I errors. As there is no gold standard for how to handle multiple comparisons when considering partially dependent variables a significance level of 0.05 was used. Due to limited numbers of samples and PBMC's available, we were not able to perform sorting of Tregs with more in-depth mechanistic studies of the potential impact of the observed decrease of indomethacin treated Tregs. For the same reason all analysis could not be performed in the latent TB group. Thus, future studies on COX-i should include latent TB controls and also investigate the *in vivo* effects of COX-i in clinical trials of patients in different stages of *Mtb* infection.

Conclusions

In conclusion, our data indicate that indomethacin may be used to modulate immune responses in TB infection by reducing the fraction of *Mtb* specific Tregs with a concomitant reduction of *Mtb* specific cytokine responses and T cell proliferation in active TB disease. Still, the effects of COX-i in TB infection need further evaluation in human models and future clinical trials should be performed to explore the effects of COX-i as HDT options in different settings of TB infection and disease.

Abbreviations

COX: Cyclooxygenase; COX-i: Cyclooxygenase inhibitor/s; HDT: Host-directed therapies; LPS: Lipopolysaccharide; MDR-TB: Multi drug resistant TB; MFI: Median fluorescence intensity; *Mtb*: Mycobacterium tuberculosis; PBMC(s): Peripheral blood mononuclear cells; PGE2: Prostaglandin E2; SEB: Staphylococcal enterotoxin B; TB: Tuberculosis

Acknowledgements

We would like to thank all study participants, Siri Feruglio, Mette Sannes, Helene Gjelsås and Linda Gail Skeie at Oslo University Hospital and Christel Gill-Haanshuus, Steinar Sørnes and Tehmina Mustafa at Haukeland University Hospital for assistance during our study.

Funding

The study was funded by the University of Oslo, Oslo University Hospital and the University of Bergen. The authors also gratefully acknowledge funding by the "Blakstad and Maarschalk Tuberkulosefond".

Availability of data and material

Data are available on request to the authors, but personal details and individual clinical characteristics of the participants will not be made available in order to protect the participants' identity.

Authors' contributions

Conceived and designed the experiments: KT, IW, NVL, KjT, DK, AMDR. Recruited the patients: KT, AMDR. Performed the experiments: KT, IW, NVL. Analyzed the data: KT, IW, NVL, AMDR. Contributed reagents/materials/analysis tools: KT, IW, AMDR, DK. Drafted and reviewed the manuscript: KT, IW, NVL, KjT, AMDR, DK. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

Ethics approval and consent to participate

Written informed consent was obtained from all participants with specific information given to the patients about the use of blood samples. The study was approved by the Regional Committees for Ethics in Medical Research (REK-Sør-Øst and REK Vest). All experiments were performed in accordance with relevant guidelines and regulations.

Author details

[1]Institute of Clinical Medicine, University of Oslo, Oslo, Norway. [2]Department of Infectious Diseases, Oslo University Hospital, Oslo, Norway. [3]Department of Clinical Science, University of Bergen, Bergen, Norway. [4]Centre for Molecular Medicine Norway, Nordic EMBL Partnership, University of Oslo, Oslo, Norway. [5]K.G. Jebsen Inflammation Research Centre, University of Oslo, Oslo, Norway. [6]Biotechnology Centre, University of Oslo, Oslo, Norway.

Received: 12 April 2016 Accepted: 18 October 2016
Published online: 24 October 2016

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