

Asthma is induced by intranasal coadministration of allergen and natural killer T-cell ligand in a mouse model

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Background: Allergic asthma is an inflammatory lung disease caused by a T_H2-driven immune response. However, intranasal exposures to soluble antigen lead to mucosal tolerance, and the mechanism involved in generation of T_H2 responses to inert inhaled allergens is unknown.

Objective: The aim of this study was to investigate whether CD1d-restricted natural killer T (NKT) cells can contribute to the induction of T_H2-dependent allergic asthma in a mouse model.

Methods: To investigate the effect of NKT cells on the development of asthma, NKT cell ligand, α -galactosylceramide (α GC), was used with antigens. We intranasally sensitized Balb/c mice with various combinations of antigen and α GC for 3 consecutive days and challenged them 2 weeks later with an aerosol of ovalbumin. NKT cell-deficient or T_H cell-deficient mice were immunized by administering ovalbumin and α GC together, and ovalbumin inhalation.

Results: Only when immunized with ovalbumin plus α GC, airway hyperreactivity, airway eosinophils, elevated IgE level, and T_H2-cytokine production were observed in Balb/c mice. Ovalbumin alone, α GC alone, or BSA plus α GC-immunized mice did not induce asthma. Studies in NKT cell-deficient, or CD4⁺ T-cell-deficient mice intranasally exposed to ovalbumin plus α GC did not show the development of asthma. An increase of NKT cells in bronchoalveolar lavage was observed in the pathologic states.

Conclusion: These data demonstrate that NKT cells can play crucial roles in allergen sensitization and pathologic states in asthma. Furthermore, our new asthma model using α GC will be very useful to induce asthma and to dissect the role of NKT cells and other cells in asthma. (*J Allergy Clin Immunol* 2004;114:1332-8.)

Key words: Natural killer T cell, α -galactosylceramide, asthma

Abbreviations used

α GC: α -Galactosylceramide
AHR: Airway hyperresponsiveness
BAL: Bronchoalveolar lavage
BALF: Bronchoalveolar lavage fluid
BLN: Bronchial lymph node
MLN: Mesenteric lymph node
NKT: Natural killer T
V α 14 ι : Invariant V α 14

Asthmas are believed to be caused by inappropriate T_H2 immune responses to commonly inhaled antigens.¹ However, only a subset of people exposed to these aeroallergens develops pathological T_H2 responses, and the mechanisms that determine whether tolerance or immunity develops in the respiratory mucosa in response to inhaled allergen are not well defined. Recently, the role of adjuvants and the innate immune system has been considered important in the induction of T_H2 responses.²

Natural killer T (NKT) cells are a unique subset of T cells that exist in both mice and human beings.³⁻⁵ NKT cells express an $\alpha\beta$ T-cell receptor as well as markers usually associated with natural killer cells and activated/memory T cells. A major subset of NKT cells is restricted by the MHC class I-like molecule CD1d.⁶⁻⁸ CD1d-restricted NKT cells mainly express a semi-invariant $\alpha\beta$ T-cell receptor composed of an invariant V α 14-J α 281 chain (also called J α 18 or J α 15), paired preferentially with V β 8.2 or V β 7 chains in mice, or V α 24-J α 18 and V β 11 in human beings with diverse CDR3 sequences.^{9,10} These invariant V α 14 (V α 14 ι) or V α 24 T cells recognize α -galactosylceramide (α GC), a specific marine sponge glycolipid, after activation, but the specific glycolipid antigens *in vivo* are not known.^{11,12} *In vivo*, α GC induces activation of NKT cells, which release both T_H1 and T_H2 cytokines, notably IFN- γ and IL-4, respectively, and elicit a downstream cascade of activation spreading to dendritic cells, natural killer cells, and B cells.¹³⁻¹⁶

To date, 3 reports have addressed that V α 14 ι NKT cells are required for the development of allergen-induced T_H2 airway inflammation through a CD1d-dependent mechanism.¹⁷⁻¹⁹ However, these previous studies induced asthma via sensitization by using alum as an adjuvant. In contrast, our current study is the first to induce asthma via the intranasal administration of α GC as an adjuvant. We here demonstrate the essential roles played by NKT

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cells in both the initial phase of antigen sensitizations and the pathological state by showing the kinetic changes in NKT cells in bronchoalveolar lavage (BAL) after aero-allergen challenges.

METHODS

Animals

Female BALB/c mice 6 to 8 weeks old were purchased from Daehan Biolink (Chungbuk, Korea) or Seoul National University Laboratory Animal Center. *CD1d*^{+/-}, *CD1d*^{-/-}²⁰ (backcrossed with BALB/c at least 12 times), and *MHC class II*^{-/-} mice (on a C57BL/6 background) were bred under aseptic conditions in facilities at Korea University. The studies reported here conformed to the principles for laboratory animal research outlined by Seoul National University.

Synthesis of α GC

α -Galactosylceramide was prepared by coupling of phytosphingosine with hexacosanoic acid followed by the protection/deprotection and galactosylation scheme as described.²¹

Antigen immunization and airway challenge

Mice were anesthetized with ketamine (Yuhan, Seoul, Korea) and xylazine (Bayer, Seoul, Korea) and then sensitized intranasally with 100 μ g ovalbumin (grade V; Sigma-Aldrich) $\pm$ 1 μ g α GC, 100 μ g BSA (Sigma-Aldrich, St Louis, Mo) plus α GC, or α GC alone in 20 μ L PBS on days 0, 1, and 2. Then mice were challenged on days 14, 15, 18, and 19 with an aerosol of 1% ovalbumin in PBS for 30 minutes by using the ultrasonic nebulizer NE-U12 (Omron, Tokyo, Japan). Nil group was naive for all experiments.

Measurement of airway hyperresponsiveness

One day after the last aerosol challenge (day 20), airway responsiveness was assessed as described.²² Airway hyperresponsiveness (AHR) was calculated in enhanced pause (Penh).

Determination of serum antibody concentrations

Mice were bled on day 21, and ovalbumin-specific IgG and IgE were determined by ELISA as previously described.²³

Analysis of bronchoalveolar lavage fluids

On day 21, mice were killed, and BAL cells were obtained and analyzed as previously described.²³ Differential cell counts were performed by counting at least 300 cells on cytocentrifuged preparations followed by staining with Diff-Quick (DADE Behring, Duding, Switzerland).

Measurement of cytokine production

At day 21, lung draining lymph nodes (peribronchial) were isolated and were prepared to single-cell suspensions. Per well, 2×10^5 cells were plated in 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark) and were cultured for 3 day with or without ovalbumin (50 μ g/mL) in 200 μ L RPMI 1640 medium containing 10% FBS (Biowhittaker, Walkersville, Md). Supernatants were analyzed for IL-4, IL-5, IFN- γ (BD Pharmingen, San Diego, Calif) and IL-13 (R&D Systems, Minneapolis, Minn) by ELISAs according to the manufacturer's directions. Then, the ovalbumin-specific cytokine levels were deduced.

Proliferation assay

Isolated bronchial lymph nodes, mesenteric lymph nodes, and spleen were prepared to single-cell suspensions and erythrocytes were depleted. Per well, 2×10^5 cells of lymph nodes and 5×10^5

cells of splenocytes were cultured with or without 50 μ g/mL ovalbumin. After 96 hours of incubation, including a final pulse of 14 to 16 hours with [³H] thymidine (1 μ Ci per well), the cultured cells were harvested, and [³H] thymidine incorporation was measured by using a liquid scintillation counter (Wallac, Turku, Finland). The results were expressed as ovalbumin-specific proliferative ability.

Lung histology

On day 21, lungs were fixed in 4% formalin. Formalin-preserved lungs were embedded in paraffin wax, sectioned at 5- μ m thicknesses, and stained with periodic acid Schiff.

Flow-cytometric analysis of NKT cells

α -Galactosylceramide was incubated with CD1d: Ig Recombinant Fusion Protein (BD Pharmingen, number 557599) at a molar ratio of 10:1. α GC-loaded CD1d complex was labeled with antimouse IgG1-FITC (SouthernBiotechnology Associates, Birmingham, Ala) at a weight ratio of 1:2 and incubated at 4°C overnight. The same amount of mouse IgG1 isotype control was added to block free antimouse IgG1-FITC. Then the mixture was used with phycoerythrin-conjugated antimouse CD3 mAb (BD Pharmingen) to stain the isolated single-cell suspensions. α GC-unloaded CD1d dimer was used as a control. The cells were pooled if the cell number of individual mice were below 5×10^5 and were analyzed by gating the lymphocytes by using a flow-cytometer, the FACScaliber (BD, Franklin Lakes, NJ).

Statistics

Results are expressed as the means $\pm$ SEMs. Each experiment was repeated at least twice ($n = 3$ -10). Values of $P < .05$ were considered statistically significant.

RESULTS

Both NKT cell activation and antigen specificity are required in the induction of asthma

To determine whether CD1d-restricted NKT cells can play a role in the induction of asthma, we divided mice into 4 sensitization groups: ovalbumin + α GC, ovalbumin, α GC, and BSA + α GC. In all experiments, mice were immunized as described in the Methods section (see Fig E1 in the Journal's Online Repository at www.mosby.com/jaci).

We first examined AHR by methacholine inhalation. When sensitized with ovalbumin antigen plus α GC, Balb/c mice developed severe AHR, whereas in contrast, the mice sensitized with ovalbumin or α GC alone did not develop AHR (Fig 1, A). To show that this effect was antigen-dependent, we included another group of mice that were sensitized with BSA plus α GC followed by the ovalbumin inhalation. That group of mice did not develop AHR. Collectively, these data indicate that NKT cell activation is necessary but not sufficient to induce allergen-mediated AHR.

The AHR in the ovalbumin + α GC group was also associated with a substantial increase in airway eosinophilia, as indicated by an increase of eosinophils in

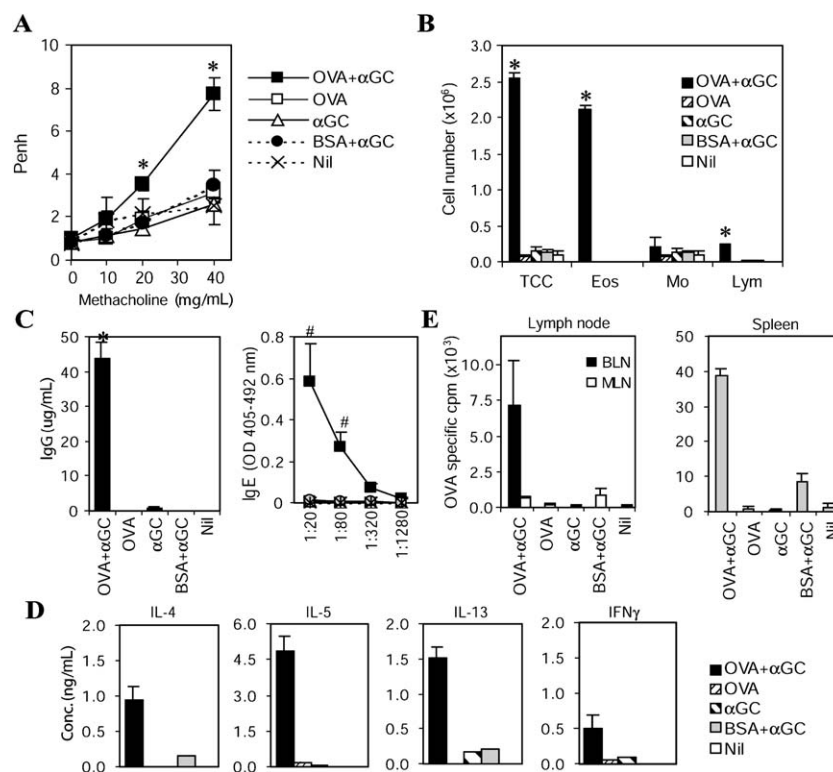


FIG 1. Analysis of antigen + α GC-induced airway responses in BALB/c mice. **A**, AHR reactivity to nebulized methacholine. **B**, The number of total cell count (TCC), eosinophils (Eos), monocyte/macrophage (Mo), and lymphocytes (Lym) in BALF. **C**, Ovalbumin (OVA)-specific IgG and IgE levels in serum. In the IgE graph, the x-axis represents the dilution of serum. **D**, Comparison of the OVA-specific cytokine production of BLN cells. **E**, Comparison of the OVA-specific proliferative ability of each group. $n = 3-5$ mice in each group. Conc, Concentration. $*P < .01$ compared with all other groups.

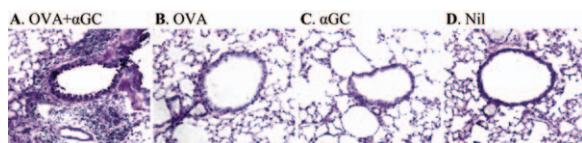


FIG 2. Histologic examination of lungs. On day 21, lung tissue was removed from individual mice, formalin-fixed and paraffin-embedded, and periodic acid Schiff-stained ($\times 200$). Lung tissue from ovalbumin (OVA) + α GC (**A**), OVA (**B**), α GC (**C**), and Nil (**D**).

bronchoalveolar lavage fluid (BALF; Fig 1, B) as well as of lung tissue infiltrates and airway mucus secretion (Fig 2). Bronchial lymph node (BLN) cells in this ovalbumin + α GC group showed the most marked increase of all section (see Table EI in the Online Repository at www.mosby.com/jaci). Mice exposed to BSA + α GC or to ovalbumin or α GC alone did not develop pulmonary inflammation after ovalbumin challenge (Fig 1, B; Fig 2). We measured IgE levels in the sera by using ELISA. Only the ovalbumin + α GC group showed an elevated ovalbumin-specific IgE level as well as an IgG level comparable with that seen in other groups of mice (Fig 1, C). It is well known that NKT cells release

IL-4 and IFN- γ immediately after activation and favor T_H2 or T_H1 protective immune responses in experimental autoimmune disease or tumors, respectively.²⁴ Accordingly, we analyzed the cytokine profiles of bronchial lymph node cells via *in vitro* ovalbumin stimulation. High levels of IL-4, IL-5, and IL-13 production confirmed the T_H2 nature of the inflammatory response in the ovalbumin + α GC group, although IFN- γ was detected in this group (Fig 1, D). The BSA + α GC group of mice showed a low level of IL-4 and IL-13 production. Other groups exposed to ovalbumin or α GC alone showed levels of cytokine production that were either low or below detection limits. We also analyzed the proliferative ability of BLN, mesenteric lymph node (MLN), and splenocytes in response to ovalbumin. BLN cells and splenocytes showed high ovalbumin-specific proliferation, whereas MLN cells did not. This finding suggests that ovalbumin + α GC sensitization and ovalbumin challenge affected the immune system systemically but that this effect did not reach the MLN (Fig 1, E). In summary, antigen-specific T_H2 immune responses and airway inflammation were induced in the presence of α GC with ovalbumin.

To determine whether the effect of α GC described was general to other antigens, we sensitized and challenged the

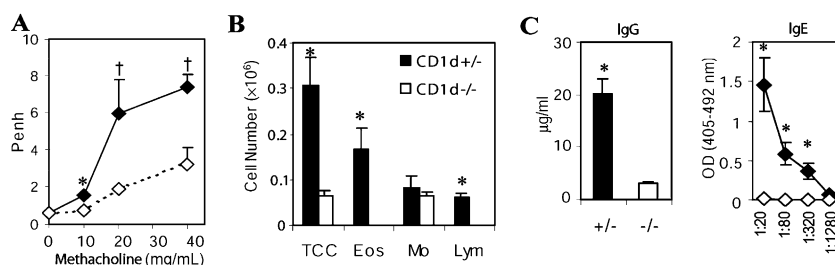


FIG 3. Analysis of antigen + αGC-induced airway responses in CD1d-deficient mice. **A**, The airway reactivity to nebulized methacholine. **B**, The number of total cell count (TCC), eosinophils (Eos), monocytes/macrophages (Mo), and lymphocytes (Lym) in BALF on day 21. **C**, Ovalbumin-specific IgG and IgE levels in serum. In the IgE graph, the x-axis indicates the dilution of serum. ♦, CD1d^{+/-}; ◇, CD1d^{-/-}. **P* < .01; †*P* < .05 compared with the CD1d^{-/-} group. Penh, Enhanced pause.

mice with BSA by using similar approaches. However, the mice were challenged with BSA intranasally (20 μg/mouse on days 14, 15, 18, and 19) rather than inhalation, because BSA vaporization was impossible even at a 0.1% concentration. These BSA-treated mice showed AHR, eosinophilia in BALF, BSA-specific IgE and IgG, and BSA-specific T-cell responses (data not shown). These findings indicate that the effect of αGC is not ovalbumin-specific but general to other antigens.

To isolate the effects caused by sensitization alone, the mice were exposed to 3 intranasal administrations of ovalbumin + αGC (day 0, 1, and 2) without further inhalation of ovalbumin. These mice did not develop airway inflammatory responses such as eosinophilia in BALF and AHR, but very low levels of ovalbumin-specific IgG and IgE were detected in the serum on day 21, and ovalbumin-specific T-cell responses were detected beginning on day 7 (data not shown). These results confirm that sensitization alone (with antigen plus αGC) does not elicit inflammatory responses.

CD1d-deficient mice do not develop airway responsiveness when exposed to ovalbumin + αGC

To demonstrate directly that NKT cells are involved in inducing asthma, we used CD1d deficient mice, which lack the class I restricting element of NKT cells and therefore lack NKT cells.²⁰ CD1d^{+/-} mice have shown normal expression ranges of CD1d compared with the wild-type mice (data not shown). CD1d^{+/-} and CD1d^{-/-} mice were sensitized with 100 μg ovalbumin + 0.5 μg αGC and were exposed to ovalbumin via inhalation. Unlike CD1d^{+/-} littermates, CD1d^{-/-} mice did not develop AHR. In addition, eosinophils in BALF were rarely detected in CD1d^{-/-} mice (Fig 3, B). In the CD1d^{-/-} mice, the ovalbumin-specific IgG level (3.0 μg/mL) was significantly lower than that of CD1d^{+/-} littermates (20 μg/mL). Also, the T_H2 cell-dependent IgE level of the CD1d^{-/-} mice remained below the detection limits even in a 1:20 serum dilution (Fig 3, C). However, the failure to produce high levels of IgE was not caused by the inability of CD1d^{-/-} mice to produce this isotype, because previous studies have reported that the immunized

CD1d^{-/-} mice produced antigen-specific IgE.²⁵ IFN-γ remained below detection limits (15 pg/mL) in CD1d^{-/-} as well as CD1d^{+/-} mice, as did BLN IL-4, IL-5, and IL-13 production in CD1d^{-/-} mice. However, the cytokine production of CD1d^{+/-} mice confirmed the T_H2 nature of the inflammatory response to ovalbumin + αGC (Fig E2, A). Interestingly, the level of cytokine production in the case of 0.5 μg αGC exposure was lower than in the case of 1.0 μg αGC (Fig 1, D), although preliminary data showed no dose-dependent variations in AHR, ovalbumin-specific IgG and IgE levels for doses ranging from 0.125 μg to 1.0 μg (data not shown). BLN cells of the CD1d^{-/-} mice did not show the proliferation ability in response to ovalbumin. The proliferation level of splenocytes was also lower than in CD1d^{+/-} littermates (see Fig E2, B, in the Online Repository at www.mosby.com/jaci). In addition, the substantial diminishment in cellularity ($8.3 \pm 5.7 \times 10^5$ in CD1d^{+/-} vs $3.0 \pm 1.2 \times 10^5$) also pointed to a reduced proliferative response in the BLN of CD1d^{-/-} mice. The CD1d^{-/-} mice exposed to ovalbumin + αGC showed a normal airway phenotype; in contrast, similarly exposed CD1d^{+/-} mice showed significant lung tissue infiltrates (data not shown). Thus, CD1d^{-/-} mice sensitized with ovalbumin + αGC and challenged with ovalbumin had normal airway responsiveness, which is related to the deficiency of NKT cells.

MHC class II^{-/-} mice do not develop airway responsiveness when exposed to ovalbumin + αGC

Earlier reports by other groups¹⁷⁻¹⁹ have demonstrated the requirement of NKT cells in asthma. Therefore, we next investigated whether asthma can be induced with the activation of NKT cells alone, in the absence of T_H2 cells. We used MHC class II-deficient mice, which do not have conventional CD4⁺ T cells.²⁶ Wild-type littermates and MHC class II-deficient mice were immunized as described in the Methods section. The increase of AHR in wild-type C57BL/6 mice was not significant (*P* > .05) in our system (data not shown), which might be a result of the genetic background.²⁷ However, wild-type mice had significant increases of BAL eosinophils and lymphocytes compared with the MHC class II-deficient mice (Fig 4, A).

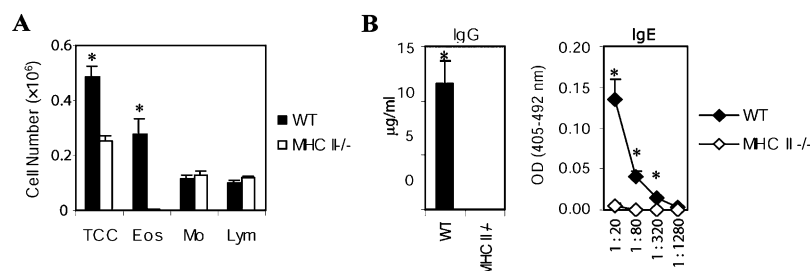


FIG 4. Analysis of antigen + α GC-induced airway responses in MHC class II-deficient mice. **A**, The number of total cell count (TCC), eosinophils (Eos), monocytes/macrophages (Mo), and lymphocytes (Lym) in BALF. **B**, Ovalbumin-specific IgG and IgE levels in serum. In the IgE graph, the x-axis indicates the dilution of serum. $n = 6$ per group. WT, Wild-type mice. * $P < .01$ compared with the MHC II^{-/-} group.

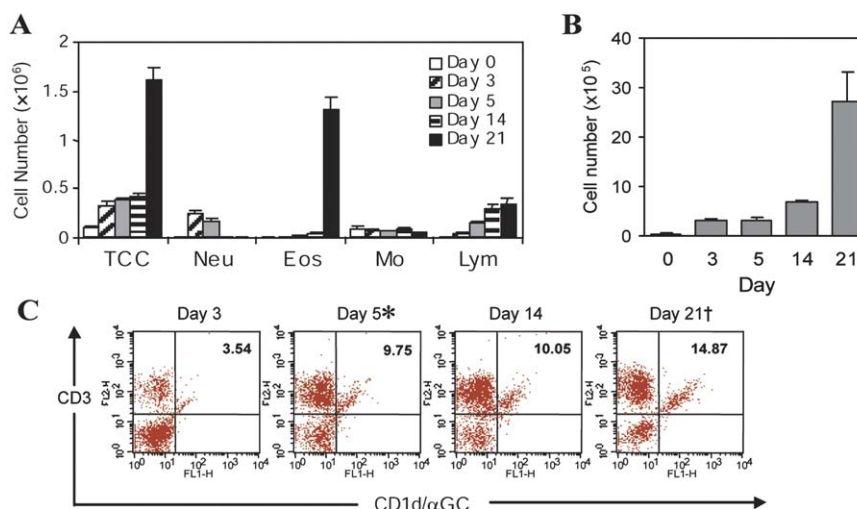


FIG 5. Changes in the NKT cell ratio in BLN and BALF of BALB/c mice. **A**, Comparison of BALF cells at indicated time points (day 0, before sensitization; day 3, 5, and 14, before inhalation of ovalbumin). **B**, The number of BLN cells at indicated time points. **C**, The increase of NKT cells in BALF. $n = 5-10$ mice per group. * $P < .01$ compared with day 3; † $P < .05$ compared with day 14. TCC, Total cell count; Neu, neutrophils; Eos, eosinophils; Mo, monocytes/macrophages; Lym, lymphocytes.

Serum IgG and IgE levels were detected in wild-type littermates but not in *MHC class II*^{-/-} mice (Fig 4, B). In C57BL/6 background mice, the numbers of BLN cells were too small to allow for analysis of proliferative ability and cytokine production. The splenocytes of wild-type mice immunized with ovalbumin + α GC proliferated in response to ovalbumin antigen *in vitro*, but those of *MHC class II*^{-/-} mice did not (data not shown). Collectively, these findings demonstrate that asthma cannot be induced by NKT cell activation alone but requires the presence of antigen-specific T_H2 cells as well.

NKT proliferation occurs in the airway mucosa but not in the draining lymph nodes after antigen challenges

We next analyzed the changes in NKT cell ratios in spleen, BLN, and BAL of BALB/c mice at the indicated time points (days 0, 3, 5, 14, and 21) by using flow cytometry. As shown in Fig 5, A, neutrophil recruitment into the airways was observed on day 3 or 5. This suggests

that the activation of NKT cells in airways under the condition with antigen can induce the T_H1-driven neutrophilia in an early phase. As shown in Fig 5, B, the cell numbers of BLN on day 14 ($6.76 \pm 0.32 \times 10^5$ cells), before the inhalation of ovalbumin, were greater than on day 3 or 5, suggesting that the cells participating in the initial antigen + α GC sensitization had moved from the airway into the draining lymph nodes by day 14 (Fig 5, B). The greatest cellularity was observed on day 21, after the aerosol ovalbumin antigen inhalation ($P < .05$ compared with day 14). We checked the frequency of NKT cells in spleen, BLN, and BALF on the indicated days (day 3, 5, 14, and 21). However, the frequency of NKT cell population was not significantly changed in spleen or BLN (data not shown), but did increase in BAL (Fig 5, C), reaching maximum levels on day 21 (14.24 ± 1.08 ; $P < .05$ compared with day 14). In each case, the frequency of α GC-unloaded CD1d dimer staining was $0.61 \pm 0.14\%$ ($n = 3$). Our finding of increased number of NKT cells in airway mucosa suggests that the NKT cells may play

a direct role in the AHR, confirming the results of Akbari et al.¹⁷

DISCUSSION

Our experiments with ovalbumin plus α GC clearly show that both CD1d-restricted V α 14 μ NKT cells and antigen-specific CD4⁺ T cell help are required for the induction of experimental allergic asthma in mice. Mice injected intranasally with both ovalbumin and α GC before ovalbumin challenge did not become tolerant but developed experimental allergic asthma. Moreover, NKT cell frequency in BAL was increased during experimental allergic asthma, suggesting that NKT cells may play a critical role in the pathology of asthma. The results presented here also support a new experimental model of sensitization to inhaled antigens that require NKT cell activation.

Administration of soluble antigens to the oral or nasal mucosa is well known to lead to systemic unresponsiveness on rechallenge with the same antigens.²⁸ As expected, ovalbumin alone priming in this study did not develop airway inflammation. However, intranasal co-injection of ovalbumin and α GC led to the development of the characteristic features of asthma (Fig 1). It is possible, therefore, that the use of α GC as an adjuvant might be required to break tolerance to ovalbumin.

The failure of the intranasal administration of α GC alone or α GC together with BSA followed by ovalbumin challenge to induce any of the parameters of asthma indicated that NKT cell activation alone was not sufficient for the development of asthma pathology. These findings suggest that α GC acted as adjuvant and that V α 14 μ NKT cells alone are not able to function as effector cells in the absence of conventional CD4⁺ T lymphocytes. Ultimately, antigen-specific T_H2-driven allergic responses are vitally important in the development of asthma,^{29,30} though not in themselves sufficient to induce asthma. Therefore, we hypothesized that the activation of both NKT cells and antigen-specific T_H2 cells was indispensable for the induction of asthma. Mice that had been intranasally administered ovalbumin plus α GC alone without ovalbumin challenge failed to develop an inflammatory response, signaling that sensitization alone is not sufficient to induce inflammation. Thus, we next sought to determine whether NKT cell activation played a role beyond that of initial antigen sensitization. As shown in Fig 5, NKT cell activation in the initial allergen sensitization is not itself sufficient to induce allergic responses in the airways. On day 21, 2 days after the last inhalation of ovalbumin, eosinophils in BAL increased to 77.4% $\pm$ 3.7% of total cells. Surprisingly, the NKT cell ratio in lymphocytes also significantly increased to 14.24% $\pm$ 1.08% (P < .05 compared with day 14), although α GC was not administered as part of the ovalbumin challenge (Fig 5, C). The significant increase in NKT cells occurred in BAL but not in sites distant from the airway, such as spleen and even BLN (data not shown). These findings suggest that NKT cells may play a direct

role in AHR and in the recruitment of eosinophils into the airway. They also suggest that various cell types in the airway, including NKT and conventional CD4⁺ memory T cells, engage in complex interactions that ultimately lead to eosinophilic T_H2 responses. Antigen-specific T_H2 cells may augment the number of NKT cells through production of cytokines or costimulatory molecules or by other mechanisms in the absence of the ligand of NKT cells. In addition, it is possible that self-antigen such as glycolipid may be recognized by NKT cells through CD1d, triggering NKT cell proliferation.

Recent reports underlining the importance of NKT cells in asthma support our findings.¹⁷⁻¹⁹ These studies did not use α GC to induce asthma. Without direct-activating NKT cells of mice, which were sensitized intraperitoneally to ovalbumin plus alum after intranasal challenge with ovalbumin, their data showed that the V α 14 μ NKT cells may mediate T_H2 eosinophil airway inflammation and AHR in an allergen-induced asthma model. An explanation for the possibility of NKT cell activation in the absence of α GC was offered by another report, although the data were limited to T_H1-directed responses.³¹ Therefore, it is possible that in pathological states, endogenous antigens associated with CD1d molecules can be recognized by NKT cells and that the activation of NKT cells is mediated and amplified by the signal from ovalbumin-specific T_H2 cell activation. It is also possible that asthma development is triggered by the inhalation of a naturally occurring NKT cell ligand, for example, by bacterial cell walls containing putative ligand, phosphatidylinositol mannoside.³² Because no ligand other than α GC has yet been identified for V α 14 μ NKT cells, it will be necessary to identify naturally occurring exogenous or endogenous ligands, which can lead to a better understanding of the role of NKT cells in asthma.

In summary, the results presented here provide further evidence that V α 14 μ NKT cells are critical not only to primary antigen sensitization and but also to the pathological state of asthma. In addition, these cells are essential for amplifying T_H2 development in the lung. In conclusion, our studies represent a new experimental allergic asthma model in mice, one that might mimic natural exogenous antigen inhalation. Our results also suggest that therapies targeting NKT cell function could contribute to ameliorating asthma.

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REFERENCES

1. Liu AH. Endotoxin exposure in allergy and asthma: reconciling a paradox. *J Allergy Clin Immunol* 2002;109:379-92.
2. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002; 196:1645-51.

3. Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol* 1997;15:535-62.
4. Hong S, Scherer DC, Singh N, Mendiratta SK, Serizawa I, Koezuka Y, et al. Lipid antigen presentation in the immune system: lessons learned from CD1d knockout mice. *Immunol Rev* 1999;169:31-44.
5. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NK T cells: facts, functions and fallacies. *Immunol Today* 2000;21:573-83.
6. Exley M, Garcia J, Balk SP, Porcelli S. Requirements for CD1d recognition by human invariant Valpha24+ CD4-CD8- T cells. *J Exp Med* 1997;186:109-20.
7. Bendelac A. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J Exp Med* 1995;182:2091-6.
8. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. *Science* 1995;268:863-5.
9. Matsuda JL, Gapin L, Fazilleau N, Warren K, Naidenko OV, Kronenberg M. Natural killer T cells reactive to a single glycolipid exhibit a highly diverse T cell receptor beta repertoire and small clone size. *Proc Natl Acad Sci U S A* 2001;98:12636-41.
10. Ronet C, Mempel M, Thieblemont N, Lehuen A, Kourilsky P, Gachelin G. Role of the complementarity-determining region 3 (CDR3) of the TCR-beta chains associated with the V alpha 14 semi-invariant TCR alpha-chain in the selection of CD4+ NK T cells. *J Immunol* 2001;166:1755-62.
11. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 1997;278:1626-9.
12. Spada FM, Koezuka Y, Porcelli SA. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J Exp Med* 1998;188:1529-34.
13. Singh N, Hong S, Scherer DC, Serizawa I, Burdin N, Kronenberg M, et al. Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J Immunol* 1999;163:2373-7.
14. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y, et al. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol* 1999;163:4647-50.
15. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, et al. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NK T cells. *J Exp Med* 1999;189:1121-8.
16. Eberl G, MacDonald HR. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. *Eur J Immunol* 2000;30:985-92.
17. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* 2003;9:582-8.
18. Lisbonne M, Diem S, de Castro Keller A, Lefort J, Araujo LM, Hachem P, et al. Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *J Immunol* 2003;171:1637-41.
19. Bilenki L, Yang J, Fan Y, Wang S, Yang X. Natural killer T cells contribute to airway eosinophilic inflammation induced by ragweed through enhanced IL-4 and eotaxin production. *Eur J Immunol* 2004;34:345-54.
20. Park SH, Weiss A, Benlagha K, Kyin T, Teyton L, Bendelac A. The mouse CD1d-restricted repertoire is dominated by a few autoreactive T cell receptor families. *J Exp Med* 2001;193:893-904.
21. Takikawa H, Muto S-E, Mori K. Diastereoselective epoxidation of the double bond at C-4 of sphingosines to provide phytosphingosine relatives such as alpha-galactosylceramide KRN7000. *Tetrahedron* 1998;54:3141-50.
22. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156:766-75.
23. Chung Y, Cho J, Chang YS, Cho SH, Kang CY. Preventive and therapeutic effects of oral tolerance in a murine model of asthma. *Immunobiology* 2002;206:408-23.
24. Chen YH, Chiu NM, Mandal M, Wang N, Wang CR. Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. *Immunity* 1997;6:459-67.
25. Smiley ST, Kaplan MH, Grusby MJ. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science* 1997;275:977-9.
26. Cardell S, Tangri S, Chan S, Kronenberg M, Benoist C, Mathis D. CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. *J Exp Med* 1995;182:993-1004.
27. Zhang Y, Lamm WJ, Albert RK, Chi EY, Henderson WR Jr, Lewis DB. Influence of the route of allergen administration and genetic background on the murine allergic pulmonary response. *Am J Respir Crit Care Med* 1997;155:661-9.
28. Strobel S, Mowat AM. Immune responses to dietary antigens: oral tolerance. *Immunol Today* 1998;19:173-81.
29. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N Engl J Med* 1995;332:133-8.
30. Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 1989;320:271-7.
31. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol* 2003;4:1230-7.
32. Apostolou I, Takahama Y, Belmant C, Kawano T, Huerre M, Marchal G, et al. Murine natural killer T(NKT) cells [correction of natural killer cells] contribute to the granulomatous reaction caused by mycobacterial cell walls. *Proc Natl Acad Sci U S A* 1999;96:5141-6.