Original Article

Natural Killer T-Cells Participate in Rejection of Islet Allografts in the Liver of Mice

Atsushi Toyofuku,[1,2] Yohichi Yasunami,[1] Kentaroh Nabeyama,[1] Masahiko Nakano,[1] Masayuki Satoh,[1] Nobuhide Matsuoka,[1] Junko Ono,[3] Toshinori Nakayama,[4] Masaru Taniguchi,[5] Masao Tanaka,[2] and Seiyo Ikeda[1]

A role of natural killer T (NKT) cells in transplant rejection remains unknown. Here, we determined whether NKT cells participate in rejection of islet allografts, using NKT cell–deficient mice. Survival of islet allografts in streptozotocin-induced diabetic CD1d$^{-/-}$ mice or Vα14 NKT cell$^{-/-}$ mice was significantly prolonged without immunosuppression when grafted into the liver, but not beneath the kidney capsule, compared with wild-type mice. Acceptance of intrahepatic islet allografts was achieved in CD1d$^{-/-}$ mice by a subtherapeutic dose of rapamycin, which was abrogated in conjunction with the transfer of hepatic mononuclear cells from wild-type, but not from CD1d$^{-/-}$, mice at islet transplantation. The second islet grafts from a donor-specific, but not from a third-party, strain in CD1d$^{-/-}$ mice bearing functional islet allografts were accepted without immunosuppression at 120 days after the initial transplantation. These findings demonstrate that NKT cells play a significant role in rejection of islet allografts in the liver of mice, but that NKT cells are not essential for induction of donor-specific unresponsiveness in this model. The current study indicates that NKT cells might be considered as a target for intervention to prevent islet allograft rejection when the liver is the site of transplantation. *Diabetes* 55:34–39, 2006

Pancreatic islet transplantation has become a procedure of choice for treatment of insulin-dependent diabetes (type 1 diabetes) since Shapiro et al. (1,2) reported that successful islet transplantation in patients with type 1 diabetes can be achieved by introduction of a novel steroid-free immunosuppressive regime different from that of organ transplantation and designed for islet transplantation. Currently, however, the rate of insulin independence in patients with type 1 diabetes declines with time to <10% by 5 years after transplantation, although it is >70% at 1 year after islet transplantation (3). This indicates that the functional mass of islet grafts in recipients decreases with time, resulting in a requirement for treatment with exogenous insulin after transplantation (4). Among the factors producing the loss of functional islet graft mass, the continuous administration of immunosuppressive agents might be a potential cause because it has been well known that calcineurin inhibitors, such as FK506 and cyclosporin A, which are currently used for prevention of islet allograft rejection in a clinical setting, are toxic to β-cells of islets (5–7), and their long-term use may lead to deterioration of islet grafts without rejection. To overcome this, a novel immunosuppressive regime for islet transplantation that is nontoxic to β-cells needs to be developed, and for this purpose, a precise dissection of cellular mechanisms involved in islet allograft rejection is essential to help provide potential targets for intervention.

Natural killer T (NKT) cells have been recently identified as a novel lymphoid subset distinct from conventional T-cells, coexpressing an invariant T-cell receptor and natural killer cell–related surface markers, including NK1.1 (8). Unlike conventional T-cells, NKT cells are specific for glycolipid antigen bound with the major histocompatibility complex class I-like molecule CD1d (9,10). NKT cells produce large amounts of γ-interferon (IFN-γ) on activation (11,12), mediating protective functions, including prevention of infectious diseases (13,14), experimental tumor metastasis (15), and chemical-induced tumor development (16). NKT cells also mediate regulatory functions in the protection against autoimmune disease development (14), such as experimental allergic encephalitis (17,18) and type 1 diabetes in NOD mice (19–21).

In the current study, we hypothesized that NKT cells play a significant role in islet allograft rejection because the liver, the site of clinical islet transplantation, contains relatively abundant NKT cells (8) and because NKT cells have been reported to activate CD4 Th1 (T helper 1) cells and CD8 cytotoxic T-cells in the acquired immune system after activation (14,22). To prove this, we used NKT cell–deficient mice as recipients, and we demonstrated that NKT cells are activated after islet transplantation, leading to rejection of islet allografts in the liver of mice. The current study suggests that NKT cells might be considered as a target for intervention for prevention of islet allograft rejection when the liver is the site of transplantation.

RESEARCH DESIGN AND METHODS

Male C57BL/6 (H-2^b, 12–15 weeks old), BALB/c (H-2^d, 9–12 weeks old), and C3H/HeN (H-2^k, 9–12 weeks old) mice were purchased from Charles River

From the [1]Department of Surgery I, Fukuoka University, Fukuoka, Japan; the [2]Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; the [3]Department of Laboratory Medicine, Fukuoka University, Fukuoka, Japan; the [4]Department of Molecular Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan; and the [5]Laboratory for Immune Regulation, Riken Research Center for Allergy and Immunology, Yokohama, Japan.

Address correspondence and reprint requests to Yohichi Yasunami, MD, Associate Professor, Department of Surgery I, Fukuoka University School of Medicine, 7-45-1, Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. E-mail: yasunami@fukuoka-u.ac.jp.

Received for publication 30 May 2005 and accepted in revised form 5 October 2005.

α-GalCer, α-galactosylceramide; IFN-γ, γ-interferon; IL, interleukin; mAb, monoclonal antibody; NKT, natural killer T; STZ, streptozotocin.

TABLE 1
Islet allograft survival without immunosuppression

Group	Mice	Site	n	Survival (individual days)	Mean survival time (means ± SE)
I	Wild type	Kidney capsule	4	13, 15, 18, 24	17.5 ± 2.4
II	Wild type	Portal vein	9	7, 7, 9, 9, 9, 9, 11, 11, 11	9.2 ± 0.5
III	CD1d$^{-/-}$	Kidney capsule	3	12, 16, 17	15.0 ± 1.5
IV	CD1d$^{-/-}$	Portal vein	7	13, 16, 16, 26, 30, 31, 34	23.7 ± 3.2*
V	Vα14NKT$^{-/-}$	Kidney capsule	5	11, 15, 15, 16, 18	15.0 ± 1.1
VI	Vα14NKT$^{-/-}$	Portal vein	6	15, 17, 19, 20, 26, 52	24.8 ± 5.6*

BALC/c islets were transplanted beneath the kidney capsule or into the liver via the portal vein of STZ-induced diabetic C57BL/6 wild-type, CD1d$^{-/-}$, or Vα14 NKT cell$^{-/-}$ mice. *The difference is statistically significant ($P < 0.01$, log rank test) compared with the corresponding control (II).

Japan (Kanagawa, Japan) and used as recipients, donors, and third-party donors, respectively. CD1d-deficient (CD1d$^{-/-}$) mice and Vα14NKT cell–deficient (Jα281$^{-/-}$) mice (15) with a C57BL/6 background were also used as recipients. Vα14NKT cells are a major component of NKT cells reactive to CD1d (23). CD1d$^{-/-}$ mice, originally developed by Dr. Luc Van Kaer (Vanderbilt University), were provided by Dr. Moriya Tsuji (Rockefeller University). All experiments were performed in accordance with the institutional animal care and use committee.

Induction of diabetes in mice. Diabetes was induced in recipient mice by an intravenous injection of streptozotocin (STZ; 180 mg/kg; Sigma, St. Louis, MO). Plasma glucose levels were measured with a glucose analyzer (Beckman Instruments, Tokyo). After the STZ injection, nonfasting plasma glucose levels exceeded 400 mg/dl by day 2, and mice remained hyperglycemic at the time of islet transplantation.

Islet isolation and transplantation. Islets were isolated using static collagenase (type S-1; Nitta Gelatin, Osaka, Japan) digestion (24) and Ficoll-Conray gradient separation (25). Isolated islets were incubated overnight at 24°C in a 5% CO_2 incubator and then handpicked using a Pasteur pipette with the aid of a dissecting microscope. We transplanted 600 islets beneath the left kidney capsule (26) or into the liver via the portal vein (27) of diabetic mice 3 days after STZ injection. Nonfasting plasma glucose levels and body weight were monitored three times a week, and rejection was defined when two consecutive plasma glucose levels exceeded 200 mg/dl after islet transplantation.

Administration of rapamycin. Rapamycin was generously provided by Wyeth-Ayerst Research (Princeton, NJ). A solution of 1.0 mg/ml rapamycin in 0.2% carboxymethylcellulose (Sigma) was dispersed after ultrasonic disruption by a Bioruptor UCD-200T (Cosmo Bio, Tokyo) for 5 min and then diluted to the specified concentrations for the experimental groups. Rapamycin was administered intraperitoneally for 7 consecutive days, starting on the day of transplantation.

Morphological study. For light microscopic examination, the livers were removed and fixed with Bouin's solution, processed, and embedded in paraffin. The sections were stained with hematoxylin and eosin or aldehyde and fuchsin.

Preparation of hepatic mononuclear cells. Hepatic mononuclear cells were prepared as described previously (28). In brief, an excised liver was pressed through a stainless steel mesh, and the resulting dissociated liver tissues were suspended in Dulbecco's modified Eagle medium (DMEM/F-12; Life Technologies, Tokyo) and washed twice. The mixture was resuspended in an isotonic 33% Percoll solution containing heparin (67 units/ml) and centrifuged at 2,000*g* at 4°C for 15 min. The resulting pellet was suspended in 0.83% ammonium chloride solution to lyse erythrocytes. After counting, these hepatic mononuclear cells were washed twice in PBS and used for further analysis.

Antibodies and flow cytometry analysis. The antibodies used for flow cytometry analysis were as follows: Fc block (anti-mouse FcRγII/III monoclonal antibody [mAb], 2.4G2), fluorescein isothiocyanate–conjugated anti-CD3ε (clone 145-2C11, American hamster IgG1κ), allophycocyanin-conjugated anti–IFN-γ mAb (clone XMG1.2, rat IgG1), anti–interleukin (IL)-4 mAb (clone 11B11, rat IgG1), anti–IL-2 receptor α-chain (CD25; clone PC61, rat IgG1), and their isotype control (clone R3-34, rat IgG1) were purchased from PharMingen (San Diego, CA). Phycoerythrin-conjugated α-galactosylceramide (α-GalCer)-loaded CD1d tetramers were prepared as described previously (29).

The stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and the data were processed by CELLQuest software (Becton Dickinson). We collected 10,000 viable hepatic mononuclear cells for dot plot to analyze each cell population and 1,000 for histogram plot for each fraction.

Preparation of hepatic mononuclear cells for adoptive transfer experiment. Hepatic mononuclear cells were prepared from wild-type or CD1d$^{-/-}$ mice in the same way as flow cytometry analysis under aseptic conditions. We transferred 5×10^6 hepatic mononuclear cells in 200 μl Hanks' balanced salt solution via the portal vein of CD1d$^{-/-}$ mice at the time of islet transplantation.

Statistical analysis. Statistical significance of graft survival was determined by log rank test or Fisher's exact probability test, and that of flow cytometry data was examined by unpaired Student's t test. Results were considered statistically different at $P < 0.05$.

RESULTS

Prolongation of islet allograft survival in the liver but not renal subcapsular space of NKT cell–deficient mice without immunosuppression. First, we determined whether NKT cells are involved in acute rejection of islet allografts with the use of CD1d$^{-/-}$ mice and Vα14 NKT cell$^{-/-}$ mice as recipients. Furthermore, the effect of transplant sites, namely the renal subcapsular space versus the liver, on survival of islet allografts was examined because the liver contains relatively abundant NKT cells (8). When BALB/c islets were grafted beneath the kidney capsule of diabetic C57BL/6 mice, all islet allografts were rejected within 3 weeks after transplantation irrespective of the presence or absence of NKT cells (Table 1). Rejection was confirmed morphologically, in which only accumulations of mononuclear cells were seen at the site of islet transplantation, namely beneath the kidney capsule (histology not shown). In contrast, when BALB/c islets were grafted into the liver of diabetic mice, there was a significant prolongation of islet allograft survival in CD1d$^{-/-}$ and Vα14 NKT cell$^{-/-}$ mice compared with wild-type mice (Table 1). The difference in survival between wild-type and CD1d$^{-/-}$ mice and between wild-type and Vα14 NKT cell$^{-/-}$ mice was statistically significant ($P < 0.05$, log rank test) (Table 1). Morphologically, islet grafts infiltrated with mononuclear cells in the liver of recipient mice were seen at the time of rejection (Fig. 1, *right panels*).

Upregulation of IFN-γ production and CD25 expression in NKT cells and T-cells in the liver of wild-type mice rejecting islet allografts. To evaluate a role of NKT cells in the liver of mice rejecting islet allografts, hepatic mononuclear cells of mice were isolated for examination by flow cytometry. The yield of mononuclear cells from the liver of naïve untreated wild-type mice was $25.8 \pm 1.2 \times 10^5$ ($n = 3$), and that from the liver of wild-type mice that had received isografts or allografts was 40.3 ± 1.3 ($n = 3$) or $56.7 \pm 2.7 \times 10^5$ ($n = 3$), respectively, at 8 days after transplantation, when islet allografts are rejected. The difference in the yield of hepatic mononuclear cells between wild-type mice receiving isografts and those receiving allografts was statistically significant ($P < 0.05$, Student's t test). The yield of hepatic mononuclear cells from CD1d$^{-/-}$ mice was 27.3 ± 1.0

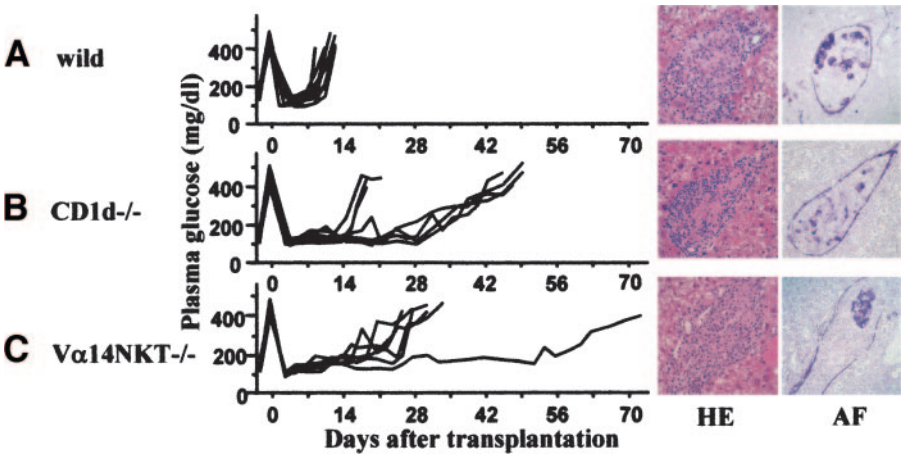

FIG. 1. Islet allograft rejection in the liver of mice without immunosuppression. We grafted 600 BALB/c islets into the livers of STZ-induced diabetic C57BL/6 wild-type (*A*, $n = 9$), CD1d$^{-/-}$ (*B*, $n = 5$), or Vα14 NKT cell$^{-/-}$ mice (*C*, $n = 6$). Individual lines of the left panels represent nonfasting plasma glucose levels of each animal. Photomicrographs of islet allografts in each group of mice at the time of rejection are shown (*right panels*). Original magnification ×200. AF, aldehyde and fuchsin stain; HE, hematoxylin and eosin stain.

$\times 10^5$ ($n = 3$) and 54.2 ± 4.6 $\times 10^5$ ($n = 3$) at 8 and 16 days, respectively, after islet transplantation, when islet allografts maintained or ceased to function without or with rejection, respectively.

We then determined whether NKT cells in the liver of wild-type mice are activated in association with rejection. To detect NKT cells, α-GalCer–loaded CD1d tetramers (29) were used for fluorescence-activated cell sorting analysis. The percentage of tetramer$^+$ CD3$^+$ NKT cells in the liver of naïve wild-type mice was 10.2 ± 1.6% ($n = 3$), and that in the liver of wild-type mice receiving isografts and allografts was 1.5 ± 0.3% ($n = 3$) and 5.9 ± 0.6% ($n = 3$), respectively, at 8 days after transplantation. Tetramer$^+$ CD3$^+$ NKT cells and tetramer$^-$ CD3$^+$ T-cells were further gated and examined with respect to the production of IFN-γ and IL-4 as well as CD25 expression. NKT cells are known to secrete IFN-γ and IL-4 on activation. The percentage of IFN-γ–producing tetramer$^+$ CD3$^+$ NKT cells in the liver of naïve wild-type mice was 2.5 ± 0.2% ($n = 3$), and that of wild-type mice receiving isografts or allografts was 6.6 ± 2.5% ($n = 3$) or 20.6 ± 2.9% ($n = 3$), respectively, at 8 days after islet transplantation. In contrast, there was no upregulation of IL-4 production in NKT cells in association with rejection. The CD25 expression of NKT cells in naïve wild-type mice was 15.2 ± 1.1% ($n = 3$), and that of wild-type mice receiving isografts or allografts was 3.4 ± 1.4% ($n = 3$) or 42.8 ± 3.7% ($n = 3$), respectively, at 8 days after transplantation. Representative data of three experiments is shown in Fig. 2*A*.

Regarding CD3$^+$ T-cells, an analysis similar to that of NKT cells was performed. It was found that IFN-γ production and CD25 expression of T-cells in the liver of wild-type mice receiving islet allografts became upregulated from 3.6 ± 0.2% ($n = 3$) to 21.9 ± 2.8% ($n = 3$) and from 7.4 ± 0.5% ($n = 3$) to 23.1 ± 1.7% ($n = 3$), respectively, at 8 days after transplantation, and that those of mice receiving isografts were from 3.6 ± 0.2% ($n = 3$) to 8.4 ± 3.7% ($n = 3$) and from 7.4 ± 0.5% ($n = 3$) to 8.5 ± 1.6% ($n = 3$), respectively. In marked contrast, the upregulation of IFN-γ production and CD25 expression of T-cells in the liver of CD1d$^{-/-}$ mice receiving islet allografts was not seen at 8 days after transplantation, when the islet grafts were rejected in wild-type mice. Representative data of three experiments is shown in Fig. 2*A* and *B*.

Acceptance of islet allografts by a subtherapeutic dose of rapamycin in the absence of NKT cells. We next determined the effect of NKT cells on rejection of islet allografts in the liver of mice treated with rapamycin. The survival rate of islet allografts in wild-type recipient mice treated with 0.2, 1.0, or 3.0 mg/kg rapamycin once a day for 6 days after transplantation was found to be 0% (0 of 9), 12.5% (1 of 8), and 71.7% (5 of 7), respectively, at 90 days after transplantation (Table 2). In marked contrast to wild-type mice, 91.0% (10 of 11) of CD1d$^{-/-}$ mice and 60% (3 of 5) of Vα14NKT cell$^{-/-}$ mice receiving islet allografts

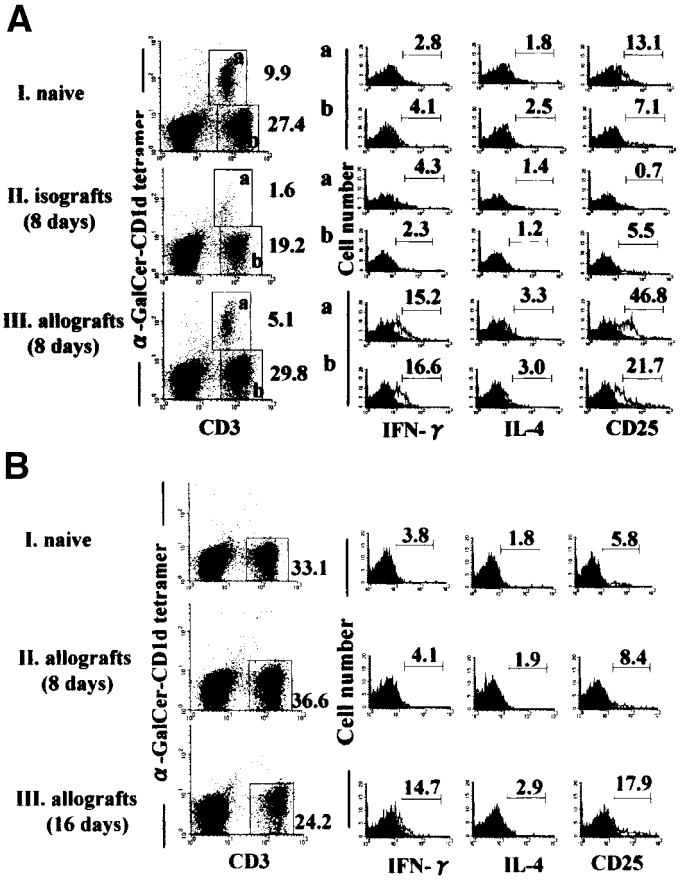

FIG. 2. Flow cytometry of mononuclear cells in the liver of mice. Mononuclear cells in the liver of wild-type (*A*) or CD1d$^{-/-}$ mice (*B*) were stained with phycoerythrin-α-GalCer–loaded CD1d tetramers and fluorescein isothiocyanate-CD3 and examined by flow cytometry (*left panels*). The figures show the percentage of the cells in the corresponding area. Tetramer$^+$ CD3$^+$NKT cells and tetramer$^-$ CD3$^+$ T-cells in wild-type mice (*A*) and tetramer$^-$ CD3$^+$ T-cells in CD1d$^{-/-}$ mice (*B*) were further gated to evaluate the production of IFN-γ as well as IL-4 and the expression of CD25 (*right panels*).

TABLE 2
Islet allograft survival in mice treated with rapamycin

Group	Mice	Site	Rapamycin (mg/kg)	n	Survival (days)	% Survival at 90 days
I	Wild type	Portal vein	—	9	7, 7, 9, 9, 9, 9, 11, 11, 11	0
II	Wild type	Portal vein	0.2	9	8, 9, 10, 13, 15, 18, 22, 25, 26	0
III	Wild type	Portal vein	1.0	8	14, 27, 33, 36, 39, 45, 46, >90	12.5
IV	Wild type	Portal vein	3.0	7	39, 54, >90 (×5)	71.4
V	CD1d$^{-/-}$	Portal vein	1.0	11	54, >90 (×10)	91.0*
VI	Vα14NKT$^{-/-}$	Portal vein	1.0	5	41, 54, >90 (×3)	60.0

BALB/c islets were grafted into the liver of STZ-induced diabetic C57L/6 mice treated with rapamycin, which was administered intraperitoneally once a day for 6 days after transplantation. *$P < 0.01$ vs. the corresponding control (III) by Fisher's exact probability test.

and treated with 1.0 mg/kg rapamycin were normoglycemic at 90 days after transplantation (Table 2 and Fig. 3, *left panels*). There was a statistical significance in the survival rate between islet allografts in CD1d$^{-/-}$ mice treated with 1.0 mg/kg rapamycin and those in wild-type mice treated similarly ($P < 0.01$, Fisher's exact probability test). Morphologically intact islets with well-granulated β-cells were seen in the liver of CD1d$^{-/-}$ mice at 120 days after islet transplantation (Fig. 3, *right panels*).

Acceptance of islet allografts in CD1d$^{-/-}$ mice is abrogated by the adoptive transfer of hepatic mononuclear cells from wild-type but not from CD1d$^{-/-}$ mice. To further confirm the role of NKT cells in rejection of islet allografts in the liver of mice treated with rapamycin, cell transfer experiments were performed. As described above, rejection of islet allografts is prevented in CD1d$^{-/-}$ mice treated with 1 mg/kg rapamycin, and therefore CD1d$^{-/-}$ mice were used for the following study. When 5×10^6 hepatic mononuclear cells from wild-type mice were transferred via the portal vein into the liver of CD1d$^{-/-}$ mice receiving islets and treated with rapamycin at the time of islet transplantation, all recipient mice ($n = 3$) became hyperglycemic again after transplantation (Fig. 4*A*). In marked contrast, when the same number of hepatic mononuclear cells from CD1d$^{-/-}$ mice was transferred, recipient CD1d$^{-/-}$ mice ($n = 3$) remained normoglycemic (Fig. 4*B*). Morphologically intact or infiltrated islets were seen in the livers of CD1d$^{-/-}$ mice receiving the transfer of cells from CD1$^{-/-}$ or wild-type mice, respectively (histology not shown).

Donor-specific unresponsiveness is induced in CD1d$^{-/-}$ mice treated with rapamycin and accepting islet allografts. Finally, we determined whether donor-specific unresponsiveness is induced in CD1d$^{-/-}$ mice treated with rapamycin and accepting islet allografts. For this purpose, third-party or donor-specific islets were grafted into the liver of CD1d$^{-/-}$ mice that had been made diabetic again with intravenous injection of STZ at 120 days after the initial islet transplantation. No immunosuppressive agent was administered at the time of the second transplantation. CD1d$^{-/-}$ mice receiving third-party C3H islets ($n = 3$) became hyperglycemic at 22, 26, and 36 days (Fig. 5*A*) because of rejection, whereas CD1d$^{-/-}$ recipient mice ($n = 3$) with donor-specific BALB/c islets remained normoglycemic after the second transplantation (Fig. 5*B*).

DISCUSSION

These findings clearly demonstrate that NKT cells play a significant role in rejection of islet allografts in the liver of mice without immunosuppressive treatment and with immunosuppression by rapamycin, which is currently used in clinical islet transplantation. In the current study, two kinds of NKT cell–deficient mice, namely CD1d$^{-/-}$ and Vα14 NKT cell$^{-/-}$ mice, were used as recipients to elucidate a role of NKT cells in islet allograft rejection. Vα14 NKT cell$^{-/-}$ mice lack only Vα14NKT cells, which are a major population of CD1d-reactive NKT cells in mice (23). The important finding is that survival of islet allografts is prolonged in CD1d$^{-/-}$ and Vα14NKT cell$^{-/-}$ mice without immunosuppression compared with wild-type mice when the liver is the site of transplantation versus the renal subcapsular space. The finding suggests that NKT cells play a significant role in acute rejection of islet allografts in the liver of mice. Flow cytometry analysis revealed that not only NKT cells but also T-cells in the liver of wild-type mice became activated at the time of rejection. In marked contrast, CD3$^+$ T-cells in the liver of CD1d$^{-/-}$ mice at 8 days after transplantation, when islet allografts were rejected in wild-type mice but not in CD1d$^{-/-}$ mice, was not activated. Consequently, CD1d$^{-/-}$ mice rejected intrahepatic islet allografts with the upregulation of IFN-γ production and CD25

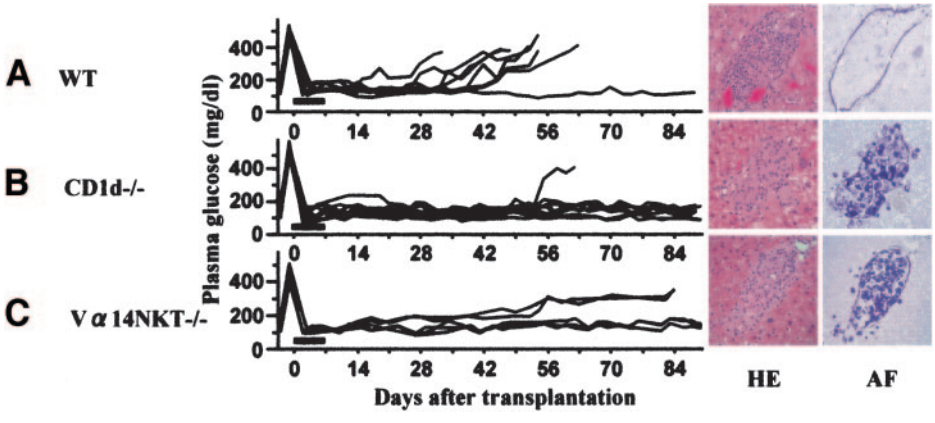

FIG. 3. Acceptance of islet allografts by a subtherapeutic dose of rapamycin in CD1d$^{-/-}$ mice. BALB/c islets were grafted into the liver of STZ-induced diabetic C57BL/6 wild-type (*A*, $n = 8$), CD1d$^{-/-}$ (*B*, $n = 11$), and Vα14 NKT cell$^{-/-}$ mice (*C*, $n = 5$) treated with rapamycin. The bars in the figure represent the period of treatment. *Right panels*: photomicrographs of islet allografts in wild-type (*A*), CD1d$^{-/-}$ (*B*), and Vα14 NKT cell$^{-/-}$ mice (*C*) at 56, 125, and 102 days after transplantation, respectively. AF, aldehyde and fuchsin stain; HE, hematoxylin and eosin stain.

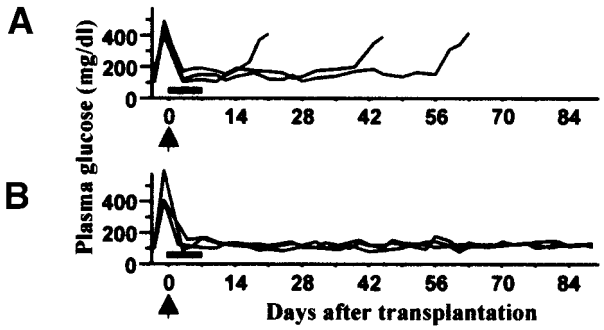

FIG. 4. Acceptance of islet allografts in CD1d$^{-/-}$ mice is abrogated by the transfer of hepatic mononuclear cells from wild-type but not from CD1d$^{-/-}$ mice. Freshly isolated 5 × 10^6 hepatic mononuclear cells from either wild-type (*A*) or CD1d$^{-/-}$ mice (*B*) were adoptively transferred (arrows) into the liver of CD1d$^{-/-}$ mice at the time of islet transplantation. The bars in the figures represent the period of treatment with rapamycin.

expression in CD3$^+$ T-cells in the liver. These findings indicate that T-cell activation is delayed in the absence of NKT cells after islet transplantation, which may reflect the prolonged survival of islets in the liver of CD1d$^{-/-}$ mice, although the precise role of NKT cells in T-cell activation in response to islet allografts remains unclear.

Regarding a role of NKT cells in islet allograft rejection under the immunosuppressive agent rapamycin, rejection was prevented in the absence of NKT cells with a subtherapeutic dose of rapamycin. The frequency of rejection appears to be high in Vα14 NKT cell$^{-/-}$ mice compared with CD1d$^{-/-}$ mice, although the difference has no statistical significance. The potential difference may rely on the disparity between CD1d$^{-/-}$ and Vα14 NKT cell$^{-/-}$ mice (23), in that CD1d$^{-/-}$ mice lack not only Vα14NKT cells, a major component of NKT cells, but also the other CD1d-reactive cells, which are less well characterized and whose role in transplant immunity remains unknown. Furthermore, the exact mechanisms involved in the response of NKT cells to donor islets leading to rejection, in relation to cell components other than NKT cells, remain undetermined, which is a matter of interest for future studies.

The current finding that the dosage of rapamycin can be reduced to prevent rejection of islet allografts in the absence of NKT cells raises a possibility that the downregulation of NKT cells may help prevent islet allograft rejection in wild-type mice with reduced dosage of rapamycin. Currently, there is limited number of reports with respect to suppression of NKT cell function. NKT cells secrete large amounts of IFN-γ on activation (11,12). IFN-γ production from NKT cells is modulated by the use of their synthetic ligand α-GalCer (30), in that single administration of α-GalCer upregulates IFN-γ production, whereas repeated administration downregulates IFN-γ production (31). Recently, natural ligands of NKT cells have been reported (32–34). Thus, it is interesting to learn whether procedures targeting NKT cells with the use of their synthetic or natural ligands have any beneficial effects on prevention of islet allograft rejection.

In the current study, it was found that donor-specific unresponsiveness is induced and maintained in CD1d$^{-/-}$ mice treated with rapamycin and accepting islet allografts. This finding indicates that NKT cells are not required for induction of donor-specific unresponsiveness in this model. Previously, we have shown that CD4$^+$ Vα14 NKT cells are essential for acceptance of rat islet xenografts in mice treated with anti-CD4 mAb (35). In a cardiac allograft model, tolerance induction after the blockade of CD28/B7 interaction in conjunction with that of LFA-1/ICAM-1 (lymphocyte function–associated antigen 1/intercellular adhesion molecule 1) interaction is dependent on NKT cells (36). NKT cells are reported to be required for induction of allospecific T-regulatory cells and are essential for survival of cornea allografts (37). In contrast to these findings, human CD1d (hCD1d) has been reported to function as a transplantation antigen, and skin grafts are rejected by expression of human CD1d in the major histocompatibility complex–matched mouse model (38). Survival of porcine neural xenografts is shown to be enhanced in the brain of CD1d$^{-/-}$ mice compared with wild-type controls (39). Recently, Oh et al. (40) reported that rejection of H-Y mismatched skin grafts is accelerated in CD1d$^{-/-}$ mice as recipients compared with wild-type mice. Thus, these findings indicate that NKT cells may have a diverse effect as regulatory and/or effector cells on the outcome of transplants, depending on the type of graft (allograft versus xenograft, organ versus tissue [cell]) and/or the type of immunosuppression.

In summary, the current study affords evidence that NKT cells play a significant role in islet allograft rejection in the liver of mice without immunosuppressive treatment and with immunosuppression by rapamycin, providing a new insight into cellular immune response in association with rejection. Currently, a major obstacle facing clinical islet transplantation is that the rate of insulin independence in patients with type 1 diabetes after islet transplantation declines with time after transplantation (3). Current immunosuppressive regimes include FK506 and rapamycin, which are known to be toxic to β-cells of islets and are considered as a major cause of islet graft failure after transplantation. Thus, the current study raises the possibility that introduction of a novel immunosuppressive regime targeting NKT cells may lead to a beneficial outcome of clinical islet transplantation with reduction and/or replacement of FK506 and rapamycin.

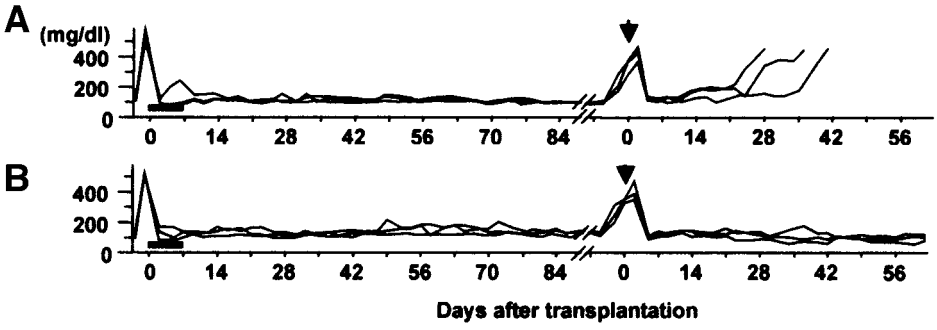

FIG. 5. Donor-specific unresponsiveness in CD1d$^{-/-}$ mice accepting islet allografts. Normoglycemic CD1d$^{-/-}$ mice receiving islet allografts and treated with rapamycin (bars) were made diabetic again at 120 days after the initial islet transplantation, and third-party C3H (*A*, *n* = 3) or donor-specific BALB/c (*B*, *n* = 3) islets were grafted into the liver (arrows). No immunosuppressive agent was administered at the time of the second islet transplantation.

ACKNOWLEDGMENTS

This work was supported by funds (0211001) from the Central Research Institute of Fukuoka University, by health science research grants from the Ministry of Health, Labor, and Welfare, Japan, and a grant-in-aid for scientific research (B) from the Japan Society for the Promotion of Science.

We thank Dr. Moriya Tsuji, Rockefeller University, for providing CD1d$^{-/-}$ mice.

REFERENCES

1. Shapiro AMJ, Lakey JRT, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238, 2000
2. Ricordi C, Strom TB: Clinical islet transplantation: advances and immunological challenges. *Nat Rev Immunol* 4:259–268, 2004
3. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JR, Shapiro AM: Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060–2069, 2005
4. Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, Rabinovitch A, Elliott JF, Bigam D, Kneteman NM, Warnock GL, Larsen I, Shapiro AM: Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 50:710–719, 2001
5. Yasunami Y, Ryu S, Kamei T: FK-506 as a sole immunosuppressive agent for prolongation of islet allograft survival in rat. *Transplantation* 49:682–686, 1990
6. Drachenberg CB, Klassen DK, Weir MR, Wiland A, Fink JC, Bartlett ST, Cangro CB, Blahut S, Papadimitriou JC: Islet cell damage associated with tacrolimus and cyclosporine: morphological features in pancreas allograft biopsies and clinical correlation. *Transplantation* 68:396–402, 1999
7. Fabian MC, Lakey JR, Rajotte RV, Kneteman NM: The efficacy and toxicity of rapamycin in murine islet transplantation. In vitro and in vivo studies. *Transplantation* 56:1137–1142, 1993
8. Bendelac A, Rivera MN, Park SH, Roark JH: Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol* 15:535–562, 1997
9. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR: CD1 recognition by mouse NK1+ T lymphocytes. *Science* 268:863–865, 1995
10. Hong S, Scherer DC, Singh N, Mendiratta SK, Serizawa I, Koezuka Y, Van Kaer L: Lipid antigen presentation in the immune system: lessons learned from CD1d knockout mice. *Immunol Rev* 169:31–44, 1999
11. Arase H, Arase N, Nakagawa K, Good RA, Onoe K: NK1.1^{+} CD4^{+} CD8^{-} thymocytes with specific lymphokine secretion. *Eur J Immunol* 23:307–310, 1993
12. Yoshimoto T, Paul WE: CD4^{pos}, NK1.1^{pos} T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J Exp Med* 179:1285–1295, 1994
13. Gonzalez-Aseguinolaza G, de Oliveira C, Tomaska M, Hong S, Bruna-Romero O, Nakayama T, Taniguchi M, Bendelac A, Van Kaer L, Koezuka Y, Tsuji M: α-Galactosylceramide-activated Vα 14 natural killer T cells mediate protection against murine malaria. *Proc Natl Acad Sci U S A* 97:8461–8466, 2000
14. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H: The regulatory role of Vα14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* 21:483–513, 2001
15. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M: Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278:1623–1626, 1997
16. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, Kawano T, Pelikan SB, Crowe NY, Godfrey DI: Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* 191:661–668, 2000
17. Jahng AW, Maricic I, Pedersen B, Burdin N, Naidenko O, Kronenberg M, Koezuka Y, Kumar V: Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* 194: 1789–1799, 2001
18. Singh AK, Wilson MT, Hong S, Olivares-Villagomez D, Du C, Stanic AK, Joyce S, Sriram S, Koezuka Y, Van Kaer L: Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* 194:1801–1811, 2001
19. Kukreja A, Cost G, Marker J, Zhang C, Sun Z, Lin-Su K, Ten S, Sanz M, Exley M, Wilson B, Porcelli S, Maclaren N: Multiple immuno-regulatory defects in type-1 diabetes. *J Clin Invest* 109:131–140, 2002
20. Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, Kronenberg M, Koezuka Y, Delovitch TL, Gombert JM, Leite-De-Moraes M, Gouarin C, Zhu R, Hameg A, Nakayama T, Taniguchi M, Lepault F, Lehuen A, Bach JF, Herbelin A: Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune type 1 diabetes. *Nat Med* 7:1057–1062, 2001
21. Hong S, Wilson MT, Serizawa I, Wu L, Singh N, Naidenko OV, Miura T, Haba T, Scherer DC, Wei J, Kronenberg M, Koezuka Y, Van Kaer L: The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat Med* 7:1052–1056, 2001
22. Denkers EY, Scharton-Kersten T, Barbieri S, Caspar P, Sher A: A role for CD4+ NK1.1+ T lymphocytes as major histocompatibility complex class II independent helper cells in the generation of CD8+ effector function against intracellular infection. *J Exp Med* 184:131–139, 1996
23. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Kaer LV NKT cells: what's in a name? *Nat Rev Immunol* 4:231–237, 2004
24. Sutton R, Peters M, McShane P, Gray DW, Morris PJ: Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation* 42:689–691, 1986
25. Okeda T, Ono J, Takaki R, Todo S: Simple method for the collection of pancreatic islets by the use of Ficoll-Conray gradient. *Endocrinol Jpn* 26:495–499, 1979
26. Yasunami Y, Lacy PE, Davie JM, Finke EH: Prolongation of islet xenograft survival (rat to mouse) by in vitro culture at 37°C. *Transplantation* 35:281–284, 1983
27. Kemp CB, Knight J, Scharp DW, Lacy PE, Ballinger WF: Transplantation of isolated pancreatic islets into the portal vein of diabetic rats. *Nature* 244:447, 1973
28. Ohtsuka K, Yasunami Y, Ikehara Y, Nagai T, Kodama S, Maki T, Tomita A, Abo T, Ikeda S: Expansion of intermediate T cell receptor cells expressing IL-2Rα^{-}β^{+}, CD8α^{+}β^{+}, and lymphocyte function-associated antigen-1+ in the liver in association with intrahepatic islet xenograft rejection from rat to mouse: prevention of rejection with anti-IL-2R β monoclonal antibody treatment. *Transplantation* 64:633–639, 1997
29. Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, Koezuka Y, Kronenberg M: Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* 192:741–754, 2000
30. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M: CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278:1626–1629, 1997
31. Burdin N, Brossay L, Kronenberg M: Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur J Immunol* 29:2014–2025, 1999
32. Zhou D, Mattner J, Cantu C 3rd, Schrantz N, Yin N, Gao Y, Sagiv Y, Hudspeth K, Wu YP, Yamashita T, Teneberg S, Wang D, Proia RL, Levery SB, Savage PB, Teyton L, Bendelac A: Lysosomal glycosphingolipid recognition by NKT cells. *Science* 306:1786–1789, 2004
33. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C 3rd, Zhou D, Saint-Mezard P, Wang V, Gao Y, Yin N, Hoebe K, Schneewind O, Walker D, Beutler B, Teyton L, Savage PB, Bendelac A: Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434:525–529, 2005
34. Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, Tsuji M, Kawahara K, Wong CH, Kronenberg M: Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 434:520–525, 2005
35. Ikehara Y, Yasunami Y, Kodama S, Maki T, Nakano M, Nakayama T, Taniguchi M, Ikeda S: CD4^{+}Vα14 natural killer T cells are essential for acceptance of rat islet xenografts in mice. *J Clin Invest* 105:1761–1767, 2000
36. Seino KI, Fukao K, Muramoto K, Yanagisawa K, Takada Y, Kakuta S, Iwakura Y, Van Kaer L, Takeda K, Nakayama T, Taniguchi M, Bashuda H, Yagita H, Okumur K: Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. *Proc Natl Acad Sci U S A* 98:2577–2581, 2001
37. Sonoda KH, Taniguchi M, Stein-Streilein J: Long-term survival of corneal allografts is dependent on intact CD1d-reactive NKT cells. *J Immunol* 168:2028–2034, 2002
38. Wang B, Chun T, Rulifson IC, Exley M, Balk SP, Wang CR: Human CD1d functions as a transplantation antigen and a restriction element in mice. *J Immunol* 166:3829–3836, 2001
39. Larsson LC, Anderson P, Widner H, Korsgrent O: Enhanced survival of porcine neural xenografts in mice lacking CD1d1, but no effect of NK1.1 depletion. *Cell Transplant* 10:295–304, 2001
40. Oh K, Kim S, Park S-H, Gu H, Roopenian D, Chung DH, Kim YS, Lee D-S: Direct regulatory role of NKT cells in allogeneic graft survival is dependent on the quantitative strength of antigenicity. *J Immunol* 174:2030–2036, 2005