Monocytes subtypes from pleural effusion reveal biomarker candidates for the diagnosis of tuberculosis and malignancy

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Abstract

Background: Pleural effusion is a common clinical condition caused by several respiratory diseases, including tuberculosis and malignancy. However, rapid and accurate diagnoses of tuberculous pleural effusion (TPE) and malignant pleural effusion (MPE) remain challenging. Although monocytes have been confirmed as an important immune cell in tuberculosis and malignancy, little is known about the role of monocytes subpopulations in the diagnosis of pleural effusion.

Methods: Pleural effusion samples and peripheral blood samples were collected from 40 TPE patients, 40 MPE patients, and 24 transudate pleural effusion patients, respectively. Chemokines (CCL2, CCL7, and CX3CL1) and cytokines (IL-1β, IL-17, IL-27, and IFN-γ) were measured by ELISA. The monocytes phenotypes were analyzed by flow cytometry. The chemokines receptors (CCR2 and CX3CR1) and cytokines above in different monocytes subsets were analyzed by real-time PCR. Receiver operating characteristic curve analysis was performed for displaying differentiating power of intermediate and nonclassical subsets between tuberculous and malignant pleural effusions.

Results: CCL7 and CX3CL1 levels in TPE were significantly elevated in TPE compared with MPE and transudate pleural effusion. Cytokines, such as IL-1β, IL-17, IL-27, and IFN-γ, in TPE were much higher than in other pleural effusions. Moreover, CD14+CD16++ nonclassical subset frequency in TPE was remarkably higher than that in MPE, while CD14++CD16+ intermediate subset proportion in MPE was found elevated. Furthermore, CX3CL1-CX3CR1 axis-mediated infiltration of nonclassical monocytes in TPE was related to CX3CL1 and IFN-γ expression in TPE. Higher expression of cytokines (IL-1β, IL-17, IL-27, and IFN-γ) were found in nonclassical monocytes compared with other subsets. Additionally, the proportions of intermediate and nonclassical monocytes in pleural effusion have the power in discriminating tuberculosis from malignant pleural effusion.

Conclusions: CD14 and CD16 markers on monocytes could be potentially used as novel diagnostic markers for diagnosing TPE and MPE.
INTRODUCTION

Pleural effusion remains a common medical problem encountered in clinical practice, including tuberculosis, malignancy, infection, and systemic autoimmune disease. Currently, clinical identification methods exhibit various limitations in efficiently discriminating pleural fluid. Therefore, a novel biomarker is required for an accurate and objective diagnostic method for TPE and MPE.

As essential effectors of the immune system, monocytes contribute to the pathogenesis of inflammatory diseases. Human blood monocytes are heterogeneous and conventionally subdivided into three subsets based on surface CD14 and CD16 expression: the classical (CD14++ CD16−), intermediate (CD14++CD16+), and nonclassical (CD14−CD16++) monocytes. A recent study revealed that the CD163+CD14++ monocytes could be a potential immune diagnostic marker for MPE and has better assay sensitivity than that of cytological analysis. Our previous study found that nonclassical monocytes were significantly increased in patients with tuberculosis pleurisy. However, the role of monocytes subtype in differentiating the etiology of pleural effusion has not been thoroughly explored.

It is well documented that chemokines bind to their respective receptors and then mediate different monocytes recruited to the site of infection: the classical monocytes respond to CCL2 and CCL7 signals via CCR2, the nonclassical monocytes express CX3CR1, which pairs with CX3CL1, while the intermediate monocytes express all of these chemokines above. Antonia et al. demonstrated that CCL2 blockade was effective against experimental MPE via inhibiting immune and vascular MPE-related phenomena, such as inflammation, new blood vessel assembly, and plasma extravasation into the pleural space. Georgios et al. revealed that CCL2 was a potent inducer of mononuclear recruitment, vascular permeability, and angiogenesis in the formation of MPE. A recent study revealed that CX3CL1 in plasma was associated with an increased risk of unfavorable outcomes in pulmonary tuberculosis. Moreover, CX3CR1 was also reported to mediate the recruitment of monocytes during respiratory infections with Mycobacterium tuberculosis. However, the relationship between monocytes subtype and different chemokines axis in MPE and TPE has been poorly examined.

The intermediate monocytes numbers appear to be positively associated with disease severity based on clinical scores or tissue damage, particularly in inflammatory diseases. Studies verified that the intermediate subset was generally termed “pro-inflammatory” monocytes because of their ability to produce high amounts of TNF-α, IL-1β, and IL-6. Our previous study also found that cytokines (IL-1β, IL-17, and IL-27) release declined when blocked with anti-CCL2, CCL7, and CX3CL1 in nonclassical monocytes isolated from TPE, which suggested that chemokines may be associated with the cytokines produced by monocytes in pleural effusion. Thus, the relationship between chemokines in pleural fluids and cytokines released by monocyte subtype needs further elucidated.

In the present study, we examined the concentrations of chemokines (CCL2, CCL7, and CX3CL1) and cytokines (IL-1β, IL-17, IL-27, and IFN-γ) in tuberculosis and non-tuberculosis pleural fluids and analyzed the distribution of different monocytes phenotypes in both MPE and TPE. We aimed to investigate whether analyzing the proportion of monocytes subset help differentiate the etiologies of pleural effusion.

MATERIALS AND METHODS

2.1 Subjects

The protocol was approved by the Ethics Committee of Xiangya Hospital, Changsha, China (201703581). All the enrolled patients signed informed consent forms. 40 TPE, 40 MPE, and 24 transudate pleural effusion patients were recruited from December 2018 to April 2022.

The diagnosis and exclusion criteria referred to previous studies. All TPE patients were diagnosed by the growth of Mtb from their pleural fluid or by demonstration of granulomatous pleurisy in closed pleural biopsy specimens, without evidence of other granulomatous diseases. All MPE patients were evidenced by the demonstration of malignant cells in pleural fluid or/and on the closed pleural biopsy specimen. The heart failure diagnosis was confirmed according to the European Society of Cardiology (ESC) guidelines by clinical examination, medical history, and echocardiographic examination by one of four experienced cardiologists. Patients were excluded if they had received any invasive procedures directed into the pleural cavity within 3 months before hospitalization. At the time of sample collection, none of the patients had received any anti-cancer therapy, corticosteroids, or other nonsteroid anti-inflammatory drugs. None of these patients had any other system diseases, such as renal, hepatic, or connective tissue diseases.

2.2 Sample collection and process

Patients enrolled received a standard thoracentesis technique within 24 h after hospitalization. 500–1000 ml of pleural fluid samples were collected in heparin-treated tubes during this procedure. Also, 10 ml of peripheral blood were drawn simultaneously. The supernatants of pleural fluid and serum were stored at −80°C after centrifuged at 1200 g for 5 min for later ELISA assays. Cell pellets of pleural fluid were then resuspended in PBS for the following flow

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cytometry assay and cell sorting. Cell pellets of peripheral blood were washed with PBS and then resuspended in red blood cell lysis buffer for 5 min. After PBS wash, cells in the blood were resuspended in PBS for the following flow cytometry.

2.3 | Flow cytometry

The expression markers on monocytes from pleural effusion and blood were determined by flow cytometry after surface staining with APC-Cy7 mouse anti-human CD45 mAb, FITC mouse anti-human CD14 mAb, and PE-Cy7 mouse anti-human CD16 mAb (BD Biosciences). Different monocyte subsets were identified within the SSC-A^hi CD45^+ cells and gated for the CD14^++CD16^- classical, CD14^++CD16^- intermediate and CD14^CD16^++ nonclassical subsets. Flow cytometry was performed on a BD FACS CantollFlow cytometer and analyzed by Flowjo 10.0.

2.4 | Cell isolation

Cell pellets were overlaid on Ficoll Paque-PLUS (GE Healthcare) and centrifuged at 800g for 10 min. The pleural effusion mononuclear cells (PFMCs) at the interface of supernatant and Ficoll were isolated. BD FACS Aria II (BD Biosciences) was then used to sort different monocyte subsets from PFMCs. Isolated monocytes were incubated in RPMI 1640 (Gibco, Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco) at 37°C in 5% CO₂.

2.5 | Measurement of chemokines and cytokines

The concentrations of chemokines (CCL2, CCL7, and CX3CL1) and cytokines (IL-1β, IL-17, IL-27, and IFN-γ) in pleural fluid were measured by ELISA kits according to the manufacturer’s protocols (All kits were purchased from Raybiotech, eBioscience or Elabscience).

2.6 | PCR

Monocytes were lysis by Trizol (Life Technologies), and total RNA was isolated by standard methods. Takara First Strand cDNA Synthesis Kit was applied to reverse transcription according to the instruction. Then cDNA was subjected to real-time qPCR analysis, using Power SYBR Green (Applied Biosystems, ABI 7100, Darmstadt, Germany). Primers are listed below: IL-1β: forward, ATGATGCGTTATACAGTTGCAA; reverse, GTGGGAGATCGTAGCTGGA; IL-17: forward, GCAATGAGGACCCTGAGAGA; reverse, TGGATGGGGACAGAGTTCAT; IL-27: forward, CTGTGCACTCTCTGCTATGG; reverse, GGTGCGCTGGCCCTGAC; IL-17: forward, ATGATGGCTTATTACAGTGGCAA; reverse, GTCGGAGATCGTAGCTGGA. δCT = CT - CT_GAPDH, ΔΔCT = CT - CT_mean GAPDH of transudate relative expression = 2^-ΔΔCT.

2.7 | Monocytes chemotaxis assays

The 8-μm Transwell chambers (Corning Costar) were used to perform the migration assay. 600μl TPE supernatant was added to the bottom chamber. 2×10^5 purified nonclassical monocytes isolated from TPE were suspended in 100μl RPMI 1640 medium and plated into the top chamber. After incubating at 37°C in 5% CO₂ atmosphere for 3 h, the cells through the membrane to the lower membrane surface were fixed in 4% paraformaldehyde for 10 min and stained with Wright-staining, then viewed and photographed under a digital microscope (Olympus BX51). To investigate whether the CX3CL1-CX3CR1 axis contributed to nonclassical monocyte migration, blocking experiments were performed by adding 1 μg/ml anti-CX3CL1 mAb (MAB3652R-100, R & D Systems).

2.8 | Statistics

Data are expressed as mean±SD. The Student t test was used to compare two groups. Comparisons of the data between different groups were performed using one-way ANOVA. The area under the curve (AUC) was calculated as a measure of discriminative ability. Correlations between numeric variables were measured with Pearson’s correlation coefficient. All statistical analyses were performed using GraphPad Prism 10.0 software.

3 | RESULTS

3.1 | Chemokines and cytokines released in TPE and MPE

Firstly, we assayed monocyte-related chemokines, including CCL2, CCL7 and CX3CL1 levels in different pleural fluids by ELISA. As Figure 1A indicated, CCL2, known as a chemokine for CD14 positive cells, was significantly elevated in the MPE group compared with TPE and transudate pleural effusion (158.85±50.74 ng/ml vs. 57.61±29.65 ng/ml, and 39.19±15.12 ng/ml, respectively). However, CCL7 and CX3CL1, mainly expressed in CD16 positive monocytes, showed higher levels in TPE (477.29±298.81 pg/ml and 460.29±12.38 pg/ml), compared with MPE (137.45±33.01 pg/ml and 192.63±51.80 pg/ml) and transudate pleural effusion (108.65±18.15 pg/mL and 184.35±35.48 pg/mL). Moreover, we detected increased release of cytokines, such as IL-1β, IL-17, IL-27, and IFN-γ, in the pleural fluid from patients with TPE compared with MPE and the
transudate effusion (Figure 1B). Furthermore, the concentrations of IL-1β in the pleural fluid were significantly increased in the MPE group compared with those in the transudate group, while other cytokines showed no significant differences.

### 3.2 | CD14 and CD16 expressions on monocytes from different pleural fluids

We then investigated the distribution of monocytes subsets in peripheral blood and pleural fluid of TPE, MPE, and transudate patients. Our results showed that there was a significant difference in monocyte subtypes amongst the pleural effusion samples, with TPE having a significantly higher percentage of CD14⁺CD16⁻ nonclassical monocytes than MPE and transudate pleural effusion (24.73 ± 6.43% vs. 14.63 ± 3.63% and 4.56 ± 0.16%, respectively), while the CD14⁺CD16⁻ intermediate subset proportion in MPE was elevated when compared with the other two subgroups (20.03 ± 4.52% vs. 0.99 ± 0.74% and 1.00 ± 0.36%, respectively) (Figure 2). The assays of monocytes subset in peripheral blood had the similar trend: classical monocytes were observed decreased in TPE patient compared with transudate pleural effusion patients (82.79 ± 5.32% vs 55.21 ± 7.18%), nonclassical monocytes were accumulated in TPE and MPE patient (9.64 ± 0.44% vs 10.14 ± 0.12% and 2.68 ± 0.30%, respectively), and intermediate monocytes were significantly increased in the blood of MPE patients (3.867 ± 1.26% vs. 0.48 ± 0.04% and 0.51 ± 0.28%, respectively). Compared with the corresponding peripheral blood samples, the percentage of classical monocytes in TPE and MPE was declined, while nonclassical mono- cytes were found elevated in both TPE and MPE, and intermediate monocytes were significantly increased. Our data indicated the accumulation of nonclassical monocytes in TPE while CD14⁺⁺CD16⁻ intermediate monocytes in MPE, compare with the corresponding peripheral blood and transudate pleural effusion, which raised our
attention to explore the differentiating power of monocytes subset in TPE and MPE.

3.3 | CX3CL1-CX3CR1 axis mediated the recruitment of CD14⁺CD16++ nonclassical monocytes in TPE

Our data showed that the concentrations of CCL7 and CX3CL1 in the TPE were much higher than those in MPE and transudate pleural effusion (Figure 1A). In addition, nonclassical monocytes were observed accumulated in TPE (Figure 2). Taking this information together with our data, significant correlation were found between the concentration of CX3CL1 and the proportion of nonclassical monocytes in TPE (Figure 3A), we hypothesized that CD14⁺CD16++ nonclassical monocytes could migrate into the pleural space in response to CX3CL1-CX3CR1 axis. In addition, significant correlation was revealed between the concentration of CCL2 and the percentage of intermediate monocytes in MPE (Figure 3B). We further explored the correlations between the level of cytokines (IL-1β, IL-17, IL-27, and IFN-γ) and nonclassical monocytes percentages in TPE and found that the expression of IL-1β and IFN-γ were positively correlated with the proportion of nonclassical monocytes in TPE (Figure 3C).

By real-time PCR, we detected significantly higher expression of chemokine receptor CX3CR1 and cytokines (IL-1β, IL-17, IL-27, and IFN-γ) in nonclassical monocytes than classical and intermediate subsets (Figure 3D-E) in TPE. Furthermore, the mRNA level of CCR2 in intermediate monocytes in MPE was elevated when compared to classical and nonclassical monocytes (Figure 3F). To further evaluate the chemotaxis effect of nonclassical monocytes via the CX3CL1-CX3CR1 axis, we tested the chemotaxis index of nonclassical monocytes in the TPE supernatant and found it exerted a potent chemotactrant activity for circulating nonclassical monocytes, whereas anti-CX3CL1 mAbs significantly suppressed monocytes chemotaxis (Figure 3G).

3.4 | Monocytes subsets in differentiating TPE and MPE

To compare nonclassical and intermediate proportions in differentiating MPE from TPE, ROC curve analyses were performed. For pleural effusion specimens, the area under the curve (AUC) was 0.830 for the nonclassical subset and 0.906 for the intermediate. Cutoff points selected for nonclassical and intermediate percentages were 16.87% (sensitivity 55%; specificity 95%) and 11.65% (sensitivity 85%; specificity 85%), respectively (Figure 4).

4 | DISCUSSION

Pleural effusions, including MPE and TPE, were characterized by intense chronic accumulation of inflammatory cells into the pleural space and increased vascular permeability. Our previous study has reported that, as a vital effector of the innate immune system, monocytes were infiltrated into pleural space and participated in the pathogenesis of TPE. However, the different distributions of monocytes subtypes between MPE and TPE are poorly characterized. Therefore, the purpose of this study was to determine whether the monocytes subtype could be used as an effective and objective biomarker for discriminating between MPE and TPE.

The previous study revealed that CD14⁺ monocytes in MPEs were significantly elevated when compared to levels of monocytes found in benign pleural effusions. Furthermore, Wang F et al. reported that CD163⁺CD14⁺ cell frequency in MPE was remarkably higher than that in NMPE. When compared to tuberculous patients, it was previously reported that the percentages of monocytes/macrophages did not differ between tuberculous patients and lung adenocarcinoma patients, while TNF-α-producing CD14⁺ cells were significantly lower in lung cancer patients than in tuberculous patients. Our data were consistent with this study, indicating that the percentage of CD14⁺CD16⁺ classical monocytes and CD14⁺CD16⁺ intermediate has a lower level in MPE (Figure 2). It was well documented that in tuberculous pleural effusions, CD16⁺ is the major monocytes subset, and the expansion of the CD16⁺ subset was founded correlated with disease severity. From our data, we observed an accelerated percentage of CD14⁺CD16⁺ nonclassical subset in the pleural fluids of TPE patients while higher proportion of CD14⁺CD16⁺ intermediate monocytes in MPE patients (Figure 2). However, the reasons and the sequences for these differences in the distribution of monocytes subset in pleural fluids are relatively unknown.

As an important chemokine for monocytes, CCL2 contributed to the formation of pleural fluids not only by inducing tPA but also by promoting inflammation, new blood vessel assembly, and plasma extravasation into the pleural space. It was reported that the level of CCL2 in pleural effusion was significantly higher in patients with lung adenocarcinoma than in those with tuberculosis. The level of CCL2 in PE appears to be a reliable surrogate marker for evaluating the therapeutic efficacy in the control of MPE and predicting survival in lung adenocarcinoma patients with MPE. Our present study was consistent with previous studies, showing a higher concentration of CCL2 in MPE when compared to TPE and transudate pleural patients (Figure 1A). CX3CL1, mainly expressed on CD16⁺ nonclassical monocytes, exerting its role in inducing the infiltration of this subset of monocytes, was reported to be associated with the progress of tuberculosis infection. J D Hall et al. demonstrated the importance of the CX3CL1 receptor, CX3CR1, which regulates the function and trafficking of macrophages and dendritic cells, in the host’s ability to control respiratory infections with Mycobacterium tuberculosis. Recent study also reported that CX3CL1 was associated with an increased risk of unfavorable outcomes in pulmonary tuberculosis. Bronchoalveolar lavage fluid from TB patients had elevated levels of CX3CL1 compared with non-TB patients. While in the tumor environment, it was reported that expression levels of CX3CL1 and CX3CR1 in the primary lung cancer were significantly
CX3CL1-CX3CR1 is related to the infiltration of nonclassical monocytes in TPE. (A) The Pearson correlation coefficient (r) and the significance for it (P) between monocytes subtype percentage and chemokines (CCL2, CCL7, and CX3CL1) expression in TPE (n = 24). (B) The correlation between monocytes subtype percentage and chemokines (CCL2, CCL7, and CX3CL1) expression in MPE (n = 24). (C) The correlation between nonclassical monocytes proportion and cytokines (IL-1β, IL-17, IL-27, and IFN-γ) expression in TPE (n = 24). (D) Representative mRNA levels of cytokines in different monocytes subsets from TPE (n = 10). Expression was normalized to the housekeeping gene GAPDH. *p < 0.05, **p < 0.01, ***p < 0.001. (E) Representative mRNA levels of chemokines receptors in different monocytes subsets from TPE (n = 10). (F) Representative mRNA levels of chemokines receptors in different monocytes subsets from MPE (n = 10). (G) Representative the chemotaxis of nonclassical monocytes in the presence of supernatants of TPE with or without anti-CX3CL1, or PBS in the bottom chamber of transwell. The migratory nonclassical monocytes on the bottom surface of the transwell membrane were visualized by Wright-staining and microscopic pictures were taken (magnification, ×400).
higher than those in the adjacent tissues, as well as positively correlated with the degree of lung cancer progression. In the present study, we demonstrated that the concentrations of CCL7 and CX3CL1 were significantly elevated in the pleural fluids of TPE patients. It is reported that, the predominant cytokine in TPE, IFN-γ, could induce the expression of CCL7 in monocytes, which may explain why CCL7 is enriched in TPE. Also, CCL7 mainly acts as a chemoattractant for monocytes, eosinophils, basophils, dendritic cells (DCs), neutrophils, NK cells, and activated T lymphocytes, while CCL2 recruits monocytes, memory T cells, and DCs. There is a larger proportion of neutrophils in TPE subjects compared with MPE. Therefore, it is plausible to speculate that CCL7 may act as a chemoattractant for neutrophils in TPE.

From our data, the expression level of CCL2 is much higher than CCL7 (almost 1000 times, ng/ml vs. pg/ml). Moreover, CCL2 is elevated in MPE compared with TPE, while CX3CL1 is higher in TPE. Furthermore, our previous chemotaxis experiment verified that the infiltration of intermediate monocytes was reduced with anti-CCL2 mAb in the presence of MPE supernatants. Taken together, CCL2 in MPE recruits more CCR2 positive classical and intermediate monocytes to pleural space than TPE, while in TPE, CX3CL1 recruits more CX3CR1 nonclassical monocytes, which may explain the reason why the proportion of classical or intermediate monocytes was not increased in TPE. In addition, mRNA levels of cytokines (IL-1β, IL-17, IL-27, and IFN-γ) and chemokines receptor CX3CR1 in nonclassical monocytes from TPE were significantly higher than those of the classical and classical subsets. In vitro chemotaxis assay further suggested that the CX3CL1-CX3CR1 axis was responsible for the accumulation of nonclassical monocytes in TPE (Figure 3G), which revealed the different distributions of monocytes subsets in TPE, and MPE may be because of the different expressions of chemokines and their receptors in TPE and MPE.

Our current study confirmed that nonclassical and intermediate monocytes subsets can discriminate between tuberculosis and malignant pleural effusion (AUC = 0.830 and 0.906, respectively). By using 16.87% nonclassical as a cutoff, we found the specificity and sensitivity were 55% and 95%. When choosing 11.65% intermediate monocytes as a cutoff value, the specificity and sensitivity were 85% and 85% (Figure 4). It was suggested intermediate monocytes as a better diagnostic marker to differentiate TPE and MPE.

However, our study has several limitations. First, we need to conduct a larger-scale study to validate their monocytes subsets in TPE and MPE. In addition, other exudate pleural effusion diseases were not included in our study to test their discrimination power of monocytes subsets.

In conclusion, we found higher levels of cytokines (IL-1β, IL-17, IL-27, and IFN-γ) and chemokines CCL7 and CX3CL1 in tuberculosis pleural effusions compared with malignant and transudate pleural effusions. CX3CL1-CX3CR1 axis mediated the recruitment of CD14+CD16++ nonclassical monocytes in TPE. Furthermore, the proportion of intermediate and nonclassical monocytes in pleural effusion may be a novel, reliable biomarker assay for the differentiation of pleural effusion especially tuberculosis from the malignant pleural effusion.
AUTHOR CONTRIBUTIONS
L. LUO performed the experimental work, analyzed the data, and wrote the article. S. DENG, W. TANG, and X. HU helped performed experiment and analyzed the data. F. YIN, H. GE, J. TANG, and Z. LIAO helped collecting samples. B. MO, X. LI, and J. FENG designed the study, supervised the study, and critically revised the article. All authors read and approved the final article.

CONFLICT OF INTEREST
All authors: No potential 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 article have been disclosed.

DATA AVAILABILITY STATEMENT
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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