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Impact of Treg on other T cell subsets in progression of fibrosis in experimental lung fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis is an irreversible, progressive and lethal lung disease. Regulatory T cells (Tregs) and Th17 cells both are involved in lung fibrosis. But there are only few reports regarding the effect of Treg on other T cell subsets in experimental lung fibrosis. The aim of this study was to investigate the impact of Treg on Th17, CD4 + CD28-T, CD4 + CD28 + T and CD8 + T cell subsets that could drive lung fibrosis. To reach the goal of our study, first we depleted Tregs by anti-CD25 mAb injection in experimental C57BL/6 mice model. It has been demonstrated in our study that depletion of Treg ameliorates bleomycin-induced lung fibrosis by immune modulating Th17 and other important T cell subsets response in lung. Our flow cytometry data revealed that the percentages of Th17, CD4 + CD28-T, CD4 + CD28 + T and CD8 + T cell subsets were decreased in experimental lung fibrosis after Treg depletion. We also observed significant downregulation of IL-17 A in Treg-depleted mice after bleomycin delivery. In addition, the study also suggested that Treg depletion led to considerable upregulation of IFN-γ after bleomycin administration. Therefore, Th17 cells, CD8 + T cells, CD4 + CD28- and CD4 + CD28 + T cell subsets all are controlled by regulatory T cell, help in progression of fibrosis in experimental lung fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is an irreversible, progressive and lethal disease of lung, characterised by extreme deposition of extracellular matrix and destruction of the normal lung architecture (Rogliani et al., 2008). Bleomycin (BM)-induced lung fibrosis in animals is a well-characterized histological and biochemical model of human pulmonary fibrosis (Yasui et al., 2001; Peng et al., 2013). In pulmonary fibrosis, the innate immune system triggers a pre-dominantly Th1-mediated acute inflammatory response and there are multiple mechanisms work to resolve this acute inflammatory response (Nathan and Ding, 2010). Those mechanisms include altered immune cells phenotype, switch in immunomodulatory molecules from pro-inflammatory to anti-inflammatory, and the activation of many anti-fibrotic mediators. Ultimately, Th2-mediated adaptive immune response contributes to and sustains non-resolving inflammation in the injured/damaged tissue that leads to the development of pulmonary fibrosis (Wynn, 2005). There are few reports about the involvement of T cells in pulmonary fibrosis which are largely conflicting and support either a pro-fibrotic or an anti-fibrotic nature in pulmonary fibrosis (Luzina et al., 2008).

Regulatory T cells are often described as CD4 + CD25 + T cells, which are a stable lineage of T cells that play a suppressive function in the maintenance of immunological tolerance and immune homeostasis (Sakaguchi et al., 2009). Kotsianidis et al. (2009) reported the phenomenon of global Treg impairment in patients with IPF, suggesting a role for Tregs in the fibrotic process (Kotsianidis et al., 2009). Other groups observed an increased frequency of Tregs in the lungs of irradiation-induced pulmonary fibrosis in mice model (Xu et al., 2014). According to Birjandi et al. (2016), CD4 + CD25hiFoxP3 + cells exacerbated the pulmonary fibrosis induced by bleomycin (Birjandi *et al.*, 2016). Another important subset of T lymphocyte, Th17 cells which produce IL-17 A (also termed IL-17) play crucial roles by recruiting neutrophils and other cytokines in lung inflammatory diseases (Alcorn et al., 2010; Ouyang et al., 2008).

Th17 cells and Tregs are thought to promote and inhibit inflammatory responses, respectively (Song et al., 2012). Tregs have the bi-functional ability to inhibit (Rangachari et al., 2006; Chaudhry et al., 2011) or endorse Th17 response (Pandiyan et al., 2011; Xu et al., 2007; Veldhoen et al., 2006). Various investigators reported the role of Tregs

Abbreviations: BM, bleomycin; BIPF, bleomycin-induced pulmonary fibrosis; IPF, idiopathic pulmonary fibrosis; Treg, regulatory T cell; ROR-γT, retinoid-related orphan receptor γT; IFN-γ, interferon-γ; IL-, interleukin-; BW, body weight

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in different types of pulmonary fibrosis such as irradiation-induced pulmonary fibrosis and silicosis (D'Alessio et al., 2009; Trujillo et al., 2010; Liu et al., 2010). However, the impact of Tregs on Th17, CD8 + T, CD4 + CD28- and CD4 + CD28 + T cells expression in pulmonary fibrosis remains unexplored. In this study, we used anti-CD25 mAb to neutralize Tregs continuously and then assessed the immune responses of bleomycin-induced lung fibrosis. The objective of this study is to investigate the modulatory effect of Treg on Th17, CD8 + T, CD4 + CD28- and CD4 + CD28 + T cell subsets in progression of lung fibrosis.

2. Materials and methods

2.1. Animals and ethics statement

Healthy adult C57BL/6 mice of age 8–10 weeks were purchased from NIN, Hyderabad, India. Mice were housed for at least one week in an environmentally controlled room (temperature, 25–28 ± 2 °C; humidity, 55 ± 5%; 12-h dark/light cycle) with access to food and water *ad libitum* before use. Animal experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India (Registration No: 885/GO/Re/S/05/CPCSEA), and as approved by the Institutional Animal Ethics Committee (IAEC) University of Calcutta, and confirmed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.2. Mice model of bleomycin(BM)-induced pulmonary fibrosis

Sixty *C57BL/6* mice were randomly divided into four groups (n = 15 per group): (i) control group, (ii) control + Treg-depleted group, (iii) bleomycin (BM)-treated group, (iv) bleomycin + Treg-depleted group. Animals were anesthetized with intraperitoneal injection of 2% pentobarbital sodium. After being anesthetized, mice were intra-tracheally injected with 50 µL of bleomycin (5 mg/kg body weight) diluted in normal saline on Day 0 (Chakraborty et al., 2017). For experiments, mice were sacrificed on day 21 and lung samples were collected. For control group, the sterile 0.9% normal saline was administrated.

2.3. Treg depletion

To deplete regulatory T cells, mice were injected intra-peritoneally (i.p.) with 100 µg anti-CD25 antibody (PC61; BioLegend, San Diego, CA, USA) one day before bleomycin instillation, and repeatedly treated i.p. with 100 µg anti-CD25 antibody every 7 days after the bleomycin exposure for continuing depletion (Liu et al., 2016).

2.4. Bronchoalveolar lavage fluid (BALF) collection and cytokine assay

After sacrifice, trachea of mice was exposed and a plastic cannula was inserted into the trachea. 1 ml of 0.9% saline solution was injected into the lungs by a syringe and was then withdrawn. This injection procedure was repeated five times. The BALF was centrifuged at 1500 rpm for 8 min at 4 °C. The BALF supernatant was collected after centrifugation and stored at −80 °C before the cytokine assay (Liu et al., 2013). IL-17 A and IFN-γ levels in BALF supernatants were measured using ELISA kits according to the manufacturer's protocol (RayBio® Mouse ELISA kit). The absorbance at 450 nm (A450) was determined using a 96-well bichromatic microplate reader (eBioscience, USA).

2.5. Histopathological evaluation

The lung samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Sections were stained with Masson's trichrome (MTS). The Ashcroft score was used for the quantitative histological analysis (Ashcroft et al., 1988).

2.6. Lung single-cell suspension preparation

Mouse lungs were minced into 1-mm pieces with scissors and digested with 2 mg/ml collagenase IV (Himedia) and 0.5 mg/ml DNase (Sigma-Aldrich) for 1 h at 37 °C on a rocking platform. Lung digests was filtered through a 40 µm nylon cell-strainer and red blood cells will be removed by centrifuging the lung digests at 2000 rpm for 5 min s using RBC lysis buffer solution. Next cells were washed, counted and re-suspended in PBS to make single-cell suspension.

2.7. Flow cytometry

Lungs were dissected out from the respective mice group, and single-cell suspension was prepared as described earlier. After accurate cell count, cells were divided into 10^6cells/100 µl in cell staining buffer (CSB), containing 3% FCS. These cells were then incubated with anti-mouse CD16/32 (TruStain fcX^{TM}, BioLegend) for 5 min at 4 °C to block nonspecific binding. Cell surface staining was performed with FITC-conjugated CD4 (Biolegend), PE-conjugated CD25 (Biolegend) and PE-conjugated CD28 (Biolegend) and incubated for 30 min at 4 °C. These cells were then fixed with 4% paraformaldehyde and permeabilization was done with 0.1% saponin. Cells were then washed twice with CSB and intracytoplasmic staining was performed using PE-conjugated IL-17 (BioLegend) for Th17 cells and AlexaFluor647-conjugated FOXP3 (BioLegend) for Treg cells respectively along with respective isotype controls (Keswani and Bhattacharyya, 2014). Finally the cells were washed, the pellets were resuspended in 100 µl CSB and data were acquired using BD Acquri C6 flow cytometer and analysed with BD Acquri C6, FlowJo version vX.0.7 software.

2.8. Confocal immunofluorescence assay

For the localization study of Th17 cells in lung, tissue sections were permeabilized with 0.2% Triton X-100 in PBS for 45 min. Next the slides of lung tissue were blocked with goat serum for 30 min to reduce nonspecific binding, and then incubated with Alexa Fluor®647-conjugated anti-mouse CD4 antibody (BioLegend) 10 mg/ml at 4 °C overnight. After washing with PBS, the slides were incubated with FITC-conjugated anti-mouse IL-17 antibody (BioLegend) 10 mg/ml for 2 h at room temperature. The localization of CD4 + IL-17 + Th17 cells were captured by a confocal laser scanning microscope (OLYMPUS) and analyzed with the FV10-ASW 2.0 Viewer software (Chakraborty et al., 2017).

2.9. Hydroxyproline assay

The collagen content in the lung homogenates was examined by a hydroxyproline (HYP) colorimetric assay kit (BioVision). All steps of the HYP assay were performed according to the manufacturer's instructions. The absorbance of each sample at 560 nm wavelength was read by a microplate reader (Thermo Fisher Scientific, USA).

2.10. RNA extraction and real-time PCR

Total RNA was extracted from lung tissues using an RNAase Mini Kit (Promega). The RNA was then reverse transcribed to cDNA according to Chakraborty et al. (2014) (Chakraborty et al., 2014). Real-time PCR amplifications were performed in triplicate using the PowerUp^{TM}SYBR Green Master Mix and were carried out using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The threshold cycle (Ct) was obtained from the PCR curves and expression levels of the target genes were quantified in terms of the Ct values corresponding to the untreated and treated samples and were normalized against GAPDH