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The predominance of Th1 Immune Response in Pleural Effusion of Patients with Tuberculosis Among Other Exudative Etiologies

Running title: **Inflammatory mediators in pleural tuberculosis**

Vinícius da Cunha Lisboa[1], Marcelo Ribeiro-Alves[2], Raquel da Silva Corrêa[1], Isabelle Ramos Lopes[1], Thiago Thomaz Mafort[3], Ana Paula Gomes dos Santos[3], Thaís Porto Amadeu[1], Rogério Rufino[3], Luciana Silva Rodrigues[1*]

[1] Laboratory of Immunopathology, Medical Science Faculty, Rio de Janeiro State University (UERJ), Rio de Janeiro, RJ, Brazil; [2] Laboratory of Clinical Research on STD/AIDS, National Institute of Infectology Evandro Chagas, Oswaldo Cruz Institute (FIOCRUZ), Rio de Janeiro, RJ, Brazil; [3] Department of Pulmonary and Tisiology Care, Pedro Ernesto University Hospital (HUPE)/UERJ, Rio de Janeiro, RJ, Brazil.

[*] Corresponding author
E-mail: lrodrigues.uerj@gmail.com

ABSTRACT

Pleural tuberculosis (PlTB), a common form of extrapulmonary TB, remains as a challenge in the diagnosis among many causes of pleural effusion. We recently reported that the combinatorial analysis of interferon-gamma (IFN-γ), IFN-γ-inducible protein 10 (IP-10), and adenosine deaminase (ADA) from the pleural microenvironment was useful to distinguish pleural effusion caused by TB (microbiologically or not confirmed cases) among other etiologies. In this cross-sectional cohort study, a set of inflammatory mediators was quantified in blood and pleural fluid (PF) from exudative pleural effusion cases, including PlTB (n = 27) and non-PlTB (nTB; n = 25) patients. The levels of IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF, IP-10, TGF-β1, and ADA were determined using cytometric bead assay, ELISA or biochemical tests. IFN-γ, IP-10, TNF, TGF-β, and ADA quantified in PF showed significantly higher concentrations in PlTB patients when compared to nTB. When blood and PF were compared, we have identified significantly higher concentrations of IL-6 and IL-10 in PF, in both groups. TGF-β, solely, showed significantly increased levels in PF and blood from PlTB when both clinical specimens were compared to nTB patients. Principal components analysis (PCA) revealed a T helper type 1 (Th1) pattern mainly attributed to higher levels of IP-10, IFN-γ, TGF-β, and TNF in pleural cavity, which was distinct between PlTB and nTB. In conclusion, our findings showed a predominantly cellular immune response in PF from TB cases rather than other causes of exudative effusion, commonly considered in the differential diagnosis of PlTB.

KEYWORDS: pleural tuberculosis, pleural effusion, adenosine deaminase, Th1 response, cytokines in pleural effusion.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is currently endemic in the world and represents an important public health problem. Globally, in 2017, more than 10 million new cases of TB were reported with an estimated 1.3 million deaths. Among infectious diseases, TB is the leading cause of death from a single agent, surpassing human immunodeficiency virus (HIV) infection (1). Although TB affects mainly the lungs, extrapulmonary forms can appear as an initial manifestation in approximately 25% of adults with the disease, of which the pleural space is one of the most affected sites (2), although the incidence varies between regions and/or due to the HIV infection, as recently reviewed by Shaw et al (3). In Brazil, a high burden TB country, pleural tuberculosis (PlTB) is responsible for more than 40% of cases among many clinical sites of extrapulmonary TB (4) and still imposes a challenging diagnosis mainly due to its paucibacillary nature and the need of invasive procedures (5).

Cellular immune response involving $CD4^+$ T-lymphocytes (T-helper type 1, Th1), classically studied and associated with the containment of Mtb in pulmonary parenchymal TB, is also predominant in tuberculous pleuritis, which is confirmed by the higher levels of interferon-gamma (IFN-γ) and other inflammatory cytokines (e.g., IL-12) in pleural fluid in comparison to peripheral blood (2,6–8). IFN-γ promotes cell differentiation, stimulates an increased phagocytic activity and intermediate nitrogen and oxygen species production, which are bactericidal and participate in resistance to Mtb infection (9,10). In addition, other T-cell effector patterns are involved in Mtb control in the pleural microenvironment, such as Th17, which express the retinoic acid-related orphan receptor gamma t (RORγt), and are characterized by secretion of large

74 quantities of IL-17 (also known as IL-17A), IL-21, and IL-22 (10,11). Th17 cells induce
75 the expression of many pro-inflammatory factors, chemokines, ultimately involved in
76 granulopoiesis and recruitment of innate cells, mainly neutrophils, especially in the
77 early stages of infection (12,13). It is well described that patients at early stages of PlTB
78 (less than 2 weeks duration) or those who present pleural effusion with high complexity
79 (e.g., loculated pleural effusion, TB empyema) are more likely to have neutrophilic
80 exudates (14), which may contribute to injuries and decrease on pleuro-pulmonary
81 functions.

82 The phenomenon termed “compartmentalization” has been well documented in
83 PlTB given the marked accumulation of Th-lymphocytes in pleural cavity rather than
84 peripheral blood (6,7,15). It was shown that PlTB patients present an increased
85 frequency of Th17 and polyfunctional effector memory T cells in the pleural cavity in
86 comparison to blood (16,17). Apart from this effector response, regulatory T cells
87 (Tregs), which act bringing down the enhanced immune-mediated damage (18), have
88 also been reported in pleural fluid from PlTB patients (16,19,20). These observations
89 provide strong pieces of evidence that cytokine producing-T cells are capable to migrate
90 into the pleural space, not only favoring accumulation of many products and
91 components of the immune response against Mtb, as well as contributing for the
92 paucibacillary nature of the disease or yet reducing tissue damage.

93 In the present study, we aimed to identify immunological response patterns
94 represented by Th1, Th2, Th17 T-cells subsets in peripheral blood and pleural fluid
95 among exudative pleural effusion which could contribute to a better understanding of
96 PlTB immunopathology and also present high potential of utility in the clinical
97 management of TB.

MATERIAL AND METHODS

Study population and settings. Patients aged ≥ 18 years with pleural effusion under investigation and with thoracentesis indication were recruited in this cross-sectional study, which was conducted at the Pulmonology and Tisiology Service, Pedro Ernesto University Hospital/Rio de Janeiro State University (HUPE/UERJ), a tertiary care center at Rio de Janeiro, RJ, Brazil. Patients who were under 18 years of age, pregnant, or refused consent were not recruited. Of 62 recruited patients, 10 were excluded: 8 patients had transudative pleural effusion (cardiac or renal failure), and 2 patients were HIV-seropositive. Thus, 52 patients with exudative pleural effusion were enrolled and grouped as described: 27 PlTB and 25 non-TB patients. ***PlTB cases*** were defined by the reviewed patient history, followed by a detailed physical examination, and at least one diagnostic criteria: i) positive results in the microbiological tests (acid-fast bacilli smear microscopy, mycobacterial culture, or Xpert MTB/RIF®) on pleural fluid or pleural tissue; ii) histopathological analysis showing the presence of granuloma with or without caseous necrosis; iii) clinical manifestations suggesting TB (fever, pain, dyspnea, cough, night sweats, hyporexia, and/or weight loss) in combination with a lymphocytic pleural effusion, followed by a full recovery after at least six months of anti-TB treatment. ***Non-TB cases*** consisted of patients with pleural or pleuro-pulmonary diseases, excluding active TB, based on clinical, laboratory, radiological, microbiological, and/or pathological features. Malignant pleural effusions were diagnosed by a positive pleural fluid cytology result or malignant cells identified in the pleural fragment. Even when both of these test results were negative, malignant effusion was diagnosed when a primary cancer was known to have disseminated and no other cause of pleural effusion was identified. Patients who did not fit the criteria used for

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123 PlTB diagnosis as above and with unknown causes of pleural effusion were classified as
124 "undefined" pleural effusion and considered as non-PlTB. Medical information,
125 peripheral blood, and pleural fluid sample collection were obtained from all recruited
126 subjects after signing a written consent. The study protocol was approved by the
127 respective institutional ethics committee (HUPE/UERJ; number 1.100.772).
128
129 **Sample collection.** Ultrasound-guided thoracentesis was performed by a trained
130 pulmonologist who collected pleural fluid which was directly drawn into collection
131 tubes for routine diagnostic tests, including chemistry panel, total and differential cell
132 count, ADA measurement by Hermes Pardini Laboratory according Giusti´s method
133 (21), cytopathology, microbiological analysis (bacteria, fungi and mycobacteria), and
134 inflammatory biomarkers for the purpose of the present study. Whole blood and pleural
135 fluid were appropriately collected in tubes without anticoagulant, immediately after
136 thoracentesis procedure and before any treatment. After collection, whole blood and
137 pleural fluid tubes were centrifuged at 1000 x g for 10 min and 25 °C or 4 °C,
138 respectively. Then, serum and pleural fluid (without cells) samples were aliquoted and
139 stored frozen at -20 °C until cytokine quantification.
140
141 **Cytokine assays.** Cytokine levels in clinical samples were assessed using the following
142 commercially available kits: i) human Th1/Th2/Th17 Cytokine Kit (BD Bioscience, San
143 Jose, CA, USA) based on the principle of cytometric bead array (CBA) technology for
144 simultaneous detection of seven cytokines (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-
145 17A). Briefly, capture beads labeled with distinct fluorescence intensity
146 (allophycocyanin; APC) conjugated to specific antibodies for cytokines were incubated
147 for about 3 hours in the dark at room temperature with undiluted samples, and

148 fluorescent detection antibody (phycoerythrin; PE). All unbound antibodies were
149 washed, and data acquired on a BD fluorescence-activated cell sorting (FACS) analyzer
150 FACSCanto II. Cytokine standard curves ranged from 0-5,000 pg/mL. ii) IP-10 and
151 TGF-β levels were measured by sandwich enzyme-linked immunosorbent assay
152 (ELISA) using human IP-10 DuoSet ELISA (R&D Systems Inc, MN, USA) and
153 human/mouse TGF beta 1 ELISA Ready-SET-Go! Kit (2nd Generation; Affymetrix,
154 eBioscience), respectively, following the manufacturer's instruction. The range of these
155 assays was 31.3-20,000 pg/mL for IP-10 and 15.6-1,000 pg/mL for TGF-β. Readings
156 higher than the upper limit were set at 20,000 (IP-10) or 1,000 (TGF-β) pg/mL for
157 analytical purposes.
158
159 **Statistical analysis**. For the description of the population included in the study,
160 according to their sociodemographic and clinical characteristics among the individuals
161 with exudative pleural effusion due to PlTB or other causes (non-TB), non-parametric
162 Mann-Whitney test was used for continuous variables or Fisher's exact test for
163 comparison of the relative frequencies of the different levels of nominal/categorical
164 variables. In the comparison between the levels of log-transformed expression (base 10)
165 of proteins in peripheral blood/serum and pleural fluid (tissue effect) between
166 individuals with or without TBPl (TB effect), the expected mean marginal values
167 obtained from multiple linear regression (log-linear) models of fixed effects were used
168 with the inclusion of first-order interactions between the main tissue and TB effects. For
169 the adjusted models, graphical analysis of residuals was performed to confirm their
170 randomness. In the comparisons between expected mean marginal values obtained from
171 linear regression models, adjustments of the confidence level were made by Sidak's
172 method and p-value adjustments by multiple comparisons by Tukey's method. Finally,

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for log10-transformed protein and ADA expression data, a multivariate principal component analysis (PCA) was performed to visualize the distribution of sample individuals in 2D dimensional spaces. After imputation of missing data by a k-Nearest Neighbor (k = 10) algorithm, we proceeded with a greedy iterative data reduction until finding a conventionally acceptable level of 0.8 for the standardized Cronbach's coefficient alpha (22) for scale reliability, selecting a subset of "highly predictive" variables. The proportion of variation explained was calculated after each eigenvalue. The cumulative percentage explained is obtained by adding the successive proportions of variation explained to obtain the running total. The contributions (in percentage) of the variables to the principal components were calculated as (var. cos2 x 100)/(total cos2 of the component); where cos2 indicates square cosine or squared coordinates. Accordingly, the contributions (in percentage) of individuals to the principal components were calculated as (ind. cos2 x 100)/(total cos2 of the component). Ellipses of the quantiles 66% of the normal distribution adjusted to the individuals of the different interest groups in these new dimensional spaces are presented. The level of significance, $P \leq 0.05$, was used in the analysis, and all analyses were performed in R software version 3.6.1.

RESULTS

Patients and characteristics. The study population was composed by 52 individuals who were diagnosed as PlTB (n = 27) or non-TB (n = 25), according to the previously described criteria (8). Their sociodemographic and clinical data are shown in Table 1. We observed a significant difference between the age distributions between the groups, which presented medians corresponding to 63 years (IQR: 18) in the non-TB group, and

198 40 years (IQR: 20.5) in the PlTB group ($p < 0.0001$). Smoking and alcoholic habits
199 among participants did not show statistical differences. Likewise, signals and symptoms
200 were not dissimilar among groups. Fourteen patients in the non-TB group (14/25) had
201 one or more associated diseases, showing that this group had a significantly higher
202 number of patients with comorbidities than observed in the P1TB group, which had 6
203 individuals (6/27) with other diseases ($p = 0.0217$). The most prevalent comorbidity was
204 hypertension, which was reported in 9 (36%) non-TB patients and 3 (11%) P1TB
205 patients. A significant mononuclear cell count in pleural fluid was observed in PlTB
206 group in comparison to non-TB ($p = 0.0148$), while polymorphonuclear cells were
207 significantly higher in non-TB patients ($p = 0.021$). Regarding the biochemical panel,
208 glucose concentration was significantly increased in non-TB patients in comparison to
209 PlTB ($p = 0.0288$). The majority of the PlTB patients had diagnostic confirmation based
210 on microbiological and/or histopathological criteria. Among non-TB patients, 18
211 presented malignancies (10 adenocarcinomas, 2 lymphomas, 6 unspecified neoplasms),
212 2 autoimmune diseases (systemic lupus erythematosus), 1 bacterial parapneumonic
213 effusion and 4 undefined pleural effusion.
214
215 **Cytokine measurement in blood and pleural fluid from PlTB and non-TB patients.**
216 In order to evaluate the pattern of Th1/Th2/Th17 cytokines and other inflammatory
217 mediators such as IP-10, TGF-β and ADA in exudative cases of pleural effusion, serum
218 and pleural fluid samples from PlTB and non-TB patients were analyzed. As recently
219 reported by our group (7) and others (21–23), IFN-γ and IP-10 levels were significantly
220 increased ($p < 0.0001$ in both) in pleural fluid comparison to serum in PlTB group
221 (Figure 1A and H). As shown in Figure 1B, TNF concentration also showed a

222 significant increase in the pleural fluid when compared to serum in PlTB patient ($p = 0.0016$).

224 When cytokines were compared with discriminatory objectives between PlTB and non-TB patients, significant differences in the pleural fluid were predominantly observed. IL-6 and IL-10 levels presented the same behavior when serum and pleural fluid were compared in PlTB or non-TB groups (Figure 1G and E, respectively). Both IL-10 and IL-6 concentrations show that patients in both PlTB ($p < 0.0001$ in both) and non-TB ($p < 0.0001$ in both) groups show increased concentrations of this cytokine in pleural fluid when compared to serum in their respective groups. As expected, ADA levels were significantly higher in the pleural fluid of PlTB patients compared to non-TB ($p < 0.0001$). Interestingly, TGF-β concentrations were significantly higher in the serum ($p < 0.0001$) and pleural fluid of P1TB patients, compared to concentrations found in non-TB patient samples ($p < 0.0001$). Concentrations of TGF-β showed no significant serum and pleural fluid differences when compared in the same group (Figure 1I).

237 Finally, IFN-γ, TNF, IP-10, TGF-β and ADA concentrations in the pleural fluid presented a differentiated profile between PlTB and non-TB patients. Cytokines IL-17A, IL-4, and IL-2 did not show significant differences in their concentrations.

240

241 **Principal component analysis of pleural fluid cytokines.** Finally, it was examined whether PlTB would be associated with a particular inflammatory pattern against other causes of exudative pleural effusion. Since that our most significative results were observed in the pleural microenvironment, all subsequent analyses were performed in pleural fluid. A Principal Components Analysis (PCA) plot illustrated 66.7% of the total variance in response to 8 biomarkers was expressed by 2 principal components. The

first component accounted for a total of 47.8%, while the second accounted for 18.9% of the total variance (Table 2; Figure 2A). Altogether, these 8 biomarkers were able to partially discriminate PlTB and non-TB cases. The most determinant variables of each of these two principal components were ADA, IP-10, TGF-β, IFN-γ, and TNF, for the first principal component (Dim1), and IL-2, and IL-4 for the second principal component (Dim2) (Table 2). Curiously, 2 clusters were identified among the main inflammatory mediators with discriminative potential between PlTB and non-TB (Figure 2B). IP-10, IFN-γ, TGF-β, and TNF were considered the main contributors for the observed variance and were capable to show a clear separation between PlTB and non-TB groups (Figure 2C). Additionally, the individual mean variation of the study population was analyzed showing that among top-ten cases with the highest contributions were identified PlTB patients who presented diagnostic confirmation based on microbiological and/or histopathological criteria (3/5), while non-TB patients consisted of confirmed cases of malignant effusion (5/5) (Figure 2D).

DISCUSSION

Among many known causes of pleural effusion, heart failure, malignant conditions, pneumonia, and PlTB are responsible for three-quarters of all cases (23). The present work extends previous data of our group, which proposed a model where IFN-γ and ADA can be used in the differential diagnosis of P1TB with high performance in microbiologically unconfirmed cases of PlTB (8). Herein, we demonstrate that among Th1/Th2/Th17 and other inflammatory mediators, such as IP-10, TGF-β and ADA, there is a predominant inflammatory pattern associated to cellular (Th1) immune response in PlTB patients in comparison to other common exudative

causes of pleural effusion, which, to our knowledge, have not been previously described. PCA analysis revealed that IP-10, IFN-γ, TGF-β, and TNF showed the largest variations associated with a clear distinction between PlTB and non-TB patients.

In the clinical practice, values > 40 IU/L of adenosine deaminase (ADA) in pleural effusion, a purine-degrading enzyme found predominantly in T-lymphocytes, associated with a lymphocytic exudate, and clinical suspicious of TB, altogether, indicates that the most likely diagnosis is tuberculosis (24–26). However, high pleural fluid ADA values can also be found in certain conditions, such as adenocarcinoma, lymphoma, mesothelioma, rheumatoid arthritis, and pleural empyema of bacterial etiology, making the differential diagnosis very hard (27,28), once that the gold standard for the diagnosis of PlTB, that is, the detection of Mtb in the sputum, pleural fluid or pleural biopsy has a discrete and variable yield (29,30).

Currently, IFN-γ measurement (a classical Th1 response) in pleural effusion has raised its importance as an auxiliary method for the diagnosis of PlTB, becoming an example of a test used for this purpose, since this cytokine is at high levels during the active phase of the disease (31,32). Therefore, the IFN-γ-release assay (IGRA) has also been highlighted in this context. This test evaluates the activity of T lymphocytes under the stimulation of Mtb ESAT-6 and CFP-10 antigens. However, as reviewed by Aggarwal and collaborators (2015), there are many conflicting results regarding this diagnostic method of active TB, both in pulmonary and pleural forms (33). Moreover, as recently delineated by our group, IGRA has a poor clinical meaning in PlTB (8), perhaps due to their paucibacillary nature or due to the enrichment of inflammatory mediators in pleural space, without needing of an additional antigen-stimuli. TNF is another important mediator in the response against Mtb and it is directly related to the maintenance of the granuloma structure, the colonization bacillus and necrosis area

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297 (34). Li and collaborators (2014) found a higher diagnostic value in TNF measurements
298 than in ADA (35). IP-10 is well studied as a possible biomarker in TB and is directly
299 associated with IFN-γ since its production is mainly induced by this cytokine. As
300 revised by Porcel (36), IP-10 is not an essential biomarker for the PlTB diagnosis but
301 has been the subject of several studies in this context, based on its participation in the
302 immunopathogenesis of the disease and its correlation with IFN-γ (8,37). In the present
303 study, these three biomarkers (IFN-γ, TNF and IP-10) were found in significantly
304 higher levels in pleural fluid from PlTB patients and were identified as the main
305 contributors for the variance observed in PCA analysis.

306 The cytokine pattern related to the Th2 effector phenotype was also evaluated.
307 In the methodology employed, we did not detect significant levels of IL-4. This finding
308 confirms the literature data that show little influence of this effector phenotype in TB
309 cases (2,36), although IL-4 concentrations in miliary TB have already been reported (6).
310 In addition, our study has shown higher concentrations of IL-10 in the pleural fluid of
311 patients with PlTB compared to serum, and in the same way in the non-TB group.
312 However, the methodology used in this study was not able to identify which cells
313 present in the pleural fluid were responsible for the increase of IL-10 concentrations, as
314 well as the other cytokines. Geffner et al. (2013) showed an increased IL-10 production
315 after stimulation of mononuclear cells in pleural fluid and peripheral blood with Mtb
316 antigens, and the decrease of this cytokine after removal of Tregs cells providing
317 evidences that Tregs is also responsible for the production of IL-10 from the pleural
318 cavity (20).

319 Another important finding in our study was the quantification of TGF-β in serum
320 and pleural fluid. This growth factor, secreted by monocytes, is a chemotactic agent for
321 fibroblasts and plays an important role in extracellular matrix remodeling (38). One of

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the possible contributions of TGF-β to the pathophysiology of PlTB is its ability to induce fibrosis, as shown in the study by Sasse and collaborators (2003), where animals infected with Mtb showed increased pleural thickening in proportion to the increase in TGF- β (39). Seiscento and collaborators (2007) also found elevated TGF-β levels in serum and pleural fluid of PlTB patients, associating with the degree of pleural thickening in these patients (40). Our findings, together with the evidence found in the literature, reinforce the hypothesis that this mediator may be related to the development of pleural effusions in TBP1 patients since TGF-β levels were found to be significantly higher in the pleural fluid of these patients, compared to the results found in non-TB patients. Although the cited studies found a significant increase of TGF-β in pleural fluid and serum, the comparison group in the experimental model of these studies was composed of patients with transudative pleural effusion. Our work was able to detect the increase of TGF-β in the serum and pleural fluid of PlTB patients, compared to blood and pleural fluid in patients with other causes of exudative effusion. This finding may contribute to future investigations, associating TGF-β as a possible biomarker to aid in the differential diagnosis of PlTB.

Malignancy is the most prevalent etiology of exudative pleural effusion, preceded by TB (23,41). In our study, the non-TB group was composed by around 70% (18/25) of malignancies among exudative cases excluding TB, which in part could be explained by the characteristic of the recruitment unit (a Tertiary Care Hospital). Atef et al (2016) have shown that although the TNF levels are significantly high in pleural fluid from exudative cases in comparison to transudative ones, there was a significant increase of TNF levels in pleural fluid from PlTB patients versus malignant effusion (42). Our work is in accordance with these data, since we did not find significant variations of TNF levels in the blood and pleural fluid from non-TB patients. In another

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report, it was shown a prevalence of Th17 response in the pleural liquid from patients with lung cancer in comparison to those with TB. IFN-γ, IL-6, IL-10 and IL17A production by $CD4^+$ T-cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin showed significant differences in lung cancer group compared to PlTB (43). However, transcriptional analysis of cytokine genes highlighted an increased expression of Th17 pattern in PlTB patients against common causes of exudative pleural effusion, including malignancies and parapneumonic effusion (44). Our presented results show high concentrations of IL-6 and TGF-β in pleural compartment of PlTB patients compared to serum. These two biomarkers are critical in the differentiation of Th17 cells (45). Therefore, although our study did not focus on the characterization of Th17 cells, it is quite probable that the microenvironment, through the high concentration of IL-6, TGF-β, and the low concentrations of IL-2, might favor the differentiation of this T-lymphocytes effector phenotype in PlTB.

Some limitations should be considered in our study. First, it was conducted in a single center, imposing a validation in other reference centers and in different populations. Another consideration is the relatively low number of patients included per group. However, patients were included prospectively, in a real routine of clinical practice in a tertiary reference center, which reflected in variable clinical characteristics inherent of each group of study, as can be observed in Table 1. Moreover, we have excluded transudative cases which could add some bias in our analysis, and we have analyzed only exudative cases of pleural effusion, the main confounders in the differential diagnosis of TB.

In summary, the pleural fluid screening for a panel of inflammatory mediators was useful to provide new hypotheses and better comprehension about the microenvironment of the pleural cavity during the immunopathology of Mtb infection.

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372 Based on this approach we could identify a predominance of cellular (Th1) immune-
373 related response pointing biomarkers with high potential for clinical use, which may
374 increase the sensitivity of diagnosis and prompt the TB treatment, especially in cases of
375 difficult identification and distinction by conventional diagnostic methods.
376

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Author Contributions

Conceptualization: Luciana Silva Rodrigues.

Data curation: Vinicius da Cunha Lisboa, Raquel da Silva Corrêa, Isabelle Ramos Lopes.

Formal analysis: Vinicius da Cunha Lisboa, Raquel da Silva Corrêa, Marcelo Ribeiro-Alves.

Funding acquisition: Rogério Lopes Rufino Alves, Luciana Silva Rodrigues.

Investigation: Vinicius da Cunha Lisboa, Thiago Thomaz Mafort, Ana Paula Gomes dos Santos, Rogério Lopes Rufino Alves, Luciana Silva Rodrigues.

Methodology: Vinicius da Cunha Lisboa, Raquel da Silva Corrêa, Isabelle Ramos Lopes, Thaís Porto Amadeu.

Project administration: Luciana Silva Rodrigues.

Resources: Rogério Lopes Rufino Alves, Luciana Silva Rodrigues.

Software: Vinicius da Cunha Lisboa, Marcelo Ribeiro-Alves.

Supervision: Luciana Silva Rodrigues.

Validation: Marcelo Ribeiro-Alves.

Writing – original draft: Vinicius da Cunha Lisboa, Raquel da Silva Corrêa, Marcelo Ribeiro-Alves, Luciana Silva Rodrigues.

Writing – review&editing: Vinicius da Cunha Lisboa, Marcelo Ribeiro-Alves, Luciana Silva Rodrigues.

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548

549 **FIGURE LEGENDS**

550

551 **Figure 1. Inflammatory mediators in serum and/or pleural fluid from PlTB and**
552 **nTB patients.** Cytokines were measured in serum and pleural fluid by CBA (IL-2, IL-4,
553 IL-6, IL-10, TNF, IFN-γ, and IL-17A), ELISA (IP-10 and TGF-β) and biochemical test
554 (ADA). The levels obtained from each inflammatory mediator were analyzed on a
555 logarithmic (base = 10) scale and illustrated using boxplots to compare serum (S) and
556 pleural fluid (PF) data between the non-TB (nTB) and PlTB groups. The small grey
557 dots represent individual cases and the boxplots represent the interquartile range and the
558 median of the sample (solid grey central line). Larger black dots and vertical bars
559 represent expected mean marginal values estimated by the linear model and its 95%
560 confidence intervals (95% CI). Comparisons of means between groups were performed
561 by contrasts/differences obtained after linear bi and multivariate models, adjusted by
562 regressions by ordinary least squares. * $p < 0.05$; ** $p < 0.01$.

563

564 **Figure 2. The pattern of inflammatory biomarkers in pleural fluid discriminates**
565 **between PlTB and non-TB patients.** The analysis of variance of cytokine
566 concentrations by CBA (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A), and ELISA
567 (TGF-β and IP-10) and a biochemical test (ADA) were evaluated in PlTB (n = 27) and
568 non-TB (n = 25) patients. All but IL-6 and IL-17A, with reliability (standardized
569 Cronbach's coefficient alpha) of 0.81, were included in a Principal Component Analysis
570 (PCA). (A) A 2D representation given by the two first principal components with
571 47.78% and 18.9% variation explained (66.68% cumulative percentage explained) of
572 PlTB (yellow dots) and non-TB (blue dots) with dot sizes proportional to individuals
573 mean contribution to either principal components, while variables (biomarkers) are

expressed by colored vectors also indicating their mean contribution to the principal components. (B) A representation where the vector represents the correlation between a variable (biomarker) and a principal component (PC) is used as the coordinates of the variable on the PC. Variables are colored accordingly to the results of a divisive k-means (k = 2) clustering. (C) Bar graphs indicating the top-five variables (biomarkers) according to their mean contribution to either principal components. (D) Bar graphs indicating the top-ten individuals (patients) according to their mean contribution to either principal components.

583 **Table 1. Sociodemographic and clinical characteristics of the study population.**

Characteristics / Group	nTB (N = 25)	PlTB (N = 27)	*P* value
Age, years median (IQR)	63 (18)	40 (20)	0.0001
Gender (%)			
Female	10 (19.2)	9 (17.3)	0.7743
Male	15 (28.8)	18 (34.6)	
Current smoker (%)	2 (3.8)	2 (5.8)	0.2119
Alcohol use (%)	3 (5.8)	11 (21.2)	0.0542
Comorbidities, yes (%)	14 (26.9)	6 (11.5)	0.0217
Hypertension	9 (17.3)	3 (5.8)	0.049
Diabetes	5 (9.6)	2 (3.8)	0.2407
Cardiac insufficiency	3 (5.8)	0 (0)	0.1041
Hepatitis	3 (5.8)	1 (1.9)	0.3409
Signals and symptoms (%)			
Fever	5 (9.6)	11 (21.2)	0.1318
Cough	19 (36.5)	12 (23.1)	0.0792
Chest pain	8 (15.4)	12 (23.1)	0.3991
Dyspnea	18 (34.6)	15 (28.8)	0.3823
Night sweats	5 (9.6)	4 (7.7)	0.7224
Weight loss	9 (17.3)	10 (19.2)	1
Pleural fluid characteristics (IQR)			
Total cells/mm^3	1600 (2048)	3000 (3075)	0.1479
Mononuclear cells, %	70 (45)	92 (26.5)	0.0148
Polymorphonuclear cells, %	30 (45)	8 (26.5)	0.021
Glucose, mg/dL	99 (43)	88 (43)	0.0288
Total proteins, g/dL	5.3 (1.1)	5.7 (0.85)	0.0129
Albumin, g/dL	3.1 (0.6)	3 (0.5)	0.6982
LDH, IU/L	464 (853)	387 (444.5)	0.8835
PlTB diagnostic criteria (%)[#]			
Microbiology		5 (18.5)	
Histopathology		11 (40.7)	
Clinical findings and full recovery after anti-TB treatment		11 (40.7)	
Cause of effusion (%)			
Tuberculosis		27 (51.9)	
Malignancy	18 (34.6)		
Autoimmune disease	2 (0.04)		
Parapneumonic effusion	1 (0.02)		
Undefined	4 (0.07)		

584 PlTB, Pleural tuberculosis; nTB, non-TB; IQR, Interquartile range; LDH, lactate
585 dehydrogenase. Values expressed as n (%; from the total population) unless otherwise stated.[#,]
586 percentage from the PlTB group.
587

588 **Table 2. Principal components analysis.**

Component	Eigenvalue	Variance Percent	Cumulative
Dim.1	3.822383375	47.77979218	47.77979218
Dim.2	1.512311812	18.90389764	66.68368983
Dim.3	0.935411982	11.69264978	78.3763396
Dim.4	0.698769724	8.73462155	87.11096115
Dim.5	0.585125808	7.314072601	94.42503376
Dim.6	0.346572257	4.33215321	98.75718697
Dim.7	0.097384212	1.217302645	99.97448961
Dim.8	0.002040831	0.025510389	100

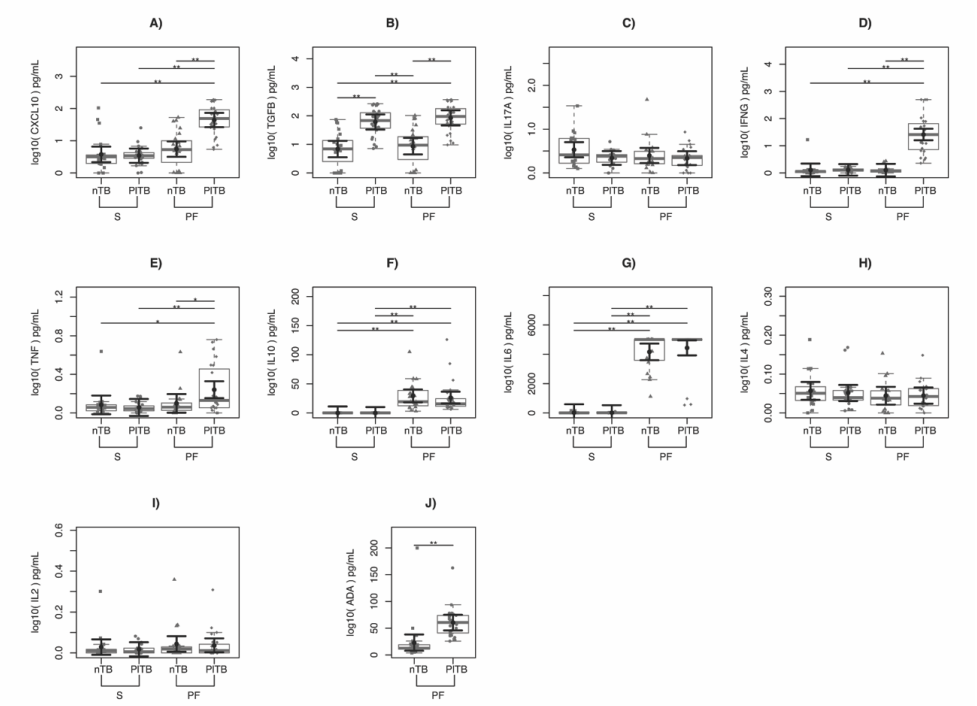
Variable	Dim. 1	Dim. 2
ADA	0.702175941	-0.162822821
IP-10	0.884960511	-0.308501329
TGF-β	0.873625099	-0.304114026
IFN-γ	0.900769802	-0.229363506
TNF	0.803134056	0.374374796
IL-10	0.337137736	0.300322131
IL-4	0.365873564	0.679167637
IL-2	0.281105917	0.744255307

589 Principal component analysis of inflammatory biomarkers in pleural fluid from patients with
590 pleural effusion by PlTB and other diagnoses. Shaded values represent the most important
591 biomarkers in the component definition.
592

593

594 **FIGURE 1**

595



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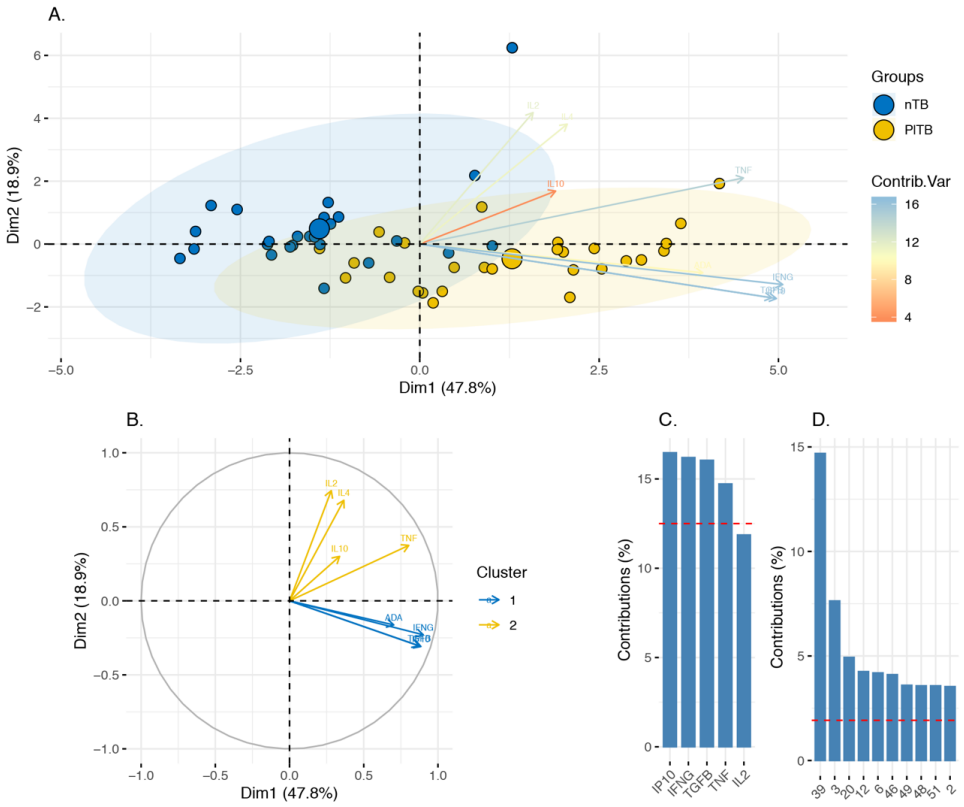
598 FIGURE 2

599



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