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CUTTING EDGE

Cutting Edge: Invariant Vα14 NKT Cells Are Required for Allergen-Induced Airway Inflammation and Hyperreactivity in an Experimental Asthma Model[1]

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Airway hyperreactivity (AHR), eosinophilic inflammation with a Th2-type cytokine profile, and specific Th2-mediated IgE production characterize allergic asthma. In this paper, we show that OVA-immunized Jα18$^{-/-}$ mice, which are exclusively deficient in the invariant Vα14^{+} (iVα14), CD1d-restricted NKT cells, exhibit impaired AHR and airway eosinophilia, decreased IL-4 and IL-5 production in bronchoalveolar lavage fluid, and reduced OVA-specific IgE compared with wild-type (WT) littermates. Adoptive transfer of WT iVα14 NKT cells fully reconstitutes the capacity of Jα18$^{-/-}$ mice to develop allergic asthma. Also, specific tetramer staining shows that OVA-immunized WT mice have activated (CD69^{+}) iVα14 NKT cells. Importantly, anti-CD1d mAb treatment blocked the ability of iVα14 T cells to amplify eosinophil recruitment to airways, and both Th2 cytokine and IgE production following OVA challenge. In conclusion, these findings clearly demonstrate that iVα14 NKT cells are required to participate in allergen-induced Th2 airway inflammation through a CD1d-dependent mechanism. The Journal of Immunology,* 2003, 171: 1637–1641.

Allergic asthma is a chronic inflammatory disease which is increasing throughout the Western world. This airway inflammation is associated with a predominant Th2 response generating eosinophilia, increased mucus production in the lung, airway hyperresponsiveness, and specific IgE Ab synthesis (1, 2). The production of Th2 cytokines, such as IL-4 and IL-5, is considered pivotal for the recruitment of eosinophils to the airways and for airway hyperreactivity (AHR),[4] which are characteristic of asthma. Adoptive transfer of effector Th2 cells into naive mice followed by exposure to inhaled Ags triggers eosinophilic inflammation, mucus hypersecretion, and airway hyperresponsiveness, definitively demonstrating the capacity of immune Th2 cells to induce the asthma phenotype (3). It is generally known that rapid generation of IL-4 supports the crucial Ag-specific Th2 cell differentiation. The exact source of this required IL-4 is not known, but besides Th2 cells themselves, cells like mast cells, basophils, γ/δ T cells, and NKT lymphocytes are thought to be contributors to Th2 immune responses.

NKT cells are a distinct subset of lymphocytes with NK markers and αβ TCR, that regulate immune responses in several models of autoimmunity, infection, and cancer (4). They are selected and are restricted by the nonpolymorphic MHC class I-like molecule, CD1d, and express an invariant Vα14 (iVα14)-Jα18 TCR chain (4, 5). Since the stimulation of their TCR by glycolipid ligands, such as exogenous α-galactosylceramide (α-GalCer) presented in the context of CD1d molecules, promptly induces secretion of high concentrations of IL-4 (6), it has been proposed that they may participate in the differentiation of Th2 cells. Indeed, α-GalCer-stimulated NKT lymphocytes, in some cases, promote a protective Th2 immune response (7, 8). However, even though Th2 differentiation can be accomplished normally in NKT cell-deficient mice (9, 10), it cannot be excluded that NKT cells influence the development of some immune responses involving Th2 cells.

In the present study, we demonstrate that the iVα14 NKT cell subset is required for airway eosinophilia, hyperresponsiveness, Th2 cytokine production, and elevated levels of IgE Abs, in an experimental model of allergic asthma.

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[4] Abbreviations used in this paper: AHR, airway hyperreactivity; BALF, bronchoalveolar lavage fluid; Penh, enhanced pause; iVα14, invariant Vα14^{+}, WT, wild type; α-GalCer, α-galactosylceramide.

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Materials and Methods

Mice

Six- to 8-wk-old male C57BL/6 wild-type (WT) and J$\alpha18^{-/-}$ (backcrossed at least 10 times in C57BL/6) (11) mice were bred in our own facilities. All mice were kept in well-controlled animal housing facilities and had free access to tap water and pellet food throughout the experimental period.

Ag immunization and airway challenge

At 1-wk intervals animals were immunized by an i.p. (day 0) and a s.c. (day 7) injection with 4 μg OVA (ICN Biomedicals) adsorbed on 1.6 mg of aluminium hydroxide gel (Merck, Darmstadt, Germany) in 0.4 ml of sterile saline. Beginning on day 14 following the initial immunization, mice were anesthetized with ketamine anesthesia (Imalgene 1000, 35 mg/kg; Merial, Lyon, France) and airway challenged twice (days 14 and 21) intranasally with 10 μg of OVA in 50 μl of saline. Control mice received only saline at these times. Twenty-four hours after the last intranasal challenge (day 22), each group consisting of 6–20 mice was analyzed for eosinophil infiltration, airway hyperresponsiveness, cytokine levels, and Ab production.

In some experiments, at 1-wk intervals, animals were immunized by three i.p. injections (days 0, 7, and 14) with 10 μg OVA adsorbed on 1.6 mg of aluminum hydroxide gel in 0.4 ml of sterile saline. Sera were collected on day 15 and storaged for IgE measurement.

Adoptive transfer

Spleen cells from WT or J$\alpha18^{-/-}$ mice were depleted of CD8$^+$ T cells, CD62L$^+$, and CD19$^+$ B cells labeled with the corresponding mAbs with anti-rat Ig-coated magnetic beads (Dynal, Compiègne, France). Among the mAbs used for cell depletion CD8 (clone 53.67) and CD62L (clone Mel14) were purified in our laboratory while the anti-CD19 (clone 1D3) was purchased from BD PharMingen (San Diego, CA). The enriched negative selected NKT spleen cell population (containing 10^5 iVα14 NKT cells, as assessed by CD1d/α-GalCer tetramers staining), as well as a negative control (equivalent numbers of similarly depleted spleen cells from J$\alpha18^{-/-}$ mice) were injected i.v. into J$\alpha18^{-/-}$ mice that had been immunized twice with OVA.

Blocking of NKT/CD1d cell interaction in vivo

In some experiments, in addition to OVA immunization and challenge (as described above), separate groups of mice were also treated i.p. with 0.15 mg of blocking rat anti-CD1d mAb (clone 20H2) or control rat IgG (Sigma-Aldrich, Stonheim, Germany) per mouse on days -1, 0, $+2$, $+6$, $+7$, $+9$, $+13$, $+14$, $+16$, $+20$, and $+21$. No differences in the percentage of iVα14 NKT splenocytes were observed following anti-CD1d or Ig treatment on day 22 when mice were sacrificed (data not shown).

Determination of airway hyperresponsiveness

Airway responsiveness was assessed as previously described (12). Briefly, 24 h after the last challenge, conscious mice were placed into a plethysmograph chamber (Buxco Eletronics, Sharon, CT), and respiratory parameters were measured after 10 min of aerosol administration of 150 mM methacholine (Sigma-Aldrich) delivered for 60 s. Airflow obstruction was expressed as enhanced pause (Penh), calculated as: Penh = [*Te* (expiratory time)/*Tr* (relaxation time)] $-$ 1 $\times$ [*Pef* (peak expiratory flow)/*Pif* (peak inspiratory flow)]. The values of Penh expressed per minute were averaged from three determinations recorded every 20 s.

Bronchoalveolar lavage fluid (BALF)

Immediately after assessment of AHR, mice were deeply anesthetized by i.p. injection of urethane (15 mg/10 g body weight) (Sigma-Aldrich), blood was collected, and the resulting serum was stored. Airways were washed twice with saline, and the BALF cell differential counts and percentages were determined by Diff-Quik (Baxter-Dale, Dudingen, Germany) staining of cytospin slides. Two-hundred cells per slide were counted. Aliquots of BALF were stored for cytokine measurement.

Determination of cytokines in BALF and of serial OVA-specific IgE

The levels of IL-4, IL-5, IL-13, and IFN-γ in BALF and OVA-specific IgE serum levels were assessed by ELISA as previously described (6, 12) OVA-specific IgE levels of samples were related to an internal standard from pooled sera of hyperimmunized BALB/c mice. Data were expressed as IgE index calculated as follows: IgE index = [OD sample $-$ OD buffer only]/[OD positive control $-$ OD buffer only].

Flow cytometric analysis

Kinetic analysis of NKT cell activation after exposure to OVA was performed. In brief, 24 h after immunizations and/or intranasal challenges, mice were sacrificed, and splenocytes were recovered and stained for 30 min in PBS containing 2% FCS and 0.01 M sodium azide and mCD1d-α-GalCer tetramer-APC. Tetramers were prepared in our laboratory from mCD1d/mβ2m expression vector (13). Cells were then incubated with appropriate dilutions of anti-CD4 PerCP-Cy-5.5 (clone RM4-5), anti-TCR$\alpha\beta$-FITC (clone H57-597) and anti-CD69-PE (H1.2F3), all purchased from BD PharMingen. Dead cells were excluded on the basis of forward and side scatter. At least 5×10^5 live lymphoid cells were acquired in each run. Samples were analyzed on a FACSCalibur (BD Biosciences, Mountain View, CA) using CellQuest software.

Statistical analysis

Nonparametric Mann-Whitney test was used to calculate significance levels for all measurements. Values of $p < 0.05$ was considered statistically significant.

Results

AHR and eosinophilic inflammation is reduced in J$\alpha18^{-/-}$ mice

Marked features in this model of allergen-induced asthma are AHR and massive airway inflammation observed following OVA challenge. Our findings clearly demonstrate that OVA-sensitized and -challenged J$\alpha18^{-/-}$ mice developed decreased AHR after methacholine inhalation as compared with OVA-sensitized and -challenged control mice (Fig. 1), implicating iVα14 NKT cells in AHR. The total cell number in BALF of J$\alpha18^{-/-}$ mice following two intranasal OVA challenges was decreased by 42% compared with WT mice (Fig. 2*A*). The percentage of eosinophils, which account for the majority of cells in BALF from OVA-challenged mice (up to 65%), was decreased by 58% in iVα14 NKT cell-deficient animals (Fig. 2*B*). Both macrophages and neutrophils also were reduced accounting for the overall decrease ($2.6 \pm 0.2 \times 10^5 \pm 0.2$ vs $1.7 \pm 0.2 \times 10^5$ macrophages/ml and $2.2 \pm 0.6 \times 10^5$ vs $1.1 \pm 0.1 \times 10^5$ neutrophils/ml in OVA-challenged WT vs J$\alpha18^{-/-}$ mice, respectively). Conversely, lymphocyte recruitment in BALF was not modified $7 \pm 2 \times 10^3$ vs $4.7 \pm 2 \times 10^3$ lymphocytes/ml in OVA-challenged WT and J$\alpha18^{-/-}$ mice, respectively). Among these lymphocytes, iVα14 NKT cells were also recruited to the lung of OVA-treated WT mice as assessed by RT-PCR analysis (data not shown).

Both IL-4 and IL-5 production are impaired in BALF of immunized J$\alpha18^{-/-}$ mice

IL-4 and IL-5 levels were markedly reduced in BALF collected 24 h after challenge from NKT cell-deficient mice as compared with WT littermates (Fig. 2, *C* and *D*). Impaired IL-5 production

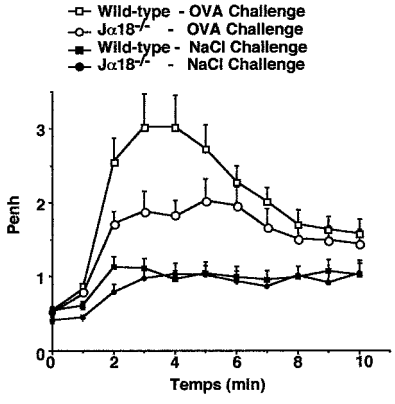

FIGURE 1. Decreased AHR development in J$\alpha18^{-/-}$ mice. Airway reactivity to methacholine was measured 1 day after the last challenge with OVA (○ and □) or NaCl (● and ■) in both WT (squares) and J$\alpha18^{-/-}$ (circles) mice. Data represent the mean ± SEM Penh values from 6 to 20 mice.

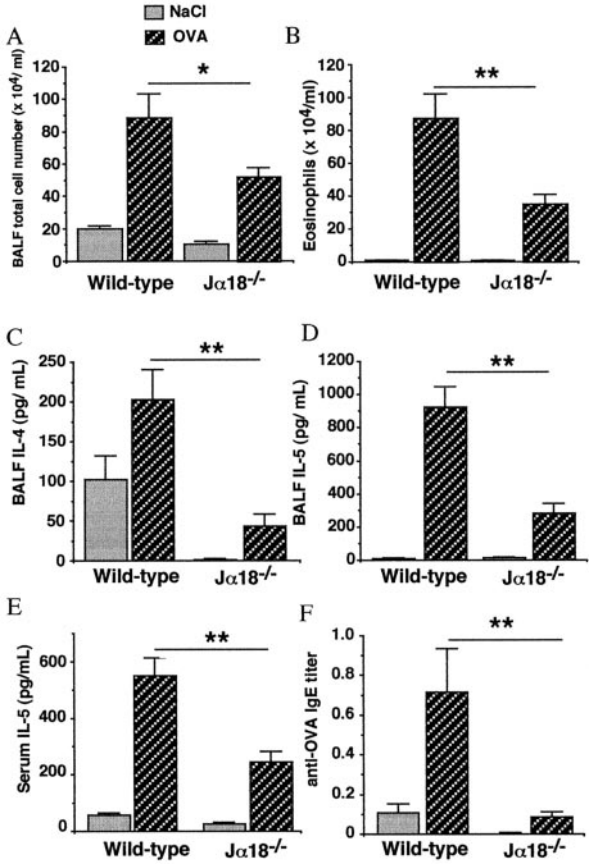

FIGURE 2. Marked decrease in specific airway eosinophilia, IL-4, IL-5, and anti-OVA IgE production in OVA-challenged Jα18$^{-/-}$ mice. Total (*A*) and eosinophil cell counts (*B*) were determined in the BALF of Jα18$^{-/-}$ and WT mice 24 h after the second NaCl or OVA challenge on day 22. Levels of IL-4 (*C*) and IL-5 (*D*) in BALF as well as IL-5 (*E*) and specific anti-OVA IgE Abs (*F*) in serum were also measured 24 h after the second NaCl or OVA challenge. Data are means ± SEM from 6 to 20 mice per group. *, $p < 0.05$; **, $p < 0.01$.

in response to OVA challenge was also observed in the serum of Jα18$^{-/-}$ mice (Fig. 2*E*), showing a systemic defect in the production of this cytokine in the absence of NKT cells. IL-4 levels in the serum were below detection limits (<10 pg/ml).

OVA-specific IgE secretion is markedly decreased in Jα18$^{-/-}$ mice

To assess the peripheral response to immunization and subsequent allergen challenge, we measured OVA-specific IgE levels in the sera using ELISA. OVA-immunized and -challenged Jα18$^{-/-}$ mice had one-eighth (12.3 ± 9.7%) OVA-specific IgE compared with WT littermates, indicating that IgE production was severely affected (Fig. 2*F*). The failure to produce high levels of anti-OVA IgE was not due to the inability of Jα18$^{-/-}$ mice to produce this isotype, because in response to three OVA immunizations but without intranasal challenges they did effectively produce anti-OVA IgE (0.83 ± 0.10 and anti-OVA IgE titer for Jα18$^{-/-}$ and 1.53 ± 0.22 for WT mice).

Adoptive transfer of iVα14 NKT cells restores AHR development, airway eosinophilia, as well as IL-4, IL-5, and IgE production in Jα18$^{-/-}$ mice

To confirm the implication of iVα14 NKT cells in the severity of allergic asthma, we reconstituted OVA-sensitized Jα18$^{-/-}$ mice with iVα14 NKT lymphocytes 24 h before the first OVA airway challenge. Adoptive transfer of iVα14 NKT cells, but not of spleen cells from Jα18$^{-/-}$ mice, fully restored the capacity of Jα18$^{-/-}$ mice to develop AHR (Fig. 3*A*). Moreover, airway eosinophilia (Fig. 3*B*), both IL-4 (Fig. 3*C*) and IL-5 (Fig. 3*D*) production in the BALF as well as specific anti-OVA IgE (Fig. 3*E*) in the serum of OVA-sensitized and -challenged Jα18$^{-/-}$ mice were restored by the adoptive transfer of iVα14 NKT cells.

iVα14 NKT cells are activated in OVA-treated mice

We evaluated whether iVα14 NKT lymphocytes were activated during OVA-induced allergic asthma, as assessed by expression

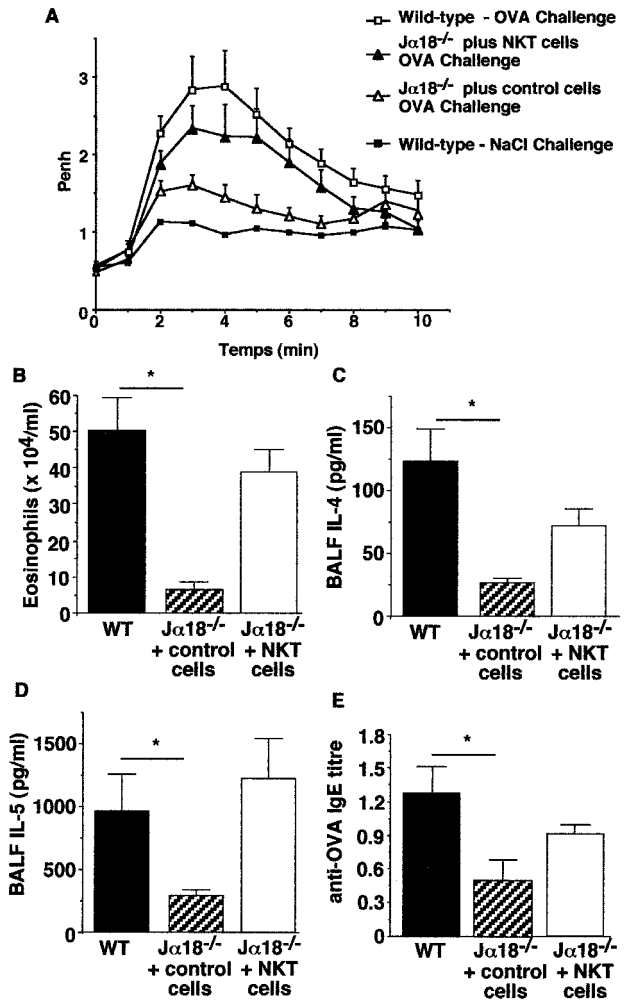

FIGURE 3. Decreased AHR development, eosinophilia as well as IL-4, IL-5, and anti-OVA IgE production in Jα18$^{-/-}$ mice are restored by adoptive transfer of NKT cells before challenge. Groups of OVA-immunized and -challenged Jα18$^{-/-}$ mice were adoptively transferred with enriched CD1d/α-GalCer tetramer positive splenocytes. Control Jα18$^{-/-}$ mice were adoptively transfered with splenocytes from Jα18$^{-/-}$ animals. *A,* Airway reactivity to methacholine was measured 1 day after the last challenge with OVA or NaCl in both WT (squares) and Jα18$^{-/-}$ (triangles) mice. Enriched CD1d/α-GalCer tetramer positive splenocytes (▲) were adoptively transferred into OVA-immunized and challenged Jα18$^{-/-}$ mice. Control Jα18$^{-/-}$ mice were adoptively transfered with splenocytes obtained from Jα18$^{-/-}$ animals (△). Data represent the mean ± SEM Penh values from 5 to 10 mice. *B–E,* Twenty-four hours after the last OVA challenge, airway eosinophil (*B*) numbers were evaluated and both IL-4 (*C*) and IL-5 (*D*) were measured in BALF. Serum levels of OVA-specific IgE (*E*) were determined by ELISA. Results represent means ± SEM from 5 to 10 mice. *, $p < 0.05$.

of the early activation marker CD69. Indeed, CD69 expression was up regulated on tetramer CD1d/α-GalCer$^+$TCRαβ$^+$ splenocytes from OVA-immunized and -challenged mice on day 21 compared with those immunized with OVA but challenged with NaCl (Fig. 4*A*), demonstrating the activation of these cells following the OVA treatments. Similar results were obtained on day 14 after the first OVA challenge (data not shown).

iVα14 NKT cells mediate Th2 allergen-induced airway inflammation in a CD1d-dependent manner

We have previously reported that NKT activation of iVα14$^+$ NKT cells does not necessarily occur in a CD1d-dependent manner, but can also be induced by the pro-inflammatory cytokines IL-12 and IL-18 (14). To evaluate possible CD1d involvement in NKT cell participation in Th2 asthmatic responses, WT mice were treated with blocking anti-CD1d mAb, or with control rat IgG at several time points during OVA immunization and challenge. Fig. 4*B* clearly shows that anti-CD1d treatment prevents the characteristic eosinophil recruitment to BALF observed after OVA challenge. Similarly, the secretion of both IL-4 (Fig. 4*C*) and IL-5 (Fig. 4*D*) levels in the BALF and of specific serum anti-OVA IgE Abs (Fig. 4*E*) were diminished when CD1d-dependent interactions were blocked.

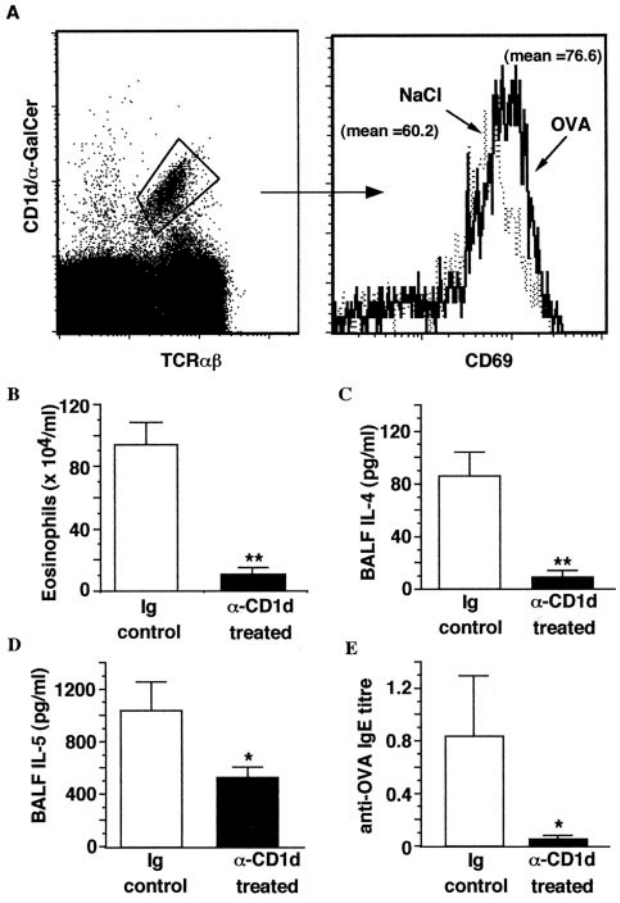

FIGURE 4. OVA immunization and challenge activates iVα14 NKT cells, while anti-CD1d mAb treatment diminishes airway eosinophilic recruitment, Th2 cytokine levels and OVA-specific IgE production. *A,* Representative FACS profiles showing up-regulation of CD69 expression on gated iVα14 CD1d/α-GalCer$^+$ TCRαβ$^+$ splenocytes recovered 24 h after the last OVA or NaCl challenge. *B–E,* Groups of C57BL/6 mice were treated with anti-CD1d mAb or control rat IgG (see *Materials and Methods*) and immunized with OVA and challenged with OVA or NaCl. Twenty-four hours later, serum and BALF were collected. Eosinophil numbers (*B*) and both IL-4 (*C*) and IL-5 (*D*) were measured in BALF while OVA-specific IgE (*E*) were determined in the serum. Results represent means ± SEM from 5 to 10 mice. *, $p < 0.05$; **, $p < 0.01$.

Discussion

Our experiments with Jα18$^{-/-}$ mice clearly show that iVα14 NKT lymphocytes are required for the characteristic features of experimental allergic asthma, namely airway inflammation, BALF Th2 cytokine production and the secretion of high levels of specific anti-OVA IgE, that will ultimely induce AHR.

The failure of Jα18$^{-/-}$ mice to develop the typical Th2 inflammation, AHR and IgE responses following OVA airway challenge is not due to a deficiency in Th2 responsiveness since these mice produce high levels of IgE in another protocol using three systemic immunizations with 2.5 higher doses of OVA. These data concur with previous reports showing that Jα18$^{-/-}$ mice are not defective in IgE production (15). In this study we used a protocol where all mice were similarly immunized with OVA and alum, and airway OVA challenge resulting in Th2 inflammation and AHR. Because we found that iVα14 NKT cells are present in the lung of OVA-challenged mice, it is possible that they are involved during challenge rather than necessarily during priming. According to this postulate, we adoptively transferred WT iVα14 NKT cells just before OVA challenge, and then sufficed to restore the capacity of Jα18$^{-/-}$ mice to fully develop airway eosinophilia, AHR, BALF cytokine, and anti-OVA IgE production.

Our results differ from those of a previous study using another type of NKT cell-deficient mice, lacking CD1d molecules (16), which demonstrated that allergen-specific IgE levels and eosinophilia were not different from those observed in WT mice. Discrepancies observed may be explained by the different experimental protocols (two vs multiple challenges, timing of mice sacrifice) as well as the genetic background of the mutant mice (129/Sv × C57BL/6 for CD1d$^{-/-}$ and C57BL/6 for Jα18$^{-/-}$ mice). It is unlikely that the genotype (CD1d$^{-/-}$ vs Jα18$^{-/-}$ mice) is responsible for these differences since CD1d$^{-/-}$ mice (backcrossed with C57BL/6) and Jα18$^{-/-}$ mice were similarly deficient in response to our protocol of immunization and challenge (data not shown). Moreover, in agreement with our results, it has recently been reported that both CD1d$^{-/-}$ and Jα18$^{-/-}$ mice, backcrossed with BALB/c, develop neither AHR nor airway eosinophilia, Th2 cytokine production and high levels of IgE following OVA challenge (17). Taken together, these results clearly demonstrate the requirement of iVα14 NKT cells in allergen-induced asthma.

Additionally, our findings show that iVα14 NKT cells are present in lungs of OVA-treated mice, that they are systemically activated (up-regulation of CD69), and that their implication in this model is mediated through CD1d interactions. We and others have already demonstrated that NKT cells stimulated with α-GalCer favor Th2 or Th1 protective immune responses in experimental autoimmune diseases or tumors, respectively (4). In contrast, the present study underscores the capacity of activated iVα14 NKT cells to mediate a pathological Th2 allergy immune response in asthma. It is probable that this activation involves recognition of endogenous Ags associated with CD1d molecules, because OVA itself was unable to stimulate NKT cells neither from naive nor from OVA-treated mice (day 22) (data not shown). Endogenous Ags capable of stimulating

iVα14 NKT cells remain to be determined, but it has been suggested that the self-Ags, presumably glycolipids, that bind CD1d are recognized by V$\alpha14^+$ TCR of these cells.

In conclusion, we have demonstrated that iVα14 NKT cells mediate Th2 eosinophil airway inflammation and AHR in an allergen-induced asthma model. Thus, NKT cells may mediate or amplify the pathogenic Th2 inflammatory response and crucial AHR in allergic asthma, suggesting that depletion or blockade of NKT cells might be a possible option for the treatment of this disease.

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