Presence of a novel subset of NKT cells bearing an invariant Vα19.1–Jα26 TCR α chain

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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 Vα19.1–Jα26 (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 Vα19.1–Jα26 invariant TCR α chain. The expression of the invariant Vα19.1–Jα26 mRNA was absent in β2-microglobulin-deficient mice. Collectively, the present findings suggest the presence of a second NKT cell repertoire characterized by an invariant TCR α chain (Vα19.1–Jα26) that is selected by an MHC class I-like molecule other than CD1d.

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 Vα14-Jα281 chain [3,4] paired preferentially with a polyclonal Vβ8, Vβ7 and Vβ2 [3,5,6]. This highly skewed TCR αβ repertoire is positively selected by the non-polymorphic MHC class I-like CD1d molecule in association with β2-microglobulin (β2m) [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].

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Abbreviations: NKT, natural killer T; β2m, β2-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 Vα14–Jα281$^+$, CD1d-restricted, $CD4^+$ or DN NKT cell repertoire (Va14 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 Vα19 NKT cell) characterized by the expression of a Vα19.1–Jα26 (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 × 129SV F_2) mice were obtained from Dr. M.J. Grusby [9]. They were backcrossed to C57BL/6 mice for several generations and $CDld^{-/-}$, H-2^b, $NK1.1^+$ mice were selected and used. $\beta2m^{-/-}$ 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×*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-FcγR 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-Vβ2)^{FITC}, KJ25 (anti-Vβ3)^{FITC}, KT4 (anti-Vβ4)^{FITC}, MR9-4 (anti-Vβ5.1, 5.2)^{FITC}, RR4-7 (anti-Vβ6)^{FITC}, TR310 (anti-Vβ7)^{FITC}, F23.1 (anti-Vβ8)^{FITC}, MR10-2 (anti-Vβ9)^{FITC}, B21.5 (anti-Vβ10)^{FITC}, RR3-15 (anti-Vβ11)^{FITC}, MR11-1 (anti-Vβ12)^{FITC}, MR12-3 (anti-Vβ13)^{FITC}, and 14-2 (anti-Vβ14)^{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

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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 Cβ mAb (H57-597, 1 μg/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 αβ 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′Vα19.1) 5′-GCTTCTGACAGAGCTCCAG-3′; (3′Jα26)5′-CTTGGTCCCAGAGCCCC-3′; (5′Cα) 5′-GAACCCAGAACCTGCTGTGT-3′; (3′Cα) 5′-TGGCGTTGGTCTCTTTTGAAG-3′; (5′-hypoxanthine phosphoribosyltransferase (HPRT)) 5′-GTTGGATACAGGCCAGACTTTGTTG-3′; (3′-HPRT) 5′-GAGGGTAGGCTGGCCTATAGGCT-3′. The RT-PCR products obtained with the 5′Vα19.1 and 3′Jα26 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*I/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 circulization of the cDNA, the following pairs of primers were used for the first and the second PCR. First PCR: 5′-AAGTCGGTGAACAGGCAGAG-3′, 5′-GACCTTGCAAGTAGTGACTG-3′. Second PCR: 5′-CTGGTACACAGCAGGTTCTG-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 oligonucleotide complementary to T7 and/or Sp6 promoter) and a DNA sequencer (Model 3000, Hitachi, Japan).

Table 1
Vα and Vβ usage of the hybrid lines derived from NKT cells isolated from CD1-deficient mice

Cell line	Vα19.1–Jα26[a]	Vβ
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

[a]Vα usage of the cell lines was determined by the cDNA sequencing (11/21 showed expression).
[b]Vβ other than Vβ 2–14.

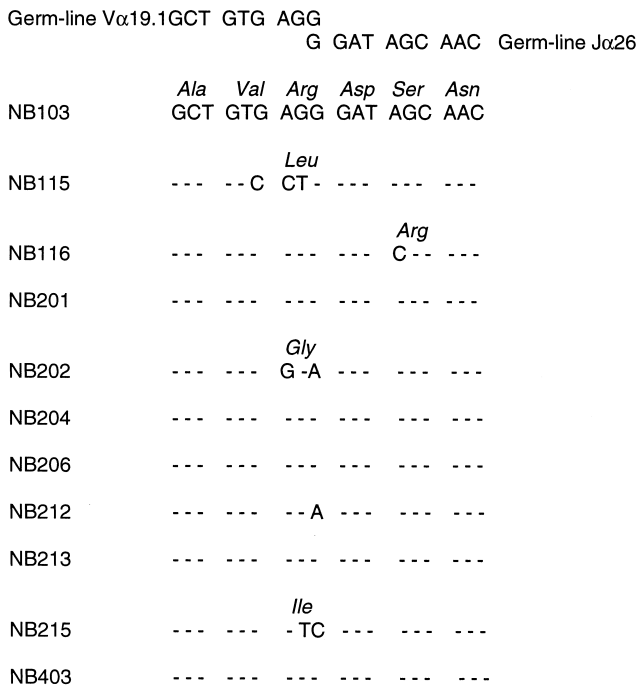

Fig. 1. Vα19.1–Jα26 junctional sequences of the hybrid lines produced from CD1d$^{-/-}$ NKT cells. The sequences were obtained from analysis of the 5′ RACE and/or RT-PCR products. The 3′-end Vα19.1 and the 5′-end Jα26 germ-line sequences are shown as references. The 'canonical sequence' produced from the Vα19.1 and Jα26 germ-line sequences without any modifications is shown as the sequence for NB103. Changes relative to it are shown in some cell lines.

3. Results

3.1. Vα19.1–Jα26 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 $\beta2m^{-/-}$ mice revealed that NKT cell repertoires other than the major CD1d-dependent repertoire with the Vα14–Jα281$^+$ invariant α chain (Vα14 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 Jα281$^{-/-}$ [21] mice was strongly skewed to CD8. These findings also support the existence of NKT cells other than Vα14 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 Vα and Vβ 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 Vα19.1 (AV19) and Jα26 (AJ33), which was originally found as the second invariant TCR α chain in human, bovine and murine peripheral blood by PCR technology [24].

The Vα19.1–Jα26 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-

ation in the junction, thus, the Vα19.1–Jα26 junctional sequences are quite homogeneous similar to the Vα14–Jα281 invariant TCR α chain [28]. These findings indicate that an NKT cell repertoire bearing an invariant Vα19.1–Jα26 receptor (Vα19 NKT cell) is present as a major component of NKT cells in CD1d$^{-/-}$ mice.

Next, attempts were made to determine whether the invariant Vα19.1–Jα26 α chain is expressed mainly by Vα19 NKT cells or also used by conventional T cells. Both NK1.1^{+} TCR αβ^{+} and NK1.1^{-} TCR αβ^{+} cells were enriched from CD1d$^{-/-}$ livers and the junctional sequences between Vα19.1 and Cα in both fractions were analyzed (Table 2). The ratio of invariant Vα19.1–Jα26^{+} sequences to total Vα19.1^{+} 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 Vα19.1–Jα26 chain is predominantly used by Vα19 NKT cells.

3.2. *Vβ usage of the Va19^{+} hybridomas*

Vβ usage of the Vα19 NKT cell lines obtained from CD1d$^{-/-}$ liver was found to be skewed to Vβ8 (3/11) and Vβ6 (3/11) (Table 1). Furthermore, the Vα19.1–Jα26 junctional sequences of Vβ8^{+} Vα19 NKT cell lines were those made from the intact germ-line sequence, but in contrast, the sequences of Vβ6^{+} Vα19 NKT cell lines were subjected to junctional modification, thus suggesting the preferential combination of the invariant Vα19.1–Jα26 α chain with a certain β chain depending on the micro-heterogeneity of the α chain. As a result, most Va19 NKT cells seem to express a quite homogeneous TCR αβ heterodimer.

3.3. *Tissue distribution of the expression of the invariant Vα19.1–Jα26 mRNA*

The expression of the invariant Vα19.1–Jα26 mRNA was analyzed by RT-PCR in the lymphoid organs to examine tissue distribution of Vα19 NKT cells. The invariant Vα19.1–Jα26 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 β2m$^{-/-}$ mice (Fig. 2B). Collectively, it is suggested that Vα19 NKT cells are abundant in bone marrow and that they are positively selected by β2m-associated MHC class I-like molecules other than CD1d.

4. Discussion

The presence of a new NKT cell bearing an invariant Vα19.1–Jα26 α chain is demonstrated. Next to the Vα14 NKT cell, the Vα19 NKT cell is the second NKT cell subset characterized by a strictly distinct TCR repertoire.

Table 2
Abundance of the invariant TCR Vα19.1–Jα26 receptor-bearing cells in the NK.1.1^{+} cell fraction among CD1$^{-/-}$ liver lymphocytes

		Invariant Vα19.1–Jα26/total Vα19^{+} sequences
NK1.1^{+} fraction		
Experiment	1	3/8
	2	11/13
	3	5/9
NK1.1^{-} fraction		
Experiment	1	1/10
	2	1/15

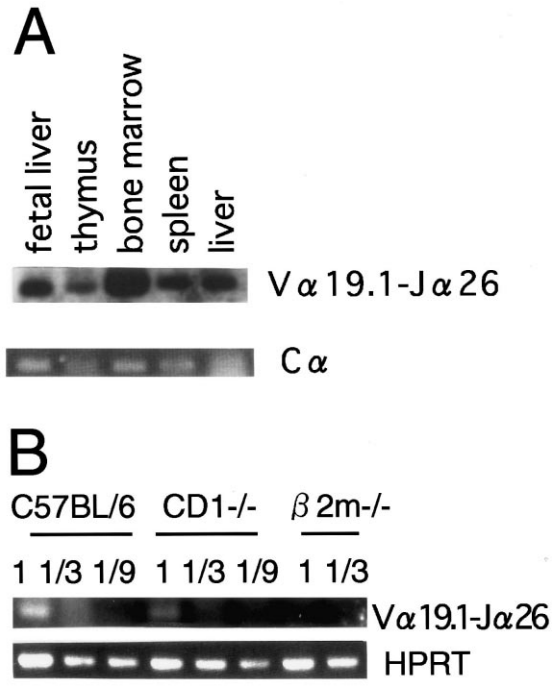

Fig. 2. Expression of the invariant Vα19.1–Jα26 α chain mRNA determined by RT-PCR analyses. A: Distribution in normal mouse lymphoid tissues. The expression of the Vα19–Jα26 mRNA was normalized by the expression of the TCR α constant region mRNA to estimate the proportion of Vα19 NKT cells to total T cells. B: The expression of Vα19.1–Jα26 mRNA in normal, CD1d$^{-/-}$, and β2m$^{-/-}$ bone marrow. The expression of HPRT mRNA is shown as a reference. cDNA prepared from 1, 1/3, or 1/9 μg total RNA was used as template.

It has been revealed by quantitative PCR analyses that cells bearing mRNA of the human counterpart of the murine invariant Vα19.1–Jα26 (human Vα7.2–Jα33) 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 Vα19 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 Vα14 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 Vα19 NKT cell lines chiefly represent both the DN and CD8^{+} but not CD4^{+} NKT cell subsets and that the Vα19 NKT cell lines derived from the CD8^{+} subset might lose the CD8 expression during the establishment of the cell lines.

Development of Vα19 NKT cells is dependent on β2m-associated MHC class I-like molecules other than CD1d. Judging from the expression of the invariant Vα19.1–Jα26 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, Vα19 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 Vα19 NKT cells are restricted by an MHC class Ib molecule other than TL antigens.

The specific antigens recognized by Vα19 NKT cells have not been identified. The stringent requirement of the TCR α chain structure (Fig. 2) combined with the preferential usage of certain Vβs (Table 1) suggests the formation of semi-invariant TCR αβ receptors in Vα19 NKT cells. Taking into account the Tap-independency for antigen processing, the specific antigens for Vα19 NKT cells are assumed to be certain limited substances other than peptides, as similar to the specific antigens for Vα14 NKT cells such as α-GalCer [12].

A panel of Vα19 NKT cell hybridomas apparently exhibited Th2-type profiles of cytokine secretion following TCR–CD3 complex stimulation (data not shown). Thus, it is possible that Vα19 NKT cells keep a certain content in the NKT cell population and share immune-regulatory roles with Vα14 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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