DIABETES MELLITUS DIRECTS NKT CELLS TOWARD TYPE 2 AND REGULATORY PHENOTYPE

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DIABETES MELITUS USMERAVA DIFERENCIJACIJU NKT ĆELIJA U PRAVCU TIP 2 I REGULATORNOG FENOTIPA

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ABSTRACT

Diabetes mellitus is chronic disorder characterized by hyperglycaemia. Hyperglycaemia induces mitochondrial dysfunction, enhances oxidative stress and thus promotes reactive oxygen species (ROS) production. Earlier studies suggested that reactive oxygen species (ROS) are involved in the pathogenesis of many diseases. Previous studies have revealed that hyperglycaemia changes the functional phenotype of monocytes, macrophages, neutrophils, NK cells and CD8⁺ T cells. The aim of this study was to investigate whether diabetes affects the functional phenotype of NKT cells.

Diabetes mellitus was induced in BALB/c mice by intraperitoneal injection of streptozotocin at a single dose of 170 mg/kg body weight. The number and functional phenotype of splenic NKT cells was assessed by flow cytometry, 28 days after diabetes induction.

The diabetic condition facilitated the production of antioxidant enzymes, including catalase (p<0.05) and superoxide dismutase. Hyperglycaemia enhanced oxidative stress and thus decreased the number of splenic NKT cells but did not change the percentage of splenic CD3⁺CD49⁺ NKT cells that express the activatory receptor NKP46 or produce IFN- γ . However, hyperglycaemia increased the frequency of splenic NKT cells that express KLRG-1 and produce TGF- β , IL-4, and IL-5, and it decreased the frequency of IL-17⁺ NKT cells.

Our study indicates that diabetes mellitus induces oxidative stress and switches the functional phenotype of NKT cells towards type 2 (IL-4 and IL-5 producing NKTs) and regulatory (TGF- β producing NKTs) phenotypes. These findings are correlated with the clinical observation in humans that diabetic patients are more prone to infections and tumours.

Keywords: diabetes, hyperglycaemia, oxidative stress, NKT cells

SAŽETAK

Dijabetes melitus je hronično oboljenje koje se karakteriše hiperglikemijom. Hiperglikemija utiče na funkciju mitohondrija, pojačava oksidativni stres i time podstiče produkciju kiseoničnih slobodnih radikala. Ranije studije su pokazale da kiseonični slobodni radikali igraju važnu ulogu u razvoju mnogih bolesti. Hiperglikemija utiče na funcionalni fenotip monocita, makrofaga, NK ćelija i CD8+T limfocita. Cilj istraživanja je bio ispitati da li hiperglikemija utiče na funcionalni fenotip NKT ćelija.

Dijabetes melitus je indukovan BALB/C miševima jednom dozom streptozotocina intraperitonealno u dozi od 170 mg/kg. Broj i funkcionalni fenotip NKT ćelija je analiziran protočnom citometrijom 28. dana nakon indukcije dijabetesa.

Dijabetes je povećao produkciju antioksidantnih enzima, katalaze i superoksid dizmutaze. Dijabetes i pojačan oksidativni stres su smanjili ukupan broj NKT ćelija u slezini hiperglikemičnih miševa, dok se procenat NKp46⁺NKT ćelija i NKT ćelija koje produkuju IFN- γ u slezini nije značajno razlikovao u poređenju sa normoglikemičnim miševima. Međutim, hiperglikemični miševi su imali veću procentualnu zastupljenost NKT ćelija koje eksprimiraju KLRG-1 i produkuju TGF- β , IL-4, and IL-5, dok je učestalost IL-17⁺ NKT ćelija bila značajno manja u poređenju sa normoglikemičnim miševima.

Rezultati ukazuju da dijabetes melitus pojačava oksidativni stres i usmerava polarizaciju NKT ćelija ka tipu 2 i regulatornom fenotipu, što je u skladu sa kliničkim studijama koje potvrđuju da su osobe sa dijabetesom sklone razvoju infekcija i tumora.

Ključne reči: *dijabetes, hiperglikemija, oksidativni stres, NKT ćelije*





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INTRODUCTION

Diabetes mellitus, one of the most common chronic diseases, increases the susceptibility to obesity and many other diseases (1). The major characteristic of diabetes mellitus is hyperglycaemia (2). Hyperglycaemia is usually caused by low insulin levels or insulin resistance and is associated with the damage of many tissues and organs, especially nerves, blood vessels, kidneys and eyes (2). One of the major effects of hyperglycaemia is increased production of reactive oxidative species (3, 4). Excess glucose in cells is involved in glucose oxidation and the nonenzymatic glycation of proteins (5). The final products of these pathways are reactive oxidative species (5). Increases of reactive oxidative species harm cellular organelles and increase lipid peroxidation (6).

Many studies have shown that immune system function is impaired in individuals with diabetes mellitus (7). The immune system can be subdivided to innate and acquired immunity (8). The innate immune system is characterized by rapid responses to pathogens and is mediated mainly by macrophages, dendritic cells, granulocytes, natural killer (NK) cells and natural killer T (NKT) cells, while the acquired immune system is composed of T and B lymphocytes (8). Earlier studies have shown that hyperglycaemia changes effector functions of innate immune cells. The decreased expression of MHC class II on circulating monocytes, reduced response of macrophages to multiple TLR ligands, reduced neutrophil degranulation, impaired $\gamma\delta$ T cell proliferation (9), significant decrease in the expression of activating receptors NKG2D, NKp30, and NKp46, and interferon-y (IFNy) and perforin production in NK cells (10, 11) are all described as phenomena that accompany diabetes.

Natural killer T (NKT) cells constitute a subset of T cells that serve as a bridge between innate and adaptive immunity (12-14). NKT cells recognize exogenous and endogenous lipid antigens presented in the context of the MHC class Ilike molecule CD1d (15). One of the main functions of NKT cells is cytokine production (13). Thus, NKT cells play an important modulatory role in the induction or prevention of many pathogenic conditions (12-14). NKT cells can be subdivided according to transcriptional factor expression and subsequent cytokine production, such as IFN γ , interleukin IL-4, IL-10, IL-13, IL-17, and IL-22; tumour necrosis factor- α (TNF α); and granulocyte-macrophage colonystimulating factor (GM-CSF), which modulate the innate and adaptive immune response (13, 16).

In the available literature, there is no evidence of the effect of diabetes on NKT cells. The aim of our study is to investigate the effect of the diabetic condition on the functional phenotype of NKT cells in mice.

MATERIAL AND METHODS

Animals

BALB/C mice (female, 6-8 weeks old) were used in all experiments. Animals were maintained under standard lab-

oratory conditions. The protocols for animal experiments were approved by the Animal Ethics Board of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Induction of diabetes

Mice were divided randomly into two groups: the experimental and control group. Diabetes was induced in the experimental group by the intraperitoneal injection of streptozotocin dissolved in a sodium citrate buffer (pH=4.5) at a single dose of 170 mg/kg body weight, while the control group was given a sodium citrate buffer (pH=4.5). Blood samples were obtained from the lateral tail vein after four hours of starvation. Blood glucose levels were determined twice a week with *Accu-Chek Performa*, *Roche*.

Determination of antioxidant enzymes

Isolated RBCs were washed three times with 3 volumes of ice-cold 0.9 mmol/l NaCl and haemolysates containing approximately 50 g of Hb/l (17), which were used for the determination of catalase (CAT) and superoxide dismutase (SOD) activity by spectrophotometry. According to Beutler, for the determination of CAT activity, lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin. Then, 100 μ l of a sample and 1 ml of 10 mM H₂O₂ were added to a 50 µl catalase buffer (18). Detection was performed at 360 nm. According to the methods of Misra and Fridovich, superoxide dismutase (SOD) activity was determined using epinephrine. Approximately 100 µl of lysate and 1 ml of carbonate buffer were mixed, and then 100 μ l of epinephrine was added (19). Detection was performed at 470 nm. The activities of SOD and CAT in red blood cells (RBCs) are presented in units per gram of haemoglobin x 10^3 (U/g Hb x 10^3).

Cell preparation

Mice were sacrificed on day 28 after diabetes induction, and their spleens were isolated. Single-cell suspensions were obtained from the spleens by mechanical dispersion through a cell strainer (BD Pharmingen, USA) in a complete growth medium (Dulbecco's-Modified Eagles Medium supplemented with 10% foetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L penicillin–streptomycin, 1 mmol/L mixed nonessential amino acids (Sigma, USA)). Erythrocytes were removed from the splenocyte cell suspension by a lysing solution (BD Pharmingen), and cells were resuspended in complete growth medium. The number of viable cells was determined by trypan blue staining, and only cell suspensions with > 90% viable cells were used.

Flow cytometry

Single-cell suspensions from spleens were incubated with mAbs that were specific for mouse CD3, CD49, NKp46, IFN γ , KLRG1, IL-4, IL-5, IL-17 and TGF- β or isotype-matched controls (BD Pharmingen/BioLegend); they were then analysed using a FACSCalibur flow cytometer



Figure 1. STZ application increases glycaemia and oxidative stress. Experimental diabetes was induced in BALB/c mice by intraperitoneal injection of streptozotocin dissolved in sodium citrate at a single dose of 170 mg/kg body weight. Hyperglycaemia was measured twice a week. Mice were sacrificed on the 28th day after streptozotocin application. The activity of antioxidative enzymes was measured in the isolated RBCs. Data are presented as the mean+SEM from two experiments. Statistical significance was tested by Mann–Whitney rank-sum test or Student's unpaired t-test where appropriate (p<0,05).

(BD). Dead cells were excluded from the analysis by positive propidium-iodide staining. The gate used for FACS analysis was the mononuclear cell region in FSC/SSC plots (20000 events were acquired). Data were analysed using CELLQUEST (BD) and FlowJo (Tristar) software.

Intracellular cytokine staining

For the analysis of IFN γ , IL-4, IL-5, IL-17 and TGF- β expression, splenocytes were stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma), ionomycin (500 ng/ml, Sigma) with GolgiStop (BD Pharmingen) and incubated for 4 h at 37°C, 5% CO₂. After fixation and permeabilisation, intracellular staining was performed using anti-IFN- γ , anti-IL-4, anti-IL-5, anti-IL-17 and anti-TGF- β anti mAb (BD Pharmingen) and analysed by flow cytometry (20).

Statistical analysis

The data were analysed using the statistical package SPSS version 20. The normality of the distribution was tested by the Kolmogorov–Smirnov test. The two-tailed Student's t-test or the nonparametric Mann–Whitney U test were used. The results were considered significantly different when p <0.05.

RESULTS

Diabetes increases the production of catalase and superoxide dismutase

Glycaemia was measured twice a week during all experiments. As shown in figure 1, the blood glucose level was significantly increased in mice treated with streptozotocin compared to CB-treated mice at day 28. Twenty-eight days after diabetes induction, we measured antioxidant enzyme activity in erythrocytes. The activity of antioxidant enzymes did not differ among the experimental and control groups on day 0 (data not shown). Hyperglycaemic mice had significantly increased activity of catalase compared to normoglycaemic mice (p=0,03, Figure 1). The activity of superoxide dismutase was also measured; our data showed that hyperglycaemic mice have increased activity of superoxide dismutase, but the difference did not reach statistical significance (Figure 1).



Figure 2. Diabetes decreases the total number of splenic NKT cells and increases the number of KLRG1⁺NKT cells in the spleen. Mononuclear cells were isolated from spleens of streptozotocin-injected mice, and non-treated mice were determined on day 28 of the experiment. Mononuclear cells were labelled with fluorochrome-conjugated antimouse antibodies and analysed by flow cytometry. Data are presented as the mean+SEM of two separate experiments, and each was carried out with seven mice per group. Statistical significance was tested by the Student's unpaired t-test (p<0,05).



Diabetes decreases the total number of spleen NKT cells and increases the number of KLRG-1⁺ NKT cells

We assessed the frequency and functional phenotype of NKT cells in the spleens of hyperglycaemic and normoglycaemic mice at the 28th day after streptozotocin induction. Our results showed that the frequency of CD3⁺CD49⁺ NKT cells was decreased in spleens of hyperglycaemic mice compared to normoglycaemic mice, but the difference did not reach statistical significance (figure 2). Further, we analysed the expression of activatory and inhibitory receptors on NKT cells. Diabetes increased the incidence of CD3⁺CD49⁺ NKT cells expressing KLRG1⁺ (which did not reach statistical significance), but it did not affect the percentage of NKP46⁺ CD3⁺CD49⁺ NKT cells (Figure 2).

Diabetes increases IL-4⁺, IL-5⁺ and TGF- β^+ NKT cells and decreases IL-17⁺NKT cells

To further determinate the functional phenotype of NKT cells, we analysed cytokine production. As shown in

figure 3, hyperglycaemia increased the frequency of splenic IL-4⁺ and IL-5⁺ NKT cells (which did not reach statistical significance, respectively) and NKT cells producing TGF- β^+ (p=0.004). Diabetic conditions also decreased the percentage of IL-17⁺-producing NKT cells (p=0.004), while it did not affect the number of IFN- γ^+ NKT cells (Figure 3).

DISCUSSION

The aim of this study was to investigate whether diabetic conditions changed the functional phenotype of NKT cells. For this purpose, hyperglycaemia was induced in one group of mice by intraperitoneal injection of streptozotocin, while the other group served as healthy controls. Streptozotocin-treated mice exhibited significantly higher levels of glycaemia in comparison to CB-treated mice on the 28th day of the experiment (Figure 1). We also reported that hyperglycaemic mice had increased systemic levels of catalase (p=0,03) and superoxide dismutase (level of superoxide dismutase was not statistically significant) in



Figure 3. Diabetes increases IL-4⁺ and IL-5⁺ and TGF- β ⁺NKT cells and decreases IL-17+NKT cells in the spleen. Mononuclear cells isolated from spleens of streptozotocin-injected mice (7 mice per group) and non-treated mice (7 mice per group) were determined on day 28 of the experiment using fluorochrome-labelled Abs and analysed on a FACS Aria. Mononuclear cells were gated by size and granularity on FSC/SSC. Results are presented as the mean+SEM of two separate experiments. Statistical significance was tested by the Student's unpaired t-test (p<0,05).



comparison to normoglycaemic mice (Figure 1). The frequency of CD3⁺CD49⁺ NKT cells was decreased, while the incidence of NKT subpopulations that express KLRG-1 or produce IL-4, IL-5 and TGF- β were higher in the spleens of hyperglycaemic mice (Figure 2 and 3). Furthermore, the percentage of IL-17-producing NKT cells was lower in mice injected with streptozotocin (Figure 3).

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia (5). One of the major phenomena caused by hyperglycaemia is the production of reactive oxidative species (21). Excess glucose in cells activates the polyol pathway, the hexosamine pathway, protein kinase C (PKC) activation, and the formation of advanced glycation end products; it thus accelerates the production of reactive oxygen species, hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide (21). Oxidative stress occurs when the production of free radicals exceeds the antioxidant defence mechanism (6). If cellular antioxidants do not remove free radicals, abnormally high levels of ROS harm DNA, lipids, and proteins, which leads to the accumulation of damaged molecules (6, 22). Additionally, ROS are known as inducers of cell apoptosis and regulators of gene expression (23). Although hyperglycaemia increases the production of free radicals, it also aggravates the endogenous antioxidant system (5). Antioxidants, such as the enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, as well as vitamins A, C, and E, work in synergy against different free radicals (24). The focus of our investigation was on two important antioxidant enzymes: catalase and glutathione reductase. Catalase enzyme, present in the peroxisome, converts hydrogen peroxide to water and oxygen and thus neutralises its toxic effects (25). Superoxide dismutase converts superoxide anion radicals to hydrogen peroxide, which is further detoxified to water (H₂O) by catalase or glutathione peroxidase (26). In this study, we reported the increased activity of antioxidant enzymes, catalase and superoxide dismutase, which was derived from erythrocytes of streptozotoin-treated mice on the 28th day of the experiment in comparison to CB-treated mice (Figure 1). Our results are in line with other studies that confirmed that hyperglycaemia increases the production of reactive oxidative species and thus increases the production of antioxidative enzymes (27).

Natural killer T (NKT) cells lie at the interface between innate and adaptive immunity and are important mediators of immune responses and tumour immunosurveillance (28). Two major subsets of NKT cells can be distinguished based on their TCR repertoire and lipid reactivity (12). Type I or invariant NKT (iNKT) cells express an invariant TCR α paired with a restricted repertoire of V β chains (12), while type II NKT cells express a more variable TCR repertoire (29, 30) and can modulate immune responses, suppress autoimmunity and inhibit tumour rejection (16). Additionally, functionally heterogeneous NKT cells (28) can be subdivided according to the expression of transcription factors and subsequent cytokine production (31). T-bet^{high} NKT1 cells are capable of

producing large amounts of IFN-y, while alternatively polarized NKT2 cells express a GATA3 transcription factor and produce IL-4, IL-5 and IL13 (31, 32). Recent studies have revealed a new type NKT17 cell that, like CD4⁺Th17 cells, constitutively expresses the RORy-t transcription factor and IL-23R and produces high levels of IL-17 (33). The other studies defined Foxp3-type INKT cells that, similarly to Tregs, suppress the proliferation of CD4⁺ T cells (28). To evaluate the influence of diabetic conditions, we analysed the expression of NKp46 and KLRG-1 receptors on NKT cells. NKp46, a transmembrane type I glycoprotein, is a major activating receptor that is important in the elimination of virally infected cells and tumour cells (34, 35). NKp46 triggers lysis by recognizing membrane ligands on infected and tumour cells (34, 35). KLRG1 is an inhibitory lectin-like receptor, predominantly expressed on NK cells that produce lower levels of IFN-y (36). Our results show that hyperglycaemia decreased the percentage of total CD3+CD49+ NKT. Further, hyperglycaemia did not affect the percentage of splenic CD3+CD49+ NKT cells that express activatory receptor NKP46, while it increased the frequency of NKT cells that express inhibitory receptor KLRG1 (Figure 2). Shimizu et al. showed that KLRG1⁺ iNKT cells coexpress CD49d and granzyme A live longer than conventional NKT cells and have the potential to be involved in a second immune response on the same antigen (37). In our study, we focused on all subpopulations of NKT cells, not only iNKT cells. Thus, our data suggest that hyperglycaemic mice have a lower percentage of highly active and functional NKT cells capable of dealing with infections or tumour cells.

In further analyses of the functional phenotype of NKT cells, we investigated the production of cytokines IFN- γ , IL-4, IL-5, IL-17 and TGF- β by NKT cells. There was no statistically significant difference in the percentage of IFNγ-producing NKT cells between hyperglycaemic and normoglycaemic mice on the 28th day of the experiment (Figure 3). We also measured the production of cytokines that are markers of type 2 immune responses. Hyperglycaemic mice had higher frequencies of splenic CD3+CD49+NKT cells that produce IL-4 and IL-5 compared to normoglycaemic mice (Figure 3). Finally, we analysed the production of IL-17 and TGF- β by NKT cells. Diabetic conditions significantly decreased the percentage of IL-17-producing NKT cells in the spleen, while it significantly increased the percentage of TGF-β-producing NKT cells in comparison to normoglycaemic animals.

Earlier studies have shown that immune deviation towards a type 1 response and the production of IFN- γ promotes tumour rejection, while a type 2 immune response prevents tumour rejection (38, 39). Additionally, IFN- γ is an important cytokine in combating intracellular pathogens (8). IL-4 can be marked as the most critical cytokine in the induction of type 2 immune responses (38, 39). The development of a type 2 immune response is followed by GATA3 expression, and GATA3 inhibits type 1 immune responses by the down-regulation of the STAT 4 tran-



scriptional factor (40). Thus, our data suggest that diabetic conditions facilitate the development of type 2 NKT cells, which suppress the type 1 immune response and make these mice more *prone* to developing *cancer and more susceptible to infections with intracellular microorganisms*.

IL-17 plays a vital role in protecting the host from infection, primarily extracellular bacterial infections and fungal infections, but it is also important for protection against intracellular bacteria and some viruses (41). IL-17 has potent pro-inflammatory functions, including the induction of IL-6 and TNF-a, that increase the recruitment of neutrophils and regulate the production of anti-microbial peptides, which contribute to the host defence (28, 41, 42). Our data indicate that hyperglycaemic mice with a significantly lower percentage of type 17 NKT can be highly susceptible to infection by extracellular pathogens.

Earlier studies showed that NKT cells produce TGF-β and thus suppress anti-tumour immunity (43). NK1.1⁺ T cells in TIL show immunosuppressive activity in the antitumour immune response through the production of TGFbeta and the preferential cytolysis of B7-expressing cells (44). Earlier studies have shown that TGF- β plays an important role in tumour escape from immune surveillance via the down-regulation of CD8+CTL and the suppression of antitumour cell activity, which results in the uncontrolled growth of tumour cells (45). TGF- β also affects myeloid cells, which modulate host immune surveillance and the tumour microenvironment and thus facilitate tumour growth and metastasis (46). TGF- β also inhibits the proliferation and effector functions of macrophages, neutrophils and T lymphocytes and thus supresses the innate and adaptive immune responses (8).

In line with these studies, our results revealed that diabetic mice had higher percentages of TGF- β -producing NKT cells, which can make them more susceptible to developing cancer and infections in comparison to normo-glycaemic mice.

Conclusion

Collectively, diabetes mellitus can modulate NKT cells' functional phenotype in at least two ways: through enhanced expression of the inhibitory receptor KLRG1 and direction toward type 2 and regulatory phenotypes. These findings are in line with data that show that diabetic patients are more prone to infections and tumours.

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Conflicts of interest

The authors declare no financial or commercial conflicts of interest.

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