

Airway Exposure Levels of Lipopolysaccharide Determine Type 1 versus Type 2 Experimental Asthma¹

Yoon-Keun Kim,^{2*} Sun-Young Oh,[†] Seong Gyu Jeon,^{*} Heung-Woo Park,[‡] Soo-Yeon Lee,[‡] Eun-Young Chun,[‡] Boram Bang,[‡] Hyun-Seung Lee,[‡] Min-Hee Oh,^{*} You-Sun Kim,^{*} Jong-Hoon Kim,[§] Yong Song Gho,^{*} Sang-Heon Cho,[‡] Kyung-Up Min,[‡] You-Young Kim,[‡] and Zhou Zhu^{2†}

Allergic asthma is characterized by airway inflammation initiated by adaptive immune responses to aeroallergens. Recent data suggest that severe asthma may be a different form of asthma rather than an increase in asthma symptoms and that innate immune responses to LPS can modulate adaptive immune responses to allergens. In this study, we evaluated the hypothesis that airway exposure to different doses of LPS induces different form of asthma. Our study showed that neutrophilic inflammation and IFN- γ expression were higher in induced sputum from severe asthma patients than from mild to moderate asthmatics. Animal experiments indicated that allergen sensitization with low-dose LPS (0.1 μ g) induced type 2 asthma phenotypes, i.e., airway hyperresponsiveness, eosinophilic inflammation, and allergen-specific IgE up-regulation. In contrast, allergen sensitization with high-dose LPS (10 μ g) induced asthma phenotypes, i.e., airway hyperresponsiveness and noneosinophilic inflammation that were not developed in IFN- γ -deficient mice, but unaffected in the absence of IL-4. During the allergen sensitization period, TNF- α expression was found to be enhanced by both low- and high-dose LPS, whereas IL-12 expression was only enhanced by high-dose LPS. Interestingly, the asthma phenotypes induced by low-dose LPS, but not by high-dose LPS, were completely inhibited in TNF- α receptor-deficient mice, whereas the asthma phenotypes induced by high-dose LPS were abolished in the homozygous null mutation of the *STAT4* gene. These findings suggest that airway exposure levels of LPS induces different forms of asthma that are type 1 and type 2 asthma phenotypes by high and low LPS levels, respectively. *The Journal of Immunology*, 2007, 178: 5375–5382.

Asthma is characterized as a chronic inflammatory disorder of the airways associated with reversible airway obstruction and airway hyperresponsiveness (AHR)³ (1, 2). In terms of the immunological pathogenesis of asthma, Th2 responses to aeroallergens are considered to play a central role in the initiation and orchestration of inflammatory responses in asthmatic airways (3, 4). The Th2 hypothesis of asthma pathogenesis

was first suggested by Mosmann in 1989, who had earlier discovered the presence of two distinct subtypes of Th cells in mice: Th1 and Th2 (5, 6). The Th2 hypothesis states that asthma is caused by an increase in Th2 response in combination with a decrease in Th1 response. Although a wealth of animal data supports the Th2 hypothesis, studies in humans, however, have found that both Th1 and Th2 cytokines are elevated in the blood and airways of asthma patients (7, 8).

It is clear that the default immune response to the inhalation of inert proteins (allergens) is nonresponsiveness, i.e., “tolerance” (9, 10). Because inhaled allergens are ubiquitous in nature, it remains unclear why some individuals develop adaptive immune responses to allergens while others do not. In this context, respiratory infections have been associated with asthma in both the preventative and facilitative sense (11, 12), and LPS, a cell wall component of Gram-negative bacteria ubiquitously encountered in the environment and in household dust allergens, including house dust mites, is known to induce the production of IFN- γ and IL-12 (9, 13) and has been suggested to enhance Th1 priming and reduce Th2 cell priming to allergens (14, 15). Interestingly, a recent study indicated that low (0.1 μ g) and high (100 μ g) levels of inhaled LPS in a mouse model of allergic sensitization induces Th2 and Th1 responses to allergens, respectively (16), suggesting that airway allergen sensitization by LPS induces asthma that is differentially regulated according to LPS levels present at the time of exposure to aeroallergens. However, the exact mechanisms by which LPS induces polarized Th1 vs Th2 responses to allergens are not completely understood.

In this study, we evaluated the hypothesis that allergen sensitization with different doses of LPS induces differential adaptive

*Department of Life Science, Postech Biotech Center, Pohang University of Science and Technology, Namgu, Pohang, Gyeongbuk, Republic of Korea; [†]Division of Allergy and Clinical Immunology, Asthma and Allergy Center, Johns Hopkins University, Baltimore, MD 21224; [‡]Department of Internal Medicine, Institute of Allergy and Clinical Immunology, Seoul National University College of Medicine, Seoul, Republic of Korea; and [§]Division of Biotechnology, College of Life Science and Biotechnology, Korea University, Seoul, Republic of Korea

Received for publication September 13, 2006. Accepted for publication January 19, 2007.

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¹ This work was supported in part by grants from the Ministry of Health and Welfare, Republic of Korea (03-PJ10-PG13-GD01-0002, A050193, and A050288), and in part by the Postech Research Fund (1RC0660301).

² Address correspondence and reprint requests to Dr. Zhou Zhu, Division of Allergy and Clinical Immunology, Asthma and Allergy Center, Johns Hopkins University, 5501 Hopkins Bayview Circle/1A.2, Baltimore, MD 21224; E-mail address: zzhu@jhmi.edu or Dr. Yoon-Keun Kim, Department of Life Science, Postech Biotech Center, Pohang University of Science and Technology, San 31 Hyojadong, Namgu, Pohang, Gyeongbuk 790-784, Republic of Korea; E-mail address: juinea@postech.ac.kr

³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; FEV1, forced expiratory volume at 1 s; PFT, pulmonary function test; DRS, dose-response curve; Penh, enhanced pause; WT, wild type; BAL, bronchoalveolar lavage; IP-10, IFN- γ -inducible protein 10.

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Table I. Characteristics of adult asthma patients according to severity^a

	Asthma Severity		
	Mild	Moderate	Severe
Age	53.6 ± 12.7	55.6 ± 11.3	58.8 ± 10.6
Male/female	15/41	35/64	7/20
Sx ^b duration (year)	3.4 ± 3.8	3.9 ± 8.7	7.9 ± 10.1
FEV1 (% predicted)	98.8 ± 11.9	88.3 ± 15.6	76.4 ± 24.3 ^c
FEV1/FVC	96.7 ± 7.18	94.3 ± 8.23	87.7 ± 15.1 ^c
FVC (% predicted)	101.8 ± 14.2	94.1 ± 14.3	85.3 ± 17.7 ^c

^a Data are expressed as mean ± SEM unless otherwise indicated.

^b Sx, asthma symptoms; FVC, forced vital capacity.

^c Value of $p < 0.05$ vs mild and moderate severity by ANOVA linearity testing.

immune responses to inhaled allergens. To test this hypothesis, mouse models of asthma were prepared by airway allergen sensitization using different LPS doses. We then evaluated the roles of Th1 and Th2 pathways in the development of adaptive immune responses to inhaled allergens in these mouse asthma models and determined the expressions of Th1 and Th2 cytokines in the sputum samples of asthma patients.

Materials and Methods

Patients

One hundred eighty-two adult asthma patients (Table I) with reversible airway obstruction (percent increase of forced expiratory volume at 1 s (FEV1) >12% and an absolute FEV1 increase of >400 ml) after the use of asthma medications and without any remarkable lung abnormalities were enrolled in this study. Patients provided sputum samples at their initial visits and underwent spirometry and methacholine bronchial challenge tests under well-controlled conditions, as previously described (17). Asthma severity was classified as mild, moderate, or severe after at least 3 mo of regular antiasthma medications, according to the Global Initiative for Asthma (GINA) guidelines concerning symptom and medication scores (18). All study protocols were approved by the ethics committee of Seoul National University Hospital, Korea.

Mice

IL-4-, IFN- γ -, TNFR1-deficient, and wild-type (WT) control mice on a C57BL/6 background, and STAT4-deficient and WT control mice on a BALB/c background were purchased from The Jackson Laboratory. The generation and breeding of null mutant and WT control mice were performed in specific pathogen-free condition as previously described (19). Animal study protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University, Korea.

A protocol for mouse models of asthma

LPS-depleted OVA was prepared as previously described (16) (Fig. 1). To generate a mouse model of LPS-enhanced asthma, 6-wk-old mice were sensitized intranasally four times with 75 μ g of OVA on days 0, 1, 2, and 7 with or without different doses of LPS (Sigma-Aldrich), and then challenged four times intranasally with 50 μ g of OVA on days 14, 15, 21, and 22. The innate immune effects of LPS on the allergen sensitization were evaluated after allergen sensitization on days 0, 1, and 2. To evaluate asthma phenotypes, methacholine AHR (pulmonary function test (PFT)) and lung inflammation were evaluated on days 23 and 24, respectively.

Measurement of methacholine AHR

Methacholine AHR in asthma patients was expressed as slope of dose-response curve (DRS) of methacholine challenge which was defined by the percentage of fall of FEV1 divided by log (total dose of applied methacholine, milligrams per milliliter).

Pulmonary function testing in mice was assessed using conscious, unrestrained mice by noninvasive whole body plethysmography (Allmedicus). Measurements were performed as previously described (20). Briefly, mice were placed in a plethysmograph chamber and exposed to an aerosol of PBS (basal readings) and then to methacholine at 6.25, 12.5, 25, and 50 mg/ml. Aerosols were generated using an ultrasonic nebulizer and drawn through the chamber for 3 min. Enhanced pause (Penh) readings were taken for 3 min and averaged. We previously confirmed a direct

correlation between Penh and airway resistance in response to methacholine challenge (20).

Evaluations of lung inflammation

Lung and bronchoalveolar lavage (BAL) samples were obtained and histological evaluations were performed as previously described (20). Briefly, mice were anesthetized and their trachea were isolated by blunt dissection. A small caliber tube was inserted and secured in the airway. Two successive volumes of 0.75 ml of PBS were instilled and gently aspirated and these two volumes were pooled. Each BAL sample was centrifuged and the supernatants were stored at -70°C until use. The total numbers of inflammatory cells were counted after dilution of cell pellets with 50 μ l of PBS. After Diff-Quick staining (Dade Behring) of BAL pellets in a cytospin preparation, types of inflammatory cells were determined by counting 300 cells, which were classified as macrophages, lymphocytes, neutrophils, or eosinophils. The lung was perfused with cold PBS through the right ventricle until the pulmonary vasculature was clean. The whole lung was inflated with fixatives for histology. H&E stains were used on lung sections after pressure fixation with Streck solution (Streck Laboratories). The same microscopic magnification was used for all sample slides for comparison.

Evaluations of immunologic parameters

mRNA was quantified in the induced sputum samples of asthma patients and BAL protein and OVA-specific IgG2a and IgE levels in BAL and serum samples obtained during animal experiments were determined as previously described (19). For FACS analysis, single-cell suspensions from lungs were prepared as previously described (19). Briefly, cells isolated from lung tissues and BAL fluids were washed three times with PBS containing 1% BSA, counted, and distributed into staining tubes (1×10^6 cells/tube) for cell surface marker analysis. They were then stained with FITC-conjugated anti-CD11c, anti-CD11b, anti-CD3, and anti-CD8 (all conjugated Abs were purchased from BD Biosciences). Intracellular cytokine staining was performed using Abs against PE-conjugated anti-IL-4, anti-IL-10, anti-IL-12, and anti-IFN- γ (BD Biosciences). Cells were analyzed using a FACSCalibur unit (BD Biosciences), and results were processed using CellQuest software (BD Biosciences).

Statistical analysis

Significant differences among groups were assessed using the Student t test, ANOVA, or a Wilcoxon rank sum test. For multiple comparisons, ANOVA was initially used and if significant differences were found individual two-tailed unpaired t or Wilcoxon rank sum tests between pairs of groups were used.

Results

Association between sputum neutrophils and disease severity in asthma patients and enhanced IFN- γ mRNA expression in these patients

A total of 182 adult asthmatic patients were enrolled in this study as shown in Table I. We defined asthma severity based on symptom and

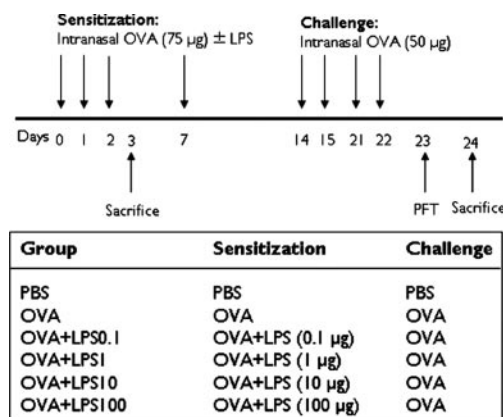


FIGURE 1. Asthma mouse model protocol. Mice were sensitized with an intranasal application of LPS-depleted OVA and different doses of LPS (0.1, 1, 10, and 100 μ g), and then challenged intranasally with OVA alone. The sensitization effects of LPS were assessed 3 days after airway allergen sensitization. AHR (PFT) and lung inflammation were assessed 24 and 48 h after the final allergen challenge, respectively.

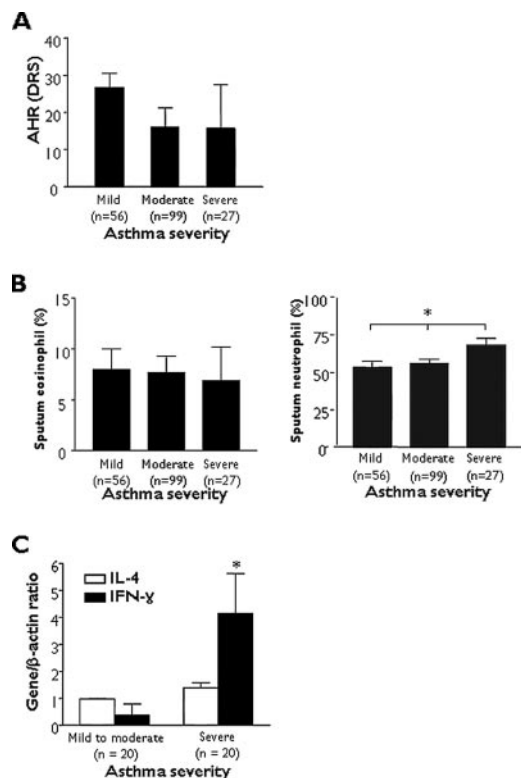


FIGURE 2. AHR and lung inflammation according to asthma severity in patients. *A*, AHR values, expressed as slopes of DRS to methacholine in which lower values mean higher AHR, were similar for patients with mild, moderate, and severe asthma. Statistical analysis was performed by ANOVA linearity testing. *B*, Eosinophil and neutrophil numbers in induced sputum: sputum neutrophil numbers were found to be positively associated with asthma severity, whereas eosinophil numbers were not. $^*p < 0.05$ by ANOVA linearity testing. *C*, Sputum IL-4 and IFN- γ mRNA expressions: IFN- γ was markedly more enhanced in severe asthmatics than in mild-to-moderate asthmatics, although IL-4 mRNA expressions were similar in the two groups. $^*p < 0.05$ vs mild-to-moderate asthmatics by Student's *t* test.

medication scores over a period of at least 3 mo during which anti-asthma medications were used and found that a reduced baseline lung function was positively associated with disease severity (Table I). However, methacholine AHR was not found to be associated with asthma severity, although showed a tendency to be increased in mild asthmatics compared with other groups (Fig. 2*A*). Interestingly, we found that the neutrophil percentage in all sputum inflammatory cells was positively associated with asthma severity, but that sputum eosinophil percentage was not (Fig. 2*B*). Moreover, our data indicate that sputum IFN- γ mRNA expression was significantly higher in severe asthma patients than in mild-to-moderate patients, although IL-4 mRNA expressions were similar in these groups (Fig. 2*C*).

Airway allergen sensitization with LPS induces enhanced AHR and lung inflammation

To test the notion that innate immune responses induced by LPS modulate adaptive immune responses to inert inhaled proteins, C57BL/6 WT mice were sensitized intranasally with LPS-depleted OVA in combination with 0.1, 1, 10, or 100 μ g of LPS at days 0, 1, 2, and 7, and then challenged intranasally with OVA alone 7 days after the final allergen sensitization as shown in Fig. 1. Methacholine AHR following allergen (OVA) challenge was more enhanced in mice sensitized with OVA plus LPS, independent of LPS dose, than in those sensitized with OVA or PBS alone (Fig. 3*A*). In terms of lung inflammation (Fig. 3*B*), we also found that

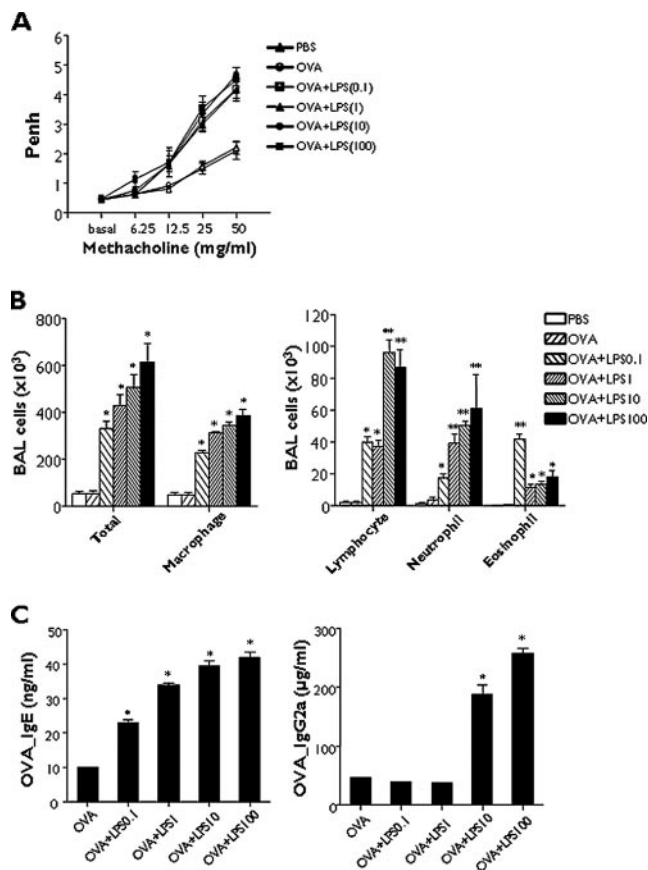


FIGURE 3. AHR and lung inflammation induced by allergen sensitization with LPS. *A–C*, One experiment representative of five identical runs is shown. *A*, AHR after allergen challenge: PFT was measured by unstrained noninvasive whole body plethysmography, and airway obstructions are expressed as Penh. Allergen sensitization by LPS, regardless of LPS dose, enhanced AHR induced by allergen challenge. *B*, BAL cellularity after allergen challenge: total bar heights represent total and individual inflammatory cell numbers in BAL. LPS enhanced BAL cellularity in a dose-dependent manner. Low (0.1 μ g) and high doses (10 and 100 μ g) of LPS enhanced eosinophil and neutrophil numbers in BAL, respectively. $^*p < 0.05$ vs OVA and PBS groups; $^{**}p < 0.05$ vs the other groups. *C*, Serum OVA-specific IgE and IgG2a levels after subsequent OVA challenge: OVA-specific IgE production was enhanced by LPS, independently of LPS dose, whereas OVA-specific IgG2a was more enhanced by high doses (10 and 100 μ g) LPS. $^*p < 0.05$ vs OVA group.

total inflammatory cell counts in BAL fluids after allergen challenge were enhanced in mice sensitized with OVA plus LPS in a dose-dependent manner. However, mice sensitized with OVA alone showed no enhanced BAL cellularity after OVA challenge and had total BAL cell counts equivalent to PBS-sensitized mice. BAL neutrophil counts were greater in mice sensitized with OVA plus high-dose (10 or 100 μ g) LPS than in mice sensitized with OVA plus low-dose (0.1 μ g) LPS, whereas BAL eosinophil counts were higher in mice sensitized with OVA plus low-dose LPS than in those sensitized with OVA plus high-dose LPS. With respect to allergen-specific Ab production (Fig. 3*C*), serum OVA-specific IgE levels were higher in mice sensitized with OVA plus LPS (independent of LPS dose) than in those sensitized with OVA alone. In contrast, serum OVA-specific IgG2a levels were significantly higher in mice sensitized with OVA plus high-dose (10 or 100 μ g) LPS than in those sensitized with OVA alone or OVA plus low-dose LPS.

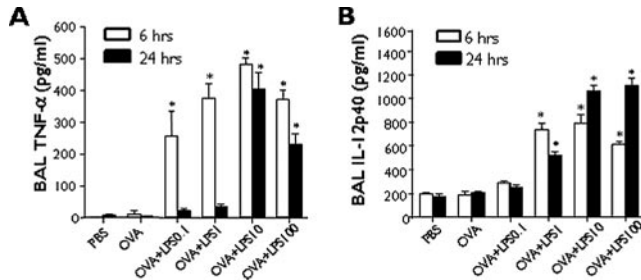


FIGURE 4. Differential expressions of TNF- α and IL-12 by LPS doses during allergen sensitization. *A* and *B*, One experiment representative of five is shown after allergen sensitization. *A*, BAL TNF- α levels after allergen sensitization: TNF- α production was enhanced in LPS-sensitized mice, regardless of dose, and this enhancement was prominent 6 h after allergen sensitization. *, $p < 0.05$ vs the other groups. *B*, BAL IL-12p40 production after allergen sensitization: BAL IL-12p40 levels 6 and 24 h after allergen sensitization with high-dose LPS, but not with low-dose (0.1 μ g), up-regulated IL-12p40 production. *, $p < 0.05$ vs the other groups.

Differential expressions of TNF- α and IL-12 by LPS doses during allergen sensitization period

During the sensitization period, we found that TNF- α levels in BAL fluids were significantly higher in C57BL/6 WT mice sensitized with OVA plus LPS, regardless of LPS dose, than in those sensitized with OVA or PBS alone (Fig. 4A). Meanwhile, BAL TGF- β 1 levels were found to be enhanced in mice sensitized with OVA alone vs PBS alone; however, the enhanced TGF- β 1 production was inhibited by the additions of LPS, independent of LPS dose (data not shown). Interestingly, BAL IL-12p40 levels 6 or 24 h after 3 days of allergen sensitization were found to be significantly higher in mice sensitized with OVA plus high-dose (1, 10, or 100 μ g) LPS than in those sensitized with OVA plus low-dose (0.1 μ g) LPS or OVA alone (Fig. 4B).

TNF- α receptor-mediated pathway plays a key role in the development of Th2 response in the low-dose LPS model

We hypothesized that adaptive immune responses enhanced by LPS are dependent on the TNF- α receptor-mediated pathway. To test this, we applied the above-described mouse asthma models to TNFR1-deficient and same-aged WT control mice (C57BL/6 background). In this experiment, low and high doses of LPS were defined as 0.1 and 10 μ g, respectively, which are levels of contamination consistent with house dust allergens (13). AHR induced by allergen challenge did not develop in TNFR1^{-/-} mice sensitized with OVA plus low-dose LPS, but developed in TNFR1^{-/-} mice sensitized with OVA plus high-dose LPS when compared with WT mice sensitized in the same manner (Fig. 5A). Moreover, BAL cellularity was significantly lower in TNFR1-deficient mice sensitized with OVA plus low-dose LPS, but similar in these mice when sensitized with OVA plus high-dose LPS, when compared with WT mice sensitized in the same manner (Fig. 5B). In terms of the role of the TNF- α receptor-mediated pathway on Ab production, our data show that OVA-specific IgE production enhanced by low-dose LPS in WT mice was not observed in TNFR1-deficient mice, whereas OVA-specific IgE production enhanced by high-dose LPS was similar in TNFR1-deficient and WT mice (Fig. 5C). In addition, IL-4 secretion enhanced by low-dose LPS was not observed in TNFR1-deficient mice, but IL-4 secretion was similar in TNFR1-deficient and WT mice sensitized with allergen plus high-dose LPS (Fig. 5D).

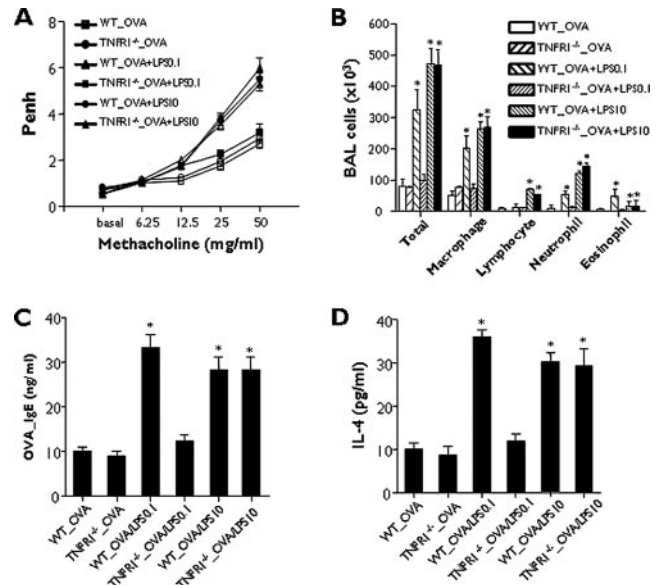


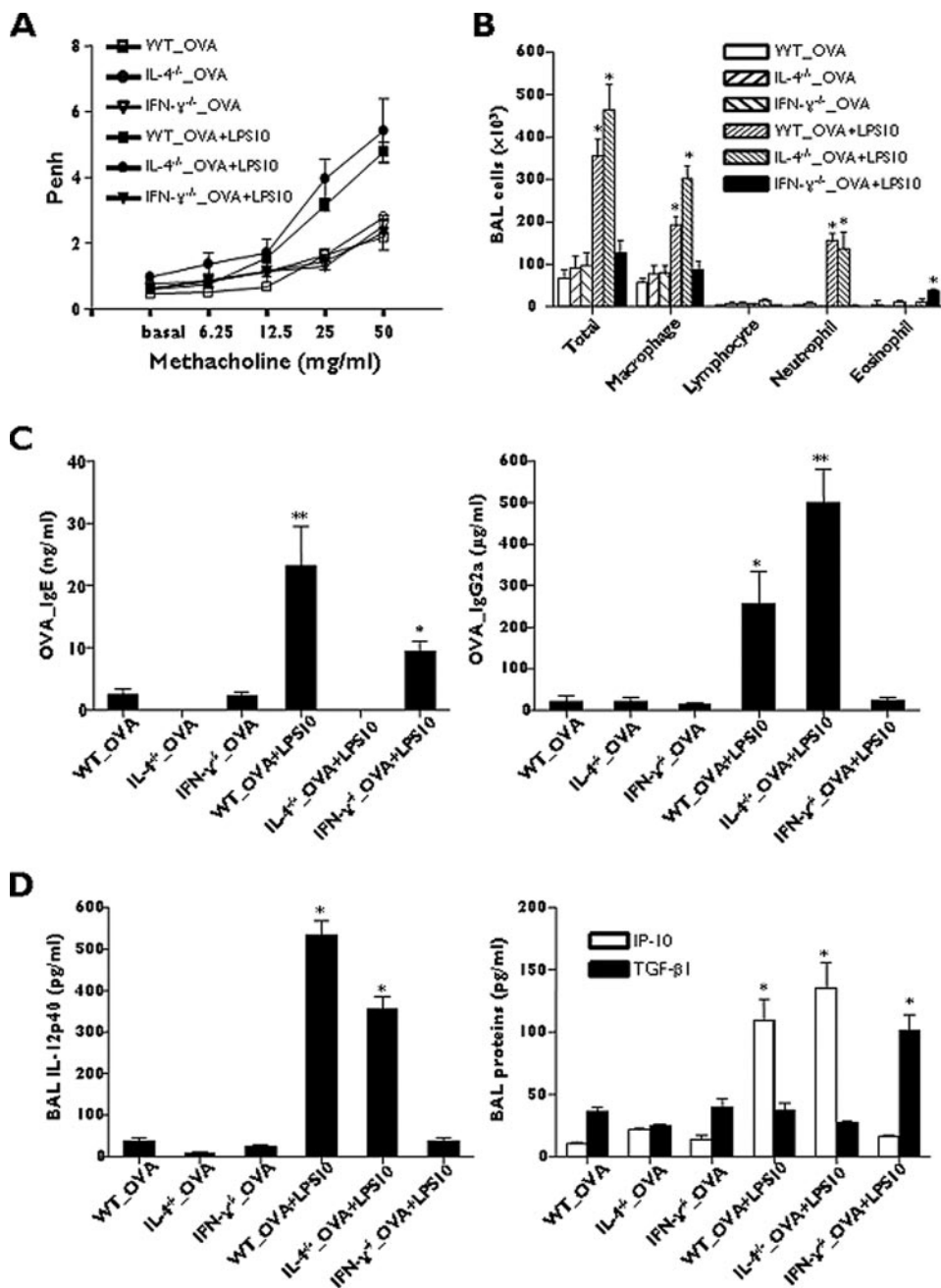
FIGURE 5. AHR, lung inflammation, and immunologic parameters in TNF- α receptor (TNFR1)-deficient mice. *A–D*, One experiment representative of five is shown. *A*, AHR after allergen challenge: AHR enhanced by low-dose LPS did not occur in TNFR1-deficient mice, whereas AHR developed in these mice sensitized with OVA plus high-dose LPS. *B*, Lung inflammation after allergen challenge: lung inflammation induced by low-dose LPS did not develop in TNFR1-deficient mice, but lung inflammation induced by high-dose LPS developed. *, $p < 0.05$ vs OVA groups. *C*, Serum OVA-specific IgE levels after allergen challenge: OVA-specific IgE production induced by low-dose LPS was inhibited in the absence of TNFR1-signaling pathway, whereas IgE production induced by high-dose LPS was not. *, $p < 0.05$ vs the other groups. *D*, BAL IL-4 levels after allergen challenge: IL-4 production enhancement by low-dose LPS was inhibited in the absence of TNFR1 gene. *, $p < 0.05$ vs the other groups.

IFN- γ plays a key role in the development of AHR and noneosinophilic lung inflammation in the high-dose LPS mouse model

Based on our data showing that AHR and lung inflammation enhanced by high-dose LPS were accompanied by enhanced allergen-specific IgE and IgG2a productions after OVA challenge, we evaluated the roles of Th1 and Th2 cytokines in the development of the asthma phenotypes enhanced by high-dose LPS. To test this, the high-dose (10 μ g) LPS-enhanced asthma model was applied to IFN- γ - and IL-4-deficient and same-aged WT control mice (C57BL/6 background). AHR induced by allergen challenge did not develop in IFN- γ ^{-/-} mice, but developed in IL-4^{-/-} mice, sensitized with OVA plus high-dose LPS relative to WT mice sensitized in the same manner (Fig. 6A). In terms of the role of IL-4 and IFN- γ on the development of lung inflammation (Fig. 6B), airway infiltration of macrophages, lymphocytes, and neutrophils after allergen challenge did not develop in IFN- γ ^{-/-} mice sensitized with OVA plus high-dose LPS relative to in WT mice sensitized in the same manner. Eosinophil infiltration, however, was aggravated in IFN- γ ^{-/-} mice sensitized with OVA plus high-dose LPS vs the WT. In contrast, BAL cellularity enhanced by high-dose LPS was not affected in the absence of IL-4.

In terms of the role of IL-4 and IFN- γ on Ab production (Fig. 6C), the levels of serum OVA-specific IgE were significantly lower in both IL-4 and IFN- γ -deficient mice sensitized in OVA plus high-dose LPS than in WT mice sensitized in the same manner. In contrast, OVA-specific IgG2a production was significantly lower in IFN- γ -deficient mice, but aggravated in IL-4-deficient

FIGURE 6. AHR, lung inflammation, and immunologic parameters in IL-4- and IFN- γ -deficient mice. *A–D*, One experiment representative of five is shown. *A*, AHR after allergen challenge: methacholine AHR enhancement by high-dose (10 μ g) LPS was inhibited in the absence of IFN- γ , but not of IL-4. *B*, Lung inflammation after allergen challenge: lung inflammation enhanced by high-dose LPS was inhibited in the absence of IFN- γ , but not of IL-4; however, eosinophilic infiltration was enhanced in IFN- γ -deficient mice. *, $p < 0.05$ vs the other groups. *C*, Serum OVA-specific IgE and IgG2a levels after allergen challenge: OVA-specific IgE production enhanced by high-dose LPS were inhibited in the absence of IFN- γ or IL-4, whereas IgG2a inhibited in the absence of IFN- γ , but aggravated in the absence of IL-4. *, $p < 0.05$ vs OVA groups; **, $p < 0.05$ vs the other groups. *D*, BAL Th1- and Th2-related cytokines levels after allergen challenge: IL-12p40 and IP-10 production enhanced by high-dose LPS were completely inhibited in the absence of IFN- γ , but not of IL-4. In contrast, TGF- β 1 production was enhanced in IFN- γ -deficient mice, but not in IL-4-/- mice, sensitized with OVA plus LPS. *, $p < 0.05$ vs the other groups.



mice, sensitized with OVA plus high-dose LPS when compared with WT mice sensitized in the same manner. Moreover, we found that the enhanced production of Th1-related cytokines, namely IL-12 and IFN- γ -inducible protein 10 (IP-10), after allergen challenge in WT mice did not occur in IFN- γ -deficient mice, but occurred in IL-4-/- mice, sensitized with OVA plus high-dose LPS (Fig. 6D). In contrast, BAL TGF- β 1 levels after allergen challenge were found to be higher in IFN- γ -deficient mice, but not in IL-4-/- mice, sensitized with OVA plus high-dose LPS relative to WT mice sensitized in the same manner (Fig. 6D).

AHR and lung inflammation in the high-dose LPS asthma model are developed mainly via the STAT4-signaling pathway

IL-12 is known to be potent Th1 skewing cytokines that are mainly mediated by the STAT4-signaling pathway (14). Based on our finding that high-dose LPS enhances IL-12 production during allergen sensitization (Fig. 4B), we hypothesized that the STAT4-

signaling pathway might play an essential role in the development of asthma phenotypes enhanced by high-dose LPS. To test this, we applied the high-dose LPS mouse asthma model to STAT4-deficient and same aged WT control BALB/c mice. AHR induced by allergen challenge was not enhanced in STAT4-/- mice sensitized with OVA plus high-dose LPS (10 μ g), but enhanced in WT mice sensitized in the same manner (Fig. 7A). Moreover, lung inflammation enhanced by high-dose LPS was found to be markedly inhibited in STAT4-deficient mice (Fig. 7B). We also found that OVA-specific IgG2a production enhanced by high-dose LPS was significantly inhibited in STAT4-deficient mice (Fig. 7C). BAL cells were examined by intracellular cytokine analysis for the ability to produce IFN- γ and showed that airway infiltration by IFN- γ -producing cells, which were only CD8⁺ cells but not CD8⁻ cells, after allergen challenge was significantly lower in STAT4-deficient mice sensitized with OVA plus high-dose LPS than in WT mice sensitized in the same manner (Fig. 7D). In addition, we

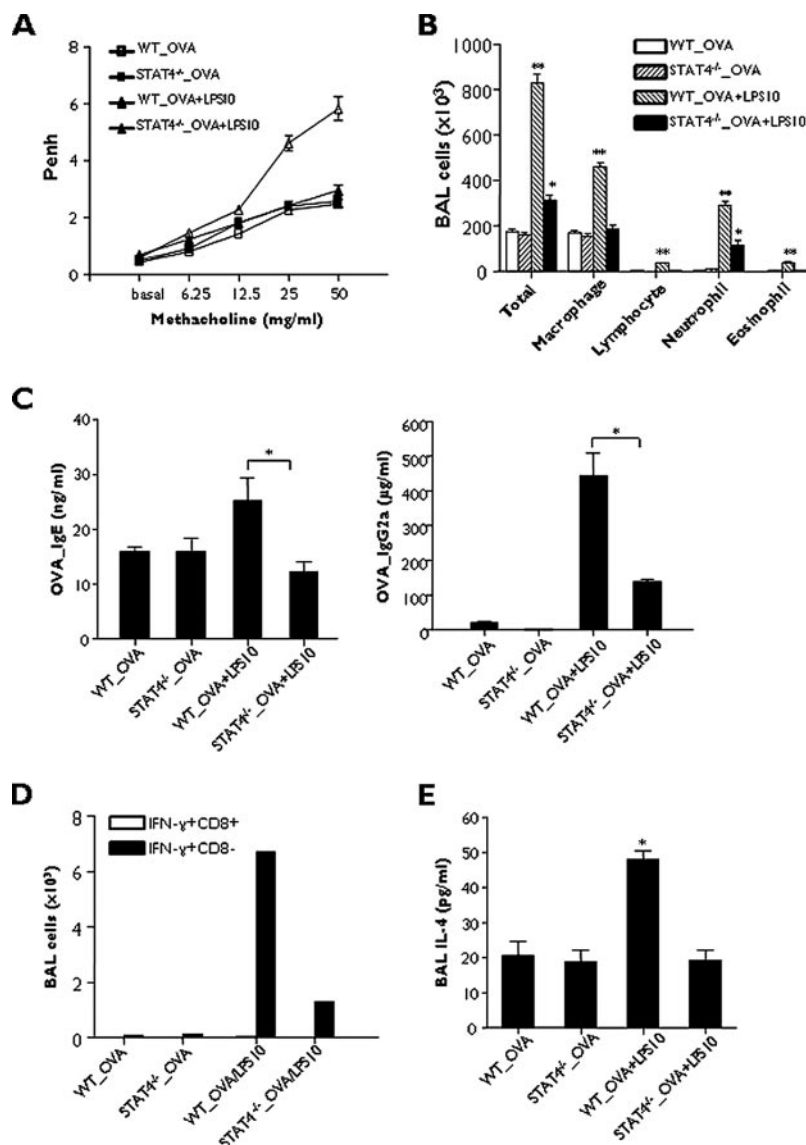


FIGURE 7. AHR, lung inflammation, and immunologic parameters in STAT4-deficient mice. *A–D*, One experiment representative of five is shown after allergen challenge. *A*, Methacholine AHR: AHR enhancement by high-dose LPS was completely inhibited in the absence of STAT4-signaling pathway. *B*, Lung inflammation: lung inflammation enhancement by high-dose LPS was also markedly inhibited in the absence of the STAT4-signaling pathway. *, $p < 0.05$ vs OVA group; **, $p < 0.05$ vs the other groups. *C*, Serum OVA-specific IgE and IgG2a levels: both OVA-specific IgE and IgG2a production enhanced by high-dose LPS were inhibited in the absence of STAT4 signaling. *, $p < 0.05$. *D*, BAL IFN- γ -producing cell numbers after allergen challenge: bar heights are mean numbers of CD8⁺IFN- γ ⁺ (□) and CD8⁺IFN- γ ⁻ (■) cells in BAL. *E*, BAL IL-4 levels after allergen challenge: IL-4 production enhancement by high-dose LPS was also inhibited in STAT4^{-/-} mice. *, $p < 0.05$ vs the other groups.

found that OVA-specific IgE production enhanced by high-dose LPS was completely inhibited in STAT4-deficient mice, and that this inhibition was positively associated with the down-regulation of IL-4 production in the absence of a STAT4-signaling pathway (Fig. 7, *C* and *E*).

Discussion

Our understanding of the pathogenesis of asthma has changed dramatically over time, and it has been proposed that immune deviation toward Th1 response can protect against asthma, because Th1 cells antagonize the functions of Th2 cells (21, 22). The “Th2 asthma hypothesis” is based on the assumption that IgE and eosinophils play crucial roles in the pathogenesis of asthma; Th2 cytokines are believed to regulate IgE synthesis and eosinophil numbers and activity are thought play a major role in driving its pathogenesis (23, 24). Mouse models of asthma based on sensitization with i.p. administered OVA in alum support this concept by demonstrating that Th2 cytokines are major contributors to its pathogenesis (3, 4), although these observations are not consistent with findings from other studies (25–27). In particular, an abundance of human data raises questions concerning any concept that IFN- γ levels are reduced in asthma. Corrigan and Kay (28) demonstrated increased circulating blood levels of IFN- γ in patients

with acute severe asthma and Magnan et al. (7) also showed that the numbers of IFN- γ ⁺ cells in whole bloods are correlated with asthma severity and AHR. In addition, our data showed that IFN- γ mRNA expression in sputum was positively associated with asthma severity. Moreover, therapeutic interventions based on the Th2 hypothesis have indicated that directing the T cell response from Th2 or to Th1 does not affect asthma properties in humans (29, 30); several studies have reported that glucocorticoids, the most potent anti-inflammatory drugs available for the treatment of asthma, guide the differentiation of Th cells toward the Th2 phenotype (31). In addition, our data indicated that airway sensitization with high-dose LPS induced asthma phenotypes, such as AHR and lung inflammation, and that these were accompanied by the up-regulation of allergen-specific IgG2a, which is induced by IFN- γ . AHR and lung inflammation (except eosinophil infiltration) enhanced by high-dose LPS were found to be completely inhibited in the absence of IFN- γ . Taken together, these findings suggest that the Th2 hypothesis provides an oversimplified description of the pathogenesis of asthma and that IFN- γ plays a key role in the development of some asthma subtypes.

Advances in medical research methodologies, such as bronchoscopy and studies on induced sputum in severe asthma, have demonstrated that noneosinophilic and even eosinophilic forms of

severe asthma are associated with airway neutrophilia (32, 33). Our study also found that sputum neutrophils, and not eosinophils, are positively associated with disease severity in asthma patients, and that in a mouse model, BAL neutrophils were induced by LPS in a dose-dependent manner. A previous transgenic mouse experiment clearly demonstrated that high levels of IFN- γ in airways induce noneosinophilic (or neutrophilic) lung inflammation (34), and a separate study showed that IFN- γ inhibits allergen-induced eosinophil recruitment into mouse lung tissue (21). It is well-known that TGF- β 1 is a key mediator in the development of eosinophilic inflammation and tissue fibrosis induced by Th2 cytokines (35) and our present data show that TGF- β 1 production and eosinophilic inflammation after an allergen challenge are more enhanced in IFN- γ -deficient mice sensitized with high-dose LPS. Moreover, by placing a focus on the presence or absence of eosinophils in patients with severe asthma, Wenzel et al. (36) found that subbasement membrane thickening and TGF- β 1-producing cell numbers in airways were higher in patients with severe eosinophilic asthma than with noneosinophilic asthma. Taken together, these findings suggest that IFN- γ plays an essential role in the development of noneosinophilic severe asthma and that IFN- γ is a key negative regulator of the development of eosinophilic inflammation during the pathogenesis of asthma.

It has been well-documented that IL-12 is produced by the innate arm of the immune system and it is an important regulator of Th1 cell development (37–39). Our study showed that LPS dose dependently enhanced IL-12 expression during allergen sensitization. In addition, because it is accepted that the biological effects of Th1-polarizing cytokines, such as IL-12 and IL-18, depend strongly on the STAT4-signaling pathway (40), we hypothesized that Th1 response enhanced by high-dose LPS is dependent on this pathway. Indeed, we have shown that AHR and lung inflammation enhanced by high-dose LPS were completely inhibited in STAT4-deficient mice. Our data also indicate that the inhibition of asthma phenotypes in STAT4-deficient mice is positively associated with the down-regulations of IFN- γ and IL-12 after allergen challenge. These findings suggest that the STAT4-signaling pathway mediates a positive feedback loop between IL-12 and IFN- γ during Th1 recall in response to inhaled allergens.

It is well-known that the default immune response to inert inhaled proteins is the development of immune tolerance (9, 10) and this study also showed that AHR and lung inflammation are not induced by airway sensitization with allergen alone. Eisenbarth et al. (16) showed that TNF- α coadministration with inhaled allergens restores Th2 response. Our present data also indicate that allergen sensitization with low-dose LPS induces the Th2 asthma phenotypes associated with the up-regulation of TNF- α production during allergen sensitization. Based on these findings, we hypothesized that the TNF- α receptor-mediated pathway plays a critical role in the development of Th2 response to allergens. Indeed, our study found that AHR and lung inflammation with IL-4 up-regulation and allergen-specific IgE production (as enhanced by low-dose LPS) were completely inhibited in TNFR1-deficient mice. In contrast, our data showed that Th1 asthma phenotypes induced by high-dose LPS were not affected by the absence of TNF- α receptor-mediated pathway. Taken together, these findings suggest that TNF- α secretion induced by low-dose LPS is a key upstream molecule of Th2 polarization.

Our data support a model of sensitization to inert inhaled proteins that requires LPS. LPS is ubiquitously present in the environment and it has even been detected in house dust (41). We are therefore constantly exposed to at least low levels of environmental LPS and it is well-known that house dust LPS is associated with asthma severity in both adults and children (13, 42). The “hygiene

hypothesis” states that the rising incidence of allergic asthma is causally related to reducing infectious burden in Westernized countries (11). A growing amount of evidence, however, supports a positive correlation between infectious burden and the development of allergic asthma (43–45). It has been reported that LPS concentrations in house dust fall in the range of 0.59–41.04 ng/mg (46). We can speculate that the LPS levels and genetic susceptibility to LPS may determine type 1 vs type 2 adaptive immune responses to inhaled aeroallergens in subjects exposed to house dust allergens contaminated with LPS during early life. This study demonstrated that airway allergen sensitization by differential doses of LPS induces different forms of asthma that are type 1 and type 2 asthma phenotypes by high and low doses of LPS, respectively.

Acknowledgments

We thank Yu-jin Choi and Areum Kim for their excellent secretarial assistance.

Disclosures

The authors have no financial conflict of interest.

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