

Natural killer T cells are dispensable in the development of allergen-induced airway hyperresponsiveness, inflammation and remodelling in a mouse model of chronic asthma

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Summary

Natural killer T (NK T) cells have been shown to play an essential role in the development of allergen-induced airway hyperresponsiveness (AHR) and/or airway inflammation in mouse models of acute asthma. Recently, NK T cells have been reported to be required for the development of AHR in a virus induced chronic asthma model. We investigated whether NK T cells were required for the development of allergen-induced AHR, airway inflammation and airway remodelling in a mouse model of chronic asthma. CD1d^{−/−} mice that lack NK T cells were used for the experiments. In the chronic model, AHR, eosinophilic inflammation, remodelling characteristics including mucus metaplasia, subepithelial fibrosis and increased mass of the airway smooth muscle, T helper type 2 (Th2) immune response and immunoglobulin (Ig)E production were equally increased in both CD1d^{−/−} mice and wild-type mice. However, in the acute model, AHR, eosinophilic inflammation, Th2 immune response and IgE production were significantly decreased in the CD1d^{−/−} mice compared to wild-type. CD1d-dependent NK T cells may not be required for the development of allergen-induced AHR, eosinophilic airway inflammation and airway remodelling in chronic asthma model, although they play a role in the development of AHR and eosinophilic inflammation in acute asthma model.

Keywords: airway inflammation, airway remodelling, airway responsiveness, asthma, natural killer T cells

Introduction

Recently, it has been reported that natural killer T (NK T) cells may play an important role in the pathogenesis of asthma [1]. NK T cells are unique CD1d-restricted T cells with NK cell surface markers; they act as a bridge between the innate and adaptive immune responses. NK T cells secrete very large amounts of T helper type 1 (Th1) cytokines, including interferon (IFN)- γ or Th2 cytokines, such as IL-4 and IL-13, immediately following activation [2,3]. NK T cells have been reported to play an essential role in the development of ovalbumin (OVA)-induced [4,5], α -GalactosylCeramide (GalCer)-induced [6], ozone-induced [7] and virus-induced [8] airway hyperresponsiveness (AHR) and/or airway inflammation in experimental studies using mouse models of asthma. Recent clinical studies [9,10] have reported that NK T cells are increased in the airways of patients with chronic asthma, suggesting that NK T cells might play a role in the pathogenesis of chronic asthma in humans.

However, previous animal studies, with the exception of the investigation by Kim *et al.* [8], that have focused on the role of NK T cells in asthma have been performed in mouse models of acute, but not chronic, asthma. Results from the studies using a mouse model for acute asthma may not be appropriate for the explanation of the pathogenesis of AHR and airway inflammation caused by chronic asthma. In addition, chronic persistent airway inflammation may lead to structural changes known as airway remodelling, another central feature of asthma, which is characterized by mucus metaplasia, subepithelial fibrosis and smooth muscle hyperplasia and hypertrophy [11,12]. Th2 immune responses have been reported to contribute to the development of airway remodelling [13,14], although the precise mechanisms are unknown. Thus, it is possible that NK T cells might contribute to the development of airway remodelling in asthma by enhancing the Th2 response in addition to producing Th2 cytokines [2,3]. Recently, NK T cells have been reported to be required for the development of virus induced asthma in a murine model of chronic asthma [8]. Therefore, in the

present study, we investigated whether NK T cells were required for the development of allergen-induced AHR, airway inflammation and airway remodelling in a mouse model of chronic asthma. We used CD1d^{-/-} mice that lack the restriction element of NK T cells and therefore lack NK T cells. The results suggest that NK T cells may be dispensable with regard to the development of allergen-induced asthma in the chronic asthma model, although they play an essential role in the pathogenesis of asthma in the acute asthma model.

Materials and methods

Mice

Wild-type (WT) BALB/c mice and CD1d^{-/-} mice on a BALB/c background were purchased from Orient Bio INC (Seungnam, Korea) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively. The animals were used between 6 and 14 weeks of age and were age- and sex-matched. The mice were maintained in a laminar flow holding unit and housed in autoclaved cages on autoclaved bedding in an air conditioned room. Autoclaved food and water were provided throughout. All animal protocols were approved by the Chonnam National University Medical School Committee on animal welfare.

Flow cytometry analysis of NK T cells

We collected cells from the spleen and stained them with anti-T cell receptor (TCR)β-fluorescein isothiocyanate (FITC) (BD Biosciences, San Jose, CA, USA) and a-GalCer-loaded CD1d tetramer-phycoerythrin (PE). a-GalCer (Alexis Biochemicals, Lausen, Switzerland) was conjugated with PE-CD1d-tetramer (Proimmune Ltd, Oxford, UK), according to the manufacturer's instructions. A control sample was labelled with unloaded tetramer-PE. Analytical flow cytometry was performed on a four-colour BD fluorescence activated cell sorter (FACS)Calibur (BD Biosciences). Analysis was performed with FlowJo software (Tree star, Ashland, OR, USA).

Mouse model of chronic and acute asthma

To develop a mouse model of chronic asthma, mice were sensitized with 100 µg of OVA (Sigma, St Louis, MO, USA) adsorbed to 2 mg of an aqueous solution of alum intraperitoneally (i.p.) on days 0 and 14, followed by 50 µg of OVA in 50 µl of phosphate-buffered saline (PBS) administered intranasally (i.n.) 3 days per week from day 21 to day 60. Control mice were non-immunized and non-exposed (i.e. naive). AHR was measured on day 61 (Fig. 1a).

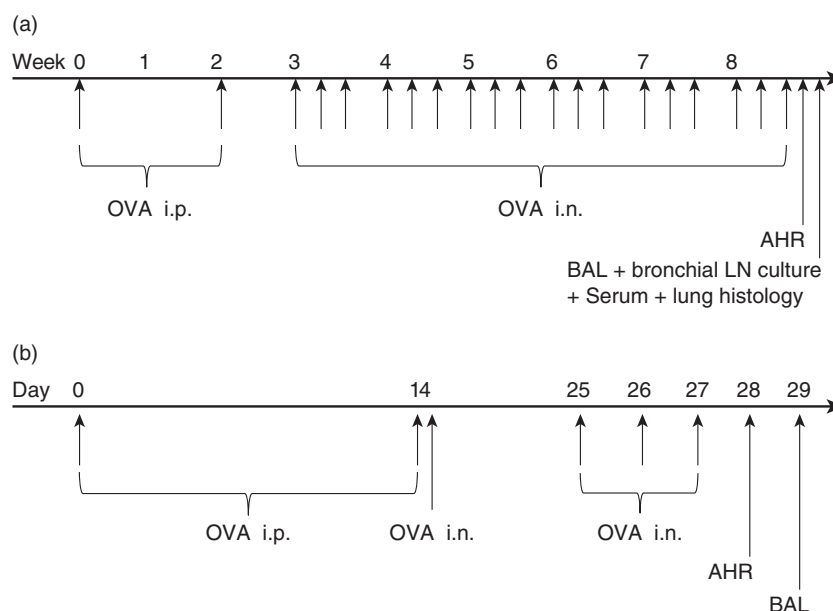


Fig. 1. Experimental protocol for the development of a mouse model of chronic or acute asthma. (a) Development of the mouse model for chronic asthma. Mice were sensitized with 100 µg of ovalbumin (OVA) adsorbed to 2 mg of an aqueous solution of alum intraperitoneally (i.p.) on days 0 and 14, followed by 50 µg of OVA in 50 µl of phosphate-buffered saline (PBS) administered intranasally (i.n.) 3 days per week from day 21 to day 60. Airway hyperresponsiveness (AHR) was measured on day 61 and bronchoalveolar lavage (BAL), bronchial lymph node (LN) cultures, collecting serum for the determination of OVA-specific immunoglobulin (Ig)E and taking lung tissues for histological examinations were performed on day 62. (b) Development of acute asthma. Mice were sensitized with 100 µg of OVA adsorbed to 2 mg of an aqueous solution of alum i.p. on days 0 and 14, followed by 50 µg of OVA in 50 µl of PBS administered i.n. on days 14 and 25–27. AHR was measured on day 28 and BAL was performed on day 29.

Some mice were used to develop a mouse model of acute asthma [15]. Mice were sensitized with 100 µg of OVA adsorbed to 2 mg of an aqueous solution of alum i.p. on days 0 and 14, followed by 50 µg of OVA in 50 µl of PBS administered i.n. on days 14 and 25–27. Naive mice were used as controls. AHR was measured on day 28 (Fig. 1b).

Measurement of AHR

The mice were placed in a barometric plethysmographic chamber (OCP 3000; Allmedicus, Anyang, Korea). Methacholine was used at concentrations of 6.25, 12.5, 25 and 50 mg/ml. Each concentration was inhaled for 3 min as an aerosol prepared by NE-U17 (OMRON, Milton Keynes, UK); enhanced pause (Penh) values were measured for 5 min. Airway resistance was expressed as the Penh, which was calculated as follows, according to the manufacturer's protocol: $\text{Penh} = (\text{expiratory time/relaxation time} - 1) \times (\text{peak expiration flow/peak inspiration flow})$. Data were expressed as the percentage increase in the Penh after challenge with each concentration of methacholine, where the baseline Penh (after PBS challenge) was expressed as 100%.

Collection and analysis of bronchoalveolar lavage (BAL) fluid

Twenty-four hours after the AHR measurement, the trachea was cannulated, the lungs were lavaged twice with 0.5 ml of PBS, the fluid was pooled, and then the volume of the yield was recorded. BAL fluid was centrifuged at 456 g for 5 min at 4°C and the supernatant was stored at –20°C. The pellet was resuspended with 0.5 ml of PBS and the total cell number was counted with a haematocytometer. The relative number of different types of leucocytes was determined from slide preparations of the BAL fluid with a cytocentrifuge that was stained with Diff-Quik solution (Sysmex, Kobe, Japan). Data were expressed as the number of cells per ml of BAL fluid.

Restimulation of bronchial lymph node (LN) cells *in vitro*

Bronchial LN cells were obtained by mechanical disruption and were restimulated *in vitro* (5.0×10^5 cells per well in a round 96-well plate) with 50 µg/ml OVA. The supernatants were collected after 96 h and kept at –20°C until assayed for the determination of the secretion of cytokines in the cultures.

Measurement of cytokines and OVA-specific serum IgE

The concentrations of IL-4 and IL-13 in the LN culture supernatant and the BAL fluid supernatant were measured using commercial enzyme-linked immunosorbent assay

(ELISA) kits (R&D Systems, Minneapolis, MN, USA). For the OVA-specific IgE assay, the mice were bled and the OVA-specific IgE antibodies were measured in the serum using ELISA kits (DS Pharma Biomedical Co., Osaka, Japan).

Histology

Whole lungs were flushed with PBS injected into the right ventricle, removed and fixed in 10% formaldehyde and then embedded in paraffin. Haematoxylin and eosin (H&E) stains were performed for general morphology, periodic acid-Schiff staining for identification of goblet cells in the epithelium, Masson's trichrome staining for airway fibrosis and actin staining for airway smooth muscle hyperplasia. The bronchioles in which the total length of the epithelial basement membrane was 1–1.5 mm were selected for analysis.

To quantify the mucus metaplasia, we used the method described by Kim *et al.* [16]. Briefly, the pathological changes were evaluated according to a modified five-point scoring system (grade 0–4), and were then expressed by scores according to the percentage of goblet cells among the epithelial cells: grade 0 (no goblet cells); grade 1, < 25%; grade 2, 25–50%; grade 3, 51–75%; grade 4, > 75%. The mean scores for the mucus metaplasia in each mouse were calculated.

The areas of airway fibrosis beneath the epithelial basement membrane in the Masson's trichrome-stained tissue sections and the areas of peribronchial smooth muscle were calculated using a Polaroid DMC1e digital microscope colour camera (Nikon Corporation, Tokyo, Japan) connected with a computerized image analyser program, Image Analysis (analysis version 3.2; Soft-Imaging System GmbH, Munster, Germany). The mean areas of subepithelial fibrosis and peribronchial smooth muscle in each mouse were calculated.

Statistical analysis

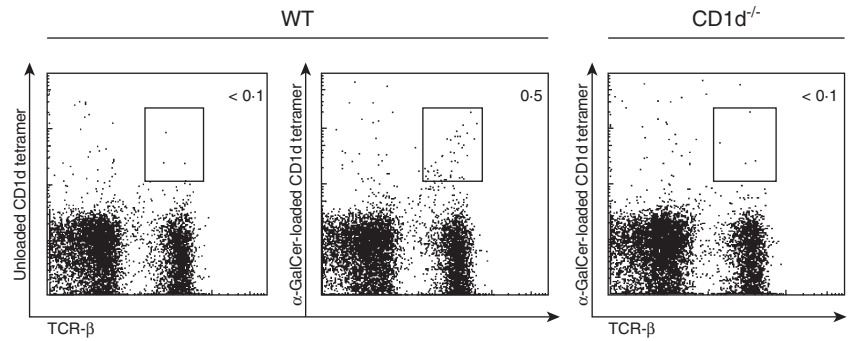
Data were expressed as the mean $\pm$ standard error of the mean (s.e.m.). Statistical analysis was performed using unpaired Student's *t*-tests or Mann–Whitney *U*-tests (SPSS version 17.0 for Windows; SPSS Inc., Chicago, IL, USA). A *P*-value less than 0.05 was considered significant.

Results

Absence of NK T cells in CD1d^{–/–} mice

We found that CD1d^{–/–} mice were deficient in NK T cells. The spleen NK T cells were stained with a-GalCer-loaded CD1d tetramer. a-GalCer-loaded CD1d tetramer⁺ NK T cells were detected in 0.5% of spleen lymphocytes from the BALB/c WT mice, but not the CD1d^{–/–} mice (Fig. 2).

Fig. 2. Natural killer T (NK T) cells were absent in $CD1d^{-/-}$ mice. Splenocytes were stained with anti-T cell receptor (TCR)- β and α -GalactosylCeramide (GalCer)-loaded CD1d tetramer or unloaded CD1d tetramer, and flow cytometry was performed. α -GalCer-loaded CD1d tetramer $^{+}$ NK T cells were present in wild-type (WT) mice, but were absent in $CD1d^{-/-}$ mice. The number of NK T cells is shown as a percentage of total lymphocytes.



NK T cell-deficient mice were not impaired in the development of AHR in a model of chronic asthma

In the mouse model of chronic asthma, the OVA-induced WT mice showed significantly increased AHR to methacholine compared to the naive WT mice ($P < 0.05$). OVA-induced $CD1d^{-/-}$ mice also showed significantly increased AHR compared to the naive $CD1d^{-/-}$ mice ($P < 0.05$); the degree of AHR did not differ from that in the OVA-induced WT mice ($P > 0.05$) (Fig. 3a).

NK T cell-deficient mice were not impaired in the development of airway inflammation in the chronic asthma model

In the mouse model of chronic asthma, the OVA-induced WT mice showed a significantly higher number of total cells ($P < 0.001$), macrophages ($P < 0.05$), lymphocytes ($P < 0.001$), neutrophils ($P < 0.001$) and eosinophils ($P < 0.001$) in the BAL fluid compared to the naive WT mice. OVA-induced $CD1d^{-/-}$ mice also showed a significantly higher number of total cells ($P < 0.01$), macrophages ($P < 0.05$), lymphocytes ($P < 0.01$), neutrophils ($P < 0.001$) and eosinophils ($P < 0.01$) in the BAL fluid compared to the naive $CD1d^{-/-}$ mice, in which the number of cells was equal to that of OVA-induced WT mice ($P > 0.05$, respectively) (Fig. 3b).

Histological examination of the lungs showed that peribronchial and perivascular inflammation was induced in the OVA-induced WT and $CD1d^{-/-}$ mice compared to the naive WT and $CD1d^{-/-}$ mice, respectively. There was no difference in the degree of inflammation between the OVA-induced WT and $CD1d^{-/-}$ mice (Fig. 3c).

NK T cell-deficient mice were not impaired in the development of airway remodelling in the chronic asthma model

In the mouse model of chronic asthma, we examined the characteristics of airway remodelling in lung tissue. OVA-induced WT mice had significantly increased scores for mucus metaplasia ($P < 0.001$, Fig. 4a), areas of subepithelial

fibrosis ($P < 0.001$, Fig. 4b) and areas of peribronchial smooth muscle ($P < 0.001$, Fig. 4c) compared to the naive WT mice. The OVA-induced $CD1d^{-/-}$ mice also showed significantly increased scores for mucus metaplasia ($P < 0.01$, Fig. 4a), areas of subepithelial fibrosis ($P < 0.01$, Fig. 4b) and areas of peribronchial smooth muscle ($P < 0.01$, Fig. 4c) compared to the naive $CD1d^{-/-}$ mice. The degree of mucus metaplasia ($P > 0.05$), subepithelial fibrosis ($P > 0.05$) and smooth muscle hyperplasia ($P > 0.05$) in the OVA-induced $CD1d^{-/-}$ mice was equal to that of the OVA-induced WT mice.

NK T cell-deficient mice were not impaired in the development of the Th2 immune response and IgE production in the chronic asthma model

To determine whether NK T cell-deficient mice were impaired in inducing OVA-specific Th2 responses in the chronic asthma model, IL-4 and IL-13 were measured in the supernatant obtained from the OVA-stimulated bronchial LN cultures. IL-4 ($P < 0.01$) and IL-13 ($P < 0.01$) were significantly higher in the OVA-induced WT mice than in the naive WT mice. The OVA-induced $CD1d^{-/-}$ mice also showed increased production of IL-4 ($P < 0.01$) and IL-13 ($P = 0.05$) compared to the naive $CD1d^{-/-}$ mice. The amounts of IL-4 ($P > 0.05$) and IL-13 ($P > 0.05$) in the OVA-induced $CD1d^{-/-}$ mice were similar to those of the OVA-induced WT mice (Table 1). IL-4 and IL-13 were measured in the supernatant of the BAL fluid. IL-4 ($P < 0.001$) and IL-13 ($P < 0.01$) were significantly higher in the OVA-induced WT mice than in the naive WT mice. OVA-induced $CD1d^{-/-}$ mice also showed increased production of IL-4 ($P < 0.001$) and IL-13 ($P < 0.01$) compared to the naive $CD1d^{-/-}$ mice. The amounts of IL-4 ($P > 0.05$) and IL-13 ($P > 0.05$) in the OVA-induced $CD1d^{-/-}$ mice were similar to those of the OVA-induced WT mice (Table 1). Next, we determined whether NK T cell-deficient mice were impaired in producing serum OVA-specific IgE in the chronic asthma model. OVA-specific IgE levels were increased significantly in the OVA-induced WT ($P < 0.01$) and $CD1d^{-/-}$ mice ($P < 0.05$) compared to the naive WT and $CD1d^{-/-}$ mice, respectively. The amounts of OVA-specific IgE in the OVA-induced $CD1d^{-/-}$ mice were

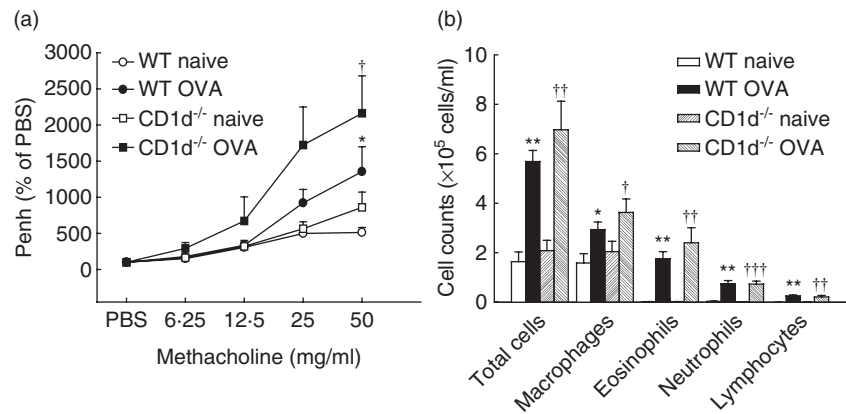


Fig. 3. CD1d^{-/-} mice were not impaired in inducing ovalbumin (OVA)-induced airway hyperresponsiveness (AHR) and airway inflammation in the chronic asthma model. Wild-type (WT) and CD1d^{-/-} mice were sensitized and challenged as in Fig. 1a. (a) Development of AHR in CD1d^{-/-} mice. AHR was determined by non-invasive measurement of airway resistance [enhanced pause (Penh)] in response to increasing doses of methacholine 24 h after the last OVA challenge. Data represent the mean $\pm$ standard error of the mean (s.e.m.) from two experiments ($n = 6-7$). Statistical analysis was performed using the Mann-Whitney *U*-test. * $P < 0.05$ versus WT naive. † $P < 0.05$ versus CD1d^{-/-} naive. (b) Development of airway inflammation in CD1d^{-/-} mice. Bronchoalveolar lavage (BAL) fluid was obtained 24 h after the AHR measurement. Data represented the number of cells/ml in BAL fluid and are the mean $\pm$ s.e.m. from three experiments ($n = 10-11$). Statistical analysis was performed using the unpaired *t*-test. * $P < 0.05$ and ** $P < 0.001$ versus WT naive. † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$ versus CD1d^{-/-} naive. (c) A representative of OVA-induced peribronchial and perivascular inflammation in CD1d^{-/-} mice from the chronic asthma model. Lungs were obtained immediately after BAL, fixed and stained with haematoxylin and eosin ($\times 100$).

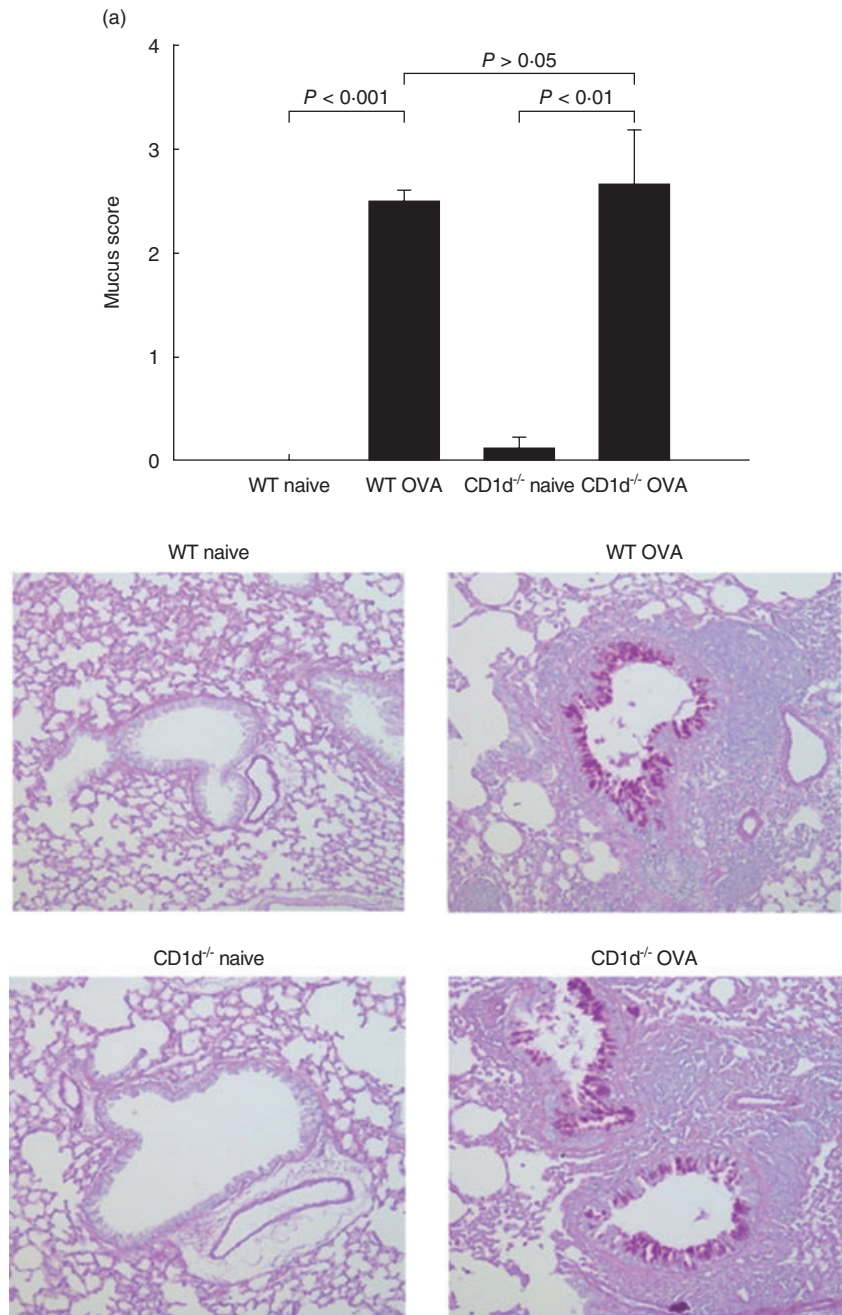
similar to those of the OVA-induced WT mice ($P > 0.05$) (Table 1).

NK T cell-deficient mice were impaired in the development of AHR and eosinophilic airway inflammation in the mouse model of acute asthma

Because NK T cells have been reported to play a critical role in the development of AHR and eosinophilic airway inflammation in mouse models of acute asthma [4,5], we studied

whether CD1d^{-/-} mice were impaired in producing OVA-induced AHR and eosinophilic airway inflammation in a mouse model of acute asthma. The OVA-induced WT mice showed significantly increased AHR to methacholine compared to the naive WT mice ($P < 0.01$). However, the OVA-induced CD1d^{-/-} mice did not show increased AHR compared to the naive CD1d^{-/-} mice ($P > 0.05$). The OVA-induced AHR was reduced significantly in the CD1d^{-/-} mice compared to the WT mice ($P < 0.05$) (Fig. 5a). The OVA-induced WT mice showed a significantly higher number of total cells ($P < 0.05$), eosinophils ($P < 0.01$), neutrophils

Fig. 4. CD1d^{-/-} mice were not impaired in inducing ovalbumin (OVA)-induced airway remodelling in the chronic asthma model. The wild-type (WT) and CD1d^{-/-} mice were sensitized and challenged as in Fig. 1a. The lungs were obtained immediately after bronchoalveolar lavage (BAL), fixed and stained. (a) Mucus metaplasia was induced in OVA-induced CD1d^{-/-} mice. Goblet cells in the epithelium were stained with periodic acid-Schiff and were scored. Data represent the mean $\pm$ standard error of the mean (s.e.m.) ($n = 3-4$), representative of three experiments. Statistical analysis was performed by the unpaired *t*-test. An example of OVA-induced mucus metaplasia in the epithelium in CD1d^{-/-} mice from the chronic asthma model was shown ($\times 100$). (b) Airway fibrosis was induced in OVA-induced CD1d^{-/-} mice. Airway fibrosis was stained with Masson's trichrome and the area of subepithelial fibrosis was calculated. Data represent the mean $\pm$ s.e.m. ($n = 3-4$), representative of three experiments. Statistical analysis was performed using the unpaired *t*-test. A representative of OVA-induced subepithelial fibrosis in the CD1d^{-/-} mice from chronic asthma model was shown ($\times 100$). (c) Airway smooth muscle hyperplasia was induced in OVA-induced CD1d^{-/-} mice. Airway smooth muscle hyperplasia was stained with actin and the area of peribronchial smooth muscle was calculated. Data represent the mean $\pm$ s.e.m. ($n = 3-4$), and are representative of three experiments. Statistical analysis was performed using the unpaired *t*-test. An example of OVA-induced peribronchial smooth muscle hyperplasia in CD1d^{-/-} mice from the chronic asthma model was shown ($\times 100$).



($P < 0.05$) and lymphocytes ($P < 0.05$) in the BAL fluid compared to the naive WT mice. The number of total cells ($P = 0.06$), eosinophils ($P = 0.09$) and neutrophils ($P = 0.07$) in the BAL fluid was increased in the OVA-induced CD1d^{-/-} mice compared to the naive CD1d^{-/-} mice; however, the differences failed to reach statistical significance. The number of lymphocytes was increased significantly in the OVA-induced CD1d^{-/-} mice compared to the naive CD1d^{-/-} mice ($P < 0.05$). The number of eosinophils was reduced significantly in the OVA-induced CD1d^{-/-} mice compared to the OVA-induced WT mice ($P < 0.05$) (Fig. 5b).

NK T cell-deficient mice were impaired in the development of the Th2 immune response and IgE production in the acute asthma model

To determine whether NK T cell-deficient mice were impaired in inducing OVA-specific Th2 responses in the acute asthma model, IL-4 and IL-13 were measured in the supernatant obtained from the OVA-stimulated bronchial LN cultures. IL-4 ($P < 0.001$) and IL-13 ($P < 0.05$) were higher in the OVA-induced WT mice than in the naive WT mice. The OVA-induced CD1d^{-/-} mice did not show

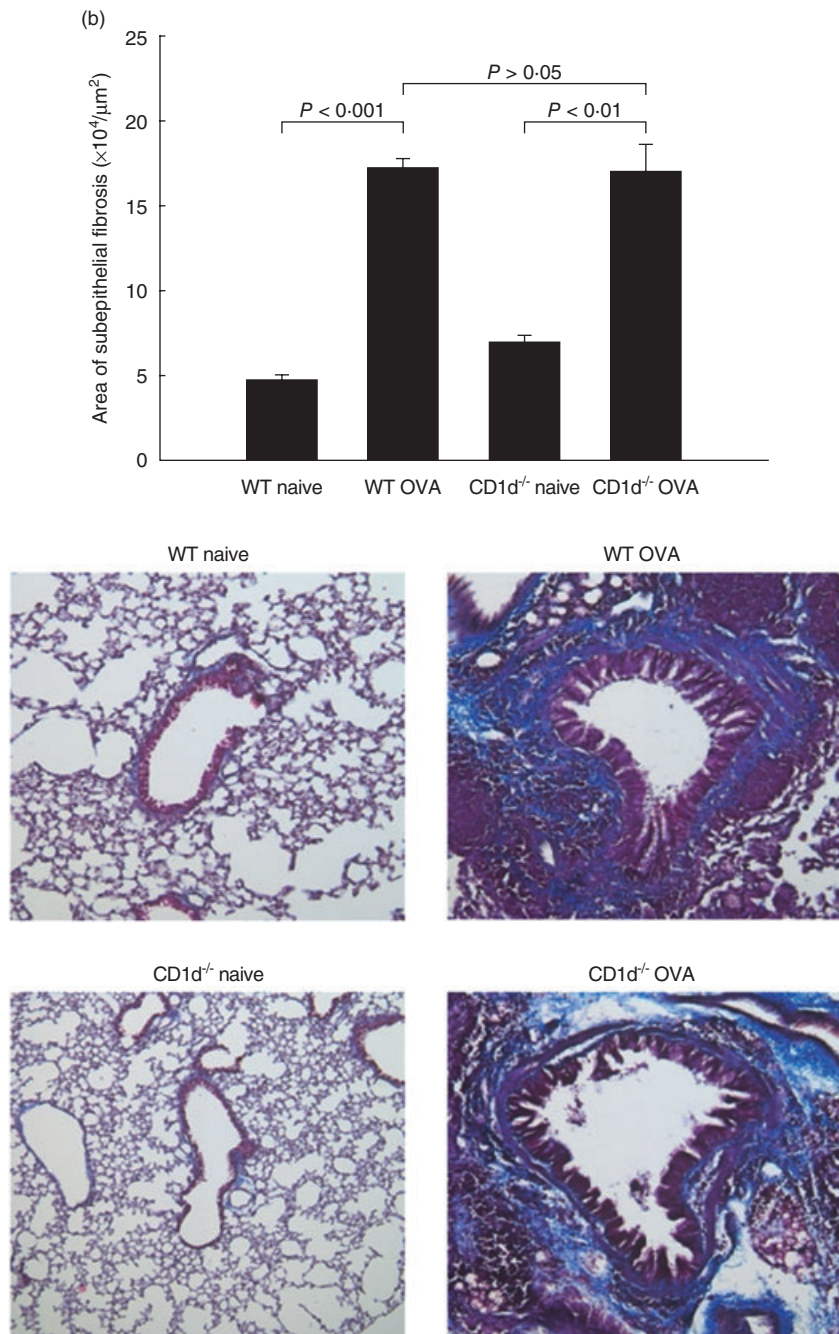
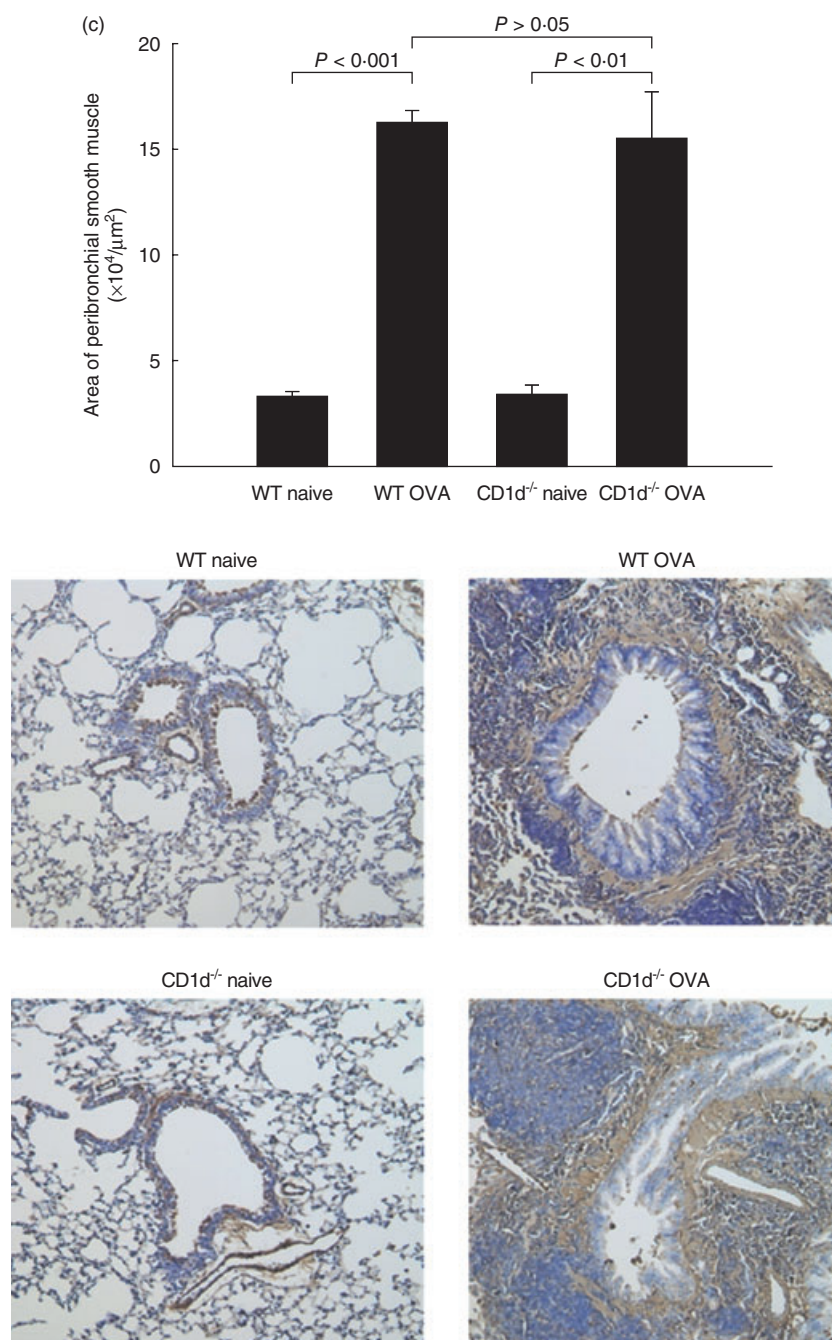


Fig. 4. Continued

increased production of IL-4 ($P > 0.05$) and IL-13 compared to the naive CD1d^{-/-} mice. The amounts of IL-4 ($P < 0.001$) and IL-13 ($P < 0.001$) were lower in the OVA-induced CD1d^{-/-} mice than in the OVA-induced WT mice (Table 2). IL-4 and IL-13 were measured in the supernatant of the BAL fluid. IL-4 ($P < 0.01$) and IL-13 ($P < 0.01$) were higher in the OVA-induced WT mice than in the naive WT mice. The OVA-induced CD1d^{-/-} mice did not show increased production of IL-4 and IL-13 ($P > 0.05$) compared to the naive CD1d^{-/-} mice. The amounts of IL-4 ($P < 0.01$) and IL-13 ($P < 0.01$) were lower in the OVA-

induced CD1d^{-/-} mice than in the OVA-induced WT mice (Table 2). Next, we determined whether NK T cell-deficient mice were impaired in producing serum OVA-specific IgE in the acute asthma model. The OVA-specific IgE levels were higher in the OVA-induced WT mice than in the naive WT mice ($P < 0.001$). The OVA-induced CD1d^{-/-} mice did not show increased production of OVA-specific IgE ($P > 0.05$) compared to the naive CD1d^{-/-} mice. The amounts of OVA-specific IgE were lower in the OVA-induced CD1d^{-/-} mice than in the OVA-induced WT mice ($P < 0.01$) (Table 2).

Fig. 4. *Continued*

Discussion

The present study showed that AHR, eosinophilic airway inflammation, airway remodelling, IgE production and Th2 responses in a model of OVA-induced chronic asthma were not impaired in NK T cell-deficient mice. These findings suggest that NK T cells are not required for the development of the allergen-induced features of chronic asthma.

Characteristics of asthma include AHR, eosinophilic airway inflammation and airway remodelling. These features

may be independent, although one may be associated with the other. It has been recognized that AHR may be divided into at least two semi-independent components [17], with variable and persistent AHR. Variable or acute AHR is episodic or transient and may be associated with acute airway inflammation. By contrast, persistent or chronic AHR probably reflects, at least in part, chronic airway inflammation and may be linked to the structural changes in chronic asthma referred to collectively as airway remodelling [18,19]. In the present study, we investigated the role of NK T cells in the development of chronic AHR using a mouse model of

Table 1. T helper type 2 (Th2) cytokines in the supernatant from bronchial lymph node (LN) cultures and bronchoalveolar lavage fluids (BALF) and serum ovalbumin (OVA)-specific IgE in a mouse model of chronic asthma.

	WT naive	WT OVA	CD1d ^{-/-} naive	CD1d ^{-/-} OVA
Bronchial LN cultures				
IL-4 (pg/ml)	9.6 ± 0.3	19.8 ± 2.8**	9.4 ± 0.3	42.5 ± 2.4 ^{††}
IL-13 (pg/ml)	4.1 ± 0.4	63.5 ± 1.1**	4.2 ± 0.5	55.6 ± 4.6
BALF				
IL-4 (pg/ml)	7.8 ± 0.5	22.4 ± 1.6***	8.2 ± 0.5	19.7 ± 1.8 ^{†††}
IL-13 (pg/ml)	6.8 ± 0.4	17.6 ± 2.7**	7.6 ± 1.7	18.9 ± 2.9 ^{††}
OVA-IgE (ng/ml)	22.7 ± 4.0	49.9 ± 8.0**	25.2 ± 3.9	46.6 ± 6.8 [†]

Data represent the mean ± standard error of the mean (s.e.m.). BALF and sera were obtained from 10–11 mice and eight to 11 mice per group, respectively. ** $P < 0.01$ and *** $P < 0.001$ versus wild-type (WT) naive. [†] $P < 0.05$, ^{††} $P < 0.01$ and ^{†††} $P < 0.001$ versus CD1d^{-/-} naive. Interleukin (IL)-4 levels in the supernatant from bronchial LN cultures were analysed statistically by the Mann–Whitney *U*-test. Otherwise, statistical analyses were performed by unpaired *t*-tests.

Table 2. T helper type 2 (Th2) cytokines in the supernatant from bronchial lymph node (LN) cultures and bronchoalveolar lavage fluids (BALF) and serum ovalbumin (OVA)-specific IgE in a mouse model of acute asthma.

	WT naive	WT OVA	CD1d ^{-/-} naive	CD1d ^{-/-} OVA
Bronchial LN cultures				
IL-4 (pg/ml)	4.6 ± 0.2	8.1 ± 0.2***	5.1 ± 0.1	5.2 ± 0.1 ^{†††}
IL-13 (pg/ml)	29.2 ± 1.4	34.9 ± 0.9*	20.7 ± 0.1	19.3 ± 0.5 ^{†††}
BALF				
IL-4 (pg/ml)	12.9 ± 1.6	20.1 ± 1.4**	11.4 ± 1.3	11.2 ± 1.6 ^{††}
IL-13 (pg/ml)	7.3 ± 1.0	12.0 ± 1.1**	5.9 ± 0.2	6.3 ± 0.4 ^{††}
OVA-IgE (ng/ml)	24.1 ± 3.4	56.5 ± 6.3***	28.7 ± 2.6	26.8 ± 4.2 ^{††}

Data represent the mean ± standard error of the mean (s.e.m.). BALF and sera were obtained from eight to 10 mice per each group, respectively. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus wild-type (WT) naive. ^{††} $P < 0.01$ and ^{†††} $P < 0.001$ versus WT OVA. Statistical analyses were performed by unpaired *t*-tests. IL: interleukin.

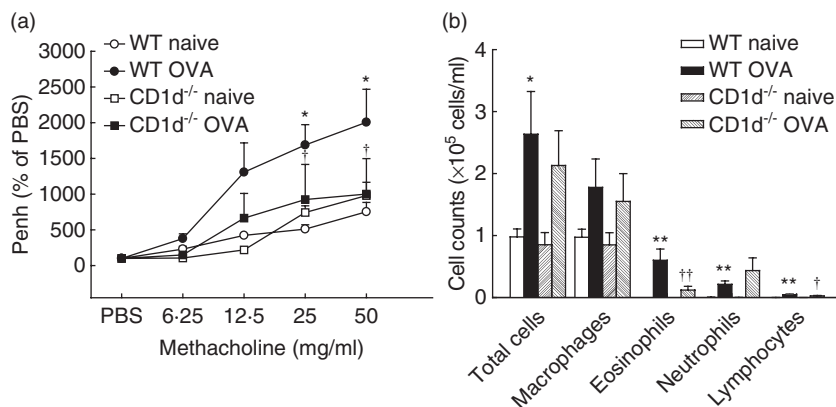


Fig. 5. CD1d^{-/-} mice were impaired in inducing OVA-induced airway hyperresponsiveness (AHR) and eosinophilic airway inflammation in the acute asthma model. Wild-type (WT) and CD1d^{-/-} mice were sensitized and challenged as in Fig. 1b. (a) CD1d^{-/-} mice were impaired in the development of AHR. AHR was determined by non-invasive measurement of airway resistance [enhanced pause (Penh)] 24 h after the last OVA challenge. Data represent the mean ± s.e.m. from two experiments ($n = 6–10$). Statistical analysis was performed using the Mann–Whitney *U*-test. * $P < 0.01$ versus WT naive. [†] $P < 0.05$ versus WT OVA. (b) CD1d^{-/-} mice were impaired in the production of OVA-induced airway eosinophilia. BAL fluid was obtained 24 h after the AHR measurement. Data represent the number of cells/ml in the BAL fluid and were the mean ± s.e.m. from two experiments ($n = 8–10$). Statistical analysis was performed using the unpaired *t*-test. * $P < 0.05$ and ** $P < 0.01$ versus WT naive; [†] $P < 0.05$ versus CD1d^{-/-} naive; ^{††} $P < 0.05$ versus WT OVA.

chronic asthma. We found that NK T cells were not required for the development of chronic AHR. Conversely, it has been reported that airway remodelling is dissociated from AHR, and that mast cell localization to the airway smooth muscle bundle is associated with AHR [20]. Based on the findings of this study, it could be hypothesized that lung NK T cells might not affect mast cell localization in the chronic asthma model. In addition, we determined whether acute AHR developed in NK T cell-deficient mice, because previous studies have demonstrated that NK T cells play a critical role in the development of acute AHR [4,5]. Consistent with previous findings [4,5], in a model of acute asthma, OVA-induced acute AHR did not develop in the NK T cell-deficient mice. The pathogenesis of chronic AHR may differ from that of acute AHR in asthma, and NK T cells may be dispensable in the development of allergen-induced chronic AHR, while they are indispensable for the development of acute AHR.

Indeed, OVA-induced chronic airway inflammation developed in NK T cell-deficient mice in a chronic asthma model, suggesting that NK T cells were not required for the development of allergen-induced chronic inflammation in the mouse model of asthma. However, in the model of acute asthma, OVA-induced eosinophilic airway inflammation was reduced in the NK T cell-deficient mice. Thus, NK T cells were not required for the development of allergen-induced chronic eosinophilic inflammation, although they played a role in the development of acute eosinophilic inflammation in asthma. A single activation of NK T cells rapidly produces very large amounts of Th1/Th2 cytokines; however, NK T cells are known not to respond to repeated activation. Prolonged stimulation may render NK T cells anergic to more stimulation [21]. It is possible that the anergic NK T cells might be useless in a mouse model of chronic asthma. This probable phenomenon might explain in part why NK T cells are dispensable for the development of allergen-induced chronic asthma, but not acute asthma. This might explain why there is a different requirement for NK T cells in acute and chronic asthma. Instead, conventional Th2 cells may be sufficient for the development of chronic eosinophilic airway inflammation [22,23]. We measured Th2 cytokines in supernatants obtained from OVA-stimulated bronchial lymph node cultures and BAL fluids in the chronic asthma model. The production of Th2 cytokines was not impaired in the OVA-induced NK T cell-deficient mice compared to the OVA-induced WT mice. By contrast, the cytokine production was impaired in the NK T cell-deficient mice from the acute asthma model. These findings suggest that NK T cells are required for the development of Th2 cells in the acute asthma model, but not in the chronic asthma model. The NK T cells may contribute in part to the development of OVA-induced asthma by enhancing the response of Th2 cells in the acute asthma model, but not in the chronic asthma model. Therefore, both NK T cells and Th2 cells may be required for the

development of allergen-induced acute inflammation; however, Th2 cells alone may be sufficient for the development of allergen-induced chronic airway inflammation in asthma. Nevertheless, the role of the NK T cells in the development of acute inflammation might be minor. Mice depleted of NK T cells and CD8⁺ T cells have been reported to develop acute airway inflammation [24]. In the model of acute asthma, airway inflammation was not inhibited completely in the NK T cell-deficient mice.

It has been reported that chronic allergen challenges induce tolerance and the total number of cells in BAL fluids decrease as allergen challenges are prolonged [25]. However, in the present study the total number of cells in BAL fluids from the OVA-induced WT mice was higher in the chronic asthma model than in the acute asthma model. These BALs and counts were performed using the same method. The inflammation of the lung tissues was analysed and compared between acute and chronic asthma models. The degree of peribronchial and perivascular inflammation in the OVA-induced WT mice was higher in the chronic asthma model than in the acute asthma model (data not shown). In the present study, the inflammation of the airways and lungs might have been more severe in the chronic asthma model than in the acute asthma model, in contrast to the findings of others [25]. The inflammatory response of the airways and lung might differ according to sensitization and challenge protocols in the murine models used for allergic asthma.

In the chronic asthma model, remodelling features, including mucus metaplasia, subepithelial fibrosis and an increased smooth muscle mass, were induced significantly in the NK T cell-deficient mice as well as in the WT mice. These findings suggest that NK T cells were not required for the development of allergen-induced airway remodelling. Airway remodelling is attributed commonly to the underlying chronic inflammatory process, although this continues to be debated. Our finding that allergen-induced chronic airway inflammation developed in NK T cell-deficient mice may explain in part the reason why airway remodelling occurred without NK T cells in the mouse asthma model. Conventional CD4⁺ cell-derived Th2 cytokines have been reported to contribute to the development of airway remodelling [13,14]. IL-13, together with IL-4, may stimulate the production of transforming growth factor (TGF)- β by bronchial epithelial cells and their release determines the direct activation of fibroblasts, which results in the indirect effects on subepithelial fibrosis and airway remodelling [26]. However, NK T cell-derived Th2 cytokines did not appear to affect the development of airway remodelling in the mouse model of chronic asthma. Conventional Th2 cells alone might be sufficient for the development of allergen-induced remodelling. However, it has been suggested that the airway remodelling observed in asthmatic airways is the consequence of abnormal injury and repair responses stemming from the susceptibility of bronchial epithelia to components

of the inhaled environment [11,12]. Airway remodelling might develop independently of the Th2-derived airway inflammation. Doherty *et al.* [27] showed that CD4⁺ T cells, and other CD4⁺ cells, including NK T cells, play a role in ongoing eosinophilic airway inflammation, but are not required for airway remodelling. In any case, lung NK T cells may be dispensable for the development of airway remodelling in asthma.

The role for NK T cells in the pathogenesis of IgE production remains unclear. Murine NK T cells have been reported to be essential [28] or non-essential [29] for IgE production. α -GalCer-activated murine NK T cells were reported to inhibit IgE production by producing a large amount of IFN- γ [30]. In the present study, using a mouse model of chronic asthma, serum OVA-specific IgE production was not impaired in the NK T cell-deficient mice. Therefore, NK T cells may be dispensable for IgE production in chronic asthma. By contrast, the OVA-specific IgE were impaired in the NK T cell-deficient mice from the acute asthma model. The findings suggest that NK T cells are required for the production of IgE in the acute asthma model, but not in the chronic asthma model.

In the present study, the development of AHR, airway inflammation and remodelling was not impaired in the chronic OVA-induced CD1d^{-/-} mice. One possible explanation for these findings could be as follows. It has been reported that type 1 NK T cells, which are absent in CD1d^{-/-} mice, have a regulatory capacity [31], and might play a role in the suppression of inflammation and AHR. Thus, their absence could contribute potentially to the increased AHR and inflammation that counters any deficiency due to the absence of type 1 NK T cells.

In the mouse model of asthma, AHR measurement may be more accurate using invasive methods than by non-invasive methods [32]. Invasive AHR measurement may be much less likely to be affected by upper airway resistance or by other artefacts. Unfortunately, the invasive method has not been available in our laboratory. However, additional AHR measurement by the invasive method should be needed to confirm our AHR data.

NK T cells have been reported to play a role in the development of AHR in distinct models of asthma, such as ozone-induced [7] or virus-induced [8] induced asthma. In these models, a different subset of invariant NK T cells was required for the development of AHR. In addition, even in a murine model of chronic asthma, NK T cells were required for the development of virus-induced asthma [8]. In the virus-induced chronic asthma model, NK T cells were required for the development of AHR and chronic NK T cell activation was triggered by viral infection [8]. Therefore, it could be hypothesized that chronic NK T cell activation may be induced by virus, but not allergen, in a murine model of chronic asthma.

In the chronic asthma model of the present study, the levels of Th2 cytokines and OVA-specific IgE were low. The

degree of Th2 response and IgE production may depend upon the type and dose of antigen, duration of antigen exposure, route of antigen administration, the use of systemic sensitization and animal strain [33]. The levels of Th2 cytokines and IgE were correlated inversely with the dose of sensitizing antigen [34]. It is possible that the Th2 cytokine and IgE responses may be attenuated in part by the relatively higher dose of sensitizing antigen. In addition, in the acute asthma model of the present study, the Th2 cytokine and IgE responses were not at all induced in NK T cell-deficient mice, unlike the other previous study [5]. One possible explanation could be that the degree of Th2 cytokine and IgE production may be low in the acute asthma model for such reasons as mentioned above. Indeed, the levels of Th2 cytokines and IgE were low in the OVA-induced WT mice of the acute asthma model.

In conclusion, in a mouse model of chronic asthma, asthma features such as AHR, eosinophilic airway inflammation, airway remodelling, IgE production and Th2 response were not impaired in NK T cell-deficient mice. However, in the mouse model of acute asthma, AHR and eosinophilic airway inflammation were impaired in the NK T cell-deficient mice, consistent with the findings reported in other studies using mouse models of acute asthma [4,5]. The present study suggests that NK T cells are not required for the development of allergen-induced asthma in the chronic asthma model, although they play an essential role in the pathogenesis of asthma in the acute asthma model. However, the lack of a requirement for NK T cells has to be put into a much larger context. AHR and airway inflammation were induced even in RAG^{-/-} mice, lacking any T cells, in response to IL-13 [35] or IL-33 [36]. Under different circumstances, asthma might be induced in the absence of any T cells.

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Disclosure

None.

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