

IL-6 Induced by Double-Stranded RNA Augments Allergic Inflammation via Suppression of Foxp3⁺ T-Cell/IL-10 Axis

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Activation of innate immunity against viruses in the respiratory tracts affects the development of asthma. Most respiratory viruses generate double-stranded (ds)RNA during their replication. We recently showed that a low-dose administration of polyinosinic polycytidylic acid (poly IC), a mimetic of viral dsRNA, during allergen sensitization augments airway eosinophilia and hyperresponsiveness in mice via enhanced production of IL-13 from T cells. However, a phenotype of asthma under severer load of dsRNA remains unknown. D-galactosamine (D-GalN) is known as a strong sensitizer of poly IC. Mice were treated with poly IC plus D-GalN during allergen sensitization. A sublethal dose of poly IC/D-GalN augmented airway eosinophilia and CD4⁺ T-cell accumulation in the lungs but not airway hyperresponsiveness. The augmented inflammation was associated with decreased IL-10 in the bronchoalveolar lavage fluid and decreased Foxp3⁺ regulatory T cells in the lungs. Serum IL-6 was prominently higher in the mice treated with poly IC/D-GalN than in that with poly IC alone or D-GalN alone. Poly IC/D-GalN did not affect IL-17-producing T cells in the lungs. Poly IC/D-GalN failed to augment airway eosinophilia after anti-IL-10 receptor monoclonal antibody treatment during allergen challenge. Finally, anti-IL-6 receptor monoclonal antibody treatment before poly IC/D-GalN completely prevented the decrease of IL-10 and Foxp3⁺ regulatory T cells and the augmentation of airway inflammation. These results indicate that enhanced production of IL-6 by poly IC/D-GalN induces the augmentation of allergic inflammation via suppression of Foxp3⁺ regulatory T-cell/IL-10 axis. IL-6 may be a target for preventing asthma augmentation related to severe-virus infection.

Keywords: IL-10; Foxp3; regulatory T cell; D-galactosamine

The natural course of asthma is affected by various environmental factors, including airway infection (1). Respiratory syncytial virus (RSV) and rhinovirus are well known to contribute to the development of asthma and its acute exacerbation (1, 2). The virulence of those viruses is generally modest, because a lethal outcome of the infected hosts is rare. In addition, some reports

CLINICAL RELEVANCE

The prominent action of IL-6 proposes a mechanism for the virus-associated pathophysiology of asthma. Our model may provide a novel strategy targeting IL-6 for preventing asthma development related to severe virus infection.

showed that more virulent viruses, including influenza and measles, are associated with the development of asthma (3, 4). The other reports, however, showed that viral infections protect against asthma (5–7), which is associated with “the hygiene hypothesis.” There may be complex relationships between viruses and the host immune status in individuals with asthma. It is important to elucidate how underlying mechanisms differ among virus species or the magnitudes of viral load onto the host immune system.

Many viruses pathogenic for the respiratory tracts have single-stranded RNA as their genome. The influenza virus contains a unique triphosphate-ended double-stranded (ds)RNA-like structure at the 5' terminus of genomic single-stranded RNA. The others generate dsRNA in their host cells. dsRNA triggers innate immune responses via its binding to pattern recognition receptors (PRRs), including Toll-like receptor 3 (TLR3) and the family of RNA helicase, namely the retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated gene (Mda)-5 (8–10). TLR3 recognizes dsRNA in the endosome and initiates signaling through an adaptor, the Toll/IL-1R domain-containing adapter inducing IFN- β (TRIF) (also known as TICAM-1) (11, 12). RIG-I and Mda5 recognize dsRNA in the cytoplasm and initiate signaling through IFN- β promoter stimulator (IPS)-1 (also known as MAVS/Cardif/VISA) (13–16). These signaling cascades lead to the production of IFN- α/β and proinflammatory cytokines such as IL-6 and IL-12. These cytokines may have roles in the virus-associated pathophysiology of asthma.

The main purpose of this study was to investigate whether severe dsRNA load is protective or harmful for the development of asthma phenotype. We have recently reported that a low-dose (10 μ g/mouse) administration of polyinosinic polycytidylic acid (poly IC), a mimetic of viral dsRNA, during allergen sensitization augments airway eosinophilia and airway hyperresponsiveness (AHR) in allergen-challenged mice (17). The augmentation is mediated via the TRIF-mediated pathway leading to enhanced production of IL-13 mainly from CD8⁺ T cells. On the other hand, establishing a model of severer load has been unfeasible because of the resistance of mice to a high dose of poly IC. Hence, we examined the effect of poly IC in combination with D-galactosamine (D-GalN), a strong sensitizer of PPR agonists including LPS, cytosine-phosphate-guanine DNA, and dsRNA (8, 18–20), on the asthma phenotypes.

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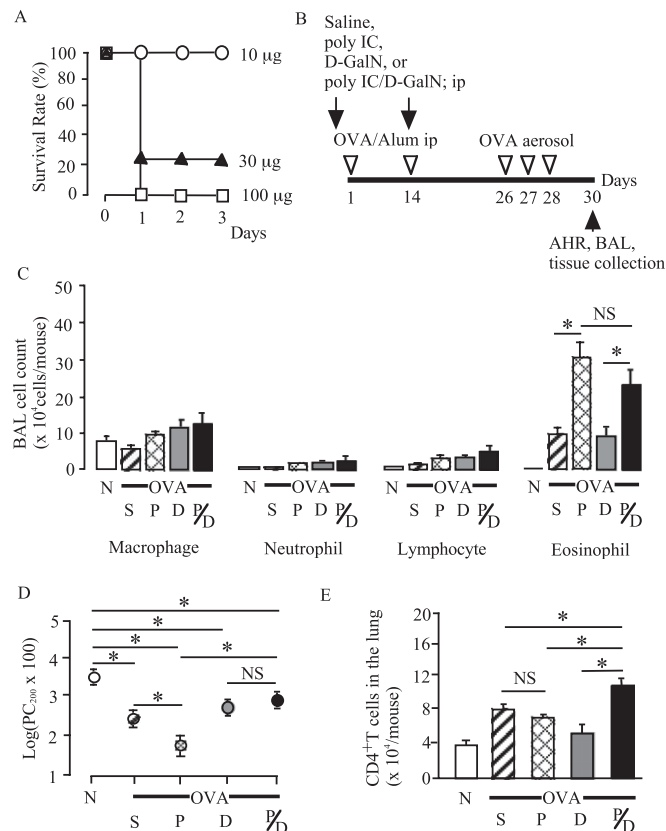


Figure 1. Effect of polyinosinic polycytidylic acid (poly IC) or poly IC/d-galactosamine (D-GalN) on allergen-induced asthma phenotype. (A) Survival rates of poly IC/D-GalN-treated mice. BALB/c mice were treated with different doses of poly IC in combination with 20 mg of D-GalN. Each group consisted of four mice. (B) Protocol for assessing the effect of poly IC or poly IC/D-GalN on OVA-induced asthma phenotype. Saline- or D-GalN-treated mice served as controls. Each group consisted of 8 to 12 mice. Effects of saline (hatched bar), poly IC (cross-hatched bar), D-GalN (shaded bar), or poly IC/D-GalN (solid bar) (C) on the eosinophilia in bronchoalveolar lavage fluid, (D) on the airway hyperresponsiveness, and (E) on the CD4⁺ T-cell accumulation into the lungs. Data are expressed as the means $\pm$ SEM. * $P < 0.05$ between the indicated two groups. AHR = airway hyperresponsiveness; BAL = bronchoalveolar lavage; D = D-GalN; OVA = ovalbumin; P = poly IC; PC₂₀₀ = provocative concentration 200; S = saline.

MATERIALS AND METHODS

Protocols

Mice were intraperitoneally sensitized with 10 µg of ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) and 0.3 mg of Al(OH)₃ (SERVA Electrophoresis, Heidelberg, Germany) on Days 1 and 14 and challenged with 5% OVA in saline mist for 20 minutes on Days 26, 27, and 28. Measurements were conducted on Day 30. Mice received an intraperitoneal injection of poly IC (Sigma-Aldrich) with or without 20 mg of D-GalN 1 hour before sensitization. Mice receiving saline served as controls. To block IL-10 signaling in the challenge phase, several mice received intraperitoneal injections of (250 µg/animal) anti-mouse IL-10 receptor (IL-10R) monoclonal antibody (mAb) (1B1.3a; BD Biosciences, San Jose, CA) 30 minutes before OVA challenge on days 26 and 28 (21, 22). To block IL-6 signaling in the sensitization phase, other mice received intraperitoneal injections of (1 mg/animal) anti-mouse IL-6 receptor (IL-6R) mAb (Chugai Pharmaceuticals, Tokyo, Japan) 30 minutes before each poly IC/D-GalN administration (23). Mice receiving rat IgG served as controls. To assess the acute effect of dsRNA, naive mouse blood was sampled 5 hours after an injection with saline, poly IC, D-GalN, or poly IC/D-GalN.

AHR and Bronchoalveolar Lavage

Anesthetized mice were ventilated to measure airway responsiveness to acetylcholine via tracheostomy (24, 25). The data were expressed as the provocative concentration 200, the concentration at which airway pressure was 200% of its baseline. Lungs were lavaged with 1 ml of saline via a tracheal cannula. Total cell counts were performed.

Measurement of Cytokines

IL-4, IL-5, IL-10, IL-13, IL-17, and IFN- γ in the bronchoalveolar lavage fluid (BALF) and IFN- β , IL-6, and IL-12p70 in the serum were

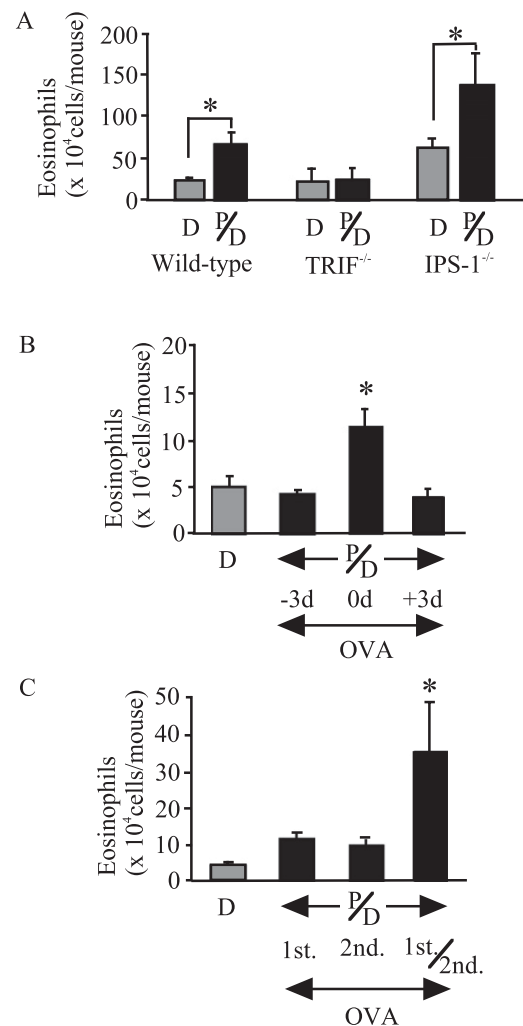


Figure 2. Effect of polyinosinic polycytidylic acid (poly IC)/d-galactosamine (D-GalN) on airway eosinophilia in various settings. (A) TRIF^{-/-} mice, IPS-1^{-/-} mice, and wild-type C57BL/6 mice were treated with poly IC/D-GalN (solid bar) or D-GalN (shaded bar) before each OVA sensitization. Following OVA challenge, the eosinophilia in BALF was measured. Each group consisted of seven to nine mice. (B) BALB/c mice were treated with poly IC/D-GalN on the same day, or 3 days before or after each sensitization. Following OVA challenge, the eosinophilia in BALF was measured. D-GalN-treated mice served as controls. Each group consisted of five mice. (C) BALB/c mice were treated with poly IC/D-GalN at the first or second sensitization or both. Following OVA challenge, the eosinophilia in BALF was measured. D-GalN-treated mice served as controls. Each group consisted of five mice. Data are expressed as the means $\pm$ SEM. * $P < 0.05$ between the indicated two groups. D = D-GalN; IFN- β promoter stimulator; P = poly IC; TRIF = Toll/IL-1R domain-containing adapter inducing IFN- β .

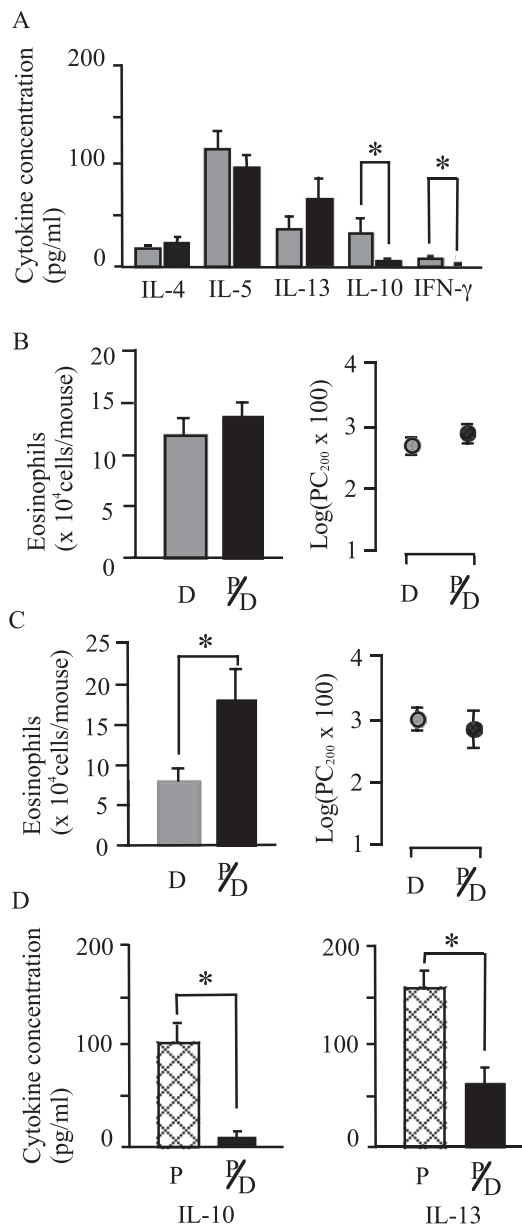


Figure 3. Effect of polyinosinic polycytidylic acid (poly IC)/D-galactosamine (D-GalN) or poly IC on cytokine profiles in the lungs. (A) BALB/c mice were treated with poly IC/D-GalN (solid bar) or D-GalN (shaded bar) before each sensitization. Following OVA challenge, cytokine contents in bronchoalveolar lavage fluid (BALF) were measured. Each group consisted of seven to eight mice. (B) BALB/c mice were treated with poly IC/D-GalN or D-GalN before each sensitization. During OVA challenge, mice were treated with anti-IL-10 receptor monoclonal antibody, and the eosinophilia in BALF and the airway hyperresponsiveness were measured. Each group consisted of five to seven mice. (C) IFN- γ -deficient mice were treated with poly IC/D-GalN or D-GalN before each sensitization. Following OVA challenge, the eosinophilia in BALF and the airway hyperresponsiveness were measured. Each group consisted of six mice. (D) BALB/c mice were treated with poly IC/D-GalN (solid bar) or poly IC (cross-hatched bar) before each sensitization. Following OVA challenge, IL-10 and IL-13 in BALF were measured. Each group consisted of 9 to 10 mice. Data are expressed as the means $\pm$ SEM. * $P < 0.05$ between the indicated two groups. D = D-GalN; P = poly IC; PC₂₀₀ = provocative concentration 200.

quantified using ELISA kits (Invitrogen Corporation, San Francisco, CA or R&D Systems, Minneapolis, MN).

Collection of Lung Cells

Lungs were cut and minced. Complete RPMI-1640 (Gibco BRL, Paisley, UK) containing 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin was added and incubated for 60 minutes. The samples were filtered through a cell strainer. The erythrocytes were lysed with an NH₄Cl-Tris buffer. Cells were suspended in complete RPMI-1640.

Flow Cytometry

Cells were preincubated with Fc Block to prevent nonspecific binding via Fc γ R, and surface staining was performed using FITC-labeled anti-cluster designation antigen (CD)25 (7D4) and peridinin-chlorophyll-protein complex (PerCP)-labeled anti-CD4 (RM4-5) mAbs. Intracellular staining for Foxp3 was performed using phycoerythrin (PE)-labeled anti-Foxp3 mAb (FJK-16s) and a commercially available kit (eBioscience, San Diego, CA). To detect IL-17-producing helper T-cell subsets (Th17), lung cells (2×10^6 cells/well) were stimulated with phorbol 12-myristate 13-acetate (10 ng/ml, Sigma-Aldrich) and 2 μ g/ml of ionomycin (Sigma-Aldrich) for 5 hours in the presence of brefeldin A (1 μ l/well; eBioscience) (26). The cells were harvested and preincubated with Fc Block and stained with PerCP-labeled anti-CD4 mAb. Intracellular staining was performed by using PE-labeled anti-IL-17 mAb (eBio1787) or isotype rat IgG and a commercially available kit. All antibodies, except for anti-Foxp3 and anti-IL-17 mAbs, were purchased from BD Biosciences. The cells were assessed using a FACSCalibur (BD Biosciences).

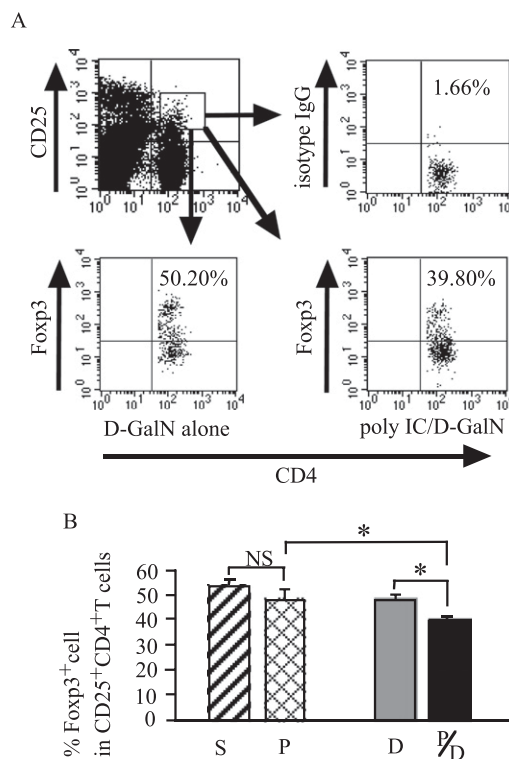


Figure 4. Effect of polyinosinic polycytidylic acid (poly IC)/D-galactosamine (D-GalN) or poly IC on Foxp3⁺ cells. (A) Representative dot plots showing Foxp3⁺ cells in the lungs of OVA-challenged BALB/c mice. CD4⁺CD25⁺ T-cell population was assessed for Foxp3 expression by percentage. (B) Effect of poly IC or poly IC/D-GalN on the percentage of Foxp3⁺ cells. BALB/c mice were treated with saline (hatched bar), poly IC (cross-hatched bar), D-GalN (shaded bar), or poly IC/D-GalN (solid bar) before each sensitization. Following OVA challenge, the percentages of Foxp3⁺ cells in the lungs were measured. Each group consisted of six to eight mice. Data are expressed as the means $\pm$ SEM. * $P < 0.05$ between the indicated two groups. D = D-GalN; P = poly IC; S = saline.

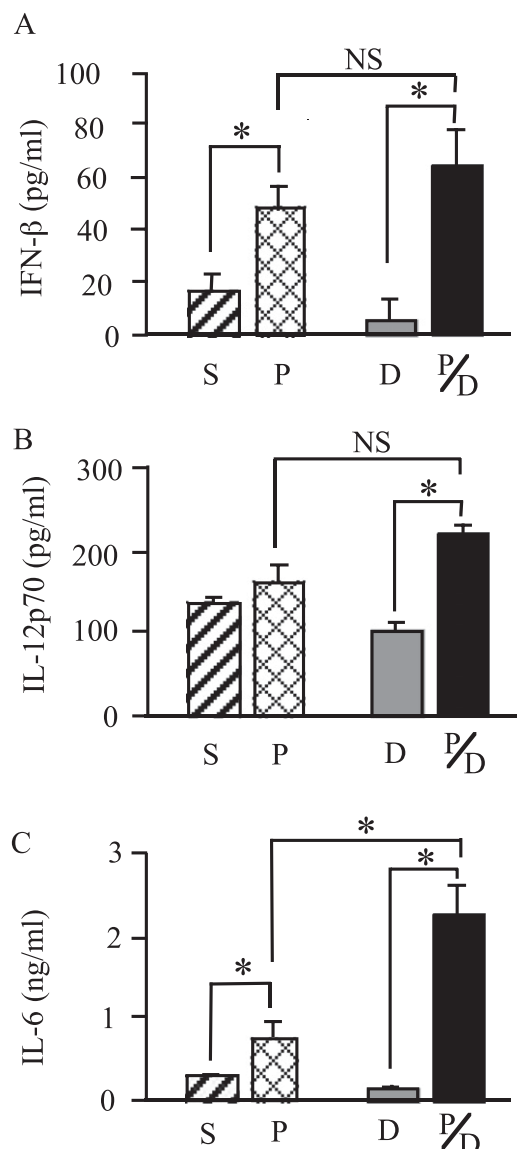


Figure 5. Different productions of type 1 IFN and proinflammatory cytokines between polyinosinic polycytidylic acid (poly IC) and poly IC/D-galactosamine (D-GalN) treatments. Naive BALB/c mice were treated with saline (hatched bar), poly IC (cross-hatched bar), D-GalN (shaded bar), or poly IC/D-GalN (solid bar). The sera were collected 5 hours after the treatment, and the (A) IFN- β , (B) IL-12p70, and (C) IL-6 were measured. Each group consisted of five mice. Data are expressed as the means $\pm$ SEM. * $P < 0.05$ between the indicated two groups. D = D-GalN; P = poly IC; S = saline.

Data Analysis

Parametric data were analyzed using the unpaired *t* test or an ANOVA with Bonferroni's correction. Nonparametric data were analyzed using the Mann-Whitney *U* test or the Kruskal-Wallis test. *P* values less than 0.05 were accepted as significant.

RESULTS

Asthma Phenotype by Poly IC/D-GalN Is Different from That by Poly IC Alone

In a dose-ranging study (Figure 1A), an administration of 100 μ g of poly IC in combination with 20 mg of D-GalN resulted in the death of all treated BALB/c mice within 24 hours, while 30 μ g of poly IC with D-GalN caused the death of three-quarters of

the treated mice. No death was induced in the mice treated with 10 μ g of poly IC with D-GalN, corresponding with a sublethal dose. Therefore, 10 μ g of poly IC with D-GalN (poly IC/D-GalN) was chosen for the following study to simulate a severe load of viral dsRNA on the immune system.

BALB/c mice were treated with saline, poly IC alone, D-GalN alone, or poly IC/D-GalN 1 hour before each sensitization with OVA (Figure 1B). The eosinophilia in BALF after OVA inhalation challenge was more significantly augmented in poly IC-treated mice and poly IC/D-GalN-treated mice than in saline-treated mice and D-GalN-treated mice, respectively (Figure 1C). There was no significant difference in the magnitude of eosinophilia between poly IC/D-GalN-treated mice and poly IC-treated mice. AHR after OVA challenge was more significantly augmented in poly IC-treated mice than in saline-treated mice, but no augmentation was induced in poly IC/D-GalN-treated mice relative to D-GalN-treated mice (Figure 1D). On the other hand, the number of CD4⁺ T cells in OVA-challenged lungs was more significantly augmented in poly IC/D-GalN-treated mice than in D-GalN-treated mice but not in poly IC-treated mice relative to saline-treated mice (Figure 1E). These results suggest that the asthma phenotype induced by severe

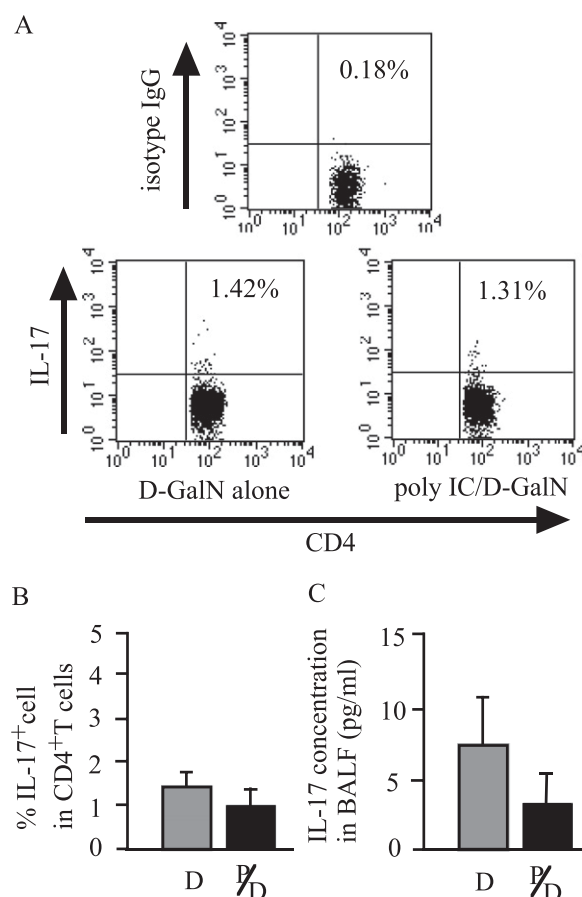


Figure 6. Effect of polyinosinic polycytidylic acid (poly IC)/D-galactosamine (D-GalN) on IL-17 production. (A) Representative dot plots showing IL-17⁺CD4⁺ T cells in the lungs of OVA-challenged BALB/c mice. CD4⁺ T-cell population was assessed for IL-17 expression by percentage. The percentage of (B) IL-17⁺CD4⁺ T cells and (C) IL-17 in BALF were compared between poly IC/D-GalN-treated (solid bar) and D-GalN-treated (shaded bar) mice. Each group consisted of five mice. Data are expressed as the means $\pm$ SEM. * $P < 0.05$ between the indicated two groups. BALF = bronchoalveolar lavage fluid; D = D-GalN; P = poly IC.

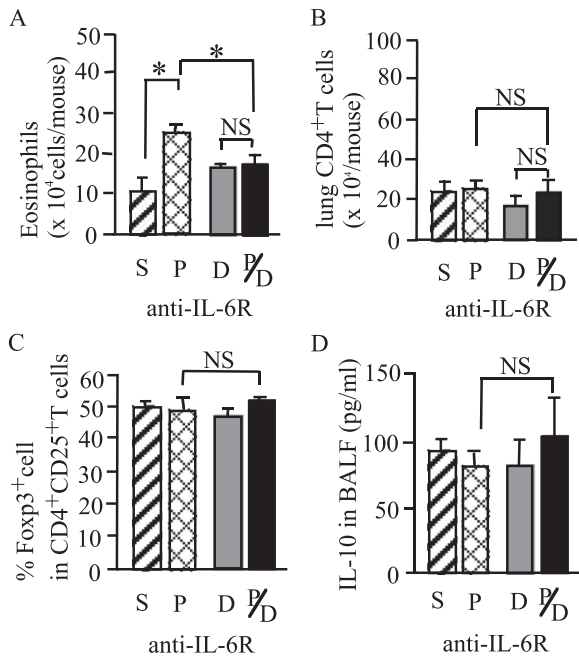


Figure 7. Effect of blocking IL-6/IL-6 receptor (IL-6R) signaling on the asthma phenotypes induced by polyinosinic polycytidylic acid (poly IC)/D-galactosamine (D-GalN) or poly IC. BALB/c mice were pretreated with anti-IL-6R monoclonal antibody, then treated with saline (hatched bar), poly IC (cross-hatched bar), D-GalN (shaded bar), or poly IC/D-GalN (solid bar) before each OVA sensitization. Following OVA challenge, (A) the eosinophilia in BALF, (B) the accumulation of CD4⁺ T cells in the lungs, (C) the percentage of Foxp3⁺ cells, and (D) IL-10 in BALF were measured. Each group consisted of seven to eight mice. Data are expressed as the means $\pm$ SEM. * P < 0.05 between the indicated two groups. D = D-GalN; P = poly IC; S = saline.

dsRNA load (poly IC/D-GalN) is partly different from that induced by mild dsRNA load (poly IC alone). Thus, the former shows reduced AHR and enhanced accumulation of CD4⁺ T cells in the lungs despite that both have comparable airway eosinophilia.

Effect of Poly IC/D-GalN Is Due to Activation of Immune Systems

To elucidate whether the effects of poly IC/D-GalN were due to the triggering of innate immune systems via binding of dsRNA to PRRs and subsequent activation of their downstream molecules, the outcomes of poly IC/D-GalN were compared among TRIF^{-/-} mice, IPS-1^{-/-} mice, and wild-type C57BL/6 mice (Figure 2A). In wild-type mice and IPS-1^{-/-} mice, poly IC/D-GalN treatment significantly augmented the eosinophilia in BALF but not in TRIF^{-/-} mice. Thus, the TRIF-mediated activation of innate immune systems is essential for the augmentation. Typical activation of innate immune systems occurs within a few hours and resolves within a few days (27). The augmentation of eosinophilia was observed in BALB/c mice that had been treated with poly IC/D-GalN on the same day at each sensitization but not 3 days before or after it (Figure 2B), consistent with the kinetics of innate immune responses.

An adaptive immune system is characterized by a phenomenon in which repeated antigenic stimuli amplify the magnitude of effector phase responses, which is derived from immune memory (28). If dsRNA affects the process of adaptive immune responses, its effect should be associated with the above phenomenon. Indeed, the augmentation was shown in mice that had been treated

with poly IC/D-GalN at both the first and second sensitization but not at one or the other alone (Figure 2C). These results suggest that the poly IC/D-GalN-triggered activation of innate immune systems affects the ongoing adaptive immune responses and results in the augmentation.

Effect of Poly IC/D-GalN Is Due to Decreased Production of IL-10

The concentrations of IL-10 and IFN- γ in the BALF of poly IC/D-GalN-treated mice were significantly lower than those in D-GalN-treated mice, while the concentrations of IL-4, IL-5, and IL-13 were comparable (Figure 3A). IL-10 and IFN- γ are known as cytokines inhibitory for asthma development (29–32). Under the treatment with anti-IL-10R mAb during the OVA challenge, the eosinophilia in BALF and the AHR in poly IC/D-GalN-treated mice were not different from those in D-GalN-treated mice (Figure 3B). When the experiments were conducted using IFN- γ ^{-/-} mice on a BALB/c background, the eosinophilia in BALF but not AHR was significantly augmented in poly IC/D-GalN-treated mice (Figure 3C). These results indicate that impaired production of IL-10 but not IFN- γ is responsible for the augmentation of the airway eosinophilia in poly IC/D-GalN-treated mice. We have reported that the administration of poly IC alone augments airway eosinophilia via the enhanced production of IL-13 but not IL-10 (17). The concentrations of IL-10 and IL-13 in the BALF of poly IC/D-GalN-treated mice were significantly lower than those in poly-IC-treated mice (Figure 3D), suggesting that the cytokines responsible for the augmentation of airway eosinophilia may be different between poly IC/D-GalN treatment and poly IC treatment alone.

Effect of Poly IC/D-GalN Is Associated with Decreased Frequency of Lung Foxp3⁺ T Cells

IL-10 is abundantly produced from subsets of regulatory T cells (Tregs) (21, 33, 34). Given that both naturally occurring and inducible Foxp3⁺CD4⁺CD25⁺ T cells are representative Tregs, the frequency of Foxp3⁺ cells in total CD4⁺CD25⁺ T cells (percentage of Foxp3⁺ cells) in the OVA-challenged lungs was assessed by flow cytometry (Figure 4A). The percentage of Foxp3⁺ cells was significantly lower in poly IC/D-GalN-treated mice than in D-GalN-treated mice, whereas there was no significant difference between poly IC-treated mice and saline-treated mice (Figure 4B). In addition, the percentage of Foxp3⁺ cells was significantly lower in poly IC/D-GalN-treated mice than poly IC-treated mice. A lower percentage of Foxp3⁺ cells also presented in the mice treated with anti-IL-10R mAb during OVA challenge (data not shown). These results suggest that the activation of an innate immune system by poly IC/D-GalN suppresses the development of a subset of Foxp3⁺ Tregs and the subsequent production of IL-10 (Foxp3⁺ Treg/IL-10 axis).

Prominent Production of IL-6 by Poly IC/D-GalN

Although the treatment with poly IC/D-GalN augmented the asthma phenotype, the associated cytokine and T-cell subset were different from those with poly IC alone. To address this difference, the concentrations of IFN- β , IL-12p70, and IL-6 in the sera were measured after a single administration of saline, poly IC, D-GalN, or poly IC/D-GalN. IFN- β in poly IC-treated mice and poly IC/D-GalN-treated mice was significantly higher than that in saline-treated mice and D-GalN-treated mice, respectively (Figure 5A). However, the concentrations were not different between poly IC-treated and poly IC/D-GalN-treated mice. IL-12p70 in poly IC/D-GalN-treated mice

was significantly higher than that in D-GalN-treated mice, whereas the concentration was not different between poly IC-treated and poly IC/D-GalN-treated mice (Figure 5B). IL-6 in poly IC-treated mice and poly IC/D-GalN-treated mice was significantly higher than that in saline-treated mice and D-GalN-treated mice, respectively (Figure 5C). Notably, the concentration in poly IC/D-GalN-treated mice was prominently higher than that in poly IC-treated mice.

Lung Th17 Is Not Affected by Poly IC/D-GalN

IL-6 is known as a crucial cytokine for the development of Th17 (35, 36). Thus, the frequency of Th17 in total CD4⁺ T cells in the OVA-challenged lungs was assessed by flow cytometry (Figure 6A). There was no difference in the frequency of Th17 cells in total lung CD4⁺ T cells between D-GalN-treated mice and poly IC/D-GalN-treated mice (Figure 6B). Consistently, there was no difference in the concentrations of IL-17 in BALF after OVA challenge between the two groups (Figure 6C). Hence, the treatment with poly IC/D-GalN may not have a significant effect on the Th17 in this model.

IL-6 Is Responsible for Augmentation of Allergic Airway Inflammation

Finally, the effect of blocking IL-6/IL-6R signaling was examined. The treatment with anti-IL-6R mAb before administration of poly IC alone did not affect the augmentation of airway eosinophilia (Figure 7A), the number of lung CD4⁺ T cells (Figure 7B), the percentage of Foxp3⁺ cells (Figure 7C), or the concentration of IL-10 in BALF (Figure 7D). Anti-IL-6R mAb also did not affect poly IC-induced augmentation of IL-13 production in BALF, as presented in Figure E1 in the online supplement. In contrast, the treatment with anti-IL-6R mAb before each administration of poly IC/D-GalN completely prevented the augmentations of airway eosinophilia and allergen-induced accumulation of CD4⁺ T cells to the lungs. Consistently, anti-IL-6R mAb completely prevented the decreases in the percentage of Foxp3⁺ cells and the concentration of IL-10 in BALF. Control rat IgG treatment had no effect on the asthma phenotypes described above (data not shown). These results indicate that the prominent production of IL-6 by poly IC/D-GalN induces the augmentation of airway inflammation presumably via suppression of Foxp3⁺ Treg/IL-10 axis.

DISCUSSION

It is reasonable to speculate that severe infection may have more harmful effects on asthma development than mild infection. Although poly IC/D-GalN treatment induced more enhanced accumulation of CD4⁺ T cells in the lungs, the augmentation of airway eosinophilia was comparable, and AHR was less than those induced by poly IC alone. These results suggest that severe viral load during allergen sensitization might not be more harmful but substantially affect the development of asthma phenotypes via mechanisms different from those in mild viral load.

Tregs are distinguished from conventional effector CD4⁺ T cells by their capacity to limit T-cell proliferation and effector function. The best characterized are naturally occurring and inducible Foxp3⁺ Tregs (33–35). In the present study, both the frequency of Foxp3⁺ Tregs and the IL-10 content were decreased in the severe infection model by poly IC/D-GalN treatment, which was consistent with our previous study, in which the frequency of Foxp3⁺ Tregs was

correlated with IL-10 in the allergen-induced asthma model of BALB/c mice (22). In addition, the augmentation of airway eosinophilia was deficient in anti-IL-10R mAb-treated mice. These results provide evidence that the suppression of Foxp3⁺ Treg/IL-10 axis is responsible for the poly IC/D-GalN-induced augmentation.

The suppression of Foxp3⁺ Treg/IL-10 axis prompted us to elucidate the underlying mechanism. The production of IL-6 was prominently enhanced in the severe model by the poly IC/D-GalN treatment. IL-6 is a crucial cytokine regulating the differentiation of Foxp3⁺ Tregs and Th17 (34–36). TGF- β -guided differentiation of Foxp3⁺ Tregs is suppressed by IL-6. Instead, IL-6 and TGF- β together induce the differentiation of pathogenic Th17 cells. A previous study showed that treatment with poly IC in combination with thymic stromal lymphopoietin promotes differentiation of Th17 under Th2-polarizing conditions *in vitro* (37). However, poly IC/D-GalN did not increase IL-17 in BALF or Th17 in the lungs. Notably, poly IC/D-GalN induced the production of IFN- β . Type 1 IFNs reportedly suppress IL-17 expression and Th17 differentiation *in vitro* and *in vivo* (38). IL-6 may inhibit the induction of Foxp3⁺ Tregs without affecting Th17. In the absence of poly IC/D-GalN treatment, anti-IL-6R mAb did not affect airway inflammation or Foxp3⁺ Treg/IL-10 axis (unpublished observation). This result is consistent with that of a previous study, in which intraperitoneal anti-IL-6R mAb before sensitization did not increase Tregs in OVA-challenged mice (23). The effect of IL-6 on Foxp3⁺ Tregs induction in the allergen sensitization phase may be confined to a poly IC/D-GalN-dependent process. On the other hand, the previous study reported that intranasal anti-IL-6R mAb just before OVA challenge enhanced the expression of Foxp3 mRNA and the production of IL-10 in the lungs and inhibited airway eosinophilia. Hence, endogenous IL-6 in the allergen challenge phase may suppress Foxp3⁺ Treg/IL-10 axis in a different mode of action.

Poly IC/D-GalN did not affect AHR. We consider that the finding is associated with the decrease of endogenous IL-10 signaling. Thus, IL-10-deficient mice sensitized and challenged with OVA developed robust airway eosinophilia but not AHR (30). Another study showed an increase in AHR despite a decrease in airway eosinophilia when allergen-sensitized and -challenged mice were administered recombinant IL-10 (31). Moreover, Tregs reportedly attenuated airway eosinophilia but not AHR (39). These reports well explain no augmentation of AHR in this model. Another possibility may be associated with the no augmentation of IL-13 production in poly IC/D-GalN treatment. IL-13 is known as a potent cytokine inducing AHR in mice (24). Indeed, the concentration of IL-13 in BALF was lower in poly IC/D-GalN-treated mice than in poly IC-treated mice, consistent with the lower AHR in the former than the latter. Notably, a recent study has shown that infection of naive mice with influenza virus causes AHR by activating “natural helper cells,” a recently identified innate lymphoid cell population, and the IL-33/IL-13 axis (40, 41). This AHR develops independently of Th2 cells and adaptive immunity. Accumulating knowledge about the complex interactions between the virus and the host immune systems may build up the integrated understanding of virus-associated problems in asthma pathophysiology.

The lethal effect of PRR agonists in combination with D-GalN is partly due to the outcome of acute hepatic injury (8, 18–20). D-GalN induces depletion of hepatic uridine triphosphate, leading to hepatocyte apoptosis. Although the doses of D-GalN and poly IC in this study were far less than those in previous studies, our approach using poly IC/D-GalN might not fully mimic the effect of a severe viral load on the development of asthma.

The prominent action of IL-6 proposes a mechanism for the virus-associated pathophysiology of asthma. A humanized anti-IL-6R mAb (tocilizumab) has been applied for the treatment of inflammatory diseases such as rheumatoid arthritis, Castleman disease, and systemic juvenile idiopathic arthritis (42–44). Our model may provide a novel strategy targeting IL-6 for preventing asthma development related to severe virus infection.

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References

- Global Initiative for Asthma. Global strategy for asthma management and prevention. NHLBI/WHO Workshop report. Bethesda, MD: National Heart, Lung, and Blood Institute; 2006.
- Kusel MM, de Klerk NH, Kebadze T, Vohma V, Holt PG, Johnston SL, Sly PD. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. *J Allergy Clin Immunol* 2007;119:1105–1110.
- Paunio M, Heinonen OP, Virtanen M, Leinikki P, Patja A, Peltola H. Measles history and atopic diseases. *JAMA* 2000;283:343–346.
- Dahl ME, Dabbagh K, Liggett D, Kim S, Lewis DB. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat Immunol* 2004;3:337–343.
- McIntire JJ, Umetsu SE, Macaubas C, Hoyte EG, Cinnoglu C, Cavalli-Sforza LL, Barsh GS, Hallmayer JF, Underhill PA, Risch NJ, et al. Immunology: hepatitis A virus link to atopic disease. *Nature* 2003;425:576.
- Ramsey CD, Celedon JC. The hygiene hypothesis and asthma. *Curr Opin Pulm Med* 2005;11:14–20.
- Chang YJ, Kim HY, Albacker LA, Lee HH, Baumgarth N, Akira S, Savage PB, Endo S, Yamamura T, Maaskant J, et al. Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity. *J Clin Invest* 2011;121:57–69.
- Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001;413:732–738.
- Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 2004;5:730–737.
- Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006;441:101–105.
- Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003;301:640–643.
- Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 2003;4:161–167.
- Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 2005;6:981–988.
- Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 2005;122:669–682.
- Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* 2005;19:727–740.
- Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005;437:1167–1172.
- Matsumoto K, Kan-O K, Eguchi-Tsuda M, Fukuyama S, Asai Y, Matsumoto T, Moriaki A, Matsunaga Y, Tsutsui H, Kawai T, et al. Essential role of B7-H1 in double-stranded RNA-induced augmentation of an asthma phenotype in mice. *Am J Respir Cell Mol Biol* 2011;45:31–39.
- Galanos C, Freudenberg MA, Reutter W. Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc Natl Acad Sci USA* 1979;76:5939–5943.
- Sparwasser T, Miethke T, Lipford G, Borschert K, Hacker H, Heeg K, Wagner H. Bacteria DNA causes septic shock. *Nature* 1997;386:336–337.
- Dejager L, Libert C. Tumor necrosis factor alpha mediates the lethal hepatotoxic effects of poly (I:C) in D-galactosamine-sensitized mice. *Cytokine* 2008;42:55–61.
- Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005;202:1539–1547.
- Matsumoto K, Inoue H, Fukuyama S, Kan-o K, Eguchi-Tsuda M, Matsumoto T, Moriaki A, Nakano T, Nakanishi Y. Frequency of Foxp3⁺CD4⁺CD25⁺ T cells is associated with the phenotypes of allergic asthma. *Respirology* 2009;14:187–194.
- Doganci A, Eigenbrod T, Krug N, De Sanctis GT, Hausding M, Erpenbeck VJ, Haddad E, Schmitt E, Bopp T, Kallen K-J, et al. The IL-6R α chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *J Clin Invest* 2005;115:313–325.
- Kibe A, Inoue H, Fukuyama S, Machida K, Matsumoto K, Koto H, Ikegami T, Aizawa H, Hara N. Differential regulation by glucocorticoid of interleukin-13-induced eosinophilia, hyperresponsiveness, and goblet cell hyperplasia in mouse airways. *Am J Respir Crit Care Med* 2003;167:50–56.
- Matsumoto K, Inoue H, Nakano T, Tsuda M, Yoshiura Y, Fukuyama S, Tushima F, Hoshino T, Aizawa H, Akiba H, et al. B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism. *J Immunol* 2004;172:2530–2541.
- Tanaka K, Ichihama K, Hashimoto M, Yoshida H, Takimoto T, Takaesu G, Torisu T, Hanada T, Yasukawa H, Fukuyama S, et al. Loss of suppressor of cytokine signaling 1 in helper T cells leads to defective Th17 differentiation by enhancing antagonistic effects of IFN- γ on STAT3 and Smads. *J Immunol* 2008;180:3746–3756.
- Traynor TR, Majde JA, Bohnet SG, Krueger JM. Intratracheal double-stranded RNA plus interferon-gamma: a model for analysis of the acute phase response to respiratory viral infections. *Life Sci* 2004;74:2563–2576.
- Wille-Reece U, Flynn BJ, Loré K, Koup RA, Miles AP, Saul A, Kedl RMA, Mattapallil JJ, Weiss WR, Roederer M, et al. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *J Exp Med* 2006;203:1249–1258.
- Kapsenberg ML, Jansen HM, Bos JD, Wierenga EA. Role of type 1 and type 2 T helper cells in allergic diseases. *Curr Opin Immunol* 1992;4:788–793.
- Makela MJ, Kanehiro A, Borish L, Dakhama A, Loader J, Joetham A, Xing Z, Jordana M, Larsen GL, Gelfand EW. Interleukin-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization. *Proc Natl Acad Sci USA* 2000;97:6007–6012.
- van Scott MR, Justice JP, Bradfield JF, Enright E, Sigounas A, Sur S. IL-10 reduces Th2 cytokine production and eosinophilia but augments airway reactivity in allergic mice. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L667–L674.
- Yoshida M, Leigh R, Matsumoto K, Wattie J, Ellis R, O'Byrne PM, Inman MD. Effect of interferon- γ on allergic airway responses in interferon- γ -deficient mice. *Am J Respir Crit Care Med* 2002;166:451–456.
- McGee HS, Agrawal DK. Naturally occurring and inducible T-regulatory cells modulating immune response in allergic asthma. *Am J Respir Crit Care Med* 2009;180:211–225.
- Ray A, Khare A, Krishnamoorthy N, Qi Z, Ray P. Regulatory T cells in many flavors control asthma. *Mucosal Immunol* 2010;3:216–229.

35. Romagnani S. Regulation of the T cell response. *Clin Exp Allergy* 2006; 36:1357–1366.
36. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T cells. *Nature* 2006;441:235–238.
37. Tanaka J, Watanabe N, Kido M, Saga K, Akamatsu T, Nishio A, Chiba T. Human TSLP and TLR3 ligands promote differentiation of Th17 cells with a central memory phenotype under Th2-polarizing conditions. *Clin Exp Allergy* 2009;39:89–100.
38. Moschen AR, Geiger S, Krehan I, Kaser A, Tilg H. Interferon-alpha controls IL-17 expression in vitro and in vivo. *Immunobiology* 2008; 213:779–787.
39. Hadeiba H, Locksley RM. Lung CD25 CD4 regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. *J Immunol* 2003;170:5502–5510.
40. Chang Y-J, Kim HY, Albacker LA, Baumgarth N, McKenzie ANJ, Smith DE, DeKruyff RH, Umetsu DT. Innate lymphoid cells mediated influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol* 2011;12:631–638.
41. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, Furusawa J, Ohtani M, Fujie H, Koyasu S. Innate production of T(H) 2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 2010;463:540–544.
42. Frosch M, Roth J. New insights in systemic juvenile idiopathic arthritis—from pathophysiology to treatment. *Rheumatology* 2008; 47:121–125.
43. Ding C, Cicuttini F, Li J, Jones G. Targeting IL-6 in the treatment of inflammatory and autoimmune diseases. *Expert Opin Investig Drugs* 2009;18:1457–1466.
44. Nishimoto N, Terao K, Mima T, Nakahara H, Takagi N, Kakehi T. Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. *Blood* 2008;112:3959–3964.

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