Successful Islet Transplantation to Two Recipients From a Single Donor by Targeting Proinflammatory Cytokines in Mice

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Background. Currently, the inability to achieve successful islet transplantation from one donor to one recipient is a major obstacle facing clinical islet transplantation. We herein determined whether this limitation could be overcome by targeting pro-inflammatory cytokines with the prevention of immediate islet graft loss in association with engraftment in mice.

Methods. Isolated islets were grafted into the liver of streptozotocin-induced diabetic mice and the role of proinflammatory cytokines in the engraftment of islets was evaluated with the use of interferon (IFN)-$\gamma^{-/-}$ mice and monoclonal antibodies against proinflammatory cytokines.

Results. Hyperglycemia in streptozotocin-induced diabetic mice receiving 200 syngenic islets, which were isolated from a single mouse pancreas, was ameliorated when IFN-$\gamma^{-/-}$, but not wild-type mice, were used as recipients. The treatment with anti-IFN-γ antibody produced normoglycemia in diabetic wild-type mice receiving 200, but not 100 islets. However, when anti–tumor necrosis factor-α and anti-interleukin-1β antibodies were administered in conjunction with anti-IFN-γ antibody, wild-type diabetic mice receiving 100 islets became normoglycemic after transplantation. In addition, the favorable effect of the combined use of antibodies was similarly achieved in mice receiving islet allografts when rejection was prevented with anti-CD4 antibody treatment.

Conclusions. These findings clearly demonstrate that successful islet transplantation from one donor to two recipients is feasible by targeting pro-inflammatory cytokines in mice, thus suggesting a potential application in clinical islet transplantation if similar mechanisms of islet graft loss could be mediated in humans.

Keywords: Islet transplantation, Proinflammatory cytokine, Engraftment.

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Shapiro et al. recently reported that patients with insulin-dependent diabetes mellitus (IDDM) do not need exogenous insulin after islet transplantation with the introduction of a novel immunosuppressive regime distinct from that of pancreatic organ transplantation (*1*). Since then, pancreatic islet transplantation has been considered a feasible procedure for the treatment of IDDM. Currently, however, sequential islet transplantations with the use of two to three donors are required to produce insulin-independence in a diabetic recipient after islet transplantation (*1,2*). This implies that the amount of insulin released from islet grafts from a single donor is not sufficient to maintain normoglycemia in recipients without the administration of exogenous insulin after transplantation, thus necessitating additional islet transplantation to produce insulin independence. Ryan et al. reported that islet graft mass in diabetic patients receiving sequential islet transplantations is approximately 36% of that of normal individuals, even though these patients received almost the equivalent number of islets as normal individuals (*3*). Therefore, these findings indicate that islet grafts are lost after transplantation with unknown etiology, resulting in the inability to achieve insulin-independence after islet transplantation from a single donor.

One factor responsible for the decreased islet mass after transplantation may be the destruction of the islet grafts by inflammatory responses caused by islet transplantation in the liver of the recipients. Earlier studies suggested that islets exposed to allogeneic blood by grafting to the liver via the portal vein of recipients are subject to an immediate blood-mediated inflammatory reaction (IBMIR) that involves the activation of coagulation and complement systems resulting in islet graft destruction (*4–6*). However, the exact cellular and molecular mechanisms involved in islet graft loss remain unclear. We previously demonstrated that natural killer T (NKT) cells, which are a recently identified novel lymphoid subset distinct from conventional T cells (*7*), play an essential role in islet graft loss soon after transplantation in mice. Within 6 hr after islet transplantation, Gr-1$^+$CD11b$^+$ cells, which are neutrophils in morphology, accumulate in the liver, which is the site of islet transplantation, and infiltrate into grafted islets with upregulation of interferon (IFN)-γ production. In the liver of NKT cell-deficient mice, the IFN-γ production of these Gr-1$^+$CD11b$^+$ cells is downregulated facilitating the prevention of islet graft loss, which leads to the amelioration of hyperglycemia in diabetic mice with islet transplantation from one

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donor to one or even two recipients (*8*). These findings prompted us to determine whether the treatment targeting pro-inflammatory cytokines including IFN-γ could help prevent islet graft loss, thus leading to successful islet cell transplantation from one donor to one recipient. The present study demonstrates that islet transplantation from one donor to two recipients thus becomes feasible in mice with the combined use of anti-IFN-γ, anti–tumor necrosis factor (TNF)-α, and anti-interleukin (IL)-1β antibodies at the time of islet transplantation.

MATERIALS AND METHODS

Animals

Male BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from Charles River Japan (Kanagawa, Japan) and used for the experiments. IFN-γ deficient (IFN-γ KO) mice with a C57BL/6 background were provided by Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan) (*9*). Because it was found that the severity of diabetes made with streptozotocin (STZ) injection differed depending upon the weight of the mice, only mice weighing 23–25 g were used as recipients. Mice weighing 25–30 g served as donors. Diabetes was induced in the recipients by the intravenous injection of STZ (180 mg/kg) (Sigma, St. Louis, MO). The plasma glucose levels of the mice exceeded 400 mg/dL at 2 to 3 days after the STZ injection and the mice remained hyperglycemic at the time of islet transplantation. All experiments were performed in accordance with the Institutional Animal Care and Use Committee.

Islet Isolation and Transplantation

Islets were isolated by the static digestion method using collagenase (*10*) and then separated by centrifugation on Ficoll-Conray gradients (*11*). Islets of 150 to 250 μm in diameter were hand-selected using a Pasteur pipette with the aid of a dissecting microscope, since it was critical to minimize the size variation of individual islets to compare the effects of the difference in the number of donor islets. The size of individual islets in each islet isolation procedure was confirmed by using a phase-contrast microscope equipped with a scale in the eyepiece. Hand-picked islets were transplanted into the liver via the recipient's portal vein (*12*) at 3 days after the induction of diabetes with STZ injection.

Monitoring Plasma Glucose and Body Weight

The nonfasting plasma glucose levels and body weight were monitored three times a week in all the recipients for 60 days after islet transplantation. The plasma glucose was measured using a Beckman glucose analyzer (Beckman Japan, Tokyo, Japan). Normoglycemia after transplantation was defined as two consecutive plasma glucose level readings below 200 mg/dL.

Exogenous Administration of IFN-γ

Human recombinant IFN-γ was kindly supplied by Shionogi Pharmaceutical Co. (Osaka, Japan). Human recombinant IFN-γ (50,000 units) was administered IP into an appropriate group of diabetic IFN-$\gamma^{-/-}$ mice once per day for 7 days after islet transplantation.

Treatment With Monocloncal Antibodies

Antimouse IFN-γ monoclonal antibody (mAb; R4-6A2; rat IgG1κ), antimouse TNF-α mAb (MP6-XT3; rat IgG1κ), and antimouse IL-1β mAb (B122; Armenian Hamster IgG) were purchased from e-Bioscience (Kyoto, Japan) and were administered IP three times after transplantation at days 0, 2, and 4. Nondepleting anti-CD4 mAb (200 μg/injection/mouse, YTS177, rat IgG1; R&D, Minneapolis, MN) was administered IP once at the time of islet transplantation.

Intraperitoneal Glucose Tolerance Test

Intraperitoneal glucose tolerance test (IPGTT) was performed in recipient mice at 60 days after islet transplantation. The mice were fasted for 8 hr prior to the examination. Blood samples were obtained from the orbital sinuses of recipient mice at 0, 30, and 120 min after the IP injection of glucose (1 g/kg body weight), and the plasma glucose was measured as previously described.

Morphological Study

The livers bearing islet grafts were examined morphologically at 60 days after transplantation in appropriate groups of mice, and the pancreases of recipient mice were also examined simultaneously. The liver and the pancreas were fixed with Bouin's solution, processed, and embedded in paraffin. The sections were prepared for light microscopy and stained with hematoxylin and eosin (HE), and aldehyde and fuchsin (AF).

Preparation of Hepatic Mononuclear Cells

Hepatic mononuclear cells (HMNCs) were prepared as described previously (*13*). Briefly, an excised liver was pressed through a stainless steel mesh, and the resulting dissociated liver tissues were suspended in Dulbecco's modified Eagle medium (D-MEM/F-12; Life Technologies, Tokyo, Japan) and washed twice. The mixture was re-suspended in an isotonic 33% Percoll solution containing heparin (67 U/mL), and centrifuged 2,000×*g* at 4°C for 15 min. The resulting pellet was suspended in a 0.83% ammonium chloride solution to lyse erythrocytes. After counting, these HMNCs were washed twice in phosphate-buffered saline (PBS) and used for further analysis.

Flow Cytometry Analysis

The following mAbs were used: antimouse FcRγII/III (2.4G2), fluorescein isothiocyanate (FITC)-conjugated anti-CD3ε (145-2C11), FITC- or phycoerythrin (PE)-conjugated anti-CD11b (M1/70), allophycocyanin-conjugated anti-IFN-γ (XMG1.2), anti–tumor necrosis factor (TNF)-α (MP6-XT22), PerCP-conjugated anti–Gr-1 (Rb6-8c5), and isotype control (clone R3-34, Rat IgG1) and were purchased from BD Biosciences (San Jose, CA). PE–α-galactosylceramide(α-GalCer)–CD1d tetramers were prepared as previously described (*14*). For intracellular staining, cells were incubated with anti-FcRγII/III and neutravidin (Invitrogen), surface stained, fixed, permeabilized, stained with mAbs, and analyzed on a flow cytometer (FACSCalibur; Becton Dickinson). A total of 10,000 viable cells were analyzed.

Statistical Analysis

The statistical significance with respect to the rate of euglycemia in streptozotocin-induced diabetic mice after is-

let transplantation and that of plasma glucose levels during IPGTT was determined by Fisher's exact test and Student's *t* test, respectively. Differences were considered significant when the *P* values were less than 0.05.

RESULTS

Amelioration of Hyperglycemia in Streptozotocin-Induced Diabetic IFN-γ-Deficient Mice Receiving Islets From a Single Donor

Previously, we have shown that the rate of normoglycemia in STZ-induced diabetic C57BL/6 mice receiving 200 and 400 syngenic islets after transplantation into the liver was 0 or 100%, respectively (*8*). In the present study, we used 200 islets, which is the number of islets isolated from a single mouse pancreas, as the marginal mass of donor islets.

First, we examined the effect of IFN-γ on engraftments of islets with the use of IFN-$\gamma^{-/-}$ mice as recipients. Diabetic mice (n=7) receiving 200 syngeneic islets remained hyperglycemic after transplantation (Fig. 1, I). In marked contrast to wild-type mice, hyperglycemia of STZ-induced diabetic IFN-$\gamma^{-/-}$ mice (n=5) was ameliorated after the transplantation of 200 syngeneic islets into the liver (Fig. 1, II). Morphologically, de-granulated and well-granulated β cells of islet grafts were seen in the liver of wild-type and IFN-$\gamma^{-/-}$ mice, respectively, at 60 days after transplantation (histology not shown). The difference in the rates of normoglycemia between diabetic wild-type and IFN-$\gamma^{-/-}$ mice receiving 200 islets was statistically significant ($P<0.05$). When IFN-γ (50,000 unit, human recombinant) was administered IP once per day for 6 days from day 0 to day 5, four out of five IFN-$\gamma^{-/-}$ mice receiving 200 islets were hyperglycemic at 60 days after transplantation (Fig. 1, III). Therefore, the exogenous administration of IFN-γ induced hyperglycemia in IFN-$\gamma^{-/-}$ mice with 200 islets, which otherwise were normoglycemic after transplantation.

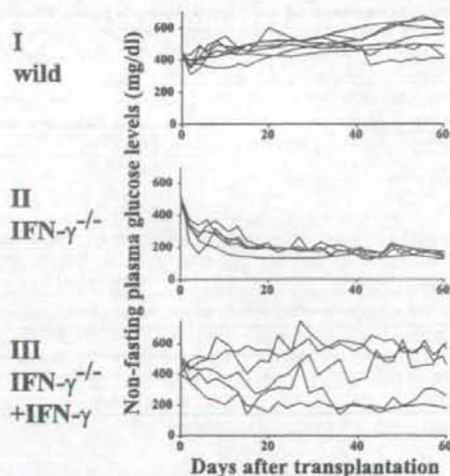

FIGURE 1. Deleterious effects of IFN-γ on the engraftment of islets in the liver of mice. Two hundred syngenic islets were grafted into the liver of STZ-induced diabetic wild-type (I) or IFN-γ-deficient (II and III) C57BL/6 mice. Human recombinant INF-γ (50,000 U) was administered into INF-γ-deficient mice 7 times after islet transplantation from day 0 to 6 (III). Individual lines represent plasma glucose levels of each animal.

Beneficial Effects of Anti-Proinflammatory Cytokine Antibodies on Engraftments of Islets

We next examined the effects of the treatment with anti-pro-inflammatory cytokine antibodies including anti-IFN-γ, anti-TNF-α and anti-IL-1β antibodies on engraftment of islets. First, we determined the effect of anti-IFN-γ antibody since the deleterious role of IFN-γ in engraftment of islets became evident with the use of IFN-γ-deficient mice. When STZ-induced diabetic mice receiving 200 islets into the liver were treated with 1, 10 (Fig. 2, II), or 100 μg anti-IFN-γ antibody for three times after islet transplantation at day 0, 2 and 4, 0/3, 5/5, or 4/5 recipient mice, respectively, became normoglycemic. Diabetic mice (n=4) with 200 islets and treated with control antibody remained hyperglycemic after transplantation (Fig. 2, I). The difference in the rate of normoglycemia at 60 days was statistically significant between the mice treated with 1 μg and those treated with 10 μg anti-INF-γ antibody ($P<0.05$), but it was not statistically significant between the mice treated with 10 and those treated with 100 μg anti-INF-γ antibody. Therefore, the dosage of 10 μg IFN-γ /injection was used for the following experiments. When the number of donor islets was reduced to 100 and diabetic mice were treated with IFN-γ (10 μg/injection), none of recipient mice (n=5) became normoglycemic after islet transplantation (Fig. 2, V).

To further determine the role of anti-IFN-γ antibody on the engraftment of islets, mononuclear cells in the liver of mice receiving 200 syngeneic islets and treated with anti-IFN-γ antibody were isolated and examined by flow cytometry. We found that not only IFN-γ, but also the TNF-α production of Gr-1$^+$CD11b$^+$ cells, accumulated in the liver of wild-type mice receiving 200 islets, was up-regulated at 6 hr after islet transplantation (Fig. 3, lower panel). In the diabetic mice receiving 200 islets and treated with anti-IFN-γ antibody, the accumulation of Gr-1$^+$CD11b$^+$ cells in the liver after islet transplantation also occurred (Fig. 3, middle panel), but the TNF-α production of Gr-1$^+$CD11b$^+$ cells was down-regulated at 6 hr after transplantation (Fig. 3, lower panel).

A similar beneficial effect of anti-TNF-α and anti-IL-1β antibodies to that of anti-IFN-γ antibody on engraftments of islets was noted, in which either the treatment with anti-TNF-α (n=3) or anti-IL-1β antibody (n=3) alone ameliorated hyperglycemia of diabetic mice receiving 200 islets (Fig. 2, III and IV) but not that of mice receiving 100 islets and treated with anti-TNF-α antibody (n=6) or anti-IL-β antibody (n=5) (Fig. 2, VI and VII). We further determined whether the beneficial effects on islet engraftments were achieved when three kinds of antibodies were administered simultaneously after islet transplantation. As shown above, neither the treatment with anti-IFN-γ antibody (n=6), anti-TNF-α antibody (n=6), nor anti-IL-1β antibody (n=5) alone ameliorated hyperglycemia of diabetic mice when 100 donor islets were grafted into the liver (Fig. 2, V, VI, and VII). In marked contrast, all diabetic mice (n=5) receiving 100 islets and treated with a combination of the three kinds of antibodies became normoglycemic after transplantation (Fig.

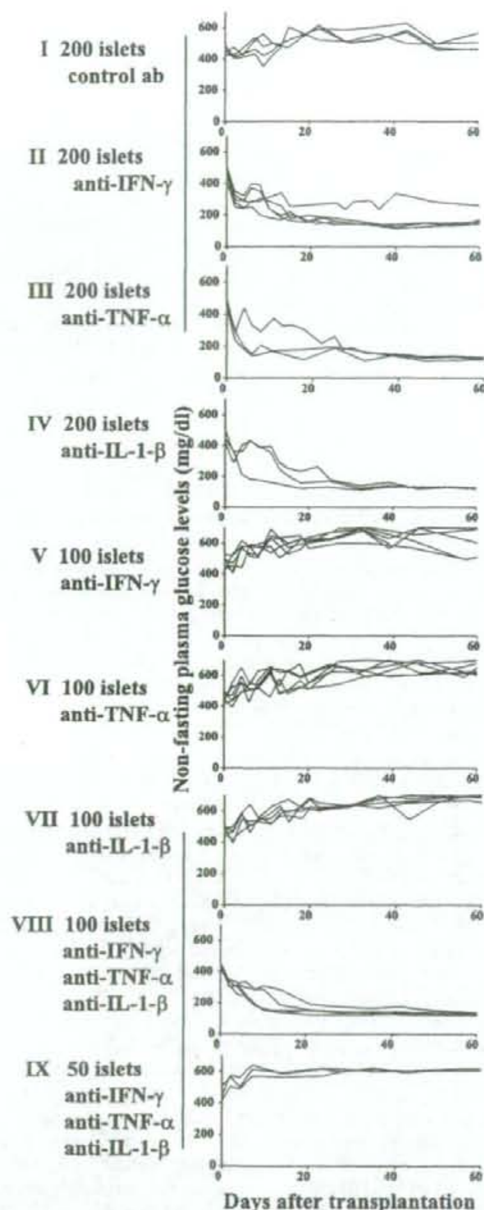

FIGURE 2. Beneficial effects of anti-proinflammatory cytokine antibodies on the amelioration of hyperglycemia in diabetic mice receiving 200 syngenic islets. Wild-type diabetic mice receiving syngenic 200 islets were treated with control (I), anti-IFN-γ (II), anti-TNF-α (III), or anti-IL-1β antibody (IV) and those receiving 100 islets were treated with anti-IFN-γ (V), anti-TNF-α (VI), or anti-IL-1β antibody (VII). Diabetic mice receiving 100 (VIII) or 50 islets (IX) were treated with the combination of three antibodies. Each antibody (10 μg/injection/mouse) was administered IP three times at day 0, 2, and 4 after transplantation. Individual lines represent the nonfasting plasma glucose levels of each animal.

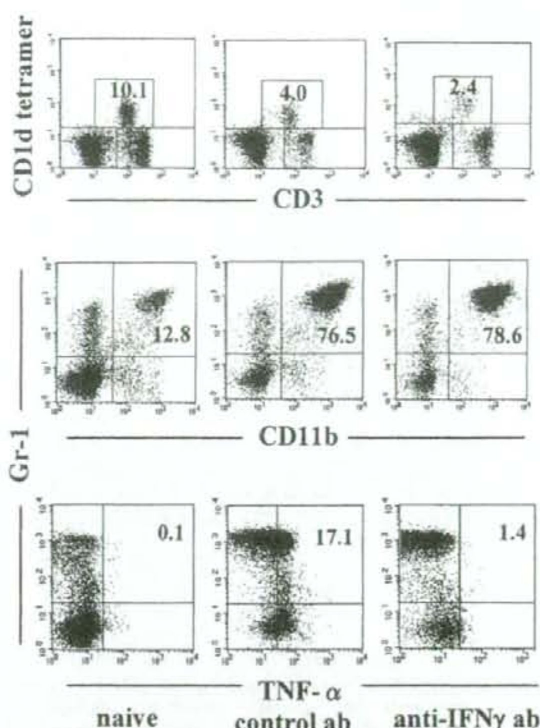

FIGURE 3. The downregulation of TNF-α production in Gr-1⁺CD11b⁺ cells accumulated in the liver of mice receiving islets and treated with anti-IFN-γ antibody. Mononuclear cells in the liver of naïve mice (left panel) were isolated and examined by flow cytometry. Mononuclear cells in the liver of mice receiving islets and treated with control antibody (middle panel) or anti-IFN-γ antibody (right panel) were isolated at 6 hr after transplantation and examined by flow cytometry. The figures show the percentage of the cells in the corresponding area. Representative data of two to three experiments are shown.

2, VIII). However, when the number of donor islets was further reduced to 50 and the mice (n=3) were treated with the combined three antibodies, the mice remained hyperglycemic after islet transplantation (Fig. 2, IX). These findings indicate that the amelioration of hyperglycemia with the combined use of three antibodies in STZ-induced diabetic mice can only be achieved when more than 100 donor islets are grafted into the liver, and that treatment alone is not sufficient to affect the plasma glucose levels of STZ-induced diabetic mice.

Glucose Tolerance in Mice Grafted With 100 Islets and Treated With Anti-Pro-Inflammatory Cytokine Antibodies Is Superior to That of Those Grafted With 400 Islets Without Treatment

To evaluate the functional mass of islet grafts in the livers of recipient mice, an intraperitoneal glucose tolerance test (IPGTT) was performed. The plasma glucose levels of naïve untreated C57BL/6 mice (n=4) were 59.1±3.1 (mean±SD),

249.8±5.6 and 133.3±4.8 mg/dl at 0, 30 and 120 min, respectively, after the IP injection of 1.0 g/kg glucose (Fig. 4A), and those of diabetic mice (n=3) without islet transplantation at 60 days after the injection of STZ were 460.0±57.0, 658.3±16.6, 539.0±7.4 mg/dl, respectively (Fig. 4A).

The plasma glucose levels of the diabetic mice (n=5) receiving 200 islets were 308.8±69.0, 657.8±42.2 and 544.4±40.2 mg/dL, those of mice (n=6) with 400 were 66.5±4.1, 413.3±17.4 and 260.0±18.7 mg/dL, and those of mice (n=4) with 700 islets were 58.5±7.6, 252.8±27.9 and 147.8±20.1 mg/dL at 0, 30 and 120 min, respectively (Fig. 4A).

The plasma glucose levels of diabetic mice (n=5) receiving 200 islets and treated with anti-INF-γ antibody were 80.0±18.2, 352.4±57.7 and 229.8±85.7 mg/dL and those of mice (n=4) treated with control antibody were 440.5±75.8, 634.5±80.1 and 456.0±164.8, respectively at 0, 30 and 120 min after the injection of glucose (Fig. 4B). The difference in the plasma glucose levels at 30 and 120 min between the mice with 200 islets and treated with anti-IFN-γ antibody and those with 400 islets without the treatment was statistically significant ($P<0.05$ by Student's t test). The plasma glucose levels of diabetic mice (n=4) with 100 islets and treated with anti-IFN-γ antibody alone were 385.5±188.5, 590.0±155.4 and 514.3±190.2 mg/dL and those of mice (n=5) treated with the combined antibodies were 67.8±8.3, 298.6±37.9 and 160.8±17.2, respectively at 0, 30 and 120 min after the injection of glucose (Fig. 4B). Therefore, the glucose tolerance of diabetic mice receiving 100 islets and treated with the combined antibodies was similar to that of mice grafted with 200 islets and treated with anti-IFN-γ antibody alone, and superior to that of mice receiving 400 islets without any antibody treatment.

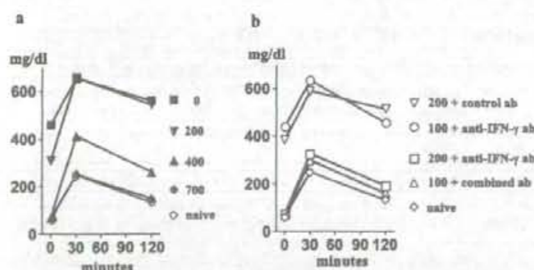

FIGURE 4. Intraperitoneal glucose tolerance test in mice. Intraperitoneal glucose tolerance test (IPGTT) in STZ-induced diabetic mice was performed at 60 days after islet transplantation. Mice were fasted for 8 hr prior to IPGTT and glucose (1g/kg) was injected IP. Blood samples were taken from the orbital sinuses at 0, 30 and 120 min after the glucose injection. (A) Experimental groups include diabetic mice without islet transplantation (closed square, n=3), those receiving 200 (closed downward triangle, n=5), 400 (closed upward triangle, n=6), or 700 syngeneic islets (closed diamond, n=4) without antibody treatment. Age-matched naïve untreated mice (open diamond, n=4) served as controls. (B) Diabetic mice receiving 200 syngenic islets and treated with either control antibody (open circle, n=4) or anti-IFN-γ antibody (open square, n=5). Diabetic mice receiving 100 syngenic islets were treated with anti-IFN-γ antibody alone (downward open triangle, n=4) or the three combined antibodies (upward open triangle, n=5).

Beneficial Effects of Anti-proinflammatory Cytokine Antibodies on the Engraftment of Islet Allografts

Finally, we determined whether the beneficial effect of anti-proinflammatory cytokine antibodies on the engraftment of islets seen in islet isografts was also observed in allografts. Preliminary experiments revealed that STZ-induced diabetic C57BL/6 mice receiving 400 BALB/c islets into the liver became normoglycemic within a few days after transplantation and hyperglycemic again at 9 days (median survival days) without immunosuppression. Rejection was confirmed histologically, in which fragmented islet grafts infiltrated with mononuclear cells were seen in the livers of recipient mice. When recipient mice with 400 islet allografts were treated with anti-CD4 antibody (IP, 200 μg) once at the time of transplantation, islet allografts were accepted and the mice remained normoglycemic for more than 60 days after transplantation (data not shown). Morphologically, intact islets with well-granulated β cells were seen in the livers of recipient mice and no accumulations of mononuclear cells were identified. Therefore, the treatment with anti-CD4 antibody was thus found to prevent islet allograft rejection in this model.

When the number of BALB/c donor islets was reduced from 400 to 200, recipient mice (n=5) did not become normoglycemic at all after transplantation (Fig. 5, I, left panel). Histologically, infiltrated islet grafts with mononuclear cells were seen in the liver at 14 days after transplantation (Fig. 5, I, right panel). When diabetic mice receiving 200 allogeneic islets and treated with anti-IFN-γ antibody (10 μg/injection/day) three times, at day 0, 2, and 4 after islet transplantation, all mice (n=5) became normoglycemic at 2 to 3 days and hyperglycemic again by 10 days (Fig. 5, II, left panel). Rejection was confirmed histologically, in which islet allografts infiltrated with mononuclear cells were seen (Fig. 5, II, right panel). When diabetic mice with 200 allogenic islets were treated with anti-CD4 antibody, recipient mice (n=4) remained hyperglycemic at 60 days after transplantation (Fig. 5, III, left panel). However, islet allografts were seen morphologically in the liver of mice, and found to be de-granulated (Fig. 5, III, right panel). These findings indicate that the rejection of 200 islet allografts was prevented by the treatment with anti-CD4 antibody and that grafted islets failed to produce normoglycemia in recipients. In contrast, hyperglycemia in diabetic mice receiving 200 islet allografts and treated with anti-CD4 antibody (n=5) was ameliorated when anti-IFN-γ antibody (10 μg/injection) was administered at the time of islet transplantation (Fig. 5, IV, left panel). Morphologically, intact islets with well-granulated β cells were seen (Fig. 5, IV, right panel). When the number of allogeneic donor islets was further reduced to 100 and recipient mice were treated with anti-CD4 antibody in conjunction with anti-IFN-γ antibody, all mice (n=5) remained hyperglycemic (Fig. 5, V, left panel). Morphologically, degranulated islets without accumulation of mononuclear cells were seen in the liver of mice at 60 days after transplantation (Fig. 5, V, right panel). When diabetic mice receiving 100 allogeneic islets and treated with anti-CD4 antibody in conjunction with the com-

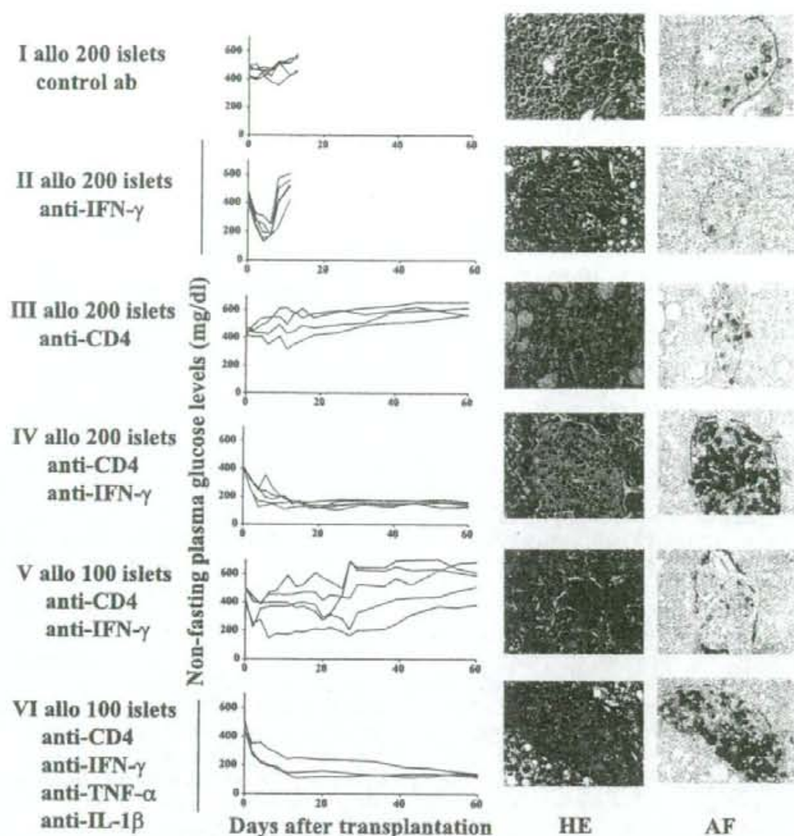

FIGURE 5. Effects of anti-proinflammatory cytokine antibodies on the engraftment of islet allografts. Two hundred (I, II, III, and IV) or 100 (V and VI) BALB/c islets were grafted into the liver of STZ-induced diabetic C57BL/6 mice. Nondepleting anti-CD4 antibody (200 μg/injection/mouse) was administered IP into recipient mice (III, IV, V, and VI) once at the time of islet transplantation. Anti-IFN-γ antibody (10 μg/injection) was administered IP at days 0, 2, and 4 after islet transplantation (II, IV, V and VI). Anti-IFN-γ, anti-TNF-α (10 μg/injection), and anti-IL-1β (10 μg/injection) were administered simultaneously into diabetic mice receiving 100 allogeneic islets in conjunction with anti-CD4 antibody (VI). Individual lines represent the nonfasting plasma glucose levels of each animal. In the right panels, photomicrographs of islet allografts at 14 (I and II) and 60 days after transplantation (II, IV, V and VI) were shown. The sections were stained with hematoxylin & eosin (HE) and aldehyde & fuchshin (AF). Original magnification, ×200.

bined use of three antibodies including anti-IFN-γ, anti-TNF-α and anti-IL-1β, mice (n=3) became normoglycemic after islet transplantation (Fig. 5, VI, left panel). Intact islets with well-granulated β cells were seen in the liver (Fig. 5, VI, right panel). These findings show that the beneficial effect of the combined use of antibodies on islet isografts engraftment is also observed in islet allotransplantation when rejection is prevented with an appropriate immunosuppressive agent.

DISCUSSION

These findings clearly demonstrate that pro-inflammatory cytokines, including IFN-γ, TNF-α, and IL-1β play a deleterious effect on islets in the liver, which is the site of clinical islet transplantation. In addition, the concurrent treatment with monoclonal antibodies against these cytokines has a favorable effect on the amelioration of hyperglycemia in diabetic mice receiving 100 islets, which is half the number of islets isolated from a single mouse pancreas.

IFN-γ has been reported to be an essential molecule for the destruction of islets, resulting in the development of diabetes in a mouse model with virus-induced IDDM (*15*). IFN-γ is well known to be toxic to β cells of islets in vitro (*16–18*), which is mediated by nitric oxide in combination with IL-1 (*19*). Our previous study demonstrated that the IFN-γ production of Gr-1$^+$CD11b$^+$ cells dependent on NKT cells is an essential component of islet graft loss in the liver soon after transplantation (*8*). Therefore, these findings indicate that pro-inflammatory cytokines are essentially involved in islet graft destruction in the liver in association with engraftments, and that they might be targets for intervention to increase islet graft mass after transplantation. In order to prove this, we first examined the role of IFN-γ in engraft-

ments of islets with the use of INF-$\gamma^{-/-}$ mice as recipients. As expected, diabetic INF-$\gamma^{-/-}$ mice receiving 200 syngenic islets became normoglycemic after transplantation (Fig. 1, II) which is in contrast to wild-type diabetic mice, which remained hyperglycemic despite receiving the same number of islets (Fig. 1, I). Furthermore, exogenous administration of IFN-γ failed to induce normoglycemia in diabetic IFN-$\gamma^{-/-}$ mice with 200 islets (Fig. 1, III). These findings indicate that IFN-γ plays an essential role in the destruction of islet grafts after transplantation. Among cellular populations regarding the IFN-γ production in the liver, we have previously shown that Gr-1^{+}CD11b^{+} cells play an essential role in islet graft loss in association with engraftments and that the IFN-γ production of Gr-1^{+}CD11b^{+} cells is dependent on NKT cells (*8*). Soon after activation, NKT cells have been reported to produce a large amount of IFN-γ (*20,21*) which, in turn, acts upon other cell populations such as NK cells, macrophages, or CD8 T cells to produce IFN-γ (*7*). Therefore, it remains unknown whether IFN-γ from NKT cells or Gr-1^{+}CD11b^{+} cells or both are responsible for the destruction of islets in vivo.

We herein found that besides anti-IFN-γ antibody, anti-TNF-α and anti-IL-1β antibodies also have beneficial effects on the amelioration of hyperglycemia in diabetic mice receiving 200 islets, which were otherwise hyperglycemic without the treatment. It would be interesting to learn whether these cytokines are produced from the same population of cells or from a different one to serve as regulatory and/or effecter molecules. In addition, we found that the production of TNF-α is also upregulated in Gr-1^{+}CD11b^{+} cells in the liver of mice receiving islets and that it is down-regulated in mice treated with anti-IFN-γ antibody treatment (Fig. 3). It remains undetermined whether the suppressive effect of anti-IFN-γ antibody on the TNF-α production of Gr-1^{+}CD11b^{+} cells is direct or indirect and whether it is mediated by other cell populations such as NKT cells.

The most impressive finding in the present study is that the glucose tolerance of normoglycemic recipients receiving 100 islets and treated with the combined use of anti-IFN-γ, anti-TNF-α and anti-IL-1β antibodies is significantly superior to that of normoglycemic mice receiving 400 islets without the antibody treatment (Fig. 4). This finding indicates that the islet graft mass in mice receiving 100 islets and treated with the combined use of antibodies is greater than that in mice receiving 400 islets without the treatment. Therefore, the present study shows that the number of donor islets can be reduced to more than one-fourth to produce an amelioration of hyperglycemia in diabetic mice after islet transplantation with the simultaneous administration of three kinds of monoclonal antibodies against proinflammatory cytokines. Importantly, the effect of the combined use of anti-proinflammatory antibodies on the engraftment of islets was found to also be true in islet allotransplantation when rejection is prevented by an appropriate immunosuppressive regimen such as treatment with anti-CD4 antibody.

Previously, we have shown that nicotinamide (*22*), troglitazone (*23*), hepatocyte growth factor (*24*) and α-galactosylceramide, a synthetic ligand of NKT cells (*8*), have favorable effects on the amelioration of hyperglycemia in STZ-induced diabetic rats and mice receiving a marginal mass of islets into the liver. We herein afford another promising approach to prevent islet graft loss in association with engraftments in the liver after transplantation. Since the treatment targeted pro-inflammatory cytokines such as monoclonal antibodies and receptor antagonists have been introduced into clinics for the treatment of inflammatory diseases including inflammatory bowel disease (*25,26*), rheumatoid arthritis (*27*) and neonatal-onset multisystem inflammatory disease (*28*), the issue of safety has been cleared and it seems ready to apply these to clinical islet transplantation. In fact, a clinical trial with the use of anti-TNF-α antibody has been initiated (*29*). Therefore, when the beneficial effects of the procedure targeting pro-inflammatory cytokines can be demonstrated in humans, then an enhanced success in clinical islet transplantation may be seen.

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The Immunoregulatory Roles of Natural Killer T Cells in Cyclophosphamide-Induced Tolerance

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Background. Recent studies have indicated that natural killer T (NKT) cells are essential for the establishment of transplantation tolerance. In the present study, we have elucidated the role of recipient and donor NKT cells in cyclophosphamide (CP)-induced tolerance.

Method. DBA/2 (DBA; H-2^d) mice were used as donors and BALB/c (BALB; H-2^d) wild-type (WT) or Vα14 NKT-knockout (KO, BALB/c background) mice were used as recipients. Recipients were treated with CP-induced tolerance regimen, which consists of donor spleen cells (SC) on day 0 and CP on day 2. In some experiments, NKT KO mice, which received NKT cells from either WT, inferon-γ KO, or interleukin-4 KO mice, were treated with tolerant regimen. To deplete Ly49 inhibitory receptors on NKT cells in the recipient mice, anti-Ly49 monoclonal antibody cocktails were injected on day −1 when indicated.

Results. Donor skin graft was permanently accepted in recipient BALB WT mice with induction of donor mixed chimerism. On the contrary, donor DBA skin allografts were chronically rejected in NKT KO recipient. Lower levels of mixed chimerism were observed in NKT KO recipients comparing to the WT recipients. The production of interferon-γ or interleukin-4 from NKT cells did not affect the induction of tolerance. Depletion of Ly49 positive NKT cells abrogated the induction of skin graft tolerance.

Conclusion. Recipient NKT cells, but not donor NKT cells, were dominantly required for the induction of allograft tolerance. Our results indicated that the single cytokine produced by NKT cells did not mediate the regulatory function in the induction of allograft tolerance.

Keywords: Cyclophosphamide, Tolerance, Transplantation, NKT.

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Natural killer T (NKT) cells, which are characterized by co-expression of natural killer (NK) cell receptors and a single invariant T-cell antigen receptor (TCR) encoded by Vα14 and Jα281 gene segments, have been identified as a novel lymphoid lineage distinct from conventional T cells or NK cells. CD1 is related to major histocompatibility complex (MHC) I antigen, and classified to group I of CD1a, CD1b, and CD1c, and group II of CD1d (*1*). Group I of CD1 molecule is expressed on human, and present lipid-containing antigens to T cells with diverse TCRs (*2*). CD1d form a dimmer with β2-microgroblin, which is expressed in both human and mice. Humans have invariant Vα24 NKT cells, and the antigen recognition by TCR of NKT cell is highly restricted to group II CD1d in both human and mice (*3*). Thus, Vα14 NKT cell recognize endogenous antigen as well as exogenous antigen expressed via CD1d of antigen-presenting cells (APC) (*4, 5*). Although the physiological role of NKT cells remains obscure, Vα14 NKT cells have been demonstrated to play an important role in tumor immunity (*6*), suppression of autoimmune disease (*7*), and infectious immunity (*8, 9*), or suppress graft-versus-host disease (GVHD) (*10*) via the dominant production of Th1 cytokine interferon (IFN)-γ and Th2 cytokine interleukin (IL)-4. With regard to transplantation immunity, it has been indicated that NKT cells have a regulatory role in both allogeneic and xenogeneic organ transplantation (*11–13*).

We have investigated cyclophosphamide (CP)-induced tolerance, which consists of an intravenous (i.v.) injection of 1×10^8 allogeneic spleen cells (SC; day 0) followed by an intraperitoneal (i.p.) administration of 200 mg/kg of CP on day 2 (*14–25*). Using this method, long-lasting skin allograft tolerance was induced in the recipient received graft from H-2-matched and minor antigen mismatched donors (*17–19*). Our previous studies have elucidated the two major mechanisms of tolerance induction (*18–21*). The first mechanism is the clonal deletion of the donor reactive T-cell populations, which proliferate in the periphery after donor SC injection, and deleted by CP treatment (*21*). The second mechanism is the establishment of intrathymic chimerism and intrathymic clonal deletion of donor-reactive T cells at 4–6 weeks after treatment (*19, 20*).

The aim of the present study was to investigate the regulatory role of NKT cells in our CP-induced tolerance system by using Vα14 NKT-knockout (KO) mice. Although an essential role of NKT cells for the induction of transplantation tolerance has been suggested in two previous reports (*11, 12*),

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the detailed mechanisms have not been clarified. Here, we evaluated the role of both recipient and donor NKT cells, and which cytokine generated by NKT cells is essential in the CP-induced tolerance system. The results suggested that the NKT cells from recipient mice play an important role for the induction of tolerance; however, donor NKT cells do not. In addition, Ly49 inhibitory receptors on NKT cells played an important role for its regulatory function. The lack of each single cytokine production from NKT cells (such as IFN-γ, IL-4, or IL-10) did not affect the induction of graft tolerance. The role of NKT cells in transplantation tolerance is discussed.

MATERIALS AND METHODS

Animals

Inbred mice of the Balb/c AnNCrj (BALB; H-2^d, Lyt-1.2, Mls-1^b, Mls-2^a) and DBA/2 NCrj (DBA; H-2^d, Lyt-1.1, Mls-1^a, Mls-2^a) strains were obtained from Charles River Japan Inc. (Yokohama, Kanagawa, Japan). Inbred mice of the B10.D2 SnSlc (B10.D2; H-2^d, Mls-1^b, Mls-2^b) strain were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). Ja281 knockout (Vα14 NKT KO) mice with a BALB background were also used as recipients (*6*). IFN-γ and IL-4 knockout (KO) mice with a BALB background were purchased from Jackson Laboratory (Ann Arbor, MI) and maintained in the Animal Facility of Kyushu University. The recipients were used at 12–16 weeks of age. All animals received humane care in compliance with the Guidelines for Animal Experiments of Kyushu University and the Law (no. 105) and Notification (no. 6) of the Japanese government.

Cell Preparation

Mice were sacrificed by decapitation. The spleens were collected and kept on ice in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Grand Island, NY) supplemented with antibiotics (100 u/mL penicillin and 100 μg/mL streptomycin). Spleens were disrupted in the medium by pressing spleen fragments between two glass slides. Cell suspensions were filtered through cotton gauze and washed three times with the RPMI medium. Viable nucleated cells were counted and usually adjusted to 2×10^8/ml.

Conditioning of CP-Induced Tolerance

A 0.5-mL aliquot containing 1×10^8 SC from donor mice was injected into the tail vein of recipient mice. Two days later, CP (Endoxan, Shionogi, Osaka, Japan) dissolved in phosphate-buffered saline at a concentration of 10 mg/mL was injected i.p. at a dose of 200 mg/kg. The day of the injection of DBA SC is referred to as day 0 throughout this report.

Reconstitution of NKT Cells in NKT KO Mice

To reconstitute NKT cells in NKT KO mice, recipient BALB NKT KO mice were irradiated with 3 Gy on day −28 and then received 1×10^7 SC and 5×10^6 bone marrow cells (BMC) from wild-type (WT), IFN-γ KO, or IL-4 KO mice on the same day. The preparation of BMC was performed according to a previous method (*26*). Briefly, the bone marrow in the femoral and tibial bones was flushed out using a 5-mL syringe with a 26-G needle (Terumo, Tokyo, Japan).

Skin Grafting

Skin grafting was performed using our previously reported procedure (*27*). Briefly, a square full-thickness skin graft (1 cm^2) was prepared on the right lateral thoracic wall of the recipient mouse. The graft was fixed to the graft bed with eight interrupted sutures of 5–0 silk thread and covered with protective tape. The first inspection was carried out on the seventh day, followed by daily inspection for 3 weeks. Grafts were considered as rejected at the time of complete sloughing or when they formed a dry scar. Survival was expressed as the median survival time (median) and the mean survival time (MST)±standard deviation (SD).

Flow Cytometry

Phenotyping was performed at various times, beginning at 2 weeks after the injection of SC. Recipients were tail-bled and white blood cells (WBC) were prepared by hypotonic shock (*28*). Staining with both donor-specific and T-cell-specific monoclonal antibodies (mAbs) was performed on each recipient and control mouse. Cells were incubated with phycoerythrin (PE)-conjugated anti-Lyt-1 (Lyt-1.1+Lyt-1.2; PharMingen, San Diego, CA) mAb and fluorescein isothiocyanate (FITC)-conjugated Lyt-1.1 (PharMingen) mAb for 30 min at 4°C and then washed twice. To block nonspecific FcγR binding of labeled antibodies, 10 μL of an undiluted culture supernatant of 2.4G2 (rat antimouse FcγR mAb) was added to the first incubation. All data were collected and analyzed by a FACSCalibure and CellQuest software (Becton Dickinson, Sunnyvale, CA). Dead cells were excluded by gating out low forward scatter-high propidium iodide-retaining cells.

For the analysis of TCR expression on T cells of WBC, two-color analysis was performed (*28*). WBC were labeled with FITC-conjugated anti-Vβ6, Vβ3, or Vβ8.1/8.2 mAb (PharMingen), and PE-conjugated anti-CD4 (PharMingen) mAb. To determine the percentage of $CD4^+$ T cells that were $V\beta6^+$, Vβ3 or $V\beta8.1/8.2^+$, 10,000 to 20,000 gated $CD4^+$ cells were collected.

For the staining NKT cells, SC or liver mononuclear cells (LMNC) were stained with PE-conjugated α-galactosyl ceramide (αGalCer)/CD1d-tetramers and FITC-conjugated anti-CD3 mAb (PharMingen). PE-conjugated αGalCer/CD1d-tetramers were prepared as previously described (*29*). The liver was disrupted in the RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) by pressing liver fragments between two-glass slides, washed, resuspended in a 40% isotonic Percoll solution (Amersham Biosciences, Piscataway, NJ), and underlaid with a 67.5% isotonic Percoll solution. Centrifugation for 30 min at 3000 rpm at room temperature isolated the liver mononuclear cells (LMNC) at the interface. Cells were washed two times with Hank's balanced salt solution containing 2% FCS and resuspended in it.

Monoclonal and Polyclonal Antibodies

To elucidate the role of IL-10 in NKT cell-mediated immunoregulation, anti-IL-10 mAb (100 μg; PharMingen Co, San Diego, CA) was injected into NKT cell-reconstituted NKT KO recipients on day −28, and then weekly until the end of the experiment. Anti-Ly-49A (YE1.48), C/I (5E6), and G2 (4D11) mAbs, which were kindly provided by Dr. William J. Murphy (Intramural Research Support Program, Frederick, MD), were injected i.p. into recipients on day −1 (each mAb: 25 μg) and then weekly until the end of the experiment. The depletions of Ly-49A-, C/I-, and G2-positive cells were con-

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firmed by a flow cytometry by both direct staining with Ly49 mAb, and indirect staining with PE-conjugated antimouse immunoglobulin (Ig), κ-light chain, and PE-conjugated antirat Ig, κ-light chain Abs. Rabbit polyclonal antiasialo GM1 was purchased from Wako Chemical Industries (Osaka, Japan).

Statistics

The statistical significance of the data was determined by a Mann-Whitney *U* test when the data were nonparametric, or a Student's *t* test when the data were parametric. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Skin Allograft Prolongation in H2-Matched DBA (H-2^d) → BALB WT (H-2^d) or BALB Background Va14 NKT KO (H-2^d) Combination Mice by Using 1×10^8 DBA SC Followed by 200 mg/kg CP

In the first experiment, DBA/2 (DBA, H-2^d) mice were used as donors, and BALB/c (BALB, H-2^d) mice or NKT KO (H-2^d, BALB background) mice were used as recipients. H2 matched DBA skin allografts (H-2^d) were rejected within 14 days in untreated BALB WT or NKT KO recipients (Fig. 1A, B). DBA skin grafts were rejected within 14 days in BALB WT or NKT KO mice treated with DBA WT SC alone or 200 mg/kg CP alone (data not shown). All of the DBA skin allografts survived for more than 100 days in the recipient BALB WT mice treated with DBA WT SC followed by CP (n=6; MST >100 days; median >100 days). On the other hand, the survival of DBA skin grafts was only partially prolonged, and all grafts were rejected within 48 days in the recipient NKT KO mice treated with DBA WT SC followed by CP (n=10; MST=36.4±7.1 days, median 39 days). This skin allograft prolongation was tolerogen-specific since the third-party skin graft of the B10.D2 strain (H-2^d) was rejected in a normal fashion (Fig. 1B).

Skin Allograft Prolongation in H2-Matched BALB WT or NKT KO → DBA WT or B10.D2 WT (H-2^d) Combination Mice by Using 1×10^8 Donor SC Followed by 200 mg/kg CP

In the next experiment, BALB NKT KO mice were used as donors, and DBA or B10.D2 mice were used as recipients. Donor BALB skins survived permanently in recipient DBA or B10.D2 WT mice treated with BALB WT SC and CP (n=6 in each group; Fig. 2). All of the BALB skin grafts were accepted in DBA or B10.D2 WT mice treated with BALB NKT KO SC and CP (n=10 in each group). BALB skin grafts were rejected within 14 days in DBA or B10.D2 WT mice treated with BALB NKT KO SC alone or 200 mg/kg CP alone (data not shown). This skin allograft prolongation was tolerogen-specific since the third party skin grafts were rejected in a normal fashion (Fig. 2A[ii] and 2B[ii]; n=5 in each group).

Chimerism and Reduction of Mls-1^a-Reactive $CD4^+V\beta6^+$ or Mls-2^a-Reactive $CD4^+V\beta3^+$ T Cells of WBC in Recipient Mice Treated With Donor SC Plus CP

The kinetics of the donor mixed chimerism after the treatments with donor SC and CP were examined by flow

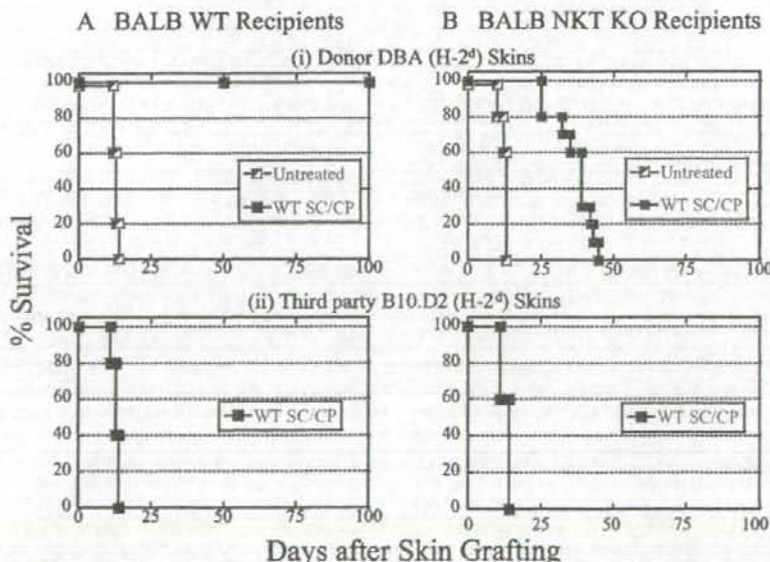

FIGURE 1. (i) Donor DBA skin allograft survival in the recipient BALB WT mice treated with DBA WT SC and CP (WT SC/CP) (A; n=6; MST >100 days; median >100 days) or BALB NKT KO mice treated with WT SC/CP (B; n=10; MST 36.4±7.1 days, median 39 days). Untreated BALB WT or NKT KO recipient mice rejected donor DBA skin grafts within 14 days (n=5 in each groups). (ii) Third-party B10.D2 skin grafts were rejected within 14 days after grafting in all groups. Recipient mice received donor or third mice skin grafts at 4 weeks after treatments.

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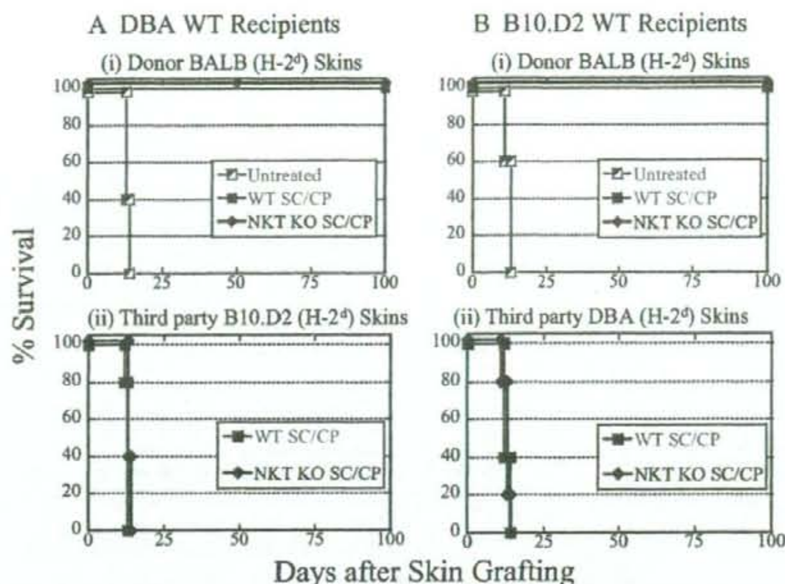

FIGURE 2. (A) (i) Donor BALB skin allograft survival in the recipient DBA WT mice treated with BALB WT SC and CP (WT SC/CP) (n=6, MST >100 days, median >100 days) or BALB NKT KO SC/CP (n=10, MST>100 days, median >100 days). (ii) Third party B10.D2 skin grafts were rejected within 14 days after grafting in all groups. Recipient mice received donor or third mice skin grafts at 4 weeks after treatments. (B) (i) Donor BALB skin allograft survival in the recipient B10.D2 WT mice treated with BALB WT SC/CP (n=6, MST >100 days, median >100 days) or BALB NKT KO SC/CP (n=10, MST >100 days, median >100 days). (ii) Third-party DBA skin grafts were rejected within 14 days after grafting in all groups. Untreated BALB WT or NKT KO recipient mice rejected donor DBA skin grafts within 14 days (n=5 in (A)i and (B)i). Recipient mice received donor or third mice skin grafts at 4 weeks after treatments.

cytometry. WBCs were obtained from the recipient mice at 2 and 8 weeks after tolerance induction (Table 1).

In the first experiment (EXP1), where BALB WT or NKT KO mice were used as recipient and DBA as donors, 2–4% of Lyt-1.1 + donor DBA cells were detected in the recipient peripheral WBC after tolerance induction (Table 1, EXP1, Group 4). In contrast, the level of the mixed chimerism was significantly low in the NKT KO recipient treated with donor DBA SC and CP (EXP1, Group 4 vs. Group 5, 3.5 ± 1.2 vs. 1.3 ± 0.3, and 4.0 ± 0.5 vs. 0.9 ± 0.3, at 2 weeks and 8 weeks, respectively; $P<0.01$ in both time point). These results were reproducible in five independent experiments (data not shown).

Donor Mls-1^a-reactive TCR Vβ6 in BALB WT or NKT KO (Mls-1^b) mice were also examined after treatments with DBA (Mls-1^a) WT SC and CP (first experiment). The WBC from the recipients was stained with FITC-conjugated anti-Vβ6 mAb and PE-conjugated anti-CD4 mAb (EXP1, Table 1). In the WBC of untreated BALB WT and NKT KO mice, CD4$^+$Vβ6$^+$ T cells were detected (EXP1, Groups 1 and 2, respectively), whereas they were hardly detectable in the WBC of untreated DBA mice (EXP1, Group 3). In all of the BALB WT mice treated with DBA WT SC and CP (EXP1, Group 4), CD4$^+$Vβ6$^+$ T cells were significantly reduced at 3 weeks. The same results were obtained in the WBC of NKT KO mice treated with DBA SC and CP (EXP1, Group 5). There was no significant difference between Groups 4 and 5. The disappearance of T cells from the WBC was specific for Vβ6$^+$ T cells, and the percentage of nondonor reactive Vβ8.1/8.2$^+$ T-cells population were similar (EXP1, Groups 4 vs. 5).

The level of the mixed chimerism was also analyzed in DBA WT recipient mice treated with BALB WT or NKT KO SC and CP (EXP2, Groups 4 and 5). There was no statistical significance between the level of mixed chimerism in DBA WT mice treated with SC from either BALB WT or NKT KO mice and CP (EXP2, $P>0.05$, Groups 4 vs. 5).

In the third experiment (EXP3), in which B10.D2 mice were used as recipients and BALB WT or NKT KO mice used as donors, the expression of donor Mls-2^a-reactive TCR Vβ3 was examined by flow cytometry (Table 1, EXP3). Certain level of CD4$^+$Vβ3$^+$ T cells were detected in the WBC of untreated B10.D2 WT mice (EXP3, Group 1), which was eliminated in the WBC of untreated BALB WT and NKT KO mice (EXP2, Groups 1 and 2, respectively). In all of the B10.D2 WT mice treated with BALB WT SC and CP (EXP3, Group 2), CD4$^+$Vβ3$^+$ T cells were significantly reduced at 3 and 9 weeks. The same results were obtained in B10.D2 WT mice treated with BALB NKT KO SC and CP (EXP3, Group 3). The disappearance of T cells from the WBC was specific for Vβ3$^+$ T cells, since the percentage of nondonor reactive Vβ8.1/8.2$^+$T cells was observed in the similar level throughout the experiment. These results indicated that the NKT cells in the recipient, but not the donor NKT cells, were critical for the induction and the subsequent maintenance of donor mixed chimerism.

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TABLE 1. Chimerism and clonal destruction in recipients treated with WT or NKT KO SC and CP

Exp.	Group	Recipient	Treatments[a] SC (day 0)	CP(mg/kg) (day 2)	No. of mice	Chimeric analysis (% positive cells±SD) %Ly1.1$^+$/Ly1$^+$ 2W	8W	Analysis of TCR expression (% positive cells±SD) CD4$^+$Vβ6$^+$/CD4$^+$ 3W	9W	CD4$^+$Vβ8$^+$/CD4$^+$ 3W	9W
1	1	BALB WT	(−)	(−)	6	0.0±0.0		9.5±1.1		16.7±2.1	
	2	BALB NKT KO	(−)	(−)	6	0.0±0.0		11.2±0.5		12.5±1.9	
	3	DBA WT	(−)	(−)	6	97.3±2.5		0.0±0.0		12.3±1.6	
	4	BALB WT	DBA WT	200	6	3.5±1.2[b]	4.0±0.5[b]	1.4±0.7[c]	1.1±0.7[c]	16.8±1.9	16.3±2.0
	5	BALB NKT KO	DBA WT	200	6	1.3±0.3	0.9±0.3	1.3±0.3	0.9±0.4	14.5±3.1	14.3±3.2
						Chimeric analysis (% positive cells±SD) %Ly1.1$^-$/Ly1$^+$ 2W	**8W**	**Analysis of TCR expression (% positive cells±SD) CD4$^+$Vβ3$^+$/CD4$^+$ 3W**	**9W**	**CD4$^+$Vβ8$^+$/CD4$^+$ 3W**	**9W**
2	1	BALB WT	(−)	(−)	6	100±0.0		0.0±0.0		18.7±2.1	
	2	BALB NKT KO	(−)	(−)	6	100±0.0		0.2±0.1		16.4±0.9	
	3	DBA WT	(−)	(−)	6	0.8±0.1		ND (not done)		ND	
	4	DBA WT	BALB WT	200	6	32.3±5.6[d]	27.4±5.3[d]	ND		ND	
	5	DBA WT	BALB NKT KO	200	6	30.4±13.5	24.1±6.7	ND		ND	
3	1	B10.D2 WT	(−)	(−)	6	ND (not done)		6.2±1.2		20.6±1.5	
	2	B10.D2 WT	BALB WT	200	6	ND	ND	0.3±0.2[e]	0.3±0.1[e]	21.8±1.9	22.7±3.2
	3	B10.D2 WT	BALB NKT KO	200	6	ND	ND	0.3±0.1	0.2±0.1	20.6±1.5	20.9±2.2

[a] Recipient mice were primed i.v. with 1×108 SC on day 0, and given 200 mg/kg CP on day 2.
[b] $P<0.01$ compared with group 5 of exp. 1.
[c] Not significant compared with group 5 of exp. 1.
[d] Not significant compared with group 5 of exp. 2.
[e] Not significant compared with group 3 of exp. 2.

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Induction of DBA Skin Graft Prolongation in NKT KO Mice Reconstituted With NKT Cells From BALB WT Mice

To clarify whether NKT cells were involved in the limitation of skin graft tolerance in CP-induced tolerance, NKT cells were reconstituted into NKT KO mice. When SC and liver mononuclear cells (LMNC) were stained with PE-conjugated αGalCer/CD1d-tetramers and FITC-conjugated anti-CD3 mAb, αGalCer/CD1d-tetramers$^+$CD3$^+$ cells accounts for about 1.0±0.3 and 19.5±5.4% of SC and LMNC in untreated BALB WT mice (n=3), respectively, and 0.3±0.1 and 1.2±0.2% in SC and LMNC of untreated NKT KO mice (n=3), respectively. Small percentage of αGalCer/CD1d-tetramers$^+$CD3$^+$ cells were detected in NKT KO mice because NKT KO mice in this study were generated by the disruption of Jα18 gene (6).

On the other hand, αGalCer/CD1d-tetramers$^+$CD3$^+$ cells account for about 0.4±0.1 and 4.3±0.5% in SC and LMNC of NKT KO mice (n=3) injected with BALB WT SC 7 days earlier, respectively. When NKT KO mice were injected with 1×10^8 SC from BALB WT mice on day −7 and treated with SC on day 0 and CP on day 2, the survival of DBA skin grafts was significantly prolonged, but 3 of 7 recipients chronically rejected (n=7; MST >100 days).

We planned the following experiment. To further reconstitute NKT cells in NKT KO mice, recipient NKT mice were irradiated with 3 Gy on day −28 and then injected with 1×10^7 SC and 5×10^6 untreated BMC from WT mice on the same day. In NKT KO mice (n=5) irradiated and injected with BALB WT SC and BMC 28 days earlier, αGalCer/CD1d-tetramers$^+$CD3$^+$ cells account for about 0.7±0.1% and 9.5±2.6% of SC and LMNC, respectively. DBA skin grafts were accepted for more than 100 days in all the NKT KO mice irradiated with 3 Gy on day −28, reconstituted with 1×10^7 SC and 5×10^6 BMC from BALB WT mice on day −28, and then treated with DBA SC on day 0 and CP on day 2 (Fig. 3A; n=6: MST >100 days). This skin allograft prolongation was tolerogen-specific since the third-party skin of the B10.D2 strain (H-2^d) was rejected in a normal fashion (Fig. 3A and B; n=5 in each group).

NKT cells have been well documented to produce large amounts of both IL-4 and IFN-γ upon activation, which are considered to play a role in both immunoactivation and immunoregulation (6–9). More recent studies have suggested that IL-10 produced by NKT cells mediates immunoregulation (30). To elucidate the role of cytokines produced form NKT cells, BALB NKT KO mice were reconstituted with NKT cells from IFN-γ or IL-4 KO mice and received tolerance conditioning. In the other group, BALB NKT KO mice were reconstituted NKT cells from WT mice and treated with anti-IL-10 mAb weekly. As shown in Figure 3A, all of the donor DBA skin grafts were accepted more than 100 days in irradiated BALB NKT KO mice reconstituted NKT cells from IFN-γ or IL-4 KO mice and received tolerance conditioning (each group, n=6; MST >100 days). On the other hand, only partial prolongation of donor skin graft survival was observed in NKT KO mice reconstituted from NKT KO mice and received tolerance conditioning (n=6; MST=51.5±13.8 days, median 54.5 days). In the BALB NKT KO mice reconstituted with WT NKT cells and received tolerance conditioning together with anti-IL-10 neutralizing mAb weekly, all of the donor skin grafts survived more than 100 days (n=6; MST >100 days). This skin allograft prolongation was tolerogen-specific because the third-party skin of the B10.D2 strain (H-2^d) was rejected in a normal fashion (Fig. 3A; n=5 in each group). Thus, each single cytokine from NKT cells, such as IFN-γ, IL-4, IL-10, was dispensable to induce tolerance in CP-induced tolerance conditioning.

Anti-Ly-49 Inhibitory Receptor mAbs Can Attenuate Graft Prolongation

NKT cells express −49 inhibitory receptors, such as Ly-49A, G2, and C/I, and do not express activation receptors, such as Ly-49D and Ly-49H molecules (31). Next study was designed to determine the regulatory function of the NKT cells via Ly49 receptors. WT or NKT KO recipients were treated with anti-Ly49A (A-1), Ly49C/I (5E6), and Ly49G2 (4D11) mAb cocktail from day −1 (each mAb: 25 μg) and then weekly, and received tolerance conditioning. Depletion of Ly49-positive cells were confirmed by flow cytometry as described in Materials and Methods. As shown in Figure 3B, DBA skin allografts survived for more than 100 days in WT mice treated with SC and CP without mAbs (n=6; MST >100 days). In Ly49-depleted WT mice treated with SC and CPs, however, the survival of DBA skin grafts was prolonged, but 4 of 6 recipient WT mice rejected donor grafts within 100 days after grafting (n=6; MST=57.8±36.2 days, median=52.5 days). DBA skin grafts were rejected within 52 and 45 days in NKT KO mice treated with SC and CP with or without mAbs, respectively (n=6; MST=33.7±11.8 days or 38.3±7.6 days, median=31.5 or 41 days, respectively). NK cells were selectively depleted, but not NKT cells, by the treatment with antiasialo GM1, because of the marginal expression of asialo GM1 on NKT cells as a surface molecule (32, 33). Thus, NK cells of recipient BALB WT mice were depleted by antiasialo GM1 injected on day −1 and then weekly, and then treated with donor DBA SC and CP. Donor DBA skin grafts were permanently accepted (n=6) in the NK-depleted WT recipients. However, DBA skin grafts were rejected within 70 days in WT mice treated with DBA SC, antiasialo GM1, and anti-Ly49 mAbs (n=6; MST=55.1±30.5 days, median ≥57 days). Thus the donor skin graft tolerance was abrogated by the depletion of Ly49-positive NKT cells, but not by the depletion of NK cells. This skin allograft prolongation was tolerogen-specific since third-party skin B10.D2 (H-2^d) allografts were rejected in a normal fashion (Fig. 3B; n=6 in each group). Thus, NKT cell, but not NK cells, was required to induce skin graft tolerance. The results also indicated that NKT cells with Ly49 inhibitory receptors were the major population for induction of tolerance.

DISCUSSION

Using the H2-matched murine combination of DBA into BALB and mAbs against T-cell markers (Lyt-1.1 and Thy-1.2) and TCR Vβ6, we have demonstrated the sequential mechanisms of cyclophosphamide-induced tolerance (18–20). These are as follows: 1) clonal destruction of antigen-stimulated T cells followed by destruction of proliferating T cells by CP; 2) establishment of stable mixed chimerism; 3) intrathymic clonal deletion; and 4) regulatory mechanisms at the late stage of tolerance. These four conditions are achieved

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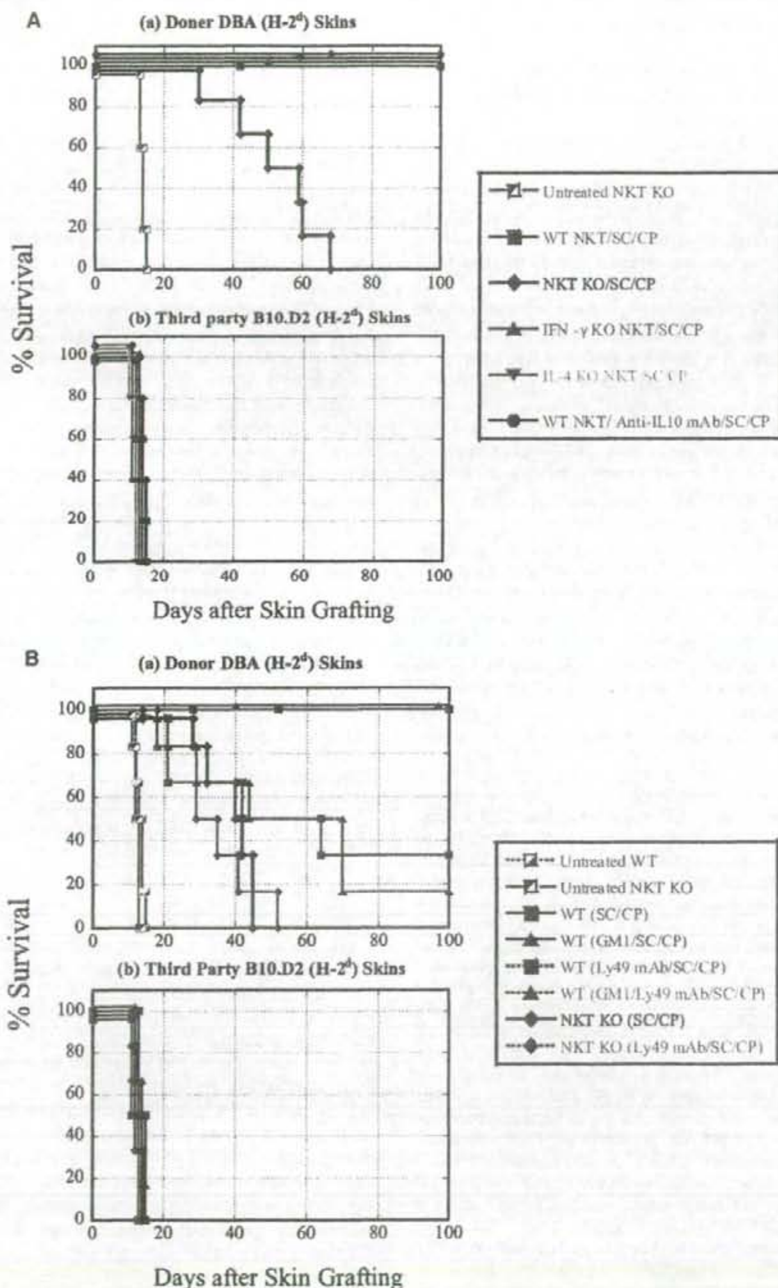

FIGURE 3. (A) (a) Donor DBA skin allograft survival in recipient BALB NKT KO mice reconstituted with NKT cells from cytokine knockout mice. Recipient BALB NKT KO mice were irradiated with 3 Gy and received both SC and BMC from BALB WT (red square, n=6; MST >100 days), BALB NKT KO (black diamond, n=6, MST 51.5±13.8 days, median 54.5 days), BALB IFN-γ KO (green triangle, n=6, MST >100 days), or BALB IL-4 KO (yellow inverted triangle, n=6, MST >100 days), and then treated with DBA SC/CP. In another group, irradiated BALB NKT KO mice were reconstituted with SC and BMC from BALB WT mice, administered with anti-IL10 mAb, and then treated with DBA SC and CP (purple circle, n=6, MST >100 days). NKT

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by our tolerance inducible conditioning, which consist of donor SC and 200 mg/kg CP in the recipient received grafts form H2-matched donors. In the present study, we have elucidated the role of donor and recipient NKT cells in the induction of skin allograft tolerance, mixed chimerism, and clonal destruction in CP-induced tolerance.

Clonal destruction of Mls-1^a-reactive $CD4^+V\beta6^+$ T cells were observed in WBC of BALB WT mice treated with DBA SC and CP (EXP1, Table 1). Similarly, clonal destruction was observed when BALB NKT KO mice were used as recipients. Concerning establishment and maintenance of mixed chimerism, permanent mixed chimerism (Lyt-1.1^+ cells) could be clearly detected in BALB WT mice treated with DBA SC and CP (EXP1, Table 1). On the other hand, a lower degree of mixed chimerism was detectable at 2 weeks but did not seem to terminate at 8 weeks in BALB WT mice treated with DBA SC and CP. Skin allograft tolerance was induced in BALB WT mice treated with DBA SC and CP, but not in BALB NKT KO mice treated with DBA SC and CP (Fig. 1). Our previous study indicated that a higher level of chimerism is required for the induction of skin allograft tolerance (*34*). These results showed that NKT cells in recipients are essential for the induction of transplantation tolerance.

Another interesting observation is that there is no requirement for donor NKT cells in the induction and maintenance of CP-induced tolerance. Clonal destruction of Mls-2^a-reactive $CD4^+V\beta3^+$ T cells were observed in WBC of B10.D2 WT mice treated with BALB WT SC and CP (EXP3, Table 1). Similarly, clonal destruction was observed when BALB NKT KO mice were used as donors. With regard to the establishment and maintenance of mixed chimerism, permanent mixed chimerism (Lyt-1.1^- cells) could be clearly detected in DBA WT mice treated with BALB WT SC and CP (EXP2, Table 1). The same degree of mixed chimerism was detectable at 2 and 8 weeks in DBA WT mice treated with BALB NKT KO SC and CP. Skin allograft tolerance was induced in DBA or B10.D2 WT mice treated with BALB WT or NKT KO SC and CP (Fig. 2).

Two reports have previously described the critical role of recipient NKT cells for inducing transplantation tolerance (*11, 12*). However, the precise mechanisms at the cellular and molecular level have remained unclear. It has been well documented that NKT cells produce large amounts of both IL-4 and IFN-γ upon activation (*35–37*). Given that IL-4 and IFN-γ have opposite effects on the development of Th1 and Th2 cells, extensive analyses have been performed with various experimental systems, and conflicting results have been reported (*38–40*). Using IL-4 KO and IFN-γ KO mice, the mechanisms of the NKT-mediated role in transplantation tolerance induction have been investigated in two different studies and produced conflicting results (*11, 12*). Ikehara et al. (*12*) suggested that there was little involvement of these two cytokines in C57BL/6 mice injected with anti-CD4 mAb and grafted with rat islets. On the other hand, Seino et al. suggested that IFN-γ partially contributes to tolerance induction in C57BL/6 mice injected with anti-LFA-1 and ICAM-1 mAbs and grafted with BALB (H-2^d) mice (*11*). However, these results do not seem to be definitive, since they could not clearly show whether IFN-γ produced by NKT cells was involved in one or more of the steps that induce and maintain transplantation tolerance, such as activation of effector T cells, apoptosis of effector T cells, reprogramming of effector T cells (anergy induction), and the generation of regulatory T cells. In the present study, we showed that skin allograft tolerance can be easily induced in NKT KO mice reconstituted with NKT cells from IFN-γ or IL-4 KO mice (Fig. 3A). The present results strongly suggested that our CP-induced tolerance is mediated by NKT cells with involvement of neither IFN-γ nor IL-4.

More recently, two reports have described the mechanisms of NKT-mediated immunoregulation in transplant immunity (*13, 41*). One study reported the critical role of CXCL16/CXCR6 in NKT-dependent transplantation tolerance (*13*). The other study showed that NKT cells have limited ability to suppress skin graft rejection (*41*). As shown in Figure 1, DBA skin grafts were rejected within 14 days in all of the untreated BALB WT and NKT KO mice, suggesting that NKT cells have no effect on rejection of multiminor histocompatibility antigen-mismatched combination of DBA and BALB mice. In the H-Y antigen-mismatched combination of B6 male with untreated B6 female mice, however, the immunoregulation by NKT cells could mildly prolong male skins (*41*). The investigators showed that this H-Y graft prolongation depends on IL-10 generated by NKT cells. In the present study, we showed that administration with anti-IL-10 mAb did not abrogate skin allograft tolerance in BALB NKT KO mice reconstituted with WT NKT cells (Fig. 3A). Our results strongly suggested that our CP-induced tolerance is mediated by NKT cells without involvement of IL-10.

NKT cells express both invariant Vβ14 NKT-specific antigen receptors as well as an NK marker. Regarding the ligand for NKT cell receptors, however, the glycolipid antigen does not seem to be implicated in allogeneic transplant im-

FIGURE 3. (Continued) KO mice rejected DBA donor skins within 14 days (blue square, n=5). (b) Third-party B10.D2 skin grafts were rejected within 14 days after grafting in all groups. Recipient mice received donor skins at 4 weeks after treatments. (B) Administration of anti-Ly49A (YEL48), C/I (5E6), and G2 (4D11) mAbs against inhibitory receptors attenuated the induction of skin allograft tolerance. (a) Groups and mean and median DBA skin graft survival time were as follows: WT mice treated with SC and CP without mAbs (red square, n=6, MST >100 days). In WT mice treated with SC and CP and anti-Ly49 mAbs (pink square with dotted line, n=6; MST 57.8±36.2 days, median=52.5 days). Donor DBA skin grafts were chronically rejected in NKT KO mice treated with SC and CP, and there was no difference after depletion of Ly49-positive cells (gray diamond, n=6, MST 33.7±11.8 days) or without depletion (black diamonds, 38.3±7.6 days, median=31.5 days or 41 days), respectively. Donor skin graft was permanently accepted in WT mice treated with DBA SC and CP after NK cell depletion (green triangle, n=6, MST >100 days). On the contrary, graft was chronically rejected in WT mice treated with DBA SC and CP after combined depletion of NK cells and Ly49-positive cells (orange triangle, n=6, MST 55.1±30.5 days, median ≥57 days). Untreated BALB WT or NKT KO recipient mice rejected donor DBA skin grafts within 14 days (n=6). (b) Third-party B10.D2 skin grafts were rejected within 14 days after grafting in all groups. Recipient mice received skin grafts at 4 weeks after treatments.

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munity. Recently, on the other hand, NK cells have been clarified to recognize the class I molecule of target cells via CD94/NKG2A and Ly-49 receptors (*42*). The mouse Ly-49 families are composed of at least nine highly related genes designated Ly-49A to I (*43*). Ly-49 receptors are expressed on NK cells, which recognize MHC class I molecules and deliver either inhibitory or activating signals. They are expressed on overlapping subsets of NK cells and NKT cells. NKT cells have been reported to express inhibitory receptors for Ly-49A, G2, and C/I molecules, but do not express activation receptors for Ly-49D and Ly-49H molecules (*31, 44*). We found that the administration of mAbs against Ly-49A, C/I, and G2 inhibitory receptors that recognize MHC class I D^d limited graft prolongation in WT mice treated with DBA SC and CP but did not affect graft prolongation in NKT KO mice treated with DBA SC and CP (Fig. 3B). We found that the administration of mAbs against Ly-49A, C/I, and G2 inhibitory receptors which recognize MHC class I D^d limited graft prolongation in NK-depleted WT mice treated with DBA SC and CP but did not affect graft prolongation in NKT KO mice treated with DBA SC and CP (Fig. 3B). Since NKT cells contain high frequencies of Ly49 inhibitory receptors (*45*), it is still possible that reduced number of NKT cell in the Ly49-depleted recipient affected the poor outcome of the skin graft survival. The recent study reported that some T cells express Ly-49 molecules (*46*). Thus, there is some possibility that as the T cells were depleted with Ly-49 mAbs, tolerance was broken. Further investigations will clarify the immunoregulatory ligands of NKT cells in transplantation immunity.

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Regulation of MyD88-Dependent Signaling Events by S Nitrosylation Retards Toll-Like Receptor Signal Transduction and Initiation of Acute-Phase Immune Responses[▽]

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Nitric oxide (NO) has been thought to regulate the immune system through S nitrosylation of the transcriptional factor NF-κB. However, regulatory effects of NO on innate immune responses are unclear. Here, we report that NO has a capability to control Toll-like receptor-mediated signaling through S nitrosylation. We found that the adaptor protein MyD88 was primarily S nitrosylated, depending on the presence of endothelial NO synthase (eNOS). S nitrosylation at a particular cysteine residue within the TIR domain of MyD88 resulted in slight reduction of the NF-κB-activating property. This modification could be restored by the antioxidant glutathione. Through S nitrosylation, NO could negatively regulate the multiple steps of MyD88 functioning, including translocation to the cell membrane after LPS stimulation, interaction with TIRAP, binding to TRAF6, and induction of IκBα phosphorylation. Interestingly, glutathione could reversely neutralize such NO-derived effects. We also found that an acute febrile response to LPS was precipitated in eNOS-deficient mice, indicating that eNOS-derived NO exerts an initial suppressive effect on inflammatory processes. Thus, NO has a potential to retard induction of MyD88-dependent signaling events through the reversible and oxidative modification by NO, by which precipitous signaling reactions are relieved. Such an effect may reflect appropriate regulation of the acute-phase inflammatory responses in living organisms.

It is increasingly becoming evident that nitric oxide (NO) regulates a broad spectrum of protein functions through S nitrosylation, a posttranscriptional modification that forms *S*-nitrosothiol by covalent addition to cysteine residues of an NO moiety (14, 42, 43). Through S nitrosylation, NO is thought to exert a physiological inhibitory effect on nuclear factor κB (NF-κB) (25, 32, 33, 39), the major transcriptional factor family deeply associated with regulation of the immune system through transcription of a wide range of genes, including cytokines, adhesion molecules, antimicrobial molecules, and antiapoptotic molecules (10, 13, 24). S nitrosylation of NF-κB inhibits its DNA binding, promoter activity, and subsequent transcription (25, 33). It has been known that S nitrosylation targets a particular cysteine residue of the NF-κB p50 and p65 subunits located in the N-terminal DNA binding loop within the Rel homology domain (25, 32, 33). This residue is conserved in other NF-κB subunits, including p52, p100, p105, and c-Rel, and other Rel homology domain-containing molecules. Upstream of NF-κB, IκB kinase β (IKKβ), a catalytic subunit of the IκB (inhibitor of NF-κB) kinase complex, also undergoes S nitrosylation, resulting in reduction of its kinase function on phosphorylation of IκB (39). Such reduction of the IKKβ function leads to reduced IκB ubiquitinylation and proteasomal degradation, resulting in NF-κB inhibition (14, 32, 39).

Toll-like receptors (TLRs) are the central innate immune sensors for a broad array of pathogen-associated molecular patterns, ranging from bacterial constituents to viral genomes (2, 35). TLRs initiate early processes of proinflammatory immune responses that help to strengthen the processes of innate and adaptive immunity (2, 20), in which NF-κB plays many important roles (13, 24). TLRs utilize MyD88, a Toll/interleukin-1 receptor (IL-1R) homology (TIR) domain-containing adaptor molecule, to activate the NF-κB pathway through IL-1R-associated kinases (IRAKs) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (1). It has been thought that TLR agonistic molecules, such as lipopolysaccharide (LPS), can regulate NO generation through upregulation of expression of all NO synthase (NOS) isoforms through NF-κB activation (4, 9, 32). TLR stimulation can directly activate an antimicrobial property through inducible NOS (iNOS) expression and NO generation in macrophages (46). NO generation is a general feature of immune cells, including neutrophils, monocytes, macrophages, dendritic cells, and NK cells, as well as other cells, including endothelial cells, epithelial cells, and fibroblasts (4), all of which express multiple members of the TLR family. However, it has remained obscure whether generated NO exerts any regulatory effects on TLR signaling or subsequent processes of innate immune responses.

There has been an accumulation of biochemical evidence indicating that TLR signaling components, including IKKβ

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and NF-κB, might be regulated by S nitrosylation. S nitrosylation inhibits the kinase activity of apoptosis signal regulation kinase 1 (ASK1) through inhibition of its binding to substrates (38). ASK1 is known as an important regulator of the TRAF6-p38 mitogen-activated protein kinase (MAPK) pathway downstream of TLR4 and is also involved in modulation of both the NF-κB and apoptotic pathways downstream of TLR2 (19, 34). Caspse-1 was recently found to be involved in TLR2- and TLR4-mediated signal transduction of the MyD88-dependent pathway through the cleavage of the TIR domain-containing adaptor protein TIRAP (also known as Mal) (37). Caspase-1 also undergoes S nitrosylation at a cysteine residue within the enzymatic active site, suppressing its proteolytic activity (6, 31). Thus, it is possible that NO provides regulatory effects on the multiple steps of TLR-mediated innate immune signaling through S nitrosylation. In this study, we therefore designed experiments to determine the effect of S nitrosylation on TLR signaling. We further investigated how S nitrosylation affects TLR-initiated immune responses in vivo. We report here that S nitrosylation controls TLR signaling through redox-sensitive and reversible suppression of the MyD88 pathway, which facilitates appropriate control of acute-phase inflammatory responses in vivo.

MATERIALS AND METHODS

Reagents and cell culture. N^G-Monomethyl-L-arginine monoacetate (L-NMMA), *S*-nitrosoglutathione (GSNO), glutathione (GSH), *N*-ethylmaleimide, coumermycin A, *N*-acetyl L-cysteine (NAC), ascorbic acid, and diphenyleneiodonium (DPI) were obtained from Sigma-Aldrich. SNAP (5-nitroso-*N*-acetyl-D,L-penicillamine) was purchased from Cayman Chemical. ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one) and KT5823 were obtained from Calbiochem. Preparation of TLR ligands, including highly purified *Escherichia coli* LPS, *Salmonella* LPS, Pam_3CSK_4, macrophage-activating lipopeptide 2 (MALP-2), and *Salmonella enterica* serovar Typhimurium flagellin, was as described previously (18). Recombinant human IL-1β was from R&D Systems. Human aortic endothelial cells (HAECs) and human embryonic kidney 293 (HEK293) cells were maintained as described previously (18). HEK293 cells stably expressing human TLR4, MD2, and CD14 (293-TLR4 cells) and HEK293 cells stably expressing human TLR2 and CD14 (293-TLR2 cells) were obtained from InvivoGen.

Mice. iNOS-deficient ($iNOS^{-/-}$) mice and endothelial-NOS (eNOS)-deficient ($eNOS^{-/-}$) mice were from The Jackson Laboratories. C57BL/6J control (wild-type) mice were obtained from Japan SLC. All mice were kept under specific pathogen-free conditions. Male mice between 6 and 10 weeks of age were used for all of experiments. All animal protocols were approved by the National Institute for Longevity Sciences Animal Experimentation Committee at the National Center for Geriatrics and Gerontology (Aichi, Japan).

For LPS-induced acute lung injury, anesthetized mice received *Escherichia coli* LPS dissolved in pyrogen-free phosphate-buffered saline (PBS) containing 1 mg/ml Evans Blue intratracheally immediately after mechanical ventilation. After 30 min of administration, lung was excised and then lysed for immunoblot analysis. The febrile responses in mice treated with *E. coli* LPS were tested according to a protocol described previously (45, 49). Mice ($n = 6$) were maintained at a neutral ambient temperature of 31°C and challenged by intraperitoneal (i.p.) injection of 5 mg LPS/kg of body weight dissolved in pyrogen-free PBS. A high dose of LPS (more than 50 mg/kg) was fatal within 90 min in $eNOS^{-/-}$ mice. A colonic thermocouple was inserted and fixed to the base of the tail with adhesive tape. The change in temperature was monitored at 5-min intervals during a period of 120 min after LPS administration. All of the tests were performed at the temperature of 31°C. After 2 h or 12 h of LPS administration, 2 ml of PBS was injected into the abdominal cavity of each mouse. Then, fluids were collected and centrifuged for assessment of cytokine production by an enzyme-linked immunosorbent assay (ELISA). Preparation of peritoneal macrophages was as described previously (28).

Plasmids. The DNA construct encoding 3× Flag-tagged MyD88 fused to the B subunit of the bacterial DNA gyrase (MyD88-GyrB) was as described previously (11). Plasmids encoding human MyD88 and TIRAP were kind gifts from Margaret K. Offermann (Emory University School of Medicine). The cDNAs of N-terminal Flag-tagged and Myc-tagged MyD88, Myc-tagged TIRAP, and IRAK-1 were amplified by PCR and cloned into the pcDNA3.1 vector (Invitrogen). The construct encoding human TLR2 was as described previously (17). Constructs encoding mutated Flag-MyD88 were obtained using a QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Protein purification. Recombinant Flag-MyD88 proteins were prepared using a FLAG M purification kit (Sigma-Aldrich) from HEK293 cells stably expressing Flag-MyD88 constructs, according to the manufacturer's instructions. Purity of recombinant proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining and immunoblotting with anti-Flag antibody.

Detection of S-nitrosylated proteins. To detect S-nitrosylated MyD88 from lung lysates from wild-type mice and $eNOS^{-/-}$ mice, we referred to the protocol described by Jaffrey et al. (21). Several experiments were performed using a NitroGlo nitrosylation detection kit (PerkinElmer) according to the manufacturer's instructions. Lung lysates from wild-type and $eNOS^{-/-}$ mice were subjected to the biotin switching S-nitrosylation assay, and then biotinylated proteins were purified on streptavidin-agarose. Purified proteins eluted by 2-mercaptoethanol were detected by immunoblotting with anti-MyD88 antibody.

The quantitative measurement of S-nitrosylated recombinant MyD88 by ELISA was performed as follows. Briefly, recombinant Flag-MyD88 (150 μg) was treated with or without SNAP for 30 min at 37°C in the dark. Then, the free sulfides of Flag-MyD88 were blocked with 4 mM methylmethanethionsulfonate for 15 min. After purification by using Micro Bio-Spin chromatography columns (Bio-Rad Laboratories), Flag-MyD88 was reacted with 25 mM ascorbate to be completely denitrosylated. Free sulfides were then labeled with a biotin-conjugated maleimide, using a biotin labeling kit (SH; Dojindo Laboratories) according to the manufacturer's instructions. The diluents of biotinylated Flag-MyD88 proteins dissolved in Tris-buffered saline (pH 7.2) were stabilized in the wells of immobilizer streptavidin plates (Nunc). Flag-MyD88 proteins in the wells were detected by using anti-Flag antibody and a secondary antibody conjugated with horseradish peroxidase. Colorimetric reaction was detected by absorbance on a spectrophotometer at 450 nm. Results were expressed as means ± standard deviations (SD) of three determinations.

Photolysis of S-nitrosylated proteins. Mouse lung lysates were exposed for 3 min to a UV-visible light mercury vapor lamp according to a protocol recently described (8). The samples were then subjected to the biotin switch technique as described above.

Luciferase reporter assay. 293-TLR2 cells were transiently transfected with wild-type MyD88-GyrB or MyD88-GyrB mutants, each with a cysteine residue replaced with a serine residue, together with 50 ng of an NF-κB (5×) luciferase reporter plasmid (pNF-κB-Luc; Stratagene) and 5 ng of an internal control luciferase reporter plasmid (pRL-TK; Promega) and incubated for 16 h. At 6 h before the end of incubation, cells were treated with or without 250 μM SNAP. Cells were then stimulated with 100 ng/ml Pam_3CSK_4 for 6 h. HEK293 cells stably expressing MyD88-GyrB were transfected with pNF-κB-Luc and pRL-TK. After 24 h of incubation, cells were stimulated with coumermycin A in the presence and absence of 250 μM SNAP. The dual luciferase activity was measured as described previously (19).

Immunoblot analysis of IRAK-1 and IκBα. HAECs were stimulated with 10 ng/ml of LPS for 0 to 90 min. HEK293 cells stably expressing MyD88-GyrB were stimulated with 1 μM coumermycin for 20 min. Cells were lysed in the presence of protease inhibitor and phosphatase inhibitor cocktails (Roche) at 4°C. Cell lysates or lysates from the mouse lungs were separated by SDS-PAGE, followed by immunoblot analyses using anti-IRAK-1, anti-IκBα, and phosphorylation-specific anti-IκBα (Ser32/Ser36) antibodies (Cell Signaling Technology).

RNA extraction and reverse transcription-PCR. Total RNA was isolated from mouse peritoneal macrophages stimulated with 100 ng/ml LPS and 10 ng/ml gamma interferon, and transcripts were quantified by real-time quantitative reverse transcription-PCR on a LightCycler ST300 system (Roche). All values were normalized to the level of β-actin mRNA. The primer sets used are as follows: for mouse macrophage inflammatory protein 2 (MIP-2), 5′-ATCCAG AGCTTGAGTGTGACGC-3′ (sense) and 5′-AAGGCAAACTTTTTGACCG AA-3′ (antisense); for mouse IL-6, 5′-CCACGGCCTTCCCTAC-3′ (sense) and 5′-AGTGCATCATCGTTGTTC-3′ (antisense); and for mouse β-actin, 5′-AA ATCGTGCGTGACATCAAA-3′ (sense) and 5′-AAGGAAGGCTGGAAAAG AGC-3′ (antisense).

Cytokine ELISA. Concentrations of human IL-8, mouse MIP-2, and mouse IL-6 were determined using a Cytoset ELISA kit (Biosource) according to the manufacturer's instructions.

Subcellular fractionation. Subcellular fractionation of HEK293 cells stably expressing Flag-MyD88 and 293-TLR4 cells stably expressing Flag-MyD88-GyrB