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The role of IL-10-producing B cells in repeated implantation failure patients with cellular immune abnormalities

Ladan Koushaeian[a,1], Farzaneh Ghorbani[a,b,1], Majid Ahmadi[c], Shadi Eghbal-Fard[d], Majid Zamani[d], Shahla Danaii[e], Bahman Yousefi[f], Farhad Jadidi-Niaragh[d,g], Kobra Hamdi[h], Mehdi Yousefi[c,d,*]

[a] Aging Research Institute, Tabriz University of Medical Sciences, Tabriz, Iran
[b] Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
[c] Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[d] Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[e] Gynecology Department, Eastern Azerbaijan ACECR ART Center, Eastern Azerbaijan Branch of ACECR, Tabriz, Iran
[f] Department of Clinical Biochemistry and Laboratory Medicine, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[g] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[h] Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

There are a few data of the role of B cells in RIF pathogenesis. Accordingly, the objective of the current study was to determine the role of IL-10-producing B cells in RIF. Twenty-three RIF women with cellular immune abnormalities and 25 normal controls were enrolled in this experiment. Isolated naïve B cells from peripheral blood of the subjects were cultured *in vitro*, divided into two parts and activated by CpG ODN and imiquimod as TLR agonists. Afterwards, the number of $CD19^+$ $IL\text{-}10^+$ B cells was evaluated by flow cytometry and their related IL-10 cytokine level was assessed by ELISA. The mRNA expression levels of related genes in just CPG stimulated B cell population were also analyzed using real-time PCR. RIF patients exhibited a decreased level of the cells ($P = 0.014$, $P = 0.023$, respectively) and IL-10 cytokine ($P = 0.009$, $P = 0.045$, respectively) in both CPG and imiquimod stimulated B cell groups. IL-10 serum level was also lower in these patients ($P = 0.0014$). Additionally, we found a negative relationship between the frequency of these cells with the number of failed ET and total IgG titers in RIF patients. The mRNA levels of IL-10-producing B cells related genes (IL-10 and PD-L1) was also significantly lower in RIF women, whereas the expression of plasma cells-associated transcriptional factors (BLIMP1, IRF4, and XBP1) was higher. Summing up the obtained results, we concluded that peripheral blood IL-10-producing B cells down-regulation might result in RIF pathogenesis. It is further suggested that these cells can suppress autoantibody generation and contribute to a successful implantation.

1. Introduction

Implantation is a complex process in which a foreign embryo must be accepted by the mother's endometrium [1]. In spite of the progresses in fertility treatment procedures, repeated implantation failure (RIF) remains a main challenge in human reproduction [2]. RIF is defined as unsuccessful implantation of high-grade embryos after at least three In vitro fertilization (IVF) treatment cycles [3]. It is apparent that the underlying cause of RIF is complex that cannot be associated with a particular abnormality. Uterine anomalies, hormonal disturbances, immunological factors as well as thrombophilia and infection are different maternal factors which cause implantation failure [4].

According to the existing literature, various maternal immune cell subsets play major roles in embryo implantation and participate in all procedures associated with maintenance and completion of a successful pregnancy [5]. In recent years, remarkable progress has been achieved

Abbreviations: RIF, repeated implantation failure; ODN, oligodeoxynucleotide; TLR, toll like receptor; IL-10, interlukin-10; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; ET, embryo transfer; PD-L1, Programmed death-ligand 1; BLIMP1, B lymphocyte-induced maturation protein 1; IRF4, Interferon Regulatory Factor 4; XBP1, x binding protein 1

* Corresponding author at: Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.
E-mail address: yousefime@tbzmed.ac.ir (M. Yousefi).

[1] Co-first authors (Ladan Koushaeian and Farzaneh Ghorbani contributed equally to this work).

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in understanding the role of immunological factors in RIF pathogenesis but the complete process is not understood. An efficient implantation requires maternal immune system to tolerate the semiallogenic embryo [6]. This needs a fine equilibrium between the effector and regulatory immune cells [7]. Several studies have been demonstrated that regulatory T (Treg) cells, which play crucial roles in immune tolerance, are extremely important for achieving a successful implantation [7,8].

Over the past few years, a new subset of B cells with immunosuppressive properties have also been identified which are known as regulatory B cells (Bregs) [9]. Interleukin (IL)-10, however, seems to be the most important immunosuppressive molecule which mediate the regulatory functions of Bregs [10]. Similar to Tregs, IL-10-producing B cells such as Bregs are thought to play critical roles during maintenance of the pregnancy by induction of immunologic tolerance [11]. Recently, some studies reported the down-regulation of a subset of IL-10-producing B cells in patients with recurrent pregnancy loss (RPL) [12]. However, to our knowledge there is no existing information on the involvement of these cells in RIF pathogenesis. Since a number of common immunological changes have been investigated in both RPL and RIF pathogenesis [6], we assumed that IL-10-producing B cells might be reduced in RIF patients. Moreover, it has been acknowledged that different autoantibodies, especially antiphospholipid antibodies (APAs), are associated with increased risk of implantation failure [13]. Since, IL-10-producing Bregs have been suggested as important players in suppressing the autoantibodies production [14], we examined whether IL-10-producing B cells population can reduce the number of failed embryo transfer (ET) by suppressing the generation of these autoantibodies in RIF patients.

Considering these observations, we decided to investigate the possible role of IL-10-producing B cells in RIF patients with cellular immune abnormalities for the first time, through evaluating the ratio of these cells and corresponding Programmed death-ligand 1 (PD-L1) expression level concomitant with associated cytokine (IL-10) in peripheral blood of RIF patients and healthy women. We also enquired if these cells can suppress the production of autoantibodies in women with RIF. To further support this correlation, we measured the mRNA expression of transcriptional factors related to plasma cells including B lymphocyte-induced maturation protein 1 (BLIMP1), Interferon Regulatory Factor 4 (IRF4) and x binding protein 1 (XBP1). The results from this study could bring promising insights to the IL-10-producing B cells population as a new immunotherapeutic strategy for women suffering from RIF.

2. Materials and methods

2.1. Study subjects

Twenty-three RIF patients referred to infertility center of Al-Zahra Hospital of Tabriz, Iran, between July 2018 and December 2018, were included in this experiment. As the control individuals, 25 healthy women who had experienced at least one successful pregnancy were recruited. All of the participants signed a written informed consent prior to entering the study. The study patients were selected if they had a history of three or more failed implantations with age between 18 to 41. All of the patients had also elevated levels of NK cells frequency and cytotoxicity and/or Th1:Th2 ratio as described in our previous study [15] and those with infection, anatomic, genetic or endocrine etiologies for implantation failure were excluded from the study. The partners were also checked for smoking and semen quality tests according to World Health Organization (WHO, 2012) guidelines. Table 1 lists the clinical characteristics of RIF patients and healthy control group. The study was approved by the Research Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1397.138).

2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

About 12 mL of whole blood samples were taken from both RIF women and normal control individuals using venipuncture. A volume of 8 ml of fresh samples was collected in tubes with anticoagulant for the PBMCs isolation by a Ficoll-Hypaque density gradient (lymphosep, Biosera, UK) separation method. The protocol was completely explained in our previous study [16]. The serum of remaining blood samples was separated and stored at −70 °C for subsequent cytokine analysis and assessment of autoantibodies and total IgG levels.

2.3. Isolation and activation of peripheral blood B cells

Magnetic-activated cell sorting (MACS) Naïve B Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), was used for negative selection of naïve B cells from the blood-derived PBMCs according to the instructions of the manufacturer and purity of higher than 95% was determined by flow cytometry. A total of 2×10^5 isolated B cells were re-suspended in total volume of 200 µL of medium and incubated in U-bottom 96-well plates at 37 °C with 5% CO2. The cultured cells were then separated into two groups: the first group was activated by CpG-oligodeoxynucleotide (ODN) (2.5 µg/ml; invivoGen), which is the agonist of TLR9, and imiquimod (invivoGen), TLR7 agonist, was used as stimulant in the other group at the concentration of 1 µg/ml. After stimulation of the cluttered cells for 48 h, they were used for subsequent analysis.

2.4. Flow cytometry

Enumeration of IL-10^+ $CD19^+$ B cells was evaluated among stimulated and non-stimulated total naïve B cell populations using flow cytometry analysis. Briefly, cells were washed in fluorescence-activated cell sorting (FACS) buffer and then incubated with FITC-labeled anti-human CD19 monoclonal antibodies (BD Biosciences, San Jose, CA, USA). Subsequently, the cells were washed twice with permeabilization or fixation solution (eBioscience) and incubated with PE-labeled antibody against intracellular IL-10 (BD Biosciences, San Jose, CA, USA). The cells were counted using a FACS Calibur (BD Biosciences) flow cytometer and analyzed with FlowJo software (Tree Star, Ashland, OR).

2.5. Determination of the cytokine level

The concentration of IL-10 was measured in serum and supernatant of activated and non-activated B cell populations from RIF patients and compared to normal women using ELISA kits (Mybiosource, San Diego, USA), according to the manufacturer's instructions. All samples were analyzed in duplicate manner to enhance the accuracy.

2.6. RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was isolated from the homogenized CPG stimulated naïve B cells subpopulation using Total RNA Purification Mini kit (YTA, Tehran, Iran). Concentration and quality of RNA content was assessed using NanoDrop spectrophotometer (Agilent Technologies, USA) and cDNA synthesis was performed using Revert Aid Reverse Transcriptase kit (Thermo Fisher, Waltham, MA, USA).

2.7. Real-Time quantitative polymerase chain reaction (RT-qPCR)

To determine the gene expression levels of IL-10, PD-L1, BLIMP1, XBP1 and IRF4 in RIF and healthy women, RT-qPCR was performed on a Light Cycler 2.0 Real-Time PCR System machine (Roche Applied Science, Germany) using specific primers and SYBR Green Master Mix (Roche, Germany). β-actin gene was used as the reference gene. Melting curves were analyzed to ensure that a single product was amplified for each primer set. PCR products were further confirmed using

Table 1
Demographic data of patients.

Variable	RIF patient	Control group	p-value
No. of patient	23	25	NS
Maternal age (years, range)	33.9 ± 5.7(18-41)	34.6 ± 4.9(21-40)	NS
Body mass index (kg/m2)	28.5 ± 3.6	27.9 ± 4.6	NS
No. of smoking patient	0	1	NS
No. of smoking partners	5	7	NS
No. of previous pregnancy history (percent)	7 (30.4%)	25 (100%)	< 0.0001
No. of previous live birth (percent)	4(17.39%)	25 (100%)	< 0.0001
No. of primary infertility (percent)	16(69.56%)	0 (0%)	< 0.0001
No. of secondary infertility (percent)	7 (30.44%)	0 (0%)	< 0.0001
No. of previous ET attempts (range)	4.75 ± 2.25 (3–11)	0	< 0.0001
Total No. of previous transferred embryos	7.5 ± 4.75	0	< 0.0001
No. of MGEs transfers in this study (range)	1.75 ± 0.5 (1–2)	0	< 0.0001
NK-cell levels (mean ± SD)	17.83 ± 4.52	9.1 ± 3.10	0.0001
Abnormal NK-cell levels (N, %)	18, (78%)	0	< 0.0001
NK-cell cytotoxicity (mean ± SD)	19.3 ± 3.9	10.8 ± 4.8	0.0001
Abnormal NK-cell cytotoxicity (N, %)	11, (47%)	0	< 0.0001
Th1/Th2 cell ratio (mean ± SD)	16.63 ± 5.93	7.91 ± 2.98	0.0001
Abnormal Th1/Th2 cell ratio (N, %)	13(56%)	0	< 0.0001
Anti TPO (N, %)	6(26.08%)	–	–
Anti TG (N, %)	5(21.73%)	–	–
ANA (N, %)	2(8.6%)	–	
ACA (N, %)	1(4.34%)	–	–
APA (N, %)	1(4.34%)	–	–
Total Autoantibody	10(43.47%)	0(0%)	< 0.0001
Total antibody, IgG (mg/dl)	1511.5 ± 329	1128 ± 402	< 0.0001

NS: not significant, ET: embryo transfer, MGEs: morphologically good-quality embryos, NK-cell: natural killer cell, Th: T helper, Anti-TPO; antithyroid Peroxidase, Anti-TG; antithyroglobulin, ANA; antinuclear antibody, ACA; anticardiolipin antibody, APA; antiphospholipid antibody.

Table 2
Primer sequences for real-time PCR.

Gene	Primer	Sequence (5′ → 3′)
β-actin	Forward	TCCCTGGAGAAGAGCTACG
	Reverse	GTAGTTTCGTGGATGCCACA
IL-10	Forward	CAT CGA TTT CTT CCC TGT GAA
	Reverse	TCT TGG AGC TTA TTA AAG GCA TTC
PD-L1	Forward	GGTGAGGATGGTTCTACACAG
	Reverse	GAGAACTGCATGAGGTTGC
BLIMP1	Forward	CGAAATGCCCTTCTACCCTG
	Reverse	GCGTTCAAGTAAGCGTAGGAGT
IRF4	Forward	CGTTCATTGCTCTCCAGTCAC
	Reverse	GCCTTCACGCACCATTCAG
XBP1	Forward	TTACGAGAGAAAACTCATGGCC
	Reverse	GGGTCCAAGTTGTCCAGAATGC

IL-10: Interleukin-10, PD-L1: Programmed death-ligand 1, BLIMP1: B lymphocyte-induced maturation protein 1, IRF4: Interferon Regulatory Factor 4, XBP1: x binding protein 1.

electrophoresis on 2% agarose gel and the DNA sequencing (SEQLAB, Germany). Sequences of the primers are summarized in Table 2.

2.8. Measurement of the autoantibodies level

The serum levels of several autoantibodies including antithyroid Peroxidase (TPO), antithyroglobulin (TG), antinuclear antibody (ANA), anticardiolipin antibody (ACA) and antiphospholipid antibody (APA) were determined using ELISA method (Mybiosource, San Diego, USA), according to the manufacturer's instructions. Furthermore, total serum IgG levels was assessed in both groups by nephelometric technique (MININEPH, The Binding Site, San Diego, California, USA) according to the manufacturer recommendation.

2.9. Statistical analysis

GraphPad Prism software for personal computers (GraphPad software Inc, version 7.01, San Diego, CA, USA, www.graph pad.com) was used for statistical evaluations and drawing the graphs. Scale variables were assessed for normal distribution using the Kolmogorov–Smirnov test. Independent *t*-test for normally distributed data was employed in order to compare the quantitative variables between the two studied groups. Pearson's correlation coefficient (r) was used for measuring the association between the variables. The data were shown as mean ± standard deviation and *P* value under 5% were reported to be significant.

3. Results

3.1. Frequency of IL-10-producing B cells subpopulation in RIF patients

To evaluate the role of IL-10-producing B cells in the pathophysiology of RIF, the frequency of $CD19^+$ $IL\text{-}10^+$ B cells was assessed among non-activated and activated cultured naïve B cells population from RIF patients and healthy controls using flow cytometry. As shown in Fig. 1, no significant differences were observed in the frequency of non-stimulated $CD19^+$ $IL\text{-}10^+$ B cells population between RIF and normal groups (0.7139 ± 0.4884 *vs.* 0.8744 ± 0.4084, $P = 0.22$). Furthermore, our findings indicated that the frequency rate of these cells were significantly lower in both CPG and imiquimod stimulated B cell population from the patients group than that of controls (4.733 ± 1.524 *vs.* 5.918 ± 1.690, $P = 0.014$ and 4.007 ± 1.652 *vs.* 5.174 ± 1.781, $P = 0.023$, respectively; Fig. 1).

3.2. Concentration of IL-10 in RIF patients

The IL-10 serum levels were measured in both studied groups using ELISA. Comparison of the serum level of this cytokine between the patients and control individuals indicated a significant decrease in women with RIF (24.43 ± 10.31 vs. 38.43 ± 14.91, $P = 0.0014$; Fig. 2).

In a complementary set of study, the concentration of this cytokine was evaluated in the supernatant of non-stimulated and stimulated cultured naïve B cells using ELISA. According to the Fig. 2, no statistically significant difference was found between the levels of IL-10 in supernatant of non-stimulated B cells in these two groups

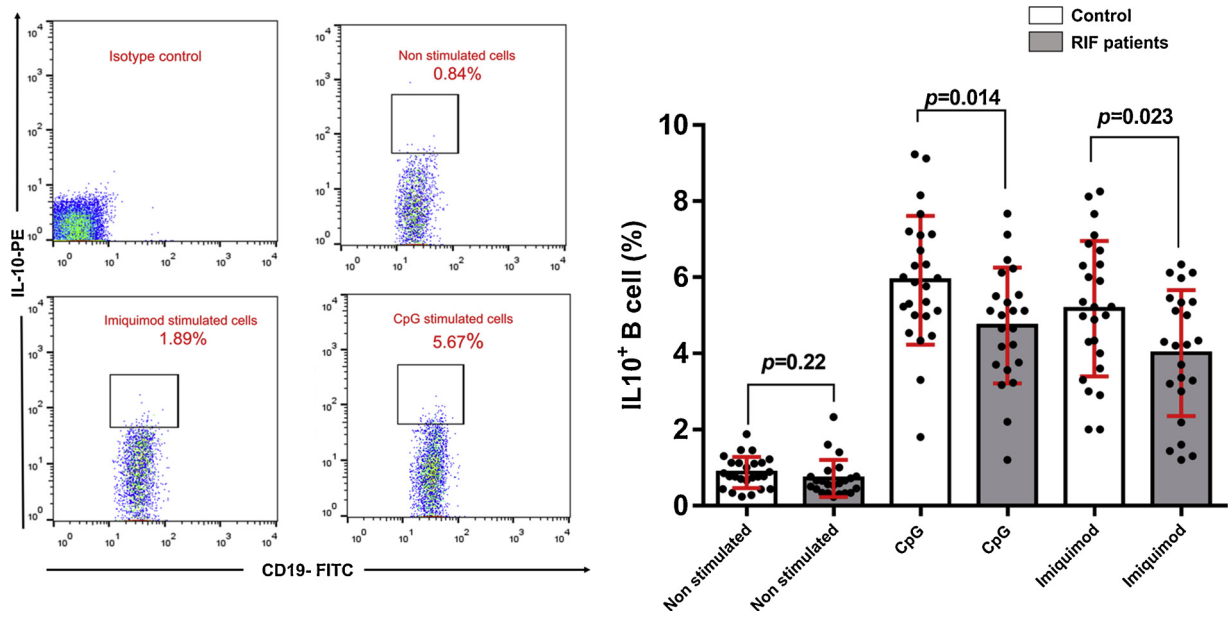

Fig. 1. The frequency of IL-10-producing B cells among non-stimulated and stimulated naïve B cells population from RIF patients and healthy control group. Representative dot plots illustrate the analyzing method used for enumeration of IL-10-producing B cells. No significant difference in percentage of non-stimulated $CD19^+$ $IL\text{-}10^+$ B cells was detected between RIF and healthy women ($P = 0.22$). however, the frequency of $CD19^+$ $IL\text{-}10^+$ B cells was lower among total naïve B cell population from RIF patients stimulated with CPG ($P = 0.014$) and imiquimod ($P = 0.0023$) as compared to the control group. Results are given as mean ± SD (RIF women, n = 23, Healthy control group, n = 25). $P < 0.05$ was considered as statistically significant.

(23.4 ± 17.95 vs. 24.13 ± 15.22, $P = 0.9$). It was also revealed that, IL-10 levels have declined in supernatant of B cells population after stimulation with CPG and imiquimod in RIF patients compared to healthy control group (154.7 ± 118.4 vs. 277.8 ± 177.9, $P = 0.009$ and 87.40 ± 66.44 vs. 144.3 ± 100.6, $P = 0.045$, respectively; Fig. 2).

3.3. IL-10-producing B cells/plasma cells-associated cytokine and genes expression pattern in enriched B cell population from RIF patients

Following CPG stimulation of purified B cells which is the most potent B cell stimulator, the mRNA expression levels of IL-10 ($P = 0.0002$) and PD-L1 ($P < 0.0001$) were significantly reduced in RIF patients as compared with the healthy control group. In contrast, expression levels of plasma cells-related transcriptional factors including BLIMP1 ($P = 0.0012$), XBP1 ($P = 0.0016$), and IRF4 ($P < 0.0001$) were noticeably elevated in these patients (Fig. 3).

3.4. Autoantibodies in RIF patients

As shown in Table 1, RIF patients were analyzed for the presence of several autoantibodies including Anti-TPO, Anti-TG, ANA, ACA and APA which are associated with the pathogenesis of RIF. Totally, ten patients were positive for these autoantibodies ($p < 0.0001$). Moreover, the results showed that the level of total serum IgG is significantly increased in the patients group as compared with the healthy controls (1511.5 ± 329 vs. 1128 ± 402.8, $p < 0.0001$).

3.5. Association between the rate of IL-10-producing B cells, number of failed ET, and total IgG concentrations in women with RIF

For further analysis of IL-10-producing B cells role in the RIF patients, a negative correlation was presented between their frequency rate with the number of failed ET in women with RIF ($r = -0.5266$, $P = 0.0098$; Fig. 4A). It was also found that the percentage of IL-10-producing B cells is inversely associated with the IgG levels in serum of

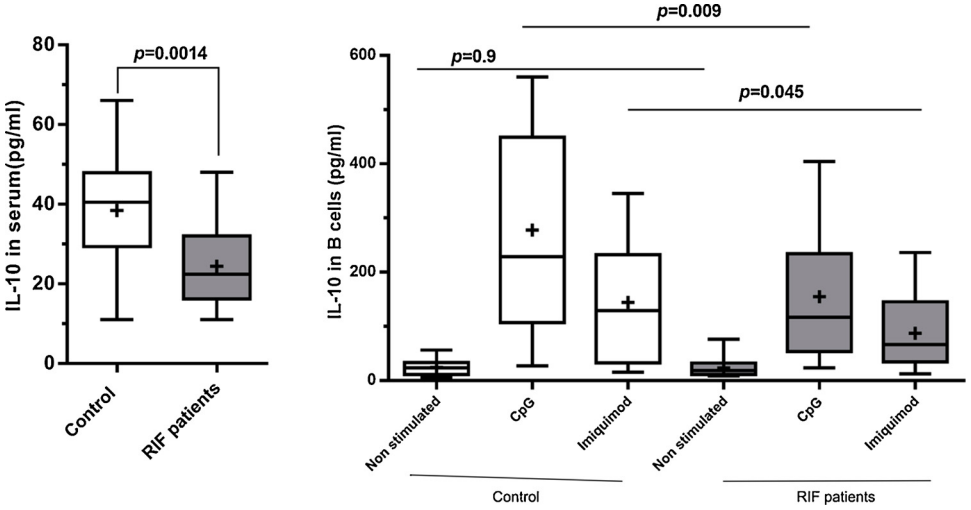

Fig. 2. The level of IL-10 in the serum and supernatant of non-stimulated and stimulated B cells from RIF patients and healthy control group. Reduced levels of IL-10 serum level was observed in RIF patients in comparison with the control group ($P = 0.0014$). Compared to RIF women, the levels of IL-10 in supernatant of non-stimulated B cells remained unchanged in control group ($P = 0.9$). However, the secretion level of IL-10 was evidently lower in the supernatant of stimulated B cells with CPG (2.5 μg/ml) and imiquimod (1 μg/ml) ($P = 0.009$, $P = 0.045$, respectively). Results are given as mean ± SD (RIF women, n = 23, Healthy control group, n = 25). $P < 0.05$ was considered as statistically significant.

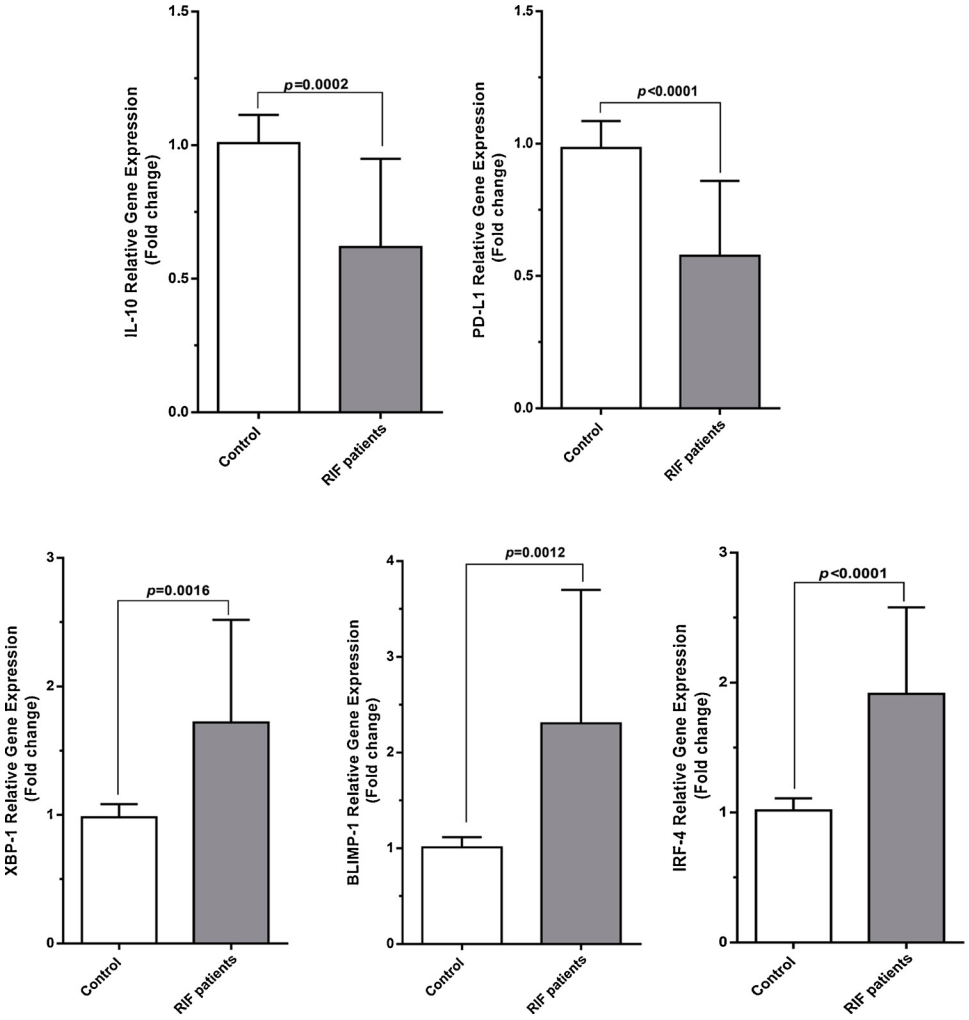

Fig. 3. mRNA expression of IL-10-producing B cells/plasma cells-associated factors in CPG stimulated naïve B cell population from RIF patients and healthy control group. Real Time-PCR method was used for IL-10, PD-L1, BLIMP1, XBP1 and IRF4 relative mRNA expression measurement in RIF patients and normal females. IL-10 and PD-L1 mRNA expression were evidently lower in RIF women ($P = 0.0002$ and $P < 0.0001$, respectively), whereas the expression levels of BLIMP1, XBP1 and IRF4 were significantly augmented after CPG stimulation of purified B cells from RIF patients when compared to the controls ($P = 0.0012$, $p = 0.0016$, $P < 0.0001$, respectively). The results were normalized to β-actin. Results are given as mean ± SD (RIF women, n = 23, Healthy control group, n = 25). $P < 0.05$ was considered as statistically significant.

RIF patients ($r = -0.5155$, $P = 0.011$) (Fig. 4B). Additionally, no statically significant relationship was found between the number of failed ET and serum levels of IgG in the patients ($r = 0.356$, $P = 0.086$) (Fig. 4C).

4. Discussion

Human reproduction inefficiency is highlighted when the probability of an successful embryo implantation is only about 30% [17]. Among the various causes which have been implicated in RIF pathogenesis, the role of immunologic factors is remarkable. The mother's immune system is comprised of various immune cell populations which serve different roles in both embryo implantation success and failure. The T helper 1 (Th1) dominance in Th1/Th2 paradigm, decreased number of Treg cells, and especially natural killer (NK) cells augmented quantity and activity are among the well-studied causes which reduce the likelihood of a successful implantation [13,18]. Nonetheless, the role of B cells, as one of the major cellular components of adaptive immune system, has been less studied in RIF patients. B cells indirectly play role in pregnancy by their ability to generate antibody [12]. It has been reported that protective antibodies help to pregnancy establishment, whereas autoantibodies are account for obstetric complications [19].

Beyond the principle function of B cells in antibody generation, IL-10-producing B lymphocytes population such as Breg cells, are identified which can negatively regulate immune responses in autoimmune diseases, cancer, transplantation, and infection [11]. Recently some studies have acknowledged that the level of these IL-10-producing B cells was reduced in recurrent miscarriage in comparison with normal pregnant controls, indicating the important role of these cells in RPL development [12,20]. However, the role of these cells in RIF pathogenesis has not been studied yet. In this experiment we demonstrated for the first time that the percentage of IL-10^+ $CD19^+$ B cells, which were stimulated with either CPG or imiquimod, was lower in peripheral blood of RIF women with cellular immune abnormalities than the healthy control group. However, no significant changes were detected in the frequency of non-stimulated B cells between these two groups. Additionally, we illustrated a negative association between the percentage of IL-10-producing B cells and number of failed ET in these patients. Thus, our results support the concept that down-regulation of IL-10-producing B cells can contribute to embryo implantation failure.

Furthermore, it has been demonstrated that the negative regulatory function of these cells is mainly mediated by the secretion of IL-10 cytokine; therefore, we decided to investigate the mRNA levels of IL-10-producing B cells-derived IL-10 in RIF patients. As its described, IL-10 is a key cytokine with the capacity to prevent inflammatory responses which is generated by a wide range of immune cells [21]. Breg cells can lead to a successful pregnancy by secreting this cytokine which inhibit the differentiation of Th1 and Th17 cells and activate the Treg cell population [22]. It has been demonstrated that the reduction of IL-10 cytokine occurs in RIF patients in comparison with normal females, showing the principle role of this cytokine in a successful implantation [8]. However, in another study, no significant differences were found between the levels of IL-10 in RIF and normal women [23]. The results from our study also demonstrated that mRNA expression level of IL-10 cytokine is lower in B cell population from RIF patients with cellular

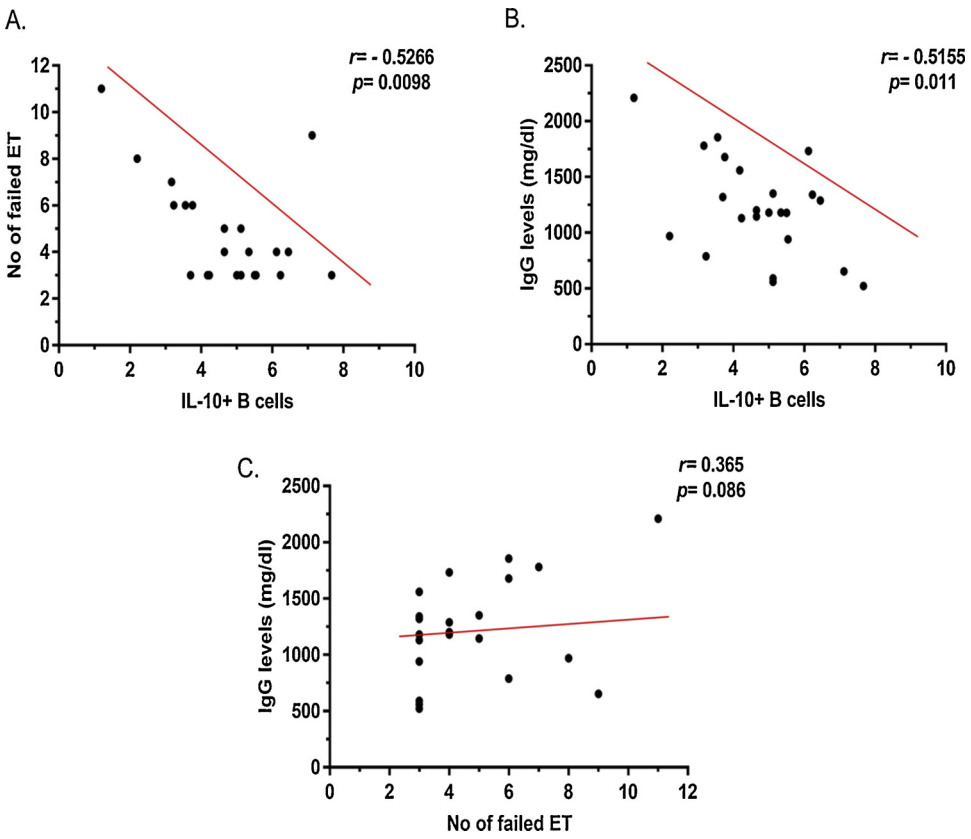

Fig. 4. Relationship between the number of IL-10-producing B cells with the number of failed ET and total IgG concentration in RIF patients. (A) An inverse correlation was found between the IL-10-producing B cells population and number of failed ET ($r = -0.5266$, $P = 0.0098$). (B) and the levels of IgG (mg/dl) in serum of RIF patients ($r = -0.5155$, $P = 0.011$). (C) no significant relationship was also found between the number of failed ET and serum IgG levels ($r = 0.356$, $P = 0.086$). Relationship variables were determined by calculation of Pearson's correlation coefficient (r). $P < 0.05$ was considered as statistically significant.

immune abnormalities than normal individuals. These findings were in line with the results obtained from the analysis of the cytokine level in serum and supernatant of activated B cell population from peripheral blood of these patients. Thus, not only the number of IL-10-producing B cells but also the down-regulation of their-derived IL-10 may play role in RIF pathogenesis.

In addition to the important role of IL-10 in negative regulation of IL-10-producing B cells function, surface molecules such as PD-L1 can also suppress inflammation upon direct interaction with pro-inflammatory cells such as Th1 and Th17 [24,25]. This inhibitory costimulatory molecule down-modulate T cell activity and promotes the Tregs induction [9]. Therefore, we measured the mRNA expression level of this molecule among CPG stimulated cultured B cell population which was lower in RIF patients. Analyzing the results, it can be concluded that IL-10-producing B cells population can regulate immune response in embryo implantation through its PD-L1 molecule.

The other finding highlighted in our experiment was the existence of Anti-TPO, Anti-TG, ANA, ACA and APA in peripheral blood of some RIF patients. Additionally, the level of total serum IgG was elevated in the patients group that could be associated with the production of serum autoantibodies in these women. We illustrated a negative relationship between the frequency of IL-10-producing B cells and the level of serum IgG. Therefore, our data suggested that IL-10-producing B cells can lead to a successful implantation through controlling the production of these autoantibodies from plasma cells. To further support this negative association, we assessed the mRNA expression of three main transcriptional factors related to plasma cells development including *IRF4, BLIMP1* and *XBP1* among CPG stimulated B cells. As expected, the expression level of these triad factors was higher in our patients as compared with the healthy controls. Based on these achievements and also down-regulation of IL-10 and PD-L1 genes, we further confirmed the mentioned negative correlation. We also demonstrated that the number of failed ET is not associated with the level of autoantibodies in patient's serum.

5. Conclusion

Taken together, we concluded that IL-10-producing B cells may increase the probability of a successful embryo implantation through increase in frequency, production of IL-10 cytokine and also up-regulation of PD-L1 molecule. Additionally, these cells may have role in suppressing the autoantibodies production which may cause implantation failure. Ultimately, results achieved in this experiment can be useful in the prediction and treatment of human implantation failure.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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References

[1] M.S. van Mourik, N.S. Macklon, C.J. Heijnen, Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment, J. Leukoc. Biol. 85 (1) (2009) 4–19.
[2] A. Maleki-Hagiagha, M. Razavi, M. Rezaeinejad, S. Rouholamin, A. Almasi-Hashiani, R. Pirjani, et al., Intrauterine administration of autologous peripheral blood mononuclear cells in patients with recurrent implantation failure: a systematic review and meta-analysis, J. Reprod. Immunol. 131 (2019) 50–56.
[3] C. Huang, H. Sun, Z. Wang, Y. Liu, X. Cheng, J. Liu, et al., Increased Krüppel-like factor 12 impairs embryo attachment via downregulation of leukemia inhibitory factor in women with recurrent implantation failure, Cell Death Discov. 4 (2018) 23.
[4] T. Timeva, A. Shterev, S. Kyurkchiev, Recurrent implantation failure: the role of the endometrium, J. Reprod. Infertil. 15 (4) (2014) 173–183.
[5] S.S. Morelli, M. Mandal, L.T. Goldsmith, B.N. Kashani, N.M. Ponzio, The maternal immune system during pregnancy and its influence on fetal development, Res. Rep.

Biol. (2015) 171–189.
[6] S. Liu, H. Wei, Y. Li, C. Huang, R. Lian, J. Xu, et al., Downregulation of ILT4(+) dendritic cells in recurrent miscarriage and recurrent implantation failure, Am. J. Reprod. Immunol. 80 (4) (2018) e12998.
[7] M. Ahmadi, S. Abdolmohammadi-Vahid, M. Ghaebi, L. Aghebati-Maleki, S. Dolati, L. Farzadi, et al., Regulatory T cells improve pregnancy rate in RIF patients after additional IVIG treatment, Syst. Biol. Reprod. Med. 63 (6) (2017) 350–359.
[8] M. Ghaebi, S. Abdolmohammadi-Vahid, M. Ahmadi, S. Eghbal-Fard, S. Dolati, M. Nouri, et al., T cell subsets in peripheral blood of women with recurrent implantation failure, J. Reprod. Immunol. 131 (2018) 21–29.
[9] C.M. Wortel, S. Heidt, Regulatory B cells: phenotype, function and role in transplantation, Transpl. Immunol. 41 (2017) 1–9.
[10] F. Fettke, A. Schumacher, S.D. Costa, A.C. Zenclussen, B cells: the old new players in reproductive immunology, Front. Immunol. 5 (2014) 285.
[11] F. Fettke, A. Schumacher, A. Canellada, N. Toledo, I. Bekeredjian-Ding, A. Bondt, et al., Maternal and fetal mechanisms of B Cell regulation during pregnancy: human chorionic gonadotropin stimulates B cells to produce IL-10 while alpha-fetoprotein drives them into apoptosis, Front. Immunol. 7 (2016) 495.
[12] L. Rolle, M. Memarzadeh Tehran, A. Morell-Garcia, Y. Raeva, A. Schumacher, R. Hartig, et al., Cutting edge: IL-10-producing regulatory B cells in early human pregnancy, Am. J. Reprod. Immunol. (New York, NY: 1989) 70 (6) (2013) 448–453.
[13] J.M. Franasiak, R.T. Scott, Contribution of immunology to implantation failure of euploid embryos, Fertil. Steril. 107 (6) (2017) 1279–1283.
[14] K. Fujio, T. Okamura, S. Sumitomo, K. Yamamoto, Regulatory cell subsets in the control of autoantibody production related to systemic autoimmunity, Ann. Rheum. Dis. 72 (Suppl. 2) (2013) ii85-9.
[15] S. Abdolmohammadi Vahid, M. Ghaebi, M. Ahmadi, M. Nouri, S. Danaei, L. Aghebati-Maleki, et al., Altered T-cell subpopulations in recurrent pregnancy loss patients with cellular immune abnormalities, J. Cell. Physiol. 234 (4) (2019) 4924–4933.
[16] M. Ahmadi, S. Abdolmohammadi-Vahid, M. Ghaebi, L. Aghebati-Maleki, A. Afkham, S. Danaii, et al., Effect of Intravenous immunoglobulin on Th1 and Th2 lymphocytes and improvement of pregnancy outcome in recurrent pregnancy loss (RPL), Biomed. Pharmacother. 92 (2017) 1095–1102.
[17] C. Coughlan, W. Ledger, Q. Wang, F. Liu, A. Demirol, T. Gurgan, et al., Recurrent implantation failure: definition and management, Reprod. Biomed. Online 28 (1) (2014) 14–38.
[18] S. Saito, A. Nakashima, T. Shima, M. Ito, Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy, Am. J. Reprod. Immunol. (New York, NY: 1989) 63 (6) (2010) 601–610.
[19] D. Muzzio, A.C. Zenclussen, F. Jensen, The role of B cells in pregnancy: the good and the bad, Am. J. Reprod. Immunol. (New York, NY: 1989) 69 (4) (2013) 408–412.
[20] F. Jensen, D. Muzzio, R. Soldati, S. Fest, A.C. Zenclussen, Regulatory B10 cells restore pregnancy tolerance in a mouse model, Biol. Reprod. 89 (4) (2013) 90.
[21] J.E. Thaxton, S. Sharma, Interleukin-10: a multi-faceted agent of pregnancy, Am. J. Reprod. Immunol. (New York, NY: 1989) 63 (6) (2010) 482–491.
[22] A. Deya-Martinez, A. Esteve-Sole, Y. Luo, A. Vlagea, J. Yague, A.M. Plaza-Martin, et al., B regulatory cells: players in pregnancy and early life, Int. J. Mol. Sci. 19 (7) (2018).
[23] P.Y. Liang, L.H. Diao, C.Y. Huang, R.C. Lian, X. Chen, G.G. Li, et al., The pro-inflammatory and anti-inflammatory cytokine profile in peripheral blood of women with recurrent implantation failure, Reprod. Biomed. Online 31 (6) (2015) 823–826.
[24] M. Nouri, M. Ghaebi, A. Ghasemzadeh, L. Farzadi, F. Jadidi-Niaragh, M. Ahmadi, et al., Immune regulatory network in successful pregnancy and reproductive failures, Biomed. Pharmacother. 88 (2017) 61–73.
[25] R.M. Guzman-Genuino, K.R. Diener, Regulatory B cells in pregnancy: lessons from autoimmunity, graft tolerance, and cancer, Front. Immunol. 8 (2017) 172.