

**Invariant NKT cell anergy is induced by a strong TCR-mediated signal  
plus costimulation**

Contributors:

Tomonori Iyoda<sup>1,2</sup>, Maki Ushida<sup>1</sup>, Yukino Kimura<sup>1</sup>, Kento Minamino<sup>1,2</sup>, Akiko Hayuka<sup>1</sup>,  
Shoichi Yokohata<sup>1</sup>, Hiromi Ehara<sup>1,3</sup>, and Kayo Inaba<sup>1,2</sup>

<sup>1</sup>Laboratory of Immunobiology, Department of Animal Development and Physiology,  
Division of Systemic Life Science, Graduate School of Biostudies, Kyoto University

<sup>2</sup>CREST, Kyoto University

<sup>3</sup>Immunology Group, Innovative Drug Research Laboratories, Research Division, Kyowa  
Hakko Kirin Co., Ltd

The corresponding author:

Kayo Inaba

Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

E-mail: [kayo@lif.kyoto-u.ac.jp](mailto:kayo@lif.kyoto-u.ac.jp)

Tel: +81 75 753 4088, Fax: +81 75 753 4112

**Running title:** NKT cell anergy induced by TCR and co-stimulation

Key word: NKT cells,  $\alpha$ -GalCer, dendritic cell, costimulation, ionomycin, CD28, *in vivo*

This manuscript consists of 34 pages of text and 5 figures.

### Abstract

V $\alpha$ 14 TCR expressing invariant natural killer T (iNKT) cells recognize  $\alpha$ -galactosylceramide ( $\alpha$ GC)/CD1d complex and produce large amounts of various cytokines before the onset of the adaptive immunity. After stimulation with a high dose (2- 5  $\mu$ g) of  $\alpha$ GC *in vivo*, iNKT cells in the spleen and liver become anergic in terms of the proliferation and cytokine production to subsequent stimulation. In this study, we monitor how iNKT anergy is induced.

Anergized iNKT cells dramatically reduced the expression of IL-2R $\alpha$ , and exogenous IL-2 restored the ability to proliferate and produce IL-4, but not to produce IFN- $\gamma$ . Anergized iNKT cells expressed high levels of PD-1. However, iNKT cells in PD-1-deficient mice became anergic as a result of  $\alpha$ GC injection, as do normal mice. Furthermore, anti-PD-1 blocking mAb was unable to restore their responsiveness. When iNKT cells were stimulated with immobilized anti-CD3 in the presence or absence of anti-CD28, they produced cytokines in a dose-dependent manner. Unlike in naïve CD4 T cells, the strong TCR-mediated signaling with costimulation render them anergic to any subsequent stimulation with  $\alpha$ GC and spleen dendritic cells (DCs). Moreover, iNKT cells also became anergic after stimulation with PMA + ionophore. Finally, the injection of  $\alpha$ GC-pulsed DCs was more potent in inducing anergy than B cells. These results indicate that strong TCR-mediated activation with costimulation provides signals that induce the anergic state in iNKT cells.

## Introduction

NKT cells that express both  $\alpha\beta$  TCR and NK1.1, an NK cell marker in mouse (1), recognize glycolipid, which is presented by major histocompatibility complex (MHC) class I-like molecules, CD1d. Although there are several candidates of natural ligand for NKT cells (2), invariant V $\alpha$ 14-expressing NKT (iNKT) cells rapidly produce large amounts of cytokines, such as IFN- $\gamma$  and IL-4, after recognizing a synthetic glycolipid antigen derived from marine sponges called  $\alpha$ -galactosylceramide ( $\alpha$ GC) (3).

CD1d is expressed on a broad range of cells. Even though marginal zone B (MZB) cells express the highest level of CD1d (4), dendritic cells (DCs) are much more potent antigen presenting cells (APCs) for iNKT cells than MZB cells both *in vitro* and *in vivo* (5). When iNKT cells recognize  $\alpha$ GC/CD1d complex, they upregulate CD40 ligand expression. Through the CD40/CD40 ligand-interactions, activated DCs produce IL-12 and upregulate the expression of co-stimulatory molecules such as CD80 and CD86 (6-9). Thus, mutual interactions between iNKT cells and DCs reciprocally activate each other and bring about the cytokine storm, leading to the effective immune responses (7,8).

It has also been reported that  $\alpha$ GC treatment accelerates Th2-type cytokine production (10) and prevents the development of type 1 diabetes in NOD mice (11,12). However, a single injection of  $\alpha$ GC at 100  $\mu$ g/kg rendered iNKT cells unresponsive (anergic) to re-stimulation

*in vitro*, although they activated iNKT cells to produce cytokines (13-17). In conventional T cells, it has been considered that TCR signaling without the co-stimulatory signal induces anergy without producing IL-2 or other cytokines (18-20). Thus, the mechanisms by which anergy is caused appear to be different for T cells and iNKT cells. However, it is not yet clear how the co-stimulatory signal is involved in the induction of iNKT cells. Recently, immunosuppressive PD-1 molecule has been shown to play a role in the induction and/or maintenance of the anergic state in iNKT cells (15,17).

Here we show that, in contrast to naïve T cells, iNKT cells become anergic only when they are given strong activation signals through TCR along with costimulation, such as with anti-CD3 + anti-CD28, and phorbol 12-myristate 13-acetate (PMA) + ionomycin. This was also confirmed by experiments in which iNKT cell anergy was induced when DCs were pulsed with a high dose of  $\alpha$ GC and a large number of these  $\alpha$ GC-pulsed DCs were then injected. Consistent with these findings,  $\alpha$ GC-loaded DCs were much more potent in rendering iNKT cells anergic than B cells. Therefore, it is conceivable that DCs are primary APCs and not only activate iNKT cells but also drive them into the anergic state.

## Methods

### Mice

C57BL/6 mice were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). OT-II mice from Dr. F. Carbone (University of Melbourne, Parkville, Victoria, Australia) and PD-1 knockout (KO) mice from Dr. T. Honjo (Kyoto university, Kyoto, Japan) were maintained under specific pathogen-free conditions in our animal facility and used at 8-12 wks of age. Animal care and use were as per the institutional guidelines of Kyoto University.

### Antibodies and reagents

Purified anti-mouse IFN- $\gamma$  and IL-2, FITC-conjugated anti-mouse CD45R, PE-conjugated anti-mouse IgG<sub>1</sub>, biotin-conjugated anti-mouse CD28 and TCR  $\beta$  chain, biotin-conjugated isotype control IgG, streptavidin-conjugated PE-Cy5, CD1d-dimer XI, anti-CD4 IMag-MS, and recombinant mouse IL-4, IL-12p70 and IFN- $\gamma$  were purchased from BD Biosciences (San Diego, CA). Purified IL-4, anti-CD3 $\epsilon$  and CD28, FITC-conjugated anti-mouse CD19, biotin-conjugated Abs for mouse IL-4, IFN- $\gamma$ , IL-2, CD25, CD122, PD-1 and BTLA were from e-Bioscience (San Diego, CA). Recombinant mouse IL-2 and IL-15, and biotinylated anti-mouse IL-15 receptor  $\beta$  (IL-15R $\beta$ ) were obtained from R&D systems (Minneapolis, MN). MACS microbeads conjugated with CD11c, CD19 and PE were from

Miltenyi Biotec GmbH (Bergisch Gladbach, Germany), and Dynabeads conjugated with sheep anti-rat IgG were from Invitrogen Life Technologies (Carlsbad, CA). mAbs for CD16/32 (2.4G2), B220 (RA3-6B2.1), MHC class II (M5/114.15.2), CD11b (M1/70), CD8 $\alpha$  (53-6.72), Gr-1 (RB6-8C5), and CD62L (Mel-14.d54) were culture supernatants of hybridoma (American Type Culture Collection, Manassas, VA).  $\alpha$ GC was provided by Kirin Brewery Co., Ltd. (Gunma, Japan). Purified anti-PD-1 mAb (RMP1-14) was a kind gift from Dr. H. Yagita (Juntendo University School of Medicine, Tokyo, Japan).

### **iNKT cell anergy induction, cell preparation and culture**

Mice were intravenously (iv) inoculated with 5  $\mu$ g of  $\alpha$ GC 7-9 d in advance. In some cases, spleen DCs and B cells pulsed with graded doses of  $\alpha$ GC, as indicated, for 1 h at 37°C were injected iv into the mice in the quantities indicated.

iNKT cells were enriched as cells binding  $\alpha$ GC/CD1d-Ig by MACS sorting using PE-conjugated anti-mouse IgG<sub>1</sub> and anti-PE mAb-coupled magnetic beads (purity >85%) from spleen or liver cells depleted of cells bearing CD8 $\alpha$ , CD11b, CD16/CD32, CD45R, CD62L and MHC class II using sheep anti-rat IgG Dynabeads (Dyna, Oslo, Norway). For *in vitro* restimulation experiments, iNKT cells were further purified as PE- $\alpha$ GC/CD1d-Ig<sup>+</sup> FITC-CD19<sup>-</sup> cells by FACS Vantage (purity >98%).

CD11c<sup>+</sup> spleen and bone marrow (BM)-derived DCs were prepared as described

previously (21,22). CD19<sup>+</sup> spleen B cells were obtained by MACS sorting.

Purified iNKT cells prepared from normal and pretreated mice with 5 µg of αGC were cultured at 0.5-1 × 10<sup>4</sup>/well in round-bottomed 96-well culture plates with 1 × 10<sup>4</sup> of splenic CD11c<sup>+</sup> cells in the presence or absence of 100 ng/ml of αGC in RPMI1640 medium supplemented with 5% FCS, 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin (R5) for 3 d. In some experiments, 10 µg/ml of blocking mAbs or 100 ng/ml of recombinant mouse IL-2 (rIL-2) or IL-15 were added to culture.

In order to induce anergy *in vitro*, iNKT cells seeded at 2 × 10<sup>5</sup> cells/well in 96-well round-bottomed plates were stimulated with graded doses of immobilized anti-CD3 mAb in the presence or absence of graded doses of anti-CD28 mAb (0-10 µg/ml) for 16 h. After washing with R5 3 times, the cells were re-plated in new vessels and allowed to rest for 2 d. In some experiments, iNKT cells and OT-II CD4 T cells were treated with 1000 ng/ml of ionomycin (Sigma-Aldrich, St. Louis, MO) with or without 50 ng/ml of PMA (Sigma-Aldrich) for 16 h. After collecting, viable cells at 5 × 10<sup>3</sup> cells were re-stimulated with 10<sup>4</sup> splenic DCs in the presence or absence of 100 ng/ml αGC or 1 µg/ml of OT-II specific OVA peptide for 3 d. At the end of culture, supernatants were collected and cytokine production was determined by sandwich ELISA. In some experiments, cytokine production in sera was determined at various time points after the injection of αGC-loaded cells using cytometric beads array (Mouse Inflammation Kit and Mouse Th1/Th2 Kit: BD Biosciences).

### Quantitative RT-PCR

Total RNA was extracted using TRIzol (Invitrogen Life Technologies). Real-time PCR was performed with QuantiTect SYBR Green PCR kit (QIAGEN GmbH, Hilden, Germany) for IL-4, IFN- $\gamma$ , and hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) as internal control. Reverse-transcription and PCR amplification were performed according to the manufacturer's instructions. Cycling was performed at 50°C for 30 min, 95°C for 15 min, 60 cycles at 94°C for 15 sec, at 60°C for 30 sec, and at 72°C for 30 sec. The amount of amplicon generated during the PCR was monitored using a Rotor-Gene 2000 (Corbett Research, Mortlake, Australia) apparatus. Analysis was performed on a Rotor-Gene version 5.0 (Corbett Research). Primer sequences were as follows: IL-4, sense 5'-CTTCCAAGGTGCTTCGCATATTTTA-3' and anti-sense 5'-CATGATGCTCTTTAGGCTTTCCAG-3'; IFN- $\gamma$ , sense 5'-GCCTAGCTCTGAGACAATGAACGC-3' and anti-sense 5'-AGATATCCAAGAAGAGACTCTTTTC-3'; HPRT, sense 5'-CTTCAGGGATTTGAATCACGTTTG-3' and anti-sense 5'-TAATTTTACTGGCAACATCAACAG-3'.



**Statistical analysis**

Data are expressed as the mean  $\pm$  SD of triplicate cultures. Statistical significances were determined by the Student's *t*-test. Differences were considered to be significant for *p* values less than 0.05. All experiments were performed at least three times and representative results are shown.

## Results

### Function and phenotype of anergized iNKT cells by soluble $\alpha$ GC.

It has been reported that the injection of a high dose of  $\alpha$ GC induces unresponsiveness of iNKT cells to any subsequent stimulation (13-17). Thus, we first confirmed the anergy of the iNKT cells that had been stimulated with 5  $\mu$ g of  $\alpha$ GC 9 d previously. iNKT cells obtained from the spleen and liver were re-stimulated by  $\alpha$ GC in the presence of spleen DCs *in vitro*, and cytokine production was then measured. Not only spleen but also liver iNKT cells produced remarkably low amounts of IFN- $\gamma$  and IL-4 (Fig. 1A).

In conventional T cells, it is widely accepted that IL-2 can reverse the anergic state (23). IL-2 shares its IL-2/IL-15 receptor  $\beta$  chain (IL-2/IL-15R $\beta$ , CD122) and common  $\gamma$  chain with IL-15. Moreover, IL-15 is known to be important for both iNKT cell development and homeostasis (24,25). Thus, we examined the receptor expression for these cytokines. Compared with iNKT cells from normal mice, iNKT cells pre-exposed to  $\alpha$ GC reduced IL-2R $\alpha$ , but did not reduce either IL-15R $\alpha$  or IL-2/IL-15R $\beta$  (Fig. 1B). In addition, anergized iNKT cells slightly upregulated IL-2R $\alpha$  expression upon the restimulation with anti-CD3 and anti-CD28 (Fig. 1C). Furthermore, exogenous IL-2 was incapable of restoring IFN- $\gamma$  production by anergized iNKT cells, although it effectively restored proliferation and IL-4 production (Fig. 1D), as reported previously (14). Unlike IL-2, exogenous IL-15 had marginal

effect on the proliferation and IL-4 production. iNKT cells as well as NK cells expressed cytokine mRNAs, such as IFN- $\gamma$  and IL-4, in the steady state (26). Thus, we used quantitative PCR to measure the mRNA levels of the respective cytokines. The results demonstrated that anergized iNKT cells contained decreased amounts of IFN- $\gamma$  mRNA, but increased amounts of IL-4 mRNA compared with unstimulated iNKT cells (Fig. 1E), which is consistent with the different role of IL-2 on IFN- $\gamma$  and IL-4 production and suggests that preactivation of iNKT cells skews the cytokine production potential.

#### **Role of PD-1 in the induction and maintenance of iNKT cell anergy.**

Other investigators have recently reported that anergic iNKT cells upregulated inhibitory molecules such as PD-1 and B and T lymphocyte attenuator (BTLA), and that PD-1 is responsible for the induction of the iNKT cell anergy at the time of activation (15,17). Therefore, we also monitored the expression of PD-1 and BTLA in comparison with TCR and CD28 expression, and confirmed a remarkable upregulation of PD-1 at 9 d after  $\alpha$ CG injection (Fig. 2A). The effect of blocking mAb to PD-1 was then examined on anergized iNKT cell responsiveness to produce cytokines upon the restimulation *in vitro*. As with the results of Parekh *et al* (17), but not of Chang *et al* (15), blocking mAb to PD-1 had almost no impact on the reversal of the hyporesponsiveness of anergized iNKT cells (Fig. 2B).

To further examine the involvement of PD-1 in the induction of iNKT cell anergy,

we measured the serum cytokine activity of PD-1 KO mice. The injection of  $\alpha$ GC was as effective in the induction of iNKT cell anergy to any subsequent stimulation in PD-1 KO mice as in WT mice (Fig. 2C) and such an anergy lasted at least a month in PD-1 KO mice as well as WT mice (data not shown). When these anergized iNKT cells were restimulated *in vitro*, they produced only a minimal amounts of cytokines, especially IFN- $\gamma$  (Fig. 2D). These results indicate that PD-1 is not a primary contributor in the induction and maintenance of iNKT cell anergy.

#### **Induction of iNKT cell anergy *in vitro*.**

Activation of iNKT cells by  $\alpha$ GC is dependent on the recognition of the  $\alpha$ GC/CD1d complex via invariant TCR. In order to mimic the activation of iNKT cells to become anergic, purified iNKT cells were stimulated with graded doses of immobilized anti-CD3 mAb, followed by resting for 2 d, and then restimulated with  $\alpha$ GC in the presence of spleen DCs. After the primary stimulation, iNKT cells produced IFN- $\gamma$ , IL-4 and IL-2 in a dose-dependent manner (Fig. 3A, left panels). However, cytokine production by pre-activated iNKT cells decreased as the dose of anti-CD3 mAb in the primary stimulation was increased (Fig. 3A, right panels), showing the inverse correlation between the magnitude of the primary response and the responsiveness to the secondary stimulation.

Costimulation is well known to be required for priming conventional T cells in

response to the specific antigen through the recognition by TCR (27,28). Therefore, the effect of CD28-mediated costimulation together with CD3-mediated TCR signaling was examined on the primary and secondary cytokine production in comparison with the production in CD4<sup>+</sup> OT-II T cells. Cytokine production by anti-CD3 was enhanced along with the increase in the dose of anti-CD28 mAb in both iNKT cells (Fig. 3B, left panels) and OT-II T cells (Fig. 3C, left panel). However, pre-activated iNKT cells inversely reduced the response upon the restimulation with  $\alpha$ GC in the presence of spleen DCs (Fig. 3B, right panels). Moreover, these pre-activated iNKT cells by anti-CD3 + CD28 were incapable of producing IFN- $\gamma$  even in the presence of IL-2, as in those anergized *in vivo* (data not shown). In contrast, pre-activated OT-II T cells with a high dose of anti-CD28 produced comparable amounts of IFN- $\gamma$  to non-activated cells, but those without anti-CD28 were refractory to the restimulation with specific OVA-peptide and spleen DCs (Fig. 3C, right panel). Similar tendencies were also observed using liver iNKT cells (data not shown).

Moreover, both iNKT cells and OT-II T cells produce IFN- $\gamma$  in response to ionomycin + PMA, but not ionomycin alone (Fig. 4A). To the subsequent stimulation with spleen DCs in the presence of appropriate respective antigen, only iNKT cells showed anergic state after the stimulation with ionomycin + PMA, whereas OT-II cells pretreated with ionomycin alone became anergic (Fig. 4B), as previously reported (29). These results together clearly demonstrate the different mechanism of anergy induction between iNKT cells and

conventional CD4<sup>+</sup> T cells, and suggest that the costimulation facilitates the induction of iNKT cell anergy.

**$\alpha$ GC-loaded DCs induced iNKT cell anergy *in vivo*.**

DCs are well documented to be a primary APC for iNKT cell activation *in vivo* as well as *in vitro*. The inefficiency of B cells as APCs was supposed to be due to the lack of costimulatory molecules (30). However,  $\alpha$ GC-loaded B cells were able to induce IL-4 production, but little IFN- $\gamma$  production, from iNKT cells only when injected *in vivo* (5,31). The results shown in Fig. 3 indicating the efficient induction of iNKT cell anergy along with costimulation raise the possibility that DCs are potent APCs in the activation of iNKT cells but also in the induction of the subsequent anergy.

To address this possibility, DCs were pulsed with graded doses of  $\alpha$ GC and injected into normal mice, and the responsiveness of spleen iNKT cells was analyzed *in vitro* 9 d later. The iNKT cell anergy was evidently induced by DCs in an  $\alpha$ GC dose-dependent manner (Fig. 5A). Next, we compared DCs with B cells in terms of their iNKT cell activating and anergy inducing ability. Although DCs were much more potent in terms of activating iNKT cells to produce cytokines, B cells also induced significant amounts of cytokines in a dose-dependent manner (Fig. 5B). It was also shown that DCs were much more potent in inducing iNKT cell anergy than B cells (Fig. 5C). Similar results were obtained in liver iNKT cells (data not

shown). These results confirmed that iNKT cells lapsed into anergy *in vivo* even when they received signals through both TCR and co-stimulatory molecules on professional APCs.

## Discussion

A single injection of a high dose of  $\alpha$ GC induced long-lasting iNKT cell anergy following the robust production of cytokines, such as IFN- $\gamma$  and IL-4 (13-17). In contrast, conventional CD4 T cells are shown to become anergic without producing cytokines when stimulated via TCR in the absence of costimulation (32). Therefore, in this study we monitored the consequences of activated iNKT cells and the requirement for costimulation in order to induce iNKT cell anergy compared with conventional CD4 T cells.

As in conventional T cells, exogenous IL-2, but not IL-15, restored the proliferation of anergic iNKT cells (Fig. 1D), as has been reported by Parekh (14). Our results additionally showed that IL-2-rescued anergic iNKT cells so that they produced more IL-4 than normal iNKT cells. However, IFN- $\gamma$  production by anergic iNKT cells was not restored. This polarized cytokine production by anergized iNKT cells seems be related to respective mRNA levels, in which there were increased and decreased amounts of IL-4 and IFN- $\gamma$  mRNAs, respectively. Recently, Kojo *et al* clearly demonstrated that the anergized iNKT cells increase the expression level of an E3 ubiquitin ligase, Cbl-b, and that Cbl-b deficiency largely rescues the decreased IFN- $\gamma$  production by  $\alpha$ GC-pretreated iNKT cells (16). They also showed that the decreased proliferative and IL-4 producing activity of  $\alpha$ GC-pretreated iNKT cells is not



restored by Cbl-b deficiency. Since it has been reported that IL-4 production is reliant on the  $\text{Ca}^{2+}$ -dependent NFAT2 mediated signaling pathway (33), and that Erg2, a target of NFAT, supports iNKT cell survival (34), the regulatory mechanisms of hyporesponsiveness of anergized iNKT cells to produce cytokines may be distinct.

It has been well documented that PD-1 expression induced by antigenic stimulation plays an important role in halting the excessive responses concomitant with the upregulation of PD-1 ligands, such as PD-L1 and PD-L2, on various cell types, including DCs (35). Recently, two groups have reported that PD-1 also regulates iNKT cell anergy induction and maintenance (15,17). However, the involvement of PD-1 in the maintenance of the anergic state of pre-activated iNKT cells is controversial in these studies. Our result showing that anti-PD-1 mAb has only a minimal effect on the cytokine production by anergized iNKT cells is consistent with that by Parekh *et al* (17), but we were unable to confirm the contribution of PD-1 in iNKT cell anergy induction *in situ*. In addition, stimulation with immobilized anti-CD3 mAb and anti-CD28 mAb induced PD-1 KO iNKT cell anergy comparable to that of WT iNKT cells *in vitro* (data not shown), demonstrating that PD-1 is not involved in iNKT cell anergy induction. It is not clear why the results are inconsistent. In previous studies, mice were treated ip with 2  $\mu\text{g}$  of  $\alpha\text{GC}$  a month before the start of the experiment, instead of iv injection with 5  $\mu\text{g}$  of  $\alpha\text{GC}$  9 d previously as in our experiments. Therefore, the anergic state

induced by a weaker stimulation may not last long, and responsiveness may be rescued by blocking PD-1/PD-L interaction, leading to the apparent recovery from anergy.

Like PD-1, BTLA is a co-inhibitory molecule belonging to the Ig superfamily (36). BTLA deficiency counteracts the induction of oral tolerance mediated by T cells (37) and also enhances the iNKT cell response to produce cytokines (38). Experiments still need to be carried out to elucidate the involvement of BTLA in the induction and maintenance of iNKT cell anergy. However, we suspect that BTLA is involved, since strong stimulation via TCR with costimulation is required for the induction of iNKT cell anergy, which is shown in the present study. It may be possible that BTLA interferes with CD28-mediated signaling in the activation of iNKT cells as PD-1 (36).

Unlike naïve T cells, iNKT cells express cytokine mRNAs when in the steady state and secrete them immediately after the antigenic stimulation, followed by the anergy to the further activating stimuli (26). Our *in vitro* culture system to induce iNKT cell anergy clearly demonstrated that, in contrast to naïve T cells, iNKT cells required a strong activation signal together with TCR and costimulation. There appeared to be a correlation between anergy induction of iNKT cells *in vitro* and the production of cytokines *in vivo*. In addition, although naïve T cells stimulated by ionomycin alone, which induces  $\text{Ca}^{2+}$  influx, became anergic

without IFN- $\gamma$  production, iNKT cells did not become anergic without concomitant stimulation with PMA. These results strengthen the possibility of the requirement for costimulation.

Parekh *et al* have reported that  $\alpha$ GC-loaded DCs did not induce NKT cell anergy, whereas  $\alpha$ GC-loaded B cells were potent inducers of iNKT cell anergy (14). Our results also demonstrated that  $\alpha$ GC-loaded B cells induced iNKT cell anergy after a single injection. However,  $\alpha$ GC-loaded spleen DCs were much more potent than B cells. In addition, similar results were obtained when LPS-activated BM-DCs were used (data not shown). The discrepancy with the results by Parekh *et al* is not clear. We thus conducted experiments using exactly the same protocol, but the results showed again that  $\alpha$ GC-loaded spleen DCs were able to induce iNKT cell anergy. Using marginal zone B cells that expressed the highest level of CD1d (4), Bialecki *et al* have recently reported that a single injection of  $\alpha$ GC-loaded cells induced cytokine production by iNKT cells *in vivo*, although they were unable to activate iNKT cells *in vitro* (31). Therefore,  $\alpha$ GC could be released from B cells and presented by endogenous DCs *in vivo* (39). This is evidently feasible, since IL-12p70 production was induced, possibly from DCs, when a large number of  $\alpha$ GC-loaded B cells was injected. Intriguingly, it has been shown that iNKT cells become anergic after the rapid activation and proliferation by BCG infection, although specific T cell priming is not affected (40). When

microbes are injected *in vivo*, phagocytic APCs, such as DCs, present protein antigens to T cells and lipid antigens iNKT cells. Microbes containing various kinds of pathogen-associated molecular patterns activate DCs, leading to upregulation of presentation of microbial-derived lipid antigens and self-antigen, such as iGb3, to iNKT cells (41). These results are consistent with our results presented in this study and support our hypothesis regarding the role of DCs in the induction of iNKT cell anergy.

All in all, the present study clearly demonstrates that, in contrast to conventional T cells, DCs are the most potent APCs not only for activating iNKT cells but also for subsequently inducing their unresponsiveness to the following antigenic stimulation., particularly the production of IFN- $\gamma$ . Further studies will be needed to clarify the exact regulatory mechanism for IFN- $\gamma$  and IL-4 production in anergized iNKT cells and the anergy-inducing mechanism by strong TCR signals together with costimulation.

### **Abbreviations**

$\alpha$ GC: alpha-galactosylceramide, BM: bone marrow, BTLA: B and T lymphocyte attenuator, CBA: cytometric bead array, Cbl-b: Casitas B-lineage Lymphoma-b, DC: dendritic cell, Erg2: early growth response 2, IM: ionomycin, iGb3: isoglobotrihexosylceramide, MACS: magnetic cell sorter, MZB: marginal zone B, NFAT: nuclear factor of activated T cells, NKT: natural killer T, PD-1: programmed death-1

### **Funding**

This work was supported by Japan Science and Technology Agency [17790263 to T.I.], a Grant-in-Aid for Scientific Research [18390121 and 20390109 to K.I.], a Grant-in-Aid for Scientific Research on Priority Area [20060013 to K.I.] from the Ministry of Education, Science, Sports and Culture of Japan, and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency. KM is a recipient of the Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

### **Acknowledgements**

We thank Drs T. Honjo (Kyoto University) and H. Yagita (Juntendo University) for providing PD-1 KO mice and purified anti-PD-1 mAb, respectively.

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### Figure legends

**Fig. 1. Exogenous IL-2 rescues IL-4, but not IFN- $\gamma$  production, of pre-activated iNKT cells.**

(A) Purified spleen and liver iNKT cells ( $10^4$ ) obtained from the mice that had been injected with 5  $\mu$ g of  $\alpha$ GC or vehicle 9 d beforehand were cultured with the same number of spleen DCs in the presence or absence of 100 ng/ml  $\alpha$ GC for 3 d, and cytokine production was then measured. (B) Spleen iNKT cells from mice treated as (A) were stained with the indicated mAbs (shadow). Open histograms indicate staining with isotype matched mAbs. (C) Purified spleen iNKT cells from mice treated as (A) were cultured with or without stimuli for 1 d and stained with anti-IL-2 receptor  $\alpha$  or isotype match mAb. (D) The cytokine production ability of pre-activated iNKT cells in the presence or absence of IL-2 and IL-15. iNKT cells were cultured as in (A) and cytokine production was then assessed 3 d later. Proliferation was determined by  $^3$ H-TdR incorporation between 60-72 h of culture. (E) The cytokine production ability of pre-activated iNKT cells was compared with non-stimulated iNKT cells by real-time PCR. All data are representative of three or four independent experiments.

**Fig. 2. PD-1 is not essential for inducing iNKT cell anergy.**

(A) Phenotype change of iNKT cells by *in vivo* stimulation. Spleen cells gated on



$\alpha$ GC/CD1d-Ig<sup>+</sup> CD19<sup>-</sup> were analyzed for the expression of the surface molecules indicated in the histogram. (B) As in Fig. 1, purified spleen iNKT cells were stimulated in the presence of anti-PD-1 mAb (RMP1-14, 10  $\mu$ g/ml) or isotype matched mAb for 3 d, and cytokine production was then measured. (C) Nine days after injecting 5  $\mu$ g of  $\alpha$ GC or vehicle, WT and PD-1 KO mice were challenged with the same dose of  $\alpha$ GC and bled at the indicated time points. Serum cytokines were measured by CBA kit (BD Biosciences). (D) Reduced responsiveness of iNKT cells in PD-1-deficient mice by the injection of 5  $\mu$ g  $\alpha$ GC 9 d previously. Spleen iNKT cells in PD-1-deficient mice that had been treated as in Fig. 1 were restimulated *in vitro* with spleen DCs and  $\alpha$ GC for 3 d, and cytokine production was then assessed by ELISA. All data are representative of three or four independent experiments.

**Fig. 3. Induction of spleen iNKT cell unresponsiveness *in vitro*.**

(A) Spleen NKT ( $2 \times 10^5$ /well) cells were stimulated with graded doses of immobilized anti-CD3 mAb in 96-well round-bottomed culture plates for 16 h and allowed to rest for 2 d after the wash, followed by restimulation at  $5 \times 10^3$  cells/well with  $10^4$  spleen DCs in the presence or absence of 100 ng/ml of  $\alpha$ GC for 3 d. Cytokines in the culture supernatants at 16 h of the first culture (3 left panels) and 3 d of the restimulation culture (3 right panels) were assessed. As in (A), spleen NKT cells (B) and OT-II T cells (C) were stimulated with immobilized anti-CD3 mAb at 10  $\mu$ g/ml in the presence of graded doses of anti-CD28 mAb

for 16 h. After 2 d rest, recovered NKT and OT-II T cells were cultured with spleen DCs in the presence or the absence of 100 ng/ml  $\alpha$ GC or 1  $\mu$ g/ml of OT-II specific OVA peptide for 3 d, and cytokines in the culture supernatants were then measured.

**Fig. 4. Requirement of complete stimulation to induce iNKT cell unresponsiveness.**

Spleen NKT cells and OT-II T cells were stimulated with 1 mg/ml of ionomycin with or without 50 ng/ml PMA for 16 h (A), and then restimulated with spleen DCs in the presence or absence of  $\alpha$ GC or OVA peptide for 3 d (B). Cytokine production was determined as in Fig. 3.

All data are representative of two or three independent experiments.

**Fig. 5. Requirement for APCs to anergize iNKT cells *in vivo*.**

(A) Spleen CD11c<sup>+</sup> DCs were pulsed with or without graded doses of  $\alpha$ GC for 1 h at 37°C.

After washing,  $2.5 \times 10^6$  cells were injected iv, and iNKT cell responsiveness was determined

by restimulation, as in Fig. 1. (B) Spleen CD11c<sup>+</sup> DCs and CD19<sup>+</sup> B cells were pulsed with or

without 2.5  $\mu$ g/ml of  $\alpha$ GC for 1 h at 37°C. After washing, graded numbers of them were

injected iv and the cytokines produced in the sera were determined at various time points

using CBA. Each dot represents the mean cytokine activity of 3 mice. (C) Nine days after the

injection of  $\alpha$ GC-pulsed DCs and B cells as in (B), the responsiveness of spleen iNKT cells

was measured as in (A). A representative result of three independent experiments is shown.

Fig. 1  
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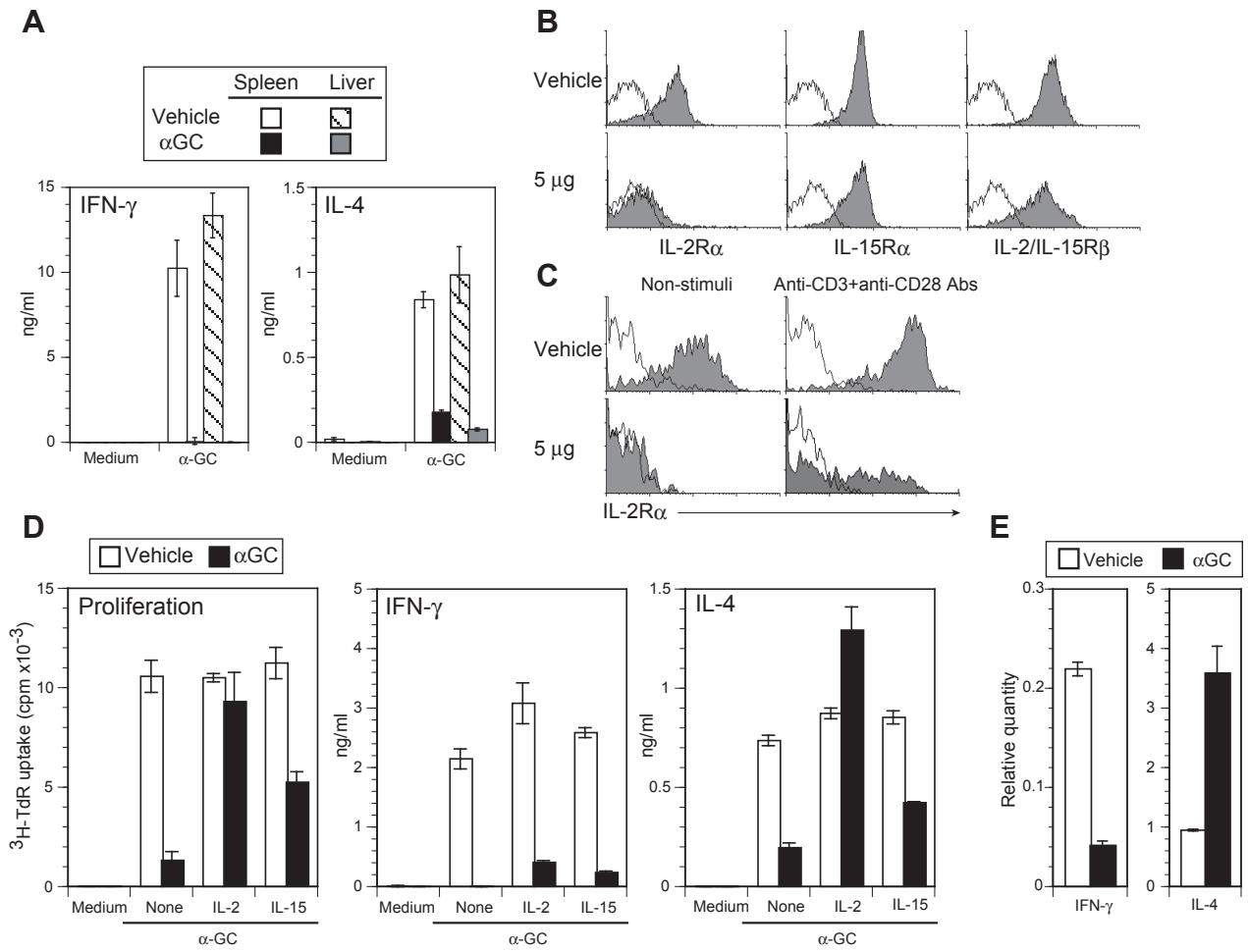


Fig. 2  
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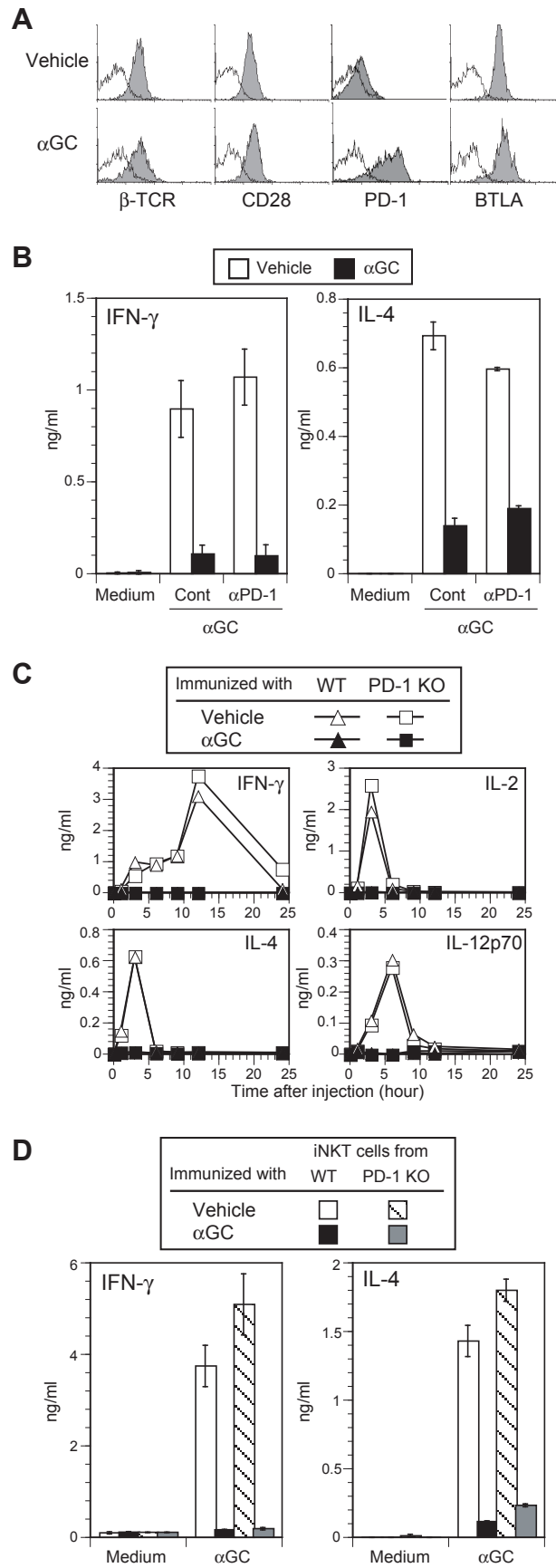


Fig. 3  
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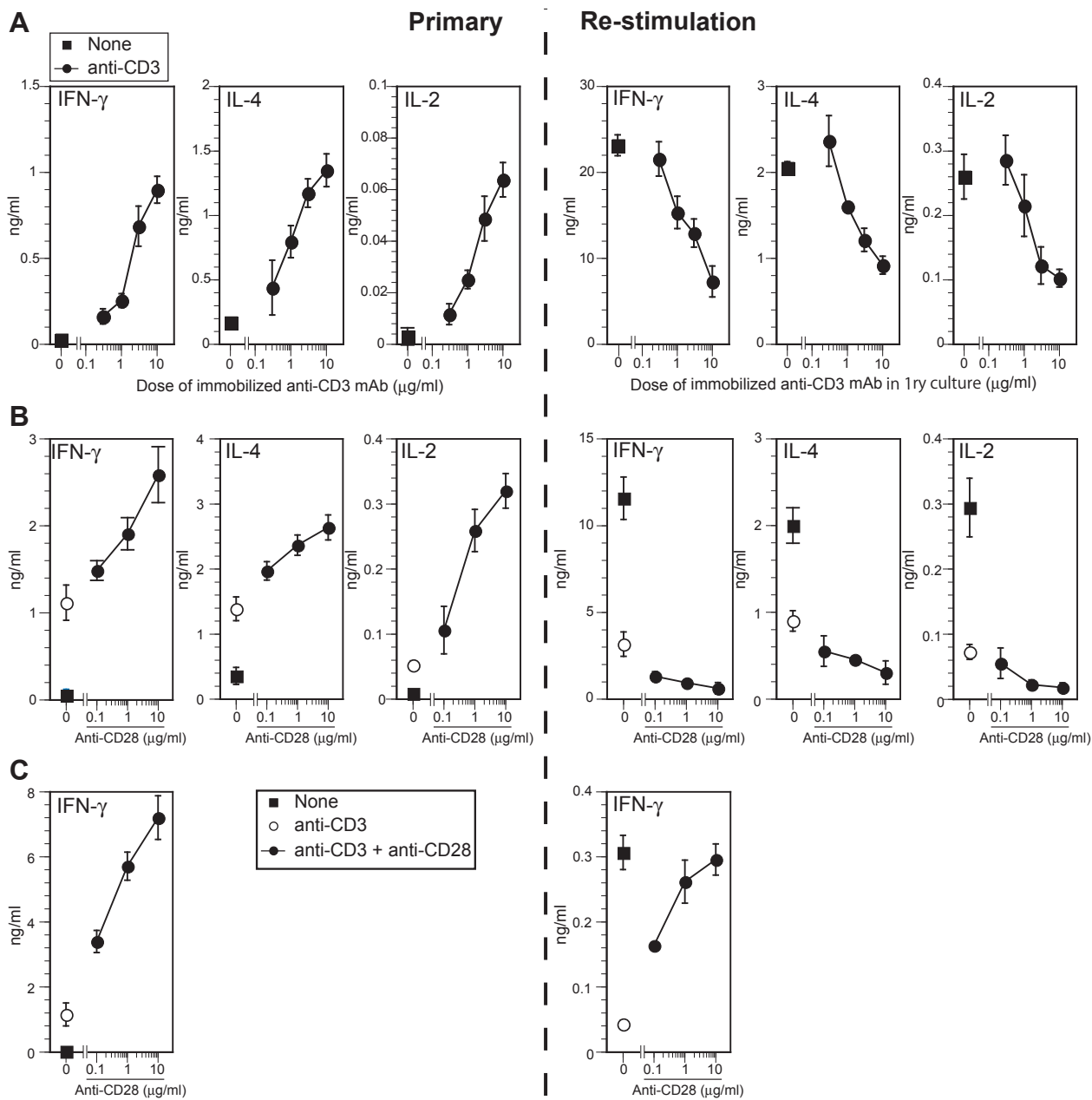


Fig 4  
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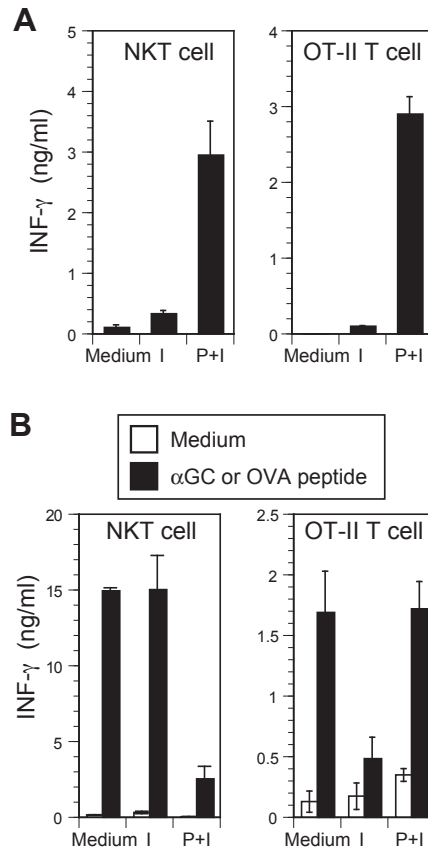


Fig. 5

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