# Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity

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**Using a model of natural killer T (NKT)-cell deficiency, we show here that allergen-induced airway hyperreactivity (AHR), a cardinal feature of asthma, does not develop in the absence of Vα14*i* NKT cells. The failure of NKT cell–deficient mice to develop AHR is not due to an inability of these mice to produce type 2 T-helper (Th2) responses because NKT cell–deficient mice that are immunized subcutaneously at non-mucosal sites produce normal Th2-biased responses. The failure to develop AHR can be reversed by the adoptive transfer of tetramer-purified NKT cells producing interleukin (IL)-4 and IL-13 to Jα281$^{-/-}$ mice, which lack the invariant T-cell receptor (TCR) of NKT cells, or by the administration of recombinant IL-13 to CD1d-deficient mice, which directly affects airway smooth muscle cells. Thus, pulmonary Vα14*i* NKT cells crucially regulate the development of asthma and Th2-biased respiratory immunity against nominal exogenous antigens. Therapies that target Vα14*i* NKT cells may be clinically effective in limiting the development of AHR and asthma.**

Asthma is a major public health problem that has increased markedly in prevalence in the past two decades[1]. Asthma is caused by Th2-driven inflammatory responses, which enhance airway and peripheral blood eosinophilia, induce AHR and elevate serum IgE[2]. Although Th2-driven immune responses are vitally important in the development of asthma[3,4], in itself a Th2 response is not sufficient to induce asthma. Th2-biased allergen sensitization can occur independently of asthma, which explains why only about a third of individuals with allergic rhinitis (caused by Th2 responses in the upper respiratory tract) develop asthma (caused by Th2 responses in the lower airways)[5].

Additional elements that are intrinsic to the lower respiratory tract may be required for localizing the Th2 response in the lungs, in a process that is distinct from allergen sensitization *per se*, and for the full development of asthma. **[AUTHOR: sentence OK as edited?]** These required specific local elements in the respiratory tract have not been identified but may involve either structural responses of the lower respiratory tract to repeated injury (for example, airway remodeling)[6,7] or immune system components that are localized to the lower respiratory tract.

NKT cells constitute a lymphocyte subpopulation that are abundant in the thymus, spleen, liver and bone marrow and are also present in the lung[8,9]. NKT cells express surface markers that are characteristic of both natural killer cells (such as NK1.1 and CD161) and conventional T cells (such as TCRs). **[AUTHOR: sentence OK as edited?]** Several NKT cells (referred to as Vα14*i* NKT cells) recognize glycolipid antigens presented by the non-polymorphic major histocompatibility complex (MHC) class I–like protein CD1d and express an invariant Vα14-Jα281 (also called Jα18 or Jα15) TCR in mice, or an invariant Vα24-Jα15 TCR in humans[10].

A specific marine sponge glycolipid, α-galactosyl ceramide (α-GalCer), binds to CD1d and is recognized by and activates mouse Vα14*i*$^{+}$ and human Vα24*i*$^{+}$ T cells[11,12], but the specific glycolipid antigens that are normally recognized by NKT cells *in vivo* are not known. When activated, however, NKT cells rapidly produce large quantities of IL-4 and interferon (IFN)-γ, which seem to influence subsequent adaptive immune responses and the polarization of conventional αβ-TCR T cells[13–15]. NKT cells therefore seem to have important regulatory functions, although a clear role for NKT cells in the respiratory tract has not been shown.

In both human and mouse models of type 1 diabetes mellitus, NKT cells regulate the development of disease. In individuals with diabetes mellitus, both the number of NKT cells and their capacity to produce IL-4 have been shown to be reduced[16], although this finding is controversial[17]. In mice, overexpression of NKT cells, adoptive transfer of NKT cell enriched populations and activation of Vα14*i* NKT cells with α-GalCer protects against the development of diabetes[18–21]. In models of experimental autoimmune encephalomyelitis, another Th1-mediated autoimmune disease, activation of Vα14*i* NKT cells with an analog of α-GalCer protects susceptible mice against disease[22].

Although IL-4 production is required for these protective effects to occur, the precise mechanisms by which NKT cells are able to protect against autoimmune inflammatory diseases are

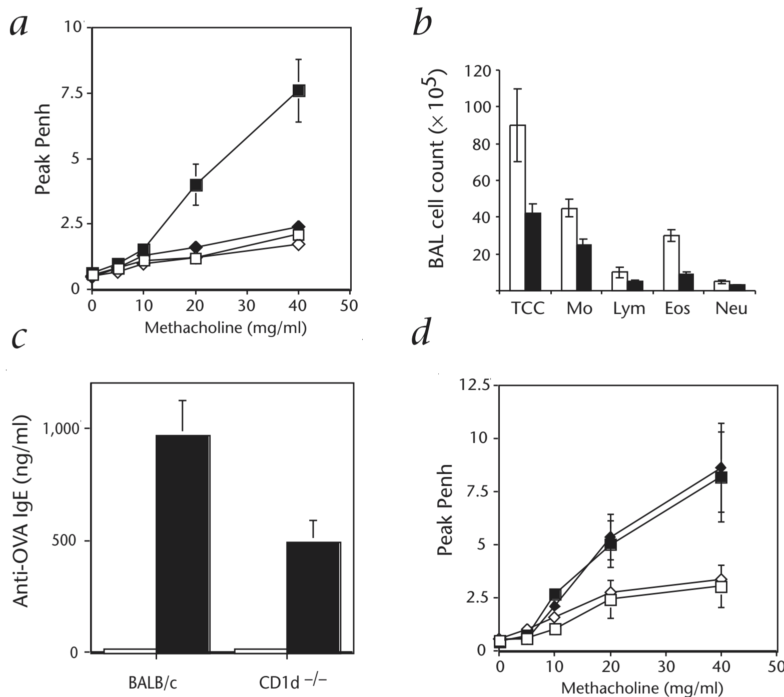


**Fig. 1** Analysis of antigen-induced airway responses in CD1-deficient mice. ***a***, CD1d-deficient mice do not develop antigen-induced AHR. Increasing concentrations of methacholine were used to measure AHR in sensitized mice 1 d after the last challenge with OVA. Data are the mean ± s.e.m. enhanced pause (Penh) values from 4 sensitized mice in each group. ■, OVA-challenged BALB/c; □, PBS-challenged BALB/c; ◆, OVA-challenged CD1d-deficient; ◇, PBS-challenged CD1d-deficient. ***b***, Airway eosinophilia is reduced in CD1d-deficient mice. BAL fluid from the mice in (*a*) was analyzed 24 h after AHR measurement. □, BALB/c; ■, CD1d-deficient. Results are shown as the number of cells per ml in BAL fluid. Eos, eosinophils; Lym, lymphocytes; Mo, monocyte/macrophage; Neu, neutrophils; TCC, total cell count. ***c***, OVA-specific IgE in CD1d-deficient mice. OVA-specific IgE was measured by ELISA in sera from mice sensitized and challenged with OVA (■) or from naive mice (□), 24 h after AHR measurement. **[AUTHOR: sentence OK as edited?]** Data are the mean ± s.d. of 3 mice. ***d***, rIL-13 induces AHR in CD1d-deficient mice. Mice received 2 μg rIL-13 or PBS intranasally daily for 3 days and AHR was measured on day 4. ■, recombinant IL-13–treated BALB/c; □, PBS-treated BALB/c; ◆, rIL-13–treated CD1d-deficient; ◇, PBS-treated CD1d-deficient. **[AUTHOR: please note that legend has been cut and details moved where possible to the Methods section, in accordance with *Nature Medicine* style; please check that meaning is OK]**

not known, particularly because the activation of NKT cells with α-GalCer is required to observe the protective effects of Vα14*i* NKT cells in these models. The production of large quantities of IL-4 by Vα14*i* NKT cells, although associated with protection against Th1-mediated disease, may also exacerbate the development of Th2-biased immune responses by providing a source of IL-4 for Th2 differentiation. However, several studies have not found a role for Vα14*i* NKT cells in enhancing Th2-biased immune responses *in vivo*[23–25], and the activation of Vα14*i* NKT cells has been shown to inhibit Th2 cell differentiation and IgE production[15].

To examine more clearly whether Vα14*i* NKT cells in the lung affect the development of Th2-biased responses in the respiratory tract, we have studied the induction of allergen-induced AHR in the presence or absence of NKT cells in CD1d-deficient and Jα281$^{-/-}$ mouse strains, which both lack Vα14*i* NKT cells. Our studies show that in the absence of Vα14*i* NKT cells, allergen-induced AHR does not develop in either CD1d-deficient or Jα281$^{-/-}$ mice (BALB/c background). We reconstituted AHR in Jα281$^{-/-}$ mice by adoptively transferring Vα14*i* NKT cells isolated with α-GalCer–loaded CD1d tetramers and found that Vα14*i* NKT cells isolated from wild-type mice, but not mice deficient in

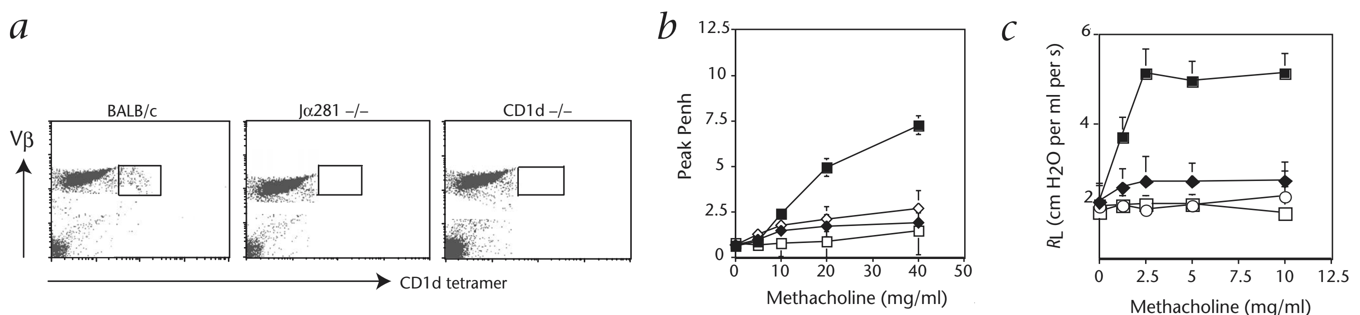


**Fig. 2** Jα281$^{-/-}$ mice do not develop AHR. ***a***, Jα281$^{-/-}$ and CD1d-deficient mice lack CD1d tetramer–staining cells. Spleen cells from naive BALB/c, Jα281$^{-/-}$ and CD1d-deficient mice were stained with α-GalCer–loaded PE-conjugated CD1d tetramers and a monoclonal antibody against Vβ and analyzed for double-positive cells. Neither Jα281$^{-/-}$ nor CD1d-deficient mice contained tetramer-positive cells, whereas ~4% of the BALB/c cells were double positive. **[AUTHOR: sentence OK as edited?]** ***b***, AHR does not develop in Jα281$^{-/-}$ mice. AHR was assessed as described in Fig. 1*a*. **[AUTHOR: OK?]** Data are the mean ± s.e.m. of peak enhanced pause (Penh) values from 5 sensitized mice in each group and are representative of 4 similar experiments. ***c***, NKT cell–deficient mice do not develop AHR, as assessed by invasive measurement of airway resistance (see Methods). **[AUTHOR: OK?]** Results are shown as $R_L$ (top, in cm $H_2O$ per ml per s) and $C_{dyn}$ (bottom, in ml per cm $H_2O$). Data are the mean ± s.e.m. of results from 4 mice in each group. *b* and *c*, ■, OVA-treated Balb/c; □, PBS-treated Balb/c; ◆, OVA-treated Jα281$^{-/-}$ (*b*) or OVA-treated CD1d-deficient (*c*); ◇, PBS-treated Jα281$^{-/-}$ (*b*) or OVA-treated Jα281$^{-/-}$ (*c*).

IL-4 and IL-13, restore AHR. Our studies indicate that the development of AHR after respiratory exposure requires the activation of pulmonary NKT cells that produce IL-4 and IL-13. **[AUTHOR: sentence OK as edited?]** They also indicate that pulmonary NKT cells may provide an intrinsic element in the lungs that permits Th2 responses to develop and to drive the development of AHR and asthma.

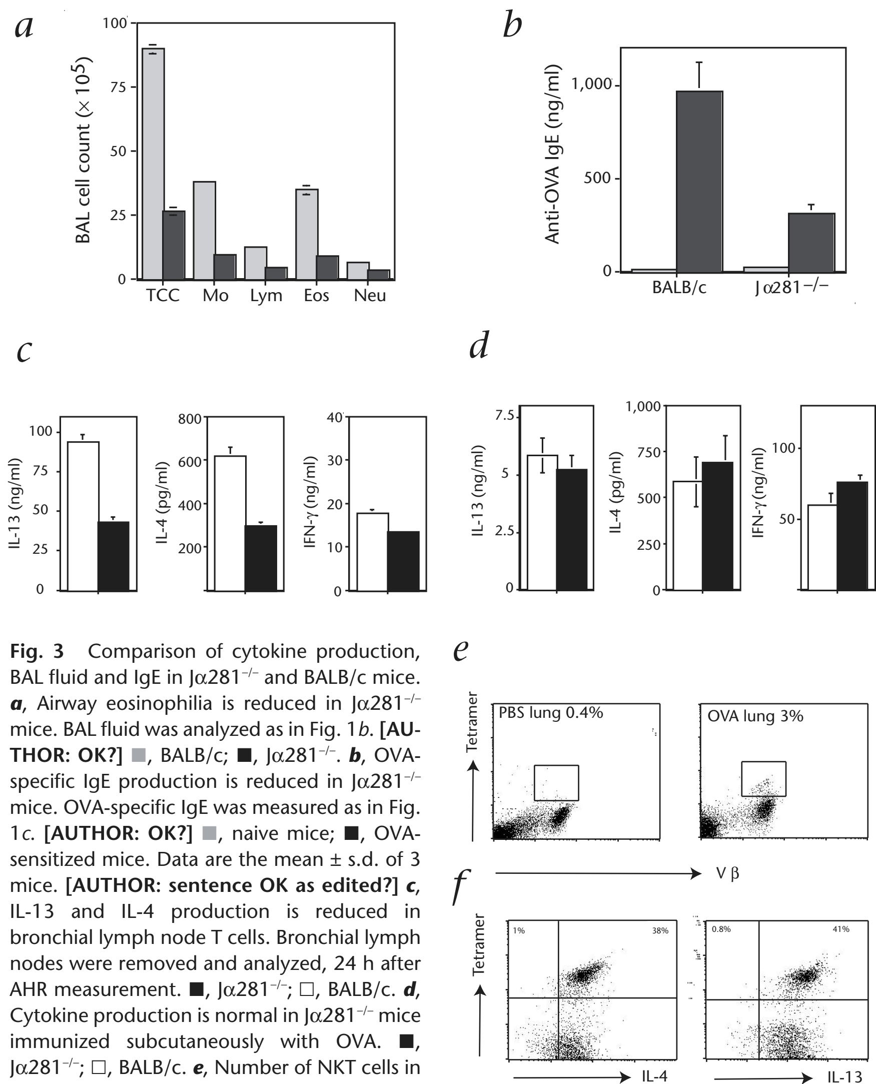


**Fig. 3** Comparison of cytokine production, BAL fluid and IgE in Jα281$^{-/-}$ and BALB/c mice. ***a***, Airway eosinophilia is reduced in Jα281$^{-/-}$ mice. BAL fluid was analyzed as in Fig. 1*b*. **[AUTHOR: OK?]** ■, BALB/c; ■, Jα281$^{-/-}$. ***b***, OVA-specific IgE production is reduced in Jα281$^{-/-}$ mice. OVA-specific IgE was measured as in Fig. 1*c*. **[AUTHOR: OK?]** ■, naive mice; ■, OVA-sensitized mice. Data are the mean ± s.d. of 3 mice. **[AUTHOR: sentence OK as edited?]** ***c***, IL-13 and IL-4 production is reduced in bronchial lymph node T cells. Bronchial lymph nodes were removed and analyzed, 24 h after AHR measurement. ■, Jα281$^{-/-}$; □, BALB/c. ***d***, Cytokine production is normal in Jα281$^{-/-}$ mice immunized subcutaneously with OVA. ■, Jα281$^{-/-}$; □, BALB/c. ***e***, Number of NKT cells in the lungs of wild-type BALB/c mice increases on airway challenge with OVA. BALB/c mice were sensitized and challenged with OVA (right) or PBS (left). Lung cells were isolated and stained with α-GalCer–loaded CD1d tetramers and a monoclonal antibody against TCR Vβ. ***f***, Pulmonary NKT cells produce IL-4 and IL-13. Lung cells isolated from the OVA-challenged mice in (*e*) were enriched for CD1d tetramer–positive NKT cells by MACS. NKT cells were stimulated with PMA and ionomycin, and stained with α-GalCer–loaded CD1d tetramers and monoclonal antibodies against IL-4 or IL-13.

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To determine the specific role of NKT cells in the development of AHR, we examined the development of AHR in CD1d-deficient mice, which lack the class I restricting element of NKT cells and therefore lack NKT cells. When sensitized and challenged with antigen, control BALB/c mice developed severe AHR, as expected; in contrast, CD1d-deficient mice that were sensitized and challenged with antigen did not develop AHR but showed normal airway responsiveness (Fig. 1*a*).

The defect in the CD1d-deficient mice was associated with a substantial reduction in airway eosinophilia, as indicated by a reduction of eosinophils in bronchoalveolar lavage (BAL) fluid as compared with BAL fluid from Balb/c mice sensitized and challenged with ovalbumin (OVA; Fig. 1*b*). **[AUTHOR: sentence OK as edited?]** Neither PBS-challenged (negative control) BALB/c mice nor PBS-challenged CD1d-deficient mice developed airway eosinophilia or abnormal cellular infiltrates in BAL fluid (data not shown).

The failure of CD1d-deficient mice to develop AHR was not due to a deficiency in Th2 responsiveness or to suboptimal stimulation or immunization with antigen, because previous studies have shown that CD1d-deficient mice have normal Th2 responses[26], and because more intensive immunization with multiple intraperitoneal immunizations with OVA in alum and intranasal challenges with antigen did not induce AHR in the CD1d-deficient mice (see Supplementary Fig. 1 online). Indeed, as previously reported[26] the immunized CD1d-deficient mice produced OVA-specific IgE (Fig. 1*c*), although the amounts of IgE observed in CD1d-deficient mice were less than those observed in wild-type control BALB/c mice. **[AUTHOR: sentence OK as edited?]**

The lack of AHR in CD1d-deficient mice was also not due to a specific unresponsiveness to OVA, because sensitization with other antigens, such as bovine serum albumin (BSA) or *Aspergillus fumigatus* antigen, did not induce AHR in CD1d-deficient mice but did induce severe AHR in BALB/c mice (see Supplementary Fig. 2 online). The absence of AHR in CD1d-deficient mice was also not due to an intrinsic inability of the mice to develop AHR, because administration of recombinant IL-13, which causes AHR though direct effects on airway smooth muscle cells and mucus gland secretion[27,28], resulted in severe AHR in CD1d-deficient mice (Fig. 1*d*).

Although the immune system in CD1d-deficient mice is thought to be normal outside the NKT cell compartment, it is possible that in the absence of CD1d molecules, defects in other non-NKT cell compartments might occur. To confirm that the failure of CD1d-deficient mice to develop AHR was due specifically to the absence of NKT cells, we examined the development of allergen-induced AHR in Jα281$^{-/-}$ mice, which lack the invariant TCR of NKT cells. The spleens of Jα281$^{-/-}$ mice, like those of CD1d-deficient mice, did not contain cells that stained with α-GalCer–loaded CD1d tetramers, indicating that both Jα281$^{-/-}$ and CD1d-deficient mice lack Vα14*i* NKT cells (Fig. 2*a*). **[AUTHOR: sentence OK as edited?]** In Jα281$^{-/-}$ mice, as in CD1d-deficient mice, sensitization and challenge with OVA did not

induce AHR, whereas similar immunization in wild-type BALB/c mice caused severe AHR (Fig. 2*b*). The lack of measurable AHR was not due to a lack of sensitivity in our method of detection, because airway reactivity to methacholine was essentially normal, measured as airway resistance ($R_L$) and dynamic compliance ($C_{dyn}$) in anesthetized, tracheostomized and mechanically ventilated CD1d-deficient and Jα281$^{-/-}$ mice that had been previously sensitized and challenged with OVA (Fig. 2*c*).

As shown by a reduction of eosinophils in BAL fluid, the absence of AHR in Jα281$^{-/-}$ mice was associated with reduced airway eosinophilia as compared with sensitized and challenged BALB/c mice (Fig. 3*a*). **[AUTHOR: sentence OK as edited?]** Neither PBS-challenged BALB/c nor PBS-challenged Jα281$^{-/-}$ mice developed airway eosinophilia or abnormal cellular infiltrates in BAL fluid (data not shown). Sensitized and challenged Jα281$^{-/-}$ mice also produced less OVA-specific IgE in their sera (Fig. 3*b*) and less IL-4 and IL-13 in their bronchial lymph nodes (Fig. 3*c*) than did similarly sensitized and challenged BALB/c mice. **[AUTHOR: sentence OK as edited?]**

The reduction in Th2 cytokine production after pulmonary exposure to antigen was restricted to responses in the lung, because subcutaneous immunization of the footpads of Jα281$^{-/-}$ mice resulted in normal production of IL-4, IL-13 and IFN-γ as compared with immunized wild-type control BALB/c mice (Fig. 3*d*). This result may reflect the fact that NKT cells, although present only in small numbers in bronchial lymph nodes (0.05–0.12%; see Supplementary Fig. 3 online), constitute as much as 3% of the pulmonary mononuclear cells after antigen challenge (Fig. 3*e*) but may not be present in subcutaneous tissue. The NKT cells found in the lungs produced large amounts of IL-4 and IL-13, as shown by intracellular cytokine staining (Fig. 3*f*), which most probably enhanced the subsequent development of AHR.

To show that the lack of Vα14*i* NKT cells specifically caused the failure of Jα281$^{-/-}$ mice to develop AHR, we reconstituted OVA-sensitized Jα281$^{-/-}$ mice with Vα14*i* NKT cells before challenge with OVA. Adoptive transfer of a cell population enriched for cells that bound α-GalCer–loaded CD1d tetramer (>75% tetramer positive; Fig. 4*b*) fully reconstituted the capacity of Jα281$^{-/-}$ mice to develop AHR (Fig. 4*a*). **[AUTHOR: sentence OK as edited?]** Binding of α-GalCer–loaded CD1d tetramer to the NKT cells was not required for reconstitution of AHR, because the adoptive transfer of unmanipulated spleen cells from wild-type BALB/c mice, but not from Jα281$^{-/-}$ mice, also reconstituted the Jα281$^{-/-}$ recipients (Fig. 4*c*). Because Jα281$^{-/-}$ mice lack only Vα14*i* NKT cells and are otherwise identical to wild-type mice, these studies verify that Vα14*i* NKT cells are required for the development of allergen-induced AHR.

To evaluate the mechanisms by which Vα14*i* NKT cells mediate the development of AHR, we reconstituted Jα281$^{-/-}$ mice with cells selected for binding to α-GalCer–loaded CD1d tetramers that we purified from cytokine knockout mice. **[AUTHOR: sentence OK as edited?]** Tetramer-binding cells from IL-4-deficient mice and IL-13-deficient mice were much less effective than tetramer-binding cells from wild-type BALB/c mice in reconstituting AHR when adoptively transferred to Jα281$^{-/-}$ mice (Fig. 5*a*). Tetramer-binding cells from mice deficient in IL-4 and IL-13 did not reconstitute AHR in the Jα281$^{-/-}$ mice (Fig. 5*a*). By contrast, tetramer-binding cells from IFN-γ–deficient mice fully restored AHR (data not shown). These results indicate that the induction of AHR may require Vα14*i* NKT cells that produce both IL-4 and IL-13, but not IFN-γ. **[AUTHOR: sentence OK as edited?]**

We next examined whether Vα14*i* NKT cells were required for the development of Th2 cells or more specifically for permitting Th2 cells to drive the development of AHR. **[AUTHOR: sentence OK as edited?]** We therefore isolated OVA-primed CD4$^+$ T cells from the spleens of wild-type BALB/c or Jα281$^{-/-}$ mice that had been immunized intraperitoneally with OVA in alum to induce polarized Th2 responses. These cells were adoptively transferred into severe combined immunodeficient (SCID) mice, which were then challenged with OVA intranasally and examined for the development of AHR. Whereas OVA-primed CD4$^+$ T cells from wild-type BALB/c mice (containing NKT cells) transferred AHR into the SCID mice, OVA-primed CD4$^+$ cells from Jα281$^{-/-}$ mice (containing no NKT cells), or CD4$^+$ cells from naive BALB/c mice, did not (Fig. 5*b*).

When Vα14*i* NKT cells from naive BALB/c mice, but not from mice deficient in IL-4 and IL-13, were transferred with the OVA-primed CD4$^+$ T cells from Jα281$^{-/-}$ mice, however, AHR was restored in the SCID mice recipients. Transfer of purified NKT cells by themselves into SCID mice did not induce AHR. Taken together, these studies indicate that Th2-biased cells can develop in the absence of Vα14*i* NKT cells, but Th2 cells that develop in Jα281$^{-/-}$ mice are not

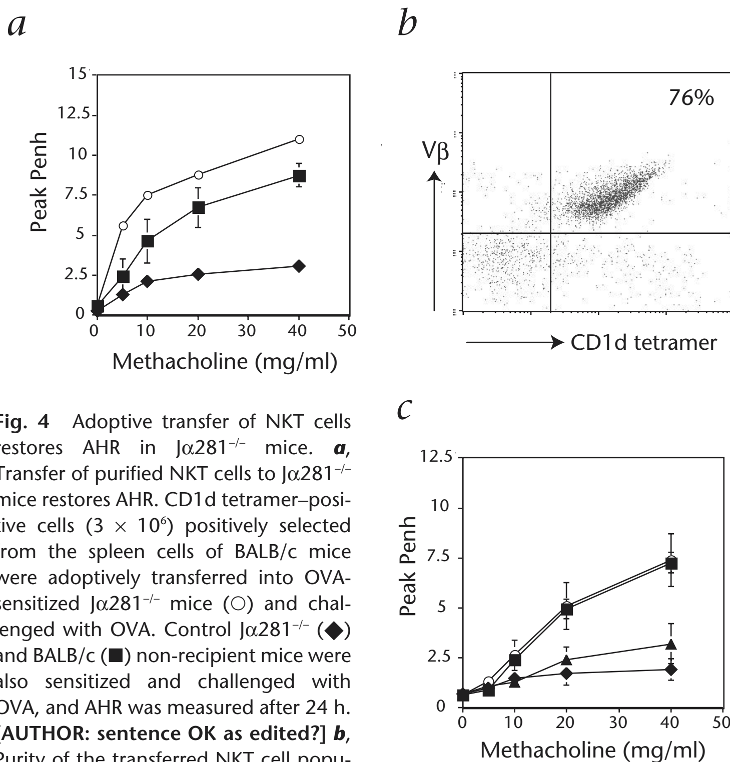


**Fig. 4** Adoptive transfer of NKT cells restores AHR in Jα281$^{-/-}$ mice. ***a***, Transfer of purified NKT cells to Jα281$^{-/-}$ mice restores AHR. CD1d tetramer–positive cells (3 × 10$^6$) positively selected from the spleen cells of BALB/c mice were adoptively transferred into OVA-sensitized Jα281$^{-/-}$ mice (○) and challenged with OVA. Control Jα281$^{-/-}$ (◆) and BALB/c (■) non-recipient mice were also sensitized and challenged with OVA, and AHR was measured after 24 h. **[AUTHOR: sentence OK as edited?]** ***b***, Purity of the transferred NKT cell population. Flow cytometry showed that 76% of the transferred cells in (*a*) stained with both α-GalCer–loaded CD1d tetramer and a monoclonal against Vβ. ***c***, Transfer of spleen cells to Jα281$^{-/-}$ mice restores AHR. Transfer of spleen cells (4 × 10$^6$) from naive BALB/c mice (○), but not from Jα281$^{-/-}$ mice (▲), into OVA-sensitized Jα281$^{-/-}$ recipients restored AHR. **[AUTHOR: sentence OK as edited?]** Recipients were given OVA and assessed for AHR as in (*a*). Control BALB/c mice (■) and Jα281$^{-/-}$ mice (◆) were also sensitized and challenged with OVA.

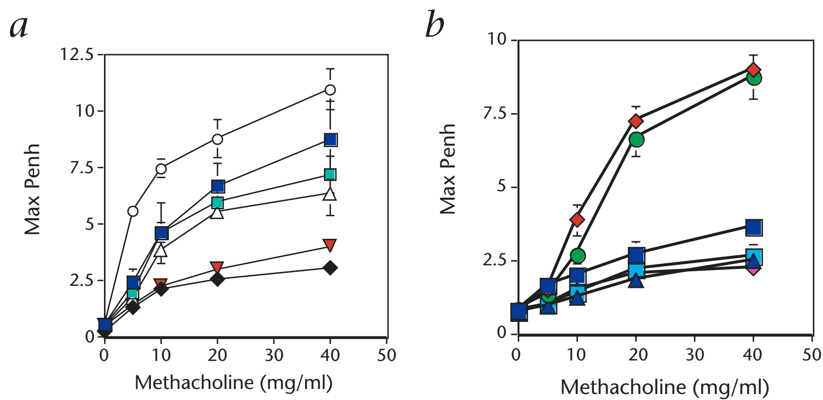


**Fig. 5** IL-4 and IL-13 production by NKT cells is required for development of AHR. ***a***, NKT cells from wild-type BALB/c mice, but not mice deficient in IL-4 and IL-13, restore AHR. CD1d tetramers–positive cells were positively selected from spleen cells of mice deficient in IL-4 (■), IL-13 (△) or both IL-13 and IL-4 (▼), or wild-type BALB/c (○) mice and adoptively transferred ($3 \times 10^6$) into OVA-sensitized Jα281$^{-/-}$ mice, which were then challenged and assessed for AHR, together with non-recipient BALB/c (■) and Jα281$^{-/-}$ (◆) mice, as in Fig. 4*a*. **[AUTHOR: sentence OK as edited? Also please check that symbols have been inserted correctly]** ***b***, OVA-sensitized T cells from Jα281$^{-/-}$ mice require NKT cells to induce AHR. MACS-purified CD4$^+$ cells from spleens of OVA-sensitized BALB/c (◆) or Jα281$^{-/-}$ (■) mice, or naive BALB/c mice (▲), were adoptively transferred into SCID mice ($8 \times 10^6$ cells/mouse). **[AUTHOR: sentence OK as edited?]** Some mice also received tetramer-purified NKT cells ($3 \times 10^6$) from naive wild-type BALB/c (●) or mice deficient in IL-4 and IL-13 (■), or received wild-type NKT cells alone (◆). Recipients were challenged with OVA and AHR was measured 24 h after the last challenge.

competent to induce AHR except in the presence of Vα14*i* NKT cells.

## Discussion

Using both CD1d-deficient mice, which lack the MHC restriction element required by NKT cells, and Jα281$^{-/-}$ mice, which lack the invariant TCR of NKT cells, we have shown that allergen-induced AHR does not develop in the absence of Vα14*i* NKT cells. The lack of AHR in these mice was not due to structural abnormalities in the pulmonary parenchyma because AHR was reconstituted in CD1d-deficient mice by the administration of recombinant IL-13, which induces AHR though direct effects on airway smooth muscle cells and mucus glands, and because AHR was reconstituted in Jα281$^{-/-}$ mice by the adoptive transfer of Vα14*i* NKT cells. Our study therefore shows that Vα14*i* NKT cells are required for the development of AHR.

Previous studies have shown that CD1d-deficient mice that are immunized with antibodies against IgD **[AUTHOR: OK?]** produce normal amounts of IgE[26], and that CD1d-deficient mice on a 129/C57BL/6 background develop airway eosinophilia[23]. We confirmed here that CD1d-deficient mice can produce IgE and also showed that Jα281$^{-/-}$ mice have the capacity both to produce IgE on immunization with antigen and to produce normal quantities of IL-4 and IL-13 when immunized subcutaneously. In addition, our assessment of AHR in CD1d-deficient and Jα281$^{-/-}$ mice has shown that AHR, a cardinal feature of asthma that does not always correlate with airway eosinophilia[29,30], does not occur in mice deficient in NKT cells.

Although β2 microglobulin–deficient mice, which lack all class I molecules including CD1d, have been shown to develop airway eosinophilia[24] and AHR[25], the absence of CD8$^+$ T cells in these mice may cause immune dysregulation in several tissue compartments that affect airway responses, making interpretation of the results difficult. Taken together, however, these studies indicate that Th2-biased immune responses can occur in mice deficient in NKT cells, and that Th2 cell differentiation and production of IgE do not require the presence of Vα14*i* NKT cells.

Because a Th2 response is not sufficient in itself to induce the full development of asthma and AHR (that is, a Th2 response in peripheral lymph nodes or in the upper airways is not always accompanied by asthma and a Th2 response in the lower airways)[5,6], Vα14*i* NKT cell must regulate additional elements in the lower respiratory tract that are required for the development of AHR. In other words, pulmonary NKT cells, which represent as much as 3% of lung mononuclear cells and have a crucial role in resistance to pathogenic bacteria in the respiratory tract[31], seem to become activated by antigens encountered in the lung, providing a necessary prerequisite for the development of AHR. By contrast, antigens encountered in subcutaneous tissue do not seem to activate NKT cells, presumably because NKT cells are present in much lower numbers in these tissues, where production of the NKT cell growth factor IL-15 (ref. 32) may be limited.

The precise mechanisms by which antigen administration into the lungs activates NKT cells to induce AHR are not clear. We found here that when transferred with wild-type NKT cells, allergen-sensitized CD4$^+$ αβ-TCR T cells from NKT cell–deficient mice could induce AHR in SCID mice, whereas the transfer of either cell population on its own could not, indicating that NKT cells have the capacity to localize the Th2 response to the lungs and to induce AHR. Because the immunization of NKT cell–deficient mice with more aggressive protocols and with different antigens did not induce AHR, we do not think that NKT cells simply influence the magnitude of the pulmonary Th2 response, but rather that they 'license' Th2 cells that enter the pulmonary compartment to become competent to induce AHR. Although the specific signals provided by Vα14i NKT cells to the allergen-sensitized CD4$^+$ αβ-TCR T cells are unknown, our model is unique in that Vα14i NKT cell function can be induced without the administration of α-GalCer; this contrasts with most other models of autoimmunity or infection, in which the role of NKT cells requires their activation by α-GalCer[31,33–36].

In our system, we propose that antigen encountered in the lung alters the mucosal environment of the respiratory tract, exposing self glycolipid antigens that are recognized by self agonist–selected NKT cells[8,37]. Although the glycolipid antigen in our system, which activates NKT cells *in vivo* when OVA, BSA or *Aspergillus* antigen is administered into the lungs, has not been identified, agonist-selected T cells in the mucus membranes are thought to look for cells that express enhanced quantities of self antigens in response to stress induced by environmental stimuli. Such agonist-selected T cells, as part of the innate immune system, then respond rapidly by producing cytokines[37]. Our studies therefore suggest that antigens other than α-GalCer can indeed activate Vα14*i* NKT cells, and that there are innate immune mechanisms involving Vα14*i* NKT cells in the lung that respond rapidly to inhaled antigens.

V$\alpha$14*i* NKT cells induced AHR through mechanisms that involved the production by NKT cells of both IL-4 and IL-13, because NKT cells deficient in IL-4 and IL-13 production could not reconstitute AHR in J$\alpha$281$^{-/-}$ mice. We confirmed that NKT cells produce both IL-4 and IL-13 (refs. 38, 39) and showed that both IL-4–deficient and IL-13–deficient NKT cells could partially reconstitute AHR in J$\alpha$281$^{-/-}$ mice, suggesting that IL-4 and IL-13 possess partly overlapping functions in this process. IFN-$\gamma$–deficient NKT cells were as effective as wild-type NKT cells in reconstituting AHR in J$\alpha$281$^{-/-}$ mice, indicating that NKT cell production of IFN-$\gamma$ has no role in the development of AHR, although IFN-$\gamma$ production by NKT cells is important in the clearance of *Pseudomonas aeruginosa* from the lung[31].

We suggest that IL-4 and IL-13 produced by V$\alpha$14*i* NKT cells potentiate the development and action of Th2-biased OVA-specific T cells in the lungs, which drives the development of asthma. There is no clear consensus, however, on how NKT cells function in the prevention or cause of any disease; precisely how the IL-4 and IL-13 released into the respiratory tract by NKT cells enable Th2 cells to induce AHR is not known.

In summary, we have identified a role for V$\alpha$14*i* NKT cells in the regulation of allergen-induced AHR and have shown that AHR does not develop in the absence of V$\alpha$14*i* NKT cells. Our studies indicate that V$\alpha$14*i* NKT cells that produce IL-4 and IL-13 actively regulate Th2-biased immune responses in the respiratory tract when antigen is encountered in the lungs, but not when antigen is administered subcutaneously at non-mucosal sites. Administration of nominal antigen into the lungs seems to activate NKT cells, which then license Th2 cells that enter the lung to induce AHR through mechanisms that are independent of $\alpha$-GalCer. Our studies indicate that V$\alpha$14*i* NKT cells in the respiratory tract may have a crucial role in the development of asthma, and that therapies targeted at limiting or altering NKT cell function may selectively restrict the development of AHR and asthma.

## Methods

**Mice and antigens.** BALB/c wild-type, IL-4-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). CD1.1 (CD1-deficient) mice[26] (backcrossed with BALB/c) were a gift from M. Grusby, **[AUTHOR: for consistency, please give institution of M. Grusby]** and J$\alpha$281$^{-/-}$ mice[36] (backcrossed with BALB/c) were a gift from M. Taniguchi (Chiba University, Chiba, Japan) and S. Balk (Brigham and Women's Hospital, Boston, Massachusetts). Mice deficient in IL-13 or both IL-4 and IL-13 (backcrossed with BALB/c) were a gift from A. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK)[28,40]. We immunized the mice with OVA (ICN, Aurora, Ohio), BSA (Sigma, St. Louis, Missouri) and *A. fumigatus* antigen (National Institutes of Health, Bethesda, Maryland). The Stanford University Committee on Animal Welfare (Administration Panel of Laboratory Animal Care) approved all mouse protocols used in this study.

**Adoptive transfer.** Spleen or NKT cells ($3 \times 10^6$), purified from spleen using $\alpha$-GalCer–loaded CD1d tetramers (purity was judged to be about 76% by flow cytometry for tetramer-positive cells double-stained with a monoclonal antibody against TCR V$\beta$; Pharmingen, San Diego, California), were adoptively transferred intravenously into J$\alpha$281$^{-/-}$ or SCID recipients (on a BALB/c background) that had been sensitized 8 d earlier by intraperitoneal administration of OVA in alum. **[AUTHOR: OK as edited? Information moved here from figure legends]** The donors of spleen cells were wild-type BALB/c mice or mice deficient in IL-13, IL-4 or both IL-4 and IL-13.

**Restimulation of lymph node cells *in vitro*.** Cells isolated from lymph nodes of OVA-primed mice were restimulated *in vitro* ($5.0 \times 10^4$ cells per well in a 96-well plate) with 62.5 µg/ml of OVA. **[AUTHOR: OK as edited?]** We collected the supernatants after 4 d and assayed them for IL-4, IL-13 and IFN-$\gamma$ by ELISA.

**Cytokine ELISAs and OVA-specific IgE assay.** ELISAs were done as described[41]. We used the following pairs of monoclonal antibodies for capture and biotinylated detection, respectively: R4-6A2 and XMG1.2 for IFN-$\gamma$ and 11B11 and BVD6-24G2 for IL-4. Reagents for detecting mouse IL-13 were purchased from R&D Systems (Minneapolis, Minnesota). For the OVA-specific IgE assay, mice were bled and OVA-specific IgE antibodies were measured in serum using a modified OVA-specific ELISA as described[41].

**Identification of NKT cells.** Splenocytes or lymph nodes cells were pre-incubated with monoclonal antibodies against Fc$\gamma$ receptor (2.4G2 culture supernatant), washed and incubated with $\alpha$-GalCer–loaded CD1d tetramers or control tetramers, prepared with phycoerythrin (PE)-streptavidin as described[42], for 30 min in 100 µl of PBS containing 1% FCS. The cells were washed and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey). **[AUTHOR: OK?]**

**Induction of AHR and measurement of airway responsiveness.** To induce AHR, we sensitized mice with 100 µg of OVA, 100 µg of BSA or 20 µg of *A. fumigatus* antigen in alum administered intraperitoneally. After 8 d, mice were exposed to intranasal antigen (50 µg/d for OVA and BSA; 20 µg/d for *A. fumigatus*) or PBS on 3 consecutive days, as described[41,43]. AHR responses were assessed by methacholine-induced airflow obstruction in conscious mice placed in a whole-body plethysmograph (Buxco Electronics, Troy, New York) as described[41]. In some experiments we assessed AHR by invasive measurement of airway resistance, in which anesthetized and tracheostomized mice were mechanically ventilated using a modified version of a described method[44]. **[AUTHOR: sentence OK as edited? Information moved here from figure legends]** Aerosolized methacholine was administered for 20 breaths in increasing concentrations (1.25, 2.5, 5 and 10 mg/ml of methacholine). We continuously computed $R_L$ and $C_{dyn}$ by fitting flow, volume and pressure to an equation of motion. In other experiments, naive CD1d-deficient mice were treated with 2 µg of recombinant IL-13 (a gift from the Genetics Institute) intranasally for 3 consecutive days in 50 µl of normal saline before measurement of AHR.

**Subcutaneous immunization of mice.** J$\alpha$281$^{-/-}$ or wild-type BALB/c mice were immunized subcutaneously with OVA in alum once in the footpads. After 8 d, the draining lymph nodes were collected, and the T cells were cultured *in vitro* with OVA. Supernatants were assessed for cytokines 4 d later by ELISA. **[AUTHOR: moved here from Fig. 3: OK?]**

**Collection and analysis of BAL fluid.** A lethal dose of phenobarbital (450 mg per kg body weight) was administered intraperitoneally to mice. After the trachea was cannulated, the lungs were lavaged 3 times with 0.3 ml of PBS, and the fluid was pooled. We counted and analyzed cells in BAL fluid as described[41]. The relative number of different types of leukocyte (lung cell differential) was determined from slide preparations of BAL fluid stained with H&E. **[AUTHOR: moved here from the figure legends: OK?]**

**Flow cytometry and fluorescence-activated cell sorting (FACS) analysis.** Analytical flow cytometry was done on a FACScan instrument (Becton Dickinson). We collected cells from lymph nodes, spleen or lungs and incubated them on ice with CD1d tetramer and a monoclonal antibody against V$\beta$ (PharMingen) using standard procedures. Flow cytometric measurements of cytokine production in NKT cells was done according to a modified protocol[45,46]. In brief, we stimulated NKT cells with phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) and ionomycin (500 ng/ml) for 3 h. Fixation and permeabilization was done on collected cells using Cytofix/Cytoperm and Perm/Wash (PharMingen) according to the manufacturer's instructions. Cytoplasmic IL-4 was stained with a FITC-conjugated labeled monoclonal antibody (PharMingen) and cytoplasmic IL-13 was stained with a mouse monoclonal antibody against IL-13 (R&D Systems). **[AUTHOR: sentence OK as edited?]**

**Lung cell isolation.** The aorta and the inferior vena cava were sectioned and the lungs were perfused with PBS through the right ventricle until white. We sliced the lobes of the lungs into small cubes and incubated them in 10 ml of solution containing 0.1% DNase I (fraction IX; Sigma) and

1.6 mg/ml of collagenase (CLS4; Worthington Biochemicals, Lakewood, New Jersey) at 37 °C for 2 h. **[AUTHOR: OK as edited?]** Mononuclear cells were separated by centrifugation on discontinuous Percoll gradients (70%, 50%, 30% and 20% Percoll; Pharmacia, Uppsala, Sweden). We isolated NKT cells by positive selection using magnetic cell sorting (MACS) after incubating the cells first with PE-labeled CD1d tetramer and then with magnetic beads conjugated to antibodies against PE. **[AUTHOR: sentence OK as edited?]** The Auto-MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) system was used in accordance with the manufacturer's instructions.

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*Competing interests statement*

*The authors declare that they have no competing financial interests.*

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