Premature Expression of T Cell Receptor (TCR) $\alpha\beta$ Suppresses TCR $\gamma\delta$ Gene Rearrangement but Permits Development of $\gamma\delta$ Lineage T Cells

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Abstract

The T cell receptor (TCR) $\gamma\delta$ and the pre-TCR promote survival and maturation of early thymocyte precursors. Whether these receptors also influence $\gamma\delta$ versus $\alpha\beta$ lineage determination is less clear. We show here that TCR $\gamma\delta$ gene rearrangements are suppressed in TCR $\alpha\beta$ transgenic mice when the TCR $\alpha\beta$ is expressed early in T cell development. This situation offers the opportunity to examine the outcome of $\gamma\delta$ versus $\alpha\beta$ T lineage commitment when only the TCR $\alpha\beta$ is expressed. We find that precursor thymocytes expressing TCR $\alpha\beta$ not only mature in the $\alpha\beta$ pathway as expected, but also as CD4$^+$CD8$^-$ T cells with properties of $\gamma\delta$ lineage cells. In TCR $\alpha\beta$ transgenic mice, in which the transgenic receptor is expressed relatively late, TCR $\gamma\delta$ rearrangements occur normally such that TCR $\alpha\beta$-CD4$^+$CD8$^-$ cells co-express TCR $\gamma\delta$. The results support the notion that TCR $\alpha\beta$ can substitute for TCR $\gamma\delta$ to permit a $\gamma\delta$ lineage choice and maturation in the $\gamma\delta$ lineage. The findings could fit a model in which lineage commitment is determined before or independent of TCR gene rearrangement. However, these results could be compatible with a model in which distinct signals bias lineage choice and these signaling differences are not absolute or intrinsic to the specific TCR structure.

Key words: lineage commitment • TCR transgenic mice • thymus • differentiation • positive selection

Introduction

The thymus is able to generate distinct types of mature T cells that are differentiated for specific TCR recognition and effector functions. Early in development, precursor thymocytes rearrange and express the genes encoding TCRs and mature as either $\alpha\beta$ or $\gamma\delta$ lineage T cells (for reviews, see references 1, 2). The first T cells are $\gamma\delta$ lineages that arise only in the fetal thymus. Each of these bears a unique, canonical TCR and colonizes distinct epithelial tissues of the periphery. The $\gamma\delta$ T cells that populate the lymphoid organs have more diverse receptors and develop in both the fetal and adult thymus. Lymphoid $\gamma\delta$ T cells and precursors to the $\alpha\beta$ T cell lineage (bearing the pre-TCR) appear roughly around the same time in the adult thymus and are thought to derive from a common CD4$^+$CD8$^-$ precursor. The productive rearrangement and expression of the TCR $\gamma\delta$ or of the pre-TCR (a heterodimer of TCR $\beta$ with invariant pT$\alpha$) is critical for survival and further differentiation of these early thymocytes (3). Of major interest is whether these receptors play a role in $\alpha\beta$ versus $\gamma\delta$ lineage determination or only in the progression of already committed precursors (4, 5).

The pathways of $\alpha\beta$ and $\gamma\delta$ T cell development are quite distinct. Although discrete stages of $\gamma\delta$ development have not been identified, most $\gamma\delta$ lineage T cells never express the CD4 or CD8$\alpha\beta$ coreceptors and have no requirement for MHC for maturation (6, 7). In contrast, precursor CD4$^+$CD8$^-$ thymocytes expressing the pre-TCR proliferate, upregulate TCR$\alpha$ rearrangement, and progress to a CD4$^+$CD8$^-$ intermediate stage (3). If rearrangement of TCR$\alpha$ is productive, TCR$\alpha$ replaces pT$\alpha$ to form the mature TCR $\alpha\beta$. Recognition of MHC by TCR $\alpha\beta$ is required for the development of mature $\alpha\beta$ lineage T cells, expressing either CD4 or CD8. The development of an additional subset of $\alpha\beta$ T cells, the so-called NK T cells, is $\beta_2$-microglobulin (B$\beta$2m) dependent (8, 9). This minor popula-

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tion of T cells expresses either CD4 or no coreceptor, a restricted TCR repertoire, and is not detected until after birth. Although the lineage relationship of NK T cells to conventional αβ T cells is somewhat controversial, NK T cells have characteristic phenotypic and functional properties that clearly distinguish them from other T cell subsets (9).

With the advent of TCR αβ transgenic mice, a novel population of TCR αβ+CD4−CD8− (TCR αβDN) T cells was observed (10–13). These cells appear early in the fetal thymus, colonize both epithelial and lymphoid tissues, and are especially prominent in TCR αβ transgenic mice undergoing strong negative selection. Naturally, questions arose as to their origin and lineage relationship to other T cells. There was speculation that these cells could be related to the TCR αβ+CD4−CD8− cells of wild-type mice (NK T cells) or to the abnormal TCR αβ+CD4−CD8− cells observed in lpr mutant mice (14). Others suggested that they derive from conventional αβ T cells after the downregulation of CD4 or CD8 (15, 16) or that they mature in the αβ lineage without ever expressing the CD4/CD8 coreceptors (16).

Evidence that the TCR αβDN T cells mature in a lineage separate from conventional αβ T cells came from studies of transgenic HY TCR mice. In contrast to the CD8 T cells of these mice, the TCR αβDN cells do not express endogenous TCR α genes, their TCR β gene segments are not deleted (17), and they do not develop in mice deficient for the common cytokine receptor γ chain (18). TCR αβDN cells mature in the absence of the selecting MHC and, most notably, in HY TCR mice with a pTα null mutation (pTα−/−), a few TCR αβDN cells coexpress TCR αγ and the transgenic TCR αβ (17). Given these characteristics, it was proposed that TCR αβDN cells of TCR αβ transgenic mice belong to the γδ lineage. In this model, the transgenic TCR αβ replaces TCR γδ while still allowing γδ lineage development. This model was contested, however, in an additional report using DO.11.10 TCR transgenic mice (16). Since TCR αβDN cells required specific MHC for development, the authors hypothesized that these cells were αβ lineage T cells that mature without passing through the CD4+CD8+ intermediate stage of development.

In previous studies, there was only limited characterization of TCR αβDN cells of TCR αβ transgenic mice, making it difficult to determine their relationship to conventional T cell subsets. As no single marker can distinguish γδ lineage T cells (with the exception of the TCR itself), we examined TCR αβDN cells using a number of criteria (phenotype, function, development, and localization). An analysis of several strains of TCR αβ transgenic mice reveals that TCR αβDN cells clearly exhibit characteristics of γδ lineage T cells. The MHC requirements for maturation and the regulation of TCR gene rearrangement are distinctively different in TCR αβDN cells than in conventional αβ lineage T cells. The results indicate that the premature expression of TCR γδ allows thymocyte precursors to mature in the γδ lineage. These findings have implications for models of γδ/αβ lineage determination.

Materials and Methods
Mice. C57BL/6 (B6), C57BL/10 (B10), B10.A, B10.Q, and B10.D2 mice were obtained from a National Institutes of Allergy and Infectious Diseases contract to Taconic Farms, Inc., and B10. BR and BALB/c, from The Jackson Laboratory. TCR αβ transgenic mice were backcrossed, intercrossed, and selected as described previously (19) to obtain H-2b, H-2d, H-2e, H-2f, H-2q RAG-2+ recombination activating gene (RAG-2+), HY TCR mice (20–23) and H-2q HA TCR mice (24). H-2b and H-2d HY TCR mice (26) were obtained by backcrossing 12 times to B10 and then to B10.D2, H-2b and H-2k 5CC7 TCR mice, by crossing B6 5CC7 TCR mice (27) to B10 or B10.A, and H-2b and H-2d class I−/− P14 TCR mice (28), by backcrossing 10 times to B6 and then to βm−/− (29). Except where noted, all TCR αβ transgenic mice were on the positive-selecting MHC background: AND TCR (H-2b or H-2e), 5CC7 TCR (H-2d), DO.11.10 TCR (H-2b), HY TCR (H-2e), and P14 TCR (H-2d). TCR γδ transgenic mice included the G8 TCR mice (H-2b βm−/−) crossed and selected as described (7), or H-2b 5G78 TCR mice, backcrossed eight times to B6.

Fetal mice were obtained from timed matings. The day of finding a vaginal plug was designated as day 0 of embryonic development. Mice were bred and maintained in a National Institutes of Allergy and Infectious Diseases Research Animal Facility or on a National Institutes of Allergy and Infectious Diseases contract to Taconic Farms, Inc., according to American Association for the Accreditation of Laboratory Animal Care specifications. All protocols for animal studies were approved by the National Institutes of Allergy and Infectious Diseases Animal Care and Use Committee.

Cell Preparation, Antibodies, and Flow Cytometry. Cultured cell lines used for these studies included: DT 73 (TCR Vγ2/Vδ5), a mouse CD4−CD8+ T cell/BW 5147 hybridoma, and DCEK, a mouse L cell fibroblast line transfected with E20. Thymocytes, LN, and NT cells were prepared in single cell suspensions as described previously (31). For enrichment of heat stable antigen (HSA) (CD24+) thymocytes, a culture supernatant of anti-HSA (J11d) antibody was used with a 1:10 dilution of Lo-Tox-M rabbit complement (Cedarlane) and DNase (106 U/ml; Calbiochem). For magnetic bead isolation of CD4+CD8+ thymocytes or LN T cells, 107 cells were reacted with 250 μl of purified H-2919 and 53-67.6 (and R A3.682, for LN T cells) antibodies (30 min, 4°C). CD4+CD8+ cells were removed by treatment with sheep anti-rat IgG-coated magnetic beads (30 min, 4°C) at a 5:1 bead to cell ratio, using an MPC-1 magnetic particle concentrator (Dynal). This process was repeated at a 10:1 bead to cell ratio. Epidermal lymphocytes were isolated and prepared in a single cell suspension as described (32). Trypsinized surface antigens were resynthesized in overnight culture with 20 U/mi recombinant IL-2 (Genzyme). To enrich for viable cells, harvested cells were incubated with biotin-labeled goat anti-hamster IgG (CalTag) (30 min, 4°C), washed twice, and bound to streptavidin-coated magnetic beads (Miltenyi Biotech) (30 min, 4°C). Cells were passed over a MACS column (Miltenyi Biotech) and the nonadherent fraction was collected.
Antibodies and staining reagents included: anti-TCR $\beta$-FITC, -PE or -allophycocyanin (APC) (H57-597), anti-γδ TCR -FITC, -PE, or unlabeled (GL3), anti-CD4–FITC, -PE, -APC, or -CyChrome (R M 4-5), anti-CD8a–CyChrome or unlabeled (53-6-7), anti-CD8b.2–FITC (53-5.8), anti-IL-2R β-FITC (TM- b1), anti-NK 1.1–PE (PK136), anti-CD5–FITC (53-7.3), anti-Vα11 TCR–FITC or unlabeled (R R8-1), anti-Vα2–FITC or -PE (B20.1), anti-Aα–FITC or -PE (AF6-120.1), anti-Eκ–FITC (14-4-45), anti-H-2Kd–FITC (SF1-1.1), anti-H-2Kk–FITC (36-7-5), anti-H-2Kd–FITC (AF6-88.5), anti-H-2Kk–FITC (KH-114), and anti-CD45R/B220–FITC, -PE, or unlabeled (Becton Dickinson). Dead cells were excluded by light scatter (TM-b1). 150,000 events were collected for cell sorting. 105 sorted cells were digested using 1 mg/ml proteinase K, 0.5 M NaOH and propidium iodide gating. 10,000 CD4$^+$ and 20,000 CD8$^+$ cells were electronically sorted using standard protocols (33). For some analyses, cells were pretreated with an unlabeled anti-Fc receptor to block Fc receptor binding of the labeled antibodies. Multicolor flow cytometry was performed on a FACSCaliburTM, FACStarPlus™, or EPICS 753 FACS® (Becton Dickinson). 150,000 cells were collected for three- and four-color analyses. For live-gated samples, 10,000–20,000 CD4$^+$CD8$^+$ events were collected. Isolation of thymocyte and LN T cell subsets by electronic cell sorting was performed on a FACStarPlus™ (Becton Dickinson) or an EPICS 753 (Beckman Coulter).

For typing of transgenic or mutant mice, peripheral blood lymphocytes were stained with labeled antibody to the appropriate surface antigen, counterstained with Thy1.2 or B220 (used for live gating for T or B cells, respectively). After staining, samples were depleted of red blood cells with ACK lysis buffer (pH 7.4) and analyzed by flow cytometry.

In Vitro TCR Stimulation for Proliferation, Induction of CD8αα Expression, and IL-4 Secretion. For TCR stimulation, cells were added to U-bottomed 96-well plates coated with anti-TCR antibodies as described (31). Proliferation was determined on day 3 of culture, measuring $[^3]H$ thymidine incorporation (1 μCi/ml pulse for 18 h). Coexpression of CD8a and CD8b was assessed on day 4 of culture by flow cytometry, IL-4 production was assayed by ELISA (34) using 100 μl of supernatant collected at day 3 of culture and stored at −20°C.

Radiation Bone Marrow Chimeras. Bone marrow chimeras were made as described by reconstituting irradiated recipients (1,000 rads, Cs source) with T-depleted bone marrow (19). For the cyclosporine A (CSA) experiments, reconstituted mice received daily intraperitoneal injections of 0.4 or 0.6 mg Sandimmune™ (Sandoz) in 100 μl olive oil (Bertoli Classico) or of 100 μl olive oil only, starting on day 3 after reconstitution.

Quantitative PCR. T cell subsets were isolated by electronic cell sorting. 105 sorted cells were digested using 1× PCR Buffer (PerkinElmer), 2.5 mM MgCl2, 20 mg/ml proteinase K, 0.05% Tween 20, and 20% InstaGene Matrix (Bio-Rad Laboratories) at 56°C (2 h), followed by boiling (10 min). PCR was performed using a reaction mixture containing 1× PCR Buffer (PerkinElmer), 2.5 mM MgCl2, 200 μM each dNTP, 12.5 pmol each primer, and 0.25 U native Taq polymerase (PerkinElmer) bound to anti-Taq (CLONTECH Laboratories, Inc.). The total reaction volume was 50 μl with 5 μl of DNA. Samples were incubated at 95°C (5 min); amplified for 40 cycles at 94°C (30 s), 56°C (1 min), and 72°C (1.5 min), and incubated at 72°C (10 min) using a 96-well plate in a PTC-100 thermocycler (MJ Research, Inc.). Aliquots of 5 μl were removed every three cycles beginning at cycle 18.

The following primers and probes were used: Vγ2, TGTCCT-TGCAACCCCTTACC; j3-T, TTGTTCTCTGCAAATACCTTG; Vγ2 probe, GAGGAAAGGAAGCAAGCTATC; 5′ Cγ1, TTACAGACAAAGGCTTGGTC; 3′ Cγ1, GTTCT-GAGCTATGACACATC; and Cγ1 probe, CTGAA-GACTAAGACACATAC.

Quantitation was performed using a modified ELISA as described (35). In brief, one primer for each gene was labeled with a 5′ biotin moiety allowing capture of the PCR product on an avidin-coated plate. The second strand was denatured with 0.1 M NaOH and an FITC-labeled probe was bound to the captured strand. Bound probe was detected with an anti-FITC labeled with alkaline phosphatase in the presence of substrate, CSPD (Tropix). Chemiluminescence was measured using a luminometer (Dynatech).

To estimate the relative frequency of Vγ2-Jγ1 rearrangements in the experimental populations, a standard curve generated by biotraping DN7.3 cells (containing three Vγ2-Jγ1 rearrangements per cell [36]) with DCEK fibroblast cells and amplifying the serially diluted samples in the same PCR. For each sample of 105 cells, a PCR ELISA was performed and the quantity of PCR product (in light units) was determined as a function of cycle number (18–39 cycles). Primers and probes specific for Cγ1 were used to normalize the amount of DNA present. Data from the luminometer were fit to a logistic equation, and the parameters used to calculate the cycle value at half-maximum (C50) of amplification (35). C50 values were plotted against the corresponding log10 cell number of DN7.3 cells in each input sample and a best-fit line was generated. C50 values for experimental samples obtained in the same assay could be matched to this best-fit line to estimate the relative frequency of Vγ2-Jγ1 rearrangements.

Results

CD4$^+$ CD8$^-$ T Cells of TCRαβ Transgenic Mice Have Properties of γδ Lineage T Cells. Wild-type mice bear two CD4$^+$ CD8$^-$ subpopulations of mature T cells, one bearing TCRαβ (referred to as NK T cells) and the other, TCRγδ. In contrast, an analysis for TCR on CD4$^+$ CD8$^-$ T cells of HY TCR (TCRαβ) transgenic mice reveals no TCRγδ and a larger than usual population of TCRαβ$^+$ cells (37). Also in contrast to CD4 or CD8 αβ lineage T cells, the CD4$^+$ CD8$^-$ T cells of HY TCR and 2B4 TCR transgenic mice express only the transgenic TCRαβ and no endogenous TCRα (17, 38). Because of these unusual features, we further characterized the TCRαβDN subset of AND TCR and other TCR transgenic mice to assess lineage properties relative to normal T cell subsets.

TCRαβDN cells were analyzed for phenotype and function and compared with the NK T, γδ T, and the major CD4 and CD8 αβ T cell subsets of wild-type mice, as well as CD4$^+$ CD8 TCRγδ$^+$ cells of TCRγδ transgenic mice (TG78) (30). As shown previously (8), freshly isolated NK T cells of B6 mice (TCRαβDN) express IL-2R
Figure 1. Phenotypic markers distinguish the TCR αβDN T cell subset of TCR αβ transgenic mice from αβ lineage T cells (CD4 and CD8), NKT, but not from γδ lineage T cells. Thymocytes (A and B) or B-cell-depleted lymph node T cells (C) from B6, transgenic TCR γδ (TG78), or transgenic TCR αβ (AND and P14) mice were each stained for TCR αβ, TCR γδ, CD4 and/or CD8, and a fifth marker (IL-2R α or CD5), and analyzed by flow cytometry. The mean fluorescence intensity for the specified markers was determined for CD4/CD8 single positive (SP) T cells by software gating for CD4+CD8− TCR αβ+ and CD4+CD8− TCR αβ−, or for CD4+CD8+ T cells, by live gating for CD4+CD8+ followed by software gating for TCR αβ+ or TCR γδ+. Each bar represents the means (with SE bars) collected from analysis of three individual mice with the exception of B6 (two mice).

Figure 2. TCR αβDN T cells respond to anti-TCR stimulation by proliferating but do not produce an IL-4 response. Lymph node CD4+CD8− or CD4+CD8+ T cells from AND TCR (Vα11/Vβ3), CD4+CD8− TCR γδ+ T cells from G8 TCR mice (purified using magnetic beads and electronic cell sorting), or HSA+ B6 thymocytes (an enriched source of NKT cells), plated at 5 × 10^5 cells/well, were stimulated with 10 ng/ml immobilized anti-TCR antibody (anti-Vα11 for AND TCR, anti-TCR γδ for G8 TCR, and anti-TCR αβ for B6 TCR) and assayed for (A) IL-4 production where 1 unit = 0.5 pg of IL-4, and for (B) proliferation. Data are representative of two experiments, averaging values from triplicate wells, and are derived from dose-response curves using 0.1–100 μg/ml of antibody.
TCR \(\alpha\beta\)DN cells to make this response. As shown in Fig. 4, a and b, \(V_{\alpha 11}\)CD4-CD8\^-, but not \(V_{\alpha 11}\)CD4\^- T cells, are induced to express CD8\^{a and b} in response to anti-TCR stimulation. Similar responses have been obtained from TCR \(\alpha\beta\)DN splenocytes of TCR \(\alpha\) transgenic mice (39). Thus, by all of the criteria we examined, TCR \(\alpha\beta\)DN cells are clearly distinguished from conventional \(\alpha\beta\) lineage and NKT cells, and most resemble \(\gamma\delta\) lineage T cells.

TCR \(\alpha\beta\)DN cells of TCR \(\alpha\beta\) transgenic mice do not require MHC for development. One of the hallmarks of T cell development is the requirement for MHC-specific positive selection (47). In contrast, \(\gamma\delta\) T cells fully mature in the absence of MHC (6, 7). Since there are conflicting reports on the selection requirements of TCR \(\alpha\beta\)DN cells (10, 12, 16), we tested several strains of TCR \(\alpha\beta\) transgenic mice, bearing MHC class I- or class II-specific TCRs. As shown in Fig. 5, the TCR \(\alpha\beta\)DN cells of five different strains of TCR \(\alpha\beta\) mice develop equally well in the positively selecting or in the neutral (nonselecting) MHC background. Development is comparable both in percentage (Fig. 5) and in absolute number (data not shown).

**Table I.** Effect of CsA on Developing Thymocyte Subsets

| Treatment      | No. of thymocytes |
|----------------|-------------------|
|                | Total | CD4\^-CD8\^- | CD4\^-CD8\^+ | CD4\^+CD8\^- | CD4\^+CD8\^+ |
| O\^i l         | 3.1 \times 10^6 | 2.25 \times 10^6 | 3.8 \times 10^5 | 3.2 \times 10^5 |
| 0.4 mg CsA/oil | 1.4 \times 10^6 | 1.1 \times 10^6 | 1.7 \times 10^5 | 3.2 \times 10^4 |
| Fold reduction  | 2 \times     | 2 \times     | 2 \times     | 10 \times     |
| O\^i l         | 16.2 \times 10^6 | 5.2 \times 10^6 | 2.9 \times 10^6 | 7.6 \times 10^6 |
| 0.6 mg CsA/oil | 1.5 \times 10^6 | 1.1 \times 10^6 | 2.6 \times 10^5 | 2.0 \times 10^4 |
| Fold reduction  | 11 \times     | 5 \times     | 11 \times     | 380 \times     |

Thymocytes were harvested at 5 wk after reconstitution, stained with antibodies to CD4, CD8, and V\(^{a11}\), and analyzed by three-color flow cytometry. The data are obtained from a minimum of four pooled thymi and are representative of three experiments. Values are given in absolute number of V\(^{a11}\) thymocytes recovered from chimeras (irradiated B10.BR or B10.A recipients reconstituted with H\^-2\^h AND TCR bone marrow) after daily in vivo treatment with CsA.

TCR \(\alpha\beta\)DN cells show no MHC dependence for development, in clear contrast to mainstream \(\alpha\beta\) lineage T cells (CD4\^-CD8\^+ or CD4\^+CD8\^-) of the same mice that show an absolute requirement for specific MHC. These findings argue against the view that TCR \(\alpha\beta\)DN cells derive from conventional CD4 or CD8 T cells by the downregulation of a coreceptor.

TCR \(\alpha\beta\)DN cells of some strains coexpress endogenous TCR \(\gamma\delta\) and transgenic TCR \(\alpha\beta\). The analyses above indicate that TCR \(\alpha\beta\)DN cells have \(\gamma\delta\) lineage properties. Therefore, CD4\^-CD8\^- T cells of several strains of TCR \(\alpha\beta\) transgenic mice were analyzed for expression of TCR \(\gamma\delta\). An obvious population of CD4\^-CD8\^- thymocytes and peripheral T cells bearing only the transgenic 

**Figure 3.** TCR \(\alpha\beta\)DN cells appear early in thymic development. Thymocytes were harvested day 15 after reconstituting B10.BR or B10.A RAG-2\^-/- irradiated recipients with T-depleted H\^-2\^h AND TCR bone marrow. (a) Cells were stained for CD4, CD8, and V\(^{a11}\)TCR and analyzed by three-color flow cytometry. (b) Thymocytes were electronically sorted for V\(^{a11}\)CD4\^-CD8\^- and V\(^{a11}\)CD4\^-CD8\^+. Sorted cells (12 \times 10^6/well) were tested in a proliferation assay for response to plate-bound anti-V\(^{a11}\) (R R 8-1) antibody. Proliferation data are representative of two sorting experiments, and the cytometric analysis on day 15 is representative from several series of analyses performed on thymocytes from chimeric mice on days 10–20 after reconstitution.

**Figure 4.** TCR stimulation can induce CD8\^{a and b} expression on TCR \(\alpha\beta\)DN T cells (a) V\(^{a11}\)CD4\^-CD8\^-, (b) V\(^{a11}\)CD4\^- lymph node T cells from AND TCR mice (isolated by electronic cell sorting and cultured at 4 \times 10^8/well on 30 \mu g/ml plate-bound anti-V\(^{a11}\), R R 8-1, in the presence of recombinant IL-1 and IL-2, 100 U/ml, each), and (c) CD4\^-CD8\^- thymocytes of day 1 neonatal mice (isolated by magnetic bead depletion and cultured at 10 \times 10^9/well on 24 \mu g/ml immobilized anti-\(\gamma\delta\), GL-3, in the presence of rIL-1 and rIL-2, 100 U/ml, each). Day 1 CD4\^-CD8\^- thymocytes were isolated by magnetic bead depletion and cultured at 10 \times 10^9/well on 24 \mu g/ml immobilized anti-\(\gamma\delta\), GL-3, in the presence of rIL-1 and rIL-2, 100 U/ml, each) were assayed for expression of CD8\^{a and b} by flow cytometry. The data are representative of three or more experiments. B6 LN T cells were used as a positive control for CD8\^{a and b} staining (data not shown).
TCR αβ was apparent in all of the mice analyzed. In some strains, however, there existed a second subset of CD4+CD8+ T cells coexpressing the transgenic TCR αβ and endogenous TCR γδ (Fig. 6 and Table II). This latter subset bearing both TCRs was most prominent in the P14 TCR mice. It is noteworthy that like the TCR αβDN subset of AND TCR mice, both TCR αβDN-bearing subsets of P14 TCR mice exhibited properties of γδ lineage T cells (Fig. 1 and data not shown). It was previously reported that TCR γδ+ cells develop in P14 TCR mice (48); however, it was not appreciated that these T cells coexpress the transgenic TCR αβ.

These different patterns of TCR expression prompted us to investigate the timing of transgenic TCR αβ expression during fetal thymic ontogeny, using the AND and P14 TCR mice as prototypes. As shown in Fig. 7, AND TCR is expressed early on a majority of E14 thymocytes. In contrast, the P14 TCR is first detected around E15–16, and then only on a minor subset of fetal thymocytes. These data, considered together with the data from adult thymocytes in Fig. 6, suggest that very early expression of the transgenic TCR αβ inhibits endogenous TCR γδ gene rearrangement and/or expression.

Endogenous TCR γδ gene rearrangements are suppressed in TCR αβDN cells of AND, but not in TCR αβDN cells of P14 TCR mice. To determine the basis for differences in TCR expression in TCR αβDN cells of AND and P14 TCR mice, TCR γδ gene rearrangements were examined using a quantitative PCR assay. Since TCR Vγ2 is commonly used by lymphoid γδ T cells (49), the frequency of TCR Vγ2→γδ1 rearrangement was determined in mature T cell subsets (Fig. 8). The analyses indicate that this gene rearrangement is much more suppressed in TCR αβDN cells.
CD8- (TCRαβDN) cells of AND TCR than of P14 TCR mice. Similar differences between AND TCR and P14 TCR mice were observed with other Vγ and Vδ gene segments, although the rearrangement frequencies were much lower (data not shown). Of note, the occurrence of Vγ2→Vγ1 rearrangement in TCR+CD4+CD8+ T cells of P14 TCR mice is equivalent to those of TCRγδ+ cells of G8 TCR mice (Vγ2+ mouse strain). Thus, in the P14 TCR mice that express the transgenic receptor relatively late, TCRγδ rearrangement is uninhibited and TCRαβDN cells bearing TCRγδ are observed (Figs. 6-8).

Interestingly, an analysis of the frequency of Vγ2→Vγ1 rearrangements in CD4+ T cells of AND TCR mice is increased in the semiselecting (class I+/–, CD4+/–) or nonselecting (H-2b) MHC background in comparison over the frequency in the selecting MHC (H-2d) background (Fig. 8). These results fit with the notion that MHC engagement terminates RAG expression during αβ development (50).

In contrast, the TCRαβDN cells developing in the CD4+CD8– (γδ) pathway follow different rules since rearrangement frequency is independent of MHC (Fig. 8a). These findings suggest that TCR gene rearrangement is differentially regulated in the γδ and αβ lineages.

TCR γδ Is Expressed by Skin Lymphocytes of AND TCR Mice. TCR γδ gene rearrangements in thymocyte precursors that localize to skin epithelium occur much earlier in fetal development than those destined for migration and residence in the lymphoid tissues (2). Therefore, there was the possibility that the dendritic epidermal T lymphocytes of AND TCR mice would express TCRγδ since some of their thymic precursors may have rearranged TCRγδ before transgenic TCRαβ expression. In contrast to the lymphoid CD4+CD8+ T cells that fail to express TCRγδ (Fig. 6), skin lymphocytes express two subsets of T cells (Fig. 9), one expressing TCRαβ alone and the second expressing both TCRαβ and TCRγδ. Thus, when TCRαβ transgene expression occurs after endogenous TCRγδ rearrangements, rearrangement is not suppressed, and TCRγδ and TCRαβ can be expressed by the same cells.

In the periphery of normal mice, the canonical Vγ3+ TCR is expressed exclusively on skin lymphocytes (2). The finding that these cells, bearing the AND TCR and coexpressing the expected TCRγδ, can home at the right time to what is normally a γδ-specific site, provides additional evidence that TCRαβDN cells are γδ lineage T cells. Presumably, the skin lymphocytes expressing only the transgenic TCRαβ have an out of frame TCRγδ or, alternatively, some cells express the transgenic TCR early enough to suppress endogenous TCRγδ rearrangements. In any case, the finding that even the TCRαβ+TCRγδ- subpopulation is able to traffic to this traditionally γδ-specific site demonstrates that skin homing is not dependent on the canonical TCR.

These data, like those above, reveal that when the TCR is expressed early (regardless of whether it is TCRγδ or TCRαβ), the receptor allows γδ lineage commitment and maturation in the γδ lineage.

Discussion

These studies examine T cell development in transgenic mice with premature expression of TCRαβ. An interesting feature of the mice is a population of mature CD4+CD8+ thymocytes and peripheral T cells expressing only the transgenic TCRαβDN. To determine whether TCRαβDN cells belong to the αβ or γδ lineage, we analyzed these cells in several TCR transgenic strains and compared them to the T cell subsets of normal mice. By all criteria examined, the TCRαβDN cells clearly exhibit characteristics of γδ lineage T cells. The lack of a coreceptor, the level of CD5, and the early maturation delineate TCRαβDN cells from the major TCRαβ+CD4 and CD8 T cell subsets. TCRαβDN cells do not express NK1.1 or IL-2Rβ (CD122) or produce IL-4, distinguish-
ing them from the NK T cells of wild-type mice. In contrast, TCR αβDN cells are similar to γδ T cells since their development is early, is relatively insensitive to CsA, and is MHC independent. Also, like γδ lineage cells, TCR αβDN cells can be induced to express CD8αα homodimers in response to anti-TCR stimulation. Most notable, in TCR αβ strains where the transgenic receptor is expressed later in development, CD4–CD8– T cells arise coexpressing the transgenic TCR αβ and endogenous TCR γδ (Table II, and Figs. 6 and 7). TCR αβDN cells with both receptors exhibit the same phenotype and properties as those lacking TCR γδ expression. These findings provide the most direct evidence that TCR αβDN cells are γδ lineage T cells.

The different patterns of TCR expression in CD4–CD8– T cells of TCR αβ mice appear to be related to the timing of TCR αβ transgene expression with respect to endogenous TCR γδ gene rearrangement. As modeled in Fig. 10, the early expression of transgenic TCR αβ in precursor thymocytes of AND TCR mice causes suppression of endogenous TCR γδ gene rearrangement; nevertheless, the transgenic receptor allows continued maturation in the CD4–CD8– (γδ) pathway. In P14 TCR mice, the transgenic receptor is expressed later such that TCR γδ gene rearrangements occur normally. If rearrangements are productive, mature CD4–CD8– T cells emerge coexpressing the TCR αβ (P14 TCR) and endogenous TCR γδ (Figs. 6–8, and Table II). The different TCR expression patterns in skin versus lymph node CD4–CD8– T cells of AND TCR mice also can be explained by this model. A subset of epidermal lymphocytes coexpresses the transgenic TCR αβ and endogenous TCR γδ (Fig. 9), but lymph node T cells bear only the transgenic TCR αβ (Fig. 6). Thus, the rearrangements of genes encoding the lymphoid type TCR γδ are suppressed by AND TCR expression, whereas rearrangements that occur early in the fetal thymus, encoding the TCR γδ of skin lymphocytes, are not suppressed. Of significance, either TCR expression pattern allows development in the CD4–CD8– (γδ) pathway.

We have considered these and previous results for understanding the role of the TCR in αβ versus γδ lineage determination. Evidence exists for an instructional model in which successful rearrangement of TCR γδ or TCR β genes biases the decision of a precursor to become a γδ or αβ lineage T cell. Of note, αβ lineage T cells are depleted of productive TCR γ and -δ rearrangements, suggesting that the production of a functional TCR γδ favors a γδ lineage decision (51–53). In addition, mice deficient for the α component of the pre-TCR show an increase in the number of γδ lineage T cells, implying that normally pre-TCR signals inhibit γδ lineage development (54). Other studies, however, have prompted speculation that γδ/αβ
mice (59), and these cells are enriched for in-frame TCR rearrangements. Samples of 10^5 each of TCR mice. Rearrangements are suppressed, independent of MHC haplotype, in AND TCR/H-2d, P14 TCR, G8 TCR, and B6 mice were isolated by CD4 TCR gene rearrangement (55–58). Of relevance, a few lineage determination may occur before or independent of TCR gene rearrangement (55–58). Of relevance, a few CD4^+CD8^+ thymocytes arise in TCRβ^-/- null mutant mice (59), and these cells are enriched for in-frame TCRγδ rearrangements (60, 61), indicating that TCRγδ, in some circumstances, can promote αβ development. Moreover, CD4^+CD8^+ cells develop, although inefficiently, in TCRγδ transgenic mice when endogenous TCRβ recombination is diminished or suppressed (56, 62). Even in normal mice, a minor population of TCRγδ-bearing CD4^+CD8^+ cells has been observed (63). Complicating the issue further are reports that the majority of TCRβ rearrangements are productive in TCRγδ^+ T cells (51, 64). Others disagree, finding that these rearrangements are predominantly out of frame (65). Clearly, the data on this question are mixed and the issue is unresolved.

Since a transgenic TCRαβ permits both γδ and αβ development, our results and those of others (17, 38, 39) could fit a model in which γδ/αβ fate is predetermined, before or independent of TCR rearrangement/expression (4, 66). In this scenario, the TCR plays no role in lineage commitment but is needed only for survival and/or lineage progression. While this model would not always couple the appropriate TCR with lineage commitment, it is noteworthy that additional mechanisms operate to correct TCR expression in the wrong lineage. In the αβ lineage, TCRγ is downregulated at the CD4^+CD8^- stage (67) and TCRα rearrangement results in the deletion of the TCRδ locus. In the γδ pathway, pTα is turned off (68) and TCRα rearrangement is not upregulated (69).

At first glance, the finding that premature expression of TCRαβ can permit both a γδ and αβ cell fate appears to be inconsistent with an instructional mechanism for lineage commitment. However, one version of an instructional model proposes that TCRγδ and pre-TCR signals influence lineage commitment, but does not necessarily imply that signaling differences are absolute or inherent in the TCR structure. Thus, quantitative differences in TCRγδ and pre-TCR signaling could bias lineage choice. Perhaps signals generated by the prematurely expressed transgenic TCRαβ quantitatively mimic TCRγδ signals. An additional possibility is that the timing of TCR expression influences the lineage decision. Recent evidence indicates that TCRγδ rearrangements occur slightly ahead of TCRβ in adult thymopoiesis (41, 42). Conceivably, these ordered rearrangements could be coordinated with developmentally regulated changes in TCR signal transduction such that the earliest TCR signals promote a γδ fate, whereas later TCR signals promote αβ development.

Figure 8. Vγ2-Jγ1 gene rearrangements are suppressed in TCRγδ^+CD4^-CD8^- (TCRαβDN) cells of AND TCR, but not of P14 TCR mice. Rearrangements are suppressed, independent of MHC haplotype, in the TCRαβDN but not the TCRγδ^+CD4^-CD8^- subset of AND TCR mice. Samples of 10^5 each of TCRγδ^+ (Vα11+ for AND, Vα2+ for P14, TCRγδ^- for G8 TCR, and TCRγδ^+ or TCRαβ^+ for B6) (a) CD4^+CD8-, (b) CD4^+CD8+, and (c) CD4^-CD8^- lymph node T cells from AND TCR/H-2d, P14 TCR/H-2d (MHC class II^+/-, CD4^+/-), AND TCR/H-2d, P14 TCR, G8 TCR, and B6 mice were isolated by electronic sorting. The relative frequency of Vγ2-Jγ1 rearrangements per sample was determined using a PCR ELISA as described in Materials and Methods. Bars represent the mean values (with SEs) of three individual sorts, using a total of five to eight mice per sort.

Figure 9. In contrast to lymphoid T cells, skin dendritic epithelial lymphocytes of AND TCR mice contain two subsets of T cells, one bearing only the transgenic TCRαβ and a second coexpressing the transgenic TCRαβ with endogenous TCRγδ. Isolated epidermal lymphocytes of (a) H-2^b AND TCR transgenic (tg) or (b) nontransgenic (non tg) B10.D2 mice were stained for TCRαβ (H57-597) and TCRγδ (GL3) and analyzed by flow cytometry. The numbers inside the quadrant represent the percentage of cells in each population. The data are representative of several analyses of AND TCR mice of H-2^b or other H-2 haplotypes.
signals favor an $\alpha\beta$ fate. Our data could fit with such a sequential model since distinct TCR signals regulating lineage choice would be generated as a function of time, irrespective of TCR substitutions. In some sense, this sequential model can be seen as both predetermined and instructional: predetermined, since changes in intracellular TCR signals over time are developmentally programmed, and instructional, since distinct signals mediate lineage commitment. However, such signals are not inherent to the TCR structure. In any case, the previous results demonstrating that $\alpha\beta$ T cells are depleted of in-frame TCR $\gamma\delta$ rearrangements (51–53) and the low frequency of productive TCR $\beta$ rearrangements in $\gamma\delta$ T cells (65) support a sequential model.

TCR $\alpha\beta$ transgenic mice are widely used to study antigen-specific immune responses in vivo. The studies reported here should send a note of caution regarding the use of such mice for this purpose. If, as we conclude, the transgenic TCR $\alpha\beta$ receptor can substitute for the TCR $\gamma\delta$ in $\gamma\delta$ lineage T cells, cells that would normally be immunologically silent can now participate in an antigen-specific response. Because $\gamma\delta$ T cells have unique developmental, functional, and homing properties, they could contribute to the response in nonphysiological ways. Thus, difficulties with these mice could be related to the large number of mature T cells expressing a single TCR, but also because $\gamma\delta$ lineage cells (bearing transgenic TCR $\alpha\beta$) contribute to the antigenic response in unpredictable ways. Even sorting for CD4$^+$ cells may not help, since a few $\gamma\delta$ T cells express CD4 (57). A new generation of TCR transgenic mice, with delayed TCR $\alpha\beta$ expression, may provide a solution to this problem.

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