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$^+$Vβ6$^+$ 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$^+$Vβ3$^+$ 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.

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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Effects of Cyclosporin A on the Activation of Natural Killer T Cells Induced by α-Galactosylceramide

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Background. Natural killer T (NKT) cells play crucial roles in preventing autoimmune diseases and inducing transplantation tolerance. We investigated whether cyclosporin A (CsA), which is generally used in clinical transplantation and autoimmune disease therapy, could modulate the NKT cell activation induced by α-galactosylceramide (α-GalCer) treatment.

Methods. C57BL/6 (B6) mice were given daily intraperitoneal injections of CsA (30 or 50 mg/kg) from day −1 and injected intravenously with α-GalCer (2 μg/mouse) on day 0. The kinetics of $NK1.1^+CD3^+$ or $NK1.1^+Thy1.2^+$ cells in the liver and spleen were analyzed by flow cytometry. Apoptosis of $NK1.1^+CD3^+$ cells, cytokine levels (interleukin [IL]-2, IL-4, IL-10 and interferon [IFN]-γ) in the recipient serum and changes in dendritic cell activation in the spleen were analyzed.

Results. In B6 mice treated with α-GalCer, $NK1.1^+CD3^+$ cells rapidly decreased in both the liver and spleen, and repopulated to their normal levels by day four, while $NK1.1^+Thy1.2^+$ cells rapidly decreased, expanded by day four and reduced to their normal level by day 15. When B6 mice were treated with α-GalCer plus 30 or 50 mg/kg CsA, $NK1.1^+CD3^+$ or $NK1.1^+$ $Thy1.2^+$ cells were similarly decreased and then expanded via extensive proliferation by day seven or four, respectively. When B6 mice were treated with α-GalCer, substantial amounts of IL-2, IL-4 and IFN-γ were produced, and the surface markers of dendritic cells were upregulated. However, these cytokine productions and maturation of dendritic cells were profoundly suppressed after treatment with α-GalCer and CsA. Apoptosis of $NK1.1^+CD3^+$ cells was not affected in mice treated with α-GalCer or α-GalCer and CsA.

Conclusions. CsA suppresses α-GalCer-induced cytokine productions and dendritic cell maturation of mouse NKT cells but does not decrease $NK1.1^+CD3^+$ cells on day one. The modulation of NKT-mediated immunoregulatory functions by CsA requires careful consideration in clinical transplantation and autoimmune disease therapy.

Keywords: Natural killer T cells, Cyclosporin A, α-galactosylceramide.

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Natural killer T (NKT) cells have been characterized as cells that coexpress the natural killer (NK) cell marker NK1.1 and the T-cell receptor (TCR) (*1–4*). Although their natural ligands have not been well characterized, NKT cells recognize and are strongly stimulated by a glycolipid antigen, α-galactosylceramide (α-GalCer), presented by the major histocompatibility complex (MHC) class I-like molecule CD1d (*5*). By using flow cytometry (FCM) and RNA extraction assays, initial studies have revealed that α-GalCer quickly activates NKT cells, which then become undetectable. This finding is correlated with increased apoptosis and increased expression of Fas and FasL by NKT cells (*6–8*). Another study reported a similar disappearance of NKT cells in vivo after stimulation with an anti-CD3 mAb or IL-12 (*9*). This demise of NKT cells after treatment with an anti-CD3 mAb or interleukin (IL)-12 was usually followed by repopulation within two to three days after the stimulation due to homeostatic proliferation in the bone marrow. On the other hand, more recent studies have suggested that receptor down-regulation is the primary cause of the NKT cell disappearance and reappearance following α-GalCer treatment (*10–12*).

After recognition of α-GalCer, NKT cells activate and rapidly secrete large amounts of both Th1 and Th2 cytokines, such as IL-4 and interferon (IFN)-γ (*5*). The activation of NKT cells has been considered to develop their immunoregulatory functions through Th1 and/or Th2 cytokines. NKT cells activated by α-GalCer have been shown to play important roles in preventing autoimmune diseases and enhancing anti-tumor cytotoxicity (*13–16*). In transplant immunity, NKT cells play vital roles in the induction of not only allograft tolerance but also xenograft tolerance, although the precise mechanisms for these effects have not yet been clarified (*17, 18*).

Cyclosporin A (CsA) is a popular immunosuppressive drug that is widely used in organ transplantation and autoimmune disease therapy. In mice, a middle dose (30 mg/kg) of CsA suppresses IL-2 production by $CD4^+$ helper T cells, whereas a high dose (75 mg/kg) suppresses that by both $CD4^+$ and $CD8^+$ helper T cells (*19*). Paradoxically, however, CsA can cause autoimmune diseases (*20, 21*) and a graft-versus-host (GVH)-like syndrome in syngeneic bone marrow transplantation (*22, 23*), and interfered with the induction of allograft tolerance in rodents (*24*). Because NKT cells play

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essential roles in the maintenance of tolerance and prevention of autoimmune diseases, breakdown of the transplantation tolerance or autoimmune disease prevention induced by CsA may be caused via the suppression of NKT cell functions.

Therefore, in the present study, we investigated whether middle and high doses of CsA (30 and 50 mg/kg, respectively) could modulate the activation of NKT cells following treatment with α-GalCer on day 0. After treatment with α-GalCer and CsA, a similar rapid disappearance of $NK1.1^+CD3^+$ cells was observed on day one, but these cells subsequently increased to a higher level than that after treatment with α-GalCer alone. Cytokine productions were completely suppressed and $CD11c^+$ dendritic cells did not become mature after treatment with α-GalCer and CsA. These results indicate that CsA could completely suppress the cytokine productions by NKT cells, but did not down-regulate their surface markers. Therefore, the results of the present study suggest that suppression of the immunoregulatory functions of NKT cells by CsA may be one of the causes of autoimmune disease development and interfere with tolerance induction.

MATERIALS AND METHODS

Animals

Inbred female mice of the C57BL/6 SnSlc (B6; H-2^b) strain were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan), and used at 8–16 weeks of age. All animals received humane care in compliance with both the Guidelines for Animal Experiments of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese government.

Reagents

α-GalCer (KRN7000) was kindly provided by Kirin Brewery (Takasaki, Japan), dissolved in 0.5% polysorbate 20 at a concentration of 200 μg/ml and then further diluted with 0.9% NaCl. CsA (Novartis Pharmaceuticals, Basel, Switzerland) was dissolved in 0.9% NaCl at a concentration of 2 mg/ml.

In Vivo Treatments

Mice were injected intravenously (i.v.) with 2 μg of α-GalCer. As a control, mice were injected with an equivalent amount of vehicle, namely 0.5% polysorbate 20 and 0.9% NaCl. From one day before the α-GalCer treatment, mice received daily intraperitoneal (i.p.) injections of CsA (30 or 50 mg/kg). The day of the α-GalCer injection is referred to as day 0 throughout this report.

Cell Preparation

Mice were sacrificed by decapitation, and cell suspensions were prepared from the liver and spleen. The liver was disrupted in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) by pressing the liver fragments between two glass slides, then washed, resuspended in 40% isotonic Percoll solution (Amersham Biosciences, Piscataway, NJ) and overlaid on 67.5% isotonic Percoll solution. Following centrifugation at 3000 rpm for 30 min at room temperature, liver mononuclear cells (LMNC) were isolated from the interface, washed twice with Hanks balanced salt solution (HBSS) containing 2% FCS and then resuspended in the same solution. The spleen was disrupted in RPMI 1640 medium in the same manner as the liver, and then washed with HBSS containing 2% FCS. The spleen cell (SC) suspensions obtained were filtered through cotton gauze and washed twice with HBSS containing 2% FCS. Viable nucleated cells were counted and usually adjusted to 1×10^7 cells/mL.

Thymectomy

Recipients were anesthetized by an i.p. administration of 50 mg/kg phenobarbital (Nembutal; Shionogi, Osaka, Japan). After a partial sternotomy, a thymectomy was performed via an en bloc excision using two forceps (25). The absence of thymic tissue was always confirmed when the thymectomized animals were sacrificed, and animals showing the presence of residual thymic tissue were excluded from the analysis.

Flow Cytometry

The surface phenotypes of the LMNC and SC were identified by two-color FCM. Cells were incubated with phycoerythrin (PE)-conjugated anti-NK1.1 or anti-CD5 (BD PharMingen, San Diego, CA), biotin-conjugated anti-CD3e or anti-NK1.1 (BD PharMingen) monoclonal antibodies (mAbs) and fluorescein isothiocyanate (FITC)-conjugated anti-Thy1.2 (BD PharMingen) for 60 min at 4°C and then washed twice with HBSS containing 2% FCS. Biotin-conjugated reagents were developed with FITC- or allophycocyanin (APC)-conjugated streptavidin (SA; BD PharMingen).

To detect the NKT cells undergoing apoptosis, three-color FCM was used. LMNC were isolated at three hr after the α-GalCer injection. The cells were stained with PE-conjugated anti-NK1.1 (BD PharMingen) and biotin-conjugated anti-CD3e (BD PharMingen) mAbs. Biotin-conjugated reagents were developed with APC-SA (BD PharMingen). Cells were washed twice in annexin V binding buffer (BD PharMingen) before labeling with FITC-conjugated annexin V (BD PharMingen) for 30 min at room temperature in the dark.

To analyze the maturation of dendritic cells (DC), SC were isolated at 24 hr after the α-GalCer injection. The cells were stained with FITC-conjugated anti-CD11c (BD PharMingen) and PE-conjugated anti-I-A^b (BD PharMingen), anti-CD40 (BD PharMingen), anti-CD80 (BD PharMingen) or anti-CD86 (BD PharMingen) mAbs for 30 min at 4°C. To block nonspecific FcγR receptor binding of the labeled antibodies, 10 μL of an undiluted culture supernatant containing 2.4G2 (a rat antimouse FcγR mAb) was added to the first incubation, and then washed. All data were analyzed with a FACSCalibur (Becton Dickinson, Sunnyvale, CA). Dead cells were excluded by gating out low forward scatter and high propidium iodide-retaining cells.

Cytokine Secretion Following In Vivo α-GalCer Treatment

Mice were injected with either α-GalCer or vehicle alone and then bled after 2 or 18 hr. The cytokine levels (IL-2, IL-4, IL10 and IFN-γ) in the serum were determined using a standard sandwich enzyme-linked immunosorbent assay (ELISA) (BioSource International Inc., Camarillo, CA).

Statistics

The statistical significance of the data was determined using Students *t* test. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Kinetics of the Percentages of NK1.1$^+$CD3$^+$ Cells in the Liver and Spleen of Recipient Mice Treated with α-GalCer and CsA

When α-GalCer is administered to B6 mice, a rapid reduction followed by restoration has been shown for NK1.1$^+$CD3$^+$ cells among the LMNC and SC (*6*, *26*). Here, we examined the effects of CsA on the kinetics of NK1.1$^+$CD3$^+$ cells induced by α-GalCer. A middle or high dose of CsA (30 or 50 mg/kg, respectively) was injected i.p. daily from one day before the α-GalCer treatment. As shown in Figure 1A, the percentage of NK1.1$^+$CD3$^+$ cells in the LMNC rapidly decreased to less than 2% on day one, was restored to around 8% by day four and then remained constant until day 15 in mice treated with α-GalCer. In the LMNC of mice treated with α-GalCer plus 30 mg/kg CsA, the NK1.1$^+$CD3$^+$ cells were reduced to less than 2% on day one, restored to the normal level by day seven and then reduced to about 13% by day 15. In the LMNC of mice treated with α-GalCer plus 50 mg/kg CsA, the NK1.1$^+$CD3$^+$ cells were similarly reduced to less than 2% on day one, restored to above the normal level by day seven and then reduced to the normal level by day 15. Similar results were observed for the SC of mice treated with α-GalCer plus 30 or 50 mg/kg CsA, and representative data are shown in Figure 1A. We analyzed the early response of NKT cells after treatment with α-GalCer or α-GalCer and CsA by using other T-cell markers such as CD5 or Thy1.2. The kinetics of the NK1.1$^+$CD5$^+$ cells after treatment with α-GalCer or α-GalCer and CsA were same as those observed for the NK1.1$^+$CD3$^+$ cells (data not shown). The kinetics of the NK1.1$^+$Thy1.2$^+$ cells in the liver and spleen of recipient mice treated with α-GalCer and CsA are shown in Figure 1B. In the liver of mice treated with α-GalCer alone, NK1.1$^+$Thy1.2$^+$ cells rapidly decreased on day one and repopulated by day four. In the liver of mice treated with α-GalCer plus 30 or 50 mg/kg CsA, NK1.1$^+$Thy1.2$^+$ cells also decreased on day one and then gradually increased by days 7 to 15. In the spleen of mice treated with α-GalCer alone or α-GalCer plus 30 or 50 mg/kg CsA, NK1.1$^+$Thy1.2$^+$ cells rapidly decreased on day one, increased to above the normal level by days four and seven and then returned to the normal level on day 15.

Kinetics of the Numbers of NK1.1$^+$CD3$^+$ Cells among the LMNC and SC of Mice Injected with α-GalCer and CsA

We further examined the numbers of total cells and NK1.1$^+$CD3$^+$ cells in the liver after treatment with α-GalCer and CsA. The LMNC counts did not change significantly on day one, increased by day four and then decreased to the normal level by day 15 in mice treated with α-GalCer alone or α-GalCer plus 30 or 50 mg/kg CsA (Fig. 2a). The LMNC dramatically increased in mice treated with α-GalCer alone (~fourfold) and α-GalCer plus 30 or 50 mg/kg CsA (~six- to eight-fold) on day four. These expansions of the LMNC number on day four showed significant differences between mice treated with α-GalCer alone and those treated with α-GalCer plus 30 or 50 mg/kg CsA. Similar results were observed for the changes in the SC counts, although the LMNC were more extensively increased than the SC.

In the liver of mice treated with α-GalCer alone, the NK1.1$^+$CD3$^+$ cell counts rapidly decreased on day one, were restored to the normal level by day four and then gradually decreased by day 15 (Fig. 2b). On the other hand, the NK1.1$^+$CD3$^+$ cell counts in the liver of mice treated with α-GalCer plus 30 or 50 mg/kg CsA showed similar decreases on day one, then increased to above the normal level by day four, further increased on day seven (~fourfold) and gradually decreased by day 15. There were significant differences in the NK1.1$^+$CD3$^+$ cell numbers between mice treated with α-GalCer alone and those treated with α-GalCer plus 30 or 50 mg/kg CsA on day seven. Similar results were observed for the changes in the spleen NKT cell counts. As shown in Figure 1A, NK1.1$^+$CD3$^+$ cells in mice treated with α-GalCer plus 30 or 50 mg/kg CsA were still decreased on day four and repopulated by day seven. Therefore, the number of NK1.1$^+$CD3$^+$ cells showed the largest increase on day seven, while the total number of LMNC showed the largest increase on day four. NK1.1$^+$Thy1.2$^+$ cells in the liver and spleen of mice treated with α-GalCer alone or α-GalCer plus 30 or 50 mg/kg CsA showed rapid decreases on day one and expansion on day four (Fig. 2c).

Effects of Thymectomy on the Kinetics of the NK1.1$^+$CD3$^+$ Cell Counts After Treatment with α-GalCer and CsA

Many studies have suggested that the thymus is involved in the normal development of NKT cells. Therefore, we investigated whether the thymus was involved in the repopulation of NK1.1$^+$CD3$^+$ cells after treatment with α-GalCer and CsA by using thymectomized B6 mice. In thymectomized mice treated with α-GalCer alone or α-GalCer plus 30 or 50 mg/kg CsA, the percentage of NK1.1$^+$CD3$^+$ cells in the LMNC rapidly decreased on day one, remained the same on day four and was then restored to above the normal level by day seven. Therefore, the NK1.1$^+$CD3$^+$ cells in thymectomized mice showed the same kinetics as those in nonthymectomized mice (Fig. 3).

In Vivo Cytokine Responses at 2 or 18 Hours After Injection of α-GalCer and CsA

To further examine the effects of CsA on the α-GalCer-induced activation of NKT cells, the productions of Th1 (IL-2 and γ-IFN) and Th2 (IL-4 and IL-10) cytokines were measured. At 2 and 18 hr after the administration of α-GalCer, serum was collected from the mice and the level of each cytokine was determined using a standard sandwich ELISA. Consistent with previous studies (*7*, *13*, *14*), α-GalCer treatment induced the productions of IL-2, IL-4 and γ-IFN (Fig. 4). However, these cytokine productions were almost abrogated in the serum of mice treated with α-GalCer plus 30 or 50 mg/kg CsA.

Maturation of DC After Intravenous Injection of α-GalCer and CsA

A recent report (*27*) demonstrated that α-GalCer treatment induces the maturation of splenic CD11c$^+$ DC, as indi-

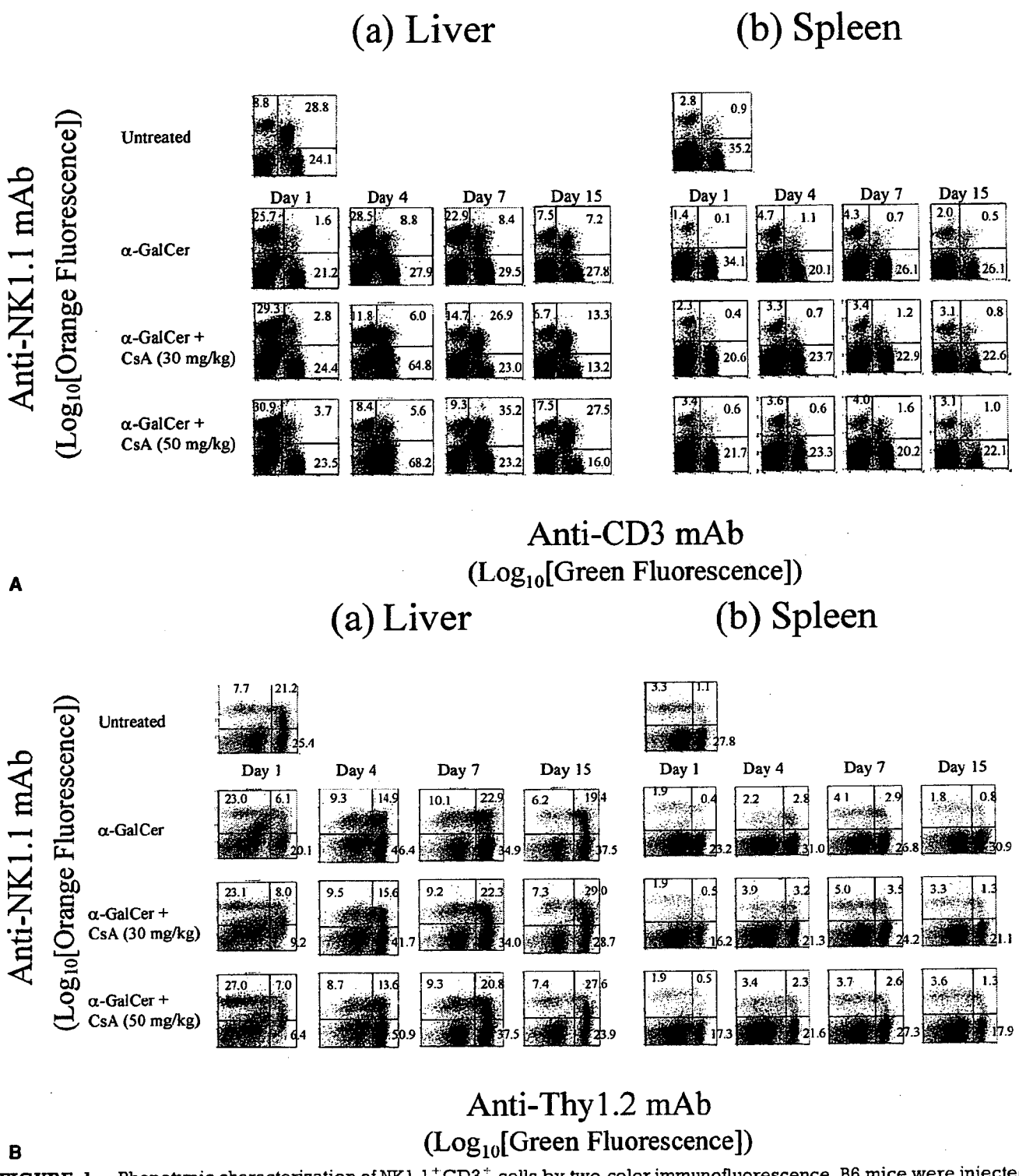

FIGURE 1. Phenotypic characterization of NK1.1$^+$CD3$^+$ cells by two-color immunofluorescence. B6 mice were injected with α-GalCer on day 0 and CsA (30 or 50 mg/kg) daily from one day before the α-GalCer treatment. The cells were labeled with PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3 mAbs (A) or PE-conjugated anti-NK1.1 and FITC-conjugated anti-Thy1.2 mAbs (B). Liver mononuclear cells (a) and spleen cells (b) were isolated after the indicated times and analyzed by flow cytometry. The numbers indicate the percentage of cells in the quadrant relative to the total cell population. The experiment shown is representative of four independent experiments.

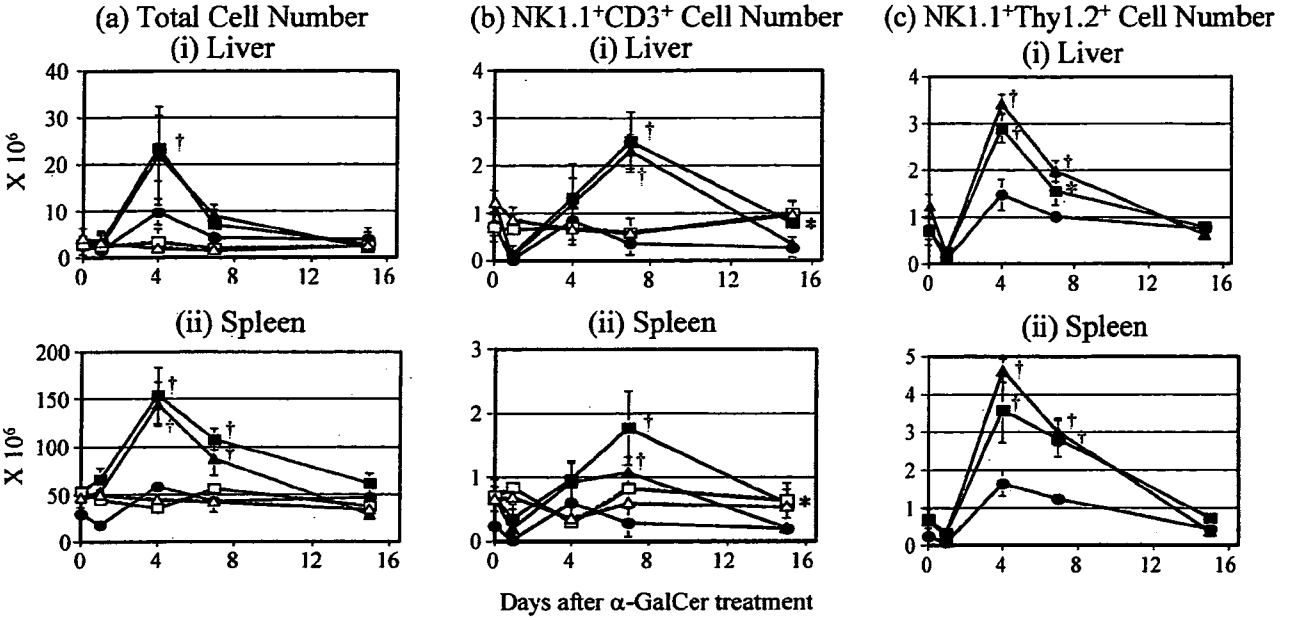

FIGURE 2. Influence of CsA on the induction of cell proliferation after α-GalCer treatment. Cells were labeled with PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3 mAbs or PE-conjugated anti-NK1.1 and FITC-conjugated anti-Thy1.2 mAbs. The kinetics of the total cell numbers (a), $NK1.1^+CD3^+$ cells (b) and $NK1.1^+Thy1.2^+$ cells (c) in the liver mononuclear cells and spleen cells of B6 mice treated with α-GalCer and CsA are shown. B6 mice were injected with α-GalCer on day 0 and CsA (30 or 50 mg/kg) daily from one day before the α-GalCer treatment. Liver and spleen cells were obtained from B6 mice treated with α-GalCer alone (● n=4), α-GalCer plus 30 mg/kg CsA (▲ n=4), α-GalCer plus 50 mg/kg CsA (■ n=4), 30 mg/kg CsA alone (Δ n=4) or 50 mg/kg CsA alone (□ n=4). Vertical bars represent the SD. *$P<0.05$, †$P<0.01$ vs. mice treated with α-GalCer alone.

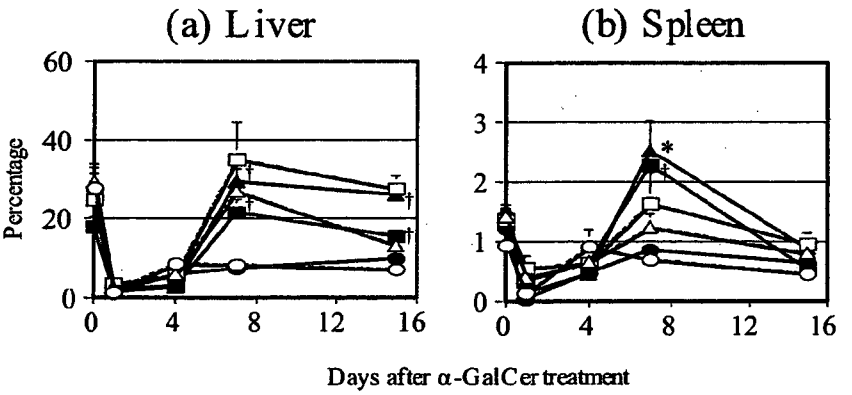

FIGURE 3. Kinetics of $NK1.1^+CD3^+$ cells in thymectomized mice treated with α-GalCer and CsA. The kinetics of the mean percentages of $NK1.1^+CD3^+$ cells in the liver mononuclear cells (a) and spleen cells (b) of thymectomized B6 mice treated with α-GalCer and CsA are shown. Cells were labeled with PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3 mAbs. B6 mice were thymectomized at four weeks before the α-GalCer treatment. The thymectomized mice were i.v. injected with α-GalCer (2 μg) on day 0 and i.p. injected with CsA (30 or 50 mg/kg) daily from one day before the α-GalCer treatment. Liver and spleen cells were obtained from non-thymectomized B6 mice treated with α-GalCer alone (○ n=4), α-GalCer plus 30 mg/kg CsA (Δ; n=4) or α-GalCer plus 50 mg/kg CsA (□ n=4), and from thymectomized B6 mice treated with α-GalCer alone (● n=4), α-GalCer plus 30 mg/kg CsA (▲ n=4) or α-GalCer plus 50 mg/kg CsA (■ n=4). Vertical bars represent the SD *$P<0.05$, †$P<0.01$ vs. mice treated with α-GalCer alone.

cated by the up-regulation of $I\text{-}A^b$, CD40, CD80 and CD86. At 24 hours after α-GalCer treatment, SC were stained with FITC-conjugated anti-CD11c and PE-conjugated anti-$I\text{-}A^b$, CD40, CD80 or CD86 mAbs, and the $CD11c^+$ cells were analyzed. As shown in Table 1, the expressions of $I\text{-}A^b$, CD40, CD80, and CD86 were clearly augmented in the $CD11c^+$ SC of mice treated with α-GalCer alone compared with those of untreated mice. The increases in the CD40, CD80, and CD86 expressions were lower in the $CD11c^+$ SC of mice treated with α-GalCer plus 30 or 50 mg/kg CsA than in those of mice treated with α-GalCer alone. On the other hand, the expression of $I\text{-}A^b$ was not augmented in the $CD11c^+$ SC of mice

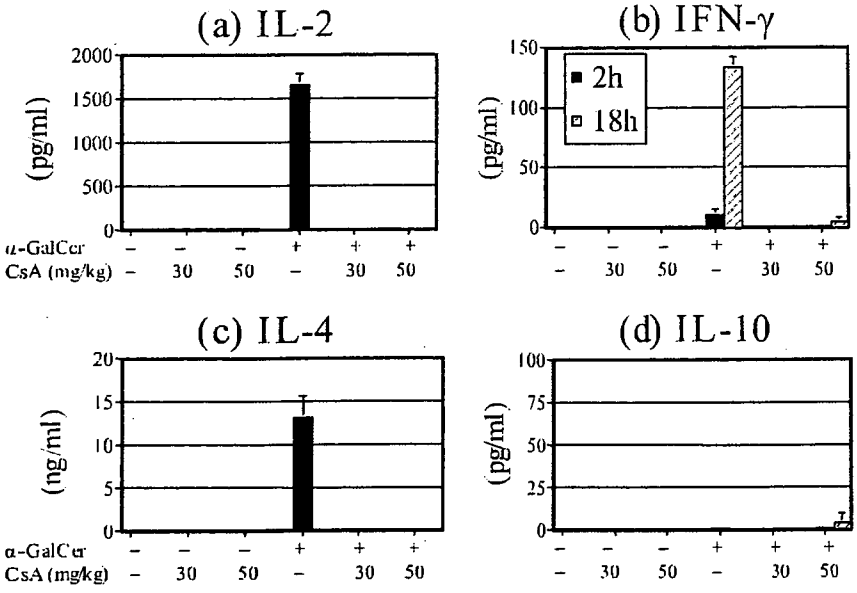

FIGURE 4. Production of Th1 and Th2 cytokines in B6 mice at 2 and 18 hr after α-GalCer treatment. B6 mice were injected with CsA (30 or 50 mg/kg) one day before the α-GalCer treatment. The serum levels of IL-2 (a), IFN-γ (b), IL-4 (c), and IL-10 (d) at 2 and 18 hr after injection of α-GalCer or vehicle were determined by ELISA. The data shown are the mean±SD of three mice for each group.

TABLE 1. Maturation of surface markers on splenic dendritic cells at 24 hours after treatment with α-GalCer and CsA

Group	Treatment[a]		No. of mice	Median fluorescence intensity (mean values±SD)			
	α-GalCer (2 μg)	CsA (mg/kg)		I-A[b]	CD40	CD80	CD86
1	−	—	4	1174.6±74.2	46.8±6.2	51.5±2.5	40.2±5.5
2	−	30	4	848.1±82.2	37.9±4.7	49.0±5.1	38.4±3.2
3	−	50	4	633.8±83.7	36.7±3.6	40.7±5.4	37.7±4.6
4	+	—	4	2714.4±51.8[b]	151.2±9.8[b]	186.8±17.5[b]	413.6±32.6[b]
5	+	30	4	865.4±310.9[c]	66.5±16.6[c,c]	59.0±6.5[c]	72.2±16.7[c,e]
6	+	50	4	681.8±91.6[e]	67.1±6.6[d,e]	53.1±4.1[d,e]	71.7±18.5[d,e]

[a] Mice received CsA on days −1 and 0 and were injected with α-GalCer on day 0.
[b] $P<0.01$ vs. Group 1.
[c] $P<0.05$ vs. Group 2.
[d] $P<0.05$ vs. Group 3.
[e] $P<0.01$ vs. Group 4.

treated with α-GalCer plus 30 or 50 mg/kg CsA compared with those of mice treated with CsA alone. However, the augmentations of all these molecules were suppressed in the $CD11c^+$ SC of mice treated with α-GalCer plus 30 or 50 mg/kg CsA compared with those of mice treated with α-GalCer alone.

α-GalCer-Induced Apoptosis of $NK1.1^+CD3^+$ Cells After Treatment with α-GalCer and CsA

Depletion of $NK1.1^+CD3^+$ cells among the LMNC and SC is already observed at three hours after the administration of α-GalCer, and most of the $NK1.1^+CD3^+$ cells become undetectable at 24 hours after the α-GalCer injection. Previous reports have shown that liver $NK1.1^+CD3^+$ cells from α-GalCer-treated mice exhibit a significant increase in annexin V binding at three hours after the injection compared to those from control mice (*6, 7*). This observation indicates that the rapid depletion of $NK1.1^+CD3^+$ cells after α-GalCer treatment is primarily due to apoptotic cell death. However, a recent study showed no significant increase in annexin V binding in liver $NK1.1^+CD3^+$ cells in α-GalCer-treated mice (*11*). Therefore, we tested whether liver NKT cells exhibited an increase in annexin V binding at three hours after the injection of α-GalCer and CsA (Table 2). The median fluorescence intensity (MFI) and percentage of annexin V-positive $NK1.1^+CD3^+$ cells in the LMNC of mice treated with CsA alone did not differ significantly from those in untreated mice. Moreover, the MFI and percentage of annexin V-positive $NK1.1^+CD3^+$ cells in the LMNC of mice treated with α-GalCer alone did not increase significantly compared with those in untreated mice. Similarly, there were no significant changes in the MFI values and percentages of annexin V-positive $NK1.1^+CD3^+$ cells of mice treated with α-GalCer plus 30 or 50 mg/kg CsA.

DISCUSSION

NKT cells have been characterized as cells that express an invariant Vα14 TCR together with the NK cell marker NK1.1. To date, a number of studies have reported on the unique characteristics and physiological functions of NKT cells (*28*). NKT cells recognize a glycolipid antigen, α-GalCer,

TABLE 2. Apoptosis of NKT cells after treatment with α-GalCer and CsA

Group	Treatment[a]		No. of mice	Analysis of annexin V binding in LMNC (means±SD)	
	α-GalCer (2 μg)	CsA (mg/kg)		Percent positive cells	Median fluorescence intensity
1	−	—	3	11.6±4.4	51.2±6.4
2	−	30	3	12.9±1.5[b]	46.2±16.5[b]
3	−	50	3	18.9±5.4[b]	55.4±11.4[b]
4	+	—	3	17.6±4.9[b]	63.1±7.4[b]
5	+	30	3	16.0±2.3[c,e]	61.9±4.4[c,e]
6	+	50	3	13.9±3.5[d,e]	55.9±7.2[d,e]

[a] Mice received CsA on day −1 and 0 and were injected with α-GalCer on day 0.
[b] No significant difference vs. Group 1.
[c] No significant difference vs. Group 2.
[d] No significant difference vs. Group 3.
[e] No significant difference vs. Group 4.

presented by CD1d. After this recognition, NKT cells rapidly produce large amounts of Th1 and Th2 cytokines (*5*). It has been reported that α-GalCer induces NKT cells to exert antitumor cytotoxicity and prevent the onset of autoimmune diseases. In transplant immunity, NKT cells also play a vital role in the induction of transplantation tolerance (*17, 18*). In addition, NKT cells activated by α-GalCer subsequently activate other cell types, such as NK cells, macrophages, CD4 T cells and CD8 T cells through their secretion of IFN-γ or IL-12, thereby promoting a functional bridge between innate and acquired immunity (*29*). NKT cells have potent immunoregulatory functions, and are therefore expected to be useful for new immunoregulatory protocols or medical therapies for the suppression of autoimmune diseases (*13, 14*). Indeed, administration of α-GalCer was found to prevent NOD mice from suffering autoimmune diabetes. However, the physiological functions and immune system roles of NKT cells are not yet completely understood. Therefore, studies on NKT cells are vigorously being pursued at the present time.

CsA is a very important immunosuppressive drug that is widely used to suppress graft rejection or treat autoimmune diseases in clinical practice. CsA binding to a cellular protein called cyclophilin blocks activation of the transcription factor, nuclear factor of activated T cells (NFAT) and the transcription of IL-2 and other cytokine genes (*30, 31*). As a result of these actions, CsA blocks IL-2-dependent growth and differentiation of T cells. On the other hand, CsA inhibits the development of mature single positive $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes and activation-induced cell death (AICD) in T cells (*32–34*). Consequently, CsA interferes with the deletion of autoreactive T cells during ontogeny in the thymus. These effects of CsA treatment induce the development of autoimmune diseases or auto graft-versus-host disease (GVHD) and increase tumor growth (*35, 36*). The achievement of tolerance induction requires AICD during the early period of induction protocols (*37, 38*). The administration of CsA and FK-506, which bind calcineurin and inhibit its activity in the early period of such protocols, interferes with the induction of tolerance (*39–41*). We also reported that administration of CsA interferes with cyclophosphamide-induced tolerance (*24*). However, it has not yet been reported whether CsA can modulate the immunoregulatory functions of NKT cells, which play essential roles in autoimmunity and transplantation immunity. Therefore, we investigated the effects of CsA administration on the production of large amounts of cytokines and the activation of DC by NKT cells. In other words, we investigated whether NKT cells, which regulate the onset of autoimmune diseases and the induction and maintenance of tolerance, show more or less intense immunological effects after CsA treatment. If NKT cells show reduced immunoregulatory functions, this implies that patients with autoimmune diseases or transplant recipients who are treated with CsA in clinical practice may also suffer from diminished functions of NKT cells in a similar manner.

T cells undergo AICD in the context of anti-CD3 stimulation (*42*). Some studies have shown that administration of α-GalCer results in rapid death of activated NKT cells by apoptosis within 24 hours, followed by their repopulation (*6, 7*). However, it has recently been reported that anti-CD3 mAb- and α-GalCer-induced apoptotic cell death of NKT cells is hard to be detected, and that this rapid disappearance is mainly caused by the downregulation of surface markers (*9–12*). In the current study, we observed that the percentage and absolute number of liver $NK1.1^+CD3^+$ cells rapidly decreased on day one after treatment with α-GalCer and then returned to the normal level by day four. After treatment with both α-GalCer and CsA, we found a similar rapid disappearance and repopulation of liver $NK1.1^+CD3^+$ cells to that observed following treatment with α-GalCer alone. Since the spleen $NK1.1^+CD3^+$ cells showed the same dynamics after administration of α-GalCer or α-GalCer and CsA, this indicates that CsA cannot prevent the α-GalCer-induced downregulation of $NK1.1^+CD3^+$ cells.

Next, we investigated the apoptosis of liver $NK1.1^+CD3^+$ cells after α-GalCer treatment. The apoptosis of liver $NK1.1^-CD3^+$ cells treated with α-GalCer did not differ significantly from that of untreated cells. After treatment with α-GalCer and CsA, the liver $NK1.1^+CD3^+$ cells undergo apoptosis, similar to the case for treatment with α-GalCer alone. To date, it has been reported that α-GalCer rapidly induces liver $NK1.1^+CD3^+$ cells to undergo apoptotic cell death (*6, 7*). However, some studies have reported that α-GalCer-induced apoptosis of $NK1.1^+CD3^+$ cells is not obviously detectable by FCM after staining with annexin V (*11*). In addition, the rapid disappearance of NKT cells after α-GalCer treatment is due not only to apoptotic cell death but

also to surface receptor downregulation (*10, 12*). In our study, CsA did not significantly suppress the apoptosis of $NK1.1^+CD3^+$ cells after α-GalCer treatment. However, it is possible that NKT cells which were undetectable by staining with anti-NK1.1 and CD3 mAbs were induced to undergo apoptosis after α-GalCer treatment. In mice treated with α-GalCer and CsA, the liver $NK1.1^+CD3^+$ cells disappeared on day one, were repopulated to the control level by day four and showed more intensive expansion by day seven than those in α-GalCer-treated mice. Spleen $NK1.1^+$ $CD3^+$ cells showed the same kinetics after treatment with α-GalCer and CsA. On the basis of these results, the expansion of $NK1.1^+CD3^+$ cells by day seven may indicate that CsA suppresses the apoptosis of $NK1.1^+CD3^+$ cells which were undetectable at three hours after the α-GalCer treatment as well as the apoptosis of other phenotypes of NKT cells after α-GalCer treatment.

It is interesting that the total numbers of LMNC and SC were markedly increased in mice on day four after treatment with α-GalCer and CsA. Since CsA inhibits AICD (*34*), we can speculate that this increase consisted of activated but undetectable NKT cells which escaped from apoptosis and bystander lymphocytes which were activated by NKT cell activation and escaped from apoptosis. Furthermore, there was a marked increase in the percentage of $NK1.1^-CD3^+$ cells on day four in the liver of mice treated with α-GalCer and CsA. Previous studies have shown that NKT cells rapidly downregulate NK1.1 and αβTCR until 24 hr after α-GalCer stimulation. Subsequently, re-expression of αβTCR occurs within two to three days after the treatment, but the NK1.1 molecule remains at a lower level for about seven days (*10, 11*). On the other hand, CsA inhibits AICD (*34*). Thus, we strongly suggest that the increased $NK1.1^-CD3^+$ cells may represent some NKT cells which are downregulated and escape from apoptosis. In the liver of mice treated with α-GalCer and CsA, NKT cells may start to re-express NK1.1 and CD3 on day seven and the number $NK1.1^+CD3^+$ cells may reach its peak. This increase in $NK1.1^-CD3^+$ cells on day four was not observed in the spleen. This discrepancy may be explained by the difference in the absolute numbers of NKT cells between the liver and spleen. Furthermore, there were dramatic increases in the total numbers of mononuclear cells in the liver and spleen on day 4 in mice treated with α-GalCer and CsA.

NKT cell development takes place in the thymus (*43–45*). Therefore, we used thymectomized mice to investigate the relationship between the thymus and the kinetics of NKT cells after α-GalCer treatment. When B6 mice are treated with an anti-CD3 mAb, NKT cells ($NK1.1^+CD3^+$) are rapidly depleted, followed by repopulation within two to three days, and the bone marrow is the major site of this NKT cell proliferation (*9*). In the current study, thymectomized mice showed similar depletion and repopulation of $NK1.1^+CD3^+$ cells to that observed in nonthymectomized mice. These data indicate that the repopulation of $NK1.1^+CD3^+$ cells in α-GalCer-treated mice is not dependent on the thymus.

After administration of α-GalCer, NKT cells produce large amounts of Th1 and Th2 cytokines. In tumor immunity, NKT cells play important roles mediated by IFN-γ (*7*). IL-4 produced by NKT cells is very important for preventing the occurrence of autoimmune diseases (*13, 14*). The results of the present study have shown that CsA completely suppresses the production of both Th1 and Th2 cytokines. Therefore, CsA interferes with the activation of innate cytotoxicity and acquired immunity of NKT cells. This suppression of the immunoregulatory functions of NKT cells may cause the onset of autoimmune diseases, induce progressive tumor growth and inhibit the induction of immunologic tolerance. These results imply that the effects on the immunological functions of NKT cells require careful consideration when immunosuppressive therapies with CsA are used for autoimmune diseases and organ transplantation. In the current study, the two doses of CsA (30 and 50 mg/kg) used had the same suppressive effects on the cytokine productions by NKT cells. If we can specifically suppress the production of either Th1 or Th2 cytokines by NKT cells, we can modulate the immunologic functions of NKT cells or DC and potentially develop new immunosuppressive methods or therapies.

Activation of NKT cells by α-GalCer induces the maturation of DC, thereby increasing their expressions of several surface markers. This maturation of DC induces not only the activation of T cells and B cells but also potent immunoregulatory functions via the activation of NKT cells after α-GalCer treatment. We investigated whether CsA modulated the expressions of $I\text{-}A^b$, CD40, CD80, and CD86 by DC after injection of α-GalCer, and found that the α-GalCer-induced maturation of surface markers on DC was diminished by CsA. There were no significant differences between the doses of CsA investigated. Therefore, these findings indicate that CsA interferes with the potent immunoregulatory functions of NKT cells, DC and other cells induced after α-GalCer treatment.

In conclusion, the present results indicate that CsA modifies the potent immunoregulatory functions of NKT cells induced by α-GalCer. CsA completely suppressed the productions of both Th1 and Th2 cytokines and DC maturation, but did not affect the decrease in $NK1.1^+CD3^+$ cells on day one. These findings provide us with important information regarding consideration of the influences of immunosuppressive drugs on NKT cell functions in clinical transplantation and autoimmune disease therapy.

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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.

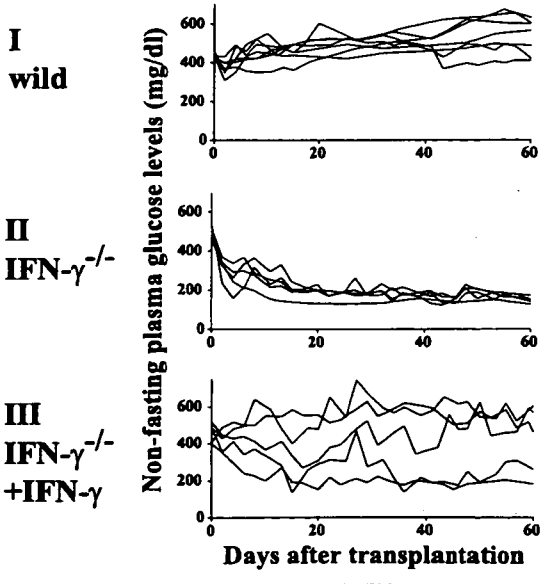

FIGURE 1. Deleterious effects of IFN-γ on the engraftment of islets in the liver of mice. Two hundred syngenic islets were grafted into the liver of STZ-induced diabetic wild-type (I) or IFN-γ-deficient (II and III) C57BL/6 mice. Human recombinant INF-γ (50,000 U) was administered into INF-γ-deficient 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^+CD$11b^+$ 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^+CD$11b^+$ cells in the liver after islet transplantation also occurred (Fig. 3, middle panel), but the TNF-α production of Gr-1^+CD$11b^+$ 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.

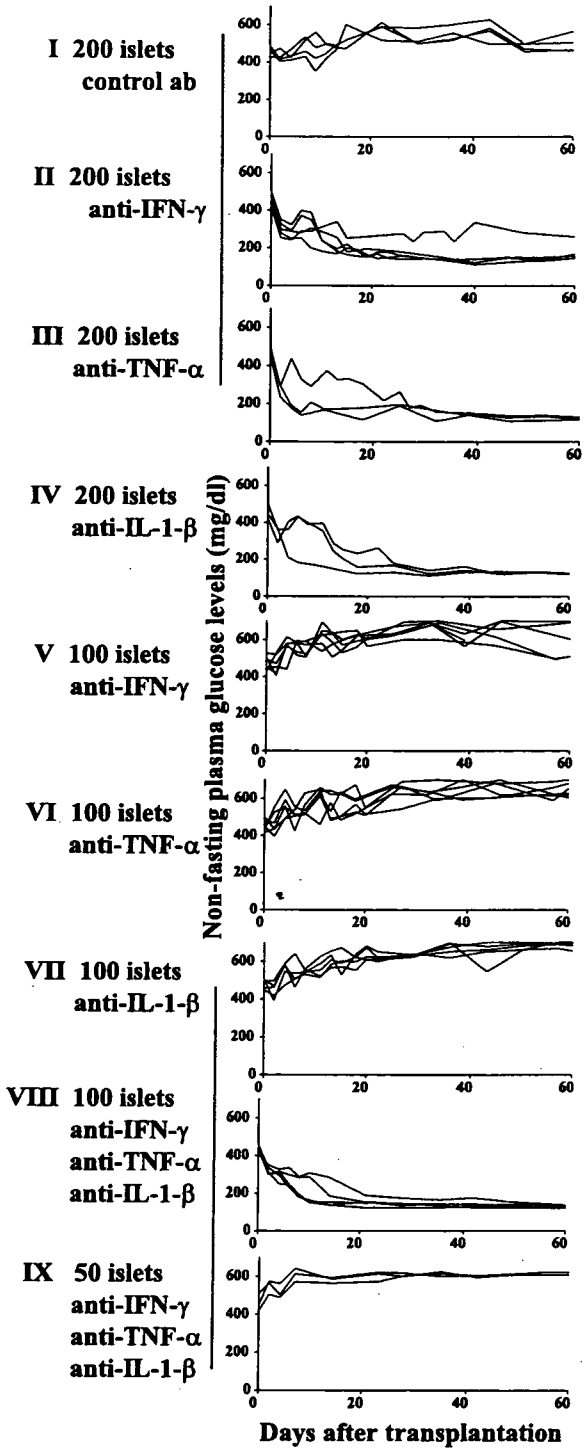

FIGURE 2. Beneficial effects of anti-proinflammatory cytokine antibodies on the amelioration of hyperglycemia in diabetic mice receiving 200 syngenic islets. Wild-type diabetic mice receiving syngenic 200 islets were treated with control (I), anti-IFN-γ (II), anti-TNF-α (III), or anti-IL-1β antibody (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.

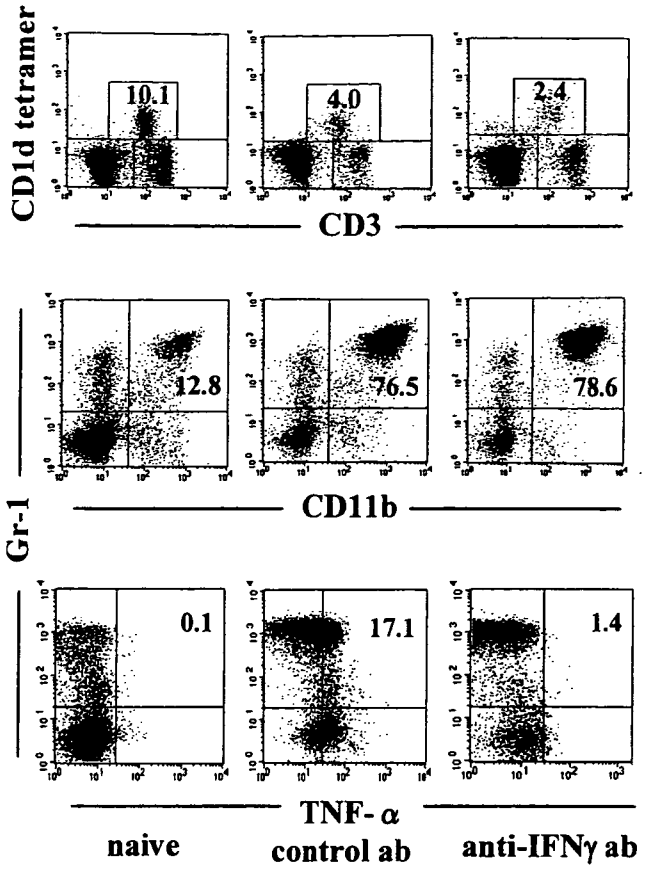

FIGURE 3. The downregulation of TNF-α production in Gr-1$^+$CD11b$^+$ cells accumulated in the liver of mice receiving islets and treated with anti-IFN-γ antibody. Mononuclear cells in the liver of naïve mice (left panel) were isolated and examined by flow cytometry. Mononuclear cells in the liver of 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.

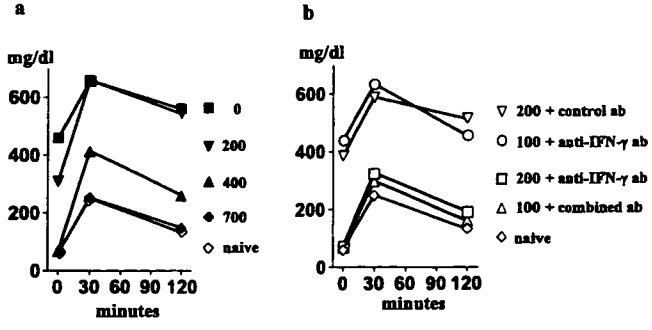

FIGURE 4. Intraperitoneal glucose tolerance test in mice. Intraperitoneal glucose tolerance test (IPGTT) in STZ-induced diabetic mice was performed at 60 days after islet transplantation. Mice were fasted for 8 hr prior to IPGTT and glucose (1g/kg) was injected IP. Blood samples were taken from the orbital 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-

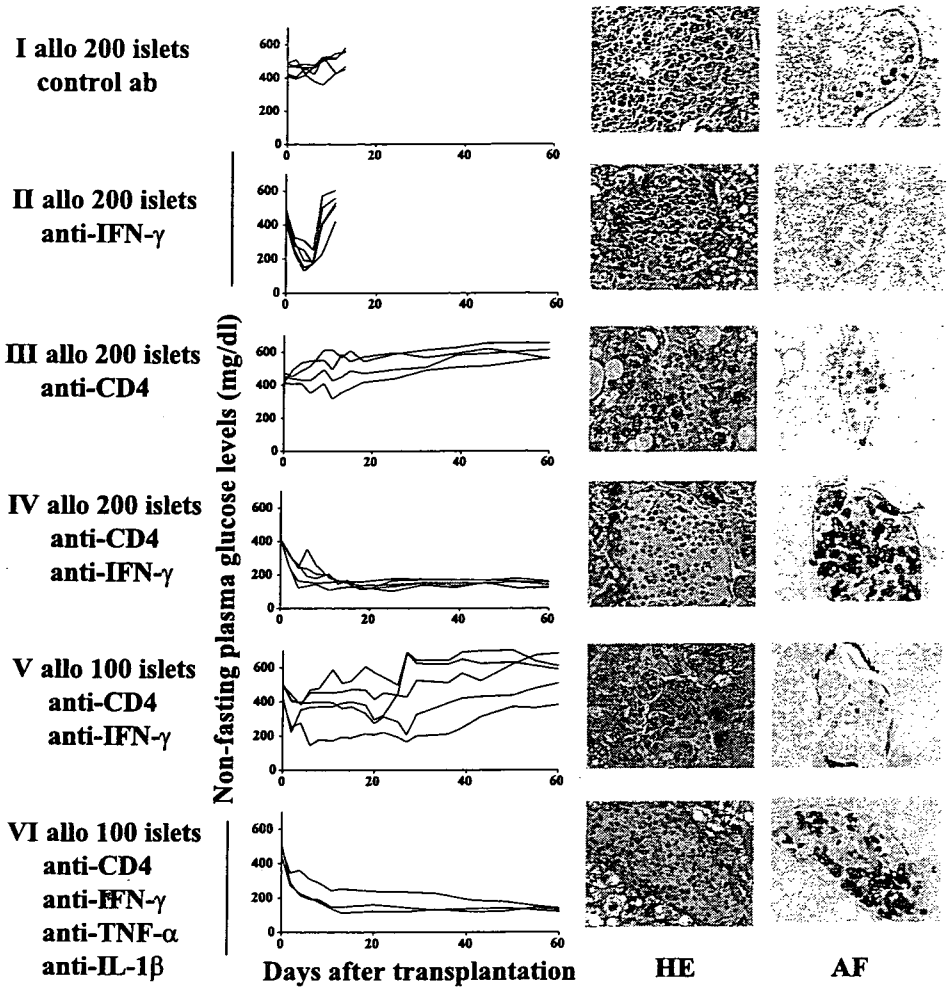

FIGURE 5. Effects of anti-proinflammatory cytokine antibodies on the engraftment of islet allografts. Two hundred (I, II, III, and IV) or 100 (V and VI) BALB/c islets were grafted into the liver of STZ-induced diabetic C57BL/6 mice. Nondepleting anti-CD4 antibody (200 μg/injection/mouse) was administered 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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