

Natural killer T cells contribute to airway eosinophilic inflammation induced by ragweed through enhanced IL-4 and eotaxin production

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Although NKT cells have been found to be capable of modulating immune responses in several model systems, the role of NKT cells in allergy remains unclear. Using CD1 gene knockout (KO) mice, which lack NKT cells, we examined the function of NKT cells in the development of allergic inflammation induced by a common airborne human allergen, ragweed. The data showed that airway eosinophilia and mucus overproduction induced by ragweed were significantly reduced in CD1 KO mice, which was correlated with significantly lower allergen-driven IL-4 production and lower eotaxin responses in the airways of CD1 KO mice. Moreover, both ragweed-specific and total serum IgE levels in CD1 KO mice were significantly lower than those in control BALB/c mice. The reduced allergic reaction in CD1 KO mice is not due to intrinsic deficiency because they showed normal levels of immune cells and function. In addition, *in vivo* stimulation of NKT cells using their natural ligand, α -galactosylceramide, enhanced ragweed-induced airway eosinophilia, IL-4, and eotaxin production in control, but not CD1 KO mice. These data provide *in vivo* evidence for the involvement of NKT cells in the allergic mechanisms responsible for allergen-driven cytokine and chemokine production and airway inflammation.

Key words: Allergy / Ragweed / Natural killer T cells / Eosinophilia / CD1 knockout

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1 Introduction

Natural killer T (NKT) cells constitute a novel type of T cells of the lymphoid lineage and are conserved across many species. They are characterized by a TCR with limited diversity, which does not appear to be expressed by conventional α/β T cells [1–5]. In addition, they are phenotypically distinct as they simultaneously express TCR and NK cell markers. NKT cell subpopulations may be double-negative, single-positive or double-positive with regards to CD4 and CD8 molecule expression [4, 6, 7–9]. Recently, the involvement of NKT cells in modulating immune responses to various antigens has been implicated [7, 10–19]. They have been found to be essential in tumor rejection as well as in the prevention of autoimmune diseases, such as multiple sclerosis and chronic inflammatory demyelinating polyneuropathy [12–14].

Interestingly, NKT cells are reported to be involved in the promotion of both Th1-like reactions in response to intracellular pathogens [15] as well as the production of Th2 cytokines [16–19].

Allergy involves a state of immediate hypersensitivity to antigens that normally do not represent a threat to the normal human host. Airway eosinophilic inflammation and IgE response represent the hallmark pathological changes in the asthma-like reaction. The mechanism underlying the initiation and development of allergic responses remains unclear. Due to the fact that NKT cells are capable of rapidly secreting key immunoregulatory cytokines such as IL-4 and IFN- γ [10, 11], they are likely to play a role in modulating allergic diseases. However, comprehensive studies are lacking regarding the role of NKT cells and CD1 molecules in allergic responses especially in asthmatic reactions. In particular, the limited studies on NKT cells in allergic responses generated very controversial data on the potential role of NKT cells in the development of allergic reaction, especially IgE responses [20–27]. Some studies suggest that NKT cells are not essential for the allergic reaction and their activation can even inhibit allergen-specific IgE

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Abbreviations: **RW:** Ragweed **BAL:** Bronchoalveolar lavage **HMI:** Histological mucus index **KO:** Gene knockout **α -GalCer:** α -Galactosylceramide

responses [20, 23–27]. Akbari et al. reported that NKT cells play an essential role in the development of ovalbumin-induced airway hyperreactivity [28].

In the present study, we intended to perform a comprehensive investigation of the role of NKT cells in the development of the asthma-like reaction using a common environmental allergen, ragweed (RW). Since CD1 is constitutively expressed by cortical double-positive CD4⁺CD8⁺ thymocytes, which positively select for developing NKT cells, CD1 gene knockout (KO) mice are deficient in NKT cells [1, 4, 8, 21, 22].

Using a well-characterized asthma-like reaction model [29, 30], we found that, in comparison with BALB/c control mice, the NKT cell-deficient CD1 KO mice (backcrossed to BALB/c 11 times) showed significantly lower pulmonary eosinophilia and bronchial mucus production following RW sensitization and local challenge. In addition, the levels of both RW-specific and total serum IgE antibody responses in the CD1 KO mice were significantly lower than control BALB/c mice following RW sensitization and challenge. The reduction of allergic reaction in CD1 KO mice was associated with an impaired allergen-driven IL-4 production by spleen and draining lymph node cells and reduced eotaxin production in the lung. Moreover, stimulation of NKT activity with α -galactosylceramide (α -GalCer) enhanced airway eosinophilia, IL-4, and eotaxin production in wild-type BALB/c mice, but not in CD1 KO mice. These data provide evidence for the involvement of NKT cells in allergic reaction and in the development of Th2-type immune responses induced by environmental allergens such as RW.

2 Results

2.1 CD1 KO mice display reduced pulmonary eosinophilia and bronchial mucus production

CD1 KO mice and BALB/c control mice were analyzed for airway inflammatory reaction and mucus secretion following RW sensitization and challenge. These two parameters were analyzed because eosinophilic infiltration and mucus oversecretion by goblet cells in the airway epithelium are the classical hallmarks of an asthma-like reaction. The results showed that CD1 KO mice demonstrated significantly less cellular infiltration into the lung following RW challenge than the control BALB/c mice with the same treatment (Fig. 1), particularly with regards to the number of infiltrating eosinophils ($4.1 \times 10^5 \pm 0.5 \times 10^5$ in CD1 KO mice vs. $1.6 \times 10^6 \pm 0.6 \times 10^6$ in BALB/c mice; $p < 0.05$).

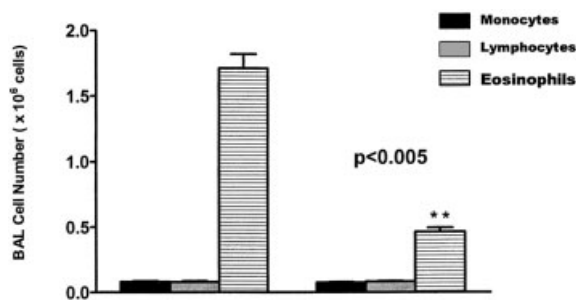


Fig. 1. CD1 KO mice show decreased pulmonary eosinophilic infiltration induced by RW. Mice were sensitized i.p. with 100 μ g RW in alum. The mice were intranasally challenged with 150 μ g RW at day 14 following RW sensitization and were killed 6 days post-challenge. Pulmonary cellular infiltration was examined by differential cell counts of the BAL fluid cells. Data presented represent three independent experiments. ** $p < 0.05$, CD1 KO vs. BALB/c mice.

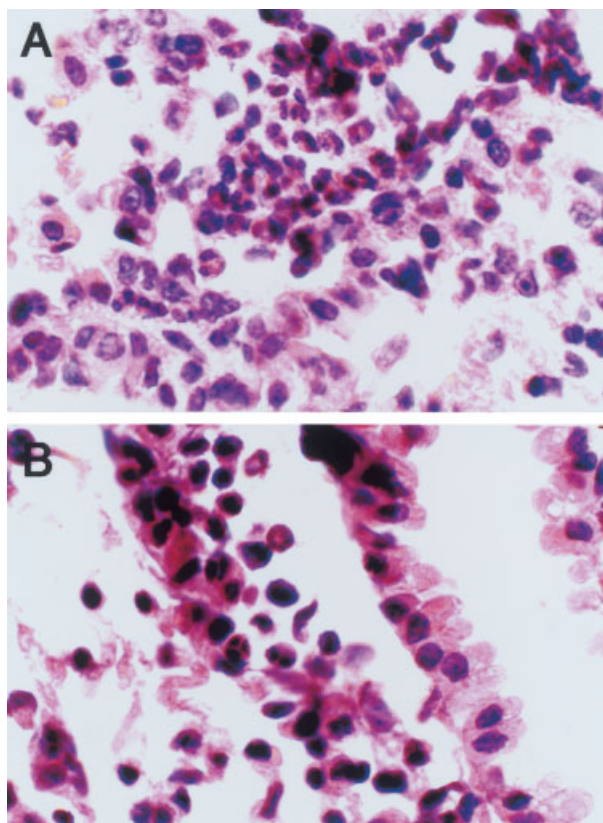


Fig. 2. RW-treated CD1 KO mice display a dramatic reduction in infiltrating eosinophils into the lung tissues compared to RW-treated BALB/c mice. Lungs from BALB/c (A) and CD1 KO mice (B) were fixed in 10% buffered formalin. Lungs were cut into 10- μ m-thick sections, stained with H&E, and examined at 400 $\times$ magnification.

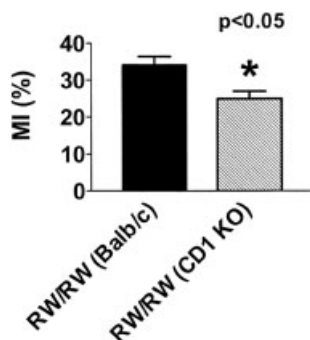


Fig. 3. CD1 KO mice show reduced mucus production induced by RW exposure in comparison with BALB/c mice. Mice were treated as described in the legend to Fig. 3. Lung sections were stained with thionin for mucus and mucus-containing goblet cells. HMI was calculated based on the percentage of the mucus-positive area over the total area of the airway epithelium. Each group was composed of four mice. Data represent means $\pm$ SD. One representative experiment of three independent experiments is shown. * $p < 0.05$, HMI in RW-treated CD1 KO mice vs. that in RW-treated BALB/c mice.

In addition, histological analysis also showed remarkable differences in cellular infiltration to the lung between CD1 KO and control BALB/c mice following RW treatment (Fig. 2). BALB/c control mice displayed massive infiltration of eosinophils in the bronchial submucosa, alveolar, and perivascular sheaths, whereas CD1 KO mice showed significantly lower levels of infiltrating cells, especially eosinophils. In addition, the levels of mucus production within the bronchial epithelium of RW-treated CD1 KO mice were significantly lower than those found in RW-treated BALB/c mice (Fig. 3). These results demonstrate that CD1 KO mice mount less asthma-like reaction to a natural allergen, implicating a role of NKT cells in the development of allergic pathological reactions in the lung.

2.2 CD1 KO mice exhibit reduced circulating eosinophils following RW exposure

Because the airway eosinophilia in asthma is often associated with elevated peripheral blood eosinophil levels, resulting from increased eosinophil production and/or release from the bone marrow, we further tested the impact of NKT deficiency on the development of systemic eosinophilia caused by RW exposure. As seen in Table 1, although RW exposure induced blood eosinophilia in both BALB/c and CD1 KO mice, the latter showed significantly less increase in blood eosinophils than the former. These results demonstrate that NKT cells are involved in the development of the allergic reaction at both local and systemic levels.

Table 1. Circulating eosinophils in peripheral blood of RW-treated CD1 KO mice versus BALB/c mice treated with RW

	BALB/C	CD1 KO
Lymphocytes	60.0 $\pm$ 0.05	64.8 $\pm$ 0.37
Neutrophils	38.1 $\pm$ 0.69	30.4 $\pm$ 0.21
Monocytes	2.91 $\pm$ 0.37	4.33 $\pm$ 0.42
Eosinophils	0.650 $\pm$ 0.183	0.333 $\pm$ 0.0318*

* = $p < 0.05$

2.3 The absence of NKT cells results in altered IL-4 and eotaxin production induced by RW allergen exposure

In order to examine the mechanism by which NKT cells regulate allergic responses to the RW allergen, we studied the cytokine and chemokine response in RW-treated BALB/c and CD1 KO mice. The results, as shown in Fig. 4, demonstrated that upon restimulation with RW allergen, both spleen and draining lymph nodes from RW-treated CD1 KO mice produced significantly lower levels of IL-4, a stereotypic Th2-like cytokine, compared with those from RW-treated BALB/c mice. In addition, CD1 KO mice exhibited significantly lower levels of eotaxin in their bronchoalveolar lavage (BAL) fluids following RW challenge (Fig. 4). Interestingly, IL-13 and IL-5, which have also been shown to play a role in the maintenance of the allergic response, did not appear to be significantly affected in the CD1 KO mice (Fig. 4). The results suggest that NKT cells play a critical role in allergen-driven IL-4 and eotaxin production.

2.4 CD1 KO mice show a significant reduction in serum IgE levels

As elevated serum IgE levels are another hallmark characteristic of atopic individuals, we also measured serum IgE in both BALB/c and CD1 KO mice. As expected, both groups of mice displayed undetectable levels of RW-specific IgE and low levels of total IgE in their sera before RW exposure. When mice were sensitized and subsequently challenged with RW, the levels of both RW-specific and total IgE responses in RW-treated BALB/c and CD1 KO mice were significantly increased. However, the levels of both RW-specific and total serum IgE levels in RW-treated CD1 KO mice were significantly ($p < 0.05$) lower compared to those measured in RW-treated BALB/c mice (Fig. 5). Similarly, RW-specific IgG1, but not IgG2a, produced by CD1 KO mice was significantly lower than that produced by wild-type control mice (Fig. 5). The data suggest an important role played

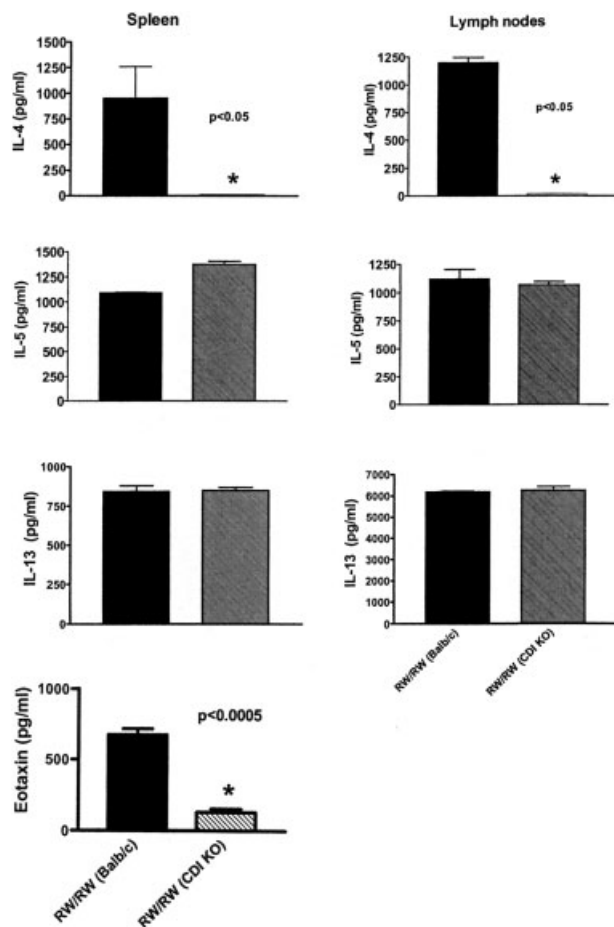


Fig. 4. Dramatic reduction in allergen-driven IL-4 production and local eotaxin levels in CD1 KO mice compared to BALB/c mice. Mice (four mice per group) were treated as described in the legend to Fig. 3. Spleen and lymph node cells were cultured at 7.5×10^6 cells/ml and 5.0×10^6 cells/ml, respectively, in the presence of RW (0.1 mg/ml), and the culture supernatants were harvested at 72 h. Th2 cytokines in the culture supernatants were determined by ELISA. Data are presented as the means $\pm$ SD of each group. * $p < 0.05$, cytokine production in RW-treated CD1 KO mice vs. that in RW-treated BALB/c mice. For the determination of eotaxin, lungs were washed with PBS. BAL fluid was centrifuged and supernatants were collected. Eotaxin levels were measured by ELISA. * $p < 0.0005$, eotaxin production in RW-treated CD1 KO mice vs. that in RW-treated BALB/c mice. One representative experiment of three independent experiments is shown.

by NKT cells in the development of Th2-related antibody responses such as IgE and IgG1, the pertinent parameters of the allergic response.

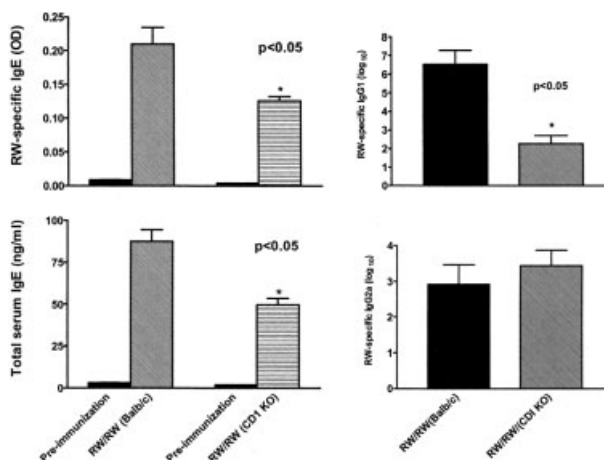


Fig. 5. Significant decrease in both RW-specific and total serum IgE levels induced by RW sensitization/challenge in CD1 KO mice compared to control BALB/c mice. Mice (four mice per group) were bled before treatment with RW as described in the legend to Fig. 3 and were bled 6–8 days post-RW challenge. ELISA titers for RW-specific antibodies were converted to log₁₀ and presented as the means $\pm$ SEM. * $p < 0.05$, RW-treated CD1 KO mice vs. RW-treated BALB/c control mice. Pooled data for three experiments are presented.

2.5 CD1 KO and wild-type BALB/c mice display comparable levels of lymphocyte number and function

To exclude the possibility that the difference observed between CD1 KO and BALB/c mice is caused by an intrinsic defect in immune cells of the former mice rather than due to NKT deficiency, we analyzed the intrinsic levels of immune cells including conventional T lymphocytes, namely CD3⁺, CD4⁺, CD8⁺ cells, and B lymphocytes; *i.e.* CD19-expressing cells, in NKT cell-deficient CD1 KO mice. As shown in Fig. 6, the percentage of CD3⁺, CD4⁺, CD8⁺, and CD19⁺ cells found in the spleen of CD1 KO mice was comparable to those found in BALB/c control mice. In addition, the levels of NK (DX5⁺) cells and $\gamma\delta$ T cells were also comparable between these mice (Fig. 6). Similar results were obtained in analysis using peripheral blood cells (data not shown).

We next examined whether the functions of lymphocytes from CD1 KO mice are functionally intact. Spleen cells from both naive BALB/c wild-type mice and CD1 KO mice were cultured alone or in the presence of a polyclonal activator, Con A. Following Con A stimulation, spleen cells from CD1 KO and wild-type mice showed comparable levels of proliferative responses (Fig. 7A) and IL-4 production (Fig. 7B). Therefore, although the CD1 KO mice are deficient in NKT cells, their key

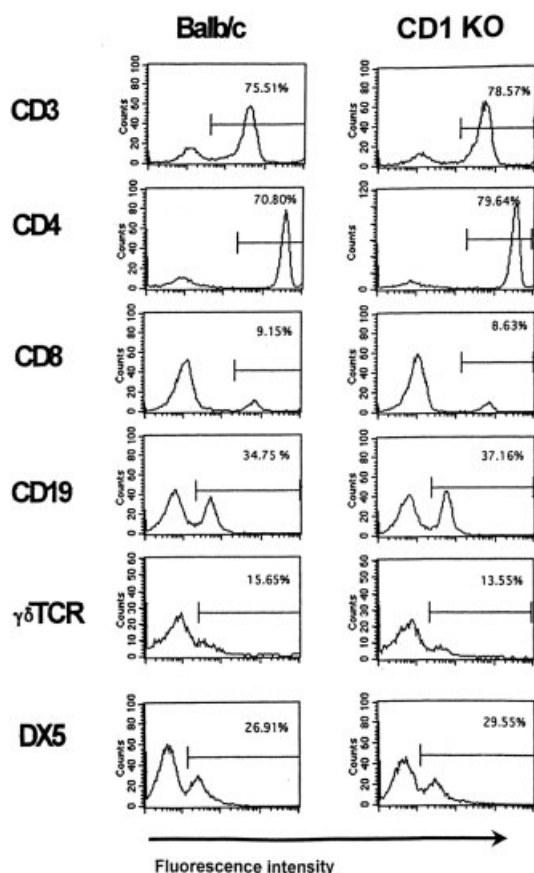


Fig. 6. Naive CD1 KO mice display comparable levels of CD3⁺, CD4⁺, CD8⁺, CD19⁺ (B cell), $\gamma\delta$ TCR⁺, and DX5⁺ (NK cell) positive cells compared to BALB/c control mice. Single-spleen-cell suspensions from naive CD1 KO and BALB/c mice were stained for T, B and NK cell markers using fluorescence-labeled monoclonal antibodies, followed by fixation with 4% paraformaldehyde. Cells were analyzed by flow cytometry. Data presented represent three independent experiments using a FACSCalibur II and CellQuest program. The figure shows the percentage of each cell type.

immune cells, namely CD3⁺, CD4⁺, CD8⁺, and CD19⁺-expressing lymphocytes appear normal in amount and function. The data suggest that the reduction of allergic reaction in CD1 KO mice is due to the deficiency of NKT cells rather than a defect in conventional immune cells.

2.6 Stimulation of NKT activity with α -GalCer enhanced allergic reactions to RW exposure, which was correlated with enhanced IL-4 and eotaxin production

To confirm the role of NKT cells in the development of allergic reaction, we further tested the effect of α -GalCer treatment on the allergic reaction in wild-type BALB/c

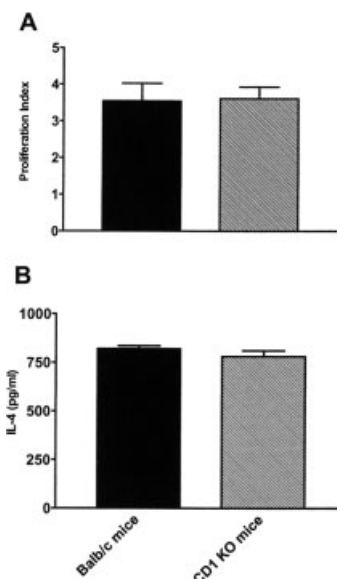


Fig. 7. Lymphocytes from CD1 KO mice show normal levels of cellular proliferation and IL-4 production following polyclonal stimulation. Splens from naive CD1 KO and BALB/c mice were aseptically removed. Spleen cells were cultured in the presence or absence of Con A stimulation. (A) Con A-stimulated cells were pulsed with [³H]thymidine and [³H]thymidine incorporation was assessed using a TopCount NXT microplate scintillation and luminescence counter. Stimulation index (S.I.) was calculated based on the fold of counts in Con A-stimulated cells over the control wells. (B) IL-4 levels in the 72-h culture supernatants were measured by ELISA.

and CD1 KO mice. α -GalCer is a natural ligand of NKT cells, which can specifically stimulate NKT cell activity *in vivo* [31, 32]. As shown in Fig. 8, BALB/c mice treated by α -GalCer (KRN7000) showed dramatically enhanced allergen-driven IL-4 production by lymphocytes, eotaxin expression by airway tissues, RW-specific IgE production, and eosinophil infiltration in the BAL. Similar responses to α -GalCer stimulation were observed in wild-type 129 mice (data not shown). All of these differences observed in wild-type (BALB/c and 129) mice with or without α -GalCer treatment were statistically significant. In contrast, CD1 KO mice, which are deficient in NKT cells, failed to respond to α -GalCer treatment in the perspectives of IL-4, eotaxin, and RW-specific IgE production and airway eosinophilic infiltration.

Therefore, the enhanced effect on the allergic reaction and cytokine (IL-4)/chemokine (eotaxin) production seen in wild-type mice following α -GalCer treatment reflects the *in vivo* function of NKT cells. Moreover, the highly associated increase in IL-4 and eotaxin production and enhancement of the allergic reaction (eosinophilia and IgE production) by *in vivo* activation of NKT cells demon-

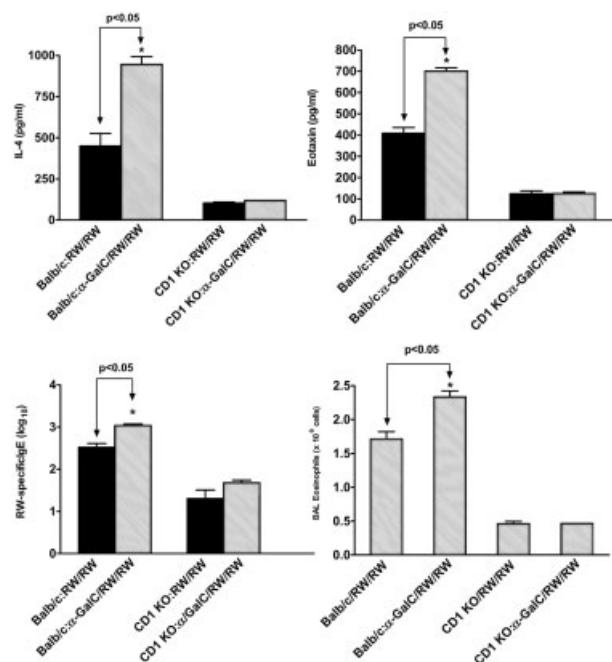


Fig. 8. *In vivo* activation of NKT cells results in exacerbated allergen-driven IL-4 production, eotaxin expression, RW-specific IgE measurements, and eosinophilia in wild-type BALB/c mice compared to CD1 KO mice. Naive CD1 KO and BALB/c mice were injected i.v. with 4 μ g α -GalCer in PBS or polysorbate vehicle control. Mice were sensitized 2 h following α -GalCer or vehicle injection with RW and challenged. Mice were sacrificed 6–8 days post-challenge and spleens were cultured in the presence of RW for allergen-driven IL-4 measurements. BAL fluid was collected and used to measure eotaxin and eosinophil levels, and sera were measured for RW-specific IgE levels. Data are presented as the means $\pm$ SD of each group. One representative experiment of four independent experiments is shown.

strate that NKT cells play a role in the development of allergic response, possibly mainly via enhancing IL-4 and eotaxin production.

3 Discussion

Our present study using a common environmental allergen, RW, provides evidence that NKT cells play an important role in the outcome of the allergic responses. The data show that airway eosinophilic inflammation induced by RW was significantly reduced in NKT-deficient CD1 KO mice, which is correlated with impaired IL-4 and eotaxin production. The CD1 KO mice also displayed a significant decrease in serum allergen-specific and total IgE levels in their sera compared to BALB/c mice. Similar difference in allergic responses was

observed in the study comparing wild-type 129 mice and CD1 KO mice (data not shown).

The difference between CD1 KO and wild-type BALB/c mice is unlikely due to potential intrinsic deficiencies of CD1 KO mice in the development and function of immune cells, because T, B and NK cell, levels and function in the two types of mice are comparable (Fig. 6, 7). The inability of CD1 KO mice to produce IL-4 in response to allergen-specific restimulation provides evidence that NKT cells are responsible for the development of IL-4 responses to natural allergen, thus capable of affecting later downstream allergic disease parameters. In contrast, the Th1 arm of the immune response (IFN- γ) appears unaffected by the lack of NKT cells in our research model (data not shown). The explanation for such a phenomenon may be twofold. Firstly, the study model is an asthma-like reaction, which is dominated by Th2-like responses; therefore the impact of NKT deficiency may be more visible with regards to the parameters of Th2-type responses. Secondly, it is possible that NKT cells play a more dominant role in IL-4 responses, although they are capable of producing IFN- γ in certain circumstances. Since Th2 cytokines other than IL-4, such as IL-5 and IL-13, remain largely unaffected by the absence of NKT cells in the allergen-treated CD1 KO mice (Fig. 4), it is likely that NKT cells are particularly critical for allergen-driven IL-4 responses.

A novel finding in this study is the dependence of eotaxin production on NKT cells. Several Th2 cytokines such as IL-4, IL-5, and IL-13 may be able to promote eotaxin production. The present study, however, showed that allergen-driven IL-5 and IL-13 productions in CD1 KO mice were normal, although eotaxin production in these mice was significantly reduced, which paralleled with significantly decreased allergen-driven IL-4 production (Fig. 4). The association between NKT cells and reduced allergen-driven IL-4 and eotaxin production was also confirmed in the study involving α -GalCer treatment, which showed that *in vivo* stimulation of NKT cells with α -GalCer significantly increased both IL-4 and eotaxin responses (Fig. 8). It has been reported that IL-4 can bind to its receptors expressed on the surface of airway epithelial cells and induce eotaxin production [33]. The results in the present study suggest that NKT cells play a particularly important role in eotaxin production through either a direct effect or indirect mechanisms such as enhancing IL-4 production.

The significant reduction in eotaxin expression may be helpful in explaining a paradoxical finding in the present study that comparable levels of IL-5 were produced by CD1 KO mice and wild-type BALB/c mice but the levels of airway eosinophilia were very different between these

mice. There are numerous reports, which have shown that IL-5 plays an important role in the maturation, release and recruitment of eosinophils in allergic and parasitic infection models [33, 34]. However, there exists some debate regarding the link between IL-5 production and eosinophilia in various allergic diseases such as asthma. Although there is no doubt that IL-5 can promote eosinophilia, the level of eosinophils may be independently regulated by multiple factors including IL-4 and chemokine expression. Notably, it has been reported that pulmonary eosinophilia can occur in IL-5 KO mice following local antigen challenge [35]. In addition, a previous study showed that the use of a corticosteroid, budesonide, reduced bone marrow eosinophil progenitors, circulating eosinophils, and airway eosinophilic infiltration, but failed to alter allergen-induced increases in IL-5 [36].

Since no significant change in IL-5 production was found, the remarkable reduction in airway eosinophilia seen in the present study may be largely attributed to the decrease in airway expression of eotaxin in the airway as shown in Fig. 4. The mechanism is likely to be that the reduced IL-4 production in CD1 KO mice results in lower levels of eotaxin secretion, which subsequently leads to reduced eosinophil recruitment to the site of local allergen exposure. Moreover, the present study also showed that the levels of eosinophils in the peripheral blood were significantly lower in CD1 KO mice compared to BALB/c mice, which indicates that NKT cells play an important role in eosinophilia, not only in the phase of recruitment, but also in the phase of production/release of eosinophils from bone marrow.

Another novel finding in this *in vivo* study is the association between NKT cells and mucus overproduction by epithelial goblet cells following allergen exposure. Mucus overproduction by goblet cells within the airway epithelial layer is a classical hallmark of the asthma-like reaction and a major factor in human asthmatic deaths. There are no previous links established between mucus production by epithelial goblet cells and NKT cells in an allergic response; thus the present data represent a key piece of evidence that implicates the role played by NKT cells in the development of the multiple components of allergic disease pathology. This finding is particularly interesting because previous studies have shown that IL-13 plays a critical role in mucus production [37, 38]. The data suggest that factors other than IL-13 that are directly or indirectly related to NKT cells may independently enhance allergen-driven mucus overproduction.

Our data are in contrast with a previous report, which shows that NK cells, rather than NKT cells, play a significant role in allergic reaction in a mouse model [20]. We

have shown in the present study that, although the immune cells including T, B and NK cells in CD1 KO and wild-type control mice are comparable, the NKT-deficient CD1 KO mice exhibit much less airway allergic reaction, indicating the critical importance of NKT cells in allergic reaction. The reason for this discrepancy between the studies is unclear. However, our data collected from a comprehensive study analyzing airway eosinophilia, mucus secretion, antibody production, and cytokine/chemokine responses speak for themselves. More importantly, the study using α -GalCer treatment, which showed enhanced allergic reaction in wild-type (BALB/c and 129) but not NKT-deficient mice (Fig. 8), provided convincing evidence that confirms the involvement of NKT cells in the allergic reaction. Moreover, the use of RW, a common environmental human allergen, in the present study further emphasizes the relevance of this finding to human asthma-like reaction.

The results are also inconsistent with another study, which showed α -GalCer treatment inhibits IgE production [31]. Notably, the strains of mice (C57BL/6 vs. BALB/c) and the allergens (ovalbumin vs. RW) used in these studies were different. However, it should be pointed out that the present data are consistent with the original finding that NKT cells can promote IgE responses [32].

Taken together, the findings in the present study have provided *in vivo* evidence that NKT cells play an important role in the development of allergic responses. The use of the RW allergen in this model is particularly significant as many atopic individuals suffer seasonal allergies as a result of the abundance of RW in the environment, and some studies on NKT cells have used less relevant allergens.

4 Materials and methods

4.1 Animals

BALB/c mice (7–10 weeks old) were purchased from Charles River Canada (Montreal, Canada). Breeding pairs of CD1 KO mice, which were originally developed in 129 background and had been backcrossed to BALB/c 11 times (C,129-*Cd1^{tm1Gru}*, 7–10 weeks old), were purchased from Jackson Laboratories (Bar Harbor, ME). The CD1 KO mice used in the study were bred at the University of Manitoba breeding facility (Winnipeg, Canada). Animals were used in accordance with the guidelines issued by the Canadian Council on Animal Care.

4.2 Sensitization and challenge with allergen

Naive BALB/c and CD1 KO mice were immunized intraperitoneally (i.p.) with 100 µg of RW extract (Hollister-Stier Canada Co., Toronto, Canada) in 2 mg of Al(OH)₃ (alum) adjuvant. On day 14 post-RW sensitization, mice were challenged intranasally with 150 µg of RW extract (40 µl) and were killed at day 6–8 following challenge.

4.3 BAL and blood differential cell counts

The mouse trachea was cannulated and lungs were washed with 1 ml sterile PBS to collect BAL fluids. The fluids were centrifuged and cell pellets were resuspended to prepare BAL smears. The slides were air-dried, fixed with ethanol, and then stained with the Fisher Leukostat Stain Kit (Fisher Scientific, Nepean, Canada). The numbers of neutrophils, monocytes, lymphocytes, and eosinophils per 200 cells were counted based on their morphology and staining characteristics. Blood smears from mice were prepared and stained for leukocytes using a Hema 3 Stain Set (Fisher Scientific) containing a cell fixative solution and eosin Y stain.

4.4 Histopathological analysis

Lungs of RW-exposed mice were collected and fixed in 10% buffered formalin. Lung tissues were embedded, sectioned and stained by hematoxylin and eosin as described [39]. Bronchial mucus and mucus-containing goblet cells were stained by thionin using the method of Mallory and quantified as histological mucus index (HMI) as previously described [39]. Briefly, the stained slides were analyzed at a final magnification of 400× with a rectangular 10-mm² reticule grid inserted into one eyepiece. Intersections of airway epithelium with the reticule were counted, distinguishing mucus-containing and mucus-non-containing epithelium. The HMI represents the ratio of the total number of mucus-positive intersections and the total number of all intersections.

4.5 Cell culture

Mice were killed at day 6–8 following intranasal RW challenge and examined for cytokine production patterns by both spleen and lymph node cells. Spleens and draining (mediastinum) lymph nodes were aseptically removed and single-cell suspensions were cultured as previously described [29]. Briefly, spleen and lymph node cells were cultured at a concentration of 7.5×10⁶ cells/ml and 5.0×10⁶ cells/ml, respectively, in the presence or absence of RW antigen (0.1 mg/ml). The complete culture medium was RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 25 µg/ml gentamycin, 2 mM L-glutamine, and 5×10⁻⁵ M 2-mercaptoethanol (Kodak, Rochester, NY). Culture supernatants were harvested at 72 h for the measure-

ment of cytokines. For lymphocyte proliferation measurements, spleen cells from naive BALB/c and CD1 KO mice (3.0×10⁵ cells/well) were cultured in the presence or absence of 4 µg/ml Con A (Sigma-Aldrich, Toronto, Canada) for 48 h in 96-well cell culture plates. The culture proceeded for another 6 h after adding 1 µCi [³H]thymidine (Sigma) to each well. [³H]Thymidine incorporation was analyzed thereafter using a TopCount NXT microplate scintillation and luminescence counter (Canberra Packard Biosciences, Mississauga, Canada).

4.6 Flow cytometry

Cell surface markers were analyzed by flow cytometry. Single-spleen-cell suspensions from naive BALB/c and CD1 KO mice were washed and red blood cells were lysed using NH₄Cl. Cells (2×10⁶ cells) were stained using fluorescence-labeled monoclonal antibodies purchased from PharMingen (San Diego, CA). The antibodies include PE-anti-mouse CD3 (clone 145-2C11, hamster IgG1, κ), PE-anti-mouse CD4 [clone GK1.5, rat (Lewis) IgG2b, κ], FITC-anti-mouse CD8 [clone 53-6.7, rat (LOU/Ws1/M) IgG2a, κ], FITC-anti-mouse CD19 [clone ID3, rat (Lewis) IgG2a, κ], FITC-anti-mouse pan-NK (clone DX5, rat IgG2a, κ), FITC-anti-mouse TCR δ chain (clone GL3, Armenian Hamster IgG2, κ), and corresponding isotype controls. Following incubation with antibody, cells were fixed using 4% paraformaldehyde, washed, resuspended in 0.2% BSA/PBS, and analyzed using a FACSCalibur flow cytometer and CellQuest program (BD Biosciences, San Diego, CA).

4.7 Determination of cytokine and chemokine production

Cytokines in the supernatants of spleen and lymph node cell cultures were analyzed by ELISA using purified (capture) and biotinylated (detection) antibodies as previously described [29, 39]. Antibodies purchased from PharMingen were used for ELISA measuring IL-4, IL-5, IL-12, and IFN-γ. IL-13 and eotaxin were determined using paired antibodies purchased from R&D Systems (Minneapolis, MN).

4.8 Determination of serum antibodies

RW-specific IgE as well as total IgE were measured by ELISA using antibodies purchased from PharMingen. For the measurement of RW-specific IgE, serum samples were pretreated by incubating twice with 50% slurry of protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) to remove IgG [29]. The treated samples were added to ELISA plate wells coated overnight with RW (20 µg/ml). Following extensive washing, biotinylated anti-IgE antibody was added. The plates were developed using avidin-conjugated alkaline phosphatase and measured by a microplate reader (Versamax; Molecular Devices, Sunnyvale, CA). For mea-

surement of total IgE, ELISA plates were coated with purified anti-mouse IgE capture antibody and detected using biotin-labeled anti-mouse IgE antibody as described [39]. RW-specific IgG1 and IgG2a antibodies in the sera were determined as described [29].

4.9 Treatment with α -galactosylceramide (KRN7000)

α -GalCer, which is 2S, 3S, 4R, 1-O-(α -galactopyranosyl)-2-(N-hexasosanoylamino)-1,3,4-octadecanetriol, was synthesized by Pharmaceutical Research Laboratories, Kirin Brewery (Gunma, Japan). The 200 μ g/ml stock solution was dissolved to a final concentration of 4 μ g with PBS. BALB/c and CD1 KO mice received a single i.v. injection of 2–4 μ g α -GalCer diluted in PBS. Control mice were injected with an identical volume of vehicle solution alone (0.025% polysorbate-20). Mice were sensitized with RW 2 h following α -GalCer or polysorbate injection.

4.10 Statistical analysis

Antibody titers (ELISA) were converted to logarithmic values and analyzed using the unpaired Student's *t*-test. Differential cellular counts, total IgE, and cytokine production levels were analyzed using the unpaired Student's *t*-test.

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