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

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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].

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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

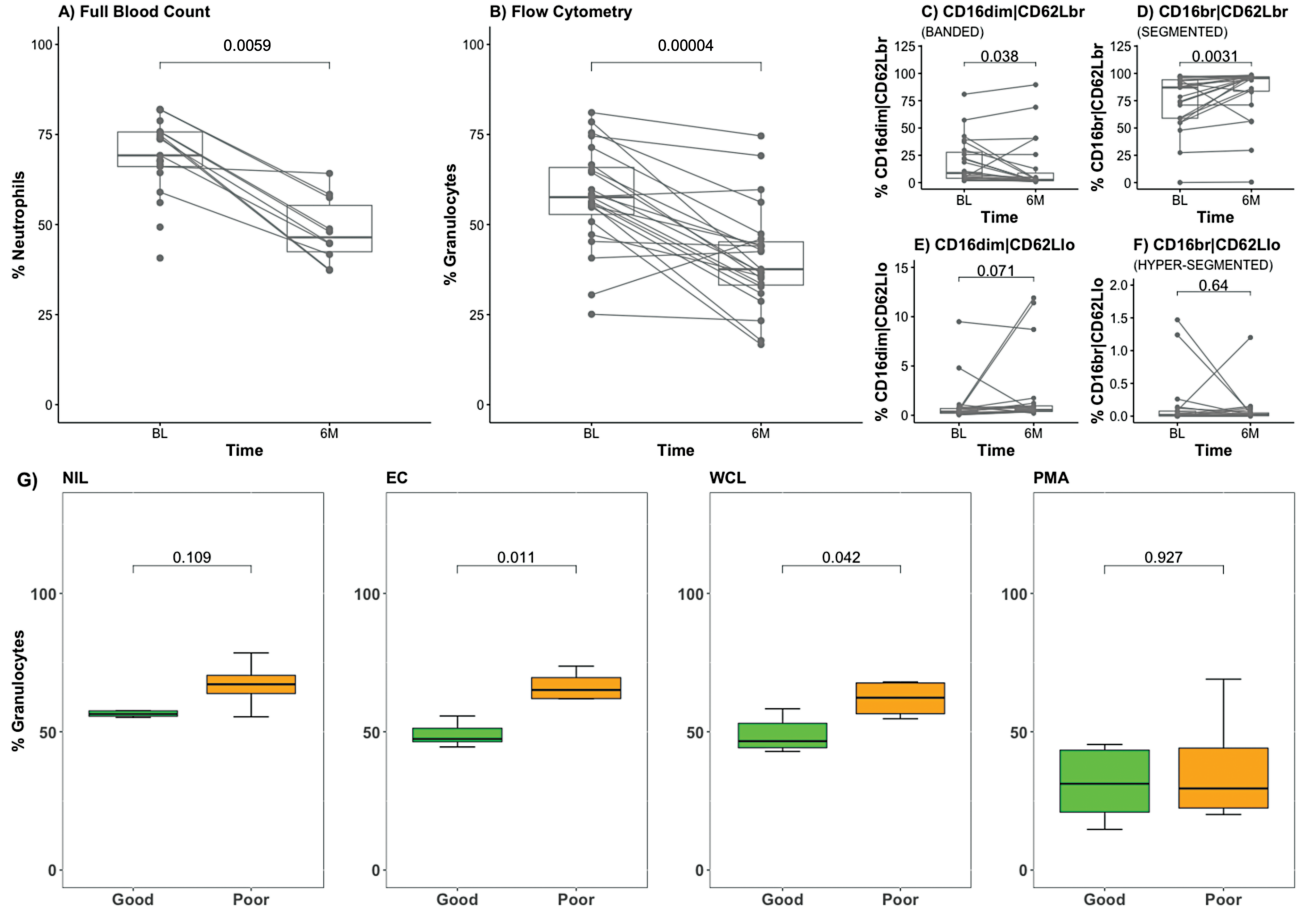

Figure 1. Longitudinal measurement of percentage blood neutrophil during antituberculosis treatment. *A*, Boxplots show median (horizontal line in box) and interquartile range of frequencies of granulocytes before and at treatment completion. The neutrophil counts in active TB patients were measured 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).

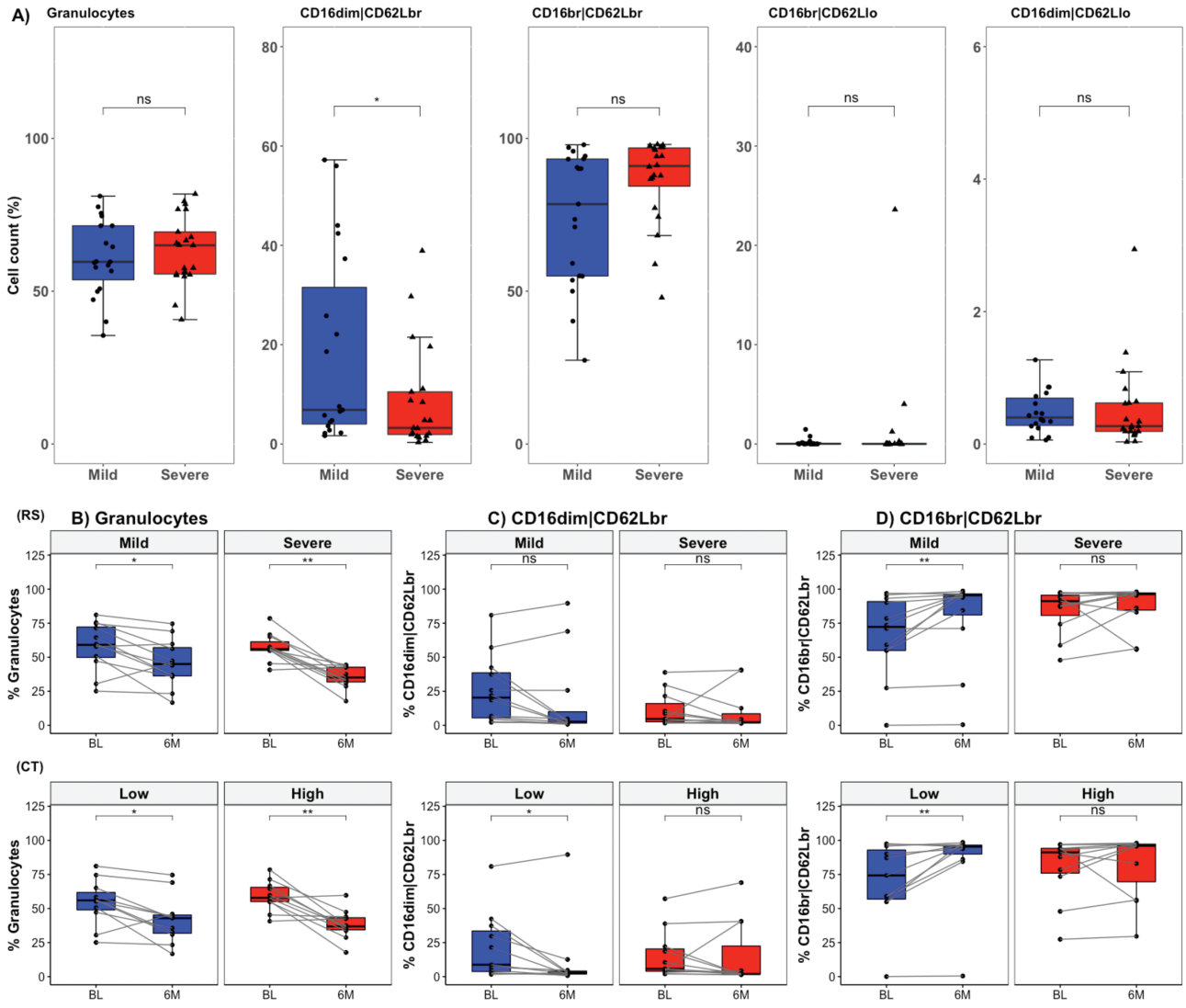

Figure 2. Percentage of neutrophils in severity groups before and after treatment. *A*, CD16/CD62L-defined neutrophil frequencies in active TB patients at baseline were analyzed by flow cytometry within Ralph score–defined mild (n = 21, O) and severe (n = 21, Δ) lung disease groups. Banded ($CD16^{dim}CD62L^{br}$) 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),

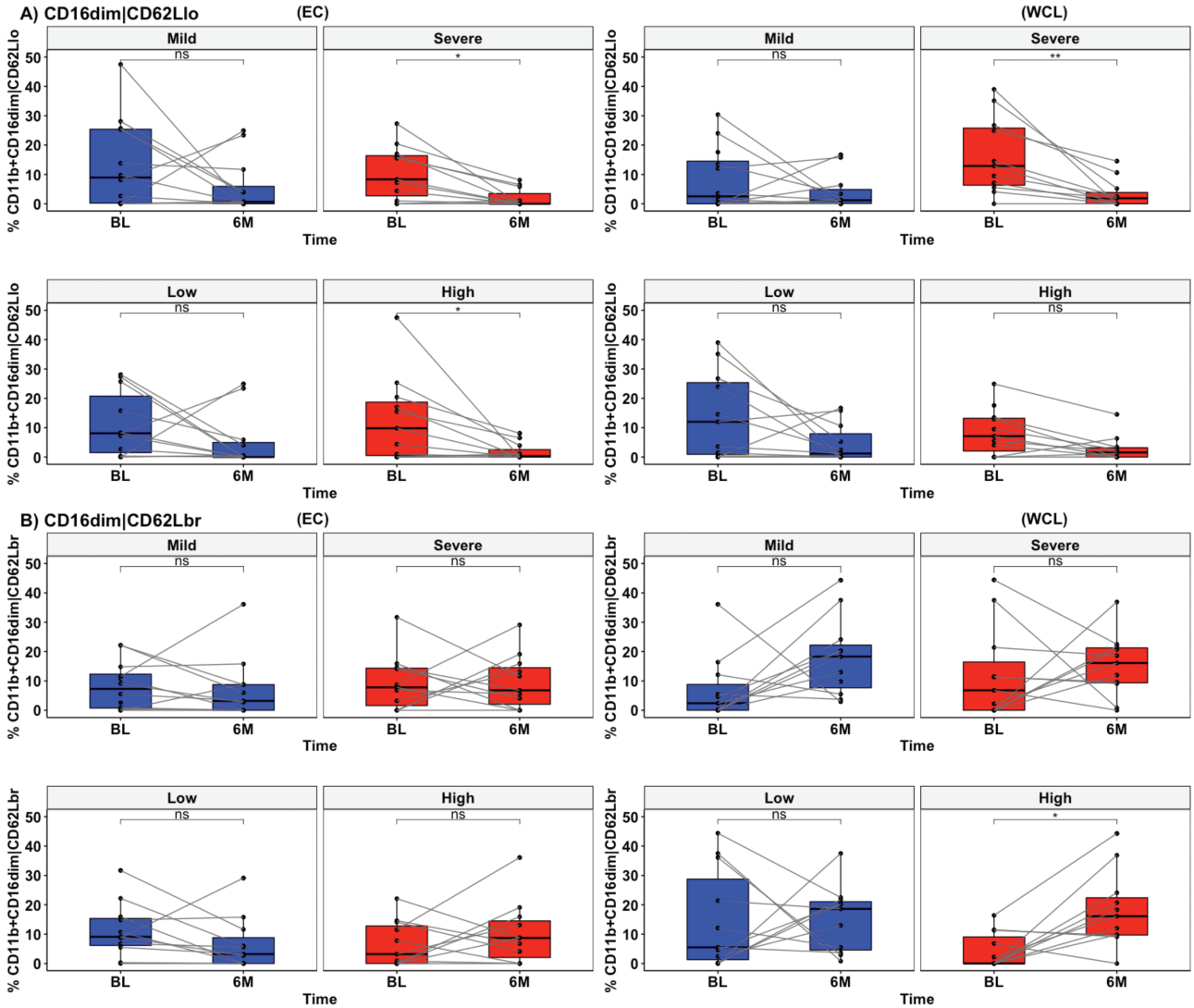

Figure 3. CD11b expression by neutrophils before and after treatment. *A*, Patients with severe lung damage (top panel, n = 11) or high *Mycobacterium tuberculosis* (*Mtb*) load (n = 11, middle panel) showed higher frequencies of $CD11b^{+}CD16^{dim}CD62L^{lo}$ neutrophils upon 2-hour stimulation with *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

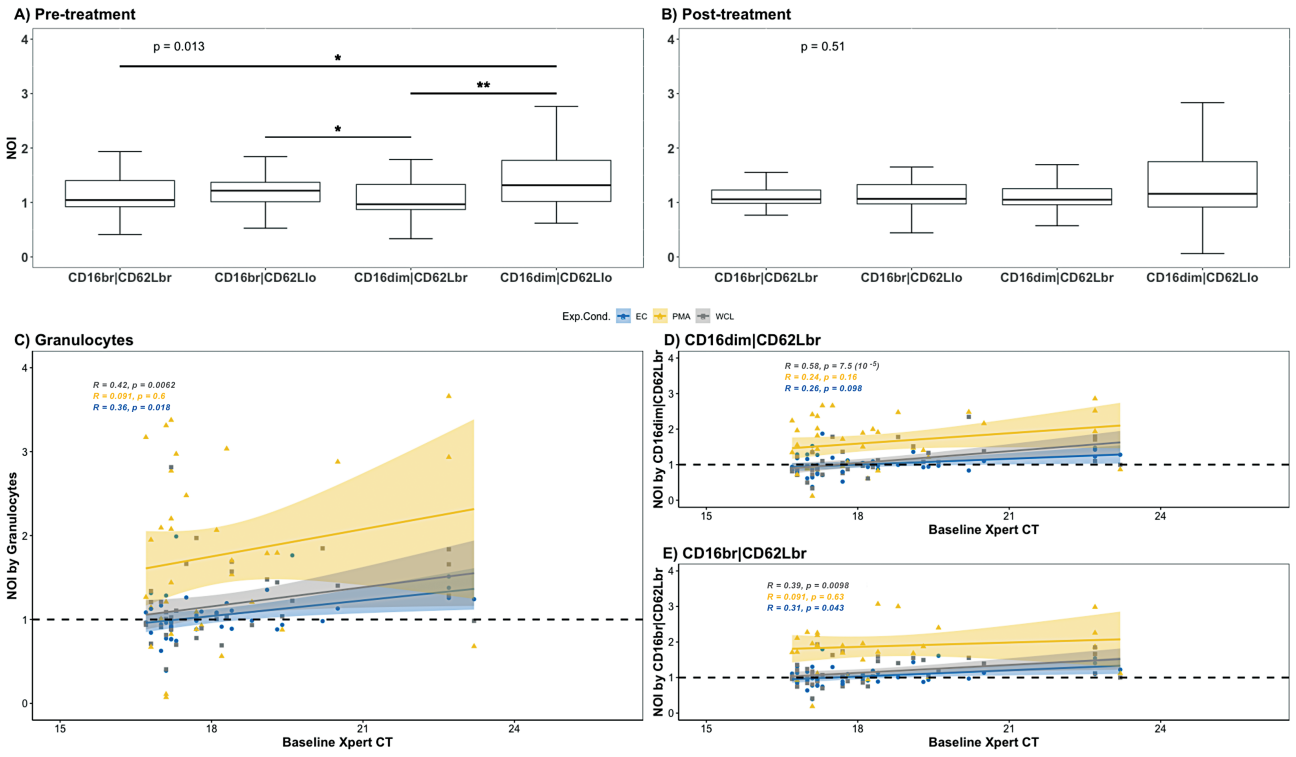

Figure 4. Neutrophil oxidative indices (NOIs) of neutrophil subsets. *A*, Pretreatment: low CD62L-expressing subsets $CD16^{dim}CD62L^{lo}$ (n = 42) and $CD16^{br}CD62L^{lo}$ (hypersegmented neutrophils, n = 27) produced more reactive oxygen species (ROS) than $CD16^{dim}CD62L^{br}$ (banded, n = 42) 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

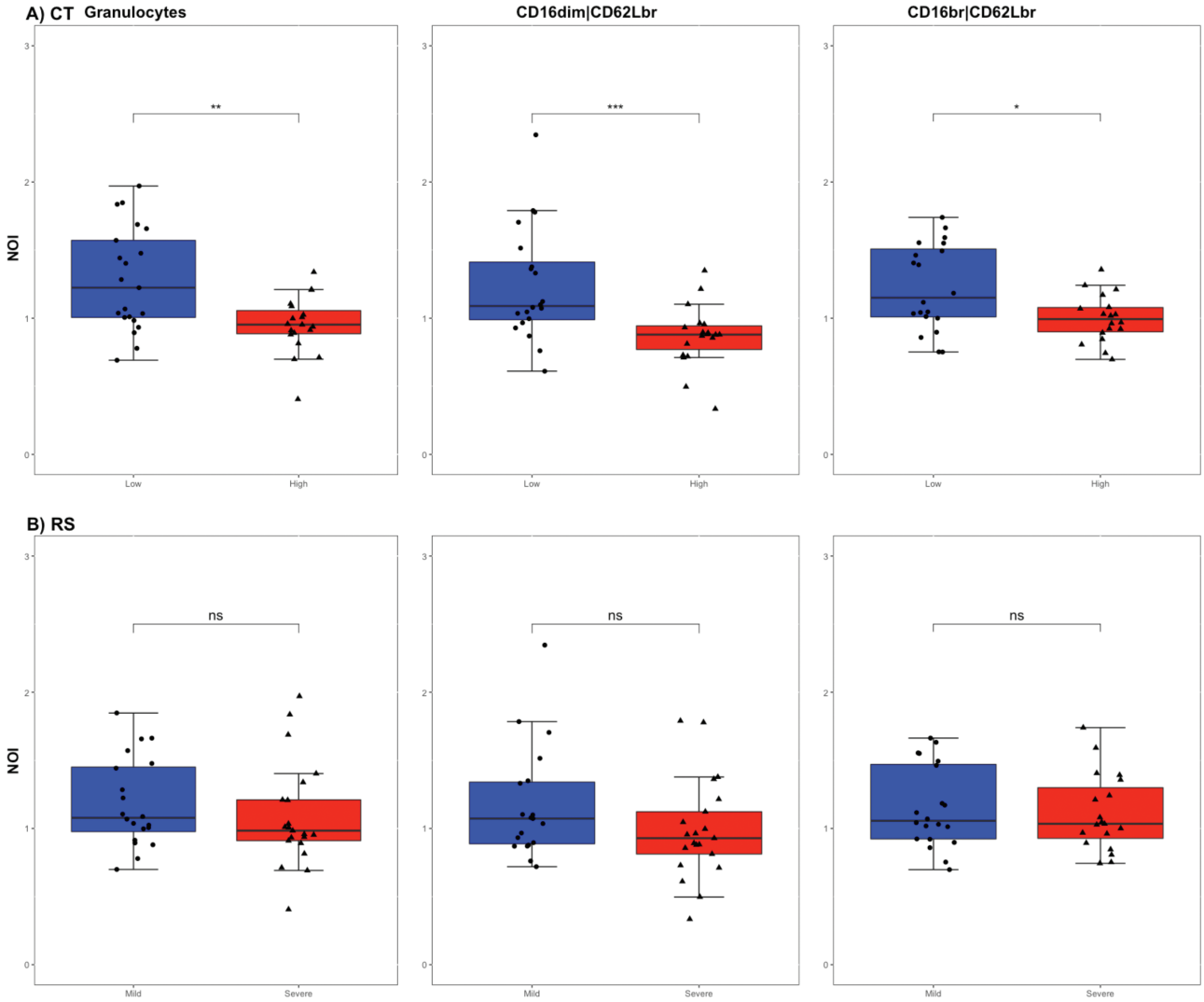

Figure 5. Neutrophil oxidative indices (NOIs) of neutrophil subsets in cycle threshold value–defined severity groups at diagnosis. *A*, At baseline, granulocytes, $CD16^{dim}CD62L^{br}$ neutrophils (banded), and $CD16^{br}CD62L^{br}$ neutrophils (segmented) had higher NOIs following 2-hour whole cell 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.

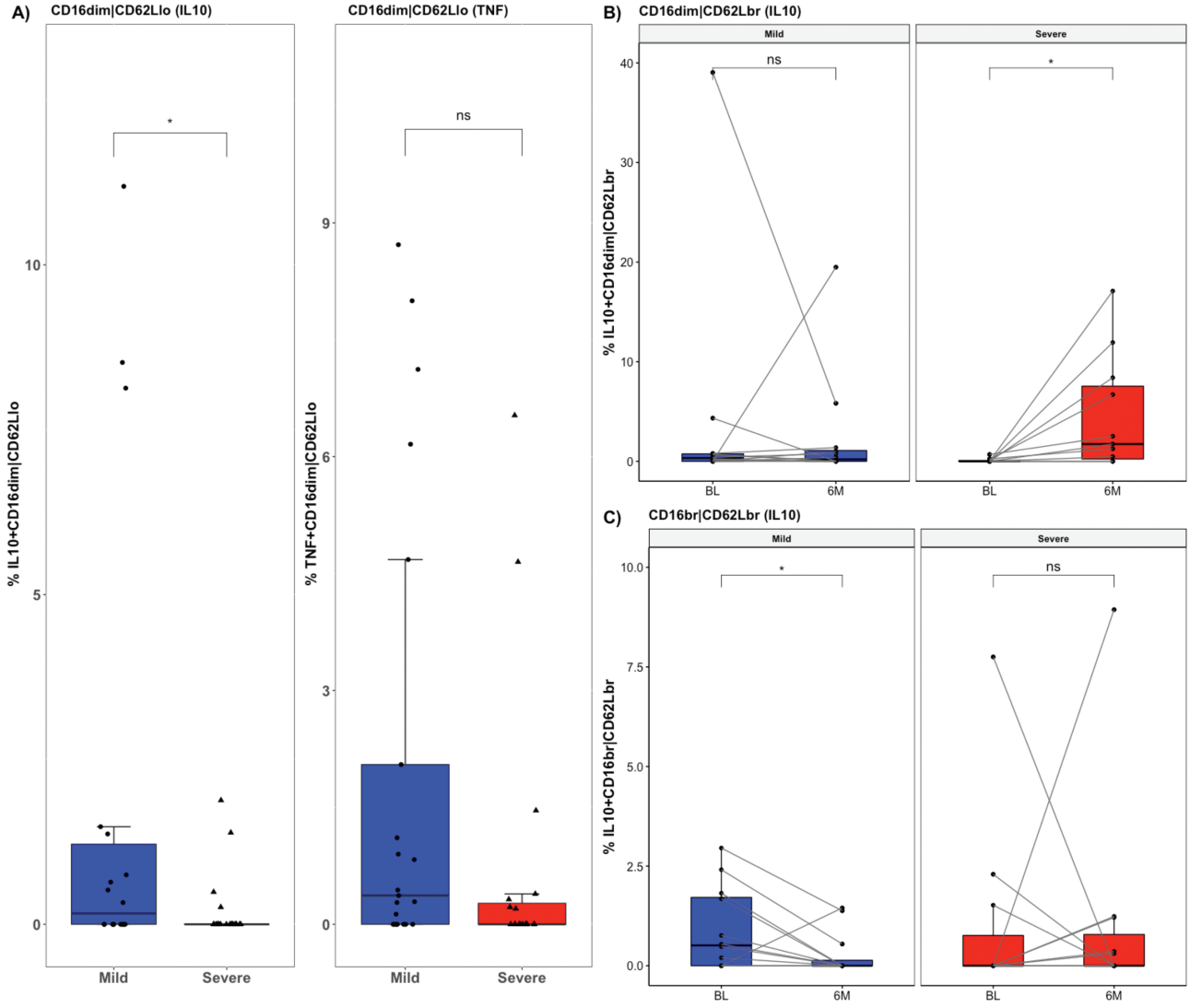

Figure 6. Frequency of interleukin 10 (IL-10)$^{+}$ neutrophils pretreatment following whole cell lysate (WCL) stimulation. *A*, Following 2-hour stimulation with WCL, the baseline (BL) frequencies of IL-10–expressing CD16^{dim}CD62L^{lo} neutrophils were higher in patients with mild damage (n = 19) 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.

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