Predominance of Th1 Immune Response in Pleural Effusion of Patients with Tuberculosis among Other Exudative Etiologies

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ABSTRACT Pleural tuberculosis (PITB), a common form of extrapulmonary TB, remains 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 confirmed or not) 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 PITB ($n = 27$) and non-PITB (nTB) ($n = 25$) patients. The levels of interleukin-2 (IL-2), IL-4, IL-6, IL-10, IL-17A, IFN- γ , tumor necrosis factor (TNF), IP-10, transforming growth factor β 1 (TGF- β), and ADA were determined using cytometric bead assay, enzyme-linked immunosorbent assay (ELISA), or biochemical tests. IFN- γ , IP-10, TNF, TGF- β , and ADA quantified in PF showed significantly higher concentrations in PITB patients than in nTB patients. When blood and PF were compared, significantly higher concentrations of IL-6 and IL-10 in PF were identified in both groups. TGF- β , solely, showed significantly increased levels in PF and blood from PITB patients when both clinical specimens were compared to those from nTB patients. Principal-component analysis (PCA) revealed a T helper type 1 (Th1) pattern attributed mainly to higher levels of IP-10, IFN- γ , TGF- β , and TNF in the pleural cavity, which was distinct between PITB 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 PITB.

KEYWORDS pleural tuberculosis, pleural effusion, adenosine deaminase, Th1 response, cytokines in pleural effusion, IP-10, transforming growth factor-beta

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, 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 HIV infection, as recently reviewed by Shaw et al. (3). In Brazil, a country with a high TB burden, pleural tuberculosis (PITB) is responsible for more than 40% of cases among many clinical sites of extrapulmonary TB (4), and it still imposes a challenging diagnosis mainly due to its paucibacillary nature and the need for invasive procedures (5).

The cellular immune response involving CD4⁺ T lymphocytes (T-helper type 1 [Th1]),

Citation da Cunha Lisboa V, Ribeiro-Alves M, da Silva Corrêa R, Ramos Lopes I, Mafort TT, Santos AP, Porto Amadeu T, Rufino R, Silva Rodrigues L. 2020. Predominance of Th1 immune response in pleural effusion of patients with tuberculosis among other exudative etiologies. *J Clin Microbiol* 58:e00927-19. <https://doi.org/10.1128/JCM.00927-19>.

Editor Geoffrey A. Land, Carter BloodCare & Baylor University Medical Center

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Received 8 June 2019

Returned for modification 16 July 2019

Accepted 6 October 2019

Accepted manuscript posted online 16 October 2019

Published 23 December 2019

classically studied and associated with the containment of *M. tuberculosis* 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., interleukin-12 [IL-12]) in pleural fluid than in peripheral blood (2, 6–8). IFN- γ promotes cell differentiation and stimulates an increased phagocytic activity and intermediate nitrogen and oxygen species production, which are bactericidal and participate in resistance to *M. tuberculosis* infection (9, 10). In addition, other T-cell effector patterns are involved in *M. tuberculosis* control in the pleural microenvironment, such as Th17, which express the retinoic acid-related orphan receptor gamma t (ROR γ t), and are characterized by secretion of large quantities of IL-17 (also known as IL-17A), IL-21, and IL-22 (10, 11). Th17 cells induce the expression of many proinflammatory factors (cytokines, chemokines, and growth factors), which are ultimately involved in granulopoiesis and recruitment of innate cells, mainly neutrophils, especially in the early stages of infection (12, 13). It is well described that patients in early stages of PITB (duration of less than 2 weeks) or those who present pleural effusion with high complexity (e.g., loculated pleural effusion or TB empyema) are more likely to have neutrophilic exudates (14), which may contribute to injuries and decrease of pleuro-pulmonary functions.

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

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

MATERIALS AND METHODS

Study population and settings. Patients aged ≥ 18 years with pleural effusion under investigation and with an indication for thoracentesis 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 in Rio de Janeiro, Rio de Janeiro, 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 27 PITB patients and 25 non-TB patients. PITB cases were defined by the reviewed patient history, followed by a detailed physical examination and at least one diagnostic criterion: (i) positive results in the microbiological tests (acid-fast bacillus smear microscopy, mycobacterial culture, or Xpert MTB/RIF) of pleural fluid or pleural tissue; (ii) histopathological analysis showing the presence of granuloma with or without caseous necrosis; and (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 6 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 PITB diagnosis as described above and with unknown causes of pleural effusion were classified as having “undefined” pleural effusion and considered non-PITB. Medical information, peripheral blood, and pleural fluid samples were obtained from all recruited subjects after they signed a written consent form. The study protocol was approved by the institutional ethics committee (HUPE/UERJ; number 1.100.772).

Sample collection. Ultrasound-guided thoracentesis was performed by a trained pulmonologist who collected pleural fluid which was directly drawn into collection tubes for routine diagnostic tests, including chemistry panel, total and differential cell count, adenosine deaminase (ADA) measurement by Hermes Pardini Laboratory according to Giusti's method (21), cytopathology, microbiological analysis (bacteria, fungi, and mycobacteria), and inflammatory biomarkers for the purpose of the present study. Whole blood and pleural fluid were appropriately collected in tubes without anticoagulant immediately after the thoracentesis procedure and before any treatment. After collection, whole blood and pleural fluid tubes were centrifuged at $1,000 \times g$ for 10 min and 25°C or 4°C , respectively. Serum and pleural fluid (without cells) samples were then aliquoted and stored frozen at -20°C until cytokine quantification.

Cytokine assays. Cytokine levels in clinical samples were assessed using the following commercially available kits. (i) The human Th1/Th2/Th17 cytokine kit (BD Bioscience, San Jose, CA, USA), based on the principle of cytometric bead array (CBA) technology, was used for simultaneous detection of seven cytokines (IL-2, IL-4, IL-6, IL-10, tumor necrosis factor [TNF], IFN- γ , and IL-17A). Briefly, capture beads labeled with a distinct fluorescence intensity (allophycocyanin [APC]) conjugated to specific antibodies for cytokines were incubated for about 3 h in the dark at room temperature with undiluted samples and fluorescent detection antibody (phycoerythrin [PE]). All unbound antibodies were washed, and data were acquired on a BD FACSCanto II fluorescence-activated cell sorting (FACS) analyzer. Cytokine standard curves ranged from 0 to 5,000 pg/ml. (ii) IFN- γ -inducible protein 10 (IP-10) and transforming growth factor β 1 (TGF- β) levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using the human IP-10 DuoSet ELISA (R&D Systems Inc, MN, USA) and the human/mouse TGF β 1 ELISA Ready-SET-Go! kit (second generation) (Affymetrix; eBioscience), respectively, following the manufacturer's instruction. The ranges of these assays were 31.3 to 20,000 pg/ml for IP-10 and 15.6 to 1,000 pg/ml for TGF- β . Readings higher than the upper limit were set at 20,000 (IP-10) or 1,000 (TGF- β) pg/ml for analytical purposes.

Statistical analysis. For the description of the population included in the study according to their sociodemographic and clinical characteristics among the individuals with exudative pleural effusion due to PITB or other causes (non-TB), the nonparametric Mann-Whitney test was used for continuous variables or Fisher's exact test for comparison of the relative frequencies of the different levels of nominal/categorical variables. In the comparison between the levels of log-transformed expression (base 10) of proteins in peripheral blood/serum and pleural fluid (tissue effect) between individuals with or without PITB (TB effect), the expected mean marginal values obtained from multiple linear regression (log-linear) models of fixed effects were used, with the inclusion of first-order interactions between the main tissue and TB effects. For the adjusted models, graphical analysis of residuals was performed to confirm their randomness. In the comparisons between expected mean marginal values obtained from linear regression models, adjustments of the confidence level were made by Sidak's method and *P* value adjustments by multiple comparisons by Tukey's method. Finally, for log₁₀-transformed protein and ADA expression data, a multivariate principal-component analysis (PCA) was performed to visualize the distribution of sample individuals in two-dimensional (2D) 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 $(\text{var. cos}^2 \times 100)/(\text{total cos}^2 \text{ of the component})$, where cos² indicates square cosine or squared coordinates. Accordingly, the contributions (in percentage) of individuals to the principal components were calculated as $(\text{ind. cos}^2 \times 100)/(\text{total cos}^2 \text{ of the component})$. 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. A *P* value of ≤ 0.05 was used as the level of significance in the analysis, and all analyses were performed in R software version 3.6.1.

RESULTS

Patient characteristics. The study population was composed of 52 individuals who were diagnosed as having PITB (*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 in the age distributions between the groups, which presented medians corresponding to 63 years (interquartile range [IQR], 18) in the non-TB group and 40 years (IQR, 20.5) in the PITB group (*P* < 0.0001). Smoking and alcohol use among participants did not show statistical differences. Likewise, signs and symptoms were not dissimilar among groups. Fourteen patients in the non-TB group (14/25) had one or more associated diseases, showing that this group had a significantly higher number of patients with comorbidities than observed in the PITB group, which had 6 individuals (6/27) with other diseases (*P* = 0.0217). The most prevalent comorbidity was hypertension, which was reported in 9 (36%) non-TB patients and 3 (11%) PITB patients. A significant mononuclear cell count in pleural fluid was observed in the PITB group in comparison to the non-TB group (*P* = 0.0148), while polymorpho-

TABLE 1 Sociodemographic and clinical characteristics of the study population^a

Characteristic	Value ^b for group		P value
	nTB (n = 25)	PITB (n = 27)	
Median (IQR) age, yr	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	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
Signs 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
Wt loss	9 (17.3)	10 (19.2)	1
Pleural fluid characteristics (IQR)			
Total cells/mm ³	1,600 (2,048)	3,000 (3,075)	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
PITB diagnostic criteria ^c			
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)		

^aPITB, pleural tuberculosis; nTB, non-TB; IQR, interquartile range; LDH, lactate dehydrogenase.^bValues are number (% from the total population) unless otherwise stated.^cValues in parentheses are percentages from the PITB group.

nuclear cells were significantly higher in non-TB patients ($P = 0.021$). Regarding the biochemical panel, glucose concentration was significantly increased in non-TB patients in comparison to PITB patients ($P = 0.0288$). The majority of the PITB patients had diagnostic confirmation based on microbiological and/or histopathological criteria. Among non-TB patients, 18 presented malignancies (10 adenocarcinomas, 2 lymphomas, and 6 unspecified neoplasms), 2 had autoimmune disease (systemic lupus erythematosus), 1 had bacterial parapneumonic effusion, and 4 had undefined pleural effusion.

Cytokine measurement in blood and pleural fluid from PITB and non-TB patients. In order to evaluate the pattern of Th1/Th2/Th17 cytokines and other inflammatory mediators such as IP-10, TGF- β , and ADA in exudative cases of pleural effusion, serum and pleural fluid samples from PITB and non-TB patients were analyzed. As recently reported by our group (8) and others (21–23), IP-10 and IFN- γ levels were

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

significantly increased ($P < 0.0001$ for both) in pleural fluid in comparison to serum in the PITB group (Fig. 1A and D). As shown in Fig. 1E, the TNF concentration also showed a significant increase in the pleural fluid compared to serum in PITB patients ($P = 0.0016$).

When cytokines were compared with discriminatory objectives between PITB 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 the PITB or non-TB groups (Fig. 1G and F, respectively). Both IL-10 and IL-6 concentrations show that patients in both the PITB ($P < 0.0001$ for both) and non-TB ($P < 0.0001$ for both) groups had increased concentrations of this cytokine in pleural fluid compared to serum in their respective groups. As expected, ADA levels were significantly higher in the pleural fluid of PITB patients than in that of non-TB patients ($P < 0.0001$). Interestingly, TGF- β concentrations were significantly higher in the serum and pleural fluid of PITB patients than in non-TB patient samples ($P < 0.0001$). Concentrations of TGF- β showed no significant serum and pleural fluid differences in the same group (Fig. 1B).

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

TABLE 2 Principal-component analysis of inflammatory biomarkers in pleural effusion from patients with PITB and other diagnoses^a

Component	Eigenvalue	Variance (%)	Cumulative variance (%)
Dim1	3.822383375	47.77979218	47.77979218
Dim2	1.512311812	18.90389764	66.68368983
Dim3	0.935411982	11.69264978	78.3763396
Dim4	0.698769724	8.73462155	87.11096115
Dim5	0.585125808	7.314072601	94.42503376
Dim6	0.346572257	4.33215321	98.75718697
Dim7	0.097384212	1.217302645	99.97448961
Dim8	0.002040831	0.025510389	100

^aThe proportion of variation and cumulative percentage are shown for each eigenvalue.

PCA of pleural fluid cytokines. Finally, we examined whether PITB was associated with a particular inflammatory pattern against other causes of exudative pleural effusion. Since that our most significant results were observed in the pleural microenvironment, all subsequent analyses were performed in pleural fluid. A principal-component analysis (PCA) plot illustrated that 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; Fig. 2A). Altogether, these 8 biomarkers were able to partially discriminate PITB 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 3). Curiously, 2 clusters were identified among the main inflammatory mediators with discriminative potential between PITB and non-TB (Fig. 2B). IP-10, IFN- γ , TGF- β , and TNF were considered the main contributors for the observed variance and were able to show a clear separation between PITB and non-TB groups (Fig. 2C). Additionally, the individual mean variation of the study population was analyzed, showing that among the top 10 cases with the highest contributions were identified PITB patients who had diagnostic confirmation based on microbiological and/or histopathological criteria (3/5), while non-TB patients consisted of confirmed cases of malignant effusion (5/5) (Fig. 2D).

DISCUSSION

Among many known causes of pleural effusion, heart failure, malignant conditions, pneumonia, and PITB are responsible for three-quarters of all cases (23). The present work extends previous data from our group, which proposed a model where IFN- γ and ADA can be used in the differential diagnosis of PITB with high performance in microbiologically unconfirmed cases of PITB (8). Here 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 with the cellular (Th1) immune response in PITB patients in comparison to those with other common exudative causes of pleural effusion, which, to our knowledge, has not been previously described. PCA revealed that IP-10, IFN- γ , TGF- β , and TNF showed the largest variations associated with a clear distinction between PITB and non-TB patients.

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

Currently, measurement of IFN- γ (a classical Th1 response) in pleural effusion has

FIG 2 The pattern of inflammatory biomarkers in pleural fluid discriminates between PITB and non-TB patients. Analyses of variance of cytokine concentrations by CBA (IL-2, IL-4, IL-6, IL-10, TNF, IFN- γ , and IL-17A), ELISA (TGF- β and IP-10), and a biochemical test (ADA) were evaluated for PITB ($n = 27$) and non-TB ($n = 25$) patients. All but IL-6 and IL-17A, with reliability (standardized Cronbach coefficient alpha) of 0.81, were included in a principal-component analysis (PCA). (A) A 2D representation, given by the two first principal components with 47.78% and 18.9% variation explained (66.68% cumulative percentage explained), of PITB (yellow dots) and non-TB (blue dots), with dot sizes proportional to the individual mean contribution to either principal component. 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 according to the results of a divisive k -means ($k = 2$) clustering. (C) Bar graph indicating the top five variables (biomarkers) according to their mean contribution to either principal component. (D) Bar graph indicating the top 10 individuals (patients) according to their mean contribution to either principal component.

raised its importance as an auxiliary method for the diagnosis of PITB, 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 *M. tuberculosis* ESAT-6 and CFP-10 antigens. However, as reviewed by Aggarwal and collaborators, there are many conflicting results regarding this diagnostic method of active TB, in both pulmonary and pleural forms (33). Moreover, as recently delineated by our group, IGRA has a poor clinical performance in PITB (8), perhaps due to its paucibacillary nature or due to the enrichment of inflammatory mediators in the pleural space, without needing an additional antigen stimulus. TNF is another important mediator in the response against *M. tuberculosis*, and it is directly related to the maintenance of the granuloma structure, the colonization bacillus, and the necrosis

TABLE 3 Contributions of the biomarkers in pleural effusion from patients with PITB and other diagnoses to components 1 and 2 in the principal-component analysis^a

Variable	Eigenvalue	
	Dim1	Dim2
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

^aShaded values represent the eigenvalues in dimensions 1 (Dim1) and 2 (Dim2) of the most important biomarkers in the component definition.

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

The cytokine pattern related to the Th2 effector phenotype was also evaluated. With the methodology employed, we did not detect significant levels of IL-4. This finding confirms the literature data that show little influence of this effector phenotype in TB cases (2, 36), although IL-4 concentrations in miliary TB have already been reported (6). In addition, our study has shown higher concentrations of IL-10 in the pleural fluid of patients with PITB than in serum, and the same was found for the non-TB group. However, the methodology used in this study was not able to identify which cells present in the pleural fluid were responsible for the increase of IL-10 concentrations, as well as the other cytokines. Geffner et al. showed an increased IL-10 production after stimulation of mononuclear cells in pleural fluid and peripheral blood with *M. tuberculosis* antigens and the decrease of this cytokine after removal of Tregs, providing evidence that Tregs are also responsible for the production of IL-10 from the pleural cavity (20).

Another important finding in our study was the quantification of TGF- β in serum and pleural fluid. This growth factor, secreted by monocytes, is a chemotactic agent for fibroblasts and plays an important role in extracellular matrix remodeling (38). One of the possible contributions of TGF- β to the pathophysiology of PITB is its ability to induce fibrosis, as shown in a study by Sasse and collaborators, where animals infected with *M. tuberculosis* showed increased pleural thickening in proportion to the increase in TGF- β (39). Seiscento and collaborators also found elevated TGF- β levels in serum and pleural fluid of PITB patients, which was associated 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 PITB patients, since TGF- β levels were found to be significantly higher in the pleural fluid of these patients than in that of 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 PITB patients compared to blood and pleural fluid in patients with other causes of exudative effusion. This finding may contribute to future investigations, indicating TGF- β as a possible biomarker to aid in the differential diagnosis of PITB.

Malignancy is the most prevalent etiology of exudative pleural effusion, preceded by TB (23, 41). In our study, the non-TB group was composed of around 70% (18/25) malignancies among exudative cases excluding TB, which in part could be explained by the characteristics of the recruitment unit (a tertiary care hospital). Atef et al. 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 PITB patients versus those with 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 report, a prevalence of the Th17 response in the pleural liquid from patients with lung cancer in comparison to those with TB was shown. 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 the lung cancer group compared to the PITB group (43). However, transcriptional analysis of cytokine genes highlighted an increased expression of the Th17 pattern in PITB patients versus those with common causes of exudative pleural effusion, including malignancies and parapneumonic effusion (44). Our results show high concentrations of IL-6 and TGF- β in the pleural compartment of PITB 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 concentrations of IL-6 and TGF- β and the low concentrations of IL-2, might favor the differentiation of this T-lymphocyte effector phenotype in PITB.

Some limitations in our study should be considered. First, it was conducted in a single center, imposing the need for 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 is reflected in the variable clinical characteristics inherent to each group in the study (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 *M. tuberculosis* infection. Based on this approach we could identify a predominance of cellular (Th1) immune-related response-pointing biomarkers with high potential for clinical use, which may increase the sensitivity of diagnosis and prompt TB treatment, especially in cases of difficult identification and distinction by conventional diagnostic methods.

ACKNOWLEDGMENTS

We thank the physicians of the Tuberculosis Outpatient Clinics of HUPE/UERJ, who contributed to the medical care of the patients included in the study, and we thank Cristiana Macedo for the revision of the manuscript.

Author contributions are as follows: conceptualization, Luciana Silva Rodrigues; data curation, Vinicius da Cunha Lisboa, Raquel da Silva Corrêa, and Isabelle Ramos Lopes; formal analysis, Vinicius da Cunha Lisboa, Raquel da Silva Corrêa, and Marcelo Ribeiro-Alves; funding acquisition, Rogério Lopes Rufino Alves and Luciana Silva Rodrigues; investigation, Vinicius da Cunha Lisboa, Thiago Thomaz Mafort, Ana Paula Gomes dos Santos, Rogério Lopes Rufino Alves, and Luciana Silva Rodrigues; methodology, Vinicius da Cunha Lisboa, Raquel da Silva Corrêa, Isabelle Ramos Lopes, and Thaís Porto Amadeu; project administration, Luciana Silva Rodrigues; resources, Rogério Lopes Rufino Alves, and Luciana Silva Rodrigues; software, Vinicius da Cunha Lisboa and Marcelo Ribeiro-Alves; supervision, Luciana Silva Rodrigues; validation, Marcelo Ribeiro-Alves; writing the original draft, Vinicius da Cunha Lisboa, Raquel da Silva Corrêa, Marcelo Ribeiro-Alves, and Luciana Silva Rodrigues; review and editing, Vinicius da Cunha Lisboa, Marcelo Ribeiro-Alves, and Luciana Silva Rodrigues.

We confirm that all relevant data are included in the article.

This work was supported by the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (grant no. 261101792014) (<http://www.faperj.br/>).

The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript. We declare that no competing interests exist.

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