Journal of Inflammation Research

Dovepress
Taylor & Francis Group

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ORIGINAL RESEARCH

RETRACTED ARTICLE: IFN-γ Induces Pleural Mesothelial Cells to RecruitImmune Cells via CXCL10-CXCR3 Axis ina Mouse Pleurisy Model

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Background: Pleural mesothelial cells (PMCs) form the entire surface of the pleural cavity and interact with microorganisms in the thorax. Although PMCs are known to exert multiple immune functions, their role in pleurisy remains unclear.

Methods: Pleurisy model was induced by intrapleural injection of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) into wild-type (WT) C57BL/6 mice. The pleural cavity was washed with Phosphate Buffered Saline (PBS) to get the immune cells. Flow cytometry was performed to identify the characteristics of the target cells.

Results: We found that IFN-γ prompts PMCs to act a surveillance role for the recruitment of inflammatory cells in pleurisy model. Our data showed that $CD4^+$ T cells were the main producer of IFN-γ in the pleurisy model, and IFN-γ stimulated PMCs to recruit immune cells into the pleural cavity through the CXCL10-CXCR3 axis. In addition, IFN-γ can reshape PMCs to display macrophage-like polarization. These results revealed some new immune roles of PMCs in pleurisy.

Conclusion: In a mouse model of pleurisy, IFN-γ, which is mainly derived from $CD4^+$ T cells, promoted PMCs to recruit of immune cells into the pleural cavity and exhibited macrophage-like polarization.

Keywords: IFN-γ, pleurisy, pleural mesothelial cells, macrophages

Introduction

Tuberculosis is one global health problem, which causes heavy burden.[1] Tuberculous pleurisy is reported to be one of the most common extrapulmonary tuberculosis.[2] Th-1 type immune response plays an important role in controlling tuberculosis infection,[3] and IFN-γ can regulate both innate and acquired immunity to infection and exert a host's protective response against mycobacterial infection to promote the elimination of pathogens.[4]

Pleural mesothelial cell (PMC) is a kind of special epithelial cell that covers the lung and inner wall of thorax.[5] PMC plays a key role in maintaining normal homeostasis of the pleural space and is a central component of pathophysiological processes in the pleural cavity.[6] PMC plays a critical role in initiating inflammatory responses in the pleural cavity because it is the first kind of cell to recognize perturbations in the pleural space.[6] When tuberculous pleurisy occurs, several kinds of immune cells enter the subtly regulated space and form a relatively independent inflammatory environment. It is already known that PMCs are able to secret cytokines and chemokines, and adhere with immune cells in an inflammatory environment.[7,8] However, the pathways that connect PMCs and immune cells remain unclear.

Macrophages are innate immune cells that digest and engulf pathogens via phagocytosis. Macrophages are highly plastic and can be polarized into different phenotypes in response to peripheral cytokines. M1 polarization results in a pro-inflammatory type, which is called "classically activated" phenotype while M2 polarization results in an anti-

Received: 1 November 2024
Accepted: 30 January 2025
Published: 20 February 2025

inflammatory type, which is called "alternatively activated" phenotype.[9–11] IFN-γ is a cytokine which could drive macrophages to differentiate into the M1 phenotype.[10] Here, we found that IFN-γ could also induce PMCs to exhibit macrophage-like polarization.

Materials and Methods

Mice and BCG-Induced Pleurisy

Six- to eight-week-old wild-type (WT) C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). The mice were housed in an environment maintained at 25°C under a 12-h light / 12-h dark cycle in the Animal Center of Capital Medical University under high health status conditions. The animal study protocol was approved by the Institutional Animal Care and Utilization Committee of Capital Medical University.

For the pleurisy models, WT mice were prepared by intrapleural injection of 5×10^6 bacillus Calmette-Guerin (BCG) in 100 μL saline as previously reported.[12,13] Fourteen days after injection, the mice were sacrificed, and the thoracic cavities were washed with 1 mL of phosphate-buffered saline (PBS) to get the pleural lavage. The pleural lavage and blood samples were collected, and mononuclear cells were collected using Ficoll-Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden) method. The samples were then resuspended in lysis buffer (BD Biosciences, NJ, USA) and after washing with PBS, the target cells were obtained.

Isolation and Culture of Primary Pleural Mesothelial Cells

C57BL/6 mice were sacrificed and subjected to intrapleural injection of 700 μL of trypsin. Five minutes later, the trypsin was recovered and the process was repeated three times for each mouse. The samples were then centrifuged at 300× g for 6 min, and the target cells were isolated and cultured in F12k medium with 10% fetal bovine serum, as previously reported.[7]

For PMCs stimulation, the cells were incubated in the presence of mouse IFN-γ (20 ng/mL; PeproTech, NJ, USA) for three days, and the cells were then trypsinized and harvested for following experiments.

Cells

The Met-5A cell line was purchased from American Type Culture Collection (ATCC, VA, USA). The cells were cultured in M199 complete growth medium according to the manufacturer's instructions and were sub-cultured at 1:2 ratios when the cells were confluent. The culture medium was changed every other day.

Flow Cytometry

The antibodies used for flow cytometry, including anti-CD3, anti-CD8, anti-CD4, anti-NK1.1, anti-B220, anti-Ly6C, anti-F4/80, anti-iNOS, anti-Ang-1, anti-calretinin, and anti-CXCR3 monoclonal antibodies were purchased from Invitrogen (Carlsbad, CA, USA) and Abcam (Cambridge, UK). The flow cytometry methods are stated in detail in previous publications.[14] The FACS Canto II system (BD Biosciences) and FCS Express 5 software program (De Novo Software, CA, USA) were used to collect and analyze the data.

Next-Generation Sequencing

After stimulated with or without mouse IFN-γ (50 ng/mL) for 48 h, the PMCs culture medium supernatants were collected and frozen at −80°C, and the cells were harvested, and total RNAs were extracted using TRIzol (Invitrogen, CA, USA) following the manufacturer's protocol. Using the Illumina NovaSeq 6000 protocol, mRNA expression profile was performed by CapitalBio Technology (Beijing, China). Two-fold change was chosen for threshold values, and 0.05 was chosen for *p* value to select significantly differentially expressed genes.

Concentration of CXCL10

CXCL10 levels in PMCs culture medium supernatants were tested by enzyme-linked immunosorbent assay (ELISA) kits (Laizee, Shanghai, China) in accordance with the manufacturer's specifications.

Chemotaxis Assays

Chemotaxis assays were performed using 8-μm-pore polycarbonate filters in 24-well Transwell chambers (Corning, NY, USA). Mononuclear cells from pleural lavage were added into the upper chamber. The lower chambers were supplemented with the supernatants of IFN-γ stimulated PMCs culture medium or PBS. The cells were incubated at 37°C in 5% CO_2 for 2 h. The migratory index was calculated by dividing the number of cells that migrated in response to PMCs culture medium by the number of cells that migrated in response to PBS. To investigate the role of CXCL10 in migration of mononuclear cells, 1 μg/mL anti-CXCL10 neutralizing mAb (BioLegend, CA, USA) or IgG isotype control was added to the lower chambers.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software version 8.0 (GraphPad Software, La Jolla, CA). Data were presented as means ± SEM. Student's *t* test was used to perform statistical differences between groups, and $p < 0.05$ was considered statistically significant.

Results

$CD4^+$ T Cells are the Main Producer of IFN-γ in BCG Induced Pleurisy

IFN-γ is an important cytokine during the protective process of microbicidal actions.[15] To verify the source of IFN-γ in pleural cavity, WT mice were intrapleural injected with 5×10^6 BCG to induce a pleurisy model. Fourteen days later, pleural lavage was collected and centrifuged to obtain the cells for further analysis of the proportion of IFN-γ-positive cells. Flow cytometry analysis showed that $CD4^+$ T cells accounted for approximately 65% of the IFN-γ-positive cells (Figure 1A), and the percentage of $CD4^+$ T cells was significantly higher than that of $CD8^+$ T cells, NK cells, and other cells (Figure 1B), indicating that $CD4^+$ T cells were the main producers of IFN-γ in our pleurisy model. Considering that $CD4^+$ T cells and $CD8^+$ T cells account for approximately 80% of IFN-γ positive cells, therefore we then examined changes in IFN-γ production by $CD4^+$ T cells and $CD8^+$ T cells before and after BCG injection. We found that the administration of BCG injection significantly increased the IFN-γ production by $CD4^+$ T cells and $CD8^+$ T cells (Figure 2A and B). In addition, previous studies also showed that BCG injection significantly increases the concentration of IFN-γ in pleural lavage in this murine pleurisy model.[3]

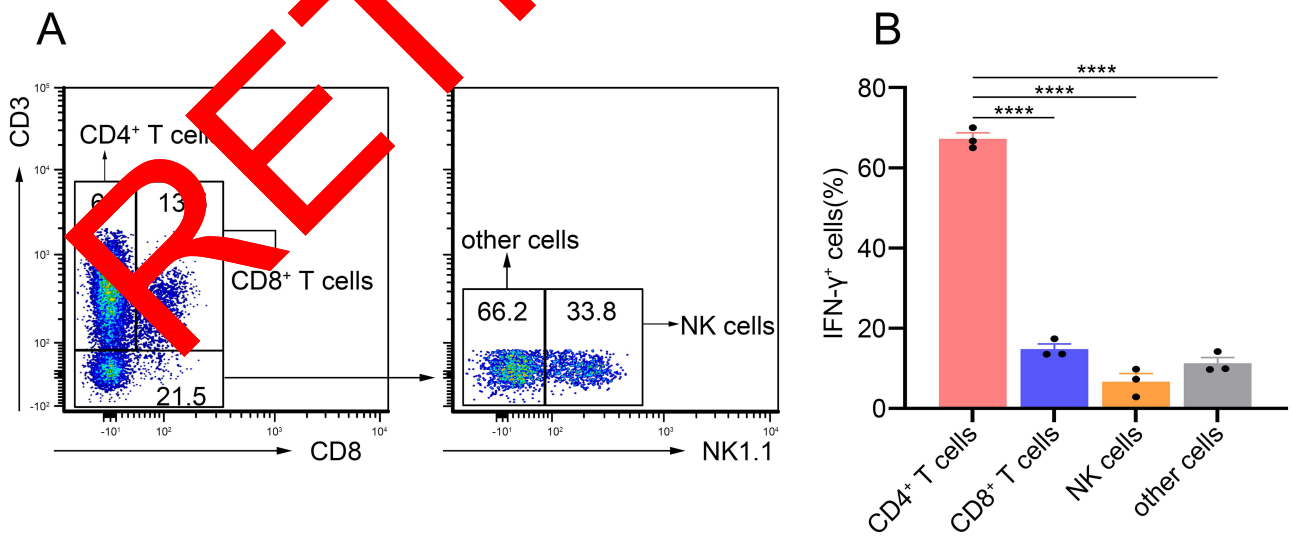

Figure 1 $CD4^+$ T cells are the main producer of IFN-γ in BCG induced pleurisy. (**A**) Flow cytometric dot plots show the constituent of IFN-γ positive cells in BCG induced pleurisy. We first gated the IFN-γ positive cells, and then defined the $CD3^+$ $CD8^-$ cells as $CD4^+$ T cells, $CD3^+$ $CD8^+$ cells as $CD8^+$ T cells and $CD3^-$ $NK1.1^+$ cells as NK cells. Representative flow cytometric dot plots are shown from three independent experiments. (**B**) Column chart show the percentages of $CD4^+$ T cells, $CD8^+$ T cells, NK cells and other cells in IFN-γ positive cells. ****$p < 0.0001$ compared with control (Student's *t* test). Data are shown as means ± SEM.

https://doi.org/10.2147/JIR.S496037

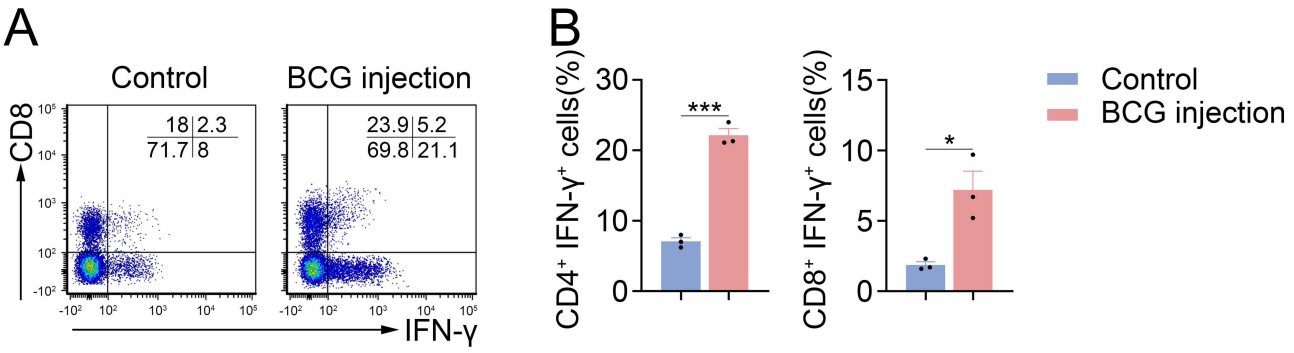

Figure 2 $CD4^+$ T cells ($CD3^+$ $CD8^-$) and $CD8^+$ T cells ($CD3^+$ $CD8^+$) from pleural lavage of BCG injected mice expressed more IFN-γ than those from control mice. (**A**) Flow cytometric dot plots show the percentages of IFN-γ positive cells of $CD4^+$ T cells and $CD8^+$ T cells before and after BCG injection. We gated the $CD3^+$ T cells, and then defined the $CD3^+$ $CD8^-$ cells as $CD4^+$ T cells, $CD3^+$ $CD8^+$ cells as $CD8^+$ T cells. Representative flow cytometric dot plots are shown from three independent experiments. (**B**) Column charts show the administration of BCG injection significantly increased the IFN-γ production by $CD4^+$ T cells and $CD8^+$ T cells. *p < 0.05, ***p < 0.001 compared with control (Student's *t* test). Data are shown as means ± SEM.

IFN-γ Induces Pleural Mesothelial Cells to Secret CXCL10

It is reported that PMCs are able to coordinate the process of pleural inflammatory,[6] so we examined the immune role of PMCs in the presence of IFN-γ. We purified PMCs from WT mice, and the PMCs showed a classic cobblestone morphology as previously reported[7] (Figure 3A). The purity of PMCs was greater than 90% identified by anti-calretinin monoclonal antibody (Figure 3B and 3C). After culturing with or without IFN-γ for 2 days, the PMCs were collected and extracted total RNA for mRNA sequencing. The result showed that 548 genes were upregulated, and 586 genes were downregulated (Figure 3D) after stimulated with IFN-γ. CXCL10, an upregulated chemokine in IFN-γ stimulation group, was noticed according to the remarkable *p* value and its role in the persistence of inflammation.[16,17] To verify the sequencing results, we used ELISA method to confirm the expression level of CXCL10 and we found a significantly elevated level of CXCL10 in the culture supernatants of both primary PMCs and Met-5A cell line after IFN-γ stimulation (Figure 3E and F).

Expression of CXCR3 in Different Kinds of Immune Cells in BCG Induced Pleurisy

Previous research reported that the CXCL10-CXCR3 signaling axis could recruit Th1 and Th17 cells into MPE and regulate the immune response.[18] Since we found that PMCs were able to secrete CXCL10 in the presence of IFN-γ, we then examined the expression of CXCR3 in immune cells from pleural lavage and blood 14 days after BCG injection (Figure 4A–E). And we found that $CD4^+$ T cells ($CD3^+CD8^-$), $CD8^+$ T cells ($CD3^+CD8^+$), B cells ($CD3^-B220^+$), macrophages ($CD11b^+F4/80^+$) and NK cells ($CD3^-NK1.1^+$) from pleural lavage all expressed significantly higher percentages of CXCR3 than their counterparts from blood (all $p < 0.05$). Based on this signaling axis, PMC-derived CXCL10 may recruit immune cells from blood into pleural cavity to exert immune functions. To verify the signaling axis, we processed a migration assay. Since it is unlikely to separate enough different kinds of immune cells from the mouse pleurisy model to carry out this experiment, we used mononuclear cells from pleural lavage of pleurisy instead. Our migration assay showed that IFN-γ stimulated PMCs culture medium supernatant attracted mononuclear cells, and the migration of the cells was significantly suppressed when anti-CXCL10 mAb was added to the medium (Figure 4F).

These data indicated that PMCs may recruit $CXCR3^+$ inflammatory cells to the pleural cavity through secreting CXCL10 induced by IFN-γ.

IFN-γ Induces PMCs to Display a Macrophage-Like Polarization

In addition to the recruitment of $CXCR3^+$ inflammatory cells, we wondered if IFN-γ stimulation had any other effects on PMCs. Purified PMCs were isolated from WT mice and cultured with or without IFN-γ for three days. After the cells were harvested, flow cytometry data demonstrated a significantly increased expression of iNOS and decreased expression of Arg-1 in the IFN-γ stimulation group (Figure 5A and B). In this way, PMCs exhibited an M1-like

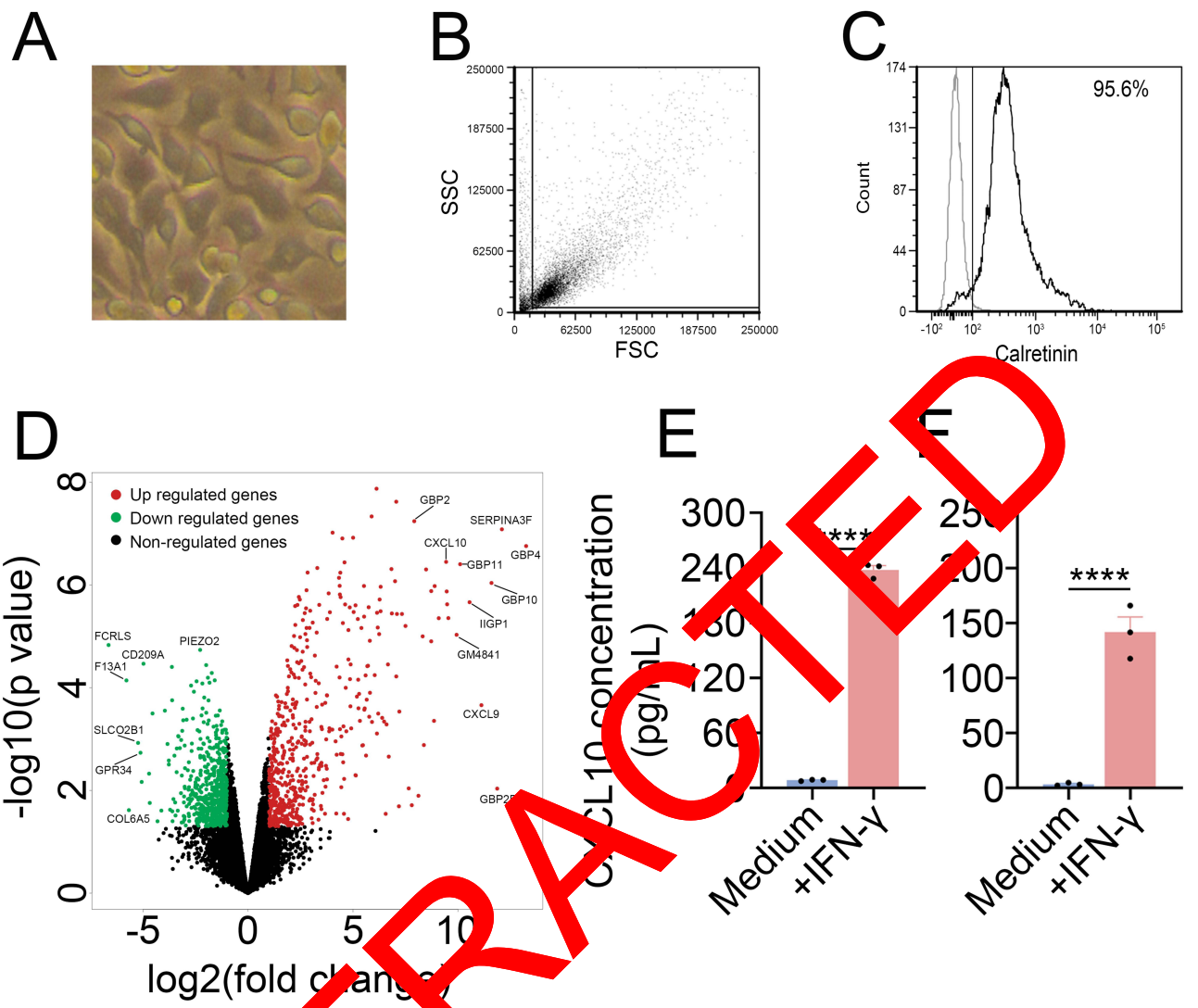

Figure 3 IFN-γ induces pleural mesothelial cells to secrete CXCL10. (**A**) Pleural mesothelial cells (PMCs) exhibited cobblestone morphology. (**B**) and (**C**) Flow cytometric dot plots show the purity of the PMCs. (**D**) Volcano plots show the differently expressed genes between the two groups. (**E**) and (**F**) Purified mouse PMCs and MeT-5A cell line were cultured with or without IFN-γ, and the CXCL10 concentrations in culture supernatant was measured by ELISA. Data are shown from three independent experiments. ****$p < 0.0001$ compared with control (Student's *t* test). Data are shown as means ± SEM.

polarization in the presence of IFN-γ and executed a pro-inflammatory function of recruiting immune cells via the CXCL10-CXCR3 signaling axis.

Discussion

Tuberculosis is still a leading cause of morbidity and mortality worldwide.[19] Tuberculous pleurisy, caused by Mycobacterium tuberculosis, is a kind of inflammatory lesion of the pleura, often accompanied by chest pain and pleural effusion.[20,21] Large numbers of cytokines have been studied in immune response of tuberculous pleurisy; however, a key character among these, IFN-γ, is required for protection against tuberculosis progression,[22] and genetic defects in IFN-γ production or signaling are related to increased susceptibility to tuberculosis.[23]

IFN-γ is the only member of the type II interferon family, which is a pleiotropic cytokine. IFN-γ plays an important role in host defense against Mycobacterium tuberculosis, which enhances proinflammatory signaling by stimulating the antimicrobial action of monocytes and macrophages through initiating producing microbicidal active nitric oxide and oxygen intermediates and stimulating the production of TNF, which plays a key role in intracellular Mycobacterium tuberculosis killing.[24] In addition, IFN-γ has also been shown to promote the granuloma formation and phagosome-

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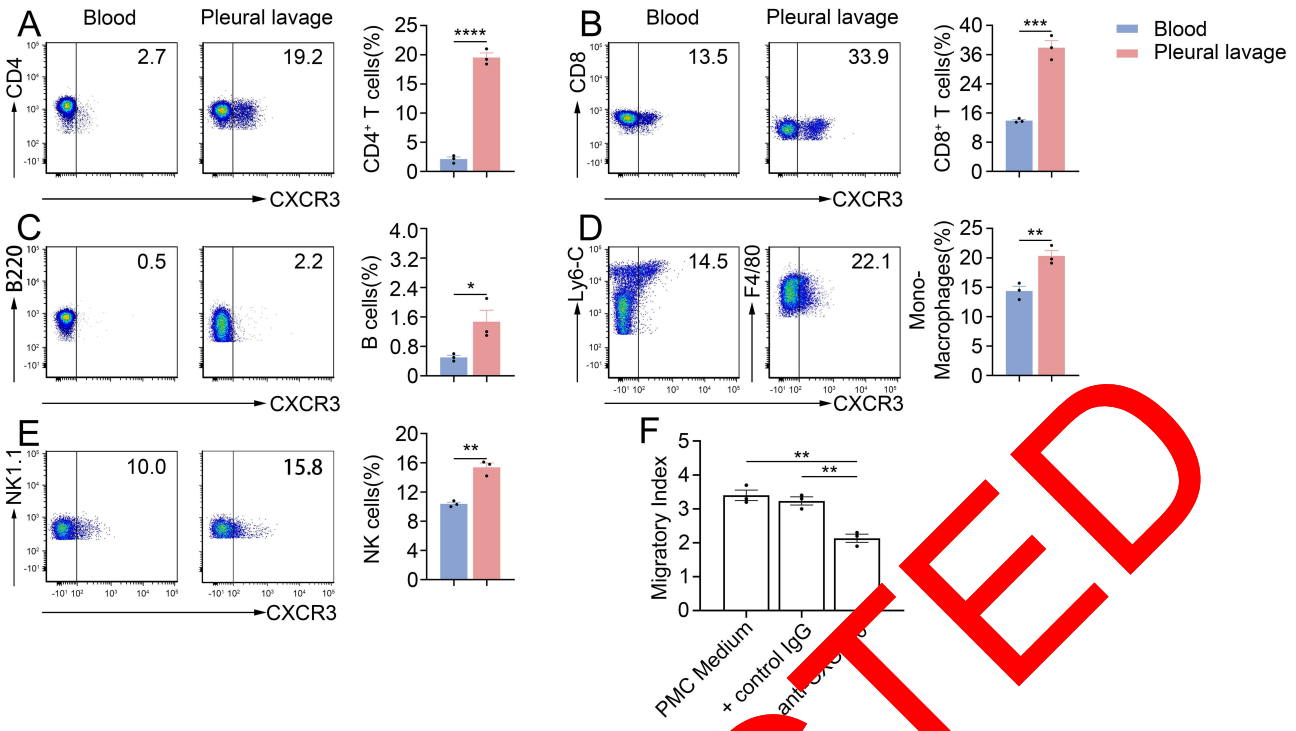

Figure 4 Expression of CXCR3 in different kinds of immune cells. Flow cytometric dot plots of CXCR3 expression on (**A**) $CD4^+$ T cells, (**B**) $CD8^+$ T cells, (**C**) B cells, (**D**) Mono-macrophages and (**E**) NK cells in blood and pleural lavage (left panel) 14 days after intrapleural injection of BCG. The immune cells from pleural lavage express higher percentages of CXCR3 than those from blood (right panel). Representative flow cytometric dot plots are shown from three independent experiments. (**F**) The mononuclear cells were seeded into the top chamber, and the supernatants of PMCs culture medium with or without anti-CXCL10 mAb or an irrelevant isotype control were placed in the lower chamber of the transwell system. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 with blood or control (Student's *t* test). Data are shown as means ± SEM.

lysosome fusion, which facilitates the death of intracellular Mycobacterium.[25] In this study, we investigated the source of IFN-γ in a mouse pleurisy model and we found that $CD4^+$ T cells make up the most constituent in producing IFN-γ, in accordance with the established idea that Th lymphocytes play an essential role in the cure of tuberculosis infection.[26,27] In addition to that, HIV patients with reduced $CD4^+$ T cell lymphocytopenia are highly susceptible to MTB, whereas $CD4^+$ T cell-deficient mice die quickly from uncontrolled bacterial replication and, apparently, $CD4^+$ T cells are required to slow MTB growth.[28] A recent single cell transcriptome analysis showed that Th1, $CD8^+$ T cells and NK cells were extensively immune exhaustion in patients with severe tuberculosis, indicating that $CD8^+$ T cells and NK cells also played a regulatory role in the progress of tuberculosis.[29]

PMCs physically surround the entire surface of the pleural cavity. Attached to the pleural basement membrane, the PMCs provide a frictionless surface between the parietal and visceral serosa by secreting glycosaminoglycans and surfactants.[30] Tight intercellular junctions between PMCs guarantee a protective barrier and a relatively isolated environment.[31] Pleural mesothelial integrity plays an important role in limiting the spread of various pathogens. Previous studies have revealed that PMCs can produce a diverse array of mediators, such as IL-18, endostatin, β-defensin, and multiple chemokines, in response to external signals, thereby initiating and regulating inflammatory responses in an inflammatory state.[32–35]

In the current study, we found that IFN-γ stimulates PMCs to secrete CXCL10. CXCL10, known as IP-10 (Interferon-gamma inducible Protein 10 kDa), is secreted by various types of cells, such as T lymphocytes, neutrophils, eosinophils, macrophages, neurons, and glial cells.[36] CXCL10 belongs to the CXC chemokine family and is a ligand for the CXCR3 (C-X-C motif chemokine receptor 3) receptor.[37] In a published study, stimulated with Mycobacterium tuberculosis antigens, B cells were also able to produce CXCL10 by IFN-γ and T cell contact manner, which implies that CXCL10 participates in the immune response to Mycobacterium tuberculosis.[38] In another study, exogenous IFN-γ induced CXCL10 production by $CD3^+$, $CD14^+$ and $CD16^+$ cells from both peripheral blood mononuclear cells and pleural

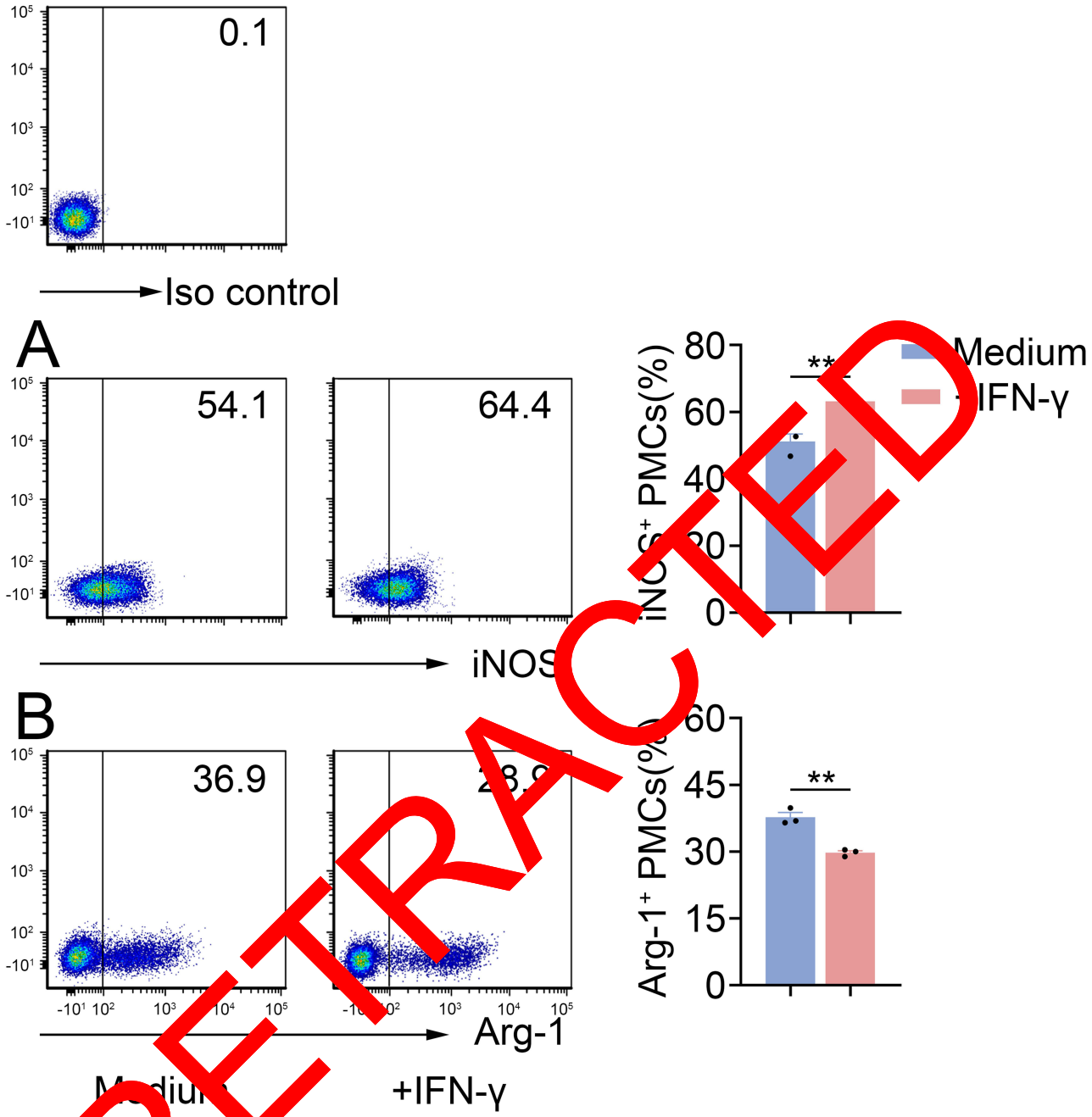

Figure 5 IFN-γ induced PMCs display a macrophage-like polarization. Purified mouse PMCs were cultured with or without IFN-γ and flow cytometric dot plots showed the expression of (**A**) iNOS and (**B**) Arg-1 (left panel). Representative flow cytometric dot plots are shown from three independent experiments. PMCs expressed higher percentages of (**A**) iNOS and lower percentages of (**B**) Arg-1 (right panel) after IFN-γ treatment. **p < 0.01 compared with control (Student's *t* test). Data are shown as means ± SEM.

fluid mononuclear cells of patients with tuberculous pleurisy in a concentration-dependent manner.[39] Our research has to some extent supplemented the sources of CXCL10, however, there are relative limitations of methodology in this study, as there is no pleural effusion in this mouse model, so we were unable to obtain the concentration of IFN-γ in the pleural cavity and conduct in vitro stimulation experiments based on this. But the stimulation experiments we conducted in vitro have been mutually confirmed with the subsequent experiments, so we believe that the results are reliable. Reviewing our results, we found that $CD4^+$ T cells were the main source of IFN-γ in this pleurisy model, suggesting that $CD4^+$ T cells may play a major role in activating PMCs through the IFN-γ pathway.

CXCL10 has been extensively studied for its role in regulating the chemotaxis of $CXCR3^+$ immune cells.[37,40] Upon binding to CXCR3, the CXCL10 / CXCR3 axis plays a pro-inflammatory role in various disease.[41] In a cardiac pressure overload model, $Cxcr3^{-/-}$ mice prevented adverse cardiac remodeling by disrupting the infiltration of $CD4^+$ T cells into the heart.[42] This axis not only regulates immune cell migrate to the inflamed sites,[43,44] but also regulates immune response in tumors. Previous research suggested that CXCL10 / CXCR3 signaling recruited tumor antigen specific $CD8^+$ T cells to melanoma.[45] In the pleurisy model, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and NK cells in pleural cavity all expressed a higher percentage of CXCR3 than the corresponding cells in peripheral blood. We speculated that a portion of immune cells in peripheral blood respond to PMC derived CXCL10 by upregulating CXCR3 and then chemotaxis into the thoracic cavity. This may provide a new explanation for how PMCs recruit immune cells to the thorax in this pleurisy model.

A previous report revealed that PMCs have not only secretion functions but also phagocytic functi[46] which act like macrophages. Our results showed that in the presence of IFN-γ, the iNOS expression of PMCs in[illegible]eased, sh[illegible]ving a M1-like polarization, and IFN-γ is also one of the inducers of M1 polarization in macrophages. In[illegible]ible NO sy[illegible]hase may contribute to the control of infections in the pleural space,[47] and thus M1-like polarizatio[illegible]f PMC[illegible] may be [illegible]volved in pleural inflammation events. Our previous data demonstrated that PMCs can function[illegible] antigen-pres[illegible]in[illegible] cells.[35] M1-like polarization may also be beneficial for achieving antigen presentation. Further s[illegible]dies s[illegible]uld pay more attention to the mechanism of how IFN-γ inducing up-regulated expression of CXCL10 in[illegible]MCs. [illegible]brief, th[illegible]esults of this study reveal a role for PMCs in recruiting immune cells in the presence of IFN-γ, [illegible]provide a n[illegible]l insight into the function of PMCs in pleurisy environments.

Conclusion

These results suggested that $CD4^+$ T cells are the main source of [illegible]FN-γ in pleu[illegible]sy, and IFN-γ could stimulate PMCs to produce CXCL10, thereby attracting T cells, B cells, NK cells, [illegible]d monocy[illegible]es from peripheral blood to the pleural cavity. IFN-γ can also cause PMCs to exhibit a maci[illegible]e-like po[illegible]ed phenotype. These findings provide new insights into the PMCs' involvement in the progression[illegible]f ple[illegible]and might offer novel therapeutic targets for the development of pleurisy.

Data Sharing Statement

The data used to support the findings[illegible]f this [illegible]dy are available from the corresponding author Feng-Shuang Yi upon reasonable request.

Ethical Approval

The animal study protoc[illegible] was approved by the Institutional Animal Care and Utilization Committee of Capital Medical University (AEEI-202[illegible]215). [illegible]he animal experiments were conducted in compliance with the Guidelines for the Care and Use of Labor[illegible]tory An[illegible]als publ[illegible]ed by National Institutes of Health.

Author Cont[illegible]ibutions

All authors m[illegible]a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisi[illegible]n of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by grants from National Natural Science Foundation of China (no. 81900094, 82000098), Reform and Development Program of Beijing Institute of Respiratory Medicine (Ggyfz202420, Ggyfz202518).

Disclosure

The authors declare that they have no conflicts of interest in this work.

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