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IL-12p40 is not required for islet allograft rejection[1]

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Key words

Th1 cells; Th2 cells; islets of Langerhans transplantation; homologous transplantation; IL-12p40-deficient mice

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Abstract

Aim: To investigate whether IL-12p40 plays a crucial role in regulating islet allograft rejection in a streptozotocin (STZ)-induced diabetes mouse model. **Methods:** C57BL/6 and IL-12p40 gene knockout mice were selected as recipient mice, to which the diabetes was induced with a treatment of STZ (150–200 mg/kg) by a single ip injection. BALB/c mice were selected as donor mice and islet cells were isolated from the mice. The 500 islets were transplanted into recipient mice beneath the capsule of the left kidney. Following the islet transplantation the glucose from the mice sera was monitored and the rejection rate of islets was analyzed. **Results:** STZ could induce diabetes in the recipient mice within 1 week. After transplantation of allograft islets, the increased glucose in wild-type (WT) mice returned to normal level and was maintained for 10 d. Unexpectedly, the rejection rate of islet allograft between IL-12p40-deficient mice and WT mice was similar. **Conclusion**: The results suggested that, although islet allograft rejection is believed to be Th1-cell predominant, the Th1 response inducer, IL-12 and IL-23 are not essential to induce islet allograft rejection.

Introduction

$CD4^+$ Th cells can differentiate into two subsets, Th1 and Th2 cells. Th1 cells produce cytokines such as interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α and often orchestrate the cellular immune responses noted in certain organ specific autoimmune diseases, allograft rejection, and delayed-type hypersensitivity responses. In contrast, Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 cytokines and lead to the production of antibodies contributing to humoral immunity. The balance between Th1 and Th2 cells determines an overcome of immune response[1].

Previous studies have shown that allograft rejections including the heart, skin and islet are usually associated with a Th1-type response. In contrast, the tolerant allograft in recipients often manifested a Th2-type response[2–5]. When IFN-γ, gene knockout (KO) mice were used as graft recipients, the allograft survival was found to be highly conditional and both the host strain and experimental conditions could influence the results[6]. When IL-4-deficient mice were used as an allograft recipient accompanied by CTLA4/Fc treatment, the allograft rejection was inhibited as compared to the control, wild-type (WT) mice[7].

IL-12 and IL-23 are believed to be Th1 initiators playing an essential role in Th1 development[8–10]. The cytokines are composed of a p35 and p40 subunit for IL-12 and p19 and p40 subunit for IL-23. They are produced primarily by activated antigen-presenting cells, such as dendritic cells and macrophages, proinflammatory natural-killer (NK) and activated T cells, and induced cell-mediated immunity[11]. The p40 subunit KO mice results in a lack of IL-12 and IL-23 and consequently leads to a defect in inducing Th1 cell response in autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE)and experimental autoimmune uveoretinitis (EAU)[12,13]. The data demonstrate that in IL-12p40 transgenic mice the development of diabetes was exacerbated in non-obese diabetic (NOD) mice[14]. The heart allograft rejection was accelerated when recipient mice were

injected with dendritic cell (DC) expressing IL-12p40[15]. It is unclear whether islet allograft survival is postponed in IL-12p40-deficient mice.

In this study, we investigated whether IL-12p40-deficient mice prevented islet allograft rejection in the streptozotocin (STZ)-induced diabetes mouse model. Our data showed that in an STZ-induced type-1 diabetes model, the survival time of islet allograft was similar in IL-12p40-deficient mice and WT mice.

Materials and methods

Mice Six-to eight-week-old female C57BL/6 ($H2^b$), BALB/c ($H2^d$), IL-12p40-deficient (P40KO,C57B6.129S1-$Il12b^{tm1Jm}$/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were kept in a specific pathogen-free facility at the Chinese Academy of Sciences. Animal care and use were in compliance with institutional guidelines.

STZ treatment and diabetes induction Recipient mice were given diabetes by a single ip injection of STZ (150 mg/kg for C57 and 200 mg/kg for IL-12p40 deficient mice). STZ (Fluka, St Louis, MO) was dissolved in sodium citrate buffer (pH 4.5). Diabetes was defined as a plasma concentration of glucose >16.7 mmol/L within 2 d after the end of STZ treatment[16]. Plasma glucose concentration was measured using a Medisense Optium blood detector (Abbott Laboratories, MediSense products, Bedfold, USA).

Islet isolation and transplantation Islets were isolated from female BALB/c ($H\text{-}2^d$) donors by using a filtered method as described in a previous study[17]. The pancreas was digested by collagenase P (Roche Diagnostics Corporation Indianapolis, IN, USA) for 12 min, and followed by filtering with a 300 μm strainer. The filtered part was poured through a 100 μm cell strainer (BD Falcon, Bedfold, USA) to enrich the islets. Finally, 500 islets (diameter varied between 100 μm and 300 μm) were handpicked and transplanted into the renal subcapsular space of STZ-induced diabetic recipients[18].

A graft transplantation was considered successful when the plasma glucose concentration was reduced to 16.7 mmol/L within 48 h. If not, the mouse was considered to be a technical failure and was excluded from analysis. Graft rejection was defined as the recurrence of a plasma glucose concentration >16.7 mmol/L on 2 successive days. Islet allograft function was confirmed by unilateral nephrectomy of the kidney bearing the transplant and documentation of the reappearance of diabetes.

ELISA The sample of mouse serum was collected and frozen at -70 °C until it was detected. Briefly, 20 μg/mL of EQ31-BSA (the C-peptide conjugated with BSA carrier) was coated with 100 μL/well in a 96-well Nunc Immunoplate (Nunc, Roskilde, Denmark) overnight at 4 °C. The plates were blocked with blocking buffer (PBS with 3% gelatin) for 2 h at 37 °C. Sample 100 μL (containing 90 μL serum and 10 μL C-peptide antibody) was added and incubated at 37 °C for 2 h. Then a second antibody horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig)-G (BD PharMingen, San Diego, CA, USA) at 100 μL/well was added and *OD* values were measured with a microplate autoreader (Biotek, Vermont,USA) at 450 nm. The standard curve for C peptide was created with different concentrations of C peptide and was used to measure C peptide concentrations in tested sera samples by competitive ELISA.

Immunohistopathology Islet graft was embedded in optimal cutting temperature compound (OCT) and frozen at -20 °C. The sections of 10-μm thickness were kept at -70 °C until staining. CD4+ and CD8+ cells were determined by immunohistochemistry with the specific antibodies (BD PharMingen).

Real-time PCR Total RNA was isolated from graft. An mRNA expression of IFN-γ and IL-4 was determined by real-time PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, USA) as described in a previous study[19]

Statistical analysis The log-rank test of nonparametric analysis (Instat software; Graph Pad, San Diego, CA, USA) was used to analyze graft survival data. The differences in graft survival were analyzed by a Kaplan-Meier test. The experiments were usually repeated three times.

Results

Establishment of islet allograft model Initially, C57BL/6 and IL-12p40-deficient mice served as recipient mice and STZ was used to induce diabetes in the mice. We found that the different genetic background had a great impact on diabetes induction, which was consistent with other's reports[20]. Based on our primary experiments, the optimal dose of STZ to induce diabetes in C57BL/6 mice is 150 mg/kg, but it was 200 mg/kg in IL-12p40-deficient mice.

After STZ ip injection, the glucose in the blood increased rapidly and remained at a relative high level. However, it remained normal in the vehicle control group. Based on this protocol, the diabetes was developed within a few days in STZ-treated mice. If the sick mice were not treated with islet transplantation, the mice began to die on d 10 after STZ treatment (Figure 1A).

Five hundred islets freshly isolated from BALB/c or C57BL/6 mice were transplanted into the renal subcapsular space of C57BL/6 mice. Then the glucose in the blood was detected. The data showed that, after transplantation of the islets, the enhanced glucose in the recipient mice dropped to

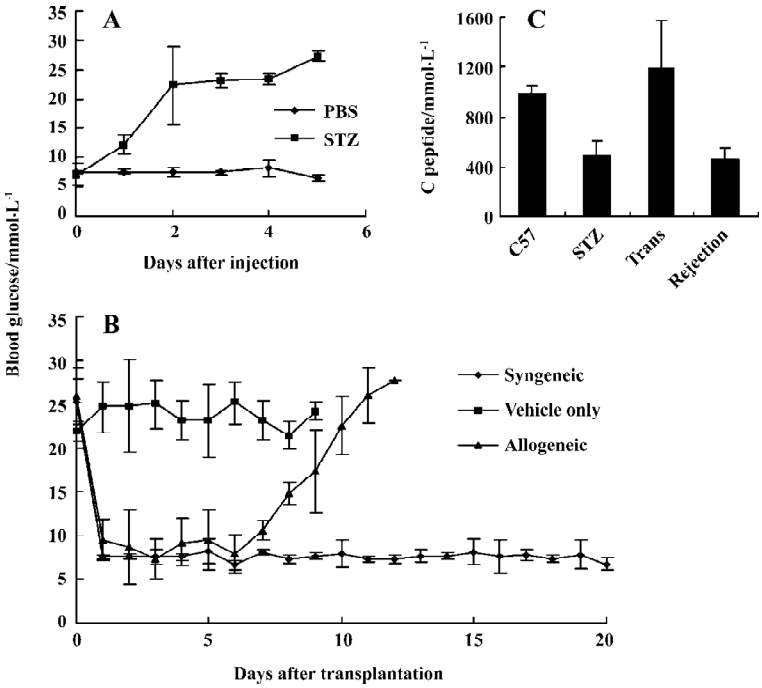

Figure 1. Establishment of the mice model of islet allograft transplantation. A, Streptozotocine induce diabetes in C57 mice with a dose of 150 mg/kg (n=6, P<0.01). B, Islet transplantation. The glucose concentration in the blood was measured kinetically by Medisense Optium blood detector in three groups (vehicle, allograft and syngeneic groups, n=7). C, C-peptide concentration in the mice sera from normal C57BL/6 mice, STZ-treated mice, diabetic mice with islet transplantation and allograft islets rejected mice (n=6).

the normal level within 2 d (Figure 1B). It was obvious that the glucose concentration was increased and exceeded 16.7 mmol/L on d 10 following the islet transplantation and remained hyperglycemic when the islet allograft from Balb/c mice (allogeneic group) was rejected completely. But the islets from C57BL/6 mice (syngeneic group) functioned well and the blood glucose remained normal until 20 d after islet transplantation. In contrast, when Hanks' solution served as a control (vehicle group) in the transplantation, the hyperglycemia was not changed.

To confirm the islet rejection, the left kidneys from islet transplanted mice were removed on d 5 (non-rejected islet) and d 12 following the transplantation. The tissue histopathology was stained with HE. Only intact islets could be seen in the tissues of non-rejected islets, but could not be seen in rejected islet (data not shown).

It was reported that the concentration of C-peptide in the blood was reduced when the diabetes occurred[21]. The concentration of C-peptide was detected in the mice serum from different types of the disease. The C-peptide concentration was consistent with diabetes. In control C57BL/6 mice, the C-peptide secretion was normal. After diabetes was induced, the C-peptide concentration was decreased. When islet transplantation was successful, the secretion of the C-peptide was rescued and then it returned to low levels after the allograft was rejected completely. The data indicates that C-peptide concentration is a good marker to reflect the stature of insulin secretion *in vivo* (Figure 1C).

Role of IL-12p40 gene in islets allograft transplantation Islets were isolated from major histocompatibility complex (MHC)-mismatched BALB/c mice and then they were transplanted into IL-12p40- deficient or wild-type C57BL/6 mice. As shown in Figure 2A and 2B, the rejection rate and survival mean days for islet transplantation were similar

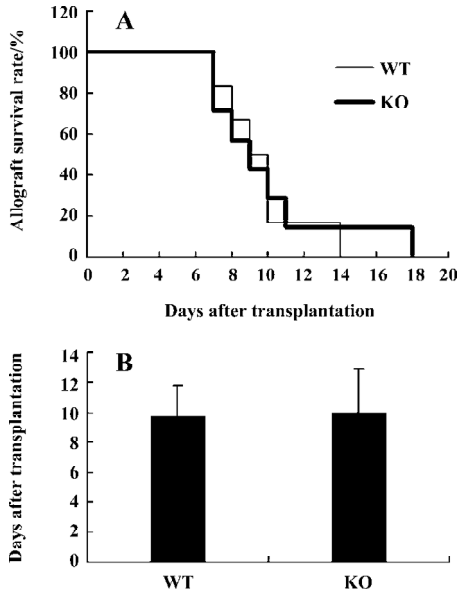

Figure 2. Analysis of islet allograft rejection in WT and IL-12p40-deficinet mice. A, The rejection rate is expressed by kinetic curve (WT, n=6; KO, n=7). B, The rejection rate is expressed by the mean days.

between WT and IL-12p40-deficient mice. In the control group, after islet transplantation, the mean survival time was 9.7±2.1 d (n=6). Similarly, it was 10.0±2.9 d (n=7) in the IL-12p40-deficient mice. The results suggested that after islet transplantation, the C57BL/6 WT and IL-12p40-deficient mice rejected the allograft at a similar rate, which was not as anticipated.

Immunohistopathology Previous studies have revealed that T cells play an important role during the islet allograft rejection and that the deficiency of IL-12p40 can affect the function of $CD4^+$ and $CD8^+$ T cells. In this study, we observed the infiltration of $CD4^+$ and $CD8^+$ T cells in the allograft. On d 9 after transplantation, the infiltration of $CD4^+$ and $CD8^+$ cells in the grafts was similar in the recipients of WT and IL-12p40-deficient mice (Figure 3).

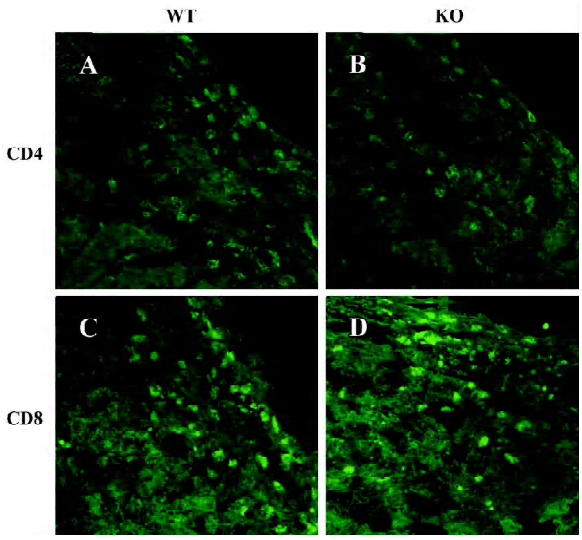

Figure 3. CD4+ and CD8+ T cell in islet grafts were analyzed by immune histopathology staining on d 9 after islet transplantation. Photomicrographs (×200) of (A) and (C) are from control WT mice, and the pictures of (B) and (D) are from IL-12p40 KO recipient mice. CD4+ T cells in infiltrated grafts are shown in (A) and (B). CD8+ T cells are shown in (C) and (D).

IL-4 and IFN-γ expression in the graft As Th1 and Th2 cells are believed to play a crucial role in determining the islet rejection, IL-4 and IFN-γ expressions in infiltrating T cells in the grafts were measured by real-time PCR. As we expected, IL-4 and IFN-γ expressions were similar in the grafts from WT and IL-12p40-deficient mice (Figure 4).

Discussion

Since the discovery of the cross-regulating Th1 and Th2

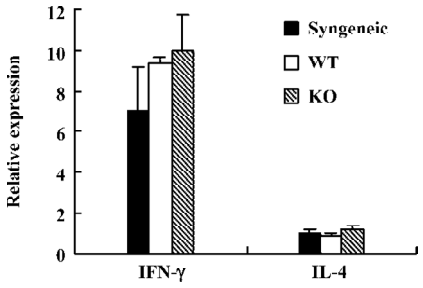

Figure 4. IFN-γ and IL-4 expression detected by real-time PCR in graft tissues. RNA from islet grafts were extracted on d 9 after islet transplantation. n=4 per group, Data represent mean±SEM.

phenotypes, there has been great anticipation that a Th1 to Th2 immune deviation may be critical in the acquisition of transplantation tolerance. This is because IL-12 is a major inducer and a pivotal regulator in the generation of Th1 cells[22]. It is also believed that IL-12 plays a key role in the destruction of insulin-producing cells during the development of autoimmune diabetes with a higher production of IFN-γ and lower production of IL-4, which is representative of a Th1-type response[23–25]. We are interested in studying the roles of IL-12 and IL-23 in allograft rejection, which is regarded as a Th1 cell-mediated response[26,27]. Allograft rejection in unmodified recipients is often associated with a Th1-type response. Moreover, allograft recipients that are treated with tolerant immunosuppressive regimens often manifest a Th2-type response during the treatment period[3–5].

In the present study, we addressed whether Th1 to Th2 immune deviation in IL-12p40-deficient mice could eliminate the severity of allograft rejection based on the fact that Th1 cells are essential in induced autoimmune diseases[28] and the observation in $CCR5^{-/-}$ mice of an obvious prolongation of islet allograft survival and a switch to Th2 response[18]. We predicted that IL-12p40 might play a determining role in islet allograft rejection in a STZ-induced diabetic mouse model. Surprisingly, islet allograft from BALB/c mice survived well in IL-12p40- deficient mice (C57BL/6 background) as compared to wild-type control mice, their mean survival time was similar and the values were 10.0±2.9 d and 9.7±2.1 d, respectively. In addition, there was no significant difference in the infiltration of CD4+ and CD8+ T cell and the cytokines expression (IFN-γ and IL-4) in transplanted grafts.

According to our observations, although tolerance therapy in some animal models often skewed the immune activation toward a Th2-dominated response[26,27], a Th1 to Th2 immune deviation does not uniformly permit the acquisition of transplant tolerance. This new finding leads us to make an explanation about the mechanisms of allograft re-

jection[29,30]. First, we have noticed that the induction of permanent engraftment or a state of allograft tolerance through the administration of long-acting Th2 cytokines (ie, IL-4-Ig and IL-10-Ig fusion proteins) or immune deviation via the application of IL-12 antagonists have failed in the heart allograft[30,31]. It is obvious that the strategies, which are extremely effective in dampening autoimmunity, have not been proven to be effective in transplantation[32–36]. In accordance with our results, although anti-IL-12 was used to deviate Th1 to Th2, anti-IL-12 treatment was totally ineffective in prolonging the engraftment of MHC-mismatched islet allografts. While the impact of anti-IL-12 upon the pattern of cytokine expression was consistent, the differences were noted in the impact of such therapy upon the duration of engraftment in MHC-mismatched versus MHC-matched conditions. It is concluded that regarding to the model of Ag presentation and the responding T cell clone number, the allograft response to MHC-matched rather than minor Ag-mismatched allografts may more closely resemble the response to auto-antigens[37]. It should be pointed out that a manifestation of a Th2-type response is not equal to an induced Th1 to Th2 immune deviation manipulation. If they have equal effects *in vivo*, logically, the Th2 biased polarization is only to induce weak tolerance rather than a sufficient condition to block immune response.

The evidence indicates that Th2 cytokine production to either graft survival or rejection is based on assessing intragraft cytokine gene expression by RT-PCR or identifying graft infiltrating cells that stain for cytokine protein by immunohistochemistry within allografts. As mentioned by Piccotti *et al*[29], these assays do not take into account the antigen specificity of the cells producing cytokines. Limiting dilution analysis studies have revealed that the frequency of donor-specific T cells infiltrating grafts is very low. Hence, RT-PCR may detect cytokine mRNA produced by irrelevant cells that are trafficking through the graft. We also found that the cytokines such as IFN-γ and IL-4 expressed by the grafts had no difference. This observation suggested that the evaluation of intragraft cytokine profiles for Th1/Th2 dominance should be viewed carefully. We accepted that all of the cytokine profile reflected a Th2 bias in the graft location, there was no solid evidence to indicate that Th2 cells are really protective as we had previously thought.

It should be pointed out that IL-12p40 is the common-chain of IL-12 and IL-23. Thus, the IL-12p40-deficient mice have both cytokines knocked out. Recently, the important role of IL-23 in autoimmune diseases and pathological inflammatory processes has been unraveled. IL-23 drives the development of a novel T-cell subset characterized by the production of IL-17 (Th_{IL-17}), which plays a central role in mediating chronic inflammatory responses[38]. It is not clear whether IL-23 will have any impact on allograft rejection. Our results suggested that a lack of IL-23 in p40-deficient mice did not inhibit islet transplantation.

In conclusion, the islet allograft survival is not prolonged in IL-12p40-deficient mice as compared to WT controls. We conclude that Th cell activation rather than Th1/Th2 polarization plays the major role in controlling allograft rejection. The precise mechanism for the failure of Th1 to Th2 immune deviation in allograft rejection in MHC-mismatched allograft recipients will require further investigation.

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