Increased B cell activating factor is associated with B cell class switching in patients with tuberculous pleural effusion

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Abstract. B cell activating factor (BAFF), a member of the tumor necrosis factor family, is a key cytokine for B cell survival, a function that is essential for B cell maturation and memory. The expression levels of BAFF and its potential contribution to B cell maturation remain elusive in patients with tuberculous pleural effusion (TPE). The present study enrolled 40 healthy controls (HC) and 45 TPE patients, and investigated the levels of BAFF in the plasma and pleural effusion. Concomitantly, B cell subsets including naïve B cell ($CD19^+IgD^+CD27^-$), unswitched B cell ($CD19^+IgD^+CD27^+$), switched B cell ($CD19^+IgD^-CD27^+$), total memory B cell ($CD19^+CD27^+$), plasma B cell ($CD19^+IgD^-CD38^+CD27^+$) and transitional B cell ($CD19^+IgD^{dim}$ $CD38^+$) in peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) were assessed using multicolor flow cytometry. Finally, the associations between BAFF and each sub-group of B cells in TPE patients were analyzed. Compared with HC cases, an increased BAFF level and elevated frequency of switched B cell were observed in the blood and pleural effusion from patients with TPE. The proportions of naïve B cell, plasma B cell and transitional B cell were lower in the PFMCs of TPE patients. Furthermore, a significant correlation was observed between the level of BAFF, and the proportion of switched B cell in the peripheral blood and pleural effusion of TPE patients. These findings indicated that the B cell profile may be different in the pleural effusion, and BAFF may activate switched B cells to enhance the humoral immune responses in patients with TPE. Further studies are required to elucidate the underlying mechanisms and determine the potential immunotherapy of the BAFF-switched B cell axis.

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Abbreviations: BAFF, B cell activating factor; TPE, tuberculous pleural effusion; HC, health control; PBMC, peripheral blood mononuclear cell; PFMC, pleural fluid mononuclear cell; TB, tuberculosis; MTB, *Mycobacterium tuberculosis*; TST, tuberculin skin test; FBS, fetal bovine serum

Key words: B cell activating factor, BAFF, switched B cell, tuberculosis, TB, tuberculous pleural effusion, TPE

Introduction

Tuberculosis (TB) is a contagious disease and continues to be a major heath issue worldwide, especially in Asia and Africa (1,2). Although the global incidence of pulmonary TB has been reported to reduce over time, approximately 17% of the relapse and new cases of TB develop extra-pulmonary TB (3). Among all types of emerging extra-pulmonary TB, tuberculous pleural effusion (TPE) is the most frequent manifestation, accounting for about 5% of all forms of TB, and is the leading etiology of pleural effusion in many high TB prevalence areas (4,5). Our understanding of the pathogenesis of TPE has evolved. TPE once was thought to be an effusion resulting from delayed hypersensitivity reaction. Recent evidences suggest that it is a result of direct infection of *Mycobacterium tuberculosis (M. tuberculosis)* in the pleura which leads to the infiltration of inflammatory cells and chronic accumulation of fluid in pleural space (6-8). The pathogenesis of TPE involves intricate cellular and humoral immune responses, although the exact underlying mechanisms are not completely understood.

B cell activating factor (BAFF) is a novel member of the tumor necrosis factor family, a homotrimer expressed by T cells, dendritic cells and macrophages (9-11). BAFF is initially expressed on the cell surface and subsequently released as a soluble form after enzymatic cleavage (12). In *in vivo* and *in vitro* experiments, BAFF has been confirmed as a key cytokine in B cell homeostasis. BAFF deficient mice lack a mature B cell component (13). Recent evidence has indicated that it's indispensable for peripheral B cell survival (14), while excessive BAFF stimulation in humans contributes to the development of a variety of autoimmune diseases (15,16). The level of BAFF has been reported to increase in human active pulmonary TB (17). However, its potential contribution to the modulation of B cell maturation in patients with TPE remains elusive. We therefore detected the level of BAFF and B cell

compositions, and further investigated whether such changes are linked to *M. tuberculosis*-induced immune response.

Materials and methods

Study population and ethics statement. A total of 45 cases of TPE were enrolled from Shenzhen Third People's Hospital (Shenzhen, China). TPE was diagnosed if i) acid fast bacilli (AFB) staining or *M. tuberculosis* (MTB) cultures or MTB-DNA polymerase chain reaction of pleural effusion or pleural biopsy specimens showed positive; ii) or if parietal pleural biopsy specimens present typical histopathology characterized with tuberculous granuloma or caseous necrosis (18). A total of 40 cases of HC subjects who had received BCG vaccination at birth and showed a negative tuberculin skin test (TST) were recruited. All subjects were recruited from January 2016 to November 2016 in Shenzhen Third People's Hospital. Subjects with HIV infection, diabetes, cancer and autoimmune diseases were excluded from the study. At the time of sample collection, all of the TPE patients had not received any anti-TB therapy, corticosteroids or other non-steroidal anti-inflammatory drugs. The characteristics of both study cohorts are shown in Table I, there was no significant differences in terms of age range and gender ratio were noted between TPE patients (age range: 18-63 years; male/female: 1.0) and HCs (age range: 20-54; male/femal: 1.2). The study was approved by the Ethics Committee of Guangdong Medical University and Shenzhen Third People's Hospital, and written informed consent was obtained from all study subjects before their participation.

Isolation and preparation of peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs). PBMCs and PFMCs were isolated and prepared as previously reported (19). Briefly, approximately 10 ml pleural effusion collected from TPE patients and 5 ml peripheral blood samples from TPE patients and HCs were centrifuged at 2000 x g for 10 min at 4˚C. The supernatants were stored at -80˚C for future analysis. Cell pellets from pleural effusion were suspended in PBS and cellular components of the blood samples were used for the PBMC isolation by standard Ficoll-Hypaque (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) density gradient centrifugation (2000 x g for 20 min at 4˚C). PBMCs and PBFCs were then washed twice with pre-cooled PBS (pH 7.4; 4˚C), and then re-suspended in complete RPMI-1640 medium with 20% heated-inactivation fatal calf serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A cell viability of >95% was seen in all experiments as determined by try-pan blue exclusion.

ELISA. For quantitative ELISA assay, BAFF in plasma and supernatants of pleural effusion was tested using the Human BAFF Quantikine ELISA Kit (SBLYS0B; R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's protocol.

Flow cytometry analysis. The freshly isolated PBMCs and PFMCs were washed with PBS (4% FBS) and suspended at a concentration of $1x10^7$/200 μl, followed by staining with CD19-APC, IgD-FITC, CD27-PE-Cy7, CD38-APC-Cy7 (Biolegend, San Diego, CA, USA) for 30 min at 4˚C in the dark. Cells were then washed twice and re-suspended in 200 μl PBS (4% FBS). Within 2 h, the samples were acquired on a modified BD Canto II™ flow cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was performed using FlowJo software (Tree Star, Inc., Ashland OR, USA).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences in sex ratio of the two study cohorts were compared by Pearson's χ^2 test. Differences in age between the two study cohorts were evaluated by Student's t-test. Differences in BAFF level and the proportion of each B cell subset were evaluated by analysis of variance with Tukey's post hoc test for multiple comparisons. Correlations between two variables were analyzed by Spearman's analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

BAFF is increased in the plasma and pleural effusion of patients with TPE. BAFF were previously reported to increase in the development of human active pulmonary TB (17). It's uncertain whether BAFF is similarly increased in the patients with TPE. In this study, we detected the level of BAFF in plasma from 40 cases HCs and 45 cases TPE patients using a sandwich ELISA kit. We found that the level of plasma BAFF in TPE patients was 2.8-fold higher than that in HCs (Fig. 1). Concomitantly, we investigated the levels of BAFF in pleural effusion of these TPE patients, and BAFF level was higher in pleural effusion compared to that in plasma (Fig. 1).

Alteration of B subsets in PBMCs and PFMCs of patients with TPE. Gating strategies were set to evaluate B cell subsets (Fig. 2). Naïve B cells were classified as $CD19^+IgD^+CD27^-$, while total memory B cells were defined as $CD19^+CD27^+$, including an unswitched IgD^+ population and a switched IgD^- population. Plasma cells were identified as $CD19^+IgD^-CD38^+CD27^+$and transitional B cell as $CD19^+IgD^{dim}$ $CD38^+$. Definitions of B cell subsets are also listed in Table II (18,20).

We analyzed B cell profile to evaluate whether B cell subsets in peripheral blood or pleural effusion were altered among the study groups. In PBMCs, the proportions of naïve B cells, total memory B cell, unswitched B cell, plasma B cell and transitional B cell were all similar between the two study groups (Fig. 3). Compared to PBMCs in TPE patients, the proportions of total memory B cell and unswitched B cell were similar in PFMCs of TPE patients, but the proportions of naïve B cells, plasma B cell and transitional B cell were much lower in PFMCs of TPE patients. It is noteworthy that the proportion of switched B cell was increased in PBMCs of patients with TPE, and higher switched B cell proportion in PFMCs than that in PBMC was also seen in these patients (Fig. 3C). Thus, these results suggest that the B cell compartment were different in the peripheral blood of TPE patients, especially in pleural effusion of TPE. The increased switched B cell may play a major role in acquired immunity against *M. tuberculosis*.

Table I. Demographic characteristics of the study groups.

Characteristics	Healthy control group	Tuberculous pleural effusion group	P-value
Patient number	40	45	-
Male/female, n (ratio)	22/18 (1.2:1)	22/23 (1.0:1)	0.57
Age, years [medium (range)]	33 (20-54)	36 (18-63)	0.22

Table II. Definitions of B cell subsets.

Subset	Parameter
Naïve B cell	$CD19^+IgD^+CD27^-$
Unswitched B cell	$CD19^+IgD^+CD27^+$
Switched B cell	$CD19^+IgD^-CD27^+$
Total memory B cell	$CD19^+CD27^+$
Plasma B cell	$CD19^+IgD^-CD38^+CD27^+$
Transitional B cell	$CD19^+IgD^{dim}CD38^+$

CD, cluster of differentiation; Ig, immunoglobulin.

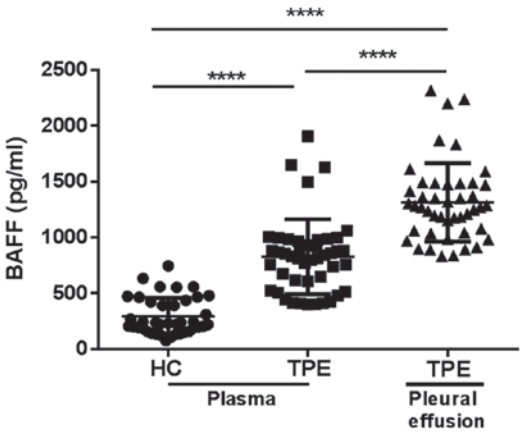

Figure 1. BAFF levels in plasma and pleural effusion of HCs and TPE patients. BAFF levels were measured by ELISA in the plasma of HCs (black circle, n=40) and patients with TPE (black square, n=45), and the pleural effusion of patients with TPE (black triangle, n=45). ****P<0.0001, as indicated. BAFF, B cell activating factor; HC, healthy controls; TPE, tuberculous pleural effusion.

BAFF level was corrected with the increased proportion of switched B cell in both blood and pleural effusion of patients with TPE. BAFF is a fundamental survival factor for the maturation and differentiation of B cell (13,14,21). To investigate how increased BAFF affects B cell survival in patients with TPE, we further analyzed the correlation of the BAFF level with the proportions of each B cell subset in PBMCs, PFMCs and both together (Fig. 4). We found that BAFF level had no correlation with naïve B cells, total memory B cell, unswitched B cell and transitional B cell in both PBMCs and PFMCs. Interestingly, BAFF level negatively correlated with plasma B cell when combining PBMCs and PFMCs, despite there was no significant correlation with either proportion alone (Fig. 4E). Moreover, the BAFF level had a high degree of correlation with the proportions of switched B cell both in PBMCs and PFMCs (Fig. 4C). These findings suggest an important role of BAFF in facilitating switched B cell proliferation and redistribution, and potentially inhibiting plasma B cell differentiation as a consequence of *M. tuberculosis*-induced immune activation in patients with TPE.

Discussion

Early researches on BAFF focused more on autoimmune diseases. It has been reported that up-regulated BAFF is involved in autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, and autoimmune encephalomyelitis (15,16,22). In human active pulmonary TB, the levels of BAFF and a proliferation-inducing ligand (APRIL) were markedly increased. The elevation of BAFF was closely related to the Th1 immune response (17). When co-infected with *Strongyloides stercoralis*, BAFF and APRIL level significantly diminished in comparison to these patients with latent TB (23). In our study, we found that BAFF levels were dramatic increased in TPE patients, particularly in the pleural effusion of these patients.

M. tuberculosis infection is well-known to influence T cell responses, whether such infection also modulates the maturation, differentiation and redistribution of B cell is worth being revealed. Li *et al* scanned the profiling B cell immune responses in TB patients, and they found the percentage of tissue-like memory B cells ($CD19^+CD10^-CD27^-CD21^-CD20^+$) was lower in the TB group than that in the HC group (24). Active TB has also been reported to be directly associated with high frequencies of Bregs ($CD19^+CD1d^+CD5^+$), which selectively inhibit Th17 activation by direct cell contact (25). In this study, we classified B cell subsets based on the expressions of surface cell markers, including CD19, IgD, CD27 and CD38. Compared with PBMCs from HC, we only found the proportion of switched B cell was significantly different, higher in patients with TPE. These apparent discrepancies reported across studies are most likely due to the use of an imperfect panel of markers to characterize the B cell subsets. Our study included previously unreported components of B cell in the pleural effusion of TPE patients. We found the proportion of switched B cell was significantly increased, while naïve B cells, plasma B cell and transitional B cell decreased in pleural effusion in comparison to peripheral blood of TPE patients. The different B cell compartments may be affected by selective activation and proliferation of B cell subsets during *M. tuberculosis* infection or redistributions of individual circulating B cell subsets between blood and pleural space.

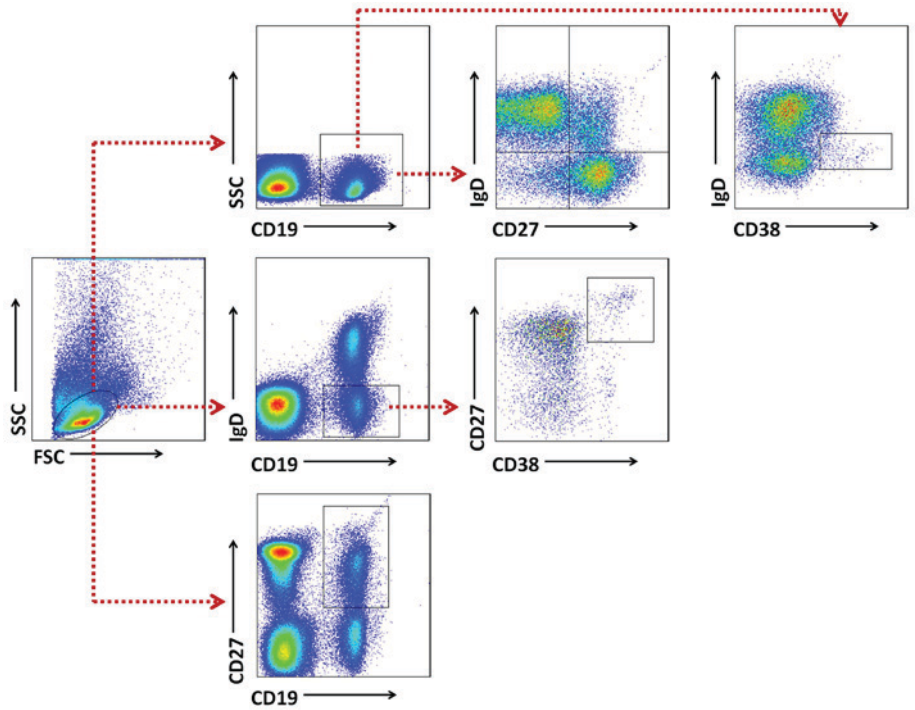

Figure 2. Representative flow cytometry gating strategy for identification of B cell subsets: Naïve B cell ($CD19^+IgD^+CD27^-$), unswitched B cell ($CD19^+IgD^+CD27^+$), switched B cell ($CD19^+IgD^-CD27^+$), total memory B cell ($CD19^+CD27^+$), plasma B cell ($CD19^+IgD^-CD38^+CD27^+$) and transitional B cell ($CD19^+IgD^{dim}CD38^+$). Ig, immunoglobulin; CD, cluster of differentiation.

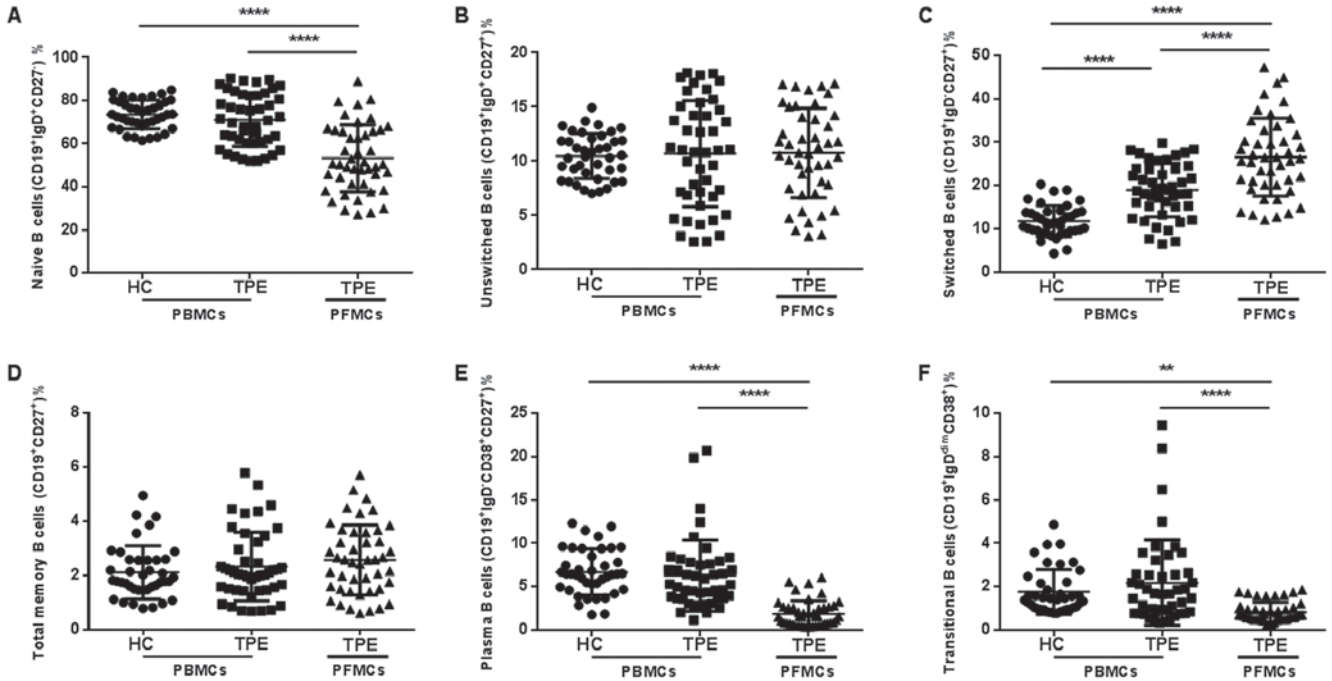

Figure 3. Proportion of each B cell subset in PBMCs and PFMCs. (A) Naïve B cell, (B) unswitched B cell, (C) switched B cell, (D) total memory B cell, (E) plasma B cell and (F) transitional B cell. Data are presented as the mean ± standard error of the mean. $^{**}P<0.01$ and $^{****}P<0.0001$, as indicated. PBMCs, peripheral blood mononuclear cells; PFMCs, pleural fluid mononuclear cells; HC, healthy controls; TPE, tuberculous pleural effusion.

The survival function of BAFF on B cells has been well documented, but which B cell subsets are benefited from the survival effect of BAFF is not clearly described. In the spleen of $BAFF^{-/-}$ mice, B cells fail to proceed from naïve B cells to the transitional type B cells (26,27), consistent with the idea that transitional B cells are exquisitely dependent on the activity of BAFF *in vitro* (28). Jaime and his colleagues reported for the first time that BAFF could considerably attenuate plasma B cell ($CD27^+CD38^+$ B cell) differentiation in response to T cell-independent activation (29). Currently, we found BAFF level was negatively correlated with plasma B cell when combining PBMCs and PFMCs, which may validate Jaime's stand point

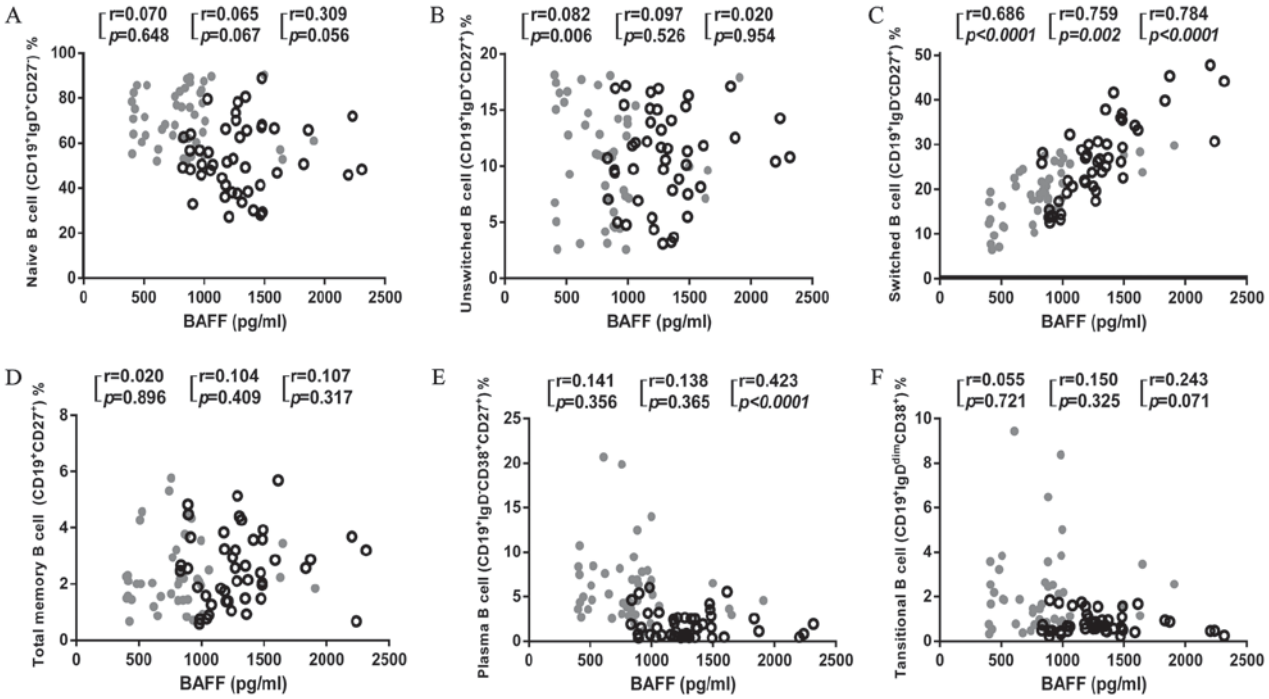

Figure 4. Correlations between BAFF levels and the proportions of B cell subsets in PBMCs and PFMCs. Graphical results of linear regression analysis of BAFF level vs. (A) naïve B cells, (B) unswitched B cells, (C) switched B cells, (D) total memory B cells, (E) plasma B cells and (F) transitional B cells in PBMCs (gray dots; r and P-values are those in the left column), PFMCs (black circle; data in the middle column) and the two combined (data is presented in the right-hand column). BAFF, B cell activating factor; PBMCs, peripheral blood mononuclear cells; PFMCs, pleural fluid mononuclear cells.

to a certain extent. Furthermore, BAFF level presented a high degree of correlation with the proportions of switched B cell in both PBMCs and PFMCs. It suggests that BAFF may facilitate switched B cell proliferation in patients with TPE.

Our study had several limitations. First, a marked limitation in the current study is the fact that we just found the correction of increased BAFF level and the proportions of switched B cell in PBMCs and PFMCs. The derivation that BAFF promote witched B cell proliferation in TPE patients were not proved *in vivo* or *in vitro*. Second, not all the B cell subset was covered in our research, such as the regulatory B cell, a subset has been reported to increase in active TB (24). Third, those pleural effusions with the other aetiologies should also been enrolled in the sample groups.

In conclusion, our study, for the first time, demonstrate a clear alteration of B cell composition in pleural effusion of TPE patients and that BAFF may activate switched B cell to enhance the humoral immune response to *M. tuberculosis* infection. The BAFF-switched B cell axis may be helpful to reveal the pathogenesis and provide a potential immunotherapy for TPE. But how BAFF affects switched B cell proliferation still need to be elucidated.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW, JFX, KDL, JAZ and GBL conceived and designed the experiments. XW, KDL, ZC, CC, ZGZ, YQL, HLL, RXL and BYZ performed the experiments, and XW and JFX analysed the data. XW wrote the paper, and XW and JFX critically reviewed the manuscript for intellectual content.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Guangdong Medical University and Shenzhen Third People's Hospital, and written informed consent was obtained from all study subjects prior to their participation.

Consent for publication

Written informed consent was obtained from all study subjects for the publication of any associated data.

Competing interests

The authors declare that they have no competing interests.

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