ВЛИЯНИЕ ГАЛЕКТИНА-9 НА ЭКСПРЕССИЮ МОЛЕКУЛЫ TIM-3 В РАЗНЫХ СУБПОПУЛЯЦИЯХ НАТУРАЛЬНЫХ КИЛЛЕРОВ

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Резюме. Галектин-9 является β-галактозид-связывающим лектином и обладает выраженной иммунорегуляторной активностью. Во время беременности галектин-9 вырабатывается клетками трофобласта и модулирует функции естественных киллеров (NK) на границе мать-плод посредством связывания с молекулами Tim-3 (Т-клеточный Ig и белок 3, содержащий домен муцина). NK-клетки периферической крови экспрессируют молекулы Tim-3. Концентрация галектина-9 повышается в периферической крови во время физиологической беременности. При беременности фенотип и функции периферических NK-клеток изменяются для поддержания толерантности иммунной системы матери к генетически чужеродному плоду. Периферические NK-клетки мигрируют к границе раздела мать-плод и трансформируются в децидуальные NK-клетки. Концентрация галектина-9 снижается у женщин с осложненной беременностью и выкидышами. Однако влияние галектина-9 на различные субпопуляции NK-клеток периферической крови не изучено. Поэтому целью работы являлось изучение влияния галектина-9 на трансформацию фенотипа и экспрессию Tim-3 NK-клетками, выделенными из периферической крови здоровых небеременных фертильных женщин. $CD56^{+}$NK-клетки получали методом иммуномагнитной сепарации и культивировали *in vitro* в течение 72 часов с цитокинами (IL-2 и IL-15), галектином-9 (5 нг/мл). Концентрация галектина-9 соответствует его уровню в периферической крови в первом триместре физиологической беременности. Количество регуляторных NK ($CD16^{-}CD56^{bright}$), цитотоксических NK ($CD16^{+}CD56^{dim/-}$) клеток и экспрессию Tim-3 на них оценивали методом проточной цитометрии. Показано, что Tim-3 экспрессировался на всех субпопуляциях NK-клеток периферической крови ($CD16^{-}CD56^{bright}$NK, $CD16^{+}CD56^{dim}$NK, $CD16^{+}CD56^{-}$NK). Инкубация с галектином-9 увеличивала экспрессию Tim-3 на регуляторных клетках $CD16^{-}CD56^{bright}$NK и не влияла присутствие Tim-3 на цитотоксических $CD16^{+}CD56^{dim/-}$NK-клетках. Галектин-9 снижал процент цитотоксических $CD16^{+}CD56^{dim}$NK в культуре, но не влиял на количество регуляторных

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CD16$^-$CD56^{bright} NK и цитотоксических CD16$^+$CD56$^-$NK-клеток. Таким образом, галектин-9 регулирует экспрессию молекулы Tim-3 и соотношение субпопуляций NK-клеток в культуре *in vitro*.

Ключевые слова: галектин-9, Tim-3, цитотоксические NK, регуляторные NK, беременность, in vitro

GALECTIN-9 INFLUENCES THE TIM-3 MOLECULE EXPRESSION IN NATURAL KILLER DIFFERENT SUBPOPULATIONS

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Abstract. Galectin-9 is a β-galactoside binding lectin with expressed immunoregulatory activity. During pregnancy galectin-9 is produced by trophoblast cells and regulates the function of natural killer (NK) cells at the maternal-fetal interface via binding to Tim-3 (T-cell Ig and mucin domain-containing protein 3) molecules. Natural killer (NK) lymphocytes belong to the innate lymphoid cells, which have a cytotoxic effect on target cells and are capable of producing a large number of regulatory factors (cytokines, chemokines). Decidual NK have a tolerant phenotype and play a leading role in the regulation of invasive trophoblast growth and provide peripheral immune tolerance in the area of uteroplacental contact. Peripheral NK cells express Tim-3 molecules. Galectin-9 concentration is increased in peripheral blood during physiologic pregnancy. At pregnancy phenotype and functions of peripheral NK cells are changed to maintain the maternal–fetal immune tolerance. Peripheral NK cells migrate to the maternal-fetal interface and are transformed into a decidual NK-like phenotype cells. Galectin-9 concentration is decreased in women with a complicated pregnancy and miscarriage. However the galectin-9 effects on different NK cell subpopulations of peripheral blood are not investigated. Therefore, we studied the galectin-9 influence on phenotype transformation and Tim-3 expression of NK cells isolated from peripheral blood of healthy non-pregnant fertile women. CD56$^+$NK cells were obtained by immunomagnetic separation and cultivated *in vitro* during 72 hours with cytokines (IL-2 and IL-15). Galectin-9 (5 ng/mL) and anti-Tim-3 (10 mg) antibodies were added to the NK cultures. Galectin-9 concentration is corresponded to its level during first trimester of physiologic pregnancy. The number of regulatory NK (CD16$^-$CD56^{bright}), cytotoxic NK (CD16$^+$CD56$^{dim/-}$) cells and Tim-3 expression on different NK subpopulations were assessed by flow cytometry. It was found that Tim-3 was expressed on all subpopulations of peripheral blood NK cells (CD16$^-$CD56^{bright}NK, CD16$^+$CD56^{dim}NK, CD16$^+$CD56$^-$NK). Incubation with galectin-9 increased the expression of Tim-3 on regulatory CD16$^-$CD56^{bright}NK cells and did not change on cytotoxic CD16$^+$CD56$^{dim/-}$NK cells. Galectin-9 reduced the percentage of cytotoxic CD16$^+$CD56^{dim}NK in culture, but did not influence the number of regulatory CD16$^-$CD56^{bright} NK and cytotoxic CD16$^+$CD56$^-$NK cells. Thus, galectin-9 regulates Tim-3 molecule expression and NK cell subpopulation distributions *in vitro* culture.

Keywords: galectin-9, Tim-3, cytotoxic NK, regulatory NK, pregnancy, in vitro

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Introduction

Galectin-9 is a β-galactoside binding lectin with expressed immunomodulatory activity produced by many type of cells. Galectin-9 participates in regulation of many physiological processes such as cell growth, differentiation, adhesion, communication and death [6]. During pregnancy trophoblast cells secrete galectin-9 and regulate the function of decidual natural killer (NK) cells at the maternal-fetal interface via binding to Tim-3 (T cell Ig and mucin domain-containing protein 3) receptor molecules [9]. Natural killer (NK) lymphocytes belong to the innate lymphoid cells, which have a cytotoxic effect on target cells and are capable of producing a large number of regulatory factors (cytokines, chemokines). NK eliminate virus-infected and tumor cells by releasing cytotoxic granules containing granzyme B or by engaging death receptors that initiate caspase cascades.

During pregnancy, the NK cytotoxic potential in peripheral blood decreases due to a diminish in the percentage of cytotoxic $CD16^{+}CD56^{dim}$NK and elevation of regulatory $CD16^{-}CD56^{bright}$NK cells [4]. At the same time, the increased number of $CD16^{+}56^{dim}$NK in the peripheral blood of women is associated with spontaneous pregnancy loss, because $CD16^{+}56^{dim}$NK are able to lyse of trophoblast cells [4]. Recent studies have shown that there is also a subpopulation of cytotoxic exhausted $CD16^{+}CD56^{-}$ NK cells in peripheral blood, which are characterized by reduced cytotoxic and secretory activity [3]. The number of $CD16^{+}CD56^{-}$NK increases in severe viral infections and is associated with a decrease in antiviral immunity, but changes in their number and functions during physiological pregnancy have not been studied [3]. It is generally accepted that the peripheral blood $CD56^{bright}CD16^{-}$NK cells migrate to the uterus and transform into decidual NK cells during early pregnancy [4]. Decidual NK have a tolerant phenotype and play a leading role in the regulation of invasive trophoblast growth and provide peripheral immune tolerance in the area of utero-placental contact [4]. However, the mechanisms of the NK phenotype shift from cytotoxic towards the tolerant during pregnancy remain poorly understood.

Tim-3 is a marker of NK cells activation and maturation and plays a critical role in the NK function regulation including degranulation, cytotoxicity, cytokine production, fetal trophic functions [5]. Galectin-9/Tim-3 signaling suppress decidual NK cells cytotoxicity and regulate cytokine productions [8]. Tim-3-positive decidual NK cells display higher interleukin (IL)-4 and lower tumor necrosis factor (TNF)-α and perforin production [8]. Tim-3 blockade on decidual NK results in fetal loss in mice [10]. In contrast, other reports have provided evidence that Tim-3 functions as a NK-cell coreceptor to enhance interferon-gamma production, which has important implications for control of infectious disease and cancer [5]. Other authors have shown that increased Tim-3 expression on NK cells leads to NK cell dysfunction in chronic virus infections, such as hepatitis B and HIV infection [9]. However, the functions of Tim-3 in peripheral blood NK function regulation during pregnancy are not elucidated. NK cells of peripheral blood constitutively express Tim-3 molecules [9]. Galectin-9 concentration is gradually increased in peripheral blood during physiologic pregnancy, but the decrease of galectin-9 level is associated with a complicated pregnancy and miscarriage [1]. All mentioned above emphasizes the important galectine-9/Tim-3 role in NK cells phenotype and function regulation associated with successful pregnancy. However the galectin-9 effects on different NK cell subpopulations of peripheral blood are not investigated. Therefore **the aim of this work** was studied the galectin-9 influence on phenotype and Tim-3 expression of peripheral blood NK cells.

Materials and methods

Peripheral blood of healthy non-pregnant fertile women (in follicular phase of the menstrual cycle) aged 21-29 years was studied (n = 12). The inclusion criteria were the absence of acute and chronic somatic, endocrine, autoimmune, genetic diseases; compliance with diet, treatment with contraceptive and hormonal, anti-inflammatory or antibacterial drugs. This study was approved by the local ethics committee of the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Sciences in accordance with the Helsinki Declaration. Written informed consent was received from all participants.

Peripheral blood samples were collected in sodium heparin vacutainer tubes. Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood by ficoll-verografin (1.077g/cm^3) density gradient centrifugation.

NK cells were obtained from PBMC by the method of negative immunomagnetic separation (depletion of the PBMC population from T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes and erythroid cells, using the NK cell isolation kit (Miltenyi Biotec, USA). The purity of the isolated $CD56^{+}CD3^{-}$NK was more than 95% (estimated as the number of $CD56^{+}$ in a gate of $CD3^{-}$ cells measured by flow cytometry with CD56-BV605 and CD3-PE (Figure 1). Purified NK cells (1 10^5) were cultured in 0,3 mL of complete media (RPMI-1640 (Gibco, UK); with 10% fetal bovine serum (Biolot, Russia); 1 мМ HEPES (Biolot, Russia); 2 мМ L-glutamine (ServiceBio, China), penicillin G (100 U/mL) – streptomycin (0.1 mg/mL) (Biolot, Russia) in 96-well cell culture plates (Eppendorf, USA) during 72 hours at 37 °C in a humid atmosphere with 5% CO_2. Cultures were supplemented with IL-15 (10 ng/mL) and IL-2 (500 ng/mL) (Cloud-Clone Corp, USA) [2]. The purified NK cells were subjected to galectin-9 (5 ng/mL; Cloud-Clone Corp, USA) and anti-human Tim-3 (CD366) (10 mg/mL; ultra-LEAFO blocking antibodies, BioLegend, USA) [8]. Galectin-9 concentration was corresponded to its level during first trimester of physiologic pregnancy [11]. The NK cells were harvested for flow cytometry after 72 h of incubation.

NK cell phenotype was assessed by flow cytometry using the following antibodies: CD3 (PE anti-human CD3, clone OKT3, eBioscience), CD56 (Brilliant Violet 605™ anti-human CD56 (NCAM), clone HCD56, BioLegend), CD366 (APC anti-human CD366 (Tim-3), clone F38-2E2, BioLegend). Cells were labeled with Zombie UV™ (Zombie UV™

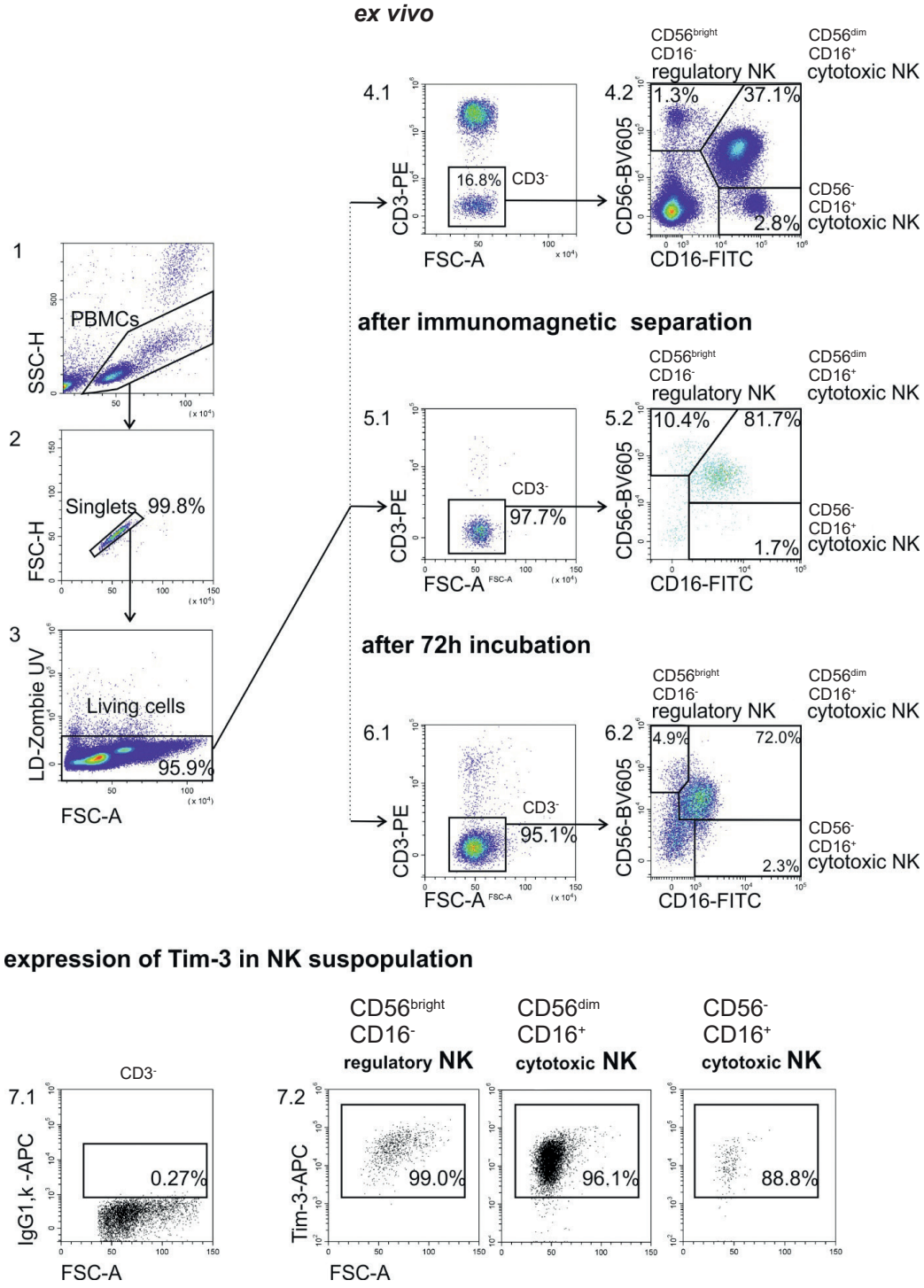

Figure 1. Gating strategy for NK cell phenotype and Tim-3 expression assessment

Note. 1, PBMC gate selection according to the forward (FSC-A) and side (SSC-H) scattering parameters; 2, discrimination of doublets according to the FSC-A/FSC-H parameters; 3, discrimination between dead and live cells by LIVE/DEAD-ZOMBIE UV stained; 4.1, selection of $CD3^-$ and $CD3^+$ cells in the peripheral blood PBMC living cell gate; 4.2, the number of the regulatory subpopulation of NK was determined as a percentage of $CD16^-CD56^{bright}$ and cytotoxic subpopulation of NK as a percentage of $CD16^+CD56^{dim}$ and $CD16^+CD56^-$ in the gate of CD3-negative PBMC (*ex vivo* – before immunomagnetic separation; 5.1, selection of $CD3^-$ and $CD3^+$ cells in the living cell PBMC gate after immunomagnetic separation; 5.2, the percentage of the NK cell different subpopulations according to CD16 and CD56 coexpression in the CD3-negative gate in after immunomagnetic separation; 6.1, selection of $CD3^-$ and $CD3^+$ cells in the living cell PBMC gate after immunomagnetic separation and 72 h cultivation; 6.2, the percentage of the NK cell different subpopulations according to CD16 and CD56 coexpression in the CD3-negative gate after immunomagnetic separation and 72 h cultivation; 7.1, the level of non-specific binding was determined using isotyping mAbs (isotype) for assessment of Tim-expression in the studied subpopulations (7.2).
Figure 1 shows histograms of one representative experiment.

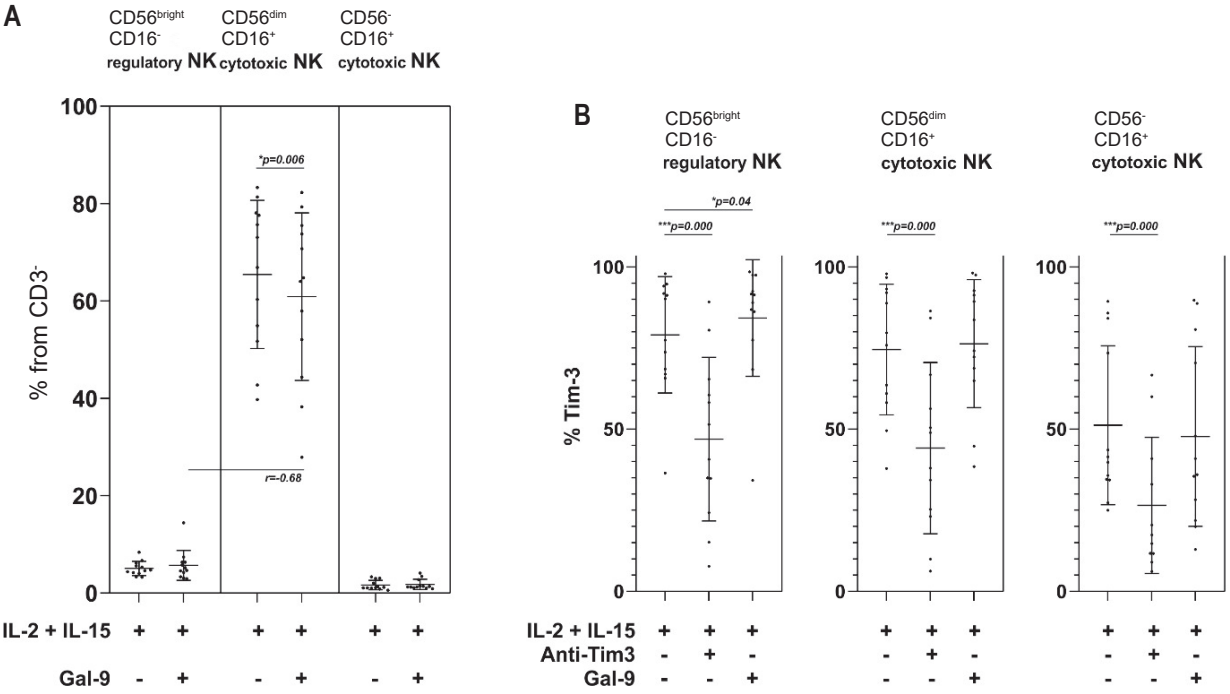

Figure 2. Assessment of the NK subpopulations

Note. (A) regulatory NK ($CD3^-CD16^-CD56^{bright}$) and cytotoxic NK ($CD3^-CD16^+CD56^{dim}$) and ($CD3^-CD16^+CD56^-$) after 72 h incubation *in vitro* with cytokines (IL-15, IL-2) and galectin-9 (Gal-9). (B) Assessment of Tim-3 expressions in different NK subpopulations – regulatory NK ($CD3^-CD16^-CD56^{bright}$) and cytotoxic NK ($CD3^-CD16^+CD56^{dim}$) and ($CD3^-CD16^+CD56^-$) after 72h incubation *in vitro* with cytokines (IL-15, IL-2) and galectin-9 (Gal-9).

In Figures 2A and 2B, data are presented as median and the lower and upper quartiles, Me ($Q_{0.25}$-$Q_{0.75}$); *, p value by two-tailed paired t-test in comparison to the NK cell cultures treated with cytokines only; r, Pearson's correlation coefficient.

Fixable Viability Kit, BioLegend) to assess cell viability.

NK subpopulations were determined by the co expression of CD56 and CD16 molecules in the gate of CD3-negative mononuclear cells: regulatory NK – $CD16^-CD56^{bright}$, cytotoxic NK – $CD16^+CD56^{dim}$ and $CD16^+CD56^-$. Gating strategy was presented in Figure 1.

The data were presented as median and the lower and upper quartiles, Me ($Q_{0.25}$-$Q_{0.75}$). Statistical analyses were performed using "GraphPad Prism version 8.01" (StatSoft, USA). The Kolmogorov-Smirnov test was used for verifying normal distribution. The significance of the difference between two groups was determined using the two-tailed paired t-test. Spearman rank correlation (r) test was used for correlation analysis. The differences were considered as significant at $p < 0.05$.

Results and discussion

To investigate the galectin-9 role in the phenotype regulation of the peripheral blood NK cells, PBMC were isolated from a peripheral blood of healthy fertile non-pregnant women. $CD56^+$NK cells were isolated by immunomagnetic separation. The purity of $CD56^+$NK cells was more than 95% confirmed using flow cytometric analysis after magnetic bead sorting (Figure 1B). Purified $CD56^+$NK were incubated with IL-15 and IL-2 during 72h. IL-15 and IL-2 have been reported to have stimulating effects on NK cell survival *in vitro* [2]. NK cell viability was assessed by Zombie staining in cultures and was more than 95% after incubation with IL-15, IL-2 and less than 50% without cytokines (data not shown).

Purified NK cells were cultured with galectin-9 in concentration corresponding to its level during first trimester of physiologic pregnancy [11]. It is known that in first trimester of pregnancy NK cytotoxic potential in peripheral blood decreases due to a diminish in the percentage of cytotoxic $CD16^+CD56^{dim}$NK and elevation of regulatory $CD16^-CD56^{bright}$NK cells [4]. At the same time, peripheral blood $CD56^{bright}CD16^-$NK cells migrate to the uterus and transform into decidual NK cells during early pregnancy [4]. The percentage of regulatory $CD16^-CD56^{bright}$ NK, cytotoxic $CD16^+CD56^{dim}$ NK and $CD16^+CD56^-$ NK were estimated in galectine-9-primed NK cultures. The results showed that galectin-9 reduced the percentage of cytotoxic $CD16^+CD56^{dim}$NK in cultures, but did not influence the percentage of regulatory $CD16^-CD56^{bright}$NK and cytotoxic $CD16^+CD56^-$NK cells (Figure 2A).

According to literature, the percentage of cytotoxic $CD16^{+}CD56^{dim}$NK may reduce due to their transformation into $CD16^{-}CD56^{bright}$NK or cytotoxic $CD16^{+}CD56^{-}$NK cells or undergo apoptosis *in vitro* [7]. Therefore the percentage of Zombie-negative $CD16^{+}CD56^{dim}$NK cells was assessed after galecting-9 incubation. Zombie UV™ is a fluorescent dye that is non-permeant to live cells, but permeant to the cells with compromised membranes that help to discriminate apoptotic/necrotic cells from live cells. There were no significant differences in the percentage of Zombie-negative $CD16^{+}CD56^{dim}$NK cells in cultures with cytokines (control) and in cultures with galecting-9 (% of Zombie-negative $CD16^{+}CD56^{dim}$NK cells in control = 79.04 (69.0-88.3); in cultures with galecting-9 = 82.4 (70.5-87.5); $p > 0.05$). We suggested that galecting-9 influence NK phenotype transformation due to CD16 and CD56 expressions regulation *in vitro* cultures. This suggestion is confirmed by the correlation analysis. The percentage of $CD16^{+}CD56^{dim}$NK cells decreased proportionally to $CD16^{-}CD56^{bright}$ NK increased after galectin-9 incubation ($r = -0.68$; $p < 0.05$) (Figure 2A). Thus, galectin-9 in concentration at first trimester of pregnancy regulates peripheral blood NK cell subpopulation distributions *in vitro* culture. Other authors had shown that during pregnancy trophoblast cells secrete galectin-9 and reduce cytotoxicity, TNFα and perforin production by decidual NK cells at the maternal-fetal interface [8, 9].

It is known that galectin-9 realizes immunomodulatory activity due to Tim-3 interactions on NK cells [8, 9]. The Tim-3 expression was analyzed on different subpopulations of peripheral blood NK cells after 72h incubation with cytokines *in vitro*. It was found that Tim-3 was expressed in all subpopulations of peripheral blood NK cells ($CD16^{-}CD56^{bright}$NK, $CD16^{+}CD56^{dim}$NK, $CD16^{+}CD56^{-}$NK) (Figure 2B). Incubation with galectin-9 increased the percentage of Tim-3-positive regulatory $CD16^{-}CD56^{bright}$NK cells and did not influence on cytotoxic Tim-3-positive $CD16^{+}CD56^{dim}$NK and $CD16^{+}CD56^{-}$NK cells (Figure 2B). It should be noted that obtained data in agreement with the results of another authors that galectin-9 affects the formation of a regulatory phenotype of decidual NK with increased Tim-3 expression [8, 9]. The Tim-3 blockade by anti-Tim-3 antibodies expected decreased Tim-expression on all investigated subpopulations of peripheral blood NK cells ($CD16^{-}CD56^{bright}$, $CD16^{+}CD56^{dim}$, $CD16^{+}CD56^{-}$) (Figure 2B).

Conclusion

Thus, galectin-9 regulates the expression of Tim-3 molecules on $CD16^{-}CD56^{bright}$NK cells and NK cell subpopulation distributions *in vitro* culture. The obtained results are important for understanding the underlying mechanism of immune dysfunctions during pregnancy and could have significant value in treating reproductive disorders associated with NK cells, including intrauterine growth restriction and repeated miscarriages.

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