Aus der Abteilung für Infektions- und Tropenmedizin
Klinik der Universität München

Direktor: Prof. Dr. med. Michael Hoelscher

Defining The Inflammatory Pathway And Prognostic Neutrophil-derived Biomarkers Associated With Pulmonary Morbidity And Long-Term Outcome Of Patients Following TB Therapy

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von

Caleb Nwongbouwoh Muefong

aus

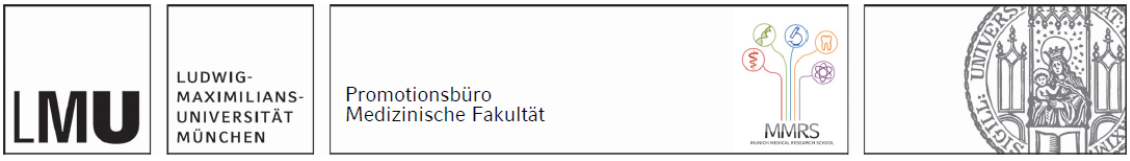
Yaoundé, Cameroon

2022

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	PD Dr. Christof Geldmacher
Mitberichterstatter:	Prof. Dr. Dennis Nowak
	Prof. Dr. Josef Eberle
	Prof. Dr. Rainer Haas
Mitbetreuung durch den promovierten Mitarbeiter:	Prof. Dr. Jayne Sutherland
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	12.07.2022

Affidavit



Affidavit

Nwongbouwoh Muefong, Caleb

Surname, first name

Street

St. Louis

P.O. Box, town

Missouri, United States of America

Country

I hereby declare, that the submitted thesis entitled:

"Defining The Inflammatory Pathway And Prognostic Neutrophil-derived Biomarkers Associated With Pulmonary Morbidity And Long-Term Outcome Of Patients Following TB Therapy"

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

St. Louis, Missouri, 08/08/2022
Place, date

Nwongbouwoh Muefong Caleb
Signature doctoral candidate

Table of content

List of abbreviations

ATB: Active Tuberculosis

ATT: Antibiotic TB Treatment

CD: Cluster of Differentiation

CXCL: Chemokine (C-X-C motif) Ligand

DAMPs: Damage-Associated Molecular Patterns

DHR: Dihydrorhodamine 123

EC: ESAT-6/CFP-10

FCM: Flow Cytometry

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HDT: Host-Directed Therapy

IFNγ: Interferon gamma

IL: Interleukin

MMP: Matrix Metalloproteinase

MPO: Myeloperoxidase

Mtb: *Mycobacterium tuberculosis*

PAMPs: Pathogen- Associated Molecular Patterns

PMA: Phorbol 12-Myristate 13-Acetate

PPD: Purified Protein Derivative

ROS: Reactive Oxygen Species

S100A8/9: Calprotectin

TNF: Tumour-Necrosis-Factor alpha

WCL: H37Rv Whole Cell Lysate

List of publications

In this doctoral thesis, the following publications are summarized to a cumulative thesis according to the examination rules of the faculty of medicine of the LMU, Munich:

Publication I:

Neutrophils contribute to severity of tuberculosis pathology and recovery from lung damage pre- and post-treatment. **Caleb Nwongbouwoh Muefong,** Olumuyiwa Owolabi, Simon Donkor, Salome Charalambous, Abhishek Bakuli, Andrea Rachow, Christof Geldmacher, Jayne S. Sutherland. Clin Infect Dis. **2021**:ciab729. doi: 10.1093/cid/ciab729. PMID: 34427644.

Publication II:

Major neutrophil-derived soluble mediators associate with baseline lung pathology and post-treatment recovery in tuberculosis patients. **Caleb Nwongbouwoh Muefong,** Olumuyiwa Owolabi, Simon Donkor, Salome Charalambous, Joseph Mendy, Isatou C M Sey, Abhishek Bakuli, Andrea Rachow, Christof Geldmacher, Jayne S. Sutherland. Front Immunol. **2021;** 12:740933.

Publication III:

Monitoring Anti-tuberculosis Treatment Response Using Analysis of Whole Blood Mycobacterium tuberculosis Specific T Cell Activation and Functional Markers. Molly A. Vickers, Fatoumatta Darboe, **Caleb N. Muefong**, Georgetta Mbayo, Amadou Barry, Awa Gindeh, Sainabou Njie, Abi-Janet Riley, Binta Sarr, Basil Sambou, Hazel M. Dockrell, Salome Charalambous, Andrea Rachow, Olumuyiwa Owolabi, Shamanthi Jayasooriya and Jayne S. Sutherland et al. Front Immunol. **2020**; 11:572620.

Additional contribution:

Neutrophils in Tuberculosis-Associated Inflammation and Lung Pathology. **Muefong CN**, Sutherland JS. Front Immunol. **2020**; 11:962. doi: 10.3389/fimmu.2020.00962. PMID: 32536917; PMCID: PMC7266980.

Your contribution to the publications

Caleb Nwongbouwoh Muefong was responsible for the following:

1.1 Contribution to paper I

The aim of this study was to monitor the dynamics of CD16|CD62L-defined neutrophil phenotypes and their activities on TB disease severity at presentation and lung pathology outcomes after standard TB treatment. I conceived the study by selecting the phenotypic classification criteria to be used and the neutrophil functional attributes to be measured by flow cytometry. I also optimised the experimental protocol and conducted the experiments (as described in the relevant Methodology section). I also performed data curation and analysis using R statistical software. I presented the data and results at local and international conferences/seminars, drafted the manuscript (including main text, figures, references, etc.) and coordinated communications between co-authors. Finally, I revised the manuscript, submitted it and integrated reviewers' comments towards publication.

1.2 Contribution to paper II

The aim of this study was to assess the effect of neutrophil-derived mediators on TB disease severity at baseline and recovery upon TB treatment initiation. I helped conceive the study approach by identifying the relevant neutrophilic biomarkers to be measured. I also designed the Luminex panel, optimised the experimental protocol and conducted the experiments (as described in the Methodology section). Additionally, I performed the data curation and formal analysis using R statistical software. I presented the data and results at local and international conferences/seminars, drafted the manuscript (including main text, figures, references, etc.) and coordinated communications between co-authors. Finally, I revised the manuscript, submitted it for publication and integrated reviewer comments towards publication with the journal.

1.3 Contribution to paper III

The objective of this study was to measure T-cell subsets, their activation markers and cytokine markers in relation to treatment response in TB patients using Flow cytometry. I helped optimise the flow cytometry antibody panel and staining protocol to the flow cytometer and trained the primary author on the procedure. I also assisted with raw flow cytometry analyses using FlowJo software. Finally, I contributed to editing the manuscript.

1.4 Contribution to paper IV (Appendix)

I wrote, revised the manuscript, submitted it for publication and integrated reviewer comments towards publication with the journal.

All co-authors confirmed with their signature that Caleb Nwongbouwoh Muefong has the permission to use the three publications for his thesis. Furthermore, they confirmed that the publications are not part of other doctoral theses. Caleb Nwongbouwoh Muefong submitted the list with all signatures separately with this medical thesis.

2. Introduction

2.1 Rationale and Objectives

2.1.1 Rationale

Despite microbiological cure, up to 50% of patients with active Tuberculosis (ATB) suffer from long-term lung damage after successful therapy [1]. This leads to reduced quality of life and a loss of 4.89 potential years of life [2]–[4]. Host-directed therapies (HDT) could reduce the burden of post-TB lung pathology but development of these requires a better understanding of the underlying pathological mechanisms in order to identify optimal targets for therapeutic intervention [5]–[10]. Severe lung damage is caused by exacerbated inflammatory responses in some patients; often linked to pro-inflammatory neutrophil activity [11]–[14]. However, recent studies have revealed significant neutrophil phenotypic and functional diversity with both pro-inflammatory and immunosuppressive sub-types [15]–[21]. This suggests that analysis of specific neutrophil subsets/mediators during ATB could enable identification of targets for novel host-directed therapies [7], [13], [22], [23].

Neutrophils are the first cells recruited to the lungs during ATB. While neutrophilic responses are generally pro-inflammatory, some neutrophil phenotypes have been shown to possess both pro- and anti-inflammatory functions in different disease states. It is possible that these differences in neutrophil functions could be exploited in host-directed therapies to reduce the burden of post-TB lung disease. This is the first study to assess the different neutrophil phenotypes, functional markers and their derived metabolites (pro- and anti-inflammatory) in relation to TB disease severity and lung recovery.

2.1.2 Study aims and objectives

The overarching aim of these doctoral studies was to determine if different neutrophil subsets play a role in lung damage at diagnosis, and recovery post-therapy. In addition, I aimed to identify specific neutrophil mediators that could potentially be harnessed in HDTs to reduce post-TB lung burden.

The specific objectives of my PhD studies were:

Objective 1: To determine the phenotype and function of neutrophil subsets in active TB and to correlate these with baseline lung pathology, bacterial load and post-TB lung recovery.

Objective 2: To determine the activation and functional dynamics of these subsets before and after TB treatment.

Objective 3: To identify predominant neutrophil-derived inflammatory mediators contributing to baseline lung damage and recovery after treatment.

Objective 4: To validate (in whole blood) previously described phenotypic, activation and cytokine markers of treatment response in our Gambian cohort.

2.2 Clinical severity of TB lung disease

ATB associated lung pathology can be determined by analysis of lung cavities through chest x-rays (CXR) (reviewed by [24]) and monitoring of pulmonary function using spirometry [25]. Disease severity may also be measured by the bacterial burden in lungs prior to initiation of treatment. Interestingly, CXR abnormalities are more reliable than spirometry for assessing lung impairment in ATB [26]. Moreover, lung damage is only weakly associated with high Mtb loads [27] and inversely proportional to spirometry evaluations [26]. Studies evaluating TB disease severity using multiple criteria are lacking which limits our capacity to draw holistic conclusions. To address these limitations, a multisite trial (TB Sequel) is underway to monitor host-pathogen and socioeconomic factors that influence the development of pulmonary sequelae in ATB patients [1]. It is within this cohort that we assessed the role of neutrophils in the severity of TB-indued lung pathology using CXR-defined Ralph score [28] and the GeneXpert-defined cycle threshold (Ct) values [29].

2.3 Heterogeneity of neutrophil response in lung pathology

I have published a review article on the role of neutrophils in ATB-induced inflammation and pulmonary pathology [30]. In this “additional contribution”, I showed that neutrophil activity during ATB could inform us on long-term lung disease outcomes in ATB and identified suitable neutrophil profiles for improved TB-focused HDT.

Additional contribution: Neutrophils in Tuberculosis-Associated Inflammation and Lung Pathology (see Appendix A).

In this review, I set a prelude for my PhD research work by describing the central role played by neutrophils in driving tissue damage in chronic inflammatory diseases and compiling information from the literature which suggests that neutrophils could play both pro- and anti-inflammatory roles, specifically in TB disease. I also highlighted the growing need to standardize neutrophil classification based on functional outcomes in diseased states and assessed previous studies for the most promising criteria for classifying neutrophils into either protective or destructive subtypes. CD16 and CD62L-defined neutrophils were found to be the most suitable as they not only allowed for separating neutrophils into subtypes with observed pro-inflammatory or immunosuppressive functions, but also ascribed to functional nuclear segmentation-defined classification (with banded ($CD16^{dim}CD62L^{br}$), segmented ($CD16^{br}CD62L^{br}$) and hyper-segmented

($CD16^{br}CD62L^{low}$) phenotypes). Additionally, I discussed the issues with existing methods for categorising TB lung pathology including structural and functional classifications. Finally, I concluded by proposing neutrophil-derived mediators and pathways (notably, MMPs, ROS, MPO, S100A8/A9 and Glutathione) which could be targeted to develop host-directed therapies for reducing ATB-associated lung pathology.

2.4 Monitoring neutrophil activation and function

Neutrophils react to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) [31] which trigger proinflammatory and antimicrobial responses in innate immune cells [32]. Thus, the use of Mtb-H37Rv whole cell lysate (WCL) or the early secretory antigenic target protein 6 and culture filtrate protein 10 complex (ESAT-6/CFP-10) fusion protein which contain Mtb-specific molecular structures and nucleic acids (PAMPs) can be used to elicit neutrophilic Mtb-specific responses. These suggest that measuring neutrophil phenotypic and functional variations in ATB patients using stimulated whole blood will provide insight into how neutrophil activity is regulated during Mtb infection; thereby capitalizing on research developments in neutrophil biology. *In vitro*, whole blood has an added advantage over cell isolates in the fact that it reduces the probability of observing non-physiological immune responses which could be introduced by sample processing. In neutrophil assays specifically, using whole blood is advantageous as it reduces the risks of neutrophil activation and necrosis due to their high susceptibility to stimuli and short life span, respectively.

Publication I: Neutrophils contribute to severity of tuberculosis pathology and recovery from lung damage pre- and post-treatment

As shown in *publication I* [33] neutrophil subtypes contribute to ATB-related disease severity. In the first part of this study, we confirmed the phenotypic and functional heterogeneity of CD16|CD62L-defined neutrophils. We observed an overall decrease in neutrophil levels at the end of treatment compared to baseline with frequencies of segmented ($CD16^{br}CD62L^{br}$) neutrophils increasing and banded ($CD16^{dim}CD62L^{br}$) neutrophils decreasing. We also found a lower frequency of banded neutrophils in patients with severe compared to mild lung damage at baseline. Following WCL stimulation, the neutrophil oxidative indices of segmented, banded and hyper-segmented neutrophils were higher in patients with low Mtb loads while IL10-expressing $CD16^{dim}CD62L^{lo}$ neutrophils were higher in patients with mild damage at baseline. Additionally, patients with good lung recovery had higher baseline granulocyte frequencies with WCL and EC stimulation than those with poor lung recovery.

Our findings show that high ROS generation capacity, low levels of banded neutrophils and high levels of IL10-expressing $CD16^{dim}CD62L^{lo}$ neutrophils result in reduced lung pathology during ATB at diagnosis. Furthermore, we demonstrate that baseline granulocyte levels are useful in distinguishing patients with good lung recovery from those with poor recovery post-treatment. Hence, neutrophils are potential early indicators of TB severity and CD16|CD62L-derived neutrophil subtypes are promising targets for TB host-directed therapy. To our knowledge, this is the first study to assess the function of neutrophils and their subsets in TB. We obtained these results in a cohort of 40 participants which we would have preferred to be larger, but the number of month 6 patient follow-ups were limited by participant dropouts and lockdowns imposed during the

Covid-19 pandemic. Smoking increases lung damage severity; however, we did not adjust for smoking habits due to limited number of samples. We plan to build-up on these initial observations with functional analysis on a larger cohort of ATB patients. It would also be of interest to perform mycobacterial killing assays to confirm the protective role of ROS on Mtb-burden and TB disease progression as well as banded neutrophils on lung damage. Analysing myeloid-derived suppressive cells, a newly identified group of granulocytes with T-cell and natural killer cell-targeted immunosuppressive functions, would also be of particular importance in describing the protective role of neutrophils in TB pathogenesis and related-lung pathology.

Publication II: Major neutrophil-derived soluble mediators associate with baseline lung pathology and post-treatment recovery in tuberculosis patients

Having shown that neutrophil subsets had differential impacts on lung pathology, *publication II* (*Muefong et al. Front Imm, 2021; In Press*) sought to determine which specific mediators in sputum and plasma could differentiate between patients with severe and mild lung damage and between high and low Mtb load groups before and after treatment. While the levels of most neutrophil mediators were considerably higher in sputum samples, the differences in sputum or plasma analyte concentrations between severity groups were generally consistent (irrespective of statistical significance). This suggests that plasma could be used as a suitable surrogate to sputum in monitoring neutrophil-specific biomarker levels in TB patients. By focusing on neutrophil-derived inflammatory mediators with known regulatory functions in TB inflammation, I demonstrated that major neutrophilic mediators are associated differentially with TB-induced lung pathology and recovery. I also found that patients with high Mtb load or severe lung damage at baseline present with similar sputum Myeloperoxidase (MPO) profiles. This is a unique observation which not only underscores the protective role of MPO in ATB lung pathology but also describes a potential link between these two clinically relevant parameters used for assessing the severity of TB-related lung pathology. Furthermore, we observed that male patients were associated with lower sputum MPO levels than females whilst the former were also associated with higher levels of proinflammatory mediators like MMP3, IL8, IL10, IL12/23(p40), GM-CSF and TNF. This also supports the protective role played by MPO given that males generally mount more severe inflammatory responses than females. We also made similar observations to previous studies by showing an association between Neutrophil collagenase (MMP8), Calprotectin (S100A8/9) and TNF levels in sputum and plasma with severe lung damage. Additionally, ATT led to an overall decrease in inflammatory mediator levels compared to baseline values with the decrease being significantly higher in (and sometimes exclusive to) patients with severe forms of ATB. Moreover, we found that patients who had severe lung disease or high Mtb loads at baseline also had significantly higher levels of S100A9 and IL8 in the sputum samples, suggesting that severe lung damage increases the likelihood of unresolving inflammatory response even after treatment. Furthermore, unresolved lung damage after treatment was linked to higher MMP9 and S100A9 sputum levels at month 6, suggesting that persistent neutrophil activity also promotes unresolved lung damage even after treatment. While supernatants generated following antigen stimulation showed fewer differences between the groups, we nevertheless found that levels of MPO, S100A8 and MMP9 at month 6 following WCL stimulations could differentiate between patients with good and poor lung recovery. Additionally, H37Rv WCL stimulation resulted in increased levels of GM-CSF, IFNγ, TNF, MPO, S100A8 and MMP9 after treatment compared to baseline. This suggests an increased sensitivity of neutrophils and potentially other immune cells to stimulation.

Hence, this study suggests that S100A8/9, MMP8, MPO and TNF may serve as targets for host-directed therapies to reduced long-term lung damage in TB patients. However, mechanistic studies such as *in vitro* or *ex vivo* experiments and animal models specifically targeting central pathways that modulate one or more of these metabolites would need to be performed to determine the optimal therapy. This study also demonstrated the ability to perform simultaneous monitoring of pro-inflammatory and immunosuppressive neutrophil subtypes to address their specific contributions to lung pathology outcomes in ATB patients. Our study could have been improved by assessment of neutrophil extracellular trap (NET) formation, given that it is a major neutrophil functional attribute associated with increased inflammation in ATB. Measuring levels of other neutrophilic inflammatory mediators associated with tissue damage (e.g., IL17 and serine proteases such as proteinase 3, neutrophil elastase and cathepsin G) would also be important to draw a more wholistic picture of their effect on TB lung pathology.

Publication III: Monitoring Anti-tuberculosis Treatment Response Using Analysis of Whole Blood Mycobacterium tuberculosis Specific T Cell Activation and Functional Markers.

In a separate study, *publication III* [34], we measured T cell specific markers in two patient groups defined by their response to treatment in order to assess clinical utility of T cell activation markers in whole blood samples from TB patients in a West African cohort for the first time. We showed that T-cell phenotypes and functional responses could be effectively monitored in whole blood serving as an alternative proxy to sputum-based methods for assessing treatment responses during anti-TB treatment (ATT) [34]. This study also validated the use of whole blood-based flow cytometry assessment of Mtb-induced immune response in our west-African cohort of ATB patients. Having shown that standard TB therapy (in addition to neutrophil activity) influences lung recovery, we complemented this with evidence that activated T cell subsets could predict treatment response speed. This pilot study confirmed that T-cell activity following Mtb-specific stimulation in whole blood samples from TB patients in The Gambia could be used to monitor treatment response rates during ATT. We showed that following purified protein derivative (PPD) stimulation, baseline levels of $CD8^{+}CD27^{-}IFN\gamma^{+}$ and $CD4^{+}CD27^{+}HLA^{-}DR^{+}CD38^{+}$ T-cell subsets were predictive of the of treatment response rates at 80% sensitivity and 70% and 100% specificities, respectively. Additionally, we observed a decrease in levels of T-cell activation markers (CD38 and HLA-DR) in $CD4^{+}CD27^{+}$ T-cells at 2 months compared to baseline. Hence, these T-cell subsets could be used to improve the positive predictive value for risks of culture positivity after 2 months of ATT. This drop in T-cell activation markers coupled to decreased neutrophil activation with treatment suggests that standard ATT contributes to the resolution of Mtb-induced inflammation by reducing both innate and adaptive immune responsiveness. Pending confirmation of these findings in a larger cohort and adjustment for possible confounders like BMI, diabetes mellitus, alcohol abuse and delay in presentation, this study is very promising and attests of the need to study subsets of T- and other major immune cell types as diagnostic tools in TB treatment response and lung outcomes. We are currently analysing levels of immune cell subsets in frozen whole blood from a larger cohort TB patients.

3. Summary (in English)

Exacerbated neutrophil activity is generally linked to Tuberculosis (TB) disease severity. However, TB-induced lung severity is dynamic with equal proportions of patients showing mild vs severe forms of lung damage after successful treatment. With recent studies revealing previously unseen neutrophil heterogeneity, it is likely that differential neutrophil profiles in patients contribute to the extent of disease severity and lung recovery. Hence, monitoring neutrophil responses could allow identification of target molecules for host-directed therapies which can be coupled to antibiotic TB treatment (ATT) to limit lung damage and promote good lung recovery. The underlying hypothesis for this thesis was that neutrophil heterogeneity is associated with differences in severity of lung pathology pre- and post-TB treatment in ATB patients. The main aim was to assess neutrophil phenotypes and function, and their soluble mediators in patients with differential lung function before and after TB therapy in The Gambia.

First, focusing on ATB-related lung pathology, TB patients were grouped based on two clinically relevant parameters of ATB severity: chest x-ray scores (based on the well-defined Ralph Score incorporating extent of lung infiltrate and presence or absence of cavities) and GeneXpert Ultra bacterial load (Cycle threshold (Ct) value). For patients with severe lung damage at baseline, Ralph scores were also used to determine if they had good or poor recovery of lung pathology post treatment. We are currently analysing lung function recovery post treatment using spirometry readouts to complement the present findings. Analysis of neutrophils using stimulation of fresh whole blood was performed for the first time in The Gambia to monitor neutrophil function and determine differences between groups at baseline and following treatment. Considering that neutrophils react to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) which trigger proinflammatory and antimicrobial responses in innate immune cells, I used Mtb H37Rv whole cell lysate (WCL) and ESAT-6/CFP-10 fusion protein which contain Mtb-specific molecular structures and nucleic acids (PAMPs) to elicit neutrophilic Mtb-specific response. Unstimulated blood samples were used to account for basal inflammatory levels and phorbol, 12-myristate, 13-acetate (PMA) was used as a positive control.

Neutrophil subsets have been identified based on their CD16 and CD62L expression levels and shown to have varying functional attributes. Activated, they secrete differing levels of pro- and anti-inflammatory cytokines and regulate the inflammatory response to Mtb. In this study, we reported the relationship between functionality of the subsets following *in-vitro* activation and TB disease severity. At baseline, flow cytometry revealed WCL-specific activation of multiple neutrophil subsets, increased ROS generation capacity, increased levels of banded neutrophils ($CD16^{dim}CD62L^{br}$) and higher levels of IL10-expressing $CD16^{dim}CD62L^{lo}$ neutrophils in patients with mild lung pathology compared to severe pathology. However, TB-specific stimulation did not result in any differences in frequencies of these phenotypes with treatment time. Meanwhile, there was heterogeneity in frequencies of CD16|CD62L-defined neutrophil subsets in unstimulated samples with treatment compared to baseline.

Having observed that neutrophil subsets play a role in ATB-related lung damage severity at baseline, we measured the concentrations of major neutrophil-derived mediators in antigen-stimulated WBA supernatants, together with *ex vivo* plasma and sputum samples from the same patients. This downstream assay involved analysis of a comprehensive neutrophil-focused panel from priming/activation markers like GM-CSF, IL8/CXCL8, TNF and IFNγ, through to damage-associated molecular pattern (DAMP), Calprotectin (S100A8/9), to IL12/23(p40) and IL10 which possess regulatory roles and finally matrix metalloproteinases (MMP1, MMP3, MMP9, MMP8) and

myeloperoxidase (MPO) which regulate tissue damage. Overall, patients with severe lung pathology at baseline also had higher levels of S100A8/9, MMP8 and TNF. High S100A8/9 levels in patients with severe lung damage is in accordance with the increased neutrophil levels and neutrophil activation seen by flow cytometry. S100A8/A9 has been previously shown to regulate CD11b expression and neutrophil recruitment. Moreover, our data showed that MMP8 association with TB disease severity is on par with that of S100A8/9, which to our knowledge is novel and underscores the importance of targeting this protein in further mechanistical studies.

Interestingly, MPO was the only neutrophil-derived marker to be associated with all three disease severity criteria and the only one (together with IL10) to be associated with a protective effect against lung damage. Lower levels of MPO in sputum were associated with more severe lung damage, higher Mtb burden and poorer lung recovery after treatment. Lower levels of MPO were also found in females: who generally develop less severe inflammatory responses compared to males. MPO is also closely linked to ROS metabolism as it catalyses peroxide degradation. Whilst both MPO and ROS are usually linked to increased acute inflammation, our observations reveal their unexpected protective role—in limiting pulmonary pathology and resolving inflammation—during ATB. This supports investigating their potential for host-directed therapies. Finally, unresolved lung damage after treatment was associated with persistently high sputum MMP9 and S100A9 levels at month 6 despite microbiological clearance. MMP9 and S100A8/9 are neutrophil-derived molecules reported to exacerbate tissue damage in chronic TB, which suggests that unresolved lung damage even after treatment is also influenced by persistent neutrophil activity.

In conclusion, our study contributes first-hand knowledge to the field by demonstrating that unlike other major neutrophil-derived inflammatory mediators (S100A8/9 and MMP8) and neutrophil subtypes, MPO, ROS (against Mtb burden), IL10 and banded ($CD16^{dim}CD62L^{br}$) neutrophils, play a protective role against TB-related lung pathology. These observations support the hypothesis that neutrophils harbour both pro-inflammatory and immunosuppressive characteristics in chronic TB pathogenesis. It also supports targeting neutrophils in further studies aimed at developing host-directed therapies against severe pathology in chronic ATB patients.

4. Zusammenfassung (deutsch)

Eine erhöhte Neutrophilenaktivität wird häufig mit der Schwere der Tuberkulose (TB) in Verbindung gebracht. Die Schwere der TB Lungenerkrankung ist jedoch dynamisch, wobei der Anteil der Patienten, die nach erfolgreicher Behandlung leichte und schwere Formen der Lungenschädigung aufweisen, in etwa gleich groß ist. Studien, welche eine hohe Heterogenität der Neutrophilen aufzeigen, lassen vermuten, dass unterschiedliche Neutrophilenpopulationen bei den Patienten das Ausmaß der Krankheitsschwere und der Lungenheilung beeinflussen. Daher könnte das Monitoren verschiedener Neutrophilenpopulationen und deren Effektormoleküle die Identifizierung von Zielmolekülen für wirtsspezifische Therapien ermöglichen. Solche wirtsspezifischen Therapien könnten dann mit einer antibiotischen Tuberkulosebehandlung (ATT) kombiniert werden, um wiederum Lungenschäden zu begrenzen und eine gute Lungenheilung zu fördern. Die dieser Arbeit zugrunde liegende Hypothese war, dass Unterschiede in neutrophilen Zellpopulationen und Effektormolekülen mit Unterschieden in der Lungenpathologie vor und nach der Tuberkulosebehandlung einhergehen. Das Hauptziel bestand also darin, Phänotyp und die Funktion der Neutrophilen Zellpopulationen sowie ihre löslichen Mediatoren bei Patienten mit unterschiedlicher Lungenfunktion vor und nach einer TB-Therapie in Gambia zu untersuchen.

Zunächst wurden die TB-Patienten auf der Grundlage von zwei klinisch relevanten Parametern für den Schweregrad der ATB in Gruppen eingeteilt: Thoraxröntgen-Scores (basierend auf dem gut definierten Ralph-Score, der das Ausmaß des Lungeninfiltrats und das Vorhandensein oder Fehlen von Hohlräumen (engl: „Cavities“) und die GeneXpert-Ultra-Bakterienlast (PCR Cycle-Schwellenwert (Ct)). Bei Patienten mit schweren Lungenschäden zu Beginn der Studie wurden die Ralph-Scores auch verwendet, um zu untersuchen ob sich die Lungenpathologie nach der Behandlung verbesserte. Derzeit analysieren wir die Erholung der Lungenfunktion nach Behandlung anhand von Spirometrie-Messungen, um die vorliegenden Ergebnisse weiter zu ergänzen. Die Analyse der Neutrophilen durch Stimulation von frischem Vollblut wurde durchgeführt, um die Neutrophilenfunktion zu studieren und Unterschiede zwischen den Gruppen zu Beginn und nach der Behandlung festzustellen. In Anbetracht der Tatsache, dass Neutrophile auf pathogen- und schadensassoziierte molekulare Muster (*PAMPs* bzw. *DAMPs*) reagieren, die proinflammatorischen und antimikrobiellen Reaktionen in angeborenen Immunzellen auslösen, wurden Mtb H37Rv-Vollzelllysat (WCL) und ESAT-6/CFP-10-Fusionsprotein verwendet, die Mtb-spezifische molekulare Strukturen und Nukleinsäuren (PAMPs) enthalten. Unstimulierte Blutproben wurden verwendet, um basale Entzündungswerte zu berücksichtigen, und Phorbol-12-Myristat-13-Acetat (PMA) diente als Positivkontrolle.

Unterschiedliche neutrophile Zellpopulationen wurden auf der Grundlage ihrer CD16- und CD62L-Expressionsmuster identifiziert, welche unterschiedliche funktionelle Eigenschaften aufweisen. Wenn sie aktiviert sind, scheiden sie unterschiedliche Mengen an pro- und anti-inflammatorischen Zytokinen aus und regulieren die Entzündungsreaktion auf Mtb auf unterschiedliche Art und Weise. In dieser Studie untersuchten wir den Zusammenhang zwischen der Funktionalität der unterschiedlichen neutrophilen Zellpopulationen nach in-vitro-Aktivierung und dem Schweregrad der TB-Erkrankung. Zu Beginn der Studie zeigten die Durchflusszytometrieergebnisse eine WCL-spezifische Aktivierung mehrerer neutrophiler Subpopulationen, eine erhöhte ROS-Erzeugungskapazität, erhöhte Mengen an gebänderten Neutrophilen (CD16dimCD62Lbr) und höhere Mengen an IL10-exprimierenden CD16dimCD62Llo-Neutrophilen bei Patienten mit leichter Lungenpathologie im Vergleich zu solchen mit schwerer Pathologie. Die TB-spezifische Stimulation führte jedoch nicht zu Unterschieden in der Häufigkeit dieser Phänotypen mit der Behandlungsdauer. Die Häufigkeit der CD16|CD62L-definierten neutrophilen

Untergruppen in den nicht stimulierten Proben war im Vergleich zum Ausgangswert vor der Behandlung heterogen.

Nachdem wir festgestellt hatten, dass unterschiedliche neutrophile Zellpopulationen bei der Schwere der ATB-bedingten Lungenschädigung vor Behandlungsbeginn eine Rolle spielen, haben wir die Konzentrationen der wichtigsten neutrophilen Effektormediatoren in antigenstimulierten Vollblut-Überständen zusammen mit Plasma- und Sputumproben derselben Patienten gemessen. Unser Assay beinhaltete die Analyse von Neutrophilen-assoziierten Zytokinen und weiteren Effektormolekülen wie GM-CSF, IL8/CXCL8, TNF und IFNγ, Calprotectin (S100A8/9), IL12/23(p40) und IL10, und schließlich Matrix-Metalloproteinasen (MMP1, MMP3, MMP9, MMP8) und Myeloperoxidase (MPO), die Gewebeschäden regulieren. Insgesamt wiesen Patienten mit schwerer Lungenpathologie bei Studienbeginn höhere Werte von S100A8/9, MMP8 und TNF auf. Die hohen S100A8/9-Werte bei Patienten mit schweren Lungenschäden stehen im Einklang mit den erhöhten Neutrophilenspiegeln und deren Aktivierung. Es wurde bereits gezeigt, dass S100A8/A9 die CD11b-Expression sowie die Rekrutierung von Neutrophilen reguliert. Darüber hinaus zeigten unsere Daten, dass die MMP8 Konzentration mit dem Schweregrad der TB-Erkrankung und der von S100A8/9 korreliert, was neu ist und in weiteren mechanistischen Studien untersucht werden sollte.

Interessanterweise war MPO der einzige von Neutrophilen stammende Marker, der mit allen drei Kriterien für den Schweregrad der Erkrankung assoziiert war, und zusammen mit IL10 der einzige Marker, der mit geringen Lungenschäden assoziiert war. Niedrigere MPO-Werte im Sputum wurden mit schwereren Lungenschäden, einer höheren Mtb-Belastung und einer schlechteren Erholung der Lunge nach der Behandlung in Verbindung gebracht. Niedrigere MPO-Werte wurden auch bei Frauen festgestellt, die im Allgemeinen eher weniger schwere Entzündungsreaktionen entwickeln als Männer. MPO ist auch eng mit dem ROS-Stoffwechsel verbunden, da es den Peroxidabbau katalysiert. Während MPO und ROS in der Regel mit einer verstärkten akuten Entzündung in Verbindung gebracht werden, zeigen unsere Beobachtungen eine unerwartete schützende Rolle - die Begrenzung der TB Lungenpathologie und das Abklingen der Entzündung. Dies spricht dafür, das Potenzial von MPO auch für wirtsspezifische Therapien zu untersuchen. Schließlich wurden Lungenschädigungen, welche nach der erfolgreichen 6-monatigen Behandlung und mikrobiologischer *Clearance* immer noch evident waren, mit hohen Sputum MMP9 and S100A9 Werten assoziiert. MMP9 und S100A8/9 sind von Neutrophilen stammende Moleküle, welche die Gewebeschäden bei chronischer Tuberkulose verschlimmern. Dies deutet darauf hin, dass diese persistierenden Lungenschäden auch nach der Behandlung durch die anhaltende Aktivität der Neutrophilen beeinflusst werden.

Zusammenfassend lässt sich sagen, dass unsere Studie neue Erkenntnisse aus erster Hand liefert. Sie weisen darauf hin, dass im Gegensatz zu anderen wichtigen neutrophilen Entzündungsmediatoren (S100A8/9 und MMP8) und Neutrophilen-Subtypen, MPO, ROS (gegen die Mtb-Belastung), IL10 und stabkernige (CD16dimCD62Lbr) Neutrophile eine schützende Rolle gegen TB-bedingte Lungenpathologie spielen. Diese Beobachtungen stützen die Hypothese, dass Neutrophile bei der chronischen TB-Pathogenese sowohl entzündungsfördernde als auch immunosuppressive Eigenschaften aufweisen können. Sie sprechen auch dafür, neutrophile Zellpopulationen und Effektormoleküle in weiteren Studien zur Entwicklung wirtsspezifischer Tuberkulosetherapien zu berücksichtigen.

5. Paper I

Clinical Infectious Diseases

MAJOR ARTICLE

Neutrophils Contribute to Severity of Tuberculosis Pathology and Recovery From Lung Damage Pre- and Posttreatment

Caleb Nwongbouwoh Muefong,[1,2] Olumuyiwa Owolabi,[1] Simon Donkor,[1] Salome Charalambous,[3] Abhishek Bakuli,[4] Andrea Rachow,[4,5] Christof Geldmacher,[4] and Jayne S. Sutherland[1]

[1]Vaccines and Immunity Theme, Medical Research Council Unit The Gambia, London School of Hygiene and Tropical Medicine, Fajara, The Gambia; [2]University of Munich, Munich, Germany; [3]School of Public Health, Aurum Institute, Johannesburg, South Africa; [4]Division of Infectious Diseases and Tropical Medicine, University Hospital, University of Munich, Munich, Germany; and [5]German Centre for Infection Research, partner site Munich, Germany

Background. Despite microbiological cure, about 50% of tuberculosis (TB) patients have poor lung recovery. Neutrophils are associated with lung pathology; however, CD16/CD62L-defined subsets have not been studied in TB. Using flow cytometry, we monitored frequencies, phenotype, and function of neutrophils following stimulation with *Mycobacterium tuberculosis* (*Mtb*) whole cell lysate (WCL) and ESAT-6/CFP-10 fusion protein (EC) in relation to lung pathology.

Methods. Fresh blood from 42 adult, human immunodeficiency virus (HIV)–negative TB patients were analyzed pre- and post-therapy, with disease severity determined using chest radiography and bacterial load. Flow cytometry was used to monitor frequencies, phenotype, and function (generation of reactive oxygen species [ROS], together with CD11b, tumor necrosis factor, and interleukin 10 [IL-10] expression) of neutrophils following 2-hour stimulation with *Mtb*-specific antigens.

Results. Total neutrophils decreased by post-treatment compared to baseline ($P = .0059$); however, $CD16^{br}CD62L^{br}$ (segmented) neutrophils increased ($P = .0031$) and $CD16^{dim}CD62L^{br}$ (banded) neutrophils decreased ($P = .038$). Banded neutrophils were lower in patients with severe lung damage at baseline ($P = .035$). Following WCL stimulation, ROS from segmented neutrophils was higher in patients with low *Mtb* loads even after adjusting for sex ($P = .038$), whereas IL-10–expressing $CD16^{dim}CD62L^{lo}$ cells were higher in patients with mild damage ($P = .0397$) at baseline.

Conclusions. High ROS generation, low levels of banded neutrophils, and high levels of IL-10–expressing $CD16^{dim}CD62L^{lo}$ neutrophils are associated with reduced lung pathology at diagnosis. Hence, neutrophils are potential early indicators of TB severity and promising targets for TB host-directed therapy.

Keywords. tuberculosis; neutrophils; immunosuppression; inflammation; lung damage.

Mycobacterium tuberculosis (*Mtb*) causes tuberculosis (TB) which, despite being curable, is the single deadliest infectious disease known to humans, with about 10 million cases in 2019 and 1.4 million deaths [1]. While there is an 85% treatment success rate in human immunodeficiency virus (HIV)–negative patients, about 50% of treated individuals suffer from any type of post-TB lung disease, irrespective of smoking habits [2–4].

Patients with severe lung damage at diagnosis are more likely to experience lasting pulmonary disability [5, 6], suggesting that early diagnosis and treatment initiation are important in limiting residual impairment. It is likely that exacerbated inflammatory response against *Mtb* prior to diagnosis results in more severe lung pathology [7]. Hence, understanding the heterogeneity from host–*Mtb* interactions and infectious outcomes [8] is crucial to improving treatment outcomes. This may be possible by reducing tissue damage and enhancing *Mtb* clearance with host-directed therapies [9, 10].

Neutrophils are a heterogeneous population whose combined activity results in different extent of inflammation and disease outcomes [11–17]. Pillay and colleagues [18] ascribed $CD16^{br}CD62L^{br}$, $CD16^{dim}CD62L^{br}$, and $CD16^{br}CD62L^{low}$ neutrophils to segmented, banded, and hypersegmented subsets, respectively. Recently, Tak and collaborators [19] showed that neutrophils exhibiting low CD62L expression are distinct from segmented and banded subsets. Indeed, segmented neutrophils are the only phenotype circulating in homeostatic conditions and hypersegmented neutrophils suppress T-cell activation [18]; meanwhile, banded neutrophils exhibit efficient migration [20], exhibit superior bacterial containment in acute inflammation [13], and are more abundant in patients who develop infectious complications [21]. Moreover, the percentage and absolute number of banded neutrophils correlate positively with TB-induced lung damage [22, 23].

Received 23 May 2021; editorial decision 20 August 2021; published online 24 August 2021.
Correspondence: C. Nwongbouwoh Muefong, Medical Research Council Unit The Gambia, London School of Hygiene and Tropical Medicine, Fajara site, Flat 3D, Atlantic Blvd, PO Box 273, Banjul, The Gambia (caleb.muefong@lshtm.ac.uk).
Clinical Infectious Diseases® 2021;XX(XX):0–0

https://doi.org/10.1093/cid/ciab729

Neutrophils are reactive to a wide range of stimuli, especially pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [24], and exposure to these enhances inflammatory and antibacterial responses and cell recruitment [25]. Kroon and collaborators [26] have revealed that increased neutrophil numbers are linked to excessive inflammation and severe Active TB (ATB) pathology. However, neutrophil numbers alone do not account for differences in disease severity observed in patients at diagnosis. Hence, the frequencies, levels of activation, and functionality of different neutrophil phenotypes may be determinant factors of ATB severity.

Studies on the influence of different neutrophil subsets on specific inflammatory pathologies are scarce. CD16 and CD62L expression appear to provide a common ground for neutrophil identification, reconciling neutrophil granularity, density, and expression of key activation markers. Furthermore, functional attributes of different neutrophil subsets (based on CD16 and CD62L expression) have not been studied in TB to date. We hypothesize that variable frequencies and functionality of these subsets may explain the different levels of lung damage seen in ATB patients at baseline, and lung recovery after therapy. Thus, the aim of this study was to determine the dynamics of these neutrophil subsets at presentation and through standard treatment and to correlate these with severity of lung pathology based on chest radiograph (CXR) scores and *Mtb* load (GeneXpert cycle threshold [Ct] value).

MATERIALS AND METHODS

Participants

A full scope of the methods used are provided in the Supplementary Data. Ethical approval was obtained from the Medical Research Council/The Gambia government joint ethics committee (SCC1523). Adult, HIV-negative TB patients with GeneXpert Ultra (Cepheid)–positive results were recruited from the TB clinic at the Medical Research Council Unit The Gambia at the London School of Hygiene and Tropical Medicine between April 2018 and October 2019 as part of a parent study, TB Sequel [27], after providing written informed consent. Sputum liquid mycobacterial growth indicator tube culture was performed at baseline, 2 months, and 6 months after TB treatment initiation, and blood samples were collected at all time points. All patients were culture negative by 6 months. CXRs were scored using the Ralph score (RS) [28] and patients were classified into mild and severe lung damage groups. Ct values were determined from the GeneXpert readings at baseline and patients were classified into high and low *Mtb* load groups. Meanwhile, lung recovery was based on the ratio of RS at baseline to RS at 6 months for patients with initially severe lung damage, and the patients were classified into good and poor lung recovery groups.

Whole Blood Processing and Stimulation

Venous blood was collected in sodium heparin vacutainer tubes (Becton Dickinson). Full blood counts were performed using a Medonic M-series cell counter (Boule Medical AB, Sweden) and antigen stimulation was performed within 2 hours of collection. Two hundred microliters of whole blood was either left unstimulated (Nil) or stimulated with ESAT-6/CFP-10 (EC) fusion protein, H37Rv whole cell lysate (WCL), or phorbol 12-myristate 13-acetate (PMA). Absolute granulocyte counts were also obtained from whole blood prior to stimulation. Details on the methodology are provided in the Supplementary Data.

Statistical Analysis

For cytokine responses, background was subtracted using the unstimulated (Nil) samples. Differences between baseline, month 2, and month 6 samples within each group were analyzed using a Kruskal-Wallis test followed by Dunn multiple comparisons test as indicated [29]. Differences between paired baseline and month 6 samples were analyzed using a Wilcoxon matched-paired rank test. For comparisons between lung pathology groups, a Wilcoxon rank-sum test was used. The Benjamini-Hochberg test was used to adjust for multiple comparisons. A P value < .05 was considered to be statistically significant. All statistical analyses were performed using R software version 3.5.2 [30].

RESULTS

Patient Demographics

Details about patient demographics are provided in the Supplementary Data. A total of 42 HIV-negative adults with pulmonary TB were recruited, of whom 71% were males (Supplementary Table 1). The median CXR score at baseline was 57.5 (interquartile range [IQR], 25–65) with 21 patients in each of the groups: mild (RS <57.5), of which 38.1% had 1 or more cavities, and severe (RS ≥57.5), of which 85.71% had cavities. For patients with severe damage at baseline, the median change in CXR score (ΔRS) from baseline to 6 months was 6.5 (IQR, 1.59–16.5) with 7 patients in the good recovery group (ΔRS ≥6.5) and 4 in the poor recovery group (ΔRS <6.5). The median CXR scores for the mild and severe groups posttreatment (6 months) were 5 (IQR, 0–10) and 5 (IQR, 5–13.5), respectively. For bacterial load calculations, we analyzed the GeneXpert Ct values for all participants. The median Ct value was 17.6 (IQR, 17.1–18.7) with 20 patients in the high bacterial load group (Ct <17.6) and 21 patients in the low bacterial load group (Ct >17.6). We found no correlation between CXR-derived RS and GeneXpert MTB/RIF Ct (R = –0.23, P = .15) at baseline (data not shown). There was no difference in age between the severity groups in any category (Supplementary Table 1). However, there was a significant association between sex and lung damage (P = .0006) and between sex and *Mtb* load (P = .05) at baseline.

Changes in Neutrophil Frequencies Upon ATB Treatment Reveal Phenotype Heterogeneity

Neutrophil frequencies were determined using a hemoanalyzer, while granulocyte frequencies were obtained by computing the percentage of the granulocyte population from all single cells acquired by flow cytometry (Supplementary Figure 1*D*). The median granulocyte frequencies were 73.9 (IQR, 64.8–78.7) and 59.4 (IQR, 55.2–71.4) at baseline and 48.4 (IQR, 41.7–57.8) and 40.4 (IQR, 30.9–47.4) at 6 months for blood counts and flow cytometry, respectively. Total neutrophil frequencies (using full blood counts: $P = .0059$) and granulocyte frequencies (using flow cytometry: $P = .00004$) in unstimulated samples decreased significantly after treatment (Figure 1A and 1B).

Interestingly, while total neutrophil frequencies decreased, this was subset specific: Frequencies of banded neutrophils decreased whereas segmented neutrophils increased by 6 months of treatment ($P = .038$ and $P = .0031$, respectively; Figure 1C and 1D). There were no significant differences in frequencies of hypersegmented and $CD16^{dim}CD62L^{lo}$ subsets (Figure 1E and 1F). Patients with good lung recovery following treatment had significantly higher granulocyte frequencies following both EC ($P = .011$) and WCL ($P = .042$) stimulation than patients with poor lung recovery (Figure 1G).

Banded and Segmented Neutrophil Levels Correlate With Baseline Lung Pathology

At baseline and in the absence of stimulation, the frequencies of banded neutrophils were significantly lower ($P = .0395$), whereas segmented frequencies were higher but not significant ($P = .0721$) in patients with severe damage compared to those with mild damage (Figure 2A). Unadjusted and adjusted logistic regression modeling of the effect of neutrophil phenotypes on

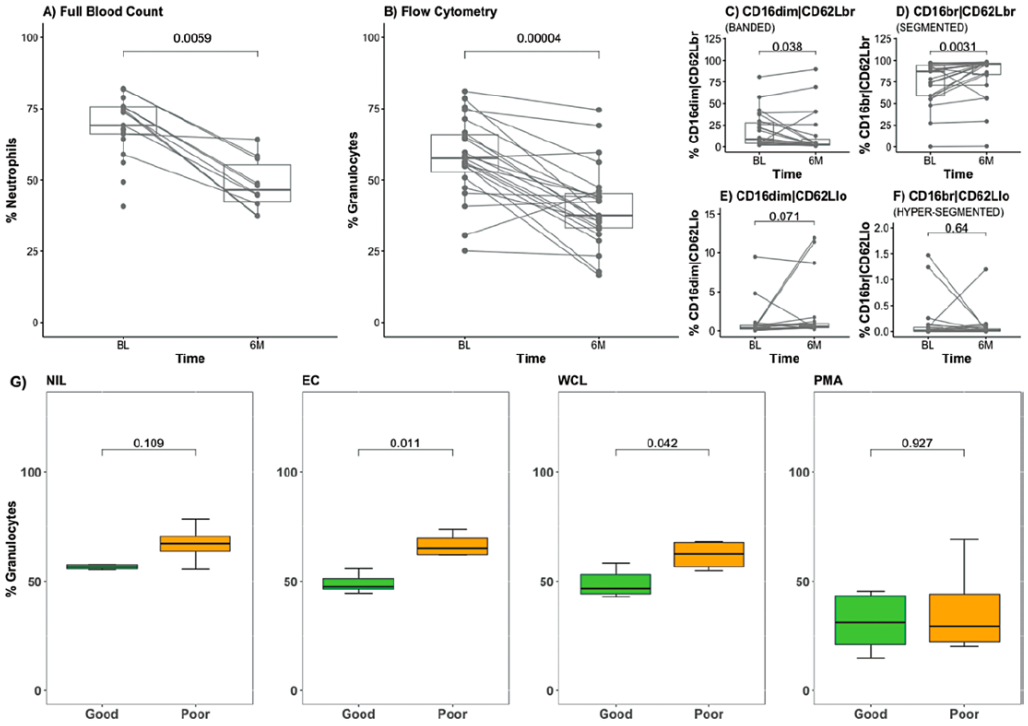


Figure 1. Longitudinal measurement of percentage blood neutrophil during antituberculosis treatment. *A*, Boxplots show median (horizontal line in box) and interquartile range of frequencies of granulocytes before and at treatment completion. The neutrophil counts in active TB patients were measured at baseline and at treatment completion (month 6). Neutrophil frequencies decreased upon treatment completion compared to baseline (n = 42). *B*, Similarly, granulocyte frequencies determined by flow cytometry also decreased after treatment. Immunofluorescence staining following 2-hour stimulation (or unstimulated [Nil]) with ESAT-6/CFP-10 fusion protein (EC), whole cell lysate (WCL), or phorbol 12-myristate 13-acetate (PMA) allowed for subset identification based on CD16 and CD62L expression levels. *C* and *D*, Segmented neutrophils decreased (*C*) and banded neutrophils increased (*D*) as homeostatic conditions were restored in the mild (not severe) group. *E* and *F*, $CD16^{dim}CD62L^{lo}$ (*E*) and hypersegmented neutrophil frequencies (*F*) were unchanged between baseline and treatment completion. Each dot represents 1 individual patient. *P* values were obtained using the Wilcoxon signed-rank test. *G*, Boxplots show frequencies of granulocytes at baseline in good (n = 7) and poor (n = 4) recovery groups. The Wilcoxon signed-rank test was used to analyze differences between lung recovery groups at baseline. Abbreviation: TB, tuberculosis.

lung damage provided further evidence of lower banded neutrophil levels ($P = .035$) in the severe damage group than in the mild damage group (Supplementary Table 2). Conversely, there was no difference in neutrophil frequencies between patients with high vs low *Mtb* loads at baseline (data not shown). At the end of treatment, we found no differences in proportions of neutrophil subsets between either the mild and severe damage or the low and high *Mtb* load groups (data not shown).

The decrease in granulocyte frequencies from baseline to month 6 was more significant in patients with severe damage ($P = .0029$) or high *Mtb* load ($P = .0098$) compared to mild damage ($P = .0161$) or low *Mtb* load ($P = .0186$), respectively (Figure 2B and Supplementary Table 4), as confirmed by coefficients of x being further from the value 1 for both severe/high groups than mild/low groups (Supplementary Figure 2*A* and 2*B*). We also found that the decrease in banded and concomitant increase in segmented neutrophil frequencies from baseline to treatment completion (Figure 1C and 1D) were exclusive to patients in the mild lung damage group ($P = .0024$ and XXX [ns], respectively) and low *Mtb* load group ($P = .032$ and $P = .0098$, respectively) (Figure 2C and 2D).

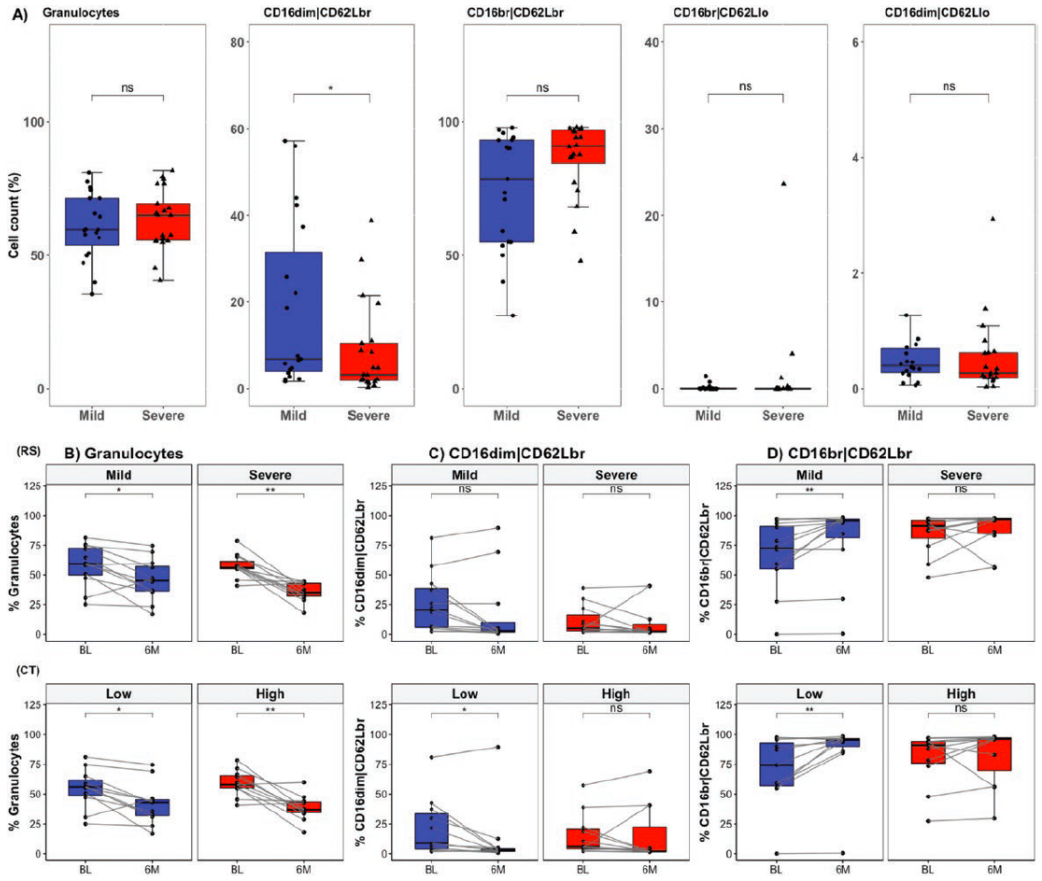


Figure 2. Percentage of neutrophils in severity groups before and after treatment. *A*, CD16/CD62L-defined neutrophil frequencies in active TB patients at baseline were analyzed by flow cytometry within Ralph score–defined mild (n = 21, O) and severe (n = 21, Δ) lung disease groups. Banded ($CD16^{dim}CD62L^{br}$) and segmented ($CD16^{br}CD62L^{br}$) neutrophils were lower and higher, respectively, in the severe group compared to the mild group. Values 2 standard deviations above/below the mean cell count were considered outliers and excluded from analysis. Data are presented as boxplots and analyzed using Wilcoxon rank-sum test. *B*, Granulocyte frequencies decreased with treatment completion. This decrease was more significant in the severe damage and high *Mycobacterium tuberculosis* (*Mtb*) load groups compared to the mild damage and low *Mtb* load groups, respectively. *C*, Compared to baseline, banded neutrophils decreased significantly after treatment in the mild damage (n = 12) and low *Mtb* load (n = 11) groups, but not in the severe damage (n = 11) and high *Mtb* load groups (n = 11). *D*, In contrast, segmented neutrophils increased after treatment compared to baseline in the mild damage and low *Mtb* load groups but not in the severe damage and high *Mtb* load groups. Wilcoxon signed-rank test was used to analyze differences between treatment time within groups. Each dot represents a single patient. $*P < .05$; $**P < .01$. Abbreviations: 6M, month 6; BL, baseline; Ct, cycle threshold; ns, not significant; RS, Ralph score; TB, tuberculosis.

Treatment Leads to Changes in CD11b Expression by Neutrophil Subsets at Baseline

We monitored activation of the different neutrophil subsets by determining CD11b expression before and after treatment. Generally, following *Mtb*-specific stimulation, $CD11b^{+}CD16^{dim}CD62L^{lo}$ neutrophil frequencies decreased after treatment. This trend in $CD11b^{+}CD16^{dim}CD62L^{lo}$ frequency was significant in patients with severe damage ($P = .011$ and $P = .0059$; with EC and WCL, respectively) and low *Mtb* load ($P = .019$ and ns; with EC and WCL, respectively) (Figure 3A). Also, WCL stimulation led to significant increase in $CD11b^{+}$ banded neutrophil frequencies from baseline to treatment completion in patients with high *Mtb* loads ($P = .014$; Figure 3B).

Patients With Low *Mtb* Load at Baseline Have Higher Neutrophil Oxidative Indices

While changes in the frequency of specific neutrophil subsets following treatment appeared to be exclusive to individuals with mild lung damage at diagnosis, we also found that reactive oxygen species (ROS) generation at diagnosis was associated with differences in bacterial load but not lung damage. ROS generation is measured as previously described [31, 32], using the neutrophil oxidative index (NOI),

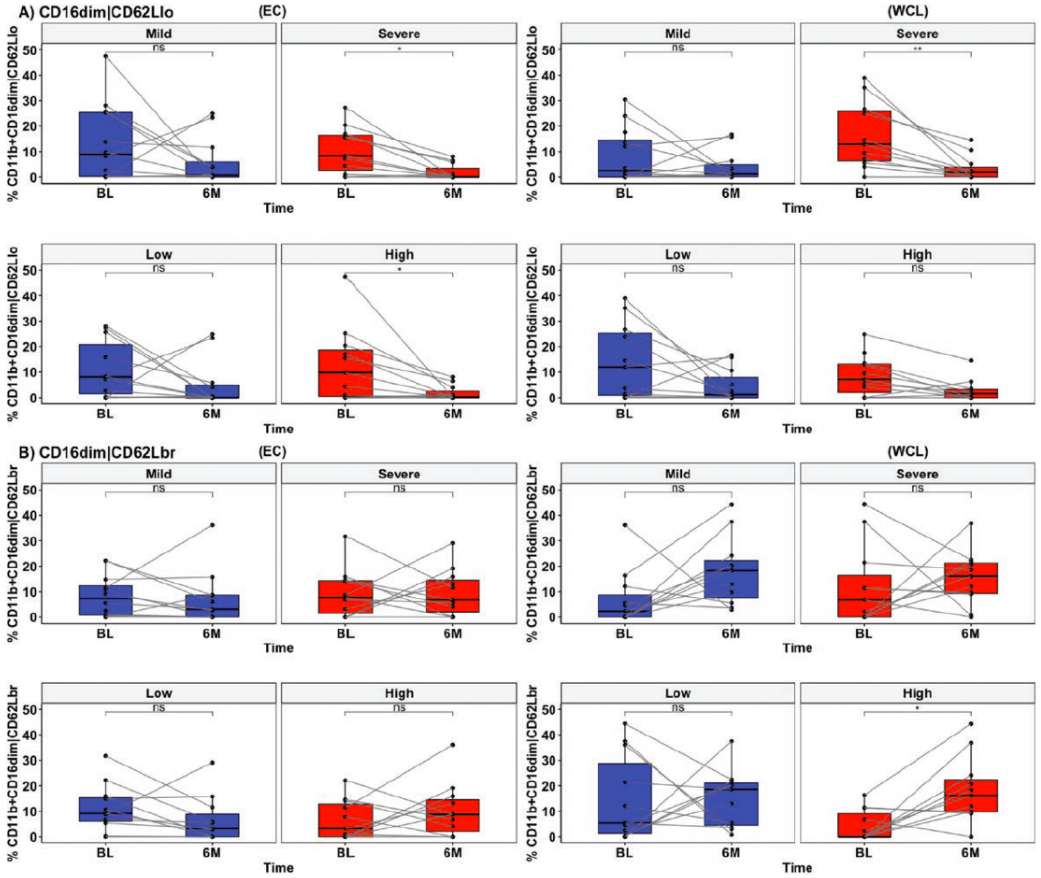


Figure 3. CD11b expression by neutrophils before and after treatment. *A*, Patients with severe lung damage (top panel, n = 11) or high *Mycobacterium tuberculosis* (*Mtb*) load (n = 11, middle panel) showed higher frequencies of $CD11b^{+}CD16^{dim}CD62L^{lo}$ neutrophils upon 2-hour stimulation with *Mtb*-specific antigens (ESAT-6/CFP-10 fusion protein and whole cell lysate [WCL]) at baseline compared to treatment completion; meanwhile, patients with mild lung damage (n = 12) and low *Mtb* load (n = 11) showed no significant difference in the frequency of this phenotype with treatment time. *B*, Patients with high *Mtb* load (n = 11) showed significant difference in the frequency of CD11b-expressing banded neutrophils ($CD11b^{+}CD16^{dim}CD62L^{br}$) with WCL stimulation following treatment compared to baseline; the low *Mtb* load group (n = 11) showed none. Wilcoxon signed-rank test was used to analyze differences between treatment time within groups. $^{*}P < .05$; $^{**}P < .01$. Abbreviations: 6M, month 6; BL, baseline; EC, ESAT-6/CFP-10 fusion protein; ns, not significant; WCL, whole cell lysate.

which is the ratio of the median fluorescence intensity of DHR in stimulated samples (EC, WCL, or PMA) to that of unstimulated controls (Nil). $CD16^{dim}CD62L^{lo}$ neutrophils displayed the highest NOI, yielding on average more ROS than banded ($P = .0017$), segmented ($P = .0172$), or hypersegmented (ns) neutrophils following WCL stimulation at baseline (Figure 4A). The levels of WCL-stimulated ROS generated posttreatment between these subsets were similar (Figure 4B). Furthermore, NOIs of granulocytes, segmented, banded, and hypersegmented neutrophil phenotypes correlated negatively with *Mtb* load following *Mtb*-specific stimulations at diagnosis. These correlations were only significant upon WCL stimulation in total granulocytes ($R = 0.42$, $P = .0062$; Figure 4C), banded neutrophils ($R = 0.58$, $P < .0001$; Figure 4D), and segmented neutrophils ($R = 0.39$, $P = .0098$; Figure 4E). Granulocytes, banded neutrophils, and segmented neutrophils generated relatively higher levels of ROS ($P = .0044$, $P = .0007$, and $P = .0222$, respectively) upon WCL stimulation in patients with low compared to high *Mtb* loads at baseline (Figure 5A). Moreover, sex-adjusted logistic regression modeling supported higher NOI ($P = .038$) in the low *Mtb* load group compared with the high *Mtb* load group only for segmented neutrophils (Supplementary Table 3). Meanwhile, there was no difference in NOI by any subset between the mild and severe lung damage groups at baseline (Figure 5B).

Interleukin 10^+ Neutrophil Frequencies Vary With ATB Severity and Treatment

Overall, neutrophils portrayed monofunctional cytokine expression profiles and we observed higher levels of interleukin 10 (IL-10) and tumor necrosis factor (TNF) production from $CD16^{dim}CD62L^{lo}$ neutrophils following EC and WCL stimulation compared with unstimulated samples (Supplementary Figure 3). At baseline, the frequency of $IL\text{-}10^+CD16^{dim}CD62L^{lo}$ neutrophils were higher following WCL stimulation in patients with mild lung damage ($P = .0397$) compared to those with severe damage; however, this was not significant after adjusting for sex (Supplementary Table 3). Meanwhile there was no difference in the frequency of TNF-expressing neutrophils between severity groups (Figure 6A). We also found that $IL\text{-}10^+$ hypersegmented neutrophil frequencies were significantly

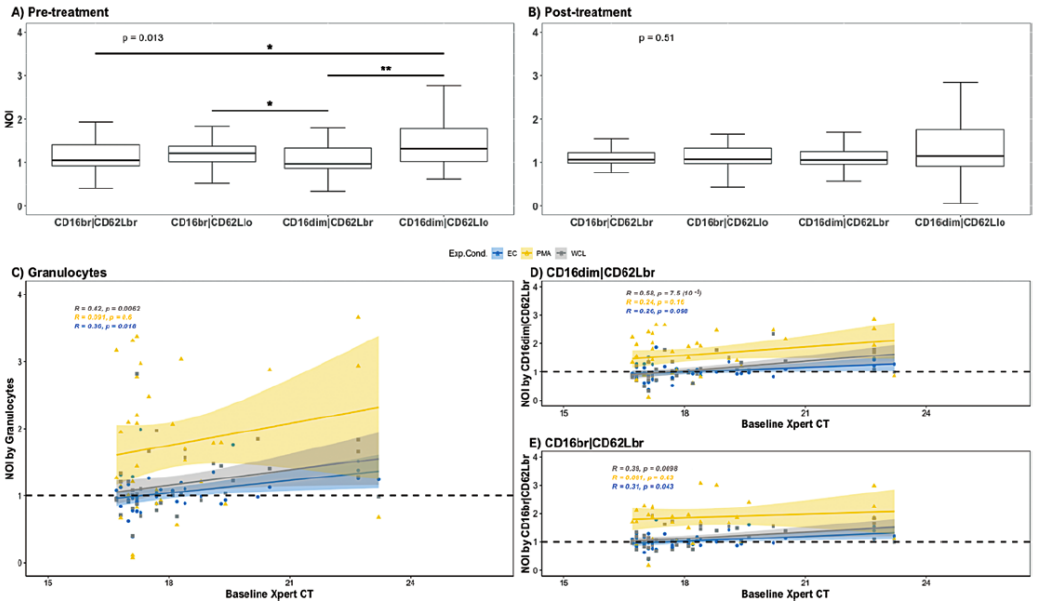


Figure 4. Neutrophil oxidative indices (NOIs) of neutrophil subsets. *A*, Pretreatment: low CD62L-expressing subsets $CD16^{dim}CD62L^{lo}$ (n = 42) and $CD16^{br}CD62L^{lo}$ (hypersegmented neutrophils, n = 27) produced more reactive oxygen species (ROS) than $CD16^{dim}CD62L^{br}$ (banded, n = 42) or $CD16^{br}CD62L^{br}$ (segmented, n = 42) neutrophils at diagnosis following 2 hours of stimulation with whole cell lysate (WCL). *B*, After treatment, the WCL-stimulated neutrophil subsets had relatively similar ROS generation capacities. *P* values were obtained using the Kruskal-Wallis test with Dunn posttest comparison. $*P < .05$; $**P < .01$. *C*, Spearman rank correlation of NOI of total granulocytes with bacterial burden at baseline. There was a weak correlation of the NOI of WCL-stimulated granulocytes with *Mycobacterium tuberculosis* loads in active TB patients, which was not observed with ESAT-6/CFP-10 fusion protein or with phorbol 12-myristate 13-acetate. *D* and *E*, $CD16^{dim}CD62L^{br}$ (banded, *D*) and $CD16^{dim}CD62L^{br}$ (segmented, *E*) neutrophils showed a similar correlation (moderately and weakly, respectively) as granulocytes. Abbreviations: Ct, cycle threshold; EC, ESAT-6/CFP-10 fusion protein; NOI, neutrophil oxidative index; PMA, phorbol 12-myristate 13-acetate; TB, tuberculosis; WCL, whole cell lysate.

higher ($P = .00857$; data not shown) in patients with low *Mtb* load compared to high *Mtb* load at the 6-month time point. Interestingly, we found that IL-10^{+} banded neutrophil frequencies increased in patients with severe lung damage ($P = .014$), whereas IL-10^{+} segmented neutrophils decreased in patients with mild lung damage ($P = .044$) from baseline to treatment completion with WCL stimulation (Figure 6B and 6C, respectively). We also found no differences in either IL-10+ or TNF^{+} neutrophil frequencies between severity groups at the 6-month time point (data not shown).

DISCUSSION

Our aim was to determine if variations in neutrophil-related immunological correlates could explain the difference in ATB lung pathology at baseline and after successful therapy. We show that blood neutrophils from ATB patients show different phenotypes and functionality when exposed to *Mtb*-specific antigens before and after therapy. Notably, most patients with severe lung pathology at baseline were males. Hence, for the recovery analysis, the majority of women were excluded.

Low banded (CD16^{dim}CD62L^{br}) and high segmented (CD16^{br}CD62L^{br}) neutrophil frequencies at diagnosis were associated with more severe lung pathology. A previous study reported significantly higher levels of segmented neutrophils in patients with greater areas of affected lungs, which supports our findings [22]. Additionally, we found no differences in neutrophil levels between the high and low *Mtb* load groups, which is consistent with previous observations from Scott et al, where

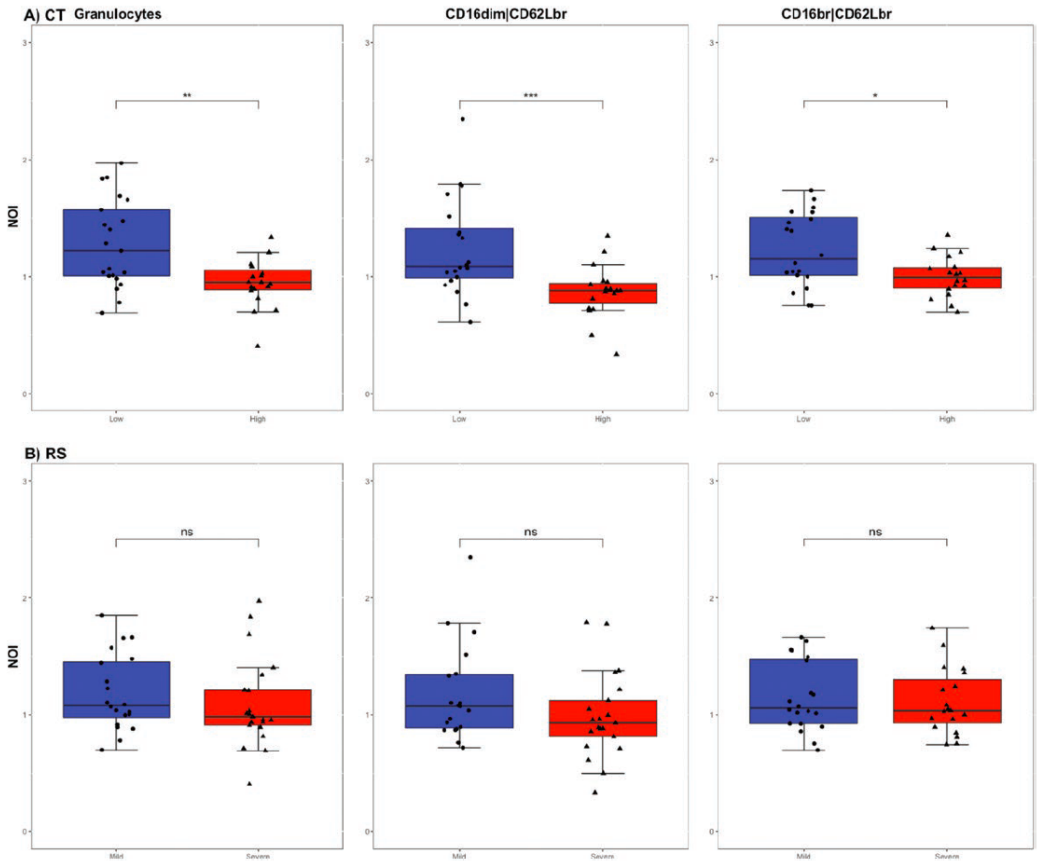


Figure 5. Neutrophil oxidative indices (NOIs) of neutrophil subsets in cycle threshold value–defined severity groups at diagnosis. *A*, At baseline, granulocytes, CD16^{dim}CD62L^{br} neutrophils (banded), and CD16^{br}CD62L^{br} neutrophils (segmented) had higher NOIs following 2-hour whole cell lysate stimulation in patients with low *Mycobacterium tuberculosis* (*Mtb*) load (n = 20) compared to those with high *Mtb* load (n = 18). *B*, There were no significant differences in NOI between mild (n = 20) and severe (n = 21) lung damage groups at baseline. Wilcoxon rank-sum test was used to analyze differences between groups at baseline. *$P < .05$; **$P < .01$; ***$P < .005$. Abbreviations: Ct, cycle threshold; NOI, neutrophil oxidative index; ns, not significant; RS, Ralph score.

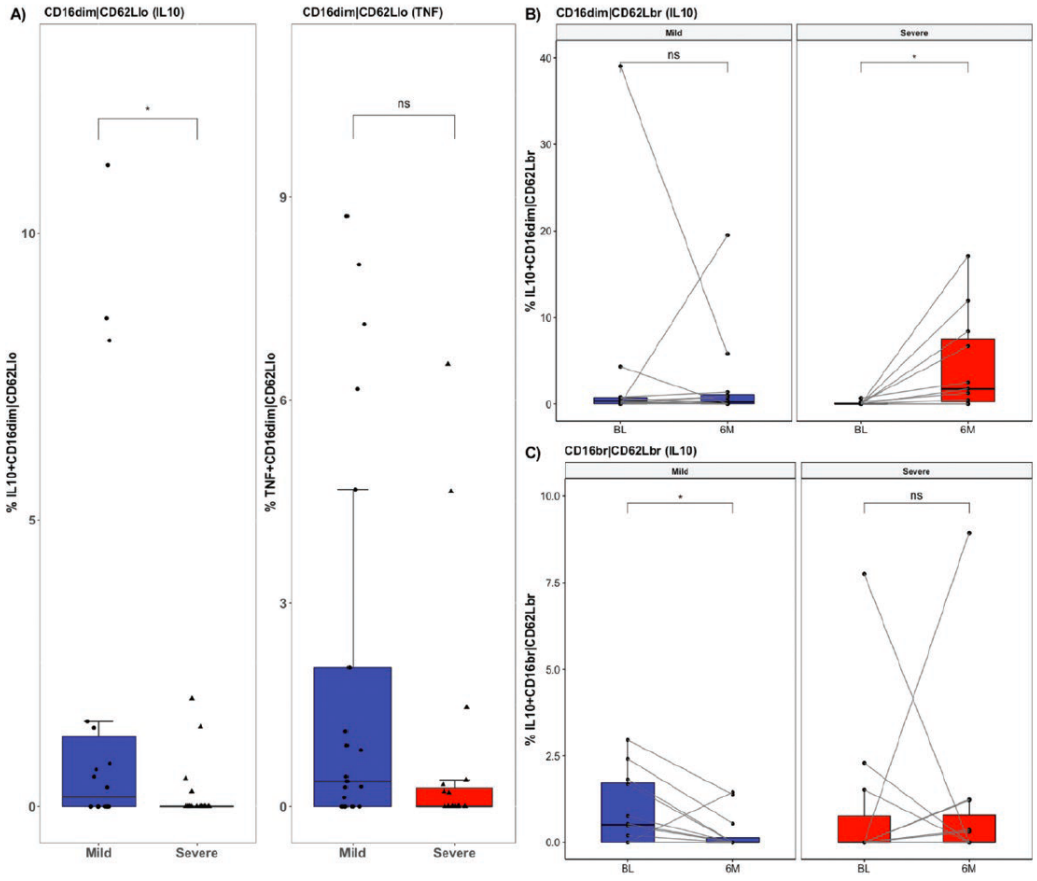


Figure 6. Frequency of interleukin 10 (IL-10)$^{+}$ neutrophils pretreatment following whole cell lysate (WCL) stimulation. *A,* Following 2-hour stimulation with WCL, the baseline (BL) frequencies of IL-10–expressing CD16^{dim}CD62L^{lo} neutrophils were higher in patients with mild damage (n = 19) than in patients with severe damage (n = 20). There were no significant differences in tumor necrosis factor (TNF)–expressing neutrophil frequencies between ATB severity groups at diagnosis. Wilcoxon rank-sum test was used to analyze differences between groups at BL. *B,* Frequency of IL-10–expressing banded (IL-10^{+}CD16^{dim}CD62L^{br}) neutrophils is higher at month 6 (6M) compared to BL in patients with severe lung damage. *C,* IL-10–expressing segmented (IL-10^{+}CD16^{br}CD62L^{br}) neutrophil frequencies are higher at BL compared to 6M in the mild lung damage group. Wilcoxon signed-rank test was used to analyze differences between treatment time within groups. *$P < .05$. Abbreviation: ns, not significant.

mice depleted of neutrophils showed no differences in lung and spleen *Mtb* burden [33].

We also observed decreased frequencies in total neutrophils after treatment, which supports previous findings by Ndlovu and colleagues [34]. This decrease was more pronounced in patients with severe lung damage or high *Mtb* load compared with those with mild damage or low *Mtb* load, respectively. Frequencies of banded neutrophils decreased while segmented neutrophil frequencies increased. These changes in segmented neutrophils (characteristic of homeostatic conditions) and banded neutrophils (which circulate following inflammation) [18] occurred only in patients with mild lung damage or low *Mtb* loads, suggesting that homeostatic conditions are restored in patients with mild but not severe pathology soon after standard treatment completion. This supports previous suggestions that functional pulmonary impairment only begins to improve several months after the end of standard TB therapy [35, 36].

We also observed higher baseline granulocyte frequencies in patients with poor recovery than in those showing good recovery. This is the first time that such evidence is shown and supports the argument that heightened neutrophil levels contribute to lung damage. It also suggests that exacerbated neutrophil levels in circulation reduce lung recovery potential.

The frequency of activated *Mtb*-specific CD11b^{+}CD16^{dim}CD62L^{lo} neutrophils was reduced after

treatment in patients with initially severe damage, suggesting that this subset may be preferentially activated pretreatment in ATB patients. While this supports previous findings that improved *Mtb* control in mice is associated with reduced lung neutrophil accumulation within TB granulomas and decreased expression of CD11b on neutrophils [33], it also reveals a direct proinflammatory role played by $CD16^{dim}CD62L^{lo}$ neutrophils in ATB pathogenesis, which is subsequently dampened following treatment and resolution of *Mtb*-induced lung inflammation/pathology.

IL-10 is an immunomodulatory cytokine, usually associated with immunosuppressive outcomes in ATB [37]. At diagnosis, the proportion of IL-10–expressing $CD16^{dim}CD62L^{lo}$ neutrophils were higher in patients with mild lung damage. To our knowledge, this is the first study to show circulating neutrophil–specific IL-10 expression during ATB disease in humans. Our results suggest that $CD16^{dim}CD62L^{lo}$ neutrophils autoregulate their activated proinflammatory potential by expressing IL-10, which results in milder pathology. Hence, neutrophil-related IL-10–mediated immunosuppression may contribute to limiting ATB severity. Interestingly, our data reveal that levels of TNF-expressing neutrophils are similar between CXR or *Mtb* burden groups. This suggests an imbalance between neutrophilic pro- and anti-inflammatory cytokines, which results in immunosuppressive outcomes (in patients with mild pathology) mediated by $CD16^{dim}CD62L^{lo}$ neutrophils pretreatment. Treatment completion resulted in increased and decreased IL-10 expression by banded (in patients with severe lung damage at baseline) and segmented (in patients with mild damage at baseline) neutrophils, respectively, further highlighting the opposing ways in which these 2 subtypes act.

While we found no differences in ROS generation levels between lung damage groups, NOIs of total, segmented, and banded neutrophils correlated positively with Ct values (ie, negatively with *Mtb* load) at baseline. To our knowledge, this is the first study that shows a relationship between neutrophilic ROS generation and *Mtb* load in ATB. This aligns with claims by others that reduced ROS is linked to increased susceptibility to bacterial and fungal infections in elderly individuals [38]. Furthermore, this correlation was only significant following WCL (not EC or PMA) stimulation. This is presumably because WCL contains more DAMPs and PAMPs, which neutrophils can recognize via pattern recognition receptors such as Toll-like receptors or C-type lectin receptors [39, 40]. This is also the only instance where we see a similar functional outcome between segmented and banded neutrophil activities. It suggests that ROS generation by neutrophils is a basic functional attribute, whereas cytokine expression in neutrophils may be more adaptable (leading to varied attributes in different phenotypes or immune conditions). Hence, coupled to frequencies of neutrophil subsets and IL-10 expression levels, NOI could be useful as a biomarker for predicting the likelihood of developing severe ATB as well as for evaluating ATB severity at diagnosis.

In summary, we show that frequencies of segmented and banded neutrophil phenotypes and their capacity to generate ROS are linked to disease severity in HIV-negative ATB patients in The Gambia. Our data also show that increased ROS generation, high levels of banded neutrophils, and high frequencies of IL-10–expressing $CD16^{dim}CD62L^{lo}$ all play a protective role in ATB pathogenesis by limiting lung impairment or reducing *Mtb* burden. Tuberculosis remains a global health threat, and we show that *Mtb* infection leads to neutrophil recruitment and differentiation into functional subsets. Our data suggest that while some neutrophil subsets are proinflammatory, others can be protective with immunosuppressive functions. Identifying the mechanism(s) that limit or promote neutrophil differentiation into either subset can directly aid in the design of novel targeted therapeutics against *Mtb*. Thus, neutrophil-mediated (subtypes and their immunosuppressive or ROS generation capacities) TB susceptibility and disease severity can be targeted as immune and host-directed therapies for TB progression and severity based on the findings of this study.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author Contributions. C. N. M.: Conceptualization, data curation, formal analysis, investigation, methodology, and writing of the manuscript. O. O.: Patient recruitment and follow-up, clinical data, review of the manuscript. S. D.: Data management. S. C.: Funding acquisition, review of the manuscript. A. B.: Data curation and formal analysis. A. R.: Conceptualization, funding acquisition, data analysis, review of the manuscript. C. G.: Supervision, methodology, manuscript review. J. S. S.: Supervision, conceptualization, data curation, methodology, funding acquisition, review of the manuscript.

Acknowledgments. The authors thank the patients who volunteered for this study and the Medical Research Council The Gambia tuberculosis research group for technical support.

Disclaimer. A. R. served as co-coordinator of the main cohort study (protocol writing, clinical data analysis, review of the manuscript), during the conduct of the study.

Financial support. This work was supported by TB Sequel (grant number 66.3010.7–002.00) with funding from the German Ministry for Education and Research.

Potential conflicts of interest. The authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

References

1. World Health Organization. Global tuberculosis report 2020. Geneva, Switzerland: WHO, **2020**.
2. Chushkin MI, Ots ON. Impaired pulmonary function after treatment for tuberculosis: the end of the disease? J Bras Pneumol **2017**; 43:38–43.
3. Allwood BW, Byrne A, Meghji J, et al. Post-tuberculosis lung disease: clinical review of an under-recognised global challenge. Respiration **2021**; 100:751–63.
4. Allwood BW, van der Zalm MM, Amaral AFS, et al. Post-tuberculosis lung health: perspectives from the First International Symposium. Int J Tuberc Lung Dis **2020**; 24:820–8.

5. Ralph AP, Kenangalem E, Waramori G, et al. High morbidity during treatment and residual pulmonary disability in pulmonary tuberculosis: under-recognised phenomena. PLoS One **2013**; 8:1–11.
6. Khosa C, Bhatt N, Massango I, et al. Development of chronic lung impairment in Mozambican TB patients and associated risks. BMC Pulm Med **2020**; 20:1–11.
7. Cadena AM, Fortune SM, Flynn JL. Heterogeneity in tuberculosis. Nat Rev Immunol **2017**; 17:691–702.
8. Pai M, Behr MA, Dowdy D, et al. Tuberculosis. Nat Rev Dis Primers **2016**; 2:16076.
9. Young C, Walzl G, Du Plessis N. Therapeutic host-directed strategies to improve outcome in tuberculosis. Mucosal Immunol **2020**; 13:190–204.
10. Ahmed S, Raqib R, Guðmundsson GH, et al. Host-directed therapy as a novel treatment strategy to overcome tuberculosis: targeting immune modulation. Antibiotics **2020**; 9:1–19.
11. Lyadova IV. Neutrophils in tuberculosis: heterogeneity shapes the way? Mediators Inflamm **2017**; 2017:8619307.
12. Hellebrekers P, Vrisekoop N, Koenderman L. Neutrophil phenotypes in health and disease. Eur J Clin Invest **2018**; 48(Suppl 2):e12943.
13. Leliefeld PHC, Pillay J, Vrisekoop N, et al. Differential antibacterial control by neutrophil subsets. Blood Adv **2018**; 2:1344–55.
14. Wang X, Qiu L, Li Z, et al. Understanding the multifaceted role of neutrophils in cancer and autoimmune diseases. Front Immunol **2018**; 9:2456.
15. Rosales C. Neutrophil: a cell with many roles in inflammation or several cell types? Front Physiol **2018**; 9:113.
16. Yang P, Li Y, Xie Y, Liu Y. Different faces for different places: heterogeneity of neutrophil phenotype and function. J Immunol Res **2019**; 2019:8016254.
17. Perobelli SM, Galvani RG, Gonçalves-Silva T, Xavier CR, Nóbrega A, Bonomo A. Plasticity of neutrophils reveals modulatory capacity. Braz J Med Biol Res **2015**; 48:665–75.
18. Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. J Clin Invest **2012**; 122:327–36.
19. Tak T, Wijten P, Heeres M, et al. Human CD62L dim neutrophils identified as a separate subset by proteome profiling and in vivo pulse-chase labeling. Blood **2019**; 129:3476–86.
20. van Grinsven E, Textor J, Hustin LSP, Wolf K, Koenderman L, Vrisekoop N. Immature neutrophils released in acute inflammation exhibit efficient migration despite incomplete segmentation of the nucleus. J Immunol **2019**; 202:207–17.
21. Spijkerman R, Hesselink L, Bongers S, et al. Point-of-care analysis of neutrophil phenotypes: a first step toward immuno-based precision medicine in the trauma ICU. Crit Care Explor **2020**; 2:e0158.
22. Panteleev AV, Nikitina IY, Burmistrova IA, et al. Severe tuberculosis in humans correlates best with neutrophil abundance and lymphocyte deficiency and does not correlate with antigen-specific CD4 T-cell response. Front Immunol **2017**; 8:963.
23. Tak T, Rygiel TP, Karnam G, et al. Neutrophil-mediated suppression of influenza-induced pathology requires CD11b/CD18 (MAC-1). Am J Respir Cell Mol Biol **2018**; 58:492–9.
24. Jones HR, Robb CT, Perretti M, Rossi AG. The role of neutrophils in inflammation resolution. Semin Immunol **2016**; 28:137–45.
25. Spahn JH, Kreisel D. Monocytes in sterile inflammation: recruitment and functional consequences. Arch Immunol Ther Exp **2014**; 62:187–94.
26. Kroon EE, Coussens AK, Kinnear C, et al. Neutrophils: innate effectors of TB resistance? Front Immunol **2018**; 9:1–12.
27. Rachow A, Ivanova O, Wallis R, et al. TB sequel: incidence, pathogenesis and risk factors of long-term medical and social sequelae of pulmonary TB—a study protocol. BMC Pulm Med **2019**; 19:1–9.
28. Ralph AP, Ardian M, Wiguna A, et al. A simple, valid, numerical score for grading chest x-ray severity in adult smear-positive pulmonary tuberculosis. Thorax **2010**; 65:863–9.
29. Pohlert T. The pairwise multiple comparison of mean ranks package (PMCMR). Vienna, Austria: R Project, **2018**.
30. R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, **2018**.
31. Elbim C, Chollet-Martin S, Bailly S, Hakim J, Gougerot-Pocidalo MA. Priming of polymorphonuclear neutrophils by tumor necrosis factor alpha in whole blood: identification of two polymorphonuclear neutrophil subpopulations in response to formyl-peptides. Blood **1993**; 82:633–40.
32. Won DI, Kim S, Lee EH. Neutrophil oxidative burst as a diagnostic indicator of IgG-mediated anaphylaxis. Blood Res **2018**; 53:299–306.
33. Scott NR, Swanson RV, Al-Hammadi N, et al. S100A8/A9 regulates CD11b expression and neutrophil recruitment during chronic tuberculosis. J Clin Invest **2020**; 130:3098–112.
34. Ndlovu LN, Peetluk L, Moodley S, et al. Increased neutrophil count and decreased neutrophil CD15 expression correlate with TB disease severity and treatment response irrespective of HIV co-infection. Front Immunol **2020**; 11:1–11.
35. Hnizdo E, Singh T, Churchyard G. Chronic pulmonary function impairment caused by initial and recurrent pulmonary tuberculosis following treatment. Thorax **2000**; 55:32–8.
36. Long R, Maycher B, Dhar A, Manfreda J, Hershfield E, Anthonisen N. Pulmonary tuberculosis treated with directly observed therapy: serial changes in lung structure and function. Chest **1998**; 113:933–43.
37. Moreira-Teixeira L, Redford PS, Stavropoulos E, et al. T cell-derived IL-10 impairs host resistance to *Mycobacterium tuberculosis* infection. J Immunol **2017**; 199:613–23.
38. Sauce D, Dong Y, Campillo-Gimenez L, et al. Reduced oxidative burst by primed neutrophils in the elderly individuals is associated with increased levels of the $CD16^{bright}/CD62L^{dim}$ immunosuppressive subset. J Gerontol A Biol Sci Med Sci **2017**; 72:163–72.
39. Liu CH, Liu H, Ge B. Innate immunity in tuberculosis: host defense vs pathogen evasion. Cell Mol Immunol **2017**; 14:963–75.
40. van Rees DJ, Szilagyi K, Kuijpers TW, Matlung HL, van den Berg TK. Immunoreceptors on neutrophils. Semin Immunol **2016**; 28:94–108.

6. Paper II

frontiers in Immunology

ORIGINAL RESEARCH
published: 23 November 2021
doi: 10.3389/fimmu.2021.740933

Major Neutrophil-Derived Soluble Mediators Associate With Baseline Lung Pathology and Post-Treatment Recovery in Tuberculosis Patients

Caleb Nwongbouwoh Muefong[1,2*], *Olumuyiwa Owolabi*[1], *Simon Donkor*[1], *Salome Charalambous*[3], *Joseph Mendy*[1], *Isatou C. M. Sey*[1], *Abhishek Bakuli*[2,4], *Andrea Rachow*[2,4], *Christof Geldmacher*[2,4] *and Jayne S. Sutherland*[1]

[1] Vaccines and Immunity Theme, Medical Research Council (MRC) Unit The Gambia at London School of Hygiene and Tropical Medicine (LSHTM), Fajara, Gambia, [2] Division of Infectious Diseases and Tropical Medicine, University Hospital, Ludwig Maximilian University (LMU) Munich, Munich, Germany, [3] School of Public Health, Aurum Institute, Johannesburg, South Africa, [4] International Clinical Trials Unit, German Centre for Infection Research (DZIF), Partner Site Munich, Munich, Germany

OPEN ACCESS

Edited by:
Rabindra Tirouvanziam,
Emory University, United States

Reviewed by:
Nathalie Winter,
Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), France
Dhiraj Kumar Singh,
Southwest National Primate Research Center (SNPRC), United States

***Correspondence:**
Caleb Nwongbouwoh Muefong
Caleb.Muefong@lshtm.ac.uk

Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: 13 July 2021
Accepted: 01 November 2021
Published: 23 November 2021

Citation:
Muefong CN, Owolabi O, Donkor S, Charalambous S, Mendy J, Sey ICM, Bakuli A, Rachow A, Geldmacher C and Sutherland JS (2021) Major Neutrophil-Derived Soluble Mediators Associate With Baseline Lung Pathology and Post-Treatment Recovery in Tuberculosis Patients. Front. Immunol. 12:740933. doi: 10.3389/fimmu.2021.740933

Background: The inflammatory response to *Mycobacterium tuberculosis* results in variable degrees of lung pathology during active TB (ATB) with central involvement of neutrophils. Little is known about neutrophil-derived mediators and their role in disease severity at baseline and recovery upon TB treatment initiation.

Methods: 107 adults with confirmed pulmonary TB were categorised based on lung pathology at baseline and following successful therapy using chest X-ray scores (Ralph scores) and GeneXpert bacterial load (Ct values). Plasma, sputum, and antigen-stimulated levels of MMP1, MMP3, MMP8, MMP9, MPO, S100A8/9, IL8, IL10, IL12/23(p40), GM-CSF, IFNγ, and TNF were analysed using multiplex cytokine arrays.

Results: At baseline, neutrophil counts correlated with plasma levels of MMP8 (rho = 0.45, p = 2.80E–06), S100A8 (rho = 0.52, p = 3.00E–08) and GM-CSF (rho = 0.43, p = 7.90E–06). Levels of MMP8 (p = 3.00E–03), MMP1 (p = 1.40E–02), S100A8 (p = 1.80E–02) and IL12/23(p40) (p = 1.00E–02) were associated with severe lung damage, while sputum MPO levels were directly linked to lung damage (p = 1.80E –03), Mtb load (p = 2.10E–02) and lung recovery (p = 2.40E–02). Six months of TB therapy significantly decreased levels of major neutrophil-derived pro-inflammatory mediators: MMP1 (p = 4.90E–12 and p = 2.20E–07), MMP8 (p = 3.40E–14 and p = 1.30E–05) and MMP9 (p = 1.60E–04 and p = 1.50E–03) in plasma and sputum, respectively. Interestingly, following H37Rv whole cell lysate stimulation, S100A8 (p = 2.80E–02), MMP9 (p = 3.60E–02) and MPO (p = 9.10E–03) levels at month 6 were significantly higher compared to baseline. Sputum MMP1 (p = 1.50E–03), MMP3 (p = 7.58E–04), MMP9 (p = 2.60E–02) and TNF (p = 3.80E–02) levels were lower at month 6 compared to baseline in patients with good lung recovery.

Conclusion: In this study, patients with severe lung pathology at baseline and persistent lung damage after treatment were associated with higher plasma and sputum levels of major pro-inflammatory neutrophil-derived mediators. Interestingly, low sputum MPO

levels were associated with severe lung damage, higher Mtb burden and low recovery. Our data suggest that therapeutic agents which target these mediators should be considered for future studies on biomarkers and host-directed therapeutic approaches against TB-related lung pathology and/or lung recovery.

Keywords: tuberculosis, neutrophils, myeloperoxidase, S100A8/9, MMP8, lung pathology

INTRODUCTION

Tuberculosis (TB) caused 1.2 million deaths from HIV-negative individuals in 2019 (1). While treatment is available, former patients are more likely to experience long-term pulmonary disability (2) and only about 50% of patients achieve full recovery from lung damage (3). It has been suggested that higher initial inflammatory responses against *Mycobacterium tuberculosis* (Mtb) lead to more severe lung damage prior to treatment initiation (4) and thus reduced of recovery following treatment.

Inflammatory mediators generated during the natural immune response to Mtb (5) are linked to increased disease severity, bacterial burden and delayed culture conversion. However, the overall inflammatory response depends on the interplay between pro- and anti-inflammatory mediators (6). Interestingly, reports show that severe inflammation and lung damage following *Mycoplasma pneumoniae* infection in mice may be a result of oxidant–antioxidant imbalances which can be reduced by immunosuppression (6). Similarly, certain neutrophil sub-types have been shown to express immunosuppressive functions including: $CD16^{bright}CD62L^{low}$ neutrophils (7) and granulocytic myeloid-derived suppressor cells (G-MDSCs) (8, 9). These support the idea that an equilibrium between neutrophilic pro- and anti-inflammatory functions (10–14) determines the extend of inflammation and lung damage in TB patients.

Previous studies have investigated a broad range of biomarkers for TB disease progression and lung damage severity in humans (15–19), recently reviewed (20–22). While some neutrophilic activities have been tested, major neutrophil-derived mediators have not been the main focus. Neutrophils are mainly pro-inflammatory but recent studies reveal that different subtypes also display anti-inflammatory functions (11, 14, 23, 24) depending on the type and quantity of inflammatory mediators produced. A current challenge is to elucidate which neutrophil subtypes and mediators are predominantly pro- or anti-inflammatory during active TB and to determine the underlying immunological mechanisms involved in protective outcomes. Muefong and Sutherland reviewed (12) promising neutrophil-derived targets for developing host directed therapies (HDTs) against TB-induced lung pathology. We also recently showed, in a smaller group of participants from this Gambian cohort, that immature (banded) neutrophils and IL10-expressing $CD16^{dim}CD62L^{low}$ neutrophils are associated with reduced lung damage in active TB patients pre-treatment (13). Additionally, MDSCs are currently considered in the development of HDTs against TB progression and Mtb control (9, 25, 26) due to their role as effectors of Mtb pathogenesis and their modulatory role on T-cell function.

Studies in mice (27), macaques (28, 29) and humans (30, 31) suggest that heightened neutrophil function correlates with tissue injury. For example, during hypoxic conditions, human neutrophils have been shown to drive tissue destruction during ATB by secreting matrix metalloproteinases (MMPs) like MMP8 and MMP9 (32). Sputum MMP levels have also been associated with disease severity in ATB patients pre-treatment (33) and excess MMP activity enhances tissue injury in clinical studies and preclinical models of pulmonary pathology (34). S100A8/9 is another neutrophil-derived mediator known to exacerbate the inflammatory response to Mtb infection and it is currently targeted in Mtb control studies (28, 35).

On the other hand, recent studies highlight an immunoregulatory effect of granulocytes (36). In mice exposed to zymosan, deficiency in myeloperoxidase (MPO)—a major constituent of neutrophil granules—results in severe lung inflammation (37), suggesting that MPO could play immunomodulatory functions; an observation which has not been made in TB. Hence, different neutrophil-related mediators could differentially influence ATB-related lung pathology.

We contribute to the field of TB biomarkers by focusing on major neutrophil-derived inflammatory mediator levels in ATB patients and relate this to chest X-ray (CXR) based lung pathology scores and bacterial load before and after TB therapy. We address gaps in our understanding of TB pathogenesis by monitoring the impact of neutrophil-derived mediators on the severity of TB-induced lung pathology to inform future experiments in controlled animal models investigating TB HDTs.

METHODS

Ethics Approval

Ethical approval was obtained from the Medical Research Council/Gambia government joint ethics committee (SCC1523). All study participants provided written informed consent prior to collection of samples.

Participants

Adult, TB patients with positive GeneXpert (Cepheid, USA) results were recruited from the TB clinic at the MRC Unit The Gambia at LSHTM between April 2018 and October 2019 as part of a parent study, TB Sequel (3). This study was conducted on a sub-cohort of TB Sequel and patients were selected based on their lung recovery outcome post-treatment. All participants

were later confirmed to have a positive mycobacteria growth indicator tube (MGIT) culture result at baseline, were drug sensitive and had not previously received anti-TB therapy (ATT). They were given standard TB therapy consisting of 2 months intensive phase and 4 months continuation phase. Sputum liquid MGIT culture was performed at baseline (BL), 2 months (2M) and 6 months (6M) after ATT initiation. Heparinised blood and sputum samples were collected and processed at BL, 2M and 6M of standard treatment. All patients were culture negative by 6M and HIV positive patients were excluded from analysis.

Scoring of Chest Radiographs

Chest radiographs were analysed based on the Ralph score (RS) (38). Briefly, posteroanterior chest radiographs were assessed for the percentage of the lung fields affected by known ATB features. When at least one cavity could be identified, 40 points were added to the value of percentage lung affected. The median [interquartile range (IQR)] RS score at baseline (RS_{Med}) of all participants in our cohort was determined, 65 [29–80]. Lung damage severity (pre- and post-treatment) groupings were defined as follows: "mild pathology" ($RS < RS_{Med}$) and "severe pathology" ($RS \geq RS_{Med}$).

GeneXpert MTB/RIF Results

The GeneXpert® machine (Cepheid, USA) was used to determine cycle threshold (Ct) values at baseline. The lowest Ct value generated among the five rpoB probes of Xpert MTB/RIF (or of the four rpoB probes in the nested-PCR stage for GeneXpert Ultra) was taken as a measure of the Mtb cell number (39). The median [interquartile range (IQR)] Ct value (Ct_{Med}) of all participants was computed, 17.4 [17.1–18.4]. Patients were grouped into: "high Mtb load" ($Ct < Ct_{Med}$) and "low Mtb load" ($Ct > Ct_{Med}$) groups.

Recovery from Severe Lung Pathology After Treatment

For each patient, RS changes (ΔRS) from BL to 6M (ΔRS = RS at BL/RS at 6M) and median change [IQR] in RS of the entire cohort (ΔRS_{Med}) were computed, 6.5 [1.6–14]. Patients were grouped into: "Good" ($\Delta RS \geq \Delta RS_{Med}$) and "Poor" ($\Delta RS < \Delta RS_{Med}$) lung recovery groups. Nine participants had a ΔRS equal to the ΔRS_{Med}.

Sputum Sample Supernatant Collection

Aliquots of sputum were digested with an equal volume of Sputolysin (MerckMillipore, USA) for 15 min and centrifuged. The supernatant was harvested and stored at −80°C until use.

Whole Blood Processing, Storage and Stimulation

Plasma was obtained from blood vials by centrifugation at 2,500 rpm and stored at −80°C prior to use. Approximately 500 µl of whole blood was stimulated with either ESAT-6/CFP-10 peptide pool (EC; at 2.5 µg/ml/peptide), purified protein derivative (PPD at 10 µg/ml; Staten Serum Institute, Denmark), H37Rv whole cell lysate (WCL; at 10 µg/ml; BEI Resources, USA) or phorbol 12-myristate 13-acetate (PMA; positive control; 10 µg/ml) along with co-stimulatory antibodies (anti-CD28, anti-CD49d; Becton Dickinson, USA); or unstimulated/cultured with medium alone (NIL; negative control). Following overnight incubation at 37°C, 5% CO_2, plates were spun (1,500 rpm, 5 min) and 200–250 µl of supernatant was harvested from each well into 0.5 ml Sarstedt tubes prior to storage at −80°C for multiplex cytokine assays.

Multiplex Cytokine Arrays

Multiplex cytokine arrays were performed using a customised 13-plex inflammatory marker panel (R&D Systems, USA) according to the manufacturer's instructions. The 13 analytes measured were GM-CSF, IL8/CXCL8, IL12/23(p40), MMP3, MMP9, S100A8, S100A9, TNF, IFNγ, IL10, MMP1, MMP8, and MPO. The minimum levels of detection for these were: 11.52, 2.96, 383.13, 78.48, 128.31, 74.86, 8.44, 42.35, 3.70, 40.95, 241.11, 113.00, and 26.87 pg/ml, respectively. Briefly, lyophilised standards were reconstituted and serial dilutions performed. Coupled beads were diluted in assay buffer and 50 µl were added to each well of the assay plate. Approximately 50 µl of diluted standards, blanks, samples (plasma, ag-stimulated supernatants or *ex vivo* sputum) and controls were added per well. Plates were then incubated at room temperature (RT), with shaking (350 rpm, 2 h) followed by three washes in wash buffer. Detection antibodies were diluted in detection antibody diluent as recommended and 50 µl added to each well followed by another 1 h incubation period. Following three washes, 100× streptavidin-PE was diluted in wash buffer (one in 100) and 50 µl added to each well. Plates were then incubated for 30 min and washed three times. Approximately 100 µl of assay buffer were then added to each well, plates were shaken for 2 min and read using Bioplex 200 plate reader with Bio-Plex Manager Software (version 6.1; Bio-Rad, Belgium).

Data Analysis

All statistical analyses were performed using R version 3.5.2 (40). For antigen-specific blood responses, background was subtracted using the unstimulated (NIL) samples. Non-parametric tests were used for all comparisons. Differences between BL, 2M and 6M samples within each group were analysed using a Kruskal–Wallis test with Dunn's post-test comparison. For comparisons between severity, treatment response and recovery groups, a Wilcoxon rank sum test was performed. The Benjamini–Hochberg test (41) was used to adjust for multiple comparisons throughout. Adjusted p values (q values) of less than 0.05 were considered statistically significant. Linear regression models were used to determine significant differences after adjusting for sex.

RESULTS

Patient Demographics

We analysed pre-selected plasma and sputum samples from 107 adult HIV negative, pulmonary TB patients of which 77% were males (**Table 1**). The median [interquartile range (IQR)] CXR score at baseline was 65 [29–80] with 46 patients in the mild (RS <65) and 61 patients in the severe group (RS ≥65). The median [IQR] CXR

TABLE 1 | Patient demographics.

	Total	CXR-defined		GeneXpert-defined			Lung Recovery	
		Mild N = 46	Severe N = 61	Low N = 53	High N = 45	NA N = 9	Good N = 30	Poor N = 22
Age	32 [23–40]	29.5 [21–39]	32 [26–41]	29 [22–40]	31 [25–40]	32 [30–34]	32 [24–41]	32 [26–37]
Male n (%)	82 (77)	29 (63)	53 (87)	34 (64)	40 (89)	8 (89)	26 (87)	19 (86)
		p = 3.90E−03			p = 4.50E−03		ns	

ns, not significant; CXR, chest X-ray; age = median [interquartile range].

score for the mild and severe groups at baseline was 25 [16.2–51.5] and 75 [65–90] respectively. For patients with severe damage at baseline, the median [IQR] change in CXR scores (ΔRS) from baseline to 6 months was 6.5 [1.6–14] with 30 patients in the good recovery (ΔRS ≥6.5) and 22 in the poor recovery group (ΔRS <6.5). Within the good and poor recovery groups, the median [IQR] ΔRS was 14 [9.5–18] and 1.5 [1.3–2.6], respectively. Nine of the patients with severe damage at BL could not be classified into either good or poor recovery groups due to missing month 6 CXR scores (NA). At the end of treatment (6M) the median [IQR] CXR for the mild and severe groups was 5 [0–10] and 5 [5–13.5], respectively. For bacterial load calculations, we analysed the Xpert Ct values for all participants. The median [IQR] Ct value was 17.4 [17.1–18.4] with 45 patients in the high bacterial load group (Ct <17.4) and 53 patients in the low bacterial low group (Ct >17.4). The median [IQR] Ct values for the high and low bacterial load groups were 17.0 [16.8–17.1] and 18.4 [17.8–19.6], respectively. CXR-derived Ralph scores and Xpert MTB/RIF cycle threshold weakly correlated (rho = −0.24, p = 1.40E−02) at baseline. No differences in age were observed in the mild *vs.* severe lung damage, low *vs.* high Mtb load and good *vs.* poor recovery groups (**Table 1**). Male sex was associated with higher levels of lung damage (p = 3.90E−03) and Mtb loads (p = 4.50E−03) but not with reduced lung recovery (ns).

Analysis of ATB Severity

The two measures of ATB severity that we used were sputum GeneXpert Ct values and CXR Ralph scores. There was a weak negative correlation between patient Ct values and Ralph scores at baseline (rho = −0.24, p = 1.40E−02) (**Supplementary Figure 1A**) as previously reported (42). We also observed a weak positive correlation between baseline Ralph scores and neutrophil counts (rho = 0.22, p = 2.50E−02) but not between baseline Ct values and baseline percentage neutrophil counts (**Supplementary Figures 1B, C**).

Neutrophil levels are associated with higher risk of lung damage (10) and death in TB patients (30) and we recently showed that neutrophil activation and function vary in ATB patients based on the severity of the lung pathology (43). Hence, we monitored the impact of neutrophil counts and neutrophilic inflammatory mediator levels on lung damage severity or Mtb burden.

Association Between Neutrophil Count and Analyte Concentrations in Plasma at Baseline

At baseline, plasma concentrations of all inflammatory mediators, excluding MPO and MMP9, correlated with absolute neutrophil counts (**Table 2**). The strongest correlations were observed for MMP8 (rho = 0.45, p = 2.80E−06), S100A8 (rho = 0.52, p = 3.00E−08), S100A9 (rho = 0.33, p = 6.30E−04) and GM-CSF (rho = 0.43, p = 7.90E−06). Analysis within groups showed that plasma MPO was associated with neutrophil counts in patients with high Mtb load only (rho = 0.37, p = 1.50E−02) and MMP9 was associated with neutrophil counts in patients with severe lung damage only (rho = 0.26, p = 4.10E−02) (**Table 3**). S100A8, MMP8, S100A9, IL10, GM-CSF, TNF, and IFNγ correlated with neutrophil count in patients with both severe lung damage and high Mtb load at baseline (**Table 3**).

For patients with mild lung damage at baseline, correlations were weaker and only significant for MMP8, S100A8, and S100A9 (**Table 3**). Likewise, within the low Mtb load group, significant correlations were only observed for S100A8, TNF, and GM-CSF (**Table 3**). These values reveal that while most plasma neutrophilic inflammatory marker levels are generally associated with neutrophil counts irrespective of the severity of lung pathology, MPO and MMP9 are only associated with neutrophil counts in patients with a severe form of lung pathology.

Analysis of Neutrophil Mediators in Relation to Lung Pathology and Sex at Baseline

Plasma concentrations of MMP8, MMP1, S100A8, IL12/23(p40), IFNγ, IL8, and TNF were significantly elevated in patients with severe lung damage at baseline compared to those with mild damage (p = 9.00E−04, p = 9.30E−03, p = 2.50E−03,

TABLE 2 | Correlation between neutrophil count and analyte concentrations at baseline.

	rho	p-value
MMP1	0.20	ns
MMP3	0.23	2.20E−02
MMP8	0.45	2.80E−06
MMP9	0.16	ns
MPO	0.021	ns
S1000A8	0.52	3.00E−08
S100A9	0.33	6.30E−04
IL8	0.22	3.00E−02
IL10	0.32	1.30E−03
IL12/23(p40)	0.32	1.10E−03
GM-CSF	0.43	7.90E−06
TNF	0.38	1.00E−05
IFNγ	0.36	2.40E−04

ns, not significant; rho, spearman's rank correlation coefficient.

TABLE 3 | Correlation between neutrophil count and analyte concentrations in plasma for patients with different degree of lung damage (CXR) and Mtb load (GeneXpert) at baseline.

Analyte	Lung damage				Mtb load			
	Mild		Severe		Low		High	
	rho	p-value	rho	p-value	rho	p-value	rho	p-value
MMP1	0.16	ns	0.16	ns	0.20	ns	0.18	ns
MMP3	0.13	ns	0.26	ns	0.26	ns	0.11	ns
MMP8	0.46	2.00E−03	0.40	2.10E−03	0.27	ns	0.65	2.80E−06
MMP9	−0.13	ns	0.26	4.10E−02	0.04	ns	0.29	ns
MPO	−0.13	ns	0.10	ns	−0.18	ns	0.37	1.50E−02
S1000A8	0.39	9.70E−03	0.49	8.20E−05	0.41	3.50E−03	0.59	2.60E−05
S100A9	0.39	1.00E−02	0.27	4.00E−02	0.26	ns	0.47	1.40E−03
IL8	0.06	ns	0.26	4.70E−02	0.09	ns	0.30	ns
IL10	0.28	ns	0.38	3.80E−03	0.20	ns	0.42	6.40E−03
IL12/23(p40)	0.18	ns	0.35	6.40E−03	0.13	ns	0.50	7.20E−04
GM-CSF	0.27	ns	0.50	5.40E−05	0.34	1.50E−02	0.51	5.50E−04
TNF	0.18	ns	0.45	4.10E−04	0.38	7.30E−03	0.37	1.50E−02
IFNγ	0.18	ns	0.43	8.20E−04	0.21	ns	0.46	1.90E−03

ns, not significant; rho, spearman's rank coefficient.

p = 3.50E−03, p = 7.70E−03, p = 2.10E−02, and p = 4.20E−02, respectively; **Figure 1A**).

Severe lung damage was associated with plasma MMP8, MMP1, S100A8, IL12/23(p40), and IFNγ (p = 3.00E−03, p = 1.40E−02, p = 1.80E−02, p = 1.00E−02, and p = 1.90E−02, respectively) even after adjusting for sex (**Supplementary Table 5** and **Supplementary Figure 2A**). With respect to Mtb burden at baseline, the only difference in plasma inflammatory mediator levels between high Mtb and low Mtb load groups was observed for S100A9 (p = 4.60E−02, adjusted for sex) (**Supplementary Table 5**).

In sputum, MMP8 (p = 7.40E−03) levels were higher in patients with severe lung damage at baseline (**Figure 1B**) however, this was not significant after adjusting for sex. TNF levels were associated with lung damage severity only after adjusting for sex (p = 5.70E−02 and p = 4.50E−02; unadjusted

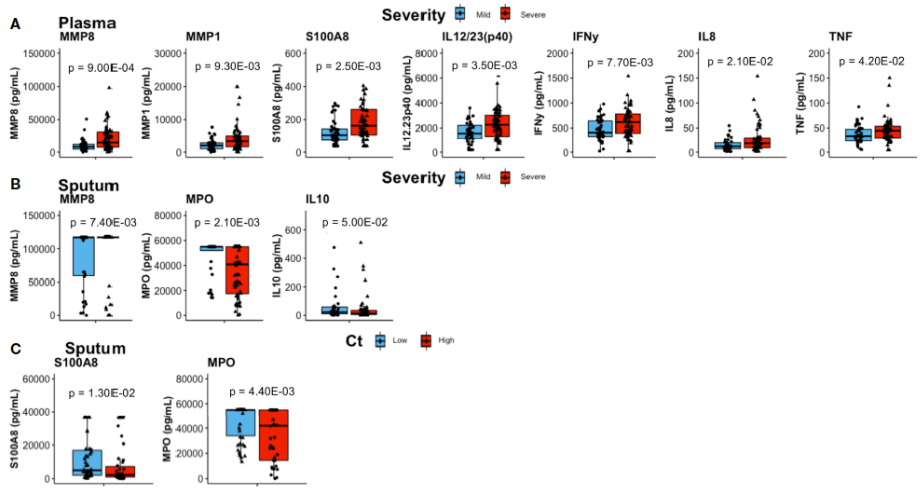


FIGURE 1 | Comparisons of inflammatory mediator concentrations at baseline. **(A)** In plasma, patients with severe lung damage (n = 61) had higher levels of proinflammatory mediators than those with mild lung damage (n = 46). **(B)** Sputum levels of mmp8 were higher whilst MPO and IL10 were lower in patients with severe (n = 60) compared to mild lung (n = 36) damage. **(C)** Whereas sputum S100A8 was higher and MPO was lower in patients with high Mtb loads (n = 45) compared to low Mtb loads (n = 53). Boxes represent the first and third quartiles and horizontal bars within indicate median concentration. Whiskers indicate minimum and maximum values. Each dot represents one individual patient. P-values were obtained using the Wilcoxon signed rank test.

and adjusted for sex, respectively) (**Supplementary Table 6**). In contrast, baseline sputum levels of IL10 (p = 5.00E−02) and MPO (p = 1.20E−03) were significantly lower in severe lung damage compared to mild lung damage group (**Figure 1B**). For MPO, the association with lung damage was significant (p = 1.80E−02) even after adjusting for sex (**Supplementary Table 6** and **Supplementary Figure 2B**). Additionally, sputum MPO (p = 4.40E−03) and S100A8 (p = 1.30E−02) concentrations were significantly lower and higher, respectively in patients with high Mtb load compared to those with low Mtb load (**Figure 1C**). This association between MPO levels and Mtb load was significant (p = 2.10E−02) even after adjusting for sex (**Supplementary Table 6**). For whole blood stimulated samples (EC, PPD, WCL, and PMA), there was no significant difference in baseline inflammatory mediator levels between either severe and mild lung damage or high and low Mtb loads (not shown).

We also observed that sputum MMP1 (p = 2.70E−02) and plasma concentrations of MMP3, IL8, IL10, IL12/23(p40), GM-CSF, and TNF (p = 3.00E−03, p = 3.76E−02, p = 4.03E−02, p = 3.30E−02, p = 2.65E−02, and p = 3.36E−02, respectively) were higher in males compared to females (**Supplementary Table 1**). Interestingly, sputum MPO concentrations were higher females (p = 1.85E−02) (**Supplementary Table 1**).

Changes in Neutrophil Mediator Concentrations Post-Treatment

The majority of pro-inflammatory mediators in plasma decreased during TB treatment except for MMP3, MPO, and IL8 (**Supplementary Table 2**). Compared to baseline, plasma levels were lower at month 2 and month 6, respectively for MMP1, MMP8, MMP9, S100A8, S100A9, TNF, IFNγ, GM-CSF, IL10, and IL12/23(p40) (**Supplementary Table 2**). In sputum, concentrations of MMP1, MMP3, MMP8, MMP9, and TNF were significantly lower at both month 2 and month 6, when compared with baseline (**Supplementary Table 2**).

Additionally, sputum GM-CSF (p = 5.50E−07), TNF (p = 2.10E−05), IFNγ (ns), S100A8 (ns), and MPO (ns) were higher at month 6 compared to baseline (**Supplementary Table 2**). Interestingly, the concentrations of these specific mediators in whole blood stimulated samples were also higher after treatment compared to baseline. Notably, this increase was significant at month 6 for GM-CSF [EC, p = 2.70E−02; PPD, p = 1.50E−09; WCL, p = 2.80E−05, and PMA, p = 6.70E−11], TNF [EC, p = 2.80E−02; PPD, p = 2.00E−03; WCL, p = 2.00E−04 and PMA, p = 2.00E−02], IFNγ [EC, (ns); PPD, p = 3.20E−08; WCL, p = 6.30E−03 and PMA, p = 1.00E−11], S100A8 [EC, (ns); PPD, (ns); WCL, p = 2.80E−02 and PMA, (ns)), MPO (EC, (ns); PPD, (ns); WCL, p = 9.10E−03 and PMA (ns)] and MMP9 [EC, (ns); PPD, p = 1.90E−08; WCL, p = 3.60E−02 and PMA, p = 5.40E−07] (**Supplementary Table 2**).

The decrease in plasma and sputum concentrations of these mediators at month 6 compared to baseline was more pronounced in patients with initially (at baseline) severe lung damage (**Supplementary Table 3**) or initially high Mtb loads (**Supplementary Table 4**). Interestingly, this decrease in concentrations was exclusive to the initially severe lung damage group for S100A8 (p = 4.61E−09), MMP9 (p = 1.26E−02), IL10 (p = 3.86E−04), TNF (p = 3.77E−06), IFNγ (p = 3.84E−07) and GM-CSF (p = 4.54E−05) levels in plasma; and for MMP1 (p = 1.90E−05), MMP8 (p = 4.99E−05) and TNF (p = 1.31E−03) levels in sputum (**Supplementary Table 3**). No such analogy was observed when groups defined by Mtb burden were considered (**Supplementary Table 4**).

Patients with good lung recovery had higher baseline sputum MPO (p = 4.70E−02) and IL10 (p = 2.70E−02) levels compared to patients with poor recovery (**Figure 2A**). For MPO, the association with lung recovery was significant (p = 2.40E−02) after adjusting for sex (**Supplementary Table 6**). Additionally, logistic regression revealed significant associations between and lung recovery and levels of plasma MMP8 (**Supplementary Table 5**) and sputum TNF (**Supplementary Table 6**) after adjusting for sex (p = 3.90E−02 and p = 3.80E−02, respectively).

Additionally, sputum MMP1, MMP3, MMP9, and TNF levels decreased significantly (p = 1.50E−03, p = 7.58E−04, p = 2.06E−02, and p = 3.81E−02, respectively) from baseline to month 6 in patients with good lung recovery but not in those with poor recovery (**Figure 2B**). We also saw significantly higher levels of MMP1 (p = 4.40E−02), MMP9 (p = 2.90E−02) and IL8 (p = 3.50E−02) in sputum from patients with poor lung recovery compared to good lung recovery at month 6 (**Figure 2C**).

In whole blood stimulated supernatants, with the exception of IFNγ (WCL at BL; p = 1.60E−02), IL12/23(p40) (WCL at 6M; p = 4.80E−02) and S100A8 (EC at BL; p = 3.00E−02) concentrations which were higher in mild compared to severe lung damage; MMP1 (PPD at BL; p = 5.00E−03) which was higher in high compared to low Mtb load and; S100A8 (EC at BL; p = 3.00E−02) which was higher in low compared to high Mtb load, there were no other significant differences in inflammatory mediator levels of whole blood stimulated samples (EC, PPD and WCL) from patients in severe *vs* mild lung damage or high *vs* low Mtb loads at any time point (not shown).

Finally, S100A9 concentrations at month 6 were significantly higher in severe lung damage and high Mtb load groups compared to mild damage and low Mtb load groups, respectively (p = 3.00E−02, and p = 2.10E−02, respectively) at month 6 (**Figures 3A, B**). Additionally, sputum levels of MMP9 declined significantly from baseline to month 6 in patients with low Mtb load (p = 3.20E−03) but not in patients with high Mtb load (**Figure 3C**). Moreover, these sputum concentrations of MMP9 remained significantly higher in the high Mtb load compared to the low Mtb load group at month 6 (p = 2.00E−02) (**Figure 3C**).

DISCUSSION

The aim of this study was to analyse neutrophil-associated soluble mediators in lung and blood samples from patients with different levels of lung pathology at baseline and recovery following treatment. We report stronger correlations between neutrophil counts and primarily neutrophil-derived mediators like MMP8, S100A8/A9, TNF, and GM-CSF as compared to

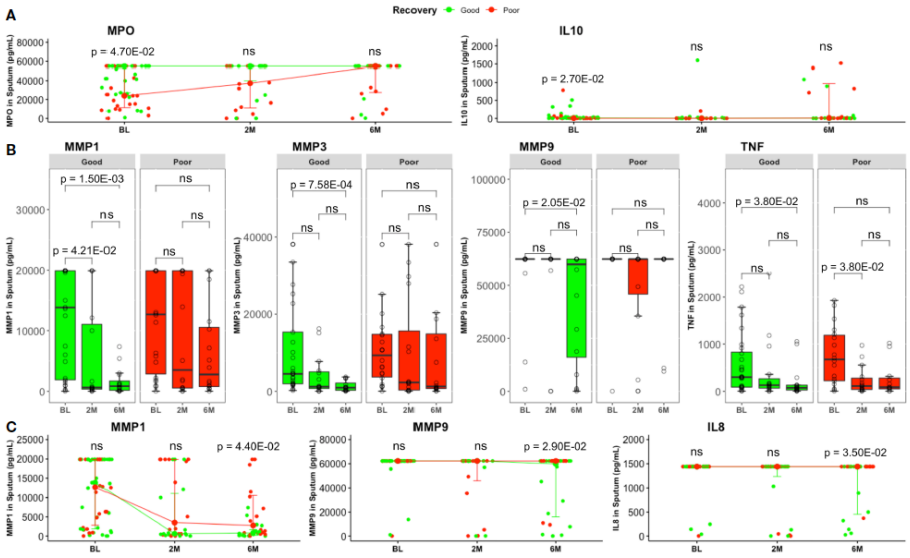


FIGURE 2 | Comparison of sputum inflammatory mediator levels between good and poor lung recovery groups with treatment time. **(A)** MPO and IL10 were higher in good (BL, n = 29; 2M, n = 16; 6M, n = 16) compared to poor (BL, n = 22; 2M, n = 16; 6M, n = 16) lung recovery groups at baseline. Data represent median [IQR]. Differences between lung pathology groups at any given time point were compared using the Wilcoxon signed rank test. **(B)** Most MMP1, 3, 9, and TNF levels were significantly lower at month 6 compared to baseline in patients with good lung recovery but not in those with poor lung recovery. Boxes represent the first and third quartiles and horizontal bars within indicate median concentration. Whiskers indicate minimum and maximum values. Each dot represents one individual patient. Kruskal–Wallis test with Dunn's post-test comparison was performed to analyse differences between time points. **(C)** At the end of standard TB treatment, MMP1, MMP9 and IL8 concentrations were still higher in patients with poor lung recovery compared to those with poor lung recovery. Groups were compared using the Wilcoxon signed rank test. Data represent median [IQR]. ns, not significant.

other known TB-related inflammatory markers like IFNγ, IL10 or IL12/23(p40), for which neutrophils are not necessarily the major sources.

GM-CSF is a known neutrophil primer and MMP8 concentrations have previously been linked to clinical and radiological TB severity (44, 45), while S100A8/9 regulates CD11b expression and accumulation in chronic TB mouse models (28, 35). Our data supports these previous observations. We show that pre-treatment plasma levels of S100A8/9, MMP8 and GM-CSF correlate strongly with neutrophil counts and lung damage severity. Interestingly, while sputum levels of MMP8 correlate positively, MPO correlates negatively with lung damage and Mtb burden at baseline. This suggest that lung pathology results from increased systemic and pulmonary inflammation. It also hints that MPO could dampen the inflammatory response in ATB, thereby preventing excessive bacterial load and lung damage. We recently revealed that neutrophil subsets are associated with protective or detrimental effects on the severity of TB-linked lung pathology (13). Gideon and collaborators also showed pro-(IFNγ) and anti-inflammatory (IL10) cytokine expression by different neutrophil subsets in granulomas from Mtb-infected cynomolgus macaques (24), suggesting an immunoregulatory function of neutrophils in TB granulomas. Also, neutrophil elastase dissociation is triggered by reactive oxygen species (ROS) in an MPO-dependent manner during NETosis (46, 47), suggesting that NETs are involved in an MPO-related protective mechanism. Additionally, Mtb control by HIV-coinfected macrophages is enhanced by apoptotic neutrophils in *via* an MPO-dependent process (48). Whilst MPO inhibition is reported to block Mtb-induced necrotic cell death (49), MPO-deficient mice develop severe lung inflammation following exposure to zymosan (37). In fact, a recent review details the numerous protective and harmful functions of MPO in human disease (50). Whilst IL10 is released by several immune cell types during TB and monocytes/macrophages also produce MPO, neutrophil granules are the main source of MPO (48). This to our knowledge, is the first report of an MPO-related beneficial role in TB-related lung pathology and recovery.

The current literature overwhelmingly supports a detrimental effect of neutrophils on lung pathology in TB patients, however, some neutrophil subsets are protective. Specific neutrophil subsets were associated with protective outcomes against TB lung pathology suggesting that the variations observed in disease outcomes may be driven by different immunomodulatory mediators or interactions with other immunocytes (13).

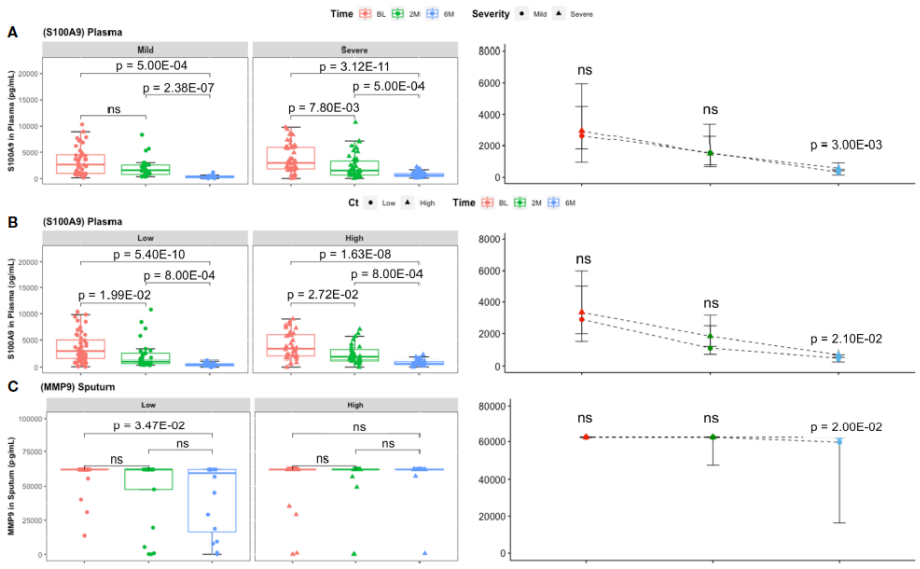


FIGURE 3 | Comparison of inflammatory mediator levels between lung damage and Mtb burden defined groups with treatment time. **(A)** In plasma, the decrease in S100A9 concentrations was comparable between lung damage groups (mild: BL, n = 46; 2M, n = 25; 6M, n = 14 and severe: BL, n = 61; 2M, n = 42; 6M, n = 43) over time. However, at month 6, S100A9 was significantly higher in patients with initially severe lung damage compared to mild lung damage. **(B)** The same observation made between initially high Mtb and low Mtb loads with S100A9 levels being higher in the former at month 6. Boxes represent the first and third quartiles and horizontal bars within indicate median concentration. Whiskers indicate minimum and maximum values. Each dot represents one individual patient. Kruskal–Wallis test with Dunn's post-test comparison was performed to analyse differences between time points. Differences between lung pathology groups at any given time point were compared using the Wilcoxon signed rank test. Data represent median [IQR]. **(C)** Sputum (low: BL, n = 46; 2M, n = 21; 6M, n = 16 and high: BL, n = 42; 2M, n = 22; 6M, n = 17) MMP9 levels were significantly lower at month 6 compared to baseline in patients with low but not in those with high Mtb loads. Additionally, S100A9 concentration at month 6 was significantly higher in patients with high Mtb compared to low Mtb load. ns, not significant.

Additionally, a neutrophil-driven regulatory effect is not unheard of. In fact, neutrophils (*via* CD11b-dependent responses) and endothelial cells have been shown to cooperate in detection and capture of pathogens in lung capillaries (51). Also, neutrophils play a central role in controlling human metapneumovirus-induced inflammation by regulating γδ T cell recruitment to the lung (52). Meanwhile, neutrophils were found to suppress of lymphocyte function by secreting MPO and hydrogen peroxide (53) and Mtb-specific stimulation of neutrophils inhibits antigen-specific T-cell production of IFNγ (24). More recently, this neutrophil-related immunosuppressive function on lymphocytes has been attributed to hyper-segmented subsets (7) and to the neutrophil-like MDSCs (54, 55) (in cancer (56, 57), leukaemia (58) and lately TB (9, 59). While the immunosuppressive roles of MDSCs on Mtb pathogenesis are still under investigation, recent experimental models show benefits in limiting their accumulation during TB HDTs (25, 60). We suggest that MPO could be protective against TB progression and lung damage.

We understand that functional analysis of neutrophils is technically difficult considering that they are short-lived, easily activated by laboratory processing methods and cannot be cryopreserved. Nevertheless, we support future investigation of mechanistical pathways that promote the secretion of these mediators or increased production of neutrophil subsets that produce them to achieve desirable outcomes during ATB. We could not confirm the link between MPO and bacterial burden using Mtb killing assays *in-vitro*. However, prospective studies within the TB sequel project are being designed to achieve that by assessing the levels of these mediators in the presence/absence of TB-targeted HDTs. We also recommend studies using isolated neutrophils from patients within these different lung pathology and gender groups to address this gap in knowledge (potentially also in animal models).

Variability in immune responses between genders have been linked to: specific immune cell types, age, levels of sex hormones, environmental factors (e.g., nutritional status or microbiome composition) and disease states (61). In accordance with previous studies (62, 63), ATB prevalence in our Gambian cohort is higher in males. Also similar to previous studies on chronic inflammatory diseases (64, 65), we observe that the pro-inflammatory response in males is higher than that in females. We report higher plasma levels of notoriously pro-inflammatory mediators like TNF, MMP3, GM-CSF, and IL8 in males

compared to females. This is in accordance with the observation that male neutrophils are more responsive to LPS and IFNγ stimulation than female neutrophils; with the former expressing higher levels of toll-like receptor 4 (TLR4) and producing more TNF (66). Meanwhile, we also observe higher sputum MPO levels in females, supporting the idea that increased MPO concentration is linked to suppressed inflammation.

Moreover, patients showing good lung recovery had higher sputum MPO concentrations at baseline. In contrast, MMP8 and sputum TNF levels were positively associated with poor recovery after adjusting for sex. These suggest that MPO, MMP8, and TNF play a considerable role in determining the degree of recovery from severe TB-related lung damage after treatment. It also supports future investigation of these mediators as proxies for predicting lung recovery following injury.

As treatment progresses, sputum and plasma concentrations of MMP1, 8, 9 and plasma levels of S100A8/9 and MMP3 decrease rapidly, suggesting that the neutrophil-related inflammatory response and matrix-degrading activity are not only fuelled by MMPs ((32, 33, 67)) and calprotectin (28) activity but also potentially resolved by variations in levels of these mediators with treatment. In contrast, sputum levels of IL8 and MPO remain fairly constant, suggesting that variations in neutrophil (and potentially monocyte) recruitment and overall activity during ATB treatment may be more complexly regulated. This is supported by our other observation that the decrease in concentrations of S100A8, MMP9, IL10, TNF, IFNγ, and GM-CSF in plasma and MMP1, MMP8, and TNF in sputum are exclusive to the severe lung damage group; suggesting that these inflammatory mediators are major contributors to severe TB-related lung pathology pre-treatment. The fact that no such analogy was observed when groups defined by Mtb burden were considered also supports the idea that high Mtb loads are not necessarily ascribed to severe lung damage outcomes (42).

Post-therapy, we observed high plasma S100A8 levels in severe compared to mild lung damage group, meanwhile plasma S100A8 and sputum MMP9 were significantly higher in patients with initially high Mtb load compared to the initially low Mtb load group. S100A9 and MMP9 are neutrophil-derived mediators, suggesting that severe lung damage at presentation may contribute to heightened residual neutrophil activity after treatment. Also, post-treatment levels of sputum MMP1, MMP9, and IL8 were higher in patients with poor lung recovery compared to those with good lung recovery. This suggests that unresolved lung damage is linked to continuous neutrophil activity and persistent leucocyte infiltration in the lungs post-treatment. While, previous studies suggested that for patients with severe lung damage, recovery may only begin many months after the end of standard ATT (68, 69), a possible reason for this was not provided. This, to our knowledge is the first report of several major neutrophil-derived mediators (in plasma and sputum) being directly linked to TB lung pathology and unresolved lung damage. Furthermore, higher levels of MMP1, MMP9, and IL8 in sputum from patients with poor compared to good lung recovery at month 6 suggest that poor lung recovery results from continuous neutrophil activity and persistent leucocyte infiltration in the lungs even after treatment completion.

For whole blood stimulated supernatants (notably with H37Rv whole cell lysate), the increased levels of GM-CSF (also increased in sputum), TNF (also increased in sputum), IFNγ, S100A8, MPO and MMP9 after treatment compared to baseline hint at an enhanced sensitivity of immune cells to pathogen stimulation. Previous studies have reported lower cytokine production by T-cells pre-treatment, suggesting that continual pathogen stimulation results in T-cell exhaustion which is then restored after treatment (reviewed in (70). To our knowledge, this is the first report of increased concentrations of major neutrophil-derived mediator levels in ATB post-treatment compared with pre-treatment levels. These suggest that chronic TB could directly (or indirectly, *via* T-cell exhaustion which leads to either higher levels of immune-inhibitory molecules like PD-1 (71, 72) and TIM3 (73) or reduced release of innate immune cell activators like IFNγ and TNF) result in reduced neutrophil activity pre-treatment. It also highlights the need to monitor the impact of neutrophil interactions with other immunocytes on TB pathogenesis. Finally, we suggest that toll-like receptor (TLR)-mediated pathogen sensing by lung epithelial/innate immune cells, MPO-regulated NET formation, neutrophil migration/activation following increased secretion of inflammatory mediators (e.g., S100A8/9, MMP8, GM-CSF, TNF, IFNγ and potentially IL17/IL17R, RANTES, IL6, ICAM1, etc.) and ROS release/NADPH-dependent leucocyte recruitment (74–76) are immune pathways potentially involved in balancing the neutrophilic inflammatory response during ATB.

CONCLUSION

We show that S100A8/9 and MMP8 contribute to increased lung damage and that MPO acts as an anti-inflammatory agent which potentially regulates TB-related lung pathology and promotes lung recovery. We also suggest that increased MPO-mediated immunosuppression could limit lung pathology in females. Treatment results in decreased inflammation characterised by lower sputum and plasma concentrations of neutrophil-derived pro-inflammatory mediators especially in patients with severe lung pathology (but not High Mtb load) at presentation. We hereby highlight the relationship between neutrophil-derived inflammatory mediator levels and radiological disease severity irrespective of Mtb burden. We also report that S100A8/9 and other neutrophilic mediators like MMP9 and IL8 may be responsible for unresolving lung damage in patients with poor lung recovery. Finally, we recommend targeting S100A8/9, MMP8, and MPO for developing host-directed therapies against TB-induced lung pathology and to promote recovery.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Research Council/Gambia government joint ethics committee (SCC1523). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CN: Conceptualisation, Data curation, Formal analysis, Investigation, Methodology and writing of manuscript. OO: Patient recruitment and follow-up, clinical data, and review of manuscript. SD: Data Management. JM: Assisted with wet-lab experiments. IS: Assisted with wet-lab experiments. SC: Funding acquisition and review of manuscript. AB: Data curation and Formal analysis. AR: Conceptualisation, Funding acquisition, data analysis, and review of manuscript. CG: Supervision, Methodology, and manuscript review. JS: Supervision, Conceptualisation, Data curation, Methodology, Funding acquisition, and review of manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a PhD student stipend from TB Sequel (grant number 66.3010.7-002.00) funded by the German Ministry for Education and Research (BMBF).

ACKNOWLEDGMENTS

We thank the patients and their families who volunteered for this study and The MRC Unit The Gambia at LSHTM for facilitating access to laboratory, clinical and other facilities. We are thankful to all the staff at The MRC Unit The Gambia at LSHTM's TB research group for technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.740933/full#supplementary-material

REFERENCES

1. WHO. *Global Tuberculosis Report 2020* Vol. 66. Geneva: World Health Organization (2020) p. 37–9.
2. Ralph AP, Kenangalem E, Waramori G, Pontororing GJ, Sandjaja, Tjitra E, et al. High Morbidity During Treatment and Residual Pulmonary Disability in Pulmonary Tuberculosis: Under-Recognised Phenomena. *PloS One* (2013) 8(11):1–11. doi: 10.1371/journal.pone.0080302
3. Rachow A, Ivanova O, Wallis R, Charalambous S, Jani I, Bhatt N, et al. TB Sequel: Incidence, Pathogenesis and Risk Factors of Long-Term Medical and Social Sequelae of Pulmonary TB - A Study Protocol. *BMC Pulm Med* (2019) 19(1):1–9. doi: 10.1186/s12890-018-0777-3
4. Cadena AM, Fortune SM, Flynn JL. Heterogeneity in Tuberculosis. *Nat Rev Immunol* (2017) 17(11):691–702. doi: 10.1038/nri.2017.69
5. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MPR. The Immune Response in Tuberculosis. *Annu Rev Immunol* (2013) 31 (1):475–527. doi: 10.1146/annurev-immunol-032712-095939
6. Shi S, Zhang X, Zhou Y, Tang H, Zhao D, Liu F. Immunosuppression Reduces Lung Injury Caused by Mycoplasma Pneumoniae Infection. *Sci Rep* (2019) 9 (1):1–8. doi: 10.1038/s41598-019-43451-9
7. Pillay J, Kamp VM, Van Hoffen E, Visser T, Tak T, Lammers J, et al. A Subset of Neutrophils in Human Systemic Inflammation Inhibits T Cell Responses Through Mac-1. *J Clin Invest* (2012) 122(1):327–36. doi: 10.1172/JCI57990
8. Pillay J, Tak T, Kamp VM, Koenderman L. Immune Suppression by Neutrophils and Granulocytic Myeloid-Derived Suppressor Cells: Similarities and Differences. *Cell Mol Life Sci* (2013) 70(20):3813–27. doi: 10.1007/s00018-013-1286-4
9. Dorhoi A, Kotzé LA, Berzofsky JA, Sui Y, Gabrilovich DI, Garg A, et al. Therapies for Tuberculosis and AIDS: Myeloid-Derived Suppressor Cells in Focus. *J Clin Invest* (2020) 130(6):2789–99. doi: 10.1172/JCI136288
10. De Melo MGM, Mesquita EDD, Oliveira MM, Da Silva-Monteiro C, Silveira AKA, Malaquias TS, et al. Imbalance of NET and Alpha-1-Antitrypsin in Tuberculosis Patients is Related With Hyper Inflammation and Severe Lung Tissue Damage. *Front Immunol* (2019) 10(JAN):1–17. doi: 10.3389/fimmu.2018.03147
11. Lyadova IV. Neutrophils in Tuberculosis: Heterogeneity Shapes the Way? *Mediators Inflamm* (2017) 2017:8619307. doi: 10.1155/2017/8619307
12. Muefong CN, Sutherland JS. Neutrophils in Tuberculosis-Associated Inflammation and Lung Pathology. *Front Immunol* (2020) 11(May):1–9. doi: 10.3389/fimmu.2020.00962
13. Nwongbouwoh Muefong C, Owolabi O, Donkor S, Charalambous S, Bakuli A, Rachow A, et al. Neutrophils Contribute to Severity of Tuberculosis Pathology and Recovery From Lung Damage Pre- and Post-Treatment. *Clin Infect Dis* (2021) ciab729:962. doi: 10.1093/cid/ciab729
14. Borkute RR, Woelke S, Pei G, Dorhoi A. Neutrophils in Tuberculosis: Cell Biology, Cellular Networking and Multitasking in Host Defense. *Int J Mol Sci* (2021) 22(9):4801. doi: 10.3390/ijms22094801
15. Kumar NP, Moideen K, Nancy A, Viswanathan V, Shruthi BS, Sivakumar S, et al. Plasma Chemokines are Biomarkers of Disease Severity, Higher Bacterial Burden and Delayed Sputum Culture Conversion in Pulmonary Tuberculosis. *Sci Rep* (2019) 9(1):1–8. doi: 10.1038/s41598-019-54803-w
16. Riou C, Du Bruyn E, Ruzive S, Goliath RT, Lindestam Arlehamn CS, Sette A, et al. Disease Extent and Anti-Tubercular Treatment Response Correlates With *Mycobacterium Tuberculosis*-Specific CD4 T-Cell Phenotype Regardless of HIV-1 Status. *Clin Transl Immunol* (2020) 9(9):e1176. doi: 10.1002/cti2.1176
17. Khosa C, Bhatt N, Massango I, Azam K, Saathoff E, Bakuli A, et al. Development of Chronic Lung Impairment in Mozambican TB Patients and Associated Risks. *BMC Pulm Med* (2020) 20(1):1–11. doi: 10.1186/s12890-020-1167-1
18. Leem AY, Song JH, Lee EH, Lee H, Sim B, Kim SY, et al. Changes in Cytokine Responses to TB Antigens ESAT-6, CFP-10 and TB 7.7 and inflammatory markers in peripheral blood during therapy. *Sci Rep* (2018) 8(1):4–11. doi: 10.1038/s41598-018-19523-7
19. Cho Y, Park Y, Sim B, Kim J, Lee H, Cho SN, et al. Identification of Serum Biomarkers for Active Pulmonary Tuberculosis Using a Targeted Metabolomics Approach. *Sci Rep* (2020) 10(1):1–11. doi: 10.1038/s41598-020-60669-0
20. MacLean E, Broger T, Yerlikaya S, Fernandez-Carballo BL, Pai M, Denkinger CM. A Systematic Review of Biomarkers to Detect Active Tuberculosis. *Nat Microbiol* (2019) 4(5):748–58. doi: 10.1038/s41564-019-0380-2
21. Yong YK, Tan HY, Saeidi A, Wong WF, Vignesh R, Velu V, et al. Immune Biomarkers for Diagnosis and Treatment Monitoring of Tuberculosis: Current Developments and Future Prospects. *Front Microbiol* (2019) 10 (December). doi: 10.3389/fmicb.2019.02789
22. Morrison H, McShane H. Local Pulmonary Immunological Biomarkers in Tuberculosis. *Front Immunol* (2021) 12(March):5–10. doi: 10.3389/fimmu.2021.640916
23. Kroon EE, Coussens AK, Kinnear C, Orlova M, Möller M, Seeger A, et al. Neutrophils: Innate Effectors of TB Resistance? *Front Immunol* (2018) 9 (NOV):1–12. doi: 10.3389/fimmu.2018.02637

24. Gideon HP, Phuah J, Junecko BA, Mattila JT. Neutrophils Express Pro- and Anti-Inflammatory Cytokines in Granulomas From Mycobacterium Tuberculosis-Infected Cynomolgus Macaques. *Mucosal Immunol* (2019) 12 (6):1370–81. doi: 10.1038/s41385-019-0195-8
25. Parveen S, Lun S, Urbanowski ME, Cardin M, Shen J, Murphy JR, et al. Effective Host-Directed Therapy for Tuberculosis by Depletion of Myeloid-Derived Suppressor Cells and Related Cells Using a Diphtheria Toxin Fusion Protein. *J Infect Dis* (2021) jiab235. doi: 10.1093/infdis/jiab235
26. Young C, Walzl G, Du Plessis N. Therapeutic Host-Directed Strategies to Improve Outcome in Tuberculosis. *Mucosal Immunol* (2020) 13(2):190–204. doi: 10.1038/s41385-019-0226-5
27. Mishra BB, Lovewell RR, Olive AJ, Zhang G, Wang W, Eugenin E, et al. Nitric Oxide Prevents a Pathogen Permissive Granulocytic Inflammation During Tuberculosis. *Nat Microbiol* (2017) 2:17072. doi: 10.1038/nmicrobiol.2017.72
28. Gopal R, Monin L, Torres D, Slight S, Mehra S, McKenna KC, et al. S100A8/A9 Proteins Mediate Neutrophilic Inflammation and Lung Pathology During Tuberculosis. *Am J Respir Crit Care Med* (2013) 188(9):1137–46. doi: 10.1164/rccm.201304-0803OC
29. Mattila JT, Maiello P, Sun T, Via LE, Flynn JL. Granzyme B-Expressing Neutrophils Correlate With Bacterial Load in Granulomas From Mycobacterium Tuberculosis-Infected Cynomolgus Macaques. *Cell Microbiol* (2015) 17(8):1085–97. doi: 10.1111/cmi.12428
30. Lowe DM, Bandara AK, Packe GE, Barker RD, Robert J. Neutrophilia Independently Predicts Death in Tuberculosis. *Eur Respir J* (2014) 42 (6):1752–7. doi: 10.1183/09031936.00140913
31. Berry MPR, Graham CM, McNab FW, Xu Z, Bloch SAA, Oni T, et al. An Interferon-Inducible Neutrophil-Driven Blood Transcriptional Signature in Human Tuberculosis. *Nature* (2010) 466(7309):973–7. doi: 10.1038/nature09247
32. Ong CWM, Elkington PT, Brilha S, Ugarte-Gil C, Tome-Esteban MT, Tezera LB, et al. Neutrophil-Derived MMP-8 Drives AMPK-Dependent Matrix Destruction in Human Pulmonary Tuberculosis. *PloS Pathog* (2015) 11 (5):1–21. doi: 10.1371/journal.ppat.1004917
33. Ugarte-Gil CA, Elkington P, Gilman RH, Coronel J, Tezera LB, Bernabe-Ortiz A, et al. Induced Sputum MMP-1, -3 & -8 Concentrations During Treatment of Tuberculosis. *PloS One* (2013) 8(4):2–9. doi: 10.1371/journal.pone.0061333
34. Elkington PTG, Friedland JS. Matrix Metalloproteinases in Destructive Pulmonary Pathology. *Thorax* (2006) 61(3):259–66. doi: 10.1136/thx.2005.051979
35. Scott NR, Swanson RV, Al-Hammadi N, Domingo-Gonzalez R, Rangel-Moreno J, Kriel BA, et al. S100A8/A9 Regulates CD11b Expression and Neutrophil Recruitment During Chronic Tuberculosis. *J Clin Invest* (2020) 130(6):3098–112. doi: 10.1172/JCI130546
36. Munder M, Schneider H, Luckner C, Giese T, Langhans CD, Fuentes JM, et al. Suppression of T-Cell Functions by Human Granulocyte Arginase. *Blood* (2006) 108(5):1627–34. doi: 10.1182/blood-2006-11-010389
37. Takeuchi K, Umeki Y, Matsumoto N, Yamamoto K, Yoshida M, Suzuki K, et al. Severe Neutrophil-Mediated Lung Inflammation in Myeloperoxidase-Deficient Mice Exposed to Zymosan. *Inflamm Res* (2012) 61(3):197–205. doi: 10.1007/s00011-011-0401-y
38. Ralph AP, Ardian M, Wiguna A, Maguire GP, Becker NG, Drogumuller G, et al. A Simple, Valid, Numerical Score for Grading Chest X-Ray Severity in Adult Smear-Positive Pulmonary Tuberculosis. *Thorax* (2010) 65(10):863–9. doi: 10.1136/thx.2010.136242
39. Chakravorty S, Simmons AM, Rowneki M, Parmar H, Cao Y, Ryan J, et al. The New Xpert MTB/RIF Ultra: Improving Detection of Mycobacterium Tuberculosis and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing. *MBio* (2017) 8(4):1–12. doi: 10.1128/mBio.00812-17
40. Team RC. R: A Language and Environment for Statistical Computing. In: *R Foundation for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing (2018). Available at: https://www.R-project.org/https://www.r-project.org/.
41. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate : A Practical and Powerful Approach to Multiple Testing. *J R Stat Soc Ser B Methodol* (1995) 57(1):289–300. Publi. J R Stat Soc. doi: 10.1111/j.2517-6161.1995.tb02031.x
42. Murthy SE, Chatterjee F, Crook A, Dawson R, Mendel C, Murphy ME, et al. Pretreatment Chest X-Ray Severity and Its Relation to Bacterial Burden in Smear Positive Pulmonary Tuberculosis. *BMC Med* (2018) 16(1):1–11. doi: 10.1186/s12916-018-1053-3
43. Nwongbouwoh Muefong C, Owolabi O, Donkor S, Charalambous S, Bakuli A, Rachow A, et al. Neutrophils Contribute to Severity of Tuberculosis Pathology and Recovery From Lung Damage Pre- and Posttreatment. *Clin Infect Dis* (2021) 2021:1–10. doi: 10.1093/cid/ciab729
44. Sigal GB, Segal MR, Mathew A, Jarlsberg L, Wang M, Barbero S, et al. Biomarkers of Tuberculosis Severity and Treatment Effect: A Directed Screen of 70 Host Markers in a Randomized Clinical Trial. *EBioMedicine* (2017) 25:112–21. doi: 10.1016/j.ebiom.2017.10.018
45. Ong CWM, Fox K, Ettorre A, Elkington PT, Friedland JS. Hypoxia Increases Neutrophil-Driven Matrix Destruction After Exposure to Mycobacterium Tuberculosis. *Sci Rep* (2018) 8(1):1–11. doi: 10.1038/s41598-018-29659-1
46. Metzler KD, Goosmann C, Lubojemska A, Zychlinsky A, Papayannopoulos V. Myeloperoxidase-Containing Complex Regulates Neutrophil Elastase Release and Actin Dynamics During NETosis. *Cell Rep* (2014) 8(3):883–96. doi: 10.1016/j.celrep.2014.06.044
47. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil Elastase and Myeloperoxidase Regulate the Formation of Neutrophil Extracellular Traps. *J Cell Biol* (2010) 191(3):677–91. doi: 10.1083/jcb.201006052
48. Andersson AM, Larsson M, Stendahl O, Blomgran R. Efferocytosis of Apoptotic Neutrophils Enhances Control of Mycobacterium Tuberculosis in HIV-Coinfected Macrophages in a Myeloperoxidase-Dependent Manner. *J Innate Immun* (2020) 12(3):235–47. doi: 10.1159/000500861
49. Corleis B, Korbel D, Wilson R, Bylund J, Chee R, Schaible UE. Escape of Mycobacterium Tuberculosis From Oxidative Killing by Neutrophils. *Cell Microbiol* (2012) 14(7):1109–21. doi: 10.1111/j.1462-5822.2012.01783.x
50. Arnhold J. The Dual Role of Myeloperoxidase in Immune Response. *Int J Mol Sci* (2020) 21(21):8057. doi: 10.3390/ijms21218057
51. Yipp BG, Kim JH, Lima R, Zbytnuik LD, Petri B, Swanlund N, et al. The Lung is a Host Defense Niche for Immediate Neutrophil-Mediated Vascular Protection. *Sci Immunol* (2017) 2(10):1–14. doi: 10.1126/sciimmunol.aam8929
52. Cheemarla NR, Baños-Lara MDR, Naidu S, Guerrero-Plata A. Neutrophils Regulate the Lung Inflammatory Response *via* γδ T Cell Infiltration in an Experimental Mouse Model of Human Metapneumovirus Infection. *J Leukoc Biol* (2017) 101(6):1383–92. doi: 10.1189/jlb.4A1216-519RR
53. el-Hag A, Clark RA. Immunosuppression by Activated Human Neutrophils. Dependence on the Myeloperoxidase System. *J Immunol* (1987) 139(7):2406–13.
54. Yang P, Li Y, Xie Y, Liu Y. Different Faces for Different Places: Heterogeneity of Neutrophil Phenotype and Function. *J Immunol Res* (2019) 2019:8016254. doi: 10.1155/2019/8016254
55. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic Diversity and Plasticity in Circulating Neutrophil Subpopulations in Cancer. *Cell Rep* (2015) 10(4):562–74. doi: 10.1016/j.celrep.2014.12.039
56. Cassetta L, Bruderek K, Skrzeczynska-Moncznik J, Osiecka O, Hu X, Rundgren IM, et al. Differential Expansion of Circulating Human MDSC Subsets in Patients With Cancer, Infection and Inflammation. *J Immunother Cancer* (2020) 8(2):e001223. doi: 10.1136/jitc-2020-001223
57. Yang Y, Li C, Liu T, Dai X, Bazhin AV. Myeloid-Derived Suppressor Cells in Tumors: From Mechanisms to Antigen Specificity and Microenvironmental Regulation. *Front Immunol* (2020) 11(July):1–22. doi: 10.3389/fimmu.2020.01371
58. Ferrer G, Jung B, Chiu PY, Aslam R, Palacios F, Mazzarello AN, et al. Myeloid-Derived Suppressor Cell Subtypes Differentially Influence T-Cell Function, T-Helper Subset Differentiation, and Clinical Course in CLL. *Leukemia* (2021) 35(11):3163–75. doi: 10.1038/s41375-021-01249-7
59. Magcwebeba T, Dorhoi A, Du Plessis N. The Emerging Role of Myeloid-Derived Suppressor Cells in Tuberculosis. *Front Immunol* (2019) 10(APR). doi: 10.3389/fimmu.2019.00917
60. Leukes V, Walzl G, du Plessis N. Myeloid-Derived Suppressor Cells as Target of Phosphodiesterase-5 Inhibitors in Host-Directed Therapeutics for Tuberculosis. *Front Immunol* (2020) 11(March):1–7. doi: 10.3389/fimmu.2020.00451
61. Klein SL, Flanagan KL. Sex Differences in Immune Responses. *Nat Rev Immunol* (2016) 16(10):626–38. doi: 10.1038/nri.2016.90
62. Neyrolles O, Quintana-Murci L. Sexual Inequality in Tuberculosis. *PloS Med* (2009) 6(12):e1000199. doi: 10.1371/journal.pmed.1000199

63. Horton KC, MacPherson P, Houben RMGJ, White RG, Corbett EL. Sex Differences in Tuberculosis Burden and Notifications in Low- and Middle-Income Countries: A Systematic Review and Meta-Analysis. *PloS Med* (2016) 13(9):1–23. doi: 10.1371/journal.pmed.1002119
64. Gemmati D, Bramanti B, Serino ML, Secchiero P. COVID-19 and Individual Genetic Susceptibility/Receptivity: Role of ACE1/ACE2 Genes, Immunity, Inflammation and Coagulation. Might the Double X-Chromosome in Females Be Protective Against SARS-CoV-2 Compared to the Single X-Chromosome in Males? *Int J Mol Sci* (2020) 21(3474):1–23. doi: 10.3390/ijms21103474
65. Doss PMIA, Umair M, Baillargeon J, Fazazi R, Fudge N, Akbar I, et al. Male Sex Chromosomal Complement Exacerbates the Pathogenicity of Th17 Cells in a Chronic Model of Central Nervous System Autoimmunity. *Cell Rep* (2021) 34(10):108833. doi: 10.1016/j.celrep.2021.108833
66. Aomatsu M, Kato T, Kasahara E, Kitagawa S. Gender Difference in Tumor Necrosis Factor-α Production in Human Neutrophils Stimulated by Lipopolysaccharide and Interferon-γ. *Biochem Biophys Res Commun* (2013) 441(1):220–5. doi: 10.1016/j.bbrc.2013.10.042
67. Kumar NP, Moideen K, Viswanathan V, Shruthi BS, Sivakumar S, Menon PA, et al. Elevated Levels of Matrix Metalloproteinases Reflect Severity and Extent of Disease in Tuberculosis-Diabetes Co-Morbidity and are Predominantly Reversed Following Standard Anti-Tuberculosis or Metformin Treatment. *BMC Infect Dis* (2018) 18(1):1–10. doi: 10.1186/s12879-018-3246-y
68. Hnizdo E, Singh T, Churchyard G. Chronic Pulmonary Function Impairment Caused by Initial and Recurrent Pulmonary Tuberculosis Following Treatment. *Thorax* (2000) 55(1):32–8. doi: 10.1136/thorax.55.1.32
69. Long R, Maycher B, Dhar A, Manfreda J, Hershfield E, Anthonisen N. Pulmonary Tuberculosis Treated With Directly Observed Therapy: Serial Changes in Lung Structure and Function. *Chest* (1998) 113(4):933–43. doi: 10.1378/chest.113.4.933
70. Khan N, Vidyarthi A, Amir M, Mushtaq K, Agrewala JN. T-Cell Exhaustion in Tuberculosis: Pitfalls and Prospects. *Crit Rev Microbiol* (2017) 43(2):133–41. doi: 10.1080/1040841X.2016.1185603
71. Day CL, Moshi ND, Abrahams DA, Van Rooyen M, O'Rie T, De Kock M, et al. Patients With Tuberculosis Disease Have Mycobacterium Tuberculosis-Specific CD8 T Cells With a Pro-Apoptotic Phenotype and Impaired Proliferative Capacity, Which is Not Restored Following Treatment. *PloS One* (2014) 9(4):1–12. doi: 10.1371/journal.pone.0094949
72. Day CL, Abrahams DA, Lerumo L, Janse van Rensburg E, Stone L, O'rie T, et al. Functional Capacity of Mycobacterium Tuberculosis -Specific T Cell Responses in Humans Is Associated With Mycobacterial Load. *J Immunol* (2011) 187(5):2222–32. doi: 10.4049/jimmunol.1101122
73. Jayaraman P, Jacques MK, Zhu C, Steblenko KM, Stowell BL, Madi A, et al. TIM3 Mediates T Cell Exhaustion During Mycobacterium Tuberculosis Infection. *PloS Pathog* (2016) 12(3):1–21. doi: 10.1371/journal.ppat.1005490
74. Greene CM, Hiemstra PS. Innate Immunity of the Lung. *J Innate Immun* (2020) 12(1):1–3. doi: 10.1159/000504621
75. Kumar V. Pulmonary Innate Immune Response Determines the Outcome of Inflammation During Pneumonia and Sepsis-Associated Acute Lung Injury. *Front Immunol* (2020) 11(August):1722. doi: 10.3389/fimmu.2020.01722
76. Galeas-Pena M, McLaughlin N, Pociask D. The Role of the Innate Immune System on Pulmonary Infections. *Biol Chem* (2019) 400(4):443–56. doi: 10.1515/hsz-2018-0304

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Paper III

frontiers in Immunology

ORIGINAL RESEARCH
published: 09 September 2020
doi: 10.3389/fimmu.2020.572620

Monitoring Anti-tuberculosis Treatment Response Using Analysis of Whole Blood *Mycobacterium tuberculosis* Specific T Cell Activation and Functional Markers

*Molly A. Vickers[1,2], Fatoumatta Darboe[2], Caleb N. Muefong[2], Georgetta Mbayo[2], Amadou Barry[2], Awa Gindeh[2], Sainabou Njie[2], Abi-Janet Riley[2], Binta Sarr[2], Basil Sambou[2], Hazel M. Dockrell[1], Salome Charalambous[3], Andrea Rachow[4,5], Olumuyiwa Owolabi[2], Shamanthi Jayasooriya[2,6] and Jayne S. Sutherland[2]**

[1] Department of Infection Biology, London School of Hygiene and Tropical Medicine, London, United Kingdom, [2] Vaccines and Immunity Theme, MRC Unit The Gambia at the London School of Hygiene and Tropical Medicine, Fajara, Gambia, [3] School of Public Health, Aurum Institute, Johannesburg, South Africa, [4] Division of Infectious Diseases and Tropical Medicine, University Hospital, LMU Munich, Munich, Germany, [5] German Centre for Infection Research (DZIF), Partner Site Munich, Munich, Germany, [6] Academic Unit of Primary Care, University of Sheffield, Sheffield, United Kingdom

OPEN ACCESS

Edited by:
Paul Laszlo Bollyky, Stanford University, United States

Reviewed by:
Carmen Judith Serrano, Mexican Social Security Institute (IMSS), Mexico
Giulia Carla Marchetti, University of Milan, Italy

***Correspondence:**
Jayne S. Sutherland
jsutherland@mrc.gm

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *15 June 2020*
Accepted: *17 August 2020*
Published: *09 September 2020*

Citation:
Vickers MA, Darboe F, Muefong CN, Mbayo G, Barry A, Gindeh A, Njie S, Riley A-J, Sarr B, Sambou B, Dockrell HM, Charalambous S, Rachow A, Owolabi O, Jayasooriya S and Sutherland JS (2020) Monitoring Anti-tuberculosis Treatment Response Using Analysis of Whole Blood Mycobacterium tuberculosis Specific T Cell Activation and Functional Markers. Front. Immunol. 11:572620. doi: 10.3389/fimmu.2020.572620

Background: Blood-based biomarkers have been proposed as an alternative to current sputum-based treatment monitoring methods in active tuberculosis (ATB). The aim of this study was to validate previously described phenotypic, activation, and cytokine markers of treatment response in a West African cohort.

Methods: Whole blood immune responses to *Mycobacterium tuberculosis* ESAT-6/CFP-10 (EC) and purified protein derivative (PPD) were measured in twenty adults at baseline and after 2 months of standard TB treatment. Patients were classified as fast or slow responders based on a negative or positive sputum culture result at 2 months, respectively. Cellular expression of activation markers (CD38, HLA-DR), memory markers (CD27), and functional intracellular cytokine and proliferation (IFN-γ, Ki-67, TNF-α) markers were measured using multi-color flow cytometry.

Results: There was a significant increase in the proportion of $CD4^+CD27^+$ cells expressing CD38 and HLA-DR following EC stimulation at 2 months compared to baseline ($p = 0.0328$ and $p = 0.0400$, respectively). Following PPD stimulation, slow treatment responders had a significantly higher proportion of $CD8^+CD27^-$IFN-γ^+ ($p = 0.0105$) and $CD4^+CD27^+$HLA-DR^+CD38^+ ($p = 0.0077$) T cells than fast responders at baseline. Receiver operating curve analysis of these subsets resulted in 80% sensitivity and 70 and 100% specificity, respectively (AUC of 0.82, $p = 0.0156$ and 0.84, $p = 0.0102$).

Conclusion: Our pilot data show reductions in expression of T cell activation markers were seen with treatment, but this was not associated with fast or slow sputum conversion at 2 months. However, baseline proportions of activated T cell subsets are potentially predictive of the subsequent speed of response to treatment.

Keywords: tuberculosis, treatment, activation markers, cytokines, immunity

INTRODUCTION

The World Health Organization's (WHO) 2018 Global Tuberculosis (TB) report estimated that 10 million people developed active TB (ATB) disease in 2018 resulting in 1.6 million deaths (1). One hurdle inhibiting control over the TB epidemic is the challenge of accurately monitoring and predicting treatment responses in a timely, efficient, reliable, and cost-effective manner. Nucleic acid amplification-based tests lack the ability to discriminate between DNA from viable and dead *Mycobacterium tuberculosis* (Mtb). Sputum smear and microscopy lacks sensitivity (2–4) and the time-lag in receiving Mtb sputum culture results limits their clinical application in identifying those that are not responding to treatment. Critically, all of these techniques require sputum samples, which are often difficult to obtain from individuals after 2 months of treatment (5, 6) at a time when there is the potential for modifying treatment regimens. Additionally, sputum samples from individuals with extra-pulmonary TB and/or HIV co-infection are often paucibacillary (7–9).

Blood-based biomarkers have been proposed as an attractive tool to diagnose, monitor and predict treatment response in ATB. Blood (particularly fingerstick) can be taken from any individual and biomarkers can be pooled to improve predictive power and create one biosignature, resulting in the creation of an inexpensive assay that could be used in the field by unskilled personnel (5). This would also enable faster TB drug development and personalized treatment regimens (5).

Previous studies have shown an ability to diagnose and distinguish ATB and latent tuberculosis infection (LTBI) using Mtb-specific $CD4^+$ T-cell activation markers including; CD27, IFN-γ, CD38, HLA-DR, and Ki67 (3, 10–17). Prior to treatment initiation, high frequencies of activated Mtb-specific $CD4^+IFN\text{-}\gamma^+$ T-cells are seen in ATB patients compared to healthy controls and LTBI participants after either ESAT-6/CFP-10 (EC) or purified protein derivative (PPD) stimulation (3, 10). CD38 and HLA-DR expression on $CD4^+$ cells declines rapidly within the first month of treatment below the cut-off for ATB, while CD27 and Ki67 expression declined more slowly and this is correlated with mycobacterial load (3, 10). PPD-specific $CD4^+CD27^-$ T-cell frequencies have also been shown to distinguish healthy BCG vaccinated individuals, LTBI and ATB patients, suggesting exposure associated differentiation (14, 18).

Whole blood cellular staining using a limited panel of fluorescent parameters (CD3, HLA-DR, TNF-α, IFN-γ), using a basic flow cytometer, demonstrated specificity at 100% and sensitivity at 86% when distinguishing LTBI and ATB patients. Thus implying feasibility for use in a resource-constrained setting (11). $CD4^+Ki67^+HLA\text{-}DR^-$ T regulatory cells have also demonstrated their potential use in predicting time to culture conversion in multidrug resistant tuberculosis (MDR-TB) (19). Receiver operating curve (ROC) analysis demonstrated that at baseline, this T reg population could predict treatment response with 81.2% sensitivity and 85% specificity (19).

The aim of this study was to determine the potential use of activation markers expressed on both $CD4^+$ and $CD8^+$ T-cells for monitoring ATB treatment response in a longitudinal cohort of ATB adults from West Africa.

MATERIALS AND METHODS

Patients

We analyzed samples from 20 HIV-negative adult patients prospectively recruited from the Medical Research Council at The Gambia (MRCG) TB clinic with confirmed ATB (sputum GeneXpert positive) following written informed consent. Patients were recruited as part of the TB sequel project (20). Heparinized blood samples were collected and processed at diagnosis (baseline) and following 2 months of standard TB treatment. All participants were mycobacteria growth indicator tube (MGIT) sputum Mtb culture positive and drug sensitive at baseline. Based on sputum culture positivity at 2 months participants were grouped into either slow responders (culture positive at 2 months but negative by 6 months) or fast responders (culture negative by 2 months).

Processing and Storage of Stimulated Whole Blood

Five hundred microliter of whole blood was stimulated with either ESAT-6/CFP-10 peptide pool [EC; overlapping 15mer peptides reconstituted in 5% DMSO and H_2O and topped up to 1 mg/ml with PBS; final concentration 2.5 μg/ml/peptide; Peptides & Elephants, Germany (**Supplementary Table S1**)], PPD (10 μg/ml; Staten Serum Institute, Denmark) or phorbol 12-myristate 13-acetate (PMA; positive control; 10 μg/ml) along with co-stimulatory antibodies (anti-CD28, anti-CD49d; Becton Dickinson, United States) or unstimulated, cultured with medium alone (negative control). Each tube was vortexed for 10 s and 1 μl of 500× protein transport inhibitor was then added (eBioscience, United Kingdom). Tubes were incubated overnight at 37°C, 5% CO_2 with loose lids. After incubation, 50 μl of 20 mM EDTA was added, vortexed and incubated for 15 min at room temperature (RT). Cells were then lysed and fixed with 4.5 ml of 1× FACS lysing solution (Becton Dickinson, United States) and incubated for a further 9 min in the dark. Vials were then centrifuged at 1500 rpm for 5 min, decanted and placed on ice. One milliliter of cryosolution (20% DMSO, 80% FCS) was added and cells were transferred into 1.8 ml cryovials and stored in liquid nitrogen.

Sample Thawing

Patients' samples from both groups (fast and slow responders) and time points (baseline and 2 months) were processed simultaneously, limiting batch to batch variation. Cryovials were retrieved from liquid nitrogen, placed on dry ice and semi-thawed in a 37°C water bath. Samples were then transferred to a Falcon tube (Becton Dickinson, United States) containing 10 ml of PBS and mixed with a pasteur pipette. Tubes were centrifuged at 1500 rpm for 5 min, supernatants discarded, and pellets resuspended in 1 ml of 1× PBS. About 0.5 ml of the solution was then transferred into 5 mm polystyrene tubes, centrifuged at 1500 rpm for 5 min and supernatants discarded carefully.

Cell Surface Staining

Anti-CD3 BV786 (clone SP34-2), anti-CD4 BV605 (clone RPA-T4), anti-CD27 APC (clone M-T271), anti-CD38 PE-CF594 (clone HIT2) (BD Biosciences, United Kingdom), and anti-HLA-DR BV421 (clone L243) and anti-CD8a BV510 (clone RPA-T8) (BioLegend, United Kingdom) antibodies were diluted in FACS buffer (1% FBS, 0.1% EDTA, 0.05% sodium azide) to create a surface staining cocktail. Titrations were conducted beforehand to determine the optimal dilution for each antibody. Twenty microliter of the cocktail was added per tube, vortexed, and incubated for 30 min at RT in the dark. Cells were then washed with 1 ml of FACS buffer, centrifuged at 1800 rpm for 5 min and supernatants discarded.

Permeabilization and Intracellular Cytokine Staining

Five hundred microliter of 1× BD FACS™ Permeabilizing Solution 2 (Perm2) (BD Biosciences, United Kingdom) was added to each tube at a 1:10 dilution, vortexed for 10 s and tubes incubated for 20 min at RT in the dark. Cells were then centrifuged at 1800 rpm for 5 min and supernatant was carefully removed using a pipette. Twenty microliter of the intracellular cytokine cocktail consisting of anti-Ki-67 PE (clone Ki67) (BioLegend, United Kingdom), anti-IFN-γ AF700 (clone B27) (BD Biosciences, United Kingdom), and anti-TNF-α Pe-Cy7 (clone MAb11) (Invitrogen), diluted in Perm2 solution was added per tube. Samples were incubated for 30 min at RT in the dark, washed and resuspended in 300 μl of FACS buffer prior to acquisition.

Flow Cytometry Acquisition

Flow-cytometry acquisition was performed using a LSR Fortessa (BD Biosciences, United States). A minimum of 150,000 lymphocytes were acquired per tube. Positive and negative ArC™ Amine Reactive Compensation Beads (Life Technologies), BD CompBeads or UltraComp eBeads (Invitrogen) were stained with a fluorochrome-conjugated antibody to apply compensation. Data files were acquired with FACSDiva™ software (BD Biosciences, United States), analyzed using FlowJo software version 10.6 (Treestar, United States) and tables were exported into Excel for statistical analysis. Polyfunctional cells were analyzed using SPICE software (21).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.1.2 software (Software MacKiev, United States). For cytokine responses, background was subtracted using the unstimulated samples. Differences between paired baseline and 2-month samples were analyzed using a Wilcoxon matched-paired rank test. For analysis of fast and slow treatment responders, a Kruskal–Wallis or Mann–Whitney U Test was used for each time-point. Receiver operating characteristic (ROC) curve analysis was conducted to determine the cut-offs with the maximum sensitivity and specificity of statistically significant markers to discriminate between fast and slow responders. A p value less than 0.05 was considered to be statistically significant.

RESULTS

Patient Demographics

There was no significant difference in age between the fast and slow responders, with a median[interquartile range (IQR)] of 25 [22–31] and 32 [28–35] years, respectively (**Table 1**). 80% were male in both groups and all were HIV negative. Importantly the GeneXpert cycle threshold (Ct) values did not significantly differ between the groups ($p = 0.23$).

Flow Cytometry Gating Strategy

Lymphocytes were first gated based on forward and side scatter (**Supplementary Figure S1A**). Doublets were then excluded (**Supplementary Figure S1B**) and $CD4^+$ and $CD8^+$ T-cells were gated (**Supplementary Figure S1C**). Within both $CD4^+$ and $CD8^+$ T-cell populations, $CD27^{\pm}$ subsets were gated (**Supplementary Figure S1D**) followed by Boolean gating analysis of activation markers (HLA-DR/CD38), Ki-67 and cytokines (TNF-α, IFN-γ) (**Supplementary Figure S1E**). The validity of the gates for the activation and functional markers was established using fluorescence minus one control (data not shown). This gating strategy was implemented due to the small proportion of IFN-γ T cells observed. Total $CD4^+$ and $CD8^+$ T-cell populations did not change between time-points for any of the stimulatory conditions (data not shown).

Changes in Cell Surface Activation Marker Expression With Treatment

In the absence of stimulation, $CD4^+CD27^-$ T-cells showed a significant decrease in CD38 expression between baseline and 2 months of treatment ($p = 0.0328$; **Figure 1B**). The converse was true for the $CD4^+CD27^+$ T-cells, with a significant increase in CD38 expression over time ($p = 0.0120$; **Figure 1E**). No difference in the expression of CD38 was seen on $CD8^+$ T-cells (**Figures 1H,K**). There was also no significant change in the proportion of CD27 (**Figures 1A,D,G,J**) and HLA-DR expressing (**Figures 1C,F,I,L**) $CD4^+$ and $CD8^+$ T-cells between the two time points.

Following EC stimulation, there was a significant increase in HLA-DR expression both in $CD4^+CD27^-$ and $CD4^+CD27^+$ T-cell compartments ($p = 0.0328$ and $p = 0.0400$, respectively; **Figures 2C,F**). Within the $CD4^+CD27^+$ T-cell compartment CD38 expression simultaneously increased over time ($p = 0.0328$;

TABLE 1 | Patient demographics.

Covariate	Fast responders ($n = 10$)	Slow responders ($n = 10$)	*P*-value
Age (median [IQR])	25 [22–31]	32 [28–35]	0.11
Male (%)	80	80	
HIV positive (%)	0	0	
GeneXpert Ct (median [IQR])	17.8 [16.8–19.0]	16.8 [15.6–18.4]	0.23

HIV, human immunodeficiency virus; IQR, interquartile range; Ct, cycle theshold.

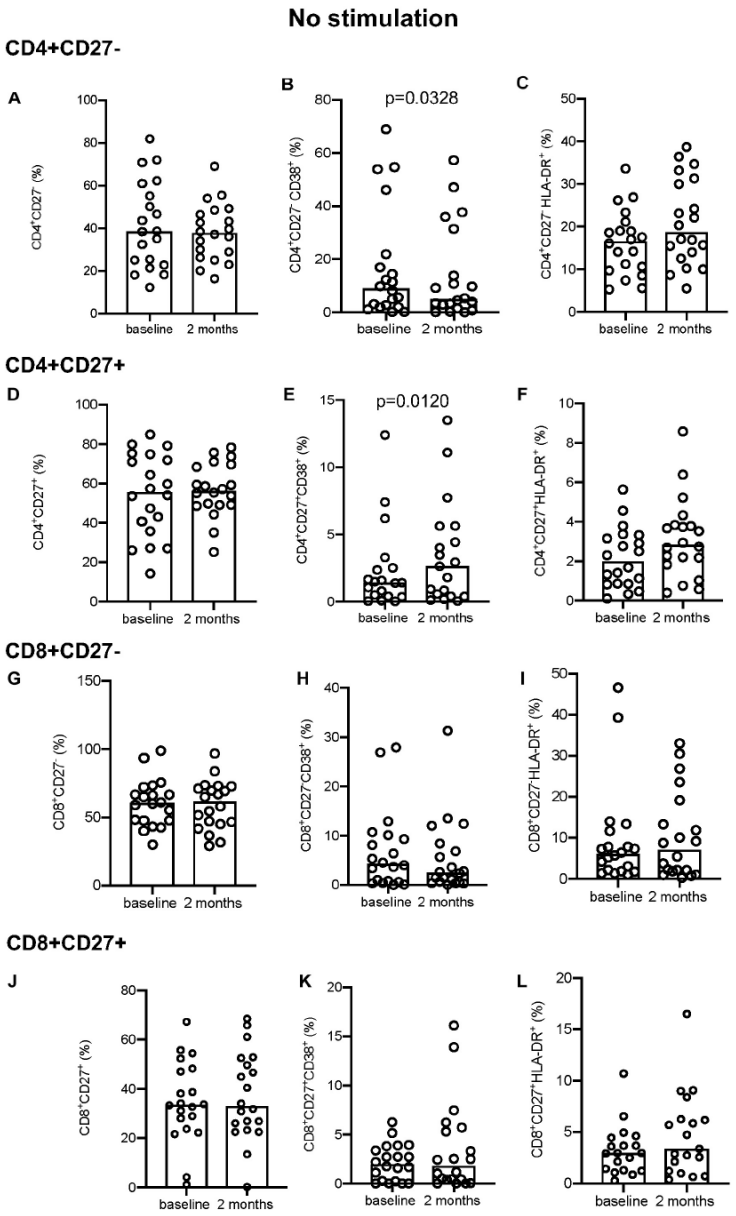


FIGURE 1 | Activation marker expression in unstimulated samples. **(A–C)** $CD4^+CD27^-$ total subset **(A)**, $CD38^+$ **(B)**, and HLA-DR^+ **(C)**. **(D–F)** $CD4^+CD27^+$ total subset **(D)**, $CD38^+$ **(E)**, and HLA-DR^+ **(F)**. **(G–I)** $CD8^+CD27^-$ total subset **(G)**, $CD38^+$ **(H)**, HLA-DR^+ **(I)**. **(J–L)** $CD8^+CD27^+$ total subset **(J)**, $CD38^+$ **(K)**, and HLA-DR^+ **(L)**. Columns indicate median. Data were analyzed using Wilcoxon matched-pairs rank test.

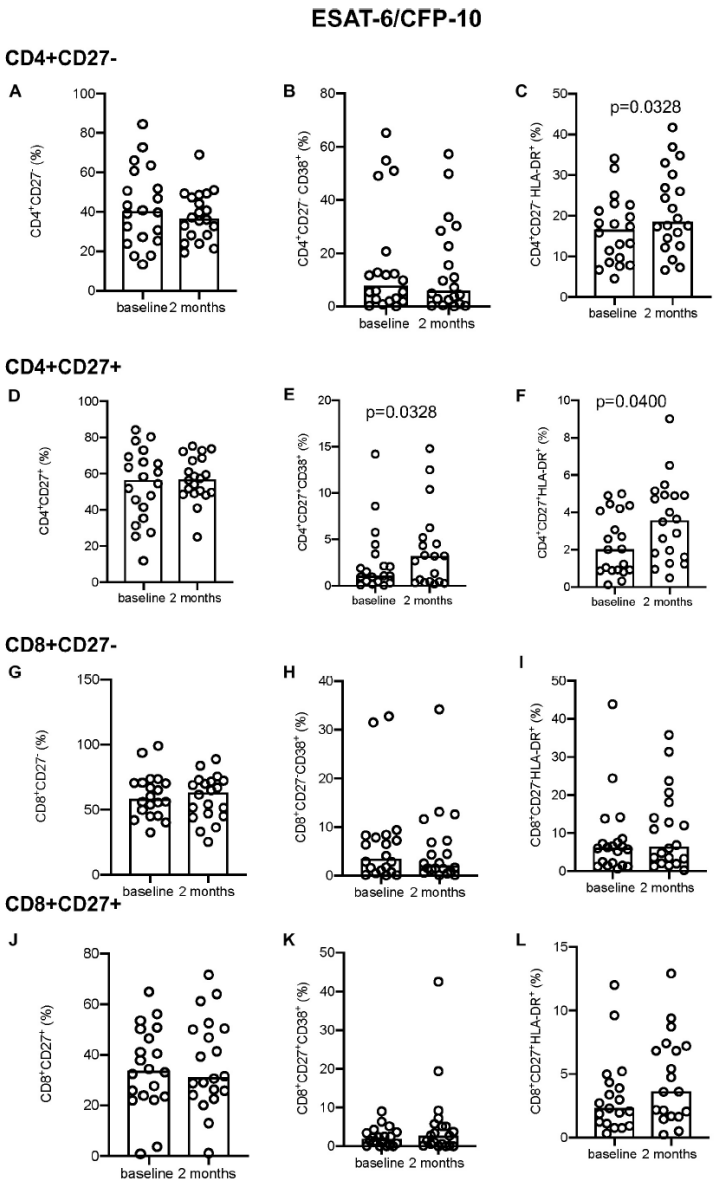


FIGURE 2 | Activation marker expression in ESAT-6/CFP-10 stimulated samples. **(A–C)** $CD4^+CD27^-$ total subset **(A)**, $CD38^+$ **(B)**, and $HLA\text{-}DR^+$ **(C)**. **(D–F)** $CD4^+CD27^+$ total subset **(D)**, $CD38^+$ **(E)**, and $HLA\text{-}DR^+$ **(F)**. **(G–I)** $CD8^+CD27^-$ total subset **(G)**, $CD38^+$ **(H)**, and $HLA\text{-}DR^+$ **(I)**. **(J–L)** $CD8^+CD27^+$ total subset **(J)**, $CD38^+$ **(K)**, and $HLA\text{-}DR^+$ **(L)**. Columns indicate median. Data were analyzed using Wilcoxon matched-pairs rank test.

Figure 2E), but no difference in CD38 expression within the $CD4^+CD27^-$ (**Figure 2B**) or in but no difference in CD27 expression in either compartment was seen (**Figures 2A,D,G,J**). No differences in CD38 and HLA-DR expression were seen within the $CD8^+CD27^-$ and $CD8^+CD27^+$ subsets (**Figures 2H,I,K,L**). No significant differences in levels of CD27, HLA-DR and CD38 expression on $CD4^+$ and $CD8^+$ CD27 expressing T-cell subsets were seen over time following PPD and PMA stimulation (**Supplementary Figures S2**, **S3**).

Changes in Cytokine and Proliferation Markers With Treatment

In the absence of stimulation there was no difference in IFN-γ^+ (**Figure 3A**) but a significant decrease at 2 months in the proportion of TNF-α^+ (p = 0.0172; **Figure 3B**) and Ki-67^+ (p = 0.0400; **Figure 3C**) producing cells within the $CD4^+CD27^-$ and $CD4^+CD27^+$TNF-α^+ T-cell subpopulations (p = 0.0204; **Figure 3E**). No difference in IFN-γ or Ki-67 expression was seen within the $CD4^+CD27^+$ subset (**Figures 3D,F**). Within the $CD8^+CD27^-$ subset, there was a significant decline in the proportion of IFN-γ^+ and Ki-67^+ producing cells (p = 0.0494 and p = 0.0007, respectively) but not TNF-α^+ producing cells (**Figures 3G–I**). Within the $CD8^+CD27^+$ subset there was also a significant decrease in the proportion of IFN-γ^+ producing cells (p = 0.0225) but not TNF-α^+ or Ki-67^+ producing cells (**Figures 3J–L**).

Following EC stimulation, the $CD4^+CD27^-$ subset showed a significant decrease in IFN-γ^+ expressing cells and a significant increase in $Ki67^+$ producing cells (p = 0.0351 and p = 0.0400, respectively; **Figures 4A–C**). No significant changes in intracellular marker expression was seen within the $CD4^+CD27^+$ compartment (**Figures 4D–F**). Within the $CD8^+CD27^-$ subset, a significant decrease in IFN-γ (p = 0.0019; **Figure 4G**) and TNF-α (p = 0.0034; **Figure 4H**) production over time was seen, with no significant variation in intracellular marker expression in the $CD8^+CD27^+$ T-cell compartment (**Figures 4J–L**).

No significant differences were seen following PPD stimulation (**Supplementary Figure S4**) but a significant increase in IFN-γ and TNF-α production was seen following PMA stimulation at 2 months compared to baseline for all T-cell subsets described (**Figure 5**). Conversely, Ki-67 expression in the $CD8^+CD27^-$ T cell compartment decreased significantly from baseline to 2 months (p = 0.0056, **Figure 5L**).

Comparison of Slow Versus Fast Treatment Responders

The majority of significant differences seen were in the kinetics of change over time within the groups. Slow responders showed a significant increase in both CD38 and HLA-DR expression from baseline to 2 months in the unstimulated cells within the $CD4^+CD27^+$ T cell population, that was not seen in the fast responder group (p = 0.0273 and p = 0.0371, respectively; **Figures 6A,B**). The proportion of $CD4^+CD27^+HLA\text{-}DR^+$ cells also increased only in the slow responder group by 2 months after both PPD (p = 0.0273) and EC stimulation (p = 0.0273); **Figures 6C,D**). Levels of PPD-stimulated $CD4^+CD27^-$IFN-γ^+ cells significantly increased at 2 months compared to baseline, in only the fast responders (p = 0.0020; **Figure 6E**). Following EC stimulation there was a significantly lower proportion of $CD8^+CD27^-$IFN-γ^+ and $CD8^+CD27^+$TNF-α^+ cells at 2 months compared to baseline in the slow responders (p = 0.0096 and p = 0.0137, respectively; **Figures 6F,G**).

In unstimulated, EC and PMA conditions, no difference between treatment response groups were seen at baseline or 2 months (data not shown). Nonetheless, two discriminatory subsets were found following PPD stimulation; levels of $CD4^+CD27^+HLA\text{-}DR^+CD38^+$ and $CD8^+CD27^-$IFN-γ^+ T cell populations were significantly higher in the slow responder group compared to the fast responder group at baseline (p = 0.0077 and p = 0.0105, respectively; **Figures 6H,I**). When ROC analysis was performed, baseline frequencies of $CD8^+CD27^-$IFN-γ^+ and $CD4^+CD27^+HLA\text{-}DR^+CD38^+$ T cells could predict treatment response with a 80% sensitivity and 70 and 100% specificity, respectively (AUC of 0.82, p = 0.0156 and 0.84, p = 0.0102) (**Figures 6J,K**).

Polyfunctional T-Cell Changes With TB Treatment

We also analyzed qualitative responses before and at 2 months of TB treatment using SPICE analysis of activation and cytokine marker combinations within each subset (**Figure 7**). Following PMA stimulation, there were no differences in any subset between baseline and 2 months with the majority of cells positive for TNF-α, IFN-γ, and/or Ki-67 but not HLA-DR nor CD38 (purple/pink categories). Following EC stimulation, diverse cell populations were present with the predominant population positive for all markers except CD38 (population 17). The overall polyfunctionality was not significantly different between fast and slow responders but slow responders showed a significantly different qualitative profile in response to EC stimulation at 2 months compared to baseline in the $CD8^+CD27^+$ subset, which was not seen in the fast responders (p = 0.0231; **Figure 7D**). At baseline, the predominant subset expressed TNF-α only (subset 31) whereas at 2 months, the predominant subset were cells producing TNF-α and IFN-γ together with Ki-67 but with the absence of CD38 and HLA-DR (population 25).

DISCUSSION

This study looked at the use of activation and functional markers for monitoring and predicting treatment responses. Overall, activation marker expression (particularly CD38) decreased in the $CD4^+CD27^-$ subset but increased in the $CD4^+CD27^+$ subset by 2 months of therapy compared to baseline. In addition, cytokine responses to EC stimulation were significantly reduced, but increased following PMA stimulation. This is consistent with our previous unpublished findings demonstrating a general reduction in overall immune responsiveness in T-cells from active TB patients, which is restored post treatment. When patients were analyzed based on response to therapy, slow responders had significantly more PPD-specific $CD8^+CD27^-$IFN-γ^+ and $CD4^+CD27^+HLA\text{-}DR^+CD38^+$ T-cells than fast responders at baseline. Receiver operating characteristics curve analyses showed that baseline PPD-stimulated $CD8^+CD27^-$IFN-γ^+ and

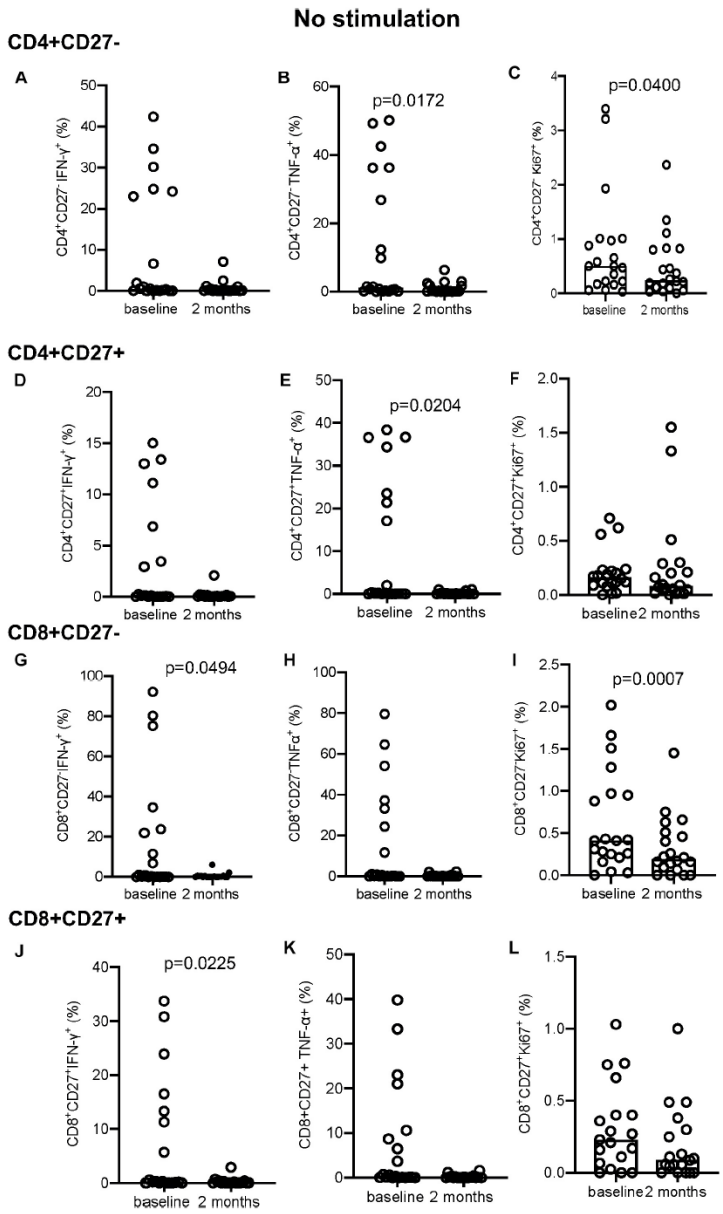


FIGURE 3 | Intracellular cytokine and proliferation marker expression in unstimulated samples. **(A–C)** $CD4^+CD27^-$ IFN-γ^+ **(A)**, TNF-α^+ **(B)**, and Ki-67^+ **(C)**. **(D–F)** $CD4^+CD27^+$ IFN-γ^+ **(D)**, TNF-α^+ **(E)**, and Ki-67^+ **(F)**. **(G–I)** $CD8^+CD27^-$ IFN-γ^+ **(G)**, TNF-α^+ **(H)**, Ki-67^+ **(I)**. **(J–L)** $CD8^+CD27^+$ IFN-γ^+ **(J)**, TNF-α^+ **(K)**, and Ki-67^+ **(L)**. Columns indicate median. Data were analyzed using Wilcoxon matched-pairs rank test.

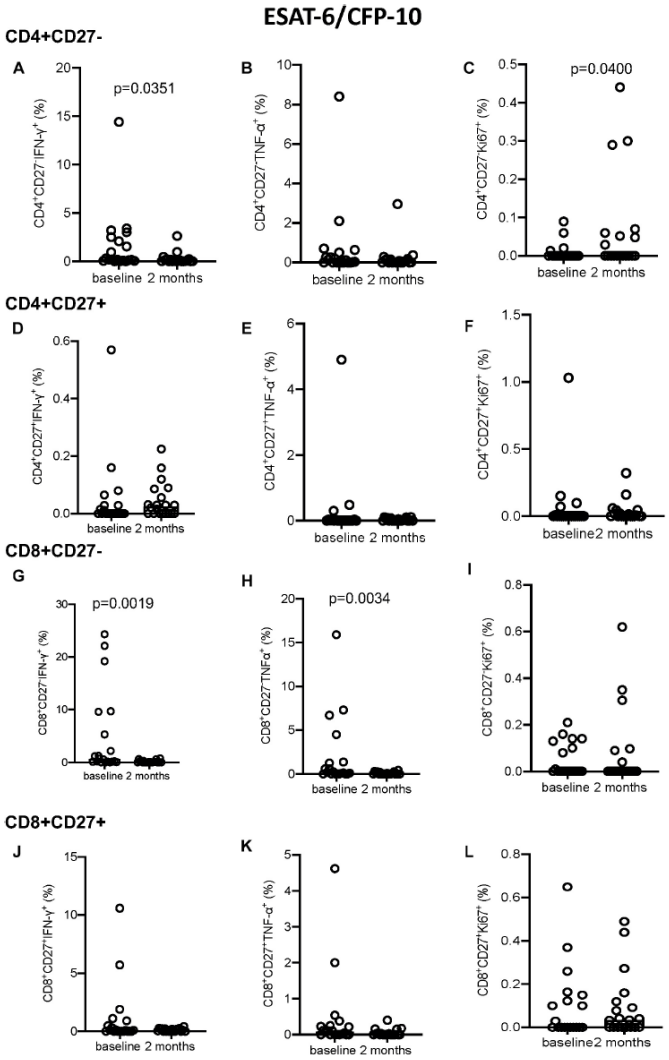


FIGURE 4 | Intracellular cytokine and proliferation marker expression in EC stimulated samples. **(A–C)** $CD4^+CD27^-$ IFN-γ^+ **(A)**, TNF-α^+ **(B)**, and Ki-67^+ **(C)**. **(D–F)** $CD4^+CD27^+$ IFN-γ^+ **(D)**, TNF-α^+ **(E)**, and Ki-67^+ **(F)**. **(G–I)** $CD8^+CD27^-$ IFN-γ^+ **(G)**, TNF-α^+ **(H)**, and Ki-67^+ **(I)**. **(J–L)** $CD8^+CD27^+$ IFN-γ^+ **(J)**, TNF-α^+ **(K)**, and Ki-67^+ **(L)**. Columns indicate median. Data were analyzed using Wilcoxon matched-pairs rank test.

$CD4^+CD27^+HLA\text{-}DR^+CD38^+$ T cells could predict treatment response with 80% sensitivity and specificity of 70 and 100%, respectively.

Our aim was to see if blood-based biomarkers could be used at 2 months rather than sputum culture as an indication of response to therapy and at baseline as prognostic markers for response to therapy. Previous studies have gated on IFN-γ^+ T cells (both $CD4^+$ and $CD8^+$) prior to activation marker analysis (3, 10, 11). However, this was not possible in our study due to the low level of IFN-γ^+ cells following both EC and PPD stimulation. This reduced responsiveness has often been observed between East and West Africans (unpublished data) and highlights the

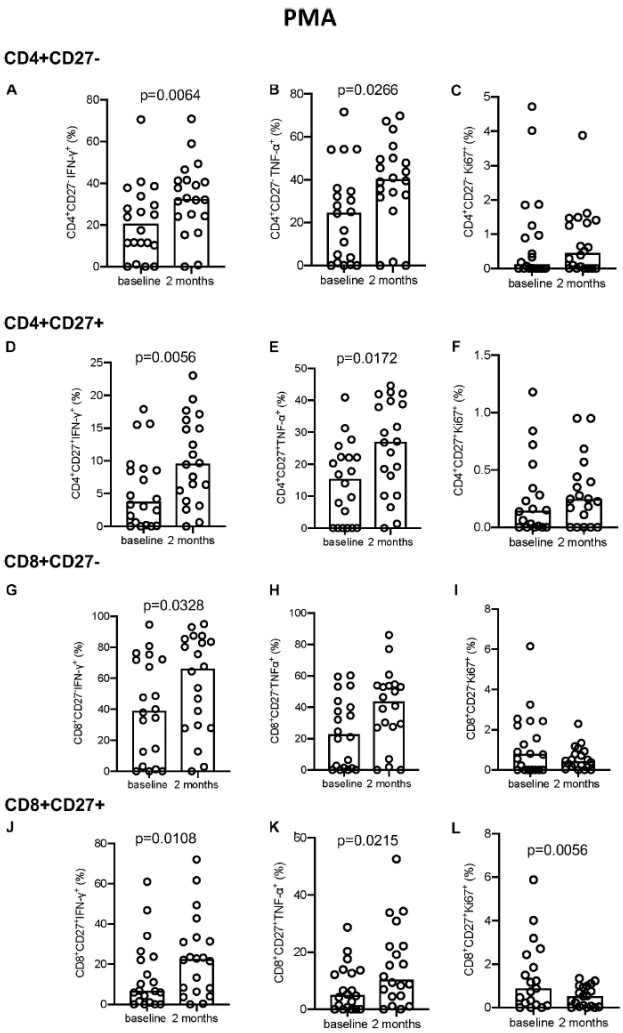


FIGURE 5 | Intracellular cytokine and proliferation marker expression in PMA stimulated samples. **(A–C)** $CD4^+CD27^-$ IFN-γ^+ **(A)**, TNF-α^+ **(B)**, and Ki-67^+ **(C)**. **(D–F)** $CD4^+CD27^+$ IFN-γ^+ **(D)**, TNF-α^+ **(E)**, and Ki-67^+ **(F)**. **(G–I)** $CD8^+CD27^-$ IFN-γ^+ **(G)**, TNF-α^+ **(H)**, and Ki-67^+ **(I)**. **(J–L)** $CD8^+CD27^+$ IFN-γ^+ **(J)**, TNF-α^+ **(K)**, and Ki-67^+ **(L)**. Columns indicate median. Data were analyzed using Wilcoxon matched-pairs rank test.

requirements for identification of global biomarkers, validated in multiple contexts. Nonetheless, our results suggest gating on IFN-γ^+ T cells is not strictly necessary and may be limited by low relevant cell counts.

CD27 acts as a T-cell differentiation marker; expression is gradually lost as the T-cell transitions from naïve or memory to effector and differentiation state is dictated by strength and duration of antigen stimulation (22). Consequently, CD27 is expressed on central memory (CM) T-cells, variably expressed on effector memory (EM) T-cells and is not expressed on terminal effector memory (TEMRA) T-cells (22–25). As we expected, the $CD4^+CD27^-$ T-cell subset was the predominantly activated population for all stimulation conditions, as demonstrated through HLA-DR, CD38, IFN-γ, and TNF-α expression levels,

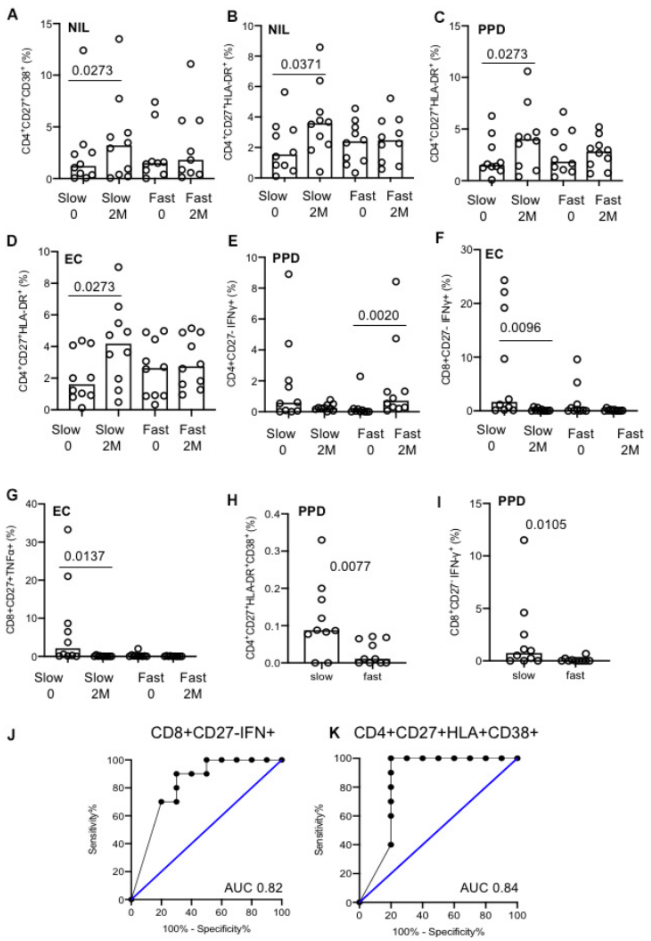


FIGURE 6 | Analysis of slow versus fast treatment responders. **(A–G)** Activation and cytokine marker analysis with different stimulation conditions (indicated). The majority of differences were seen were within groups. **(H)** $CD4^+CD27^+HLA\text{-}DR^+CD38^+$ cells at baseline between fast and slow responders. **(I)** $CD8^+CD27^-IFN\text{-}\gamma^+$ cells at baseline between fast and slow responders. **(J)** ROC curve of $CD8^+CD27^-IFN\text{-}\gamma^+$ cells at baseline. **(K)** ROC curve of $CD4^+CD27^+CD38^+HLA\text{-}DR^+$ cells at baseline. Columns indicate median. Data were analyzed using Kruskal–Wallis or Wilcoxon matched-pairs rank test **(A–G)**.

which is consistent with previous studies (18, 21). $CD4^+$ T-cells are important in controlling Mtb infection and those that are $CD27^-$ are mostly TEMRA, EM, and effector cells which exert the quickest and strongest activated effector response (18, 26). However, this subset is also the most likely to undergo activation induced cell death and to be exhausted from persistent antigen stimulation *in vivo* which will reduce their frequency (27).

Overall, a general decrease in cytokine expression in unstimulated samples from baseline to 2 months was witnessed alongside general cell responsiveness to PMA. These results suggest that rather than general T-cell anergy, persistent MTB-antigen stimulation (*in vitro* and *in vivo*) in these ATB patients has led to dysfunction of these antigen-specific T-cells, resulting in apoptosis and exhaustion (27). Earlier studies have revealed that persistent antigen stimulation in ATB results in an upregulation of inhibitory receptors such as programmed cell death protein 1 (PD-1), resulting in the inhibition and exhaustion of MTB-specific $CD4^+$ T cell (27–29). Therefore, alongside terminally differentiated T-cells, these exhausted populations demonstrate much

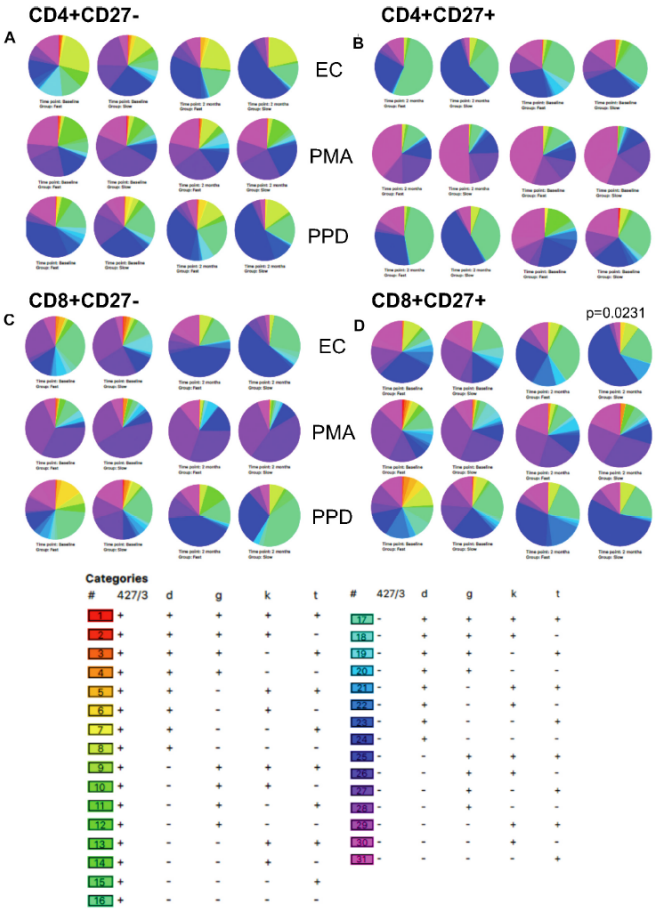


FIGURE 7 | SPICE analysis. Combinatorial analysis of activation, cytokine and proliferation markers was performed at baseline and 2 months of therapy. Pie graphs illustrate differences in each category for each subset. **(A)** $CD4^+CD27^-$; **(B)** $CD4^+CD27^+$; **(C)** $CD8^+CD27^-$; **(D)** $CD8^+CD27^+$.

lower levels of cytokine production compared to effector T cells (22).

When analyzing changes from baseline to 2 months our findings were mostly consistent with previous studies (3, 10, 30). Elevated levels of IFN-γ, TNF-α, CD38, and Ki67 decreased in the $CD4^+CD27^-$ population from baseline to 2 months, likely due to a diminishing bacterial burden (3). However, a significant increase in HLA-DR expression in the $CD4^+CD27^-$ subset after 2 months of treatment was not anticipated. A potential explanation for this phenomenon is a reduction in T regulatory cells (Tregs) (19, 31, 32). Nevertheless, these results reveal that whole blood samples from ATB patients may not require *in vitro* Mtb-specific stimulation to deliver valuable information. The significant decrease in activation marker expression within the $CD4^+CD27^-$ T cell population from baseline to 2 months suggests the potential ability to monitor treatment adherence using unstimulated whole blood. Additionally, measuring activation at baseline may prove beneficial as a point-of-care diagnostic tool.

When participants were stratified based on treatment response, the majority of the changes from baseline to 2 months occurred within the slow responder group in the absence of *in vitro* stimulation. These included an increase in expression of CD38 and HLA-DR expression on the $CD4^+CD27^+$ subset. Interestingly, specific $CD4^+$ and $CD8^+$ T-cell subsets were able to predict treatment response at baseline: slow responders had

significantly more $CD8^+CD27^-$ T cells producing IFN-γ than fast responders with PPD stimulation at baseline. $CD8^+$ T cells have been shown to play a role in MTB immunity in more severe disease (33) but $CD8^+CD27^-$ T cells producing IFN-γ and TNF-α are also associated with protection in Mtb infection (33). However, this implies a difference in disease severity/inflammation between treatment response groups which is not supported by our findings. Nonetheless, this suggests that if an individual is diagnosed with ATB and is showing high production levels of IFN-γ by the $CD8^+CD27^-$ subset, they may be at greater risk of still being culture positive at 2 months and thus the CD8+CD27-IFN-γ+ subset could be used as a predictive marker. This could be used in conjunction with $CD4^+CD27^+$ T cells, co-expressing CD38 and HLA-DR to improve positive predictive value.

There are several limitations of this study, mainly due to small sample size making it difficult to adjust for possible confounders such as BMI, alcohol abuse, diabetes mellitus, and delay in presentation. Another possible explanation for the difference in treatment response could be a higher bacterial burden at baseline, however, there was no difference in GeneXpert Ct between the groups suggesting that a slow response was not simply due to higher bacterial load at baseline. Future work should corroborate these findings in a larger cohort of ATB patients using fresh cells to progress toward real-time monitoring and application in the field. Analysis of Treg cells from other T cell subsets would also be important together with addition of an exhaustion marker such as PD-1 to help prove our hypothesis on T cell exhaustion in patients at baseline (34). It would also be of interest to analyze a post-treatment time-point to determine the stability of our findings.

In summary, our pilot data suggest there is potential for use of activation and cytokine markers for predicting and monitoring treatment response in HIV negative ATB patients in The Gambia. However, this requires validation in a larger cohort. Analysis of baseline levels of IFN-γ production from the $CD8^+CD27^-$ subset and HLA-DR and CD38 co-expression in the $CD4^+CD27^+$ subset after PPD stimulation has the potential to predict response to treatment at 2 months. Further, our results demonstrate the ability of analyzing unstimulated samples for diagnosis and monitoring treatment adherence – warranting further evaluation for the development of a point of care test.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by MRC and Gambian Government Joint Ethics Committee and the London School of Hygiene and Tropical Medicine Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MV performed the experiments, analyzed the data, and wrote the manuscript. FD and CM provided training for FACS and analysis. GM, AB, AG, and SN processed blood samples. A-JR, BiS, and BaS processed the sputum cultures. HD provided supervision, training, and the manuscript critique. OO and SJ provided all clinical evaluation of the patients. SC and AR obtained funding, developed protocols, and provided critique of data. JS conceived the idea and provided supervision, training, critique of data, and manuscript review.

FUNDING

This study was nested within the TB sequel project, funded by the German Ministry for Education and Research (BMBF, 01KA1613) and is a part of the Research Networks for Health Innovations in Sub-Saharan Africa.

ACKNOWLEDGMENTS

We thank all study participants and their families, TB clinic, field and laboratory staff, and the Gambian National TB Control Program.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.572620/full#supplementary-material

FIGURE S1 | Gating strategy for flow cytometry.

FIGURE S2 | Activation marker analysis following PPD stimulation.

FIGURE S3 | Activation marker analysis following PMA stimulation.

FIGURE S4 | Cytokine expression following PPD stimulation.

REFERENCES

1. World Heatlh Organisation Global Tuberculosis Report. (2019). Available online at: https://www.who.int/tb/publications/global_report/en/ (accessed August 5, 2020).
2. Babu S. Biomarkers for treatment monitoring in tuberculosis: a new hope. *EBioMedicine.* (2017) 26:13–4. doi: 10.1016/j.ebiom.2017.11.002
3. Adekambi T, Ibegbu CC, Cagle S, Kalokhe AS, Wang YF, Hu Y, et al. Biomarkers on patient T cells diagnose active tuberculosis and monitor treatment response. *J Clin Invest.* (2015) 125:1827–38. doi: 10.1172/JCI77990
4. Malherbe ST, Shenai S, Ronacher K, Loxton AG, Dolganov G, Kriel M, et al. Persisting positron emission tomography lesion activity and Mycobacterium tuberculosis mRNA after tuberculosis cure. *Nat Med.* (2016) 22:1094–100.
5. Sigal GB, Segal MR, Mathew A, Jarlsberg L, Wang M, Barbero S, et al. Biomarkers of tuberculosis severity and treatment effect: a directed screen of

70 host markers in a randomized clinical trial. *EBioMedicine*. (2017) 25:112–21. doi: 10.1016/j.ebiom.2017.10.018
6. Cliff JM, Kaufmann SHE, Mcshane H, van Helden P, O'Garra A. The human immune response to tuberculosis and its treatment: a view from the blood. *Immunol Rev*. (2015) 264:88–102. doi: 10.1111/imr.12269
7. Denkinger CM, Kik SV, Cirillo DM, Casenghi M, Shinnick T, Weyer K, et al. Defining the needs for next generation assays for tuberculosis. *J Infect Dis*. (2015) 211:S29–38. doi: 10.1093/infdis/jiu821
8. Getahun H, Harrington M, O'Brien R, Nunn P. Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes. *Lancet*. (2007) 369:2042–9. doi: 10.1016/S0140-6736(07)60284-0
9. Ahmed MIM, Ziegler C, Held K, Dubinski I, Ley-Zaporozhan J, Geldmacher C, et al. The TAM-TB assay—a promising TB immune-diagnostic test with a potential for treatment monitoring. *Front Pediatr*. (2019) 7:27. doi: 10.3389/fped.2019.00027
10. Musvosvi M, Duffy D, Filander E, Africa H, Mabwe S, Jaxa L, et al. T-cell biomarkers for diagnosis of tuberculosis: candidate evaluation by a simple whole blood assay for clinical translation. *Eur Respir J*. (2018) 51:1800153. doi: 10.1183/13993003.00153-2018
11. Wilkinson KA, Oni T, Wilkinson RJ. Activation profile of *Mycobacterium tuberculosis*-specific CD4+ T cells reflects disease activity irrespective of HIV status. *Am J Respir Crit Car Med*. (2016) 193:1307–10. doi: 10.1164/rccm.201601-0116LE
12. Nikitina IY, Kondratuk NA, Kosmiadi GA, Amansahedov RB, Vasilyeva IA, Ganusov VV, et al. Mtb-specific CD27 low CD4 T cells as markers of lung tissue destruction during pulmonary tuberculosis in humans. *PLoS One*. (2012) 7:e43733. doi: 10.1371/journal.pone.0043733
13. Schuetz A, Haule A, Reither K, Ngwenyama N, Rachow A, Meyerhans A, et al. Monitoring CD27 expression to evaluate *Mycobacterium tuberculosis* activity in HIV-1 infected individuals in vivo. *PLoS One*. (2011) 6:e27284. doi: 10.1371/journal.pone.0027284
14. Riou C, Berkowitz N, Goliath R, Burgers WA, Wilkinson RJ. Analysis of the phenotype of *Mycobacterium tuberculosis*-specific CD+ T cells to discriminate latent from active tuberculosis in HIV-Uninfected and HIV-Infected individuals. *Front Immunol*. (2017) 8:968. doi: 10.3389/fimmu.2017.00968
15. Portevin D, Moukambi F, Clowes P, Bauer A, Chachage M, Ntinginya NE, et al. Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study. *Lancet Infect Dis*. (2014) 14:931–8. doi: 10.1016/S1473-3099(14)70884-9
16. Halliday A, Whitworth H, Kottoor SH, Niazi U, Menzies S, Kunst H, et al. Stratification of latent *Mycobacterium tuberculosis* infection by cellular immune profiling. *J Infect Dis*. (2017) 215:1480–7. doi: 10.1093/infdis/jix107
17. Streitz M, Tesfa L, Yildirim V, Yahyazadeh A, Ulrichs T, Lenkei R, et al. Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. *PLoS One*. (2007) 2:e735. doi: 10.1371/journal.pone.0000735
18. Ferrian S, Ross M, Conradie F, Omar JV, Ismail N, Little F, et al. Frequency of circulating CD4+KI67+HLA-DR-T regulatory cells prior to treatment for multidrug resistant tuberculosis can differentiate the severity of disease and predict time to culture conversion. *Front Immunol*. (2018) 9:2438. doi: 10.3389/fimmu.2018.02438
19. Roederer M, Nozzi J, Nason M. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytom Part A*. (2011) 79:167–74. doi: 10.1002/cyto.a.21015
20. Rachow A, Ivanova O, Wallis R, Charalambous S, Jani I, Bhatt N, et al. TB sequel: incidence, pathogenesis and risk factors of long-term medical and social sequelae of pulmonary TB–a study protocol. *BMC Pulm Med*. (2019) 19:4. doi: 10.1186/s12890-018-0777-3
21. Lyadova I, Nikitina I. Cell differentiation degree as a factor determining the role for different T-helper populations in tuberculosis protection. *Front Immunol*. (2019) 10:972. doi: 10.3389/fimmu.2019.00972
22. Schiött Å, Lindstedt M, Johansson-Lindbom B, Roggen E, Borrebaeck CAK. CD27- CD4+ memory T cells define a differentiated memory population at both the functional and transcriptional levels. *Immunology*. (2004) 113:363–70. doi: 10.1111/j.1365-2567.2004.01974.x
23. Larbi A, Fulop T. From "truly naïve" to "exhausted senescent" T cells: when markers predict functionality. *Cytom Part A*. (2014) 85:25–35. doi: 10.1002/cyto.a.22351
24. Willinger T, Freeman T, Hasegawa H, McMichael AJ, Callan MFC. Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets. *J Immunol*. (2005) 175:5895–903. doi: 10.4049/jimmunol.175.9.5895
25. Arrigucci R, Lakehal K, Vir P, Handler D, Davidow AL, Herrera R, et al. Active tuberculosis is characterized by highly differentiated effector memory Th1 cells. *Front Immunol*. (2018) 9:2127. doi: 10.3389/fimmu.2018.02127
26. Prezzemolo T, Guggino G, La Manna MP, Di Liberto D, Dieli F, Caccamo N. Functional signatures of human CD4 and CD8 T cell responses to *Mycobacterium tuberculosis*. *Front Immunol*. (2014) 5:180. doi: 10.3389/fimmu.2014.00180
27. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: human memory T-cell subsets. *Eur J Immunol*. (2013) 43:2797–809. doi: 10.1002/eji.201343751
28. Day CL, Abrahams DA, Bunjun R, Stone L, de Kock M, Walzl G, et al. PD-1 expression on *Mycobacterium tuberculosis*-specific CD4 T cells is associated with bacterial load in human tuberculosis. *Front Immunol*. (2018) 9:1995. doi: 10.3389/fimmu.2018.01995
29. Shen L, Gao Y, Liu Y, Zhang B, Liu Q, Wu J, et al. PD-1/PD-L pathway inhibits M.tb-specific CD4+ T-cell functions and phagocytosis of macrophages in active tuberculosis. *Sci Rep*. (2016) 6:38362. doi: 10.1038/srep38362
30. Jean Bosco M, Wei M, Hou H, Yu J, Lin Q, Luo Y, et al. The exhausted CD4+CXCR5+ T cells involve the pathogenesis of human tuberculosis disease. *Int J Infect Dis*. (2018) 74:1–9. doi: 10.1016/j.ijid.2018.06.011
31. Ahmed MIM, Ntinginya NE, Kibiki G, Mtafya BA, Semvua H, Mpagama S, et al. Phenotypic changes on *Mycobacterium Tuberculosis*-specific CD4 T cells as surrogate markers for tuberculosis treatment efficacy. *Front Immunol*. (2018) 9:2247. doi: 10.3389/fimmu.2018.02247
32. Pathakumari B, Devasundaram S, Raja A. Altered expression of antigen-specific memory and regulatory T-cell subsets differentiate latent and active tuberculosis. *Immunology*. (2018) 153:325–36. doi: 10.1111/imm.12833
33. Lewinsohn DM, Zhu L, Madison VJ, Dillon DC, Fling SP, Reed SG, et al. Classically restricted human CD8 + T lymphocytes derived from *Mycobacterium tuberculosis* -infected cells: definition of antigenic specificity. *J Immunol*. (2001) 166:439–46. doi: 10.4049/jimmunol.166.1.439
34. Ahmed A, Adiga V, Nayak S, Uday Kumar JAJ, Dhar C, Sahoo PN, et al. Circulating HLA-DR+CD4+ effector memory T cells resistant to CCR5 and PD-L1 mediated suppression compromise regulatory T cell function in tuberculosis. *PLoS Pathog*. (2018) 14:e1007289. doi: 10.1371/journal.ppat.1007289

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

[1] A. Rachow *et al.*, “TB sequel: Incidence, pathogenesis and risk factors of long-term medical and social sequelae of pulmonary TB - A study protocol,” *BMC Pulm. Med.*, vol. 19, no. 1, pp. 1–9, 2019.

[2] G. P. Maguire *et al.*, “Pulmonary tuberculosis, impaired lung function, disability and quality of life in a high-burden setting,” *Int. J. Tuberc. Lung Dis.*, vol. 13, no. 12, pp. 1500–1506, 2009.

[3] A. P. Ralph *et al.*, “High morbidity during treatment and residual pulmonary disability in pulmonary tuberculosis: Under-recognised phenomena,” *PLoS One*, vol. 8, no. 11, pp. 1–11, 2013.

[4] S. Hoger, K. Lykens, S. F. Beavers, D. Katz, and T. L. Miller, “Longevity loss among cured tuberculosis patients and the potential value of prevention,” *Int. J. Tuberc. Lung Dis.*, vol. 18, no. 11, pp. 1347–1352, 2014.

[5] M. Rao *et al.*, “Improving treatment outcomes for MDR-TB — Novel host-directed therapies and personalised medicine of the future,” *Int. J. Infect. Dis.*, vol. 80, pp. S62–S67, 2019.

[6] K. D. Mayer-Barber *et al.*, “Host-directed therapy of tuberculosis based on interleukin-1 and type i interferon crosstalk,” *Nature*, vol. 511, no. 7507, pp. 99–103, 2014.

[7] N. R. Scott *et al.*, “S100A8/A9 regulates CD11b expression and neutrophil recruitment during chronic tuberculosis,” *J. Clin. Invest.*, vol. 130, no. 6, pp. 3098–3112, 2020.

[8] A. Kolloli and S. Subbian, “Host-directed therapeutic strategies for tuberculosis,” *Front. Med.*, vol. 4, no. 171, 2017.

[9] H. Ndlovu and M. J. Marakalala, “Granulomas and inflammation: Host-directed therapies for tuberculosis,” *Front. Immunol.*, vol. 7, no. OCT, 2016.

[10] S. Ahmed, R. Raqib, G. H. Guðmundsson, P. Bergman, B. Agerberth, and R. S. Rekha, “Host-directed therapy as a novel treatment strategy to overcome tuberculosis: Targeting immune modulation,” *Antibiotics*, vol. 9, no. 1, pp. 1–19, 2020.

[11] K. Moideen, N. P. Kumar, D. Nair, V. V. Banurekha, R. Bethunaickan, and S. Babu, “Heightened systemic levels of neutrophil and eosinophil granular proteins in pulmonary tuberculosis and reversal following treatment,” *Infect. Immun.*, vol. 86, no. 6, pp. 1–8, 2018.

[12] Y. Chen and W. G. Junger, “Leucocytes,” vol. 844, no. 4, pp. 115–124, 2012.

[13] C. W. M. Ong *et al.*, “Neutrophil-Derived MMP-8 Drives AMPK-Dependent Matrix Destruction in Human Pulmonary Tuberculosis,” *PLoS Pathog.*, vol. 11, no. 5, pp. 1–21, 2015.

[14] A. V. Panteleev *et al.*, “Severe tuberculosis in humans correlates best with neutrophil abundance and lymphocyte deficiency and does not correlate with antigen-specific CD4 T-cell response,” *Front. Immunol.*, vol. 8, no. 963, 2017.

[15] J. Y. Sagiv *et al.*, “Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer,” *Cell Rep.*, vol. 10, no. 4, pp. 562–574, 2015.

[16] T. N. Mayadas, X. Cullere, and C. A. Lowell, “The multifaceted functions of neutrophils,” *Annu Rev Pathol*, vol. 9, pp. 181–218, 2014.

[17] C. Rosales, “Neutrophil: A cell with many roles in inflammation or several cell types?,” *Front. Physiol.*, vol. 9, no. 113, 2018.

[18] P. Yang, Y. Li, Y. Xie, and Y. Liu, “Different faces for different places: Heterogeneity of neutrophil phenotype and function,” *J. Immunol. Res.*, vol. 2019, p. 8016254, 2019.

[19] P. H. C. Leliefeld *et al.*, “Differential antibacterial control by neutrophil subsets,” *Blood Adv.*, vol. 2, no. 11, pp. 1344–1354, 2018.

[20] J. Pillay *et al.*, “A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1,” *J. Clin. Invest.*, vol. 122, no. 1, pp. 327–336, 2012.

[21] C. Young, G. Walzl, and N. Du Plessis, “Therapeutic host-directed strategies to improve outcome in tuberculosis,” *Mucosal Immunol.*, vol. 13, no. 2, pp. 190–204, 2020.

[22] N. F. Walker *et al.*, “Matrix Degradation in Human Immunodeficiency Virus Type 1-Associated Tuberculosis and Tuberculosis Immune Reconstitution Inflammatory Syndrome: A Prospective Observational Study,” *Clin. Infect. Dis.*, vol. 65, no. 1, pp. 121–132, 2017.

[23] T. Dallenga *et al.*, “M. tuberculosis-Induced Necrosis of Infected Neutrophils Promotes Bacterial Growth Following Phagocytosis by Macrophages,” *Cell Host Microbe*, vol. 22, no. 4, pp. 519-530.e3, 2017.

[24] J. J. Yeh, “Predictors of Initial Smear-Negative Active Pulmonary Tuberculosis with Acute Early Stage Lung Injury by High-Resolution Computed Tomography and Clinical Manifestations: An Auxiliary Model in Critical Patients,” *Sci. Rep.*, vol. 9, no. 1, pp. 1–13, 2019.

[25] R. Pellegrino *et al.*, “Interpretative strategies for lung function tests,” *Eur. Respir. J.*, vol. 26, no. 5, pp. 948–968, 2005.

[26] R. Báez-Saldaña *et al.*, “A novel scoring system to measure radiographic abnormalities and related spirometric values in cured pulmonary tuberculosis,” *PLoS One*, vol. 8, no. 11, pp. 1–12, 2013.

[27] S. E. Murthy *et al.*, “Pretreatment chest x-ray severity and its relation to bacterial burden in smear positive pulmonary tuberculosis,” *BMC Med.*, vol. 16, no. 1, pp. 1–11, 2018.

[28] A. P. Ralph *et al.*, “A simple, valid, numerical score for grading chest x-ray severity in adult smear-positive pulmonary tuberculosis,” *Thorax*, vol. 65, no. 10, pp. 863–869, 2010.

[29] S. E. Dorman *et al.*, “Xpert MTB/RIF Ultra for detection of Mycobacterium tuberculosis and rifampicin resistance: a prospective multicentre diagnostic accuracy study,” *Lancet Infect. Dis.*, vol. 18, no. 1, pp. 76–84, 2018.

[30] C. N. Muefong and J. S. Sutherland, “Neutrophils in Tuberculosis-Associated Inflammation and Lung Pathology,” *Front. Immunol.*, vol. 11, no. May, pp. 1–9, 2020.

[31] H. R. Jones, C. T. Robb, M. Perretti, and A. G. Rossi, “The role of neutrophils in inflammation resolution,” *Semin. Immunol.*, vol. 28, no. 2, pp. 137–145, 2016.

[32] R. Medzhitov, “Recognition of microorganisms and activation of the immune response,” *Nature*, vol. 449, no. 7164, pp. 819–826, 2007.

[33] C. Nwongbouwoh Muefong *et al.*, “Neutrophils Contribute to Severity of Tuberculosis Pathology and Recovery From Lung Damage Pre- and Posttreatment,” *Clin. Infect. Dis.*, vol. ciab729, pp. 1–10, 2021.

[34] M. A. Vickers *et al.*, “Monitoring Anti-tuberculosis Treatment Response Using Analysis of Whole Blood Mycobacterium tuberculosis Specific T Cell Activation and Functional Markers,” *Front. Immunol.*, vol. 11, no. September, pp. 1–13, 2020.

Appendix A: Paper IV

frontiers in Immunology

REVIEW
published: 27 May 2020
doi: 10.3389/fimmu.2020.00962

Neutrophils in Tuberculosis-Associated Inflammation and Lung Pathology

Caleb N. Muefong and Jayne S. Sutherland*

Vaccines and Immunity Theme, Medical Research Council Unit, The Gambia at the London School of Hygiene and Tropical Medicine, Fajara, The Gambia

Protective immunity to *Mycobacterium tuberculosis (Mtb)*—the causative agent of tuberculosis (TB)—is not fully understood but involves immune responses within the pulmonary airways which can lead to exacerbated inflammation and immune pathology. In humans, this inflammation results in lung damage; the extent of which depends on specific host pro-inflammatory processes. Neutrophils, though increasingly linked to the development of inflammatory disorders, have been less well studied in relation to TB-induced lung pathology. Neutrophils mode of action and their specialized functions can be directly linked to TB-specific lung tissue damage observed on patient chest X-rays at diagnosis and contribute to long-term pulmonary sequelae. This review discusses aspects of neutrophil activity associated with active TB, including the resulting inflammation and pulmonary impairment. It highlights the significance of neutrophil function on TB disease outcome and underlines the necessity of monitoring neutrophil function for better assessment of the immune response and severity of lung pathology associated with TB. Finally, we propose that some MMPs, ROS, MPO, S100A8/A9 and Glutathione are neutrophil-related inflammatory mediators with promising potential as targets for developing host-directed therapies for TB.

Keywords: tuberculosis, neutrophils, inflammatory mediators, lung damage, sequelae

OPEN ACCESS

Edited by:
Mary Jane Thomassen, East Carolina University, United States

Reviewed by:
Niaina Rakotosamimanana, Institut Pasteur de Madagascar, Madagascar
Jose Roberto Lapa E. Silva, Federal University of Rio de Janeiro, Brazil

****Correspondence:***
Caleb N. Muefong
cmuefong@mrc.gm

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *02 March 2020*
Accepted: *23 April 2020*
Published: *27 May 2020*

Citation:
Muefong CN and Sutherland JS (2020) Neutrophils in Tuberculosis-Associated Inflammation and Lung Pathology. Front. Immunol. 11:962. doi: 10.3389/fimmu.2020.00962

INTRODUCTION

Tuberculosis (TB) is the single deadliest infectious disease known to man with 10 million new cases and 1.6 million deaths (including 300,000 HIV coinfected) in 2018 (1). This report does not account for health impairment nor deaths during and following TB treatment; which is suggested to be about three times higher than those observed in the general population or suitably matched controls (2). It is known today that despite being diagnosed as microbiologically cured from TB, about 50% of patients still suffer from some form of pulmonary impairment after tuberculosis (PIAT), irrespective of smoking habits (3). The definition of PIAT encompasses several clinical conditions; which in former TB patients is suggested to result from chronic inflammation, characterized by disrupted pulmonary structure and function (residual lung deficits) (4, 5); a state described as thoracic/TB sequelae (6). These include parenchymal, airway, vascular and mediastinal lesions manifested mainly through structural [cicatrization, calcification, fibrosis and reduction in cavity wall thickness (6)] and functional [deficit in forced expiratory volume (4)] damage; the establishment (7) and severity (8–10) of which, are associated to neutrophil abundance and (hyper-)activity.

As compelling as these effects of TB may be, PIAT is presently not included in global estimates of TB burden despite increasing scientific interest and evidence of associated morbidity and mortality (3–5, 11–14).

A recent cohort study showed that subjects with a history of fully treated active TB (ATB) lost 3.6 years on average of disability-adjusted life expectancy compared to subjects with latent TB infection (LTBI) who did not progress to the active state (12). Despite the lack of data to support disease burden resulting from long-term sequelae (11), the above reduced life expectancy is a direct result of TB sequelae and suggests that a considerable proportion of the TB disease burden is contributed by subjects who have successfully cleared Mtb. Indeed, a study in Texas, USA reported that the number of years lived with chronic TB disability accounts for 75% of non-fatal health effects of TB (11). This same study suggests that the most health and financial savings may be achieved by preventing rather than shortening therapeutic strategies. Additionally, a recent retrospective study reveals the negative effect of drug-resistance and disease recurrence on PIAT (15). Consequently, early detection of parameters which increase the likelihood of ATB complication into chronic inflammation and long-term sequelae would inform clinicians on the need for case-specific treatment measures and contribute to minimizing the global TB burden (13). Such parameters can be realistically linked to neutrophil function and/or interaction with other immune cell populations in the view of their specific activities described in subsequent sections below.

Generally, protective immunity to *Mycobacterium tuberculosis* (Mtb)—the causative agent of TB—is a combination of innate and adaptive immune responses within the pulmonary airways via which this pathogen gains entrance into the human host (16, 17). This immune response to TB is described as a chronic granulomatous inflammation; caused by close interaction between Mtb bacilli and host immune agents at the infection site (18). Indeed, the term "chronic granulomatous" draws from a condition, chronic granulomatous disease (CGD), with similar inflammatory outcomes; resulting from genetic mutations of reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) oxidase-encoding genes (19, 20). Disruption in the production of this enzyme; which normally catalyzes the synthesis of reactive oxygen species (ROS) used by phagocytes to destroy bacteria during phagocytosis, leads to enhanced susceptibility to infectious pathogens and granuloma formation; particularly in the lungs (21). Despite several gaps in knowledge, the contribution of adaptive immune responses: particularly T-cells [reviewed in Jasenosky et al. (22)] and to a lesser extent B-cells [reviewed in Achkar et al. (23)] have been addressed. Furthermore Dyatlov et al. recently reviewed the role of B cells on reducing neutrophil influx to infection sites (24) and; these Mtb-specific immune responses having been studied extensively and will not form a focus of this review.

Recent studies have revealed that the innate arm of the immune system plays a bigger role in the onset and regulation of inflammatory processes during ATB than previously thought. ROS-generating cells are central to Mtb-induced inflammatory response; and that they are main actors of relevant cell death processes (i.e., apoptosis, necrosis, pyroptosis, necroptosis, pyronecrosis, NETosis, and autophagy) that influence TB disease progression [reviewed by Mohareer et al. (25)], suggests that their activity contributes considerably to destructive immunity to Mtb infection. The aim of this review is to provide an update on the importance of neutrophils during ATB and to identify related immune mediators associated with anti-TB treatment response and lung damage.

TB-INDUCED INFLAMMATORY RESPONSE

Innate immune responses play a central role in the pathology of infectious and inflammatory diseases including acute abdominal inflammation (26), cancers (27, 28) and respiratory tract disorders (29, 30). Phagocytic cells (i.e., neutrophils and macrophages) are the predominant components of this response in TB (17). In collaboration with inflammatory mediators like cytokines (31) and proteases, they are key contributors to the host interaction with Mtb, in a process which generally ends with the destruction of the pathogen and resolution of inflammation (32). In many cases, however, the inflammatory response is relatively ineffective and can lead to destruction of host tissues as reviewed by Fullerton and Gilroy (33). Such an unwanted scenario is characterized by a constant influx of inflammatory mediators and innate immune cells to the site of infection with progressive deterioration of the affected tissue. The end result is the formation of tuberculous granulomas whose structure, immune/pathogen cell balance (34), and intrinsic T-cell activity (35) ultimately determine the degree of formation of tissue lesions (36).

Defining and Assessing Lung Impairment

In order to understand the role of neutrophils in lung pathology, we need consensus on structural versus functional impairment. There are currently no international guidelines describing how to classify levels of structural impairment following TB as well as identifying TB sequelae in general (37). ATB is increasingly further classified with respect to disease severity into the extent of functional and/or structural lung damage, however, a decisive classification of TB patient pathology has not been reached at this time. Nonetheless, certain criteria have allowed the severity of active pulmonary TB to be determined following assessment of impaired pulmonary function via spirometry testing (38) and the observation of lesions and/or lung cavities through chest x-rays (CXR) and computed tomography (CT) (39).

Structural lung abnormalities determined by x-ray or computed topography (CT) scores have been observed to correlate to a degree with lung function in pulmonary TB (40). Reports also suggest that functional pulmonary impairment at diagnosis only begins to improve significantly several months after the end of successful TB therapy (4, 40). Saldana et al. observed that CXR abnormalities are inversely proportional to and more reliable than spirometry evaluations when assessing severity of lung impairment in cured ATB patients (41). An even earlier study by Plit et al. showed that the change in CXR score (pre- vs. post-treatment) is the most reliable predictor of the severity of functional lung impairment in ATB: here too, an inverse proportionality was observed between CXR

TABLE 1 | Clinical studies assessing TB sequelae.

References	Study site	Study type and design post-treatment commencement	ATB sample size, *n*	Nature of residual lung impairment	Associated inflammatory response
Ngahane et al. (46)	Cameroon	Cross-sectional Includes HIV$^+$	269	Structural (CXR lesions) and Functional (dyspnoea and spirometry)	No
Ralph et al. (13)	Indonesia	Longitudinal [Baseline (BL), 6M & over 6M] Includes HIV$^+$	200	Structural (CXR score of lesions and cavitation) and Functional (dyspnoea, SGRQ, spirometry)	No
Kumar et al. (47)	India	Longitudinal (BL & 6M) No HIV$^+$ cases Part of larger study involving patients with co-morbidities	24	Structural (cavitation; no CXR-score)	Yes
Ravimohan et al. (48)	USA	Prospective (over 6M) All TB/HIV$^+$	14	Functional (spirometry)	Yes (MMPs)
Pasipanodya et al. (11)	USA	Longitudinal (BL, 6M & over 6M) Includes HIV$^+$	177	Functional (SGRQ and spirometry)	No
Plit et al. (42)	South Africa	Longitudinal (BL & 6M) Includes HIV$^+$	76	Structural (CXR score of lung infiltrates) Functional (spirometry)	Yes (c-reactive protein (CRP) and serum α1-protease inhibitor (α1-PI)
Cole et al. (43)	South Africa	Cross-sectional Includes HIV$^+$	55	Functional (SGRQ and spirometry)	No
Patil and Patil (44)	India	Longitudinal (6M, 9M, & 12M) No HIV$^+$ cases	118	Functional (dyspnoea and spirometry)	No
Hnizdo et al. (4)	South Africa	Retrospective (BL-–375M) Includes HIV$^+$	2,599	Functional (spirometry)	No
Maguire et al. (45)	Indonesia	Longitudinal (BL, 2M, & 6M) Includes HIV$^+$	115	Functional (dyspnoea, SGRQ, spirometry)	No
Saldana et al. (41)	Mexico	Cross-sectional Includes HIV$^+$	127	Functional (Spirometry) and Structural (CXR abnormalities)	No
Vecino et al. (14)	USA	Longitudinal (BL, 6M, & over 6M)	123	Functional (Spirometry)	No
Chushkin et al. (3)	Russia	Prospective (Over 12M) Undetermined HIV status	214	Functional (Spirometry)	No

scores and forced expiratory volume (FEV1; a spirometric parameter) (42). These suggest that monitoring variations in structural impairment during TB therapy is essential (or at least of significant added value) when attempting to determine the extent of TB sequelae. However, whilst most relevant clinical studies have generally attempted to monitor ATB-linked signs of TB sequelae via assessment of dyspnoea and disrupted lung function by spirometry (3, 4, 11, 14, 43–45), fewer cases have accounted for both structural and functional damage (13, 41, 42, 46), and none focussing on the former exclusively (see **Table 1**). Relevant follow-up parameters, where available (especially involving longitudinal cohort studies), appear to have relied on the researchers' study objective and understanding of TB sequelae—variably assessing different forms of pulmonary damage, lung rehabilitation and even treatment responses but not the potential inflammatory triggers of these events as the Ravimohan group's latest review hints (49). This is probably owing to absence of a referential guideline as mentioned above. At this time, a few studies: Ravimohan et al. (48) and Plit et al. (42) have assessed severity of lung impairment in ATB in relation to the expression of inflammatory mediators: matrix metalloproteinases (MMPs) in the former and; serum c-reactive protein (CRP), serum α1-protease inhibitor (α1-PI) and urine cotinine in the latter. To account for these limitations, a multisite trial is currently underway to monitor host-pathogen and socioeconomic factors that influence the development of pulmonary sequelae in ATB patients (50).

EVIDENCE OF NEUTROPHIL IMPACT ON DESTRUCTIVE TB INFLAMMATION

Neutrophilia and Hyperinflammation

Polymorphonuclear neutrophils are the most abundant type of white blood cells and play a central role in the immune response to bacterial pathogens (51). The protective activity of neutrophils in TB infection is observed during granuloma formation where mycobacteria are phagocytosed from infected macrophages by oxidative killing (52).

Previous work indicates that the levels of granulocytes (neutrophils and eosinophils) in circulation are higher in patients with ATB disease than those with latent TB infection (LTBI); with levels decreasing significantly following successful TB treatment (53). It has also been demonstrated that neutrophilia independently associates not only with increased risk of cavity formation and lung tissue damage (54), but also mortality in patients undergoing TB therapy (55), suggesting that the neutrophil count in tuberculosis positively correlates with bacillary load and disease outcome. Recently, Leem et al. (56) monitored inflammatory markers in TB patients and found that the neutrophil counts and neutrophil to lymphocyte ratios (NLR) were decreased following a 6-months anti-TB drug therapy compared to baseline. These results hint that inflammation might be resolved only following the 6-month treatment completion, suggesting that progress to chronic inflammation and development of pulmonary lesions is a silent process potentially mediated by secondary products of inflammatory response whose activity persist in tissue long after mycobacterial clearance.

Despite the lack of a consensus on neutrophil classification, varying attributes: granule content (cytotoxic species/enzyme concentration), density (low or normal density granulocytes), nuclear segmentation [banded or (hyper)-segmented], tumor suppressive/enhancing functions (N1/N2), to surface antigen expression [CD177 (7, 57, 58); CD16, CD62L and CD11b] and cytokine/chemokine secretion levels have been associated to disease and immunoregulation [reviewed in Hellebrekers et al. (59), Perobelli et al. (60), and Wang (61)]. It is therefore arguable that a combination of these attributes could constitute a neutrophil profile suggestive of disease severity at an early stage as well as anticipated development of sequalae if chronic conditions (in TB potentially) were to be established. However, given the vast discrepancies in markers, experimental conditions and disease models investigated by previous studies as described in the reviews cited above, these functional differences in neutrophil subsets will not constitute a focus here. Nevertheless, the severity of ATB is linked to neutrophilia as discussed above; but also a specific hyperactivated profile of the circulating neutrophils; which has predominantly been associated with immature banded (or non-segmented) neutrophils (8). Interestingly, the bulk of neutrophil cytotoxic (and antibacterial) molecules are concentrated in their granules. Hence, neutrophil degranulation and exocytosis: processes requiring phosphatidylinositol 3-kinase, (PI3-K) (62); are closely related to the severity of neutrophil-mediated inflammation. We therefore anticipate that a potential neutrophil bio-signature of ATB would encompass enhancement/inhibition of some specific chemokines and increased neutrophil-specific enzyme concentrations. In fact, a recent review by Leisching (7) exposes the regulatory role of PI3-K on enhanced neutrophil mobility and hyperactivity and; the effect on neutrophil-driven TB inflammation. This hyperactivity is equally suggested to be at play in chronic periodontis where it is associated with increased migratory capacity as well as pro-inflammatory cytokine (IL-8, TNF, and IL-1, notably) production by circulating neutrophils (63). Taken together, neutrophil relative abundance (in circulation and at infection sites) and cytokine/enzyme release are potentially major agents of hyperinflammatory conditions observed in ATB.

The mechanisms responsible for this inflammatory response mainly result from three neutrophil functions: oxidative burst, necrosis and NETosis.

Oxidative Burst Capacity

Although neutrophils have the capacity to protect against Mtb infection, if left uncontrolled their collective activity may produce pathogenic effects through different functions (64). One such phagocytic function is oxidative burst, which is the release of reactive oxygen species (ROS) mainly by neutrophils and to a lesser extent, macrophages during phagocytosis, a process which is mediated by nicotinamide adenine dinucleotide phosphate (NADP) oxidase (65). This antibacterial activity is performed by a myeloperoxidase system composed mainly of reduced NADP ($NADPH_2$), reduced glutathione (GSH), azide, cyanide, thiocyanate, Tapazole, thiourea, cysteine, ergothioneine, thiosulfate, reduced nicotinamide adenine dinucleotide ($NADH_2$), and tyrosine (66).

GSH levels have been shown to reduce significantly in PMBCs and red blood cells isolated from tuberculosis patients compared to healthy controls (67), while increased GSH levels are reported to enhance T-cell capacity to inhibit Mtb growth inside macrophages (68). Also, ROS produced by neutrophils during oxidative burst have been reported to drive Mtb-induced necrosis; which in turn promotes Mtb growth (69). It has also been suggested that rapid assessment of individual neutrophil oxidative burst capacity could distinguish patients at risk of excessive immune responses and thus could potentially guide therapy (70). Hence, correlating neutrophil oxidative burst capacity with GSH and/or $NADPH_2$ levels in TB patients may provide avenues for novel host-directed therapies.

Neutrophil Extracellular Traps (NETs)

In-vitro studies by Brinkmann (71) revealed that neutrophil activation with lipopolysaccharide (LPS), interleukin 8 or phorbol myristate acetate (PMA) led to the release of cell components, which form an extracellular fibril matrix called neutrophil extracellular traps (NETs). These components are proteins [namely neutrophil elastase (NE) and myeloperoxidase (MPO)], DNA and chromatin-derived fibers; which destroy bacteria extracellularly (71, 72). This process, NETosis, is a powerful neutrophil-mediated response to a range of infections but also acts as a double-edged sword during inflammatory diseases (73). Interestingly, neutrophils can sense pathogen size and can produce more NETs in presence of larger pathogens like *Mycobacterium bovis* (74). Although aggregated NETs are reported to degrade neutrophil-derived inflammatory mediators in an attempt to resolve inflammation (75), NETs also stimulate unwanted immune reactions and trigger tissue injury (73, 76).

In TB pathogenesis, Mtb is reported to induce the formation of NETs, which trap Mycobacteria *in vitro* but are unable to kill them (77). This may be partially explained by the fact that expression of enzyme systems such as those required in inflammatory pathways [i.e., to degrade proteins within

the phagolysosome (e.g., MPO) and for the phagocytic burst, NADPH-oxidase complex and the generation of ROS] are suppressed (78). Furthermore, these Mtb-induced NETs are also associated with macrophage activation in humans (79) and could thus help elucidate specific inflammatory mechanisms of lung damage in TB pathogenesis. Indeed, a recent study by De Melo et al. revealed high levels of citrullinated H3—a common NET marker—in serum samples from TB patients with extensive pulmonary damage (54). Although this marker is usually measured in combination with others (i.e., MPO and NE) to specifically identify NETs, this study suggests that NET formation is centrally linked with severe lung tissue damage in TB patients and could be implicated in subsequent pulmonary pathology.

Neutrophil-mediated lung injury is not just restricted to Tuberculosis. For example, excessive neutrophil recruitment and NETosis was linked to acute lung injury in a mouse model of Influenza pneumonitis (80). Additionally, more recent studies reveal that reduced neutrophil recruitment into infected tissue promotes resolution of inflammation (81). Hence, monitoring NETosis and neutrophil-associated inflammatory mediators within inflamed tissue could be useful in developing therapeutic targets against chronic inflammatory conditions like TB (72).

Metalloproteinases in Destructive TB Immunity

A group of molecules increasingly associated with excessive lung inflammation is the matrix metalloproteinases (MMPs). In the case of cystic fibrosis, which results in loss of pulmonary architecture, Pardo et al. described in a review (82) the essential role played by MMPs in modifying the tissue microenvironment and modulating cell signaling through their ability to degrade constituents of the extracellular matrix. Although the origin of most MMPs cannot be directly linked to neutrophils, MMP-9 is known to be secreted rapidly by neutrophils in whole blood from healthy volunteers following proinflammatory stimulus (83) and is suggested to facilitate transmembrane neutrophil migration (84); also reviewed in Pardo et al. (82). Similar to MMP-9, MMP-8 synthesis in ATB patients is also suspected to be of neutrophil origin (85). In effect, Ravimohan et al. (48) assessed the role of MMPs on TB-immune reconstitution inflammatory syndrome and observed increased MMP-8; whilst MMP-2, -3 and -9 levels reduced (MMP-1 did not vary significantly) in patients with impaired lung function post-TB cure following antiretroviral therapy. MMP-1 and MMP-8 have previously been shown to correlate with pulmonary tissue damage (PTD) in patients with ATB (85, 86) while MMP-14 has been shown to play a central role in TB pathogenesis by provoking collagen degradation and regulating monocyte migration (87). Interestingly, a more recent study by De Melo et al. found lower levels of serum MMP-8 in TB patients with severe PTD showing no radiological improvement after 60 days of anti-TB treatment (54). Whether this change in trend is related to plasma vs serum or due to the presence/absence of HIV infection is unknown, however, the latter observation is inconsistent with that from the previous studies reported above and suggests that MMP regulation in TB patients might differ between the circulation and the lung as well as in the presence of coinfection. Nonetheless, de Melo et al. (54) did find higher MMP-1 levels in these patients compared to those with improved chest-x rays. Hence, whilst there is clearly a role for MMPs in TB-linked tissue damage, more detailed studies, with assessment of coinfections, are required to ascertain which MMPs are predominant mediators. This will help to determine potential host-directed therapeutic strategies.

Furthermore, a few clinical studies on major TB comorbidities have recently emerged. One shows that sputum levels of MMP-1, -2, -3 and -9 are higher in HIV negative TB patients than in TB healthy controls (HC) and HIV positive TB patients with a correlation found between the degree of chest x-ray inflammation and both MMP-1 and MMP-3 levels in HIV negative TB patients (88). Moreover, the clinical severity of TB is known to increase in TB patients with diabetes mellitus (DM). Kumar et al. have shown that circulatory levels of MMP-1, -2, -3, -8, and -13 in these patients decrease following successful TB treatment and that MMP-1 (in sputum) and MMP-1, -2, -3, -9, and -12 levels (in serum) were higher in patients with more severe structural lung damage at baseline (47) as determined from chest x-rays.

These findings suggest that MMPs (MMP-1, -2, -3, -8, and -9, particularly) may promote tissue injury following Mtb infection. Hence, monitoring the correlation of these particular MMPs together with the downregulation of other neutrophil-related inflammatory proteins and pro-inflammatory cytokines associated with intracellular killing pathways during TB infection would increase our understanding of the active inflammatory pathways which enhance susceptibility to development of PIAT and subsequent sequelae. Importantly, natural regulation of MMP activity is performed by tissue inhibitors of metalloproteinases (TIMPs). The levels of TIMPs have not yet been monitored in TB patients; an aspect of TB research which should be addressed for optimal understanding of inflammatory mechanisms involved in development and host control of TB-related PTD.

NEUTROPHIL-RELATED TB HDT

With increasing cases of co-infections, co-morbidities, drug resistance; as well as the cost associated with the relatively long standard antibiotic TB-treatment, new treatment regiments like host directed therapies (HDT) could complement existing Mtb-targeted approaches. Meanwhile biomarkers for efficiently identifying and treating TB disease progressors at an early stage are being actively researched (89), those that could single out individuals who develop unresolving inflammation-induced lung damage are still greatly under-investigated. This means that research on HDTs should ideally focus on diagnosis and prevention of the latter long-lasting condition as well. Recent reviews have highlighted various established as well as promising host directed adjuvant therapies against the development of TB disease (90), TB-linked inflammation (91, 92) and lung damage (93). Drugs that potentially inhibit pulmonary damage and/or promote lung repair range from steroids to nonsteroidal anti-inflammatory drugs, statins, metformin, dietary supplements,

TNF blockers etc. (10). Of these, we observe that those suppressing pro-inflammatory aspects of the disease appear to be potent targets in preclinical and clinical trials. In fact, Young et al. have recently reviewed current targets in TB HDT with some of the most advanced ATB-relevant in clinical trials being modulators of pro-inflammatory mediators which: dampen inflammatory responses, curb immunopathology and resolve lung damage (93). These include the phase 3 drugs: cox-2 inhibitor (Meloxicam) and corticosteroids (Prednisolone and Dexamethasone) amongst others. This HDT potential of inflammatory mediators has also been addressed with inhibitory effects on neutrophil recruitment (Ibuprofen) and neutrophil-derived inflammatory mediators such as ROS and MPO as reviewed by Dallenga et al. (94).

Also, a combination of the anti-inflammatory drug, zileuton (an inhibitor of the synthesis of pro-inflammatory eicosanoid; already approved against asthma) and prostaglandin E2 (95) is reported to reduce bacillary load and TB-induced lung damage in mice. Statins are also interesting HDT targets against destructive lung pathology following ATB (96); with a promising phase 2 trial using pravastatin being investigated in South Africa (ClinicalTrials.gov Identifier: NCT03456102). Moreover, a phase 2b trial testing the effect of atorvastatin against PIAT in patients with or without HIV is about to begin in South Africa (ClinicalTrials.gov Identifier: NCT04147286); underlining the potential of these agents.

Potentially, some mediators of neutrophil function (mentioned in previous sections) could provide suitable HDT targets. Amongst others, these involve: vitamin D which is reported to inhibit Mtb-induced expression of MMP-7 and−10 as well as MMP-9 gene expression, secretion and activity by peripheral blood mononuclear cells (PBMCs) (97). Although the authors reported that the latter inhibition occurs irrespective of infection, MMP-9 is reported to be of neutrophil origin and in-depth investigation may be warranted. Doxycycline is also a known MMP-inhibitor which in TB-HIV co-infection particularly, is shown to suppress the secretion of TNF, MMP-1 and−9 by primary human macrophages while reducing Mtb growth in the guinea pig model of TB (98). Also, Allen et al. reviewed the importance of considering GSH as HDT against TB and TB/HIV co-infection (99). Furthermore, it is important to note that N-acetylated proline glycine proline (ac-PGP) induces neutrophil chemotaxis and neutrophil production of MMP-9 and IL-8 (100, 101) which has led this molecule to be suggested as potential HDT-target against chronic neuroinflammatory diseases (102) and cystic fibrosis (103); which result in MMP activation and result in considerable tissue damage like TB.

Besides these, calprotectin, a hetero-dimer made up of proteins S100A8 and S100A9 is a mediator of inflammatory responses and a potent diagnostic and HDT target against inflammatory diseases (104). Actually, Gopal et al. (9) reported that S100A8/A9 accumulates in TB-induced granulomas. The authors showed that this accumulation was neutrophil-driven (in humans) and the animal models they employed suggested an association between S100A8/A9 and the degree of inflammation and lung pathology during ATB. Recently, it has been shown that these high levels of S100A8/A9 as well as an S100A8/A9-mediated enhanced accumulation of neutrophils in lungs of mice and macaques are associated with Mtb proliferation in chronic TB disease (105). Both studies reveal a close interaction between neutrophils and S100A8/A9 in ATB suggesting that neutrophils and S100A8/A9, particularly could be targeted in TB HDT.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion, we believe that observation and monitoring of neutrophil subsets and related inflammatory mediators is important not only for studies aiming at developing novel therapeutic targets against TB (72) but also for improved estimation of host immuno-modulatory effects on the severity of TB sequalae. It is foreseeable that the extent of long-term pulmonary injury sustained and potentially resorbed following TB therapy (irrespective of HIV coinfection) could be correlated to a specific neutrophil function. It is also likely that ATB patients who express a specific form of neutrophil-mediated inflammatory response over the period from diagnosis through treatment are more susceptible to developing chronic PIAT than otherwise. A major challenge will be harmonizing the categorization of disease severity (structural and functional) to ease comparison between clinical studies. Moreover, we believe that prediction of treatment response and residual pulmonary impairment in future clinical studies would be made more effective and reproduceable by evaluating inflammatory responses as well as simultaneously monitoring variations in pulmonary structure and function during and months after treatment completion. Finally, prospective HDTs; which rely on inflammatory mediators of neutrophil activity particularly, should be investigated further.

AUTHOR CONTRIBUTIONS

CM was the primary author of the manuscript. JS provided substantial writing support, edits, and suggestions.

FUNDING

CM was supported with a Ph.D student stipend from TB-Sequel (grant number 66.3010.7-002.00) funded by the German Ministry for Education and Research (BMBF).

REFERENCES

1. WHO. *Global Tuberculosis Report 2018*. Geneva: World Health Organization (2018).

2. Romanowski K, Baumann B, Basham CA, Khan FA, Fox GJ, Johnston JC. Articles long-term all-cause mortality in people treated for tuberculosis: a systematic review and meta-analysis. *Lancet Infect Dis*. (2019) 3099:1–9. doi: 10.1016/S1473-3099(19)30309-3

3. Chushkin MI, Ots ON. Impaired pulmonary function after treatment for tuberculosis: the end of the disease? *J Bras Pneumol.* (2017) 43:38–43. doi: 10.1590/s1806-37562016000000053
4. Hnizdo E, Singh T, Churchyard G. Chronic pulmonary function impairment caused by initial and recurrent pulmonary tuberculosis following treatment. *Thorax*. (2000) 55:32–8. doi: 10.1136/thorax.55.1.32
5. Chakaya J, Kirenga B, Getahun H. Long term complications after completion of pulmonary tuberculosis treatment: a quest for a public health approach. *J Clin Tuberc Other Mycobact Dis.* (2016) 3:10–2. doi: 10.1016/j.jctube.2016.03.001
6. Kim HY, Song K.-S, Goo JM, Lee JS, Lee KS, Lim T.-H. Thoracic sequelae and complications of tuberculosis. *RadioGraphics*. (2001) 21:839–58. doi: 10.1148/radiographics.21.4.g01jl06839
7. Leisching GR. Susceptibility to Tuberculosis is associated with PI3K-dependent increased mobilization of neutrophils. *Front Immunol.* (2018) 9:1669. doi: 10.3389/fimmu.2018.01669
8. Panteleev AV, Nikitina IY, Burmistrova IA, Kosmiadi GA, Radaeva TV, Amansahedov RB, et al. Severe tuberculosis in humans correlates best with neutrophil abundance and lymphocyte deficiency and does not correlate with antigen-specific CD4 T-cell response. *Front Immunol.* (2017) 8:963. doi: 10.3389/fimmu.2017.00963
9. Gopal R, Monin L, Torres D, Slight S, Mehra S, McKenna KC, et al. S100A8/A9 proteins mediate neutrophilic inflammation and lung pathology during tuberculosis. *Am. J Respir Crit Care Med.* (2013) 188:1137–46. doi: 10.1164/rccm.201304-0803OC
10. Stek C, Allwood B, Walker NF, Wilkinson RJ, Lynen L, Meintjes G. The immune mechanisms of lung parenchymal damage in tuberculosis and the role of host-directed therapy. *Front Microbiol.* (2018) 9:2603. doi: 10.3389/fmicb.2018.02603
11. Pasipanodya JG, McNabb SJ, Hilsenrath P, Bae S, Lykens K, Vecino E, et al. Pulmonary impairment after tuberculosis and its contribution to TB burden. *BMC Public Health*. (2010) 10:259. doi: 10.1186/1471-2458-10-259
12. Hoger S, Lykens K, Beavers SF, Katz D, Miller TL. Longevity loss among cured tuberculosis patients and the potential value of prevention. *Int J Tuberc Lung Dis.* (2014) 18:1347–52. doi: 10.5588/ijtld.14.0242
13. Ralph AP, Kenangalem E, Waramori G, Pontororing GJ, Tjitra E, Maguire GP, et al. High morbidity during treatment and residual pulmonary disability in pulmonary tuberculosis: under-recognised phenomena. *PLoS ONE.* (2013) 8:1–11. doi: 10.1371/journal.pone.0080302
14. Vecino M, Pasipanodya JG, Slocum P, Bae S, Munguia G, Miller T, et al. Evidence for chronic lung impairment in patients treated for pulmonary tuberculosis. *J Infect Public Health.* (2011) 6:244–52. doi: 10.1016/j.jiph.2011.08.005
15. Vashakidze SA, Kempker JA, Jakobia NA, Gogishvili SG, Nikolaishvili KA, Goginashvili LM, et al. Pulmonary function and respiratory health after successful treatment of drug-resistant tuberculosis. *Int J Infect Dis.* (2019) 82:66–72. doi: 10.1016/j.ijid.2019.02.039
16. Scriba TJ, Coussens AK, Fletcher HA. Human immunology of tuberculosis. *Tuberc Tuber Bacillus.*. (2016) 5:213–37. doi: 10.1128/9781555819569.ch11
17. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MPR. The immune response in tuberculosis. *Annu Rev Immunol.* (2013) 31:475–527. doi: 10.1146/annurev-immunol-032712-095939
18. Kiran D, Podell BK, Chambers M, Basaraba RJ. Host-directed therapy targeting the Mycobacterium tuberculosis granuloma: a review. *Semin Immunopathol.* (2016) 38:167–83. doi: 10.1007/s00281-015-0537-x
19. Holland SM. Chronic granulomatous disease. *Hematol Oncol Clin North Am.* (2013) 27:89–99. doi: 10.1016/j.hoc.2012.11.002
20. Heyworth PG, Cross AR, Curnutte JT. Chronic granulomatous disease. *Curr Opin Immunol.* (2003). 15:578–584. doi: 10.1016/S0952-7915(03)00109-2
21. Roos D, de Boer M. Molecular diagnosis of chronic granulomatous disease. *Clin Exp Immunol.* (2014) 175:139–49. doi: 10.1111/cei.12202
22. Jasenosky LD, Scriba TJ, Hanekom WA, Goldfeld AE. T cells and adaptive immunity to *Mycobacterium tuberculosis* in humans. *Immunol Rev.* (2015) 264:74–87. doi: 10.1111/imr.12274
23. Achkar JM, Chan J, Casadevall A. B cells and antibodies in the defense against *Mycobacterium tuberculosis* infection. *Immunol Rev.* (2015) 264:167–81. doi: 10.1111/imr.12276
24. Dyatlov V, Apt AS, Linge IA. B lymphocytes in anti-mycobacterial immune responses : Pathogenesis or protection? *Tuberculosis*. (2018) 114:1–8. doi: 10.1016/j.tube.2018.10.011
25. Mohareer K, Asalla S, Banerjee S. Cell death at the cross roads of host-pathogen interaction in Mycobacterium tuberculosis infection. *Tuberculosis*. (2018) 113:99–121. doi: 10.1016/j.tube.2018.09.007
26. Bilyy R, Fedorov V, Vovk V, Leppkes M, Dumych T, Chopyak V, et al. Neutrophil extracellular traps form a barrier between necrotic and viable areas in acute abdominal inflammation. *Front Immunol.* (2016) 7:1–7. doi: 10.3389/fimmu.2016.00424
27. Liu J, Geng X, Li Y. Milky spots: omental functional units and hotbeds for peritoneal cancer metastasis. *Tumor Biol.* (2016) 37:5715–26. doi: 10.1007/s13277-016-4887-3
28. Clark R, Krishnan V, Schoof M, Rodriguez I, Theriault B, Chekmareva M, et al. Milky spots promote ovarian cancer metastatic colonization of peritoneal adipose in experimental models. *Am J Pathol.* (2013) 183:576–91. doi: 10.1016/j.ajpath.2013.04.023
29. Robb CT, Regan KH, Dorward DA, Rossi AG. Key mechanisms governing resolution of lung inflammation. *Semin Immunopathol.* (2016) 38:425–48. doi: 10.1007/s00281-016-0560-6
30. Porto BN, Stein RT. Neutrophil extracellular traps in pulmonary diseases: too much of a good thing? *Front Immunol.* (2016) 7:1–13. doi: 10.3389/fimmu.2016.00311
31. Etna MP, Giacomini E, Severa M, Coccia EM. Pro-and anti-inflammatory cytokines in tuberculosis: A two-edged sword in TB pathogenesis. *Semin Immunol.* (2014) 26:543–51. doi: 10.1016/j.smim.2014.09.011
32. Newson J, Stables M, Karra E, Arce-Vargas F, Quezada S, Motwani M, et al. Resolution of acute in fl ammation bridges the gap between innate and adaptive immunity. *Blood.* (2015) 124:1748–65. doi: 10.1182/blood-2014-03-562710
33. Fullerton JN, Gilroy DW. Resolution of inflammation: a new therapeutic frontier. *Nat Rev.* (2016) 15:551–67. doi: 10.1038/nrd.2016.39
34. Marakalala MJ, Raju RM, Sharma K, Zhang YJ, Eugenin EA, Prideaux B, et al. Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nat Med.* (2016) 22:531–8. doi: 10.1038/nm.4073
35. Gideon HP, Phuah J, Myers AJ, Bryson BD, Rodgers MA, Coleman MT, et al. Variability in tuberculosis granuloma T cell responses exists, but a balance of pro- and anti-inflammatory cytokines is associated with sterilization. *PLoS Pathog.* (2015) 11:1–28. doi: 10.1371/journal.ppat.1004603
36. Lin PL, Ford CB, Coleman MT, Myers AJ, Gawande R, Ioerger T, et al. Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. *Nat Med.* (2014) 20:75–9. doi: 10.1038/nm.3412
37. van Kampen SC, Wanner A, Edwards M, Harries AD, Kirenga BJ, Chakaya J, et al. International research and guidelines on post-tuberculosis chronic lung disorders: a systematic scoping review. *BMJ Glob Heal.* (2018) 3:1–8. doi: 10.1136/bmjgh-2018-000745
38. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi RE, et al. Interpretative strategies for lung function tests. *Eur Respir J.* (2005) 26:948–68. doi: 10.1183/09031936.05.00035205
39. Yeh JJ. Predictors of initial smear-negative active pulmonary tuberculosis with acute early stage lung injury by high-resolution computed tomography and clinical manifestations: an auxiliary model in critical patients. *Sci Rep.* (2019) 9:1–13. doi: 10.1038/s41598-019-40799-w
40. Long R, Maycher B, Dhar A, Manfreda J, Hershfield E, Anthonisen N. Pulmonary tuberculosis treated with directly observed therapy: serial changes in lung structure and function. *Chest.* (1998) 113:933–43. doi: 10.1378/chest.113.4.933
41. Báez-Saldaña R, López-Arteaga Y, Bizarrón-Muro A, Ferreira-Guerrero E, Ferreyra-Reyes L, Delgado-Sánchez G, et al. A novel scoring system to measure radiographic abnormalities and related spirometric values in cured pulmonary tuberculosis. *PLoS ONE.* (2013) 8:1–12. doi: 10.1371/journal.pone.0078926
42. Plit ML, Anderson R, Van Rensburg CE, Page-Shipp L, Blott JA, Fresen JL, et al. Influence of antimicrobial chemotherapy on spirometric parameters and pro-inflammatory indices in severe pulmonary tuberculosis. *Eur Respir J.* (1998) 12:351–6. doi: 10.1183/09031936.98.12020351

43. Cole G, Miller D, Ebrahim T, Dreyden T, Simpson R, Manie S. Pulmonary impairment after tuberculosis in a South African population. *South Afr J Physiother.* (2016) 72:1–6. doi: 10.4102/sajp.v72i1.307
44. Patil P, Patil S. A six-month follow-up study to evaluate changes of pulmonary function test in Category I pulmonary tuberculosis treatment completed patient. *Natl J Physiol Pharm Pharmacol.* (2017) 8:31–6. doi: 10.5455/njppp.2018.8.0724121072017
45. Maguire GP, Anstey NM, Ardian M, Waramori G, Tjitra E, Kenangalem E, et al. Pulmonary tuberculosis, impaired lung function, disability and quality of life in a high-burden setting. *Int J Tuberc Lung Dis.* (2009) 13:1500–6.
46. Ngahane BH, Nouyep J, Motto MN, Njankouo YM, Wandji A, Endale M, et al. Post-tuberculous lung function impairment in a tuberculosis reference clinic in Cameroon. *Respir Med.* (2016) 114:67–71. doi: 10.1016/j.rmed.2016.03.007
47. Kumar NP, Moideen K, Viswanathan V, Shruthi BS, Sivakumar S, Menon PA, et al. Elevated levels of matrix metalloproteinases reflect severity and extent of disease in tuberculosis-diabetes co-morbidity and are predominantly reversed following standard anti-tuberculosis or metformin treatment. *BMC Infect Dis.* (2018) 18:1–10. doi: 10.1186/s12879-018-3246-y
48. Ravimohan S, Tamuhla N, Kung SJ, Nfanyana K, Steenhoff AP, Gross R, et al. Matrix metalloproteinases in tuberculosis-immune reconstitution inflammatory syndrome and impaired lung function among advanced HIV/TB co-infected patients initiating antiretroviral therapy. *EBioMedicine.* (2016) 3:100–7. doi: 10.1016/j.ebiom.2015.11.040
49. Ravimohan S, Kornfeld H, Weissman D, Bisson GP. Tuberculosis and lung damage: from epidemiology to pathophysiology. *Eur Respir Rev.* (2018) 27:170077. doi: 10.1183/16000617.0077-2017
50. Rachow A, Ivanova O, Wallis R, Charalambous S, Jani I, Bhatt N, et al. TB sequel: incidence, pathogenesis and risk factors of long-term medical and social sequelae of pulmonary TB - A study protocol 11 Medical and Health Sciences 1117 Public Health and Health Services. *BMC Pulm Med.* (2019) 19:1–9. doi: 10.1186/s12890-018-0777-3
51. Jenne CN, Wong CH, Zemp FJ, McDonald B, Rahman MM, Forsyth PA, et al. Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. *Cell Host Microbe.* (2013) 13:169–80. doi: 10.1016/j.chom.2013.01.005
52. Yang CT, Cambier CJ, Davis JM, Hall CJ, Crosier PS, Ramakrishnan L. Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. *Cell Host Microbe.* (2012) 12:301–12. doi: 10.1016/j.chom.2012.07.009
53. Moideen K, Kumar NP, Nair D, Banurekha VV, Bethunaickan R, Babu S. Heightened systemic levels of neutrophil and eosinophil granular proteins in pulmonary tuberculosis and reversal following treatment. *Infect Immun.* (2018) 86:1–8. doi: 10.1128/IAI.00008-18
54. de Melo MG, Mesquita ED, Oliveira MM, Silva-Monteiro CD, Silveira AK, Malaquias TS, et al. Imbalance of NET and alpha-1-antitrypsin in tuberculosis patients is related with hyper inflammation and severe lung tissue damage. *Front Immunol.* (2019) 9:1–17. doi: 10.3389/fimmu.2018.03147
55. Lowe DM, Bandara AK, Packe GE, Barker RD, Robert J. Europe PMC Funders Group Neutrophilia independently predicts death in tuberculosis. *Eur Respir J.* (2014) 42:1752–7. doi: 10.1183/09031936.00140913
56. Leem AY, Song JH, Lee EH, Lee H, Sim B, Kim SY, et al. Changes in cytokine responses to TB antigens ESAT-6, CFP-10 and TB 7.7 and inflammatory markers in peripheral blood during therapy. *Sci Rep.* (2018) 8:4–11. doi: 10.1038/s41598-018-19523-7
57. Zhou G, Yu L, Fang L, Yang W, Yu T, Miao Y, et al. CD177+ neutrophils as functionally activated neutrophils negatively regulate IBD. *Gut.* (2018) 67:1052–63. doi: 10.1136/gutjnl-2016-313535
58. Wang X, Qiu L, Li Z, Wang XY, Yi H. Understanding the multifaceted role of neutrophils in cancer and autoimmune diseases. *Front Immunol.* (2018) 9:1–10. doi: 10.3389/fimmu.2018.02456
59. Hellebrekers P, Vrisekoop N, Koenderman L. Neutrophil phenotypes in health and disease. *Eur J Clin Invest.* (2018) 48:e12943. doi: 10.1111/eci.12943
60. Perobelli SM, Galvani RG, Gonçalves-Silva T, Xavier CR, Nóbrega A, Bonomo A. Plasticity of neutrophils reveals modulatory capacity. *Braz J Med Biol Res.* (2015) 48:665–75. doi: 10.1590/1414-431x20154524
61. Wang J. Neutrophils in tissue injury and repair. *Cell Tissue Res.* (2018) 371:531–9. doi: 10.1007/s00441-017-2785-7
62. Lacy P. Mechanisms of degranulation in neutrophils. *Aller Asthma Clin Immunol.* (2006) 2:98–108. doi: 10.1186/1710-1492-2-3-98
63. Ling MR, Chapple ILC, Matthews JB. Peripheral blood neutrophil cytokine hyper-reactivity in chronic periodontitis. *Innate Immun.* (2015) 21:714–25. doi: 10.1177/1753425915589387
64. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, et al. Netting Neutrophils Induce Endothelial Damage, Infiltrate Tissues, and Expose Immunostimulatory Molecules in Systemic Lupus Erythematosus. *J Immunol.* (2011) 187:538–552. doi: 10.4049/jimmunol.1100450
65. Babior BM. Oxidants from phagocytes: agents of defense and destruction. *Blood.* (1984) 64:959–66. doi: 10.1182/blood.V64.5.959.959
66. Klebanoff SJ. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J Bacteriol.* (1968) 95:2131–8. doi: 10.1128/JB.95.6.2131-2138.1968
67. Venketaraman V, Millman A, Salman M, Swaminathan S, Goetz M, Lardizabal A, et al. Glutathione levels and immune responses in tuberculosis patients. *Microb Pathog.* (2008) 44:255–61. doi: 10.1016/j.micpath.2007.09.002
68. Guerra C, Morris D, Sipin A, Kung S, Franklin M, Gray D, et al. Glutathione and adaptive immune responses against mycobacterium tuberculosis infection in healthy and HIV infected individuals. *PLoS ONE.* (2011) 6:e28378. doi: 10.1371/journal.pone.0028378
69. Dallenga T, Repnik U, Corleis B, Eich J, Reimer R, Griffiths GW, et al. *M. tuberculosis*-induced necrosis of infected neutrophils promotes bacterial growth following phagocytosis by macrophages. *Cell Host Microbe.* (2017) 22:519–30.e3. doi: 10.1016/j.chom.2017.09.003
70. Vernon PJ, Schaub LJ, Dallelucca JJ, Pusateri AE, Sheppard FR. Rapid detection of neutrophil oxidative burst capacity is predictive of whole blood cytokine responses. *PLoS ONE.* (2015) 10:1–13. doi: 10.1371/journal.pone.0146105
71. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Sciene.* (2004) 3033:1532–5. doi: 10.1126/science.1092385
72. Gupta S, Kaplan MJ. The role of neutrophils and NETosis in autoimmune and renal diseases. *Nat Rev Nephrol.* (2016) 12:402–13. doi: 10.1038/nrneph.2016.71
73. Kaplan JM. Neutrophil extracelullar traps (NETs): double-edged swords of innate immunity 1. *J Immunol.* (2013) 189:2689–95. doi: 10.4049/jimmunol.1201719
74. Branzk N, Lubojemska A, Hardison SE, Wang Q, Gutierrez MG, Brown GD, et al. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat Immunol.* (2014) 15:1017–25. doi: 10.1038/ni.2987
75. Schauer C, Janko C, Munoz LE, Zhao Y, Kienhöfer D, Frey B, et al. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat Med.* (2014) 20:511–7. doi: 10.1038/nm.3547
76. Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. *Annu Rev Pathol.* (2014) 9:181–218. doi: 10.1146/annurev-pathol-020712-164023
77. Ramos-Kichik V, Mondragón-Flores R, Mondragón-Castelán M, Gonzalez-Pozos S, Muñiz-Hernandez S, Rojas-Espinosa O, et al. Neutrophil extracellular traps are induced by *Mycobacterium tuberculosis*. *Tuberculosis.* (2009) 89:29–37. doi: 10.1016/j.tube.2008.09.009
78. von Both U, Berk M, Agapow PM, Wright JD, Git A, Hamilton MS, et al. *Mycobacterium tuberculosis* exploits a molecular off switch of the immune system for intracellular survival. *Sci Rep.* (2018) 8:1–17. doi: 10.1038/s41598-017-18528-y
79. Braian C, Hogea V, Stendahl O. *Mycobacterium tuberculosis*-induced neutrophil extracellular traps activate human macrophages. *J Innate Immun.* (2013) 5:591–602. doi: 10.1159/000348676
80. Narasaraju T, Yang E, Samy RP, Ng HH, Poh WP, Liew AA, et al. Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am J. Pathol.* (2011) 179:199–210. doi: 10.1016/j.ajpath.2011.03.013

81. Sugimoto MA, Vago JP, Teixeira MM, Sousa LP. Annexin A1 and the resolution of inflammation: modulation of neutrophil recruitment, apoptosis, and clearance. *J Immunol Res.* (2016) 2016:8239258. doi: 10.1155/2016/8239258
82. Pardo A, Cabrera S, Maldonado M, Selman M. Role of matrix metalloproteinases in the pathogenesis of idiopathic pulmonary fibrosis. *Respir Res.* (2016) 17:23. doi: 10.1186/s12931-016-0343-6
83. Pugin J, Widmer MC, Kossodo S, Liang CM, Preas HL, Suffredini AF. Human neutrophils secrete gelatinase B *in vitro* and *in vivo* in response to endotoxin and proinflammatory mediators. *Am J Respir Cell Mol Biol.* (1999) 20:458–64. doi: 10.1165/ajrcmb.20.3.3311
84. Delclaux C, Delacourt C, D'Ortho MP, Boyer V, Lafuma C, Harf A. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol.* (1996) 14:288–95. doi: 10.1165/ajrcmb.14.3.8845180
85. Ong CW, Elkington PT, Brilha S, Ugarte-Gil C, Tome-Esteban MT, Tezera LB, et al. Neutrophil-derived MMP-8 drives AMPK-dependent matrix destruction in human pulmonary tuberculosis. *PLoS Pathog.* (2015) 11:1–21. doi: 10.1371/journal.ppat.1004917
86. Elkington PTG, Friedland JS. Matrix metalloproteinases in destructive pulmonary pathology. *Thorax.* (2006) 61:259–66. doi: 10.1136/thx.2005.051979
87. Sathyamoorthy T, Tezera LB, Walker NF, Brilha S, Saraiva L, Mauri FA, et al. Membrane type 1 matrix metalloproteinase regulates monocyte migration and collagen destruction in tuberculosis. *J Immunol.* (2015) 195:882–91. doi: 10.4049/jimmunol.1403110
88. Walker NF, Wilkinson KA, Meintjes G, Tezera LB, Goliath R, Peyper JM, et al. Matrix degradation in human immunodeficiency virus type 1-associated tuberculosis and tuberculosis immune reconstitution inflammatory syndrome: a prospective observational study. *Clin Infect Dis.* (2017) 65:121–32. doi: 10.1093/cid/cix231
89. Sumner T, Scriba TJ, Penn-Nicholson A, Hatherill M, White RG. Potential population level impact on tuberculosis incidence of using an mRNA expression signature correlate-of-risk test to target tuberculosis preventive therapy. *Sci Rep.* (2019) 9:11126. doi: 10.1038/s41598-019-47645-z
90. Kolloli A, Subbian S. Host-directed therapeutic strategies for tuberculosis. *Front Med.* (2017) 4:171. doi: 10.3389/fmed.2017.00171
91. Ndlovu H, Marakalala MJ. Granulomas and inflammation: host-directed therapies for tuberculosis. *Front Immunol.* (2016) 7:434. doi: 10.3389/fimmu.2016.00434
92. Ahmed S, Raqib R, Guð*mundsson GH, Bergman P, Agerberth B, Rekha RS. Host-directed therapy as a novel treatment strategy to overcome tuberculosis: targeting immune modulation. *Antibiotics.* (2020) 9:1–19. doi: 10.3390/antibiotics9010021
93. Young C, Walzl G, Du Plessis N. Therapeutic host-directed strategies to improve outcome in tuberculosis. *Mucosal Immunol.* (2019) 13:190–204. doi: 10.1038/s41385-019-0226-5
94. Dallenga T, Linnemann L, Paudyal B, Repnik U, Griffiths G, Schaible UE. Targeting neutrophils for host-directed therapy to treat tuberculosis. *Int J Med Microbiol.* (2017) 308:142–7. doi: 10.1016/j.ijmm.2017.10.001
95. Mayer-Barber KD, Andrade BB, Oland SD, Amaral EP, Barber DL, Gonzales J, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature.* (2016) 5117:99–103. doi: 10.1038/nature 13489
96. Hu Z, Lowrie DB, Fan X-Y. Statins as adjunctive therapy against tuberculosis (TB): the balance between statin-induced anti-TB effect and trained immunity suppression, *J. Infect. Dis.* (2019) jiz675. doi: 10.1093/infdis/jiz675
97. Coussens A, Timms PM, Boucher BJ, Venton TR, Ashcroft AT, Skolimowska KH, et al. 1α,25-dihydroxyvitamin D3 inhibits matrix metalloproteinases induced by *Mycobacterium tuberculosis* infection. *Immunology.* (2009) 127:539–48. doi: 10.1111/j.1365-2567.2008.03024.x
98. Walker NF, Clark SO, Oni T, Andreu N, Tezera L, Singh S, et al. Doxycycline and HIV infection suppress tuberculosis-induced matrix metalloproteinases. *Am J Respir Crit Care Med.* (2012) 185:989–97. doi: 10.1164/rccm.201110-1769OC
99. Allen M, Bailey C, Cahatol I, Dodge L, Yim J, Kassissa C, et al. Mechanisms of control of *Mycobacterium tuberculosis* by NK cells: role of glutathione. *Front Immunol.* (2015) 6:1–9. doi: 10.3389/fimmu.2015.00508
100. Xu X, Jackson PL, Tanner S, Hardison MT, Roda MA, Blalock JE, et al. A self-propagating matrix metalloprotease-9 (MMP-9) dependent cycle of chronic neutrophilic inflammation. *PLoS ONE.* (2011) 6:1–12. doi: 10.1371/journal.pone.0015781
101. Overbeek SA, Henricks PA, Srienc AI, Koelink PJ, de Kruijf P, Lim HD, et al. N-acetylated Proline-Glycine-Proline induced G-protein dependent chemotaxis of neutrophils is independent of CXCL8 release. *Eur J Pharmacol.* (2011) 668:428–34. doi: 10.1016/j.ejphar.2011.03.022
102. Hill JW, Nemoto EM. Matrix-derived inflammatory mediator N-acetyl proline-glycine-proline is neurotoxic and upregulated in brain after ischemic stroke. *J Neuroinflammation.* (2015) 12:1–7. doi: 10.1186/s12974-015-0428-z
103. Gaggar A, Rowe SM, Hardision M, Blalock JE. Proline-glycine-proline (PGP) and high mobility group box protein-1 (HMGB1): potential mediators of cystic fibrosis airway inflammation. *Open Respir Med J.* (2010) 4:32–8. doi: 10.2174/1874306401004020032
104. Wang S, Song R, Wang Z, Jing Z, Wang S, Ma J. S100A8/A9 in inflammation. *Front Immunol.* (2018) 9:1298. doi: 10.3389/fimmu.2018.01298
105. Scott NR, Zuñiga J, Khader S. S100A8/A9 regulates CD11b expression and neutrophil recruitment during chronic tuberculosis. *J Clin Invest.* (2020). doi: 10.1172/JCI130546. [Epub ahead of print].

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix B: Ethical Approval

C/o MRC Unit: The Gambia @ LSHTM, Fajara
P.O. Box 273, Banjul
The Gambia, West Africa
Fax: +220 – 4495919 or 4495513
Tel: +220 – 4495442-6 Ext. 2308
Email: ethics@mrc.gm

The Gambia Government/MRCG Joint
ETHICS COMMITTEE

7 January 2019

Mr. Caleb Nwongbouwoh Muefong
MRCG at LSHTM
Fajara

Dear Mr. Muefong,

SCC 1657, Describing Neutrophil Function In TB-induced Pulmonary Morbidity And Chronic Lung Pathology Following Therapy

Thank you for submitting your proposal dated 4 December 2018 for consideration by the Gambia Government/MRCG Joint Ethics Committee at its meeting held on 18 December 2018.

Our Committee is pleased to approve your proposed study.

With best wishes,

Yours sincerely,

Dr. Mohammadou Kabir Cham
Chair, Gambia Government/MRCG Joint Ethics Committee

Documents submitted for review:

- SCC approval letter – 12 December 2018
- Response letter – 4 December 2018
- SCC application form, version 1.1 – 4 December 2018
- ICD (SCC 1523), version 2.0 – 27 March 2018
- CV – Christof Geldmacher

Acknowledgements

I am heartily thankful to my supervisors, Prof. Dr. Jayne Sutherland and PD Dr. rer. nat. Christof Geldmacher, whose trust, support and guidance enabled me to grow as a scientist and as a person through every step of my research work. Thanks for giving me the opportunity to walk with you on this journey and for being absolutely great mentors. Your words of encouragement and support gave me confidence to keep on pushing for the end goal.

I would also like to express my gratitude to my friends/colleagues from MRCG at LSHTM, LMU, Germany and abroad for providing me with encouragements to strive through the challenges that came my way. The welcoming and friendly environment that you provided became my home and I hope your hard work, expertise and great personalities are reflected in this thesis.

I am also thankful to TB Sequel and the German Ministry for Education and Research (BMBF) for providing the funds for this study and for my studentship. Similarly, I am grateful to all the patients and their families who volunteered for this study and to The MRC Unit The Gambia at LSHTM for facilitating access to laboratory, clinical and other facilities.

A special thanks to my father (Capt. Mongkuo Salomon), my mother (Mrs Mongkuo Rebecca) and my siblings for their unconditional love, patience and loving support throughout the years. Beyond family; you have been my friends throughout this lifechanging journey.

My sincerest thanks and blessings are for Metang Cannelle with whom I started on this once-in-a-lifetime journey; her gentleness and optimism in the face of life's challenges were, once, a great source of motivation. Also, to all who never ceased to believe in me throughout the completion of this Doctoral thesis, I wish you peace, life and love.

Lastly, I thank my God and faithful companion for being my flashlight through every single moment. Words cannot express how much your presence made and continue to make the overwhelming difference in my life.