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T cell Subsets in Peripheral Blood of Women with Recurrent Implantation Failure

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ABSTRACT

The objective of this study was to determine whether there are any differences in the T cell composition and the expression of specific factors (i.e., IRF4, TBX21, GATA3, and GITR) of T cells between women with Repeated Implantation Failure (RIF) and fertile women. We observed a decrease in circulating Tregs and exhausted CD8 + T cells in RIF patients when compared to the controls whereas exhausted Treg and Th17 cells were more frequent. Using real-time PCR, we determined that the expression of IRF-4 and TBX21 was significantly elevated in the cases. In contrast, mRNAs encoding GATA3 and GITR were reduced. Furthermore, the expression of some miRNAs involved in T cell differentiation and their target gene candidates were examined in T cells from women with RIF and fertile control women. The patients showed significant up-regulation of miR-25, miR-93, and miR-326. miR-155 and miR-146a demonstrated significant down-regulation in RIF patients. The results revealed that the expression pattern of target genes was in line with data for miRNAs expression from purified Treg and Th17 cells. The findings of real-time PCR analysis provided insights into the genetic pathways underlying this aberration in the proportions of T cell subsets. Our data suggest that a combination of higher pro-inflammatory Th17 and exhausted Treg cells, and lower Treg and exhausted CD8 + T cells may co-exist in the peripheral blood of women with RIF. Moreover, the expression level of transcription factors and miRNAs controlling T cell differentiation may differ in women with RIF influencing pregnancy outcomes in these women.

1. Introduction

Human embryo implantation is an early stage of pregnancy at which the blastocyst adheres to the endometrial epithelium to provide a nourishing blood supply for embryo growth (Koot et al., 2012). Failure of implantation during the first step in human reproduction can interfere with development, leading to embryo loss (Cross et al., 1994). Implantation as a rate limiting factor has gained much attention in assisted reproduction. Assisted Reproductive Technology (ART) is utilized to treat fertility problems. ART involves various techniques to help the couples who have trouble in conceiving. Among these techniques, the most effective is *In Vitro* Fertilization (IVF) procedure (Malina and Pooley, 2017). Repeated Implantation Failure (RIF) is defined as failure to achieve pregnancy following at least 3 embryo transfers of high quality embryos in IVF cycles (Margalioth et al., 2006).

Various immune cell types, particularly special subsets of T cells, have different functions in successful pregnancy and reproductive failures. The regulatory T (Treg) cells with suppressive properties are

Abbreviations: NK cell, natural killer cell; IRF4, Interferon Regulatory Factor 4; TBX21, T-box transcription factor TBX21; GATA3, GATA-binding protein 3; GITR, Glucocorticoid-induced TNF receptor family-related protein; IVIG, intravenous immunoglubolin

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Table 1
Demographic data of study populations.

Variable	RIF patients	Fertile women	*P* value
No. of subjects	40	50	-
Maternal age (years, range)	33.42 ± 3.1 (24-44)	32.64 ± 2.8 (23-42)	NS*
Body mass index (kg/m2)	24.8 ± 1.4	25.7 ± 2.2	NS
Duration of infertility	9.52 (1.5-20)	-	-
(years, range)	0	0	NS
No. of smoking patient	14	18	NS
No. of smoking partners			NS
No. of previous ET attempts	5.23 ± 2.2	-	-
abnormal karyotype	0	-	-
Male infertility	0	-	-
%NK cells	16.11 ± 2.46%	9.21 ± 3.18	0.0001
T17/Treg cell ratio	1.37 ± 0.43	0.77 ± 0.14	0.0035
exhausted Treg/Treg ratio	2.17 ± 0.87	1.24 ± 0.75	0.0009
%exhausted T cells	6.637 ± 3.23	8.297 ± 3.752	0.029

* NS = not significant.

crucial in embryo implantation and development. Treg cells are actively engaged in maternal immune adaptation by suppressing the generation and function of Th1 lymphocytes, the major mediator of type I inflammatory responses. Down-regulation of the Th1-type immunity is an essential requirement for clinically normal pregnancies as the cellular immunity induced by these effectors and/or their cytokines have been shown to be deleterious for conceptus growth. In contrast, the Th2-mediated immune response tends to be predominated during healthy pregnancy. In comparison to normal pregnancy, recurrent miscarriage and RIF in humans are associated with significant and higher levels of Th1 cytokines, such as interferon (IFN)-γ and a low serum level of Th2 cytokines like IL-6 and IL-10 (Raghupathy et al., 1999, Ng et al., 2002). Th17 cells are an inflammatory subset of T cells which produce IL-17 but not IFN-γ or IL-4. Th17-type cytokines play critical roles in the induction of chronic inflammation (Peck and Mellins, 2010). Their contribution in human reproduction is reported in women with Recurrent Pregnancy Loss (RPL), whose peripheral blood show increased level of IL-17$^+$ T cells when compared to the fertile controls (Lee et al., 2011a).

The state of immune response or tolerance is characterized by distinct subsets of Treg cells. Dysfunctional or exhausted Treg cells, as a unique subset, have been previously described to be developed in chronic disease and infectious conditions (Shen et al., 2011). Exhausted Treg cells up-regulate the expression of PD-1. The expression of PD-1 (Programmed cell death protein 1; CD279) on T cells is linked to T cell exhaustion, which is characterized by reduced proliferation and limited immunosuppressive capacity (Barber et al., 2006). A growing body of evidence indicates that exhausted Treg cells may play role in adverse pregnancy outcomes. Besides, CD8 + T cells as a distinct lineage different from helper T CD4+ cells are shown to be important in reproductive biology, while their role is still less understood. Human studies have reported higher peripheral blood CD8$^+$ T cells in women with recurrent miscarriage (Ghafourian et al., 2014). However, their suppressive or cytotoxic properties remained to be explored.

MiRNAs, a class of short non-coding RNA molecules, are the master regulators of gene expression. They are known to have critical functions in a variety of biological processes (Hwang and Mendell, 2006) as well as in human disorders (Skaftnesmo et al., 2007). Exploring for miRNAs that are aberrantly expressed in the endometrium of cases with RIF can contribute in better clarifying of disease pathogenesis (Revel et al., 2011).

Despite a number of proposed mechanisms that accounts for immunopathogenesis of implantation failure, the complete mechanisms are still unknown. Several strategies have been proposed to predict pregnancy success in ART. Immunodiagnostic evaluation in women with RIF can be a promising method. Recently, with the demonstration of some supposed casual factors of implantation failure, immunologic studies have become an important type of observational studies in the field of obstetrics and gynecology. Having this in mind, we turned our attention in studying new diagnostic methods of implantation failure in the peripheral blood of cases with RIF. In this study, we aimed to compare the prevalence of different subsets of T cells including Treg, Th17, exhausted CD8+ and exhausted Treg cells in peripheral blood of non-pregnant women with a history of multiple implantation failure after IVF with that of normal fertile controls. We have further assessed the expression level of functional markers and the transcription factors crucial for the immune cell types as well as specific miRNAs involved in T-cell differentiation.

2. Materials and methods

2.1. Study population

In this study, 90 subjects were recruited from Al-zahra state Hospital of Tabriz University of Medical Sciences from May; 2017 to September; 2017. The study was approved by the Research Ethics Committee of Tabriz University of Medical Sciences (No. IR.TBZMED.Rec.1396.103). A total of 40 women with a history of three or more IVF-embryo transfer failures after transfer of at least 6 high quality embryos and 50 healthy female volunteers aged 23-42 years with a history of at least one successful pregnancy were included in the experiment. Women with RIF were investigated for anatomic abnormalities, endocrine disorders, infectious, and genetic etiologies prior to their enrollment in the study and nothing was found. The patients with a history of RIF were chosen according to the immune cell abnormalities; the elevated blood CD3-CD56+CD16$^+$ NK cell count (≥12%), which is the clinical immune marker previously reported for such cases. The peripheral blood was collected from the subjects at the luteal phase of menstrual cycle. Demographics and clinicopathological properties such as age, the number of previous IVF cycles, and obstetrical history of patient population are listed in Table 1. All participants signed a written informed consent before the study and peripheral blood samples were collected in sterile heparinized tubes to evaluate the percentages of lymphocytes.

2.2. Separation of Peripheral Blood Mononuclear Cells and T cell subsets

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood by Ficoll–Hypaque 1.077 g/ml (Biosera, UK) gradient centrifugation (25 min, 450 g). The protocol has been completely described in our previous study (Ahmadi et al., 2017). Cells were then washed twice with RPMI-1640 medium (Sigma, Germany). T cells were isolated from blood-derived PBMCs by magnetic activated cell sorting (MACS) (Miltenyi Biotec, Germany) method according to the manufacturer's instructions. For T cell isolation by negative selection, cells

were incubated with an antibody cocktail provided in the Pan T-cell isolation kit (Miltenyi Biotec). Cryopreserved peripheral blood mononuclear cells were used to isolate Treg and Th17 subpopulations. For isolation of the indicated T cell subpopulation the following kits were used: EasySep Human Th17 Cell Enrichment Kit II (from STEMCELL, Japan) and CD4+ CD25+ Regulatory T Cell Isolation Kit, human (Miltenyi Biotec). Purified CD4+CD25+ Tregs and Th17 cells were used for total RNA extraction and analyzed by quantitative real time PCR for miRNAs and their target genes expression.

2.3. RNA extraction and reverse transcription

Total RNA was extracted from the isolated PBMCs and purified T cells using Total RNA Purification Mini kit (YTA, Tehran, Iran) according to the manufacturer protocol. RNA concentration and purity was measured by NanoDrop spectrophotometer. The extracted RNA was first reverse transcribed into complementary DNA (cDNA) with oligo dT and random hexamer primers using M-MLV (H-) Reverse Transcriptase kit (Thermo Fisher, Waltham, MA, USA). cDNA synthesis for miRNA quantification was performed by using MicroRNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA) and High Capacity RNA-to-cDNA Master Mix.

2.4. Quantitative Real-time PCR

The analysis of gene expression was carried out by relative quantification using Real-time PCR method. mRNA expression levels of *IRF4*, *TBX21*, *GATA3*, and *GITR* genes were detected by specific primers and SYBER Green Master Mix (Roche, Germany). The levels of target gene expressions were normalized to β-actin as the housekeeping gene. Then data were presented as relative mRNA expression using the $2^{-\Delta CT}$ method in a way that was previously described by Schmittgen and Livak (Schmittgen and Livak, 2008). The amplification was carried out as the following: an initial denaturation step for 10 min at 95 ℃ and subsequent denaturation at 95 ℃ for 10 s, followed by annealing and extension at 72 ℃ for 20 s for 45 cycles. Reactions were analyzed by LightCycler 2.0 Real-Time PCR System machine (Roche Applied Science, Germany). A panel of six miRNAs including miR-25, miR-93, miR-106b, miR-146a, miR-155 and miR-326 were also examined by Real-time PCR. mRNA levels of miRNA's target genes were also assessed in PBMCs, purified T cells and isolated Treg cells and Th17 cells from PBMCs by real time PCR. Primer sequences are listed in Tables 2 and 3.

2.5. Fluorescence-activated cell sorter staining

The isolated PBMCs (1×10^6/sample) were washed once with FACS buffer (PBS containing 0.1% sodium azide and 0.5% BSA) and surface stained for 45 min at 4 ℃ in dark with FITC conjugated anti-CD4 (BD Biosciences, San Jose, CA, USA), PE conjugated anti-CD25, and PerCP-Cy5.5 conjugated anti-CD-127 (BD Biosciences) antibodies to determine $CD4^+CD25^+CD127^{-/low}$ Treg cells. Cells were then re-suspended in 1 ml of PBS for flowcytometry analysis. The following antibodies were used to examine exhausted $CD8^+$ T cell population: anti-CD8-FITC (BD Biosciences), anti-PD-1- PerCP-Cy5.5 (BD Biosciences), and anti-Tim3-PE (BD Biosciences). Flow cytometry was also used to assess $CD4^+CD25^+PD\text{-}1^+$ exhausted Treg cells using anti-CD4-FITC (BD Biosciences), anti CD25-PE and anti-PD1-PerCP-Cy5.5.

To analyze Th17 cells, samples were stimulated for 5 hrs with 1 μl/ml phorbol myristate acetate (PMA) (eBioscience, San Diego, CA, USA) at 37 ℃ in a 5% CO_2 humidified incubator for intracellular staining. After incubation, the suspension was centrifuged at 300 g for 10 min to obtain a cell pellet. The cells were re-suspended in FACS buffer and incubated for 30 min at room temperature. Subsequently, the cells were washed and first incubated with PE-conjugated antibody (BD Biosciences, San Jose, CA, USA) against intracellular IL-17 at 4 ℃ for 30 min followed by surface staining with anti-CD4-FITC (BD Biosciences, San Jose, CA, USA). Then they were analyzed using FACS Calibur (BD Biosciences) flow cytometer and obtained data were processed using FlowJo software (Tree Star FlowJo X 10.0.7 R2 / 10.0.4 Win/Mac/Linux).

Table 2
Primer sequences used for quantification of target genes in RIF patients and control subjects.

Gene	Primer	Sequence (5′ → 3′)	Amplicon size (bp)
IRF4	Forward	CGTTCATTGCTCTCCAGTCAC	150
	Reverse	GCCTTCACGCACCATTCAG	
TBX21	Forward	GGTGAACGACGGAGAGC	106
	Reverse	TCGGCATTCTGGTAGGC	
GATA3	Forward	GCCTCAGCCACTCCTAC	119
	Reverse	CCTGACCGAGTTTCCGTAG	
GITR	Forward	TGGGTCGGGATTCTCAGGTC	100
	Reverse	TTTCAAGAGCCCACAGCCAG	
β-actin	Forward	TCCCTGGAGAAGAGCTACG	131
	Reverse	GTAGTTTCGTGGATGCCACA	
STAT1	Forward	TCCATGCGGTTGAACCCTAC	193
	Reverse	TGCTTGCCCCACTTACCTTC	
SOCS1	Forward	GAGACAAAGAGGTGAGCTGGG	157
	Reverse	TCTAGCTGTGTCCACTGAGG	
Ets1	Forward	CCATGGCTTATGCGGGTACA	199
	Reverse	CTGACAACAGCCAAAACGGG	
P21	Forward	AGAATCCATGGTCCAAGGGC	168
	Reverse	AAGGGCCTGGCATAATGAACA	
BIM	Forward	GAGCCTTGCAAAGCCTGACA	224
	Reverse	GGGAATGACACTGCATCGGG	

IRF4, Interferon Regulatory Factor 4; TBX21, T-box transcription factor TBX21; GATA3, GATA-binding protein 3; GITR, Glucocorticoid-induced TNF receptor family-related protein; STAT1, Signal transducer and activator of transcription 1; SOCS1, suppressor of cytokine signaling 1.

2.6. Statistical analysis

Statistical evaluations and plotting were done using the GraphPad software (version 7.01). Scale variables were assessed for normal distribution using the Kolmogorov–Smirnov test. If normal, independent sample *t*-test or Mann-Whitney nonparametric test was used to compare the groups. All data were expressed as mean ± SD with statistical significance set of $P < 5\%$.

3. Results

3.1. mRNA expression level of transcription factors

Women with RIF showed significant overexpression of *IRF4* (Fold change = 1.7, $p = 0.0003$) and *TBX21* (Fold change = 1.4, $p = 0.013$) when compared to the fertile cases. There was a decreased level of *GATA3* in PBMCs from women with RIF when compared to fertile controls (Fold change = 0.6, $p = 0.0015$). Moreover, GITR expression in PBMCs was down-regulated in RIF group than in controls (Fold change = 0.7, $p = 0.055$), but the difference was not significant (Fig. 1.A). *Fig* 1.B shows the real time PCR amplification results obtained from total RNA extracted from isolated T cells. Comparison of results from samples of women with RIF with those obtained from control subjects revealed the same pattern in mRNA expression level of these genes in purified T cell population. IRF4 and TBX21 mRNA expression levels were increased in purified T cell population in women with RIF ($p < 0.0001$), but GATA3 and GITR mRNA expression levels were decreased in women with RIF compared with the control group ($p < 0.0001$).

3.2. miRNA transcription levels

Comparing miRNA expression profile in the PBMCs demonstrated significant differences between the two groups (Fig. 2.A-1). There was

Table 3
Primer sequences used for quantification of miRNAs in RIF patients and control subjects.

Gene	Primer	Sequence (5′ → 3′)
miR155	Forward	CTAGCCTGCAGGTATTCAAATATTTCCACAGA
	Reverse	ATCCGGCCGGCCTGAAGATGGTTATGAACATA
miR146a	Forward	CTAGCCTGCAGGCTGCCCTTGGACCAGCAGTC
	Reverse	ATCCGGCCGGCCGCTCTCTTTTTCTTCTTGAC
miR106b	Forward	CTAGCCTGCAGGGCCTGCTTTCCCGCTTTCCC
	Reverse	ATCCGGCCGGCCGAGACCAGACCCTTCTGAAC
miR93	Forward	CTAGCCTGCAGGGGTGAGTGGTGGGTCCCTGT
	Reverse	ATCCGGCCGGCCCTTTCTTTGCTCCAGCTTCA
miR25	Forward	CATTGCACTTGTCTCGGT
	Reverse	GGTCCAGTTTTTTTTTTTTTTTCAGA
miR326	Forward	ACACTCCAGCTGGGCCTCTGGGCCCT
	Reverse	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTGGAGGA

significant up-regulation of transcription levels of miR-25 (Fold change = 1.5, $p = 0.033$), miR-93 (Fold change = 1.6, $p = 0.013$), and miR-326 (Fold change = 1.5, $p = 0.02$) in RIF patients compared to the controls. However, miR-106b was overexpressed insignificantly ($p = 0.083$). On the other hand, both miR-155 (Fold change = 0.5, $p = 0.0006$) and miR-146a (Fold change ~ 0.5, $p = 0.01$) transcript levels demonstrated significant down-regulation in RIF patients than controls. Table 4 shows the results obtained by real-time PCR analysis of miRNA expression in the blood samples of RIF patients and healthy controls. miRNAs expression levels in total RNA samples from whole T cells and from isolated T cell subpopulation were similar to PBMCs (*Fig* 2.B-1). The expression levels of miR-25, miR-93, and miR-106b were significantly up-regulated in purified Treg cell population in women with RIF ($p < 0.0001$) but expression levels of miR-155 and miR-146a were significantly decreased in Treg cells from women with RIF compared to the controls ($p = 0.0008$, and $p < 0.0001$, respectively) (*Fig* 2.C-1). To further analyze the potential link between miR-326 and Th17 cells in women with RIF, we evaluated the expression of this miRNA in the cellular fraction enriched for Th17 cells. Th17 cells from women with RIF expressed high amount of miR-326 compared to normal fertile women (*Fig* 2.D-1).

3.3. mRNA expression level of miRNA's target genes

The six-miRNA panel analyzed in our experiment has been reported to be involved in T cell differentiation. We, therefore, next investigated expression of their target genes in various differentiation pathways in T cells from women with RIF and fertile control women. miR-155 has been detected to regulate expression of suppressor of cytokine signaling 1 (SOCS1) and miR-146a targets signal transducer and activator of transcription 1 (STAT1) (Zhou et al., 2014). mRNA expression data showed that STAT1 (Fold change = 2.6, $p < 0.0001$) and SOCS1 (Fold change = 2.4, $p < 0.0001$) mRNA were significantly up-regulated in PBMCs from women with RIF. By contrast, expression of cell cycle inhibitor CDKN1A (p21) and pro-apoptotic gene BCL2L11 (BIM), two main effectors in TGF-β signaling pathway that are regulated by miR-106b/miR-93 and miR-25, respectively, were significantly (Fold change ~ 0.4, Fold change = 0.4, respectively, $p < 0.0001$) decreased in PBMCs of patients when compared to controls. In addition, as suggested by previous studies that miR-326 regulates the expression of Ets-1- the negative regulator of Th17 differentiation- a significant correlation was observed between the expression level of Ets-1 and miR-326. Ets-1 expression was significantly (Fold change = 0.3, $p < 0.0001$) down-regulated in PBMCs from women with RIF (*Fig* 2.A-2). Similar results were obtained from Th17 cell fraction isolated from PBMCs of patients (*Fig* 2.D-2). The STAT1 and SOCS1 mRNA expression levels, were increased in purified Treg cells from women with RIF when compared to the control group ($p < 0.0001$), but P21 and BIM expression levels were decreased in Tregs from women with RIF compared with control group ($p < 0.0001$) (*Fig* 2.C-2).

3.4. Frequency of T cell subsets in peripheral blood

To elucidate the respective importance of different subsets of T cells,

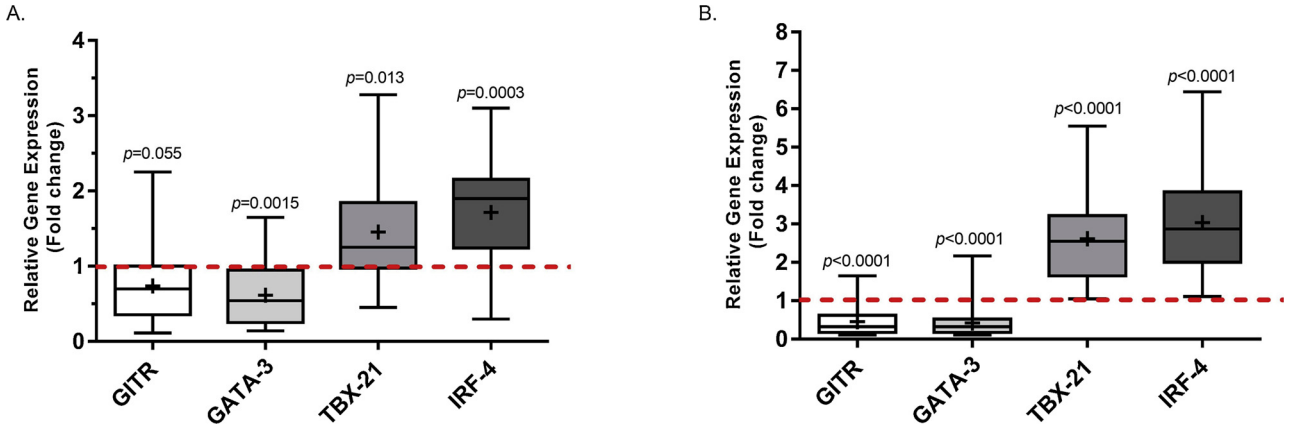

Fig. 1. Ratios of selected transcription factors and surface marker expression levels in women with RIF and non-pregnant fertile women **A.** Relative gene expression in PBMCs from RIF patients. IRF4 and TBX21 mRNA expression levels were up-regulated in women with RIF ($P = 0.0003$), and ($P = 0.013$), respectively, when compared to that of healthy fertile women. The results of Real time PCR showed that GATA3 and GITR mRNA expression levels were down-regulated in women with RIF when compared to the control group ($P = 0.0015$), and ($P = 0.055$), respectively. **B.** Relative gene expression in purified T cells from women with RIF. The mRNA expression levels of IRF4 and TBX21 were also up-regulated in purified T cell in RIF patients ($P < 0.0001$), but GATA3 and GITR mRNA expression levels were down-regulated in women with RIF in compared to the control group ($P < 0.0001$). Results are given as, mean ± SD (RIF group, n = 40; control group, n = 50). $P < 0.05$ was considered statistically significant.

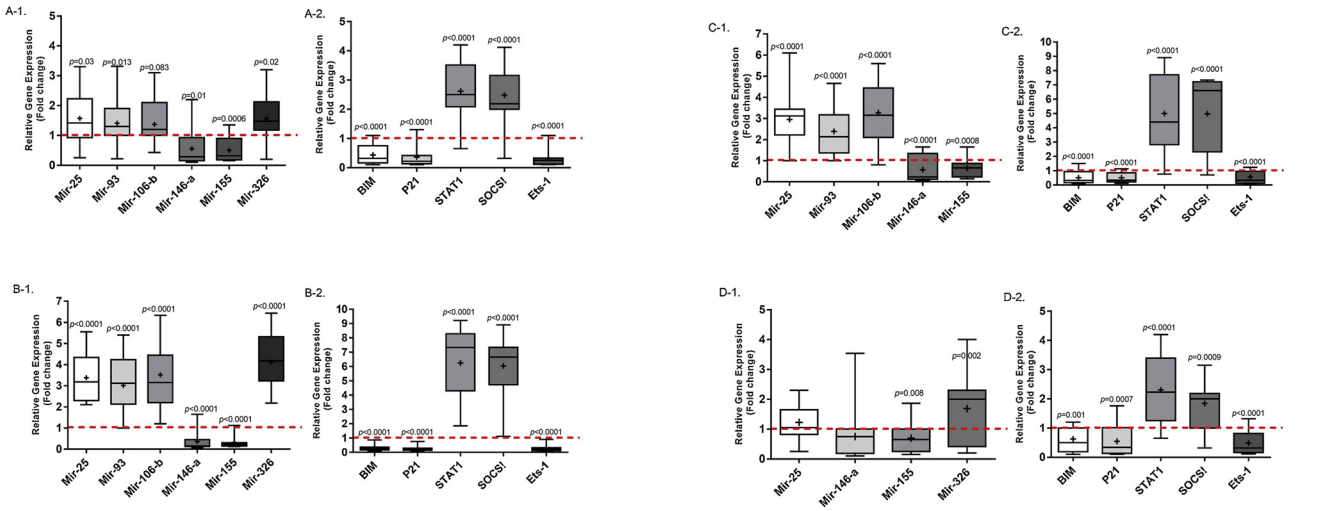

Fig. 2. miRNAs and their target genes expression in PBMCs, T cells, purified Treg and Th17 cells from women with RIF and non-pregnant fertile women. **A-1.** The relative expression levels of miR-93, miR-326, miR-106b, miR-25, miR-155, and miR-146a in PBMCs of RIF patients compared to healthy control. Significantly higher levels of miR-25, miR-93 and miR-326 was observed in RIF patients ($P = 0.03$), ($P = 0.013$), and ($P = 0.02$), respectively, however miR-106b was over-expressed insignificantly ($P = 0.083$). The expression levels of miR-155 and miR-146a were significantly down-regulated, ($P = 0.0006$) and ($P = 0.01$), respectively **A-2.** The expression levels of miRNAs-dependent targets genes in PBMCs from women with RIF. STAT1 and SOCS1, the target genes for miR-146a and miR-155, respectively, were up-regulated in women with RIF compared to control group ($P < 0.0001$). P21, BIM and Ets-1 expression levels were down-regulated in PBMCs from RIF patients when compared to the control group ($P < 0.0001$). **B-1.** The relative expression levels of miR-93, miR-326, miR-106b, miR-25, miR-155, and miR-146a in isolated T cells from women with RIF. The expression levels of miR-25, miR-93, miR-106b and miR-326 were significantly increased in RIF patients ($P < 0.0001$) but expression levels of miR-155 and miR-146a were significantly down-regulated ($P < 0.0001$) in purified T cell in women with RIF. **B-2.** The results of miRNAs-dependent targets genes expression levels in isolated T cells from women with RIF. The expression levels of STAT1 and SOCS1, were up-regulated in T cells purified from women with RIF when compared to the control group ($p < 0.0001$), but P21, BIM and Ets-1 expression levels were down-regulated, ($p < 0.0001$). The relative gene expression showed higher differences in T cells from women with RIF when compared to that in PBMCs from the same group. **C-1.** miRNA expression profile from purified Treg cells of women with RIF compared to the control group. **C-2.** Target genes expression in purified Treg cells. **D-1.** Results from miRNA expression level of Th17 cells and **D-2.** Target gene expression from Th17 cells. Results are given as, mean ± SD (RIF group, n = 40; control group, n = 50). $P < 0.05$ was considered statistically significant.

Table 4
miRNAs of women with a history of RIF and non-pregnant fertile women.

miRNA		*P* value	*miRNA target genes*		*P* value
In PBMCs:					
	Upregulated in RIF patients			**Downregulated in RIF patients**	
miR-25	1.566 ± 0.8013	0.033	BIM	0.4338 ± 0.3605	< 0.0001
miR-93	1.602 ± 1.037	0.013	P21	0.3631 ± 0.3395	< 0.0001
miR-106b	1.372 ± 0.673	NS*	P21	0.3631 ± 0.3395	< 0.0001
miR-326	1.56 ± 0.7166	0.02	Ets-1	0.3177 ± 0.2762	< 0.0001
	Downregulated in RIF patients			**Upregulated in RIF patients**	
miR-155	0.4993 ± 0.3971	0.0006	SOCS1	2.479 ± 1.018	< 0.0001
miR-146a	0.5596 ± 0.5117	0.01	STAT1	2.621 ± 1.054	< 0.0001
In total T cells:					
	Upregulated in RIF patients			**Downregulated in RIF patients**	
miR-25	3.381 ± 0.9878	< 0.0001	BIM	0.3069 ± 0.2039	< 0.0001
miR-93	3.013 ± 1.186	< 0.0001	P21	0.2538 ± 0.1772	< 0.0001
miR-106b	3.515 ± 1.466	< 0.0001	P21	0.2538 ± 0.1772	< 0.0001
miR-326	4.132 ± 1.186	< 0.0001	Ets-1	0.2908 ± 0.2374	< 0.0001
	Downregulated in RIF patients			**Upregulated in RIF patients**	
miR-155	0.316 ± 0.2873	< 0.0001	SOCS1	6.035 ± 2.342	< 0.0001
miR-146a	0.3573 ± 0.4393	< 0.0001	STAT1	6.245 ± 2.486	< 0.0001

miRNA, microRNA; PBMC, peripheral blood mononuclear cell; RIF, recurrent implantation failure; NS, not significant.

four subsets of T cells, characterized as CD4 + CD25 + CD127- regulatory T (Treg) cell, CD4 + CD25 + CD279 + exhausted Treg cells, CD8 + CD279 + Tim-3 + exhausted T cells, and CD4 + IL-17 + helper T (Th) 17 cells were evaluated by flowcytometry. The percentage of CD4 + IL-17 + T cells in peripheral blood of RIF group was significantly increased when compared to the healthy controls (4.354 ± 1.654 vs. 3.32 ± 1.708, respectively, p = 0.0048, Fig. 3 A). As shown in *Fig* 3B, the frequency of CD4 + CD25 + CD127- Treg cells in RIF women (3.174 ± 149) was significantly ($p = 0.0022$) lower than that of controls (4.296 ± 1.817). The frequency of CD8 + PD-1 + Tim-3 + exhausted T cells in circulation of RIF patients (6.637 ± 3.23) was significantly lower ($p = 0.029$, *Fig* 3C) than healthy volunteers (8.297 ± 3.752). An increased count of CD4 + CD25 + PD-1 + subset was observed in RIF patients when compared to the controls (6.899 ± 2.99 vs. 5.355 ± 1.845, $p = 0.0034$, *Fig* 3D).

4. Discussion

The relatively low efficiency of human reproduction in successful pregnancy is highlighted when an approximately 30% of conceptions

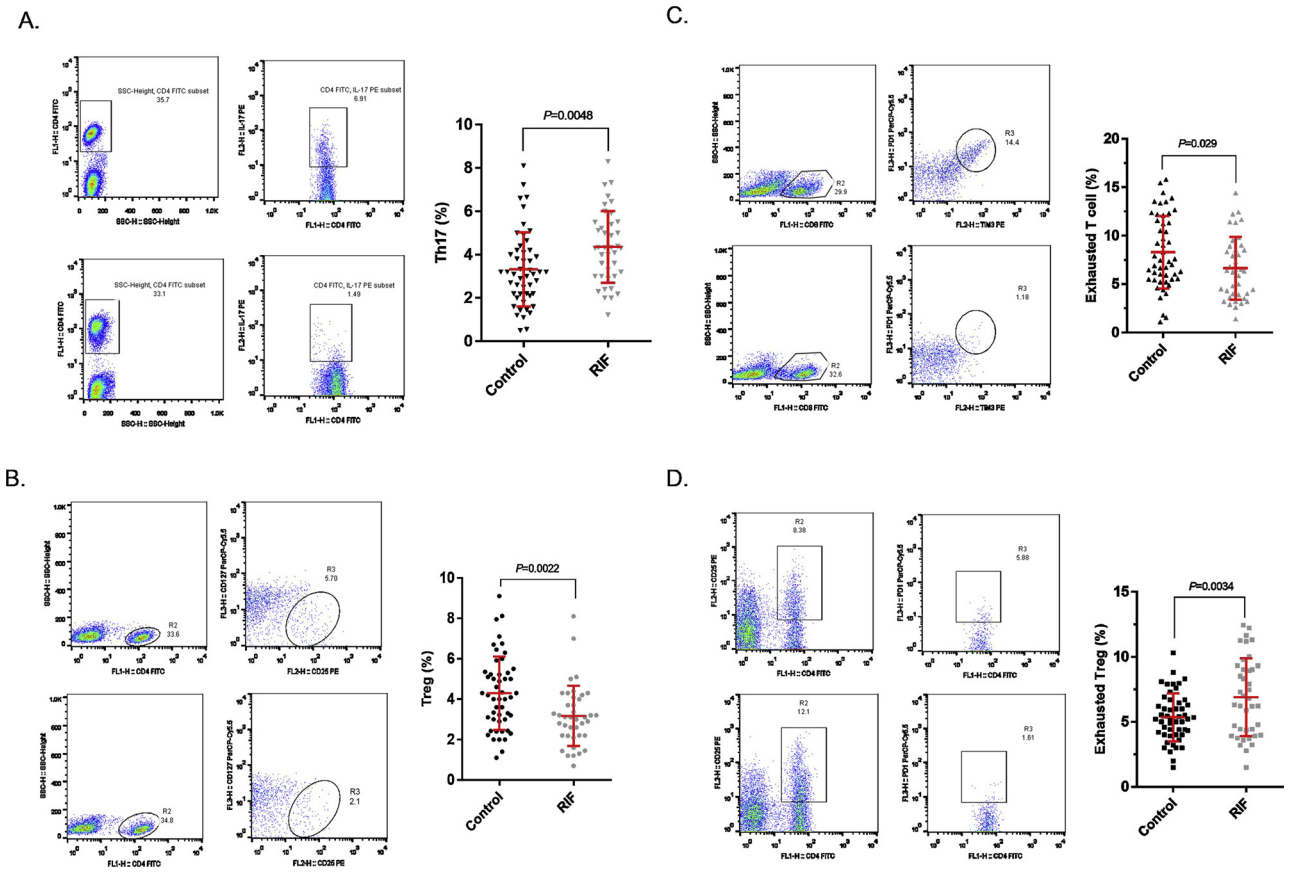

Fig. 3. Flow cytometry analysis of T cell subsets in the peripheral blood of women with RIF and fertile women. **A.** Isolated PBMCs were stained with conjugated antibodies against CD4 and intracellular IL-17 to assess Th17 cells by flow cytometry. Th17 frequency was higher in peripheral blood of RIF patients when compared to the controls ($P = 0.0048$). **B.** representative flow cytometric dot plots (right) and statistical analysis (left) of $CD4^+CD25^+CD127$- Treg cells in samples of women with RIF and control group. The results showed a lower percentage of Treg cells in RIF patients when compared to the controls ($P = 0.0022$). **C.** The frequency of $CD8^+$ T cells expressing PD-1 and Tim-3 (exhausted T cells) from PBMCs of RIF patients was lower than that of control ($P = 0.029$). **D.** The percentage of $CD4^+CD25^+CD279^+$ exhausted Treg cells was higher in peripheral blood of RIF patients ($P = 0.0034$). Results are given as, mean ± SD (RIF group, n = 40; control group, n = 50). $P < 0.05$ was considered statistically significant.

are lost before implantation (Larsen et al., 2013). Failure of most embryos to implant is the principal cause of repeated IVF failure.

As a subgroup of recurrent IVF failure, RIF is primarily attributed to uterine factors. During normal pregnancy the implanting embryo escapes immunological rejection by the hostile maternal immune responses. Cellular immune responses mediated by T lymphocytes are one of the main mechanisms responsible for human embryo transfer failures. Treg cells play a significant role in the establishment and maintenance of tolerance in pregnancy (Shima et al., 2010). In early pregnancy, the proportion of CD4+ CD25+ Tregs in bulk CD4 + T cells increase in both decidua and periphery. Several studies have reported reduced frequency of Treg cells within peripheral blood and decidual tissues in women experiencing implantation failure or recurrent miscarriage (Sasaki et al., 2004). Our previous study also demonstrated the putative effects of Treg cells in implantation process, as treatment with IVIG could improve pregnancy outcome in RIF women via the mechanisms involving the induction of Treg cells (Ahmadi et al., 2017). It has been shown that reduced suppressive capability of regulatory T cells is associated with implantation failure due to the lack of control over activated T cells (Yang et al., 2008). Several lines of evidence indicated that the inhibitory receptor pathway is a pivotal factor that greatly influences the responsiveness of Treg cells *in vivo* (Franceschini et al., 2009, Barber et al., 2006). CD279/PD-1 expression on Treg cells is associated with the reduced suppressive activity (Franceschini et al., 2009). PD-1/PD-L1 pathway was proved to have negative regulatory effects on Treg proliferation and suppressive functions through the mechanism involving the prevention of IL-2 induced STAT-5 phosphorylation in chronic infections (Franceschini et al., 2009). Many publications have characterized PD-1 as an exhaustion marker that determine the poor effector functions of T cells Wherry et al. (2011). PD-1 expression on Tregs is associated with the delivery of inhibitory signals that may suppress Treg function in the setting of pregnancy complications (Toldi et al., 2015). Toldi et al. reported that elevated $CD4^+CD25^{high}FoxP3^+CD279^+$ Treg cells may account for decreased functionality of these cells in preeclampsia (PE) (Toldi et al., 2015). In line with these findings about PE, we observed higher numbers of $CD4^+CD25^+CD279^+$ "exhausted" Treg cells and decreased $CD4^+CD25^+CD127$- Treg cells in RIF when compared to the controls. Since, the inhibition of Th1- and Th17-cell responses by Tregs is a classical mechanism involved in protecting the fetus from the immune attack (Sasaki et al., 2004), increased population of Tregs expressing PD-1 (dysfunctional exhausted Tregs) in peripheral blood of women with pregnancy failure may be informative for decreased frequency and/or suppressive activity of Tregs, that is the abnormality critical in development of implantation failures with immune etiology. However, it is clear that further studies will definitely elucidate T cells variations in leukocyte populations at human decidua and also will fully understand the role of PD-1 molecule on Tregs in pregnancy outcome in women with RIF and healthy cases. Although immunoregulatory functions of Treg cells are considered to be local at the maternal-fetal interface, systemic immune state may reflect fetomaternal environment in the uterus around the implantation period. Circulating immune cells

appear to communicate with immune cells in the decidua and regulate tissue remodeling and trophoblast invasion and, thereby, contribute to feto-maternal cross-talk at the embryo implantation site (Fujiwara, 2009). Furthermore, reportedly, a higher percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in the peripheral blood lymphocytes of women undergoing IVF treatment is predictive of better IVF outcome (Zhou et al., 2012). Therefore, measuring these circulating factors may provide a prognostic biomarker for implantation failure.

Another contributing factor to fetal-maternal tolerance may be a subset of CD8 + T cells expressing both PD-1 and Tim-3 that are described as 'exhausted' T cells. CD8 + T cells can elicit cytotoxic responses with deleterious impacts on fetal survival (Wang et al., 2015). The activation or tolerance mode in CD8 + T cells has been largely associated with inhibitory signals that are mediated by various regulatory molecules. Tim-3 has been regarded as a marker for dysfunctional CD8 + T cells, since blockade of Tim-3 signal cascade has been shown to rescue exhausted CD8 + T cells (Golden-Mason et al., 2009). PD-1 is similarly known to inhibit T cell functions, such as cytokine production and cytotoxic activity (Keir et al., 2008). The impaired number and function of $PD\text{-}1^+Tim\text{-}3^+CD8^+$ T cells in miscarriage was verified in a comprehensive study by Wang et al. He showed that PD-1 and Tim-3 co-expression on decidual and peripheral blood CD8 + T cells favor normal pregnancy *in vivo* in mice and human. He also identified that the population of $PD\text{-}1^+Tim\text{-}3^+CD8^+$ T cell is affected in women who had miscarriage (Wang et al., 2015). Moreover, blocking Tim-3 and/or PD-1 pathways caused fetal loss in pregnant CBA/J mice due to impaired CD8+ activity, which implies the significant role of $PD\text{-}1^+Tim\text{-}3^+CD8^+$ T cells in maternal-fetal tolerance *in vivo* (Wang et al., 2015). Our study also demonstrated that $PD\text{-}1^+Tim\text{-}3^+CD8^+$ T cells were less abundant in peripheral blood of women with RIF than normal non-pregnant subjects. Co-expression of PD-1 and Tim-3 on CD8 + T cells is preferentially correlated to the production of Th2 cytokines representing a specific subset of CD8 + T cells with regulatory functions that are hypotoxic, and thereby contribute in immune responses in normal pregnancy. More clearly, Th2-biased responses divert the adaptive immunity away from Th1/Th17-cell mediated immune responses that are harmful to the fetus. One recent study described the characteristics of PD-1 and Tim-3 double positive CD8 + T cells in pregnancy. The authors demonstrated that PD-1+ Tim-3+ CD8 + T cells in decidua display higher expression of Th2 cytokines, IL-10 and IL-4 when compared to the single positive CD8 + T cells (Wang et al., 2015). They also detected the lowest amounts of Th2 cytokines release by PD-1- Tim-3- CD8 + T cells.

We also examined IL-17-producing $CD4^+$ T cells in the blood samples. In parallel with the previous findings, the results showed that during preconception, the number of $IL\text{-}17^+$ T cells was much higher in RIF patients than healthy controls. These findings suggest that preconception T cell abnormalities over different phases of menstrual cycle in women with RIF are considerable and changes in the composition of circulating T cell subtypes is skewed toward a hazardous condition for pregnancy. Herein, we showed that, compared to controls, peripheral blood Treg cells were significantly lower in women who experienced RIF with elevated peripheral blood NK cells. Assessing circulating NK cells and their predictive value in reproductive failure of women with a history of RIF was first documented in 1996 (Beer et al., 1996). Various subpopulations of Tregs have been reported to interact with other lymphocytes such as NK cells (Satoguina et al., 2008). The level of Treg cells may be negatively correlated to CD3-CD56+ NK cells in peripheral blood. A previous prospective research study demonstrated significant correlation between Foxp3+ T cells and NK cells *in vivo* in normal population. Our results showed a profound decrease in Treg levels while NK cells were at a high level in patients. According to the previous literatures, a decrease in Tregs together with NK cell expansion indicates that NK cell proliferation is regulated by Tregs (Lee et al., 2011b). On the other hand, NK cells have the capacity to affect Foxp3+ Treg cell expansion by cell lysis mediated by NKG2D and NKp46 receptors (Roy et al., 2008). Therefore, NK and Treg cells may influence each other by a mutual control of their levels. The findings in this study can help in developing approaches to effectively manage couples with RIF. Despite the fact that results have suggested misbalanced immune profile in RIF patients, it is still unclear that whether the cellular immune status in the circulation could present the immunological status in the uterus or not. However, it must be notified that studying the implantation events *in vivo* in women is virtually difficult.

We further achieved more detailed information on the molecular basis underlying this imbalance. We evaluated the mRNA expression of *IRF4*, *TBX21*, *GATA3*, and *GITR* in PBMCs and in isolated T cell subsets that are important factors in transcriptional programs of T cells. The transcription factors TBX-21 and GATA-3 are essential in the differentiation of Th1 and Th2 lineages, respectively (Mullen et al., 2001, Lee et al., 2000). IRF-4 is a member of the Interferon Regulatory Factor (IRF) family of transcription factors, which is a signature of Th17 cells (Brüstle et al., 2007). We found elevated levels of *TBX21* and *IRF4* transcripts in PBMCs from RIF patients, while the expression of *GATA3* was reduced in PBMCs of these patients. Exhausted $CD8^+$ cells appear to bias toward Th2 cell by producing critical cytokines like IL-4 and IL-10 (Wang et al., 2015). GATA-3 expression is responsible for this polarization in a way that experiments showed treating with anti-Tim3 and/or anti-PD-1 blocking antibodies resulted in decreased Th2-type cytokine production concomitant with markedly reduced level of GATA-3 expression (Wang et al., 2015). Therefore, the observed low frequency of exhausted CD8+ cells in RIF patients might be attributed to decreased levels of GATA-3 expression. Furthermore, there have been studies suggesting an essential role for GATA-3 in Treg cell functions (Wang et al., 2011). The Tumor-necrosis-factor receptor (TNFR)-family member glucocorticoid induced TNFR-related protein (GITR) has attracted attention as a marker that is constitutively expressed at high levels by Treg cells (Shevach and Stephens, 2006). Our results also support this view point, as lower expression of GITR mRNA in PBMCs and in T cell population from patients was consistent with low $CD4^+CD25^+$Treg cell count.

miRNA profiling has been regarded as an active research area in the field of reproductive biology in recent years. Identification of miRNAs which are differentially expressed in the endometrium of women with recurrent failure of implantation is of particular relevance for pathogenesis (Revel et al., 2011). Herein, we explored the miRNA expression profiles in peripheral blood from women with RIF compared to the fertile women. miR-106b-25 cluster (miR-93, miR-106b, and miR-25), miR326, miR155 and miR146a are known to be crucial in immune system. Several results suggested that miR-106b-25 cluster, in particular miR-25 and miR-106b, are involved in interrupting Transforming Growth Factor (TGF)-β signaling (Petrocca et al., 2008, De Santis et al., 2010). TGF-β is an immunoregulatory cytokine involved in controlling immune reactions by promoting Treg cell development (Fantini et al., 2004). In the course of Multiple Sclerosis (MS), it has been shown that up-regulation of miR-25 and miR-106b may play a role in the pathogenesis of MS by disrupting TGF-β signaling and loss of suppressive ability of Treg cells (De Santis et al., 2010). We speculated that deregulation of miR-25 and miR-106b levels may exert a function in repeated failure of embryo implantation by affecting Treg cells. We found overexpression of miR-25, miR-106b and miR-93 in PBMCs and purified T cells from RIF patients. But the results for miR-106b were not statistically significant. It has been reported that miR-106b-25 cluster operate in inactivating the TGFβ pathway by suppressing p21 and BIM, the two main effectors in TGFβ pathway (Petrocca et al., 2008). Our data indicate that p21 and BIM genes were down-regulated in PBMCs and T cell populations from RIF patients. All the isolated subsets of T cells were assessed for relevant miRNAs and their target genes by the use of real-time PCR. The results showed that the expression of miR-25, miR-93 and miR-106b were specifically up-regulated in Treg cells isolated from RIF patients compared with normal controls, whereas the expression of miR-25 in Th17 subset of T cells was approximately at the

same level as control group. Consistently, their target genes BIM and p21 were decreased significantly in Treg cells of patients. miR-326, a Th17 cell-associated miRNA, was significantly up-regulated in peripheral blood leukocytes as well as in isolated T cells of patients with RIF when compared to the controls. Based on previous published works that suggested the regulation of Th17 by miR326 (Du et al., 2009), increased expression of miR326 was in line with increased number of Th17 cells as detected in patients with RIF. Consistently, in our results, Ets-1 that is a functional target of miR-326 was found to be reduced in PBMCs and T cells from women with RIF when compared to the controls. To address the increased percent of Th17 cells in patients, we examined the expression of this miRNA in Th17 cells isolated from T cell population. As expected, a higher level of miR-326 and a lower expression of its target gene, Ets1 was detected in Th17 cells of patients. The expression of miR-155 and miR-146a showed the most significant difference between the two groups. As shown in patients with rheumatoid arthritis, decreased expression of both miR-155 and miR-146a contributes in a pro-inflammatory phenotype of Treg cells in spite of their suppressive activity (Zhou et al., 2014). Decreased expression of miR-155 and miR-146a in regulatory T cells from RIF were accompanied by an up-regulation of their target genes, SOCS1 and STAT1, respectively. These data allow us, therefor, to suggest that the pattern of miRNA expression is associated with acquisition of the given cellular composition in women with RIF.

Taken together, this study suggests that a combination of higher pro-inflammatory Th17 and exhausted Treg cells and lower Treg and exhausted $CD8^+$ T cells with immunosuppressive properties may co-exist in the peripheral blood immune cell repertoire of women with a history of RIF after IVF treatment. These may result from abnormal expression of transcription factors. In addition, T cell-associated miRNAs are dysregulated and might be potential candidates for diagnosis and, hopefully, be exploited as treatment targets for RIF therapy.

5. Declarations of interest

none

Conflict of Interest

The authors declare no conflicts of interests.

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