



# Breakdown in peripheral immune tolerance in experimental Diabetes Mellitus

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## ABSTRACT

**Background:** Peripheral tolerance can be mediated by extrathymic Aire-expressing cells (eTACs) and lymph node stromal cells (LNSCs) have recently been shown to induce T-cell tolerance by ectopically expressing and presenting range of peripheral tissue antigens (PTAs). New evidence shows that all types of LNSCs—including fibroblastic reticular cells, follicular DCs, and lymphatic endothelial cells - express PTAs. Ectopic expression of genes encoding PTAs in lymph nodes (LN) controlled by transcriptional regulators Aire and deformed autoregulatory factor 1 (Deaf1). Therefore, the aim of our study was to determine the effect of the levels of Deaf1 and Aire mRNA expression on the nature of Foxp3<sup>+</sup> Treg cells differentiation during experimental STZ-induced diabetes mellitus (EDM) in rats pancreatic lymph nodes (PLN). **Methods.** To determine the level of mRNA Deaf1 and Aire expression was performed RT-PCR in real-time by thermocycler CFX96™ Real-Time PCR Detection Systems. The relative level of gene expression were studied with rat reference genes *GAPDH* by the method  $\Delta\Delta Ct$ . Statistical analysis were conducted using available software «Bio-Rad CFX Manager 3.1». The Foxp3<sup>+</sup>-immunopositive lymphocytes were determined using an indirect immunofluorescence technique with using a monoclonal rat antibody. **Results:** Development of EDM was accompanied by decreased the expression levels of the transcriptional regulator Deaf1 and Aire in rats PLN. So, Deaf1 expression is decreased 4,2-fold in rats PLN with 3-week EDM and 2,5-fold in rats with 5-week EDM. Aire expression is decreased 2-fold in rats PLN with 3-week EDM and 50-fold in rats with 5-week EDM. Reduced Deaf1 and Aire mRNA expression during EDM associated with an decreased of total amount of Treg in the PLN, led to changes of distribution into individual classes of FoxP3<sup>+</sup> lymphocytes and FoxP3 concentration in immunopositive cells. **Conclusions:** development of EDM due to a breakdown in peripheral immune tolerance. This contributes to progression of diabetes mellitus.

**KEY WORDS:** Experimental diabetes mellitus; Deformed autoregulatory factor 1 (Deaf1); Autoimmune regulator (Aire); Foxp3; Treg.

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## INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a T-cell mediated autoimmune disease characterized by the destruction of pancreatic  $\beta$ -cells due to a breakdown in central and/or peripheral tolerance [1]. Tolerance mechanisms that operate in the thymus before the maturation and circulation of T cells are referred to as “central tolerance”. Thymic medullary epithelial cells (mTECs) ectopically express a range of peripheral tissue antigens (PTAs) under the transcriptional control of the autoimmune regulator, *Aire* [2]. However, not all antigens that T cells need to be tolerant to are expressed in the thymus, and thus central tolerance mechanisms alone are insufficient. This has led to a number of studies examining Aire expression and function at these extra-thymic sites [3, 4]. Peripheral tolerance can be mediated by extrathymic Aire-expressing cells (eTACs) in addition, lymph node stromal cells (LNSCs) have recently been shown to induce T-cell tolerance by ectopically expressing and presenting self-antigens in a manner comparable to thymic epitheliocytes [5, 6]. New evidence shows that all types of LNSCs—including fibroblastic reticular cells (FRCs), follicular DCs

(FDCs), and lymphatic endothelial cells (LECs) - express TSA [7]. Ectopic expression of genes encoding PTAs is not controlled by Aire in LNSCs but instead is regulated in part by the transcriptional regulator deformed autoregulatory factor 1 (Deaf1) [8].

Besides, peripheral self-tolerance and immune homeostasis are maintained, at least in part, by the balance between T regulatory cells (Tregs) and effector T cells [9]. Treg cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. Numerous studies have demonstrated the key role of FoxP3<sup>+</sup> Tregs in the development of T1DM [10]. Most studies of the role of Tregs in T1DM were performed on peripheral blood rather than pancreas or pancreatic lymph nodes. The autoimmune cascade that culminates in diabetes initiates within pancreatic lymph nodes (PLNs). At present all indications are that the PLN are essential in the initial activation of diabetogenic T-cells, prior to their islet migration [11]. The importance of the PLN in the development of diabetes was shown in two experimental settings. In the first setting, surgical excision from NOD mice resulted in the absence of diabetes without apparent

priming of T cells [12]. In the second setting, offspring of pregnant NOD mothers injected with lymphotoxin- $\beta$  receptor fused to human Ig Fc lacked lymph nodes and did not develop diabetes [13]. The level of autoreactivity was limited in these mice lacking PLN. In both situations, transfer of *activated* diabetogenic T-cells resulted in diabetes. LNSCs also have been suggested to mediate the conversion of autoreactive CD4<sup>+</sup> T cells to Tregs [14]. Yip L. (2015) showed that *Deaf1* controls the transcription of hundreds of genes in the pancreatic lymph nodes (PLNs) and regulates the processing and presentation of PTA genes in LNSCs [15]. Therefore, the aim of our study was to determine the effect of the levels of *Deaf1* and *Aire* mRNA expression on the nature of Foxp3<sup>+</sup> Treg cells differentiation during experimental STZ-induced diabetes mellitus (EDM) in rats PLN.

## METHODS AND MATERIAL

### Animals and experimental design

Six-month-old male Wistar rats were purchased from Veterinary Medicine Association Ltd. "Biomodelservis" (Kiev) and kept in a 12-h light/dark cycle with controlled humidity (60–80%) and temperature (22 $\pm$ 1 $^{\circ}$ C). All experiments on animals were performed according to international principles "of the European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 1986) and "General ethical principles of animal research" (Ukraine, 2001). Test animals were divided into 3 experimental groups: control rats, which were administered once intraperitoneally (i.p.) with 0.5 ml of 0.1 M citrate buffer (pH = 4.5) (group 1); rats with 21-day and 35-day STZ-induced diabetes mellitus (EDM) (group 2 and 3).

### Induction of experimental diabetes

Streptozotocin (STZ) (SIGMA Chemical, USA) was injected intraperitoneally at a dose of 50 mg/kg dissolved in 0.5 ml of 0.1 M citrate buffer (pH 4.5). The time elapsed since the date of introduction of the drug in the future presentation of the material has been interpreted as the duration of diabetes. Determination of glucose concentration in blood which was collected from the tail vein was performed by the glucose-oxidase method using the instrument "BIONIME Rightest<sup>TM</sup> GM 110" (Switzerland) in 12 hours on the 3d, 7th, 14th, 21st and 35th

days after injection of STZ. Measurement of blood glucose levels was performed after 6 hours from the last meal on 3d day after injection of STZ. For further studies animals with fasting glucose level > 8.0 mmol/l were selected.

### Fixation and deparaffinization

After removal of the PLN, the tissue was flushed with cold phosphate buffered saline and segments were placed into Bouin's solution. After fixation, the samples were dehydrated, incubated in xylene, embedded in paraffin, and sectioned using standard histological protocols. The ages of the fixed tissues analyzed ranged from 3 years. RNA was extracted from 15  $\mu$ m thick sections of Bouin's fixed tissues. For this study, paraffin-embedded tissue blocks were cut with a disposable microtome blade into 15 $\mu$ m sections and placed in Eppendorf tubes. Tissues were deparaffinized by incubation in two consecutive baths of xylenes for 5 minutes each, then in two consecutive baths of 100% ethanol for 5 minutes each. After deparaffinization and centrifugation, the pellets were air-dried.

### Analysis of mRNA by real-time RT-PCR

Total RNA was extracted from PLN tissue by Trizol RNA Prep 100 (Isogen, Russia), according to the manufacturer's instructions. RNA was resuspended in RNase free water, quantified and subjected to RT-PCR reaction using RT-PCR kit, RT-1 (Syntol, Russia). RT-PCR was performed on the final volume of 25  $\mu$ l containing 10  $\mu$ l ready 2,5X reaction mixture, 11  $\mu$ l of ddH<sub>2</sub>O, 1  $\mu$ l of Random-6 primers, 1  $\mu$ l of reverse transcriptase and 2  $\mu$ g of RNA. Reverse transcription was performed at 45 $^{\circ}$ C for 45 minutes, followed by heating for 5 min at 92 $^{\circ}$ C. For real-time RT-PCR with gene-specific primers, we using an CFX96<sup>TM</sup> Real-Time PCR Detection Systems (Bio-Rad Laboratories Inc., USA) according to the manufacturer's recommendations, with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, USA) for detection. The Master Mix includes Maxima Hot Start Taq DNA Polymerase and dNTPs in an optimized PCR buffer. Samples were amplified in a volume of 25 $\mu$ l reaction mix, with a concentration of 0.3 $\mu$ M of forward and reverse primer, 12.5  $\mu$ l of Maxima SYBR Green/ROX qPCR Master Mix (2X), Template DNA  $\leq$ 500 ng/reaction, nuclease-free water to 25  $\mu$ l. All primers were designed by using Primer-BLAST design software (NIH, USA) and were synthesized by Metabion (Germany). The primers that we used are listed in Table 1.

**Table 1.** The design of primers.

Gene	Primer	Tm, $^{\circ}$ C	Product length (bp)	Exon junction
<i>Deaf1</i>	F = GCAGAGAGGAAGGAGCAGTC	60	59	1605/
	R = GTGCACTCACTCATGGCCT	60		1606
<i>Aire</i>	F = GCCTAAAGCCAGTGATCCGA	60	43	850/
	R = TCTCTACCCTGGGTTCCCTTT	60		851
<i>GAPDH</i>	F = GCCTGGAGAAACCTGCCAAG	61	52	825/
	R = GCCTGCTTCACCACCTTCT	60		826

Deaf1, deformed autoregulatory factor 1; Aire, autoimmune regulator; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

All PCR was performed using the following parameters. After initial denaturation for 10 min at 95°C, 50 cycles of sequential steps denaturation was performed at 95°C for 15 s, annealing at 60 to 61°C for 1 min, extension at 72°C for 30 s, followed by a final incubation at 72°C for 7 minutes. Each sample was tested in triplicate, and results were normalized using amplification of the same cDNAs with reference genes GAPDH using  $\Delta\Delta C_t$  calculations. Real-time PCR analysis of Deaf1 was expressed as the relative normalized expression of the indicated mRNA.

### Immunohistochemical staining

Population structure of FoxP3<sup>+</sup> Treg-cells was studied on the basis of analysis of serial histological sections and their data of morphometric and densitometric characteristics. For this study a rotary microtome MICROM HR-360 (Microm, Germany) did 5 micron serial sections of PLN. They were deparaffinized in xylene, rehydrated in a descending carried concentrations of ethanol (100%, 96%, 70%), washed in 0.1M phosphate buffer (pH = 7.4) and stained with a rabbit polyclonal primary antibodies (PABs) to the transcription factor FoxP3 (Santa Cruz Biotechnology, USA) for 18 hours in a humid chamber at  $t = 4^\circ\text{C}$ . After washing of the excess primary antibody in a 0.1 M phosphate buffer, sections were incubated for 60 minutes ( $t = 37^\circ\text{C}$ ) with a secondary antibody molecule to the total rabbit IgG (Santa Cruz Biotechnolog, USA), conjugated with FITC. After incubation, sections were washed with 0.1 M phosphate buffer and embedded in a mixture of glycerol and a phosphate buffer (9:1) for the subsequent fluorescence microscopy. Treated histological sections were studied using computer software ImageJ (NIH, USA). Images which were obtained on the microscope PrimoStar (ZEISS, Germany) in the ultraviolet spectrum of excitation 390 nm (FITC) with using a highly sensitive camera AxioCam 5c (ZEISS, Germany) and the software package for receiving, archiving, and preparing images for publication AxioVision 4.7.2 (ZEISS, Germany) were

immediately entered into the computer. In the automatic mode, areas with the statistically significant fluorescence characteristics of cells that express FoxP3 were identified. Morphometric and densitometric characteristics of immunopositive cells were determined. When painting the PABs FoxP3<sup>+</sup>-cells in the paracortical zone and medullary cords of PLN was examined.

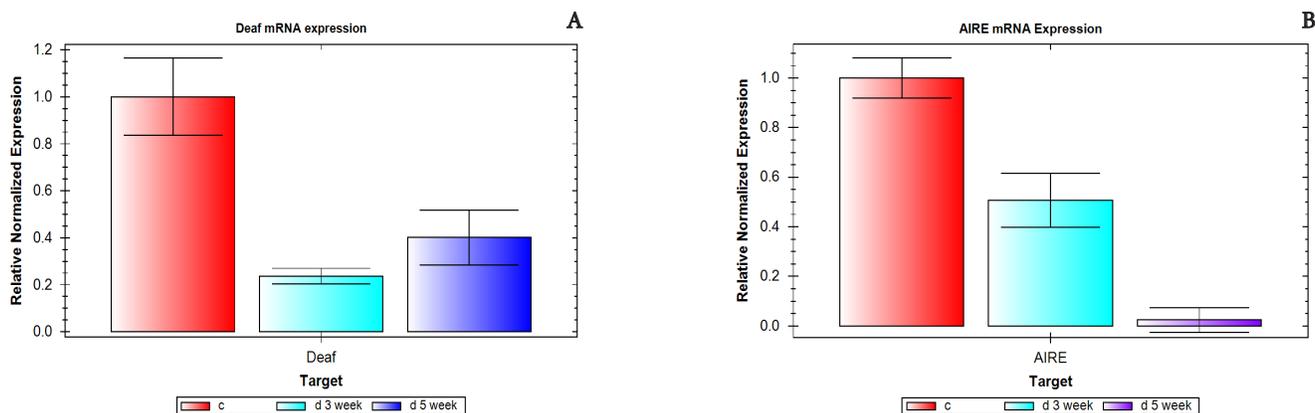
### Statistical analysis

All the experimental data were processed on a personal computer with using application package statistical programs EXCEL of the MS Office 2010 (Microsoft Corp., USA), STATISTICA 6.0 (Stat-Soft, 2001). For all indicators the arithmetic mean value of the sample ( $m$ ), its variance and the standard error of the mean (SEM) were calculated. To identify the significance of differences in the results of studies of experimental and control groups of animals Student's coefficient ( $t$ ) was determined, after which the sample was determined by the possibility of difference ( $p$ ) and the average confidence interval. Critical significance level when testing statistical hypotheses assumed was equal to 0.05.

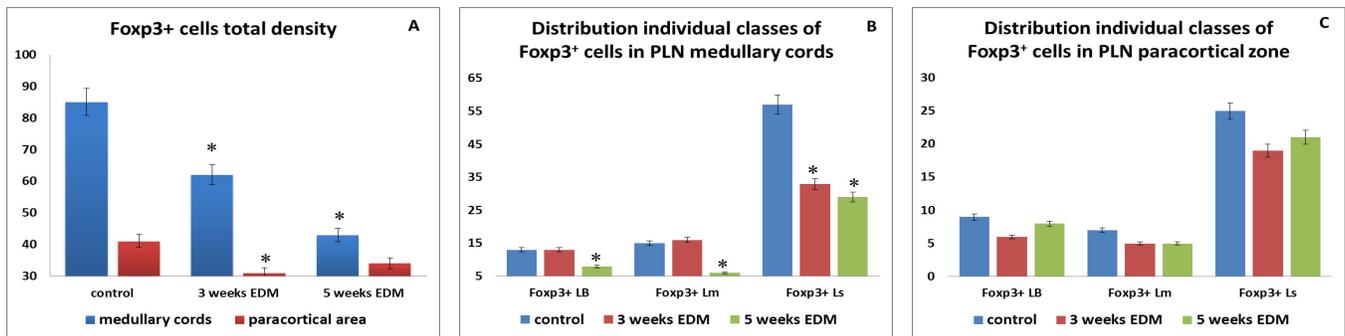
### RESULTS

Administration of streptozotocin to the experimental animals caused development of the EDM: concentration of blood glucose level increased 3,6-fold at the end of the 3d week ( $12,23 \pm 0,4$  mmol/L,  $p < 0,05$ ) compared with the control group ( $3,37 \pm 0,08$  mmol/L). Concentration of blood glucose level reached ( $14,39 \pm 0,7$  mmol/L) up to the 5<sup>th</sup> week. Dipsesis, hyperphagia and polyuria have been observed, which constituted all the main symptoms of insulin-dependent diabetes mellitus.

Firstly, we determined that the expression levels of the transcriptional regulators Deaf1 and Aire are decreased in PLN samples of rats with EDM. Deaf1 expression is decreased 4.2-fold ( $P < 0.05$ ) in rats PLN with 3-week EDM and 2.5-fold ( $P < 0.05$ ) in rats with 5-week EDM compared to control group ( $P < 0.05$ ) (Fig. 1A and 1B). Aire expression is decreased 2-fold ( $P < 0.05$ ) in rats PLN with 3-week EDM and 50-fold ( $P < 0.05$ ) in rats with 5-week EDM (Fig. 1B).



**Fig. 1.** Relative Deaf1 (A) and Aire (B) mRNA expression during EDM development. Target transcription factor were normalized to GAPDH. Data expressed as means  $\pm$  SEM, \*  $P < 0.05$ , vs. control



**Fig. 2.** The total number (on 1 mm<sup>2</sup>) of Foxp3<sup>+</sup> cells (A) and distributions Treg individual classes in medullary cords (B) and paracortical area (C) of PLN. Note: \* P<0.05, vs. control. LB: Lymphoblast's, LM: Medium Lymphocytes and LS: Small Lymphocytes.

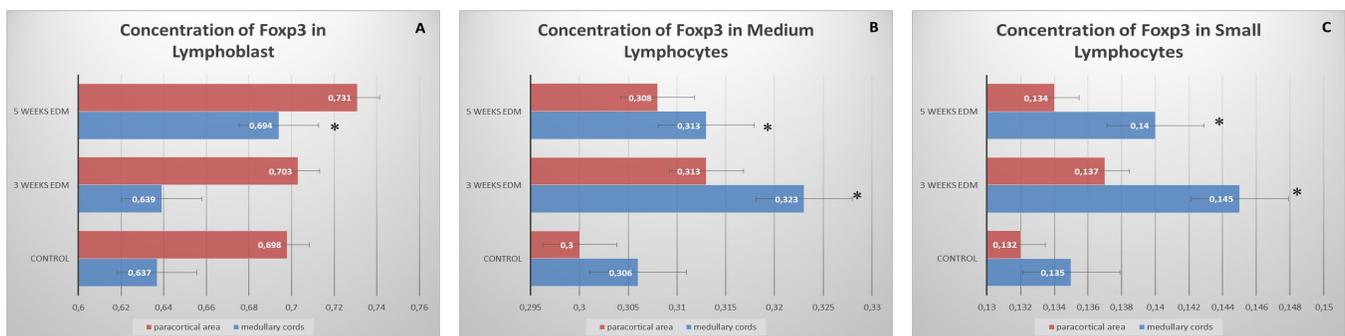
Development of experimental STZ-induced diabetes mellitus (3-week EDM) led to a change in representation of FoxP3<sup>+</sup>-lymphocytes in paracortical zone and medullary cords of pancreatic lymph nodes (PLN), in which the total density decreased by 25% (p < 0,05) and 28% (p < 0,05) as compared to control group (Fig.2 A). Indicators in the group of rats with 5-week EDM decreased by 50% (p <0,05) only in medullary cords of PLN. The distribution into individual classes of FoxP3<sup>+</sup> lymphocytes in the PLN group of experimental animals with a 3-week EDM showed an increase in medullary cords PLN percentage of FoxP3<sup>+</sup>-lymphoblasts by 40% (p <0,05), FoxP3<sup>+</sup>-medium lymphocytes by 42% (p <0,05), respectively, the percentage of FoxP3<sup>+</sup>-small lymphocytes decreased by 21% (p <0,05), and their population density by 42% (p <0,05), respectively (Fig.2 B, C).

Measurement of the fluorescence intensity of FoxP3<sup>+</sup>-cells showed a significant increase in these parameters in medullary cords PLN as at 3-week EDM and at 5-week EDM, namely FoxP3<sup>+</sup>-medium and small lymphocytes. At 5-week EDM FoxP3<sup>+</sup>-lymphoblasts concentration of transcription factor FoxP3 by 10% (p <0,05) increased (Fig.3 A-C).

## DISCUSSION

Research into how self-reactive T cells are tolerized in lymph nodes has focused largely on dendritic cells (DCs) [16]. Today we know that LNSCs are important mediators of deletional tolerance to PTAs, which are constitutively

expressed and presented by LNSCs [17, 18]. Recently a role was described for Deaf1 in regulating PTAs expression in the LNSCs of PLN of NOD mice [19]. To more directly test the role of Deaf1 in TSAs expression, Yip et al. (2009) analyzed by microarray whole PLN from wild-type and Deaf1-deficient mice. This analysis indicated that Deaf1 regulates the expression of a host of genes including a significant number of TSAs in the PLN. Particularly, 22 of the 30 most highly Deaf1-1-induced genes are tissue-specific, and the set of Deaf1-regulated TSAs identified by microarray shares some overlap with AIRE-regulated genes in the thymus, though Aire transcription itself is unchanged in Deaf1-deficient LN. On the other hand, the set of Deaf1-regulated genes in the PLN is almost completely different from the set of genes regulated by AIRE. As well as, knockdown of Deaf1 in vitro caused reduced expression of candidate TSAs. Yip L. et al. (2014) found that the expression of the gene encoding the transcriptional regulator Deaf1 changed in parallel with the expression of genes encoding a number of islet-specific tissue antigens including insulin 1 (*Ins1*), insulin 2 (*Ins2*), glucagon (*Gcg*) and other in the PLN [20]. The expression of *Deaf1* and genes encoding these islet-specific antigens was significantly downregulated in the PLN of NOD mice at the age of 12 weeks, a time coincident with the onset of destructive insulinitis. In humans, *INS* was not detected in the PLN of T1D patients, but was expressed in the PLN of healthy individuals and spleens of both control and T1D samples. In 12-week old NOD PLN, *Ins2* gene expression was also reduced, but no difference in *Ins2*



**Fig. 3.** Concentration of transcription factor FoxP3 (fluorescence intensity in arbitrary units, AU) in FoxP3<sup>+</sup>- Lymphoblast's (A), FoxP3<sup>+</sup>- Medium Lymphocytes (B) and FoxP3<sup>+</sup>- Small Lymphocytes (C). Note: \* P<0.05, vs. control.

mRNA expression in the PLN of *Deaf1*-KO mice compared to BALB/c control mice was noticed [21].

On the other hand, functional defects in T regulatory cells are key features of the pancreatic lymph nodes in patients with type 1 diabetes and experimental animals [22-25]. Ferraro A. et al. (2011) phenotypically and functionally characterized Tregs and Th17 cells residing in the pancreatic-draining lymph nodes (PLNs) of 19 patients with type 1 diabetes [22]. Authors have

shown that the main features of the PLN of diabetic subjects are unbalanced Treg/Th17 cell ratio and reduced FoxP3 expression in Tregs.

Data concerning *Deaf1* influence on the generation of Tregs are negligible in the current literature. Most available literature deals with the influence of another expression regulator PTAs – Aire. Yang S. et al. (2015) report that Aire promotes the perinatal generation of a distinct compartment of FoxP3<sup>+</sup>Treg cells, which stably persists in adult mice [26]. Extrathymic Aire-expressing cells (eTACs) are a distinct bone marrow-derived population that induce functional inactivation of CD4<sup>+</sup> T cells. Gardner J. et al. (2013) demonstrated that eTACs can functionally inactivate CD4<sup>+</sup> T cells through a mechanism that does not require regulatory T cells (Treg) and is resistant to innate inflammatory stimuli [4,27]. Aire upregulates Foxp3 mRNA expression in splenocytes and increases CD4<sup>+</sup>FoxP3<sup>+</sup> T cell production [28].

We only can guess about role for LNSCs in Tregs differentiation. So, LNSCs in mesenteric lymph nodes produce retinoic acid, which promotes the development of Tregs [29]. Supposedly, there are several mechanisms by which LNSCs can cooperate to induce CD4 T-cell tolerance in LN: LNSCs transcriptionally express a variety of PTA, which are directly presented to T-cells [30]; LNSCs transfer the PTA to DC, which induce CD4 T-cell anergy [31]; LNSCs can acquire peptide/MHC II complexes generated by DC, which are then presented by the LNSCs, leading to CD4 T-cell apoptosis; LNSCs can cross-present soluble antigens from the lymph, leading to CD8 activation and increased apoptosis [5].

## CONCLUSION

1. Development of diabetes was accompanied by decreased the expression levels of the transcriptional regulator *Deaf1* 4,2-fold in rats PLN with 3-week EDM and 2,5-fold in rats with 5-week EDM. Aire expression is decreased 2-fold in rats PLN with 3-week EDM and 50-fold in rats with 5-week EDM.
2. Reduced *Deaf1* and Aire mRNA expression during EDM associated with an decreased of total amount of Treg in the PLN, led to changes of distribution into individual classes of FoxP3<sup>+</sup> lymphocytes and FoxP3 concentration in immunopositive cells.

## CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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