# Characterization of Monocytes and Blood Plasma Pathology in Humans with Tuberculosis Disease

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Düsseldorf, October 2024

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Gedruckt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf

Berichterstatter:

1. Prof. Dr. Marc Jacobsen

2. Prof. Dr. Rainer Kalscheuer

Tag der mündlichen Prüfung: 10.03.2025

**List of publications**

**Paper I:**

**Ahor HS**, Vivekanandan M, Harelimana JD, Owusu DO, Adankwah E, Seyfarth J, Phillips R, Jacobsen M. (2024). Immunopathology in human pulmonary tuberculosis: Inflammatory changes in the plasma milieu and impaired host immune cell functions. *Immunology*. 172(2):198-209.

**Paper II:**

**Ahor HS**, Schulte R, Adankwah E, Harelimana JD, Minadzi D, Acheampong I, Vivekanandan MM, Aniagyei W, Yeboah A, Arthur JF, Lamptey M, Abass MK, Kumbel F, Osei-Yeboah F, Gawusu A, Debrah LB, Owusu DO, Debrah A, Mayatepek E, Seyfarth J, Phillips RO, & Jacobsen M. (2023). Monocyte pathology in human tuberculosis is due to plasma milieu changes and aberrant STAT signaling. *Immunology.* 170(1):154-166.

**Paper III (preliminary):**

**Ahor HS**, Vivekanandan MM, Adankwah E, Minadzi D, Acheampong I, Aniagyei W, Yeboah A, Arthur JF, Lamptey M, Abass MK, Kumbel F, Osei-Yeboah F, Gawusu A, Petzsch P, Köhrer K, Debrah LB, Owusu DO, Debrah A, Mayatepek E, Seyfarth J, Phillips RO, & Jacobsen M. Monocyte transcriptome signatures of inflammation and enhanced neutrophil recruitment characterize immunopathology in the blood of tuberculosis patients.

**Publications not included in this thesis**

Harelimana JD, **Ahor HS**, Benner B, Hellmuth S, Adankwah E, Minadzi D, Aniagyei W, Lamptey MNK, Arthur J, Yeboah A, Abass MK, Debrah LB, Owusu DO, Mayatepek E, Seyfarth J, Phillips RO, Jacobsen M. (2022).Cytokine-induced transient monocyte IL-7Ra expression and the serum milieu in tuberculosis. *Eur J Immunol.* 52(6):958-969.

Aniagyei W, Mohayideen S, Sarfo-Kantanka O, Bittner S, Vivekanandan MM, Arthur, JF, Boateng AO, Yeboah A, **Ahor HS,** Asibey SO, Owusu E, Herebian D, Huttasch M, Burkart V, Wagner R, Roden M, Adankwah E, Owusu DO, Mayatepek E, Jacobsen M, Phillips RO, Seyfarth J.(2024). BCG Vaccination-Associated Lower HbA1c and Increased CD25 Expression on $CD8^{+}$T Cells in Patients with Type 1 Diabetes in Ghana. *Vaccines*, *12*(5), 452.

**Summary**

Monocytes and their derived cells are critical in immune surveillance against *Mycobacterium tuberculosis* infection. However, alterations in the phenotypes and functions of monocytes in peripheral blood have been observed in tuberculosis (TB) patients. The potential impact of the changes in the plasma milieu driving the immunopathology of TB patients has been suggested but has not been fully characterized. A review of the literature on the plasma pathology of TB patients revealed significant heterogeneity, highlighting the complexity of the disease. However, signatures of inflammation in the plasma of TB patients were also identified. To investigate the hypothesis that plasma changes may contribute to the altered immune response in TB patients, an *in vitro* plasma milieu assay was established to analyze the influence of plasma samples from TB patients on monocytes. This assay involved an overnight culture of healthy reference monocytes in media supplemented with plasma samples from TB patients or their asymptomatic contacts (controls). Subsequent analysis of the phenotype, transcription profile, and cytokine signal transduction was performed using flow cytometry and RNA sequencing. Analyses of monocytes, along with other immune cell subsets in TB patients (before and after treatment) and in healthy controls, were conducted simultaneously to confirm changes in monocytes and to determine the effects of plasma immunopathology on the anti-mycobacterial host response in TB. This study observed that the plasma of TB patients induced monocyte phenotypes resembling the phenotype of monocytes in TB patients. Notably, STAT-mediated signal transduction was shown to play a critical role in the plasma-induced phenotype differences. Proinflammatory cytokines secreted by monocytes amplified the effects of TB plasma endogenously via STAT pathways. High concentrations of chemokines induced by TB plasma were functionally associated with the recruitment and mobilization of neutrophil granulocytes and CCR2+ inflammatory monocytes from the bone marrow. The biological relevance of this finding was underpinned by the fact that the distribution of immune cells in the peripheral blood of TB patients showed a higher proportion of neutrophil granulocytes and a shift towards an inflammatory monocyte subset. Interestingly, interleukin (IL)-6 secreted by reference monocytes and IL-6 concentrations in plasma were key factors that positively correlated with the enhanced effects of TB plasma on reference monocytes and the proportion of neutrophils in the blood of TB patients respectively. In summary, this study observed a clear overlap between plasma effects on reference monocytes

and monocyte phenotype changes in TB patients. The effects of TB plasma on monocytes promote both inflammatory disturbances and immune modulation, potentially leading to the suppression of the immune responses in TB patients. Cytokines or plasma factors that signal via STAT pathways may play a crucial role in boosting inflammation, which could contribute to the pathology of TB in humans.

**Zusammenfassung**

Monozyten und von ihnen abgeleitete Zellen spielen eine entscheidende Rolle bei der Immunabwehr gegen den Erreger *Mycobacterium tuberculosis*. Bei Patienten mit Tuberkulose (TB) wurden in bisherigen Studien Phänotyp- und Funktionsveränderungen der Monozyten im peripheren Blut beobachtet. Der potenzielle Einfluss von Plasmamilieuveränderungen auf die Immunpathologie von TB-Patienten konnte bisher jedoch noch nicht vollständig charakterisiert werden. Eine Überprüfung der Literatur zur Plasmapathologie von TB-Patienten legen eine erhebliche Heterogenität nahe, was die Komplexität der Krankheit verdeutlicht. Es wurden jedoch auch Entzündungssignaturen im Plasma von TB-Patienten festgestellt. Um zu beleuchten, ob Plasmaveränderungen zu einer veränderten Immunantwort bei TB-Patienten beitragen können, wurde ein In-vitro-Plasmamilieu-Assay entwickelt, der den Einfluss von Plasmaproben von TB-Patienten auf Monozyten untersucht. Im Versuch wurden gesunde Referenzmonozyten über Nacht kultiviert, die mit Plasmaproben von TB-Patienten oder ihren asymptomatischen Kontaktpersonen (Kontrollen) supplementiert wurden. Anschließend erfolgte die Analyse des Phänotyps, des Transkriptionsprofils und der Zytokinsignaltransduktion mittels Durchflusszytometrie und RNA-Sequenzierung. Analysen von Monozyten und anderen Untergruppen von Immunzellen bei TB-Patienten (vor und nach der Behandlung) und bei gesunden Kontrollpersonen wurden zeitgleich durchgeführt, um Veränderungen bei Monozyten zu bestätigen und die Auswirkungen der Immunpathologie des Plasmas auf die antimykobakterielle Wirtsantwort bei TB zu untersuchen. In dieser Studie wurde festgestellt, dass das Plasma von TB-Patienten Monozytenphänotypen hervorruft, die dem Phänotyp der Monozyten von TB-Patienten ähneln. Insbesondere wurde gezeigt, dass die STAT-vermittelte Signaltransduktion eine entscheidende Rolle bei den plasmainduzierten Phänotypunterschieden spielt. Verstärkten von Monozyten ausgeschüttete proinflammatorische Zytokine die Wirkung von TB-Plasma endogen über STAT-Signalwege. Hohe Konzentrationen von Chemokinen, die durch TB-Plasma induziert wurden, waren funktionell mit der Rekrutierung und Mobilisierung von neutrophilen Granulozyten und CCR2+ Entzündungsmonozyten aus dem Knochenmark assoziiert. Die biologische Relevanz dieses Ergebnisses wurde durch die Tatsache untermauert, dass die Verteilung der Immunzellen im peripheren Blut von TB-Patienten einen

höheren Anteil an neutrophilen Granulozyten und eine Verschiebung hin zu einer entzündlichen Monozytenuntergruppe zeigte. Interessanterweise waren das von Referenzmonozyten sezernierte Interleukin (IL)-6 und die IL-6-Konzentration im Plasma Schlüsselfaktoren, die positiv mit den verstärkten Auswirkungen von TB-Plasma auf Referenzmonozyten bzw. den Anteil der Neutrophilen im Blut von TB-Patienten korrelierten. Zusammenfassend lässt sich sagen, dass in dieser Studie eine deutliche Überschneidung zwischen den Auswirkungen des Plasmas auf Referenzmonozyten und den Veränderungen des Monozytenphänotyps bei TB-Patienten beobachtet wurde. Die Auswirkungen von TB-Plasma auf Monozyten fördern sowohl entzündliche Störungen als auch die Immunmodulation, was bei TB-Patienten möglicherweise zur Unterdrückung der Immunantwort führt. Zytokine oder Plasmafaktoren, die über STAT-Signalwege wirken, könnten eine entscheidende Rolle bei der Verstärkung von Entzündungen spielen, die zur TB-Pathologie beim Menschen beitragen könnten.

**List of abbreviations**

| | |
|---|---|
| **BCG** | Bacillus Calmette–Guérin |
| **CCL** | Chemokine (C-C motif) ligand |
| **CCR** | Chemokine (C-C motif) receptor |
| **CD** | Cluster of differentiation |
| **CXCL** | Chemokine (C-X-C motif) ligand |
| **G-CSF** | Granulocyte colony stimulating factor |
| **HLA-DR** | Human leukocyte antigen-DR isotype |
| **IFN-γ** | Interferon-gamma |
| **IGRAs** | Interferon-gamma release assays |
| **IL** | Interleukin |
| **LTBI** | Latent tuberculosis infection |
| **MMP** | Matrix metalloproteinase |
| **mRNA** | Messenger ribonucleic acid |
| **Mtb** | *Mycobacterium tuberculosis* |
| **MTBC** | *Mycobacterium tuberculosis* Complex |
| **PD1** | Programmed cell death protein 1 |
| **PDL1** | Program death Ligand 1 |
| **PHA** | Phytohemagglutinin |
| **SOCS3** | Suppressor of cytokine signaling 3 |
| **STAT** | Signal transducer and activator of transcription |
| **TB** | Tuberculosis |
| **TGF-β** | Transforming growth factor beta |
| **Th1** | T-helper 1 |
| **TNF-α** | Tumor necrosis factor-alpha |
| **WHO** | World Health Organization |

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# 1. Introduction

## 1.1 Overview of human tuberculosis disease

### 1.1.1 Epidemiology of tuberculosis

Tuberculosis (TB) is a significant global health issue and the second leading cause of death from a single infectious organism, following the coronavirus disease (COVID-19) (World Health Organization, 2023). The World Health Organization (WHO) reports that one-fourth of the world's population is infected with the causative agent of TB. In 2022, approximately 10.6 million individuals were reported to have developed TB, resulting in 1.3 million deaths globally. The highest disease burden is reported by WHO, primarily from the Southeast, Africa, and Western Pacific regions (Figure 1). Countries such as China, Indonesia, India, Mongolia, the Philippines, Pakistan, Bangladesh, South Africa, the Democratic Republic of the Congo, and Nigeria collectively account for two-thirds of all global cases (World Health Organization, 2023).

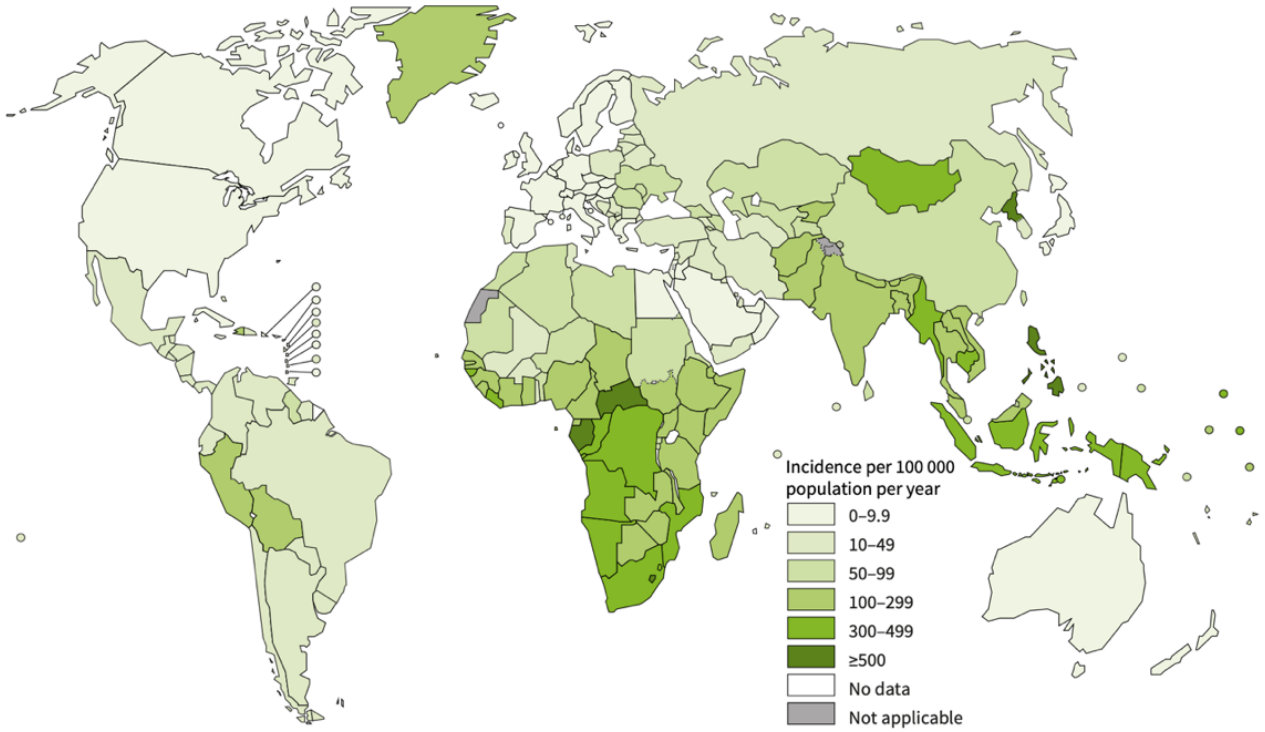


**Figure 1**: Estimated TB Incidence Rates in 2022. Source: World Health Organisation Global TB Report 2023.

### 1.1.2 Mode of transmission and symptoms of tuberculosis

TB in humans is caused by *Mycobacterium tuberculosis* (Mtb), a member of the *Mycobacterium tuberculosis Complex* (MTBC) (Pai et al., 2016). The bacilli are transmitted through aerosols from individuals with active pulmonary TB to close contacts. Following exposure to Mtb, individuals may either develop active TB disease, exhibiting symptoms such as fatigue, night sweats, fever, weight loss, lack of appetite, hemoptysis, and persistent cough, or they may have a latent TB infection (LTBI) without noticeable symptoms. Approximately 5-10% of individuals with LTBI will develop active TB disease later in life (Pai et al., 2016, Chandra et al., 2022). While TB primarily affects the lungs (pulmonary TB), it can also affect other tissues and organs (extrapulmonary TB).

### 1.1.3 Diagnosis and treatment of tuberculosis

Accurate diagnosis of TB requires a comprehensive approach that includes clinical history and various diagnostic tests. These tests include sputum smear for acid-fast bacilli, mycobacterial culture, chest X-ray, and nucleic acid amplification tests such as Xpert MTB/RIF Ultra, Xpert MTB/RIF, and line probe assays (Pai et al., 2016). Additionally, immune-based assays, including interferon-gamma release assays (IGRAs) and tuberculin skin tests, are employed to identify Mtb infection (Pai et al., 2016). However, current immune-based tests have low sensitivity in immunocompromised individuals and cannot effectively differentiate between latent infection and active TB (Adankwah et al., 2019, Hamada et al., 2023).

Standard treatment for TB involves a 6-month regimen, comprising an intensive two-month treatment phase with rifampicin, pyrazinamide, isoniazid, and ethambutol, followed by a 4-month phase with isoniazid and rifampicin (Nahid et al., 2016). However, antibiotic resistance has been reported in all WHO regions, complicating TB management (Nahid et al., 2016, Pai et al., 2016, World Health Organization, 2023). The only licensed vaccine against TB by WHO, the Bacillus Calmette–Guérin (BCG) vaccine, offers partial protection against disseminated TB in children but not against pulmonary TB in adults (Wolf et al., 2008, Pai et al., 2016). Therefore, there is a critical need for the development of new therapies in the fight against TB (Pai et al., 2016).

### 1.2 Pathophysiology of tuberculosis

Mtb infection begins when Mtb in aerosols enters the human body through the respiratory tract. Once inside the lungs, it is engulfed by alveolar macrophages and transported to the lung interstitium (Cohen et al., 2018). Within the lung interstitium, Mtb is phagocytosed by macrophages, monocytes, neutrophils, and dendritic cells. This triggers antimycobacterial killing and strong inflammatory responses resulting in the recruitment of diverse immune cells via cytokines and chemokines (Chandra et al., 2022, Cronan, 2022). Approximately 3 to 8 weeks after infection, T cells primed by infected dendritic cells and monocytes in the lymph nodes, proliferate and migrate into the lungs, initiating a delayed type IV hypersensitivity response primarily driven by CD4+ T helper 1 (TH1) cells (Samstein et al., 2013, Chandra et al., 2022, Cronan, 2022). Interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) secreted by Mtb-specific T cells activate macrophages to contain and eliminate Mtb via phagocytosis, phagosome maturation, apoptosis, and autophagy (Carabali-Isajar et al., 2023). Activated macrophages secrete pro-inflammatory and regulatory cytokines and chemokines, which further drive the recruitment of more cells into the lungs to control Mtb infection (Chandra et al., 2022, Cronan, 2022). Although these responses are directed to protect the host, chronic activation may exacerbate inflammation and drive TB immunopathology (Chandra et al., 2022, Cronan, 2022).

Despite the diverse antimycobacterial immune responses, Mtb manages to survive due to host evasion mechanisms that hinder phagosome-lysosome fusion, maturation, and antigen presentation to T cells (Houben et al., 2012, Peddireddy et al., 2017, Chandra et al., 2022). The persistence of Mtb and its tuberculin-like products leads to the formation of granulomas, a consequence of the delayed-type hypersensitivity response against mycobacteria (Peddireddy et al., 2017, Chandra et al., 2022, Cronan, 2022). Granulomas consist of a fibrotic matrix and clusters of uninfected or infected macrophages and specialized monocyte-macrophage-derived cells (epithelioid cells and multinucleated giant cells), surrounded by aggregates of diverse immune cells (Figure 2) (Peddireddy et al., 2017, Chandra et al., 2022, Cronan, 2022). Granulomas restrict and control Mtb infection by concentrating immune responses within a localized region to promote immune surveillance. This may lead to different outcomes, including the complete eradication of the bacteria or latent infection (Peddireddy et al., 2017, Chandra et al., 2022, Cronan, 2022). Several host and

pathogen factors determine granuloma stability (Chandra et al., 2022, Cronan, 2022). When containment of Mtb infection fails during primary infection, Mtb replicates and spreads in the lung resulting in active TB disease with clinical symptoms (Chandra et al., 2022, Cronan, 2022). In individuals with latent TB infection, reactivation of a previous infection or reinfection may lead to the development of active disease, referred to as secondary TB/Post-primary TB. Secondary TB is said to account for the majority of cases in adults and almost all transmission of the infection (Hunter et al., 2014).

### 1.3 Lung immunopathology in tuberculosis

Host immune surveillance is crucial for protection against Mtb and for preventing progression to TB. However, aberrantly strong antimycobacterial immune responses are associated with the pathology seen in TB patients (Peddireddy et al., 2017, Chandra et al., 2022). Macrophages and other phagocytic cells, through phagocytosis and apoptosis, limit the spread of Mtb. However, Mtb utilizes its virulence factors to induce necrosis, particularly in macrophages, which enhances bacterial survival, proliferation, and infection of additional cells. These events drive strong innate and T-cell-mediated responses, leading to chronic inflammation and subsequent lung tissue damage (Chandra et al., 2022, Cronan, 2022, Peddireddy et al., 2017). Chronic inflammatory responses against Mtb and its tuberculin products disrupt the delicate balance of immune surveillance and contribute to (and potentially trigger) lung pathology. Elevated levels of IL-1β and TNF-α, considered host-protective cytokines, are associated with airway pathology (Chandra et al., 2022). Additionally, increased TNF-α can exacerbate tissue destruction by inducing necrotic cell death and promoting the production of tissue-degrading matrix metalloproteinases (MMPs) (Chandra et al., 2022, Cronan, 2022, Peddireddy et al., 2017). High levels of inflammatory cytokines/chemokines (IL-1β, TNF-α, IL-4, TGF-β, and CCL-4) and MMPs (MMP 1, 3,8, and 9) associated with TB disease severity, cavity formation, and fibrosis in the lungs of TB patient have been reported (Ameglio et al., 2005, Stek et al., 2018, Sabir et al., 2019, Herrera et al., 2022).

Cavity formation, a prominent pathological feature of secondary TB, is usually preceded by caseous pneumonia, which is driven by the intracellular or extracellular accumulation of host lipids and mycobacterial antigens. This accumulation leads to caseous necrosis and fragmentation of granulomas, causing the liquefied content of

granulomas to be discharged into the bronchi and airways during coughing (Hunter et al., 2014, Weeratunga et al., 2024). This facilitates Mtb proliferation and further contributes to chronic lung tissue inflammation, necrosis, fibrosis, tissue remodeling, and damage, in addition to exacerbating clinical symptoms and facilitating Mtb transmission (Hunter, 2011, Hunter et al., 2014, Basaraba and Hunter, 2017, Weeratunga et al., 2024). These events occurring in the lung result in a highly oxidative and hypoxic environment, which further causes massive tissue damage (Belton et al., 2016, Amaral et al., 2021). In summary, the lung pathology in TB is characterized by the disruption of the finely-tuned host immune response aimed at controlling Mtb infection, leading to chronic inflammation, aberrant immune cell function, cavity formation, and massive remodeling/ damage of lung tissue resulting in impaired lung function (Basaraba and Hunter, 2017, Hunter et al., 2014, Chandra et al., 2022). Consequently, the development of novel antimicrobials and host-directed treatments that positively influence immune responses to Mtb would enhance clinical outcomes, shorten treatment durations, and improve the long-term effect of TB (Cubillos-Angulo et al., 2022).

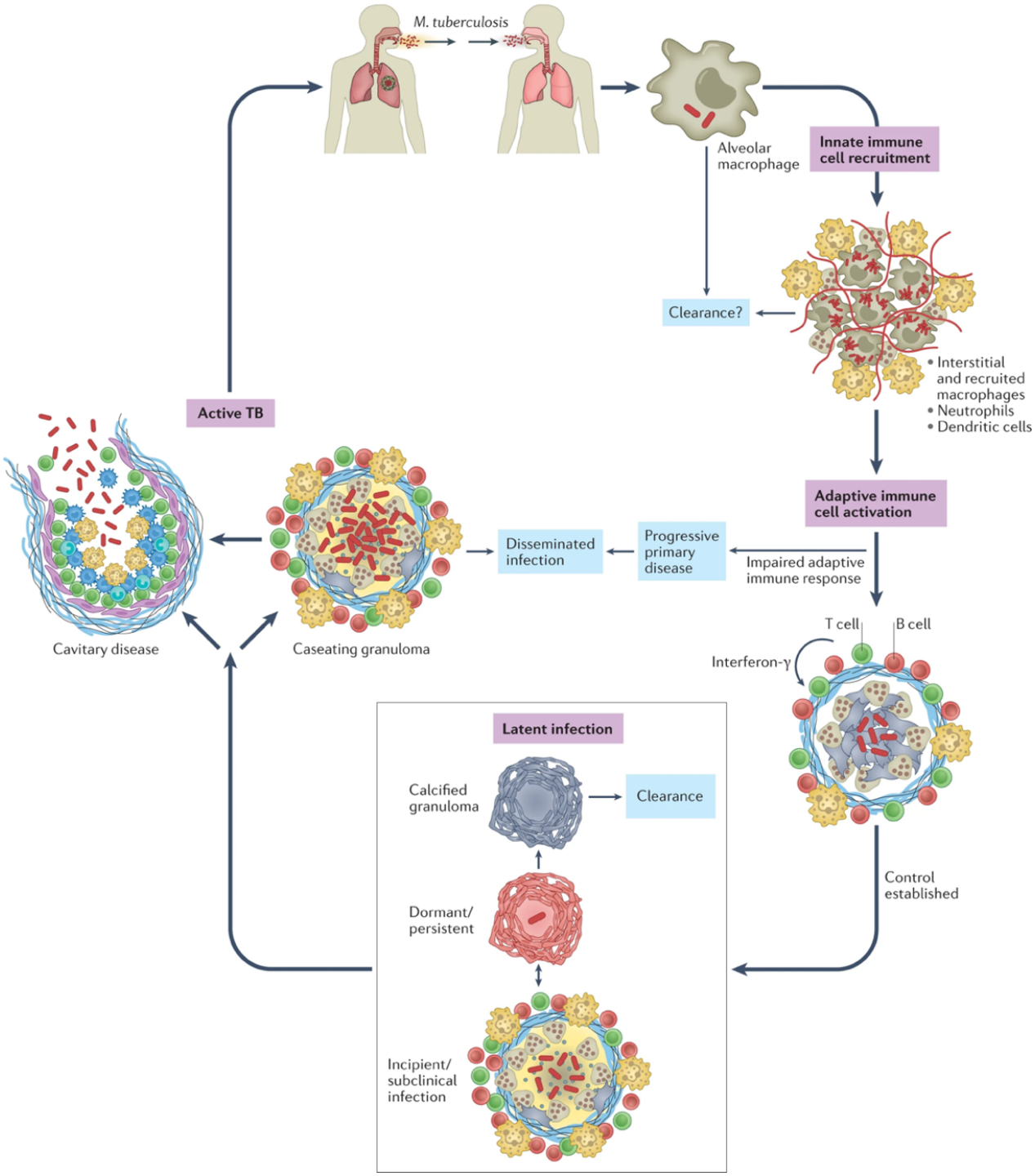


**Figure 2:** *Mycobacterium* infection model, Figure adapted from Chandra et al., (2022) and reprinted with permission from *Springer Nature: Nature Reviews Microbiology*. © 2024.

### 1.4 Immunopathology in peripheral blood of pulmonary tuberculosis

Immunopathological features of pulmonary TB extend beyond the lung to the peripheral blood of patients, as documented in previous studies (Chandra et al., 2022). This thesis, Paper I, presents a comprehensive review of alterations in the plasma milieu and their connection to immune cell responses and clinical characteristics in TB patients. Notably, aberrant levels of plasma factors including inflammatory cytokines (IL-1β, IL-6, IFNγ, and TNF-α), chemokines (CXCL-8, CXCL-9, and CXCL-10), eicosanoids (Lipoxin A4), metalloproteinases (MMP-1,-8, and -9) and heme oxygenase-1 (which induces hypoxia) were identified in the peripheral blood of TB patients. These factors are associated with dysregulated immune cell function and lung tissue damage, highlighting the connection between lung and blood immunopathology in TB patients. Additionally, the aberrant plasma milieu in TB patients was implicated in altering the immune responses of immune cells in the peripheral blood (Adankwah et al., 2021b).

#### 1.4.1 Tuberculosis plasma milieu effect on immune cells

Initial evidence of the role of TB plasma in influencing immune cells comes from the work of Ellner's group (Kleinhenz et al., 1981, Ellner et al., 1981). Kleinhenz *et al.* demonstrated, using an *in vitro* culture of immune cells from healthy individuals in media supplemented with plasma from anergic TB patients, that TB plasma could suppress both antigen-specific and phytohemagglutinin (PHA) induced T-cell responses. This finding suggests that the immunosuppressive effects of TB plasma could also be transferred to healthy reference immune cells (Kleinhenz et al., 1981). Interestingly, the antigen-specific immune suppression characteristics of TB plasma were found to depend on the presence of monocytes that secrete suppressive factors, highlighting the central role of this immune cell subset and the influence of TB plasma (Ellner et al., 1981, Kleinhenz et al., 1981). Further research indicated that host immune responses, particularly TGF-β and IL-10 from suppressive immune cells, as well as immunoglobulins, inhibit T-cell responses in anergic TB patients, although the precise underlying mechanisms remain elusive (Taylor et al., 1995, Hirsch et al., 1996, Vanham et al., 1997, Ellner, 1997).

More recent studies have reported changes in inflammatory mediators in the plasma of TB patients and have postulated the immune effects of these alterations (Adankwah et al., 2021b). For T cells, increased plasma levels of IL-6 and IL-10 in TB patients

were associated with constitutive phosphorylation of signal transducer and activator of transcription 3 (STAT3) and high levels of the suppressor of cytokine signaling 3 (SOCS3), a critical regulator of STAT signaling that suppresses T cell responses (Harling et al., 2019). Additionally, elevated levels of inflammatory cytokine/chemokine in TB plasma were linked with hypo-responsiveness of TB patients (Vivekanandan et al., 2023a, DiNardo et al., 2022). It was demonstrated in an *in vitro* assay that plasma from TB patients causes nuclear alterations and changes in the ability of neutrophils to perform antimicrobicidal functions (Juárez-Ortega et al., 2018, Rojas-Espinosa et al., 2021). Furthermore, increased frequency and phenotypic changes in blood monocytes have been linked to inflammatory plasma changes in TB, although the precise mechanism of the plasma effect is unknown (Balboa et al., 2011, Tamene et al., 2021, Harelimana et al., 2022). These studies suggest that changes in the plasma milieu may influence alterations in peripheral blood immune cells.

### 1.4.2 Monocytes in tuberculosis

Monocytes play a crucial role in innate immunity. They are highly plastic and serve as precursor cells for macrophages and dendritic cells. Monocytes are produced in the bone marrow and released into the bloodstream to perform various functions, including phagocytosis, antigen presentation, and the secretion of chemokines and cytokines, which influence the activity of other immune cells (Sampath et al., 2018, Ozanska et al., 2020). In the bloodstream, monocytes are categorized into three subsets based on the expression of cluster of differentiation (CD)14 and CD16: classical (M1) monocytes ($CD14^{high}$/ $CD16^{low}$), alternative/non-classical (M2) monocytes ($CD14^{low}$/ $CD16^{high}$), and intermediate (M1/2) monocytes ($CD14^{high}$/ $CD16^{high}$). These subsets comprise 80-95%, 2-11%, and 2-8% of circulating monocytes, respectively. Classical monocytes are highly phagocytic and produce pro-inflammatory cytokines. Non-classical monocytes are motile cells that engage in transendothelial crawling during injury and are involved in the secretion of inflammatory cytokines, antiviral responses, and antigen presentation. Intermediate monocytes have strong antigen presentation capabilities and also contribute to inflammatory responses (Sampath et al., 2018, Ozanska et al., 2020). The distribution and function of these monocytes subsets are altered under inflammatory conditions (Ozanska et al., 2020).

Monocytes and monocyte-derived cells are essential for initiating immune responses following Mtb infection. When Mtb enters the body, monocytes are recruited from the

bloodstream to the lungs, where they differentiate into monocyte-derived macrophages and dendritic cells. This process replaces short-lived cells and ensures effective immune surveillance (Chandra et al., 2022, Weeratunga et al., 2024). Consequently, any alterations in monocyte phenotype or function could significantly impact immune surveillance in the lungs.

#### 1.4.2.1 Monocyte immunopathology in tuberculosis

TB has been linked to alterations in the proportions, phenotype, and function of monocytes in peripheral blood. Previous studies have found that increased frequencies of monocytes or a high monocyte-to-lymphocyte ratio in TB patients are associated with disease severity (Wang et al., 2015, Wang et al., 2019, Adankwah et al., 2021a). The monocyte-to-lymphocyte ratio has also been proposed as a potential diagnostic and monitoring tool for assessing the effectiveness of anti-TB treatment (Wang et al., 2015, Wang et al., 2019, Adane et al., 2022).

In TB, emphasis has been placed on the enrichment of CD16+ monocytes, also known as “small-sized” monocytes, which are described as a pathognomonic feature of the disease. These monocytes are characterized by their hyperinflammatory properties and their ability to migrate to inflamed tissues (Castano et al., 2011, Balboa et al., 2011, Adankwah et al., 2021a). Impaired monocyte differentiation into macrophages or dendritic cells, along with altered phenotypes and reduced capacity to induce T-cell responses, have been associated with the enrichment of CD16+ monocytes (Rajashree et al., 2009, Castano et al., 2011, Balboa et al., 2013). Changes in cytokine production have also been observed in monocytes and monocyte-derived cells in TB (Castano et al., 2011, Restrepo et al., 2021). Monocytes in TB patients have been shown to exhibit inflammatory signatures and dysregulated genes linked to cytokine/chemokine production, signaling pathways, apoptosis, and antigen presentation (Li et al., 2021, Hillman et al., 2022). In addition, alterations in the expression levels of key functional surface receptors have been reported in TB patients (Balboa et al., 2011, Castano et al., 2011, Sampath et al., 2018, Adankwah et al., 2021a). These studies highlight the systemic impact of TB in peripheral blood, which may alter monocyte phenotype and function, and significantly impact immune surveillance.

## 2. Research Objectives

Changes in the functions and phenotypes of peripheral blood monocytes have been described in acute TB, with initial evidence suggesting an important role of the plasma milieu. However, the exact mechanisms underlying the impact of TB plasma pathology on monocytes have not been characterized. In this thesis, a comprehensive literature search was conducted to understand the immunopathology of TB in peripheral blood. The underlying hypothesis posits that alterations in the plasma milieu may directly influence monocyte responses in TB patients. To investigate this, an *in vitro* plasma culture assay, originally designed by Kleinhenz *et al.* (1981), was modified and used to examine the impact of plasma samples from TB patients on reference monocytes. Additionally, ex vivo characterization of immune cell distribution and monocyte phenotypes in TB patients and asymptomatic contacts (controls) was performed.

The objectives of this study were:

- To identify biomarkers of immunopathology in the blood of TB patients.
- To characterize plasma invitro-induced effects on reference monocyte transcriptome, phenotype, and function.
- To compare the effect of TB plasma on reference monocytes with aberrant monocyte phenotype and function in TB patients.
- To characterize the effect of TB plasma on immune cell distribution and the recruitment of innate immune cells in TB patients.
- To determine the effect of treatment on monocyte phenotypes.

**3.1 Paper I:** Immunopathology in human pulmonary tuberculosis: Inflammatory changes in the plasma milieu and impaired host immune cell functions.

**Ahor HS,** Vivekanandan M, Harelimana JD, Owusu DO, Adankwah E, Seyfarth J, Phillips R, Jacobsen M. (2024). Immunopathology in human pulmonary tuberculosis: Inflammatory changes in the plasma milieu and impaired host immune cell functions. *Immunology*. 172(2):198-209.



Contribution to this publication: 40%

- Literature search
- Data compilation and analyses
- Writing of the manuscript

Received: 25 September 2023 | Accepted: 17 January 2024

DOI: 10.1111/imm.13761

REVIEW

immunology
insights into immunity in health and disease

# Immunopathology in human pulmonary tuberculosis: Inflammatory changes in the plasma milieu and impaired host immune cell functions

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**Funding information**
Deutsche Forschungsgemeinschaft, Grant/Award Numbers: 1479/9-1, 1479/14-1; Jürgen Manchot Stiftung

## Abstract

Host immune response is key for protection in tuberculosis, but the causative agent, *Mycobacterium (M.) tuberculosis,* manages to survive despite immune surveillance. Key mechanisms of immune protection have been identified, but the role of immunopathology in the peripheral blood of tuberculosis patients remains unclear. Tuberculosis immunopathology in the blood is characterised by patterns of immunosuppression and hyperinflammation. These seemingly contradictory findings and the pronounced heterogeneity made it difficult to interpret the results from previous studies and to derive implications of immunopathology. However, novel approaches based on comprehensive data analyses and revitalisation of an ancient plasma milieu in vitro assay connected inflammation with immunosuppressive factors in tuberculosis. Moreover, interrelations between the aberrant plasma milieu and immune cell pathology were observed. This review provides an overview of studies on changes in plasma milieu and discusses recent findings linking plasma factors to T-cell and monocyte/macrophage pathology in pulmonary tuberculosis patients.

**KEYWORDS**
activation, inflammation, macrophage

## BACKGROUND

Immunopathology is a common feature of chronic infectious diseases. Recently, this topic received a surge of interest when the important role of inflammatory syndromes in patients with severe COVID-19 became apparent [1, 2]. Aberrant high levels of pro-inflammatory mediators (e.g., interleukin [IL]-6) in the plasma were identified in COVID-19 patients with severe disease manifestation and the relevance for pathology has been clearly shown [3]. Consequently, host-directed therapies (HDTs) with corticosteroids or anti-inflammatory biologicals have become part of the standard treatment regimen in severe cases. Cytokine patterns seen in these patients with COVID-19 are

similar to inflammatory diseases (e.g., rheumatoid arthritis) and other inflammatory syndromes [4].

In tuberculosis, a clinical relevant role of inflammatory immunopathology has been mainly attributed to the Immune Reconstitution Inflammatory Syndrome (IRIS) in patients with concomitant HIV infection [5]. However, inflammatory plasma cytokine patterns have also been observed in tuberculosis patients without HIV co-infection, but the relevance of these findings and potential clinical implications, for example, for treatment response and recovery, are unclear [6]. Two main reasons may contribute to this lack of knowledge. First, the marked heterogeneity of plasma markers found in different studies makes it difficult to identify common factors and decipher the underlying mechanisms of immunopathology in tuberculosis. Influential factors causative for heterogeneity and recent approaches to deduce common patterns as well as disease endotypes are discussed in this review. On the other hand, a possible interrelation between pathological changes of the plasma milieu and impaired immune cell functions in tuberculosis has been neglected in recent years. A probable reason for this was seemingly contradictory findings on immunosuppressive and pro-inflammatory signatures in the blood of tuberculosis patients. Here, we discuss recent studies that shed light on these seemingly controversial findings using a plasma milieu in vitro assay combined with phenotype and functional analyses of immune cells from tuberculosis patients. These studies validated the relevance of the plasma milieu in tuberculosis and identified pathways involved in immunopathology. Key factors, like IL-6, were found and the complexity of underlying mechanisms was demonstrated. Moreover, hyporesponsive immune responses in a subgroup of tuberculosis patients—detected as an impaired T-cell response to phytohemagglutinin (PHA)—were found to be associated with hyperinflammatory cytokines and blood signatures. We decided to review the current knowledge on plasma and cellular immunopathology in tuberculosis to facilitate the conduct of future studies and to support considerations for usage of host-directed treatment (HDT) regimens in tuberculosis.

## ABERRANT PLASMA CYTOKINES IN TUBERCULOSIS PATIENTS REFLECT PATHWAYS INVOLVED IN LUNG PATHOLOGY

Cytokines and other humoral factors (for simplicity termed cytokines) in plasma samples from tuberculosis patients have been analysed as part of several studies. A major challenge in the selection of studies for this overview was the heterogeneity of designs. Hypothesis- as well as data-driven approaches were performed, and this resulted in high variability in the selection of analysed plasma components. However, to provide an overview of candidates, we screened studies based on a case/control design comparing 'plasma' or 'serum' samples from patients with pulmonary tuberculosis (before treatment) and matched controls. Sixty studies were identified that meet these criteria [7–66]. The selection criteria used for the definition of 'controls' varied between the studies, and this is at least partially due to a missing gold standard for the classification of 'controls' in human tuberculosis. Asymptomatic potentially latent *M. tuberculosis*-infected individuals (diagnosed by IFN-γ release assays or tuberculin skin test), household contacts, and community controls were included in different studies. Only a few studies included more than one control group and several studies did not provide details on how controls were selected. Since immunopathology of pulmonary tuberculosis disease was the focus of this review, we ignored these differences in control groups. We designated all participants without tuberculosis symptoms as 'controls', regardless of how they were selected. Co-infections and genetic background of included individuals were only partly described and, therefore, the influence of these parameters could not be evaluated here.

All analysed candidates were deduced and the factors showing significant differences in any study were identified. We excluded those candidates for which less than three independent studies were performed as well as those either 'not different' or 'not detectable' in most studies (>75%). Based on these preselection steps, the final list comprised 37 plasma cytokines (Figure 1a). Many of these cytokines could be assigned to processes that play an important role in granulomatous lung tissue from tuberculosis patients. These were (i) inflammation, (ii) immune cell recruitment and chemotaxis, (iii) cell growth, (iv) tissue remodelling and (v) hypoxia (Figure 1a). In addition, several members of the common-γ cytokine receptor family, cytokines with described immune-suppressive functions, molecules with endocrine function, and cytokines with no clear assignment to one of the abovementioned processes or families were identified (Figure 1a). The predominance of factors with a role in granulomatous lung pathology was not surprising. Systemic inflammation as a consequence of pathology in affected tissues has been described and tissue remodelling as well as oxidative stress were also found to be crucial [67]. Three candidates within the list (i.e., TGF-β, IL-10 and soluble IL-2 receptor [sIL-2R]) were assumed to have anti-inflammatory and/or immune inhibitory functions. sIL-2R and TGF-β showed higher plasma levels in tuberculosis patients in most of the studies [12, 17, 19, 20, 23, 44, 51, 52, 58], but

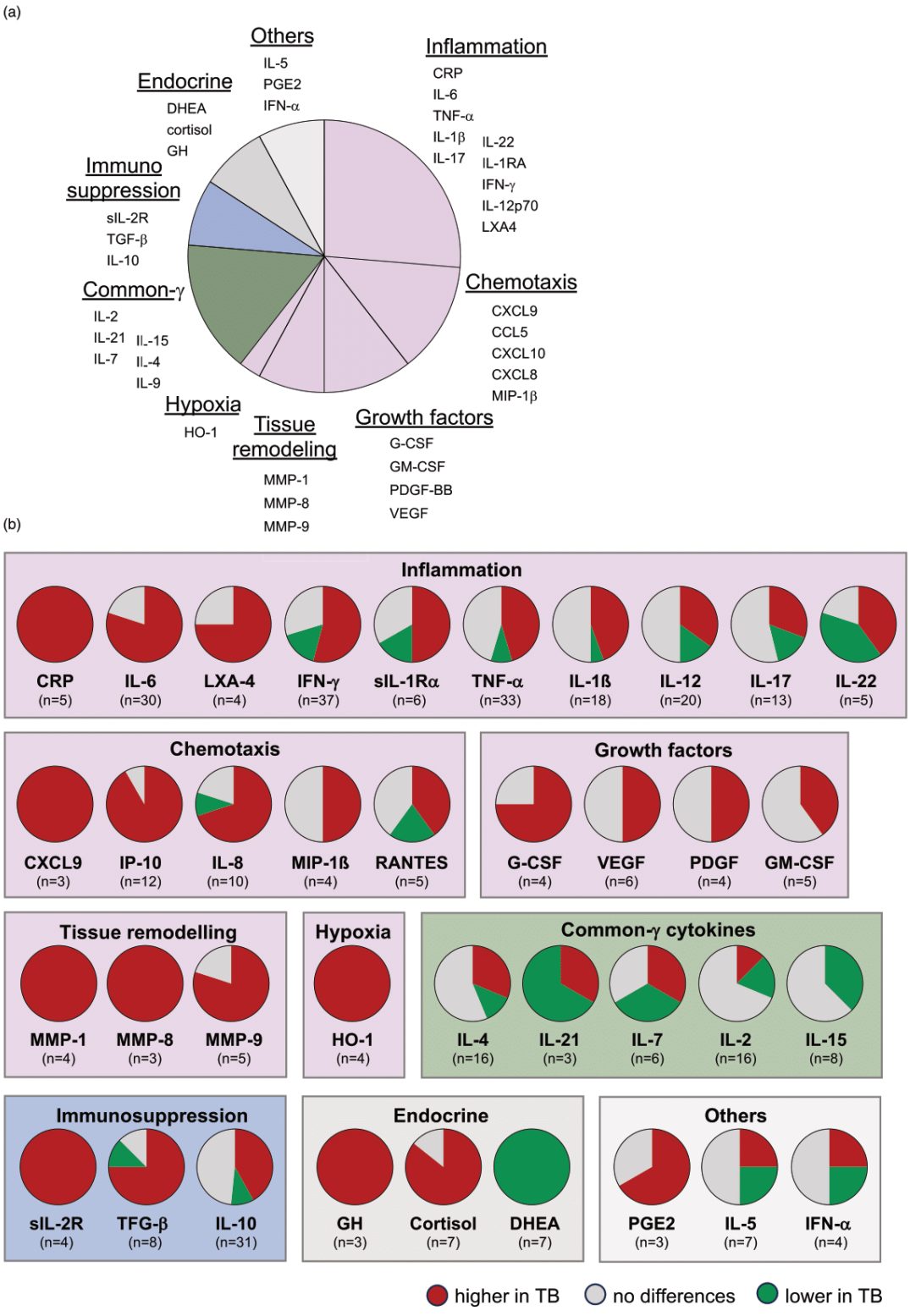


**FIGURE 1** Plasma and serum cytokines with significantly different concentrations between patients with pulmonary tuberculosis and control. Cytokines and other humoral factors identified as significantly different in at least three of 60 included studies are shown. (a) A pie chart indicating the proportional distribution of identified groups is depicted. Functional similarities and groups associated with lung pathology are indicated by the same colour. (b) Pie charts indicate the distribution of results from different studies for each candidate. Red colour indicates higher concentration and green colour indicates lower concentration in plasma/serum of patients with tuberculosis as compared to controls. Grey indicates no difference between plasma/serum samples of the study groups. The number of studies performed for each candidate is given. Candidates with the same function and groups associated with lung pathology are indicated by boxes and the same background colour.

the total number of studies was moderate (Figure 1b). IL-10 was frequently tested and was higher in plasma from tuberculosis patients in approx. 50% of the studies (Figure 1b). Similarly, for other functional clusters, where candidates with invariable differences were included in a limited number of studies (i.e., CRP, CXCL-9, MMP-1, MMP-8, HO-1, GH and DHEA). These analyses showed that inflammatory plasma signatures were dominant but only a few candidates, which were frequently analysed, like IP-10 and IL-6, showed clear results of higher plasma levels in tuberculosis patients (Figure 1b).

## HETEROGENEITY OF PLASMA CYTOKINE EXPRESSION IN PULMONARY TUBERCULOSIS

Only a few cytokines show an unambiguous picture of being either higher or lower in tuberculosis patients' plasma. Particularly, frequently studied candidate cytokines, such as TNF-α or IFN-γ, were elevated in the plasma of tuberculosis patients in several studies [20, 23–26, 29, 30, 32, 37, 44, 45, 47, 57, 60, 62, 68–71], but either or both did not differ in other studies [7–10, 14, 15, 18, 20, 21, 23–26, 29–32, 37, 40, 42, 44–47, 51–54, 57, 60–64, 68–71] (Figure 1b). Insufficient study cohort sizes and low statistical power in some studies likely contribute to heterogeneity, but this does not sufficiently explain differences. The complexity of host/pathogen interaction at different stages of *Mycobacterium tuberculosis* infection/disease is certainly an influential factor. The current 'gold standard' for the classification of patients with symptoms and asymptomatic—but latently *M. tuberculosis*-infected—individuals may underestimate the complexity and may not sufficiently reflect disease stages [72]. Differences in the severity and stages of tuberculosis disease may also contribute to inconsistent results of previous studies. Inflammatory cytokines (e.g., IL-6, TNF-α and IFN-γ) were shown to be associated with disease severity and/or higher mycobacterial burden [20, 37, 43, 59, 73, 74]. In accordance, several studies detected decreasing plasma levels of inflammatory cytokines during treatment [20, 21, 37, 44, 59, 68, 73, 75–77]. Such markers may also provide clinically relevant information as some were described to predict treatment outcomes [78–83] and others may indicate the risk of recurrent tuberculosis [70, 84, 85].

Helpful approaches to investigate plasma heterogeneity in pulmonary tuberculosis were contributed by Andrade et al. This group compared multiple cytokines in similar study populations of tuberculosis patients and controls from China and India [50]. Although differentially expressed candidates varied markedly between tuberculosis patients from both sites, features of inflammatory perturbation were seen in patients from both populations and the level of perturbation distinguished tuberculosis patients from controls [50]. Consistent with this, key markers of immunopathology in tuberculosis patients, for example, may differ by region of recruitment. Two factors associated with tissue remodelling and oxidative stress, matrix metalloproteinase (MMP)-1 and heme oxygenase-1 (HO-1), were among the candidates with higher plasma concentrations in tuberculosis patients (Figure 1). Andrade et al. demonstrated that distinct expression patterns of MMP-1 and HO-1 characterised different clinical manifestations of tuberculosis [9]. Moreover, tuberculosis patients with either high HO-1 or MMP-1 levels displayed distinct plasma inflammatory marker profiles (e.g., CRP, IFN-γ and IL-10) [9]. These findings provide possible explanations for differences in identified plasma cytokine candidates from previous studies and strengthen the hypothesis that the complexity of host/pathogen interaction in tuberculosis requires comprehensive analyses of multiple factors. Moreover, different plasma cytokine patterns seen in different populations also render the likelihood of different subtypes of pulmonary tuberculosis disease possible.

## DISTINCT ENDOTYPES CHARACTERISED BY HYPERINFLAMMATION AND IMMUNE HYPO-RESPONSIVENESS MAY CONTRIBUTE TO HETEROGENEITY IN TUBERCULOSIS

Different disease subtypes in tuberculosis may contribute to observed heterogeneity. This assumption was strengthened by a recent study from DiNardo et al., who compared global mRNA data in the blood from several tuberculosis patient cohorts. This study performed unsupervised clustering to identify subgroups of tuberculosis patients with differential gene expression patterns [86]. A signature of 40 genes was identified that classified two endotypes (endotypes A and B) within tuberculosis patients. These two endotypes were detectable in most cohorts of tuberculosis patients and differed also from healthy controls [86]. Different levels of inflammatory mediators were among the most influential factors for discrimination of endotypes. Endotype A had the strongest inflammatory signature and concomitantly showed signs of reduced proliferation [86]. Tuberculosis patients classified as endotype A were shown to have slower treatment response and a higher frequency of treatment failures [86]. Notably, plasma samples from a selected cohort of tuberculosis patients differed in inflammatory cytokines and chemokines (i.e., IL-6, TNF-α, IL-1β,

CXCL-9 and IP-10) and this was accompanied by hyporesponsiveness of whole blood samples stimulated with the mitogen PHA [86]. This study is trendsetting in different ways. It showed that the heterogeneity in tuberculosis can be partly explained by different disease endotypes. Moreover, it suggested that signatures of hyperinflammation seen in the plasma are reflected by changes of the cellular immune response including an impaired T-cell response in tuberculosis patients.

## IMPAIRED IMMUNE RESPONSES IN TUBERCULOSIS PATIENTS ARE CAUSED BY CHANGES OF THE PLASMA MILIEU

The impaired immune response to PHA in a subgroup of tuberculosis patients is well described and has already been identified in the last century [87]. PHA hyporesponsiveness is consistently seen in several studies from different regions and qualifies as a common feature of immunopathology in a subgroup of tuberculosis patients [88–93]. Importantly, pioneering research from the group of J.J. Ellner demonstrated that plasma samples from pulmonary tuberculosis patients were sufficient to transfer hypo-responsiveness to immune cells from healthy individuals [94]. Similar plasma effects were seen for different infectious diseases at this time and this demonstrated the relevance of plasma pathology [94–98]. Since inhibitory effects dominated, humoral factors with suppressive function were assumed to be causative for plasma effects in tuberculosis and other mycobacterial diseases [99–101]. J.J. Ellner's group also investigated the role of different immune cell subsets in tuberculosis patients with impaired immune response. This work formed the basis for several studies that characterised plasma effects and cellular immunopathology of tuberculosis patients thereafter.

## THE INFLAMMATORY PHENOTYPE OF MONOCYTES AND IMPAIRED FUNCTIONS IN TUBERCULOSIS

Monocytes were identified early to play an important role in tuberculosis immunopathology since this subset was crucial for the plasma-mediated inhibition of T-cell responses in tuberculosis patients [94]. Monocytes were found at higher proportions in the blood of patients with tuberculosis and depletion of monocytes in vitro partially recovered antigen-specific T-cell functions in tuberculosis patients [99, 102]. Moreover, monocytes from tuberculosis patients were characterised by low expression of HLA-DR molecules ex vivo [103], low IL-12 secretion [104], as well as increased expression of immunosuppressive TGF-β (spontaneously and activation-induced) [105]. These initial findings strengthened the assumption about a functional impairment of blood monocytes from tuberculosis patients. Already at this time, however, several studies also identified features of inflammation in tuberculosis and showed that monocytes from tuberculosis patients secreted high amounts of pro-inflammatory cytokines (i.e., TNF-α, IL-1β, IL-6 and IL-8) upon activation (reviewed in [106]).

Characteristics of monocyte phenotype and functional changes in tuberculosis were investigated by more recent studies [107, 108]. Large-sized monocytes make up the vast majority of blood monocytes in healthy individuals but under certain pathologic conditions, the proportion of small-sized monocytes is increased [109]. Small monocytes are characterised by high concomitant expression of polarisation markers (i.e., CD14 and CD16) and have been found to be enriched in the blood of tuberculosis patients [110–112]. Small monocytes secrete predominantly IL-1β and TNF-α (and less IL-10) and were, therefore, also termed 'inflammatory' monocytes [113]. Several studies found changes in the monocyte phenotype from tuberculosis patients and strengthened the assumption of functional implications for monocyte differentiation [110–112, 114–117].

## PLASMA MILIEU EFFECTS ON MONOCYTE PHENOTYPES AND FUNCTIONS IN TUBERCULOSIS

Initial evidence suggests that changes in the plasma milieu in tuberculosis may contribute to monocyte phenotype changes. Balboa et al. detected an association between the proportions of inflammatory monocytes with TNF-α plasma levels in tuberculosis patients. In addition, constitutive phosphorylation of the p38 MAPK signalling pathway in $CD16^{+}$ inflammatory monocytes was seen and p38 inhibition restored the capacity of monocytes to differentiate into DCs [112]. Although a direct interaction between TNF-α and the MAPK pathway is unlikely, these results connected the inflammatory plasma milieu with constitutive cytokine signalling in monocytes from tuberculosis patients [112]. Against this background, the effects of the plasma milieu from tuberculosis patients on monocytes were recently analysed in two studies [27, 111], which used a modified version of the plasma milieu in vitro assay originally established by the group of J. J. Ellner [94]. Reference monocytes from healthy individuals were cultured in a medium supplemented with plasma from patients with tuberculosis or contacts to determine changes in phenotype and cytokine signalling [27, 111]. Altered Signal Transducers and Activators of Transcription (STAT) pathways and phenotype changes were detected in the presence of plasma samples

from tuberculosis patients [27, 111]. STAT3 as well as STAT5 were strongly phosphorylated in monocytes by tuberculosis patients' plasma [111]. Constitutive STAT3 phosphorylation of monocytes from tuberculosis patients was shown previously [118]. Lastrucci et al. characterised influential factors produced by *M. tuberculosis*-infected monocyte-derived macrophages (MDMs) on reference monocytes. This study detected high pSTAT3 levels together with the upregulation of CD16 in monocytes [111]. Interestingly, monocytes treated with *M. tuberculosis*-infected MDM supernatants were prone to become anti-inflammatory macrophages and IL-10 was key for these effects [111]. In addition, impaired anti-mycobacterial effector functions of treated monocytes as well as worsened interaction with *M. tuberculosis*-specific T-cells were found [111]. Further studies are needed to determine if tuberculosis plasma affects monocyte differentiation and function in a similar way.

In addition to the activation of cytokine signalling pathways, the plasma samples led to changes in the monocyte phenotype. Reference monocytes showed higher expression of CD33, CD40 and CD64 in the presence of plasma from tuberculosis patients [111]. These plasma-induced phenotype changes resembled the immunopathology of monocytes found in the same patient study group [111]. Higher expression of CD40 and CD64 on monocytes in respective tuberculosis patients was seen and regulation of CD33 expression on monocytes during treatment was detected [111]. The assumption of an interrelation between plasma components and phenotype changes was strengthened by the finding that distinct STAT pathways were positively correlated with candidate markers (i.e., pSTAT3 with CD33; pSTAT5 with CD40 and CD64) [111]. High CD64 expression on monocytes was identified in previous studies as part of a biomarker signature for acute tuberculosis and was highly efficient for classifying tuberculosis patients [119, 120]. Taken together, the characterisation of plasma in vitro effects resembled the aberrant monocyte phenotype of patients with tuberculosis. Cytokines using STAT3 and STAT5 signalling pathways are potentially involved in the immunopathology of the plasma from tuberculosis patients.

## ABERRANT CONSTITUTIVE STAT3 PHOSPHORYLATION AND HIGH SOCS3 EXPRESSION AFFECT T-CELL FUNCTIONS IN TUBERCULOSIS PATIENTS

Evidence for the role of plasma-mediated T-cell modulation via STAT pathways in tuberculosis was provided by a study from Harling et al. [121]. This study found high constitutive STAT3 phosphorylation levels in $CD4^+$ T-cells from tuberculosis patients [121]. Similar signatures of high pSTAT3 levels in $CD4^+$ T-cells were previously seen in patients with rheumatoid arthritis (RA). These RA patients had high IL-6 plasma levels associated with constitutive STAT3 phosphorylation [122]. In accordance, high IL-6 plasma levels in tuberculosis patients were positively correlated with STAT3 phosphorylation in $CD4^+$ T-cells from tuberculosis patients [43, 121]. High pSTAT3 levels were accompanied by high expression of its key regulator SOCS-3 in tuberculosis patients [121]. SOCS-3 and an IL-6-dependent signature of gene expression were found for T-cells from both, RA and tuberculosis patients, using global transcriptome analyses [123, 124].

The altered phenotype of T-cells from tuberculosis patients has potential functional implications. An association of high SOCS3 levels with low *M. tuberculosis*-specific IFN-γ and IL-2-producing T-cell proportions were seen in tuberculosis patients [121]. Inhibition of IL-2-induced STAT5 phosphorylation and T-cell proliferation by SOCS3 has been shown before [125, 126]. Interference of SOCS3 with IL-2 signalling was suggested as the underlying mechanism of impaired T-cell response in tuberculosis patients [121]. Although unclear until now, induced high SOCS3 expression levels may also be involved in inhibitory plasma effects and impaired PHA response seen in tuberculosis patients. Kleinhenz et al. found that plasma effects on PHA-induced T-cell response were independent of antigen-presenting cells and this rendered direct effects of tuberculosis plasma on T-cells likely [94]. In summary, previous studies suggested plasma milieu effects on T-cells in tuberculosis. IL-6-induced aberrant STAT3 signalling as well as high SOCS3 levels are potential mechanisms underlying impaired T-cell response in tuberculosis patients.

## THE ROLE OF IL-6 ON IMPAIRED T-CELL RESPONSE AND IMMUNOPATHOLOGY OF TUBERCULOSIS

IL-6 is a pleiotropic cytokine and several cell types produce IL-6. In tuberculosis patients, high spontaneous secretion of IL-6 during whole blood in vitro culture is seen and antigen-specific T-cell stimulation induces higher IL-6 expression in tuberculosis patients as compared to controls [121]. Hence, T-cells themselves contribute to high IL-6 plasma levels in tuberculosis patients and may, therefore, contribute to their aberrant phenotype. This is an example of a self-reinforcing mechanism and shows that immune cells in tuberculosis patients are

not only influenced by the plasma environment but also contribute to aberrant plasma cytokine levels.

High IL-6 plasma levels decrease rapidly under treatment [20, 59], and normalisation was also seen for PHA-induced response [92, 127]. In accordance, a recent study by Vivekanandan et al. found that impaired PHA response in tuberculosis patients before treatment was associated with high IL-6 plasma levels [92]. This rendered a causative relationship between aberrant high IL-6 plasma levels with impaired T-cell functions in patients with tuberculosis likely. There is initial evidence that not all T-cells are affected to the same extent by impaired PHA response and a negative correlation with plasma IL-6 levels was solely seen for IFN-γ, IL-22 and IL-10 [92]. As expected, these three cytokines showed increasing response to PHA during treatment, whereas other cytokines, including IL-6 and IP-10, did not change during treatment [92]. Further studies are needed to characterise the differences between these subsets and to determine potential implications for T-cell function in tuberculosis.

## CONCLUSIONS AND OUTLOOK

This review summarises the results of previous studies on immunopathology in the blood of pulmonary tuberculosis patients. Plasma and immune cells show features of immunopathology and despite considerable heterogeneity, common features, like inflammatory signatures and immune cell hypo-responsiveness, were identified. A summary of the main findings is depicted in Figure 2. Comprehensive candidate marker analyses, for example, based on preselected plasma cytokines deduced as part of this review, should be applied in future studies to confirm common patterns of immunopathology in tuberculosis. These studies should also scrutinise the role and relevance of common tuberculosis endotypes. Furthermore, concomitant measurement of plasma cytokines, immune cell phenotype and plasma-dependent effects on reference immune cells in vitro can be seen as a promising approach to decipher complex mechanisms underlying immunopathology of tuberculosis.

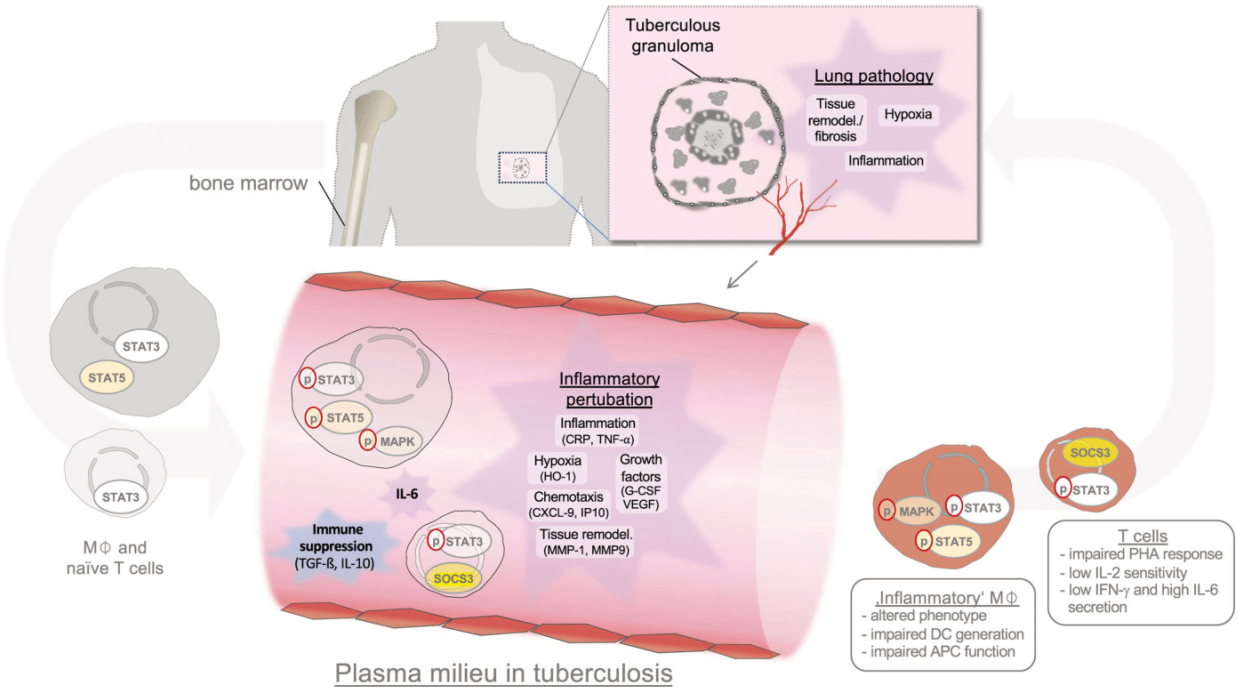


**FIGURE 2** Immunopathology in the blood of patients with tuberculosis. Aberrant cytokine (and non-cytokine humoral factor) signatures in the plasma largely reflect the mechanisms seen in tuberculous lung tissue pathology. Aberrant high expression of candidates involved in inflammation, tissue remodelling/fibrosis, chemotaxis, cell growth and hypoxia are detectable, whereas fewer markers of immune regulation/inhibition were found in plasma/serum by previous studies. A plasma milieu in vitro assay demonstrated immunomodulatory effects of tuberculosis patients' samples, which strongly induced activation of STAT (i.e., STAT3 and STAT5) pathways in healthy immune cells. Phenotypic and functional changes of monocytes and T-cells are induced by tuberculosis plasma samples and these are reflected by immunopathology seen in T-cells and monocytes from patients with tuberculosis. IL-6 has been identified as a potential mediator of immunopathology in tuberculosis. The results from these descriptive and in vitro functional studies strongly suggest implications of blood immunopathology for disease outcomes in human tuberculosis.

The immunopathology of the plasma milieu and immune cells in tuberculosis exhibits high similarity to inflammatory diseases and syndromes. Importantly, several studies demonstrated functional implications of tuberculosis pathology on immune cells and found associations with disease severity and course. These results support the use of HDT to supplement antimycobacterial treatment. Anti-inflammatory drugs in particular are promising candidates for HDT, and several clinical trials are underway (reviewed in [128]). Besides shortening of treatment duration, future trials should also determine the implications of HDT on described long-term consequences of tuberculosis, like Post Tuberculosis Lung Disease [129] and the risk of recurrent tuberculosis [70, 85]. Plasma cytokine signatures and plasma effects on reference monocytes can provide promising candidates as biomarkers for monitoring the efficacy of HDT treatment.

## ACKNOWLEDGEMENTS

M.J. and R.P. were supported by the German Research Foundation (DFG, JA 1479/9-1 and 1479/14-1). M.J. and H.S.A. were supported by the graduate school molecules of infection (MOI)-4 funded by the Jürgen Manchot Foundation. Open Access funding enabled and organized by Projekt DEAL.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**How to cite this article:** Ahor HS, Vivekanandan M, Harelimana JDD, Owusu DO, Adankwah E, Seyfarth J, et al. Immunopathology in human pulmonary tuberculosis: Inflammatory changes in the plasma milieu and impaired host immune cell functions. Immunology. 2024;172(2): 198–209. https://doi.org/10.1111/imm.13761

**3.2 Paper II:** Monocyte pathology in human tuberculosis is due to plasma milieu changes and aberrant STAT signaling.

**Ahor HS**, Schulte R, Adankwah E, Harelimana JD, Minadzi D, Acheampong I, Vivekanandan MM, Aniagyei W, Yeboah A, Arthur JF, Lamptey M, Abass MK, Kumbel F, Osei-Yeboah F, Gawusu A, Debrah LB, Owusu DO, Debrah A, Mayatepek E, Seyfarth J, Phillips RO, & Jacobsen M. (2023). Monocyte pathology in human tuberculosis is due to plasma milieu changes and aberrant STAT signaling. *Immunology.* 170 (1):154-166



Contribution to this publication: 80%

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

Received: 15 March 2023 | Accepted: 29 April 2023

DOI: 10.1111/imm.13659

ORIGINAL ARTICLE

immunology
Insights into immunity in health and disease

# Monocyte pathology in human tuberculosis is due to plasma milieu changes and aberrant STAT signalling

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**Funding information**
Deutsche Forschungsgemeinschaft, Grant/Award Number: JA 1479/9-1; Jürgen Manchot Stiftung

## Abstract

Monocyte-derived macrophages contribute centrally to immune protection in *Mycobacterium tuberculosis* infection and changes in monocyte phenotype characterize immunopathology in tuberculosis patients. Recent studies highlighted an important role of the plasma milieu in tuberculosis immunopathology. Here, we investigated monocyte pathology in patients with acute tuberculosis and determined tuberculosis plasma milieu effects on phenotype as well as cytokine signalling of reference monocytes. Patients with tuberculosis ($n = 37$) and asymptomatic contacts (controls $n = 35$) were recruited as part of a hospital-based study in the Ashanti region of Ghana. Multiplex flow cytometry phenotyping of monocyte immunopathology was performed and effects of individual blood plasma samples on reference monocytes prior to and during treatment were characterized. Concomitantly, cell signalling pathways were analysed to elucidate underlying mechanisms of plasma effects on monocytes. Multiplex flow cytometry visualization characterized changes in monocyte subpopulations and detected higher expression of CD40, CD64 and PD-L1 in monocytes from tuberculosis patients as compared to controls. Aberrant expression normalized during anti-mycobacterial treatment and also CD33 expression decreased markedly. Notably, higher CD33, CD40 and CD64 expression was induced in reference monocytes when cultured in the presence of plasma samples from tuberculosis patients as compared to controls. STAT signalling pathways were affected by the aberrant plasma milieu and higher levels of STAT3 and STAT5 phosphorylation was found in tuberculosis plasma-treated reference monocytes. Importantly, high pSTAT3 levels were associated with high CD33 expression and pSTAT5 correlated with CD40 as well as CD64 expression. These results suggested plasma milieu effects with potential implications on monocyte phenotype and function in acute tuberculosis.

KEYWORDS
cytokine signalling, monocytes, plasma milieu, tuberculosis

## INTRODUCTION

Immune surveillance in pulmonary granulomas is central for protection against *Mycobacterium tuberculosis* and to prevent tuberculosis disease progression after infection [1]. The granuloma is characterized by complex cellular composition including several specialized monocyte-derived macrophage subsets. Both, tissue resident macrophages as well as circulating monocyte-derived macrophages (MDM), contribute to local anti-mycobacterial host response, and MDM require steady replacement of short-lived macrophages from the blood monocyte pool [2]. Changes in blood monocyte phenotype and function would, therefore, likely also affect local MDM subsets with potential implications for immune surveillance.

Immunopathology of peripheral blood immune cells in acute tuberculosis are well described. These include alterations in the distribution of immune cell populations as well as changes in phenotype and function [2–4]. Amongst others, higher monocyte proportions (and increased monocyte/T-cell ratios) have been described [4]. In addition, aberrant expression of monocyte subset markers CD14/CD16 was detected, and a subset of small 'inflammatory' monocytes were found in acute tuberculosis [3, 5–8]. Furthermore, functional relevant receptors (e.g., Programmed cell Death Ligand (PD-L)1, FC-gamma receptor 1 (CD64)) were differentially expressed in monocytes of tuberculosis patients [9, 10]. Higher PD-L1 expression was shown to have implications on antimycobacterial effector functions [11, 12]. High CD64 expression was detected in some studies and was identified as a potent marker that may contribute to diagnosis of tuberculosis disease [10, 13–15]. Own previous studies showed that monocytes and T cells of tuberculosis patients had lower Interleukin (IL)-7 receptor expression and impaired response to IL-7 [5, 16]. These findings raised the question about a common cause for changes in different cell subsets and mechanisms that affect immune cells from the peripheral blood without direct contact to the pathogen. For T cells, we could show that cytokine signalling was affected in tuberculosis. High constitutive STAT3 phosphorylation as well as high Suppressor of Cytokine Signalling (SOCS)3 levels were found in acute tuberculosis patients and aberrant pSTAT3 levels were associated with high IL-6/IL-10 plasma concentrations [17]. In addition, we could show recently that aberrant high IL-6 plasma levels were associated with impaired mitogen-induced IFN-γ expression by T cells in patients with tuberculosis [18]. Changes in the plasma milieu and aberrant cytokine concentrations in tuberculosis patients have been described [19–21]. These results rendered a role of the plasma milieu in tuberculosis immunopathology likely and suggested similarity to inflammatory syndromes in different diseases (reviewed in Ref. [22]). Recently, we established an in vitro assay based on tuberculosis patients' plasma samples, which were added to the culture medium of reference monocytes and compared to matched control plasma samples [23]. This assay confirmed tuberculosis plasma specific effects on monocyte HLA-DR/IL-7 receptor expression and strengthened the hypothesis that the plasma milieu in tuberculosis is responsible for immunopathology seen for T cells and monocytes [23].

Against this background, we investigated if the aberrant monocyte phenotype in tuberculosis patients is caused by plasma milieu factors in the present study. Several monocyte markers were included for phenotyping of monocytes from tuberculosis patients and controls as well as for analysing the effects of plasma samples on reference monocytes. STAT1, STAT3 and STAT5 phosphorylation was determined to characterize the underlying mechanisms.

## MATERIALS AND METHODS

### Study cohorts and clinical characterization

We recruited tuberculosis patients ($n = 40$) and asymptomatic contacts of indexed tuberculosis patients (controls, $n = 35$) from July 2019 to March 2022 at the Agogo Presbyterian Hospital, the St Mathias Catholic Hospital, the Atebubu District Hospital, and the Sene West District Hospital in Ghana. Diagnosis of active tuberculosis was based on patient history, clinical examination, chest x-ray and sputum smear test. GeneXpert (Cepheid) analyses were done for all tuberculosis patients. All patients were included prior to initiation of treatment and blood was taken at baseline (BL), as well as 6 and 16 weeks after treatment onset. EDTA plasma samples were collected at baseline as well as during treatment and were cryopreserved in −80°C freezer until used. Controls were close relatives living in the same household with indexed tuberculosis patients according to self-report and direct observation. Controls had no history of tuberculosis and showed no symptoms at recruitment. None of the controls presented with symptoms of tuberculosis during the study period at one of the participating hospitals. Study

**TABLE 1** Study group characteristics.

| | Ex vivo phenotyping | | Plasma reference monocyte assay | | Total recruit | |
|---|---|---|---|---|---|---|
| | TB | Controls | TB | Controls | TB | Controls |
| Number of Study cohort (*n*) | 19 | 19 | 33[a] | 30 | 40 | 35 |
| Mean age, years (range) | 55.3 (23–79) | 47.3 (32–73) | 45.5 (18–79) | 45.5 (21–75) | 46.8 (18–79) | 45.2 (21–75) |
| Male/female | 9/10 | 9/10 | 17/16 | 14/16 | 22/18 | 16/19 |
| HIV-Status | 0/19 | 0/19 | 0/33 | 0/30 | 0/40 | 0/35 |
| Diagnostic tests | | | | | | |
| GeneXpert (pos/neg/nd) | 19/0/0 | | 31/1/1 | | 38/1/1 | |
| Sputum culture (pos/neg) | 16/3 | | 25/8 | | (32/8) | |
| Sputum smear (pos/neg) | 15/4 | | 24/9 | | (31/9) | |
| Symptoms | | | | | | |
| Cough >2 weeks (%) | 90 | | 70 | | 73 | |
| Fever (%) | 47 | | 70 | | 68 | |
| Chest pain (%) | 32 | | 49 | | 48 | |
| Hemoptysis (%) | 47 | | 73 | | 70 | |
| Weight loss (%) | 42 | | 64 | | 60 | |

Abbreviations: *n*, number; nd, not done; neg, negative; pos, positive.
[a]Three tuberculosis patients were only included for time course STAT analyses in reference monocytes.

group details are summarized in Table 1. Due to restrictions in sample availability not all experiments included all samples. Numbers of subsets are given in Table 1. Buffy coat cells of healthy individuals retrieved from the Transfusion Medicine Department at the Heinrich-Heine-University in Duesseldorf, Germany, were used for all in vitro monocyte experiments. The present study received approval from the Committee on Human Research, Publication and Ethics (CHRPE/AP/023/18) at the School of Medicine and Dentistry at the Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, Ghana as well as the ethics committee of the Medical Faculty of the Heinrich-Heine-University Duesseldorf (ID: 5445).

## Whole blood monocyte phenotype characterization by flow cytometry

Monocyte phenotype differences between tuberculosis patients and controls as well as phenotype changes during treatment were characterized using peripheral blood collected in heparin tubes. Whole blood was diluted (1:1) with RPMI1640 media (supplemented with Penicillin/Streptomycin [100 U/mL] and L-glutamine [2 mM]). Cells were pelleted and stained with fluorescently labelled antibodies (i.e., anti-CD11c [Alexafluor488, clone: Bu15; BioLegend], anti-HLA-DR [PerCp-Cy5.5, Clone: L243; BioLegend], anti-CD33 [Alexafluor700, clone: WM-53; Invitrogen], anti-CD16 [APC, clone: 3G8; BioLegend], anti-CD70 [PE-CF594, clone: 113–16; BioLegend], anti-CD11b [PE-Cy7, clone: ICRF44; BioLegend], anti-CD40 [BV421, clone: 5C3; BioLegend], anti-CD64 [BV510, clone: 10.1; BioLegend], anti-CD14 [BV605, clone: M5E2; BioLegend], anti-PD-L1 [BV650, clone:29EA3; BioLegend]) and viability dye (eFluor780; eBiosciences) on ice in the dark for 30 min. After staining, red blood cells were lysed with Red Blood Cell lysis buffer (RBC; Roche), washed and then fixed with Fixation buffer (BioLegend). Cells were subsequently washed and measured using a four-laser flow cytometer (CytoFlex S; Beckman Coulter). FlowJo software (Version 10, Becton Dickinson) was used to analyse the data. A representative gating strategy is depicted in Figure S1.

## Plasma milieu effects on reference monocyte phenotypes and cytokine signalling

Using our previously established monocyte in vitro assay [23], we characterized the effect of plasma milieu on monocyte phenotypes and cytokine signalling. A schematic depiction of the assay is shown in Figure 3. Monocytes were enriched (using magnetic cell sorting (EasySept Monocyte negative selection kit; Stemcell Technology) as described before [23]) from peripheral blood mononuclear cells (PBMCs) purified from

peripheral blood of healthy individuals by density gradient centrifugation (Histopaque-1077; Sigma-Aldrich) according to manufacturers' guidelines. Enriched monocytes ($5 \times 10^4$ per well) were cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with L-Glutamine (2 mM, Sigma Aldrich), Hepes (10 mM, Thermo Fisher Scientific) and 10% of heterologous plasma either from a patient with tuberculosis or a control. After overnight incubation (24 h at 37°C, 5% $CO_2$), monocytes were incubated in ice-cold PBS containing 10 mM EDTA and 0.5% BSA for 30 min to detach adherent cells. Cells were then stained on ice for 30 min in the dark using the same antibody panel as for ex vivo phenotyping (see above). Thereafter, cells were washed and measured using LSR-Fortessa flow cytometer (BD Bioscience). Analyses were performed with FlowJo software (Version 10, Becton Dickinson). A representative gating strategy is depicted in Figure S2.

## STAT phosphorylation analysis

After culture of reference monocytes with medium containing 10% of heterologous plasma (from a tuberculosis patient or control), STAT phosphorylation was measured as described previously [17]. In brief, cultured cells were fixed with 100 µL of true nuclear fixation buffer (BioLegend) for 15 min at 37°C, 5% $CO_2$ and then permeabilized with pure methanol (130 µL) for 30 min in the dark on ice. Cells were then washed and stained with antibodies against human pSTAT1 (PE-CY7, clone: KIKSI0803; eBioscience), pSTAT3 (APC, clone LUVNKLA; eBioscience), pSTAT5 (PE, clone SRBCZX; eBioscience), CD14 (BV605, clone M5E2; BioLegend) for 30 min on ice. Phosphorylated STAT molecules were measured using the LSR-Fortessa Flow Cytometer (BD Bioscience) and analysed using FlowJo software (Version 10, Becton Dickinson).

## Data visualization and clustering for identification of putative monocytes subsets and candidate marker analysis

Data analysis was performed using FlowJo software (version 10; BD Biosciences, Franklin Lakes). For phenotyping of monocytes from study groups of tuberculosis patients and controls, monocyte changes under antimycobacterial treatment as well as reference monocytes treated with tuberculosis patients' or controls' plasma supplemented media, we performed combined multiplex data analyses. The following steps (shown as part of Figures S1 and S2) were done. Initially, IDs were assigned to each study group and time points during treatment. Next, viable HLA-DR positive cells were down-sampled to a maximum of 7000 cells (for ex vivo phenotyping) or 2000 cells (for reference monocytes) per replicate using the Downsample v3.3 plugin for FlowJo. All samples were then concatenated to one sample for comparison of study groups (Figures 1a,b and 4b,c) and time points (Figure 2b). We then performed fast interpolation-based t-distributed Stochastic Neighbour Embedding (fitSNE) [24] for reduction of data complexity and visualization of cell phenotype pattern. We applied default settings of the fitSNE FlowJo plugin for all parameters but set the number of iterations to 600 (default 1000). FitSNE depicts similarity of cells based on two parameters (fitSNE parameters 1 and 2; Figures 1a and 4b) and the similarity of cells is illustrated by their distances in the respective graphs. Smoothed density plots (Figures 1a and 4b), colour density plots (Figure 1a) and contour plots (Figure 4c) were used to depict different data sets. Algorithm supported unbiased identification of monocyte subsets was done using flow cytometry self-organizing maps (FlowSOM) algorithm (FlowJo plugin downloaded from www.flowjo.com/exchange/). The number of clusters was arbitrarily set to five. All other parameters were left at manufacturer's default settings. Analyses of the three main subsets predicted by FlowSOM was performed by back-gating clustered cells based on assigned IDs for individuals, study groups and replicates.

## Graphical depiction and statistics

GraphPad Prism v9 software (GraphPad Software) was used for all statistical analyses. Due to the non-normal distribution of data (tested by Kolmogorov–Smirnov and ShapiroWilk test), nonparametric tests were used throughout. Study group comparisons were performed by the Mann–Whitney *U*-test while the Wilcoxon matched-pairs signed rank test was used for paired comparisons. Spearman rank correlation was used to assess association between phenotype marker expression and plasma sample induced candidate markers and STAT phosphorylation levels. A *p*-value below 0.05 was considered statistically significant.

# RESULTS

## Increased frequency of putative M1 cells and high CD64, CD40 and PD-L1 expression in monocytes from tuberculosis patients

Initially, we characterized monocytes from the peripheral blood of tuberculosis patients and healthy controls from

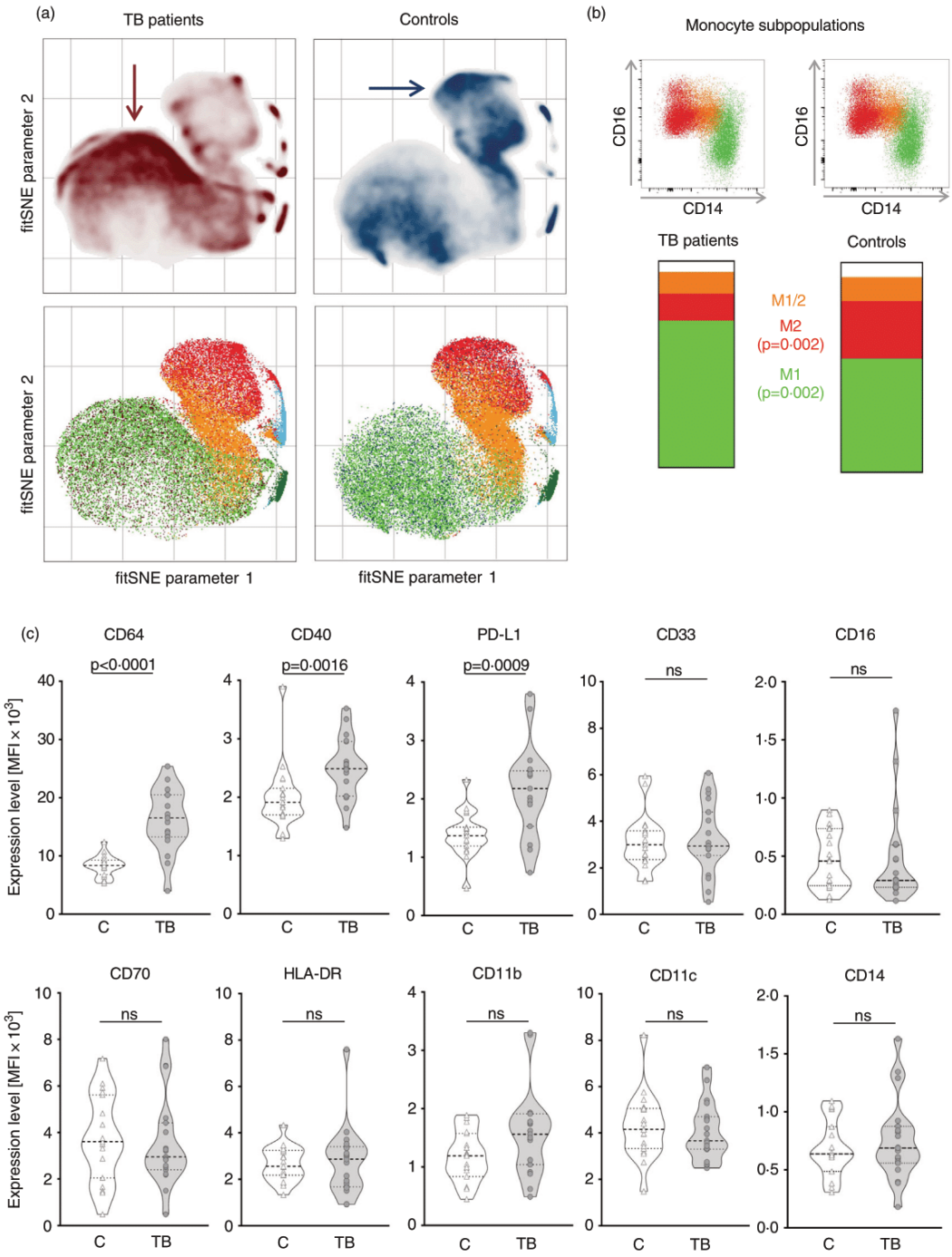


**FIGURE 1** Monocyte marker expression differences and visualization of expression pattern in predicted subsets from tuberculosis patients and healthy controls. Ex vivo phenotyping of peripheral blood monocytes by flow cytometry from tuberculosis patients ($n = 19$) and healthy controls ($n = 19$) for CD64, CD40, PD-L1, CD33, CD16, CD70, HLA-DR, CD11b, CD11c and CD14 is shown. (a) FitSNE-based visualization of concatenated data sets including all markers for tuberculosis patients and healthy controls was performed. Smoothed density plots of tuberculosis patients (red) and healthy controls (blue) depicted for fitSNE parameters 1 and 2 are shown in the upper graphs. FlowSOM clustering of fitSNE visualized data from tuberculosis patients and controls are presented in the lower graphs. Five populations are highlighted by different colours. (b) Colour density plots and stacked colour bar charts depict CD14/CD16 expression pattern for concatenated data from tuberculosis patients and controls. Each dot represents a single monocyte from a tuberculosis patient or a control. (c) Violin plots including 25, 50 and 75 percentiles (as dotted or dashed lines) are given. Each symbol represents the mean of duplicates from an individual donor. Study group comparisons were performed and *p*-values were calculated using the two-tailed Mann–Whitney *U*-test. Nominal *p*-values are given for significant differences. ns: not significant.

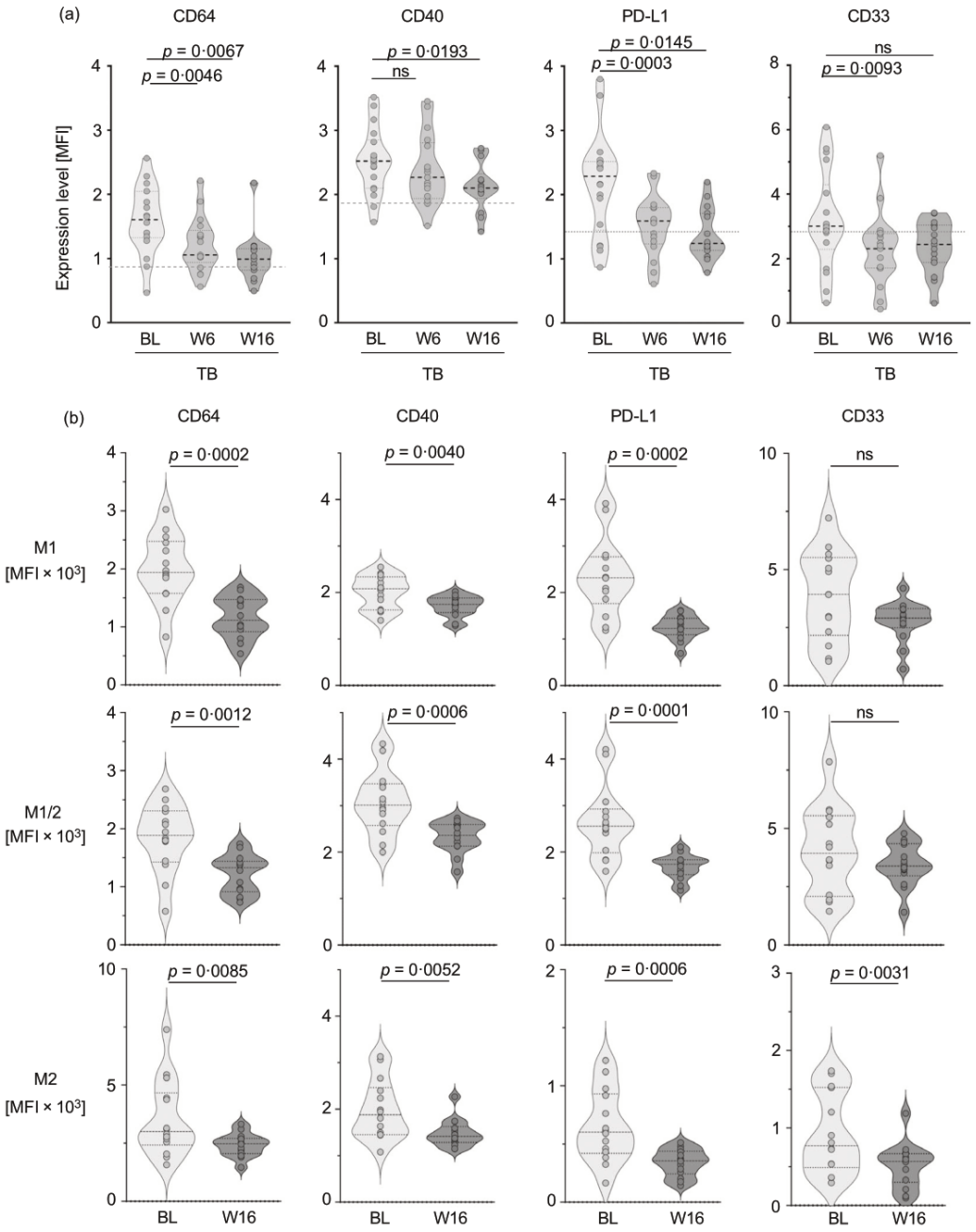


**FIGURE 2** Time course comparisons of candidate marker expression in monocytes and monocyte subsets during treatment of tuberculosis patients. Ex vivo phenotyping of peripheral blood monocytes by flow cytometry from tuberculosis patients prior to treatment (BL), week 6 (W6) and week 16 (W16) after treatment start was performed. Analyses for selected markers (i.e., CD64, CD40, PD-L1, CD33) are shown. (a) Violin plots for BL (bright grey violin, $n = 18$), W6 (medium grey violin, $n = 17$) and W16 (dark grey violin, $n = 15$) including 25, 50 and 75 percentiles (as dotted or dashed lines) are given. Each circle represents the mean of duplicates from an individual donor. The dotted lines indicate candidate marker expression in controls. Paired comparisons were performed for individuals between the time points and *p*-values are calculated using the Wilcoxon-signed rank test. Nominal *p*-values are given. ns: not significant. (b) Candidate marker expression of concatenated data sets for the three main monocytes subsets (i.e., putative M1, M1/2, M2) from tuberculosis patients at BL and W16.

Ghana using a comprehensive antibody panel of markers (including CD11b, CD11c, CD14, CD16, CD33, CD40, CD64, CD70, HLA-DR, PD-L1) previously found to be affected in tuberculosis immunopathology. Individual measurements were concatenated to generate combined datasets for study groups of tuberculosis patients and

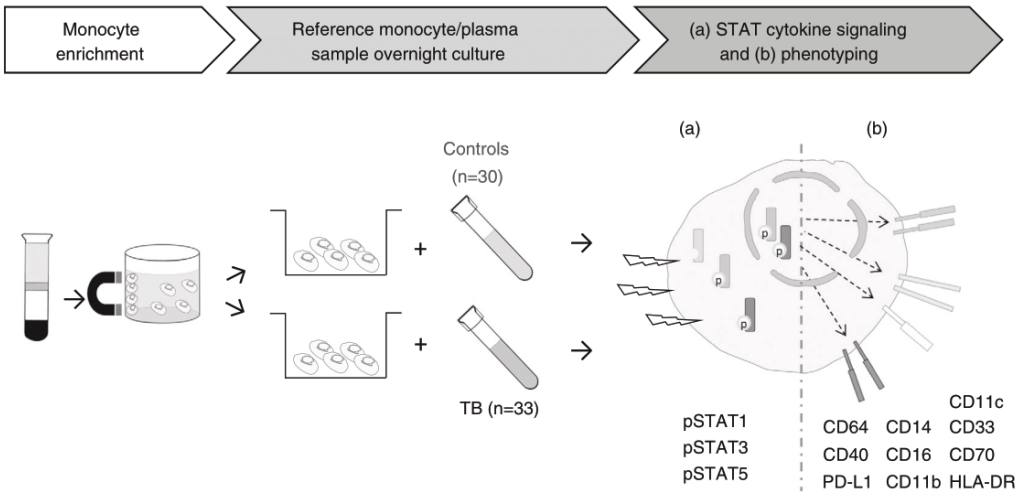


**FIGURE 3** The experimental scheme of the reference monocyte/plasma in vitro assay. Reference monocytes were enriched by density gradient centrifugation and non-touch magnetic beads-based purification. Reference monocytes ($5 \times 10^4$) were then co-cultured in medium containing either plasma from a tuberculosis patient or a control. After overnight culture, monocytes were harvested and directly stained with (a) pSTAT antibodies (i.e., pSTAT1, pSTAT3, pSTAT5) for intracellular analyses of cytokine receptor signalling or (b) monocyte markers (i.e., CD64, CD40, PD-L1, CD33, CD16, CD70, HLA-DR, CD11b, CD11c and CD14). Multicolor flow cytometry analysis was then performed to determine mean protein expression differences of markers on reference monocytes in response to individual donor plasma samples.

controls. For data complexity reduction and visualization of study group differences, we applied fast interpolation-based t-Stochastic Neighbourhood Embedding (fitSNE) (see Methods section and Figure S1 for details). FitSNE calculates two parameters from all included markers and density plots depict distribution of monocytes with high similarity for tuberculosis patients and controls (Figure 1a). FitSNE plot comparison revealed marked differences between the study groups with distinct regions showing high density for tuberculosis patients (dark red arrow; left graph) and controls (dark blue arrow; right graph) (Figure 1a). Self-organizing map clustering by FlowSOM was then applied to classify potential monocyte subpopulations based on similarities. Starting with an arbitrarily set number of five, three subsets (i.e., bright green, orange, red) classified by FlowSOM algorithm made up more than 95% of monocytes in tuberculosis patients and controls (Figure 1a; lower graphs). The bright green subpopulation had a phenotype similar to M1 ($CD14_{high}$), was largely distinct from putative M2 (red; $CD16_{high}$) and showed some overlap with putative M1/2 ($CD14_{medium}/CD16_{high}$; orange) (Figure 1b). Distribution of monocyte subpopulation showed marked differences. Whereas the putative M1 population was more frequent in tuberculosis patients (71.2%) as compared to controls (54.2%) ($p = 0.002$), the putative M2 subset was higher in controls (TB: 13.1%; Controls: 27.5%; $p = 0.002$; Figure 1b). No differences were detected for the putative M1/2 subpopulation (Figure 1b).

Next, we compared protein expression level of candidate markers on monocytes between the study groups. Notably, three markers, CD64, CD40, and PD-L1, were significantly higher in monocytes from tuberculosis patients whereas CD33, CD16, CD70, HLA-DR, CD11b, CD14 and CD11c showed no differences between the study groups (Figure 1c). Phenotype comparisons between putative M1, M1/2 and M2 subpopulations also showed differential expression for identified markers (Figure S3a). Higher CD40, CD64 and PD-L1 expression was seen for all monocyte subpopulations in tuberculosis patients except putative M2 cells where CD40 differences did not reach significance levels (Figure S3a). Interestingly, whereas CD16 expression was not generally different between the study groups (Figure 1b), higher CD16 was detected for putative M1 cell from tuberculosis patients but not for the other subpopulations (Figure S3b). We concluded that tuberculosis patients were characterized by high expression of CD64, CD40 and PD-L1 as well as increased proportions of putative M1 cells with higher CD16 expression.

## CD64, CD40, CD33 and PD-L1 expression in monocytes decreased during treatment of tuberculosis patients

Next, we compared monocyte phenotypes from tuberculosis patients prior to treatment (baseline, BL) with an

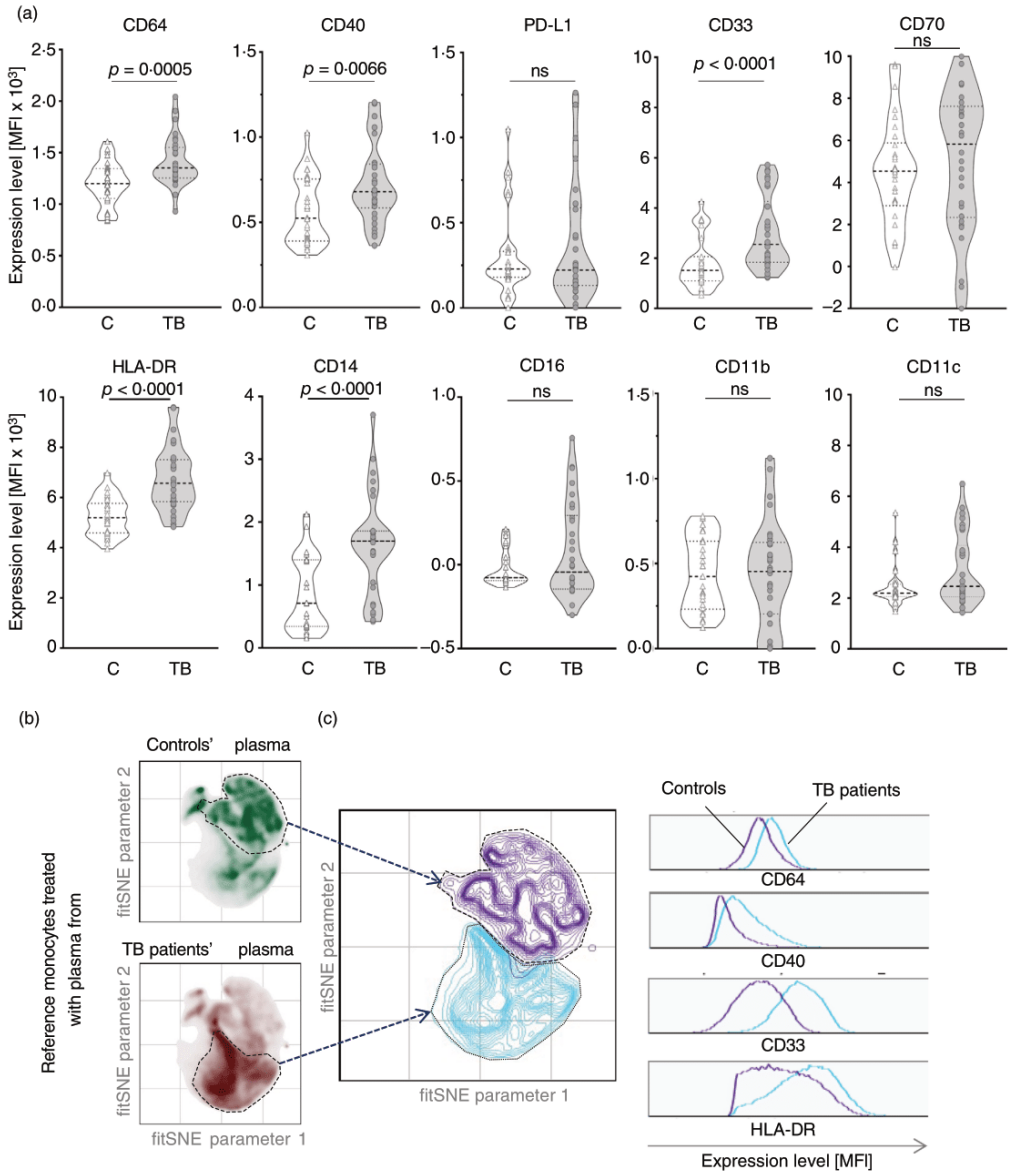


**FIGURE 4** Effects of plasma samples from tuberculosis patients/controls on reference monocyte marker expression. Flow cytometry phenotyping of reference monocytes after overnight culture with medium containing either tuberculosis patients' (grey circles, $n = 30$) or healthy controls' (open triangles, $n = 30$) plasma samples were determined for CD64, CD40, PD-L1, CD33, CD16, CD70, HLA-DR, CD11b, CD11c and CD14 expression. (a) Violin plots (grey background colour for patients and open for controls) including 25, 50 and 75 percentiles (as dotted or dashed lines) are given. Each symbol represents the mean of duplicates from an individual donor. A representative gating example of viable HLA-DR$^+$ cells is shown in Figure S2. Study group comparisons were performed and $p$-values were calculated using the two-tailed Mann–Whitney $U$-test. Nominal $p$-values are given. ns: not significant. (b) FItSNE-based visualization of concatenated data sets including all markers measured on reference monocytes after culture with medium containing tuberculosis patients' or healthy controls' samples. Smoothed density plots of tuberculosis patients (red) and healthy controls (green) depicted for FItSNE parameters 1 and 2 are shown. (b, c) Dashed and dotted lines indicate the two main subsets induced by plasma samples from tuberculosis patients and controls, respectively. (c) FlowSOM based identification of two main phenotype subsets induced by plasma samples from tuberculosis patients and controls. Two dominant clusters (violet for controls; bright blue for tuberculosis patients) were detected by FlowSOM and histograms show the expression of candidate markers for both clusters.

early timepoint (i.e., 6 weeks, W6) and a late timepoint (i.e., 16 weeks, W16) after treatment start. Monocyte markers with high expression at BL (i.e., CD40, CD64 and PD-L1; Figure 1a) decreased significantly under treatment (Figure 2a). For CD64 and PD-L1, a rapid decrease until W6 was detected (CD64, $p = 0.0046$; PD-

L1, $p = 0.0003$) whereas a significant CD40 decline was only seen between BL and W16 ($p = 0.0193$). At W16, CD64, PD-L1 and CD40 expression largely normalized and median values were comparable to healthy controls (dotted line; Figure 2a). Interestingly, CD33 (an inhibitory receptor of the Siglec family) although not differentially expressed between tuberculosis patients and controls (Figure 1a), showed decreased expression between BL and W6 (Figure 2a; $p = 0.0093$). CD11b, CD11c and CD70 were not different between the time points (Figure S4). Next, we analysed putative monocyte subpopulations for candidate marker expression differences during treatment. Putative M1, M1/2, M2 subpopulations showed significant decrease in CD64, PD-L1 and CD40 expression between BL and W16 (Figure 2b). For CD33, however, significant differences were only detected for putative M2 between BL and W16 (Figure 2b). These results suggested normalization of the aberrant monocyte phenotype during treatment and strengthen the assumption that differential monocyte marker expression is a specific feature of immunopathology in human tuberculosis.

## Overnight culture of reference monocytes supplemented with plasma samples from tuberculosis patients induced high CD64, CD40 and CD33 expression

Previous studies suggested plasma milieu effects on immune cell phenotypes in tuberculosis [17, 23]. In order to directly test plasma effects on monocyte phenotypes in tuberculosis, we performed cell culture of reference monocytes in medium supplemented with plasma samples from either a tuberculosis patient ($n = 30$) or a control ($n = 30$) (a schematic depiction is shown as Figure 3). After overnight culture, we analysed the monocyte phenotype by flow cytometry. Notably, three of four candidates (i.e., CD64, CD33 and CD40) showed higher expression in reference monocytes cocultured with plasma samples from tuberculosis patients as compared to healthy controls (CD64: $p = 0.0005$; CD33: $p < 0.0001$; CD40: $p = 0.0066$; Figure 4a). PD-L1 was not different between plasma samples from the study groups (Figure 4a). Higher expression was also detected for HLA-DR and CD14 in the presence of tuberculosis patients' plasma (both $p < 0.0001$). This confirmed previous results for plasma-induced HLA-DR expression [23], and indicated that plasma effects on reference monocytes may not completely resemble monocyte phenotype changes in acute tuberculosis.

Next, we visualized phenotypic changes of treated reference monocytes and identified marked differences between reference monocytes treated with plasma from tuberculosis patients or controls (Figure 4b). Two main populations, one dominant in reference monocytes cultured with plasma from tuberculosis patients and one in reference monocytes cultured with plasma from controls, were identified (Figure 4b, density plots). Notably, FlowSOM confirmed the visual impression and classified these two main subsets as independent clusters, which comprised approximately 90% of all cells (Figure 4c). Notably, differences in HLA-DR, CD33, CD40 and CD64 expression were the main factors for classification and this confirmed concomitant upregulation of these markers on reference monocytes treated with tuberculosis patients' plasma (Figure 4c; histograms). These results suggested causative effects of the plasma milieu in acute tuberculosis for immunopathology seen in monocytes.

## STAT3 and STAT5 signalling is higher in reference monocytes cocultured with plasma samples from tuberculosis patients

To characterize underlying mechanisms of plasma milieu effects, we next analysed STAT mediated signalling in reference monocytes cocultured overnight with plasma samples from both study groups. STAT1, STAT3 and STAT5 phosphorylation was determined directly thereafter. Whereas STAT1 showed comparable phosphorylation levels between the study groups, both, STAT3 and STAT5, showed significantly higher phosphorylation in the presence of plasma samples from tuberculosis patients as compared to controls (Figure 5a). Next, we compared plasma samples from tuberculosis patients prior to treatment with samples taken at W6 and W16 after start of therapy. The capacity of plasma samples to induce STAT1 phosphorylation was similar at different time points whereas STAT5 phosphorylation decreased significantly already within 6 weeks of antimycobacterial treatment (Figure 5b). For pSTAT3, a tendency of decreased levels was detected for both W6 and W16 (Figure 5b). These results suggested a role of plasma cytokine signalling via STAT3 and/or STAT5 as underlying mechanisms for the aberrant monocyte phenotype.

To determine a possible association between plasma-induced STAT3/STAT5 signalling and phenotypic changes, we correlated CD64, CD40 and CD33 expression with pSTAT3/pSTAT5 levels in reference monocytes incubated with respective plasma samples. CD40 and CD64 showed a positive correlation with pSTAT5 for both, plasma samples from tuberculosis patients and controls (Figure 5b). For pSTAT3, no correlation was seen for CD64 and only in controls plasma samples for CD40 (Figure 5b). Notably, CD33 expression showed strong positive correlation with pSTAT3 in both cohorts but not with pSTAT5 in

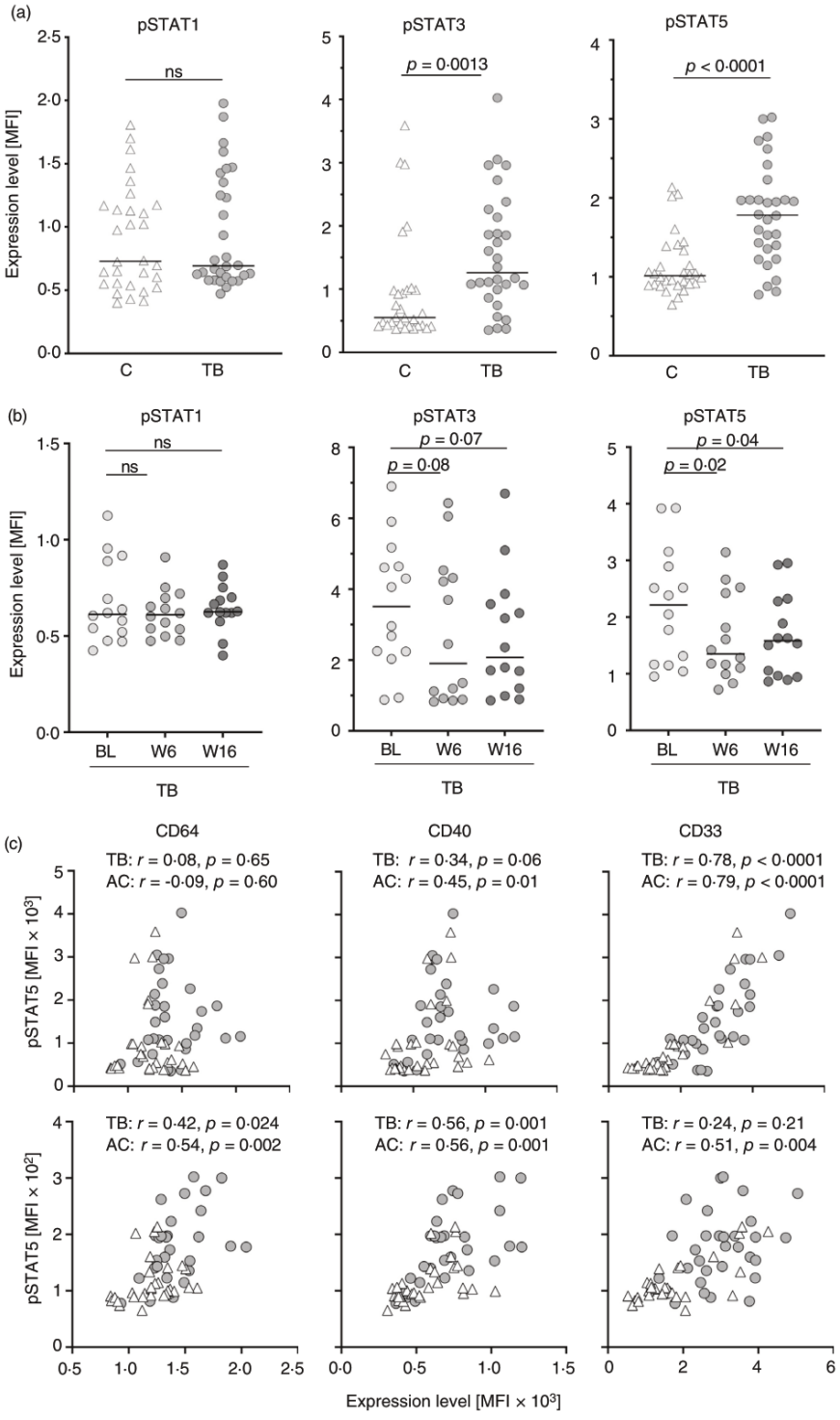


**FIGURE 5** Plasma sample culture effects on reference monocyte STAT phosphorylation. Flow cytometry analysis of STAT1, STAT3 and STAT5 phosphorylation in reference monocytes after overnight culture with medium containing either plasma from a tuberculosis patient (grey circles, $n = 30$) or a control (open triangles, $n = 30$). (a) Symbol plots including median values as straight lines are given. Each symbol represents the mean of duplicates from an individual donor. Study group comparisons were performed and $p$-values were calculated using the two-tailed Mann–Whitney $U$-test. Nominal $p$-values are given for significant differences. ns: not significant. (b) Time course comparisons are shown as symbol plots for BL (bright grey circles, $n = 14$), W6 (medium grey circles, $n = 14$) and W16 (dark grey circles, $n = 14$). Each symbol represents the mean of duplicates from an individual donor. Study group comparisons were performed and $p$-values are calculated using the Wilcoxon-signed rank test. Nominal $p$-values are given. (c) Correlation of candidate marker mean fluorescence intensity with pSTAT3 and pSTAT5 at BL are shown. The Spearman Rank test was applied to determine significant correlations for both study groups separately. Correlation coefficients (rho) and nominal $p$-values are given.

tuberculosis patients (Figure 5b). This suggested an association of CD33 expression with induced STAT3 phosphorylation. In summary, different pathways and causative plasma cytokines affect monocyte phenotype in tuberculosis and strengthened the hypothesis that the plasma milieu in acute tuberculosis exerted immunopathology via candidate cytokines, which signal via the STAT3 or STAT5 pathway.

## DISCUSSION

The present study provided evidence that the plasma milieu in acute tuberculosis contributes to the immunopathology phenotype of monocytes seen in tuberculosis patients. Higher STAT3 and STAT5 phosphorylation was induced in reference monocytes by plasma samples from

tuberculosis patients as compared to controls and associations were seen with distinct phenotypic markers. This finding suggested that different plasma cytokines are involved in tuberculosis immunopathology. In accordance, a previous study failed to identify individual plasma cytokines from tuberculosis patients associated with induced IL-7Rα expression in monocytes [23]. Distinct association pattern seen for pSTAT3, which correlated positively with CD33 expression, and pSTAT5, which correlated with CD40 and CD64 in the present study, narrowed down potential causative factors.

Evidence for functional relevance of plasma immunopathology in tuberculosis has been provided by previous studies in T cells and monocytes from tuberculosis patients [17, 22]. Our group detected constitutive STAT3 phosphorylation in T cells associated with high IL-6 and IL-10 plasma levels [17]. Consequently, increased SOCS3 expression and inhibition of IL-2 induced STAT5 mediated signalling affected *M. tuberculosis* specific T cells [17]. Constitutive STAT3 phosphorylation of monocytes was detected in a previous study, by Lastrucci et al., that characterized influential factors produced by *M. tuberculosis* infected monocyte-derived macrophages (MDMs) in reference monocytes [25]. This study detected high pSTAT3 levels together with upregulation of CD16 in monocytes and this resembled monocyte immunopathology features seen in tuberculosis [25]. Functionally, monocytes treated with *M. tuberculosis* infected MDM supernatants were prone to become anti-inflammatory macrophages in this study and IL-10 was key for these effects [25]. In addition, they detected impaired anti-mycobacterial effector functions of treated monocytes as well as worsened interaction with *M. tuberculosis* specific T cells [25]. A role of IL-10 was also seen in previous studies on tuberculosis plasma cytokine levels [19] and pathognomonic effects on T cells were found [17]. These findings possibly indicate immunomodulatory mechanisms but since IL-10 is accompanied by aberrant high IL-6 levels in acute tuberculosis [17, 19], our findings may alternatively be interpreted as a feature of inflammation [22]. We did not characterize the function of MDM derived from plasma treated monocytes and this was partly due to negative effects of non-inactivated heterologous plasma on monocyte culture. However, others detected functional effects of plasma from tuberculosis patients on monocyte derived dendritic cells [26]. This rendered our assumption likely that aberrant cytokine signalling and accompanied monocyte phenotype changes have functional implications on derived macrophage populations.

Previous studies showed high complexity of peripheral blood monocytes displayed by differences in phenotype, size, and functions [3]. A subpopulation of 'small' monocyte has been identified and characterized by specific effector functions including increased production of pro-inflammatory cytokines [27, 28]. This subset of so-called 'inflammatory monocytes' was found to be increased in inflammatory diseases, sepsis and infections [3]. We and others found higher proportions of 'inflammatory monocytes' in tuberculosis [2, 5, 8, 25, 29]. Self-organizing map clustering performed in the present study indicated that this subset, which is characterized by concomitant expression of CD14 and CD16, is more similar to M1 (or classical monocytes) and less to M1/2 (or intermediate monocytes characterized by $CD14_{medium}/CD16_{high}$ expression). In addition, we showed that M1 proportions are higher in tuberculosis patients as compared to healthy controls. This argued for upregulation of CD16 in enriched putative M1 cells as the mechanisms underlying generation of inflammatory monocytes in tuberculosis. More importantly, the differences in monocyte phenotype from tuberculosis patients were detected for all main monocyte subsets. This suggested subset-independent immunopathology in monocytes and plasma milieu effects on references monocytes strengthened this assumption. In addition, we provided initial evidence for complex mechanisms of plasma and monocyte pathology in acute tuberculosis. Two markers, CD64 and CD40, were strongly induced by tuberculosis plasma samples and also showed higher expression in monocytes from acute tuberculosis patients. Since CD64/CD40 expression was positively correlated with plasma induced pSTAT5 levels, it is likely that pSTAT5 signalling dependent plasma cytokines are causative for aberrant high levels in acute tuberculosis. Notably, and in contrast to pSTAT3, previous studies did not detect higher constitutive pSTAT5 levels in monocytes (or T cells) from tuberculosis patients [5, 17]. This could either be explained by impaired response to causative cytokine(s) or negative regulation of STAT5 phosphorylation. Both has been described for IL-7-mediated T-cell response in tuberculosis [16]. T cells from tuberculosis patients are characterized by low IL-7 receptor expression and impaired response to high IL-7 plasma levels [16]. In addition, the key regulator of STAT signalling SOCS3 was increased in T cells from tuberculosis patients [30] with potential negative effects on pSTAT5 induction [17] and IL-7 receptor expression [31]. Monocytes from tuberculosis patients also have low IL-7 receptor expression and show impaired IL-7-induced pSTAT5 [5]. These findings render IL-7 a promising plasma cytokine candidate involved in the induction of STAT5 phosphorylation.

Another tuberculosis plasma-induced factor in the present study was CD33. Monocytes from tuberculosis patients showed similar CD33 expression as compared to healthy controls but expression decreased in monocytes during treatment. CD33 was strongly induced by plasma samples from tuberculosis patients and this effect was associated with high STAT3 phosphorylation. Although

the role of CD33 is not finally defined, its expression on suppressive monocyte subsets [32] may also indicate that alternative monocyte polarization is induced by the plasma milieu in tuberculosis and normalized during antimycobacterial treatment.

In summary, we provided evidence for plasma milieu effects on monocyte signalling and pathology in acute tuberculosis. Further studies are needed to investigate the functional implications in monocytes. The application of identified candidates as markers for tuberculosis diagnosis and treatment is promising since CD64 has already been proven as part of a biomarker signature of tuberculosis [10]. Future studies will investigate the capacity of identified monocyte markers for monitoring treatment efficacy in tuberculosis patients. Furthermore, these studies should also analyse associations between plasma induced signalling pathways and monocyte pathology during treatment and recovery of patients with tuberculosis.

## AUTHOR CONTRIBUTIONS

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## ACKNOWLEDGEMENTS

We would like to thank all study participants, study nurses and physicians supporting this investigation. Open Access funding enabled and organized by Projekt DEAL.

## FUNDING INFORMATION

This study was supported by the German Research Foundation (DFG, JA 1479/9-1) and the graduate school molecules of infection (MOI)-4 funded by the Jürgen Manchot foundation. The funders had no role in study design, data collection, data analysis, decision to publish, or preparation of the manuscript.

## CONFLICT OF INTEREST STATEMENT

The authors declare that no conflict of interest exists.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Ahor HS, Schulte R, Adankwah E, Harelimana JDD, Minadzi D, Acheampong I, et al. Monocyte pathology in human tuberculosis is due to plasma milieu changes and aberrant STAT signalling. Immunology. 2023;170(1):154–66. https://doi.org/10.1111/imm.13659

**3.3 Paper III:** Monocyte transcriptome signatures of inflammation and enhanced neutrophil recruitment characterize immunopathology in the blood of tuberculosis patients.

**Ahor HS**, Vivekanandan MM, Adankwah E, Minadzi D, Acheampong I, Aniagyei W, Yeboah A, Arthur JF, Lamptey M, Abass MK, Kumbel F, Osei-Yeboah F, Gawusu A, Petzsch P, Köhrer K, Debrah LB, Owusu DO, Debrah A, Mayatepek E, Seyfarth J, Phillips RO, & Jacobsen M. Monocyte transcriptome signatures of inflammation and enhanced neutrophil recruitment characterize immunopathology in the blood of tuberculosis patients **(Preliminary Manuscript).**

Contribution to this publication: 80%

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

# Monocyte transcriptome signatures of inflammation and enhanced neutrophil recruitment characterize blood plasma immunopathology in tuberculosis patients

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**Running title:** TB plasma induced signatures in monocytes.

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**Abstract**

Tuberculosis is characterized by immunopathology in the blood and monocytes are highly sensitive to plasma environment changes in tuberculosis patients. Here, we investigated TB plasma effects on ‘reference monocytes’ using RNA sequencing to characterize a potential immunomodulatory role of monocytes in tuberculosis. Candidate pathways induced by plasma samples from tuberculosis patients (n=99) as compared to healthy contacts (controls; n=62) were analyzed for changes in signal transduction, phenotype, and secreted cytokines by flow cytometry. Finally, potential implications were characterized in blood samples from corresponding patients and controls.

Reference monocytes treated with TB plasma showed an enrichment of pathways involved in inflammation and chemotaxis. Inflammatory cytokines were accompanied by enhanced phosphorylation of STAT molecules (i.e., STAT1/3/5), and strong positive correlations were detected for IL-6 only in TB plasma-treated monocytes. Moreover, monocyte chemokine receptors (i.e., CCR1, CCR5) and pro-inflammatory chemokines (i.e., CXCL-1, CXCL-2, CXCL-8, G-CSF, CCL-2) that attract granulocytes and monocytes were significantly higher in TB plasma-treated monocytes. Notably, corresponding clinical samples also showed higher plasma levels for a subset of inflammatory cytokines/chemokines. In particular, high IL-6 levels positively correlated with the accumulation of neutrophil granulocytes in the blood of tuberculosis patients. Finally, monocytes from tuberculosis patients were characterized by increased chemokine receptor expression, higher proportions of a $CCR2^{+}$ subpopulation, and aberrant high SOCS3 expression.

These results indicate an important role of monocytes as potential amplifiers of plasma immunopathology, causing sustained mobilization and accumulation of neutrophil granulocytes and chronic inflammation in the blood of tuberculosis patients.

## Introduction

Monocytes and derived macrophage/dendritic cell subsets play a critical role in immune protection against *Mycobacterium (M.) tuberculosis* infection. As the primary host cells of *M. tuberculosis* and because of their involvement in granuloma formation, monocyte-derived cells fulfill crucial functions in the immune defense. Against this background, it is important to note that tuberculosis patients show changes in the monocyte population in the peripheral blood. Amongst others, monocytes differ in phenotype and secreted cytokines in acute tuberculosis [1-4]. Due to these differences, which have been described to occur under inflammatory conditions, they were also referred to as 'inflammatory monocytes' [5,6]. Functional implications of tuberculosis pathology on blood immune cells were already documented in the last century [7]. The group of J.J. Ellner conducted ground-breaking studies demonstrating an impaired immune response in a subgroup of tuberculosis patients [8, 9]. Importantly, they showed that plasma samples from tuberculosis patients can transfer immunosuppression *in vitro* to healthy reference immune cells [9]. Monocytes and secreted soluble factors were shown to be essential for immunosuppression in these studies [9], highlighting the central role of this immune cell subset.

Own previous study confirmed the effects of the TB plasma environment on reference monocytes and provided evidence for functional implications [10]. Moreover, we demonstrated that plasma-induced monocyte phenotype changes largely reflect the inflammatory monocyte phenotype seen in tuberculosis patients [4]. Signal Transducer and Activator of Transcription (STAT) 3 and STAT5 pathways were shown to correlate with monocyte phenotype changes, and this suggested that host cytokines play a role in the plasma-dependent immunopathology of tuberculosis [4]. The present study is based on the assumption that monocytes contribute to blood pathology in tuberculosis by acting as sensors and amplifiers of *Mycobacterium tuberculosis*-triggered changes in the plasma milieu. To investigate this question, we conducted a combined study based on the *in vitro* effects of tuberculosis/control plasma on reference monocytes and phenotyping of immune cells and peripheral blood plasma from the respective tuberculosis patient and contact study groups. *In vitro* effects of blood plasma on reference monocytes were interpreted as early/initial events, while blood signatures were thought to reflect the chronic effects of tuberculosis immunopathology.

Initially, we performed global messenger RNA sequencing of TB/control plasma-treated reference monocytes to identify involved pathways and functional implications of the aberrant plasma milieu in tuberculosis patients. Selected candidates were then confirmed on the protein level on plasma-treated reference monocytes and culture supernatants. Finally, phenotyping and quantification of immune cell subsets in corresponding blood samples were performed to identify potential chronic implications on immunopathology in tuberculosis patients.

## Material and Methods

### *Study cohorts and clinical characterization*

Between July 2019 and March 2022, tuberculosis patients (n=99) and asymptomatic contacts of indexed patients (controls, n=62) were recruited at four hospitals in Ghana (i.e., Agogo Presbyterian Hospital, St. Mathias Catholic Hospital, Atebubu District Hospital, Sene West District Hospital). Diagnosis of active tuberculosis was based on the patient's history, clinical examination, chest X-ray, sputum smear test, and GeneXpert analyses as described [4]. All patients were included before initiation of treatment. Controls were close relatives living in the same household with indexed tuberculosis patients according to self-report and direct observation. Controls had no history of tuberculosis and showed no symptoms of tuberculosis at recruitment. Each participant donated blood for immune cell phenotyping (10mL sampled in BD Vacutainer®heparin tubes, BD) and for generation of plasma (5mL sampled in BD Vacutainer® EDTA Tubes, BD) according to manufacturers' instructions. Due to the limitations in sample availability, not all experiments included all samples. Samples were selected randomly and according to sex and matching. The characteristics of study participants and the subsets included in different experiments are provided in Table 1.

To conduct the *in vitro* reference monocyte experiments, buffy coat cells were retrieved from healthy individuals at the Transfusion Medicine Department of Heinrich-Heine-University in Duesseldorf, Germany. Ethics approval for the study was obtained from the Committee on Human Research, Publication and Ethics (CHRPE/AP/023/18) at the School of Medicine and Dentistry at Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, Ghana as well as the ethics committee of the Medical Faculty of Heinrich-Heine-University Duesseldorf (ID: 5445).

### *The plasma milieu reference monocyte assay*

The plasma milieu response of reference monocytes has been described before [4, 10]. In brief, monocytes were enriched by magnetic cell sorting (EasySept Monocyte negative selection kit; Stemcell Technology) from peripheral blood mononuclear cells (PBMCs) purified from peripheral blood of healthy individuals by density gradient centrifugation (Histopaque-1077; Sigma-Aldrich) according to manufacturers' guidelines. Enriched monocytes ($5x10^4$ per well) were cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with L-Glutamine (2 mM, Sigma Aldrich), Hepes (10 mM, Thermo Fisher Scientific), and 10% of a heterologous plasma sample from a tuberculosis patient or a control. Monocytes were then cultured overnight at 37°C and 5% CO2. Thereafter, supernatants (80µL) were harvested and stored at -80°C (i.e., supernatant samples) until usage. Reference monocytes were incubated in 170 µL of ice-cold PBS containing 10 mM EDTA and 0.5% BSA for 30 min to detach adherent cells.

### *Messenger (m)RNA sequencing of plasma milieu-treated reference monocytes*

Total RNA was extracted from reference monocytes after culture using the NucleoSpin RNA kit (Macherey-Nagel) following the manufacturer's instructions. Selected plasma samples from age and sex-matched tuberculosis patients (n=9) and controls (n=9), previously described to have differential effects on STAT signaling (4), were included. All samples were simultaneously processed to minimize variability. The concentration of RNA samples was determined using the Qubit RNA HS Assay (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. RNA quality was assessed by capillary electrophoresis using the 'Total RNA Standard Sensitivity Assay' (Agilent Technologies, Inc. Santa Clara, USA). All samples used for sequencing had an RNA quality number (RQN) ≥ 8. Library preparation was carried out using the VAHTS Universal stranded mRNA library prep kit V6 with polyA-capture, following the manufacturers' instructions. Briefly, 100 ng of total RNA was used for mRNA capturing, fragmentation, cDNA synthesis, adapter ligation, and library amplification. The bead-purified libraries were normalized and then sequenced on the NextSeq2000 system (Illumina Inc., San Diego, CA, USA) with a read setup of SR 1×100 bp. Sequencing output was between 20.1 and 28.5 million reads per sample with a mean of 23.9 million reads per sample. Conversion of BCL files to fastq files, adapter trimming, and demultiplexing were performed using the Illumina BCL Convert tool (version 4.0.3).

Data analyses of fastq files were conducted using CLC Genomics Workbench (version 23.0.4, QIAGEN, Venlo, Netherlands). The reads from all probes were subjected to adapter trimming (Illumina TruSeq) and quality trimming, using the default parameters (trimming bases below Q13 from the end of the reads, allowing a maximum of 2 ambiguous nucleotides). Mapping was performed against the Homo sapiens GRCh38.107 (hg38) genome sequence. The Wald test was used in the CLC Differential Expression for RNA-Seq tool (version 2.8) for the comparison of differentially expressed genes between pairs of groups (patient and control). The resulting P-values were corrected for multiple tests using FDR and Bonferroni correction. A p-value below 0.05 was considered significant. Ingenuity Pathway Analysis (IPA, QIAGEN, Venlo, Netherlands) and GraphPad Prism v10 software (GraphPad Software, La Jolla CA, USA) were used to depict gene expression as Z scores. For functional annotation of the differentially expressed genes, we used the Database for Annotation, Visualization, and Integrated Discovery (DAVID; https://david.ncifcrf.gov). Significantly enriched pathways ($p<0.05$; Bonferroni corrected) in the categories 'UniProtKB Keywords (UP_KW) Biological Process' and 'Gene Ontology (GO)_Biological Process' were included.

***Reference monocyte phenotype and STAT phosphorylation analyses by flow cytometry***

Reference monocyte phenotyping and STAT phosphorylation analyses were performed after overnight culture as described previously [4]. In brief, detached monocytes were stained on ice for 30 minutes in the dark in PBS/FCS 10% medium (25 µL) using fluorescently labelled antibodies against HLA-DR (PerCp-Cy5.5, Clone L243; BioLegend), CD14 (BV605, clone: M5E2; BioLegend), CCR-1 (AF488, clone 5F10B29; BioLegend), CCR-5 (PE, clone J418F1; BioLegend), CCR-2 (PE-Cy7, clone K036C2; BioLegend), and a viability dye (eFluor780; eBiosciences).

For measurement of STAT phosphorylation, monocytes were fixed using true nuclear fixation buffer (100µL; BioLegend) for 15 minutes at 37°C, 5% CO2, and permeabilized with pure methanol (130 µL) for 30 minutes in the dark on ice. Cells were then washed and stained with antibodies against human phosphorylated (p)STAT1 (PE-CY7, clone KIKSI0803; eBioscience), pSTAT3 (APC, clone LUVNKLA; eBioscience), pSTAT5 (PE, clone SRBCZX; eBioscience), anti-HLA-DR (PerCp-Cy5.5, Clone L243; BioLegend] for 30 min on ice. After incubation, cells were washed and measured using

an LSR-Fortessa flow cytometer (BD Bioscience). FlowJo software (Version 10, Becton Dickinson) was used to analyze the data. Reference monocyte samples treated with plasma from age and sex-matched tuberculosis patients (n=30) and controls (n=30) were included in these experiments.

### *Quantification of immune cell subsets in whole blood samples by flow cytometry*

Whole blood quantification of immune cell subsets was done by multi-color flow cytometry as described before [11]. In brief, whole blood was diluted (1:1) with RPMI1640 media. Samples were then centrifuged and stained with fluorescently labelled antibodies against human CD3 (AF700, clone SK7; BioLegend), CD45RA (APC, clone HI100; BioLegend), CD45RO (BV605, clone UCHL1; BioLegend), HLA-DR (PE-Dazzle594, clone L243; BioLegend), CD16 (APC, clone 3G8; BioLegend) and viability dye (eFluor780; eBiosciences) on ice in the dark for 30 minutes. After staining, red blood cells were lysed, washed, and fixed as described previously. The cells were measured using a CytoFlex S cytometer (Beckman Coulter), and the data were analyzed using FlowJo software. A representative example of the gating strategy is shown in Supplementary Figure 4. Tuberculosis patients (n=89) and controls (n=50) with comparable age and sex distributions were included in these experiments.

### *Monocyte phenotype characterization of tuberculosis patients and controls by multi-color flow cytometry*

Cryopreserved PBMC samples were thawed, washed, and stained with fluorescence-labelled antibodies, as described before [4]. For characterization of monocyte phenotype and subpopulations, the following panel of antibodies against human monocyte markers was used: CCR-1 (AF488, clone 5F10B29; BioLegend), HLA-DR (PerCp-Cy5.5, Clone L243; BioLegend), CCR-5 (PE, clone J418F1; BioLegend), CCR-2 (BV421, clone K036C2; BioLegend), CD14 (BV605, clone M5E2; BioLegend), CD16 (APC, clone 3G8; BioLegend), anti-CD68/SR-D1 [Alexafluor700, clone: 298807; R and D systems], anti-CD80 [PE-CF594, clone: 2D10; BioLegend], anti-CD11b [PE-Cy7, clone: ICRF44; BioLegend], anti-CD163 [BV510, clone: GHI/61; BioLegend] anti-CD86 [BV 650, clone: IT2.2; BioLegend] and viability dye (eFluor780; eBiosciences). After incubation, cells were washed and measured using LSR-Fortessa flow cytometer

(BD Bioscience). Samples from age and sex-comparable tuberculosis patients (n=52) and controls (n=45) were included in these experiments.

For SOCS3 quantification, the cells were stained (30min, on ice and protected from light) with the following panel of antibodies: HLA-DR (PerCp-Cy5.5, Clone L243; BioLegend), CD14 (BV605, clone M5E2; BioLegend), CD16 (AF488, clone 3G8; BioLegend). The samples were then washed in PBS supplemented with 10% Fetal bovine serum (Sigma Aldrich). After fixation and permeabilization (with Fixation Buffer and Intracellular Staining permeabilization wash buffer (1X), BioLegend) according to manufacturers' instructions, the cells were stained with an antibody against human SOCS3 (C204, Immuno-Biological Laboratories, Inc.) labelled with Dy650 (DyLight™ 650 microscale antibody labeling kit; ThermoFisher). After washing in permeabilization buffer, the samples were resuspended in PBS and measured using LSR-Fortessa flow cytometer (BD Bioscience). FlowJo software (Version 10, Becton Dickinson) was used to analyze the data. A representative gating strategy is depicted in Supplementary Figure 5. Samples from age and sex-comparable tuberculosis patients (n=42) and controls (n=42) were included in these experiments.

***Quantification of cytokines/chemokines in plasma and reference monocyte supernatants using flow cytometry-based bead assays***

Frozen culture supernatants from plasma-treated reference monocytes or plasma samples of tuberculosis patients and controls were analyzed using a customized LEGENDplex™ Multi-Analyte Flow Assay kit (13-plex Panel). The following cytokines/chemokine candidates were measured (i.e., IL-6, IL-10, IL-1$\alpha$, IL-1$\beta$, CXCL-1, CXCL-2, CXCL-8, CXCL-13, G-CSF CCL-7, CCL-20, CCL-22, CCL-24). CCL-2 measurement was performed separately using a LEGENDplex™ Human Proinflammatory Chemokine mix-and-match Panel. The samples were prepared and processed according to the manufacturer's instructions. Briefly, 12.5 µL of samples were diluted two-fold in assay buffer and incubated with premixed antibody-labeled beads for 2 h at room temperature. Streptavidin-PE was added to samples and incubated for an additional 30 min. Samples were washed and analyzed with a BD LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed using LEGENDplex Data Analysis Software (Qognit. Inc). Concentrations were calculated using the respective cytokine standard. Values below the standard curve were set to the detection limit of each analyte.

### *Data visualization of CCR-2 expressing monocytes and candidate marker analysis*

For the characterization of CCR-2 positive monocytes from tuberculosis patients and controls, multiplex data analyses were performed as described in a previous study [4]. Briefly, study groups and each participant were initially assigned IDs. Viable HLA-DR positive cells were then gated and down-sampled to a maximum of 2,000 cells per replicate measure using the Downsample v3.3 plugin for FlowJo, excluding CD16 and CD14 double-negative cells. All cells were included in samples with less than 2000 cells. All samples were then concatenated into one sample to compare study groups (Supplementary Figure 5). TriMap, a method for large-scale dimensionality reduction and visualization of cell phenotype patterns, was applied. Default settings of the TriMap FlowJo plugin were used for all parameters. Analyses of CCR-2 positive subsets based on density plots were performed by back-gating clustered cells based on assigned IDs for individuals and study groups.

### *Graphical depiction and statistics*

GraphPad Prism v10 software (GraphPad Software, La Jolla CA, USA) and R (version 4.3.2) were utilized for statistical analyses and graphical depictions. Nonparametric tests were employed due to the non-normal distribution of data, as determined by the Kolmogorov-Smirnov and Shapiro-Wilk tests. The Mann-Whitney U test was used to compare study groups. Spearman rank correlation was employed to assess the association between the parameters measured and Benjamini-Hochberg multiple correction was performed. Principal components analysis (PCA) was performed using the plotPCA function in the DESeq2 package in R. A p-value less than 0.05 was considered statistically significant.

## Results

### *Plasma from tuberculosis patients induced higher transcription rates and distinct gene expression patterns in reference monocytes*

To characterize monocyte response to plasma environment changes in human tuberculosis, we determined the RNA expression of reference monocytes after overnight culture with plasma samples from either a tuberculosis patient (n=9) or a healthy contact (n=9). For the sake of simplicity, we refer to the samples as TB or control plasma-treated monocytes throughout. RNA quantification revealed increased concentrations in monocytes treated with TB plasma (as compared to controls). This suggested generally higher transcription activity of monocytes induced by TB plasma (Figure 1a). Comparison of gene expression patterns revealed marked differences between the study groups. 1932 genes showed significant differences between TB and control plasma-treated monocytes after adjustment for multiple testing (≥2-fold change, $p<0.05$, Bonferroni; Figure 1b). Most genes (n=1343) were downregulated in monocytes treated with TB plasma, possibly indicating the suppressive effects of TB plasma. However, this result may also be partly due to bias from equal RNA concentrations used for sequencing because higher total RNA concentrations were detected in patient plasma-treated monocytes (Figure 1a). Signatures of regulated genes showed marked differences between the study groups and unsupervised clustering revealed high similarity among TB plasma-treated monocytes (Supplementary Figure 1a). Interestingly, two control plasma-treated samples induced monocyte gene expression patterns more like TB plasma (Supplementary Figure 1a). Principal component analyses confirmed high concurrence of TB plasma-treated samples and the similarity of two contact samples to tuberculosis patients (Figure 1c). These results suggested distinct and homogenous signatures induced by plasma from tuberculosis patients.

### *Enrichment of gene signatures associated with inflammation and chemotaxis in TB plasma-treated monocytes*

We focused on transcripts with higher expression after TB plasma culture to identify pathways induced in tuberculosis disease pathology. Five hundred eighty-nine (589) genes were significantly upregulated and we used two platforms (i.e., UniProtKB, and GeneOntology) to identify functionally related gene groups. Both platforms identified

several gene groups to be enriched in TB plasma-treated monocytes (Figure 1d). Notably, genes assigned to chemotaxis and inflammation were detected by both platforms among those pathways with the highest significance in TB plasma-treated monocytes (Figure 1d). Several chemokines were identified that contributed to the assignment of chemotaxis and a subset of chemokines involved in the chemotaxis of granulocytes and/or monocytes under inflammatory conditions was part of both biological processes/pathways (Figure 1e). Moreover, key cytokines and Toll-like receptor pathways contributed to the enrichment of inflammation-related factors in TB plasma-treated monocytes (Figure 1e). Among the cytokines were members of the IL-1, IL-6, and IL-10 families (Figure 1e). Interestingly, IL-10 was among the most significantly induced genes in TB plasma-treated monocytes (Figure 1b, e), and other cytokines of the IL-10/IL-20 family as well as their receptors also showed higher levels in TB plasma-treated monocytes (Supplementary Figure 1b).

***TB plasma-induced cytokines amplify an inflammatory response via STAT pathways in monocytes***

Since RNA sequencing indicated strong effects of TB plasma on cytokines secreted by monocytes, we next analyzed the supernatants of treated monocytes to measure the cytokine candidates (i.e., IL-1$\alpha$, IL-1$\beta$, IL-6, IL-10). Supernatants of TB plasma-treated (n=30) or control plasma-treated (n=30) monocytes were analyzed using a custom-designed cytometric beads assay. All four cytokines had significantly higher concentrations in the supernatants of TB plasma-treated monocytes (Figure 2a). Higher cytokine concentrations in supernatant may affect reference monocytes in an autologous manner. This was even more likely as the receptors specific for IL-1, IL-6, and IL-10 also showed increased RNA expression in monocytes treated with TB plasma (Figure 1e; Supplementary Figure 1b). IL-6 and IL-10 induce Jak/STAT signaling pathways that have been shown to contribute to the effects of plasma milieu before (4). Therefore, we measured STAT1, STAT3, and STAT5 phosphorylation in treated reference monocytes after overnight culture. TB plasma induced significantly higher levels of phosphorylated (p)STAT1, pSTAT3, and pSTAT5, and the strongest induction was seen for pSTAT3 and pSTAT5 (Figure 2b). STAT3 and STAT5 phosphorylation was concomitantly detected in a subset of monocytes (Supplementary Figure 2a) and proportions of pSTAT3/pSTAT5 double-positive monocytes were also significantly higher in TB plasma-treated monocytes (Figure 2c).

To address the question of potential autologous cytokine effects on signaling pathways, we next determined associations between cytokine concentrations and STAT phosphorylation in both study groups. TB plasma-treated samples showed a strong positive correlation for almost all comparisons (Figure 2d). However, only moderate correlations were detected between cytokines and pSTATs in control plasma-treated monocytes (Figure 2d). In particular, IL-6 concentrations were strongly associated with pSTAT1 and pSTAT3 expression in the presence of TB plasma but not control plasma (Figure 2d). These results indicated autologous effects of secreted cytokines on STAT phosphorylation in TB plasma-treated reference monocytes.

***Monocyte chemokine receptors and chemokines for the recruitment of granulocytes are induced by TB plasma***

Chemotaxis pathways were strongly involved in monocyte transcriptome changes and aberrant chemokine receptor expression has been described to characterize monocyte pathology in tuberculosis patients [2]. Therefore, we next compared the influence of TB plasma on the expression of monocyte chemokine receptors and secreted chemokines in supernatants of reference monocytes. TB plasma induced higher expression of CCR-1 and CCR-5 on reference monocytes as compared to control plasma (both $p<0.0001$) whereas CCR-2 was not different (Figure 3a). Several chemokines associated with the recruitment of immune cells under inflammatory conditions were found to be higher in the monocyte transcriptome of TB plasma-treated monocytes (Figure 1b, e). CBA-based analyses of selected chemokine candidates by flow cytometry confirmed differences in the supernatants between the study groups (Supplementary Figure 3a). In particular, three chemokines involved in the recruitment of granulocytes, namely CXCL-1, CXCL-2, and CXCL-8, showed markedly higher concentrations (in some cases above the maximum detection limit) in the supernatants of monocytes treated with TB plasma (Figure 3b). Moreover, two key factors of bone marrow granulocyte and monocyte mobilization under inflammatory conditions, i.e., G-CSF and CCL-2, showed higher concentrations in supernatants from monocytes treated with TB plasma as compared to controls (Figure 3c).

***Correlation between inflammatory signatures and chemokine receptor expression/chemokine secretion in TB plasma-treated monocytes***

Next, we analyzed possible interrelations between differentially expressed chemokines and chemokine receptors for monocytes treated with plasma samples from both study groups. CXCL-8 showed a positive correlation with all other regulated chemokines and receptors (Figure 3e). CXCL-1 and CXCL-2 correlated strongly with each other independent of TB or control plasma sample treatment (Figure 3d). In contrast, correlations of CXCL-1 and CXCL-2 with CCR-1 expression were only detected in monocytes treated with TB plasma (Figure 3d). Moreover, CXCL-1 correlated with the mobilizing factors, G-CSF and CCL-2, only in the presence of TB plasma (Figure 3d). Interestingly, the inclusion of IL-6 – which was associated with STAT signaling only in TB plasma-treated monocytes (Figure 2d) – also showed strong correlations with CXCL-1 and CXCL-2 only in the presence of TB plasma (Figure 3e). These results rendered an amplifying role of IL-6 secreted by monocytes in the presence of TB plasma and induced expression of inflammatory chemokines likely.

***Inflammation and granulocyte chemotaxis signatures in plasma samples from tuberculosis patients***

The plasma milieu assay determined early mechanisms in monocytes induced by TB plasma. To determine whether the identified pathways and/or potential implications on immune cell subsets are detectable in the blood of tuberculosis patients, we next analyzed the candidate cytokines/chemokines in plasma samples of tuberculosis patients (n=30) and controls (n=30). Inflammatory signatures were detected in plasma and the key cytokines, i.e., IL-6, IL-10, IL-1$\alpha$, and IL-1$\beta$, showed higher concentrations in plasma samples from tuberculosis patients (Figure 4a). Chemokines expressed under inflammatory conditions, which were all markedly different in the plasma milieu assay, showed a more heterogeneous picture in plasma samples. Of the chemokines that recruit granulocytes, only CXCL-8 showed higher concentrations in the plasma of tuberculosis patients compared to healthy controls, while CXCL-1 and CXCL-2 were similar between the study groups (Figure 4b). In addition, there were significant differences, particularly in the chemokines involved in the mobilization of granulocyte/monocyte populations from the bone marrow. CCL-2, the crucial chemokine for the mobilization of monocytes under inflammatory conditions, had

comparable concentrations between the study groups (Figure 4c). In contrast, higher G-CSF concentrations were seen in the plasma of tuberculosis patients as compared to healthy contacts (Figure 4c). These results suggested chronic inflammation as well as ongoing mobilization of granulocytes from the bone marrow in tuberculosis patients.

***Higher proportions of neutrophil granulocytes in the blood of tuberculosis patients***

To address this question of the ongoing mobilization of granulocytes affecting the blood picture of tuberculosis patients, we next performed whole blood phenotyping using flow cytometry to quantify the different immune cell populations in corresponding study groups of tuberculosis patients (n=89) and controls (n=50). Gating procedures are provided in Supplementary Figure 4. Immune cell subsets differed markedly between the study groups (Figure 5a). Granulocyte proportions were significantly higher in the blood of tuberculosis patients, and this was accompanied by lower proportions of lymphocytes (CD3 positive and CD3 negative; Figure 5b). Monocyte proportions were comparable between the study groups (Figure 5a). Granulocyte subset analyses revealed that mainly CD16 positive (i.e., neutrophil granulocytes) were enriched in tuberculosis patients whereas CD16 negative granulocyte subpopulations showed similar proportions between the study groups (Figure 5c). Correlation analyses were then performed between the altered plasma cytokine/chemokine concentrations and the proportion of granulocytes. CXCL-8 and G-CSF concentrations did not show significant correlations with the proportions of granulocytes in the subgroups (Supplementary Figure 4c). In contrast, IL-6 concentrations correlated with the proportion of granulocytes only in the cohort of tuberculosis patients (Figure 5d). This suggested that chronic inflammation - characterized by persistently high IL-6 plasma levels - contributes to the ongoing mobilization and accumulation of neutrophil granulocytes in tuberculosis patients.

***An inflammatory phenotype and enriched proportions of CCR-2/CCR-5 positive cells characterize monocytes from tuberculosis patients***

Monocyte proportions in the blood were comparable between the study groups although higher CCL-2 concentrations were found in TB plasma-treated reference monocyte supernatants. To analyze if induced CCL-2 was of biological relevance, we next characterized the phenotype of monocytes with a special focus on chemokine

receptors in tuberculosis patients (n=52) and healthy contacts (n=45). As in the plasma assay, CCR-1 and CCR-5 expression levels were significantly higher on monocytes from tuberculosis patients as compared to controls (Figure 6a). Notably, and in contrast to plasma-treated reference monocytes, we also detected increased expression of CCR-2 on monocytes from tuberculosis patients (Figure 6a). Since CCR-2 is the exclusive ligand of CCL-2, we considered recruitment of CCR-2 positive monocytes at early stages of *M. tuberculosis* infection (before clinical onset) a possible explanation. Hence, we next classified monocytes based on CCR-2 expression and found higher proportions of CCR-2 positive cells within monocyte populations from tuberculosis patients as compared to controls (Figure 6b). These results suggested an accumulation of CCR-2-positive monocytes recruited before the onset of symptomatic tuberculosis.

To further characterize this monocyte subset, we analyzed the expression of selected monocyte markers described to play a role in tuberculosis and performed data complexity reduction and visualization of study group differences by TriMap (for details see methods section). The gating procedure of CCR-2 positive monocytes is shown in Supplementary Figure 5. Interestingly, two populations (termed CCR-$2_{Pop1}$ and CCR-$2_{Pop2}$) were identified and these contributed to approx. 90% of all CCR-2 positive monocytes (Figure 6c). Concatenated data sets suggested higher proportions of CCR-$2_{Pop2}$ within monocytes from tuberculosis patients whereas the CCR-$2_{Pop1}$ was seemingly more frequent in controls (Figure 6c). Comparison of study groups showed that only subgroups of tuberculosis patients and controls had high proportions of CCR-$2_{Pop2}$ and these were not significantly different (Figure 6d). However, phenotype comparison of CCR-$2_{Pop2}$ monocytes between the study groups showed markedly higher expression of several markers with relevance in tuberculosis immunopathology on CCR-$2_{Pop2}$ monocytes from tuberculosis patients (Figure 6e). CCR-$2_{Pop1}$ monocytes showed comparable phenotypes between the study groups (Figure 6e). These results suggested that mobilization of CCR-2 positive inflammatory monocytes by CCL-2 is a transient feature that happens at early stages of *M. tuberculosis* infection.

### *Higher SOCS3 expression in monocytes from tuberculosis patients*

Our results indicate a continuous mobilization and accumulation of neutrophil granulocytes in acute tuberculosis patients, whereas the mobilization of monocytes in tuberculosis patients is not continuous. Since the enrichment of neutrophils is associated with high IL-6 plasma levels, we assumed that the sensitivity of monocytes from tuberculosis patients to IL-6 might be impaired. A similar mechanism has been described for T cells in tuberculosis patients and aberrant high SOCS3 expression has been identified as the potential inhibitor [12]. Hence, we analyzed SOCS3 protein levels in monocytes from tuberculosis patients (n=42) and healthy controls (n=42). We detected higher SOCS3 expression in monocytes from patients as compared to healthy contacts (Figure 6f). These results suggested that high SOCS3 expression and likely effects on IL-6-dependent STAT3 signaling may impair the recruitment of CCR-2 positive monocytes in tuberculosis patients.

## Discussion

In the present study, global changes in gene transcription, phenotype, and cytokine secretion were analyzed in monocytes treated with TB (or control) plasma samples. The *in vitro* response of monocytes to the TB plasma milieu was then compared with the phenotype and composition of immune cell subsets in corresponding samples from tuberculosis patients and healthy contacts. Inflammatory cytokines and chemokines were strongly induced by TB plasma and especially markers of chronic inflammation, like IL-6, showed aberrant high expression in the plasma of tuberculosis patients. Ongoing granulocyte mobilization and enrichment in the blood of tuberculosis patients was seen and this was associated with high IL-6 plasma levels.

Plasma pathology in a subset of patients with severe tuberculosis has been described already in the last century [9]. The group of J.J. Ellner demonstrated that *in vitro* culture of immune cells from healthy individuals with plasma from tuberculosis patients conferred impaired functional responses, including proliferation and T-cell cytokine expression [13]. Interestingly, monocytes were identified as central effector cells, and suppressive features were found in monocytes from tuberculosis patients [9, 14-17]. The mechanisms underlying TB plasma effects on monocytes, however, remain elusive. Mycobacterial components with immunomodulatory functions were identified [8] but the detection and functional assignment of such a component in plasma remains to be done. If mycobacterial components are responsible for the effect, these

must also be effective at very low concentrations, as only a small amount of plasma is required to modulate the reference monocytes. In the present study, a 1 in 10 dilution of TB plasma in a medium was sufficient to confer monocyte modulation. This argued for a highly active molecule that can induce an amplifying reaction in reference monocytes. The fact that diluted plasma was effective also excluded a direct effect of increased TB plasma cytokines (e.g., IL-1$\alpha$, IL-1$\beta$, IL-6, IL-10) since the dilution will result in a final concentration at or below physiological cytokine plasma levels. Hence, these host cytokines are unlikely triggers of plasma pathology but our results suggested that induced secretion of inflammatory cytokines in reference monocytes potentially boosted the effect in an autologous way. In accordance, STAT signaling pathways were shown to be strongly associated with monocyte phenotype changes and secreted cytokines. IL-6 and IL-10 are well-known inducers of STAT3 signaling and both were associated with STAT3/5 signaling in reference monocytes. These results were in accordance with previous studies that showed high constitutive STAT3 phosphorylation in T cells and monocytes from tuberculosis patients [12, 18]. High expression levels of the STAT3 regulator SOCS3 in monocytes from tuberculosis patients were detected and this strengthened the assumption of a role of aberrant STAT pathway signaling in tuberculosis pathology. The central role of STAT3 and SOCS3-mediated regulation for immune protection against *M. tuberculosis* infection is well established [19]. Own previous studies suggested a role in T-cell polarization [20] and impaired T-cell response of tuberculosis patients [12]. Notably, both STAT3 and SOCS3 were part of hyperinflammation-associated gene signatures in resected pulmonary tissue from tuberculosis patients [21]. This finding strengthened the assumption that the TB plasma-induced signatures reflected immunopathology in pulmonary tissue from tuberculosis patients.

Chemotaxis pathways – and especially chemokines secreted under inflammatory conditions – were strongly induced in reference monocytes by TB plasma. Among these were candidates involved in the mobilization and recruitment of granulocytes. Granulocyte chemoattractant proteins (i.e., CXCL-1, CXCL-2, CXCL-8) and G-CSF, a key factor of granulocyte mobilization in the bone marrow, were strongly induced and secreted by monocytes treated with TB plasma. Higher CXCL-8 and G-CSF concentrations were also detected in the plasma from tuberculosis patients as expected [22, 23]. The functional relevance of these chemokine differences was

confirmed by the finding of higher granulocyte proportions in the blood of tuberculosis patients. Granulocyte enrichment in the blood of tuberculosis patients was shown before [24]. The role of granulocytes in tuberculosis has long been controversial and these innate immune cells have been described as contributing to both immune protection and disease progression [25]. Controversial results may at least partly reflect the differential roles of granulocyte subsets since the contribution of eosinophils to host resistance and neutrophils to disease pathology were described [26]. The role of neutrophilia in tuberculosis disease pathology, however, has been clearly shown [27]. Neutrophilia is associated with hyperinflammation and severe lung damage in tuberculosis patients [28]. Moreover, mortality rates are increased in tuberculosis patients with neutrophilia [29]. Here we provide evidence that especially neutrophil ($CD16^+$) granulocyte proportions were higher in tuberculosis patients. Interestingly, we found that IL-6 plasma concentrations showed a strong correlation with granulocyte proportions in tuberculosis patients. Own previous studies showed that high IL-6 levels in the plasma and IL-6 secretion by *M. tuberculosis*-specific T cells are features of acute tuberculosis [12, 30, 31]. Notably, IL-6 plasma levels were associated with impaired T-cell functions in tuberculosis patients [32] and this suggested the interrelation between hyperinflammation and immune suppression in tuberculosis [33]. Recently, a meta-analysis of transcriptome studies strengthened this assumption and provided evidence for distinct tuberculosis endotypes in humans [34].

Phenotype changes of blood monocytes in human tuberculosis are well described and our previous studies demonstrated the important role of the aberrant plasma environment for monocyte pathology [4, 10]. Higher expression of CCR-1 and CCR-5 on monocytes has been demonstrated [2] and this has been confirmed for TB plasma-treated reference monocytes and monocytes from tuberculosis patients in the present study. Notably, higher CCR-2 expression was not induced by TB plasma but a subset with high CCR-2 expression was enriched within the monocyte population in the blood of tuberculosis patients. CCL-2 is the key chemokine for mobilization of monocytes from the bone marrow under inflammatory conditions and CCR-2 is the exclusive receptor for CCL-2 (reviewed in [35]). Since CCL-2 was strongly induced in reference monocytes treated with TB plasma but showed no differences between plasma samples from the study groups, we assumed that mobilization of monocytes from the bone marrow by CCL-2 was induced previously but was not ongoing in tuberculosis patients. This could explain the fact that although a general enrichment of granulocytes

was observed in tuberculosis patients, the general monocyte percentage did not differ between the study groups. A potential mechanism underlying changes in the mobilization could involve IL-6 and its feedback inhibitor SOCS3. Since granulocyte recruitment was associated with IL-6 plasma levels, higher SOCS3 levels detected in monocytes may cause impaired IL-6 response, as shown for T cells from tuberculosis patients [12]. Future studies will have to address the question of whether the mobilization of CCR-2 positive monocytes is occurring early during *M. tuberculosis* infection causing changes in monocyte subpopulations detectable in tuberculosis patients at clinical onset.

Taken together, the present study strengthened the hypothesis that changes in the plasma milieu are drivers of a hyperinflammatory immune response in tuberculosis. Monocytes contribute to the harmful recruitment of neutrophils and secret key factors of inflammation, which, like IL-6, are associated with impaired T-cell response and disease severity. This study strongly argues for host-directed treatment to modulate the harmful effects of chronic inflammation with the potential long-term effects of tuberculosis.

## Author Contributions

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## Acknowledgments

We would like to thank all study participants, study nurses, and physicians for supporting this investigation. Computational infrastructure and support were provided by the Centre for Information and Media Technology at Heinrich Heine University Düsseldorf.

## Funding

This study was supported by the German Research Foundation (DFG, JA 1479/9-1) and the graduate school molecules of infection (MOI)-4 funded by the Jürgen Manchot Foundation. The funders had no role in study design, data collection, data analysis, decision to publish, or preparation of the manuscript.

## Conflict of interest

The authors declare that no conflict of interest exists.

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**Legends to Figures**

***Figure 1: Comparison of TB and control plasma cultured reference monocytes by RNA sequencing and identification of pathways enriched in the presence of plasma from tuberculosis patients.*** Concomitant analysis of reference monocytes cultured with samples from tuberculosis patients (n=9) and controls (n=9) has been performed for RNA sequencing. **(a)** Quantification of RNA concentrations from reference monocytes after culture containing different plasma samples. **(a, c)** Differences in symbols and colors indicate monocytes cultured with samples from tuberculosis patients (red triangles) and controls (blue circles). **(b)** A Volcano plot depicts differences in sequenced mRNAs between the study groups. Fold changes and p-values adjusted for multiple testing are shown. Thresholds of two-fold changes and p-values of <0.05 have been applied (dashed lines) and differentially expressed mRNAs in TB sample cultured monocytes are marked (i.e., up-regulated in red, down-regulated in blue). The candidates selected by functional annotation are highlighted by enlarged dots and labeled with the gene name. **(c)** Results from Principal Component Analysis (PCA) are shown as a symbol plot. Principle components 1 and 2 and the respective contribution to the variance (in brackets) are depicted. Two controls, which showed high similarity to tuberculosis patients, are highlighted by a circle. **(d)** Functional annotation of candidate genes up regulated in TB plasma treated reference monocytes using Gene Ontology (GO) and UniProt Knowledgebase (UniProtKB). Only significantly enriched pathways are shown ($p<0.05$, adjusted for multiple testing, Benjamini-Hochberg). The exact p-values (x-axis), the numbers of contributing genes (circle size), and fold enrichment (shades of red) are depicted. **(e)** Differential expression (z-scores) is shown for candidate pathways of chemotaxis, and inflammation and both are shown for individual TB/control plasma samples as a heatmap. Color shading depicts individual z-scores for each gene being up-regulated (red) or down-regulated (blue).

***Figure 2: Inflammatory cytokine signatures in the supernatant and induced STAT phosphorylation in TB/control plasma cultured reference monocytes.*** Concomitant characterization of reference monocytes (n=2) each cultured with different samples from tuberculosis patients (n=30) and controls (n=30) has been performed. **(a)** Selected candidate cytokines released under inflammatory conditions were measured in the supernatant of TB/control plasma cultured reference monocytes

using a custom-designed cytometric bead assay and flow cytometry. **(b, c)** Phosphorylation of STAT family members measured in plasma cultured reference monocytes is depicted. Median fluorescence intensities (MFI) of phosphorylated (p)STAT1, STAT3, and STAT5 **(b)** and proportions of pSTAT3/pSTAT5 positive monocytes **(c)** are provided. **(a, b, c)** Symbol plots are depicted and differences in symbols and colors indicate monocytes cultured with samples from tuberculosis patients (red triangles) and controls (blue circles). Nominal p-values calculated with the Mann-Whitney U-test are given. P-values below 0.05 were considered significant. **(d)** Correlation matrices of candidates are shown for both study groups. Symbol sizes and color shading indicate correlation indices (R-values) calculated using the Spearman rank test. P-values were adjusted for multiple testing according to Benjamini-Hochberg and are indicated as asterisks with *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

***Figure 3: Chemokines and chemokine receptors expressed under inflammatory conditions in TB/control plasma cultured reference monocytes and supernatants.*** Concomitant characterization of reference monocytes (n=2) each cultured with different samples from tuberculosis patients (n=30) and controls (n=30) has been performed. **(a, b)** Chemokine receptors were measured in reference monocytes cultured with TB/control plasma samples by flow cytometry. MFI values for individual samples are given for both study groups. Chemokines involved in the recruitment **(b)** or mobilization **(c)** of granulocytes/monocytes under inflammatory conditions were measured in the supernatant of TB/control plasma cultured reference monocytes by flow cytometry using a custom-designed cytometric bead assay. **(a, b, c)** Symbol plots are depicted and differences in symbols and colors indicate monocytes cultured with samples from tuberculosis patients (red triangles) and controls (blue circles). Nominal p-values calculated with the Mann-Whitney U-test are given. P-values below 0.05 were considered significant. **(d)** Correlation matrices of chemokines/chemokine receptors and the key inflammatory cytokine IL-6 are shown for both study groups. Symbol sizes and color shading indicate correlation indices (R-values) calculated using the Spearman rank test. P-values adjusted for multiple testing according to Benjamini-Hochberg and are indicated as asterisks with *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

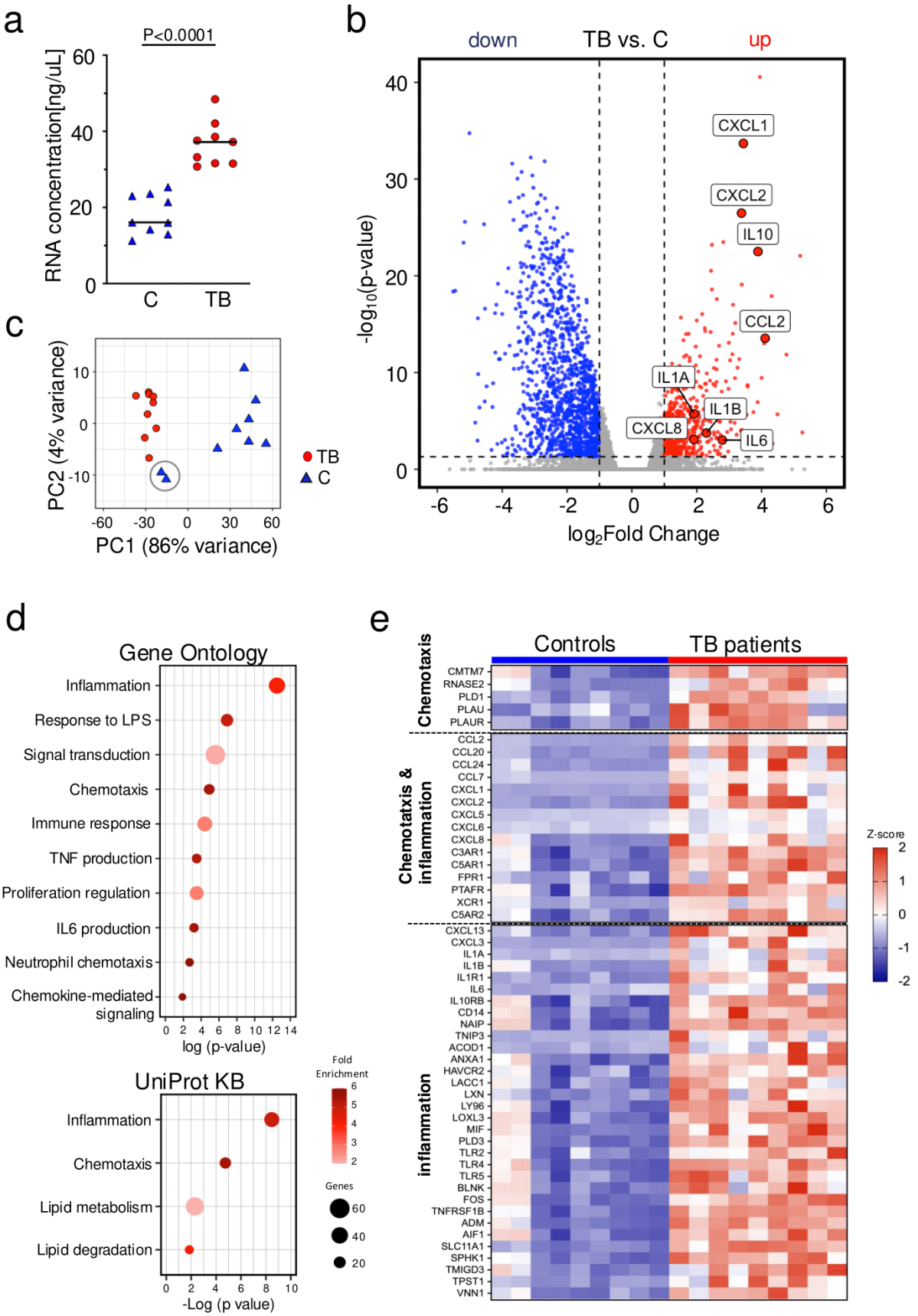
***Figure 4: Cytokines and Chemokines expressed under inflammatory conditions in plasma samples from tuberculosis patients and controls.*** Selected candidate cytokines and chemokines released under inflammatory conditions were measured in plasma samples by flow cytometry using a custom-designed cytometric bead assay. Symbol plots are depicted, and different symbols/colors indicate plasma samples from tuberculosis patients (red triangles; n=30) and controls (blue circles; n=30). Nominal p-values calculated with the Mann-Whitney U-test are given. P-values below 0.05 were considered significant.

***Figure 5: Differences in immune cell subsets in the peripheral blood from tuberculosis patients and controls.*** Proportions of different immune cell populations (i.e., granulocytes, monocytes, lymphocytes) as well as subpopulations were measured by flow cytometry and are depicted as pie charts **(a)**, violin plots **(b, c)**, or symbols **(d)**. **(b, c, d)** Different symbols/colors indicate samples from tuberculosis patients (red triangles; n=89) and controls (blue circles; n=50). Nominal p-values calculated with the Mann-Whitney U-test are given. **(e)** Correlations between granulocyte (left graph) and CD16 positive (neutrophil) granulocyte proportions with IL-6 plasma concentrations for both study groups (TB patients, n=28; controls, n=28) are depicted. The Spearman Rank Test has been used for correlation analyses and nominal p-values have been given. P-values below 0.05 were considered significant.

***Figure 6: Characterization of monocyte phenotype and subsets in the peripheral blood of tuberculosis patients.*** Monocyte phenotype analyses were done using flow cytometry in PBMCs from tuberculosis patients and controls. **(a, b)** Chemokine receptor expression patterns were measured and depicted as MFI **(a)** or proportions of CCR-2 positive cells within all monocytes **(b)**. **(c)** For further characterization of CCR-2 positive monocytes, additionally, measured monocyte markers (i.e., HLA-DR, CD11b, CD14, CD16, CD68, CD80, CD86) were included. After combined down-sampling of all samples, CCR-2 positive monocytes were visualized using TriMap-based complexity reduction for each study group separately. Density plots are depicted for both study groups and the main two subpopulations within CCR-2 positive cells were gated (i.e., CCR-$2_{Pop1}$, CCR-$2_{Pop2}$). **(d)** Relative frequencies of CCR-$2_{Pop2}$ within CCR-2 are shown for each participant and both study groups as a symbol plot. (e) Stacked histograms show MFI expression differences for all included markers on

both, CCR-$2_{Pop1}$ and CCR-$2_{Pop2}$, subsets. **(f)** Intracellular measurement of Suppressor Of Cytokine (SOCS)-3 expression in monocytes in both study groups is depicted. Samples from tuberculosis patients (n=42) and controls (n=42) have been included. Nominal p-values calculated with the Mann-Whitney U-test are given. P-values below 0.05 were considered significant.

# Figure 1


a
P<0.0001
RNA concentration[ng/uL]
C
TB
b
down
TB vs. C
up
-log10(p-value)
log2Fold Change
CXCL1
CXCL2
IL10
CCL2
IL1A
IL1B
CXCL8
IL6
c
PC2 (4% variance)
PC1 (86% variance)
TB
C
d
Gene Ontology
Inflammation
Response to LPS
Signal transduction
Chemotaxis
Immune response
TNF production
Proliferation regulation
IL6 production
Neutrophil chemotaxis
Chemokine-mediated signaling
log (p-value)
UniProt KB
Inflammation
Chemotaxis
Lipid metabolism
Lipid degradation
-Log (p value)
Fold Enrichment
Genes
e
Controls
TB patients
Chemotaxis
Chemotatxis & inflammation
inflammation
CMTM7
RNASE2
PLD1
PLAU
PLAUR
CCL2
CCL20
CCL24
CCL7
CXCL1
CXCL2
CXCL5
CXCL6
CXCL8
C3AR1
C5AR1
FPR1
PTAFR
XCR1
C5AR2
CXCL13
CXCL3
IL1A
IL1B
IL1R1
IL6
IL10RB
CD14
NAIP
TNIP3
ACOD1
ANXA1
HAVCR2
LACC1
LXN
LY96
LOXL3
MIF
PLD3
TLR2
TLR4
TLR5
BLNK
FOS
TNFRSF1B
ADM
AIF1
SLC11A1
SPHK1
TMIGD3
TPST1
VNN1
Z-score

# Figure 2

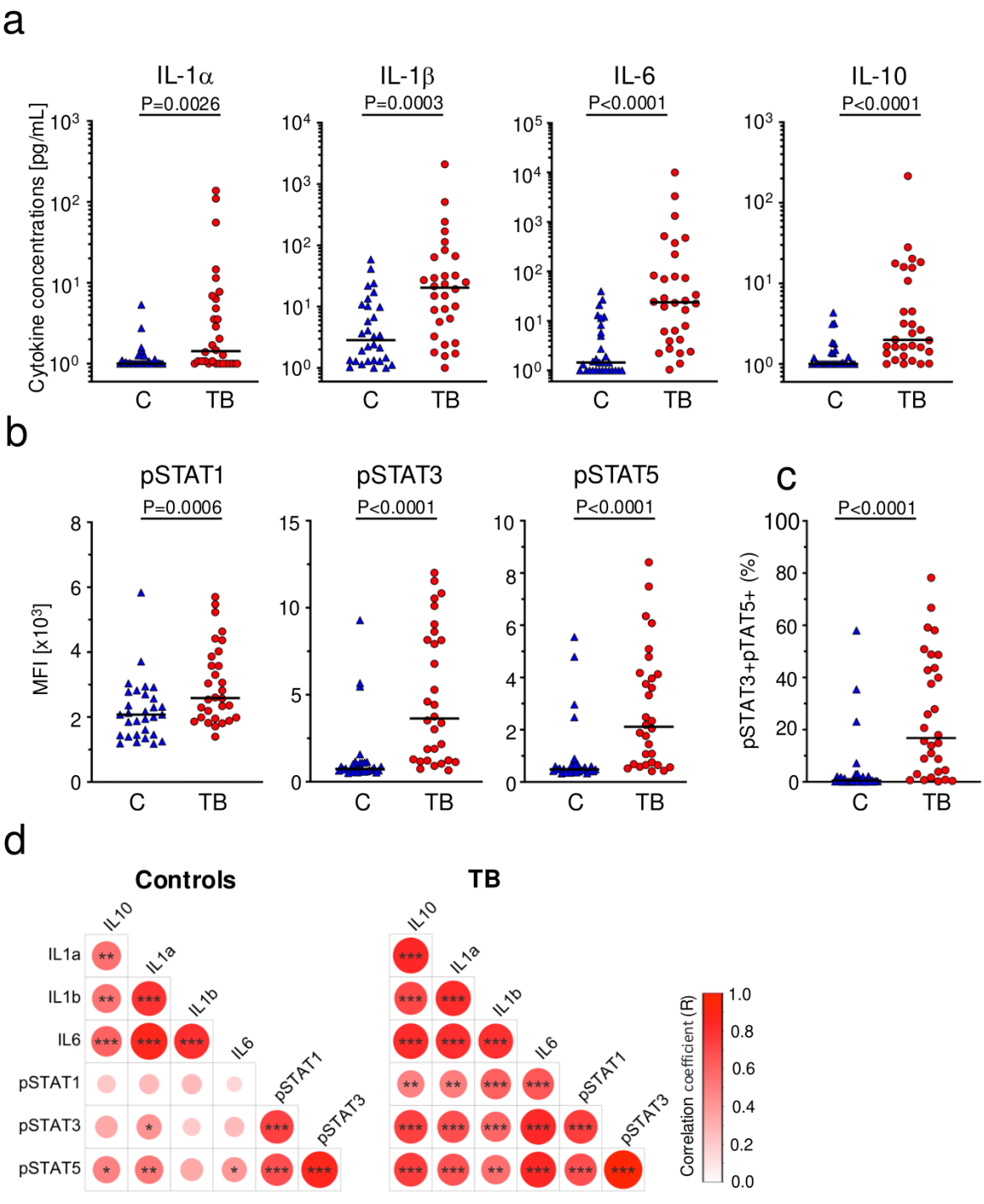
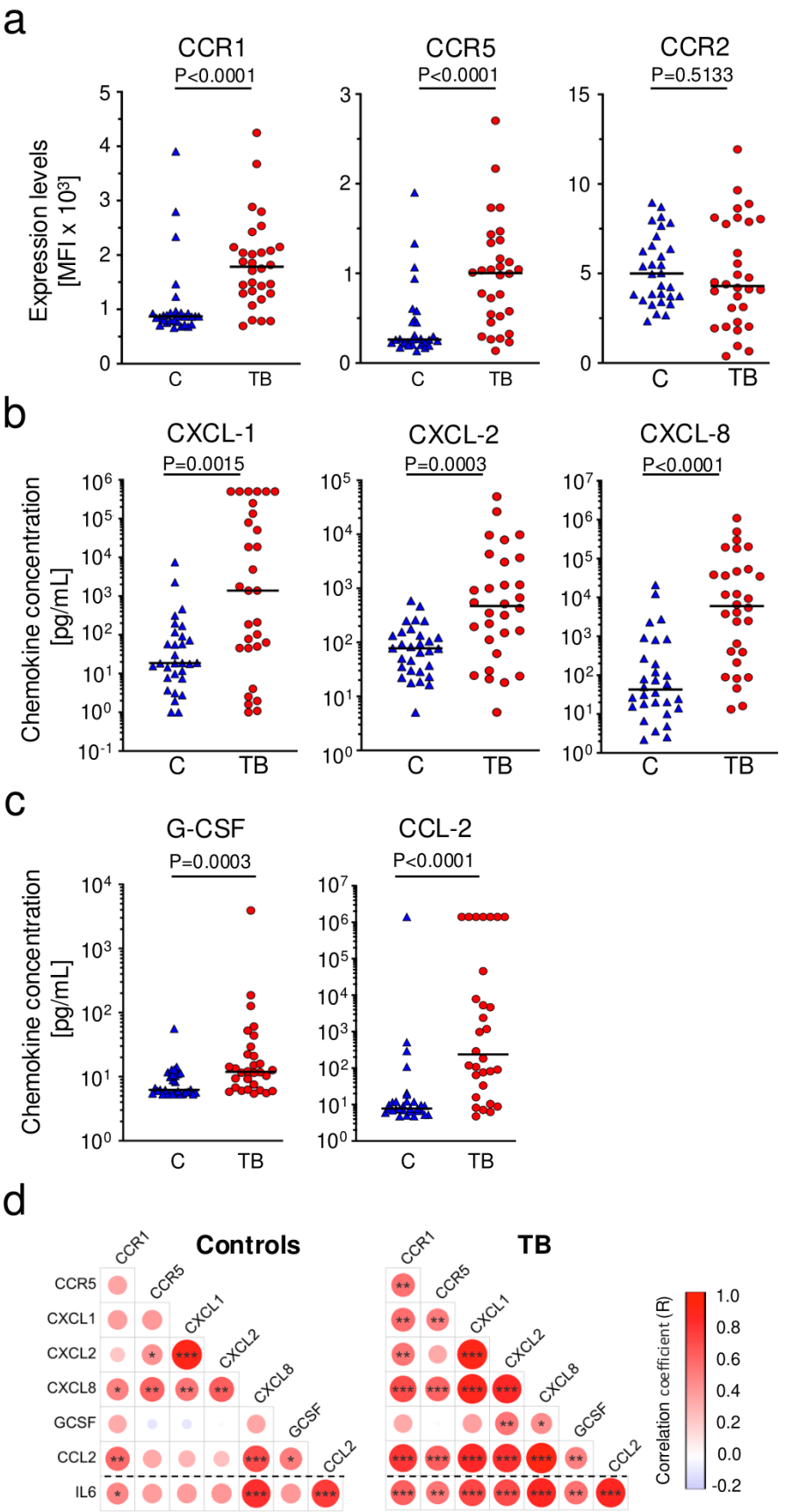

a
IL-1α
P=0.0026
IL-1β
P=0.0003
IL-6
P<0.0001
IL-10
P<0.0001
Cytokine concentrations [pg/mL]
C
TB
b
pSTAT1
P=0.0006
pSTAT3
P<0.0001
pSTAT5
P<0.0001
MFI [x10³]
c
P<0.0001
pSTAT3+pTAT5+ (%)
d
Controls
TB
IL10
IL1a
IL1b
IL6
pSTAT1
pSTAT3
pSTAT5
Correlation coefficient (R)
1.0
0.8
0.6
0.4
0.2
0.0

Figure 3


a
CCR1
P<0.0001
CCR5
P<0.0001
CCR2
P=0.5133
Expression levels [MFI x 10³]
C
TB
b
CXCL-1
P=0.0015
CXCL-2
P=0.0003
CXCL-8
P<0.0001
Chemokine concentration [pg/mL]
c
G-CSF
P=0.0003
CCL-2
P<0.0001
d
Controls
TB
CCR1
CCR5
CXCL1
CXCL2
CXCL8
GCSF
CCL2
IL6
Correlation coefficient (R)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2

# Figure 4

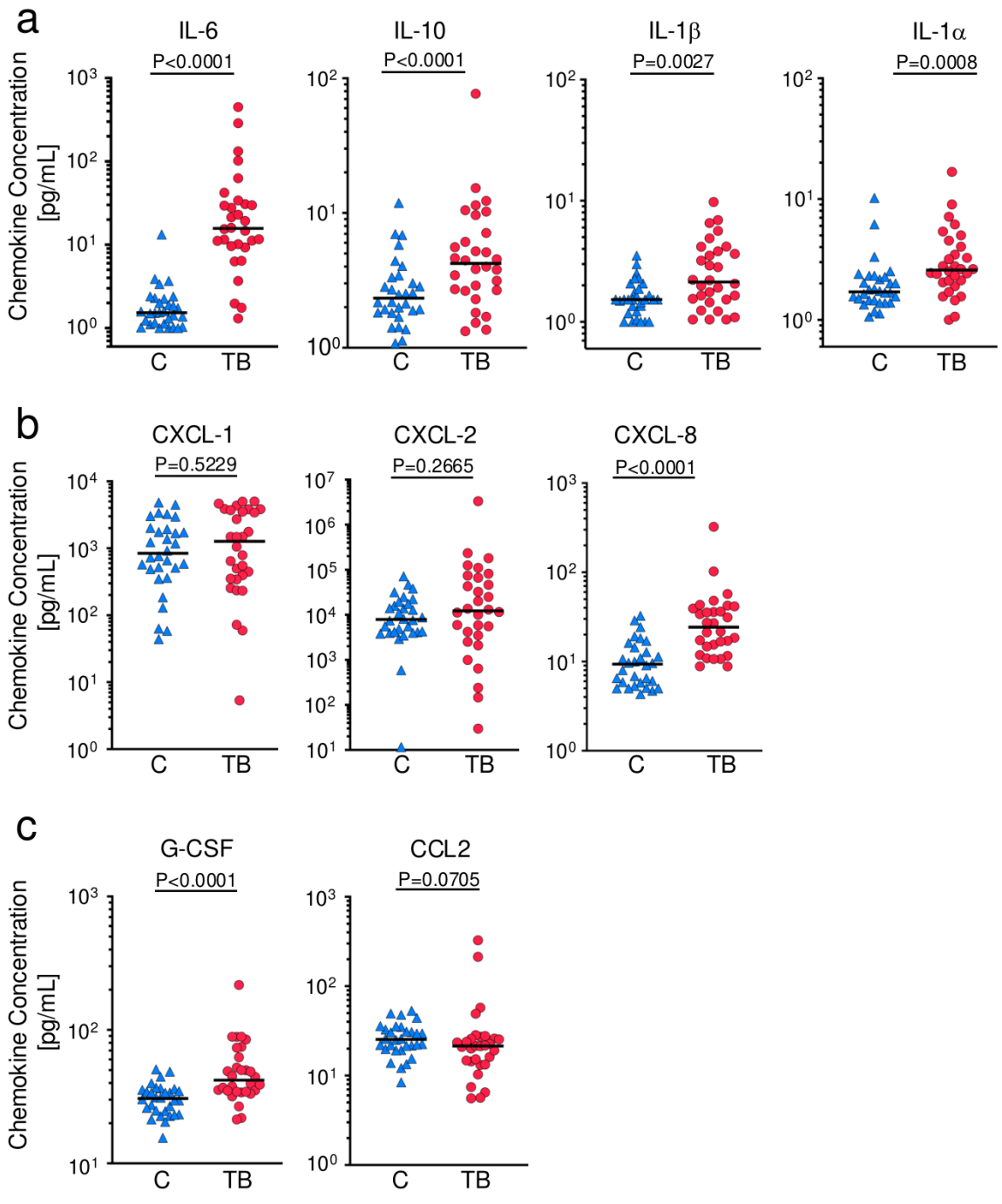
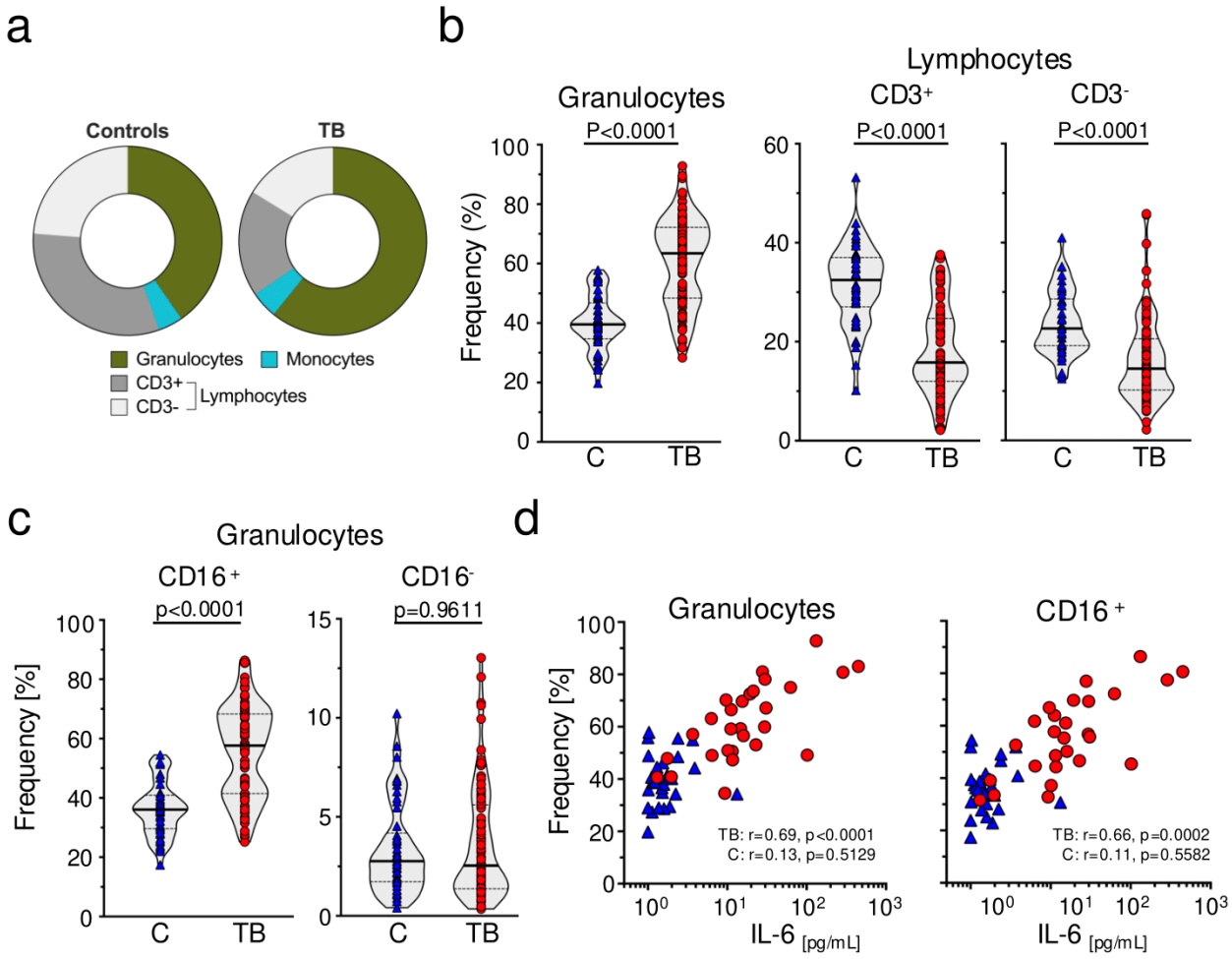

a
IL-6
P<0.0001
IL-10
P<0.0001
IL-1β
P=0.0027
IL-1α
P=0.0008
Chemokine Concentration [pg/mL]
C
TB
b
CXCL-1
P=0.5229
CXCL-2
P=0.2665
CXCL-8
P<0.0001
c
G-CSF
P<0.0001
CCL2
P=0.0705

Figure 5


a
Controls
TB
Granulocytes
Monocytes
CD3+
CD3-
Lymphocytes
b
Granulocytes
P<0.0001
Lymphocytes
CD3+
P<0.0001
CD3-
P<0.0001
Frequency (%)
C
TB
c
Granulocytes
CD16+
p<0.0001
CD16-
p=0.9611
Frequency [%]
d
Granulocytes
CD16+
Frequency [%]
TB: r=0.69, p<0.0001
C: r=0.13, p=0.5129
TB: r=0.66, p=0.0002
C: r=0.11, p=0.5582
IL-6 [pg/mL]

# Figure 6

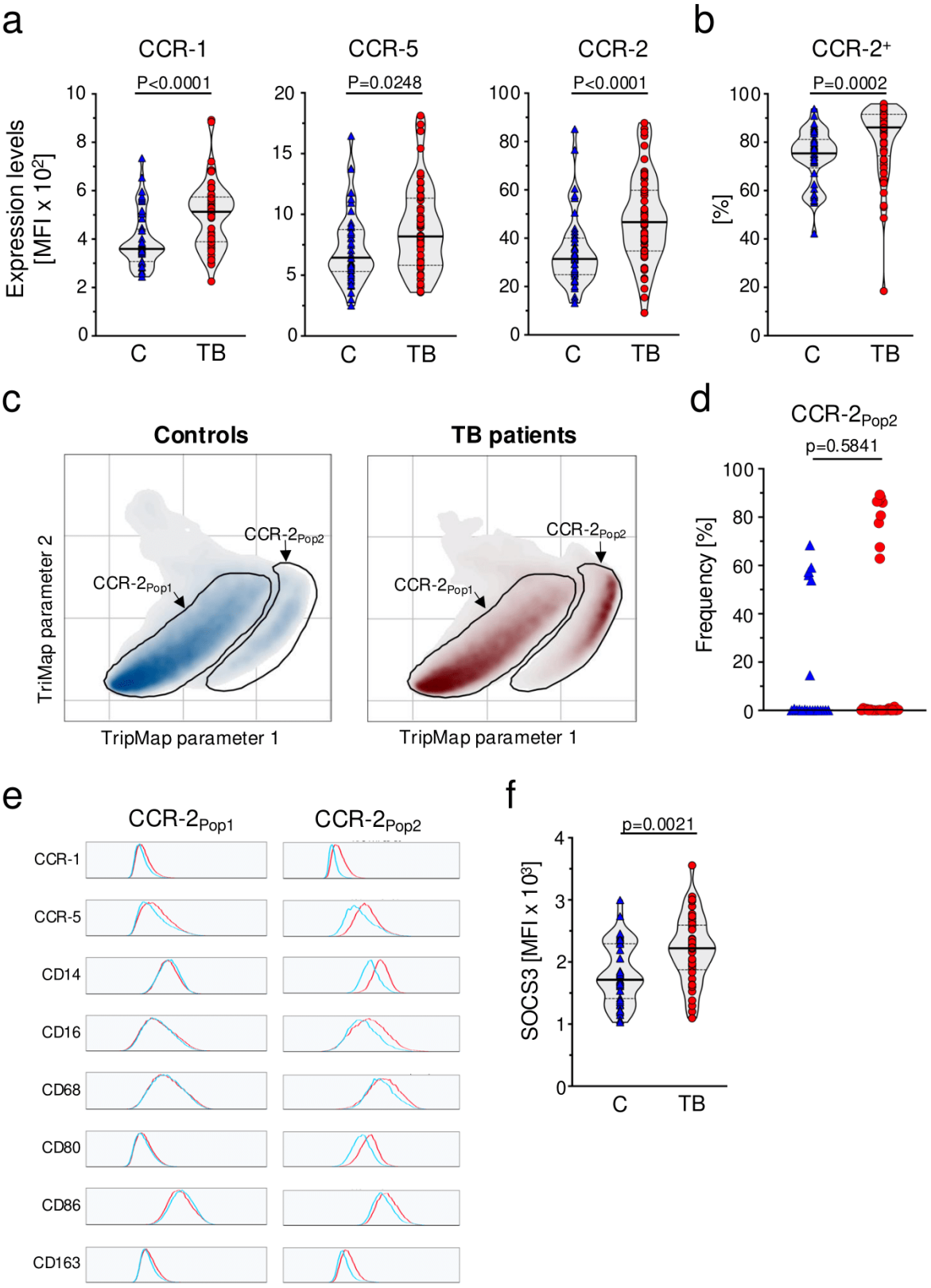

a
CCR-1
P<0.0001
CCR-5
P=0.0248
CCR-2
P<0.0001
Expression levels [MFI x 10²]
C
TB
b
CCR-2⁺
P=0.0002
[%]
c
Controls
TB patients
CCR-2Pop1
CCR-2Pop2
TriMap parameter 2
TripMap parameter 1
d
CCR-2Pop2
p=0.5841
Frequency [%]
e
CCR-2Pop1
CCR-2Pop2
CCR-1
CCR-5
CD14
CD16
CD68
CD80
CD86
CD163
f
p=0.0021
SOCS3 [MFI x 10³]

**Table 1: Characteristics of study groups**

| | Total recruit | | | Ex vivo monocyte Phenotyping | | | Ex-vivo whole blood phenotyping | | | Plasma assay (CCR,chemokine and cytokines) | | | Plasma Transcriptome Assay | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB | C | p | TB | C | p | TB | C | p | TB | C | p | TB | C | p |
| **Number (n)** | 99 | 62 | | 52 | 45 | | 89 | 50 | | 30 | 30 | | 9 | 9 | |
| **Age (years) *** Mean±sd | 45±15 | 44±13 | 0.64 | 48±14 | 44±14 | 0.12 | 45±15 | 46±13 | 0.56 | 44±14 | 45±13 | 0.96 | 47±18 | 47±17 | 0.88 |
| **Gender** Male/Female [#] | 67/32 | 30/32 | 0.02 | 35/17 | 25/20 | 0.21 | 60/29 | 26/24 | 0.10 | 18/12 | 18/12 | 1 | 3/6 | 3/6 | 1 |
| **Diagnosis** | | | | | | | | | | | | | | | |
| X-ray (suggestive/nd) | 24/75 | | | 7/45 | | | 24/65 | | | 5/25 | | | 0/9 | | |
| GeneXpert (pos/neg/nd) | 76/1/22 | na | | 43/9/0 | na | | 67/1/21 | na | | 24/6/0 | na | | 7/1/1 | na | |
| Sputum culture (pos/neg/nd) | 69/27/3 | na | | 37/13/2 | na | | 61/27/1 | na | | 19/11/0 | na | | 5/4/0 | na | |
| Sputum smear AFB (neg/scanty/1+/2+) [§] | 42/7/2/48 | | | 30/5/1/16 | | | 38/4/1/46 | | | 17/4/0/9 | | | 5/2/0/2 | | |

*The Mann-Whitney U-test was used for group comparisons; [#]the *Fisher's exact test was used for gender distribution comparisons;* [§]The degree of sputum smear AFB burden was assigned to one of the four categories (neg: no AFB, scanty: 1-9 AFB, 1+: 10-99 AFB, 2+: >100 AFB; no. per 100 fields of observation).

Abbreviations: TB, tuberculosis patient; C, contact; CCR, Chemokine (C-C motif) receptor; n, number; nd, not done; neg, negative; pos, positive; AFB, Acid Fast Bacilli; no, number; *na: not applicable.*

## 4. Discussion

This thesis investigated monocyte phenotypes, immune cell distribution in the peripheral blood of pulmonary TB patients, and the effect of TB plasma milieu on monocytes. This study found notable changes in monocyte phenotypes, including an increased frequency of highly motile inflammatory monocytes in TB patients. Using an *in vitro* plasma assay, it was demonstrated that TB plasma altered the phenotype of reference monocytes through STAT pathways, making them resemble the monocyte phenotypes in TB patients. Furthermore, TB plasma induces the secretion of inflammatory cytokines and the recruitment of granulocytes/neutrophils via monocytes, potentially exacerbating inflammatory processes and contributing to pathology in TB patients.

### 4.1 Phenotype and functional alterations of monocytes in tuberculosis

Alterations in monocyte phenotypes and the enrichment of inflammatory monocytes characterized by CD16 expression are pathognomonic features observed in TB patients (Sampath et al., 2018). An unbiased clustering algorithm revealed a high frequency of putative M1 monocytes (classical monocytes) in TB patients [Paper II]. Surprisingly, these M1 monocyte subsets exhibited elevated CD16 expression [Paper II]. This indicates a gradual shift from an M1 subpopulation to an M1/2 monocyte subset, which exhibits enhanced motility and pro-inflammatory capabilities (Idzkowska et al., 2015, Ozanska et al., 2020). This phenotype change was also characterized by high expression levels of CD40, CD64, and the immune modulating marker, program death ligand 1(PDL1). Interestingly, these aberrant receptor expressions normalize during treatment [Paper II]. The expression of CD16 on myeloid cells under inflammatory conditions has been observed in various inflammatory diseases, including TB (Ozanska et al., 2020, Sampath et al., 2018). The functional implications of CD16 expression on monocytes in TB patients have been characterized *in vitro* (Rajashree et al., 2009, Castano et al., 2011, Balboa et al., 2013, Lastrucci et al., 2015). Impaired dendritic cell and macrophage differentiation, along with reduced antigen presentation, phagocytic ability, antimycobacterial function, and allostimulatory activity, have been associated with the enrichment of CD16+ monocytes in TB (Rajashree et al., 2009, Castano et al., 2011, Balboa et al., 2013, Lastrucci et al., 2015). From these studies, it can be deduced that the enrichment of

M1 putative monocytes with concomitant CD16 expression in TB patients may alter monocyte functions, potentially enhancing Mtb survival and contributing to the pathology of TB (Lugo-Villarino and Neyrolles, 2013).

Differences in monocyte phenotypes were observed across all major monocyte subsets in TB patients [Paper II], consistent with findings from other studies (Jacobsen et al., 2007, Castano et al., 2011, Singh et al., 2013, Shen et al., 2016, Tamene et al., 2021, Adankwah et al., 2021a). This suggests that the immunopathology of monocytes is independent of specific subsets. The high expression levels of the surface markers CD40, CD64, and PDL1 on monocytes point to potential interactions with T cells and suggest roles in antibody-mediated cytotoxicity and immune modulation, respectively (Shen et al., 2016, Akinrinmade et al., 2017, Chauhan et al., 2020). Additionally, elevated CD40 levels indicate a heightened activation status of monocytes in TB patients [Paper II].

The role of CD40 signaling in monocytes in the context of TB has not been well characterized. However, in Mtb-infected monocyte-derived macrophages and dendritic cells, CD40 stimulation with its ligand induces the production of inflammatory cytokines and chemokines that enhance T cell-mediated antimycobacterial effects (Chauhan et al., 2020). CD40-deficient ($CD40L^{-/-}$) mice were found to be susceptible to Mtb infection due to low production of IL-12 and IFN-γ, while CD40 ligand deficient ($CD40L^{-/-}$) mice exhibited resistance to Mtb infection (Lazarevic et al., 2003). This suggests an important role for CD40 signaling in TB. The potential use of CD40 and Toll-like receptor 4(TLR4) stimulation of dendritic cells and macrophages as adjunct therapy alongside antimycobacterial drugs has been proposed (Khan et al., 2016). However, there are suggestions of possible paradoxical functions of CD40 signaling (Lazarevic et al., 2003, Chauhan et al., 2020). While CD40 is an important co-stimulatory receptor, its stimulation may produce IL-10 and TGF-β, which could antagonize protective host immune responses (Chauhan et al., 2020). This suggests that excessive CD40 signaling in TB (as a consequence of increased levels of CD40 in monocytes) may impair host immune responses. Nonetheless, further studies are needed to confirm this hypothesis.

Increased expression of PDL1 and programmed cell death 1 (PD1) on monocytes and monocyte-derived cells has been found to impair the differentiation of monocytes, diminish antimycobacterial function, and reduce T-cell response to Mtb (Singh et al.,

2013, Shen et al., 2016). However, blocking PDL1 and PD1 interaction restores the antimycobacterial function of monocytes/macrophages as well as the effector function of Mtb-specific T cells (Singh et al., 2013, Shen et al., 2016, Suarez et al., 2019). This emphasizes the significance of this co-stimulatory receptor in modulating immune cell functions in TB and positions it as a potential target for host-directed therapy, as noted in other diseases such as HIV/AIDS and cancer (Gubser et al., 2022, Yamaguchi et al., 2022).

The role of CD64 (an Fc-gamma receptor 1) expression in TB has not been fully explored although it has been associated with disease severity and proposed as a reliable biomarker for TB diagnosis (Jacobsen et al., 2007, Liu et al., 2020a, Gatti et al., 2020). CD64 is highly induced under inflammatory conditions and plays a vital role in antibody-dependent cellular cytotoxicity, antigen presentation, immune complex clearance, and phagocytosis (Akinrinmade et al., 2017). In a murine model, the activation of Fc-gamma receptors influences cytokine production and triggers respiratory bursts in phagocytes to control Mtb infection (Maglione et al., 2008). This suggests that CD64 expression on monocytes may be essential in TB pathology. However, further research is necessary to determine the functional significance of CD64 expression on monocytes in TB patients.

### 4.1.1 Mobilization and recruitment of monocytes in tuberculosis

Appropriate gradients of chemokines and cytokines, with specific receptor expression patterns, regulate the recruitment and mobilization of immune cells. Several factors influence these processes during inflammations. The differential expression of chemokines, cytokines, and their receptors ensures that unique subsets of immune cells are recruited in response to inflammatory conditions (Griffith et al., 2014). In Paper III of this thesis, monocytes from TB patients were characterized by high expression of C-C chemokine receptors (CCR1, CCR2, and CCR5), which are essential for their mobilization and recruitment in TB patients (Balboa et al., 2011, Tamene et al., 2023). An increased frequency of inflammatory monocytes expressing CCR2 was also observed in TB patients [Paper III] (Balboa et al., 2011). These monocytes contribute to inflammation and are described to play a critical role in presenting Mtb to dendritic cells in the lymph nodes (Samstein et al., 2013). Additionally, CCR2+ monocyte-derived macrophages and dendritic cells are important for mediating T cell responses during Mtb infection (Slight and Khader, 2013). In

murine models, CCR2$^{-/-}$ mice infected with Mtb exhibited a defective accumulation of antigen-presenting cells and delayed T-cell responses (Samstein et al., 2013, Domingo-Gonzalez et al., 2016, Dunlap et al., 2018). These findings suggest a functional relevance of CCR2 expression during Mtb infection. CCL-2, an important ligand for CCR2, is essential for recruiting monocytes (Slight and Khader, 2013). However, elevated levels of CCL-2 in the plasma of TB were not observed in the plasma of TB patients [Paper III], as confirmed by other studies (Lee et al., 2003, Kumar et al., 2019b). Nonetheless, the increased expression of CCR2 in monocytes of TB patients [Paper III] suggests high responsiveness of TB monocytes to CCL-2/CCL-7. In CCR5-deficient mice challenged with Mtb, increased immune cell infiltration and effective control of Mtb infection were observed (Algood and Flynn, 2004, Slight and Khader, 2013). However, Mtb-infected CCL-5$^{-/-}$ mice exhibited altered recruitment of antigen-presenting cells and a transient impairment of T-cell response (Vesosky et al., 2010). This demonstrates that other chemokine receptors and their ligands can compensate for CCR5 function in cell recruitment, while its main ligand (CCL-5) is essential in the early immune response against Mtb (Slight and Khader, 2013, Domingo-Gonzalez et al., 2016). Although the functional role of CCR1 has not been fully investigated in the context of TB disease, the expression of CCR1 and CCR5 on monocytes enhances adhesion and movement across endothelial cells (Griffith et al., 2014). The importance of these chemokine receptors in TB disease has been further highlighted as genetic polymorphisms of CCR2 and CCR5 genes, as well as their ligands, have been associated with increased susceptibility to Mtb infection (Flores-Villanueva et al., 2005, Mishra et al., 2012, Liu et al., 2020b). Taken together, the high expression of chemokine receptors on monocytes in TB patients suggests that TB disease induces increased motility in monocytes and recruitment of CCR2+ inflammatory monocytes from the bone marrow, which may drive inflammatory processes against Mtb.

It is important to note that while monocytes rarely encounter Mtb in the bloodstream, the immunopathology of pulmonary TB is evident in the phenotype and functional dysregulation of monocytes, as well as other immune cells in peripheral blood [Paper I, II, and III]. Furthermore, circulating monocytes from TB patients are characterized as highly activated and displaying a more mature phenotype, highlighting the influence of the plasma microenvironment on this immune subset (Balboa et al., 2011).

### 4.2 Plasma milieu in tuberculosis and its effects on monocytes

Plasma changes in TB patients, as reviewed in Paper I of this thesis, primarily involve factors that drive inflammation, immune cell chemotaxis, tissue remodeling, and immune suppression. These changes mirror a similar phenomenon occurring in the lungs of TB patients, highlighting the connection between lung and plasma pathology (Belton et al., 2016, Stek et al., 2018, Sabir et al., 2019, Herrera et al., 2022). Studies have suggested that these changes in the plasma environment can affect immune cells in the peripheral blood of TB patients, potentially impacting their ability to control TB disease [Paper I] (Adankwah et al., 2021b, Harelimana et al., 2022). However, the mechanisms by which plasma changes influence monocytes and other immune cells crucial for immune surveillance in TB remain elusive.

Using an *in vitro* plasma assay to assess monocyte responses to the plasma of pulmonary TB patients, it was observed that TB plasma induced a high expression of functional markers (CD40, CD64, CD33, and HLA-DR) and chemokine receptors (CCR1 and CCR5) on reference monocytes [Paper II and Paper III]. Additionally, TB plasma induced messenger ribonucleic acid (mRNA) transcripts and cytokines/ chemokines in reference monocytes, which are related to inflammation and chemotaxis of immune cells [Paper III]. The changes observed in reference monocytes following exposure to TB plasma closely align with alterations in both the phenotype (Jacobsen et al., 2007, Sampath et al., 2018, Tamene et al., 2021) and transcriptome changes of monocytes (Li et al., 2021, Hillman et al., 2022) in TB patients. These findings provide direct evidence of the plasma environment's influence on monocytes. The chemokines and cytokines induced by TB plasma *in vitro* via monocytes [Paper II and III] also largely reflect the plasma milieu in TB patients [Paper I] (Chowdhury et al., 2014, Kumar et al., 2019a, Kumar et al., 2019b, Vivekanandan et al., 2023b).

The potential mechanism by which TB plasma affects monocytes involves STAT signaling, particularly STAT3 and STAT5, which correlated with plasma-induced monocyte phenotypes, cytokines, and chemokines [Paper II and III]. This suggests that cytokines or plasma factors activating these pathways could be key mediators of plasma-induced monocyte immunopathology in TB. The correlation between TB plasma-induced inflammatory cytokines and STAT signaling [Paper III], also indicates an autologous effect. This suggests that exposure to TB plasma enhances the inflammatory properties of immune cells, especially monocytes, which may

subsequently alter their phenotypes and functions. These alterations may have a consequence on monocyte-derived cells. Although this thesis did not characterize the phenotype/function of monocyte-derived cells from plasma-treated reference monocytes, it has been demonstrated that dendritic cells differentiated from healthy control monocytes in TB plasma-supplemented media exhibited altered phenotypes and defective allogeneic T-cell stimulation (Wu et al., 2008).

The study by Harling *et al.* demonstrated how an inflammatory plasma environment affects immune cells. It showed that constitutive STAT 3 signaling driven by increased plasma levels of IL-6 and IL-10 led to increased expression of SOCS3, which in turn impaired T-cell responses (Harling et al., 2019). Furthermore, elevated levels of inflammatory cytokines and chemokines (IL-1β, TNF-α, IL-6, CXCL-9, and CXCL10) in plasma were associated with impaired mitogen responses in TB patients. This highlights the impact of hyperinflammation in TB plasma on immune cell responses (Vivekanandan et al., 2023a, DiNardo et al., 2022). In Paper III of this thesis, TB patients with elevated plasma levels of cytokines and chemokines also exhibited high levels of SOCS3 in their monocytes. This suggests that chronic activation driven by the inflammatory plasma milieu in TB patients, which leads to dysregulated cytokine signaling in T cells, as reported by Harling *et al*., may also occur in monocytes. Supporting this, the study by Lastrucci *et al*. indicates that the altered phenotypes and functions of monocytes/macrophages in TB patients may be driven by aberrant IL-10/STAT3-dependent pathways (Lastrucci et al., 2015). Constitutive STAT3 signaling, along with the induction of SOCS 3, has been described to diminish the antimycobacterial functions of monocytes (Rottenberg and Carow, 2014, Lastrucci et al., 2015, Gao et al., 2018). While the functional implication of constitutive STAT5 signaling in TB has not been documented, lower or impaired STAT5 signaling in T cells and monocytes of TB patients is linked to aberrant cytokine signaling (Lundtoft et al., 2017, Harling et al., 2019, Adankwah et al., 2021a). The effects of aberrant STAT signaling in immune cells have also been observed in inflammatory diseases such as rheumatoid arthritis (Isomaki et al., 2015, Anderson et al., 2016) and Crohn's disease (Lovato et al., 2003). Overall, the findings in this thesis support the hypothesis that TB has a systemic impact on immune cells through hyperinflammation of TB plasma. This plasma milieu changes modifies the phenotype and function of key sensory immune cells such as monocytes, through abnormal cytokine signaling driven

by aberrant host responses. These modifications may affect the effectiveness of immune surveillance in the lungs, thereby contributing to disease pathology.

The findings of this thesis support the hypothesis that aberrant host immune responses drive plasma immunopathology in pulmonary TB patients [Paper I]. However, the initial triggers of these responses in peripheral blood remain unknown. The use of diluted plasma (1:10 dilution) in the *in vitro* assays, which induced reactions in reference monocytes, suggests that the initial triggers of plasma effects on monocytes are highly potent, even at very low concentrations. This eliminates plasma cytokines as the initial triggers for plasma pathology, as their concentrations in diluted plasma may fall below physiological levels in humans. Instead, the results of this thesis indicate that the secretion of inflammatory cytokines/chemokines by reference monocytes upon exposure to TB plasma may boost the plasma effect in an autologous manner [Paper III]. The presence of Mtb in the peripheral blood of pulmonary TB patients without HIV co-infection or extra-pulmonary disease remains inconclusive (Rees et al., 2024). Therefore, the ability of TB plasma to trigger changes in reference monocytes [Paper II and III] suggests the presence of a highly potent molecule in circulation, potentially secreted by immune cells or Mtb. In the studies conducted by Rojas-Espinosa and colleagues, it was demonstrated *in vitro* that TB serum induces nuclear and morphological changes in the neutrophils of healthy donors (Juárez-Ortega et al., 2018, Rojas-Espinosa et al., 2021). These studies implicated soluble mycobacterial antigens such as early secreted antigenic target 6 kDa (ESAT-6) and 10 kDa culture filtrate antigen (CFP-10), not cytokines or mycobacterial antibodies in TB serum as factors triggering the plasma effect on neutrophils. The presence of circulating immune complexes containing mycobacterial antigens in the plasma of pulmonary TB patients supports this assumption (Bentley-Hibbert et al., 1999, Goyal et al., 2017). However, further studies are needed to characterize the role of soluble mycobacterial antigens in the plasma of TB patients. The immunomodulatory effects of mycobacteria components/products are well-known (Ellner and Daniel, 1979, Chandra et al., 2022), suggesting that these factors, if present in circulation, could be triggers of plasma effects. In addition to mycobacterial antigens, other mediators, such as circulating exosomes containing Mtb components, may also play a role in the plasma immunopathology of TB (Sun et al., 2021).

### 4.2.1 Tuberculosis plasma induces granulocytes and neutrophils recruitment via monocytes

In Paper III of this thesis, high levels of chemokines, which mediate the recruitment and mobilization of granulocytes and neutrophils, were observed in the peripheral blood of TB patients (Griffith et al., 2014, Alcantara et al., 2023). Additionally, the plasma from TB patients induced monocyte mRNA transcripts and proteins that promote the recruitment and movement of granulocytes and neutrophils [Paper III]. These findings may account for the increased frequency of granulocytes and CD16+ neutrophils observed in TB patients [Paper III].

Neutrophilia has been observed in TB patients and is associated with disease severity and lung tissue damage (Mayer-Barber, 2023, Alcantara et al., 2023). This enrichment of neutrophils in TB patients is accompanied by an interferon (IFN)-induced gene profile linked to the exacerbation of TB disease (Berry et al., 2010). Neutrophilia does not only exacerbate inflammation but also facilitates the mobilization of inflammatory monocytes, further contributing to TB pathology (Prame Kumar et al., 2018). Therefore, it can be suggested that changes in the plasma environment may influence neutrophil recruitment and chemotaxis through monocytes or vice versa. Interestingly, the increase of granulocytes and neutrophils in TB patients correlated with plasma IL-6 levels [Paper III]. This correlation suggests that IL-6 may play a role in neutrophil chemotaxis or that heightened levels of granulocyte neutrophils could exacerbate inflammation through increased IL-6 production. The involvement of IL-6 in mediating neutrophil trafficking or chemotaxis during inflammatory conditions has been previously described (Hashizume et al., 2011, Wright et al., 2014, Florentin et al., 2021). IL-6 has been shown to enhance IL-8-dependent chemotaxis of neutrophils (Wright et al., 2014). In a murine model, Florentina *et al*. demonstrated that IL-6 facilitates the egress of neutrophils from the bone marrow by mediating CX3CR1 expression through interferon regulatory factor 4 (Florentin et al., 2021). Similarly, Fielding *et al.* found that IL-6 induces neutrophil movement during inflammation through STAT-dependent signaling (Fielding et al., 2008). Notably, the use of siltuximab (anti-IL-6) and tocilizumab (anti-IL-6 receptor) in patients with type 1 diabetes and rheumatoid arthritis resulted in neutropenia, suggesting a significant role for IL-6 in neutrophil chemotaxis and recruitment (Speake et al., 2022). While these studies indicate an important role of IL-6 in granulocytes and neutrophils chemotaxis,

further research is necessary to confirm IL-6 involvement in neutrophil recruitment in the context of TB disease.

### 4.3 Conclusion and Outlook

Monocytes and their derived cells are essential in establishing immune surveillance against Mtb infection. As a result, any changes to these immune cells can impact disease outcomes. This thesis focused on changes in monocytes in the blood of pulmonary TB patients and the influence of plasma milieu on the immune responses of monocytes. It highlights the enrichment of highly motile inflammatory monocytes with altered phenotypes, which could affect monocyte-T cell interactions and the function of monocyte-derived cells, driving TB pathology. Changes in the plasma milieu of TB patients primarily involve factors that promote inflammation and chemotaxis. Notably, exposure of monocytes to the plasma of TB patients leads to alterations in their phenotypes and functions, exacerbating inflammation through the secretion of inflammatory mediators and the recruitment of neutrophils. Cytokines, particularly IL-6, or plasma factors signaling via STAT3/STAT5 are potential drivers behind these changes in monocytes. These findings highlight the importance of host-directed therapy targeting plasma and monocyte changes to modulate the harmful effects of chronic inflammation in TB patients, with potential long-term benefits. Furthermore, this thesis demonstrated that aberrant monocyte phenotypes (CD40, CD64, and PDL1) normalize during antimycobacterial therapy, suggesting that these changes in monocyte phenotype could be used as diagnostic tools to distinguish different stages of TB and monitor treatment outcomes. However, further studies with larger cohort sizes are needed to validate the diagnostic potential of these biomarkers.

## 5. Acknowledgements

Glory and Honour to the Lord Almighty for His abundant Grace and Mercies throughout my PhD studies.

I sincerely thank Prof. Dr. Marc Jacobson, my esteemed supervisor, for his invaluable help and guidance throughout my Doctoral studies. Our numerous scientific conversations have greatly enriched my knowledge and benefited me immensely. Without this, my PhD studies would not have been a success. I sincerely appreciate the confidence and trust you placed in me. I would also like to express my gratitude to Prof. Rainer Kalscheuer, who served as my mentor and provided valuable advice and training opportunities in his laboratory. Lastly, I extend my heartfelt thanks to Dr. Julia Seyfarth, whose assistance and expert advice were invaluable precisely when I needed them.

I am also incredibly appreciative of the current and previous members of the AG Jacobsen such as Jean, Ju-Young, Monika, Wilfred, Rebecca, Sophie, Maryam, Steffen, Yoshi, Sarah, Souhaila, and Katharina. You all provided excellent support and made the laboratory welcoming for fruitful research. To the whole 'Stoffwechsellabor' colleagues, I say a big thank you. You were all very supportive and created a hospitable environment for conducive research.

A special thank you to Prof. Dr. Richard Phillips Odame, the Scientific Director of Kumasi Center for Collaborative Research into Tropical Medicine (KCCR), Ghana, whose collaboration with Prof. Dr. Marc Jacobson, made it possible to work with patients' data and materials. I also had a lot of assistance from the whole Immunology against Mycobacteria group at KCCR, whose contributions, particularly in terms of patient recruitment and experiment assistance, during my time at KCCR cannot be overstated. I sincerely appreciate all of you.

To all study participants, I say a very big THANK YOU for being part of this study.

I would also like to express my appreciation to the "Molecule of Infection-IV" graduate school and the Juergen-Manchot Foundation for providing the scholarship for my Ph.D.

The last but not the least, I would like to extend my sincere gratitude to my parents, siblings, and my beloved wife, Morrah, for their unwavering emotional support during difficult times. Your support is deeply cherished and truly invaluable.

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## 7. Statutory declaration

I declare under oath that I have compiled my dissertation independently and without any undue assistance by third parties under consideration of the ‘Principles for the Safeguarding of Good Scientific Practice at Heinrich Heine University Düsseldorf’.
The dissertation has not been presented to other faculties, nor do I have any record of previous successful or unsuccessful attempts to obtain a doctorate.

_____

Name

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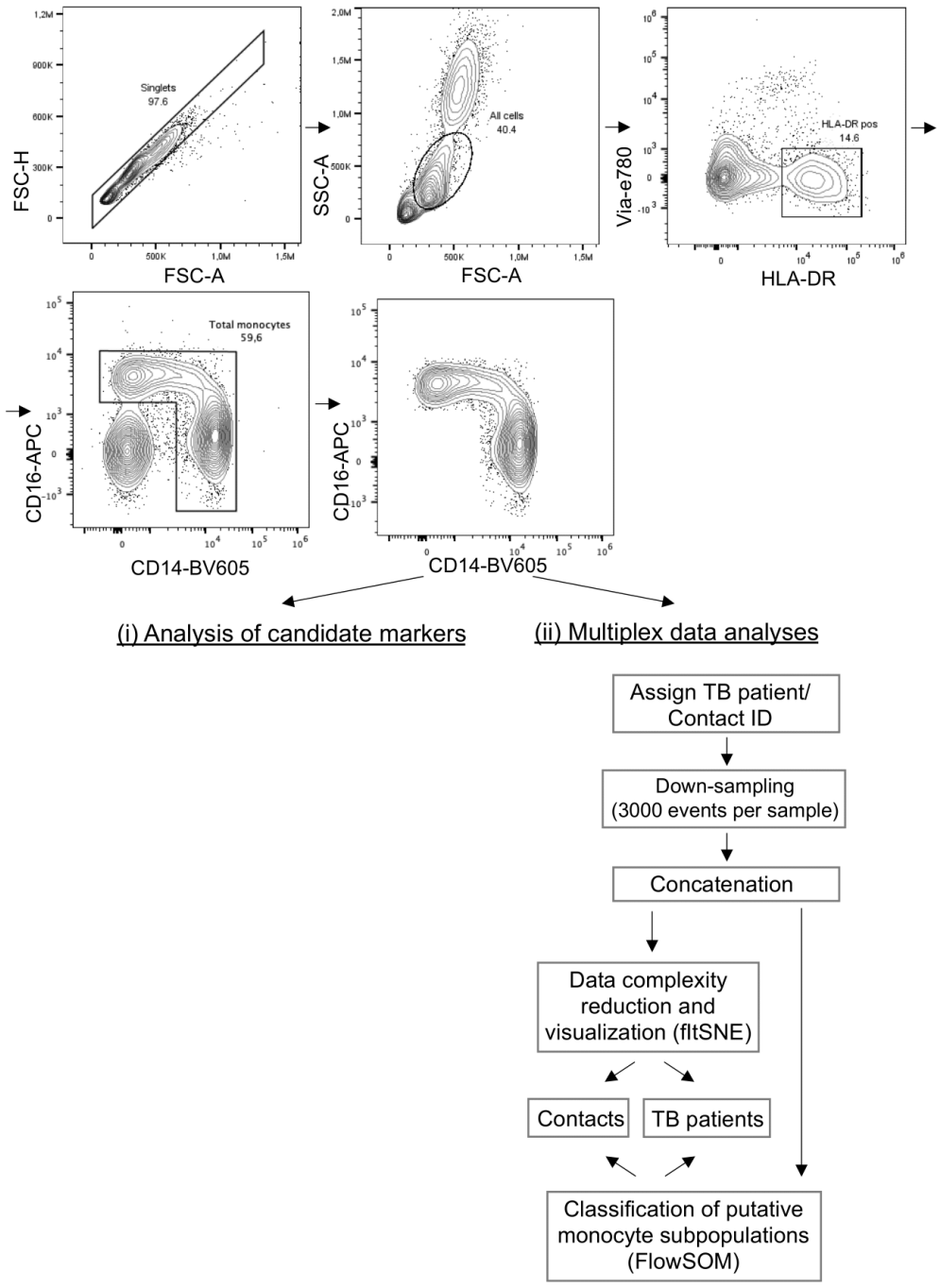
Date / Signature

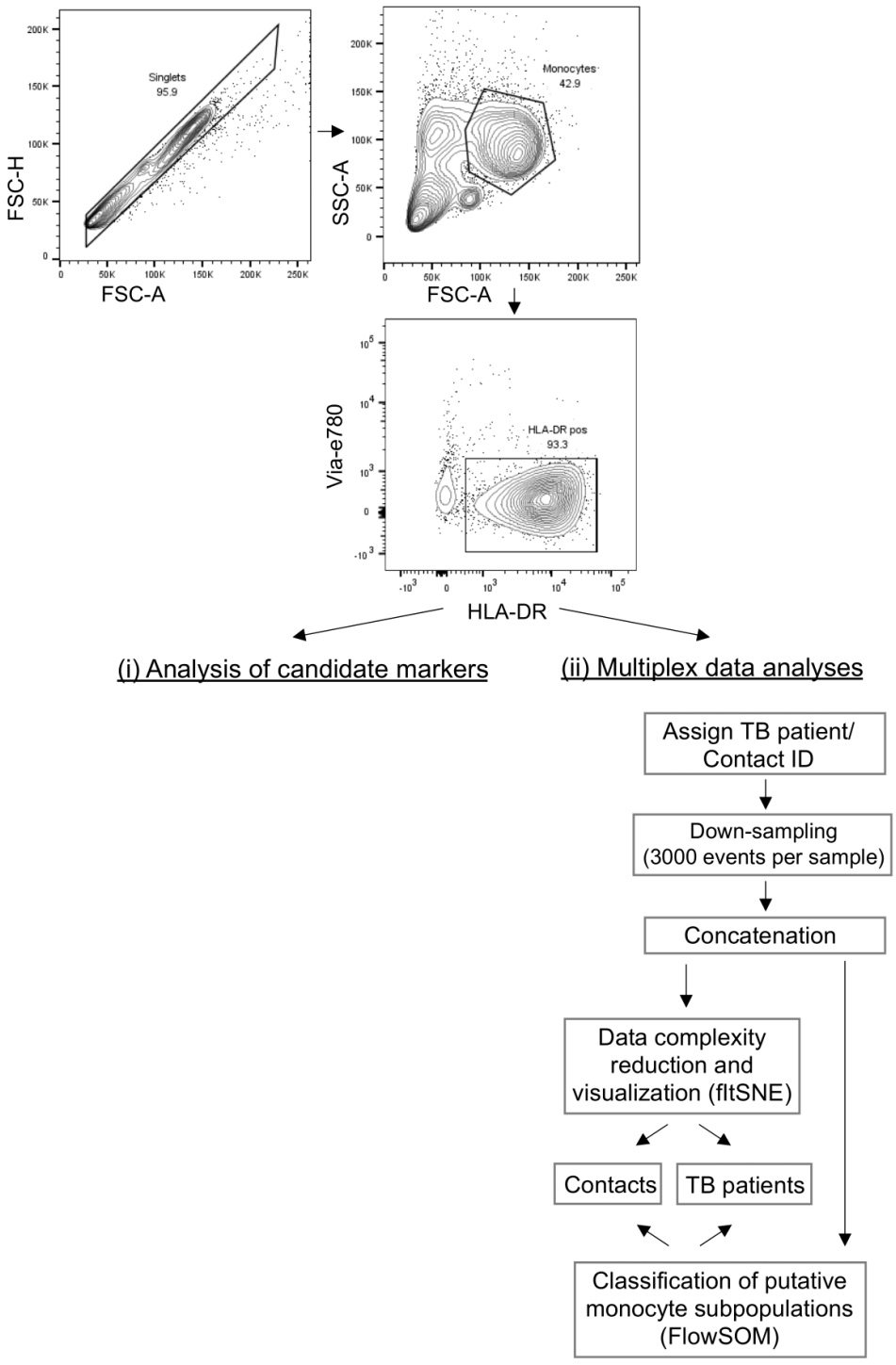
## Supplementary information | paper II

Supplementary Figure 1



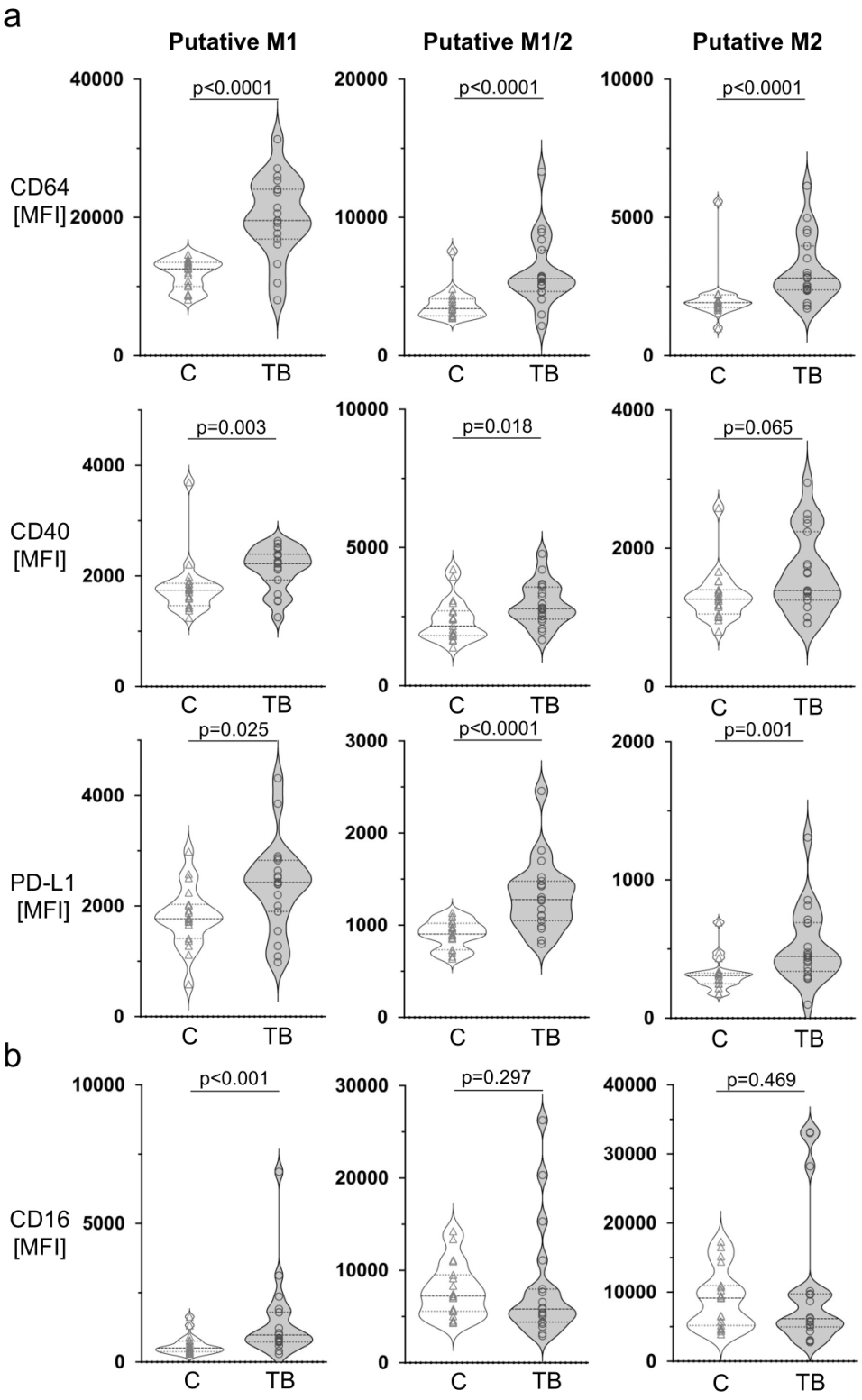

***Flow cytometry gating procedure and data processing.*** Initially cell duplets were excluded using a forward scatter height (FSC-H) vs. FSC area (FSCA-A) zebra plot with outliers. Next, monocyte-like cells were selected based on size (FSC-A) and granularity (Side scatter area, SSC-A)). Then HLA-DR positive monocytes were gated excluding viability dye (via-e780) positive (i.e., dead) cells. Then CD14/CD16 double-negative cells were excluded. The selected cell subset was then used for (i) single candidate comparisons of mean fluorescence intensities (MFI) between study group individuals and time points; (ii) multiplex data analyses. Detailed description is provided in the respective methods section.

# Supplementary Figure 2



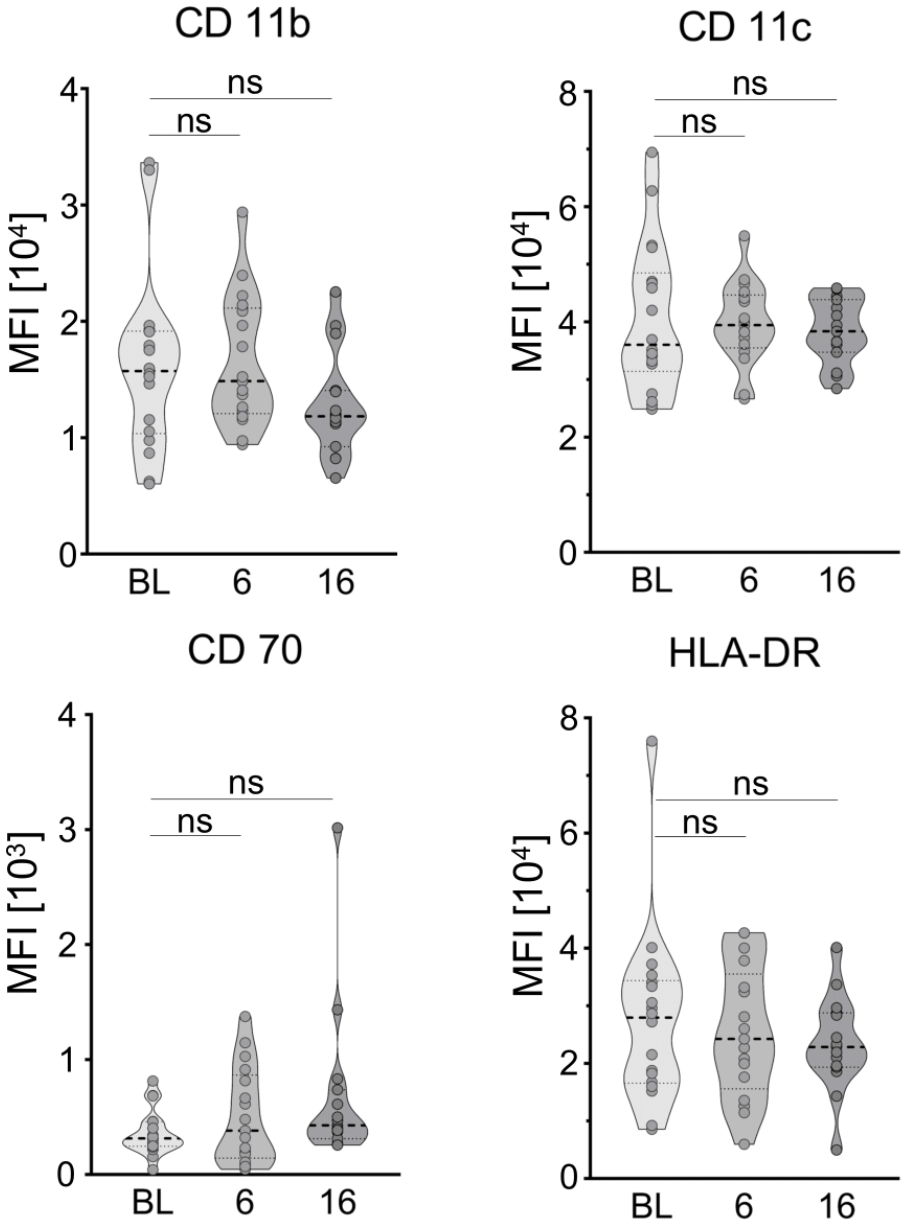

***Flow cytometry gating procedure and data processing.*** Initially cell duplets were excluded using a forward scatter height (FSC-H) vs. FSC area (FSCA-A) zebra plot with outliers. Next, monocyte-like cells were selected based on size (FSC-A) and granularity (Side scatter area, SSC-A)). Then HLA-DR positive monocytes were gated excluding viability dye (Via) positive (i.e., dead) cells. The selected cell subset was then used for (i) single candidate comparisons of mean fluorescence intensities (MFI) between study group individuals and time points; (ii) multiplex data analyses. Detailed description is provided in the respective methods section.

# Supplementary Figure 3



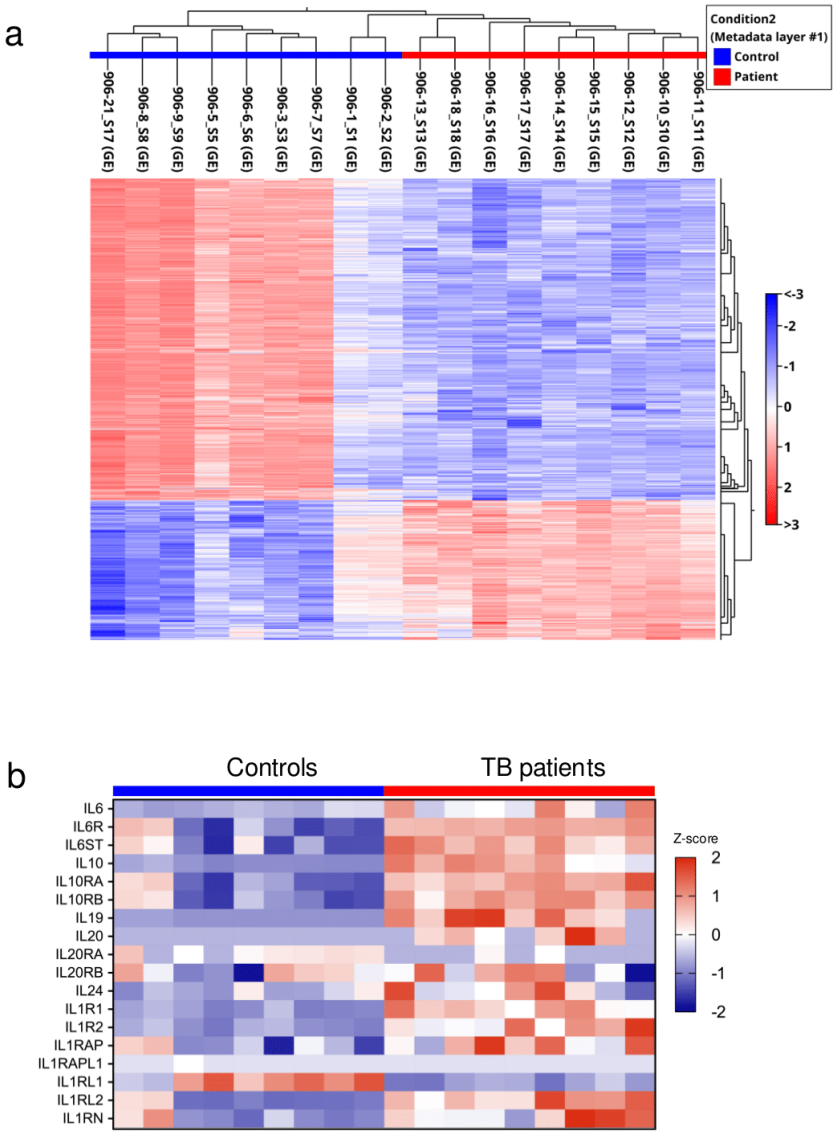

Monocyte subsets (i.e., M1, M1/2, M2) between TB patients (TB) and controls (C) for **(a)** CD64, CD40, PD-L1, CD33 and **(b)** CD16 expression. Violin plots for BL (bright grey violin, n=18), and w16 (dark grey violin, n=16) including 25, 50, and 75 percentiles (as dotted or dashed lines) are given. Each symbol represents the mean of duplicates from an individual donor. P-values are calculated using the Wilcoxon-signed rank test. MFI: Mean Flourescence Intensity.

# Supplementary Figure 4



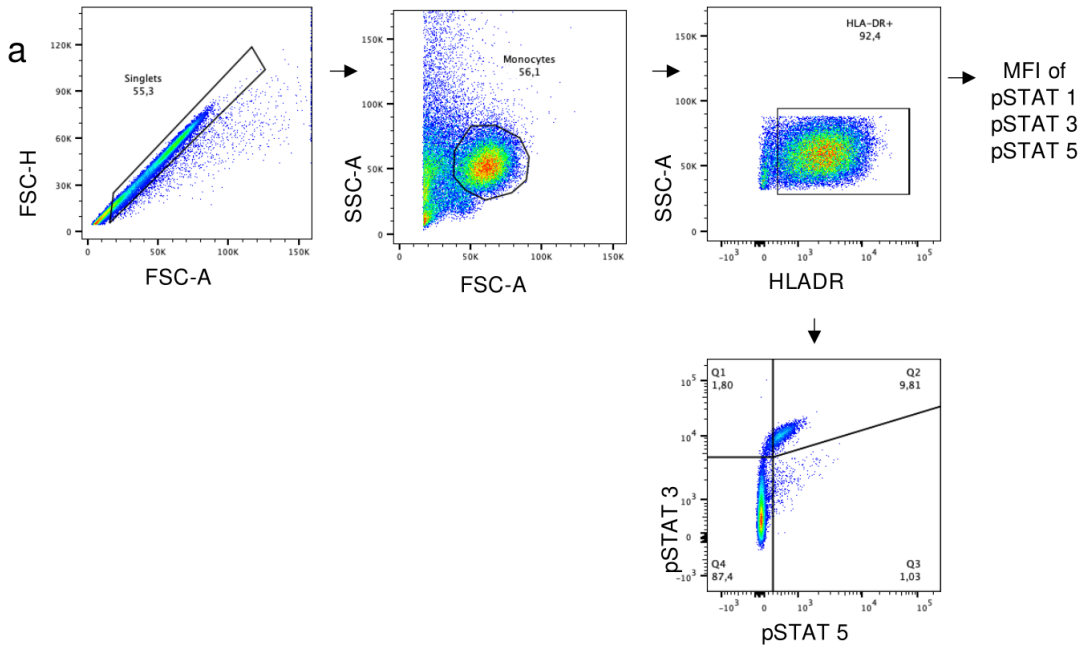

Time course comparisons of selected monocyte marker expression during treatment and recovery of tuberculosis patients. Ex vivo phenotyping of peripheral blood monocytes by flow cytometry from tuberculosis patients prior to treatment (BL), week 6 (W6), and week 16 (W16) after treatment start was performed. Analyses for CD11b, CD11c, CD70, and HLA-DR are shown as violin plots for BL (bright grey violin, n=18), W6 (medium grey violin, n=17), and W16 (dark grey violin, n=16) including 25, 50, and 75 percentiles (as dotted or dashed lines) are given. Each symbol represents the mean of duplicates from an individual donor. Study group comparisons were performed and p-values are calculated using the Wilcoxon-signed rank test. ns: not significant.

# Supplementary Figure 1



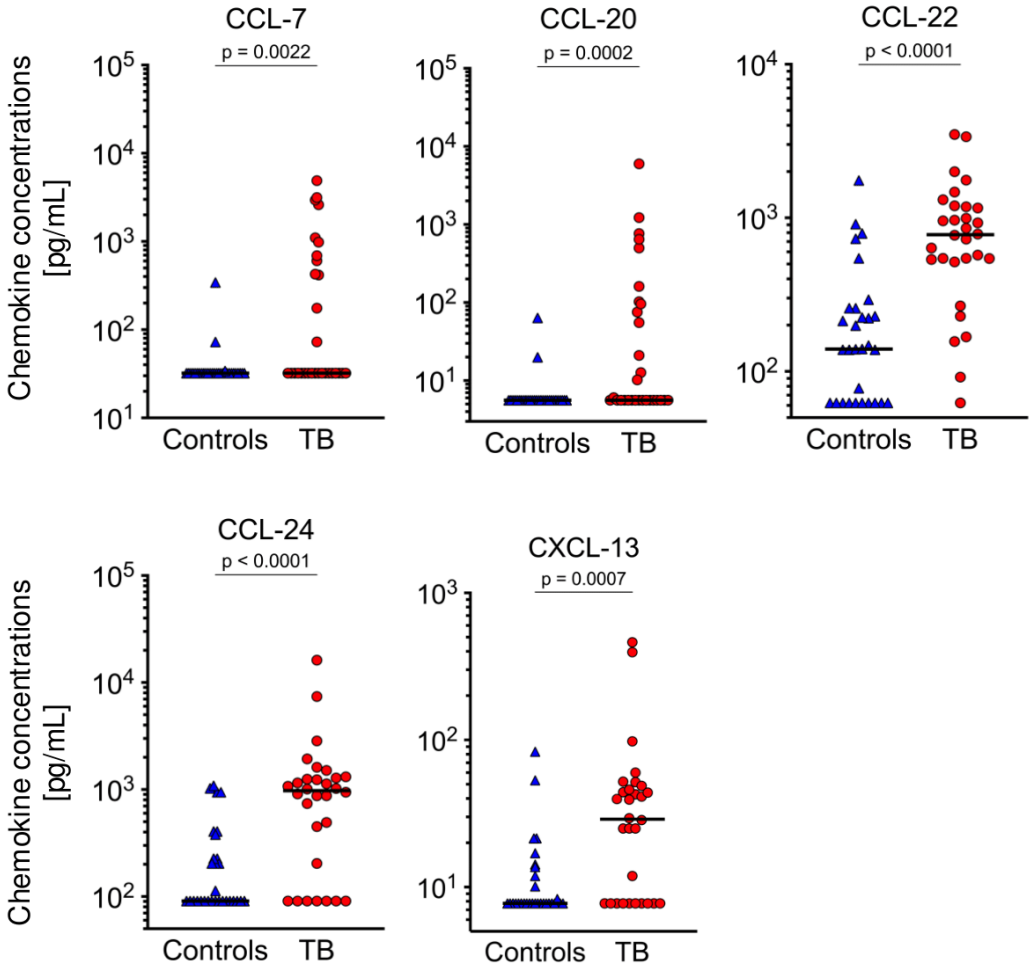

a) Unsupervised clustering of study cohort using differentially expressed genes (Fold change≥2, Bonferroni p value< 0.05). b) Heatmap of z scores of IL1, IL6, and IL10 family cytokine transcript in plasma-treated monocytes

# Supplementary Figure 2



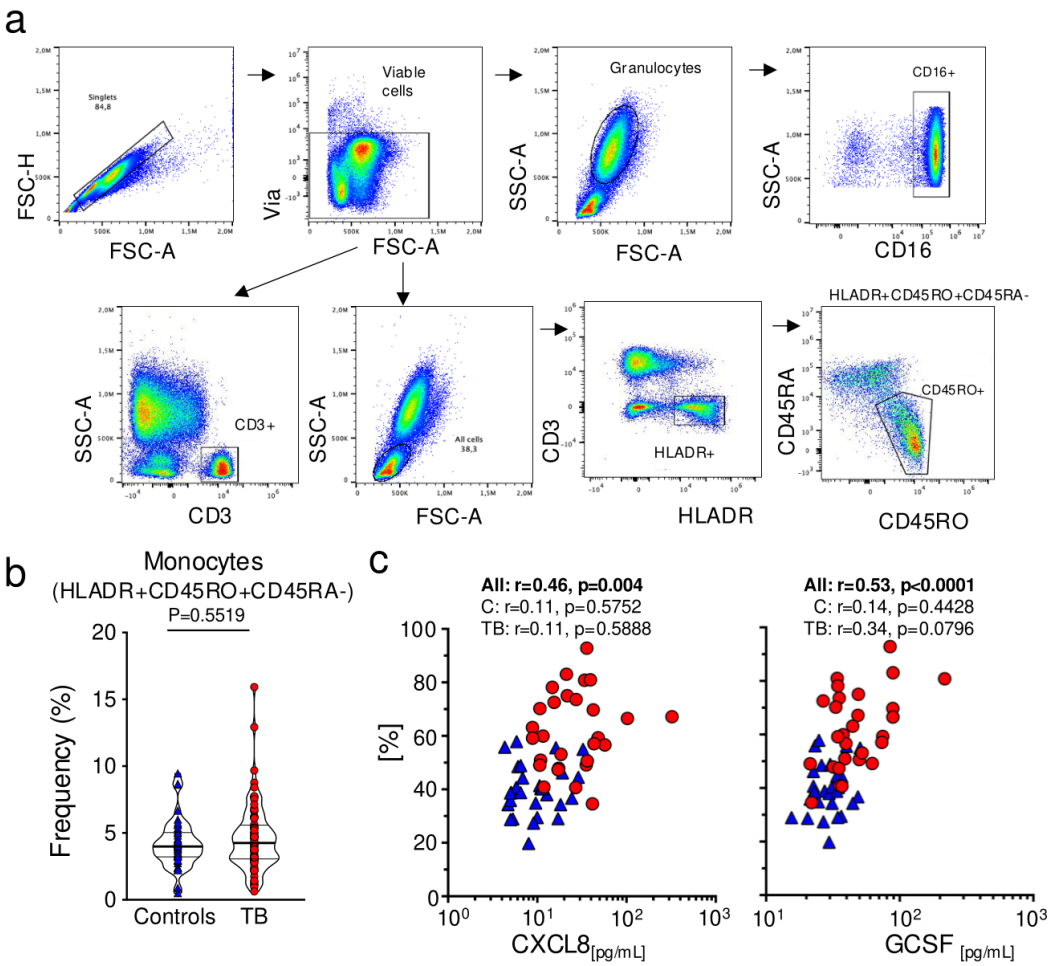

***pSTAT Flow cytometry gating procedure and data processing.*** Duplet cells were excluded using a forward scatter height (FSC-H) vs. FSC area (FSCA-A) dot plot. Next, Monocyte-like cells were selected based on size (FSC-A) and granularity (Side scatter area, SSC-A)). Then HLADR positive monocytes were gated and proportions of pSTAT 3 and pSTAT 5 double-positive cells were determined. The mean fluorescence intensity (MFI) of pSTAT 1, 3, and 5 were also determined from HLADR-positive monocytes.

# Supplementary Figure 3



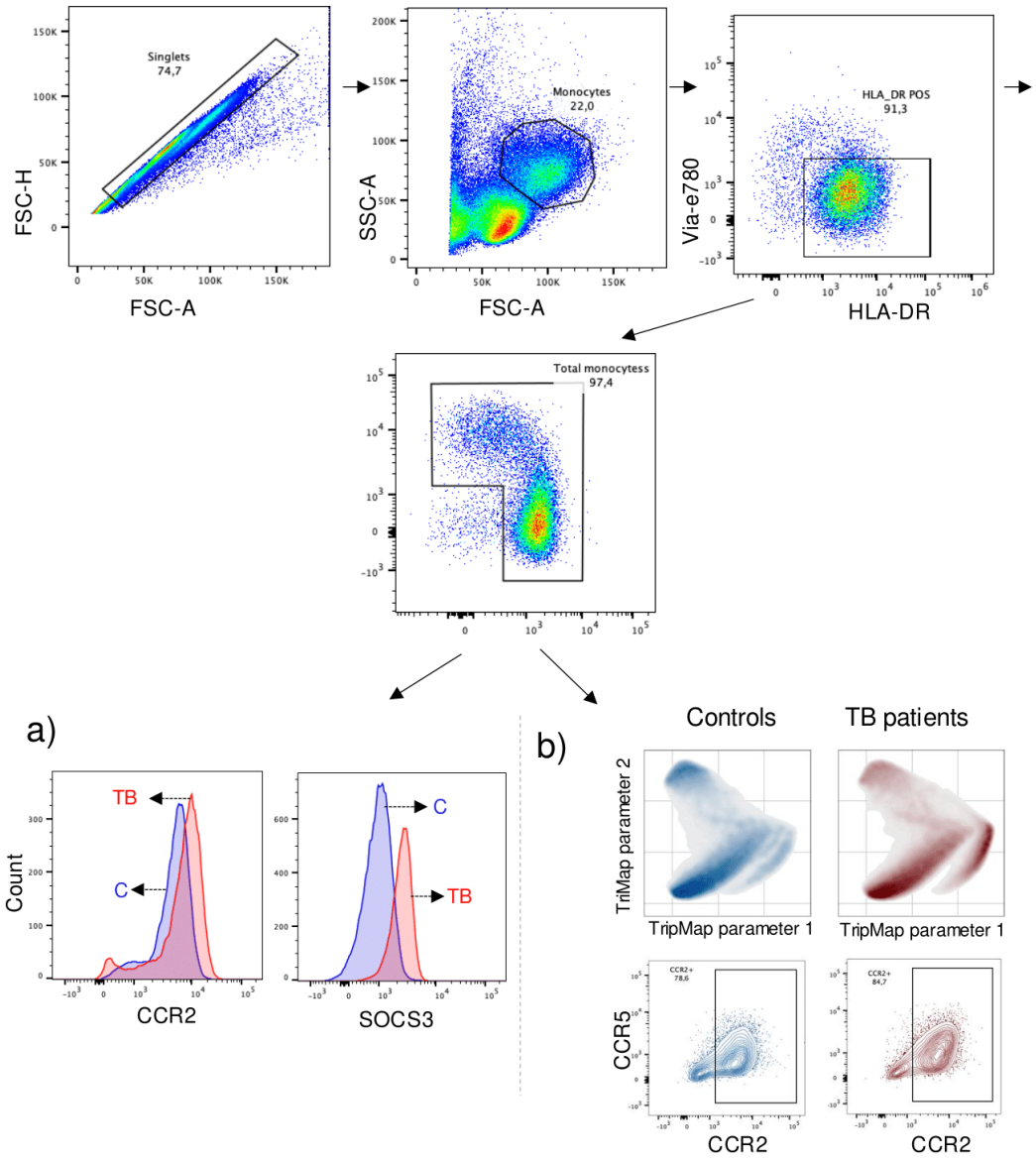

Chemokine levels in culture supernatants of Tuberculosis patients and controls. Each symbol represents the mean of duplicates from an individual donor. P-values are calculated using the using the two-tailed Mann-Whitney U-test. A *p*-value < 0.05 was considered significant. Detailed descriptions of chemokine measurements are provided in the methods section.

# Supplementary Figure 4




***Flow cytometry gating procedure and data processing.*** Duplet cells were excluded using a forward scatter height (FSC-H) vs. FSC area (FSCA-A) dot plot. Then viable cells were gated by excluding viability dye (Via) positive (i.e., dead) cells. (a) Next, Granulocytes-like cells were selected based on size (FSC-A) and granularity (Side scatter area, SSC-A)). Then CD16 positive or negative were gated. The CD3-positive cells were gated from all viable cells. Monocyte was determined by gating on the lymphocyte cloud based on size and granularity. Then after HLADR positive and CD45RO positive- CD45RA negative were gated. The final proportion of immune cell subsets was calculated from the total viable cells. (b) The proportion of monocytes is compared between study groups. (c) Frequencies of granulocytes were correlated with plasma levels of CXCL8 and GCSF. Each symbol represents the mean of duplicates from an individual donor. Dark red circles and dark blue triangles represent TB patients and controls respectively. P-values are calculated using the using the two-tailed Mann-Whitney U-test for study group comparison. Correlations were performed using Spearman correlation. A *p*-value < 0.05 was considered significant.

# Supplementary Figure 5




***Flow cytometry gating procedure and data processing.*** Initially, cell duplets were excluded using a forward scatter height (FSC-H) vs. FSC area (FSCA-A) dot plot. Next, monocyte-like cells were selected based on size (FSC-A) and granularity (Side scatter area, SSC-A)). Then HLA-DR positive monocytes were gated excluding viability dye (via) positive (i.e., dead) cells and CD14/CD16 double-negative cells excluded. The total monocyte subset was used for a) comparisons of mean fluorescence intensities (MFI) and proportions of chemokine receptors. SOCS 3 mean fluorescence intensities (MFI) between study groups (TB patients (TB) and controls were also compared. (b) Dimensionality reduction and visualization of CCR2+ expressing monocytes using TriMap. Detailed descriptions are provided in the respective methods section.