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Original Article

Evaluation of Regulatory B Cells and Serum IL-10 Concentration in Peripheral Blood of Women with Recurrent Pregnancy Loss

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Abstract

Background: Although the role of B cells in normal pregnancy has been recently highlighted, their importance and function are not completely clarified. Until now, some investigations have shown that during pregnancy, regulatory B cells (Breg), a subset of B cells, are one of the key players in immune regulation by both producing IL-10 and cell-cell interactions. Therefore, any decrease in the number or function of these cells may lead to recurrent pregnancy loss (RPL). Thus, the objective of this study was to characterize Breg cell frequency and function in women who suffered from RPL in comparison with healthy non-pregnant and pregnant women (under twenty weeks of gestational age) as controls.

Method: In this study, peripheral blood samples of women suffering from RPL (n=8), women with normal pregnancy under 20 weeks of gestational age (n=14), and healthy nonpregnant women (n=10) were collected. The frequency of Breg cells ($CD19^{+}CD24^{hi}CD38^{hi}$) was measured by flow cytometry. The serum level of the IL-10 cytokine, as a marker of Breg cell function, was measured by ELISA.

Results: The Percentage of Breg cells in women who suffered from RPL was significantly lower than that of women who had normal pregnancies (*P=0.0016*). The percentages of Breg cells in women who suffered from RPL were also significantly lower than in non-pregnant women (*P=0.0001*). Furthermore, no significant differences were observed in Breg cell percentages between normal pregnant and non-pregnant women. Evaluation of IL-10 concentration in the serum of women who had participated in this study showed no significant differences between the three groups.

Conclusion: Based on our results, the number of Breg cells was significantly lower in RPL women than in healthy non-pregnant and normal-pregnant women, which shows the significance of these cells in the maintenance of normal pregnancy. However, we could not detect significant differences in the serum levels of IL-10, bringing to mind the notion that the beneficial and supportive function of these cells during pregnancy might be independent of IL-10 secretion. by these cells. Thus, screening of Breg cells in women with pregnancy complications, especially RPL, could be helpful for predicting a healthy pregnancy.

Keywords: Regulatory B cell; Reproductive Immunology; IL-10; RPL

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Introduction

The field of reproductive immunology and the importance of tolerance in the progression of healthy pregnancy were established more than 50 years ago when Medawar et al. proposed the paradigm of why the fetus, as a semi-allograft, is not rejected by the maternal immune system (1). Due to this main concept, the progress of normal pregnancy is controlled by different parameters, such as immune system cell types and their related signaling pathways (2).

Recurrent pregnancy loss (RPL) is defined as three miscarriages before the 20th week of gestation without any ectopic, molar, and biochemical factors(3). Females with recurrent miscarriages have higher numbers of decidual $CD83^+$ DCs than normal controls, which may suggest that activation of DCs has a negative impact on implantation. Moreover, it has been shown that T lymphocytes play an important role in establishing immune tolerance for maintenance of the fetal semi-allograft due to their broad subtypes and their diversity in function (2). During the last decades, the exact role of some of the immune cells in normal pregnancy has been verified; for instance, 70% of decidua lymphocytes are $CD56^{bright}$ $CD16^-$ NK cells, and the substitution of these NK cells with a high amount of $CD56^{bright}CD16^+$ NK cells results in miscarriage (2). Furthermore, macrophages are involved in the implantation process, but unlike NK cells, their numbers in the uterus increase in the first trimester and remain elevated throughout pregnancy (2).

On the other hand, B lymphocytes, which are the most important arm of the humoral immune system, not only by producing antigen-specific antibodies but also by acting as antigen-presenting cells with the great capacity to release many cytokines (4), have been neglected during pregnancy, and in recent years researchers have paid attention to their possible important roles in pregnancy success through the antibody production and regulatory capacities (5) but the exact role of this cells in pregnancy is still a matter of controversy (4).

Emerging observations supporting the presence of regulatory B (Breg) cells in a B cells subpopulation with immune suppressive capacities. Although different phenotypes for Bregs have been described in human, for example, $CD19^+CD24^{hi}CD38^{hi}$ as immature Breg cells, $CD19^+CD24^{hi}CD27^+$ as B10 Breg cells, and also iBregs, their prominent regulatory feature is likely related to the high capacity of IL-10 production (6). The immunosuppressive functions of Breg cells have been shown in several murine and human studies of chronic inflammatory diseases, including collagen-induced arthritis(7), inflammatory bowel disease(8), and systemic lupus erythematosus (9).

The concept that Breg cells immunomodulate inflammatory processes and participate in the maintenance of tolerance through IL-10 production proposes a role for them in controlling inflammatory processes during pregnancy (10). Thus, the aim of this study was to investigate the frequency and function of Bregs during the first trimester of pregnancy between RPL patients and matched control women.

Materials and Methods

Study Population

In general, 32 subjects were enrolled in this study, including 8 RPL patients, 14 healthy pregnant (under 20 weeks of gestational age), and 10 nonpregnant (without any history of abortion) women who were attending Erfan Niyayesh and Taleghani hospitals Tehran –Iran, from July 2017 until April 2018. All individuals were informed about the purpose of our research and gave their written consent before sampling. The study groups were age-matched. All experiment protocols, including samples from human subjects, were reviewed and approved by the Shahid Beheshti Medical of Science University Ethics Committee, Tehran-Iran (IR.SBMU.MSP.REC.1395.536). 10 ml heparinized peripheral blood samples were collected from each individual. The exclusion criteria of this study were diabetes, hypertension, autoimmune and infectious disease, any type of cancer, and smoking during six months prior to sampling. None of the patients received specific medications with specific effects on gene expression or cytokine production. The clinical characteristics of patients and control women are summarized in **Table 1.**

Cell Staining and Flow Cytometry

PBMCs of blood samples were separated by Ficoll-Hypaque density gradient (lymphosep, bios-

era, UK). The isolated PBMCs were subjected to further analysis by flow cytometry. Immediately, freshly isolated cells were prepared to be analyzed using four-color BD flow cytometry (BD FACS Calibur, USA). After two steps of washing with PBS, leukocytes were incubated for 30 min at 4°C with ani-CD19 PercP clone SJ25C1 (BD Biosciences, USA) and anti-CD24 APC clone ML5 (BD Biosciences, USA), and anti-CD38 FITC clone HIT2 (BD Biosciences, USA). Immunoglobulin isotype control was used to detect and remove background staining. About 10,000 cells were analyzed in all cases. The lymphocytes were gated on CD19/SSC, and within this population, CD24 and CD38 double-positive cells were analyzed. The FlowJo software (7.6.1 version) was used to analyze data.

Measurement of serum IL-10

The serum samples of all individuals were stored at -80°C for a maximum of 6 months. According to the manufacturer's instructions, Serum IL-10 cytokine was assayed by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Mabtech, Nacka Strand, Sweden). To ensure accuracy, samples were tested in duplicates. The detection limit of this kit was 0.5 pg/ml.

Statistical analysis

Since data was not normally distributed, non-parametric tests were used for statistical analysis. Bregs percentages and IL-10 concentrations are presented as means of ±standard deviation. GraphPadPrism 6 software was used for graph presentations. Mann-Whitney U and Kruskal-Wallis tests were used for comparisons between two and more than two groups, respectively. The correlation of variables was evaluated by the Spearman test. Also, $P < 0.05$ was defined as statistically significant.

Results

Breg cell subpopulation frequency dramatically decreased in RPL patients

As shown in figure1, the CD19+ cells population **(Figure 1.a,b,)** which were CD24+ CD38+ **(Figure 1.c)**, were considered as Breg cells in non-pregnant, healthy pregnant, and RPL individuals. The comparison of Breg cells between study groups was illustrated in Figure .1.d. Bregs in women who suffered from RPL (1.11±0.59, *P*=0.001) represented a dramatic decline compared to non-pregnant (6.13±6.01, *P*=0.001) and normal pregnant groups (7.09±8.01, *P*=0.0016), respectively. The frequency of Breg cells between nonpregnant and normal pregnant individuals was not statistically significant (*P*=0.3384).

IL-10 serum concentration was not significantly increased in RSA patients

Although there was a slight increase in serum level IL-10 (5.14±5.18) (pg/ml) in women with normal pregnancy, in comparison with non-pregnant (2.25±1.68) (pg/ml) and RPL patients (2.35±1.76) (pg/ml) but these differences were statistically insignificant (*P*=0.092). **(Figure 2)**

Correlation of Breg cells frequency and IL-10 serum concentration with gestational age

Due to spearman analysis, the correlation between selected groups were as following: Breg frequency with IL-10 concentrations ($P>0.05$ rs = 0.139) and IL-10 serum concentration with gestational weeks ($P>0.05$, rs = 0.135) showed very weak correlation.

"On the other hand, Breg percentages with gestational week showed a positive correlation ($P>0.05$, rs = 0.647); however, missed statistical significance."

Discussion

In the current study we showed that percentages of Bregs in RPL patients were significantly lower in comparison to normal pregnant or healthy non-pregnant women. Besides, there were a little decline in IL-10 serum concentration of RPL patients which was not statistically different in comparison with healthy non-pregnant or normal pregnant women.

Based on previous studies, the immunoregulatory role of Bregs in pregnancy was reported (11). Also, the association of Bregs with pregnancy has been described in animal models (12). Jensen et al. demonstrated that the frequency of B10 cells increases during the progression of murine normal pregnancies, while the mice model of spontaneous pregnancy abortion lacks the expected increasing pattern of regulatory B10 cells during pregnancy progress (12). Furthermore, Muzzio et

Table 1. Demographic and clinical characteristics of participated women in this study

Groups	Sample code	Age	Race	Childbearing history	Spontaneous abortion history	Duration after last abortion (days)
RPL(n=8)	1	29	Iranian	0	3	37
	2	30	Iranian	0	4	64
	3	31	Iranian	0	3	53
	4	33	Iranian	0	4	75
	5	28	Iranian	0	3	49
	6	32	Iranian	0	3	50
	7	29	Iranian	0	3	70
	8	32	Iranian	0	4	63
Age (Mean±SD): 30.33 ± 1.72						
Healthy pregnant women (n=14)						Gestational age (week)
	10	30	Iranian	1	0	8
	11	24	Iranian	2	0	6
	12	33	Iranian	1	0	22
	14	29	Iranian	1	0	16
	15	30	Iranian	2	0	22
	16	31	Iranian	3	0	12
	17	32	Iranian	1	0	10
	18	33	Iranian	1	0	9
	19	29	Iranian	2	0	13
	20	22	Iranian	2	0	4
	21	23	Iranian	1	0	7
	22	26	Iranian	1	0	10
	23	31	Iranian	3	0	15
	24	19	Iranian	1	0	13
Age (Mean±SD): 27.93±4.28 **Gestational weeks (Mean±SD): 7.26±1.43**						
Healthy non-pregnant women (n=10)						
	25	29	Iranian	1	0	-
	26	33	Iranian	0	0	-
	27	26	Iranian	1	0	-
	28	32	Iranian	0	0	-
	29	35	Iranian	1	0	-
	30	29	Iranian	0	0	-
	31	25	Iranian	0	0	-
	32	32	Iranian	1	0	-
	33	28	Iranian	1	0	-
	34	27	Iranian	1	0	-
Age (Mean±SD): 29.60±3.80						

al. have shown that marginal zone (MZ) B cells, another phenotype of Breg cells, emerge as a critical component of a healthy pregnancy. They showed that, unlike normal pregnant mice, MZB cells in mice with pregnancy complications did not expand in the spleen (13).

The low percentages of Bregs in RPL patients in the current study have been already described by Rolle et al. (6). Rolle et al. demonstrated that Bregs with CD19+CD24hiCD27+ surface markers were decreased in RPL patients compared to normal pregnant women (6). Indeed, according

to Lima et al. CD24hiCD38hi regulatory B cells are reduced in late pregnancy (between 3rd trimester and delivery) compared to post-partum and non-pregnant women. But, unlike CD24hiCD38hi Bregs, there were no significant differences in CD19+CD24hiCD27+ Bregs frequency between non-pregnant and pregnant women also from 3rd trimester of pregnancy to post-partum, the same pattern of results has been detected (5). Additionally, it is important to exclusively identify Bregs with specific markers. However, there is no unique marker for the characterization of these cells (14). Our findings showed that CD24hiCD38hi Bregs were significantly higher in both normal and healthy non-pregnant women compared with women with a history of RPL. A decrease in CD24hiCD38hi Bregs frequency in RPL patients could be considered as a possible diagnostic/prognostic marker of abortions, an issue that needs further investigations to be successfully approved.

IL-10, as an anti-inflammatory cytokine, is compatible with pregnancy and plays a vital role in maintaining immune tolerance against semi-allograft fetuses (15). There are different types of immune and non-immune cells that can produce IL-10 (15). IL-10 mainly plays its regulatory role by inhibiting pro-inflammatory cytokines such as IL-1a, IL-6, IL-8, IL-12, and TNF-α production. Also, this cytokine down-regulates major histocompatibility complex II (MHC II) expression that results in inhibition of antigen presentation. These reports introduce IL-10 as a critical player in healthy pregnancy outcomes (11, 15). (16-18). Based on Piccinni et al. IL-10 production by decidua T-cells was decreased in RPL-suffered women (19). Reyes-Lagos and coworkers demonstrated that in normal pregnant women, IL-10 sera concentration decreased at delivery compared to 3rd trimester of gestation (20). On the other hand, Bates et al. found that mitogen-stimulated peripheral blood mononuclear cells (PBMCs), isolated from RPL patients, produced more IL-10 than the control group (21). It is worth mentioning that Isazadeh et al. have shown that in a population of 300 Iranian women with RPL and their matched controls, the polymorphism of IL-10 did not affect the healthy progression of pregnancy, whereas IL-18 and IL-33 polymorphisms had possible associations with the risk of RPL incidence. This study highlighted the importance of genetic /ethnic background in the interpretation of studies(22). Mor et al. reported that in RPL patients, IL-10/TNF-α ratio in the first trimester is a more reliable prognostic factor than the evaluation of changes in each cytokine separately(23). In our study, although IL-10 serum concentration in healthy pregnant women was higher than in non-pregnant and RPL women, this difference was statistically insignificant. Based on different and controversial results obtained in the mentioned studies, the exact role of IL-10 in RPL needs to be highlighted by studies in large populations with different genetic backgrounds.

Primarily, Bregs are defined as IL-10-produced cells, but it was shown that they can also play their regulatory function in an IL-10-independent manner (24). Pers et al. revealed that iBregs are activated by CTLA-4, producing TGF-β and indoealamine2, 3-dioxygenase (IDO), which induced natural Tregs and IL-10 and TGF-β secreting CD4+ T cells. They also mentioned that B cells induced regulatory T cells are alloantigen-specific, which can become really helpful for pregnancy maintenance(25). (26). (27). Another possible IL-10 independent pathway of Breg function was mentioned by Dittel et al. They introduce glucocorticoid-induced TNFR ligands (GITRL) on B cells for maintenance of the Tregs population(28). Surprisingly, PD-1 high Bregs (CD5hiCD24−/+CD27hi/+CD38dim)have been identified by Dong-Ming et al. in hepatoma patients, which are IL-10-producing cells and suppress tumor-specific T cells(29), although these cells are IL-10-dependent they are phenotypically different from the cells we had evaluated. These studies, in general, bring up this theory that Bregs are different due to several factors, such as surface markers, tissue distribution, and pattern of action, which until now have not been completely separated (29).

Conclusion

The results of this investigation show that measurement of the frequency of Breg cells under 20 weeks of gestational age, could potentially anticipate the outcome of pregnancy. According to our findings, low frequency of Breg cells during the first trimester of pregnancy may influence fe-

tus tolerance by the immune system and thereby lead to pregnancy failure. It is unfortunate that the study did not include measurement of other major Breg cell subsets simultaneously. However, with a small sample size, caution must be taken into consideration, as the findings might not be extrapolated to the clinic. So, further research in this field would be of great importance in the understanding of the exact mechanisms and role of Breg cells during pregnancy.

Conflict of interests

The authors have no conflict of interest.

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