

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

TNF- α is a key mediator in the development of Th2 cell response to inhaled allergens induced by a viral PAMP double-stranded RNA

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To cite this article: Choi J-P, Kim Y-S, Kim OY, Kim Y-M, Jeon SG, Roh T-Y, Park J-S, Gho YS, Kim Y-K. TNF- α is a key mediator in the development of Th2 cell response to inhaled allergens induced by a viral PAMP double-stranded RNA. *Allergy* 2012; DOI: 10.1111/j.1398-9995.2012.02871.x.

Keywords

dsRNA; IL-4; Th2 cell response; tumor necrosis factor- α ; virus-associated asthma.

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Accepted for publication 03 June 2012

DOI:10.1111/j.1398-9995.2012.02871.x

Edited by: Angela Haczku

Abstract

Background: Viral pathogen-associated molecular patterns, such as dsRNA, disrupt airway tolerance to inhaled allergens. Specifically, the Th2 and Th17 cell responses are induced by low-dose dsRNA and the Th1-dominant response by high-dose dsRNA.

Objective: In this model, we evaluate the role of TNF- α in the development of adaptive immune dysfunction to inhaled allergens induced by airway sensitization with dsRNA-containing allergens.

Methods: A virus-associated asthma mouse model was generated via simultaneous airway administration of ovalbumin (OVA) and low (0.1 μ g) or high (10 μ g) doses of polyinosine-polycytidylic acid (poly[I:C]). The effect of TNF- α on Th2 airway inflammation was evaluated using TNF- α -deficient mice and recombinant TNF- α .

Results: TNF- α production was enhanced by airway exposure to low and high doses of poly[I:C]. After airway sensitization with OVA plus low-dose poly[I:C], TNF- α -deficient mice exhibited less OVA-induced airway inflammation than did wild-type (WT) mice. However, this did not occur upon sensitization with high-dose poly[I:C]. In terms of T-cell response, the production of IL-4 from lung T cells after OVA challenge was enhanced by airway sensitization with OVA plus low-dose poly[I:C] in WT mice, and this phenotype was inhibited by the absence of TNF- α . Moreover, the Th2 cell response induced by sensitization with OVA plus low-dose poly[I:C], which was abolished in TNF- α -deficient mice, was restored in these mice upon addition of recombinant TNF- α .

Conclusion: The results of this study suggest that TNF- α produced by airway exposure to low-dose dsRNA is a key mediator in the development of Th2 cell response to inhaled allergens.

Asthma is a common chronic airway disease in which the airways occasionally constrict and become inflamed, often in response to inhaled allergens (1). Wheezing in children may be related to atopic asthma or may be virus-associated (2), while viral infection may worsen existing asthma. Viral infection itself or with innocuous antigens sufficiently induces all three subtypes of the adaptive immune response (Th1, Th2, and Th17) (3). Virus-associated asthma is phenotypically characterized by neutrophil-dominant inflammation with

little eosinophil recruitment and a significantly smaller number of eosinophils than in atopic asthma (4, 5). Recent experimental data indicate that airway sensitization of allergens plus double-stranded RNA (dsRNA) induces neutrophilic airway inflammation, which reflects virus-associated asthma in humans (6, 7).

A child that has suffered from viral respiratory infections before the age of 3 years is at a greater risk of developing asthma (8–10). During their replicative cycles, viruses, such

as influenza virus, rhinovirus, and respiratory syncytial virus, produce dsRNA (11–13), which stimulate innate immune responses via pattern recognition receptors, resulting in the production of immune-modulating mediators (14–16). Our experimental data revealed that airway exposure to allergens with low doses of dsRNA induces an asthma phenotype resulting from both Th2 and Th17 cell responses (6), whereas high doses of dsRNA induce Th1 cell response through interferon gamma (IFN- γ) (7). In the case of low-dose dsRNA, vascular endothelial growth factor (VEGF) and interleukin (IL)-6 were found to be key mediators that induce the development of Th17 cell response in the virus-associated asthma model (6). Although IL-4 is the key Th2-polarizing cytokine, the exact molecular mechanism of how dsRNA activates the Th2 immune response has not yet been fully clarified.

Tumor necrosis factor- α (TNF- α), an important mediator of inflammatory response, is produced by innate immune cells, including macrophages, mast cells, and lung epithelial cells. TNF- α induces the infiltration of inflammatory cells to the inflamed site through up-regulation of adhesion molecules and increased cytokine production (17). TNF- α activities have been well studied in terms of pro-inflammatory effects, but the exact functions of TNF- α in adaptive immune dysfunction are disputed. According to some reports, TNF- α acts like Th1 cytokine in lung inflammation; however, others have reported that Th2 inflammation is induced by recombinant TNF- α (rTNF- α) administration and diminished through TNF- α signaling inhibition (18–21).

In the present study, we hypothesized that TNF- α , produced by dsRNA during allergen sensitization, is the key mediator of T-cell priming to inhaled allergens and polarization to Th2 cells in the development of allergic airway inflammation. To test this hypothesis, the effect of TNF- α in the development of Th2 airway inflammation was evaluated using a virus-associated asthma model in TNF- α -deficient mice, and mice were sensitized with allergens together with dsRNA. Furthermore, to elucidate the direct role of TNF- α in the development of the Th2 cell response, dsRNA was administered in combination with rTNF- α in the virus-associated asthma model in TNF- α -deficient mice.

Methods

Mice

Tumor necrosis factor- α -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 the POSTECH Ethics Committee.

Reagents

Synthetic dsRNA polyinosine–polycytidylic acid (poly[I:C]) was purchased from Calbiochem (La Jolla, CA, USA). Ovalbumin (OVA) was obtained from Sigma-Aldrich

(St. Louis, MO, USA). Mouse rTNF- α was purchased from R&D Systems (Minneapolis, MN, USA).

Generation of a virus-associated asthma mouse model

A virus-associated asthma mouse model was generated by co-sensitization of mice with poly[I:C] and OVA as described previously (6, 7). Briefly, 6-week-old mice were treated intranasally with 75 μ g OVA plus 0.1 or 10 μ g poly[I:C] on days 0, 1, 2, and 7 and challenged with OVA alone on days 14, 15, 21, and 22. Innate immune response induced by poly[I:C] was evaluated after sensitization on day 0, and allergen-specific adaptive immune response was examined 6 h after allergen challenge on day 21. Allergen-induced lung inflammation was examined 24 h after the final OVA challenge on day 22.

Administration of rTNF- α

To evaluate *in vivo* effects of rTNF- α on the development of adaptive immune dysfunction, mice were sensitized with OVA and rTNF- α (100 ng) on days 0, 1, 2, and 7 and then challenged with OVA alone on days 14, 15, 21, and 22.

Cellularity in bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) cellularity was analyzed as described previously (7). Briefly, cell pellets were diluted in 50 μ l phosphate-buffered saline (PBS), and 300 inflammatory cells were counted and classified as macrophages, lymphocytes, neutrophils, or eosinophils.

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 collagenase 200 unit/ml (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 (StemCell Technologies, Vancouver, 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.

Lung tissue 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. Lung inflammation was measured by assessing the degree of peribronchiolar and perivascular inflammation as described previously (6).

Immune response in the lung and lung-draining lymph nodes

After harvest, lung-draining LNs 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). Cytokine levels produced by the restimulated T cells were determined from culture supernatant fractions collected 12 h after CD3/CD28 antibody stimulation.

Cytokine measurement

Cytokine levels in BAL fluid and culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA) in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Fluorescence-activated cell sorting analyses

For intracellular cytokine staining, isolated cells from lung-draining LN (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). Two hours before harvest, Brefeldin A (10 μ g/ml; Sigma-Aldrich) was added. After harvest, cells were stained with surface antibodies (CD3-APC and CD4-FITC; BD Biosciences, San Jose, CA, USA) 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-17-PE, and anti-IFN- γ -PE; BD Biosciences) for 30 min at room temperature and then analyzed on a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences) using CellQuest Pro software.

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

Effects of airway application of rTNF- α on the development of adaptive immune response to inhaled allergens

Lung epithelial cells and alveolar macrophages are the first-line cells responding to inhaled agents. This study showed that the production of TNF- α after *in vitro* stimulation with dsRNA (poly[I:C]) was more enhanced from alveolar macrophages than from lung epithelial cells (Fig. S1). In addition, we found that TNF- α levels in BAL fluid were up-regulated in mice sensitized with dsRNA (poly[I:C]), regardless of the poly[I:C] dose (Fig. 1A). Thus, we evaluated the effect of TNF- α on the development of allergic airway inflammation. To test this, WT mice were sensitized intranasally with OVA (75 μ g) and rTNF- α (100 ng) and

then challenged four times with OVA (50 μ g) alone. Evaluation of BAL cellularity 24 h after the last OVA challenge revealed that lung infiltrations of neutrophils and eosinophils were significantly increased in mice sensitized with OVA plus rTNF- α (OVA/rTNF- α) compared with those sensitized with OVA or rTNF- α alone (Fig. 1B). Additionally, the inflammatory score, based on histologic findings, indicated that both perivascular infiltration and peribronchiolar infiltration of inflammatory cells were enhanced by sensitization with OVA/rTNF- α compared with the other groups (Fig. 1C). In terms of pro-inflammatory mediator production, the levels of IP-10 (a Th1 mediator) and IL-17 (a Th17 mediator) in BAL fluid were enhanced by sensitization with OVA/rTNF- α compared with the OVA group. Additionally, the production of Th2 mediators, including IL-4 and eotaxin, was significantly increased in mice sensitized with OVA/rTNF- α (Fig. 1D). Regarding the production of OVA-specific antibodies, the results revealed that both OVA-specific IgG1 and IgE levels in serum were significantly increased in mice sensitized with OVA/rTNF- α compared with mice from the other groups (Fig. 1E). Taken together, these findings suggest that TNF- α enhances allergic airway inflammation to inhaled allergens.

Next, we evaluated the effects of TNF- α on the development of a T-cell response to inhaled allergens. To test this, WT mice were sensitized with rTNF- α (100 ng) plus OVA (75 μ g) and then challenged three times with OVA (50 μ g) alone. T-cell responses were evaluated 6 h after the third OVA challenge. The data indicate that the production of IFN- γ (a Th1 cytokine), IL-17 (a Th17 cytokine), and IL-4 (a Th2 cytokine) by T cells from lung-draining LN after stimulation with CD3/CD28 antibodies was significantly increased by sensitization with OVA/rTNF- α compared with OVA or rTNF- α alone (Fig. 1F). Similarly, the proportion of IFN- γ -, IL-17-, and IL-4-positive CD4+ T cells in the lung-draining LN was enhanced by sensitization with OVA/rTNF- α compared with OVA or rTNF- α alone (Fig. 1G). As for T cells infiltrating to the lung tissues, production of IFN- γ , IL-17, and IL-4 from lung cells after the CD3/CD28 stimulation was enhanced by sensitization with OVA/TNF- α (Fig. 1H). Collectively, these findings suggest that TNF- α induces Th2 as well as Th1 and Th17 cell responses to inhaled allergens.

Effects of TNF- α deficiency in the development of airway inflammation and allergen-specific antibody production induced by sensitization with allergens containing low- or high-dose dsRNA

Based on the above observations that rTNF- α can disrupt airway tolerance to inhaled allergens, we evaluated the role of TNF- α in the development of lung inflammation and antibody production by dsRNA-containing allergens. To test this, TNF- α -deficient and WT mice were sensitized intranasally with OVA (75 μ g) plus a low dose (0.1 μ g) or a high dose (10 μ g) of poly[I:C] and then challenged four times with OVA (50 μ g) alone. Bronchoalveolar lavage cellularity was evaluated 24 h after the last OVA challenge. The results

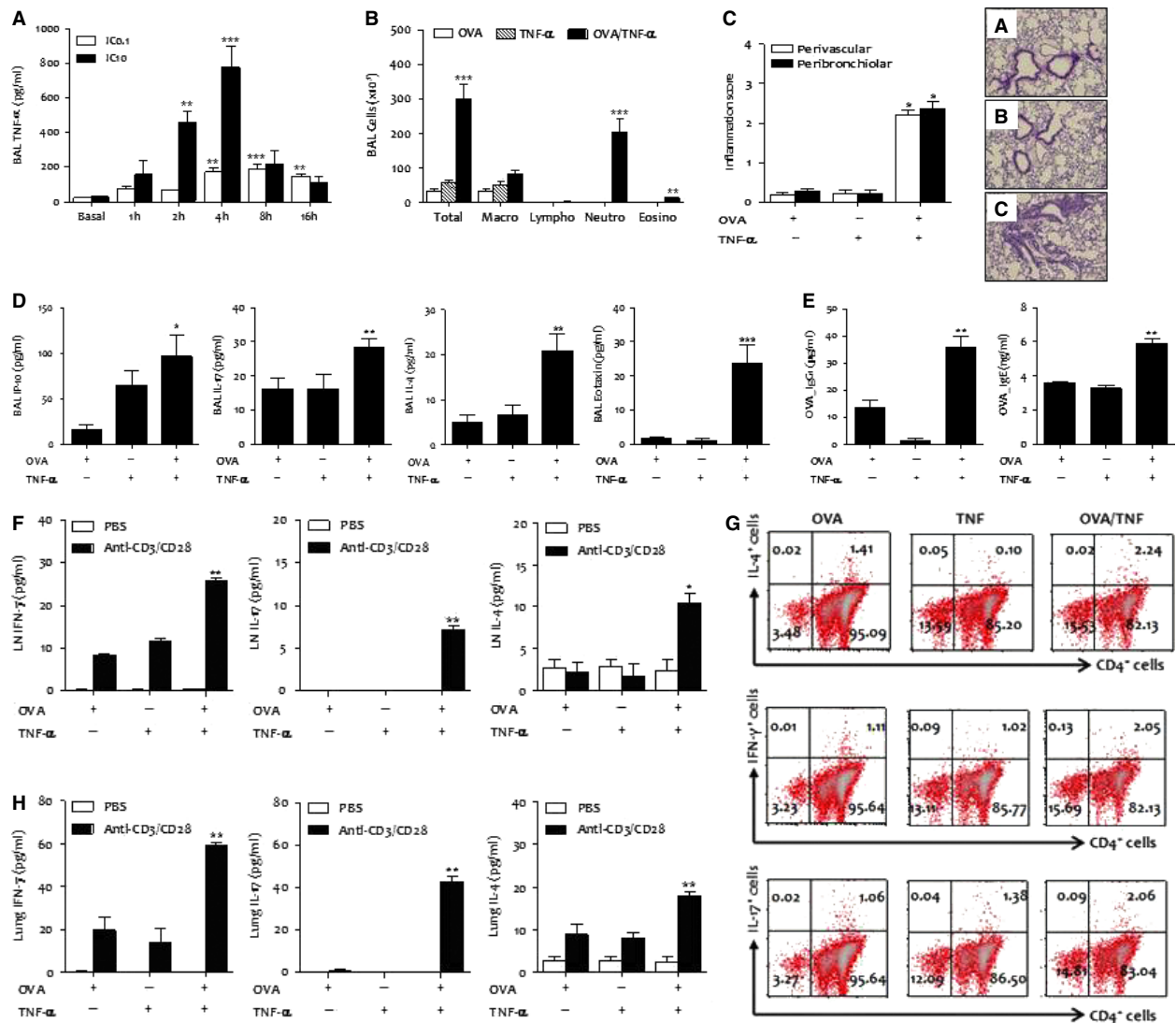


Figure 1 Airway sensitization with recombinant tumor necrosis factor- α (rTNF- α) plus allergens disrupts airway tolerance to inhaled allergens. For panel (A), 0.1 and 10 μ g of poly[I:C] (IC) were administered to the wild-type (WT) mouse airways ($n = 4$ for each group) and evaluated at different time points. (A) The levels of TNF- α in bronchoalveolar lavage (BAL) fluids. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ relative to basal level. For panels (B)–(H), WT mice ($n = 5$ for each group) were sensitized with ovalbumin (OVA, 75 μ g), rTNF- α (100 ng), or OVA + TNF- α and then challenged with OVA (50 μ g) alone; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ relative to the OVA group. For panels (B)–(E), evaluation was performed 24 h after the last OVA challenge. (B) BAL

cellularity. (C) Inflammatory score (left panel) and representative lung histologic findings (right panel: a, OVA; b, TNF- α ; c, OVA + TNF- α , H&E stain, 200 $\times$ magnification). (D) Levels of IP-10, IL-17, IL-4, and eotaxin in BAL fluid. (E) Serum levels of OVA-specific IgG1 and IgE. For panels (F)–(H), evaluation was performed 6 h after the third OVA challenge. Cells were isolated from lung-draining lymph nodes (LN) (F) and lung tissues (H) and incubated with phosphate-buffered saline or CD3 and CD28 antibodies for 12 h. Levels of each cytokine were evaluated in supernatant fraction. (F) Levels of interferon gamma (IFN- γ), IL-17, and IL-4 from lung-draining LN cells. (G) The proportion of CD3⁺CD4⁺ T cells in lung-draining LN. (H) The levels of IFN- γ , IL-17, and IL-4 from lung cells.

revealed that lung infiltration of neutrophils induced by sensitization with OVA plus low-dose poly[I:C] was partially abolished in the absence of TNF- α , whereas neutrophil infiltration induced by OVA plus high-dose poly[I:C] was not affected (Fig. 2A). Additionally, the inflammatory score, based on lung histology, revealed that both perivascular

infiltration and peribronchiolar infiltration of inflammatory cells induced by sensitization with OVA + low-dose poly[I:C] were significantly decreased in TNF- α -deficient mice compared with WT mice (Fig. 2B). Again, this was unaffected in the high-dose-dsRNA-treated mice. Regarding the production of T-cell downstream mediators, the results revealed that

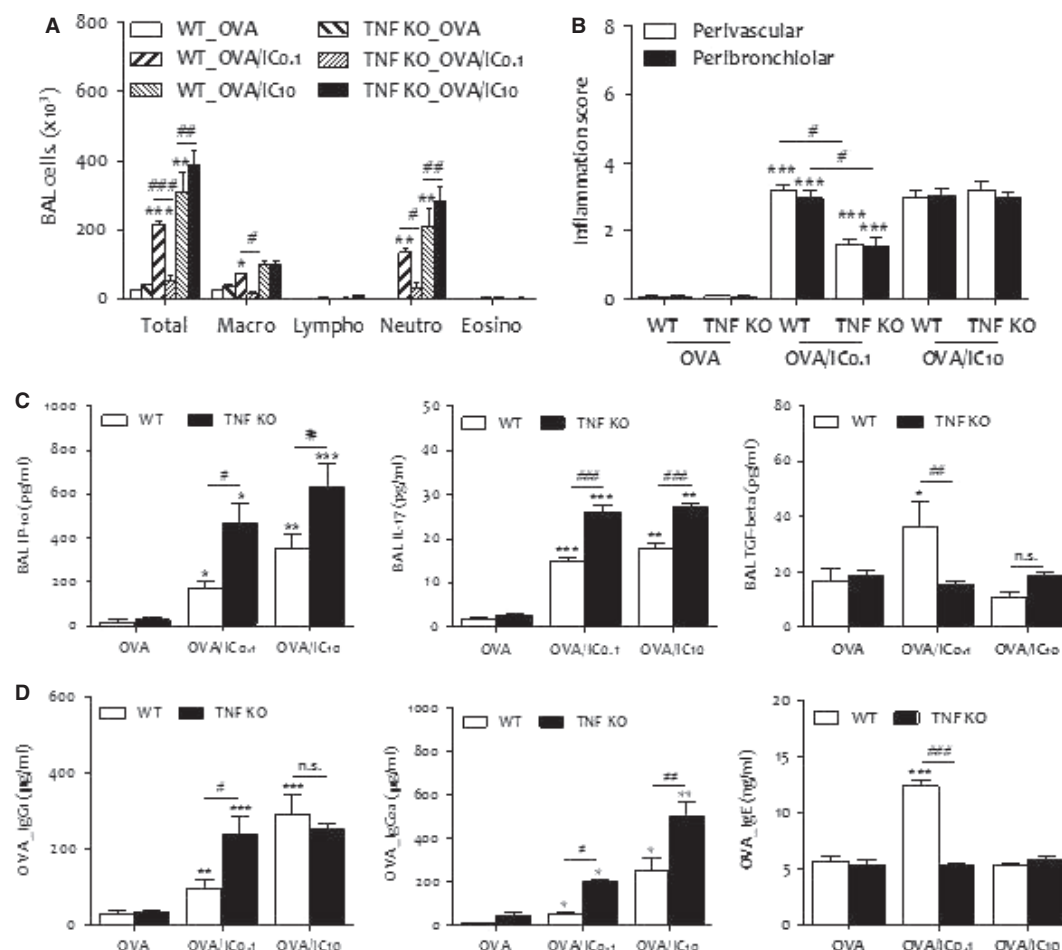


Figure 2 Allergic inflammation induced by airway sensitization with allergen plus low-dose dsRNA is diminished in the absence of tumor necrosis factor- α (TNF- α). For all panels, TNF- α -deficient and wild-type (WT) mice ($n = 5$ for each group) were sensitized with 75 μ g of ovalbumin (OVA), OVA + 0.1 μ g of poly[I:C], or OVA + 10 μ g of poly[I:C], then challenged with OVA (50 μ g) alone,

and evaluated 24 h after the last OVA challenge; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to OVA groups; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; n.s.: not significant. (A) bronchoalveolar lavage (BAL) cellularity. (B) Inflammatory score based on lung histology findings. (C) Levels of IP-10, IL-17, and TGF- β 1 in BAL fluid. (D) Levels of OVA-specific IgG1, IgG2a, and IgE in serum.

the levels of IP-10 and IL-17 (Th1 and Th17 mediators, respectively) in BAL fluid were enhanced in the absence of TNF- α , regardless of poly[I:C] doses. However, TGF- β 1 levels (a Th2 mediator) in BAL fluid were significantly decreased in TNF- α -deficient mice sensitized with OVA + low-dose poly[I:C] compared with WT mice sensitized in the same manner (Fig. 2C). In terms of allergen-specific antibody production, the serum levels of OVA-specific IgG1 and IgG2a were found to be significantly increased in TNF- α -deficient mice sensitized with OVA + low-dose poly[I:C] compared with WT mice sensitized in the same manner. However, serum OVA-specific IgE levels, enhanced by sensitization with OVA + low-dose poly[I:C], were down-regulated by the absence of TNF- α (Fig. 2D). Taken together, these findings suggest that TNF- α is a key mediator in the development of allergic inflammation and allergen-specific IgE production induced by sensitization with low-dose-dsRNA-containing allergens.

Effects of TNF- α deficiency in the development of T-cell immune dysfunction induced by airway sensitization with allergens containing low-dose dsRNA

Based on the above findings, we evaluated the role of TNF- α in the development of T-cell immune dysfunction induced by airway sensitization with allergens containing low-dose dsRNA. To test this, T-cell response was evaluated in TNF- α -deficient and WT mice 6 h after the third OVA challenge. The results revealed that the production of IL-4 from LN cells after CD3/CD28 stimulation, enhanced by sensitization with low-dose-dsRNA-containing OVA, was decreased in the absence of TNF- α , whereas IFN- γ and IL-17 production was reversed (Fig. 3A). Similarly, IL-17 and IFN- γ production from lung cells after CD3/CD28 stimulation was significantly higher in TNF- α -deficient mice than in WT mice (Fig. 3B). In contrast, IL-4 production from lung T cells after CD3/CD28 treatment was diminished by

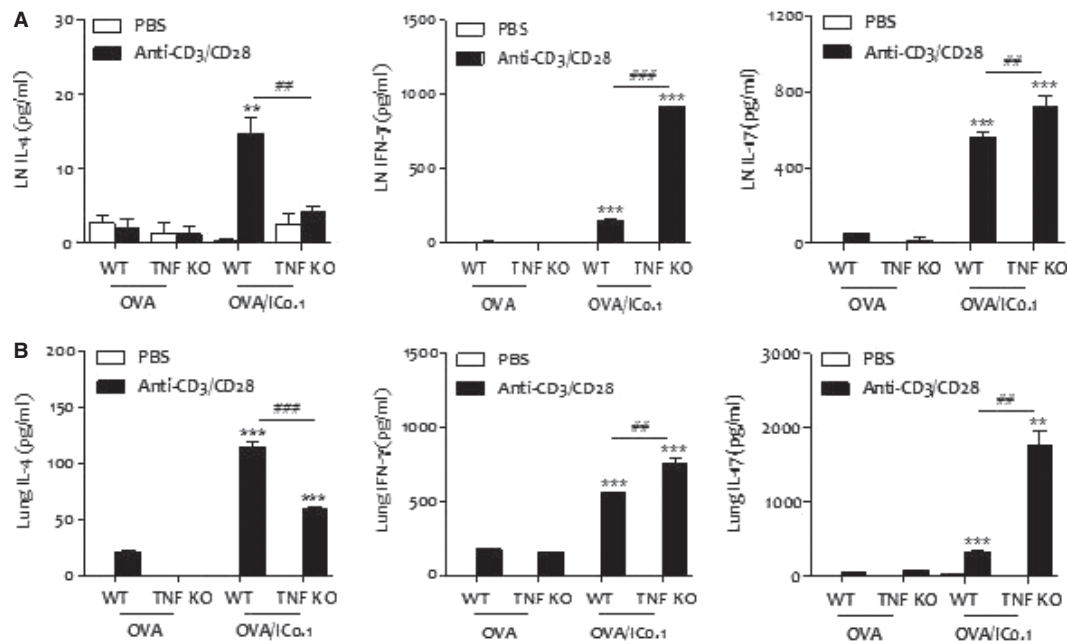


Figure 3 The Th2 cell response induced by airway sensitization with allergen plus low-dose dsRNA is abolished in the absence of tumor necrosis factor- α (TNF- α), whereas the Th1 and Th17 cell responses are enhanced. For all panels, TNF- α -deficient and wild-type (WT) mice ($n = 5$ for each group) were sensitized with 75 μ g of ovalbumin (OVA) or OVA + 0.1 μ g of poly[I:C] and then

challenged with OVA (50 μ g) alone; evaluation was performed 6 h after three times of OVA challenge. $**P < 0.01$; $***P < 0.001$ relative to OVA groups; $##P < 0.01$; $###P < 0.001$. Cells were isolated from lung-draining lymph nodes (LN) (A) and lung tissue (B) and incubated with phosphate-buffered saline or CD3 and CD28 antibodies for 12 h. Cytokine levels were evaluated in the supernatant fraction.

the absence of TNF- α (Fig. 3B). These data suggest that TNF- α produced by dsRNA during sensitization is the key mediator in the development of the Th2 cell response to inhaled allergens, but this mediator inhibits both Th1 and Th17 cell responses.

Effects of TNF- α deficiency in the development of the innate immune response induced by low-dose dsRNA

Previous evidence indicates that innate immune responses induced by dsRNA modulate the subsequent adaptive immune responses. Airway exposure to low-dose dsRNA up-regulates VEGF and IL-6, which subsequently leads to the Th17 cell response to inhaled allergens (6). In addition to the induction of TNF- α by low-dose dsRNA, the results of the present study indicate that airway exposure to 0.1 μ g of poly[I:C] enhances the production of Th2-polarizing cytokines [IL-4 and thymic stromal lymphopoietin (TSLP)] as well as Th1 (IL-12p70) and Th17 (IL-6)-polarizing cytokines (Fig. 4A). Therefore, we evaluated the role of TNF- α in the development of innate immune responses modulating T-cell priming and polarization. To test this, TNF- α -deficient and WT mice were sensitized intranasally with OVA + 0.1 μ g poly[I:C] and evaluated 12 h after this sensitization. Bronchoalveolar lavage cellularity revealed that sensitization with OVA + poly[I:C] induced lung infiltration of inflammatory cells, which was abolished in the absence of TNF- α (Fig. 4B). As for the expression of co-stimulatory molecules, which

determine T-cell priming, the expression of co-stimulatory molecules, such as CD40, CD80, and CD86 in lung F4/80-CD11c+MHCII+ cells (dendritic cells, DC), was enhanced by sensitization with OVA + poly[I:C] in WT mice compared with OVA alone. This enhanced expression was reversed in the absence of TNF- α (Fig. 4C). With regard to the expression of T-cell-polarizing cytokines, the production of IL-4 and TSLP in lung tissue exposed to low-dose dsRNA was markedly decreased in TNF- α -deficient mice sensitized with OVA + poly[I:C] compared with WT mice sensitized in the same manner (Fig. 4D). However, IL-12p70 and IL-6 were up-regulated in WT mice by sensitization with OVA poly[I:C], and this enhanced production was not diminished in the absence of TNF- α (Fig. 4E). Taken together, these data suggest that TNF- α signaling is critical to co-stimulatory molecule expression and Th2-polarizing cytokine production induced by low-dose dsRNA.

Role of TNF- α in the development of Th2 inflammation and OVA-specific IgE production induced by sensitization with allergens containing low-dose dsRNA

Based on the findings that the Th2 cell response induced by sensitization with allergens containing low-dose dsRNA was abolished in the absence of TNF- α , we evaluated the recovery of Th2 cell response upon addition of rTNF- α during sensitization in the low-dose dsRNA model. To test this, TNF- α -deficient mice were sensitized intranasally with OVA (75 μ g)

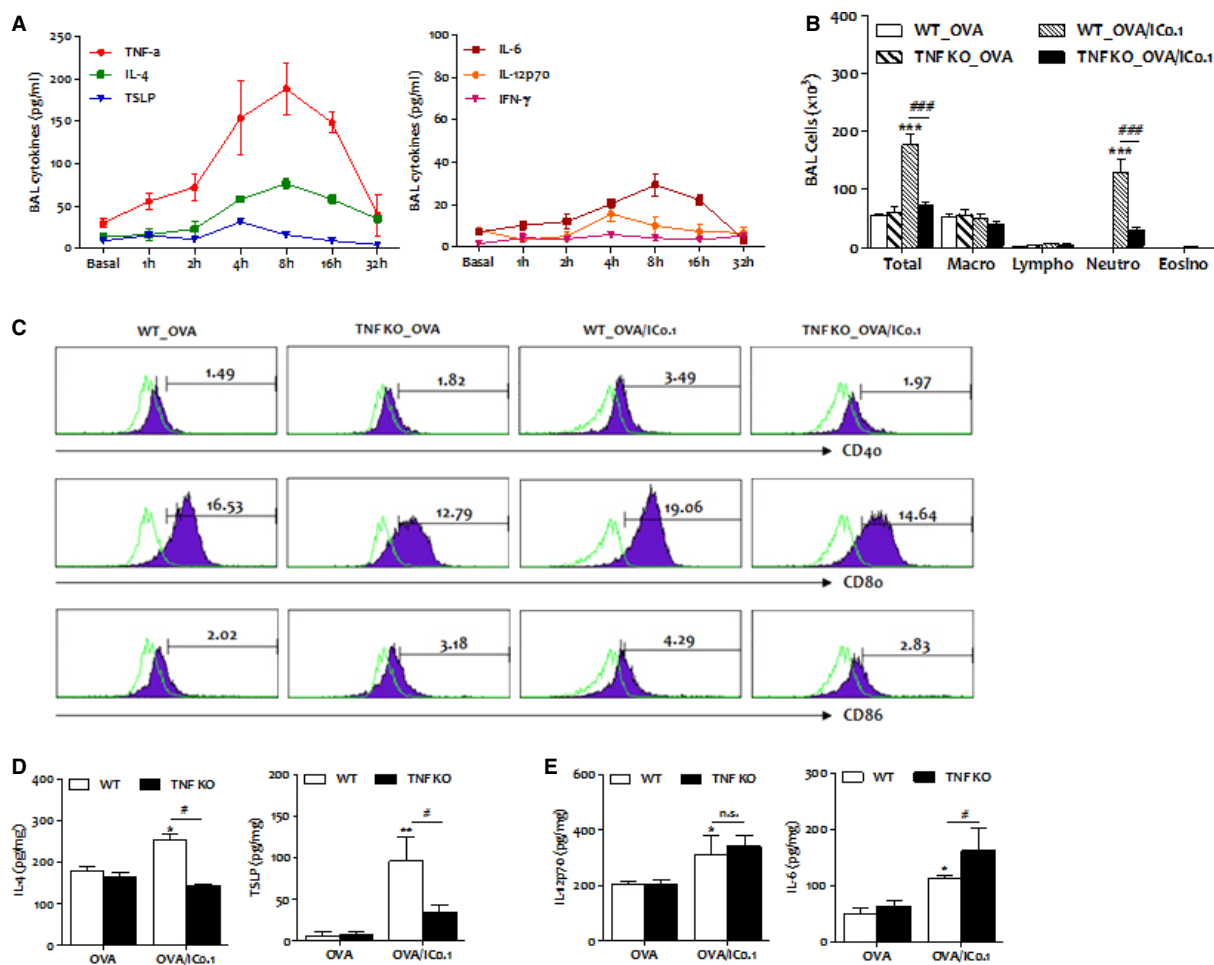


Figure 4 Dendritic cell (DC) maturation and Th2-polarizing cytokine production enhanced by low-dose dsRNA are abolished in the absence of tumor necrosis factor- α (TNF- α). For panel (A), 0.1 μ g of poly[I:C] was administered to the wild-type (WT) mouse airways ($n = 4$ for each group) and then evaluated at various time points. (A) The levels of TNF- α , IL-4, thymic stromal lymphopoietin (TSLP), IL-6, IL12p70, and interferon gamma (IFN- γ) in bronchoalveolar lavage (BAL) fluids. For panels (B)–(E), TNF- α -deficient and WT mice ($n = 5$ for each group) were sensitized

once with 75 μ g of ovalbumin (OVA) in the presence or absence of 0.1 μ g of poly[I:C] and then evaluated 12 h after the sensitization. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to ovalbumin (OVA) group; # $P < 0.05$; ### $P < 0.001$; n.s.: not significant. (B) Cellularity in BAL fluid. (C) Expression levels of co-stimulatory molecules, such as CD40, CD80, and CD86 on lung DCs. (D) Levels of Th2-polarizing cytokines (IL-4 and TSLP) in BAL fluid. (E) Levels of Th1 (IL-12p70)- and Th17-polarizing (IL-6) cytokines in BAL fluid.

+ poly[I:C] (0.1 μ g) + rTNF- α (100 ng) and then challenged four times with OVA (50 μ g) alone. Mice were evaluated 24 h after the last OVA challenge. Bronchoalveolar lavage cellularity revealed that the lung infiltration of inflammatory cells diminished in TNF- α -deficient mice was recovered by the addition of rTNF- α (Fig. 5A). Similarly, the inflammatory score, based on the lung histologic findings, indicated that the addition of rTNF- α during sensitization in TNF- α -deficient mice recovered lung inflammation induced by sensitization with OVA-containing poly[I:C] (Fig. 5B). Additionally, the results revealed that the production of the Th2 mediators, IL-4 and TGF- β 1, which was abolished in TNF- α -deficient mice sensitized with OVA + poly[I:C], was completely recovered upon the addition of TNF- α (Fig. 5C). However, Th1

(IP-10) and Th17 (IL-17) mediator production, augmented in TNF- α -deficient mice sensitized with OVA + poly[I:C], was inhibited by the addition of TNF- α (Fig. 5D). As for allergen-specific antibody production, the results revealed that the serum OVA-specific IgE levels, which were diminished in TNF- α -deficient mice sensitized with OVA + poly[I:C], were recovered by the addition of TNF- α , whereas OVA-specific IgG1 and IgG2a levels were reversed (Fig. 5E).

In addition, we evaluated the effects of rTNF- α addition during sensitization on the development of OVA-specific adaptive immune response in the absence of TNF- α . The present data showed that cellularity and Th2 mediators (IL-4, TGF- β , and eotaxin) levels in BAL fluid were similar in TNF- α -deficient and WT mice sensitized with rTNF- α

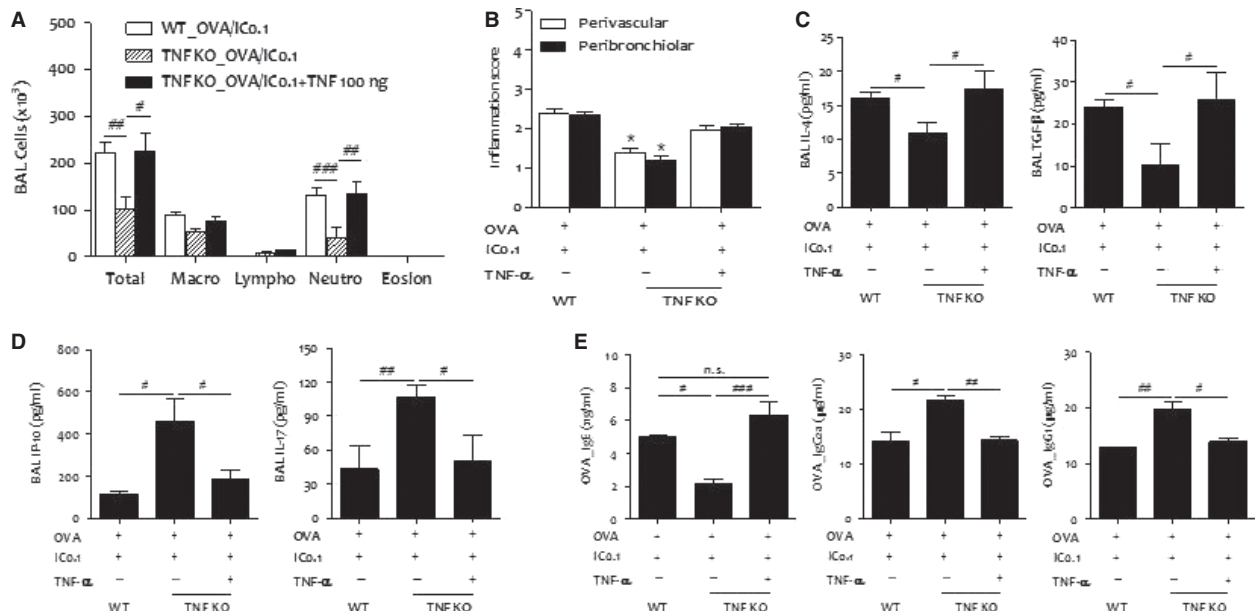


Figure 5 Tumor necrosis factor- α (TNF- α) plays a key role in the development of allergic airway inflammation induced by sensitization with allergen plus low-dose dsRNA. For all panels, TNF- α -deficient and wild-type (WT) mice ($n = 5$ for each group) were sensitized with ovalbumin (OVA, 75 μ g) + 0.1 μ g poly[I:C] (IC0.1) and then challenged with OVA (50 μ g) alone; evaluation was performed 24 h after

the fourth OVA challenge. * $P < 0.05$ relative to WT_OVA/IC0.1 group; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; n.s.: not significant. (A) Cellularity in bronchoalveolar lavage (BAL) fluid. (B) Inflammatory score. (C) IL-4 and TGF- β levels in BAL fluid. (D) IP-10 and IL-17 levels in BAL fluid. (E) Serum levels of OVA-specific IgE, IgG2a, and IgG1.

plus OVA (Fig. S2A, B). In addition, there is no difference in the Th2 immune response between the two groups (Fig. S2C, D). However, in terms of Th1 and Th17 immune responses, the present study showed that levels of both Th1 and Th17 mediators (IP-10 and IL-17, respectively) in BAL fluid and anti-CD3/CD28-stimulated production of IFN- γ and IL-17 from lung and lung-draining LN cells were higher in the TNF- α -deficient mice than in the WT mice (Fig. S2B–D).

To sum up, these findings suggest that the TNF- α produced by low-dose dsRNA is critical for the development of Th2 inflammation and allergen-specific IgE production in the low-dose-dsRNA-induced asthma model.

Role of TNF- α in the development of the Th2 cell response induced by sensitization with allergens containing low-dose dsRNA

Finally, we evaluated whether the Th2 cell response is restored by the addition of TNF- α during sensitization in TNF- α -deficient mice sensitized with allergens containing low-dose dsRNA. To test this, TNF- α -deficient mice were sensitized with OVA + poly[I:C] (0.1 μ g) and then challenged three times with OVA alone. Lung and lung-draining LN cells were isolated 6 h after the OVA challenge, and then, these cells were stimulated with the CD3 and CD28 antibodies. The results revealed that the production of IL-4 from T cells in lung-draining LN, abolished in TNF- α -deficient mice sensitized with OVA + poly[I:C], was recovered by the addi-

tion of TNF- α , whereas IFN- γ and IL-17 production was reversed (Fig. 6A). Similarly, the proportion of IL-4-positive CD4+ T cells (Th2) in the lung-draining LN, diminished in TNF- α -deficient mice sensitized with OVA + poly[I:C], was completely recovered by the addition of TNF- α . In contrast, the proportion of IFN- γ -positive CD4+ T (Th1) and IL-17-positive CD4+ T (Th17) cells, which was enhanced in TNF- α -deficient mice sensitized with OVA + poly[I:C] compared with WT mice, was diminished by the addition of TNF- α (Fig. 6B). In terms of T-cell subsets infiltrating lung tissues, the results revealed that the production of IL-4 from lung T cells, abolished in TNF- α -deficient mice sensitized with OVA + poly[I:C], was recovered by the addition of TNF- α , whereas IFN- γ and IL-17 production was reversed (Fig. 6C). Overall, these results indicate that TNF- α , produced by low-dose dsRNA during sensitization, plays an essential role in the development of the Th2 cell response in the low-dose dsRNA-induced asthma model.

Discussion

Virus-associated asthma is characterized by noneosinophilic or neutrophilic airway inflammation, whereas atopic asthma is characterized by eosinophilic inflammation (2). Our previous experimental data indicated that VEGF and IL-6, induced by low-dose dsRNA, induce Th17 cell response to inhaled allergens (6). The present study demonstrated that TNF- α is a key mediator in development of the Th2 cell response in the virus-associated asthma model mainly via

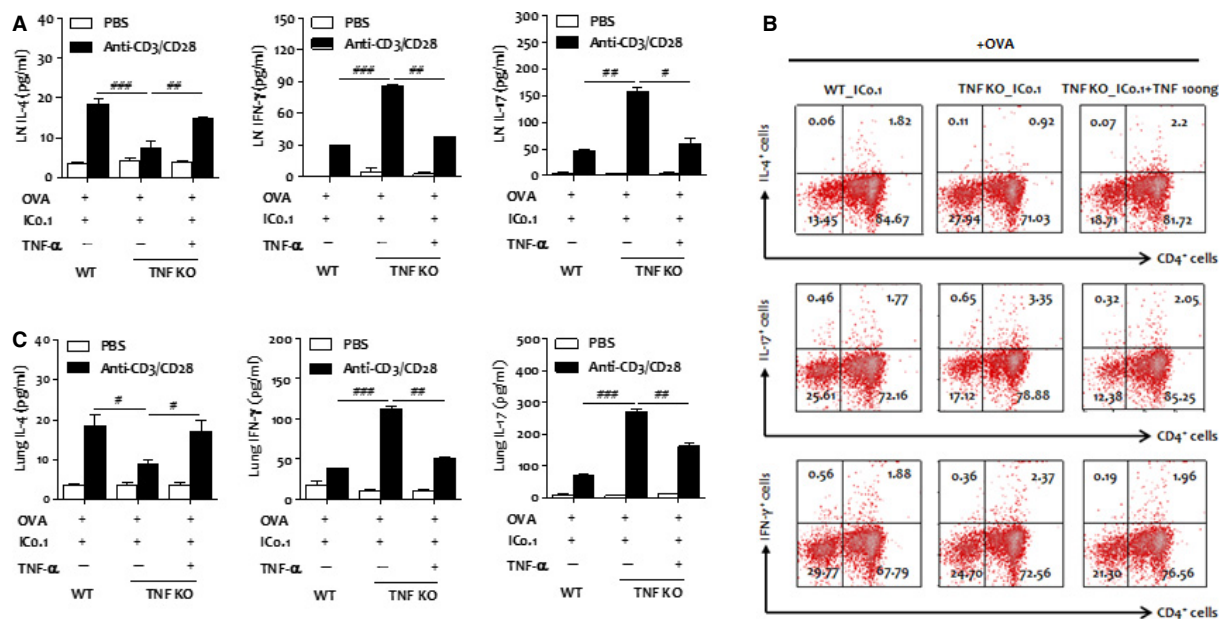


Figure 6 Tumor necrosis factor-alpha (TNF- α) plays a key role in development of the Th2 cell response induced by sensitization with allergen plus low-dose dsRNA. For all panels, TNF- α -deficient and wild-type (WT) mice ($n = 5$ for each group) were sensitized with ovalbumin (OVA, 75 μ g) + 0.1 μ g poly[I:C] (IC0.1) and then challenged with OVA (50 μ g) alone; evaluation was performed 6 h after the third OVA challenge. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$;

n.s.: not significant. Cells were isolated from lung-draining lymph nodes (LN) (A) and lung tissue (C) and incubated with phosphate-buffered saline or CD3 and CD28 antibodies for 12 h. The levels of each cytokine were evaluated in the supernatant fraction. (A) IL-4, interferon gamma (IFN- γ), and IL-17 levels from lung-draining LN cells. (B) The proportion of CD3⁺ CD4⁺ T cells in lung-draining LN. (C) IL-4, IFN- γ , and IL-17 levels from lung cells.

enhancement of DC maturation and Th2-polarizing cytokine production.

Tumor necrosis factor-alpha is a well-defined pro-inflammatory mediator produced by innate immune cells. TNF- α accelerates the inflammatory response to increase the expression of adhesion molecules and other pro-inflammatory cytokines (17, 22). The present study demonstrated that intranasal rTNF- α administration with allergens disrupts airway tolerance to inhaled allergens and induces Th2 as well as Th1 and Th17 cell responses in the lung. TNF- α is known to enhance DC maturation by modulating co-stimulatory molecule expression (23, 24). Lack of co-stimulatory molecule signaling induces insufficient T-cell stimulation and causes down-regulation of the adaptive immune response (25). Similarly, in a TNF- α -deficient state, DC maturation and T-cell proliferation were down-regulated when infected with virus (25). The current data also demonstrate that co-stimulatory molecule expression by dsRNA-containing allergen sensitization was abolished in the absence of TNF- α . Collectively, these data suggest that TNF- α produced during allergen sensitization is a key mediator in the development of T-cell priming to inhaled allergens.

The role of TNF- α has only been phenotypically studied in the development of Th2 airway inflammation, for example IL-13 production or eosinophil recruitment (18, 19, 21, 26, 27). In the present study, IL-4 and TSLP, well-known Th2-polarizing cytokines (28), were induced after rTNF- α administration during allergen sensitization. The results of

the present study also indicate that the production of IL-4 and TSLP induced by low-dose dsRNA during sensitization is significantly down-regulated in the absence of TNF- α . Moreover, the current study showed that Th2 cell response induced by airway sensitization with low-dose-dsRNA-containing allergens, which was abolished in TNF- α -deficient mice, is restored by the addition of rTNF- α during sensitization in the virus-associated asthma model. Based on these data, we postulate that TNF- α , induced by low-dose dsRNA, is a key mediator in the development of Th2 cell response via up-regulation of Th2-polarizing cytokine production.

In the case of TNF- α production, airway epithelial cells and alveolar macrophages are both known as the TNF- α producer at the front line of the lung (17). In the present study, we could confirm that alveolar macrophages are more potent TNF- α producers than epithelial cells after low-dose dsRNA stimulation. These data indicated that during sensitization, TNF- α produced by alveolar macrophage plays a more important role in the Th2 immune responses than those produced by airway epithelial cells when exposed to low-dose dsRNA.

The current study showed that IL-4 was enhanced during the sensitization. Among the innate immune cell, NKT cell is known as a potent IL-4-producing cell (29). According to our previous study, NK1.1⁺ cells were infiltrated in the lung and produced IL-4 by low-dose dsRNA sensitization (7). Based on these results, we postulated that IL-4 production by low-dose dsRNA is induced by NK1.1⁺ cell, such as

NKT cell. However, the relationship between TNF- α and elaboration of Th2 cytokine in sensitization is not defined clearly; therefore, we need to investigate more on the exact mechanism.

The results of the present study demonstrated that TNF- α deficiency led to enhancement of the Th17 cell response but abolished the Th2 cell response. IL-6 and TGF- β 1 are known to play an important role for Th17 polarization (30). Much evidence indicates that TNF- α and TGF- β 1 suppress each other (31–33). The results of the present study demonstrated that the IL-6 production was elevated in the TNF- α -deficient state during sensitization in the virus-associated asthma model. These findings indicate that the absence of TNF- α enhances Th17 polarization, possibly via up-regulation of IL-6 production and/or enhancement of TGF- β 1 activity.

In the case of Th1 polarization, it was also enhanced in TNF- α -deficient mice when stimulated with low-dose dsRNA plus OVA. In Th1 polarization, STAT4 signaling, induced by IL-12p70, is known to play a key role. However, GATA3, induced by IL-4, is an important factor for Th2 polarization and suppresses Th1 polarization (34, 35). In the present study, no significant enhancement of IL-12p70 production was observed during sensitization in the TNF- α -deficient state, although Th1 polarization was up-regulated. Additionally, the results revealed that IL-4 production during sensitization was diminished in the TNF- α -deficient state. These data suggest that Th1 polarization, enhanced in TNF- α -deficient mice, is mediated by the absence of Th2-polarizing cytokine production, rather than the suppression of Th1-polarizing cytokine production.

In summary, the present study directly proved that TNF- α plays a key role in the development of the Th2 cell response, but not in the development of the Th1 and Th17 responses, in a virus-associated allergic asthma model. Additionally, the results indicate that TNF- α produced during respiratory viral infections can disrupt airway tolerance to inhaled allergens

via up-regulation of DC maturation and also enhances the Th2 response via up-regulation of Th2-polarizing cytokine production.

Acknowledgments

We thank Seo-Hyun Lim for providing secretarial assistance and members of the POSTECH animal facility for their experimental expertise. This study was supported by the National Research Foundation of Korea Grant funded by the Korean Government (No. 2011-0000879).

Author contributions

J-P.C., Y-S.K., Y-M.K., and O-Y.K. designed and conducted experiments, analyzed and interpreted results, and wrote the manuscript; S-G.J., T-Y.R., J-S.P., and Y-S.G. designed experiments and wrote the manuscript; Y-K.K. directed the study and wrote the manuscript.

Conflict 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. The production of TNF- α by dsRNA stimulation is enhanced from alveolar macrophages than from lung epithelial cells.

Figure S2. TNF- α plays an important role in the development of Th2 cell and Th2 immune response in the lung.

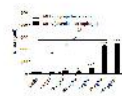
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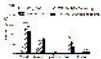
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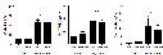
Abstract



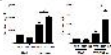
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