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CD4$^+$Foxp3$^+$regulatory T cells induced by TGF-β, IL-2 and all-trans retinoic acid attenuate obliterative bronchiolitis in rat trachea transplantation

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ABSTRACT

Obliterative bronchiolitis (OB) is the major obstacle for long-term allograft survival in lung transplantation, and the underlying mechanism is still not well understood. Regulatory T cells (Tregs) have been shown to be essential in the maintenance of immune tolerance. In this study we investigated the role of Tregs in protecting OB in rat. We show that the combination of TGF-β, Interleukin (IL)-2, and all-trans retinoic acid (atRA) could induce naïve rat CD4$^+$CD25$^-$ T cells to differentiate into CD4$^+$CD25$^+$Foxp3$^+$ T cells in vitro, and they acquired suppressive function. In a rat orthotopic tracheal transplantation OB model, the adoptive transfer of the induced Tregs reduced symptoms of airway obliteration and fibrication of grafts when compared with adoptive transfer of control cells without suppressive property. Moreover, recipients treated with the induced Tregs secreted high level of immunosuppressive cytokine TGF-β and IL-10, and low level of pro-inflammatory cytokines IL-17, IFN-γ, IL-6, and MCP-1, and had fewer effector T cells including Th17 cells and Th1 cells in the graft. Taken together, these findings suggest that in vitro induced Tregs by the combination of TGF-β, IL-2, and atRA are effective in protecting rat trachea allograft rejection through the inhibition of effector T cells and their function. These datas implicate new therapies to prevent OB and allograft rejection in human lung transplantation.

1. Introduction

Lung transplantation is the only curative treatment for many end-stage pulmonary diseases including cystic fibrosis, pulmonary fibrosis, and chronic obstructive pulmonary disease [1]. The long-term allograft survival in lung transplantation has been hampered by chronic graft dysfunction, which is manifested as obliterative bronchiolitis (OB) referring to inflammatory and fibroproliferative obliteration of bronchioles [2]. Despite the success of immunosuppressive drugs in preventing acute allograft rejection, chronic obliteration of bronchioles remains a risk factor for long-time survival of the graft [3]. CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs) maintain immune tolerance, suppress inflammation [4], inhibit autoimmune disease [5–7], and prevent allograft rejection [8,9].The transcription factor Foxp3 is not only the marker of Tregs, it also programs the regulatory function [10–12]. There are two subpopulations of Foxp3$^+$CD4$^+$ Tregs: naturally occurring Treg (nTreg) which are generated in the thymus; and induced Tregs (iTregs) which are generated in the peripheral lymphoid tissues from naïve CD4$^+$CD25$^-$ T cells in the presence of TGF-β and IL-2 [13,14]. The conversion of naïve CD4$^+$CD25$^-$ T cells into iTregs can be enhanced by atRA in mouse and human [15–17].

It has been established that interleukin (IL)-17 producing Th17 cells, a newly identified subset of effect T cells characterized by the expression of transcript factor RORγt [18], play an important role in inflammation and in autoimmune diseases including asthma, rheumatoid arthritis, experimental autoimmune encephalitis [19,20], and they have been implicated in the progression of bronchiolitis obliterans syndrome (BOS) after human lung transplant [21–24]. It was also reported that nTregs and iTregs are able to inhibit the differentiation and function of Th17 cells so as to ameliorate many forms of inflammatory diseases mediated by Th17 cells [25]. However, whether Th17 cells are involved in the development of OB and whether Tregs could lessen OB by suppressing Th17 cell differentiation and function have not been well established.

Here, we used a rat allo-orthotopic tracheal transplantation model to investigate the development of OB after lung transplantation, and to test whether adoptive transfer of iTregs would attenuate OB. We

Abbreviations: OB, Obliterative bronchiolitis; SPF, Specific pathogen free; BN, Brown-Norway rat; Lew, Lewis rat; FACS, Fluorescence-activated cell sorting; Elisa, Enzyme-linked immunosorbent assay; IL, Interleukin; IFN-γ, Interferon-γ; TGF-β, Transforming growth factor-β; POD, Postoperative day; OTT, Orthotopic tracheal transplantation; atRA, all-trans Retinoic acid.

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report that adoptive transfer of iTregs induced by TGF-β, IL-2, and atRA was able to attenuate the progression of fibration and inflammation of lung allograft by blocking the function and differentiation of Th17 cells and Th1 cells. These data could provide new therapeutic avenues to promote long term allograft survival after lung transplantation.

2. Materials and methods

2.1. Animals

Eight- to ten-week-old specific pathogen-free (SPF), male, Lewis, Brown Norway (BN) rats weighing 200–250 g were purchased from the Beijing Vital River Experimental Animal and Technology Co., Ltd. (Beijing, China). All rats were used in the SPF animal care facilities according to the guidelines of institutional animal care and use committee of Shanghai Public Health Clinical Center Affiliated to Fudan University.

2.2. Antibodies

Anti-rat mAbs for CD4-APC and –FITC (OX-35), CD8-PerCP (OX-8), CD3-PE (G4.18), CD25-PE and –FITC (OX-39), CD54-PE (ICAM-1,1A29), Purified CD11b/c (OX-42), CD25(OX-39), CD8 (OX-8), CD28 (JJ319), CD3 (G4.18), CD45RA (OX-33), DC (OX-62), CD161a (NKR-P1A, 10/78) and isotype controls were purchased from BD PharMingen (San Diego, CA).Anti-rat CD62L-PerCP (OX-85), CD134-FITC (OX-40), CD152-PE (CTLA-4, WKH203), and IFN-γ-PE (DB-1) were from BioLegend. Antibodies for Foxp3-PE-Cy7 (FJK-16 s), IL-17-APC and –FITC (eBio17B7) were obtained from eBioscience (San Diego, CA). Carboxy-fluoresceindiacetate succinimidyl ester (CFSE) was from Molecular Probes (Eugene, OR). All-trans retinoic acid, Ionomycin and PMA were from Sigma-Aldrich (St Louis, MO).

2.3. Cells isolation

Spleens were removed from Lewis rats, teased into cell-single suspensions and filtered through a 70 μm cell strainer (BD Falcon, USA). After removing red blood cells by RBC lysing buffer (R&D Systems), $CD4^+CD25^-$ T cell were negatively separated after incubation with a mixture of mouse IgG anti-rat mAbs: OX42 (CD11b/c), 3.2.3 (NKR-P1A), OX62 (integrin E2 chain or CD103), OX39 (CD25, IL-2Rchain), CD8a (OX-8), CD45RA (OX-33), followed by anti-mouse IgG-coated magnetic beads (Invitrogen Dynal AS, Oslo, Norway) [26]. The purity of cells was routinely 90%.

2.4. Cell culture and in vitro T cell stimulation

$CD4^+CD25^-$ T Cells were cultured at 37 °C and under 5% CO_2 in AIM-V serum free medium supplemented with 10% (vol/vol) FBS, 1% penicillin-streptomycin, 10 mM HEPES, 1 mM pyruvate and 0.1 mM nonessential amino acids and 5 mM β-mercaptoethanol (Gibco, USA) at a concentration of 2×10^5 cells/200 μl per well in 96-well flat bottom plates and stimulated by pre-coated anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml). Exogenous cytokines used were IL-2 (100 μ/ml), TGF-β1 (10 ng/ml)(R&D Systems, USA). In some experiments, all-trans retinoic acid were added to the cultures at a concentration of 10 nM. Cells were collected on day 4 for Flow Cytometry analysis or for the suppression and proliferation assays.

2.5. Surface and intracellular staining and Flow Cytometry analysis

Prior to cell staining, cells were washed and re-suspended in staining buffer containing 1x PBS, 2% FCS and 0.01%NaN_3.Cells were incubated for 30 min at 4 °C after adding fluorescently labeled antibodies for cell surface markers. An Aria II (BD-Bioscience, USA) and FlowJo software (Tree Star) were used for FACS. For intracellular cytokine staining, cells were incubated for 4–5 h with 100 ng/ml PMA, 1 μg/ml Ionomycin and 1× Monensin in a tissue culture incubator at 37 °C. After surface cell staining, Cytofix/Cytoperm kit (BD PharMingen, USA) was used for intracellular cytokine staining according to the manufacturer's protocol. Intracellular Foxp3 staining was performed according to the Foxp3-staining kit protocol (eBioscience) [27].

2.6. In vitro suppression and proliferation assay

1×10^5 freshly isolated $CD4^+CD25^-$ T cells labelled with 2 μM CFSE were used as effectors and cultured in 96-well round-bottom plates. Tregs or control cells stimulated with varies cytokines in vitro as suppressors were added in the wells and co-cultured with CFSE-$CD4^+CD25^-$T cells at graded concentrations (effect T cells:supressors = 1:0, 1:0.5, 1:1, 1:2, 1:4). Cells were stimulated with 10 μg/ml anti-CD3 and 5 μg/ml anti-CD28 coated Dynal beads at a cell:beads rate of 1:2 in 200 μl AIM-V serum free medium. After 4 days, CFSE dilution was analysed with Flow Cytometer and the suppressive capacity of Tregs to responder cells in co-culture was expressed as the relative inhibition of the percentage of $CFSE^{low}$ cells [100 × (1-%$CFSE^{low}$effectors in coculture ÷ %$CFSE^{low}$effectors alone)] for CFSE based measurement of proliferation [28]. For proliferation assay, Tregs or control cells were labelled with 2 μM CFSE and stimulated with anti-CD3 and anti-CD28 coated Dynal beads at a cell:beads rate of 1:2 in RPMI1640 media containing 10% FCS for 4 days in the presence or absence of IL-2 (100U/ml), followed by Flow Cytometer analysis.

2.7. Trachea transplantation

Rat orthotopic tracheal transplantation was performed with male Brown-Norway as donor and Lewis rats as recipient weighing 200–250 g as published previously [29]. Briefly, five or six rings of donor trachea were implanted end-to-end into the recipient via a midline cervical incision [30].

2.8. Graft homogenate

Tracheal grafts in each group were deposited in 0.5 ml PBS and minced into small pieces with scissors, then dissociated with a Medmachine (BD Bioscience, Rockville, MD) according to the manufacturer's protocol. The homogenate were filtered with 70um Nylon strainers (BD Falcon, USA). After centrifugation, cells were collected for Flow Cytometer analysis and supernatants for ELISA analysis.

2.9. Measurement of cytokines and chemokines by ELISA

Serum and graft homogenate supernatants were harvested and stored at −80 °C until measurement. The total protein concentrations in homogenate supernatants were measured and adjusted to 1 mg/ml using PBS, followed by detecting the relative concentrations of IL-17A, IL-6, IFN-γ, MCP-1, IL-10, TGF-β1 by ELISA according to the protocol provided by the manufacturers (Rat IL-6, IL-10, and IFN-γ ELISA Kits were purchased from BD Pharmingen, CA; rat IL-17, MCP-1 were from eBioscience, CA; and TGF-β1 were from R&D Minneapolis, MN). All experiments are performed at least in triplicate samples. A standard curve using recombinant cytokine was generated for each assay.

2.10. Micro-CT scan and three-dimensional reconstruction

After anesthetizing, grafts of recipients were scanned on by Micro-CT (GE company, USA), then the three-dimensional images were reconstructed, and the obliteration areas were eventually measured by GEHC MicroView software (GE company, USA). Luminal obliteration was quantified as previously described [31]. Briefly, luminal obliteration was defined as the area containing tissue inside the