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Original Research

Changes in the Transcriptional Activity of the Lymphocyte Homing Regulatory Genes Madcam1, Cxcr3, Ccr7 and S1pr1 Affect Structure of the Population of T-bet+, Rorγt+ and Foxp3+ cells in Mesenteric Lymph Nodes in Offspring of Rats with Experimental Gestational Diabetes

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Abstract

Background and aims: Maternal hyperglycemia may violate peripheral immunological tolerance (PIT) formation to pancreatic antigens; antigen administration stimulates PIT. Mesenteric lymph nodes (MLN) are transition point for recirculating lymphocytes of gastrointestinal associated lymphoid tissue (GALT) and the location for PIT induction. Homing of lymphocytes in MLN is regulated by MAdCAM-1, CXCR4 and CCR7, while S1PR1 activates T-cell exit from MLN. We aimed to evaluate mRNA expression of these genes and their effect on the distribution of Th1-, Th17-, and Treg-cells in MLN of the rat offspring with experimental gestational diabetes (EGD) with oral insulin administration. **Material and method:** We studied one and six months old male offspring of Wistar rats with EGD, which received insulin orally for the first 14 days of life. RT-PCR method was used for Madcam1, Cxcr4, Ccr7 and S1pr1 mRNA expression measurements in MLN. The structure of T-bet+, Rorγt+, Foxp3+ cell population was studied using histological sections of MLN, morphometric and densitometric characteristics. **Results:** An increase of CCR7 and MAdCAM-1 mRNA in offspring of animals with EGD indicated activation of the GALT immune cells, which is accompanied by intensification of lymphocytes homing, while no changes were observed in CXCR4 expression. Increased expression of S1PR1 confirms its role in diabetes. Chemokine receptors affect the activation of different Th cells subsets with their pivotal role in diabetes type 1, through violation of oral tolerance. A significant increase in Th1 and Th17 cell population was observed in the offspring of rats with EGD. **Conclusions:** The changes in mRNA expression of the lymphocyte "homing" regulators in MLN affected the distribution of Th1-, Th17- and Treg-cells in the offspring of rats which parents had EGD. The development of oral insulin tolerance is accompanied by a decrease in the relative normalized mRNA number of Ccr7, Madcam1 and S1pr1 genes in MLN cells.

Keywords: Experimental gestational diabetes, mesenteric lymph nodes, *Madcam1, Cxcr4, Ccr7, S1pr1.*

Introduction

Mesenteric lymph nodes (MLN) are the main site of induction of peripheral immunological response to a variety of antigens, including pancreatic [1], as well as a major "transitional" point for the pool of recirculating lymphocytes of the gut-associated lymphoid tissue (GALT). The main regulators of lymphocyte "homing" in MLN are addressin MAdCAM-1 (mucosal addressin cell adhesion molecule 1, an adhesive protein of high endothelial venules of GALT lymphoid formations), CXCR4 chemokine receptors (CXC chemokine receptor type 4) and CCR7 (chemokine receptor type 7), the ligands for which are CXCL12, CCL19, and CCL21, respectively. The chemokine-receptor interaction between the CCL19/21-CCR7 and CXCL12-CXCR4 pairs, as well as MAdCAM-1 and its ligand integrin α4β7, regulate the transfer of most immune cells to the lymph nodes. Thus, the entry of T cells into lymph nodes is impaired in the absence of CCR7 signaling, including the *plt/plt* line mice model, lacking CCL21 and CCL19 [2]. In turn, the exit of lymphocytes from MLN is regulated by sphingosine-1-phosphate (S1P) receptors (S1PR1-S1PR5) [3], among which lymphocytes are the most actively expressing type 1 receptor, S1PR1 [4]. After the activation of immune cells in MLN, the expression of S1PR1 on their membrane increases, allowing lymphocytes to respond to changes in the S1P gradient and leave the lymph nodes [5].

The objectives of this work were to evaluate the levels of mRNA expression of MAdCAM-1, S1PR1, CXCR4, and CCR7 genes and their effect on the distribution of Th1-, Th17-, and Treg-cells in mesenteric lymph nodes in the offspring of rats with experimental gestational diabetes (EGD) and under conditions of oral insulin administration.

Material and Methods

Study design

The male Wistar rats (N=120) were divided into 6 experimental groups, 20 rats per each group: the offspring of intact rats, 1-month-old (group 1) and 6 months old (group 2), female parents of which were administered 0.5 ml of 0.1 M citrate buffer (pH = 4.5) intraperitoneally on the 15th day after pregnancy; the offspring of rats with experimental gestational diabetes (EGD) at 1 month (group 3) and 6 months age (group 4), female parents of which were once injected streptozotocin at a dose of 45 mg/kg on the 15th day of pregnancy; offspring of rats with EGD at 1 month of age (group 5), female parents of which were administered oral short-acting human insulin (ACTRAPID® HM, NOVO NORDISK, Denmark) at a dose of 30 IU (1.05 mg, 1 IU corresponds to 35 μg of anhydrous human insulin) orally during the first 14 days of life; offspring of rats with EGD at 6 months of age (group 6), female parents of which were given 30 IU insulin orally during the first 14 days of life.

Molecular genetics experiments

Molecular genetics experiments using the real-time reverse transcription-polymerase chain reaction (RT-PCR) method were performed on isolated MLN of experimental rats. The tissue was placed in a Bouin's fixative solution (15 parts of the saturated aqueous picric acid solution, 5 parts of formaldehyde, 1 part of acetic acid), followed by tissue dehydration using ascending concentrations of ethanol, and then placed in paraffin blocks. The experiments were conducted on archival material not older than 2 years. RNA was obtained from histological sections with a thickness of 15 μm, by their deparaffinization in xylene followed by rehydration in descending concentrations of ethanol (100%, 96%, 70%). Total RNA was isolated using a Trizol RNA Prep 100 kit (Isogen Lab., LTD, Russian Federation) containing Trizol reagent (a lysing reagent comprising guanidine thiocyanate as denaturing agent and phenol with pH = 4.0) and ExtraGene E (suspension of ion exchange mixture). The RNA was isolated according to the manufacturer's instructions.

cDNA synthesis was carried out by reverse transcription reaction using a commercial kit OT-1 (Synthol, Russian Federation). The reaction

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Table 1: List and description of genes and corresponding primers used during experiments

Gene	Primer	Tm,°C	Product length (bp)	Exon junction
Cxcr4	F =TGCCATGGAAATATACACTTCGG R = TCCAGACCCTACTTCTTCGGA	58.87 59.64	52	81/82
Ccr7	F = CTGGTCATTTTCCAGGTGTGC R = TGGTGTTCTCGCCGATGTAG	59.73 59.83	67	138/139
Madcam1	F = CCAAACTGGTGCTGACACAT R = TCTGGCTCTGTAGGACTGGAA	58.68 59.92	51	662/663
S1pr1	F= CGGATCGCGCGGTGTAG R= GAAACAGCAGCCTCGCTCAA	60.73 61.23	70	61/62
GAPDH	F = GCCTGGAGAAACCTGCCAAG R =GCCTGCTTCACCACCTTCT	61 60	52	825/826

mixture with a total volume of 25 µl contained 1 µl of Random-6 primer, 2 µl of total RNA, 8.5 µl of deionized H2O, 12.5 µl of 2.5x reaction mixture, and 1 µl of MMLV-reverse transcriptase. Reverse transcription was carried out at 45°C for 45 min, followed by MMLV-RT heat inactivation (92°C, 5 min), as suggested by the manufacturer.

The CFX96 ™Real-Time PCR Detection Systems Amplifier (Bio-Rad Laboratories, Inc., USA) and Maxima SYBR Green/ROX qPCR MasterMix (2X) reagent kit (ThermoScientific, USA) were used to determine expression levels of mRNAs of the investigated genes, namely, Madcam1 (NM_019317.1), Cxcr4 (NM_022205.3), S1pr1 (NM_017301.2), Ccr7 (NM_199489.4). The final reaction mixture for amplification included SYBR Green dye, Maxima HotStartTaq DNA Polymerase, 0.2 µl of forward and reverse specific primers, 1 µl of the template (cDNA). The reaction mixture was brought to a total volume of 25 µl with the addition of deionized H2O. Gene identification numbers (NM_019317.1, NM_022205.3, NM_017301.2, NM_199489.4) were derived from the US National Library of Medicine, National Center of Biotechnology Information website (https://www.ncbi.nlm.nih.gov/). Specific primer pairs (forward and reverse) for amplification of the investigated and reference genes were selected using the PrimerBlast software (www.ncbi.nlm.nih.gov/tools/primer-blast) and manufactured by Metabion, Germany (Table 1). After the initial denaturation for 10 min at 95°C, the amplification consisted of 45 cycles and each cycle was performed under the following conditions: denaturation - 95°C for 15 sec, annealing –59-61°C for 30-60 sec, elongation –72°C for 30 sec. Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as a reference gene to determine the relative values in changes in the expression level of the studied genes and normalize results. The relative normalized number of cDNA target genes was determined by the ΔΔCt method.

Immunohistochemistry

The population structure of T-bet + - (Th1), Rorγt + - (Th17), and Foxp3 + - (Treg) lymphocytes was studied by analyzing serial histological sections of MLN and their morphometric and densitometric characteristics. MICROM HR-360 rotary microtome (Microm, Germany) was used to make 5 micron serial MLN sections. The histological sections were dewaxed in xylene, followed by rehydration in decreasing concentrations of ethanol (100%, 96%, 70%), then washed in 0.1 M phosphate buffer (pH = 7.4), and treated with primary rabbit polyclonal antibodies (PRPA) to T-bet, Rorγt or Foxp3 (Santa Cruz Biotechnology, USA, sc-1550-R) for 18 hours in a humid chamber at 4°C. After washing off the excess of primary antibodies in 0.1 M phosphate buffer, histological sections were incubated for 60 min at 37°C with secondary antibodies to the full rabbit IgG molecule, conjugated to FITC (Santa Cruz Biotechnology, USA). After incubation, all sections were washed with 0.1 M phosphate buffer and placed in a mixture of glycerol and phosphate buffer (1:9) for further fluorescence microscopy.

Images were taken with a Primo Star microscope (ZEISS, Germany) in a 390 nm ultraviolet excitation spectrum (FITC) using a high-sensitivity Axio Cam 5c camera (ZEISS, Germany) and processed with a software package for obtaining, archiving and preparing images (Axio Vision 4.7). The processed histological sections were examined using a computer program ImageJ (National Institute of Health, USA). Image regions with statistically significant fluorescence were determined in the automatic mode using specific software. The morphometric and densitometric characteristics of immunopositive cells were calculated. The absolute number of immunopositive cells of different classes as the number of cells per 1 mm^2 slice area and relative (%) density of their distribution in the studied areas of MLN histological sections were determined.

Statistical analysis

For molecular genetics experiments, three sets of histological sections from MLN of each rat were used for RNA extraction. Following cDNA synthesis from each set, PCR amplification reactions were performed on individual samples in three repetitions. Negative controls were included in all experiments, including the absence of MMLV-RT in cDNA synthesis, absence of mRNA in the matrix of cDNA synthesis, and absence of cDNA matrix in the real-time PCR reaction. Student's t-test ($p<0.05$) was used to determine if the means of two sets of data are significantly different from each other (control vs. EGD, EGD vs. EGD+insulin). Statistical analysis of PCR data was performed using the CFX Manager ™ software (Bio-Rad, USA) with a statistical significance threshold set at $p<0.05$.

For immunochemistry experiments, three histological sections were used from the cortical plateau and medullar cords of MLN of each rat to determine the absolute number of immunopositive cells (number of cells per 1 mm^2 slice area) and relative (%) density of their distribution from five studied areas of MLN histological sections. Student's t-test ($p<0.05$) was used to determine if the means of two sets of data are significantly different from each other (control vs. EGD, EGD vs. EGD+insulin). Statistical analysis of immunochemistry data was performed using Statistica ver. 10 (StatSoft, Inc., Tulsa, OK, USA).

Results

The results of the Ccr7 gene expression study in MLN tissues showed that there was a significant increase in mRNA content of this chemokine receptor in offspring of EGD rats, with a 26.8-fold increase in 1-month and a 21-fold increase in 6-month-old offspring compared with the control group of animals of the corresponding age ($p<0.05$, Figure 1 A, B). As for the expression of CXCR4 receptor mRNA, its content did not change significantly in both age groups compared to control ($p>0.05$, Figure 1 C, D). The relative normalized adressin MAdCAM-1 mRNA expression increased 2.4 times and 2.3 times in the age group of 1 month and 6 months offspring of EGD rats, respectively ($p<0.05$, Figure 1 E, F). Regarding S1pr1 gene expression, the content of S1PR1 mRNA increased 3.6 times ($p<0.05$) in the 1-month group of offspring rats with EGD; in the 6-months group, a 5.0-fold increase was observed compared to the control group ($p<0.05$, Figure 1 G, H).

Expression of mRNA of the Ccr7 gene in 1-month-old offspring of EGD rats after oral administration of insulin during the first 14 days of life decreased by 81% compared to offspring of untreated EGD rats of the same age as the control group ($p<0.05$). However, no significant difference was observed between corresponding groups at 6-months age ($p>0.05$) (Figure 2 A, B). Cxcr4 gene expression did not change significantly in both age groups while comparing the offspring of EGD rats treated with insulin and the offspring of untreated EGD rats (Figure 2 C, D). Contrary, the MAdCAM-1 mRNA content decreased by 95% at 1 month of age as a result of insulin administration ($p<0.05$), whereas in 6-month-old rats, a 3.9-fold increase was noticed ($p<0.05$) (Figure 2 E, F). The mRNA content of the S1pr1 gene decreased by 65% and 95% for the 1-month and 6-months offspring of EGD rats treated orally with insulin ($p<0.05$) (Figure 2 G, H).

The changes detected in the level of expression of the regulators of lymphocyte

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"homing" in MLN influenced the distribution of individual subpopulations of T-helper cells. For example, the total density of T-bet+-cells in the cortical plateau in the 1-month-old offspring of animals with EGD compared to the control group increased 2-fold; in 6-months rats, we observed an increase of 56%. Regarding medullary cords (chordae medullares), this index increased by 2.6 and 2.8 times in 1-month and 6-months rats, in offspring of EGD rats against control rats, respectively ($p < 0.05$). In the group of animals receiving oral insulin, a decrease in the total density of T-bet+ lymphocytes in the cortical plateau was observed by 33% ($p < 0.05$) at 1-month and by 41% ($p < 0.05$) in 6-month-old rats. In the medullary cords, the total amount of Th1 after insulin administration decreased only in animals aged 6 months - by 44% ($p < 0.05$).

Analysis of the distribution of Rorγt+ -cells in MLN tissue of rats showed that in the 1-month-old offspring of rats with EGD, the total Th17 cell density in cortical plateau was significantly higher - by 48% ($p < 0.05$) -than the control group of offspring from intact rats. In 6 months-old animals, the number of Rorγt+ lymphocytes in this zone increased by 2.4 times. In medullary cords, the total Th17 density in the 1-month-old offspring of EGD animals increased by 51% ($p < 0.05$); in 6-month-old rats – by 2.4 times ($p < 0.05$), compared to the offspring of intact animals of corresponding age group. After insulin administration, the total lymphocyte density of Rorγt + lymphocytes in the cortical plateau of 1-month-old animals did not change significantly, and at 6 months of age, it decreased ($p < 0.05$). In the medullary cords, the number of Rorγt + -cells decreased by 52% ($p < 0.05$) in the 1-month-old offspring and by 46% ($p < 0.05$) in the 6-month-old offspring.

The number of Foxp3 + lymphocytes in the cortical plateau of the offspring of rats with EGD was lower by 57% in 1-month-old animals ($p < 0.05$), and lower by 54% ($p < 0.05$) in 6-months old animals, compared with the control group. A decrease in T-regulatory cells by 76% and 64% ($p < 0.05$) was also observed in medullary cords. In rats treated with insulin, the following results were found: the total density of Foxp3 + cells in 1-month-old rats in the cortical plateau did not change significantly. Still, it increased 2.2-fold in 6 months-old animals ($p < 0.05$). The number of Treg increased in the medullary cords by 2.3 - 2.6 times ($p < 0.05$).

Discussion

Earlier, we showed that under conditions of diabetes, there are changes in the transcriptional activity of some genes and the subpopulation composition of T-lymphocytes [6-8]. CCR7-mediated signals affect T-cell homeostasis in lymph nodes at different levels, as well as the activation and polarization of different T-helper subpopulations [9]. Thus, CCR7 plays an essential role in the function of T-regulatory cells, while performing a dual role: for Treg-induced expression, CCR7 expression is required for their efficient priming in lymph nodes [10]; while effector Treg apparently uses CCR7 to exit tissues and, therefore, CCR7 limits their excessive accumulation in peripheral tissues [11]. Although the total number of Treg in the thymus in CCR7 knockout mice and wild-type mice is the same, their suppressor functions in the periphery are impaired due to their inability to enter the lymph nodes and position themselves within the T-cell zones of LN [12]. A recent study by Mackley et al. showed that migration of a group of congenital RORγ-lymphocytes from the intestine into MLN is also CCR7-dependent [13], and such cells intentionally migrate precisely to MLN and not to other groups of LN. CCR7 -/- mice are more susceptible to infections caused by *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Leishmania donovani* and *Toxoplasma gondi* [14], showing an increase in lymphocytic infiltration of peripheral tissue and pancreatic islets, disturbances in central and peripheral tolerance to their own antigens and defects in the functioning of Treg cells, leading to the development of multiple organ autoimmune reactions [15]. This is accompanied by an increase in the titer of circulating autoantibodies to a large number of tissue-specific antigens and the formation of ectopic tertiary lymphoid structures on the mucous membranes [16]. Shan et al. established the important role of CCR7 in engaging T cells in pancreatic islets in

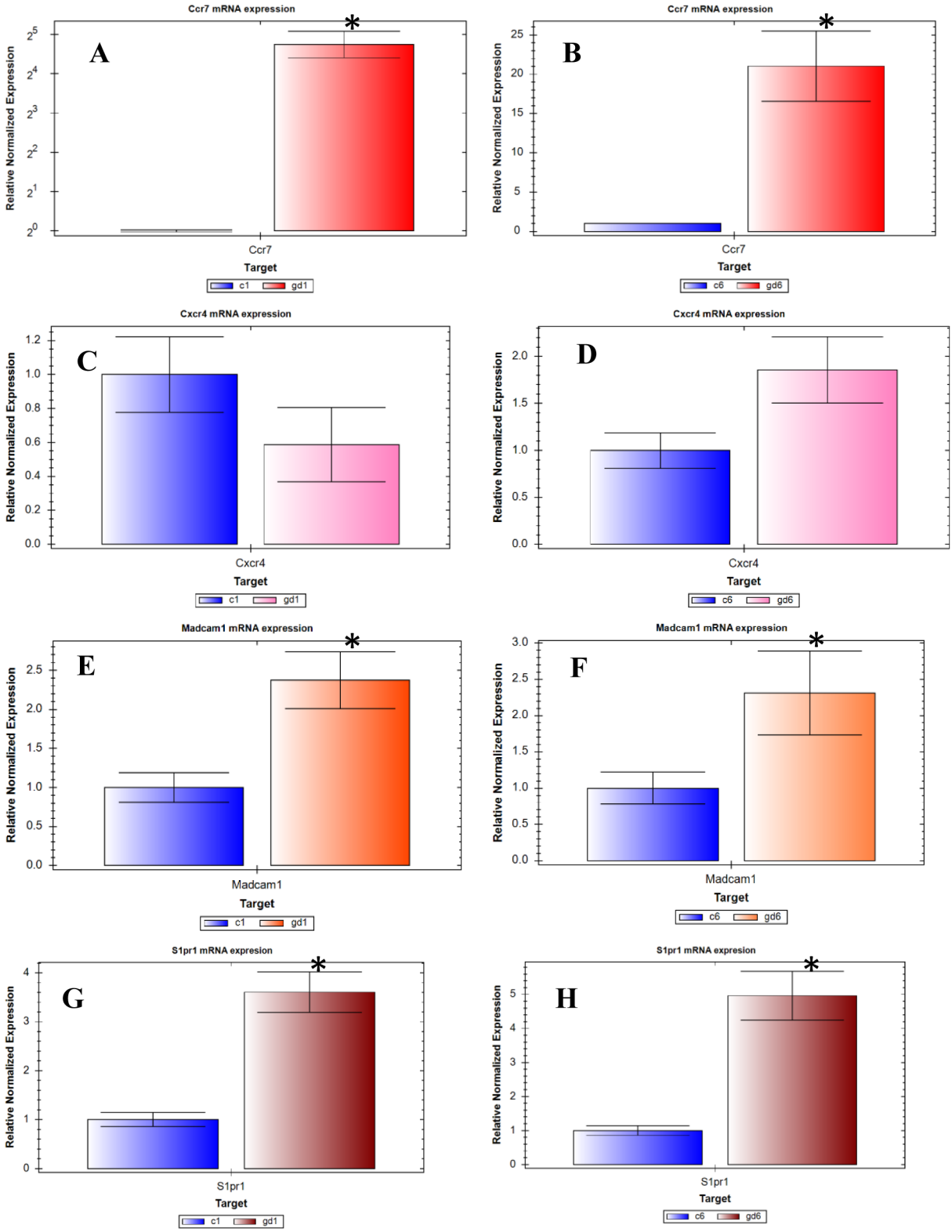

Figure 1: Relative normalized mRNA number of genes Ccr7 (A, B), Cxcr4 (C, D), Madcam1 (E, F) and S1pr1 (G, H) in the cells of MLN tissue. Normalization performed by $_{\Delta\Delta}$Ct method with GAPDH as reference gene (c1 – 1-month old control offspring from intact rats, c6 – 6-months old control offspring from intact rats, gd1 – 1-month old offspring from EGD rats, gd6 – 6-months old offspring from EGD rats). Asterisk (*) indicates a significant difference ($p < 0.05$).

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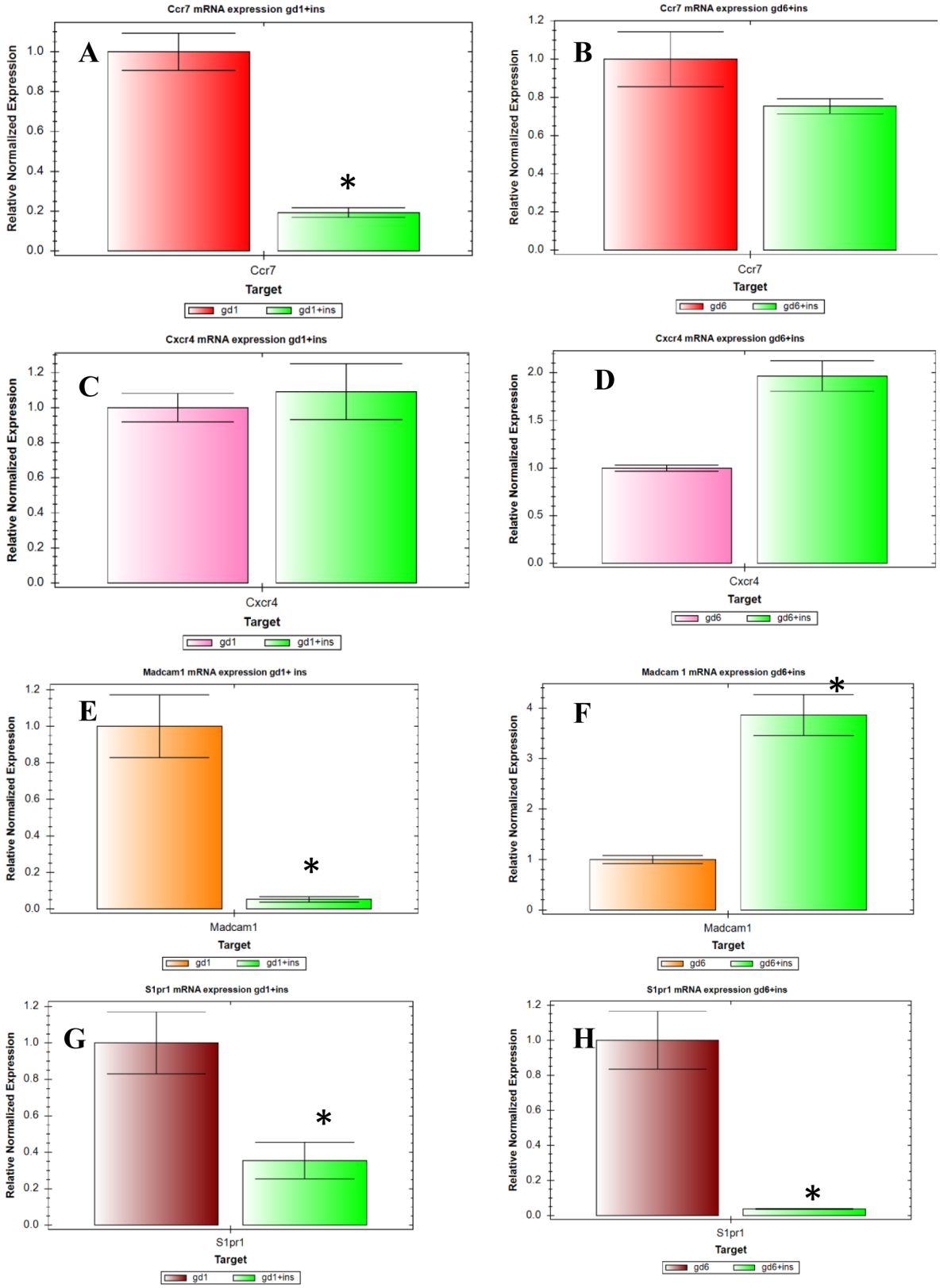

Figure 2: Relative normalized mRNA number of genes Ccr7 (A, B), Cxcr4 (C, D), Madcam1 (E, F) and S1pr1 (G, H) in the cells of MLN tissue. Normalization performed by $_{\Delta\Delta}$Ct method with reference gene of GAPDH. (gd1 – 1-month old control offspring from EGD rats, gd6 – 6-months old control offspring from EGD rats, gd1+ins – 1-month old offspring from EGD rats given insulin orally, gd6+ins – 6-months old offspring from EGD rats given insulin). Asterisk (*) indicates a significant difference ($p < 0.05$).

NOD-mice [17], using RT-PCR showed intensive expression of CCR7 mRNA and its CCL19 and CCL21 ligands in inflamed, but not intact islets. Turning off CCR7 signaling blocked the migration of about 75% of T cells from the blood to the pancreatic islets, but this did not affect the migration of B lymphocytes to the islets. The received results indicate that CCR7 and its ligands play an essential role in T-cell entrance in the islets and, thus, in the pathogenesis of type I diabetes mellitus. Some studies have demonstrated the role of CCR7 signaling in the formation of oral tolerance to various antigens. However, oral tolerance cannot be induced in CCR7-deficient mice due to impaired migration of dendritic cells, which carry those antigens from the intestinal mucosa of the intestine into the MLN [18].

The increase in the level of mRNA of another of the regulators of "homing" of lymphocytes - MAdCAM-1 in MLN is confirmed by the results of previous research in which it is shown that RNA transcripts of MAdCAM-1 are well expressed in the endothelium of both lymphoid and non-lymphoid tissues, including small and large intestines, mesenteric and pancreatic lymph nodes, spleen [19]. Characteristically, this expression is increased in response to the action of proinflammatory cytokines, in animal models of inflammatory bowel disease, and patients with Crohn's disease and ulcerative colitis [20]. MAdCAM-1 is exclusively expressed in high endothelial venules MLN, but not peripheral LN [21]. There are no data on the expression of MAdCAM-1 in MLN in offspring of parent rats having EGD. Only lymphocyte adhesion, regulated by the α4β7 integrin/MAdCAM-1 system, is known to be essential for B-cell migration into pancreatic lymph nodes (PLN) in non-obese diabetic (NOD) mice. Thus, B-lymphocytes from PLN of 3 to 4-week old NOD mice are characterized by high expression of integrin α4, LFA-1, and intermediate level of integrin β7. In-vivo studies showed that B cells migrated from the bloodstream to PLN more efficiently than other peripheral LN. Also, antibodies to MAdCAM-1 and integrin α4β7 inhibited the B lymphocyte migration into PLN by more than 90% [22]. Single intraperitoneal injection of anti-MAdCAM-1 antibodies to 3-week-old NOD mice significantly slowed B-lymphocyte entry into the PLN for at least 2 weeks. These results demonstrate that the α4β7 integrin/MAdCAM-1 adhesion pathway plays a vital role in the migration of B lymphocytes into PLV in NOD mice and might be a potential target for T1DM therapy. The role of MAdCAM-1 in the development of type 1 diabetes mellitus was shown by other researchers as well. Thus, CD4 + cells reactive to pancreatic islets are able to initiate the development of type 1 diabetes when transferred to newborn NOD mice, but not in adult animals. These differences are due to unequal levels of MAdCAM-1 expression in pancreatic islets in newborn and adult mice [23]. Besides, blockade of this adresin interferes with the induction of diabetes in newborn mice.

In the final stage of lymphocyte homing inside LN, another chemokine receptor, namely CXCR4, also known as fusin or CD184, plays an important role. Specifically, it binds to the ligand - the stromal-derived factor (SDF-1) molecule. Under normal physiological conditions, SDF-1 is produced by stromal cells of many tissues, and CXCR4 is expressed on the surface of CD4+ T-cells, dendritic cells and B lymphocytes. We did not detect changes in CXCR4 mRNA levels in MLN in the offspring of rats with EGD, although other studies have shown that the CXCR4/CXCL12 signaling pathway protects NOD mice from autoimmune diabetes [24]. CXCR4 mRNA levels were increased in the PLN of 4-week NOD mice compared with Balb/C mice [25]. A significant decrease in the level of CXCR4 expression was observed in 12 weeks-old animals regarding both mRNA expression and protein levels, while their content in pancreatic islets increased. The introduction of AMD3100, a CXCR4 antagonist, reduced the number of CXCR4+ and SDF1+ positive cells in inflamed islets and provided protective effects against autoimmune diabetes. Simultaneously, the transcriptional level of CXCL12, a Cxcr4 ligand, is significantly increased in the bone marrow of NOD mice compared to Balb/c and C57BL/6 mice. The introduction of AMD3100 into NOD mice mobilizes regulatory T cells and hematopoietic bone marrow stem cells to the periphery and suppresses insulitis and slows the onset of diabetes [26]. Transgenic mice,

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which are overexpressing SDF-1, are resistant to streptozocin-induced β-cell apoptosis and diabetes, whereas MIN6, another CXCR4 antagonist, causes their apoptosis, increases the production of ROS, reduces the expression of anti-apoptotic protein BCL-2 and phosphorylation of the proapoptotic BAD protein [27].

Inhibition of S1PR on T or B lymphocytes (FTY720, fingolimod) renders these cells insensitive to a change in the S1P concentration gradient between lymph nodes and biological fluids; thus, provides CCR7 receptor-mediated lymphocyte retention in lymph nodes [28]. FTY720 has demonstrated its effectiveness in several models of immunological diseases, including rheumatoid arthritis, myasthenia gravis, multiple sclerosis, and type 1 diabetes mellitus [29]. The increased level of S1PR1 mRNA expression in MLN lymphocytes, which we found, may play an important role in the development of diabetes. Thus, the cessation of lymphocytes exits from PLN by the S1P agonist FTY720 receptors in NOD mice prevents further progression of diabetes even during significant insulitis [30]; after its use, no increase in expression levels of cytotoxic molecules of granzyme B, interferon γ, and CD107a in islet CD8-lymphocytes was observed [31]. Mesenteric lymphadenectomy significantly reduces the size of the population of Th17 lymphocytes in the intestine. The introduction of FTY720 induces the accumulation of α4β7+ IL-17A+ proliferating cells in MLN [32].

Conclusion

The changes we detected in the level of expression of the regulators of lymphocyte "homing" in MLN affect the distribution of Th1-, Th17- and Treg-cells in the offspring of rats who had EGD. The development of oral insulin tolerance is accompanied by a decrease in the relative normalized mRNA number of Ccr7, Madcam1 and S1pr1 genes in MLN cells, total Rorγt+ - and T-bet+ lymphocyte density.

Conflict of Interest

The authors declare no conflict of interest.

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