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CTLA4-Ig-modified dendritic cells inhibit lymphocyte-mediated alloimmune responses and prolong the islet graft survival in mice

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ABSTRACT

The induction of antigen specific tolerance is critical for prevention and treatment of allograft rejection. In this study, we transfected CTLA4-Ig gene into dendritic cells (DCs), and investigated their effect on inhibition of lymphocyte activity in vitro and induction of immune tolerance on pancreatic islet allograft in mice. An IDDM C57BL/6 murine model induced by streptozotocin is as model mouse. The model mice were transplanted of the islet cells isolated from the BALB/c mice to their kidney capsules, and injected of CTLA4-Ig modified DCs (mDCs). The results showed that mDCs could significantly inhibit T lymphocyte proliferation and induce its apoptosis; whereas, unmodified DCs (umDCs) promoted the murine lymphocyte proliferation. Compared with injection of umDCs and IgG1 modified DCs, the injection of mDCs prolonged IDDM mice's allograft survival, and normalized their plasma glucose (PG) levels within 3 days and maintained over 2 weeks. The level of IFN-γ was lower and the level of IL-4 was higher in mDCs treated recipient mice than that in control mice, it indicated that mDCs led to Th1/Th2 deviation. After 7 days of islet transplantation, HE stain of the renal specimens showed that the islets and kidneys were intact in structure, and islet cells numbers are increased in mDCs treated mice. Our studies suggest that DCs expressing CTLA4-Ig fusion protein can induce the immune tolerance to islet graft and prolong the allograft survival through the inhibition of T cell proliferation in allogeneic mice.

1. Introduction

The American Diabetes Association (ADA) estimates that more than 20 million people in the United States, or 7% of the population, have diabetes mellitus(DM), and that one in three Americans born in 2000 will develop diabetes sometime during their lifetime. Currently more than 230 million people live with DM worldwide. The current therapeutic protocols of DM include exogenous insulin supplement and stimulation of endogenous insulin. Pancreatic islet cell transplantation for the treatment of DM is still a great challenge until now, but it is a promising therapy for IDDM [1]. It is well known that the transplanted islet cells are very sensitive to various stimulations and easily rejected by recipient immune systems. Therefore, a new technique that can only induce the recipient immune tolerance to transplanted islet but not affect the immune response to other antigens is anticipated.

Dendritic cells (DCs) have several functions in innate and adaptive immunity, they play a major role in antigen presentation and T cell priming and are therefore crucial in stimulating the primary response of transplant immune [2–4]. They are potentially powerful tools for the therapeutic manipulation of immune reactivity in allergic diseases, cancers, infectious diseases, and allograft rejection [2–7]. The antigen presentation may be blocked or inhibited by the modification of the genes encoding costimulatory molecules and cytokines, thus, the allotype immune tolerance is induced. CTLA4-Ig is a recombinant fusion protein of the ectodomain of cytotoxic T lymphocyte associated antigen (CTLA4) and the constant domain of human immunoglobulin. *In vitro* and *in vivo* studies have demonstrated that CTLA4-Ig can block the B7/CD28 costimulatory pathway and prevent the rejection of xenogeneic islets and/or allogeneic corneal, skin, liver, cardiac and islet graft [8–11].

In this study, we transfected CTLA4-Ig gene into DCs through liposome Lipofectamine to obtain gene modified DCs (mDCs) that could stably express fusion protein CTLA4-Ig. Then we evaluated their

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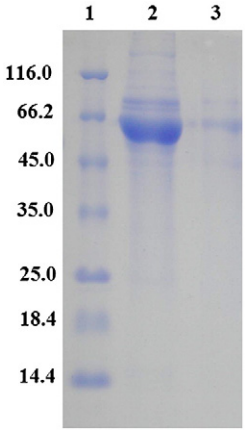

Fig. 1. SDS-PAGE analysis of CTLA4-Ig expression in DC culture supernatant. 1: protein marker (M_r); 2: culture supernatant of pG/CTLA4-Ig-transfected DCs; 3: culture supernatant of untransfected DCs.

impacts on lymphocyte proliferation and apoptosis in vitro, investigated their effects on allogeneic islet transplant in IDDM mice.

2. Materials and methods

2.1. The animals and their induction of hyperglycemia by STZ

Eight- to 10-week-old BALB/c and C57BL/6 male mice, weighing 18–22 g, were purchased from the Center of Experimental Animals, Hubei Academy of Medical Science. BALB/c mice were used as donors, C57BL/6 mice as recipients. Mice were housed in an air-conditioned environment, with a 12-hour light-dark cycle, and fed a regular unrestricted diet. All experiments were approved by the local government authorities.

Diabetes was induced in C57BL/6 mice by an intravenous injection of streptozotocin (Sigma, St. Louis, MO) at a concentration of 220mg/kg [12]. The plasma glucose (PG) levels were measured by a Beckman glucose analyzer (Roche Accu Check III). Three days later, the non-fasting PG was measured daily for 5 days. The IDDM C57BL/6 model was thought to be successfully created if the PG level was over 16.65 mmol/L for at least 3 consecutive days.

2.2. Transfection of CTLA4-Ig gene into DCs

The mouse thymic DCs were kindly provided by Prof. Chen Weifeng (Department of Immunology, Beijing Medical University). Plasmid pG/CTLA4-Ig was transfected into the DCs by liposome Lipofectamine (Invitrogen). The positive cells were screened by mycophenolic acid (Sigma) and cloned by limiting dilution. The CTLA4-Ig fusion protein was identified by SDS-PAGE and indirect enzyme-linked immunoabsorbent assay (ELISA).

2.3. Detection of lymphocyte apoptosis

The lymphocytes of healthy C57BL/6 mice were prepared by a sterile method and were used as responders (1×10^7/ml). CTLA4-Ig modified DCs (mDCs) or unmodified DCs (umDCs) pretreated with mitomycin C (Sigma) were employed as stimulators (1×10^5/ml). After the lymphocytes were cocultured with pretreated mDCs or umDCs for 6 days, the apoptosis was detected by ELISA (Roche) as described previously [13]. In brief, the lymphocytes, cultured in 6-well culture plates, were directly lysed with a lysis buffer, after which the cytosolic oligonucleosome was quantified using biotin-labeled mouse monoclonal anti-histone antibody as the capturing antibody, peroxidase-conjugated mouse monoclonal anti-DNA antibody as the detecting antibody, and ABTC (2,2'-azino-di[3-ethylbenzthiazolin-sulfonate]) as the developing agent. The relative increase in nucleosomes in the cytoplasm was expressed as an enrichment factor, which was calculated as the ratio of the specific absorbance of lysates at 405 nm to that of untreated cells.

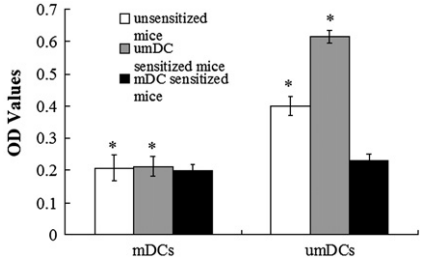

Fig. 2. The lymphocyte proliferation in different sensitized mice (umDCs and mDCs) and unsensitized mice when stimulated by mDCs and umDCs. The results are shown as mean ± SD; *OD mean values as stimulated by mDCs were significantly less than these as stimulated by umDCs(*$p<0.01$).

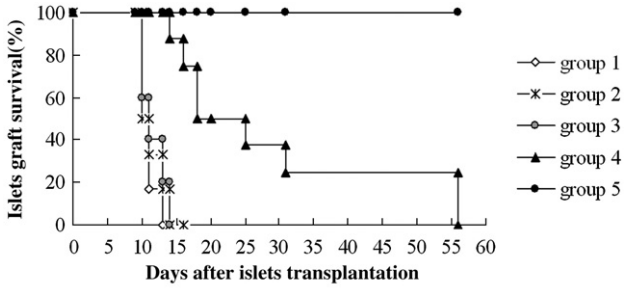

Fig. 3. Survival days of transplanted mice (survival rate, %) Group 1: islets graft control; Group 2: umDCs treated recipients; Group 3: IgG1 modified DCs treated recipients; Group 4: mDCs treated recipients; Group 5: healthy mice.

2.4. Lymphocyte proliferations induced by different DCs

Modified DCs or umDCs were intraperitoneally injected into C57BL/6 mice at a dose of 1×10^7/mouse. Five days later, the animals were sacrificed and their spleen cells were harvested to isolate mononuclear cells. 100 μl mononuclear cells (5×10^6/ml) and 100 μl mitomycin C pretreated mDCs or umDCs (5×10^4/ml) were planted into 96-well plates, and incubated at 37 °C for 7 days. Proliferation was assessed by methyl thiazolyl tetrazolium (MTT) chromatometry. The OD value was measured at 570 nm by a microtiter plate reader (Bio-Rad, Tokyo, Japan).

2.5. Islet transplantation

Pancreatic islets were isolated from the male BALB/c mice by collagenase technique [14]. After washed twice, the islets were cultured in 1 ml Krebs-Ringer bicarbonate buffer with HEPES and their insulin secretory function was assayed by immunoradioassay [15]. Male IDDM model C57BL/6 mice were served as recipients. The islet cells were grafted under the left kidney capsule of recipient mice at a dose of 600 islets/mouse. This technique is followed from the renal subcapsular islet transplantation as reported previously [16]. Briefly, after an appropriate anesthesia with pentobarbital sodium, the mice were placed on the right lateral position, by which the left kidney contour was visualized. A small skin incision was made and the abdomen was opened. The left kidney was protruded extraperitoneally through the incision manually. A small horizontal incision was made on the capsule close to the lower pole of the kidney. A pocket space beneath the kidney capsule was created toward the upper pole of the kidney, where pancreatic grafts were introduced. The kidney bearing the grafts was placed back into the abdomen after the completion of transplantation, and the incision was closed by layers.

Grouping of transplanted mice: the islet recipient mice were allocated into four groups and treated with: I) islet transplant only (6 mice); II) islet transplant followed by injection of mDCs through the vena caudalis at the same day (8 mice); III) islet transplant followed by injection of IgG1 modified DCs (5 mice); IV) islet transplant followed by injection of umDCs (6 mice). The number of DCs in above groups was 1×10^7 per mouse. Eight healthy C57BL/6 mice without islet transplant were employed as controls.

2.6. Observation and cytokine quantification in the transplanted mice

After transplantation, the survival time of recipient mice was recorded. Body weights and PG levels were monitored on a daily basis. Normoglycemia was defined as

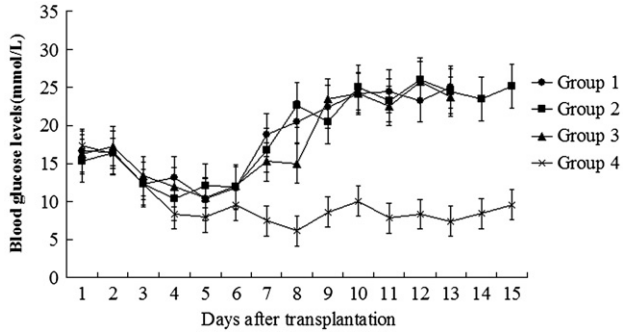

Fig. 4. The profiles of PG levels in different islet recipient mice. Group 1: graft control mice; Group 2: umDCs treated recipients; Group 3: mDC-IgG1 treated recipients; Group 4: mDCs treated recipients.

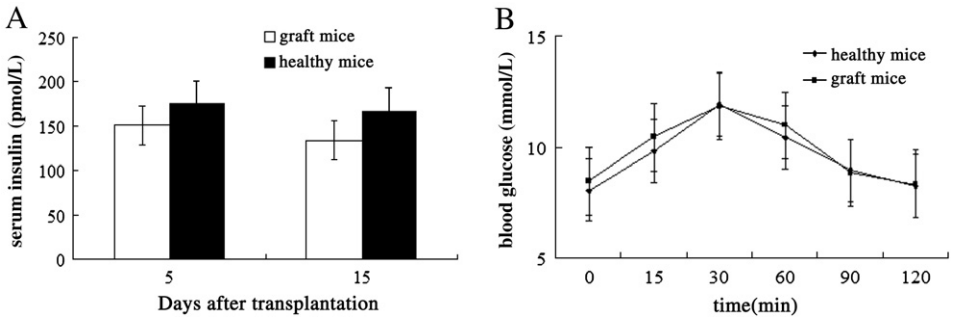

Fig. 5. The serum insulin levels and glucose tolerance test after ip injection of glucose in mDC treated grafted mice. A: serum insulin levels in mDC treated graft mice and healthy control mice; B: blood glucose profiles in both groups on days 5 and 15.

PG no greater than 8.4mmol/L. In normoglycemic recipients, insulin secretions were assessed by radioimmunoassay on days 5 and 15, and glucose tolerance tests were performed on day 15 according to the procedure reported previously [17]. Briefly, the animals were fasted one night before the experiment. 1.0g/kg of glucose was administered intraperitoneally, and serum insulin concentration was measured at 10 min and plasma glucose was detected at 0, 15, 30, 60, 90 and 120 min after glucose challenge. On days 5 and 15, serum IL-4 and IFN-γ levels in mDCs treated recipient mice and umDCs treated mice were measured using ELISA kit (Jingmei Biotech Co., Ltd), according to the manufacturer's instructions.

2.7. Immunohistochemical stain of the transplanted islets

The kidneys from the recipient mice were embedded in paraffin and sectioned at 4 μm intervals. The sections were stained with hematoxylin-eosin or processed for immuno-peroxidase histochemistry with antibodies specific for insulin, then studied under the microscope.

2.8. Statistics analysis

The data were shown as mean±standard deviations. *t*-test was used to compare the difference between two groups. Differences between groups were considered significant at $p<0.05$.

3. Results

3.1. Screening of transfected DCs and the expression of fusion protein in the supernatant

Plasmid pG/CTLA4-Ig was transfected into DCs via liposome Lipofectamine, 72 h later, the culture wells were added 100 mg/L mycophenolic acid to screen transfected DCs. Two weeks later, we obtained the positive clones of pG/CTLA4-Ig transfected DCs. ELISA showed that the OD value of mDC supernatant was similar to that of human IgG which served as positive control. A specific protein band at 55–66 kD, identical to CTLA4-Ig fusion protein, was displayed on the gel when positive DC supernatant was analyzed by SDS-PAGE; no corresponding band was displayed when untransfected DC supernatant was analyzed (Fig. 1). It indicated that expression vector pG/CTLA4-Ig had been transfected into DCs and the transfected cells were able to secrete the CTLA4-Ig fusion protein.

3.2. Effects of mDC on apoptosis and proliferation of allotype lymphocytes

The lymphocyte apoptosis was measured by ELISA. The enrichment factor was proportional to the apoptotic cells. The results showed that the enrichment factor was 2.37±0.38 when umDCs was used as stimulatory cells and 4.26±0.53 when mDCs were used as stimulatory cells, indicating that mDCs induce significant higher lymphocyte apoptosis than umDCs ($p<0.05$, $n=4$).

The lymphocyte proliferations induced by mDCs decreased markedly in both sensitized mice and unsensitized mice, their OD_{570} values were lower than those in mice induced by the umDCs. OD_{570} value was the lowest in mDC sensitized mice when the lymphocytes were induced by mDCs, and highest in umDC sensitized mice when the lymphocytes were induced by umDCs (Fig. 2).

3.3. The survival of transplanted mice and their PG levels

Transplanted IDDM mice injected with umDCs, IgG1 modified DCs and mDCs survived for 10.17±1.60 days, 10.40±1.67 days and 25.50±13.60 days, respectively. Allograft control group survived for 10.15±2.07 days (Fig. 3). The results demonstrated that mDCs injection prolonged the graft survival evidently, compared with umDCs and IgG1 modified DCs injection ($p<0.01$).

The transplantation outcome was evaluated by monitoring PG levels daily. It was considered successful if PG remained lower than 11.1mmol/L for over 3 days. The graft failure was diagnosed by relapse of hyperglycemia (>11.1 mmol/L) for more than 3 consecutive days. The PG levels in mDCs treated mice decreased to less than 11.1mmol/L since 3 days after transplantation and remained in normal range (6.11–9.50 mmol/L) for over 2 weeks. But those in the mice treated with umDCs and IgG1 modified DCs elevated to 11.89±2.86 mmol/L and 12.10±2.58 mmol/L on day 5 respectively; indicative of a graft failure. (Fig. 4).

3.4. The results of insulin secretion test and glucose tolerance test in transplanted mice

The insulin secretion test was carried out in the mice whose PG normalized 5 and 15 days after transplantation and the healthy control mice. The insulin secretion reached the peak at 10 min after injection of glucose. The results showed that the insulin secretion in PG recovery recipients was similar to the healthy mice on day 5 and was slightly lower than that in healthy ones on day 15 ($p>0.05$). The glucose tolerance test displayed that PG profiles resembled in both groups after glucose challenge (Fig. 5).

3.5. Cytokines in the transplanted mice

The production of IFN-γ was obviously inhibited in mDC mice with normal PG level. The level of IFN-γ in mDC mice was significantly lower than that in umDC mice; whereas, the level of IL-4 was higher than that in umDC mice ($p<0.05$)(Fig. 6).

3.6. The histochemical stain of the allografts

HE stain of renal specimens was conducted in IDDM mice 7 days after transplantation. In the islet specimens from the IDDM mice treated with umDCs and IgG1 modified DCs, there were numerous inflammatory cells infiltrating and the islets were destroyed. However, in mice treated with mDCs, the islet structures remained intact, the grade of mononuclear cell infiltrations into the grafts was minor, and their kidney structure was normal. The kidneys to immunohistochemical staining with anti-insulin showed that more cells stained positively for insulin in mDCs treated mice than that in IgG1 modified DCs treated mice (Fig. 7).

4. Discussion

Insulin-dependent type 1 diabetes mellitus (IDDM) is caused by autoimmune destruction or dysfunction of the insulin secreting cells mediated by T lymphocytes and antibodies recognizing pancreatic islet cell antigens. In this disease, the insulin secretion decreases or

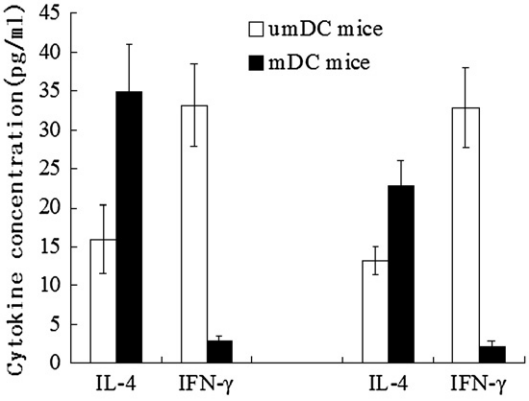

Fig. 6. Cytokines production in islet allografts. The expressions of IFN-γ and IL-4 in serum from recipient C57BL/6 mice treated with mDCs and umDCs were assessed by ELISA on days 5 and 15. Results are expressed as mean±SD for three individual experiments.

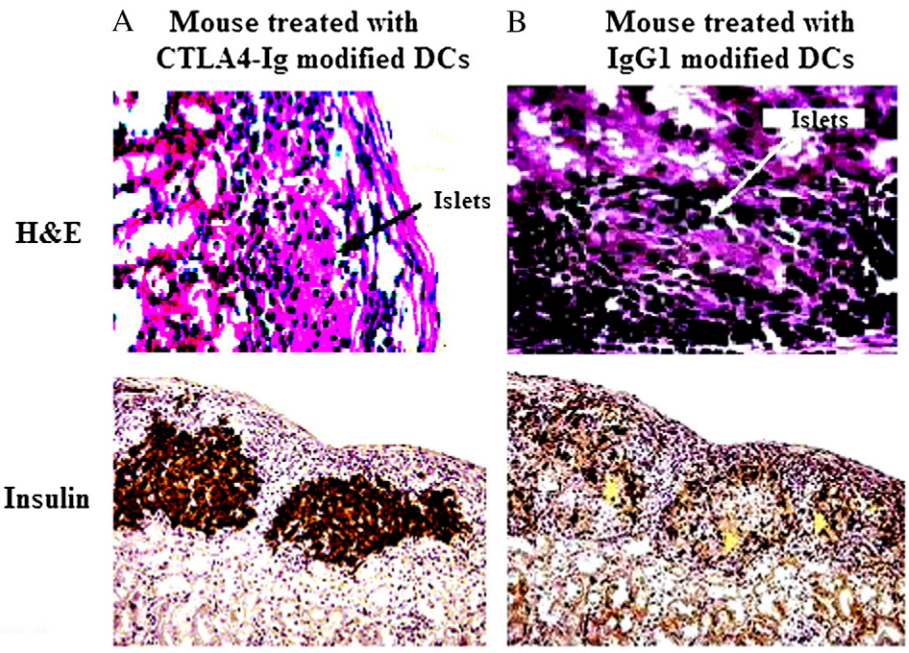

Fig. 7. Comparison of islet allograft histology in recipients from mDCs and IgG1-DCs treatment groups. Line 1, hematoxylin/eosin staining (H&E). Line 2, immunoperoxidase staining for antibodies to insulin (magnification×100).

suspends because the islet β cells are impaired by pathogenic autoimmune CD4+Th1 and CD8+T recognizing islet β cell autoantigens, such as glutamic acid decarboxylase (GAD65), islet antigen-2 or islet cell antibody 512 (ICA512) and insulin related antigen [18–20].

T cells are major effector cells in the allograft rejection. CD28 on T cells and B7-1/B7-2 on APCs are two important costimulatory molecules to activate T lymphocytes [21,22]. Blocking or inhibiting the transmission of costimulatory receptor signals and antigen presentation are efficient ways to induce the specific immune tolerance. CTLA4 is a negative-regulator of T cell activation by inhibiting T lymphocyte proliferation and inducing its apoptosis by blocking the co-stimulatory pathway [8]. Evidences have demonstrated that the immunosuppressive molecules modified DCs could induce the alloantigen-specific T cell anergy and prolong allograft survival [23–25]. In this study, we demonstrated that CTLA4-Ig modified DCs were able to stably express the CTLA4-Ig fusion protein. As a result, the lymphocyte proliferation induced by mDCs was obviously less than that induced by umDCs, because the activation of the allotype antigen-specific T cells was inhibited and followed by a long-term immune tolerance. The responsibility of murine T cells sensitized by mDCs to allotype DC stimulation in vitro was compromised, whereas, the lymphocytes from umDC-sensitized mice displayed a strong response for the stimulation of allotype cells in vitro, these data demonstrated that CTLA4-Ig modified DCs could induce T cell anergy and evoke the peripheral tolerance against specific antigens.

In order to assess the effect of mDCs on allotype islet transplantation, the islet cells of BALB/c mice were planted into the kidney capsules of C57BL/6 mice and the gene-modified DCs were intravenously administered simultaneously. Consequently, injection of mDCs could evidently prolong the graft survival. By daily monitoring PG levels, we found that the PG levels could decrease to lower than 11.1 mmol/L from a higher level. These phenomena happened in the mice treated with mDC 3 days after islet transplantation and maintained more than 2 weeks. However, in the control mice, 5 days after transplantation, their PG continued increase gradually to the level more than 11.1 mmol/L. In addition, we detected serum IFN-γ and IL-4 on days 5 and 15, the results showed that the level of IFN-γ decreased and IL-4 increased in mDC treated recipient mice. These indicated that a deviation of Th1/Th2 occurred. The HE stain of the IDDM murine renal specimens after 7 days islet transplantation showed that there were numerous islet cells in the renal slices in the normoglycemic mice and their cellular structure was intact with a sparse inflammatory cells infilitration. These results suggested that administration of mDCs would improve the outcome of islet transplantation by inhibiting T-lymphocyte proliferation and inducing the immune tolerance.

Injection of mDCs after transplantation could induce the immune tolerance against allotype antigens effectively. The mechanisms may include: mDCs directly present the antigens to cytotoxic T lymphocytes (CTL), MHC homologous CTL recognizes antigens and obtains the first signal. The mDCs secrete CTLA4-Ig to block CD28/B7 costimulatory pathway or to supply a negative costimulatory signal, then inhibit the further activation of allotype T cells or induces their anergy; the negative costimulatory molecules and cytokines which down-regulate the Th1 activity may inhibit the activation and proliferation of specific MHC-restricted T cells, then inhibit the recipient immune response to allotype antigens. By secreting the negative costimulatory molecules, mDCs inhibit the activation and proliferation of allotype lymphocytes as well as autotype T cells against the islet β cells, thereby induce the allotype tolerance and restructure the autotolerance [26–28].

In summary, our studies demonstrate that CTLA4-Ig modified DCs can prolong graft survival in islet transplantation by the inhibition of T cell proliferation and induction of T cell apoptosis. It may lead a novel strategy to treat diabetes.

Acknowledgement

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