ВЛИЯНИЕ ГЛИКОДЕЛИНА НА CCR6$^+$ СУБПОПУЛЯЦИИ Т-ХЕЛПЕРОВ, ПОЛЯРИЗОВАННЫХ В ФЕНОТИП Th17

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Резюме. Гликоделины, белки репродуктивного тракта, отличающиеся гликозилированием и обладающие иммуномодулирующими функциями, представляют интерес в связи с их ролью в формировании иммунной толерантности. Интерлейкин-17-продуцирующие Т-хелперы (Th17), несущие поверхностный маркер CCR6, являются гетерогенной клеточной популяцией с повышенной пластичностью и функциональной дихотомией. С одной стороны, эти клетки поддерживают антимикробный и противогрибковый иммунитет, состав микробиоты, а с другой – участвуют в патогенезе аутоиммунных заболеваний, отторжении трансплантата и осложнениях беременности. Несмотря на достаточно высокий научный интерес к гликоделину как к иммуномодулятору, его прямые эффекты на провоспалительные Th17 не изучались. Поэтому целью нашей работы было исследовать влияние рекомбинантного гликоделина человека на Th17-поляризацию наивных Т-хелперов человека, оценивая экспрессию ими поверхностных молекул CCR6, CCR4 и CXCR3. Наивные Т-хелперы поляризовали в Th17 *in vitro* при помощи активатора TCR и цитокинов в течение 7 дней с добавлением гликоделина в концентрациях, характерных для первого-второго триместров беременности. После этого определяли процент популяции CD4$^+$CCR6$^+$ клеток (Th17), и ее CCR4$^+$CXCR3$^-$ (Th17/Th22) и CCR4$^-$CXC3$^+$ (Th17.1) субпопуляций. Кроме того, при помощи мультиплексного анализа определяли концентрацию цитокинов и хемокинов в супернатантах культур Th17-поляризованных Т-хелперов. Рекомбинантный гликоделин в концентрациях, соответствующих таковым при беременности (0,2, 2 и 10 мкг/мл), не изменял процент CD4$^+$CCR6$^+$ клеток в культуре и продукцию ими IL-17 и других исследуемых цитокинов. Однако в концентрации 10 мкг/мл он снижал долю Th17.1 (CCR6$^+$CCR4$^-$CXCR3$^+$) и увеличивал концентрацию IL-2 в культуре Т-хелперов. Помимо этого, установлено, что гликоделин в концентрации 2 мкг/мл проявляет избирательную апоптотическую активность в от-

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ношении Th17.1. В силу описанной вовлеченности этих клеток в патологические процессы, данный эффект гликоделина может представлять интерес с точки зрения биофармацевтики, но механизм обнаруженного избирательного действия этого белка беременности требует дальнейшего исследования.

Ключевые слова: гликоделин, IL-17, Th17, CCR6⁺ Т-хелперы, PP14, PAEP

EFFECTS OF GLYCODELIN ON CCR6⁺ CELL SUBPOPULATIONS OF Th17-POLARIZED HELPER T CELLS

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Abstract. Glycodelins, the glycosylated proteins of reproductive tract are characterized by immunomodulatory functions, are of interest because of their role in the development of immune tolerance. Interleukin-17-producing T helpers (Th17) bearing the surface marker CCR6, are a heterogeneous cell population with increased plasticity and functional dichotomy. On the one hand, these cells support antimicrobial and antifungal immunity and microbiota composition; on the other hand, they are involved in the pathogenesis of autoimmune diseases, graft rejection, and pregnancy complications. Despite the scientific interest in glycodelin as an immunomodulator, its direct effects on pro-inflammatory Th17 have not been studied. Therefore, the aim of our work was to investigate the effect of recombinant human glycodelin on Th17 polarization of naïve human T helper cells cells by assessing surface expression of CCR6, CCR4, and CXCR3 molecules. Naïve T helper cells were polarized for 7 days *in vitro* to Th17 cells with a TCR activator and cytokines for 7 days, supplemented with glycodelin at concentrations appropriate for the 1st and 2nd trimesters of pregnancy. The percentages of $CD4^+CCR6^+$ cell population (Th17 cells), and their $CCR4^+CXCR3^-$ (Th17/Th22) and $CCR4^-CXC3^+$ subpopulations (Th17.1) was then determined. Moreover, the levels of IL-17, IL-2, and other cytokines/chemokines were determined in the culture supernatants of Th17-polarized T helper cells. Treatment with recombinant glycodelin at concentrations equivalent to those in pregnancy (0.2, 2, and 10 μg/mL) did not alter the percentage of $CD4^+CCR6^+$ cells in culture, or their IL-17 production. However, at a concentration of 10 μg/mL, it caused a decrease in Th17.1 ($CCR6^+CCR4^-CXCR3^+$) percentage in the T helper culture, and increased the production of IL-2. In addition, glycodelin was found to have selective pro-apoptotic activity against Th17.1 if applied at 2 μg/mL. Given the known involvement of these cells in pathological processes, the observed effect of glycodelin could be of interest from a biopharmaceutical perspective. However, the mechanism of the revealed selective effects of this pregnancy protein needs further investigation.

Keywords: glycodelin, IL-17, Th17, CCR6⁺ T helpers, PP14, PAEP

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Introduction

Scientific interest in pregnancy (placental) glycoproteins has not diminished for decades [10, 17, 49]. First of all, these molecules arefascinating in the context of basic research on the phenomenon of immunological tolerance between mother and fetus. Moreover, the effects of these molecules on various cells of the immune system open the way to the world of a promising industry – biopharmacy [39]. Glycodelins are human glycoproteins that belong to the lipocalin superfamily [63] and bind small hydrophobic molecules, cell surface receptors, and soluble macromolecules [12]. Four isoforms of glycodelin (A, C, F, S) are secreted in reproductive organs and exhibit sex-specific glycosylation that regulates important phases of human reproduction [34, 61, 62, 79]. The immunosuppressive effect of glycodelin has been known for a long time and is interpreted by researchers as one of the mechanisms that allow to protect a semi-allogenic fetus from the mother's immune system [5]. However, the discovery of previously unknown

subpopulations of immunocompetent cells involved in pro- and anti-inflammatory responses, including those at the maternal-fetal interface, presents researchers with new challenges. In addition, the demand for mild, side-effect-free immunosuppressants and the booming biopharmaceutical market are opening the way for new research on glycodelin. Since bacterial protein expression systems are still the most productive and available, recombinant forms of glycodelin are more promising for use as biopharmaceuticals [27].

IL-17 producing helper T cells (Th17) differs from other subpopulations of T helper cells by a certain dichotomy of their functions [59, 67]. On the one hand, they protect against mucosal pneumococcal infections, eliminate extracellular infections, maintain the composition of the microbiota and provide antifungal immunity [8, 19, 30, 35, 36, 80]. On the other hand, Th17 are involved in the pathology of autoimmune diseases (psoriasis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease) and transplant rejection, and are associated with pregnancy complications and tumor development [18, 19, 58, 70]. An increase in Th17 count during pregnancy indicates pathological changes and may lead to preterm delivery or miscarriage [58]. It has been shown that the inflammatory hypoxic conditions that led to neuropathology in mouse offspring were due to IL-17RA signaling, as IL-17RA-/- mice were protected from disease, supported by clinical data of high Th17 cell numbers in children diagnosed with autism spectrum disorder [23]. This duality is largely determined by the so-called "plasticity" of these cells. The observed plasticity of Th17 is determined by the epigenetic regulation of key transcription factors and cytokines that set the polarization state [55]. When the cytokine milieu changes, they can transdifferentiate into other Th-like subpopulations. For example, when they begin to produce IFNγ, Th17 take on the characteristics of Th1 cells [66]. Now such cells are referred to as pathogenic Th17 [73]. There is evidence that these cells (also called Th17-derived Th1) play an important role in the pathogenesis of chronic inflammatory diseases with autoimmune character [15, 31].

Although there are many articles on the immunomodulatory effects of glycodelin, its impact on the Th17 proinflammatory subpopulation have been studied in only a few research papers. For example, in a 2012 study, macrophages treated with GdA in co-culture with autologous lymphocytes had no significant effect on intracellular IL-17 levels of T helper cells [32]. Studies on the direct effect of glycodelin on Th17 and its subpopulations according to the expression of chemokine surface receptors have not been performed yet.

If glycodelin retains its immunosuppressive properties, it could be used in biomedicine as a biopharmaceutical for the treatment of post-transplant complications [9, 56] and autoimmune diseases [46]. Therefore, the aim of this study was to investigate the direct effects of the recombinant form of glycodelin on Th17 polarization of $CD4^+$ cells.

Materials and methods

The research was conducted in accordance with the Declaration of Helsinki of the World Medical Association and the Council of Europe Protocol to the Convention on Human Rights and Biomedicine and approved by the Ethics Committee of the Institute of Ecology and Genetics of Microorganisms, Ural Branch of the Russian Academy of Sciences (IRB00010009) on August 30, 2019. Written informed consent was obtained from all participants.

The main stages of the experiment were as follows: Isolation of naïve T helpers from peripheral blood mononuclear cells, their cultivation under Th17-polarizing conditions (TCR activator$^+$ a complex of cytokines and antibodies) with the addition of glycodelin (or glycerol) at various concentrations, analysis of lymphocyte viability and proliferation, determination of the percentage of the Th17-enriched population and its subpopulations, determination of the cytokine profile in the culture supernatants.

Study groups

Written informed consent was obtained from all subjects who participated in the study. Venous blood samples were obtained from healthy donors (nonpregnant women, n = 6, 25-39 years old) by venipuncture with vacuum tubes (BD Vacutainer™, Greiner-bio-one, Austria).

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Diacoll 1077, Dia-m, Russia, = 1.077 g/cm^3). The maximum time between blood sample collection and density gradient separation was 30 minutes.

Glycodelin

We used recombinant human glycodelin (PAEP) protein from *E. coli*, MBS718444, with His tag, synthesized on demand from the website https://www.mybiosource.com/recombinant-protein/glycodelin-paep/718444.

For the experiment, we used the concentration of glycodelin corresponding to its level in the peripheral blood of a woman during physiological pregnancy (0.2 μg/mL – I and III trimesters; 2 μg/mL – II trimester), as well as in a concentration of 10 μg/mL corresponding to its concentration in the amniotic fluid (I trimester) and endometrial tissues [14, 24].

Isolation and culture of naïve $CD4^+$ cells

Naïve $CD4^+$ cells were obtained from PBMCs by negative immunomagnetic separation (MACS® MicroBeads and MS Columns, Miltenyi Biotec, Germany). The purity of isolated naïve T cells was confirmed by CD45R0, CD45RA and CD62L staining (CD45RA-FITC, CD45R0-PE (BioLegend,

USA) and CD62L-APC (Miltenyi Biotec, Germany) and a CytoFLEX S Flow Cytometer (Beckman Coulter, USA). The average percentage of naïve ($CD45R0^-CD45RA^+CD62L^+$) cells was ~70% from single lymphocytes.

Th17 polarization

Isolated naïve $CD4^+$ cells (1×10^6 cells/mL, 200 μL) in serum-free complete medium (TexMACS™ medium (Miltenyi Biotec) supplemented with 10 mM HEPES, 2 mM L-glutamine (both from ICN Pharmaceuticals, USA) and penicillin-streptomycin-amphotericin B (BI, Israel)) were cultured in 96-well plates in a humidified CO_2 incubator at 37 °C and 5% CO_2 for 7 days without medium change.

Cultures without glycodelin served as controls. The purchased drug (initial concentration 0.83 mg/mL) contained 50% glycerol. Purification of the drug by dialysis could lead to a loss of protein, a change in the concentration of the solution, and a reduction in the storage time of the drug, which was highly undesirable. It was decided not to purify the drug from glycerol, but to perform controls for each glycodelin concentration with the corresponding glycerol concentration. The glycerol concentrations in the controls were 0.01, 0.14, and 0.73%, respectively.

The viability of the cells after 7 days of incubation, as determined by zombie aqua staining (ZA) (Invitrogen, USA), was 73.28±8.36%. Glycerol and glycodelin did not affect cell number or viability.

To polarize $CD4^+$ cells into Th17 cells, we used TCR activator (T Cell Activation/Expansion Kit human, Miltenyi Biotec, Germany), IL-1β (20 ng/mL), IL-6 (30 ng/mL), IL-23 (30 ng/mL), TGF-β (2.25 ng/mL) cytokines; anti IL-4 (2.5 μg/mL) and anti IFNγ (1 μg/mL) antibodies (Miltenyi Biotec, Germany) as recommended by Miltenyi Biotec.

Flow cytometry

After 7 days of culture, we determined the frequency of Th17-enriched population as a percentage of $ZA^-CD3^+CD4^+CCR6^+$ cells. To evaluate the rate of subpopulations within this population, we assessed the percentage of $CCR4^+CXCR3^-$, $CCR4^+CXCR3^+$, $CCR4^-CXCR3^-$ $CCR4^-CXCR3^+$ cells in the $ZA^-CD3^+CD4^+CCR6^+$ gate (Figure 1).

Sample preparation for surface staining was performed according to the antibody manufacturer's instructions (Miltenyi Biotec, Germany). Stained samples were analyzed by a six-color flow cytometry assay using a CytoFLEX S (Beckman Coulter, USA). The antibodies used were mouse IgG1 anti-human CXCR3-PE-Vio615 (clone REA 232), CD4-PerCP (clone VIT4), CCR4-PE-Vio 770 (clone REA279), CCR6-APC (REA190) (all Miltenyi Biotec, Germany) and CD3-Pacific Blue™ (clone UCTH1) (BioLegend, USA).

The threshold between positive and negative cells was determined using fluorescence minus one (FMO) controls. Flow cytometry data were analyzed using Kaluza Analysis 2.0 software (Beckman Coulter, USA).

Proliferation analysis

A differential gating method was used to determine the proliferation status of cells [74]. The differential gating method is based on the simple idea that cells, whether proliferating or apoptotic, change their size and granularity and thus the light scattering parameters [4, 74]. Thus, in the two-parameter light scatter plot (FSC-A / SSC-A), proliferating cells shift to the right and upward, while apoptotic cells shift to the left. The cells form fairly distinct populations that can be gated, and you can calculate what percentage of the total number of cells ends up in each gate. This method was previously described in our article [71], and in a subsequent article [72], we showed that the percentage of proliferating cells obtained by differential gating correlated with the percentage of $Ki67^+$ cells.

Data were collected using a CytoFLEX S flow cytometer and analyzed using Kaluza Analysis 2.0 software (Beckman Coulter, USA).

Determination of the cytokine profile of culture supernatants

To determine the level of cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, I IL-8, L-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFNγ, MCP-1, MIP-1α, TNFα in culture supernatants used a commercial Bio-Plex Pro™ Human Cytokine Grp I Panel 17-Plex kit (BioRad, USA).

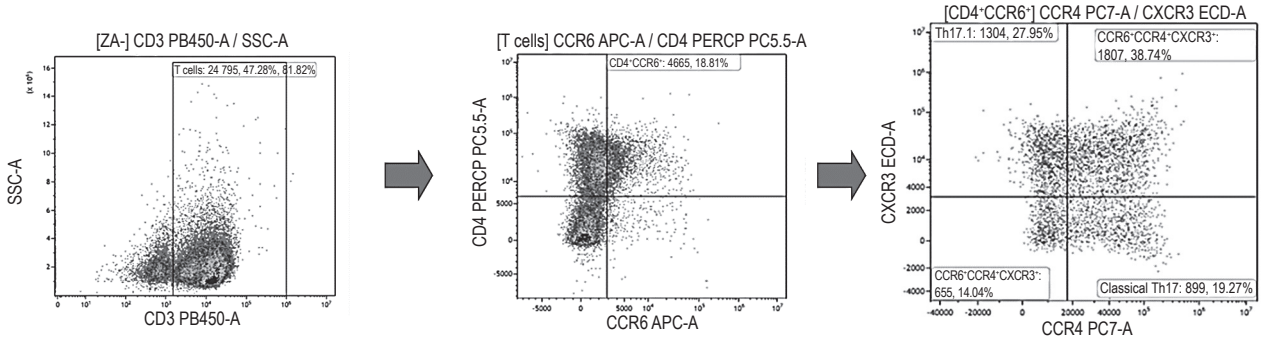

Figure 1. Example of successive gating of Th17-enriched subset and its subpopulations

The results were read on a MAGPIX multiplex analyzer (BioRad, USA) using Luminex xMAP technology using xPONENT 3.1 software. Standard curves were constructed using a five-parameter logistic (5PL) analysis method. The obtained data were processed using the Belysa™ Immunoassay Curve-Fitting Software (Merck KGaA, Germany).

The results for the cytokines IL-1β and IL-6 are not presented in the article because these cytokines were introduced into the cultures for Th17 polarization. The concentrations of IL-4 and IFNγ may also not reflect the true picture because antibodies against these cytokines were added to the cultures according to the cell polarization protocol.

Statistics

Statistical data analysis was performed with GraphPad Prism 8 using the Friedman test with Dunn's multiple comparisons test. Data are presented as medians and first and third quartiles — Me ($Q_{0.25}$-$Q_{0.75}$). Differences were considered significant at $p < 0.05$.

Results

The direct effect of glycodelin on viability, proliferation and apoptosis of Th17-enriched population of Th17-polarized CD4$^+$ cells

Glycodelin at physiological (0.2, 2 and 10 μg/mL) concentrations [14, 24] had no effect on cell viability as determined by Zombie Aqua (ZA) staining and flow cytometry analysis. The average percentage of viable (ZA-) cells in all cultures with GD was 73.22-75.74 and was not significantly different from that in negative control cultures, which was 76.04 (65.2-80.52) (Me ($Q_{0.25}$-$Q_{0.75}$)). The differential gating data are consistent with these results. There were no differences in the number of apoptotic and dead (with reduced size and granularity) cells between control and experimental cultures (data not shown). Controls mimicking the concentration of glycerol impurity in glycodelin (0.015, 0.15, and 0.73% glycerol corresponding to 0.2, 2, and 10 μg/mL glycodelin, respectively) also showed no differences.

When the effect of glycodelin on the proliferation of CD4$^+$T cells associated with their Th17 polarization was evaluated, no changes in the frequency of proliferating T helpers were detected in GdA-treated cultures according to the differential gating method. Importantly, no changes in the percentage of proliferating cells were detected in glycerol-treated controls. There is evidence that glycerol can reduce the proliferation activity and viability of cultured cells [77], but apparently the percentages included in the GD preparation that we used as additional controls were low enough for such effects to occur.

Although glycodelin did not affect the proliferation of CCR6$^+$Th17-enriched T helper cells, there was a statistically significant decrease in the percentage of proliferating cells in the CCR4$^-$CXCR3$^+$Th17.1-enriched subpopulation in cultures with 2 μg/mL glycodelin compared with the control with glycerol (Figure 2). On the contrary, the percentage of apoptotic cells in this subpopulation increased significantly (Table 1). Thus, in our study, glycodelin at a concentration of 2 μg/mL had a selective apoptotic effect on the Th17.1-enriched subpopulation. Interestingly, the higher concentration of glycoprotein studied did not maintain this effect (Table 1).

The effect of glycodelin on percentage of IL-17-producing T helpers and their subpopulations

In 2005, IL-17 producing T helpers were separated into a distinct subpopulation and shortly thereafter

TABLE 1. PERCENTAGE OF APOPTOTIC CELLS IN DIFFERENT SUBPOPULATIONS OF CCR6$^+$ T HELPERS, Me ($Q_{0.25}$-$Q_{0.75}$), n = 6

Subpopulations of CD4$^+$CCR6$^+$ cells	Controls with glycerol			Glycodelin (μg/mL)		
	0.2	2	10	0.2	2	10
Th17/Th22 (CCR4$^+$CXCR3$^-$)	8.458 (7.143-12.720)	10.53 (6.907-13.330)	7.717 (7.157-14.500)	9.435 (7.166-11.110)	11.04 (7.232-15.380)	10.38 (6.955-13.370)
Th17.1 (CCR4$^-$CXCR3$^+$)	24.06 (9.580-31.060)	20.67 (9.458-25.380)	19.19 (10.22-24.09)	25.04 (9.482-38.460)	28.55 (12.88-38.74) p = 0.015	22.12 (8.575-35.410)
CCR4$^-$CXCR3$^-$	10.20 (7.151-13.730)	9.899 (6.477-11.970)	9.839 (7.481-11.840)	9.351 (7.672-15.060)	10.23 (8.774-12.660)	8.849 (7.553-11.630)
CCR4$^+$CXCR3$^+$	51.92 (35.34-60.51)	53.34 (28.25-68.76)	53.08 (33.25-68.20)	52.35 (37.16-62.41)	51.58 (45.00-69.22)	55.89 (26.24-60.37)

Note. Controls with glycerol: 0.2, 2, and 10, controls with glycerol concentrations corresponding to the indicated glycodelin concentrations. $p < 0.05$ value according to the Friedman test with Dunn's multiple comparison test is shown.

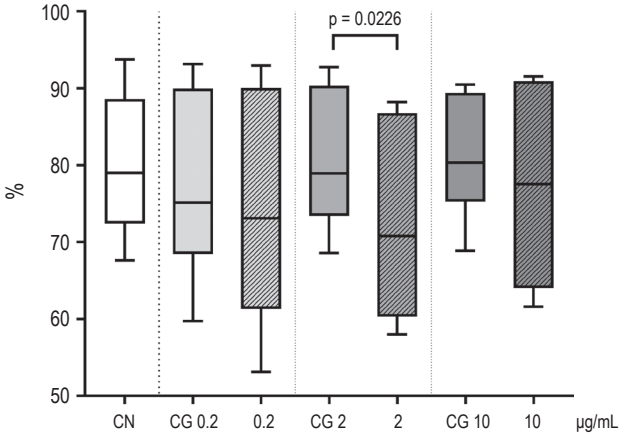

Figure 2. Percentage of proliferating cells in the CCR4$^-$CXCR3$^+$ gate of CD4$^+$CCR6$^+$ cells according to differential gating data

Note. n = 6; medians, Me ($Q_{0.25}$-$Q_{0.75}$), and maxima are shown. The x-axis, glycodelin concentration (µg/mL). The y-axis, percentage of live (ZA$^-$) proliferating cells according to the differential gating method. CN (negative control), culture without GD and glycerol. CG 0.2, CG 2 and CG 10, corresponding controls with glycerol. p-values < 0.05 according to the Friedman test with Dunn's multiple comparison test are shown.

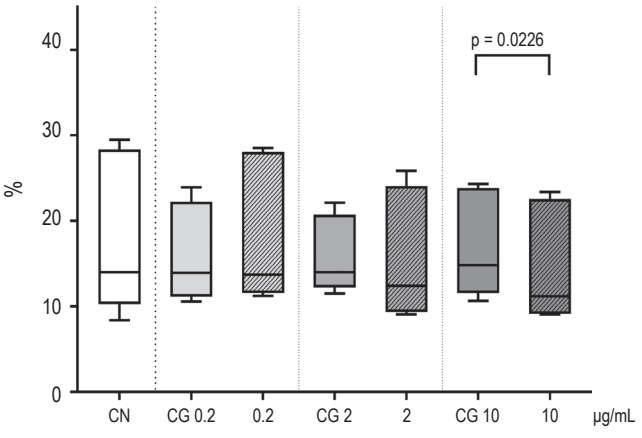

Figure 4. Effect of glycodelin on the percentage of CCR4$^-$CXCR3$^+$ (Th17.1) cells within the CCR6$^+$ population of Th17-polarized CD4$^+$ cells

Note. n = 6; Me ($Q_{0.25}$-$Q_{0.75}$), minima, and maxima are shown. The x-axis, glycodelin concentration (µg/mL). The y-axis, the percentage of Th17.1 from ZA$^-$CD3$^+$CD4$^+$CCR6$^+$ gate. CN (negative control), culture without GD and glycerol. CG 0.2, CG 2 and CG 10, corresponding controls with glycerol. p-value according to the Friedman test with Dunn's multiple comparison test is shown.

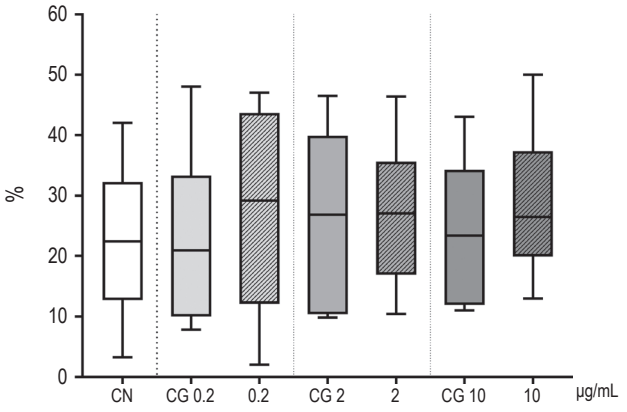

Figure 3. Percentage of CCR6$^+$T helpers in a Th17-polarized culture with different concentrations of glycodelin

Note. n = 6; Me ($Q_{0.25}$-$Q_{0.75}$), minima and maxima are shown. The x-axis, glycodelin concentration (µg/mL). The y-axis, percentage of CCR6$^+$ cells in ZA$^-$CD3$^+$CD4$^+$ gate. CN (negative control), culture without GD and glycerol. CG 0.2, CG 2 and CG 10, corresponding controls with glycerol.

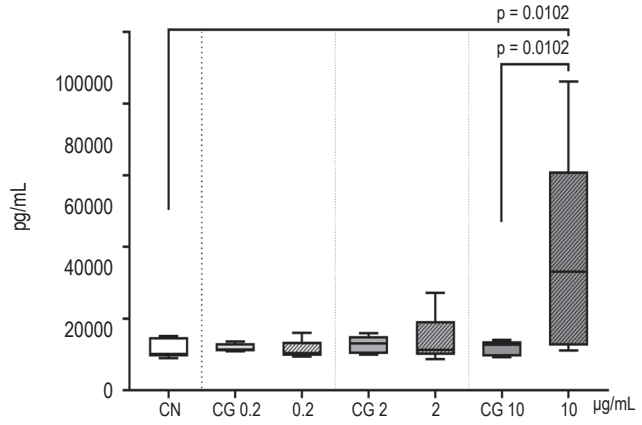

Figure 5. Effect of glycodelin on IL-2 concentration in supernatants of Th17-polarized T helper cultures

Note. n = 6; Me ($Q_{0.25}$-$Q_{0.75}$), minima, and maxima are shown. The x-axis, glycodelin concentration (µg/mL). The y-axis, IL-2 concentration (pg/mL). CN (negative control), culture without glycerol and glycodelin. CG 0.2, CG 2 and CG 10, corresponding controls with glycerol. p-values according to the Friedman test with Dunn's Multiple Comparison Test are shown.

were found to carry a CCR6 molecule on their surface that serves as a receptor for the chemokine CCL20 and promotes cell migration to sites of inflammation [1, 16, 47, 76]. Moreover, regulatory T cells also express CCR6 on their surface [78]. However, when these cells enter the proinflammatory milieu, they begin to express the transcription factor RORgt (RORC), develop a Th17-like phenotype, and produce IL-17 [11, 13, 28, 29]. Since the link between the ability to produce IL-17 and the expression of CCR6 in human T cells has been established, this marker could indicate the pro-inflammatory function of all T helpers *in situ* [2, 64].

In a heterogeneous population of CCR6$^+$T helpers, subpopulations can be distinguished based on

the expression of the chemokine receptors CCR4 and CXCR3 [57].

$CCR6^+CCR4^+CXCR3^-$ (Th17/Th22) cells produce high levels of IL-17A and little IFNγ, express RORC, and produce IL-22. $CCR6^+CCR4^+CXCR3^+$ (double-positive) cells produce both IL-17A and IFNγ. $CCR6^+CCR4^-CXCR3^+$ (Th17.1) cells produce high levels of IFNγ and low levels of IL-17A. Th17.1 ($CCR6^+CCR4^-CXCR3^+$) cells have been reported to be present both in peripheral blood and at sites of inflammation in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease [7, 44, 54]. There is also a subpopulation within $CCR6^+$Th cells that do not express CCR4 or CXCR3 receptors (double-negative cells) [48]. The double-positive and double-negative ($CCR4^+CXCR3$ and $CCR4^-CXCR3^-$) cells within the $CCR6^+$ subset are thought to be Th17 progenitor cells or Th17 in a transitional stage [6].

We evaluated the effect of glycodelin on Th17 polarization of T helper cells by determining the percentage of $CD4^+CCR6^+$ cells in the total $CD3^+$ population. We also determined the percentage of CCR6-expressing cell subpopulations based on their CCR4 and CXCR3 surface molecule expression.

No effect of GD on the number of $CD4^+$ $CCR6^+$T lymphocytes was detected at any of the concentrations studied (Figure 3). The proportion of $CD3^+CD4^+$ cells, $CD3^+CD4^+CCR4^+$ cells, and $CD3^+CD4^+CXCR3^+$ cells was also not altered (data not shown).

However, a significant ($p = 0.0226$) decrease in the percentage of $CCR6^+CXCR3^+CCR4^-$ (Th17.1-enriched) subpopulation was observed in the culture with 10 μg/mL of GD compared with the control with glycerol (Figure 4). The same concentration of glycodelin resulted in a significant increase in the proportion of double-positive $CXCR3^+CCR4^+$ cells, but only compared with the negative control (data not shown).

As for the Th17/Th22-enriched $CCR4^+CXCR3^-$ and DN $CCR4^-CXCR3^-$ subpopulations of $CCR6^+$T helpers, their abundance was not affected by the addition of GD to $CD4^+$ cell cultures.

The influence of glycodelin on cytokine and chemokine production of Th17-polarized T helpers

The concentrations of cytokines, chemokines, and colony-stimulating factors in Th17-polarized T helper culture supernatants with glycodelin are shown in Table 2. The polarization scheme we used resulted in enrichment in culture supernatants of mainly IL-2, IL-17, TNFα, MIP-1b, and IL-8 (data not shown). The effect of glycodelin on cytokine production was manifested only in a statistically significant increase in the concentration of IL-2 in the culture with 10 μg/mL glycodelin compared with the corresponding control (Table 2, Figure 5).

Discussion

The suppressive effect of native glycodelin on proliferation has been demonstrated previously [50, 53]. In 2001, direct antiproliferative and apoptotic effects of glycodelin from amniotic fluid and Pichia pastoris on PBMC and T lymphocyte cell lines Jurkat и MOLT-4 were demonstrated. Moreover, glycodelin had an apoptotic effect mainly on activated T lymphocytes [42].

Later, it was shown that the presence of sialic acids in the structure of oligosaccharides and the size of glycans, as well as the availability of the protein backbone of the molecule are responsible for the apoptotic activity of glycodelin [21, 22, 25, 41]. In addition, the apoptotic effect of glycodelin A is thought to be related to its lectin activity and mediated by the CD7 galectin-1 receptor [68].

However, there is evidence that recombinant glycodelin from *E. coli* does not bind to T cells and has no apoptotic effect on T lymphocytes, likely due to an incorrect conformation of the molecule [25, 37].

In our study, this recombinant glycodelin had no pronounced apoptotic and antiproliferative effect on the total population of T lymphocytes (data not shown) and on their $CCR6^+$ subpopulation. However, a weak apoptotic effect was observed on Th17.1, a similar subpopulation of these cells. In 2011, Lee and colleagues showed that glycodelin induces death only in Th1 cells by binding to Th1 and Th2 [33]. Thus, we see that the effect of glycodelin on different subpopulations of T cells can be differentiated. Probably, the tertiary structure of glycodelin from *E. coli* does not correspond to that of the native protein, but we cannot exclude the possibility that the binding sites that determine its apoptotic activity are partially conserved.

There are data that Th17.1 enriched and DP subpopulations of $CCR6^+$ cells produce more IFNγ and express a higher proportion of the transcription factor TBX1 than Th17/Th22 enriched and DN subpopulations [7]. While there is some evidence for a negative role of the Th17.1 subpopulation in the development of autoimmune diseases, reducing the number of these cells could potentially be beneficial [15, 31].

Previously, under the influence of native glycodelin, the effect of reducing the expression of the chemokine receptor CXCR3, which is expressed in Th1, but not CXCR4, which is typical of Th2, was shown. It has also been shown that the selective effect of glycodelin abolishes the suppression of Th2 transcription factor GATA3 expression [38]. In our study, the recombinant variant of glycodelin did not affect the expression of CCR6, CCR4, CXCR3, CXCR4 by T cells in general, but in partial agreement with the above data, it decreased the expression of CXCR3 in the $CCR6^+CCR4^-$ cell population.

TABLE 2. GD EFFECT ON CYTOKINE/CHEMOKINE PROFILE OF Th17-POLARIZED CD4+ CELL CULTURE SUPERNATANTS, Me ($Q_{0.25}$-$Q_{0.75}$), n = 6

Cytokine (pg/mL)		Controls with glycerol			Glycodelin (μg/mL)		
		0.2	2	10	0.2	2	10
Regulatory	IL-2	11255 (11204-12702)	13046 (10337-14406)	12766 (9614.7-13326.0)	10385 (9643.8 11080.0)	11272 (11131-11501)	33115 (13859-36098) p = 0.03
	IL-7	263.33 (260.00-268.57)	258.03 (212.59-278.84)	268.57 (187.27-268.57)	247.19 (224.51-247.19)	247.19 (241.66-258.03)	268.57 (247.19-273.74)
	IL-12p70	95.00 (84.01-105.15)	100.10 (97.53-119.55)	89.55 (86.81-110.07)	94.91 (92.25-112.48)	102.65 (84.01-110.07)	102.65 (92.25-102.65)
Colony-stimulating	G-CSF	300.00 (285.38-322.48)	297.32 (282.38-372.68)	299.93 (246.05-329.83)	416.38 (389.26-593.26)	466.46 (410.69-533.63)	364.78 (258.24-510.37)
	GM-CSF	2268.30 (2058.6-2273.4)	1725.56 (1603.2-2273.8)	2101.72 (1656.0-2571.9)	2356.94 (1977.7-2966.5)	2078.65 (1610.1-2536.6)	1567.45 (1456.1-1616.9)
	IL-5	1612.5 (1585.5-1678.1)	1558.3 (1551.0-1646.3)	1560.1 (1556.5-1617.9)	1614.3 (1589.1-1621.4)	1589.1 (1460.0-1664.0)	1499.3 (1495.6-1583.7)
Proinflammatory	IFNγ	890.20 (518.25-1151.80)	923.88 (505.18-936.50)	860.67 (500.81-925.98)	953.30 (604.90-1003.60)	775.99 (524.78-902.84)	660.79 (587.63-890.20)
	IL-17	2500.0 (2326.5-2981.7)	3229.6 (1767.6-4016.3)	2068.5 (1508.9-2706.9)	2935.64 (1948.5-3654.7)	2716.45 (1703.9-4018.9)	2905.13 (2029.2-3667.9)
	TNFα	172678 (164210-248920)	208941 (140581-253531)	197352 (176613-234335)	195926 (184072-247644)	213213 (212575-239306)	243328 (213239-248172)
Anti-inflammatory	IL-4	120.00 (116.65-124.64)	123.81 (123.57-124.88)	123.23 (118.87-129.59)	121.49 (117.77-128.46)	120.90 (116.48-128.55)	120.73 (120.23-124.56)
	IL-10	180.00 (133.23-220.92)	186.27 (120.32-244.58)	189.32 (108.84-190.08)	228.35 (93.80-283.85)	177.09 (102.20-247.52)	149.11 (128.41-312.51)
	IL-13	5353.3 (4500.0-16836.0)	7157.9 (4023.3-16823.0)	4103.7 (3573.1-9351.1)	6977.7 (6033.4-7183.6)	7500.0 (6314.9-8648.8)	4890.8 (4171.2-6172.2)
Chemokines	MCP-1	536.13 (414.41-556.08)	541.00 (317.47-585.81)	546.29 (409.52-591.78)	483.54 (414.65-544.94)	460.59 (436.77-545.47)	602.59 (392.83-615.72)
	MIP-1β	29194 (22657-32102)	29825 (28441-31651)	28194 (27632-33352)	30388 (27769-31909)	31001 (25242-31564)	27265 (24643-29731)
	IL-8 (CXCL8)	46993 (40000-50724)	50122 (43563-50801)	51563 (44962-54544)	51303 (49107-55412)	58329 (51019-59923)	59368 (58199-65101)

Note. As for Table 1.

Our data contradict previous studies on GD describing the inhibitory effect of this protein on IL-2 production. In particular, it has been shown that in $CD4^+$T lymphocytes, deprivation of the growth factor IL-2 leads to inhibition of proliferation, a decrease in Bcl-2 expression, or an increase in Bax, resulting in mitochondrial stress and cell death [65].

IL-2, a potent growth factor for lymphocytes, has long been considered only to enhance the functions of these cells. Blocking antibodies against IL-2R have been used to suppress graft rejection [40]. However, later studies have shown the important role of IL-2 in the development, expansion and functioning of regulatory T cells responsible for suppressing the body's autoimmune reactions [43]. Thanks to these studies, a different approach to the therapy of GVHD and autoimmune diseases has been formed, based not on blocking the production of IL-2 or signal transduction pathways from receptors to this cytokine, but, on the contrary, on therapy with low doses of IL-2 [69]. Thus, an increase in IL-2 under the effect of a high concentration of recombinant glycodelin may favor the formation/expansion of Tregs, which is a favorable factor from the standpoint of transplantation immunity. However, this effect of glycodelin needs further investigation because of the discrepancy between our data and the studies of other teams.

The effects of glycodelin on T cells are not limited to its apoptotic activity. This protein has a pleiotropic effect on various subpopulations of T cells [33, 38]. The recombinant GD, which is derived from the Human Embryonic Kidney 293 (HEK 293) cell line and has a similar structure to amniotic glycodelin, increased the number of myelin-specific Tregs and their expression of the transcription factor FoxP3, while suppressing effector T cell differentiation *in vitro*. It also doubled the number of $CD25^{high}GITR^{high}FoxP3^+$T cells [45]. Studies on the immunosuppressive effects of glycodelin have shown that it can suppress T cell receptor signaling [51, 52]. GdA also suppresses the surface expression of CD25 (IL-2Rα) and consequently the production of IL-2, which together with the decreased expression of eomesodermin (Eomes) contributes to the attenuation of CTL cytolytic activity [65].

The only molecular target identified for GdA on T cells is CD45 tyrosine phosphatase [51]. Glycodelin-A binds to the CD45 receptor on T lymphocytes and inhibits Th1 cytokine secretion and T cell differentiation [20, 38, 51]. It is suggested that the mechanism of its action may not be related to the oligosaccharides on the surface of the molecule [22, 25, 41].

Summing up, we studied for the first time the direct effect of recombinant *E. coli*-derived glycodelin on the Th17-enriched lymphocyte subpopulation. We found a selective apoptotic effect of glycodelin at a concentration of 2 μg/mL on the Th17.1 cell subpopulation. Interestingly, when glycodelin was added to the culture at a concentration of 10 μg/mL, a decrease in the percentage of the same cell subpopulation was observed in the culture of Th17-polarized naïve T lymphocytes. However, no effects of this molecule were observed on the percentage of the total $CCR6^+$ subpopulation of T helpers, on their apoptosis, and on the production of IL-17 by these cells.

Previous studies have not demonstrated binding of the recombinant form of *E. coli*-derived glycodelin to T cells or its apoptotic effect [25, 37, 47]. As mentioned above, the protein backbone of the glycodelin molecule is involved in the implementation of its immunosuppressive effects [22, 25]. Therefore, the reason for the absence of pronounced effects might be not so much the absence of glycosylation as defective disulfide bonds leading to altered folding of the protein molecule [27]. The fact that some effects are nevertheless present could be due to the fact that the structure of the native molecule is partially preserved by recombinant glycodelin, allowing it to interact with T cells. In our opinion, there is also the possibility that the observed effects are mediated by residual monocytes present in the T helper culture, because it has been shown that a similar glycodelin can bind to human monocytes via a specific receptor [32, 37].

This indirectly confirms the fact that we observed a significant decrease in IL-17 and abrogation of an increase in concentrations of other proinflammatory cytokines in the blood serum of glycodelin-injected rats in the rat model of local allograft transplantation [3]. There are also promising studies on the use of soluble mutant recombinant glycodelin in the allograft nude mouse model [9, 60]. Glycodelin has been shown to reduce the number of activated $CD4^+$ and $CD8^+$ cells and suppress the expression of granzyme-B, EOMES, IL-2 and pro-inflammatory cytokines [9]. As we can see, a protein thought to be unique to primates also has immunosuppressive effects in rats, supporting the suggestion by Keil and colleagues that glycodelin is a highly conserved glycoprotein in mammals [26]. Thus, the great prospects for the use of glycodelin as a biopharmaceutical cannot be dismissed. Work should continue to clarify the dependence of the immunosuppressive properties of this molecule on its structural features.

Conclusion

Recombinant glycodelin at concentrations equivalent to those during pregnancy (2, 0.2, and 10 μg/mL) did not alter the frequency of $CD4^+CCR6^+$ cells, IL-17 and other cytokines/chemokines studied that are produced by Th17-polarized helper T cells. However, glycodelin at a concentration of 2 μg/mL

increased the proportion of apoptotic Th17.1 cells and 10 μg/mL decreased the proportion of Th17.1 cells ($CCR6^+CCR4^-CXCR3^+$) and increased IL-2 production in $CD4^+$Th17-polarized cell cultures. The Th17.1 subpopulation is considered to be one of those involved in pathological processes in autoimmune diseases and in complications of pregnancy. Thus, this effect of glycodelin could be of interest from a biopharmaceutical point of view, but the mechanism of the demonstrated selective action of this pregnancy protein requires further investigation.

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