



Progesterone-induced blocking factor increases the frequency of Foxp3⁺CD4⁺CD25⁺ regulatory T cells in human peripheral blood

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ABSTRACT

Background: Pregnancy is a unique immunological state in which the semi-allogeneic fetus must be tolerated by the maternal immune system. Progesterone-induced blocking factor (PIBF) contributes to sustaining this immune balance through its regulatory effects, particularly those involving CD25⁺ T cells and maternal immune tolerance mechanisms.

Objective: To investigate whether PIBF influences the proportion of Foxp3⁺ cells among activated human peripheral CD4⁺ CD25⁺ T cells.

Materials and methods: We isolated peripheral blood mononuclear cells (PBMCs) were isolated from 15 healthy women of reproductive-age, all sampled during the follicular phase of their menstrual cycles. The cells were stimulated with anti-CD3, anti-CD28 and IL-2 and cultured for 5 days in the presence or absence of PIBF at concentrations of 200 or 1000 ng/mL. The proportion of CD4⁺CD25⁺Foxp3⁺T cells was quantified by flow cytometry.

Results: Treatment with PIBF increased the percentage of CD4⁺CD25⁺Foxp3⁺ T cells compared with untreated controls (mean $\pm$ SE: control $2.7 \pm 1.18\%$; PIBF 200 ng/mL $3.05 \pm 1.06\%$, $p = 0.008$; PIBF 1000 ng/mL $3.50 \pm 1.39\%$, $p = 0.002$). Although the increases were significant relative to the control group, no clear dose-dependent trend was observed between the two PIBF concentrations. No adverse effects on cell viability were detected under the experimental conditions.

Conclusion: In vitro treatment of activated human peripheral CD4⁺CD25⁺ T cells with PIBF increased the percentage of Foxp3⁺ cells, suggesting that PIBF may promote regulatory T cell phenotypes in the context of maternal–fetal tolerance.

Keywords: Fetal-maternal tolerance; Forkhead box P3 (Foxp3); Progesterone-induced blocking factor; Regulatory T cells

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INTRODUCTION

The fetus is a semi-allogeneic graft within the maternal uterus. Several immunoregulatory factors and mechanisms contribute to the maintenance of maternal–fetal tolerance and ensure fetal survival during pregnancy (1-6). Steroid hormones, particularly progesterone, play a crucial role in the development and maintenance of normal pregnancy (7). In addition to its classical physiological functions, progesterone also exerts significant immunomodulatory effects that are vital for gestational success (8).

Progesterone stimulates lymphocytes to express and secrete progesterone-induced blocking factor (PIBF), a protein that has no significant sequence homology with known proteins (9-11). The immunomodulatory effects of PIBF contribute to progesterone-related immune modulation through both direct and indirect mechanisms (12-14). During normal pregnancy, PIBF concentrations increase from the first trimester to the third trimester and fall rapidly toward the end of pregnancy (15). Reduced levels of PIBF have been reported in women with pregnancy complications, including recurrent spontaneous abortion (RSA) (16, 17), pre-eclampsia (15), and preterm birth (18).

Regulatory T cells (Tregs) play an important role in the induction and maintenance of maternal immune tolerance to the fetus. Their numbers gradually increase from the onset of pregnancy and decrease during the third trimester (19, 20). In women with RSA and pre-eclampsia, both the frequency and functional activity of Foxp3⁺ Treg cells are lower than in healthy pregnancies (21, 22).

Forkhead box P3 (Foxp3) is a transcription factor required for the development, function, and homeostasis of Treg cells (23, 24). Although the presence of Foxp3 is not exclusive to Treg cells (25), mutations in the Foxp3 gene lead to fatal autoimmune disease in both humans and mice (26, 27). Progesterone has been shown to promote Treg cell polarization (28); however, the effect of

PIBF on Treg cell development has not yet been established. Given the essential role of Treg cells in maintaining pregnancy and immune tolerance, this study investigated whether PIBF influences the frequency of CD4⁺CD25⁺FoxP3⁺ T cells, to determine if PIBF is linked to changes in the Treg cell population.

MATERIALS AND METHODS

Study Population

This study was conducted at Isfahan University of Medical Sciences and included fifteen healthy women of reproductive age (22-34 years). To avoid hormonal interference, blood samples were collected from all subjects during the follicular phase of the menstrual cycle. Written informed consent was obtained from all participants. This study protocol was approved by the Institutional Ethics Committee of Isfahan University of Medical Sciences (ethics code: 1395030632).

Blood collection and peripheral blood mononuclear cells (PBMC) isolation

We collected 6 mL of fresh blood samples from each participant in tubes containing 0.2 mL of sodium heparin to prevent clotting. PBMCs were then isolated from the whole blood using density gradient centrifugation with Ficoll-Hypaque (Bio Sera, France).

Cell culture

PBMCs (7×10^4 cells per 200 μ L) were cultured in 96-well plates and stimulated with anti-CD3 (0.2 μ g/mL; Mabtech, Sweden) and anti-CD28 (0.1 μ g/mL; Mabtech) monoclonal antibodies. Cells were maintained in RPMI-1640 medium supplemented with 2 g/mL sodium bicarbonate, 10% fetal bovine serum, 16 mM HEPES, and 1% penicillin/streptomycin. IL-2 (25 U/mL) was added to promote Treg polarization. Cells were then incubated in the presence or absence of recombinant human protein PIBF (Abnova, Taiwan) at concentrations of 200 or 1000 ng/mL.

for 5 days at 37°C in a humidified atmosphere containing 5% CO₂. After 3 days of incubation, 100 µL of fresh RPMI-1640 medium was added to each well. Finally, cells were harvested, washed with phosphate buffered saline (PBS), and analyzed by flow cytometry.

Analysis of CD4⁺ CD25⁺ Foxp3⁺ T cells

For flow cytometric evaluation of Treg cells, PBMCs were incubated with anti-human CD4 antibody conjugated to phycoerythrin–cyanine dye (PE Cy5) (clone RPA T4, BioLegend, USA) and anti-human CD25 antibody conjugated to phycoerythrin (PE) (clone BC96, BioLegend,). The stained cells were incubated on ice in the dark for 30 minutes and then washed with PBS at

300 × g for 5 minutes. Cell fixation and permeabilization were performed using Cell Fixation & Permeabilization Kit (Abcam, UK) according to the manufacturer's instructions. After that, the permeabilized cells were subsequently incubated with Alexa Fluor 488-conjugated anti-mouse/rat/human FOXP3 antibody (clone 150D, BioLegend,) for 1 hour at room temperature. Flow cytometric analysis was then carried out using a FACSCalibur flow cytometer (BD Biosciences, USA). Data were analyzed using FlowJo software (version 7.6, FlowJo, LLC, USA). The evaluation of CD4⁺CD25⁺Foxp3⁺ cells was performed using a sequential gating strategy. First, lymphocytes were gated based on forward scatter (FSC) and side scatter (SSC). Then, within the gated

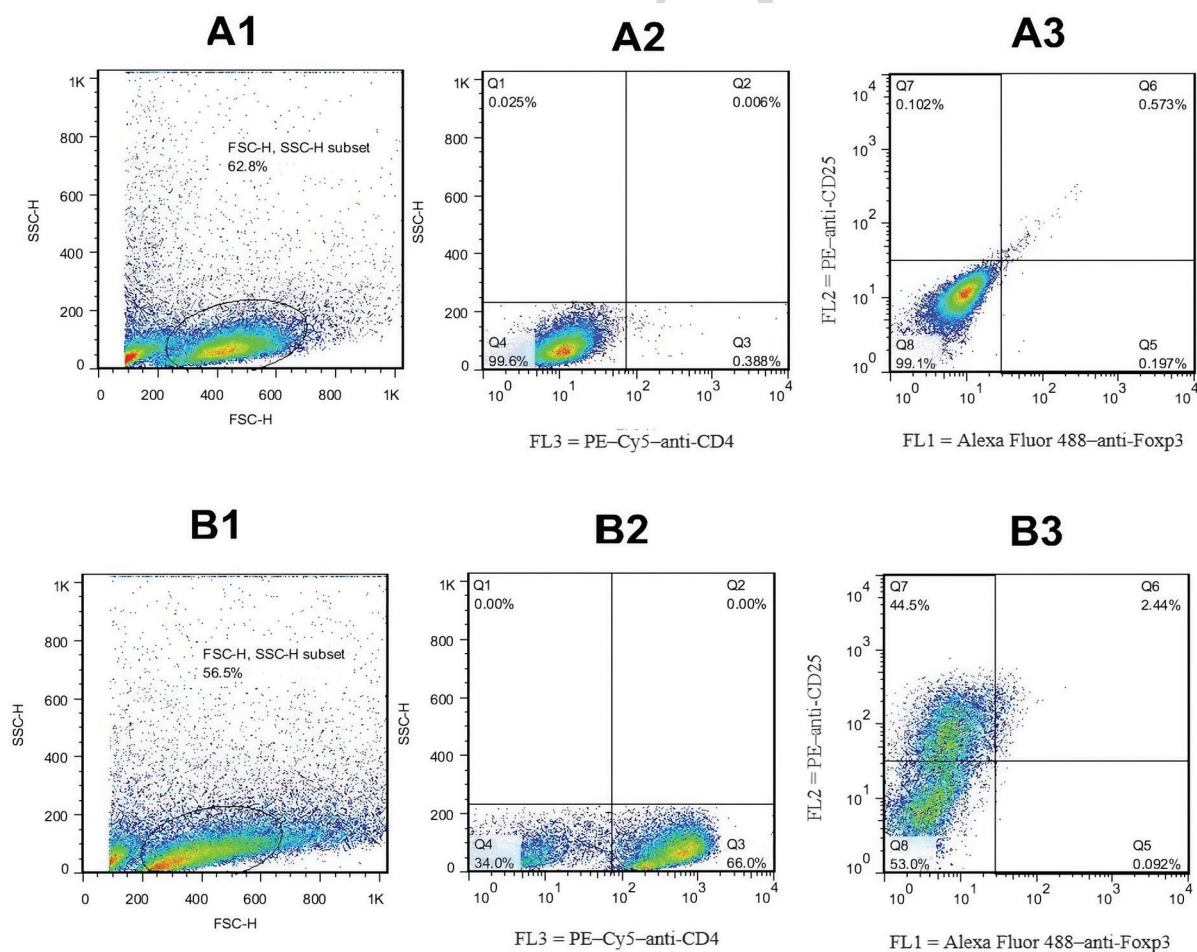


Figure 1. Flow cytometric gating strategy for analysis of Foxp3⁺ cells within the CD4⁺ CD25⁺ T cell population. (A1, A2 and A3): showing isotype controls. (B1) Lymphocyte population was identified based on forward scatter (FSC) and side scatter (SSC) parameters. (B2) CD4⁺ T cells were subsequently gated using SSC versus FL3 (PE-Cy5; CD4). (B3) Within the CD4⁺ population, the percentage of CD25⁺Foxp3⁺ Treg cells was determined using FL2 (PE; CD25) versus FL1 (Alexa Fluor 488; Foxp3) gating.

lymphocyte population, CD4⁺ T cells were identified (using SSC/FL3). Finally, within the CD4⁺ T cell population, the percentage of CD25⁺Foxp3⁺ cells was determined using FL2 versus FL1 gating. Figure 1 illustrates the flow cytometric gating strategy used for the analysis of the CD4⁺CD25⁺Foxp3⁺ T cell population.

Statistical analysis

Data analysis was performed using SPSS software (version 20). Quantitative results

are reported as mean $\pm$ standard error of the mean (SEM). Differences between untreated samples and those treated with various concentrations of PIBF were analyzed using repeated-measures ANOVA with Bonferroni's post-hoc test. A p-value of <0.05 was considered statistically significant.

RESULTS

PBMCs were isolated from the peripheral

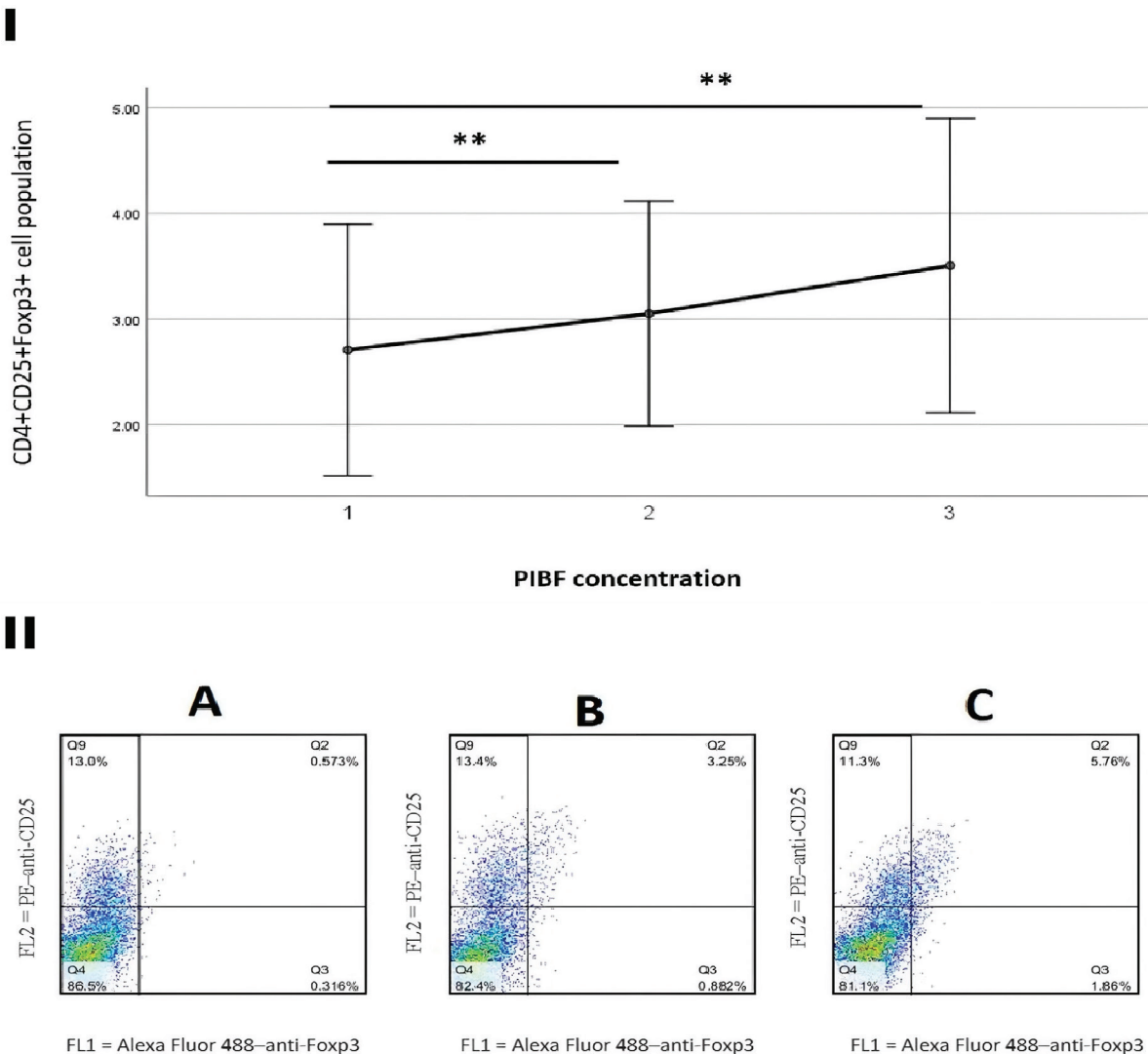


Figure 2. Effect of PIBF on the frequency of Foxp3⁺ cells among CD4⁺CD25⁺ T cells. (I) Chart showing the percentage of Foxp3⁺CD4⁺CD25⁺ T cells at different concentrations of PIBF. The PIBF concentrations were defined as follows: 1, control (0 ng/mL PIBF); 2, PIBF 200 ng/mL; and 3, PIBF 1000 ng/mL. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical significance is indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. (II) Representative flow cytometric analysis of Foxp3⁺ cells among CD4⁺ CD25⁺ T cells in the absence or presence of PIBF. (A) Untreated control (0 ng/mL PIBF). (B) Treatment with 200 ng/mL PIBF. (C) Treatment with 1000 ng/mL PIBF. The percentage of CD25⁺Foxp3⁺ cells within the CD4⁺ gate is shown in each plot.

Table 1: Frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells following PIBF treatment. CD4⁺CD25⁺Foxp3⁺ T cells

	Mean $\pm$ SD	N	P value
Untreated cells	2.7 $\pm$ 1.18	15	
Cells treated with PIBF (200 ng/ml)	3.05 $\pm$ 1.06	15	0.008**
Cells treated with PIBF (1000 ng/ml)	3.50 $\pm$ 1.39	15	0.002**

Peripheral blood mononuclear cells (PBMCs) from healthy fertile women (n = 15) were cultured with or without PIBF at concentrations of 200 and 1000 ng/mL for 5 days. The percentage of Foxp3⁺ cells within the CD4⁺CD25⁺ T cell population was determined by flow cytometry. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical significance was assessed using repeated-measures ANOVA with Bonferroni's post-hoc test. *p < 0.05, **p < 0.01 vs. untreated control.

blood of 15 healthy women (mean age: 26.2 $\pm$ 6.9 years). The cells were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies in the presence of IL-2 for 5 days. Subsequently, the cells were cultured with or without different concentrations of PIBF. The percentage of CD4⁺CD25⁺FoxP3⁺ was then evaluated by flow cytometry.

Frequency of Foxp3⁺CD4⁺CD25⁺ T cells

Treatment with PIBF significantly increased the frequency of Foxp3⁺CD4⁺CD25⁺ T cells compared to the untreated controls (200 ng/mL: 3.05 $\pm$ 1.06% vs. 2.7 $\pm$ 1.18%, p = 0.008; 1000 ng/mL: 3.50 $\pm$ 1.39% vs. 2.7 $\pm$ 1.18%, p = 0.002). However this increase was not dose-dependent, as no significant difference was observed between the two PIBF concentrations (p > 0.05). These findings are summarized in Table 1 and illustrated in Figure 2.

DISCUSSION

The present study demonstrates that treatment with PIBF significantly increased the frequency of Foxp3⁺CD4⁺CD25⁺ T cells obtained from healthy fertile women. This observation is most reasonably interpreted as the activation of Treg-associated immunoregulatory programs, suggesting that PIBF may promote the expansion or polarization of the Treg lineage. These findings point to a potential mechanism through which PIBF could contribute to the

establishment and maintenance of maternal immune tolerance during pregnancy, thereby complementing the well-characterized immunomodulatory actions of progesterone (P4) (4, 8).

This interpretation is biologically plausible. Progesterone is well-established to shift the maternal immune balance away from Th1-dominated responses toward Th2 dominance and to promote anti-inflammatory cytokine milieu (23). In line with this, progesterone has been shown to enhance IL-4 production by CD4⁺ T cells (8). PIBF signals through a heterodimeric receptor composed of a glycosylphosphatidylinositol (GPI)-anchored protein and the IL-4 receptor α chain (IL-4R α); activation of this receptor initiates STAT-6 signaling, which favors Th2-type responses and promotes IL-4 production (12). In addition, both PIBF and progesterone suppress natural killer (NK) cell activity. Progesterone can induce apoptosis in NK cells expressing progesterone receptors (24) whereas, PIBF blocks NK cell degranulation (25, 26). Mechanistically, PIBF also reduces prostaglandin synthesis by inhibiting phospholipase A₂ (29). Given that pro-inflammatory cytokines such as IL-1, TNF- α , and IL-6 stimulate phospholipase A₂ activity and subsequent prostaglandin production (27), this inhibitory pathway may further contribute to the establishment of an anti-inflammatory immune environment.

In support of PIBF's physiological relevance, a previous study demonstrated that administration of rabbit anti-PIBF IgG on

gestation day 15 in pregnant Sprague-Dawley rats induced signs of pre-eclampsia (PE), including elevated blood pressure, increased pro-inflammatory cytokine production (TNF- α), decreased anti-inflammatory cytokines such as IL-4 and IL-10, altered NK cell activity, underscoring the essential role of PIBF in maintaining healthy pregnancy (30).

Through these mechanisms, PIBF likely contributes to the establishment of an immunological milieu that favors Treg induction and expansion. Progesterone has also been shown to promote Treg development by suppressing the mTOR (mammalian Target of Rapamycin) pathway in mice and by directing human fetal naïve T cells toward Treg rather than Th17 differentiation (28, 31). Collectively, these findings provide mechanistic context suggesting that steroid-regulated pathways and PIBF signaling may converge to influence the frequency of CD4⁺CD25⁺FoxP3⁺ regulatory T cells. Our results, demonstrating a significant increase in Foxp3⁺CD4⁺CD25⁺ T cells following PIBF treatment, are consistent with these pathways and support the notion that PIBF can modulate adaptive immunity toward a more tolerogenic profile.

Foxp3 is a well-recognized transcription factor and master regulator of regulatory T cells, encompassing both naturally occurring Tregs (nTregs) and certain induced Tregs (iTregs), and is essential for their development and suppressive function (32, 33). As a lineage-defining transcription factor, Foxp3 regulates a broad array of target genes involved in immune regulation (34); accordingly, its upregulation provides a plausible molecular mechanism linking PIBF signaling to enhanced Treg frequency and functional activity. However, several limitations of the present study should be considered. In humans, transient Foxp3 expression can occur in activated conventional T cells, in contrast to the stable regulatory phenotype typically observed in mice (32). Because our experiments were performed using bulk peripheral CD4⁺ T cells, the observed increase

in Foxp3 could reflect induction within naïve cells, memory cells, recently activated effector cells, or preexisting Tregs. Nevertheless, Foxp3 expression in non-regulatory T cells is usually low and transient (34), which provides some support for a regulatory interpretation of the findings. Further experiments would help clarify the functional significance and underlying mechanisms of this effect. Specifically, functional suppression assays—evaluating the ability of PIBF-treated Foxp3⁺CD4⁺CD25⁺ cells to inhibit responder T cell proliferation or cytokine production—would determine whether these cells possess typical Treg suppressive activity. Assessment of phenotypic stability, such as analysis of FOXP3 TSDR (Treg-specific demethylated region) methylation status and longitudinal culture to examine sustained expression of Foxp3, CTLA-4, and CD25, would help distinguish transient activation-associated expression from stable induction of iTregs. Expanded flow cytometry panels including markers such as CD25, CD127, and Helios or Neuropilin 1, together with sorting of naïve and memory/effector subsets prior to PIBF exposure, could identify the T cell populations most responsive to PIBF. Mechanistic studies examining signaling pathways such as STAT-6 or mTOR, as well as blockade experiments targeting the PIBF receptor or prostaglandin synthesis pathways, would further elucidate the molecular basis of PIBF activity. Finally, in vivo studies evaluating circulating PIBF levels, Foxp3⁺ Treg frequencies, cytokine profiles (e.g., IL-4, IL-10, and IL-12), and NK cell activity across different stages of pregnancy would help determine the physiological relevance of these findings in the context of human gestation.

CONCLUSION

In summary, this study demonstrates that PIBF exerts significant immunomodulatory effects on peripheral CD4⁺ T cells derived

from healthy fertile women, as evidenced by a significant increase in the frequency of Foxp3⁺CD4⁺CD25⁺ Treg cells following PIBF treatment. The observed upregulation of Foxp3 supports a potential role for PIBF in promoting Treg-associated regulatory responses, thereby contributing to the establishment and maintenance of maternal-fetal immune tolerance during pregnancy.

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AVAILABILITY OF DATA AND MATERIALS

All data generated and analyzed during this study are included in this published article.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the study design, experimental work, statistical analysis and manuscript preparation. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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