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Immunomodulation of human CD19^{+}CD25^{high} regulatory B cells via Th17/Foxp3 regulatory T cells and Th1/Th2 cytokines

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ABSTRACT

Regulatory B (Breg) cells are a special subset of immunoregulatory cells with unique phenotypes and functions. In this study, human CD19^{+}CD25^{high} Breg cells were purified from human peripheral blood. Based on the coculture system of Breg cells and CD4^{+} T cells in vitro, Breg cells were found to promote the increase in regulatory T (Treg) cells while decreasing the number of Th17 cells. Breg cells regulate Treg cells through two processes: cell-cell contact and cytokines. TGF-βsRII, a blocker of transforming growth factor-β (TGF-β), can attenuate the effects of Treg elevation, suggesting that TGF-β is the main cytokine, while Breg cells rather than interleukin-10 (IL-10) regulate the differentiation of Treg cells. However, Th17 cells were mainly regulated by cytokines, without an obvious regulatory effect on cell-cell contacts. Breg cells may regulate Th17 cells by a pathway independent of TGF-β and IL-6. The coculture of Breg cells and CD4^{+} T cells led to changes in the cytokine spectrum, which included significant increases in IL-4, IL-6 and IL-10 but not obvious changes in IL-2, IFN-γ and TNF. The inhibitory effect of Breg cells was weakened by blocking cell-cell contacts in cultures separated with the Transwell chamber because IL-10 decreased while IL-6 increased when compared with co-cultured Breg and CD4^{+} T cells. When the IL-10 inhibitor IL-10sRα was added, IL-6 and TNF levels significantly increased, while treatment with the TGF-β inhibitor TGF-βsRII did not result in similar changes, suggesting that IL-10 is an important molecule to inhibit the proinflammatory factors IL-6 and TNF in this culture system.

1. Introduction

In healthy individuals, inflammation is self-limiting, and its resolution is controlled by the release of anti-inflammatory mediators and cytokines. Regulatory B (Breg) cells are a special subset of immunoregulatory cells with unique phenotypes and functions and play an immunosuppressive role that is not only involved in maintaining the normal balance and stability of the immune system but also closely related to infectious diseases, immune-related diseases, tumors, etc. Although a partial consensus regarding the effector function of Breg cells has been reached, the field has yet to obtain a unified view concerning their phenotype [1]. In mice, multiple subsets of Breg have been described, including B10 (CD1d^{hi}CD5^{+}) [2–4], transitional 2 marginal zone precursor B cells (CD1d^{hi}CD23^{+}IgM^{+}) [5,6], marginal zone B cells (CD1d^{hi}CD23^{-}IgM^{hi}) [7], Tim-1^{+} B cells (Tim-1^{+}CD19^{+}) [8], plasma cells (CD138^{+}MHC-11^{lo} B220^{+}) [9,10] and plasmablasts (CD138^{+}CD44^{hi}) [11]. In humans, a perplexing number of Breg cell types based on surface marker combinations has also been reported, such as immature cells (CD19^{+}CD24^{hi}CD38^{hi}) [12,13], plasmablasts (CD19^{+}CD24^{hi}CD27^{int}) [11], Br1 cells (CD19^{+}CD25^{hi}CD71^{hi}) [14] and B10 cells (CD24^{hi}CD27^{+}) [15]. Thus, the described human IL-10-producing B cells show a resemblance to immature, transitional, marginal

Abbreviations: Breg, Regulatory B; IL-10, interleukin-10; Treg, regulatory T; Th, helper T cell; CBA, cytometric bead array; PBMCs, Peripheral blood mononuclear cells; TGF-β, transforming growth factor-β

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zone, activated and memory B cells and even plasma cells, which seems to indicate that Breg cells exist at all stages of B cell differentiation. Therefore, it is difficult to define the types of Breg cells.

Another difficulty in researching Breg cells is that the number of Breg cells is extremely low in peripheral blood, and these cells are difficult to enrich for functional research. Interleukin-10 (IL-10) production seems to be the only distinguishing feature of Breg cells described in previous reports. However, because this cytokine is an intracellular factor, it is almost impossible to adopt it as a target or marker to enrich and purify active Breg cells for functional research in vitro. A feasible solution of purifying Breg cells was reported using the cellular surface markers $CD19^+$ and $CD25^+$. A human $CD19^+CD25^{high}$ Breg subset was defined by its ability to suppress $CD4^+$ T cell proliferation and enhance Foxp3 and CTLA-4 expression in regulatory T (Treg) cells [16,17]. Therefore, this study was performed to focus on the immune regulation of $CD19^+CD25^{high}$ Breg relating to Th17 cells, Foxp3 Treg cells and Th1/Th2 cytokines. A coculture system of $CD19^+CD25^{high}$ Breg cells and $CD4^+$ T cells was established, and the pattern and mechanism of Breg cell regulation on $CD4^+$ T cells were recognized. This is a preliminary discussion regarding $CD19^+CD25^{high}$ Breg cells in human peripheral blood, which may provide a feasible way to research functional Breg cells in vitro.

2. Materials and methods

2.1. Study material

Peripheral blood was obtained from 20 healthy volunteers (10 female and 10 male) aged 25–40 years. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by Ficoll-Paque Plus (Invitrogen) density gradient centrifugation. All volunteers signed an agreement before participating in this study, which was approved by the hospital's ethics committee.

2.2. Purification of $CD19^+CD25^{high}$Breg cells

$CD19^+CD25^{high}$ Breg cells were purified by two steps. First, total B lymphocytes were enriched by negative selection from PBMCs using a B cell isolation kit II for magnetic cell separation (MACS system; Miltenyi Biotec, Germany) following the manufacturer's instructions. Second, $CD19^+CD25^{high}$ Breg cells were purified by positive selection from enriched B lymphocytes that were incubated with $CD25^+$ microbeads (20 μl/10^7 cells; Miltenyi Biotec, Germany). Positive selection columns (Miltenyi Biotec, Germany) were used to separate $CD19^+CD25^{high}$ B lymphocytes and $CD19^+CD25^{low}$ B lymphocytes. Purified $CD19^+CD25^{high}$ Breg cells were stained for the surface markers CD45 (PerCP), CD19 (PE-Cy7) and CD25 (BB515). The achieved purity of $CD19^+CD25^{high}$ Breg cells was approximately 93%, while that of $CD19^+CD25^{low}$ B lymphocytes was approximately 90.6%.

2.3. Purification of $CD4^+$ T cells

$CD4^+$T cells were purified from PBMCs by positive selection with CD4 microbeads (20 μl/10^7 cells; Miltenyi Biotec, Germany) according to the manufacturer's instructions. Purified $CD4^+$ T cells were stained for the surface markers CD45 (PerCP), CD4 (PE-Cy7) and CD3 (FITC). The achieved purity of $CD4^+$ T cells was approximately 93%.

2.4. Coculture of Breg and $CD4^+$ T cells

$CD19^+CD25^{high}$ Breg and $CD4^+$ T cells were plated in 96-well U-bottom plates at 1.0×10^5 cells/well (1:1 ratio) in 200 μl of medium. The cells were cultured in complete medium (RPMI 1640, Invitrogen) supplemented with 10% FCS (Gibco), 200 mg/ml penicillin (Gibco), 200 U/ml streptomycin (Gibco), 4 mM L-glutamine (Gibco) and HEPES in the presence of 1 μM ODN2006 (human TLR9 ligand, InvivoGen, San Diego, CA, USA), 5 μg/ml CD40 ligand (CD40L, Peoprotech, USA) and 5 μg/ml IL-4 (Peoprotech, USA) in duplicate for 48 h at 37 °C. Then, PMA (50 ng/ml; Sigma-Aldrich), ionomycin (1 μg/ml; Sigma-Aldrich) and brefeldin A (BFA; 1 μl/ml; Golgi Plug, BD Biosciences) were added for the last 6 h at 37 °C. Supernatant samples were collected for cytokine analysis, and cell pellets were prepared for Treg/Th17 flow cytometry analysis.

2.5. Transwell cultures

A Transwell culture system (0.4 μm pore size membrane, Corning, USA) was used to block direct contact between Breg and Treg cells. However, cytokines can freely pass through the transwell membrane. In this assay, $CD4^+$ T cells were plated in the lower chamber of transwell inserts in 24-well cell culture plates at 1×10^5 cells/well. Meanwhile, Breg cells were added to the upper chamber at 1×10^5 cells/well. Both transwell-cultured and cocultured cells were stimulated with the same concentrations of ODN, CD40L and IL-4 for 48 h at 37 °C. Then, PMA, ionomycin and brefeldin A were added for the last 6 h at 37 °C. Supernatant samples were collected for cytokine analysis, and cell pellets were prepared for Treg/Th17 flow cytometry analysis.

2.6. Functional assays for blocking cytokine activity

To determine the contribution of IL-10 and TGF-β in the coculture system, Breg and $CD4^+$ T cells were cultured in the presence or absence of the IL-10 blocker IL-10sRα (Recombinant Human soluble IL-10 Rα, R &D systems) at a concentration of 1 μg/ml and/or the TGF-β blocker TGF-βsRII (Recombinant Human TGF-β RII, R&D systems) at a concentration of 1 μg/ml. Then, the numbers of Treg and Th17 cells as well as cytokine levels were analyzed by flow cytometry.

2.7. Flow cytometry analysis of Treg/Th17 cells and cytokines

Th17 and Treg cells were stained by using a HumanTh17/Treg Phenotyping Kit (BD Pharmingen, USA) containing CD45-PerCP-Cy5.5, Foxp-3-Alexa Fluor 647 and IL-17-PE. The staining procedure, including harvesting, fixing and permeabilizing the cells as well as staining with the cocktail, was performed following the manufacturer's instructions. Stained cells were analyzed by a BD Canto II flow cytometer (BD Biosciences, USA).

2.8. Cytometric bead array (CBA)

The concentrations of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF were measured by means of a human cytometric bead array (CBA) using a Human Th1/Th2 Cytokine Kit II (BD Biosciences, USA). The theoretical detection limits of these kits were 2.6 pg/ml for IL-2, 2.6 pg/ml for IL-4, 3.0 pg/ml for IL-6, 2.8 pg/ml for IL-10, 2.8 pg/ml for TNFα and 7.1 pg/ml for IFN-γ. The maximum detection limit of all the kits was 2,500 pg/ml. Data were acquired on a BD Canto II flow cytometer (BD Biosciences, USA).

2.9. Statistical analysis.

Flow cytometry data were analyzed by FlowJo software (version X 10.0.7r2). Comparison analysis between two conditions was performed using the *unpaired Student's t-test*. A *one-way ANOVA* was used to analyze the differences between multiple groups, followed by the Tukey *post-hoc*. P value < 0.05 was considered statistically significant. The statistical program GraphPad Prism 6.0 was used to analyze the data and get the graphs.

3. Results

In coculture, $CD19^+CD25^{high}$ Breg cells can remarkably upregulate

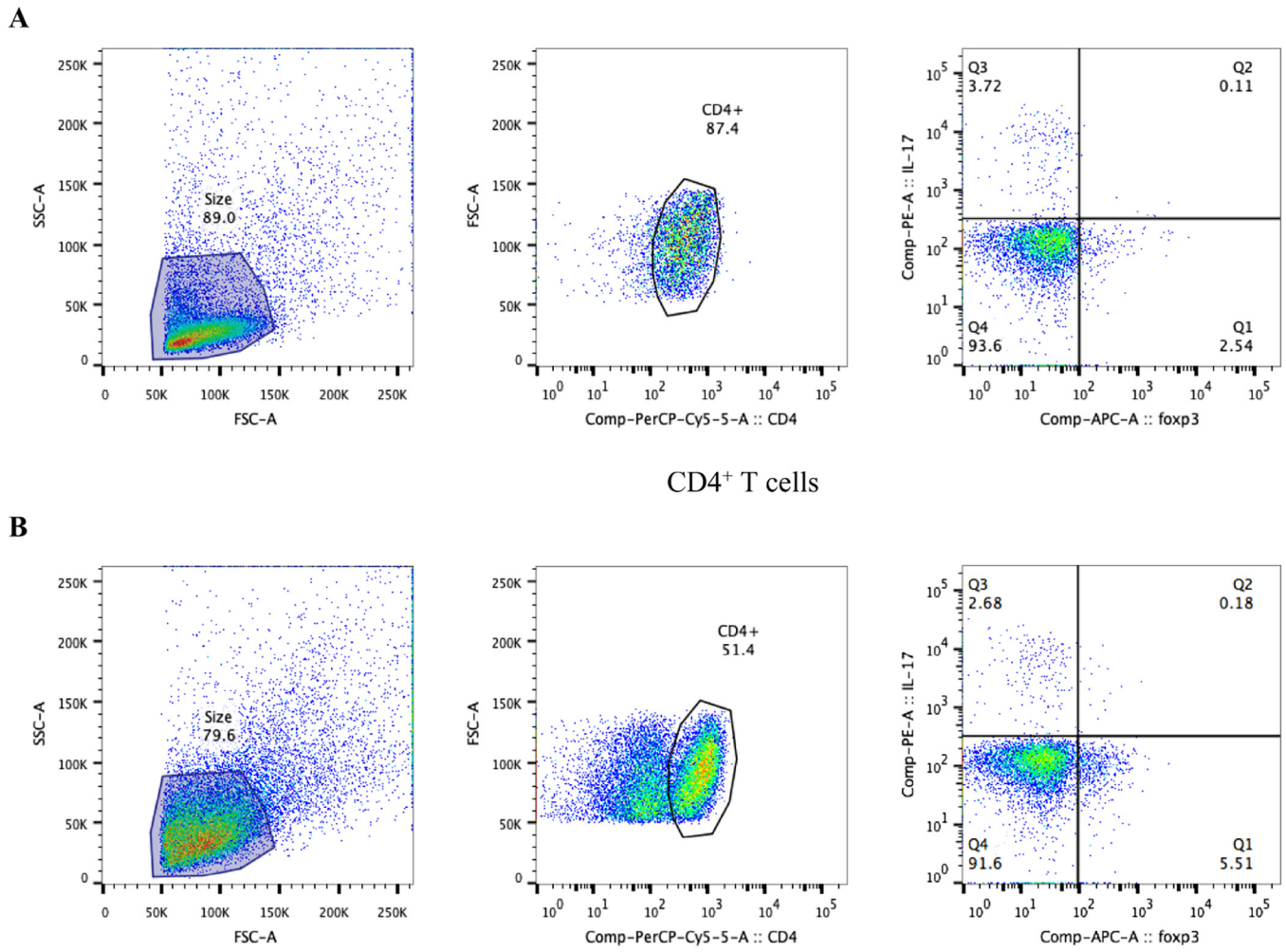

Fig. 1. The percentage of Treg and Th17 cells in $CD4^+$ T cells was changed in cocultures containing $CD19^+CD25^{high}$ Breg cells. Purified $CD19^+CD25^{high}$ Breg and $CD4^+$ T cells were cocultured at 1.0×10^5 cells/well in a 1:1 ratio with stimulators ODN, CD40L, and IL-4 and the cytokine secretion blockers PMA, ionomycin, and BFA. Treg and Th17 cells were analyzed by flow cytometry using Foxp-3 and Th17 as featured markers. (A) Gating strategy for Treg and Th17 cells in $CD4^+$ T cell. (B) Gating strategy for Treg and Th17 cells in $CD19^+CD25^{high}$ and $CD4^+$ T cell coculture. (C) $CD19^+CD25^{high}$ Breg cells induced a significant increased percentage of Treg cells ($p < 0.0001$) and a significant decreased percentage of Th17 cells ($p = 0.0007$) as determined by *unpaired Student's t-test*. (D) $CD19^+CD25^{high}$ Breg cells induced a significant increase in Treg cells number ($p < 0.0001$) and a significant decrease in Th17 cells ($p = 0.0141$) as determined by *unpaired Student's t-test*.

Foxp3-Treg cells and downregulate Th17 cells. When $CD4^+$ T cells were cultured alone, the cell number and percentage of Treg and Th17 cells were (180.50 ± 40.65) and (2.78 ± 0.48) %, (202.40 ± 24.83) and (3.10 ± 0.37) % respectively. However, when $CD19^+CD25^{high}$ Breg cells were cocultured with $CD4^+$ T cells, the cell number and percentage of Treg cells increased to (367.60 ± 51.85) and (5.35 ± 0.53) %, and that of Th17 cells decreased to (171.9 ± 25.37) and (2.53 ± 0.24) %. This result suggested that $CD19^+CD25^{high}$ Breg cells induce an increase in Treg cell number and percentage ($p < 0.0001$ and $p < 0.0001$) and a decrease in Th17 cell number and percentage ($p = 0.0141$ and $p = 0.0007$) to modify the immune system to be tolerant (Fig. 1A–D).

$CD19^+CD25^{high}$ Breg cells and $CD4^+$ T cells were placed in the upper and lower chamber of a Transwell insert, respectively, and separately cultured. Cell-cell contact was blocked, but cytokines could still pass through the chamber septum and distribute freely and evenly in the upper and lower chambers. The result indicated the following. ① Treg cells number was (291.5 ± 33.42) accounted for (4.32 ± 0.38) %, which was between the result of $CD4^+$ T cells cultured alone (180.50 ± 40.65) accounted for (2.78 ± 0.48) % and that of $CD19^+CD25^{high}$ Breg and $CD4^+$T cells cocultured (367.60 ± 51.85) accounted for (5.35 ± 0.53) %. These results indicated that cell-cell contacts were necessary for Breg cells to regulate Treg cells. However, the regulation was not completely blocked by the physical separation of the chamber. ②Th17 cells number was (166.3 ± 21.16) accounted for (2.45 ± 0.23) %, indicating almost no difference with that from $CD19^+CD25^{high}$ Breg and $CD4^+$ T cell cocultures (171.9 ± 25.37) accounted for (2.53 ± 0.24) %. This result suggested that although cell-cell contact was blocked by the chamber, Th17 production was still inhibited, and the degree of inhibition was not significantly different with that in the cocultured cells (Fig. 2A–C).

When $CD19^+CD25^{high}$ Breg and $CD4^+$ T cells were cocultured, the cell number and percentage of Treg cells were (367.60 ± 51.85) and (5.35 ± 0.53) %. After an IL-10 blocker (IL-10sRα) was added to the system, the cell number and percentage of Treg cells were (348.40 ± 52.76) and (5.23 ± 0.72) %, which was not significantly different from that in the untreated coculture group. In contrast, when a TGF-β blocker (TGF-βsRII) was added to the system, the cell number and percentage of Treg cells decreased to (289.20 ± 38.71) and (4.27 ± 0.48) %. When IL-10sRα and TGF-βsRII were simultaneously added into the system, the cell number and percentage of Treg cells were still (276.40 ± 43.65) and (4.20 ± 0.54) %. Therefore, it is implied that the main cytokine influencing $CD19^+CD25^{high}$ Breg-mediated upregulation of Treg cells is TGF-β. At the same time, these

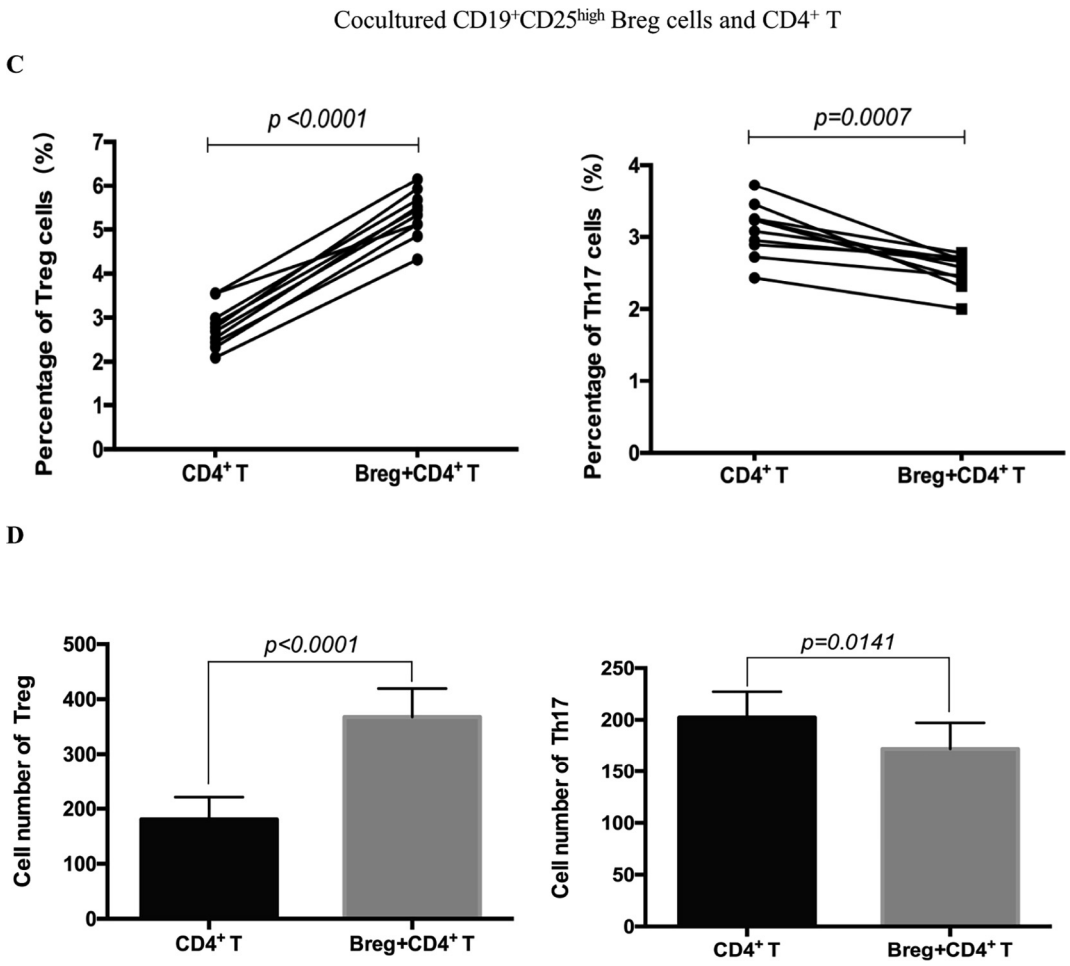

Fig. 1. (*continued*)

data suggest again that cell- cell contact is another factor affecting $CD19^+CD25^{high}$ Breg-mediated upregulation of Treg cells. However, the cell number and percentage of Th17 cells were (179.20 ± 25.42) and (2.66 ± 0.31) % in the IL-10sRα group, (169.10 ± 23.24) and (2.50 ± 0.23) % in the TGF-βsRII group and (157.80 ± 21.70) and (2.39 ± 0.28) % in the IL-10sRα + TGF-βsRII group. Therefore, neither IL-10sRα nor TGF-βsRII can effectively block the inhibitory effect of $CD19^+CD25^{high}$ Breg cells on Th17 cell production; thus, the regulatory effect of $CD19^+CD25^{high}$ Breg cells on Th17 cells is not achieved by either TGF-β or IL-10 (Fig. 3A–C).

The CBA technique was used to detect the Th1/Th2 cytokine, and the results revealed a remarkable increase in the levels of IL-4, IL-6 and IL-10, while the levels of IL-2, IFN-γ and TNF showed no apparent changes after coculture of $CD19^+CD25^{high}$ Breg and $CD4^+$T cells. When these cells were cultured separately in the Transwell chamber, IL-10 decreased from (98.6 ± 9.9) μg/ml to (74.8 ± 10.0) μg/ml, while IL-6 increased from (377.4 ± 35.9) μg/ml to (734.4 ± 34.8) μg/ml. Therefore, blocking cell-cell contact partially weakened the expression of the anti-inflammatory factor IL-10, thereby promoting the expression of the proinflammatory factor IL-6.

When IL-10sRα and/or TGF-βsRII were added into the cocultured $CD19^+CD25^{high}$ Breg and $CD4^+$T cells, significant changes were observed in the cytokine spectrum. The details are as follows: ① Compared with the untreated cells, the IL-10sRα and IL-10sRα + TGF-βsRII groups showed an increase in IL-6 by more than 3.6-fold, but no change was observed in the TGF-βsRII group. ② TNF increased more than 2-fold in both the IL-10sRα and IL-10sRα + TGF-βsRII groups compared with the untreated groups, yet no change was detected in the TGF-βsRII group (Table 1).

4. Discussion

$CD4^+$ T cells, also known as T helper (Th) cells, are key in the response to infectious agents and in the plasticity of the immune system. When naïve $CD4^+$ T cells (Th0) recognize antigens and are exposed to various cytokines in the microenvironment, they are activated and differentiate into the various subtypes Th0, Th1, Th2, Th17, Tfh, Th9, iTreg, and Foxp3-independent Treg cells [18]. In this study, a coculture system of $CD19^+CD25^{high}$ Breg and $CD4^+$ T cells enriched by immunomagnetic beads was established to clarify the regulatory effect of $CD19^+CD25^{high}$ Breg cells on $CD4^+$ T cell differentiation. $CD19^+CD25^{high}$ Breg cells increased the cell number and percentage of Tregs and decreased the cell number and percentage of Th17 cells. Tregs are responsible for the maintenance of self-tolerance, thus inhibiting autoimmunity, whereas proinflammatory Th17 cells contribute to the induction and propagation of inflammation. A shift in the Th17/Treg equilibrium toward the proinflammatory Th17 has been reported in several autoimmune disorders, including rheumatoid arthritis (RA), ankylosing spondylitis (AS), psoriasis and psoriatic arthritis (PsA), multiple sclerosis (MS), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), and Crohn's disease (CD) [19–21]. Therefore, $CD19^+CD25^{high}$ Breg cells may exert its immunosuppressive effect by inhibiting Th17 cells and activating Treg cells.

To clarify the immunoregulatory effect of $CD19^+CD25^{high}$ Breg cells on $CD4^+$T cells, $CD19^+CD25^{high}$ Breg cells and $CD4^+$ T cells were added to the upper chamber and lower chamber of a Transwell insert,

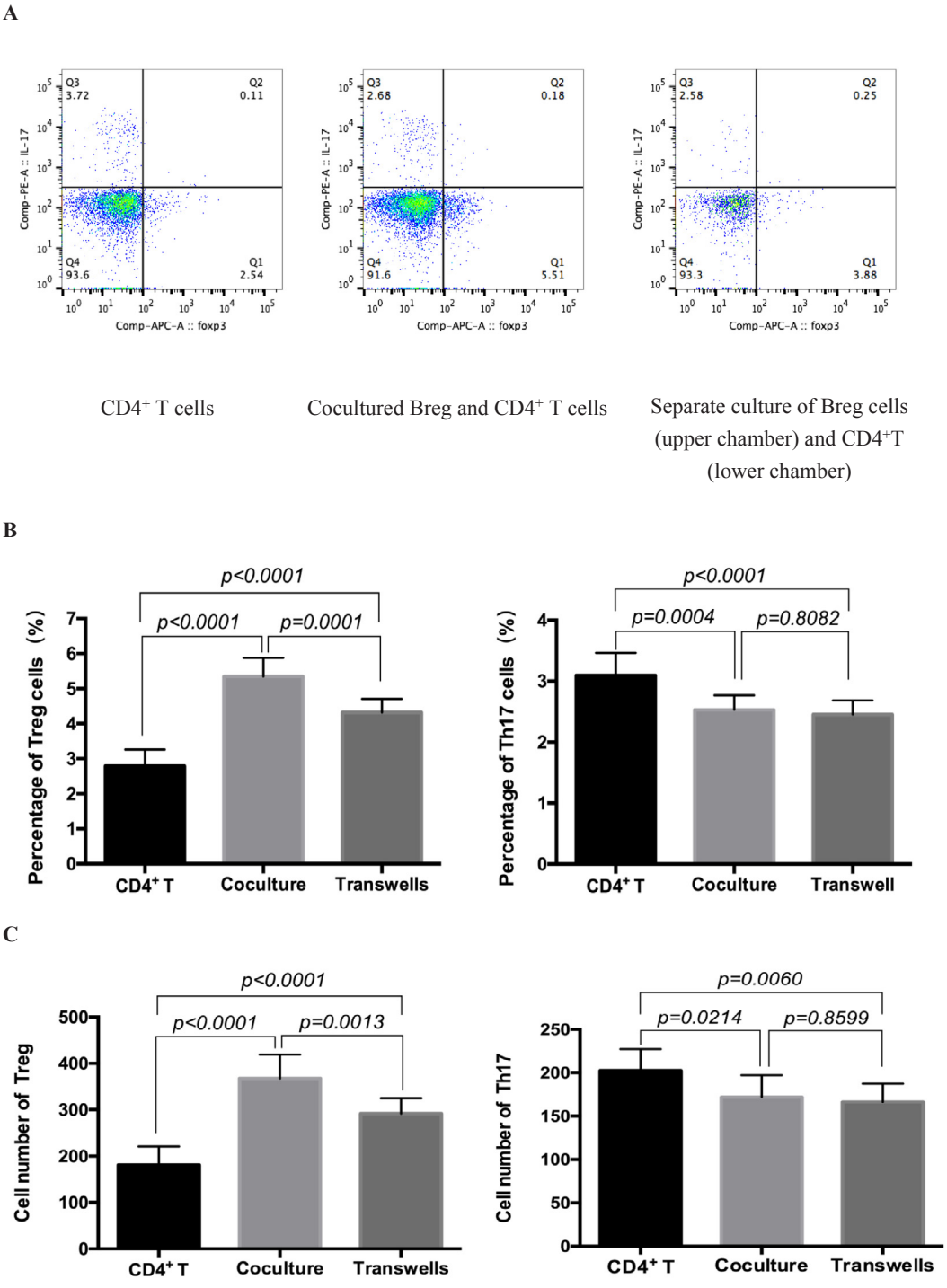

Fig. 2. Cell-cell contacts between $CD19^{+}CD25^{high}$ Breg and $CD4^{+}$ T cells affected Treg and Th17 cell production. Cell-to-cell contact was blocked by the transwell. The percentage of Treg cells and Th17 cells were analyzed by flow cytometry. (A) Gating strategy for Treg cells and Th17 cells in the $CD4^{+}$ T cell culture, $CD4^{+}$ T and Breg cell coculture and Transwell culture. (B) The percentages of Treg and Th17 cells in the difference culture conditions (n = 10). (C) The cell number of Treg and Th17 cells in the difference culture conditions (n = 10). Bars represent median values, and lines indicate the ± SD. The *p* values were obtained by *one-way ANOVA* statistical analysis.

respectively, and cultured separately. Cytokines could pass through the compartment membrane and distribute in the upper and lower chambers freely despite the lack of cell-cell contact. The elevated cell number and percentage of Treg cells observed in the coculture were partially inhibited in the Transwell culture, the value of which was ranked between that from $CD4^{+}$ T cells cultured alone and that from cocultured $CD19^{+}CD25^{high}$ Breg and $CD4^{+}$ T cells. This result suggested that the interaction between Breg cells and $CD4^{+}$ T cells was to change the subsets pattern of $CD4^{+}$ T cell. Meanwhile, the effect of Breg cells was not completely blocked in the transwell culture, which indirectly implied that cytokines may partially upregulate Treg cells. Therefore, $CD19^{+}CD25^{high}$ Breg cells may upregulate Treg cells by both cell-cell

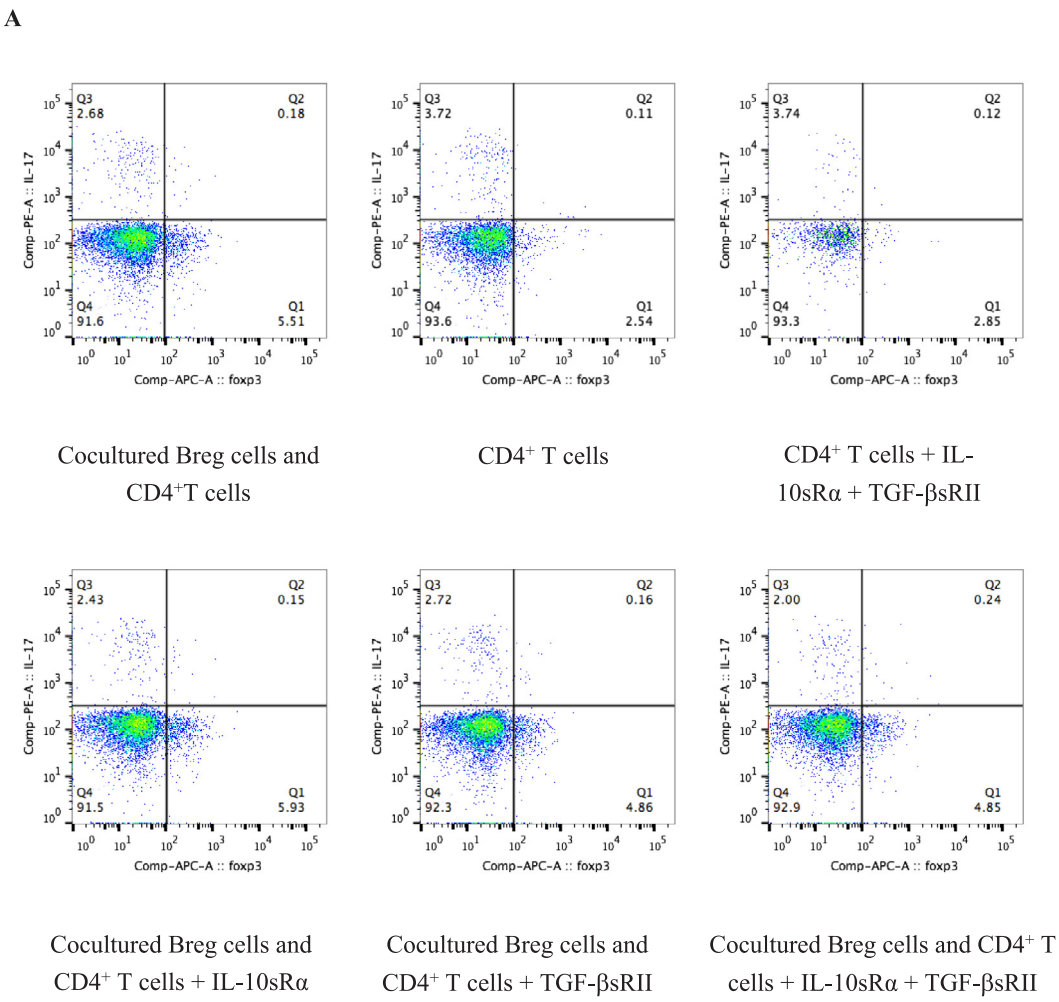

Fig. 3. The effects of IL-10 and/or TGF-β on Treg/Th17 production in cocultured cells. $CD19^+CD25^{high}$ Breg and $CD4^+$ T cells were cultured in the presence or absence of an IL-10 blocker (IL-10sRα) and/or TGF-β blocker (TGF-βsRII) in the above groups. $CD4^+$ T cells only and $CD4^+$ T cells + IL-10sRα + TGF-βsRII served as controls. The percentages of Treg cells and Th17 cells were analyzed by flow cytometry. (A) Gating strategy for Treg and Th17 cells in cocultures with or without cytokine blockers. (B) The percentages of Treg and Th17 cells from cocultures with or without cytokine blockers (n = 10). (C) The cell number of Treg and Th17 cells from cocultures with or without cytokine blockers (n = 10). Bars represent median values, and lines indicate the ± SD. The *p* values were obtained by *one-way ANOVA* statistical analysis.

contact and cytokines. Furthermore, the suppressive effect of $CD19^+CD25^{high}$ Breg cells on Th17 cells was not weakened by transwell culture, suggesting that the regulation of Breg cells on Th17 cells mainly relies on cytokines.

$CD4^+$ T helper cells are the major source for cytokine production and regulation, and these cells produce two types of cytokines: type 1 (Th1) and type 2 (Th2) [22]. Based on the research above, it was suggested that the cytokine milieu plays an important role in the regulatory mechanism of $CD19^+CD25^{high}$ Breg cells. In this study, the CBA technique was used to detect Th1/Th2 cytokines. After coculture of $CD19^+CD25^{high}$ Breg cells with $CD4^+$ T cells, IL-2, IFN-γ and TNF showed no marked changes, while IL-4, IL-6 and IL-10 levels increased significantly, suggesting that the Th1/Th2 cytokine ratio shifted toward Th2. After blocking cell-cell contact between $CD19^+CD25^{high}$ Breg cells and $CD4^+$T cells, the anti-inflammatory factor IL-10 decreased but the proinflammatory factor IL-6 increased when compared with the respective levels in the coculture. As previously shown, the cell number and percentage of Treg cells were also decreased at that point, while the cell number and percentage of Th17 cells did not change significantly when cell -cell contact was blocked. In this regard, cell -cell contact is critical for Breg cell regulation on immunocytes and cytokine production.

Breg cells may suppress the immune response by secreting IL-10 and/or TGF-β [23–25], which were also significantly higher in Breg cells than in non-Breg cells. To define the IL-10 and TGF-β function in $CD19^+CD25^{high}$ Breg, an IL-10 blocker (IL-10sRα) and TGF-β blocker (TGF-βsRII) were added into the coculture system of $CD19^+CD25^{high}$ Breg cells and $CD4^+$ T cells either individually or simultaneously to detect the changes in the cell number and percentages of Treg cells and Th17 cells and cytokine levels. The Treg cell number and percentage showed no remarkable changes in the IL-10sRα group but were reduced in both the TGF-βsRII and IL-10sRα + TGF-βsRII groups, which showed a similar degree of decline. TGF-β is initially required for the development of Treg cells and Th17 cells by triggering their differentiating transcription factors, Foxp3 and RORγ t, respectively. The subsequent differentiation of the cells depends mainly on the surrounding cytokine milieu. TGF-β, in combination with IL-2, induces $CD4^+$ T cell differentiation into Treg cells via induction of Foxp3 expression [26]. Once TGF-βsRII was added to the $CD19^+CD25^{high}$ Breg and $CD4^+$T cell coculture, the cell number and percentage of Treg cells decreased significantly, indicating that TGF-β rather than IL-10 is the main cytokine for $CD19^+CD25^{high}$ Breg-mediated Treg differentiation. This conclusion has also been observed in a previous study [16]. Furthermore, there was a significant increase in IL-6 and TNF in the IL-10sRα group. IL-10 has been proven to be a powerful anti-inflammatory factor that binds to the IL-10 receptor and induces sustained activation of STAT3 and

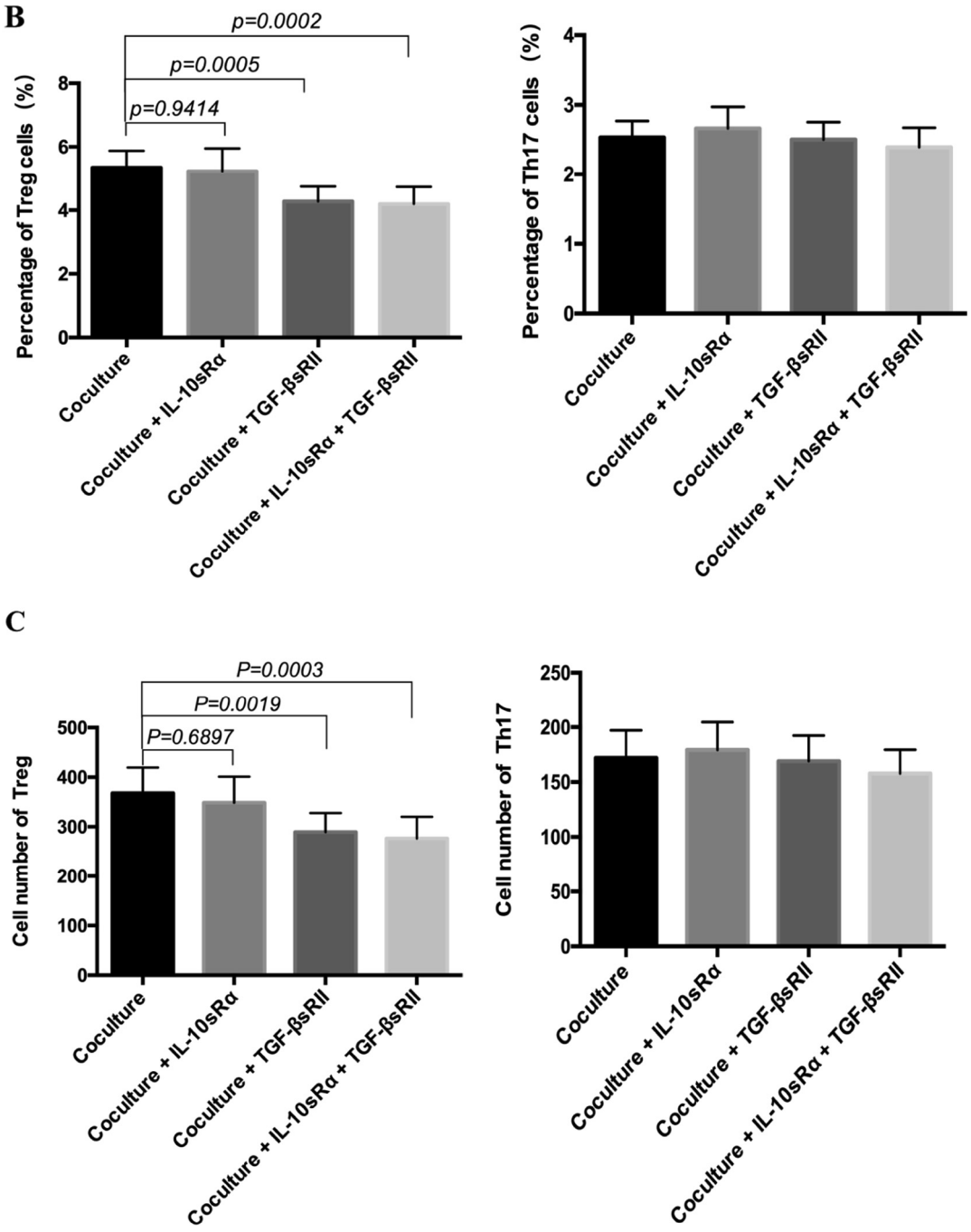

Fig. 3. (*continued*)

Table 1
Cytokine spectrum under different culture conditions (n = 10).

	IL-2	IL-4	IL-6	IL-10	IFN-γ	TNF
$CD4^+$ T cells	4.9 ± 1.6	305.3 ± 34.2	133.2 ± 19.7	21.9 ± 5.3	14.8 ± 3.3	20.4 ± 4.1
Breg + $CD4^+$ T	4.0 ± 1.1	475.1 ± 40.0*	377.4 ± 35.9*	98.6 ± 9.9*	15.2 ± 3.1	24.6 ± 4.3
Breg (upper) + $CD4^+$ T (lower)	4.2 ± 1.0	494.6 ± 31.0	734.4 ± 34.8**	74.8 ± 10.0	14.0 ± 2.9	17.6 ± 4.0
Breg + $CD4^+$ T + IL-10sRα	4.2 ± 1.1	599.2 ± 33.3	1028.0 ± 45.8***	83.4 ± 10.7	15.4 ± 3.4	55.0 ± 4.3***
Breg + $CD4^+$ T + TGF-βsRII	5.0 ± 1.3	530.1 ± 38.4	385.1 ± 32.7	91.0 ± 10.1	17.8 ± 3.8	23.7 ± 3.9
Breg + $CD4^+$ T + IL-10sRα + TGF-βsRII	5.7 ± 1.3	470.8 ± 30.3	1002.0 ± 36.2***	57.2 ± 10.6	21.6 ± 4.0	50.6 ± 4.5***
$CD4^+$ T + IL-10sRα + TGF-βsRII	4.5 ± 1.3	382.4 ± 33.5	139.4 ± 24.5	18.7 ± 5.1	14.3 ± 3.2	18.0 ± 3.8

P values were obtained by *one-way ANOVA* statistical analysis.
*In comparison with the $CD4^+$ T cells, $p < 0.0001$.
**In comparison with Breg + $CD4^+$ T, $p < 0.0001$.
***In comparison with Breg + $CD4^+$ T, $p < 0.0001$.

SOCS3 transcription, which contributes to transcriptional regulation of proapoptotic genes, inhibition of cell activation, cytokine production and proliferation. Additionally, IL-10 inhibits the production of proinflammatory cytokines, including IL-1β, IL-6, IL-12, IL-18, GM-CSF and TNFα. In our research, regardless of the presence of absence of a TGF-β inhibitor, IL-6 and TNF obviously increased once IL-10sRα was added into the system, while no changes in IL-6 and TNF were found in the TGF-β inhibitor group. Consequently, IL-10 may be an important molecule that inhibits the significant increase in the proinflammatory factors IL-6 and TNF to prevent an excessive immune response. Therefore, it is speculated that IL-10 is the main effector in this immunoregulatory system.

Cytokines derived from $CD19^+CD25^{high}$ Breg cells to drive Th17 cell differentiation seemed much more complex. Because IL-10sRα and/or TGF-βsRII cannot effectively block the inhibitory effect of $CD19^+CD25^{high}$ Breg cell on Th17 cell differentiation, neither TGF-β nor IL-10 may be the main cytokine of $CD19^+CD25^{high}$ Breg to regulate Th17 cells. Many reports have proven that the generation of Th17 cells is promoted by the combination of TGF-β and IL-6 [26–28]. In the IL-10sRα single inhibitor group, an increase in IL-6 was detected in the absence of a TGF-β inhibitor. It seemed that the number of Th17 cells should be elevated in this group. Nevertheless, there was no noticeable increase in Th17 cells in our research. In fact, another study showed that Th17 differentiation can occur in the absence of TGF-β signaling [29]. Neither IL-6 nor IL-23 alone could efficiently generate Th17 cells. However, these cytokines in combination with IL-1β effectively induced IL-17 production in naïve precursors independent of TGF-β. Thus, it was presumed that $CD19^+CD25^{high}$ Breg cells regulate Th17 cells in a TGF-β- and IL-6-independent manner.

Breg cells are a special subset of immunoregulatory cells with unique phenotypes and functions. In this study, the pattern and mechanism of $CD19^+CD25^{high}$ Breg cell regulation on $CD4^+$ T cells were recognized. Although this is a preliminary discussion regarding $CD19^+CD25^{high}$ Breg cells in human peripheral blood, but our findings would provide new clues to understand the function of Breg cells in vitro.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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