Commitment toward the natural T (iNKT) cell lineage occurs at the CD4⁺8⁺ stage of thymic ontogeny

Jelena S. Bezbradica*, Timothy Hill*, Aleksandar K. Stanic, Luc Van Kaer, and Sebastian Joyce†

Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232

Edited by Philippa Marrack, National Jewish Medical and Research Center, Denver, CO, and approved February 22, 2005 (received for review November 13, 2004)

T lineage commitment occurs in a discrete, stage-specific manner during thymic ontogeny. Intrathymic precursor transfer experiments and the identification of CD4⁺8⁺ double-positive (DP), Vα14Jα18 natural T (iNKT) cells suggest that commitment to this lineage might occur at the DP stage. Nevertheless, this matter remains contentious because others failed to detect Vα14Jα18-positive iNKT cells that are CD4⁺8⁺. In resolution to this issue, we demonstrate that retinoic acid receptor-related orphan receptor γ (RORγ)^{0/0} thymi, which accumulate immature single-positive (ISP) thymocytes that precede the DP stage, do not rearrange Vα14-to-Jα18 gene segments, suggesting that this event occurs at a post-ISP stage. Mixed radiation bone marrow chimeras revealed that RORγ functions in an iNKT cell lineage-specific manner. Further, introgression of a Bcl-x_L transgene into RORγ^{0/0} mice, which promotes survival and permits secondary rearrangements of distal Vα and Jα gene segments at the DP stage, rescues Vα14-to-Jα18 recombination. Similarly, introgression of a rearranged Vα14Jα18 transgene into RORγ^{0/0} mice results in functional iNKT cells. Thus, our data support the “T cell receptor-instructive (mainstream precursor) model” of iNKT cell lineage specification where Vα14-to-Jα18 rearrangement, positive selection, and iNKT cell lineage commitment occur at or after the DP stage of ontogeny.

RORγ | Vα14-to-Jα18 rearrangement | lineage specification | T cell ontogeny

Commitment and differentiation to T lineage lymphocytes occur in the thymus gland from a pluripotent lymphocyte progenitor. The T lineage consists predominantly of αβ T cells and a minor population of γδ cells. Amongst the αβ T cells are the conventional CD4⁺8[−] and CD4[−]8⁺ lymphocytes as well as several minor subsets, including CD4⁺25⁺ T_{reg} and NK1.1⁺ natural T cells. Lineage decision (i.e., the decision to become γδ or αβ) occurs early, whereas commitment to CD4⁺8[−] and CD4[−]8⁺ lineages, which are specified by interactions with antigen-presenting molecules, occurs late in thymic ontogeny. These cell-fate specifications during thymic ontogeny of T lymphocytes depend on specific environmental cues, which signal heritable activation and repression of genes.

Natural T cells comprise a heterogeneous subset amongst which Vα14Jα18 T (iNKT) lymphocytes predominate. They are CD1d-restricted, innate-like T lymphocytes that express a restricted T cell receptor (TCR) repertoire, which consists of the invariant Vα14Jα18 α-chain that predominantly pairs with the Vβ8.2 β-chain (1). iNKT cell function, albeit elusive, appears to be immunoregulatory in nature (2, 3). Its ontogeny proceeds through the very same early stages (CD3[−]4[−]8[−] triple-negative, TN1–4) as do the developing conventional T lymphocytes (4–9). Although it is contentious at which stage commitment to iNKT cell lineage occurs (discussed below), positive selection of this T cell subset relies on the interaction of precursor cells with CD1d1 expressed by CD4⁺8⁺ double-positive (DP) thymocytes (10–13). Immature iNKT cells express their TCR and undergo maturation-dependent expression of NK1.1 and DX5, yielding two immediate precursors (DX5[−]NK1.1[−] and DX5⁺NK1.1[−]) of mature (DX5⁺NK1.1⁺ and DX5[−]NK1.1⁺) iNKT cells (5–7).

TCR Vα and Jα gene segments undergo ordered primary and secondary recombination events during immature CD8 single-positive (ISP) and DP stage, with proximal segments rearranging before the distal ones (14). Initiation of TCR rearrangement is a tightly regulated process and is kept under the control of two cis-acting elements, the T early α promoter (15, 16) and the TCRα enhancer (17). Selection-induced programmed cell death of TCR-positive, DP thymocytes expressing the proximal Vα and Jα recombinants, however, limits the progression of rearrangements along the Jα locus and constricts the repertoire of positively selected T cells in RORγ^{0/0} thymocytes (14).

Retinoic acid receptor-related orphan receptor γ (RORγ) and its thymus-specific isoform (RORγt), which differ only in their 5′ exons, owing to their expression from distinct promoters, are critical for thymocyte development (18, 19). RORγ is expressed at low levels in ISP cells but primarily in immature DP thymocytes (14, 16, 20). It binds to the T early α promoter and is predicted to regulate TCRα gene rearrangement (16). RORγ^{0/0} mice exhibit ≈9-fold expansion of ISP cells, as well as a reduction in DP (60–80%) and CD4⁺8[−] and CD4[−]8⁺ single-positive (>90%) thymocytes (14, 18). RORγ regulates the survival window of DP thymocytes through induction of antiapoptotic Bcl-x_L and allows rearrangement through more distal 5′ Vα-to-3′ Jα segments (14, 18).

Numerous studies using various mutant mouse models have demonstrated unique requirements for the development of iNKT cells when compared with conventional T lymphocytes, suggesting that they may constitute a distinct T cell lineage (8, 21–27). Although much has been learned regarding the factors that drive differentiation, the developmental origin of this cell lineage remains less clearly defined. Purified, genetically tagged CD3[−] DP thymocytes from either WT or CD1d-deficient mice, when injected intrathymically into WT hosts, beget Vα14Jα18⁺CD4⁺ and CD4[−]8[−] double-negative iNKT cells. Further, CD1 tetramer [tetrameric, α-galactosylceramide (αGalCer)-loaded CD1d1]-positive, DP iNKT cells were identified in this study (4). Our studies of thymocyte-specific NF-κB signaling-deficient mice revealed a CD24^{HIGH} (HSA) CD1 tetramer-positive thymocyte, which also expressed Nur77, a marker for negative selection (23). These data suggest that iNKT cells may be derived from the DP compartment but could also reflect the presence of an already precommitted precursor in the sorted CD3[−] DP population. Nevertheless, other studies have identified CD4⁺ thymocytes as the first CD1 tetramer-positive cells and failed to identify DP iNKT cells (5, 6). Thus, whether iNKT cells are derived from a distinct precursor or are selected from a common T cell precursor pool still remains unclear. Conse-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: RORγ, retinoic acid receptor-related orphan receptor γ; DP, double positive; iNKT, Vα14Jα18 natural T; TCR, T cell receptor; αGalCer, α-galactosylceramide; BM, bone marrow; RT, reverse transcription.

*J.S.B. and T.H. contributed equally to this work.

†To whom correspondence should be addressed. E-mail: sebastian.joyce@vanderbilt.edu.

© 2005 by The National Academy of Sciences of the USA

quently, we have sought to resolve this issue by using ROR $\gamma^{0/0}$ mice as a model.

We hypothesized that the thymocyte precursor in which *Vα14*-to-*Jα18* rearrangement occurs would specify the iNKT cell lineage. Our data are consistent with the “TCR-instructive (mainstream precursor) model” (reviewed in ref. 28) of iNKT cell development in which *Vα14*-to-*Jα18* rearrangement, and hence commitment to the iNKT cell lineage, occurs at the DP stage.

Experimental Procedures

Mice. C57BL/6 mice were purchased from The Jackson Laboratory. B6-V α 14^{tg} (29), B6-J α 18^{0/0} (30), B6.129-ROR γ ^{0/0}, B6-ROR γ ^{0/0};Bcl-X_L^{tg} (18), and B6-I κ B α N^{tg} (23, 31) mice were generously provided by A. Bendelac (University of Chicago, Chicago), M. Taniguchi (Chiba University, Chiba, Japan), D. R. Littman (New York University, New York), and M. R. Boothby (Vanderbilt University), respectively. B6.129-CD1d1^{0/0} mice are described in ref. 12. B6.129-ROR γ ^{0/0};V α 14^{tg} mice were generated by introgressing the rearranged *V α 14 α 18* transgene into the knockout animal through breeding. All mice were bred and maintained in compliance with Vanderbilt's Institutional Animal Use and Care Committee regulations.

Antibodies and Reagents. All antibodies were purchased from BD Biosciences. α GalCer was generously provided by Kirin Brewery (Gunma, Japan). Preparation and use of CD1 tetramer are described in ref. 32.

Flow Cytometry. Splenocytes of individual, age-matched (3–7 weeks old) mice were stained for four-color flow cytometric analysis by using the following antibodies: anti-B220-FITC, anti-CD8 α -FITC, anti-CD4-PE, anti-CD3 ϵ -PerCP-Cy5.5, anti-CD1d (1B1)-biotin, streptavidin-allophycocyanin, and CD1 tetramer-allophycocyanin. iNKT cells were analyzed within electronically gated B220[−] splenic and hepatic or CD8 α [−] thymic populations. Flow cytometry was performed with a FACSCalibur instrument (Becton Dickinson), and the data were analyzed by using FLOWJO software (Treestar, Ashland, OR).

ELISA. Each mouse was injected i.p. with 5 μ g of α GalCer or with vehicle (0.1% Tween 20 in PBS) as the control. Two hours later, serum was collected, and IL-2 and IL-4 responses were monitored by ELISA as described in refs. 24 and 32.

Generation of Radiation Bone Marrow (BM) Chimeras. Recipient mice were irradiated twice with 500 rads from a Cs source 4 hr apart, rested for 6 hr, and transfused i.v. with $2\text{--}2.5 \times 10^7$ donor BM cells in PBS. Mice were maintained under antibiotic treatment (sulfamethoxazole and trimethoprim oral suspension, Al-Pharma, Baltimore) from 2 days before transfer until 2 weeks after transfer. Animals were kept under sterile conditions and analyzed 4–8 weeks posttransplantation.

Real-Time Quantitative PCR. Total thymic RNA was extracted by using TRIzol reagent (Invitrogen) per the manufacturer's instructions. Reverse transcription (RT) was performed on 1 μ g of RNA by using the Omniscript RT Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Quantitative PCR was conducted by using 1 μ l of cDNA resulting from the above RT reaction. TaqMan MGB (Applied Biosystems) forward *Va14* (5'-CACTGCCACCTACATCTGTGT-3'), reverse *Ja18* (5'-AGTCCCAGCTCCAAAATGCA-3'), and *Va14Ja18* reporter (5'-CCTAAGGCTGAAC-CTC-3') primers, as well as the proprietary β -actin primers (Applied Biosystems assay Mn00607939_s1), were used. Data were collected on a Stratagene Mx3000P thermocycler. Cycle threshold values were obtained by using Stratagene MX3000P

Fig. 1. ROR γ -deficient mice lack iNKT cells. (A) Thymic, splenic, and liver ROR $\gamma^{+/0}$ and ROR $\gamma^{0/0}$ iNKT cells were identified by using CD3 ϵ -specific mAb in conjunction with CD1 tetramer and analyzed by flow cytometry. CD8 α^{LOW} thymocytes as well as B220 LOW splenic and hepatic leukocytes were electronically gated, and CD3 ϵ^{+} CD1 tetramer-positive cells were identified. Numbers within plots refer to the percentage of iNKT cells among total leukocytes. The data represent at least three independent experiments. (B) Thymic ROR $\gamma^{+/0}$ and ROR $\gamma^{0/0}$ cells were reacted with CD1d-, CD4-, and CD8 α -specific mAbs and analyzed by flow cytometry. DP cells were gated (*Left*), and the level of CD1d1 expression was analyzed (*Right*). Numbers within plots refer to the percentage of DP thymocytes and the mean fluorescence intensity (MFI) of CD1d1. (C) Freshly isolated ROR $\gamma^{+/0}$ and ROR $\gamma^{0/0}$ thymocytes were incubated with N38-2C12 iNKT hybridoma. After 12–15 hr, IL-2 secreted into the coculture medium was monitored as a function of iNKT hybridoma activation by using ELISA. Plate-bound anti-CD3 ϵ mAb and CD1d $^{0/0}$ stimulators were used as positive and negative controls, respectively. Data in B and C are representative of two independent experiments. stim:resp, stimulator-to-responder ratio.

software. Data are represented as percent *Vα14/α18* expression levels in each sample relative to β -actin transcripts detected in the same sample.

RT-PCR. RT was performed on total RNA as described above. PCR was performed by using the cDNA template resulting from the above RT reaction and forward (^F) and reverse (^R) primers: Vα14^F (5'-TGGGAGATACTCAGCAACTCTGG-3'), Jα18^R (5'-CAGGTATGACAATCGCTGAGTCC-3'), Vα3^F (5'-CCCAGTGGTTCAAGGAGTGA-3'), Vα8^F (5'-TCACAGACAACAAG-AGGACC-3'), Cα^R (5'-TGCGCTGTGGTCTCTT-TGAAG-3'), β-actin^F (5'-AGAGGGAAATC-GTGCCTGAC-

Fig. 3. *Vα14-to-Jα18* rearrangement occurs within DP thymocytes. (A) Schematic rendition of the germ-line organization of mouse *Vα* locus. Note that the *Vα* and *Jα* segments are numbered 5' to 3' in a descending order (adapted from ref. 46). (B) *Vα14-to-Jα18* rearrangement was assessed by quantitative real-time PCR by using TaqMan MGB primers and probes as described in *Materials and Methods*. Positive and negative controls were a *Vα14* iNKT hybridoma DN32.D3 and *Jα18*^{0/0} thymocytes, respectively. *β*-actin was used as an endogenous control for each sample. Error bars represent standard errors of data collected from three independent experiments. (C) Conventional RT-PCR was used to assess the indicated *Vα*-to-*Cα* and *Vα14-to-Jα18* rearrangements. *β*-actin was used as the positive control. Data are representative of at least three independent experiments.

ontogeny, we concluded that ROR γ must play a haematopoietic cell-intrinsic role during development. In support of this conclusion, ROR $\gamma^{0/0} \rightarrow$ J α 18 $^{0/0}$ (Fig. 2, third row) BM chimeras did not develop iNKT cells. To determine whether ROR γ plays an iNKT cell or T lymphocyte-autonomous role, we generated mixed ROR $\gamma^{0/0} +$ J α 18 $^{0/0} \rightarrow$ ROR $\gamma^{0/0}$ (Fig. 2, fourth row) and ROR $\gamma^{0/0} +$ J α 18 $^{0/0} \rightarrow$ J α 18 $^{0/0}$ (Fig. 2, fifth row) BM chimeras. In this system, J α 18 $^{0/0}$ BM chimeras (which develop ROR γ -competent T cells but do not develop iNKT cells) should provide any T cell-derived, ROR γ -induced factor(s). We found that the mixed BM chimeras do not reconstitute iNKT cell development (Fig. 2). Similar data were obtained when B6-I κ B α Δ N 1g mice [which, akin to J α 18 $^{0/0}$ mice, do not develop iNKT cells (23)] were used as donors for mixed radiation BM chimeras generated in ROR $\gamma^{0/0}$ animals (data not shown). Therefore, we conclude that ROR γ plays an iNKT cell-autonomous role in this ontogenetic process.

ROR γ Deficiency Impairs iNKT Cell Ontogeny. ROR γ plays an important role in conventional T cell development (14, 18). Its role in iNKT cell ontogeny hitherto remains undefined. Lineage-specific phenotyping (23) using CD1 tetramer revealed that ROR $\gamma^{0/0}$ mice do not contain any detectable iNKT cells in the thymus, spleen, and liver (Fig. 1A). This deficit could be a consequence of altered CD1d1 expression on DP ROR $\gamma^{0/0}$ thymocytes, thereby disrupting positive selection of iNKT cells. Our analysis of CD1d1 expression (Fig. 1B) and function (Fig. 1C) indicates that these aspects are intact in ROR $\gamma^{0/0}$ mice. Note that the ROR $\gamma^{0/0}$ thymus, as compared with the WT thymus, contains only ≈ 40 –50% DP thymocytes (refs. 14 and 18 and Fig. 1B) and, hence, requires twice as many cells to activate iNKT hybridomas (Fig. 1C). Because ROR $\gamma^{0/0}$ mice express functional CD1d1 molecules, we conclude that ROR γ deficiency impairs iNKT cell development by a mechanism distinct from defective positive selection of this lineage.

ROR γ Plays a Cell-Autonomous Role in iNKT Cell Ontogeny. To determine the mechanism by which ROR γ regulates iNKT cell ontogeny, we prepared a series of reciprocal BM chimeras by using ROR $\gamma^{0/0}$ and J $\alpha 18^{0/0}$ mice (which develop conventional T cells but do not develop iNKT cells). Because ROR $\gamma^{+/+} \rightarrow$ ROR $\gamma^{0/0}$ (Fig. 2, first row) and ROR $\gamma^{+/+} \rightarrow$ J $\alpha 18^{0/0}$ (Fig. 2, second row) radiation BM chimeras reconstitute iNKT cell

Fig. 4. Introgression of Bcl-x_L and the Vα14Jα18 transgene into RORγ^{0/0} mice rescues iNKT cell development. (A and B) RORγ⁺⁰, RORγ^{0/0}, and RORγ^{0/0};Bcl-x_L^{tg} (A) and RORγ⁺⁰;Vα14^{tg} and RORγ^{0/0};Vα14^{tg} (B) iNKT cells were analyzed as described in the legend of Fig. 1A. Absolute numbers of thymic iNKT cells (B Right) were calculated from total leukocyte number and percentage of gated CD3ε⁺CD1 tetramer-positive cells (B Left). Vα14^{tg} (29) thymii are lymphopenic; therefore, RORγ^{0/0};Vα14^{tg} mice have up to 3- to 5-fold lower thymic cellularity when compared with RORγ⁺⁰ control. (C) Serum cytokine response of RORγ⁺⁰, RORγ^{0/0}, RORγ^{0/0};Vα14^{tg}, and RORγ⁺⁰;Vα14^{tg} mice to *in vivo* activation of iNKT cells was measured by ELISA after 2 hr of αGalCer or vehicle administration. Data are representative of three independent experiments.

ROR γ Deficiency Precludes *V α 14-to-J α 18* Gene Rearrangement. ROR $\gamma^{0/0}$ mice rearrange only the proximal, not the distal, *V α* and *J α* gene segments because of premature death of DP thymocytes (14). Whether ROR $\gamma^{0/0}$ thymocytes recombine *V α 14-to-J α 18* gene segments hitherto remains unknown. Quantitative RT-PCR analysis using specific primers for the *V α 14-to-J α 18* rearrangement found in iNKT cells revealed that ROR $\gamma^{0/0}$ thymocytes, akin to *J α 18 $^{0/0}$* thymocytes, do not rearrange *V α 14* and *J α 18* gene segments (Fig. 3*B*). To exclude the possibility that the *V α 14-to-J α 18* rearrangement occurs in ROR $\gamma^{0/0}$ thymocytes but is undetectable because the lineage is not further expanded by positive selection, we used CD1d1 $^{0/0}$ thymocytes as a sensitivity control for the assay to detect preexisting but unselected *V α 14-to-J α 18* rearrangements. The results using whole CD1d1 $^{0/0}$ thymus (whose DP thymocyte content is >80%) show that *V α 14-to-J α 18* rearrangement is detectable in CD1d1 $^{0/0}$ thymocytes at levels similar to WT (Fig. 3*B*), which is consistent with a published report (4). Thus, the *V α 14J α 18* iNKT cell precursor is less likely to exist in ROR $\gamma^{0/0}$ thymocytes. Nevertheless, although *V α 14*, as well as *V α 3* and *V α 8*, segments recombine with perhaps more proximal *J α* segments (Fig. 3*C*), the *V α 14* segment does not recombine with the more distal *J α 18* element (Fig. 3*B*). This finding is consistent with an earlier report that demonstrated that the major limitation to successful *V α -to-J α* rearrangement in ROR $\gamma^{0/0}$ mice is not the *V α* element but the proximity of a particular *J α* segment to *V α* elements (14) (see Fig. 3*A* for a schematic rendition of the *V α* locus). This study, in which recombination as far upstream as the *V α 19* locus to all *J α* segments was analyzed, revealed that rearrangement occurred

as far as the *Jα30* segment (ref. 14 and Y.-W. He, personal communication). Note that *Jα18* is farther away from the *Vα* locus than is *Jα30* (Fig. 3A). Thus, we conclude that RORγ deficiency precludes *Vα14-to-Jα18* gene rearrangement and impairs iNKT cell ontogeny before lineage specification.

***Vα14-to-Jα18* Gene Rearrangement Occurs at the DP Stage and Specifies the iNKT Cell Lineage.** Previous studies have shown that enforced expression of the Bcl-x_L transgene in RORγ^{0/0} (RORγ^{0/0};Bcl-x_L^{tg}) mice promotes survival of TCR-positive DP thymocytes, restoring them to numbers equivalent to those in RORγ^{+/+} mice (14, 18). The resulting RORγ^{0/0};Bcl-x_L^{tg} thymocytes have rearranged *Vα* elements to the most distal *Jα* segments in addition to the proximal ones (14). We found that RORγ^{0/0};Bcl-x_L^{tg} thymocytes recombine *Vα14-to-Jα18* gene segments (Fig. 3*B*). Consequently, iNKT cell ontogeny is also restored in RORγ^{0/0};Bcl-x_L^{tg} mice (Fig. 4*A*). These data suggest an essential role of RORγ in the survival of the cellular substrate in which *Vα14-to-Jα18* gene recombination occurs.

To affirm the conclusion that impaired iNKT cell ontogeny in $\text{ROR}\gamma^{0/0}$ mice is owed exclusively to the lack of $V\alpha 14$ -to- $J\alpha 18$ gene rearrangement, we introgressed the rearranged $V\alpha 14J\alpha 18$ transgene into $\text{ROR}\gamma^{0/0}$ ($\text{ROR}\gamma^{0/0}; V\alpha 14^{\text{tg}}$) mice. Akin to $\text{ROR}\gamma^{0/0}; \text{Bcl-xL}^{\text{tg}}$ mice but unlike $\text{ROR}\gamma^{0/0}$ animals, $\text{ROR}\gamma^{0/0}; V\alpha 14^{\text{tg}}$ mice develop functional iNKT cells (Fig. 4 B and C). Based on these findings and the fact that $\text{ROR}\gamma$ is a highly DP stage-specific transcription factor, we propose that $V\alpha 14$ -to- $J\alpha 18$ rearrangement and iNKT cell lineage specification are DP stage-specific events.

Fig. 5. A putative ontogenetic pathway for iNKT cells. Early steps in the ontogeny of iNKT lymphocytes and conventional T cells are common to both lineages. The ontogenetic programming of the unique features of iNKT cell function occurs at the CD4⁺8⁺ DP stage and begins with the rearrangement of the Vα14Jα18 TCR α-chain and after its interaction with the positively selecting ligand CD1d-self lipid complex. Stage-specific iNKT cell markers (gray boxes) and lineage-specific differentiation signals are indicated. Several iNKT cell markers, e.g., CD4, CD44, CD122 (IL-2Rβ), CD127 (IL-7Rα), and CD161 (NK1.1), indicated in gray boxes, as well as CD5, CD24 (HSA), DX5, and Ly49A/D and C/I (data not shown), are developmentally regulated.

Concluding Remarks

It is currently thought that the first iNKT cells to emerge in the thymus are CD4⁺ and CD24^{LOW} (5, 6). That conclusion notwithstanding, intrathymic transfer of CD3ε⁺CD4⁺8⁺ DP thymocytes from WT or CD1d1^{0/0} mice into CD1d1^{+/+} recipients yields iNKT cells (4). We previously identified a putative CD1 tetramer-positive thymic precursor that expresses CD24 in mice severely deficient in thymocyte-specific NF-κB signaling (23). These latter studies suggest that commitment to the iNKT cell lineage might occur at the DP stage. Nevertheless, interpretation of these findings is complicated by inherent limitations of adoptive cell transfer and immunophenotyping experiments. The genetic system described here overcomes the limitations of previous studies, and the data support the hypothesis that the iNKT cell precursor emerges from CD4⁺8⁺ DP thymocytes. Thus, our findings are consistent with the “TCR-instructive (mainstream precursor) model” (reviewed in ref. 28) in which commitment to the CD1d1-restricted iNKT cell lineage, akin to MHC class II-restricted CD4⁺8⁺ and MHC class I-restricted CD4⁺8⁺ T lymphocyte lineages, occurs at or after the DP stage through positive selection by specific self-ligands.

Early steps in the ontogeny of iNKT lymphocytes and conventional T cells are common to both lineages (Fig. 5). The common progression of the two lineages through early ontogenesis is also reflected in similar-to-identical signaling requirements (IL-7Rα, Jak3, and common γ-chain) for the development of their precursors before *Tcrα* rearrangement (reviewed in refs. 4 and 27). Our data further suggest that Vα14-to-Jα18 rearrangement represents part of a general rearrangement process along the *Tcrα* locus that is independent of precommitment to the iNKT cell lineage itself. Nevertheless, it is after Vα14 and Jα18 rearrangement and Vα14Jα18 TCR expression on the cell surface that the iNKT cell lineage begins to acquire unique TCR signaling features, which are clearly distinct from the ones acquired by conventional T cells. Fyn, which is required for iNKT cell ontogeny,

is dispensable for conventional T cell development. Further, Fyn appears to be the first iNKT cell-specific signal that acts in a distinct manner within this lineage during thymic development (33–35). RORγ and Fyn perhaps act on the same precursor, which we predict to be the DP thymocytes, based on previous data (33–35) and data presented here.

We propose that the ontogenetic programming of the unique features of iNKT cell function occurs at the stage that begins with the rearrangement of the Vα14Jα18 TCR α-chain and after its interaction with the positively selecting ligand, CD1d-self lipid complex (Fig. 5). The distinct requirement for IL-15/IL-15 receptor (36–38), lymphotoxin-β (39), Fyn (33–35), SAP (40–42), and PKCθ (24) signaling, as well as Ets (43), T-bet (26), AP-1 (44), and NF-κB (21–23) transcription factors for iNKT but not for conventional T lymphocyte ontogeny, underscores the impact of Vα14Jα18 TCR/CD1d interaction in this process. Our previous data have suggested that the Vα14Jα18 iNKT cell receptor is organized and/or interfaces its ligand(s) in a manner distinct from the αβ TCR of conventional T lymphocytes (45). Thus, the identification of the earliest precursor that begets the iNKT cell lineage will allow the dissection of the unique cellular, biochemical, and molecular programming of this T lymphocyte subset.

Note Added in Proof. During the review of this manuscript, it came to our attention that T. Egawa *et al.* (47), using an identical and complementary system, reached a conclusion similar to the one described here regarding the origin and lineage commitment of iNKT cells.

We thank Drs. A. Bendelac, M. R. Boothby, D. R. Littman, and M. Taniguchi for genetically altered mice; Drs. A. Bendelac and K. Hayakawa (Fox Chase Cancer Center, Cheltenham, PA) for iNKT cell hybridomas; Kirin Brewery for generous supplies of αGalCer; Y. A. Oh and K. Richter for help with RNA preparation and RT-PCR; and A. J. Joyce and M. McReynolds for technical assistance. This work was supported by National Institutes of Health Grants AI49460 (to M. R. Boothby); AI050953, NS044044, and HL068744 (to L.V.K.); and AI042284 (to S.J.); and grants from the Juvenile Diabetes Research Foundation (to S.J.) and the Human Frontiers in Science Program (to S.J.).

- PNAS | April 5, 2005 | vol. 102 | no. 14 | 5119