

# Tacrolimus Improves the Implantation Rate in Patients with Elevated Th1/2 Helper Cell Ratio and Repeated Implantation Failure (RIF)

## Tacrolimus verbessert die Implantationsrate bei Patientinnen mit einem erhöhten Verhältnis von T-Helferzellen des Typs 1 zum Typ 2 und wiederholtem Implantationsversagen

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### Key words

repeated implantation failure, embryo implantation, tacrolimus, immune system, assisted reproductive technology (ART), intracytoplasmic sperm injection (ICSI), in vitro fertilization (IVF), leukemia inhibitory factor (LIF), interleukin (IL)-4, IL-10, IL-17, interferon gamma (IFN- $\gamma$ )

### Schlüsselwörter

wiederholtes Implantationsversagen, Embryotransfer, Tacrolimus, Immunsystem, assistierte Reproduktionstechnologie (ART), intrazytoplasmatische Spermieninjektion (ICSI), In-vitro-Fertilisation (IVF), leukämiehemmender Faktor (LIF), Interleukin (IL)-4, IL-10, IL-17, Interferon gamma (IFN- $\gamma$ )

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### ABSTRACT

**Introduction** An abnormal endometrial immune response is involved in the pathogenesis of repeated implantation failure (RIF), so we investigated the effectiveness of tacrolimus treatment on the endometrium of RIF patients.

**Materials and Methods** Ten RIF patients with elevated T-helper 1/T-helper 2 (Th1/Th2) cell ratios were recruited into a clinical study. The expression of p53, leukemia inhibitory factor (LIF), interleukin (IL)-4, IL-10, IL-17, and interferon gamma (IFN- $\gamma$ ) in the endometrium of patients with and without tacrolimus treatment and the association of these factors with assisted reproductive technology (ART) outcomes were investigated.

**Results** Tacrolimus significantly increased the expression of LIF, IL-10, and IL-17 and decreased the expression of IL-4, IFN- $\gamma$ , and the IFN- $\gamma$ /IL-10 ratio in RIF patients. Tacrolimus treatment resulted in an implantation rate of 40%, a clinical pregnancy rate of 50%, and a live birth rate of 35% in RIF patients with elevated Th1/Th2 ratios who had previously failed to become pregnant despite at least three transfers of embryos. We also found a significant positive correlation between IL-10 levels and the implantation rate.

**Conclusions** Our findings suggest that RIF patients with a higher Th1/Th2 ratio could be candidates for tacrolimus therapy and that this immunosuppressive drug could be acting through upregulation of LIF, IL-10, and IL-17.

## ZUSAMMENFASSUNG

**Einleitung** Da eine abnorme Immunantwort des Endometriums möglicherweise an der Pathogenese von wiederholtem Implantationsversagen (RIF) beteiligt ist, haben wir die Auswirkungen einer Tacrolimus-Behandlung auf das Endometrium von Patientinnen mit RIF untersucht.

**Material und Methoden** Eine klinische Studie wurde bei 10 RIF-Patientinnen mit einem erhöhten Verhältnis der T-Helferzellen des Typs 1 zum Typ 2 (Th1/Th2) durchgeführt. Alle in der Studie aufgenommenen Frauen hatten mindestens 3 er-

folglose Implantationsversuche hinter sich. Die Exprimierung von p53, leukämiehemmendem Faktor (LIF), Interleukin (IL)-4, IL-10, IL-17 und Interferon gamma (IFN- $\gamma$ ) in der Gebärmutter-schleimhaut dieser Patientinnen vor und nach der Behandlung mit Tacrolimus und der Zusammenhang zwischen diesen Faktoren und dem Outcome nach assistierten Reproduktionstechnologien (ART) wurden geprüft.

**Ergebnisse** Die Behandlung mit Tacrolimus führte zu einer signifikanten Steigerung der Exprimierung von LIF, IL-10 und IL-17, verringerte die Exprimierung von IL-4 und IFN- $\gamma$  und senkte das Verhältnis von IFN- $\gamma$  zu IL-10 bei Patientinnen mit RIF. Die Tacrolimus-Behandlung führte zu einer Implantationsrate von 40%, einer 50%igen klinischen Schwangerschaftsrate und einer Lebendgeburtensrate von 35% bei Patientinnen mit RIF und einem erhöhten Th1/Th2-Verhältnis. Es gab auch eine signifikante positive Korrelation zwischen den IL-10-Konzentrationen und der Implantationsrate.

**Schlussfolgerungen** Unsere Ergebnisse lassen darauf schließen, dass RIF-Patientinnen mit einem erhöhten Th1/Th2-Verhältnis geeignete Kandidaten für eine Behandlung mit Tacrolimus sein könnten und dass dieses Immunsuppressivum möglicherweise durch eine Hochregulierung von LIF, IL-10 und IL-17 seine Wirkung entfaltet.

## Introduction

While assisted reproductive technology (ART) has made considerable advances in the decades since its inception, the current success rate is still under 30% [1] and implantation failure remains a significant obstacle [2]. Repeated implantation failure (RIF) is diagnosed when a woman under the age of 40 is unable to achieve a pregnancy after the transfer of at least three good-quality embryos over a minimum of three fresh or frozen cycles [3]. Dysregulation of the endometrial immune profile results in a decrease in endometrial receptivity and is thought to be responsible for the failure of embryo implantation [2,4,5]. It has been reported that endometrial immune profiles appear to be dysregulated in 81.7% of RIF patients. Studies have demonstrated an overactivation of the immune system in 56.6% and low activation in 25% of RIF patients [2]. Thus, resolving this mechanism may improve the success rates for RIF patients.

During the window of implantation (WOI), a massive influx of immune cells induces maternal immune tolerance to protect the embryo from rejection [6]. Immune cells such as T-helper cells (Th1, Th2, and Th17) and regulatory T-cells (Treg) contribute to maternal immune tolerance and the precise balance of cytokine signals needed to maintain pregnancy [7]. Th1 cells, which contribute to cell-mediated immunity, produce inflammatory cytokines such as interleukin-2 (IL-2), interferon gamma (IFN- $\gamma$ ), and tumor necrosis factor-alpha (TNF- $\alpha$ ), while Th2 cells are a source of anti-inflammatory cytokines such as IL-4 and IL-10 which contribute to humoral responses. Successful implantation shifts the response of the endometrial lining from a cell-mediated to a humoral response, but an imbalance between Th1 and Th2 cells

and their cytokines can result in implantation failure [4]. Pro- and anti-inflammatory cytokine ratios in peripheral blood which reflect the Th1/Th2 ratio (e.g., IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10) are significantly higher in RIF patients compared to fertile controls [4, 8, 9]. Th2 cytokines such as IL-4 can induce the expression of leukemia inhibitory factor (LIF) [10], a pro-inflammatory cytokine essential for blastocyst implantation and stromal decidualization [11]. The tumor suppressor p53 plays a crucial role in maintaining genomic stability and for tumor prevention [12]. Furthermore, p53 plays a role regulating maternal reproduction through regulation of LIF gene expression [13]. An embryo carries both paternal and maternal antigens, and embryo implantation is considered an allograft [14]. It has been proved that Th17 cells contribute to allograft rejection [15, 16], and it has been demonstrated that the RIF mechanism is similar to that of allograft rejection. During the transient immune switch, 65–70% of the immune cells are uterine natural killer (uNK) cells in the endometrium that belong to the innate immunity compartment [17]. The activation of these cells plays a fundamental role in Th2 cytokine production and the establishment of local maternal tolerance [18]. Interestingly, it has been demonstrated that the endometrium of women with RIF contains abnormal levels of uNK cells [19]. Regulation of the immune system by therapeutic agents may improve implantation and pregnancy rates in women with a history of RIF. Nakagawa et al. [4, 8] demonstrated that when RIF patients with an elevated Th1/Th2 ratio were treated with tacrolimus, an immune-suppressive agent, the pregnancy rate increased significantly compared to a non-treated group. Tacrolimus is a T-cell-specific, anti-inflammatory agent [20] that suppresses the immunological rejection of an allograft and promotes its survival by inhibiting lymphocyte prolifera-

tion [21]. Moreover, it has been documented that tacrolimus inhibits the transcription and release of both Th1 and Th2 cytokines, such as IL-2, IL-4, IL-5, IFN- $\gamma$ , and TNF- $\alpha$  [22], but primarily suppresses Th1 cells [8]. Tacrolimus can also inhibit the expression of IL-17 and induce the expression of both p53 and LIF, reducing allograft rejection [15, 16, 22–26].

Given the involvement of tacrolimus in immune regulation, we investigated the effect of tacrolimus treatment on endometrial immune response, as well as endometrial receptivity markers in RIF patients. To do this, we evaluated protein and mRNA expression of p53, LIF, IL-4, IL-10, IL-17, and IFN- $\gamma$  in the endometrium of RIF patients with and without tacrolimus treatment and also investigated the association between these factors and ART outcomes. To our knowledge, this is the first study to investigate the effects of tacrolimus on the cytokine expression profile and endometrial receptivity markers in RIF patients.

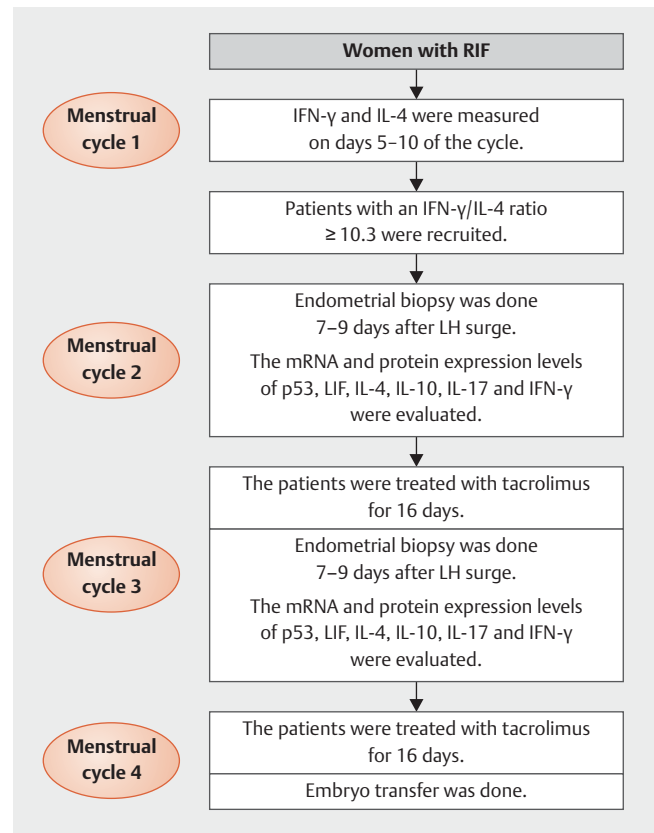
## Materials and Methods

The study was carried out at the Infertility and IVF division of AL-Zahra Hospital at the Tabriz University of Medical Sciences in Tabriz, Iran from May 2017 to December 2018 in collaboration with the Department of Reproductive Biology at the Shahid Beheshti University of Medical Sciences in Tehran, Iran. This study was approved by the Research Ethics Committee of the Shahid Beheshti University of Medical Sciences (approval code: IR. SBMU. MSP. REC.1395.580). The study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and a signed informed consent form was obtained from all patients prior to entering the study. The study has been registered in the Iranian Registry of Clinical Trials (<https://www.irct.ir>; Registry code: IRCT20171007036623N1).

### Study population

Ten women diagnosed with RIF and eligible for intracytoplasmic sperm injection (ICIS)/embryo transfer (ET), were selected for participation. Each participant also had a history of three or more failed ART cycles with morphologically good-quality embryos. Patients were assessed by transvaginal ultrasound, hysterosalpingography, and hysteroscopy before they were entered in the study to exclude patients with submucosal fibroids, endometrial polyps, intrauterine adhesions, congenital anomalies of the uterus, and/or hydrosalpinges. Furthermore, none of the participants had any history of autoimmune disease. Women with a history of chronic inflammatory conditions, acquired or inherited thrombophilia, previous immunotherapy, previous miscarriage, irregular menstrual cycles, or who were vaccinated in the last three months were excluded from the study. To determine the presence of immunological disorders, IFN- $\gamma$  and IL-4 levels were measured on days 5–10 of the menstrual cycle and an IFN- $\gamma$ /IL-4 ratio  $\geq 10.3$  was classified as an elevated Th1/Th2 ratio [4]. These patients were recruited into the study. A schema of the in-vivo study design is presented in ► Fig. 1.

To determine the baseline Th1/Th2 ratio, 10 mL of venous blood was collected into sodium heparinized tubes on days 5–10 of the menstrual cycle. Th1 and Th2 cells were determined by detecting intracellular interferon (IFN)- $\gamma$  and IL-4 production. Periph-



► Fig. 1 Schema of the in-vivo study design. RIF: repeated implantation failure, IFN- $\gamma$ : interferon gamma, IL: interleukin, LH: luteinizing hormone, LIF: leukemia inhibitory factor.

eral blood mononuclear cells (PBMCs) were isolated from blood samples by adding 1.077 g/mL of ficoll (Lymphosep, Biosera, UK) followed by centrifugation. Before flow cytometry, samples were incubated with 10 ng/mL of phorbol 12-myristate 13-acetate (PMA) and 0.5 mM ionomycin at 37 °C in a 5% CO<sub>2</sub> humidified incubator for 5 h. Chemical stimulators such as monensin (eBioscience, San Diego, CA, USA) were used to improve the staining of intracellular cytokines. Before staining, cells were washed and incubated with monoclonal antibodies including anti-CD4-APC (BD Biosciences), fluorescein isothiocyanate (FITC)-labeled anti-IFN- $\gamma$  (Becton Dickinson, Franklin Lakes, NJ, USA), and anti-IL-4-phycoerythrin (PE) (Becton Dickinson). Th1 and Th2 cells were detected among PBMCs using the BD FACSCalibur™ flow cytometer (San Jose, CA, USA). Th1 cells were defined as CD4+ lymphocytes with intracellular IFN- $\gamma$  but without IL-4. Th2 cells were detected as CD4+ lymphocytes with intracellular IL-4 but without IFN- $\gamma$ . The ratio of IFN- $\gamma$  to IL-4 positive Th cells was expressed as the Th1/Th2 ratio.

### Controlled ovarian stimulation and embryo transfer (ET)

Applied ovarian stimulation was completed as follows: According to the age and ovarian reserve of patients, 150–225 IU of rFSH (Gonal-F, Merck Serono, Tokyo, Japan) was administered on day 2

or 3 of the menstrual cycle. Follicular growth was assessed by transvaginal sonography. When follicles reached 12–13 mm in diameter, 0.25 mg/day of GnRH antagonist (Cetrotide, Merck Serono, Darmstadt, Germany) and 75 IU/day of human menopausal gonadotropin ([hMG], Menopur, Ferring, Switzerland) was administered until at least one follicle reached a diameter of 17–18 mm. At this point, 10 000 IU hCG (Pregnyl, Merck Serono) was injected and ovum pick-up (OPU) was performed 34–36 hours later. Intracytoplasmic sperm injection (ICSI) was utilized for all patients. For luteal phase support, hormone replacement cycle (HRC) was used, starting with the administration of an oral contraceptive (Ovocept-HD, Abureihan Co., Iran) and a GnRH agonist (Dipheline 3.75 mg, Ipsen, France), and in the next cycle, treatment was continued with conjugated estrogens (1.25 mg equilenin, Grupo Ferrer Internacional SA, Spain) until an appropriate endometrial thickness was achieved (at least 7 mm under transvaginal sonography). Frozen ET was performed on day 19–21 of the menstrual cycle and all patients received two morphologically good quality blastocysts. From the day of ET until pregnancy testing, patients were treated with Cyclogest® (400 mg pessary, Actoverco, Karaj, Iran). A biochemical pregnancy test was performed 14 days after ET. Clinical pregnancy was recognized when the development of a gestational sac was detected on transvaginal ultrasound 21 days after ET. Ongoing pregnancy was recognized when a normal fetus was seen on transvaginal ultrasound at 12 weeks of gestation. The primary and secondary endpoints were clinical pregnancy and ongoing pregnancy, respectively.

### Tacrolimus treatment

Patients were treated with two cycles of tacrolimus (Prograf, Astellas Pharma Ltd, Staines, UK), one for the endometrial biopsy (second endometrial biopsy) and one for the embryo transfer. In the first treatment cycle, patients were treated with tacrolimus for 16 days before undergoing endometrial biopsy. Similarly, in the second treatment cycle, we administered tacrolimus for 16 days (two days before ET and up to 14 days after ET). The daily dosage of tacrolimus was 1–3 mg, depending on the degree of Th1/Th2 elevation; patients with a mild increase in the Th1/Th2 ratio ( $\geq 10.3$  and  $< 13.0$ ) were treated with 1 mg of tacrolimus, patients with a moderate increase in the Th1/Th2 ratio ( $\geq 13.0$  and  $< 15.8$ ) were treated with 2 mg of tacrolimus, and patients with high increase in the Th1/Th2 ratio ( $\geq 15.8$ ) were treated with 3 mg of tacrolimus.

### Steroid hormone evaluation

Serum estradiol and progesterone were evaluated using commercial ELISA kits (MyBioSource, San Diego, USA) according to the manufacturer's instructions. Both hormones were analyzed in cycles with and without tacrolimus treatment 7 to 9 days after the LH surge.

### Endometrial biopsy

The endometrium was gently aspirated by rotating a Pipelle de Cornier (Laboratoire CCD, Paris, France) in the endometrial cavity during the window of implantation (LH + 7 to LH + 9) in two different consecutive cycles. The Pipelle contents were emptied onto a gauze compress and divided into two parts, one of which was

placed in 4% formaldehyde for routine evaluation, endometrial dating, and CD56 immunolabeling. The second part was placed in RNAlater™ stabilization solution for molecular analysis. A urinary LH kit (Abon Biopharma Company, China) was used to determine the day of the LH surge. Twenty samples of endometrial tissue from 10 patients were obtained in two subsequent menstrual cycles with and without tacrolimus treatment. The first biopsy was done in a cycle without treatment and the other biopsy in a second cycle with tacrolimus treatment. Samples were histologically assessed to match the secretory phase based on the appearance of tortuous glands, enlarged stromal cells, and the presence of subnuclear cytoplasmic glycogen vacuoles in the lumen of the glands. Data from one patient was excluded due to non-histo-compatibility with secretory phase features. Endometrial samples were used to evaluate the protein and mRNA levels of cytokines using immunohistochemistry and real time-PCR techniques, respectively.

### mRNA expression of p53, LIF, IL-4, IL-10, IL-17, and IFN- $\gamma$

Total RNA was extracted from 20 endometrial tissues obtained from ten RIF patients using an RNeasy Plus Kit (QIAGEN, USA) according to the manufacturer's instructions. The concentration of each RNA sample was measured using a NanoDrop 2000 Spectrophotometer (ND-1000, NanoDrop technology, Australia). Complementary DNA (cDNA) was synthesized using a PrimerScript RT reagent kit (Takara Bio, Otsu, Japan). SYBR Green was employed for the analysis of p53, LIF, IL-4, IL-10, IL-17, and IFN- $\gamma$  expression. Sequences of the study primer sequences are summarized in ► **Table 1**. In the PCR program, after 2 min at 50°C and 2 min at 95°C as an activation step, 40 cycles comprised of 15 s denaturation at 95°C, 15 s annealing at 60°C, and extension at 72°C for 1 min were performed using a StepOnePlus real-time PCR system (Applied Biosystems, Fisher Scientific, US). Gene expression was analyzed by comparison to a reference gene in each PCR run. Relative gene expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method and expressed as a fold change of the control sample. All reactions were performed in triplicate.

### Protein expression of LIF, IL-4, IL-10, IL-17, and IFN- $\gamma$

One part of the endometrial biopsies was immediately placed into 10% neutral buffered formalin for overnight fixation and then embedded in paraffin wax. LIF, IFN- $\gamma$ , IL-4, IL-10, and IL-17 protein expression as well as CD56+ cells (uNK cells) were determined using fluorescent immunohistochemistry. Endometrial sections with a thickness of 5  $\mu$ m were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 20 min and permeabilized by 0.3% Triton X100 for 30 min and blocked in 10% normal goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature. Samples were incubated overnight at 4°C with the corresponding primary antibodies for LIF (ab113262, Abcam, Cambridge, UK), IFN- $\gamma$  (ab25101, Abcam), IL-4 (ab2503, Abcam), IL-10 (ab34843, Abcam), and IL-17 (ab79056, Abcam) diluted in PBS. Samples were then incubated with FITC-conjugated secondary antibody (ab6717, Abcam) diluted with PBS at room temperature and kept in the dark for 1 h. Sections were subsequently mounted with 4'6-diamidino-2'-phenylindole dihydrochloride (DAPI, Roche Di-

► **Table 1** Primer sequences for real-time PCR.

		Sequence (5' → 3')	TM (°C)	Product size (bp)
TP53	Forward	AGCCACATTCTAGGTAGGGGC	61.78	130
	Reverse	TGCACTCTGTGAGGTAGGTGC	61.78	
LIF	Forward	GAAGCTGGGCTGTCAACTCCT	61.78	99
	Reverse	CCTCGGTTACAGCACACTTC	61.78	
IL-10	Forward	TCCCCTAACCTCATTCCCAA	59.82	90
	Reverse	GCCCGGCTAGAACCAATTT	59.82	
IL-17	Forward	CCGGCTGGAGAAGATACTGGT	61.78	126
	Reverse	CTGGGTCGGCTCTCCATAGTC	63.73	
IL-4	Forward	GCTGATCCGATTCTGAAA	54.51	89
	Reverse	TACTCTGGTTGGCTTCCT	53.69	
IFN-γ	Forward	CTTTAAAGATGACCAGAGCATCCA	59.30	94
	Reverse	ATCTCGTTCTTTTTTGTGCTATTGA	56.92	
β-actin	Forward	GCATGGGTCAGAAGGATTCTCT	61.88	106
	Reverse	TCGTCCCACTGGTGACC	61.83	

LIF: leukemia inhibitory factor, IL-10: interleukin-10, IL-17: interleukin-17, IL-4: interleukin-4, IFN-γ: interferon gamma, β-actin: beta-actin

agnostics, Germany) to stain the nuclei and preserve fluorescence signals. Fluorescent signals were analyzed with fluorescence microscopy (Olympus BX51, Olympus Optical Co., Tokyo, Japan). For each patient, endometrial tissue samples were analyzed in triplicate. Triplicate tissue was a serial section from the same paraffin tissue block. All samples were imaged using a 400× objective lens.

### Immunohistochemistry of uterine NK cells

Immunohistochemistry (IHC) with pre-diluted anti-CD56 (clone 123C3) mouse monoclonal primary antibody (Ventana Medical Systems, Roche Diagnostics, Germany) was performed on twenty endometrial biopsies obtained from ten RIF patients to detect uterine NK cells (uNKs). CD56-positive cells were counted per one mm<sup>2</sup> of the tissue slide. Images were taken and prepared with an on-board camera (Cyber-Shot, Zeiss, Japan). Scale bars were produced using a morphometric lens (CH-2, Olympus, Germany) and proofed with Measure software (C Thing Software, Sunnyvale, CA, USA). All images were captured under 100× magnification. Pixel-based frequency values were obtained using Image-Pro-insight software (Version 9.0, Media Cybernetics, USA).

### Statistical analysis

Statistical analysis was performed using SPSS-PC statistics software (version 23; SPSS Inc., Chicago, IL, USA). Since the study data were normally distributed, paired t-test and student's t-test were applied to compare data before and after tacrolimus treatment and between pregnant and non-pregnant patients, respectively. Because of the normality of distribution, we used Pearson or Spearman tests to evaluate correlation. A p-value < 0.05 was considered statistically significant.

## Results

### Patient hormone levels

Estradiol and progesterone levels in peripheral blood were measured on days 7–9 after the LH surge in cycles with and without tacrolimus treatment. Both estradiol (125.70 ± 74.13 vs. 117.46 ± 91.86 pg/ml, p = 0.84) and progesterone (5.21 ± 3.29 vs. 3.90 ± 2.58 ng/ml, p = 0.36) were lower in the cycle with tacrolimus treatment compared to the cycle without tacrolimus. However, the differences did not reach statistical significance.

### mRNA and protein expression of endometrial markers in endometrial samples

The expression of IL-4, IL-10, IL-17, IFN-γ, LIF, and p53 mRNA in the endometrial samples was not statistically different with and without tacrolimus treatment (► **Fig. 2a**). Although the expression of IL-10, IL-17, and p53 decreased following tacrolimus treatment, statistical significance was not reached (p > 0.05). The expression ratios of IFN-γ/IL-4 and IFN-γ/IL-10 also did not show a significant change following tacrolimus treatment (p > 0.05, ► **Fig. 2a**).

In the cycle without tacrolimus treatment, positive correlations were found between p53 and LIF, IL-10, IL-17, IFN-γ, and the IFN-γ/IL-4 ratio ( $r_s = 0.793, 0.794, 0.937, 0.694,$  and  $0.772$ , respectively). LIF mRNA was positively correlated with IL-10 and IL-17 expression ( $r_s = 0.716$  and  $0.896$ , respectively). Furthermore, IL-10 expression showed positive correlations with IL-17 and IFN-γ expression as well as with the IFN-γ/IL-4 ratio in the cycle without tacrolimus treatment ( $r_s = 0.872, 0.816,$  and  $0.857$ , respectively). IL-17 expression was positively correlated with the IFN-γ/IL-4 ratio (p = 0.032 and  $r_s = 0.712$ ). In the cycle with tacrolimus treatment, we observed positive correlations between p53 and LIF and IL-17

expression ( $r_s = 0.972$  and  $0.885$ , respectively). IL-4 was significantly correlated with IFN- $\gamma$  in the treated cycle ( $r_s = 0.812$ ).

Protein expressions of endometrial IL-4, IL-10, IL-17, IFN- $\gamma$ , and LIF in the cycles with and without tacrolimus treatment are illustrated in ► Fig. 2b. Significant decreases in IL-4 and IFN- $\gamma$  protein, as well as a decrease in the IFN- $\gamma$ /IL-10 ratio, were observed following tacrolimus administration ( $p < 0.05$ ). IL-10, IL-17, and LIF protein were significantly higher in the endometrial tissues treated with tacrolimus than those without treatment ( $p < 0.05$ , ► Fig. 2b).

Without tacrolimus treatment, a negative correlation was found between IL-10 and the IFN- $\gamma$ /IL-10 ratio ( $r_s = -0.875$ ). Also, IFN- $\gamma$  protein was positively correlated with IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10 ratios ( $r_s = 0.851$  and  $0.795$ , respectively). Similarly, with tacrolimus treatment, a negative correlation between IL-10 and the IFN- $\gamma$ /IL-10 ratio ( $r_s = -0.718$ ) was observed, while positive correlations between IFN- $\gamma$  and the IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10 ratios were observed ( $r_s = 0.835$  and  $0.866$ , respectively).

### Protein and mRNA levels of endometrial markers in pregnant and non-pregnant women

Cytokine mRNA and protein levels were compared in pregnant and non-pregnant women (► Fig. 3a and b, respectively). There was no significant difference in mRNA (► Fig. 3a) and protein (► Fig. 3b) expression between pregnant and non-pregnant women with tacrolimus treatment ( $p > 0.05$ ).

We also compared mRNA and protein levels in the previous cycle (without tacrolimus treatment) in pregnant and non-pregnant participants. The mRNA expression of IL-4 and IFN- $\gamma$  was significantly higher in the endometrial tissue of pregnant compared to non-pregnant women in the cycle without treatment ( $p = 0.03$  and  $0.05$ , respectively). Protein levels did not show any significant difference between pregnant and non-pregnant patients.

The mRNA expression of IL-4, IL-10, IL-17, IFN- $\gamma$ , LIF, and p53, as well as the IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10 ratios in endometrial tissues on days 7–9 after the LH surge did not have a significant association with the implantation rate (► Table 2). However, there was a significant positive correlation between IL-10 protein levels and implantation rates ( $p = 0.03$ , ► Table 2).

### Tacrolimus treatment improved RIF patient outcomes

Embryo transfer was carried out in ten patients who received tacrolimus. The treatment resulted in an implantation rate of 40%, a clinical pregnancy rate of 50% and a live birth rate of 35% in RIF patients with elevated Th1/Th2 ratios who had previously failed to become pregnant after at least three separate embryo transfers.

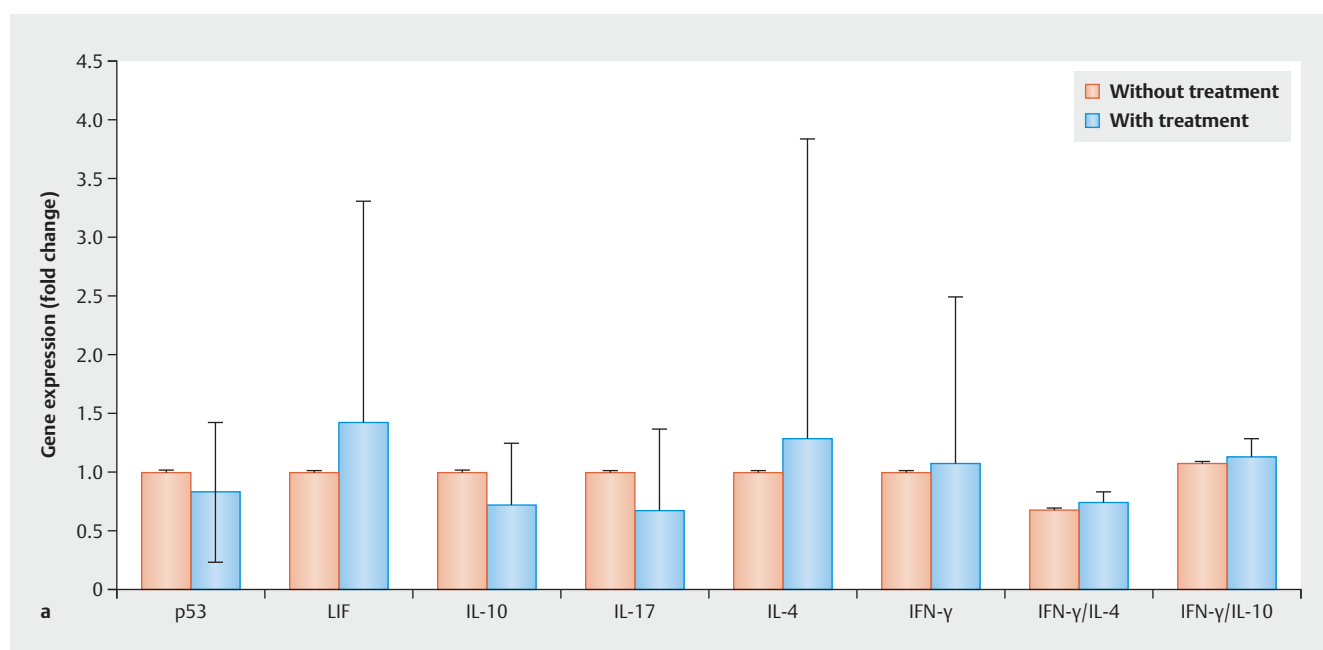
### Effect of tacrolimus on uNK in endometrial tissue

The number of uNK cells was higher in the cycle without tacrolimus treatment compared to the cycle with tacrolimus; however, this difference did not reach statistical significance ( $p = 0.06$ , ► Fig. 4a). We did not find a difference in uNK cell counts between pregnant and non-pregnant women ( $p > 0.05$ , ► Fig. 4b).

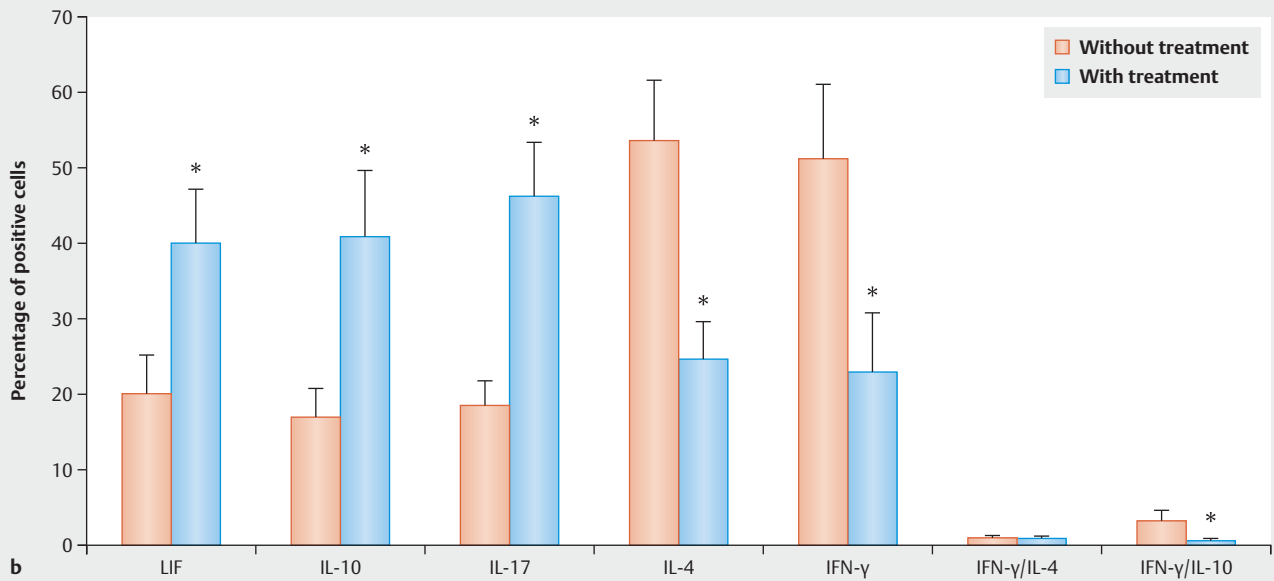
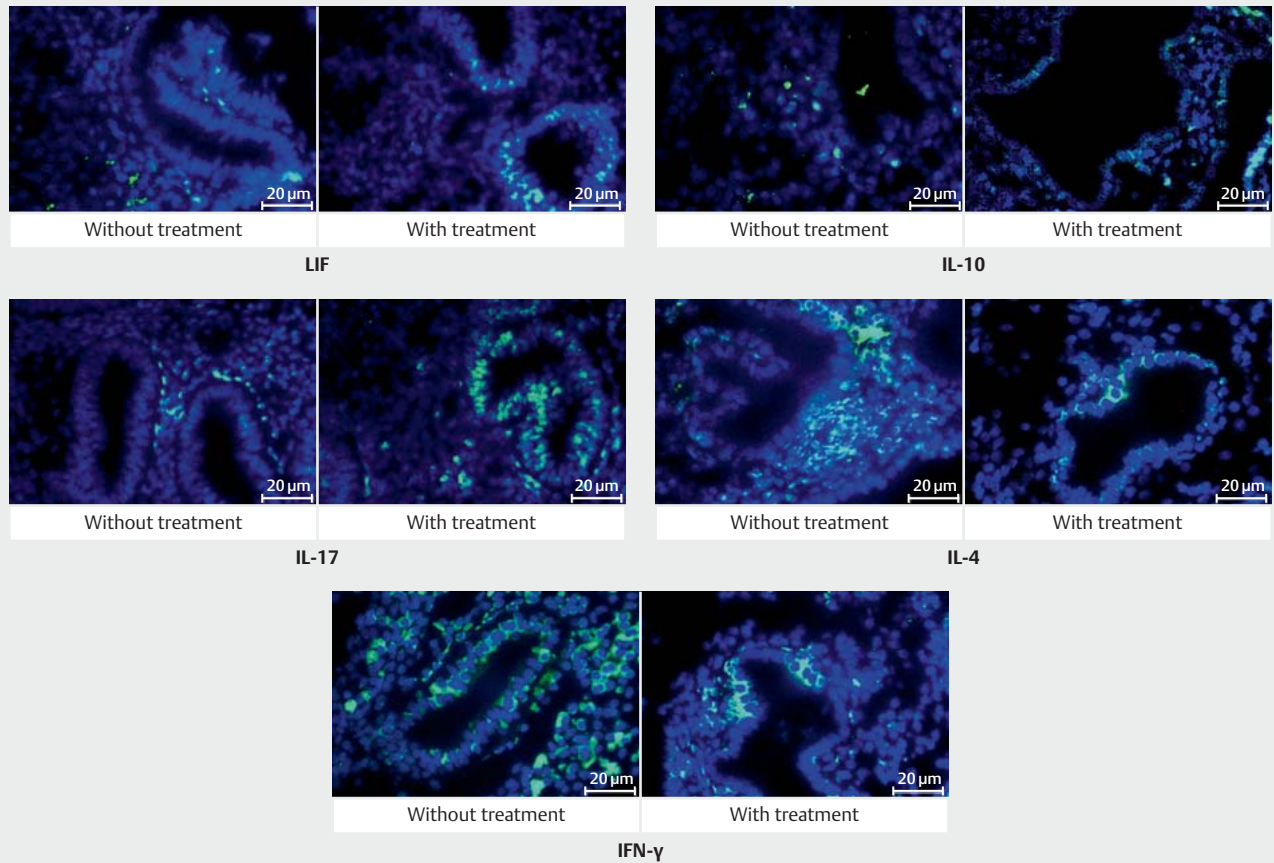
## Discussion

Given the ability of tacrolimus to increase implantation and pregnancy rates in RIF patients with an elevated Th1/Th2 ratio [4, 8], the effects of tacrolimus administration on the expression of endometrial factors (p53, LIF, IFN- $\gamma$ , IL-4, IL-10, and IL-17) were assessed along with ART outcomes in RIF patients. The in-vitro expression of these factors in human endometrial epithelium cells (EECs) and endometrial stromal cells (ESCs) after treatment with different doses of tacrolimus was also assessed.

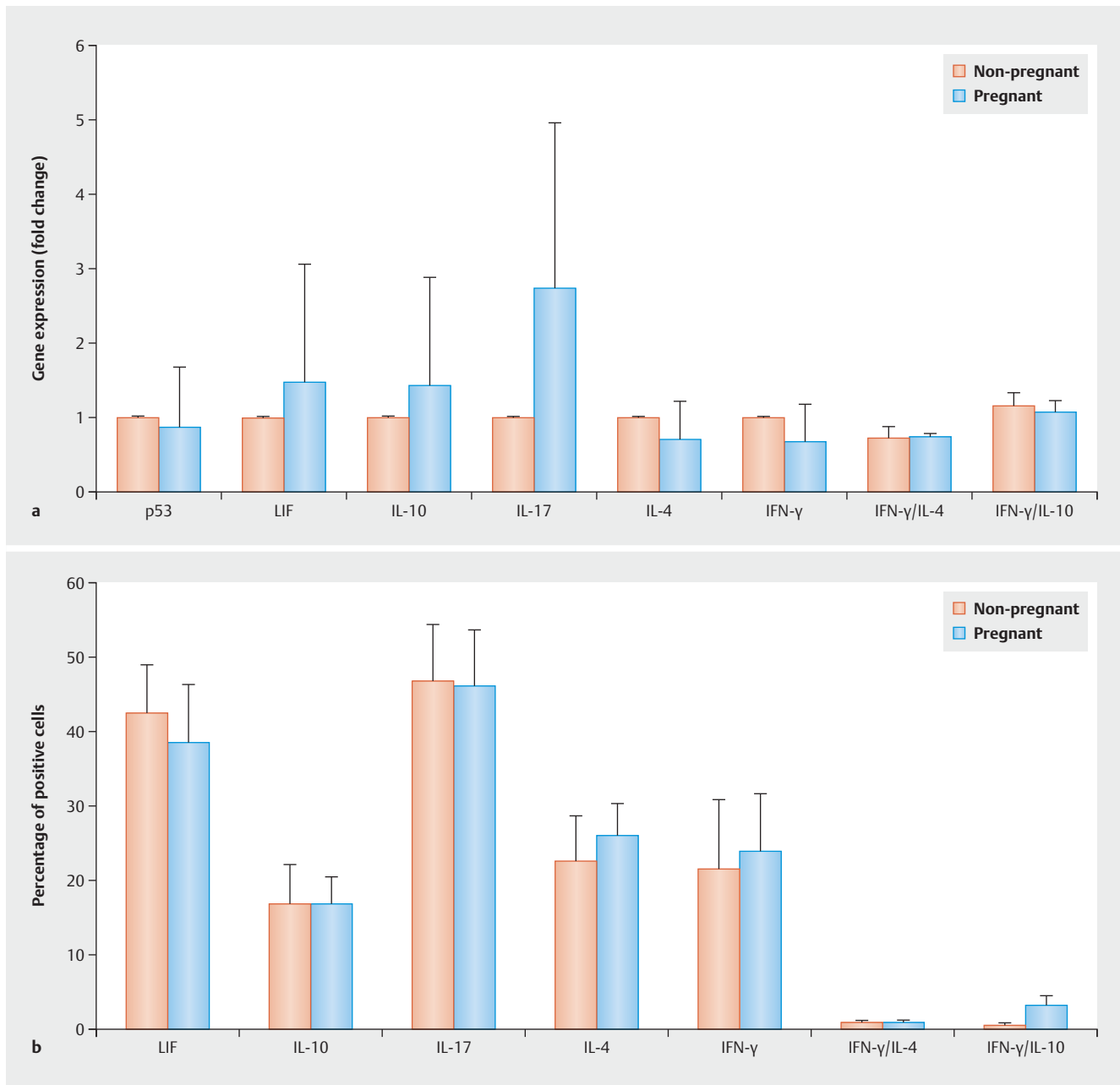
Endometrial LIF protein levels increased significantly after treatment with tacrolimus. To the best of our knowledge, this is



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**Fig. 2** p53, LIF, IL-10, IL-17, IL-4, and IFN- $\gamma$ , mRNA (a) and protein (b) levels in the endometrial tissue of women with RIF with and without tacrolimus treatment. Fluorescent immunohistochemistry was conducted using primary antibodies against IL-4, IL-10, and IL-17, IFN- $\gamma$ , LIF, and fluorescein isothiocyanate-conjugated secondary antibody. Nuclei are stained with DAPI. All images were captured under 400 $\times$  magnification. LIF: leukemia inhibitory factor, IL: interleukin, IFN- $\gamma$ : interferon gamma. The nuclei are shown in blue (stained with DAPI) and the evaluated factors in green (fluorescence signals). \* = Statistically different in compared to without treatment group.

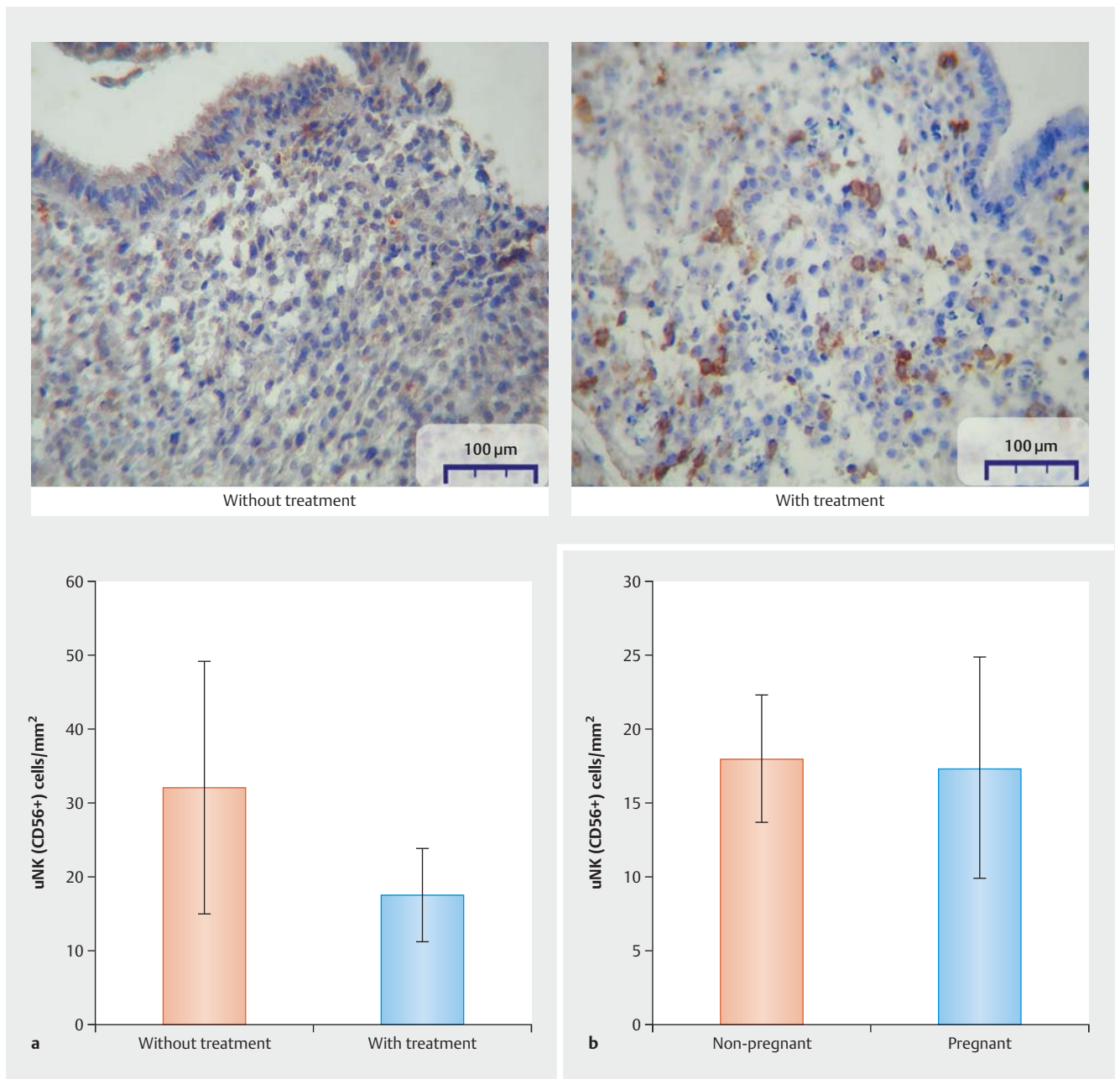


► **Fig. 3** p53, LIF, IL-10, IL-17, IL-4, and IFN- $\gamma$ , mRNA (a) and protein (b) levels in the endometrial tissue of pregnant and non-pregnant women with RIF who had tacrolimus treatment. LIF: leukemia inhibitory factor, IL: interleukin, IFN- $\gamma$ : interferon gamma.

the first description of the effect of tacrolimus on LIF. LIF is a critical parameter in embryonic development and implantation [27, 28], trophoblast growth and differentiation [29, 30], luminal epithelial pinopode formation [31], and extravillous trophoblast (EVT) invasion [32]. Moreover, LIF enhances decidualization of stromal cells [33] and contributes to the blastocyst hatching process [34, 35]. The role of LIF in maternal immune tolerance during pregnancy through the regulation of human leukocyte antigen G (HLA-G) [36], which suppresses immune reactions and re-modulates spiral arteries by changing cytokine secretion, consequently facilitates embryo implantation and pregnancy [37]. HLA-G is also

involved in establishing a balance between Th1 and Th2 cells that is important for a successful pregnancy [38]. Our data also showed that the mRNA expression of LIF in the pregnant group was higher compared to the non-pregnant group in both tacrolimus treated and non-treated cycles, demonstrating the importance of LIF for endometrial receptivity and embryo implantation. Higher levels of LIF expression were observed in the endometrial tissue of pregnant compared to non-pregnant women during the WOI, and tacrolimus appeared to have a stimulatory effect. Therefore, tacrolimus may improve implantation via the induction of LIF. However, LIF protein levels were lower in pregnant women





► **Fig. 4** Number of uterine natural killer cells (uNKs) in the endometrial tissue of women with RIF. **a** uNKs in the endometrial tissues with and without tacrolimus treatment. **b** uNKs in the endometrial tissues of pregnant and non-pregnant women receiving tacrolimus treatment. Immunohistochemistry staining for CD56+ (a marker of uNKs) allowed for counting per one mm<sup>2</sup> of the tissue slide. Scale bars were produced using a morphometric lens (CH-2, Olympus, Germany) and proofed with Measure software. All images were captured under 100× magnification.

compared to non-pregnant women, which could be due to the high rate of LIF secretion into the uterine lumen [39, 40].

During pregnancy, the maternal immune system shifts toward the production of Th2-type cytokines such as IL-10 [2, 41] in both the maternal circulation and the endometrium. IL-10 plays an important role in maternal immune tolerance [42, 43] via several mechanisms, including the inhibition of pro-inflammatory Th1-type cytokines (e.g. IFN-γ), macrophage production [44, 45], up-regulation of soluble HLA-G [46], and the induction of angiogen-

esis and placental growth [2]. Interestingly, we found that tacrolimus treatment significantly increased endometrial IL-10, which can improve maternal immune tolerance and the chances of implantation. There are several studies demonstrating elevated IL-10 levels after treatment with immunosuppressant agents such as tacrolimus [47–49].

The role of IL-17 in embryo implantation has not yet been determined. However, due to its pro-inflammatory properties [50], it may have a role in embryo rejection. Since embryo implantation

► **Table 2** Association between embryo implantation rate and cytokine mRNA and protein levels with tacrolimus treatment.

	p53	LIF	IL-4	IL-10	IL-17	IFN- $\gamma$	IFN- $\gamma$ /IL-4	IFN- $\gamma$ /IL-10
Embryo implantation rate	mRNA levels							
	$r_s = -0.351$ $p = 0.394$	$r_s = -0.169$ $p = 0.688$	$r_s = -0.365$ $p = 0.374$	$r_s = -0.052$ $p = 0.902$	$r_s = 0.266$ $p = 0.524$	$r_s = -0.240$ $p = 0.568$	$r_s = -0.128$ $p = 0.762$	$r_s = -0.247$ $p = 0.556$
	Protein levels							
	–	$r_s = 0.341$ $p = 0.409$	$r_s = -0.323$ $p = 0.436$	$r_s = 0.735$ $p = 0.038$	$r_s = 0.048$ $p = 0.910$	$r_s = -0.219$ $p = 0.602$	$r_s = -0.115$ $p = 0.786$	$r_s = -0.537$ $p = 0.170$

$r_s$ : Spearman's correlation coefficient, LIF: leukemia inhibitory factor, IL: interleukin, IFN- $\gamma$ : interferon gamma

is a semi-allograft, implantation failure may be similar to organ transplant rejection, in which IL-17 plays an important role [15, 16]. On the other hand, IL-17 may pave the way for implantation and pregnancy by inducing angiogenesis [51] and progesterone secretion [52]. We found that tacrolimus significantly increased IL-17 protein levels in the endometrium. However, other studies have reported that tacrolimus and other immunosuppressive agents such as cyclosporine A may decrease IL-17 levels in peripheral blood [20, 53–55]. There are no previous reports of the effect of tacrolimus on IL-17 in the endometrium. Interestingly, we found respectively higher and lower mRNA and protein levels of IL-17 in the pregnant group compared to the non-pregnant group, which may be due to the high degradation rate of IL-17 protein in a receptive endometrium or the high rate of IL-17 secretion by the endometrial cells of a receptive uterus. In the present study, both treated and non-treated cycles showed a strong positive correlation between IL-17 and LIF, an important receptivity marker, indicating a positive association between IL-17 and endometrial receptivity. Thus, upregulation of IL-17 following tacrolimus administration in RIF patients could improve endometrial receptivity and, consequently, implantation.

Elevated levels of Th2 cytokines lead to successful implantation, whereas a Th1-dominant environment causes implantation failure [41]. Our study showed that the protein levels of both IL-4 and IFN- $\gamma$  (Th1-related cytokines) significantly decreased following tacrolimus administration. Furthermore, tacrolimus reduced IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10 ratios, favoring embryo implantation in RIF patients. These findings are aligned with those of other studies which reported an inhibitory effect of tacrolimus on Th1 cytokines [56, 57]. In accordance with our results, Nakagawa et al. [8] also stated that tacrolimus could suppress Th1 but not Th2 cells in the uterus. Since we found that in the non-treated cycles the IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10 ratios were lower in the pregnant group compared to the non-pregnant group, RIF patients with a low Th1/Th2 ratio may be good candidates for tacrolimus therapy. According to our results, patients with low ratios had better outcomes while tacrolimus was less effective in patients with higher ratios. However, further studies with larger sample sizes are required to define a cut-off value for the ratio as an index figure for tacrolimus therapy.

uNKs are also necessary for the establishment of local maternal tolerance and the survival of the fetus [2]. These cells are different from blood NKs as they have no cytotoxicity potential. uNKs play

important roles in the production of Th2 cytokines [18], angiogenic factors, and spiral artery remodeling [58, 59]. We observed that in the cycle before treatment with tacrolimus, levels of uNKs were higher in the pregnant group compared to the non-pregnant group, hinting at the importance of these cells in endometrial receptivity. The number of uNKs decreased after treatment with tacrolimus. Previous studies have also pointed to a reduction in the number of NKs and the impairment of their function following tacrolimus administration [60, 61]. Due to a lack of information about the effect of tacrolimus and other immunosuppressive agents on uNKs, it is not known how tacrolimus could decrease the number of uNKs in the endometrium and how such an inhibitory effect might influence implantation and pregnancy rates. Further studies are required to clarify whether a reduction in uNK cells results from tacrolimus treatment and impacts endometrial receptivity and implantation.

To the best of our knowledge, this is the first study to evaluate the effect of tacrolimus on endometrial receptivity, implantation, and pregnancy outcomes. It should be mentioned that our findings demonstrated that tacrolimus significantly affects the protein levels of IL-10, IL-17, and LIF rather than their mRNA expression. There are two possible explanations for this effect. First, tacrolimus could induce translation of the genes mRNAs or stability of their protein. Secondly, we evaluated the percentage of positive cells for the protein, and it is possible that tacrolimus increases the number of endometrial cells that express these factors instead of increasing the expression of the factors in each cell. However, the main limitation of this study is its small sample size and the unwillingness of patients to undergo two endometrial biopsies. Further studies with larger sample sizes are required to confirm our findings. Moreover, more studies are needed to investigate other immunosuppressive agents such as cyclosporine A or sirolimus and their potential impact for improving implantation rates in RIF patients.

In conclusion, tacrolimus significantly increased the expression of LIF, IL-10, and IL-17 and decreased the expression of IL-4, IFN- $\gamma$  and the IFN- $\gamma$ /IL-10 ratio in RIF patients. Tacrolimus treatment led to an implantation rate of 40%, clinical pregnancy rate of 50% and a live birth rate of 35% in RIF patients with elevated Th1/Th2 ratios who had previously failed to become pregnant despite at least three transfers of embryos. Our findings suggest that RIF patients with higher Th1/Th2 ratios could be candidates for tacrolimus therapy. Furthermore, we found a reduced estradiol and progesterone

terone serum levels after treatment with tacrolimus, suggesting that luteal phase support should be considered in the ART cycle of patients receiving tacrolimus.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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