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De novo generation of CD1d1-deficient NKT-cell hybridomas from CD1d$^{-/-}$ mice or by gene editing of CD1d^{+} hybridomas

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Objective: NKT cell hybridomas have provided a useful platform to exploit ligands and to examine cellular signaling, function, and interactions. These hybridomas express the antigen-presenting molecule CD1d and thus permit autopresentation of the ligands. This property provides an advantage when an exogenous ligand is tested, but it is a disadvantage when testing cells that express endogenous antigen in the context of CD1d. To overcome this limitation, we attempted the *de novo* generation of CD1d^{-} NKT-cell hybridomas to analyze the functions of autoreactive NKT cells in a controlled manner.
Methods: To obtain hybridomas that do not express CD1d, two methods were employed: 1. standard cell fusion of CD1d^{-} NKT cells and CD1d^{-} BW1100 cells, and 2. disruption of the *cd1d1* gene in 2E10 NKT hybridoma cells. For both procedures, the CRISPR/Cas9 technique was utilized.
Results: CD1d^{-} NKT-cell hybridomas lost the capacity of autopresentation but could be stimulated with α-galactosylceramide (α-GC) in the presence of antigen-presenting cells to produce IL-2.
Conclusion: For the first time, CD1d^{-} NKT-cell hybridomas were established and characterized for their application in ligand discovery and studies of cellular interactions in the absence of autopresentation.

Key words: NKT cells, T cell hybridomas, CD1d, lipid antigens, gene editing, CRISPR/Cas9

Abbreviations: Ag, antigen; APC; antigen-presenting cells; CD, cluster of differentiation; NKT, natural killer T; dNKT, diverse NKT; iNKT, invariant NKT; vNKT, variant NKT; α-GC, α-galactosylceramide; IL-2, interleukin-2; CRISPR/Cas9, clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9; TCR; T-cell antigen receptor; iTCR, invariant TCR; GFP, green fluorescent protein; mAb, monoclonal antibody

Introduction

Natural killer T (NKT) cells recognize glycolipid antigens (Ag) in a CD1d-restricted manner and modulate immune response through production of various cytokines.[1] NKT-cells consist of two subsets. The first is an invariant NKT (iNKT)-cell subset that expresses invariant Vα14Jα18 chains in mice or Vα24Jα18 chains in humans, and recognizes α-GC as a prototype glycolipid Ag. The second is a diverse (dNKT)- or variant NKT (vNKT)-cell subset that expresses diverse or oligoclonal T cell receptors (TCR) and recognizes glycolipid Ags such as sulfatide or lysophosphatidylcholine, or, in some cases, peptides.[2,3] Although iNKT-cells constitute about 60% of all NKT cells, it is difficult to be obtain sufficient numbers of these cells to perform functional analyses because NKT-cells are a relatively rare subset of T cells.[4] iNKT-cell hybridomas (hereafter called NKT hybridomas for short) serve as an alternative to perform functional studies by gene transduction[5] and to search for novel ligands.[6]

A fusion partner thymoma cell line, BW1100 (TCR$\alpha\beta^{-}$), which was derived from BW5147 thymoma (TCR$\alpha\beta^{+}$) cells[7] and has been used for preparing T-cell

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hybridomas, is CD1d positive, and is thus able to present glycolipid Ags to NKT cells. Because NKT cells also express low levels of CD1d,[8] the resultant NKT hybridomas express CD1d and are able to autopresent Ag. The capacity for autopresentation is useful when testing ligand activity with potential therapeutic activities in bioassays because there is no need to use specific antigen presenting cells (APC). In contrast, to study interactions between NKT hybridoma cells and CD1d-expressing cells that may express endogenous ligand(s), autopresentation may make it challenging to distinguish between direct activation of NKT by CD1d-lipid complexes on APC or via the ligands produced by the APCs and presented on CD1d in the hybridomas. In the present study, we attempted to prevent this autopresentation pathway by disrupting the CD1d gene in NKT cell hybridomas in two ways: 1. with the use of radiation bone-marrow chimeras to generate CD1d-deficient NKT cells followed by fusing with CD1d-deficient lymphoma cells, and 2. with a recent gene-editing technique[9] so as to induce CD1d-deficiency in established CD1d-expressing hybridomas. We discuss the possible utility of the hybridomas for NKT-cell research.

Materials and Methods

Cells

A thymoma cell line, BW1100 cells[7] which were provided by Willi Born, PhD, (National Jewish Medical and Research Center, CO, USA), CD1d-disrupted BW1100 cells, and established hybridomas were cultured in a complete medium (RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M β-mercaptoethanol) under 5% CO_2 at 37℃.

Transfection of CRISPR/Cas9 plasmid and establishment of CD1d-negative (CD1d-) BW1100 cells and 2E10 NKT hybridoma

Cas9-expressing plasmid pCMV-Cas9-GFP, which also expresses CD1d1/d2 guide RNA under the U6 promoter, was purchased from Sigma (St. Louis, MO, USA) and introduced to BW1100 by electroporation (Bio-Rad Laboratories Inc., Hercules, CA, USA) with 200V/975 μF. After purification of the GFP^+ cells using cell sorting (FACS Aria, BD Biosciences, NJ, USA), the $CD1d^-$ BW1100 clone was selected by limiting dilution, and was confirmed using flow cytometric and sequence analyses. The disruption of CD1d gene in 2E10 NKT hybridomas[8] were also performed by transfection of CRISPR/Cas9 plasmid as described above.

Mice and irradiated bone-marrow chimeras

B6-GFP mice[10] and $B6.CD1d^{-/-}$ mice[11] were provided by Prof. Masaru Okabe, PhD, (Osaka University) and Prof. Luc Van Kaer, PhD, (Vanderbilt University, TN, USA), respectively, and maintained in specific pathogen-free conditions at the animal facility of the Center for Genetic Studies of Integrated Biological Functions at Kitasato University School of Medicine. Irradiation bone marrow chimeras in mice were prepared by transferring bone-marrow cells into irradiated host mice as previously described.[12] In brief, bone marrow cells from donor mice were treated with anti-Thy1.2 mAb + complement (Cedarlane, Ontario, Canada) to remove mature T cells and intravenously transferred to lethally irradiated (9 Gy) host mice. Irradiation bone-marrow chimeras prepared as above were represented as [donor strain → host strain] and chimeras were administered via drinking water with oxytetracycline for 4 weeks. All experimental procedures on mice conformed to the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine (2017-143, 2018-119, 2019-024).

Establishment of hybridomas

For fusion with BW1100 cells, $CD1d^-$ iNKT cells were prepared from thymi and spleens obtained from irradiated bone-marrow chimeras, [$B6.CD1d^{-/-}$ + B6-GFP → $B6.CD1d^{-/-}$]. The thymocytes and splenocytes were cultured in the presence of α-GC (Funakoshi, Tokyo) and the activated iNKT cells were further expanded in the presence of IL-2 (300 U/ml; PeproTech Inc, NJ, USA). Then, the cells were fused with $CD1d^-$ BW1100 clone 1 (#1) at a 10:1－5:1 ratio with PEG1500 (Roche, Basel, Switzerland) and the hybrid cells were cultured for HAT selection. The selected cells were cloned with limiting dilution and tested for the negative expression of both GFP and CD1d, implying that the NKT cells were of the $B6.CD1d^{-/-}$ origin. The sequences of TCR V (TRAV/TRBV) and J (TRAJ/TRBJ) genes were determined for each clone (Repertoire Genesis, Osaka, Japan).

Flow cytometry

BW1100 and hybridoma cells were first incubated with 2.4G2 mAb (anti-FcγRIII/II) to block non-specific binding of antibodies, and later stained with the following mAb: allophycocyanin/Cy7-conjugated anti-TCRβ (H57-597, BioLegend, CA, USA), allophycocyanin-conjugated anti-NK1.1 (PK136, BioLegend), anti-CD44 (IM7, BioLegend), phycoerythrin-conjugated anti-α-GalCer: CD1d complex mAb (L363, BioLegend), α-GalCer

(PBS-57)-loaded CD1d tetramer that was kindly provided by NIH Tetramer Core Facility at Emory University (Atlanta), and FITC-conjugated anti-CD69 (H1.2.F3, BioLegend). Stained cells were assessed using FACS Verse flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (FlowJo, LLC, CA, USA).

Sequence analyses

The disrupted allele of *cd1d1* was analyzed using genomic sequencing with primer pairs that cover the target sequence to confirm the deletion: forward, *cagagcctttgtgtaccagtccg*; reverse, *agtcttctttaggtgacatcatt*. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on Genetic Analyzer 3100 (Applied Biosystems, MA, USA).

Functional analyses of NKT hybridomas

For autopresentation of ligands, 2E10 or CD1d$^-$ NKT hybridomas were cultured in complete medium at a density of 1×10^5 or 2×10^5 cells/well in a 96-well flat-bottomed culture plate (BD Bioscience), with α-GC (0-100 ng/ml for dose escalation experiments or 100 ng/ml for usual experiments) for 24 hours. The hybridomas were also co-cultured with α-GC-pulsed RBL-CD1d cells as APC, with phorbol 12-myristate 13-acetate (PMA) at 50 ng/ml and ionomycin at 500 ng/ml, or stimulated for 24 hours with immobilized α-GC-loaded CD1d-Ig fusion protein (Dimer X; BD Bioscience) on a well of a flat-bottomed 96-well plate at 10 μg/ml in 0.1 M $NaHCO_3$ for coating. The latter two methods depend on APC-free stimulation for NKT hybridomas. The concentration of IL-2 in the culture supernatant was quantified using the BD Cytometric Bead Array (CBA) according to the manufacturer's protocol.

Results

CD1d$^+$ NKT hybridoma cells are activated with α-GC in the absence of APCs.

The 2E10 NKT hybridoma cells were readily stimulated by the addition of α-GC in culture. 2E10 expressed CD69 and produced IL-2 in a dose-dependent manner with increasing concentration of α-GC added to the cultures (Figure 1A, B). Although it is convenient to assay iNKT-cell ligands without the need for exogenous APC, autopresentation may, at least in some cases, be unfavorable in such assays. To avoid this potential concern, we initiated experiments to remove CD1d expression from hybridomas.

Generation of CD1d-negative (CD1d$^-$) NKT cells

1. Preparation of CD1d$^-$ BW1100 thymoma cells

To prepare the CD1d$^-$ NKT hybridomas, we employed two different ways of establishing the cell lines. One method was to utilize iNKT cells derived from CD1d$^{-/-}$ mice, and the other was to directly disrupt CD1d in an NKT hybridoma, 2E10, that is already available. For the former method, we needed to prepare CD1d$^-$ BW1100

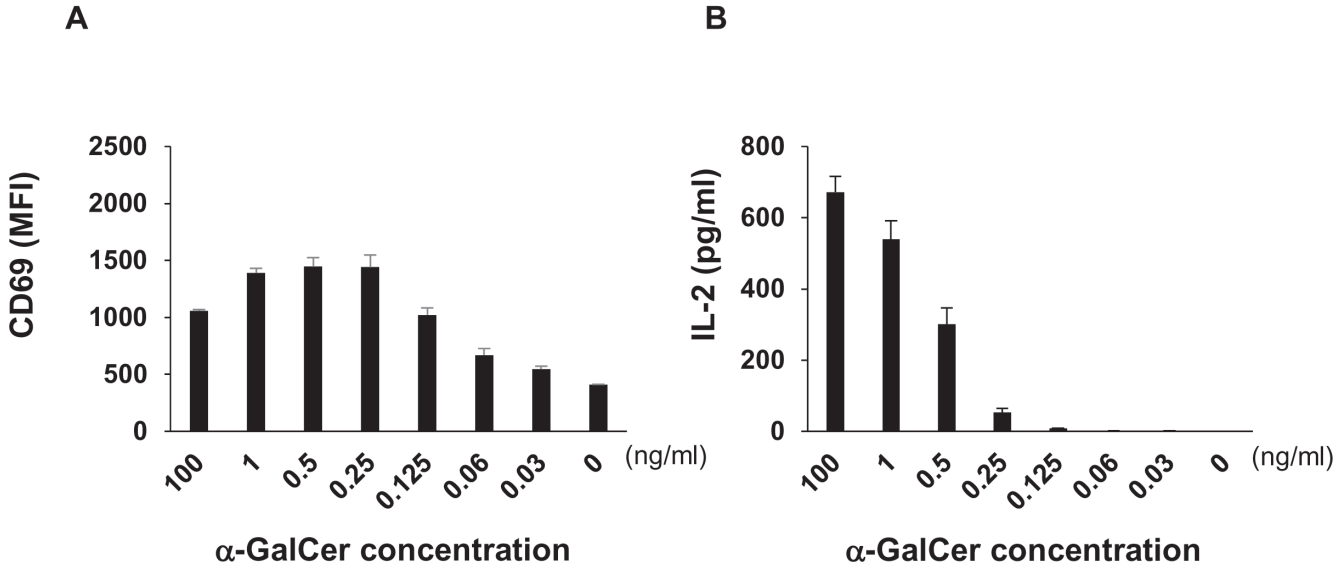

Figure 1. Antigen presenting cell-free stimulation of an NKT cell hybridoma, 2E10

CD69 expression (**A**) and IL-2 production (**B**) of 2E10 were assayed at each concentration of α-GC (0－100 ng/ml) cultured for 24 hours. CD69 expression was analyzed by flow cytometry and IL-2 production was quantified with CBA (Materials and Methods). Representative data of three similar results are presented. Representative data from at least three independent experiments are shown. Data are shown as mean ± SD from triplicate.

cells to fuse with CD1d^{-} iNKT cells. For this purpose, CD1d1 guide RNA and CRISPR/Cas9 plasmids were introduced into BW1100 cells, and CD1d^{-} clones were obtained by limiting dilution. Each deletion and/or addition of bases in the *cd1d1* genome was confirmed using sequencing (Figure 2). The BW1100 clones 1 (#1) and 4 (#4) both have a premature stop codon that destroys the CD1d1 molecules due to frameshifts on both alleles. Clones #2 and #3 had deletions of 3 ×N bases indicating that the products were 'in-frame', but just lacked one amino acid, or contained an insertion of different amino acids that presumably led to protein degradation, reflecting the lack of surface expression of CD1d on each clone (Figure 3A). Meanwhile, the morphology and proliferation of the cells appeared indistinguishable to the parent BW1100 cell line (data not shown). Ag presenting capacity was examined by culturing 2E10 hybridoma cells and parental BW1100 or CD1d^{-} BW1100 clones pulsed with α-GC. The parent BW1100 cells could present α-GC to 2E10 hybridoma cells to induce IL-2 production, whereas none of the CD1d^{-} BW1100 clones (#1－4) were able to activate 2E10 (Figure 3B). The interaction between 2E10 and α-GC-pulsed BW1100 was inhibited by a monoclonal antibody, L363, which binds the α-GC/CD1d complex, suggesting that the interaction indeed was CD1d-restricted. The above results clearly demonstrated the absence of CD1d expression in the functional assays of BW1100 cells, although the sequence information (deletion and/or addition of bases) of the *cd1d2* gene remained to be determined.

2. Establishment of CD1d^{-} NKT cells and hybridomas from irradiation bone-marrow chimeras via *de novo* cell fusion

To prepare NKT cells that do not express CD1d, bone-marrow chimeras were established by transferring 1:1 mixture of bone marrow cells obtained from B6.CD1d$^{-/-}$ and B6-GFP Tg mice into lethally-irradiated (9 Gy of X-ray) B6.CD1d$^{-/-}$ mice. Following this transfer, the thymocytes that underwent Vα14Jα18-rearrangement

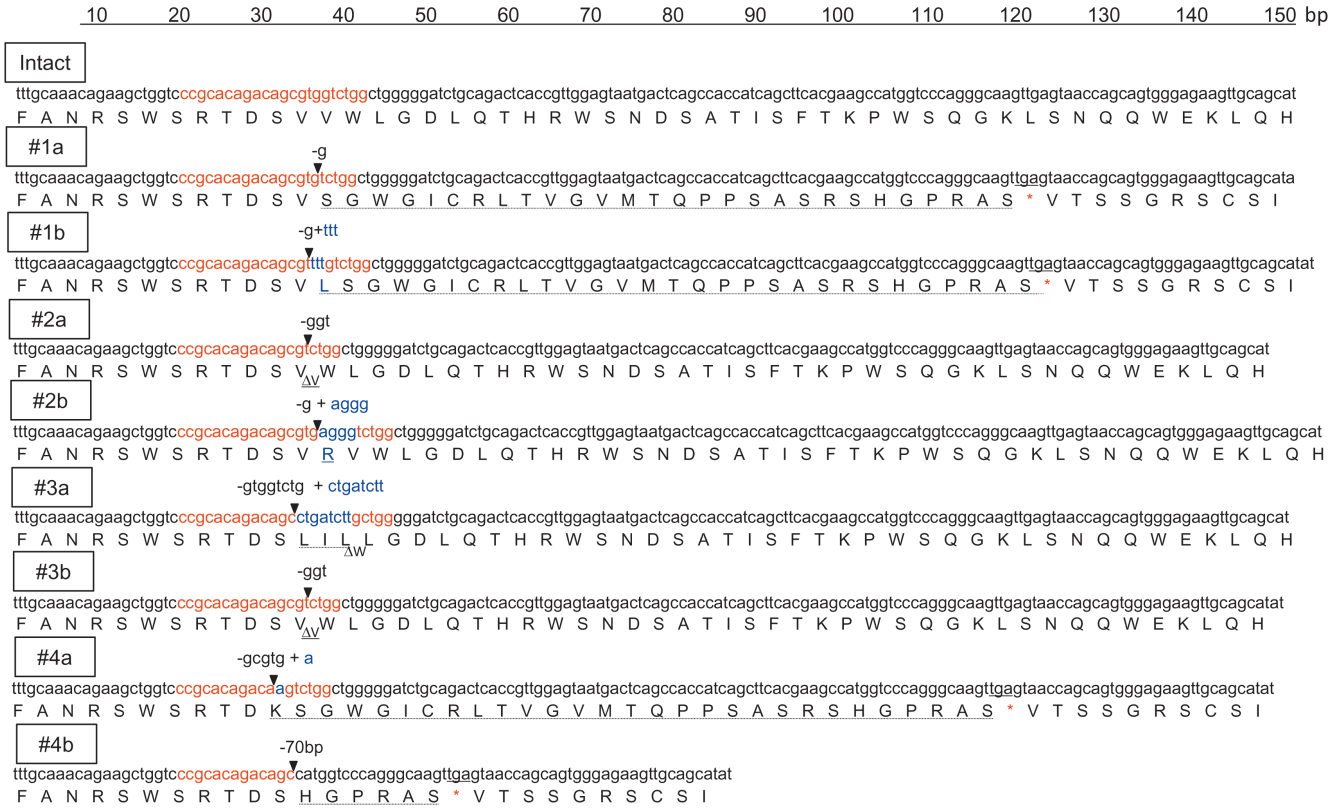

Figure 2. Sequence analyses of *cd1d1* gene disrupted in CD1d^{-} BW1100 clones

Intact genome sequence in parent BW1100 is described as "Intact" on top and red letters correspond to the target site in gRNA for Cas9 nuclease of pCMV-Cas9-GFP to disrupt the *cd1d1* gene. Deletions and/or insertions with variable numbers in each clone on both alleles (a and b) were demonstrated. Blue letters correspond to inserted base as shown + nnn and deleted bases are shown in black as - nnn. A single deletion of an amino acid (Aa) residue deleted by the gene editing approach is shown as ΔX below. The Aa sequence with a dotted underline corresponds to one generated by a frame shift; *: Stop (underlined base sequence = stop codon); arrowhead: site of base deletion/addition.

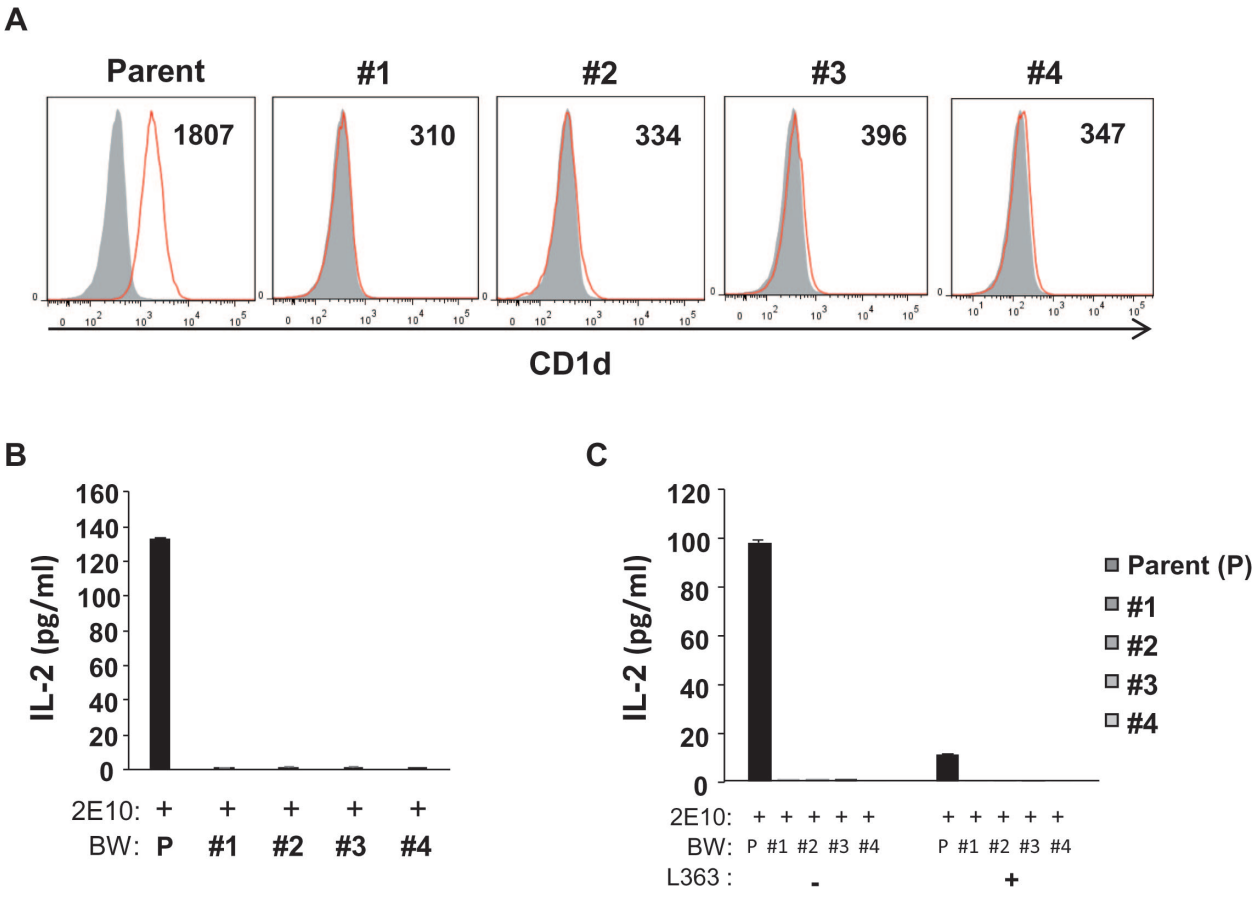

Figure 3. Characterization of CD1d$^-$ BW1100 clones

A. CD1d expression in parent BW1100 and CD1d1-disrupted clones (#1－4) of BW1100. The number in the panel is mean fluorescence intensity of surface CD1d expressed by each clone. Gray histograms represents isotype control and red lines represent CD1d surface expression by each clone. **B**. IL-2 production by 2E10 cells cultured with parental (P) BW1100 (BW) or CD1d1-disrupted clones (#1－4) of BW1100 as APCs. Parental cells and cells of clones #1－4 were incubated with α-GC for 2－3 hours, then thoroughly washed and cultured with 2E10 for 24 hours. IL-2 in the culture supernatant was quantified with CBA. **C**. IL-2 production by 2E10 cells cultured with parental (P) BW1100 (BW) or CD1d1-disrupted clones (#1－4) of BW1100 as APCs in the absence (-) or presence of a monoclonal antibody, L363. 2E10 and BW1100 cells as APCs were cultured and IL-2 concentration in the culture supernatant was quantified as described above. Representative data from at least two independent experiments are shown. Data are shown as mean ± SD.

Table 1. Sequence analyses of CD1d$^-$ NKT-cell hybridomas of *de novo* origin

Clone	TRAV/TRAJ	CDR3 Sequence	TRBV/TRBJ	CDR3 Sequence
T8	11/18	CVVGDRGSALGRLHF	13-1/1-1	CASRRTGPTEVFF
S16			5/2-3	CASSQDWGGAETLYF
S25			13-2/2-7	CASGDKGASSYEQYF
S26			13-2/1-3	CASGRDGNTLYF

Representative clones were analyzed for T cell antigen receptor (TCR) rearrangement. TRAV/TRAJ and TRBV/TRBJ reads are summarized. All clones expressed TRAV11/TRAJ18 (Vα14Jα18) as invariant Vα chain. Three clones employed TRBV13 (Vβ8) and one employed TRBV5 (Vβ5) as Vβ chain to constitute invariant TCR. The CDR3 of 2E10 Vβ chain was previously reported as: CASSTGTEVFF (13-3/1-1 in the present nomenclature).[8]

on the Vβ8/7/2$^+$ cells from the CD1d$^{-/-}$ mice were selected by CD1d expressed on CD4$^+$8$^+$ (double positive; DP) thymocytes from the B6.GFP mice to become iNKT cells (Figure 4A). First, we attempted to purify CD1d$^-$ iNKT cells from the thymus of chimeric mice at more than 8 weeks after transfer, and then stimulated them with α-GC. However, the level of iNKT cell purity obtained was not sufficient. Therefore, we employed a more straightforward method by using whole thymocytes or splenocytes to stimulate with α-GC and to perform fusion experiments with CD1d$^-$ BW1100 clone #1 that harbored a premature stop codon on both alleles of *cd1d1*, followed by limiting dilution of the hybrid cells. GFP- hybridoma cells were selected and confirmed for negative CD1d expression (Figure 4B). Both NKT hybridomas of thymic (T#) and splenic (S#) origin were generated and cloned by limiting dilution. Representative profiles of flow cytometric analyses of NKT hybridomas are shown in

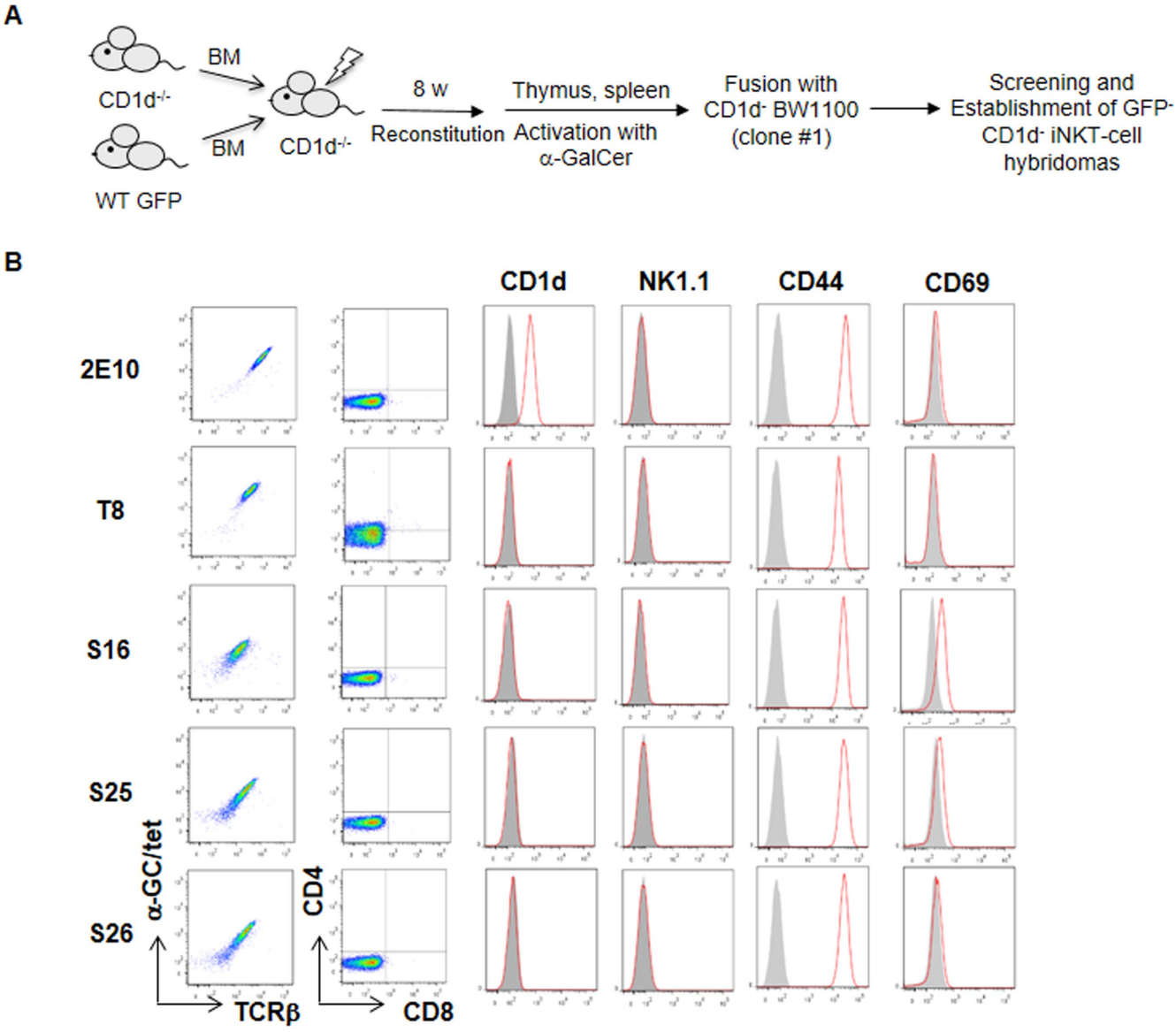

Figure 4. Generation and characterization of GFP$^-$CD1d$^-$ NKT-cell hybridomas

A. Establishment of CD1d$^-$ NKT-cell hybridomas with the use of mixed radiation bone marrow chimeras [CD1d$^{-/-}$ (donor) + B6. GFP (donor) $\rightarrow$ CD1d$^{-/-}$ (host)]. Lethally irradiated CD1d$^{-/-}$ mice were reconstituted with T-cell-depleted bone-marrow cells of CD1d$^{-/-}$ and B6.GFP mice (1:1 mixture). After 8 weeks thymocytes and splenocytes were prepared, stimulated *in vitro* with α-GC, and fused with CD1d$^-$ BW1100 cells. **B**. Flow cytometric analyses of 2E10 and each clone of GFP-CD1d- NKT-cell hybridomas. T8 clone was derived from thymocytes and S16, S25, S26 were derived from splenocytes respectively. Representative clones were also stained with fluorochrome-conjugated anti-NK1.1, anti-CD44, and anti-CD69 antibodies. All clones were positive for both anti-Vβ (H57-597) and α-GC-loaded-CD1d tetramer staining and lacked both CD4 and CD8 expression (double negative; DN). Representative data from at least 3 independent experiments are shown. Data are shown as mean $\pm$ SD.

Figure 4B. The α-GC/CD1d-tetramer+TCRVβ+ NKT hybridomas were CD4-8- NK1.1 (CD161c)- CD44+ CD69-~lo and all of them expressed the characteristic Vα14Jα18 invariant chain together with Vβ8.1 for two clones, or with Vβ8.3 or Vβ5 chains for one clone each (Table 1).

3. Establishment of CD1d- NKT cells and hybridomas using direct gene editing of 2E10 hybridoma cells

Another method to generate CD1d- NKT hybridomas is to directly disrupt the CD1d genome in a CD1d+ NKT hybridoma, 2E10, which we have previously employed in CRISPR/Cas9-mediated gene modification experiments as BW1100 cells. 2E10 cells were transfected with the same plasmids used to disrupt CD1d in BW1100 cells, followed by cloning out CD1d- 2E10 cells by limiting dilution. Two clones (B29 and G33) were selected for further experiments. The selected clones exhibited the same phenotype as 2E10, except for the lack of CD1d surface expression (data not shown) and harbored premature stop codons in *cd1d1* on both alleles (Figure 5).

4. Functional analyses of CD1d- NKT hybridomas

Two types of CD1d- iNKT hybridomas, prepared from either CD1d-/- iNKT cells fused with CD1d- BW1100 cells (T8, S16, S25, S26) or 2E10-derived CD1d- 2E10 hybridomas generated via CRISPR/Cas9-mediated disruption of the *cd1d* gene (B28, G33), were obtained. To evaluate reactivity of each clone, hybridoma cells (2 × 10^5 cells/well) were cultured with α-GC (100 ng/ml) for 24 hours in the absence of APC (Figure 6A) or in the presence of RBL-CD1d transfectants as APC (Figure 6B). CD1d+ 2E10 cells were activated to produce IL-2 either with or without APC, whereas CD1d- NKT hybridomas were able to produce IL-2 with α-GC only in the presence of APC but not in the absence of APC. In the case where the cells were activated with α-GC-loaded/CD1d dimers immobilized on culture plates, CD1d- NKT hybridomas produced IL-2 in the absence of APCs (Figure 6C). When CD1d- hybridomas were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, which directly activate intracellular signaling molecules, all the cells responded irrespective of CD1d expression, but responses remained variable (Figure 6D). Notably, the amount of IL-2 produced in the supernatant appeared variable among clones in the respective experiments and this variation appeared to be independent of their origin, i.e., *de novo* generation or direct disruption, in comparison with CD1d+ 2E10 cells as a control. Meanwhile, the response of clone T8 was always low, regardless of the method employed for stimulation.

Discussion

We were able to successfully establish CD1d- NKT hybridoma cells in two ways: 1. with the use of CD1d- BW1100 cells and CD1d- iNKT cells from irradiation

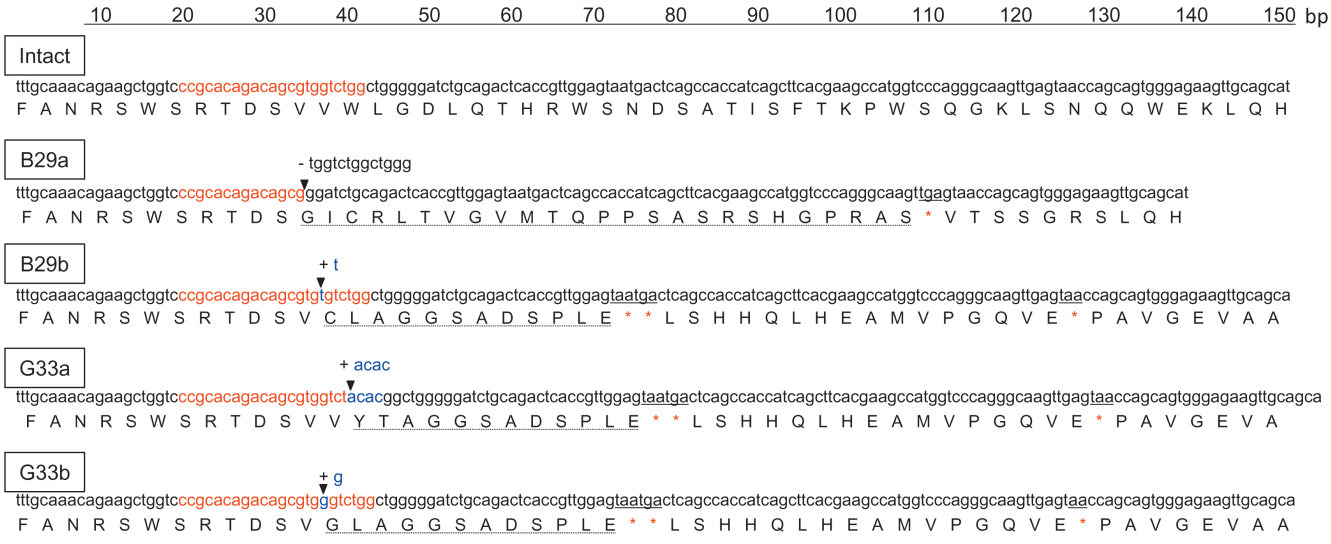

Figure 5. Sequence analyses of *cd1d1* gene disrupted in CD1d- 2E10 clones

Intact gene sequence of *cd1d1* in parental 2E10 cells is described as "Intact" on top and the red letters correspond to the target site in gRNA for Cas9 nuclease of pCMV-Cas9-GFP to disrupt the *cd1d1* gene. Explanations of deletions and/or insertions of bases with variable numbers and the consequences in each clone on both alleles (described as **a** and **b**) were demonstrated as those shown with BW1100 in Figure 2.

bone-marrow chimeras, and 2. by direct CD1d disruption in 2E10 NKT hybridoma cells with gene editing. To our knowledge, this is the first report of establishing CD1d- NKT hybridomas in the literature. The CD1d- NKT hybridomas were unable to present α-GC without APCs but were able to respond to α-GC in the context of CD1d on APCs to produce IL-2 as anticipated. Therefore, the CD1d- NKT hybridomas may provide a useful platform to test potential reactivity of NKT cells against CD1d-expressing cells *in vivo*, e.g., hepatocytes, intestinal epithelial cells, and tumor cells.

Before the CD1d- NKT hybridomas were obtained, we assumed that CD1d- hybridomas are different in terms of growth and reactivity, as a consequence of activation

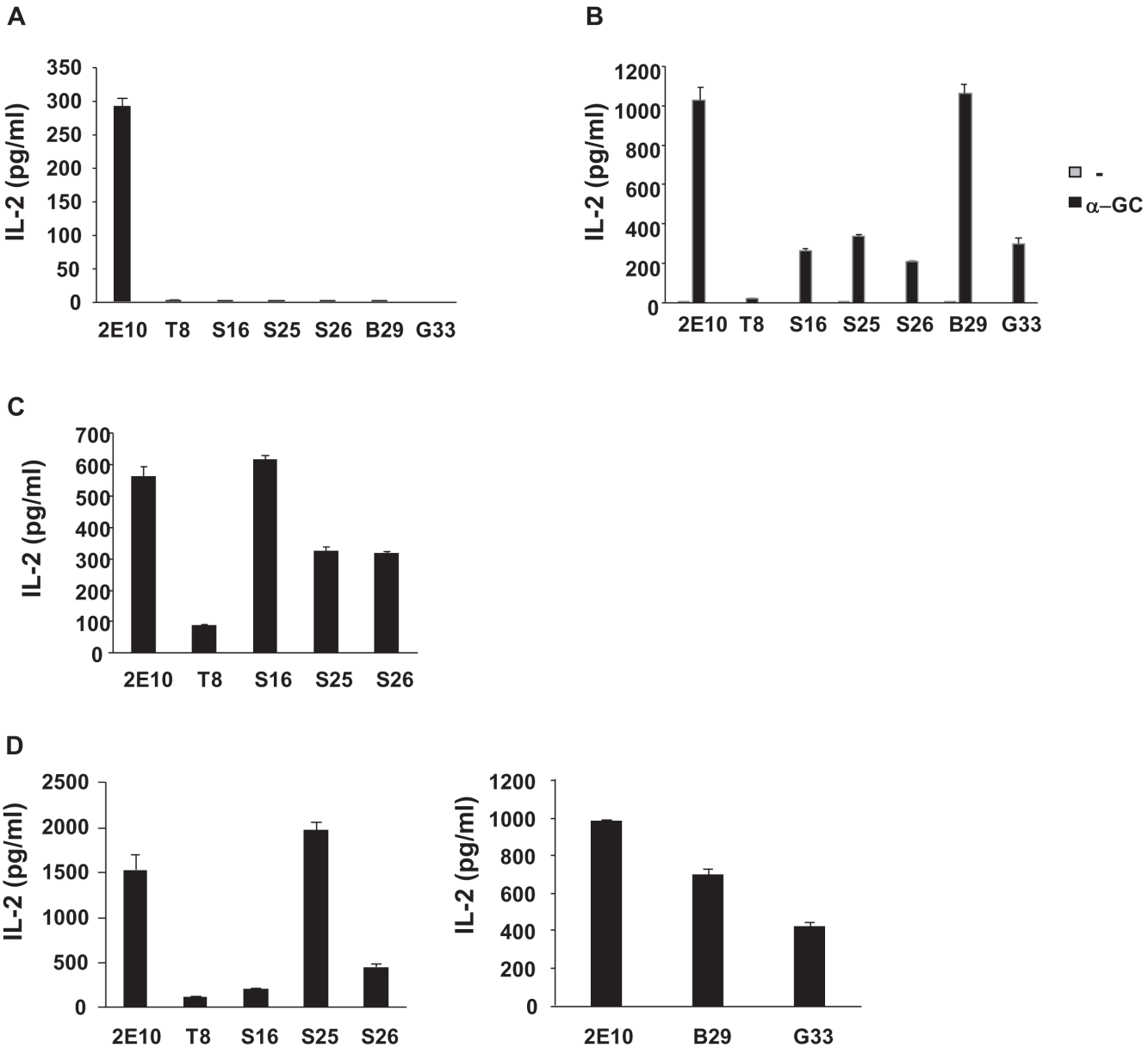

Figure 6. IL-2 production by 2E10 and CD1d- NKT-cell hybridomas obtained in two different ways with different methods of activation

A, **B**. CD1d+ 2E10 hybridomas or CD1d- NKT-cell hybridomas that were established with either fusion of CD1d- NKT and CD1d- BW1100 cells (T8, S16, S25, S26) or direct CD1d1-disruption of 2E10 with gene-editing by CRISPR/Cas9 methodology (B28, G33) (2×10^5 cells/well in triplicate) were cultured with α-GC for 24 hours in the absence of APC (**A**) or in the presence of RBL-CD1d transfectants pulsed with α-GC as APC (**B**). **C**. 2E10 and CD1d- NKT hybridomas (1×10^5 cells/well) were stimulated with α-GC-loaded CD1d-Ig fusion protein (α-GC-Dimer X) coated on flat-bottomed wells (96-well plate). **D**. The combinations of either [2E10 and CD1d- NKT hybridomas] (left panel) or [2E10 and CD1d- 2E10] (right panel) were stimulated (2×10^5 cells/well in triplicate) with PMA (50 ng/ml) and ionomycin (500 ng/ml). Representative data from at least two independent experiments are shown. Data are shown as mean ± SD from triplicate.

with ligand, as compared with CD1d$^+$ hybridomas. For example, we assumed that CD1d$^+$ NKT hybridomas were activated through recognition of endogenous ligand(s) and undergo fratricide[13,14] or activated-induced cell death.[15] However, this assumption turned out to be incorrect because we observed only small differences in growth and cell death in cultures between CD1d$^+$ and CD1d$^-$ NKT hybridomas (data not shown).

In the present study, the disruption of the *cd1d1* gene in BW1100 cells was demonstrated, whereas the *cd1d2* gene was left intact. Because CD1d2 expression is very limited, it has been reported that CD1d2 had a minimal influence on NKT cell development.[16] Functionally, the autopresentation of α-GC was not observed for CD1d$^-$ NKT hybridomas obtained in the present study, suggesting that disruption of CD1d1 with gene-editing was sufficient to impair NKT cell activation. Notably, CD1d2 has a distinct spectrum for binding of α-GC with short (C_{10}) acyl chains such as *N*-acylsphingosine, whereas CD1d1 binds α-GC with longer acyl chains (C_{26}).[17] If the autopresentation of such a ligand was seen in the CD1d1$^-$ NKT hybridomas established here, then the *cd1d2* gene would need additional characterization.

There were variations in the reactivity and production of IL-2 upon stimulation with α-GC-pulsed APCs or with alternative ways of activation among clones of CD1d- NKT hybridomas, even between parental 2E10 and 2E10 derivatives (Figure 6). *De novo* generation of hybridomas involved an unusual selection process of NKT cells in the thymus, because CD1d molecules were expressed on the CD4$^+$8$^+$ thymocytes but not on the precursor thymocytes to be selected for NKT cells. This process may skew the repertoire, although the population we examined is essentially invariant in nature. However, the difference in TCR Vβ chain usage in '*de novo'* clones vs. 2E10 derivatives may give rise to a different response because Vβ chain usage alters the avidity[18] or fine-reactivity to ligands.[19] These issues should be pursued in further comparative studies using other clones that have not yet been characterized.

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Conflicts of Interest: None

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