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Original Article

Characterization of NKT-cell Hybridomas Expressing Invariant T-cell Antigen Receptors

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Two natural killer T (NKT)-cell hybridomas were established by fusing sorted NKT cells with BW1100 thymoma cells. The first hybridoma line, 1B6, was $CD4^+8^-$, whereas the second one, 2E10, was $CD4^{low}8^-$. Initial characterizations revealed that both cell lines expressed an invariant T cell antigen receptor, which could be readily detected with α-galactosylceramide-loaded CD1d : Ig fusion protein (α-GalCer/CD1d). Sequence analyses of the α and β chains of the T cell receptor V genes revealed that 1B6 and 2E10 cells expressed $V\alpha14J\alpha18/V\beta8.2D\beta2J\beta2.7$ and $V\alpha14J\alpha18/V\beta8.1D\beta1J\beta1.1$, respectively. When these hybridoma cells were stimulated with immobilized anti-CD3 monoclonal antibodies, α-GalCer/CD1d, or α-GalCer in the presence of antigen-presenting cells, they produced IL-4 and IFN-γ. The expression levels of CD69, CD154, and CD178 were concomitantly up-regulated on both hybridomas upon stimulation. Because it is difficult to isolate a sufficient number of NKT cells, these hybridomas should provide useful platforms to study a variety of functions of NKT cells. 〔*J Clin Exp Hematopathol 47 (1) : 1-8, 2007*〕

Keywords: NKT-cell, Hybridoma, Invariant TCR, IL-4, IFN-γ.

INTRODUCTION

NKT cells constitute a unique subset of T lymphocytes that recognize non-peptide antigens such as glycosphingolipids and phospholipids in the context of the non-classical class I major histocompatibility complex (MHC) molecule CD1d[1,2]. In mice, a major subpopulation of NKT cells expresses the $V\alpha14J\alpha18$ invariant α chain, which is preferentially associated with the $V\beta8$, $V\beta7$, or $V\beta2$ chains of the T-cell antigen receptor (TCR). To date, NKT cells have been reported to be active in various host responses against pathogenic microbes and tumors, and in inflammatory and autoimmune diseases[3,4]. Thus, examination of the ligand-based modulation of NKT cells has been expected to provide new therapeutic targets for these diseases[5,6].

The paucity of available NKT cells, however, has proved to be an obstacle during *in vitro* functional studies of these cells. Thus, the utilization of NKT-cell hybridomas may offer a convenient platform to examine specific functions of NKT cells and to screen arrays of natural products or synthetic compounds for potential therapeutic agents. Thus far, a number of NKT-cell hybridomas have been established in several laboratories[7-9]. In the present study, two NKT-cell hybridomas were established and the characteristics of these cell lines were examined.

MATERIALS AND METHODS

Cells

A fusion partner, the BW1100.129.237 cell line ($TCR\alpha^-\beta^-$)[10], was kindly provided by Dr. Willi Born (Department of Immunology, National Jewish Medical and Research Center, Denver, CO). BW1100 cells and established hybridomas were cultured in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (FCS ; Invitrogen Corp., Carlsbad, CA), benzylpenicillin potassium (100 U/ml ; Meiji Seika Kaisha Ltd., Tokyo, Japan), strepto-

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mycin sulfate (100 μg/ml ; Meiji Seika), and β-mercaptoethanol (1×10^{-5} M ; LifeTechnologies, Grand Island, NY).

Preparation and activation of NKT cells

NKT cells were purified from the thymi of C57BL/ 6 mice purchased from Japan SLC (Hamamatsu, Japan) as previously described[11]. In brief, thymocytes were incubated with anti-CD24 monoclonal antibodies (mAbs) (J11d) and anti-CD8 mAbs (53.6.7) followed by an incubation with goat anti-rat immunoglobulin (Ig) G microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Then, the $CD24^-$ $CD8^-$ thymocytes that passed through an LS column (Miltenyi) were labeled with phycoerythrin (PE)-conjugated anti-NK1.1 (BD Biosciences Pharmingen, San Diego, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-TCRβ antibodies (BD Biosciences). After the $NK1.1^+TCR\beta^+$ cellular population was isolated as NKT cells with a FACS (fluorescence-activated cell sorter) Vantage SE (BD Biosciences Immunocytometry Systems, Mountain View, CA), the sorted cells were cultured overnight in medium containing recombinant human (rh)IL-2 (300 U/ml ; kindly provided by Takeda Chemical Industries, Osaka, Japan). Then, the NKT cells were activated with α-GalCer (100 ng/ml ; kindly provided by Kirin Brewery Company, Takasaki, Japan) in the presence of irradiated (30 Gy) splenocytes that were enriched for $CD11c^+$ cells. The activated NKT cells were expanded in the presence of rhIL-2 (300 U/ml)[12] and later employed in the cell fusion experiments.

Establishment of the hybridoma lines

A standard protocol for establishing B- cell hybridomas[13] was employed for the fusion of the NKT cells and the BW1100 cells. In brief, the sorted NKT cells and the BW1100 cells were washed three times with RPMI-1640 medium without FCS and pelleted. Polyethylene glycol 1500 (Roche Diagnostics, Ingelheim, Germany) was added to the resulting pellets dropwise followed by a dilution with RPMI-1640. Then, the cells were spun down and resuspended in HAT medium (RPMI-1640 containing hypoxanthine, aminopterin, and thymidine ; Roche Diagnostics) for culture. Colonies were picked and transferred to HT medium (RPMI-1640 containing hypoxanthine and thymidine ; Roche Diagnostics) and then to RPMI-1640 containing 10% FCS for further analysis.

Antibodies and flow cytometry

Hybridoma cells were first incubated with 2.4G2 mAbs (anti-FcγR) to block non-specific binding, and then labeled with a combination of the following mAb conjugates : biotinylated (biotin)-anti-Vβ8.1/8.2 (F23.1, mouse IgG_{2a} isotype) ; FITC-anti-TCRβ (H57- 597, hamster IgG_2 isotype) ; FITC-anti-CD1d (1B1, rat IgG_{2b} isotype), FITC-anti-CD49b (DX5, rat IgM isotype) ; FITC-anti-CD69 (H1.2F3, hamster IgG_1 isotype) ; FITC-anti-CD95 (Jo2) ; PE-anti-NK-T/NK cell antigen (U5A2- 13, rat IgG_{2a} isotype) ; PE-anti-CD154 (MR1, hamster IgG_3 isotype) ; PE-anti-CD178 (MFL3) ; and allophycocyanin (APC)-CD161 (PK136, mouse IgG_{2a} isotype). Preparation of α-GalCer/CD1d was performed according to the manufacturer's instructions by incubating CD1d : Ig fusion protein with α-GalCer at 37℃ overnight. For the detection of invariant TCR (iTCR), cells were incubated with α-GalCer/CD1d for 60 min at 4℃ followed by incubation with PE-anti-mouse IgG_1. All reagents for flow cytometry were purchased from BD Biosciences Pharmingen. Propidium iodide-positive (Sigma) cells were electronically gated out from the analysis, and cells were analyzed using a FACScalibur flow cytometer (BD Biosciences Immunocytometry Systems) with CellQuest software (BD Biosciences).

Sequencing the iTCRs from the hybridomas

Total RNA was extracted from 1B6 and 2E10 cells using the acid guanidium thiocyanate- phenol- chloroform method[14]. Reverse transcription was performed as previously described[11]. The invariant Vα chain was amplified from complemantary DNA using the polymerase chain reaction with the Vα14 (5'-CAC/AGC/CAC/CCT/GCT/GGA/T-3') and Jα18 (5'-CCA/AAA/TGC/AGC/CTC/CCT/AA-3') primer pair. The Vβ chain was amplified with the primer pairs of either Vβ8.1 with an EcoRI site (5'-*CTC/ GAA/ TTC*/ ATG/ GGC/ TCC/ AGA/ CTC/ TTC/ TT-3') or Vβ8.2 with an EcoRI site (5'-*CTC/ GAA/ TTC*/ TAA/ CAC/ TGC/ CTT/ CCC/ TGA-3') and a common Cβ primer with a BamHI site (5'- *CTC/ GGA/ TCC*/ TTT/ TGT/ TTG/ TTT/ GCA/ AT-3')[15]. Each amplified band was directly sequenced with the dye terminator method by Hokkaido System Science Co. (Sapporo, Japan). Genomic DNA sequences for the assignment of rearranged TCR genes were retrieved from National Center for Biotechnology Information (NCBI ; www.ncbi.nlm.nih.gov/).

Activation of the hybridomas

Each well of a 24-well plate was coated with α- GalCer/ CD1d (10 μg/ ml in PBS) and was used for cross-linking the NKT- cell hybridomas *in vitro*. The hybridomas were harvested after 12-hr incubations and used for FACS analysis as described above. Supernatants were also collected for the quantification of the production of IL-4 and IFN-γ. In some experiments, hybridomas were cultured with the BC1 murine dendritic cell line[16] or a rat basophilic leukemia cell

line transfected with murine CD1d1, RBL-CD1d[11] (kindly provided by Dr. Albert Bendelac, University of Chicago), as antigen-presenting cells in the presence of α-GalCer.

Quantification of cytokine levels

The levels of IFN-γ and IL-4 in the culture supernatants were quantified with an OptEIA enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences) according to the manufacturer's protocol[17]. A cytometric bead array (BD Biosciences) was also employed for the quantification of the cytokine levels in the supernatant using the manufacturer's protocol.

RESULTS AND DISCUSSION

Characterization of the surface markers of the NKT-cell hybridomas

Two hybridoma cell lines, 1B6 and 2E10, were established and characterized in the present study. First, we examined the expression of iTCR and several surface markers. Because the source NKT cells for fusion were derived from α-GalCer/CD1d-expressing cells, it was not surprising that both 1B6 and 2E10 were α-GalCer/CD1d$^+$TCRβ^+(Fig. 1A). 1B6 cells uniformly expressed CD4 at a high level, whereas the expression of CD4 in 2E10 cells was either undetectable or observed at a low level (Fig. 1A).

Next, typical molecular markers of NKT cells were examined. It was found that both 1B6 and 2E10 did not express a detectable level of NK1.1 (CD161c) or DX5 (CD49b) (Fig. 1B). It has been reported that the expression of NK1.1 is lost during activation of the cells[18]. Thus, it appeared that the source NKT cells collected from the thymus stopped expressing NK1.1 during activation with α-GalCer and expansion with rhIL-2 prior to fusion with the BW1100 cells. Because thymic NK1.1$^+$TCRβ^- cells, but not NK1.1$^+$TCRβ^+ cells, express DX5[11], it is likely that the resultant hybridoma cells are indeed DX5$^-$. 1B6 cells were weakly labeled by the pan-NK/T marker U5A2-13, whereas 2E10 cells were not labeled with this antibody. U5A2-13 antibodies recognize a conformational epitope of intercellular adhesion molecule-1[19]. Both hybridoma cell lines demonstrated enhanced expression of the U5A2-13 epitope after anti-CD3 crosslinking (data not shown). Additionally, both hybridoma cell lines expressed the restriction molecule CD1d (Fig. 1B).

Morphology of the NKT-cell hybridomas

Forward light scatter (FSC) analysis demonstrated that the 2E10 cells were larger than the 1B6 cells (Fig. 1C). When the cells were examined under a light microscope, both the 1B6 and 2E10 cells had a T cell blast-like morphology, similar to the original NKT cells (Fig. 1D). Upon activation with immobilized anti-CD3 mAbs, a significant extension of the uropod-like structures was observed (Fig. 1D). These findings demonstrate that both hybridoma cell lines retained the morphological traits of NKT cells even after fusion with the thymomas.

Sequences of TCR genes in the NKT-cell hybridomas

Next, we sequenced the $V\alpha$ and $V\beta$ TCR genes expressed by the 1B6 and 2E10 cells. Both cell lines expressed invariant $V\alpha$ chains with identical amino-acid sequences, including a Gly residue in the complementarity-determining region (CDR) 3 (Fig. 2A). 2E10 cells had a G as non-coding (N) sequence, whereas 1B6 cells had no N nucleotide but did have an A after $J\alpha 18$; both of these sequences result in a codon for a Gly residue at a position that typically is conserved in the $V\alpha$ chain of iTCR[7,20].

As expected, 1B6 and 2E10 cells were labeled with anti-$V\beta 8.1/8.2$ mAbs (F23.1) (data not shown). PCRs with the $C\beta$ common primer and either the $V\beta 8.1$ primer or $V\beta 8.2$ primer demonstrated that the 1B6 and 2E10 cell lines carried $V\beta 8.2$ and $V\beta 8.1$, respectively (data not shown). Further analyses revealed that 1B6 carried $V\beta 8.2D\beta 2J\beta 2.7$, whereas 2E10 carried $V\beta 8.1D\beta 1J\beta 1.1$ with N nucleotides as shown in Fig. 2B. 1B6 cells had a TGA sequence as N nucleotides that resulted in an Asp residue between $D\beta 2$ and $J\beta 2.7$. On the other hand, 2E10 cells had a C as an N nucleotide after joining $V\beta 8.1$ with $D\beta 1$; changing the T to C, however, did not change the corresponding Ser residue. As previously reported with the variety of $V\beta 8$ chains expressed by iNKT cells[7], the two hybridoma cell lines in this study employed different VDJ sets and expressed differently sized variants of CDR3; the 2E10 cells expressed a shorter CDR3 than the 1B6 cells.

Surface molecules on the activated NKT-cell hybridomas

We then examined the surface markers that were expressed following activation through the TCR/CD3 complex. Unstimulated 1B6 and 2E10 cells were negative for CD69, CD154, and CD178. These markers were markedly upregulated 12 hr after iTCRs were crosslinked with immobilized α-GalCer/CD1d (Fig. 3A, B). Of note, these hybridomas, which were weakly positive for CD95, expressed substantial levels of CD95 upon stimulation (Fig. 3A, B). These results clearly indicate that the activation markers were expressed on the hybridoma cells only after stimulation, and suggest that these hybridomas retained characteristics that were typical of normal unstimulated NKT cells in the absence of stimulation. The expression levels of CD154 and CD178 were reduced to the background levels when the cells were stimulated for 12 hr and then cultured for 24 hr without any stimulation. By contrast, the 1B6 and 2E10 cells were still CD69$^+$ under the

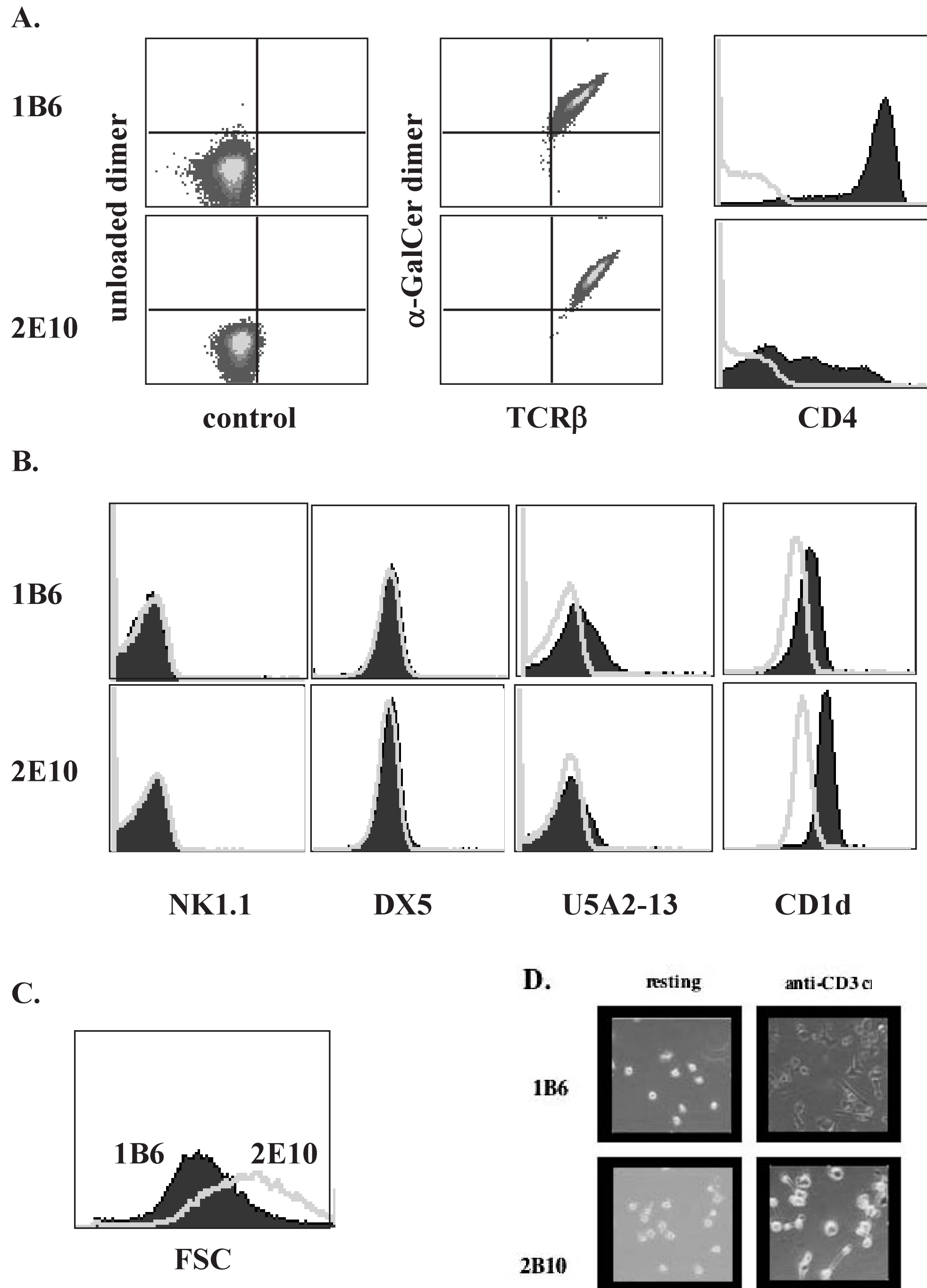


Fig. 1. Expression levels of iTCR, CD4 and NK markers on 1B6 and 2E10 cells, and the morphologies of these cells. **A**. Expression levels of unloaded CD1d versus isotype controls (*left panel*), α-GalCer/CD1d versus TCRβ (*middle panel*), and CD4 (*right panel*). The histogram of an isotype control (rat IgG2b) for CD4 is overlaid as thin lines in the right panels. **B**. Histograms for NK markers: NK1.1 (CD161c), DX5 (CD49b), and U5A2-13 (pan-NK/T), and CD1d. The histograms (*filled*) are overlaid with their isotype controls (*thin lines*). **C**. Forward light scatter data for 1B6 (*filled histogram*) and 2E10 (*thin line*) cells. **D**. Morphologies of 1B6 and 2E10 cells. Hybridomas were observed under a phase contrast microscope. Resting cells (*left panel*) and cells stimulated with immobilized anti-CD3 mAbs (*right panel*) are shown. Original magnification × 200.

A

Germline$V_{\alpha}14$: ...ATC/ TGT/ GTG/ GTG/ GG			
		TA/ GAT/ AGA/ GGT/ TCA/ GCC... : germline $J_{\alpha}18$	
	$V_{\alpha}14$	N	$J_{\alpha}18$
1B6 :	...ATC/ TGT/ GTG/ GTG/ GG		A/ GAT/ AGA/ GGT/ TCA/ GCC...
2E10 :	...ATC/ TGT/ GTG/ GTG/ GG	**G**	/ GAT/ AGA/ GGT/ TCA/ GCC...
	I C V V	**G**	D R G S A

B.

$V_{\beta}8.2$: ...TGT/ GCC/ AGC/ GGT/ GAT/ *G*				
		$D_{\beta}2$: *G*/ GGA/ CTG/ GG*GGGC*		
				$J_{\beta}2.7$: *CTCC*/ TAT/ GAA/ CAG/ TAC/ TTC...
	$V_{\beta}8.2$	$D_{\beta}2$	N	$J_{\beta}2.7$
1B6 :	...TGT/ GCC/ AGC/ GGT/ GAT/	GGA/ CTG/ GG	**T/ GA**	C/ TAT/ GAA/ CAG/ TAC/ TTC...
	C A S G D	G L G	**D**	Y E Q Y F

$V_{\beta}8.1$: ...TGT/ GCC/ AGC/ AGT/ GAT/ *G*				
		$D_{\beta}1$: *GGG*/ ACA/ GG *GGGC*		
				$J_{\beta}1.1$: *CAAA*C/ ACA/ GAA/ GTC/ TTC/ TTT...
	$V_{\beta}8.1$	N	$D_{\beta}1$	$J_{\beta}1.1$
2E10 :	...TGT/ GCC/ AGC/ AG	***C/***	ACA/ GG	C/ ACA/ GAA/ GTC/ TTC/ TTT...
	C A S S	**T**	G	T E V F F

Fig. 2. Sequence analyses of the V_{α} and V_{β} chains of the iTCRs in the NKT-cell hybridomas. **A**. Nucleotide sequences of the rearranged iTCR V_{α} chains in the 1B6 and 2E10 cell lines and the deduced amino-acid sequences. Germline V_{α} and $J_{\alpha}18$ sequences are also aligned to compare the rearranged sequences. *T* (*italicized*) in the 5' end of $J_{\alpha}18$ was excised in the 1B6 cells and *TA* in 2E10. The N nucleotide is shown in bold font. **B**. Nucleotide sequences of the rearranged V_{β} chains in the 1B6 and 2E10 cell lines and the deduced amino-acid sequences. Germline $V_{\beta}8.2$, $D_{\beta}2$, and $J_{\beta}2.7$ sequences for 1B6, and $V_{\beta}8.1$, $D_{\beta}1$, and $J_{\beta}1.1$ sequences for 2E10 (*italicized bases* are excised on joining) are also aligned to compare the rearranged sequences. N nucleotides are shown in bold font. The amino-acid residue that is coded for due to the N-nucleotide addition is also shown in bold font.

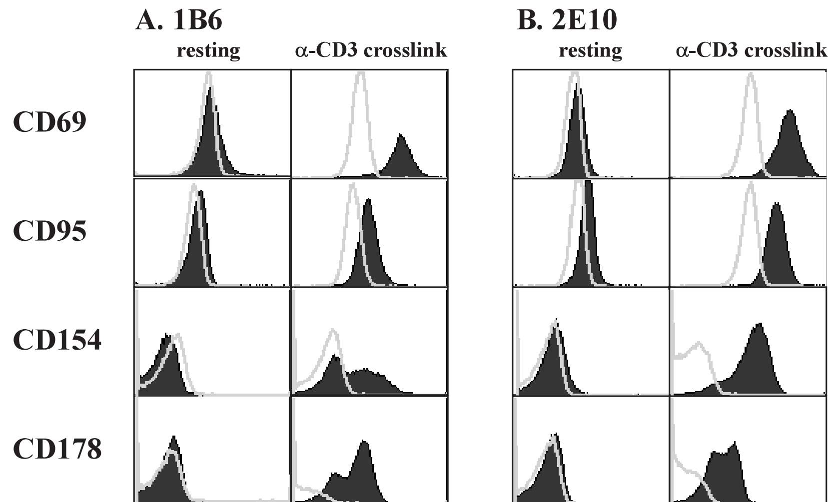
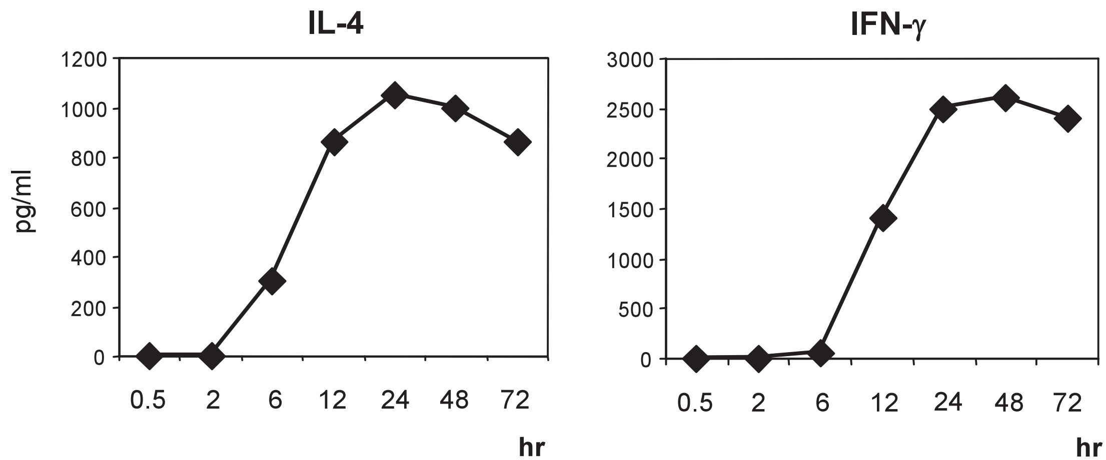


Fig. 3. Expressions of activation markers on NKT-cell hybridomas before and after activation. **A**. Expression levels of CD69, CD95 (Fas), CD154 (CD40L), and CD178 (FasL) on 1B6 cells before (resting) and after activation (anti-CD3 crosslinking). **B**. Expression levels of CD69, CD95, CD154, and CD178 on 2E10 cells before (resting) and after activation (anti-CD3 crosslinking). In (**A**) and (**B**), the histogram for each mAb has been filled in and the respective isotype control histogram is shown as a thin line.

same conditions (data not shown). Nevertheless, the reversible upregulation of these surface molecules indicated that these hybridoma cells were immunologically regulated, which will be important when these cells are used to screen reagents for activities that inhibit NKT-cell activation.

Cytokine production by the NKT-cell hybridomas

After crosslinking with α-GalCer/CD1d or anti-CD3 mAbs, these hybridomas also produced significant levels of IFN-γ and IL-4 that were similar to those produced in normal NKT cells. When 1B6 hybridoma cells were stimulated with immobilized anti-CD3 mAbs, IL-4 was detected in the supernatant after 6 hr of culture, whereas IFN-γ was detected only after 12 hr of culture (Fig. 4A). This pattern of cytokine production was consistent with the previous findings that showed the peak level of IL-4 production in normal NKT cells preceded the peak level of IFN-γ production[21]. 2E10



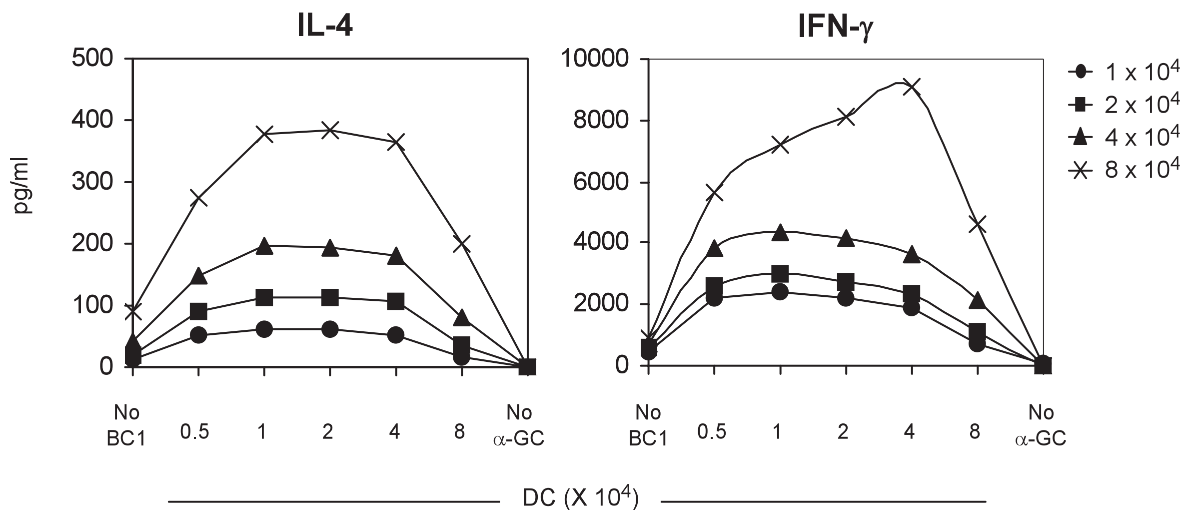


Fig. 4. IL-4 and IFN-γ production by 1B6 and 2E10 cells. **A**. Time course of IL-4 (*left panel*) and IFN-γ (*right panel*) production by 1B6 upon crosslinking with anti-CD3 mAbs. Cytokines in the supernatant were quantified as described in the *MATERIALS and METHODS* section. **B**. The production of IL-4 (*left panel*) and IFN-γ (*right panel*) by 2E10 cells stimulated with α-GalCer in the presence of the BC1 murine dendritic cell line. Different ratios of 2E10 and BC1 cells were cocultured. The symbols in the graph (right) represent the number of responder hybridoma cells in each culture, which ranged from 1×10^4 to 8×10^4. The numbers of BC1 cells varied between 0.5×10^4 and 8×10^4 cells/culture, whereas cultures lacking BC1 cells (NoBC1) or α-GalCer (No α-GC) served as controls. Thus, the ratio of responder cells to BC1 cells varied from 16 : 1 to 1 : 8, not including the no-BC1 cell control culture.

cells demonstrated similar kinetics for the production of these cytokines (data not shown).

When 2E10 cells and BC1 dendritic cells were co-cultured at different cellular ratios (the ratios of hybridoma cells to BC1 cells were between 1 : 8 and 16 : 1), both the levels of IL-4 and IFN-γ production were dependent on the number of hybridoma cells (Fig. 4B). It seemed that optimal cellular ratio (hybridoma cell number to BC1 cell number) fell between 1 and 4. Of note, 2E10 cells produced small but significant amounts of IL-4 and IFN-γ even in the absence of BC1 cells, especially when the cellular density of the culture was high. This result suggests that CD1d expressed on 2E10 cells is directly involved in presenting the α-GalCer ligand (see Fig. 1B). 1B6 cells demonstrated the same reactivity (data not shown). Furthermore, IL-2 and TNF-α were produced by α-GalCer-stimulated 1B6 cells (data not shown). Therefore, our system should be convenient and useful for screening compounds that directly act on NKT cells and modulate cytokine production in the absence of antigen-presenting cells.

The involvement of NKT cells in various diseases has been reported and the functional modulation of these cells can alter the development of these diseases[3-6]. For example, we have reported that NKT cells accelerate atherogenesis[22] and are involved in the development of a murine model of autoimmune hepatitis[23]. Thus, it may be possible to treat these diseases by regulating certain functions of NKT cells. To this end, the stable NKT-cell hybridomas established in the present study, which retain essential characteristics of NKT cells, should help with future investigations, allowing the effects of inhibitors and modulators on the expression levels of genes involved in the immunological responses of NKT cells to be examined *in vitro*.

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