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HOST RESPONSE AND INFLAMMATION

Heightened Systemic Levels of Neutrophil and Eosinophil Granular Proteins in Pulmonary Tuberculosis and Reversal following Treatment

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ABSTRACT Granulocytes are activated during *Mycobacterium tuberculosis* infection and act as immune effector cells, and granulocyte responses are implicated in tuberculosis (TB) pathogenesis. Plasma levels of neutrophil and eosinophil granular proteins provide an indirect measure of degranulation. In this study, we wanted to examine the levels of neutrophil and eosinophil granular proteins in individuals with pulmonary tuberculosis (PTB) and to compare them with the levels in individuals with latent TB (LTB). Hence, we measured the plasma levels of myeloperoxidase (MPO), neutrophil elastase, proteinase 3, major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPX) in these individuals. Finally, we also measured the levels of all of these proteins in PTB individuals following antituberculosis treatment (ATT). Our data reveal that PTB individuals are characterized by significantly higher plasma levels of MPO, elastase, proteinase 3, as well as MBP and EDN in comparison to those in LTB individuals. Our data also reveal that ATT resulted in the reversal of all of these changes, indicating an association with TB disease. Finally, our data show that the systemic levels of MPO and proteinase 3 can significantly discriminate PTB from LTB individuals. Thus, our data suggest that neutrophil and eosinophil granular proteins could play a potential role in the innate immune response and, therefore, the pathogenesis of pulmonary TB.

KEYWORDS neutrophils, tuberculosis, eosinophils, granular proteins

Granulocyte activation is a hallmark of tuberculosis (TB), and neutrophils are thought to play an important role in *Mycobacterium tuberculosis* infection and disease. Neutrophils are the first cells to infiltrate the lungs after *M. tuberculosis* infection and are the predominant cell type present in the lungs of active pulmonary tuberculosis (PTB) patients (1). Their recruitment to the lung is regulated by various cytokines and chemokines (2, 3), alarmins (such as S100A8/A9 proteins), and intrinsically expressed microRNAs (such as microRNA-223) (4, 5). Neutrophils are efficient at phagocytosis and can engulf and kill microorganisms by producing reactive oxygen intermediates in phagolysosomes. In addition, the factors released by neutrophils during the respiratory burst, such as elastase, collagenase, and myeloperoxidase (MPO), indiscriminately damage bacterial and host cells. Pulmonary TB is typically characterized by peripheral neutrophilia (6) and is associated with elevated levels of neutrophil extracellular traps (7, 8). In contrast, neutrophil numbers and signatures exhibit a positive correlation with active disease in humans (6) and are known to contribute to

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TABLE 1 Demographics of the study population

Characteristic	Value(s) for the following group:	
	PTB	LTB
No. of subjects	44	44
No. of males/no. of females	29/15	27/17
Median (range) age (yr)	41 (24–67)	44 (25–65)
Median (range) ht (cm)	159 (133–173)	164 (140–184)
Median (range) wt (kg)	45 (30–81)	49 (32–90)

lung pathology in animal models (9, 10). Thus, neutrophils constitute a potent population of effector cells that can mediate both antimycobacterial activity and immunopathology during *M. tuberculosis* infection (11). However, the role of neutrophil granular proteins in human TB is not well studied.

Eosinophilia is typically a hallmark of parasitic infections (12). Eosinophils have bilobed nuclei, and eosinophil-specific toxic proteins are stored in granules. These are eosinophil cationic protein (ECP), eosinophil peroxidase (EPX), eosinophil-derived neurotoxin (EDN), and major basic protein (MBP) (13). ECP, EPX, and MBP are potent pathogen toxins, and MBP can induce histamine release from mast cells, while both EDN and ECP can act as ribonucleases (14, 15). However, the role of eosinophils in *M. tuberculosis* infection or disease has been poorly studied, although the rapid influx of eosinophils and subsequent degranulation have been described in the guinea pig model of *M. tuberculosis* infection (16).

In this study, we wanted to determine the role of neutrophil and eosinophil granular proteins in pulmonary TB (PTB). To this end, we measured the plasma levels of neutrophil granular proteins (elastase, MPO, and proteinase 3) and eosinophil granular proteins (ECP, EDN, EPX, and MBP) in individuals with PTB or latent TB (LTB). Our data suggest that neutrophil and eosinophil granular proteins are present at enhanced levels in individuals with PTB and that this is reversed following treatment.

RESULTS

Study population characteristics. The demographic features of the study population are shown in Table 1. No significant differences in age, sex, height, or weight were observed between PTB and LTB individuals. In addition, the hematological parameters of the study groups are shown in Fig. 1. As shown, PTB individuals exhibited significantly higher numbers of platelets and neutrophils and significantly lower numbers of red blood cells and lymphocytes than LTB individuals.

PTB is associated with enhanced levels of MPO, elastase, and proteinase 3 and reversal following ATT. To determine the role of neutrophil granular proteins in PTB, we measured the plasma levels of MPO, elastase, and proteinase 3 in PTB and LTB individuals. As shown in Fig. 2A, PTB individuals had significantly higher levels of MPO (geometric mean [GM], 10,365 pg/ml in PTB individuals versus 6,651 pg/ml in LTB individuals), elastase (GM, 12.02 pg/ml in PTB individuals versus 9.3 pg/ml in LTB individuals), and proteinase 3 (GM, 13,917 pg/ml in PTB individuals versus 3,460 pg/ml in LTB individuals) than LTB individuals. To determine the effect of antituberculosis treatment (ATT) on the systemic levels of neutrophil granular proteins in PTB, we measured the levels of neutrophil granular proteins in PTB individuals before and after treatment. As shown in Fig. 2B, the systemic levels of MPO (GM, 10,365 pg/ml pretreatment versus 5,821 pg/ml posttreatment), elastase (GM, 12.02 pg/ml pretreatment versus 6.9 pg/ml posttreatment), and myeloperoxidase (GM, 13,917 pg/ml pretreatment versus 3,219 pg/ml posttreatment) at 6 months of ATT (posttreatment) were significantly decreased from the pretreatment levels. Next, the relationship between absolute neutrophil counts and the levels of the neutrophil granular proteins MPO, elastase, and proteinase 3 was assessed. As shown in Fig. 2C, the absolute count of neutrophils exhibited a modest, albeit significant, negative correlation with the levels of proteinase 3 ($r = -0.2318$; $P = 0.0328$) but did not exhibit any significant relationship with the

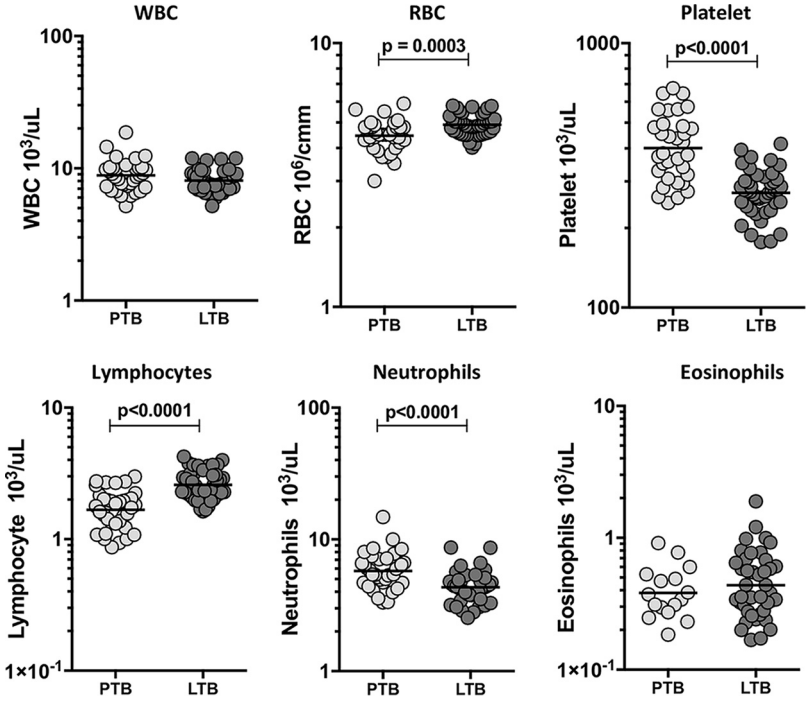

FIG 1 PTB is associated with elevated platelet and neutrophil counts. The absolute counts of white blood cells (WBCs), red blood cells (RBCs), platelets, lymphocytes, neutrophils, and eosinophils in PTB and LTB individuals were determined by use of a hematology analyzer. Data are shown as scatter plots, with the bars representing the geometric means. *P* values were calculated using the Mann-Whitney U test.

levels of MPO or elastase in all individuals. Finally, to determine the discriminatory power of neutrophil granular proteins in differentiating PTB from LTB, we performed receiver operator characteristic (ROC) curve analysis of the MPO, elastase, and proteinase 3 levels in PTB versus LTB individuals. As shown in Fig. 2D, MPO, elastase, and proteinase 3 exhibited a significant area under the curve (AUC), sensitivity, and specificity in discriminating PTB from LTB individuals.

PTB is associated with enhanced levels of MBP and EDN and reversal following ATT. To determine the levels of eosinophil granular proteins in PTB, we measured the plasma levels of MBP, EDN, ECP, and EPX in PTB and LTB individuals. As shown in Fig. 3A, PTB had significantly higher levels of MBP (GM, 6.08 ng/ml in PTB individuals versus 2.3 ng/ml in LTB individuals) and EDN (GM, 3.4 ng/ml in PTB individuals versus 2.5 ng/ml in LTB individuals) than LTB individuals. To determine the effect of ATT on the systemic levels of eosinophil granular proteins in PTB individuals, we measured the levels of eosinophil granular proteins in PTB individuals before and after treatment. As shown in Fig. 3B, the systemic levels of MBP (GM, 6.08 ng/ml pretreatment versus 2.09 ng/ml posttreatment), EDN (GM, 3.4 ng/ml pretreatment versus 2.4 ng/ml posttreatment), ECP (GM, 5.6 ng/ml pretreatment versus 3.4 ng/ml posttreatment), and EPX (GM, 4.5 ng/ml pretreatment versus 3.6 ng/ml posttreatment) at 6 months of ATT (posttreatment) were significantly decreased from the pretreatment levels. Next, the relationship between absolute eosinophil counts and eosinophil granular protein levels were assessed. As shown in Fig. 3C, the absolute eosinophil counts exhibited a modest, albeit significant, positive correlation with the levels of MBP ($r = 0.3217$; $P = 0.0024$) and EDN ($r = 0.2628$; $P = 0.0134$) but not the levels of ECP or EPX in all individuals. Finally, to determine the discriminatory power of neutrophil granular proteins to differentiate PTB from LTB, we performed ROC analysis of the MBP, EDN, ECP, and EPX levels in PTB versus LTB individuals. As shown in Fig. 3D, none of the eosinophil granular proteins exhibited a significant AUC, sensitivity, or specificity in discriminating PTB from LTB individuals.

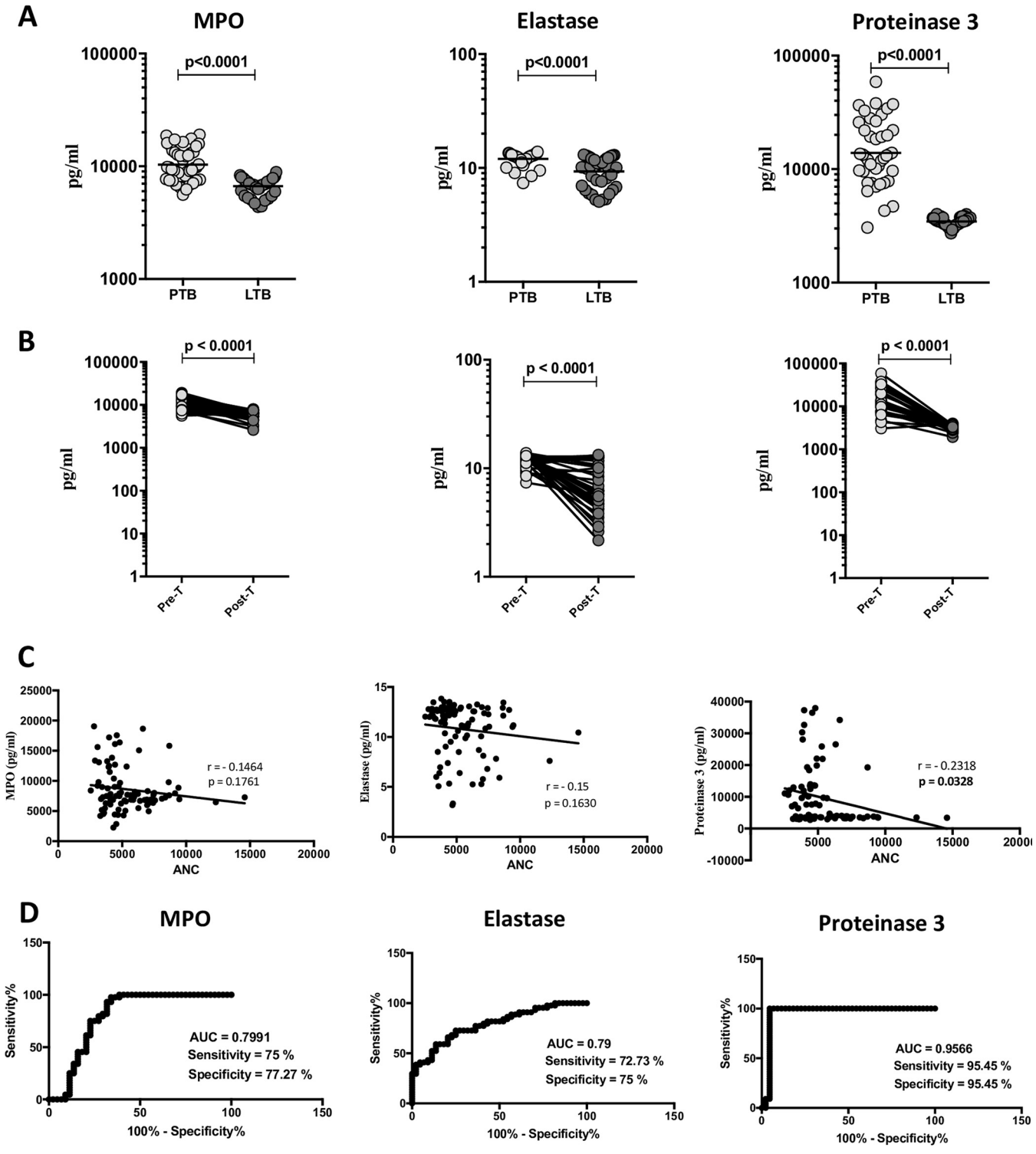

FIG 2 PTB is associated with enhanced levels of neutrophil granular proteins and reversal following ATT. (A) The plasma levels of myeloperoxidase (MPO), neutrophil elastase, and proteinase 3 from PTB ($n = 44$) and LTB ($n = 44$) individuals were measured by ELISA. Data are shown as scatter plots, with the bars representing the geometric means. *P* values were calculated using the Mann-Whitney U test. (B) The plasma levels of MPO, elastase, and proteinase 3 from PTB individuals at the baseline (pretreatment [pre-T]) and following 6 months of ATT (posttreatment [post-T]) were measured by ELISA. *P* values were calculated using the Wilcoxon matched-pair test. (C) Absolute neutrophil counts (ANC) were correlated with the plasma levels of MPO, elastase, and proteinase 3 in all individuals. *P* and *r* values were calculated using the Spearman rank correlation test at the 95% confidence intervals. (D) ROC analysis to estimate the sensitivity, specificity, and area under the curve was performed using neutrophil granular proteins to estimate the capacity of these factors to distinguish PTB individuals from LTB individuals.

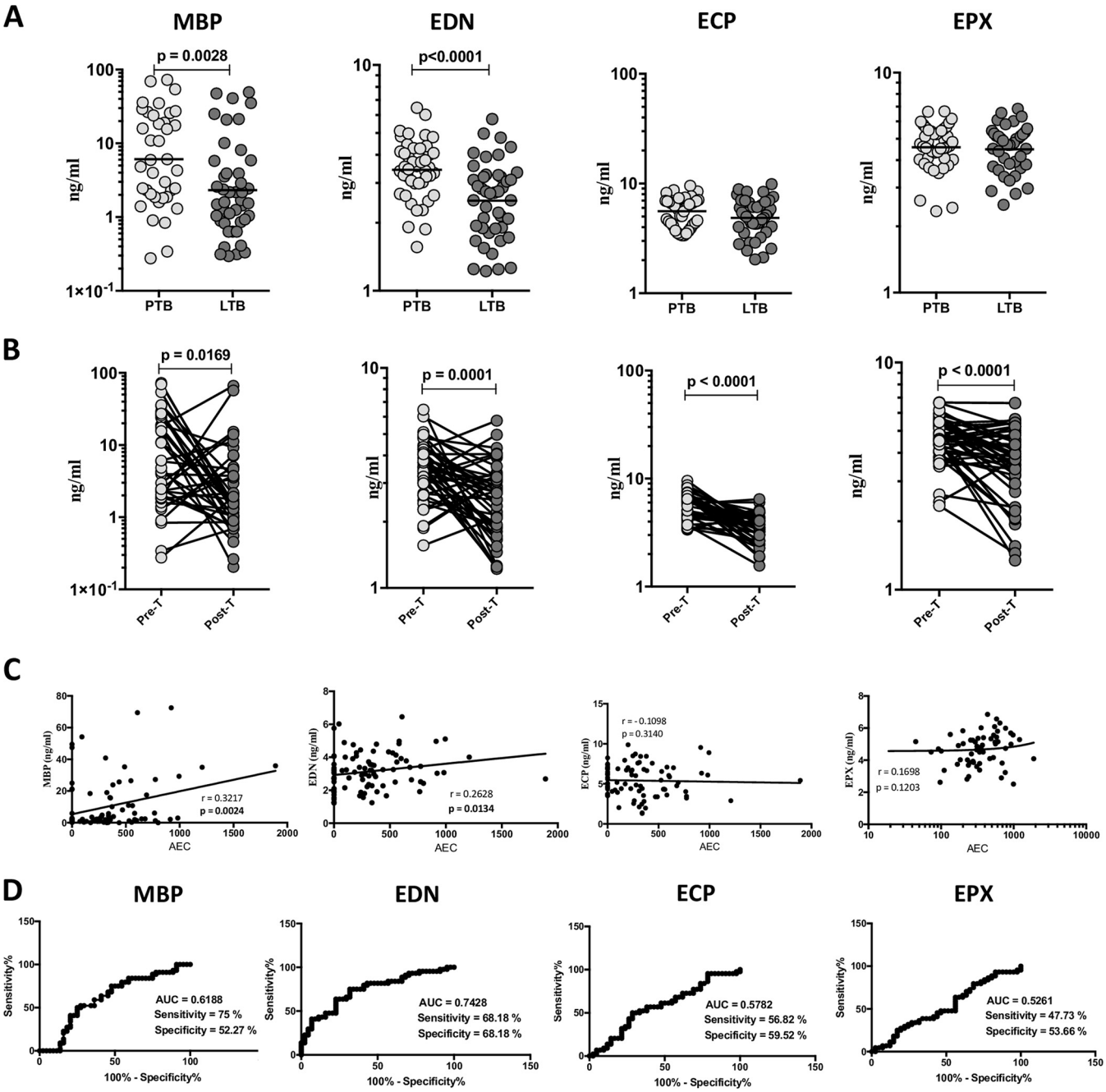

FIG 3 PTB is associated with enhanced levels of eosinophil granular proteins and reversal following ATT. (A) The plasma levels of major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPX) from PTB and LTB individuals were measured by ELISA. Data are shown as scatter plots, with the bars representing the geometric means. *P* values were calculated using the Mann-Whitney U test. (B) The plasma levels of MBP, EDN, ECP, and EPX from PTB individuals at the baseline (pretreatment [pre-T]) and following 6 months of ATT (posttreatment [post-T]) were measured by ELISA. *P* values were calculated using the Wilcoxon matched-pair test. (C) Absolute eosinophil counts (AEC) was correlated with the plasma levels of MBP, EDN, ECP, and EPX in all individuals. *P* and *r* values were calculated using the Spearman rank correlation test at the 95% confidence interval. (D) ROC analysis to estimate the sensitivity, specificity, and area under the curve was performed using eosinophil granular proteins to estimate the capacity of these factors to distinguish PTB individuals from LTB individuals.

DISCUSSION

Neutrophils are involved in the activation, regulation, and effector functions of innate and adaptive immune cells (17). Neutrophils have been implicated in both host resistance mechanisms and the promotion of pathology in *M. tuberculosis* infection and disease (18, 19). Neutrophilia is typically a hallmark of TB disease (18, 20). Our data reconfirm these findings by showing an increase in the absolute counts of neutrophils

in PTB individuals compared to LTB individuals. Neutrophils can phagocytose bacteria and subsequently kill them via various processes, including degranulation (11). However, more recent reports indicate that human neutrophils fail to kill *M. tuberculosis* after phagocytosis and instead undergo necrotic cell death, which contributes to pathology (21, 22). Neutrophil granular products include MPO, elastase, and proteinase 3. Neutrophil elastase is known to contribute to host-protective immunity against mycobacterial infections in mice (23). Neutrophil elastase and myeloperoxidase levels are elevated in TB pleural effusions (24). Furthermore, these proteins form an important component of neutrophil extracellular traps, which are involved in host defense against TB (25). Our study is novel in that it examines the association of these granular proteins with PTB before and after treatment. Our data clearly reveal a major elevation in the levels of MPO, elastase, and proteinase 3 in the PTB group. This indicates heightened neutrophil activity in PTB individuals. Whether this contributes to increased immune-mediated pathology in PTB remains to be determined. Of additional interest, ATT significantly reverses this enhanced induction of neutrophil granular proteins in the PTB group. This suggests that the presence of bacteria in the lungs clearly modulates the systemic levels of neutrophil granular proteins. Finally, the levels of these proteins did not exhibit any significant association (or only a mild association) with absolute neutrophil counts. Our interpretation of these data suggests that the neutrophil granular proteins are perhaps of greater importance at the site of infection, and therefore, future studies examining the local levels of these markers either in sputum or in bronchoalveolar lavage fluid would reveal important insights. Finally, our study also delineates the utility of neutrophil granular proteins as biomarkers distinguishing PTB from LTB, with MPO and proteinase 3 serving as the main factors of distinction. These could be assessed in future studies for their utility in discriminating PTB from other pulmonary diseases.

Eosinophils are one of the major components of the immune system responsible for combating infections, specifically, parasitic infections (26). More recently, eosinophils have been shown to play an important role in metabolism. However, the role of eosinophils in *M. tuberculosis* infection or disease has not been fully explored. Eosinophils carry toxic granules, which contain MBP, ECP, EDN, and EPO, which are directly toxic to a variety of pathogens (13). The plasma levels of eosinophil granule proteins provide an indirect measure of degranulation in the tissues and are markedly increased in many parasitic infections (12). Our study revealed a significant elevation in the levels of MBP and EDN in the PTB group. In addition, our results also revealed a significant decrease in the levels of all the eosinophil granular proteins following ATT. Moreover, there was a moderate but significant correlation between the levels of MBP and EDN and the absolute eosinophil counts. Thus, eosinophil granular proteins do appear to play an important role in the innate immune response to PTB. While the presence of allergies or helminths can also influence the levels of these proteins, we do not have any reason to assume an increased prevalence of either condition in the PTB group compared to the LTB group. Also, the changes following ATT suggest that bacterial loads can significantly influence the systemic levels of eosinophil granular proteins. This clearly suggests an important effect of TB disease directly on eosinophil granular protein upregulation. Our study has certain weaknesses, including being descriptive and describing an association and not a cause and effect. Moreover, we did not include a healthy control group in our analysis. In addition, it is possible that the PTB individuals had secondary coinfections that could have contributed to the results.

Our study provides new insights into the regulation of neutrophil and eosinophil granular proteins in TB disease. Our study adds to the growing body of literature showing the importance of neutrophils and neutrophil granular proteins in the innate immune response to TB. Our study also provides novel data on the role of eosinophils and eosinophil granular proteins in *M. tuberculosis* infection and disease. Finally, our study also provides insight into the regulation of these molecules following antituberculosis treatment of PTB. Moreover, our study derives strength from the fairly large sample size and the homogeneity of the population studied. Further studies exploring

the exact role of these granular proteins should provide valuable insight into the regulation of the protective or pathogenic immune response in *M. tuberculosis* infections at large.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Internal Ethics Committees of NIRT. Informed written consent was obtained from all participants.

Study population. Plasma samples were collected from 88 individuals: 44 with PTB and 44 with LTB. The diagnosis of PTB was based on smear and culture positivity for *M. tuberculosis*. At the time of enrollment, all active TB cases had no record of prior TB disease or anti-TB treatment (ATT). All individuals had been vaccinated with *M. bovis* BCG, were HIV negative, and had a normal body mass index. The study groups were similar with regard to age and gender, and the baseline characteristics of the study participants are shown in Table 1. Standard antituberculosis treatment (ATT) was administered to PTB individuals using the directly observed treatment, short course (DOTS), strategy. At 6 months following ATT initiation, fresh plasma samples were obtained. All PTB individuals were culture negative at the end of ATT. LTB diagnosis was based on the tuberculin skin test (TST) and QuantiFERON TB-Gold in tube enzyme-linked immunosorbent assay (ELISA) positivity, the absence of chest radiograph abnormalities or pulmonary symptoms, and a negative sputum smear. A positive TST result was defined as an induration of at least 12 mm in diameter to minimize false positivity due to exposure to environmental mycobacteria. Only individuals positive by both TST and the QuantiFERON TB Gold in-tube ELISA were considered to have LTB.

Plasma processing. Venous blood (10 ml) was collected in lithium heparin tubes. Plasma was separated by centrifugation at 3,000 × *g* for 10 min at 4°C, aliquoted, and stored at −80°C until required.

Measurement of hematological parameters. The hematological parameters in the two groups were measured at baseline using an AcT 5 Diff hematology analyzer.

Measurement of neutrophil granular proteins. The plasma levels of myeloperoxidase (MPO) and proteinase 3 (R&D Systems, Minneapolis, MN, USA) and elastase (Cell Sciences Hycult Biotech, Canton, MA, USA) were measured using ELISA kits, according to the manufacturers' instructions.

Measurement of eosinophil granular proteins. The plasma levels of ECP, EDN, EPX, and MBP were measured using MyBioSource ELISA kits (MyBioSource, Inc., San Diego, CA, USA), according to the manufacturer's instructions.

Statistical analysis. Data analyses were performed using GraphPad Prism (version 6) software (GraphPad Software, Inc., San Diego, CA, USA). Geometric means (GM) were used for measurements of central tendency. Statistically significant differences were analyzed using either the Mann-Whitney U test or the Wilcoxon matched-pair test. Correlations were calculated by the Spearman rank correlation test. Receiver operator characteristic (ROC) curves were designed to test the power of each candidate granular protein to distinguish PTB from LTB.

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