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ORIGINAL ARTICLE *Early pregnancy*

An imbalance in interleukin-17-producing T and Foxp3$^+$ regulatory T cells in women with idiopathic recurrent pregnancy loss

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BACKGROUND: T cells which produce interleukin (IL)-17 are involved in chronic inflammatory processes and regulatory T (Treg) cells are possibly the most important immune regulators. We aimed to investigate peripheral blood IL-17$^+$ T and Foxp3$^+$ Treg cells in women with idiopathic recurrent pregnancy loss (RPL).

METHODS: The study design is a cross-sectional evaluation of Th1, Th2, IL-17$^+$ T and Treg cells in women with idiopathic RPL ($n = 42$) and age-matched parous controls ($n = 24$). Flow cytometric analysis was performed to measure IL-17$^+$ T and Foxp3$^+$ Treg cells, and ratios of Th1/Th2 cells using anti-IL-17A and anti-Foxp3 antibodies, and monoclonal antibodies to tumor necrosis factor (TNF)-α, interferon (IFN)-γ and IL-10. Student's *t*-test and partial correlations were applied for statistical analysis.

RESULTS: TNF-α-/IL-10-producing CD3$^+$CD4$^+$ T cell ratio was higher in women with RPL than controls ($P = 0.048$). Levels of IL-17$^+$ T cells ($P = 0.021$) and the IL-17$^+$ T/CD4$^+$Foxp3$^+$ Treg cell ratio ($P = 0.001$) were increased, whereas Foxp3$^+$ ($P = 0.035$), Foxp3^{low} ($P = 0.032$) and CD4$^+$Foxp3$^+$ T cell ($P = 0.037$) levels were decreased in women with RPL, compared with controls. Levels of IL-17$^+$ T cells were correlated with TNF-α-producing CD3$^+$CD4$^+$ T cells ($r = 0.269$, $P = 0.033$), and with ratios of TNF-α/IL-10 ($r = 0.276$, $P = 0.027$) and IFN-γ/IL-10 ($r = 0.266$, $P = 0.035$)-producing CD3$^+$CD4$^+$ cells. Furthermore, the ratio of IL-17$^+$ T cells to CD4$^+$Foxp3$^+$ Treg cells showed a positive correlation with TNF-α-producing CD3$^+$CD4$^+$ T cells ($P = 0.047$) and IFN-γ-producing CD3$^+$CD4$^+$ T cells ($P = 0.048$) as well as a ratio of IFN-γ/IL-10-producing CD3$^+$CD4$^+$ T cells ($P = 0.037$).

CONCLUSIONS: Enhanced pro-inflammatory immune responses with suppressed immune regulation may be an important immune mechanism involved in RPL.

Key words: interleukin-17 / Th17 / Foxp3 / Treg / recurrent pregnancy loss

Introduction

Immune regulation entails homeostasis between Th1 and Th2 activity, and polarization to Th1 or Th2 immune response has been associated with various clinical diseases. Th1 cells secrete interleukin (IL)-2 and interferon (IFN)-γ and lead to cell-mediated responses, whereas Th2 cells mediate humoral immune responses by secreting IL-4, IL-5 and IL-13 (Mosmann and Coffman, 1989). Th1-Th2 paradigm was considered as a key theory to explain tissue damage caused by pathogen-activated immune system and autoimmunity (Weaver *et al.*, 2006). Actually, some autoimmune diseases result from an imbalance in favor of a Th1 response and are negatively regulated by Th2 cells. In contrast, there are some Th2-dependent autoimmune diseases also. While numerous studies support this theory, data are accumulating that do not fit with the original Th1-Th2 paradigm (Sheikh *et al.*, 2003; Van Oosterhout and Motta, 2005). Autoimmune diseases, such as insulin-dependent diabetes mellitus, systemic lupus erythematosus and myasthenia gravis, were explained by Th1-Th2

hypothesis, however, experimental autoimmune encephalitis and collagen-induced arthritis cannot be explained by this theory (Weaver *et al.*, 2006; Steinman, 2007). Recently, a novel subset of T cells, called Th17 cells, has been reported to induce experimental autoimmune encephalomyelitis and adjuvant arthritis in mice models (Harrington *et al.*, 2005; Steinman, 2007). Th17 cells are directly involved in chronic inflammatory processes, by secreting IL-17, which recruits neutrophils to tissue through induction of granulocyte colony-stimulating factor and IL-8 (Kolls and Linden, 2004). In patients with rheumatoid arthritis, Th17 cells were found in peripheral blood, synovial fluid and biopsies of synovium (Kotake *et al.*, 1999; Heo *et al.*, 2010) and contributed to the erosion of cartilage in an animal model of rheumatoid arthritis (Lubberts, 2010). Th17 cells were also found in higher numbers in cerebrospinal fluid than in peripheral blood of patients with multiple sclerosis (Matusevicius *et al.*, 1999) and were believed to promote disruption of the blood-brain barrier in central nervous system inflammation (Kebir *et al.*, 2007).

Th17 cells have a distinct developmental lineage, which is different from Th1 and Th2 cells (Harrington *et al.*, 2005; Ivanov *et al.*, 2006). Transforming growth factor (TGF)-β was suggested as a crucial cytokine for Th17 cell development, in conjunction with IL-6 and IL-21 in humans (Laurence *et al.*, 2007; Manel *et al.*, 2008; O'Garra *et al.*, 2008; Yang *et al.*, 2008). Th17 cells secret IL-17A, IL-17F and other cytokines, such as IL-21 in mice and IL-22 and IL-26 in humans, which are associated with chronic inflammation. $CD8^+$ T cells are also known to produce IL-17 and have a role in allergic contact dermatitis (Zhao *et al.*, 2009) and in human immunodeficiency virus infection (Maek *et al.*, 2007). $CD4^+$ and $CD8^+$ T cells producing IL-17 were called Th17/Tc17 cells and a role for these IL-17-producing T cells in autoimmune diseases was proposed. On the other hand, cytokines produced by Th1 cells are mainly involved in cytotoxicity (Weaver *et al.*, 2006). There is a body of evidence that the inflammatory autoimmune response is associated with the pathogenesis of recurrent pregnancy loss (RPL). Increased prevalence of antiphospholipid antibodies and other autoimmune abnormalities have been reported in women with RPL, and pregnant women with a history of RPL showed significantly increased $CD56^+$ and $CD56^+CD16^+$ Natural Killer cells when compared with those of pregnant controls (Kwak *et al.*, 1995). In addition, women with RPL demonstrated significantly higher Th1/Th2 cell ratios, including increased ratios of IFN-γ/IL-4, tumor necrosis factor (TNF)-α/IL-4 and TNF-α-/IL-10-producing $CD3^+CD4^+$ T cells, in peripheral blood when compared with those of controls (Kwak-Kim *et al.*, 2003). Additionally, more women with RPL had detectable levels of TNF-α, IFN-γ, IL-2 and IL-12 in endometrium but fewer women had detectable levels of IL-6, compared with control fertile women (Lim *et al.*, 2000).

Regulatory T (Treg) cells may be the most important immune regulator in peripheral immune system. Foxp3, encoding a forkhead-winged-helix transcription factor designated Scurfin, was identified as a master gene for generation and development of Treg cells and considered as the most significant marker for Treg cells among other markers. Many autoimmune diseases in rodents and humans are linked to abnormal function or decreased number of Treg cells. The presence of antigen-specific Treg cells was reported in autoimmune ovarian disease of mice (Samy *et al.*, 2005). Only the Treg cells retrieved from ovary-draining lymph nodes adaptively suppressed autoimmune ovarian disease (Samy *et al.*, 2005). A few reports have shown that the number of Treg cells was decreased in peripheral blood and endometrium/deciduas of women with RPL (Sasaki *et al.*, 2004; Jin *et al.*, 2009; Schumacher *et al.*, 2009). Sasaki *et al.* demonstrated that deciduas in early human pregnancy contain abundant $CD4^+CD25^{bright}$ Treg cells, which inhibit the proliferation of autologous $CD4^+CD25^-$ T cells in a dose-dependent fashion (Sasaki *et al.*, 2004). Patients having miscarriages or an ectopic pregnancy presented significantly decreased hCG levels associated with decreased Foxp3, neuropilin-1, IL-10 and TGF-β mRNA levels in the decidual and placental tissue when compared with those of normal pregnant women (Schumacher *et al.*, 2009). In the decidua, $CD4^+CD25^{bright}$ T cells and Foxp3 expression were significantly decreased in women with unexplained RPL with early miscarriages when compared with early pregnant women with no history of RPL (Mei *et al.*, 2010). Thus, we aimed to investigate peripheral blood Th1, Th2, IL-17- producing T cells (Th17/Tc17) and Treg cells in women with idiopathic RPL, and evaluate the correlations between these cells.

Materials and Methods

Study population

All study populations and controls were recruited at the Department of Obstetrics and Gynecology, Konyang University Hospital, Korea. Women with idiopathic RPL had a history of two or more spontaneous abortions and no abnormalities in genetic, anatomic, endocrine and autoimmune factors or any sign of infection. Controls consisted of healthy parous women of reproductive age, who have no history of spontaneous abortions. No one had a history of or active autoimmune disease, endometriosis, acute inflammatory diseases or vaccination within 2 months prior to study enrollment. The study received local Institutional Review Board approval and participants signed informed consent before enrollment.

In total, 42 women with idiopathic RPL and 24 fertile controls were enrolled in this study. Age (mean ± SD) of women with RPL (32.5 ± 4.0 years) was not different from that of controls (32.3 ± 3.1 years). Parity of women with RPL (mean ± SD) was 0.1 ± 0.3 (range 0–1) and controls was 1.7 ± 0.7 (range 1–3) ($P < 0.001$). Number of spontaneous pregnancy losses was significantly higher in women with RPL (3.3 ± 1.5, range 2–10) compared with that of controls (0.0 ± 0.0, range 0) ($P < 0.001$).

Separation of peripheral blood mononuclear cells

Peripheral blood was sampled between 9 a.m. and 11 a.m. during the early to mid-follicular phase of the menstrual cycle, because proportions of peripheral blood lymphocytes fluctuated between follicular and luteal phases in our previous study (Lee *et al.*, 2010). Measurement of Treg or Th17 cells in the follicular phase may reduce any influence of sex steroid hormones and gonadotrophins on immune cells, leading to more reliable data when compared with blood sampling in the luteal phase. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Hypaque (Biotech, Uppsala, Sweden) density centrifugation.

Study of IL-17-producing T cells

To activate the PBMCs, 1 ml of 5×10^6/ml of cell suspension was incubated with 10 ng/ml phorbol myristate acetate (Sigma, St-Louis, MO, USA) and 0.5 μM ionomycin (Sigma) for 5 h at 37°C in a 5% CO_2 humidified incubator. Monensin (1 μl of a ×1000 solution) (eBioscience,

San Diego, CA, USA) was also applied to enhance intracellular cytokine staining. After incubation, PBMCs were washed in phosphate-buffered saline (PBS) with 0.09% (w/v) sodium azide (Sigma) twice, followed by staining with the anti-CD3-peridinin chlorophyl (PerCP), and then incubated for 15 min at 4°C. The cells were washed in PBS and fixed with IC Fixation buffer (eBioscience). The cells were washed twice with 1 × permeabilization buffer (eBioscience) and incubated with 0.25 μg phycoerythrin (PE)-conjugated anti-human IL-17A (eBioscience) at room temperature for 20 min. After intracellular staining, the cells were washed with 1 × permeabilization buffer and resuspended in 0.5 ml of permeabilization buffer. The proportion of IL-17-producing T cells in peripheral blood lymphocytes were enumerated by flow cytometry (Fig. 1A).

Study of Treg cells

To identify Treg cells, PBMCs were stained with anti-CD4-fluorescein isothiocyanate (FITC) monoclonal antibody (BD biosciences, Franklin Lakes, NJ, USA) for surface antigens, and anti-Foxp3-PE monoclonal antibody (eBioscience) for intracellular molecules, according to the manufacturer's instruction. Briefly, 1 × 10^6 PBMCs were washed twice in PBS following staining with the fluorochrome-conjugated antibodies specific for cell surface antigen markers for 20 min in the dark at 4°C. To stain an intracellular molecule, Foxp3, cells were permeabilized with permeabilization/fixation buffer and stained with anti-Foxp3 antibody following the surface staining. PE-Rat immunoglobulin G2a was used as an isotype control for anti-Foxp3-PE antibody. Cells were resuspended in 0.5 ml of staining buffer for subsequent flow cytometric analysis.

Study of type 1 and type 2 intracellular cytokines

We performed cytokine study as previously reported (Kwak-Kim *et al.*, 2003). Briefly, to activate PBMCs, 1 ml of 5 × 10^6 cells/ml of cell suspension was incubated with 25 ng/ml 4β-phorbol-12-myristate-13-acetate (Sigma) and 1 μM ionomycin (Sigma) for 16 h at 37°C in a 5% CO_2 humidified incubator. Golgiplug (BD biosciences) was also applied to a final concentration of 0.2 μM in order to inhibit cytokine secretion. Cell staining procedure was carried out according to the manufacturer's instructions with the Cytofix/Cytoperm kit (BD biosciences). Anti-CD3-PerCP and anti-CD8-FITC were used to identify T cell populations. To detect intracellular cytokines, 0.2 μg of monoclonal antibodies for TNF-α, IFN-γ and IL-10 were applied. For determining ratios of TNF-α-/IL-10-producing cells, levels of TNF-α-producing cells were

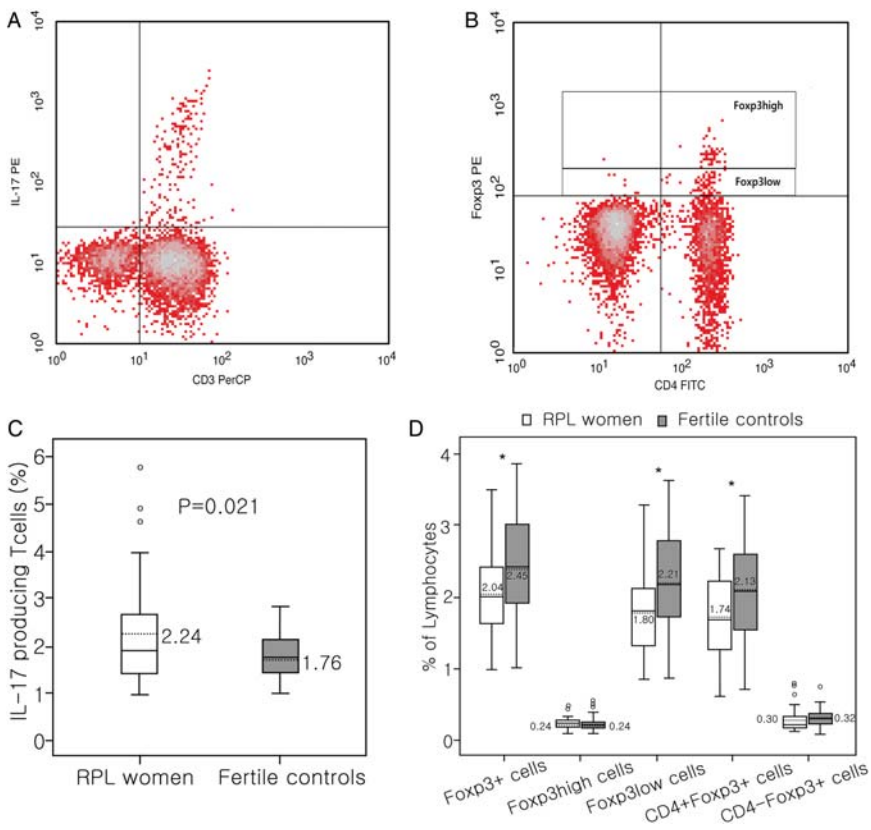

Figure 1 Populations of IL-17-producing T (**A**) and Fopx3 expressing T (**B**) cells. Foxp3$^+$ T regulatory (Treg) cells can be classified into Foxp3^{high} and Foxp3^{low} according to the level of Foxp3 expression. Comparison of proportion of peripheral blood IL-17-producing T cells (**C**) and Foxp3$^+$ Treg cell subsets (**D**) between women with idiopathic RPL ($n = 42$) and fertile controls ($n = 24$). The level of IL-17-producing T cells was increased in women with RPL when compared with that of controls ($P = 0.021$). The proportions of Foxp3$^+$ ($P = 0.035$), Foxp3^{low} ($P = 0.032$) and CD4$^+$Foxp3$^+$ Treg cells ($P = 0.037$) were decreased in women with RPL when compared with those of controls. However, proportions (%) of Foxp3^{high} and CD4$^-$Foxp3$^+$ Treg cells did not differ between women with RPL and controls. Each box shows the first and third quartiles, and a horizontal bar in the box represents the median. Dotted lines indicate the mean values. Whiskers at the top and bottom of the box represent the range of typical data values. Outliers are plotted as circles. *$P < 0.05$ for RPL versus control.

divided by levels of IL-10-producing cells in each cell subset, including $CD3^+$, $CD3^+CD8^-$ (namely $CD3^+CD4^+$) and $CD3^+CD8^+$ cells. The same calculation was made for the ratios of IFN-γ/IL-10 producing lymphocytes. We have used negative and positive selection for the CD4+ T cell population: this may raise a concern that one may not be comparing exactly the same cell populations; however, negative selection of CD4+ cells has been utilized previously (Kwak-Kim *et al.*, 2003).

Flow cytometric analysis

The stained cells were analyzed on a fluorescence-activated cell sorter Calibur flow cytometer (BD Biosciences) and CellQuest Pro software (BD Biosciences) was used for data analysis. A total of 10,000 cells were counted. Viable lymphocytes were gated based on their forward- and side-scatter profile. In Treg cell analysis, $Foxp3^+$ Treg cells were classified into $Foxp3^{high}$ and $Foxp3^{low}$ cells, according to the levels of Foxp3 expression (Fig. 1B).

Statistical analysis

The primary objectives of this study were to estimate proinflammatory immune responses using Th1, Th2, IL-17 producing T and Treg cells as the primary measure and to compare the women with RPL to age-matched healthy parous controls. Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (version 15.0, SPSS Inc., Chicago, IL, USA). Normality of the data was checked prior to the data analysis. Student's *t*-test was applied to compare the levels of type 1 and type 2 cytokine-producing cells, IL-17-producing T and Treg cells and ratios of IL-17-producing T to Treg cells between study group and controls. Mean values were presented with SD. All proportions are presented as percentages. Correlations between IL-17-producing T and Treg cell populations, type 1 and type 2 cytokine-producing T cell populations and Th1/Th2 cell ratios were further explored using partial correlations, controlled for age. Since we aimed to determine differences of immune variables which are mutually correlated, we determined that the Bonferroni correction method is inappropriate for this study as it will be highly conservative and may not detect real differences. Statistical significance was set at $P < 0.05$.

Results

Levels of IL-17-producing T and $Foxp3^+$ Treg cells

In women with idiopathic RPL, the proportion (%) of $IL\text{-}17^+$ T cells in peripheral blood lymphocytes (2.2 ± 1.1) was increased compared with that of controls (1.8 ± 0.5) ($P = 0.021$) (Fig. 1C). The proportion of $Foxp3^+$ lymphocytes was decreased in women with RPL (2.0 ± 0.6) compared with controls (2.5 ± 0.8) ($P = 0.035$) (Fig. 1D). Proportions (%) of $Foxp3^{low}$ and $CD4^+Foxp3^+$ cells in women with RPL (1.8 ± 0.6 and 1.7 ± 0.5, respectively) were lower than controls (2.2 ± 0.8 and 2.1 ± 0.8, respectively) ($P = 0.032$ and 0.037, respectively). However, proportions of $Foxp3^{high}$ and $CD4^-Foxp3^+$ Treg cells did not differ in women with RPL (0.2 ± 0.1 and 0.3 ± 0.2, respectively) versus controls (0.2 ± 0.1 and 0.3 ± 0.1, respectively).

Type 1 and type 2 cytokine-producing lymphocytes in women with idiopathic RPL

The ratio of TNF-α-/IL-10-producing $CD3^+CD4^+$ T lymphocytes was higher in women with RPL (29.5 ± 22.1) than in controls (21.2 ± 11.3) ($P = 0.048$) (Table I). Furthermore, ratios of TNF-α

Table I Comparison of proportions (%) of type 1 and type 2 cytokine-producing T cells in women with idiopathic RPL and fertile controls (mean ± SD).

	RPL women ($n = 42$)	Controls ($n = 24$)	*P*-value
Cytokine-producing T cells			
$CD3^+TNF\text{-}\alpha^+$	55.5 ± 8.2	58.3 ± 9.6	NS
$CD3^+IFN\text{-}\gamma^+$	28.9 ± 12.7	28.5 ± 9.3	NS
$CD3^+IL\text{-}10^+$	3.4 ± 2.2	4.0 ± 1.9	NS
$CD3^+CD4^+TNF\text{-}\alpha+$	73.0 ± 11.1	71.7 ± 7.8	NS
$CD3^+CD4^+IFN\text{-}\gamma^+$	28.8 ± 11.4	30.1 ± 9.1	NS
$CD3^+CD4^+IL\text{-}10^+$	3.5 ± 1.8	4.0 ± 1.6	NS
$CD3^+CD8^+TNF\text{-}\alpha^+$	45.9 ± 9.2	48.1 ± 12.0	NS
$CD3^+CD8^+IFN\text{-}\gamma^+$	37.6 ± 12.8	39.6 ± 10.8	NS
$CD3^+CD8^+IL\text{-}10^+$	1.9 ± 0.8	1.7 ± 0.8	NS
Ratios of type 1 to type 2 cytokine-producing T cell			
$CD3^+TNF\text{-}\alpha^+/CD3^+IL\text{-}10^+$	24.8 ± 16.5	17.5 ± 7.4	0.016
$CD3^+IFN\text{-}\gamma^+/CD3^+IL\text{-}10^+$	12.6 ± 11.1	8.4 ± 4.0	0.029
$CD3^+CD4^+TNF\text{-}\alpha^+/CD3^+CD4^+IL\text{-}10^+$	29.5 ± 22.1	21.2 ± 11.3	0.048
$CD3^+CD4^+IFN\text{-}\gamma^+/CD3^+CD4^+IL\text{-}10^+$	11.5 ± 9.0	8.8 ± 4.7	NS
$CD3^+CD8^+TNF\text{-}\alpha^+/CD3^+CD8^+IL\text{-}10^+$	28.5 ± 15.4	32.6 ± 15.5	NS
$CD3^+CD8^+IFN\text{-}\gamma^+/CD3^+CD8^+IL\text{-}10^+$	23.0 ± 11.7	26.3 ± 12.5	NS

TNF, tumor necrosis factor; IFN, interferon; IL, interleukin.

to IL-10 and IFN-γ to IL-10-producing $CD3^+$ T lymphocytes were also increased in women with RPL compared with controls ($P = 0.016$ and 0.029, respectively). However, the ratios of cytokine-producing $CD3^+CD8^+$ T cells did not differ between women with RPL and controls. There were no differences in proportions of TNF-α, IFN-γ- and IL-10-producing peripheral blood T lymphocyte subsets, such as $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, between women with RPL and controls.

IL-17-producing T cell/Treg cell ratio

To see a balance of IL-17-mediated immune response, ratios of $IL\text{-}17^+$ T cells to Treg cell subpopulations were calculated and compared in women with RPL versus controls. For $IL\text{-}17^+$ T /Treg cell ratio calculation, the percentage of $IL\text{-}17^+$ T cells was divided by the percentage of Treg cell subsets, including $Foxp3^+$, $Foxp3^{high}$, $Foxp3^{low}$, $CD4^+Foxp3^+$ and $CD4^-Foxp3^+$.

Ratios of $IL\text{-}17^+$ T cells to $Foxp3^+$ ($P = 0.001$), to $Foxp3^{high}$ ($P = 0.036$), to $Foxp3^{low}$ ($P = 0.001$), to $CD4^+Foxp3^+$ ($P = 0.001$) and to $CD4^-Foxp3^+$ ($P = 0.020$) Treg cells were higher in women with RPL (1.3 ± 0.7, 12.3 ± 6.9, 1.6 ± 0.9, 1.6 ± 0.9 and 10.8 ± 6.8, respectively) when compared with those of normal fertile controls (0.8 ± 0.3, 8.7 ± 4.3, 0.9 ± 0.3, 0.9 ± 0.3 and 6.7 ± 4.6, respectively) (Fig. 2).

Correlation of IL-17-producing T cells with other T cell subsets

IL-17-producing T cell level was not correlated with any $Foxp3^+$ Treg cell subpopulations including $Foxp3^+$, $Foxp3^{high}$, $Foxp3^{low}$, $CD4^+Foxp3^+$ and $CD4^-Foxp3^+$ cells (Table II). The level of TNF-α-producing $CD3^+CD4^+$ T cells was correlated with that of $IL\text{-}17^+$ T cells ($r = 0.269$, $P = 0.033$). However, IFN-γ- and IL-10-producing $CD3^+CD4^+$ T cell levels were not correlated with $IL\text{-}17^+$ T cell levels. TNF-α-, IFN-γ- and IL-10-producing $CD3^+$ and $CD3^+CD8^+$ T cells were not correlated with $IL\text{-}17^+$ T cell levels. The levels of $IL\text{-}17^+$ T cells demonstrated significant positive correlations with ratios of TNF-α/IL-10 ($r = 0.276$ and $P = 0.027$) and IFN-γ-/IL-10-producing $CD3^+CD4^+$ T cells ($r = 0.266$ and $P = 0.037$). Ratios of type 1/2 cytokine-producing $CD3^+$ and $CD3^+CD8^+$ T cells showed no correlation with levels of IL-17-producing T cells.

To determine whether there was any relationship between the type 1/2 cytokine ratios and $IL\text{-}17^+$ T/Treg cell balance, we analyzed correlations of these two immune cell systems. Type 1 and type 2 cytokine production from T lymphocytes was correlated with a ratio of $IL\text{-}17^+$ T cells to $CD4^+Foxp3^+$ Treg cells (Table II). TNF-α and IFN-γ-producing $CD3^+CD4^+$ T cells showed a positive correlation with a ratio of $IL\text{-}17^+$ T/$CD4^+Foxp3^+$ Treg cells ($P = 0.047$ and 0.048, respectively). Positive correlations between $IL\text{-}17^+$ T/Treg cell ratio and ratios of IFN-γ to IL-10-producing $CD3^+$ and $CD3^+CD4^+$ T lymphocytes ($P = 0.010$, and 0.037, respectively) were also demonstrated. However, there was no correlation between TNF-α/IL-10 ratio and $IL\text{-}17^+$T/$CD4^+Foxp3^+$ T cell ratio in $CD3^+CD4^+$ Th cells ($P = 0.112$). However, TNF-α/IL-10 ratio and $IL\text{-}17^+$T/$CD4^+Foxp3^+$ T cell ratio in $CD3^+$ T cells showed a weak correlation ($P = 0.072$). A lack of correlation might be related to type II errors.

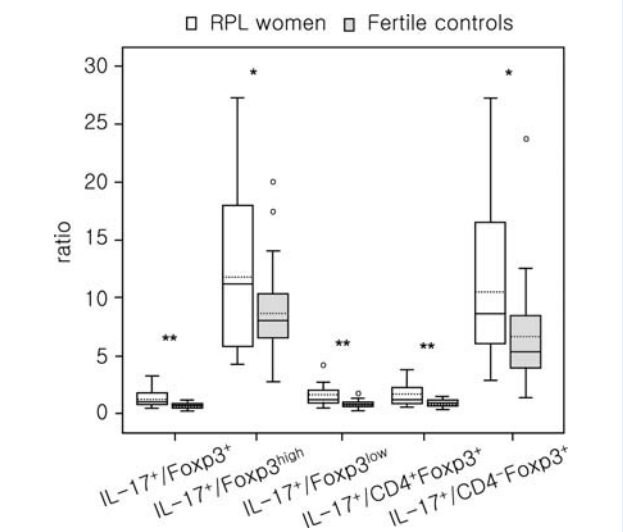

Figure 2 Ratios of $IL\text{-}17^+$ T cells to $Foxp3^+$ Treg subpopulations in women with idiopathic RPL ($n = 42$) and fertile controls ($n = 24$). All ratios were increased in women with RPL when compared with controls. Each box shows the first and third quartiles, and a horizontal bar in the box represents the median. Dotted lines indicate the mean values. Whiskers at the top and bottom of the box represent the range of typical data values. Outliers are plotted as circles. $^*P < 0.05$, $^{**}P < 0.01$ for RPL versus control.

Discussion

In this study, we demonstrated that the level of $IL\text{-}17^+$ T cells and ratios of $IL\text{-}17^+$ T/Treg cells were significantly increased in peripheral blood from non-pregnant women with idiopathic RPL when compared with fertile controls. Recent studies reported that proportions of Th17 cells in the peripheral blood and deciduas were increased in pregnant women with unexplained RPL when compared with normal early pregnant women (Wang *et al.*, 2010), and non-pregnant women with unexplained RPL had higher Th17 cell levels in circulating blood than parous controls (Liu *et al.*, 2011). In addition, Th17 cells were increased in the deciduas of inevitable abortions, such as progression stage of abortion (Nakashima *et al.*, 2010). Therefore, a Th17 cell-mediated inflammatory immune response is associated with RPL in addition to predominant Th1 immunity (Kwak-Kim *et al.*, 2003).

Several studies have elucidated that Treg cells are involved in abnormal pregnancy (Arruvito *et al.*, 2007; Yang *et al.*, 2009). In this study, $Foxp3^+$, $Foxp3^{low}$ and $CD4^+Foxp3^+$ lymphocytes were decreased in non-pregnant women with idiopathic RPL compared with controls; however, $Foxp3^{high}$ and $CD4^-Foxp3^+$ cells did not differ. This suggests that women with RPL are unlikely to be able to induce and maintain the immune tolerance regulated by Treg cells. Our study is in line with a previous report of Sasaki *et al.* (2004) in which $CD4^+CD25^+$ Treg cells were increased in peripheral blood and deciduas of normal pregnancy, and decreased in peripheral blood and decidua in cases of spontaneous abortion. Arruvito *et al.* (2007) reported that Treg cells from women with RPL were not only decreased in number but also functionally deficient when compared

Table II Correlations between IL-17-producing T cells and the ratio of IL-17$^+$ T/CD4$^+$Foxp3$^+$ T cells, and Foxp3$^+$ Treg cells and cytokine-producing T cell subpopulations.

	IL-17$^+$ T cells		Ratio of IL-17$^+$ T/CD4$^+$Foxp3$^+$ T cells	
	R	*P*-value	*R*	*P*-value
Regulatory T cell subsets				
Foxp3$^+$ cells	−0.053	NS	−0.540	0.000
Foxp3^{high} cells	0.053	NS	−0.106	NS
Foxp3^{low} cells	−0.063	NS	−0.536	0.000
CD4$^+$Foxp3$^+$ cells	−0.074	NS	−0.580	0.000
CD4$^-$Foxp3$^+$ cells	0.075	NS	−0.053	NS
Cytokine-producing T cells				
CD3$^+$TNF-α $^+$ cells	0.115	NS	−0.001	NS
CD3$^+$IFN-γ $^+$ cells	−0.015	NS	0.224	NS
CD3$^+$IL-10$^+$ cells	−0.098	NS	−0.118	NS
CD3$^+$CD4$^+$TNF-α $^+$ cells	0.269	0.033	0.292	0.047
CD3$^+$CD4$^+$IFN-γ $^+$ cells	0.114	NS	0.289	0.048
CD3$^+$CD4$^+$IL-10$^+$ cells	−0.118	NS	−0.130	NS
CD3$^+$CD8$^+$TNF-α $^+$ cells	0.033	NS	−0.065	NS
CD3$^+$CD8$^+$IFN-γ $^+$ cells	−0.017	NS	0.111	NS
CD3$^+$CD8$^+$IL-10$^+$ cells	0.187	NS	0.126	NS
Ratios of type 1 to type 2 cytokine-producing T cells				
CD3$^+$TNF-α $^+$/CD3$^+$IL-10$^+$ cell ratio	0.136	NS	0.265	NS
CD3$^+$IFN-γ $^+$/CD3$^+$IL-10$^+$ cell ratio	0.059	NS	0.374	0.010
CD3$^+$CD4$^+$TNF-α $^+$/CD3$^+$CD4$^+$IL-10$^+$ cell ratio	0.278	0.027	0.235	NS
CD3$^+$CD4$^+$IFN-γ $^+$/CD3$^+$CD4$^+$IL-10$^+$ cell ratio	0.266	0.035	0.305	0.037
CD3$^+$CD8$^+$TNF-α $^+$/CD3$^+$CD8$^+$IL-10$^+$ cell ratio	−0.116	NS	−0.188	NS
CD3$^+$CD8$^+$IFN-γ $^+$/CD3$^+$CD8$^+$IL-10$^+$ cell ratio	−0.105	NS	−0.064	NS

R, partial correlation coefficient adjusted by age.

with fertile women, and they also showed that Foxp3$^+$ Treg cell expansion following T cell activation was limited *in vitro* (Arruvito *et al.*, 2010). Additionally, it has been reported that the proportion of CD4$^+$CD25^{bright} Treg cells in peripheral blood from patients with unexplained RPL is increased after paternal or third-party lymphocyte immunotherapy, and the proportion of CD4$^+$CD25^{bright} Treg cells is higher in women with a successful pregnancy than in those with pregnancy loss after lymphocyte immunotherapy (Yang *et al.*, 2009). Furthermore, the results of a recent study suggest that low levels of circulating CD4$^+$CD25$^+$Foxp3$^+$ Treg cells could be a poor prognostic factor of index pregnancy in pregnant women with a history of pregnancy loss or immunologic infertility (Winger and Reed, 2011). Therefore, it is promising that a potential therapy to augment Treg cell levels may enhance pregnancy outcome.

The function of effector T cells, such as Th1, Th2 and Th17 cells, is regulated by Treg cells, and Treg cells are important for the maintenance of peripheral tolerance. In this study, we report that IL-17$^+$ T/Treg cell ratios are higher in women with RPL compared with controls. Since the IL-17$^+$ T/Treg cell ratio reflects both Th17/Tc17 inflammatory and Treg immune regulatory responses, this ratio may serve as a novel biomarker for diagnosis (and thereby treatment) of women with RPL.

Many researchers have reported that women with idiopathic RPL show Th1 cytokine dominance (Raghupathy *et al.*, 1999; Makhseed *et al.*, 2001). Previously, we reported that patients with RPL demonstrated higher Th1/Th2 ratios of IFN-γ/IL-4, TNF-α/IL-4 and TNF-α/IL-10-producing CD3$^+$CD4$^+$ T cells than those of controls (Kwak-Kim *et al.*, 2003). In this study, we confirmed a significant increase of Th1/Th2 cytokine-producing cell ratios, including TNF-α/IL-10 and IFN-γ-/IL-10-producing CD3$^+$ and CD3$^+$CD4$^+$ T cells, in women with RPL compared with controls. As CD3$^+$CD4$^+$ T cells are the major lymphocytes producing type 1 and type 2 cytokines, (Lee *et al.*, 2010), the type 1 immune response of T cells seems to be mostly dependent on CD3$^+$CD4$^+$ T cells.

Our study clearly elucidated that a Th1 bias was closely correlated with the level of IL-17-producing T cells in peripheral blood. A positive correlation was present between the level of IL-17-producing T cells and ratios of TNF-α/IL-10 and IFN-γ-/IL-10-producing CD3$^+$CD4$^+$ cells. We also found that the IL-17$^+$ T/Treg cell ratio had a significant positive correlation with type 1 cytokine production. In a study of patients with immune thrombocytopenic purpura, the levels of Th17 and Th1 cells were significantly increased in the peripheral blood of patients when compared with controls, and the proportion of Th17 cells was positively

correlated with Th1 cells (Zhang et al., 2009): this represents the simultaneous activation of a Th17 and Th1 immune response, which is in line with our data. Thus, our results suggest that an imbalance between Th1 and Th2 cells and additional biological effects of increased numbers of IL-17$^+$ T cells may induce an inflammatory immune response which contributes to the development of RPL.

Th17 cells were proposed to have a link with Foxp3$^+$ Treg cells in the early stage of Th17 differentiation. TGF-β is essential to induce Th17 cells and Treg cells. In the presence of IL-6, CD4$^+$ T cells were differentiated to Th17 cells by TGF-β1 or co-culture with naturally occurring Treg cells (Veldhoen et al., 2006). However, the low concentration of TGF-β that could induce Th17 cells and inhibit Th1 and Th2 cell differentiation was not enough to stimulate Treg cells (Harrington et al., 2006). IL-2 and retinoid acid negatively regulate the transcription factor retinoid-related orphan receptor γt and inhibit the induction of Th17 cells, while combined with TGF-β, they drive differentiation of Foxp3$^+$ T cells (Laurence et al., 2007; Mucida et al., 2007). IL-21, a Th1 cytokine, is known to promote Th17 development along with TGF-β, and to suppress Foxp3 expressing T cells in mice (Fantini et al., 2007). Therefore, these findings may suggest that the induction of Th17 and Treg cells is determined by the local concentration of TGF-β or other factors/cytokines in the milieu, although the underlying mechanism involved in the Treg cell to Th17 cell shift has not yet been elucidated (Bettelli et al., 2006). However, in a study of obesity, increased Th17 cell level in obese mice was not related to IFN-γ-producing T cells, CD4$^+$Foxp3$^+$ Treg cells or IL-6 production (Winer et al., 2009). In our study, we found no correlation between the levels of IL-17$^+$ T and Foxp3$^+$ Treg cells. Contrary to our result, a recent report demonstrated an inverse relationship between percentages of CD4$^+$IL-17A$^+$ T cells and CD4$^+$CD25$^+$CD127$^{low/-}$ Treg cells in the peripheral blood and decidua of women with unexplained RPL (Wang et al., 2010). This discrepancy might be related to differences in study design and sample size, as the Wang et al. (2010) study involved a relatively small number of pregnant women and controls, and different cell markers.

We report elevated IL-17$^+$ T cells in peripheral blood of non-pregnant women with a history of RPL. Therefore, we speculate these cells actively induce pro-inflammatory immune responses at the maternal-fetal junction at the time of implantation, and lead to RPL. Previously, decidual T cells in women with inevitable abortions were shown to have increased IL-17 expression but not in missed abortion and it was suggested that IL-17$^+$ cells might be involved in the induction of inflammation in the late, but not the early, stage of abortion (Nakashima et al., 2010). Further study is needed to explore the interaction between peripheral blood and decidual IL-17$^+$ T cells in the early stage of pregnancy loss with inflammatory immune responses.

In this study, women with RPL have an immunological alteration in peripheral blood effectors, such as Th1, Th2, IL-17$^+$ T and Treg cells. These findings suggest that women with RPL have a propensity to inflammatory immune response and decreased immune regulatory function, which negatively affect reproductive outcome, including RPL. This study demands a paradigm shift from Th1/Th2 theory to a broader concept of pro-inflammatory immune responses, which involve increased IL-17$^+$ T cells (Th17/Tc17), increased Th1/Th2 cell ratios and suppressed Treg cell immune responses as the underlying immune pathology for RPL and, potentially, other obstetrical complications.

Authors' roles

S.K.L. a main author who designed this study, analyzed the results and wrote the manuscript. J.Y.K. a significant contributor who performed most part of experiments. S.E.H. a significant contributor who helped recruiting subjects and advised this study. C.J.K. a significant contributor who helped recruiting subjects and advised this study. B.J.N. a significant contributor who designed statistical methods for this study and helped interpretation of the results. M.L. a significant contributor who helped study design and advised whole process of the experiment. A.G.-S. a significant contributor who advised the process of the experiment and helped interpretation of the results. J.K.-K. a corresponding author who proposed concept of this study and gave directions for this study.

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