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The role of regulatory B cells (Bregs) in the Tregs-amplifying effect of Sirolimus

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ABSTRACT

Sirolimus can significantly amplify regulatory T cells (Tregs) in vivo and in vitro, but the specific mechanism of this has not been well documented. The role of regulatory B cells (Bregs) in the Tregs-amplifying effect of Sirolimus was investigated in peripheral blood mononuclear cells (PBMCs) in vitro in this study. The results showed that the percentages of both CD19 + CD24 + CD38 + TGF-β1 + Bregs and CD19 + CD24 + CD38 + IL-10 + Bregs to B cells were elevated by Sirolimus in PBMCs including B cells. Sirolimus significantly enhances the cytokine production of transforming growth factor-β1 (TGF-β1) and interleukin-10 (IL-10) in PBMCs with B cells, and the enhancement significantly decreased in PBMCs without B cells. The percentage of CD4 + CD25 + Foxp3 + Tregs to T cells was also elevated by Sirolimus in PBMCs including B cells. The elevation of Tregs percentage decreased in PBMCs without B cells and recovered when additional TGF-β1 and IL-10 were added. The amplification of Tregs by Sirolimus was partially inhibited when either TGF-β1 or IL-10 was neutralized, and it even disappeared when these two cytokines were both neutralized. These results indicate that Sirolimus can amplify Bregs and Tregs in PBMCs in vitro, and Bregs may be the why Sirolimus amplifies Tregs.

1. Introduction

Immune tolerance has been an interesting topic in organ transplantation research since the first organ transplant operation was performed. Immunosuppressive agents, stem cell transplantation, and other methods have been tried to induce long-term, stable immune tolerance [1–3]. Nevertheless, no reliable method has been found until now. Currently, immunosuppressive agents still play the most important role in suppressing the immune response of the host to the graft [4]. Immunosuppressive agent development has mainly focused on inhibiting T cell function or depleting T cells [4]. However, the roles of regulatory lymphocyte subsets and humoral factors are being increasingly recognized in both acute and chronic graft injury [5–7]. Thus, the effects of present immunosuppressive agents on regulatory lymphocyte subsets and humoral rejection are receiving much attention [8].

Sirolimus, a macrolide antibiotic, was found in the late 20th century, and its immunosuppressive function has been noted in the last two decades [9–11]. The revealed immunosuppressive mechanisms of Sirolimus include inhibiting the activation of Ca^{2+} dependent or independent T and B cells [12–15], inducing autophagy and inhibiting some non-immune cells including vascular endothelial cells and fibroblasts [16]. However, detailed study on the mechanism of Sirolimus has not been well documented. In our center, we found that the conversion of Tacrolimus to Sirolimus in liver transplant receipts was an effective treatment for steroid-resistant rejection. B cells, plasma cells, and even C4d were detected in the portal tracts of these graft liver biopsy specimens, which indicate that humoral factors had participated in these rejections [17]. Many other transplant centers also reported that Sirolimus was helpful in inducing long-term graft tolerance in solid organ transplantation [18]. Further studies have revealed that Sirolimus could significantly amplify Tregs in vivo and in vitro [18–21], which was achieved by Sirolimus producing TGF-β [22,23]. Where the TGF-β produced by Sirolimus comes from has not been expounded.

Regulatory B cells (Bregs) can induce amplification of Tregs. Bregs are characterized by IL-10 and TGF-β production. They play a negative regulatory role and can even induce immune tolerance through inducing effector T cells to disable and amplify Tregs in transplant receipts [24,25]. Does Sirolimus amplify Bregs and stimulate the secretion of IL-10? If so, does the TGF-β that Sirolimus produces come mainly from Bregs? These questions remain to be answered. In this study, the effect of Sirolimus on Bregs was investigated by co-culturing Sirolimus and PBMCs in vitro. In addition, the amplifying efficiency of Sirolimus to Tregs was also investigated in different Bregs conditions.

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2. Materials and methods

2.1. Subjects

Mononuclear cell-rich blood was obtained from 7 healthy blood donors (5 male and 2 female) in the Chinese People's Liberation Army General Hospital blood bank. The average age of blood donors was 30.9 ± 7.1 years (range: 22–43 years). Written informed consent was obtained from each donor. The research protocol was approved by the Ethics Committee of the 309th Hospital of Chinese People's Liberation Army in accordance with the regulations of China and the guidelines of the Declaration of Helsinki.

2.2. Isolation of PBMCs

The mononuclear cell-rich blood was diluted 1:1 with 0.9% salt solution, layered smoothly on Lymphoprep™ solution (TBD, Tianjin, China), and centrifuged for 20 min at 500 g. The purified mononuclear cell-rich band was absorbed and washed twice by RPMI 1640 (Gibco, USA). Then the cells were resuspended in RPMI 1640 and counted. The percentages of Bregs and Tregs of the isolated PBMCs were analyzed by using flow cytometric (FCM; BD, USA).

2.3. Elimination of B cells from PBMCs

The isolated PBMCs were centrifuged and resuspended at a concentration of 1×10^7 cells/ml in IMag™ buffer (BD, USA). Then the cells were labeled using Biotinylated Human B Lymphocyte Enrichment Cocktail (BD, USA) at 5 μl per 1×10^6 cells. Next, the cells were incubated at 4 °C for 30 min, and washed with excess volume of IMag™ buffer. After being centrifuged at 500 g for 5 min and aspirated in the supernatant, the sample was added to full-mixed IMag™ Streptavidin Particles Plus-DM (BD, USA) at 5 μl per 1×10^6 cells and incubated at room temperature for another 30 min. Then IMagnet™ (BD, USA) was used to separate non-B PBMCs from B cells at a concentration of 2×10^7 cells/ml.

2.4. Cells culture and grouping

Isolated PBMCs and non-B PBMCs were respectively suspended at a concentration of 1×10^6/ml in culture medium containing 90% RPMI 1640, 10% heat-inactivated fetal bovine serum (FBS), streptomycin 100 μg/ml, and penicillin 100 U/ml. Then, the cells were maintained on 6-well culture plates (2 ml per well) at 37 °C in humidified air with a 5% CO_2 atmosphere. Both TGF-β1 (R&D, USA) and IL-10 (Stemlmmune, USA) were added into half of the non-B PBMCs at 20 pg/ml. Thus, the cultured cells were divided into three series: PBMCs including B cells series, a non-B PBMCs series, and non-B PBMCs including cytokines series. Each series was divided into 3 groups. Sirolimus was added into each group at 0 ng/ml, 2 ng/ml, and 10 ng/ml, respectively.

2.5. Flow cytometric analysis of Bregs and Tregs

The percentages of Bregs to B cells in the series of PBMCs including B cells and the percentages of Tregs to T cells in all series were analyzed with FCM at the 24th, 48th, and 72nd hours, respectively. The detection method of Bregs was as follows. Each well of cultured cells was stimulated by 2 μg lipopolysaccharide (LPS, Sigma-Aldrich, USA) for 4 h and then blocked by 1.4 μl GolgiStop (BD, USA) for 4 h. After being washed twice with phosphate buffered saline solution (PBS), the cells were stained with 20 μl CD19-FITC (BD, USA), 5 μl CD24-PE (BD, USA), and 20 μl CD38-APC (BD, USA) for 20 min [26,27], and then washed again. Fixation and permeabilization solution (BD, USA) was used to treat the cells for 20 min, and BD Perm/Wash™ Buffer (BD, USA) was used to wash the cell twice. Next, 5 μl TGF-β1-PE (BD, USA) was used to stain TGF-β1 + Bregs and 20 μl IL-10-PE (BD, USA) was used to stain IL-10 + Bregs. After treated by BD Perm/Wash™ Buffer again and washed, the treated cells were assessed by FCM. The Tregs were washed using PBS twice, and the cells were stained with 5 μl CD4-FITC (BD, USA) and CD25-PE (BD, USA) for 15 min at room temperature in the dark and then treated with fixation/permeabilization (Concentrate:Diluent = 1:3) (eBioscence, USA) for 30 min and washed with permeabilization buffer (eBioscence, USA). After centrifugation, the sample was incubated with 2 μl FoxP3-APC for 30 min at room temperature in the dark and then washed with permeabilization buffer and assessed using FCM. The results were analyzed using CellQuest™ pro software (BD, USA).

2.6. Cytokines assay

To examine whether the cytokines associated with Bregs were influenced by Sirolimus, supernatants from cultured PBMCs with and without B cells were collected at the 48th hour, and the levels of TGF-β1 and IL-10 were quantified using ELISA kits (BD, USA) according to manufacturer's instructions.

2.7. The roles of TGF-β1 and IL-10 in Sirolimus amplifying Tregs

Mononuclear cell-rich blood from another 7 donors was used to investigate the effect of TGF-β1 and IL-10 on Tregs proliferation. PBMCs were isolated from mononuclear cell-rich blood using the above-described method and resuspended at a concentration of 1×10^6 cells/ml in culture medium. The percentage of Tregs to T cells was detected using FCM before culture. Then the PBMCs were divided into five culture wells (2 ml/well) at 37 °C in humidified air with a 5% CO_2 atmosphere. One of the five wells was set as the control sample. Sirolimus was added into the other 4 wells at 10 ng/ml, into 3 of which were added anti-TGF-β1 (1 μg/ml) (Abcam, UK), anti-IL-10 (0.8 μg/ml) (Abcam, UK), and anti-TGF-β1 (1 μg/ml) + anti-IL-10 (0.8 μg/ml), respectively. The percentages of Tregs to T cells in the 5 wells were detected using FCM after 72 h.

2.8. Statistical evaluation

Statistical analyses were performed with SPSS 19.0 statistical software (SPSS, Inc., USA). The data were graphed with GraphPad Prism™ 5 software (GraphPad Software, Inc., USA). Continuous data were tested for normal distribution and expressed as the mean ± standard deviation. Data were analyzed using one-way ANOVA with post-hoc Bonferroni's correction. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Sirolimus amplifies Bregs in vitro

The percentages of Bregs to B cells in the series of PBMCs including B cells were measured using FCM at the 0th, 24th, 48th, and 72nd hours (Fig. 1). The procedure for analyzing TGF-β1 + Bregs is shown in Fig. 1A, and a typical result from one blood donor is shown in Fig. 1B. Statistical results showed that the percentage of both TGF-β1 + Bregs and IL-10 + Bregs were elevated in the 2 ng/ml Sirolimus group and in the 10 ng/ml Sirolimus group with by prolonging the time (Fig. 1C, E). Moreover, at each time point, the percentage of both TGF-β1 + Bregs and IL-10 + increased with increasing Sirolimus concentration (Fig. 1D, F). These results suggest that Sirolimus elevated the expression of Bregs in PBMCs in vitro.

3.2. Sirolimus enhanced B cells secreting TGF-β1 and IL-10

We compared cytokines TGF-β1 and IL-10 levels in culture supernatants among different Sirolimus concentration groups in PBMCs with and without B cells series at the 48th hour. It was observed that Sirolimus obviously enhanced the production of both TGF-β1 and IL-