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The Link Between Seminal Cytokine Interleukin 18, Female Circulating Regulatory T Cells, and IVF/ICSI Success

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Abstract

Seminal plasma (SP) is thought to be a crucial factor which affects the expansion of regulatory T cells (Tregs) in female reproductive tract during embryo implantation. We propose that seminal transforming growth factor (TGF) β1 is responsible for local accumulation of circulating Tregs, which manifests as changes in Treg frequency in peripheral blood, whereas seminal interleukin (IL) 18 interferes with TGF-β1-dependent cellular reactions. The purpose of the present study is to determine whether the frequency of circulating Tregs is associated with the levels of seminal cytokines and pregnancy establishment in women exposed to partner's SP during in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycle. Twenty-nine women were exposed to SP via timed intercourse before the day of ovum pickup (day-OPU) and also subjected to intravaginal SP application just after OPU. Measurements of seminal TGF-β1 and IL-18 were made by FlowCytomix technology. The percentage of $CD4^+CD25^+CD127^{low+/-}$ Tregs among total circulating $CD4^+$ T cells was determined by flow cytometry and the difference between Treg values on the day of embryo transfer and day-OPU was calculated. The percentage of Tregs on the day-OPU, identified as a predictive factor of clinical pregnancy after IVF/ICSI, showed a positive correlation with IL-18 concentration and content of this cytokine per ejaculate ($P < .001$ and $P < .004$, respectively) and negative correlation with the TGF-β1/IL-18 ratio ($P < .014$).These findings indicate that the adverse effect of seminal IL-18 excess on implantation may be realized by the prevention of postcoital TGF-β1-related migration of circulating Tregs, which clearly manifests as elevated level of Treg frequency in peripheral blood.

Keywords

seminal plasma, transforming growth factor-β1, interleukin 18, regulatory T cells, IVF/ICSI outcome

Introduction

It has become increasingly evident that seminal plasma (SP) is a key factor involved in the immunoregulation of female reproductive functionality.[1] Many studies have shown that SP deposition in the female reproductive tract (FRT) induces an active inflammatory response that is required to accommodate pregnancy in several animal species,[2-4] as well as humans.[5]

It has also been documented that SP causes local postcoital expansion of regulatory T cells (Tregs) within the FRT and/or draining lymph nodes (LNs) of experimental animals.[6-10] Regulatory T cells represent a subset of $CD4^+$ T cells that specifically express the master transcription factor, Foxp3,[11] and play a key role in suppressing inappropriate inflammatory responses and in the generation of immune tolerance.[12] Moreover, many studies in both mice,[13-16] and humans[17-21] have demonstrated that Tregs are important for the regulation of fetal and antipaternal immune responses, which are essential for a successful pregnancy.

Available data in the literature concerning animal model systems clearly indicate that the role of Tregs during implantation may be much more crucial than that exhibited at later stages of pregnancy.[14,15,22] Furthermore, the role of Tregs during implantation in humans is supported by the association of

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unexplained infertility with reduced Foxp3 messenger RNA (mRNA) expression within the endometrial tissue.[23]

Previous reports have concluded that the migration of thymic-derived Tregs plays a crucial role in the preparation of the uterus for implantation.[24] Moreover, self Ag-specific Tregs[25] as well as paternal antigen-specific Tregs[10] may be recruited to the pregnant uterus from the uterine-draining LNs.

The accumulation of Tregs in the uterus, potentially in response to the production of several chemokines, has been observed before mating in mice.[9,26] Recently, it was shown that low level of CC chemokine receptor 4 expression on Tregs is associated with recurrent pregnancy failure in women who underwent in vitro fertilization (IVF).[27] In addition, human chorionic gonadotropin (hCG) attracts Tregs into the fetomaternal interface during the early stages of human pregnancy via the upregulation of the luteinizing hormone/choriogonadotropin receptor on the surface of Tregs.[28] Thus, the migratory capacity of Tregs is thought to be crucial to their regulatory function during the implantation period, while SP can be one of the important factors providing postcoital accumulation of circulating Tregs in the uterus.

Global transcriptomic profiling demonstrated that SP contains bioactive factors capable of eliciting chemotactic responses in the uterus, which can lead to recruitment of leukocytes to the endometrium.[29] Several experimental studies have identified transforming growth factor (TGF) β1 as a key player in the induction, resolution, and control of the local inflammatory response to SP. It was shown that TGF-β1 is critical to the generation of $CD4^+CD25^+Foxp3^+$-Tregs from naive $CD4^+$ T cells,[30] as well as the proliferation of Treg cells.[9] Along with that, TGF-β1-mediated postcoital migration of Tregs can be the determining factor of modulation of uterine immunosuppressive environment required for successful pregnancy. Transforming growth factor β stimulates the release of granulocyte-macrophage colony-stimulating factor (GM-CSF), thereby activating the GM-CSF-dependent migration of the myeloid cells,[31,32] which in turn may be involved in attracting $CD4^+CD25^+$ T cells due to the production of chemokines.[33]

Moreover, TGF-β1 can indirectly influence Treg migration by upregulating the expression of CC chemokine ligand 19 (CCL19) mRNA in murine uterine glandular and luminal epithelial cells.[8] CCL19 can signal via the CC chemokine receptor 7 (CCR7) receptor to regulate Treg recruitment and retention in peripheral tissues.[9] It was shown that exogenous TGF-β3, delivered vaginally at mating, was accompanied by accumulation of $Foxp3^+$ T cells in the murine vaginal lumen, which led to the enhancement of the implantation rates as well as the reduction of the abortion rates in the mouse model of recurrent spontaneous abortion.[34]

In addition to TGF-β1, the SP of fertile men also contains a broad array of pro-inflammatory cytokines with the potential for antagonizing TGF-β1 signaling.[35] We have previously shown that a high level of seminal interleukin (IL) 18, originally identified as an Interferon gamma (IFN-γ)-inducing factor,[36] negatively impacts the pregnancy rate of IVF/intracytoplasmic sperm injection (ICSI)-treated women, who came into contact with SP during the IVF/ICSI cycle.[37] Therefore, the presence of excessive levels of IL-18 in the SP may potentially prevent TGF-β1-related cellular reactions, including the migration of Tregs into the FRT.

Thus, these findings led us to hypothesize that one of the mechanisms responsible for the adverse effects of seminal IL-18 on implantation may be the prevention of the TGF-β1-related local postcoital cellular reaction, including the migration of Tregs. Regulatory T-cell migration may be manifested both locally and systemically in response to changes in the percentage of these cells within the peripheral blood.[19,21] Therefore, the aim of the present study was to elucidate the relationship between the seminal TGF-β1 and IL-18 cytokine content, frequency of circulating Tregs following SP transmission, and pregnancy establishment in women having unprotected sexual relations until several days before the day of ovum pickup (day-OPU) and subsequently exposed to an intravaginal SP application just after OPU during IVF/ICSI treatment. Since the effect of SP transmitted during human sexual relations may manifest as the frequency of circulating Tregs during the preconception period and the intravaginal application of SP may result in the subsequent redistribution of Tregs, we analyzed the frequency of peripheral blood Tregs on the day-OPU and day of embryo transfer (day-ET), evaluated the changes in the content of Tregs in the period between these events, and correlated all of these parameters with the levels of TGF-β1 and IL-18 in the partner's SP.

Materials and Methods

Study Participants

Twenty-nine female patients diagnosed with tubal factor infertility and undergoing IVF/ICSI treatment were recruited into this study. The study also included the sexual partner for each patient. Ethical approval was obtained from the National Medical Research Center for Obstetrics, Gynecology and Perinatology Ethics Committee. Informed consent was given by all patients at the time of recruitment. The criteria for female patient enrollment into the study were as follows: age $\leq$41 years; tubal factor infertility; 0 to 2 previous IVF attempts; normal ovarian reserve testing by evaluation of serum levels of anti-Müllerian hormone and follicle-stimulating hormone (FSH) and antral follicular count with transvaginal sonography; normal anatomy of the uterus, estimated by transvaginal sonography combined with saline contrast sonohysterography; and the absence of pelvic inflammatory disorders or autoimmune diseases. No detectable pelvic pathology based on history of any previous surgery and history of symptoms such as pelvic pain, severe dysmenorrhea, and dyspareunia were also considered as criteria for patient selection. The exclusion criteria were also TORCH (TOxoplasmosis, Rubella, Cytomegalovirus, and Herpes) infections and sexually transmitted diseases with *Ureaplasma urealyticum, Mycoplasma, Chlamydia*, gonococcus, fungi, *Trichomonas vaginalis*, human immunodeficiency virus, *Treponema pallidum*, and hepatitis B. Male patients with

clinical signs of genital tract inflammation, varicocele, or autoimmune diseases were also excluded from the study.

Stimulation Protocol

Female patients included in the study were treated with a controlled ovarian hyperstimulation protocol for IVF with a gonadotropin-releasing hormone antagonist. All patients received recombinant FSH or highly purified human menopausal gonadotrophin. When at least 3 follicles reached preovulatory size (17-18 mm), 10 000 IU of hCG was administered. Transvaginal oocyte retrieval was performed ~36 hours later. Just after oocyte pickup, 0.5 mL fresh undiluted SP was injected into the vaginal vault of each patient. All embryos were allowed to cleave and the best 2 embryos were transferred into the uterus on the fifth day after oocyte collection. Micronized progesterone was used for luteal support. Patients had unprotected sexual relations until the third to fifth day prior the day-OPU. The couples were then advised not to have intercourse until the pregnancy results were known. Clinical pregnancy was defined as a visible intrauterine pregnancy sac, as estimated by ultrasound at 3 weeks' gestational age.

Specimen Collection and Preparation

Venous blood samples from female patients were collected into K2 EDTA tubes within 1 hour before the OPU. Semen samples were collected at the day-OPU by masturbation following a 3- to 5-day period of sexual abstinence. Each ejaculate was collected into sterile containers for conventional semen analysis, according to the World Health Organization (WHO) protocol.[38] DNA flow cytometry was used to distinguish leukocytes from immature sperm present in the ejaculate.[39] The direct mixed agglutination reaction (MAR) test was performed using a SpermMar Test (FertiPro, Beernem, Belgium) according to the WHO protocol.[38] Only specimens with an MAR percentage $\leq 10\%$ were used. After centrifugation, 0.5 mL SP was used for the intravaginal application. The remaining SP was aliquoted and stored at $-80^{\circ}C$.

Flow Cytometry Analysis of Treg Cells

PerCP-conjugated anti-human CD4, phycoerythrin-conjugated anti-human CD127, fluorescein isothiocyanate-conjugated anti-human CD25 (BD Bioscience, San Jose, California) were mixed with 100 μL of fresh anticoagulated whole blood. The reaction mixture was incubated for 30 minutes in the dark and gently stirred. Red blood cells were lysed with lysing solution (BD Biosciences) for 10 minutes, and samples were washed with phosphate-buffered saline. Flow cytometric analysis was performed using BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, California). The gate was set for both forward and side scatter parameters and included lymphocytes. After gating on $CD4^+$ T cells, the percentage of $CD4^+CD25^+CD127^{low+/-}$ Tregs within the $CD4^+$T cell was measured. Statistical analysis was based on at least 10 000 gated $CD4^+$ cells. The difference between Treg values on the day-ET and day-OPU (ΔTreg) and the absolute value of ΔTreg (|Δ|Treg) were calculated.

Measurements of TGF-β1 and IL-18 in SP

Quantifications of total TGF-β1 (active plus latent) as well as IL-18 were performed using a FlowCytomix Human TGF-β1 and IL-18 Simplex Kits (Bender MedSystems, Vienna, Austria). Acidic activation of the samples at the time of TGF-β1 testing was performed according to the manufacturers' instructions. Seminal plasma was tested at 1:120 and 1:4 dilutions for TGF-β1 and IL-18 analysis, respectively. All samples were analyzed by FACSCalibur cytometer. The concentrations of TGF-β1 (the sum of free TGF-β1 and TGF-β1 released from latent complexes at acidification, ng/mL) and IL-18 (pg/mL) were calculated using FlowCytomix Pro 3.0 Software (Bender MedSystems).The ratio of TGF-β1 to IL-18 concentration (TGF-β1/IL-18), the content of TGF-β1 and IL-18 in the intravaginally applied SP (calculated as the concentration of TGF-β1 or IL-18 × 0.5 mL), and the TGF-β1 and IL-18 content per ejaculate (calculated as the concentration of TGF-β1 or IL-18 × volume of ejaculate) were also determined.

Statistical Analysis

Statistical analysis was performed using MedCalc 12.3.0 software. Deviation from normality was tested using Shapiro-Wilk test. Mann-Whitney U test was used to compare data between pregnant and nonpregnant groups (unpaired test) and Wilcoxon test was used to compare data between day-OPU and day-ET (paired test). Differences between categorical variables were analyzed using the χ^2 test. Continuous variables were presented as medians (range, min to max). Categorical variables were presented as frequencies (%). The Spearman rank order correlation was applied to calculate correlation coefficients. Differences were considered to be statistically significant when $P \leq .05$.

Results

Baseline Characteristics

The clinical pregnancy rate in 29 patients exposed to SP during IVF/ICSI cycle was 48.3%. The clinical profiles of pregnant (n = 14) and nonpregnant (n = 15) groups were not significantly different (Table 1). Of the clinical pregnancies, 11 pregnancies yielded live births (37.9% live birth rate per transfer) and 3 ended in spontaneous abortions up to 12th week of gestation. As shown in Table 2, no differences were detected between conventional sperm parameters in partners of pregnant and nonpregnant patients.

Characterization of Seminal Cytokines

The results of quantification of cytokines in the partners' SP in both pregnant and nonpregnant groups are shown in Table 3.

Table 1. Demographic and Clinical Data of Study Participants.[a,b]

Characteristics	Pregnant (n = 14)	Nonpregnant (n = 15)	*P* Value
Age, years	31.5 (26-37)	33.0 (25-40)	1.000
Age of husband, years	34.5 (28-43)	34.0 (27-43)	.677
BMI, kg/m^2	22.28 (17.78-32.01)	21.97 (23.94-37.26)	.810
Duration of infertility, years	3.0 (1-8)	4.0 (1-11)	.393
Basal FSH levels, IU/L	5.98 (3.89-9.27)	6.25 (4.4-9.7)	.679
Basal LH levels, IU/L	5.8 (2.1-13.1)	4.37 (2.95-12.50)	.769
No. of oocytes collected	7.0 (3-15)	9.0 (2-15)	.726
Fertilization method			
IVF, n (%)	10 (71.4)	10 (66.7)	.782[c]
ICSI, n (%)	4 (28.6)	5 (33.3)	

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; LH, luteinizing hormone.
[a]Values are median (range) or number percentages.
[b]*P* Value for between-group difference from Mann-Whitney *U* tests unless otherwise stated.
[c]*P* Value for between-group difference from χ^2 tests.

Table 2. Standard Semen Parameters Analysis.[a,b]

Semen Parameters	Pregnant (n = 14)	Non-pregnant (n = 15)	*P* Value
Volume, mL	2.7 (1.2-4.2)	3.2 (1.4-6.0)	.198
Sperm concentration ($\times 10^6$/mL)	59 (6-218)	76 (17-142)	.326
Progressive motility (%)	51 (41-78)	44 (17-90)	.163
Sperm morphology (normal forms, %)	6 (3-9)	5 (2-9)	.465
Leukocyte ($\times 10^6$/mL)	0.1-2.4 (0.7)	0.1-2.3 (0.9)	.458

[a]Data are presented as median (range).
[b]*P* Value for between-group difference from Mann-Whitney *U* tests.

There was no difference in TGF-β1 levels between the 2 groups, whereas the concentration of IL-18 was significantly lower in pregnant group ($P = .020$). The content of IL-18 per ejaculate was also significantly lower in pregnant group ($P = .025$). The TGF-β1/IL-18 ratio was significantly higher in pregnant group ($P = .033$).

Characterization of Tregs

The percentage of Tregs on the day-OPU was analyzed in peripheral blood of all 29 patients included in this study, and 17 patients had 2 sequential Treg measurements on the day-OPU and on the day-ET.

The results of retrospective analysis of Treg percentage in peripheral blood of female patients in both pregnant and nonpregnant groups are shown in Figure 1A. The percentage of Tregs on the day-OPU was significantly lower in pregnant group than in nonpregnant group, with a median value of 4.99% (range: 3.01%-7.62%) in patients with IFV/ICSI success compared to 6.74% (range: 3.78%-9.06%) in patients with unsuccessful IVF/ICSI cycle ($P = .016$). The percentage of Tregs on the day-ET was also lower in pregnant group, with a median value of 4.93% (range: 2.49%-6.69%), compared to nonpregnant group, with a median value of 6.26% (range: 4.29%-8.90%); however, this difference was very close to the threshold of statistical significance ($P = .078$).

Analysis of temporal dynamic of Tregs revealed the bidirectional changes of Treg percentage in both pregnant and nonpregnant groups (Figure 1B). We have not revealed the statistically significant difference between the Treg percentage on the day-OPU and the day-ET in pregnant and nonpregnant groups ($P = .413$ and $P = .562$, respectively).

The difference between Treg percentage on the day-ET and day-OPU (ΔTreg) was calculated. An increase as well as a decrease in the percentage of Tregs on the day-ET compared to the day-OPU is indicated by positive or negative values of ΔTreg, respectively. There was no association between ΔTreg and pregnancy establishment (Figure 1C).

Analysis of absolute values of Treg changes—|Δ| Treg—revealed a significantly higher level of this parameter in pregnant group in comparison with nonpregnant group, with median value of 2.12% (range: 0.70%-3.68%) and 0.36% (range: 0.15%-1.43%), respectively, $P < .003$ (Figure 1D).

Table 3. Cytokine Levels in Partners' Seminal Plasma of Pregnant and Nonpregnant Patients.[a,b]

Parameters	Pregnant (n = 14)	Nonpregnant (n = 15)	*P* Value
Cytokine concentration			
TGF-β1, ng/mL	121.8 (74.1-167.0)	115.9 (70.4-211.6)	.777
IL-18, pg/mL	290.1 (181.4-555.3)	420.9 (188.7-602.3)	.020
Cytokine content per ejaculate			
TGF-β1, ng	299.8 (181.5-634.4)	379.2 (161.2-705.2)	.234
IL-18, pg	770.1 (325.3-1879.3)	1489.7 (572.1-2595.6)	.025
Ratio of TGF-β1-to-IL-18 concentration	476 (206-657)	288 (190-555)	.033

Abbreviations: IL, interleukin; TGF-β1, transforming growth factor β1.
[a]Data are presented as median (range).
[b]*P* Value for between-group difference from Mann-Whitney *U* tests.

Association Between Tregs and Seminal Cytokines

No correlation was revealed between the percentage of Tregs on the day-OPU and the concentration of TGF-β1, as well as

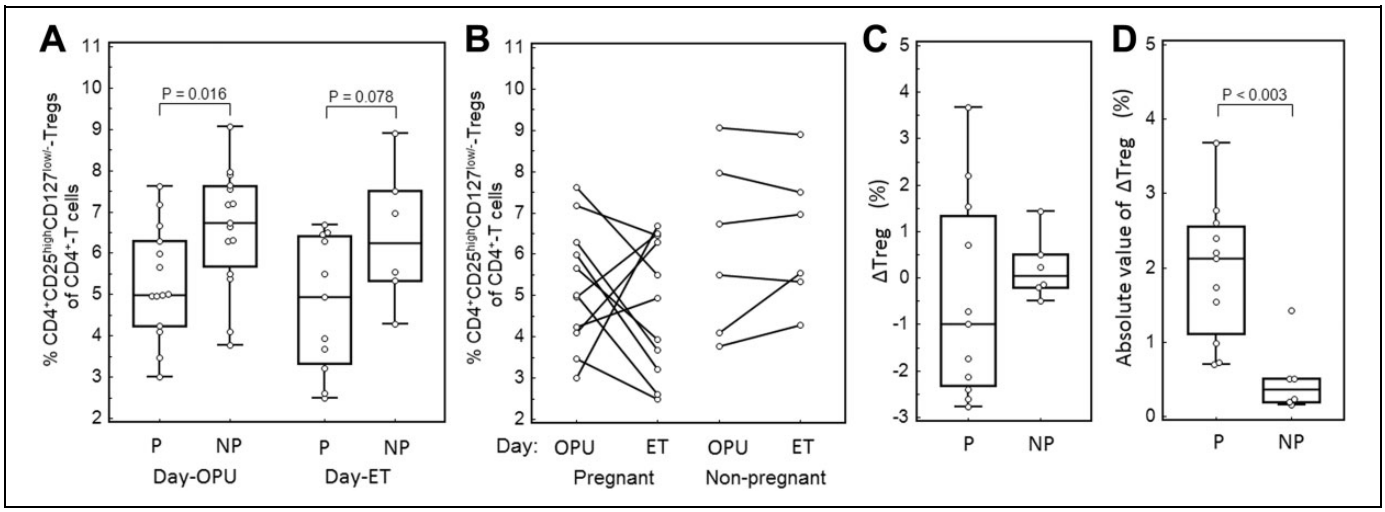

Figure 1. Frequency and dynamics of regulatory T cells (Tregs) in the peripheral blood of women from the pregnant (P) and nonpregnant (NP) groups exposed to partners' seminal plasma during in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycle. A, Comparison of Treg frequency in women on the day of ovum pickup (day-OPU) and day of embryo transfer (day-ET) using Mann-Whitney *U* Test. B, Comparison of Treg frequency in women on the day-OPU and day-ET using Wilcoxon test. C, The difference between Treg values in the female peripheral blood on the day-ET and day-OPU (ΔTreg). D, The absolute values of ΔTreg between Treg values on the day-ET and day-OPU. Horizontal lines indicate the median values.

Table 4. Correlations Between Cytokines in Partners' Seminal Plasma and Percentage of Tregs Among Total CD4+ T Cells in Peripheral Blood of Women on the Day-OPU and Day-ET, ΔTreg[a], and |Δ|Treg.[b,c]

	Percentage of Tregs							
	Day-OPU		Day-ET		ΔTreg		|Δ|Treg	
Parameters	ρ	*P* Value	ρ	*P* Value	ρ	*P* Value	ρ	*P* Value
Concentration								
TGF-β1, ng/mL	0.083	.670	−0.132	.613	−0.336	.188	−0.301	.240
IL-18, pg/mL	0.582	.0009	0.238	.358	−0.282	.273	−0.199	.445
Cytokine content per ejaculate								
TGF-β1, ng	0.298	.116	0.206	.428	−0.199	.445	−0.088	.736
IL-18, pg	0.524	.004	0.282	.273	−0.216	.406	0.022	.933
Ratio of cytokines concentration								
TGF-β1/IL-18	−0.451	.014	−0.252	.328	0.071	.786	0.007	.978

Abbreviations: day-OPU, day of ovum pickup; day-ET, day of embryo transfer; IL, interleukin; TGF-β1, transforming growth factor β1; Treg, regulatory T cell; ρ, Spearman rank correlation coefficient.
[a] ΔTreg was calculated as the difference between Treg values on the day-ET and day-OPU.
[b] |Δ|Treg, modulus or absolute value of ΔTreg.
[c] *P* Value for the Spearman rank statistic.

the content of TGF-β1 per ejaculate (Table 4 and Figure 2A and B). At the same time, Treg percentage showed a positive and significant correlation with IL-18 concentration and content of this cytokine per ejaculate (Table 4 and Figure 2C and D). Notably, the percentage of Tregs showed a negative correlation with TGF-β1/IL-18 ratio in the partners' SP (Table 4 and Figure 2E). No correlations between seminal cytokines and the percentage of Tregs on the day-ET, ΔTreg, and |Δ|Treg were revealed (Table 4).

Receiver Operating Characteristic Analysis, Sensitivity, Specificity, Cutoff Values

The receiver operating characteristic (ROC) curve analysis of seminal cytokines and Treg characteristics as potential predictors of the chance of clinical pregnancy after IVF/ICSI cycle was performed. It was found that the percentage of Tregs on the day-OPU, |Δ|Treg, the content of IL-18 per ejaculate, the concentration of IL-18 and TGF-β1/IL-18 ratio were significant predictors of IVF/ICSI success. The sensitivity, specificity, the cutoff values for Treg characteristic and seminal cytokine

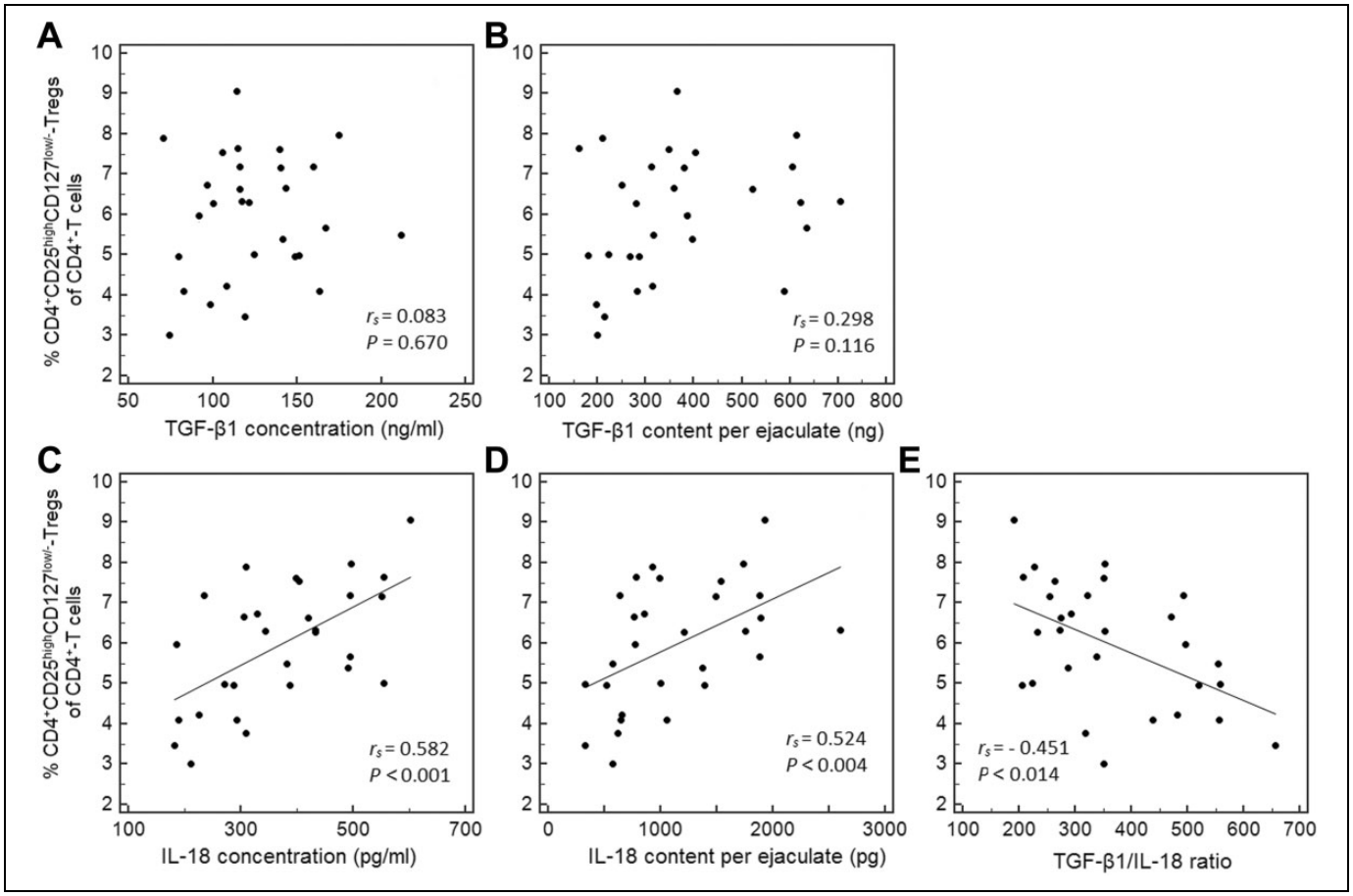

Figure 2. Associations between regulatory T-cell (Treg) frequency on the day of ovum pickup (day-OPU) in the peripheral blood of women exposed to partners' seminal plasma during in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycle and cytokines in seminal plasma. Correlation between Tregs and concentration of transforming growth factor β1 (TGF-β1; A), content of TGF-β1 per ejaculate (B), concentration of interleukin (IL)-18 (C), content of IL-18 per ejaculate (D), and TGF-β1/IL-18 ratio (E).

Table 5. ROC Curve Analysis for Measurements of Regulatory T Cells in Peripheral Blood of Female Patients and Seminal Cytokines in Male Partners' Seminal Plasma to Predict IVF/ICSI Success.

Parameters	AUC Value	95% CI	Cutoff Value	SN (%)	SP (%)	*P* Value
Tregs on the Day-OPU[a]	0.762	0.568-0.899	≤6.3	78.6	73.3	.0045
|Δ|Treg[b]	0.955	0.732-1.000	>0.51	100.0	83.3	<.0001
IL-18 (concentration)	0.755	0.560-0.894	≤305.2 pg/mL	64.3	93.3	.008
IL-18 (total content in SP)	0.745	0.550-0.888	≤1392.9 pg	92.9	53.3	.009
TGF-β1/IL-18 ratio	0.733	0.537-0.879	>322	78.6	73.3	.024

Abbreviations: AUC, area under the receiver operating characteristic curve; CI, confidence interval; day-OPU, day of ovum pickup; IL, interleukin; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; ROC, receiver operating characteristic; SN, sensitivity; SP, specificity; TGF-β1, transforming growth factor β1; Treg, regulatory T cell.

[a] Percentage of $CD4^+$ $CD25^+$ $CD127^{low+/-}$ Tregs within the $CD4^+$ T cell.

[b] Absolute value of ΔTreg, calculated as difference between the percentage of Tregs on the day-ET and day-OPU.

parameters, and the area under the curve obtained by the ROC analysis are shown in Table 5.

Discussion

In the present study, we have demonstrated for the first time that distinct levels of the components of SP delivered into the FRT during sexual relations may manifest systemically by the frequency of Tregs in the female peripheral blood. We discovered that low levels of IL-18 and a high TGF-β1/IL-18 ratio in the partner's SP from couples who had unprotected sexual relations until several days before the day-OPU are associated with a low percentage of Tregs in the female peripheral blood on the day-OPU, which may predict IVF success.

In contrast to murine pregnancy, in which the immunoregulatory potential of SP occurs during the postconception period, recurrent contact with SP during sexual relations in humans may activate the redistribution of Tregs during the preconception period. Thus, it appears that the effect of seminal cytokines is primarily responsible for the postcoital migration of Tregs to the LNs and uterus, which clearly manifested on the day-OPU by the low frequency of circulating Tregs. Based on the estrogen-dependent systemic expansion of Tregs during the late follicular phase,[40] it should be noted that when the estradiol level plateaus or decreases on the day-OPU, it is unlikely to interfere with the SP-dependent attenuation of circulating Tregs.

In the present study, we also revealed that prominent bidirectional changes occur in the frequency of Tregs in the group of fertile women between the day-OPU and the day-ET. Moreover, the absolute values of these changes (|Δ|Treg) in the group of patients with successful pregnancies were almost 6 times higher than in the group that experienced reproductive failure; thus, |Δ|Treg may represent a significant prognostic indicator of pregnancy establishment for IVF/ICSI treatment. Similarly, fluctuations in the number of circulating Tregs during the IVF cycle were previously noted in pregnant women in contrast to nonpregnant patients.[41]

During an immune response, many studies suggest that Treg migration is accelerated from peripheral tissues to the draining LNs followed by the rapid trafficking of Tregs from the LNs and blood to peripheral inflamed tissues.[42] It was shown that female lymphocyte populations are activated and expanded after mating in para-aortic LN cells, and lymphocytes are subsequently recruited from the circulation into the uterus during early pregnancy in mice.[43] Thus, it is highly likely that the different directions of Treg fluctuation observed after the day-OPU represent the result of the course of diverse phases of Treg relocation—the accumulation of circulating Tregs in the uterus and LNs and the egress of Tregs from the LNs. It seems that between-person differences in the time frame and intensity of Treg egress may lead to the detection of different values of Treg frequency on the day-ET; nevertheless, an appropriate amount of functionally competent Tregs required for the establishment of pregnancy accumulated in the uterus.

Based on the present data and previous findings obtained using animal models, as well as the results from human studies, we hypothesize that the transmission of SP with a pregnancy-favorable balance of TGF-β1 and IL-18 into the FRT during the IVF/ICSI cycle may result in well-timed immunoendocrine interactions intended to control the inflammatory status of the endometrium during the implantation stage. This control of inflammation is mediated by the redistribution of Tregs, which manifests as the frequency of circulating Tregs (Figure 3).

It is important to note that we did not identify a correlation between the level of analyzed seminal cytokines and Treg changes as well as the percentage of circulating Tregs on the day-ET. So, we believe that the frequency of circulating Tregs on the day-ET may be determined by the bidirectional processes mentioned previously, which may be regulated by different seminal and endocrine factors, and thus, it is difficult to assess the impact of seminal levels of TGF-β1 and IL-18 on the relocation of Tregs around the day-ET.

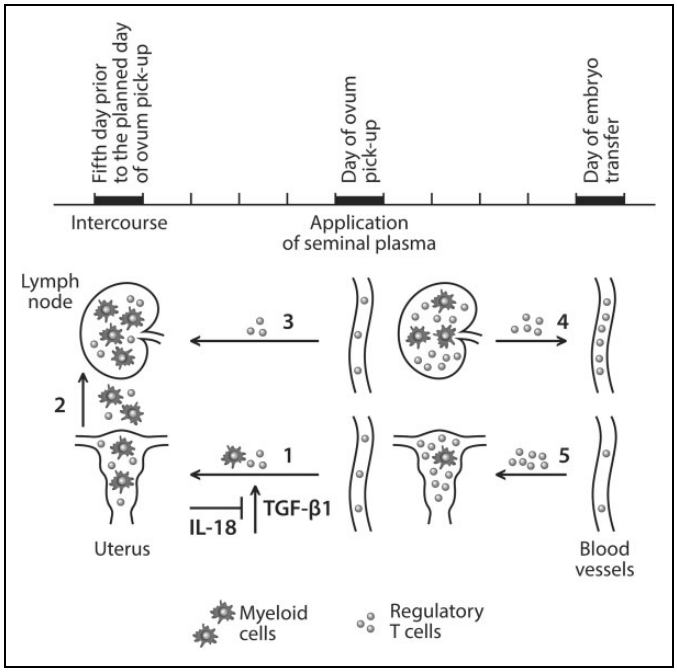

Figure 3. A putative model of regulatory T-cell (Treg) dynamics in peripheral blood during in vitro fertilization/intracytoplasmic sperm injection (IFV/ICSI) cycle in women exposed to partners' seminal plasma. (1). Recruitment of myeloid cells (neutrophils, macrophages, and dendritic cells) and Tregs to the endometrium of animals[3,4,31] as well as human cervix[5] as a result of postcoital reaction caused by the intromission of seminal plasma (SP) into the female reproductive tract (FRT). Seminal transforming growth factor β1 (TGF-β1) is considered a key factor that enhances the recruitment of myeloid cells[31-32] and Tregs,[8] whereas the excessive level of IL-18 in SP prevents the TGF-β1-related cellular reactions. (2) Postcoital reaction is followed by the myeloid cells migration from the uterus to the uterine draining lymph nodes (LNs) and T-, B-, and NK cells activation in the LNs.[43](3) Migration of thymus-derived Tregs to the periphery including the LNs. As a result of the stages (1-3), a significant amount of circulating Tregs accumulates in the LNs and uterus by the day-OPU that is evident as a remarkable decrease in the content of cells circulating in the peripheral blood around the day-OPU. (4) Functionally competent Tregs egress the uterine LNs via the efferent lymphatics and enter the circulation.[10,19,43] (5) The process of Treg recruitment is amplified by cumulative influence of the events 1 to 3 that occur after the intravaginal SP application on the day-OPU and the uterine expression of chemokines, which reaches its peak during implantation.[26] Administration of exogenous human choriogonadotropin (hCG) as a component of the pregestational therapy enhances the accumulation of Tregs within the uterus due to the hCG-dependent upregulation of the luteinizing hormone/choriogonadotropin receptor on the surface of Tregs.[28] High intensity of Treg egress from the LNs occurring on the day-ET leads to an increased circulating Treg percentage compared to this parameter determined on the day-OPU. When a low intensity of Treg egress from the LNs takes place on the day-ET, the migration and retention of Tregs into the uterus becomes detectable, that manifests by a decrease in circulating Treg percentage.

According to our results, a negative correlation between the TGF-β1/IL-18 ratio in the SP and circulating Tregs on the

day-OPU may reflect a key role played by TGF-β1 in the local accumulation of Tregs, whereas an excessive level of seminal IL-18 may interfere with TGF-β1 signaling. Binding of IL-18 to its cognate receptors inhibits TGF-β signaling by inducing the transcription of the SMAD-7 intracellular protein, which blocks the extracellular signal transduction of TGF-β to the nucleus.[35] In this manner, seminal IL-18 may prevent the TGF-β1-induced postcoital reaction, migration of circulating Tregs, and their local accumulation around the day-OPU, thereby interfering with the chain of sequential interrelated events (eg, morphological and functional changes to the endometrium and reducing the fertility-promoting effects of SP). In addition, these results are consistent with a previous study which demonstrated that although IL-18 is essential for endometrial angiogenesis and implantation, elevated secretion of this cytokine is deleterious and can potentially lead to implantation failure.[44]

It has been well established that IL-18 and IL-12 are essential cytokines which synergistically stimulate IFN-γ production by T cells and natural killer cells.[45] In turn, IFN-γ is able to antagonize TGF-β1 signaling in the human FRT[46] and interfere with the beneficial effect of TGF-β1 on the local expansion of Tregs and implantation.[47] Interleukin 18 also triggers a cascade of pro-inflammatory cytokines in the absence of a co-stimulus,[48] which may significantly attenuate the effects of TGF-β1. Therefore, the positive correlation between the frequency of circulating Tregs and seminal IL-18 may reflect the potential of IL-18 to reduce the TGF-β1-dependent local accumulation of Tregs by enhancing the pro-inflammatory cytokine expression.

Previous studies have shown that the level of IL-18 in SP is elevated in men with urogenital infections and patozoospermia.[49,50] Moreover, urogenital infections are often caused by bacteria, which leads to inflammation and leukocytospermia. However, no differences between the conventional semen parameters and leukocyte content were found in the pregnant and nonpregnant groups in this study. There was also no correlation between IL-18 concentration and concentration of leukocytes in semen samples (the data are not shown). Additionally, the association between seminal IL-18 and the establishment of pregnancy also manifested in patients without leukocytospermia.[37] Although the male patients were not tested for urogenital infections, female patients with TORCH infections and other types of sexually transmitted diseases were excluded from the present study. In addition, the concentration of seminal IL-18 in the partners of the women from both the pregnant and nonpregnant groups was similar to those previously defined in the SP of fertile men.[49] Therefore, it is unlikely that the observed increase in the level of seminal IL-18 in the partners of women from the nonpregnant group reflects an infection and/or sperm abnormality. It should be noted that IL-18 is considered to be a stress-related cytokine, the level of which can increase with stress even in the absence of an infection.[51] Moreover, recent data have indicated that the extent of stress response during the IVF/ICSI cycle correlates with the level of TGF-β1 in blood serum of both partners, as well as with cervicovaginal IL-6 and IL-1β in women.[52] Therefore, it cannot be excluded that psychological factors may also affect a cytokine profile of SP in partners of IVF/ICSI-treated women.

In the present study, the ROC analysis revealed that the frequency of circulating Tregs, absolute value of ΔTreg, concentration of IL-18, total content of IL-18 per ejaculate, and TGF-β1/IL-18 ratio are significant predictors of IVF/ICSI success. In particular, we found an association between low levels of peripheral Tregs on the day-OPU with IVF/ICSI success. Our findings are in contrast to that of Zhou et al,[53] in which the authors argued that there was an association between high levels of peripheral Tregs on the day-OPU with IVF/ICSI success. These contradictive results can be explained by the fact that in our study, the patients were exposed to SP via sexual contact prior to the day-OPU and at the additional application on the day-OPU, whereas no application of SP was performed during the IVF/ICSI cycle in the study by the authors mentioned previously and no information regarding sexual contact before and during the IVF/ICSI cycle was presented. Another possible explanation for these contradictions may be the diverse approaches of Treg identification using flow cytometry. In their study, Dr Zhou and colleagues isolated peripheral blood mononuclear cells (PBMCs) using Ficoll-Hypaque, then permeabilized the cells for intracellular Foxp3 staining, revealing a percentage of 0.77% and 0.59% of Tregs in pregnant and nonpregnant women, respectively. These values are almost 10 times lower than that of the data we obtained. In the present study, whole blood staining was performed to avoid alterations in leukocyte composition and activation, which may occur during PBMC isolation. Moreover, the simpler method of staining with CD127 (IL-7 receptor), an extracellular marker, rather than for intracellular Foxp3 avoids the requirement for permeabilization and optimizes the identification of live Tregs.[54] Using this marker, our data are in accordance with the reference values of $CD25^{+}CD127^{+/low}$ percentages among the total $CD4^{+}$ T cells in healthy donors.[54]

These results support the hypothesis that TGF-β1-rich SP entering the FRT during chronic sexual relations or the occurrence of singular intercourse during the preconception period initiates a maternal immune adaptation to pregnancy.[55] The presence of an adequate number of Tregs within the uterine LNs and/or uterus before implantation appears to be required to establish pregnancy. In addition, SP with an optimal cytokine balance may be responsible for the accumulation of this "strategic reserve" of Tregs via their migration and local retention during the preconception period. However, it remains unclear whether the application of SP on the day-OPU contributes to the effect of seminal cytokines on the redistribution of Tregs and subsequent success of IVF/ICSI. Thus, further investigation is needed to assess the relationship between seminal cytokines and IVF/ICSI effectiveness, as well as Treg redistribution following each type of SP application, including studies without an SP application on the day-OPU.

Taken together, an analysis of an individual's seminal cytokine status represents a promising personalized approach for predicting the success of implantation following IVF/ICSI

treatment. However, this strategy is only possible for patients with cytokines exhibiting a low level of within-subject variability. Indeed, seminal cytokine parameters fluctuate substantially in response to multiple factors.[56] As a marker of female reactivity to the immunomodulatory activity of SP, the frequency of circulating Tregs on the day-OPU can be considered a more useful predictor of IVF/ICSI success compared to seminal cytokine parameters.

We have also shown that the absolute value of ΔTreg is a strong prognostic factor for IVF/ICSI success, which does not depend on the level of seminal cytokines. However, other constituents of SP may be responsible for the relocation of Tregs in the FRT. Thus, one of the limitations of the present study is that no other seminal cytokines were included in our analysis. Another disadvantage of our study appears to be an insufficient amount of data regarding the frequency of Tregs on the day-ET, which is explained by the refusal of some patients to repeatedly take a blood test on crucial days of the IVF cycle. Therefore, the results obtained in the current study should be confirmed in a larger data set, including the measurement of a broader spectrum of cytokines.

In summary, this study demonstrates that the transmission of seminal cytokines into the FRT during sexual relations can be manifested systemically by the frequency of Tregs in the peripheral blood. Such levels of Tregs may be considered a marker of SP-dependent migration of Tregs from the circulation to the LNs and uterus. Moreover, several studies have confirmed that a successful pregnancy requires a fine balance between maternal and fetal cytokines and growth factors. In addition, our results also support the role of paternal cytokines in the regulation of female reproductive functionality. These findings provide a foundation for further investigation into the role of paternal factors in the immunoregulation of implantation and pregnancy and suggest a novel approach for overcoming infertility.

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