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ИММУНОРЕГУЛЯТОРНЫЙ ПОТЕНЦИАЛ ТРОФОБЛАСТИЧЕСКОГО β1-ГЛИКОПРОТЕИНА

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Резюме. Эмбрион, являясь наполовину «чужеродным» в антигенном отношении организмом, должен вызывать ответную реакцию иммунной системы матери. Однако в процессе эволюции сформировались механизмы, обеспечивающие успешное развитие беременности. В частности, одним из факторов, обеспечивающим иммунную толерантность при беременности, являются белки, ассоциированные с беременностью. Трофобластический β1-гликопротеин (PSG, PSG1; SP1; PSβG1) является доминантным фетоплацентарным белком, который продуцируется клетками цито- и синцитиотрофобласта и обладает иммуносупрессивными свойствами. Наш авторский коллектив владеет собственной запатентованной методикой получения нативного препарата PSG человека из сыворотки крови беременных женщин, который представляет собой смесь PSG1, PSG3, PSG7, PSG9, а также их изоформ и прекурсоров. В данном обзоре представлен анализ собственных результатов за период с 2015 по 2020 г. Изучали иммунорегуляторный эффект полученного препарата PSG в концентрациях, сопоставимыми с беременностью (1, 10, 100 мкг/мл), объектами исследования служили клетки периферической крови, полученные от небеременных женщин. Было установлено, что PSG достоверно увеличивал уровень адаптивных Treg *in vitro*, а также экспрессию этими клетками CTLA-4 и GITR и продукцию IL-10. Показано, что в отношении активности индоламин-2,3-диоксигеназы (IDO) на уровне периферических моноцитов реализуется стимулирующий эффект PSG. В отношении Th17-клеток было продемонстрировано, что PSG способен подавлять дифференцировку и пролиферацию этих клеток, а также продукцию ими ключевых провоспалительных цитокинов (IL-8, IL-10, IL-17, IFNγ, MCP-1, TNFα). На уровне Т-клеток иммунной памяти PSG подавлял экспрессию CD25 и продукцию IL-2 этими клетками, одновременно снижая экспрессию генов *Gfi1, hnRNPLL,* препятствуя таким образом формированию «зрелой» изоформы CD45R0. Было показано, что на уровне Т-хелперов PSG препятствовал конверсии наивных Т-клеток в терминально-дифференцированную эффекторную субпопуляцию Т-хелперов. При анализе влияния PSG на цитокиновый профиль иммунокомпетентных клеток было установлено, что белок преимущественно подавляет продукцию Th1-цитокинов исследуемыми типами клеток и разнонаправленно регулирует продукцию Th2-цитокинов. Полученные результаты согласуются с общим вектором иммуносупрессии в период беременности.

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Таким образом, PSG является одним из факторов, не позволяющим сформироваться и реализоваться иммунному ответу на фетоплацентарные антигены.

Ключевые слова: трофобластический β1-гликопротеин, иммуномодулирующие эффекты, иммунокомпетентные клетки, иммунная толерантность при беременности

IMMUNOREGULATORY POTENTIAL OF PREGNANCY-SPECIFIC β1-GLYCOPROTEIN

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Abstract. The embryo, being half an antigenically "foreign" organism, should elicit a maternal immune response. During evolution, however, the mechanisms ensuring successful development of pregnancy have been formed. In particular, among factors providing immune tolerance during pregnancy are some proteins associated with pregnancy. The pregnancy-specific β1-glycoprotein (PSG, PSG1; SP1; PSβG1) is a dominant fetoplacental protein produced by cyto- and syncytiotrophoblast cells, and it exhibits immunosuppressive properties. Our team of authors possesses a patented method for obtaining native human PSG preparation from blood serum of pregnant women, a mixture of PSG1, PSG3, PSG7, PSG9, and their isoforms and precursors. This review presents an analysis of our results for the period from 2015 to 2020. We studied the immunoregulatory effects of the obtained PSG preparation at concentrations comparable to those observed in pregnancy (1, 10, 100 μg/mL). The study was performed with peripheral blood cells obtained from non-pregnant women. It was found that PSG significantly increased the percentage of adaptive Tregs *in vitro*, as well as expression of CTLA-4, GITR, and production of IL-10 by these cells. It has been shown that PSG has a stimulating effect upon indoleamine-2,3-dioxygenase (IDO) activity of peripheral blood monocytes. For Th17 cells, we have demonstrated that PSG can suppress differentiation and proliferation of these cells, along with reduced production of critical proinflammatory cytokines (IL-8, IL-10, IL-17, IFNγ, MCP-1, TNFα). As for the memory T cells, PSG suppressed CD25 expression and IL-2 production by them, along with simultaneous decreased expression of *Gfi1*, *hnRNPLL* genes, thus preventing the formation of the "mature" CD45R0 isoform. PSG has been shown to inhibit naive T cells' conversion to the terminally differentiated effector subpopulation of helper T cells. When analyzing PSG effects upon cytokine profile of immunocompetent cells, it was found that the protein predominantly suppresses the Th1 cytokine production by the studied cell types, and regulates the Th2 cytokine production in divergent manner. The results obtained are consistent with general concept of immunosuppression during pregnancy. Thus, PSG could be one of the factors preventing formation and implementation of immune response to placental antigens.

Keywords: pregnancy-specific β1-glycoprotein, immunomodulatory effects, immunocompetent cells, fetomaternal immune tolerance

Abbreviations

APC, antigen-presenting cell; CD45, cluster of differentiation 45, leukocyte common antigen; CD45R0, low molecular weight isoform of CD45 receptor; CD45RA, high molecularweight isoform of CD45 receptor; cDNA, complementary deoxyribonucleic acid; CTLA-4, cytotoxic T lymphocyte-associated protein 4; CD152; DNA, deoxyribonucleic acid; GfI1, growthfactor independent 1; GITR, glucocorticoid-induced tumour necrosis factor receptor; hCG, human chorionic gonadotropin; hnRNPLL, heterogeneous nuclear ribonucleoprotein L-like; IDO, indoleamine 2,3-dioxygenase; LPS, lipopolysaccharide; MHC, major histocompatibility complex; AFP, alpha-fetoprotein; FoxP3, forkhead box P3, transcriptional factor; ROR-γt, (RORC2), RAR (retinoic acid receptor)-related orphan receptor gamma; TGF-β1, transforming growth factor beta; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; PSG1, pregnancy specific beta-1-glycoprotein; PTPRC, protein tyrosine phosphatase, receptor type, C; RNA, ribonucleic acid; TCM, central memory T cell ($CD45RA^-CD45R0^+CD62L^+$); TCR, T cell receptor; TEM, effector memory T cell ($CD45RA^-$

CD45R0+CD62L-); TEMRA, terminally differentiated effector memory T cell (CD45RA+CD45R0-CD62L-); Th, helper T cell; Th17, IL-17-producing cells; Treg, regulatory T cells; U2af1l4, U2 smallnuclear RNA auxiliary factor 1 like 4.

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Introduction

The embryo, which carries half of the “foreign” to the mother’s body molecules, must cause a response from her immune system. However, in evolution, mechanisms have been formed that ensure the successful development of pregnancy as a phenomenon of genetically different organisms’ coexistence. In particular, the factors providing immune tolerance during pregnancy are proteins associated with pregnancy.

Pregnancy-specific beta1-glycoprotein (PSG, PSG1; SP1; PSβG1) is a dominant fetoplacental protein produced by cyto- and syncytiotrophoblast cells. It plays a significant role in embryonic development, trophoblast engraftment, hemostasis regulation, and placental angiogenesis [34, 39, 52]. A successful pregnancy implies serious vascular adaptation, including angiogenesis during pregnancy, reconstruction of the maternal decidual arteries, and dilation of the uterine arteries [62].

In humans, the dominant expression product is PSG-1 (PSG1), which was discovered and identified in 1970 by a group of Russian researchers [50]. In pregnancy dynamics, the PSG level gradually increases and reaches 200-400 μg/mL by the third trimester, while in the fetal serum, its level does not exceed 1-2 μg/L [3, 26, 49].

PSG is an expression product of the PSG (pregnancy-specific glycoprotein) genes. It is a member of the CEA (carcinoembryonic antigen) protein family, which, in its turn, is a member of the immunoglobulin superfamily [19]. PSG is a protein family of more than 30 molecular forms, including precursors, glycoisoforms, and catabolic products [35]. Eleven glycoproteins can be referred to as PSG, the protein part of each represented by a single polypeptide chain with a high degree of homology and a molecular weight of 37 to 49 kDa. The carbohydrate portion of PSG can account for 21 to 32% of the protein molecule’s total molecular weight. Thus, glycosylated molecules can have a 46 to 72 kDa [31, 52].

Previous studies have reported abnormal PSG levels in complicated pregnancies and demonstrated the importance of this protein for maintaining healthy pregnancies [13]. Thus, it is known that the level of PSG in the blood serum decreases with spontaneous abortion, ectopic pregnancy, intrauterine growth retardation, preeclampsia, and fetal hypoxia [15, 28]. In 2020, M. Temur and colleagues confirmed that circulating PSG1 levels were significantly lower in women with preeclampsia than in healthy pregnant women [51]. Thus, this protein is vital for the successful development of pregnancy.

The complex structure and diversity of PSG forms give rise to specific difficulties associated with obtaining a pure preparation of native PSG. Only recombinant forms of PSG are available for research, which have their disadvantages (structural differences, incomplete folding, unequal post-translational modification, etc.). Our authors’ team owns a patented method for obtaining a native human PSG preparation, prioritizing research [41].

So, over the past five years, we have demonstrated the effects of a native human PSG preparation, obtained according to the author’s method, on the expression of IDO by antigen-presenting cells, T regulatory lymphocytes, Th17 cells, T cells of immune memory, as well as the regulation of the cytokine profile of these cells. In this review, the obtained immunomodulatory effects of native PSG are characterized.

Characteristic of PSG

First of all, it is worth characterizing the PSG preparation used in the experiments. Human PSG was obtained by the author’s patented immunopurification method using a biospecific sorbent with subsequent release from immunoglobulin contamination on a HiTrap™ Protein G HP column (Amersham Biosciences, Sweden) [41]. The purity of the preparation was confirmed by electrophoresis, molecular heterogeneity – by LC/MS. The preparation obtained by this method contained at least four molecular forms of the protein: PSG-1, PSG-3, PSG-7, PSG-9 [69]. The obtained PSG has apparent advantages over the recombinant forms of the protein and is as close as possible in its composition to the pregnant woman’s PSG. The experiments used physiological PSG concentrations corresponding to its level in the mother’s peripheral blood during pregnancy: 1, 10, and 100 μg/mL (I, II, III trimester, respectively). The research objects were cells of the immune system obtained from the peripheral blood of healthy non-pregnant women of reproductive age. The choice of such an experimental approach is because, during pregnancy, over 400 new proteins that have biological effects appear in the mother’s bloodstream [8]. When choosing pregnant cells as objects, we would be faced with the impossibility of assessing the effect of a specific protein (PSG). Firstly, it is already present in the body of pregnant women. Secondly, its impact is summed up with the effects of other pregnancy proteins.

Pregnancy is a state of immune tolerance to the embryo – the role of pregnancy-associated proteins

During pregnancy, the maternal organism is “immunized” with fetoplacental alloantigens. As a result,

a dynamic state of immune tolerance is formed, and pregnancy-associated proteins play an essential role in its maintenance. In 2008, proteomics methods demonstrated that only a few molecules regulate fetomaternal immune tolerance. Among them PSG, along with chorionic gonadotropin (hCG), alpha-fetoprotein (AFP), glycodelin, and chorionic somatomammotropin [8].

It is known that one of the most significant mechanisms of immunological tolerance formation is the shift in the emphasis of systemic immune responses towards humoral ones (the so-called “phenomenon of Th2 bias”) [57]. In addition to the dominant Th1/Th2 subpopulations, the generation of antigen-specific clones of regulatory T lymphocytes (Treg) is of great importance during pregnancy [43]. The maintaining of peripheral tolerance is also carried out due to the expression by antigen-presenting cells (APCs) of the indolamine 2,3-dioxygenase (IDO) enzyme. IDO is involved in the biotransformation of L-tryptophan with the formation of toxic metabolites [9]. Cells expressing increased levels of IDO promote the generation of adaptive Tregs [10]. These Tregs are considered the critical subpopulation of T helper cells responsible for self-tolerance, forming the so-called “immune regulatory memory” [25].

The balance between Tregs and subpopulations of IL-17-producing lymphocytes (Th17) is of great importance during gestation. It is known that normal pregnancy is accompanied by a decrease in Th17 in the peripheral blood compared to non-pregnant women and an increase in Treg level in the endometrium and the periphery. A reduction of decidual and peripheral Tregs and an increase in the Th17 percentage are accompanied by spontaneous abortion and preeclampsia and can lead to premature birth [43].

Memory T cells, which are generated under constant exposure to antigens of embryonic origin, play an essential role in fetomaternal immune tolerance forming [23, 48]. It has recently become known that long-lived memory T cells are generated during pregnancy, specific to placental antigens, vital for repeated pregnancies [20, 21]. Probably, during uncomplicated pregnancy in the peripheral blood, some factors lead to a decrease in the circulating pool of effector memory T cells capable of carrying out antigen-specific cytotoxic reactions of adaptive immunity against fetal antigens.

The cytokine network is also directly involved in immunological tolerance formation, performing intercellular communication [60]. In general, the modern concept of immunological tolerance is that changes occur in the mother’s immune system during normal pregnancy, accompanied by Th2 and Treg’s dominance over Th1 and Th17, following which circulating spectrum cytokines also changes [43]. Violation of the adequate restructuring of the cytokine balance can cause pregnancy complications. Thus, the study of the fetoplacental proteins regulating the phenomenon of fetomaternal tolerance is a topical area of reproductive immunology.

The role of PSG in the regulation of Treg differentiation

Treg’s primary role is associated with controlling the immune response, contributing to the maintenance of tolerance during pregnancy. In 2012, in an experimental model using mice, it was shown that PSG increased Treg activity, realizing its effects through dendritic cells [29].

The effect of PSG on Treg differentiation was studied *in vitro*, where T helpers were subjected to targeted induction into the Th17 phenotype by cytokines (IL-2+TGF-β1) and polyclonal activation by phytohemagglutinin [69].

Our studies have found that the daily incubation of lymphocytes with PSG (1, 10, and 100 μg/mL) significantly increased the percentage of $CD4^+FoxP3^+$ and $CD4^+CD25^{bright}FoxP3^+$ cells in mononuclear cell culture [67]. Considering that the result was assessed after one day of incubation, and the proliferation of natural Tregs takes several days [38], we interpret the obtained effect as the protein’s ability to increase the proportion of adaptive Tregs. Further studies have shown that PSG at high concentrations (10 and 100 μg/mL) can induce an increase in FoxP3 expression in 72 h monocultures of T helpers ($CD4^+$ cells) [69]. It is important to clarify that these cells were activated by cytokines (IL-2 and TGF-β1), which involve JAK/STAT kinase transduction pathways. The induction of JAK kinases is accompanied by dimerization of STAT5 molecules, which directly enter the nucleus and cause expression of the FoxP3 gene [6].

Because Treg’s functional activity is associated with the surface expression of CTLA-4 and GITR molecules, we also assessed these molecules’ surface expression. PSG at high concentrations (10 and 100 μg/mL) was shown to increase the level of active Tregs expressing GITR. However, only high (100 μg/mL) PSG concentration increased CTLA-4 expression [69]. In the supernatants of T helper cultures polarized in the Treg phenotype, we assessed the level of IL-10 by enzyme immunoassay. It is known that Treg secret this cytokine to suppress the immune response [43]. It was found that PSG (10 and 100 μg/mL) increased the production of IL-10 [68]. The significance of IL-10 for the normal development of pregnancy is confirmed by the fact that spontaneous abortions and ectopic pregnancy are accompanied by a significant decrease in IL-10 mRNA levels [46].

Interestingly enough, the low PSG concentration (1 μg/mL) did not have a similar effect. Extrapolating the obtained data to the *in vivo* situation, we can say that when the PSG level is low (1 μg/mL) in the first trimester, it does not affect Treg. Simultaneously, in

the II-III trimesters, when the level of PSG is significantly increased, this protein effectively increases the amount of Treg and the surface membrane expression of CTLA-4 and GITR markers by these cells, and the production of IL-10.

To confirm the obtained effects at the Treg level, we carried out some PCR experiments to assess PSG's role in the regulation of FoxP3 mRNA expression by the T helpers [63]. It was found that PSG (1 and 100 μg/mL) enhances spontaneous expression of FoxP3 mRNA, assessed after 18 h without additional inducers. As a result, we demonstrated that PSG increases the expression of the FoxP3 transcription factor by T helpers both spontaneously and under Treg polarizing conditions, participating in immunological tolerance formation during pregnancy.

Thus, PSG can increase the level of Tregs as well as their activity *in vitro*. If these effects of PSG are extrapolated *in vivo*, Tregs, in turn, suppress proliferation, activation, and effector functions of a wide range of immunocompetent cells, including $CD4^+$, $CD8^+$ lymphocytes, NK, NKT, B cells, and APC [45]. In general, this leads to suppressing the immune response to fetoplacental antigens and contributes to the successful development of pregnancy.

Effect of PSG on indoleamine-2,3-dioxygenase expression and activity

Indoleamine-2,3-dioxygenase (indoleamine-2,3-dioxygenase, IDO) is an enzyme that initiates the oxidative degradation of L-tryptophan along the kynurenine pathway with the formation of toxic products such as L-formyl kynurenine, L-kynurenine, 3-hydroxykynurenine, and others [22]. These metabolites are involved in the induction of immune tolerance in various physiological and pathological conditions, including pregnancy. IDO is widely expressed in most organs and tissues, including the chorion, placenta, decidua, and APC [7, 33].

First of all, we assessed the effect of PSG on the IDO activity in monocytes by the spectrophotometric method based on the change in kynurenine concentration in a short-term 4-hour culture of mononuclear cells. The stimulating effect of PSG (1, 10, 100 μg/mL) on IDO activity in the LPS-induced test was demonstrated [67].

We confirmed the obtained data by flow cytometry, examining the intracellular expression of IDO in peripheral monocytes after 24 h incubation with PSG. It was shown that PSG at all studied concentrations increased the expression of IDO in IFNγ-induced probes. At the same time, only a low concentration of PSG increased the expression of IDO in LPS-induced samples [70]. It is known that stimulation of cells with IFNγ triggers the STAT1-dependent pathway of IDO expression, and LPS, through a signaling pathway from Toll-like receptors, converts inactive IDO into a biologically active enzyme [4]. Thus, PSG increased the activity of IDO; however, depending on the type of inducer, and its effects were concentration-depended.

Thus, it was found that PSG stimulates the expression of IDO by female monocytes, contributing to the formation of peripheral immunological tolerance during pregnancy. In addition, the regulation of IDO activity is essential in the processes of carcinogenesis, transplant rejection and plays a critical role in autoimmune diseases' pathogenesis.

The role of PSG in the regulation of Th17 differentiation

Th17 is a subset of T helper cells that produces large amounts of the IL-17 proinflammatory cytokine, which plays an important role in inflammation induction, the development of autoimmune diseases, and acute transplant rejection. The main Th17 transcription factor is ROR-γτ (RORC2) (RAR (retinoic acid receptor)-related orphan receptor gamma) [43]. We studied the effect of PSG on Th17 differentiation *in vitro*, where helper T cells were polarized into the Th17 phenotype using proinflammatory cytokines (IL-6, IL-1β) and a T cell activator (T Cell Activation/Expansion Kit human, Miltenyi Biotec, Germany) [69].

As a result, our studies found that PSG at high concentrations (10 and 100 μg/mL) reduced the expression of $ROR\text{-}\gamma\tau^+$ in $CD4^+$ lymphocytes. In addition, PSG (100 μg/mL) also inhibited the number of double-positive $ROR\text{-}\gamma\tau^+IL\text{-}17A^+CD4^+$ lymphocytes [69]. The concentration of IL-17A in supernatants of Th17-induced $CD4^+$ lymphocyte culture was assessed in parallel by the enzyme immunoassay. It was found that PSG (100 μg/mL) reduced the level of IL-17A, thus suppressing the functional activity of Th17 [69].

We extended our results on the role of PSG in the regulation of Th17 cells with a series of similarly designed experiments, where this aspect of PSG action was studied in more detail. In particular, we investigated the effect of PSG on Th17 differentiation, simultaneously assessing the proliferation of these cells (by Ki-67 expression) and the cytokine profile of culture supernatants using a multiplex method [54].

As a result of the research, we found that PSG suppressed the expression of both ROR-γτ and Ki-67 in $CD4^+$ cells (10 and 100 μg/mL). Overall, we confirmed that PSG is capable of inhibiting Th17 cell differentiation and proliferation. When analyzing the cytokine profile, we found that PSG suppressed the production of IL-5, IL-7, IL-8, IL-10, IL-12, IL-17, IFNγ, MCP-1, TNFα, as well as G-CSF, and GM-CSF [54]. In the context of the studied subpopulation, it is crucial for us that PSG (10 and 100 μg/mL) reduced the production of IL-17, which is consistent with the expression of ROR-γτ and our previous studies [69]. Thus, in the experimental model used, PSG had a pronounced suppressive effect on

the differentiation and cytokine production of Th17-polarized helper T cells. PSG likely has a fetoprotective role *in vivo,* reducing the activity of these cells. It is important to note that a normal pregnancy is accompanied by a decrease in peripheral blood Th17, while an increase in Th17 may lead to premature birth or spontaneous abortion [43]. Thus, PSG inhibits Th17 functional activity, contributing to a successful pregnancy outcome.

The role of PSG in the regulation of immune memory T cells differentiation

PSG in the regulation of molecular genetic factors of naive and immune memory T cells differentiation

It is known that the functional activity of T lymphocytes is closely related to the CD28 and CD25 surface markers expression. The CD28 molecule is the primary coreceptor mediating positive costimulation of T cells, participating in forming an immune synapse through interaction with CD80/86 on the surface of APC. CD25 (the α-chain of the IL-2 receptor) is an early activation marker that is functionally associated with the production of IL-2 and reflects the ability of cells to differentiate and proliferate [27].

The study of memory T cell differentiation is currently associated with assessing the expression of various isoforms of the CD45 molecule, regulating T cell receptor (TCR) signaling [32]. In the T cell differentiation process, the structure of the extracellular domain of CD45 changes: for example, in naive T cells, it is the full form (CD45RA, 220 kDa). During antigen-dependent differentiation, several domains are lost, and the product of the final modification expressing on primed T cells of immune memory is designated as CD45R0 (180 kDa) [24]. To understand the role of PSG in the differentiation of memory cells, we studied both naive T cells and T cells of immune memory proper [42].

In general, CD45 is a transmembrane tyrosine-protein phosphatase encoded by the *Ptprc* gene [61]. By the mechanism of alternative splicing, as a result of the differential use of three exons (4, 5, and 6) of the *Ptprc* gene, it is possible to generate eight different isoforms of the CD45 molecule, five of which are present on lymphocytes (R0, RA, RB, RBC, and RABC) and determine the stages of their differentiation [5]. After activation of T cells, skipping of the variable exons of CD45 leads to homodimerization of the receptor on the cell surface and forming an inactive form of phosphatase with a decrease in signaling through the TCR. Currently, three genes have been identified (*U2af1l4, Gfi1, hnRNPLL*), whose products interact to modulate the differentiation of immunocompetent cells by regulating alternative splicing of the *Ptprc* gene [16].

That is why we studied the maturation and differentiation of T cells with a simultaneous assessment of the levels of relative expression of the *U2af1l4*, *Gfi1*, and *hnRNPLL* genes, which regulate alternative splicing of the *Ptprc* gene in the studied subpopulations of T cells ($CD45R0^+$, $CD45RA^+$).

As a result of the research, it was found that PSG inhibited the expression of CD28 and CD25 on naive T cells without affecting the production of IL-2 by them. At the same time, PSG suppressed the expression of CD25 on the immune memory T cells and the production of IL-2 by them. In parallel, the expression of genes *U2af1l4, Gfi1, hnRNPLL*, regulating alternative splicing of the Ptprc gene encoding CD45, was assessed. It was found that PSG decreased the expression of the *Gfi1* and *hnRNPLL* genes, but increased the expression of the *U2af1l4* gene in the studied T cell subpopulations, thus preventing the formation of the “mature” CD45R0 isoform [42].

It is known that the products of the *hnRNPLL* gene coordinate the work of many transcription factors in the process of alternative splicing of T lymphocytes. The functional activity of the *hnRNPLL* gene is associated with the expression of CD28, and it is assumed that this relationship is an additional mechanism for regulating alternative splicing of CD45 [5]. In particular, increased expression of the *hnRNPLL* gene causes exon 4 skipping, which leads to the formation of the short isoform CD45R0 [55]. In the context of our work, the downregulation of the *hnRNPLL* gene under the influence of PSG seems to block the trans-differentiation of naive T cells into memory T cells ($CD45R0^+$).

In addition to the involvement of the *hnRNPLL* gene products in the regulation of alternative splicing of the CD45 molecule, an essential role in this process is attributed to the joint actions of the auxiliary splicing factor *U2AF26* and the transcription factor *Gfi1*. It was shown that antagonistic interactions between *U2AF26* and *Gfi1* determine the ratio of CD45: *U2AF26* isoforms contribute to the fourth exon exclusion, which leads to the formation of short isoforms – CD45R0, while Gfi1 promotes the formation of a more active, high molecular weight form of the receptor – CD45RB or RA [16]. Thus, the effects of PSG revealed by us seem to prevent the generation of a more active, high-molecular form of the receptor, CD45RB or RA, due to decreased expression of *Gfi1*. At the same time, an increase in the expression of *U2af1l4*, which can promote the formation of CD45R0, is compensated by a decreased expression of the *hnRNPLL* gene, which has the opposite effect on the formation of the “mature” form of CD45R0.

Thus, PSG reduces the functional activity of naive T cells and immune memory T cells associated with the expression of CD25 and CD28. The data obtained expand the understanding of the role of PSG in the regulation of molecular genetic mechanisms of differentiation of “naive” T cells and T cells of immune memory.

PSG in the regulation of the conversion of naive T cells to effector subpopulations of T cells

As mentioned above, the question of the linearity of memory T cells differentiation is not fully understood; nevertheless, it is believed that changes in the expression of CD45 isoforms are directly associated with the passage of cells at various stages of differentiation [24]. In addition, cell transformation is associated with changes in the expression of L-selectin (CD62L) molecules, which are involved in cell translocation into lymphoid tissue [44]. Thus, a part of naive T cells ($CD45RA^+CD45R0^-CD62L^+$) after contact with the antigen undergoes conversion into central memory T cells (TCM; $CD45RA^-CD45R0^+CD62L^+$), which do not exhibit effector functions but can quickly respond to the antigen after re-stimulation. Another part of the memory cells pool is transformed into pre terminally differentiated memory effector T cells (TEM; $CD45RA^-CD45R0^+CD62L^-$) and terminally differentiated memory T cells (TEMRA; $CD45RA^+CD45R0^-CD62L^-$). Both TEM and TEMRA secrete cytokines, primarily IL-4 and IFNγ, and other biologically active molecules [44]. Taking into account the fact that physiological pregnancy does not affect the number of peripheral $CD8^+$ memory lymphocytes ($CD45R0^+CD8^+$) but significantly regulates the functions of memory $CD4^+$ lymphocytes [20, 21], we conducted a series of experiments to study the effect of PSG on the conversion of naive T helpers into effector subpopulations.

Thus, it was shown that the introduction of PSG at concentrations of 1, 10, and 100 μg/mL into cultures of activated T helpers did not affect the conversion of naive helper T cells to TCM and TEM. However, PSG at high concentrations significantly reduced the percentage of TEMRA while increasing the level of naive helper T cells in culture [53]. It is known that TEMRA cells are resistant to apoptosis and have a powerful potential for cytokine production upon repeated contact with the antigen [44]. Probably, a decrease of TEMRA percentage under the influence of PSG has a fetoprotective effect *in vivo*. In the supernatants of activated helper T cells, the level of the central cytokines determining the direction of the immune response, IL-4 and IFNγ, was assessed. These cytokines are produced mainly by the effector populations of T lymphocytes – TEM and TEMRA [44]. In the peripheral blood of healthy people, TEM cells have the highest percentage of IFNγ-producing cells [12]. It was shown that PSG reduced the production of IL-4 and IFNγ in culture supernatants of activated T helpers [53]. Obviously, it is TEM and TEMRA that make the main contribution to the production of IL-4 and IFNγ. In the context of our data, the decrease of TEMRA percentage under the influence of PSG is logically associated with a reduction in the production of IL-4 and IFNγ. It is known that an increase of effector $CD4^+$ cells (TEM and TEMRA) percentage occurs during the development of preeclampsia when the circulation of the fetoplacental complex antigens increases due to the gain in placental permeability [23]. Thus, PSG may be one of the factors preventing the formation and implementation of an immune response to placental antigens. In general, the obtained data expand our understanding of the role of PSG in the formation of immune tolerance during pregnancy.

The role of PSG in the regulation of the cytokine and chemokine profiles of immunocompetent cells

The cytokine network is directly involved in immunological tolerance formation, performing intercellular communication function [61]. In general, the modern concept of immunological tolerance is that changes occur in the mother's immune system during normal pregnancy, accompanied by the dominance of Th2 and Treg over Th1 and Th17, following which the spectrum of circulating cytokines also changes. Violation of an adequate restructuring of the cytokine balance can cause a complicated pregnancy.

We assessed the role of PSG in the regulation of the production of basic cytokines and chemokines by immunocompetent human blood cells without additional inducers under conditions of 18-hour incubation. After incubation, the content of 27 cytokines and chemokines was determined by flow fluorimetry (multiplex analysis, Luminex xMAP) in culture supernatants: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, TNFα, IFNγ, IP-10, G-CSF, GM-CSF, Eotaxin, FGF-β, PDGF-BB, RANTES, VEGF, MCP-1, MIP-1α, MIP-1β.

We found that when introduced into the culture of mononuclear cells, PSG reduced the production of proinflammatory cytokines IL-6, IL-8, IL-17, IFNγ, TNFα [40]. In general, the integral cytokine profile under PSG's action is formed as an anti-inflammatory (Th2).

We also found that PSG at high concentration had an inhibitory effect on IL-9 and IL-13 production. It is known that IL-13 stimulates the differentiation of T cells and the production of antibodies, and IL-9 is associated with the development of autoimmune reactions. Therefore, a decrease in their levels under the influence of PSG in the context of pregnancy plays a prominent role in fetoprotection. Also, we found that PSG (100 μg/mL) inhibits the production of G-CSF and GM-CSF by mononuclear cells. These hematopoietic colony-stimulating factors are necessary for the onset and development of pregnancy. It is possible that *in vivo,* their synthesis is stimulated by other factors; in particular, it is known that the expression of GM-CSF is triggered by chorionic gonadotropin [37].

What concerns chemokines, PSG at a high concentration was shown to suppress CCL2/MCP-1 pro-

duction and CCL4/MIP-1β, and its low concentrations were shown to decrease the production of CCL3/MIP-1α. These chemokines realize regular cell transit and cell migration during inflammation, and their increased levels during pregnancy are associated with the development of spontaneous abortions [58]. Regarding the production of VEGF by mononuclear cells, we registered the stimulating effect of PSG at a 100 μg/mL concentration, which *in vivo* can contribute to blood vessels' genesis.

It was found that PSG reduced the production of proinflammatory cytokines with autocrine regulation function (IL-1β, IL-1ra, IL-6, IL-8, IL-9, IL-15, IFNγ, IL-2, TNFα) in isolated $CD4^+$ cell culture [66].

It is important to note that the effect of a high PSG concentration (100 μg/mL) was more evident and affected all of the listed cytokines. A middle PSG concentration (10 μg/mL) inhibited only IL-1ra, IL-6, IL-2, TNFα, while PSG (1 μg/mL) did not affect the concentration of the listed cytokines at all. In addition, it was shown that PSG inhibited the production of G-CSF (100 μg/mL) and GM-CSF (1, 10, 100 μg/mL). About chemokine synthesis, PSG was found to suppress the production of CCL3/MIP-1α, CCL4/MIP-1β, PDGF-BB (all doses), CCL5/RANTES (100 and 10 μg/mL) and CCL2/MCP-1 (10 and 100 μg/mL) [64]. Also, we demonstrated the inhibitory effect of PSG (100, 10 μg/mL) on the production of VEGF by $CD4^+$ cells. A stimulating effect of PSG on IL-5 production was found – at a concentration of 10 μg/mL, a similar trend for a high dose of PSG.

Thus, PSG had a predominantly suppressive effect on the production of the studied cytokines and chemokines, and the effect of a high protein concentration was universal. In general, PSG exerted a predominantly inhibitory effect on the production of proinflammatory cytokines and chemokines, suppressing the generation of Th1 and Th17. The revealed results can be interpreted as the contribution of PSG to the formation of immunological tolerance during pregnancy.

Conclusion

It is known that the most crucial factor in the protection of the fetus is the maternal immunological tolerance to the antigens of paternal origin. Taken together, the effects of hormones and specific proteins of the placenta are synergistic and form a network of biological protection of the fetoplacental complex from the action of the cellular and humoral components of the mother's immune system. Obtained by us immunomodulatory effects of PSG coincide with the general vector of immunosuppression *in vivo*. Thus, it can be assumed that PSG is one of the factors that form immune tolerance during pregnancy.

The figure shows the final diagram summarizing the data obtained (Fig.1, see 3rd page of cover). PSG significantly increases the amount of Tregs in culture, thus increasing the proportion of adaptive Tregs. In turn, Tregs suppress proliferation, activation, and effector functions of a wide range of immunocompetent cells, including $CD4^+$, $CD8^+$ lymphocytes, NK, NKT, B cells. In addition, PSG increases the expression of CTLA-4 and GITR on the Treg surface. It is known that CTLA-4 suppresses the T cell response by competing for the binding of the same ligands (CD80/CD86) to the positive co-stimulatory CD28 receptor. CTLA-4 accumulates in lysosomes and is secreted to the T lymphocyte site with APC after stimulation of the T cell receptor. Also, PSG increased the level of IL-10 in the culture of helper T cells targeting the Treg phenotype.

We confirmed the Treg level both by flow cytometry by intranuclear expression of FoxP3 and by PCR by the expression of FoxP3 mRNA. At the same time, we evaluated the expression of TGF-β1 mRNA, which was also increased under PSG's influence [65]. The following relationship is quite interesting: PSG promotes the production of TGF-β1 [38], which is a key cytokine that induces the development of Tregs, which suggests that PSG can induce Treg differentiation through TGF-β1.

It was shown that the stimulating effect of PSG is realized in relation to IDO activity. Cells expressing increased IDO levels further promote the generation of adaptive Tregs, which ultimately leads to immunosuppression. At the same time, it is evident that PSG is involved in the regulation of IDO via the involvement of CTLA-4^+ Tregs too. Contact of CTLA-4 molecules with CD80/CD86 ligands of antigen-presenting cells leads to increased IDO expression in the latter. In turn, cells expressing increased levels of IDO further facilitate the generation of adaptive Tregs. Thus, it was found that PSG stimulates the expression of IDO by female monocytes, contributing to peripheral immunological tolerance during pregnancy.

As a result of our studies, we found that PSG suppressed the proliferation and differentiation of the Th17 proinflammatory subpopulation. When analyzing the cytokine profile, it was found that PSG inhibited the production of primarily proinflammatory cytokines (IL-8, IL-17, IFNγ, MCP-1, TNFα), as well as G-CSF and GM-CSF. It is likely that PSG, reducing these cells' activity, exerts a fetoprotective effect *in vivo*.

As a result of the research, it was found that PSG inhibited the expression of CD28 and CD25 activation markers on the naive T cells without affecting the IL-2 production by them. At the same time, at the level of immune memory T cells, PSG suppressed the expression of CD25 and the production of IL-2 by these cells. It was found that PSG reduced gene expression that regulates alternative CD45 splicing (*Gfi1, hnRNPLL*). In the

context of our work, downregulation of the *hnRNPLL* gene under the influence of PSG seems to block the transdifferentiation of naive T cells into memory T cells.

In 2017 Keiffer T.E. and colleagues showed that physiological pregnancy does not affect the number of peripheral $CD8^+$ memory lymphocytes ($CD45R0^+CD8^+$) but significantly regulates the function of memory $CD4^+$ lymphocytes [20, 21]. To clarify the data obtained, we conducted a series of experiments to study the effect of PSG on the conversion of naive helper T cells to effector ones. It was found that PSG did not affect the conversion of naive helper T cells to TCM and TEM; however, it significantly reduced the TEMRA level at high concentrations. It is known that these cells are resistant to apoptosis and have a strong potential for the production of cytokines upon repeated contact with the antigen [44]. In supernatants of activated T helpers, PSG decreased the level of IL-4 and IFNγ, the central cytokines that determine the direction of the immune response. These cytokines are produced mainly by the effector populations of T cells – TEM and TEMRA. Probably a decrease in the percentage of TEMRA under the PSG influence has a fetoprotective effect *in vivo*.

As a result of our experiments, we found that when introduced into mononuclear cells' culture, PSG reduced the production of proinflammatory cytokines IL-6, IL-8, IL-17, IFNγ, TNFα. It was found that in T helper culture, PSG reduced the production of proinflammatory cytokines that play the role of autocrine regulation – IL-1β, IL-1ra, IL-6, IL-8, IL-9, IL-15, IFNγ, IL-2, TNFα. At the same time, PSG suppressed chemokines' production, an increased level of which is associated with spontaneous abortion: IL-8, MIP-1α, MIP-1β, RANTES, and MCP-1. As a result, it can be concluded that PSG forms the fetoprotective chemokine profile of the studied peripheral cells of the immune system. In general, PSG creates an anti-inflammatory cytokine profile (Th2).

Interestingly enough, in our study, the effects of PSG are more pronounced in high concentrations extrapolated from the II-III trimesters of pregnancy. It can be assumed that this is related to the fact that with an increase in gestational age, the number of antigenic determinants of the fetoplacental unit increases in parallel. An active sensitization of the mother with antigens of the fetus and trophoblast occurs. As a result, this leads to the development of immune reactions directed against alloantigens of the fetoplacental complex. However, a parallel increase in PSG level in the mother's peripheral blood suppresses these reactions, protecting the semi-allogenic embryo. It can be assumed that the lowered PSG levels, which are associated with certain pathological conditions during pregnancy, will, to some extent, cancel the immunosuppression necessary for fetus protection.

In conclusion, it is worthwhile to analyze the study of PSG in a global context briefly. Thus, several researchers study the immunomodulatory effects of PSG in models with experimental animals, using mainly recombinant forms of the protein. For example, it is known that recombinant PSG-23 induces the synthesis of IL-10, IL-6, TGF-β1, and VEGF cytokines by mouse macrophages, thus contributing not only to immunosuppression but also angiogenesis [59]. Recombinant PSG1, PSG6, PSG6N, and PSG11 induce dose-dependent secretion of IL-10, TGF-β1, and IL-6 cytokines by human monocytes and RAW 264.7 mouse cells, demonstrating interspecies activity [47]. It is known that recombinant PSG1a induces alternative activation of human and mouse monocytes, associated with arginase activity, while simultaneously suppressing the proliferation of T cells [36].

In an experimental mouse model, it was shown that recombinant PSG1a promotes the formation of a unique DC phenotype, which secretes IL-6, IL-10, TGF-β1; stimulates the formation of a Th2 cytokine profile, and increases Treg and Th17 percentage [30]. In general, PSG contributes to the modulation of both innate and adaptive immune responses [29]. The immunomodulatory effects of PSG1 were investigated in a mouse model of collagen-induced arthritis (CIA). It was found that recombinant PSG1a improved the clinical symptoms of arthritis while simultaneously increasing the level of Treg in the spleen and also suppressed the Th1 and Th17 responses [11]. In 2015, it was shown that recombinant PSG suppressed the development of DSS-induced colitis in mice, increasing the Treg percentage while simultaneously decreasing the level of proinflammatory cytokines [2]. The same authors found that recombinant and native PSG1 activate TGF-β1 and TGF-β2 *in vitro*. It is known that TGF-β is secreted as latent complexes, and its activity is regulated through the activation of these complexes. In general, the authors identify PSG1 as one of the few known biological activators of TGF-β2 [2]. In 2018, it became known that all human PSG and mouse PSG23 activate latent TGF-β1. Apparently, PSG can potentially increase the availability of active TGF-β1 from soluble and matrix-bound latent cytokine forms *in vivo*, contributing to creating a tolerogenic environment during pregnancy [56].

Moreover, a little earlier, the specific mechanism of PSG1 domains binding to TGF-β1 was determined, which, in general, provides a mechanistic basis for how exactly PSG modulates the immunoregulatory environment in the fetomaternal interface [1]. In 2019, the role of PSG in the prevention of acute GVHD in mice was investigated. It was found that in mice receiving recombinant PSG1, the level of Treg increased, and the level of inflammatory T lymphocytes infiltrating the tissue decreased. In addition, the

PSG1 administration significantly reduced weight loss and mortality associated with a GVHD [18].

Interestingly, and consistent with our data, PSG9 induced Treg differentiation *in vitro* both at the level of human and mouse cells [17]. The same authors found that PSG9 binds to LAP and activates the latent form of TGF-β1. In addition, PSG9 induces the secretion of TGF-β1 by macrophages, but not by helper T cells, while simultaneously decreasing the production of proinflammatory cytokines in cell cultures. The authors suggest that PSG9, due to the activation of TGF-β1, can be a potent inducer of immune tolerance [17]. The mechanism of PSG action is, in some cases, associated with the CD9 molecule, the expression of which affects the realization of the protein's effects [14, 69].

Thus, our results demonstrate previously unknown immunomodulatory effects of PSG, which may contribute to immune tolerance formation during pregnancy. In general, our results coincide with the general vector of immunosuppression during pregnancy and quite logically correlate with other researchers' results. Further study of the influence of the PSG on the formation of immune tolerance will open up the possibilities of its use as a promising agent for treating autoimmune diseases.

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