A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity

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Airway hypersensitive reaction (AHR) is an animal model for asthma, which is caused or enhanced by environmental factors such as allergen exposure. However, the precise mechanisms that drive AHR remain unclear. We identified a novel subset of natural killer T (NKT) cells that expresses the interleukin 17 receptor B (IL-17RB) for IL-25 (also known as IL-17E) and is essential for the induction of AHR. IL-17RB is preferentially expressed on a fraction of $CD4^+$ NKT cells but not on other splenic leukocyte populations tested. $IL\text{-}17RB^+$ $CD4^+$ NKT cells produce predominantly IL-13 and Th2 chemokines upon stimulation with IL-25 in vitro. $IL\text{-}17RB^+$ NKT cells were detected in the lung, and depletion of $IL\text{-}17RB^+$ NKT cells by IL-17RB–specific monoclonal antibodies or NKT cell–deficient $J\alpha18^{-/-}$ mice failed to develop IL-25–dependent AHR. Cell transfer of $IL\text{-}17RB^+$ but not $IL\text{-}17RB^-$ NKT cells into $J\alpha18^{-/-}$ mice also successfully reconstituted AHR induction. These results strongly suggest that $IL\text{-}17RB^+$ $CD4^+$ NKT cells play a crucial role in the pathogenesis of asthma.

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Airway hypersensitive reaction (AHR) (1) is known to be associated with Th2 cytokines—including IL-4, IL-5, and IL-13—regulating effector functions (2). Indeed, overexpression of these Th2 cytokines results in the development of AHR (3). However, efforts to ameliorate experimental asthma with antibodies against Th2 cytokines have generally proven unsuccessful. Among these, only IL-13 seems to be a key cytokine responsible for goblet cell hyperplasia, airway remodeling, and AHR (4), because inhibition of IL-13 activity, but not that of other Th2 cytokines, by a blocking antibody suppresses both AHR and airway inflammation.

IL-25 (also known as IL-17E), a member of the structurally related IL-17 cytokine family (5–7), has recently been reported to be produced by activated Th2 cells (5) and mast cells (8), resulting in enhancement of AHR (9, 10). Administration of a blocking antibody against IL-25 (11) or IL-25–deficient mice (12) eliminates Th2 responses. Conversely, systemic expression of either human (7) or mouse (11) IL-25, or administration of recombinant IL-25 (5), induces Th2-type immune responses, including increased serum IgE levels, blood eosinophilia, and pathological changes in the lung and other tissues. These findings clearly demonstrate a pivotal role of IL-25 as a mediator of Th2 responses, suggesting that IL-25 lies upstream of the classical Th2 cytokine responses (5, 11).

NKT cells characterized by the expression of an invariant antigen receptor encoded by *Vα14Jα18* in mice or *Vα24Jα18* in humans are also involved in the development of asthma, because NKT cell–deficient $J\alpha18^{-/-}$ mice fail to develop antigen-induced AHR (13). Th2 cells are not always essential for NKT cell–mediated AHR development, because activation of NKT cells induces AHR in the absence of $CD4^+$ T

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cells in *MHC class II*–deficient mice (14). These findings suggest that NKT cells are directly involved in the development of AHR independent of Th2 responses in some conditions. In this report, we investigated the role of IL-25 in NKT cell–dependent AHR induction in mouse models and found that IL-17RB, a receptor for IL-25, was selectively expressed on a fraction of mouse NKT cells, which preferentially produced IL-13 and induced the development of AHR upon stimulation with IL-25.

RESULTS AND DISCUSSION

We first investigated the role of IL-25 in the development of AHR in relation to NKT cells because of previous findings that IL-25 induces Th2-biased responses (5–7). The receptor for IL-25, termed IL-17RB or EVI27/IL-17BR, was originally found to bind IL-17B (15). Interestingly, however, the receptor binds IL-25 with higher affinity than IL-17B. Therefore, this receptor is now termed IL-17RB/IL-25R (15). To identify mouse IL-17RB$^+$ cells, we generated specific mAbs (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20080698/DC1) by immunization with a recombinant IL-17RB–Ig fusion protein (Fig. S2). We first investigated IL-17RB$^+$ cells in the spleen by mouse IL-17RB mAb to confirm the previous findings that IL-17RB expression is detected on a fraction of non–B/non–T (NBNT), c-kit$^+$, FcεRI$^-$ cells in the mesenteric lymph node (Fig. S3), which has identified as IL-4–, IL-5–, IL-13–producing cells in response to IL-25 (12). NKT cells were distinct from the NBNT c-kit$^+$ cells based on the expression of c-kit$^-$, FcεRI$^-$, Vα14-Jα18$^+$ transcripts detected by RT-PCR (Fig. S4). Moreover, IL-17RB was preferentially expressed on a fraction of α-galactosylceramide (α-GalCer)/CD1d dimer$^+$ NKT cells but not on other cell types, including NBNT c-kit$^+$ cells, CD4$^+$ T cells, CD8$^+$ T cells, $\gamma\delta^+$ T cells, CD19$^+$ B cells, CD11c$^+$ DCs, DX5$^+$ NK cells, or noninvariant NKT cells in the spleen (Fig. 1 A).

The preferential expression of IL-17RB in a fraction of NKT cells raises a question about the phenotypic and functional

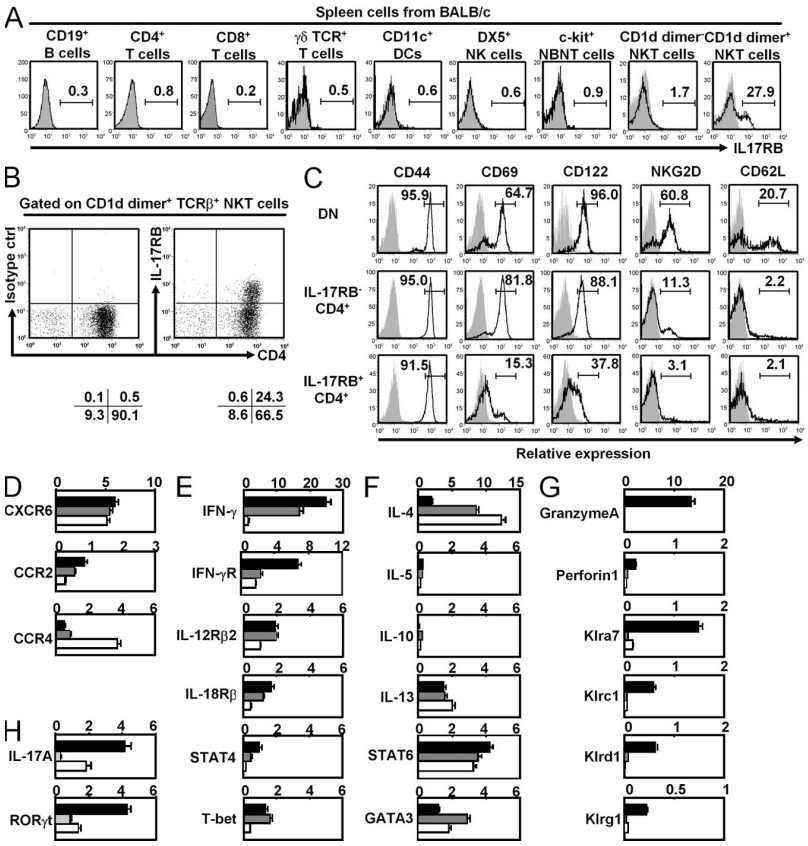

Figure 1. Phenotypic and gene expression profiles of splenic IL-17RB$^+$ NKT cells. (A) Identification of IL-17RB$^+$ cells from BALB/c mice. The indicated populations were gated and analyzed by FACS using F(ab')$_2$ fragments of the anti–IL-17RB mAb B5F6. Shaded profiles in the histograms indicate the background staining with rat F(ab')$_2$ IgG2a. (B) FACS profile of CD1d dimer$^+$ NKT cells stained with anti-CD4 and anti–IL-17RB. (C) Surface phenotype of DN, IL-17RB$^-$ CD4$^+$, and IL-17RB$^+$ CD4$^+$ NKT cells indicated in B. Shaded profiles in the histograms indicate the background staining with isotype-matched control antibody. Percentages are shown. (D–H) Quantitative analyses of genes for chemokine receptors (D), Th1-related molecules (E), Th2-related molecules (F), cytotoxic molecules (G), and Th17-related molecules (H) in isolated DN (black bars), IL-17RB$^-$ CD4$^+$ (gray bars), and IL-17RB$^+$ CD4$^+$ (white bars) NKT cells. Expression of each mRNA was determined by quantitative real-time PCR using the primer sets shown in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20080698/DC1) and is depicted as the number of transcripts per one copy of the housekeeping gene HPRT. One representative out of three experiments is shown (means ± SEM).

characteristics of these cells. Based on our current understanding, NKT cells are divided into two populations based on CD4/CD8 expression: a $CD4^+$ and a $CD4^-CD8^-$ double-negative (DN) population (16). Using an IL-17RB mAb, we found that one third of the $CD4^+$ but none of the DN NKT cells expressed IL-17RB, indicating the existence of three subpopulations: DN, IL-17RB$^-$ $CD4^+$, and IL-17RB$^+$ $CD4^+$ NKT cells (Fig. 1 B). Moreover, the majority of IL-17RB$^+$ NKT cells were dimly positive for CD69 and CD122 compared with brightly positive DN NKT and IL-17RB$^-$ $CD4^+$ NKT cells (Fig. 1 C). The expression level of CD62L by IL-17RB$^+$ NKT cells was low, comparable to that on IL-17RB$^-$ $CD4^+$ NKT cells and similar to that of conventional memory T cells, whereas it was quite high on DN NKT cells. In addition, IL-17RB$^+$ NKT cells (3%), like IL-17RB$^-$ $CD4^+$ NKT cells (11%), barely expressed an NK receptor, NKG2D, which was highly expressed on the majority (>60%) of DN NKT cells, suggesting their cytotoxic nature (17).

To investigate cytokine and chemokine gene expression profiles on IL-17RB$^+$ NKT cells, we performed quantitative real-time PCR using primer sets as shown in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20080698/DC1). The expression of the Th2 chemokine receptor *CCR4* was several times higher on IL-17RB$^+$ NKT cells than on the other subsets, whereas no significant differences were found in *CXCR6* expression (Fig. 1 D), which is important for NKT cell migration (18). The results are consistent with the previous findings that NKT cells require CCR4 to localize to the airways and to induce AHR (19).

Concerning cytokine production of IL-17RB$^+$ NKT cells, it is reported that NKT cells produce both Th1 and Th2 cytokines at the same time upon stimulation with their ligand, α-GalCer (20). Surprisingly, IL-17RB$^+$ NKT cells expressed lower levels of Th1-related transcripts, such as *IFN-γ*, *T-bet*, *Stat4*, *IL-18Rβ*, and *IL-12Rβ2* (Fig. 1 E), whereas higher levels of the Th2-related transcript *IL-4* were detected (Fig. 1 F). In contrast, transcripts for cytotoxic effector molecules, such as *Granzyme*, *Perforin*, and *killer receptors* (*KIRa1*, *KIRc1*, *KIRd1*, and *KIRg1)*, were expressed mainly in DN NKT cells (Fig. 1 G), supporting the previous findings that DN NKT but not $CD4^+$ NKT cells predominantly mediate antitumor immunity (19) and also that NKG2D was predominantly expressed on DN NKT cells, as shown in Fig. 1 C. The expression levels of *IL-17A* and *RORγt* transcripts in IL-17RB$^+$ NKT cells, which are high in Th17 cells, were lower than those in DN NKT cells (Fig. 1 H). These results on surface phenotypes and mRNA expression profiles clearly indicate that IL-17RB$^+$ NKT cells are Th2-type NKT cells and are distinct from other NKT cells, such as DN NKT cells or IL-17–producing NKT cells.

Next, we analyzed the function of IL-17RB$^+$ NKT cells in response to IL-25 in vitro. IL-17RB$^+$ NKT but not $CD4^+$ IL-17RB$^-$ nor DN NKT cells responded to IL-25 in a dose-dependent manner only in the presence of APCs (Fig. 2 A), which is similar to previous findings on the requirement of two signals, such as IL-12 and CD1d on APCs for IFN-γ production (21) and for IL-21 production (22), in NKT cell activation. Under these conditions, IL-25–activated IL-17RB$^+$ NKT cells mainly produced IL-13, along with modest production of IL-4, but barely produced IFN-γ (Fig. 2 B). Moreover, IL-17RB$^+$ NKT cells produced Th2 chemokines such as thymus and activation-regulated chemokine/CCL17, macrophage-derived chemokine/CCL22, and C10/CCL6 as well as eosinophil chemotactic factor–L (ECF-L) upon stimulation with IL-25 (Fig. 2 C). These results indicate that IL-25 triggers IL-17RB$^+$ NKT cells to preferentially produce the IL-13, Th2 chemokines, and ECF-L important for recruitment of eosinophils.

Recently, other IL-17 family members, IL-17A or IL-17F, have been shown to be involved in chronic inflammatory and allergic lung diseases (23, 24). The expression levels of *IL-17A* mRNA in IL-17RB$^+$ NKT cells were slightly elevated after treatment with IL-25 (Fig. 2 C). However, levels of *IL-17A* and *RORγt* mRNA in IL-17RB$^+$ NKT cells were lower than those in DN NKT cells (Fig. 1 H). Because high expression of *RORγt* is one of the markers for Th17 cells, low expression of *RORγt* in IL-17RB$^+$ NKT cells (Fig. 1 H) indicates that IL-17RB$^+$ NKT cells are distinct from IL-17–producing NKT cells. In addition, even though NK1.1-negative NKT cells represent IL-17–producing cells in C57BL/6 mice (25, 26), it

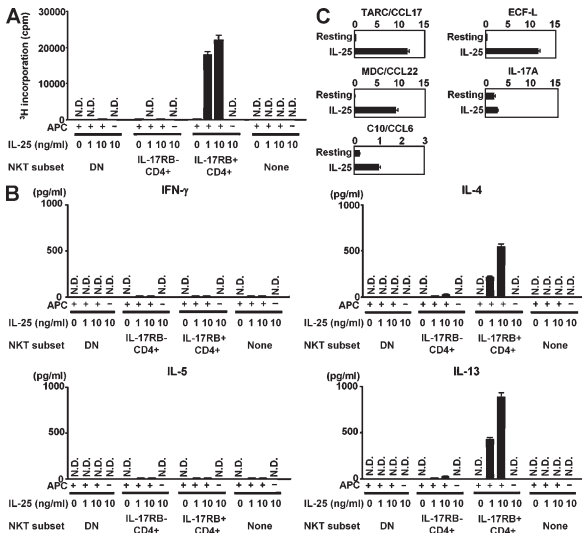

Figure 2. Properties of splenic IL-17RB$^+$ $CD4^+$ NKT cells. (A and B) Proliferation (A) and cytokine production (B) of DN, IL-17RB$^-$ $CD4^+$, and IL-17RB$^+$ $CD4^+$ NKT cells. Indicated NKT cell subpopulations (10^6 cells/ml) were co-cultured for 3 d with or without splenic $CD11c^+$ DCs (10^5 cells/ml) in the absence or presence of IL-25 (0, 1, and 10 ng/ml), and proliferation was assayed by [^{3}H]thymidine incorporation (A) or cytokine production by cytokine bead array (B). The data are shown as the means ± SD of three cultures. The data are representative of four independent experiments. (C) Quantitative analysis of genes in IL-17RB$^+$ $CD4^+$ NKT cells. IL-17RB$^+$ $CD4^+$ NKT cells (10^6 cells/ml) were co-cultured with splenic $CD11c^+$ DCs (10^5 cells/ml) in the presence of 10 ng/ml IL-25 for 48 h. IL-17RB$^+$ $CD4^+$ NKT cells were sorted and analyzed for their gene expression by quantitative real-time PCR, as described in Materials and methods. The data are representative of three independent experiments (means ± SEM).

has also been reported that IL-17–producing NKT cells are not restricted to a particular NKT cell subset (27). Another report has also revealed that ozone- but not allergen-induced AHR requires IL-17A produced from both NKT and T cells (28). These results suggest that IL-17RB$^+$ NKT cells described in this report are not equivalent to IL-17A–producing NKT cells reported by others.

We further examined whether the frequency of IL-17RB$^+$ NKT cells differs among mouse strains because allergic responses are strain dependent. Intriguingly, IL-17RB$^+$ NKT cells were fairly abundant in Th2-prone BALB/c and DBA2/cr mice but were barely detectable in Th1-prone C57BL/6 and C3H/HeN mice (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20080698/DC1). We also found that IL-17RB$^+$ NKT cells were detected more in the lung and spleen than the thymus but were almost undetectable in the liver. Although the number of NKT cells in the lung was one tenth of that in the spleen or the thymus, IL-17RB$^+$ NKT cells make up a higher proportion in the total NKT cells in the lung (Fig. 3 A). Similar to the splenic NKT cells shown in Fig. 1 A, the selective expression of IL-17RB on NKT cells was also detected in the lung, whereas conventional CD4$^+$ T, γδ$^+$ T, c-kit$^+$ NBNT, and noninvariant NKT cells in the lung were negative or barely detectable (Fig. 3 B).

To determine whether IL-17RB$^+$ NKT cells are required for IL-25 in the development of AHR, WT mice were

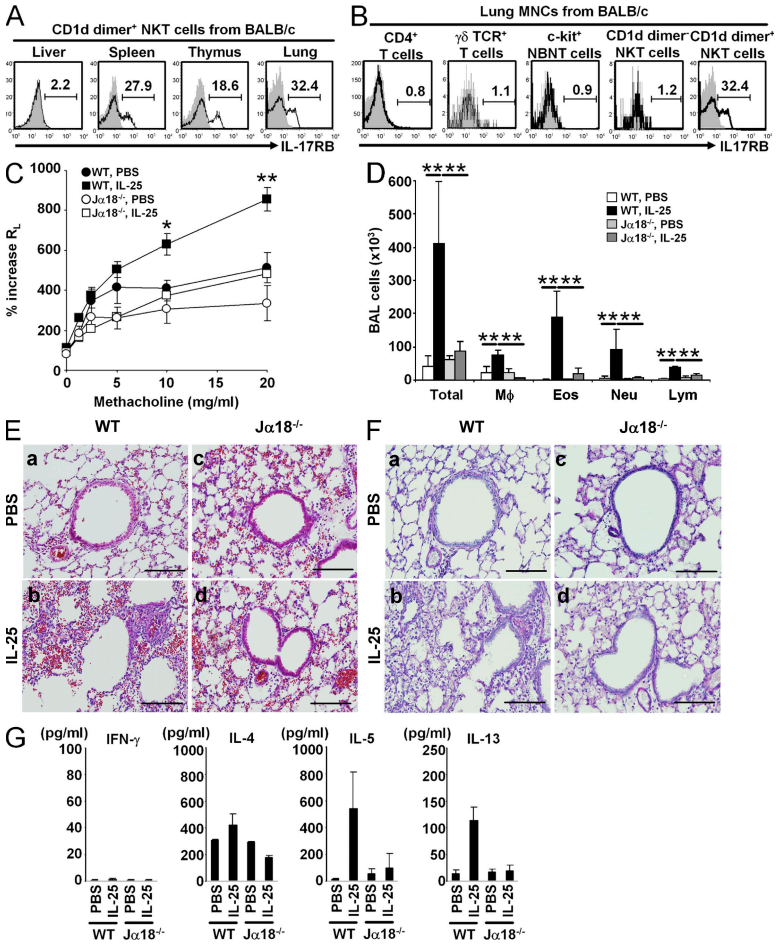

Figure 3. Involvement of IL-17RB$^+$ NKT cells in the development of IL-25–induced AHR. (A and B) Tissue distribution of IL-17RB$^+$ NKT cells (A) and IL-17RB expression among lung mononuclear cell populations (B) in BALB/c mice. α-GalCer/CD1d dimer$^+$ NKT cells in the indicated organs (A) and mononuclear cell populations in the lung (B) from BALB/c mice were gated and analyzed by FACS using B5F6 F(ab')$_2$ IL-17RB mAb. Shaded profiles in the histograms indicate the background staining with rat F(ab')$_2$ IgG2a (n = 3). Percentages are shown. (C) Development of AHR. The changes in R_L were measured. The detailed method for development of OVA/IL-25–induced AHR is described in Materials and methods. Results are expressed as the mean ± SEM. The group of IL-25–treated WT mice was compared with three other groups. *, $P < 0.05$; and **, $P < 0.01$ calculated by ANOVA. The results represent one out of three experiments with five mice in each group. (D and G) Total and differential cell counts (D) and cytokines (G) in BAL fluid. BAL fluid was collected 24 h after challenge with intranasal OVA of the mice depicted in C. IL-25–induced pulmonary inflammation (D) and IL-13 and IL-5 production (G) were reduced in Jα18$^{-/-}$ mice. The data on cytokines in G are expressed as the amounts detected in the 10-fold PBS-diluted BAL samples. Results are expressed as means ± SEM. *, $P < 0.05$; **, $P < 0.01$. The group of IL-25–treated WT mice was compared with three other groups. These results represent one out of four experiments with five mice in each group. (E and F) Histological analysis of lung tissues with hematoxylin and eosin (E) and periodic acid Schiff (F) staining. IL-25–treated WT (c) or Jα18$^{-/-}$ (d) mice were compared with WT (a) or Jα18$^{-/-}$ (b) mice from control (n = 4). Bars,100 μm.

immunized with a suboptimal dose of OVA/alum twice and were subsequently treated with PBS before a single intranasal OVA challenge (see Materials and methods). Under these conditions, the mice failed to develop AHR. When the mice were subsequently treated with IL-25 instead of PBS, they developed AHR (Fig. 3 C). In contrast, even after treatment with IL-25, NKT cell–deficient Jα18$^{-/-}$ mice failed to develop significant AHR, comparable to that seen in the PBS-treated control mice (Fig. 3 C). Unlike WT mice, Jα18$^{-/-}$ mice treated with IL-25 significantly reduced numbers of airway macrophages, eosinophils, neutrophils, and lymphocytes in the lung (Fig. 3 D).

Hematoxylin and eosin staining of the lung tissue of IL-25–treated WT mice revealed that the levels of infiltration of inflammatory mononuclear cells into the peribronchiolar region were higher in WT mice with severe tissue destruction compared with those in Jα18$^{-/-}$ mice. No inflammatory cell infiltration was detected in untreated WT or Jα18$^{-/-}$ mice (Fig. 3 E). By periodic acid Schiff staining, mucus-producing cells were abundant only in IL-25–treated WT but not Jα18$^{-/-}$ mice (Fig. 3 F). To further investigate the effects on allergic responses mediated by IL-17RB$^+$ NKT cells, we examined cytokine production in the bronchoalveolar lavage (BAL) fluid of IL-25–treated WT or Jα18$^{-/-}$ mice and controls. The production of IL-5 and IL-13, which plays a crucial role in the recruitment of eosinophils and Th2 cells, respectively, were detected only in IL-25–treated WT mice (Fig. 3 G). Even though IL-17RB$^+$ NKT cells did not produce IL-5 upon IL-25 stimulation (Fig. 2 B), they produced ECF-L (Fig. 2 C), which is important for the recruitment of eosinophils producing IL-5. These results strongly suggest that IL-25 acts directly on NKT cells and induces AHR.

We then investigated whether IL-17RB$^+$ NKT cells are involved in the development of IL-25–dependent AHR, and we depleted IL-17RB$^+$ cells with 3H8 IL-17RB mAb. Among the NKT cell populations, the majority of IL-17RB$^+$ NKT cells were α-GalCer/CD1d dimer^{hi} TCRβ^{hi}, indicating that the expression level of TCRα and TCRβ on IL-17RB$^+$ NKT cells was higher than that on other IL-17RB$^-$ NKT cells (Fig. 4 A). IL-17RB$^+$ NKT depletion persisted for at least 5 d after 3H8 IL-17RB mAb injection (Fig. 4 B), whereas no effects were detected on other cell types, such as CD4$^+$ T, $\gamma\delta^+$ T, and NBNT c-kit$^+$ cells (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20080698/DC1). As expected, treatment of 3H8 IL-17RB mAb significantly induced suppression of AHR (Fig. 4 C), which was tightly correlated with the reduction of the number of IL-17RB$^+$ NKT cells (Fig. 4 B). Administration of an isotype-matched control mAb did not suppress AHR (Fig. 4 C), and the number of IL-17RB$^+$ NKT cells remained unchanged (Fig. 4 B).

To confirm the findings that IL-17RB$^+$ NKT cells are essential for the development of IL-25–dependent AHR, we transferred enriched splenic IL-17RB$^+$ NKT cells into Jα18$^{-/-}$ mice and tested their ability to develop IL-25–dependent AHR (Fig. 4 D). The cell transfer of IL-17RB$^+$ NKT cells, but not IL-17RB$^-$ NKT cells nor PBS alone, restored AHR induced by OVA plus IL-25, indicating that IL-17RB$^+$ NKT cells in the lung are functionally equivalent to those in the spleen. In addition, when equal numbers of cells were transferred, the severity of AHR induced by enriched IL-17RB$^+$ NKT cells was almost three times higher than the total spleen NKT cells (Fig. 4 D), consistent with the ratio of IL-17RB$^+$ NKT cells (Fig. 1 B). This indicates that the severity of AHR depends on the cell numbers of IL-17RB$^+$ NKT cells transferred (Fig. 4 D) and, thus, that IL-17RB$^+$ NKT cells contribute to the development of IL-25–dependent AHR.

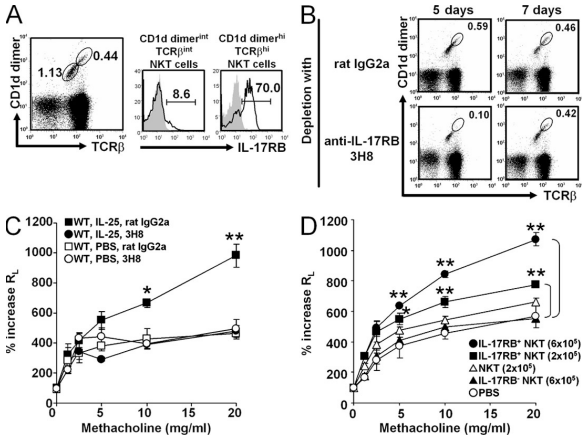

Figure 4. Requirement of IL-17RB$^+$ NKT cells in the development of IL-25–induced AHR. (A) FACS profiles of NKT cells in the expression of IL-17RB. α-GalCer/CD1d dimer^{hi} TCRβ^{hi} NKT cells and α-GalCer/CD1d dimer^{int} TCRβ^{int} NKT cells in the spleen from BALB/c mice were gated, and IL-17RB expression was analyzed using F(ab')$_2$ IL-17RB mAb B5F6. IL-17RB was preferentially expressed in α-GalCer/CD1d dimer^{hi} TCRβ^{hi} NKT cells (n = 3). (B) FACS profiles of NKT cells in mice treated with IL-17RB mAb. α-GalCer/CD1d dimer$^+$ TCRβ^+ NKT cells in the spleen from BALB/c mice at the indicated days after injection of 3H8 IL-17RB mAb or control rat IgG2a (1 mg/mouse) were gated and analyzed by FACS. Depletion of IL-17RB$^+$ NKT cells persisted for at least 5 d after 3H8 injection because of the absence of α-GalCer/CD1d dimer^{hi} TCRβ^{hi} NKT cells in A (n = 2). Percentages are shown. (C) Development of AHR in mice treated with IL-17RB mAb. The detailed method for development of OVA/IL-25–induced AHR is described in Materials and methods. In brief, OVA/alum-sensitized mice were injected with 3H8 IL-17RB mAb or rat IgG2a (1 mg/mouse) 24 h before 2 µg IL-25 or PBS treatment and were challenged with OVA after 24 h, and R_L was determined after 48 h. The group of rat IgG2a–treated WT mice was compared with three other groups. Results are expressed as means ± SEM. The results represent one out of three experiments with five mice in each group. *, $P < 0.05$; and **, $P < 0.01$ calculated by ANOVA. (D) AHR development after cell transfer of spleen IL-17RB$^+$ NKT cells into Jα18$^{-/-}$ mice. The detailed method for development of OVA/IL-25–induced AHR is described in Materials and methods. Indicated cell numbers of sorted IL-17RB$^+$, IL-17RB$^-$ NKT, or total NKT cells from spleen or PBS control were intravenously transferred into OVA/alum-sensitized Jα18$^{-/-}$ mice 24 h before treatment with 3 µg IL-25 and were challenged with OVA after 24 h, and R_L was measured after 48 h. Each group of IL-17RB$^+$ NKT cell–transferred mice was compared with three other groups. *, $P < 0.05$; and **, $P < 0.01$ calculated by the Kruskal-Wallis test. The results represent one out of four experiments with five mice in each group. Results are expressed as means ± SEM.

Although we have not identified the cells producing IL-25 in the present report, it is detected in lung biopsy samples from patients with asthma (29). In addition, IL-25 has been reported to induce inflammatory cytokine and chemokine production by human lung fibroblasts, and components of extracellular matrix by airway smooth muscle cells (29). These reports have also suggested that IL-25 plays a role in human asthma (29). Our findings clearly revealed IL-17RB$^+$ NKT cells as target cells of IL-25 in the development of AHR or asthma. The efficacy with which IL-17RB antibodies prevent AHR and reduce Th2-cytokine–induced inflammation in vivo suggests that IL-17RB is an ideal therapeutic target for asthma.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from Charles River Laboratories or Clea Japan, Inc. Jα18-deficient mice were generated as previously described (30) and were backcrossed >10 times to BALB/c mice. 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.

Generation of mouse IL-17RB–specific mAbs. The IL-17RB–Ig fusion gene was created by fusing the cDNA of the extracellular domain of mouse IL-17RB in frame to the CH2/CH3 domains of human IgG1 in the pIRES2-EGFP expression vector (Clontech Laboratories, Inc.). IL-17RB–Ig was purified from the culture supernatants of transfected HEK293 cells using a protein A–sepharose column (GE Healthcare; Fig. S2). Mouse IL-17RB mAbs were produced by immunizing Wister rats with IL-17RB–Ig. After initial screening by ELISA on IL-17RB–Ig fusion protein, 100 hybridoma clones were further characterized by flow cytometry on IL-17RB transfectants (Fig. S1).

OVA/IL-25–induced AHR model. The original protocol for induction of AHR by OVA sensitization (100 μg three times) and challenge (100 μg OVA/alum three times), as previously described (16), was modified in the present study. In our modified method, mice were intraperitoneally immunized with a suboptimal dose of OVA/alum twice and were subsequently challenged once with intranasal OVA at the same time points as described. In this model, BALB/c mice failed to induce development of AHR, cell infiltration, and histological changes in the lung without IL-25 but induced AHR with intravenous injection of IL-25 (Fig. 3). In brief, mice were intraperitoneally immunized with 50 μg/2 mg OVA/alum twice at a 1-wk interval. 7 d later, mice were treated intravenously with 2 μg/200 μl IL-25 or 200 μl of control PBS at 2 d before intranasal challenge with 50 μg OVA. 24 h later, AHR responses were measured. For transfer of IL-17RB$^+$, IL-17RB$^-$ NKT, or total NKT cells, cells were sorted by a FACSAria (BD). Sorted cells or PBS alone were intravenously injected 1 d before IL-25 treatment (Fig. 4 D). For depletion of IL-17RB$^+$ cells, 1 mg 3H8 IL-17RB mAb was intraperitoneally injected 5 d before AHR measurement (Fig. 4 C).

Measurement of airway responsiveness. Airway function was measured for changes in lung resistance (R_L) 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 death, the lung of the mouse trachea canulated was lavaged twice with 1 ml PBS (~10-fold PBS dilution), and the BAL fluid was pooled as previously described (13). Spleen, blood, and lung lymphocytes were isolated as described previously (31).

Cytokine measurement. BAL fluid and culture supernatants were collected and analyzed by cytometric bead array (BD) according to the manufacturer's protocol.

Flow cytometry. Antibodies used for flow cytometric analysis were as follows: FITC anti–mouse TCRβ (H57-597; BD), Pacific blue anti–mouse CD4 (RM4-5; BD), PerCP-Cy5.5 anti–mouse CD8α (53-6.7; BD), PE anti–mouse CD44 (IM7; BD), PE anti–mouse CD122 (TM-β1; BD), PE anti–mouse CD62L (MEL-14; BD), and PE anti–mouse NKG2D (C7; eBioscience).

Proliferation assay. Proliferation assays were done in 96-well U-bottomed plates. The spleen cell cultures were incubated for 3 d and pulsed with 0.037 MBq/well of [^{3}H]thymidine (GE Healthcare) for the last 16 h. Radioactivity was measured using a MicroBeta (PerkinElmer).

Quantitative real-time PCR. The PCR was performed with the Platinum SYBR Green qPCR SuperMix-UGD kit with ROX (Invitrogen) according to the protocol provided. A sequence detection system (ABI PRISM 7900HT; Applied Biosystems) 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 using the internal control gene HPRT. Sequences of PCR primers (Table S1) were designed with Primer Express software (Applied Biosystems) for optimal product length, germinal center content, and Tm value.

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

Online supplemental material. Fig. S1 shows generation of mouse IL-17RB–specific mAbs. Fig. S2 shows expression and purification of mouse IL-17RB–Ig fusion protein. Fig. S3 shows FACS profiles of cells stained with mouse IL-17RB–specific mAbs. Fig. S4 shows RT-PCR analysis on c-kit$^+$ NBNT cells and NKT cell subsets. Fig. S5 provides IL-17RB$^+$ NKT cells in different mouse strains. Fig. S6 shows FACS analysis on CD4$^+$ T, γδ$^+$ T, and c-kit$^+$ NBNT cells in mice treated with anti–IL-17RB mAb. Table S1 lists primers used for quantitative real-time PCR analysis. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080698/DC1.

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