

Presence of a novel subset of NKT cells bearing an invariant V α 19.1–J α 26 TCR α chain

Michio Shimamura*, Yi-Ying Huang

Laboratory of Developmental Immunology, Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194-8511, Japan

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Abstract CD1d-deficient (CD1d^{−/−}) mouse lymphocytes were analyzed to classify the natural killer T (NKT) cells without reactivity to CD1d. The cells bearing a V α 19.1–J α 26 (AV19–AJ33) invariant TCR α chain, originally found in the peripheral blood lymphocytes, were demonstrated to be abundant in the NK1.1⁺ but not NK1.1[−] T cell population isolated from CD1d^{−/−} mice. Moreover, more than half (11/21) of the hybrid cell lines established from CD1d^{−/−} NKT cells expressed the V α 19.1–J α 26 invariant TCR α chain. The expression of the invariant V α 19.1–J α 26 mRNA was absent in β 2-microglobulin-deficient mice. Collectively, the present findings suggest the presence of a second NKT cell repertoire characterized by an invariant TCR α chain (V α 19.1–J α 26) that is selected by an MHC class I-like molecule other than CD1d. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Natural killer T cell; Invariant TCR α chain; V–J junction; Hybridoma; Interleukin-4 secretion

1. Introduction

Natural killer T (NKT) cells are defined as lymphocytes bearing both the common NK marker NK1.1, a member of the NKR-P1 gene family, and TCR–CD3 complex [1,2]. The TCR repertoire of the majority of NKT cells consists of an invariant V α 14–J α 281 chain [3,4] paired preferentially with a polyclonal V β 8, V β 7 and V β 2 [3,5,6]. This highly skewed TCR $\alpha\beta$ repertoire is positively selected by the non-polymorphic MHC class I-like CD1d molecule in association with β 2-microglobulin (β 2m) [7–11], and is shown to be reactive to certain glycolipids in the context of CD1d [12,13]. The CD4/CD8 coreceptor phenotype of this major NKT cell repertoire is CD4⁺ or CD4[−]CD8[−] double negative (DN) [7,8]. An important feature of NKT cells is their ability to secrete both pro-inflammatory (Th1) cytokines such as interferon (IFN)- γ and anti-inflammatory (Th2) cytokines such as interleukin (IL)-4 and IL-10 upon stimulation through the semi-invariant TCR [14–17], suggesting their pivotal roles in immuno-regulatory functions including tumor immunity [18,19].

*Corresponding author. Fax: (81)-42-724 6317.

E-mail address: michio@libra.ls.m-kagaku.co.jp (M. Shimamura).

Abbreviations: NKT, natural killer T; β 2m, β 2-microglobulin; DN, CD4[−]CD8[−] double negative; Tap, transporter associated with antigen processing; RACE, rapid amplification of cDNA ends; DIG, digoxigenin; HPRT, hypoxanthine phosphoribosyltransferase

Recent studies revealed that NKT cells are heterogeneous in terms of TCR structure, CD1d-reactivity, and CD4/CD8 co-receptor expression although the V α 14–J α 281⁺, CD1d-restricted, CD4⁺ or DN NKT cell repertoire (V α 14 NKT cell) is most abundant [20–23]. For instance, CD1-independent CD8⁺ NKT cells are relatively common in spleen and bone marrow. Classification of NKT cell subsets is necessary for understanding the immuno-regulatory functions of NKT cells, but such attempts have just started and further investigations are required.

In the current study, we demonstrate the presence of a novel NKT cell repertoire (designated as V α 19 NKT cell) characterized by the expression of a V α 19.1–J α 26 (AV19–AJ33) invariant TCR α chain that was first found in human, bovine, and a transporter associated with antigen processing (Tap)-1-deficient mouse peripheral blood by PCR techniques [24].

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Sankyo Service Co. (Tokyo, Japan). CD1d^{−/−} (BALB/c $\times$ 129SV F₂) mice were obtained from Dr. M.J. Grusby [9]. They were backcrossed to C57BL/6 mice for several generations and CD1d^{−/−}, H-2^b, NK1.1⁺ mice were selected and used. β 2m^{−/−} mice (C57BL/6 background) were obtained from Jackson Laboratory (Bar Harbor, ME, USA).

2.2. Cell preparation

Single cell suspensions were prepared from the liver, spleen, bone marrow, and thymus. Total liver cells were resuspended in a 40% isotonic Percoll solution (Pharmacia, Uppsala, Sweden), and underlaid with an 80% Percoll solution. Mononuclear cells were isolated from the 40–80% interface after centrifugation for 20 min at 900 $\times$ g. Spleen, and bone marrow (femur, tibia) mononuclear cells were enriched by density gradient centrifugation using Lymphosepar II ($d = 1.090$, IBL, Gunma, Japan).

2.3. Flow cytometry and antibodies

Cells were pretreated with rat IgG_{2b} mAb, 2.4 G2 (anti-Fc γ R II, III), to saturate FcR binding. Specific staining was then performed with a combination of the following conjugated mAbs: H57-597 (anti-TCR β)^{PE}, PK136 (anti-NK1.1)^{biotin}, GK1.5 (anti-CD4)^{PE}, 53-6.7 (anti-CD8)^{PE}, B20.6 (anti-V β 2)^{FITC}, KJ25 (anti-V β 3)^{FITC}, KT4 (anti-V β 4)^{FITC}, MR9-4 (anti-V β 5.1, 5.2)^{FITC}, RR4-7 (anti-V β 6)^{FITC}, TR310 (anti-V β 7)^{FITC}, F23.1 (anti-V β 8)^{FITC}, MR10-2 (anti-V β 9)^{FITC}, B21.5 (anti-V β 10)^{FITC}, RR3-15 (anti-V β 11)^{FITC}, MR11-1 (anti-V β 12)^{FITC}, MR12-3 (anti-V β 13)^{FITC}, and 14-2 (anti-V β 14)^{FITC}. Biotinylated mAbs were revealed with streptavidin-FITC or PE. The stained cells were analyzed on a FACScan flow cytometer equipped with the Cell Quest software (Becton Dickinson, San Jose, CA, USA).

2.4. Preparation of NKT cell hybridomas

NKT cell hybridomas were produced by fusing NK1.1⁺ T cells with

the TCR $\alpha^- \beta^-$ variant of the BW5147 thymoma [25] essentially as described previously [26]. Briefly, NK1.1⁺ TCR $\alpha\beta^+$ cells were sorted from liver lymphocytes of CD1d^{-/-} mice on a FACStar (Becton Dickinson). Their purity for several sortings was more than 95%. They were cultured with anti-TCR C β mAb (H57-597, 1 μ g/ml), and rIL-7 (20 ng/ml, Pharmingen, San Diego, CA, USA) in the presence of irradiated (2000R) spleen cells. rIL-2 (25 U/ml) was added after 2 days. On day 4, the recovered cells were fused with BW5147 $\alpha^- \beta^-$ by the standard technique. Growing hybridomas were screened for expression of TCR $\alpha\beta$ by flow cytometry and positive wells were subcloned by limiting dilution.

2.5. RT-PCR

Total RNA was extracted from mouse tissues. It was digested with DNase I, extracted with phenol, and precipitated with ethanol. Then, cDNA was prepared from total RNA using RT-PCR kits (Takara, Tokyo, Japan). RT-PCRs were performed using the following primers: (5'V α 19.1) 5'-GCTTCTGACAGAGCTCCAG-3'; (3'J α 26) 5'-CTTGGTCCCAGAGCCCC-3'; (5'C α) 5'-GAACCCAGAACC-TGCTGTGT-3'; (3'C α) 5'-TGGCGTTGGTCTCTTTGAAG-3'; (5'-hypoxanthine phosphoribosyltransferase (HPRT)) 5'-GTTGGA-TACAGGCCAGACTTTGTTG-3'; (3'-HPRT) 5'-GAGGGTAGG-CTGGCCTATAGGCT-3'. The RT-PCR products obtained with the 5'V α 19.1 and 3'J α 26 primers were analyzed by Southern blot using a digoxigenin (DIG)-labeled probe. A probe was prepared from the PCR products obtained with these primers (5' *Pst*II/3' *Eco*RI digest, 360 bp), and labeled using a DIG High Prime DNA labeling kit (Roche, Switzerland).

2.6. 5' RACE (rapid amplification of cDNA ends) analysis

RACE was performed on cDNAs prepared from the CD1d^{-/-} liver NKT cell hybridomas using a 5'-full RACE core set (Takara, Japan). A 5'-end phosphorylated reverse Caprimer (5'-ATCTTGGCAGGT-3') was used as a reverse transcription primer. After circularization of the cDNA, the following pairs of primers were used for the first and the second PCR. First PCR: 5'-AAGTCGGTGAACAGGCAGAG-3', 5'-GACCTTGCAAGTAGTACTG-3'. Second PCR: 5'-CTGG-TACACAGCAGGTTCTG-3', 5'-CGATACCTAAGGTTCTCGTT-3'.

2.7. DNA sequencing

The PCR products were cloned into the pCRII vector (Invitrogen, USA). DNA sequencing was performed using sequencing kits (Amersham Life Science, USA), a sequencing primer (FITC-labeled oligo-

	Germ-line V α 19.1GCT GTG AGG						G GAT AGC AAC Germ-line J α 26					
	Ala	Val	Arg	Asp	Ser	Asn						
NB103	GCT	GTG	AGG	GAT	AGC	AAC						
				Leu								
NB115	---	--C	CT-	---	---	---						
					Arg							
NB116	---	---	---	---	C--	---						
NB201	---	---	---	---	---	---						
				Gly								
NB202	---	---	G-A	---	---	---						
NB204	---	---	---	---	---	---						
NB206	---	---	---	---	---	---						
NB212	---	---	--A	---	---	---						
NB213	---	---	---	---	---	---						
				Ile								
NB215	---	---	-TC	---	---	---						
NB403	---	---	---	---	---	---						

Fig. 1. V α 19.1–J α 26 junctional sequences of the hybrid lines produced from CD1d^{-/-} NKT cells. The sequences were obtained from analysis of the 5' RACE and/or RT-PCR products. The 3'-end V α 19.1 and the 5'-end J α 26 germ-line sequences are shown as references. The 'canonical sequence' produced from the V α 19.1 and J α 26 germ-line sequences without any modifications is shown as the sequence for NB103. Changes relative to it are shown in some cell lines.

nucleotide complementary to T7 and/or Sp6 promoter) and a DNA sequencer (Model 3000, Hitachi, Japan).

3. Results

3.1. V α 19.1–J α 26 invariant α -bearing cells as one of the major components of NKT cells in CD1d^{-/-} mice

Analysis of NK1.1⁺ TCR $\alpha\beta^+$ cells in CD1d^{-/-} and β 2m^{-/-} mice revealed that NKT cell repertoires other than the major CD1d-dependent repertoire with the V α 14–J α 281⁺ invariant α chain (V α 14 NKT cell) are present ([27]; M. Shimamura, unpublished results). The expression of CD4/CD8 coreceptors by the residual NKT cells in CD1d^{-/-} ([20,27], data not shown) and J α 281^{-/-} [21] mice was strongly skewed to CD8. These findings also support the existence of NKT cells other than V α 14 NKT cells.

Hybrid cell lines were produced from CD1d^{-/-} liver NKT cells by fusion with thymoma, BW5147, to characterize further the NKT cell repertoires. The TCR V α and V β usage of the hybrid lines thus established were determined by cDNA sequencing and FACS analysis, and are summarized in Table 1. Astonishingly, more than half of the hybrid lines expressed TCR α chain with rearrangement between V α 19.1 (AV19) and J α 26 (AJ33), which was originally found as the second invariant TCR α chain in human, bovine and murine peripheral blood by PCR technology [24].

The V α 19.1–J α 26 junctional sequences of these hybrid lines are shown in Fig. 1. A few nucleotide conversions from the germ-line sequence were observed in some cell lines. However, they were limited to those causing only one amino acid alter-

Table 1

V α and V β usage of the hybrid lines derived from NKT cells isolated from CD1-deficient mice

Cell line	V α 19.1–J α 26 ^a	V β
NB102	–	2
NB103	+	2
NB104	–	^b
NB110	–	4
NB115	+	^b
NB116	+	6
NB201	+	7
NB202	+	6
NB204	+	8
NB206	+	4
NB208	–	8
NB209	–	6
NB211	–	8
NB212	+	5
NB213	+	8
NB215	+	6
NB308	–	8
NB403	+	8
NB404	–	6
NB405	–	8
NB408	–	8

^aV α usage of the cell lines was determined by the cDNA sequencing (11/21 showed expression).

^bV β other than V β 2–14.

ation in the junction, thus, the V α 19.1–J α 26 junctional sequences are quite homogeneous similar to the V α 14–J α 281 invariant TCR α chain [28]. These findings indicate that an NKT cell repertoire bearing an invariant V α 19.1–J α 26 receptor (V α 19 NKT cell) is present as a major component of NKT cells in CD1d^{-/-} mice.

Next, attempts were made to determine whether the invariant V α 19.1–J α 26 α chain is expressed mainly by V α 19 NKT cells or also used by conventional T cells. Both NK1.1⁺ TCR $\alpha\beta$ ⁺ and NK1.1⁻ TCR $\alpha\beta$ ⁺ cells were enriched from CD1d^{-/-} livers and the junctional sequences between V α 19.1 and C α in both fractions were analyzed (Table 2). The ratio of invariant V α 19.1–J α 26⁺ sequences to total V α 19.1⁺ sequences obtained from the NK1.1⁺ fraction was 63% on the average of the independent experiments, whereas the ratio from NK1.1⁻ fraction was 8%, thus indicating that the invariant V α 19.1–J α 26 chain is predominantly used by V α 19 NKT cells.

3.2. V β usage of the V α 19⁺ hybridomas

V β usage of the V α 19 NKT cell lines obtained from CD1d^{-/-} liver was found to be skewed to V β 8 (3/11) and V β 6 (3/11) (Table 1). Furthermore, the V α 19.1–J α 26 junctional sequences of V β 8⁺ V α 19 NKT cell lines were those made from the intact germ-line sequence, but in contrast, the sequences of V β 6⁺ V α 19 NKT cell lines were subjected to junctional modification, thus suggesting the preferential combination of the invariant V α 19.1–J α 26 α chain with a certain β chain depending on the micro-heterogeneity of the α chain. As a result, most V α 19 NKT cells seem to express a quite homogeneous TCR $\alpha\beta$ heterodimer.

3.3. Tissue distribution of the expression of the invariant V α 19.1–J α 26 mRNA

The expression of the invariant V α 19.1–J α 26 mRNA was analyzed by RT-PCR in the lymphoid organs to examine tissue distribution of V α 19 NKT cells. The invariant V α 19.1–J α 26 mRNA was produced more or less in all the organs examined, but the expression was markedly enhanced in the adult bone marrow of normal mice (Fig. 2A). The mRNA was found in the CD1d^{-/-} as well as normal bone marrow, but not in the β 2m^{-/-} mice (Fig. 2B). Collectively, it is suggested that V α 19 NKT cells are abundant in bone marrow and that they are positively selected by β 2m-associated MHC class I-like molecules other than CD1d.

4. Discussion

The presence of a new NKT cell bearing an invariant V α 19.1–J α 26 α chain is demonstrated. Next to the V α 14

Table 2

Abundance of the invariant TCR V α 19.1–J α 26 receptor-bearing cells in the NK1.1⁺ cell fraction among CD1^{-/-} liver lymphocytes

Invariant V α 19.1–J α 26/total V α 19 ⁺ sequences		
NK1.1 ⁺ fraction		
Experiment 1	3/8	
2	11/13	
3	5/9	
NK1.1 ⁻ fraction		
Experiment 1	1/10	
2	1/15	

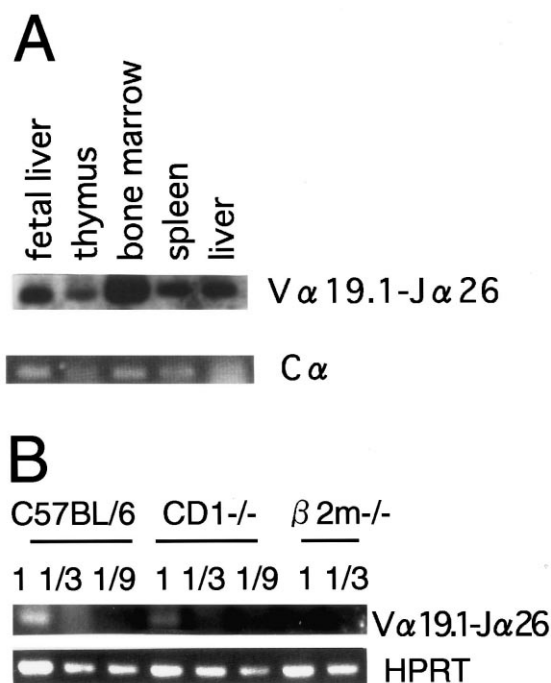


Fig. 2. Expression of the invariant V α 19.1–J α 26 α chain mRNA determined by RT-PCR analyses. A: Distribution in normal mouse lymphoid tissues. The expression of the V α 19–J α 26 mRNA was normalized by the expression of the TCR α constant region mRNA to estimate the proportion of V α 19 NKT cells to total T cells. B: The expression of V α 19.1–J α 26 mRNA in normal, CD1d^{-/-}, and β 2m^{-/-} bone marrow. The expression of HPRT mRNA is shown as a reference. cDNA prepared from 1, 1/3, or 1/9 μ g total RNA was used as template.

NKT cell, the V α 19 NKT cell is the second NKT cell subset characterized by a strictly distinct TCR repertoire.

It has been revealed by quantitative PCR analyses that cells bearing mRNA of the human counterpart of the murine invariant V α 19.1–J α 26 (human V α 7.2–J α 33) account for 0.1–0.2% of peripheral blood lymphocytes [24]. If the tissue distribution of the invariant α chain-bearing cells in human is common to other species, the newly identified NKT cell subset may not be a minor cell component. Establishment of specific antibodies against the invariant TCR α chain is required to exactly determine their frequency in vivo by FACS analysis.

The CD4/CD8 coreceptor phenotype of the V α 19 NKT cell lines, which represent more than half of the NKT cell hybridomas examined here (Table 1), was DN; only NB213 expressed low levels of CD4 (data not shown). This phenotype does not reflect the phenotype of the NKT cells in CD1d^{-/-} mouse livers where the ratio of CD4⁺, DN and CD8⁺ NKT cells in CD1d^{-/-} mouse livers was about 1:2:3 ([20]; data not shown). In contrast, the CD4/CD8 phenotype of a panel of thymic V α 14 NKT cell hybrid lines [28] produced by the same protocols used in this study (CD4⁺, 60%; DN, 40%; M. Shimamura, unpublished results) well corresponds to the phenotype of the intact thymic NKT cells [20–22]. Thus, one possible interpretation of these findings is that V α 19 NKT cell lines chiefly represent both the DN and CD8⁺ but not CD4⁺ NKT cell subsets and that the V α 19 NKT cell lines derived from the CD8⁺ subset might lose the CD8 expression during the establishment of the cell lines.

Development of V α 19 NKT cells is dependent on β 2m-associated MHC class I-like molecules other than CD1d. Judging from the expression of the invariant V α 19.1–J α 26 mRNA in Tap-1^{−/−} mouse peripheral blood [24], the selection molecule is one of the MHC class Ib molecules. Joyce et al. suggested the presence of TL (H-2T18^d)-dependent NKT cells [29]. However, V α 19 NKT cell hybridomas were not responsive to H-2T3^b (homologous to H-2T18^d)-transfectants in vitro (data not shown). Thus, it is likely that V α 19 NKT cells are restricted by an MHC class Ib molecule other than TL antigens.

The specific antigens recognized by V α 19 NKT cells have not been identified. The stringent requirement of the TCR α chain structure (Fig. 2) combined with the preferential usage of certain V β s (Table 1) suggests the formation of semi-invariant TCR $\alpha\beta$ receptors in V α 19 NKT cells. Taking into account the Tap-independency for antigen processing, the specific antigens for V α 19 NKT cells are assumed to be certain limited substances other than peptides, as similar to the specific antigens for V α 14 NKT cells such as α -GalCer [12].

A panel of V α 19 NKT cell hybridomas apparently exhibited Th2-type profiles of cytokine secretion following TCR–CD3 complex stimulation (data not shown). Thus, it is possible that V α 19 NKT cells keep a certain content in the NKT cell population and share immune-regulatory roles with V α 14 NKT cells under the control of MHC class Ib molecules other than CD1d. Determination of the MHC restriction for this new NKT cell repertoire is required to understand their specific roles in the immune system.

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