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ORIGINAL CONTRIBUTION

Vitamin C supplementation reconstitutes polyfunctional T cells in streptozotocin-induced diabetic rats

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Abstract
Background Studies have demonstrated that vitamin C supplementation enhances the immune system, prevents DNA damage, and decreases the risk of a wide range of diseases. Other study reported that leukocyte vitamin C level was low in diabetic individuals compared with nondiabetic controls.
Aim of the work To study the effect of vitamin C on oxidative stress, blood lipid profile, and T-cell responsiveness during streptozotocin (STZ)-induced type I diabetes mellitus.
Methods Thirty male Sprague–Dawley rats were randomly split into three groups. The first served as a control group ($n = 10$) in which rats were injected with the vehicle alone. The second ($n = 10$) and the third groups ($n = 10$) were rendered diabetic by intraperitoneal (i.p.) injection of single doses of STZ (60 mg/kg body weight). The third group was supplemented with vitamin C (100 mg/kg body weight) for 2 months.
Results T lymphocytes from the diabetic rats were found to be in a stunned state, with a decreased surface expression of the CD28 costimulatory molecule, low levels of phosphorylated AKT, altered actin polymerization, diminished proliferation and cytokine production, and, eventually, a marked decrease in abundance in the periphery. Vitamin C was found to significantly decrease the elevated levels of blood hydroperoxide, glucose, cholesterol, triglycerides and low-density lipoprotein (LDL) in diabetic rats. Furthermore, it was found to restore CD28 expression, AKT phosphorylation, actin polymerization, and polyfunctional T cells (IFN-γ- and IL-2-producing cells that exhibit a high proliferation capacity).
Conclusion Vitamin C treatment restores and reconstitutes polyfunctional, long-lived T cells in diabetic rats.

Keywords Diabetes mellitus · Oxidative stress · T-cell exhaustion and functions

Abbreviations

CFSE	Carboxyfluorescein diacetate succinimidyl ester
HDL-C	High-density lipoprotein-cholesterol
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
IL-2	Interleukin 2
LDL-C	Low-density lipoprotein-cholesterol
PKB	Protein kinase B (AKT)
STZ	Streptozotocin

Introduction

Streptozotocin-induced type I diabetes is characterized by loss of weight, polydipsia, polyuria, glucosuria, polyphagia, hypoinsulinemia, and hyperglycemia [1]. Diabetes mellitus is usually associated with many metabolic

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complications [2–5]. These complications are caused among others by the action of free radicals [6, 7], which damage cellular components such as lipids, proteins, and DNA. Therefore, continuous hyperglycemia is a life-threatening risk factor for infection and diseases because it causes a functional decline in immune cells by increasing oxidative stress and blood lipids.

Although there is a large body of literature addressing the immune responses that cause type I diabetes, the suppression of T-cell immune response during diabetes is poorly understood. The proliferative response of T cells is a crucial step in cell-mediated immunity. In type I diabetes, the mechanisms leading to impaired proliferative response of T cells are still poorly defined. A previous study has reported a marked reduction in the proliferative response and IL-2 production of $CD4^+$ T cells among patients with type I diabetic [8]. A recent study has revealed that monocytes from patients with type I diabetes spontaneously secrete proinflammatory cytokines leading to altered T-cell response [9]. Moreover, modulation in the expression of CD28/CTLA-4 costimulatory molecules known to be involved in T-cell immune response was detected in patients with type I diabetes [10]. The CD28/CTLA-4–B7 system plays an important role in initiating and controlling T-cell immune response [11, 12]. CD28 signals are required to protect T cells from Fas-mediated apoptosis by activating PI3K/Akt pathway. Additionally, it was found that activation of the Fas ligand induced lymphocyte apoptosis in diabetic mice [13].

Improvement on immune function with dietary antioxidants can play an important role in preventing many human diseases [14–16] and diabetic complications. Vitamin C has attracted great interest for its useful physiologic and pharmacological properties. Vitamin C counteracts the potential damage caused by reactive oxygen species to cellular tissues. It modulates immune cell function by regulating redox-sensitive transcription factors and affecting the production of cytokines [17, 18]. Numerous studies have demonstrated that vitamin C supplementation enhances the immune cells response, influences the outcome and progression of diabetic complications [6], prevents DNA damage, and significantly decreases the risk of a wide range of pathologies such as cancers and degenerative and chronic diseases [19]. Moreover, previous studies suggest that the vitamin C levels in the plasma and tissues of diabetic patients and animals are lower than in those of normal groups [20, 21].

To assess whether vitamin C supplementation could influence the outcome and progression of diabetic complications, we investigated the impact of vitamin C on the improvement on T-cell immune response. We therefore focused on the influence of vitamin C supplementation on the T-cell activation, proliferation, and functions in an animal model of type I diabetes.

Materials and methods

Chemicals

Streptozotocin (STZ) was obtained from Sigma Chemical Co., St. Louis, MO, USA. STZ was dissolved in cold 0.01 M citrate buffer, pH 4.5, and was always freshly prepared for use within 5 min. Vitamin C was purchased from Sigma, France.

Experimental methods

Thirty male Sprague–Dawley laboratory rats weighing 160–190 g were obtained from the Central Animal House, Faculty of Pharmacy, King Saud University. All animal procedures were in accordance with the standards set forth in the guidelines for the care and use of experimental animals by the Committee for Purpose of Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH) protocol. The study protocol was approved by the Animal Ethics Committee of the Zoology Department, College of Science, King Saud University. Animals were allowed to acclimatize in metal cages inside a well-ventilated room for 2 weeks prior to the experiment. They were maintained under standard laboratory conditions—temperature 23 °C, relative humidity 60-70% and a 12-h light/dark cycle—and were fed a standard commercial pellet diet and water. All rats were fasted for 20 h before diabetes was induced with STZ. Male rats ($n = 20$) 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) [22]. Male rats 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 nondiabetic rats; group 2, diabetic rats; and group 3, diabetic rats orally supplemented with vitamin C (100 mg/kg body weight/day) [23] for 2 months. Blood glucose levels were measured 4 days after STZ injection by cutting off the tip of the tail, squeezing it gently and using OneTouch Ultra (LifeScan, Paris, France). Rats were considered diabetic if glycemia was higher than 220 mg/dl.

Hydroperoxide levels

Blood levels of hydroperoxide were evaluated using a free-radical analytical system (FRAS 2, Iram, Parma, Italy). The test is a colorimetric test that takes advantage of the ability of hydroperoxide to generate free radicals after reacting with transitional metals [24].

Plasma glucose, protein, and lipid profile

Blood glucose levels were measured with the glucose oxidase method [25] using BioMerieux kits (France); protein levels were measured by the cupric ion reaction [26]. The intensity of the coloration was measured using a UV/visible Model 80-2106-00 spectrophotometer at 546 nm (Pharmacia Biotech, Cambridge, England).

Lipid profiles were determined colorimetrically with BioMerieux kits and a standard assay method. Cholesterol levels were evaluated using the cholesterol esterase method [27]. Triglycerides were measured using lipase method [28]. In the HDL assay, performed according to Lopez-Virella [29], LDL, and chylomicrons were precipitated with phosphotungestic acid. The amount of cholesterol bound to HDL was determined using the cholesterol oxidase method and the phosphotungstate-magnesium salt method using a Cholesterol E-Test Kit (Wako, Osaka, Japan).

Blood samples, plasma, PBMCs, and $CD3^+$ T-cell isolation

At the end of the experiment, animals were anesthetized with pentobarbital (60 mg/kg body weight). Whole blood was drawn from the abdominal aorta. After plasma isolation, half of the obtained blood cells were subjected to peripheral blood mononuclear cell (PBMCs) isolation using a Ficoll gradient method; the other half was subjected to $CD3^+$ T-cell purification according to the manufacturer's protocol for the MagCellect Rat $CD3^+$ T-cell isolation kit (R&D Systems Europe Ltd, Abingdon, UK). The final purity was 92–95% (as assessed by flow cytometry). Isolated PBMCs and purified $CD3^+$ T cells were either freshly used for experiments or cryopreserved in liquid nitrogen for later use. Frozen PBMCs or $CD3^+$ T cells were thawed and cultured in RPMI 1640 supplemented with 10% FCS and HEPES (R-10 media) for at least 4 h. Cells were then washed twice in prewarmed R-10 to exclude the dead cells before starting the experiments.

Cell surface and intracellular phospho-specific flow cytometry

Directly conjugated antibodies against CD3 (pan T cells), CD28, and CTLA4 (costimulatory) surface molecules were used; these antibodies included CD3-allophycocyanin (APC) (clone 1F4) and CD28-phycoerythrin (PE) (clone JJ319), all from BD Biosciences, San Jose, CA), as well as CTLA4-fluorescein isothiocyanate (FITC) from abcam (ab33967). Control stains were used to determine the background levels of staining. Four-color flow cytometry analysis was performed using a standard FACSCalibur (BD Biosciences) with FACSDiva software (BD Biosciences). Compensation was performed with single fluorochromes and BD CompBeads (BD Biosciences). Data files were analyzed using FlowJo software, version 9 for Mac (Tree Star, Inc., Ashland, OR). All flow cytometry assays were performed on freshly isolated PBMCs. For surface expression of CD28 within the $CD3^+$ T-cell population, 1×10^6 PBMCs were stained with the freshly prepared surface antibodies anti-CD28-PE and anti-CD3-APC in fluorescence-activated cell sorting (FACS) buffer (1× phosphate-buffered saline [PBS], 1% fetal calf serum [FCS], 0.02% NaN_3); for 30 min at 4 °C. For surface expression of CLTA-4 in the T-cell population, PBMCs were stimulated for 1 h prior to staining with anti-CTLA-4-FITC and anti-CD3-APC. Samples were then washed twice in FACS buffer and fixed in FACS fixation buffer (1× PBS, 1% formaldehyde).

For intracellular phospho-AKT, PBMCs were costimulated with anti-CD3 mAb (2 μg/mL; BD Biosciences) anti-CD28 mAb (2 μg/mL; BD Biosciences) or incubated without stimulation for 10 min at 37 °C and then fixed for 10 min in pre-warmed cytofix buffer (BD Cytofix #554655). Cells were then permeabilized for 30 min on ice in PERM III buffer (BD PERM-III buffer #558050). Permeabilized PBMCs were washed twice and then re-suspended in staining buffer (phosphate-buffered saline plus 0.5% bovine serum albumin) and stained in a final volume of 100 μl for 30 min at room temperature. The directly conjugated antibodies used were specific for rat CD3-APC (BD Biosciences), phospho-AKT (S472, S473, S474), and control IgG (R&D systems, France). Cells were fixed and analyzed for phospho-AKT in the $CD3^+$ population. Flow cytometry was used to evaluate phospho-AKT-positive and -negative cells within the $CD3^+$ T-cell population.

Western blot analysis

Purified $CD3^+$ T cells were re-suspended at a density of 1×10^7 cells/mL in pre-warmed RPMI 1640 without FCS and stimulated for 5 min at 37 °C with medium or a mix of anti-CD3 mAb (2 μg/mL) and anti-CD28 mAb (2 μg/mL). Lysates were prepared as previously described [30]. Equal amounts of total cellular protein were subjected to SDS–PAGE and blotted onto a nitrocellulose membrane (MilliPore, Bedford, MA, USA). The primary antibodies recognizing phospho-AKT (S473) and AKT (MilliPore, Bedford, MA, USA) were diluted 1:1000. After incubation with secondary antibodies conjugated to HRP (rabbit anti-mouse (DAKO) or goat anti-rabbit (Santa Cruz Biotechnology), the antigens were visualized using chemiluminescence (ECL, Supersignal West Pico chemiluminescent substrate, Perbio, Bezons, France) and acquired with X-ray film (Amersham Biosciences, France). The ECL signal was recorded on ECL hyperfilm.

To quantify band intensities, films were scanned, saved as TIFF files and analyzed with NIH ImageJ software.

F-actin polymerization assay

Intracellular F-actin polymerization was assessed as previously described [31]. PBMCs from each group (8×10^6/mL) were incubated in HEPES-buffered RPMI 1640 at 37 °C, with or without anti-CD3/CD28. At the indicated times, cells (100 µl) were added to 400 µl of assay buffer containing 4×10^{-7} M FITC-labeled phalloidin, 0.5 mg/mL L-α-lysophosphatidylcholine (both from Sigma–Aldrich) and 4.5% formaldehyde in PBS. Fixed cells were analyzed by flow cytometry, and the mean fluorescence intensity (MFI) was determined for each sample. The percent change in MFI was calculated for each sample at each time point as follows: [1-(MFI before addition of anti-$CD3^+$ CD28/MFI and after addition of anti-$CD3^+$ CD28)] × 100.

CFSE proliferation assays

PBMCs were resuspended at 20×10^6/mL PBS and stained with 0.63 mM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) for 8 min at room temperature. The reaction was stopped with FBS; cells were washed three times in PBS and then resuspended at 2×10^6 cells/mL in prewarmed R-10 medium. CFSE-labeled cells were stimulated for 6 days with medium, a mix of anti-CD3 mAb (2 µg/mL) and anti-CD28 mAb (2 µg/mL) or Concanavalin A (ConA; 2 µg/mL) as a positive control at 37 °C and 5% CO_2. On day 6, cells were stained for surface antigens with anti-CD3-APC, fixed, and directly analyzed by flow cytometry.

Detection of apoptotic cells

PBMCs were fixed and permeabilized via incubation in 70% ice-cold ethanol for at least 1 h and then washed twice in PBS. DNA was stained by incubating cells at 37 °C for 1 h in 40 µg/mL propidium iodide and 100 µg/mL DNase-free RNase in PBS. Samples were analyzed by assessing FL2 red fluorescence on a linear scale. The percentage of cells undergoing apoptosis was determined as the percentage of hypodiploid cells (sub G0/G1 peak). Dead cells were determined with the Trypan blue exclusion test.

Intracellular cytokine staining (ICS) assay

PBMCs (2×10^6/mL R-10) were stimulated with a mix of anti-CD3 mAb (2 µg/mL) and anti-CD28 mAb (2 µg/mL) for 1 h at 37 °C and 5% CO_2 in 5 mL snap-cap polypropylene tubes set at an angle of 5°. Following 1 h of either stimulation or lack of stimulation, 10 µg/mL of brefeldin A (Sigma–Aldrich, France) and 6 µg/mL of monensin sodium salt (Sigma–Aldrich, France) were added, and cells were incubated for a total of 12 h. Cells were then washed with FACS buffer, stained for 30 min at 4 °C CD3-APC, and permeabilized using BD Cytofix/Cytoperm solution (BD Bioscience, France). Intracellular staining with anti-IL-2-FITC (Abcam) and anti-IFN-γ-PE (BD Biosciences) was performed for 30 min, and then the cells were washed twice in BD Perm/Wash buffer (BD Biosciences, France) and fixed in 200 µl of FACS fix buffer. Polyfunctional data were exported using Boolean gates in FlowJo and further analyzed using PESTLE software (version 1.5.4) and SPICE software (version 4.1.6) obtained from M. Roederer, National Institutes of Health, Bethesda, MD. For analysis, cells were gated on viable $CD3^+$ T cells. The percent specific expression was calculated as a background-adjusted function in the presence or absence of CD3/CD28 stimulation. Polyfunctional T cells were defined as viable $CD3^+$ T cells that produce IFN-γ and IL-2 simultaneously (IFN-γ^+ IL-2), in contrast to monofunctional T cells, which produce either IL-2 or IFN-γ alone.

Statistical analysis

The data are expressed as means ± SEM (standard error of mean). Statistical differences between groups were analyzed with a one-way analysis of variance (for more than two groups) followed by Tukey post-test using SPSS software version 17. Differences were considered statistically significant at $P < 0.05$.

Results

Vitamin C treatment improves complications of diabetes

We monitored changes in body weight and plasma parameters in all animal groups throughout the experiment period. We first observed a significant decrease in the total body weight (32%) in the diabetic group compared with the control. A significant increase in the body weight was observed in vitamin C-treated diabetic group compared to diabetic group (Table 1). We observed a significant 3.35- and 2.53-fold elevation in blood glucose levels in the diabetic and vitamin C-treated diabetic groups, respectively, compared to the control group. The glucose levels in the vitamin C-treated diabetic group were found to be significantly lower than those in the diabetic group ($^+P < 0.05$) (Table 1). Blood hydroperoxide levels were also found to be significantly lower in the control and vitamin C-treated groups compared to the diabetic group. The levels of cholesterol, triglycerides, and LDL in the

Table 1 Effect of vitamin C supplementation during diabetes on body weight, blood glucose level, total blood protein, and plasma lipid profile

Parameters	Time post-treatment	Control	Diabetic	Diabetic + vitamin C
Body weight (g)	Zero	168. 7 ± 8.89	179.1 ± 8.82	182.2 ± 12.4
	2 Months	297. 9 ± 11.76	202.4 ± 12.73*	263.2 ± 17.1$^{+,\#}$
Plasma glucose (mg/100 mL)	Zero	87.03 ± 2.58	268.3 ± 28.40*	250.4 ± 13.9$^{+,\#}$
	2 Months	90.2 ± 1.57	301.9 ± 19.64*	228.9 ± 16.8$^{+,\#}$
Cholesterol (mg/100 mL)		73.7 ± 3.8	93.8 ± 5.6*	80.7 ± 2.9$^{+,\#}$
Triglycerides (mg/100 mL)		55.3 ± 3.8	90.5 ± 5.7*	53.8 ± 3.02^{+}
HDL-C (mg/100 mL)		18.7 ± 1.2	15.1 ± 1.6	17.3 ± 1.2
LDL-C (mg/100 mL)		49.6 ± 2.1	69.2 ± 4.6*	58.7 ± 2.8^{+}
Hydroperoxide (mg/100 mL)		26.4 ± 1.5	40.8 ± 2.4*	31.8 ± 1.4^{+}
Total protein (g/100 mL)		7.3 ± 0.24	6. ± 0.06*	6.9 ± 0.19

Results are expressed as means ± SEM. * $P < 0.05$, diabetic vs. control; $^{+}$ $P < 0.05$, VC-treated diabetic vs. diabetic; $^{\#}$ $P < 0.05$, VC-treated diabetic vs. control (ANOVA with the Tukey post-test)

diabetic group were found to be significantly ($*P < 0.05$) increased by 1.3-, 1.6-, and 1.4-fold over the control values, respectively (Table 1). The administration of vitamin C significantly restored the levels of cholesterol, triglycerides, and LDL in diabetic rats. Nevertheless, the levels of HDL and total protein were not significantly altered among the groups.

Vitamin C reverses CD28 down-regulation during diabetes

We next investigated the potential impact of vitamin C on the expression of costimulatory molecules in T lymphocytes. CD28 was found to be strongly expressed in control rats (MFI > 350). We observed a significantly lower surface expression of CD28 on the T cells of diabetic rats. During diabetes, vitamin C was found to partially (MFI > 280) but not completely restore the surface expression of CD28 on T lymphocytes (Fig. 1a). In contrast, the administration of vitamin C did not alter the surface expression of CTLA4 in diabetic rats (Fig. 1b). Accumulated data from nine rats in each group revealed that the MFI of CD28 surface expression on T cells was significantly lower in diabetic rats than in control rats (354 ± 9.6 in control rats versus 18 ± 4.9, $*P < 0.05$ in diabetic rats, Fig. 1c). Vitamin C-treated rats exhibited a significantly higher level of CD28 expression than untreated diabetic rats (MFI = 281 ± 8.9), $^{+}P < 0.05$.

Vitamin C restores CD28/PI3K/AKT signaling during diabetes

We investigated whether CD28 signaling, which is important for T-cell survival and long-term responsiveness, was impaired in diabetic rats. We found that the phosphorylation of AKT underlying TCR/CD28 ligation was significantly reduced ($*P < 0.05$) in the diabetic group (MFI = 110 ± 17.8) by 4.38 fold compared to the control group (MFI = 482 ± 19) (Fig. 2a). Impaired signaling from TCR/CD28 to PI3K and hence impaired phosphorylation of AKT were found to be significantly restored (MFI = 319 ± 21) in the vitamin C-treated rats ($^{+}P < 0.05$). Because antibodies directed against rat molecules vary in their sensitivity depending on the technique used, we confirmed our results using Western blot analysis. When the levels of phosphorylated AKT were normalized to the total amount of AKT, we observed that AKT phosphorylation was 8.74-fold lower in diabetic rats (213 ± 20.9) than in the control group (1,465 ± 29) with $*P < 0.05$ (Fig. 2c). In the vitamin C-treated group, the phosphorylation of AKT was found to be significantly restored (MFI = 849 ± 31, $^{+}P < 0.05$).

Vitamin C sustains TCR-mediated actin polymerization and T-cell responsiveness

Actin and microtubules provide the dynamic cellular framework to orchestrate and ultimately control T-cell activation. Therefore, actin polymerization upon TCR/costimulation was monitored in T cells in the three animal groups. PBMCs were stimulated every 15 s with anti-CD3/CD28 mAbs, and the degree of F-actin polymerization was determined by flow cytometry. In the control group, the percentage of F-actin polymerization was 95 ± 12, 75 ± 11, 17 ± 4.3 and 1 ± 1.8 at 15, 30, 45 and 60 s, respectively (Fig. 3). Similarly, the percentage of F-actin polymerization was 70 ± 11, 50 ± 11.4, 20 ± 7.6, and 10 ± 3.9 at 15, 30, 45, and 60 s, respectively, in the vitamin C-treated group. In contrast, in the diabetic group, the percentage of F-actin polymerization was decreased to 42 ± 8.6, 9 ± 6.6, 1 ± 4, and 0 at 15, 30, 45, and 60 s, respectively.

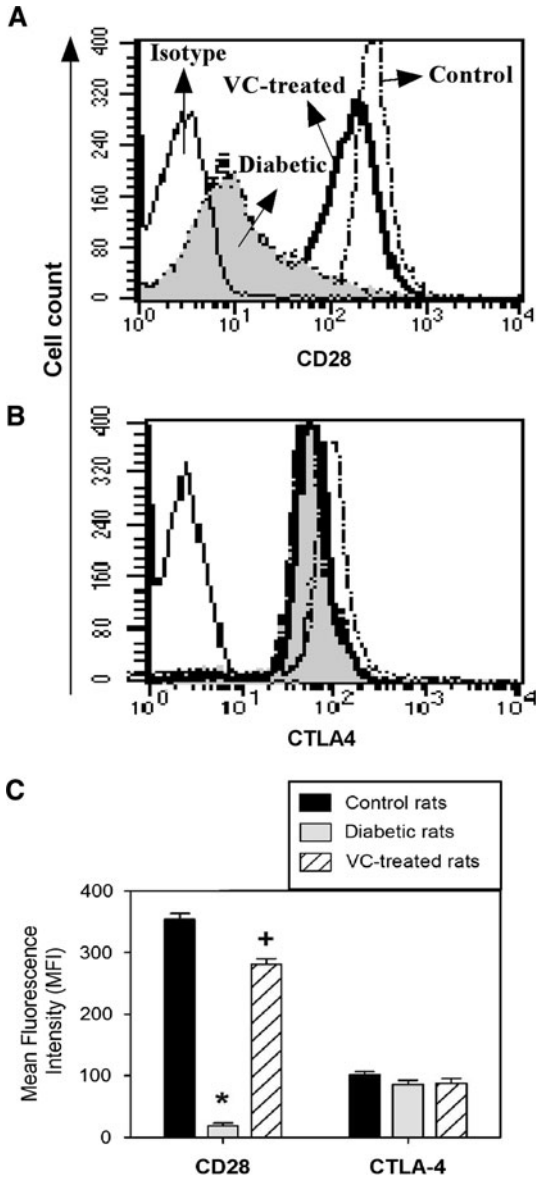

Fig. 1 Vitamin C treatment during diabetes rescues the surface expression of CD28 but not that of CTLA-4 on T lymphocytes. Surface expression of CD28 and CTLA4 on T cells of the PBMCs was analyzed by flow cytometry using directly conjugated mAbs: anti-CD3-APC and anti-CD28-PE or anti-CTLA4-FITC. Mean fluorescence intensity (*MFI*) of specific staining (CD28-positive population minus IgG isotype control) for surface expression of CD28 was evaluated on the T cells of control (*open*, *dotted line histogram*), diabetic (*closed*, *gray histogram*), and vitamin C-treated (*open*, *bold line histogram*) groups. One representative experiment out of nine is shown. **a** For the surface expression of CTLA-4, PBMCs were cultured for 3 h and stimulated with CD3/CD28 for 1 h before staining with anti-CD3-APC and anti-CTLA-4-FITC. The MFI of specific surface staining (CTLA4-positive population minus IgG control) for CTLA-4 was evaluated for T cells of the control (*open*, *dotted line histogram*), diabetic (*closed*, *gray histogram*), and vitamin C-treated (*open*, *bold line histogram*) groups. One representative experiment out of nine is shown. **b** The experiment was performed on nine rats from each group, and results are expressed as the mean percentage ± SEM of MFI values (**c**). $^{*}P < 0.05$, diabetic vs. control; $^{+}P < 0.05$, VC-treated diabetic vs. diabetic (ANOVA with the Tukey post-test)

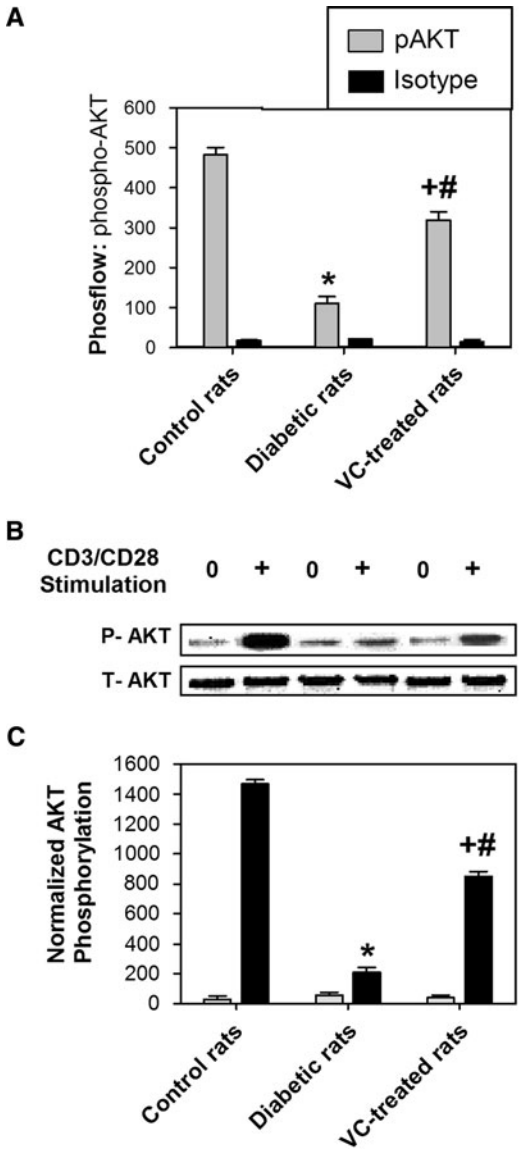

Fig. 2 TCR/CD28/AKT signaling in T cells is impaired in diabetic rats. We analyzed the phosphorylation of AKT (pAKT), the main signaling pathway downstream of TCR/CD28 ligation, using flow cytometry with Phosflow mAbs anti-pAKT-PE and anti-CD3-APC or an isotype control. PBMCs with or without a 10-min stimulation with anti-CD3/CD28 were subjected to the Phosflow technique as described in the "Materials and methods". The MFIs of phospho-AKT (*gray bars*) and the isotype control (*black bars*) were monitored in a T-cell population; results are expressed as the mean percentage ± SEM of MFI values (**a**) $^{*}P < 0.05$, diabetic vs. control; $^{+}P < 0.05$, VC-treated diabetic vs. diabetic; $^{\#}P < 0.05$, VC-treated diabetic vs. control (ANOVA with the Tukey post-test). To further confirm the impairment of TCR/CD28 signaling during diabetes, PBMCs were stimulated with anti-CD3/CD28, lysed, and analyzed by Western blotting as described in the "Materials and methods". One representative experiment out of three is shown (**b**). The phosphorylation of AKT was normalized against the total relevant protein (pan AKT) on stripped blots. The experiment was conducted for nine rats from each group, and results are expressed as the mean ± SEM normalized phosphorylation values (**c**). $^{*}P < 0.05$, diabetic vs. control; $^{+}P < 0.05$, VC-treated diabetic vs. diabetic; $^{\#}P < 0.05$, VC-treated diabetic vs. control (ANOVA with the Tukey post-test)

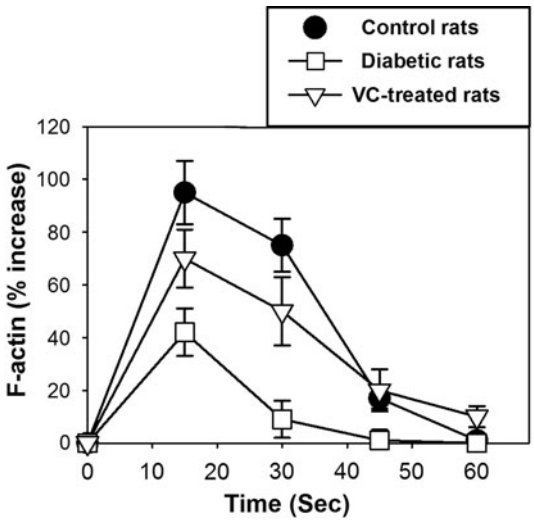

Fig. 3 Vitamin C supplementation reverses impairment of TCR-mediated F-actin polymerization in diabetic rats. PBMCs were isolated from control (*closed symbols*), diabetic (*open squares*), and vitamin C-treated groups (*open triangles*). F-actin polymerization was measured in response to anti-CD3/CD28. Results are expressed as the percentage change in MFI ($n = 6$) ± SD as described in the "Materials and methods"

Vitamin C enhances TCR-mediated proliferation and renders T cells resistant to apoptosis during diabetes

We also monitored T-cell function during diabetes and after vitamin C administration using CFSE proliferation and ICS assays. As shown in Fig. 4a, the percentage of proliferating T cells was found to be reduced from 64% in the control rats to 23% in the diabetic rats. In the vitamin C-treated rats, however, the percentage of T-cell proliferation was found to be 55%. Data revealed that the proliferative capacity of TCR/CD28-stimulated T cells was significantly reduced from 50 ± 5.9% in the control group to 25 ± 6.9% in the diabetic group ($^{*}P < 0.05$; Fig. 4b). The administration of vitamin C significantly rescued T-cell proliferation to 42 ± 6.7% compared to untreated diabetic group, $^{+}P < 0.05$. A similar result was obtained when PBMCs were stimulated with a mitogen (ConA) as a positive control.

After 6 days in culture for the CFSE assay, PBMCs were counted using the Trypan blue exclusion test. The number of dead cells (Trypan blue positive cells) was evaluated in each experiment. We found a twofold increase in the percentage of dead PBMCs in the diabetic group (45 ± 9.7%) compared to the control group (22 ± 7.7%), $^{*}P < 0.05$ (Fig. 4c). The percentage of dead PBMCs in the vitamin C-treated diabetic group was found to be partially and significantly reduced to 32 ± 6.8% compared to untreated diabetic group ($^{+}P < 0.05$). Other cells were stained in parallel for propidium iodide (PI) and CD3 to evaluate the percentage of apoptotic cells within the T-cell

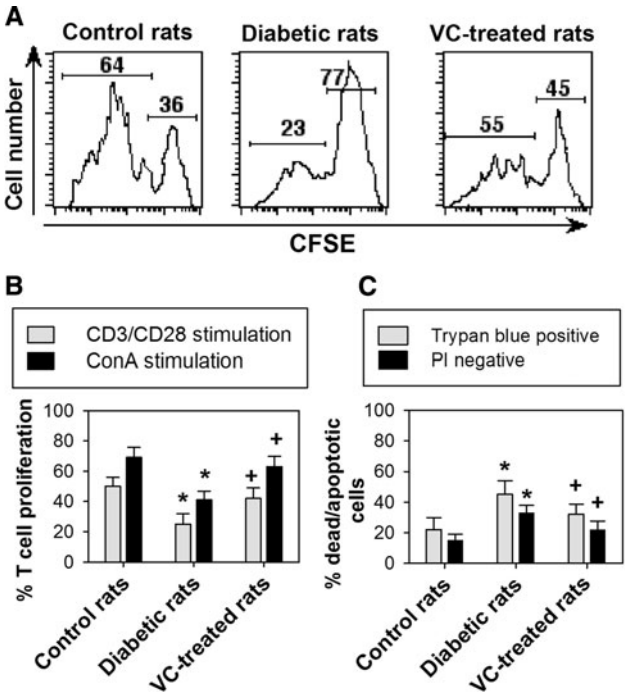

Fig. 4 Vitamin C supplementation restores the ability of exhausted T cells to proliferate following TCR/CD28 or mitogen stimulation during diabetes. The ability of T cells to proliferate in response to TCR/CD28 or mitogen stimulation was evaluated using the CFSE assay and flow cytometry. CFSE-labeled PBMCs, with or without a stimulation with anti-CD3/CD28 or ConA, were cultured for 6 days. Cells were then stained with anti-CD3-APC to monitor T-cell proliferation. Histograms were gated on viable $CD3^{+}$ T cells of control, diabetic, and vitamin C-treated diabetic groups. The mean of the *left histograms* represents the percentage of CFSE-lo (proliferating cells), while the mean of the *right histograms* represents the percentage of CFSE-high (nonproliferating cells) within the T-cell population. One representative experiment is shown (**a**). Data are expressed as the percentage of CFSE negative cells (% of proliferating T cells) ± SEM in response to CD3/CD28 (*gray bars*) or ConA (*black bars*); $n = 9$ (**b**). In each experiment, two parallel tests were conducted: the Trypan blue exclusion test (*gray bars*) to discriminate between dead and viable cells and PI and CD3 APC staining to evaluate the apoptotic T cells (PI-negative cells; *black bars*) (**c**). $^{*}P < 0.05$, diabetic vs. control and $^{+}P < 0.05$, VC-treated diabetic vs. diabetic (ANOVA with the Tukey post-test)

population. The percentage of apoptotic T cells (PI-negative cells) was found to be elevated from 15 ± 3.99% in the control group to 33 ± 4.9% in diabetic group, $^{*}P < 0.05$. In the vitamin C-treated group, the percentage of apoptotic T cells was found to be partially and significantly decreased to 22 ± 5.6% compared to the untreated diabetic group ($^{+}P < 0.05$).

Vitamin C converts exhausted T cells to polyfunctional cells during diabetes

We analyzed cytokine production as a major T-cell function following TCR/CD28 stimulation and found that diabetic rats exhibited decreased IL-2 or IFN-γ production as

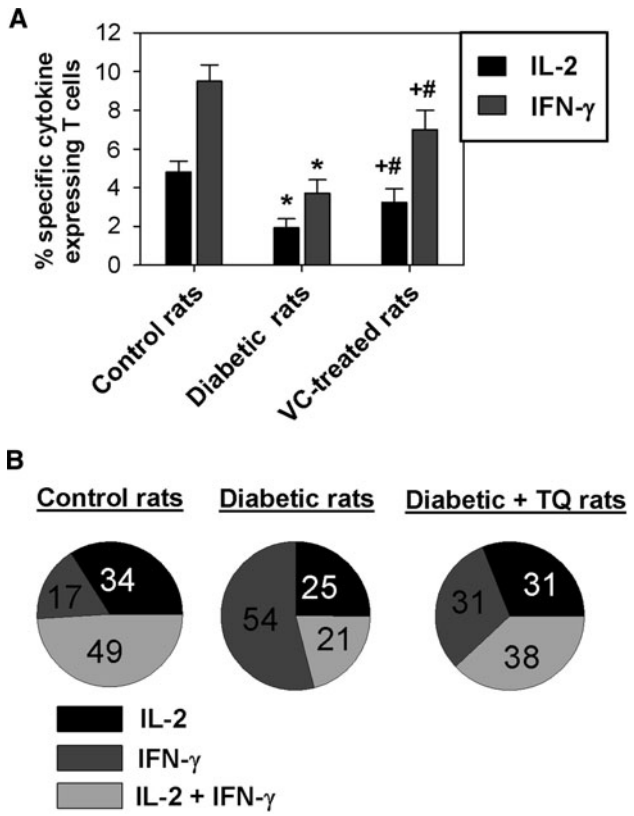

Fig. 5 Vitamin C supplementation rescues polyfunctional T-cell production during diabetes. The ability of activated T cells to produce cytokines was monitored in the three animal groups. Analysis of cytokine production by ICS assay following anti-CD3/CD28 stimulation, represented as the percentage of specific expression for each individual cytokine ± SEM, i.e., the percentage of IL-2 (*black bars*) and IFN-γ (*dark gray bars*), were gated on viable $CD3^+$ T cells (**a**). $^*P < 0.05$, diabetic vs. control; $^+P < 0.05$, VC-treated diabetic vs. diabetic; $^\#P < 0.05$, VC-treated diabetic vs. control (ANOVA with the Tukey post-test). Polyfunctionality of T cells was monitored on viable $CD3^+$ T cells, where *light gray color* represents two functions simultaneously (IFN-γ and IL-2 production), *dark gray color* represents one function (IFN-γ production), and *black color* represents one function (IL-2 production). *Numbers* on the *pie* chart represent the percentage of cells in each population

demonstrated by ICS (Fig. 5a). The percentage of T cells that were producing IL-2 was significantly decreased from 4.8 ± 0.56% in the control group to 1.92 ± 0.48% in the diabetic group ($^*P < 0.05$). In the vitamin C-treated group, the percentage of T cells that were producing IL-2 was significantly restored to 3.22 ± 0.72% ($^+P < 0.05$) compared to diabetic group. Similarly, the percentage of T cells producing IFN-γ was significantly reduced from 9.5 ± 0.84% in the control group to 3.7 ± 0.70% in the diabetic group ($^*P < 0.05$). In the vitamin C-treated group, the percentage of T cells that were producing IFN-γ was significantly restored to 7 ± 0.99% compared to the diabetic group ($^+P < 0.05$). The T cells of the diabetic rats were primarily monofunctional (IFN-γ producers). Notably, the generation of polyfunctional T cells was robust in the control and vitamin C-treated groups. Polyfunctional cells constituted 21% of all T cells in the diabetic group versus 49% and 38% in the control and vitamin C-treated groups, respectively (Fig. 5a).

Discussion

Vitamin C is an essential nutritional component and an important therapeutic supplement in normal and acute conditions because of its many biochemical functions [19, 32]. It is a strong aqueous antioxidant and a cofactor for several enzymes. Severely critical illness may require increased levels of vitamin C owing to higher levels of oxidative stress [32]. Although the daily requirement of a human person for vitamin C has now been established at 100 mg, there is no evidence for any adverse effects at the intakes up to 4 g/day [33]. We used a daily dose (100 mg/kg body weight) of vitamin C which equivalents to 1 g for adult human based on data from Food and Drug Administration (FDA) guidelines to convert drug dose from human to animal. Here, we found that the concentration of lipid hydroperoxides, which is an effective index of oxidative stress, is elevated in diabetic rats. Similar results were published by Yadav et al. [34], who found that hyperoxide levels increase in streptozotocin-induced diabetic rats. Our data reveal that vitamin C supplementation improves diabetic complications because it significantly decreases the elevated levels of blood hydroperoxide, glucose, cholesterol, triglycerides, and LDL. A significant increase in arterial elasticity index, a significant improvement in glucose and lipid metabolism and a significant increase in HDL-cholesterol were also observed in patients who were treated with antioxidants including vitamin C [35]. Additionally, the beneficial effect of antioxidant supplementation on LDL oxidation has been demonstrated [36, 37]. The mechanisms by which the antioxidants inhibit atherosclerotic factors and improved glucose metabolism remain to be clarified. Combined supplementation of vitamins E and C have been shown to inhibit DNA oxidation by H_2O_2 in human lymphocytes, to enhance endogenous plasma and tissue antioxidant defenses and restore endothelium-dependent vasoactivity [36, 38, 39].

Recently, an inverse correlation was demonstrated between blood lipid profile and T-cell proliferative capacity in dogs, where the reduction in total blood cholesterol, LDL, and non-HDL-cholesterol levels was correlated with an increase in T-cell proliferation [30]. Another study revealed that the improvement in T-cell function during diabetes was probably partially a result of the reduction in total blood cholesterol and LDL [40]. Ascorbic deficiency, in turn, impairs T-cell-mediated immunity [21].

The B7/CD28 pathway provides critical costimulatory signals required for complete T-cell activation and has served as a potential target for immunotherapeutic strategies designed to regulate autoimmune diseases [41]. Blockade of the CD28/B7 interaction resulted in an inhibition of induction and progression of autoimmunity in a number of experimental models [42]. However, mice with defect in CD28/B7 costimulatory pathway have exacerbated autoimmune diabetes [43]. Our data demonstrate that diabetic rats have a significantly lower level of CD28 surface expression on T cells. Similarly, the expression of co-stimulatory molecule CD28 was decreased in children with type I diabetes [10]. Additionally, decreased CD28 expression has been observed in dysfunctional peripheral T-lymphocytes from patients with hairy-cell leukemia, chronic lymphocytic leukemia, colorectal cancer, and breast cancer [44]. T-cell activation requires at least two distinct signals, signaling via the Ag-specific TCR and a co-stimulatory pathway [45–47], for proliferation, differentiation, and acquisition of effector functions. CD28 signals are required to protect T cells from apoptosis mediated by the death receptor Fas [48, 49]. Therefore, without co-stimulatory signals, i.e., the T-cell receptors on T cells or APC ligands, T cells will undergo apoptosis or become anergic [50, 51]. CD28 may also contribute to T-cell viability by increasing glucose metabolism in activated T cells [45]. Thus, the lower level of CD28 surface expression on T cells from diabetic rats could explain the observed two-fold higher level of dead cells in the diabetic group.

Vitamin C-treated rats were found to have a significantly higher level of CD28 expression on T cells compared with the untreated diabetic group. Co-stimulatory signals play crucial roles in all phases of T-cell activation [46]. Activation signals are transmitted by receptor-associated protein tyrosine kinases and phosphatases through calcium mobilization to a secondary cascade of kinases. These kinases activate transcription factors that lead to actin polymerization, cell proliferation, and cytokine production. In this study, we found that the impaired signaling from TCR/CD28 to PI3K and resulting reduction in AKT phosphorylation in diabetic rats was significantly rescued by vitamin C treatment. These results were confirmed by Western blot analysis. PI3K activation is a pre-requisite for CD28-mediated protection against Fas [44, 47]. Signaling downstream of the IL-2 receptor can also act through the PI3K/AKT pathway [51]. Through this pathway, co-stimulation controls T-cell expansion and proliferation; this pathway is also utilized for long-term T-cell survival and memory development [47]. Unlike CD28, CTLA4 is a negative regulator of T-cell activation because it is not expressed on resting T cells but is induced after the initial steps of T-cell activation. CD28 and ICOS provide positive signals that promote and sustain T-cell responses, while CTLA-4 and PD-1 limit such responses. The balance between the two co-signals determines the ultimate nature of the T-cell response [49]. Our data show that CTLA-4 expression on T cells is not significantly altered by vitamin C administration, although it is slightly lower in diabetic rats.

Actin polymerization plays a critical role in activated T lymphocytes by regulating TCR-induced immunological synapse formation and signaling, cell migration, morphogenesis, cellular trafficking, cytokinesis, and cell proliferation [52]. In the vitamin C-treated group, T cells were found to sustain long-term F-actin polymerization after TCR/CD28 ligation, suggesting that these T cells sustain long-term responsiveness. T cells in the diabetic group displayed transient F-actin polymerization that peaked at 15 s and declined rapidly thereafter. Defective actin polymerization leads to long-term T-cell unresponsiveness or anergy [53]. We found that the percentage of proliferating T cells was significantly reduced in diabetic rats and increased in vitamin C-treated rats. Therefore, the level of CD28 surface expression in vitamin C-treated and untreated diabetic rats could be the potential determinant of T-cell survival. Trypan blue discriminates only between dead and viable cells and does not distinguish between cell types; thus, CD3-APC and propidium iodide staining of PBMCs was employed to determine the percentage of apoptotic T cells in PBMCs. Overall, the percentage of apoptotic T cells in all animal groups was found to be lower than the percentage of dead cells detected by Trypan blue, suggesting that diabetes complications can affect other types of cells that are also protected by vitamin C.

In the present study, the analysis of intracellular cytokine expression as a major T-cell function following TCR/CD28 stimulation revealed that T cells from diabetic rats exhibit decreased IL-2 or IFN-γ expression. Moreover, the T cells of diabetic rats were found to be primarily monofunctional (IFN-γ producers). Unlike the diabetic group, the generation of polyfunctional T cells (IFN-γ- and IL-2-double positive cells) was detected in the vitamin C-treated group. Furthermore, in this group, a higher percentage of T cells produced IL-2. Kahwa et al. [54] demonstrated that administration of a mega-dose of vitamin C shifts the immunity of Balb/c mice toward Th1 (increased IL-2, IFN-γ, and TNF-α production). Co-stimulation is essential for optimal cytokine production; in the absence of co-stimulation, cytokine secretion and T-cell expansion, proliferation, survival, and memory development are affected [55, 56]. Taken together, these results explain the significant restoration of IL-2 levels in vitamin C-treated rats, which have a high expression of CD28. There are currently no data on the treatment of the diabetic impairment of immune function with vitamin C that would indicate whether this intervention has a significant clinical

impact. The potential role of this substance in the treatment of diabetic immune impairment needs to be intensively investigated in future research focusing on patient-oriented outcomes. We have shown here that there is a strong, positive correlation between vitamin C and T-cell-mediated immune function during diabetes in an animal model.

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