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Down-regulation of the invariant Vα14 antigen receptor in NKT cells upon activation

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Abstract

NKT cells expressing the invariant Vα14 antigen receptor constitute a novel lymphocyte subpopulation with immunoregulatory functions. Stimulation via their invariant Vα14 receptor with anti-CD3 or a ligand, α-galactosylceramide (α-GalCer), triggers activation of Vα14 NKT cells, resulting in a rapid cytokine production such as IFN-γ and IL-4. Soon after their receptor activation, Vα14 NKT cells disappeared as judged by staining with CD1d tetramer loaded with α-GalCer (α-GalCer/CD1d tetramer), which has been believed to be due to apoptotic cell death. Here we show that such a disappearance was largely attributed to down-regulation of the Vα14 receptor. In fact, Vα14 NKT cells were relatively resistant to apoptosis compared to the conventional T cells as evidenced by less staining with Annexin-V, a limited DNA fragmentation, and their preferential expression of anti-apoptotic genes such as NAIP and MyD118. Furthermore, they did not become tolerant, and maintained their proliferative capacity and cytokine production even after their receptor down-regulation. These as yet unrecognized facets of Vα14 NKT cells are discussed in relation to their regulatory functions.

Introduction

Effector T cells are activated and proliferate to initiate immune responses upon antigen stimulation, whereas in certain milieux, they become anergic or doomed to die by apoptosis (1–3). In contrast, regulatory T cells, such as $CD4^+CD25^+$ cells that mediate immunosuppressive functions, have been shown to be relatively resistant to apoptosis induced by TCR stimulation (4). Moreover, stimulation of a co-receptor molecule, CTLA-4, elicits negative signals and results in the suppression of effector T cell functions, while it leads to the activation of $CD4^+CD25^+$ regulatory T cells (2,5,6). These unique features of effector and regulatory cells can be explained by cell-type-specific outcomes in response to extracellular stimuli, such as TCR engagement and/or cytokines.

Vα14 NKT cells, another type of immunoregulatory cell, possess solely an invariant Vα14 antigen receptor, encoded by Vα14 and Jα281 gene segments, that is reactive to a glycolipid antigen, α-galactosylceramide (α-GalCer), presented by CD1d (7–10). Vα14 NKT cells play crucial immunoregulatory roles in a variety of immune responses, including protection from autoimmune disease development, maintenance of transplantation tolerance and immunological surveillance for tumors [reviewed in (11)]. However, little is known about the molecular events operating in activated Vα14 NKT cells and about the destiny of Vα14 NKT cells upon stimulation. Understanding the comportment of Vα14 NKT cells after activation will help to elucidate the mechanisms

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through which Vα14 NKT cells exert regulatory functions in the immune system.

In this report we show that Vα14 NKT cell activation results in the rapid down-regulation of the Vα14 receptor. Subsequently, Vα14 NKT cells become undetectable by flow cytometry with α-GalCer/CD1d tetramer staining. Activated Vα14 NKT cells remain quiescent for a while, but eventually proliferate and continue to produce cytokines. Furthermore, these cells show a resistance to apoptosis induced by TCR stimulation relative to conventional T cells. These data indicate that Vα14 NKT cells neither become anergic nor are eradicated by apoptosis upon activation.

Methods

Mice

Eight- to 10-week-old female C57BL/6 mice were from SLC (Hamamatsu, Japan). C57BL/6 Ly-5.1 congenic mice were from Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions in our animal facility; they were treated in accordance with guidelines for animal care at Chiba University.

Reagents and antibodies

α-GalCer as previously described (10) was kindly provided by Kirin Brewery (Takasaki, Japan). The following mAb were purchased from BD PharMingen (San Diego, CA): anti-CD16/32 (2.4G2) and anti-CD3 (2C11), FITC-conjugated anti-TCRβ (H57-597) and anti-NK1.1 (PK136), and biotin-conjugated anti-TCRβ (H57-597), Ly-5.1 (A20) and Ly-5.2 (104). Anti-FITC microbeads were purchased from Miltenyi Biotec (Auburn, CA). Phycoerythrin-labeled α-GalCer/CD1d tetramer was prepared in our laboratory using a baculovirus expression system kindly provided by Dr M. Kronenberg (La Jolla Institute, La Jolla, CA). Cells were cultured in complete RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol (Invitrogen, Carlsbad, CA).

Preparation of dendritic cells (DC) and liver mononuclear cells

Mouse splenic DC were prepared as described previously (10). Total liver cells were suspended in a 33% Percoll solution (Pharmacia, Uppsala, Sweden) containing heparin (100 U/ml) and centrifuged at 1000 *g* for 15 min at room temperature. Pellets were used as liver mononuclear cells for subsequent studies after lysing red blood cells.

Stimulation with α-GalCer or α-GalCer-DC

For *in vivo* stimulation, 100 μg/kg of α-GalCer was i.p. injected into mice. For *in vitro* stimulation, DC were pulsed with 100 ng/ml of α-GalCer or vehicle alone for 24 h. After extensive washing, pulsed DC (10×10^3 cells/well) were added to stimulate spleen cells (0.2×10^6 cells/well) cultured in 96-well plates.

Flow cytometric analysis and cell sorting

Single-cell suspensions were prepared in PBS supplemented with 2% FCS and 0.05% sodium azide, pre-incubated with anti-CD16/CD32 mAb to prevent non-specific binding via Fc receptor interactions, and incubated with the appropriate mAb on ice for 30 min. Flow cytometric analysis and cell sorting were performed with Coulter Epics XL or Epics Elite cell sorters (Beckman Coulter, Palo Alto CA). Purity of sorted cells was estimated to be >98%.

Cell labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE)

Electronically sorted Vα14 NKT cells from Ly-5.2 mice liver (10×10^6) were incubated with 1 μM CFSE (Molecular Probes, Eugene, OR) in PBS for 8 min at room temperature. Labeling was stopped by the addition of FCS at equal volume. Labeled cells were washed extensively with medium prior to addition of Ly-5.1$^+$ spleen cells pulsed with α-GalCer or vehicle.

Cytokine concentration measurement by ELISA

Two hundred thousand fresh spleen cells, or spleen cells incubated with α-GalCer-DC for the indicated time periods, were co-cultured for an additional 72 h with α-GalCer-DC or vehicle-DC. At the end of the incubation period, culture supernatants were collected and the concentration of IFN-γ and IL-4 was measured using an ELISA kits (BD PharMingen).

Quantification of genomic DNA by PCR

Genomic DNA was extracted from cells using the QiaAmp DNA blood kit (Qiagen, Hilden, Germany). The amount of amplicons generated during PCR was monitored using the iCycler iQ Detection System (Bio-Rad, Hercules, CA). This method is based on the 5′ to 3′ nuclease activity of Taq polymerase, which allows the release of a fluorescent reporter during the PCR. The sequences of the primers and Taqman probes used in this study were as follows: Vα14: 5′-TGGGAGATACTCAGCAACTCTGG-3′; Jα281: 5′-CAGGTATGACAATCAGCTGAGTCC-3′;TCR Cα exon I forward: 5′-CAGAACCCAGAACCTGCTGT-3′; TCR Cα exon I reverse: 5′-TAGGTGGCGTTGGTCTCTTT-3′; Vα14 probe FAM: 5′-FAM-CACCCTGCTGGATGACACTGCCAC-TAMRA-3′; and TCR Cα exon I-probe FAM: 5′-FAM-CTCCCAAATCAATGTGCCGAAAACCA-TAMRA-3′.

Apoptosis detection assays

Electronically purified T (TCRβ$^+$NK1.1$^-$) and α-GalCer/CD1d tetramer-reactive Vα14 NKT cells (TCRβ$^+$NK1.1$^+$) from liver mononuclear cells were stimulated with plate-bound anti-CD3 mAb (10 μg/ml) for the indicated periods of time. Cells were subsequently stained with FITC-labeled Annexin-V (BD PharMingen) and analyzed with a flow cytometer. DNA fragmentation during apoptosis was monitored using the Cell Death Detection ELISA Plus kit (Roche, Mannheim, Germany).

DNA microarray analysis

Total RNA was prepared from electronically sorted hepatic T and α-GalCer/CD1d tetramer-reactive Vα14 NKT cells stimulated with plate-bound anti-CD3 mAb (10 μg/ml) for 2 h. cDNA was synthesized according to the manufacturer's instructions and labeled antisense RNA was prepared by *in vitro* transcription using T7 RNA polymerase (MessageAmp aRNA kit; Ambion, Austin, TX). Mouse Apoptosis Expression Arrays (R & D Systems, Minneapolis, MN) were used to interrogate the

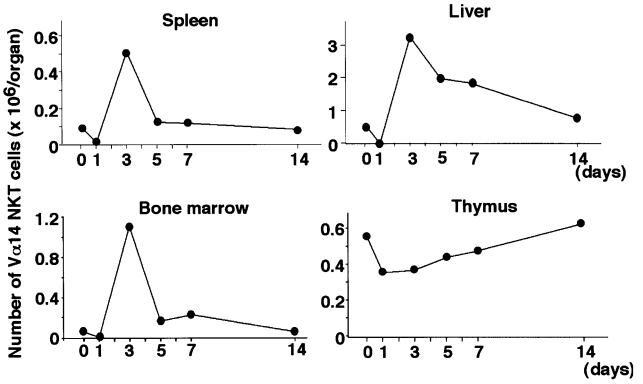

Fig. 1. Kinetics of Vα14 NKT cells from different organs after stimulation with α-GalCer *in vivo*. Mice were injected with α-GalCer (100 μg/kg). After different periods of time, spleen, thymus, liver and bone marrow were removed, and the cells stained with anti-TCRβ and α-GalCer/CD1d tetramer. Absolute numbers of Vα14 NKT cells were calculated from the flow cytometric data. Mean values from three independent mice are indicated.

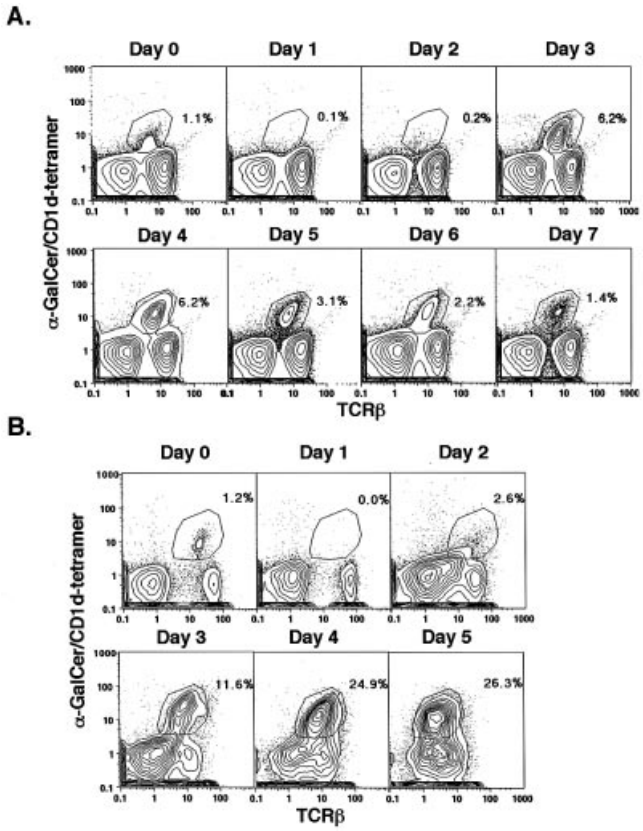

Fig. 2. Comportment of Vα14 NKT cells upon α-GalCer stimulation. (A) Kinetics of disappearance and repopulation of Vα14 NKT cells after α-GalCer stimulation *in vivo*. Spleen cells from C57BL/6 mice before and after administration of α-GalCer (100 μg/kg) were analyzed by flow cytometry at the indicated time. The percentage of Vα14 NKT cells, defined as TCRβ and α-GalCer/CD1d tetramer-reactive cells, is indicated. Representative data from three independent experiments are shown. (B) Kinetics of cell disappearance and repopulation of Vα14 NKT cells after α-GalCer stimulation *in vitro*. Spleen cells from C57BL/6 mice were co-cultured with α-GalCer-DC for the indicated periods of time *in vitro*. Vα14 NKT cells were identified as described in (A). Representative data from three independent experiments are shown.

expression of murine apoptosis-related genes. Radioactive signals were detected with FLA 8000 and the quantitative analysis was performed with Image-Gage software (Fuji Film, Tokyo, Japan).

RT-PCR

cDNA was synthesized from RNA used in DNA microarray analysis with oligo-dT primer. The following primer sets were used for the RT-PCR: NAIP, 5′-TCATGACTGTGCTTGCTTCC-3′ and 5′-CCAGTGGGAACGAACAGTTT-3′; MyD118, 5′-CTC-CTGGTCACGAACTGTCA-3′ and 5′-GGGTAGGGTAGCCTTT-GAGG-3′; IL-1β, 5′-GCCCATCCTCTGTGACTCAT-3′ and 5′-AGGCCACAGGTATTTTGTCG-3′; HPRT, 5′-AGCGTCGTGA-TTAGCGATG-3′ and 5′-CTTTTATGTCCCCCGTTGAC-3′. The number of PCR cycles to detect the above transcripts was as follows: 28 cycles for HPRT, 35 cycles for NAIP, and 37 cycles for MyD118 and IL-1β. PCR products were visualized by ethidium bromide staining and subjected to DNA sequencing to verify authenticity.

Results

Down-regulation of the invariant Vα14 receptors upon activation

To monitor the behavior of Vα14 NKT cells upon receptor activation, α-GalCer was injected i.p. into C57BL/6 mice, and the number of Vα14 NKT cells in the spleen, liver, bone marrow and thymus was examined (Fig. 1). Vα14 NKT cells detected by α-GalCer/CD1d tetramer represented ~1% of the total spleen cells prior to α-GalCer administration (Fig. 2A, day 0). However, the injection of α-GalCer made Vα14 NKT cells virtually undetectable within 24 h (Figs 1 and 2A, *cf.* day 0 and 1, 1.1–0.1%). It was not until day 3 that Vα14 NKT cells robustly reappeared, occupying up to 6.2% of spleen cells. After day 4, the number and proportion of Vα14 NKT cells gradually declined, and reached normal levels by day 14 (Figs 1 and 2A). Such a homeostasis of Vα14 NKT cells after α-GalCer stimulation *in vivo* was also observed in other organs such as liver and bone marrow, but not in the thymus (Fig. 1). Challenging spleen cells with α-GalCer-pulsed DC (α-GalCer-DC) *in vitro* resulted in a similar behavior of Vα14 NKT cells (Fig. 2B), whilst vehicle-pulsed DC (vehicle-DC) had no effect (data not shown). The apparent disappearance of Vα14 NKT cells *in vitro* occurred within 120 min after the addition of α-GalCer-DC (see Supplementary data available at *International Immunology* online). Thus, the receptor-mediated disappearance of Vα14 NKT cells was observed in a manner dependent on an antigen *in vivo* and *in vitro*.

To preclude the possibility that the invariant Vα14 receptor was occupied by α-GalCer on DC and thereby could not be detected by α-GalCer/CD1d tetramer, Vα14 NKT cells were stimulated with plate-bound anti-CD3 mAb and subjected to the same experiments. It turned out that anti-CD3 mAb stimulation also culminated in similar results (see Supplementary data). These data indicate that the apparent disappearance of Vα14 NKT cells was not caused by

occupation of the invariant Vα14 receptor with α-GalCer on DC. The tonic expansion observed in the *in vitro* culture

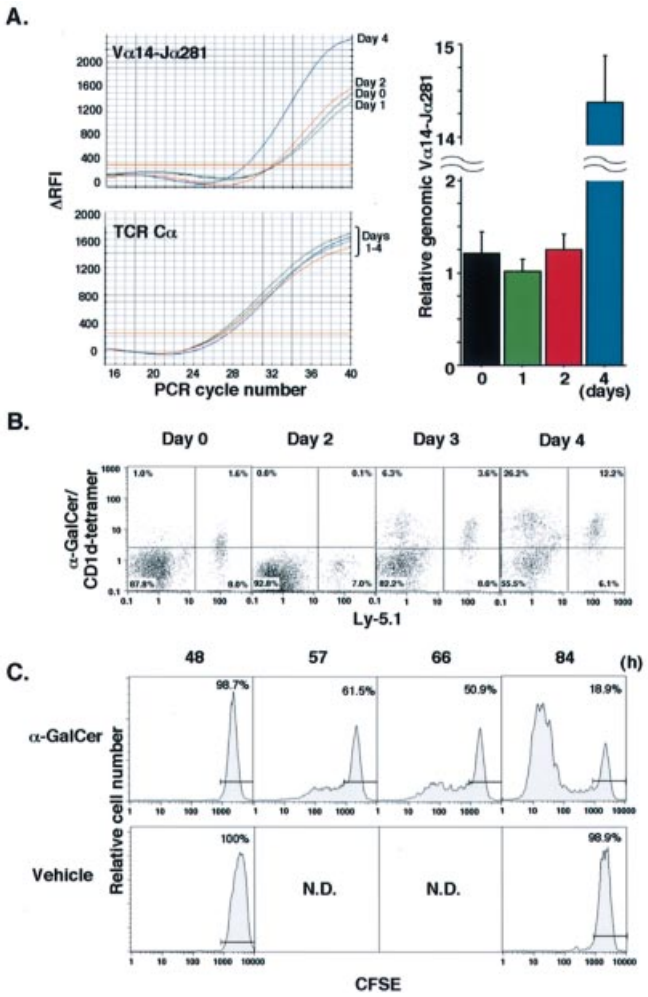

Fig. 3. Down-regulation of the invariant Vα14 receptor and survival of Vα14 NKT cells after α-GalCer stimulation. (A) Quantification of rearranged Vα14–Jα281 genomic DNA. The relative amounts of Vα14–Jα281 (left panel, upper) and TCR Cα (left panel, lower) genomic DNA from spleen cells stimulated with α-GalCer-DC were quantified by means of real-time PCR using Taqman probes. The amounts of rearranged Vα14–Jα281 genomic DNA were first compensated with that of TCR Cα and relative amounts of Vα14–Jα281 genes were calculated using an external standard prepared from Vα14 NKT hybridoma cells (data not shown). Relative amounts of genomic Vα14–Jα281 are plotted (right panel). Representative data from three independent experiments are shown with SD. (B) Study of Vα14 NKT cell destiny using Ly-5.1 and Ly-5.2 C57BL/6 mice. Electronically sorted liver Vα14 NKT cells from Ly-5.1 mice were mixed with spleen cells from Ly-5.2 mice at a ratio of 1:9 and co-cultured with α-GalCer-DC. The expression of the invariant Vα14 receptor was monitored with α-GalCer/CD1d tetramer. The percentage of cells in each subset is indicated. Representative data from two independent experiments are shown. (C) Cell division of Vα14 NKT cells after α-GalCer stimulation. Electronically sorted liver Vα14 NKT cells from Ly-5.2 mice were labeled with CFSE, mixed with Ly-5.1$^+$ spleen cells at a ratio of 1:9 and pulsed with α-GalCer or vehicle. The cell division of Ly-5.2$^+$ Vα14 NKT cells was monitored at the indicated time points after stimulation. Representative data from two independent experiments are shown. N.D. = not determined

(Fig. 2B), where there was no supply of Vα14 NKT cells, prompted us to hypothesize that α-GalCer/CD1d tetramer-reactive Vα14 NKT cells disappear, causing down-regulation of the Vα14 receptor, but are not eliminated.

To examine this hypothesis, we carried out a kinetic PCR analysis to rigorously measure the quantity of rearranged genomic Vα14–Jα281 upon receptor activation (Fig. 3A). When the relative numbers of Vα14–Jα281 genomic copies were compared after an appropriate compensations had been made using Cα, there was no change up to day 2, whereas the curve for the day 4 culture shifted to the left (equivalent to 3.5 PCR cycles). These results showed that there was no significant decrease in the number of Vα14–Jα281 genomic copies during the culture periods from day 0 to 2, implying that most of the Vα14 NKT cells were alive without significant cell death (Fig. 3A, left panel). The net increase in Vα14 NKT cell number upon stimulation was estimated to be in the range of 11-fold over 4 days (Fig. 3A, right panel).

The above data suggested that Vα14 NKT cells survive after stimulation rather than succumbing to death. To further explore this possibility, liver NK1.1$^+$ TCRβ$^+$ cells (of which >90% are α-GalCer/CD1d tetramer-reactive Vα14 NKT cells) from Ly-5.1 mice were purified by electronic cell sorting, mixed with Ly-5.2$^+$ spleen cells and stimulated with α-GalCer-DC. While Ly-5.1$^+$ Vα14 NKT cells expressed the invariant Vα14 receptor prior to stimulation, administration of α-GalCer-DC led to down-regulation of their invariant Vα14 receptor by day 2, resulting in a significant decrease in the number of Ly-5.1$^+$ Vα14 NKT cells (Fig. 3B, *cf.* day 0 and 2).

It is, however, of importance to note that the Ly-5.1$^+$ Vα14 NKT cells stayed alive even after α-GalCer-DC stimulation (Fig. 3B, day 0–4). As observed previously, α-GalCer/CD1d tetramer-reactive Vα14 NKT cells reappeared on day 3 (Fig. 3B, day 3) and the number of Ly-5.1$^+$ Vα14 NKT cells subsequently expanded to account for >18% of total cultured cells (Fig. 3B, day 4). These results clearly demonstrated that Ly-5.1$^+$ Vα14 NKT cells down-regulate their invariant Vα14 receptor upon α-GalCer stimulation, stay quiescent and eventually proliferate.

We also examined whether the observed cellular expansion accompanied cell division by labeling cells with CFSE. Electronically sorted liver NK1.1$^+$TCRβ$^+$ cells from Ly-5.2 mice were labeled with CFSE and mixed with unfractionated Ly-5.1$^+$ spleen cells. These mixtures were stimulated with α-GalCer or vehicle. It was not until 57 h after stimulation that cell division could be detected only in cells activated with α-GalCer (Fig. 3C, upper panel and data not shown). After that period, α-GalCer-stimulated cells continued to divide. In marked contrast, vehicle-stimulated Vα14 NKT cells showed little, if any, cell division (Fig. 3C, lower panel). These results indicated that α-GalCer promotes Vα14 NKT cell proliferation and further underpinned the above hypothesis.

Vα14 NKT cells are relatively resistant to apoptosis induced by receptor activation

The above data, however, did not exclude the possibility that a limited number of Vα14 NKT cells undergo apoptosis upon activation. We thus examined, by Annexin-V staining, whether Vα14 NKT cells underwent apoptosis upon stimulation. A comparison of Annexin-V staining upon anti-CD3 mAb stimu-

lation showed less staining for Vα14 NKT cells relative to conventional T cells (TCRβ$^+$NK1.1$^-$) at all time points examined (Fig. 4A, left panel), suggesting that, albeit weakly, Vα14 NKT cells underwent apoptosis upon receptor-mediated activation. The number of cells that underwent apoptosis was further evaluated by measuring the amount of released DNA fragments by ELISA. As in the case of Annexin-V staining, fragmented DNA was observed in some fractions of Vα14 NKT cells, but the degree of fragmentation was less than that in conventional T cells (Fig. 4A, right panel).

To explore the molecular mechanisms underlying resistance to apoptosis in Vα14 NKT cells, we compared the profile of apoptosis-related gene expression in Vα14 NKT and conventional T cells through a DNA microarray study (Fig. 4B, upper panel and see Supplementary data). After stimulation with anti-CD3 mAb for 2 h, RNA was extracted from Vα14 NKT cells and from conventional T cells, and subjected to differential hybridization. To compare the signal intensity, we used scattered plot analysis (Array-Gage; Fuji Film). When the signal was normalized with HPRT, several molecules showed increased hybridization signal in Vα14 NKT cells relative to conventional T cells (Fig. 4B, upper and lower left panel). Subsequent semiquantitative RT-PCR analyses confirmed that NAIP and MyD118 were indeed up-regulated in Vα14 NKT cells relative to conventional T cells (Fig. 4B, lower right panel). We have also examined several genes that showed reduced expression in Vα14 NKT cells based on scattered plot data. It turned out that IL-1β, an indicator of apoptosis, showed reduced hybridization signal concomitant with decreased mRNA expression as revealed by RT-PCR analysis when compared to conventional T cells (Fig. 4B, lower panels) (12). All gene expression profiles in Vα14 NKT cells and in conventional T cells are available in the Supplementary data.

These differences in expression of apoptotic genes may reflect the intrinsic nature of each cell type. We also compared the expression of apoptosis-related genes in Vα14 NKT cells and conventional T cells prior to receptor activation. It was found that the expression profile of these cells prior to receptor activation mirrored that of post-receptor stimulation (*cf.* Fig. 4B, lanes 3 and 4, and C, lanes 1 and 2). Further kinetic studies on the expression of the indicated genes in Vα14 NKT cells *in vivo* revealed that, upon receptor activation, NAIP expression increased up to day 3, while expression thereafter gradually declined. MyD118 showed a similar expression pattern over time as NAIP, while the level of IL1-β expression remained constant (Fig. 4C).

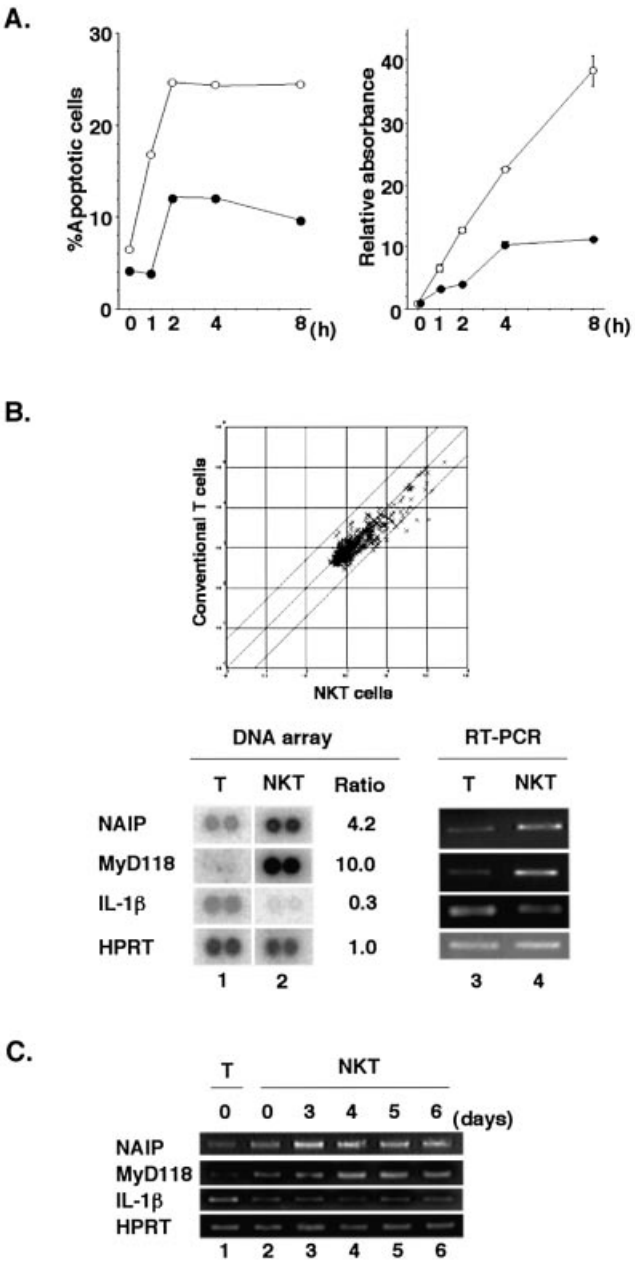

Fig. 4. Vα14 NKT cells are resistant to apoptotic cell death induced by receptor-mediated activation relative to conventional T cells. (A) Kinetics of apoptotic cell death induced by activation in Vα14 NKT cells and conventional T cells. Annexin-V expression levels were analyzed in electronically sorted Vα14 NKT cells and conventional T cells from the liver after stimulation with anti-CD3 mAb for the indicated periods of time. The percentage of Annexin-V-stained cells is shown as open circles for conventional T cells and closed circle for Vα14 NKT cells (left panel). Apoptosis was also quantified by measuring the amounts of fragmented DNA from the cells using an ELISA kit (right panel). Representative data from two independent experiments are shown. (B) Apoptosis-related genes are differentially expressed in Vα14 NKT cells stimulated for 2 h. Vα14 NKT cells and conventional T cells were isolated and stimulated as described in (A). Total RNA was extracted and subjected to RNA amplification. After random priming, differential hybridization was performed. Upper panel: scattered plot analysis between NKT and conventional T cells. Lower left panel: differential hybridization signal on DNA array. The ratio represents the fold change of the hybridization signal between two groups in duplicate experiments after normalizing with the signal of HPRT. Lower right panel: RT-PCR detection for NAIP, MyD118 and IL-1β in cells used for DNA array experiments. HPRT serves as a control to assure the equal amount of the recovered RNA. (C) Up-regulation of anti-apoptotic genes upon receptor activation *in vivo*. After *in vivo* stimulation with α-GalCer for the indicated periods of time, NKT cells from the spleen were electronically sorted. RNA was extracted from spleen NKT cells at the indicated time, and subjected to RT-PCR with primers for NAIP, MyD118 and IL-1β. HPRT serves as control to ensure equal cDNA input in each lane. For comparison, RNA from the same number of conventional spleen T cells was subjected to RT-PCR without any stimulation.

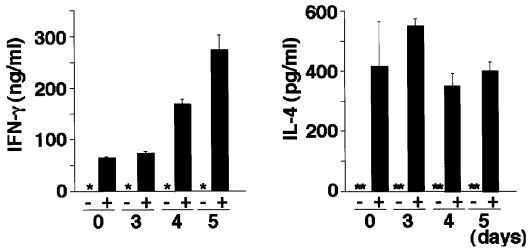

Fig. 5. Cytokine production from re-stimulated Vα14 NKT cells. Total spleen cells were cultured in the presence of α-GalCer-DC as described in Fig. 2(B) for the indicated periods of time (day 0–5). Cells were then collected, washed and stimulated with vehicle-DC (–) or re-stimulated with α-GalCer-DC (+) for another 72 h. The concentration of IFN-γ and IL-4 in the supernatant was determined by ELISA. Data from three independent experiments are shown with ± SD. The asterisks (* and **) indicate that the concentration was below the detection threshold for IFN-γ (<0.16 ng/ml) or IL-4 (<15.6 pg/ml), respectively.

Vα14 NKT cell resistance to tolerance induction after receptor down-regulation

Since the activation of T cells often results in the loss of their function and becomes tolerant (2), the ability of Vα14 NKT cells to secrete IFN-γ and IL-4 upon re-stimulation with α-GalCer-DC or vehicle-DC was examined at the indicated time points following the initial α-GalCer-DC stimulation. At any given time point, even after the down-regulation of the Vα14 receptor, activated Vα14 NKT cells produced both IFN-γ and IL-4 upon re-stimulation with α-GalCer-DC (Fig. 5). In contrast, neither IFN-γ nor IL-4 production could be observed following vehicle-DC re-stimulation (Fig. 5). These results indicate that the activation of Vα14 NKT cells does not affect cytokine production in these cells after re-stimulation.

Discussion

We have demonstrated that Vα14 NKT cell activation results in the subsequent down-regulation of the Vα14 receptor. Activated Vα14 NKT cells remain quiescent for a while, but eventually proliferate. However, the effector functions of activated Vα14 NKT cells, such as cytokine production, remain intact even after receptor down-regulation. These unique features distinguish Vα14 NKT cells from other types of effector T cells which are readily subjected to clonal deletion or anergy upon TCR-mediated activation.

Our present data are somewhat contradictory to the previous reports claiming that NKT cells, defined as NK1.1$^+$TCRβ^{dim} cells, are extremely sensitive to IL-12- and TCR-induced apoptosis (13). This is most evident in our *in vitro* experiments where there is no provision of exogenous Vα14 NKT cells or Vα14 NKT cell progenitors (Fig. 2B). Under such conditions, the rapid disappearance and subsequent expansion of Vα14 NKT cells upon α-GalCer challenge cannot be attributed to the immediate eradication of the cells by apoptosis. Rather, the seeming disappearance of Vα14 NKT cells is better explained by the down-regulation of the Vα14 receptor. This interpretation is further supported by transfer experiments using Ly-5.1$^+$ cells and by kinetic PCR (Fig. 3). Still, more detailed studies are needed to elucidate the precise mechanism of Vα14 NKT cell number reduction following receptor stimulation *in vivo* (Figs 1 and 2A). Apoptotic cell death may play a role in this process. However, since the expression of certain anti-apoptotic genes is up-regulated following receptor activation, it appears likely that NKT cells become less susceptible to apoptosis over time (see below, Fig. 4C).

The molecular mechanism underlying the resistance of Vα14 NKT cells to TCR-mediated apoptosis was further explored by DNA microarray studies combined with RT-PCR analysis (Fig. 4B). Our results indicate that the resistance to apoptosis of Vα14 NKT cells relative to conventional T cells is primarily due to the preferential expression of anti-apoptotic genes, such as NAIP and MyD118. NAIP belongs to the inhibitor of apoptosis (IAP) that regulates lymphocyte sensitivity to Fas-mediated apoptosis (3,14). IAP bind activated caspases, and target their ubiquitination and degradation via the baculovirus IAP repeat (BIR) motif, which eventually allows the cells to survive upon Fas-induced signals. MyD118, also known as Gadd45β, was first identified as a member of the Gadd45 family associated with cell-cycle control and DNA repair (15,16). MyD118/Gadd45β is induced by an apoptotic factor, tumor necrosis factor-α, and acts as an anti-apoptotic protein by inhibiting JNK activity (17). Since continuous activation of JNK activity is tightly correlated with apoptosis (18), its inhibition results in the blocking of apoptosis. In summary, the increased expression of anti-apoptotic genes post-receptor activation together with the unaltered expression of IL-1β in Vα14 NKT cells may explain, at least in part, why these cells are more resistant to apoptosis than conventional T cells. In the light of these data, it is not surprising that Vα14 NKT cells are relatively resistant to apoptosis induced by irradiation and glucocorticoid treatment (19,20). Since conventional T cells comprise naive and memory cells, a more fine comparison in the expression of anti-apoptotic genes between Vα14 NKT cells and conventional T cells will provide more insight into the anti-apoptotic mechanism in Vα14 NKT cells.

Another unique facet of Vα14 NKT cells is that its effector function, the production of cytokines, is kept intact even after activation (Fig. 5). This indicates that Vα14 NKT cells do not become anergic upon receptor activation. In line with this, priming α-GalCer-pulsed DC *in vivo* followed by re-stimulation with α-GalCer-pulsed DC *in vitro* does not induce anergy in Vα14 NKT cells (21). However, we have to mention that the amount of IL-4 produced by activated NKT cells was relatively constant regardless of the different NKT cell number (Figs 2A and 5). This may be explained, at least in part, by the fact that IL-4 produced from the activated NKT cells is consumed by NKT cells *per se* or B cells, thus culminating in the similar net production of IL-4 (22,23).

These distinct features of Vα14 NKT cells may be tightly linked to the role of Vα14 NKT cells in controlling and suppressing autoreactive effector T cells. Collectively, it is conceivable that the nature of Vα14 NKT cells to be resistance to tolerance induction as well as apoptosis after down-regulation of the invariant Vα14 receptor confers on these cells the ability to mediate a long-lasting regulatory function under any given circumstances.

These findings will open the door to the elucidation of the homeostasis of immunoregulatory cells.

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Abbreviations

α-GalCer	α-galactosylceramide
CFSE	carboxyfluorescein diacetate succinimidyl ester
DC	dendritic cell
IAP	inhibitor of apoptosis

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