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ORIGINAL ARTICLE

Diabetes-induced alteration of F4/80$^+$ macrophages: a study in mice with streptozotocin-induced diabetes for a long term

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Abstract Macrophages as an early stage of immune responses form a bridge between innate and acquired immunity and shape the adaptive immune response. The immunoregulatory functions of macrophages in hosts with a prolonged exposure to a diabetic milieu remain to be determined. The levels, phenotype, and immunity including antigen-presenting ability, phagocytosis and immunogenicity of F4/80$^+$ splenic macrophages (SPMs), and peritoneal exudates macrophages (PEMs) were detected in age-matched control mice and mice with streptozotocin (STZ)-induced diabetes for 16 weeks. The numbers of F4/80$^+$ SPMs and PEMs significantly decreased in STZ-induced diabetic mice, compared with age-matched non-diabetic mice (control) at 16 weeks after diabetes induction. Functional analysis showed that F4/80$^+$ SPMs and PEMs in STZ-induced diabetic mice exhibit significantly lower immunogenicity and nonopsonic phagocytosis to allogeneic T cells than those of control mice both in vitro and in vivo. Coincidently, the antigen-presenting capacity of F4/80$^+$ PEMs, but not F4/80$^+$ SPMs, in mice with STZ-induced

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diabetes for 16 or more weeks is also significantly lower than that of control mice. Our results showed that total cell number and immune function of F4/80$^+$ macrophages were significantly defective in mice with a prolonged exposure to a diabetic milieu, which may be a mechanism responsible for the increased macrophage-related complications in diabetic patients such as the high prevalence of infection and cardiovascular mortality.

Keywords Monocytes/macrophages · Diabetes · Autoimmune · Immunodeficiency · Diabetic complications

Abbreviations

APCs	antigen-presenting cells
DCs	dendritic cells
DTH	delayed-type hypersensitivity
FCM	flow cytometry
IFN-γ	interferon-γ
IL-2	interleukin-2
MFI	median fluorescence intensity
MLR	mixed leukocyte reactions
PEMs	peritoneal exudate macrophages
SPMs	splenic macrophages

Introduction

Insulin-dependent diabetes mellitus, also known as type 1 diabetes (T1D), is an organ-specific autoimmune disease resulting from the destruction of insulin-producing pancreatic beta cells. Macrophages, dendritic cells (DCs), B cells, and T cells have been shown to be involved in the pathogenesis of autoimmune diabetes [1]. Macrophages, which are the first cell type to infiltrate the pancreatic islets, serve as antigen-presenting cells (APCs), and are important as effector cells, are critical in the development and pathogenesis of autoimmune diabetes [2, 3]. Altered monocytes/macrophages in NOD mice have been reported in the initial or early stage of diabetes [4–7]. Although we have understood the critical role of the altered macrophages in the development of T1D, we have known much less for the chronic effects of diabetes on the phenotype and immunity of host macrophages, which may be related with the occurrence of the complications and high risk of infections of patients with a prolonged exposure to a diabetic milieu [8]. A few studies showed that the cytokine production by macrophages is significantly impaired in diabetic animal models [9–11].

Several previous studies [4–7] on T1D were done in small animal models that develop diabetes spontaneously, such as NOD mice [12]. Despite the fact that the pancreas is the main target organ severely affected by the immune system, extrapancreatic signs of autoimmunity such as autoimmune thyroiditis and adenosialitis are observed in NOD mice, and also frequently diagnosed in T1D patients [13, 14]. The NOD mouse as a genetic deficient mouse model is not the appropriate model to be used for studies on the impacts of diabetes itself on the immunological system and on the function of other organs. Therefore, in previous studies [4, 5, 12, 15], it is hard to explain the defects of macrophages in NOD mice due to the genetic effects or the subsequent effects of diabetes in this model [12, 16]. Streptozotocin (STZ)-induced diabetic mice could be used as a diabetes experimental model to specifically study the effects of diabetes on macrophages or other cells [17–19]. Although a few studies have used the STZ-induced mouse model to investigate the diabetes-caused impairment on the cytokine production by macrophages at a short period (10 to 14 days) after STZ treatment [9–11], the antigen-presenting ability and phagocytosis of macrophages in mice with a prolonged exposure to a diabetic milieu has not been addressed.

In the present study, we utilized a STZ-induced diabetic mouse model to systemically investigate the effects of diabetes on the cell numbers, phenotypes, and functions of macrophages from spleen and peritoneal exudates as late as 16 weeks after STZ-induced diabetes. These data offered basic information for the altered macrophage immunity in hosts with a prolonged exposure to a diabetic milieu which may help us understand the susceptibility of diabetic patients to infectious diseases and other complications at the late stage of diabetes.

Materials and methods

Mice Five- to 7-week-old C57BL/6 (B6, H-2^b), BALB/c (H-2^d), and TCR-transgenic DO11.10 mice, in which all TCRs recognize OVA323-339 epitope presented by H-2^d [20], obtained from Beijing University Experimental Animal Center (Beijing, China), were used. All mice were maintained in a specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

Multiple doses of STZ-induced diabetes in C57BL/6 Mice were treated with intraperitoneal injections of STZ (Sigma, 40 mg/kg body weight, daily) dissolved in citrate buffer (pH 4.5) for 5 consecutive days. Day 1 was defined as the day of the first injection of STZ. Blood glucose concentrations were measured on blood samples taken from the tail tip on days 0, 7, and 14 and more time points when necessary using a blood glucose meter. Hyperglycemia was

defined as a non-fasting blood glucose level higher than 11.1 mM as in our previous studies [21, 22].

Monoclonal antibodies (mAbs) The following mAbs were purchased from BD Biosciences Pharmingen (San Diego, CA, USA): fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD40 mAb (3/32; IgG2a), FITC-labeled hamster anti-mouse CD80 (B7-1) mAb (16-10A1; IgG2b), FITC-conjugated rat anti-mouse CD86 (B7-2) mAb (GL1, IgG2a), FITC-labeled mouse anti-mouse H-$2K^b$ mAb (AF6-88.5; IgG2a), FITC-labeled mouse anti-mouse I-A^b mAb (AF6-120.1; IgG2a), and FITC-conjugated anti-mouse F4/80^+ mAb (BM8). Rat anti-mouse FcR mAb (2.4G2, IgG2b) was produced by 2.4G2 hybridoma (ATCC, Rockville, MD, USA) in our laboratory.

Preparation of PEMs and SPMs Mouse peritoneal exudate cells were obtained from the peritoneal exudates of mice. Mouse splenocytes were prepared, and red blood cells were lysed with ACK lysis buffer (Invitrogen, San Diego, CA, USA) as described previously [23]. After the cells were washed twice with cold Hanks' solution, these cells were adjusted to 5×10^6 cells/ml in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) and cultured in 2% gelatin (Sigma)-pretreated six-well plates (Costar, Cambridge, MA, USA) for 3–4 h at 37°C and 5% CO_2. The non-adherent cells were removed by washing them with warm RPMI 1640 medium. The adherent cells were harvested with 5 mM EDTA (Sigma) in ice-cold PBS (pH 7.2) and readjusted to 1×10^6 cells/ml. The cell viability was usually more than 95% as determined by trypan blue exclusion. The macrophage purity was analyzed by a two-proton microscope LSM510 (Zeiss, Germany) and flow cytometry (FCM; Becton Dickinson, Mountain View, CA, USA), using macrophage marker F4/80^+. The adherent cells constituted more than 90% of F4/80^+ macrophages, as reported previously [23].

Immunofluorescence staining and FCM BALB/c (5×10^5) or B6 PEMs and SPMs were washed once with FACS buffer (PBS, pH 7.2, containing 0.1% NaN_3 and 0.5% bovine serum albumin). For two-color staining, cells were stained with PE-labeled anti-mouse F4/80^+ mAb versus FITC-labeled anti-I-A^b (39-10-8), CD80 (16-10A1), CD86 (GL1), CD40 (3/23) mAb, or the isotype control Ab, respectively. Nonspecific FcR binding was blocked by anti-mouse FcR mAb 2.4G2. At least 10,000 cells were assayed by two-color FCM using a FASCalibur flow cytometry (Becton Dickinson, CA, USA), and data were analyzed with CellQuest software (Becton Dickinson, Mountain View, CA, USA). Non-viable cells were excluded using the vital nucleic acid stain propidium iodide. The percentage of cells stained with a particular reagent or reagents was determined by subtracting the percentage of cells stained nonspecifically with the negative control mAb from staining in the same dot-plot region with the anti-mouse mAbs. Certain molecule expression levels were determined as the median fluorescence intensity (MFI) of the cells positively stained with the specific mAb. The cell number of F4/80^+ cells in each spleen = the total cell number of the splenocytes × the percentage of F4/80^+ cells in splenocytes detected by a FCM.

Detection of IFN-γ and IL-2 production by enzyme-linked immunosorbent assay (ELISA) For determination of cytokine production, co-cultures of CD4 T cells with allogeneic macrophages were established in 96-well plates. After 3- or 4-day incubation, the supernatant was preserved for further analysis. Amounts of IFN-γ and IL-2 were analyzed using specific ELISA kits (R&D Systems and Bioscience, respectively).

Allogeneic mixed leukocyte reactions (MLR) Murine splenocytes were prepared using the sterile technique as described previously [24]. CD4 T cells were purified by negative selection of mouse splenocytes using mouse CD4 T lymphocyte enrichment set-DM (BD Biosciences Pharmingen). Cells were suspended in RPMI 1640 medium supplemented with 10% (vol/vol) mouse serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids (Gibco BRL), 1 mM sodium pyruvate, 10 U/ml penicillin, 10 μg/ml streptomycin, 10 mM HEPES buffer (Gibco BRL), and 10 μM 2-mercaptoethanol. Triplicate wells containing 2×10^5 responders with 1×10^5 or with the indicated doses of allogeneic macrophage stimulators (pre-treated with 50 μg/ml mitomycin C) in a total volume of 0.2 ml of medium were incubated in U-bottomed 96-well microplates (Costar) at 37°C in 5% CO_2. Duplicate plates were pulsed with 1 μCi of ^{3}H-labeled thymidine (radioactivity, 185 GBq/mmol; Atomic Energy Research Establishment, China) per well on days 3 and 4 and, after 18 h of further incubation, were harvested onto glass fiber filters with an automatic cell harvester (Tomtec, Toku, Finland). Samples were assayed in a liquid scintillation analyzer (Beckmon Instruments, USA). Values are expressed as counts per minute (cpm) from triplicate wells and are the results after subtracting counts per minute from wells in the absence of stimulator cells [25].

The phagocytosis of allogeneic T cells by macrophages BALB/c splenic lymphocytes were obtained freshly. Naïve CD4 T cells were purified by negative selection of splenocytes of BALB/c and B6 mice using mouse CD4 T lymphocyte enrichment set-DM (BD Biosciences Pharmingen). Following two washes with PBS, 1×10^7 cells/ml CD4 T cells in PBS were labeled with 5.0 μM 5- (and 6-) carboxyfluorescein

diacetate succinimidyl ester (CFSE; Molecular Probes, Inc. Eugene, OR, USA) for 15 min at 37°C. These cells were then washed thoroughly and re-suspended at a concentration of 1×10^6 cells/ml. Cell viability was determined by trypan blue exclusion. Cell viability was usually more than 95%. Macrophages (2×10^5; F4/80$^+$ PEMs or SPMs) were co-incubated with 1×10^6 CFSE-labeled CD4 T cells in six-well plates (Costar, Cambridge, MA, USA) that had been preset with cover glass at 37°C and 5% CO_2 for 3–4 h. The cover glasses were washed once and adherent cells were blocked with anti-mouse FcγR mAb (clone 2.4G2) and stained with PE-conjugated anti-H-2D^b (34-2-12) mAb. Three-channel images were taken with a two-photon laser scanning microscope (LSM510, Zeiss). Individual macrophages were isolated from Z stacks with the extract region feature and further analyzed using the ortho and gallery displays of the LSM510 imaging software [26].

Delayed-type hypersensitivity (DTH) Sensitized effector T cells were generated by immunizing BALB/c mice with allogeneic B6 splenocytes. Ten days after immunization, BALB/c CD4 T cells were enriched using the negative selecting MACS kit for CD4 T lymphocytes (BD Biosciences Pharmingen). B6 or STZ-induced diabetic B6 F4/80$^+$ PEMs or SPMs were used as stimulator cells. Sensitized BALB/c effector CD4 T cells and allogeneic or syngeneic macrophage stimulators (5×10^5 cells each) in 10 μl RPMI 1640 medium were injected intradermally into the pinnate of naïve BALB/c mice. The changes in ear thickness were measured using an engineer's micrometer at 24 or 48 h after challenge [23]. The ear thickness change was calculated by subtracting the thickness of the same ear before injection from the thickness of the ear after injection.

Antigen-presenting ability of mouse F4/80$^+$ SPMs or PEMs in vitro OVA-specific T cell receptor (TCR)-transgenic mice (DO11.10 mice) were immunized with 500 μg of OVA in CFA. At 14 days after immunization, the splenic CD4 T cells in these mice were purified using mouse CD4 T lymphocyte enrichment set-DM (BD Biosciences Pharmingen). The purified CD4 T cells (2×10^5) were cultured with 1 mg/ml OVA protein in the presence of different cell numbers of F4/80$^+$ SPMs or PEMs separated from diabetes or control mice in a total volume of 200 μl medium in U-bottomed 96-well plates. After incubation at 37°C for 3 days, assays were pulsed with 0.5 μCi of ^{3}H-TdR for 18 h and performed as described above.

Statistical analysis All data are presented as the mean ± SD. Student's unpaired *t* test for comparison of means was used to compare groups. A *P* value less than 0.05 was considered to be statistically significant.

Results

Insignificantly altered cell numbers and functions of F4/80$^+$ PEMs and SPMs in mice with STZ-induced diabetes for 2 weeks

C57BL/6 mice were treated with low-dose STZ daily for 5 days. By week 1 onwards, these mice displayed hyperglycemia as indicated by the significantly enhanced blood glucose levels, whereas control mice that received citrate buffer alone had normal blood glucose ($P<0.001$, Fig. 1). Consistent with our previous studies [21, 22], STZ-treated mice progressively showed typical diabetes symptoms including elevated glycemia (>11.1 mM), glycosuria, polyuria, weight loss, and ketonuria (data not shown).

To determine the early changes of macrophages in STZ-induced diabetes in C57BL/6, we evaluated the cell numbers and immunity of PEMs and SPMs in mice with or without STZ-induced diabetes for 2 weeks. As shown in Fig. 2, no significant difference for the cell numbers, phagocytosis of allogeneic T cells, and immunogenicity to allogeneic T cells of PEMs and SPMs was observed between diabetic and control mice, except that SPMs in diabetic mice showed a decreased immunogenicity to allogeneic T cells ($P<0.05$; Fig. 2c (A)).

Significant alteration of cell numbers and phenotypes of F4/80$^+$ PEMs and SPMs in mice with STZ-induced diabetes for more than 16 weeks

As shown in Fig. 3, significantly decreased cell numbers of PEMs, F4/80$^+$ PEMs, and the percentage and cell number of

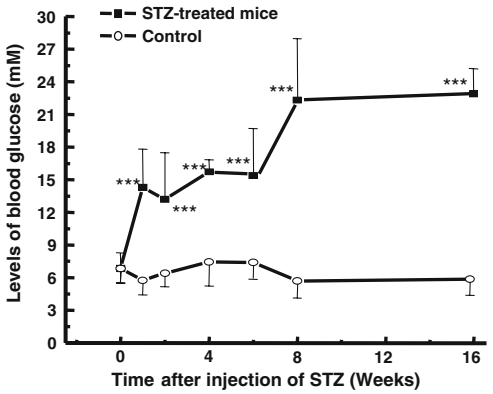

Fig. 1 STZ-induced diabetes in C57BL/6 mice. C57BL/6 mice were treated with intraperitoneal injections of STZ (Sigma, 40 mg/kg body weight) dissolved in citrate buffer, pH 4.5 during 5 consecutive days. Day 1 was defined as the day of the first injection of STZ. Blood glucose concentrations were measured on blood samples taken from the tail tip on days 0, 7, 14, 28, 56, and 112, using a blood glucose meter. Hyperglycemia was defined as a non-fasting blood glucose level higher than 11.1 mM. ***$P<0.001$ compared with the corresponding groups. Data were presented as mean ± SD. At least ten mice in each group were examined

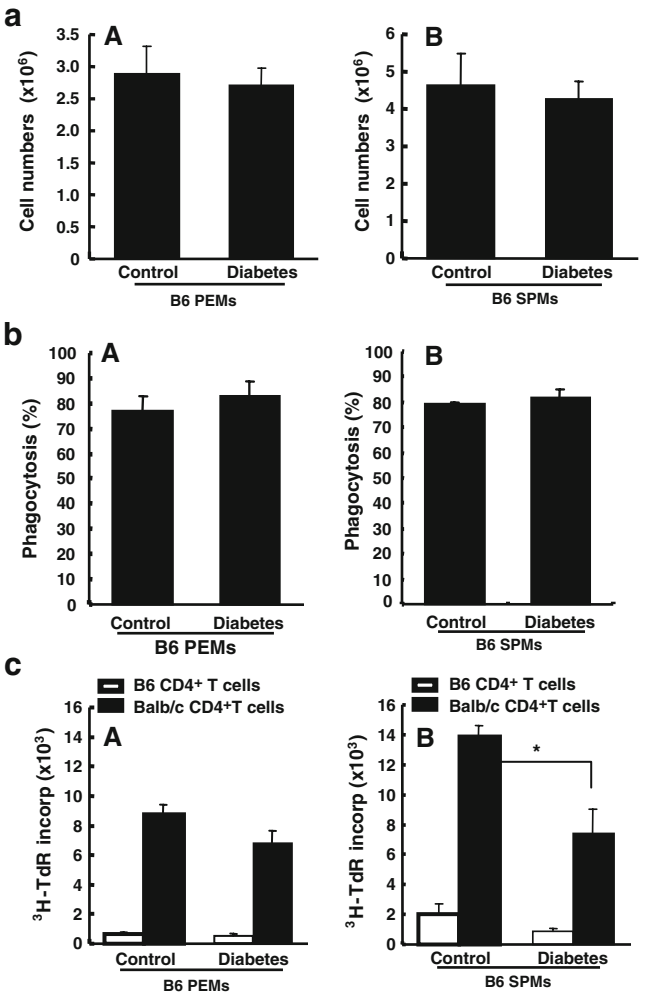

Fig. 2 The cell numbers and function of F4/80$^+$ PEMs and SPMs in mice with STZ-induced diabetes for 2 weeks. **a** Cell numbers of F4/80$^+$ PEMs and SPMs in diabetes mice. More than eight mice in each group were examined. **b** The in vitro phagocytosis effects of B6 F4/80$^+$ PEMs in STZ-induced diabetes mice against BALB/c T cells as detected with a FCM. **c** The proliferation of BALB/c CD4 T cells induced by allogeneic B6 PEMs or SPMs from mice with or without STZ-induced diabetes in vitro. Data were presented as mean ± SD of triplicate wells. *P<0.05 compared with the corresponding groups. One representative of the four independent experiments with similar data was shown

F4/80$^+$ SPMs in mice with STZ-induced diabetes for at least 16 weeks were observed compared with control mice (P< 0.05, or P<0.01, respectively). When the phenotypes of PEMs and SPMs were detected using a two-color FCM, significantly lower percentages of F4/80$^+$ PEMs expressed CD86 and significantly higher percentages of F4/80 PEMs expressed MHC II molecules in diabetes mice than those in control mice (P<0.01; Fig. 3c (A)). Significantly higher percentages of F4/80$^+$ SPMs expressed CD80 molecules in diabetes mice compared with control mice (P<0.01; Fig. 3c (B)). Thus, a significant alteration of cell numbers and phenotype of F4/80$^+$ PEMs and SPMs occurred in the late stage of diabetes mice.

Nonopsonic phagocytosis deficiency of F4/80$^+$ macrophages in mice with STZ-induced diabetes for 16 weeks

Phagocytosis represents an early and crucial event in triggering host defenses against invading pathogens. The phagocytic capacity of F4/80$^+$ PEMs and SPMs against allogeneic T cells was assessed using a two-photon microscope and FCM. Both B6 F4/80$^+$ PEMs and SPMs have the ability to engulf allogeneic BALB/c T lymphocytes but not syngeneic T cells as detected with a two-

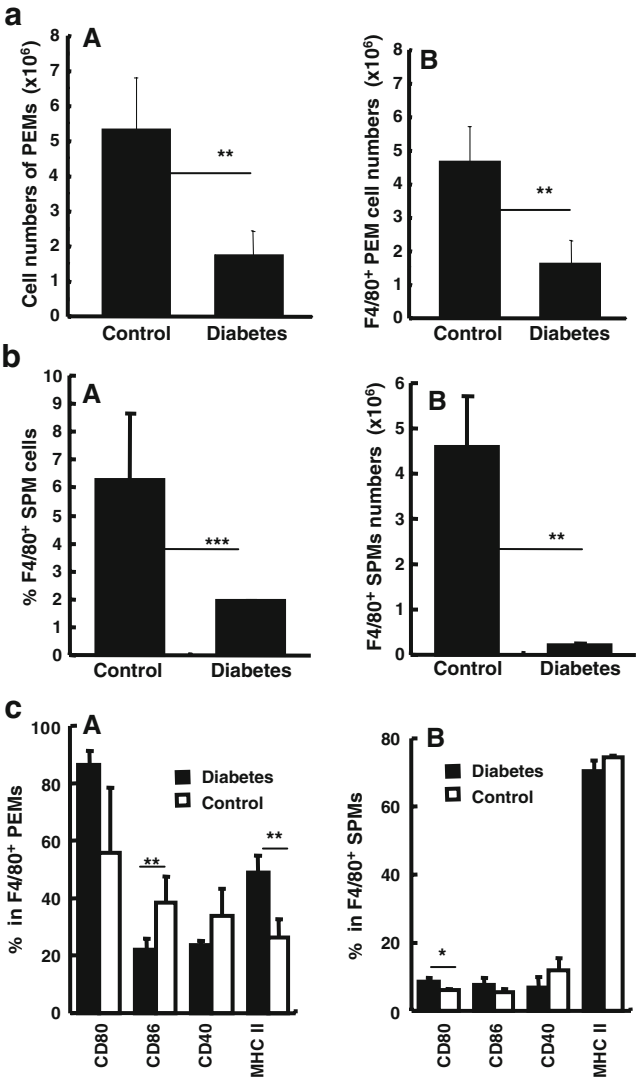

Fig. 3 Cell numbers and phenotype changes of F4/80$^+$ PEMs and SPMs in mice with or without STZ-induced diabetes for 16 weeks. **a** Cell numbers of PEMs (*A*) and F4/80$^+$ PEMs (*B*) in diabetes and control mice. **b** Mean percentages of F4/80$^+$ SPMs (*A*) and cell numbers of F4/80$^+$ SPMs (*B*) in diabetes and control mice. **c** Phenotype characteristics of F4/80$^+$ PEMs or SPMs in STZ-induced diabetes or control mice. The percentages of I-A^b, CD80, CD86, or CD40-positive cells in B6 F4/80$^+$ PEMs (*A*) or SPMs (*B*) in STZ-induced diabetes or control mice were detected by a FCM. Data were presented as mean ± SD. *P<0.05; **P<0.01; ***P<0.001 compared with the corresponding groups. More than eight mice in each group were examined

photon microscope (data not shown). However, B6 F4/80$^+$ PEMs in mice with STZ-induced diabetes for more than 16 weeks showed decreased ability to engulf allogeneic BALB/c T lymphocytes compared with control mice (data not shown). Importantly, when the phagocytosis of macrophages was quantitatively assayed using a FCM, B6 F4/80$^+$ PEMs in diabetic mice had a significantly lower phagocytic rate to allogeneic BALB/c T cells than those of control mice ($P<0.001$, Fig. 4a and b). B6 F4/80$^+$ SPMs in diabetic mice had normal phagocytosis as the control mice ($P>0.05$, Fig. 4b (B)).

Decreased immunogenicity of F4/80$^+$ macrophages in mice with STZ-induced diabetes for 16 weeks

Macrophages, as one of the important APCs, play a critical role in the initiation of adaptive immune responses. The allogeneic immunogenicity of F4/80$^+$ PEMs and SPMs in diabetic and control mice was evaluated in vitro and in vivo. Both B6 F4/80$^+$ SPMs and PEMs could efficiently induce the proliferative reaction of allogeneic BALB/c T cells in MLR assays (Fig. 5a (A) and (B)). However, F4/80$^+$ SPMs and PEMs in diabetic B6 mice had significantly less ability to stimulate the proliferation of allogeneic BALB/c CD4 T cells than those of control mice respectively (Fig. 5a).

Furthermore, the cytokine products (IL-2 and IFN-γ) of BALB/c CD4 T cells co-cultured with allogeneic B6 F4/80$^+$ SPMs or PEMs from diabetic and control mice were assayed by ELISA methods. As shown in Fig. 5b,

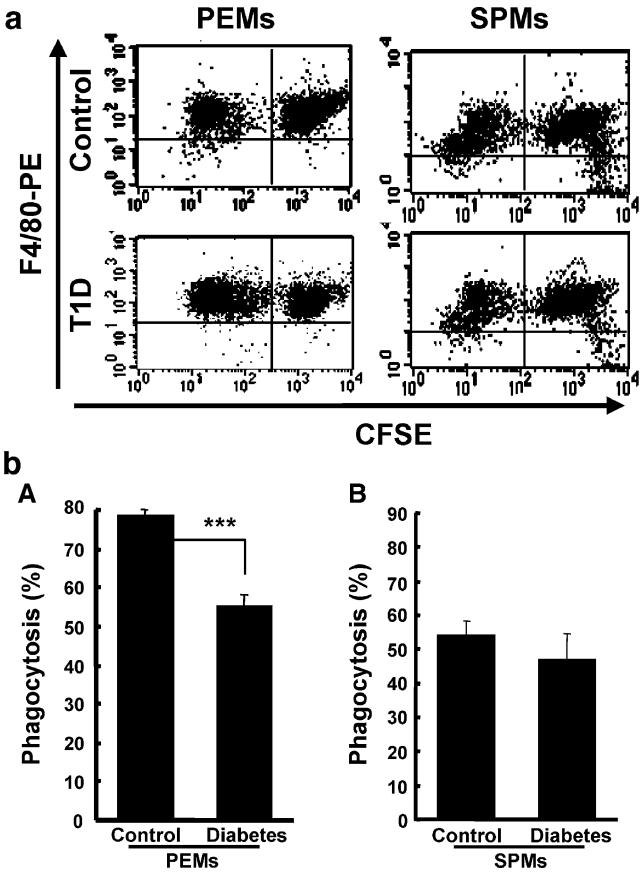

Fig. 4 Decreased phagocytosis of allogeneic T cells by F4/80$^+$ PEMs in mice with STZ-induced diabetes for 16 weeks. **a** One representative showing B6 F4/80$^+$ PEM or SPM phagocytosis of allogeneic BALB/c T cells as detected by a FCM. B6 PEMs or SPMs were stained with PE-labeled anti-F4/80$^+$ mAb, whereas BALB/c T cells were labeled with CFSE. B6 PEMs or SPMs were co-cultured with BALB/c T cells as described in the "Materials and methods" section. **b** Diabetes or control B6 F4/80$^+$ PEMs phagocyted allogeneic BALB/c T cells as determined by FCM. Data were presented as mean ± SD. Six mice in each group were used. ***$P<0.001$ compared with the indicated groups

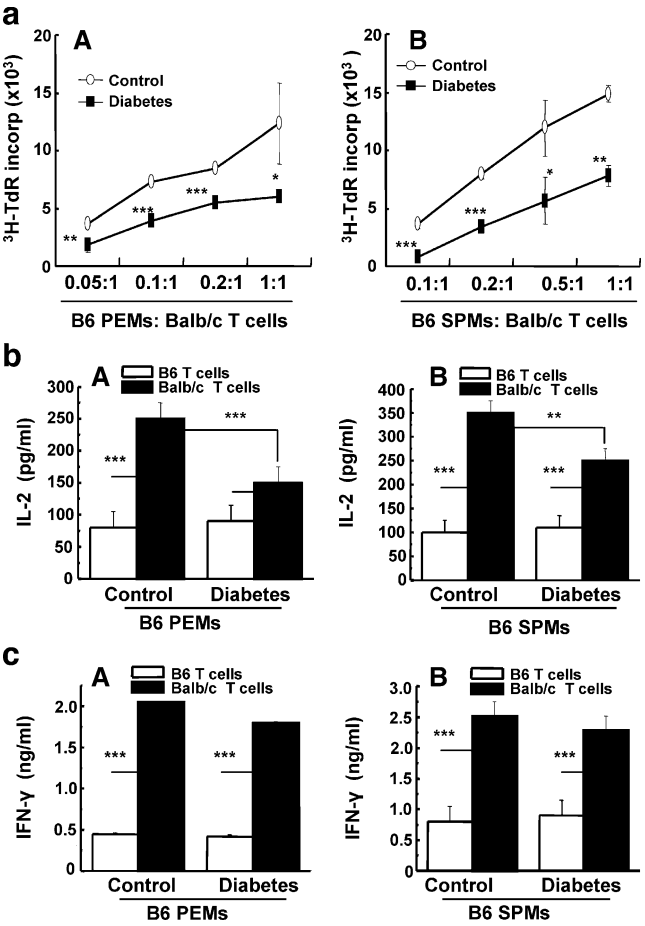

Fig. 5 Markedly reduced immunogenicity of F4/80$^+$ PEMs and SPMs in mice with STZ-induced diabetes for 16 weeks. **a** The proliferation of allogeneic CD4 T cells induced by B6 F4/80$^+$ PEMs (*A*) or SPMs (*B*) in STZ-induced diabetes or control mice in vitro as determined by ^{3}H-TdR incorporation. **b** IL-2 production by allogeneic CD4 T cells stimulated by B6 F4/80$^+$ PEMs (*A*) or PMs (*B*) in STZ-induced diabetes or control mice was determined by ELISA. **c** IFN-γ production by allogeneic CD4 T cells stimulated by B6 F4/80$^+$ PEMs (*A*) or SPMs (*B*) in STZ-induced diabetes or control mice was determined by ELISA. Data were presented as mean ± SD of triplicate wells. One representative of four independent experiments with similar data was shown. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with the indicated groups

significantly higher levels of IL-2 in BALB/c CD4 T lymphocytes cultured with allogeneic B6 F4/80$^+$ SPMs or PEMs were detected no matter whether B6 F4/80$^+$ SPMs or PEMs were from diabetic or control mice. However, F4/80$^+$ SPMs or PEMs from B6 mice with diabetes for 16 weeks stimulated allogeneic BALB/c CD4 T cells to produce significantly lower levels of IL-2 than control B6 F4/80$^+$ SPMs or PEMs did (P<0.01 and P<0.001, respectively; Fig. 5b). Although significantly higher levels of IFN-γ in BALB/c CD4 T cells cultured with allogeneic B6 F4/80$^+$ SPMs or PEMs were detected in all groups (P<0.001), no significant differences for IFN-γ products induced by B6 F4/80$^+$ SPMs or PEMs in diabetic mice and control mice were observed (P>0.05; Fig. 5c).

To further determine the immunogenicity of F4/80$^+$ macrophages of diabetic mice in vivo, DTH reaction induced by allogeneic F4/80$^+$ PEMs or SPMs was performed. Sensitized BALB/c CD4 T cells were co-injected intradermally with allogeneic B6 F4/80$^+$ PEMs or SPMs, respectively, into the pinnate of naïve BALB/c mice. The changes in ear thickness were measured at 24 and 48 h after challenge. As shown in Fig. 6, significant DTH responses were observed if sensitized BALB/c T cells were stimulated by allogeneic B6 F4/80$^+$ PEMs or SPMs as reported previously. However, F4/80$^+$ SPMs or PEMs from B6 mice with diabetes for more than 16 weeks induced significantly lower DTH responses of sensitized BALB/c T cells than those of control mice did (P<0.001, Fig. 6).

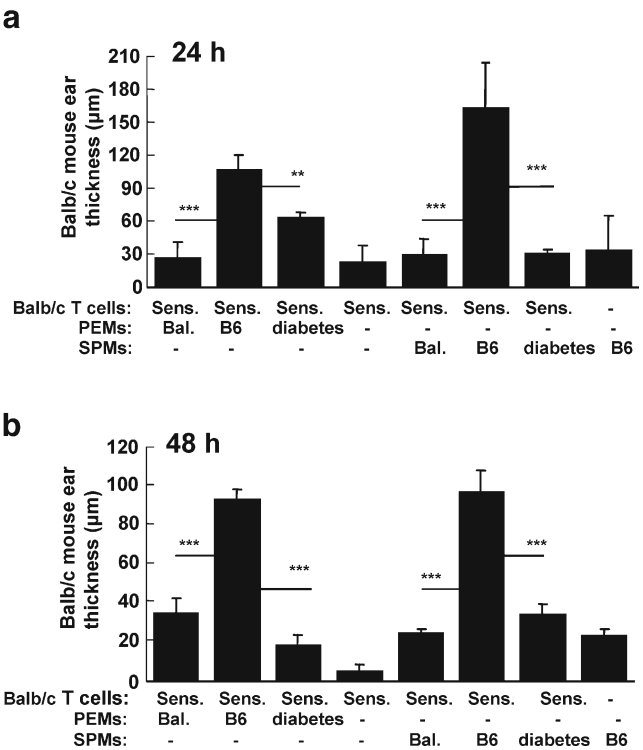

Fig. 6 Significantly reduced allo-DTH induction by F4/80$^+$ PEMs and SPMs separated from mice with STZ-induced diabetes for 16 weeks. DTH responses induced by B6 F4/80$^+$ PEMs or SPMs in STZ-induced diabetes or control mice were detected at 24 h (**a**) or 48 h (**b**) after injection into the pinnate. Data were presented as mean ± SD. More than nine mice in each group were assayed. Data was a summary of four independent experiments. **P<0.01, ***P<0.001 compared with the indicated groups

Antigen-presenting deficiency of F4/80$^+$ macrophages in mice with STZ-induced diabetes for 16 weeks

To determine whether F4/80$^+$ macrophages in STZ-induced diabetic mice are capable of processing antigen with the same efficiency as those in control mice, we studied the T cell proliferation induced by antigens presented by macrophages using a TCR-transgenic mouse model. At 14 days after DO11.10 transgenic mice in which all T cells express a TCR recognizing OVA323-339 epitope were immunized with OVA in CFA, the splenic CD4 T cells (2×10^5) were separated and cultured with 1 mg/ml OVA protein in the presence of different cell numbers of F4/80$^+$ PEMs or SPMs in diabetes or control mice in U-bottomed 96-well plates. As shown in Fig. 7, F4/80$^+$ PEMs or SPMs could efficiently present OVA antigen to and induce the proliferative reaction of DO11.10 CD4 T cells. However, F4/80$^+$ PEMs from mice with diabetes for 16 weeks stimulated the proliferation of DO11.10 CD4 T cells less efficiently than those of control mice in the presence of OVA antigens (Fig. 7a). Otherwise, F4/80$^+$ SPMs from mice with diabetes showed similar ability to stimulate the proliferation of DO11.10 CD4 T cells as those of control mice in the presence of OVA antigen (Fig. 7b).

Discussion

Diabetes mellitus has a dramatic impact on human health, and its severe complications including impaired wound healing, accelerated atherosclerosis, a high incidence of cardiovascular diseases, nephropathy, and other inflammatory or degenerative manifestations contribute to a high mortality of diabetic patients [8, 16]. These patients also exhibit increased susceptibility to infection and greater tissue loss following infection, as noted by more severe periodontitis [8]. A likely common denominator in all these abnormalities is the dysfunction of the macrophage system. Macrophages are the body's primary scavenger cells and are responsible for clearing pathogens and apoptotic cells by phagocytosis [27, 28]. They also modulate the immune response by antigen presentation, cytokine production, and activation of other immune cells [29]. Numerous phenotypic abnormalities have been found in peripheral blood mononuclear cells (PBMCs) from diabetic patients, although it is unclear whether these changes of PBMCs were the reason to cause diabetes or the subsequent impacts of diabetes [5, 30]. In the present study, there were almost no significant changes of SPMs and PEMs in diabetic mice at

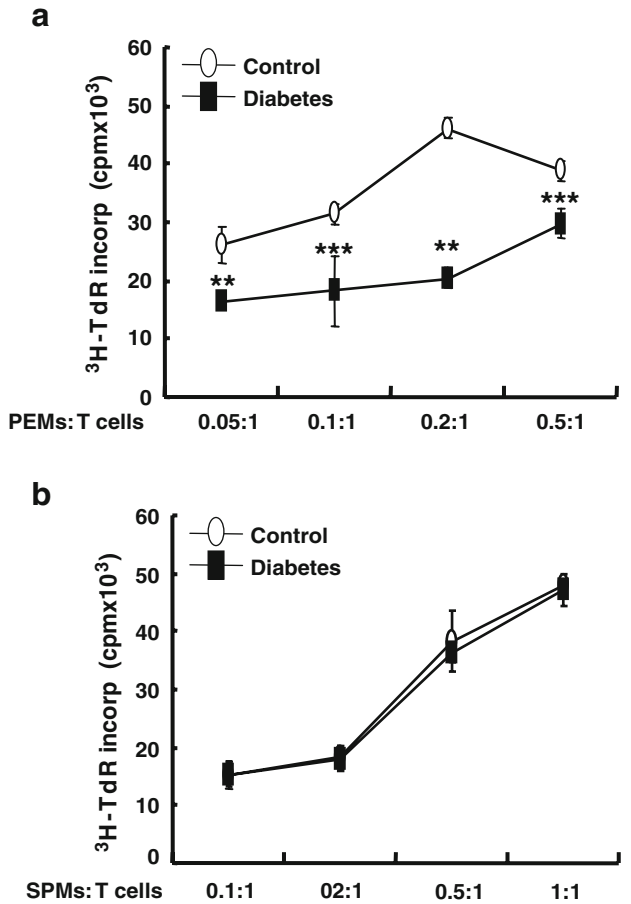

Fig. 7 Significantly reduced antigen-presenting ability of F4/80$^+$ PEMs in mice with STZ-induced diabetes for 16 weeks. The antigen-presenting capacity by F4/80$^+$ PEMs (**a**) or SPMs (**b**) in STZ-induced diabetes or control mice was determined by their presenting OVA antigens to TCR-transgenic CD4 T cells in vitro. T cell proliferation was detected by a ^{3}H-TdR incorporation assay. Data were presented as mean ± SD of triplicate wells. One representative of the four independent experiments with similar data was shown. $^{**}P<0.01$, $^{***}P<0.001$ compared with the identical group, respectively

2 weeks after STZ treatment, except for the decreased immunogenicity of SPMs to allogeneic T cells. However, dramatic changes in cell numbers, phenotypes, and functions were detected in F4/80$^+$ PEMs and F4/80$^+$ SPMs of diabetic mice at 16 weeks after STZ treatment. These observations indicate that the alteration of host macrophages may mainly be due to STZ-induced diabetes but not caused by STZ treatment in these mice. Thus, we provided for the first time direct evidence for the effects of diabetes on the cell number, phenotype, and function of host-resident macrophages in mice.

Significantly decreased cell numbers of F4/80$^+$ PEMs and F4/80$^+$ SPMs in mice with STZ-induced diabetes were detected at 16 weeks after diabetes induction. The mechanisms for this alteration were unclear at this moment. It may be caused by the shortened life span of macrophages and/or the blocking differentiation of macrophages from monocytes or bone marrow cells (BMCs). The capacity of BMCs in diabetes to generate macrophages in vitro has been studied previously and a low yield of cells in NOD BMCs stimulated with GM-CSF was observed [31]. Consistent with this report, our preliminary data showed that BMCs from STZ-induced diabetic mice produced a significantly lower number of F4/80$^+$ macrophages than those from non-diabetic mice (Ma H and Zhao Y, unpublished data), which is consistent with the decreased ability of other stem cells including endothelial progenitor cells, cardiac stem cells, and embryonic neural stem cells in diabetes or in high glucose situations [32–34]. These data support the possibility that the decreased macrophage number in diabetes mice, at least in part, is due to the deficient differentiation of macrophages from BMCs, though other possibilities were not excluded.

Macrophages are a major component of the mononuclear phagocyte system that consists of closely related cells of bone marrow origin, including blood monocytes and tissue macrophages [35]. Phagocytosis by macrophages is an essential step and a part of innate immunity for protection against foreign pathogens, microorganisms, or dead cells [36, 37]. F4/80$^+$ PEMs but not F4/80$^+$ SPMs in diabetic mice have significantly lower phagocytosis capacity against allogeneic T cells than those of control mice in the later stage of disease as assayed by a two-photon microscope or FCM. The significantly decreased phagocytosis capability of host macrophages may be related to the increased susceptibility to infection as shown by previous reports regarding their relation with the occurrence of autoimmune diseases and susceptibility to microorganism infections in other models [38–42]. We also demonstrated the enhanced cardiac injury in diabetic mice at 2 months after STZ treatment as compared with non-diabetic mice when they were challenged with LPS [43].

Due to their endocytic and phagocytic capacity as well as their ability to up-regulate MHC-II and co-stimulatory molecules upon activation [44], macrophages, as one of the important APCs, play a critical role in the immune response. Furthermore, we have studied their ability to stimulate the immune responses of allogeneic T cells and their phagocytosis on allogeneic target cells. The ability of F4/80$^+$ PEMs and F4/80$^+$ SPMs in STZ-induced diabetic mice to stimulate cytokine productions (IL-2 and IFN-γ), cell proliferation, and DTH reaction of allogeneic CD4 T cells was significantly lower than those of control mice at 16 weeks after diabetes induction, as determined in vitro and in vivo. Furthermore, the antigen-presenting capacity of F4/80$^+$ PEMs was significantly decreased in these diabetes mice as detected by their presenting OVA antigen to T cells with a transgenic TCR recognizing OVA peptide. The reasons for the poor antigen-presenting ability of macrophages in diabetes mice were not addressed in this study. It

may be related with the phenotype alteration, decreased phagocytosis function as well as the impaired cytokine productions of these cells in hosts with STZ-induced diabetes [8–10].

It is noted that somewhat different alterations between $F4/80^+$ PEMs and $F4/80^+$ SPMs present in mice with STZ-induced diabetes at 16 weeks after diabetes induction, including phenotype and phagocytosis changes. Macrophages in a different microenvironment display a wide diversity in terms of their function and morphology [23, 45]. Recent studies have shown that macrophage phenotypic and functional heterogeneity might be related to a wide diversity of factors, including different precursor cells, different differentiation stages, different microenvironments, or even different stimulatory factors [46]. Thus, it is interesting to determine whether and why macrophages in different tissues in diabetes hosts display different changes in the future.

In summary, significantly alterations of $F4/80^+$ PEMs and $F4/80^+$ SPMs in mice with STZ-induced diabetes for 16 weeks have been detected concerning cell numbers, phenotype, phagocytosis, immunogenicity, and antigen-presenting ability. These data indicated that remarkable dysfunctions of macrophages might occur in diabetes hosts. The present study offered evidence for the effects of diabetes on host macrophages, which may be related to the severe complications and susceptibility to infections in patients with a prolonged exposure to a diabetic milieu.

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