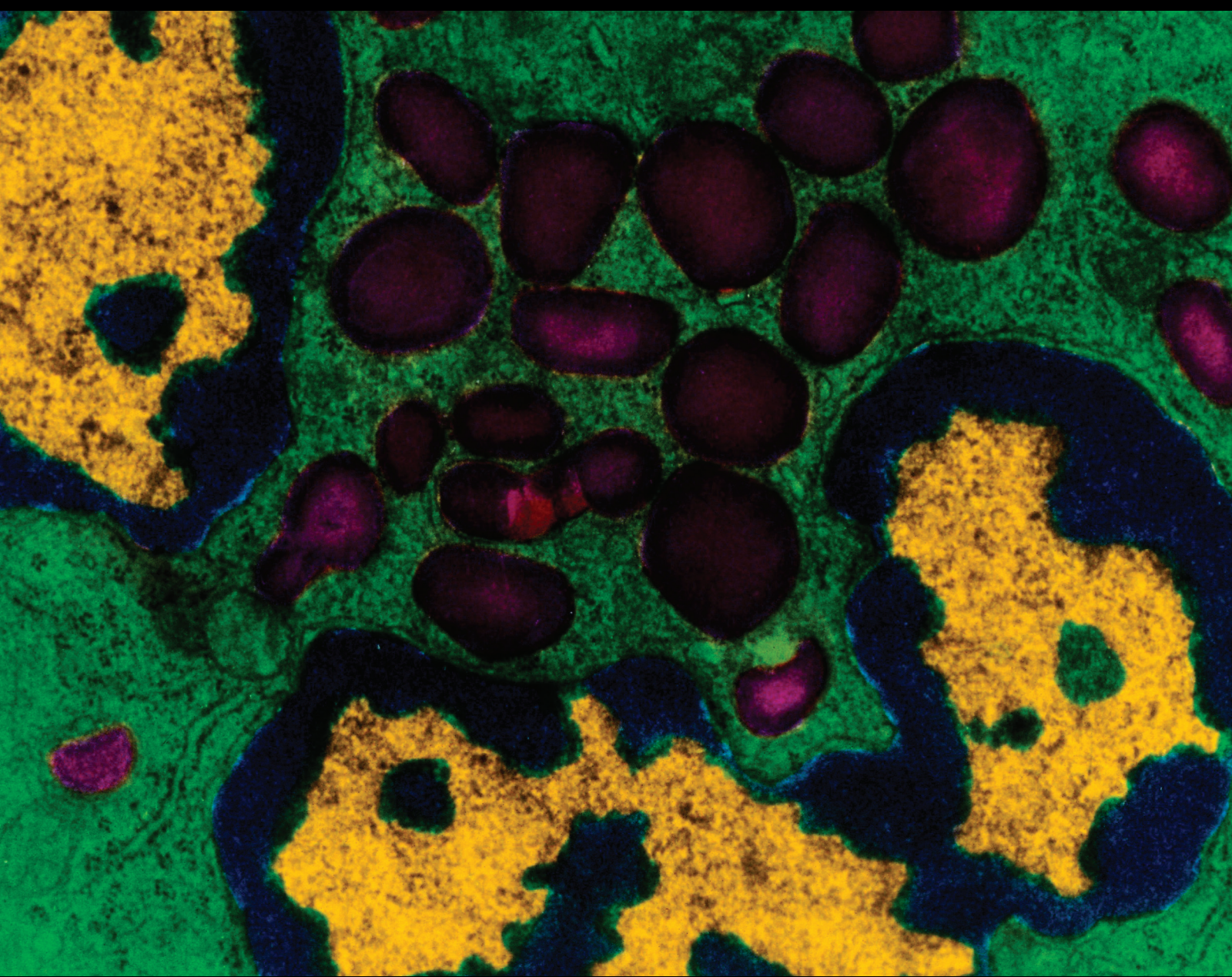
Mediators of Inflammation

Immune Regulatory Cells in Inflammation, Infection, Tumor, Metabolism, and Other Diseases

Lead Guest Editor: Qingdong Guan
Guest Editors: Cheng Xiao and Minggang Zhang



Immune Regulatory Cells in Inflammation, Infection, Tumor, Metabolism, and Other Diseases

Mediators of Inflammation

Immune Regulatory Cells in Inflammation, Infection, Tumor, Metabolism, and Other Diseases

Lead Guest Editor: Qingdong Guan
Guest Editors: Cheng Xiao and Minggang Zhang

Copyright © 2018 Hindawi. All rights reserved.

This is a special issue published in “Mediators of Inflammation.” All articles are open access articles distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Editorial Board

Anshu Agrawal, USA
Muzamil Ahmad, India
Maria Jose Alcaraz, Spain
Simi Ali, UK
Amedeo Amedei, Italy
Oleh Andrukhov, Austria
Emiliano Antiga, Italy
Zsolt J. Balogh, Australia
Adone Baroni, Italy
Jagadeesh Bayry, France
Jürgen Bernhagen, Germany
Tomasz Brzozowski, Poland
Philip Bufler, Germany
Elisabetta Buommino, Italy
Daniela Caccamo, Italy
Luca Cantarini, Italy
Raffaele Capasso, Italy
Calogero Caruso, Italy
Maria Rosaria Catania, Italy
Carlo Cervellati, Italy
Cristina Contreras, Spain
Robson Coutinho-Silva, Brazil
Jose Crispin, Mexico
Fulvio D'Acquisto, UK
Eduardo Dalmarco, Brazil
Pham My-Chan Dang, France
Wilco de Jager, Netherlands
Beatriz De las Heras, Spain
Chiara De Luca, Germany
James Deschner, Germany
Clara Di Filippo, Italy
Carlos Dieguez, Spain
Agnieszka Dobrzyn, Poland
Elena Dozio, Italy
Emmanuel Economou, Greece
Ulrich Eisel, Netherlands
Giacomo Emmi, Italy
Fabíola B Filippin Monteiro, Brazil
Antonella Fioravanti, Italy
Stefanie B. Flohé, Germany
Jan Fric, Czech Republic
Tânia Silvia Fröde, Brazil
Julio Galvez, Spain
Mirella Giovarelli, Italy
Denis Girard, Canada
Ronald Gladue, USA
Markus H. Gräler, Germany
Hermann Gram, Switzerland
Francesca Granucci, Italy
Oreste Gualillo, Spain
Elaine Hatanaka, Brazil
Nobuhiko Kamada, USA
Yoshihide Kanaoka, USA
Yasumasa Kato, Japan
Yona Keisari, Israel
Alex Kleinjan, Netherlands
Marije I. Koenders, Netherlands
Elzbieta Kolaczkowska, Poland
Vladimir A. Kostyuk, Belarus
Dmitri V. Krysko, Belgium
Sergei Kusmartsev, USA
Martha Lappas, Australia
Philipp M. Lepper, Germany
Eduardo López-Collazo, Spain
Andreas Ludwig, Germany
Ariadne Malamitsi-Puchner, Greece
Francesco Marotta, Italy
Joilson O. Martins, Brazil
Donna-Marie McCafferty, Canada
Barbro N. Melgert, Netherlands
Paola Migliorini, Italy
Vinod K. Mishra, USA
Eeva Moilanen, Finland
Alexandre Morrot, Brazil
Jonas Mudter, Germany
Kutty Selva Nandakumar, China
Hannes Neuwirt, Austria
Nadra Nilsen, Norway
Daniela Novick, Israel
Marja Ojaniemi, Finland
Sandra Helena Penha Oliveira, Brazil
Olivia Osborn, USA
Carla Pagliari, Brazil
Martin Pelletier, Canada
Vera L. Petricevich, Mexico
Sonja Pezelj-Ribarić, Croatia
Phileno Pinge-Filho, Brazil
Michele T. Pritchard, USA
Michal A. Rahat, Israel
Zoltan Rakonczay Jr., Hungary
Marcella Reale, Italy
Alexander Riad, Germany
Carlos Rossa, Brazil
Settimio Rossi, Italy
Bernard Ryffel, France
Carla Sipert, Brazil
Helen C. Steel, South Africa
Jacek Cezary Szepietowski, Poland
Dennis D. Taub, USA
Taina Tervahartiala, Finland
Kathy Triantafilou, UK
Fumio Tsuji, Japan
Giuseppe Valacchi, Italy
Luc Vallières, Canada
Elena Voronov, Israel
Kerstin Wolk, Germany
Soh Yamazaki, Japan
Shin-ichi Yokota, Japan
Teresa Zelante, Singapore

Contents

Immune Regulatory Cells in Inflammation, Infection, Tumor, Metabolism, and Other Diseases
Qingdong Guan, Cheng Xiao, and Minggang Zhang
Editorial (2 pages), Article ID 4170780, Volume 2018 (2018)

Expansion of a Population of Large Monocytes (Atypical Monocytes) in Peripheral Blood of Patients with Acute Exacerbations of Chronic Obstructive Pulmonary Diseases
Jing Yang, Man Qiao, Yanxia Li, Guohua Hu, Chunxia Song, Liping Xue, Hong Bai, Jie Yang, and Xi Yang
Research Article (13 pages), Article ID 9031452, Volume 2018 (2018)

Downregulation of DJ-1 Fails to Protect Mitochondrial Complex I Subunit NDUFS3 in the Testes and Contributes to the Asthenozoospermia
Yupeng Wang, Yi Sun, Xin Zhao, Renpei Yuan, Hui Jiang, and Xiaoping Pu
Research Article (10 pages), Article ID 6136075, Volume 2018 (2018)

Regulatory T Lymphocytes in Periodontitis: A Translational View
Carla Alvarez, Carolina Rojas, Leticia Rojas, Emilio A. Cafferata, Gustavo Monasterio, and Rolando Vernal
Review Article (10 pages), Article ID 7806912, Volume 2018 (2018)

Evaluation of Regulatory Immune Response in Skin Lesions of Patients Affected by Nonulcerated or Atypical Cutaneous Leishmaniasis in Honduras, Central America
Gabriela Venicia Araujo Flores, Carmen Maria Sandoval Pacheco, Thaise Yumie Tomokane, Wilfredo Sosa Ochoa, Concepción Zúniga Valeriano, Claudia Maria Castro Gomes, Carlos Eduardo Pereira Corbett, and Marcia Dalastra Laurenti
Research Article (7 pages), Article ID 3487591, Volume 2018 (2018)

The Immunoregulation of Th17 in Host against Intracellular Bacterial Infection
Yonghong Li, Chaojun Wei, Hui Xu, Jing Jia, Zhenhong Wei, Rui Guo, Yanjuan Jia, Yu Wu, Yuanting Li, Xiaoming Qi, Zhenhao Li, and Xiaoling Gao
Review Article (13 pages), Article ID 6587296, Volume 2018 (2018)

Intestinal Immunomodulatory Cells (T Lymphocytes): A Bridge between Gut Microbiota and Diabetes
Qingwei Li, Zezheng Gao, Han Wang, Haoran Wu, Yanwen Liu, Yingying Yang, Lin Han, Xinmiao Wang, Linhua Zhao, and Xiaolin Tong
Review Article (8 pages), Article ID 9830939, Volume 2018 (2018)

Emerging Roles of Immune Cells in Postoperative Cognitive Dysfunction
Yue Liu and Yiqing Yin
Review Article (8 pages), Article ID 6215350, Volume 2018 (2018)

Vγ4+ T Cells: A Novel IL-17-Producing $\gamma\delta$ T Subsets during the Early Phase of Chlamydial Airway Infection in Mice
Li-da Sun, Sai Qiao, Yue Wang, Gao-ju Pang, Xiao-yu Zha, Teng-Li Liu, Hui-Li Zhao, Ju-You Liang, Ning-bo Zheng, Lu Tan, Hong Zhang, and Hong Bai
Research Article (10 pages), Article ID 6265746, Volume 2018 (2018)

Phenotypic and Functional Properties of Tumor-Infiltrating Regulatory T Cells
Gap Ryol Lee
Review Article (9 pages), Article ID 5458178, Volume 2017 (2018)

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 4170780, 2 pages
https://doi.org/10.1155/2018/4170780

Editorial

Immune Regulatory Cells in Inflammation, Infection, Tumor, Metabolism, and Other Diseases

Qingdong Guan,[1,2] **Cheng Xiao**,[3] **and Minggang Zhang**[4]

[1]*Cellular Therapy Laboratory, CancerCare Manitoba, Winnipeg, MB, Canada*
[2]*The Institute of Clinical Research and Translational Medicine, Gansu Provincial Hospital, Lanzhou, China*
[3]*Institute of Clinical Medicine, China-Japan Friendship Hospital, Beijing, China*
[4]*Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*

Correspondence should be addressed to Qingdong Guan; qguan@hsc.mb.ca

Received 2 April 2018; Accepted 2 April 2018; Published 12 June 2018

Copyright © 2018 Qingdong Guan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The immune system is the host defense against infection, inflammation, tumor, and other diseases. The regulatory arm of immune system, including regulatory immune cells, regulatory complement, and regulatory cytokines, plays important roles in controlling immune responses and shutting down inflammation. However, cancer and some pathogens may escape and/or limit immune responses by driving immune regulatory cells. The balance of regulatory arm and effector arm of immune system maintains immune homeostasis. The recent advance has greatly increased our understanding of immune regulatory cells in health and pathogenesis of inflammation, infection, tumor, and other diseases. Immune regulatory cells are a large group of cells, such as regulatory T cells, myeloid-derived suppressor cells, mesenchymal stromal/stem cells, regulatory dendritic cells, and regulatory eosinophils. One of the keys to control infection, inflammation, tumor, and other diseases is to improve the understanding of immune regulatory cells in these situations.

In this special issue, we present original research articles as well as review papers on the role of immune regulatory cells with other immune cells in inflammation, infection, tumor, and other diseases. *Leishmania (L.) infantum chagasi* causes both visceral leishmaniasis and nonulcerated or atypical cutaneous leishmaniasis (NUCL). NUCL is characterized by mononuclear inflammatory infiltration of the dermis. By far, little is known about the pathogenesis of NUCL. G. V. A. Flores et al. evaluated the regulatory response in situ in skin lesions of patients affected by NUCL. By immunohistochemistry, $CD4^+$, $FoxP3^+$, TGF-β^+, and IL-10^+ cells were found in the dermis with inflammatory infiltration in all studied cases and at higher densities compared to the normal skin controls. A strong correlation was observed between $CD4^+$ and $FoxP3^+$ cells, and a moderate correlation was observed between $FoxP3^+$ and TGF-β^+, suggesting that T regulatory $FoxP3^+$ cells and the regulatory cytokines, especially TGF-β, play an important role in the immunopathogenesis of NUCL, modulating a cellular immune response in the skin, avoiding tissue damage, and leading to low tissue parasitic persistence.

In recent years, studies have generated an accumulating wealth of evidence on the role of IL-17-producing cells in protective immunity to intracellular bacterial pathogens, such as *Mycobacterium tuberculosis* and *Chlamydia trachomatis*, which are one of the most important types of pathogens that inflict significant socioeconomic burden across the globe. Y. Li et al. summarized the recent progress on the functions and mechanisms by which Th17/IL-17 responds to intracellular bacterial infections. L. Sun et al. showed that $V\gamma1^+$ T and $V\gamma4^+$ T cells were the major proliferative cell subsets of $\gamma\delta$ T cell during *Chlamydia muridarum* lung infection in mice; moreover, $V\gamma4^+$ T cells were the major IL-17 and IFNγ-producing $\gamma\delta$ T cell subsets at the early period of *Chlamydia muridarum* lung infection.

These findings provide new insights into the mechanisms bridging innate and adaptive immunity during lung chlamydial infections.

Recent advances have demonstrated that microbiota play important roles in many kinds of diseases. Q. Li et al. summarized how changes of the gut microbiota affect the physiological and pathological properties of the intestinal immunomodulatory cells, thus regulating diabetes mellitus. Understanding this bridge role of intestinal immunomodulatory cells may clarify the mechanisms by which the gut microbiota contributes to diabetes mellitus. Periodontitis is a chronic immunoinflammatory disease, in which the disruption of the balance between host and microbiota interactions is the key to the onset and progression of the disease. The immune homeostasis associated with periodontal health requires a regulated immunoinflammatory response, during which the presence of regulatory T cells (Treg) is essential to ensure a controlled response that minimizes collateral tissue damage. C. Alvarez et al. presented a comprehensive summary of regulatory T lymphocytes in periodontitis from bench to bedside.

Understanding the phenotypic and functional properties of tumor infiltrating Treg is essential to effectively and specifically target these cells in cancer therapy without compromising immune homeostasis in general. G. R. Lee summarized recent advances relating to tumor-infiltrating Treg in the tumor microenvironment, with particular emphasis on their accumulation, phenotypic, and functional properties, and targeting to enhance the efficacy of antitumor treatment.

Accumulating evidence suggests that neuroinflammation plays a critical role in postoperative cognitive dysfunction (POCD). Y. Liu and Y. Yin summarized the roles of immune regulatory cells with other immune cells and crosstalk between them in the pathogenesis of POCD, which may uncover promising therapeutic targets for POCD treatment and prevention.

Monocytes play an essential role in the pathogenesis of acute exacerbation of chronic obstructive pulmonary disease (AECOPD). J. Yang et al. analyzed monocyte subpopulation in the peripheral blood of healthy volunteers and AECOPD patients at the stages of admission and remission after clinical therapy, and they found a dramatic increase of a previously unreported population of large circulating atypical monocytes in AECOPD patients, characterized by higher forward scatter and lower side scatter, higher expression of CD16, intercellular adhesion molecule 1 (ICAM-1) and chemotactic protein-1 receptor-2 (CCR2), and lower expression of MHC class II antigen (HLA-DR) than typical monocytes. Further, it was found that the percentage of circulating atypical monocytes in total monocytes correlated with the length of hospital stay and disease duration. The circulating atypical monocytes might have the potential to serve as a biomarker in the diagnosis and prognosis of AECOPD.

Y. Wang et al. showed that the expression of mitochondrial protein nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) Fe-S protein 3 (NDUFS3) was significantly decreased in the sperm of patients with Asthenozoospermia (AS), and DJ-1 may play a role in maintaining mitochondrial function by means of the association with NDUFS3 during spermatogenesis in the testes. The interaction between DJ-1 and NDUFS3 in rat testes was weakened by ornidazole treatment. It showed that downregulation of DJ-1 and NDUFS3 expression likely contributes to mitochondrial dysfunction, which may underlie AS pathogenesis.

Overall, we believe that these articles may contribute to improve our knowledge of immune regulatory cell and other immune cell-mediated immune mechanisms in infection, inflammation, tumor, and other diseases, to provide insights into designing of effective immunodiagnostic tools, and to present rational basis for the development of potential therapeutic strategies.

Acknowledgments

We would like to thank the authors for their cutting-edge research data and thought-provoking reviews. We also express our gratitude to all the reviewers for their kind assistance and valuable insights.

Qingdong Guan
Cheng Xiao
Minggang Zhang

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 9031452, 13 pages
https://doi.org/10.1155/2018/9031452

Research Article

Expansion of a Population of Large Monocytes (Atypical Monocytes) in Peripheral Blood of Patients with Acute Exacerbations of Chronic Obstructive Pulmonary Diseases

Jing Yang[1,2] **Man Qiao**[3] **Yanxia Li**[3] **Guohua Hu**[3] **Chunxia Song**[3] **Liping Xue**[3] **Hong Bai**[1] **Jie Yang**[1] **and Xi Yang**[1,4]

[1]*Department of Immunology, Tianjin Key Laboratory of Cellular and Molecular Immunology and Key Laboratory of Educational Ministry of China, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China*
[2]*Department of Pharmacology, Institute of Acute Abdominal Diseases, Tianjin Nankai Hospital, Tianjin, China*
[3]*Department of Respiration, Tianjin Nankai Hospital, Tianjin, China*
[4]*Department of Immunology, Max Rady College of Medicine, University of Manitoba, Winnipeg, MB, Canada*

Correspondence should be addressed to Xi Yang; x.yang@umanitoba.ca

Received 27 December 2017; Accepted 27 February 2018; Published 17 May 2018

Academic Editor: Minggang Zhang

Copyright © 2018 Jing Yang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Acute exacerbation of chronic obstructive pulmonary disease (AECOPD) is closely associated with airway inflammation including monocytes, lymphocytes, and neutrophils. Monocytes play an essential role in the pathogenesis of chronic obstructive pulmonary disease (COPD). To elucidate the association of circulating monocyte alteration with AECOPD, we analyzed monocyte subpopulation in the peripheral blood of 16 healthy volunteers and 22 AECOPD patients at the stages of admission and remission after clinical therapy. We found a dramatic increase of a previously unreported population of large size circulating atypical monocytes (A Mo) in AECOPD patients, characterized by higher forward scatter and lower side scatter values than the typical monocytes (T Mo) which were observed predominantly in healthy individuals. Further analysis showed that A Mo expressed higher levels of CD16, intercellular adhesion molecule 1 (ICAM-1), and chemotactic protein-1 receptor-2 (CCR2) than T Mo. In contrast, the expression of class II antigen (HLA-DR) by A Mo was lower than T Mo. More importantly, we observed that the percentage of circulating A Mo among total monocytes correlated with the length of hospital stay (time to remission) and disease duration. The data suggest that circulating A Mo might have the potential to serve as a biomarker in the diagnosis and prognosis of AECOPD.

1. Introduction

COPD is defined as a progressive and irreversible decline in lung function caused by airflow obstruction which is associated with chronic airway inflammation in response to cigarette smoke or other noxious particles. The chronic inflammation often persists despite smoking cessation. Altered innate immune cell function appears to play a key role in the development and progression of COPD [1, 2]. On the one hand, increased numbers of monocytes, macrophages, and neutrophils traffic prominently into a patient airway; on the other hand, these invasive leukocytes exhibit defective function resulting in chronic bacterial colonization and perpetual infection. The dysregulation of the innate immune system in COPD is even more serious in the status of acute exacerbation [3].

AECOPD could have significant detrimental effects on patients, leading to the loss of lung function [4] and a reduced quality of life [5, 6] with a poorer survival rate [7]. The exacerbations are often caused by viral and/or bacterial infections in the respiratory tract accompanied by extravagant inflammatory responses [8, 9]. The influx of leukocytes in the lung of COPD/AECOPD patients is comprised of a variety of cells, including lymphocytes, neutrophils, and cells from the mononuclear phagocytic system (MPS), including monocytes, macrophages, and DCs [10]. Macrophages are

professional antigen-presenting cells and are specialized to maintain airway sterility. However, monocytes/macrophages in COPD patients, although increasing in number, show reduction in the phagocytic and killing function for clearing infectious agents and apoptotic cells [11–13], resulting in persistent bacterial colonization, necrotic material accumulation, and subsequent perpetuation of inflammation.

Accumulated macrophages in the airway originate from both tissue-resident macrophages and alveolar macrophages and from monocyte-derived macrophages recruited from the circulation [14, 15]. Blocking CC chemokine receptor 2 (CCR2), a critical regulator of monocyte trafficking, reduced monocyte accumulation and neutrophil influx in the airway in a mouse model [16], suggesting that newly recruited monocytes might participate in the inflammatory process. Similarly, the depletion of monocytes but not neutrophils could prevent lung emphysema induced by cigarette smoke exposure in rats [17]. The relationship between the circulating monocytes and airway inflammation raises the question whether it is possible to predict the inflammation in the lung by studying peripheral blood. Recent studies have established the heterogeneity and plasticity of circulating monocytes in animals and humans [18, 19]. Data from several groups also indicated that the varying patterns of monocyte subsets were associated with disease progression or prognosis [20–22]. However, it remains unclear for the changes in macrophage subpopulations especially circulating monocytes in a relationship with COPD or AECOPD.

In the present study, we evaluated the number, size, granularity, and surface markers of circulating monocytes in the peripheral blood of AECOPD patients in comparison with healthy controls. Our data show a dramatic increase of a novel A Mo population of monocytes in AECOPD patients. This novel A Mo population is characterized by a larger size and higher expression of surface CD16, ICAM, and CCR2 markers than T Mo in the healthy controls. About 60% of the monocytes in AECOPD patients were A Mo while A Mo was nearly negligible in the healthy controls. In addition, the A Mo population appeared expressing lower levels of HLA-DR, suggesting alteration in immune function. More importantly, we found that the percentage of A Mo in AECOPD patients correlated with hospital stay and disease duration of the patients. The finding suggests a novel classification strategy for monocyte subsets in the blood of AECOPD patients and provides the first proposal regarding the presence and definition of atypical monocytes, which provides new insights into the underlying roles of monocytes in the pathogenesis and prognosis of AECOPD. It also suggests that A Mo in circulation might have the potential to be a biomarker for the diagnosis and prognosis of COPD/AECOPD.

2. Materials and Methods

2.1. Study Subjects. This study population consisted of 16 healthy never-smoking donors (H), 22 hospitalized AECOPD patients at admission (Admission) and 17 AECOPD patients at remission (Remission) in Tianjin Nankai Hospital. All subjects provided fully informed consent in this study.

Cases of COPD were defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [23]. AECOPD was diagnosed as acute altered respiratory symptoms requiring additional treatments including oxygen, antibiotics, or systemic corticosteroids to meet the Anthonisen diagnostic criteria [24]. Lung function and chest radiography results were obtained for all participants. None of the patients had other serious diseases, such as asthma, allergic rhinitis, tuberculosis, or cancer. General data including age, gender, routine blood tests, and serum C-reactive protein (CRP) levels were recorded (Table 1).

2.2. Sample Preparation and Flow Cytometry Analysis. Peripheral venous blood samples were collected from healthy donors and AECOPD patients. Flow cytometric analysis of monocyte subsets was performed according to the forward scatter (FSC) and side scatter (SSC) as well as surface marker expression. Briefly, 100 ml whole blood was collected from each individual into an anticoagulant-coated tube (EDTA). Subsequently, the following antibodies were added into the blood sample and incubated for 15 minutes at room temperature in the dark: anti-CD14-PercP-Cy™5.5 (clone MφP9), anti-CD16-FITC (clone 3G8), and anti-CD54-APC (ICAM-1) (clone HA58), and Ig-matched isotypes were all purchased from BD Pharmingen; anti-HLA-DR-PE (clone LN3) and the corresponding isotype were purchased from eBioscience; anti-CCR2-APC (clone K036C2) and the corresponding isotypes were purchased from BioLegend. Then, 1 ml of lysis solution (BD Biosciences) was added to lyse erythrocytes. Finally, harvested cells were washed and analyzed with flow cytometry immediately. The four-color analysis was performed, and the expression levels of the surface markers mentioned above were measured on a FACSCalibur flow cytometer (BD Biosciences) equipped with 488 nm blue and 633 nm red lasers and analyzed by FlowJo software (Tree Star Inc., Ashland, OR). Surface molecule levels were expressed as the percentages and mean fluorescence intensity (MFI) values. A minimum of 100,000 events of the total cells was acquired.

2.3. Collection and Analysis of Laboratory and Inflammatory Parameters. Venous blood samples were collected and centrifuged for 10 min at 3000 rpm/min at 4°C. Serum CRP levels were determined by an ADVIA2400 Chemistry System (Siemens AG, Germany) according to the manufacturer's instructions. A routine test of blood leukocytes was done using the Sysmex XE-2100-automated blood cell counter (Sysmex, Kobe, Japan). Serum cytokine levels of IL-6 and IL-8 were measured by a Cytometric Bead Array kit (CBA, BD Pharmingen) according to the instructions. Briefly, serum samples (50 μL) were inoculated with 50 μL of capture microbeads and 50 μL of PE-conjugated detection reagents (anti-human IL-6 and anti-human IL-8) in the dark at room temperature. Three hours later, samples were washed and collected. Data were analyzed using the BD FCAP Array Software version 1.0.1 (BD Biosciences). All AECOPD patients

Table 1: Study population.

	Healthy	Admission	Remission
Age (yr)	56.23 ± 8.60	74.83 ± 8.92	72.35+ 7.98
Gender (M/F)	8/8 (16)	13/9 (22)	9/8 (17)
FEV1/FVC (%)	80.39 ± 7.50	52.10 ± 12.45***	
FEV1/pred (%)	90.57 ± 7.01	49.53 ± 11.63***	
WBC ($\times 10^9$/L)	5.48 ± 1.50	9.60 ± 3.58**	8.10 ± 2.01*
Neutrophil ($\times 10^9$/L)	4.51 ± 1.07	7.46 ± 3.27**	5.77 ± 2.14**
Monocyte ($\times 10^9$/L)	0.33 ± 0.10	0.64 ± 0.30*	0.59 ± 0.21*
Lymphocyte ($\times 10^9$/L)	1.97 ± 0.73	1.38 ± 0.95	1.80 ± 0.51
Neutrophil (%)	51.10 ± 6.30	76.46 ± 14.44***	68.00 ± 9.94***,▲
Monocyte (%)	5.54 ± 1.01	6.57 ± 3.39	7.61 ± 2.17
Lymphocyte (%)	32.14 ± 5.38	15.92 ± 10.93***	21.65 ± 8.19**,▲
CRP (mg/L)	<8.3	39.93 ± 28.89	9.94 ± 7.91▲▲
IL-6 (pg/mL)	3.76 ± 1.21	14.24 ± 11.07**	11.75 ± 9.79*
IL-8 (pg/mL)	25.43 ± 4.75	77.12 ± 37.96***	70.08 ± 33.44**

M: male; F: female; FEV1/pred: forced expiration volume in one second % predicted FEV1; FEV1/FVC: forced expiratory volume in one second/forced vital capacity; CRP: C-reactive protein; WBC: white blood cells. Data are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus healthy. ▲$P < 0.05$ and ▲▲$P < 0.01$ versus AECOPD.

underwent chest radiography to characterize the nature of their lung disease.

2.4. Statistical Analysis. The data are represented as the mean ± standard deviation (SD). For comparing distributions between groups, the nonparametric Newman-Keuls test was used, and for two independent groups, Student's *t*-test was performed. A *P* value less than 0.05 was considered to be statistically significant. A linear regression analysis was performed to examine the relationship between atypical monocyte count and total monocytes. All initial statistical calculations were done with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Patients with AECOPD Exhibit Systemic Inflammation. Clinical features, spirometric data, and laboratory parameters of AECOPD patients and healthy controls are detailed in Table 1. Compared to the values in healthy controls, the forced expiratory volume in one second/forced vital capacity (FEV1/FVC) and forced expiration volume in one second % predicted FEV1 (FEV1% predicted) values were dramatically lower in patients with AECOPD ($P < 0.001$). All patients were given routine blood examination twice, once at admission (AECOPD) and again at remission (Remission). The results showed that at admission, the absolute numbers of white blood cells (WBCs), neutrophils, and monocytes, as well as the percentages of neutrophils, were significantly increased in AECOPD patients compared with healthy controls, while the percentage of lymphocytes was lower in patients compared to controls ($P < 0.01$). Additionally, serum C-reactive protein (CRP) and IL-6 and IL-8 levels were also significantly higher in AECOPD patients than in the control group. At remission, the increased neutrophil count and percentage of neutrophils observed in AECOPD patients were decreased significantly compared to the values at admission (neutrophil count: 5.77 ± 2.14 versus 7.46 ± 3.27, resp., $P < 0.05$; neutrophil %: 68.00 ± 9.94 versus 76.46 ± 14.44, resp., $P < 0.05$). In contrast, the previously decreased lymphocyte percentage was elevated at remission compared to at admission (lymphocyte %: 21.65 ± 8.19 versus 15.92 ± 10.93, resp., $P < 0.05$), although it had still not returned to normal levels. Notably, although the number of monocytes was significantly increased in AECOPD patients compared with healthy controls ($P < 0.01$), there was no marked decline in monocyte numbers at remission. Additionally, the serum CRP level was reduced nearly five folds at remission compared to at admission (9.94 ± 7.91 versus 39.93 ± 28.89, resp., $P < 0.01$).

3.2. Atypical Monocytes Expand Dramatically in Patients with AECOPD. We further focused on the monocytes in AECOPD patients at admission and at remission after clinical therapy. As shown in Figure 1, scatter profiling clearly distinguished monocytes from bigger neutrophils (Neu) and smaller lymphocytes (Lym) by flow cytometry. Unlike the healthy controls that showed a rather universal monocyte population (T Mo), the monocytes in AECOPD patients were divided into two subpopulations of different sizes. The larger one with higher forward scatter (FSC) values was designated as "atypical monocytes or A Mo," and the smaller one which was similar as those in healthy controls was designated as "typical monocytes or T Mo." When we gated $CD14^+$ cells (monocytes) for scatter profiling, it also showed the separate A Mo population in AECOPD patients which was not seen in healthy controls (Figure 2(a)). We also examined the percentages and absolute numbers of these

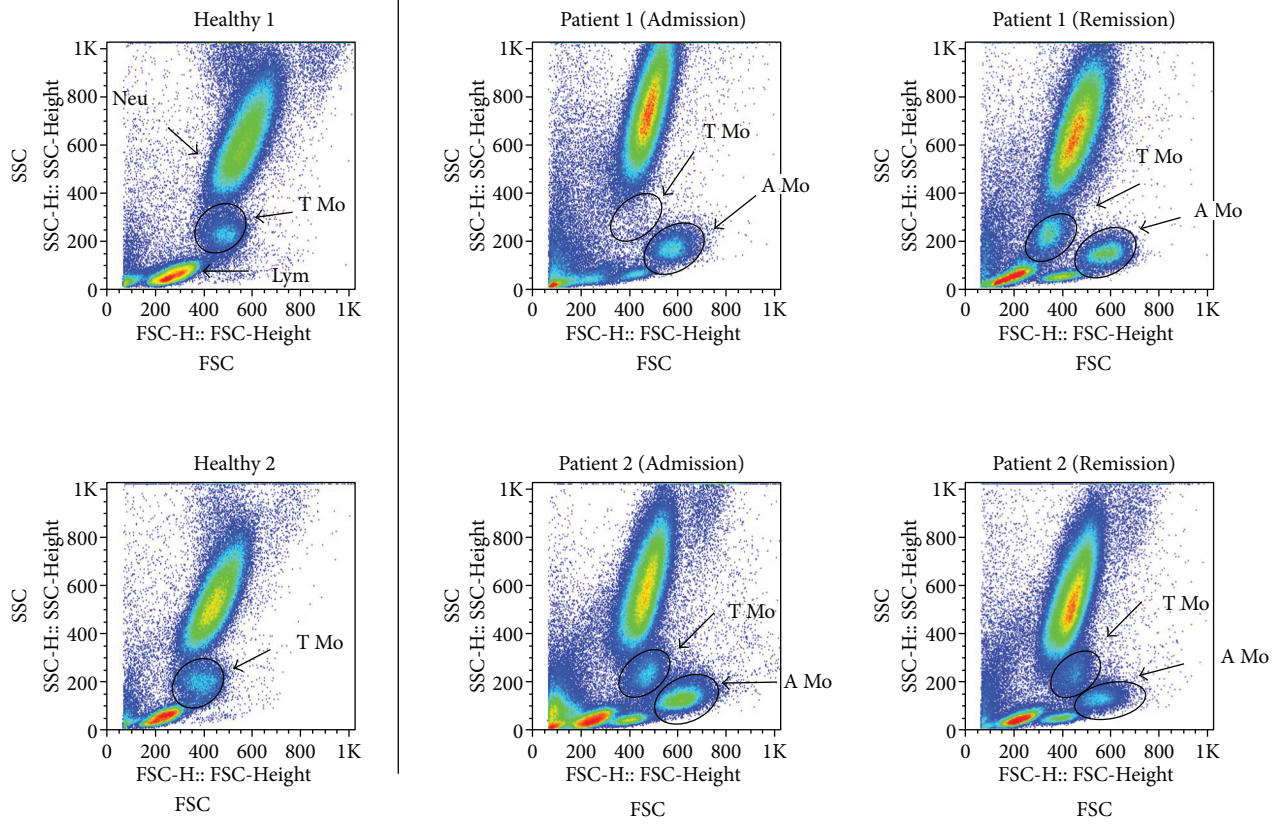


Figure 1: Gating strategy for the characterization of monocyte population. Representative dot plot of the whole blood cells from normal healthy donors and patients with AECOPD at admission by flow cytometry. Mo: monocytes; Neu: neutrophils; Lym: lymphocytes.

subpopulations (T Mo and A Mo) in AECOPD patients before (Admission) and after (Remission) successful clinical therapy in comparison with healthy subjects. As summarized in Figure 2(b), about 60% of monocytes in AECOPD patients were A Mo while virtually all the monocyte healthy controls were T Mo. When the absolute number of monocyte subsets was examined, it was found that the T Mo population in AECOPD was similar to that in healthy controls, so the difference of monocytes between the patient and the control was mainly in the A Mo population. The results suggest that expansion of A Mo is a characteristic change in the blood of AECOPD patients. Furthermore, although the patients at remission have achieved clinical improvement, the percentage of A Mo among total monocytes, on average, had no significant changes (Figure 2(c)). Although some individuals had changes in the percentage of Mo at remission, the trend was not consistent, namely, both increases and decreases being found (Figure 2(d)).

3.3. Differential Surface Marker Expression by A Mo and T Mo. To unravel the difference in the phenotypic signature between T Mo and A Mo, we compared the levels of CD14, CD16, ICAM-1, CCR2, and HLA-DR on these two subpopulations in patients and controls (Figure 3(a), Table 2). The expression levels of these molecules on the typical monocytes of healthy controls (H T Mo) were taken as baselines to which the levels on T Mo and A Mo of AECOPD patients at admission and at remission were compared (Figure 3(b)).

The data showed that the density (MFI) of CD14 was lower on T Mo and A Mo of AECOPD patients compared with monocytes (T Mo) from the healthy group ($P < 0.01$). The expression level of CD16 (% and MFI) increased dramatically on A Mo but not on T Mo of patients with AECOPD compared with those from healthy controls.

Reduced expression of HLA-DR molecules on monocytes is associated with a depressed immune status, especially in critically ill patients [25]. We found that both the percentage and the MFI of HLA-DR on T Mo were significantly lower in AECOPD patients than in healthy donors. Similar results could be observed for A Mo, but to a relatively lower degree. Increased ICAM-1 levels (MFI) on both subpopulations were found in patients compared with controls, and this upregulation was more pronounced on A Mo than on T Mo ($P < 0.001$).

CCR2 is an important chemokine receptor that is involved in the chemotaxis of specific monocyte subsets [26, 27]. We observed that only A Mo expressed higher levels of CCR2 than T Mo from healthy controls.

Notably, there were no significant differences in the expression of CD14, CD16, ICAM-1, HLA-DR, and CCR2 on any subset when cells from the same patient were compared before (at admission) and after clinical treatment (at remission).

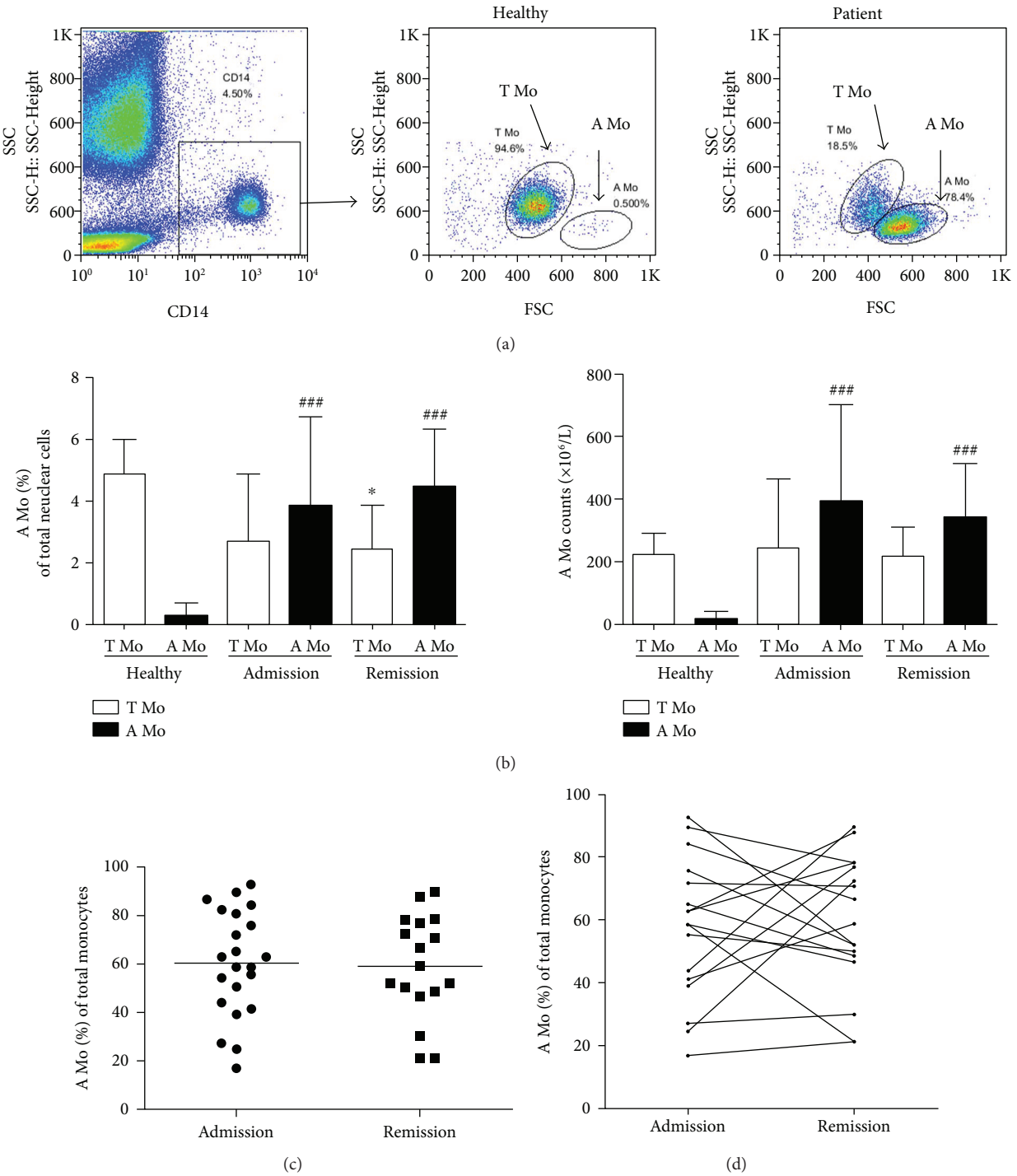
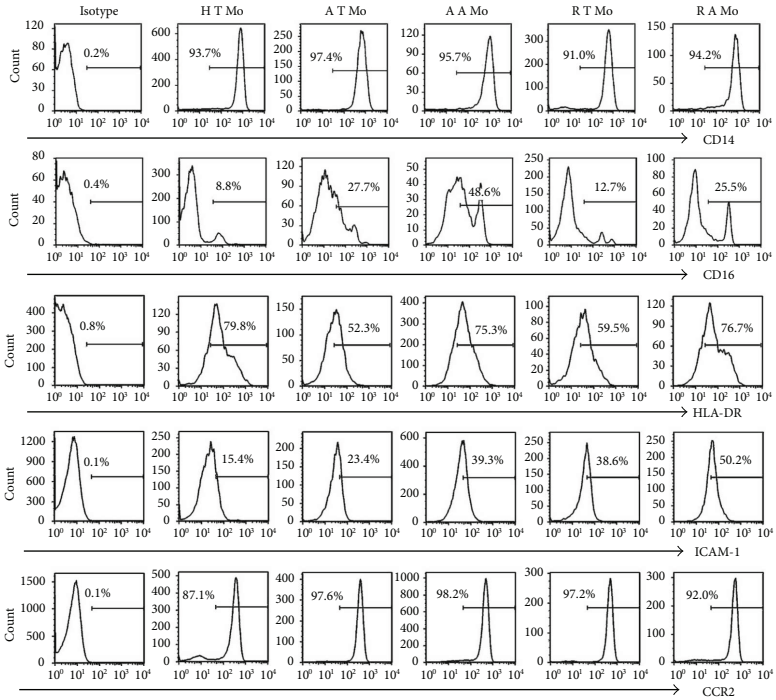
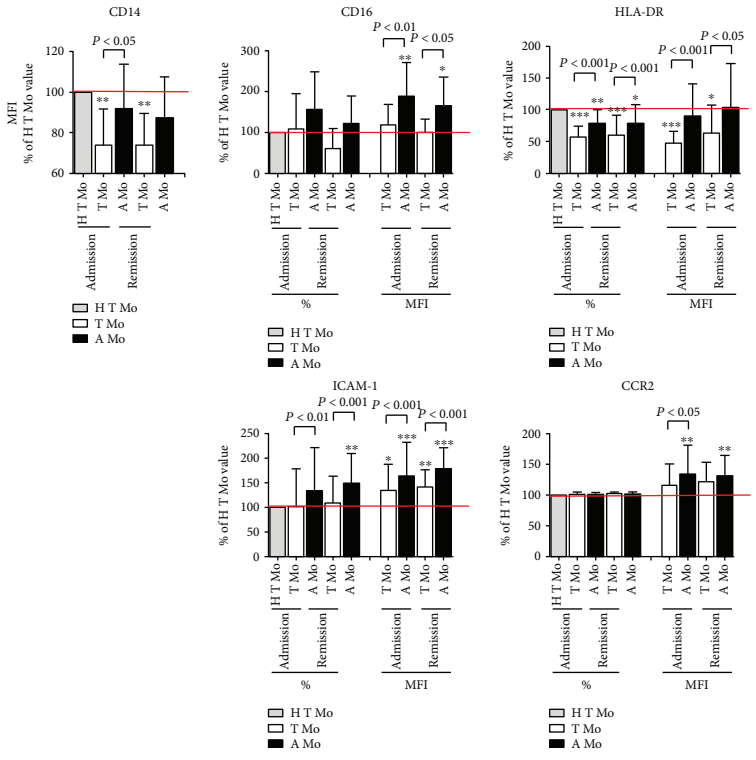


FIGURE 2: Light scatter distribution for T Mo and A Mo of healthy donors and AECOPD patients at admission and remission. (a) T Mo and A Mo in the $CD14^+$ population of AECOPD patients. (b) Summary of monocyte subsets in AECOPD patients and control subjects. The percentage (left) and absolute number (right) of T Mo (open bar) and A Mo (black bar) monocyte subset from the healthy control group, and patients at admission and remission are shown as the mean ± SD. Comparisons were performed by ANOVA with the nonparametric Newman-Keuls test. $^*P < 0.05$, compared with T Mo of healthy individuals. $^{\#\#\#}P < 0.001$, compared with A Mo of healthy subjects. (c) Frequency of A Mo in total $CD14^+$ monocytes from AECOPD patients ($n = 17$) at admission and remission. (d) Comparison of the percentage of A Mo in total $CD14^+$ monocytes from each AECOPD patient at admission and remission. The results were analyzed by Student's *t*-test.



(a)



(b)

Figure 3: Surface phenotype of T Mo and A Mo from AECOPD patients at admission ($n = 22$) and at remission ($n = 17$) as well as from the control group ($n = 16$). (a) Representative shows altered expression of surface markers of CD14/CD16/HLA-DR/ICAM-1/CCR2 in T Mo of healthy donors (H T Mo), from AECOPD patients at admission (A T Mo) and remission (R T Mo), as well as in A Mo of AECOPD patients at admission (A A Mo) and remission (R A Mo). (b) Mean data of the percentage and MFI of surface marker expression of T Mo (white bar) and A Mo (black bar) from patients at admission and at remission are represented. Values are expressed as the mean ± SD, and Student's t-test and the nonparametric Newman-Keuls test were used for comparisons between groups. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with T Mo of healthy subjects.

Table 2: Surface marker expression of A Mo and T Mo from AECOPD patients and controls.

	%					MFI				
	Healthy	AECOPD		Remission		Healthy	AECOPD		Remission	
	T Mo	T Mo	A Mo	T Mo	A Mo	T Mo	T Mo	A Mo	T Mo	A Mo
CD14	92.79 ± 2.09	97.47 ± 1.45***	96.66 ± 2.06***	98.28 ± 0.81***	96.88 ± 2.04***	607.00 ± 84.73	441.68 ± 115.60**	551.59 ± 149.01	441.41 ± 93.74**	521.47 ± 120.94
CD16	13.84 ± 4.83	13.14 ± 11.36	18.76 ± 12.62	8.43 ± 7.14	16.10 ± 9.80	8.21 ± 4.16	8.81 ± 5.07	12.74 ± 7.07**	6.41 ± 2.06	10.53 ± 4.49
HLA-DR	83.69 ± 8.95	49.27 ± 12.65***	66.42 ± 17.92***	54.05 ± 25.26***	69.83 ± 22.82**	53.71 ± 22.13	28.82 ± 10.25***	53.44 ± 29.25	39.73 ± 25.32	65.46 ± 39.66
ICAM-1	23.84 ± 4.65	25.14 ± 17.52	33.19 ± 19.44*	26.75 ± 12.16**	36.84 ± 12.83***	21.53 ± 2.89	28.20 ± 8.51*	34.59 ± 12.34***	31.53 ± 6.92***	39.59 ± 8.63***
CCR2	94.69 ± 1.40	96.19 ± 2.67	95.27 ± 3.35*	97.08 ± 2.10	95.30 ± 4.18	252.63 ± 32.97	292.00 ± 75.13	327.68 ± 96.90**	307.47 ± 76.62	339.41 ± 81.98**

Data are expressed as the mean ± SD, and Student's t-test and a nonparametric Newman-Keuls test were used for comparisons between groups. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus healthy.

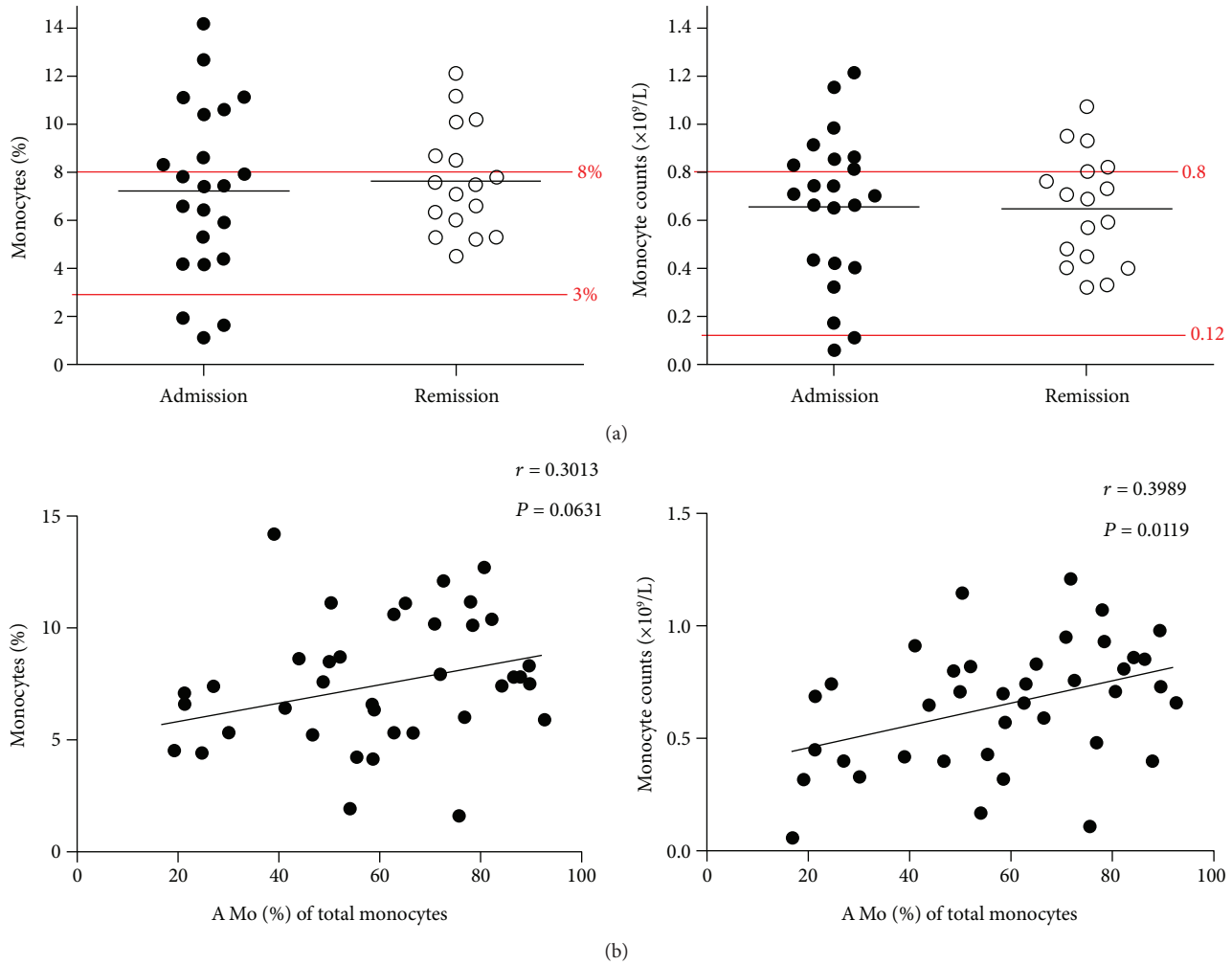


Figure 4: Relationships between A Mo and total monocytes. (a) Frequency (left panel) and absolute number (right panel) of circulating CD14 + monocytes in AECOPD patients at admission ($n = 17$) and remission ($n = 17$). The normal range of the percentage and the absolute number of monocytes were labeled in red line. (b) Correlation analyses of the proportion of A Mo with CD14+ monocytes in the peripheral blood of patients with AECOPD at admission ($n = 22$) and remission ($n = 17$). Each dot represents one individual. Simple linear regressions are shown.

Taking together, the phenotypic analysis showed increased expression of CD16, ICAM-1, and CCR-2, but decreased CD14 (density) and HLR-DR on A Mo.

3.4. Correlation of A Mo Amounts (Percentage and Absolute Number) with Disease Duration. To study the relationship between circulating monocytes and the disease status, we examined total and A Mo monocytes with several parameters. First, we examined the total monocyte ($CD14^+$) levels of AECOPD patients at admission and remission. As shown in Table 1 and Figure 4(a), there was no significant difference in the frequency and absolute number of $CD14^+$ total monocytes in AECOPD patients before and after clinical treatment. Not surprisingly, a simple linear regression analysis revealed that there was a trend for a positive correlation between the percentage of A Mo among total monocytes and the frequency of total monocytes among blood nuclear cells ($P = 0.0631$). There was additionally a strong positive correlation between the percentage of A Mo among monocytes and the absolute number of monocytes ($P = 0.0119$, Figure 4(b)). The results confirm the finding in Figure 2(b) that the predominant increase of monocytes in AECOPD patients was the A Mo subpopulation.

We further examined the relationship of total monocyte and A Mo with patient hospital stay and disease duration. Interestingly, we found that the proportion of A Mo among total $CD14^+$ monocytes, but not the proportion of total monocytes or the monocyte count, among the blood nuclear cells correlated positively with the length of hospital stay ($r = 0.4836$, $P = 0.0492$) and the length of disease duration ($r = 0.4952$, $P = 0.0433$). The results suggest that A Mo proportion might be a better marker for predicting AECOPD outcomes, particularly hospital stay and disease duration (Figure 5).

4. Discussion

In this study, for the first time, we demonstrated a high count of a population of large size monocytes, termed A Mo in the blood of AECOPD patients. These cells can be distinguished

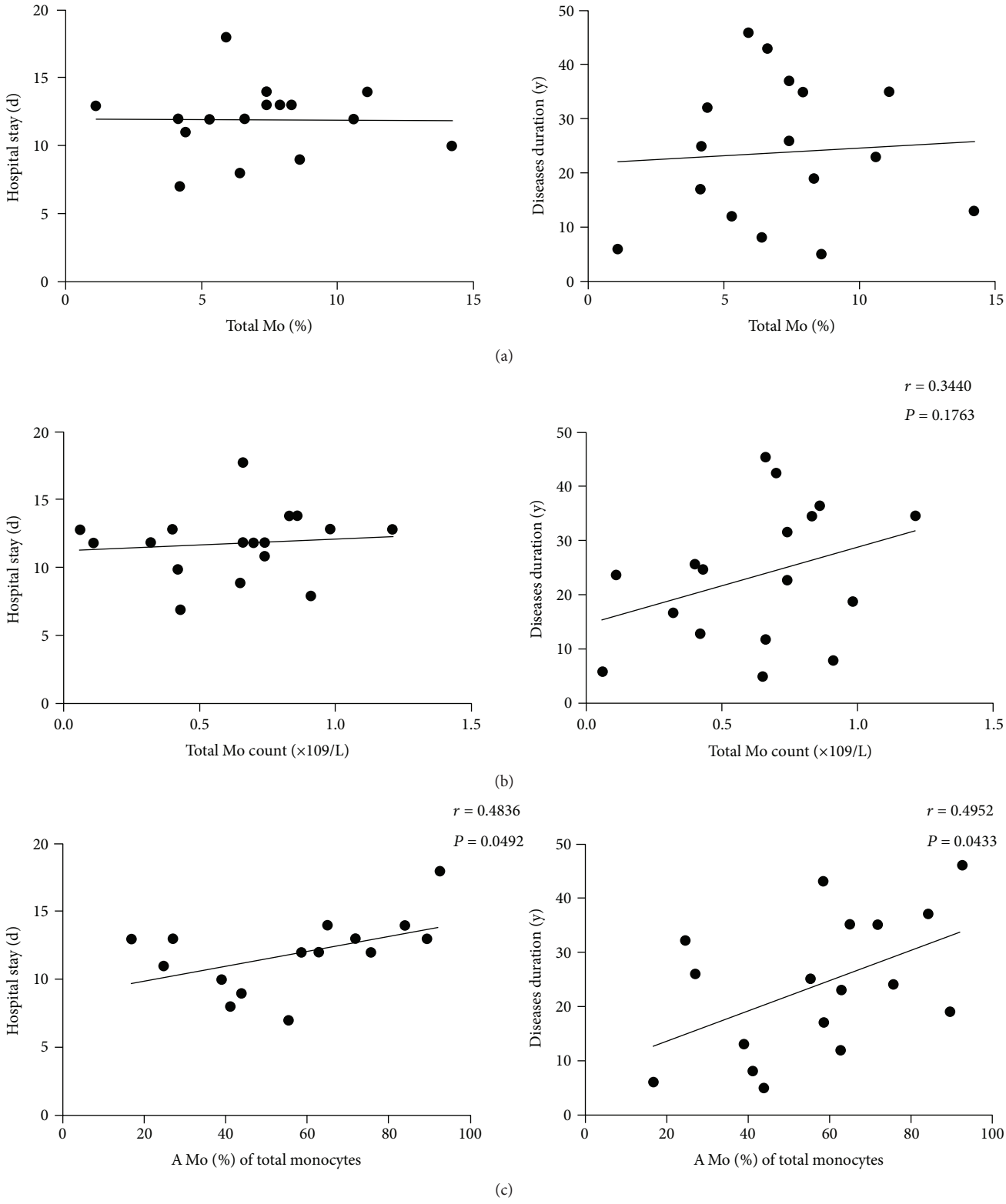


Figure 5: Correlations between numbers of A Mo and clinical parameters of patients with AECOPD. Relationships between the percentage of total monocytes (a) and total monocyte counts (b) and the percentage of A Mo in total monocytes (c) with the length of stay in the hospital of current acute exacerbate of COPD patients and duration of COPD ($n = 17$). Each dot represents one individual. Simple linear regressions are shown.

from the smaller typical monocytes that prevail in healthy individuals. About 60% of the monocytes in AECOPD patients were A Mo, which was negligible in healthy controls. In comparison with T Mo, A Mo exhibited higher levels of surface marker expression (CD14, CD16, HLA-DR, ICAM-1, and CCR2) in AECOPD patients, suggesting higher biological activity of A Mo. More importantly, we found that the proportion of A Mo showed a significantly positive correlation with the length of hospital stay and the years (duration) of the COPD history of AECOPD patients, which suggested that A Mo might be a predictive parameter for disease assessment of AECOPD in diagnosis and prognosis, at least for short-term outcomes.

In the 1980s, after monocytes were purified by density gradient, several groups found that human monocytes were comprised of two populations that differed in size and density [28–30]. Recently, based on the recognition of surface antigens by CD14 and CD16 monoclonal antibodies, three monocyte subsets were identified: a $CD14^{++}CD16^{-}$ classical subset, a $CD14^{++}CD16^{+}$ intermediate subset, and a $CD14^{+-}CD16^{++}$ nonclassical subset [31]. Using two-color fluorescence and morphological analysis, Ziegler et al. found that the three subsets differed in size: $CD14^{++}CD16^{-} > CD14^{++-}CD16^{+} > CD14^{+}CD16^{++}$ [32]. Another more recent study of an animal model also demonstrated that the Ly-$6C^{high}$ subset was larger than the Ly-$6C^{low}$ subset with higher FSC/SSC values [33]. Correspondingly, our study confirmed the inhomogeneous size of monocytes. In addition, we found that A Mo and T Mo in AECOPD patients were different not only in physical properties (size and granularity) but also in surface marker expression.

CD14, CD16, and CCR2 are the best-known surface markers for circulating monocyte identification. The proportion of $CD16^{+}$ monocytes is less than 5% in healthy individuals but rises with age [34], during infections [35, 36], and in patients with coronary artery disease [37] or periodontitis [38]. The interaction of chemokine receptor CCR2 with its ligand chemoattractant protein-1 (MCP-1, CCL2) is responsible for the migration of monocytes from circulation into a local inflammatory site [39–41]. ICAM-1 is constitutively expressed at low levels on the surface of epithelial cells, endothelial cells, and human monocytes under normal conditions but is increased during infection and inflammation, one of the primary functions of which is to regulate leucocyte infiltration and migration during respiratory infections [42, 43]. In this study, we found that the expression of CD16, CCR2, and ICAM-1 increased significantly on the surface of A Mo in patients with AECOPD compared with healthy controls, which implied that A Mo but not T Mo from AECOPD patients might exhibit stronger migratory ability. Therefore, we postulate that in contrast to the smaller T Mo that circulate in the peripheral blood, A Mo might contribute to the mobilization and migration of monocytes into local lung tissues in AECOPD. In fact, there are some clues to support our hypothesis. For example, a group described the percentage of a population of small macrophages that was significantly increased in induced sputum of COPD patients; these cells were considered monocyte/macrophage lineage cells based on the presence of CD14 and HLA-DR antigens [44]. This was consistent with the study by Rosseau et al., who reported that alveolar cells in acute respiratory distress syndrome (ARDS) patients were newly settled from the blood and shared a phenotype with circulating monocytes [45]. Correspondingly, several groups have demonstrated higher MCP-1 concentrations in sputum, plasma, and bronchoalveolar lavage (BAL) of both acute exacerbation and stable stage COPD patients compared to healthy controls [46, 47], and MCP-1 and CCR2 polymorphisms are considered new risk factors for COPD [48].

The expression of HLA-DR on monocytes/macrophages as measured by flow cytometry has been considered to be an indicator for predicting the occurrence of infections and to be related with outcomes [49]. Therefore, it is likely that monocytes, both T Mo and A Mo, are defective in AECOPD patients, and decreased HLA-DR level might contribute, at least in part, to the immunosuppressed or immune tolerant status of COPD patients [50] and even result in the frequent occurrence of AECOPD.

Considering the positive correlation between the percentage of A Mo and the total $CD14^{+}$ monocytes in circulation, our data indicate the possibility that increased total monocytes in the blood of AECOPD patients might be due to the occurrence of atypical monocytes. A question is then raised: where do atypical monocytes originate from? Are they released directly from the bone marrow or from other reservoirs such as the spleen, or do they differentiate from circulating monocytes in the blood? We cannot yet propose a conclusive answer to this question in the present research. It is well known that monocytes do not proliferate. In an inflammatory condition, the influx of monocytes in the bloodstream and lung might likely depend on mobilization from the bone marrow mediated by some critical molecules, such as CCR2 [39–41, 51]. Additionally, there has been a general agreement that monocytes develop from the haematopoietic stem and progenitor cells in the bone marrow via several sequential steps [52, 53]. Compared with mature monocyte in circulation, immature precursors such as monoblasts and promonocytes, which could not be observed in the peripheral blood of healthy individuals, are larger and exhibit lower granularity [54, 55], which seemed in line with the features of A Mo in our study. Notably, after clinical management, neither the increased number nor the altered expression levels of surface molecules on A Mo were observed to recover. These data might possibly explain why COPD patients with a history of AECOPD seem more inclined to frequently experience exacerbations [56].

However, there are no effective biomarkers to distinguish the diagnosis and outcome predictions of COPD/AECOPD. Molecular changes and gene expression profiling in the lung tissue might directly reflect lung pathology in the progression of COPD; however, lung tissue samples are not routinely accessible [57]. Hence, until now, the best predictor of exacerbations in stable COPD has been based on previous exacerbation events [58]. Whether inflammatory biomarkers can be applied in evaluating or predicting exacerbation events or outcomes in AECOPD patients is still a topic of debate. Previous studies have determined that some inflammatory biomarkers such as CRP, inflammatory cells, and fibrinogen

are associated with poor outcomes and an increased onset risk of exacerbations in patients with AECOPD [59–61]. In contrast, other studies have found that these biomarkers are helpful in clinical practice but are far from ideal in AECOPD assessments and prognoses, because most of them are nonspecific. However, the combination of several inflammatory biomarkers is still important and has been recommended [58]. Unlike the other two inflammatory immune cells that are predominant in the circulation, neutrophils that are related to innate immune response to bacterial infection and lymphocytes related to adaptive immune responses against the detailed biological effects of monocytes remain relatively ambiguous [62]. Additionally, there is still controversy regarding whether monocytes can be used as a valuable clinical indicator. Similarly, we also found that the numbers of total monocytes varied in AECOPD patients, but dramatically elevated A Mo numbers could be seen in nearly all the patients enrolled in this study. More importantly, A Mo but not total monocytes were positively associated with short-term outcomes (the length of hospital stay and disease duration) in AECOPD patients. A Mo might be a more sensitive and specific biomarker than total monocytes.

Although the finding of the predominant expansion of A Mo as a potential biomarker in predicting AECOPD hospital stay is encouraging, much more study is needed before it becomes a reality in clinical use. One of the major limitations of the study is the relatively small size of the samples, especially the control group, which were from healthy donors of younger ages than the patients. Second, although the phenotypic changes have provided some clue of the functional difference of A Mo from T Mo of healthy individuals and patients, there was no experimental study to confirm this. Thirdly, further characterization including the transcriptome study of the novel A Mo population is needed for its origin, function, migration, and distribution. Notably, we found that there are no significant correlations between the percentage of A Mo among monocytes with the systemic inflammatory parameters in the stages of admission and remission. As shown in Table 1, many of the inflammation parameters were improved at remission, but the A Mo population in average had no significant changes including their tested surface markers. The phenomena in one way might suggest the lack of correlation between A Mo with systemic inflammation but in the other way might be because of the smaller sample size of the study. Indeed, nearly half of the patients showed a reduction of A Mo at remission compared to admission (Figure 2(d)), which might be more consistent when large samples are tested. Therefore, further study is needed to properly assess the significance of the finding.

5. Conclusion

In conclusion, in this human study, through the comparison of a healthy individual with AECOPD patients at the time of hospital admission and remission, we observed a dramatic expansion of a novel monocyte population, A Mo, in the peripheral blood of AECOPD patients. More importantly, we found the percentage of the A Mo population at admission correlated with hospital stay and disease duration of AECOPD patients. The results suggest that A Mo level may potentially be a biomarker in disease diagnosis and short-term outcome prediction in AECOPD patients. In addition to a much larger size and multicenter study to confirm the finding, further studies should focus on the signature and biological function of A Mo in patients, as well as the relationship of A Mo with disease progression and long-term outcome.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This work was supported by research grants from Tianjin Medical University (116001-20100066) and Canadian Institute of Health Research (MOP 315712) to Xi Yang.

References

[1] G. G. Brusselle, G. F. Joos, and K. R. Bracke, "New insights into the immunology of chronic obstructive pulmonary disease," *The Lancet*, vol. 378, no. 9795, pp. 1015–1026, 2011.

[2] R. A. Holloway and L. E. Donnelly, "Immunopathogenesis of chronic obstructive pulmonary disease," *Current Opinion in Pulmonary Medicine*, vol. 19, no. 2, pp. 95–102, 2013.

[3] A. J. Mackay and J. R. Hurst, "COPD exacerbations: causes, prevention, and treatment," *Medical Clinics of North America*, vol. 96, no. 4, pp. 789–809, 2012.

[4] G. C. Donaldson, T. A. Seemungal, A. Bhowmik, and J. A. Wedzicha, "Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease," *Thorax*, vol. 57, no. 10, pp. 847–852, 2002.

[5] T. A. R. Seemungal, G. C. Donaldson, E. A. Paul, J. C. Bestall, D. J. Jeffries, and J. A. Wedzicha, "Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 157, no. 5, pp. 1418–1422, 1998.

[6] R. Kessler, E. Ståhl, C. Vogelmeier et al., "Patient understanding, detection, and experience of COPD exacerbations: an observational, interview-based study," *Chest*, vol. 130, no. 1, pp. 133–142, 2006.

[7] J. J. Soler-Cataluña, M. A. Martínez-García, P. Román Sánchez, E. Salcedo, M. Navarro, and R. Ochando, "Severe acute exacerbations and mortality in patients with chronic obstructive pulmonary disease," *Thorax*, vol. 60, no. 11, pp. 925–931, 2005.

[8] A. Papi, C. M. Bellettato, F. Braccioni et al., "Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations," *American Journal of Respiratory and Critical Care Medicine*, vol. 173, no. 10, pp. 1114–1121, 2006.

[9] A. Singanayagam, P. V. Joshi, P. Mallia, and S. L. Johnston, "Viruses exacerbating chronic pulmonary disease: the role of immune modulation," *BMC Medicine*, vol. 10, no. 1, p. 27, 2012.

[10] R. van Furth, Z. A. Cohn, J. G. Hirsch, J. H. Humphrey, W. G. Spector, and H. L. Langevoort, "The mononuclear phagocyte system: a new classification of macrophages, monocytes, and

their precursor cells," *Bulletin of the World Health Organization*, vol. 46, no. 6, pp. 845–852, 1972.

[11] L. E. Donnelly and P. J. Barnes, "Defective phagocytosis in airways disease," *Chest*, vol. 141, no. 4, pp. 1055–1062, 2012.

[12] A. E. Taylor, T. K. Finney-Hayward, J. K. Quint et al., "Defective macrophage phagocytosis of bacteria in COPD," *The European Respiratory Journal*, vol. 35, no. 5, pp. 1039–1047, 2010.

[13] S. Hodge, G. Hodge, R. Scicchitano, P. N. Reynolds, and M. Holmes, "Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells," *Immunology & Cell Biology*, vol. 81, no. 4, pp. 289–296, 2003.

[14] C. S. Berenson, M. A. Garlipp, L. J. Grove, J. Maloney, and S. Sethi, "Impaired phagocytosis of nontypeable *Haemophilus influenzae* by human alveolar macrophages in chronic obstructive pulmonary disease," *The Journal of Infectious Diseases*, vol. 194, no. 10, pp. 1375–1384, 2006.

[15] R. Vlahos and S. Bozinovski, "Role of alveolar macrophages in chronic obstructive pulmonary disease," *Frontiers in Immunology*, vol. 5, p. 435, 2014.

[16] U. Maus, K. von Grote, W. A. Kuziel et al., "The role of CC chemokine receptor 2 in alveolar monocyte and neutrophil immigration in intact mice," *American Journal of Respiratory and Critical Care Medicine*, vol. 166, no. 3, pp. 268–273, 2002.

[17] A. F. Ofulue and M. Ko, "Effects of depletion of neutrophils or macrophages on development of cigarette smoke-induced emphysema," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 277, no. 1, pp. L97–105, 1999.

[18] H. W. L. Ziegler-Heitbrock, "Definition of human blood monocytes," *Journal of Leukocyte Biology*, vol. 67, no. 5, pp. 603–606, 2000.

[19] F. Ginhoux and S. Jung, "Monocytes and macrophages: developmental pathways and tissue homeostasis," *Nature Reviews Immunology*, vol. 14, no. 6, pp. 392–404, 2014.

[20] J. Han, B. Wang, N. Han et al., "CD14highCD16+ rather than CD14lowCD16+ monocytes correlate with disease progression in chronic HIV-infected patients," *Journal of Acquired Immune Deficiency Syndromes*, vol. 52, no. 5, pp. 553–559, 2009.

[21] V. G. Valcour, B. T. Shiramizu, and C. M. Shikuma, "HIV DNA in circulating monocytes as a mechanism to dementia and other HIV complications," *Journal of Leukocyte Biology*, vol. 87, no. 4, pp. 621–626, 2010.

[22] G. H. Heine, C. Ulrich, E. Seibert et al., "$CD14^{++}CD16^{+}$ monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients," *Kidney International*, vol. 73, no. 5, pp. 622–629, 2008.

[23] J. Vestbo, S. S. Hurd, A. G. Agustí et al., "Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary," *American Journal of Respiratory and Critical Care Medicine*, vol. 187, no. 4, pp. 347–365, 2013.

[24] B. Q. Cai, S. X. Cai, R. C. Chen et al., "Expert consensus on acute exacerbation of chronic obstructive pulmonary disease in the People's Republic of China," *International Journal of Chronic Obstructive Pulmonary Disease*, vol. 9, pp. 381–395, 2014.

[25] K. Tschaikowsky, M. Hedwig-Geissing, A. Schiele, F. Bremer, M. Schywalsky, and J. Schüttler, "Coincidence of pro- and anti-inflammatory responses in the early phase of severe sepsis: longitudinal study of mononuclear histocompatibility leukocyte antigen-DR expression, procalcitonin, C-reactive protein, and changes in T-cell subsets in septic and postoperative patients," *Critical Care Medicine*, vol. 30, no. 5, pp. 1015–1023, 2002.

[26] A. I. P. M. Smits, V. Ballotta, A. Driessen-Mol, C. V. C. Bouten, and F. P. T. Baaijens, "Shear flow affects selective monocyte recruitment into MCP-1-loaded scaffolds," *Journal of Cellular and Molecular Medicine*, vol. 18, no. 11, pp. 2176–2188, 2014.

[27] P. Ancuta, R. Rao, A. Moses et al., "Fractalkine preferentially mediates arrest and migration of $CD16^{+}$ monocytes," *The Journal of Experimental Medicine*, vol. 197, no. 12, pp. 1701–1707, 2003.

[28] C. G. Figdor, W. S. Bont, I. Touw, J. de Roos, E. E. Roosnek, and J. E. de Vries, "Isolation of functionally different human monocytes by counterflow centrifugation elutriation," *Blood*, vol. 60, no. 1, pp. 46–53, 1982.

[29] T. Yasaka, N. M. Mantich, L. A. Boxer, and R. L. Baehner, "Functions of human monocyte and lymphocyte subsets obtained by countercurrent centrifugal elutriation: differing functional capacities of human monocyte subsets," *The Journal of Immunology*, vol. 127, no. 4, pp. 1515–1518, 1981.

[30] Y. Akiyama, P. J. Miller, G. B. Thurman et al., "Characterization of a human blood monocyte subset with low peroxidase activity," *The Journal of Clinical Investigation*, vol. 72, no. 3, pp. 1093–1105, 1983.

[31] E. F. Devêvre, M. Renovato-Martins, K. Clément, C. Sautès-Fridman, I. Cremer, and C. Poitou, "Profiling of the three circulating monocyte subpopulations in human obesity," *The Journal of Immunology*, vol. 194, no. 8, pp. 3917–3923, 2015.

[32] B. Passlick, D. Flieger, and H. W. Ziegler-Heitbrock, "Identification and characterization of a novel monocyte subpopulation in human peripheral blood," *Blood*, vol. 74, no. 7, pp. 2527–2534, 1989.

[33] F. K. Swirski, M. Nahrendorf, M. Etzrodt et al., "Identification of splenic reservoir monocytes and their deployment to inflammatory sites," *Science*, vol. 325, no. 5940, pp. 612–616, 2009.

[34] N. Glezeva, S. Horgan, and J. A. Baugh, "Monocyte and macrophage subsets along the continuum to heart failure: Misguided heroes or targetable villains?," *Journal of Molecular and Cellular Cardiology*, vol. 89, Part B, pp. 136–145, 2015.

[35] D. Castano, L. F. Garcia, and M. Rojas, "Increased frequency and cell death of $CD16^{+}$ monocytes with *Mycobacterium tuberculosis* infection," *Tuberculosis*, vol. 91, no. 5, pp. 348–360, 2011.

[36] Y. Rodríguez-Muñoz, S. Martín-Vílchez, R. López-Rodríguez et al., "Peripheral blood monocyte subsets predict antiviral response in chronic hepatitis C," *Alimentary Pharmacology & Therapeutics*, vol. 34, no. 8, pp. 960–971, 2011.

[37] T. Tallone, G. Turconi, G. Soldati, G. Pedrazzini, T. Moccetti, and G. Vassalli, "Heterogeneity of human monocytes: an optimized four-color flow cytometry protocol for analysis of monocyte subsets," *Journal of Cardiovascular Translational Research*, vol. 4, no. 2, pp. 211–219, 2011.

[38] T. Nagasawa, H. Kobayashi, M. Aramaki, M. Kiji, S. Oda, and Y. Izumi, "Expression of CD14, CD16 and CD45RA on monocytes from periodontitis patients," *Journal of Periodontal Research*, vol. 39, no. 1, pp. 72–78, 2004.

[39] N. V. Serbina and E. G. Pamer, "Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2," *Nature Immunology*, vol. 7, no. 3, pp. 311–317, 2006.

[40] L. Landsman, C. Varol, and S. Jung, "Distinct differentiation potential of blood monocyte subsets in the lung," *The Journal of Immunology*, vol. 178, no. 4, pp. 2000–2007, 2007.

[41] C. L. Tsou, W. Peters, Y. Si et al., "Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites," *The Journal of Clinical Investigation*, vol. 117, no. 4, pp. 902–909, 2007.

[42] A. G. Frick, T. D. Joseph, L. Pang, A. M. Rabe, J. W. St. Geme, and D. C. Look, "*Haemophilus influenzae* stimulates ICAM-1 expression on respiratory epithelial cells," *Journal of Immunology*, vol. 164, no. 8, pp. 4185–4196, 2000.

[43] R. Arnold and W. Konig, "Respiratory syncytial virus infection of human lung endothelial cells enhances selectively intercellular adhesion molecule-1 expression," *The Journal of Immunology*, vol. 174, no. 11, pp. 7359–7367, 2005.

[44] M. Frankenberger, M. Menzel, R. Betz et al., "Characterization of a population of small macrophages in induced sputum of patients with chronic obstructive pulmonary disease and healthy volunteers," *Clinical & Experimental Immunology*, vol. 138, no. 3, pp. 507–516, 2004.

[45] S. Rosseau, P. Hammerl, U. Maus et al., "Phenotypic characterization of alveolar monocyte recruitment in acute respiratory distress syndrome," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 279, no. 1, pp. L25–L35, 2000.

[46] W. I. de Boer, J. K. Sont, A. van Schadewijk, J. Stolk, J. H. van Krieken, and P. S. Hiemstra, "Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD," *The Journal of Pathology*, vol. 190, no. 5, pp. 619–626, 2000.

[47] P. G. Woodruff, A. Ellwanger, M. Solon, C. J. Cambier, K. E. Pinkerton, and L. L. Koth, "Alveolar macrophage recruitment and activation by chronic second hand smoke exposure in mice," *COPD*, vol. 6, no. 2, pp. 86–94, 2009.

[48] J. Bai, H. Song, C. Cai, M. Zhang, S. Xu, and J. Tan, "The association of monocyte chemotactic protein-1 and CC chemokine receptor 2 gene variants with chronic obstructive pulmonary disease," *DNA and Cell Biology*, vol. 31, no. 6, pp. 1058–1063, 2012.

[49] C. Landelle, A. Lepape, N. Voirin et al., "Low monocyte human leukocyte antigen-DR is independently associated with nosocomial infections after septic shock," *Intensive Care Medicine*, vol. 36, no. 11, pp. 1859–1866, 2010.

[50] G. Monneret, F. Venet, A. Pachot, and A. Lepape, "Monitoring immune dysfunctions in the septic patient: a new skin for the old ceremony," *Molecular Medicine*, vol. 14, no. 1-2, pp. 64–78, 2008.

[51] G. Conductier, N. Blondeau, A. Guyon, J. L. Nahon, and C. Rovère, "The role of monocyte chemoattractant protein MCP1/CCL2 in neuroinflammatory diseases," *Journal of Neuroimmunology*, vol. 224, no. 1-2, pp. 93–100, 2010.

[52] C. Auffray, M. H. Sieweke, and F. Geissmann, "Blood monocytes: development, heterogeneity, and relationship with dendritic cells," *Annual Review of Immunology*, vol. 27, no. 1, pp. 669–692, 2009.

[53] F. K. Swirski, I. Hilgendorf, and C. S. Robbins, "From proliferation to proliferation: monocyte lineage comes full circle," *Seminars in Immunopathology*, vol. 36, no. 2, pp. 137–148, 2014.

[54] A. Kitami, T. Suzuki, S. Suzuki, G. Hori, H. Mori, and T. Mitsuya, "Effective treatment of thymic carcinoma with operation and combination chemotherapy against acute monocyte leukemia: case report and review of the literature," *Japanese Journal of Clinical Oncology*, vol. 28, no. 9, pp. 555–558, 1998.

[55] D. G. Tenen, "Disruption of differentiation in human cancer: AML shows the way," *Nature Reviews Cancer*, vol. 3, no. 2, pp. 89–101, 2003.

[56] G. C. Donaldson and J. A. Wedzicha, "COPD exacerbations .1: Epidemiology," *Thorax*, vol. 61, no. 2, pp. 164–168, 2006.

[57] J. G. Shaw, A. Vaughan, A. G. Dent et al., "Biomarkers of progression of chronic obstructive pulmonary disease (COPD)," *Journal of Thoracic Disease*, vol. 6, no. 11, pp. 1532–1547, 2014.

[58] M. Thomsen, T. S. Ingebrigtsen, J. L. Marott et al., "Inflammatory biomarkers and exacerbations in chronic obstructive pulmonary disease," *JAMA*, vol. 309, no. 22, pp. 2353–2361, 2013.

[59] M. Dahl, A. Tybjœrg-Hansen, J. Vestbo, P. Lange, and B. G. Nordestgaard, "Elevated plasma fibrinogen associated with reduced pulmonary function and increased risk of chronic obstructive pulmonary disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 164, no. 6, pp. 1008–1011, 2001.

[60] M. Dahl, J. Vestbo, P. Lange, S. E. Bojesen, A. Tybjærg-Hansen, and B. G. Nordestgaard, "C-reactive protein as a predictor of prognosis in chronic obstructive pulmonary disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 175, no. 3, pp. 250–255, 2007.

[61] A. Agustí, L. D. Edwards, S. I. Rennard et al., "Persistent systemic inflammation is associated with poor clinical outcomes in COPD: a novel phenotype," *PLoS One*, vol. 7, no. 5, article e37483, 2012.

[62] A. Mantovani, M. A. Cassatella, C. Costantini, and S. Jaillon, "Neutrophils in the activation and regulation of innate and adaptive immunity," *Nature Reviews Immunology*, vol. 11, no. 8, pp. 519–531, 2011.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 6136075, 10 pages
https://doi.org/10.1155/2018/6136075

Research Article

Downregulation of DJ-1 Fails to Protect Mitochondrial Complex I Subunit NDUFS3 in the Testes and Contributes to the Asthenozoospermia

Yupeng Wang,[1,2] Yi Sun,[1,2] Xin Zhao,[1,2] Renpei Yuan,[3] Hui Jiang,[3] and Xiaoping Pu[1,2]

[1]*National Key Research Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100191, China*
[2]*Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China*
[3]*Department of Urology, Peking University Third Hospital, Beijing 100191, China*

Correspondence should be addressed to Yi Sun; sunyi@bjmu.edu.cn, Hui Jiang; jianghui55@163.com, and Xiaoping Pu; pxp123@bjmu.edu.cn

Received 1 November 2017; Revised 29 December 2017; Accepted 14 January 2018; Published 3 April 2018

Academic Editor: Cheng Xiao

Copyright © 2018 Yupeng Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Asthenozoospermia (AS), an important cause of male infertility, is characterized by reduced sperm motility. Among the aetiologies of AS, inflammation seems to be the main cause. DJ-1, a conserved protein product of the *PARK7* gene, is associated with male infertility and plays a role in oxidative stress and inflammation. Although our previous studies showed that a reduction in DJ-1 was accompanied by mitochondrial dysfunction in the sperm of patients with AS, the specific mechanism underlying this association remained unclear. In this study, we found that compared to the patients without AS, the expression of mitochondrial protein nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) Fe-S protein 3 (NDUFS3) was also significantly decreased in the sperm of patients with AS. Similarly, decreased expression of DJ-1 and NDUFS3 and reduced mitochondria complex I activity were evident in a rat model of AS. Moreover, we showed that the interaction between DJ-1 and NDUFS3 in rat testes was weakened by ORN treatment. These results suggest that the impaired mitochondrial activity could be due to the broken interaction between DJ-1 and NDUFS3 and that downregulation of DJ-1 in sperm and testes contributes to AS pathogenesis.

1. Introduction

Asthenozoospermia (AS) is a common cause of human male infertility [1], characterized by reduced sperm motility (grade A + B sperm motility < 50% or A < 25%). It is involved in more than 40% of infertility in men [2]. Inflammation is a largely reversible cause of male infertility. Neutrophils and macrophages can damage spermatozoa by generating pro-inflammatory cytokines and reactive oxygen species (ROS), which leads to oxidative stress and DNA fragmentation that may affect sperm motility and metabolism, consequently causing infertility [3, 4]. Mitochondria have been proposed as major contributors to oxidative stress, which can result in defective sperm in humans [5]. Electron microscopy showed that sperm from patients with AS have disordered mitochondria, with significantly shorter midpieces and fewer mitochondrial gyres than their normozoospermic counterparts [6]. Furthermore, sperm quality, particularly motility, is positively correlated with the enzymatic activity of electron transfer chain (ETC) complexes [7, 8] and the expression of ETC subunits [9]. Mitochondrial protein nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) Fe-S protein 3 (NDUFS3) is a core component of complex I (CI) in the respiratory chain of the mitochondrial matrix for CI assembly and activity [10] and was found to be associated with DJ-1 in NIH3T3 and HEK293 cells [11].

DJ-1, the *PARK7* gene product, is a ubiquitous protein of 189 amino acids that belongs to the Thi/PfpI protein superfamily of molecular chaperones. It is highly conserved in a variety of mammalian tissues, and mutations or deletions in *PARK7* have been found associated with many diseases, including male infertility. DJ-1 has been proposed

to function as a survival factor and antioxidant [12, 13]. Furthermore, the function of DJ-1 in inflammation has been elucidated. DJ-1 regulates the expression of proinflammatory cytokines by regulating NF-κB transcriptional activity in macrophages [14]. Additionally, DJ-1 has been shown to play a pivotal modulatory role by triggering inflammation and subsequently enhancing the secretion of IL-6 and TNF-α in liver progenitor cells [15].

DJ-1 and its homologues, sperm protein 22 (SP22) and contraception-associated protein 1 (CAP1), were the first proteins found to be correlated with male infertility [16–18]. DJ-1 is expressed after second spermatocytes appear during spermatogenesis and is ultimately mainly located in the sperm head, suggesting an important function for DJ-1 in spermatogenesis [17, 18]. Downregulation of DJ-1 was found in ejaculated sperm from Chinese patients with AS, and DJ-1 was shown to translocate into sperm mitochondria during oxidative stress to maintain mitochondrial structure [19, 20].

Ornidazole- (ORN-) treated rats have been a common animal model to study AS in the last several decades [21]. This model is established by daily intragastric administration of ORN to rats for 10~14 days [22]. Although DJ-1 has been found to be associated with NDUFS3 in some somatic cells, this interaction in male germ cells has not yet been reported, and its expression during spermatogenesis in AS is still unknown. In this study, we analyzed changes in the expression of and interaction between DJ-1 and NDUFS3 in sperm and testes to understand AS pathogenesis.

2. Material and Methods

2.1. Ethics Statement

2.1.1. Participants. We enrolled 10 males (aged 21~45 years) diagnosed with infertility in the Department of Andrology, Peking University Third Hospital, Beijing, China, and 10 age-matched control subjects with normal semen parameters. Semen samples of patients with pyospermia or varicocele or with a history of smoking were not obtained for this study. All participants provided informed consent for participation in the study, and the study was approved by the Ethics Institutional Review Board of Peking University Third Hospital, under protocol number 2011SZ016.

2.1.2. Animals. Sexually mature male Sprague-Dawley rats weighing 330~370 g at the beginning of the experiment were obtained from Charles River Laboratories Inc. (SCXK (Jing) 2012-0001, Beijing, China). The rats were maintained at a controlled temperature ($24 \pm 2^{\circ}$C) and housed by group in separate cages (12 h light/dark cycle) with access to food and water ad libitum. All experimental procedures involving the use of animals were approved by the Animal Care and Use Committee of Peking University.

2.2. Experimental Procedures with Human Sperm

2.2.1. Western Blotting. Total sperm protein was extracted as described previously [19]. In brief, the sperm were washed twice with Hanks balanced salt solution with 4.2 g/L hydroxyethyl piperazine ethanesulfonic acid (HEPES), 0.35 g/L $NaHCO_3$, 0.9 g/L D-glucose, 0.1 g/L sodium pyruvate, 0.025 g/L soybean trypsin inhibitor, and freshly added EDTA-free halt protease inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, USA) by centrifugation (2500 ×g, 10 min). The combined sperm pellets, resuspended in 80 mM Tris-HCl buffer (pH 7.4) with 150 mmol/L NaCl, 2 mmol/L ethylenediamine tetraacetic acid, 0.4 mmol/L dithiothreitol, and 0.1% sodium dodecyl sulfate (SDS), were ultrasonically oscillated and then homogenized for 1 h on ice. Finally, the mixtures were centrifuged at 12,000 ×g for 20 min to remove insoluble debris, and the supernatant fraction was frozen at −80°C until use. The protein concentration was measured using an enhanced bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China). Proteins (60 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, USA). Membrane was blocked with 5% nonfat milk in Tris-buffered solution with Tween-20 under agitation. Proteins were sequentially incubated with anti-NDUFS3 monoclonal antibody (1 : 3000, Abcam, Cambridge, UK) and horseradish peroxidase- (HRP-) conjugated IgG (1 : 5000, KPL, Gaithersburg, USA). GAPDH was used as an internal standard. Protein bands were visualized using an enhanced chemiluminescence detection kit (ECL; Applygen Technologies Inc., Beijing, China) and a Molecular Imager ChemiDoc XRS+ Imaging System (Bio-Rad, Hercules, USA). Quantity One software (Bio-Rad, Hercules, USA) was used to quantify the optical density of each band, and the amount of NDUFS3 was normalized to that of GAPDH.

2.2.2. Immunofluorescence Assay. Spermatozoa were collected by density gradient centrifugation, using Percoll solution (Sigma, St. Louis, USA) as a medium [23]. All Percoll solutions were buffered with Biggers, Whitten, and Whittingham medium (BWW). Following centrifugation, sperm that accumulated at the bottom of the tube were collected.

According to the operation manual recommended by Life Technologies Corporation, spermatozoa were resuspended in prewarmed (37°C) BWW containing 200 nM MitoTracker Deep Red FM (MT-DR FM) (Invitrogen, Carlsbad, USA) and incubated at 37°C for 45 min. After staining, the spermatozoa were washed, spotted on glass slides, and air-dried. These preparations were fixed with 4% paraformaldehyde and then permeabilized with ice-cold acetone. After blocking with 10% goat serum, spermatozoa were sequentially incubated with anti-DJ-1 monoclonal antibody (1 : 200, Abcam, Cambridge, UK) or anti-NDUFS3 monoclonal antibody (1 : 200, Abcam, Cambridge, UK) and then goat anti-rat lgG secondary antibody (1 : 200, Alexa Fluor 555 conjugate) or goat anti-mouse lgG secondary antibody (1 : 200, Alexa Fluor 488 conjugate) (Thermo Fisher Scientific Inc., Rockford, USA). Normal rabbit or mouse IgG was used as a negative control. Nuclei were counterstained with Hoechst 33342. All samples were observed by laser scanning confocal microscopy (TCS SP8-Confocal-MP-FLIM, Leica, Mannheim, Germany).

Table 1: General seminal parameters.

	Age	pH	Ejaculate volume (mL)	Sperm density (10^6/mL)	Grade A sperm (%)	Grade A + B sperm (%)
Control	29.90 ± 1.233	7.420 ± 0.08406	4.140 ± 0.4110	57.23 ± 6.306	37.75 ± 1.984	60.39 ± 2.951
AS	30.30 ± 1.221	7.390 ± 0.05859	3.640 ± 0.4031	59.53 ± 11.85	11.95 ± 1.753****	22.47 ± 2.139****

****$P < 0.0001$, one-way ANOVA test, $n = 10$ in each group.

2.3. Experimental Procedures with Rat Samples

2.3.1. Rat Model of AS. A rat model of AS was generated by intragastric administration of ORN, according to a previously described method with some modifications [24]. Briefly, twenty sexually mature (330~370 g) male Sprague-Dawley rats were randomly assigned to two groups (ORN and control) with ten rats per group. In the ORN group, ORN was dissolved in 1% (*w*/*v*) sodium carboxymethylcellulose (CMC-Na) in water, and a dose of 400 mg/kg body weight was fed to adult male rats once a day by oral gavage for a period of 14 days. The control rats received 1% (*w*/*v*) CMC-Na in water without ORN throughout the experiment.

2.3.2. Sperm Motility and Count. After the last intragastric administration of ORN or the control solution on day 14, the animals were anesthetized with intraperitoneal (i.p.) administration of 0.6% pentobarbital sodium (10 mL/kg). To assess sperm motility, sperm in the cauda epididymides were collected and prepared as described elsewhere [25]. In brief, each caudal epididymis was placed in 4 mL of saline prewarmed to 34°C and then incised in several places to allow the semen to ooze out. After this, an aliquot of 10 μL was transferred to a histological slide. Under a light phase contrast microscope (200x magnification, Binocular, Olympus IX71, Tokyo, Japan), 400 sperm were analyzed and classified into three types on the basis of their motility: progressive motility (PR), nonprogressive motility (NP), and immotility (IM). Sperm motility data were expressed as the percentage of sperm that were progressively motile.

Sperm counts were evaluated according to an established method [26]. Semen obtained from cauda epididymides was diluted, thoroughly mixed, and transferred to a hemocytometer with a cover slip overlay. The semen was observed under a light microscope at 200x, and the sperm in each specimen were counted.

2.3.3. Western Blotting. Rats were deeply anesthetized with 0.6% pentobarbital sodium (10 mL/kg i.p.) and cardiac perfused with normal saline. The testes and sperm were immediately homogenized in ice-chilled lysis RIPA buffer. The following steps were the same as those used for patients' samples. β-Actin was used as an internal standard. The standardized ratio of DJ-1 and NDUFS3 to β-actin band density was used to indicate change in expression.

2.3.4. Sperm Mitochondrial Membrane Potential (MMP) Analysis. Spermatozoa collected from cauda epididymides were resuspended in prewarmed (37°C) phosphate-buffered saline (PBS) containing 200 nM MT-DR FM and incubated at 37°C for 45 min. After staining, the sperm were pelleted again by centrifugation and resuspended in prewarmed PBS. This spermatozoon suspension was spotted on glass slides and air-dried. The nuclei of cells on the slides were counterstained with Hoechst 33342 and mounted with antifade mounting medium. All samples were observed by laser scanning confocal microscopy (TCS SP8-Confocal-MP-FLIM, Leica, Mannheim, Germany).

2.3.5. CI Enzyme Activity Assay. The analysis of CI enzyme activity in sperm and testes was performed using a CI Enzyme Activity Microplate Assay Kit (Abcam, Cambridge, UK) following the kit protocol. In short, CI was extracted and introduced into a microplate. After incubation at room temperature for 3 h, the microplate was washed, and assay solution was added. Absorbances of samples were tested at 450 nm for 30 min with an interval of 20 s, following the kinetic program in a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, USA). Activity was expressed as the change in absorbance per minute per amount of sample loaded into a well. The data are shown as the standardized ratio of the activity in the ORN-treated rats to that in the control animals.

2.3.6. Coimmunoprecipitation (Co-IP). Using the method described above, the testes of rats were removed and homogenized in a glass homogenizer. The protein extract was preincubated with Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) and normal goat IgG (M&C Gene Technology Ltd.) to remove nonspecifically adhered proteins. One milliliter of the above tissue lysate (approximately 500 μg total cellular protein) was transferred to a microcentrifuge tube, and goat antibody to DJ-1 (1 : 100, Abcam, Cambridge, UK) was added for co-IP. Another 1.0 mL of lysate with normal goat IgG was used as the negative control group. The samples were incubated for 4 h at 4°C, and then Protein A/G PLUS-Agarose was added to each. After incubation on a rotating device at 4°C overnight, the immunoprecipitates were collected by centrifugation. The pellets were then washed and resuspended in electrophoresis sample buffer. After boiling, the samples were centrifuged to obtain the supernatant for SDS-PAGE analysis.

2.4. Statistical Analysis. Statistical analyses were performed with GraphPad Prism 6 for Windows. All data were expressed as means ± standard errors of the mean (SEM). One-way analysis of variance (ANOVA) was used to make comparisons between groups. P values < 0.05 were considered indicative of significant differences.

3. Results

3.1. General Parameters of Semen from Participants. General parameters, including age of participants, ejaculate volume,

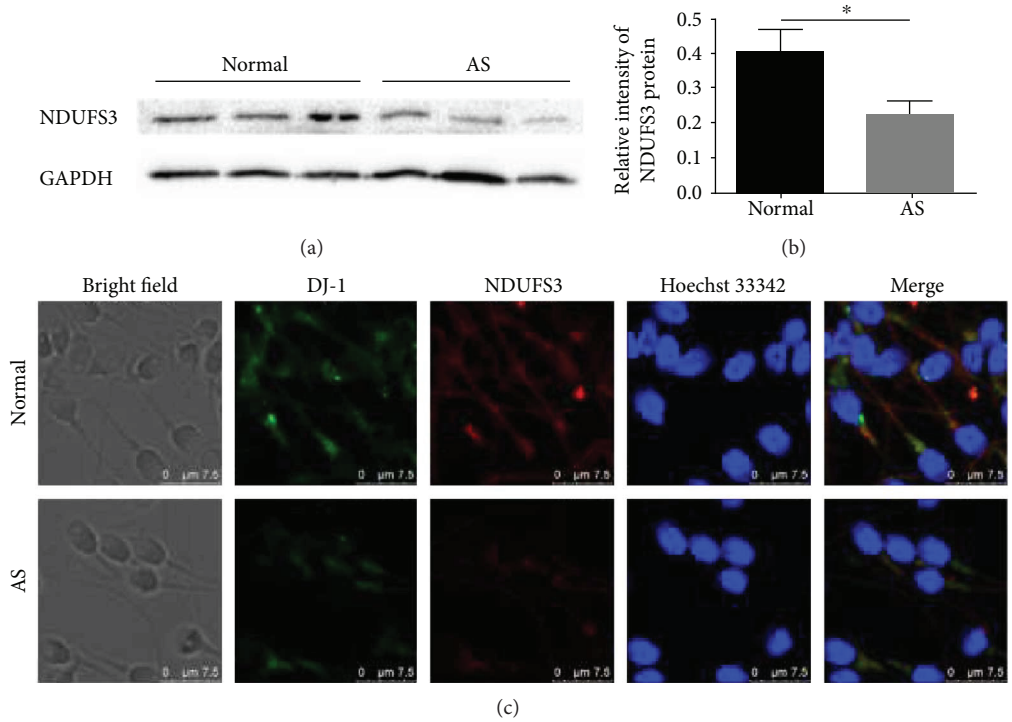


Figure 1: Downregulation of NDUFS3 in the sperm of patients with AS. (a) Representative immunoblot showing NDUFS3 expression in human sperm. (b) Quantification of "a." NDUFS3 expression was normalized to that of GAPDH as a loading control. The results showed that NDUFS3 expression was reduced significantly in the sperm of patients with AS. (c) Immunofluorescence of DJ-1 (green) and NDUFS3 (red) in sperm from patients with and without AS. Sperm nuclei were stained with Hoechst 33342 (blue). Note that NDUFS3 were both downregulated in the sperm of patients with AS and their colocalization in the midpiece of sperm. $^{*}P < 0.05$, one-way ANOVA test, $n = 10$ in each group. Scale bar = 7.5 μm. AS: asthenozoospermia.

sperm density, and percentage of grade A and grade A + B sperm, are listed in Table 1. Ten males (aged 21~45 years), diagnosed with infertility as described above, were enrolled, and 10 age-matched males with normal semen parameters were registered as control subjects. No participants exhibited pyospermia or varicocele or had a history of smoking.

3.2. Change in NDUFS3 Expression in Sperm of Participants with AS. Our previous studies confirmed the significant reduction in DJ-1 in the sperm of patients with AS [19, 20]. NDUFS3 was identified as a single band of approximately ~30 kDa by Western blot (Figure 1(a)). Quantification of the NDUFS3 bands revealed that NDUFS3 expression decreased along with DJ-1 expression in patients with AS compared to that in control subjects (NDUFS3/GAPDH: 0.40 ± 0.07 versus 0.23 ± 0.04, $P < 0.05$; Figure 1(b)). In the immunofluorescence images, we found that both DJ-1 and NDUFS3 were expressed mostly in the midpiece of sperm (Figure 1(c)). These results suggested that the expression of DJ-1 and NDUFS3 correlated positively with low motility in AS sperm.

3.3. MMP Analysis in the Sperm of Participants. In human sperm, the mitochondrial sheath is organized in a helix of approximately 13 gyres surrounding the axoneme in the midpiece. Mitochondria in sperm were stained by MT-DR FM (Figure 2(a)). The percentage of MT-DR FM-positive sperm was measured in each of the two groups ($72.13 \pm 5.05\%$ for normal samples, $42.90 \pm 7.08\%$ for AS samples, Figure 2(b)). The AS group contained a lower percentage of MT-DR FM-positive sperm than that in the normal group ($P < 0.001$), indicating that mitochondrial function was damaged in patients with AS.

3.4. Sperm Motility and Concentration in the AS Rat Model. Sperm motility and concentration were analyzed to determine whether the AS rat model was successfully established. A statistically significant decrease in the proportion of sperm with progressive motility was found in the ORN-treated rats ($19.8 \pm 3.3\%$) compared to that in the control rats ($47.7 \pm 3.1\%$, $P < 0.001$; Figure 3(a)). Moreover, we found that ORN had no influence on rat sperm concentrations (Figure 3(b)). These results showed that ORN-treated rats displayed characteristics of AS [27, 28].

3.5. Reduced Expression of DJ-1 and NDUFS3 in AS Rats. To investigate whether ORN-treated AS in rats was associated with DJ-1 and NDUFS3 expression during spermatogenesis, we examined DJ-1 and NDUFS3 expression levels in AS and control rats. In the Western blotting analysis, DJ-1 was identified as a single band at ~20 kDa (Figure 4(a)). Quantification of the DJ-1 bands revealed that the level of DJ-1 was reduced in the testes of AS rats (1.66 ± 0.07) compared to those in control rats (3.13 ± 0.18, $P < 0.01$; Figure 4(b)). Western blotting analysis detected DJ-1 as a single band at ~30 kDa in the rat testes (Figure 4(c)). The relative intensity of NDUFS3 protein was also deceased in the testes of ORN-

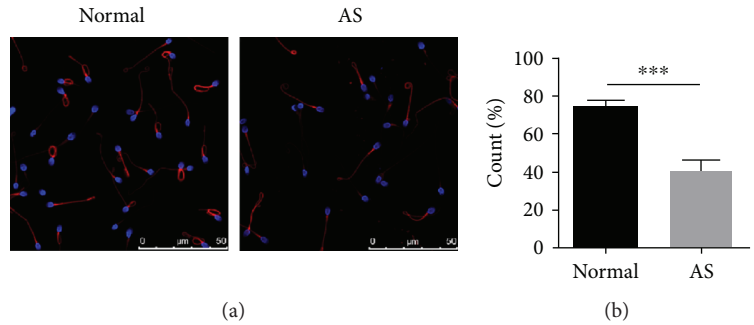


Figure 2: Decreased MMP in the sperm of patients with AS. (a) Immunofluorescence of mitochondria in human sperm, shown by MT-DR FM. Mitochondria in the midpiece of sperm were dyed red. Sperm nuclei were stained with Hoechst 33342 (blue). (b) Percentage MT-DR FM-positive sperm. Note that the sperm of patients with AS showed a significant reduction in MMP. $^{***}P < 0.001$, one-way ANOVA test, $n = 10$ in each group. Scale bar = 50 μm. AS: asthenozoospermia; MT-DR FM: MitoTracker Deep Red FM.

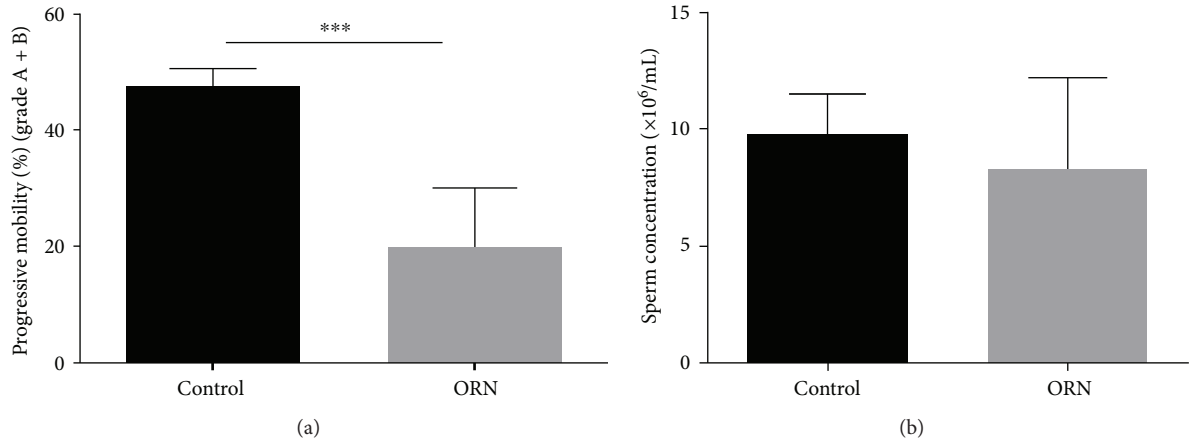


Figure 3: Analysis of sperm motility and concentration in control and ORN-treated rats. (a) Progressive motility (grade A + B) of sperm. The percentage of sperm with progressive motility was quantified and is showed in the histogram. Note that ORN induced a statistically significant reduction in the progressive motility of sperm. (b) Sperm concentration. There was no difference in the effect of ORN treatment on sperm concentration between the two groups. $^{***}P < 0.001$, one-way ANOVA test, $n = 10$ rats in each group.

treated rats (1.00 ± 0.11) compared to that in the control group (1.62 ± 0.20, $P < 0.05$; Figure 4(d)). DJ-1 was also identified as a single band at ~20 kDa in rat sperm (Figure 4(e)). The relative intensity of DJ-1 protein was decreased in the sperm of ORN-treated rats (0.50 ± 0.12) compared to that in the control group (1.00 ± 0.12, $P < 0.05$; Figure 4(f)). These results indicated that DJ-1 and NDUFS3 were affected by ORN treatment during spermatogenesis in rat sperm and testes.

3.6. Deceased MMP in the Sperm of AS Rats. Similar to the results of the MMP assay of human samples, the mitochondria in the midpiece of sperm showed different amounts of staining (Figure 5(a)). An analysis of the images showed that the percentage of MT-DR FM-positive sperm in ORN-treated rats (88.85 ± 4.81) was statistically significantly reduced compared to that in the control group (13.75 ± 6.49, $P < 0.001$; Figure 5(b)), suggesting mitochondrial dysfunction in the sperm of AS rats.

3.7. Depressed CI Activity in the Sperm and Testes of AS Rats. Mitochondrial CI is the first catalytic system in the respiratory chain. A specific reduction in mitochondrial CI activity inhibits sperm motility by regulating the NAD^+/NADH redox balance. Thus, we tested CI enzyme activity in sperm and testes of AS rats using an assay kit. The CI activity in the sperm of AS rats (37.93 ± 21.51) was significantly reduced compared to that in the control rats (100.0 ± 15.92, $P < 0.05$; Figure 6(a)). The CI activity in the testes of AS rats showed the same tendency toward decrease (9.91 ± 5.54), unlike that observed in normal rats (100.0 ± 16.97, $P < 0.05$; Figure 6(b)). The data showed reduced mitochondrial activity in the sperm and testes of AS rats.

3.8. Weakened Interaction between DJ-1 and NDUFS3 in Rat Testes. To assess the endogenous association of DJ-1 with the mitochondria complex, proteins from the rat testes were immunoprecipitated with anti-DJ-1 antibody, and the precipitates were analyzed by Western blotting with anti-NDUFS3 and anti-DJ-1 antibodies. Western blotting analysis identified DJ-1 as a single band at ~20 kDa and NDUFS3 as a single band at ~30 kDa (Figure 7(a)). The result confirmed NDUFS3 to be a DJ-1-binding protein in the rat testes. Moreover, the interaction between these proteins was weakened in the AS rat testes (0.37 ± 0.095) compared to that in normal rats (0.046 ± 0.022, $P < 0.05$; Figure 7(b)). Thus, DJ-1, an

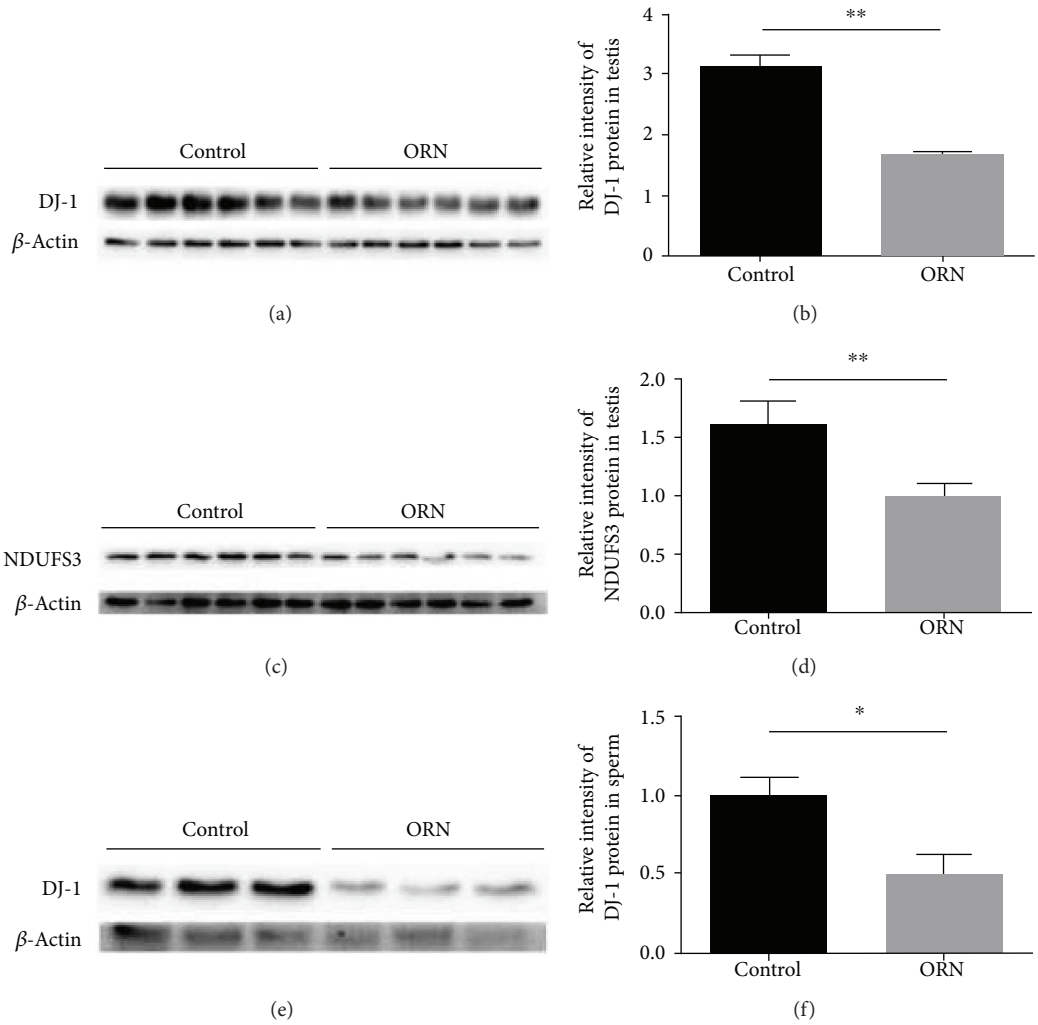


Figure 4: Decreased expression of DJ-1 and NDUFS3 in ORN-treated rat testes and sperm. (a) Western blotting detection of DJ-1 protein expression in rat testes. (b) Expression of DJ-1 was normalized against that of β-actin. Note that there was a reduction in DJ-1 in the testes from ORN-treated rats. (c) Western blotting detection of NDUFS3 protein expression in rat testes. (d) Expression of NDUFS3 was normalized against that of β-actin. These results showed that the expression of NDUFS3 decreased significantly in the testes of ORN-treated rats compared to that in control subjects. (e) Western blotting detection of DJ-1 protein expression in rat sperm. (f) Expression of DJ-1 was normalized against that of β-actin. These results showed that the expression of DJ-1 decreased significantly in the sperm of ORN-treated rats compared to that in control subjects. $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA test, $n = 6$ rats in each group.

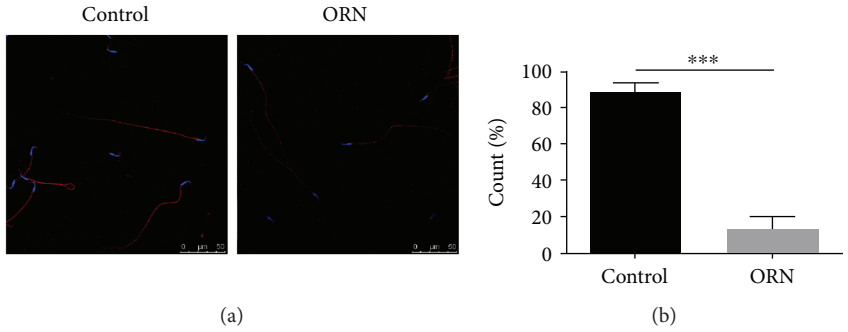


Figure 5: Decreased MMP in the sperm of ORN-treated rats. (a) Immunofluorescence of mitochondria in rat sperm, shown by MT-DR FM. Mitochondria in the midpiece of sperm were dyed red, and sperm nuclei were stained with Hoechst 33342 (blue). (b) Histogram showing the percentage of MT-DR FM-positive sperm. Note that the sperm in ORN-treated rats showed a significant reduction in MMP. $^{***}P < 0.001$, one-way ANOVA test, $n = 3$ rats in each group. Scale bar = 50 μm. MT-DR FM: MitoTracker Deep Red FM.

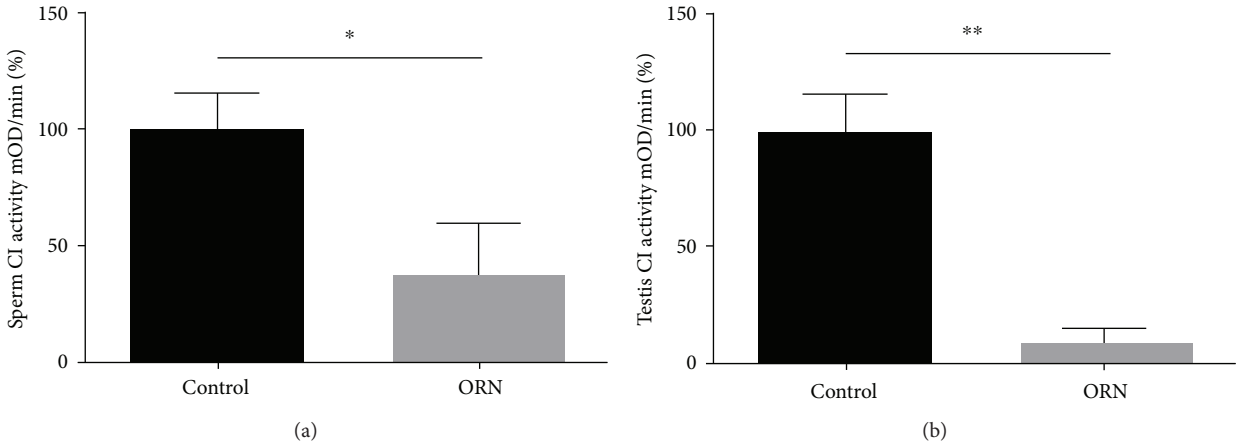


Figure 6: Decreased CI enzyme activity in the testes and sperm of ORN-treated rats. (a) Analysis of the CI activity of sperm in control and ORN-treated rats determined by CI enzyme activity microplate assay kit. CI enzyme activity in sperm was expressed in mOD/min. The histogram shows the ratio of the normalized mOD/min of rats in the ORN group compared to that in the control group. Note that CI activity in AS rat sperm was reduced relative to that in the control subjects. (b) Analysis of CI activity in testes of rats in the control and AS groups. The data indicate that CI activity in the testes of ORN-treated rats decreased significantly compared to that in the control group. $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA test, $n = 6$ rats in each group. CI: complex I.

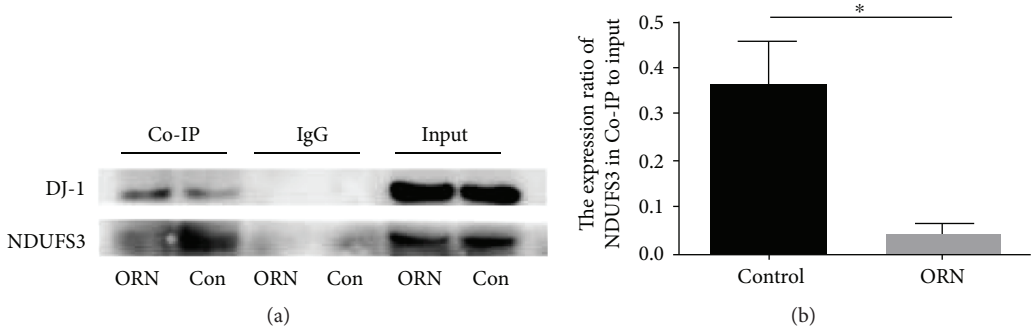


Figure 7: Direct interactions between DJ-1 and NDUFS3 demonstrated by coimmunoprecipitation. (a) The protein extracts were immunoprecipitated with an anti-DJ-1 antibody or IgG (negative control), and the precipitates were analyzed by Western blotting with anti-DJ-1 and anti-NDUFS3 antibodies. (b) The final coimmunoprecipitation result was obtained by subtracting the amount obtained from that of the negative control. The histogram shows the amount of DJ-1-NDUFS3 complex (presented as the ratio of the amount of normalized coimmunoprecipitated NDUFS3 versus that in the input) in rat testes. Note that DJ-1 and NDUFS3 were shown to interact in rat testes, and the ability of these two proteins to bind was significantly decreased in the testes of ORN-treated rats. $^{*}P < 0.05$, one-way ANOVA test, $n = 3$ rats in each group.

integral mitochondrial protein, appears to play a role in maintaining CI activity, in conjunction with NDUFS3, in the rat testes.

4. Discussion

In the present study, we found that the expression of NDUFS3 and DJ-1 was both decreased in the sperm of patients with AS. We established an AS rat model to investigate this association further. CI activity was decreased in the testes and sperm of AS rats. DJ-1 and NDUFS3 expression levels were similarly reduced in the AS rat testes. The expression of DJ-1 was decreased in AS rat sperm. Moreover, a protein interaction between DJ-1 and NDUFS3 was demonstrated in the rat testes for the first time, and this interaction was weakened in AS.

Although the exact etiology of diminished sperm motility is still generally unexplained, ultrastructural defects of the sperm flagellum due to congenital defects and sperm degeneration caused by genital infections, oxidative stress, antisperm antibodies, cryopreservation, or metabolic disorders have been implicated [29]. Among the possible causes of AS, mitochondrial dysfunction in sperm is one of the most relevant causes, because sperm need large amounts of energy for mobility of their flagella and activity during fertilization. The generation of cellular energy for sperm motility can be accomplished through both oxidative phosphorylation and glycolysis in various regions of the sperm flagellum [30]. MMP is a key parameter that represents mitochondrial function. Its decrease can imply the disruption of the mitochondrial electron transport chain, which results in cellular dysfunction and even death [31, 32]. Our results showed

mitochondrial dysfunction in the sperm of patients with AS. Several other researchers have found defects in mitochondrial respiratory activity in idiopathic as well as varicocele-related cases of AS [33, 34]. Very recently, less motile sperm from infertile patients were shown to exhibit lower MMP. With an increase in sperm MMP, sperm motility and fertility potential also increase [35]. In our study, ORN-treated rat sperm as well as testes showed decreased mitochondrial activity compared to that in the control group, suggesting that mitochondrial dysfunction in AS sperm might occur in the testes during spermatogenesis.

DJ-1, a protein related to male reproduction and infertility, has pleiotropic functions, ranging from a role as a chaperone with protease activity to that of a transcriptional regulator, redox sensor, and antioxidant scavenger [36]. In 1997, DJ-1 was first identified as a putative oncogene product that transformed mouse NIH3T3 cells in cooperation with activated ras [37]. Studies conducted in rats and mice have shown that DJ-1 expression is highly correlated with male infertility. When exposed to sperm toxicants such as ORN or epichlorohydrin, male rats and mouse showed reversible infertility, with reduced sperm motility and decreased expression of DJ-1 in sperm and epididymides [17, 38]. The fact that DJ-1 can serve as a biomarker for male fertility in both of these animals and in humans has been noted previously [39]. In 2011, downregulation of DJ-1 in sperm ejaculated from patients with AS was identified in a proteomic study comparing normal motile human sperm and that in idiopathic AS [19, 40]. Another study suggested that oxidation modification of DJ-1 was intensified in sperm from patients with AS compared to that in subjects without AS [20]. The distribution of DJ-1 in ejaculated sperm is in the surface of the posterior part of the head, the anterior part of the midpiece, and spermatozoa flagella. Our study showed that downregulation of DJ-1 may occur during spermatogenesis because decreased levels of DJ-1 were observed in the testes of AS rats by Western blotting. And the localization of DJ-1 in the midpiece of sperm indicated its function in mitochondria of sperm. These findings suggest that DJ-1 plays an essential role in sperm motility and AS.

Mitochondria generate adenosine triphosphate by oxidative phosphorylation (OXPHOS) via the mitochondrial respiratory chain, which consists of five multisubunit complexes (CI–V) composed of at least 75 nuclear DNA-encoded and 13 mitochondrial DNA- (mtDNA-) encoded proteins [41]. A high correlation between sperm motility and the activity of some mitochondrial enzymes (citrate synthase and respiratory CI, II, I + III, II + III, and IV) has been found [42]. NDUFS3 is a poorly characterized component of CI in the mitochondrial respiratory chain that is localized in the matrix portion of this multisubunit complex. Cleavage of NDUFS3, caused by lymphocyte protease granzyme A (GzmA), can lead to the production of ROS and ultimately cell death [43]. This study is the first to show the involvement of NDUFS3 in AS. We discovered reduced NDUFS3 expression in the sperm of patients with AS and in the testes of AS rats for the first time. This phenomenon suggests that a reduction in NDUFS3 in the testes and sperm may be to blame for low sperm motility. The reduced expression of NDUFS3 may inhibit the normal function of mitochondrial CI in spermatogenic cells of the testes, leading to decreased CI activity and sperm motility in AS.

The similar trends in the expression of NDUFS3 and DJ-1 in the sperm of patients with AS and the testes of AS rats indicated a correlation between these proteins in germ cells. In fact, colocalization of DJ-1 and NDUFS3 has been shown in NIH3T3 and HEK293 cells [11]. However, mitochondria in sperm are significantly different from those in somatic cells. The difference between mitochondria in sperm and somatic cells is reflected in both their morphology and biochemistry [44]. In our study, the protein interaction between DJ-1 and NDUFS3 was first confirmed in rat testes by coimmunoprecipitation. Moreover, this interaction was reduced in AS. Decreased expression of DJ-1 after knockdown of *PARK7* results in reduced CI activity in cells [45], and numerous studies have supported the role of DJ-1 in protecting cells against oxidative stress and maintaining mitochondrial structure. Recently, DJ-1 was found to translocate into sperm mitochondria in conditions of oxidative stress in Chinese patients with AS [20]. Thus, mitochondrial dysfunction caused by decreased levels of DJ-1 in sperm is a possible etiology of AS. DJ-1 can maintain mitochondrial function during oxidative stress by working with Pink1/Parkin pathway. An increase in mitochondrial DJ-1, regulated by Pink1/Parkin, can reduce ROS-induced damage in mitochondria [46]. Protection against mitochondrial damage of DJ-1 protein is probably promoted by the oxidation of C106, a cysteine residue in DJ-1 [47]. The correlation of DJ-1 and NDUFS3 suggests that NDUFS3 is a target of CI that assists DJ-1 in protecting mitochondria function. Decreased expression of DJ-1 as well as reduced DJ-1 and NDUFS3 binding in AS may lead to the loss of DJ-1 protection of CI, ultimately reducing sperm motility.

Animal models, historically, have played a critical role in the exploration and characterization of disease pathophysiology, identification of drug targets, and evaluation of novel therapeutic agents and treatments in vivo [48]. In this study, we established a classical ORN-treated rat model to investigate the function of DJ-1 and NDUFS3 in AS. The significantly reduced sperm motility and unchanged sperm concentration exhibited in this rat model conformed to observations in typical AS [22]. Although the AS rat model has been used in other studies, the results obtained here should be verified in the testes of humans. In addition, the sample size and types of human sperm samples used in this study were limited, and this study should be expanded to different types of AS pathogenesis.

5. Conclusions

In summary, we have shown a statistically significant reduction in DJ-1 and NDUFS3 expression in patients with AS and in a rat model. Interactions between DJ-1 and NDUFS3 in the testes were demonstrated and suggest that DJ-1 may play a role in maintaining mitochondrial function by means of the association with NDUFS3 during spermatogenesis in the

testes. This protective function may be weakened in AS because of a reduction in binding ability as well as a decrease in the amount of DJ-1. This study suggests that downregulation of DJ-1 and NDUFS3 expression likely contributes to mitochondrial dysfunction, which may underlie AS pathogenesis, since current treatments for AS involve in vitro fertilization techniques rather than treatment of male infertility. These findings contribute to a deeper understanding of mitochondrial function in spermatogenesis in AS and may lead to the identification of a new therapeutic target for drug discovery.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Yupeng Wang and Yi Sun contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 81401259).

References

[1] S. M. Curi, J. I. Ariagno, P. H. Chenlo et al., "Asthenozoospermia: analysis of a large population," *Archives of Andrology*, vol. 49, no. 5, pp. 343–349, 2003.

[2] A. Jungwirth, A. Giwercman, H. Tournaye et al., "European Association of Urology guidelines on male infertility: the 2012 update," *European Urology*, vol. 62, no. 2, pp. 324–332, 2012.

[3] A. Fathy, S. J. Chen, N. Novak, H. C. Schuppe, G. Haidl, and J. P. Allam, "Differential leucocyte detection by flow cytometry improves the diagnosis of genital tract inflammation and identifies macrophages as proinflammatory cytokine-producing cells in human semen," *Andrologia*, vol. 46, no. 9, pp. 1004–1012, 2014.

[4] J. S. Armstrong, M. Rajasekaran, W. Chamulitrat, P. Gatti, W. J. Hellstrom, and S. C. Sikka, "Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism," *Free Radical Biology & Medicine*, vol. 26, no. 7-8, pp. 869–880, 1999.

[5] A. J. Koppers, G. N. De Iuliis, J. M. Finnie, E. A. McLaughlin, and R. J. Aitken, "Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa," *The Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 8, pp. 3199–3207, 2008.

[6] A. J. Mundy, T. A. Ryder, and D. K. Edmonds, "Asthenozoospermia and the human sperm mid-piece," *Human Reproduction*, vol. 10, no. 1, pp. 116–119, 1995.

[7] E. Ruiz-Pesini, C. Diez, A. C. Lapena et al., "Correlation of sperm motility with mitochondrial enzymatic activities," *Clinical Chemistry*, vol. 44, no. 8, Part 1, pp. 1616–1620, 1998.

[8] E. Ruiz-Pesini, A. C. Lapena, C. Diez, E. Alvarez, J. A. Enriquez, and M. J. Lopez-Perez, "Seminal quality correlates with mitochondrial functionality," *Clinica Chimica Acta*, vol. 300, no. 1-2, pp. 97–105, 2000.

[9] A. Amaral, J. Ramalho-Santos, and J. C. St John, "The expression of polymerase gamma and mitochondrial transcription factor A and the regulation of mitochondrial DNA content in mature human sperm," *Human Reproduction*, vol. 22, no. 6, pp. 1585–1596, 2007.

[10] D. Martinvalet, D. M. Dykxhoorn, R. Ferrini, and J. Lieberman, "Granzyme A cleaves a mitochondrial complex I protein to initiate caspase-independent cell death," *Cell*, vol. 133, no. 4, pp. 681–692, 2008.

[11] T. Hayashi, C. Ishimori, K. Takahashi-Niki et al., "DJ-1 binds to mitochondrial complex I and maintains its activity," *Biochemical and Biophysical Research Communications*, vol. 390, no. 3, pp. 667–672, 2009.

[12] M. Inden, T. Taira, Y. Kitamura et al., "PARK7 DJ-1 protects against degeneration of nigral dopaminergic neurons in Parkinson's disease rat model," *Neurobiology of Disease*, vol. 24, no. 1, pp. 144–158, 2006.

[13] E. Andres-Mateos, C. Perier, L. Zhang et al., "DJ-1 gene deletion reveals that DJ-1 is an atypical peroxiredoxin-like peroxidase," *Proceedings of the National Academy of Sciences*, vol. 104, no. 37, pp. 14807–14812, 2007.

[14] J. M. Kim, H. J. Jang, S. Y. Choi et al., "DJ-1 contributes to adipogenesis and obesity-induced inflammation," *Scientific Reports*, vol. 4, no. 1, 2014.

[15] L. Chen, M. Luo, X. Sun et al., "DJ-1 deficiency attenuates expansion of liver progenitor cells through modulating the inflammatory and fibrogenic niches," *Cell Death & Disease*, vol. 7, no. 6, article e2257, 2016.

[16] G. R. Klinefelter, J. W. Laskey, J. Ferrell, J. D. Suarez, and N. L. Roberts, "Discriminant analysis indicates a single sperm protein (SP22) is predictive of fertility following exposure to epididymal toxicants," *Journal of Andrology*, vol. 18, no. 2, pp. 139–150, 1997.

[17] A. Wagenfeld, C. H. Yeung, K. Strupat, and T. G. Cooper, "Shedding of a rat epididymal sperm protein associated with infertility induced by ornidazole and alpha-chlorohydrin," *Biology of Reproduction*, vol. 58, no. 5, pp. 1257–1265, 1998.

[18] J. E. Welch, R. R. Barbee, N. L. Roberts, J. D. Suarez, and G. R. Klinefelter, "SP22: a novel fertility protein from a highly conserved gene family," *Journal of Andrology*, vol. 19, no. 4, pp. 385–393, 1998.

[19] C. N. An, H. Jiang, Q. Wang et al., "Down-regulation of DJ-1 protein in the ejaculated spermatozoa from Chinese asthenozoospermia patients," *Fertility and Sterility*, vol. 96, no. 1, pp. 19–23.e2, 2011.

[20] Y. Sun, W. J. Zhang, X. Zhao, R. P. Yuan, H. Jiang, and X. P. Pu, "PARK7 protein translocating into spermatozoa mitochondria in Chinese asthenozoospermia," *Reproduction*, vol. 148, no. 3, pp. 249–257, 2014.

[21] R. M. McClain and J. C. Downing, "Reproduction studies in rats treated with ornidazole," *Toxicology and Applied Pharmacology*, vol. 92, no. 3, pp. 480–487, 1988.

[22] W. Bone, N. G. Jones, G. Kamp, C. H. Yeung, and T. G. Cooper, "Effect of ornidazole on fertility of male rats: inhibition of a glycolysis-related motility pattern and zona binding required for fertilization in vitro," *Journal of Reproduction and Fertility*, vol. 118, no. 1, pp. 127–135, 2000.

[23] World Health Organization, *WHO laboratory manual for the examination and processing of human semen*, World Health Organization, Geneva, Switzerland, 2010.

[24] G. Oberlander, C. H. Yeung, and T. G. Cooper, "Induction of reversible infertility in male rats by oral ornidazole and its effects on sperm motility and epididymal secretions," *Journal of Reproduction and Fertility*, vol. 100, no. 2, pp. 551–559, 1994.

[25] D. A. Rizzetti, C. S. Martinez, A. G. Escobar et al., "Egg white-derived peptides prevent male reproductive dysfunction induced by mercury in rats," *Food and Chemical Toxicology*, vol. 100, pp. 253–264, 2017.

[26] K. Chenniappan and K. Murugan, "Therapeutic and fertility restoration effects of ionidium suffruticosumon sub-fertile male albino Wistar rats: effects on testis and caudal spermatozoa," *Pharmaceutical Biology*, vol. 55, no. 1, pp. 946–957, 2017.

[27] Y.-N. Wang, B. Wang, M. Liang et al., "Down-regulation of CatSper1 channel in epididymal spermatozoa contributes to the pathogenesis of asthenozoospermia, whereas up-regulation of the channel by Sheng-Jing-San treatment improves the sperm motility of asthenozoospermia in rats," *Fertility and Sterility*, vol. 99, no. 2, pp. 579–587, 2013.

[28] S. Benoff, K. Auborn, J. L. Marmar, and I. R. Hurley, "Link between low-dose environmentally relevant cadmium exposures and asthenozoospermia in a rat model," *Fertility and Sterility*, vol. 89, no. 2, pp. e73–e79, 2008.

[29] C. Ortega, G. Verheyen, D. Raick, M. Camus, P. Devroey, and H. Tournaye, "Absolute asthenozoospermia and ICSI: what are the options?," *Human Reproduction Update*, vol. 17, no. 5, pp. 684–692, 2011.

[30] P. Piomboni, R. Focarelli, A. Stendardi, A. Ferramosca, and V. Zara, "The role of mitochondria in energy production for human sperm motility," *International Journal of Andrology*, vol. 35, no. 2, pp. 109–124, 2012.

[31] X. Li, M. Tian, G. Zhang et al., "Spatially dependent fluorescent probe for detecting different situations of mitochondrial membrane potential conveniently and efficiently," *Analytical Chemistry*, vol. 89, no. 6, pp. 3335–3344, 2017.

[32] D. P. Evenson, Z. Darzynkiewicz, and M. R. Melamed, "Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility," *Journal of Histochemistry & Cytochemistry*, vol. 30, no. 3, pp. 279-280, 1982.

[33] A. Ferramosca, R. Focarelli, P. Piomboni, L. Coppola, and V. Zara, "Oxygen uptake by mitochondria in demembranated human spermatozoa: a reliable tool for the evaluation of sperm respiratory efficiency," *International Journal of Andrology*, vol. 31, no. 3, pp. 337–345, 2008.

[34] F. Pelliccione, A. Micillo, G. Cordeschi et al., "Altered ultrastructure of mitochondrial membranes is strongly associated with unexplained asthenozoospermia," *Fertility and Sterility*, vol. 95, no. 2, pp. 641–646, 2011.

[35] Z. Li, Y. Zhou, R. Liu et al., "Effects of semen processing on the generation of reactive oxygen species and mitochondrial membrane potential of human spermatozoa," *Andrologia*, vol. 44, no. 3, pp. 157–163, 2012.

[36] J. Y. Chan and S. H. Chan, "Activation of endogenous antioxidants as a common therapeutic strategy against cancer, neurodegeneration and cardiovascular diseases: a lesson learnt from DJ-1," *Pharmacology & Therapeutics*, vol. 156, pp. 69–74, 2015.

[37] D. Nagakubo, T. Taira, H. Kitaura et al., "DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras," *Biochemical and Biophysical Research Communications*, vol. 231, no. 2, pp. 509–513, 1997.

[38] A. Wagenfeld, J. Gromoll, and T. G. Cooper, "Molecular cloning and expression of rat contraception associated protein 1 (CAP1), a protein putatively involved in fertilization," *Biochemical and Biophysical Research Communications*, vol. 251, no. 2, pp. 545–549, 1998.

[39] G. R. Klinefelter, "Saga of a sperm fertility biomarker," *Animal Reproduction Science*, vol. 105, no. 1-2, pp. 90–103, 2008.

[40] S. Shen, J. Wang, J. Liang, and D. He, "Comparative proteomic study between human normal motility sperm and idiopathic asthenozoospermia," *World Journal of Urology*, vol. 31, no. 6, pp. 1395–1401, 2013.

[41] R. O. Vogel, C. E. Dieteren, L. P. van den Heuvel et al., "Identification of mitochondrial complex I assembly intermediates by tracing tagged NDUFS3 demonstrates the entry point of mitochondrial subunits," *The Journal of Biological Chemistry*, vol. 282, no. 10, pp. 7582–7590, 2007.

[42] E. Ruizpesini, A. C. Lapeña, C. Díez, E. Alvarez, J. A. Enríquez, and M. J. Lópezpérez, "Seminal quality correlates with mitochondrial functionality," *Clinica Chimica Acta*, vol. 300, no. 1-2, pp. 97–105, 2000.

[43] S. Lucken-Ardjomande and J. C. Martinou, "Granzyme A, a stealth killer in the mitochondrion," *Cell*, vol. 133, no. 4, pp. 568–570, 2008.

[44] S. Rajender, P. Rahul, and A. A. Mahdi, "Mitochondria, spermatogenesis and male infertility," *Mitochondrion*, vol. 10, no. 5, pp. 419–428, 2010.

[45] L. Zhang, M. Shimoji, B. Thomas et al., "Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis," *Human Molecular Genetics*, vol. 14, no. 14, pp. 2063–2073, 2005.

[46] A. P. Joselin, S. J. Hewitt, S. M. Callaghan et al., "ROS-dependent regulation of Parkin and DJ-1 localization during oxidative stress in neurons," *Human Molecular Genetics*, vol. 21, no. 22, pp. 4888–4903, 2012.

[47] R. M. Canet-Aviles, M. A. Wilson, D. W. Miller et al., "The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 24, pp. 9103–9108, 2004.

[48] P. McGonigle and B. Ruggeri, "Animal models of human disease: challenges in enabling translation," *Biochemical Pharmacology*, vol. 87, no. 1, pp. 162–171, 2014.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 7806912, 10 pages
https://doi.org/10.1155/2018/7806912

Review Article

Regulatory T Lymphocytes in Periodontitis: A Translational View

Carla Alvarez[1,2] **Carolina Rojas**[1] **Leticia Rojas**[1,3] **Emilio A. Cafferata**[1] **Gustavo Monasterio**[1] **and Rolando Vernal**[1,4]

[1]*Periodontal Biology Laboratory, Faculty of Dentistry, Universidad de Chile, Santiago, Chile*
[2]*Oral Pathology Laboratory, Faculty of Dentistry, Universidad Andrés Bello, Santiago, Chile*
[3]*Translational Allergy and Immunology Laboratory, Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile*
[4]*Dentistry Unit, Faculty of Health Sciences, Universidad Autónoma de Chile, Santiago, Chile*

Correspondence should be addressed to Rolando Vernal; rvernal@uchile.cl

Received 27 December 2017; Accepted 20 February 2018; Published 2 April 2018

Academic Editor: Cheng Xiao

Copyright © 2018 Carla Alvarez et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Periodontitis is a chronic immuno-inflammatory disease in which the disruption of the balance between host and microbiota interactions is key to the onset and progression of the disease. The immune homeostasis associated with periodontal health requires a regulated immuno-inflammatory response, during which the presence of regulatory T cells (Tregs) is essential to ensure a controlled response that minimizes collateral tissue damage. Since Tregs modulate both innate and adaptive immunity, pathological conditions that may resolve by the acquisition of immuno-tolerance, such as periodontitis, may benefit by the use of Treg immunotherapy. In recent years, many strategies have been proposed to take advantage of the immuno-suppressive capabilities of Tregs as immunotherapy, including the *ex vivo* and *in vivo* manipulation of the Treg compartment. Ongoing research in both basic and translational studies let us gain a better understanding of the diversity of Treg subsets, their phenotypic plasticity, and suppressive functions, which can be used as a substrate for new immunotherapies. Certainly, as our knowledge of Treg biology increases, we will be capable to develop new therapies designed to enhance the stability and function of Tregs during periodontitis.

1. Introduction

Periodontitis is a chronic immuno-inflammatory disease in which the disruption of the balance between host and microbiota interactions plays a pivotal role in the onset and progression of the disease. The host immune response is disturbed by key pathogens, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, and further sustained by pathogenic microorganisms [1]. During periodontitis, the immuno-inflammatory infiltrate invades deep compartments of the periodontium, causing the destruction of the tooth supporting tissues: radicular cement, periodontal ligament, and alveolar bone. In severe cases of the disease, the tissue destruction leads to tooth mobility and ultimately tooth loss [2]. The immune homeostasis associated with periodontal health requires a regulated immuno-inflammatory response, during which the presence of immune regulatory cells is essential to ensure a response that minimizes collateral tissue damage [3]. Tregs are a subset of $CD4^+$ T lymphocytes that play an essential role in the maintenance of self-tolerance and immune homeostasis [4]. Although different T cell types with regulatory functions have been identified, the most physiologically relevant Treg population is characterized as $CD4^+$ T lymphocytes that constitutively express the IL-2 receptor α-chain, CD25, and the transcription factor Foxp3, comprising approximately 10% of the $CD4^+$ T cell compartment [5]. Since Tregs modulate both innate and adaptive immunity, pathological conditions that may resolve by the acquisition of immuno-tolerance, such as autoimmune diseases, organ transplantation, and overall inflammatory diseases—including periodontitis—may benefit by the use of Treg immunotherapy [6]. In this context, increasing evidence about *ex vivo* strategies to isolate, preserve, expand, and transfer Tregs, and new protocols to manipulate the

Treg pool *in vivo*, have led the way to new promising therapeutic approaches that may be eventually suitable for clinical use.

2. Treg Biology

2.1. Development. The two main subsets of Tregs are classified accordingly to their site of development. Firstly, natural or thymic Tregs (nTregs or tTregs) develop in the thymus through intermediate strength interactions between a self-reacting T cell receptor (TCR) and their cognate antigens, presented by medullary thymic epithelial cells and hematopoietic antigen-presenting cells, leading to upregulation of CD25 [7, 8]. In addition, costimulatory molecules such as CD28, GITR, OX40, and TNFR2 contribute to Treg development [9]. Most intercellular signals converge to the NF-κB pathway, which appears to be the main transcription factor involved in thymic generation of Tregs [10]. At the terminal stage of differentiation, the transcription factor Foxp3 is upregulated by the action of IL-2 through CD25, whose signalization induces further CD25 production and high expression of suppressor genes, rendering regulatory functions [7]. Compared with induced Tregs, nTregs exhibit a higher expression of PD-1 (programmed cell death-1), neuropilin 1 (Nrp1), Helios, and CD73 [11].

The second route for Treg generation is the differentiation from naïve $CD4^+$ T lymphocytes at the periphery, named induced or peripheral Tregs (iTregs or pTregs). iTregs are mostly present in the mucosal interface, by the action of tolerogenic antigen-presenting cells [12]. TGF-β is a major inductor of Foxp3 expression through phosphorylation and activation of the transcription factors Smad2 and Smad3, which bind the intronic enhancer CNS1 in the *foxp3* gene locus. In the gut, TGF-β is produced by $CD103^+$ mucosal dendritic cells (DCs) that also produce retinoic acid (RA), which in turn induces the binding of RA receptor (RAR) and retinoic X receptor (RXR) to CNS1, leading to increased binding of Smad3 [12, 13]. Moreover, gut commensal bacteria also promote the iTreg generation by metabolites, such as short-chain fatty acid (SCFA), secreted from bacterial fermentation of dietary fibers [13–16].

2.2. Phenotypic Characterization. The transcription factor Foxp3 is considered the main Treg phenotype marker. Foxp3 stabilized the Treg canonical genetic profile, controlling its differentiation, maintenance, and suppressive functions [17, 18]. However, Foxp3 expression is not exclusive of Tregs, especially in humans, since activated conventional T cells may transiently upregulate Foxp3 without the acquisition of suppressive functions [19]. Besides, Foxp3 is a nuclear protein that cannot be used as a marker to purify viable Tregs; thus, a number of surface phenotypic markers have been characterized to define Tregs and its subsets [20]. In humans, the markers $CD25^{high}$ and $CD127^{low/-}$ are frequently used for Treg sorting from peripheral blood and tissues [21]. Postsorting analysis of this population shows a Foxp3 expression above 87%, indicating a reliable strategy to purify viable Tregs [22]. In addition, several studies have identified phenotypic markers within the $CD25^{high}$ $CD127^{low}$ $Foxp3^+$ population that are differentially expressed by discrete Treg subsets, according to activation and memory status (CD45RA naïve and CD45RO memory), chemotactic profile (chemokine receptors like CCR4 and CCR9), suppressive functions (CTLA-4, CD39, and CD73), and more [20]. Definitely, human Tregs are phenotypically complex, and, as technology advances, even more new Treg subtypes have been identified, reaching up to 22 distinct subpopulations [22].

2.3. Phenotypic Plasticity. Recent studies suggest that Tregs retain lineage plasticity, the ability to switch their cell fate to other effector T cell subset under particular environmental conditions, such as sustained inflammation or lymphopenia [23]. iTregs have been shown to be more unstable than nTregs. Epigenetic changes in the CNS2 region of the *foxp3* locus explain at least in part this difference. In nTregs, CpG islands of the Treg-specific demethylated region (TSDR) from the CNS2 region are hypo-methylated, but in freshly generated iTregs, this region is heavily methylated; thus, important transcription factors cannot be recruited to the site, and Foxp3 expression becomes unstable [13]. Also, homeostatic proliferation in the periphery depends on cytokines, particularly IL-2 [7]. Pathogenic conversion of $Foxp3^+$ T cell into Th17 cells has been demonstrated under inflammatory conditions enriched in IL-6 *in vivo*, where $CD25^{low}$ $Foxp3^+$ $CD4^+$ T cells lose Foxp3 expression and transdifferentiate into Th17 lymphocytes [24]. Moreover, under certain circumstances, $Foxp3^+$ T cells may acquire effector T cell-like features without losing Foxp3 expression, with "hybrid" phenotypes. For example, $Foxp3^+$ $RORC2^+$ $IL\text{-}17^+$ cells have been identified in human intestine and $Foxp3^+$ $T\text{-}bet^+$ $IFN\text{-}\gamma^+$ cells in patients with chronic inflammatory diseases, although in most cases those $Foxp3^+$ T cells retain suppressive functions [23].

3. Treg Suppressive Mechanisms

The main function of Tregs is the suppression of naïve T cell activation and expansion; however, they can also inhibit activated effector T cells, memory $CD4^+$ T cells, $CD8^+$ T cells, NKs, NKTs, APCs, and B cells [7].

Tregs present a battery of suppressive mechanisms that may proceed by four distinctive ways: (1) modulation of antigen-presenting cell (APC) maturation or function, (2) suppression by killing targeted cells, (3) suppression by metabolic disruption, and (4) suppression by inhibitory cytokines [25]. An example of APC inhibition is the Treg expression of CTLA-4, an inhibitory receptor relative to the T cell costimulatory molecule CD28. While CD28 signaling promotes T cell activation, CTLA-4 suppresses the T cell response by interacting with costimulatory receptors CD80 and CD86, expressed at the APC surface. This contact leads to the downregulation and sequestration of both costimulatory molecules [26]. Additionally, CTLA-4 induces the expression of the enzyme indoleamine 2,3-dioxygenase (IDO) by DCs, which catalyzes degradation of the essential amino acid tryptophan to kynurenine, leading to effector T cell starvation [25]. Tregs may also kill their target cells

through cell contact-dependent cytolysis by granzymes A and B, in both perforin-dependent and perforin-independent manner, or induce apoptosis via the tumor necrosis factor-related apoptosis-inducing ligand-death receptor 5 (TRAIL-DR5) pathway, among other means [25]. Tregs mediate suppressive metabolic disruption of effector T cells by consumption of local IL-2, which limits T cell proliferation [27]. Another suppressive mechanism is the expression of surface ectoenzymes, CD39 and CD73, which catalyze extracellular ATP hydrolysis to ADP, AMP, and adenosine. Adenosine signals may inhibit APCs as well as activated T lymphocytes by elevation of intracellular cAMP [7, 28]. A contact-independent suppressive mechanism is the production of inhibitory cytokines, such as IL-10, IL-35, and TGF-β, which interact with their specific receptors in a wide range of cell phenotypes [7].

Another immuno-suppressive function of Tregs is the inhibition of osteoclast differentiation and their bone-resorptive activity [29]. *In vitro* studies, with human or murine Tregs, have shown that these cells can inhibit the differentiation of monocytes/macrophages to osteoclasts by the secretion of TGF-β, IL-4, and IL-10, and by the interaction of CTLA-4 with CD80/86 receptors present in osteoclasts and their precursors [30, 31]. In an *in vivo* study of osteoporosis, it was reported that the adoptive transfer of murine Tregs to $Rag1^{-/-}$ mice, deficient of T cells, increases the total bone mass associated with the decrease in the number of osteoclasts [32]. In addition, in patients with rheumatoid arthritis, nTregs secrete low levels of regulatory cytokines and have defects in the expression of CTLA-4, which is associated with increased bone destruction [29]. Therefore, Treg may present important suppressive functions during inflammation-mediated bone destruction, as well as in bone homeostasis.

Besides the immuno-suppression activity, it has been postulated that Tregs may have the capacity to directly exert tissue-repairing functions by promoting the wound healing processes at multiple tissue sites [33]. Tregs, exposed to inflammatory conditions during skin injury, express the epidermal growth factor receptor (EGFR), which plays a major role in skin wound healing by stimulating epidermal and dermal regeneration. Specific ablation of Tregs early after the skin injury resulted in delayed wound reepithelialization and closure, increased granulation tissue, and bigger overlying eschar [34]. Also, in a murine model of infectious lung injury, Tregs produce amphiregulin, an EGFR ligand, in response to the inflammatory cytokines IL-18 and IL-33. This Treg effect is independent of TCR signaling and dispensable for their suppressive functions, indicating a distinct tissue-protective function that is evoked in response to specific cues, different from those that induce suppressive functions [35] (Figure 1).

4. Tregs in Periodontitis

Although the microorganisms that comprise the pathogenic subgingival biofilm are the primary etiological agents of periodontitis, the determinant of the disease progression and clinical outcome is the host's immune response, which includes the formation of the periodontal inflammatory infiltrate and the activation of osteoclasts [36]. During periodontitis, the immune response has to be controlled to effectively avoid the pathogenic microorganism dissemination and, at the same time, prevent collateral tissue damage. Therefore, Tregs preferentially accumulate at infected tissues, limiting the immune responses and promoting pathogen survival [37]. Different studies have described the enrichment of Tregs within the infected periodontal tissues. For instance, there is a higher frequency of $CD4^+$ $CD25^+$ CTLA-4^+ Tregs in periodontitis biopsies than in gingivitis [36]. These cells exhibited phenotypic characteristics of Tregs, confirmed by the expression of CTLA-4, GITR, CD103, CD45RO, and Foxp3 [37]. Moreover, the migration of $CD4^+$ $CD25^+$ T cells to periodontitis that affected gingival tissues seemed to be dependent on CCL17 and CCL22 expression by the local inflammatory infiltrate, which recruits Treg expressing CCR4 or CCR8 [37, 38]. Despite the increase in the number of Tregs during periodontitis, it is possible that a fraction of these cells loses their suppressive functions due to the inflammatory periodontal environment enriched in IL-6 [39]. For instance, in active periodontal lesions, compared with inactive lesions, Foxp3, T-bet, RANKL, IL-17, IL-1β, and IFN-γ mRNAs were significantly overexpressed [40]. However, TGF-β1 and IL-10 mRNA expression was increased within inactive periodontal lesions compared to active ones [40]. $CD25^+$ $Foxp3^+$ Tregs are strikingly diminished in bone resorption lesions from periodontitis compared to healthy gingival tissues. Also, in periodontal tissue homogenates, the correlation between RANKL and IL-10 protein concentrations is negative, whereas the correlation between RANKL and the pro-inflammatory cytokine IL-1β is positive [41]. Furthermore, a population of $Foxp3^+$ $IL\text{-}17^+$ cells has been identified in periodontal lesions of patients with periodontitis, indicating the possible conversion of Tregs to Th17 lymphocytes [42]. However, until now, it has not been confirmed whether the periodontal inflammatory environment modifies to some degree the phenotypic or functional stability of infiltrating Tregs.

Different studies using animal models of periodontitis have ratified the importance of Treg suppressive functions during the late stages of the disease. For instance, the inhibition of Tregs with anti-GITR in an *A. actinomycetemcomitans*-induced model of periodontitis showed increased alveolar bone loss associated with the reduction of IL-10, CTLA-4, and TGF-β levels [43]. A similar effect was observed in an IDO knockout mouse model in conjunction with lipopolysaccharide- (LPS-) induced gingival inflammation [44]. In this study, the deficiency of IDO increased the number of $IL\text{-}17^+$ cells and apoptotic or necrotic gingival cells. Also, the number of Tregs was markedly reduced [44]. In a different murine study, Tregs seem to cooperate with Th2 cells, where the coexistence and expression of IL-4, Foxp3, and IL-10 correlate with attenuation of osteolysis. For instance, IL-4 induces CCL22 expression that modulates CCR4-dependent Treg migration. Specifically, experimental periodontitis in IL-4 knockout mice shows an almost total reduction of Tregs and CCL22 production/expression [38]. Therefore, Treg functionality is needed to sustain a controlled immune response that might avoid the disease progression or reactivation.

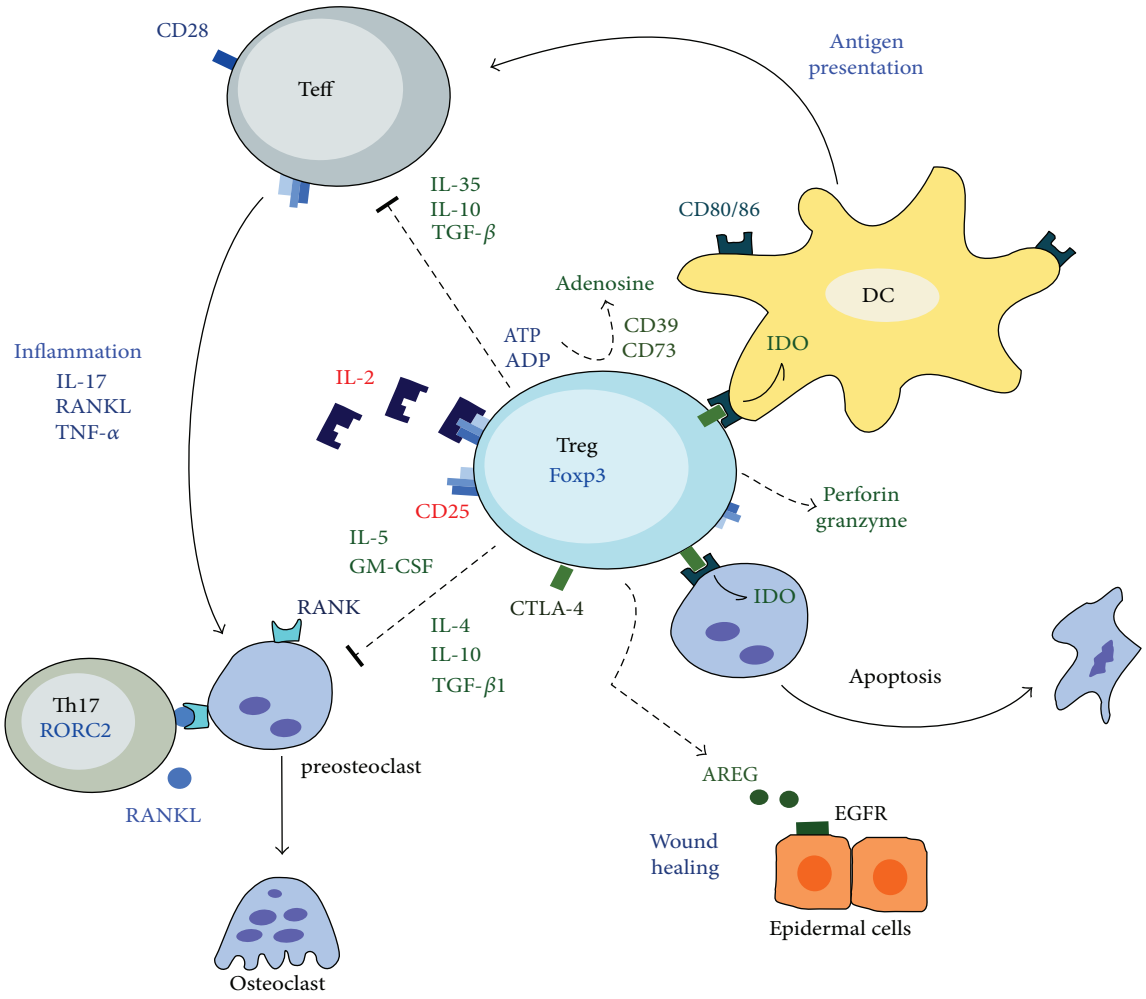


Figure 1: Treg suppressive functions. Tregs have several suppressive mechanisms that may inhibit different cell types. For instance, Tregs can directly inhibit antigen-presenting cells (APCs), such as dendritic cells (DCs), through its inhibitory receptor CTLA-4 that bounds to the costimulatory molecules CD80 and CD86, expressed on the surface of APCs. This interaction induces the production of indoleamine 2,3-dioxygenase (IDO), which in turn may provoke apoptosis of DCs and preosteoclasts. In addition, CTLA-4 competes with the CD28 receptor present on the surface of effector T cells (Teff) and inhibits costimulatory signals during antigenic presentation. Similarly, CTLA-4 directly suppresses osteoclast differentiation and activation, mechanisms potentiated by the secretion of inhibitory cytokines such as IL-4, IL-5, IL-10, TGF-β, and GM-CSF. Furthermore, Tregs suppress the pro-inflammatory functions of Teff, such as Th17 ($CD4^+$ $RORC2^+$) lymphocytes through various mechanisms such as the local consumption of IL-2; secretion of anti-inflammatory cytokines such as IL-10, IL-35, and TGF-β; inhibition of antigenic presentation; transformation of ATP and ADP to adenosine by surface ectoenzymes (CD39 and CD73), and the controlled release of perforin and granzyme. Finally, Tregs may promote tissue repair through the production of amphiregulin (AREG), ligand of the epidermal growth factor receptor (EGFR), expressed in epidermal cells and other resident cells.

5. Tregs as Therapeutic Tool

In recent years, many strategies have been proposed to take advantage of the immuno-suppressive capabilities of Tregs as immunotherapy [6]. Some of these approaches include the *ex vivo* manipulation of Tregs for adoptive-transfer purposes. In this scheme, Tregs are purified from the host's or a donor's peripheral or banked umbilical cord blood [45]. Subsequently, Tregs are expanded *in vitro* following particular protocols that may include the following: the cell expansion in presence of anti-CD3/CD28 microbeads and rhIL-2, which results in Tregs with polyclonal reactivity [45]; the cell expansion in presence of donor APCs, which generate alloantigen-specific Tregs [46]; or the cell expansion in presence of genetically modified K562-based artificial APCs, which may efficiently expand a specific Treg population [6]. Finally, the developed Tregs may be phenotyped and infused in the patient. So far, numerous clinical trials, most of them in pilot safety and feasibility phase, are analyzing the therapeutic effects of Treg infusion in patients with liver transplantation, graft versus host disease, type 1 diabetes mellitus (T1DM), lupus, and auto-immune hepatitis, showing promising results [21, 47, 48].

Another form of immunotherapy is the manipulation of the Treg compartment *in vivo*, by the use of an array of systemically or locally delivered molecules that promote Treg

proliferation, phenotype stability, and functionality [6]. Some of the molecules that might affect the Treg pool *in vivo* or *in vitro* are as follows.

5.1. Cytokines. Multiple cytokines have been associated with Treg phenotypic stability and suppressive functionality enhancement through different mechanisms. In mice and humans, IL-2 maintains Treg function by stabilizing Foxp3 expression and regulating key Treg-signature molecules such as CTLA-4 and GITR [49]. IL-2 signaling is also essential to prevent the polarization of Tregs into pro-inflammatory effector cells [50]. Besides its direct effect on Foxp3 expression, IL-2 acts indirectly as it is required for negative regulation of the TBX21 and RORC2 loci, which encode two transcription factors that feedback to diminish Foxp3 expression [50]. The potential role of exogenous IL-2 for Treg survival and Foxp3 expression maintenance has led the exploration of therapeutic approaches. Preclinical studies have shown that the delivery of IL-2/anti-IL-2-antibody complexes stimulates Treg expansion and reduces disease in models of T1DM, experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis, and angiotensin II-induced aortic stiffening [49–51]. In clinical trials, therapy with low-dose IL-2 for the treatment of graft versus host disease (GVHD) and T1DM appears to successfully expand the circulating Treg cell pool [47, 52, 53].

On the other hand, IL-33, a member of the IL-1 cytokine family, has recently gained interest as regulator of Treg biology. It binds to ST2 receptor, whose deficiency is associated with augmented inflammatory response [54]. IL-33 has shown to increase the $CD4^+$ $Foxp3^+$ Treg pool, enhance their suppressive activity, and boost ST2 surface expression [55]. It supports direct and indirect Treg cell expansion through the induction of myeloid cells to secrete IL-2, which increases ST2 expression by T lymphocytes [56]. Moreover, IL-33 has the ability to induce regulatory phenotype by promoting the expansion of $ST2^+$ Tregs [57, 58]. The upregulation of ST2 expression on Tregs increases the expression of Foxp3, and it has been suggested that genes encoding both of these molecules might depend on each other [59]. In animal models of skin transplant [60], collagen-induced arthritis [61] treated with IL-33 showed the induction of Treg proliferation and enhancement of their immuno-suppressive properties. Furthermore, in patients with type 1 diabetes, *in vitro* IL-33 treatment induced regulatory $CD4^+$ $CD25^{high}$ $FOXP3^+$ cell frequencies as well as upregulated the surface expression of ST2 molecule and Foxp3 expression [55].

5.2. All-trans Retinoic Acid. All-*trans* retinoic acid (atRA) is the main active metabolite of vitamin A, well known for playing a major role in various cellular functions, such as proliferation, embryogenesis, differentiation, inflammation, and cell death [62]. Recent studies have revealed that atRA, after binding to RAR, regulates reciprocal differentiation between Tregs and Th17 lymphocytes, reinforcing the regulatory functions of Tregs and suppressing the pro-inflammatory activities of Th17 cells [63, 64]. In conjunction with TGF-β1 and IL-2, atRA has shown to improve the differentiation of naïve T lymphocytes into Tregs, reflecting an increment in the number of these cells as well as amplified expression of Foxp3 [65, 66]. atRA enhances the differentiation and stability of iTregs, increasing the activation of the ERK1/2 signaling pathway, resulting in a more stable Foxp3 expression [67]. Moreover, it can increase the nTreg stability under inflammatory conditions through the inhibition of the methylation of the *foxp3* gene [67, 68]. In addition, atRA greatly reduces RORγt expression and Th17 cell differentiation [69].

5.3. Rapamycin. Rapamycin (RAPA) is a macrolide immunosuppressant, widely used in the treatment of organ rejection after transplantation, cancer, and autoimmune diseases [70]. RAPA binds to the mammalian target of rapamycin (mTOR) and inhibits its signaling pathway. The inhibition of mTORC1 and mTORC2 after prolonged exposure to RAPA allows preferential expansion and function of $CD4^+$ $CD25^{hi}$ $Foxp3^+$ Tregs [71] and blocks critical effector T cell functions such as proliferation, migration, and cytokine production, limiting their differentiation [72]. Conversely, Foxp3 expression by Tregs induces the serine/threonine kinase Pim-2 pathway, which permits the evasion of many RAPA-imposed signaling block [73].

It has been shown that both RAPA and atRA have similar effects on promoting and stabilizing Tregs during their expansion [74]. Although atRA, compared with RAPA, has demonstrated to be more efficient in stabilizing nTregs under inflammatory conditions [67, 75], atRA in conjunction with RAPA promotes the expansion of functional Tregs in the absence of exogenous TGF-β [76]. In fact, it has been suggested that the combined use of RAPA and atRA in Treg culture increases the percentage of Tregs with demethylated *foxp3* alleles, making them more likely to remain as Tregs once reinfused in the patient and provide long-lasting, effective control [76]. Adoptive transfer of Tregs after *ex vivo* treatments with atRA and/or RAPA has been considered a promising strategy for cell-based therapeutic treatment of transplant rejection and autoimmune diseases, such as T1DM [21], rheumatoid arthritis [77], and Crohn's disease [78].

5.4. Vitamin D. Vitamin D metabolites have long been recognized as important immuno-modulators that exert their functions by binding to the vitamin D receptor (VDR), expressed on many immune cells [79]. The active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1,25-$[OH]_2$D3, calcitriol), is a secosteroid hormone that is mainly produced by a sunlight-catalyzed biosynthesis pathway in the skin [80]. VDR is a member of the superfamily of hormone nuclear receptors that, after binding with calcitriol, has a conformational change that results in binding to RXR, forming a heterodimer that translocates to the nucleus, where it binds to vitamin D response elements [80]. Calcitriol promotes the growth of Tregs, inhibits Th17 lymphocytes, and induces the secretion of anti-inflammatory cytokines [81]. Moreover, vitamin D also inhibits effector T cell responses through modulation of APC functionality. Treatment of human DCs with calcitriol *in vitro* results in an immature phenotype, known as tolerogenic DCs, characterized by reduced expression of CD80, CD86, and HLA-DR [80, 82]. In this context,

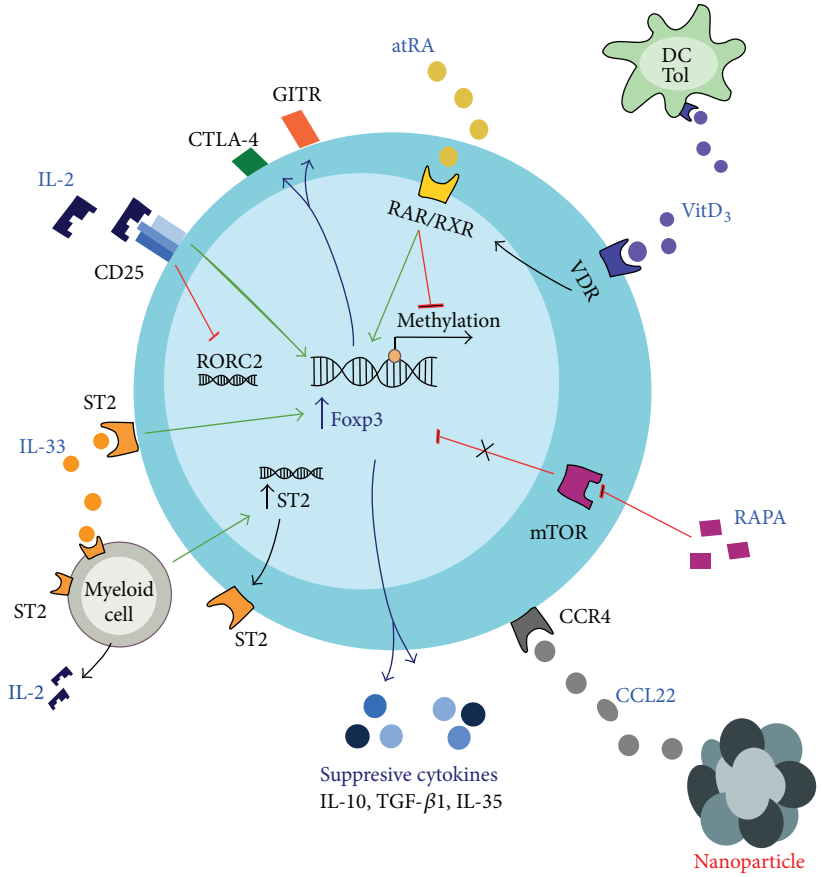


Figure 2: Therapeutic approaches for Treg enrichment *in vivo*. There is an array of systemically or locally delivered molecules that may promote Treg proliferation, phenotype stability, and functionality *in vivo*. Among them, IL-2 has been described as a major growth factor for T cells, particularly important for Treg physiology. IL-2 binds to its receptor CD25, whose signaling pathway induces Foxp3 expression on Tregs and inhibits Th17 differentiation. Foxp3 enables the expression of canonical Treg features, such as CTLA-4, GITR, and anti-inflammatory cytokines. On the other hand, all-*trans* retinoic acid (atRA) and calcitriol ($VitD_3$), active metabolites of vitamins A and D, have shown to reinforce suppressive functions of Tregs. atRA induces the binding of the RA receptor (RAR) and the retinoic X receptor (RXR) to an intronic enhancer of *foxp3* gene locus, increasing its expression. Similarly, $VitD_3$ binds to the vitamin D receptor (VDR), which later binds to RXR, forming a heterodimer that translocates towards the nucleus to promote Foxp3 expression. Also, $VitD_3$ induces tolerogenic dendritic cells (DC Tol), with an immature phenotype that may drive Treg responses. Rapamycin (RAPA) inhibits mTOR signaling pathway, allowing preferential expansion of Tregs, and blocks critical Teff functions. Additionally, IL-33 binds to ST2 receptor, promoting further Foxp3 and ST2 expression on Tregs. Also, indirectly, IL-33 supports Treg expansion, inducing IL-2 secretion by myeloid cells, which stimulates additional ST2 expression. Finally, CCL22-loaded nanoparticles may recruit $CCR4^+$ Tregs locally, decreasing Teffs and their pro-inflammatory functions.

pretreatment of human blood-derived myeloid DCs with calcitriol, and then coculture with T cells, inhibits the effector T cell cytokine production and promotes Treg suppressive functions [82].

5.5. Controlled Delivery of Treg Promoters. The use of the polymeric-nanoparticle technology has demonstrated its biomedical potential due to its ability to encapsulate and control the release of hydrophobic, small molecules [83]. In a recent report, biodegradable poly(ethylene glycol)-poly(lactic-*co*-glycolic acid) (PEG-PLGA) microparticles were engineered to release TGF-β, RAPA, and IL-2 to locally induce Treg polarization in an *in vivo* model of allergic contact dermatitis [83]. The prophylactic treatment with these microparticles increased the Treg/Teff ratio in the skin draining lymph nodes, suppressing the T cell-mediated delayed-type hypersensitivity and rendering systemic and specific tolerance to contact allergens [84]. In another study, PLGA microspheres encapsulating recombinant mouse CCL22 were formulated to enhance local recruitment of $CCR4^+$ Tregs in a murine model of dry eye disease [84]. The results showed that the microsphere treatment successfully prevents the inflammatory symptomatology by increasing the frequency of Tregs and decreasing the Teff in the lacrimal gland [85]. A different approach for Treg generation was the use of antigenic peptides conjugated to poly(lactide-*co*-glycolide) nanoparticles, which provided a platform for tolerance induction in a murine model of multiple sclerosis (relapsing-remitting experimental autoimmune encephalomyelitis). Through this system, tolerogenic antigen-polymer-conjugated nanoparticles can be formulated to incorporate multiple antigens responsible for the

pathogenesis of multiple sclerosis and other diseases [86] (Figure 2).

6. Treg Therapeutic Potential in Periodontitis

Treg immuno-suppressive mechanisms and tissue-repairing functions are necessary to sustain periodontal health, which make them an interesting potential therapeutic target. Over the recent years, different therapeutic approaches have been attempted in order to increase the number of functional Tregs in periodontal disease. One approach has been the selective chemo-attraction of Tregs to a particular diseased periodontal lesion by the use of CCL22-releasing microparticles [3]. This method successfully reduced bone resorption, as it enhanced the expression of osteogenic, regenerative, and anti-inflammatory markers in the periodontium, and diminished inflammatory cell infiltration in both murine and canine models of periodontitis [87]. Another method employed in a murine model of periodontitis was the oral administration of atRA. This treatment has shown to effectively regulate the Th17/Treg balance by increasing the percentage of $CD4^+$ $Foxp3^+$ Tregs and reducing the $CD4^+$ $ROR\gamma t^+$ Th17 lymphocyte frequency [88]. Similarly, the same group studied the effects of oral administration of tamibarotene (Am80), a synthetic RAR agonist with high specificity for RARα and RARβ. Retinoid agonists have been shown to inhibit Th17 cell polarization and to enhance Foxp3 expression during the course of inflammatory diseases; besides, they do not present the atRA limitations such as compound's instability, poor bioavailability, and unexpected side effects. In a murine model of periodontitis, Am80 reduced the percentage of $CD4^+$ $ROR\gamma t^+$ Th17 lymphocytes and increased the percentage of $CD4^+$ $Foxp3^+$ Tregs in the gingival tissues, cervical lymph nodes, and spleen. Also, Am80 downregulated the mRNA expression of IL-17A, RANKL, MCP-1, IL-6, and IL-1β and upregulated the expression of IL-10 and TGF-β1 in gingival tissues and cervical lymph nodes [89].

A different approach has been the subcutaneous vaccination with formalin-killed *P. gingivalis*, which protects mice from inflammation and alveolar bone resorption by modulating the Th17/Treg ratio. The vaccinated mice showed significant reduction in the frequencies of total $CD4^+$ T and $CD4^+$ $ROR\gamma t^+$ cells, and a significant increase in the percentage of Tregs from cervical lymph nodes and spleens [90]. Although these studies showed promising results, more research is still needed in order to apply them in humans.

7. Concluding Remarks

Treg protective functions during periodontitis have been demonstrated *in vivo*. Ongoing research in both basic and translational studies lets us gain a better understanding of the diversity of Treg subsets, their plasticity, and their function. Certainly, as our knowledge of Treg biology increases, we will be capable developing new therapies designed to enhance the stability and function of Treg during periodontitis.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Carla Alvarez and Carolina Rojas contributed equally to this work and should be considered as joint first authors.

Acknowledgments

This study was supported by grants FONDECYT 1140904 and FONDECYT 1181780 from Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) from the Chilean Government.

References

[1] G. Hajishengallis, "Periodontitis: from microbial immune subversion to systemic inflammation," *Nature Reviews Immunology*, vol. 15, no. 1, pp. 30–44, 2015.

[2] G. Hajishengallis and J. M. Korostoff, "Revisiting the Page & Schroeder model: the good, the bad and the unknowns in the periodontal host response 40 years later," *Periodontology 2000*, vol. 75, no. 1, pp. 116–151, 2017.

[3] G. P. Garlet, C. S. Sfeir, and S. R. Little, "Restoring host-microbe homeostasis via selective chemoattraction of Tregs," *Journal of Dental Research*, vol. 93, no. 9, pp. 834–839, 2014.

[4] S. Sakaguchi, N. Sakaguchi, M. Asano, M. Itoh, and M. Toda, "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases," *The Journal of Immunology*, vol. 155, no. 3, pp. 1151–1164, 1995.

[5] J. Barbi, D. Pardoll, and F. Pan, "Treg functional stability and its responsiveness to the microenvironment," *Immunological Reviews*, vol. 259, no. 1, pp. 115–139, 2014.

[6] B. D. Singer, L. S. King, and F. R. D'Alessio, "Regulatory T cells as immunotherapy," *Frontiers in Immunology*, vol. 5, 2014.

[7] F. J. Alroqi and T. A. Chatila, "T regulatory cell biology in health and disease," *Current Allergy and Asthma Reports*, vol. 16, no. 4, p. 27, 2016.

[8] R. E. Hoeppli, K. G. MacDonald, M. K. Levings, and L. Cook, "How antigen specificity directs regulatory T-cell function: self, foreign and engineered specificity," *HLA*, vol. 88, no. 1-2, pp. 3–13, 2016.

[9] S. A. Mahmud, L. S. Manlove, H. M. Schmitz et al., "Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells," *Nature Immunology*, vol. 15, no. 5, pp. 473–481, 2014.

[10] C.-S. Hsieh, H.-M. Lee, and C.-W. J. Lio, "Selection of regulatory T cells in the thymus," *Nature Reviews Immunology*, vol. 12, no. 3, pp. 157–167, 2012.

[11] X. Lin, M. Chen, Y. Liu et al., "Advances in distinguishing natural from induced $Foxp3^+$ regulatory T cells," *International Journal of Clinical & Experimental Pathology*, vol. 6, no. 2, pp. 116–123, 2013.

[12] J. L. Coombes, K. R. R. Siddiqui, C. V. Arancibia-Cárcamo et al., "A functionally specialized population of mucosal $CD103^+$ DCs induces $Foxp3^+$ regulatory T cells via a TGF-β-

and retinoic acid-dependent mechanism," *The Journal of Experimental Medicine*, vol. 204, no. 8, pp. 1757–1764, 2007.

[13] M. Kanamori, H. Nakatsukasa, M. Okada, Q. Lu, and A. Yoshimura, "Induced regulatory T cells: their development, stability, and applications," *Trends in Immunology*, vol. 37, no. 11, pp. 803–811, 2016.

[14] P. M. Smith, M. R. Howitt, N. Panikov et al., "The microbial metabolites, short-chain fatty acids, regulate colonic T_{reg} cell homeostasis," *Science*, vol. 341, no. 6145, pp. 569–573, 2013.

[15] S. Lucas, Y. Omata, J. Hofmann et al., "Short-chain fatty acids regulate systemic bone mass and protect from pathological bone loss," *Nature Communications*, vol. 9, no. 1, p. 55, 2018.

[16] N. Komatsu, M. E. Mariotti-Ferrandiz, Y. Wang, B. Malissen, H. Waldmann, and S. Hori, "Heterogeneity of natural $Foxp3^+$ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 6, pp. 1903–1908, 2009.

[17] S. Hori, T. Nomura, and S. Sakaguchi, "Control of regulatory T cell development by the transcription factor *Foxp3*," *Science*, vol. 299, no. 5609, pp. 1057–1061, 2003.

[18] L. M. Williams and A. Y. Rudensky, "Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3," *Nature Immunology*, vol. 8, no. 3, pp. 277–284, 2007.

[19] M. A. Gavin, T. R. Torgerson, E. Houston et al., "Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 17, pp. 6659–6664, 2006.

[20] S. Sakaguchi, M. Miyara, C. M. Costantino, and D. A. Hafler, "$FOXP3^+$ regulatory T cells in the human immune system," *Nature Reviews Immunology*, vol. 10, no. 7, pp. 490–500, 2010.

[21] J. A. Bluestone, J. H. Buckner, M. Fitch et al., "Type 1 diabetes immunotherapy using polyclonal regulatory T cells," *Science Translational Medicine*, vol. 7, no. 315, article 315ra189, 2015.

[22] G. M. Mason, K. Lowe, R. Melchiotti et al., "Phenotypic complexity of the human regulatory T cell compartment revealed by mass cytometry," *The Journal of Immunology*, vol. 195, no. 5, pp. 2030–2037, 2015.

[23] S. Hori, "Lineage stability and phenotypic plasticity of $Foxp3^+$ regulatory T cells," *Immunological Reviews*, vol. 259, no. 1, pp. 159–172, 2014.

[24] N. Komatsu, K. Okamoto, S. Sawa et al., "Pathogenic conversion of $Foxp3^+$ T cells into T_H17 cells in autoimmune arthritis," *Nature Medicine*, vol. 20, no. 1, pp. 62–68, 2014.

[25] R. Liberal, C. R. Grant, M. S. Longhi, G. Mieli-Vergani, and D. Vergani, "Regulatory T cells: mechanisms of suppression and impairment in autoimmune liver disease," *IUBMB Life*, vol. 67, no. 2, pp. 88–97, 2015.

[26] L. S. K. Walker, "Treg and CTLA-4: two intertwining pathways to immune tolerance," *Journal of Autoimmunity*, vol. 45, no. 100, pp. 49–57, 2013.

[27] A. Schmidt, N. Oberle, and P. H. Krammer, "Molecular mechanisms of Treg-mediated T cell suppression," *Frontiers in Immunology*, vol. 3, 2012.

[28] J. Meyle and I. Chapple, "Molecular aspects of the pathogenesis of periodontitis," *Periodontology 2000*, vol. 69, no. 1, pp. 7–17, 2015.

[29] A. Bozec and M. M. Zaiss, "T regulatory cells in bone remodelling," *Current Osteoporosis Reports*, vol. 15, no. 3, pp. 121–125, 2017.

[30] A. Bozec, M. M. Zaiss, R. Kagwiria et al., "T cell costimulation molecules CD80/86 inhibit osteoclast differentiation by inducing the IDO/tryptophan pathway," *Science Translational Medicine*, vol. 6, no. 235, article 235ra60, 2014.

[31] M. M. Zaiss, R. Axmann, J. Zwerina et al., "Treg cells suppress osteoclast formation: a new link between the immune system and bone," *Arthritis & Rheumatism*, vol. 56, no. 12, pp. 4104–4112, 2007.

[32] M. M. Zaiss, K. Sarter, A. Hess et al., "Increased bone density and resistance to ovariectomy-induced bone loss in FoxP3-transgenic mice based on impaired osteoclast differentiation," *Arthritis & Rheumatism*, vol. 62, no. 8, pp. 2328–2338, 2010.

[33] C. Zhang, L. Li, K. Feng, D. Fan, W. Xue, and J. Lu, "'Repair' Treg cells in tissue injury," *Cellular Physiology and Biochemistry*, vol. 43, no. 6, pp. 2155–2169, 2017.

[34] A. Nosbaum, N. Prevel, H.-A. Truong et al., "Cutting edge: regulatory T cells facilitate cutaneous wound healing," *The Journal of Immunology*, vol. 196, no. 5, pp. 2010–2014, 2016.

[35] N. Arpaia, J. A. Green, B. Moltedo et al., "A distinct function of regulatory T cells in tissue protection," *Cell*, vol. 162, no. 5, pp. 1078–1089, 2017.

[36] T. Nakajima, K. Ueki-Maruyama, T. Oda et al., "Regulatory T-cells infiltrate periodontal disease tissues," *Journal of Dental Research*, vol. 84, no. 7, pp. 639–643, 2005.

[37] C. R. Cardoso, G. P. Garlet, A. P. Moreira, W. M. Junior, M. A. Rossi, and J. S. Silva, "Characterization of $CD4^+CD25^+$ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis," *Journal of Leukocyte Biology*, vol. 84, no. 1, pp. 311–318, 2008.

[38] A. C. Araujo-Pires, A. E. Vieira, C. F. Francisconi et al., "IL-4/CCL22/CCR4 axis controls regulatory T-cell migration that suppresses inflammatory bone loss in murine experimental periodontitis," *Journal of Bone and Mineral Research*, vol. 30, no. 3, pp. 412–422, 2015.

[39] K. Takahashi, S. Takashiba, A. Nagai et al., "Assessment of interleukin-6 in the pathogenesis of periodontal disease," *Journal of Periodontology*, vol. 65, no. 2, pp. 147–153, 1994.

[40] N. Dutzan, J. Gamonal, A. Silva, M. Sanz, and R. Vernal, "Over-expression of forkhead box P3 and its association with receptor activator of nuclear factor-κ B ligand, interleukin (IL)-17, IL-10 and transforming growth factor-β during the progression of chronic periodontitis," *Journal of Clinical Periodontology*, vol. 36, no. 5, pp. 396–403, 2009.

[41] C. W. O. Ernst, J. E. Lee, T. Nakanishi et al., "Diminished forkhead box P3/CD25 double-positive T regulatory cells are associated with the increased nuclear factor-kB ligand ($RANKL^+$) T cells in bone resorption lesion of periodontal disease," *Clinical & Experimental Immunology*, vol. 148, no. 2, pp. 271–280, 2007.

[42] T. Okui, Y. Aoki, H. Ito, T. Honda, and K. Yamazaki, "The presence of $IL\text{-}17^+/FOXP3^+$ double-positive cells in periodontitis," *Journal of Dental Research*, vol. 91, no. 6, pp. 574–579, 2012.

[43] G. P. Garlet, C. R. Cardoso, F. S. Mariano et al., "Regulatory T cells attenuate experimental periodontitis progression in mice," *Journal of Clinical Periodontology*, vol. 37, no. 7, pp. 591–600, 2010.

[44] X. Qin, J. Y. Liu, T. Wang et al., "Role of indoleamine 2,3-dioxygenase in an inflammatory model of murine gingiva," *Journal of Periodontal Research*, vol. 52, no. 1, pp. 107–113, 2017.

[45] H. Fan, J. Yang, J. Hao et al., "Comparative study of regulatory T cells expanded *ex vivo* from cord blood and adult peripheral blood," *Immunology*, vol. 136, no. 2, pp. 218–230, 2012.

[46] H. Kasahara, T. Kondo, H. Nakatsukasa et al., "Generation of allo-antigen-specific induced Treg stabilized by vitamin C treatment and its application for prevention of acute graft versus host disease model," *International Immunology*, vol. 29, no. 10, pp. 457–469, 2017.

[47] A. Hartemann, G. Bensimon, C. A. Payan et al., "Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial," *The Lancet Diabetes & Endocrinology*, vol. 1, no. 4, pp. 295–305, 2013.

[48] N. Safinia, C. Scotta, T. Vaikunthanathan, R. I. Lechler, and G. Lombardi, "Regulatory T cells: serious contenders in the promise for immunological tolerance in transplantation," *Frontiers in Immunology*, vol. 6, 2015.

[49] J. D. Goldstein, L. Pérol, B. Zaragoza, A. Baeyens, G. Marodon, and E. Piaggio, "Role of cytokines in thymus- versus peripherally derived-regulatory T cell differentiation and function," *Frontiers in Immunology*, vol. 4, 2013.

[50] E. A. Wohlfert, J. R. Grainger, N. Bouladoux et al., "GATA3 controls Foxp3$^+$ regulatory T cell fate during inflammation in mice," *Journal of Clinical Investigation*, vol. 121, no. 11, pp. 4503–4515, 2011.

[51] S.-Y. Lee, M.-L. Cho, H.-J. Oh et al., "Interleukin-2/anti-interleukin-2 monoclonal antibody immune complex suppresses collagen-induced arthritis in mice by fortifying interleukin-2/STAT5 signalling pathways," *Immunology*, vol. 137, no. 4, pp. 305–316, 2012.

[52] A. A. Kennedy-Nasser, S. Ku, P. Castillo-Caro et al., "Ultra low-dose IL-2 for GVHD prophylaxis after allogeneic hematopoietic stem cell transplantation mediates expansion of regulatory T cells without diminishing antiviral and antileukemic activity," *Clinical Cancer Research*, vol. 20, no. 8, pp. 2215–2225, 2014.

[53] K. Matsuoka, J. Koreth, H. T. Kim et al., "Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease," *Science Translational Medicine*, vol. 5, no. 179, article 179ra43, 2013.

[54] T. Gajardo Carrasco, R. A. Morales, F. Pérez et al., "*Alarmin*' immunologists: IL-33 as a putative target for modulating T cell-dependent responses," *Frontiers in Immunology*, vol. 6, 2015.

[55] M. Ryba-Stanisławowska, P. Werner, M. Skrzypkowska, A. Brandt, and J. Myśliwska, "IL-33 effect on quantitative changes of CD4$^+$CD25^{high}FOXP3$^+$ regulatory T cells in children with type 1 diabetes," *Mediators of Inflammation*, vol. 2016, Article ID 9429760, 7 pages, 2016.

[56] F. Y. Liew, J.-P. Girard, and H. R. Turnquist, "Interleukin-33 in health and disease," *Nature Reviews Immunology*, vol. 16, no. 11, pp. 676–689, 2016.

[57] B. M. Matta, J. M. Lott, L. R. Mathews et al., "IL-33 is an unconventional alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2$^+$ regulatory T cells," *The Journal of Immunology*, vol. 193, no. 8, pp. 4010–4020, 2014.

[58] B. M. Matta, D. K. Reichenbach, X. Zhang et al., "Peri-alloHCT IL-33 administration expands recipient T-regulatory cells that protect mice against acute GVHD," *Blood*, vol. 128, no. 3, pp. 427–439, 2016.

[59] C. Schiering, T. Krausgruber, A. Chomka et al., "The alarmin IL-33 promotes regulatory T-cell function in the intestine," *Nature*, vol. 513, no. 7519, pp. 564–568, 2014.

[60] T. Gajardo, R. A. Morales, M. Campos-Mora, J. Campos-Acuna, and K. Pino-Lagos, "Exogenous interleukin-33 targets myeloid-derived suppressor cells and generates periphery-induced Foxp3$^+$ regulatory T cells in skin-transplanted mice," *Immunology*, vol. 146, no. 1, pp. 81–88, 2015.

[61] J. Biton, S. K. Athari, A. Thiolat et al., "In vivo expansion of activated Foxp3$^+$ regulatory T cells and establishment of a type 2 immune response upon IL-33 treatment protect against experimental arthritis," *The Journal of Immunology*, vol. 197, no. 5, pp. 1708–1719, 2016.

[62] K. Pino-Lagos, Y. Guo, and R. J. Noelle, "Retinoic acid: a key player in immunity," *BioFactors*, vol. 36, no. 6, pp. 430–436, 2010.

[63] P. Henning, H. H. Conaway, and U. H. Lerner, "Retinoid receptors in bone and their role in bone remodeling," *Frontiers in Endocrinology*, vol. 6, p. 31, 2015.

[64] S. Xiao, H. Jin, T. Korn et al., "Retinoic acid increases Foxp3$^+$ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-β-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression," *The Journal of Immunology*, vol. 181, no. 4, pp. 2277–2284, 2008.

[65] M. Bono, G. Tejon, F. Flores-Santibañez, D. Fernandez, M. Rosemblatt, and D. Sauma, "Retinoic acid as a modulator of T cell immunity," *Nutrients*, vol. 8, no. 6, p. 349, 2016.

[66] C. C. Brown and R. J. Noelle, "Seeing through the dark: new insights into the immune regulatory functions of vitamin A," *European Journal of Immunology*, vol. 45, no. 5, pp. 1287–1295, 2015.

[67] L. Lu, Q. Lan, Z. Li et al., "Critical role of *all-trans* retinoic acid in stabilizing human natural regulatory T cells under inflammatory conditions," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 33, pp. E3432–E3440, 2014.

[68] L. Lu, J. Ma, Z. Li et al., "*All-trans* retinoic acid promotes TGF-β-induced Tregs via histone modification but not DNA demethylation on Foxp3 gene locus," *PLoS One*, vol. 6, no. 9, article e24590, 2011.

[69] Z. Chen, A. Laurence, and J. J. O'Shea, "Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation," *Seminars in Immunology*, vol. 19, no. 6, pp. 400–408, 2007.

[70] A. W. Thomson, H. R. Turnquist, and G. Raimondi, "Immunoregulatory functions of mTOR inhibition," *Nature Reviews Immunology*, vol. 9, no. 5, pp. 324–337, 2009.

[71] G. M. Delgoffe, K. N. Pollizzi, A. T. Waickman et al., "The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2," *Nature Immunology*, vol. 12, no. 4, pp. 295–303, 2011.

[72] F. Jing, F. Yang, F. Cui, Z. Chen, L. Ling, and X. Huang, "Rapamycin alleviates inflammation and muscle weakness, while altering the Treg/Th17 balance in a rat model of myasthenia gravis," *Bioscience Reports*, vol. 37, no. 4, article BSR20170767, 2017.

[73] S. Basu, T. Golovina, T. Mikheeva, C. H. June, and J. L. Riley, "Cutting edge: Foxp3-mediated induction of Pim 2 allows

human T regulatory cells to preferentially expand in rapamycin," *The Journal of Immunology*, vol. 180, no. 9, pp. 5794–5798, 2008.

[74] C. Scotta, M. Esposito, H. Fazekasova et al., "Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human $CD4^+CD25^+FOXP3^+$ T regulatory cell subpopulations," *Haematologica*, vol. 98, no. 8, pp. 1291–1299, 2013.

[75] Z.-M. Liu, K.-P. Wang, J. Ma, and S. Guo Zheng, "The role of all-*trans* retinoic acid in the biology of $Foxp3^+$ regulatory T cells," *Cellular & Molecular Immunology*, vol. 12, no. 5, pp. 553–557, 2015.

[76] T. N. Golovina, T. Mikheeva, T. M. Brusko, B. R. Blazar, J. A. Bluestone, and J. L. Riley, "Retinoic acid and rapamycin differentially affect and synergistically promote the *ex vivo* expansion of natural human T regulatory cells," *PLoS One*, vol. 6, no. 1, article e15868, 2011.

[77] D. Cejka, S. Hayer, B. Niederreiter et al., "Mammalian target of rapamycin signaling is crucial for joint destruction in experimental arthritis and is activated in osteoclasts from patients with rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 62, no. 8, pp. 2294–2302, 2010.

[78] P. Desreumaux, A. Foussat, M. Allez et al., "Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease," *Gastroenterology*, vol. 143, no. 5, pp. 1207–1217.e2, 2012.

[79] R. E. Hoeppli, D. Wu, L. Cook, and M. K. Levings, "The environment of regulatory T cell biology: cytokines, metabolites, and the microbiome," *Frontiers in Immunology*, vol. 6, p. 61, 2015.

[80] E. S. Chambers and C. M. Hawrylowicz, "The impact of vitamin D on regulatory T cells," *Current Allergy and Asthma Reports*, vol. 11, no. 1, pp. 29–36, 2011.

[81] Q. Zhou, S. Qin, J. Zhang, L. Zhon, Z. Pen, and T. Xing, "$1,25(OH)_2D_3$ induces regulatory T cell differentiation by influencing the VDR/PLC-γ1/TGF-β1/pathway," *Molecular Immunology*, vol. 91, pp. 156–164, 2017.

[82] L. Adorini and G. Penna, "Induction of tolerogenic dendritic cells by vitamin D receptor agonists," in *Handbook of Experimental Pharmacology*, vol. 188, pp. 251–273, 2009.

[83] F. Danhier, E. Ansorena, J. M. Silva, R. Coco, A. Le Breton, and V. Preat, "PLGA-based nanoparticles: an overview of biomedical applications," *Journal of Controlled Release*, vol. 161, no. 2, pp. 505–522, 2012.

[84] S. C. Balmert, C. Donahue, J. R. Vu, G. Erdos, L. D. Falo Jr., and S. R. Little, "*In vivo* induction of regulatory T cells promotes allergen tolerance and suppresses allergic contact dermatitis," *Journal of Controlled Release*, vol. 261, pp. 223–233, 2017.

[85] M. L. Ratay, A. J. Glowacki, S. C. Balmert et al., "Treg-recruiting microspheres prevent inflammation in a murine model of dry eye disease," *Journal of Controlled Release*, vol. 258, pp. 208–217, 2017.

[86] R. M. Pearson, L. M. Casey, K. R. Hughes et al., "Controlled delivery of single or multiple antigens in tolerogenic nanoparticles using peptide-polymer bioconjugates," *Molecular Therapy*, vol. 25, no. 7, pp. 1655–1664, 2017.

[87] A. J. Glowacki, S. Yoshizawa, S. Jhunjhunwala et al., "Prevention of inflammation-mediated bone loss in murine and canine periodontal disease via recruitment of regulatory lymphocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 46, pp. 18525–18530, 2013.

[88] L. Wang, J. Wang, Y. Jin, H. Gao, and X. Lin, "Oral administration of all-*trans* retinoic acid suppresses experimental periodontitis by modulating the Th17/Treg imbalance," *Journal of Periodontology*, vol. 85, no. 5, pp. 740–750, 2014.

[89] Y. Jin, L. Wang, D. Liu, and X. Lin, "Tamibarotene modulates the local immune response in experimental periodontitis," *International Immunopharmacology*, vol. 23, no. 2, pp. 537–545, 2014.

[90] L. Wang, N. Guan, Y. Jin, X. Lin, and H. Gao, "Subcutaneous vaccination with *Porphyromonas gingivalis* ameliorates periodontitis by modulating Th17/Treg imbalance in a murine model," *International Immunopharmacology*, vol. 25, no. 1, pp. 65–73, 2015.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3487591, 7 pages
https://doi.org/10.1155/2018/3487591

Research Article

Evaluation of Regulatory Immune Response in Skin Lesions of Patients Affected by Nonulcerated or Atypical Cutaneous Leishmaniasis in Honduras, Central America

Gabriela Venicia Araujo Flores,[1] Carmen Maria Sandoval Pacheco,[1] Thaise Yumie Tomokane,[1] Wilfredo Sosa Ochoa,[1,2] Concepción Zúniga Valeriano,[3] Claudia Maria Castro Gomes,[1] Carlos Eduardo Pereira Corbett,[1] and Marcia Dalastra Laurenti[1]

[1]*Laboratory of Pathology of Infectious Diseases, Medical School, University of São Paulo, São Paulo, SP, Brazil*
[2]*Microbiology School, National Autonomous University of Honduras, Tegucigalpa, Honduras*
[3]*University School Hospital, Tegucigalpa, Honduras*

Correspondence should be addressed to Marcia Dalastra Laurenti; mdlauren@usp.br

Received 1 November 2017; Accepted 28 January 2018; Published 21 March 2018

Academic Editor: Minggang Zhang

Copyright © 2018 Gabriela Venicia Araujo Flores et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In Honduras, *Leishmania (L.) infantum chagasi* causes both visceral leishmaniasis (LV) and nonulcerated or atypical cutaneous leishmaniasis (NUCL). NUCL is characterized by mononuclear inflammatory infiltration of the dermis, composed mainly of lymphocytes followed by macrophages with discrete parasitism. Considering that little is known about the pathogenesis of NUCL, the aim of this study was to evaluate the regulatory response in situ in skin lesions of patients affected by NUCL. Biopsies ($n = 20$) from human cutaneous nonulcerative lesions were collected and processed by usual histological techniques. The in situ regulatory immune response was evaluated by immunohistochemistry using antihuman CD4, FoxP3, IL-10, and TGF-β antibodies. $CD4^+$, $FoxP3^+$, TGF-β^+, and IL-10^+ cells were observed in the dermis with inflammatory infiltration in all studied cases and at higher densities compared to the normal skin controls. A positive and strong correlation was observed between $CD4^+$ and $FoxP3^+$ cells, and a positive and moderate correlation was observed between $FoxP3^+$ and TGF-β^+ but not with IL-10^+ cells. The data suggest that T regulatory $FoxP3^+$ cells and the regulatory cytokines, especially TGF-β, play an important role in the immunopathogenesis of NUCL, modulating a cellular immune response in the skin, avoiding tissue damage, and leading to low tissue parasitic persistence.

1. Introduction

Nonulcerated cutaneous leishmaniasis (NUCL) is a rare form of leishmaniasis described in areas of visceral leishmaniasis (VL) transmission in Central America, including Honduras, Costa Rica, El Salvador, and Nicaragua. *Leishmania (L.) infantum chagasi* is implicated as the aetiological agent that is transmitted by *Lutzomyia longipalpis* sand flies that bite the vertebrate hosts [1]. The patients do not present clinical signs of VL, nor a previous history of visceral diseases. The lesions are characterized by small nonulcerative erythematous papules or erythematous plaques of chronic evolution that are between 1 to 10 mm in diameter and are located in exposed areas of the body, especially the face and extremities, often surrounded by a hypopigmented halo. The main tissue features have been characterized by a granulomatous reaction with a small number of amastigote forms of the parasite [2].

It is important to mention that the identification of *Leishmania* isolates from NUCL lesions from Honduras showed that parasites belong to the *Leishmania donovani* complex by specific monoclonal antibodies, and they were

identified as *Leishmania (L.) donovani chagasi* by isoenzyme analysis [1]. Despite NUCL, VL occurs in the same endemic areas in Honduras, and it should be noted that NUCL is the most common form of the clinical presentation of *Leishmania (L.) infantum chagasi* infection; it affects children older than 6 years and young adults more frequently, while VL occurs mainly in children younger than 5 years [1, 3, 4].

Studies have suggested the ability of *Leishmania (L.) infantum chagasi* to cause both clinical forms, while VL and NUCL could be related to the immunological and genetic backgrounds of the host, as well as parasite and sand fly vector characteristics [2–4]. However, little is known about the profile of human infection by *Leishmania (L.) infantum chagasi* in Honduras, especially of the nonulcerated or atypical form. We have observed self-limiting and nonulcerated skin lesions independent of the disease evolution time, characterized by mononuclear inflammatory infiltration composed mainly of lymphocytes, vacuolated macrophages associated with granulomatous reactions, and scarce parasites, suggesting an efficient cellular immune response in the skin of individuals affected by NUCL. However, the persistence of low tissue parasitism may be related to the regulatory immune response responsible for balanced cellular immune responses that prevent the evolution of the lesion size and lead to lasting immunity. Therefore, the aim of the present study was to characterize the immune regulatory response in skin lesions of patients affected by nonulcerated or atypical cutaneous leishmaniasis in order to better understand the pathogenesis of the infection caused by this species of parasite in Central America.

2. Material and Methods

2.1. Study Area. Two endemic areas of nonulcerated or atypical cutaneous leishmaniasis, Amapala and Orocuina municipalities, located in the southern region of Honduras, were studied. These regions have an average annual temperature of 30°C, with a maximum ranging between 34°C–35°C, a minimum between 25°C–26°C, and an annual humidity of 65% [5].

2.2. Casuistry. Twenty skin biopsies from patients with NUCL, without treatment, and with parasitological diagnosis confirmed by scraping of lesions and stained by Giemsa were used. Patients were informed about the research protocol, and those who agreed to participate signed the informed consent form. This work was approved by the Research Ethics Committee of the Master of Infectious and Zoonotic Diseases of the National Autonomous University of Honduras (Protocol number 03-2014) and by the Research Ethics Committee of the Medical School of the University of São Paulo (CAAE: 64223917.1.0000.0065, Protocol: 1.938.092).

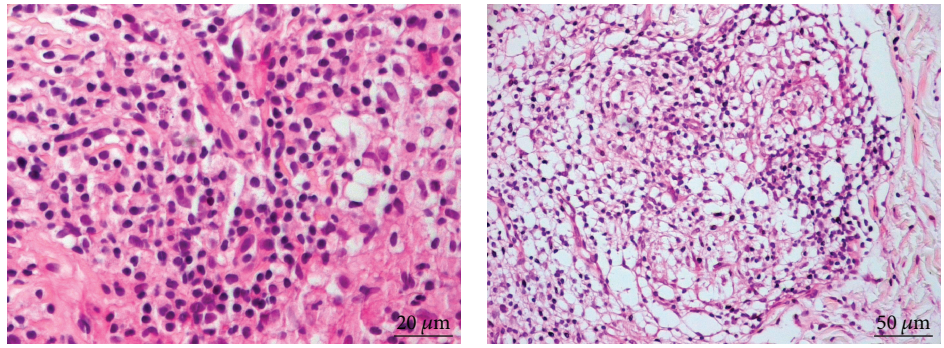
2.3. Histopathology. The biopsies of skin lesions from patients, defined as nonulcerative, erythematous papules, infiltrative plaques, or nodules, in the presence or absence of hypopigmented halos, were collected using a 3 mm punch under aseptic conditions and under local anaesthesia. These biopsies were immersed in 10% formalin solution buffered with 0.01 M phosphate and processed by the usual histological techniques to obtain the paraffin sections. Paraffin sections stained by haematoxylin-eosin (HE) were observed under an optical microscope, with the goal of characterizing histopathological changes. A semiquantitative comparative analysis of the sections stained by HE was performed according to the adaptation of Ridley and Ridley [6], assigning scores for the intensity of the different characterized processes, where (−) is negative, (+) is discrete, (++) is moderate, and (+++) is intense.

2.4. Immunohistochemistry. The in situ regulatory response was assessed by immunohistochemistry using the following markers: anti-CD4 monoclonal antibody and anti-FoxP3, anti-TGF-β1, and anti-IL-10 polyclonal antibodies. Hyperimmune serum from a mouse chronically infected with *Leishmania* (*Leishmania*) *amazonensis* was used to confirm tissue parasitism. Histological sections of 4 μm thickness were deparaffinized in xylene for 15 minutes, followed by hydration with a descending series of alcohols; endogenous peroxidase was blocked with 3% hydrogen peroxide solution. Antigen retrieval was conducted using 10 mM citrate buffer at pH 6.0 in a boiling water bath. After this step, primary antibodies were added to the tissues in the following dilutions: anti-*Leishmania* (mouse hyperimmune serum produced in our laboratory, Moreira et al. [7]) diluted at 1 : 2000; anti-CD4 (monoclonal, NCL-L-CD4-1F6, Novocastra) diluted at 1 : 20; anti-FoxP3 (polyclonal, (H-190): SC-28705, Santa Cruz Biotechnology) diluted at 1 : 250; anti-TGF-β1 (polyclonal, (V): SC-146, Santa Cruz Biotechnology) diluted at 1 : 100, and anti-IL-10 (polyclonal, ab34843, ABCAM) diluted at 1 : 1000. As a negative control, a solution containing phosphate-buffered saline (PBS) and bovine serum albumin (BSA) with the omission of the primary antibody was used. The slides were incubated in a humidified chamber overnight at 4°C. For all markers, the Novolink kit (RE7280-K—Leica) was used. The chromogenic substrate, DAB + H_2O_2 (diaminobenzidine with hydrogen peroxide—K3468—DakoCytomation), was added to the tissue, incubated for 5 minutes, and counterstained with Harris haematoxylin. Finally, the slides were dehydrated in a series of ascending alcohols and mounted with Permount and glass coverslips.

Ten skin samples obtained from healthy individuals undergoing plastic surgery were included as controls.

2.5. Quantitative Analysis of Immunostained Cells. Images were obtained using an optical microscope coupled to the microcomputer, and quantification of immunostained cells was performed using AxioVision 4.8.2 software (Zeiss, San Diego, CA, USA). Ten microscopic fields of each histological section for different markers were imaged by a 40x objective, and the cells immunostained in brown were quantified. The cellular density (number of cells per square millimetre) was determined by the ratio of the immunolabelled cells to the area of each image.

2.6. Statistical Analysis. For the statistical analysis of the results, GraphPad Prism 5.0 software was used and to analyse the difference between the different groups, the t test



(a) (b)

Figure 1: Histological section of a skin biopsy from a patient affected by nonulcerated cutaneous leishmaniasis showing intense mononuclear inflammatory infiltration in the dermis (a) and epithelioid granuloma (b).

was performed for the Gaussian distribution data, and the Mann-Whitney test was used for the non-Gaussian distribution data.

3. Results

3.1. Histopathological Features. In the histopathological analysis, the skin lesions were characterized by mononuclear inflammatory infiltration in the dermis, composed predominantly of lymphocytes, followed by vacuolated macrophages and a few plasma cells. The intensity of the inflammatory infiltration varied from discrete to intense, but in both, the parasitism was discrete. Despite the direct parasitological exam, the presence of the amastigote form of *Leishmania* was in 100% (20/20) of the cases, while histological sections stained by immunohistochemistry evidenced amastigote forms of parasites in only 55% (11/20) of the cases. Granulomas were present in 60% (12/20) of the cases and were associated with moderate to intense inflammation (Figure 1).

3.2. Immunohistochemical Analysis. The skin lesions of NUCL patients showed the presence of $CD4^+$ T, $FoxP3^+$ lymphocytes, TGF-β^+, and IL-10^+ cells, which were evidenced by the immunohistochemical reaction (Figure 2).

The quantitative morphometric analysis of the skin lesions of patients affected by nonulcerated or atypical cutaneous leishmaniasis showed that the cellular density (mean ± standard error) of $CD4^+$ T lymphocytes was 296.60 ± 53.47, that of $FoxP3^+$ cells was 168.40 ± 28.71, that of TGF-β^+ cells was 78.63 ± 16.54, and that of IL-10^+ cells was 63.72 ± 9.70 cells/mm^2. However, in skin from healthy individuals, the number of cells/mm^2 was 46.25 ± 11.55 for $CD4^+$ T lymphocytes, 3.79 ± 1.72 for $FoxP3^+$ cells, 0.11 ± 0.11 for TGF-β^+ cells, and 13.26 ± 5.05 for IL-10^+ cells. The densities of $CD4^+$ T cells and IL-10^+ cells were higher in NUCL patients when compared to healthy skin ($p < 0.01$), and the densities of $FoxP3^+$ cells and TGF-β^+ cells were higher in NUCL patients compared to healthy skin ($p < 0.001$) (Figure 3).

The ratio between regulatory $FoxP3^+$ and effector $CD4^+$ T cells, as well as the ratio between positive cytokines (TGF-β^+ and IL-10^+) and $FoxP3^+$ and $CD4^+$ T cells in NUCL and normal skin, was assessed in order to better evaluate the participation of regulatory cells in the cutaneous inflammation caused by atypical cutaneous leishmaniasis. The ratio of FoxP3 : CD4 was six times higher in NUCL (0.568) than in healthy skin (0.082), the ratio of TGF-β : CD4 was one hundred and thirty-two times higher in NUCL than in healthy skin, and the ratio of TGF-β : FoxP3 was fifteen times higher in NUCL (0.260 and 0.467, resp.) than in healthy skin (0.002 and 0.029, resp.). Already, the ratio of IL-10 : CD4 was similar between NUCL and healthy skin, at 0.3 times higher in healthy skin (0.286) than in NUCL (0.215), and the ratio of IL-10 : FoxP3 was eight times higher in healthy skin (3.500) than in NUCL (0.378).

A positive and strong correlation was observed between the density of $CD4^+$ T cells and $FoxP3^+$ ($\rho = 0.7078$, $p = 0.0007$), and a positive and moderate correlation was detected between $FoxP3^+$ and TGF-β^+ cell density ($\rho = 0.6868$, $p = 0.00465$); however, the density of IL-10^+ cells did not show correlation with any other markers (Figure 4).

4. Discussion

Nonulcerated or atypical cutaneous leishmaniasis is a rare clinical form of infection caused by *Leishmania (L.) infantum chagasi*, and interestingly, it has been described only in Central America. In Honduras, infection caused by *Leishmania (L.) infantum chagasi* is restricted to the southern region of the country, where cases of VL and NUCL occur in the same geographic area [1]. It is an intriguing fact that since the first cases of NUCL have been described in the country, a reduction in the number of cases of the visceral form of the disease has been noted, accompanied by an increase in cases of the cutaneous form, suggesting an efficient adaptation of the pathogen to the host that leads to a balanced parasite-host relationship.

In our study, the main histopathological changes observed in skin lesions of patients with NUCL were characterized by mononuclear inflammatory infiltration in the dermis formed by lymphocytes and macrophages of variable intensity and associated with the formation of epithelioid granulomas. The presence of a granulomatous reaction was associated with an inflammatory infiltration ranging from moderate to severe, mainly diffuse, with evidence of giant cells and focal necrosis in some cases. Despite very few parasites being observed in 100% of cases by direct parasitological

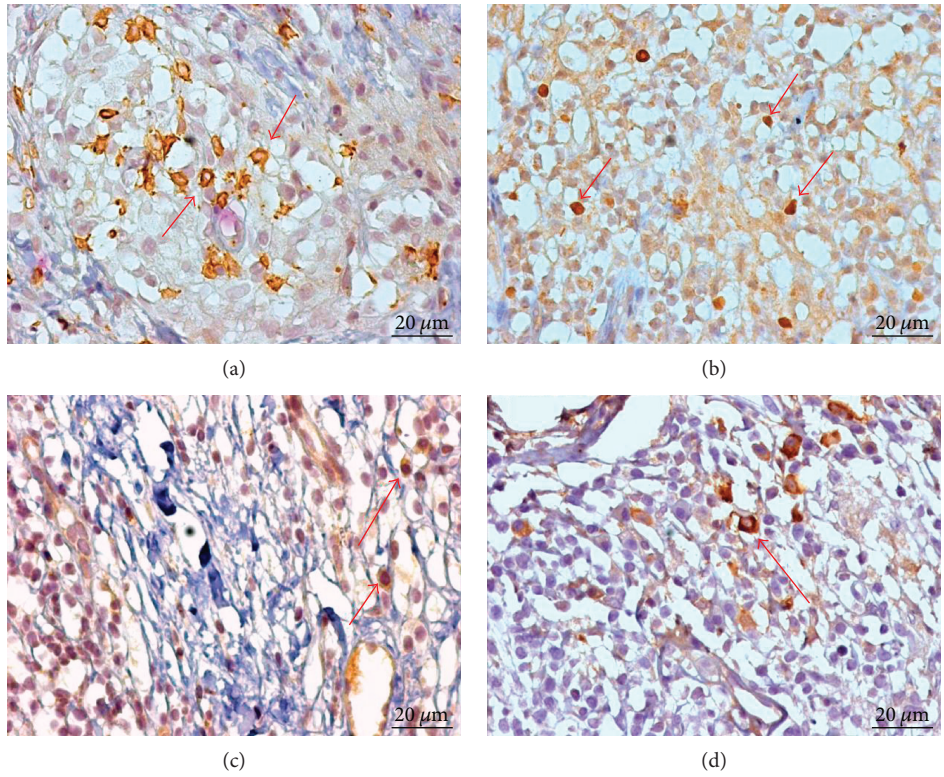


Figure 2: Immunohistochemistry of the skin of patients with nonulcerated or atypical cutaneous leishmaniasis evidenced in brown colour for $CD4^+$ T lymphocytes (a); $FoxP3^+$ cells (b); IL-10^+ cells (c), and TGF-β^+ cells (d) (×400). The red arrows signal immunostained cells for the different markers.

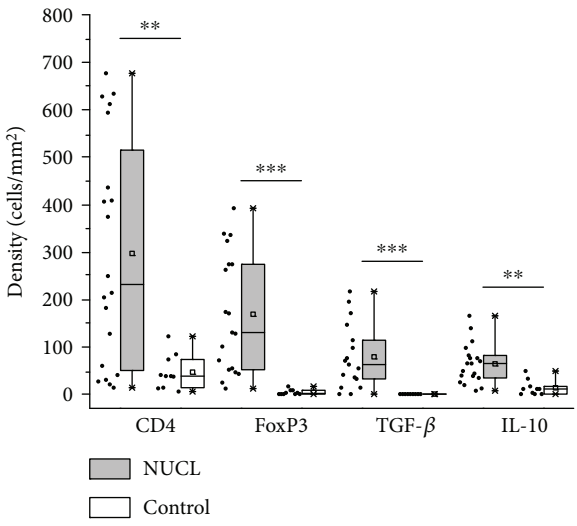


Figure 3: A dot plot showing the distribution and a box plot showing the median, mean, quartile, maximum, and minimum values for the number of positive cells per square millimetre for CD4, FoxP3, TGF-β, and IL-10 markers in the skin biopsies of nonulcerated cutaneous leishmaniasis (grey) and healthy individuals (white). $^{**}p < 0.01$; $^{***}p < 0.001$ of cellular density between NUCL and healthy controls.

exam, suggestive forms of the parasite were observed in only 55% of the cases in histological sections stained by HE or immunohistochemistry. These histopathological aspects differ from those that have been described in the Old World for cutaneous lesions caused by other viscerotropic species, such as *Leishmania* (*L.*) *donovani* and *Leishmania* (*L.*) *infantum* [8, 9]. This reinforces the role of the parasite species in determining the clinical and immune-histopathological aspects of the infection [10].

A previous study from our group on skin lesions of atypical cutaneous leishmaniasis showed self-limiting and nonulcerated skin lesions independent of the time of evolution, which were characterized by the evidence of a high density of $CD8^+$ T lymphocytes and IFN-γ^+ cells, added to the presence of $iNOS^+$ macrophages that are rarely parasitized [11]; these results suggested that those patients had efficient cellular immune responses in the skin. However, the persistence of low tissue parasitism could be associated with a regulatory immune response that leads to a balanced cellular immune response [12].

Regulatory T lymphocytes represent a subpopulation of T lymphocytes characterized phenotypically by $CD4^+$ $CD25^+$ cells expressing transcription factor forkhead box P3 (FoxP3), essential for the control of excessive immune response against microorganisms or self-antigens. Regulatory T cells (T_{reg}) act in conjunction with effector T cells on the modulation of the cellular immune response [13–16]. Therefore, the role of T_{reg} cells is mediated by the secretion of regulatory cytokines, such as IL-10 and TGF-β, which directly affect the activity of effector T cells and antigen-presenting cells [12–14]. The production of these cytokines at the site of infection can compromise the

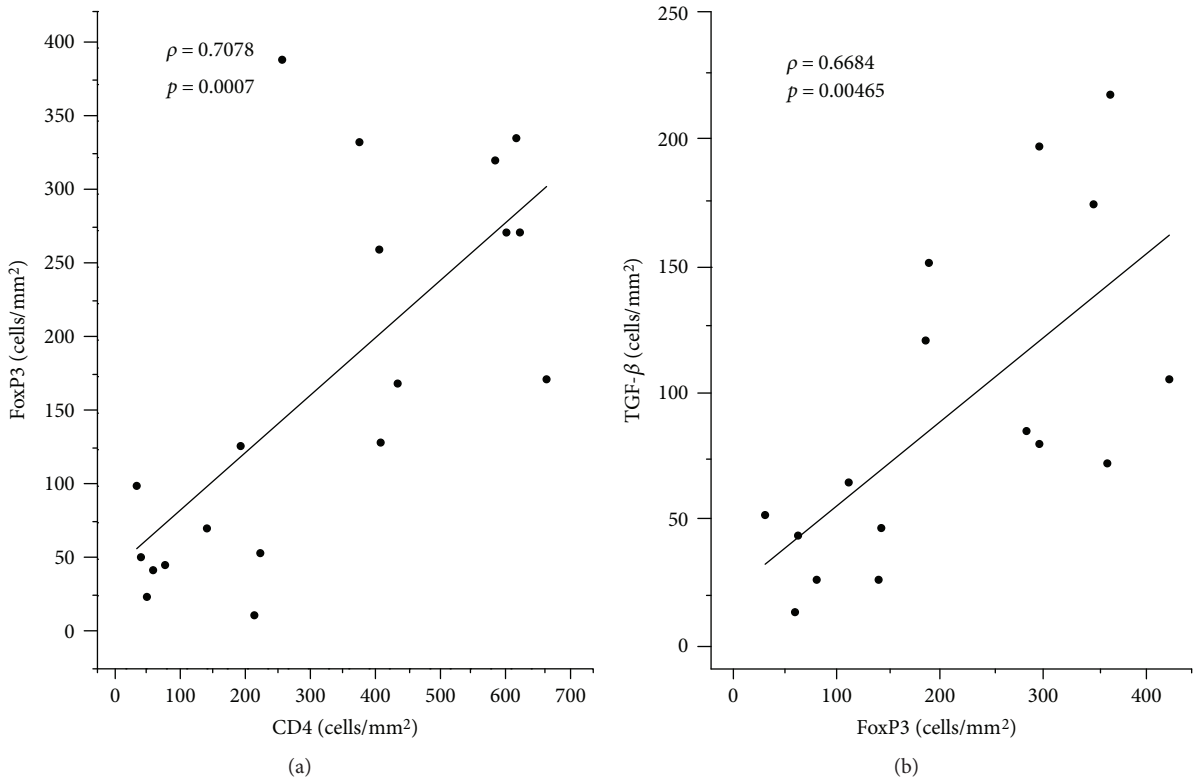


Figure 4: Graphic of dispersion showing a positive and strong correlation between the cellular density of $CD4^+$ cells and $FoxP3^+$ cells (a) and a positive and moderate correlation between $FoxP3^+$ cells and TGF-β^+ cells (b). The value of ρ is the Pearson correlation coefficient, and p is the p value.

proper proliferation of effector T cells and the production of proinflammatory cytokines, inhibiting full parasite elimination [12, 17].

Considering the morphology of lymphocytes, we estimated that approximately 10% of the lymphocytes were T_{reg} cells, characterized by $FoxP3^+$ cells in our study. In addition, a strong and positive correlation was observed between the number of $CD4^+$ T cells and the number of $FoxP3^+$ cells ($p = 0.0007$), and the ratio of FoxP3 : CD4 was six times higher in NUCL than in healthy skin, suggesting that a significant part of the $CD4^+$ T lymphocyte population was T_{reg} cells in the inflammatory infiltration in the skin lesions caused by NUCL [18].

Moreover, there was a positive and moderate correlation between the density of $FoxP3^+$ cells and the density of TGF-β^+ cells ($p = 0.00465$) but not between $FoxP3^+$ and IL-10^+ cells ($p = 0.53585$). Additionally, the ratios of TGF-β : CD4 and TGF-β : FoxP3 were higher in NUCL than in healthy skin, which did not occur with IL-10. These data suggest that in NUCL, the T_{reg} cells could regulate an effector cellular immune response, mainly through the production of TGF-β, a cytokine that depends on the environment and concentration at which it is produced to present proinflammatory or anti-inflammatory properties [19–22]. Previously, it has been shown that both T_{reg} and T effector cells are present in chronic leishmaniasis, suggesting that the persistence of *Leishmania* at the site of infection is due to the activity of T_{reg} cells, although it has been believed that the low number of parasites at the site of infection is important to produce long-lasting and protective immunity [12, 23]. Thus, these cells can control the balance of the cellular immune response established between the pathogen and its host, mediating an equilibrium that may be mutually beneficial. An imbalance in this cellular subtype may promote lesion progression and change in the immune cellular response, since $CD4^+$ $CD25^+$ IL-10^+ TGF-β^+ cells could be involved in the modulation of the effector immune response in skin lesions induced by *Leishmania* spp. [12, 17, 24]. In addition, it was demonstrated that the chronicity of the skin lesions caused by *Leishmania guyanensis* is associated with the immunosuppression due to the presence of T_{reg} cells, suggesting that T_{reg} cells could play a role in the downregulation of *Leishmania*-specific immune responses [25, 26].

The lack of correlation between $FoxP3^+$ and IL-10^+ cells suggests that other regulatory cells could be the source of IL-10, since it has been described that IL-10-producing $CD25^-$ $Foxp3^-$ T cells are involved in the pathogenesis of visceral leishmaniasis [27]. Moreover, skin lesions of patients affected by cutaneous leishmaniasis caused by *L. (V.) braziliensis* showed a stronger correlation between IL-10 expression and proinflammatory cytokines such as IFN-γ, IL-27, and IL-21, rather than with $FoxP3^+$ cells [28].

Taken together, the data obtained in this study suggest that $CD4^+$ T lymphocytes and $FoxP3^+$ T regulatory cells, as well as TGF-β^+ and IL-10^+ cells, although discrete, play an important role in the immunopathogenesis of nonulcerated

or atypical cutaneous leishmaniasis. These elements could modulate the balance in the cellular immune response, resulting in the maintenance of low tissue parasitism necessary for protective immunity that prevents the evolution of the lesion size.

Ethical Approval

This research project was approved by the Research Ethics Committee of the Master of Infectious and Zoonotic Diseases of the National Autonomous University of Honduras, (Protocol number 03-2014) and by the Research Ethics Committee of the Medical School of the University of São Paulo (CAAE: 64223917.1.0000.0065, Protocol: 1.938.092). Patients were informed about the research protocol, and those who agreed to participate signed the informed consent form.

Conflicts of Interest

The authors have no conflicts of interest concerning the work reported in this paper.

Authors' Contributions

Gabriela Venicia Araujo Flores and Carmen Maria Sandoval Pacheco equally contributed to this work.

Acknowledgments

The authors would like to thank Dr. Sara Avalos Hernandez and Dr. Mazlova Toledo for the skin biopsy procedures and Luiz Felipe Domingues Passero for the critical review of the manuscript. This research was funded by the Fundação de Amparo à Pesquisa no Estado de São Paulo (FAPESP) Grant nos. 2014/50315-0 and 2015/01154-7, Gabriela Venicia Araujo Flores Scholarship from CAPES (Social Demand) and Laboratório de Patologia de Moléstias Infecciosas (LIM50 HC-FMUSP). Marcia Dalastra Laurenti is a research fellow from the National Research Council (CNPq), Grant no. 303098/2014-7, Brazil.

References

[1] C. Ponce, E. Ponce, A. Cruz, R. Kreutzer, D. McMahon Pratt, and F. Neva, "Leishmania donovani chagasi: new clinical variant of cutaneous leishmaniasis in Honduras," *The Lancet*, vol. 337, no. 8733, pp. 67–70, 1991.

[2] A. Belli, D. García, X. Palacios et al., "Widespread atypical cutaneous leishmaniasis caused by *Leishmania* (*L.*) *chagasi* in Nicaragua," *The American Journal of Tropical Medicine and Hygiene*, vol. 61, no. 3, pp. 380–385, 1999.

[3] M. Campos-Ponce, C. Ponce, E. Ponce, and R. D. C. Maingon, "*Leishmania chagasi*/*infantum*: further investigations on *Leishmania* tropisms in atypical cutaneous and visceral leishmaniasis foci in Central America," *Experimental Parasitology*, vol. 109, no. 4, pp. 209–219, 2005.

[4] H. Noyes, M. Chance, C. Ponce, E. Ponce, and R. Maingon, "*Leishmania chagasi*: genotypically similar parasites from Honduras cause both visceral and cutaneous leishmaniasis in humans," *Experimental Parasitology*, vol. 85, no. 3, pp. 264–273, 1997.

[5] "Clima de Honduras," July 2017, http://www.clima-de.com/honduras/#Clima_deHonduras_Zona_Sur.

[6] D. S. Ridley and M. J. Ridley, "The evolution of the lesion in cutaneous leishmaniasis," *The Journal of Pathology*, vol. 141, no. 1, pp. 83–96, 1983.

[7] M. A. B. Moreira, M. C. R. Luvizotto, J. F. Garcia, C. E. P. Corbett, and M. D. Laurenti, "Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs," *Veterinary Parasitology*, vol. 145, no. 3-4, pp. 245–252, 2007.

[8] P. del Giudice, P. Marty, J. P. Lacour et al., "Cutaneous leishmaniasis due to *Leishmania infantum*. Case reports and literature review," *Archives of Dermatology*, vol. 134, no. 2, pp. 193–198, 1998.

[9] D. R. Mehregan, A. H. Mehregan, and D. A. Mehregan, "Histologic diagnosis of cutaneous leishmaniasis," *Clinics in Dermatology*, vol. 17, no. 3, pp. 297–304, 1999.

[10] F. T. Silveira, R. Lainson, C. M. De Castro Gomes, M. D. Laurenti, and C. E. P. Corbett, "Immunopathogenic competences of *Leishmania* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* in American cutaneous leishmaniasis," *Parasite Immunology*, vol. 31, no. 8, pp. 423–431, 2009.

[11] C. M. Sandoval Pacheco, *Non-Ulcerated or Atypical Cutaneous Leishmaniasis Caused by Leishmania (L.) infantum chagasi in the Municipality of Amapala, Valle, Honduras: Immunohistotopathological Characterization of Skin Lesions [Ph.D. Thesis]*, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brazil, 2017.

[12] Y. Belkaid and K. Tarbell, "Regulatory T cells in the control of host-microorganism interactions," *Annual Review of Immunology*, vol. 27, no. 1, pp. 551–589, 2009.

[13] N. Askenasy, A. Kaminitz, and S. Yarkoni, "Mechanisms of T regulatory cell function," *Autoimmunity Reviews*, vol. 7, no. 5, pp. 370–375, 2008.

[14] Y. Belkaid, "Regulatory T cells and infection: a dangerous necessity," *Nature Reviews Immunology*, vol. 7, no. 11, pp. 875–888, 2007.

[15] F. P. Carneiro, A. V. De Magalhães, M. De Jesus Abreu Almeida Couto, A. L. Bocca, M. I. Muniz-Junqueira, and R. N. Ribeiro Sampaio, "Foxp3 expression in lesions of the different clinical forms of American tegumentary leishmaniasis," *Parasite Immunology*, vol. 31, no. 10, pp. 646–651, 2009.

[16] K. M. Melo and B. T. C. Carvalho, "Células T regulatórias: mecanismos de ação e função nas doenças humanas/T regulatory cell: mechanism of action and function in human diseases," *Revista Brasileira de Alergia e Imunopatologia*, vol. 32, pp. 184–188, 2009.

[17] F. M. D. Rodrigues, G. T. Coelho Neto, J. G. P. B. Menezes et al., "Expression of Foxp3, TGF-β and IL-10 in American cutaneous leishmaniasis lesions," *Archives of Dermatological Research*, vol. 306, no. 2, pp. 163–171, 2014.

[18] G. V. Araujo Flores, *Non-ulcerated or atypical cutaneous leishmaniasis in the municipalities of Amapala (Valle) and Orocuina (Choluteca), Honduras: evaluation of the inflammatory and regulatory immune response in situ in skin lesions [Ph.D. thesis]*, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brazil, 2017.

[19] P.-Y. Mantel and C. B. Schmidt-Weber, "Transforming growth factor-beta: recent advances on its role in immune tolerance,"

in *Suppression and Regulation of Immune Responses*, pp. 303–338, Humana Press, Totowa, NJ, USA, 2010.

[20] N. L. McCartney-Francis and S. M. Wahl, "TGF-β and macrophages in the rise and fall of inflammation," in *TGF-β and Related Cytokines in Inflammation*, pp. 65–90, Birkhäuser, Basel, Switzerland, 2001.

[21] F. M. Omer, J. A. L. Kurtzhals, and E. M. Riley, "Maintaining the immunological balance in parasitic infections: a role for TGF-β?," *Parasitology Today*, vol. 16, no. 1, pp. 18–23, 2000.

[22] S. M. Wahl, "Transforming growth factor beta: the good, the bad, and the ugly," *The Journal of Experimental Medicine*, vol. 180, no. 5, pp. 1587–1590, 1994.

[23] Y. Belkaid, C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks, "$CD4^+CD25^+$ regulatory T cells control *Leishmania major* persistence and immunity," *Nature*, vol. 420, no. 6915, pp. 502–507, 2002.

[24] A. P. Campanelli, A. M. Roselino, K. A. Cavassani et al., "$CD4^+CD25^+$ T cells in skin lesions of patients with cutaneous leishmaniasis exhibit phenotypic and functional characteristics of natural regulatory T cells," *The Journal of Infectious Diseases*, vol. 193, no. 9, pp. 1313–1322, 2006.

[25] E. Bourreau, C. Ronet, E. Darcissac et al., "Intralesional regulatory T-cell suppressive function during human acute and chronic cutaneous leishmaniasis due to *Leishmania guyanensis*," *Infection and Immunity*, vol. 77, no. 4, pp. 1465–1474, 2009.

[26] D. Rodriguez-Pinto, A. Navas, V. M. Blanco et al., "Regulatory T cells in the pathogenesis and healing of chronic human dermal leishmaniasis caused by *Leishmania* (*Viannia*) species," *PLoS Neglected Tropical Diseases*, vol. 6, no. 4, article e1627, 2012.

[27] S. Nylén, R. Maurya, L. Eidsmo, K. D. Manandhar, S. Sundar, and D. Sacks, "Splenic accumulation of IL-10 mRNA in T cells distinct from $CD4^+CD25^+$ (Foxp3) regulatory T cells in human visceral leishmaniasis," *The Journal of Experimental Medicine*, vol. 204, no. 4, pp. 805–817, 2007.

[28] D. L. Costa, T. M. Cardoso, A. Queiroz et al., "Tr-1–like $CD4^+CD25^-CD127^{-/low}FOXP3^-$ cells are the main source of interleukin 10 in patients with cutaneous leishmaniasis due to *Leishmania braziliensis*," *The Journal of Infectious Diseases*, vol. 211, no. 5, pp. 708–718, 2015.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 6587296, 13 pages
https://doi.org/10.1155/2018/6587296

Review Article

The Immunoregulation of Th17 in Host against Intracellular Bacterial Infection

Yonghong Li, Chaojun Wei, Hui Xu, Jing Jia, Zhenhong Wei, Rui Guo, Yanjuan Jia, Yu Wu, Yuanting Li, Xiaoming Qi, Zhenhao Li, and Xiaoling Gao

The Institute of Clinical Research and Translational Medicine, Gansu Provincial Hospital, Lanzhou 730000, China

Correspondence should be addressed to Xiaoling Gao; gaoxl008@hotmail.com

Received 16 December 2017; Accepted 4 February 2018; Published 19 March 2018

Academic Editor: Minggang Zhang

Copyright © 2018 Yonghong Li et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

T helper 17 cells (Th17) constitute a distinct subset of helper T cells with a unique transcriptional profile (STAT3, RORγ, and RORα), cytokine production pattern (IL17 family), and requirement of specific cytokines for their differentiation (TGF-β, IL6, IL21, and IL23). Recent studies involving experimental animals and humans have shown that Th17/IL17 plays a crucial role in host defense against a variety of pathogens, including bacteria and viruses. The underlying mechanisms by which Th17 performs include dendritic cell (DC) regulation, neutrophil recruitment, Th1 modulation, and T regulatory cell (Treg) balance. In recent years, researchers have generated an accumulating wealth of evidence on the role of Th17/IL17 in protective immunity to intracellular bacterial pathogens, such as *Mycobacterium tuberculosis* and *Chlamydia trachomatis*, which are one of the most important pathogens that inflict significant socioeconomic burden across the globe. In this article, we reviewed the current literature on the functions and mechanisms by which Th17/IL17 responds to intracellular bacterial infections. A better understanding of Th17/IL17 immunity to pathogens would be crucial for developing effective prophylactics and therapeutics.

1. Background

The current field of medicine was marked not only by an increase in the endemism of socially significant infectious diseases but also by an action taken to fight against them globally. Despite active antiepidemic actions, mass vaccination campaigns, and facilities of etiotropic therapy, infectious diseases still challenge the whole world. Understanding the essence of infectious pathogenic factors of diseases at the cellular and molecular level will allow us to form a holistic view of the anti-infectious immune reactivity.

It is estimated that over one billion patients are infected with intracellular bacteria that infect and replicate inside host cells, which may be facultative or obligatory. Facultative intracellular bacteria include *Listeria monocytogenes*, *Salmonella* spp., *Francisella* spp., and *Legionella* spp., while obligate intracellular bacteria, such as *Chlamydia* spp., generally require a host cell for survival and replication. It is believed that the acquired resistance against intracellular bacteria depends on $CD8^+$ T cells. However, current scientific and technological development has deepened our understanding of host immunity against the intracellular bacteria.

After bacterial invasion, innate immunity provides the initial acute inflammatory response to microorganisms to prevent, control, and eliminate the infection and further modulate and stimulate adaptive immune responses. The acute inflammatory response is generally self-limiting and results in tissue repair, while the persistent inflammatory stimuli or dysregulation of immune mechanism results in chronic inflammation, recognized as a different kind of cytokine/chemokine secretion to activate and attract immune cells into invasive sites. Antigen-presenting cells, like DC and macrophage, are central players in the immune response. After activation, they migrate to lymphoid organs to present antigen to naive T cells and initiate Th cell differentiation [1, 2]. Classically, Th1 response (IL12/IFN-γ) is crucial for host defense against intracellular infection by activating cellular immunity to kill bacterial and infected cells [3, 4]. Current advances in the understanding of intracellular bacterial infection indicate that immune response is more complex

than the Th1/Th2 paradigm [5]; the recent recognition of Th17 cells has provided new insights into the mechanisms that are important in antimicrobial host defense [6–8].

Th17 cells potentially induce tissue damage and have been associated with many autoimmune diseases and extracellular pathogen infection [9–11]. But several lines of evidence suggested that Th17 cells are also required for host defense against intracellular bacterial infection, such as *L. monocytogenes* [12], *M. tuberculosis* [13–17], *Chlamydia* [18], *Salmonella* [19, 20], *Mycoplasma pneumoniae* [21], and *Leishmania donovani* [22]. Indeed, mice deficiency in both IL23 and IL12 are more susceptible to *M. tuberculosis* and *Toxoplasma gondii* infection compared to the IL12 knockout mice.

Th17 differentiation depends on the steroid receptor-type nuclear receptor (RORγt), which is induced by the IL6 and IL23 through activation of signal transducer and activator of transcription (STAT3) [23]. IL21 can promote Th17 cell differentiation, survival, and expansion. In addition, IL23, originally regarded as the key Th17 inducer, is only required for its expansion and maintenance [24]. In addition to STAT3 and RORγ, many other transcript factors play a critical role in Th17 differentiation, including basic leucine zipper ATF-like transcript factor (BATF), IRF4, fos-related antigen 2 (FOSL2), and RORα [25–29]. Th17 cells are reciprocally related to FoxP3$^+$ Tregs. The presence of a high dose of TGF-β activates the transcription factor FoxP3 in naïve T cells and thereby promotes Treg, whereas the presence of IL6 suppresses FoxP3. It, combined with TGF-β, induces RORγt and leads to Th17 differentiation. Thus, the balance between Th17 cells and FoxP3$^+$ Tregs is mediated by the antagonistic interaction of the transcription factors FoxP3 and RORγt [30]. A recent study has added further complexity to the Th differentiation. It indicated that the CD4$^+$ T cells could end with continuous cell fates, rather than a limited number of distinct phenotypes, when exposed to numerous combinations of cytokines [31].

Th17 cells are named from IL17 secretion by the Th17. The activation of Th17 cells results in a large amount of inflammatory cytokine production, such as IL17A, IL17F, IL21, IL22, and CCL20 [11, 32]. IL17 acts as the most critical biological effector of Th17. IL17 increases iNOS production and induces the expression of granulocyte macrophage colony-stimulating factor, IL1β, IL6, IL8, TNF, and several chemokines. IL17A and IL17F have a high similarity in sequence and share many biologic properties. Along with IL17, Th17 cells also produce IL22, IL26, and GM-CSF. IL22 and IL26 are members of the IL10 family with significantly different biological activities despite their similar structure. IL22 and IL26 can support tissue reactions of innate immunity and simulate the production of IFNγ and secretion of antimicrobial peptides, including acute phase proteins, such as serum amyloid A, A1-antichymotrypsin, and haptoglobin. Furthermore, some study shows that IL22 is a potent stimulator of Th2 responses and can directly upregulate the expression of epithelial-derived type 2 cytokines, such as IL-33, TSLP, and GRP, thus promoting a strong Th2-biased systemic immune response [33]. In contrast to IL17 and TNFα, rather work on immunocompetent cells, IL22 acts essentially on epithelial cells and hepatocytes. It is known that IL26 is involved in local mucosal immunity. Monocytes stimulated with IL26 promote Th17 cell development. GM-CSF is produced by Th17 cells to activate macrophages and play a role in immunity against anti-intracellular pathogen [34].

The biological activities of IL17 depend on their binding to its multimeric receptors [35]. The IL17 receptor family contains at least five members: IL17RA, IL17RB, IL17RC, IL17RD, and IL17RE [36]. To date, IL17A and IL17F have been shown to form either homodimers or heterodimers that can bind to the IL17RA and IL17RC receptor complex to activate downstream signaling cascades, whereas IL17E is believed to signal though the IL17RA and IL17RB receptor complex to activate its downstream pathways [37]. At present, the IL17RA and IL17RE heterodimer is considered the functional receptor for IL17C. And IL17RB has been suggested to be a receptor for IL17B [38]. Moreover, IL17RD was recently found to be a positive component in IL17 signaling and a negative suppressor for TLR signaling [39, 40]. The differences in the cytokine-receptor combination largely shape the functional diversity of this family of cytokines at distinct barrier surfaces. The necessary cytokines involved in the Th17 differentiation in mice and human are not identical. TGF-β and IL6 are required for mouse Th17 development, whereas human naive T cells differentiate into Th17 cells in the presence of IL1β, IL23, and possibly TGF-β [41]. The cooperation of all inflammatory factors potentiates tissue inflammation, and the consequences of host immune responses depend on the pathogens.

The IL17 production is multicellular in origin. $\gamma\delta$ T cells have been found to contribute to the early production of IL17 in a murine model of some intracellular bacterial infection, like *M. tuberculosis* [42], *M. bovis*, *BCG* [15], *Listeria monocytogenes* [43, 44], *S. enterica* [45], and *S. typhimurium* [46]. Even some studies suggested that dominant cellular source for IL17 production is $\gamma\delta$ T cells, rather than Th17 [42, 44]. Furthermore, a $\alpha\beta$ TCR$^+$ CD4$^-$CD8$^-$ double negative T cell population which produced IL17 has been found in *L. monocytogenes* [43] and *F. tularensis* LVS infection [47]. Invariant natural killer T (iNKT) cells were able to produce IL17 after stimulated with lipopolysaccharide [48]. iNKT cells have been reported to secrete higher quantities of IL17, in addition to IFNγ, during *Chlamydia pneumoniae* lung infections [49]. Early production of IL17 may amplify the development of Th17 response in adaptive immunity [50]. The mechanisms behind it have not been elucidated even both paracrine and/or autocrine promotion of IL17 production are suggested [51].

Several studies have focused on the role of Th17/IL17 in infectious and noninfectious diseases, while little information is available on the contribution of IL17 and Th17 to the immunopathogenesis of intracellular bacterial infections in humans. In the present review, we provided an overview of the advances of the roles and cellular mechanisms of Th17/IL17 in the host immunity against the intracellular bacterial infections (the major characteristics of pathogenic intracellular bacterial species, as well as the Th17/IL17 functions, were summarized in Table 1). A better understanding of immune

Table 1: The major characteristics of pathogenic intracellular bacterial species.

Serial number	Bacterial species	Gram staining	Facultative/ obligatory	Diseases	Function of Th17/IL17
1	*M. tuberculosis*	Positive	Obligatory	Pulmonary infection, pleurisy, tuberculous pericarditis, bone tuberculosis, tubercular meningitis, tuberculous arthritis	(i) Recruit neutrophils, macrophages, Th1 cells and IFNγ-producing cells (ii) Accelerate Th1 memory response
2	*Listeria monocytogenes*	Positive	Facultative	Septicemia, meningitis, monocytosis	(i) Promote adaptive CTL responses (ii) Enhance DC cross-presentation (iii) Accumulate innate neutrophils
3	(i) *Chlamydia trachomatis* (ii) *Chlamydia pneumoniae*	Negative	Obligatory	Pelvic inflammatory disease, trachoma, pneumoniae	Promote DC functions
4	(i) *Salmonella enterica* serovar Enteritidis (ii) *Salmonella typhimurium*	Negative	Facultative	Typhoid fever, paratyphoid fever, Enteritidis	IL23 is required for protection against the sublethal doses of S. Enteritidis
5	*Francisella tularensis*	Negative	Facultative	Tularemia	(i) Regulate the IL12-Th1 cell pathway (ii) Induce IL12 and INF-γ production

complexity will contribute to the identification of disease/resistance biomarkers and influence the development of vaccines and immunotherapies for intracellular bacteria.

2. Th17/IL17 in Intracellular Bacterial Infection

2.1. M. Tuberculosis. It has been shown that Th17 is involved in the immune response to *M. tuberculosis* [16, 52–54]. However, the exact role of IL17 in the *Mtb* infection is still unclear. It seems like the roles of Th17 are dependent on the stage of infection, bacteria strains, or its burden. And Th17 response is dispensable for protection if predominant Th1 response is present in the primary *Mtb* infection [53, 55]. In the early developmental stage of initiating a protective immune response during *Mtb* infection, Th17 cells facilitate the recruitment of neutrophils, macrophages, and Th1 cells to the area of inflammation and participate in the control of the infection process [56]. Umemura et al. showed that Th17 is critical not only in the early activation of lung neutrophils but also in the development of Th1 responses in *Mtb* infection [15]. In addition, Khader et al. found that Th17 induced the expression of CXCl9, CXCL10, and CXCL11 chemokines, recruits IFNγ-producing cells, and thus ultimately restricts the reproduction of mycobacteria in macrophage in BCG vaccination model [16]. The relatively increased levels of specific cytokines such as IL6, IL21, IL1β, and TNFα, produced by mycobacteria-infected cells, may act as cofactors for Th17 differentiation [10, 57, 58].

In other studies, on the contrary, some scientists believe that Th17 response has rather pathological than protective effects because there is a connection between the progression of pulmonary *Mtb* and the hyposecretion of IL17. IL17 appeared to enhance *Mtb* dissemination from primary pulmonary infection [53]. While, at secondary disease sites, IL17 neutralized mice had less granulocyte in the lungs and resulted in less bacterial load in the spleen. Their observations suggested that IL17 impaired the host's ability to control *Mtb* infection [59, 60]. In contrast, mice infected with BCG benefited from IL17 protection [61]. One study emphasized the role of IL17 in the granuloma formation in the BCG-infected lung. They found that IL17A$^{-/-}$ mice showed a normal level of nascent granuloma formation on day 14 but failed to develop mature granulomas on day 28 after the BCG infection in the lung. The observation implies that IL17A is required for the maturation of granuloma from the nascent to the mature stage. Furthermore, IL17A KO mice had an impaired protective response to virulent *Mtb*. So, they suggested that IL17A plays a critical role in the prevention of *Mtb* infection through the induction of mature granuloma formation [59]. In addition, IL17 was necessary for accelerated Th1 memory response and provided protection in BCG vaccinated mice [16]. IL17-produced cells responded quickly and populated the lung. That signaling was necessary for the trafficking of Th1 cells to the lungs [16]. IL17 seems important in maintaining a long lasting immune response [62]. However, recent studies showed that repeated BCG vaccination after *Mtb* infection resulted in increased IL17 production, which was responsible for the influx of granulocytes and neutrophils and lung tissue damage [63, 64]. All the data suggest that more efforts are necessary to explore the mechanisms behind the discrepancy of the role of Th17/IL17 in different *Mtb* strain infections/vaccinations.

2.2. Listeria monocytogenes. L. monocytogenes is a facultative intracellular bacterium that is one of most virulent foodborne pathogens. In addition, *L. monocytogenes* has been widely used as a model organism to illustrate the host immunity for intracellular bacterial infection. It has been shown that

IL17A- and IL17A-producing $\gamma\delta$T cells had a beneficial effect against intracellular *L. monocytogenes* infection, not only by expansion and accumulation of innate neutrophils but also by promoting adaptive CTL responses through enhancing DC cross-presentation [44, 65, 66]. IL23 signaling controls the balance between Th1 and Th17 responses. And IL23/IL17 axis is required for an optimal immune response against *L. monocytogenes* infection [67]. IL17R$^{-/-}$ and IL23$^{-/-}$ mice are more susceptible to *L. monocytogenes* infection [67, 68]. In addition, administration of exogenous rIL17A [67] or adoptive transfer of IL17-producing cells [43] reduced bacterial burden in the liver of *L. monocytogenes*-infected mouse [67]. All these data support that IL17 provides a protective immunity against *L. monocytogenes*. However, in a less virulent strain infection, the host may control bacterial growth in a fashion independent of IL17A [69].

2.3. Chlamydia. *Chlamydia trachomatis* (*Ct*) is a gram-negative pathogen which causes various diseases, including cervicitis, pelvic inflammation, ectopic pregnancy, sterility, pneumoniae, and trachoma [70]. One human study showed that in *Ct* infection, both IL23 and IL17 production were dramatically increased compared to uninfected patients and both cytokines actively participate in all processes of host defense against infection [71]. An in vivo study showed that IL23-deficient mice exhibited normal susceptibility to infection and oviduct pathology. IL23 was required for the development of a *Chlamydia*-specific Th17 response in the lymph nodes and for production of IL17 in the genital tract. It is likely that IL23 plays a minimal role in the pathogenesis of *Chlamydia* infection in the mouse model [72]. Our work confirmed that enhanced IL17 production and Th17 expansion in *Ct* infection was critical for host defense against *Ct* infection. It suggested a significant detrimental impact of in vivo IL17 neutralization by anti-IL17 mAb on disease course, immune response, and dendritic cell (DC) functions. The DC from IL17-deficient mice showed lower CD40 and MHC II expression and IL12 production [18]. Our findings have been supported by the other studies [73, 74]. However, another *Chlamydia* strain, *C. pneumoniae*, has been found which impaired IL17 signaling pathway through inhibiting Act1 recruitment to the IL17R which prevented NFκB activation [75].

2.4. Salmonella enterica. *Salmonella enterica* is a gram-negative bacteria species, including *Salmonella enterica* serovar Enteritidis (S. Enteritidis) and *Salmonella typhimurium*, two most abundant serotypes that cause gastroenteritis or systemic infection in human [76]. IL12/IFNγ and B cells contribute to host protective immunity to *Salmonella* [77, 78]. IL17 production is enhanced shortly after the *S. enterica* infection in mice model, but IL17-associated response is dispensable in the presence of an effective Th1 response. In contrast, in the absence of Th1 responses, IL23-dependent IL22 is required for protection against the sublethal doses of S. Enteritidis, but not IL17A [79, 80], while another exquisite study showed that Th17A were important for orchestrating early inflammatory responses during *S. typhimurium* colitis [19, 76].

2.5. Francisella tularensis. *F. tularensis*, a causative agent of tularemia, is ranked as a category A agent of bioterrorism by the US Center for Disease Control [81]. Inhalation is extremely dangerous and is most likely the route of bioterrorism because low doses of airborne bacteria could cause severe diseases [82, 83]. Numerous studies confirmed that IFNγ and Th1 cell responses are important for host control of *F. tularensis* infection and implicate IL17 in the regulation of Th1 cell immunity against *Francisella tularensis* [84, 85]. Further study showed that the IL23/Th17 pathway regulated the IL12/Th1 cell pathway and was required for protective immunity against *F. tularensis* live vaccine strain. Furthermore, the study found that IL17A induced IL12 production in dendritic cells and mediated Th1 responses. And IL17A also induced IL12 and INFγ production in macrophages and mediated bacterial killing [86]. So, Th17 cells play a pivotal role in immunity to *F. tularensis* infection.

3. Mechanisms of Th17/IL17 in the Intracellular Bacterial Infection

3.1. Immunomodulation of DC. Dendritic cells are professional APCs in priming CD4$^+$ and CD8$^+$ T cells [87]. After stimulation, DC could upregulate both IL17 receptor expression and the Th17 responses [50]. IL17 promoted DC differentiation through upregulating cell surface costimulatory molecule expression, such as CD40, CD80, CD86, and MHC class II, which have been reported in the organ allograft rejection [88] and airway inflammation [89, 90]. On the other hand, an exquisite study showed that mammalian sterile 20-like kinase 1 (MST1) signaling from DCs negatively regulated Th17 differentiation. MST1 deficiency in DCs increased IL17 production by CD4$^+$ T cells, whereas ectopic MST1 expression in DCs inhibited it. Notably, MST1-mediated DC-dependent Th17 differentiation regulated experimental autoimmune encephalomyelitis and antifungal immunity. Mechanistically, MST1-deficient DCs promoted IL6 secretion and regulated the activation of IL6 receptor α/β and STAT3 in CD4$^+$ T cells in the course of Th17 differentiation. Activation of the p38 MAPK signal was responsible for IL6 production in MST1-deficient DCs. Thus, the results defined the DC MST1-p38 MAPK signaling pathway in directing Th17 differentiation [91] (Figure 1).

Our lab investigated the effect of IL17 on the maturation of DC in the intracellular *C. muridarum* infection [18]. The DC isolated from IL17-neutralized mice showed lower CD40 and MHC II expression and IL12 production, but higher IL10 production compared with those from sham-treated mice. In in vitro DC-T cell coculture systems, DC isolated from IL17-neutralized mice showed less maturation and induced higher IL4, but lower IFNγ production by Ag-specific T cells than by those from sham-treated mice in cell priming and reaction settings. Adoptive transfer of DC isolated from IL17-neutralized mice, unlike those from sham-treated mice, failed to protect the recipients against challenge infection. These findings provided evidence that IL17/Th17 can modulate DC function and thus provide protection against *Cm*.

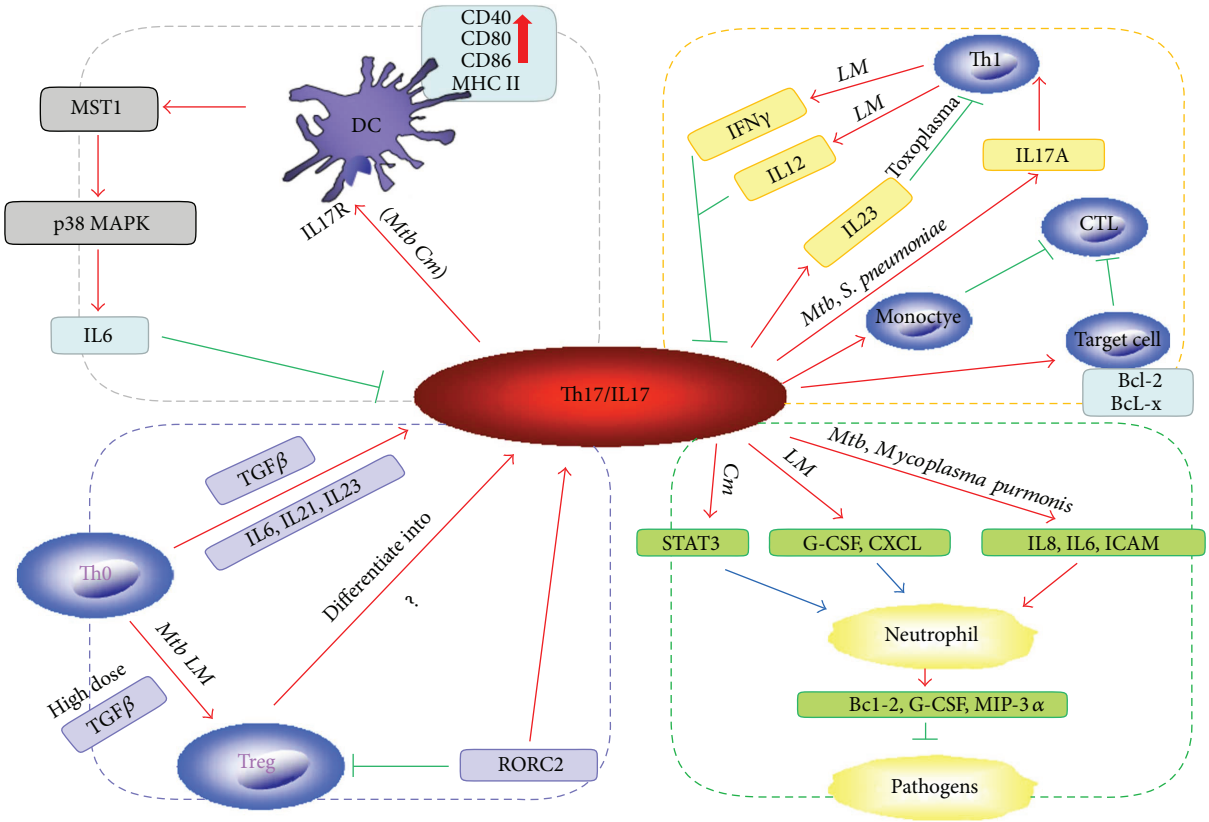


Figure 1: Schematic depiction of Th17/IL17 immunoregulation mechanisms.

Mtb vaccine study based on liposome demonstrated that Ag85B-ESAT-6/CAF01 can induce protective Th17 and Th1 immune response through prolonged DC uptake and activation [92]. Ag85B-ESAT-6/CAF01 vaccination dramatically decreased the post challenge bacterial growth of BCG and induced strong Th1 and Th17 responses in neonatal and adult groups. DC in the draining LN associated with protection were more mature regarding the high expression of CD40 and CD86, and the activated DC were recovered several days after immunization [92]. However, they did not directly examine the relationship of IL17 and maturation of DC in their model. Another study directly showed that *Mtb* induced monocyte-derived human DC maturation through increasing the expression of CD80, CD86, CD40, CD83, HLA-DR, and HLA-I, as well as cytokine production including IL23. Dectin-1 molecule engaged on DC promotes Th17 response, where DC-SIGN and MR costimulation limited Th17 generation and favored Th1 responses [93, 94].

In virulent *L. monocytogenes* infection, Xu et al. found that IL17A could upregulate the MHC class I molecular H2-K^b expressed on DC while no effects on CD40, CD80, CD86, and MHCII. They analyzed the DC phenotype and function in IL17A-deficient mice after *LM* infection. The data showed that the absolute number of DC, especially $CD8^+$ DC as the major DC subset contributing to the cross-presentation [95], was not changed. However, DC deficiency in IL17A signaling lost the potential to promote OT-I T cell activation and proliferation [44]. They implied in their study that IL17A, instead of IL17F, can enhance cross-presentation of DC in vivo and in vitro [44].

Cytokine production is an important characteristic of DC. Lin et al. compared cytokine production in *F. tularensis*-infected DC. They found that IL17A-treated *F. tularensis* LVS-stimulated BMDCs resulted in the significantly enhanced IL12 production in comparison to *F. tularensis* LVS treatment alone. However, IL17F and IL22 treatment did not impact IL12 production by the BMDC [86]. Using IL17A neutralized antibody experiment also supported that IL17A was important to promote IL12 produced by DC in *Cm* infection [18, 96].

3.2. Immunoregulatory Function of Th17. Th17 lymphocytes perform an immunoregulatory function, which produce a unique range of cytokines and chemokines as mentioned before. Besides, Th17 also induces the production of proinflammatory cytokines (TNFα, IL1β, G-CSF, and IL6) by macrophage and expression of CC and CXC chemokine receptors. Therefore, Th17 contributes to the recruitment and expansion of cells of innate immunity. Of all cytokines, IL17 more strongly stimulates the production of human Bd2, G-CSF, and MIP-3α, known to be the main humoral components of the innate immunity of the respiratory tract. It provides effective protection against pathogens. The Bd2 and MIP-3a highly expressed on neutrophil, which was migrating from peripheral blood into the tissue and backwards, were activated in response to IL17A [97, 98] (Figure 1).

3.2.1. Recruitment of Neutrophil. The crosstalk between Th17 and neutrophils has been investigated in many diseases. The fact that transfer of IL17 cDNA to the mice liver or intraperitoneal injection of IL17 in mice increased neutrophil recruitment [99] suggested that IL17 contributed to the local neutrophil accumulation [100]. The mechanisms behind the IL17-mediated recruitment of neutrophil are not fully understood, but at least three mechanisms have been reported. Firstly, indirect chemoattraction is involved. Th17 cells or $\gamma\delta$ T cells derived IL17 mobilize neutrophils via induction of chemokine/cytokines secretion by epithelial and endothelial cells. IL17 regulates granulocyte colony-stimulating factor (G-CSF) produced by the epithelial cells and thus promotes expansion of neutrophil. Secondly, IL17 can upregulate epithelial cells expressing chemokines, like CXCL1, CXCL2, and CXCL8 [101, 102]. The biologically active CXCL8 is a strong chemoattractant for neutrophils [103]. In addition, a number of cytokines released by the Th17, such as IL8, IL6, and adhesion molecule ICAM-1, are related to the maturation and activation of neutrophils [104, 105]. And thirdly, some studies showed that IL17 inducing STAT3 activation is a necessary step in neutrophil recruitment, and STAT3 acts as a link between IL17-mediated endothelial cell activation and neutrophil recruitment [106] (Figure 1).

The direct relationship of Th17 and neutrophil recruitment in *Mtb* infection has been rarely reported. And the fact showed that both IL17 production and neutrophil recruitment have been found in *Mtb* infection. The role of neutrophil in tuberculosis infection is controversial. Neutrophil might help limit bacterial spread, but intense neutrophilia is an important factor contributing to inflammatory immunopathology [16, 53, 107]. Further study showed that during chronic infection with *Mtb*, neutrophils were recruited to the lung in two waves after intranasal infection with virulent *Mtb* or the live attenuated vaccine strain BCG. A first wave of neutrophils was swiftly recruited, followed by a subsequent adaptive wave that reached the lung together with IFNγ- and IL17A-producing T cells. Interestingly, the adaptive wave was critically dependent on the expression of IL17RA, the receptor for IL17A expressed in non-hematopoietic cells [108].

It is well known that neutrophils are required for elimination of the *L. monocytogenes* and for survival of the host. Neutrophil-deficient mice have increased bacterial loads in their spleen and liver [109, 110]. Sieve et al. demonstrated that IL17A induced in the *Mycoplasma pulmonis* infection provided a cross protection against subsequent *L. monocytogenes* infection. And this IL17A-mediated protection was mediated through increased recruitment of neutrophils [111]. IL17 facilitated recruitment of neutrophils to the infective sites due to enhanced multitude of cytokine and chemokine production, like G-CSF, GM-CSF [112], CXCL1, and CXCR2 [113]. Collectively, these data suggested that IL17 was required for optimal neutrophil recruitment and host resistance in *LM* infection. Lin also found that similar mechanisms of IL17A recruited neutrophil to the lung through induction of G-CSF in *F. tularensis* pulmonary infection model [67, 86] (Figure 1).

Our study of the interaction of Th17/IL17 on the recruitment of neutrophils in *C. Muridarum* (*Cm*) infected mice showed that IL17 neutralization induced less neutrophil inflammation but suffered more severe infections. Exogenous IL17 treatment significantly enhanced the neutrophil infiltration in the lung in response to *Cm* infection [73]. Neutrophil alone may not be efficient in controlling *Cm* pulmonary infection [114]. The exact role of neutrophil in the control of *Cm* infection is not clearly understood.

The contribution of IL17 on the neutrophil recruitment is also reported in the *S. enteria* infection. *S. enteria*-infected WT mice have a relatively higher portion of $CD11b^{+}Gr1^{+}$ cells than $IL17A^{-/-}$ mice, while the portion of CD4 T cells, CD8 T cells, macrophages, and dendritic cells have no changes [19]. Neutrophils contributed to the host resistance to S. Enteritidis, too [76, 99, 115].

3.2.2. Promoting CTL Response. Cytotoxic T cells (also known as cytotoxic T lymphocytes (CTLs)), one of T cell subsets, release perforin and/or granulysin which causes the infected cells to burst or lyse. The role of IL17 in CTL function is not fully clear. Some studies showed that IL17 could activate monocytes to express B7-H1. Consequently, the $B7\text{-}H1^{+}$ monocyte cell effectively suppressed cytotoxic T cell immunity in vitro. It suggested that IL17 selectively impaired the generation and functions of CLT [116]. A similar study found that IL17 promoted the expression of Bcl-2 and Bcl-x, and thus prevented cellular apoptosis at a much lower concentration and inhibited cytotoxic T cell function [117] (Figure 1).

3.3. Crosstalk of Th1 and Th17. As professional antigen presentation cells, DCs rapidly produce both IL12 and IL23 when stimulated with antigens [53]. IL12 is known for the ability to promote Th1, while IL23 is necessary for Th17 differentiation. IL12 is comprised of IL12p40 and IL12p35, while IL23 shares an IL12p40 subunit with IL12. IL12p40 is covalently bound to p19 subunit that is implicated in the induction of Th17. The cross regulation of these two cytokines is critical for the balance of Th1 and Th17 [118] (Figure 1).

3.3.1. IL17 Promotes Th1 Responses. IL17 induces a protective Th1 response against intracellular pathogens [16, 18, 22, 86]. Th1 cells are essential for the host to control mycobacterial replication by activating macrophage and $CD8^{+}$ cytotoxic cells [119–121]. But how Th17 influences Th1 in *Mtb* infection is still an interesting question [16]. The dynamics of Th1 and Th17 in *Mtb* infection is different. IL17 is produced very early in *Mtb* infection and BCG vaccination that IL17 recall preceded Th1 responses. And Th17 populated the lung and triggered the chemokine production that recruited IFN-γ^{+} Th1 cell, which ultimately limited bacterial growth [16]. IL17 supplement can restore the Th1 recall response in IL23-deficient mice while IL17 depletion reduced the Th1 responses.

In the absence of IL12p70, IL23 is essential for the generation of Th1 cells. There are reports showing that IL23 can compensate for the absence of IL12p70 in both *Tuberculosis*

[53] and *Toxoplasma* infection [122], that exogenous IL23 was able to control pathogen burden in IL12p40-deficient mice through promoting Th1 response. Both models suggested that compensatory IL23 response may protect the host from infection. The same was in *Cm*-infected mice model [18]. Our study showed that IL17-neutralized mice exhibited reduced Th1 antigen-specific immune responses and lower Th1-promoting cytokine production in both spleen and dLN. A significant contributing role of IL17/Th17 in enhancing type 1 cytokine responses in both CD4 and CD8T cells during *Cm* infection is supported. Another intracellular infection model also confirmed that IL23-Th17 pathway regulated the IL12-Th1 cell pathway [86]. They found that Th17/IL17 was required for protective immunity against *F. tularensis* live vaccine strain. Further mechanism investigation showed that IL17A, but not IL17F or IL22, induced IL12 production in both dendritic cells and macrophages and mediated Th1 responses. Exogenous delivery of IL17A can rescue the IFNγ levels in *F. tularensis*-infected lungs [86].

Th1 and Th17 pathways are compensatory to each other in some intracellular bacterial infection. Meeks et al. proposed that both Th1 and Th17 responses are activated in the *LM* infection even if they did not directly compare the two pathways. They demonstrated that activated IL12/IFNγ axis was essential for the macrophage activation and IL23/IL17 axis influenced the neutrophil recruitment to the infection site. They suggested the IL17 functions as a complementary, but separate, branch of the immune system to the IL12 during *LM* infection [67]. Failure of either of them can increase host susceptibility to *LM* infection [67]. Schulz et al. also suggested that Th17 complements the Th1 response which is essential for protective immunity in both human and mice in their study of the *S. enterica* infection. The IL17A-deficient mice showed reduced and delayed clearance of bacteria but had no impact on the Th1 responses [19]. A further study suggested that immunization of mice with *S. pneumoniae* induced protective immunity that depended on IL17A and $CD4^+$ T cells. However, this immunity may be short-lived since IL17A-producing $CD4^+$ effector T cells did not survive to become memory cells [123] (Figure 1).

3.3.2. Th1/Th17 Cross Regulate Each Other. Other studies also suggested that Th17 cells can negatively regulate Th1 in some infections. In BCG-infected mice, IL17 limits IL12 production while it enhances IL23 production. IFNγ can increases IL12p70 but reduce IL23 [124]. Cells with the ability to secrete IFNγ and IL17 concomitantly (T_H17/T_H1 cells) have been described in inflammatory diseases in mice [125] and in humans [126]. And Yeh et al. demonstrate that IFNγ inhibits Th17 differentiation and functions in a STAT1-dependent and Tbet-independent manner [118]. Moreover, the transition from IL17-positive cells to IFNγ-positive cells in the presence of IL12 has also been reported [127, 128]. Double-positive cells have been found recently in *Cm*-infected model [129]. Together, all these findings illustrate a biological function for IL17A in regulating Th1 cell immunity and host responses to intracellular pathogens (Figure 1).

3.4. Interaction with Regulatory T Cells (Treg). The participation of TGF-β in the differentiation of Th17 cells makes Th17 lineage development closely related to the regulatory T cells since TGF-β also promotes the $FoxP3^+$ Treg differentiation. The balance between these subpopulations of lymphocytes is established due to the antagonistic interaction of RORγt (encoded by RORC2 in humans) and FoxP3 transcription factors [130, 131]. However, the antagonistic relationship does not exclude their simultaneous expression in the CD4 T cells as demonstrated that the transcript factors for Th17 (RORγt) or Treg (FoxP3) were found coexpressed in naive $CD4^+$ T cells [130]. Reciprocal regulations of Th17 and Treg cells are controlled by the presence of specific cytokines in the differential environment [130, 132]. TGF-β is required for the expression of both FoxP3 and RORγt. A high dose of TGF-β can orchestrate Treg cell differentiation through preferably inducing FoxP3 expression in T cells and thus endow their cells with regulatory/suppressor capacity [133]. IL6 also plays a pivotal role to control FoxP3/RORγt balance, thus directing the balance between the generation of Tregs and Th17. The synergization of IL6 or IL21 and IL23 with TGF-β can relieve FoxP3-mediated inhibition of RORγt [130] and promote Th17 differentiation in both in vivo and in vitro studies [24, 132, 134–137]. It was reported that RORC2 inhibited the FoxP3 expression due to the competition with the NFAT transcription factor, which is essential for the FoxP3 gene activation [138]. Koenen et al. showed that human Treg cells were capable of differentiating into IL17-producing T cells [139]. It implied that human Treg cells not only act as a suppressor but also has additional proinflammatory functions, even mechanisms that are currently unknown. And IRAK is an important intracellular kinase to direct the differentiation of naïve $CD4^+$ T cells into Th17 or Treg [140] (Figure 1).

The interdependent regulation of Treg and Th17 cells is well characterized in some human inflammatory diseases [141, 142]. Treg may restrict the overwhelming immune response to protect the host from tissue damage caused by the effector cells. But during *Mtb* infection, Treg may be deleterious since they downregulate DC antigen presentation and macrophage activity and therefore release the *Mtb* replication control [143–145]. Th17 is activated during early *Mtb* infection. But the interaction of Treg and Th17 is inconsistent. In latent *Mtb* infection (defined by TST positivity), there is a study showing a clear inverse relationship of Treg and Th17. They found that depletion of $CD4^+CD25^+$ T cells dramatically reversed *Mtb*-specific Th17 inhibition in TST-positive but not TST-negative patients. They suggested that downregulation of Th17 responses by Treg cells played a vital role in mediating resistance to latent infection [146]. However, recent human study showed that Treg only suppressed the activated Th1 immune responses, while it had no effect in the inhibition of the proinflammatory Th17 responses in activated and latent *Mtb* infection in in vitro PBMC culture [147]. A similar conclusion has been reported in *LM* infection. IL17A was excluded to interfere with Treg cells [44].

It should be noted that data on the regulation of the differentiation of Th17 and Treg now were obtained from

animal models. In the case of infectious diseases in humans, the regulatory mechanisms of these process, in general, remain unclear or are not studied at all.

4. Conclusion

The new discoveries of the functions of Th17/IL17 in host immunity have been accumulated rapidly in the last few years. Despite the importance and functional significance of Th17 lymphocytes, their clinical significance and regulatory mechanisms in the development of anti-intracellular bacterial immunity are still not well understood. An analysis of the role of Th17 cells in the immunopathogenesis in intracellular bacterial infections is of indisputable scientific interest. Subsequently, understanding the Th17/IL17 responses and its interaction and regulation with other immune repertoire provides critical insights into the host immune defense in infectious diseases. It would facilitate the development of new effective immunomodulatory strategies for the treatment and prophylactic in bacterial infection.

Conflicts of Interest

There are no conflicts of interest to declare.

Authors' Contributions

Yonghong Li and Chaojun Wei contributed equally to this work and are co-first author.

Acknowledgments

This work was supported by the National Natural Science Foundation (Grant nos. 81360264 and 31360508) and the Health Industry Research Project of Gansu Province (Grant no. GSWSKY2017-16).

References

[1] R. L. Reinhardt, S. J. Kang, H. E. Liang, and R. M. Locksley, "T helper cell effector fates — who, how and where?," *Current Opinion in Immunology*, vol. 18, no. 3, pp. 271–277, 2006.

[2] R. Medzhitov, "Recognition of microorganisms and activation of the immune response," *Nature*, vol. 449, no. 7164, pp. 819–826, 2007.

[3] J. A. L. Flynn and J. Chan, "Immunology of tuberculosis," *Annual Review of Immunology*, vol. 19, no. 1, pp. 93–129, 2001.

[4] U. Boehm, T. Klamp, M. Groot, and J. C. Howard, "Cellular responses to interferon-γ," *Annual Review of Immunology*, vol. 15, no. 1, pp. 749–795, 1997.

[5] T. R. Mosmann and R. L. Coffman, "TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties," *Annual Review of Immunology*, vol. 7, no. 1, pp. 145–173, 1989.

[6] H. Park, Z. Li, X. O. Yang et al., "A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17," *Nature Immunology*, vol. 6, no. 11, pp. 1133–1141, 2005.

[7] L. E. Harrington, R. D. Hatton, P. R. Mangan et al., "Interleukin 17–producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages," *Nature Immunology*, vol. 6, no. 11, pp. 1123–1132, 2005.

[8] B. Stockinger, M. Veldhoen, and B. Martin, "Th17 T cells: linking innate and adaptive immunity," *Seminars in Immunology*, vol. 19, no. 6, pp. 353–361, 2007.

[9] P. R. Mangan, L. E. Harrington, D. B. O'Quinn et al., "Transforming growth factor-β induces development of the T_H17 lineage," *Nature*, vol. 441, no. 7090, pp. 231–234, 2006.

[10] K. I. Happel, P. J. Dubin, M. Zheng et al., "Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*," *The Journal of Experimental Medicine*, vol. 202, no. 6, pp. 761–769, 2005.

[11] L. A. Tesmer, S. K. Lundy, S. Sarkar, and D. A. Fox, "Th17 cells in human disease," *Immunological Reviews*, vol. 223, no. 1, pp. 87–113, 2008.

[12] N. N. Orgun, M. A. Mathis, C. B. Wilson, and S. S. Way, "Deviation from a strong Th1-dominated to a modest Th17-dominated CD4 T cell response in the absence of IL-12p40 and type I IFNs sustains protective CD8 T cells," *Journal of Immunology*, vol. 180, no. 6, pp. 4109–4115, 2008.

[13] T. M. Wozniak, A. A. Ryan, and W. J. Britton, "Interleukin-23 restores immunity to *Mycobacterium tuberculosis* infection in IL-12p40-deficient mice and is not required for the development of IL-17-secreting T cell responses," *Journal of Immunology*, vol. 177, no. 12, pp. 8684–8692, 2006.

[14] P. Paidipally, S. Periasamy, P. F. Barnes et al., "NKG2D-dependent IL-17 production by human T cells in response to an intracellular pathogen," *Journal of Immunology*, vol. 183, no. 3, pp. 1940–1945, 2009.

[15] M. Umemura, A. Yahagi, S. Hamada et al., "IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guérin infection," *Journal of Immunology*, vol. 178, no. 6, pp. 3786–3796, 2007.

[16] S. A. Khader, G. K. Bell, J. E. Pearl et al., "IL-23 and IL-17 in the establishment of protective pulmonary $CD4^+$ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge," *Nature Immunology*, vol. 8, no. 4, pp. 369–377, 2007.

[17] T. J. Scriba, B. Kalsdorf, D. A. Abrahams et al., "Distinct, specific IL-17- and IL-22-producing $CD4^+$ T cell subsets contribute to the human anti-mycobacterial immune response," *Journal of Immunology*, vol. 180, no. 3, pp. 1962–1970, 2008.

[18] H. Bai, J. Cheng, X. Gao et al., "IL-17/Th17 promotes type 1 T cell immunity against pulmonary intracellular bacterial infection through modulating dendritic cell function," *Journal of Immunology*, vol. 183, no. 9, pp. 5886–5895, 2009.

[19] S. M. Schulz, G. Kohler, C. Holscher, Y. Iwakura, and G. Alber, "IL-17A is produced by Th17, $\gamma\delta$ T cells and other $CD4^-$ lymphocytes during infection with *Salmonella enterica* serovar Enteritidis and has a mild effect in bacterial clearance," *International Immunology*, vol. 20, no. 9, pp. 1129–1138, 2008.

[20] M. Raffatellu, R. L. Santos, D. E. Verhoeven et al., "Simian immunodeficiency virus–induced mucosal interleukin-17 deficiency promotes *Salmonella* dissemination from the gut," *Nature Medicine*, vol. 14, no. 4, pp. 421–428, 2008.

[21] Q. Wu, R. J. Martin, J. G. Rino, R. Breed, R. M. Torres, and H. W. Chu, "IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense

against respiratory *Mycoplasma pneumoniae* infection," *Microbes and Infection*, vol. 9, no. 1, pp. 78–86, 2007.

[22] M. G. R. Pitta, A. Romano, S. Cabantous et al., "IL-17 and IL-22 are associated with protection against human kala azar caused by *Leishmania donovani*," *The Journal of Clinical Investigation*, vol. 119, no. 8, pp. 2379–2387, 2009.

[23] R. Stadhouders, E. Lubberts, and R. W. Hendriks, "A cellular and molecular view of T helper 17 cell plasticity in autoimmunity," *Journal of Autoimmunity*, vol. 87, pp. 1–15, 2018.

[24] L. Zhou, I. I. Ivanov, R. Spolski et al., "IL-6 programs T_H-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways," *Nature Immunology*, vol. 8, no. 9, pp. 967–974, 2007.

[25] B. U. Schraml, K. Hildner, W. Ise et al., "The AP-1 transcription factor *Batf* controls T_H17 differentiation," *Nature*, vol. 460, no. 7253, pp. 405–409, 2009.

[26] A. Brustle, S. Heink, M. Huber et al., "The development of inflammatory T_H-17 cells requires interferon-regulatory factor 4," *Nature Immunology*, vol. 8, no. 9, pp. 958–966, 2007.

[27] F. J. Quintana, A. S. Basso, A. H. Iglesias et al., "Control of T_{reg} and T_H17 cell differentiation by the aryl hydrocarbon receptor," *Nature*, vol. 453, no. 7191, pp. 65–71, 2008.

[28] M. Veldhoen, K. Hirota, A. M. Westendorf et al., "The aryl hydrocarbon receptor links T_H17-cell-mediated autoimmunity to environmental toxins," *Nature*, vol. 453, no. 7191, pp. 106–109, 2008.

[29] X. O. Yang, B. P. Pappu, R. Nurieva et al., "T helper 17 lineage differentiation is programmed by orphan nuclear receptors RORα and RORγ," *Immunity*, vol. 28, no. 1, pp. 29–39, 2008.

[30] T. Korn, A. C. Anderson, E. Bettelli, and M. Oukka, "The dynamics of effector T cells and Foxp3$^+$ regulatory T cells in the promotion and regulation of autoimmune encephalomyelitis," *Journal of Neuroimmunology*, vol. 191, no. 1-2, pp. 51–60, 2007.

[31] I. Eizenberg-Magar, J. Rimer, I. Zaretsky, D. Lara-Astiaso, S. Reich-Zeliger, and N. Friedman, "Diverse continuum of CD4$^+$ T-cell states is determined by hierarchical additive integration of cytokine signals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 31, pp. E6447–E6456, 2017.

[32] W. Ouyang, J. K. Kolls, and Y. Zheng, "The biological functions of T helper 17 cell effector cytokines in inflammation," *Immunity*, vol. 28, no. 4, pp. 454–467, 2008.

[33] H. Lou, J. Lu, E. B. Choi et al., "Expression of IL-22 in the skin causes Th2-biased immunity, epidermal barrier dysfunction, and pruritus via stimulating epithelial Th2 cytokines and the GRP pathway," *Journal of Immunology*, vol. 198, no. 7, pp. 2543–2555, 2017.

[34] M. Corvaisier, Y. Delneste, H. Jeanvoine et al., "IL-26 is overexpressed in rheumatoid arthritis and induces proinflammatory cytokine production and Th17 cell generation," *PLoS Biology*, vol. 10, no. 9, article e1001395, 2012.

[35] F. Shen and S. L. Gaffen, "Structure–function relationships in the IL-17 receptor: implications for signal transduction and therapy," *Cytokine*, vol. 41, no. 2, pp. 92–104, 2008.

[36] S. L. Gaffen, "Structure and signalling in the IL-17 receptor family," *Nature Reviews Immunology*, vol. 9, no. 8, pp. 556–567, 2009.

[37] X. Song and Y. Qian, "IL-17 family cytokines mediated signaling in the pathogenesis of inflammatory diseases," *Cellular Signalling*, vol. 25, no. 12, pp. 2335–2347, 2013.

[38] Y. Shi, S. J. Ullrich, J. Zhang et al., "A novel cytokine receptor-ligand pair. Identification, molecular characterization, and in vivo immunomodulatory activity," *The Journal of Biological Chemistry*, vol. 275, no. 25, pp. 19167–19176, 2000.

[39] M. Mellett, P. Atzei, R. Bergin et al., "Orphan receptor IL-17RD regulates toll-like receptor signalling via SEFIR/TIR interactions," *Nature Communications*, vol. 6, no. 1, p. 6669, 2015.

[40] M. Mellett, P. Atzei, A. Horgan et al., "Orphan receptor IL-17RD tunes IL-17A signalling and is required for neutrophilia," *Nature Communications*, vol. 3, no. 1, p. 1119, 2012.

[41] F. Annunziato, L. Cosmi, F. Liotta, E. Maggi, and S. Romagnani, "Type 17 T helper cells—origins, features and possible roles in rheumatic disease," *Nature Reviews Rheumatology*, vol. 5, no. 6, pp. 325–331, 2009.

[42] E. Lockhart, A. M. Green, and J. L. Flynn, "IL-17 production is dominated by $\gamma\delta$ T cells rather than CD4 T cells during *Mycobacterium tuberculosis* infection," *Journal of Immunology*, vol. 177, no. 7, pp. 4662–4669, 2006.

[43] L. Riol-Blanco, V. Lazarevic, A. Awasthi et al., "IL-23 receptor regulates unconventional IL-17–producing T cells that control bacterial infections," *Journal of Immunology*, vol. 184, no. 4, pp. 1710–1720, 2010.

[44] S. Xu, Y. Han, X. Xu, Y. Bao, M. Zhang, and X. Cao, "IL-17A–producing $\gamma\delta$T cells promote CTL responses against *Listeria monocytogenes* infection by enhancing dendritic cell cross-presentation," *Journal of Immunology*, vol. 185, no. 10, pp. 5879–5887, 2010.

[45] S. Siegemund, N. Schutze, S. Schulz et al., "Differential IL-23 requirement for IL-22 and IL-17A production during innate immunity against *Salmonella enterica* serovar Enteritidis," *International Immunology*, vol. 21, no. 5, pp. 555–565, 2009.

[46] I. Godinez, M. Raffatellu, H. Chu et al., "Interleukin-23 orchestrates mucosal responses to *Salmonella enterica* serotype Typhimurium in the intestine," *Infection and Immunity*, vol. 77, no. 1, pp. 387–398, 2009.

[47] S. C. Cowley, A. I. Meierovics, J. A. Frelinger, Y. Iwakura, and K. L. Elkins, "Lung CD4$^-$CD8$^-$ double-negative T cells are prominent producers of IL-17A and IFN-γ during primary respiratory murine infection with *Francisella tularensis* live vaccine strain," *Journal of Immunology*, vol. 184, no. 10, pp. 5791–5801, 2010.

[48] M. L. Michel, A. C. Keller, C. Paget et al., "Identification of an IL-17–producing NK1.1^{neg} iNKT cell population involved in airway neutrophilia," *The Journal of Experimental Medicine*, vol. 204, no. 5, pp. 995–1001, 2007.

[49] A. G. Joyee, J. Uzonna, and X. Yang, "Invariant NKT cells preferentially modulate the function of CD8α^+ dendritic cell subset in inducing type 1 immunity against infection," *Journal of Immunology*, vol. 184, no. 4, pp. 2095–2106, 2010.

[50] C. E. Sutton, S. J. Lalor, C. M. Sweeney, C. F. Brereton, E. C. Lavelle, and K. H. G. Mills, "Interleukin-1 and IL-23 induce innate IL-17 production from $\gamma\delta$ T cells, amplifying Th17 responses and autoimmunity," *Immunity*, vol. 31, no. 2, pp. 331–341, 2009.

[51] D. J. Cua and C. M. Tato, "Innate IL-17-producing cells: the sentinels of the immune system," *Nature Reviews Immunology*, vol. 10, no. 7, pp. 479–489, 2010.

[52] C. Infante-Duarte, H. F. Horton, M. C. Byrne, and T. Kamradt, "Microbial lipopeptides induce the production

of IL-17 in Th cells," *Journal of Immunology*, vol. 165, no. 11, pp. 6107–6115, 2000.

[53] S. A. Khader, J. E. Pearl, K. Sakamoto et al., "IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-γ responses if IL-12p70 is available," *Journal of Immunology*, vol. 175, no. 2, pp. 788–795, 2005.

[54] A. M. Cooper, "Cell-mediated immune responses in tuberculosis," *Annual Review of Immunology*, vol. 27, no. 1, pp. 393–422, 2009.

[55] S. J. Aujla, P. J. Dubin, and J. K. Kolls, "Th17 cells and mucosal host defense," *Seminars in Immunology*, vol. 19, no. 6, pp. 377–382, 2007.

[56] A. Awasthi and V. K. Kuchroo, "Th17 cells: from precursors to players in inflammation and infection," *International Immunology*, vol. 21, no. 5, pp. 489–498, 2009.

[57] F. Gerosa, B. Baldani-Guerra, L. A. Lyakh et al., "Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells," *The Journal of Experimental Medicine*, vol. 205, no. 6, pp. 1447–1461, 2008.

[58] A. J. van Beelen, Z. Zelinkova, E. W. Taanman-Kueter et al., "Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells," *Immunity*, vol. 27, no. 4, pp. 660–669, 2007.

[59] Y. Okamoto Yoshida, M. Umemura, A. Yahagi et al., "Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung," *Journal of Immunology*, vol. 184, no. 8, pp. 4414–4422, 2010.

[60] P. S. Redford, A. Boonstra, S. Read et al., "Enhanced protection to *Mycobacterium tuberculosis* infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung," *European Journal of Immunology*, vol. 40, no. 8, pp. 2200–2210, 2010.

[61] R. Gopal, Y. Lin, N. Obermajer et al., "IL-23-dependent IL-17 drives Th1-cell responses following *Mycobacterium bovis* BCG vaccination," *European Journal of Immunology*, vol. 42, no. 2, pp. 364–373, 2012.

[62] A. Blauvelt, M. G. Lebwohl, and R. Bissonnette, "IL-23/IL-17A dysfunction phenotypes inform possible clinical effects from anti-IL-17A therapies," *The Journal of Investigative Dermatology*, vol. 135, no. 8, pp. 1946–1953, 2015.

[63] A. Cruz, A. G. Fraga, J. J. Fountain et al., "Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with *Mycobacterium tuberculosis*," *The Journal of Experimental Medicine*, vol. 207, no. 8, pp. 1609–1616, 2010.

[64] M. M. Trentini, F. M. de Oliveira, A. Kipnis, and A. P. Junqueira-Kipnis, "The role of neutrophils in the induction of specific Th1 and Th17 during vaccination against tuberculosis," *Frontiers in Microbiology*, vol. 7, p. 898, 2016.

[65] S. Hamada, M. Umemura, T. Shiono et al., "Importance of murine V$\delta1^{+}\gamma\delta$ T cells expressing interferon-γ and interleukin-17A in innate protection against *Listeria monocytogenes* infection," *Immunology*, vol. 125, no. 2, pp. 170–177, 2008.

[66] S. Hamada, M. Umemura, T. Shiono et al., "IL-17A produced by $\gamma\delta$ T cells plays a critical role in innate immunity against *Listeria monocytogenes* infection in the liver," *Journal of Immunology*, vol. 181, no. 5, pp. 3456–3463, 2008.

[67] K. D. Meeks, A. N. Sieve, J. K. Kolls, N. Ghilardi, and R. E. Berg, "IL-23 is required for protection against systemic infection with *Listeria monocytogenes*," *Journal of immunology*, vol. 183, no. 12, pp. 8026–8034, 2009.

[68] L. A. Zenewicz, G. D. Yancopoulos, D. M. Valenzuela, A. J. Murphy, M. Karow, and R. A. Flavell, "Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation," *Immunity*, vol. 27, no. 4, pp. 647–659, 2007.

[69] S. J. Aujla, Y. R. Chan, M. Zheng et al., "IL-22 mediates mucosal host defense against gram-negative bacterial pneumonia," *Nature Medicine*, vol. 14, no. 3, pp. 275–281, 2008.

[70] S. S. Witkin, E. Minis, A. Athanasiou, J. Leizer, and I. M. Linhares, "*Chlamydia trachomatis*: the persistent pathogen," *Clinical and Vaccine Immunology*, vol. 24, no. 10, pp. e00203–e00217, 2017.

[71] N. Shavlakadze and B. Gorgoshidze, "IL-17/IL-23 and chlamydia trachomatis," *Georgian Medical News*, vol. 183, pp. 45–51, 2010.

[72] L. C. Frazer, A. M. Scurlock, M. A. Zurenski et al., "IL-23 induces IL-22 and IL-17 production in response to *Chlamydia muridarum* genital tract infection, but the absence of these cytokines does not influence disease pathogenesis," *American Journal of Reproductive Immunology*, vol. 70, no. 6, pp. 472–484, 2013.

[73] X. Zhang, L. Gao, L. Lei et al., "A MyD88-dependent early IL-17 production protects mice against airway infection with the obligate intracellular pathogen *Chlamydia muridarum*," *Journal of Immunology*, vol. 183, no. 2, pp. 1291–1300, 2009.

[74] L. Chen, L. Lei, X. Chang et al., "Mice deficient in MyD88 develop a Th2-dominant response and severe pathology in the upper genital tract following *Chlamydia muridarum* infection," *Journal of Immunology*, vol. 184, no. 5, pp. 2602–2610, 2010.

[75] K. Wolf, G. V. Plano, and K. A. Fields, "A protein secreted by the respiratory pathogen *Chlamydia pneumoniae* impairs IL-17 signalling via interaction with human Act1," *Cellular Microbiology*, vol. 11, no. 5, pp. 769–779, 2009.

[76] A. M. Keestra, I. Godinez, M. N. Xavier et al., "Early MyD88-dependent induction of interleukin-17A expression during *Salmonella* colitis," *Infection and Immunity*, vol. 79, no. 8, pp. 3131–3140, 2011.

[77] P. Mastroeni, "Immunity to systemic salmonella infections," *Current Molecular Medicine*, vol. 2, no. 4, pp. 393–406, 2002.

[78] R. C. Alaniz, B. L. Deatherage, J. C. Lara, and B. T. Cookson, "Membrane vesicles are immunogenic facsimiles of *Salmonella typhimurium* that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity in vivo," *Journal of Immunology*, vol. 179, no. 11, pp. 7692–7701, 2007.

[79] S. M. Schulz, G. Kohler, N. Schutze et al., "Protective immunity to systemic infection with attenuated *Salmonella enterica* serovar Enteritidis in the absence of IL-12 is associated with IL-23-dependent IL-22, but not IL-17," *Journal of Immunology*, vol. 181, no. 11, pp. 7891–7901, 2008.

[80] B. A. Frolov, I. N. Chainikova, V. Filippova Iu, A. I. Smoliagin, T. V. Panfilova, and A. D. Zheleznova, "Mechanisms of realization of protective effect of miliacin during experimental *Salmonella* infection: effect of endotoxinemia and cytokine production," *Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii*, vol. 5, pp. 8–12, 2014.

[81] D. T. Dennis, T. V. Inglesby, D. A. Henderson et al., "Tularemia as a biological weapon: medical and public health management," *JAMA*, vol. 285, no. 21, pp. 2763–2773, 2001.

[82] M. D. Woolard, L. L. Hensley, T. H. Kawula, and J. A. Frelinger, "Respiratory *Francisella tularensis* live vaccine strain infection induces Th17 cells and prostaglandin E_2, which inhibits generation of gamma interferon-positive T cells," *Infection and Immunity*, vol. 76, no. 6, pp. 2651–2659, 2008.

[83] J. Ellis, P. C. F. Oyston, M. Green, and R. W. Titball, "Tularemia," *Clinical Microbiology Reviews*, vol. 15, no. 4, pp. 631–646, 2002.

[84] N. S. Duckett, S. Olmos, D. M. Durrant, and D. W. Metzger, "Intranasal interleukin-12 treatment for protection against respiratory infection with the *Francisella tularensis* live vaccine strain," *Infection and Immunity*, vol. 73, no. 4, pp. 2306–2311, 2005.

[85] R. de Waal Malefyt, "Interleukin-17 kick-starts T helper 1 cell differentiation," *Immunity*, vol. 31, no. 5, pp. 700–702, 2009.

[86] Y. Lin, S. Ritchea, A. Logar et al., "Interleukin-17 is required for T helper 1 cell immunity and host resistance to the intracellular pathogen *Francisella tularensis*," *Immunity*, vol. 31, no. 5, pp. 799–810, 2009.

[87] M. J. Wick and H. G. Ljunggren, "Processing of bacterial antigens for peptide presentation on MHC class I molecules," *Immunological Reviews*, vol. 172, no. 1, pp. 153–162, 1999.

[88] M. A. Antonysamy, W. C. Fanslow, F. Fu et al., "Evidence for a role of IL-17 in organ allograft rejection: IL-17 promotes the functional differentiation of dendritic cell progenitors," *Journal of Immunology*, vol. 162, no. 1, pp. 577–584, 1999.

[89] S. Schnyder-Candrian, D. Togbe, I. Couillin et al., "Interleukin-17 is a negative regulator of established allergic asthma," *The Journal of Experimental Medicine*, vol. 203, no. 12, pp. 2715–2725, 2006.

[90] H. Ait-Oufella, A. P. Sage, Z. Mallat, and A. Tedgui, "Adaptive (T and B cells) immunity and control by dendritic cells in atherosclerosis," *Circulation Research*, vol. 114, no. 10, pp. 1640–1660, 2014.

[91] C. Li, Y. Bi, Y. Li et al., "Dendritic cell MST1 inhibits Th17 differentiation," *Nature Communications*, vol. 8, article 14275, 2017.

[92] A. T. Kamath, A. F. Rochat, D. Christensen et al., "A liposome-based mycobacterial vaccine induces potent adult and neonatal multifunctional T cells through the exquisite targeting of dendritic cells," *PLoS One*, vol. 4, no. 6, article e5771, 2009.

[93] E. Zenaro, M. Donini, and S. Dusi, "Induction of Th1/Th17 immune response by *Mycobacterium tuberculosis*: role of dectin-1, mannose receptor, and DC-SIGN," *Journal of Leukocyte Biology*, vol. 86, no. 6, pp. 1393–1401, 2009.

[94] T. Lindenstrom, E. M. Agger, K. S. Korsholm et al., "Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells," *Journal of Immunology*, vol. 182, no. 12, pp. 8047–8055, 2009.

[95] J. M. M. den Haan, S. M. Lehar, and M. J. Bevan, "$CD8^+$ but not $CD8^-$ dendritic cells cross-prime cytotoxic T cells in vivo," *The Journal of Experimental Medicine*, vol. 192, no. 12, pp. 1685–1696, 2000.

[96] J. Celli and T. C. Zahrt, "Mechanisms of *Francisella tularensis* intracellular pathogenesis," *Cold Spring Harbor Perspectives in Medicine*, vol. 3, no. 4, article a010314, 2013.

[97] S. Bishu, E. Su, E. R. Wilkerson et al., "Rheumatoid arthritis patients exhibit impaired *Candida albicans*-specific Th17 responses," *Arthritis Research & Therapy*, vol. 16, no. 1, article R50, 2014.

[98] M. Nonaka, N. Ogihara, A. Fukumoto et al., "Synergistic induction of macrophage inflammatory protein-3α/CCL20 production by interleukin-17A and tumor necrosis factor-α in nasal polyp fibroblasts," *World Allergy Organization Journal*, vol. 2, no. 10, pp. 218–223, 2009.

[99] P. Schwarzenberger, V. La Russa, A. Miller et al., "IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines," *Journal of Immunology*, vol. 161, no. 11, pp. 6383–6389, 1998.

[100] K. R. Kasten, P. S. Prakash, J. Unsinger et al., "Interleukin-7 (IL-7) treatment accelerates neutrophil recruitment through $\gamma\delta$ T-cell IL-17 production in a murine model of sepsis," *Infection and Immunity*, vol. 78, no. 11, pp. 4714–4722, 2010.

[101] C. E. Jones and K. Chan, "Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene- α, and granulocyte–colony-stimulating factor by human airway epithelial cells," *American Journal of Respiratory Cell and Molecular Biology*, vol. 26, no. 6, pp. 748–753, 2002.

[102] S. L. Gaffen, "An overview of IL-17 function and signaling," *Cytokine*, vol. 43, no. 3, pp. 402–407, 2008.

[103] M. Pelletier, L. Maggi, A. Micheletti et al., "Evidence for a cross-talk between human neutrophils and Th17 cells," *Blood*, vol. 115, no. 2, pp. 335–343, 2010.

[104] U. Bank, D. Reinhold, D. Kunz et al., "Effects of interleukin-6 (IL-6) and transforming growth factor-β (TGF-β) on neutrophil elastase release," *Inflammation*, vol. 19, no. 1, pp. 83–99, 1995.

[105] M. F. Tosi, J. M. Stark, C. W. Smith, A. Hamedani, D. C. Gruenert, and M. D. Infeld, "Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: effects on neutrophil-epithelial cell adhesion," *American Journal of Respiratory Cell and Molecular Biology*, vol. 7, no. 2, pp. 214–221, 1992.

[106] S. Yuan, S. Zhang, Y. Zhuang, H. Zhang, J. Bai, and Q. Hou, "Interleukin-17 stimulates STAT3-mediated endothelial cell activation for neutrophil recruitment," *Cellular Physiology and Biochemistry*, vol. 36, no. 6, pp. 2340–2356, 2015.

[107] A. M. Cooper, L. B. Adams, D. K. Dalton, R. Appelberg, and S. Ehlers, "IFN-γ and NO in mycobacterial disease: new jobs for old hands," *Trends in Microbiology*, vol. 10, no. 5, pp. 221–226, 2002.

[108] R. Lombard, E. Doz, F. Carreras et al., "IL-17RA in non-hematopoietic cells controls CXCL-1 and 5 critical to recruit neutrophils to the lung of mycobacteria-infected mice during the adaptive immune response," *PLoS One*, vol. 11, no. 2, article e0149455, 2016.

[109] C. J. Czuprynski, J. F. Brown, N. Maroushek, R. D. Wagner, and H. Steinberg, "Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to *Listeria* monocytogenes infection," *Journal of Immunology*, vol. 152, no. 4, pp. 1836–1846, 1994.

[110] H. W. Rogers and E. R. Unanue, "Neutrophils are involved in acute, nonspecific resistance to *Listeria monocytogenes*

in mice," *Infection and Immunity*, vol. 61, no. 12, pp. 5090–5096, 1993.

[111] A. N. Sieve, K. D. Meeks, S. Bodhankar et al., "A novel IL-17-dependent mechanism of cross protection: respiratory infection with mycoplasma protects against a secondary listeria infection," *European Journal of Immunology*, vol. 39, no. 2, pp. 426–438, 2009.

[112] C. Cheers, A. M. Haigh, A. Kelso, D. Metcalf, E. R. Stanley, and A. M. Young, "Production of colony-stimulating factors (CSFs) during infection: separate determinations of macrophage-, granulocyte-, granulocyte-macrophage-, and multi-CSFs," *Infection and Immunity*, vol. 56, no. 1, pp. 247–251, 1988.

[113] Y. Ebe, G. Hasegawa, H. Takatsuka et al., "The role of Kupffer cells and regulation of neutrophil migration into the liver by macrophage inflammatory protein-2 in primary listeriosis in mice," *Pathology International*, vol. 49, no. 6, pp. 519–532, 1999.

[114] H. Bai, J. Yang, H. Qiu et al., "Intranasal inoculation of *Chlamydia trachomatis* mouse pneumonitis agent induces significant neutrophil infiltration which is not efficient in controlling the infection in mice," *Immunology*, vol. 114, no. 2, pp. 246–254, 2005.

[115] C. Johansson, M. Ingman, and M. Jo Wick, "Elevated neutrophil, macrophage and dendritic cell numbers characterize immune cell populations in mice chronically infected with *Salmonella*," *Microbial Pathogenesis*, vol. 41, no. 2-3, pp. 49–58, 2006.

[116] Q. Zhao, X. Xiao, Y. Wu et al., "Interleukin-17-educated monocytes suppress cytotoxic T-cell function through B7-H1 in hepatocellular carcinoma patients," *European Journal of Immunology*, vol. 41, no. 8, pp. 2314–2322, 2011.

[117] W. Hou, Y. H. Jin, H. S. Kang, and B. S. Kim, "Interleukin-6 (IL-6) and IL-17 synergistically promote viral persistence by inhibiting cellular apoptosis and cytotoxic T cell function," *Journal of Virology*, vol. 88, no. 15, pp. 8479–8489, 2014.

[118] W. I. Yeh, I. L. McWilliams, and L. E. Harrington, "IFNγ inhibits Th17 differentiation and function via Tbet-dependent and Tbet-independent mechanisms," *Journal of Neuroimmunology*, vol. 267, no. 1-2, pp. 20–27, 2014.

[119] P. Salgame, "Host innate and Th1 responses and the bacterial factors that control *Mycobacterium tuberculosis* infection," *Current Opinion in Immunology*, vol. 17, no. 4, pp. 374–380, 2005.

[120] I. Flesch and S. H. Kaufmann, "Mycobacterial growth inhibition by interferon-gamma-activated bone marrow macrophages and differential susceptibility among strains of Mycobacterium tuberculosis," *Journal of Immunology*, vol. 138, no. 12, pp. 4408–4413, 1987.

[121] N. V. Serbina, V. Lazarevic, and J. L. Flynn, "$CD4^+$ T cells are required for the development of cytotoxic $CD8^+$ T cells during *Mycobacterium tuberculosis* infection," *Journal of Immunology*, vol. 167, no. 12, pp. 6991–7000, 2001.

[122] L. A. Lieberman, F. Cardillo, A. M. Owyang et al., "IL-23 provides a limited mechanism of resistance to acute toxoplasmosis in the absence of IL-12," *Journal of Immunology*, vol. 173, no. 3, pp. 1887–1893, 2004.

[123] M. Pepper, J. L. Linehan, A. J. Pagan et al., "Different routes of bacterial infection induce long-lived T_H1 memory cells and short-lived T_H17 cells," *Nature Immunology*, vol. 11, no. 1, pp. 83–89, 2010.

[124] A. Cruz, S. A. Khader, E. Torrado et al., "Cutting edge: IFN-γ regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection," *Journal of Immunology*, vol. 177, no. 3, pp. 1416–1420, 2006.

[125] R. A. O'Connor, C. T. Prendergast, C. A. Sabatos et al., "Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis," *Journal of Immunology*, vol. 181, no. 6, pp. 3750–3754, 2008.

[126] F. Annunziato, L. Cosmi, V. Santarlasci et al., "Phenotypic and functional features of human Th17 cells," *The Journal of Experimental Medicine*, vol. 204, no. 8, pp. 1849–1861, 2007.

[127] G. Shi, C. A. Cox, B. P. Vistica, C. Tan, E. F. Wawrousek, and I. Gery, "Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells," *Journal of Immunology*, vol. 181, no. 10, pp. 7205–7213, 2008.

[128] Y. K. Lee, H. Turner, C. L. Maynard et al., "Late developmental plasticity in the T helper 17 lineage," *Immunity*, vol. 30, no. 1, pp. 92–107, 2009.

[129] H. Yu, X. Jiang, C. Shen et al., "*Chlamydia muridarum* T-cell antigens formulated with the adjuvant DDA/TDB induce immunity against infection that correlates with a high frequency of gamma interferon (IFN-γ)/tumor necrosis factor alpha and IFN-γ/interleukin-17 double-positive $CD4^+$ T cells," *Infection and Immunity*, vol. 78, no. 5, pp. 2272–2282, 2010.

[130] L. Zhou, J. E. Lopes, M. M. W. Chong et al., "TGF-β-induced Foxp3 inhibits T_H17 cell differentiation by antagonizing RORγt function," *Nature*, vol. 453, no. 7192, pp. 236–240, 2008.

[131] J. Du, C. Huang, B. Zhou, and S. F. Ziegler, "Isoform-specific inhibition of RORα-mediated transcriptional activation by human FOXP3," *Journal of Immunology*, vol. 180, no. 7, pp. 4785–4792, 2008.

[132] E. Bettelli, Y. Carrier, W. Gao et al., "Reciprocal developmental pathways for the generation of pathogenic effector T_H17 and regulatory T cells," *Nature*, vol. 441, no. 7090, pp. 235–238, 2006.

[133] W. Chen, W. Jin, N. Hardegen et al., "Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-β induction of transcription factor *Foxp3*," *The Journal of Experimental Medicine*, vol. 198, no. 12, pp. 1875–1886, 2003.

[134] S. Aggarwal, N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney, "Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17," *The Journal of Biological Chemistry*, vol. 278, no. 3, pp. 1910–1914, 2003.

[135] D. J. Cua, J. Sherlock, Y. Chen et al., "Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain," *Nature*, vol. 421, no. 6924, pp. 744–748, 2003.

[136] J. J. Letterio and A. B. Roberts, "Regulation of immune responses by TGF-β," *Annual Review of Immunology*, vol. 16, no. 1, pp. 137–161, 1998.

[137] T. Korn, E. Bettelli, W. Gao et al., "IL-21 initiates an alternative pathway to induce proinflammatory T_H17 cells," *Nature*, vol. 448, no. 7152, pp. 484–487, 2007.

[138] S. Burgler, P. Y. Mantel, C. Bassin, N. Ouaked, C. A. Akdis, and C. B. Schmidt-Weber, "RORC2 is involved in T cell

polarization through interaction with the FOXP3 promoter," *Journal of Immunology*, vol. 184, no. 11, pp. 6161–6169, 2010.

[139] H. J. P. M. Koenen, R. L. Smeets, P. M. Vink, E. van Rijssen, A. M. H. Boots, and I. Joosten, "Human $CD25^{high}Foxp3^{pos}$ regulatory T cells differentiate into IL-17–producing cells," *Blood*, vol. 112, no. 6, pp. 2340–2352, 2008.

[140] U. Maitra, S. Davis, C. M. Reilly, and L. Li, "Differential regulation of Foxp3 and IL-17 expression in CD4 T helper cells by IRAK-1," *Journal of Immunology*, vol. 182, no. 9, pp. 5763–5769, 2009.

[141] X. Cheng, X. Yu, Y. J. Ding et al., "The Th17/Treg imbalance in patients with acute coronary syndrome," *Clinical Immunology*, vol. 127, no. 1, pp. 89–97, 2008.

[142] K. Nistala, H. Moncrieffe, K. R. Newton, H. Varsani, P. Hunter, and L. R. Wedderburn, "Interleukin-17–producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers," *Arthritis & Rheumatism*, vol. 58, no. 3, pp. 875–887, 2008.

[143] M. Kursar, M. Koch, H. W. Mittrucker et al., "Cutting edge: regulatory T cells prevent efficient clearance of *Mycobacterium tuberculosis*," *Journal of Immunology*, vol. 178, no. 5, pp. 2661–2665, 2007.

[144] X. Chen, B. Zhou, M. Li et al., "$CD4^+CD25^+FoxP3^+$ regulatory T cells suppress *Mycobacterium tuberculosis* immunity in patients with active disease," *Clinical Immunology*, vol. 123, no. 1, pp. 50–59, 2007.

[145] L. S. Taams, J. M. R. van Amelsfort, M. M. Tiemessen et al., "Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells," *Human Immunology*, vol. 66, no. 3, pp. 222–230, 2005.

[146] S. Babu, S. Q. Bhat, N. P. Kumar, V. Kumaraswami, and T. B. Nutman, "Regulatory T cells modulate Th17 responses in patients with positive tuberculin skin test results," *The Journal of Infectious Diseases*, vol. 201, no. 1, pp. 20–31, 2010.

[147] N. D. Marin, S. C. Paris, V. M. Velez, C. A. Rojas, M. Rojas, and L. F. Garcia, "Regulatory T cell frequency and modulation of IFN-gamma and IL-17 in active and latent tuberculosis," *Tuberculosis*, vol. 90, no. 4, pp. 252–261, 2010.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 9830939, 8 pages
https://doi.org/10.1155/2018/9830939

Review Article

Intestinal Immunomodulatory Cells (T Lymphocytes): A Bridge between Gut Microbiota and Diabetes

Qingwei Li,[1,2] **Zezheng Gao**,[1,2] **Han Wang**,[3] **Haoran Wu**,[1,2] **Yanwen Liu**,[1,2] **Yingying Yang**,[1,2] **Lin Han**,[1] **Xinmiao Wang**,[1] **Linhua Zhao**,[1,4] **and Xiaolin Tong**[1]

[1]*Department of Endocrinology, Guang'anmen Hospital of China, Academy of Chinese Medical Sciences, Beijing 100053, China*
[2]*Beijing University of Chinese Medicine, Beijing 100029, China*
[3]*Shenzhen Hospital, Guangzhou University of Chinese Medicine, Guangzhou 518034, China*
[4]*Laboratory of Molecular and Biology, Guang'anmen Hospital of China, Academy of Chinese Medical Sciences, Beijing 100053, China*

Correspondence should be addressed to Linhua Zhao; melonzhao@163.com and Xiaolin Tong; tongxiaolin@vip.163.com

Received 2 November 2017; Revised 10 January 2018; Accepted 28 January 2018; Published 11 March 2018

Academic Editor: Minggang Zhang

Copyright © 2018 Qingwei Li et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Diabetes mellitus (DM) is one of the most familiar chronic diseases threatening human health. Recent studies have shown that the development of diabetes is closely related to an imbalance of the gut microbiota. Accordingly, there is increasing interest in how changes in the gut microbiota affect diabetes and its underlying mechanisms. Immunomodulatory cells play important roles in maintaining the normal functioning of the human immune system and in maintaining homeostasis. Intestinal immunomodulatory cells (IICs) are located in the intestinal mucosa and are regarded as an intermediary by which the gut microbiota affects physiological and pathological properties. Diabetes can be regulated by IICs, which act as a bridge linking the gut microbiota and DM. Understanding this bridge role of IICs may clarify the mechanisms by which the gut microbiota contributes to DM. Based on recent research, we summarize this process, thereby providing a basis for further studies of diabetes and other similar immune-related diseases.

1. Introduction

The gut microbiota is an important "organ" in the human body; it includes extensive microbes with a wide range of characteristics and has important physiological and pathological functions in the body. Studies of correlations between the gut microbiota and various diseases, such as diabetes, obesity, metabolic syndrome, liver disease, tumors, and functional constipation, have become increasingly popular, and diabetes has been a particular focus of the recent research. It is believed that changes in the gut microbiota significantly influence the onset and development of diabetes mellitus (DM), and increasing studies have evaluated the mechanisms underlying this interaction. Recent studies have shown that the gut microbiota has an impact on the immune system of animals, particularly on intestinal mucosal immunity [1]. Furthermore, extensive immunological and inflammatory responses are involved in the physiological and pathological processes of DM [2, 3]. Thus, we believe that the immune system is a potential link between the gut microbiota and DM.

Intestinal immunomodulatory cells (IICs), a kind of regulatory immune cell in the gastrointestinal system, are believed to be particularly important. IICs control immune responses and reduce inflammation [4]. The immune system is an organic whole, composed of various factors that are coordinated and strictly regulated. Immune effector cells are regulated and controlled by a series of complex, but precise, regulatory networks, and IICs have important roles in maintaining the balance of these networks. Research has shown that the gut microbiota influences IICs to alter immune responses, and by altering the immune responses, IICs influence diabetes development [2, 5]. The position of IICs is critical, and we refer to this key position as the "Bridge."

We summarize existing literature to explain how the Bridge, that is, the IICs, functions as a link between the gut

microbiota and diabetes. In this review, we focus on the intensively studied immunoregulatory cells in the T lymphocyte family, including T helper 1 cells (Th1), T helper 2 cells (Th2), T helper 17 cells (Th17), and regulatory T cells (Treg), to explore the mechanisms by which the gut microbiota affects DM and to clarify the Bridge role of IICs.

1.1. Gut Microbiota Closely Related to Diabetes. More than 1000 species of bacteria live in the human intestinal tract. Each species can also be classified into a number of subspecies, totaling over 10^{14}, which is almost 10 times greater than the number of cells in the human body. Human microbes weigh approximately 1.275 kg in total, of which those in the gastrointestinal tract account for about 1 kg. These bacteria constitute the intestinal microecosystem [6, 7]. An imbalance of the intestinal microecosystem can cause various diseases, such as functional constipation, Crohn's disease, ulcerative colitis, cirrhosis, fatty liver, senile dementia, and diabetes [8–10]. There are many kinds of intestinal microflora, of which 80% to 90% of taxa are Firmicutes and Bacteroidetes, followed by Actinobacteria and Proteobacteria [11].

Diabetes is a clinical syndrome characterized as a glucose metabolism disorder and is caused by a combination of genetic and environmental factors. Insulin deficiency and insulin dysfunction induce disorders of sugar, fat, protein, water, and electrolyte metabolism, alone or in combination, and chronic hyperglycemia is the main clinical feature [12]. As a multifactorial disease, diabetes requires multivariate treatments for a variety of risk factors. In recent years, the microorganisms in the intestine, also known as the gut microbiota, have been regarded as the main component of the human internal environment and one of the primary environmental factors that determines the improvement or deterioration of DM [13]. Studies have found that type 2 diabetes mellitus (T2DM) is closely related to human nutritional metabolism, which is also substantially influenced by the gut microbiota; thus, we hypothesized that diabetes and the gut microbiota are inextricably linked [14], and this prediction is supported by increasing studies. Further in-depth analyses may identify important indicators and targets for future personalized treatments and methods for the prevention of diabetes.

In early studies of nonobese diabetic (NOD) mice with congenital hypoglycemia, diabetes did not develop in normal conditions, but when mice were kept in sterile environments, they developed severe diabetes, and this was attributed to a lack of beneficial gut microbes [15]. In another study, compared with nondiabetic patients, Firmicutes and Clostridium species in the gut microbiota of patients with diabetes were significantly reduced, and the Bacteroides/Firmicutes ratio and *Pseudobacillus*/*Escherichia coli* ratio were positively correlated with blood glucose levels, without any relationship to weight. Moreover, β-Proteobacteria were significantly enriched and were positively related to blood glucose levels in patients with diabetes [16]. In another study, Cani et al. [3] showed that the combined use of antibiotics in obese (ob/ob) mice fed a high-fat diet resulted in an obvious alteration of the gut microbiota composition, thereby reducing the endotoxin, blood glucose, and glucose tolerance levels in the animal model, indicating that the regulation of the gut microbiota may be beneficial for improving diabetes.

In patients with type 1 diabetes mellitus (T1DM), the composition of the gut microbiota exhibits substantial changes; its alpha diversity is decreased by about 25%, indicating that the cause of T1DM is related not only to susceptibility genes but also to the internal intestinal environment [17]. In another study of the gut microbiota of sixteen 6–8-year-old Caucasian children with T1DM, compared with those of the control group, the number of *Enterobacter cloacae*, *Bacteroides*, and *Veillonella* increased significantly, the number of *Actinomyces*, *Firmicutes*, *Bifidobacterium*, and *Lactobacillus* species decreased, and the ratio of *Bacteroides* to *Firmicutes* increased [18]. Similar changes were observed in patients with type 2 diabetes. Larsen et al. found an obvious increase in β-Proteobacteria in patients with T2DM, with reductions in *Bifidobacterium*, *Firmicutes*, and *Clostridium* species; furthermore, the ratios of *Bacteroides*/*Firmicutes* and *Brevibacterium*/*Clostridium sphaeroides* were positively correlated with blood glucose levels, demonstrating that changes in the gut microbiota were closely related to the decrease in glucose tolerance [10]. Qin et al. studied fecal samples obtained from 342 Chinese patients with T2DM by intestinal genome sequencing and showed that the gut microbiota of patients with diabetes was changed and some butyric acid-producing bacteria were reduced, but pathogenic bacteria increased in frequency [12].

Additional studies have shown that the gut microbiota is closely linked to the development of diabetes and the underlying mechanism has received increasing interest. The inflammation hypothesis and energy storage hypothesis describe two major mechanisms [19–21]. Despite extensive research, these studies did not examine a particularly wide array of mechanisms and the mechanism is highly complex; accordingly, it is urgent to determine the key mediators that allow the gut microbiota to influence DM. Based on a literature review, we found that intestinal immunoregulatory cells (IICs) are the most common intermediate identified in most of the proposed mechanistic pathways. IICs are immune regulatory cells located in the human intestine, including T lymphocytes, innate lymphoid cells, macrophages, dendritic cells, and mesenchymal stem cells [22]. Among these, T lymphocytes could be classified from a functional point of view into various lineages, including Th1, Th2, Th17, and Treg cells [23]. Here, we define the IIC Bridge position and clarify the Bridge in detail, focusing on Th1, Th2, Th17, and Treg cells, which are the most extensively studied cell subsets. We also demonstrate that the Bridge concept clarifies the mechanism by which the gut microbiota affects DM and provides new ideas and targets for future research, clinical treatments, drug development, and so on.

1.2. Effects of the Gut Microbiota and Its Metabolites on Intestinal Immunomodulatory Cells

1.2.1. Effects of the Gut Microbiota and Its Metabolites on TH1/TH2 Cells. A healthy balance of Th1/Th2 cells is essential for immune regulation. IL-12 (p35-p40) and IL-4 are the central cytokines that control the differentiation of Th1 and

Th2 cells. These two cytokines induce the production of their respective T cell subsets, while suppressing the production of opposite subsets. IL-12 can promote interferon-γ (IFN-γ) and T-bet secretion by T cells and natural killer cells, and these two transcription factors induce the differentiation of Th1 cells via the signal transducer and activator of transcription 4 (STAT4) pathway. IL-4 promotes the secretion of Th2 cytokines by upregulating the expression of GATA binding protein 3 (GATA3) via the activation of the STAT6 pathway [24]. A large number of experiments have shown that the gut microbiota and its metabolites have an effect on the balance of Th1/Th2 cells in the intestinal tract. An experimental study indicated that the gram-negative anaerobic bacterium *Bacteroides fragilis* and its product polysaccharide A could promote the expression of proinflammatory cytokines (IL-12 and p40) and nitrogen oxide in antigen-presenting cells by Toll-like receptor (TLR), thereby activating NF-κB translocation and upregulating IFN-γ levels in the body. Under such conditions, the differentiation of Th1 cells was induced by the activation of the STAT4 pathway and major histocompatibility complex II (MHC II) expression [25, 26]. Pam3 of gram-positive bacteria can combine with TLR2 as a ligand and activate IFN-γ production via the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, including JNK, p38, and ERK. The upregulation of IFN-γ induced the differentiation of Th1 cells [27]. The induction of the gut microbiota is related not only to the differentiation of Th1 cells but also to that of Th2 cells; Wu and colleagues found that commensal A4 bacteria belonging to the Lachnospiraceae family, which produce an immunodominant microbiota CBir1 antigen, inhibit Th2 cell development and CBir1 could inhibit Th2 cell differentiation by inducing TGF-β production by dendritic cells [28]. In addition, recent research has indicated that yeast β-glucan, a polysaccharide, can promote the differentiation and secretory activity of Th2 cells by upregulating the expression of *GATA3* mRNA. Additionally, yeast β-glucan upregulates various additional anti-inflammatory cytokines, for example, IL-4, IL-5, IL-15, and IL-33, and downregulates proinflammatory cytokines, including IL-6, TNF-α, IL-1β, and IL-18, as well as some acute phase proteins, like chemokine C-C motif receptor 2 (CCR2), serum amyloid A3 (SAA3), and orosomucoid 2 (Orm2) [29].

1.2.2. Effects of the Gut Microbiota and Its Metabolites on Th17 Cells. Th17 cells, also known as inflammatory helper T cells, are derived from natural T cell precursors. They have independent mechanisms for differentiation and developmental regulation. Th17 cells mainly secrete IL-17, but not IFN-γ and IL-4, and the retinoid-related orphan receptor-γt (ROR-γt) is the specific transcriptional regulator of Th17 cells [30]. Recent studies have found that IL-23, TGF-β, and IL-6 could promote the differentiation and expression of Th17 cells; conversely, Th17 cell expression could be inhibited by IL-12, IFN-γ, IL-4, T-bet, and Socs3. Some research has shown that segmented filamentous bacteria (SFB) located in the ileum could induce the differentiation of Th17 cells; however, the induction of SFB is unrelated to TLR, NOD-RIP2, or ATP signaling pathways based on analyses in a model of MyD88/TRIF double-deficiency and RIP-2 mutant mice [31, 32]. In another experiment, Ivanov and colleagues found that the main mechanism by which SFB induced the differentiation of Th17 cells was the upregulation of serum amyloid A (SAA) levels [33]. Goto et al. found that SFB antigen presentation by dendritic cells via a major histocompatibility complex (MHC) II-dependent pathway is essential for the induction of Th17 cells, although presentation by group 3 innate lymphoid cells (ILC3) negatively regulates Th17 cell differentiation [34]. Analyses of high-fat diet-induced ROR-γt-deficient and wild-type mice showed that the abundances of Porphyromonadaceae, Peptostreptococcaceae, Comamonadaceae, and Bacteroidaceae were correlated with the expression of ileum IL17 cells and *ROR-γt* mRNA [35].

1.2.3. Effects of the Gut Microbiota and Its Metabolites on Treg Cells. Treg cells play an important role in immunoregulation; according to surface markers, the secreted cytokines, and mechanism of action, Treg cells can be divided into different subtypes. Recent studies of Treg cells have focused on $CD4^+CD25^+$ Treg cells. The differentiation and function of $CD4^+CD25^+$ Treg cells are regulated by the transcription factor Foxp3, antigen-presenting cells, and the cytokines IL-10, IL-12, and TGF-β. Additionally, several experiments have indicated that the gut microbiota induces the differentiation and function of Treg cells. *Bacteroides fragilis* and its metabolite polysaccharide A mediate the conversion of $CD4^+$ T cells into $Foxp3^+$ Treg cells, which produce IL-10 by activating the TLR2 signaling pathway [36]. Clusters IV and XIVa of the genus *Clostridium* promote the expression of $Foxp3^+$ Tregs. After the oral inoculation of *Clostridium* in mice, transforming growth factor-β (TGF-β) levels and $Foxp3^+$ Tregs increased, and systemic immunoglobulin E (IgE) responses were inhibited [33]. Tang et al. found that *Lactobacillus murinus* is able to promote the expression of TGF-β and IL-10, activating the transcription factor Foxp3 and increasing Treg and Th17 cells in the colon [37]. In addition, the intestinal flora can induce other immune cells by affecting the differentiation of Treg cells. Early in 2009, studies showed that Tregs could express high levels of ROR-γt [38]. SFB and *Lactobacillus casei* BL23 could induce the expression of ROR-γt on Treg cells, and increased ROR-γt^+ Treg cells induced the differentiation and function of Th17 cells [39, 40]. Geuking et al. found that the altered Schaedler flora, a collection of eight benign intestinal symbiotic microbiota, promoted the production of Treg cells but diminished Th1 and Th17 cell responses [41].

In addition to the gut microbiota, metabolites, especially short-chain fatty acids (SCFA), also affect the differentiation and function of Treg cells. Smith et al. found that SCFAs promoted the differentiation of Treg cells by activating G-protein-coupled free fatty acid receptor 43 (GPR43) [42]. Atarashi and colleagues verified that SCFAs induce the expression of Treg cells by stimulating epithelial cells to produce TGF-β [43]. Gpr109A (encoded by Niacr1) is a receptor for butyrate and niacin in the colon. Butyrate is able to induce the differentiation of Treg cells and IL 10-producing T cells by binding to Gpr109A. Furthermore, Gpr109a was

essential for the butyrate-mediated induction of IL-18 in the colonic epithelium. Animal experimental studies have shown that colitis and colon cancer develop easily in Niacr1$^{-/-}$ mice, which is a model of a lack of GPR109A expression [44]. The SCFA butyrate also induces an increase in ROR-γt^{+} Treg cells, dependent on dendritic cells and MHC II, and thereby affects the differentiation and function of other immune cells [39].

1.3. Effects of Intestinal Immunomodulatory Cells (T Lymphocytes) on Diabetes

1.3.1. Effects of Th1/Th2 Cells and Their Cytokines on Diabetes. The differentiation of early CD4^{+} T cells is based on a simple dichotomy between interferon- (IFN-) γ-dominated Th1 cell responses and IL-4-dominated Th2 cell responses. Th1 cells can be induced by IL-12 and are key elements for macrophage activation and the clearance of intracellular pathogens, while Th2 cells defend against helminth infections, which are related to allergic disorders, along with IgE, mast cells, and eosinophils.

Th1 cells mainly produce IFN-γ, and IL-12 could lead to the activation of CD8^{+} T cells, thus resulting in the destruction of islet β cells. The destruction of β cells occurs by the infiltration by T cells and the secretion of cytotoxic factors by Th1 cells [45]. Th1- and Th2-mediated immunities are reciprocally regulated and maintain a balance in immune-mediated diseases [46]. In Th1 cells, IFN-γ could activate macrophages to exert cytotoxic activities by the secretion of toxic cytokines [47]. Studies have indicated a direct role of IFN-γ in driving the disease process. Sarvetnick et al. found that the expression of IFN-γ under the control of the human insulin promoter is sufficient to cause the development of diabetes in mice [48] and, conversely, the blockade of IFN-γ in NOD mice could prevent diabetes [49].

IL-12 is another important regulator of Th1 cell differentiation, and it is also the primary immunoregulatory factor secreted by Th1 cells. IL-12 plays a key role in the pathogenesis of diabetes and its complications. Weaver and Nadler found that IL-12 is able to bind to IL-12 receptors on pancreatic islet β cells and activate proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ), resulting in the induction of the apoptosis of islet β cells via the STAT4 signaling pathway [50]. In addition, IL-12 contributes to the development of complications during type 2 diabetes, and angiogenesis and arteriogenesis were induced in mice with T2DM via an eNOS/Akt/VEGFR2/oxidative stress/inflammation-dependent mechanism [51].

Some Th2 cell cytokines, for example, IL-10, can specifically enhance major histocompatibility complex class II expression, thus promoting peri-insulitis. They could also activate resident immune cells, establishing anti-β cell immunity, leading to pancreatic infiltration from other types of cells [52]. Sokolova et al. verified that the serum levels of the Th2 cell cytokines IL-4 and IL-5 were significantly higher in patients with T2DM than those in a healthy control group with a BMI of 18–24.9 kg/m^2 [53]. Anand et al. detected a mixed Th1/Th2 cytokine profile in patients with T2DM, and they also found higher serum levels of the Th2 cytokines IL-4 and IL-13 in T2DM patients compared with those of the control group [54]. Kang et al. demonstrated that IL-4 is involved in metabolic control, improving insulin sensitivity and glucose tolerance [55]. The pathophysiological and immunological mechanisms linking increased serum levels of IL-4 and the occurrence of T2DM, metabolic control, and insulin resistance are still unclear. IL-4, a major immunoregulatory factor secreted by Th2 cells, plays an important role in protecting pancreatic β cells, reducing the inflammation level in insulin target organs and thus alleviating insulin resistance. IL-4 is able to improve insulin sensitivity by upregulating the level of Akt phosphorylation and attenuating the activation of GSK-3b, while IL-4 could regulate fat metabolism and inhibit fat accumulation by affecting the levels of adipokines (adiponectin and leptin) and free fatty acids [56].

IL-10 acts as an immunoregulatory factor secreted by Th2 and Treg cells and plays an important role in regulating autoimmunity, reducing inflammation levels, and alleviating insulin resistance and metabolic disorders. Studies have shown that IL-10 could bind to IL-10 receptor on macrophages, thus inhibiting macrophage activation and the secretion of inflammatory factors by activating the Jak1/STAT3 or Tyk2/STAT3 signaling pathway [57–59]. Glucose transmembrane transport is performed by glucose transporters (GLUTs) on the cell membrane. GLUT4 is widely expressed in various types of cells, such as muscle and adipose tissue cells. Skeletal muscle is an important organ of glucose metabolism, and the aggregation of lipid-mediated inflammatory factors (TNF-α, IL-1β, and IL-6) is a main cause of insulin resistance. IL-10 binds to IL-10 receptor on the membrane of skeletal muscle cells, alleviating oxidative stress and inflammation, thus promoting glucose metabolism [60].

1.3.2. Effects of Th17/Treg Cells and Their Cytokines on Diabetes. Th17 and Treg cells are two CD4^{+} T helper cells that can be derived from the same naive CD4^{+} T cells depending on the amounts of TGF-β and proinflammatory cytokines. Treg cells regulate and control immune tolerance in healthy individuals, while Treg cell dysfunction or a decrease in their amount might result in excessive immune attacks and autoimmune diseases [61]. In some animal models, CD4^{+} CD25^{+} FoxP3^{+} Tregs could stop the destruction of pancreatic islets and protect against autoimmune T1DM [62]. Yuan et al. indicated that the percentage of CD4^{+} CD25^{+} Foxp3^{+} Tregs and levels of related cytokines (mainly IL-10 and TGF-β) were precipitously decreased in patients with newly diagnosed T2DM [63]. After 2 weeks of treatment with Tregs, patients exhibit a significant decrease in the requirement of exogenous insulin and a decrease in HbA1c levels; furthermore, an increase in the percentage of Tregs in the peripheral blood was also observed since the day of treatment [64].

Th17 cells mainly secrete the signature cytokine IL-17A (commonly referred to as IL-17); they also produce IL-17F, IL-21, IL-22, and granulocyte monocyte colony-stimulating factor (GM-CSF) and potentially produce TNF and IL-6 [65]. The cytokines produced by Th17 cells have a wide range of effects on many types of cells and could induce the

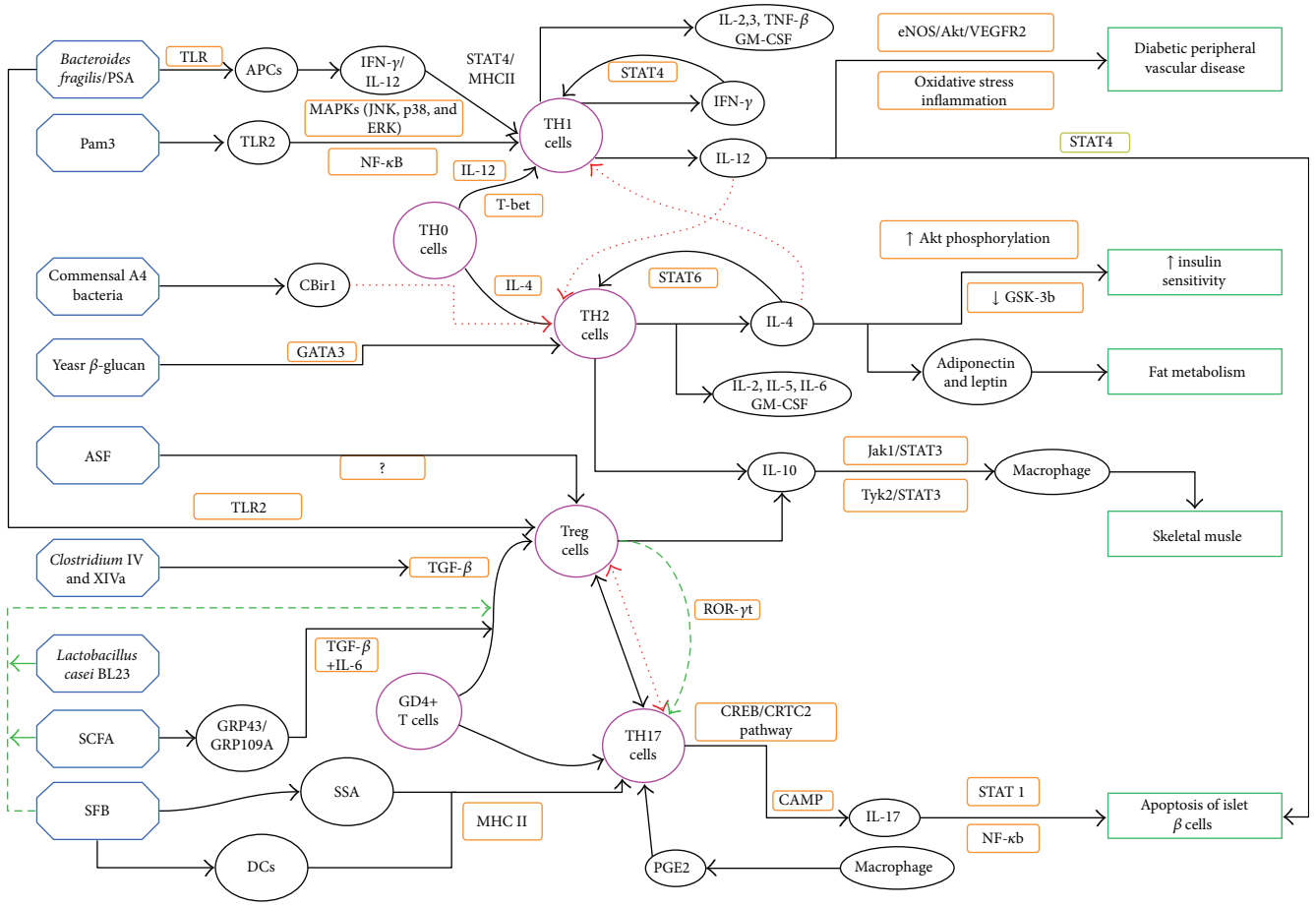


Figure 1: Blue indicates the gut microbiota, purple indicates T lymphocytes, and green indicates different targets of diabetes. Red lines represent inhibitory action. Solid black lines represent a positive effect (promotion). PSA: polysaccharide A; ASF: altered Schaedler flora; SCFA: short-chain fatty acids; SFB: segmented filamentous bacteria; APCS: antigen-presenting cells; TLR: Toll-like receptor; TH0, 1, 2, and 17 cells: T helper 0, 1, 2, and 17 cells; Treg: regulatory T cell; SAA: serum amyloid A; MAPK: mitogen-activated protein kinase; JNK: c-Jun N-terminal kinase; ERK: extracellular signal-regulated kinase; NF-κB: nuclear factor kappa B; STAT4 and 6: signal transducer and activator of transcription 4 and 6; MHC: major histocompatibility complex; eNOS: endothelial nitric oxide synthase; VEGFR2: vascular endothelial growth factor receptor 2; Jak: Janus kinase; Tyk: tyrosine kinase; CREB: cAMP-response element binding protein; CRTC2: CREB-regulated transcription coactivator 2.

secretion of proinflammatory cytokines and chemokines. However, in some instances, IL-17-driven inflammation does not have a protective effect but is a risk factor for severe immunopathology and autoimmunity [66].

Martin-Orozco et al. evaluated a spontaneous autoimmune diabetes model and showed that IL-17A and IL-17F expressions in islets are related to insulitis in NOD mice. The increased expression of Th17 cells is often accompanied by Th1 cells or IFN-γ. Moreover, islet antigen-specific Th17 cells need to be transformed into Th1-like cells to induce diabetes [67]. Arif and colleagues claimed that a feature of T1DM is the autoreaction of IL-17$^+$-specific CD4$^+$ T cells, and the inhibition of Th17 cells reduced the islet-specific inflammatory T cell infiltration [68]. Honkanen and colleagues clearly demonstrated a Th17-based response in patients with T1DM. The peripheral blood CD4$^+$ T cells from children with new onset T1DM secrete higher levels of IL-17 and IL-22 than those from healthy individuals upon polyclonal activation, and there was no increase in IFN-γ levels or T-bet expression in patients with T1DM [69]. According to another study, the cAMP-mediated production of macrophage-derived prostaglandin E2 (PGE2) stimulates Th17 cell production of IL-17A and IL-17F via the cAMP-response element binding protein/CREB-regulated transcription coactivator 2 (CREB/CRTC2) pathway [70]. IL-17 binds to IL-17 receptor of islet β cells and upregulates the expression of STAT1 and NF-κB in cells, which results in the apoptosis of islet β cells [68, 71]. In addition to direct destruction in islet cells, Saxena et al. also found that IL-17 could induce the differentiation of Th1 cells and then cooperate with Th1 cells to mediate autoimmune diseases [72].

Zhang et al. showed that the duration of diabetes was positively related to the proportion of Th17 cells and negatively related to the proportion of Treg cells. Furthermore, they did not detect significant relationships between diabetes duration and Th1 cells or Th2 cells, and they supposed that this parameter may have an impact on the Th17/Treg paradigm, rather than the Th1/Th2 paradigm [73]. The balance

between Th17 and Treg cells is a key factor in autoimmune diseases, and an imbalance typically involves an increase in Th17 cells, but a decrease in Treg cells, which leads to a failure of the regulation on ongoing immune reactions. For example, in NOD mice, a Th17/Treg imbalance weakens the ability of Treg cells to suppress self-reactive effector T cell activity and to stop the destruction of pancreatic islets, which may potentially induce or aggravate T1DM [64].

2. Conclusions

DM is a common metabolic disorder with a high morbidity and a high risk of severe complications, resulting in substantial damage to multiple organs in humans. Without any effective long-term treatment, patients with diabetes usually have a rather low quality of life, and this situation has prompted studies of diabetes from multiple perspectives. Recently, increasing studies have shown that the gut microbiota participates in the maintenance of human health and the development of diseases and is directly related to the occurrence and development of diabetes. The gut microbiota and related topics have become a focus of recent research and provide an opportunity to make great progress in our understanding of diabetes, with the potential to facilitate mechanistic research, clinical trials, drug development, and improving the efficacy of treatment strategies.

Based on a number of studies, we identified a key link between the gut microbiota and diabetes, or a Bridge material, that is, IICs. Many cell types are classified as IICs, and this review focuses on T lymphocytes, which have been evaluated extensively in recent research, to describe the mechanisms by which the gut microbiota affects diabetes. We developed a flow chart to clearly display the complex mechanisms described in this review (Figure 1). As this graph shows, many gut microbiota components regulate the differentiation and function of T lymphocytes via a variety of pathways. T lymphocytes secrete various cytokines, and these play important roles in regulating autoimmunity, protecting islet cells, improving glucose and lipid metabolism, and reducing insulin resistance, thus alleviating diabetes. Interestingly, Th1, Th2, Th17, and Treg cells come from the T lymphocytes, and they are not only able to regulate each other, but they also have antagonistic effects depending on their conditions, consequently alleviating or aggravating the syndrome of diabetes. These findings provide new targets to develop therapies. T lymphocytes are not the only IICs, and further studies are necessary to examine a wider array of cellular mechanisms and disease characteristics.

Disclosure

Dr. Linhua Zhao and Dr. Xiaolin Tong are co-corresponding authors.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Dr. Qingwei Li and Dr. Zezheng Gao contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81704067 and 81430097) and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA12030202).

References

[1] X. Yao, C. Zhang, Y. Xing et al., "Remodelling of the gut microbiota by hyperactive NLRP3 induces regulatory T cells to maintain homeostasis," *Nature Communications*, vol. 8, no. 1, p. 1896, 2017.

[2] A. M. Kabat, N. Srinivasan, and K. J. Maloy, "Modulation of immune development and function by intestinal microbiota," *Trends in Immunology*, vol. 35, no. 11, pp. 507–517, 2014.

[3] P. D. Cani, R. Bibiloni, C. Knauf et al., "Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice," *Diabetes*, vol. 57, no. 6, pp. 1470–1481, 2008.

[4] T. Tanoue, K. Atarashi, and K. Honda, "Development and maintenance of intestinal regulatory T cells," *Nature Reviews Immunology*, vol. 16, no. 5, pp. 295–309, 2016.

[5] M. Morimoto, N. Azuma, H. Kadowaki, T. Abe, and Y. Suto, "Regulation of type 2 diabetes by helminth-induced Th2 immune response," *Journal of Veterinary Medical Science*, vol. 78, no. 12, pp. 1855–1864, 2016.

[6] P. B. Eckburg, E. M. Bik, C. N. Bernstein et al., "Diversity of the human intestinal microbial flora," *Science*, vol. 308, no. 5728, pp. 1635–1638, 2005.

[7] K. Tiihonen, A. C. Ouwehand, and N. Rautonen, "Human intestinal microbiota and healthy ageing," *Ageing Research Reviews*, vol. 9, no. 2, pp. 107–116, 2010.

[8] J. Qin, R. Li, J. Raes et al., "A human gut microbial gene catalogue established by metagenomic sequencing," *Nature*, vol. 464, no. 7285, pp. 59–65, 2010.

[9] S. Macfarlane, H. Steed, and G. T. Macfarlane, "Intestinal bacteria and inflammatory bowel disease," *Critical Reviews in Clinical Laboratory Sciences*, vol. 46, no. 1, pp. 25–54, 2009.

[10] N. Larsen, F. K. Vogensen, F. W. J. van den Berg et al., "Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults," *PLoS One*, vol. 5, no. 2, article e9085, 2010.

[11] X. Wu, C. Ma, L. Han et al., "Molecular characterisation of the faecal microbiota in patients with type II diabetes," *Current Microbiology*, vol. 61, no. 1, pp. 69–78, 2010.

[12] J. Qin, Y. Li, Z. Cai et al., "A metagenome-wide association study of gut microbiota in type 2 diabetes," *Nature*, vol. 490, no. 7418, pp. 55–60, 2012.

[13] L. Mengual, P. Roura, M. Serra, M. Montasell, G. Prieto, and S. Bonet, "Multifactorial control and treatment intensity of type-2 diabetes in primary care settings in Catalonia," *Cardiovascular Diabetology*, vol. 9, no. 1, p. 14, 2010.

[14] B. Rémy, "Gut microbiota and immune crosstalk in metabolic disease," *Biologie Aujourd'hui*, vol. 211, no. 1, pp. 1–18, 2017.

[15] L. Wen, R. E. Ley, P. Y. Volchkov et al., "Innate immunity and intestinal microbiota in the development of type 1 diabetes," *Nature*, vol. 455, no. 7216, pp. 1109–1113, 2008.

[16] F. H. Karlsson, V. Tremaroli, I. Nookaew et al., "Gut metagenome in European women with normal, impaired and diabetic glucose control," *Nature*, vol. 498, no. 7452, pp. 99–103, 2013.

[17] A. D. Kostic, D. Gevers, H. Siljander et al., "The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes," *Cell Host & Microbe*, vol. 17, no. 2, pp. 260–273, 2015.

[18] M. Murri, I. Leiva, J. M. Gomez-Zumaquero et al., "Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study," *BMC Medicine*, vol. 11, no. 1, p. 46, 2013.

[19] R. Caesar, C. S. Reigstad, H. K. Bäckhed et al., "Gut-derived lipopolysaccharide augments adipose macrophage accumulation but is not essential for impaired glucose or insulin tolerance in mice," *Gut*, vol. 61, no. 12, pp. 1701–1707, 2012.

[20] M. Remely, E. Aumueller, C. Merold et al., "Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity," *Gene*, vol. 537, no. 1, pp. 85–92, 2014.

[21] M. Watanabe, S. M. Houten, C. Mataki et al., "Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation," *Nature*, vol. 439, no. 7075, pp. 484–489, 2006.

[22] T. R. Mosmann, H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman, "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins," *Journal of Immunology*, vol. 136, no. 7, pp. 2348–2357, 1986.

[23] P. P. Teh, A. Vasanthakumar, and A. Kallies, "Chapter seven - development and function of effector regulatory T cells," *Progress in Molecular Biology and Translational Science*, vol. 136, pp. 155–174, 2015.

[24] M. F. Neurath, S. Finotto, and L. H. Glimcher, "The role of Th1/Th2 polarization in mucosal immunity," *Nature Medicine*, vol. 8, no. 6, pp. 567–573, 2002.

[25] Q. Wang, R. M. McLoughlin, B. A. Cobb et al., "A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2," *Journal of Experimental Medicine*, vol. 203, no. 13, pp. 2853–2863, 2006.

[26] S. K. Mazmanian, C. H. Liu, A. O. Tzianabos, and D. L. Kasper, "An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system," *Cell*, vol. 122, no. 1, pp. 107–118, 2005.

[27] T. Imanishi, H. Hara, S. Suzuki, N. Suzuki, S. Akira, and T. Saito, "Cutting edge: TLR2 directly triggers Th1 effector functions," *The Journal of Immunology*, vol. 178, no. 11, pp. 6715–6719, 2007.

[28] W. Wu, H. P. Liu, F. Chen et al., "Commensal A4 bacteria inhibit intestinal Th2-cell responses through induction of dendritic cell TGF-β production," *European Journal of Immunology*, vol. 46, no. 5, pp. 1162–1167, 2016.

[29] Y. Cao, Y. Sun, S. Zou, B. Duan, M. Sun, and X. Xu, "Yeast β-glucan suppresses the chronic inflammation and improves the microenvironment in adipose tissues of ob/ob mice," *Journal of Agricultural and Food Chemistry*, vol. 66, no. 3, pp. 621–629, 2018.

[30] I. I. Ivanov, B. S. McKenzie, L. Zhou et al., "The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17$^+$ T helper cells," *Cell*, vol. 126, no. 6, pp. 1121–1133, 2006.

[31] K. Atarashi, J. Nishimura, T. Shima et al., "ATP drives lamina propria T_H17 cell differentiation," *Nature*, vol. 455, no. 7214, pp. 808–812, 2008.

[32] I. I. Ivanov, R. de Llanos Frutos, N. Manel et al., "Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine," *Cell Host & Microbe*, vol. 4, no. 4, pp. 337–349, 2008.

[33] I. I. Ivanov, K. Atarashi, N. Manel et al., "Induction of intestinal Th17 cells by segmented filamentous bacteria," *Cell*, vol. 139, no. 3, pp. 485–498, 2009.

[34] Y. Goto, C. Panea, G. Nakato et al., "Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation," *Immunity*, vol. 40, no. 4, pp. 594–607, 2014.

[35] L. Garidou, C. Pomié, P. Klopp et al., "The gut microbiota regulates intestinal CD4 T cells expressing RORγt and controls metabolic disease," *Cell Metabolism*, vol. 22, no. 1, pp. 100–112, 2015.

[36] J. L. Round and S. K. Mazmanian, "Inducible Foxp3$^+$ regulatory T-cell development by a commensal bacterium of the intestinal microbiota," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 27, pp. 12204–12209, 2010.

[37] C. Tang, T. Kamiya, Y. Liu et al., "Inhibition of dectin-1 signaling ameliorates colitis by inducing *Lactobacillus*-mediated regulatory T cell expansion in the intestine," *Cell Host & Microbe*, vol. 18, no. 2, pp. 183–197, 2015.

[38] M. Ayyoub, F. Deknuydt, I. Raimbaud et al., "Human memory FOXP3$^+$ Tregs secrete IL-17 ex vivo and constitutively express the T_H17 lineage-specific transcription factor RORγt," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 21, pp. 8635–8640, 2009.

[39] C. Ohnmacht, J. H. Park, S. Cording et al., "The microbiota regulates type 2 immunity through RORγt$^+$ T cells," *Science*, vol. 349, no. 6251, pp. 989–993, 2015.

[40] N. G. Cortes-Perez, D. Lozano-Ojalvo, M. A. Maiga, S. Hazebrouck, and K. Adel-Patient, "Intragastric administration of *Lactobacillus casei* BL23 induces regulatory FoxP3 +RORγt+ T cells subset in mice," *Beneficial Microbes*, vol. 8, no. 3, pp. 433–438, 2017.

[41] M. B. Geuking, J. Cahenzli, M. A. E. Lawson et al., "Intestinal bacterial colonization induces mutualistic regulatory T cell responses," *Immunity*, vol. 34, no. 5, pp. 794–806, 2011.

[42] P. M. Smith, M. R. Howitt, N. Panikov et al., "The microbial metabolites, short-chain fatty acids, regulate colonic T_{reg} cell homeostasis," *Science*, vol. 341, no. 6145, pp. 569–573, 2013.

[43] K. Atarashi, T. Tanoue, K. Oshima et al., "T_{reg} induction by a rationally selected mixture of Clostridia strains from the human microbiota," *Nature*, vol. 500, no. 7461, pp. 232–236, 2013.

[44] N. Singh, A. Gurav, S. Sivaprakasam et al., "Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis," *Immunity*, vol. 40, no. 1, pp. 128–139, 2014.

[45] W. Held, H. R. MacDonald, I. L. Weissman, M. W. Hess, and C. Mueller, "Genes encoding tumor necrosis factor alpha and granzyme A are expressed during development of autoimmune diabetes," *Proceedings of the National Academy of*

Sciences of the United States of America, vol. 87, no. 6, pp. 2239–2243, 1990.

[46] A. K. Abbas, K. M. Murphy, and A. Sher, "Functional diversity of helper T lymphocytes," *Nature*, vol. 383, no. 6603, pp. 787–793, 1996.

[47] L. Adorini, S. Gregori, and L. C. Harrison, "Understanding autoimmune diabetes: insights from mouse models," *Trends in Molecular Medicine*, vol. 8, no. 1, pp. 31–38, 2002.

[48] N. Sarvetnick, D. Liggitt, S. L. Pitts, S. E. Hansen, and T. A. Stewart, "Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma," *Cell*, vol. 52, no. 5, pp. 773–782, 1988.

[49] M. Debray-Sachs, C. Carnaud, C. Boitard et al., "Prevention of diabetes in NOD mice treated with antibody to murine IFNγ," *Journal of Autoimmunity*, vol. 4, no. 2, pp. 237–248, 1991.

[50] J. R. Weaver and J. L. Nadler, "Interleukin-12 (IL-12)/STAT4 axis is an important element for β-cell dysfunction induced by inflammatory cytokines," *PLoS One*, vol. 10, no. 11, article e0142735, 2015.

[51] M. Ali, V. Mali, S. Haddox et al., "Essential role of IL-12 in angiogenesis in type 2 diabetes," *The American Journal of Pathology*, vol. 187, no. 11, pp. 2590–2601, 2017.

[52] S. T. Azar, H. Tamim, H. N. Beyhum, M. Z. Habbal, and W. Y. Almawi, "Type I (insulin dependent) diabetes is a Th1- and Th2-mediated autoimmune disease," *Clinical and Diagnostic Laboratory Immunology*, vol. 6, no. 3, pp. 306–310, 1999.

[53] R. N. Sokolova, R. K. Yankova, T. I. Abadjieva et al., "Association between type 2 diabetes, obesity and key immunological components of IgE-mediated inflammation," *Folia Medica*, vol. 59, no. 2, pp. 159–164, 2017.

[54] G. Anand, R. Vasanthakumar, V. Mohan, S. Babu, and V. Aravindhan, "Increased IL-12 and decreased IL-33 serum levels are associated with increased Th1 and suppressed Th2 cytokine profile in patients with diabetic nephropathy (CURES-134)," *International Journal of Clinical and Experimental Pathology*, vol. 7, no. 11, pp. 8008–8015, 2014.

[55] K. Kang, S. M. Reilly, V. Karabacak et al., "Adipocyte-derived Th2 cytokines and myeloid PPARδ regulate macrophage polarization and insulin sensitivity," *Cell Metabolism*, vol. 7, no. 6, pp. 485–495, 2008.

[56] Y.-H. Chang, K.-T. Ho, S.-H. Lu, C.-N. Huang, and M.-Y. Shiau, "Regulation of glucose/lipid metabolism and insulin sensitivity by interleukin-4," *International Journal of Obesity*, vol. 36, no. 7, pp. 993–998, 2012.

[57] W. Ouyang, S. Rutz, N. K. Crellin, P. A. Valdez, and S. G. Hymowitz, "Regulation and functions of the IL-10 family of cytokines in inflammation and disease," *Annual Review of Immunology*, vol. 29, no. 1, pp. 71–109, 2011.

[58] L. Grant, K. D. Shearer, A. Czopek et al., "Myeloid-cell protein tyrosine phosphatase-1B deficiency in mice protects against high-fat diet and lipopolysaccharide-induced inflammation, hyperinsulinemia, and endotoxemia through an IL-10 STAT3-dependent mechanism," *Diabetes*, vol. 63, no. 2, pp. 456–470, 2014.

[59] K. Asadullah, W. Sterry, and H. D. Volk, "Interleukin-10 therapy—review of a new approach," *Pharmacological Reviews*, vol. 55, no. 2, pp. 241–269, 2003.

[60] S. Dagdeviren, D. Y. Jung, E. Lee et al., "Altered interleukin-10 signaling in skeletal muscle regulates obesity-mediated inflammation and insulin resistance," *Molecular and Cellular Biology*, vol. 36, no. 23, pp. 2956–2966, 2016.

[61] Y.-H. Van, W.-H. Lee, S. Ortiz, M.-H. Lee, H.-J. Qin, and C.-P. Liu, "All-*trans* retinoic acid inhibits type 1 diabetes by T regulatory (Treg)-dependent suppression of interferon-γ–producing t cells without affecting Th17 cells," *Diabetes*, vol. 58, no. 1, pp. 146–155, 2009.

[62] E. A. Green, L. Gorelik, C. M. McGregor, E. H. Tran, and R. A. Flavell, "$CD4^+CD25^+$ T regulatory cells control anti-islet $CD8^+$ T cells through TGF-β–TGF-β receptor interactions in type 1 diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 19, pp. 10878–10883, 2003.

[63] N. Yuan, H.-f. Zhang, Q. Wei, P. Wang, and W.-y. Guo, "Expression of CD4+CD25+Foxp3+ regulatory T cells, interleukin 10 and transforming growth factor β in newly diagnosed type 2 diabetic patients," *Experimental and Clinical Endocrinology & Diabetes*, 2017.

[64] N. Marek-Trzonkowska, M. Mysliwiec, A. Dobyszuk et al., "Administration of $CD4^+CD25^{high}CD127^-$regulatory T cells preserves β-cell function in type 1 diabetes in children," *Diabetes Care*, vol. 35, no. 9, pp. 1817–1820, 2012.

[65] T. Korn, E. Bettelli, M. Oukka, and V. K. Kuchroo, "IL-17 and Th17 cells," *Annual Review of Immunology*, vol. 27, no. 1, pp. 485–517, 2009.

[66] Y. Li, Y. Liu, and C.-Q. Chu, "Th17 cells in type 1 diabetes: role in the pathogenesis and regulation by gut microbiome," *Mediators of Inflammation*, vol. 2015, Article ID 638470, 7 pages, 2015.

[67] N. Martin-Orozco, Y. Chung, S. H. Chang, Y.-H. Wang, and C. Dong, "Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells," *European Journal of Immunology*, vol. 39, no. 1, pp. 216–224, 2009.

[68] S. Arif, F. Moore, K. Marks et al., "Peripheral and islet interleukin-17 pathway activation characterizes human autoimmune diabetes and promotes cytokine-mediated β-cell death," *Diabetes*, vol. 60, no. 8, pp. 2112–2119, 2011.

[69] J. Honkanen, J. K. Nieminen, R. Gao et al., "IL-17 immunity in human type 1 diabetes," *The Journal of Immunology*, vol. 185, no. 3, pp. 1959–1967, 2010.

[70] J. B. Hernandez, C. Chang, M. LeBlanc et al., "The CREB/CRTC2 pathway modulates autoimmune disease by promoting Th17 differentiation," *Nature Communications*, vol. 6, no. 1, p. 7216, 2015.

[71] F. A. Grieco, F. Moore, F. Vigneron et al., "IL-17A increases the expression of proinflammatory chemokines in human pancreatic islets," *Diabetologia*, vol. 57, no. 3, pp. 502–511, 2014.

[72] A. Saxena, S. Desbois, N. Carrie, M. Lawand, L. T. Mars, and R. S. Liblau, "Tc17 $CD8^+$ T cells potentiate Th1-mediated autoimmune diabetes in a mouse model," *The Journal of Immunology*, vol. 189, no. 6, pp. 3140–3149, 2012.

[73] C. Zhang, C. Xiao, P. Wang et al., "The alteration of Th1/Th2/Th17/Treg paradigm in patients with type 2 diabetes mellitus: relationship with diabetic nephropathy," *Human Immunology*, vol. 75, no. 4, pp. 289–296, 2014.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 6215350, 8 pages
https://doi.org/10.1155/2018/6215350

Review Article

Emerging Roles of Immune Cells in Postoperative Cognitive Dysfunction

Yue Liu and Yiqing Yin

Department of Anesthesiology, China-Japan Friendship Hospital, Beijing 100029, China

Correspondence should be addressed to Yiqing Yin; yyq518@sina.com

Received 2 November 2017; Accepted 25 December 2017; Published 18 February 2018

Academic Editor: Qingdong Guan

Copyright © 2018 Yue Liu and Yiqing Yin. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Postoperative cognitive dysfunction (POCD), a long-lasting cognitive decline after surgery, is currently a major clinical problem with no clear pathophysiological mechanism or effective therapy. Accumulating evidence suggests that neuroinflammation plays a critical role in POCD. After surgery, alarmins are leaked from the injury sites and proinflammatory cytokines are increased in the peripheral circulation. Neurons in the hippocampus, which is responsible for learning and memory, can be damaged by cytokines transmitted to the brain parenchyma. Microglia, bone marrow-derived macrophages, mast cells, and T cells in the central nervous system (CNS) can be activated to secrete more cytokines, further aggravating neuroinflammation after surgery. Conversely, blocking the inflammation network between these immune cells and related cytokines alleviates POCD in experimental animals. Thus, a deeper understanding of the roles of immune cells and the crosstalk between them in POCD may uncover promising therapeutic targets for POCD treatment and prevention. Here, we reviewed several major immune cells and discussed their functional roles in POCD.

1. Introduction

Postoperative cognitive dysfunction (POCD) refers to a long-lasting cognitive decline after surgery, characterized by impaired concentration, memory, and learning, which can be detected by a battery of neuropsychological tests [1]. The incidence of POCD is 7 to 26% after major noncardiac surgery and even higher in patients older than 60 years [1, 2]. POCD not only diminishes the patient's quality of life and imposes a serious burden on healthcare costs but also increases mortality [3]. Although several risk factors for POCD have been identified, the pathophysiological mechanism underlying POCD remains unclear and no effective therapies have been developed to date.

A large number of studies conducted in patients have revealed that POCD is associated with elevated levels of plasma inflammatory cytokines, including tumor necrosis factor- (TNF-) α and interleukin- (IL-) 6 [4–7]. IL-1β and IL-6 levels in the cerebrospinal fluid (CSF) of patients with POCD are higher than those of patients with normal cognitive function after surgery [8, 9]. The learning and memory function was also impaired by surgery and anesthesia in experimental animals, accompanied by the upregulation of proinflammatory cytokine levels in both the blood and the brain [10–13]. Neuroinflammation, particularly in the hippocampus, has been proved to be one of the main causes of POCD [14–17]. The activation of microglia and other blood-derived immune cells orchestrates neuroinflammation and subsequent neuronal damage [14–17]. In this review, we discuss the main types of immune cells involved in POCD and their possible roles. We describe their functions in neuroinflammation, put forth a possible mechanism of their involvement in POCD, and point out the fields that need further exploration.

2. Immune Cells in POCD

2.1. Microglia. Microglia are highly specialized tissue-resident macrophages in the central nervous system (CNS) and the major resident immune cells of the brain [18]. Microglia are the only CNS cells originating from

hematopoiesis. Primitive macrophage progenitors in the yolk sac colonize the CNS and differentiate into mature microglia, confined behind the blood-brain barrier (BBB) [19]. Unlike other tissue macrophages, such as Kupffer cells in the hepatic sinusoids, which need to be renewed from bone marrow progenitors, microglia are capable of local expansion and maintenance throughout life without reconstitution from the bone marrow [20].

In healthy brains, microglia are ramified and in a resting state, monitoring the local microenvironment and detecting CNS damage [21]. Danger signals, including pathogen invasion, injury, and abnormal protein accumulation, can trigger microglia transformation into an activated amoeboid shape. Activated microglia are both neuroprotective and neurotoxic. Studies in adult and neonatal hypoxic-ischemic injury models have shown that a complete blockade of microglial activity exacerbates brain damage [22, 23]. However, activated microglia can also produce excessive proinflammatory cytokines, leading to neuronal dysfunction and death. Several neurodegenerative diseases, including Alzheimer's, Huntington's, and Parkinson's diseases, have been proved to be associated with the hyperactivation of microglia [24–26]. As a specific type of macrophages, activated microglia can have one of the two different phenotypes: classically activated M1 and alternatively activated M2 microglia. M1 microglia promote inflammation by secreting proinflammatory cytokines such as IL-1α, IL-1β, and TNF. M2 microglia elicit neuroprotective effects through the release of vascular endothelial growth factor and extracellular matrix proteins [27]. In Alzheimer's disease (AD), amyloid β (Aβ) sensitizes microglia to subsequent cytokine stimulation and M1 activation [28], whereas the induction of the M2 polarization of microglia by drugs or adeno-associated viral vectors can reduce Aβ deposition and relieve AD symptoms [29, 30]. In other neurological diseases, such as Parkinson's disease (PD), chronic cerebral hypoperfusion, traumatic brain injury, and hepatic encephalopathy, the priming of microglial polarization towards the M1 phenotype plays a pivotal role in neuroinflammation [31–34].

After peripheral surgery, an immune challenge is transmitted to the brain via multiple humoral and neural routes. The integrity of the BBB can be disrupted by a systemic inflammatory response or anesthesia during and after surgery [11, 35, 36]. Adenosine triphosphate (ATP), alarmins, and cytokines, which are leaked from an injury site or increase in response to systemic inflammation, enter the brain and activate microglia [11, 36–38]. Activated microglia may impair learning and memory via the release of proinflammatory cytokines, among which IL-1β and TNF-α are particularly important [38, 39]. Mild repeated stress or systemic endotoxin challenge can trigger microglia to secrete IL-1β and TNF-α [38, 40–42]. After surgery, aged rats and mice demonstrated significant deficits in memory and learning, concurrent with the activation of microglia and increased expression of TNF-α and IL-1β in the hippocampus [43, 44]. Preemptively depleting microglia reduced surgery-induced hippocampal inflammatory cytokine secretion and attenuated the cognitive decline in mice [14]. IL-1β and TNF-α can cause neuronal cell death, reduction of acetylcholine release, and attenuation of glutamatergic transmission, resulting in learning and memory deficits [38, 40–42]. Neuroinflammation and POCD were mitigated in IL-1R knockout mice or mice pretreated with an IL-1 receptor (IL-1R) antagonist compared with control mice [10]. Furthermore, microglia can be activated by peripheral TNF-α [38]. Preemptive treatment of anti-TNF antibody is able to limit the release of IL-1 in the hippocampus and prevent cognitive decline in a mouse model of POCD [11]. Therefore, microglia may respond to peripheral TNF-α, secrete more TNF-α and IL-1β in the hippocampus, and amplify neuroinflammation in POCD. Additionally, a study also reported reduced infiltration of bone marrow-derived monocytes into the hippocampus after microglial depletion, suggesting crosstalk between microglia and bone marrow-derived macrophages (BMDMs) in POCD [14].

No studies to date have reported the polarization of microglia in POCD. However, the main cytokines secreted by activated microglia in POCD are IL-1 and TNF-α [14, 43, 44], suggesting the predominance of the M1 state of microglia in POCD. Furthermore, the M2 response of microglia was impaired after brain ischemia in aged mice [45]. Because older patients are particularly susceptible to POCD, we speculate that the M1 phenotype of microglia plays a central role in neuroinflammation in POCD. Pharmacological approaches that have been successfully used to modulate microglia polarization in other neurological diseases may hold promise for developing POCD treatments [32, 34].

In a synthesis of the existing microglia and POCD research, we can draw a picture of how microglia may orchestrate postoperative neuroinflammation in POCD. As the resident immune cells of the brain parenchyma, microglia are activated by proteins and other signals leaked from the injury sites. The cytokines secreted from the microglia can directly damage neurons and also recruit more immune cells from the blood penetrating into the brain parenchyma, further accelerating neuronal injury.

2.2. Bone Marrow-Derived Macrophages. Macrophages are present in virtually all tissues. They differentiate from circulating peripheral-blood mononuclear cells, which migrate into tissues constitutively or in response to inflammation [46]. In a healthy CNS, BMDMs are divided into three classes according to their location: choroid plexus, meningeal, and perivascular macrophages [20]. These macrophages are exterior to the brain parenchyma, and their population homeostasis is achieved by replacement from blood-born monocytes. In disease states, BMDMs respond to inflammation and migrate into the brain parenchyma from the circulation.

BMDMs are a major component of the inflammatory immune response to CNS diseases. Similar to microglia, BMDMs have a proinflammatory M1 phenotype and an anti-inflammatory M2 phenotype. M2 macrophages can be beneficial for the healing of sterilized wounds, clearing necrotic debris or abnormal proteins. In a spinal cord injury model, macrophages played an anti-inflammatory role during recovery [47]. Furthermore, numerous studies have

suggested that BMDMs can infiltrate the brain, reduce the Aβ plaque burden, and alleviate the cognitive decline in AD [48, 49]. In a clinical study, transplantation of autologous M2 macrophages significantly improved motor and cognitive activities in patients with severe cerebral palsy [50]. Other reports, however, have indicated that macrophages mainly play a detrimental role in CNS pathology. Penetration of macrophages into the brain impaired spatial learning and memory after traumatic brain injury in mice [51, 52]. In a model of intracerebral hemorrhage, mice exhibited improved motor function after the depletion of inflammatory monocytes [53]. In addition, circulating monocytes or macrophages have been implicated in the exacerbation and relapses of experimental autoimmune encephalitis (EAE) in mice [54, 55].

BMDMs were found in the hippocampi of mice with POCD [56]. Depletion of BMDMs attenuated surgery-induced increases of the IL-6 levels in serum and the hippocampus, reduced hippocampal macrophage infiltration, and prevented surgery-induced memory dysfunction [15]. Inhibiting the proinflammatory signaling pathway in BMDMs or preserving the integrity of the BBB can also reduce the infiltration of BMDMs in the hippocampus and prevent POCD [56]. Furthermore, mice deficient in IL-6 exhibited less IL-1β and TNF-α expression in the hippocampus and better working memory [57]. These findings indicate that, with the BBB integrity disrupted, BMDMs infiltrate into the hippocampus and secrete proinflammatory cytokines, exacerbating neuroinflammation in POCD.

The depletion of microglia has also been shown to prevent BMDMs infiltrating the hippocampus without impairing the capacity of monocytes to penetrate into the brain [14]. Monocyte chemotactic protein-1 (MCP-1), also known as CCL2, is a major chemoattractant to recruit BMDMs [58]. Postoperative hippocampal MCP-1 levels were reduced by the depletion of microglia [14] but not BMDMs [15], indicating that microglia are the major source of secreted MCP-1. Taken together, these studies show that microglia attract BMDMs into the brain via MCP-1 secretion after injury.

High-mobility group box 1 protein (HMGB1), a ubiquitous nucleosomal protein, is passively released into the circulation from damaged necrotic cells, and circulating HMGB1 levels increase after surgery [36, 59]. Blocking the HMGB1 function with a monoclonal antibody reduced the hippocampal expression of MCP-1 and postoperative memory decline in mice [60]. Furthermore, the depletion of BMDMs prevented an HMGB1-mediated memory decline after surgery [60]. Together with the previous studies, these results indicate that HMGB1 may stimulate hippocampal microglia to secrete MCP-1, enabling monocyte recruitment. Similar to HMGB1, many cytokines can simulate microglia. In a model of peripheral organ inflammation, microglia were stimulated by peripheral TNF-α and attracted circulating monocytes into the brain [61]. Moreover, plasma TNF-α levels were upregulated early after aseptic surgery, and a blockade of TNF-α prevented POCD in mice [11]. However, whether the TNF-α/microglia/BMDM pathway is essential in the pathogenesis of POCD is still unknown.

In summary, the activation of microglia and BMDM recruitment play important roles in POCD. However, the relationship between microglia and BMDMs in POCD needs further investigation. The possibility of BMDM infiltration into the CNS after surgery through other microglia-independent pathways also needs exploration.

2.3. Mast Cells. Mast cells (MCs) are myeloid cells originating from $CD34^+/CD117^+$ pluripotent progenitor cells [62]. MCs contain many cytoplasmic granules, which store a number of preformed mediators, including histamine, heparin, serotonin, chymase, tryptase, prostaglandins, and leukotrienes. MCs are best known for their roles in allergic disease and host defense. Crosslinking immunoglobulin E (IgE) receptors of MCs triggers the release of many allergic and inflammatory mediators [63]. MCs are abundant within tissues exposed to the external environment, such as the skin, gut, and the respiratory tract. MCs are also present in the CNS, mainly located in the perivascular spaces and along the leptomeninges [64, 65]. Upon activation, MCs can release the mediators and infiltrate into the brain parenchyma, participating in the pathophysiological processes of various neurological diseases.

It is well established that MCs contribute to general vascular permeability through the production of vasodilators, such as histamine and serotonin. Ample evidence also exists that the vasodilatory and proinflammatory mediators released by MCs contribute to the impairment of the BBB integrity (reviewed in [66]). For instance, histamine can open the tight junctions between the endothelia in the BBB [67]. Proteinases secreted by MCs, including tryptase and gelatinase, can degrade protein constituents of the neurovascular matrix, thus damaging the BBB [67]. In recent decades, studies have demonstrated that MCs play critical roles in the disruption of the BBB and associated neurological diseases. Acute stress increased the permeability of BBB through the activation of MCs [68]. Furthermore, compared with wild-type mice, MC-deficient mice showed decreased BBB permeability, reduced T cell infiltration, and, consequently, less severe EAE [69]. In addition, in a mouse model of brain ischemia, animals that were deficient in MCs or treated with the MC stabilizer Cromolyn exhibited improved BBB integrity and reduced brain edema [70].

Studies have suggested that MCs are the predominant cells that initiate glial activation. In a model of perinatal hypoxia-ischemia, MCs were found to be the "first responders," with their activation preceding that of microglia [71]. In addition, the clinical conditions of depression and mild neurocognitive disorders are closely related to the malfunction of the MC-glia crosstalk [72]. Microglia express a large variety of proteins/receptors that can be activated by MC-secreted mediators. For instance, tryptase can trigger microglia activation through the proteinase-activated receptor 2 (PAR2) [73]. Furthermore, microglia express all four histamine receptors (HRs) and can be activated by MCs via HRs [74, 75]. Astrocytes also express PAR2 and HRs and can be activated by MCs [76, 77]. The interactions between MCs and glial cells are not restricted to the receptors mentioned above (reviewed in [78]), and accumulating evidence

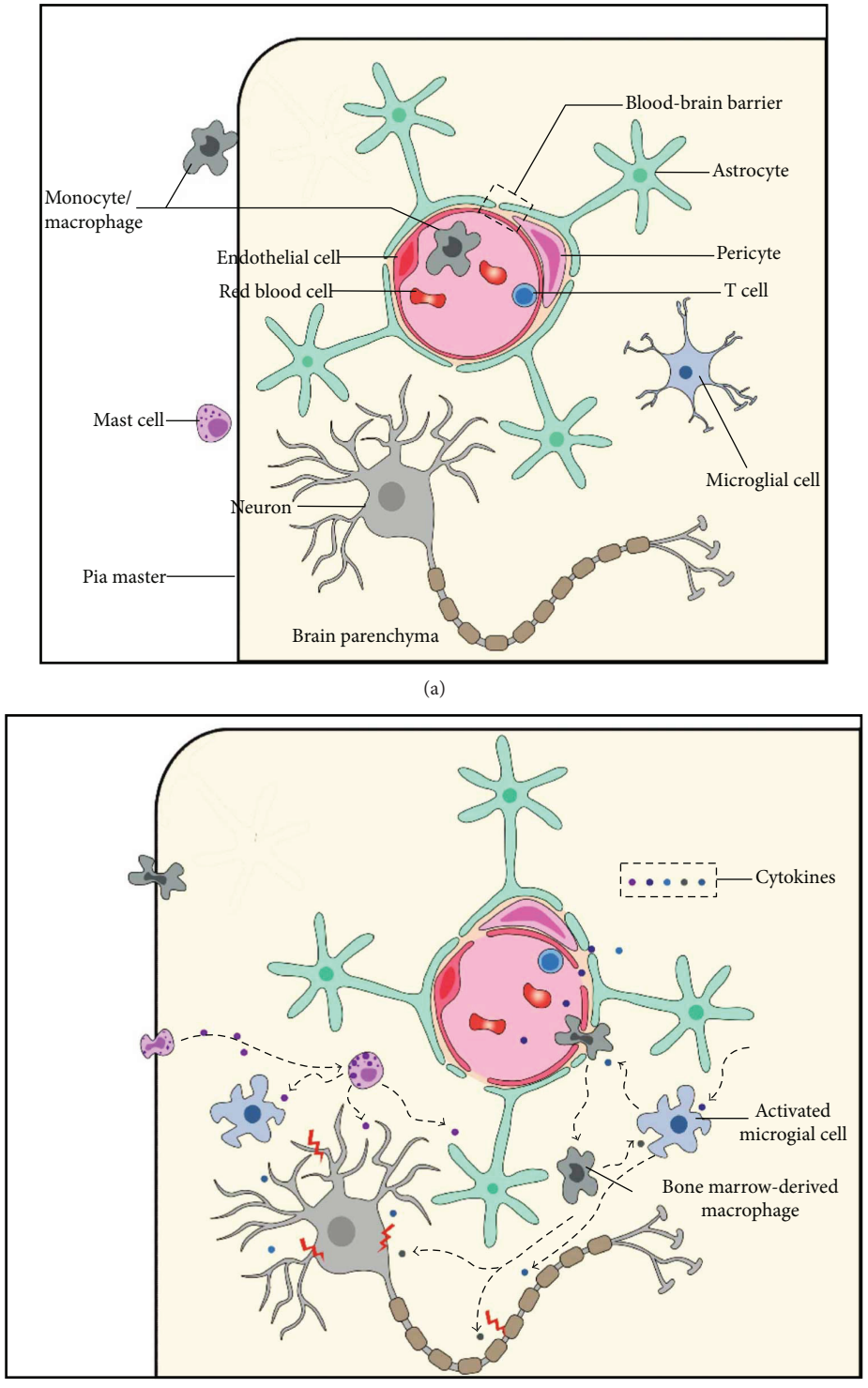


Figure 1: Schematic diagram of immune cells in POCD. (a) Under a normal condition, neurons are normally functioning. Microglia are ramified and in a resting state. The BBB is intact. Monocytes, mast cells, and T cells are restricted outside the brain parenchyma. (b) After surgery, many cytokines are released from the injured sites and damage the BBB. Microglia are triggered by these cytokines and turned into an activated, amoeboid shape. Microglia-secreted cytokines can damage neurons and also recruit BMDMs and other inflammatory cells from the blood. BMDMs and MCs infiltrate into the brain parenchyma and release more cytokines, which can directly damage neurons and also activate microglia. Cytokines secreted by T cells also participate in neuroinflammation in POCD. The immune cells and cytokines compose an inflammation network that aggravates neural damage, leading to POCD. POCD: postoperative cognitive dysfunction; BBB: blood-brain barrier; BMDM: bone marrow-derived macrophage; MC: mast cell.

indicates that MCs and glial cells work in concert to promote neuroinflammation [78].

While numerous studies in rodents have explored the role of MCs in neurological diseases, relatively few have focused on MC function in POCD. Surgery was found to induce MC degranulation in mice [79]. Rats treated with the MC stabilizer Cromolyn showed less severe cognitive deficits after surgery, accompanied by increased BBB stability [16] and reduced microglia and astrocyte activation [79, 80]. Therefore, via disrupting BBB and activating microglia, MCs promote neuroinflammation in POCD. In the studies of MCs in POCD, Cromolyn was administered intracerebroventricularly [16, 79, 80]; the therapeutic efficacy of Cromolyn administered via other routes remains to be established. Masitinib, an oral selective tyrosine-kinase inhibitor, can effectively inhibit the survival, migration, and activity of MCs. In a clinical trial, masitinib slowed the cognitive decline in patients with AD [81]. The effectiveness of masitinib in the treatment and prevention of POCD also needs further investigation.

2.4. T Cells. The thymus-derived T cells constitute key players in antigen-specific immune responses. T cells are divided into three main functional subsets: CD8 cells, also known as cytotoxic T cells; helper CD4 cells (Th cells); and regulatory CD4 cells (Treg cells). In healthy noninflamed CSF, 90% of the total cells are T cells, predominantly CD4 cells [82]. In a pathological state, T cells can penetrate into the brain parenchyma. Multiple studies have shown the importance of T cells in autoimmune and virus infectious neurological diseases, such as multiple sclerosis and herpes simplex virus encephalitis [83]. Recently, the roles of T cells in neurodegenerative diseases have also received much attention. The activation of Th cells enhances the loss of dopaminergic neurons in a mouse model of PD [84], while Treg cells provide neuroprotection through the attenuation of microglial activation in this disorder [85].

There is no direct evidence of T cells participating in the pathological process of POCD. One study demonstrated that surgery-induced cognitive impairment in mice was accompanied by upregulation of IL-17 and downregulation of IL-10 expression, mainly in Th17 (a subset of Th cells) and Treg cells, respectively [17]. This study proposed the possibility that a T cell-subtype imbalance may contribute to POCD. More evidence is needed to uncover the role of T cells in POCD.

3. Conclusion

While a plethora of studies have suggested that immune cells trigger neuroinflammation in response to surgery leading to POCD, the neurobiological basis of POCD remains unknown (Figure 1). As the major resident immune cells in the CNS, microglia are activated by proteins released from the injury sites and circulating cytokines upregulated by surgery. The activation of microglia results in neuronal damage via the release of proinflammatory cytokines. Circulating BMDMs are recruited into the brain in response to surgery, a process that may be initiated by microglia-secreted MCP-1. The degranulation of MCs contributes to BBB disruption and the activation of microglia, further aggravating POCD. T cells may also be involved in POCD.

These immune cells interact with one another in the pathogenesis of POCD. Different elements of the resulting network of neuroinflammation may serve as targets in the prevention and treatment of POCD. First, cytokines leaking from the injury site are the primary trigger of the immune response in the CNS. Thus, approaches that inhibit cytokine release may prevent POCD. Second, microglia occupy the central position of the inflammatory network; hence, drugs that stabilize microglia or promote their transition to the M2 state may have beneficial effects. Third, other circulating immune cells penetrating into the brain parenchyma and secreting inflammatory cytokines exacerbate neuroinflammation. Therefore, therapies that reduce cytokine secretion by these immune cells may also be effective for treating POCD. Studies in rodents using blocking antibodies and other agents interfering with the neuroinflammation network have provided proof of concept for these strategies as POCD treatments [10, 11, 15, 17, 56, 60, 79, 80]. However, their feasibility in humans still needs to be validated. Further research on the mechanisms of immune cell involvement in POCD is urgently required to identify other potential targets for POCD treatment and prophylaxis.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1] S. Newman, J. Stygall, S. Hirani, S. Shaefi, and M. Maze, "Postoperative cognitive dysfunction after noncardiac surgery: a systematic review," *Anesthesiology*, vol. 106, no. 3, pp. 572–590, 2007.

[2] S. Paredes, L. Cortinez, V. Contreras, and B. Silbert, "Postoperative cognitive dysfunction at 3 months in adults after non-cardiac surgery: a qualitative systematic review," *Acta Anaesthesiologica Scandinavica*, vol. 60, no. 8, pp. 1043–1058, 2016.

[3] J. Steinmetz, K. B. Christensen, T. Lund, N. Lohse, L. S. Rasmussen, and ISPOCD Group, "Long-term consequences of postoperative cognitive dysfunction," *Anesthesiology*, vol. 110, no. 3, pp. 548–555, 2009.

[4] G. X. Lin, T. Wang, M. H. Chen, Z. H. Hu, and W. Ouyang, "Serum high-mobility group box 1 protein correlates with cognitive decline after gastrointestinal surgery," *Acta Anaesthesiologica Scandinavica*, vol. 58, no. 6, pp. 668–674, 2014.

[5] X. U. Zheng, Z. Ma, and X. Gu, "Plasma levels of tumor necrosis factor-α in adolescent idiopathic scoliosis patients serve as a predictor for the incidence of early postoperative cognitive dysfunction following orthopedic surgery," *Experimental and Therapeutic Medicine*, vol. 9, no. 4, pp. 1443–1447, 2015.

[6] D. Sun, L. Yang, Y. Wu, R. Liu, J. Han, and L. Wang, "Effect of intravenous infusion of dobutamine hydrochloride on the development of early postoperative cognitive dysfunction in elderly patients via inhibiting the release of tumor necrosis factor-α," *European Journal of Pharmacology*, vol. 741, pp. 150–155, 2014.

[7] L. Peng, L. Xu, and W. Ouyang, "Role of peripheral inflammatory markers in postoperative cognitive dysfunction (POCD): a meta-analysis," *PLoS One*, vol. 8, no. 11, article e79624, 2013.

[8] M. H. Ji, H. M. Yuan, G. F. Zhang et al., "Changes in plasma and cerebrospinal fluid biomarkers in aged patients with early postoperative cognitive dysfunction following total hip-replacement surgery," *Journal of Anesthesia*, vol. 27, no. 2, pp. 236–242, 2013.

[9] A. Buvanendran, J. S. Kroin, R. A. Berger et al., "Upregulation of prostaglandin E2 and interleukins in the central nervous system and peripheral tissue during and after surgery in humans," *Anesthesiology*, vol. 104, no. 3, pp. 403–410, 2006.

[10] M. Cibelli, A. R. Fidalgo, N. Terrando et al., "Role of interleukin-1β in postoperative cognitive dysfunction," *Annals of Neurology*, vol. 68, no. 3, pp. 360–368, 2010.

[11] N. Terrando, C. Monaco, D. Ma, B. M. J. Foxwell, M. Feldmann, and M. Maze, "Tumor necrosis factor-alpha triggers a cytokine cascade yielding postoperative cognitive decline," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 47, pp. 20518–20522, 2010.

[12] X. Wu, Y. Lu, Y. Dong et al., "The inhalation anesthetic isoflurane increases levels of proinflammatory TNF-α, IL-6, and IL-1β," *Neurobiology of Aging*, vol. 33, no. 7, pp. 1364–1378, 2012.

[13] A. M. Valentim, P. Di Giminiani, P. O. Ribeiro, P. Rodrigues, I. A. S. Olsson, and L. M. Antunes, "Lower isoflurane concentration affects spatial learning and neurodegeneration in adult mice compared with higher concentrations," *Anesthesiology*, vol. 113, no. 5, pp. 1099–1108, 2010.

[14] X. Feng, M. Valdearcos, Y. Uchida, D. Lutrin, M. Maze, and S. K. Koliwad, "Microglia mediate postoperative hippocampal inflammation and cognitive decline in mice," *JCI Insight*, vol. 2, no. 7, article e91229, 2017.

[15] V. Degos, S. Vacas, Z. Han et al., "Depletion of bone marrow-derived macrophages perturbs the innate immune response to surgery and reduces postoperative memory dysfunction," *Anesthesiology*, vol. 118, no. 3, pp. 527–536, 2013.

[16] S. Zhang, H. Dong, X. Zhang, N. Li, J. Sun, and Y. Qian, "Cerebral mast cells contribute to postoperative cognitive dysfunction by promoting blood brain barrier disruption," *Behavioural Brain Research*, vol. 298, Part B, pp. 158–166, 2016.

[17] A. Tian, H. Ma, X. Cao, R. Zhang, X. Wang, and B. Wu, "Vitamin D improves cognitive function and modulates T_{h17}/T_{reg} cell balance after hepatectomy in mice," *Inflammation*, vol. 38, no. 2, pp. 500–509, 2015.

[18] R. M. Ransohoff and V. H. Perry, "Microglial physiology: unique stimuli, specialized responses," *Annual Review of Immunology*, vol. 27, no. 1, pp. 119–145, 2009.

[19] F. Ginhoux, M. Greter, M. Leboeuf et al., "Fate mapping analysis reveals that adult microglia derive from primitive macrophages," *Science*, vol. 330, no. 6005, pp. 841–845, 2010.

[20] R. M. Ransohoff and B. Engelhardt, "The anatomical and cellular basis of immune surveillance in the central nervous system," *Nature Reviews Immunology*, vol. 12, no. 9, pp. 623–635, 2012.

[21] A. Nimmerjahn, F. Kirchhoff, and F. Helmchen, "Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo," *Science*, vol. 308, no. 5726, pp. 1314–1318, 2005.

[22] J. V. Faustino, X. Wang, C. E. Johnson et al., "Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke," *The Journal of Neuroscience*, vol. 31, no. 36, pp. 12992–13001, 2011.

[23] M. Lalancette-Hebert, G. Gowing, A. Simard, Y. C. Weng, and J. Kriz, "Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain," *The Journal of Neuroscience*, vol. 27, no. 10, pp. 2596–2605, 2007.

[24] H. Akiyama, S. Barger, S. Barnum et al., "Inflammation and Alzheimer's disease," *Neurobiology of Aging*, vol. 21, no. 3, pp. 383–421, 2000.

[25] M. Politis, N. Pavese, Y. F. Tai et al., "Microglial activation in regions related to cognitive function predicts disease onset in Huntington's disease: a multimodal imaging study," *Human Brain Mapping*, vol. 32, no. 2, pp. 258–270, 2011.

[26] L. Qian, P. M. Flood, and J. S. Hong, "Neuroinflammation is a key player in Parkinson's disease and a prime target for therapy," *Journal of Neural Transmission*, vol. 117, pp. 971–979, 2010.

[27] E. Pena-Altamira, S. Petralla, F. Massenzio, M. Virgili, M. L. Bolognesi, and B. Monti, "Nutritional and pharmacological strategies to regulate microglial polarization in cognitive aging and Alzheimer's disease," *Frontiers in Aging Neuroscience*, vol. 9, p. 175, 2017.

[28] R. S. Jones, A. M. Minogue, O. Fitzpatrick, and M. A. Lynch, "Inhibition of JAK2 attenuates the increase in inflammatory markers in microglia from APP/PS1 mice," *Neurobiology of Aging*, vol. 36, no. 10, pp. 2716–2724, 2015.

[29] C. H. Latta, T. L. Sudduth, E. M. Weekman et al., "Determining the role of IL-4 induced neuroinflammation in microglial activity and amyloid-β using BV2 microglial cells and APP/PS1 transgenic mice," *Journal of Neuroinflammation*, vol. 12, no. 1, p. 41, 2015.

[30] D. Zhu, N. Yang, Y. Y. Liu, J. Zheng, C. Ji, and P. P. Zuo, "M2 macrophage transplantation ameliorates cognitive dysfunction in amyloid-β-treated rats through regulation of microglial polarization," *Journal of Alzheimer's Disease*, vol. 52, no. 2, pp. 483–495, 2016.

[31] V. Hernandez-Rabaza, A. Cabrera-Pastor, L. Taoro-Gonzalez et al., "Hyperammonemia induces glial activation, neuroinflammation and alters neurotransmitter receptors in hippocampus, impairing spatial learning: reversal by sulforaphane," *Journal of Neuroinflammation*, vol. 13, no. 1, p. 41, 2016.

[32] C. Qin, W. H. Fan, Q. Liu et al., "Fingolimod protects against ischemic white matter damage by modulating microglia toward M2 polarization via STAT3 pathway," *Stroke*, vol. 48, no. 12, pp. 3336–3346, 2017.

[33] A. Kumar, J. P. Barrett, D. M. Alvarez-Croda, B. A. Stoica, A. I. Faden, and D. J. Loane, "NOX2 drives M1-like microglial/macrophage activation and neurodegeneration following experimental traumatic brain injury," *Brain, Behavior, and Immunity*, vol. 58, pp. 291–309, 2016.

[34] T. Chen, R. Hou, S. Xu, and C. Wu, "Donepezil regulates 1-methyl-4-phenylpyridinium-induced microglial polarization in Parkinson's disease," *ACS Chemical Neuroscience*, vol. 6, no. 10, pp. 1708–1714, 2015.

[35] U. Heinemann, "New dangers of anesthesia: isoflurane induced opening of the blood-brain barrier (commentary on Tetrault et al.)," *The European Journal of Neuroscience*, vol. 28, no. 7, p. 1329, 2008.

[36] J. K. Chan, J. Roth, J. J. Oppenheim et al., "Alarmins: awaiting a clinical response," *The Journal of Clinical Investigation*, vol. 122, no. 8, pp. 2711–2719, 2012.

[37] S. Koizumi, K. Ohsawa, K. Inoue, and S. Kohsaka, "Purinergic receptors in microglia: functional modal shifts of microglia mediated by P2 and P1 receptors," *Glia*, vol. 61, no. 1, pp. 47–54, 2013.

[38] M. Pickering, D. Cumiskey, and J. J. O'Connor, "Actions of TNF-α on glutamatergic synaptic transmission in the central nervous system," *Experimental Physiology*, vol. 90, no. 5, pp. 663–670, 2005.

[39] S. Tanaka, H. Kondo, K. Kanda et al., "Involvement of interleukin-1 in lipopolysaccaride-induced microglial activation and learning and memory deficits," *Journal of Neuroscience Research*, vol. 89, no. 4, pp. 506–514, 2011.

[40] P. Taepavarapruk and C. Song, "Reductions of acetylcholine release and nerve growth factor expression are correlated with memory impairment induced by interleukin-1β administrations: effects of omega-3 fatty acid EPA treatment," *Journal of Neurochemistry*, vol. 112, no. 4, pp. 1054–1064, 2010.

[41] C. Cunningham, D. C. Wilcockson, S. Campion, K. Lunnon, and V. H. Perry, "Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration," *The Journal of Neuroscience*, vol. 25, no. 40, pp. 9275–9284, 2005.

[42] S. Tanaka, M. Ide, T. Shibutani et al., "Lipopolysaccharide-induced microglial activation induces learning and memory deficits without neuronal cell death in rats," *Journal of Neuroscience Research*, vol. 83, no. 4, pp. 557–566, 2006.

[43] H. L. Wang, R. H. Ma, H. Fang, Z. G. Xue, and Q. W. Liao, "Impaired spatial learning memory after isoflurane anesthesia or appendectomy in aged mice is associated with microglia activation," *Journal of Cell Death*, vol. 8, pp. 9–19, 2015.

[44] Y. Wang, H. He, D. Li et al., "The role of the TLR4 signaling pathway in cognitive deficits following surgery in aged rats," *Molecular Medicine Reports*, vol. 7, no. 4, pp. 1137–1142, 2013.

[45] J. Suenaga, X. Hu, H. Pu et al., "White matter injury and microglia/macrophage polarization are strongly linked with age-related long-term deficits in neurological function after stroke," *Experimental Neurology*, vol. 272, pp. 109–119, 2015.

[46] D. M. Mosser and J. P. Edwards, "Exploring the full spectrum of macrophage activation," *Nature Reviews Immunology*, vol. 8, no. 12, pp. 958–969, 2008.

[47] R. Shechter, A. London, C. Varol et al., "Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice," *PLoS Medicine*, vol. 6, no. 7, article e1000113, 2009.

[48] K. E. Bernstein, Y. Koronyo, B. C. Salumbides et al., "Angiotensin-converting enzyme overexpression in myelomonocytes prevents Alzheimer's-like cognitive decline," *The Journal of Clinical Investigation*, vol. 124, no. 3, pp. 1000–1012, 2014.

[49] O. Butovsky, G. Kunis, M. Koronyo-Hamaoui, and M. Schwartz, "Selective ablation of bone marrow-derived dendritic cells increases amyloid plaques in a mouse Alzheimer's disease model," *The European Journal of Neuroscience*, vol. 26, no. 2, pp. 413–416, 2007.

[50] E. R. Chernykh, M. Y. Kafanova, E. Y. Shevela et al., "Clinical experience with autologous M2 macrophages in children with severe cerebral palsy," *Cell Transplant*, vol. 23, Supplement 1, pp. 97–104, 2014.

[51] C. L. Hsieh, C. C. Kim, B. E. Ryba et al., "Traumatic brain injury induces macrophage subsets in the brain," *European Journal of Immunology*, vol. 43, no. 8, pp. 2010–2022, 2013.

[52] C. L. Hsieh, E. C. Niemi, S. H. Wang et al., "CCR2 deficiency impairs macrophage infiltration and improves cognitive function after traumatic brain injury," *Journal of Neurotrauma*, vol. 31, no. 20, pp. 1677–1688, 2014.

[53] M. D. Hammond, R. A. Taylor, M. T. Mullen et al., "CCR2^{+} Ly6C^{hi} inflammatory monocyte recruitment exacerbates acute disability following intracerebral hemorrhage," *The Journal of Neuroscience*, vol. 34, no. 11, pp. 3901–3909, 2014.

[54] B. Ajami, J. L. Bennett, C. Krieger, K. M. McNagny, and F. M. Rossi, "Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool," *Nature Neuroscience*, vol. 14, no. 9, pp. 1142–1149, 2011.

[55] I. L. King, T. L. Dickendesher, and B. M. Segal, "Circulating Ly-6C^{+} myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease," *Blood*, vol. 113, no. 14, pp. 3190–3197, 2009.

[56] N. Terrando, L. I. Eriksson, J. K. Ryu et al., "Resolving postoperative neuroinflammation and cognitive decline," *Annals of Neurology*, vol. 70, no. 6, pp. 986–995, 2011.

[57] N. L. Sparkman, J. B. Buchanan, J. R. Heyen, J. Chen, J. L. Beverly, and R. W. Johnson, "Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers," *The Journal of Neuroscience*, vol. 26, no. 42, pp. 10709–10716, 2006.

[58] C. Shi and E. G. Pamer, "Monocyte recruitment during infection and inflammation," *Nature Reviews Immunology*, vol. 11, no. 11, pp. 762–774, 2011.

[59] M. J. Cohen, K. Brohi, C. S. Calfee et al., "Early release of high mobility group box nuclear protein 1 after severe trauma in humans: role of injury severity and tissue hypoperfusion," *Critical Care*, vol. 13, no. 6, article R174, 2009.

[60] S. Vacas, V. Degos, K. J. Tracey, and M. Maze, "High-mobility group box 1 protein initiates postoperative cognitive decline by engaging bone marrow-derived macrophages," *Anesthesiology*, vol. 120, no. 5, pp. 1160–1167, 2014.

[61] C. D'Mello, T. Le, and M. G. Swain, "Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factorα signaling during peripheral organ inflammation," *The Journal of Neuroscience*, vol. 29, no. 7, pp. 2089–2102, 2009.

[62] S. C. Bischoff and G. Sellge, "Mast cell hyperplasia: role of cytokines," *International Archives of Allergy and Immunology*, vol. 127, no. 2, pp. 118–122, 2002.

[63] S. J. Galli and M. Tsai, "Mast cells in allergy and infection: versatile effector and regulatory cells in innate and adaptive immunity," *European Journal of Immunology*, vol. 40, no. 7, pp. 1843–1851, 2010.

[64] M. Khalil, J. Ronda, M. Weintraub, K. Jain, R. Silver, and A. J. Silverman, "Brain mast cell relationship to neurovasculature during development," *Brain Research*, vol. 1171, pp. 18–29, 2007.

[65] R. M. Ransohoff and M. A. Brown, "Innate immunity in the central nervous system," *The Journal of Clinical Investigation*, vol. 122, no. 4, pp. 1164–1171, 2012.

[66] D. Ribatti, "The crucial role of mast cells in blood–brain barrier alterations," *Experimental Cell Research*, vol. 338, no. 1, pp. 119–125, 2015.

[67] N. Li, X. Zhang, H. Dong, Y. Hu, and Y. Qian, "Bidirectional relationship of mast cells-neurovascular unit communication in neuroinflammation and its involvement in POCD," *Behavioural Brain Research*, vol. 322, Part A, pp. 60–69, 2017.

[68] P. Esposito, D. Gheorghe, K. Kandere et al., "Acute stress increases permeability of the blood–brain-barrier through activation of brain mast cells," *Brain Research*, vol. 888, no. 1, pp. 117–127, 2001.

[69] B. A. Sayed, A. L. Christy, M. E. Walker, and M. A. Brown, "Meningeal mast cells affect early T cell central nervous system infiltration and blood-brain barrier integrity through TNF: a role for neutrophil recruitment?," *Journal of Immunology*, vol. 184, no. 12, pp. 6891–6900, 2010.

[70] C. M. McKittrick, C. E. Lawrence, and H. V. Carswell, "Mast cells promote blood brain barrier breakdown and neutrophil infiltration in a mouse model of focal cerebral ischemia," *Journal of Cerebral Blood Flow & Metabolism*, vol. 35, no. 4, pp. 638–647, 2015.

[71] Y. Jin, A. J. Silverman, and S. J. Vannucci, "Mast cells are early responders after hypoxia-ischemia in immature rat brain," *Stroke*, vol. 40, no. 9, pp. 3107–3112, 2009.

[72] S. D. Skaper, L. Facci, and P. Giusti, "Neuroinflammation, microglia and mast cells in the pathophysiology of neurocognitive disorders: a review," *CNS & Neurological Disorders - Drug Targets*, vol. 13, no. 10, pp. 1654–1666, 2014.

[73] S. Zhang, X. Zeng, H. Yang, G. Hu, and S. He, "Mast cell tryptase induces microglia activation via protease-activated receptor 2 signaling," *Cellular Physiology and Biochemistry*, vol. 29, no. 5-6, pp. 931–940, 2012.

[74] X. Zhang, Y. Wang, H. Dong, Y. Xu, and S. Zhang, "Induction of microglial activation by mediators released from mast cells," *Cellular Physiology and Biochemistry*, vol. 38, no. 4, pp. 1520–1531, 2016.

[75] H. Dong, W. Zhang, X. Zeng et al., "Histamine induces upregulated expression of histamine receptors and increases release of inflammatory mediators from microglia," *Molecular Neurobiology*, vol. 49, no. 3, pp. 1487–1500, 2014.

[76] L. Dm Juri, M. Krzan, and M. Lipnik-Stangelj, "Histamine and astrocyte function," *Pharmacological Research*, vol. 111, pp. 774–783, 2016.

[77] X. Zeng, S. Zhang, L. Xu, H. Yang, and S. He, "Activation of protease-activated receptor 2-mediated signaling by mast cell tryptase modulates cytokine production in primary cultured astrocytes," *Mediators of Inflammation*, vol. 2013, Article ID 140812, 10 pages, 2013.

[78] S. D. Skaper, L. Facci, and P. Giusti, "Mast cells, glia and neuroinflammation: partners in crime?," *Immunology*, vol. 141, no. 3, pp. 314–327, 2014.

[79] X. Zhang, H. Dong, N. Li et al., "Activated brain mast cells contribute to postoperative cognitive dysfunction by evoking microglia activation and neuronal apoptosis," *Journal of Neuroinflammation*, vol. 13, no. 1, p. 127, 2016.

[80] X. Zhang, H. Yao, Q. Qian, N. Li, W. Jin, and Y. Qian, "Cerebral mast cells participate in postoperative cognitive dysfunction by promoting astrocyte activation," *Cellular Physiology and Biochemistry*, vol. 40, no. 1-2, pp. 104–116, 2016.

[81] F. Piette, J. Belmin, H. Vincent et al., "Masitinib as an adjunct therapy for mild-to-moderate Alzheimer's disease: a randomised, placebo-controlled phase 2 trial," *Alzheimer's Research & Therapy*, vol. 3, no. 2, p. 16, 2011.

[82] P. Kivisakk, D. J. Mahad, M. K. Callahan et al., "Human cerebrospinal fluid central memory $CD4^+$ T cells: evidence for trafficking through choroid plexus and meninges via P-selectin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 14, pp. 8389–8394, 2003.

[83] R. S. Liblau, D. Gonzalez-Dunia, H. Wiendl, and F. Zipp, "Neurons as targets for T cells in the nervous system," *Trends in Neurosciences*, vol. 36, no. 6, pp. 315–324, 2013.

[84] V. Brochard, B. Combadiere, A. Prigent et al., "Infiltration of $CD4^+$ lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease," *The Journal of Clinical Investigation*, vol. 119, no. 1, pp. 182–192, 2009.

[85] A. D. Reynolds, D. K. Stone, J. A. Hutter, E. J. Benner, R. L. Mosley, and H. E. Gendelman, "Regulatory T cells attenuate Th17 cell-mediated nigrostriatal dopaminergic neurodegeneration in a model of Parkinson's disease," *The Journal of Immunology*, vol. 184, no. 5, pp. 2261–2271, 2010.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 6265746, 10 pages
https://doi.org/10.1155/2018/6265746

Research Article

Vγ4+ T Cells: A Novel IL-17-Producing γδ T Subsets during the Early Phase of Chlamydial Airway Infection in Mice

Li-da Sun, Sai Qiao, Yue Wang, Gao-ju Pang, Xiao-yu Zha, Teng-Li Liu, Hui-Li Zhao, Ju-You Liang, Ning-bo Zheng, Lu Tan, Hong Zhang, and Hong Bai

Department of Immunology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin Key Laboratory of Cellular and Molecular Immunology, Key Laboratory of Educational Ministry of China, Tianjin 300070, China

Correspondence should be addressed to Hong Bai; hongbai25@163.com

Received 14 November 2017; Accepted 18 December 2017; Published 18 February 2018

Academic Editor: Qingdong Guan

Copyright © 2018 Li-da Sun et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Our previous studies showed that γδ T cells provided immune protection against Chlamydial *muridarum* (Cm), an obligate intracellular strain of chlamydia trachomatis, lung infection by producing abundant IL-17. In this study, we investigated the proliferation and activation of lung γδ T cell subsets, specifically the IL-17 and IFNγ production by them following Cm lung infection. Our results found that five γδ T cell subsets, Vγ1+ T, Vγ2+ T, Vγ4+ T, Vγ5+ T, and Vγ6+ T, expressed in lungs of naïve mice, while Cm lung infection mainly induced the proliferation and activation of Vγ4+ T cells at day 3 p.i., following Vγ1+ T cells at day 7 p.i. Cytokine detection showed that Cm lung infection induced IFNγ secretion firstly by Vγ4+ T cells at very early stage (day 3) and changed to Vγ1+ T cells at midstage (day 7). Furthermore, Vγ4+ T cell is the main γδ T cell subset that secretes IL-17 at the very early stage of Cm lung infection and Vγ1+ T cell did not secrete IL-17 during the infection. These findings provide in vivo evidence that Vγ4+T cells are the major IL-17 and IFNγ-producing γδ T cell subsets at the early period of Cm lung infection.

1. Introduction

Chlamydia, an obligated intracellular bacterium, can cause various human diseases by the two chlamydial species, Chlamydia *trachomatis* and Chlamydia *pneumoniae*. C. *pneumoniae* causes respiratory diseases like bronchitis, sinusitis, and pneumonia, whereas C. *trachomatis* is a major cause of ocular and sexually transmitted diseases [1]. The mouse pneumonitis strain of C. *trachomatis*, recently designated as Chlamydia *muridarum* (Cm), has been widely used in mouse models of respiratory and genital tract infections [2]. Th1 response has been demonstrated to be the dominant protective determinant for controlling chlamydial infection in human and mouse models [3–5]. More recently, our and others' studies indicate that Th17 plays an important role in host defense against chlamydial infection through either promoting Th1-type cell responses or working synergistically with IFNγ [6]. Therefore, the development of both Th1 and Th17 cell immune responses is optimal for host defense against chlamydial lung infections.

Although αβT cells dominate Ag-specific effector and memory stages, γδ T cells have fused adaptive and innate-like qualities to be at the forefront of immune responses. γδ T cells can directly kill infected cells, produce molecules required for pathogen clearance, and release immunomodulatory cytokines such as IFNγ, IL-17, and IL-4 [7, 8] with no MHC-limited recognition and antigen processing or presentation [9–11]. A number of recent studies using various experimental mouse models have shown that γδ T cell is also a major producer of IL-17 following intracellular pathogen infections, including H1N1 influenza virus [12], *Staphylococcus aureus* [13], *Listeria monocytogenes* [14], and Salmonella enterica enteritidis [15]. In general, activated γδ T cells mainly make resistance to pathogens by secreting IFNγ. However, a growing number of studies recently showed that γδ T cells are an important source of

proinflammatory cytokine IL-17 [16], and in some researches, IL-17-producing $\gamma\delta$ T cells expanded more faster than $\alpha\beta$T cells and worked more effectively than adaptive CD4+ Th17 cells [17, 18].

According to the difference of TCRγ, the mouse $\gamma\delta$ T cells are divided into 6 kinds of $\gamma\delta$ T cell subsets, including Vγ1+ T, Vγ2+ T, Vγ4+ T, Vγ5+ T, Vγ6+ T, and Vγ7+ T cells and lung $\gamma\delta$ T cells of naïve mice predominantly comprising Vγ1+ and Vγ4+ subsets [19, 20]. Study on a variety of disease models showed that the specific TCR-expressing VγT cells play its unique function [21]. For example, Vγ1+ T cells aggravated airway responsiveness, whereas Vγ4+ T cells reversed airway responsiveness [22]. Although the function of $\gamma\delta$ T cells has been demonstrated in a variety of mouse models such as Klebsiella pneumonia [23] and cryptococcal pneumonia [24], the subsets of $\gamma\delta$ T cells in lung inflammation were seldom investigated. Current studies have shown that Vγ4+ T cells are the dominant IL-17-producing cells in infectious or noninfectious diseases. The ability of Vγ1+ $\gamma\delta$ T cells to produce IFNγ was significantly reduced in the late phase of blood-stage Plasmodium berghei XAT (PbXAT) parasite infection [25]. In infectious model of Lester coli [26], *Escherichia coli* [27], Bacillus subtilis [28], and Vγ4+ T quickly secreted a large number of IL-17 combined with IL-23 produced by DC during infection. Vγ4+ T cells produced IL-17 but not IFNγ in a mouse model of collagen-induced arthritis (CIA) [29].

Our previous study found that depletion of $\gamma\delta$ T cells reduced IL-1α production by dendritic cells, which was associated with a reduced Th17 protective response during Cm infection [6]. Large amounts of IFNγ and IL-17 existed at the early stage of infection participate in host immune response against Chlamydia infection. However, the sources of IFNγ and IL-17 production by which of $\gamma\delta$ T cell subset in lungs and their biological activities following chlamydial infection remained unclear. Here, we will further elucidate the properties and the role of $\gamma\delta$ T cell subsets during Cm lung infection and also provide a theoretical basis for clinical diagnosis and treatment of chlamydia infectious diseases and their complications.

2. Materials and Methods

2.1. Mice and Microorganisms. Breeding pairs of TCRδ−/− mice (C57BL/6) were gifted from Nankai University, Professor Yin Zhinan. The WT control mice (C57BL/6) were purchased from Laboratory Animal Center, Academy of Military Medical Sciences. Mice were housed in specific pathogen-free conditions in Tianjin Medical University with autoclaved cage, food and water, and filtered airflow. Age- and sex-matched mice at 6–8 weeks old were used for study. Chlamydial muridarum (Cm), a mouse chlamydial strain, was reproduced and purified as previously described [30]. Briefly, Cm was grown in HeLa-229 cells in DMEM medium containing 10% fetal bovine serum (FBS) and 2 mM glutamine. Elementary bodies (EBs) were purified by discontinuous density gradient centrifugation. Titers of EBs were determined by measuring inclusion-forming units (IFUs) after immunostaining, and aliquots of the EB stock were stored at −80°C.

2.2. Infection of Mice and Quantification of Lung Chlamydial Loads. Mice were sedated with isoflurane and infected intranasally with 1×10^3 IFUs of C. muridarum in 40 μl sucrose-phosphate-glutamic acid (SPG) buffer. Mouse body weights were monitored daily. Mice were euthanized at the indicated time points, and the lungs were aseptically isolated and homogenized using a cell grinder in SPG buffer. The tissue homogenates were centrifuged, and supernatant was stored at −80°C until being tested. The bacterial loads in lungs at day 3, day 7, and day 14 after Cm infection were titrated by infection of HeLa cell monolayers as previously described [31].

2.3. Lung Mononuclear Cell Preparation. Lung mononuclear cells were prepared as described previously [9]. Briefly, the lung tissues were incubated in digestive buffer (containing 100 μg/ml DNase [Sigma-Aldrich] and 2 mg/ml collagenase type XI [Sigma-Aldrich, St. Louis, MO, USA]) for 60 min at 37°C and added 2 mM EDTA 5 min before incubation finished. Then the cell population was purified by mixing with 35% Percoll (Sigma-Aldrich) and centrifuged for 20 min at 750 g, followed by lysis of erythrocytes with ammonium chloride-potassium (ACK) lysis buffer (150 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, and 0.1 mmol/l EDTA). The cells were washed twice using RPMI 1640 with 2% fetal calf serum and resuspended in complete RPMI 1640 medium (containing 10% FBS) for further experiment.

2.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). To analyze the expressions of TCR Vγ transcripts, total RNA was extracted from frozen lung tissues using Trizol agent (Invitrogen) according to the manufacturer's instruction. The isolated total RNA was reversely transcribed into cDNA (TaKaRa). Special primers for Vγ1, Vγ2, Vγ4, Vγ5, Vγ6, and Vγ7 were used to amplify cDNA. And β-actin, a housekeeping gene, was used as a control. The primers used in the PCR analysis were as follows: Vγ1 (320 bp), forward: 5′-ACACAGCTATACATTGGTAC-3′, reverse: 5′-CTTATGGAGATTTGTTTCAGC-3′; Vγ2 (270 bp), forward: 5′-CGGCAAAAAACAAATCAACAG-3′, reverse: 5′-CTTATGGAGATTTGTTTCAGC-3′; Vγ4 (310 bp), forward: 5′-TGTCCTTGCAACCCCTACCC-3′, reverse: 5′-CTTATGGAGATTTGTTTCAGC-3′; Vγ5 (300 bp), forward: 5′-TGTGCACTGGTACCAACTGA-3′, reverse: 5′-CTTATGGAGATTTGTTTCAGC-3′; Vγ6 (300 bp), forward: 5′-TGTGCACTGGTACCAACTGA-3′, reverse: 5′-CTTATGGAGATTTGTTTCAGC-3′; Vγ7 (380 bp), forward: 5′-AAGCTAGAGGGGTCCTCTGC-3′, reverse: 5′-CTTATGGAGATTTGTTTCAGC-3′; β-actin (582 bp), forward: 5′-CTTATGGAGATTTGTTTCAGC-3′, reverse: 5′-ATGAGGTAGTCMGTCAGGT-3′. The products were electrophoresed in 1% agarose gel containing Gel-Red (0.01%). The bands were visualized and photographed by automatic gel imaging system and were analyzed for density on Image J software.

2.5. Flow Cytometry. Lung mononuclear cells were aseptically prepared from mice at different time points postinfection and incubated with anti-CD3, anti-TCR$\gamma\delta$, anti-CD69, anti-TCRVγ1, anti-TCRVγ4, and isotype control Abs for 30 min on ice for surface marker analysis. For intracellular cytokine analysis, single cell suspensions were stimulated with PMA (50 ng/ml)/ionomycin (1 μg/ml) (Sigma) for 6 hours at 37°C in the presence of 20 mg/ml brefeldin A (Sigma). After the stimulation, cells were washed with FACS buffer twice and incubated with Fc receptor (FcR) block antibodies (anti-CD16/CD32; eBioscience) for 15 min on ice to block nonspecific staining. Surface markers (CD3, TCR$\gamma\delta$, TCRVγ1, and TCRVγ4) were stained first. The cells were then fixed with 4% *w*/*v* paraformaldehyde in PBS and permeabilized with permeabilization buffer (0.1% saponin [Sigma] Sigma, 2% heat-inactivated FCS, and 0.1% NaN_3 in PBS), subsequently stained with anti-IFNγ, IL-17, or corresponding isotype control Abs (eBioscience). The raw data were collected using FACS CantoII flow cytometer (BD Biosciences) and were analyzed using Flowjo 6.0 software.

2.6. Statistical Analysis. One-way analysis of variance (ANOVA) and unpaired *t*-test were used to determine statistical significance among groups. IFUs of Cm were converted to logarithmic values and analyzed using ANOVA. The value of $p < 0.05$ was considered as a statistically significant difference.

3. Results

3.1. $\gamma\delta$ T Cells Mediated Immune Protection against Cm Infection by Expansion, Activation, and Secreting IFNγ and IL-17. $\gamma\delta$ T cells are the vital components of the innate immune system and play important roles in the early responses to pathogens. Our previous studies have shown that $\gamma\delta$ T cells are the major producer of IL-17A in the very early stages of infection and depletion of $\gamma\delta$ T cells by administration of mAb (GL3) against TCR$\gamma\delta$ i.n. exists more body weight loss following Cm lung infection. The results here keep consistent with our previous studies that the percentage and absolute number of lung $\gamma\delta$ T cells significantly increased at day 3 postinfection (p.i.) and reached to the highest level at day 7 p.i. Even though the percentage of $\gamma\delta$ T cells reduced to baseline levels, the absolute number of $\gamma\delta$ T cells still kept in a relatively higher level (Figures 1(b) and 1(c)). CD69 was generally used for indicating the activation of $\gamma\delta$ T cells. Figure 1(d) showed that Cm infection induced $\gamma\delta$ T cell activation in lungs by increased CD69 expression on $\gamma\delta$ T cells following Cm infection. Following activation, IFNγ or IL-17 secretion by $\gamma\delta$ T cells was significantly increased especially on day 3 p.i. (Figures 1(e)–1(h)). TCRδ−/− mice were used for further confirmation of the function of $\gamma\delta$ T cells during Cm lung infection in the current researches. With Cm lung infection, TCRδ−/− mice had more weight loss compared with WT mice, especiallly at day 3 to 6 p.i. (Figure 1(i)); however, the lung bacterial loads (IFUs) between TCRδ−/− and WT mice did not show a significant difference (Figure 1(j)). Furthermore, the lung tissues of TCRδ−/− mice had more inflammatory cell infiltration compared with WT mice after chlamydial lung infection (data not shown). All these results implicated that $\gamma\delta$ T cells contribute to the IFNγ and IL-17 production and reduce morbidity during Cm infection, but its role in bacterial clearance is rather limited.

3.2. Vγ1+ T and Vγ4+ T Cells Are the Major Proliferative Cell Subsets of $\gamma\delta$ T Cell during Cm Lung Infection in Mice. $\gamma\delta$ T cells are heterogeneous population that can be subdivided based on the expression of specific Vγ and Vδ TCR chains. Although we already demonstrated the importance of $\gamma\delta$ T cell in the early protection against Cm lung infection, this did not prove that $\gamma\delta$ T cell subpopulation actually contributes to the $\gamma\delta$ T cell-mediated early protection. To investigate this, we first analyzed the $\gamma\delta$ T cell subsets in lungs of naive mice. Our results by using RT-PCR detection showed that there are more than five subpopulations, Vγ1+ T, Vγ2+ T, Vγ4+ T, Vγ5+ T, and Vγ6+ T but not Vγ7+ T cells; in lungs of naive mice, the expression intensity of mRNA is Vγ2 > Vγ4 > Vγ1 > Vγ6 > Vγ5 (Figures 2(a) and 2(b)). Next, we further detected the mRNA expression of $\gamma\delta$ T cell subsets in the lungs at different time point post-Cm infection. The results showed that TCRVγ4 was significantly upregulated at day 3 p.i. while TCRVγ1 mRNA expression was significantly increased at day 7 p.i (Figures 2(c) and 2(d)). Vγ6+ mRNA also showed a relatively high expression level at day 7 p.i. Collectively, these results showed that Vγ1+ T and Vγ4+ T cells are the major proliferative cell subsets of $\gamma\delta$ T cell in lungs of mice during Cm infection.

3.3. Cm Infection Induced Dramatic Proliferation and Activation of Vγ1+ T and Vγ4+ T Cells in Lungs. Some studies have shown that Vγ1+ T and Vγ4+ T cells were induced to proliferate and activate and provide different roles in host defense against pathogen infection. To further confirm the proliferation and activation of the TCR Vγ1+ and TCR Vγ4+ $\gamma\delta$ T cells at an early stage of Cm infection, we examined the lung TCR Vγ1+ and TCR Vγ4+ $\gamma\delta$ T cell percentage and CD69 expression by FACS. As shown in Figure 3, the percentage, absolute number (Figure 3(a)), and CD69 expression (Figure 3(c)) of Vγ4+ T cell in lungs quickly reached the peak at day 3 p.i. and kept a high level in absolute number at day 7 p.i., while the percentage, absolute number (Figure 3(b)), and CD69 expression (Figure 3(d)) of Vγ1+ T cells significantly increased at day 3 p.i. and reached the peak at day 7 p.i. Taking these results together, we concluded that Cm infection induced dramatic proliferation and activation of TCR Vγ4+ and Vγ1+ $\gamma\delta$ T cells in the lungs at an early stage.

3.4. Both Vγ1+ and Vγ4+ T Cells Are the IFNγ-Producing $\gamma\delta$ T Cell Subpopulations at Different Stages of Cm Infection. IFNγ has been reported to be produced by several different $\gamma\delta$+ T cell subpopulations in different stages of disease and mediated various immune functions. As shown in Figure 4, both Vγ1+ and Vγ4+ T cells can produce IFNγ during Cm lung infection; however, Vγ4 T cells are the major sources of IFNγ at very early time p.i. (day 3) while Vγ1 T cells at midstage p.i. (day 7) (Figures 4(c) and 4(b)).

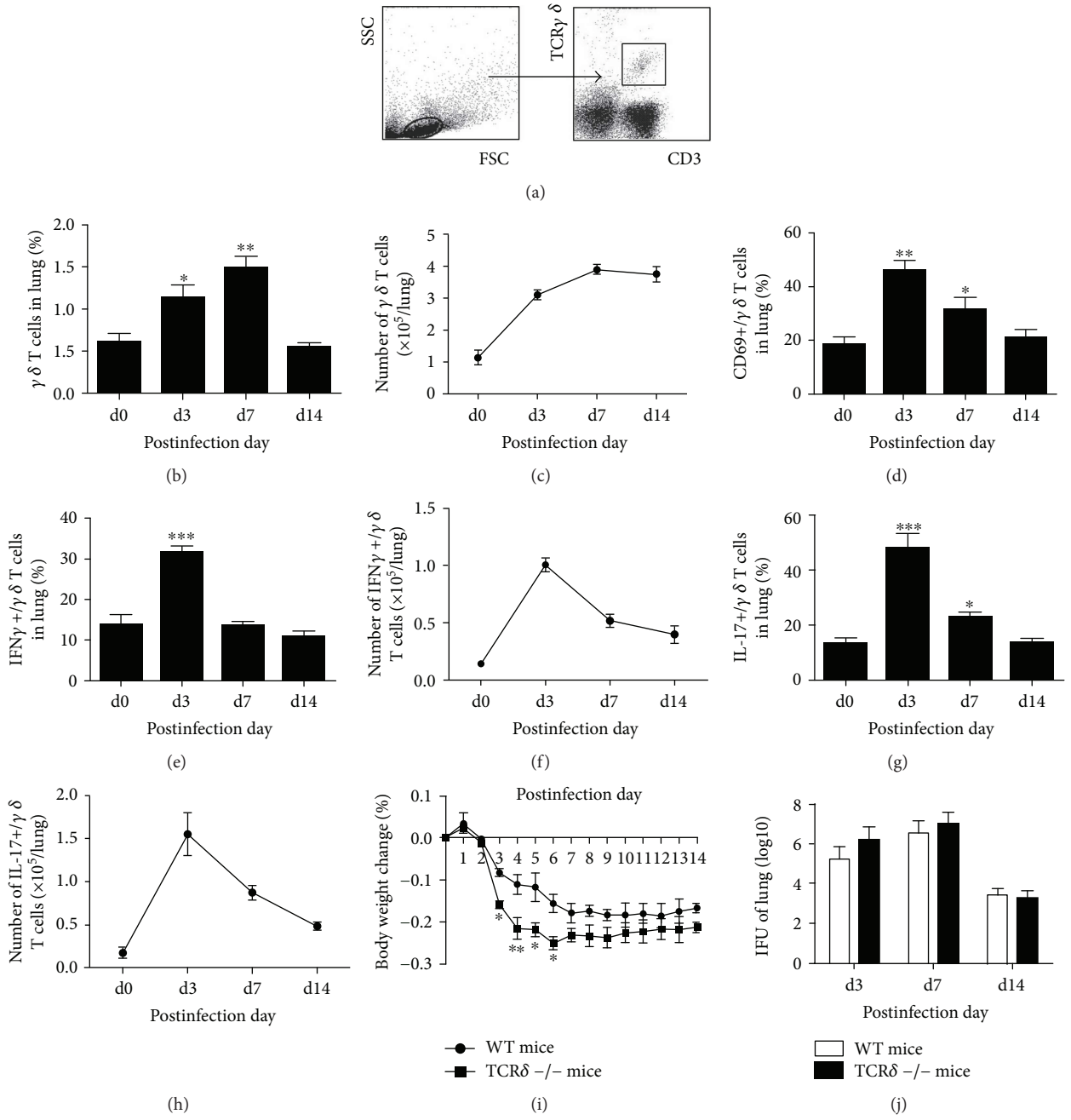


FIGURE 1: $\gamma\delta$ T cells provided immune protection against Cm infection by expansion, activation, and secreting *IFN*γ and *IL-17*. The mononuclear cells from WT mice (four/group) killed at specific time points following C. m*uridarum* infection (1×10^3 IFUs) were extracted from the lungs. In gated lymphocytes (a), percentage (b), and absolute number (c) of CD3+ TCR$\gamma\delta$+ T cells, expression level of CD69 on CD3+ T CR$\gamma\delta$+ T cells (d), percentage (e, g), and absolute number (f, h) of IFNγ/IL-17-produing $\gamma\delta$ T cells were analyzed and calculated by flow cytometry. WT and TCR$\delta^{-/-}$ mice (four/group) were infected intranasally with C. *muridarum* (1×10^3 IFUs). Body weight changes (i) were monitored daily, and pulmonary C. *muridarum* (j) were assessed at day 3, day 7, and day 14 p.i. as mentioned in Materials and Methods. Shown are the representative data of two independent experiments with similar results presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

After Cm infection, the secretion of IFNγ was gradually increased and reached the peak at day 7 p.i. which had significant difference with uninfected group then declined to the basic level at day 14 p.i. (Figure 4(b)). However, the percentage of IFNγ+ Vγ4+ T cells increased rapidly after infection and even reached the peak at day 3 p.i. then restored to the basic level at day 7 p.i. (Figure 4(c)). The absolute number of Vγ1+ T and Vγ4+ T cells in lungs (Figure 4(d)) also indicated the similar variation with their percentages. Taking these results together, we concluded that Cm lung infection induces IFNγ secretion from Vγ4+ T cells at very early stage and Vγ1+ T cells at midstage of infection.

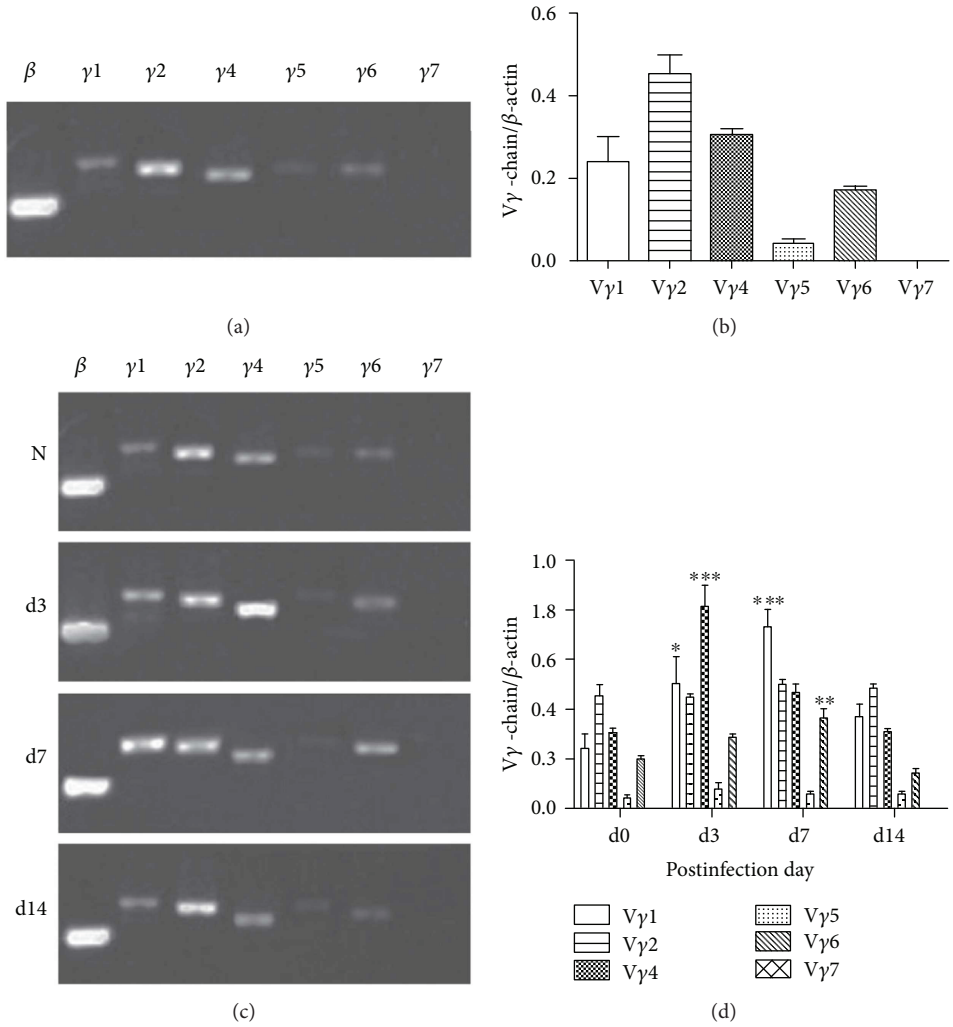


FIGURE 2: Vγ1+ T and Vγ4+ T cell are two main subsets of γδ T cells during Cm respiratory tract infection. The types of γδ T cell subsets from lung tissues in naïve (a, b) and infected mice (c, d) were defined according to the expression of TCRVγ mRNA in lungs detected by RT-PCR. Shown are the representative data of two independent experiments with similar results presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3.5. Vγ4+ T Cells Are the IL-17-Producing γδ T Cell Subpopulations at the Very Early Stage of Cm Infection. We further identified IL-17-producing γδ T cell subpopulations at different stage of Cm infection by intracellular cytokine staining. Few IL-17+ Vγ1+ T cells were detected in uninfected mice and had no significant increase following Cm lung infection (Figure 5(b)), whereas Vγ4+ T cells can secrete large quantity of IL-17 (Figure 5(c)) during Cm lung infection in mice. It was noted that the percentage of IL-17+ Vγ4+ T cells increased rapidly after infection and even reached the peak at day 3 p.i. and then quickly restored to the basic level at day 7 p.i. All these above results demonstrated that lung Cm-infected Vγ4+ T cell is the main γδ T cell subset secreting IL-17 at the very early stage of Cm lung infection. Meanwhile, there are still a small number of IL-17-producing-Vγ4-γδ T cell subsets which is not identified during Cm infection, which should be discussed further.

4. Discussion

γδ T cells provide immune protective in Chlamydia trachomatis infection. Here, we demonstrate the coincident involvement of multiple γδ T cell subsets. While a significant proportion of naive lung γδ T cells exhibited an activated phenotype, activation was clearly enhanced in infected mice, most notably in respect to Vγ1 and Vγ4 expression. Based on the kinetics of IFNγ and IL-17 production by γδ T cells, we tested the function of γδ T cell subset in the ensuing immune response against Cm infection. Surprisingly, we demonstrated that Vγ4+ T cells are the major IL-17 and IFNγ-producing γδ T cell subsets at the early period of Cm lung infection, while Vγ1+ T cells are responsible for the secretion of IFNγ at midstage.

γδ T cells express a distinct TCR composed of the TCRγ- and δ-chains [32]. Human γδ T cells can be divided into three main populations based on δ chain expression: Vδ1,

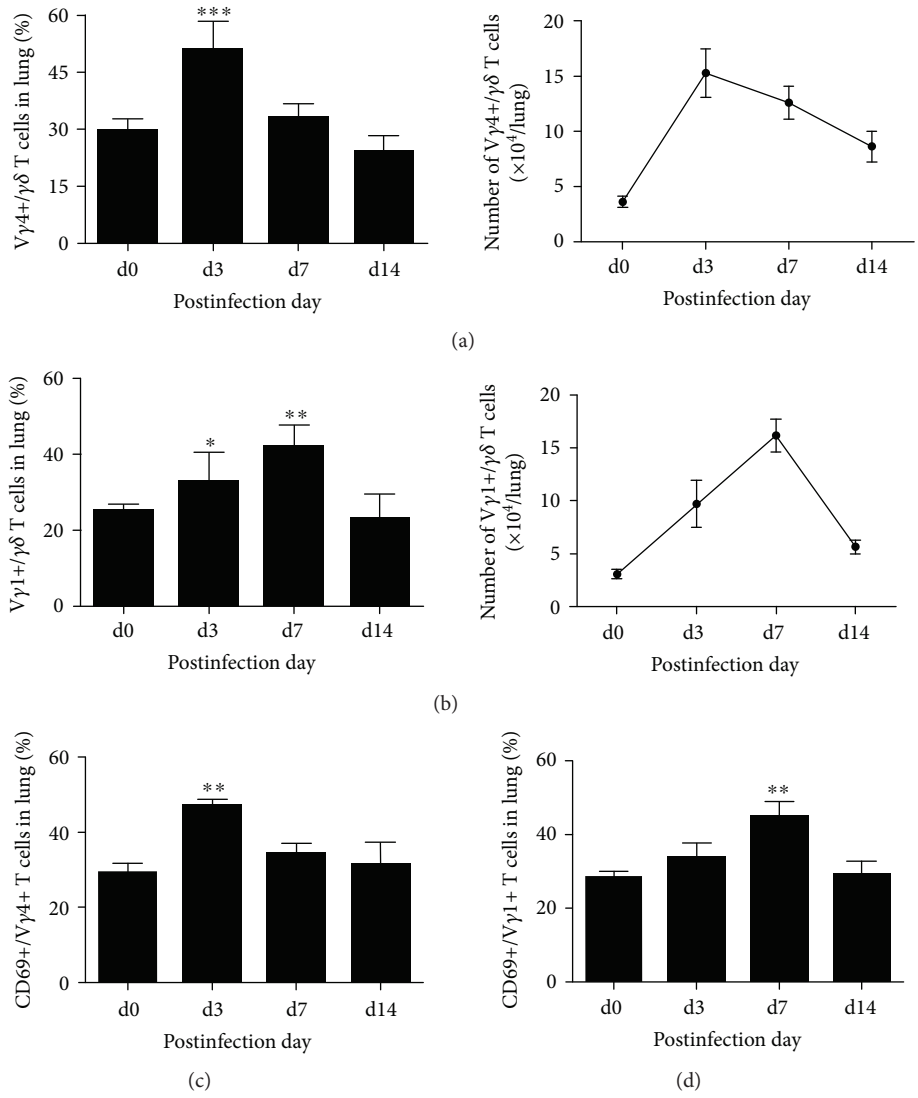


Figure 3: Vγ1+ T and Vγ4+ T cell proliferated and activated during Cm respiratory infection. Mononuclear cells in lung tissues at different time points postinfection were extracted. Staining with anti-mouse CD3, TCRγδ, TCRVγ1, and TCRVγ4 antibody to analyze the percentage and absolute number of TCRVγ1+ TCRγδ+ T cells (a) and TCRVγ4$^+$TCRγδ$^+$T cells (b) by flow cytometry. The activation extent of Vγ1+ T (c) and Vγ4+ T (d) cell was measured by the expression of CD69, staining with anti-mouse CD69 antibody. The results are presented as mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Vδ2, and Vδ3 γδ T cells. γδ T cells in lungs of infected mice are classified into six subsets, Vγ1+ T, Vγ2+ T, Vγ4+ T, Vγ5+ T, Vγ6+, and Vγ7+ γδ T cells in local responses to Streptococcus pneumoniae infection [33]. In our present study, according to distinct TCR γ chain expression, there are five subpopulations, Vγ1+ T, Vγ2+ T, Vγ4+ T, Vγ5+ T, and Vγ6+ T but not Vγ7+ T cells in lungs of naive mice. It was reported [34] that lung Vγ1+ and Vγ4+ γδ T cells proliferated significantly in pulmonary Mycobacterium bovis bacille Calmette-Guerin infection, and study on sepsis [35] showed that Vγ1+ γδ T cells preferentially expanded over time after infection with PbXAT parasites. Similarly, our results showed that the increase of CD69+ Vγ4+ T cells and CD69+ Vγ1δT cells showed to be concordant with subpopulation proliferation and infected lung γδ T cells comprising predominantly Vγ1+ and Vγ4+ subsets.

The effector functions of γδ T cells can be broadly classified by their tissue localization, status of activation, and expression of TCR variable genes [36]. IL-17-producing γδ T cells play a crucial role in innate immunity against various infections [26, 36, 37]. Our previous study has shown that γδ T cells are the major producer of IL-17 in the very early stages of infection, and the depletion of γδ T cells by administration of mAb (GL3) against TCRγδ i.n. exists more body weight loss following Cm lung infection, which suggested that γδ T cells played a protective role in mice Chlamydia lung infection [16]. These results are in accordance with our data using TCRδ−/− mice in this paper. It is worth mentioning that γδ T cell is the highest producer of IL-17A but the protection conferred by IL-17A is mainly mediated by Th17 cells following Cm infection. Therefore, the protective role of early production of IL-17 and IFNγ by Vγ4+ T and Vγ1+ T cells is not

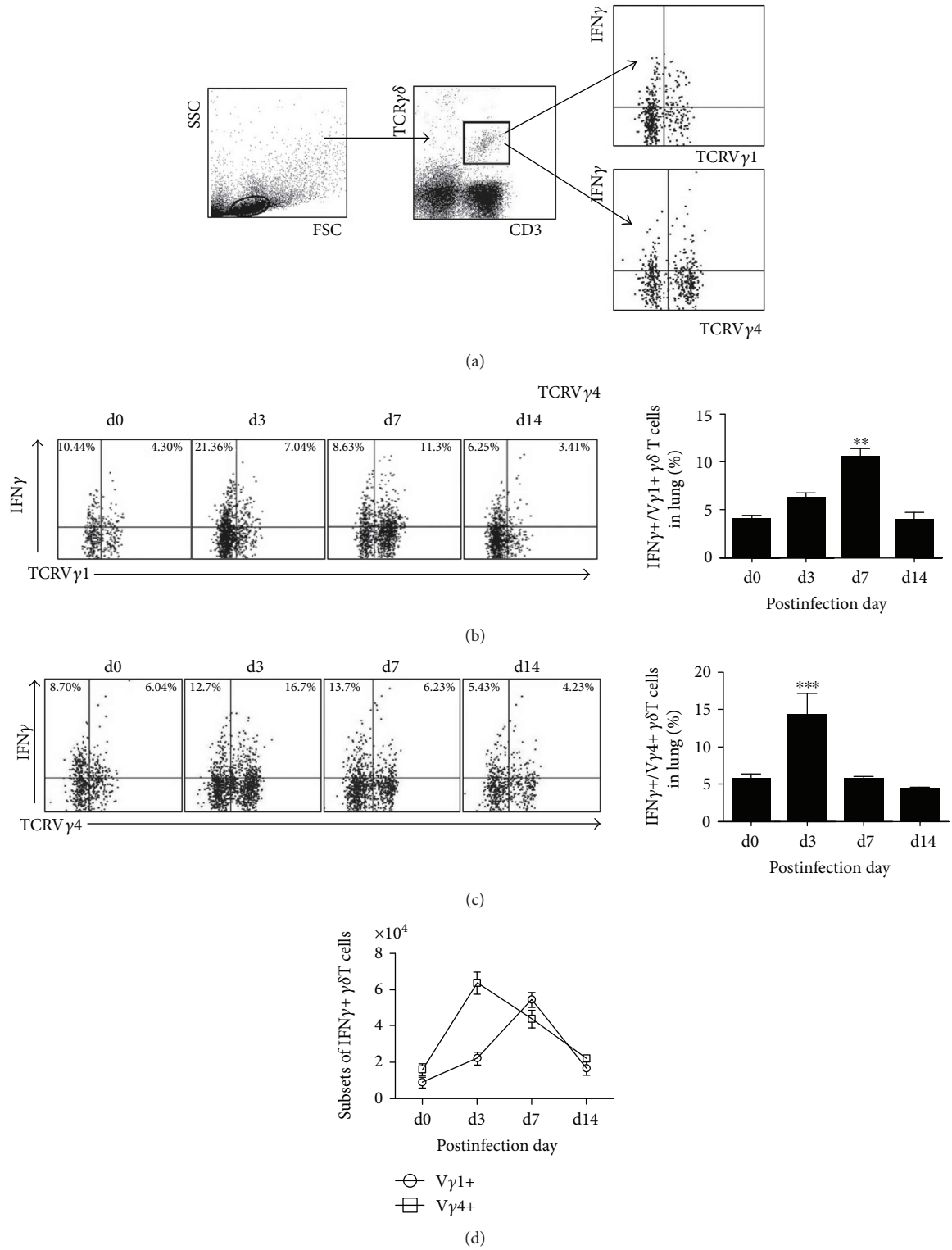


FIGURE 4: Vγ4 cells at day 3 p.i. and Vγ1 cells at day 7 p.i. are the major sources of IFNγ during Cm lung infection. IFNγ+ Vγ1+/Vγ4+ T cells were gated (a). Staining with anti-mouse CD3, TCRγδ, TCRVγ1/Vγ4, and IFNγ/IL-17 antibody to analyze the percentage and absolute number of IFNγ+ Vγ1+ T cells (b) and IFNγ+ Vγ4+ T cells (c) in lung tissues after Cm infection by flow cytometry. Comparison between IFNγ+ Vγ1+ cell and IFNγ+ Vγ4+ cell with its absolute number (d). The results are presented as mean ± SD. $^{**}p < 0.01$ and $^{***}p < 0.001$.

essential but supplementary in clearance of Chlamydia. In our present study using Cm infection model, it was found that Vγ4+ T cells were the major source of IL-17 in the early stage, and Vγ1+ T cells did not secrete IL-17. Similarly, *Listeria monocytogenes* also induces γδ T cells, especially Vγ4+ T and Vγ6+ T cells, and secretes IL-17 in infected

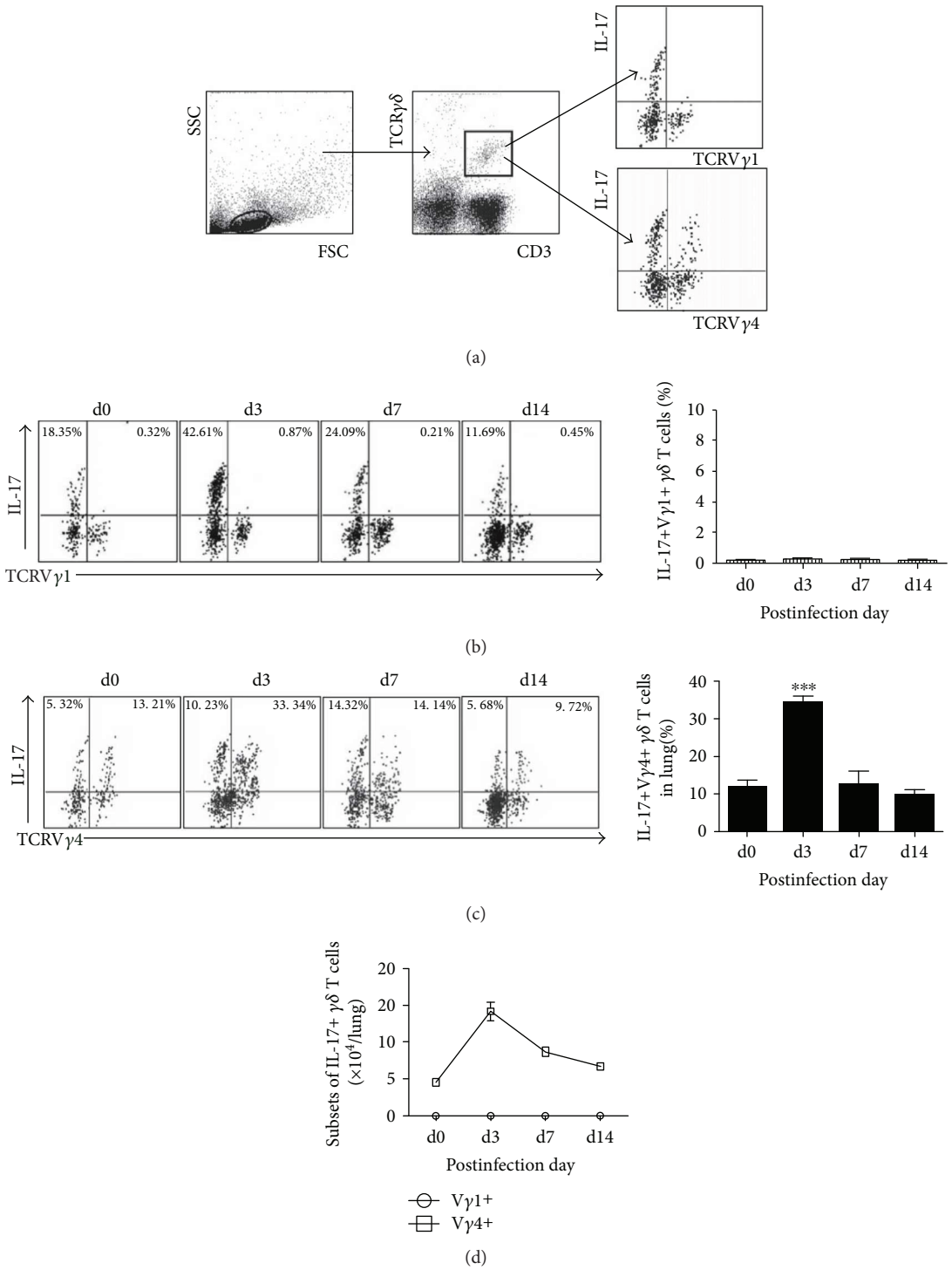


Figure 5: Vγ4 cells at day 3 p.i. are the major sources of IL-17 during Cm lung infection. IL-17+ Vγ4+/Vγ1+ T cells were gated (a), stained with anti-mouse CD3, TCRγδ, TCRVγ4, and IFNγ/IL-17 antibody to analyze percentage of Il-17+ Vγ1+ T cells (b) and IL-17+ Vγ4+ T cells (c) in lung tissues after Cm infection. Comparison between IL-17+ Vγ1+ cell and IL-17+ Vγ4+ cell with its absolute number (d). The results are presented as mean ± SD. $^{***}p < 0.001$.

liver, but more than 60 percent of the IL-17 are produced by Vγ6+ T cell, which have fast kinetic response characteristics [26]. However, the chronic granulomatous disease leads to unrestrained Vγ1+ γδ T-cell reactivity which dominantly produces IL-17. Furthermore, with anti-CD3 antibody and virus-LPS stimulation in vitro, Vγ1+ T cells dramatically produced IL-17, while only IL-10+ Vγ4+ T cells existed [38]. Unlike Th17 cells, the subsets of IL-17+ γδ T cell in varieties of pathogen infections are not always the same pattern, while these data suggest increased numbers of γδ T

cells with cytokine-producing potential during immune response; any role for $\gamma\delta$ T cell-derived cytokines in various model remains to be defined.

Notably, there are still a small number of IL-17-producing-Vγ4-$\gamma\delta$ T cell subsets which is not identified during Cm infection. Interestingly, in our model, Vγ6+ cells also present to proliferate following the Cm infection at the middle stage, which might be an important IL-17-producing cell after the early infection stage. IL-17+ Vγ6+ T cells promote cancer cell growth by mobilizing peritoneal macrophages in the mice model of ovarian cancer [39]. In *Listeria monocytogenes*, more than 60 percent of the IL-17 are produced by Vγ6+ T cell in infected liver, which have fast kinetic response characteristics [26]. In this study, we did not focus on Vγ6+ T cell because it is reported that Vγ6+ cells are the major $\gamma\delta$ T cell population in reproductive tract but not in lungs [40]. But it still can be speculated that IFNγ and IL-17 may be partially secreted by Vγ6+ T cells apart from Vγ1+ T and Vγ4+ T cells during Cm infection.

In conclusion, our data show that Vγ1+ T and Vγ4+ T cells are the major proliferative cell subsets of $\gamma\delta$ T cell during Cm lung infection in mice. Moreover, Vγ4+ T cells are the major IL-17 and IFNγ-producing $\gamma\delta$ T cell subsets at the early period of Cm lung infection. The findings in the present study provide new insights into the mechanisms bridging innate and adaptive immunity during lung chlamydial infections, which may have implications in developing effective chlamydial vaccines and in the understanding of host defense mechanisms in other lung infections.

Disclosure

Li-da Sun is the only first author in this paper.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by the grants from the National Natural Science Foundation of China (31070797) and the Key Program (15JCZDJC34900) from Tianjin Municipal Science and Technology Commission (TSTC).

References

[1] J. Schachter, "Chlamydial infections," *The New England Journal of Medicine*, vol. 298, no. 8, pp. 428–435, 1978.

[2] R. P. Morrison and H. D. Caldwell, "Immunity to murine chlamydial genital infection," *Infection and Immunity*, vol. 70, no. 6, pp. 2741–2751, 1978.

[3] R. D. Vicetti Miguel, N. E. Quispe Calla, S. D. Pavelko, and T. L. Cherpes, "Intravaginal *Chlamydia trachomatis* challenge infection elicits T_H1 and T_H17 immune responses in mice that promote pathogen clearance and genital tract damage," *PLoS One*, vol. 11, no. 9, article e0162445, 2016.

[4] X. Yang, K. T. Hay Glass, and R. C. Brunham, "Genetically determined differences in IL-10 and IFN-gamma responses correlate with clearance of chlamydia trachomatis mouse pneumonitis infection," *Journal of Immunology*, vol. 156, pp. 4338–4344, 1996.

[5] S. Wang, Y. Fan, R. C. Brunham, and X. Yang, "IFN-γ knockout mice show Th2-associated delayed-type hypersensitivity and the inflammatory cells fail to localize and control chlamydial infection," *European Journal of Immunology*, vol. 29, no. 11, pp. 3782–3792, 1999.

[6] H. Bai, J. Cheng, X. Gao et al., "IL-17/Th17 promotes type 1 T cell immunity against pulmonary intracellular bacterial infection through modulating dendritic cell function," *The Journal of Immunology*, vol. 183, no. 9, pp. 5886–5895, 2009.

[7] M. Bonneville, R. L. O'Brien, and W. K. Born, "$\gamma\delta$ T cell effector functions: a blend of innate programming and acquired plasticity," *Nature Reviews Immunology*, vol. 10, no. 7, pp. 467–478, 2010.

[8] K. D. Jensen, X. Su, S. Shin et al., "Thymic selection determines $\gamma\delta$ T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon γ," *Immunity*, vol. 29, no. 1, pp. 90–100, 2008.

[9] L. M. R. Ferreira, "Gammadelta T cells: innately adaptive immune cells?," *International Reviews of Immunology*, vol. 32, no. 3, pp. 223–248, 2013.

[10] A. C. Hayday, "$\gamma\delta$ cells: a right time and a right place for a conserved third way of protection," *Annual Review of Immunology*, vol. 18, no. 1, pp. 975–1026, 2000.

[11] N. Himoudi, D. A. Morgenstern, M. Yan et al., "Human $\gamma\delta$ T lymphocytes are licensed for professional antigen presentation by interaction with opsonized target cells," *The Journal of Immunology*, vol. 188, no. 4, pp. 1708–1716, 2012.

[12] C. Xue, M. Wen, L. Bao et al., "V$\gamma4^+\gamma\delta$T cells aggravate severe H1N1 influenza virus infection-induced acute pulmonary immunopathological injury via secreting interleukin-17A," *Frontiers in Immunology*, vol. 8, article 1054, 2017.

[13] A. G. Murphy, K. M. O'Keeffe, S. J. Lalor, B. M. Maher, K. H. G. Mills, and R. M. McLoughlin, "*Staphylococcus aureus* infection of mice expands a population of memory $\gamma\delta$ T cells that are protective against subsequent infection," *The Journal of Immunology*, vol. 192, no. 8, pp. 3697–3708, 2014.

[14] P. D. Guirnalda and Y. Paterson, "Vaccination with immunotherapeutic *Listeria monocytogenes* induces IL-17^+ $\gamma\delta$ T cells in a murine model for HPV associated cancer," *OncoImmunology*, vol. 1, no. 6, pp. 822–828, 2012.

[15] S. M. Schulz, G. Köhler, C. Holscher, Y. Iwakura, and G. Alber, "IL-17A is produced by T_h17, $\gamma\delta$ T cells and other $CD4^-$ lymphocytes during infection with *Salmonella enterica* serovar Enteritidis and has a mild effect in bacterial clearance," *International Immunology*, vol. 20, no. 9, pp. 1129–1138, 2008.

[16] H. Bai, X. Gao, L. Zhao et al., "Respective IL-17A production by $\gamma\delta$ T and Th17 cells and its implication in host defense against chlamydial lung infection," *Cellular and Molecular Immunology*, vol. 14, no. 10, pp. 850–861, 2017.

[17] K. E. Sivick, M. A. Schaller, S. N. Smith, and H. L. T. Mobley, "The innate immune response to uropathogenic *Escherichia coli* involves IL-17A in a murine model of urinary tract infection," *The Journal of Immunology*, vol. 184, no. 4, pp. 2065–2075, 2010.

[18] F. Coulter, A. Parrish, D. Manning et al., "IL-17 production from T helper 17, mucosal-associated invariant T, and $\gamma\delta$ cells in tuberculosis infection and disease," *Frontiers in Immunology*, vol. 8, p. 1252, 2017.

[19] C. L. Roark, M. K. Aydintug, J. Lewis et al., "Subset-specific, uniform activation among Vγ6/Vδ1$^+$ $\gamma\delta$ T cells elicited by inflammation," *Journal of Leukocyte Biology*, vol. 75, no. 1, pp. 68–75, 2004.

[20] J. M. Wands, C. L. Roark, M. K. Aydintug et al., "Distribution and leukocyte contacts of $\gamma\delta$ T cells in the lung," *Journal of Leukocyte Biology*, vol. 78, no. 5, pp. 1086–1096, 2005.

[21] G. K. Sim, R. Rajaserkar, M. Dessing, and A. Augustin, "Homing and *in situ* differentiation of resident pulmonary lymphocytes," *International Immunology*, vol. 6, no. 9, pp. 1287–1295, 1994.

[22] A. Kanehiro, M. Lahn, M. J. Mäkelä et al., "Requirement for the p75 TNF-α receptor 2 in the regulation of airway hyperresponsiveness by $\gamma\delta$ T cells," *The Journal of Immunology*, vol. 169, no. 8, pp. 4190–4197, 2002.

[23] T. A. Moore, B. B. Moore, M. W. Newstead, and T. J. Standiford, "$\gamma\delta$-T cells are critical for survival and early proinflammatory cytokine gene expression during murine *Klebsiella* pneumonia," *The Journal of Immunology*, vol. 165, no. 5, pp. 2643–2650, 2000.

[24] K. Uezu, K. Kawakami, K. Miyagi et al., "Accumulation of $\gamma\delta$ T cells in the lungs and their regulatory roles in Th1 response and host defense against pulmonary infection with *Cryptococcus neoformans*," *The Journal of Immunology*, vol. 172, no. 12, pp. 7629–7634, 2004.

[25] S. I. Inoue, M. Niikura, H. Asahi, Y. Iwakura, Y. Kawakami, and F. Kobayashi, "Preferentially expanding Vγ1$^+$ $\gamma\delta$ T cells are associated with protective immunity against *Plasmodium* infection in mice," *European Journal of Immunology*, vol. 47, no. 4, pp. 685–691, 2017.

[26] S. Hamada, M. Umemura, T. Shiono et al., "IL-17A produced by $\gamma\delta$ T cells plays a critical role in innate immunity against *Listeria monocytogenes* infection in the liver," *The Journal of Immunology*, vol. 181, no. 5, pp. 3456–3463, 2008.

[27] K. Shibata, H. Yamada, H. Hara, K. Kishihara, and Y. Yoshikai, "Resident Vδ1$^+$ $\gamma\delta$ T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production," *The Journal of Immunology*, vol. 178, no. 7, pp. 4466–4472, 2007.

[28] P. L. Simonian, C. L. Roark, W. K. Born, R. L. O'Brien, and A. P. Fontenot, "$\gamma\delta$ T cells and Th17 cytokines in hypersensitivity pneumonitis and lung fibrosis," *Translational Research*, vol. 154, no. 5, pp. 222–227, 2009.

[29] C. L. Roark, J. D. French, M. A. Taylor, A. M. Bendele, W. K. Born, and R. L. O'Brien, "Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing $\gamma\delta$ T cells," *The Journal of Immunology*, vol. 179, no. 8, pp. 5576–5583, 2007.

[30] L. Bilenki, S. Wang, J. Yang et al., "Adoptive transfer of CD8α^+ dendritic cells (DC) isolated from mice infected with *Chlamydia muridarum* are more potent in inducing protective immunity than CD8α^- DC," *The Journal of Immunology*, vol. 177, no. 10, pp. 7067–7075, 2006.

[31] L. Jiao, X. Gao, A. G. Joyee et al., "NK cells promote type 1 T cell immunity through modulating the function of dendritic cells during intracellular bacterial infection," *The Journal of Immunology*, vol. 187, no. 1, pp. 401–411, 2011.

[32] L. Mincheva-Nilsson, "Pregnancy and gamma/delta T cells: taking on the hard questions," *Reproductive Biology and Endocrinology*, vol. 1, no. 1, p. 120, 2003.

[33] A. C. Kirby, D. J. Newton, S. R. Carding, and P. M. Kaye, "Evidence for the involvement of lung-specific $\gamma\delta$ T cell subsets in local responses to *Streptococcus pneumoniae* infection," *European Journal of Immunology*, vol. 37, no. 12, pp. 3404–3413, 2007.

[34] M. Umemura, A. Yahagi, S. Hamada et al., "IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guérin infection," *The Journal of Immunology*, vol. 178, no. 6, pp. 3786–3796, 2007.

[35] M. A. Flierl, D. Rittirsch, H. Gao et al., "Adverse functions of IL-17A in experimental sepsis," *The Faseb Journal*, vol. 22, no. 7, pp. 2198–2205, 2008.

[36] P. Vantourout and A. Hayday, "Six-of-the-best: unique contributions of $\gamma\delta$ T cells to immunology," *Nature Reviews Immunology*, vol. 13, no. 2, pp. 88–100, 2013.

[37] M. Sheel, L. Beattie, T. C. Frame et al., "IL-17A–producing $\gamma\delta$ T cells suppress early control of parasite growth by monocytes in the liver," *The Journal of Immunology*, vol. 195, no. 12, pp. 5707–5717, 2015.

[38] L. Romani, F. Fallarino, A. De Luca et al., "Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease," *Nature*, vol. 451, no. 7175, pp. 211–215, 2008.

[39] M. Rei, N. Goncalves-Sousa, T. Lanca et al., "Murine CD27$^{(-)}$ Vγ6$^{(+)}$ $\gamma\delta$ T cells producing IL-17A promote ovarian cancer growth via mobilization of protumor small peritoneal macrophages," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 34, pp. E3562–E3570, 2014.

[40] P. Pereira, D. Gerber, S. Y. Huang, and S. Tonegawa, "Ontogenic development and tissue distribution of V gamma 1-expressing gamma/delta T lymphocytes in normal mice," *Journal of Experimental Medicine*, vol. 182, no. 6, pp. 1921–1930, 1995.

Hindawi
Mediators of Inflammation
Volume 2017, Article ID 5458178, 9 pages
https://doi.org/10.1155/2017/5458178

Review Article

Phenotypic and Functional Properties of Tumor-Infiltrating Regulatory T Cells

Gap Ryol Lee

Department of Life Science, Sogang University, 35 Baekbeom-ro, Mapo-gu, Seoul 04107, Republic of Korea

Correspondence should be addressed to Gap Ryol Lee; grlee@sogang.ac.kr

Received 31 October 2017; Revised 11 December 2017; Accepted 12 December 2017; Published 31 December 2017

Academic Editor: Qingdong Guan

Copyright © 2017 Gap Ryol Lee. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Regulatory T (Treg) cells maintain immune homeostasis by suppressing excessive immune responses. Treg cells induce tolerance against self- and foreign antigens, thus preventing autoimmunity, allergy, graft rejection, and fetus rejection during pregnancy. However, Treg cells also infiltrate into tumors and inhibit antitumor immune responses, thus inhibiting anticancer therapy. Depleting whole Treg cell populations in the body to enhance anticancer treatments will produce deleterious autoimmune diseases. Therefore, understanding the precise nature of tumor-infiltrating Treg cells is essential for effectively targeting Treg cells in tumors. This review summarizes recent results relating to Treg cells in the tumor microenvironment, with particular emphasis on their accumulation, phenotypic, and functional properties, and targeting to enhance the efficacy of anticancer treatment.

1. Introduction

Regulatory T (Treg) cells are CD4 T cells that inhibit immune responses. Treg cells express high amounts of CD25 and transcription factor Forkhead box protein 3 (Foxp3) [1, 2]. Treg cells maintain immune homeostasis by inhibiting immune responses. These cells not only protect tissues from excessive immune responses but also suppress immune responses against self-antigens, innocuous environmental antigens, antigens from food and microbiota, and fetal antigens during pregnancy.

Treg cells inhibit immune responses by a variety of mechanisms, including the secretion of anti-inflammatory cytokines such as interleukin- (IL-) 10, tumor growth factor- (TGF-) β, and IL-35 [3]. In addition, Treg cells express high levels of IL-2R, depleting IL-2, a growth factor for effector T (Teff) cells, in the surrounding environment. Treg cells also kill Teff cells directly through the FasL-Fas pathway as well as through granzyme-/perforin-mediated cytotoxicity, disrupting the metabolism of Teff cells. Moreover, Treg cells can suppress immune responses by inducing tolerogenic dendritic cells (DCs) [3].

Two types of Treg cells have been identified. Thymus-derived Treg (tTreg) cells develop in the thymus, whereas periphery-derived Treg (pTreg) cells differentiate from naive CD4 T cells in the periphery.

Incipient tumor cells are removed by immune system cells; specifically, $CD8^+$ cytotoxic T lymphocytes (CTLs) kill tumor cells, aided by $CD4^+$ T cells. Tumor cells express tumor-associated antigens (TAAs), which are newly expressed or mutated self-antigens, and are recognized and killed by CTLs, a phenomenon known as "cancer immune surveillance." Continual generation of cancer cells and removal by immune cells can be balanced and can last for a long time. Some of these cancer cells may eventually evade immune responses and grow unchecked. Thus, immune system cells are critical in keeping cancers under control.

Treg cells infiltrate tumors and inhibit antitumor immune responses by tumor antigen-specific CD8 T cells and CD4 T cells. Thus, Treg cells can block cancer immunotherapy. Because depleting Treg cells throughout the entire body cause fulminant autoimmunity, targeting tumor-infiltrating Treg (TI-Treg) cells can enhance tumor immunotherapy without inducing deleterious autoimmune

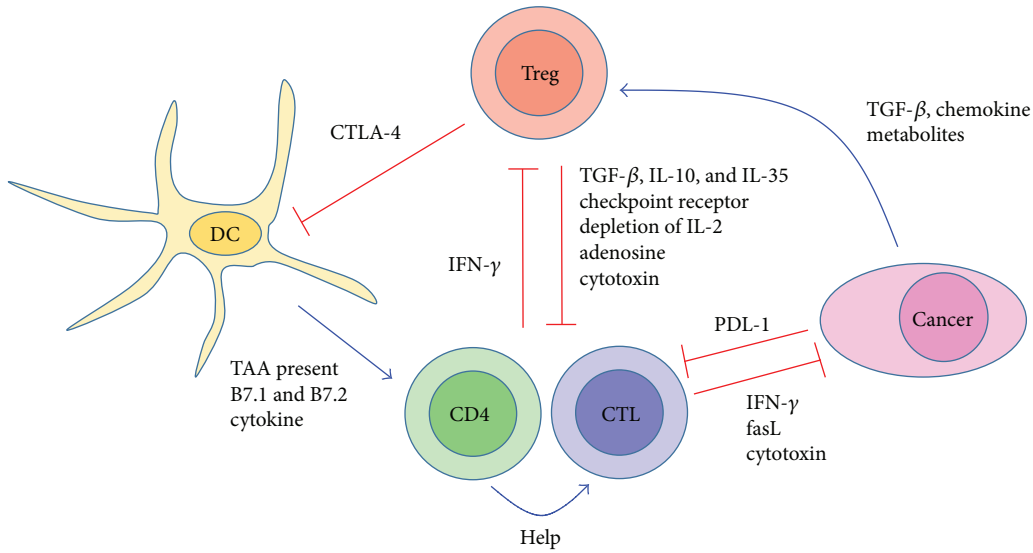


Figure 1: The role of TI-Treg cells in TME. A schematic illustration of the role of TI-Treg cells in the TME. Activation is shown as blue arrows, and inhibition is shown as red blocked lines. TI-Treg cells inhibit CTLs and CD4 Teff cells by secreting anti-inflammatory cytokines, expressing checkpoint receptors, disturbing metabolism, and killing directly. TI-Treg cells also intercept costimulatory signal on DCs by CTLA-4, preventing activation of Teff cells. Cancer cells attract Treg cells to tumor by secreting chemokines and nurture Treg cells by secreting TGF-β and immunosuppressive metabolites.

diseases. Understanding the properties of TI-Treg cells and their methods of suppressing anticancer treatment is essential to achieve this goal. This review summarizes recent findings of TI-Treg cell properties and their therapeutic application (summarized in Figure 1).

2. TI-Treg Cells

Cancer cells accumulate mutations during tumorigenesis and acquire the ability to establish their own protective environment, called the tumor microenvironment (TME). The TME contains many types of cells, including cancer cells, immune system cells, fibroblasts, pericytes, and occasionally adipocytes [4, 5]. The immune cells in the TME include CD8 T cells, CD4 T cells, Treg cells, DCs, macrophages, natural killer cells, B cells, and mast cells [4, 5]. These cells establish an environment that is highly immunosuppressive, tolerogenic, hypoxic, and rich in proangiogenic factors. Because Treg cells have immunosuppressive properties, Treg cells in the TME are generally thought to inhibit antitumor activity mediated by Teff cells and to promote tumor growth [6]. Secreted and/or surface molecules in the TME influence the growth of cancer cells. Immunosuppressive cytokines, such as TGF-β and IL-10, inhibit antitumor immunity mediated by Teff cells and boost the activity of Treg cells.

High numbers of Treg cells and low CD8 T cell to Treg cell ratios have been found to correlate with poor prognosis and reduced survival of patients with many types of cancer, including ovarian cancer [7, 8], lung cancer [9], pancreatic ductal adenocarcinoma [10, 11], non-Hodgkin's lymphoma [12], glioblastoma [13], melanoma, and other malignancies [14, 15]. By contrast, high numbers of Treg cells were found to correlate with good prognosis in patients with colorectal [16], head and neck [17], and gastric [18] cancer. One explanation of this discrepancy is that Treg cells that reduce inflammation may inhibit the growth of certain types of cancer that depend heavily on inflammation [19]. Inflammation has been shown to contribute to cancer initiation and progression, neoplastic transformation, and metastasis [20]. Alternative explanation is that the discrepancy is caused by inability to quantify heterogeneous Treg cell subsets or the concomitant inflammation in the tumors [21]. Treg cell heterogeneity has been proven in colorectal cancer [22].

3. Recruitment and Expansion of Treg Cells in the TME

Increases in the numbers of Treg cells in the TME may result from the preferential recruitment of TI-Treg cells over conventional T (Tconv) cells, increased Treg cell proliferation, and/or conversion of Tconv cells to Treg cells.

3.1. Treg Cell Recruitment into the TME. Preferential recruitment of Treg cells into the TME may result from interactions between chemokines and their receptors. Chemokines produced by tumors, including CC chemokine ligand 22 (CCL22), CCL17, CXC chemokine ligand 12 (CXCL12), and CCL28, recruit Treg cells into tumors [23]. Cancer cell-produced CCL22 or CCL17 attracts CC chemokine receptor 4-positive ($CCR4^+$) Treg cells in the TME, which seems to be the most prevalent mechanism for Treg cell migration to tumors [7, 24]. Blocking CCR4 reduces the number of intratumoral Treg cells and enhances antitumor immunity [25, 26]. The CCL5/CCR5 axis also plays a role in Treg cell recruitment [27], and hypoxia-induced CCL28 has been found to attract $CCR10^+$ Treg cells into ovarian cancers [28].

3.2. Expansion of Treg Cells in the TME. TI-Treg cells exhibit increased proliferation, as evidenced by high expression of Ki-67, compared with Treg cells from peripheral blood and healthy tissue [29]. This increased proliferation of TI-Treg cells may be related to their recognition of self-antigens and the nurturing environment in the TME. Higher numbers of prostate-specific Treg cells accumulate in the prostate than in other organs, suggesting that the presence of self-antigens may trigger the expansion of Treg cells in tumors [30]. TI-Treg cells show high surface expression of CD25 (high-affinity IL-2 receptor subunit α), allowing these cells to absorb available IL-2 in the environment. This results in the high proliferation of Treg cells but inhibits the growth of Tconv cells in the TME.

Recent evidence shows that metabolic fitness is associated with the preferential expansion of TI-Treg cells in the TME [31]. Because cancer cells preferentially acquire energy from glycolysis, the TME is rich in immunosuppressive metabolites [32–35]. These conditions suppress Teff cell function, while enhancing the function of Treg cells. The differentiation and function of Treg cells preferentially involve fatty acid oxidation and oxidative phosphorylation [36–38], as well as the uptake of lactic acid from the surrounding environment [39, 40], resulting in the metabolic fitness of TI-Treg cells. Several fatty acid-binding proteins are specifically expressed in TI-Treg cells in breast cancers, but not in Treg cells in peripheral blood and normal tissues [41]. It remains unclear, however, whether these metabolites contribute to the expansion of TI-Treg cells.

3.3. Conversion of Tconv Cells into Treg Cells. The TME is rich in immunosuppressive molecules, including TGF-β, IL-10, and VEGF, suggesting that Tconv cells are converted to Treg cells through the formation of tolerogenic antigen-presenting cells (APCs) in the TME [42–44]. Indoleamine2,3-dioxygenase- (IDO-) expressing APCs may induce the conversion of Tconv to Treg cells through an aryl hydrocarbon receptor [45]. Myeloid-derived suppressor cells (MDSCs) in the TME may also promote the differentiation of Treg cells in an IDO-dependent manner.

It remains unclear, however, whether Tconv cells can be converted to Treg cells in TME. In mouse tumor model, injection of MCA-38 colon adenocarcinoma cells causes enrichment of neuropilin-1- (Nrp1-) pTreg cells, whereas that of 4T1 breast cancer cells causes enrichment of $Nrp1^{+}$ tTreg cells, suggesting that both tTreg and pTreg cells can be enriched in the TME depending on the types of tumor [46]. Analyses showed that TI-Treg cells and Tconv cells have a largely nonoverlapping T cell receptor (TCR) repertoire and that TI-Treg cells originate from tissue-specific Treg cells generated in the thymus [30, 47, 48], suggesting that conversion did not occur. Further studies are needed to resolve this issue.

4. Phenotypes and Suppressive Mechanisms of TI-Treg Cells

4.1. Phenotypes of TI-Treg Cells. TI-Treg cells exhibit more highly activated phenotypes than Treg cells in the peripheral blood and healthy tissue [49]. TI-Treg cells express high amounts of distinct markers, including CD25, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family-related genes (GITR), programmed death-1 (PD-1), lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucin domain-containing-3 (TIM-3/HAVCR2), and inducible T cell costimulator (ICOS). These cells are $CD44^{high}$, $CD62L^{low}$, and $CCR7^{low}$, indicating an effector-memory phenotype [50]. TI-Treg cells have greater immunosuppressive activity than other Treg cells, possibly due to stimulation by TAAs. TAAs originate from self-antigens and bind more strongly by Treg cells than by Teff cells, as Treg cells have higher affinity TCR than Teff cells, leading to preferential activation of Treg cells.

The stability and suppressive function of TI-Treg are very important in tumor growth. Several factors were recently shown to be important in maintaining the stability and suppressive activity of TI-Treg cells, either positively or negatively. The stability of TI-Treg cells and their ability to potentiate immunosuppressive functions were shown to involve the Sema4a-Nrp1 pathway, specifically in tumors but not in other tissues [51]. Treg-specific deletion of Nrp1 was found to block tumor growth in several animal models of cancer [51]. Molecularly, the Sema4a-Nrp1 interaction inhibits Akt phosphorylation by phosphatase and tensin homologue (PTEN), resulting in the nuclear localization of Foxo3a [51]. PI3K is also important for the suppressive activity of TI-Treg cells. CD8 T cell activity was enhanced, and tumor burden was reduced in Treg-specific PI3K p110δ-deficient mice [52]. Foxo1 was also found to be important in regulating the generation of activated Treg cells in the TME. Treg-specific Akt-insensitive mutant mice, in which Foxo1 is not repressed, show strong antitumor activity due to lack of activated Treg cells, along with a concomitant increase in intratumoral CD8 T cells [53]. NF-κB c-Rel are also important in the suppressive activity of TI-Treg cells. Treg-specific deletion of c-Rel reduces the expression of activated Treg-specific marker genes, including *Itgae*, *Tigit*, *Klrg1*, *Il1r2*, and *Tnfsf8*, as well as inhibiting tumor growth in the B16F1 melanoma transplantation model; however, these cells do not show an overt autoimmune phenotype [54]. Helios was also shown to be important in TI-Treg cell stability and suppressive activity [55]. Treg-specific Helios-deficient cells enhanced antitumor activity in the TME, whereas systemic Helios-deficient Treg cells did not. Helios-deficient Treg cells increase IFN-γ and TNF-α expression, indicating phenotypic conversion. By contrast, TI-Treg cell activity is downregulated by IFN-γ produced by Teff cells in the TME. Nrp1-deficient Treg cells produce IFN-γ in the TME, with the resultant IFN-γ reducing the suppressive activity of Treg cells without losing Foxp3 expression, a phenomenon called "Treg cell fragility" [56].

TI-Treg cells show specific gene expression patterns. A recent study compared the gene expression profiles of breast cancer-infiltrating Treg cells with those of Treg cells in the peripheral blood and normal tissue [41]. The overall gene expression pattern of TI-Treg cells was closer to that of

normal breast tissue-resident Treg cells than that of peripheral Treg cells, suggesting that the tissue surrounding the tumor is the major determinant of Treg cell gene expression. TI-Treg cells express a few distinct genes, including those encoding chemokine receptor CCR8 and type I interferons. A similar approach showed the upregulation on Tl-Tregs in human cancers of gene-encoding surface markers [57], including those encoding several immune checkpoint receptors, such as IL1R2, PD-L1, PD-L2, and CCR8. The levels of expression of some of these gene products, including LAYN, MAGEH1, and CCR8, were found to correlate with poor prognosis. Further elucidation and characterization of TI-Treg-specific genes will help in precisely targeting these cells, without compromising general Treg cell activity in other parts of the body.

4.2. Suppressive Mechanisms of TI-Treg Cells. Although many studies have assessed the mechanisms by which Treg cells suppress immune responses in general, less is known about the mechanisms by which these cells suppress antitumor immunity. In addition, Treg cells acquire distinct immunomodulatory mechanisms when residing in different peripheral tissues [58]. Therefore, understanding TI-Treg-specific suppressive mechanisms is critical in developing therapeutic strategies to treat cancers without affecting Treg functions in general.

In many types of human cancers, including hepatocellular carcinoma, pancreatic cancer, and ovarian cancer, TI-Treg cells suppress antitumor activity by secreting the anti-inflammatory cytokines TGF-β and IL-10 and by upregulating the expression of inhibitory immune checkpoint receptors, including CTLA-4, GITR, TIM-3, and ICOS [7, 29, 59–64]. CTLA-4 has a higher avidity to B7.1 and B7.2 on DCs than CD28 does, thereby preventing Teff cell activation. TIM-3, LAG-3, and PD-1 also inhibit Teff cells and $CD8^+$ CTLs. TI-Treg cells induce the exhaustion of CTLs characterized by inefficient release of cytotoxic granules, low expression of effector cytokines, and expression of the coinhibitory receptors PD-1 and TIM-3 [65]. These results suggest that TI-Treg cells use mechanisms common to Treg cells in general, as well as preferentially involving immune inhibitory receptors [66].

IDO exerts an important immunosuppressive effect in tumors. Interactions between CTLA-4 and DCs can induce the expression of IDO, resulting in the production of the immunosuppressive metabolite kynurenine [67]. Kynurenine can support Treg cell differentiation but impairs T cell cytotoxic activity [68, 69]. IDO is expressed at high levels in tumors and other immunomodulatory cells, leading to increased kynurenine levels in the TME and possibly enhancing Treg cell activity [70].

TI-Treg cells show high expression of CD39, which converts ATP into AMP, and of CD73, which converts AMP to adenosine [66]. Adenosine is a powerful anti-inflammatory factor that inhibits the function of immune cells by binding to the adenosine receptor 2A ($A_{2A}R$) on Teff cells and upregulates intracellular cAMP level [66]. Adenosine also potentiates the differentiation, proliferation, and suppressor activities of Treg cells and MDSCs [66].

5. Immunotherapy Targeting TI-Treg Cells

Because Treg cells suppress antitumor immunity mediated by CD8 and CD4 Teff cells, immunotherapy targeting Treg cell function in TME is being actively pursued. Methods to target Treg cells include depletion of Treg cells, blocking immune checkpoint receptors, recruitment of Treg cells, and treatment of cells with inhibitory cytokines [66, 71, 72].

5.1. Depleting Treg Cells. CD25 is a well-known Treg cell marker. Depleting Treg cells by targeting CD25 has yielded conflicting results. The anti-CD25 monoclonal antibody daclizumab was reported to have beneficial effects in patients with glioblastoma and breast cancer [73, 74] but was reported to have a marginal effect in metastatic melanoma [75]. Similarly, the IL-2-diphtheria toxin fusion protein denileukin diftitox was effective in patients with renal cell carcinoma (RCC) [76] but had an adverse effect in metastatic melanoma [77]. Possible reasons for these conflicting results are the effect of these drugs on Teff cells, the rapid repopulation by Treg cells upon drug withdrawal, or the nonrecognition of Treg cells by immune conjugates following treatment with denileukin diftitox. Recently, CD25 was found to be preferentially expressed in tumors in vivo [78]. The commonly used rat IgG1-depleting antibody PC-61 does not effectively deplete Treg cells in tumors, because it binds to inhibitory FcγRIIb. Treatment with Fc-optimized anti-CD25 antibody (i.e., the Fc region of PC-61 was replaced by murine IgG2a and κ constant region) resulted in the effective depletion of Treg cells and an increase in the Teff-to-Treg ratio, leading to tumor regression and increased survival [78].

5.2. Immune Checkpoint Inhibitors. CTLA-4 is an immune checkpoint receptor highly expressed in Treg cells. Immune checkpoint receptors are immune inhibitory receptors that are often highly expressed in the TME [79]. The rationale for using immune checkpoint inhibitors is to block inhibitory signals to Teff cells and restore their antitumor activity. Immune checkpoint inhibitors showed significant activity in clinical trials of patients with melanoma, nonsmall cell lung cancer (NSCLC), and RCC [80–83]. In recent years, four immune checkpoint inhibitors have been approved by the FDA for the treatment of metastatic melanoma, NSCLC, advanced RCC, and Hodgkin's lymphoma: monoclonal antibodies targeting CTLA-4 (ipilimumab and tremelimumab) and PD-1 (nivolumab and pembrolizumab).

Mechanistically, anti-CTLA-4 was first thought to prevent Treg cells from intercepting costimulatory signals from DCs, resulting in DC-induced Teff cell activation and proliferation. Ipilimumab and tremelimumab induce significant activation and expansion of Teff and CD8 T cells [84–88]. The effect of ipilimumab was recently substantiated by depleting Treg cells via antibody-dependent cell-mediated cytotoxicity (ADCC) [89]. However, tremelimumab, which does not have ADCC activity, had a similar therapeutic effect, suggesting that Treg depletion may not be the main mechanism of ipilimumab.

Another Treg-specific marker GITR is also a target for TI-Treg cells. Unlike in Treg cells, GITR acts as a costimulatory

molecule in Teff cells, suggesting a beneficial effect in cancer therapy. In animal models, anti-GITR antibody induced antitumor activity by increasing Teff cells [90]. Combined treatment with anti-GITR and anti-CTLA-4 antibodies synergistically induced antitumor activity in human patients [91]. OX40, a member of the TNF receptor family, has a mechanism of action similar to that of GITR; that is, anti-OX40 antibody stimulates Teff cells but inhibits Treg cells. Anti-OX40 antibody enhanced CD8 T cell-mediated antitumor immunity in animal models of cancer [92]. Antibodies against GITR and OX40 are now in clinical trials [93].

Combining Treg cell depletion with immune checkpoint inhibitors resulted in a synergistic effect in an animal model of Claudin-low breast cancer, a subtype of triple-negative breast cancer [94]. Treg cell depletion and immune checkpoint inhibitors each had little effect on tumor growth, whereas their combination greatly reduced tumor burden [94].

5.3. Blocking Treg Cell Recruitment. Infiltration of Treg cells into tumors is a prerequisite for their activity. TI-Treg cells express a variety of chemokine receptors, including CCR4, CCR5, CCR6, CCR7, CCR10, CXCR3, and CXCR4, and migrate efficiently in response to tumor-derived chemokines [23, 95, 96].

CCR4 is preferentially expressed on TI-Treg cells rather than on Teff cells [25], with the CCL17/22-CCR4 axis playing an important role in lymphomas and in breast, lung, ovarian, gastric, and prostate cancers [23, 95, 96]. A monoclonal antibody targeting CCR4 has shown promising results, effectively depleting Treg cells, both in vitro and in clinical trials in human cancer patients [96, 97].

$CXCR3^+$ Treg cells selectively accumulate in ovarian cancer and block the interactions between CXCR3 and its ligands CXCL9, CXCL10, and CXCL11, thereby suppressing tumor growth [98].

5.4. Blocking Inhibitory Cytokines. Because the TME is rich in immunosuppressive cytokines that strengthen the activity of TI-Treg cells, neutralizing these cytokines may reestablish effective antitumor immunity. Genetic ablation or blocking of IL-10 or TGF-β signaling results in tumor regression [99–102]. In addition, neutralization of IL-35 or Treg-specific deletion of IL-35 was found to enhance antitumor T cell responses and reduce tumor growth in various mouse tumor models [103]. Interestingly, IL-35 produced by Treg cells promoted the expression of several inhibitory receptors, including PD-1, TIM-3, and LAG-3, leading to T cell exhaustion. The higher numbers of IL-35-expressing Treg cells present in tumors than in spleen can be exploited for tumor-specific blockade of Treg cell function without affecting Treg function in general [103].

6. Conclusions and Perspective

In recent years, tumor immunotherapy has drawn much attention because of its specific targeting ability and reduced side effects. Targeting Treg cells in cancer treatment was hampered by a lack of knowledge of the properties of TI-Treg cells. Understanding the phenotypic and functional properties of Treg cells is essential to effectively and specifically target TI-Treg cells in cancer therapy without compromising immune homeostasis in general. Future studies should include a search for TI-Treg-specific genes in human cancers and elucidate their roles in tumor progression. Treg cells are heterogeneous, with different functional properties. Similarly, TI-Treg cells likely have distinct functional properties depending on their TME, as tumors have different environments. Treg cells may preferentially use limited suppressive mechanisms that best fit their environment. Studies of cancer-specific suppressive mechanisms, including causative factors, interactions with other cells in the TME, and their functional significance, are warranted.

Conflicts of Interest

The author declares no competing financial interests.

Acknowledgments

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (NRF-2017R1A2B3008621).

References

[1] J. D. Fontenot, M. A. Gavin, and A. Y. Rudensky, "Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells," *Nature Immunology*, vol. 4, no. 4, pp. 330–336, 2003.

[2] S. Hori, T. Nomura, and S. Sakaguchi, "Control of regulatory T cell development by the transcription factor Foxp3," *Science*, vol. 299, no. 5609, pp. 1057–1061, 2003.

[3] D. A. Vignali, L. W. Collison, and C. J. Workman, "How regulatory T cells work," *Nature Reviews Immunology*, vol. 8, no. 7, pp. 523–532, 2008.

[4] D. Hanahan and L. M. Coussens, "Accessories to the crime: functions of cells recruited to the tumor microenvironment," *Cancer Cell*, vol. 21, no. 3, pp. 309–322, 2012.

[5] J. A. Joyce and J. W. Pollard, "Microenvironmental regulation of metastasis," *Nature Reviews Cancer*, vol. 9, no. 4, pp. 239–252, 2009.

[6] D. Lindau, P. Gielen, M. Kroesen, P. Wesseling, and G. J. Adema, "The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells," *Immunology*, vol. 138, no. 2, pp. 105–115, 2013.

[7] T. J. Curiel, G. Coukos, L. Zou et al., "Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival," *Nature Medicine*, vol. 10, no. 9, pp. 942–949, 2004.

[8] N. Leffers, M. J. M. Gooden, R. A. de Jong et al., "Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer," *Cancer Immunology, Immunotherapy*, vol. 58, no. 3, pp. 449–459, 2009.

[9] H. Tao, Y. Mimura, K. Aoe et al., "Prognostic potential of FOXP3 expression in non-small cell lung cancer cells

combined with tumor-infiltrating regulatory T cells," *Lung Cancer*, vol. 75, no. 1, pp. 95–101, 2012.

[10] Y. Jiang, Z. Du, F. Yang et al., "FOXP3$^+$ lymphocyte density in pancreatic cancer correlates with lymph node metastasis," *PLoS One*, vol. 9, no. 9, article e106741, 2014.

[11] Y. Tang, X. Xu, S. Guo et al., "An increased abundance of tumor-infiltrating regulatory T cells is correlated with the progression and prognosis of pancreatic ductal adenocarcinoma," *PLoS One*, vol. 9, no. 3, article e91551, 2014.

[12] Z. Z. Yang, A. J. Novak, M. J. Stenson, T. E. Witzig, and S. M. Ansell, "Intratumoral CD4$^+$CD25$^+$ regulatory T-cell-mediated suppression of infiltrating CD4$^+$ T cells in B-cell non-Hodgkin lymphoma," *Blood*, vol. 107, no. 9, pp. 3639–3646, 2006.

[13] E. J. Sayour, P. McLendon, R. McLendon et al., "Increased proportion of FoxP3$^+$ regulatory T cells in tumor infiltrating lymphocytes is associated with tumor recurrence and reduced survival in patients with glioblastoma," *Cancer Immunology, Immunotherapy*, vol. 64, no. 4, pp. 419–427, 2015.

[14] R. J. deLeeuw, S. E. Kost, J. A. Kakal, and B. H. Nelson, "The prognostic value of FoxP3$^+$ tumor-infiltrating lymphocytes in cancer: a critical review of the literature," *Clinical Cancer Research*, vol. 18, no. 11, pp. 3022–3029, 2012.

[15] B. Shang, Y. Liu, and S. J. Jiang, "Prognostic value of tumor-infiltrating FoxP3$^+$ regulatory T cells in cancers: a systematic review and meta-analysis," *Scientific Reports*, vol. 5, no. 1, p. 15179, 2015.

[16] P. Salama, M. Phillips, F. Grieu et al., "Tumor-infiltrating FOXP3$^+$ T regulatory cells show strong prognostic significance in colorectal cancer," *Journal of Clinical Oncology*, vol. 27, no. 2, pp. 186–192, 2009.

[17] C. Badoual, S. Hans, J. Rodriguez et al., "Prognostic value of tumor-infiltrating CD4$^+$ T-cell subpopulations in head and neck cancers," *Clinical Cancer Research*, vol. 12, no. 2, pp. 465–472, 2006.

[18] M. Haas, A. Dimmler, W. Hohenberger, G. G. Grabenbauer, G. Niedobitek, and L. V. Distel, "Stromal regulatory T-cells are associated with a favourable prognosis in gastric cancer of the cardia," *BMC Gastroenterology*, vol. 9, no. 1, p. 65, 2009.

[19] S. Ladoire, F. Martin, and F. Ghiringhelli, "Prognostic role of FOXP3$^+$ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer," *Cancer Immunology, Immunotherapy*, vol. 60, no. 7, pp. 909–918, 2011.

[20] G. Trinchieri, "Cancer and inflammation: an old intuition with rapidly evolving new concepts," *Annual Review of Immunology*, vol. 30, no. 1, pp. 677–706, 2012.

[21] W. H. Fridman, L. Zitvogel, C. Sautes-Fridman, and G. Kroemer, "The immune contexture in cancer prognosis and treatment," *Nature Reviews. Clinical Oncology*, vol. 14, no. 12, pp. 717–734, 2017.

[22] T. Saito, H. Nishikawa, H. Wada et al., "Two FOXP3^{+-} CD4$^+$ T cell subpopulations distinctly control the prognosis of colorectal cancers," *Nature Medicine*, vol. 22, no. 6, pp. 679–684, 2016.

[23] A. W. Mailloux and M. R. Young, "Regulatory T-cell trafficking: from thymic development to tumor-induced immune suppression," *Critical Reviews in Immunology*, vol. 30, no. 5, pp. 435–447, 2010.

[24] M. Gobert, I. Treilleux, N. Bendriss-Vermare et al., "Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome," *Cancer Research*, vol. 69, no. 5, pp. 2000–2009, 2009.

[25] T. Ishida, T. Ishii, A. Inagaki et al., "Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege," *Cancer Research*, vol. 66, no. 11, pp. 5716–5722, 2006.

[26] H. Pere, Y. Montier, J. Bayry et al., "A CCR4 antagonist combined with vaccines induces antigen-specific CD8$^+$ T cells and tumor immunity against self antigens," *Blood*, vol. 118, no. 18, pp. 4853–4862, 2011.

[27] M. C. Tan, P. S. Goedegebuure, B. A. Belt et al., "Disruption of CCR5-dependent homing of regulatory T cells inhibits tumor growth in a murine model of pancreatic cancer," *Journal of Immunology*, vol. 182, no. 3, pp. 1746–1755, 2009.

[28] A. Facciabene, X. Peng, I. S. Hagemann et al., "Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells," *Nature*, vol. 475, no. 7355, pp. 226–230, 2011.

[29] A. Pedroza-Gonzalez, C. Verhoef, J. N. Ijzermans et al., "Activated tumor-infiltrating CD4$^+$ regulatory T cells restrain antitumor immunity in patients with primary or metastatic liver cancer," *Hepatology*, vol. 57, no. 1, pp. 183–194, 2013.

[30] S. Malchow, D. S. Leventhal, S. Nishi et al., "Aire-dependent thymic development of tumor-associated regulatory T cells," *Science*, vol. 339, no. 6124, pp. 1219–1224, 2013.

[31] H. Wang, F. Franco, and P. C. Ho, "Metabolic regulation of Tregs in cancer: opportunities for immunotherapy," *Trends in Cancer*, vol. 3, no. 8, pp. 583–592, 2017.

[32] P. C. Ho, J. D. Bihuniak, A. N. Macintyre et al., "Phosphoenolpyruvate is a metabolic checkpoint of anti-tumor T cell responses," *Cell*, vol. 162, no. 6, pp. 1217–1228, 2015.

[33] C. H. Chang, J. Qiu, D. O'Sullivan et al., "Metabolic competition in the tumor microenvironment is a driver of cancer progression," *Cell*, vol. 162, no. 6, pp. 1229–1241, 2015.

[34] K. E. Scott and J. L. Cleveland, "Lactate wreaks havoc on tumor-infiltrating T and NK cells," *Cell Metabolism*, vol. 24, no. 5, pp. 649-650, 2016.

[35] D. E. Speiser, P. C. Ho, and G. Verdeil, "Regulatory circuits of T cell function in cancer," *Nature Reviews Immunology*, vol. 16, no. 10, pp. 599–611, 2016.

[36] N. J. MacIver, R. D. Michalek, and J. C. Rathmell, "Metabolic regulation of T lymphocytes," *Annual Review of Immunology*, vol. 31, no. 1, pp. 259–283, 2013.

[37] R. D. Michalek, V. A. Gerriets, S. R. Jacobs et al., "Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4$^+$ T cell subsets," *The Journal of Immunology*, vol. 186, no. 6, pp. 3299–3303, 2011.

[38] K. Fischer, P. Hoffmann, S. Voelkl et al., "Inhibitory effect of tumor cell-derived lactic acid on human T cells," *Blood*, vol. 109, no. 9, pp. 3812–3819, 2007.

[39] V. Huber, C. Camisaschi, A. Berzi et al., "Cancer acidity: an ultimate frontier of tumor immune escape and a novel target of immunomodulation," *Seminars in Cancer Biology*, vol. 43, pp. 74–89, 2017.

[40] A. Angelin, L. Gil-de-Gómez, S. Dahiya et al., "Foxp3 reprograms T cell metabolism to function in low-glucose, high-lactate environments," *Cell Metabolism*, vol. 25, no. 6, pp. 1282–1293.e7, 2017.

[41] G. Plitas, C. Konopacki, K. Wu et al., "Regulatory T cells exhibit distinct features in human breast cancer," *Immunity*, vol. 45, no. 5, pp. 1122–1134, 2016.

[42] S. Nizar, B. Meyer, C. Galustian, D. Kumar, and A. Dalgleish, "T regulatory cells, the evolution of targeted immunotherapy," *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, vol. 1806, no. 1, pp. 7–17, 2010.

[43] D. Gabrilovich, "Mechanisms and functional significance of tumour-induced dendritic-cell defects," *Nature Reviews Immunology*, vol. 4, no. 12, pp. 941–952, 2004.

[44] G. Zhou and H. I. Levitsky, "Natural regulatory T cells and de novo-induced regulatory T cells contribute independently to tumor-specific tolerance," *The Journal of Immunology*, vol. 178, no. 4, pp. 2155–2162, 2007.

[45] A. Curti, S. Pandolfi, B. Valzasina et al., "Modulation of tryptophan catabolism by human leukemic cells results in the conversion of $CD25^-$ into $CD25^+$ T regulatory cells," *Blood*, vol. 109, pp. 2871–2877, 2007.

[46] J. M. Weiss, A. M. Bilate, M. Gobert et al., "Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced $Foxp3^+$ T reg cells," *The Journal of Experimental Medicine*, vol. 209, no. 10, pp. 1723–1742, 2012.

[47] J. P. Hindley, C. Ferreira, E. Jones et al., "Analysis of the T-cell receptor repertoires of tumor-infiltrating conventional and regulatory T cells reveals no evidence for conversion in carcinogen-induced tumors," *Cancer Research*, vol. 71, no. 3, pp. 736–746, 2011.

[48] A. Sainz-Perez, A. Lim, B. Lemercier, and C. Leclerc, "The T-cell receptor repertoire of tumor-infiltrating regulatory T lymphocytes is skewed toward public sequences," *Cancer Research*, vol. 72, no. 14, pp. 3557–3569, 2012.

[49] M. Bu, Y. Shen, W. L. Seeger et al., "Ovarian carcinoma-infiltrating regulatory T cells were more potent suppressors of $CD8^+$ T cell inflammation than their peripheral counterparts, a function dependent on TIM3 expression," *Tumour Biology*, vol. 37, no. 3, pp. 3949–3956, 2016.

[50] C. Wang, J. H. Lee, and C. H. Kim, "Optimal population of $FoxP3^+$ T cells in tumors requires an antigen priming-dependent trafficking receptor switch," *PLoS One*, vol. 7, no. 1, article e30793, 2012.

[51] G. M. Delgoffe, S. R. Woo, M. E. Turnis et al., "Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis," *Nature*, vol. 501, no. 7466, pp. 252–256, 2013.

[52] K. Ali, D. R. Soond, R. Piñeiro et al., "Inactivation of PI(3)K p110δ breaks regulatory T-cell-mediated immune tolerance to cancer," *Nature*, vol. 510, pp. 407–411, 2014.

[53] C. T. Luo, W. Liao, S. Dadi, A. Toure, and M. O. Li, "Graded Foxo1 activity in Treg cells differentiates tumour immunity from spontaneous autoimmunity," *Nature*, vol. 529, no. 7587, pp. 532–536, 2016.

[54] Y. Grinberg-Bleyer, H. Oh, A. Desrichard et al., "NF-κB c-Rel is crucial for the regulatory T cell immune checkpoint in cancer," *Cell*, vol. 170, no. 6, pp. 1096–1108.e13, 2017.

[55] H. Nakagawa, J. M. Sido, E. E. Reyes, V. Kiers, H. Cantor, and H. J. Kim, "Instability of Helios-deficient Tregs is associated with conversion to a T-effector phenotype and enhanced antitumor immunity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 22, pp. 6248–6253, 2016.

[56] A. E. Overacre-Delgoffe, M. Chikina, R. E. Dadey et al., "Interferon-γ drives T reg fragility to promote anti-tumor immunity," *Cell*, vol. 169, no. 6, pp. 1130–1141.e11, 2017.

[57] M. De Simone, A. Arrigoni, G. Rossetti et al., "Transcriptional landscape of human tissue lymphocytes unveils uniqueness of tumor-infiltrating T regulatory cells," *Immunity*, vol. 45, no. 5, pp. 1135–1147, 2016.

[58] D. Burzyn, W. Kuswanto, D. Kolodin et al., "A special population of regulatory T cells potentiates muscle repair," *Cell*, vol. 155, no. 6, pp. 1282–1295, 2013.

[59] M. Scurr, K. Ladell, M. Besneux et al., "Highly prevalent colorectal cancer-infiltrating LAP^+ $Foxp3^-$ T cells exhibit more potent immunosuppressive activity than $Foxp3^+$ regulatory T cells," *Mucosal Immunology*, vol. 7, no. 2, pp. 428–439, 2014.

[60] N. Kakita, T. Kanto, I. Itose et al., "Comparative analyses of regulatory T cell subsets in patients with hepatocellular carcinoma: a crucial role of $CD25^-$ $FOXP3^-$ T cells," *International Journal of Cancer*, vol. 131, no. 11, pp. 2573–2583, 2012.

[61] A. Amedei, E. Niccolai, M. Benagiano et al., "Ex vivo analysis of pancreatic cancer-infiltrating T lymphocytes reveals that ENO-specific Tregs accumulate in tumor tissue and inhibit Th1/Th17 effector cell functions," *Cancer Immunology, Immunotherapy*, vol. 62, no. 7, pp. 1249–1260, 2013.

[62] Y. Yi, H. W. He, J. X. Wang et al., "The functional impairment of HCC-infiltrating $\gamma\delta$ T cells, partially mediated by regulatory T cells in a TGFβ- and IL-10-dependent manner," *Journal of Hepatology*, vol. 58, no. 5, pp. 977–983, 2013.

[63] J. Yan, Y. Zhang, J. P. Zhang, J. Liang, L. Li, and L. Zheng, "Tim-3 expression defines regulatory T cells in human tumors," *PLoS One*, vol. 8, no. 3, article e58006, 2013.

[64] J. D. Bui, R. Uppaluri, C. S. Hsieh, and R. D. Schreiber, "Comparative analysis of regulatory and effector T cells in progressively growing versus rejecting tumors of similar origins," *Cancer Research*, vol. 66, no. 14, pp. 7301–7309, 2006.

[65] C. A. Bauer, E. Y. Kim, F. Marangoni, E. Carrizosa, N. M. Claudio, and T. R. Mempel, "Dynamic Treg interactions with intratumoral APCs promote local CTL dysfunction," *The Journal of Clinical Investigation*, vol. 124, no. 6, pp. 2425–2440, 2014.

[66] T. L. Whiteside, "The role of regulatory T cells in cancer immunology," *ImmunoTargets and Therapy*, vol. 4, pp. 159–171, 2015.

[67] U. Grohmann, C. Orabona, F. Fallarino et al., "CTLA-4-Ig regulates tryptophan catabolism in vivo," *Nature Immunology*, vol. 3, no. 11, pp. 1097–1101, 2002.

[68] J. D. Mezrich, J. H. Fechner, X. Zhang, B. P. Johnson, W. J. Burlingham, and C. A. Bradfield, "An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells," *The Journal of Immunology*, vol. 185, no. 6, pp. 3190–3198, 2010.

[69] F. Fallarino, U. Grohmann, S. You et al., "The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells," *The Journal of Immunology*, vol. 176, no. 11, pp. 6752–6761, 2006.

[70] D. A. Wainwright, I. V. Balyasnikova, A. L. Chang et al., "IDO expression in brain tumors increases the recruitment of regulatory T cells and negatively impacts survival," *Clinical Cancer Research*, vol. 18, no. 22, pp. 6110–6121, 2012.

[71] H. Pere, C. Tanchot, J. Bayry et al., "Comprehensive analysis of current approaches to inhibit regulatory T cells in cancer," *OncoImmunology*, vol. 1, no. 3, pp. 326–333, 2012.

[72] B. Chaudhary and E. Elkord, "Regulatory T cells in the tumor microenvironment and cancer progression: role and therapeutic targeting," *Vaccines*, vol. 4, no. 3, p. 28, 2016.

[73] J. H. Sampson, R. J. Schmittling, G. E. Archer et al., "A pilot study of IL-2Rα blockade during lymphopenia depletes regulatory T-cells and correlates with enhanced immunity in patients with glioblastoma," *PLoS One*, vol. 7, no. 2, article e31046, 2012.

[74] A. J. Rech, R. Mick, S. Martin et al., "CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patients," *Science Translational Medicine*, vol. 4, no. 134, article 134ra62, 2012.

[75] J. F. Jacobs, C. J. Punt, W. J. Lesterhuis et al., "Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment: a phase I/II study in metastatic melanoma patients," *Clinical Cancer Research*, vol. 16, no. 20, pp. 5067–5078, 2010.

[76] J. Dannull, Z. Su, D. Rizzieri et al., "Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells," *The Journal of Clinical Investigation*, vol. 115, no. 12, pp. 3623–3633, 2005.

[77] P. Attia, A. V. Maker, L. R. Haworth, L. Rogers-Freezer, and S. A. Rosenberg, "Inability of a fusion protein of IL-2 and diphtheria toxin (denileukin diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma," *Journal of Immunotherapy*, vol. 28, no. 6, pp. 582–592, 2005.

[78] F. Arce Vargas, A. J. S. Furness, I. Solomon et al., "Fc-optimized anti-CD25 depletes tumor-infiltrating regulatory T cells and synergizes with PD-1 blockade to eradicate established tumors," *Immunity*, vol. 46, no. 4, pp. 577–586, 2017.

[79] E. I. Buchbinder and A. Desai, "CTLA-4 and PD-1 pathways: similarities, differences, and implications of their inhibition," *American Journal of Clinical Oncology*, vol. 39, no. 1, pp. 98–106, 2016.

[80] J. D. Wolchok, H. Kluger, M. K. Callahan et al., "Nivolumab plus ipilimumab in advanced melanoma," *The New England Journal of Medicine*, vol. 369, no. 2, pp. 122–133, 2013.

[81] S. L. Topalian, M. Sznol, D. F. McDermott et al., "Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab," *Journal of Clinical Oncology*, vol. 32, no. 10, pp. 1020–1030, 2014.

[82] M. A. Postow, M. K. Callahan, and J. D. Wolchok, "Immune checkpoint blockade in cancer therapy," *Journal of Clinical Oncology*, vol. 33, no. 17, pp. 1974–1982, 2015.

[83] S. M. Ansell, A. M. Lesokhin, I. Borrello et al., "PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma," *The New England Journal of Medicine*, vol. 372, no. 4, pp. 311–319, 2015.

[84] B. Kavanagh, S. O'Brien, D. Lee et al., "CTLA4 blockade expands FoxP3$^+$ regulatory and activated effector CD4+ T cells in a dose-dependent fashion," *Blood*, vol. 112, no. 4, pp. 1175–1183, 2008.

[85] A. V. Maker, P. Attia, and S. A. Rosenberg, "Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade," *The Journal of Immunology*, vol. 175, no. 11, pp. 7746–7754, 2005.

[86] D. O'Mahony, J. C. Morris, C. Quinn et al., "A pilot study of CTLA-4 blockade after cancer vaccine failure in patients with advanced malignancy," *Clinical Cancer Research*, vol. 13, no. 3, pp. 958–964, 2007.

[87] C. Ralph, E. Elkord, D. J. Burt et al., "Modulation of lymphocyte regulation for cancer therapy: a phase II trial of tremelimumab in advanced gastric and esophageal adenocarcinoma," *Clinical Cancer Research*, vol. 16, no. 5, pp. 1662–1672, 2010.

[88] J. S. Weber, O. Hamid, S. D. Chasalow et al., "Ipilimumab increases activated T cells and enhances humoral immunity in patients with advanced melanoma," *Journal of Immunotherapy*, vol. 35, no. 1, pp. 89–97, 2012.

[89] T. R. Simpson, F. Li, W. Montalvo-Ortiz et al., "Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma," *The Journal of Experimental Medicine*, vol. 210, no. 9, pp. 1695–1710, 2013.

[90] K. Ko, S. Yamazaki, K. Nakamura et al., "Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells," *The Journal of Experimental Medicine*, vol. 202, no. 7, pp. 885–891, 2005.

[91] J. Mitsui, H. Nishikawa, D. Muraoka et al., "Two distinct mechanisms of augmented antitumor activity by modulation of immunostimulatory/inhibitory signals," *Clinical Cancer Research*, vol. 16, no. 10, pp. 2781–2791, 2010.

[92] S. Piconese, B. Valzasina, and M. P. Colombo, "OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection," *The Journal of Experimental Medicine*, vol. 205, no. 4, pp. 825–839, 2008.

[93] A. M. Lesokhin, M. K. Callahan, M. A. Postow, and J. D. Wolchok, "On being less tolerant: enhanced cancer immunosurveillance enabled by targeting checkpoints and agonists of T cell activation," *Science Translational Medicine*, vol. 7, no. 280, p. 280sr1, 2015.

[94] N. A. Taylor, S. C. Vick, M. D. Iglesia et al., "Treg depletion potentiates checkpoint inhibition in claudin-low breast cancer," *The Journal of Clinical Investigation*, vol. 127, no. 9, pp. 3472–3483, 2017.

[95] B. Ondondo, E. Jones, A. Godkin, and A. Gallimore, "Home sweet home: the tumor microenvironment as a haven for regulatory T cells," *Frontiers in Immunology*, vol. 4, p. 197, 2013.

[96] K. Kurose, Y. Ohue, E. Sato et al., "Increase in activated Treg in TIL in lung cancer and in vitro depletion of Treg by ADCC using an antihuman CCR4 mAb (KM2760)," *Journal of Thoracic Oncology*, vol. 10, no. 1, pp. 74–83, 2015.

[97] T. Ishida, T. Joh, N. Uike et al., "Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase II study," *Journal of Clinical Oncology*, vol. 30, no. 8, pp. 837–842, 2012.

[98] N. Redjimi, C. Raffin, I. Raimbaud et al., "CXCR3$^+$ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity," *Cancer Research*, vol. 72, no. 17, pp. 4351–4360, 2012.

[99] R. S. Muraoka, N. Dumont, C. A. Ritter et al., "Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases," *The Journal of Clinical Investigation*, vol. 109, no. 12, pp. 1551–1559, 2002.

[100] L. Gorelik and R. A. Flavell, "Immune-mediated eradication of tumors through the blockade of transforming growth

factor-β signaling in T cells," *Nature Medicine*, vol. 7, no. 10, pp. 1118–1122, 2001.

[101] Z. Sun, J. Fourcade, O. Pagliano et al., "IL10 and PD-1 cooperate to limit the activity of tumor-specific $CD8^+$ T cells," *Cancer Research*, vol. 75, no. 8, pp. 1635–1644, 2015.

[102] A. P. Vicari, C. Chiodoni, C. Vaure et al., "Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody," *The Journal of Experimental Medicine*, vol. 196, no. 4, pp. 541–549, 2002.

[103] M. E. Turnis, D. V. Sawant, A. L. Szymczak-Workman et al., "Interleukin-35 limits anti-tumor immunity," *Immunity*, vol. 44, no. 2, pp. 316–329, 2016.