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An important role of tumor necrosis factor receptor-2 on natural killer T cells on the development of dsRNA-enhanced Th2 cell response to inhaled allergens

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Keywords

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Abstract

Background: Recent evidence indicates that TNF- α is a key mediator of the development of dsRNA-enhanced Th2 cell response to inhaled allergens. Natural killer T (NKT) cells may be a candidate source of Th2-polarizing cytokines.

Objective: The objective of this study was to evaluate the role of lung NKT cells on the development of TNF- α -mediated Th2 cell response.

Methods: A virus-associated asthma mouse model was generated by the administration of ovalbumin (OVA, 75 μ g) and poly[I:C] (0.1 μ g). Role of NKT and type I NKT cells was evaluated using CD1d- and J α 18-deficient mice. TNF- α receptors (TNFRs) were antagonized by using TNFR blocking peptides.

Results: The number of infiltrated NKT cells was increased in a virus-associated asthma mouse model. Increase in Th2 and Th17 cytokine levels in wild-type mice were abolished in both CD1d- and J α 18-deficient mice. *In vitro* co-culture experiments with alveolar macrophages and NKT cells showed that TNF- α produced by macrophages in the presence of poly[I:C] acts on NKT cells, inducing production of Th2-polarizing cytokines. Moreover, the induction of Th2-polarizing cytokines by poly[I:C] or recombinant TNF- α was impaired in both CD1d- and J α 18-deficient mice and that the above effect was reversed by a TNF- α receptor-2 (TNFR2) blocking peptide, but not by a TNFR1 blocker.

Conclusions: These findings suggest that NKT cells play a key role in the development of Th2 cell response to inhaled allergens and that TNF- α produced by alveolar macrophages induces Th2 cell response, via TNFR2 on NKT cells.

In airway inflammatory conditions such as asthma, T-cell memory is an important part of the response to inhaled allergens (1). The development of this kind of memory to inhaled allergens can be induced by the stimulation of allergens contaminated with viruses or viral PAMP double-stranded RNA (dsRNA) (2). Notably, in experiments using dsRNA models, airway inflammatory reactions are divided into Th1- and Th2-/Th17-dominant types according to the amount of adjuvant such as dsRNA (2, 3). Previously, we showed that the administration of low-dose dsRNA to airways during sensitization induces the production of Th2-polarizing cytokines, such as IL-4 and IL-13, while in the challenge period, it induces an allergen-specific Th2 immune response (4).

Generally, TNF- α is considered to be a pro-inflammatory cytokine that induces adhesion molecules or the production of other pro-inflammatory cytokines (5). However, there is evidence that TNF- α is related to Th2 immune response (6–9). Among the many frontline cells in the lung, alveolar macrophages are potent producers of TNF- α when exposed to dsRNA. Furthermore, our previous experimental data indicate that TNF- α plays a key role in the development of Th2 immune response to inhaled antigens by inducing IL-4 in a dsRNA-enhanced asthma model (4).

During the sensitization period in the lungs, many kinds of immune cells contribute to the cytokine environment necessary for T-cell development (10). In the case of Th2 cell development, the Th2-polarizing cytokines are crucial

for Th2 cell differentiation, acting on dendritic cells to form Th2-polarizing environment (10, 11). As innate immune cells, natural killer T (NKT) cells are a suitable candidate for the production of Th2-polarizing cytokines. These cells share some characteristics with NK and T cells, such as that existing in the lung, and may act rapidly in inflammatory states (12, 13). In addition, during acute immune responses, NKT cells produce enormous amounts of IL-4 and IL-13, and for this reason, NKT cells are regarded to be important for Th2 polarization in adaptive immunity, such as in asthma (14). α -Galactosylceramide (α -galcer) is a well-known and suitable ligand for the elaboration of IL-4 and IFN- γ (15). However, it acts on NKT cells too strongly and artificially, and there is evidence that it induces IL-4 and IFN- γ at different time points, early and late time points, respectively (15). Moreover, because α -galcer is not an endogenous ligand for NKT cells, it is hard to explain the role of NKT cells using α -galcer *in vivo*. This means that other unknown mechanisms activate NKT cells during inflammatory responses *in vivo*; thus, it is necessary to study how NKT cells activate and induce cytokines in inflammatory states *in vivo*.

In this study, we hypothesized that TNF- α , which is produced by alveolar macrophages in response to low-dose dsRNA, induces Th2 cell response via NKT cells. To test this hypothesis, we evaluated the role of NKT cells in the development of Th2 immune responses to an inhaled allergen using CD1d- and J α 18-deficient mice in a virus-associated asthma model. Additionally, we aimed to elucidate the signaling mechanism(s) of TNF- α -induced NKT activation during T-cell polarization.

Methods

Mice

CD1d-deficient, J α 18-deficient, and wild-type (WT) mice (C57BL/6 background) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were bred in a pathogen-free facility at POSTECH, and all live animal experiments were approved by POSTECH Ethics Committee.

Reagents

Synthetic dsRNA polyinosinic-polycytidylic acid (poly[I:C]) was obtained from Calbiochem (La Jolla, CA, USA). Ovalbumin (OVA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse recombinant TNF- α was obtained from R&D systems (Minneapolis, MN, USA). To inhibit the recognition of TNF- α through its receptor, mouse TNF- α receptor-1/2 blocking peptide was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Induction of lung inflammation in mice

To induce the OVA-specific acute lung inflammation model, 6-week-old mice were administrated 75 μ g of OVA with or without 0.1 μ g/10 μ g of poly[I:C] and sensitized

intranasally on days 0, 1, 2, and 7. After sensitization, 50 μ g of OVA was challenged intranasally on days 14, 15, 21, and 22, and 24 h after final OVA challenge, mice were killed for evaluation. For evaluating the role of NKT cell in early time of inflammation, mice were sensitized once with OVA in the presence or absence of 0.1 μ g of poly[I:C] and then evaluated 12 h after sensitization. The effects of TNF- α to NKT cells on innate immune response were evaluated 12 h after administration of 100 ng of recombinant mouse TNF- α in each mouse. In the case of TNF- α receptor-1 and TNF- α receptor-2 blocking peptides, mice were sensitized with OVA + 0.1 μ g of poly[I:C] and + 10 μ g of TNF- α blocking peptides 1 and 2, respectively, and then killed 12 h after sensitization to evaluate airway inflammation.

Cellularity in bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) cellularity was analyzed as described previously (2). Briefly, cell pellets were diluted in 50 μ l of phosphate-buffered saline (PBS), and 300 inflammatory cells were counted and classified as macrophages, lymphocytes, neutrophils, or eosinophils.

Lung histology

Lung sections were stained with hematoxylin and eosin (H&E) after pressure fixation with Streck solution (Streck Laboratories, La Vista, NE, USA). All slides were compared at the same magnification as described previously (2).

Single-cell preparation from lung and lung-draining lymph nodes

Briefly, for single-cell isolation from the lung tissue, tissue was chopped and incubated in 37°C with 0.05% trypsin (GIBCO, Grand Island, NY, USA) and 200 unit/ml of collagenase (GIBCO). After digestion for 10 min, tissue was ground using the cell strainer (BD Falcon, Bedford, MA, USA) and incubated in 4°C with RBC lysis buffer (Stem-Cell Technologies, Vancouver, BC, Canada). For isolating lymph node (LN), tissue was ground using the cell strainer and incubated with RBC lysis buffer as in single-cell preparation from the lung tissue.

Immune response in the lung and lung-draining lymph nodes

After harvest, lung-draining lymph nodes were cultured (2.0×10^6 /ml) in 24-well plates at 37°C in RPMI 1640 (Hyclone, UT, USA) in the presence or absence of CD3 and CD28 antibodies (1 μ g/ml each; eBioscience, San Diego, CA, USA) or OVA (100 μ g/ml). The levels of cytokines including IL-4, IL-17, and IFN- γ produced by the restimulated T cells were determined from culture supernatant fractions collected 12 h after CD3/CD28 antibody stimulation. In the case of OVA stimulation, evaluation was performed 72 h after restimulation.

Cytokine measurement

The levels of cytokines including IL-4, IL-6, IL-13, IL-17, TGF- β , IFN- γ , and IP-10 in BAL fluid and culture supernatants were measured using enzyme-linked immunosorbent assays (ELISAs) in accordance with the manufacturer's instructions (R&D Systems).

NKT cell stimulation and co-culture with alveolar macrophages

MH-S cells (mouse alveolar macrophage) were grown in RPMI 1640 medium (Hyclone) supplemented with 10% fetal bovine serum (GIBCO) and antibiotics (Hyclone). Cells (MH-S, 1×10^5 cells) were seeded in 96-well plates, and after 8 h for stabilization, DN32.D3 cells (NKT hybridoma cell, 1×10^4 or 1×10^5) were co-cultured with 100 μ g/ml of poly[I:C] for 12 h. In addition, for blockade of recognition of TNF- α on NKT cells, they were pre-incubated with TNFR 1 or 2 blocking peptide (10 μ g/ml) for 1 h and then used for *in vitro* assay. To stimulate NKT cells with TNF- α , cells were pursued with 100 ng/ml of recombinant mouse TNF- α , and receptor blockade was performed as mentioned previously.

Fluorescence-activated cell sorting analyses

For evaluating the level of NKT in inflammatory state by dsRNA, isolated lung cells were labeled with surface antibodies (TCR- β -PECy5 and NK1.1-FITC; BD Biosciences, San Jose, CA, USA). After labeling, analysis was performed on a Gallios flow cytometer (Beckman Coulter, Indianapolis, IN, USA) using Kaluza software. For intracellular cytokine staining, isolated cells from lung-draining lymph nodes (4.0×10^6 cells/ml) were incubated at 37°C for 6 h in 48-well plates coated with the CD3 and CD28 antibodies (1 μ g/ml each; eBioscience). Two hours before harvest, brefeldin A (10 μ g/ml; Sigma-Aldrich) was added. After harvest, cells will be stained with surface antibodies (CD3-APC and CD4-FITC; BD Biosciences) for 30 min at 4°C and then fixed for 10 min in 4% paraformaldehyde at room temperature. Cells were incubated with antibodies (anti-IL-4-PE, anti-IL-10-PE, anti-IL-17-PE, and anti-IFN- γ -PE; BD Biosciences) for 30 min at room temperature and then analyzed on a flow cytometer (Gallios; Beckman Coulter) using Kaluza software. Surface staining, for evaluating the levels of co-stimulatory molecules, was performed by incubating with antibodies (F4/80-FITC, CD11b-, CD11c-APC, CD40-PE, CD80-PE, and CD86-PE), and then, cells were analyzed as intracellular cytokine staining.

Statistical analyses

Analysis of variance (ANOVA) was used to determine the statistical significance of differences between all groups. Significant differences between treatments were assessed using Student's *t*-test, ANOVA, or Wilcoxon's rank sum test. For multiple comparisons, ANOVA was used first, and if significant

differences were found, individual *t*-tests or Wilcoxon's rank sum tests were performed between pairs of groups. Differences were considered statistically significant if $P < 0.05$.

Results

Role of NKT cells on the development of pulmonary inflammation in response to an inhaled allergen with low- or high-dose dsRNA

According to our previous study, sensitization with low- or high-dose dsRNA plus allergen induces Th2/Th17 and Th1 immune responses to an allergen challenge, respectively (2). In addition, we showed that TNF- α , in response to low-dose dsRNA, plays a key role in the development of allergen-specific Th2 airway inflammation (4). However, little is known about the mechanism linking TNF- α and the production of Th2 cytokines, such as IL-4. Based on the previous reports, NKT cells are known to be potent producers of the Th2 cytokine, which are important for forming the environment necessary for Th2 polarization, and that they account for about 7% of all immune cell population in the mouse lung (12, 13). For these reasons, we evaluated NKT cell numbers in the inflammatory state through stimulation with dsRNA and the allergen OVA. WT mice were sensitized intranasally with OVA with or without 0.1 μ g/10 μ g poly[I:C]. The number and proportion of CD3⁺NK1.1⁺ cells were increased in mice sensitized with low- or high-dose dsRNA compared with those treated with PBS or OVA alone (Fig. 1A,B). In the case of low-dose dsRNA sensitization, greater numbers of NKT cells were recruited to the lung as early as 6 h after sensitization. Based on these data, we postulated that NKT cells play an important role in the development of Th2/Th17 or Th1 cell responses to inhaled antigens. To test this, WT and CD1d-deficient (NKT-cell-deficient) mice were sensitized with OVA, OVA + 0.1 μ g of poly[I:C], or OVA + 10 μ g of poly[I:C] and were then challenged with OVA (50 μ g) alone. An evaluation of BAL cellularity 24 h after the last OVA challenge showed that the infiltration of lung tissue by inflammatory cells such as neutrophils and macrophages was significantly increased in WT mice sensitized with OVA and low- or high-dose poly[I:C] compared with those sensitized with OVA alone (Fig. 1C). However, in CD1d-deficient mice, neutrophil infiltration was decreased robustly only in mice sensitized with OVA plus low-dose poly[I:C]. In addition, based on lung histologic findings, the infiltration of inflammatory cells was enhanced in mice sensitized with OVA and low- or high-dose poly[I:C]. The BAL cellularity results were reversed in CD1d-deficient mice sensitized with OVA and low-dose poly[I:C], but not in mice sensitized with high-dose poly[I:C] (Fig. 1D). In terms of the production of pro-inflammatory cytokines, the levels of IL-4 and IL-17 (Th2 and Th17 cytokines, respectively) enhanced in WT mice were reversed in CD1d-deficient mice, irrespective of poly[I:C] doses (Fig. 1E). However, the levels of IP-10, a Th1 mediator, were enhanced by the absence of NKT cells (Fig. 1E). Taken together, these findings indicate that Th2

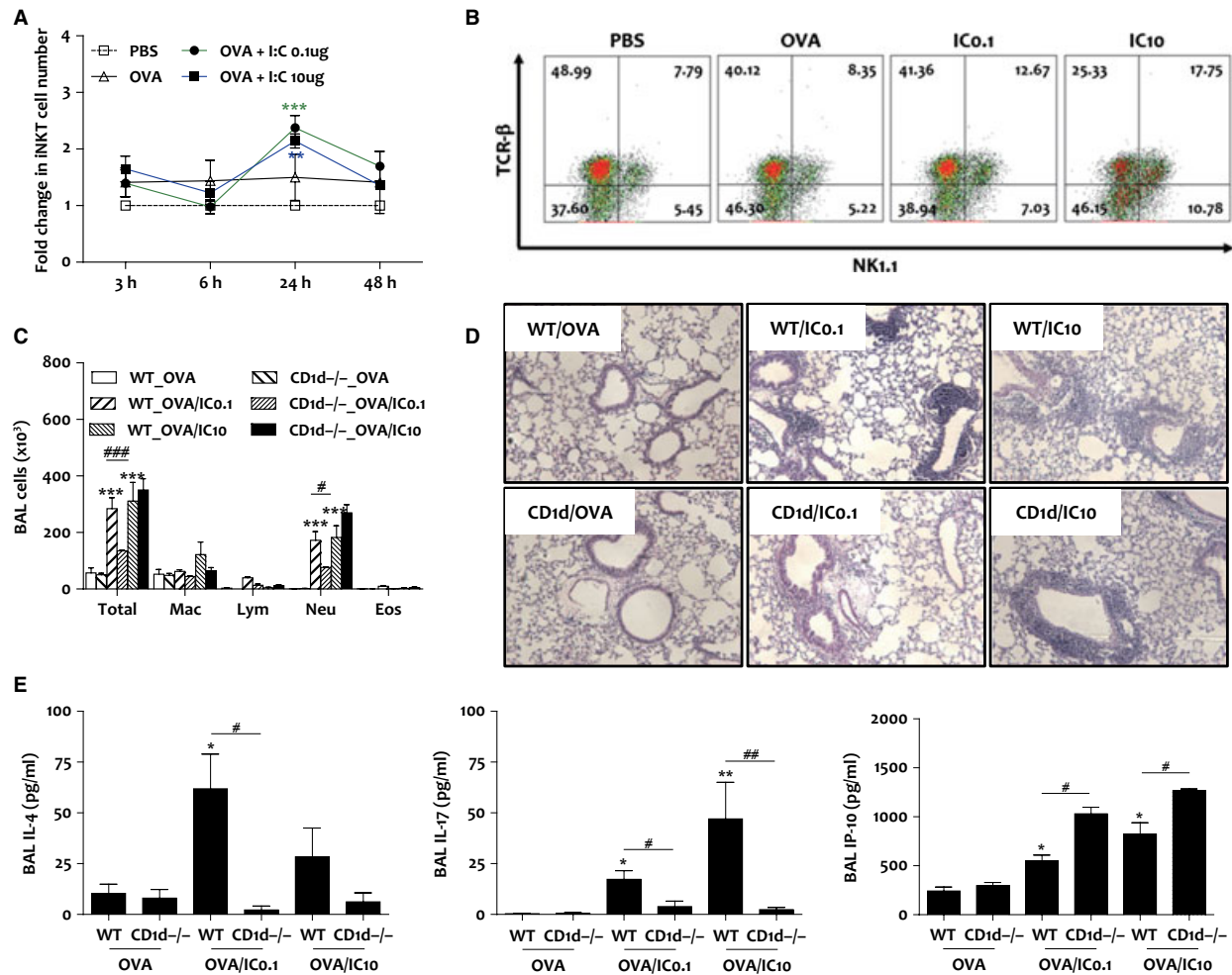


Figure 1 Airway inflammation induced by allergens plus low-dose dsRNA, but not by high-dose dsRNA, is abolished by the absence of natural killer T (NKT) cells. For all panels, each group consisted with five mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to ovalbumin (OVA) groups; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; n.s.: not significant. For panels (A)–(B), wild-type (WT) mice were administered OVA with or without 0.1 $\mu\text{g}/10 \mu\text{g}$ of poly[I:C] (IC). (A) The levels of TCR- β + NK1.1 + cell in the lung were evaluated at various time points. (B) The proportion of TCR- β +NK1.1 + cells in lung cells. For

panels (C)–(E), the airways of WT or CD1d deficiency mice was sensitized with OVA, OVA + 0.1 μg of poly I:C, or OVA + 10 μg of poly I:C and then challenged with OVA (50 μg) alone. Lung inflammation and histology were evaluated 24 h after the last allergen challenge. (C) Bronchoalveolar lavage (BAL) cellularity. (D) Lung histologic findings. (a, WT_OVA; b, CD1d^{-/-}_OVA; c, WT_OVA/ICo.1; d, CD1d^{-/-}_OVA/ICo.1; e, WT_OVA/IC10; f, CD1d^{-/-}_OVA/IC10, hematoxylin and eosin (H&E) stain, 200 $\times$ magnification). (E) Levels of IL-4, TGF- β , IL-17, and IP-10 in BAL fluid.

and Th17 allergic inflammation enhanced by low-dose poly [I:C] is mediated by NKT cells, whereas Th1 inflammation by high-dose poly[I:C] is not.

Role of NKT cells on the development of T-cell responses in response to an inhaled allergen with low-dose dsRNA

Next, we evaluated the role of NKT cells on the development of T-cell responses to inhaled allergens by the stimulation with low-dose dsRNA. OVA and low-dose (0.1 μg) poly[I:C] were applied intranasally into the airways of WT and CD1d-deficient mice. Cells were then isolated from lung-draining lymph nodes and lung tissues 6 h after the allergen challenge

on day 21 and were co-incubated with PBS or antibodies against CD3 and CD28 for 12 h. T cells from lung tissues of WT mice sensitized with OVA and low-dose poly[I:C] produced much greater amounts of IL-4, IL-17, and IFN- γ compared with WT mice sensitized with OVA alone, when stimulated with anti-CD3/CD28 antibodies (Fig. 2A). By contrast, the production of IL-4 and IL-17 from lung T cells enhanced by low-dose poly[I:C] was reversed in the absence of NKT cells, whereas IFN- γ production was enhanced (Fig. 2A). In the case of cytokine production from lung-draining lymph nodes (Fig. 2B), IL-4 and IL-17 production enhanced by low-dose poly[I:C] in WT mice was completely reversed in CD1d-deficient mice, whereas IFN- γ production

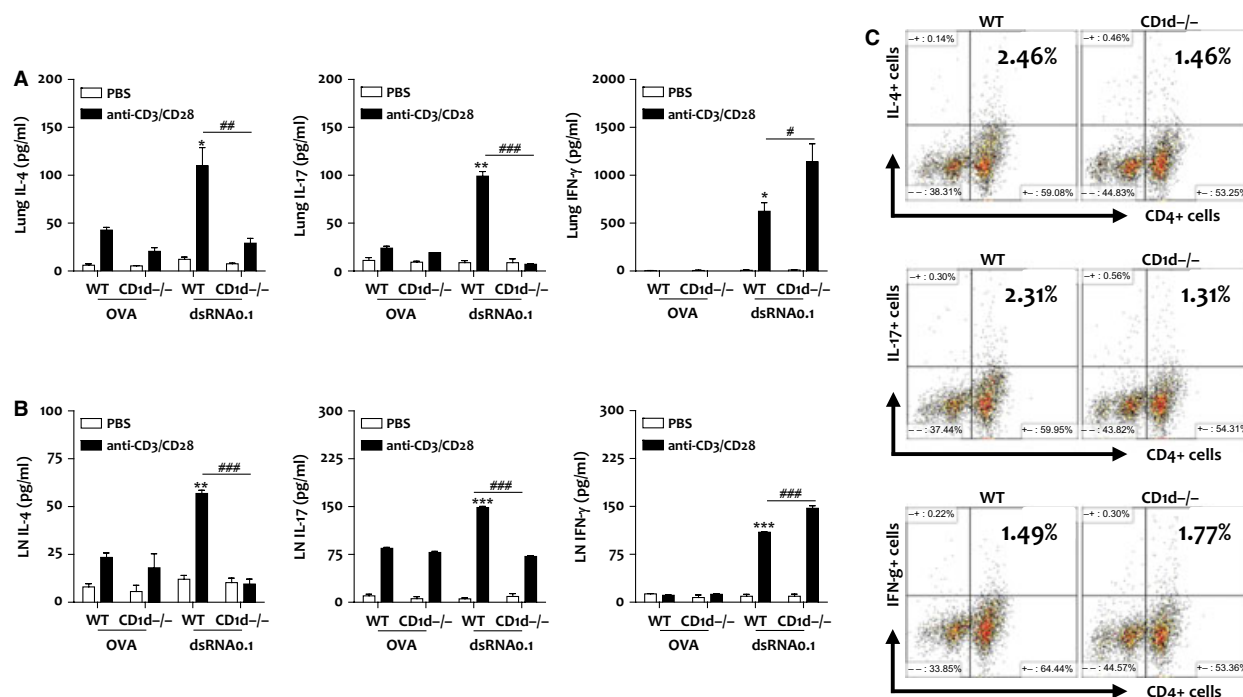


Figure 2 Allergen-specific Th2 and Th17 cell responses enhanced by low-dose dsRNA are decreased in the absence of natural killer T (NKT) cells. For panels (A)–(C), WT and CD1d-deficient mice were sensitized with ovalbumin (OVA) or OVA+ 0.1 μ g of poly[I:C], and cells, isolated from lung-draining lymph node and lung tissues 6 h after allergen challenge on day 21, were incubated with phosphate-buffered saline (PBS) or

anti-CD3 plus anti-CD28 antibodies for 12 h. Levels of each cytokine in the supernatant fraction were measured by enzyme-linked immunosorbent assay (ELISA). (A)–(B) The production of IL-4, IL-17 and IFN- γ from lung tissue and lung-draining lymph node cells, respectively. (C) The proportion of each cytokine-positive CD4 T cells in lung-draining lymph node cells from wild-type (WT) or CD1d-deficient mice.

was enhanced. In addition, the proportions of IL-4- and IL-17-positive T cells in the lung-draining lymph nodes were decreased, but IFN- γ -positive cells increased in CD1d-deficient mice compared with WT mice (Fig. 2C). These findings indicate that NKT cells play an important role in the development of Th2 and/or Th17 cell responses to inhaled allergens induced by low-dose dsRNA.

Role of type I NKT cells on the development of allergic inflammation induced by inhaled allergens containing low- or high-dose dsRNA

The previous experiment using CD1d-deficient mice showed that NKT cells play a role in the development of Th2 immune response to OVA plus low-dose dsRNA. NKT cells can be classified into three subtypes: type I, II, and III. CD1d-deficient mice show a lack of type I and II NKT cell development (16). Of these two types, type I NKT (iNKT) cells are known to be potent producers of IL-4 and IL-13, which are important for Th2 polarization during sensitization period (13). For these reasons, we hypothesized that the Th2 airway inflammation to inhaled allergens is induced by iNKT cells. To test this, WT and α 18-deficient (iNKT-cell-deficient) mice were sensitized with OVA with or without 0.1 μ g or 10 μ g of poly[I:C] and were challenged with OVA (50 μ g)

alone and then phenotypes were evaluated 24 h after the last OVA challenge. BAL cellularity showed that the lung inflammation enhanced by OVA plus low-dose poly[I:C] in WT mice was reversed in α 18-deficient mice sensitized in the same manner, whereas the inflammation enhanced by high-dose poly[I:C] was aggravated in the absence of iNKT cells (Fig. 3A). Histologic findings also indicated that lung inflammation enhanced by low-dose poly[I:C] in WT mice was markedly decreased in α 18-deficient mice, whereas the lung inflammation enhanced by high-dose poly[I:C] was similar between WT and the knockout mice (Fig. 3B). Additionally, the production of Th2 and Th17 cell downstream mediators (IL-4 and IL-17, respectively) enhanced by low-dose poly[I:C] was reversed by the absence of iNKT cells, whereas Th1 cell mediator IP-10 was enhanced (Fig. 3C). To sum up, these findings suggest that, among the NKT cell subtypes, iNKT cells play an important role in the development of allergic inflammation enhanced by low-dose dsRNA.

Role of iNKT cells on the development of T-cell responses in response to an inhaled allergen plus low-dose dsRNA

To evaluate the role of iNKT cells on the development of T-cell responses to inhaled allergens enhanced by low-dose poly[I:C], the experimental protocol described in Fig. 2 was

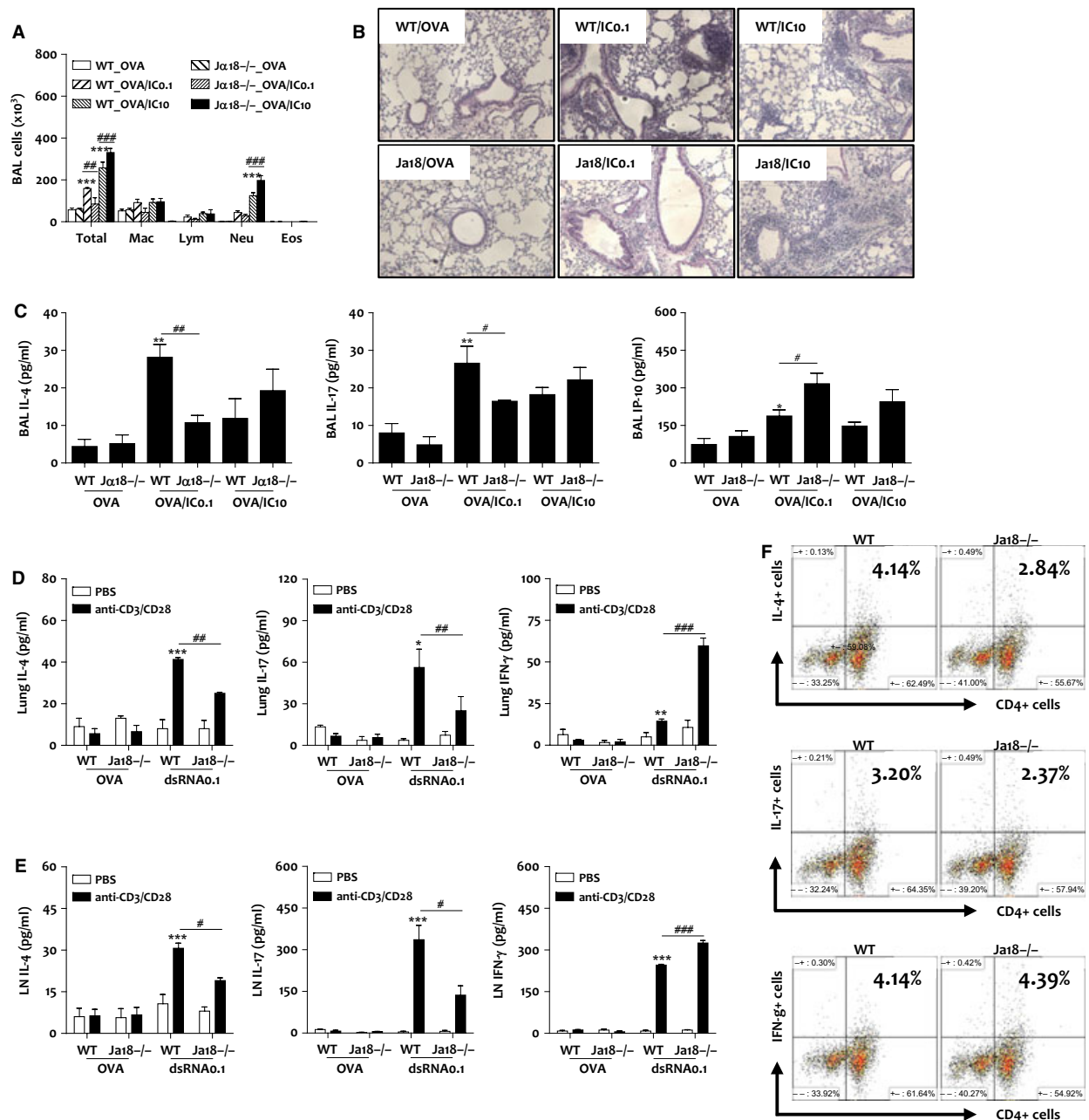


Figure 3 Allergen-specific Th2 and Th17 cell responses and lung inflammation, enhanced by low-dose dsRNA, are downregulated in the absence of type I natural killer T (NKT) cells. For all panels, each group was consisted of five mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to OVA groups; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; n.s.: not significant. For panels (A)–(C), wild-type (WT) and Jα18-deficient (type I NKT-cell-deficient) mice airways were sensitized with ovalbumin (OVA) with or without 0.1 μg or 10 μg of poly[I:C] and then challenged with OVA alone; evaluations were performed 24 h after the last OVA challenge. (A) Numbers of inflammatory cells in bronchoalveolar lavage (BAL) fluid. (B) Lung histologic findings (a, WT_OVA; b, Jα18^{-/-}_OVA; c, WT_OVA/

ICo.1; d, Jα18^{-/-}_OVA/ICo.1; e, WT_OVA/IC10; f, Jα18^{-/-}_OVA/IC10, hematoxylin and eosin (H&E) stain, 200× magnification). (C) The levels of IL-4, IL-17, and IP-10 in BAL fluid. For panels (D)–(F), cells in WT and Jα18-deficient mice that were sensitized with OVA or OVA + 0.1 μg of poly[I:C], isolated from lung-draining lymph node and lung tissues and were incubated with phosphate-buffered saline (PBS) or anti-CD3 and anti-CD28 antibodies for 12 h. The production of each cytokine was measured in supernatant fraction. (D)–(E) The levels of IL-4, IL-17 and IFN-γ from restimulated lung tissue and lung-draining lymph node cells, respectively. (F) The proportion of each cytokine-positive CD4 T cells in lung-draining lymph node cells from WT or Jα18-deficient mice.

applied to WT and *Jα18*-deficient mice. Lung tissue and lymph node cells from WT and *Jα18*-deficient mice sensitized with OVA and low-dose poly[I:C] were incubated with or without anti-CD3/-CD28 antibodies. The production of IL-4 and IL-17 from T cells, enhanced by dsRNA in WT mice, was reversed in *Jα18*-deficient mice, whereas IFN- γ production was enhanced (Fig. 3D). Similarly, the enhanced production of IL-4 and IL-17 from lymph node T cells, in WT mice, was abolished in the absence of iNKT cells, whereas IFN- γ production was enhanced (Fig. 3E). The proportions of IL-4- and IL-17-positive T cells in lung-draining lymph nodes were decreased by the absence of iNKT cells, whereas IFN- γ -positive T cells were increased (Fig. 3F). In addition, to clarify T-cell responses to allergens, we restimulated T cells with OVA *in vitro*. The levels of IL-4 and IL-17 in supernatants were decreased, whereas IFN- γ was enhanced in cells isolated from both lung and lymph nodes in *Jα18*-deficient mice compared with WT mice (Fig. S1). Based on these findings, we suggest that iNKT cells, among the different types of NKT cells, play a key role in the development of allergen-specific Th2 and/or Th17 cell responses enhanced by low-dose dsRNA.

Role of iNKT cells on the development of innate immune responses induced by low-dose dsRNA

Having shown that diminished Th2 and Th17 cell responses in response to inhaled allergens were caused by a lack of NKT cells and that iNKT cells are important for the development of these immune responses, we next sought to clarify the role of iNKT cells in the Th2 and Th17 cell polarization induced by stimulation with low-dose dsRNA. To this end, we tested

the effect of an NKT cell defect during the sensitization period. WT, CD1d-deficient, and *Jα18*-deficient mice were sensitized once with OVA and 0.1 μ g of poly[I:C] and then were evaluated 12 h after the sensitization. BAL cellularity showed that lung infiltration of inflammatory cells was decreased in both CD1d- and *Jα18*-deficient mice compared with WT mice (Fig. 4A). Dendritic cells are important for the development of adaptive immune response; thus, we evaluated the expression levels of co-stimulatory molecules and MHCII molecules on the lung F4/80⁺CD11c⁺ cells (Fig. 4B). The expression levels of CD40, CD80, CD86, and MHCII were upregulated in WT mice sensitized with OVA plus low-dose poly[I:C]. The expression of each molecule was reduced in both CD1d- and *Jα18*-deficient mice compared with WT controls. Interestingly, the expression of co-stimulatory molecules on dendritic cells was similar between CD1d- and *Jα18*-deficient mice. Next, to identify the changes in cytokine production that play an important role in T cell differentiation by forming the cytokine environment, we assessed the levels of key polarizing cytokines for Th1, Th2, and Th17 development (Fig. 4C). The production of IL-4 and IL-6 (Th2- and Th17-polarizing cytokines, respectively) was significantly decreased in both CD1d- and *Jα18*-deficient mice sensitized with OVA plus low-dose poly[I:C] compared with WT mice sensitized in the same manner, whereas IFN- γ (a Th1-polarizing cytokine) production was similar among these three groups. In addition, the production of TNF- α enhanced by low-dose dsRNA was not affected by the absence of NKT or iNKT cells. Taken together, these data suggest that iNKT cells are critical for Th2- and Th17-polarizing cytokine production and co-stimulatory molecule expression induced by low-dose dsRNA.

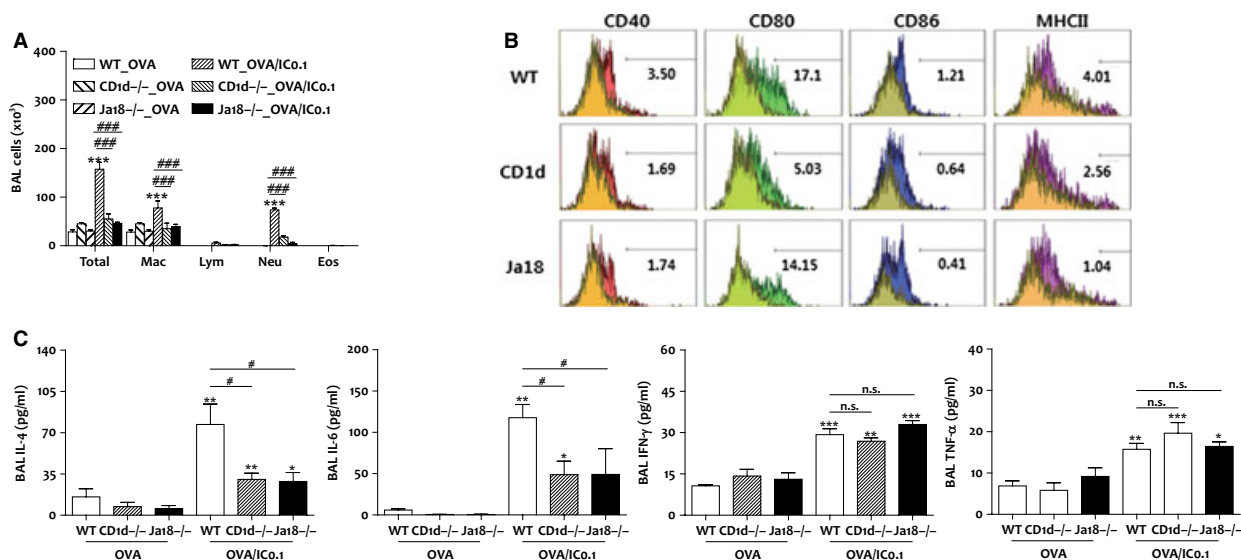


Figure 4 Natural killer T (NKT) cells, especially type I NKT cells, play an important role in dendritic cell maturation and Th2-polarizing cytokine production, enhanced by low-dose dsRNA. For all panels, CD1d-, *Jα18*-deficient, and wild-type (WT) mice were sensitized once with ovalbumin (OVA) in with or without 0.1 μ g of poly[I:C] and then evaluated 12 h after the sensitization. Each of groups was consisted with

five mice. * P < 0.05; ** P < 0.01; *** P < 0.001 relative to OVA groups; # P < 0.05; ## P < 0.01; ### P < 0.001; n.s.: not significant. (A) Bronchoalveolar lavage (BAL) cellularity. (B) Expression of co-stimulatory molecules, such as CD40, CD80, CD86, and MHC II, on lung F4/80-CD11c⁺ cells. (C) Levels of Th2 (IL-4), Th17 (IL-6), and Th1 (IFN- γ)-polarizing cytokines in BAL fluid.

Effect of TNF- α on the production of T-cell-polarizing cytokines from iNKT cells *in vitro*

Previously, we showed that allergens containing low-dose dsRNA enhanced TNF- α production by alveolar macrophages and that this response was important for inducing a Th2 immune response to inhaled allergens (4). In the present study, to confirm the direct effect of TNF- α on the production of Th2-polarizing cytokines, we administrated

recombinant mouse TNF- α to WT mice and then evaluated the production of cytokines at different time points (Fig. 5A). We found that the production of the Th2-polarizing cytokines IL-4 and IL-13 was strongly induced as early as 1 h after the TNF- α application. Additionally, the level of the Th17-polarizing cytokine IL-6 was also increased 4 h after TNF- α administration. IFN- γ production was also statistically enhanced, although the absolute amount was

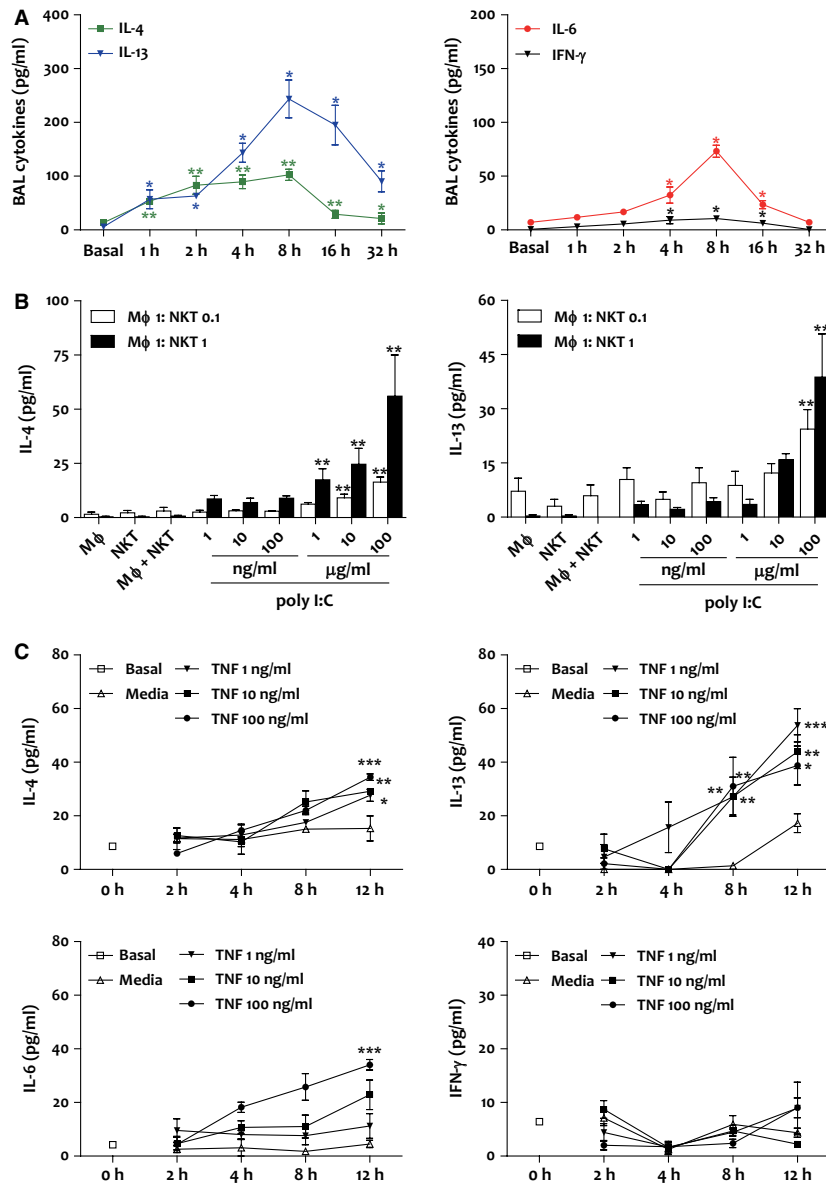


Figure 5 TNF- α directly upregulates the production of Th2-polarizing cytokines from natural killer T (NKT) cells. For all panels, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ relative to OVA groups. (A) wild-type (WT) mice were sensitized with recombinant mouse TNF- α ($n = 4$ for each group) and then the production of each cytokine was evaluated in bronchoalveolar lavage (BAL) fluid in each time points (Left panel, levels of IL-4 and IL-13; right panel,

IL-6 and IFN- γ). (B) Macrophages and NKT cells were co-cultured in different concentrations of dsRNA (poly I:C), and then the levels of IL-4 and IL-13 were measured from co-cultured supernatant. (C) NKT cells were stimulated with different concentrations of recombinant mouse TNF- α (1, 10, 100 ng/ml) and levels of IL-4, IL-13, IL-17 and IFN- γ were measured at several time points.

significantly lower than the other cytokines. Next, to evaluate whether TNF- α derived from alveolar macrophages activates iNKT cells, which are the source of the Th2- and Th17-polarizing cytokines, we co-cultured alveolar macrophages (MH-S) and iNKT cells (DN32.D3) with the stimulation of different doses of dsRNA. The levels of IL-4 and IL-13 were highly enhanced by dsRNA stimulation in a dose-dependent manner (Fig. 5B), whereas IL-6 and IFN- γ were not enhanced (data not shown). Moreover, when iNKT cells (DN32.D3) were directly treated with different doses of dsRNA, none of the above cytokines were detected (data not shown). Additionally, to elucidate the direct effect of TNF- α on the production of polarizing cytokines from iNKT cells, we stimulated iNKT cells (DN32.D3) with different doses of recombinant mouse TNF- α (Fig. 5C). This study showed that the production of IL-4 and IL-13 was enhanced with only 1 ng/ml of TNF- α , whereas IL-6 production increased with 100 ng/ml of TNF- α . By contrast, the production of IFN- γ by iNKT cells was not observed by the stimulation with TNF- α . Collectively, these data suggest that TNF- α produced by alveolar macrophages in response to low-dose dsRNA is a key mediator of the production of Th2-polarizing cytokines from iNKT cells.

Role of iNKT cells on the development of innate and adaptive immune responses induced by TNF- α *in vivo*

To evaluate the effect of TNF- α on the production of T-cell-polarizing cytokines by iNKT cells *in vivo*, 100 ng of recombinant mouse TNF- α was applied intranasally into WT, CD1d-deficient, and J α 18-deficient mice and then the immune responses were evaluated 12 h later. BAL cellularity showed that inflammatory cell infiltration induced by TNF- α was reversed in the absence of NKT cells or iNKT cells (Fig. 6A). As for the production of T-cell-polarizing cytokines, IL-4, IL-13, and IL-6 levels in BAL fluid enhanced by TNF- α in WT mice were decreased in both CD1d- and J α 18-deficient mice (Fig. 6B), whereas IFN- γ was not (data not shown). Next, to evaluate the interaction between TNF- α and iNKT cells on the development of allergen-specific T-cell responses *in vivo*, we sensitized mice with OVA plus TNF- α and then challenged them with 50 μ g of OVA. BAL cellularity showed that inflammatory cell recruitment into the lung was significantly decreased in both CD1d- and J α 18-deficient mice compared with WT mice (Fig. 6C). As for T-cell downstream mediators, the levels of IL-4, IL-17, and TGF- β (Th2 and Th17 downstream mediators) in BAL fluid were significantly lower in the knockout mice than the WT control group, whereas IP-10 (a Th1 downstream mediator) was not affected (Fig. 6D). When lung and lymph node cells were restimulated with anti-CD3/-CD28 antibodies, the production of IL-4 and IL-17 from both lung and lymph node T cells was downregulated in both CD1d- and J α 18-deficient mice compared with WT mice, whereas IFN- γ production was not (Fig. 6E–F). To sum up, these data suggest that iNKT cells play an important role in the development of allergen-specific Th2 and Th17 cell responses enhanced by TNF- α .

Role of TNFR-mediated signaling on the development of Th2 and Th17 cell responses induced by allergens plus low-dose dsRNA or recombinant TNF- α

TNF- α can be recognized by two different receptors: TNF- α receptor-1 (TNFR1, CD120a) and TNFR2 (CD120b). To determine which TNF- α receptor(s) is/are involved in the production of T-cell-polarizing cytokines induced by dsRNA, we sensitized WT mice with OVA plus low-dose poly[I:C], with or without the co-treatment of 10 μ g of an TNFR1 or TNFR2 blocking peptide, and then evaluated 12 h after the sensitization. We found that lung inflammation induced by low-dose dsRNA was reversed by the treatment with the TNFR2 blocking peptide, but not with the TNFR1 blocking peptide (Fig. 7A). With regard to the production of T-cell-polarizing cytokines, IL-4 and IL-6 levels in BAL fluid were significantly reduced in TNFR2-blocking-peptide-treated mice, but not in mice treated with the TNFR1 blocking peptide or sham, whereas IFN- γ levels were not affected by the two peptide treatments (Fig. 7B). Next, to evaluate the role of TNF- α receptor(s) on the production of T-cell-polarizing cytokines induced by TNF- α , we sensitized WT mice using OVA and recombinant mouse TNF- α with or without TNFR1 or TNFR2 blocking peptides. BAL cellularity showed that infiltration by inflammatory cells, such as macrophages and neutrophils, into the lung tissue was downregulated in mice treated with TNFR2 blocking peptide, but not TNFR1 blocking peptide (Fig. 7C). The levels of IL-4 and IL-6 in BAL fluid were decreased in mice treated with TNFR2 blocking peptide, and in this time, IFN- γ was also decreased (Fig. 7D). Collectively, these data indicate that TNFR2, rather than TNFR1, is important to the production of Th2- and Th17-polarizing cytokines in response to TNF- α , which is produced by low-dose dsRNA.

Role of TNFR2 on iNKT cells on the production of Th2-polarizing cytokines

To evaluate whether the production of Th2-polarizing cytokines by dsRNA-stimulated macrophages is mediated by TNFRs on iNKT cells, we co-cultured iNKT cells (DN32.D3) and alveolar macrophages (MH-S) with 100 μ g/ml poly[I:C] with or without TNFR1/2 blocking peptides. This experiment showed that the production of IL-4 and IL-13 from NKT cells was abolished by the treatment of TNFR2 blocking peptide only, but not by the TNFR1 blocker (Fig. 7E). Next, to clarify the direct effect of TNF- α receptor blockade on the production of TNF- α -induced Th2-polarizing cytokines, we cultured iNKT cells (DN32.D3) with recombinant mouse TNF- α (100 ng/ml) in the presence or absence of TNFR1/2 blocking peptides. The present study showed that the TNF- α -induced production of IL-4 and IL-13 from NKT cells was abolished by the TNFR2 blocking peptide, but not by TNFR1 blocker (Fig. 7F). Taken together, these data indicate that TNFR2 on NKT cells is the key signaling pathway on the production of Th2-polarizing cytokines from NKT cells, in response to TNF- α produced by dsRNA-stimulated macrophages.

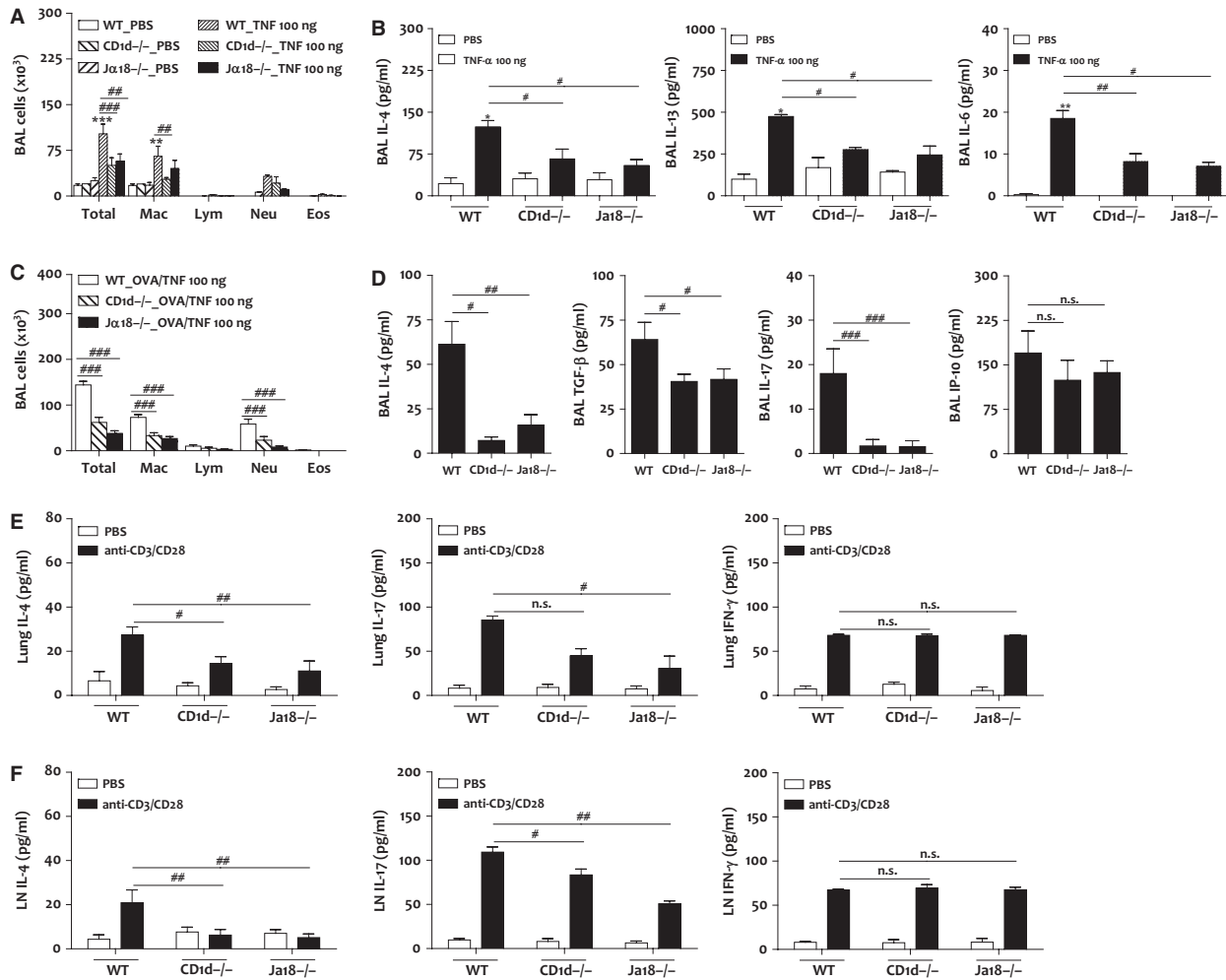


Figure 6 Type I natural killer T (NKT) cells play a key role in the development of allergen-specific Th2 cell responses enhanced by *in vivo* exposure to recombinant TNF- α . For all panels, each group was consisted with five mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to OVA groups; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; n.s.: not significant. For panels (A)–(B), CD1d^{-/-}, Jα18^{-/-} deficient, and wild-type (WT) mice were sensitized three times with OVA containing 100 ng of recombinant mouse TNF- α and then evaluated 12 h after

sensitization. (A) Bronchoalveolar lavage (BAL) cellularity. (B) Levels of IL-4, IL-6, and IL-13 in BAL fluid. For panels (C)–(F), mice were sensitized as above and then challenged with OVA (50 μ g) alone; evaluation was performed 6 h after the third OVA challenge. (C) Numbers of inflammatory cell in BAL fluid. (D) Cytokine production level in BAL fluid. For panels (E)–(F), cytokine levels in culture supernatants from isolated lung (E) and lymph node (F) cells by the stimulation of anti-CD3/CD28 antibodies.

Discussion

Our previous experimental data showed that TNF- α plays a key role in the development of Th2 cell responses to inhaled allergens induced by low-dose dsRNA. However, in those experiments, the exact mechanisms by which low-dose dsRNA-induced TNF- α elicits a Th2 immune response were not determined. In the present study, we demonstrated that TNFR2 on iNKT cells recognizes low-dose dsRNA-induced TNF- α and through this mechanism induces Th2 and/or Th17 immune responses to the inhaled allergens.

IL-4 and/or IL-13 secreted by cells such as mast cells, eosinophils, and NKT cells (17) are essential for Th2 polarization (10, 11, 18). In the present low-dose dsRNA mouse

model, low-dose dsRNA does not induce lung infiltration of eosinophils. Much evidence indicates that mast cells are not found in the lungs of naïve mice (19). Additionally, when we evaluated the lung-resident cells by FACS analysis in the dsRNA asthma model, CD117⁺ cells (mast cells) were not detected (data not shown). By contrast, CD3⁺NK1.1⁺ (NKT) cells do exist in the lung of naïve mice (12). For these reasons, we focused on the role of NKT cells on the development of allergen-specific Th2 cell response enhanced by low-dose dsRNA.

There is no evidence that NKT cells do not respond directly to dsRNA stimulation, although the present data show that NKT cells, especially iNKT cells, are a source of Th2-polarizing cytokines in inflammation induced by

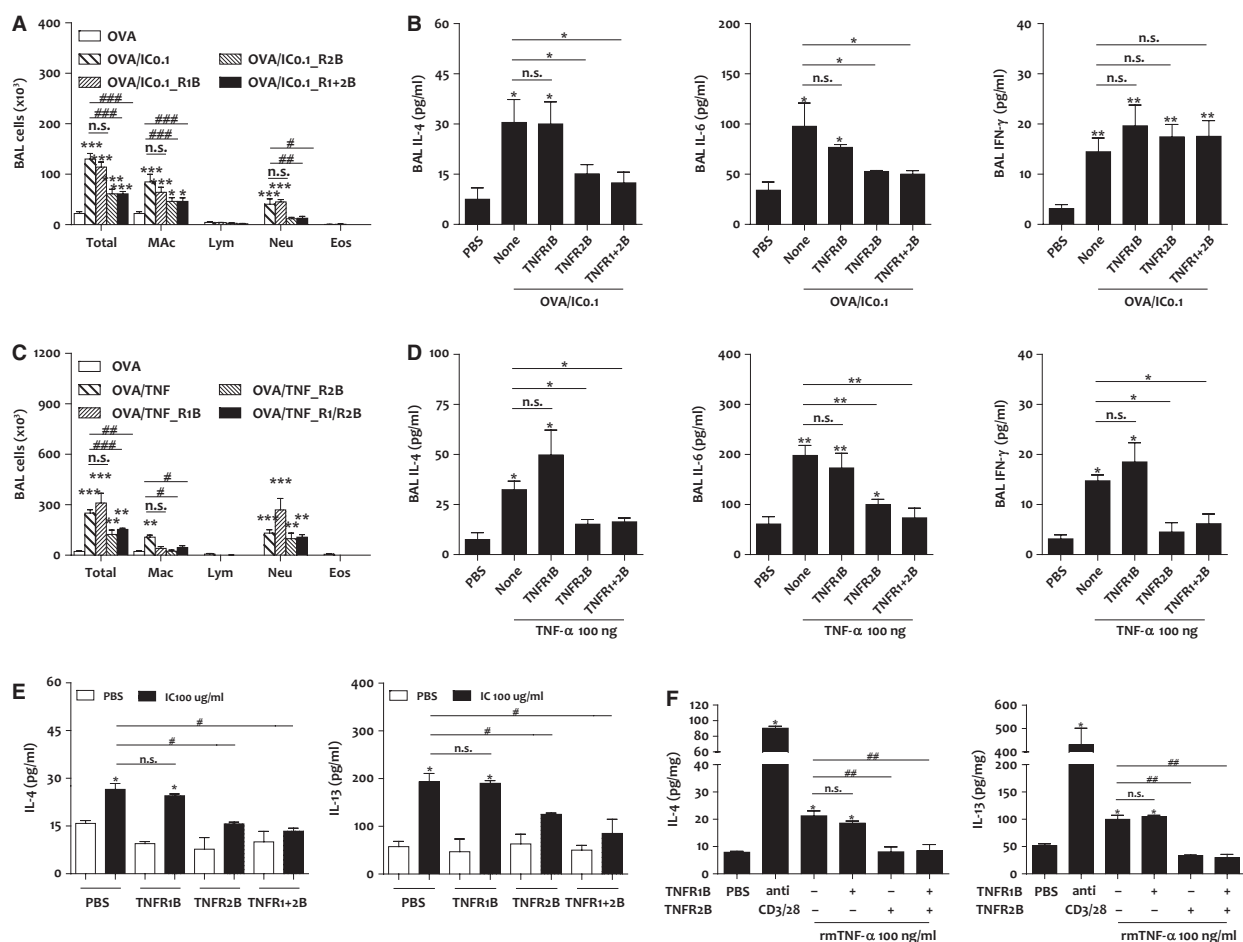


Figure 7 The production of Th2-polarizing cytokines, induced by low-dose dsRNA or TNF- α , is mainly dependent on TNF- α receptor (TNFR) 2 on natural killer T (NKT) cells. For all panels, each group was consisted with five mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to OVA groups; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; n.s.: not significant. For panels (A) and (B), wild-type (WT) mice were sensitized three times with ovalbumin (OVA) plus low dose of dsRNA with or without intranasal treatment of TNFR1 or TNFR2 blocking peptides (10 μ g of each blocking peptide was used). Evaluation was performed 12 h after the sensitization. (A) bronchoalveolar lavage (BAL) cellularity. (B) The levels of IL-4, IL-6, and IFN- γ in BAL fluid. For panels (C)–(D), WT mice were sensi-

tized three times with OVA and recombinant mouse TNF- α (100 ng) with or without intranasal treatment of TNFR1 (10 μ g) or TNFR2 blocking (10 μ g) peptides. Phenotypes were evaluated 12 h after the sensitization. (C) BAL cellularity, (D) Levels of each cytokine (IL-4, IL-6, and IFN- γ) in BAL fluid. For panel (E), macrophages and NKT cells were co-cultured with 100 μ g/ml of poly(I:C) plus TNFR1 or TNFR2 blocking peptides (10 μ g/ml) for 12 h. Levels of IL-4 and IL-13 were measured in co-culture supernatants. For panel (F), NKT cells were cultured with 100 ng/ml of recombinant mouse TNF- α with or without TNFR1 or TNFR2 blocking peptides. Levels of IL-4 and IL-13 were measured in the cell culture supernatants.

low-dose dsRNA. A recent study indicates that macrophages present antigens to CD1d on NKT cells, and subsequently, IL-13 produced by activated NKT cells elicits lung inflammation (20). When exposed to a particular ligand, the α -galcer, iNKT cells are known to be potent producers of Th2-polarizing cytokines such as IL-4 and IL-13 (16). In the present study, the stimulation of dsRNA both *in vitro* and *in vivo* elicited the production of IL-4 and IL-13, which is dependent on iNKT cells. In addition, the present data show that the stimulation of TNF- α both *in vitro* and *in vivo* also induces the production of the Th2-polarizing cytokines from iNKT

cells. These findings suggest that iNKT cells, among the different types of NKT cells, are the key player in the development of low-dose dsRNA-enhanced Th2 cell response in the lung. Taken into consideration that alveolar macrophages are the main source of TNF- α in response to dsRNA exposure (4), dsRNA-induced TNF- α is a novel activator of iNKT cells during Th2 sensitization to inhaled allergens.

iNKT cells are also known to be potent producers of the Th1-inducing cytokine IFN- γ in response to the α -galcer (16). During allergen sensitization in the lung, the present data show that the stimulation of TNF- α *in vitro* does not

elicit the production of IFN- γ from iNKT cells, although the stimulation of TNF- α and dsRNA *in vivo* enhances the production of IFN- γ in the lung (4). These data led us to postulate that iNKT cells are not the main producers of IFN- γ , when stimulated with TNF- α produced by dsRNA. Furthermore, we can also postulate that immune cells, such as NK or $\gamma\delta$ T cells, other than NKT cells are important in the development of allergen-specific Th1 immune response enhanced by dsRNA.

IL-6 is known as an important inducer of Th17 cell development (3). Although alveolar macrophages are known as potent producers of IL-6 in the lung (5, 21), sources of IL-6, iNKT cells, have not been studied in detail. Our previous data indicate that the alveolar macrophages are potent producers of IL-6, as well as TNF- α , in response to dsRNA (4). The present study shows that TNF- α also upregulates the production of IL-6 via iNKT-cell-dependent manner. These data suggest that TNF- α can amplify the production of IL-6, via iNKT cells. However, our previous study indicates that allergen-specific Th17 cell response enhanced by low-dose dsRNA arises mainly through the VEGF-IL-6 axis, and the Th17 response is enhanced in the absence of TNF- α (3). These data suggest that pathways, other than TNF- α -IL-6 axis, are mainly involved in Th17 polarization in the low-dose dsRNA-enhanced Th17 asthma model, although iNKT cells in response to TNF- α can produce IL-6 and subsequently induce Th17 cell response.

TNF- α is recognized by its receptors, TNFR1 and TNFR2 (22). While most cells express TNFR1 abundantly on their surfaces, TNFR2 largely exists on the surfaces of hematopoietic cells (23). According to previous studies, both receptors can mediate inflammation; however, which one is the main receptor for inflammation is controversial. A previous study showed that inflammation was mediated by TNFR1 (24), while others showed the opposite (22, 25, 26). In the present study, we used blocking peptides to inhibit the recognition of TNF- α by each receptor, and TNFR2 on iNKT cells is a main signaling pathway during Th2 sensitization to inhaled allergens enhanced by dsRNA. An interesting report suggests that TNFR1 is related to anti-inflammatory effects (27), and some favoring reports indicate that the expression of TNFR2 is increased during inflammation (25). In this context, we can postulate that TNF- α produced by dsRNA elicits Th2 cell responses to inhaled antigens, mainly via TNFR2 on iNKT cells.

In summary, the present study indicates that NKT cells, especially iNKT cells, play an important role in the development of Th2 immune response to inhaled allergens enhanced

by low-dose dsRNA, although lung infiltration of NKT cells was enhanced by both low- and high-dose dsRNA. In addition, we found that alveolar macrophage-derived TNF- α in response to dsRNA induces Th2 sensitization to inhaled allergens, mainly via TNFR2 on iNKT cells.

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Author contributions

The authors made the following contributions to the study. J.C., Y.J., S.J., and Y.K. contributed to study concept and design; J.C., Y.K., H.P., S.C., and W.L. contributed to acquisition of data; J.C., Y.G., S.J., and Y.K. were involved in analysis and interpretation of data; J.C., S.J., and Y.K. contributed to the drafting of the manuscript; J.C., H.C., and S.J. contributed to statistical analysis; Y.K. was involved in obtaining funding.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Allergen-specific Th2 and Th17 cytokines enhanced by low-dose dsRNA are downregulated in the absence of type I NKT cells. Cells in WT and α 18-deficient mice, that were sensitized with OVA or OVA + 0.1 μ g of poly[I:C], were isolated from lung-draining lymph node and lung tissues and then incubated with PBS or OVA for 72 h. The production of each cytokine was measured in supernatant fraction. * P < 0.05; ** P < 0.01; *** P < 0.001 relative to OVA groups; # P < 0.05; ## P < 0.01; ### P < 0.001; n.s.: not significant.

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Supplementary Fig. 1

