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Reduced deaf1 mRNA expression during STZ-induced diabetes mellitus inhibits foxp3$^+$regulatory T-cells differentiations in rat's pancreatic lymph nodes

Alex M. Kamyshnyi [1,*], Denis A. Putilin [2] and Vita A. Kamyshna [3]

[1] Zaporozhye State Medical University, department of microbiology, virology and immunology, Mayakovskiy str., 26, Zaporozhye, Ukraine
[2] Zaporozhye State Medical University, department of normal physiology, Mayakovskiy str., 26, Zaporozhye, Ukraine
[3] Zaporozhye State Medical University, department of normal anatomy, Mayakovskiy str., 26, Zaporozhye, Ukraine

Abstract: We studied to determine the effect of the levels of Deaf1 mRNA expression on the nature of Foxp3$^+$ Treg cells differentiation during experimental STZ-induced diabetes mellitus (EDM) in rats PLN. To determine the level of Deaf1 mRNA expression was performed RT-PCR in real-time by thermocycler CFX96™ Real-Time PCR Detection Systems. The Foxp3$^+$-immunopositive lymphocytes were determined using an indirect immunofluorescence technique with using a monoclonal rat antibody. We established that development of EDM 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. Reduced Deaf1 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$^+$ lymphocytes and FoxP3 concentration in immunopositive cells.

Key words: experimental diabetes mellitus; deformed autoregulatory factor 1 (Deaf1); Foxp3; Treg.

Introduction

Type 1 diabetes mellitus (T1DM) is a T-cell mediated autoimmune disease characterized by the destruction of pancreatic β-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$^+$ 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,

**Corresponding author: Alex M. Kamyshnyi*
Email address: alexkamyshny@yandex.ru
DOI: http://dx.doi.org/

offspring of pregnant NOD mothers injected with lymphotoxin-β 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 $CD4^+$ 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 mRNA expression on the nature of $Foxp3^+$ Treg cells differentiation during experimental STZ-induced diabetes mellitus (EDM) in rats PLN.

Materials and methods

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°±1°C). All experiments on animals were performed according to the 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: groups: (group 1, n=12); control rats administered once intraperitoneally (i.p.) with 0.5 ml of 0.1 M citrate buffer (pH = 4.5), (group 2, n=12) rats with 21-day and (group 3, n=12) with 35-day STZ-induced diabetes mellitus (EDM).

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 material has been interpreted as the duration of diabetes. Determination of glucose concentration in blood collected from the tail vein was performed by the glucose-oxidase method using the instrument "BIONIME RightestTM GM 110" (Switzerland) in 12 hours on the 3rd, 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 3rd 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 μm thick sections of Bouin's fixed tissues. For this study, paraffin-embedded tissue blocks were cut with a disposable microtome blade into 15μm 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 a final volume of 25 μl containing 10 μl ready 2,5X reaction mixture, 11 μl of ddH_2O, 1 μl of Random-6 primers, 1 μl of reverse transcriptase and 2 μg of RNA. Reverse transcription was performed at 45°C for 45 minutes, followed by a heating step for 5 min at 92°C. For real-time RT-PCR with gene-specific primers, we used an CFX96™ 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 25μl reaction mix, with a concentration of 0.3μM of forward and reverse primer, 12.5 μl of Maxima SYBR Green/ROX qPCR Master Mix (2X), Template DNA ≤500 ng/reaction, nuclease-free water to 25 μl. 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,°C	Product length (bp)	Exon junction
Deaf1	F = GCAGAGAGGAAGGAGCAGTC R = GTGCACTCACTCATGGCCT	60 60	59	1605/ 1606
GAPDH	F = GCCTGGAGAAACCTGCCAAG R = GCCTGCTTCACCACCTTCT	61 60	52	825/ 826

Deaf1, deformed autoregulatory factor 1; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

All PCRs were 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 ΔΔCt calculations. Real-time PCR analysis of Deaf1 was expressed as the relative normalized expression of the indicated mRNA.

Immunohistochemical staining.

Population structure of FoxP3$^+$ 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°C. After washing of the excess primary antibody in a 0.1 M phosphate buffer, sections were incubated for 60 minutes (t = 37°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 were obtained on the microscope PrimoStar (ZEISS, Germany) in the ultraviolet spectrum of excitation 390 nm (FITC) 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). 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$^+$-cells in the paracortical zone and medullary cords of PLN was examined.

Statistical analysis.

All the experimental data were processed on a personal computer 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 and discussion

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± 0,4 mmol/L, p <0,05) compared with the control group (3,37±0,08 mmol/L). Concentration of blood glucose level reached (14,39±0,7 mmol/L) up to the 5th week. Dipsesis, hyperphagia and polyuria have been observed, which constituted all the main symptoms of insulin-dependent diabetes mellitus.

Firstly, we noticed that the expression levels of the transcriptional regulator Deaf1 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. 1).

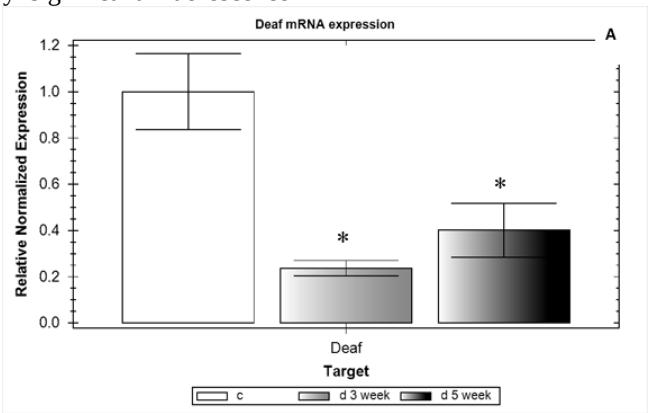

Figure 1. Relative Deaf1 mRNA expression during EDM development. Target transcription factor were normalized to GAPDH. Data expressed as means ± SEM, * P<0.05, vs. control

Development of experimental STZ-induced diabetes mellitus (3-week EDM) led to a change in representation of FoxP3$^+$-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, Fig.3 A-D). 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$^+$ lymphocytes in the PLN group of experimental animals with a 3-week EDM showed an increase in medullary cords PLN percentage of FoxP3$^+$-lymphoblasts by 40% ($p < 0,05$), FoxP3$^+$-medium lymphocytes by 42% ($p < 0,05$), respectively, the percentage of FoxP3$^+$-small lymphocytes decreased by 21% ($p < 0,05$), and their population density by 42% ($p < 0,05$), respectively (Fig.2 B, C).

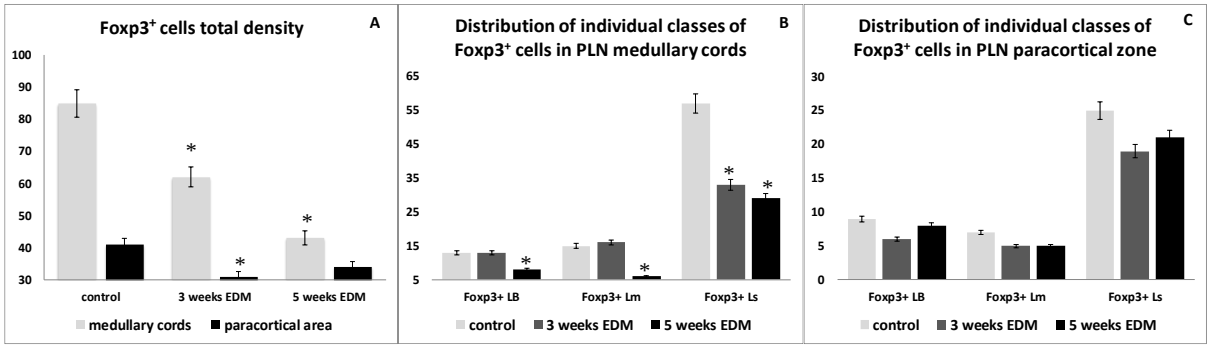

Figure 2. The total number (on 1 mm^2) of Foxp3$^+$ cells (A) and distributions Treg individual classes in medullary cords (B) and paracortical area (C) of PLN. Note: * P<0.05, vs. control. LB: Lymphoblast's, LM: Medium Lymphocytes and LS: Small Lymphocytes

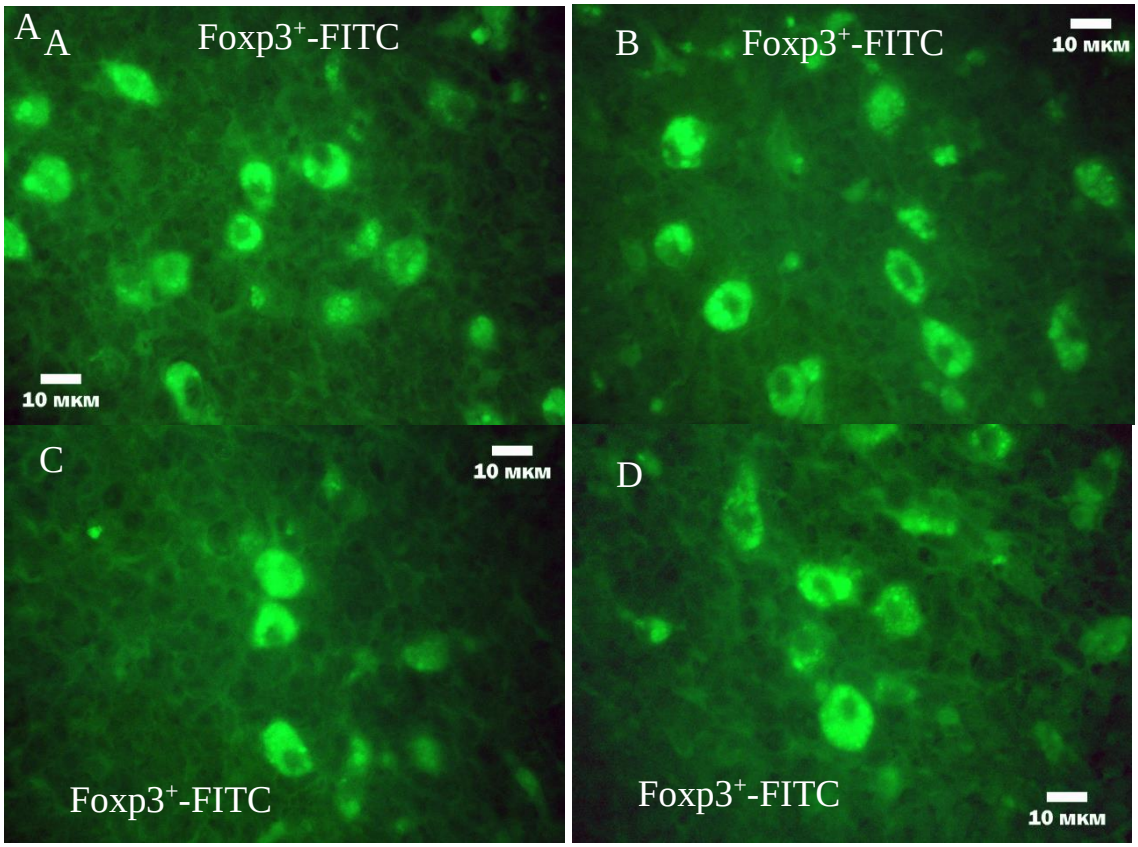

Figure 3. FoxP3$^+$cells in paracortical area of PLN. Indirect fluorescence reaction with a rabbit polyclonal primary antibodies to the transcription factor FoxP3 and secondary antibody molecule to the total rabbit IgG, conjugated with FITC. Control (A-B), 3-week EDM (C), 5-week EDM (D).

Measurement of the fluorescence intensity of FoxP3$^+$cells showed a significant increase in these parameters in medullary cords PLN as at 3-week EDM and at 5-week EDM, namely FoxP3$^+$medium

and small lymphocytes. At 5-week EDM FoxP3$^+$-lymphoblasts concentration of transcription factor FoxP3 increased by 10% (p <0,05) (Fig.4 A-C).

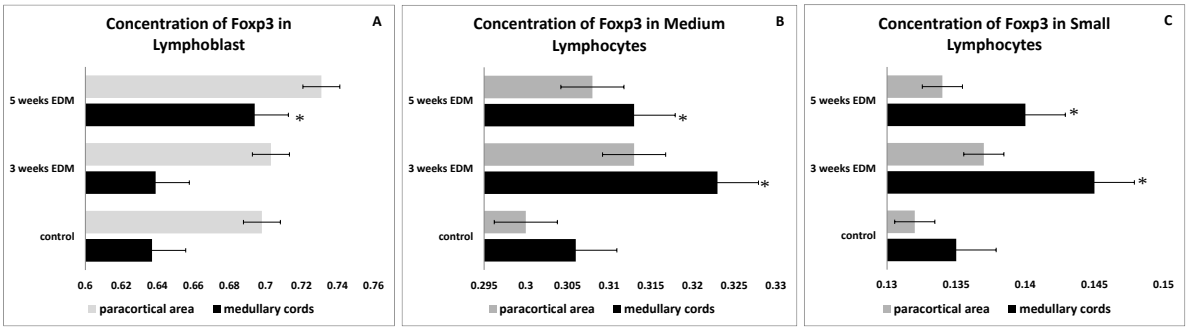

Figure 4. Concentration of transcription factor FoxP3(fluorescence intensity in arbitrary units, AU) in FoxP3$^+$- Lymphoblast's (A), FoxP3$^+$- Medium Lymphocytes (B) and FoxP3$^+$- Small Lymphocytes (C). Note: * P<0.05, vs. control.

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 Deaf-1-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 Deaf-1-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 insulitis. 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* 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$^+$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$^+$ T cells. Gardner J. et al. (2013) demonstrated that eTACs can functionally inactivate CD4$^+$ 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$^+$FoxP3$^+$ 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 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.
2. Reduced Deaf1 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 $FoxP3^+$ lymphocytes and FoxP3 concentration in immuno-positive 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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