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Research Article

Pleural Mesothelial Cells-Induced Monocytes to the Pleural Cavity through the Effect of C3 Lytic Products in Tuberculous Pleural Effusion

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Background. The activation of complement is involved in monocyte recruitment in tuberculous pleural effusion (TPE), while the role of the cleavage product of complement C3 in this process needs further research. *Methods*. The expression of complement components in pleural biopsy specimens of TPE patients was measured. The concentration of cleavage products of complement was tested in TPE by ELISA. Moreover, the colocalizations of C3b and CR1, C3d and CR3, and CXCL12 and CXCR4 in monocytes and pleural mesothelial cells (PMCs) isolated from TPE were determined by an immunofluorescent assay. Monocyte chemotaxis assay was analyzed via transwell chambers. *Results*. Three pathways of the complement system were activated in tuberculous pleurisy. In patients with TPE, C3 lysis was more active than peripheral blood in pleural cavity. Tuberculous protein Mpt64 and anaphylatoxin C3a could significantly promote CXCL12 production in human PMCs isolated from TPE. C3b-CR1, C3d-CR3, and CXCL12-CXCR4 were colocalized in PMCs and monocytes from TPE. The recruitment of monocytes into TPE mediated by PMCs could be inhibited by anti-CR1, anti-CR3, and anti-CXCL12 monoclonal antibodies (mAbs). *Conclusions*. Complement activates strongly in TPE, and PMCs induced monocytes to the pleural cavity through C3a, C3b, and C3d.

1. Introduction

As a common form of tuberculous pleurisy, tuberculous pleural effusion (TPE), is characterized by the intense chronic accumulation of fluid and inflammatory cells, including monocytes/macrophages, T cells, and natural killer cells, in the pleural space [1].

During the process of pleural infections, pleural mesothelial cells (PMCs), as the first barrier to *Mycobacterium tuberculosis* (Mtb) invasion, play an essential role in the initiation and maintenance of the innate and adaptive immune response [2]. Mohammed et al. found that PMCs stimulated by Bacille Calmette–Guerin (BCG) or interferon-γ (IFN-γ) produced MIP-1a and MCP-1, which suggested that PMCs played an important role in the recruitment of mononuclear cells to pleural space [3]. Our previous study revealed that Mpt64 stimulated PMCs to secrete the chemokines CCL2, CCL7, and CX3CL1, which recruited nonclassical monocytes from peripheral blood to the pleural cavity in TPE [4]. Collectively, PMCs, as active participants in the process of immunopathology in the tuberculous pleurisy context, are capable of secreting various chemokines to recruit monocytes to the pleural cavity. Meanwhile, leukocytes from patients with tuberculosis exhibit higher levels of chemokine receptors and migration capacity [5].

Our previous research also found that the complement system was activated and C3a was deposited and C3aR was localized in monocytes and PMCs isolated from the pleural

effusion fluid of patients with TPE [4]. In addition, we found that C3a not only stimulated PMCs to secrete chemokines that recruit monocytes [4] but also enhanced Th17 responses via promoting the production of cytokines by CD16+ monocytes in TPE [6]. Our data suggests that complement C3a plays an important role in the monocyte migration induced by PMCs in TPE.

Complement system plays an important role in protection from Mtb infection. Complement system is activated through three pathways, namely, the classical, lectin and alternative pathways. These pathways result in the generation of multiple effector molecules, including anaphylatoxin C3a and opsonins C3b and C3d, which induce monocyte/macrophage chemoattraction and phagocytosis by binding to the receptors—C3aR, CR1, and CR3, respectively [7].

Ghosh and Saxena found that the intraperitoneal administration of Mtb resulted in a marked increase in the proportion and absolute numbers of CR3 (+) cells [8]. These results also indicate that the expression of the CR3 marker was significantly enhanced on all macrophages incubated with Mtb but was more pronounced on macrophages with internalized mycobacteria, and this upregulation may favour the production of a protective immune response against Mycobacterium tuberculosis [8]. Another study suggests that absence of CR3 results in binding and ingestion Mtb reduced by macrophages [9], whether the activation of opsonins and their receptors is involved in the monocyte migration induced by PMCs in TPE is unclear.

The purpose of this study was to explore the role of the lytic products of complement C3 on the pathogenesis of tuberculous pleurisy.

2. Materials and Methods

2.1. Subjects. The study protocol was approved by the Institutional Review Boards for Human Studies of Xiangya Hospital, Changsha, China, and informed written consent was obtained from all subjects (Number: 201703581, Approval date: March 10, 2017). For follow-up *in vitro* experiments, 20 patients were recruited from the TPE group. The characteristics of all subjects: the diagnostic and exclusion criteria for patients with TPE and transudate pleural effusion, and the methods for collecting and processing pleural tissue, pleural effusion, and blood samples were consistent with a previous study [4].

2.2. Cell Isolation. Pleural effusion and peripheral blood were collected and overlayed on Ficoll Paque-PLUS (17144002, GE Healthcare) and centrifuged at 2000 rpm for 10 min. The PBMCs at the interface of plasma and Ficoll were isolated. Mononuclear cells were allowed to adhere to 24-well plates (CLS3527, Costar) at 5×10^5 cells/well for 1 h at 37°C in warm RPMI-1640 medium (11875119, Gibco). After that could obtained the monocytes. The cells were then washed with warm PBS twice to purify. And monocytes were positively selected using magnetic beads against CD14 (130-050-201, Miltenyi Biotec). Typical purity of monocytes was 96% or greater. Isolated monocytes were incubated in RPMI 1640 containing 10% fetal bovine serum (26010074, Gibco) at 37°C in 5% CO_2.

For isolating PMCs, the cell pellets of TPE were resuspended in DMEM (11965092, Gibco) containing 10% fetal bovine serum, 20 ng/ml epidermal growth factor (236-EG-200, R&D systems), and 50 mg/ml gentamycin. The cells were seeded into 25 cm^2 flasks at a density of 1×10^4 cells/cm^2 and placed in an incubator at 37°C in 5% CO_2. After 24 h, the monolayers were washed with PBS to remove nonadherent cells, and fresh media was added. The monolayers were monitored until confluent (7–10 d), then trypsinized and subcultured for 5 to 6 passages. After each passage the cells grew to confluence within 6-7 d. In general, PMCs could be maintained for 6 to 7 passages before they became senescent. PMCs were identified by anticalretinin monoclonal antibody (mAb), using confocal microscopy.

2.3. Immunohistochemistry. To study complement activation, immunohistochemical staining was performed for C1q, factor B, factor P, MBL, membrane attack complex (MAC), CD46, C3, C5, C3a, C5a, C3b, C3d, CR1, CR3, CXCL12, CXCR4 were detected in all pleura specimens examined, while the expression of targets on 4 μm deparaffinized sections of formaldehyde-fixed pleural tissue using mouse antihuman monoclonal antibody C1q (ab71089, Abcam), rabbit antihuman polyclonal antibody factor B (ab192577, Abcam), rabbit antihuman polyclonal antibody factor P (ab186834, Abcam), mouse antihuman monoclonal antibody MBL (ab23457, Abcam), rabbit antihuman polyclonal antibody MAC (ab55811, Abcam), rabbit antihuman monoclonal antibody CD46 (ab108307, Abcam), mouse antihuman monoclonal antibody C3 (sc-28294, Santa cruz), rabbit antihuman polyclonal antibody C5 (ab217027, Abcam), mouse antihuman monoclonal antibody C3a (ab37230, Abcam), mouse antihuman monoclonal antibody C5a (ab11877, Abcam), mouse antihuman monoclonal antibody C3b (ab11871, Abcam), mouse antihuman monoclonal antibody C3d (053A-1149.3.1.4(1003), Thermofisher), rabbit antihuman polyclonal antibody CR1 (PL Laboratories, PL0301925), rabbit antihuman polyclonal antibody CR3 (NB110-89474SS, Novusbio), rabbit antihuman polyclonal antibody CXCL12 (ab9797, Abcam) and mouse antihuman monoclonal antibody CXCR4 (ab189048, Abcam) as primary antibodies. Sections of pleural tissue were dewaxed and rehydrated with an alcohol gradient and PBS. Antigen retrieval was performed with citrate (pH 6.0). Endogenous peroxidase was blocked with 3% H_2O_2 in water for 20 min, and nonspecific binding was blocked with diluted normal goat serum for 60 min, and then incubated with primary antibody above for 18 h at 4°C. Labeling was identified using the SP goat IgG kit (PV-6000, ZSGB-Bio, China), according to the manufacturer's instructions. The chromogenic reaction solution contained 3, 3′-diaminobenzidine (DAB) (ZLI-9018, ZSGB-Bio, China),

and counterstaining was performed with Mayer's hematoxylin (Solarbio, Beijing, China). Slides were viewed under imaging light microscope. As negative controls, primary antibodies were replaced by normal rabbit IgG or normal mouse IgG.

2.4. Immunofluorescence Staining. The slides of cells and tissues were embedded in paraffin according to standard pathology protocols. Mouse antihuman monoclonal antibody C3b (ab11871, Abcam), mouse antihuman monoclonal antibody C3d (053A-1149.3.1.4(1003), Thermofisher), rabbit antihuman polyclonal antibody CR1 (PL Laboratories, PL0301925), rabbit antihuman polyclonal antibody CR3 (NB110-89474SS, Novusbio), rabbit antihuman polyclonal antibody CXCL12 (ab9797, Abcam), and mouse antihuman monoclonal antibody CXCR4 (ab189048, Abcam). Appropriate species-matched Abs were used as isotype controls. As secondary Abs, ALexa Fluor®594-labeled affinity purified goat antimouse IgG (ab150116, Abcam) was used for labeling the mouse antihuman mAbs, and ALexa Fluor®488-labeled affinity purified goat antirabbit IgG (ab150077, Abcam) was used for labeling the rabbit antihuman mAbs. After permeabilizing with 0.3% Triton X-100 in PBS for 15 min at room temperature and washing with PBS, slides were incubated with 10% goat serum in PBS at 4°C overnight, and then incubated at 4°C overnight with the recommended concentrations of primary Abs by the manufacturer. After washing, slides were incubated with selected secondary Abs for 40 min at room temperature in the dark, correctly matched to the appropriate species. DAPI mounting medium (Vector Laboratories, Burlingame, CA) was used for cell nuclei staining. Finally, slides were viewed under imaging fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

2.5. Measurement of Cytokines and Chemokines by ELISA. The concentrations of complement components and chemokines, including C1q (E-EL-H6053), factor B (E-EL-H6056), factor (E-EL-H0817), MBL (E-EL-H1305), MAC (E-EL-H2376), CD46 (ab283877, Abcam), C3 (E-EL-H6054), C5 (E-EL-H0810), C3a (E-EL-H0818), C5a (E-EL-H0190), C3b (E-EL-H6054), C3d (E-EL-H5457), and CXCL12 (E-EL-H0052c), in both pleural fluids and plasma were measured by ELISA kits according to the manufacturer's protocols (all kits were purchased from Elabscience and Abcam). All samples were assayed in duplicate.

2.6. Real-Time qPCR. Monocytes isolated from TPE were incubated in the presence of medium alone or with MPT64 (20 μg/ml, Goodhere Biotechnology, Hangzhou, China), MPT64 + C3a (100 nM, 3677-C3-025, R&D Systems), MPT64+C3aRA (100 nM, SB290157, Calbiochem), or MPT64 + C3a + C3aRA. Total RNA was isolated for PCR, and the culture supernatant was harvested for ELISA. Total RNA was isolated using TRIzol (10296010, Invitrogen) according to the manual. After the quantification of the RNA concentration with a Nanodrop (Thermo Scientific, Darmstadt, Germany), equal concentrations of RNA were reverse transcribed using the Takara First Strand cDNA Synthesis Kit (6110A, Takara), and then the samples were subjected to real-time qPCR analysis using Power SYBR Green (Applied Biosystems, ABI 7100, Darmstadt, Germany).

2.7. Monocyte Chemotaxis Assays. Eight-micrometer pore polycarbonate filters in 24-well transwell chambers (CLS3464, Corning Costar) were used to perform the migration assay. Purified monocytes from blood (2×10^6/L) were added into the top chamber resuspended in RPMI 1640 medium in a final volume of 100 μl, the supernatants of PMCs were placed in the bottom chamber in a volume of 600 μl, and the chambers were incubated at 37°C in a 5% CO_2 atmosphere for 12 hours. Finally, the nonmigratory cells in the upper chamber were scraped off and washed gently in PBS, and the migratory cells on the bottom surface of the trans-well membrane were fixed in 4% paraformaldehyde for 10 min, underwent Wright staining, and were viewed and photographed under a digital microscope (Olympus BX51; Olympus, Tokyo, Japan). The migratory index was calculated by dividing the number of monocytes that migrated in response to the supernatants from cultured PMCs by the number of monocytes that migrated in response to the control. To investigate whether CR1, CR3, and CXCL12 contributed to monocyte migration, blocking experiments were performed by mixing monocytes with anti-CR1 mAb (5 μg/ml, BM2364, Origene), anti-CR3 mAb (10 μg/ml, 101213, Biolegend) or rabbit IgG irrelevant isotype control. To verify whether CXCL12 contributed to monocyte migration, blocking experiments were performed by mixing the supernatants with anti-CXCL12 mAb (10 μg/ml, ab9797, Abcam) or rabbit IgG irrelevant isotype control.

2.8. Statistics. Data are expressed as the mean ± SD. Student's *t*-test was used to compare two groups. Comparisons of the data between different groups were performed using a Kruskal–Wallis one-way ANOVA. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc, La Jolla, CA). Experiments were repeated at least twice to ensure reproducibility, and differences were considered statistically significant at *P* values of <0.05.

3. Results

3.1. Complement Components Deposition in the Pleural Tissue of TPE Patients. The deposition of complement components in pleural biopsy specimens obtained from TPE patients was assessed by immunohistochemistry. As shown in Figure 1(a), we revealed that the rich infiltration of motive factors (including C1q, factor B, factor P, and MBL), C3, C5, regulatory protein (CD46), and MAC were observed in the TPE patients' pleural tissue. Furthermore, we found that the levels of complement components (C1q, factor P, MBL, CD46, and SC5b-9) are higher in pleural effusion than that in the peripheral blood (Figure 1(b)), except for factor B (the

expression level in pleural effusion is only one sixtieth of that in peripheral blood). Taken together, these findings strongly supported that local complement activation in the pleural tissue was involved in the pathogenesis of TPE.

3.2. C3 was Cleaved into C3a, C3b, and C3d Increasingly in TPE. The production of protein fragments during complement activation was further verified by immunohistochemical detection, such as anaphylatoxins (C3a and C5a), opsonins (C3b and C3d), and their corresponding receptors CR1 and CR3 were observed in the pleural tissue of patients with TPE (Figure 2(a)). Moreover, the production level of complements (C3a, C3b, and C3d) was higher in pleural effusion than that in the peripheral blood of TPE patients, which was confirmed by our ELISA results (Figure 2(b)).

3.3. C3a Enhanced Recruitment of Monocytes via CXCL12-CXCR4 Axis Produced by PMCs. Due to the positive influence of C3a on CXCR4, which involved in opsonin receptor activation in monocytes [10], we measured the expression of CXCR4 and its ligand CXCL12 in pleural tissue. As expected, positive CXCL12 and CXCR4 staining were found in the plural tissue of TPE patients (Figure 3(a)). ELISA was used to compare CXCL12 production between pleural effusion and peripheral plasma of TPE patients. Our data indicated that the expression of CXCL12 was higher in pleural effusion than in plasma in TPE patients (Figure 3(b)). To evaluate whether C3a has an influence on CXCL12 production in the context of TPE, we used recombinant human MPT64 and C3a to treat the PMCs mentioned above. The ELISA and RT-qPCR results demonstrated that MPT64 stimulated the release of chemokine CXCL12 from PMCs isolated from TPE (Figure 3(c)). Furthermore, our data revealed that C3a enhanced the effect of MPT64 on the expression of CXCL12, which can be reduced by the C3a inhibitor (Figure 3(c)). In addition, coexpression of CXCL12-CXCR4 was observed by immunofluorescence in PMCs and monocytes isolated from TPE (Figure 3(d)). A coculture system utilizing PMCs and monocytes from the pleural effusion of TPE patients was used to simulate the pleural cavity of a person with TPE. We found that the supernatant of PMCs treated with the anti-CXCL12 mAbs was significantly suppressed monocyte chemotaxis (Figure 3(e)). Taken together, our results indicated that the chemokine CXCL12 might be responsible for the recruitment of monocytes into TPE.

3.4. PMCs Recruit Monocytes via C3b-CR1 and C3d-CR3 Axes. As the complement pathway was activated in TPE, we further investigated the colocalization of C3b-CR1 and C3d-CR3 in PMCs and monocytes from TPE by immunofluorescence. C3b-CR1 and C3d-CR3 were colocalized in the cytoplasm of PMCs isolated from TPE, and the same results were also found in monocytes isolated from TPE (Figure 4(a)). This result indicated that opsonins are produced by PMCs and localized on the cell surface. We hypothesized that monocytes could migrate into the pleural space when stimulated by CR1 or CR3. To test this hypothesis, we performed an *in vitro* transwell assay to examine the monocytes' chemotaxis. A coculture system containing PMCs and monocytes from the pleural effusion of TPE patients was established in the absence or presence of anti-CR1or anti-CR3. We found that the monocytes treated with the anti-CR1 and anti-CR3 mAbs negatively affected monocyte chemotaxis (approximately a two-fold decrease). Taken together, our results indicated that the opsonins C3b and C3d might be responsible for the recruitment of monocytes into TPE (Figure 4(b)).

4. Discussion

Complements, as a crucial part of the innate immune system, are activated during Mtb infection [11]. Studies have indicated that the complement system participated in the defense against Mtb, but the exact roles of this system in TPE have not been fully established. Salomaa et al. found that the concentrations of pleural fluid C3 and C4 were significantly higher in tuberculous pleural fluid than in rheumatic pleurisy [12]. In this study, higher levels of C3 and MAC in tuberculosis effusions were detected than in plasma (Figure 1(b)). In addition, our results showed higher levels of complement components, including C3a and C5a, in pleural effusion than in peripheral blood in TPE patients (Figures 1(b) and 2(b)). In particular, our results showed that the concentration of fB in the blood was 60 times higher than that in pleural fluid, and the levels of C3 and C5 are also lower in the pleural fluid than in the plasma. We speculated that this might be due to the stronger complement activation in thoracic cavity. For example, fB cleaves to Ba and Bb, C3 cleaves to alternative pathways products (C3b and C3d), and C5 cleaves to C5a [13]. Our speculation accorded with the result of Salomaa et al. which reported that complement factor Ba was higher in pleural fluid than in plasma in TPE [12]. Cai et al. confirmed that C1q mRNA expression was significantly higher in pleural fluid mononuclear cells (PFMCs) and bronchoalveolar lavage fluid (BALF) than in matched peripheral blood mononuclear cells (PBMCs) [14]. These findings indicated that C1q expression is significantly increased at local TB lesions which were consisted with our ELISA results (Figure 1(b)). However, it remains unclear whether these increased complement components are produced locally at inflammatory sites or systemically in organs such as the liver. In the process of TB infection, Mtb releases a series of immunogenic proteins, including ESAT-6, Ag85b, and Mpt64. Mpt64 has been reported to function as a mycobacteria-specific virulence factor in the tuberculosis microenvironment. When C3 is activated, the complement signaling cascade is further activated. Complement anaphylatoxin C3a, viewed as a proinflammatory mediator, participates in inflammatory processes by binding to C3aR to recruit inflammatory cells and enhancing the inflammatory reaction [15, 16]. In fact, we observed that C3a significantly enhanced Mpt64-mediated CXCL12 production in human PMCs in the present study. This finding suggests that complement is strongly activated in localized inflammatory sites, especially in the pleural cavity.

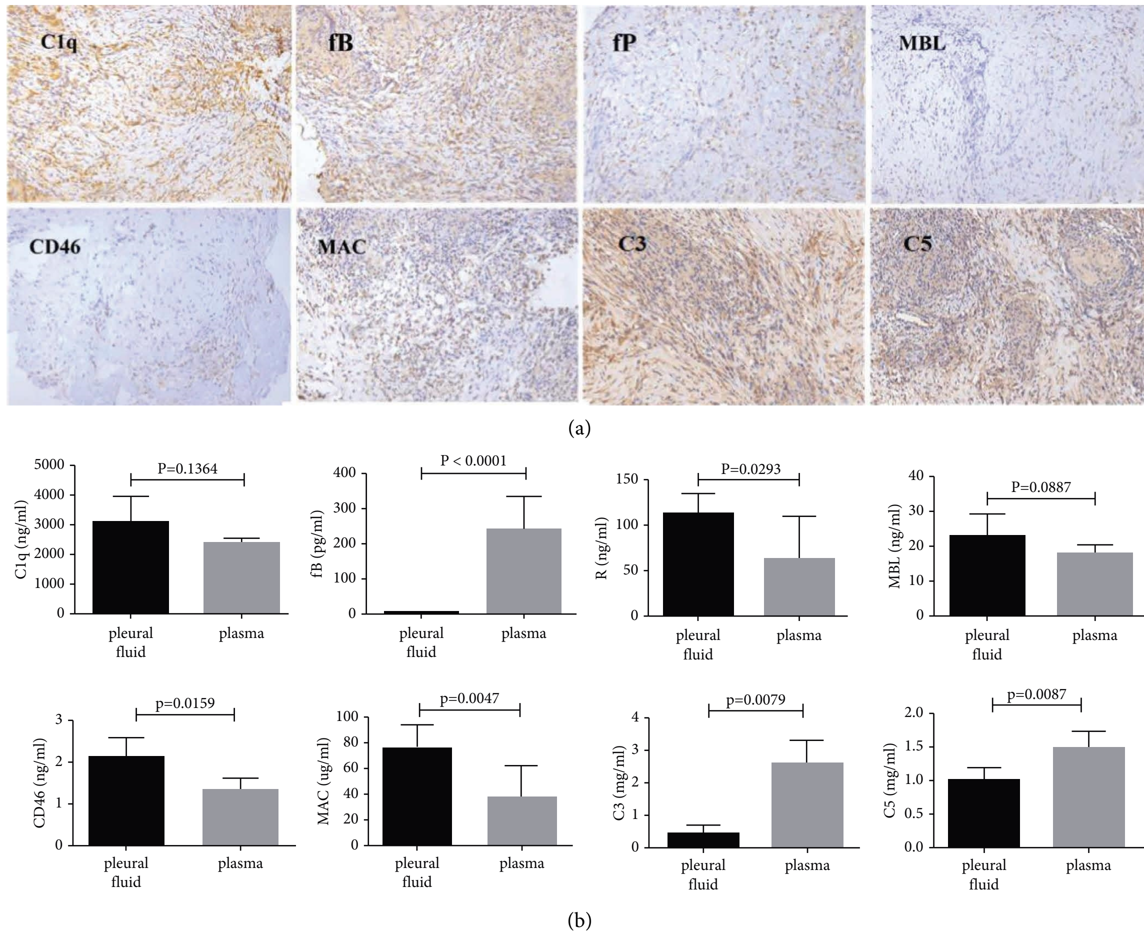

Figure 1: Complement system was activated in human tuberculous pleurisy. (a) Immunohistochemistry staining images of three complement activation pathways, including early markers in the activation pathway (C1q, factor B factor P, and MBL), the final product (MAC) a regulatory protein (CD46), effector molecules (C3 and C5) in human pleural biopsy samples from TPE patients (original magnification, ×200) ($n = 4$). (b) The concentrations of complement components in pleural fluid and plasma from TPE patients were measured by ELISA ($n = 20$).

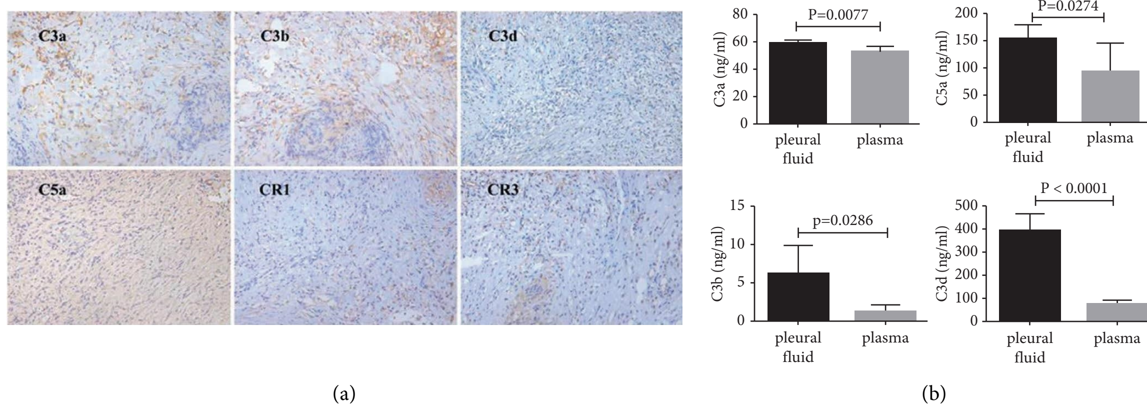

Figure 2: The concentrations of complement pyrolysis products were found higher in pleural effusion than in plasma from TPE patients. (a) Complement pyrolysis products, including C3a, C3b, C3d, C5a, and opsonin receptors (CR1 and CR3) were detected in human tuberculosis pleural biopsy samples by immunohistochemistry. (Original magnification, ×200) ($n = 4$). (b) Higher levels of complement pyrolysis products were found in pleural effusion than in plasma in TPE patients ($n = 20$). The concentrations of complement pyrolysis products in pleural fluid and plasma from TPE patients were measured by ELISA ($n = 20$).

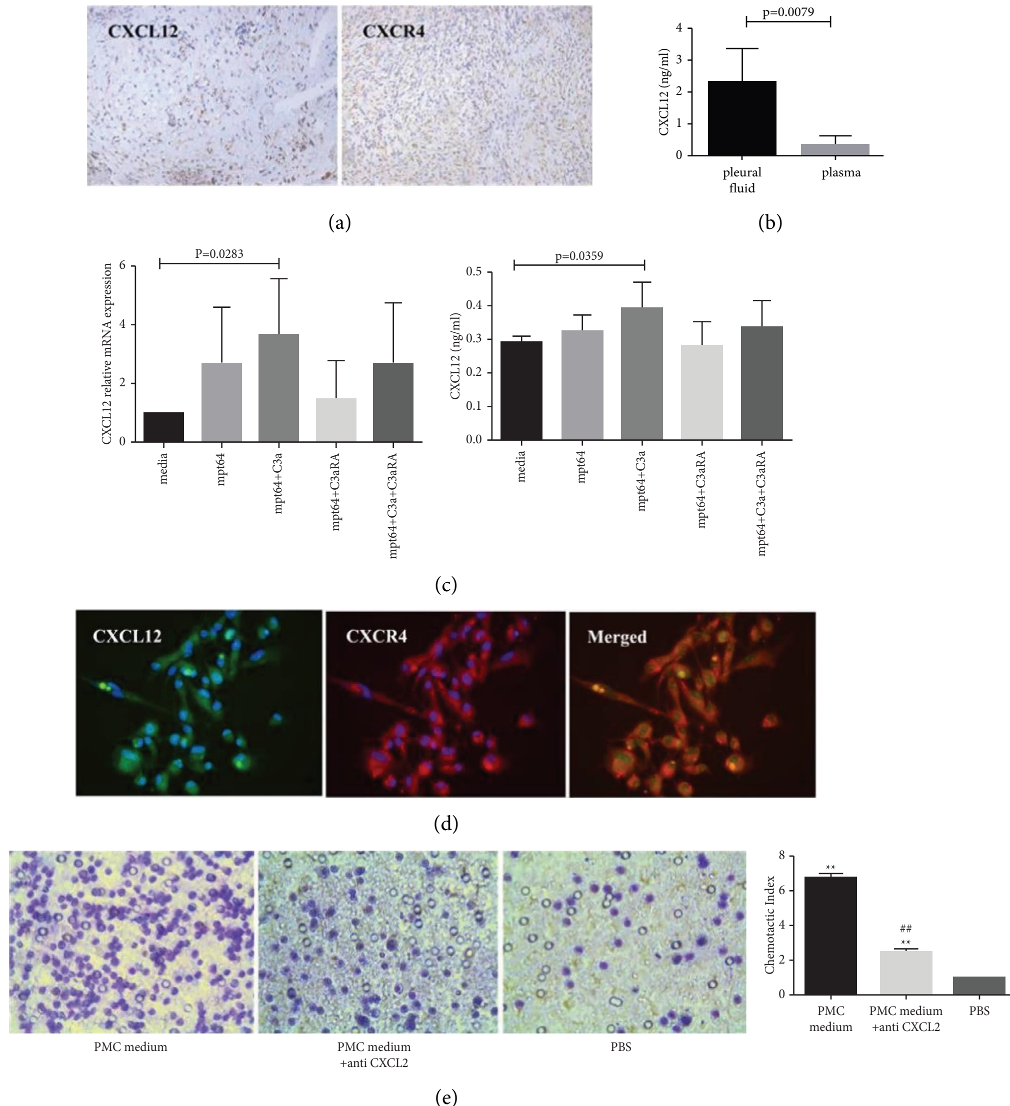

Figure 3: Monocyte migration was inhibited by antibodies that blocked CXCL12. (a) CXCL12 and CXCR4 staining by immunohistochemistry in human pleural biopsy (original magnification, ×200) ($n = 4$). (b) The concentration of CXCL12 in pleural fluid and plasma from TPE patients was measured by ELISA ($n = 20$). (c) CXCL12 produced by PMCs was measured by PCR and ELISA after Mpt64 and anaphylatoxin activation. PMCs were incubated for 24 hours in control media or in media with Mpt64 (20 μg/ml) and with or without human C3a (100 nM) or C3aRA (100 nM) ($n = 4$). (d) Coexpression of CXCL12-CXCR4 in PMCs and monocytes from TPE was detected by immunofluorescence (original magnification, ×400) ($n = 4$). (e) Monocytes were seeded into the top chamber of a transwell system, and the supernatant from PMCs cultured with anti-CXCL12 antibody or PBS were placed in the bottom chamber. The migratory index was calculated by dividing the number of monocytes that migrated in response to the supernatants from cultured PMCs by the number of monocytes that migrated in response to the control. *vs the MO-PBS group, $^{**}P < 0.01$. $^{\#}$vs the MO-PMC group, $^{\#\#}P < 0.01$ ($n = 4$).

Complement is mainly produced by liver hepatocytes and delivered to the systemic blood circulation. However, it has long been known that local complement, produced by various cell types, is also a source of complements. Monocytes and macrophages, including tissue-resident macrophages, have been shown to produce the full array of complement components [17]. Monocytes and neutrophils are characterized by infiltration from the vascular compartment to sites of inflammation within the tissue spaces [18]. Some scholars have demonstrated that complement activation participated in Mtb infection and encourages monocyte chemotaxis [19]. Our results also indicated that complement receptors, including CR1 and CR3, were expressed in monocytes and mediated the recruitment of monocytes into the pleural cavity (Figure 4). CR1, as a receptor for C3b and C4b, is expressed on the membranes of many cell types, including erythrocytes, granulocytes, monocytes, and macrophages [20]. Via binding to its receptor, erythrocytes carries immune complexes (C3b and C4b) from the circulation to the spleen and liver, where the immune complexes are transferred to phagocytic cells [21]. CR3 acts as a receptor for iC3b and C3d, which are expressed on monocytes/macrophages and granulocytes, and performs functions not only in

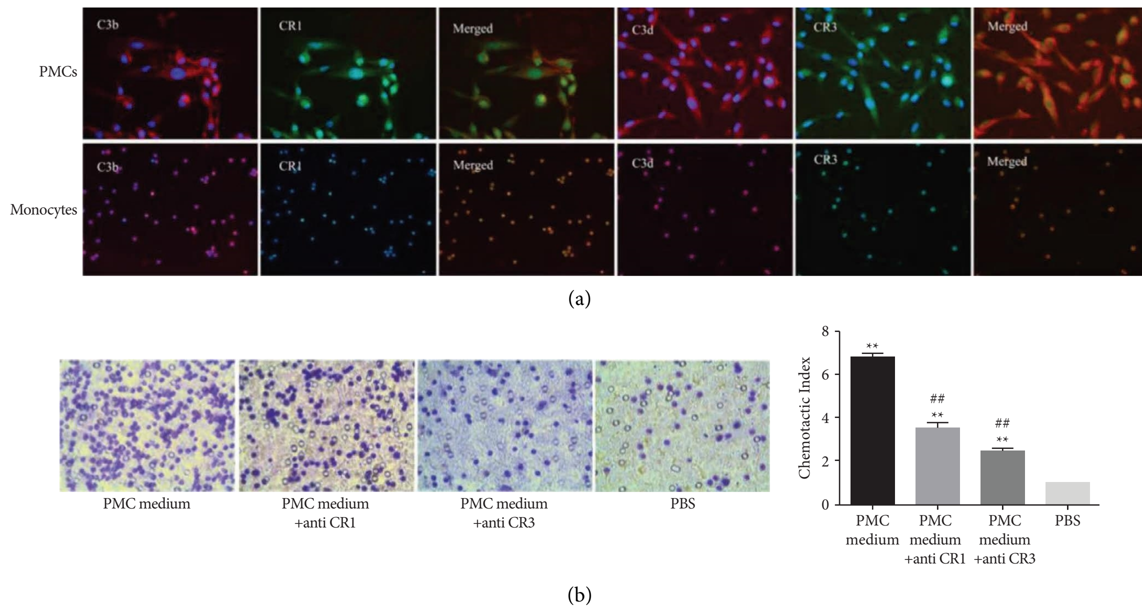

Figure 4: Monocyte migration was inhibited by antibodies that blocked CR1 and CR3. (a) Representative colocalization of C3b-CR1 and C3d-CR3 in PMCs and monocytes from TPE. The cells from TPE were immunostained with C3b/C3d (red) and CR1/CR3 (green), and the nuclei were stained with DAPI (blue) ($n = 4$). (b) To evaluate the effect of C3b-CR1 and C3d-CR3 axes on monocytes, monocytes were seeded into the top chamber with or without anti-CR1 antibody or anti-CR3 antibody, and the supernatants from cultured PMCs were placed in the bottom chamber of the transwell system. The migratory index was calculated by dividing the number of monocytes that migrated in response to the supernatants from cultured PMCs by the number of monocytes that migrated in response to the control. *vs the MO-PBS group, $^{**}P < 0.01$. $^{\#}$vs the MO-PMC group, $^{\#\#}P < 0.01$ ($n = 4$).

phagocytosis but also in leukocyte trafficking and migration, synapse formation, and costimulation [22, 23]. Previous studies have shown that CR1 and CR3 are associated with phagocyte migration [24]. By expressing opsonin receptors, peripheral blood monocytes migrate across the vascular endothelium and extracellular matrix to the alveolar epithelium and adhere to the alveolar epithelium to kill Mtb [25]. Studies have reported that the preincubation of neutrophils from healthy donors with anti-CD35 antibodies reduced the internalization of Mtb in neutrophils [26]. Rosen and Gordon found that both the accumulation of polymorphonuclear (PMN) and the increase in vascular permeability were abolished by the pretreatment of mice with a mAb against CR3 [27]. It is reported that, another function of C3b-CR1 and C3d-CR3 axes is pathogen recognition by phagocytic cells. Via binding to pathogens by receptors, opsonins such as C3b and C3d could mediate the killing of bacteria and apoptotic cells by phagocytes [28]. A study showed that pulp fibroblasts express and secrete the complement C3b fragment, which is fixed on bacteria and recognized by its CR1 receptor on macrophages, and then induce bacterial to be phagocytosed [29]. In the content of tuberculous infection, Mtb can induce an increase in opsonin receptor expression in alveolar macrophages and peritoneal macrophages, thus enhancing the recognition of opsonin-labeled bacteria and phagocytosis for killing and scavenging [8]. Similarly, our results showed that the presence of anti-CR1 and anti-CR3 monoclonal antibodies in transwell experiments abrogated the transmigration effect of CR1 and CR3 on monocytes (Figure 4(b)). According to these data, monocyte recruitment via opsonins in TPE may also be involved in the recognition and phagocytosis of Mtb.

Host immunity plays a critical role in the control of infection via granuloma formation at the site of infection, and this process may depend on the activation of T lymphocytes and the migration of monocytes and macrophages [30]. The protective immune response to Mtb is regulated by cytokines and chemokines, which play a pivotal role in determining the course of infection with Mtb [31]. CXC chemokines, such as CXCL8, CXCL10, and CXCL11, induce memory T cell proliferation and control immune reactions via the polarization of Th1 cells involved in granuloma development [32–34]. Consistent with this notion, our previous observations showed that anaphylatoxins stimulated PMCs to secrete CCL2, CCL7, and CX3CL1, which recruits CD14+CD16+ monocytes to the pleural cavity in TPE [4]. Another study indicated that CXCL12 expression levels were increased after Mtb infection, and the expression of CXCL12 is associated with the response to antitubercular treatment [35]. Luo et al. found that concentrations of CXCL12 were higher in patients with TPE than in patients with benign pleural effusion (BPE) and MPE [36]. Wysoczynski et al. found that C3a and $C3a_{des\text{-}Arg}$ enhance CXCL12-dependent megakaryocyte migration and adhesion [37]. In this study, we also found elevated CXCL12 expression in TPE and demonstrated that C3a can promote the Mpt64-induced CXCL12 expression in PMCs, which further enhanced the migration of CXCR4 expressing monocytes into the pleural cavity.

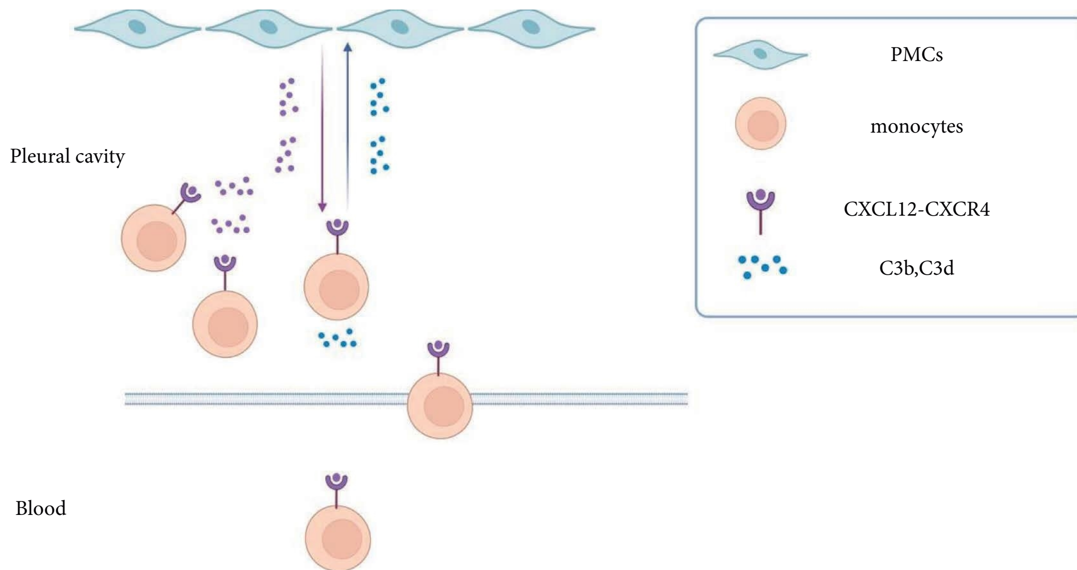

Figure 5: Overview: C3 lytic products enhanced recruitment of monocytes via CXCL12-CXCR4 axis produced by PMCs in TPE.

Previous studies have shown that opsonins can affect the production of chemokines in cells [38, 39]. Studies have reported that artificial opsonin increases neutrophil IL-8 chemokine production during phagocytosis [40], while purified iC3b and C4b were found to inhibit IFN-β-induced CXCL10 production by granulocytes [38]. In addition, the interaction between iC3b-opsonized apoptotic cells and iDCs downregulated the expression of CCR2 and CCR5 and upregulated the expression of CCR7 [39]. In this study, we demonstrated that PMCs can be labeled by opsonins and stimulated by C3a to produce chemokines and then participate in the chemotaxis of monocytes to tuberculous pleural cavity.

However, our study has several limitations. First, we just compared the complement components of pleural fluid and plasma in TPE, other exudate pleural effusion or transudate pleural effusion were not studied in our research. Second, although the important value of opsonin-mediated complement activation in the mechanism of TPE *in vitro* was shown in this study, it remains unclear whether the same function occurs *in vivo*.

In summary, our study suggests that the complement in pleural cavity is strongly activated in patients with tuberculous pleural effusion, and then the opsonins-labeled PMCs (C3b and C3d) will bind to opsonin receptors (CR1 and CR3) expressing monocytes, inducing the migration of monocytes into the pleural cavity of TPE patients. In addition, our results indicate that the CXCL12-CXCR4 axis also participates in the pathogenesis of TPE by recruiting monocytes (Figure 5). These data add to the emerging role of opsonins in the progress of TPE and blocking opsonins or CXCL12-CXCR4 axis might protect the formation of TPE by preventing monocytes infiltration. The precise functions of complement in TPE immunopathology and disease progression warrant further investigation.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Ethical Approval

This study was approved by medical Ethics Committee of Xiangya Hospital of Central South University (Number: 201703581, Approval date: March 10, 2017). All methods were performed in accordance with the Declarations of Helsinki.

Consent

All the enrolled patients signed informed consent forms.

Conflicts of Interest

All authors declare that they have no conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Authors' Contributions

Lisha Luo performed the experimental work, analyzed the data, and wrote the manuscript. Juntao Feng, Shuanglinzi Deng, Xinyue Hu, and Bingrong Zhao helped performed experiment and analyzed the data. Wei Tang and Xiaozhao Li designed the study, supervised the study, and critically revised the manuscript. All authors read and approved the final manuscript. Wei Tang and Xiaozhao Li these authors contributed equally to this work.

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