A Novel Mouse Model for Invariant NKT Cell Study

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We have generated a novel mouse model harboring the in-frame rearranged TCRVα specific for invariant NKT (iNKT) cells (Va14-Ja18) on one allele by crossing the mouse cloned from NKT cells with wild-type mice. This genomic configuration would ensure further rearrangement and expression of TCRVa14-Ja18 under the endogenous promoters and enhancers. Mice harboring such an in-frame rearranged TCRVα (Va14-Ja18 mouse) possessed an increase in iNKT cells in the thymus, liver, spleen, and bone marrow. Intriguingly, both Th1- and Th2-type cytokines were produced upon stimulation with αGalactosylceramide, an agonist of iNKT cells, and the IgE level in the serum remained unaffected in the Va14-Ja18 mouse. These features markedly distinguish the nature of iNKT cells present in the Va14-Ja18 mouse from that of iNKT cells found in the Va14-Ja18 transgenic mouse. Besides these, the expression of TCRVγδ cells remained intact, and the use of the TCRVβ repertoire in iNKT cells was highly biased to TCRVβ in the Va14-Ja18 mouse. Furthermore, αGalactosylceramide-CD1d dimer-reactive immature iNKT cells expressed less Rag2 as compared with the conventional immature T cells at the positive selection stage. Cell cycle analysis on the thymocytes revealed that no particular subset proliferated more vigorously than the others. Crossing the Va14-Ja18 mouse with the CD1d knockout mouse revealed a novel population of iNKT cells whose coreceptor expression profile was similar to that assigned to iNKT precursor cells. These mice will be useful for the study on the development of iNKT cells as well as on their functions in the immune system. The Journal of Immunology, 2007, 179: 3888–3895.

Invariant NKT (iT) cells are a subset of TCRαβ T cells possessing a set of markers for the NK cell lineage, such as NK1.1 and a member of Ly-49 family, as well as the invariant Vα14-Jβ818 TCR (1–3). They may function as regulatory cells because they produce a copious amount of cytokines. Defective development of iNKT cells has been linked to autoimmunity (4, 5). Intriguingly, the development of iNKT cells is not dependent on the classical MHCs, but is conditional upon CD1d, a MHC-like molecule, mainly expressed on double-positive (DP) thymocytes (6, 7). Furthermore, they occupy up to 0.7% of the thymocytes as revealed by CD1d tetramer or dimer loaded with a synthetic ligand, α-galactosylceramide (αGalCer) (8, 9). Such an unusual abundance in the invariant TCRVα is intriguing in that their development may be somehow unique as compared with that of the mainstream αβT cells. Many efforts have been devoted to elucidate the development of such cells and suggested that their commitment is “selected” during thymocyte development (10). According to this, iNKT cells diverge from CD4+CD8+ DP in which cells undertake random TCRVα rearrangement, and those succeeding in rearranging Va14 with Ja18 are “selected” to follow the fate to become iNKT cells. The fact that there are no iNKT cells present in CD1d knockout mice argues that CD1d is indispensable for the development of iNKT cells as well as of non-iNKT cells (8, 11–13).

The study on the Va14-Ja18 transgenic (Tg) mice demonstrates 1) a preponderance of iNKT cells in the thymocytes and 2) a biased decrease of TCRVβ7 and Vβ8 in CD8 single-positive (SP) cells that gives ground for a favorable selection of Va14-Ja18 (8, 14). Such a bias in TCRVβ repertoire, however, does not necessarily insur the lineage commitment of iNKT cells upon assemblage of Va14-Ja18 and TCRVβ or TCRVβ8, as it is conceivable that such a combination may result in an inhibition of the positive selection imposed by TCRVβ8/CD8 SP cells. Similarly, Va24-JaQ Tg/Ca−/− mice show a slight increase in iNKT cell number and an enhanced Vβ7 usage concomitant with a decrease in Vβ8.2 usage (15). Furthermore, the Va14-Ja18 Tg mouse produced only IL-4, but not IFN-γ, upon the Ag stimulation, and the mice possessed Th-2 biased Ig production in the serum (14). Although Va24-JaQ Tg/Ca−/− mice produced both cytokines, the amount of the cytokines did not reflect the increase of iNKT cells (15). Because the production of both Th1- and Th2-type cytokines is a hallmark of iNKT cells, the Va14-Ja18 or Va24-JaQ Tg mouse may not be suitable for certain experiments. These Tg mice have other disadvantages in that the spatiotemporal expression of the Va14-Ja18 or Va24-JaQ is not ensured, and that cells are not allowed to delete such a transgene by further rearrangement. It is, therefore, imperative to develop a novel mouse model in which the development and the functions of iNKT cells mimic those found in the control as faithfully as possible.

Our recent work has provided evidence that nuclei from iNKT cells are competent for reprogramming a genome in such a way that they exert the totipotency when transferred into enucleated...
oocytes (16). Because such cloned mice possess a rearranged set of the in-frame rearranged Va14-Ja18 and Vβ8-D-J, some progenies from the clone will inherit the in-frame rearranged Va14-Ja18 in one allele (hereafter referred as the Va14-Ja18 mouse). In such circumstances, expression of the Va14-Ja18 TCR would be under the control of the endogenous promoter(s) and enhancers that ensure their proper expression, and the Va14-Ja18 TCR is readily subject to further rearrangement.

We found that Va14-Ja18 mice harbored an increased population of iNKT cells in the thymus as well as liver, spleen, and bone marrow. Concomitantly, these mice produced a copious amount of Thl- and Th2-type cytokines upon an appropriate stimulus both in vitro and in vivo relative to the control. These data indicate that the Va14-Ja18 mouse would be a novel model suitable for studying the development and the functions of iNKT cells.

Materials and Methods

Generation of the in-frame rearranged Va14-Ja18 mice

The cloned mouse harboring the in-frame rearranged Va14-Ja18 locus derived from C57BL/6 was mated with female C57BL/6 (Charles River Laboratories) and resulting progenies were analyzed by Southern blot for the inheritance of the Va14-Ja18 locus (16). Mice harboring the in-frame rearranged Va14-Ja18 on one allele (Va14-Ja18 mice) were used throughout the study unless specified. Va14-Ja18 mice were mated with CD1d knockout mice to generate mice possessing the in-frame rearranged Va14-Ja18 on the CD1d−/− background in F2. Mouse colonies were maintained in a specific pathogen-free facility at the Research Center for Allergy and Immunology and all experiments were conducted in compliance with the protocol approved by the RIKEN Animal Care and Use committee.

Flow cytometry

Liver lymphocytes were prepared with the Percoll gradient method by centrifugation. Cells from 8- to 10-week-old mice were stained with mAbs, anti-CD4 (L3T4 RM-4-5), anti-CD8 (LY-2 3-5-6.7), anti-TCRVβ (H57-597), anti-TCRVβ8 (F23.1), anti-TCRVγδ (GL3), anti-NK1.1 (PK136), anti-CD24 (M1/69), anti-CD44 (Im7), anti-CD69 (H1.2F3), anti-CD1d (1B1) (BD Pharmingen). αGalCer was obtained from Kirin Brewery and CD1d:Jγ dimers were obtained from BD Biosciences. αGalCer-CD1d dimers were prepared according to the manufacturer’s protocol (BD Biosciences). For αGalCer-CD1d dimer staining, cells were blocked with anti-CD16/32 (2.4G2; BD Biosciences) for 5 min on ice. After brief centrifugation to remove supernatant, a similar increase of iNKT cells was also observed except that some progenies from such mice represented ~15% of that from the control mice (Table I). Although the absolute number of the thymocytes decreased, the net iNKT cell number was augmented six times over the control (Table I and Fig. 2a). In the spleen, the number of iNKT cells increased >20 times as compared with the control, and iNKT cells occupied ~50% of the total T lymphocytes (Fig. 2a). In the liver mononuclear cells, more than half were iNKT cells (Fig. 2a). A similar increase of iNKT cells was also observed in the bone marrow where the number of such cells exhibited almost 20 times that of the control (Fig. 2a). We then examined the CD4/CD8 expression profile in the thymocytes. CD4+/CD8− DP cells showed a decrease (47%) accompanying an increase in CD4+ (20%) or CD8+ (13%) SP cells (Fig. 2b). Concomitantly, double-negative (DN) cells represented ~20% of the total thymocytes (Fig. 2b). Because most of the iNKT cells reported to date are either CD4+/-CD8− or CD4+/-CD8−, we evaluated the percentage of iNKT cells within the Va14-Ja18 mouse thymocytes. More than 40% of CD4+/-CD8− cells were iNKT cells, while almost half of CD4+/-CD8− cells represented iNKT cells. Among CD4+/-CD8+ and CD4+/-CD8− cells, few iNKT cells were present (Fig. 2b). In the periphery, a similar tendency was observed except that some iNKT cells were CD4−/CD8+ in the liver (data not shown). The
results demonstrated that iNKT cells in Val14-Ja18 mice are essentially either CD4\(^+\)/CD8\(^-\) or CD4\(^-\)/CD8\(^+\).

We analyzed the expression of the other markers on iNKT cells in the thymus. The profile for the NKRs such as Ly49A, Ly49D, and Ly49C on Val14-Ja18 mice was almost equivalent to that found in the control thymocytes (Fig. 2c, data not shown). In contrast, CD69\(^+\) and NKI.1\(^+\) cells decreased concomitantly with an increase in CD44\(^{dim}\) cells, while CD24\(^+\) cells increased in the Val14-Ja18 mouse thymocytes (Fig. 2c).

Production of a copious amount of the cytokines upon \(\alpha\)GalCer stimulation in Val14-Ja18 mice

We next evaluated the potential of iNKT cells to respond to \(\alpha\)GalCer and the resulting production of the cytokines in the serum. Without any stimulation, no cytokine was produced in both control and Val14-Ja18 mice. Upon \(\alpha\)GalCer stimulation, however, both types of mice simultaneously produced Th1-type cytokines such as IL-2 and IFN-\(\gamma\), Th2-type cytokines, IL-4, IL-5, and IL-10, and inflammatory cytokines such as IL-1\(\beta\) and TNF-\(\alpha\) (Fig. 3a). Cytokine production peaked at 4 h poststimulation for all the cytokines. Val14-Ja18 mice produced >10 times cytokines such as IL-2, IL-4, IL-10, GM-CSF, and IFN-\(\gamma\) relative to the control at 4 h. In contrast, no significant increment in the amount of IL-1\(\beta\) and IL-5 was detected in Val14-Ja18 mice under the same conditions (Fig. 3a).

We also examined whether stimulation with \(\alpha\)GalCer-pulsed DCs resulted in enhanced cytokine production in vitro. DCs prepared from the spleen of Val14-Ja18 or control mice were cocultured with the whole spleen cells from these mice. Coculture of the control spleen cells with DCs from either control or Val14-Ja18 mice led to a modest production of IFN-\(\gamma\) and IL-4 (Fig. 3b). In contrast, irrespective of the origin of DCs, use of spleen cells from Val14-Ja18 mice resulted in a 5- and 10-fold increase for IL-4 and IFN-\(\gamma\), respectively (Fig. 3b). This increment mirrored well the abundance of iNKT cells in the Val14-Ja18 mouse spleen.

FIGURE 1. a, Inheritance of the in-frame Val14-Ja18 and \(\psi\)Bj8-DB1-\(\beta\)2S5 locus to the progeny. Genomic DNA (5 \(\mu\)g/lane) from the F\(_1\) progeny [(C57BL/6 \times 129/Sv) \times C57BL/6] (lanes 1–9) was digested with EcoRI (for TCRVal14) or BamHI (for TCRJ\(\beta\)) and subjected to Southern blot with the specific probes (16). Arrowheads show the bands indicating the TCR rearrangement. C: control DNA from C57BL/6. b, Schematic representation of the TCRVal14 locus in the C57BL/6 and Val14-Ja18 mouse. The TCRVal14 locus in nonrearranged and rearranged germline configuration is shown with EcoRI sites for C57BL/6 (upper panel) and Val14-Ja18 mice (lower panel), respectively. 

FIGURE 2. a, Predominance of \(\alpha\)GalCer-CD1d dimer-reactive NKT (iNKT) cells in Val14-Ja18 mice. Thymocytes, spleen cells, liver mononuclear cells, and bone marrow cells from the control and Val14-Ja18 mouse were stained with anti-TCRV\(\beta\) and \(\alpha\)GalCer-CD1d dimers. The number in the figure represents the percentage of the each subset (upper panel, CD4 vs CD8). The percentage of iNKT cells (bold line) over the control staining (dotted line) within the each subset is shown (lower panels). c, Surface marker expressions on iNKT cells in Val14-Ja18 mouse. Thymocytes from the control and from Val14-Ja18 mouse were stained with anti-TCRV\(\beta\) and \(\alpha\)GalCer-CD1d dimers together with the indicated mAbs. Histograms display the frequency of the cells expressing the indicated markers among TCRV\(\beta\)\(^+\)/\(\alpha\)GalCer-CD1d dimer\(^+\) gated cells (bold line) over the control staining (dotted line). The number in figure represents the percentage of iNKT cells expressing the indicated molecule.
production of IL-4 and IFN-γ from the splenic iNKT cells was confirmed by intracellular staining (Fig. 3c).

Because Va14-Jα18 Tg mice harbor a Th2-biased Ig isotype in serum, we examined whether this was the case for Va14-Jα18 mice (14). A modest increase in Th2-type IgG1 (5-fold above controls on averages) concomitant with a decrease in Th1-type IgG2a production (one-tenth relative to the controls on average) was detected. Nevertheless, there was little bias in the amount of IgE production (one-tenth relative to the controls on average) was detected. These experiments also demonstrated that γδ T cells in the thymus was due to the results of competition (17) or to exclusive transcription of the allele harboring the rearranged Va14-Jα18 whatever be the reason, γδT cells in the intestinal intraepithelial lymphocytes (iIEL) were analyzed. It turned out that the cell number of the each subset and the ratio of αβ:γδ in Va14-Jα18 mice were quasiequivalent to those of the control mice (Fig. 3e), and there were γδT cells in the thymocytes (data not shown). These experiments also demonstrated that γδ T cells stemmed from the other nonrearranged allele (Fig. 1b). When B and NK cells from Va14-Jα18 mice were examined, little difference in the cell number and in the receptor expression profile was noticed relative to the controls (data not shown). Together, these data suggested that the in-frame rearranged Va14-Jα18 locus mainly affects T lymphocyte development.

γδT cells in Va14-Jα18 mice

To exclude the possibility that the increase of iNKT cells in the thymus was due to the results of αβ vs γδ lineage commitment competition (17) or to exclusive transcription of the allele harboring the rearranged Va14-Jα18 whatever be the reason, γδT cells in the intestinal intraepithelial lymphocytes (iIEL) were analyzed. It turned out that the cell number of the each subset and the ratio of αβ:γδ in Va14-Jα18 mice were quasiequivalent to those of the control mice (Fig. 3e), and there were γδT cells in the thymocytes (data not shown). These experiments also demonstrated that γδ T cells stemmed from the other nonrearranged allele (Fig. 1b). When B and NK cells from Va14-Jα18 mice were examined, little difference in the cell number and in the receptor expression profile was noticed relative to the controls (data not shown). Together, these data suggested that the in-frame rearranged Va14-Jα18 locus mainly affects T lymphocyte development.

CD1d may be responsible for the up-regulation of CD44, CD69, and NK1.1 and down-regulation of CD24

Mice deficient for CD1d are devoid of iNKT cells, as CD1d is responsible for the development of iNKT and non-iNKT cells (11–13). iNKT cells present in the periphery express the coreceptor such as CD44, CD69, and NK1.1. Studies on the ontogeny of iNKT cells have indicated that iNKT cells emerge from CD24high/NK1.1+CD44low cells in the thymus, and they acquire the CD24low/NK1.1+CD44high phenotype upon emigration from the thymus (18, 19). We have mated Va14-Jα18 mice with CD1d−/− mice to obtain mice harboring the hemizygous Va14-Jα18 and no CD1d (Va14-Jα18/CD1d−/− mouse). If a Va14-Jα18 TCR signal is critical for iNKT cell development, an immature iNKT cell population would be tangible on the CD1d−/− background. Analyses

![FIGURE 3](image-url)

**FIGURE 3.** a, Cytokine production upon αGalCer stimulation. The concentration of cytokines in the serum was measured at the indicated time points using 3-month-old Va14-Jα18 mice and the age-matched litters (control). Four individuals were sampled at each time point. Data are means ± SD. b, IL-4 and IFN-γ production upon in vitro DC stimulation. Spleen cells from the control and from Va14-Jα18 mice were mixed with αGalCer-pulsed DC. The concentration of IFN-γ and IL-4 in the resultant supernatant is shown. Data are means from three experiments ± SD (three wells per experiment). c, Production of IL-4 and IFN-γ from the Va14-Jα18 mouse spleen cells. Predominance of iNKT cells in TCRβ+/TCRβ+ cells (upper panels). Va14-Jα18 mouse spleen cells were stained with anti-TCRβ, anti-TCRβ Abs, and αGalCer- or vehicle-loaded CD1d dimers, and examined for TCRβ vs TCRβ expression (upper left panel). The number in the figure indicates the percentage of TCRβ+/TCRβ− cells. The percentage of αGalCer-CD1d reactive cells (bold line) over control-stained cells (dotted line) among the TCRβ+/TCRβ− cells is shown (upper right panel). IL-4 and IFN-γ production from the TCRβ+ cells (lower panels). The percentage of cells positive for the indicated cytokine staining (bold line) relative to the isotype control-stained cells (dotted line) is shown. One representative data from three experiments is shown. d, Slightly Th2-biased Ig isotypes in Va14-Jα18 mice. Serum level of the each Ig isotype was measured by ELISA in 3-month-old Va14-Jα18 mice and littermate control housed under the same conditions. Four individuals were examined and means ± SD are shown. e, γδ and αβT cells in the iIEL. iIEL were prepared from the control and Va14-Jα18 mouse intestine, and stained with anti-TCRβ and anti-TCRδ. Numbers in the quadrant represent the percentage of each subset ±SD.
figure represents the percentage of immature iNKT cells expressing the TCRV
expression is most likely dependent on CD1d. In contrast, CD24-
comprising iNKT cells (Fig. 4a).

Previous studies showed that the usage of TCRV
is highly biased to TCRV8, 7, and 5 (1–3). The analysis of TCRVβ+/TCRVβ8 cells revealed that >80% of this population comprised iNKT cells (Fig. 4c, upper panel). A similar tendency was observed in TCRVβ+/TCRVβ8 cells but not in TCRVβ+/TCRVβ5+ cells (data not shown). These data indicated that TCRVβ8 was the preferred repertoire of iNKT cells in Va14-Ja18 mice. In contrast, only ~20% of TCRVβ+/TCRVβ8- cells were iNKT cells in the Va14-Ja18/CD1d−/− mice (Fig. 4c, lower panel).

Decreased further rearrangement in immature iNKT cells relative to the conventional T cells at the DP stage

We next examined whether there was a difference in further rearrangement between immature iNKT and the immature conventional T cells. Rag2 expression was examined with RT-PCR using TCRVβmed/TCRVβ8med and TCRVβmed/TCRVβ8 populations, both of which represent DP cells. A population stained with αGalCer-CD1d dimers in DP is considered as immature iNKT cells under maturation, while cells not stained with this reagent are considered as the conventional T cells under positive selection. RT-PCR analysis revealed that immature iNKT cells expressed less Rag2 than the conventional T cells in both TCRVβmed/TCRVβ8med and TCRVβmed/TCRVβ8 populations (Fig. 5a). As an internal control, there was no difference in the expression of Myd118 and Hprt between αGalCer-CD1d dimer-reactive and nonreactive cells (Fig. 5a).
No vigorous proliferation and no massive apoptosis in the Val14-Jα18 mouse thymocytes

Because the above data indicated that immature iNKT cells less frequently perform further rearrangement relative to the conventional immature T cells, it was necessary to examine whether a particular subset of the thymocytes proliferated more vigorously than the others, and/or whether there was massive apoptosis to give grounds for the preponderance of iNKT cells in the thymus. We, therefore, have examined the cell cycle status of the thymocytes. When TCRβ\textsuperscript{high}/TCRβ\textsuperscript{low} cells (mature T cells) from the control mouse thymocytes were stained with propidium iodide, >98% of cells were in the G\textsubscript{0}/G\textsubscript{1} phase (Fig. 5b, 1, control). This was also true for TCRβ\textsuperscript{high}/TCRβ\textsuperscript{low} cells (Fig. 5b, 4). In TCRβ\textsuperscript{med}/TCRβ\textsuperscript{med} cells that represent the immature T cells, ~3% of cells were actively cycling (Fig. 5b, 2). On the contrary, >9% of TCRβ\textsuperscript{high} cells (most of which represent DN cells) were cycling (Fig. 5b, 3). In Val14-Jα18 mice, the proportion of the cycling cells was almost equivalent to that of the control for each subset except TCRβ\textsuperscript{high}/TCRβ\textsuperscript{low} cells in which >15% were actively cycling (Fig. 5b, 3, Val14-Jα18). This may reflect the fact that the absolute number of DN cells increased ~2-fold in Val14-Jα18 mice (Fig. 2b and Table I). The analyses revealed that no subset of the thymocytes proliferated more vigorously than the others within the Val14-Jα18 mouse thymocytes concomitant with no extensive apoptosis (Fig. 5b, Val14-Jα18).

Discussion

The Val14-Jα18 mouse as a novel model to study the function of iNKT cells

Our present data showed that Val14-Jα18 locus has a large impact on the development of T lymphocytes in the thymus accompanying a significant increase in iNKT cells (Fig. 2a). Cytokine production experiments suggested that the function of DC remained intact in Val14-Jα18 mice. Analysis of DC markers such as CD40, CD80, CD86, CD1d, and I-A\textsuperscript{d} on DC revealed no significant difference in their expression profiles relative to the control mice (data not shown). From these data, we could conclude that the functions of iNKT cells in Val14-Jα18 mice mimic those found in the control mouse. Thus, Val14-Jα18 mice could represent a novel model to study further the roles of iNKT cells in the immune system.

The Val14-Jα18 mouse to study the development mode of iNKT cells

In line with the assumption that iNKT cells pass through DP thymocytes, the recent fate-mapping study has demonstrated that indeed these cells derive from DP (21). Nonetheless, there remains a fundamental question as to how iNKT cells develop in the thymus. The identification of immature iNKT cells in Val14-Jα18/CD1d\textsuperscript{−/−} mice underpinned the phenotype described in the other studies (Fig. 4b) (18, 19). CD1d may be responsible for the expression of NK1.1, CD44, and CD69, and for down-regulation of CD24 (compare Figs. 2c and 4b). The fact that iNKT precursor cells undergo extensive proliferation during their maturation suggests that CD1d is required for iNKT cell survival/proliferation (10, 18). Alternatively, it may serve as an extrinsic stimulus that instructs the development of iNKT cells.

Extrinsic factors can impact on the consequence of fate decision by multipotent progenitors through two distinct mechanisms. In a permissive mechanism, the cell fate decision of progenitors to differentiate into a particular lineage is made independent of extrinsic factors, and their raison-d’être is to support the survival and proliferation of the committed cells. On the contrary, in an instructive mechanism, extrinsic factors impose upon the progenitors to select one lineage at the expense of others (22). The intensive scrutiny in the neural crest stem cells has demonstrated that neural cell fate is instructively determined by the extrinsic factors such as bone marrow protein 2, neuregulin, TGFβ, and ciliary neurotrophic factor (23–25).

In lymphopoiesis, AgR signals play a pivotal role in the development of lymphocytes. However, the relative contribution of the above two mechanisms in determining the fate of progenitors has been a matter of debate. It is proposed that formation of the pre-TCR complex composed of TCRβ together with pTα and CD3ε instructively induces αβT cells over γδT cells, based on the fact that pTα knockout mice contain less in-frame rearranged TCRβ-harboring cells relative to the control (17). Nonetheless, it can be interpreted that such a pre-TCR complex formation merely supports the survival and/or proliferation of the committed αβT cells, indicating the permissive role for the above pre-TCR complex. Another report has suggested that the αβ-γδT cell lineage diversification occurs well before the TCR rearrangement, rather supporting the permissive role for TCR signals in the αβT cell lineage commitment (26).

Similarly, the mode of the cell fate decision to be helper (CD4) or cytotoxic (CD8) T cells has also been a long-standing dispute. Transgenic expression of CD8 substantially improves MHC class I-specific CD8 selection while affecting little on class II-specific CD4 selection, arguing that the CD8 cell lineage commitment is instructively determined (27). Subsequent study on MHC class I knockout mice revealed that indeed there are no CD8 cells. However, reintroduction of CD8 using the transgene into such mice restored CD8 cells and vice versa (28, 29). These studies simply denote that the lineage choice to be CD4 or CD8 cells is not dictated by CD4 or CD8 per se; it conferred room for an interpretation that signals elicited from these molecules permissively contribute to the survival and/or proliferation of the committed CD4 or CD8 cells. These seemingly conflicting interpretations stem from the fact that transgenic expression or ablation of the signaling molecules tend to support their permissive action on the cell fate decision through providing or depriving of survival signals.

Another caveat to address the above issue is that the lymphocyte progenitors proliferate more vigorously than the neural progenitors. Because the presence of precommitted and committed cells is a prerequisite for addressing the issue, such a propensity often blunts the interpretation. It is thus imperative to examine whether the lineage precommitted cells already predominate the lymphocytes early in development. Hitherto, due to lack of appropriate assays, it has not been possible to address properly whether the AgR signals instructively or permissively affect the destiny of the progenitor cells.

The analysis on the Val14-Jα18 mouse thymocytes revealed that iNKT cells comprise CD1-interacted and CD1d-noninteracted cells as evidenced by the presence of CD24\textsuperscript{high}/NK1.1\textsuperscript{−/−} and CD24\textsuperscript{high}/NK1.1\textsuperscript{−/−}CD44\textsuperscript{low} cells, respectively (Figs. 2c, 4b, and 6, a and b). This antecedent allows us to investigate further whether the development of iNKT cell is determined instructively or permissively by CD1d through the interaction with the Val14-Jα18TCR. Our present data rather support the former possibility based on the following observations. 1) The preponderance of iNKT cells in the thymus (Fig. 2a). 2) The CD1d-dependent preferential association of Val14-Jα18TCR with Vβ8 (Fig. 4c). 3) Immature iNKT cells found in
The commitment is over upon CD1d
/H11002
CTLs that happened to express the V
/H9251
tone). Permissive model (Tc) to commit to be iNKT cells at the expense of the Th and CTLs (half
CD1d-reacted iNKT cells are present in the V
middle panel
decked. Half-tone text shows that the commitment has not yet com-

In the control thymocytes, a few CD1d-nonreacted immature iNKT are
acted cells over the CD1d-reacted cells is different in each thymocyte.

CD1d-nonreacted (half tone text and open circle) and
CD1d-reacted (plain text and shaded circle) iNKT cells in the thymocytes. CD1d-nonreacted (half tone text and open circle) and
CD1d-reacted (plain text and shaded circle) iNKT cells in the thymocytes are shown. However, the relative abundance of the CD1d-nonreacted
cells over the CD1d-reacted cells is different in each thymocyte. In the control thymocytes, a few CD1d-nonreacted immature iNKT are
present (left panel), while the Vα14-Jα18 thymocytes consist of CD1d-
reacted and CD1d-reacted iNKT cells (middle panel). In contrast, no
CD1d-reacted iNKT cells are present in the Vα14-Jα18/CD1d
mouse thymocytes (right panel). Note that the number of CD1d-nonreacted and of CD1d-reacted iNKT cells depicted in the figure does not
necessarily reflect the real number of the each subset. c, Instructive vs permissive cell commitment. Instructive model (left): The (Vα14-
Jα18TCR)-CD1d signal instructs the multipotent progenitors (NKT/Th/
Tc) to commit to be iNKT cells at the expense of the Th and CTLs (half
tone). Permissive model (right): In contrast, the (Vα14-Jα18TCR)-
CD1d signal solely supports the survival and proliferation of the com-
nitted iNKT cells. Note that the commitment of iNKT cells from the
multipotent progenitors is determined independent of the (Vα14-
Jα18TCR)-CD1d signal. In this case, simultaneously generated Th and
CTLs that happened to express the Vα14-Jα18 TCR should be deleted by apoptosis, or they should alter Vα14-Jα18 TCR to change their fate
by operating recombination machinery to give ground for the predom-
nance of the thymic iNKT cells upon (Vα14-Jα18 TCR)-CD1d signal.

DP express less Rag2 relative to the conventional T cells under
positive selection (Fig. 5a). 4) Few subsets of the thymocytes
proliferate more vigorously than the others, and show a massive
apoptosis (Fig. 5b). These data are in line with the instructive
mode of iNKT cell development in that upon interaction with
CD1d, immature iNKT cells obey their fate to be iNKT cells
without striving to change the TCRα repertoire by operating the
recombination machinery, and without dying massively by ap-
optosis (Fig. 5c). Although further study has yet to be per-
formed to rigorously prove the above hypothesis, the Vα14-
Jα18 mouse is an invaluable model to shed light on the mode of
iNKT cell development.

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