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Placental growth factor exerts modulatory effects on *in vitro* activated T cells

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ABSTRACT

Background. Recent studies demonstrated immunosuppressive properties of vascular endothelial growth factor (VEGF-A) and identified VEGF-A as a key mediator of tumor-induced immunosuppression. Placental growth factor (PlGF) is another member of VEGF family in which dramatic elevation is associated with effective immune adaptation in successful pregnancy, whereas low concentrations are related to pregnancy complications resulting from the activation of immune system. Previously, we have shown that activated T cells express VEGF receptor type 1 (VEGFR-1), and PlGF inhibits T cell proliferation in VEGFR-1–dependent manner.

The aim of the present study was to further characterize PlGF effects on T cell responses *in vitro*.

Materials and methods. Peripheral blood mononuclear cells (PBMC) from healthy donors were stimulated with anti-CD3 monoclonal antibodies (a-CD3) in the absence or presence of PlGF and assessed for IL-10 production, programmed cell death and the expression of inhibitory receptors (PD-1, CTLA-4, Tim-3) in CD4+ and CD8+ T cell subsets.

Results. The addition of PlGF to PBMC cultures activated with a-CD3 resulted in increased percentages of IL-10-producing CD4+ and CD8+ T cells. Besides, PlGF promoted CD8+ T cells apoptosis while did not affect programmed cell death within CD4+ T cells. Notable, T cell activation with a-CD3 in the presence of PlGF was accompanied by the enhancement of PD-1-expressing cells in CD8+ T cell subset and Tim-3-expressing cells in both CD4+ and CD8+ T cells, and by the increased expression of PD-1 and Tim-3 on T cells.

Conclusion. Our *in vitro* findings indicate that PlGF can inhibit T cell responses due to the increasing interleukin-10 (IL-10) production, promoting CD8+ T cell apoptosis and enhancing the expression of PD-1 and Tim-3 inhibitory receptors. Given the elevated levels of PlGF in successful pregnancy and its decrease in gestation complications, the obtained data also suggest that PlGF-mediated suppression may be implicated into the governing immune evasion in pregnancy.

Key words: PlGF, T cells, apoptosis, IL-10, inhibitory receptors, PD-1, CTLA-4, Tim-3.

Conflict of interest. The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

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Фактор роста плаценты модулирует ответ активированных *in vitro* Т-клеток

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РЕЗЮМЕ

Актуальность. Недавние исследования выявили иммуносупрессивные свойства фактора роста эндотелия сосудов (VEGF-A) и его ключевую роль в опухоль-индуцированной иммуносупрессии. Плацентарный фактор роста (PlGF) является еще одним представителем семейства VEGF, резкое возрастание которого ассоциировано с эффективной иммунной адаптацией при успешной беременности, тогда как низкие концентрации PlGF являются предиктором гестационных осложнений на фоне активации иммунной системы. Ранее нами показано, что активированные Т-клетки экспрессируют рецепторы VEGF 1-го типа (VEGFR-1) и PlGF через связывание с VEGFR-1 ингибирует пролиферацию Т-клеток.

Цель. Дальнейшее изучение влияния PlGF на Т-клеточный ответ *in vitro*.

Материалы и методы. Мононуклеарные клетки (МНК) периферической крови здоровых доноров стимулировали моноклональными анти-CD3-антителами (a-CD3) в отсутствие и присутствии рекомбинантного PlGF и оценивали продукцию интерлейкина-10 (IL-10), уровень апоптоза и экспрессию ингибиторных рецепторов (PD-1, CTLA-4, Tim-3) в субпопуляциях CD4+ и CD8+ Т-клеток.

Результаты. Активация МНК a-CD3 в присутствии PlGF приводила к возрастанию относительного содержания CD4+ и CD8+ Т-клеток, продуцирующих IL-10. Кроме того, PlGF усиливал апоптоз активированных CD8+ Т-лимфоцитов, не влияя значимо на уровень программированной клеточной гибели CD4+ Т-клеток. Характерно, что активация Т-клеток a-CD3 в присутствии PlGF сопровождалась возрастанием PD-1 экспрессирующих клеток в субпопуляции CD8+ Т-клеток и Tim-3-экспрессирующих клеток среди CD4+ и CD8+ Т-клеток, а также повышением уровня экспрессии PD-1 и Tim-3 на Т-клетках.

Заключение. PlGF способен ингибировать Т-клеточный ответ посредством усиления продукции IL-10 и активационно-индуцированного апоптоза CD8+ Т-клеток, а также экспрессии ингибиторных рецепторов. Учитывая повышенный уровень PlGF при физиологической беременности и его снижение при гестационных осложнениях, полученные данные позволяют предполагать, что ингибиторный эффект PlGF на Т-клеточный ответ может являться еще одним механизмом, обеспечивающим защиту плода от иммунной системы матери.

Ключевые слова: PlGF, Т-клетки, апоптоз, IL-10, ингибиторные рецепторы, PD-1, CTLA-4, Tim-3.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Исследование выполнено при финансовой поддержке РФФИ и Правительства Новосибирской области (проект № 18-44-540005р-а).

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INTRODUCTION

Vascular endothelial growth factor (VEGF) family proteins play a pivotal role in de-novo angiogenesis in physiological and pathological conditions. VEGF-A is the most active and the best-studied VEGF family member that mediates pro-angiogenic activity via activation of two receptors with tyrosine kinase activity, i.e. VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) [1]. Placental growth factor (PlGF) is another potent pro-angiogenic factor that is ligated exclusively with VEGFR-1 [2, 3].

Recent studies showed that VEGF-A, in addition to pro-angiogenic activity, demonstrates immunomodulating properties: (i) induces accumulation of regulatory T cells and myeloid suppressor cells, (ii) inhibits maturation of dendritic cells (DCs) and T cell functions [4], and operates as a key factor of tumour-induced immunosuppression [5]. Nevertheless, PlGF-dependent immunomodulating properties have not been extensively studied. It is known though that PlGF: (i) stimulates M2 macrophage polarisation, (ii) suppresses DC maturation, and (iii) induces regulatory B-cells [3, 6]. However, the effects of PlGF on T cell functions remain largely unexplored. According to the literature data, the effect of VEGF on T cells is mediated via VEGFR-2 [7]. At the same time, PlGF is a selective ligand for VEGFR-1, and its role in regulating functional T cell activity remains unclear.

Studies of PlGF-dependent immunomodulating properties are motivated by a putative participation of this factor in tumour escape from immune surveillance, since increased PlGF levels in most tumour types are associated with immune dysfunctions and correlate with tumour progression [8, 9]. PlGF-dependent immunomodulating activity in pregnancy evoked even stronger interest owing to significantly increased serum PlGF levels in normal pregnancy, in contrast to decreased PlGF concentrations in gestational complications [10].

During pregnancy the immune system undergoes a significant rearrangement (termed "immune adaptation") [11], which is directed on inducing tolerance to paternal alloantigens and preventing excessive inflammatory reactions. Several mechanisms underlying immune adaptation have been elucidated, including depletion of alloantigen-reactive T cells, Th1→Th2 switch and induction of regulatory T cells [11]. Recent studies have also demonstrated the role of T cell exhaustion in suppressing maternal T-cell-mediated cytotoxic activity [12, 13]. From this standpoint, immunomodulating activity of PlGF could represent yet another mechanism involved in fetal protection from the maternal immune system.

Previously, we demonstrated VEGFR-1 expression on activated T cells, whereby ligation of PlGF with VEGFR-1 resulted in inhibition of CD4+ and CD8+ T cell proliferation in mononuclear cell cultures [14]. This study aimed to elucidate the effects of PlGF on T cell responses *in vitro*, with particular reference to T-cell-derived immunosuppressive cytokine production (IL-10), T cell apoptosis and expression of inhibitory receptors (PD-1, CTLA-4, Tim-3) involved in T cell exhaustion.

MATERIALS AND METHODS

This study included 35 healthy blood donors of both genders aged 20–54 years. Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood using Ficoll-Verografin (p = 1.078 g/ml) gradient centrifugation. PBMC were cultivated in round-bottomed 96-well plates in RPMI-1640 cell culture medium supplemented with 10% inactivated donor AB (blood group IV) serum, 2mM HEPES-buffer, 0.3 mg/ml L-glutamine and 100 μg/ml gentamycin (all reagents were from Sigma-Aldrich, St. Louis, MO USA) at 37°C in and 5% CO_2. PBMC were stimulated in the presence of monoclonal anti-CD3 antibodies (1 μg/ml, a-CD3, ICO-90, MedBioSpektr, Moscow), 0.1–100 ng/ml PlGF (R&D Systems, Abingdon, UK). To assess proliferation, cells were incubated for 4 days, followed by pulse-labelling with 1.0 mCi/well of ^{3}H-thymidine. Cells were harvested, and radioactivity was quantitated using a Liquid Scintillation Counter SL-30 (Intertechnic, France).

In a separate set of experiments, we studied the effect of neutralising anti-VEGFR-1 antibodies (a-VEGFR-1) on suppressive PlGF properties. To this end, PBMC were stimulated with a-CD3 in the presence of PlGF (5 ng/ml) followed by cultivation in the presence or absence of neutralising a-VEGFR-1 or a-VEGFR-2 antibodies (HumanVEGFR1/Flt-1; VEGFR2/KDR/Flk-1 antibodies, 2.5 μg/ml; R&D Systems, USA), which were added to PBMC cultures concomitantly with PlGF or 24 h later.

Intracellular IL-10 expression was analysed in 48 h PBMC cultures activated by a-CD3 in the presence or absence of PlGF. Cells were labelled by anti-CD3 (Phycoerythrin, PE), CD8+ (Fluorescein isothiocyanate, FITC), CD4 (Peridinin chlorophyll, PerCP), IL-10(PE) antibodies (BD Biosciences, San Jose, CA, USA) followed by fixation/permeabilization using fixation/permeabilization Transcription Factor

Buffer Set (BD Biosciences), according to the manufacturer's instructions. Content ratio of IL-10-secreting cells in CD4+ and CD8+ T cell gates (altogether 30,000 events were collected for each sample) was analysed by flow cytometry (BD FACSCalibur).

Apoptosis of activated T cells was analysed by flow cytometry. To this end, PBMC were stimulated by a-CD3 and PlGF, as described above, and cultivated for 48 h. Cells were labelled with anti-CD4(FITC) or anti-CD8(FITC) antibodies (BD Biosciences) followed by Annexin V/7-7-amino-actinomycin D (ADD) (PE-conjugated Annexin V/7-ADDkit), according to the manufacturer's instructions. Altogether 10,000 events were assessed for each sample, and percentages of Annexin V-positive and/or 7-ADD-positive CD4+ and CD8+ T cells were analysed using CellQuest software (BD Biosciences, USA).

Cell surface expression of inhibitory receptors (PD-1, CTLA-4, Tim-3) on T cells was analysed by flow cytometry using anti-CD4(Pe), anti-CD8(FITC), anti-CTLA-4(Phycoerythrin/Cyanine dye tandem conjugate, PE-Cy5), anti-PD-1(Allophycocyanin, APC), anti-TIM-3(PerCP/Cyanine dye tandem conjugate, PerCP/Cy5.5) and relevant isotype controls (BD PharMingen, USA). Percentages and mean fluorescence intensity (MFI) of PD-1$^+$, CTLA-4$^+$, and Tim-3$^+$ cells were analysed in CD4+ and CD8+ T cell gates. Statistical analysis was performed using an analytics software portfolio Statistica 6.0 for Windows (StatSoft Inc., USA). The data are presented as Median (*Me*) with the interquartile range [Q_1–Q_3]. Related samples were compared using a nonparametric paired difference test (Wilcoxon signed-rank test) with the Bonferroni correction. Results were considered statistically significant at $p < 0.05$.

RESULTS

PlGF-mediated inhibition of a-CD3-activated T cells is VEGF-independent.

We showed previously that VEGFR-1 blockade aborted suppressive effects of PlGF on T cell proliferation in a-CD3-stimulated PBMC cultures [14], suggesting that PlGF exerted a direct inhibitory effect on T cells via ligating with its cognate receptor VEGFR-1. PlGF is also known to activate PBMC and induce VEGF production [15], which could also inhibit T cell proliferation [7]. Suppressive effects of VEGF have been described to manifest themselves from day 7 under high VEGF concentrations [7], which significantly surpassed VEGF levels present in PBMC cultures [15].

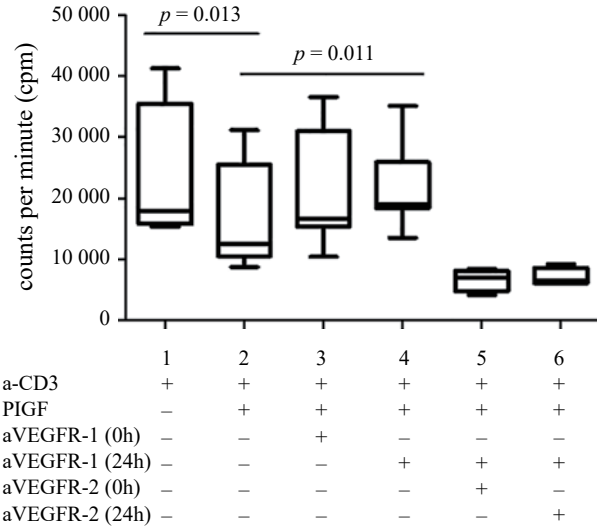

Fig. 1. Neutralising a-VEGFR-1 antibodies withdraw the inhibitory effect of PlGF on T cell proliferation: PBMC were stimulated with a-CD3-antibodies in the absence (*1*) and presence of 5 ng/ml PlGF(*2–6*); neutralising a-VEGFR-1 (*3*, *4*) or a-VEGFR-2 (*5*, *6*) antibodies were added at a dose of 2.5 μg/ml concomitantly with PlGF (*3*, *5*) or 24 h after the beginning of cultivation (*4*, *6*), counts per minute (cpm), *Me* [Q_1–Q_3], *Min–Max*, $n = 8$

In order to rule out the involvement of VEGF in the inhibitory PlGF activity, we compared blocking effects of a-VEGFR-1 and a-VEGFR-2 on PlGF-mediated suppression. Blocking antibodies were added either simultaneously with PlGF or 24 h later taking into consideration low VEGFR expression levels on unstimulated T cells followed by significant augmentation of VEGFR expression 24 h after the cultivation onset. Figure 1 shows that PlGF presence resulted in a significant (31%; 26–38%, $p = 0.013$) reduction in T cell proliferation levels in response to a-CD3 stimulation. Neutralising a-VEGFR-1 antibodies reduced PlGF suppressive activity to 9% (3–25%) if added concomitantly with PlGF and to 3% (0–16%) if added 24 h later. More pronounced inhibition of PlGF suppressive activity in the last case was likely to be due to higher VEGFR-1 expression on T cells 24 h after a-CD3 stimulation. Of note, VEGFR-2 blockade did not abrogate PlGF-mediated suppressive effects.

The effect of PlGF on IL-10 production by a-CD3-activated CD4+ and CD8+ T cells.

To study the effect of PlGF on IL-10-producing capacity of T cells, we analysed percentages of CD4+ and CD8+ T cells with intracellular IL-10 expression in cultures of a-CD3-stimulated PBMC in the presence

and absence of PlGF (Fig. 2). Activation of PBMC with a-CD3 significantly increased relative numbers of IL-10-positive cells in CD8+ T cell subpopulation ($p = 0.028$). Although we also detected an increase in IL-10-producing CD4+ T cells derived from most donors studied here, these changes were not statistically different from the baseline levels ($p = 0.06$).

The addition of PlGF to PBMC cultures increased proportion of IL-10$^+$ cells both in CD4+, and CD8+ T cell subsets, as compared to a-CD3-stimulated PBMC cultures incubated in the absence of PlGF. Of note, percentages of CD8$^+$IL-10$^+$ T cells in PBMC cultures incubated with PlGF were 2.7-fold higher, as compared to CD4$^+$IL-10$^+$ T cells ($p = 0.018$).

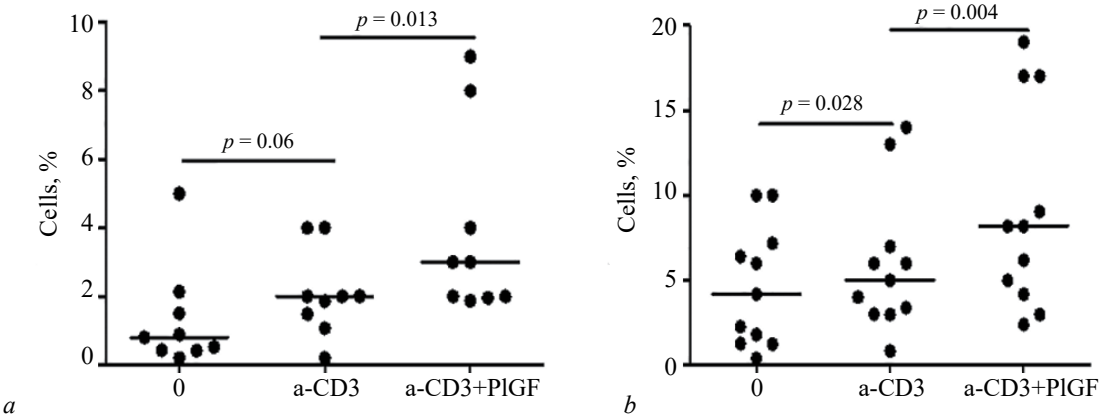

Fig. 2. PlGF increases intracellular IL-10 expression CD4+ (*a*) and CD8+ (*b*) by T lymphocytes: PBMC were cultivated in the medium (*0*) or stimulated with a-CD3 in the absence (a-CD3) and presence of 5 ng/ml PlGF (a-CD3+PlGF). After 48 h of cultivation intracellular IL-10 expression was assessed CD4+ and CD8+ T cell subpopulations using flow cytometry. The data are presented as individual values and *Me*, $n = 9–11$

PlGF enhanced apoptosis of a-CD3-activated T cells.

In order to address putative involvement of PlGF in programmed cell death regulation, we studied the effect of PlGF on the level of activation-induced apoptosis in CD4$^+$ and CD8$^+$ T cells. Apoptotic and necrotic cells were identified by a three-colour flow cytometry. Cells in early apoptosis are known to exclude a DNA-labelling vital dye 7-ADD, thus consistent with Ann$^+$/7-ADD$^-$ phenotype. Late apoptotic or necrotic cells are permeable to 7-AAD and therefore could be identified as Ann$^+$/7-ADD$^+$. Most CD4+ and CD8+ T cells in freshly isolated PBMC were viable (Ann$^-$/7-ADD$^-$ phenotype) and contained negligible proportion of apoptotic cells. Incubation of PBMC with a-CD3 for 48 h was accompanied by an increase in percentages of apoptotic cells in CD4+ and CD8+ T cell subpopulations (Fig. 3). Further supplementation of PBMC cultures with PlGF enhanced apoptosis in CD8+T cells ($p < 0.05$), but not in CD4+ T cells. Since the number of Ann$^+$/7-ADD$^-$ cells reflects only proximal (early) apoptotic events, we performed additional analysis of the total amount of Ann+ T cells (i.e. Ann$^+$/7ADD$^{-/+}$). Relative proportions of Ann+CD8+ T cells were found to be higher in a-CD3-activated PBMC incubated with PlGF than that in control a-CD3 stimulated PBMC. Meanwhile, no differences were detected in percentages of AnnV+CD4+ T cells incubated in the presence or absence of PlGF.

PlGF enhanced the expression of inhibitory receptors on activated T cells.

Overexpression of inhibitory receptors (also called inhibitory checkpoint molecules) is considered an important mechanism restraining T cell responses due to T cell exhaustion [12]. In order to assess the effects of PlGF on inhibitory receptor expression, we studied the expression of PD-1, CTLA-4, and Tim-3 on CD4+ and CD8+ T cells in 48 h PBMC cultures (Table).

PBMC stimulation with a-CD3 increased percentages of T cells expressing checkpoint molecules. Thus, relative numbers of CD4+PD-1+ and CD8+PD-1+ T cells following anti-CD3 stimulation were statistically higher, as compared to that observed in unstimulated PBMC cultures. The addition of PlGF enhanced the proportion of PD-1-positive CD8+ T cells ($p < 0.05$), with no effect on CD4+PD-1+ T cells. Ligation of T cell receptors with a-CD3 antibodies resulted in an increased proportion of CD4+ T cells co-expressing CTLA-4, while incubation with PlGF did not affect

percentages of CTLA-4-positive CD4+ or CD8+ T cells. Furthermore, a-CD3 stimulation enhanced percentages of Tim-3-positive cells in CD4+ and CD8+ T cell subpopulations, while in the presence of PlGF both CD4+Tim3+ and CD8+Tim3+ T cells showed statistically significant increase ($p < 0.05$).

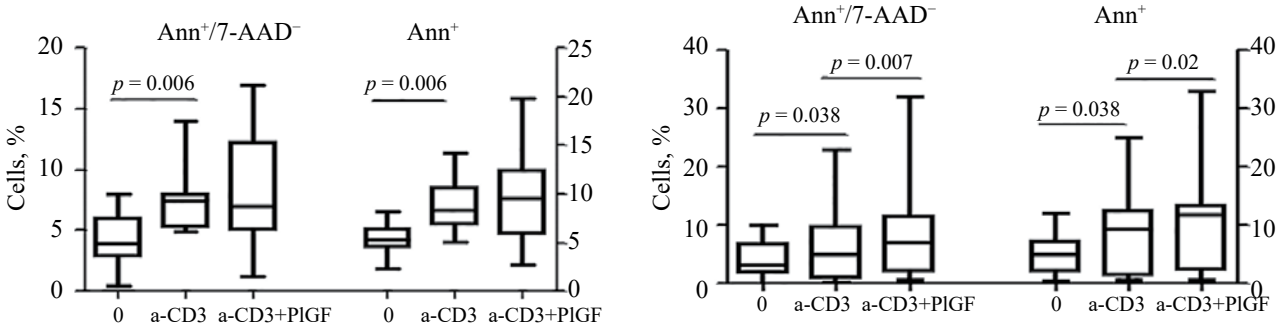

Fig. 3. PlGF enhances apoptosis of a-CD3-activated CD8+ T cells: the summarized data on the relative numbers of cells in the early apoptosis stage ($Ann^+/7ADD^-$) and the total amount of apoptotic cells (AnnV+) in CD4+ (*a*) and CD8+ (*b*) T cell gates are presented. The data from four independent experiments were analyzed using the paired Wilcoxon test, $n = 10$

Table

Effect of PlGF on the checkpoint molecules expression on a-CD3-activated CD4+ and CD8 + T cells, *Me* [Q_1–Q_3], $n = 8$

PBMC	$CD4^+$ T cells (%)			$CD8^+$ T cells, %		
	PD-1	CTLA-4	Tim-3	PD-1	CTLA-4	Tim-3
0	3.7 [2.2–4.3]	4.6 [2.1–7.1]	2.9 [2.0–3.6]	2.3 [1.6–2.8]	0.4 [0.1–0.8]	0.7 [0.1–1.6]
a-CD3	6.0* [3.0–8.5]	7.0* [2.4–10.2]	3.7* [2.7–5.3]	3.2* [2.4–4.2]	0.3 [0.1–0.7]	2.4* [1.6–3.2]
a-CD3+PlGF	5.5* [4.5–8.1]	7.3* [4.2–10.0]	5.0*# [3.3–6.6]	3.8 *# [2.9–4.5]	0.5 [0.3–0.7]	5.1*# [3.3–6.1]

Note. The percentage of PD-1, CTLA-4 and Tim-3-positive cells in the gates of CD4+ and CD8+ T lymphocytes was evaluated in 48-hour cultures of unstimulated PBMC (0) and PBMC activated by anti-CD3-antibodies in the absence of (a-CD3) and in the presence of 5 ng/ml PlGF (a-CD3+PlGF).

* $p < 0.05$ – significance of differences compared with unstimulated PBMC; # $p < 0.05$ – significance of differences compared with a-CD3-activated PBMC.

Importantly, PlGF not only increased percentages of PD-1- and Tim-3-positive T cells, but also enhanced the expression of these receptors on T cells. Thus, treatment of PBMC in the presence of PlGF facilitated the enhancement of mean fluorescence intensity (MFI) of PD-1 staining on CD4+ T cells from (37.1 ± 3.5) to (47.8 ± 5.8), while on CD8+ T cells MFI of PD-1 staining increased from (38 ± 3.3) to (47.0 ± 4.1) ($p < 0.05$). MFI of Tim-3 staining increased on CD4+ T cells in the presence of PlGF from (41.0 ± 4.2) to (49.0 ± 4.9), as well as on CD8+ T cells from (44.0 ± 4.5) to (49.0 ± 5.3) ($p < 0.05$). Taken together, activation of PBMC in the presence of PlGF resulted in moderate, but statistically significant augmentation of expression levels of PD-1 and Tim-3 *on both* CD4+ and CD8+ T cells.

DISCUSSION

The data obtained in this study showed that inhibitory effects of PlGF on T cell proliferation in a-CD3-stimulated PBMC cultures were mediated via VEGFR-1, and that these effects were not associated with a probable increase in VEGF production by activated PBMC [15], as judged from the fact that VEGFR-2 blockade did not withdraw suppressive effects of PlGF. In addition, it was demonstrated that PlGF: enhanced IL-10 production by activated CD4+ and CD8+ T cells, aggravated apoptosis of CD8+ T cells, and increased expression of inhibitory receptors PD-1 and Tim-3 on T cells, implying an important role of PlGF/VEGFR-1 signal transduction pathway in modulation of T cell functions.

VEGFR-1 has been reported to possess low tyrosine kinase activity, which for a long time supported a paradigm that considered VEGFR-1 exclusively a ligand-trapping receptor [2]. However, it was subsequently shown that this receptor was expressed on haemopoietic precursors, monocytes/macrophages and DCs, and that VEGFR-1-dependent signalling pathway was involved in mobilisation of bone marrow precursors, activation and migration of monocytes, and regulation of DC maturation and cell proliferation [3, 16, 17]. Moreover, recent studies demonstrated that VEGFR-1-mediated signalling in hypoxic condi-

tions activated STAT3 transcription factor [8], which plays an important role in regulating T cell functions by inhibiting proliferation and IL-2-producing capacity of T cells [18]. These observations are in agreement with our data that PlGF exerts inhibitory effect on T cell proliferation by binding to VEGFR-1.

This study identified an interesting property of PlGF – this factor stimulated IL-10 production by activated T cells. Y.L. Lin et al. demonstrated previously that PlGF modulated cytokine-secreting function of T cells indirectly via a DC-dependent mechanism. Thus, PlGF-modified DCs enhanced IL-13 and IL-5 production by allogeneic T cells without affecting IL-10 production in mixed leukocyte culture [6]. These results stress important differences between direct and DC-mediated effects of PlGF.

J.Y. Shin et al. described the augmentation of IL-10 production due to VEGF mediated ligation of VEGFR-1 on activated spleen T cells and CD4+ T cell line [19], which supports the ability of VEGFR-1 to exert direct effects on T cells. However, the authors did not detect suppression of T cell proliferation under conditions of VEGFR-1 activation. This fact is likely explained by PlGF and VEGF exerting different effects when binding to VEGFR-1 due to the activation of different downstream transcription factors [17].

IL-10 is known to be a key immunosuppressive cytokine involved in restricting immune responses and inducing tolerance in pregnancy [20]. Suppressive effects of IL-10 are mediated principally via generation of tolerogenic DCs, induction of regulatory T cells and activation of anti-inflammatory JAK1/STAT3 signalling pathway in T cells [21]. Inhibitory effects of IL-10 are most clearly demonstrated with regard to CD4+ T cells, in which endogenous IL-10 production constitutes an important regulatory mechanism restraining CD4+ T cell functions [22]. The effect of IL-10 on CD8+ T cell functions is far less unambiguous. Indeed, in tumour growth IL-10 could activate and stimulate expansion of cytotoxic CD8+ T cells [23], whereas in chronic infection a direct inhibitory effect of IL-10 on CD8+ T cells has been described [24]. Our data suggests that PlGF is capable of enhancing IL-10 production not only by CD4+, but also CD8+ T cells. However, whether IL-10 production underlies the suppressive effects of PlGF on T cell proliferation, with particular reference to CD8+ T cells, remains an open question and awaits further investigations.

Importantly, this study also revealed that PlGF could enhance both apoptosis of CD8+ T cells and expression of inhibitory receptors (PD-1 and Tim-3) on T cells. The ability of PlGF to modulate apoptosis levels was recently demonstrated using an experimental lung emphysema model [25]. The authors showed that PlGF increased apoptosis of lung epithelium via activation of c-Jun N-terminal kinase (JNK) and protein kinase C. Our study demonstrated that PlGF enhanced apoptosis of activated CD8+ T cells for the first time.

Interestingly, PlGF also increased the relative numbers of PD-1+ cells, and specifically in CD8+ (but not CD4+) T cell subpopulation, suggesting that PlGF-mediated augmented apoptosis of CD8+ T cells could be associated with enhanced PD-1 expression. An important role of PD-1 inhibitory receptors in suppressing cytotoxic T cell functions has been convincingly demonstrated previously in cancer [26], and recently in pregnancy [27]. PD-1-dependent signalling has been shown to inhibit T cells via various mechanisms, including apoptosis induction [28]. Furthermore, increased expression of checkpoint molecules could reflect T cell exhaustion state [12].

In addition to PD-1 up-regulation, treatment with PlGF enhanced relative numbers of Tim-3-positive cells, and notably both in CD8+ and CD4+ T cell subpopulations. Tim-3 is another checkpoint molecule involved in the formation of T cell exhaustion status, thus playing an important role in suppressing maternal immune responses against fetal alloantigens during successful pregnancy [29]. T. Voronet et al. demonstrated for the first time that *VEGF*-A strengthened expression of various inhibitory checkpoint molecules (PD-1, CTLA-4, Tim-3) on CD8+ T cells in tumour-bearing mice by engaging a VEGFR-2 signalling pathway [30]. These authors also detected simultaneous expression of several inhibitory receptors on CD8+ T cells in the presence of high VEGF concentrations.

Our data showed that angiogenic factors have stimulating effects on checkpoint molecule expression on human T cells, and, in particular, we demonstrated that PlGF enhanced the expression of PD-1 and Tim-3 on T cells. Along with this, if the stimulatory effect of PlGF on PD-1 expression was observed only in CD8+ T cell subpopulation, the influence of PlGF on Tim-3 expression was detected in both CD4+ and CD8+ T cell subpopulations. A co-expression analysis of different inhibitory receptors was beyond the scope of this investigation, which is a study limitation. However, the increase of both CD8+PD-1+ and CD8+-Tim-3 T cells associated with reduced CD8+ T cell proliferation in cultures with PlGF, implies that the up-regulation of checkpoint molecules on the surface

of T cells could mediate inhibitory effects of PlGF on T cells.

The data obtained in this study infer that PlGF could be considered a novel immunomodulator in pregnancy. Indeed, serum PlGF concentrations are known to increase during normal pregnancy, while declining PlGF levels observed in placental hypoxia serve as a predictor factor of pre-eclampsia and intrauterine growth retardation [10]. In spite of the high prognostic value of PlGF levels, its pathophysiological significance has not been fully elucidated. Taking into consideration an important role of immune adaptation in successful pregnancy paralleled by pronounced immune impairments observed in women with pre-eclampsia [31, 32], we hypothesise that PlGF is involved in immune modulation in normal pregnancy, while reduced PlGF levels facilitate immune system activation leading to the development of pregnancy complications.

CONCLUSION

Taken together, this study showed that PlGF enhanced IL-10 production by activated CD4+ and CD8+ T cells, apoptosis of CD8+ T cells, and expression of inhibitory receptors PD-1 and Tim-3 on T cells, evidencing an important role of PlGF/VEGFR-1 signalling pathway in modulating T cell functions. Taking into account enhanced PlGF levels in normal pregnancy paralleled by their reduction during pregnancy complications, we envisage that inhibitory effects of PlGF on T cell responses could constitute yet another mechanism governing immune evasion in pregnancy.

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