Premature Expression of T Cell Receptor (TCR)αβ Suppresses TCRγδ Gene Rearrangement but Permits Development of γδ Lineage T Cells

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Abstract

The T cell receptor (TCR)γδ and the pre-TCR promote survival and maturation of early thymocyte precursors. Whether these receptors also influence γδ versus αβ lineage determination is less clear. We show here that TCRγδ gene rearrangements are suppressed in TCRαβ transgenic mice when the TCRαβ is expressed early in T cell development. This situation offers the opportunity to examine the outcome of γδ versus αβ T lineage commitment when only the TCRαβ is expressed. We find that precursor thymocytes expressing TCRαβ not only mature in the αβ pathway as expected, but also as $CD4^-CD8^-$ T cells with properties of γδ lineage cells. In TCRαβ transgenic mice, in which the transgenic receptor is expressed relatively late, TCRγδ rearrangements occur normally such that $TCR\alpha\beta^+CD4^-CD8^-$ cells co-express TCRγδ. The results support the notion that TCRαβ can substitute for TCRγδ to permit a γδ lineage choice and maturation in the γδ 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 αβ or γδ lineage T cells (for reviews, see references 1, 2). The first T cells are γδ 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 γδ T cells that populate the lymphoid organs have more diverse receptors and develop in both the fetal and adult thymus. Lymphoid γδ T cells and precursors to the αβ 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 TCRγδ or of the pre-TCR (a heterodimer of TCRβ with invariant pTα) is critical for survival and further differentiation of these early thymocytes (3). Of major interest is whether these receptors play a role in αβ versus γδ lineage determination or only in the progression of already committed precursors (4, 5).

The pathways of αβ and γδ T cell development are quite distinct. Although discrete stages of γδ development have not been identified, most γδ lineage T cells never express the CD4 or CD8αβ coreceptors and have no requirement for MHC for maturation (6, 7). In contrast, precursor $CD4^-CD8^-$ thymocytes expressing the pre-TCR proliferate, upregulate TCRα rearrangement, and progress to a $CD4^+CD8^+$ intermediate stage (3). If rearrangement of TCRα is productive, TCRα replaces pTα to form the mature TCRαβ. Recognition of MHC by TCRαβ is required for the development of mature αβ lineage T cells, expressing either CD4 or CD8. The development of an additional subset of αβ T cells, the so-called NK T cells, is β_2-microglobulin (β_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 TCRαβ transgenic mice, a novel population of TCRαβ$^+$CD4$^-$CD8$^-$ (TCRαβDN)[1] 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 TCRαβ 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 TCRαβ$^+$CD4$^-$CD8$^-$ cells of wild-type mice (NK T cells) or to the abnormal TCRαβ$^+$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 (14, 15) or that they mature in the αβ lineage without ever expressing the CD4/CD8 coreceptors (16).

Evidence that the TCRαβDN 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 TCRαβDN cells do not express endogenous TCRα genes, their TCRδ gene segments are not deleted (17), and they do not develop in mice deficient for the common cytokine receptor γ chain (18). TCRαβDN cells mature in the absence of the selecting MHC and, most noteworthy, in HY TCR mice with a pTα null mutation (pTα$^{-/-}$), a few TCRαβDN cells coexpress endogenous TCRγδ and the transgenic TCRαβ (17). Given these characteristics, it was proposed that TCRαβDN cells of TCRαβ transgenic mice belong to the γδ lineage. In this model, the transgenic TCRαβ replaces TCRγδ while still allowing γδ lineage development. This model was contested, however, in an additional report using DO11.10 TCR transgenic mice (16). Since TCRαβDN 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 TCRαβDN cells of TCRαβ 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 TCRαβDN cells using a number of criteria (phenotype, function, development, and localization). An analysis of several strains of TCRαβ transgenic mice reveals that TCRαβDN cells clearly exhibit characteristics of γδ lineage T cells. The MHC requirements for maturation and the regulation of TCR gene rearrangement are distinctly different in TCRαβDN cells than in conventional αβ lineage T cells. The results indicate that the premature expression of TCRαβ allows thymocyte precursors to mature in the γδ lineage. These findings have implications for models of γδ/αβ lineage determination.

[1]*Abbreviations used in this paper:* APC, allophycocyanin; β_2m, β_2-microglobulin; B6, C57BL/6; B10, C57BL/10; CsA, cyclosporine A; HSA, heat stable antigen; RAG, recombination activating gene; TCRαβDN, TCRαβ$^+$CD4$^-$CD8$^-$.

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. TCRαβ transgenic mice were backcrossed, intercrossed, and selected as described previously (19) to obtain H-2^b, H-2^k, H-2^d, H-2^q, H-2^b recombination activating gene (RAG)-2$^{-/-}$, H-2^q RAG-2$^{-/-}$, or H-2^b MHC class II$^{+/-}$CD4$^{+/-}$ AND TCR mice (20–23); H-2^d and H-2^b class II$^{-/-}$ DO11.10 TCR mice (24); and H-2^d HA TCR mice (25). H-2^b and H-2^d HY TCR mice (26) were obtained by backcrossing 12 times to B10 and then to B10.D2; H-2^b and H-2^k 5CC7 TCR mice, by crossing B6 5CC7 TCR mice (27) to B10 or B10.A; and H-2^b and H-2^b class I$^{-/-}$ P14 TCR mice (28), by backcrossing 10 times to B6 and then to β_2m$^{-/-}$ (29). Except where noted, all TCRαβ transgenic mice were on the positive-selecting MHC background: AND TCR (H-2^b or H-2^k), 5CC7 TCR (H-2^k), DO11.10 TCR (H-2^d), HY TCR (H-2^b), and P14 TCR (H-2^b). TCRγδ transgenic mice included the G8 TCR mice (H-2^b β_2m$^{-/-}$) crossed and selected as described (7), or H-2^b TG78 TCR mice (30), 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 of 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: DN7.3 (TCRVγ2/Vδ5), a mouse CD4$^-$CD8$^-$ T cell/BW5147 hybridoma, and DCEK, a mouse L cell fibroblast line transfected with EαE^{bk}. Thymocytes, LNs, and LN T cells were prepared in single cell suspensions as described previously (31). For enrichment of heat stable antigen (HSA)^{lo} (CD24^{lo}) 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 (10^6 U/ml; Calbiochem). For magnetic bead isolation of CD4$^-$CD8$^-$ thymocytes or LN T cells, 10^7 cells were reacted with 250 μl of purified H129.19 and 53-6.7 (and RA3-6B2, 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/ml 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–TCRβ–FITC, –PE or –allophycocyanin (APC) (H57-597), anti–γδ TCR-FITC, -PE, or unlabeled (GL3), anti-CD4–FITC, –PE, –APC, or –CyChrome (RM4-5), anti-CD8α–CyChrome or unlabeled (53-6.7), anti-CD8β.2–FITC (53-5.8), anti–IL-2Rβ–FITC (TM-β1), anti-NK1.1–PE (PK136), anti-CD5–FITC (53-7.3), anti-Vα11 TCR–FITC or unlabeled (RR8-1), anti-Vα2–FITC or –PE (B20.1), anti-A^b–FITC or –PE (AF6-120.1), anti-E^k–FITC (14-4-45), anti–H-$2K^d$–FITC (SF1-1.1), anti–H-$2K^k$–FITC (36-7-5), anti–H-$2K^b$–FITC (AF6-88.5), anti–H-$2K^q$–FITC (KH-114), and anti-CD45R/B220–FITC, –PE, or unlabeled (RA3-6B2), all obtained from BD PharMingen; anti-CD8α–FITC, –PE, or –biotin (CT-CD8a), anti-CD4–biotin (YTS 191.1), Thy 1.2–FITC or –PE (5a-8), streptavidin-APC or -TriColor, goat anti–mouse IgG1–PE, goat anti–mouse IgG2a–FITC, all obtained from Caltag; goat anti–rat IgG–FITC (Kirkegaard & Perry); rat anti–mouse IgG1–FITC and streptavidin-FITC (Zymed Laboratories); and anti-CD24 (J11d), anti-HY TCR (T3.70), anti-HA TCR (6.5), and anti-DO11.10 TCR (KJ-126) culture supernatants.

Cells were stained and analyzed by flow cytometry and/or electronically sorted using standard protocols (33). For some analyses, cells were pretreated with an unlabeled anti-FcRγ culture supernatant (24G2) to block Fc receptor binding of the labeled antibodies. Multicolor flow cytometry was performed on a FACS® 440, FACSCalibur™, FACStar$^{Plus™}$, or FACS Vantage™ (Becton Dickinson). Dead cells were excluded by light scatter and propidium iodide gating. 150,000 events 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 FACStar$^{Plus™}$ (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 lysing 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 CD8α and CD8β was assessed on day 4 of culture by flow cytometry. IL-4 production was assayed by specific 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™ CsA (Sandoz) in 100 μl olive oil (Bertolli 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. 10^5 sorted cells were digested using 1× PCR Buffer (PerkinElmer), 2.5 mM $MgCl_2$, 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 $MgCl_2$, 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, TGTCCTTGCAACCCCTACCC; Jγ1, TGTTCCTTCTGCAAATACCTTG; Vγ2 probe, GAGGAAGAAGACGAAGCTATC; 5′ Cγ1, TTACAGACAAAAGGCTTGAGTC; 3′ Cγ1, GTTCTCATGTTTGACAATACATCTG; and Cγ1 probe, CTGAAGACTAACGACACATAC.

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 titrating 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 10^5 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 were used to calculate the cycle value at half-maximum (C_{50}) of amplification (35). C_{50} values were plotted against the corresponding $\log_{10}$ cell number of DN7.3 cells in each input sample and a best-fit line was generated. C_{50} 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 $TCR\gamma\delta^+$ and a larger than usual population of $TCR\alpha\beta^+$ 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 TCRα 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 $CD4^-CD8^-TCR\gamma\delta^+$ cells of TCRγδ transgenic mice (TG78) (30). As shown previously (8), freshly isolated NK T cells of B6 mice (TCRαβDN) express IL-2R

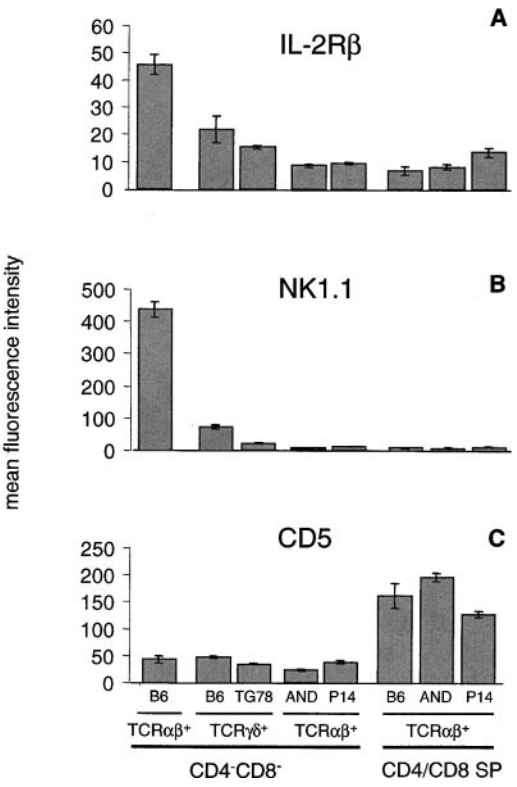

Figure 1. Phenotypic markers distinguish the TCRαβDN T cell subset of TCRαβ transgenic mice from αβ lineage T cells (CD4 and CD8), NK T, but not from γδ lineage T cells. Thymocytes (A and B) or B cell–depleted lymph node T cells (C) from B6, transgenic TCRγδ (TG78), or transgenic TCRαβ (AND and P14) mice were each stained for TCRαβ, TCRγδ, CD4 and/or CD8, and a fifth marker (IL-2Rβ, NK1.1, 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 $CD4^+CD8^-TCR\alpha\beta^{hi}$ and $CD4^-CD8^+TCR\alpha\beta^{hi}$, or for $CD4^-CD8^-$ T cells, by live gating for $CD4^-CD8^-$ followed by software gating for $TCR\alpha\beta^+$ or $TCR\gamma\delta^+$. Each bar represents the means (with SE bars) collected from analysis of three individual mice with the exception of B6 (two mice).

(CD122) and NK 1.1, and produce high amounts of IL-4 in response to in vitro TCR stimulation (Fig. 1, A and B, and Fig. 2). In contrast, the TCRαβDN population of AND TCR mice expresses lower levels of these markers and produces no IL-4 (39; Fig. 1, A and B, and Fig. 2). The TCRαβDN cells also express relatively lower levels of CD5, delineating this subset from mature CD4 and CD8 T cells, but not from $TCR\gamma\delta^+$ T cells (Fig. 1 C). Together these phenotypic and functional properties distinguish TCRαβDN cells from NK T and the major αβ lineage, but not from γδ lineage T cells.

γδ T cells appear early in adult T cell development, before the αβ lineage T cells (40–42). To assess when the TCRαβDN cells arise in thymic development, we generated hematopoietic stem cell chimeras using bone marrow from AND TCR mice to reconstitute irradiated recipients. Between days 10 and 15 after reconstitution, we observed a population of $V\alpha11^+CD4^-CD8^-$ followed by $V\alpha11^+CD4^+CD8^+$ thymocytes. By days 18–20, mature $CD4^+CD8^-$ thymocytes develop (data not shown). On day 15, transgenic TCR^+ (Vα11) $CD4^-CD8^-$, and $CD4^+CD8^+$ thymocytes were sorted and stimulated in vitro using anti-Vα11 antibody (Fig. 3, a and b). The $V\alpha11^+CD4^-CD8^-$ thymocytes are competent to incorporate [^{3}H]thymidine in response to anti-Vα11 cross-linking while Vα11-bearing $CD4^+CD8^+$ thymocytes are not. Therefore, like γδ T cells, TCRαβDN T cells appear well before the $CD4^+CD8^-$ thymocytes and much earlier than NK T cells that arise after the $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes in wild-type mice (8).

Previous studies (43–45) have indicated that the development of the major αβ T cell subsets (CD4/CD8), as well as the minor $TCR\alpha\beta^+CD4^-CD8^-$ (NK T) subset of wild-type mice, are inhibited by CsA. γδ lineage T cells are relatively less sensitive. To further assess lineage properties, TCRαβDN cells of AND TCR mice were tested for sensitivity to CsA, administered over the course of adult T cell development. Irradiated recipients reconstituted with AND TCR bone marrow were treated daily with CsA for 5 wk, after reconstitution. As shown in Table I, the development of $V\alpha11^+CD4^-CD8^-$ TCR^+ is up to 75-fold less sensitive to CsA than are $V\alpha11^+CD4^+CD8^-$ thymocytes. These data indicate that $TCR\alpha\beta^+$DN cells are relatively resistant to CsA administered during development, as are γδ lineage T cells.

Since $CD4^-CD8^-$ $TCR\gamma\delta^+$ thymocytes of wild-type mice can be induced to express CD8αα after in vitro activation (46; Fig. 4 c), we tested the ability of mature

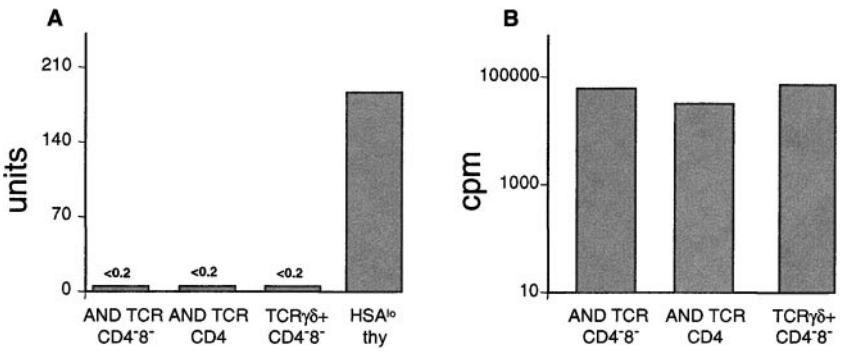

Figure 2. TCRαβDN T cells respond to anti-TCR stimulation by proliferating but do not produce an IL-4 response. Lymph node $CD4^-CD8^-$ or $CD4^+$ T cells from AND TCR (Vα11/Vβ3), $CD4^-8^-TCR\gamma\delta^+$ T cells from G8 TCR mice (purified using magnetic beads and electronic cell sorting), or HSA^{lo} B6 thymocytes (an enriched source of NK T cells), plated at 5×10^4 cells/well, were stimulated with 10 μg/ml immobilized anti-TCR antibody (anti-Vα11 for AND TCR, anti-TCRγδ for G8 TCR, and anti-TCRαβ 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.

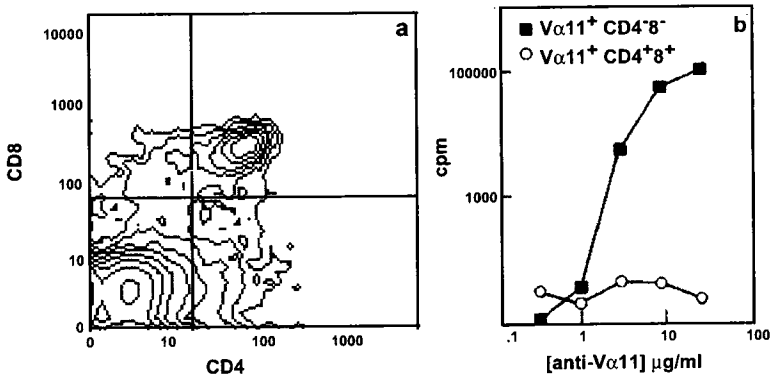

Figure 3. TCRαβDN cells appear early in thymic development. Thymocytes were harvested day 15 after reconstituting B10.BR or B10.A RAG-$2^{-/-}$ irradiated recipients with T-depleted H-2^k AND TCR bone marrow. (a) Cells were stained for CD4, CD8, and Vα11 TCR and analyzed by three-color flow cytometry. (b) Thymocytes were electronically sorted for $V\alpha11^+CD4^+CD8^+$ and $V\alpha11^+CD4^-CD8^-$. Sorted cells (12×10^4/well) were tested in a proliferation assay for response to plate-bound anti-Vα11 (RR8-1) 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.

TCRαβDN cells to make this response. As shown in Fig. 4, a and b, $V\alpha11^+CD4^-CD8^-$, but not $V\alpha11^+CD4^+$ T cells, are induced to express CD8αα in response to anti-TCR stimulation. Similar responses have been obtained from TCRαβDN splenocytes of TCRα transgenic mice (39). Thus, by all of the criteria we examined, TCRαβDN cells are clearly distinguished from conventional αβ lineage and NK T cells, and most resemble γδ lineage T cells.

TCRαβ DN Cells of TCRαβ Transgenic Mice Do Not Require MHC for Development. One of the hallmarks of αβ T cell development is the requirement for MHC-specific positive selection (47). In contrast, γδ T cells fully mature in the absence of MHC (6, 7). Since there are conflicting reports on the selection requirements of TCRαβDN cells (10, 12, 16), we tested several strains of TCRαβ transgenic mice, bearing MHC class I– or class II–specific TCRs. As shown in Fig. 5, the TCRαβDN cells of five different strains of TCRαβ 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). Thus, TCRαβDN cells show no MHC dependence for development, in clear contrast to mainstream αβ 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 TCRαβDN cells derive from conventional CD4 or CD8 T cells by the downregulation of a coreceptor.

TCRαβDN Cells of Some Strains Coexpress Endogenous TCRγδ and Transgenic TCRαβ. The analyses above indicate that TCRαβDN cells have γδ lineage properties. Therefore, $CD4^-CD8^-$ T cells of several strains of TCRαβ transgenic mice were analyzed for expression of TCRγδ. An obvious population of $CD4^-CD8^-$ thymocytes and peripheral T cells bearing only the transgenic

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

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

Thymocytes were harvested at 5 wk after reconstitution, stained with antibodies to CD4, CD8, and Vα11, 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 $V\alpha11^+$ thymocytes recovered from chimeras (irradiated B10.BR or B10.A recipients reconstituted with H-2^k AND TCR bone marrow) after daily in vivo treatment with CsA.

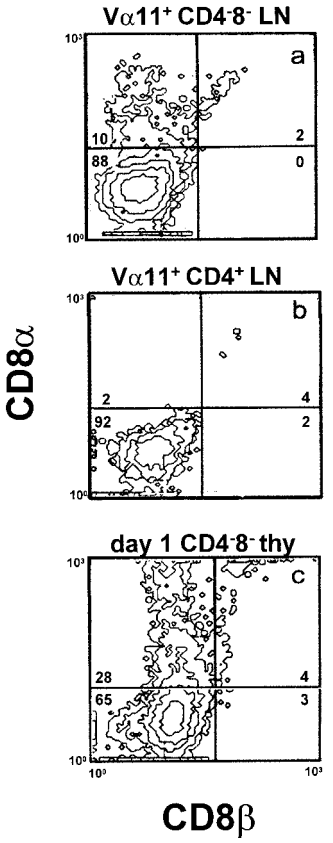

Figure 4. TCR stimulation can induce CD8αα expression on TCRαβ DN T cells. (a) $V\alpha11^+$ $CD4^-CD8^-$, (b) $V\alpha11^+CD4^+$ lymph node T cells from AND TCR mice (isolated by electronic cell sorting and cultured at 4×10^4/well on 30 μg/ml plate-bound anti-Vα11, RR8-1, in the presence of recombinant 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 10×10^4/well on 24 μg/ml immobilized anti-γδ, GL-3, in the presence of rIL-1 and rIL-2, 20 U/ml each) were assayed for expression of CD8α and CD8β by flow cytometry. The data are representative of three or more experiments. B6 LN T cells were used as a positive control for CD8β staining (data not shown).

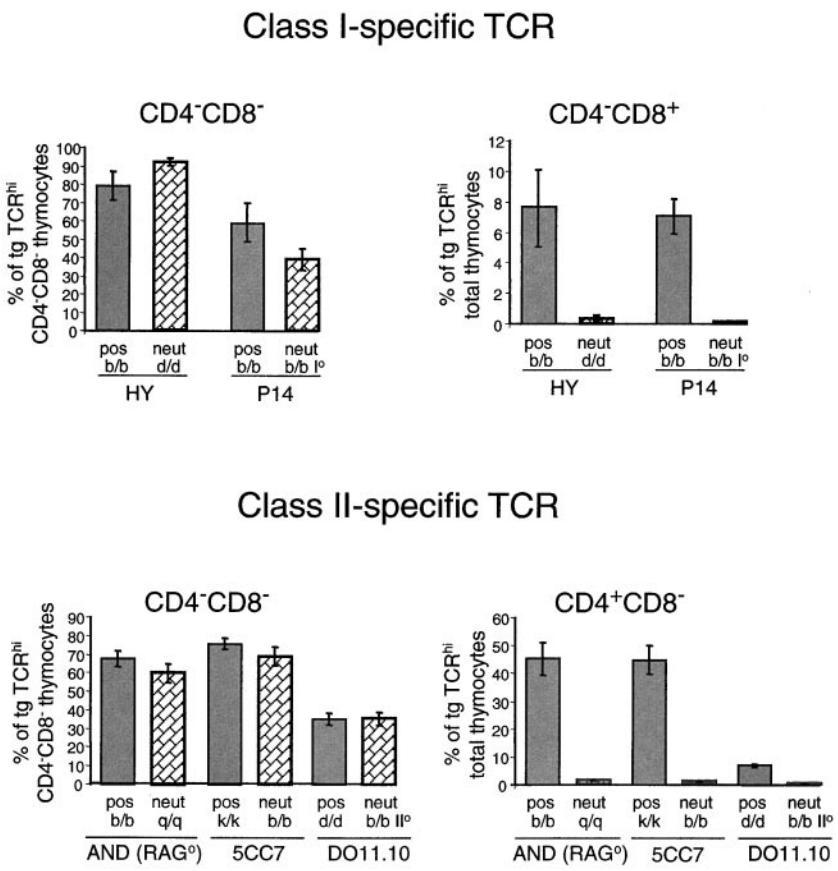

Figure 5. TCRαβDN cells do not require MHC-dependent positive selection for development. Thymocytes were isolated from MHC class I–specific (HY and P14) or class II–specific (AND, 5CC7, and DO11.10) TCR transgenic mice bred onto a positively selecting (pos) or nonselecting, neutral (neut) MHC background. Cells were stained for CD4, CD8, and TCR (using antibodies: T370 for HY, anti-Vα2 for P14, anti-Vα11 for AND and 5CC7, and KJ-126 for DO11.10 TCR). The percent of transgenic (tg) TCR^{hi} cells was determined from analysis of total thymocytes by software gating for $CD4^-CD8^-$, $CD4^-8^+$, or $CD4^+CD8^-$. Each bar represents the mean percentage (with SE bars) of TCR^{hi} of $CD4^-CD8^-$ or of total thymocytes from analyses of three to six individual mice per group.

TCRαβ 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 TCRαβ and endogenous TCRγδ (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 TCRαβDN subset of AND TCR mice, both TCRαβDN–bearing subsets of P14 TCR mice exhibited properties of γδ lineage T cells (Fig. 1 and data not shown). It was previously reported that $TCR\gamma\delta^+$ cells develop in P14 TCR mice (48); however, it was not appreciated that these T cells coexpress the transgenic TCRαβ.

These different patterns of TCR expression prompted us to investigate the timing of transgenic TCRαβ 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 TCRαβ inhibits endogenous TCRγδ gene rearrangement and/or expression.

Endogenous TCRγδ Gene Rearrangements Are Suppressed in TCRαβDN Cells of AND, but Not in TCRαβDN Cells of P14 TCR Mice. To determine the basis for differences in TCR expression in TCRαβDN cells of AND and P14 TCR mice, TCRγδ gene rearrangements were examined using a quantitative PCR assay. Since TCRVγ2 is commonly used by lymphoid γδ T cells (49), the frequency of TCRVγ2→Jγ1 rearrangement was determined in mature T cell subsets (Fig. 8). The analyses indicate that this gene rearrangement is much more suppressed in TCR^+CD4^-

Table II. *Coexpression of Transgenic TCRαβ and Endogenous TCRγδ in $CD4^-CD8^-$ Thymocytes*

	Percentage of $CD4^-CD8^-$ thymocytes		
Mice	$TCR\alpha\beta^+TCR\gamma\delta^-$	$TCR\alpha\beta^+TCR\gamma\delta^+$	*n*
B6	12.5 (2.0)	0.06 (0.02)	8
AND	94.2 (9.2)	0.05 (0.01)	7
HY	94.5 (3.0)	0.06 (0.02)	7
HA	74.8 (9.6)	0.08 (0.04)	5
DO11.10	81.8 (4.9)	0.15 (0.04)	7
5CC7	80.7 (6.6)	0.56 (0.26)	6
P14	71.3 (5.4)	2.85 (0.54)	7

Thymocytes obtained from wild-type B6 or TCRαβ transgenic mice were stained and analyzed by flow cytometry as shown in Fig. 6. Average values are given with SE in parentheses; *n* is the number of mice analyzed of each type.

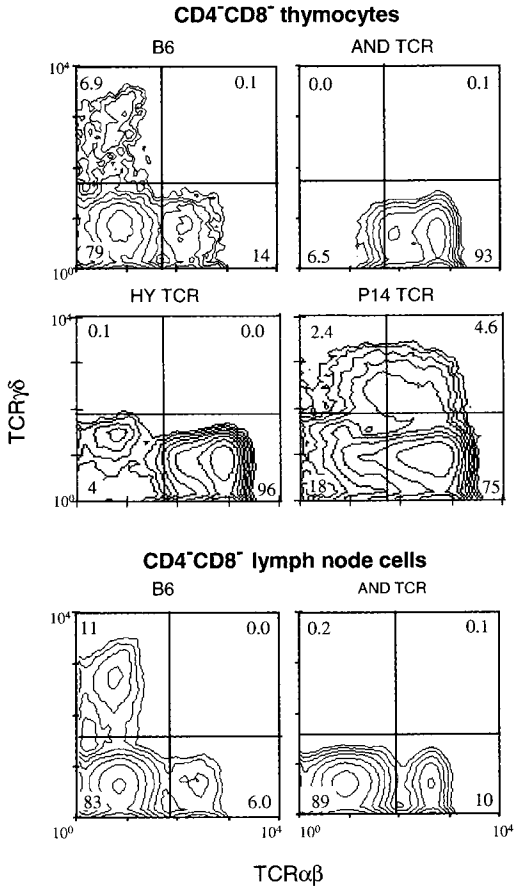

Figure 6. CD4⁻CD8⁻ T cells of AND and HY TCR mice express transgenic TCRαβ but no TCRγδ, while those of P14 TCR mice coexpress transgenic TCRαβ and endogenous TCRγδ. Thymocytes and lymph node cells, stained for TCRαβ (H57-597), TCRγδ (GL3), CD4, and CD8, were analyzed by flow cytometry using live gating to collect data only from CD4⁻CD8⁻ cells. The numbers inside the quadrants represent the percentage of CD4⁻CD8⁻ thymocytes in each population. Statistics are given in Table II.

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→Jγ1 rearrangement in TCR⁺CD4⁻CD8⁻ T cells of P14 TCR mice is equivalent to those of TCRγδ⁺ cells of G8 TCRγδ (Vγ2⁺) transgenic mice and of B6 wild-type mice (Fig. 8 a). 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→Jγ1 rearrangements in CD4 T cells of AND TCR mice is increased in the semiselecting (class $II^{+/-}$, $CD4^{+/-}$) or nonselecting ($H\text{-}2^{q}$) MHC background in comparison over the frequency in the selecting MHC ($H\text{-}2^{b}$) background (Fig. 8 b). 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. 8 a). These findings suggest that TCR gene rearrangement is differentially regulated in the γδ and αβ lineages.

TCRγδ Is Expressed by Skin Lymphocytes of AND TCR Mice. TCRγδ 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. We determined in parallel analyses that these cells are CD4⁻CD8⁻, $V\alpha11^{+}$, $V\beta3^{+}$, and $V\alpha3^{+}$.

In the periphery of normal mice, the canonical $V\gamma3^{+}$ TCR is expressed exclusively on skin lymphocytes (2). The finding that T 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 TCRαβ (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-

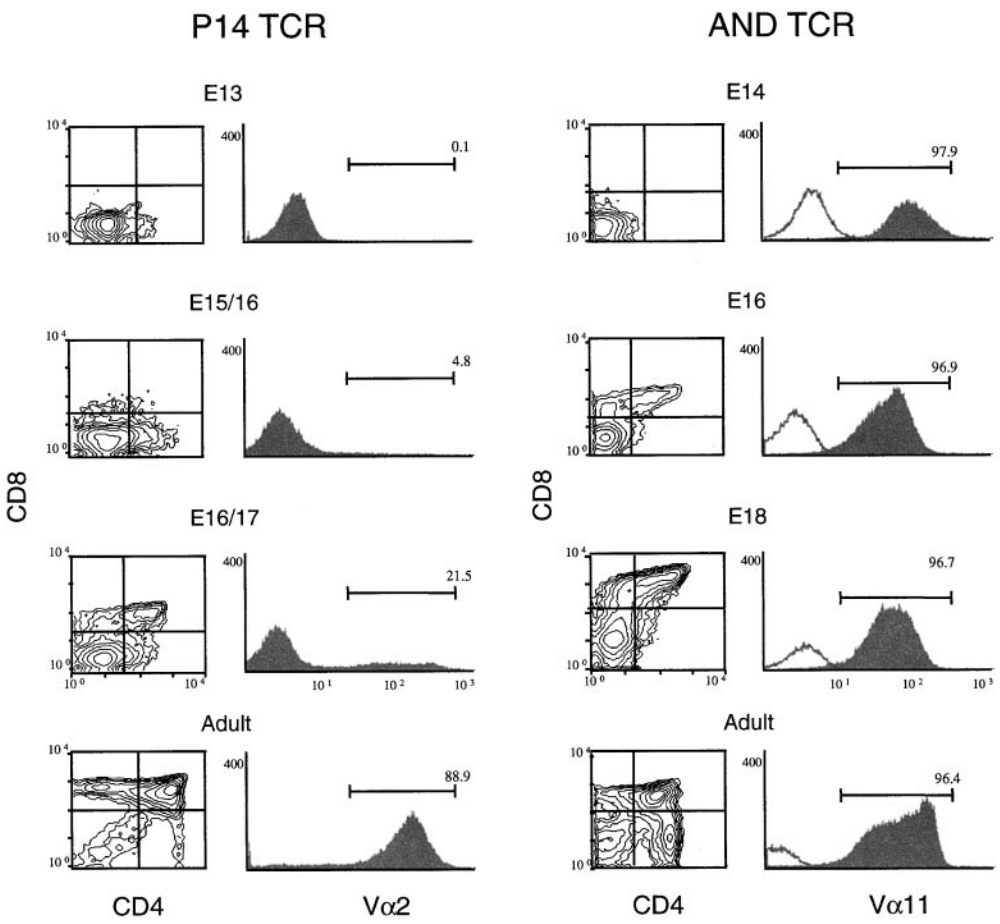

Figure 7. The transgenic AND TCR is expressed much earlier than the P14 TCR in fetal development. Thymocytes from P14 TCR or AND TCR embryos, harvested on the days indicated (E13–18), were stained for Thy 1.2, CD4, CD8, and TCR (Vα2 for P14 and Vα11 for AND) and analyzed by four color flow cytometry. Distributions, gated for total Thy1.2$^+$ thymocytes, display dual parameter, CD4 and CD8, or single parameter, TCR (shaded), overlaid with the negative control for background fluorescence (unshaded). Numbers indicate the percentage of cells within the indicated gates. Data are representative of two such experiments with similar time courses.

ing them from the NK T cells of wild-type mice. In contrast, TCRαβDN 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, TCRαβDN cells can be induced to express CD8αα homodimers in response to anti-TCR stimulation. Most notable, in TCRαβ strains where the transgenic receptor is expressed later in development, CD4$^-$CD8$^-$ T cells arise coexpressing the transgenic TCRαβ and endogenous TCRγδ (Table II, and Figs. 6 and 7). TCRαβDN cells with both receptors exhibit the same phenotype and properties as those lacking TCRγδ expression. These findings provide the most direct evidence that TCRαβDN cells are γδ lineage T cells.

The different patterns of TCR expression in CD4$^-$CD8$^-$ T cells of TCRαβ mice appear to be related to the timing of TCRαβ transgene expression with respect to endogenous TCRγδ gene rearrangement. As modeled in Fig. 10, the early expression of transgenic TCRαβ in precursor thymocytes of AND TCR mice causes suppression of endogenous TCRγδ 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 TCRγδ gene rearrangements occur normally. If rearrangements are productive, mature CD4$^-$CD8$^-$ T cells emerge coexpressing the TCRαβ (P14 TCR) and endogenous TCRγδ (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 TCRαβ and endogenous TCRγδ (Fig. 9), but lymph node T cells bear only the transgenic TCRαβ (Fig. 6). Thus, the rearrangements of genes encoding the lymphoid type TCRγδ are suppressed by AND TCR expression, whereas rearrangements that occur early in the fetal thymus, encoding the TCRγδ 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 TCRγδ or TCRβ 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 TCRγ and -δ rearrangements, suggesting that the production of a functional TCRγδ favors a γδ lineage decision (51–53). In addition, mice deficient for the pTα 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

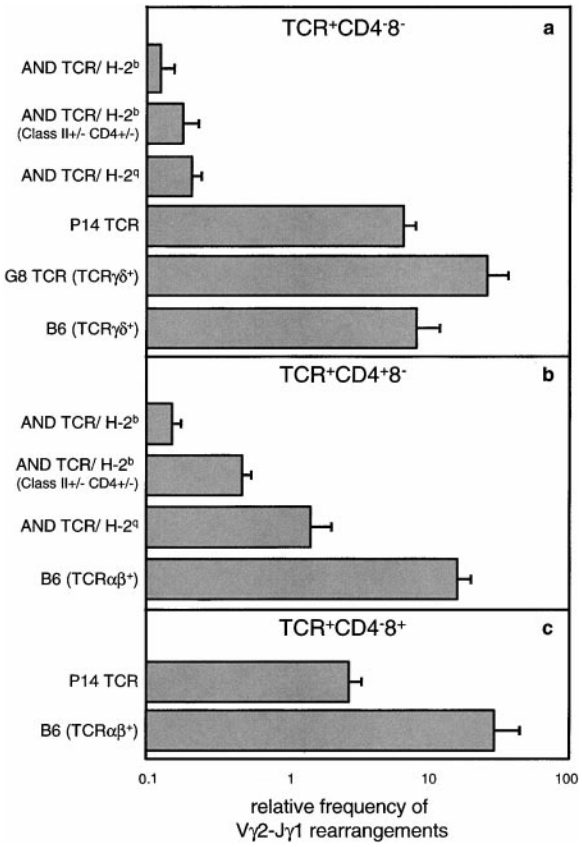

Figure 8. Vγ2–Jγ1 gene rearrangements are suppressed in TCR^+ $CD4^-CD8^-$ (TCRαβDN) cells of AND TCR but not of P14 TCR mice. Rearrangements are suppressed, independent of MHC haplotype, in the TCRαβDN but not the $TCR^+CD4^+CD8^-$ subset of AND TCR mice. Samples of 10^5 each of TCR^+ ($V\alpha11^+$ for AND, $V\alpha2^+$ for P14, $TCR\gamma\delta^+$ for G8 TCR, and $TCR\gamma\delta^+$ or $TCR\alpha\beta^+$ for B6) (a) $CD4^-CD8^-$, (b) $CD4^+CD8^-$, and (c) $CD4^-CD8^+$ lymph node T cells from AND TCR/H-2^b, AND TCR/H-2^b (MHC class $II^{+/-}$, $CD4^{+/-}$), AND TCR/H-2^q, 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.

lineage determination may occur before or independent of TCR gene rearrangement (55–58). Of relevance, a few $CD4^+CD8^+$ thymocytes arise in $TCR\beta^{-/-}$ 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 $TCR\gamma\delta^+$ 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

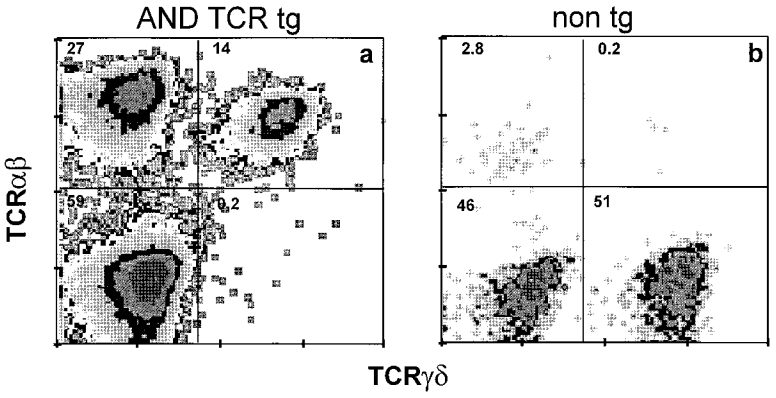

Figure 9. In contrast to lymphoid T cells, skin dendritic epithelial lymphocytes of AND TCR mice contain two subsets of T cells, one bearing only the transgenic TCRαβ and a second coexpressing the transgenic TCRαβ with endogenous TCRγδ. Isolated epidermal lymphocytes of (a) H-2^d AND TCR transgenic (tg) 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^d or other H-2 haplotypes.

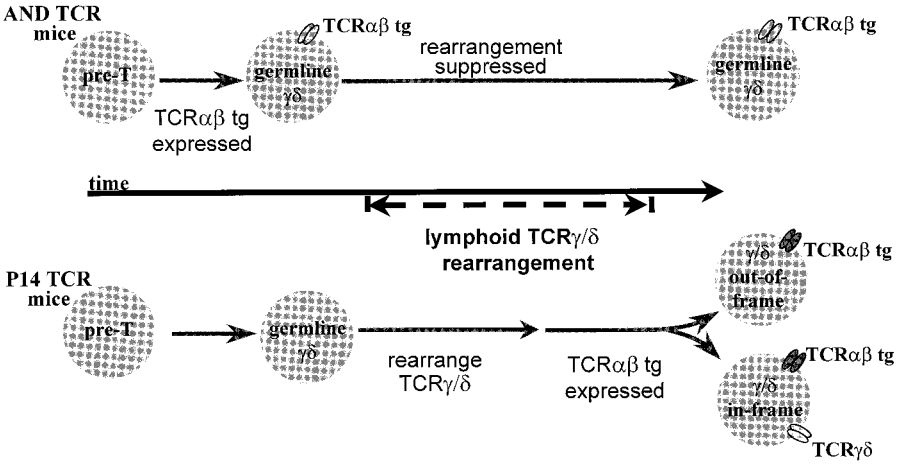

Figure 10. A model to explain the different patterns of TCR expression in $CD4^-CD8^-$ thymocytes of TCRαβ transgenic mice. Lymphoid-type TCRγδ gene rearrangements occur at a distinct time (stage) in thymocyte development. In AND TCR mice, the transgenic TCRαβ is expressed early with respect to TCRγδ gene rearrangement and rearrangement is suppressed. Since transgenic TCRαβ expression occurs somewhat later in P14 TCR mice, there is no interference with rearrangement and cells with in-frame TCRγδ rearrangements can express both the transgenic TCRαβ and endogenous TCRγδ. Irrespective of the TCR expression pattern, $CD4^-CD8^-$ cells mature with properties of γδ lineage T cells.

signals favor an αβ 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 preprogrammed, 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 αβ T cells are depleted of in-frame TCRγδ rearrangements (51–53) and the low frequency of productive TCRβ rearrangements in γδ T cells (65) support a sequential model.

TCRαβ 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 TCRαβ receptor can substitute for the TCRγδ in γδ lineage T cells, cells that would normally be immunologically silent can now participate in an antigen-specific response. Because γδ 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 γδ lineage cells (bearing transgenic TCRαβ) contribute to the antigenic response in unpredictable ways. Even sorting for $CD4^+$ cells may not help, since a few γδ T cells express CD4 (57). A new generation of TCR transgenic mice, with delayed TCRαβ expression, may provide a solution to this problem.

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