



Brilliant Violet™ Antibody Conjugates
Superior Performance for the Violet Laser



This information is current as
of January 23, 2012

Airway Activation of Formyl Peptide Receptors Inhibits Th1 and Th17 Cell Responses via Inhibition of Mediator Release from Immune and Inflammatory Cells and Maturation of Dendritic Cells

You-Me Tae, Hyun Taek Park, Hyung-Geun Moon, You-Sun Kim, Seong Gyu Jeon, Tae-Young Roh, Yoe-Sik Bae, Yong Song Gho, Sung Ho Ryu, Hyouk-Soo Kwon and Yoon-Keun Kim

J Immunol; Prepublished online 18 January 2012;
doi:10.4049/jimmunol.1102481
<http://www.jimmunol.org/content/early/2012/01/18/jimmunol.1102481>

Supplementary Data	http://www.jimmunol.org/content/suppl/2012/01/18/jimmunol.1102481.DC1.html
Subscriptions	Information about subscribing to <i>The Journal of Immunology</i> is online at http://www.jimmunol.org/subscriptions
Permissions	Submit copyright permission requests at http://www.aai.org/ji/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at http://www.jimmunol.org/etoc/subscriptions.shtml/

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
Copyright ©2012 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Airway Activation of Formyl Peptide Receptors Inhibits Th1 and Th17 Cell Responses via Inhibition of Mediator Release from Immune and Inflammatory Cells and Maturation of Dendritic Cells

You-Me Tae,* Hyun Taek Park,* Hyung-Geun Moon,* You-Sun Kim,* Seong Gyu Jeon,* Tae-Young Roh,* Yoe-Sik Bae,[†] Yong Song Gho,* Sung Ho Ryu,* Hyouk-Soo Kwon,* and Yoon-Keun Kim*

Formyl peptide receptors (FPRs) are chemoattractant receptors that mediate inflammatory cell responses to infection. Recent evidence indicates that noneosinophilic asthma phenotypes can be developed by both Th1 and Th17 cell responses when exposed to LPS-containing allergens. In this study, we evaluated the effects of airway activation of FPRs by their synthetic agonist, Trp-Lys-Tyr-Met-Val-D-Met (W-peptide), on the development of Th1 and Th17 cell responses in a noneosinophilic asthma mouse model. A noneosinophilic asthma mouse model was generated by intranasal sensitization with 10 μ g of LPS plus 75 μ g of OVA on days 0, 1, 2, and 7. Mice were then challenged with 50 μ g of OVA alone on days 14, 15, 21, and 22. W-peptide was administered during the sensitization period, and immune and inflammatory responses were evaluated after OVA challenge. Lung inflammation after OVA challenge was partly abolished by airway activation of FPRs during sensitization. Maturation of dendritic cells (DCs) and migration of DCs from the lung to lung-draining lymph nodes were inhibited by FPR activation. In addition, airway activation of FPRs inhibited allergen-specific T cell proliferation in the lymph nodes. Production of IL-12 and IL-6 (Th1- and Th17-polarizing cytokines) from lung DCs was decreased by airway activation of FPRs. This effect resulted in the inhibition of allergen-specific Th1 and Th17 cell responses. Airway activation of FPRs during sensitization effectively prevents the development of Th1 and Th17 cell responses induced by LPS-containing allergens via multiple mechanisms, such as inhibition of DC maturation and migration and the production of Th1- and Th17-polarizing cytokines. *The Journal of Immunology*, 2012, 188: 000–000.

Asthma is a chronic inflammatory disorder of the airways that is characterized by reversible airway obstruction and airway hyperresponsiveness (AHR) (1, 2). Asthma has been considered to be induced by a Th2 cell response and resultant eosinophilic inflammation in the airways (3). However, recent experimental and clinical evidence indicates that severe asthma is characterized by neutrophilic inflammation, which is related to both Th1 and Th17 cell responses (4, 5). In particular, our pre-

vious animal experiments showed that airway sensitization of LPS-containing allergens induced neutrophilic inflammation after airway allergen challenge; the neutrophilic inflammation was elicited by both Th1 and Th17 cell responses (6).

Formyl peptide receptors (FPRs) are pattern-recognition receptors to formylated peptides derived from Gram-negative bacteria and are involved in host defense against pathogens (7). Three FPRs (FPR, FPRL1, and FPRL2) have been identified in humans, and fpr1 and fpr2 have been found in mice as counterparts to human FPR and FPRL1, respectively (7, 8). FPRs are expressed mostly in phagocytes, such as neutrophils, monocytes, macrophages, and dendritic cells (DCs), as well as in immune cells, such as T cells and B cells (7–9). It was recently reported that the expression of FPRs is essential for early host defense to bacterial infections (10). Moreover, we demonstrated that FPR activation prevented the progression of sepsis in a sepsis animal model (11).

The effects of FPRs on the development of inflammation have been widely studied. It was reported that several peptides, such as serum amyloid A, which is a 42-aa form of β amyloid peptide, were major causes of amyloidogenic disease via FPRL1 (8). In addition, binding of serum amyloid A to FPRL1 contributed to the destruction of bone and cartilage via the promotion of synovio-cyte hyperplasia and angiogenesis in rheumatoid arthritis (12). In contrast, FPR knockout mice exhibited enhanced susceptibility against infection and inflammation compared with wild-type mice (10, 13). FPRL1 is also known to be a receptor for lipoxin A4 (LXA4), the endogenous agonist of this receptor (7, 14, 15). Severe asthma is reportedly associated with a loss of LXA4, and the activation of FPRs by stable analogs of LXA4 blocked AHR and

*Department of Life Science, Pohang University of Science and Technology Biotech Center, Pohang 790-784, Republic of Korea; and [†]Department of Biological Sciences, Sungkyunkwan University, Suwon 440-746, Republic of Korea

Received for publication August 29, 2011. Accepted for publication December 14, 2011.

This work was supported by grants from the Korea Ministry of Health and Welfare, Republic of Korea (A080711-0912-0000100).

Y.-M.T. designed and performed experiments, analyzed and interpreted results, and wrote the manuscript; H.T.P., H.-G.M., Y.-S.K., and S.G.J. performed animal experiments; T.-Y.R., Y.S.G., Y.-S.B., and S.H.R. designed experiments and wrote the manuscript; and H.-S.K. and Y.-K.K. directed the study and wrote the manuscript.

Address correspondence to Prof. Yoon-Keun Kim and Prof. Hyouk-Soo Kwon, Pohang University of Science and Technology, San 31, Hyojadong, Namgu, Pohang 790-784, Republic of South Korea. E-mail addresses: juinea@postech.ac.kr (Y.-K.K.) and kwonhs21@naver.com (H.-S.K.)

The online version of this article contains supplemental material.

Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; FPR, formyl peptide receptor; LN, lymph node; LXA4, lipoxin A4; POSTECH, Pohang University of Science and Technology; tBOC, *N*-t-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe; VEGF, vascular endothelial growth factor.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/\$16.00

pulmonary inflammation in a murine model of asthma (16–18). From this perspective, it can be predicted that activation of FPRs is directly related to the regulation of inflammation and that synthetic agonists of FPRs might be promising drug candidates for the treatment of asthma.

W-peptide (Trp-Lys-Tyr-Met-Val-D-Met) is well known as a synthetic ligand for FPRs (19). An *in vitro* assay demonstrated that W-peptide induced the chemotactic migration of phagocytes, enhanced the bactericidal activity of monocytes and neutrophils via the production of superoxide anions, and blocked cell apoptosis (19–22). Furthermore, W-peptide effectively prevented development of severe sepsis following microbial infection via modulation of inflammation and immune responses (11).

We hypothesized that airway activation of FPRs inhibits adaptive immune dysfunction induced via sensitization with LPS-containing allergens by modulation of LPS-induced innate immune responses. To test this, the W-peptide (an FPR agonist) was administered in a noneosinophilic asthma model that was induced by airway sensitization with LPS-containing allergens during the sensitization period, and immune and inflammatory responses were evaluated after allergen challenge.

Materials and Methods

Mice

BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were bred in a pathogen-free facility at Pohang University of Science and Technology (POSTECH), and all live animal experiments were approved by the Ethics Committee of POSTECH.

Reagents

LPS and OVA were purchased from Calbiochem (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO), respectively. W-peptide (Trp-Lys-Tyr-Met-Val-D-Met) was synthesized by Pepton (Daejeon, Republic of Korea).

Protocols for a noneosinophilic asthma mouse model and pharmacologic intervention

A noneosinophilic asthma model characterized by Th1 and Th17 cell responses was generated, as described previously (6). In brief, 6-wk-old mice were intranasally sensitized with LPS-depleted OVA (75 µg) plus LPS (10 µg) on days 0, 1, 2, and 7 and then challenged with OVA (50 µg) alone on days 14, 15, 21, and 22. To evaluate the effect of W-peptide on development of Th1 and Th17 cell responses, it was administered intranasally (200 µg/kg) on days 0, 1, 2, and 7 with LPS-containing OVA. Additionally, W-peptide was intranasally administered on days 14, 15, 21, and 22 with OVA to evaluate the therapeutic effect. OVA-specific adaptive immune responses and airway inflammation were evaluated 6 and 48 h after the last allergen challenge on day 22. To evaluate the effects of W-peptide on the innate immune response induced by LPS, mice were sensitized with LPS (10 µg) with or without W-peptide (10–1000 µg/kg).

Measurement of methacholine AHR

Pulmonary function testing in mice was assessed using conscious, unrestrained mice by noninvasive whole body plethysmography (Allmedicus, Anyang, Republic of Korea). Measurements were performed as previously described (23). 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 (23).

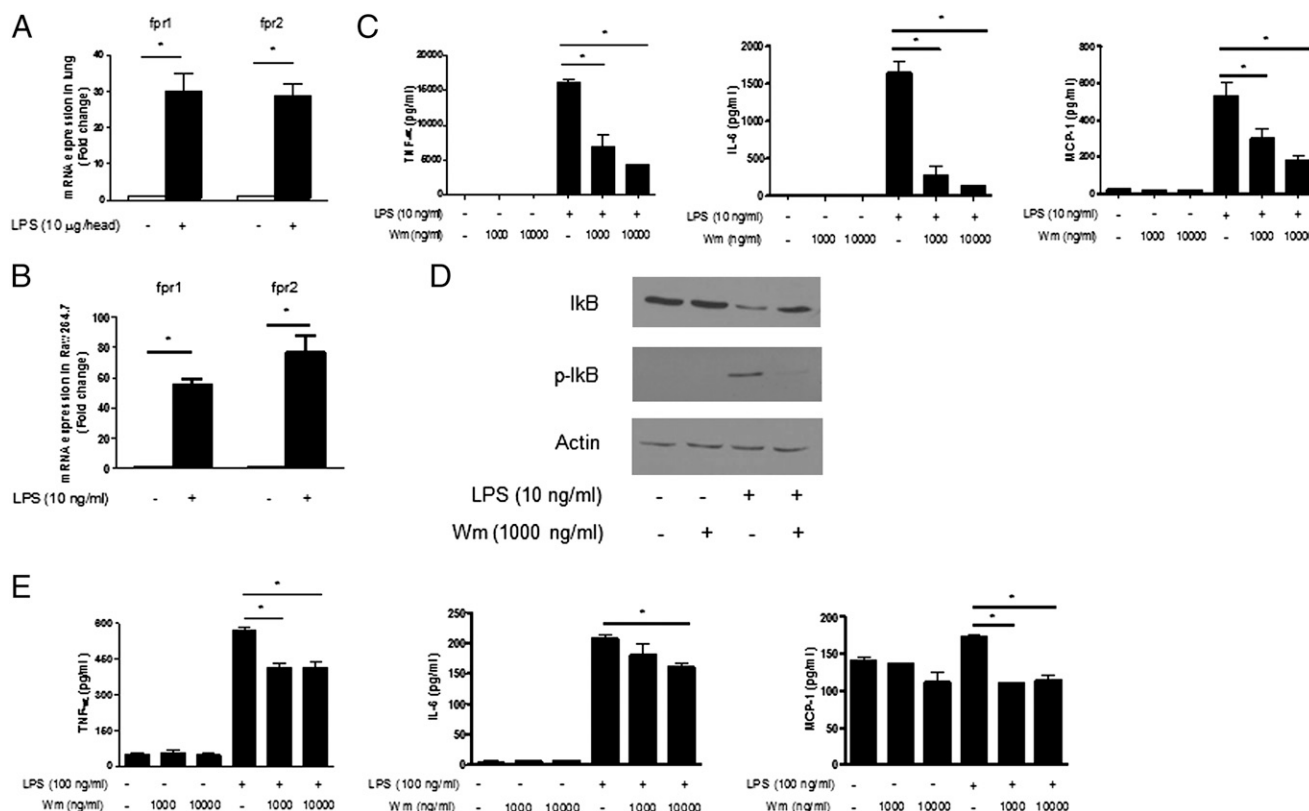


FIGURE 1. Activation of FPRs reduces release of proinflammatory cytokines and chemokines from macrophages. *A*, Expression of FPRs (fpr1 and fpr2) in mouse lung cells. Lung cells were obtained from naive mice (–) and intranasally LPS-applied mice (+). *B*, Expression of FPRs (fpr1 and fpr2) in the mouse macrophage cell line Raw264.7. Cells were stimulated or not with LPS for 12 h. *C*, Cytokine levels released from LPS-stimulated Raw264.7 cells (mouse peritoneal macrophage cell line). *D*, Protein levels of IκB and p-IκB after cotreatment with LPS and W-peptide in Raw264.7 cells. Whole-cell lysates were obtained 30 min after cotreatment of LPS and W-peptide, and protein levels of IκB and p-IκB were analyzed by Western blot. *E*, Cytokine levels released from LPS-stimulated MH-S cells (mouse alveolar macrophage cell line). In *C* and *E*, cells were pretreated with W-peptide for 12 h and then stimulated with LPS for 12 h. **p* < 0.05.

Cellularity in bronchoalveolar lavage fluids

Bronchoalveolar lavage (BAL) cellularity was analyzed, as described previously (6). Briefly, BAL cells were stained with Diff-Quik (Dade Behring, Newark, DE), and cellularity was determined by counting 300 inflammatory cells. Inflammatory cells were classified as macrophages, lymphocytes, neutrophils, and eosinophils.

Histologic examination of lung tissues

Lung sections were stained with H&E after pressure fixation with Streck solution (Streck Laboratories, La Vista, NE). All sample slides were compared at the same magnification. Lung inflammation was assessed by the degree of peribronchiolar and perivascular inflammation, as described previously (6).

Measurement of cytokines

The levels of cytokines in BAL fluids and cell culture supernatants were measured by ELISA, in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Cytokine production from T cells after allergen-specific stimuli

Single-cell suspensions from lung or lung-draining lymph nodes (LNs) were prepared. Cells were incubated with OVA (100 μ g/ml) for 72 h. The levels of cytokines in the supernatants were measured.

Intracellular staining of T cells in lung tissues

Cells isolated from lung tissues (4×10^6 /ml) were incubated in 48-well plates coated with anti-CD3 plus anti-CD28 Abs (1 μ g/ml each; eBio-

science) at 37°C for 6 h and then incubated with brefeldin A (10 μ g/ml) for 2 h. Cells were stained with surface-specific Abs (anti-CD3-allophycocyanin, anti-CD4-FITC, and anti-CD8-PE-Cy5; BD Biosciences) for 30 min at 4°C and then fixed for 10 min in 4% paraformaldehyde at room temperature. Cells were incubated with Abs to candidate cytokines (anti-IFN- γ -PE, anti-IL-17-PE; BD Biosciences) for 30 min at room temperature and analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

Intracellular staining of inflammatory cells in lung tissues

Cells isolated from lung tissues were stained with surface-specific Abs (anti-CD11b-PerCP, anti-CD11c-allophycocyanin; BD Biosciences) for 30 min at 4°C and then fixed for 10 min in 4% paraformaldehyde at room temperature. Cells were incubated with Abs to candidate cytokines (anti-IL-12-PE and anti-IL-6-PE; BD Biosciences) for 30 min at room temperature and analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

Expression of FPRs on inflammatory cells and lung tissue

Total RNA was isolated from mouse lung tissue and Raw264.7 cells. Double-stranded cDNA was synthesized from total RNA using the One Step SYBR PrimeScript RT-PCR Kit (Takara Bio). Real-time quantitative RT-PCR was performed on LightCycler 480 (Roche Diagnostics). The sequences of the primers used were as follows: fpr1: sense, 5'-CATGAACAAGTCTGCAGTGAACCT-3', antisense, 5'-AGGTTTATGTCTATTACAGTATAT-3'; fpr2: sense, 5'-TCTACCATCTCCAGAGTTC-TGTGG-3', antisense, 5'-TTACATCTACCACAATGTGAACTA-3'; and β -actin: sense, 5'-TGGAATCCTGTGGCATCCATGAAAC-3', antisense, 5'-TAAACGCGAGCTCAGTAACAGTCCG-3'. Amplification was per-

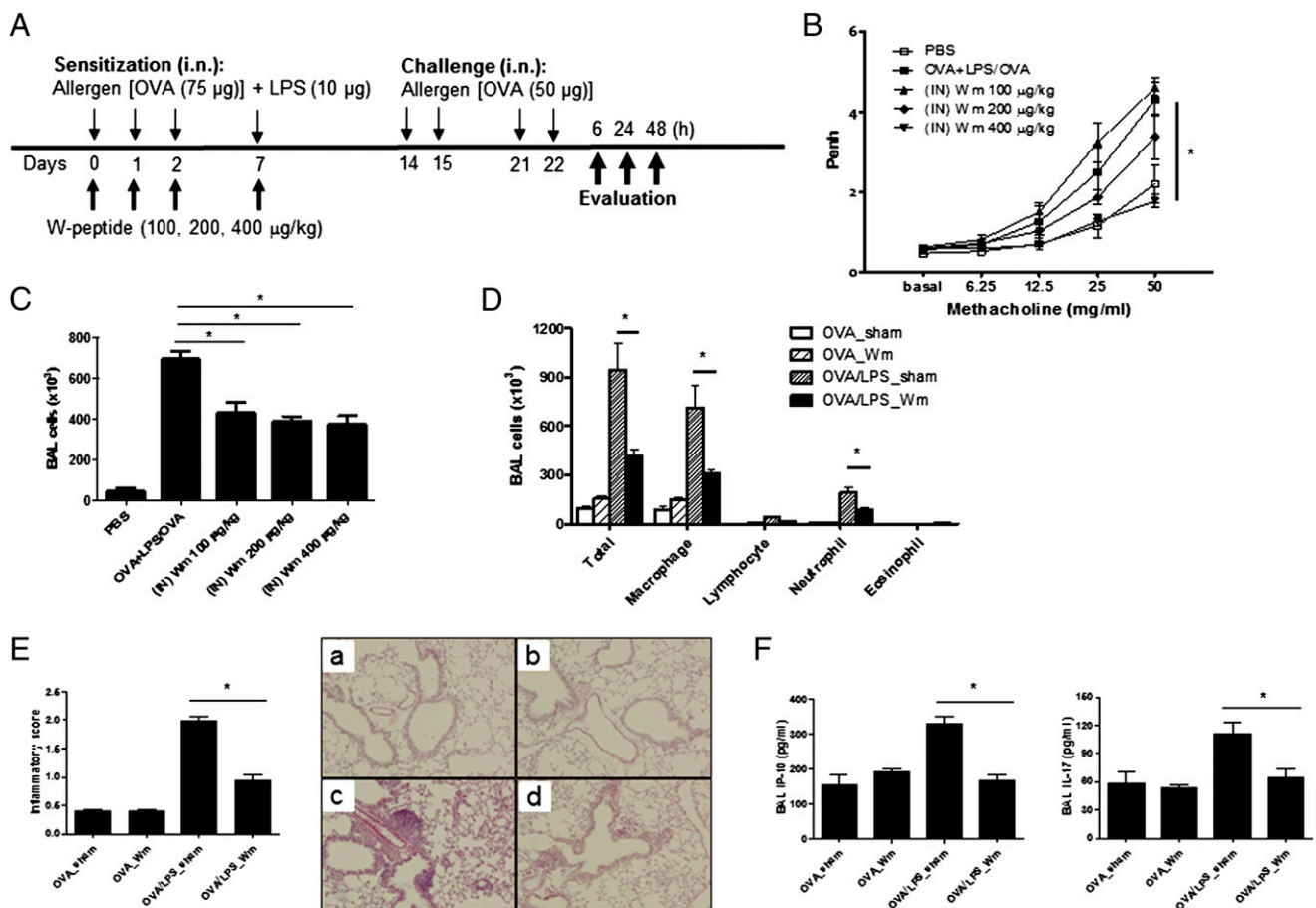


FIGURE 2. Airway activation of FPRs during sensitization inhibits the development of noneosinophilic airway inflammation induced by LPS-containing allergens. **A**, Protocol for the generation of a noneosinophilic asthma. Mice ($n = 5$ /group) were sensitized with LPS-containing allergen on days 0, 1, 2, and 7 and challenged with allergen four times on days 14, 15, 21, and 22. W-peptide (100, 200, and 400 μ g/kg) was applied intranasally during sensitization. **B**, AHR 24 h after the last OVA challenge. **C** and **D**, BAL cellularity 48 h after the last OVA challenge. **E**, Inflammatory score and representative lung histologic findings (H&E stain, original magnification $\times 100$). **a**, OVA_sham. **b**, OVA_Wm. **c**, OVA/LPS_sham. **d**, OVA/LPS_Wm. **F**, Levels of IP-10 (left panel) and IL-17 (right panel) in BAL fluids obtained 48 and 6 h after the last OVA challenge, respectively. In **D–F**, 200 μ g/kg of W-peptide was applied intranasally during sensitization. * $p < 0.05$.

formed over 45 cycles (95°C/15 s [denaturation], 55°C/15 s [annealing], and 72°C/10 s [extension]).

In vitro production of proinflammatory mediators

To evaluate the effects of FPR activation by W-peptide on the mediator release from LPS-stimulated macrophages, Raw264.7 (a mouse peritoneal macrophage cell line) and MH-S (a mouse alveolar macrophage cell line) cells were pretreated with W-peptide for 12 h. Raw264.7 and MH-S cells were then stimulated with 10 and 100 ng/ml LPS for 12 h, respectively.

Maturation of lung DCs

Single-cell suspensions isolated from lung tissues were prepared, as described previously (13). FACS analysis was performed using mAbs from BD Biosciences (San Jose, CA): anti-I-A-FITC, anti-CD40-PE, anti-CD80-PE, anti-CD83-PE, anti-CD86-PE, anti-CD11b-PerCP, and anti-CD11c-allophycocyanin. Cells were analyzed on a FACSCalibur (BD Biosciences) flow cytometer with CellQuest software.

Migration of lung DCs into lung-draining LNs

To investigate the migration of lung DCs, 60 μ l OVA-FITC (10 mg/ml; Sigma-Aldrich) was administered intranasally, along with LPS (10 μ g) or W-peptide (200 μ g/kg) (14). Twenty-four hours after administration, the migration of lung DCs was determined by detecting CD11c⁺ cells carrying FITC⁺ in lung-draining LNs by FACS.

Proliferation of T cells in lung-draining LNs

For the T cell-proliferation assay, cells from lung-draining LNs were collected, and 6×10^6 cells/ml were stained with 5 μ M CFSE (Invitrogen, Carlsbad, CA) and incubated in 24-well plates at 37°C in medium alone (RPMI 1640 containing 10% FBS and antibiotics) or with 100 μ g/ml OVA for 96 h. After incubation, cells were stained with anti-CD3-allophycocyanin (BD Biosciences). Proliferation of T cells was then determined by detecting CD3⁺ cells carrying CFSE in LNs by FACS.

Statistical analysis

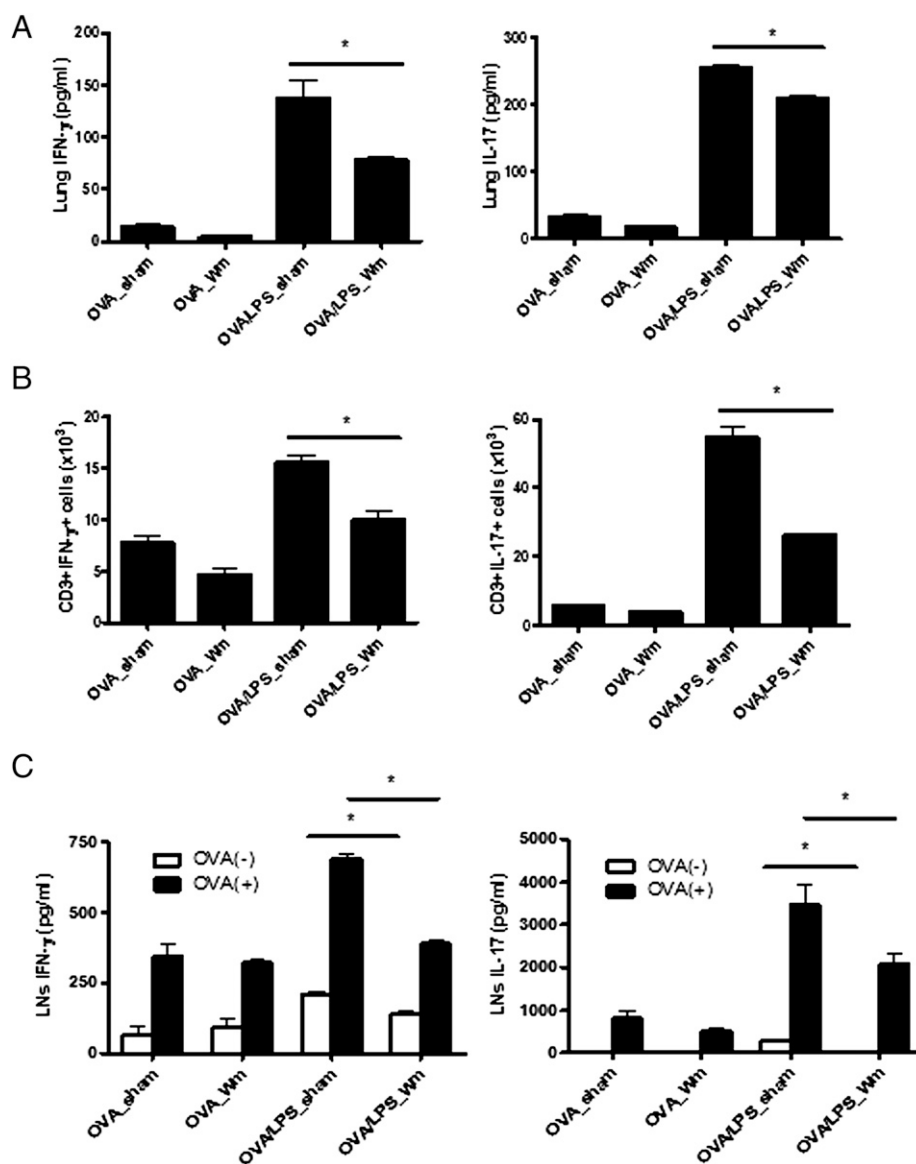
Significant differences between the treatments were assessed using the Student *t* test, ANOVA, or Wilcoxon rank-sum test. For multiple comparisons, ANOVA was used initially, and when significant differences were found, individual *t* tests or Wilcoxon rank-sum tests for pairs of groups were performed.

Results

Effects of FPR activation on mediator release from macrophages

Recent studies suggest that W-peptide treatment reduces systemic levels of proinflammatory cytokines, such as TNF- α and IL-1 β , in a sepsis mice model (11). The present study showed that FPRs (fpr1 and fpr2) were expressed in mouse lung tissues (Fig. 1A) and Raw264.7 cells, the mouse macrophage cell line (Fig. 1B). In

FIGURE 3. Airway activation of FPRs during sensitization regulates Th1 and Th17 adaptive-immune responses. *A*, OVA-specific IFN- γ (left panel) and IL-17 (right panel) production in the lung. Mice ($n = 5$ /group) were sacrificed 6 h after the last challenge on day 22. Isolated cells from the lung were stimulated with OVA (100 μ g/ml) for 72 h, and IFN- γ and IL-17 levels in the supernatant were measured. *B*, Numbers of IFN- γ -producing (left panel) and IL-17-producing (right panel) T cells in the lung. Lung cells were isolated 6 h after the last challenge and analyzed using flow cytometry. *C*, OVA-specific IFN- γ (left panel) and IL-17 (right panel) production in lymph nodes. Lung-draining LNs of mice were obtained 6 h after the last challenge on day 22. Isolated cells from lymph nodes were stimulated or not with OVA (100 μ g/ml) for 72 h. * $p < 0.05$.



addition, fpr1 and fpr2 expression on macrophages was enhanced by LPS treatment (Fig. 1A, 1B). Next, we evaluated the effects of FPR activation by W-peptide on mediator release from macrophages induced by LPS stimulation. The release of proinflammatory mediators, such as TNF- α , IL-6, and MCP-1, from Raw264.7 cells enhanced by LPS incubation was inhibited by W-peptide coinubation in a dose-dependent manner (Fig. 1C). In terms of the molecular mechanisms underlying W-peptide-mediated effects on the downregulation of proinflammatory mediators from macrophages, phosphorylated I κ B expression was enhanced by LPS treatment, whereas I κ B expression decreased; however, expression of the former was decreased by W-peptide cotreatment, whereas the latter increased (Fig. 1D). MH-S cells (a mouse alveolar macrophage cell line) were used to confirm the effects of W-peptide on the downregulation of proinflammatory mediators from lung macrophages. The production of proinflammatory mediators, such as TNF- α , IL-6, and MCP-1, enhanced by LPS incubation was reversed by W-peptide cotreatment (Fig. 1E). Taken together, these data indicated that activation of FPRs, the expression of which was enhanced by LPS, by W-peptide inhibited the release of proinflammatory mediators from inflammatory cells induced by LPS, possibly via modulation of the NF- κ B pathway.

Effects of airway activation of FPRs during sensitization on the development of allergic airway inflammation

Based on the findings that W-peptide modulates *in vitro* innate immune response induced by LPS, we evaluated the effects of airway activation of FPRs by different doses of W-peptide on the

development of AHR and airway inflammation after allergen challenge in the noneosinophilic asthma model (Fig. 2A). Intranasal application of W-peptide during sensitization reduced AHR induced by allergen challenge in a dose-dependent manner (Fig. 2B). BAL cellularity 48 h after the last allergen challenge showed that lung inflammation was significantly inhibited by W-peptide treatment, regardless of doses (Fig. 2C). Based on these findings, further experiments were performed using 200 μ g/kg of W-peptide. BAL cellularity 48 h after the last allergen challenge showed that lung infiltration of macrophages and neutrophils was significantly inhibited by W-peptide treatment compared with sham treatment (Fig. 2D). Inflammatory scores based on lung histology were also decreased by W-peptide treatment compared with sham treatment (Fig. 2E). In addition, the levels of IP-10 (a Th1-type cytokine) and IL-17 (a Th17-type cytokine) in BAL fluids were significantly decreased by W-peptide treatment compared with sham treatment (Fig. 2F). Taken together, these findings suggest that W-peptide treatment prevents the development of allergen-induced noneosinophilic inflammation in a noneosinophilic asthma mouse model.

Effects of airway activation of FPRs on the development of Th1 and Th17 cell responses

Previous evidence showed that noneosinophilic inflammation induced by sensitization with LPS-containing allergens was caused by both Th1 and Th17 cell responses (6, 24, 25). To evaluate the effects of airway activation of FPRs on the development of Th1 and Th17 cell responses, W-peptide (200 μ g/kg) was administered intranasally during sensitization, and immune responses were

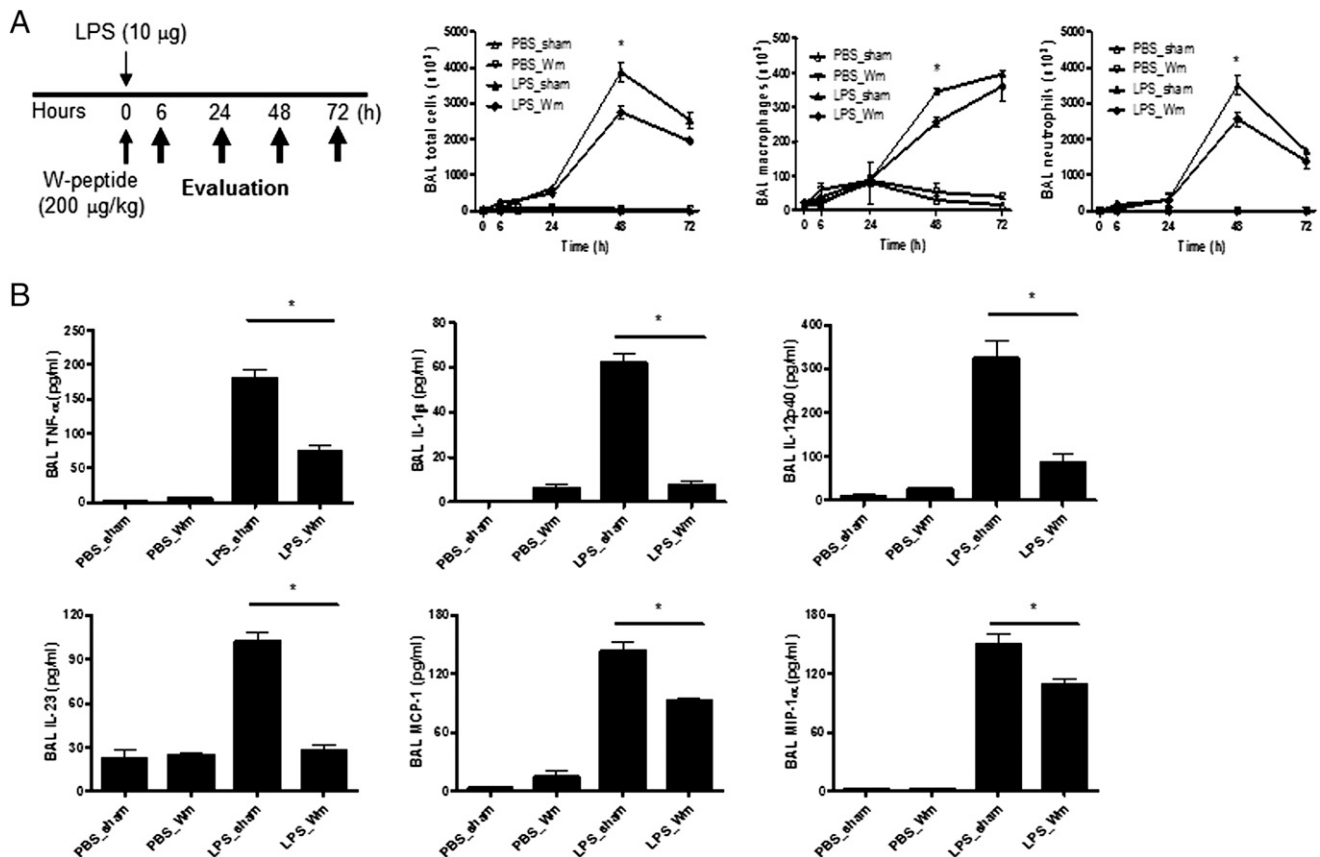


FIGURE 4. Airway activation of FPRs inhibits the infiltration of inflammatory cells induced by LPS application. *A*, Profiles of inflammatory cells, such as total cells, macrophages, and neutrophils, in BAL fluids after airway application of LPS. W-peptide (200 μ g/kg) was applied to mice ($n = 5$ /group) intranasally with LPS (10 μ g), and BAL cellularities were measured 6, 24, 48, and 72 h after administration. *B*, Levels of TNF- α , IL-1 β , IL-12p40, IL-23, MCP-1, and MIP-1 α in BAL fluids. BAL fluids were obtained 24 h after the third sensitization. * $p < 0.05$.

evaluated 6 h after the last allergen challenge (Fig. 2A). The production of IFN- γ from lung T cells 72 h after OVA stimulation was markedly increased by sensitization with LPS-containing OVA, which was partly abolished by W-peptide cotreatment (Fig. 3A). Similarly, allergen-specific IL-17 production from lung T cells enhanced by LPS-containing OVA was also inhibited by W-peptide cotreatment (Fig. 3A). With respect to lung infiltration of Th1 and Th17 cells after allergen challenge, the number of CD3⁺IFN- γ ⁺ cells (Th1 cells) was increased by sensitization with LPS-containing allergens, which was reversed by W-peptide treatment. In addition, the number of CD3⁺IL-17⁺ cells (Th17 cells) enhanced by LPS-containing allergen sensitization was significantly decreased by W-peptide treatment (Fig. 3B). With respect to the expression of Th1 and Th17 cells in lung-draining LNs, the production of both IFN- γ and IL-17 from LN T cells after allergen stimulation was enhanced by sensitization with LPS-containing allergen compared with OVA alone; the enhanced production was reversed by W-peptide cotreatment (Fig. 3C). Taken together, these data suggest that W-peptide inhibits both Th1 and Th17 cell responses induced by LPS-containing allergens.

Effects of airway activation of FPRs on lung infiltration of inflammatory cells induced by LPS inhalation

W-peptide is a well-known chemoattractant that induces chemotactic migration of various inflammatory cells (19, 26); however, its effect on cell infiltration into inflamed sites is unclear. Thus, to

elucidate the effect of W-peptide on inflammatory cell recruitment into airways, we evaluated the infiltration of inflammatory cells into the lung during the 72 h after intranasal application of LPS to mice with or without W-peptide (200 μ g/kg) treatment. The number of inflammatory cells, especially neutrophils, in BAL fluids peaked 48 h after LPS application, and the infiltration of inflammatory cells induced by LPS was significantly decreased by W-peptide treatment (Fig. 4A). In accordance with BAL cellularities, the levels of proinflammatory mediators, such as TNF- α , IL-1 β , IL-12p40, IL-23, MCP-1, and MIP-1 α , in BAL fluids 24 h after the third sensitization with LPS were enhanced by LPS; the enhanced production was significantly inhibited by W-peptide treatment in a dose-dependent manner (Fig. 4B, Supplemental Fig. 1). These data suggest that airway activation of FPRs by W-peptide inhibits lung infiltration of inflammatory cells, possibly via inhibition of the production of proinflammatory mediators.

Effects of airway activation of FPRs on DC infiltration, maturation, and migration enhanced by LPS inhalation

With respect to lung infiltration of DCs, the number of CD11b⁺CD11c⁺ cells was highly enhanced by LPS application, which was inhibited by airway activation of FPRs with W-peptide (200 μ g/kg) treatment (Fig. 5A). Previous evidence showed that W-peptide inhibited in vitro maturation of human monocyte-derived DCs by LPS (27). To further investigate the effect of airway activation of FPRs on the development of in vivo DC maturation in the lungs by LPS, LPS-exposed mice were treated with W-peptide (200 μ g/kg)

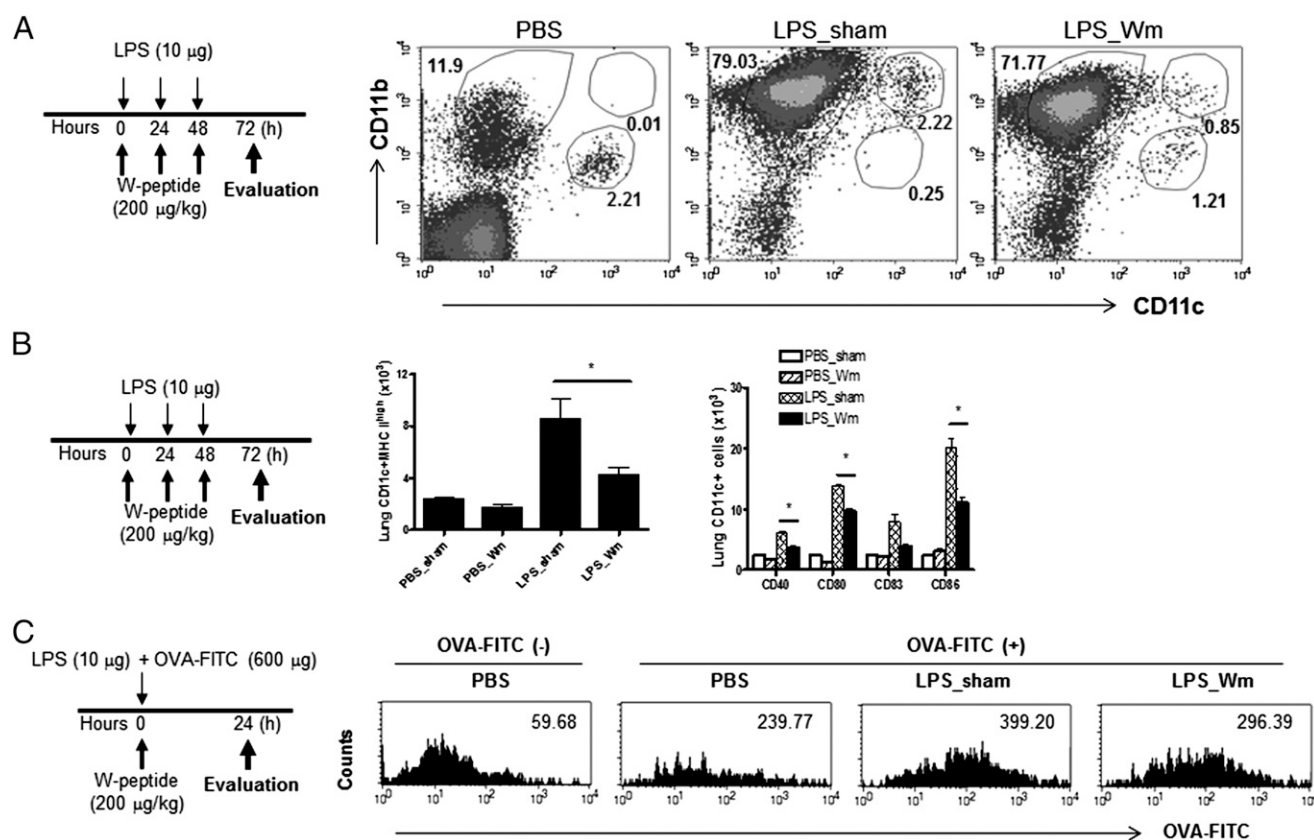


FIGURE 5. Airway activation of FPRs during sensitization inhibits infiltration and maturation of DCs. **A**, Percentage of CD11b⁺ and CD11c⁺ cells in the lung. Mice ($n = 5$ /group) were sensitized three times (once a day) with LPS (10 μ g), and W-peptide (200 μ g/kg) was administered during each sensitization. **B**, Numbers of CD11c⁺MHC class II^{high} and costimulatory molecules, including CD40-, CD80-, CD83-, and CD86-expressing cells, in the lung. In **A** and **B**, samples from mice were obtained 24 h after the third sensitization. **C**, Mean fluorescence intensity of FITC-OVA-containing CD11c⁺ cells in the LNs. OVA-FITC (10 mg/ml, 60 μ l) was applied intranasally to mice simultaneously with LPS and W-peptide. Lung-draining LNs were obtained 24 h after the first sensitization, and cells were isolated and analyzed using FACS analysis. In **A** and **C**, lungs from five mice/group were pooled for each sample. The experiment was repeated three times, and a representative blot is shown. * $p < 0.05$.

(Fig. 5B). This experiment demonstrated that the number of CD11c⁺MHC class II^{high} cells in lung tissues was enhanced by LPS application; this phenotype was significantly inhibited by W-peptide treatment (Fig. 5B). In addition, the expression of costimulatory molecules, such as CD40, CD80, CD83, and CD86, in CD11c⁺ cells in lung tissues was enhanced by airway exposure to LPS, which was reversed by W-peptide treatment (Fig. 5B). Moreover, we evaluated the effect of W-peptide treatment on the migration of lung DCs into lung-draining LNs. To accomplish this, FITC-conjugated OVA peptides were administered intranasally into the mouse airways, and lung-draining LNs were obtained 24 h after administration (Fig. 5C). This study showed that CD11c⁺ OVA-FITC⁺ cells in LNs were increased by LPS application, which was reversed by W-peptide (Fig. 5C). Taken together, these data suggest that airway activation of FPRs by W-peptide inhibits DC infiltration and maturation in the lung and subsequent migration of lung DCs into regional LNs.

Effects of airway activation of FPRs on T cell proliferation

Based on the finding that airway activation of FPRs inhibited DC maturation and migration, we investigated the effect of W-peptide treatment on Ag-specific T cell proliferation. To test this objective, LPS-containing OVA-sensitized mice were treated with W-peptide (200 µg/kg) and then challenged with OVA alone, without W-peptide treatment (Fig. 6A). Lung-draining LNs were collected 6 h after OVA challenge on day 15, and isolated single cells were then stained with CFSE and incubated with or without OVA for 96 h. This experiment showed that OVA-specific T cell proliferation

was enhanced by LPS-containing OVA sensitization compared with sensitization with OVA alone; the enhanced proliferation was decreased by W-peptide treatment (Fig. 6B). This finding indicated that airway activation of FPRs inhibited allergen-specific T cell proliferation in regional LNs in mice sensitized with LPS-containing allergens.

Effects of airway activation of FPRs on T cell polarization

In our previous study, we demonstrated that vascular endothelial growth factor (VEGF) produced by LPS plays a key role in priming T cells and that IL-12p70 and IL-6 are essential for Th1 and Th17 polarization, respectively (24, 25). To further investigate the molecular mechanisms underlying the effect of W-peptide on T cell polarization, the expression of polarizing mediators was evaluated 24 h after the third sensitization on day 2 (Fig. 6A). The levels of IL-12p70 (Th1-polarizing cytokine) and IL-6 (Th17-polarizing cytokine) in BAL fluids were enhanced by LPS-containing OVA compared with OVA alone, and this phenotype was reversed by W-peptide treatment (Fig. 6C). However, the production of VEGF enhanced by LPS was not reversed by W-peptide treatment (data not shown). Moreover, the number of IL-12p70⁺ and IL-6⁺ CD11c⁺ cells isolated from lung tissues was increased by LPS-containing OVA and reversed by W-peptide treatment (Fig. 6D). To further investigate which receptors are mainly involved in W-peptide-mediated effects, mice were treated with *N*-t-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe (tBOC; fpr1 antagonist) or Trp-Arg-Trp-Trp-Trp-Trp (WRW4; fpr2 antagonist) prior to W-peptide. The reduced levels of IL-12p70 and IL-6 in BAL fluids by W-peptide were partially reversed by tBOC, whereas

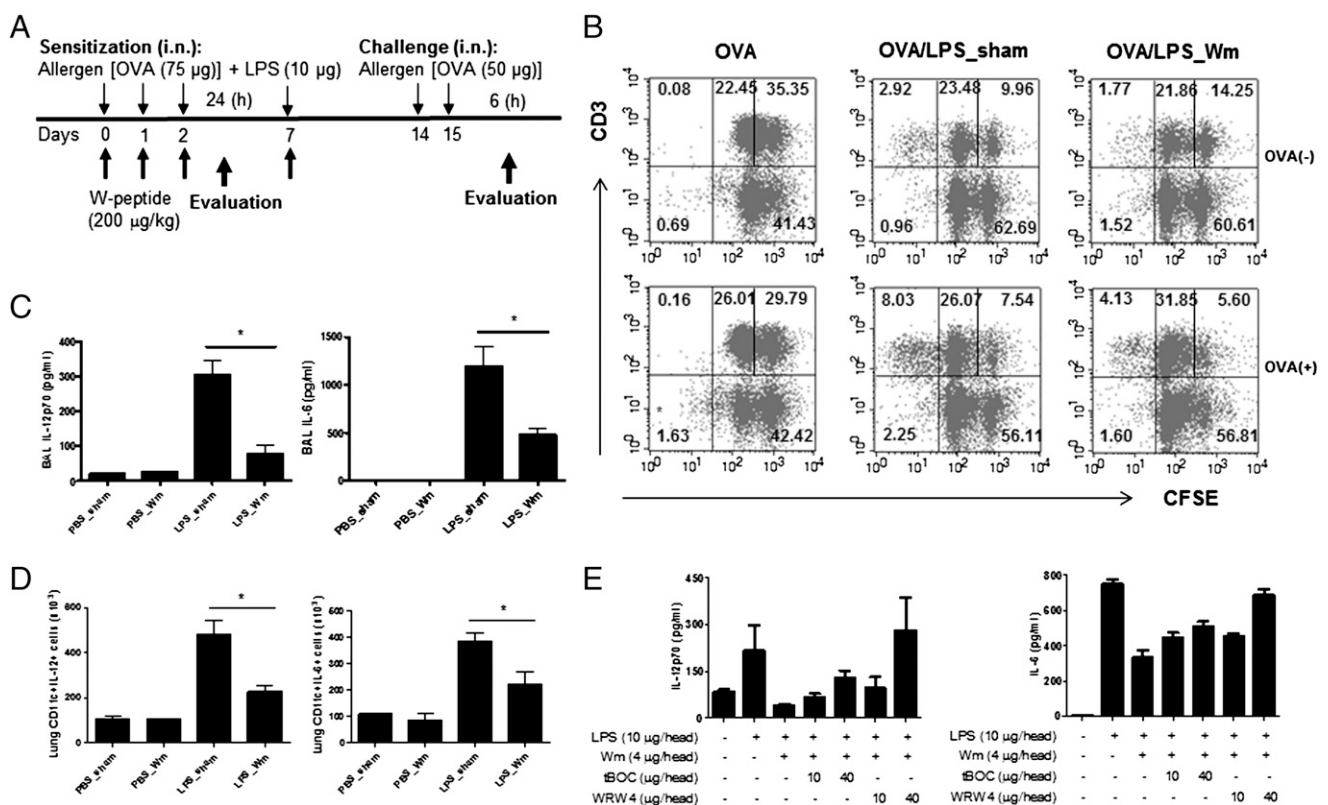


FIGURE 6. Airway activation of FPRs modulates allergen-specific T cell proliferation and polarization. **A**, Study protocol. **B**, Allergen-specific T cell proliferation in LNs. Lung-draining LNs obtained 6 h after the second challenge in a noneosinophilic asthma model. Isolated single cells were stained with CFSE (5 µM) and incubated or not with OVA (100 µg/ml) for 96 h. For each sample, lungs from five mice per group were pooled. The experiment was repeated three times, and a representative blot is shown. **C**, Levels of IL-12p70 (left panel) and IL-6 (right panel) in BAL fluids. **D**, Numbers of IL-12-producing (left panel) and IL-6-producing (right panel) CD11c⁺ cells in the lung. **E**, Levels of IL-12p70 (left panel) and IL-6 (right panel) in BAL fluids. W-peptide (fpr1 and fpr2 agonist) was administered intranasally, whereas tBOC (fpr1 antagonist) and WRW4 (fpr2 antagonist) were administered i.p. In C–E, samples from mice (*n* = 5/group) were obtained 24 h after the third sensitization. **p* < 0.05.

completely reversed by WRW4 (Fig. 6E). These data suggest that airway activation of FPRs, mainly fpr2, inhibits both Th1 and Th17 polarization induced by LPS-containing allergens partly via downregulation of the production of polarizing cytokines.

Therapeutic effects of airway activation of FPRs during allergen challenge on the development of noneosinophilic airway inflammation

To summarize the previous data, airway activation of FPRs during sensitization inhibited the development of noneosinophilic airway inflammation by inhibition of Th1- and Th17-polarizing cytokine production, DC maturation and migration, and T cell proliferation. To further investigate the effect of airway activation of FPRs during allergen challenge on the development of airway inflammation, W-peptide was applied intranasally or i.p. to the mice during allergen challenge (Fig. 7A). Interestingly, therapeutic effects were different between intranasal and i.p. routes of W-peptide application. Intranasal application of W-peptide significantly reduced both AHR and airway inflammation, whereas i.p. application had no therapeutic effects (Fig. 7B, 7C). In terms of proinflammatory mediator release, the former treatment significantly inhibited both Th1 and Th17 mediators (IP-10 and IL-17, respectively), whereas the latter treatment did not (Fig. 7D). Together, these results suggest that FPR activation through the topical route is a good therapeutic target for treatment of noneosinophilic asthma subtype.

Discussion

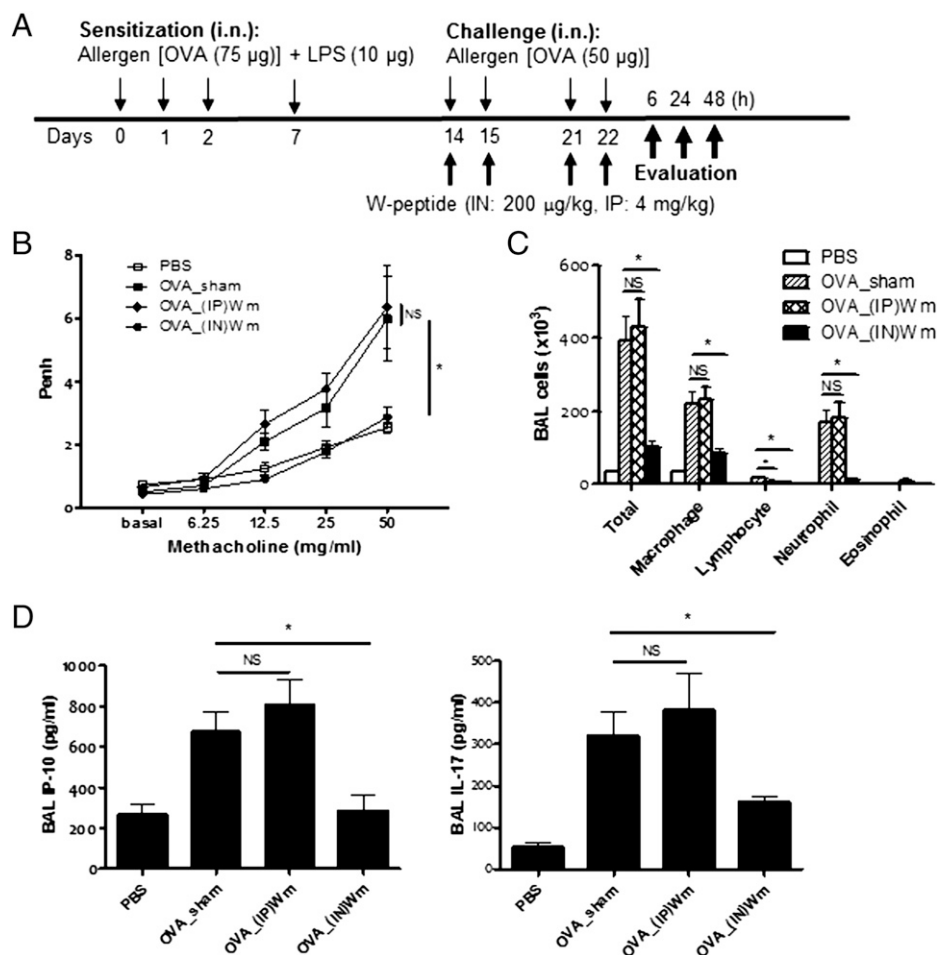
The significance of FPRs as pattern recognition receptors has been well established with respect to host defense (7, 8). Moreover, ac-

tivation of FPRs using its synthetic agonist modulated systemic inflammation and immune responses in a sepsis model (11). In the current study, we demonstrated that airway activation of FPRs during sensitization significantly inhibited lung inflammation accompanied by downregulation of mediator release, which could exacerbate inflammation. In terms of the immune response, airway activation of FPRs reduced Th1 and Th17 adaptive-immune responses in noneosinophilic asthma. These results indicated that airway activation of FPRs could modulate innate, as well as adaptive, immune responses, and synthetic agonists of FPRs, such as W-peptide, might be promising therapeutic molecules that could treat severe asthma characterized by dominant Th1 and Th17 phenotypes.

Our group previously reported that VEGF induced by LPS plays a key role in the priming and polarization of T cells (25). With respect to T cell polarization, polarizing cytokines, such as IL-12 and IL-6, released from DCs enhanced by VEGF are essential for the polarization of naive T cells into Th1 and Th17 cells, respectively (24, 25). The present study showed that airway activation of FPRs downregulated Th1 and Th17 cell responses in the lung. We also found that the production of IL-12 and IL-6 from lung DCs enhanced by LPS was reversed by W-peptide treatment, although VEGF production was not affected. These data indicated that airway activation of FPRs modulates the production of Th1- and Th17-polarizing cytokines from lung DCs, which results in downregulation of Th1 and Th17 cell responses.

In addition to the inhibitory effects of FPRs activation on polarizing cytokine production, the current study showed that airway activation of FPRs by W-peptide reduced the DC maturation induced by LPS. These results are supported by previous

FIGURE 7. Airway activation of FPRs during allergen challenge inhibits AHR and airway inflammation induced by LPS-containing allergen. **A**, Study protocol. Mice ($n = 5/\text{group}$) were sensitized with LPS-containing OVA on days 0, 1, 2, and 7 and then challenged with OVA alone on days 14, 15, 21, and 22. W-peptide was applied intranasally (200 $\mu\text{g}/\text{kg}$) or i.p. (4 mg/kg) during OVA challenge. **B**, AHR 24 h after the last challenge. **C**, BAL cellularity 48 h after the last challenge. **D**, Levels of IP-10 (left panel) and IL-17 (right panel) in BAL fluids obtained 48 and 6 h after the last challenge, respectively. * $p < 0.05$.



data showing that in vitro W-peptide treatment inhibited DC maturation (27). Interestingly, we found that W-peptide blocked lung DC migration into lung-draining LNs and that allergen-specific T cell proliferation in LNs was abolished by W-peptide treatment. These findings suggest that the inhibition of T cell proliferation by W-peptide treatment is mediated by the inhibition of Ag presentation by DCs. In the current study, we focused on the change in allergen-specific T cell responses mediated by DCs. There is a report that annexin-1, the endogenous agonist of FPRL1, enhanced T cell proliferation and skewing of Th1 cells through the direct activation of FPRL1 on T cells (28). Therefore, additional investigations are needed to examine the effects of W-peptide on T cells, because FPRs are also expressed on T cells.

FPRs are known as classical chemoattractant receptors that induce leukocyte chemotactic migration (7, 8). However, previous data showed that activation of FPRs inhibited migration of neutrophils through the desensitization of other chemokine receptors (29, 30). As endogenous agonists, LXA4 and its metabolite 15-epi-LXA4 bind to FPRL1 and act as potent anti-inflammatory molecules that inhibit neutrophil chemotaxis, adherence, diapedesis, cytokine production, and superoxide generation (16, 31). The present data indicated that the infiltration of inflammatory cells, such as neutrophils, macrophages, lymphocytes, and DCs, into the lung during innate immune responses induced by intranasal LPS application was blocked by W-peptide, with downregulation of proinflammatory cytokines in BAL fluids. Furthermore, with respect to the adaptive immune response, recruitment of T cells into the lung (both Th1 and Th17 cells) was reduced with W-peptide treatment. Taken together, these data suggest that W-peptide reduces the recruitment of inflammatory cells via downregulation of proinflammatory mediator production induced by LPS; however, W-peptide can induce infiltration of inflammatory cells.

In addition to the large amount of data on the anti-inflammatory effect of endogenous agonists of FPRs, such as LXA4 or annexin-1 (14, 15, 18, 31), research on FPR activation by W-peptide has focused on its role in microbe defense by innate immune responses (11). It has been unclear whether the activation of FPRs by W-peptide reduces Th1 and Th17 cell responses. Previous data showed that systemic application of W-peptide, as an adjuvant of vaccination, increased the number of IFN- γ -producing CD8⁺ T cells (32). However, the current study showed that airway activation of FPRs by W-peptide during both allergen sensitization and challenge inhibited both Th1 and Th17 cell responses induced by LPS-containing allergens in the lung. These data suggest that the effects of W-peptide on the modulation of adaptive immune responses may differ based on routes of administration.

In conclusion, the current study demonstrated that airway activation of FPRs with W-peptide effectively prevents the development of allergic inflammation induced by inhalation of LPS-containing allergens. This effect is mediated by multiple mechanisms: inhibition of T cell priming, which is mediated by the inhibition of DC infiltration, maturation, and migration; and inhibition of both Th1 and Th17 polarization, which is partly mediated by the downregulation of polarizing cytokine production. Thus, FPRs in the lung might be a novel therapeutic target for the treatment of noneosinophilic asthma.

Acknowledgments

We thank Jee-In Lim for providing secretarial assistance and members of the POSTECH animal facility for help with the animal experiments.

Disclosures

The authors have no financial conflicts of interest.

References

- Busse, W. W., and R. F. Lemanske, Jr. 2001. Asthma. *N. Engl. J. Med.* 344: 350–362.
- Bochner, B. S., B. J. Udem, and L. M. Lichtenstein. 1994. Immunological aspects of allergic asthma. *Annu. Rev. Immunol.* 12: 295–335.
- Cohn, L., J. A. Elias, and G. L. Chupp. 2004. Asthma: mechanisms of disease persistence and progression. *Annu. Rev. Immunol.* 22: 789–815.
- Magnan, A. O., L. G. Mély, C. A. Camilla, M. M. Badier, F. A. Montero-Julian, C. M. Guillot, B. B. Casano, S. J. Prato, V. Fert, P. Bongrand, and D. Vervloet. 2000. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN- γ -producing CD8(+) T cells in asthma. *Am. J. Respir. Crit. Care Med.* 161: 1790–1796.
- Cho, S. H., L. A. Stanciu, S. T. Holgate, and S. L. Johnston. 2005. Increased interleukin-4, interleukin-5, and interferon- γ in airway CD4+ and CD8+ T cells in atopic asthma. *Am. J. Respir. Crit. Care Med.* 171: 224–230.
- Kim, Y. K., Y. K. Oh, S. G. Jeon, H. W. Park, S. Y. Lee, E. Y. Chun, B. Bang, H. S. Lee, M. H. Oh, Y. S. Kim, et al. 2007. Airway exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma. *J. Immunol.* 178: 5375–5382.
- Migeotte, I., D. Communi, and M. Parmentier. 2006. Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. *Cytokine Growth Factor Rev.* 17: 501–519.
- Le, Y., P. M. Murphy, and J. M. Wang. 2002. Formyl-peptide receptors revisited. *Trends Immunol.* 23: 541–548.
- Panaro, M. A., A. Acquafredda, M. Sisto, S. Lisi, A. B. Maffione, and V. Mitolo. 2006. Biological role of the N-formyl peptide receptors. *Immunopharmacol. Immunotoxicol.* 28: 103–127.
- Gao, J. L., E. J. Lee, and P. M. Murphy. 1999. Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. *J. Exp. Med.* 189: 657–662.
- Kim, S. K., Y. K. Kim, H. Y. Lee, Y. S. Kim, S. G. Jeon, S. H. Baek, D. K. Song, S. H. Ryu, and Y. S. Bae. 2010. The agonists of formyl peptide receptors prevent development of severe sepsis after microbial infection. *J. Immunol.* 185: 4302–4310.
- Lee, M. S., S. A. Yoo, C. S. Cho, P. G. Suh, W. U. Kim, and S. H. Ryu. 2006. Serum amyloid A binding to formyl peptide receptor-like 1 induces synovial hyperplasia and angiogenesis. *J. Immunol.* 177: 5585–5594.
- Dufton, N., R. Hannon, V. Brancalione, J. Dalli, H. B. Patel, M. Gray, F. D'Acquisto, J. C. Buckingham, M. Perretti, and R. J. Flower. 2010. Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation. *J. Immunol.* 184: 2611–2619.
- Chiang, N., M. Arita, and C. N. Serhan. 2005. Anti-inflammatory circuitry: lipoxin, aspirin-triggered lipoxins and their receptor ALX. *Prostaglandins Leukot. Essent. Fatty Acids* 73: 163–177.
- Chiang, N., C. N. Serhan, S. E. Dahlén, J. M. Drazen, D. W. Hay, G. E. Rovati, T. Shimizu, T. Yokomizo, and C. Brink. 2006. The lipoxin receptor ALX: potent ligand-specific and stereoselective actions in vivo. *Pharmacol. Rev.* 58: 463–487.
- Planagumà, A., S. Kazani, G. Marigowda, O. Haworth, T. J. Mariani, E. Israel, E. R. Blecker, D. Curran-Everett, S. C. Erzurum, W. J. Calhoun, et al. 2008. Airway lipoxin A4 generation and lipoxin A4 receptor expression are decreased in severe asthma. *Am. J. Respir. Crit. Care Med.* 178: 574–582.
- Levy, B. D., G. T. De Sanctis, P. R. Devchand, E. Kim, K. Ackerman, B. A. Schmidt, W. Szczeklik, J. M. Drazen, and C. N. Serhan. 2002. Multi-pronged inhibition of airway hyper-responsiveness and inflammation by lipoxin A(4). *Nat. Med.* 8: 1018–1023.
- Vachier, I., C. Bonnans, C. Chavis, M. Farce, P. Godard, J. Bousquet, and P. Chané. 2005. Severe asthma is associated with a loss of LX4, an endogenous anti-inflammatory compound. *J. Allergy Clin. Immunol.* 115: 55–60.
- Bae, Y. S., Y. Kim, Y. Kim, J. H. Kim, P. G. Suh, and S. H. Ryu. 1999. Trp-Lys-Tyr-Met-Val-D-Met is a chemoattractant for human phagocytic cells. *J. Leukoc. Biol.* 66: 915–922.
- Bae, Y. S., S. A. Ju, J. Y. Kim, J. K. Seo, S. H. Baek, J. Y. Kwak, B. S. Kim, P. G. Suh, and S. H. Ryu. 1999. Trp-Lys-Tyr-Met-Val-D-Met stimulates superoxide generation and killing of *Staphylococcus aureus* via phospholipase D activation in human monocytes. *J. Leukoc. Biol.* 65: 241–248.
- Kim, H., E. K. Noh, E. J. Lee, J. H. Baek, S. J. Shin, J. H. Park, K. H. Lee, and Y. J. Min. 2008. Enhanced bactericidal function by WKYMVm in patients with acute leukemia. *Leuk. Res.* 32: 717–725.
- Dahlgren, C., T. Christophe, F. Boulay, P. N. Madianos, M. J. Rabet, and A. Karlsson. 2000. The synthetic chemoattractant Trp-Lys-Tyr-Met-Val-DMet activates neutrophils preferentially through the lipoxin A(4) receptor. *Blood* 95: 1810–1818.
- Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest.* 103: 779–788.
- Kim, Y. S., S. J. Choi, Y. M. Tae, B. J. Lee, S. G. Jeon, S. Y. Oh, Y. S. Cho, Z. Zhu, and Y. K. Kim. 2010. Distinct roles of vascular endothelial growth factor receptor-1- and receptor-2-mediated signaling in T cell priming and Th17 polarization to lipopolysaccharide-containing allergens in the lung. *J. Immunol.* 185: 5648–5655.

25. Kim, Y. S., S. W. Hong, J. P. Choi, T. S. Shin, H. G. Moon, E. J. Choi, S. G. Jeon, S. Y. Oh, Y. S. Gho, Z. Zhu, and Y. K. Kim. 2009. Vascular endothelial growth factor is a key mediator in the development of T cell priming and its polarization to type 1 and type 17 T helper cells in the airways. *J. Immunol.* 183: 5113–5120.
26. Le, Y., W. Gong, B. Li, N. M. Dunlop, W. Shen, S. B. Su, R. D. Ye, and J. M. Wang. 1999. Utilization of two seven-transmembrane, G protein-coupled receptors, formyl peptide receptor-like 1 and formyl peptide receptor, by the synthetic hexapeptide WKYMVm for human phagocyte activation. *J. Immunol.* 163: 6777–6784.
27. Kang, H. K., H. Y. Lee, M. K. Kim, K. S. Park, Y. M. Park, J. Y. Kwak, and Y. S. Bae. 2005. The synthetic peptide Trp-Lys-Tyr-Met-Val-D-Met inhibits human monocyte-derived dendritic cell maturation via formyl peptide receptor and formyl peptide receptor-like 2. *J. Immunol.* 175: 685–692.
28. D'Acquisto, F., A. Merghani, E. Lecona, G. Rosignoli, K. Raza, C. D. Buckley, R. J. Flower, and M. Perretti. 2007. Annexin-1 modulates T-cell activation and differentiation. *Blood* 109: 1095–1102.
29. Sogawa, Y., T. Ohyama, H. Maeda, and K. Hirahara. 2011. Inhibition of neutrophil migration in mice by mouse formyl peptide receptors 1 and 2 dual agonist: indication of cross-desensitization in vivo. *Immunology* 132: 441–450.
30. Sogawa, Y., T. Ohyama, H. Maeda, and K. Hirahara. 2011. Formyl peptide receptor 1 and 2 dual agonist inhibits human neutrophil chemotaxis by the induction of chemoattractant receptor cross-desensitization. *J. Pharmacol. Sci.* 115: 63–68.
31. Serhan, C. N., S. Yacoubian, and R. Yang. 2008. Anti-inflammatory and pro-resolving lipid mediators. *Annu. Rev. Pathol.* 3: 279–312.
32. Lee, C. G., S. Y. Choi, S. H. Park, K. S. Park, S. H. Ryu, and Y. C. Sung. 2005. The synthetic peptide Trp-Lys-Tyr-Met-Val-D-Met as a novel adjuvant for DNA vaccine. *Vaccine* 23: 4703–4710.

Supplementary Figure 1. Airway activation of FPRs inhibits *in vivo* production of inflammatory mediators induced by airway exposure to LPS. W-peptide (10-1000 µg/kg) was applied intranasally to mice (n=5 in each group) with LPS (10 µg), and BAL cytokines including TNF- α , IL-1 β , IL-12p40, IL-23, MCP-1, and MIP-1 α were measured 24 h after daily application for three days.

