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CD1d-Specific NK1.1+ T Cells with a Transgenic Variant TCR

Markus Sköld,* Nurun N. Faizunnessa,* Chyung-Ru Wang, † and Susanna Cardell‡*‡

The majority of T lymphocytes carrying the NK cell marker NK1.1 (NKT cells) depend on the CD1d molecule for their development and are distinguished by their potent capacity to rapidly secrete cytokines upon activation. A substantial fraction of NKT cells express a restricted TCR repertoire using an invariant TCR Vα14-Jα281 rearrangement and a limited set of TCR Vβ segments, implying recognition of a limited set of CD1d-associated ligands. A second group of CD1d-reactive T cells use diverse TCR potentially recognizing a larger diversity of ligands presented on CD1d. In TCR-transgenic mice carrying rearranged TCR genes from a CD1d-reactive T cell with the diverse type receptor (using Vα3.2/Vβ9 rearrangements), the majority of T cells expressing the transgenic TCR had the typical phenotype of NKT cells. They expressed NK1.1, CD122, intermediate TCR levels, and markers indicating previous activation and were CD4/CD8 double negative or CD4+. Upon activation in vitro, the cells secreted large amounts of IL-4 and IFN-γ, a characteristic of NKT cells. In mice lacking CD1d, TCR-transgenic cells with the NKT phenotype were absent. This demonstrates that a CD1d-reactive TCR of the “non-Vα14” diverse type can, in a ligand-dependent way, direct development of NK1.1+ T cells expressing expected functional and cell-surface phenotype characteristics. The Journal of Immunology, 2000, 165: 168–174.

Members of the CD1 family are MHC class I-like Ag-presenting molecules, found in several species including man and mouse (1). The CD1 molecules, unlike conventional MHC molecules, present lipids and glycolipids (1–3), as well as peptides (4, 5), to T lymphocytes. In the mouse, different types of TCROβ T cells have been described that recognize CD1d in the absence of added Ag (6–10). Some of these CD1d-reactive T cells express the NK1.1 marker (6). NK1.1 was first described as a NK cell marker and is present on NK cells and a subpopulation of T cells (termed NKT cells) in some mouse strains, like C57BL/6 (B6) mice (11). To date the described CD1d-reactive T cells known to derive from the NK1.1+ population had an invariant TCR α-chain consisting of a particular Vα14-Jα281 rearrangement and used TCR β-chains having primarily Vβ8.2, Vβ7, or Vβ2 segments with diverse rearrangements (12). The use of this semimvariant TCR for CD1d recognition has been conserved through evolution (13–17). A very similar human TCR, with an invariant TCR α-chain and using the corresponding human TCR Vβ segments, recognizes the human homologue CD1d, and there is cross-recognition between the murine and human system (15). Others described murine CD1d-reactive T cells used diverse TCR α- and β-chains (7–10). These cells appear to share some of the characteristic features of the NK1.1+ T cells of B6 mice (7, 9, 18), but whether these cells belong to the NK1.1+ T cell subset is not clear.

The role of NKT cells, or CD1d-reactive T cells, in the immune system is not well understood, although their potent functional capacities, like rapid production of large amounts of IL-4 and IFN-γ upon in vivo stimulation (19), have been described. Their absence or aberrant function in certain murine and human autoimmune disorders indicate that they might be involved in the regulation of harmful autoimmune reactions (20–25). There is also evidence for a role of NKT cells during bacterial infections (26, 27), in IL-12-dependent rejection of tumors (28), and as helper T cells for IgG production (3).

To analyze further the function and differentiation of CD1d-reactive T cells, we have established a TCR-transgenic system expressing as transgene-rearranged TCR genes from a CD1d-autoreactive T cell hybridoma with the diverse-type TCR. Hybridoma VIII24 had been derived from the CD4+ population in MHC class II-deficient mice, used Vα3.2 and Vβ9 TCR rearrangements, and was reactive to endogenous CD1d on splenocytes and different CD1d-transfected cell lines (7). This report describes the phenotype and functional capacity of the TCR-transgenic T cells, their appearance in the thymus, and demonstrates their dependence on the ligand CD1d.

Materials and Methods

TCR-transgenic mice

The variable regions of the TCR genes from the VIII24 hybridoma were first cloned and sequenced from mRNA as previously detailed (29). Using appropriate oligo nucleotides, genomic DNA fragments containing the rearranged variable genes, Vα3.2/Jα20 and Vβ9/Jβ1.4, were amplified by PCR from the hybridoma and cloned into TCR expression cassette vectors as described in detail (30). Linearized DNA constructs from the TCRα and TCRβ cassette vectors, with prokaryotic sequences eliminated, were injected into fertilized (B6 × SJL)F1 embryos. Transgenic founders were screened for by Southern blot hybridization of tail DNA, and progeny of

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3 Abbreviations used in this paper: NKT cell(s), T cell(s) expressing the NK1.1 marker; β2-m, β2-microglobulin; B6, C57BL/6; DN, double negative; DP, double positive; SP, single positive; TCRαβ, T cells with diverse TCR; TCRαβ14, T cells using the semimvariant Vα14-Jα281 TCR; WT, wild type; CD1d+, CD1d deficient.

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positive founders typed by flow cytometry for transgene expression on PBLs. Transgene-carrying founder mice were backcrossed to B6 mice. Mice were 5 wk to 6 mo of age unless otherwise stated, and from the first to fifth backcross generation. The NK1.1 Ag is present in both SJL and B6 mice (11). For some experiments, the 24β transgenic line had been crossed with mice lacking CD1d (31), backcrossed five generations to the B6 genetic background.

Flow cytometry

Cells were stained with conjugates and Abs of the following specificities: Vβ9-FITC, CD3-FITC, TCRαβ-PE, CD44-PE, CD24 (M1/69), CD62L (Mel-14), Vβ9 (MR10.2), Vα3.2 (RR3.16), and B220 (RA3.6B2) had been purified and conjugated to FITC or biotin using standard procedures or to Cy5 according to the manufacturers instructions (Amer sham Life Sciences, Little Chalfont, U.K.). The samples were analyzed using FACSSort or FACS Calibur flow cytometers (Becton Dickinson, Mountain View, CA) and CellQuest software. Fluorescence was displayed on a log2 scale.

T cell activation

Spleen or lymph node cells were stimulated in vitro at 10^6 cells/well in 96-well plates in complete RPMI 1640 medium supplemented with 1 mM l-glutamine, 50 μM 2-ME, 1 mM sodium pyruvate, penicillin-streptomycin, and 10% heat-inactivated FCS) in the presence of 2.5% of supernatant from the X63Ag8 cells transfected to produce IL-2. The plates (nontissue culture grade) had been precoated with KT3 (anti-CD3ε) Abs, incubated with several dilutions of each supernatant or recombinant cytokine standards (ImmunoKontact, Bioggio, Switzerland), or BVD6 (anti-IL-4, Dako, Glostrup, Denmark). IgE was assayed using monoclonal anti-IgE reagents from Serotec (Oxford, U.K.) and an IgE standard from Pharmingen. IgG1 and IgG2a were analyzed using the mouse monoclonal isotyping reagents ISO-2 (Sigma).

Results

Mice expressing a transgenic CD1d-reactive Vβ9/Vα3.2+ TCR

For the construction of mice carrying a transgenic CD1d-reactive TCR, we selected a well-characterized CD4+ T cell hybridoma, VIII24 (7), as donor of rearranged TCR genes. VIII24 belongs to a set of CD1d-reactive hybridomas generated from CD4+ T cells from MHC class II-deficient mice, which were shown to respond to CD1d in the absence of added Ags (7). The reactivity of VIII24 was dependent on the expression by the APC of β2-microglobulin (βm), but not the TAP molecule (7), consistent with the requirements for CD1d expression (32–34). Stimulation could be inhibited by Abs to CD1d, or to either of the TCR V-segments, Vα3.2 or Vβ9 (S. Cardell, unpublished data). Rearranged genomic variable region fragments from the VIII24 hybridoma were cloned into expression cassette vectors containing natural promoter and enhancer regions (30). Separate lines were established for the transgenic TCR α-chain and the TCR β-chain constructs, respectively, and the two were crossed to obtain transgenic mice expressing the full CD1d-reactive TCRαβ (Fig. 1). The two single transgenic lines are referred to as 24α and 24β below, and the double transgenic mice as 24αβ.

Transgene-expressing T cells were visualized by flow cytometry with Abs to TCR Vβ9 and Vα3.2. Either transgenic TCR chain expression alone resulted in an increased proportion of CD8+ vs CD4+ T cells in the spleen (Fig. 1a). In contrast, in 24αβ mice the CD8+ population was severely reduced, and there was a decrease in the frequencies of splenic CD4+ cells. Among CD8+ cells, <10% expressed both transgenes at high levels (Fig. 1b), while total TCRαβ levels were homogenous (Fig. 1c), indicating a pronounced expression of endogenous both TCR α- and β-chains. Also in the CD4+ population, there was a marked expression of endogenous TCR α- and β-chains (Fig. 1b). Cells expressing both TCR transgenes were primarily found in a population of CD4/CD8 double negative (DN) T cells, with no signs within this subset of endogenous TCR chains on the surface (Fig. 1b, and demonstrated by costaining for CD3 and transgenic TCR chains, data not shown). The TCR levels were slightly reduced on both CD4+ and
CD8⁺ 24αβ T cells compared with cells from single transgenic and transgenic-negative littermate mice, suggesting an influence of low levels of the transgenic CD1d-reactive TCR also on these cells. The TCR levels on the DN population were even lower and similar to those of NKT cells from wild-type (WT) mice (Fig. 1c).

Transgene-expressing T cells exhibited a surface phenotype of NKT cells

In 24αβ mice, the NK1.1⁺ TCRαβ⁺ population increased 5- to 10-fold compared with WT or B6 mice (Fig. 2a), both in frequencies and absolute numbers. NKT cells made up 12.6% (±3.1, n = 7) of total spleen cells and 35.6% (±12.5, n = 5) of the TCRαβ⁺ population. In B6 or WT mice, 1.9% (±0.5) of splenocytes were NK1.1⁺ TCRαβ⁺. In mice expressing only one of the transgenic TCR chains, the frequency of NK1.1⁺ TCRαβ⁺ cells was similar or slightly lower than in control mice. In 24αβ mice, the majority of transgene-expressing cells (Vα3.2⁺ Vβ9⁺ cells in gate R2 in the first upper panel of Fig. 2b) were TN, although a substantial population, somewhat variable between mice, expressed graded levels of CD4 (Fig. 2b). A heterogenous CD4 expression was seen also on NK1.1⁺ T cells from WT mice. This can be directly compared between WT and 24αβ CD4⁺ cells in Fig. 1 and the TCRαβ⁺ NK1.1⁺ population displayed in Fig. 2b (in Fig. 2b an additional gate has been added for CD4⁺ T cells, while the upper gate is the same as that in Fig. 1). The majority of transgene-expressing cells (R2 gate) were CD4⁺ CD8⁻, CD62Llow, and positive for the NK1.1 marker and CD122 (IL-2R β-chain) (Fig. 2b), a surface phenotype similar to that of NKT cells of normal mice (gate R3 in the lower panel), although they expressed only low levels of CD69 while NKT cells of B6 mice were positive (not shown). Thus, 24αβ T cells present in the periphery of the 24αβ-transgenic mice possess a surface marker phenotype very similar to NKT cells from normal B6 mice.

Functional characteristics of transgenic T cells

NKT cells have been shown to display a particular profile of cytokines when stimulated in vitro (12, 18, 35) and further to rapidly secrete high amounts of cytokines upon in vivo induction (19). To analyze the potential of transgenic T cells to produce cytokines, lymph node or spleen cells were polyclonally stimulated in vitro. T cells in the 24αβ-transgenic mice responded well to TCR ligation in vitro by proliferation (not shown) and secretion of large amounts of IL-4 and IFN-γ (a 15- and 2-fold increase, respectively, compared with WT, Fig. 3a), while single transgenic controls were similar to WT.

To investigate the typical NKT cell function in vivo (19), 24αβ and control (single transgenic and B6) mice were injected with anti-CD3 Ab. Ninety minutes after anti-CD3 injection, spleen cells were prepared and cultured in medium for 90 min in vitro before supernatants were harvested and analyzed for IL-4 and IFN-γ. Each circle and box represents the value from one mouse.

Transgene-expressing cells in the thymus

Thymi of 24αβ mice contained 5–10% the number of cells compared with WT. 24α thymi contained around 20%, and 24β thymi were similar to WT (not shown). The CD4/CD8 double positive (DP) population was greatly reduced in 24αβ thymi but the CD4/CD8 DN subset enlarged (Fig. 5), generally also in absolute numbers (up to 2.5-fold). A population of DN thymocytes (Fig. 5)
expressing the transgenic TCR α-chain, but lacking the NKT phenotype (not shown), was present in 24α mice. DN T cell populations have been found in some other TCR-transgenic systems and have been proposed to contain cells of the γδ lineage (37, 38). Twenty-five to 50% of the TCR1 cells in 24α thymi were NK1.1− (Fig. 5), corresponding to an ~2.5-fold increase in numbers compared with WT, while either of the two transgenic chains alone rather decreased the number of NK1.1− TCRab thymocytes. The great majority of cells in the 24α DN population, 65–75%, expressed both TCR transgenes (Fig. 6a), with very few cells expressing endogenous TCR on the surface as determined by costaining for CD3 expression and transgenic TCR chains (not shown). In the 24α DP population, low levels of the transgenic TCR could be detected on some of the cells. There was a prominent expression of endogenous TCR α- and β-chains in the thymic CD4+ and CD8+ single positive (SP) subsets, just like in the peripheral SP subsets.

Among 24α DN thymocytes, most TCRαβ+ cells were CD122+, and around 40% of the DN TCRαβ+ population was CD24+ (heat shock Ag) (Fig. 6b). NK1.1 expression was found on a subset of TCRαβ+ cells, mostly within the CD24− fraction (Fig. 6c), and their level of TCRαβ was slightly lower than that of the major DN TCRαβ+ population (Fig. 6b). CD122-
Like in adult mice, thymi of 1-wk-old 24\(\alpha\beta\) mice contained very few SP cells, a reduced DP population, and a large DN population (Fig. 7b), of which most cells expressed the transgenic TCR chains, and 10% of these were NK1.1\(^+\) (Fig. 7c). Thus, 24\(\alpha\beta\)-transgenic T cells with the typical NKT phenotype appeared in the thymus and periphery only in the presence of the CD1d ligand.

### Discussion

In 24\(\alpha\beta\) mice expressing a transgenic CD1d-reactive TCR using \(\text{Va3.2}^\text{a}^\text{b}\) and \(\text{V\(\beta\)}\text{9}\), the majority of peripheral 24\(\alpha\)\(\beta\) T cells, and a high proportion of 24\(\alpha\)\(\beta\)\(\text{CD1d}°\) thymocytes expressed the NK1.1 marker. Peripheral 24\(\alpha\)\(\beta\) T cells shared characteristic features with NKT cells, including a phenotype of previous activation (CD44\text{high}, CD62L\text{low}), expression of CD122/IL-2R, intermediate levels of the TCR, and synthesis of high amounts of cytokines, IL-4 and IFN-\(\gamma\), upon activation (12, 18, 35). The appearance of 24\(\alpha\)\(\beta\) T cells of the typical NKT phenotype required the presence of CD1d and the expression of both chains of the CD1d-reactive TCR. Further, in contrast to what was reported for mice transgenic for the V\(\alpha\text{14}\)-type TCR, may result in dissimilar behavior of the cells in vivo.

Additionally, recognition of different CD1-associated ligands, presented on different APC or in distinct locations, by the 24\(\alpha\beta\)- and Va14-type TCR, may result in dissimilar behavior of the cells in vivo.
vivo. Regardless of some functional discrepancy, our results demonstrate that CD1d-reactive T cells with diverse TCR (TCRdiv) may be contained within the NK1.1+ TCRαβ+ population and potentially share at least some immune functions with CD1d-reactive TCRVα14+ T cells.

Although α-galactosylceramide has been shown to be a common ligand for TCRVα14+ cells (2), the broad TCR repertoire of TCRdiv CD1d-reactive T cells may reflect a potential to recognize a variety of ligands presented on CD1d. In line with this, recent reports have described ligand-specific reactivity to several distinct GPI molecules by NKT cells (3) and reactivity to cellular phospholipids (40), demonstrating the existence of a diversity of ligands recognized by CD1-restricted T cells. The 24αβ TCR recognizes a putative unknown CD1-bound ligand not requiring endosomal loading (41) and distinct from the ceramide based ligands activating the Vα14-type T cells (2, 40, 42, 56).

The major 24αβ+ TCR-positive T cell population in the thymus and periphery of transgenic mice was DN, and a minor subset gand interaction (49). CD122 expression on 24α Th splenic T cells (43), and down-regulated on CD4 notype dictated by the TCR specificity. In fact, it has been demonstrated that DN, but not CD4+ cells during thymic selection due to CD1d autoreactivity of the transgenic TCR were found in the DN subset, and the absence of the ligand CD1d, compatible with a deletion of cells expressing the transgenic TCR. In 24α Th manuscrit in preparation).

Diverse TCRs recognize murine CD1. Although CD4+ NKT cells have many similarities: the reactivity to CD1d in the absence of exogenous ligands, the activated cell surface phenotype, and the profile of cytokines secreted upon activation. But there are also important distinctions, such as the recognition of distinct CD1d-bound ligands and, in the case of 24αβ NKT cells, the lack of rapid cytokine secretion in response to TCR stimulation in vivo. This implies that the TCRdiv NKT cells may have the capacity to perform the same immune regulatory functions as suggested for the TCRVα14+ NKT cells, but possibly that they are induced in different situations. Identification of the endogenous ligands activating NKT cells in vivo will shed some light on this issue, as well as further analysis of the precise functions of NKT cell subsets during the immune responses in which their importance has been implied.

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