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Essential requirement of an invariant Vα14 T cell antigen receptor expression in the development of natural killer T cells

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ABSTRACT **NK1.1$^+$ T [natural killer (NK) T] cells express an invariant T cell antigen receptor α chain (TCRα) encoded by Vα14 and Jα281 segments in association with a limited number of Vβs, predominantly Vβ8.2. Expression of the invariant Vα14/Jα281, but not Vα1, TCR in transgenic mice lacking endogenous TCRα expression blocks the development of conventional T αβ cells and leads to the preferential development of Vα14 NK T cells, suggesting a prerequisite role of invariant Vα14 TCR in NK T cell development. In Vβ8.2 but not Vβ3 transgenic mice, two NK T cells with different CD3ε expressions, CD3ε^{dim} and CD3ε^{high}, can be identified. CD3ε^{high} NK T cells express surface Vα14/Vβ8 TCR, indicating a mature cell type, whereas CD3ε^{dim} NK T cells express Vβ8 without Vα14 TCR and no significant CD3ε expression (CD3ε^{dim}) on the cell surface. However, the latter are positive for recombination activating gene (RAG-1 and RAG-2) mRNA, which are only expressed in the precursor or immature T cell lineage, and also possess CD3ε mRNA in their cytoplasm, suggesting that CD3ε^{dim} NK T cells are the precursor of Vα14 NK T cells.**

T lymphocytes and natural killer (NK) cells are distinct subsets of lymphoid cell lineage that develop from common precursors (1–3). NK cells do not possess T cell antigen receptors (TCRs) but express distinct types of NK receptors (4, 5). NK T cells are characterized by the expression of TCR composed mainly of αβ heterodimers and CD16 and NK1.1, the latter two of which are "normally" NK markers (4, 5), and form a minor subset in the thymic CD4$^-$CD8$^-$ or CD4$^+$CD8$^-$ population (<0.5%) (6–13). In the periphery, NK T cells form a relatively major subset comprising 5% of splenic T and 40% of bone marrow T cells (13). Generally, murine splenocytes contain 2–3% NK cells (NK1.1$^+$ TCRβ$^-$), 1–2% NK T cells (NK1.1$^+$ TCRβ$^+$), and 40–50% conventional αβ T cells (NK1.1$^-$ TCRβ$^+$). Contrary to the general rule that the interaction of TCR with major histocompatibility complex molecules leads to the development of conventional αβ T lymphocytes, NK T cells are selected by a nonclassical major histocompatibility complex class I molecule (14–19), recently identified as CD1 in mice (20). It has been suggested that NK T cells are involved in tumor cell killing (11, 12, 21), controlling the T cell helper type 2 response (22, 23), acute bone marrow rejection (24, 25), and autoimmunity (26, 27).

Unique features of NK T cells include the expression of an invariant TCR α chain (TCRα) encoded by Vα14 and Jα281 segments with a one-base N region in association with limited Vβs, mostly Vβ8.2 (13, 28–30). This invariant Vα14 TCRα is not found in conventional αβ T cells. We investigated the role of Vα14/Vβ8 TCR in the development of NK T cells and found that Vα14 TCR expression in the absence of endogenous TCRα expression leads to the preferential development of NK T cells but the developmental arrest of conventional αβ T cells, indicating an essential role of invariant Vα14 TCR in the development of NK T cells. Furthermore, we found CD3ε^{dim} Vβ8$^+$, Vα14$^-$ NK T cells of the precursor or immature cell type that express mRNA of recombination activating genes (RAG-1 and RAG-2) in cytoplasm. Thus, we defined CD3ε^{dim} Vβ8$^+$, Vα14$^-$ NK T cells as NK T cell precursors.

MATERIALS AND METHODS

Mice. Specific pathogen-free C57BL/6 mice were purchased from the Shizuoka Experimental Animal Co. Ltd (Hamamatsu, Shizuoka, Japan). Vα14 TCR gene transgenic (Vα14 tg) mice were established using Vα14/Jα281 cDNA (pTsα2) (28). A 1.0-kb fragment of Vα14Jα281Cα cDNA (pTsα104/46) was cloned into the *Sal*I site of pHβAPr-3-neo, which contains a 4.3-kb fragment of the human (h) β-actin promoter (pHβAPr-Tsα2-neo). The 11.0-kb *Bgl*II/*Pvu*I fragment of pHβAPr-Tsα2-neo was microinjected into C57BL/6 fertilized eggs and implanted into foster mothers. The resulting progeny were screened for the integration of Vα14 TCR transgenes (5.3 kb) by Southern blot analysis on *Eco*RI-digested tail DNA with probes for hβ-actin promoter (4.3 kb fragment) and Vα14 cDNA. One (no. 12) out of five independent lines established was used for further experiments and mated with TCRα-deficient (TCRα$^{-/-}$) mice (kindly provided by P. Mombaerts; ref. 31) to generate TCRα$^{-/-}$/Vα14 tg mice by backcrossing for five generations. The (TCRα$^{+/-}$ × Vα14 tg)F$_1$ mice were selected and crossed with TCRα$^{+/-}$ mice to generate TCRα-deficient homozygous Vα14 tg (TCRα$^{-/-}$/Vα14 tg) mice with a C57BL/6 background.

Several other TCR gene transgenic mice were obtained: Vα11 tg (B10.A) from M. Davis (32), Vβ3 tg (B10.A) from M. Davis (32), Vα11/Vβ3 tg (B10.A) derived from crossing Vα11 tg and Vβ3 tg mice, Vβ8.2 (VβHY) tg mice (C57BL/6) and VαHY/Vβ8.2 tg (C57BL/6) from H. von Boehmer (33), Vα14/Vβ8.2 tg mice (C57BL/6) obtained by mating with Vα14 tg and Vβ8.2 (VβHY) tg mice, and TCRα$^{-/-}$/Vα1 tg mice with a C57BL/6 background generated by mating Vα1 (VαDO11.10) tg mice with a C57BL/6 background from D. Loh (34) with TCRα$^{-/-}$ mice and further backcrossing for five to seven generations in S.K.'s laboratory.

Fluorescein-Activated Cell Sorter (FACS) Analysis. Splenocytes were incubated with 10 μg/ml anti-CD16 (2.4G2) to block nonspecific Fc-mediated antibody binding, followed by incubation with various combinations of antibodies. For the

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Abbreviations: NK, natural killer; RAG, recombination activating gene; TCR, T cell antigen receptor; TCRα and TCRβ, TCR α and β chains; tg, transgenic; hβ-actin, human β-actin; FACS, fluorescein-activated cell sorter.

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detection of Vα14 TCR expression, Cychrome-avidin, biotinylated anti-NK1.1 (PK136), fluorescein isothiocyanate-conjugated anti-TCRβ (H57-598), and phycoerythrin-labeled anti-Vα14 (CMS-1) (35) were used; for the detection of CD3ε in NK T cells, fluorescein isothiocyanate-anti-CD3ε (2C11), phycoerythrin-anti-NK1.1, and biotinylated anti-TCRβ mAbs together with Cychrome-avidin were used. To confirm specific staining with anti-TCRβ or anti-Vα14, cells were preincubated with excess amounts of unlabeled antibodies and reacted with labeled antibodies. At least 10^5 cells, including small size cells, were gated and analyzed by EPICS-XL as described (13).

PCR. PCR was carried out with cDNAs derived from 5000 cells using primers specific for Vα14/Cα, Vβ8/Cβ, CD3ε, RAG-1, RAG-2, and β-actin under the conditions described previously (13). PCR was performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 40 cycles. Oligonucleotides were 5′-GTACTGGTATCGGCAGGACAC-3′ and 5′-GGTAGCCTTTTGTTTGTTTGC-3′ for Vβ8; 5′-CCCAAGTGGAGCAGAGTCCTAAGG-3′ and 5′-CTGTCCTGAGACCGAGGATC-3′ for Vα14; 5′-AACACTTTCTGGGGCATCCTG-3′ and 5′-TGATGATTATGGCTACTGCTG-3′ for CD3ε; 5′-CCAAGCTGCAGACATTCTAGCACTC-3′ and 5′-CAACATCTGCCTTCACGTCGATCC-3′ for RAG-1; 5′-CACATCCACAAGCAGGAAGTACAC-3′ and 5′-GGTTCAGGGACATCTCCTACTAAG-3′ for RAG-2; and 5′-GAGAGGGAAATCGTGCGTGA-3′ and 5′-ACATCTGCTGGAAGGTGGAC-3′ for β-actin.

RNase Protection Assay. The RNase protection assay described previously (29) was performed using ^{32}P-labeled antisense probes generated by Vα14/Jα281/Cα cDNA (pTsα2) and β2 m cDNA (pMZ416). The radioactivity of the protected band was measured by automated densitometry (Bioimage Analyzer BAS 2000, Fuji) and expressed as a percentage of total Cα or β2 m bands.

RESULTS AND DISCUSSION

NK T cells are known to express only one invariant TCRα encoded by Vα14 and Jα281 gene segments with a one-base N region, indicating its essential requirement for NK T cells (28–30). To examine the role of invariant Vα14/Jα281 TCR in NK T cell development, we generated a Vα14/Jα281 transgenic (Vα14 tg) mouse strain (Fig. 1 *A* and *B*). The expression of transgenic Vα14/Jα281 TCR was investigated by RNase protection (Fig. 1*C*) and FACS analysis (Fig. 1*D*). The transgenic Vα14 TCR mRNA was detected in all tissues, including thymus, spleen, and kidney, suggesting the unrestricted tissue expression of transgenic Vα14 TCR mRNA under the control of hβ-actin promoter. The frequency of Vα14 TCR expression in lymphoid tissues was, however, about 9 times higher (3.4% and 3.7% in thymus and spleen, respectively) than in kidney (0.43%) (Fig. 1*Ca*), indicating normal lymphoid cell development in Vα14 tg mice. FACS profiles of Vα14 tg spleen (Fig. 1*Dc*) showed similar patterns to those of normal C57BL/6 spleen (Fig. 1*Da*), whereas Vα14 TCR expression was significantly increased in the Vα14 tg NK T cell population (Fig. 1*Dd*) compared with those in C57BL/6 NK T cells (Fig. 1*Db*) (31–64%). This result indicates that the transgene of the invariant Vα14 TCR gives rise to increased expression of invariant Vα14 TCR even though the absolute numbers of NK T cells (7.7×10^5) in Vα14 tg spleen are at normal levels (8×10^5).

Since allelic exclusion of the TCRα locus is usually incomplete, TCRαs other than transgenic TCRα are likely to be expressed in Vα14 tg animals. To avoid the contribution of endogenous TCRα, we mated Vα14 tg mice with TCRα$^{-/-}$ mice to generate TCRα$^{-/-}$/Vα14 tg mice. Only Vα14$^+$ NK T cells developed in these mice, but surprisingly, conventional αβ T cells were absent (Fig. 2 *Ac* and *Ad*). In fact, thymocyte development was completely disturbed, because only double-positive thymocytes with no TCR expression in the absence of CD4$^+$ or CD8$^+$ single-positive thymocytes were detected, indicating the severe developmental arrest of conventional αβ T cells (Fig. 2*Ab*). TCR Vβ repertoire analysis also supports the present data and indicates that Vα14 TCR detected in TCRα$^{-/-}$/Vα14 tg mice are selectively associated with Vβ8.2 similar to the major NK T cell repertoire in normal C57BL/6 mice (data not shown). The expression of invariant Vα14 TCR in thymocytes and spleen cells of TCRα$^{-/-}$/Vα14 tg mice was also confirmed by RNase protection assay, demonstrating that invariant Vα14 mRNA expression was 6 times (1.4% in spleen) and 20 times (5.1% in thymus) higher than in kidney (0.23%) (Fig. 1*Cb*). In contrast, TCRα$^{-/-}$/Vα1 tg mice (Fig. 2*Ag*) have conventional αβ T cells but lack NK T cells. These results indicate that the expression of invariant Vα14/Jα281, but not other TCRαs such as Vα1, leads to the development of NK T cells and suppresses conventional αβ T cell development. The essential requirement of invariant Vα14 TCR expression for NK T cell development was recently confirmed in mice lacking invariant Vα14 TCR expression generated by the homologous recombination technique. In these animals, no NK T cells were

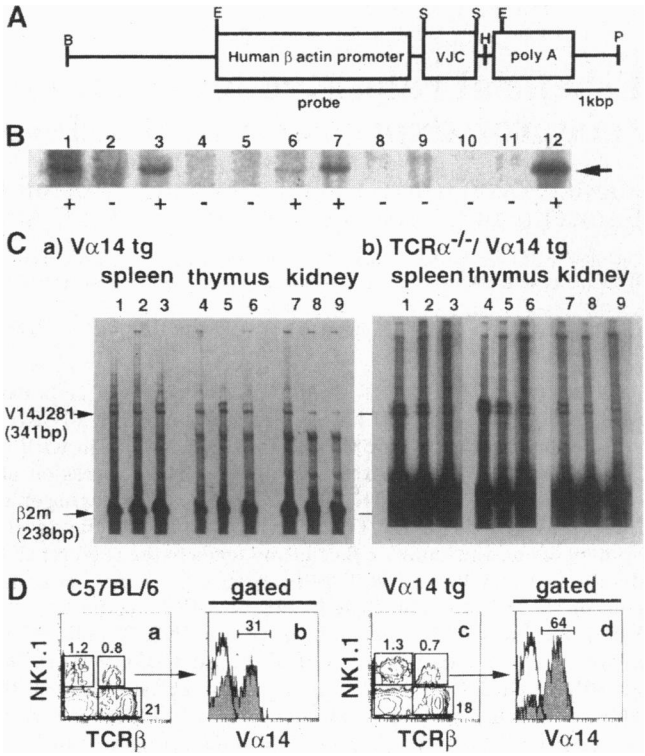

FIG. 1. Generation of Vα14 TCR gene transgenic mice and their expression. (*A*) Schematic representation of the Vα14 TCR construct. Restriction enzyme sites: B, *Bgl*II; E, *Eco*RI; S, *Sal*I; H, *Hin*dIII; P, *Pvu*I. (*B*) Detection of the transgenic hβ-actin/Vα14 gene by Southern blotting on *Eco*RI-digested tail DNA. The data show the 5.3-kb band detected by an hβ-actin probe. The Vα14 probe also detected the same band. No. 12 and its progeny were used for experiments. (*C*) Expression of invariant Vα14 TCR as detected by an RNase protection assay. The frequency of invariant Vα14 TCR is expressed as the percentage of β2m in each sample (nos. 1–9): (*a*) 5.0%, 1.2%, and 4.0% (mean 3.4%) in the spleen; 3.4%, 6.1%, and 1.5% (mean 3.7%) in the thymus; 0.7%, 0.4%, and 0.2% (mean 0.43%) in the kidney in Vα14 tg mice; (*b*) 2.5%, 0.9%, and 0.7% (mean 1.4%) in the spleen; 8.4%, 4.8%, and 2.1% (mean 5.1%) in the thymus; 0.3%, 0.1%, and 0.3% (mean 0.23%) in the kidney in TCR$^{-/-}$/Vα14 tg mice. (*D*) Cell surface expression of Vα14 TCR detected by FACS. (*a*) C57BL/6 spleen (1×10^8). (*c*) Vα14 tg spleen (1.1×10^8). (*b* and *d*) Vα14 staining patterns of NK T cells gated from *Da* and *Dc*, respectively. The numbers in parentheses represent total cell numbers in the legend and percentages in the FACS figures, respectively.

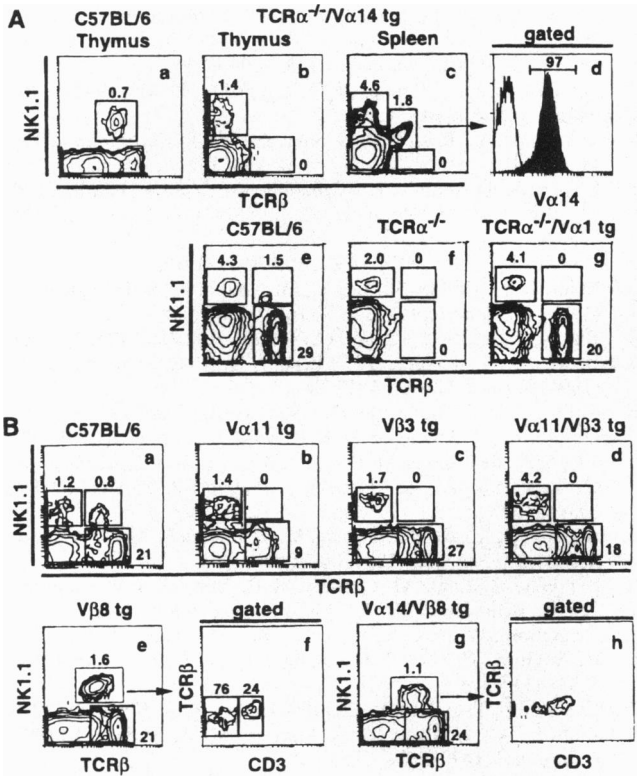

FIG. 2. FACS analysis of NK T cells in various TCR tg mice. (*A*) (*a*) C57BL/6 thymus (1.1×10^8). (*b*) C57BL/6 TCR$\alpha^{-/-}$/Vα14 tg thymus (0.85×10^8). Note that many of NK1.1$^+$ cells detected in Fig. 2*Ab* are γδ NK T cells that are not normally seen in C57BL/6 thymus but are present in TCR$\alpha^{-/-}$ mice. (*c*) C57BL/6 TCR$\alpha^{-/-}$/Vα14 tg spleen (1.1×10^8). (*d*) Vα14 TCR staining profile of NK T cells gated from *Ac*. (*e*) C57BL/6 spleen (0.8×10^8). (*f*) C57BL/6 TCR$\alpha^{-/-}$ spleen (1×10^8). (*g*) C57BL/6 TCR$\alpha^{-/-}$/Vα1 tg spleen (0.88×10^8). (*B*) (*a*) C57BL/6 spleen (1×10^8). (*b*) B10.A Vα11 tg spleen (1×10^8). (*c*) B10.A Vβ3 tg spleen (1.2×10^8). (*d*) B10.A Vα11/Vβ3 tg spleen (1×10^8). (*e*) C57BL/6 Vβ8.2 tg (1.3×10^8). (*f*) CD3ε staining patterns of NK T cells gated from *Be*. CD3ε^{high} and CD3ε$^{dim/-}$ fractions were sorted out by FACS and used for PCR analysis shown in Fig. 4. (*g*) C57BL/6 Vα14/Vβ8.2 tg spleen (1.1×10^8). (*h*) CD3ε staining profiles of NK T cells gated from *Bg*. The numbers in parentheses represent total cell numbers in this legend and percentages in the figures, respectively.

observed, whereas other lymphoid lineage cells were normal (unpublished data). These findings also confirmed that NK T cells are an independent lineage distinct from other cell types and that Vα14 TCR is essential for the development of NK T cells.

Since Vβ8.2 is predominantly used in NK T cells, we examined NK T cell development in Vβ8.2 tg mice. As shown in Fig. 2*Be*, Vβ8.2 tg mice have NK T and T cells but lack NK cells. In contrast, Vβ3 tg animals have NK and T cells but lack NK T cells (Fig. 2*Bc*). It has been shown that Vβ5 tg mice also lack NK T cells (24). It is thus likely that Vβ8.2, but not Vβ3 or Vβ5, supports the development of NK T cells. This was also confirmed in TCRα/β double-transgenic mice. Mice expressing both Vα14 and Vβ8.2 have NK T cell populations similar to that of Vβ8.2 tg mice (Fig. 2*Bg*), but the majority of NK T cells in the double-transgenic mice become Vα14$^+$ CD3ε^{high} (data not shown; see Fig. 2*Bh*). On the other hand, Vα11 tg, Vβ3 tg, or Vα11/Vβ3 tg mice do not have NK T cells showing normal NK and T cell development (Fig. 2 *Bb*, *Bc*, and *Bd*). Although the disappearance of NK cells in Vβ8 tg or Vα14/Vβ8 tg mice is intriguing, actual proof of this observation awaits further experiments. In any event, Vβ8.2 expression by itself is not sufficient to induce NK T cell development, whereas both Vα14/Vβ8.2 TCR expression are required for full NK T cell development. RNase protection analysis also supports the above notion, because invariant Vα14 expression was always detected in Vβ8.2 tg and VαHY/Vβ8.2 tg but not in Vα1 tg, Vα11 tg, Vβ3 tg, or Vα11/Vβ3 tg mice (Fig. 3). Therefore, the expression of invariant Vα14/Jα281 TCR and the development of NK T cells correlate perfectly, indicating that NK T cell development depends on invariant Vα14/Jα281 expression.

Interestingly, NK T cells in Vβ8.2 tg mice contain two populations: CD3ε^{high} and CD3ε^{dim}/CD3ε$^-$ (Fig. 2*Bf*). Thus, it is possible that the former is a mature cell type, whereas the latter is a precursor or immature cell type of the former. In fact, the transition from CD3ε^{dim} NK T cells to CD3ε^{high} NK T cells is quite likely, because the CD3ε^{dim} NK T cell fraction in Vβ8.2 tg mice, as shown in Fig. 2*Bf*, was greatly reduced, and the majority of NK T cells become CD3ε$^+$ in Vα14/Vβ8.2 double-transgenic mice (Fig. 2*Bh*). We thus attempted to investigate whether CD3ε^{dim} NK T cells possess the molecular characteristics of an immature or precursor cell type. CD3ε^{high} and CD3ε^{dim} NK T cell populations in Vβ8.2 tg mice (Fig. 2*Bf*) were isolated by FACS and analyzed by PCR. The data shown in Fig. 4 clearly demonstrate that CD3ε^{dim} NK T cells have no Vα14 TCR mRNA, although they express small but significant levels of CD3ε mRNA in the cytoplasm, even though their cell surface expression is not enough to be detected by anti-CD3ε (left rectangle in Fig. 2*Bf*). In addition, CD3ε^{dim} NK T cells possess RAG-1 and RAG-2 mRNA, indicating their immature or precursor potential in this particular cell type (Fig. 4). It is, therefore, quite likely that CD3ε^{dim} NK T cells without Vα14 TCR expression are precursor for mature Vα14$^+$ NK T cells. In fact, both RAG-1 and RAG-2 expression were negative following their expression of Vα14 TCR in CD3ε^{high} NK T cells (Fig. 4).

Based on these characteristics, we define the Vα14$^-$ Vβ8$^+$ CD3ε^{dim} NK T cell population as an NK T cell precursor. The results clearly suggest that the induction of Vα14 TCR, as well as the down-regulation of RAG expression, are essential for full NK T cell development. These events are strikingly similar to those observed in the transition from pro-Bμ$^-$ cells to large pre-Bμ$^+$ cells where RAG expression in pre-Bμ$^+$ cells becomes entirely negative along with the expression of the μ-heavy chain on the cell surface (36).

In summary, we present evidence that invariant Vα14 TCR expression in the absence of endogenous TCRα expression blocks the generation of conventional αβ T cell development

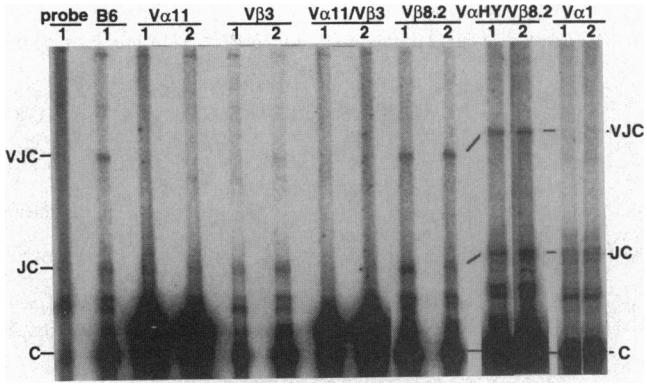

FIG. 3. RNase protection analysis of invariant Vα14/Jα281 TCR expression in various TCR tg mice. Total RNA from C57BL/6 (B6), Vα1 tg, Vα11 tg, Vβ3 tg, Vα11/Vβ3, Vβ8.2 tg, and VαHY/Vβ8.2 tg mice were used. Lanes 1 and 2 represent two individual mice in each experiment. Anti-sense riboprobes detect Vα14/Jα281/Cα (401 bp), Jα281/Cα (275 bp), and Cα (211 bp) protected bands, respectively. Note that invariant Vα14 TCR expression was detected only in Vβ8.2 tg and VαHY/Vβ8.2 tg mice, but not in other mice.

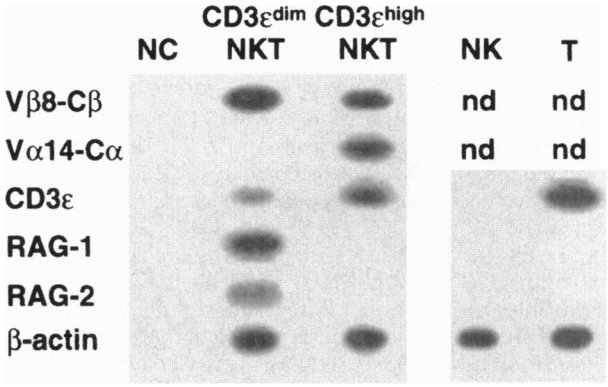

FIG. 4. Expression of TCR complex and RAG mRNA in $CD3\varepsilon^{dim}$ and $CD3\varepsilon^{high}$ NK T cells. Reverse transcription–PCR was performed on RNA from 4–5 × 10^5 cells sorted from $CD3\varepsilon^{dim}$ (lane 2) and $CD3\varepsilon^{high}$ (lane 3) fractions of C57BL/6 Vβ8.2 tg NK T cells shown in Fig. 2*Bf*, and RNA from 2 × 10^5 αβ T cells (lane 4) and 2 × 10^5 NK cells of C57BL/6 spleen (lane 5). nd, not done; NC (lane 1), negative control.

and selectively drives the NK T cell-developmental pathway, indicating the essential role of Vα14 TCR in the development of NK T cells. In addition, the expression of Vβ8.2 results in the generation of the precursor form of Vα14 NK T cells. The precursor NK T cells express small amounts of CD3ε mRNA but large amounts of RAG-1/RAG-2 mRNA in the cytoplasm, whereas no significant Vα14 TCR or CD3ε were detected on the cell surface. Interestingly, the down-regulation of RAG-1/RAG-2 expression in precursor NK T cells seems to be essential for the development of mature $V\alpha14^+$ NK T cells. Although NK T cells seem to represent a lineage distinct from conventional T cells because of their extrathymic development before thymus formation during the very early stages of embryogenesis (d9.5) (37, 38), the molecular events that occur during NK T cell development are similar to those of other lymphoid cell lineages.

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