

Glycolipid activation of invariant T cell receptor⁺ NK T cells is sufficient to induce airway hyperreactivity independent of conventional CD4⁺ T cells

Everett H. Meyer^{*†}, Sho Goya^{*}, Omid Akbari^{*}, Gerald J. Berry[†], Paul B. Savage[§], Mitchell Kronenberg[¶], Toshinori Nakayama^{||}, Rosemarie H. DeKruyff^{*†}, and Dale T. Umetsu^{*†**}

^{*}Division of Immunology, Children's Hospital, Harvard Medical School, One Blackfan Circle, Boston, MA 02115; [†]Immunology Program and School of Medicine, Stanford University, Stanford, CA 94305; [‡]Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305; [§]Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602; [¶]Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121; and ^{||}Department of Immunology, Chiba University, Chiba 260-8677, Japan

Communicated by K. Frank Austen, Harvard University, Boston, MA, December 22, 2005 (received for review October 14, 2005)

Asthma is an inflammatory lung disease, in which conventional CD4⁺ T cells producing IL-4/IL-13 appear to play an obligatory pathogenic role. Here we show, in a mouse model of asthma, that activation of pulmonary IL-4/IL-13 producing invariant TCR⁺ CD1d-restricted natural killer T (NKT) cells is sufficient for the development of airway hyperreactivity (AHR), a cardinal feature of asthma, in the absence of conventional CD4⁺ T cells and adaptive immunity. Respiratory administration of glycolipid antigens that specifically activate NKT cells (α -GalactosylCeramide and a *Sphingomonas* bacterial glycolipid) rapidly induced AHR and inflammation typically associated with protein allergen administration. Naïve MHC class II-deficient mice, which lack conventional CD4⁺ T but have NKT cells, showed exaggerated baseline AHR and, when challenged with α -GalactosylCeramide, demonstrated even greater AHR. These studies demonstrate an expanded role for NKT cells, in which NKT cells not only produce cytokines that influence adaptive immunity but also function as critical effector cells that can induce AHR. These results suggest that NKT cells responding to glycolipid antigens, as well as conventional CD4⁺ T cells responding to peptide antigens, may be synergistic in the induction of AHR, although in some cases, each may independently induce AHR.

asthma | atopy | IgE

Bronchial asthma is an immunological disease resulting from Th2-driven inflammation in the airways. It is characterized by inflammation in the peribronchial space, with increased production of airway mucus, and by airway hyperreactivity (AHR), a cardinal feature of asthma. Over the past decade, investigators focused on the role of conventional CD4⁺ Th2 cells in orchestrating inflammation in asthma. CD4⁺ Th2 cells, which are thought to be present in the airways of all patients with asthma (1), secrete key cytokines, such as IL-4 and IL-13 (2, 3), as well as IL-5 and IL-9 (4). Conventional CD4⁺ T cells recognize exogenous antigens and initiate allergic inflammation in the lungs and, in mouse models of asthma, elimination of CD4⁺ cells abrogates the development of AHR (5). Therefore, conventional CD4⁺ Th2 cells are thought to play an obligatory role in the pathogenesis of asthma (6).

CD4 is expressed not only by conventional CD4⁺ T cells but also by natural killer T cells (NKT cells), which comprise a unique and relatively rare subset of lymphocytes that have features of both T cells and NK cells. Many NKT cells express a highly restricted T cell receptor (TCR) repertoire consisting of V α 14-J α 18 (in mice) or V α 24-J α 18 (in humans) and are called invariant TCR⁺ NKT (*i*NKT) cells (7). Through this invariant TCR, *i*NKT cells recognize bacterial and endogenous glycolipid antigens presented by the nonpolymorphic MHC class I-like protein, CD1d, and rapidly produce large quantities of cytokines, including IL-4 and IFN- γ , which enhance the function of dendritic cells, NK cells, and B cells, as well as conventional CD4⁺ and CD8⁺ T cells (8–11). Rapid production of cytokines by *i*NKT cells is a manifestation of innate-

like immunity and endows *i*NKT cells with the capacity to amplify and regulate adaptive immune responses and thus link innate and adaptive immunity. Moreover, *i*NKT cells have been shown to regulate the development of autoimmune, antimicrobial, antitumor, and antitransplant immune responses (12–14).

Recently, we and others showed in standard mouse models of allergen-induced AHR that *i*NKT cells producing IL-4 and IL-13 are required for the development of allergen-induced AHR (15, 16). In the absence of *i*NKT cells, AHR failed to develop after sensitization and challenge with allergen, although Th2 responses and eosinophilia developed in these *i*NKT cell-deficient mice (15, 16). However, because conventional CD4⁺ T cells are thought to play an essential role in asthma, and because *i*NKT cells cannot recognize the protein allergens that drive allergic asthma and AHR, the precise relationship between *i*NKT cells and conventional CD4⁺ T cells in the induction of airway inflammation and AHR was not clear.

To define a specific role for *i*NKT cells in the induction of AHR, and in particular to define the relationship between *i*NKT cells and conventional CD4⁺ T cells, we studied the direct activation of *i*NKT cells by the respiratory administration of *i*NKT cell-activating glycolipid antigens, α -GalactosylCeramide (α -GalCer) and the glycolipid antigen PS-30, derived from *Sphingomonas*, which are gram⁻ LPS⁻ opportunistic bacterial pathogens (17).

Results

Activation of Pulmonary *i*NKT Cells Induces AHR and Eosinophilia. To determine the specific role of *i*NKT cells in the development of AHR, we administered to naïve *BALB/c* mice α -GalCer, a marine sponge glycolipid antigen that specifically activates CD1d-restricted *i*NKT cells. *BALB/c* mice challenged intranasally (i.n.) with α -GalCer developed severe AHR by 24 h (Fig. 1*a*). Induction of AHR by α -GalCer did not occur in *i*NKT cell-deficient *CD1d*^{-/-} and *J α 18*^{-/-} mice (*BALB/c* background) challenged with α -GalCer (Fig. 1*b*). Lower respiratory AHR was confirmed by direct measurement of airway resistance (R_L) and dynamic compliance (C_{dyn} ; a measure of the elasticity of the lung) in anesthetized, tracheostomized, intubated, and mechanically ventilated wild-type *BALB/c* and *CD1d*^{-/-} mice (Fig. 1*c*). α -GalCer-induced AHR was observed in all mouse strains tested (*BALB/c* *BYJ*, *C57BL/6*, *NZB/BINJ*, and *CBA/J*; Table 1, which is published as supporting information on the PNAS web site). The AHR induced after α -GalCer challenge occurred with i.n. but not i.v. administration (Fig. 1*d*), a defect that

Conflict of interest statement: No conflicts declared.

Abbreviations: AHR, airway hyperreactivity; NKT cell, natural killer T cell; TCR, T cell receptor; *i*NKT, invariant TCR⁺ NKT; α -GalCer, α -GalactosylCeramide; i.n., intranasal(ly); OVA; MHC Δ/Δ mice, MHC class II-deficient mice.

**To whom correspondence should be addressed. E-mail: dale.umetsu@childrens.harvard.edu.

© 2006 by The National Academy of Sciences of the USA

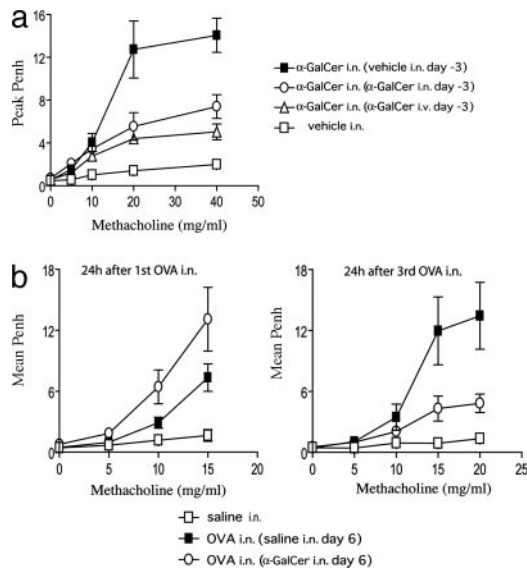


Fig. 2. AHR depends upon timing of *i*NKT cell activation. (a) Pretreatment with α -GalCer exhausts *i*NKT cell capacity to induce AHR. α -GalCer ($2 \mu\text{g}$) administered i.n. or i.v. 3 days before a second i.n. dose of α -GalCer reduces AHR. AHR was assessed as in Fig. 1a. Data are the mean \pm SEM, representative of two experiments ($n = 5$). (b) Activation of *i*NKT cells with α -GalCer before i.n. antigen challenge first enhanced (Left) and then inhibited (Right) allergen-induced AHR. α -GalCer ($2\text{--}4 \mu\text{g}$) was administered 6 days after OVA/alum-sensitized mice, 1 day before 3 consecutive days of i.n. challenge with OVA. AHR was assessed on day 8 before the second i.n. challenge and again on day 10. Data are the mean \pm SEM Penh, representative of three experiments ($n = 3\text{--}5$). The α -GalCer- and saline-treated groups have significantly different AHR ($P < 0.05$; Student's *t* test).

ment of AHR was accompanied by pulmonary eosinophilia, as well as by a rise in serum IgE and peribronchial infiltrates (Fig. 4 *c–e*).

IL-4 and IL-13 but Not IL-5 or Ig Are Required for *i*NKT Cell-Induced AHR. We evaluated the capacity of α -GalCer to induce AHR in IL-4- and IL-13-deficient mice, because IL-4 and IL-13 were present in lung lysates 24 h after challenge with α -GalCer (data not shown). We found that both *IL-4*^{-/-} and *IL-13*^{-/-} mice challenged with α -GalCer had reduced AHR and IgE responses when compared with wild-type BALB/c mice, whereas *IL-4*^{-/-}*IL-13*^{-/-} (double knockout) mice failed to develop AHR or IgE responses (Fig. 5 *a* and *b*). Together, this indicated that both IL-4 and IL-13 are critical cytokines for the induction of AHR and elevated serum IgE with α -GalCer. Production of AHR did not depend on an increase in IgE levels, because the administration of α -GalCer to B cell-deficient *JHD* mice induced significant AHR within 24 h (Fig. 5*c*).

Given the potentially controversial role of eosinophils in the development of asthma, we asked whether IL-5 and pulmonary eosinophilia were important for the induction of AHR by α -GalCer. We challenged BALB/c mice in the presence or absence of anti-mouse IL-5 mAb (TRFK5) administered before i.n. administration of α -GalCer. Treatment with anti-IL-5 mAb but not isotype control reduced eosinophil levels in the bronchiolar lavage fluid by $>85\%$ (Fig. 6*a*) but had no effect upon AHR (Fig. 6*b*). These results suggest that IL-5 and eosinophils are not critical for AHR induced with α -GalCer.

Conventional CD4⁺ T Cells Are Not Required for AHR. We examined MHC class II-deficient mice (MHC Δ/Δ mice, C57BL/6 background), which lack conventional CD4⁺ T cells but contain *i*NKT cells. MHC Δ/Δ mice have an increase in the number of pulmonary *i*NKT cells versus wild-type mice, and the majority of these *i*NKT cells are CD4⁺ (Fig. 7*a*). These mice have very few CD1d-restricted

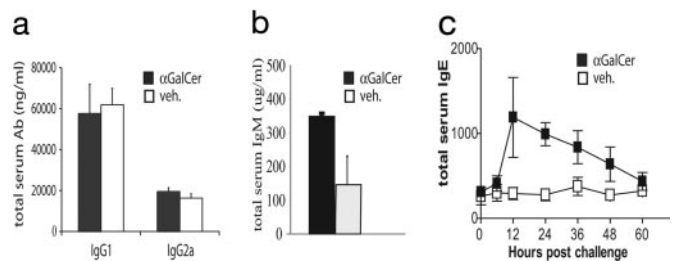


Fig. 3. Analysis of serum Ig levels after activation of pulmonary *i*NKT cells. After i.n. challenge with $1.5 \mu\text{g}$ of α -GalCer, (a) total serum IgG1 and IgG2a were unchanged at 24 h as measured by ELISA, representative of four experiments ($n \geq 4$). (b) Total serum IgM levels increase 2- to 3-fold at 24 h for mice in a. (c) Total serum IgE levels increase after $1.5 \mu\text{g}$ of α -GalCer challenge, as measured by ELISA at 6, 12, 24, 36, 48, and 60 h. Data represent the mean \pm SEM pooled from three experiments ($n = 10$).

pulmonary $\gamma\delta$ T cells (data not shown). Surprisingly, challenge of the MHC Δ/Δ mice with α -GalCer induced severe airway inflammation, eosinophilia, and severe AHR that was much greater than that observed in α -GalCer-challenged wild-type mice (Fig. 7*b*; see also Fig. 10, which is published as supporting information on the PNAS web site). Moreover, the saline-challenged MHC Δ/Δ mice showed greatly increased baseline responsiveness to methacholine, well beyond that of saline-challenged wild-type mice, although to a much lower degree than α -GalCer-challenged MHC Δ/Δ mice. Although the AHR response in the MHC Δ/Δ mice to α -GalCer was not accompanied by a detectable serum IgE response (data not shown), the development of severe AHR response in MHC Δ/Δ mice demonstrates that AHR can indeed occur in the total absence of conventional CD4⁺ T cells.

We confirmed that the exaggerated AHR response in the MHC Δ/Δ mice was due to CD1d-restricted NKT cells and not some other confounding factor due to the loss of conventional CD4⁺ T cells, because treatment of these mice with an anti-CD1d mAb completely blocked the response to α -GalCer in terms of AHR (Fig. 7*c*) and airway inflammation (Fig. 10). Importantly, the high level of baseline AHR in naïve MHC Δ/Δ mice was also due to a CD1d-restricted *i*NKT cell response (presumably in response to endogenous glycolipids), because treatment of the naïve MHC Δ/Δ mice with anti-CD1d mAb completely reversed the elevated baseline airway responsiveness (Fig. 7*d*).

Discussion

In this paper, we demonstrate that the specific and direct activation of *i*NKT cells by glycolipid antigens results in the rapid induction of AHR, airway inflammation, and IgE production, which typifies the Th2-driven response normally associated with allergen administration. The induction of AHR with direct *i*NKT cell activation was independent of eosinophils and B cells and did not occur in *i*NKT cell-deficient *Ja18*^{-/-} or *CD1d*^{-/-} mice. Surprisingly, the induction of AHR was also completely independent of conventional MHC class II-restricted CD4⁺ helper T cells and hence of adaptive immunity. In fact, airway responsiveness to methacholine was enhanced in MHC class II knockout mice, which lack conventional CD4⁺ T cells, but which have increased lung *i*NKT cells. The AHR response in MHC class II knockout mice was eliminated by the administration of anti-CD1d mAb, establishing that CD1d-restricted *i*NKT cells play an essential and critical role in the development of AHR independent of conventional CD4⁺ T cells. Because conventional CD4⁺ T cells have been thought to perform an obligatory role in the pathogenesis of asthma, our studies demonstrating that AHR can occur in the complete absence of conventional CD4⁺ T cells suggest that *i*NKT cells may mediate a previously unrecognized effector pathway contributing to the pathogenesis of AHR.

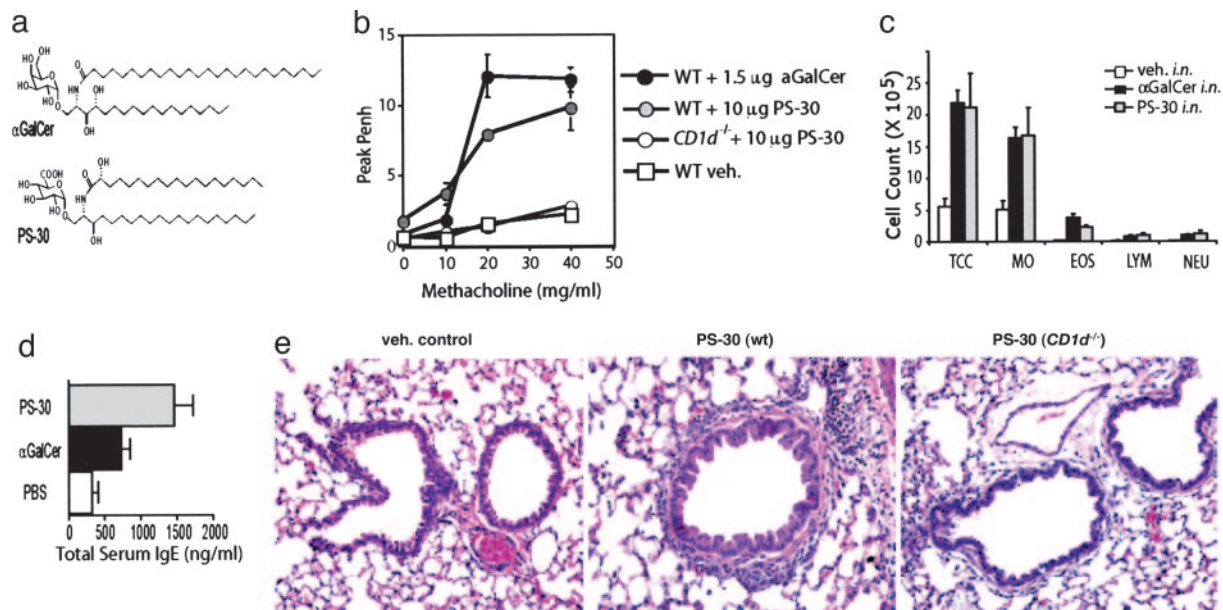


Fig. 4. Airway response after *Sphingomonas* antigen PS-30 activation of pulmonary *i*NKT cells. (a) Molecular structure of α -GalCer and PS-30. α -GalCer, with an unusual α linkage at the 1' carbon of the sugar to its sphingosine base, is a more potent activator of *i*NKT cells than PS-30, which has a biologically more common β linkage at the 1' carbon of the sugar linked at its sphingosine base. (b) Wild-type but not *i*NKT cell-deficient mice develop AHR, assessed as in Fig. 1a at 24 h after i.n. challenge with 10 μ g of PS-30 or 1.5 μ g of α -GalCer in wild-type BALB/c versus $CD1d^{-/-}$ mice. Data are the mean \pm SEM Penh, representative of three experiments ($n \geq 4$). (c) *Sphingomonas* antigen i.n. challenge induces airway eosinophilia. BAL fluid from mice in a was analyzed at 3 h after airway measurements, shown as in Fig. 1c. (d) Total serum IgE levels, measured by ELISA in the sera of mice in b, increase 2- to 3-fold after *Sphingomonas* antigen i.n. challenge, which also results in (e) eosinophilic and lymphocytic peribronchiolar inflammation and increased eosinophilic cytoplasm of bronchiolar lining cells (Center) not seen in similarly treated $CD1d^{-/-}$ mice (Right). Images are representative sections ($\times 200$).

The possibility that *i*NKT cells function as effector cells is supported by our result that *i*NKT cells induced AHR independent of conventional $CD4^+$ T cells, eosinophils, and B cells, although other cell types could serve as effector cells. In contrast, the conventional wisdom is that *i*NKT cells trigger the immune system by rapidly releasing cytokines on activation, which then influences the function of dendritic cells, macrophages, NK cells, B cells, and conventional $CD4^+$ Th2 cells (26, 27), thereby amplifying the subsequent development of adaptive autoimmune, antimicrobial, antitumor, and transplant immune responses (28–30). For example, IFN- γ release by *i*NKT cells enhances the differentiation of effector $CD4^+$ Th1 cells that mediate autoimmune disease or protection against specific infectious organisms (31–33). Similarly, we previously proposed that *i*NKT cells might “license” conventional Th2 effector cells, which would orchestrate the development of AHR and asthma (15).

We now believe, however, that direct activation of *i*NKT cells by antigen allows *i*NKT cells to mediate AHR and Th2 inflammation, suggesting that *i*NKT cells function directly as effector cells in inducing AHR, a role that is distinct from their role in autoimmunity or infection. That *i*NKT cells function as effector cells is consistent with our observation that a large fraction of $CD4^+$ cells in the lungs of human patients with asthma are *i*NKT cells and not conventional $CD4^+$ T cells (17). Moreover, because pulmonary *i*NKT cells are phenotypically similar to conventional $CD4^+$ Th2 effector cells [pulmonary *i*NKT cells are $CD4^+$ and produce Th2 cytokines when activated with α -GalCer or *Sphingomonas* glycolipids], it is possible that pulmonary *i*NKT “effector” cells may have been mistakenly identified in the past as Th2 cells.

Both α -GalCer and *Sphingomonas* glycolipid induced a very rapid *i*NKT cell-mediated rise in serum IgE. Increased serum IgE levels of 6-fold 9 days after a single dose or multiple doses of α -GalCer were reported (34), but the rapid rise we observed in serum IgE over 24 h is reminiscent of an innate immune pathway and is distinct from the induction of antigen-specific IgE, which can

occur in the absence of *i*NKT cells (35). The rapid rise in IgE may be related to the observation that *i*NKT cells activate B cells to produce IgM (14, 36), and the observation that *in vivo* treatment with rIL-18 results in the *i*NKT cell-dependent production of nonspecific IgE antibody (37). This unique innate-like *i*NKT cell-induced IgE production may represent a reflex response that might be important for host defense against parasites and bacteria that express NKT cell activating glycolipids (9, 10). Although the development of AHR in our glycolipid-induced AHR model was independent of the rise in IgE, *i*NKT cell-mediated IgE responses might contribute to the severity of atopic diseases and asthma.

Although we found that administration of α -GalCer directly induces AHR, several groups recently found that administration of α -GalCer 24 h before challenge with OVA inhibited allergen-induced AHR (19–21). In contrast, other investigators have found that coadministration of α -GalCer with antigen sensitizes mice to these antigens, thereby enhancing AHR (38, 39). These seemingly contradictory results confirm that *i*NKT cells are critically involved in the regulation of AHR and indicate that the timing of activation of the *i*NKT cells dictates their role in AHR. Thus, activation of *i*NKT cells can function as an adjuvant when *i*NKT cells are activated during the administration of protein antigen (38), can directly induce AHR when they are activated in the absence of other signals (Fig. 1), or can function to “prevent” the development of AHR, when they are rendered unresponsive (possibly anergic) at a critical time point by strong NKT cell activating agents (18). It is possible that the development of human asthma could depend upon the activation state of *i*NKT cells.

In our studies, glycolipid antigens could activate *i*NKT cells and induce AHR independent of conventional $CD4^+$ T cells, suggesting that *i*NKT cells may autonomously induce AHR, at least in some forms of asthma. However, we believe that conventional $CD4^+$ T cells are still likely to play a significant role in asthma. Conventional $CD4^+$ T cells respond to allergens important in asthma and produce IL-4 and IL-13, which are known to amplify and prolong allergic

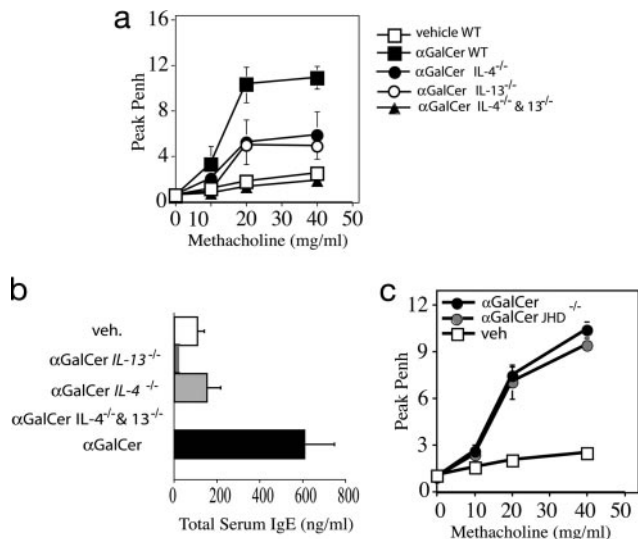


Fig. 5. IL-4 and IL-13 but not B cells or IgE are necessary for the full development of AHR. (a) AHR depends upon IL-4 and IL-13. After i.n. challenge with 1.5 μ g of α -GalCer, AHR was assessed as in Fig. 1a and was reduced in $IL-4^{-/-}$ and $IL-13^{-/-}$ single knockout and absent in $IL-4^{-/-}IL-13^{-/-}$ double knockout *BALB/c* mice. Data are the mean \pm SEM Penh, representative of four experiments ($n = 3-5$). (b) Total serum IgE, collected from mice in a 1–2 h after AHR measurement, was reduced in $IL-4^{-/-}$ and absent in $IL-13^{-/-}$ and $IL-4^{-/-}IL-13^{-/-}$ versus wild-type mice (measured by ELISA). Data are the mean \pm SEM, representative of four experiments ($n = 4-5$), two of which examined only $IL-13^{-/-}$ and $IL-4^{-/-}IL-13^{-/-}$ mice. (c) AHR does not require B cells. AHR was assessed as in Fig. 1a for B cell-deficient *JHD*^{-/-} versus wild-type *BALB/c* mice at 24 h after 1.5 μ g of α -GalCer i.n. challenge. Data are the mean \pm SEM Penh, representative of five experiments ($n \geq 4$).

inflammation. Because conventional CD4⁺ T cells alone do not appear to induce AHR in mouse models of asthma (15, 16), we suggest that in many forms of asthma, *i*NKT cells are synergistic with conventional CD4⁺ T cells in the induction of allergic pulmonary inflammation. First, *i*NKT cells may become activated early on and, by releasing IL-4 and IL-13, boost the priming of allergen-specific conventional CD4⁺ T cells (38, 39). Second, we suggest that inflammation or pulmonary injury induced by allergen-specific Th2 cells may modify or uncover self-glycolipid antigens, which then activate *i*NKT (effector) cells that directly induce AHR. Although conventional CD4⁺ T cells may be important in the initial response to exogenous allergens (for example, in determining whether *i*NKT cells are triggered), our current studies suggest that *i*NKT cells can play a major amplifying and pathogenic role once allergen recognition occurs and endogenous glycolipids are exposed.

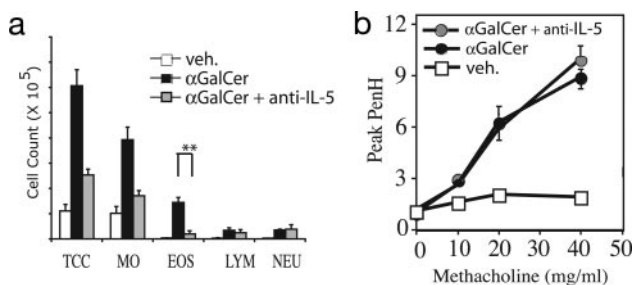


Fig. 6. IL-5 and eosinophils are not required for AHR. (a) *BALB/c* mice treated with anti-IL-5 mAb blocking antibody (described in *Materials and Methods*) and challenged with 1.5 μ g of α -GalCer i.n. show >85% reduction in BAL eosinophils at 24–27 h versus isotype control-treated mice ($P < 0.05$; Student's *t* test). Results represent three experiments, shown as in Fig. 1c. (b) Mice depleted of lung eosinophils in a show normal AHR at 24 h, assessed as in Fig. 1a. Data are the mean \pm SEM Penh, representative of three experiments ($n \geq 4$).

The specific glycolipid antigens that might activate *i*NKT cells *in vivo* to induce asthma are not yet identified. However, glycolipids that are recognized by the invariant TCR of NKT cells appear to be highly conserved and can include pollens (11, 23). As we show here, a glycolipid constituent of *Sphingomonas* cell membranes, synthesized as PS-30, activates *i*NKT cells to induce a rapid AHR response after respiratory administration in wild-type but not *i*NKT cell-deficient mice (9, 10). This pulmonary response to a *Sphingomonas* glycolipid indicates that the induction of AHR by glycolipids is not limited to the marine sponge glycolipid, α -GalCer, and suggests a pathway through which microorganisms or pollen antigens may be presented by CD1d⁺ APC to activate *i*NKT cells and induce AHR. This might play a role in some forms of asthma in humans.

In summary, we showed that *i*NKT cells activated by glycolipids can directly effect the development of AHR and airway inflammation, independent of conventional CD4⁺ T cells, eosinophils, and B cells, and hence of adaptive immunity. In view of previous reports showing that the induction of allergen-induced AHR absolutely requires *i*NKT cells, which may have been mistakenly thought in the past to be Th2 effector cells, are critical effector cells that drive the development of asthma, in concert with or instead of conventional CD4⁺ T cells in some forms of asthma. Therefore, therapies that target *i*NKT cells may be clinically effective in limiting AHR and asthma.

Materials and Methods

Mice. Wild-type *BALB/c ByJ* and *C57BL/6* mice were purchased from The Jackson Laboratory. $IL-4^{-/-}$ -deficient mice on the *BALB/c ByJ* background and *B6.129-H2 dIAb1-Ea/J* (MHC Δ/Δ mice) on the *C57BL/6* background were also purchased from The Jackson Laboratory. *CD1.1^{-/-}* (*Cd1d^{-/-}*) and *Ja18^{-/-}* on the *BALB/c* background were gifts from M. Grusby (Harvard School of Public Health, Boston) and M. Taniguchi (Chiba University), respectively. $IL-13^{-/-}$, $IL-4^{-/-}IL-13^{-/-}$ on the *BALB/c* background were a gift of A. McKenzie (Medical Research Council, Laboratory of Molecular Biology, Cambridge, U.K.). B cell-deficient *JHD* mice were obtained from D. Huszar (GenPharm, Mountain View, CA) and were backcrossed to *BALB/c ByJ* for seven generations. Animals were used between 5 and 16 wk of age and were age- and sex-matched. All animal protocols were approved by the Stanford University and Children's Hospital committee on animal welfare.

Antibodies and Reagents. Neutralizing rat anti-mouse CD1.1 mAb (hybridoma HB323, American Type Culture Collection) and rat anti-mouse IL-5 neutralizing antibody (hybridoma TRFK5, a gift of T. Mossman, DNAX) were purified by ammonium sulfate precipitation and ion-exchange chromatography. The CD1.1 blocking antibody was used at a dose of 500 μ g per mouse administered i.p. 1 day before antigen challenge. The IL-5 neutralizing antibody was used at a dose of 500 μ g per mouse administered i.p. at 5 days and again 1 day before antigen challenge. Isotype control rat mAb were administered in the same fashion (R & D Systems). α -GalCer (KRN7000) and its corresponding proprietary vehicle control were a gift of Kirin Brewery, Takasaki, Japan. *Sphingomonas* glycolipid antigen, PS-30 (also known as PBS-30), was synthesized as described (9). Both α -GalCer and PS-30 (0.2 mg each per aliquot) were provided as a lyophilized powder that included a vehicle (40). The vehicle included sucrose (56 mg/ml), L-histidine (7.5 mg/ml), cysteine (7.5 mg/ml in PS-30), and polysorbate 20 (5 mg/ml). Glycolipids were brought up in water (to a concentration of 0.2 μ g/ml), and this initial stock was further diluted in PBS just before i.n. or i.v. challenge.

Induction of AHR and Measurement of Airway Responsiveness. CD1d glycolipid antigens were administered i.n. (1.5–2 μ g of α -GalCer and 10 μ g of PS-30, both in 50 μ l) or i.v. (1.5 μ g of α -GalCer in 150

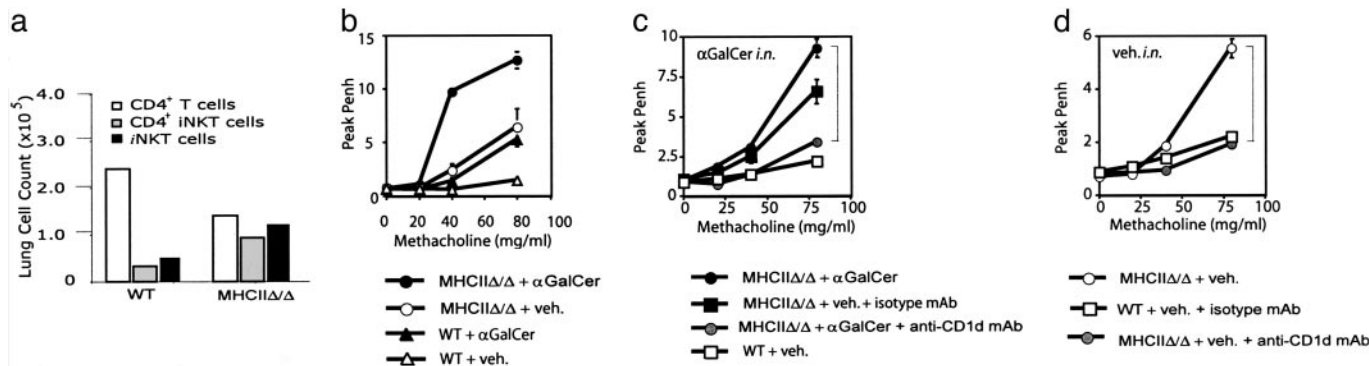


Fig. 7. Conventional MHC class II-restricted CD4 T cells are not required for AHR, and elevated AHR in *MHCII* Δ/Δ mice is CD1d-dependent. (a) *MHCII* Δ/Δ mice have more pulmonary iNKT cells. Lungs from *MHCII* Δ/Δ mice and wild-type *C57BL/6* mice were digested and stained with anti-TCR β mAb, anti-CD4 mAb, and CD1d-tetramer for evaluation by flow cytometry. TCR β^+ CD1d tetramer $^+$ cell numbers were increased in the lungs of *MHCII* Δ/Δ versus wild-type mice. Total cell counts (bar graph) show that iNKT cells are the majority of lung CD4 $^+$ cells in *MHCII* Δ/Δ (69%) versus wild-type (14%) mice, representative of three experiments ($n = 3-4$). (b) *MHCII* Δ/Δ mice had exaggerated representative of three experiments ($n = 3-4$) resting state and postchallenge AHR, assessed in wild-type *C57BL/6* versus *MHCII* Δ/Δ at 24 h after i.n. challenge with 2 μ g of α -GalCer or vehicle controls. Data are the mean \pm SEM Penh, representative of three experiments ($n = 4-5$). (c) Exaggerated AHR seen in *MHCII* Δ/Δ mice after i.n. challenge with 2.0 μ g of α -GalCer was assessed as in Fig. 1a in *C57BL/6* wild-type versus *MHCII* Δ/Δ mice at 24 h. Some *MHCII* Δ/Δ mice were treated with anti-CD1d blocking or isotype mAb before challenge. Data are the mean \pm SEM Penh, representative of three experiments ($n = 4$). (d) AHR in anti-CD1d-treated resting mice was reduced to wild-type control levels. AHR was assessed in wild-type versus *MHCII* Δ/Δ at 24 h after i.n. challenge with vehicle controls or PBS. Some *MHCII* Δ/Δ mice were treated with anti-CD1d mAb (indicated). Data are the mean \pm SEM Penh, representative of three experiments ($n = 3-4$).

μ l) to mice anesthetized with ketamine and xylazine. Glycolipid vehicles were administered as controls. Allergen-induced AHR was induced as described (15). AHR was measured as described (15). In some experiments, AHR was assessed by invasive measurement of airway resistance, in which anesthetized and tracheostomized mice were mechanically ventilated modified version of a described method (Buxo Electronics) (15). For additional materials and methods, see *Supporting Text*, which is published as supporting information on the PNAS web site.

Lymph Node Cell Restimulation and Collection of BAL Fluid and Lung Histology. Lymph node cells were isolated from the OVA-primed mice by mechanical disruption and were restimulated *in vitro* (5.0×10^5 cells per well in a 96-well plate) with 33 μ g/ml OVA. Supernatant were collected after 4 days of culture and assayed for cytokines. BAL and lung histology was collected as described (15).

Flow Cytometry and FACS. Analysis of iNKT cells was performed by preincubation of cells (2×10^6) with mAb against the Fc γ receptor (2.4G4). After washing, cells were incubated with α -GalCer-loaded mouse CD1d tetramer (16) or mouse CD1d:Ig Dimer (Becton Dickinson) for 1 h. Samples were sometimes costained with monoclonal anti-TCR β -cyochrome (PharMingen), anti-TCR β PeCy5.5 (eBioscience, San Diego, CA), or CD4-PE (PharMingen).

Cytokine and Serum Antibody Measurement. Cytokine ELISAs were completed as described (15). Serum IgE, IgG1, IgG2a, and OVA-specific IgE antibodies were obtained by ELISA as described (15).

We thank A. McKenzie, Medical Research Council, Laboratory of Molecular Biology, Cambridge, U.K., and M. Grusby, Harvard School of Public Health, for providing genetically modified mice. These studies were supported by Public Health Service Grants RO1 HL62348, RO1 HL69507, and RO1 AI26322.

- Robinson, D. S., Hamid, Q., Ying, S., Tsiopoulos, A., Barkans, J., Bentley, A. M., Corrigan, C., Durham, S. R., & Kay, A. B. (1992) *N. Engl. J. Med.* **326**, 298-304.
- Grunig, G., Warnock, M., Wakil, A. E., Venkayya, R., Brombacher, F., Rennick, D. M., Sheppard, D., Mohrs, M., Donaldson, D. D., Locksley, R. M., et al. (1998) *Science* **282**, 2261-2263.
- Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, b., Neben, T. Y., Karp, C. L. & Donaldson, D. D. (1998) *Science* **282**, 2258-2261.
- Holt, P. G., Macaubas, C., Stumbles, P. A. & Sly, P. D. (1999) *Nature* **402**, B12-B17.
- Wills-Karp, M. (1999) *Annu. Rev. Immunol.* **17**, 255-281.
- Cohn, L., Elias, J. A. & Chupp, G. L. (2004) *Annu. Rev. Immunol.* **22**, 789-815.
- Taniguchi, M., Harada, M., Kojima, S., Nakayama, T. & Wakao, H. (2003) *Annu. Rev. Immunol.* **21**, 483-513.
- Kronenberg, M. & Gapin, L. (2002) *Nat. Rev. Immunol.* **2**, 557-568.
- Mattner, J., DeBord, K. L., Ismail, N., Goff, R. D., C. C., III, Zhou, D., Saint-Mezard, P., Wang, V., Gao, Y., Yin, N., Hoehle, K., et al. (2005) *Nature* **434**, 525-529.
- Kinjo, Y., Wu, D., Kim, G., Xing, G.-W., Poles, M., Ho, D. D., Tsuji, M., Kawahara, K., Wong, C.-H. & Kronenberg, M. (2005) *Nature* **434**, 520-525.
- Zhou, D. P., Mattner, J., Cantu, C., Schrantz, N., Yin, N., Gao, Y., Sagiv, Y., Hudspeth, K., Wu, Y. P., Yamashita, T., et al. (2004) *Science* **306**, 1786-1789.
- Brigl, M. & Brenner, M. B. (2004) *Annu. Rev. Immunol.* **22**, 817-890.
- Cui, J. Q., Shin, T., Kawano, T., Sato, H., Kondo, E., Taura, I., Kaneko, Y., Koseki, H., Kanno, M. & Taniguchi, M. (1997) *Science* **278**, 1623-1626.
- Zeng, D. F., Liu, Y. P., Sidobre, S., Kronenberg, M. & Strober, S. (2003) *J. Clin. Invest.* **112**, 1211-1222.
- Akbari, O., Stock, P., Meyer, E., Kronenberg, M., Sidobre, S., Nakayama, T., Taniguchi, M., Grusby, M. J., DeKruyff, R. H. & Umetsu, D. T. (2003) *Nat. Med.* **9**, 582-588.
- Lisbonne, M., Diem, S., de Castro Keller, A., Lefort, J., Araujo, L., Hachem, P., Fourneau, J., Sidobre, S., Kronenberg, M., Taniguchi, M., et al. (2003) *J. Immunol.* **171**, 1637-1641.
- Perola, O., Nousiainen, T., Suomalainen, S., Aukee, S., Karkkainen, U., Kauppinen, J., Ojanen, T. & Katila, M. (2002) *J. Hosp. Infect.* **50**, 196-201.
- Parikh, V. V., Wilson, M. T., Olivares-Villagomez, D., Singh, A. K., Wu, L., Wang, C. R., Joyce, S. & Van Kaer, L. (2005) *J. Clin. Invest.* **115**, 2572-2583.
- Akbari, O., Faul, J. L., Hayte, E. G., Gerry, G. J., Wahlström, J., Kronenberg, M., DeKruyff, R. H. & Umetsu, D. T. (2006) *N. Engl. J. Med.*, in press.
- Matsuda, H., Suda, T., Sato, J., Nagata, T., Koide, Y., Chida, K. & Nakamura, H. (2005) *Am. J. Respir. Cell Mol. Biol.* **33**, 22-31.
- Hachem, P., Lisbonne, M., Askenase, P. W., Dy, M. & Leite-de-Moraes, M. C. (2005) *Eur. J. Immunol.* **35**, 2793-2802.
- Burdin, N., Brossay, L. & Kronenberg, M. (1999) *Eur. J. Immunol.* **29**, 2014-2025.
- Agea, E., Russano, A., Bistoni, O., Mannucci, R., Nicoletti, I., Corazzi, L., Postle, A. D., Libero, G. D., Porcelli, S. A. & Spinozzi, A. F. (2005) *J. Exp. Med.* **202**, 295-308.
- Lin, M. & Rikihisa, Y. (2003) *Infect. Immun.* **71**, 5325-5331.
- Kawahara, K., Kiraiishi, H. & Zahringer, U. (1999) *J. Industrial Microbiol. Biotechnol.* **23**, 408-413.
- Stein-Streilein, J. (2003) *J. Exp. Med.* **198**, 1779-1783.
- Godfrey, D. I., MacDonald, H. R., Kronenberg, M., Smyth, M. J. & Van Kaer, L. (2004) *Nat. Rev. Immunol.* **4**, 231-237.
- Godfrey, D. I. & Kronenberg, M. (2004) *J. Clin. Invest.* **114**, 1379-1388.
- Vincent, M. S., Gumperz, J. E. & Brenner, M. B. (2003) *Nat. Immunol.* **4**, 517-523.
- Seino, K. & Taniguchi, M. (2004) *Front. Biosci.* **9**, 2577-2587.
- Schmig, J., Yang, G. L., Franck, R. W. & Tsuji, M. (2003) *J. Exp. Med.* **198**, 1631-1641.
- Nieuwenhuis, E. E. S., Matsumoto, T., Exley, M., Schleipman, R. A., Glickman, J., Bailey, D. T., Corazza, N., Colgan, S. P., Onderdonk, A. B. & Blumberg, R. S. (2002) *Nat. Med.* **8**, 588-593.
- Levy, O., Orange, J. S., Hibberd, P., Steinberg, S., LaRussa, P., Weinberg, A., Wilson, S. B., Shaulov, A., Fleisher, G., Geha, R. S., et al. (2003) *J. Infect. Dis.* **188**, 948-953.
- Parikh, V. V., Singh, A. K., Wilson, M. T., Olivares-Villagomez, D., Bezbradica, J. S., Inazawa, H., Ehara, H., Sakai, T., Serizawa, I., Wu, L., et al. (2004) *J. Immunol.* **173**, 3693-3706.
- Smiley, S. T., Kaplan, M. H. & Grusby, M. J. (1997) *Science* **275**, 977-979.
- Campos, R. A., Szczezanik, M., Itakura, A., Akahira-Azuma, M., Sidobre, S., Kronenberg, M. & Askenase, P. W. (2003) *J. Exp. Med.* **198**, 1785-1796.
- Leite-De-Moraes, M. C., Hameg, A., Pacilio, M., Koezuka, Y., Taniguchi, M., Van Kaer, L., Schneider, E., Dy, M. & Herbelin, A. (2001) *J. Immunol.* **166**, 945-951.
- Kim, J. O., Kim, D. H., Chang, W. S., Hong, C. W., Park, S. H., Kim, S. H. & Kang, C. Y. (2004) *J. Allergy Clin. Immunol.* **114**, 1332-1338.
- Bilenki, L., Yang, J., Fan, Y. J., Wang, S. & Yang, X. (2004) *Eur. J. Immunol.* **34**, 345-354.
- Giaccone, G., Punt, C. J., Ando, Y., Ruijter, R., Nishi, N., Peters, M. & Pinedo, H. M. (2002) *Clin. Cancer Res.* **8**, 3702-3709.