Article

IL-10 Gene Modified Dendritic Cells Inhibit T Helper Type 1-Mediated Alloimmune Responses and Promote Immunological Tolerance in Diabetes

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Dendritic cells (DCs) have the potency to regulate the outcome of autoimmunity through the modulation of immune responses. The induction of antigen specific tolerance is critical for prevention and treatment of allograft rejection. In the present study, we transfected IL-10 gene into DCs and investigated their effect on inhibition of lymphocyte activity *in vitro* and induction of immune tolerance on islet allograft in mice. An IDDM C57BL/6 mouse model was induced by streptozotocin. The islet cells isolated from the BALB/c mice were transplanted into the kidney capules of the model mice followed by injection of IL-10 modified DCs (mDCs). The results showed that mDCs could significantly inhibit T lymphocyte proliferation mediated by allotype cells and induce its apoptosis, whereas, unmodified DCs (umDCs) could promote the murine lymphocyte proliferation markedly. The injection of mDCs could prolong the survival of allotype islet transplanted IDDM mice. The average plasma glucose (PG) level in mDCs treated mice returned to normal within 3 days and lasted for about 2 weeks. The rejection response in control mice occurred for 5 days after transplantation. The level of IFN-γ was lower while IL-4 was higher in mDCs treated mice than that in umDCs treated mice, which indicated that Th1/Th2 deviation occurred. Our studies suggest that IL-10 gene modified DCs can induce the immune tolerance to islet graft and prolong survival of the recipients by the inhibiting of T cell proliferation in allotype mice. *Cellular & Molecular Immunology*. 2008; 5(1):41-46.

Key Words: IL-10, dendritic cell, IDDM, islet transplantation, immune tolerance

Introduction

Diabetes mellitus (DM) is a predominant cause of end-stage renal disease, nontraumatic lower extremity amputations and adult blinding in the United States and other developed countries. DM will be a leading cause of morbidity and mortality for the foreseeable future (1). The present therapeutic procedure of DM includes exogenous insulin supplement and stimulation of endogenous insulin. Islet cell transplantation for the treatment of DM is still in experimental stage, but it is a promising therapy for Insulin-dependent type 1 diabetes mellitus (IDDM) (2). However, the transplanted islet cells are very sensitive to various stimuli and could be easily rejected by host 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 (3, 4). They are potentially powerful tools for the therapeutic manipulation of immune reactivity in cancer, infectious disease, and allograft rejection. T cells are the major responsive cells for the rejection process of allotype transplantation. The induction of antigen-specific tolerance is critical for the prevention of autoimmunity and the maintenance of immune tolerance. Presently, two different approaches for the selective enhancement of the tolerogenic properties of DCs are under investigation: (1) the use of immature DCs or the pharmacological arrest of the

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maturation of DCs; (2) the use of genetically modified DCs expressing immunosuppressive molecules (5, 6).

IL-10 is a pleiotrophic immunomodulatory cytokine that functions at different levels of the immune response (7). Especially, IL-10 induces anergy of T cells by efficiently inhibiting their proliferation and cytokine production. IL-10 can directly inhibit the phosphorylation of CD28, and thereby abrogates any further downstream signaling. In addition, when human naïve T cells were cultured with IL-10, with or without IFN-α, they became regulatory T cells that are able to suppress the activation and proliferation of other bystander T cells (8). IL-10 may also induce tolerance indirectly, as it interferes with the DC function. Therefore, IL-10 is considered as a promising therapeutic factor for autoimmune/ inflammatory diseases (9, 10). As *il-10* is a critical immune modulatory gene and modulation of *il-10* gene expression may alter DC function. In the present study, we investigated the effect of the *il-10* gene modified dendritic cells on immune tolerance of allotype islet transplantation.

Materials and Methods

Animals and induction of hyperglycemia by STZ

Eight- to ten-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. All experiments were approved by the local government authorities. BALB/c mice were used as donors, and C57BL/6 mice as recipients. Diabetes was induced in C57BL/6 mice by an intravenous injection of streptozotocin (Sigma, St. Louis, MO) at a concentration of 200 mg/kg (11). The plasma glucose (PG) levels were measured by a Beckman Glucose Analyzer (Roche Accu Check III). Three days later, the nonfasting PG was measured daily for five 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.

Transfection of IL-10 gene into DCs and cell culture

Plasmid pcDNA3.1/IL-10 was transfected into DCs by Lipofectin 2000 (Invitrogen Co., Ltd), and the positive cells were screened by G418 and identified by ELISA kit (Jingmei Biotech Co., Ltd). After cloned by limiting dilution, the positive DCs were cultured in RPMI 1640 supplented with 10% FCS and 500 μg/ml G418.

Detection of lymphocyte apoptosis

The lymphocytes of healthy C57BL/6 mice were prepared by a sterile method and were used as reactive cells (1×10^7/ml). *il-10* gene modified DCs (mDCs) or unmodified DCs (umDCs) pretreated with mitomycin C (Sigma) were employed as stimulatory cells (1×10^5/ml). After the lymphocytes were incubated with mDCs or umDCs, their apoptosis were detected by ELISA (Roche) as described previously (12), and its OD value was read at 405 nm. The specific enrichment factor of nucleosomes or oligonucleosomes delivered by lymphocytes was calculated by the following formula: enrichment factor = mU of experimental wells / mU of control wells (mU = mean OD value - OD value of substrate).

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 once a day for five days. The murine splenocytes were harvested and mononuclear cells were isolated after the animals were sacrificed. 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. The proliferation activity was tested by methyl thiazolyl tetrazolium (MTT) chromatometry. Stimulation index (SI) was calculated as the following: SI = mean OD value of experimental wells / mean OD of control wells.

Islet transplantation

Pancreatic islets were isolated from the male BALB/c mice by collagenase technique (13). After washed twice, the islets were cultured in 1 ml Krebs-Ringer bicarbonate buffer with HEPES and its insulin secretory function was assayed by immunoradio assay (14). 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 according to the technique for renal subcapsular islet transplantation as reported previously (15).

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 mDCs-pcDNA3.0 (5 mice); IV) islet transplant followed by injection of umDCs (6 mice). The number of DCs in above groups was 1×10^7/mouse. Eight healthy C57BL/6 mice without islet transplantation were employed as controls.

Observation and cytokine quantitation 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 plasma glucose no greater than 8.4 mmol/L. Insulin secretion was tested in normoglycemic recipients at days 5 and 15 after transplantation according to the procedure reported previously (16). At days 5 and 15 after transplantation, serum IL-4 and IFN-γ levels in recipient mice and healthy control mice were measured using ELISA kit (Jingmei Biotech Co., Ltd), according to the manufacturer's instructions.

Immunohistochemical stain of the transplanted islets

The kidneys from the recipient mice were sliced horizontally and embedded in paraffin. The sections were stained with hematoxylin and eosin (HE stain) 7 days after transplantation and studied under the microscope.

Statistical analysis

The data were shown as mean ± standard deviations (SD).

Table 1. Expression of hIL-10 in the culture supernatant of DCs

Culture supernatant	IL-10 concentration (ng/10^6)
Control (PBS)	0.0
umDCs	0.0
mDCs transfected for 48 h	68
mDCs positive clone	71

Plasmid IL-10 was transfected into DCs and the supernatant of positive cells was identified by ELISA kit.

The Student's *t*-test was used to compare the difference between two groups. The differences were considered statistically significant when $p < 0.05$.

Results

The expression of IL-10 in the supernatant

Plasmid pcDNA3.0/IL-10 was transfected into DCs, the supernatant was collected and its IL-10 concentration was measured by ELISA (Table 1).

Effects of mDC on apoptosis and proliferation of allotype lymphocytes

The apoptosis of the lymphocytes was measured by ELISA. The enrichment factor was proportional to the apoptotic cells. The results showed that the enrichment factor was 2.23 ± 0.41 when umDCs was used as stimulatory cells and 3.90 ± 0.64 when mDCs were used as stimulatory cells, indicating that mDCs induced significantly higher lymphocyte apoptosis than umDCs ($p < 0.05$, n = 4).

The lymphocyte proliferations induced by mDCs were 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 the highest in umDC sensitized mice when the lymphocytes were induced by umDCs (Figure 1).

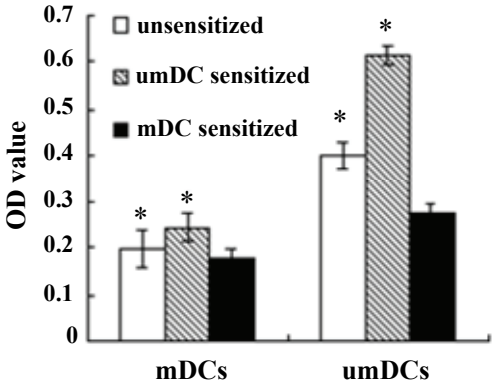

Figure 1. The lymphocyte proliferations of different groups by different DCs. Modified DCs or umDCs were intraperitoneally injected into C57BL/6 mice at a dose of 1×10^7/mouse once a day for five days. The murine splenocytes were harvested and mononuclear cells were isolated after the animals were sacrificed, then 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. The proliferation activity was tested by MTT. *$p < 0.01$, compared between mDC and umDC group. Data were shown as mean ± SD, n = 3.

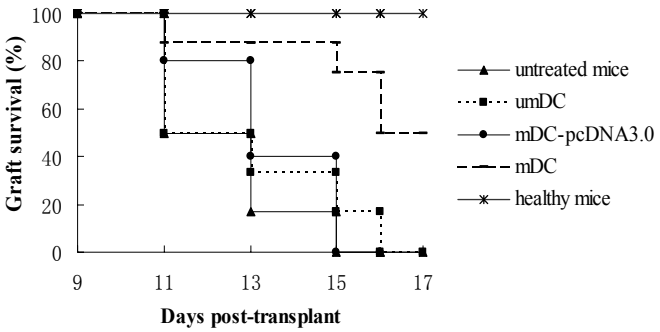

Figure 2. Survival days of transplanted mice. The islet recipient mice were allocated into four groups and treated with: 1) islet transplant only (6 mice); 2) islet transplant followed by injection of mDCs through the vena caudalis at the same day (8 mice); 3) islet transplant followed by injection of mDCs-pcDNA3.0 (5 mice); 4) islet transplant followed by injection of umDCs (6 mice). The number of DCs in above groups was 1×10^7/mouse. Eight healthy C57BL/6 mice without islet transplantation were employed as controls.

The survival of transplanted mice and their PG levels

Transplanted IDDM mice injected with umDCs, mDCs-pcDNA3.0 and mDCs-IL-10 survived for 10.17 ± 1.60 d, 11 ± 1.58 d and 19.38 ± 9.81 d, respectively (Figure 2). The results demonstrated that mDC injection prolonged the mouse survival more evidently than umDC and pcDNA3.0

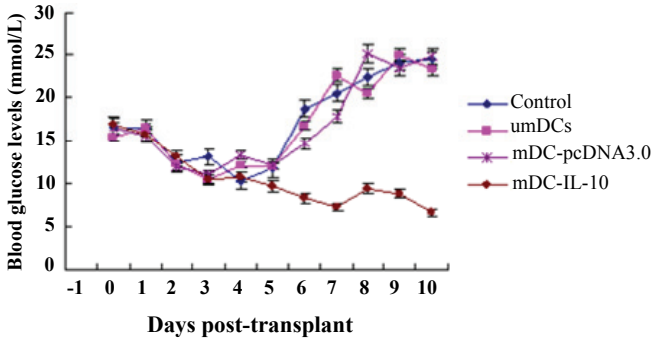

Figure 3. The profiles of PG levels in different islet recipient mice. The PG levels in the mice injected with mDCs were decreased to less than 11.1 mmol/L 3 days after transplantation, but those in the mice treated with umDCs and mDCs-pcDNA3.0 elevated to 11.89 ± 2.86 mmol/L and 11.99 ± 2.34 mmol/L at day 5 respectively, indicating the occurrence of graft rejection.

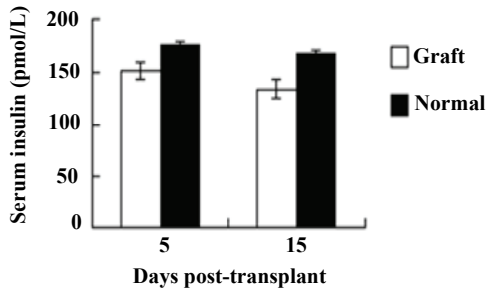

Figure 4. The serum insulin levels after *i.p.* injection of glucose in mDC treated grafted mice. The glucose tolerance tests were carried out in the mice whose PG returned to normal at days 5 and 15 after transplantation and the healthy control mice to evaluate the function of the islet graft.

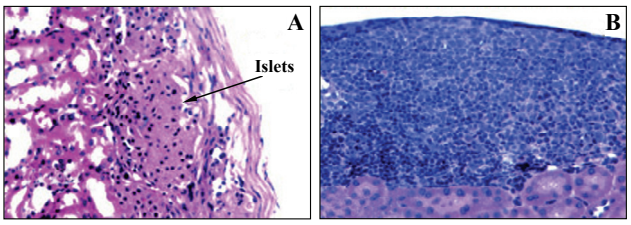

Figure 6. Hematoxylin and eosin staining of islet grafts (100×). The kidneys from the recipient mice were sliced horizontally and embedded in paraffin. The sections were stained with hematoxylin and eosin (HE stain) 7 days after transplantation and studied under the microscope. (A) Mice treated with mDCs, the islet cells remained the intact cellular structures. (B) Mice treated with mDC-pCDNA3.0, numerous inflammatory cells infiltrating and destroying the islets.

modified DC injection ($p < 0.01$).

The graft rejection was identified by monitoring the daily levels of PG. The transplantation was considered successfully if PG remained lower than 11.1 mmol/L for more than 3 days. The graft rejection was diagnosed by return of hyperglycemia (>11.1 mmol/L) for more than 3 consecutive days. The PG levels in the mice injected with mDCs decreased to less than 11.1 mmol/L at day 3 after transplantation, but those in the mice treated with umDCs and mDCs-pcDNA3.0 elevated to 11.89 ± 2.86 mmol/L and 11.99 ± 2.34 mmol/L at day 5, respectively, which indicated the occurrence of graft rejection. The PG level in mDC treated mice remained in normal range, 9.68 ± 1.82 mmol/L, for about 2 weeks (Figure 3).

The result of glucose tolerance tests in transplanted mice

The glucose tolerance tests were carried out in the mice whose PG returned to normal at days 5 and 15 after transplantation and the healthy control mice to evaluate the function of the islet graft. The insulin secretion reached the peak after 10 min of glucose injection. We found that the insulin secretion in PG recovery recipients was similar to the healthy mice at day 5 after transplantation and was slightly lower than that in healthy ones at day 15 ($p > 0.05$) (Figure 4).

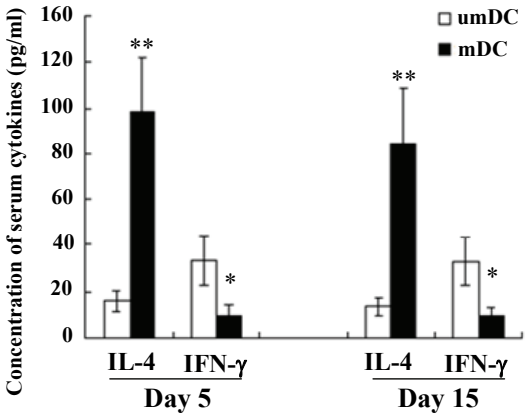

Figure 5. The level of serum cytokines of transplanted mice. At days 5 and 15 after transplantation, serum IL-4 and IFN-γ levels in mDCs and umDCs treated recipient mice were measured by ELISA. *$p < 0.05$, **$p < 0.01$, compared between mDCs and umDCs treated mice. Data were shown as mean ± SD, n = 3.

Cytokines in the transplanted mice

The production of IFN-γ was obviously inhibited in mDC mice whose PG returned to normal. The IFN-γ level in mDC mice was significantly lower than that in umDC mice; whereas, the IL-4 level was higher than that in umDC mice ($p < 0.05$) (Figure 5).

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 mDCs-pcDNA3.0, there were numerous inflammatory cells infiltrating and destroying the islets. The lamellar necrosis was observed in some areas. However, in mice treated with mDCs, the islet cells remained the intact cellular structures. The grade of mononuclear cell infiltrating into the grafts was minor. The structure of the kidney was normal (Figure 6).

Discussion

IDDM is an organ-specific autoimmune disease mediated by T lymphocytes recognizing pancreatic islet cell antigens and antibodies (17). In this disease, 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 antigens (18). As the consequence, the insulin secretion decreases or suspends. The present therapeutic procedure of IDDM includes exogenous insulin supplement and stimulation of endogenous insulin. Islet cell transplantation for the treatment of IDDM is still in experimental stage, but it is a promising therapy for IDDM (19). However, the transplanted islet cells are very sensitive

to various stimulations and easily rejected by host immune systems. Persistence of activated T cells is followed by acute rejection after allotype transplantation, implying that the risk of rejection is based on continuous T-cell activation that is not inhibited by present immunosuppressive therapy. One approach to induce immunologic hyporesponsiveness in recipients is down-regulating activated T cells by modifying donor-derived DCs, which theoretically may lead to donor-specific tolerance.

IL-10 is an inhibitor of immunity, released by immune cells, particularly, in response to antigen stimulation. IL-10 has been shown to block DC maturation *in vitro*, markedly reducing expression of MHC and costimulatory molecules on DC. It is now accepted that signaling through the T-cell antigen receptor (TCR) alone can lead to allospecific T-cell anergy or apoptosis (20). It has been reported that *il-10* gene modified DCs could induce antigenic-specific tolerance in autoimmune disease such as autoimmune myocarditis (21, 22).

In the present study, we induced specific tolerance to islet cells by intravenously injection of pcDNA3-IL-10-DCs. Injection of mDCs could evidently prolong the mouse survival. By daily monitoring PG levels, we found that the PG levels could return to less than 11.1 mmol/L, a normal level, in mDC treated mice at day 3 after islet transplantation, and lasted for about 2 weeks. However, the grafts in the control mice were rejected 5 days after transplantation. Their PG was increased gradually and surpassed 11.1 mmol/L. HE staining of the IDDM mouse renal specimens 7 days after islet transplantation showed that the kidney structure was normal. There were numerous islet cells in the renal slices of the mDCs treated mice and their cellular structures were intact and infiltrated by only a few inflammatory cells, indicating that the islet cells survived.

IL-10 modified DCs may induce immune tolerance by some pathways. Our studies demonstrated that mDCs could prevent allogeneic rejection in islet transplantation and prolong survival of the recipients by the inhibition of T cell proliferation and inducing apoptosis in these cells. We also detected serum cytokines at days 5 and 15 after transplantation. In mDCs treated mice, the levels of IFN-γ were decreased but IL-4 increased, indicating that a deviation of Th1/Th2 occurred. The Th1/Th2 deviation is beneficial to long-term amelioration of IDDM because creating a Th2 cytokine enriched environment can halt IDDM development (23).

So far, there have been a few reports that graft rejection could be inhibited by IL-4 or other inhibitory cytokines. Both IL-10 and IL-4 are synthesized by Th1 cells, but recent study showed that IL-10 up-regulated the expression of IL-4 and down-regulated the expression of IFN-γ, a rejection activator (24). So it may inhibit the immune rejection stronger than IL-4. In this study, our results also displayed that the serum IL-4 concentration in mDC mice was obviously higher than that in umDC mice, while the IFN-γ concentration in mDC mice lower than that in umDC mice. Data above may implicate that IL-10 modified DC is a more effective therapeutic strategy compared with IL-4.

Our studies demonstrated that IL-10 gene modified DCs could prevent allogeneic rejection in islet transplantation and prolong survival of the recipients by the inhibition of T cell proliferation and induction of its apoptosis, which might lead to new therapeutic strategies for IDDM.

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