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Full Length Research Paper

Increased level of type I Interferon (IFN) during type I diabetes (T1D) induces apoptosis in spleen-homing T cells

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Type I diabetes (TID) is an autoimmune disease characterized by abnormalities in the defense mechanisms against a variety of infectious agents. Susceptibility to infections occurring in diabetic individuals is attributed to the decrease in the number of lymphocytes, which is probably a clinical consequence of the occurrence of apoptosis described in diabetes. TID is associated with increased cytokines that dampen lymphocytes proliferation, functions and subsequently increase risk to infection. Previous studies have reported an increase in IFN-α level which is associated with TID pathogenesis. In the present study, we further investigated the effect of blocking type I IFN receptor signaling pathway on the lymphocyte proliferation and functions within spleen as a secondary lymphoid organ in a streptozotocin (STZ)-induced type I diabetic mouse model. Three groups of mice were used (10 mice in each group): group 1, control non-diabetic mice; group 2, diabetic mice; and group 3, diabetic mice intraperitoneal injected with anti-IFNAR1 (10 mg/kg body weight once/day for up to 20 days). We found that diabetic mice exhibited increase in the apoptosis, DNA fragmentation, chromatin condensation and cell shrinkage; prolonged elevation in IFN-α and TNF-α levels and obvious reduction in spleen-homing T lymphocytes as compared to control mice. Interestingly, blocking type I IFN receptor of diabetic mice significantly decreased (P< 0.05) apoptotic changes of the diabetic lymphocyte, significantly restored the distribution and numbers of T lymphocytes in the spleen and also decreased the level of IFN-α and TNF-α as compared to diabetic non treated mice. Our data revealed the correlation between the elevated levels of IFN-α during TID and the perturbation in lymphocyte architecture and distribution within lymphoid organs.

Key words: Diabetes mellitus, IFN-α, apoptosis, lymphocytes, spleen.

INTRODUCTION

Type I diabetes (T1D) is a chronic autoimmune disease caused by the specific destruction of pancreatic β-cells, which produce insulin (Maahs and Rewers, 2006).

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Abbreviations: TID, Type I diabetes; **IFNα, i**nterferon alpha; **TNFα,** tumor necrosis factor alpha.

Multiple complications are usually associated with diabetes mellitus (Thompson, 2008). These complications include increased susceptibility to infections occurring in a poorly controlled diabetic state (Kraine and Tisch, 1999). The incidence of a recognized group of rare infections is definitely high in diabetes mellitus or confined almost entirely to diabetic patients (Larkin et al., 1985). Moreover, infectious diseases, particularly tuberculosis, were a major cause of death among diabetic patients before the advent of insulin therapy (Eliopoulos et al., 1995). Critical evaluations suggest that infections in

general are more difficult to eliminate in the diabetic host (Garcia-Leme and Farsky, 1993).

It has been observed that increased susceptibility to frequent and prolonged infections during T1D are caused by the decrease in the number of lymphocytes which is probably a clinical consequence of the occurrence of apoptosis while a profound T lymphopenia proceeds to T1D (Jackson et al., 1981; Yale et al., 1985). In addition, peripheral T lymphocytes are not only reduced in number but also functionally impaired (Elder and Maclaren, 1992; Jung et al., 1999; Otton et al., 2002). Increased apoptosis in peripheral blood smear lymphocytes was found to be associated with diabetes (Shidham and Swami, 2000). Moreover, apoptotic cells often shrink and undergo cytoplasmic membrane blebbing, and their chromosomes rapidly condense and aggregate around the nuclear periphery, forming small apoptotic bodies (Wyllie, 1980; Vermes et al., 2000; Eizirik and Mandrup-Poulsen, 2001).

IFN-α, a group of pleiotropic cytokines in the type I family of IFNs have a dual role in the control of apoptosis, as they may both induce and block apoptosis (Ruuth et al., 2001). In particular, IFN-α exerts broad but distinct effects on innate and adaptive immune responses by signaling through a heterodimeric receptor composed of IFN-α receptor 1 (IFNAR1) and IFNAR2 (Bach et al., 1997; Heim, 1999). Many studies have suggested that IFNα is involved in the development of T1D. For example; higher levels of IFNα mRNA and protein were detected in the pancreata of T1D patients than in pancreata of non-diabetic patients (Huang et al., 1995; Foulis et al., 1987). IFNα treatment of patients with tumors or viral hepatitis is associated with an increased incidence of T1D (Guerci et al., 1994; Fabris et al., 2003). Over-expression of IFNα on β cells induced T1D in non-autoimmune-prone C57BL/6 mice (Stewart et al., 1993). Additionally, IFN regulatory factor 1-deficient NOD mice failed to develop insulitis and diabetes (Nakazawa et al., 2001). Nevertheless, Type I IFNs have a dual role in the control of apoptosis, as they may both induce and block apoptosis (Ruuth et al., 2001).

The aim of this study was to investigate the potential apoptotic effects of the alteration in signaling pathway of Type I IFNs on lymphocytes within spleen as secondary lymphoid organ using a diabetic mice model.

MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ) was obtained from Sigma Chemicals Co., St. Louis, MO, USA. The STZ was dissolved in cold 0.01 M citrate buffer (pH 4.50) and was always freshly prepared for immediate use (within 5 min).

Animals and experimental design

A total of 30 sexually mature 6-8week-old male Swiss Webster (SW) mice weighing 25 to 30 g each were obtained from the Central Animal House of the Faculty of Medicine, Assiut University. All animal procedures were conducted in accordance with the standards set forth in the guidelines for the Care and Use of Experimental Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH). The study protocol was approved by the Animal Ethics Committee of the Histology Department, Faculty of Medicine, Assiut University, according to the Helsinki principles. All animals were allowed to acclimatize to the metal cages inside a well-ventilated room for 2 weeks prior to experimentation.

Animals were maintained under standard laboratory conditions (temperature 23°C, relative humidity 60 - 70% and a 12-h light/dark cycle), fed a diet of standard commercial pellets and given water *ad libitum*. All mice were fasted for 20 h before diabetes induction. Mice (n = 20) were rendered diabetic with an intraperitoneal injection (i.p.) of a single dose of STZ (60 mg/kg body weight) in 0.01 M citrate buffer (pH 4.5) (Badr et. at., 2011). Mice in the control group (n = 10) were injected with the vehicle alone (0.01 M citrate buffer, pH 4.5). The animals were divided into three experimental groups: group 1, control non-diabetic mice; group 2, diabetic mice and group 3, diabetic mice with an intraperitoneal injection with anti-IFNAR1 at a dose of (10 mg/kg body weight once/day for up to 20 days) (Badr et al., 2010).

Blood analysis

Blood glucose levels were determined using the AccuTrend sensor (Roche Biochemicals, Mannheim, Germany). Serum insulin level was analyzed by Luminex (Biotrend, Düsseldorf, Germany) according to the manufacturer's instructions. The plasma levels of IFN-α and TNF-α were measured using commercially available ELISA kits (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemical staining for CD3 T cells was performed as described previously (Kashino et al., 2010). In brief, paraffin sections of spleen were prepared from control mice, diabetic mice and diabetic mice injected with mAb antagonist according to NIH protocol to block type I IFN receptor signaling. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized, subjected to microwave antigen retrieval and incubated using a 1/100 dilution for 1 h of anti-mouse CD3 primary antibody (ab5690; Abcam, Cambridge, MA). After washing in phosphate buffered saline (PBS), the slides were incubated with biotinylated secondary antibody, followed by avidin-biotinylated horseradish peroxidase complex (ABC) and diaminobenzidine (DAB) according to the Vectastain protocol (Vector Labs). Slides were counterstained with hematoxylin solution (DakoCytomation), dehydrated, cleared and mounted in synthetic resin (Poly-Mount; Poly Scientific).

Electron microscopic study

For the electron microscopic examination, small pieces (1×1 mm) of spleen of anti IFNα-treated diabetic ,diabetic and control mice were quickly removed, and fixed in 5% cold buffer glutaraldehyde for one week. The specimens were then washed in phosphate buffer (pH 7.2) in four changes of 15 min each with slow shaking and post fixed in 1% osmium tetroxide for 2 h. They were washed again with phosphate buffer and then dehydrated using ascending grades of 50% alcohol for 30 min, at 70% overnight, at 95% for 30 min and finally at 100%, 3 changes of 30 min each. Samples were embedded in propylene oxide for 30 min to remove remnants of

Table 1. Levels of TNF-α and IFN-α was altered during diabetes.

Parameter	Control	Diabetes	Diabetes + anti-IFNα
IFN-α (ng/ml)	1.722 ± 0.21	3.858 ± 0.586*	2.284 ± 0.264+#
TNF-α (ng/ml)	18.74 ± 1.673	38.66 ± 5.268*	29.3 ± 3.704+#
Glucose (mg/dl)	135.6 ± 12.466	433.4 ± 64.599*	260.4 ± 5.089+#
Insulin (ng/ml)	5.14 ± 0.44	1.3 ± 0.09*	2.7 ± 0.19+#

Blood levels of glucose, insulin, IFN-α and TNF-α were monitored in the 3 groups of mice. Accumulated data from 5 separate individual mice from each group are shown. Data were first tested for normality and variances homogeneity prior to any further statistical analysis. Data were normally distributed and are expressed as the mean ± SEM. *P< 0.05, diabetic vs. control; +P< 0.05, diabetic + anti IFN-α vs. control; #P< 0.05, diabetic + anti IFN-α vs. diabetic (ANOVA with Tukey's post-test).

alcohol. Afterward, samples were embedded in propylene oxide plus Epon 812 (1:1, v/v) for 30 min and finally they were embedded in Epon 812 for 4 h. Samples were finally embedded into capsules containing the embedding mixture and the tissue blocks were polymerized in an oven for 2 days at 60°C. Semi thin sections of 0.5 µM thickness were prepared using LKB ultra microtome and then stained with toluidine blue. Semi thin sections were examined for localization of the desired tissues and accordingly, ultrathin sections were prepared. Sections were stained with uranyl acetate and lead citrate and examined under transmission electron microscope (Jeol, 100 CXII) operated at 80 KV, Electron Microscopic Center, Assiut University. Electron micrographs were taken for selected semi thin regions, reconstructed and processed using Photoshop computer program to study spleen of each group. The results were presented as micrographs and discussed in view of a valuable literature (Gupta, 1983).

Statistical analysis

Data were first tested for normality (using Anderson-Darling test) and for variances homogeneity prior to any further statistical analysis. Data were normally distributed and were expressed as the mean ± SEM (standard error of the mean). Statistical differences between groups were analyzed using a one-way analysis of variance (for more than two groups) followed by Tukey's post-test using SPSS software, version 17. Data are expressed as mean ± SEM. Differences were considered statistically significant at *P< 0.05, diabetic vs. control; +P< 0.05, diabetic +anti-IFNα vs. control; #P< 0.05, diabetic + anti-IFNα vs. diabetic.

RESULTS

Treatment of diabetic mice with type I IFN receptor blocking antibody partially restored the levels of IFN-α, TNF-α, glucose and insulin

To optimize all the parameters and conditions of the animal models during the experiments, blood glucose, insulin, TNF-α and IFN-α levels were monitored in the 3 groups of mice throughout the experiment period. We found that the diabetic mice exhibited an obvious and significant (P< 0.05) increase in the levels of glucose, IFN-α and TNF-α as compared to control non diabetic mice. Moreover, these mice exhibited a marked decrease in the blood insulin level as compared to control non-diabetic mice. Interestingly, we observed that blocking type I IFN receptor in diabetic mice partially and significantly (P< 0.05) restored the altered levels of blood glucose, insulin, IFN-α and TNF-α as compared to diabetic non-treated mice (Table 1).

Blocking type I IFN receptor signaling during TID restored the number and distribution of T cells in the spleen.

Since T cells play important and central roles in the cellular immune response, the numbers of CD3-positive T cells in spleen of control, diabetic and anti IFNα-treated mice were estimated using immunohistochemical analysis. The number of T cells in the spleen of control mice was higher in the periarterial lymphatic sheath and moderate numbers were found in the marginal zone of white pulp as well as in lymph follicles (Figure 1A). In contrast, T cells number was significantly decreased in the spleen of diabetic mice (Figure 1B). Nevertheless, in diabetic mice that were intravenously injected with anti-IFNAR antagonist mAb to block the signaling pathways of type I IFN, a significant increase (P< 0.05) in the number of T cells was present in the marginal zone and the periarterial lymphatic sheath (Figure 1C).

Increased level of IFN-α during diabetes induced exhaustion and apoptosis of spleen homing lymphocytes

Electron microscopy examination of ultra sections of spleen from control non-diabetic mice demonstrated that mature and immature granulocytes, as well as lymphocytes were in a healthy state (Figure 2A). In contrast, ultra section in spleen of diabetic mice demonstrated several apoptotic changes, including lymphocytes with irregular heterochromatic nucleus, ill distinct nuclear envelope and vacuolated cytoplasm with destructed mitochondria. Moreover, plasma cell exhibited shrinkage and pyknotic nucleus with condensed chromatin, while other lymphocytes were observed with nearly total

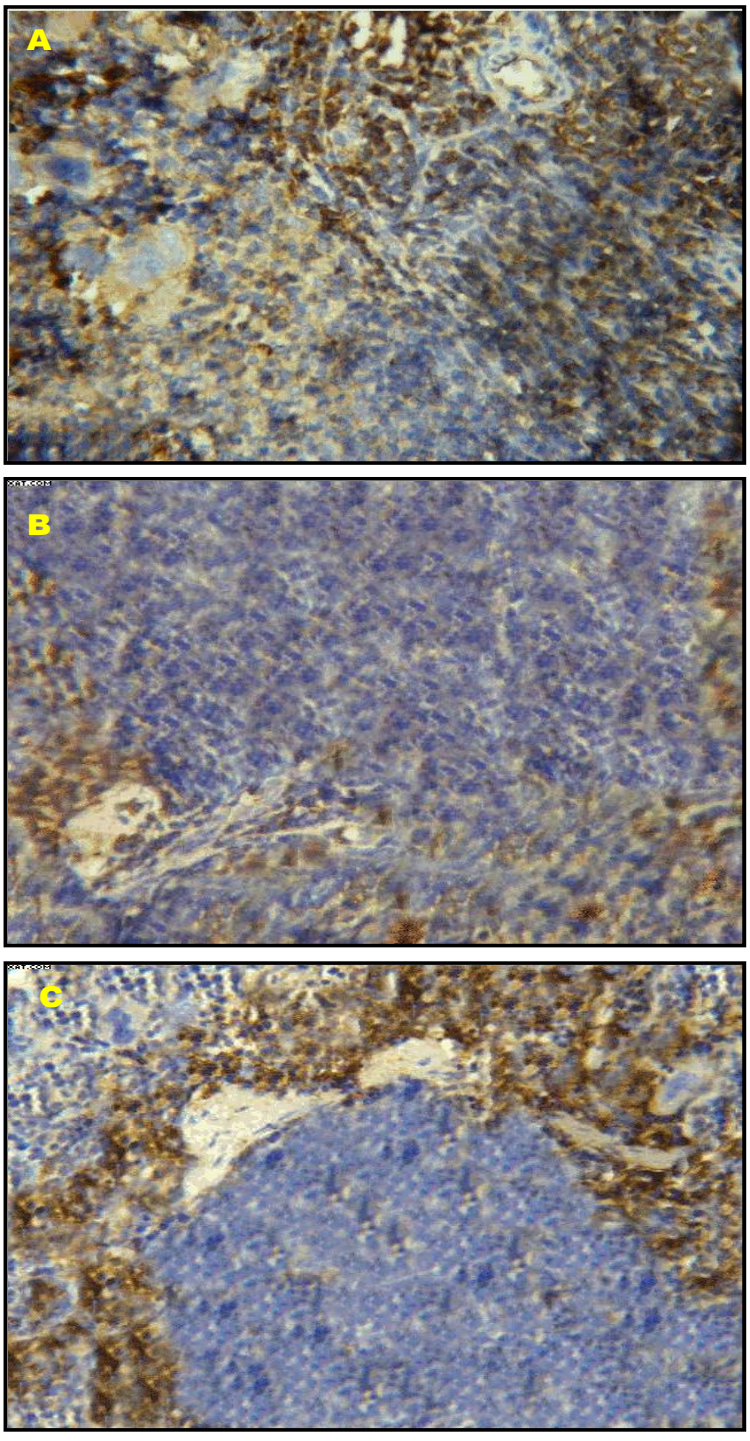

Figure 1. Altered number and distribution of T cells in the spleen of diabetic mice. Immunohistochemical analysis for demonstrating the number and distribution of CD3+ T cells (brown color) in the spleen of the: **(A)** control mice, numerous T cells are normally distributed in the marginal zone of the white pulp and the germinal center of the lymphatic follicles (immunoperoxidase 40X); **(B)** diabetic mice, a marked reduction in the numbers of T cells is shown in the marginal zone of the white pulp and the germinal center of the lymphatic follicles compared to control (immunoperoxidase 40 X); (**C)** anti-IFN-α-treated diabetic mice, restoration of T cell number is observed in the marginal zone and the periarterial lymphatic sheath appeared more or less similar to those of control (immunoperoxidase 40 X).

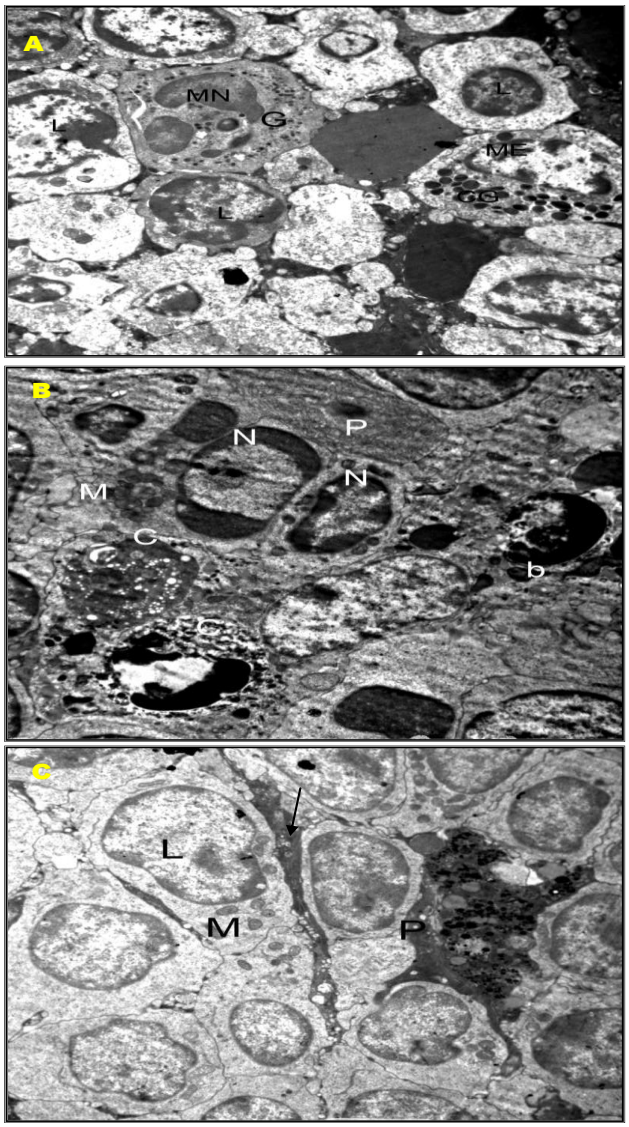

Figure 2. Increased apoptosis in the lymphocytes homing spleen of diabetic mice. Electron micrograph in the spleen of: **(A**) control mice showing mature and immature lymphocytes. Mature eosinophil (ME) has a bi-lobed nucleus and large, cytoplasmic 'specific' granules(CG) which have electron dense crystalloid cores, Mature neutrophil (MN) has small cytoplasmic granules (G) and multi-lobed nucleus, lymphocytes(L) with large, round nuclei. The nucleus occupies most of the cytoplasm, with a thin rim of cytoplasm. (3600X); (**B**) diabetic mice showing apoptosis in the form of condensation and shrinkage of nucleus with a healthy mitochondria (M). Some showed marginated nuclear chromatin (N); plasma cell looked pyknotic (shrinkage, dense nucleus) (P) with a well defined rough endoplasmic reticulum; others exhibited degenerative signs in the form electron dense vacuolated cytoplasm (C) with a healthy mitochondria, still other cells exhibited irregular margination electron chromatin and rarified cytoplasm with electron dense bodies extend of it (b). (3600X); (**C**) the anti-IFN-α-treated diabetic mice showing immature and mature lymphocytes (L) with a healthy mitochondria (M), irregular electron dense phagocyte (P) with numerous lysosomal bodies in the cytoplasm. The process of electron dense vacuolated cell is clearly observed (arrow) (3600 X).

karyolysis of the nucleus (Figure 2B). In anti IFNα treated mice, slightly enhanced number of healthy mature and immature lymphocytes, decrease severe apoptotic changes as degenerated lymphocyte were observed (Figure 2C).

DISCUSSION

Type 1 diabetes contributes to the occurrence of apoptosis and T lymphopenia which lead to increased susceptibility to frequent and prolonged infections (Elder and Maclaren, 1992; Jung et al., 1999). Although it seems that the role of IFN-α is well established in immune and inflammatory diseases, little is known about the role of the increasing level of IFNα during T1D (Huang et al., 1995) and increasing incidence of apoptosis of lymphocytes during T1D. Therefore, several attempts have been made to understand the underlying defects in lymphocyte proliferation and function. We hypothesized that blocking Type I IFN receptor in TID improves lymphocytes proliferation within secondary lymphoid organs through decrease in apoptosis, modulation of blood glucose levels and decreased inflammatory factors.

In this study, we found that increasing levels of IFN-α and TNF-α cytokines were significantly reduced in the diabetic mice injected with anti-IFNAR1 antibody compared with the diabetic mice, which also lead to a significant reduction in glucose as was in consistent with previous results. It has been shown that when IFN-α action was blocked by a monoclonal antibody specific for IFN-α receptor unit 1 (IFNAR1) at postnatal for 15 to 25 days, T1D was significantly delayed or prevented in wild type (wt) NOD mice (Devendra et al., 2004; Bach, 2005). Other findings revealed that the release of IFN-α is entirely an internal event; that is the debris of apoptotic β-cells activates pDCs, which produce large amounts of IFN-α, subsequently initiating the pathogenesis of T1D. And this concept could be strengthened by showing that complete blockade of IFN-α completely prevents T1D (Li et al., 2011).

Furthermore, immunohistochemical study demonstrated a marked reduction in the number of CD3-positive T cell numbers in the periarterial lymphatic sheath and the marginal zone of white pulp as well as in lymph follicles of spleen during T1D. These observations are in agreement with observations made by Kimura et al. (1998) who demonstrated that T lymphopenia in both spleen and thymus developed and all T-lymphocyte subsets examined were similarly reduced in T1D. While slightly increased numbers of T cells present in the marginal zone and the periarterial lymphatic sheath in anti IFNα treated mice give evidence that IFNα plays a prominent role in constitutive activation and exhaustion of T cells and subsequently these cells are subjected to die by apoptosis. Our results are in consistent with other data which demonstrated that activation-induced cell death

(AICD) may contribute to lymphopenia observed during IFN-α therapy (Kaser et al., 1999). Electron microscope investigations have confirmed the increase of apoptosis in lymphocytes in spleen of diabetic animals. Increased level of IFN-α during diabetes was accompanied by apoptotic events in the architecture of spleen of diabetic mice, including DNA fragmentation, chromatin condensation, cell shrinkage and disassembly into membrane-enclosed vesicles (apoptotic bodies). Our results are also consistent with previous studies which reported that a significant proportion of lymphocytes obtained from diabetic patients and alloxan-induced diabetic rats show apoptotic features such as DNA fragmentation and chromatin condensation without loss of cell membrane integrity, as well as decreased number of circulating lymphocytes in diabetic patients, which is possibly as a consequence of the high occurrence of apoptosis (Otton et al., 2004).

Subsequently, decrease in the number of lymphocytes might play an important role in the impaired immune function and high incidence of infections in poorly controlled diabetic patients (Pires et al., 2007). In addition to apoptosis, different degrees of degenerative changes as well as karyolysis could be observed mainly in lymphocytes of diabetic group. In the present study, blocking type I IFN receptor in TID decreases apoptotic changes observed such as DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and disassembly into membrane-enclosed vesicles. Moreover, Kudryavets (2001) reported that IFN-α has been shown to increase cytotoxicity of anticancer agents, in particular TNF and vinblastin (VBL) on tumor cell lines, and this effect seems to be due to IFN ability to enhance apoptosis, thus resulting in increasing number of apoptotic cells and increasing oligonucleosomal DNA fragmentation. Taken together, our results suggest that increased level of IFN-α during diabetes induced continuous activation and exhaustion of lymphocytes and altered the distribution of these cells in the secondary lymphoid organs (site of antigen recognition and immune response).

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