RESEARCH

Attenuation of NK cells facilitates mammary tumor growth in streptozotocin-induced diabetes in mice

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Abstract

Diabetic patients have higher incidence and mortality of cancer. Recent study revealed that hyperglycemia-induced oxidative stress is involved in the acceleration of tumor metastasis. We used model of high-dose streptozotocin-induced diabetes to investigate its effect on tumor growth and modulation of antitumor immune response of 4T1 murine breast cancer in BALB/c mice. Diabetes accelerated tumor appearance, growth and weight, which was associated with decreased NK cells cytotoxicity against 4T1 tumor cells in vitro. Diabetes reduced frequencies of systemic NKG2D+, perforin+, granzyme+, IFN- γ + and IL-17⁺ NK cells, while increased level of PD-1 expression and production of IL-10 in NK cells. Diabetes decreased percentage of NKG2D+NK cells and increased percentage of PD-1+ NK cells also in primary tumor. Diabetes increased accumulation of IL-10+ Tregs and TGF- β ⁺ myeloid-derived suppressor cells (MDSCs) in spleen and tumor. Diabetic sera in vitro significantly increased the percentage of KLRG-1+ and PD-1+ NK cells, decreased the percentage of IFN- γ^+ NK cells, expression of NKp46 and production of perforin, granzyme, CD107a and IL-17 per NK cell in comparison to glucose-added mouse sera and control sera. Significantly increased percentages of inducible nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO) producing MDSCs and dendritic cells (DC) were found in the spleens of diabetic mice prior to tumor induction. 1-methyl-DL-tryptophan, specific IDO inhibitor, almost completely restored phenotype of NK cells cultivated in diabetic sera. These findings indicate that diabetes promotes breast cancer growth at least in part through increased accumulation of immunosuppressive cells and IDO-mediated attenuation of NK cells.

Key Words

- diabetes mellitus
- breast cancer
- NK cells
- myeloid-derived suppressor cells
- indoleamine
 2,3-dioxygenase

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Introduction

Breast cancer is the most frequently diagnosed cancer in women and one of the leading causes of death related to tumors (DeSantis *et al.* 2014, Tao *et al.* 2015). Diabetes mellitus is metabolic disease, mainly characterized by hyperglycemia, resulting due to lack of insulin secretion

© 2018 Society for Endocrinology Published by Bioscientifica Ltd. Printed in Great Britain or insulin action (American Diabetes Association 2014). Beside very important role of hyperglycemia, the other factors such as oxidative stress and hyperlipidemia are also involved in the development of diabetic complications (Kangralkar *et al.* 2010).

Previous animal and human studies indicate that diabetes is associated with increased risk of breast cancer development because of many shared risk factors (Goto et al. 1995, Vigneri et al. 2009, Giovannucci et al. 2010, Cohen & LeRoith 2012, Shi & Hu 2014). Diabetes may influence tumor genesis and progression by several mechanisms (Shi & Hu 2014). According to Warburg's hypothesis, cancer cells use far more glucose for energy and survival than regular cells, implicating that hyperglycemia could facilitate tumor growth (Vander Heiden et al. 2009). Abundant reactive oxidative species, one of the main characteristics of diabetes, are involved in both, the initiation and progression of cancer (Waris & Ahsan 2006). Recent study indicated that a hyperglycemia-mediated enhanced oxidative stress increased the expression of adhesive molecules on endothelial cells thus accelerating melanoma metastasis and that nullifying systemic oxidative stress suppressed progression of tumor metastasis in diabetes (Ikemura et al. 2013). These suggested mechanisms explain the possible linkage between diabetes and accelerated genesis and progression of tumors. However, the role of diabetes in primary tumor growth and immune response to tumor is incompletely understood.

There is evidence that diabetes compromises defense to infection and immune response in general, including NK cells. Type 1 diabetic patients have decreased expression and aberrant signaling through the NKG2D receptor as well as markedlylowerexpression of p30/p46 NK-activating receptor molecules (Rodacki et al. 2007, Qin et al. 2011). Chen and coworkers have shown that stimulated streptozotocin (STZ)diabetic cytotoxic T cells produce less perforin and TNF- α and thus they have no capability to eliminate melanoma cells and improve the survival of tumor-bearing mice (Chen et al. 2014). Other studies have shown that diabetes decreases total percentage and also impairs the expression of chemokine receptors and costimulatory molecules and decreases the production of proinflammatory cytokines in professional antigen-presenting cells (Nieminen et al. 2012, Sun et al. 2012). To our knowledge, there are no data about immunosuppressive cells, important players in biology of tumor, in diabetic condition. As mammary cancer is one of the most frequent tumors in population, the aim of our study was to investigate the effects of diabetes on murine 4T1 breast cancer growth and alteration of antitumor immune response.

Herewith, we provide the evidence that diabetic condition accelerates murine breast cancer growth, promotes suppressive activity of MDSC and regulatory T cells (Treg) affecting NK cells activity at least in part by enhancing 2,3-dioxygenase (IDO) production.

Material and methods

Mice

Six-to-eight-week-old female BALB/C mice used in all experiments were obtained from Military Medical Academy, Belgrade, Serbia. Mice were housed under standard laboratory conditions $(22\pm 2^{\circ}C, relative humidity 51\pm 5\%$ and a 12-h light-dark cycle) and were administered standard laboratory chow and water *ad libitum*. All experiments were approved by the Animal Ethics Board of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Induction of diabetes

BALB/C mice received an intraperitoneal injection of streptozotocin (STZ, obtained from Sigma Chemical) dissolved in sodium citrate buffer (CB, pH 4.5) at a single dose of 170 mg/kg body weight. Control group of mice received only 100μ L of CB.

Induction of mammary carcinoma

The mouse mammary carcinoma cell line 4T1, syngenic to the BALB/c background, was purchased from the American Type Culture Collection (ATCC). 4T1 cells were maintained in culture medium (Dulbecco's-modified Eagle's medium added with 10% fetal bovine serum (FBS), 2mmol/L L-glutamine, 1mmol/L penicillin-streptomycin and 1 mmol/L mixed nonessential amino acids (Sigma). Subconfluent monolayers, in log growth phase, were harvested by brief treatment with 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS, PAA Laboratories GmbH, Etobicoke, Canada) and washed three times in serum-free PBS before use in all in vivo and in vitro experiments. The number of passage of 4T1 cell line was 7-10 in all experiments. The number of viable tumor cells was determined by trypan blue exclusion. Suspensions only with >95% viable cells were used in experiments. After 4 of weeks STZ or CB injection, each mouse was inoculated with 5×10⁴ 4T1 tumor cells orthotopically into the 4th mammary fat pad.

Estimation of *in vivo* 4T1 tumor appearance and growth

Appearance of primary tumor and tumor diameter were assessed during experiment. The size of primary 4T1 mammary tumors was assessed morphometrically

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from the day of tumor appearance, two times per week, using electronic calipers in two dimensions. Thirty-six days after tumor cell injection, mice were killed and the primary tumors were surgically removed and measured their weight. Blood was taken from the abdominal aorta.

Determination of catalase

Red blood cells (RBC) isolated from the blood of STZ-treated and CB-treated mice were used for measuring antioxidative enzyme, catalase (CAT). RBCs were treated as previously described, and the activity of CAT in RBCs are presented in units per gram of hemoglobin× 10^3 (U/g Hb× 10^3) (Gajovic *et al.* 2016).

Purification of CD8⁺ T cells

CD8⁺ T cells were isolated from spleens of tumor-bearing mice treated with STZ or CB, respectively using magnetic cell separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (Jovanovic *et al.* 2014). After negative selection, unlabeled cells were highly enriched with CD8⁺ T cells.

Purification of NK cells

NK49b⁺ cells were purified from spleens of diabetic and control group of mice using magnetic cell separation kit (Miltenyi Biotec) as previously described (Jovanovic *et al.* 2014). After positive selection, labeled cells were highly enriched with NK cells.

Cytotoxicity assay

Cytotoxicity assay was determined by the xCELLigence RTCA (Real-Time Cell Analyzer) DP (Dual Plate) Instrument (ACEA Biosciences, San Diego, CA, USA) according to manufacturer's recommendations. 100 µL of complete medium were added to each well and background impedance on the plates was measured on the xCELLigence RTCA DP instrument at 37° and 5% CO₂. The E-plate 16 were placed in the xCELLigence RTCA DP, and impedance measurements were recorded every 15 min for 60 h. 4T1 cells (1×104/well) were seeded in plate and used as target cells. After 36h, splenocytes (ratio of target to effector cells (T:E = 1:20), purified CD8+ T cells (T:E=1:5) and NK cells (T:E=1:5) derived from spleen of experimental and control group of mice, respectively, were added in plates as effector cells. Splenocytes, CD8+ T-cell and NK cell-mediated death of tumor cells were

monitored in real time and was indicated by a decrease in cell index. Data were analyzed with RTCA Software 1.2 (ACEA Biosciences).

Flow cytometric analyses of splenocytes and tumor-infiltrating leukocytes

Single-cell suspensions of spleens were obtained by mechanical dispersion. Single-cell suspensions of primary tumors were obtained by enzymatic digestion as previously described (Jovanovic et al. 2014). Fluorochrome-labeled anti-mouse mAbs specific for CD3 (145-2C11), CD49b (HMa2), CD4 (H129.19), CD8 (53-6.7), NKp46 (29A1.4), CD69 (H1.2F3), CD11b (M1/70), CD11c (N418), MHC class II (14-4-4S), CD86 (GL1), CD279 (J43), Ly-6C/G (RB6/8C5), NKG2D (CX5), KLRG-1 (2F1) or isotypematched controls (BD Pharmingen, San Diego, CA, USA; Invitrogen) were used. For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL, Sigma-Aldrich), ionomycin (500 ng/mL, Sigma-Aldrich) and GolgyStop (BD Pharmingen) for 4h and stained with fluorochrome-labeled anti-mouse mAbs specific for perforin (eBio0MAK-D), granzyme (16g6; NGZB), CD107a (1D4B), Foxp3 (MF23), IFN-γ (XMG1.2), IL-10 (JES5-16E3), IL-17 (C15.6) and TGF-β (141231), IDO (2E2/IDO1) and iNOS (CXNFT) (BD Pharmingen; BioLegend, San Diego, CA, USA; eBiosciences, San Diego, CA, USA). Flow cytometry was conducted on FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) and the data were analyzed using FlowJo (Tree Star).

In vivo depletion of NK cells

For *in vivo* depletion of NK cells, mice were treated i.p. with $100 \mu g$ of anti-GM1b anti-asialo antibody (Waco Chemicals, Richmond, VA, USA) 1 day prior and 5 days after 4T1 tumor cell application. Efficacy of depletion of NK cells was >95%.

In vitro experiments on splenocytes

Freshly isolated splenocytes derived from healthy mice were cultured for 24h in RPMI-1640 medium (Sigma-Aldrich) containing 10% of serum isolated from diabetic mice, serum isolated from control mice with added glucose (glucose level was equal $(16.7\pm0.8 \text{ mmol/L})$ to the glucose concentration in serum of diabetic mice) and serum isolated from control mice, respectively. After incubation, cells were harvested and stained with appropriate antibodies for FACS and evaluated by flow cytometry.

Measurement of cytokines

Levels of TNF- α , IFN- γ , IL-1, IL-12, IL-17 and IL-10 in the mouse serum were measured using ELISA kits specific for the mouse cytokines (R&D Systems) according to the manufacturer's instructions.

In vitro inhibition of H₂O₂

Freshly isolated splenocytes derived from healthy mice were cultured for 24h in RPMI-1640 medium (Sigma-Aldrich) containing 10% of serum isolated from diabetic mice, serum isolated from diabetic mice with added pegcatalase (Sigma-Aldrich) and serum isolated from control mice, respectively. Fifteen minutes prior the addition of serum diabetic mice, splenocytes were incubated with 2000 U/mL catalase, which converts H_2O_2 to H_2O and O_2 (Peraldi *et al.* 2009). After incubation, cells were harvested and stained with appropriate antibodies for FACS and evaluated by flow cytometry.

In vitro pharmacological inhibition of IDO

Freshly isolated splenocytes from healthy mice were cultured for 24 h in RPMI medium containing 10% of serum isolated from diabetic mice with or without addition of inhibitor of IDO enzymatic activity, 1 mM 1-methyltryptophan, (1-MT, Sigma-Aldrich) and 10% of serum isolated from healthy mice, respectively. After incubation, cells were harvested, stained with appropriate antibodies for FACS and analyzed by flow cytometry.

Statistical analysis

The data were analyzed using commercially available software (SPSS, version 20). Results were analyzed using the Student's *t*-test or Mann–Whitney *U* test where appropriate. Difference in appearance of primary tumor was assessed using Kaplan–Meier analysis and was evaluated using the log-rank test. Data are presented as mean \pm s.E.M. Statistical significance was set at *P*<0.05.

Results

Enhancement of tumor growth in hyperglycemic mice

Glycemia and body weight were measured weekly after treatment with STZ. As shown in Fig. 1a and b, glycemia was significantly increased (P=0.001) while the

body weight was significantly decreased (P=0.041) in STZ-treated group of mice compared to control group on the 28th day after STZ injection. STZ-treated mice had significantly increased catalase activity compared to control mice (P=0.043; Fig. 1c). At that time, 4T1 mammary carcinoma cells were inoculated and tumor appearance and tumor diameter were monitored three times per week while tumor weight was measured after mice were killed. The appearance of palpable tumor was significantly accelerated in STZ-treated mice (P=0.037; Fig. 1d). Log-rank test confirmed significant difference in tumor appearance between defined groups, which was illustrated using Kaplan–Meier curves (P=0.040; Fig. 1d). Primary tumor diameters were increased in STZ-treated mice in comparison to control mice and the difference reached statistical significance 16 days after tumor induction (P=0.020; Fig. 1e). Thirty-five days after tumor cell application, mice were killed, and tumors were isolated and measured. Tumor weights were significantly increased in mice treated with STZ compared to controls. (P=0.038; Fig. 1f).

Altered NK but not cytotoxic T cells activity contributes to enhanced tumor growth in diabetic mice

In order to tested cells important for enhanced tumor growth of STZ-treated animals, we evaluated the cytotoxicity of splenocytes and isolated NK cells and CD8+T cells against 4T1 tumor cell line, in vitro. The results obtained by xCELLigence system for monitoring real-time cytotoxicity showed that diabetes led to decreased cytotoxic activity of splenocytes (P=0.021; Fig. 2A). In order to define the effector cells responsible for diminished cytotoxic activity of splenocytes, we isolated splenic CD8+ T cells from tumor-bearing mice and tested their effect on 4T1 cells. There were no differences in cytotoxicity between CD8+ T lymphocytes derived from STZ-treated and control mice (Fig. 2B). Further, we tested cytotoxic activity of isolated CD49b+ NK cells against tumor cells, at same target:effector ratio as for CD8+ T cells (1:5). As shown in Fig. 2C, splenic NK cells isolated from STZ-treated mice were significantly less cytotoxic against 4T1 cells (P=0.001), indicating that diabetes significantly reduced cytotoxic potential of NK cell population.

Further, we depleted NK cells in STZ and CB-treated mice by *in vivo* administration of an antiasialo-GM1 antibody, following tumor challenge. After antiasialo-GM1 treatment, tumor appearance was significantly accelerated in both groups of mice in comparison to non-treated

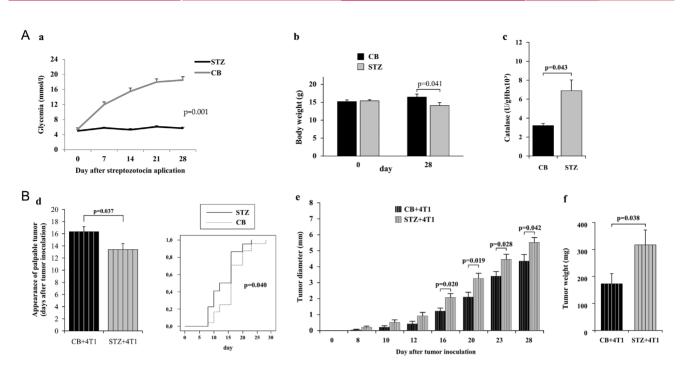


Figure 1

Diabetes accelerates tumor appearance and growth. (A) Metabolic parameters prior to tumor induction. Mice were injected intraperitonealy with STZ at a single dose of 170 mg/kg bodyweight or 100 μ L of CB only. Glycemia and body weight were measured weekly (a and b). Catalase was measured by spectrophotometry in red blood cells derived from STZ-treated and control mice (c). (B) Appearance and growth of mammary tumor inoculated 28 days after STZ-induced diabetes. Mice were inoculated with 5 × 10⁴ 4T1 cells. Palpable tumors were monitored for 36 days (d and e). Tumor weight was measured on day 36 (f). Data are presented as mean±s.E.M. of seven mice per group and are representative of three separate experiments. Statistical significance was tested by Mann–Whitney rank-sum test or Student's unpaired *t*-test and log-rank test where appropriate.

animals, which is confirmed by log-rank test (CB vs CB+AA P=0.001; STZ vs STZ+AA P=0.001; Fig. 2D). Moreover, after NK cells depletion, the difference in dynamic of tumor appearance as well as in tumor diameter and tumor weight in STZ and CB-treated mice could no longer be registered (Fig. 2D, E and F).

Diabetes alters functional phenotype of NK cells in spleen

We further examined phenotype of splenic NK cells on the 28th day after STZ induction, prior to tumor induction. Diabetic condition decreased expression of NKG2D-activating receptor on CD3-CD49b⁺NK cells compared to NK cells derived from control mice (P=0.005; Fig. 3A). There was no significant difference in percentage of PD-1⁺CD3-CD49b⁺NK cells. However, level of PD-1 expression was significantly increased on individually NK cell derived from STZ-treated mice in comparison to controls (P=0.017; Fig. 3B). Intracellular staining of NK cells revealed significantly lower percentage of perforin (P=0.045), IFN- γ (P=0.049) and IL-17 (P=0.032) producing CD3-CD49b⁺NK cells in STZ-induced mice (Fig. 3C, D and E). There was no significant difference (P>0.05) in the percentage of IL-10-producing NK cells between groups; however, we found significantly higher expression of IL-10 per NK cell isolated from STZ-treated mice (P=0.049; Fig. 3F).

Interestingly, there were no differences in the phenotype of splenic CD8⁺ T cells between STZand CB-treated tumor-bearing mice, respectively. Analyses revealed insignificant increased percentage of KLRG-1⁺CD3⁺CD8⁺ T cells in STZ-treated tumor-bearing mice while we did not find any differences in the expression of other surface markers of interest: NKG2D, CD69, Cd107a and PD-1 or in the production of IFN-γ, IL-17, perforin and granzyme (data not shown).

Diabetes facilitates accumulation of innate and acquired immunosuppressive cells in tumor-bearing mice

Analysis of serum cytokines have shown increased concentration of IL-10 in the sera of STZ-treated mice in comparison to control group (Fig. 4A), while there were no differences in the level of TNF- α , IFN- γ , IL-1, IL-12 and

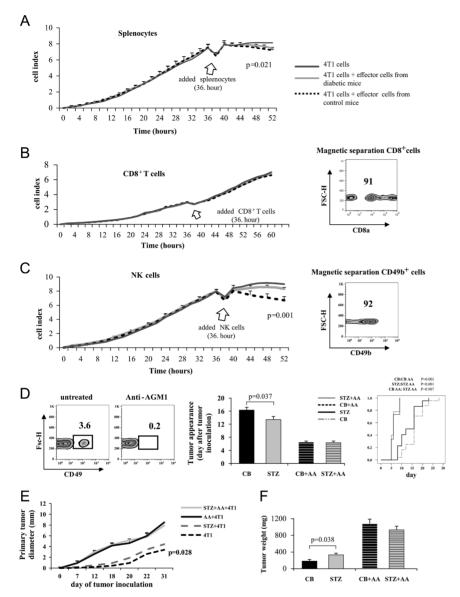


Figure 2

Diabetes diminishes NK cell cytotoxicity. The results obtained by xCELLigence system showed cytotoxic activity of splenocytes (A), CD8+T cells (B) and NK cells (C) isolated from mice treated with STZ and CB, respectively. Data are presented as means ± s.E.M. of two individual experiments, each carried out with seven mice per group. Statistical significance was tested by unpaired Student's t-test. NK cells were depleted by administration of antiasialo GM1 mAb (AA). Representative flow cytometry plots show percentage of splenic NK cells after in vivo depletion (D). NK-depleted diabetic and control mice were injected with 5 × 10⁴ 4T1 tumor cells. Palpable tumors (E), tumor diameter (F) and tumor weight (G) were measured up to day 36 from the inoculation of 4T1 tumor cells. Data are presented as mean ± S.E.M. of seven mice per group and are representative of two separate experiments. Statistical significance was tested by Student's unpaired t-test and and log-rank test, where appropriate.

IL-17 between two groups (data not shown). According to these results, we further analyzed immunosuppressive cells in spleens of STZ-treated and CB-treated mice, respectively, prior and 15 days after 4T1 tumor cell application. STZ-treated mice exhibited pronounced increase in percentage of splenic CD11b⁺CD11c⁻Gr-1⁺ MDSCs compared with control mice (P=0.029; Fig. 4B). Moreover, STZ-treated mice exhibited increased percentage of splenic CD11b⁺CD11c⁻Gr-1⁺ MDSCs that produce TGF- β (P=0.03; Fig. 4C). Percentage of CD4⁺Foxp3⁺ cells (P=0.029) as well as percentage of IL-10-producing CD4⁺ Foxp3⁺ cells (P=0.048) were significantly increased in spleens of diabetic tumor-bearing mice (Fig. 4D and E).

Analyses of dendritic cells (DCs) revealed that there were no differences in the percentage of CD11b⁺CD11c⁺ cells in spleen derived from STZ-treated and CB-treated

© 2018 Society for Endocrinology Published by Bioscientifica Ltd. Printed in Great Britain mice prior and after tumor challenge (data not shown). There were no differences in expression of MHC II and CD86⁺ on CD11b⁺CD11c⁺ cells derived from spleen of animals from previously defined groups (data not shown).

Diabetes modulates innate lymphoid and myeloid cells phenotype in primary tumor

Analysis of NK cells in tumor tissue did not show differences in percentage of CD45+NKp46+ NK cells (Fig. 5A). Analyses of functional phenotype revealed significantly lower percentage of NKG2D+ (P=0.008) and higher percentage of PD-1+NK cells (P=0.029) in the tumors of STZ-treated mice compared to animals treated with CB only (Fig. 5B and C).

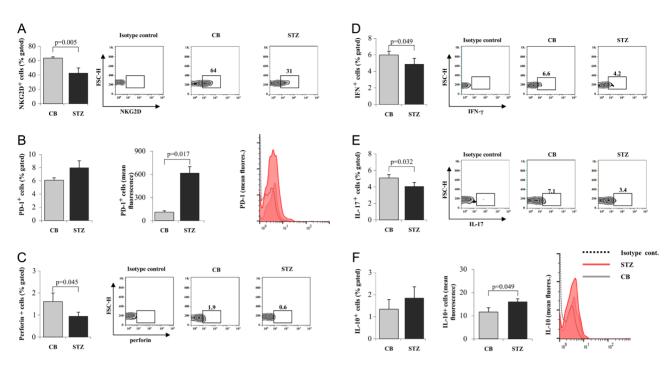


Figure 3

Diabetes alters functional phenotype of NK cells in spleen. The graphs and representative facs plots displaying the percentage of NKG2D (A), PD-1 (B), perforin (C), IFN- γ^+ (D), IL-17+ (E) and IL-10+ (F) NK cell derived from spleens of STZ and control, respectively. Data are shown as mean ±s.E.M. of seven mice per group and are representative of three separate experiments. Statistical significance was tested by Mann–Whitney rank-sum test or Student's unpaired *t*-test, where appropriate. A full color version of this figure is available at https://doi.org/10.1530/ERC-17-0529.

We found increased percentage of CD11b+CD11c-Gr-1+ MDSCs (P=0.021) as well as TGF- β + CD11b+CD11c-Gr-1+ MDSCs (P=0.039) in primary tumor of STZ-treated mice compared to control mice (Fig. 5D and E).

Diabetic serum altered NK cell phenotype in vitro

Further, we investigated the effects of diabetes-mediated soluble factors and glucose on functional phenotype of NK cells. Splenocytes isolated from healthy mouse were seeded for 24 h in medium containing serum derived from diabetic mice, serum derived from healthy mice with added glucose or serum derived from healthy mice only, and CD3-CD49+ NK cells were analyzed within cultivated population of splenocytes. Addition of glucose increased percentage of KLRG-1⁺ (P=0.029) and PD-1⁺ (P=0.016) CD3-CD49b+NK cells (Fig. 6A and B). Although there were no differences in the percentage of NKp46 and perforin, there was significant decrement in the level of expression of these molecules in NK cells treated with glucose added sera (P=0.026; P=0.017; Fig. 6C and D). Cultivation in diabetic sera additionally increased percentage of KLRG-1+ (P=0.032; P=0.016) and PD-1⁺ (P=0.036; P=0.016)CD3-CD49b+NK cells and decreased expression of NKp46 (*P*=0.038; *P*=0.014) and production of perform (*P*=0.049;

P=0.012) per CD3-CD49b+NK cell in comparison to glucose added sera and control sera (Fig. 6A, B, C and D). Diabetic sera decreased percentage of IFN- γ -producing CD3-CD49b+NK cells (*P*=0.029; *P*=0.029) decreased expression of CD107a (*P*=0.016; *P*=0.014), and production of granzyme (*P*=0.004; *P*=0.003) and IL-17 (*P*=0.048; *P*=0.032) in CD3-CD49b+NK cells compared to glucose added sera and control sera (Fig. 6E, F, G and H).

In order to nullify enhanced oxidative stress, we performed additional *in vitro* experiments. Freshly isolated splenocytes derived from healthy mice were cultivated in medium containing serum derived from diabetic mice, serum derived from diabetic mice with added peg-catalase, and serum derived from healthy mice, respectively. Addition of peg-catalase significantly increased the percentage of NKp46+CD3-CD49b+NK cells (P=0.05) but did not affect the expression of PD-1, KLRG-1 and NKG2D molecules in comparison to NK cells cultivated in diabetic sera (Table 1).

NK cell function is suppressed in IDO-dependent manner

Further, we analyzed the production of iNOS and IDO in different cell populations. Intracellular staining of

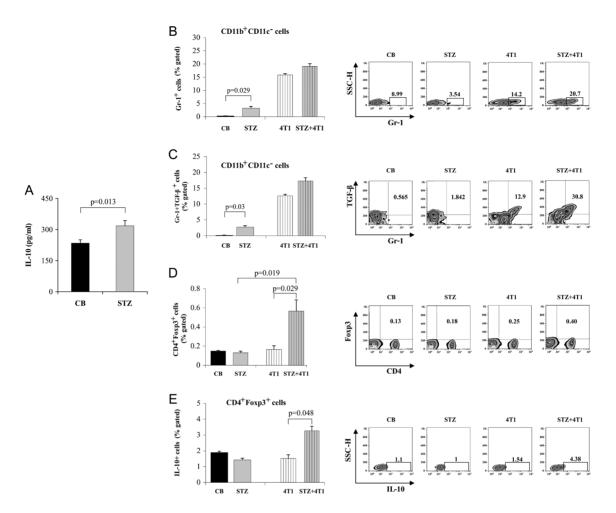


Figure 4

Diabetic condition favors immunosuppressive phenotypes of spleen cells. The level of IL-10 in the sera of diabetic and control group of mice (A). The percentage of CD11b+CD11c-Gr-1+ MDSCs (B) and TGF- β +CD11b+CD11c-Gr-1+ MDSCs (C), CD4+Foxp3+ cells (D), IL-10+CD4+ Foxp3+ cells (E) in the spleen of STZ-treated and control mice, respectively, prior and 15 days after 4T1 tumor cell application were determined by flow cytometry and displayed on graphs and representative facs plots. Data are mean ± s.E.M. from six mice per group and are representative of two separate experiments. Statistical significance was determined by Mann–Whitney rank-sum test.

splenic MDSCs and DCs revealed significantly higher percentages of iNOS⁺ (P=0.029) and IDO⁺ (P=0.031) CD11b+CD11c-Gr+MDSCs as well as iNOS+ (P=0.005) and IDO+ (P=0.026) CD11b+CD11c+ DCs in STZ-treated mice in comparison to control group (Fig. 7A, B, C and D). Next, in *in vitro* experiments, splenocytes isolated from healthy mouse have been cultivated in medium containing serum derived from diabetic mice with and without added 1-MT, selective blocker of IDO, and in sera isolated from healthy mice, respectively, for 24h. Addition of 1-MT in diabetic sera abrogates suppressive effect on percentage of NKG2D+ (*P*=0.016), NKp46⁺ (*P*=0.038) and CD69⁺ (*P*=0.004), perforin-producing (P=0.008) and granzyme-producing (P=0.032) CD3-CD49b⁺ cells compared to the same population of cells cultivated in diabetic sera without addition of 1-MT (Fig. 7E, F, G, H and I). Addition of 1-MT

decreased percentage of IL-10-producing CD3-CD49+bNK cells (P=0.041) and expression of PD-1 per CD3-CD49b+ cells (P=0.015) in comparison to NK cells cultivated in diabetic serum only (Fig. 7J and K).

Discussion

Previous evidence in mice and humans clearly demonstrated the relationship between type 1 and type 2 diabetes and malignancy and some studies have investigated the underlying mechanisms (Ikemura *et al.* 2013, Chen *et al.* 2014). The study by Goto and coworkers demonstrated that STZ-induced diabetes accelerates mammary tumor genesis; however, the cellular basis of this effect was not explored (Goto *et al.* 1995). It is also obvious that effector cells involved in antitumor immunity may

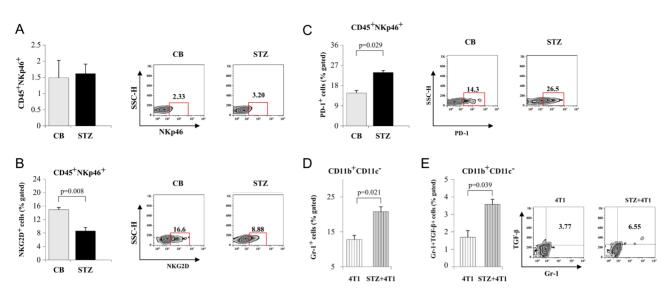


Figure 5

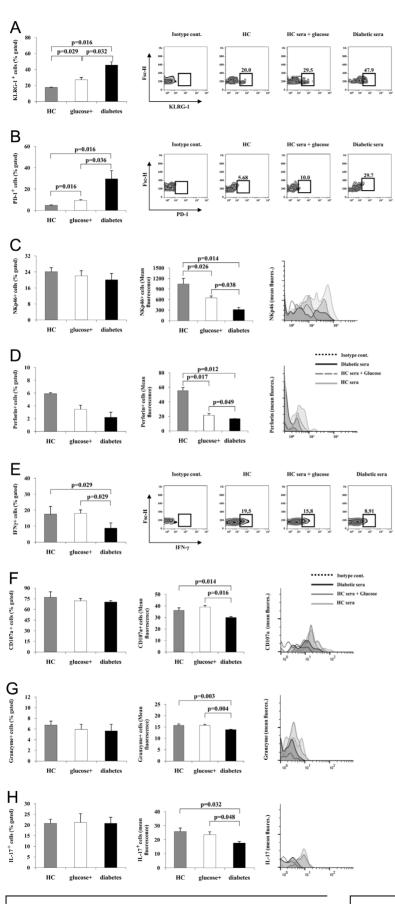
Diabetes affects the phenotype of NK cells and Tregs in primary tumor. The graphs and representative facs plots displaying the percentage of CD45+NKp46 NK cells (A) as well as NKG2D+ (B) and PD-1+ (C) NK cells, derived from primary tumor of STZ and control mice, respectively, 15 days after 4T1 tumor cell application. The percentage of CD11b+CD11c-Gr-1+ MDSCs (D) as well as TGF- β + CD11b+CD11c-Gr-1+ MDSCs (E) in the primary tumor of STZ-treated mice and control mice were determined by flow cytometry 15 days after 4T1 tumor cell application. Data are mean±s.E.M. from seven mice per group and are representative of two separate experiments. Statistical significance was determined by Mann–Whitney rank-sum test. A full color version of this figure is available at https://doi.org/10.1530/ERC-17-0529.

be differently affected by hyperglycemia and pathologies in obesity, insulin resistance and inflammation (Sell *et al.* 2012, Wrann *et al.* 2012, Berrou *et al.* 2013). In this paper, we have demonstrated the accelerated growth of mammary carcinoma in mice made hyperglycemic by a single 'high'-dose injection of STZ.

We found that splenocytes derived from tumorbearing diabetic mice had significantly lower cytotoxic activity in comparison to those isolated from control tumor-bearing mice (Fig. 2A). Further, we tested the cytotoxicity of isolated CD8+T cells and NK cells. Recently, using melanoma model, Chen and coworkers have shown that the acquisition of tumor-killing capacity of CD8+ T cytotoxic cells is diminished in melanoma-bearing STZdiabetic mice (Chen et al. 2014). However, we did not find any significant difference in cytotoxicity of CD8+ T cells to mammary tumor cells in vitro (Fig. 2B). While in these animal model, CD8+ T cells have been shown to play an important antitumor role, our previous studies revealed a nonessential role of CD8+ T cells-mediated cytotoxic antitumor activity (Jovanovic et al. 2011, 2014). The fact that 4T1 is weakly immunogenic mouse breast tumor cell line and previous findings that innate immunity is essential in antitumor response in this tumor model are in line with our results (Jovanovic et al. 2011, 2014). As we and others have shown previously that NK cells cytotoxicity is important in immunological surveillance

and immune response to established tumor, we found cytotoxic capacity and phenotypic alteration of NK cells in diabetic mice as shown prior to tumor induction (Smyth *et al.* 2005, Jovanovic *et al.* 2011, Marcus *et al.* 2014). These findings demonstrated lower cytotoxicity of NK cells of mice treated with STZ (Fig. 2C). *In vivo* depletion of NK cells nullified the difference in tumor appearance and growth which is in line with *in vitro* experiment (Fig. 2D, E and F). These findings suggest on essential role of diminished NK cells activity in accelerated tumor growth phenomenon in diabetic mice.

Rodacki and coworkers reported that NK cells in patients with long-standing type 1 diabetes had markedly lower expression of p30/p46 NK receptors compared with those of control subjects (Rodacki et al. 2007). Interestingly, both NOD mice and patients with diabetes also have slightly decreased expression of NKG2D (Ogasawara et al. 2003, Qin et al. 2011). Accordingly, our findings show that diabetic condition attenuates expression of activating receptor such as NKG2D, production of cytolytic molecules such as perforin and proinflammatory cytokines IFN-y and IL-17, while favors expression of PD-1 molecule and production of IL-10 in splenic NK cells (Fig. 3). These findings together with diminished cytotoxic activity of NK cells derived from STZ-treated mice, as previously described (Fig. 2), implicate that diabetes attenuates



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Figure 6

Diabetic serum induces alteration of NK cells phenotype, *in vitro*. Splenocytes derived from healthy mice were cultivated in medium containing diabetic sera, glucose added sera and control sera, respectively. The graphs and representative facs plots displaying the percentage of KLRG-1+ (A), PD-1+ (B), NKp46+ (C), perforin (D), IFN- γ (E), Cd107a+ (F), granzyme+ (G), IL-17+ (H) NK cells. Data are shown as mean±s.E.M. of six mice per group and are representative of three separate experiments. Statistical significance was tested by Mann–Whitney rank-sum test or Student's unpaired *t*-test, where appropriate.

	Diabetic sera	Diabetic sera+peg-catalase	Control sera	
KLRG-1	39.85±0.21	36.13±2.12	27.82±3.24	P>0.05
PD-1	31.95±2.62	29.15±2.39	13.52±3.57	P>0.05
NKG2D	8.13±0.85	9.15 ± 0.96	10.44 ± 1.33	P>0.05
NKp46	13.28±0.85*	20.02 ± 1.01*	22.36 ± 1.37	P=0.05

Table 1Effect of catalase, in vitro.

Splenocytes derived from healthy mice were cultivated in medium containing diabetic sera, diabetic sera with added peg-catalase and control sera, respectively. The table displays the percentage of KLRG-1+, NKG2D+ and NKp46+ NK cells. Data are shown as mean±s.E.M. of seven mice per group and are representative of two separate experiments. Statistical significance was tested by Mann–Whitney rank-sum test or Student's unpaired *t*-test, where appropriate. *Statistical significance in NKp46 expression between diabetic sera and diabetic sera+peg-catalase is 0.05.

tumoricidal phenotype of NK cells. Our results are in line with previously mention studies claiming alteration of NK cells phenotype in humans with diabetes (Rodacki *et al.* 2007, Berrou *et al.* 2013). Similar phenomenon was obtained in tumor microenvironment. Although we did not find difference in percentage of tumor-infiltrating NK cells, significantly lower percentage of NKG2D⁺ NK cells and higher percentage of PD-1⁺ NK cells suggest that diabetes favors accumulation of poorly functional NK cells in primary tumor (Fig. 5A, B, C and D).

As various mechanisms are proposed to be responsible for the immunosuppressive effects of diabetes, we tested several hypotheses to identify agents responsible for suppression of NK cells. First, we examined the possibility that this is mediated by hyperglycemia, which is known to directly disturb endoplasmatic reticulum (ER) function, favor accumulation of misfolded proteins in the lumen thus promoting ER stress, which in turn decreases the expression of activating receptors on NK cells (Berrou et al. 2013). Diabetic sera increased percentage of KLRG-1+ and PD-1+ NK cells, decreased the expression of NKp46, CD107a and production of perforin, granzyme, IFN-γ and IL-17 per NK cell compared to glucose added sera and control sera (Fig. 6A, B, C, D, E, F, G and H). These results suggest that hyperglycemia is not crucial for altered NK cell phenotype in diabetes and left at least two nonexclusive possibilities. First, reactive oxidative species, which are abundant in the sera of diabetic mice, may suppress NK cell activity through diminished expression of activating receptors such as NKG2D, as previously reported (Choi et al. 2008, Peraldi et al. 2009). In our study, increment in catalase concentration indicates on higher systemic H₂O₂ level, one of the major ROSs, in serum of diabetic mice (Fig. 1c). However, addition of peg-catalase and subsequently blocking of enhanced oxidative stress revealed increment of NKp46 expression only but did not affect the expression or production of other markers of NK cell activity such as NKG2D, KLRG-1 and PD-1 (Table 1). Recent study has shown that in vivo treatment with pegcatalase significantly reduced the number of tumor cells in the lung and liver in both untreated and STZ-treated mice however, our results are implicating on some other mediators that suppress tumoricidal phenotype of NK cells (Ikemura *et al.* 2013).

Level of IL-10 was significantly increased in the sera of diabetic mice (Fig. 4A). It is known that immunosuppressive cells such as MDSCs, Tregs and immature DCs are important regulators of innate and adaptive immunity and all may produce IL-10 (Dilek et al. 2012, Yang 2013). It appears that diabetic condition facilitated accumulation of suppressive CD11b+CD11c-Gr-1+ MDSCs in spleen, prior to tumor injection (Fig. 4B and C). During tumor growth, recruited MDSCs promote generation, polarization, activation and migration of Tregs (Huang et al. 2006, Hoechst et al. 2009, Vitale et al. 2014). Indeed, diabetic condition led to significant increased accumulation of TGF-B producing MDSCs both in spleen and mammary tumor (Figs 4C and 5E). Moreover, increased accumulation of IL-10 producing CD4+Foxp3+Tregs was detected in spleen of tumor-bearing diabetic mice (Fig. 4E). Sitrin and coworkers argue that Tregs control NK cell function by controlling bioavailability of limited amount of IL-2 in the islets generated mainly by CD4+ T cells infiltrating the islets in BDC2.5/NOD mice (Sitrin et al. 2013). However, in our model, this should be of very limited effect as the single high dose of STZ is toxic for β cells but does not induce significant islet infiltration.

Few possible mechanisms of MDSC-mediated NK cell suppression are known. They inhibit NK cells through a cell-contact-dependent mechanism via membrane-bound TGF- β or recognition of the NK cell-activating receptors (Ostrand-Rosenberg *et al.* 2012). It is also known that in order to make immunosuppressive tumor microenvironment, activated MDSCs produce arginase 1, inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO), NADPH oxidase and immunosuppressive cytokines (Wesolowski *et al.* 2013). iNOS is one of the products of immunosuppressive cells important for producing NO that further directly

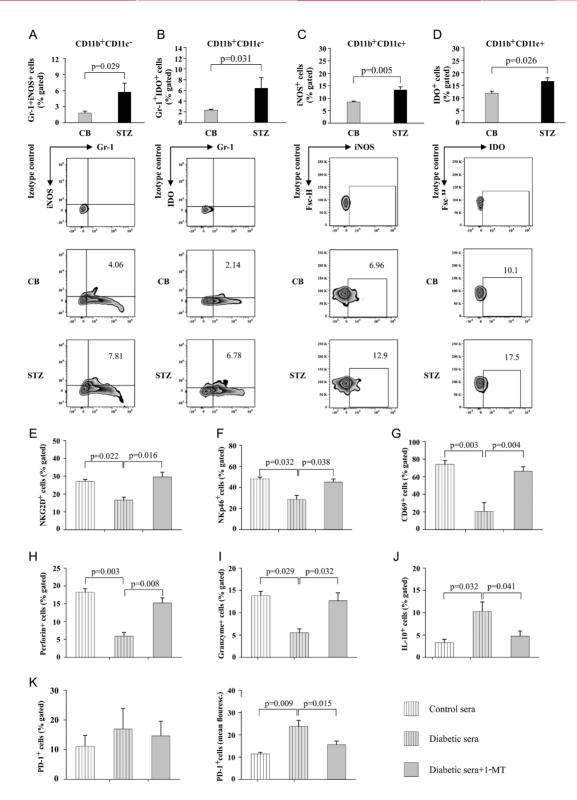


Figure 7

Diabetic condition suppresses NK cells function in IDO-dependent manner. The graphs and facs plots display percentage of iNOS⁺ MDSCs (A) and IDO⁺ (B) MDSCs, respectively, as well as iNOS⁺ (C) and IDO⁺ CD11b⁺CD11c⁺ (D) DCs, respectively, in the spleens of experimental and control mice. Splenocytes derived from healthy mice were cultivated in medium containing diabetic sera with added 1-MT, diabetic sera and control sera. The percentage of NKG2D⁺ (E), NKp46⁺ (F) and CD69⁺ (G), perforin (H) and granzyme (I), IL-10 (J) and PD-1⁺ (K) CD3⁻CD49b⁺ cells were determined by flow cytometry. Data are mean ±s.E.M. from six mice per group and are representative of two separate experiments. Statistical significance was determined by Mann–Whitney rank-sum test or Student's unpaired *t*-test, where appropriate.

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Author contribution statement

N G, M L L and I J wrote the manuscript. N G, M J and I J collected the data. N G, M J, J P, G R and I J were involved in the analysis and interpretation of the data. N G, N A, M L L and I J contributed toward conception and design. All the authors have approved the final version of this manuscript.

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higher iNOS activity in diabetic patients (Adela et al. 2015). Diabetic condition significantly increased percentage of iNOS+ MDSCs as well as iNOS+ DCs (Fig. 7A and C). Low-grade inflammation, one of the diabetes hallmarks, provoke iNOS expression and subsequent NO production that leads to increased IDO activity (Biswas 2016, Gazdic et al. 2017). IDO, an enzyme produced by variety of immune and stromal cells, has powerful immunomodulatory effects (Yu et al. 2013). IDO is involved in the catabolism of the essential amino acid L-tryptophan to L-kynurenine, while further metabolites of the L-kynurenine act in the immunosuppressive manner (Lee et al. 2002, Sucher et al. 2010, Harden & Egilmez 2012). IDO inhibits proliferation and increased apoptosis of T cells, interferes with the IL-2-induced expression of activating receptors on NK cells, inhibits IFN-y production and favors apoptosis of NK cells (Della Chiesa et al. 2006, Li et al. 2009, Cacalano 2016).

suppress proliferation of lymphocytes (Gabrilovich

& Nagaraj 2009, Bogdan 2011). Recent study revealed

Accordingly, herewith we showed significantly increment in percentages of IDO producing MDSCs and DCs in diabetic mice (Fig. 7B and D). In order to confirm possible immunosuppressive MDSCs/DCs-IDO the pathway, we blocked effect of IDO using pharmacological inhibitor 1-MT in vitro. Addition of 1-MT in diabetic serum increased percentage of NKG2D+, NKp46+, CD69+, perforin and granzyme producing NK cells (Fig. 7E, F, G, H and I). Moreover, inhibition of IDO in diabetic condition has significantly decreased percentage of IL-10 producing CD3-CD49+ NK cells as well as expression of PD-1 molecule on CD3-CD49+ cells (Fig. 7J and K). In comparison to control serum, 1-MT treatment almost completely restored the phenotype of NK cells indicating on important role of IDO signaling pathway in the suppression of NK cells in diabetic condition.

Collectively, diabetic condition promotes breast cancer growth through suppression of antitumor immunity. Diabetes favors accumulation of iNOS and IDO-producing innate immunosuppressive cells and subsequent attenuation of NK cells tumoricidal phenotype in at least in part through IDO-dependent manner. The growth of breast cancer in diabetic condition might thereby be controlled by the development of new IDO based biologics.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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