Development and Function of Invariant Natural Killer T Cells Producing TH2- and TH17-Cytokines

Hirosi Watarai1,2*, Etsuko Sekine-Kondo1, Tomokuni Shigeura1, Yasutaka Motomura3, Takuwa Yasuda4, Rumi Satoh5, Hisahiro Yoshida4, Masato Kubo3, Hiroshi Kawamoto5, Haruhiko Koseki6, Masaru Taniguchi1

1 Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan, 2 PRESTO, Japan Science and Technology Agency, Tokyo, Japan, 3 Division of Biotechnology, Research Institute for Biological Science, Tokyo University of Science, Chiba, Japan, 4 Laboratory for Immunogenetics, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan, 5 Laboratory for Lymphocyte Development, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan, 6 Laboratory for Developmental Genetics, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan

Abstract

There is heterogeneity in invariant natural killer T (iNKT) cells based on the expression of CD4 and the IL-17 receptor B (IL-17RB), a receptor for IL-25 which is a key factor in Th2 immunity. However, the development pathway and precise function of these iNKT cell subtypes remain unknown. IL-17RB+ iNKT cells are present in the thymic CD4+/− /CD8− NK1.1+ population and develop normally even in the absence of IL-15, which is required for maturation and homeostasis of IL-17RB− iNKT cells producing IFN-γ. These results suggest that iNKT cells contain at least two subtypes, IL-17RB+ and IL-17RB− subtypes. The IL-17RB+ iNKT subtypes can be further divided into two subtypes on the basis of CD4 expression both in the thymus and in the periphery. CD4+ IL-17RB+ iNKT cells produce Th1 (IL-13), Th9 (IL-9 and IL-10), and Th17 (IL-17A and IL-22) cytokines in response to IL-25 in an E4BP4-dependent fashion, whereas CD4− IL-17RB+ iNKT cells are a retinoic acid receptor-related orphan receptor (ROR)γt+ subset producing Th17 cytokines upon stimulation with IL-23 in an E4BP4-independent fashion. These IL-17RB+ iNKT cell subtypes are abundantly present in the lung in the steady state and mediate the pathogenesis in virus-induced airway hyperreactivity (AHR). In this study we demonstrated that the IL-17RB+ iNKT cell subsets develop distinct from classical iNKT cell developmental stages in the thymus and play important roles in the pathogenesis of airway diseases.

Introduction

Natural killer T (NKT) cells, unlike conventional T cells bearing diverse antigen receptors, are characterized by the expression of an invariant T cell receptor (TCR), Vα14Jα18 paired with Vβ8, Vβ7, or Vβ2 in mice [1] and the Vα24Jα18/Vβ11 pair in humans [2,3], that recognizes glycolipid antigens in conjunction with the monomorphic MHC class I-like CD1d molecule [4,5]. Therefore, these cells are termed invariant NKT (iNKT) cells. Another characteristic feature of iNKT cells is their rapid and massive production of a range of cytokines, such as those typically produced by the helper cell Th1 1, Th2, and Th17 cells [6–8], upon stimulation with their ligand, α-Galactosylceramide (α-GalCer) [9,10].

It is speculated that the ability of iNKT cells to produce these various cytokines is due either to the microenvironment in which they undergo priming or to the existence of functionally distinct subtypes of iNKT cells producing different cytokines; however, there is no clear-cut evidence to support the latter notion. It has been reported that iNKT cells include both CD4+ and CD4− subtypes [6,11], each of which produces different cytokines. Human CD4+ iNKT cells produce both Th1 and Th2 cytokines, whereas CD4− iNKT cells produce mainly Th1 cytokines [12,13]. Although such functional differences were originally less apparent in mouse CD4+ and CD4− iNKT cells, two functionally distinct subtypes of NKT cells in the mouse thymus have since been identified based on NK1.1 expression; NK1.1+ iNKT cells produce a large amount of IL-4 and little IFN-γ whereas NK1.1+ iNKT cells produce more effective in mediating tumor rejection than CD4+ iNKT cells in the liver or any other tissues [16].

There is also further heterogeneity of CD4+ iNKT cells in terms of expression of the IL-17 receptor B (IL-17RB), a receptor for IL-25 [17]. IL-25 is a key factor in Th2 immunity, including allergic reactions and airway hyperreactivity (AHR). The CD4+ IL-17RB+ iNKT cells produce large amounts of IL-13 and IL-4 but little IFN-γ in response to IL-25, mediating a key role in IL-25-driven
**T cells are a diverse group of immune cells involved in cell-mediated acquired immunity. One subset of T cells is the innate-like invariant natural killer T (iNKT) cells that recognize glycolipid ligands on target cells instead of peptides. We know that functionally distinct subtypes of iNKT cells are involved in specific pathologies, yet their development, phenotypes, and functions are not well understood. Here, we determine the relationship between various mouse iNKT cell subsets, identify reliable molecular markers for these subsets, and show that these contribute to their functional differences. We identify four iNKT cell subsets that we show arise via different developmental pathways and exhibit different cytokine profiles. Importantly, we show that these subsets can be isolated from the thymus (the organ of all T cells), as well as from peripheral tissues such as spleen, liver, lung, and lymph nodes. Contrary to the general understanding that iNKT cells mature after their exit from the thymus and their migration into peripheral tissues, we conclude that distinct phenotypic and functional IL-17RB cell subsets can be distinguished in the thymus by virtue of the absence of the cytokine receptor IL-17RB and another cell surface molecule called CD4, and these subsets then migrate to peripheral tissues where they retain their phenotypic and functional characteristics. Regarding functional significance, we show that those iNKT cell subsets that lead to airway hyper-responsiveness to respiratory viruses are different to those that lead to allergen-induced airway hyperreactivity, which will enable researchers to focus on specific subsets as potential targets for therapeutic intervention.**

**AHR** [17,18]. Another subset of newly identified iNKT cells within the NK1.1−CD4− subset is the retinoic acid receptor-related orphan receptor (ROR)γt+iNKT cells. These cells can induce autoimmune disorders by their production of IL-17A and IL-22 [8,19], even though IL-17A-producing iNKT cells are absolutely CD1d/α-GalCer dependent.

**Results**

**Identification of Two Distinct Subtypes in iNKT Cells; IL-17RB+ iNKT Cells Producing Tn12 and Tn17 Cytokines and IL-17RB− iNKT Cells Producing IFN-γ**

We previously identified a fraction of splenic CD4+iNKT cells that expresses IL-17RB and produces Tn12 cytokines after treatment with IL-25 [17]. In order to directly analyze the function of IL-17RB on iNKT cells, we generated IL-17RB-deficient mice by the disruption of exon 1 and exon 2 of the Il17rb gene (Figure S1). We then compared the number and function of iNKT cells in the spleen and the liver from Il17rb−/− mice on a C57BL/6 (B6) background to those of wild type (WT) B6 mice. We also included in our comparison Il15Rα−/− mice, in which AHR [17,18]. Another subset of newly identified iNKT cells within the NK1.1−CD4− subset is the retinoic acid receptor-related orphan receptor (ROR)γt+iNKT cells. These cells can induce autoimmune disorders by their production of IL-17A and IL-22 [8,19], even though IL-17A-producing iNKT cells are absolutely CD1d/α-GalCer dependent.

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We then analyzed the frequency of IL-17RB+ subtypes among α-GalCer/CD1d dimer+iNKT cells in the spleen and liver of WT, Il17rb−/−, and Il15Rα−/− mice (Figure 1B). The percentage of IL-17RB+ iNKT cells was increased more than 4 times in the spleen and 10 times in liver of the Il15Rα−/− mice.

By using Il17rb−/− and Il15Rα−/− mice, we further analyzed the iNKT cell subtypes in terms of their ability to produce cytokines (Figure 1C and 1D). α-GalCer/CD1d dimer+ TCRβ+iNKT cells from the spleen of WT, Il17rb−/−, and Il15Rα−/− mice (Figure 1C) and those from the liver of B6 and Il17rb−/− mice (Figure 1D) were sorted and co-cultured with GM-CSF-induced bone marrow derived dendritic cells (BM-DCs) in the presence of α-GalCer. The Il17rb−/− iNKT cells produced normal levels of IFN-γ, but this was significantly decreased in Il15Rα−/− iNKT cells. Intriguingly, there was impaired production of not only Tn12 cytokines such as IL-9, IL-10, and IL-13, but also of Tn17 cytokines IL-17A and IL-22 in Il17rb−/− iNKT cells, but not in Il15Rα−/− iNKT cells in the spleen (Figure 1C and 1D), even though the number of iNKT cells were only slightly decreased in Il17rb−/− (see Figure 1A). The iNKT cells derived from WT, Il17rb−/−, or Il15Rα−/− mice failed to produce any indicated cytokines when co-cultured with BM-DCs from Cdd1l−/− mice (unpublished data), indicating the cytokine production from iNKT cells are absolutely CD1d/α-GalCer dependent.

To examine the functional activity of Il17rb−/− iNKT cells in vivo, we administered α-GalCer (2 μg) intravenously (i.v.) and monitored serum cytokine levels (Figure 1E). The production of IFN-γ peaked normally at 12 to 24 h after stimulation in the Il17rb−/− mice. On the other hand, the production (around 1–6 h) of other cytokines, such as IL-9, IL-10, IL-13, IL-17A, and IL-22, was severely impaired in the Il17rb−/− mice. The results suggest that IL-17RB+ iNKT cells are distinct from IL-17RB− iNKT cells, which mainly produce IFN-γ, and also that IL-17RB+ iNKT cells produce IL-9, IL-10, and IL-13 among Tn12 cytokines and IL-17A and IL-22 Tn17-type cytokines.

**Development of IL-17RB+ and IL-17RB− iNKT Subtypes in the Thymus**

iNKT cells in the spleen and liver from il17rb−/− mice are defective in the production of IL-9, IL-10, IL-13, IL-17A, and IL-22, while IFN-γ production is diminished in Il15Rα−/− iNKT cells (Figure 1). We therefore attempted to identify the origin of IL-17RB+ iNKT cells in the thymus by comparing α-GalCer/CD1d dimer+ iNKT cells in B6 with those in Il17rb−/− and in Il15Rα−/− mice on a B6 background (Figure 2A). The percentage and number of iNKT cells in the thymus were severely decreased in Il15Rα−/− mice to a similar extent as previously reported in Il15−/− mice [21]. By contrast, the percentage and number of iNKT cells in Il17rb−/− mice was only slightly decreased, to a similar extent to...
Figure 1. Function of iNKT cells in the spleen and liver from Il17rb^−/− and Il15^L117P mice. (A) FACS profile of spleen and liver mononuclear cells in WT, Il17rb^−/− and Il15^L117P mice on a B6 background. Numbers are percentage of gated cells. α-GalCer/CD1d dimer^+ iNKT cells and α-GalCer/CD1d dimer^+ NK1.1^+ NK cells were slightly decreased in Il17rb^−/− mice and markedly reduced in Il15^L117P mice. (B) IL-17RB expression in spleen and liver iNKT cells of WT B6, Il17rb^−/− and Il15^L117P mice. Shaded profiles in the histograms indicate the background staining with isotype matched control mAb. (C, D) In vitro cytokine production by spleen iNKT cells from Il17rb^−/− and Il15^L117P mice (C) and by liver iNKT cells from Il17rb^−/− mice (D). Sorted iNKT cells (5 x 10^4/100 μL) from spleen and liver of WT B6 and Il17rb^−/− mice were co-cultured with BM-DCs (5 x 10^3/100 μL) for 48 h in the presence of the indicated doses of α-GalCer. The Il17rb^−/− iNKT cells produced IFN-γ at levels equivalent to WT, while Th2 and Th17 cytokine production, except for IL-4, were severely impaired. (E) iNKT cell-dependent cytokine production in WT B6 and Il17rb^−/− mice in vivo. α-GalCer (2 μg) was i.v. injected and the levels of cytokines in serum were analyzed at the indicated time points. The serum IFN-γ levels were similar in both mice, whereas production of Th2 and Th17 cytokines, except for IL-4, was significantly reduced in the Il17rb^−/− mice. Cytokines were measured by ELISA or a cytometric bead array system at the indicated time points. Data are mean ± SDs from three mice and repeated three times with similar results.

doi:10.1371/journal.pbio.1001255.g001
that seen in the spleen and liver (Figure 1C and 1D). In order to analyze their phenotype precisely, enriched α-GalCer/CD1d dimer† NKT cells were further divided based on the expression of CD44 and NK1.1 (Figure 2B), because NKT cells can be classified into developmental stages based on the cell surface expression of these molecules, i.e., CD44hi NK1.1† (Stage 1), CD44lo NK1.1† (Stage 2), and CD44+ NK1.1† (Stage 3) [14,25]. In agreement with earlier results [21], there was a decrease in the CD44+ NK1.1† (Stage 3) population of α-GalCer/CD1d dimer† NKT cells in the thymus of Il17rb−/− mice. By contrast, the percentage and number of NKT cells in Il17rb−/− mice were reduced, especially in the CD44lo NK1.1† (Stage 1) and CD44+ NK1.1† (Stage 2) populations, although the CD44lo NK1.1† (Stage 3) population was unchanged (Figure 2B). To determine whether the reduction in absolute numbers of developmental Stages 1 and 2 NKT cell populations in Il17rb−/− mice is due to a developmental defect or to bypassing of these developmental stages, we analyzed surface expression of IL-17RB and CD122, a receptor for IL-15 (Figure 2C). Consistent with the observation shown in Figure 2B, IL-17RB expression was detected mainly in the Stage 1 and Stage 2 populations in both CD4+ and CD8+ fractions (Figure 2C), whereas CD122 expression was mainly in the Stage 3 population as previously reported [21], and is inversely correlated with the expression of IL-17RB (Figure 2C). In order to investigate whether IL-17RB+NKT cells are distinct from IL-15-dependent NKT cells, thymic NKT cells from B6 and Il17rb−/− mice were divided based on the expression of IL-17RB and CD4, and were further analyzed in the expression of CD44 and NK1.1 (Figure 2D and 2E). The percentage of CD4+ and CD8+ IL-17RB+NKT cells was higher in Il17rb−/−, mice (Figure 2D), due to the reduction in the numbers of IL-17RB− NKT cells. Concerning the distribution of the expression of CD44 and NK1.1 in NKT cell subtypes, even though IL-17RB+NKT cells comprised only ~10% of the thymic NKT cells, more than half of them were Stage 2, while almost all (>97%) of the CD8+ and CD4+, IL-17RB+NKT cells were Stage 3 (Figure 2E). Furthermore, more than 80% of Stage 1/2 NKT cells were IL-17RB+NKT cells, while only ~2% of the Stage 3 NKT cells were IL-17RB+. The percentage (Figure 2F) and absolute number (Figure 2G) of IL-17RB+NKT cells among the total NKT cells and in developmental Stages 1 and 2 were similar to those of Il17rb−/− mice, while those of IL-17RB− NKT cells (i.e., CD44lo NK1.1+) among the total and in developmental Stage 3 were also comparable to those in Il17rb−/− mice, indicating that two distinct NKT cell subsets are present in the different stages of NKT cell development, i.e., the IL-17RB+ subtype in Stages 1 and 2 and the CD122+ subtype in Stage 3.

In order to determine if IL-17RB+ cell subsets arise as a distinct population in the thymus of each other, each subtype in Stage 1 or Stage 2 was sorted and co-cultured with a fetal thymus (FT) lobe from Jp18−/− mice (Figure 2H and 2I). IL-17RB+ subtype in Stage 1 gave rise to cells in Stage 2 and Stage 3 with IL-17RB− phenotype (Figure 2H and 2I, lower left), whereas IL-17RB+ subtype in Stage 1 gave rise to cells in Stage 2 but not to Stage 3 with IL-17RB+ phenotype (Figure 2H and 2I, upper left). Furthermore, IL-17RB− subtype in Stage 2 gave rise to cells in Stage 3 with IL-17RB+ phenotype (Figure 2H and 2I, lower left), whereas IL-17RB+ subtype in Stage 2 kept in Stage 2 with IL-17RB+ phenotype (Figure 2H and 2I, upper left), indicating that IL-17RB+ NKT cells arise in the thymus as distinct phenotypic subtypes from IL-17RB− NKT cells, which undergo a series of developmental stages (i.e., Stages 1–3) previously characterized [14,15].

To confirm the differences among subtypes of NKT cells, we compared global gene expression profiles in WT B6 CD4+ or CD8+, IL-17RB+ or IL-17RB− NKT cells to each other (Figure S2A), and also WT B6 CD4+ or CD8+, IL-17RB+ NKT cells to the same cell types from Il17rb−/− mice (Figure S2B). The genome-wide expression profile of the CD4+ and CD8+ IL-17RB+NKT cells were similar to each other but different from those of CD4− and CD8−, IL-17RB− NKT cells (Figure S2A). Moreover, the gene expression profiles of CD4− or CD8−, IL-17RB+ WT NKT cells were similar to those in Il17rb−/− mice (Figure S2B). Therefore, it is likely that IL-17RB+NKT cell development in the thymus is distinct from the IL-17RB− (i.e. CD122+) NKT cells. The gene expression profiles of the CD4+ IL-17RB+NKT cells were quite similar to those of the CD4− IL-17RB+ cells rather than the CD4+ or CD8+, IL-17RB− NKT cells (Figure S2A), suggesting that these two subtypes, CD4+ and CD8+, IL-17RB+NKT cells, develop from the same precursors, whereas the precursors for IL-17RB− NKT cells are distinct.

In order to investigate functional differences in the IL-17RB+ and IL-17RB− subsets of NKT cells, we analyzed the ability of thymic NKT cells in B6, Il17rb−/− and Il15−/− mice to produce cytokines in response to α-GalCer (Figure S3). IFN-γ was produced at similar levels by Il17rb−/− and WT NKT cells, but was greatly reduced in the Il15−/− NKT cells, while the production of IL-9, IL-10, IL-13, IL-17A, and IL-22 was impaired in the Il17rb−/− but not in the Il17rb−/− NKT cells, similar to what we had observed in the spleen and liver (Figure S3).

**Dominant Development of IL-17RB+NKT Subtypes in Tg2-Prone BALB/c Mice**

In a previous study, IL-17RB+NKT cells were fairly abundant in the spleen of Tg2-prone mice, but were barely detectable in Tg11-prone mice [17]. Thus, we examined whether the frequency of IL-17RB+NKT cells in the thymus of BALB/c mice is different from that of B6 mice. Intriguingly, more than one-third of thymic NKT cells were IL-17RB+ in Tg2-prone BALB/c mice, four times higher than in Tg11-prone B6 mice (Figure S4A). The genome-wide expression profiles of CD4+ or CD8+, IL-17RB+NKT cells in BALB/c were similar to each other, but different from those of CD4− or CD8+, IL-17RB− NKT cells (Figure S4B). Cluster analysis also showed that CD4+ or CD8+, IL-17RB+NKT cells in B6 and BALB/c mice were essentially equivalent (Figure S4C).

**Genetic Analysis of NKT Cell Subtypes in the Thymus**

NKT cells in the thymus can be divided into four populations based on their expression of CD4 and IL-17RB (Figures 2D and S4A), and thymic Il17rb−/− NKT cells had a decreased ability to produce Tg2 and Tg11 cytokines (Figure S3). Therefore, we analyzed the function of NKT cell subtypes in the thymus of B6 (Figure 3) and BALB/c mice (Figure S5). We first used quantitative real-time PCR to investigate Tg2/Tg2/Tg17-related gene expression patterns in FACS sorted thymic NKT subtypes. The levels of Cd4 and Il17rb transcripts were correlated with the surface expression of these molecules (Figures S3A and S5A). Il2rb (=Cd22) expression was restricted to CD4+ and CD8+, IL-17RB+NKT cell subtypes (Figures S3A and S3A) in correlation with their surface protein expression (Figure 2C). The expression levels of Tg2-related transcripts, such as Ifng, Tbx21, and Stat4, were more than 10 times higher in those of CD4+ and CD8+ IL-17RB+NKT cells. Higher levels of Tg2-related transcripts, such as Il4, were detected in CD4+ IL-17RB+NKT cells, even though Gata3, a transcription factor essential for Tg2 cytokine production, was expressed at a similar level in all subtypes (Figures 3B and
Figure 2. Profile of iNKT cells in the thymus of Il17rb<sup>−/−</sup> and Il15<sup>L117P</sup> mice. (A, B) FACS profiles of thymus (A) and enriched thymic iNKT cells (B) in WT, Il17rb<sup>−/−</sup> and Il15<sup>L117P</sup> mice on a B6 background. (A) α-GalCer/CD1d dimer<sup>+</sup> iNKT cells were slightly decreased in Il17rb<sup>−/−</sup> mice and markedly reduced in Il15<sup>L117P</sup> mice. (B) There was a loss of the NK1.1<sup>+</sup> population in Il17rb<sup>−/−</sup> thymic iNKT cells, while Il15<sup>L117P</sup> thymic iNKT cells showed impairment of the NK1.1<sup>+</sup> population. (C) IL-17RB and CD122 expression by thymic iNKT cell populations of B6 mice. IL-17RB and CD122 expression in CD4<sup>−</sup> and CD4<sup>+</sup> iNKT cells were analyzed. IL-17RB expression was observed in Stages 1/2, while CD122 expression was in the Stage 3 cells. (D, E) Profiles of thymic iNKT cell populations. IL-17RB expression was observed in Stages 1/2, while CD122 expression was in the Stage 3 cells. (D, E) Profiles of thymic iNKT cells in B6 and Il15<sup>L117P</sup> mice showing expression of IL-17RB and CD4<sup>+</sup> (D) and further divided into CD4<sup>+</sup> and NK1.1 subpopulations (E). The percentage of IL-17RB<sup>+</sup> iNKT cell was increased due to the loss of expansion of IL17RB<sup>−</sup> iNKT cells in Il15<sup>L117P</sup> mice. CD4<sup>−</sup> and CD4<sup>+</sup>, IL-17RB<sup>−</sup> iNKT cells were almost all Stage 1 and Stage 2 in both WT B6 and Il15<sup>L117P</sup> mice. On the other hand, the majority of CD4<sup>−</sup> and CD4<sup>+</sup>, IL-17RB<sup>+</sup> iNKT cells were Stage 3 in both WT B6 and Il15<sup>L117P</sup> mice. Loss of expansion of CD4<sup>−</sup> and CD4<sup>+</sup>, IL-17RB<sup>+</sup> iNKT cells was also observed in Il15<sup>L117P</sup> mice. (F, G) Percentage (F) and cell number (G) of the total iNKT cells and the four subtypes (i.e. IL-17RB<sup>−</sup> and CD4<sup>−</sup>, IL-17RB<sup>−</sup> and CD4<sup>+</sup>, IL-17RB<sup>+</sup> and CD4<sup>−</sup>, IL-17RB<sup>+</sup> and CD4<sup>+</sup>) in B6, Il17rb<sup>−/−</sup> and Il15<sup>L117P</sup> mice based on their CD44 and NK1.1 expression patterns. The number of CD4<sup>−</sup> and CD4<sup>+</sup>, IL-17RB<sup>−</sup> iNKT cells was significantly decreased especially in Stage 3 in Il15<sup>L117P</sup> mice compared to WT B6 mice. By contrast, CD4<sup>−</sup> and CD4<sup>+</sup>, IL-17RB<sup>+</sup> iNKT cells in Il15<sup>L117P</sup> mice were present in numbers comparable to WT. Results are representative of
TH2- and TH17-Cytokine-Producing iNKT Cells

those from three independent experiments. (H, l) Development of iNKT subtypes in Stages 1 and 2. Stage 1 and 2 cells in the four iNKT subtypes (i.e. IL-17RB
+ and CD4
+) from WT B6 mice were sorted and cocultured with dGuo treated 15 dpc FT lobes from Jsk18+/− mice (1,000 cells/well). 10 d after culture, cells were recovered and analyzed the surface expression pattern in CD44 versus NK1.1 (H) and CD4 versus IL-17RB (l). IL-17RB
+ precursors gave rise through Stage 2 to Stage 3 cells with IL-17RB
+, while IL-17RB
− subtypes gave rise to Stage 1 cells with IL-17RB
+. Results are representative of those from three independent experiments.

doi:10.1371/journal.pbio.1001255.g002

SSB). On the other hand, the expression of TH17-related transcripts, such as Il17a, Il22, and Ras, were restricted to the CD4
+ IL-17RB
− iNKT cells (Figures 3B and S5B).

We then investigated the gene expression level in cells derived from Stages 1 and 2 by FT organ culture (Figure 2H and 2l). Consistent with the findings above, Ilpg expression was restricted to the cells derived from CD4
− and CD4
+, IL-17RB
− iNKT precursors (Figure S6A and S6B). Higher levels of Il4 were detected in CD4
+ IL-17RB
+ derived cells and restricted expression of Il17a in cells derived from CD4
+ IL-17RB
+ precursors (Figure S6A and S6B), supporting each subtype arise from Stage 1 as a functionally distinct subtype.

Based on these findings, we analyzed potential production of cytokines from these thymic iNKT cell subtypes. Sorted iNKT cell subtypes were stimulated with PMA plus ionomycin (Figure S7A). Similar to the cytokine expression, IFN-γ was exclusively produced by the CD4
+ and CD4
+, IL-17RB
− subtypes, while IL-10 and IL-13 were mainly produced by the CD4
− IL-17RB
+ iNKT cells and IL-17A was produced predominantly by CD4
+ IL-17RB
+ iNKT cells. It should be noted that all four subtypes have a potential to produce IL-4, in correlation with their mRNA expression of Il4 and Gata3 (Figures 3B and S5B).

We then further analyzed cytokine production after α-GalCer activation. Sorted iNKT cell subtypes were co-cultured with BM-DCs in the presence of α-GalCer (Figures S3C and S5C). IFN-γ was exclusively produced by the CD4
− and CD4
+, IL-17RB
− subtypes, while IL-4, IL-9, IL-10, and IL-13 were mainly produced by the CD4
+ IL-17RB
+ iNKT cells. Similarly, IL-17A and IL-22 were produced predominantly by CD4
+ IL-17RB
+ iNKT cells. These cytokine production patterns correlated with their differential expression of Th1/Th12/Th17-related genes in the different iNKT subtypes.

We also analyzed the expression profiles of cytokine receptor genes. Il12b2 transcript was expressed in CD4
− and CD4
+, IL-17RB
− iNKT cells, and Il23r expression was restricted to CD4
− IL-17RB
+ iNKT cells (Figures 3D and S5D), suggesting that CD4
− and CD4
+, IL-17RB
− iNKT cells respond to IL-12 through IL-12Rβ2/IL-12Rβ1, while CD4
+ IL-17RB
+ iNKT cells respond to IL-23 through IL-23R/IL-12Rβ1. In fact, CD4
− and CD4
+, IL-17RB
− iNKT cells produced large amounts of IFN-γ but not Th12 and Th17 cytokines in response to IL-12 (Figures 3E and S5E), while CD4
− IL-17RB
+ iNKT cells produced large amounts of Th17 cytokines, IL-17A and IL-22, but not IFN-γ and Th12 cytokines in response to IL-23 (Figures 3F and S5F). IL-25-mediated activity requires only IL-17RB but also IL-17RA expression [26], which is expressed on all iNKT cell subtypes (Figures 3D and S5D). IL-25 acts on thymic CD4
+ IL-17RB
+ iNKT cells to induce a large amount of Th12 cytokines, along with moderate amounts of Th17 cytokines (Figures S3G and S5G) similar to previous observations in the CD4
+ IL-17RB
+ iNKT cell subtype in the spleen [17]. Interestingly, however, IL-25 does not stimulate CD4
− IL-17RB
+ iNKT cells, despite their expression of IL-17RB (Figures 3G and S5G). We also found that cytokine production from iNKT cells in these experimental settings was hardly observed when BM-DCs derived from Cdlad1+/−/− mice (unpublished data), indicating signals from TCR are also required for cytokine production from iNKT cells. These results suggest

The chemokine receptor expression patterns are also distinct among thymic iNKT cell subtypes. Ccr4 and Ccr7 expression was restricted to both CD4
− and CD4
+, IL-17RB
− iNKT cells, and Ccr6 expression was only observed on CD4
+ IL-17RB
+ iNKT cells (Figures 3H and S5H). Cxcr3 expression was several times higher on IL-17RB
− iNKT cells than on the other subtypes. Surprisingly, the expression of Cxcr6, which has been reported to be abundantly expressed by all iNKT cells [27,28], was also restricted to the IL-17RB
− iNKT cells (Figures 3H and S5H). Note that the expression patterns and levels of all of the genes tested were almost equivalent between B6 and BALB/c mice, consistent with our finding that all iNKT subtypes are present in these strains.

Distribution of iNKT Subtypes in the Periphery

Distinct expression of chemokine receptors among thymic iNKT cell subtypes (Figures 3H and S5H) may reflect the differential distribution of iNKT cell subtypes in the periphery. We thus investigated the frequency of total iNKT cells and subtypes in the spleen, liver, BM, lung, inguinal lymph node (LN), and mesenteric LN in WT B6, BALB/c, and Il17rb−/− mice (Figures 4 and S8). The absolute number and percentage of iNKT cells were slightly decreased in the spleen, lung, inguinal LN, and mesenteric LN of Il17rb−/− mice, but were unchanged compared to WT mice in liver and BMs (Figures 4A and S8A). We then gated on α-GalCer/CD1d dimer+/TCRβ
+ iNKT cells and further analyzed them for the expression of CD44 and NK1.1 in B6 background mice (Figure 4B). The percentage of NK1.1− subcell type was higher in the spleen, lung, inguinal LN, and mesenteric LN, but lower in the liver and BM, and was decreased in Il17rb−/− mice, suggesting that the majority of iNKT cell subtypes maintain surface expression of NK1.1− after emigration from the thymus (Figure 2B). Similarly, we examined the expression of CD4+ and IL-17RB on the iNKT subtypes (Figures 4C and S8B). Interestingly, IL-17RB
+ iNKT cells were abundant in the lung, inguinal LN, and mesenteric LN, but barely detectable in the liver and BM of both B6 and BALB/c mice. More than 40% of iNKT cells were IL-17RB
+ in the lung, inguinal LN, and mesenteric LN, whereas more than 90% were IL-17RB
− in the liver and BM (Figures 4C and S8B). Therefore, the distribution patterns of the iNKT cell subtypes are distinct in the tissues. In agreement with a previous study [21], we found that the number of iNKT cells was decreased in the spleen (~1/3) and liver (~1/30) in Il17rb−/− mice (Figure S9A). Reduction of iNKT cell number was also observed in BM (~1/8) in these mice (Figure S9A), probably due to the selective reduction of the IL-17RB
+ subtypes (Figure S9B). We finally compared iNKT cell subtypes in the thymus and periphery of B6 and BALB/c mice (Figure 4D). The total iNKT cell number was almost equivalent between these two strains, but BALB/c had ~4 times more CD4
+ IL-17RB
+ subtypes, but lower (~1/3) numbers of CD4
− IL-17RB
+ cells, resulting in a higher number of CD4
− IL-17RB
− cells in the spleen (~5 times), lung (~2 times), inguinal LN (~1.5 times), mesenteric LN (~4 times), and lower
numbers of CD4<sup>+</sup> IL-17RB<sup>+</sup> cells, especially in liver (~1/6) and BM (~2/5) of BALB/c mice (Figure 4D).

To confirm the distribution profiles of each subtype in the periphery, we performed intracellular cytokine staining after PMA plus ionomycin stimulation (Figure S7B) and quantitative real-time PCR analysis (Figure S10A-D) on these iNKT cells that were tested in the thymic iNKT cell subtypes (Figures 3A, 3B, 3D, 3H, S7A). The gene expression profiles and potential cytokine production in the iNKT cell subtypes were almost equivalent among those in the different peripheral tissues, but higher than those in the thymus, strongly suggesting that each iNKT subtype in the periphery is derived from the same iNKT subtypes in the thymus.

IL-17RB<sup>+</sup> iNKT Cells as T<sub>H2</sub>/T<sub>H17</sub>-Producing iNKT Subtypes

We next compared global gene expression profiles of CD4<sup>+</sup> or CD4<sup>+</sup> IL-17RB<sup>+</sup> or IL-17RB<sup>+</sup> iNKT subtypes in the thymus and spleen in order to test whether each subtype is functionally and phenotypically stable or plastic. Each of the four subtypes in spleen was highly correlated with the corresponding subtype in the thymus (Figure 5A), suggesting that iNKT subtypes can be divided by CD4 and IL-17RB expression both in the thymus and the

Figure 3. Differential gene expression and cytokine production among thymic iNKT cell subtypes from B6 mice. (A, B, D, H) Quantitative PCR analysis of thymic iNKT subtypes. Thymic iNKT cells further divided into four subtypes based on the expression of CD4 and IL-17RB (red, CD4<sup>+</sup> IL-17RB<sup>+</sup>; orange, CD4<sup>+</sup> IL-17RB<sup>+</sup>; blue, CD4<sup>+</sup> IL-17RB<sup>+</sup>; green, CD4<sup>+</sup> IL-17RB<sup>+</sup>). One representative out of three experiments is shown (mean ± SEM). (A) The purity of the sorted cells was confirmed by the relative Il17rb and Cd4 mRNA expression levels in the respective subtypes. Il2rb (= Cd122) expression was restricted to CD4<sup>+</sup> and CD4<sup>+</sup>, IL-17RB<sup>+</sup> iNKT cells. (B) Expression of T<sub>H1</sub>/T<sub>H2</sub>/T<sub>H17</sub> related genes: T<sub>H1</sub> related: Ifng, Tbx21, and Stat4; T<sub>H2</sub> related: Ifng and Gata3, and T<sub>H17</sub> related: Il17a, Il22 and Rorc transcripts were analyzed. (C) Expression of cytokine receptor genes. Receptor for IL-12, IL-23, and IL-25 were analyzed. The component chains of the various receptors are IL-12 receptor: IL-12R<sub>b2</sub>/IL-12R<sub>b1</sub>; IL-23 receptor: IL-23R/IL-12R<sub>b1</sub>; IL-25 receptor: IL-17RB/IL-17RA. (H) Expression of chemokine receptor genes. Ccr4, Ccr6, Ccr7, Cxcr3, and Cxcr6. (E, F, G) In vitro cytokine production by thymic iNKT cell subtypes (red, CD4<sup>+</sup> IL-17RB<sup>+</sup>; orange, CD4<sup>+</sup> IL-17RB<sup>+</sup>; blue, CD4<sup>+</sup> IL-17RB<sup>+</sup>; green, CD4<sup>+</sup> IL-17RB<sup>+</sup>). Sorted thymic iNKT subtypes (5 × 10<sup>5</sup> cells/100 µL) were co-cultured with BM-DCs (5 × 10<sup>5</sup>/100 µL) for 48 h in the presence of α-GalCer (100 ng/µL) (C), IL-12 (10 ng/µL) (E), IL-23 (10 ng/µL) (F), or IL-25 (10 ng/µL) (G). Levels of IFN-γ, IL-4, IL-9, IL-10, IL-13, IL-17A, and IL-22 were analyzed. The data are representative of three independent experiments (mean ± SEM).
periphery. Furthermore, NKT cell subtypes in the periphery (Figure S10) showed similar quantitative gene expression profiles as in the thymus (Figure 3).

In order to confirm the stability and plasticity of NKT cell subtypes, we sorted thymic NKT cell subtypes based on the expression of CD4 and IL-17RB from WT B6 and transferred them into NKT cell-deficient Il17rb<sup>−/−</sup> mice. Ten days after transfer, we analyzed the IL-17RB expression by NKT cell subtypes in the spleen. The results clearly showed that the majority of transferred cells maintained their surface IL-17RB expression.
Figure 5. Function of iNKT cell subtypes in the spleen. (A) Global gene expression profiles in iNKT subtypes in the thymus and spleen. Tree view representation of clustering analysis among the four iNKT subtypes in thymus and spleen from B6 and BALB/c. The values represent coefficients between the indicated panels. \( r^2 > 0.95 \) in red, 0.85 < \( r^2 < 0.95 \) in orange, and \( r^2 < 0.85 \) in blue. One representative experiment of three is shown. (B) Plasticity and stability of iNKT subtypes. The four iNKT cell subtypes in the thymus were sorted and each subtype \( (5 \times 10^6) \) was i.v. transferred into independent C.18 \( ^{-/-} \) mice \( (n = 3) \). 10 d after transfer, \( \alpha \)-GalCer/CD1d dimer+ TCR\(^{+}\) cells in spleen were analyzed by FACS for the expression of IL-17RB and CD4. Representative data from three experiments are shown. (C–F) In vitro cytokine production by splenic iNKT cell subtypes (red, CD4\(^{+}\) IL-17RB\(^{+}\); orange, CD4\(^{+}\) IL-17RB\(^{-}\); blue, CD4\(^{-}\) IL-17RB\(^{+}\); green, CD4\(^{-}\) IL-17RB\(^{-}\) ). Sorted splenic iNKT subtypes \( (5 \times 10^6 \text{cells/100 \muL}) \) were co-cultured with BM-DCs \( (5 \times 10^5/100 \text{ \muL}) \) for 48 h in the presence of \( \alpha \)-GalCer \( (100 \text{ ng/\muL}) \) (C), IL-12 \( (10 \text{ ng/\muL}) \) (D), IL-23 \( (10 \text{ ng/\muL}) \) (E), and IL-25 \( (10 \text{ ng/\muL}) \) (F). Levels of IFN-\( \gamma \), IL-4, IL-9, IL-10, IL-13, IL-17A, and IL-22 in the supernatants were analyzed by ELISA or CBA. Data are mean ± SD of triplicate wells. One representative experiment of three is shown.

doi:10.1371/journal.pbio.1001255.g005

E4BP4 Is Required for the Production of IL-9, IL-10, IL-13, IL-17A, and IL-22 Cytokines by CD4\(^{+}\) IL-17RB\(^{+}\) iNKT Cells in Response to IL-25

Both thymic and peripheral iNKT cells in the steady state contain E4BP4 mRNA in the CD4\(^{+}\) and CD4\(^{-}\) IL-17RB\(^{+}\) cells \( (Bto21 \text{ expressed, } \text{iNKT-T}_{141}, \text{IL-12 reactive}), \text{and R17a and R22 mRNA in the CD4}^{+} \text{ IL-17RB}^{+} \text{cells (Rom expressed, } \text{iNKT-T}_{1417}, \text{IL-23 reactive).} \) The expression of these cytokine transcripts is thought to result from the fact that peripheral iNKT cells are not truly quiescent, but instead appear to be continuously activated at a low level due to their recognition of endogenous self-glycolipid ligand(s) in vivo. However, the CD4\(^{+}\) IL-17RB\(^{+}\) iNKT cells do not contain I9, I10, I13 (unpublished data), I17a, or R22 mRNA \( (\text{Figures 3B, S3B, S10B}) \) in the steady state, even though these cytokines are immediately produced after activation by \( \alpha \)-GalCer, similar to cases of IFN-\( \gamma \) from IL-17RB\(^{+}\) iNKT cells. These results suggest differences in the transcriptional regulation of cytokine genes in the different iNKT cell subtypes.

One of the candidate genes is E4BP4, a mammalian basic leucine zipper transcription factor that regulates IL-10 and IL-13 production not only by CD4\(^{+}\) T cells and regulatory T cells but also by iNKT cells \[29\]. E4BP4 expression was markedly induced in IL-25-treated iNKT cells, and its expression level correlated with I10 and I13 expression \[29\]. Furthermore, iNKT cells lacking E4bp4 had reduced expression of IL-10 and IL-13 in response to either IL-25 or \( \alpha \)-GalCer stimulation, but the IFN-\( \gamma \) and IL-4 production were unaffected \[29\], indicating that E4bp4 controls the T1\( _{12} \) cytokine production in a particular iNKT cell subtype.

Therefore, we analyzed the role of E4bp4 in iNKT cell subtypes. The expression of E4bp4 was selectively and strongly induced by IL-25 treatment in CD4\(^{+}\) IL-17RB\(^{+}\) iNKT cells both from thymus...
and spleen (Figure 6B). However, CD4\(^+\) IL-17RB\(^+\) NKT cells failed to induce E4bp4 expression even after treatment with IL-25 (Figure 6B), suggesting the cell-type-specific function of E4bp4 and its possible role not only in IL10 and IL13 expression but also in E9, IL17a, and IL22 expression by IL-25-treated CD4\(^+\) IL-17RB\(^+\) NKT cells. To test this hypothesis, we analyzed cytokine production by CD4\(^+\) IL-17RB\(^+\) NKT cells lacking E4bp4 after treatment with IL-25 in the presence of BM-DCs (Figure 6C). The production of IL-9, IL-10, IL-13, IL-17A, and IL-22 cytokines by both thymic or splenic CD4\(^+\) IL-17RB\(^+\) NKT cells in response to IL-25 was completely abrogated, indicating E4BP4 turned out to be an intrinsic regulator of IL-25-mediated production, not only of IL-17A but also of other cytokines.

**Involvement of IL-17RB\(^+\) NKT Cells in the Pathogenesis of Virus-Induced AHR**

We then investigated the role of IL-17RB\(^+\) NKT cells in the pathogenesis of virus-induced AHR, which is known to be different from allergen-induced AHR [30]. Certain viruses, such as respiratory syncytial virus (RSV), Sendai virus, metapneumovirus, and parainfluenza virus, cause childhood asthma and COPD-like symptoms, which include AHR, airway inflammation, and mucus hypersecretion [31–33]. However, it has been very difficult to understand how such symptoms develop, even long after the apparent clearance of viruses. It has been reported that, in mouse models of infection with parainfluenza virus or Sendai virus, virus-induced chronic inflammation leads to asthma that resembles human asthma and COPD [34]. The chronic pulmonary symptoms evolved independently of CD4\(^+\) T cells but required CD4\(^+\) NKT cells and did not occur in Cdld\(^+/-\) and fjx18\(^+/-\) mice [34]. Therefore, we attempted to determine whether or not the CD4\(^+\) IL-17RB\(^+\) NKT cells are responsible for chronic inflammatory lung disease induced by RSV infection. We used the secreted form of recombinant G protein of RSV (rec Gs) (Figure S12) as an immunogen because priming with a recombinant vaccinia virus (rVV) expressing rec Gs induced a more Th2biased response and enhanced pulmonary eosinophil and macrophage infiltration following RSV challenge than did priming with rVV expressing either wild-type G or membrane anchored G (Gm) proteins [35,36]. Mice were inoculated i.n. with RSV (10\(^6\) pfu/100 \(\mu\)l) or PBS as a control four times at 10-d intervals and were intraperitoneally (i.p.) immunized with rec Gs/ alum (50 \(\mu\)g/2 mg) 4 d after the first RSV infection. Three days after the last RSV administration, mice were exposed i.n. to 50 \(\mu\)g rec Gs and then, 24 h later, measured for AHR (Figure 7A). In this experimental setting, RSV/rec Gs-induced AHR was observed in WT BALB/c but not in fjx18\(^-/-\) or Il17b\(^-/-\) mice, which had a similar response level as PBS/rec Gs-induced WT controls, indicating that IL-17RB\(^+\) NKT cells contribute to the development of RSV plus viral antigen-induced AHR (Figure 7B). Airway macrophage and lymphocyte numbers, which were relatively higher than eosinophils and neutrophils, were recruited into the bronchoalveolar lavage (BAL) fluid of RSV/rec Gs-induced WT mice but not the other mice (Figure 7C). These results suggest that IL-17RB\(^+\) NKT cells are required for the development of RSV-induced AHR. Low level of cytokines (IL-4, IL-9, IL-10, IL-13, IL-17A, and IL-22) in the BAL fluid was detected in this experiment (Figure 7D). The production of IL-13 and IL-22, which plays a crucial role in the activation of macrophages and neutrophils, respectively, was detected higher in RSV/rec Gs-induced WT mice. Hematoxylin and cosin (H&E) staining of the lung tissue revealed that a large number of inflammatory mononuclear cells had infiltrated into the peribronchial region, a response that was higher in RSV/rec Gs-induced WT mice compared to RSV/rec Gs-induced fjx18\(^-/-\) or Il17b\(^-/-\) mice (Figure 7E, upper panel). By periodic acid-Schiff (PAS) staining, mucus-producing cells were abundant only in RSV/rec Gs-induced BALB/c mice but not in fjx18\(^-/-\) or Il17b\(^-/-\) mice (Figure 7E, lower panel). To confirm the findings that IL-17RB\(^+\) NKT cells are essential for the development of RSV/rec Gs-induced AHR, we transfected enriched splenic IL-17RB\(^+\) NKT cells into fjx18\(^-/-\) mice and tested their ability to develop AHR (Figure 7F). The cell transfer of IL-17RB\(^+\) NKT cells, but not IL-17RB\(^+\) NKT cells nor PBS alone, restored AHR induced by RSV plus rec Gs, dependent of cell number transferred, demonstrating the important contribution of IL-17RB\(^+\) NKT cells in the pathogenesis of development in virus plus viral antigen-induced AHR.

**Discussion**

In the present study, we identified IL-17RB\(^+\) and IL-17RB\(^-\) subtypes of NKT cells both in the thymus and the periphery. The IL-17RB\(^+\) NKT cells express CD122 (IL-15R\(\beta\) chain), expand in an IL-15-dependent manner, and produce IFN-\(\gamma\) in response to IL-12. On the other hand, the IL-17RB\(^-\) NKT cells do not express CD122 or respond to IL-15. The IL-17RB\(^+\) NKT cells can be further divided into at least two subtypes: (1) CD4\(^+\) IL-17RB\(^+\) NKT cells produce T\(_{h}2\), T\(_{h}9\), and T\(_{h}17\) cytokines in an E4BP4-dependent fashion in response to IL-25, and (2) CD4\(^+\) IL-17RB\(^-\) NKT cells are ROR\(\gamma\)t and produce T\(_{h}17\) cytokines in response to IL-23, but independently of E4BP4. In the thymus, the IL-17RB\(^+\) NKT cells have a developmental pathway distinct from the IL-17RB\(^-\) NKT cells.

It has been proposed that NKT cell differentiation stages can be categorized based on the expression patterns of CD44 and NK.1.

1. for example CD44\(^{hi}\) NK1.1\(^+\) for Stage 1, CD44\(^{hi}\) NK1.1\(^-\) for Stage 2, and CD44\(^{hi}\) NK1.1\(^+\) for Stage 3 [14,25]. However, the majority (>80%) of IL-17RB\(^+\) NKT cells was present in both the Stage 1 and Stage 2 subsets, while IL-17RB\(^-\) NKT cells were enriched in Il17b\(^-/-\) mice and were mainly detected in Stage 3, suggesting that a certain but not all of the Stage 1 and Stage 2 IL-17RB\(^+\) NKT cells are not precursors for the Stage 3 cells. It is believed that NKT cells acquire their ability to produce IL-4 and IL-10, but make little IFN-\(\gamma\) in Stages 1/2 populations, whereas NKT cells in Stage 3 produce abundant IFN-\(\gamma\) but less if any IL-10 [14,15,25,37]. These findings are in agreement with the present results that the Stage 1/2 populations mainly contain IL-17RB\(^+\) NKT cells that can produce IL-4 and IL-10, but not IFN-\(\gamma\), whereas the majority of the Stage 3 NKT cells are IL-17RB\(^-\) NKT cells producing IFN-\(\gamma\) but not T\(_{h}2\) cytokines. The results shown here also indicated that all of the four NKT subtypes already existed in Stage 1 and developed into phenotypically and functionally distinct NKT cells as CD4\(^+\) or CD4\(^-\), IL-17RB\(^+\) in Stage 2 and CD4\(^+\) or CD4\(^-\), IL-17RB\(^-\) through Stage 2 to Stage 3.

It has reported that IL-15 plays an important role in the expansion of NKT cells [21]. Our present data showed that IL-15 requires only for the expansion of IL-17RB\(^+\) NKT cell subtypes but not for IL-17RB\(^-\) NKT cells, even though it has still been unclear that the cytokine(s) are required for the development and expansion of IL-17RB\(^-\) subtypes. In fact, IL-17RB\(^+\) NKT cell subtypes were greatly reduced in number among NKT cell subtypes but already had an ability to produce IFN-\(\gamma\) in Il17b\(^-/-\) mice, resulting in the reduced IFN-\(\gamma\) production after NKT cell activation due to the reduced number of these subtypes.

In the previous reports, the IL-17A-producing subtypes were proposed to be contained within the CD44\(^{hi}\) NK1.1\(^+\) CD4\(^-\)
**Figure 6. Involvement of E4bp4 in cytokine production by CD4+ IL-17RB+ iNKT cells in response to IL-25.** (A, B) Quantitative analysis of Rorc (A) and E4bp4 (B) in iNKT cell subtypes after cytokine treatment. Sorted iNKT cell subtypes (5 x 10⁶/100 μL) from thymus (left) or spleen (right) were co-cultured with BM-DCs (5 x 10⁶/100 μL) in the presence or absence of IL-25 (10 ng/ml) or IL-25 (10 ng/ml) for 24 h. The iNKT cell subtypes were then sorted again and analyzed for expression of the indicated genes by quantitative real-time PCR. The data are representative of three independent experiments (mean ± SEM). (C, D) Cytokine production by CD4+ IL-17RB+ iNKT cells in response to IL-25. Sorted CD4+ IL-17RB+ iNKT cells (5 x 10⁶/100 μL) from thymus (C) or spleen (D) of B6 or E4bp4−/− mice were co-cultured with BM-DCs (5 x 10⁶/100 μL) in the presence of IL-25 (10 ng/ml) for 48 h and then the levels of the indicated cytokines in the tissue culture media were analyzed. iNKT cells from B6 were compared to those from E4bp4−/− mice. **p < 0.01 calculated by t test. The data are representative of three independent experiments (mean ± SD). doi:10.1371/journal.pbio.1001255.g006

RORγt+ subpopulation [8,19]. In the present studies, we found that the CD4+ IL-17RB+ iNKT cell subtype is CD44hi NK1.1+ CD4+ (about 50%-70% of the cells are IL-17RB+) and has a restricted expression of Il17a, Rorc, Cerf6, and Il23r genes, for a phenotype similar to the previously reported CD44hi NK1.1+ CD4+ RORγt+ population that produces IL-17A [18,19]. These results indicate that IL-17RB (and CD4+) is a reliable and specific phenotypic marker for RORγt+ IL-17A-producing iNKT cells in the thymus.

In the periphery, the tissue distribution of the iNKT cell subtypes seems to largely depend on the expression of chemokine receptors: CCR6+, CCR4+, and CCR7+ expression by CD4+ IL-17RB+ iNKT cells, CCR4+CCR7+ expression by CD4+ IL-17RB+ iNKT cells, and CCR3+ CXCR6+ by CD4+ and CD4+, IL-17RB+ iNKT cells. Indeed, the number of liver iNKT cells, the majority of which are the CD4+ and CD4+, IL-17RB+ iNKT cells identified here, depends on the chemokine receptor CXCR6, whereas iNKT cells in other tissues are less dependent as reported [27,28]. In Cerf6−/− mice, the lung has fewer iNKT cells and a corresponding reduction in iNKT cell-mediated AHR [39], implicating the reduction of pulmonary localization of IL-17RB+ iNKT cells.

IL-17A-producing iNKT cells have been described in other studies in the thymus, liver, spleen, lung, LNs, and skin [8,19,20,38,40]. In these studies, it was suggested that all NK1.1+ iNKT cells have the potential to secrete IL-17A. However, in the present study, we show heterogeneity among NK1.1+ iNKT cells. Accordingly, CD4+ but not CD4+, IL-17RB+ iNKT cells correspond to the IL-17A-producing iNKT cells previously reported, as does the exclusive expression of Cerf6 along with Il1r1 (= Cd115) and Il12r1 (= Cd121a) in CD4+ IL-17RB+ iNKT cells (unpublished data) [40].

CD4+ IL-17RB+ iNKT cells produce not only the previously described IL-13 and IL-4 [17,18] but also IL-9 and IL-10 along with IL-17A and IL-22 in response to IL-25 [41]. This IL-9 production is IL-4 independent, highlighting the role of IL-25 in the regulation of both TH2 and TH9 cells [42]. We demonstrated here that IL-25 induces not only IL-13 and IL-4 but also IL-9 and IL-10 from CD4+ IL-17RB+ iNKT cells, which can thus be characterized as iNKT-T1[12] and iNKT-T1[19] cells. Concerning the cytokine production by CD4+ IL-17RB+ iNKT cells in response to IL-25, not only IL-10 and IL-13 but also IL-9,
IL-17A, and IL-22 were attenuated in the absence of E4bp4, recently defined as a transcription factor that regulates IL-10 and IL-13 production by CD4+ T cells and iNKT cells [29], suggesting that E4BP4 also controls IL-25-mediated production of IL-9, IL-17A, and IL-22. Although the precise mechanisms by which IL-25 mediates cytokine expression still remains unclear, E4BP4 itself directly or indirectly controls IL-9, IL-13, IL-17A, and IL-22 expression by genetic/epigenetic regulation in CD4+ IL-17RB+ iNKT subtypes. It will be of interest to determine if E4BP4 regulates IL-9, IL-17A, and IL-22 production by CD4+ TH cells. Taken collectively, our studies indicate that CD4+ or CD4+, IL-17RB+ iNKT cells become functionally stable iNKT-TH17 or iNKT-TH2/9/17, respectively, during their development.

The study described here indicates that iNKT cell-mediated AHR was not induced by viral infections in Jα18−/− or Il17rb−/− mice, suggesting that IL-17RB+ iNKT cells are responsible for the pathogenesis of many different forms of airway inflammation. Although distinct subsets of iNKT cells have been reported to be involved in different forms of asthma [17,18,34,43], they are now consolidated into CD4+ and/or CD4+ IL-17RB+ iNKT cell subsets.

iNKT cells are also known to mediate regulatory functions controlling various pathological conditions, such as infectious diseases caused by microbes [44], autoimmune diseases (colitis, lupus, diabetes) [45,46], atherosclerosis [47], and malignancy [48]. It will be interesting to elucidate whether subsets of iNKT cells...
play differential roles in mediating and controlling these diverse pathological conditions.

**Materials and Methods**

**Mice**

B6 and BALB/c mice were purchased from Charles River Laboratories or Clea Japan, Inc. Il17b-deficient mice were generated as shown in Figure S1 and were backcrossed ≥8 times to B6 or BALB/c mice. Iiβ2.1/17P mutant mice were produced by N-Ethyl-N-nitrosourea (ENU) mutagenesis by ENU administration to male C57BL/6J mice, and their sperm was mated to wild-type eggs and preserved as founder embryos [49,50]. Jfx18-deficient mice were generated as previously described [51] and were backcrossed >10 times to B6 or BALB/c mice. Cdld1-deficient mice [52] were provided by Dr. Luc van Kaer (Nashville, TN). E4bp4-deficient mice were generated as previously described and were backcrossed 8 times to B6 mice [29]. All mice were kept under specific pathogen-free conditions and were used at 8–16 wk of age. All experiments were in accordance with protocols approved by the RIKEN Animal Care and Use Committee.

**Cytokine Measurement**

Cytokines except IL-22 in culture supernatants and BAL fluids were analyzed by cytometric bead array (BD Biosciences) according to the manufacturer’s protocol. IL-22 was quantified using an ELISA reagent set (eBioscience) according to the manufacturer’s protocol.

**Flow Cytometry and Cell Sorting**

Cells were analyzed by FACS Calibur (BD Biosciences) or FACS Canto II (BD Biosciences) and sorted by FACS Aria (BD Biosciences). Antibodies (BD Biosciences or eBioscience) used for staining mouse cells were as follows: FITC or APC-Cy7 anti-TCRβ (H57-597), Pacific blue anti-CD4 (RM4-5), FITC anti-CD44 (IM7), PE-Cy7 anti-NK1.1 (PK136), PE anti-CD122 (TM-β1), FITC anti-CD8α (53-6.7), PerCP-Cy5.5 anti-CD25 (PC61), PE anti-IFN-γ (XMG1.2), PE anti-IL-4 (11B11), PE anti-IL-10 (JES5-16E3), PE anti-IL-13 (eBio13A), PE anti-IL-17A (TC11-18H10), and PE rat IgG1 (A110-1). Biotinylated anti-mouse IL-17RB (B5F6) was generated previously [17] and detected by staining with PE or PE-Cy7 Avidin (BD Biosciences). APC α-GalCer loaded CD1d dimer (BD Biosciences) for iNKT cell enrichment and detection was prepared as previously described [53].

**Coculture with a FT lobe**

The procedures for the coculture with a deoxyguanosine (dGuo)-treated FT lobe under high oxygen submerison conditions have been described in detail previously [54,55]. Basically, single dGuo-treated FT lobes from Jfx18/− mice of B6 background were placed into wells of a 96-well V-bottom plate, to which cells from B6 mice to be examined were added. Culture medium was supplemented with IL-7 (1 ng/ml), IL-15 (10 ng/ml), and soluble IL-15Rα (10 ng/ml). The plates were centrifuged at 150 x g for 5 min at room temperature, placed into a plastic bag (Ohmi Odor Air Service), the air inside was replaced by a gas mixture (70% O2, 25% N2, and 5% CO2), and incubated at 37°C. After 10 d of culture, cells were harvested from each well and analyzed by FACS and quantitative real-time PCR.

**Intracellular Cytokine Staining**

Intracellular cytokine staining was performed as described previously [53]. For cytokine production from sorted iNKT cells, Brefeldin A (Sigma-Aldrich) was added for the last 4 to 5 h of culture to accumulate intracellular cytokines after PMA (25 ng/ml, Sigma) with ionomycin (1 μg/ml, Sigma) treatment. Following fixation with Cytofix/Cytoperm plus (BD Biosciences), cells were stained for indicated intracellular cytokines for 15 min at room temperature.

**Quantitative Real-Time PCR**

PCR primers and probes were designed with Universal ProbeLibrary Assay (Roche) or with TaqMan Gene Expression Assays (Applied Biosystems). Sequence of primers and probes in the latter case are shown in Table S1. PCR was performed with the TaqMan universal master mix with ROX (Applied Biosystems) according to the protocol provided. ABI PRISM7900HT Fast system (Applied Biosystems) or Biomark system (Fluidigm) was used for quantitative real-time PCR according to the manufacturer’s instructions. To ensure the specificity of the amplification products, a melting curve analysis was performed. Results were normalized and analyzed by △Ct or △△Ct methods using the internal control gene Hprt1.

**Correlation Analysis of Microarrays**

Gene expression detected using microarrays was normalized by the quantile normalization method [56]. Pearson’s correlation values of logarithms of all signal intensities from 45,101 probes were calculated, and we performed hierarchical clustering of correlation matrices to indicate the degree of similarity between cell types. Scatter diagrams were drawn to display how similarly or differently genes were expressed in two samples. These diagrams contain only probes whose signals were present and coefficient values were shown in the figures.

**RSV-Induced AHR**

Strain A2 of human RSV was used in this study. The general protocol for analyzing airway remodeling during RSV infection in mice is as follows: AHR was induced by sensitizing and challenging with OVA/alum (3–4 times) and/or infection with RSV (3–4 times), and then challenging with OVA, resulting in the examination of various pathological endpoints as previously described [57–59]. In the present study, we modified these protocols in order to analyze the physiological role of iNKT cells in the development of AHR mediated by RSV. In brief, mice were i.n. administered with RSV (10⁶ pfu) or PBS as a control 4 times at 10-d intervals. Mice were i.p. immunized with rec Gs/α/α (50 μg/2 mg) 4 d after first RSV infections. Three days after the last RSV administration, mice were exposed i.n. to rec Gs recombinant protein and AHR responses were measured 1 d later.

**Measurement of Airway Responsiveness**

Airway function was measured for changes in lung resistance (Rl) and dynamic compliance in response to increasing doses of inhaled methacholine (1.25, 2.5, 5, 10, and 20 mg/ml) by using an invasive FlexiVent (SCIREQ Scientific Respiratory Equipment Inc.).

**Lymphocyte Isolation and Analysis of BAL Fluid**

After measurement of AHR and sacrifice, the mouse trachea was cannulated, the lungs were lavaged twice with 1 ml PBS (10-fold PBS dilution), and the BAL fluid was pooled as previously described [30]. Lymphocytes from thymus, spleen, liver, lung, BM, inguinal LN, and mesenteric LN were isolated as described previously [53].
Statistical Analysis

The statistical significance of differences was determined by t test, analysis of variance (ANOVA), or the Kruskal-Wallis test. The values were expressed as means ± SEM from independent experiments. Any differences with a p value of <0.05 were considered significant (* p<0.05; ** p<0.01).

Supporting Information

Figure S1 Generation of Il17rb−/− mice. (A) Targeting strategy to disrupt the Il17rb gene. Exons 1 and 2 were substituted with a neomycin resistance gene. Neo, neomycin; TK, thymidine kinase. (B) Genomic PCR analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, amplified with primers indicated in (A). Genomic PCR results gave a single 500 bp band for wild-type (+/+), a 300 bp band for homozygous (−/−) and both bands for heterozygous mice (+/−). (TIF)

Figure S2 Global gene expression profile in thymic iNKT subtypes. (A, B) Tree view representation of clustering analysis among the four iNKT subtypes in thymus from WT B6 (A) and between CD4+ or CD4+ IL-17RB+ cells from WT B6 or Il17rb+/- mice (B). The values represent coefficients between the indicated panels. i>0.9 in red and i<0.9 in orange. (TIF)

Figure S3 In vitro cytokine production by thymic iNKT cells from Il17rb−/− and Il17rb+/- mice. Sorted iNKT cells (5×10^5/100 μL) from thymus of WT B6, Il17rb−/−, and Il17rb+/- mice were co-cultured with BM-DCs (5×10^5/100 μL) for 48 h in the presence of indicated doses of α-GalCer. IFN-γ levels from Il17rb−/− iNKT cells were comparable to controls, whereas Th142 and Th1417 cytokine levels were severely impaired. By contrast, IFN-γ from Il17rb+/- iNKT cells was markedly reduced, whereas Th142 and Th1417 cytokine levels remained constant, which is the same outcome as in NKT cells from spleen as shown in Figure 1. (TIF)

Figure S4 Thymic iNKT cell subtypes in BALB/c mice. (A) FACS profile of MACS enriched α-GalCer/CD1d dimer+ TCRβ+ cells from B6 (upper) or BALB/c (lower) mice were further analyzed for the expression of the indicated markers. (B, C) Tree view representation of clustering analysis among the four thymic iNKT cell subtypes in BALB/c mice (B) and in comparison with thymic iNKT cell subtypes in B6 mice (C). The values represent coefficients between indicated panels. i>0.9 in red and 0.9<i<2 in orange. (TIF)

Figure S5 Differential gene expression and cytokine production among thymic iNKT cell subtypes from BALB/c mice. (A, B, D, H) Quantitative RT-PCR analysis of thymic iNKT subtypes. Thymic iNKT cells were further divided into four subtypes based on the expression of IL-17RB and CD4. The results are shown as ΔCt. One representative out of three experiments is shown (mean ± SEM). (A) Purity of sorted cells was high based on the levels of Il17rb and Cd4 mRNA expression. Il2rb (=Cd122) expression was restricted to IL-17RB+NKT cells. (B) Expression of Tq11/Tq22/Tq17 related genes. Tq11: Ifng, Tbx21 and Stat4, Tq22: B4 and Gata3, and Tq17: Iil7a, Iil22 and Iilr4. (D) Expression of cytokine receptor genes. Receptor for IL-12, IL-23, and IL-25 were analyzed. IL-12 receptor consists of IL-12Rβ2/IL-12Rβ1; IL-23 receptor: IL-23R/IL-12Rβ1; IL-25 receptor: IL-17RB/IL-17RA. (H) Expression of chemokine receptor genes. Ccr4, Ccr6, Ccr7, Ccr5, and Cxcr6 were analyzed. (C, E, F, G) Cytokine production by thymic NKT cell subtypes in vitro. Sorted thymic NKT subtypes (5×10^4 cells/100 μL) were co-cultured with BM-DCs (5×10^5/100 μL) for 48 h in the presence of α-GalCer (100 ng/μL) (C), IL-12 (10 ng/μL) (E), IL-23 (10 ng/μL) (F), or IL-25 (10 ng/μL) (G). Levels of IFN-γ, IL-2, IL-9, IL-10, IL-13, IL-17A, and IL-22 were analyzed. The data are representative of three independent experiments (mean ± SEM). (TIF)

Figure S6 Cytokine gene expression in NKT cells after being co-cultured with FT lobes. (A, B) Quantitative RT-PCR analysis of thymic NKT precursors developed from Stage 1 (A) and Stage 2 (B) precursors. Cells shown in Figure 2H and 2I were sorted and analyzed the expression of indicated genes. The results are shown as ΔCt. One representative out of three experiments is shown (mean ± SEM). (TIF)

Figure S7 Potential of cytokine production from NKT cell subtypes. (A, B) The four NKT subtypes (i.e. IL-17RB+/- and CD4+/-) from thymus (A) and spleen (B) were sorted and treated with PMA and ionomycin. Indicated cytokines produced from each subtype were analyzed by intracellular cytokine staining. IFN-γ were highly produced from IL-17RB+ cells, while IL-10, IL-13 were from CD4+ IL-17RB+ subtypes, and IL-17A was from CD4− IL-17RB+. All four subtypes had a potential to produce IL-4. The data are representative of three independent experiments. (TIF)

Figure S8 Peripheral NKT cell subtypes in BALB/c mice. (A, B) FACS profiles of peripheral NKT cells in BALB/c mice. α-GalCer/CD1d dimer+ TCRβ+ NKT cells (A), and NKT subtypes based on the expression of IL-17RB and CD4 (B) in spleen, liver, bone marrow, lung, inguinal LN, and mesenteric LN in WT and Il17rb−/− mice. Numbers indicate percentage of total mononuclear cells (A) and NKT cells (B). (TIF)

Figure S9 Peripheral NKT cell subtypes in Il17rb+/- mice. (A, B) Number of total NKT cells (A) and NKT subtypes based on the expression of IL-17RB and CD4 (B) in WT B6 and Il17rb+/- mice. Both CD4− and CD4+ IL-17RB− NKT cells in Il17rb+/- mice were dramatically reduced in number among all of the tested organs. The majority of NKT cells in liver and BM were IL-17RB− NKT cells, resulting in decreased cell numbers in Il17rb+/- mice. In contrast, both CD4− and CD4+ IL-17RB+ NKT cells in Il17rb+/- mice were present in numbers comparable to WT B6. (TIF)

Figure S10 Quantitative RT-PCR analysis of NKT subtypes in the periphery. (A–D) NKT cell subtypes from the tissues shown in Figure 4B were sorted and the same genes were analyzed as in Figures 3A, 3B, 3D, and 3H. (A) The purity of sorted cells was high based on the respective level of Cd4 and Il17rb mRNA expression. Il2rb (=Cd122) expression was restricted to IL-17RB− NKT cells. (B–D) Expression of Tq11/Tq22/Tq17 related genes (B), cytokine receptor genes (C), and chemokine receptor genes (D). (TIF)

Figure S11 Cytokine production by splenic NKT cell subtypes from BALB/c mice in vitro. (A–D) Sorted splenic NKT subtypes (5×10^4 cells/100 μL) were co-cultured with BM-DCs (5×10^5/100 μL) for 48 h in the presence of α-GalCer (100 ng/μL) (A), IL-12 (10 ng/μL) (B), IL-23 (10 ng/μL) (C), and IL-25 (10 ng/μL) (D). Levels of IFN-γ, IL-2, IL-9, IL-10, IL-13, IL-17A, and IL-22 were analyzed. (TIF)
Figure S12 Expression of recombinant RSV-Gs protein. (A) Schematic representation of RSV-G proteins. Membrane form (Gm, upper) and soluble form (Gs, middle) of RSV-G were shown. Recombinant RSV-Gs protein (rec Gs) was expressed as a fusion with a mouse IL-2 leader sequence and a C-terminal tag (BirA-Gm, upper) and soluble form (Gs, middle) of RSV-G were shown. Schematic representation of RSV-G proteins. Membrane form used in this study.

Table S1 Primers and probes for quantitative real-time PCR used in this study.

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Acknowledgments
We thank S. Inoue, K. Kakimoto, S. Sakata, and Y. Nagata for technical assistance; T. Tashiro and K. Mori for rGalcer synthesis; P. D. Burrows for comments on the manuscript; and N. Takeuchi for secretarial assistance.

Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: HW. Performed the experiments: HW ESK TS. Analyzed the data: HW. Contributed reagents/materials/analysis tools: Provided E4bp4-deficient mice: YM. Provided Il15-mutant mice: TY. HW. Wrote the paper: HW. MT. Built the initial constructs, generated mouse lines of Il17rb-deficient mice: HW. Designed FTOC experiments: HKawamoto. Generated B17rb-deficient embryonic stem cell lines: HKoseki. Supervised the project: MT.
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