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# Direct Regulatory Role of NKT Cells in Allogeneic Graft Survival Is Dependent on the Quantitative Strength of Antigenicity<sup>1</sup>

Keunhee Oh,<sup>\*‡</sup> Sanghee Kim,<sup>‡</sup> Se-Ho Park,<sup>§</sup> Hua Gu,<sup>¶</sup> Derry Roopenian,<sup>||</sup> Doo Hyun Chung,<sup>†</sup> Yon Su Kim,<sup>2#</sup> and Dong-Sup Lee<sup>2\*‡</sup>

The role of NKT cells during immune responses is diverse, ranging from antiviral and antitumor activity to the regulation of autoimmune diseases; however, the regulatory function of CD1d-dependent NKT cells in rejection responses against allogeneic graft is uncertain. In this study, we demonstrated the direct regulatory effects of CD1d-dependent NKT cells using an allogeneic skin transplantation model. H-Y-mismatched skin graft survival was shortened in CD1d<sup>-/-</sup> recipients compared with wild-type recipients. Adoptive transfer of syngeneic NKT cells via splenocytes or hepatic mononuclear cells into CD1d<sup>-/-</sup> recipients restored graft survival times to those of wild-type recipients.  $\alpha$ -Galactosylceramide, a specific activator of NKT cells, further prolonged graft survival. Although CD1d-dependent NKT cells did not extend skin graft survival in either major or complete minor histocompatibility-mismatched models, these cells affected graft survival in minor Ag mismatch models according to the magnitude of the antigenic difference. The afferent arm of NKT cell activation during transplantation required CD1d molecules expressed on host APCs and the migration of CD1d-dependent NKT cells into grafts. Moreover, the regulatory effects of CD1d-dependent NKT cells against alloantigen were primarily IL-10 dependent. Taken together, we concluded that CD1d-dependent NKT cells may directly affect the outcome of allogeneic skin graft through an IL-10-dependent regulatory mechanism. *The Journal of Immunology*, 2005, 174: 2030–2036.

Natural killer T cells have been identified as a unique population of cells that express both TCRs and NK cell receptors. They secrete large amounts of IL-4 and IFN- $\gamma$  upon stimulation with their TCRs (1). The phenotypic characteristics of NKT cells include the expression of NK1.1, IL-2R $\beta$  (CD122), and memory/activated phenotype markers such as CD44<sup>high</sup>, CD69<sup>high</sup>, and Ly6C<sup>high</sup> (2). The majority of NKT cells use a highly biased and evolutionary conserved TCR repertoire (V $\alpha$ 14-J $\alpha$ 281 in mice and V $\alpha$ 24 in human) (3). As reported in previous studies, the activation of mouse NKT cells occurs by presentation of glycolipid on CD1d molecules (4, 5). Although the nature of the natural activating ligand remains an unanswered but important issue, a marine sponge-derived glycolipid,  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer),<sup>3</sup> potently activates NKT cells (6). The role

of NKT cells during immune response has been reported to be diverse, ranging from antiviral and antitumor activity (7–9) to the regulation of autoimmune diseases (10). The numbers of NKT cells were selectively reduced in autoimmune-prone mice in association with disease development (11), and the germline deletion of the *CD1* locus exacerbated disease in NOD mice (12), whereas repeated stimulation of NKT cells with  $\alpha$ -GalCer reduced disease severity (13, 14).

NKT cells have been exploited in several organ transplantation systems. They are critical for the induction of Ag-specific tolerance to xenogeneic islet cells, induced by anti-CD4 mAbs (15). NKT cells also mediate the tolerogenic action of anti-LFA-1 and anti-ICAM-1 Ab in an allogeneic heart graft model (16). However, the underlying mechanisms of their actions are unknown. NKT cells are crucial in corneal allograft survival (17), where they constitute a key component of anterior chamber-associated immune privilege (18, 19). However, the significance of CD1d-dependent NKT cells in the modulation of rejection responses against allogeneic transplantation is uncertain and may be less potent than that previously reported for CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (20, 21).

In this study, we investigated the direct role of CD1d-dependent NKT cells in skin allotransplantation by titrating their regulatory capacity. We demonstrate that the presence of CD1d-dependent NKT cells affects allograft survival and that these cells have differential regulatory capacities that are dependent on the magnitude of the antigenic differences.

## Materials and Methods

### Animals

CD1d-deficient mice on a C57BL/6 (B6, H-2<sup>b</sup>) background (designated as B6.CD1d<sup>-/-</sup>) were produced, bred, and maintained in specific pathogen-free conditions at the animal facility of the Clinical Research Institute of Seoul National University Hospital. CD1d-deficient mice on a BALB/c (H-2<sup>d</sup>) background were purchased from The Jackson Laboratory and

\*Laboratory of Immunology, Cancer Research Institute and †Department of Pathology, Seoul National University College of Medicine, Seoul, Korea; ‡Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul, Korea; §Graduate School of Life Sciences and Biotechnology, Korea University, Seoul, Korea; ¶Department of Microbiology, Columbia University Medical Center, New York, NY 10032; ||The Jackson Laboratory, Bar Harbor, ME 04609; #Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea; and ‡Xenotransplantation Research Center, Seoul National University Hospital, Seoul, Korea  
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<sup>2</sup> Address correspondence and reprint requests to Dr. Dong-Sup Lee, Laboratory of Immunology, Cancer Research Institute, Seoul National University College of Medicine, Chongno-Gu, Seoul 110, Korea. E-mail address: dlee5522@snu.ac.kr and Dr. Yon-Su Kim, Department of Internal Medicine, Seoul National University College of Medicine, Chongno-Gu, Seoul 110, Korea, E-mail address: yonsukim@snu.ac.kr

<sup>3</sup> Abbreviations used in this paper:  $\alpha$ -GalCer,  $\alpha$ -galactosylceramide; LN, lymph node; Ct, cycle threshold.

backcrossed from 129 to BALB/c mice to create third and fourth generation BALB/c background animals with multiple minor differences compared with wild-type BALB/c (designated BALB/c CD1d<sup>-/-</sup>.N3 and BALB/c CD1d<sup>-/-</sup>.N4, respectively). B6, B6.H-2<sup>bm1</sup> (bm1), B6.H-2<sup>bm12</sup> (bm12), B10.D2, BALB.B, and BALB/c mice were either purchased from The Jackson Laboratory or obtained by breeding at our Clinical Research Institute. H13- and H28-congenic mice were originally derived from a minor Ag of BALB/c origin. The animal protocol for experiments was reviewed and approved by the Ethics Committee of the Seoul National University.

#### Abs and flow cytometry

11B11 (anti-IL-4), R46-A2 (anti-IFN- $\gamma$ ), and PK136 (anti-NK1.1) Abs were purified from ascitic fluid by affinity chromatography. The following pairs of mAbs for detecting mouse cytokines were purchased from BD Pharmingen; 11B11 and biotinylated BVD6-24G2 for IL-4, R4-6A2, and biotinylated XMG1.2 for IFN- $\gamma$ , and biotinylated SXC-1 for IL-10. The following mAbs were used for FACS staining: PE-Cy5(Cy5)-conjugated anti-CD4(H129.19), FITC- and R-PE-conjugated anti-CD8 (53-6.7), PE-anti-CD25 (PC61), PE-anti-CD44 (IM7), PE-anti-CD45RB (23G2), PE-anti-CD62L (MEL-14), PE-anti-CD69 (H1.2F3), PE-anti-NK1.1 (PK136), FITC-anti-CD1d (1B1), and Cych-anti-TCR $\beta$  (H57-597). These mAbs were purchased from BD Pharmingen.

Draining axillary lymph node (LN) and splenic cell suspensions from graft recipients were stained for T cell activation using standard procedures as previously described (22). In brief, early activation was monitored using anti-CD25 and anti-CD69 Abs. CD44, CD45RB, and CD62L staining were also included to monitor memory/activated T cells.

#### Skin graft

Donor tail skin was grafted as previously described (23). Briefly, recipient mice anesthetized with 3-bromoethanol were grafted with an  $\sim 5 \times 6$ -mm piece of donor tail skin onto the left lateral thoracic region. In most cases, single pieces of skin from two different donors were grafted alongside each other. Tapes were removed 8 days after grafting and the grafts were observed daily for up to 60 days. Some of the host mice were injected i.p. with 6  $\mu$ g of  $\alpha$ -GalCer 7 and 3 days before grafting and twice weekly after grafting. Some of the recipient mice were treated with either IL-10 blocking or IL-4 blocking Ab twice per week, starting 7 days before receiving the grafts.

#### Synthesis of $\alpha$ -GalCer

The  $\alpha$ -anomeric form of galactosylceramide ( $\alpha$ -GalCer) was synthesized using the method developed by Kim et al. (24) and dissolved in PBS containing 0.5% Tween 20 at a concentration of 220  $\mu$ g/ml.

#### Adoptive transfer of splenic lymphocytes and hepatic mononuclear cells

Recipient B6.CD1d<sup>-/-</sup> mice were lightly irradiated (600 rad), and after 1 day were injected i.v. with  $1.2 \times 10^8$  splenocytes from wild-type B6 mice. Skin grafting was performed 7 days later. Briefly,  $3.5 \times 10^6$  wild-type hepatic mononuclear cells were injected i.v. into some unirradiated recipients (B6.CD1d<sup>-/-</sup>) 3 days before skin transplantation.

#### RT-PCR for cytokines and *V $\alpha$ 14*

Spleens, LNs, and grafted skin were frozen immediately upon removal. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies), and 2  $\mu$ g of the total RNA was reverse transcribed into cDNA using Moloney murine leukemia virus-reverse transcriptase (Promega) and random primer. Primers used for the PCRs of IL-10, TGF- $\beta$ , and IFN- $\gamma$  have been previously described (25, 26). The sequences of the primers used for the PCR of TCR *V $\alpha$ 14* and GAPDH were as follows: *V $\alpha$ 14* sense, 5'-CTAAGCACA GCACGCTGCACA-3' and anti-sense, 5'-AGGTAT GACAATCAGCTGAGTCCC-3'; and GAPDH sense, 5'-CCCACTAA CATCAAATGGGG-3' and antisense, 5'-ATCCACAGTCTTCTGGGTGG-3'. PCRs were performed in 20- $\mu$ l reaction volumes over 35 amplification cycles (45 s at 95°C, 45 s at 62°C, and 45 s at 72°C).

#### Real-time PCR for cytokines

The relative mRNA expressions of cytokine genes were quantified using real-time PCR. 18S ribosomal RNA was used as an internal standard to estimate variation between samples. All primers and probes used for PCR have been described previously (27). The primer sets used are listed in Table I. PCR was performed in a 25- $\mu$ l reaction volume containing 3  $\mu$ l of cDNA, 900 nM each of sense and antisense primers, 250 nM each of labeled probes for cytokine and 18S rRNA, and 12.5  $\mu$ l of TaqMan universal PCR master mix (Roche). Relative increases in reporter fluorescent dye emission were monitored in real time during PCR amplification using the ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems). Levels of cytokine mRNA were calculated using (26) this formula: relative mRNA expression =  $2^{-(Ct \text{ of cytokine} - Ct \text{ of 18S rRNA})} \times 10^{10}$ .

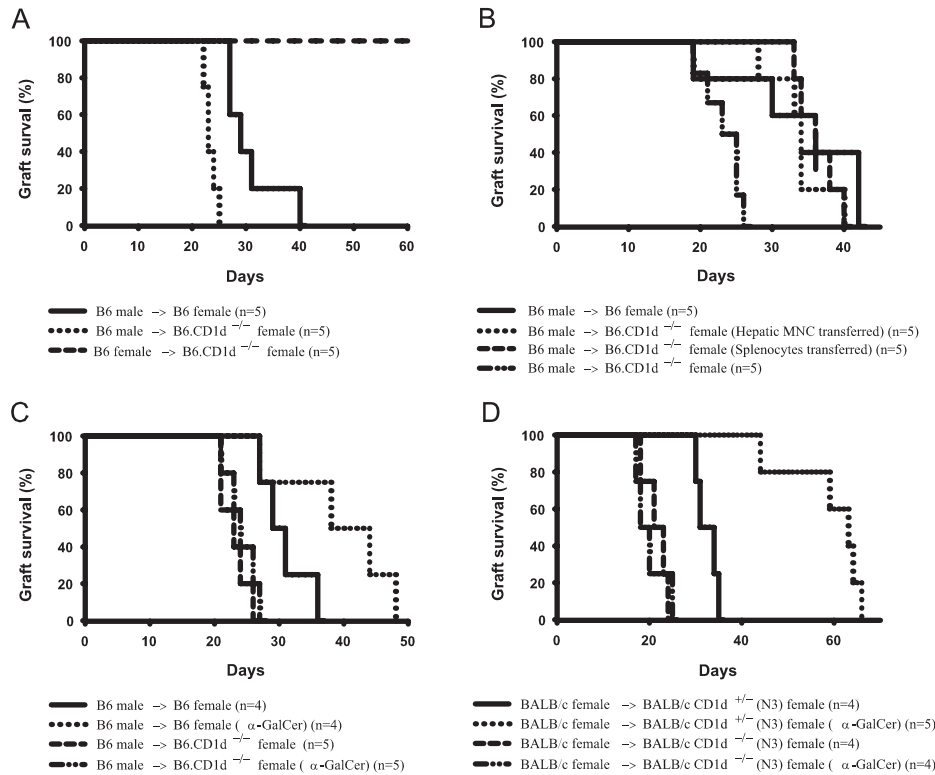
## Results

### CD1d-dependent NKT cells directly affected the outcome of allogeneic skin graft transplantation

To address the direct role of CD1d-dependent NKT cells on allograft rejection, we compared the survival of whole-thickness skin grafts (B6 male) between wild-type B6 and B6.CD1d<sup>-/-</sup> female recipient mice, where male H-Y Ag could elicit graft rejection in the recipients (28). B6.CD1d<sup>-/-</sup> mice rejected H-Y different skin grafts earlier than wild-type B6 mice (mean survival time: 24 vs 30 days, Fig. 1A). In B6.CD1d<sup>-/-</sup> mice, skin grafts were rejected rapidly, showing early scab formation and complete graft loss, whereas grafts in wild-type B6 mice were rejected gradually, showing shrinkage and fibrosis. The shortened graft survival in B6.CD1d<sup>-/-</sup> mice was restored by the adoptive transfer of normal lymphocytes containing NKT cell populations. When  $1 \times 10^8$  splenocytes from wild-type B6 mice were transferred into lightly irradiated (600 rad) B6.CD1d<sup>-/-</sup> recipients 7 days before transplantation, graft survival time were restored to that of the wild-type mice (Fig. 1B). Similar results were observed when  $3.5 \times 10^6$  hepatic mononuclear cells were transferred into nonirradiated CD1d<sup>-/-</sup> mice 3 days before transplantation (Fig. 1B).

Table I. Primer sequences

Gene	Sequence
IL-4	Probe 5'-FAM CACAGGAGAAGGGACGCCATGCA TAMRA-3'
	Sense 5'-CATCGGCATTTT GAA-3'
	Antisense 5'-CGTTTGGCACATCCATCTCC-3'
IL-10	Probe 5'-FAM TGAAGACCCTCAGGATGCGGGCTG TAMRA-3'
	Sense 5'-TTTGAATTCCTGGGTGAGAA-3'
	Antisense 5'-ACAGGGGAGAAATCGATGACA-3'
IFN- $\gamma$	Probe 5'-FAM CCTCAAACCTGGCAATACTCATGAATGCATCC TAMRA-3'
	Sense 5'-AGCAACAGCAAGGCGAAAA-3'
	Antisense 5'-CTGGACCTGTGGTTGTGTTGA-3'
TGF- $\beta$	Probe 5'-FAM ACCTTGGTAACCGGCTGCTGACCC TAMRA-3'
	Sense 5'-GCAACATGTGGAACCTACCAGAA-3'
	Antisense 5'-GACGTCAAAGACAGCCACTCA-3'
18S rRNA	Probe 5'-VIC TGCTGGCACCAGACTTGCCCTC TAMRA-3'
	Sense 5'-CGGCTACCACATCCAAGGAA-3'
	Antisense 5'-GCTGGAATTACCGCGGCT-3'



**FIGURE 1.** CD1d-dependent NKT cells affect skin allograft survival. *A*, To compare the survival of skin grafts on wild-type B6 and B6.CD1d<sup>-/-</sup> mice, the tail skin of wild-type B6 male mice was grafted onto wild-type B6 or B6.CD1d<sup>-/-</sup> female mice. Skin grafting from wild-type B6 female to B6.CD1d<sup>-/-</sup> female mice was used as a control. *B*, The repopulation of NKT cells corrected the NKT-deficient phenotype. Wild-type B6 and B6.CD1d<sup>-/-</sup> female mice were grafted with wild-type B6 male skin. Some B6.CD1d<sup>-/-</sup> recipients also received splenic or hepatic cells, including NKT cells, from wild-type B6 mice. Briefly,  $1 \times 10^8$  splenocytes from wild-type B6 mice were transferred into lightly irradiated (600 rad) B6.CD1d<sup>-/-</sup> female mice. Skin grafting was performed 7 days later. Some nonirradiated B6.CD1d<sup>-/-</sup> female mice received  $3.5 \times 10^6$  hepatic mononuclear cells from wild-type B6 mice 3 days before skin transplantation. *C*, Wild-type B6 and B6.CD1d<sup>-/-</sup> female mice were grafted with wild-type B6 male skin, and a number of B6 and B6.CD1d<sup>-/-</sup> recipients were injected i.p. with 6  $\mu$ g of  $\alpha$ -GalCer or vehicle, twice per week during the transplantation period, starting 7 days before transplantation. *D*, BALB/c CD1d<sup>+/-</sup> (N3) female and BALB/c CD1d<sup>-/-</sup> (N3) female mice were grafted with wild-type BALB/c female skin. Some recipients were injected i.p. with 6  $\mu$ g of  $\alpha$ -GalCer or vehicle, twice per week during the transplantation period, starting 7 days before transplantation. CD1d-deficient mice on a BALB/c (H-2<sup>d</sup>) background were generated by backcrossing 129  $\times$  BALB/c (N2) mice with BALB/c mice to produce third and fourth backcross generations (designated BALB/c CD1d<sup>-/-</sup>.N3 and BALB/c CD1d<sup>-/-</sup>.N4, respectively). Five to six mice per group were used. Experiments were repeated three times with similar results.

The specificity of the immunomodulatory effect of CD1d-dependent NKT cells was further confirmed by treating recipient mice with  $\alpha$ -GalCer, a specific activator of CD1d-dependent NKT cells. When we treated wild-type female recipients with  $\alpha$ -GalCer before and after transplantation (6  $\mu$ g/mouse at -7, -4, 0, 3, and 7 days of operation and twice a week), the survival of male skin grafts was prolonged vs nontreated control recipients ( $\alpha$ -GalCer-treated B6,  $40 \pm 9.1$  days; vehicle-treated B6,  $32 \pm 3.9$ ;  $\alpha$ -GalCer-treated CD1d<sup>-/-</sup>,  $25 \pm 2.4$ ; vehicle-treated CD1d<sup>-/-</sup>,  $24 \pm 2.1$ , Fig. 1C). The effect of  $\alpha$ -GalCer administration was limited to wild-type recipients, i.e., it had no effect in CD1d<sup>-/-</sup> recipients (Fig. 1C).

The effect of CD1d-dependent NKT cells on allograft survival was not restricted to B6 background recipients. Survival of grafted BALB/c tail skin on CD1d<sup>-/-</sup> mice of mixed 129  $\times$  BALB/c background (either N3 or N4 in terms of BALB/c background, designated hereafter as BALB/c CD1d<sup>-/-</sup>.N3 or BALB/c CD1d<sup>-/-</sup>.N4) was shortened compared graft survival on heterozygous littermate recipients, and  $\alpha$ -GalCer treatment prolonged skin graft survival on heterozygous littermate recipients (Fig. 1D).

#### CD1d-dependent NKT cells regulated graft rejection induced by a relatively weak Ag

To evaluate the notion that the regulatory effects of CD1d-dependent NKT cells are confined to minor histocompatibility antigenic differences, we investigated the immunomodulatory capacity of these cell populations. When we grafted MHC-different skin (BALB.B) onto BALB/c background mice, the grafts were rejected within 17 days both by BALB/c CD1d<sup>-/-</sup> mice and heterozygous littermates, and repeated injections of  $\alpha$ -GalCer did not prolong graft survival. Also, complete minor different B10.D2 skins were rejected with the same kinetics in both recipients (data not shown). To address the hypothesis that quantitative antigenic strength might be one of the critical factors affecting the regulatory functions of NKT cells in allograft rejection, we used different congenic mice on the B6 background that bear single minor histocompatibility Ags from BALB.B mice. Since it has been reported that minor histocompatibility Ags have immunologic hierarchy, i.e., H28>H13>H-Y, as defined by cytotoxic assay (29), we used this system to evaluate the regulatory capacity of NKT cells. CD1d-dependent NKT cells affected the graft survival of

H-Y (Fig. 2A), H13 (Fig. 2B), and H-Y and H13 (Fig. 2C) differences, but did not modulate that of H28-different skin grafts (Fig. 2D). In conclusion, we favor the idea that CD1d-dependent NKT cells affect immune responses regarding weak antigenic differences.

#### Activation and migration of NKT cells during skin transplantation

Because the mechanism through which CD1d-dependent NKT cells modulate graft survival is unclear, we examined the role of CD1d molecules expressed on donor Langerhans cells. Skin grafts from B6.CD1d<sup>-/-</sup> male mice were transplanted onto wild-type B6 female mice. In this case, we found that the expression of CD1d molecules on donor skin cells was not required for NKT cell activation (Fig. 3A). Since immune response during skin graft rejection is mostly confined within the draining LNs, the migration of NKT cells into the draining LNs was assessed using flow cytometric analysis. The absolute numbers of NKT cells were increased in the draining axillary LNs after transplantation (Fig. 3B), and NKT cell migration into the skin graft was confirmed using V $\alpha$ 14-specific RT-PCR (Fig. 3C) and real-time quantitative RT-PCR (data not shown). Indeed, we were able to detect V $\alpha$ 14-specific mRNA from grafted skin, but not from control skin. In addition, V $\alpha$ 14-specific mRNA was detected in the donor skin of B6.CD1d<sup>-/-</sup> mice, suggesting that CD1d-dependent NKT cells from recipients migrated into the donor skin graft, even in the absence of CD1d molecules in donor skin cells.

#### Regulatory effects of CD1d-dependent NKT cells are IL-10 dependent

To evaluate the notion that CD1d-dependent NKT cells contribute to the regulation of allograft survival by changing immunosuppressive cytokine profiles, we measured the mRNA expression of draining LNs from recipient mice. In a skin graft model of B6 male

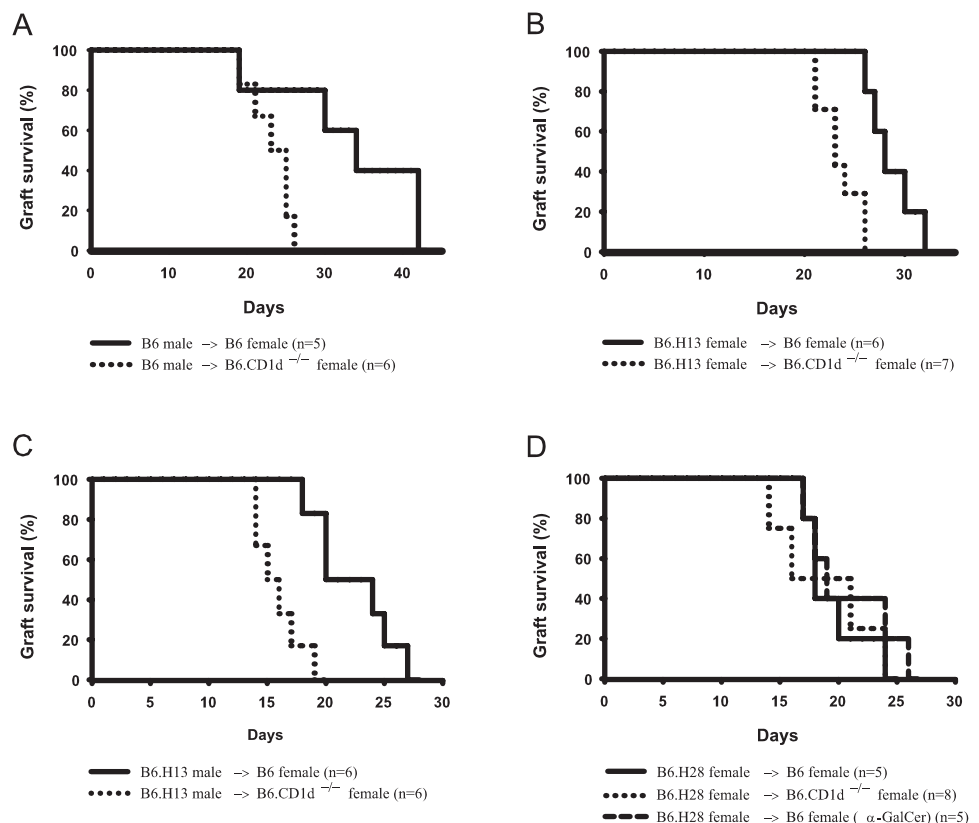
to B6 female or B6.CD1d<sup>-/-</sup> female mice, the transcription level of IL-10 was found to be higher in B6 recipients than in CD1d<sup>-/-</sup> B6 recipients (Fig. 3D), and similar patterns of cytokine milieu were confirmed by real-time quantitative RT-PCR using the TaqMan system (data not shown). After multiple injections of  $\alpha$ -GalCer, the cytokine profile was exaggerated showing IL-10 up-regulation and minimal IFN- $\gamma$  expression (Fig. 4). Blocking IL-10 pathways shortened the survival times of skin grafts in wild-type mice and prevented the prolongation of graft survival induced by  $\alpha$ -GalCer treatment (Fig. 5).

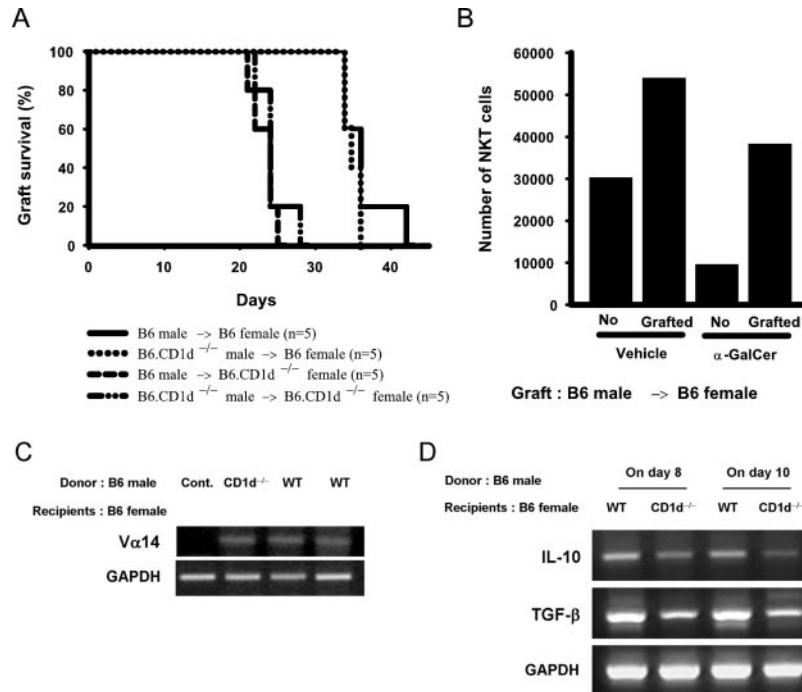
## Discussion

In the current study, we undertook to examine the distinctive role of NKT cells in alloimmune responses using a murine transplantation model. We found that CD1d-dependent NKT cells affect allogeneic skin graft survival and that this immunoregulatory property of CD1d-dependent NKT cells depends on the antigenic strength of the transplantation barrier. Furthermore, the migration of NKT cells into skin grafts and the secretion of cytokines were identified as mechanistic routes for the down-regulation of alloimmunity.

CD1d-dependent NKT cells have been identified as a novel lymphocyte lineage and are characterized by the expression of an invariant V $\alpha$ 14 Ag receptor and NK1.1 marker (30, 31). These cells play critically distinctive roles in immune responses such as in the maintenance of peripheral tolerance (32), transplantation tolerance (15), and protection from autoimmune disease (13). However, the direct role of CD1d-dependent NKT cells in a murine allograft model was uncertain, and differential immunomodulation capacities according to the degree of antigenicity have not been exploited thoroughly. The present study shows that the alloantigenic differences in which CD1d-dependent NKT cells can affect the outcome of skin graft survival are confined to several minor differences. The

**FIGURE 2.** CD1d-dependent NKT cells regulate the graft rejection induced by a relatively weak Ag. Wild-type B6 female and B6.CD1d<sup>-/-</sup> female mice were grafted with B6 male skin (A), B6.H13 female skin (B), B6.H13 male skin (C), and B6.H28 female skin (D). Some recipients were injected i.p. with 6  $\mu$ g of  $\alpha$ -GalCer or vehicle, twice per week during the transplantation period, starting 7 days before transplantation. H13- and H28-congenic mice carry a minor Ag of BALB/c origin. Five to six mice per group were used. Experiments were repeated three times with similar results.





**FIGURE 3.** Activation and migration of NKT cells during skin transplantation. *A*, The activation of NKT cells does not require donor CD1d molecule expression. Wild-type B6 female mice were grafted with wild-type B6 male and B6.CD1d<sup>-/-</sup> male skin. B6.CD1d<sup>-/-</sup> female mice were grafted with wild-type B6 male and B6.CD1d<sup>-/-</sup> male skin. Five to six mice per group were used. Experiments were repeated three times with similar results. *B*, The number of NKT cells in the draining LNs was increased by allogeneic skin transplantation and α-GalCer treatment. Wild-type B6 female mice were injected i.p. with 6 μg of α-GalCer or vehicle at -7, -4, 0, 3, and 7 days postoperation. Some mice were grafted with B6 male skin. Draining axillary LN cells were harvested from grafted and nongrafted mice 6 h after the last injection of α-GalCer on day 7 following the skin transplantation, stained for αβTCR and NK1.1, and analyzed by flow cytometry. The number of NKT cells was calculated by counting αβTCR<sup>+</sup>NK1.1<sup>+</sup> cells. The data shown represent one of five independent experiments. *C*, NKT cells migrate into grafted skin. B6 female mice were grafted with wild-type B6 male skin or B6.CD1d<sup>-/-</sup> male skin. Grafted skins were removed 7 days after skin transplantation. The frequencies of skin-infiltrating NKT cells were estimated by RT-PCR for TCR Vα14 expression. *Lane 1*, Nongrafted control; *lane 2*, B6.CD1d<sup>-/-</sup> male graft; *lanes 3 and 4*, wild-type B6 male graft. These data represent one of five independent experiments. *D*, Wild-type B6 female and B6.CD1d<sup>-/-</sup> female mice were grafted with wild-type B6 male skin. Recipients were sacrificed on days 8 and 10 after skin transplantation. Total RNA was extracted from spleen cells and the mRNA expression levels of IL-10 and TGF-β1 were measured. One microgram of total RNA was reverse-transcribed into cDNA. Three microliters of cDNA was used for PCR. The data shown are representative of five independent experiments.

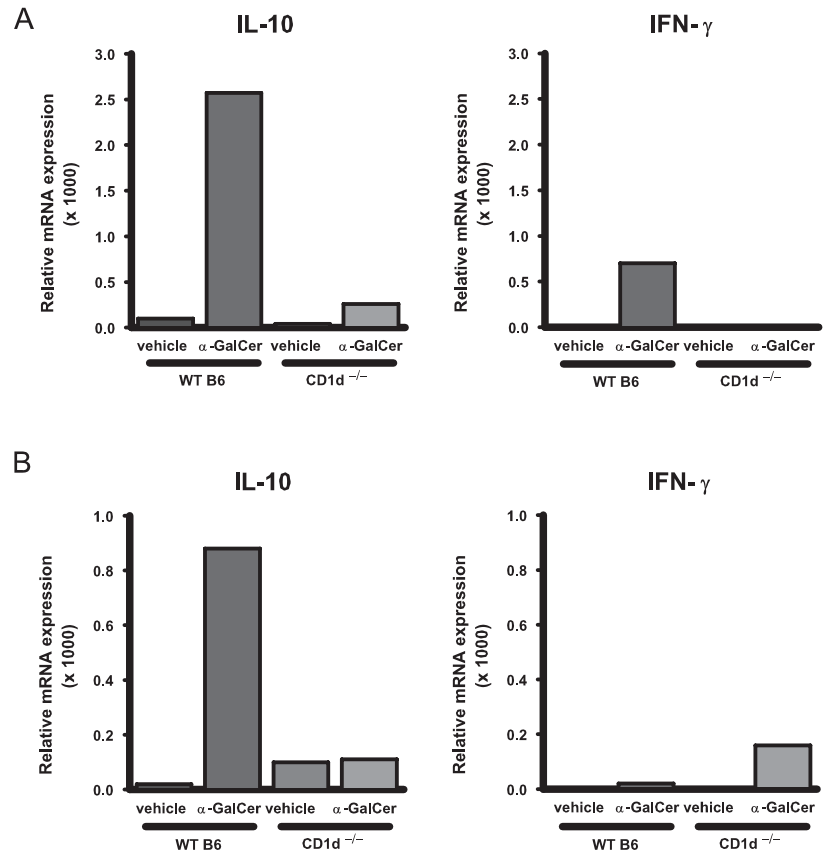
regulatory capacity of NKT cells, however, may not be trivial considering that skin grafts are considered to be highly vulnerable to rejection compared with pancreas islet or cardiac grafts (33). Skin grafts provide the strongest immune stimulus because of differences in the mode of vascularization, the presence of tissue-specific Ags, the number of APCs in the graft, and the graft size. Previous reports regarding the role of CD1d-dependent NKT cell populations in allo- or xenogeneic transplantation have shown that the beneficial effects for grafts are mediated either with the aid of costimulatory molecular blockades or in the lymphopenic hosts where aberrant activation of lymphocytes occurs (15, 16, 34). Also, in clinical transplantation where minor histocompatibility Ags are the major targets of chronic rejection and graft-versus-host disease, the regulatory capacity of NKT cells over minor histocompatibility antigenic differences might affect the outcome of the disease process.

In our experiments, NKT cells were activated by CD1d molecules expressed on the surface of host APCs. However, the ligand required for NKT cell activation during the transplantation process is unknown. As in hapten-mediated contact dermatitis (35), mediators from the grafted skin could affect the remote NKT cell population by some uncharacterized pathway or rather the process of transplantation itself might act as a so-called “danger” signal that delivers an alarm to hosts and thus activates NKT cells. These two possibilities are not mutually exclusive. In the case of tissue dam-

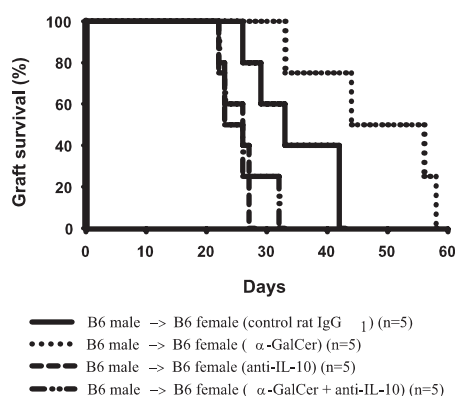
age due to injury, surgery, or others, many leukocytes are recruited into the injured site to remove tissue debris and aid the healing process. But an excessive response must be controlled to prevent an overwhelming response by potentially harmful activated leukocytes. Several lipid molecules produced during tissue damage have been suggested to behave as an endogenous danger signal for the immune system (36). Another possibility is that the endogenous self-ligand might be differentially presented to NKT cells in a CD1d-dependent manner and thus initiate the activation of NKT cells through endogenous self-ligand (37, 38). In our model, CD1d-dependent NKT cells migrated into draining LNs and target tissue, thus NKT cells could intimately affect alloreactive T cells during the initiation and effector phases of alloimmune response. We propose that the modes of immune regulation during allotransplantation are similar between CD1d-dependent NKT cells and regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells (21).

In an attempt to evaluate whether the migration of CD1d-dependent NKT cells is associated with the rejection of allogeneic grafts, we measured mRNAs specific for Vα14. As shown in Fig. 3C, we were able to detect mRNAs specific for Vα14 from the skin grafts regardless of the presence of CD1d molecules in the skin. Indeed, mRNAs for Vα14 were detectable in the grafts from CD1d<sup>-/-</sup> donors. This result suggests that the recipient CD1d-dependent NKT cells migrate into grafts and is consistent with the

**FIGURE 4.** The regulatory effects of CD1d-dependent NKT cells were primarily IL-10 dependent. Wild-type B6 female and B6.CD1d<sup>-/-</sup> female mice with no graft (A) and with B6 male skin grafts (B) were injected i.p. with 6  $\mu$ g of  $\alpha$ -GalCer or vehicle at -7, -4, 0, 3, and 7 days postoperation. Grafted and nongrafted mice were sacrificed 6 h after a final injection of  $\alpha$ -GalCer 7 days after skin transplantation. Total RNA was extracted from spleen cells. Each RNA sample was tested separately for real-time cytokine mRNA expression. One microgram of total RNA was reverse-transcribed into cDNA and tested by real-time PCR for IL-10 and IFN- $\gamma$ . The relative expression levels of cytokines were normalized vs 18S rRNA. The data shown are representative of three independent experiments.



finding that NKT cells act in peripheral tissue rather than in secondary lymphoid organs, because of the higher chemokine receptor expression on their surfaces (39). Cytokine production from CD1d-dependent NKT after multiple  $\alpha$ -GalCer injection showed a similar pattern to that of other regulatory T cells (40). Of the various cytokines, IL-10 is known to inhibit cytokine production from T cells, to exert anti-inflammatory and suppressive effects on most hemopoietic cells, and to be involved in the induction of peripheral tolerance via effects on T cell-mediated responses (23). IL-10 indirectly suppresses T cell responses by potently inhibiting the Ag-presenting capacity of APCs, which include dendritic cells (41), Langerhans cells, and macrophages (25). We found that multiple



**FIGURE 5.** Wild-type B6 female mice were grafted with wild-type B6 male skin. Some recipients were injected i.p. with 6  $\mu$ g of  $\alpha$ -GalCer and/or 50  $\mu$ g of anti-IL-10 blocking mAb (JES5-2A5) twice per week during the transplantation period, starting 7 days before the operation. Irrelevant isotype-matched Ab was used in the controls. Five to six mice per group were used. This experiment was repeated three times with similar results.

$\alpha$ -GalCer injections raised IL-10 and TGF- $\beta$  production (our unpublished data) in wild-type mice, but not in CD1d<sup>-/-</sup> mice. Moreover, blocking the IL-10 pathway inhibited the beneficial effect of NKT cells on graft survival. When we measured cytokine levels after  $\alpha$ -GalCer treatment, either in combination with blocking anti-IL-4 Ab or using IL-4-deficient mice, IL-10 secretion was found to have increased (our unpublished data), which is contrary to that reported previously (42). Thus, Th2 deviation after multiple  $\alpha$ -GalCer injections may explain the regulatory effects of NKT cells in our study. In fact, when we grafted BALB/c islet grafts into B6 mice (fully MHC mismatched; H-2<sup>d</sup> → H-2<sup>b</sup>), islet survival was increased from 10 to 30 days by repeated  $\alpha$ -GalCer injection (our unpublished data). The BALB/c strain is likely to produce Th2-associated cytokines, and in the case of *Leishmania* infection could not provide protective immunity due to insufficient Th1 development (43). On the B6 background, multiple injections of  $\alpha$ -GalCer shifted the cytokine profile toward a Th2 pattern. Large amounts of IL-10 and TGF- $\beta$  were also previously demonstrated in NOD and B6 experimental autoimmune encephalomyelitis models (13, 14). On the BALB/c background, however, cytokine secretion was predominantly Th2-like, even after a single injection of  $\alpha$ -GalCer (our unpublished data). This may be one of the reasons why the protective effects of NKT cells and  $\alpha$ -GalCer were more pronounced on the BALB/c background. Although our studies performed with CD1d<sup>-/-</sup> recipients cannot formally exclude a contribution by CD1d-dependent non-NKT cell populations (5), our data obtained with  $\alpha$ -GalCer clearly shows the involvement of CD1d-dependent NKT cells in improved graft survival. We are currently investigating the possibility of promoting another population of regulatory T cells by activating CD1d-dependent NKT cells in an allogeneic transplantation environment.

To our knowledge, this is the first report that distinct NKT cells may lead to the development of differential effects on alloimmune

responses and that their stratified regulatory capacities are related to alloantigenic strength. We believe that an understanding of the manner in which these cells work will have implications for the induction of donor-specific allograft tolerance and suggest bases for cell therapy as dependable therapeutic modalities.

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