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Effect of experimental gestational diabetes and administration of glibenclamide on mRNA level of NLRP3-inflammasome and distribution of NLRP3$^+$-cells in mesenteric lymph nodes in progeny

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A number of dysfunctions of congenital and adaptive components of the immune system are observed in progeny of rats with experimental gestational diabetes (EGD). The key link in pathogenesis of EGD and other diseases is the activation of NLRP3-inflammasome. Therefore, it is an attractive target for pharmaceutical effects. Among the numerous inhibitors of the inflammasome, glibenclamide is the most promising drug, which can effectively correct hyperglycemia in pregnant women.

The aim of the study was to determine the level of mRNA expression of NLRP3-inflammasome and the distribution of NLRP3$^+$-cells in mesenteric lymph nodes in progeny of rats with experimental gestational diabetes and after administration of glibenclamide to pregnant Wistar rats.

Materials and methods. A molecular-genetic study was carried out using polymerase chain reaction with real-time reverse transcription (RT–PCR) of mRNA expression level of the *Nlrp3* gene. The distribution of NLRP3$^+$-cells in MLN of experimental animals was investigated by immunofluorescence and immunohistochemical methods.

Results. The development of EGD is accompanied by transcriptional induction of the *Nlrp3* gene in MLN in descendants, whose mRNA level increased five-fold ($p < 0.05$) in 1-month and 3-fold ($p < 0.05$) in 6-month-old animals. The administration of glibenclamide to pregnant rats inhibited the transcription of the *Nlrp3* gene only at the age of 1 month (5.3 times, $p < 0.05$) and did not change it in the older age group. In the progeny of rats with EGD, the density of the NLRP3$^+$-lymphocyte population in the MLN increased, more clearly at early observation times. The intake of glibenclamide reduced the number of NLRP3$^+$-lymphocytes only at the age of 1 month (by 33 %, the cortex plateau), whereas their number in the medullary cords of 6-month-old progeny even increased.

Conclusion. The increased mRNA expression of NLRP3-inflammasome and density of NLRP3$^+$-cells in MLN in descendants of rats with EGD indicates activation of pro-inflammatory signaling. Glibenclamide, as an inhibitor of the activation of the NLRP3-inflammasome, demonstrated its effectiveness only at early observation times.

Key words: EGD, MLN, NLRP3-inflammasome, glibenclamide.

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Вплив експериментального гестаційного діабету та введень глібенкламіду на рівень мРНК NLRP3-інфламасоми та розподіл NLRP3$^+$-клітин у брижових лімфатичних вузлах у нащадків

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У нащадків щурів з експериментальним гестаційним діабетом (ЕГД) спостерігається ціла низка порушень функціонування вроджених й адаптивних компонентів імунної системи. При ЕГД важливою ланкою патогенезу є активація NLRP3-інфламасоми. Саме тому вона – привабливий об'єкт для фармацевтичного впливу. Серед інгібіторів інфламасоми перспективним є глібенкламід, який до того ж може ефективно коригувати гіперглікемію у вагітних.

Мета роботи – з'ясувати рівень експресії мРНК NLRP3-інфламасоми та розподіл NLRP3$^+$-лімфоцитів у брижових лімфатичних вузлах у нащадків щурів з експериментальним гестаційним діабетом і після введень глібенкламіду вагітним щурам лінії Вістар.

Матеріали та методи. Здійснили молекулярно-генетичне дослідження методом полімеразної ланцюгової реакції зі зворотною транскрипцією в режимі реального часу (ЗТ-ПЛР) рівня експресії мРНК гена *Nlrp3*, імунофлюоресцентне та імуногістохімічне дослідження розподілу NLRP3$^+$-клітин у БЛВ у експериментальних тварин.

Результати. Розвиток ЕГД супроводжується транскрипційною індукцією гена *Nlrp3* в БЛВ у нащадків, рівень мРНК котрого зростав у 5 разів ($p < 0,05$) в 1-місячних і в 3 рази – ($p < 0,05$) у 6-місячних тварин. Введення глібенкламіду під час вагітності інгібує транскрипцію гена *Nlrp3* тільки у віці 1 місяць (у 5,3 раза, $p < 0,05$) і не змінюється у старшій віковій групі. У нащадків щурів з ЕГД зростає щільність популяції NLRP3$^+$-лімфоцитів у БЛВ, виразніше – на ранніх термінах спостереження. Приймання глібенкламіду знижує кількість NLRP3$^+$-лімфоцитів тільки у віці 1 місяць (на 33 %, коркове плато), тоді як у 6-місячних нащадків їхня чисельність у м'якотних тяжах навіть зростає.

Висновки. Підвищення рівня експресії мРНК NLRP3-інфламасоми та щільності NLRP3$^+$-лімфоцитів у БЛВ у нащадків щурів з ЕГД свідчить про активацію прозапальної сигналізації. Глібенкламід як інгібітор активації NLRP3 продемонстрував свою ефективність лише на ранніх термінах спостереження.

Ключові слова: ЕГД, БЛВ, NLRP3-інфламасома, глібенкламід.

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Ключевые слова:
ЭГД, БЛУ,
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глибенкламид.

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Влияние экспериментального гестационного диабета и введений глибенкламида на уровень мРНК NLRP3-инфламмасомы и распределение NLRP3+-клеток в брыжеечных лимфатических узлах у потомства

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У потомков крыс с экспериментальным гестационным диабетом (ЭГД) наблюдается целый ряд нарушений функционирования врождённых и адаптивных компонентов иммунной системы. При ЭГД важным звеном патогенеза является активация NLRP3-инфламмасомы. Именно поэтому она является привлекательным объектом для фармацевтического воздействия. Среди ингибиторов инфламмасомы перспективным является глибенкламид, который к тому же может эффективно корректировать гипергликемию у беременных.

Цель работы – выяснить уровень экспрессии мРНК NLRP3-инфламмасомы и распределение NLRP3$^+$-лимфоцитов в брыжеечных лимфатических узлах у потомков крыс с экспериментальным гестационным диабетом и после введения глибенкламида беременным крысам линии Вистар.

Материалы и методы. Осуществлено молекулярно-генетическое исследование методом полимеразной цепной реакции с обратной транскрипцией в режиме реального времени (ОТ-ПЦР) уровня экспрессии мРНК гена *Nlrp3*, иммунофлюоресцентное и иммуногистохимическое исследование распределения NLRP3$^+$-клеток в БЛУ у экспериментальных животных.

Результаты. Развитие ЭГД сопровождается транскрипционной индукцией гена *Nlrp3* в БЛУ у потомков, уровень мРНК которого увеличился в 5 раз ($p < 0,05$) у 1-месячных и в 3 раза – ($p < 0,05$) у 6-месячных животных. Введение глибенкламида при беременности ингибирует транскрипцию гена *Nlrp3* только в возрасте 1 месяц (в 5,3 раза, $p < 0,05$) и не меняется в старшей возрастной группе. У потомков крыс с ЭГД возрастает плотность популяции NLRP3$^+$-лимфоцитов в БЛУ, более выражено – на ранних сроках наблюдения. Приём глибенкламида снижает количество NLRP3$^+$-лимфоцитов только в возрасте 1 месяц (на 33 %, корковое плато), тогда как у 6-месячных потомков их численность в мякотных тяжах даже возрастает.

Выводы. Повышение уровня экспрессии мРНК NLRP3-инфламмасомы NLRP3-инфламмасомы и плотности NLRP3$^+$-лимфоцитов в БЛУ у потомков крыс с ЭГД свидетельствует об активации провоспалительной сигнализации. Глибенкламид как ингибитор активации NLRP3 продемонстрировал свою эффективность только на ранних сроках наблюдения.

Our findings show that progeny of experimental gestational diabetes mellitus (EGD) rats have such immune violations of immunological tolerance as AIRE gene repression, reduced mRNA levels of Deaf1, transcription factor Foxp3, the latter being confirmed by decrease in Treg cells [1] and inhibition of gene expression suppressor cytokine IL-10 and negative costimulatory molecules Ctla4 [2]. However, gestational diabetes (GD) increases the genes expression of immune response in pregnant [3] and leads to violations of innate and adaptive components of immune system in progeny. Thus, Li Q. et al. (2016) showed that gestational diabetes mellitus increased interleukin-1β in progeny spleen cells [4]. This indicates activation of NLRP3-inflammasome – protein that belongs to the family of nucleotide-binding and oligomerization domain-like receptors, NLRs [5]. Activation of NLRP3-inflammasome in GD showed in other experiments [6], and the extensive involvement of the NLRP3-inflammasome in such a range of diseases makes it a highly desirable drug target. Recently, numerous promising inhibitors of NLRP3-inflammasome activation have been described [7–9], but we turned our attention to glibenclamide, which along with metformin and insulin can effectively correct hyperglycemia for pregnant women. Glibenclamide was the first oral hypoglycemic drug prospectively tested and used to manage GD [10]. A lot of meta-analysis showed that adequate glycemic control could be achieved with glibenclamide in many women with GD, and this treatment reduced hyperglycemia-associated outcomes [11,12]. But the most interesting was the ability of glibenclamide to inhibit the formation of NLRP3-inflammasome that was first detected in 2009 [13]. These results were later confirmed by other studies [14,15]. Therefore, the aim of the work was to study the expression level of mRNA NLRP3-inflammasome and distribution of NLRP3$^+$-cells in mesenteric lymph nodes in progeny of rats with experimental gestational diabetes and after glibenclamide administration to pregnant.

Materials and methods

The studied animals were divided into 6 experimental groups, each group contains 12 rats. Descendants of intact Wistar rats (males) were 1 month of age (group 1) and 6 months of age (group 2). Their mothers were administered 0.5 ml 0.1 M citrate buffer (pH = 4.5) on the 15th day of pregnancy once intraperitoneally. In descendants of Wistar rats (males) with experimental gestational diabetes (EGD) of 1 month of age (group 3) and 6 months (group 4), mothers were administered 45 mg/kg of streptozotocin once intraperitoneally on the 15th day of pregnancy. In descendants of rats with EGD of 1 month of age (group 5) and 6 month of age (group 6), mothers were administered 45 mg/kg of streptozotocin once intraperitoneally on the 15th day of pregnancy with simultaneous administration of oral intragastric Glibenclamide (Farmak, Ukraine) at a dose of 5 mg/kg for 7 days.

The objects for molecular genetic studies with using of the real-time reverse transcription polymerase chain reaction (RT-PCR) techniques were MLN of experimental animals. They were placed in the Bouin's fluid, dehydrated with graded concentrations of ethanol and embedded in paraffin. Molecular genetic studies were performed on archival material of 2 years old. RNA was extracted from histology sections of 15 μm thick. They were dewaxed in xylene and rehydrated with descending concentrations of ethanol (100 %, 96 %, 70 %). Total RNA was procured from sam-

ples by use of “NucleoZOL” (Macherey-Nagel, Germany). For reverse transcription and obtaining cDNA, we used RevertAid First Strand cDNA Synthesis Kit (ThermoScientific, USA). Reverse transcription was conducted at 42 °C for 60 min then at 70 °C for 5 min. To determine the level of mRNA *Nlrp3* (NM_001191642.1), we used thermocycler CFX96™Real-Time PCR Detection Systems (“Bio-Rad Laboratories, Inc.”, USA) and set of reagents Luminaris HiGreen Fluorescein qPCR MasterMix (Thermo Scientific, USA). The final reaction mixture for amplification includes 10 μl MasterMix (2X), 0.6 μl of direct and reverse specific primers, 1 μl cDNA. The reaction mixture was brought to total volume of 20 μl by adding deionized H_2O. Specific primer pairs (5’–3’) for analysis of target and reference genes were selected by the software PrimerBlast (www.ncbi.nlm.nih.gov/tools/primer-blast) and produced by Metabion (Germany) (*Table 1*). After initial denaturation at 95 °C for 10 min amplification consisted of 45 cycles and was conducted under the following conditions: denaturation – 95 °C for 15 sec, annealing at 59–61 °C for 30–60 sec, elongation at 72 °C for 30 sec. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as a reference gene to determine the relative value of changes in the expression level of target genes. Normalized relative quantity of cDNA target genes was determined by the method $_{\Delta\Delta}$Ct. Statistical data analysis of PCR were conducted using available software CFX Manager™ (Bio-Rad, USA).

Structure of Nlrp3$^+$-cells population was studied using analysis of serial histological sections of MLN and their morphometric and densitometric characteristics. For this study, serial sections of 5 μm thick were made on a rotary microtome MICROM HR-360 (Microm, Germany), then they were dewaxed in xylene and rehydrated with descending concentrations of ethanol (100 %, 96 %, 70 %), washed with 0.1 M phosphate buffer (pH = 7.4) and colored with Nlrp3 rabbit polyclonal antibodies (Cryopyrin, H-66) (Santa Cruz Biotechnology, USA, sc-66846) for 18 hours in a humid chamber at T = 4 °C. After washing with 0.1 M phosphate buffer, sections were incubated for 60 min at T = 37 °C with secondary antibody solution to whole molecule of rabbit IgG (Santa Cruz Biotechnology, USA), conjugated with FITC. After incubation, all sections were washed with 0.1 M phosphate buffer and placed in a mixture of glycerol phosphate buffer (1:9) for subsequent fluorescent microscopy. Prepared histological sections were studied using a computer program Image J (NIH, USA), and the morphometric and densitometric characteristics of immunopositive cells were measured. We determined the absolute (number of cells per 1 mm^2) and relative (%) density of different subsets of Nlrp3+-lymphocytes in cortex and medullary cords of MLN. For additional visualization of Nlrp3$^+$-cells, we also performed an immunohistochemical reaction with secondary rabbit antibodies ImmunoCruzTM Staining system (Santa Cruz Biotechnology, USA), conjugated to horseradish peroxidase.

Results and discussion

Investigation of *Nlrp3* gene expression in MLN showed that in the progeny of EGD rats there was a significant fivefold growth of mRNA of this protein in the 1-month-old rat ($p < 0.05$), and threefold growth ($p < 0.05$) in the 6-month-old animals compared with control groups (*Fig. 1A, B*). The study of rats whose mothers were treated during pregnancy with glibenclamide showed that the animals aged 1 month had a significant 5.3 times decrease of *Nlrp* gene expression ($p < 0.05$), but these changes were not detected in the older age group (*Fig. 1C, D*).

Table 1. Specific primer pairs for RT-PCR

Gene	Primer	Tm, °C	Product length (bp)	Exon junction
Nlrp3	F = AGCTAAGAAGGACCAGCCAG R = CGTGCATGCATCATTCCACTC	59 60	40	713/ 714
GAPDH	F = GCCTGGAGAAACCTGCCAAG R = GCCTGCTTCACCACCTTCT	61 60	52	825/ 826

Studying the distribution of specific subpopulations of Nlrp3$^+$-cells gave the following results. Total density of immunopositive cells in MLN cortical plateau of 1-month-old EGD rats increased by 49 % compared with control group ($p < 0.05$). In the next age group comparative analysis revealed no significant changes. Total number of Nlrp3$^+$-cells in MLN medullary cords of 1-month-old EGD rats was significantly increased by 44 % ($p < 0.05$) in comparison with control group. The study of materials taken from the 6-month-old animal showed an increase in the total density by 69 % ($p < 0.05$) (*Fig 2A, B*, *Fig. 3A–D*).

Analysis of MLN sections in EGD progeny of rats treated with glibenclamide during pregnancy gave the following results. In cortical plateau of MLN at 1 month of age we obtained reducing of the total number of Nlrp3$^+$-cells by 33 % ($p < 0.05$) in comparison with EGD1. At 6 months of age there were no significant changes in the number of immunopositive cells. As for medullary cords of mesenteric lymph nodes, total density of Nlrp3$^+$-cells remained unchanged at 1 months of age. And at the age of 6 months, it increased by 29 % ($p < 0.05$) (*Fig 2A, B*).

There are three main models that describe the mechanism of NLRP3-inflammasome activation. They were shown by K. Schroder et al. (2010) [16]. In the first model, extracellular adenosine triphosphate (ATP) is regarded as initiator of NLRP3 inflammasome activation and assembly with K+ efflux through a purogenic P2X7-dependent pore. The second model, special activators trigger the generation of reactive oxygen species (ROS), which in turn induce assembly of the NLRP3 inflammasome [17]. In the last model, these processes are triggered by some external irritants such as silica or alum, which are taken over by phagocytes. These aggregates induce lysosomal rupture and release of lysosomal contents via a mechanism mediated by cathepsin B [18]. Other factors can also activate the NLRP3 inflammasome such as mitochondrial damage or dysfunction caused by mitochondrial $Ca2^+$ overload, autophagic dysfunction and the activity of thioredoxin-interacting protein (TXNIP) [19].

The inflammasomes and the complement system are traditionally viewed as quintessential components of innate immunity required for the detection and elimination of pathogens. But a direct role for NLRP3 in human adaptive immune cells has not been described yet. In recent years, evidence suggested that NLRP3 could be expressed by mouse and human lymphocytes [20] and has an ability to adjust the differentiation of Th1, Th2 Th17-cells. Recently, G. Arbore et al. (2016) have shown

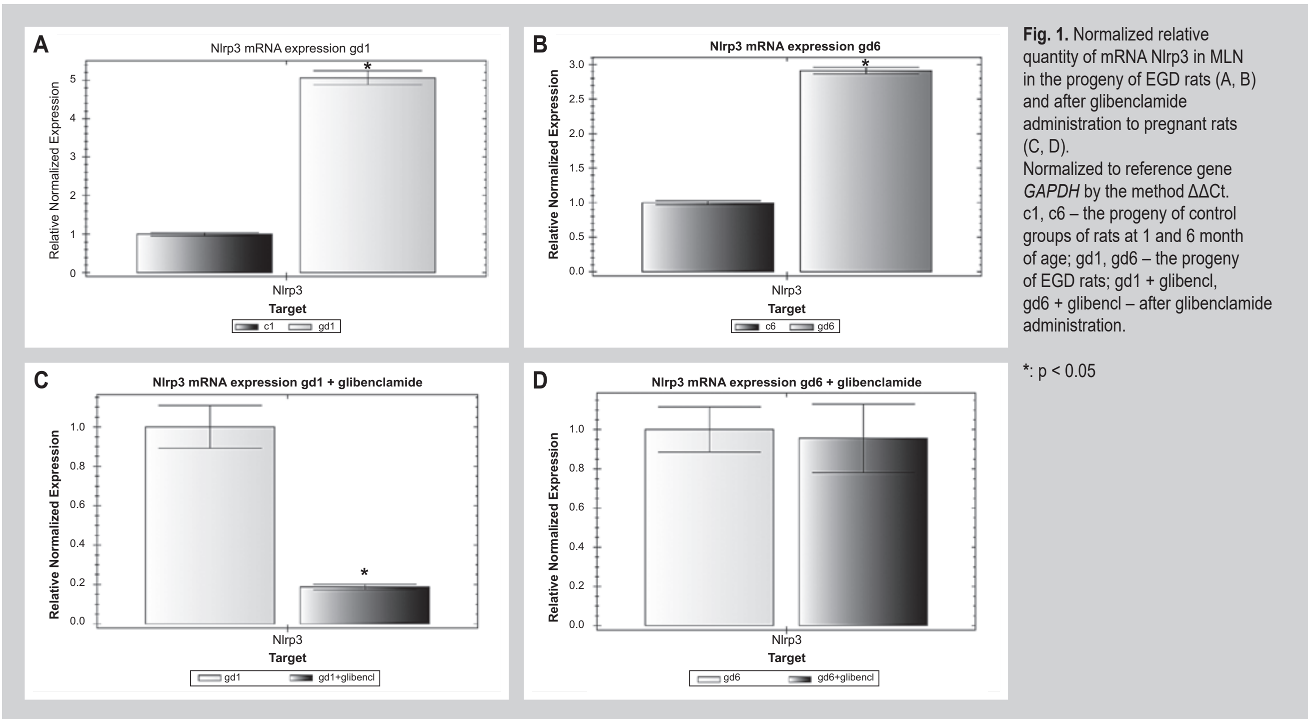

Fig. 1. Normalized relative quantity of mRNA Nlrp3 in MLN in the progeny of EGD rats (A, B) and after glibenclamide administration to pregnant rats (C, D).
Normalized to reference gene *GAPDH* by the method ΔΔCt.
c1, c6 – the progeny of control groups of rats at 1 and 6 month of age; gd1, gd6 – the progeny of EGD rats; gd1 + glibencl, gd6 + glibencl – after glibenclamide administration.

*: $p < 0.05$

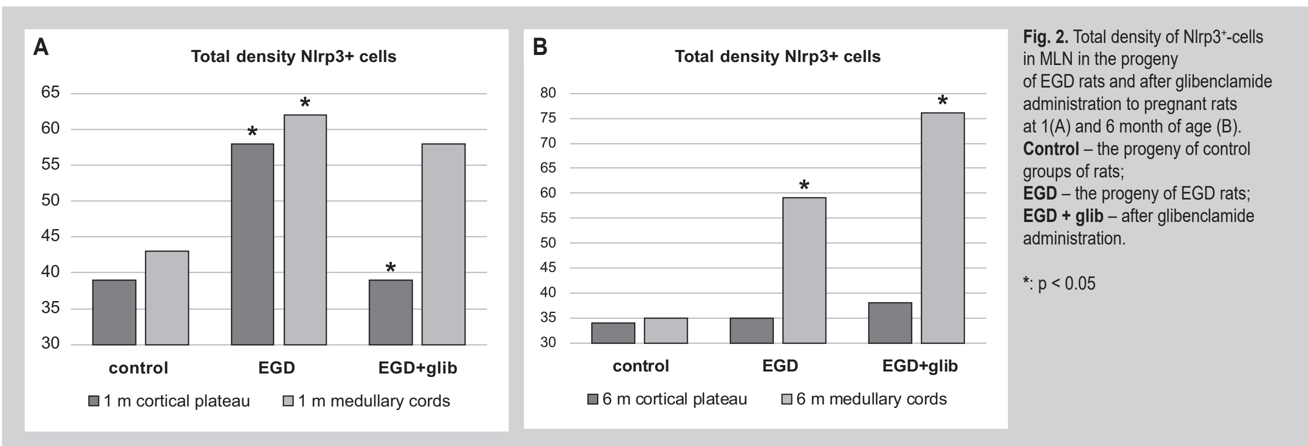

Fig. 2. Total density of Nlrp3+-cells in MLN in the progeny of EGD rats and after glibenclamide administration to pregnant rats at 1(A) and 6 month of age (B).
Control – the progeny of control groups of rats;
EGD – the progeny of EGD rats;
EGD + glib – after glibenclamide administration.

*: $p < 0.05$

that NLRP3 inflammasome assembles in human CD4+ T cells and initiates caspase-1–dependent interleukin-1β secretion, thereby promoting interferon-γ production and T helper 1 (TH1) differentiation in an autocrine fashion. [21]. Abnormal activity of NLRP3- inflammasome in T-cells affects the development of inflammatory and autoimmune diseases in humans and in mice experimental models. Obviously, NLRP3-inflammasome activity is not limited to «innate immune cells» and is an integral part of normal Th-adaptive responses. Violation of NLRP3 activity in CD4+ T cells in experimental models of colitis causes uncontrolled infiltration of Th17 cells and aggravation of bowel disease. Thus, autocrine activity of NLRP3-inflammasomes in lymphocytes controls Th1-Th17 balance in terms of experimental inflammatory bowel disease. Furthermore, M. Bruchard et al. (2015) recently showed the ability of NLRP3 to act as a key transcription factor that controls the Th2-differentiation [22]. In Th2 cells NLRP3 binds to promoter IL4 and activates it in conjunction with transcription factor IRF4 [22]. In contrast to Th1, where NLRP3 is detected mainly in the cytoplasm by methods of immunofluorescence microscopy, in the Th2-cells it is localized mainly in the nucleus. It is possible that such a nuclear localization function can promote inflammasome transcription. This work showed that NLRP3 should be seen not only as a key inflammasome component, but as a transcription factor in cells CD4+ Th2. Finally, the mechanisms of IL-1β-induced Th17 differentiation are related to the ability of TGF-β to induce expression RORγt in naive T cells [23]. Studies *in vitro* have shown that IL-1β induces the expression of IRF-4, positively regulates IL-21-mediated expression of transcription factors STAT-3 and RORγt [24]. In addition, the role of IL-1 in the i nduction of Th17 phenotype was attributed to alternative splicing of Foxp3 [25].

At the same time, NLRP3-inflammasome is one of the sensors of metabolic stress developing diabetes [26]. NLRP3–-deficient NOD-mice are protected from developing diabetes by reducing migration of diabetogenic lymphocytes in the pancreatic islets, they have reduced

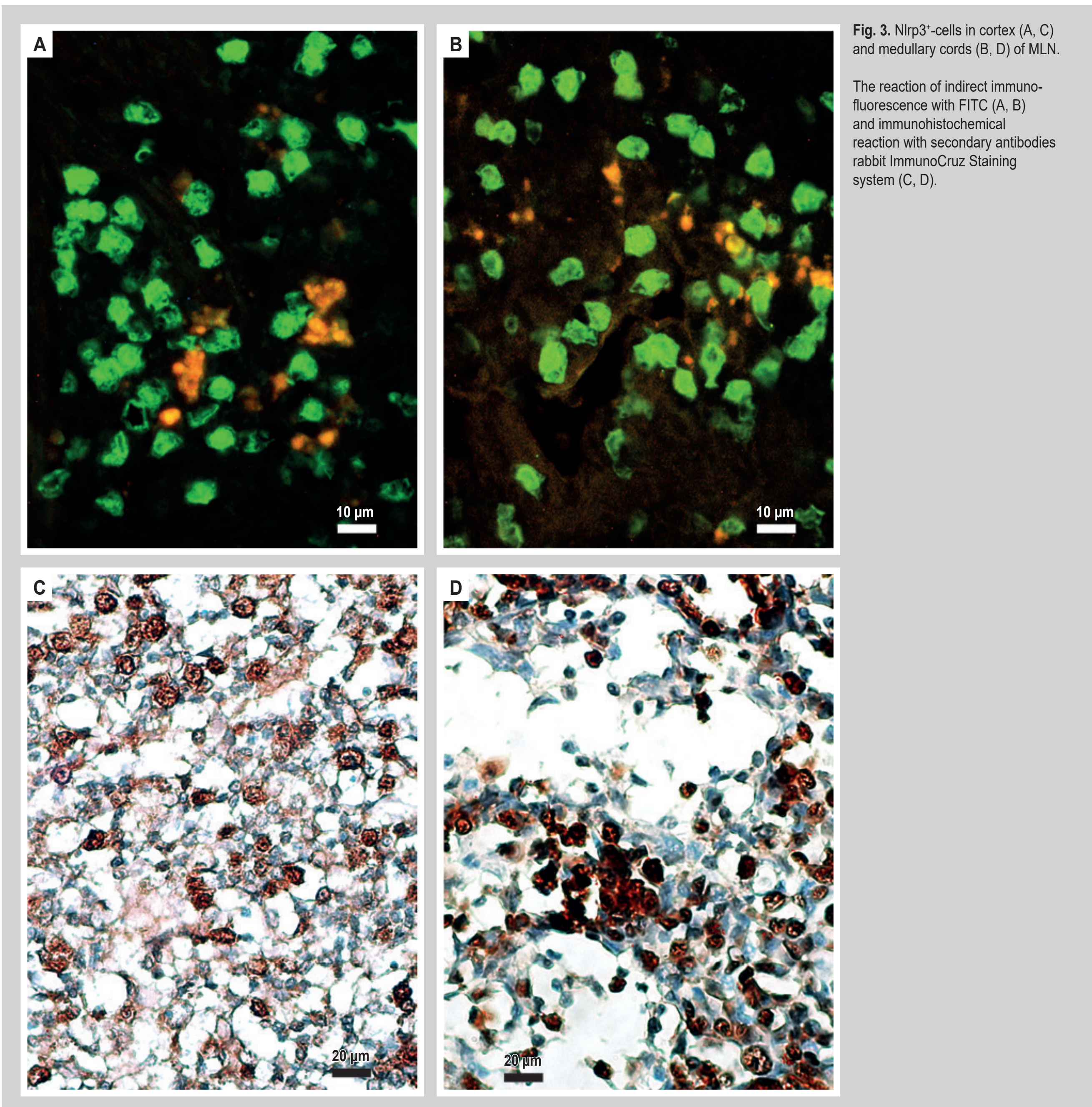

Fig. 3. Nlrp3+-cells in cortex (A, C) and medullary cords (B, D) of MLN.

The reaction of indirect immunofluorescence with FITC (A, B) and immunohistochemical reaction with secondary antibodies rabbit ImmunoCruz Staining system (C, D).

the number of CD4+ T cells, CD19+ B-cells and CD11b+ APC in spleen and PLN, but not in the thymus [27]. The study has found two single-nucleotide polymorphism in NLRP3, associated with DM type 1 [28], and D. Carlos et al. (2017) showed growth of NLRP3 expression in PLN in NOD mice [29]. A similar increase of NLRP3 gene expression was observed in STZ-induced diabetes in mice C57BL/6. Furthermore, diabetic mice *C57BL/6* also showed a decrease in IL-17-producing CD4 and CD8 T cells (Th17 and Tc17) and IFNγ-producing CD4 and CD8 T cells (Th1 and Tc1) in PLN. Interestingly, diabetic mice showed an increase in the expression of genes associated with mitochondrial DNA, such as cytochrome b and cytochrome c. Mitochondrial DNA (mDNA) of diabetic mice induced production of IL-1β and activation of caspase-1 with macrophages, but was reduced in NLRP3-/--macrophages. Finally, the administration of mDNA *in vivo* increased the number prodiabetic Th17/Tc17/Th1 cells in the PLN, but it was also canceled in NLRP3-/--mice [29].

NLRP3-inflammasome is an important pharmacological target for blocking a number of diabetes complications [30], and the ability of glibenclamide to inhibit the formation of NLRP3 can affect the risk of inflammatory and autoimmune diseases in the progeny of mothers with GD. Recent research by S. Lamprianou et al. (2016) demonstrated that glibenclamide protects NOD mice from progressing hyperglycemia and loss of insulin-producing β-cells. Although the administration of glibenclamide does not stop the development of insulitis, but induces a shift of the phenotype of immune cells and protects cells of insulinoma MIN6 from apoptosis and loss of connexin Cx36 [31].

Conclusions

1. The development of EGD is accompanied by transcriptional induction of the *Nlrp3* gene in MLN in descendants, whose mRNA level increased five-fold ($p < 0.05$) in 1-month and 3-fold ($p < 0.05$) in 6-month-old animals. The administration of glibenclamide to pregnant rats inhibited the transcription of the *Nlrp3* gene only at the age of 1 month (5.3 times, $p < 0.05$) and did not change it in the older age group.

2. In the progeny of rats with EGD, the density of the NLRP3^{+}-lymphocyte population in the MLN increased, more clearly at early observation times. The intake of glibenclamide reduced the number of NLRP3^{+}-lymphocytes only at the age of 1 month (by 33 %, the cortex plateau), whereas their number in the medullary cords of 6-month-old progeny even increased.

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