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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

You-Sun Kim,* Seng-Jin Choi,* You-Me Tae,* Byung-Jae Lee,[†] Seong Gyu Jeon,* Sun-Young Oh,[‡] Yong Song Gho,* Zhou Zhu,[‡] and Yoon-Keun Kim*

Vascular endothelial growth factor (VEGF) is a key mediator in the development of airway immune dysfunction to inhaled allergens. However, the exact role of its receptors-mediated signaling is controversial. In this study, we evaluated the role of VEGF receptor (VEGFR)-1– and VEGFR-2–mediated signaling in T cell priming and polarization in the context of inhalation of LPS-containing allergens. A murine asthma model of mixed Th1 and Th17 cell responses was generated using intranasal sensitization with LPS-containing allergens. Pharmacologic intervention was performed during sensitization. In vivo production of VEGF and Th1- and Th17-polarizing cytokines (IL-12p70 and IL-6, respectively) were upregulated by airway exposure to LPS. Pharmacological intervention with a VEGFR-2–neutralizing Ab (anti-Flk1 mAb) abolished the production of IL-6 (but not IL-12p70) and the subsequent development of allergen-specific Th17 cell response. On the other hand, blocking VEGFR-1 signaling with a VEGFR-1 antagonist (anti-Flt1 hexapeptide) did not affect the production of IL-12p70 and IL-6. However, blocking VEGFR-1 signaling resulted in T cell tolerance rather than priming, mainly by inhibiting the maturation of lung dendritic cells, and their migration into lung-draining lymph nodes. These results suggest that T cell priming to LPS-containing allergens depends on VEGFR-1–mediated signaling, and the subsequent Th17 polarization depends on VEGFR-2 signaling. *The Journal of Immunology*, 2010, 185: 000–000.

T cell priming to inhaled allergens is critical in the initiation and orchestration of the inflammatory response in asthmatic airways (1). LPS, a cell wall component of Gram-negative bacteria, is ubiquitous in the environment, including in household dust allergens such as dust mites (2). LPS is a pathogen-associated molecular pattern that induces proinflammatory and immune-modulating mediators (2, 3). Previous studies have demonstrated that inhalation of LPS-containing inert allergens induces robust allergen-specific Th1 and Th17 cell responses and subsequent inflammatory responses in the airways, whereas inhalation of allergen alone is insufficient (4).

Vascular endothelial growth factor (VEGF) was originally described as a vascular permeability factor, based on its ability to generate tissue edema (5). Increased levels of VEGF have been detected in tissues and biological samples from patients with

asthma (6). VEGF binds to three receptors: VEGF receptor (VEGFR)-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4) (7, 8). VEGFR-1 and VEGFR-2 are highly expressed on vascular endothelial cells (9). VEGFR-1 is also expressed on non-endothelial cells such as monocytes and macrophages, and its signaling may be related to the migration of inflammatory cells (10). In a previous study, we found that VEGF is a key mediator in the development of T cell priming and Th1 and Th17 cell responses in the airways upon exposure to LPS-containing allergens (11). However, the molecular mechanisms by which VEGF regulates these processes are still not elucidated. In the current study, we evaluated the role of VEGFR-1– and VEGFR-2–mediated signaling in lung dendritic cells (DCs) during the development of T cell priming and subsequent Th1 or Th17 polarization in mice after exposure to LPS-containing allergens.

Materials and Methods

Mice and reagents

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred in pathogen-free facilities at the Pohang University of Science and Technology. All live animal experiments were approved by the Pohang University of Science and Technology Ethics Committee.

LPS and OVA were purchased from Calbiochem (La Jolla, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Anti-Flt1 peptide (Gly-Asn-Gln-Trp-Phe-Ile) and reverse peptide (Ile-Phe-Trp-Gln-Asn-Gly) were purchased from Pepton (Daejeon, Korea). Anti-mouse VEGFR-2 (anti-Flk1) mAb and IgG isotype control were purchased from R&D Systems (Minneapolis, MN).

Protocol for a noneosinophilic asthma model and pharmacologic intervention

A mouse model with noneosinophilic asthma (high-dose LPS model) was generated as described previously (4). Briefly, 6-wk-old mice were sens-

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Abbreviations used in this paper: BAL, bronchoalveolar lavage; DC, dendritic cell; IP-10, IFN- γ -inducible protein-10; LN, lymph node; MFI, mean fluorescence intensity; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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itized intranasally with 75 μ g LPS-depleted OVA plus 10 μ g LPS on days 0, 1, 2, and 7 and then challenged with OVA alone on days 14, 15, 21, and 22. As a negative control, mice were sensitized with OVA and then challenged with PBS. Innate immune responses to LPS were evaluated at different time points after one or three allergen sensitizations, and allergen-specific adaptive immune responses were examined 24 h after the last allergen challenge on day 22.

Pharmacologic intervention was conducted using an anti-Flt1 hexapeptide as a VEGFR-1 antagonist (12) and an anti-Flk1-neutralizing mAb as a VEGFR-2 antagonist. To block VEGFR-1- and VEGFR-2-mediated signaling, mice were i.p. injected with 200 μ g/mouse anti-Flt1 hexapeptide, which had been selected after dose-response experiments, and were i.p. injected with 0.1, 1, or 10 μ g/mouse anti-VEGFR-2 (anti-Flk1) mAb on days 0, 1, 2, and 7, respectively.

Cellularity in bronchoalveolar lavage fluids

Bronchoalveolar lavage (BAL) cellularity for the evaluation of lung inflammation was analyzed as described previously (4). Briefly, after counting the total cells in the BAL samples, the cell pellet was diluted with 200 μ l PBS. BAL differential cell count was determined by Diff-Quik (Dade Behring, Newark, DE) staining and counting 300 inflammatory cells; inflammatory cells were classified as macrophages, lymphocytes, neutrophils, or eosinophils.

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-PE, anti-I-A-FITC, anti-CD11c-allophycocyanin, anti-CD40-PE, anti-CD80-PE, and anti-CD86-PE. Cells were analyzed on a FACSCalibur (BD Biosciences) flow cytometer with CellQuest software.

Migration of lung DCs

To investigate the migration of lung DCs, 60 μ l FITC-OVA (10 mg/ml; Sigma-Aldrich) was administered intranasally, along with the VEGFR-1 inhibitor (anti-Flt1 peptide) or sham (reverse-sequenced peptide) (14). Control mice received 60 μ l vehicle. Twenty-four hours after administration, the migration of lung DCs was determined by detecting CD11c⁺ MHC class II^{high} cells carrying FITC⁺ in lung-draining lymph nodes (LNs) by FACS.

T cell proliferation in lung-draining LNs

For the T cell proliferation assay, cells from lung-draining LNs were collected and then 4×10^6 cells/ml were incubated in 48-well plates at 37°C in medium alone (RPMI 1640 containing 10% FBS and antibiotics) or with 100 μ g/ml OVA for 72 h. [³H]thymidine (1 μ Ci/well) was added during the last 18 h, and then cellular thymidine incorporation was measured.

Characterization of T cell subsets

To characterize T cell subsets, cells were collected and then 4×10^6 cells/ml were incubated in 48-well plates at 37°C in medium alone (RPMI 1640 containing 10% FBS and antibiotics) or with 100 μ g/ml OVA for 72 h.

VEGFR-1 and VEGFR-2 expression in lung DCs

Single-cell suspensions isolated from lung tissues were prepared as described previously (13). To determine VEGFR-1 and VEGFR-2 expression in lung DCs, lung cells were stained with anti-VEGFR-1 (R&D Systems), anti-VEGFR-2 (BioLegend, San Diego, CA), and anti-CD11c Abs (BD Biosciences) and analyzed by FACS.

Ex vivo stimulation of lung CD11c⁺ DCs

Lung CD11c⁺ DCs were isolated using an AutoMACS system with anti-CD11c Abs. Isolated lung DCs were stimulated with or without LPS (1 μ g/ml) in the presence of the anti-Flt1 peptide (100 μ M), the reverse peptide (100 μ M), or PBS. Supernatants were harvested 6 h later, and the levels of IL-12p70 and IL-6 were measured.

Intracellular staining of T cell-regional LNs

Cells isolated from regional LNs (2×10^6 /ml) were incubated in 48-well plates coated with anti-CD3 plus anti-CD28 Abs (1 μ g/ml each; eBioscience, San Diego, CA) at 37°C for 6 h and then restimulated 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 and permeabilized 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, and anti-IL-4-PE; BD Biosciences) for 30 min at room temperature and analyzed on a FACSCalibur flow cytometer using CellQuest software.

Measurement of cytokine production

Cytokine levels were measured by ELISA, according to the manufacturer's instructions (R&D Systems).

Statistical analysis

ANOVA was used to determine the significant differences among all groups. Significant differences among treatments were assessed using the Student *t* test, ANOVA, or a Wilcoxon rank-sum test. ANOVA was used for multiple comparisons, and when significant differences were found, an individual *t* test or a Wilcoxon rank-sum test was performed between pairs of groups. Statistical significance was set at *p* < 0.05.

Results

Innate immune responses in the airways after LPS stimulation

To begin to understand the immunological mechanisms underlying our previous observation that inhalation of LPS (10 μ g)-containing allergens induced mixed Th1 and Th17 cell responses (4, 11), we evaluated the innate immune response induced by LPS in the airways. Lung inflammation and production of proinflammatory and immune-modulating mediators were evaluated at various time points after airway application of LPS (10 μ g). The number of inflammatory cells in the BAL fluid (BAL cellularity) significantly increased 8 h after LPS application as compared with the levels before stimulation (Fig. 1A). Analyses of proinflammatory cytokines and mediators in the airways showed that, relative to the basal levels, the levels of IFN- γ in the BAL fluids increased significantly from 2 to 8 h after LPS application but returned to the baseline in 24 h. However, BAL IL-12p70 showed a slower pattern, increasing from 8 to 24 h after LPS application (Fig. 1B). The levels of TNF- α and VEGF in BAL fluid increased significantly starting 2 h after LPS application (Fig. 1C). Additionally, the levels of Th17-polarizing cytokine IL-6 in the BAL fluid increased significantly 8 h after LPS application (Fig. 1D). These findings indicate that airway application of 10 μ g of LPS induces lung inflammation and production of proinflammatory and immune-modulating mediators, and that the production of VEGF as well as IFN- γ and TNF- α precedes that of mediators of Th1- or Th17-polarizing cytokines (IL-12p70 and IL-6, respectively).

Role of VEGFR-2-mediated signaling in the development of LPS-induced innate immune response

To evaluate the role of VEGFR-2 signaling in the LPS-induced innate immune response, we treated mice with anti-Flk1 mAb during OVA allergen sensitization in the LPS model and then evaluated the cellular and cytokine responses 24 h after the sensitization (Fig. 2A). Dosing experiments showed that lung inflammation induced by LPS was blocked by treatment with the inhibitor in a dose-related manner (Fig. 2B). To further evaluate the role of VEGFR-2 signaling, we injected mice i.p. with 10 μ g/mouse anti-Flk1 mAb during sensitization. The in vivo production of TNF- α and IL-6 from the lung was inhibited by anti-Flk1 mAb treatment in OVA/LPS-sensitized mice (Fig. 2C); however, that of IL-12p70 was unaffected by the VEGFR-2 inhibitor treatment (data not shown). Additionally, the in vitro production of IL-6 from lung DCs after LPS stimulation was inhibited by treatment of anti-Flk1 mAb compared with IgG isotype control and PBS, whereas this production was similar in IgG isotype control and PBS-treated groups (data not shown). In terms of the expression of VEGFR-2 on APCs, this expression was noted in <1% of lung DCs (CD11c⁺ cells), which is further inhibited by LPS sensitization (Fig. 2D). Additionally, the expression of costimulatory

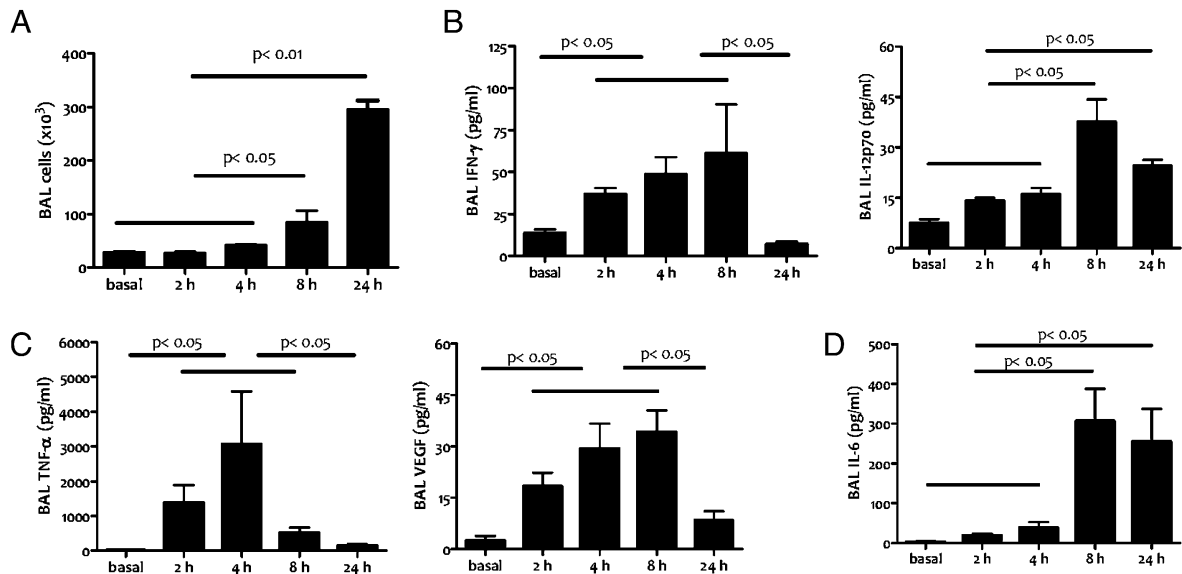


FIGURE 1. Airway exposure to LPS induces innate immune responses in the airways characterized by inflammation and production of proinflammatory and immune-modulating mediators. For all figures, evaluation ($n = 5$ mice/group) was performed at different time points after one airway application of 10 μ g of LPS. A, BAL cellularity before and different time points after the sensitization. Levels of BAL proteins before and different time points after the sensitization are described in B–D; that is, Th1-polarizing cytokines IFN- γ and IL-12p70 (B), TNF- α and VEGF (C), and Th17-polarizing cytokine IL-6 (D).

molecules, such as CD40 and CD80, on lung DCs (CD11c⁺ cells) was not affected by treatment with the VEGFR-2 inhibitor (Fig. 2E). Taken together, these findings suggest that VEGFR-2-mediated signaling is important in the development of lung inflammation and the production of proinflammatory and/or immune modulating mediators, such as TNF- α and IL-6, but not in the maturation of APCs.

Role of VEGFR-2-mediated signaling in the development of allergen-specific Th17 polarization

Based on the finding that blockade of VEGFR-2 signaling inhibits IL-6 production during sensitization, we further evaluated the role of VEGFR-2 signaling in the development of the allergen-specific Th17 cell response in the high-dose LPS model. Mice were treated with 10 μ g of anti-Flk1 mAb during allergen sensitization and then evaluated 24 h after the final allergen challenge (Fig. 3A). These experiments demonstrated that BAL cellularity decreased significantly in OVA/LPS-sensitized, inhibitor-treated mice compared with sham-treated mice sensitized in the same manner (Fig. 3B). Moreover, the numbers of IL-17-producing CD4⁺ and CD8⁺ T cells from lung-draining LNs were significantly reduced in the former group compared with the latter, although the numbers of IFN- γ -producing T cells were similar in the two groups (Fig. 3C). Moreover, the levels of IL-17 in the BAL fluid decreased significantly in the former group compared with the latter, although the BAL levels of IFN- γ -inducible protein-10 (IP-10) were similar between the two groups (Fig. 3D). These findings indicate that, upon exposure to LPS-containing allergens, VEGFR-2-mediated signaling plays a key role in the differentiation of primed T cells to Th17 cells, but not Th1 cells, mainly through regulation of IL-6 production by lung DCs during Ag presentation.

VEGFR-1-mediated signaling in the development of allergen-specific Th1 or Th17 cell responses

To evaluate the role of VEGFR-1-mediated signaling in the development of Th1 or Th17 cell responses, mice were injected i.p. with 200 μ g/mouse anti-Flt1 hexapeptide, which had been selected after dosing experiments, or with the same dose of the reverse-

sequenced hexapeptide as a control during sensitization in the LPS model. Adaptive immune responses were evaluated 24 h after the final airway challenge with OVA alone. An examination of BAL cellularity revealed that recruitment of inflammatory cells into the lung was significantly inhibited by treatment with anti-Flt1 peptide, but not with the reverse-sequenced peptide (Fig. 4A). Additionally, the levels of IP-10 (a key IFN- γ downstream mediator) and IL-17 were significantly lower in the anti-Flt1 peptide group compared with the reverse peptide control group (Fig. 4B). To further investigate the role of VEGFR-1-mediated signaling in the expression of allergen-specific Th1 and Th17 cell responses, the production of Th1 and Th17 cytokines in cells isolated from the lungs and lung-draining LNs on day 23 was determined 72 h after in vitro restimulation with OVA (100 μ g/ml). In response to OVA stimulation, production of both IFN- γ and IL-17 in lung cells and in lung-draining LN immune cells from OVA plus LPS-sensitized, anti-Flt1 peptide-treated mice was lower compared with that by cells from OVA plus LPS-sensitized, reverse peptide-treated mice (Fig. 4C, 4D). Taken together, these data suggest that blocking VEGFR-1-mediated signaling during LPS-containing allergen sensitization inhibited the development of both allergen-specific Th1 and Th17 responses.

VEGFR-1-mediated signaling in the production of Th1- and Th17-polarizing cytokines in mice exposed to LPS-containing allergen

It is well recognized that IL-12p70 and IL-6 produced by DCs are essential in the differentiation of Th1 and Th17 cells, respectively (4, 11). To investigate the involvement of VEGFRs in DCs, we determined the expression of VEGFR-1 and VEGFR-2 in lung DCs from allergen-free mice. Flow cytometry data revealed that VEGFR-1, but not VEGFR-2, was highly expressed on lung DCs (Fig. 5A). These findings led to the notion that VEGFR-1-mediated signaling in lung DCs may be important in the production of these polarizing cytokines. However, the present study showed that ex vivo production of IL-12p70 and IL-6 by lung DCs in response to LPS stimulation was not inhibited by treatment with the anti-Flt1 peptide compared with the reverse peptide and PBS

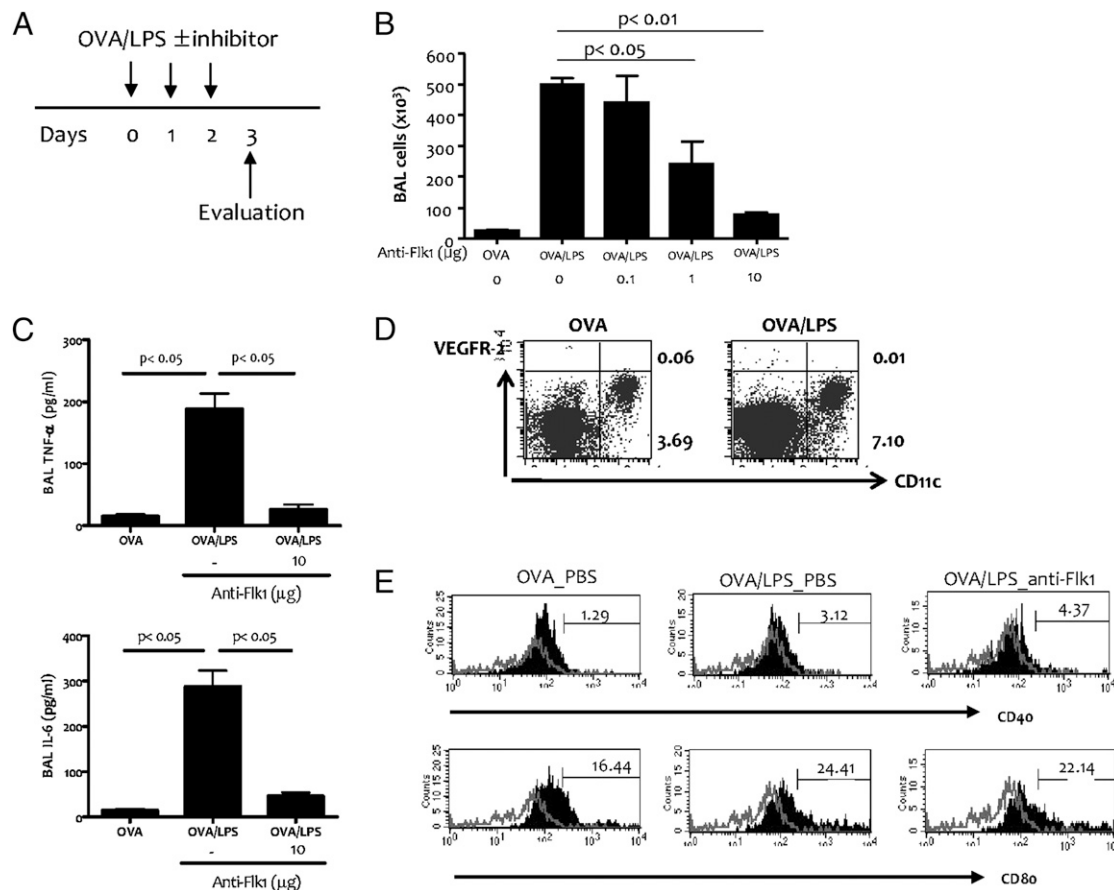


FIGURE 2. Lung inflammation and the production of proinflammatory and/or immune-modulating mediators induced by LPS depends on VEGFR-2 signaling. For all figures, evaluation ($n = 5$ mice/group) was performed 24 h after sensitization three times on days 0, 1, and 2 with or without anti-Flk1 mAb treatment. OVA indicates mice sensitized with OVA alone; OVA/LPS indicates mice sensitized with OVA plus LPS (10 μ g). **A**, Study protocol for the evaluation of innate immune response. **B**, BAL cellularity following treatment with 0.1, 1, and 10 μ g/mouse anti-Flk1 mAb. **C**, Levels of TNF- α and IL-6 in BAL fluids following treatment with 10 μ g/mouse anti-Flk1 mAb. **D**, Expression of VEGFR-2 in lung CD11c⁺ (DCs) from mice sensitized with OVA alone or OVA plus LPS. **E**, Expression of costimulatory molecules (CD40 and CD80) in lung CD11c⁺ cells (DCs).

(Fig. 5B). Moreover, in vivo production of IL-12p70 and IL-6 stimulated by sensitization with LPS-containing allergen for 3 d was not affected by treatment with anti-Flt1 peptide (200 μ g) (Fig. 5C). Additionally, lung infiltration of inflammatory cells induced by LPS exposure alone was not affected by anti-Flt1 peptide treatment (Fig. 5D). Taken together, these data suggest that neither the production of Th1- and Th17-polarizing cytokines nor lung inflammation induced by LPS exposure in the airways was related to VEGFR-1 signaling.

VEGFR-1-mediated signaling in DC maturation in the airways

DC maturation is an important initial step in priming naive T cells to a specific Ag. Anti-Flt1 (200 μ g) and the reverse peptide (200 μ g) were used to evaluate the role of VEGFR-1-mediated signaling in the maturation of lung DCs, as shown in Fig. 2A. The number of lung CD11c⁺ cells was lower in OVA plus LPS-sensitized, anti-Flt1 peptide-treated mice compared with OVA plus LPS-sensitized, reverse peptide-treated mice; furthermore, the number of MHC class II^{high}CD11c⁺ cells was also lower in OVA plus LPS-sensitized mice treated with anti-Flt1 peptide compared with those treated with the control peptide (Fig. 6A), whereas this number was similar in OVA plus LPS-sensitized mice treated with the reverse peptide and with PBS (data not shown). Moreover, the expression of costimulatory molecules such as CD40, CD80, and CD86 was lower in both lung DCs (CD11c⁺ cells) and lung myeloid DCs (CD11c⁺CD11b⁺ cells) of OVA plus LPS-sensitized mice treated with anti-Flt1 peptide

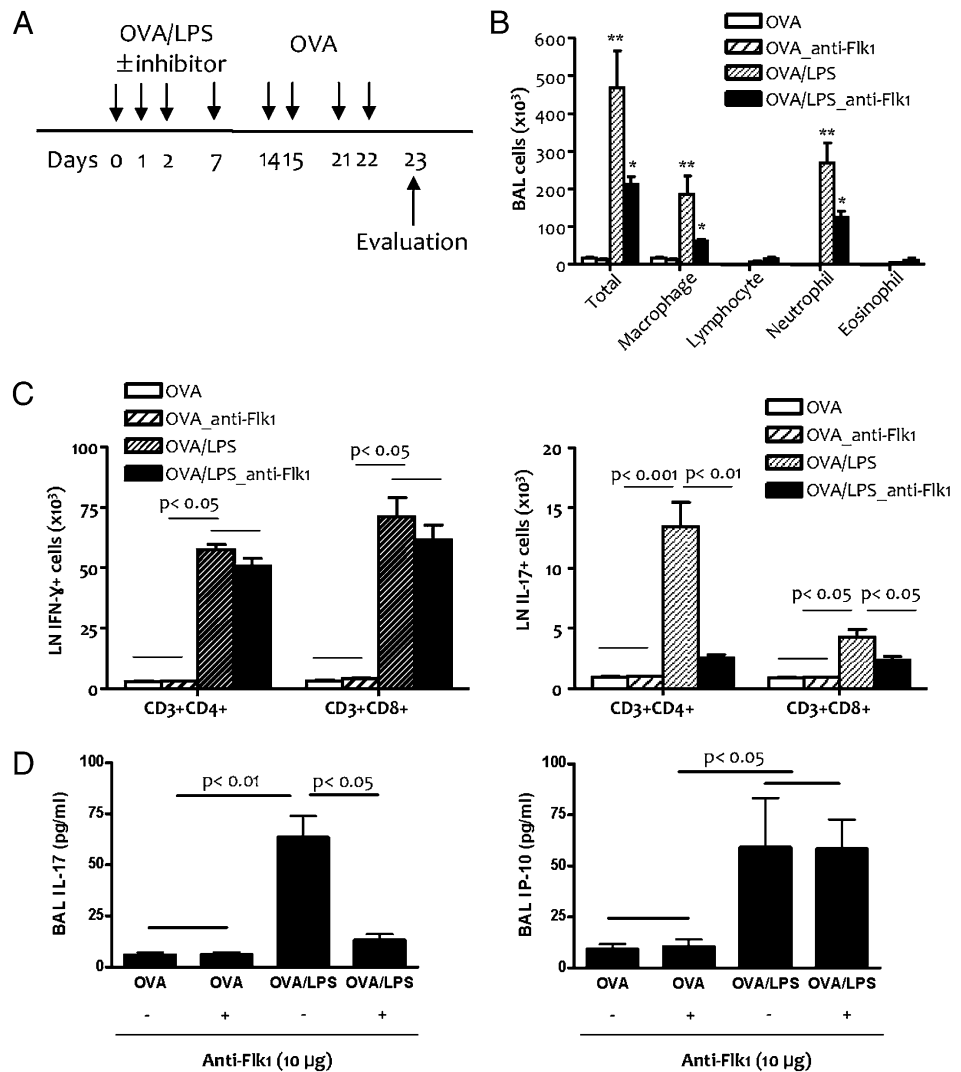
compared with those treated with the reverse peptide (Fig. 6B, 6C). These findings suggest that VEGFR-1-mediated signaling was important for the maturation of lung DCs in mice exposed to LPS-containing allergens.

VEGFR-1-mediated signaling in the migration of lung DCs into lung-draining LNs, followed by T cell priming

We focused on the expansion of naive CD4⁺ T cells in mice after exposure to LPS-containing allergen. The proliferation of OVA-specific CD4⁺ T cells in LNs was enhanced in OVA plus LPS-sensitized mice; however, proliferation was lower in OVA plus LPS-sensitized, anti-Flt1 peptide-treated mice compared with the former group (Fig. 6D). This finding suggests that VEGFR-1 signaling is important in T cell priming when exposed to LPS-containing allergens.

Previous evidence has indicated that allergen-contacted DCs in the airways mature quickly and migrate into the lung-draining LNs; the peak airway DC accumulation in regional LNs was reached 8 h after allergen uptake in the airways (13). To evaluate the role of VEGFR-1 signaling in the migration of DCs into lung-draining LNs, fluorescent-labeled OVA (FITC-OVA) and the anti-Flt1 or reverse peptide were administered intranasally to naive C57BL/6 mice. Fewer FITC-OVA-containing CD11c⁺ cells (DCs) were observed in the lung-draining LNs of mice treated with anti-Flt1 peptide than in mice treated with the reverse peptide (Fig. 6E).

FIGURE 3. VEGFR-2 signaling plays an essential role in the development of allergen-specific Th17 cell response following exposure to LPS-contaminated allergens. For all figures, evaluation ($n = 5$ mice/group) was performed 24 h after the final allergen challenge on day 22 with or without anti-Flk1 treatment (10 μ g/mouse). OVA indicates mice sensitized with OVA alone; OVA/LPS indicates mice sensitized with OVA plus LPS (10 μ g). **A**, Study protocol for the evaluation of adaptive immune response. **B**, BAL cellularity after the allergen challenge. $*p < 0.05$ compared with OVA; $**p < 0.05$ compared with the other groups. **C**, Numbers of IFN- γ - and IL-17-producing CD4 $^{+}$ and CD8 $^{+}$ from regional LN T cells 6 h after stimulation with anti-CD3 (1 μ g/ml) plus anti-CD28 Abs (1 μ g/ml). LN T cells were isolated from lung-draining LNs after the allergen challenge. **D**, Levels of IL-17 and IP-10 in BAL fluids after the allergen challenge.



These findings together suggest the importance of VEGFR-1 signaling in the migration of lung DCs into lung-draining LNs.

Discussion

Allergic inflammation can be induced by allergen inhalation, and T cell priming is critical for the initiation of airway immune dysfunction to inhaled allergens (4). Previously, we demonstrated that T cell priming and Th1 and Th17 cell responses to inhaled allergens could be induced by simultaneous inhalation of an allergen with LPS as an adjuvant (4, 11). VEGF is a potent mediator of vascular remodeling and angiogenesis in the lung (15). VEGF is found to be produced in LPS-induced innate response (16). Additionally, the overexpression of VEGF in the airways augments T cell priming to inhaled allergens along with enhanced expression of costimulatory molecules on lung DCs (15). Interestingly, our previous studies revealed that airway exposure to LPS induces the production of VEGF and that T cell priming and Th1 and Th17 responses to LPS-containing allergens depend on VEGF (11). In the present study, in the context of inhalation of LPS-containing allergens, we provided evidence that the maturation of lung DCs, the migration of DCs into lung-draining LNs, and T cell priming depend on VEGFR-1-mediated signaling, whereas Th17 polarization depends on VEGFR-2-mediated signaling.

VEGFR-1 is expressed not only in vascular endothelial cells, but also in airway epithelial cells and inflammatory cells such as mono-

cytes and macrophages (9, 10). Meanwhile, VEGFR-2 is expressed mainly in vascular endothelial cells (9). Transgenic animal experiments have demonstrated that high levels of VEGF in the airways induce lung inflammation (15). Additionally, VEGF enhances adhesion molecules such as ICAM-1 in vascular endothelial cells via VEGFR-2 (17). Our present data show that lung infiltration of inflammatory cells induced by LPS depends on VEGFR-2, but not on VEGFR-1 signaling. Additionally, the production of the proinflammatory mediators, including TNF- α , was also related to VEGFR-2 signaling. Collectively, these findings suggest that VEGFR-2 signaling is important in the recruitment of inflammatory cells via the upregulation of expression of adhesion molecules in vascular endothelial cells and/or the production of proinflammatory mediators upon exposure to LPS-containing allergens.

When LPS-containing allergens are inhaled into the airways, the maturation of lung DCs is a key initial step in the priming of naive T cells. However, the exact role of VEGF and its receptor-mediated signaling in DC maturation has been controversial. Previous evidence has demonstrated that VEGF signaling through VEGFR-1 and VEGFR-2 plays a major role in abnormal DC differentiation and function in cancer (18); other evidence suggests that DC maturation is inhibited in cancer patients by the effects of VEGF (19). However, our previous transgenic studies indicated that high levels of VEGF in the airways can enhance the maturation of lung DCs (15). Consistent with the transgenic finding, we also showed

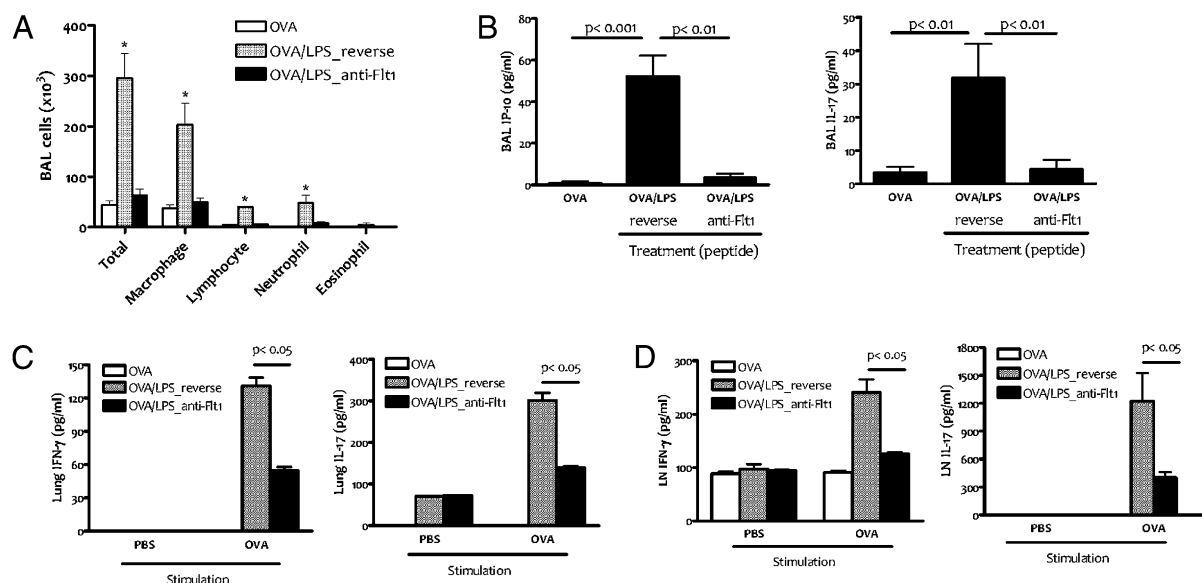


FIGURE 4. VEGFR-1 signaling is critical in the development of allergen-specific adaptive responses and subsequent inflammation. For all figures, evaluation ($n = 5$ mice/group) was performed 24 h after the final allergen challenge on day 22 with treatment of anti-VEGFR-1 inhibitor (anti-Flt1 peptide, 200 $\mu\text{g}/\text{mouse}$) or the same dose of reverse peptide during sensitization. OVA indicates mice sensitized with OVA alone; OVA/LPS indicates mice sensitized with OVA plus LPS (10 μg). **A**, BAL cellularity. $*p < 0.05$ compared with the other groups. **B**, Levels of IP-10 (a Th1-related cytokine) and IL-17 in BAL fluids. **C**, Levels of IFN- γ and IL-17 in supernatant from lung T cells 72 h after stimulation with PBS or OVA (100 $\mu\text{g}/\text{ml}$). Lung T cells were collected from total lung cells isolated after the allergen challenge. **D**, Levels of IFN- γ and IL-17 in supernatant from regional LN T cells 72 h after stimulation with PBS or OVA (100 $\mu\text{g}/\text{ml}$). LN T cells were isolated from lung-draining LNs after the allergen challenge.

that the maturation of lung DCs stimulated by LPS depends on VEGF-mediated effects (11). In the present study, we found more specifically that the maturation of lung DCs depended on VEGFR-1 rather than VEGFR-2 signaling, suggesting that VEGFR-1 may be

critical in the development of T cell priming via enhanced maturation of lung DCs.

Migration of mature DCs into regional LNs is also an essential step for the activation of naive T cells (20). During maturation in

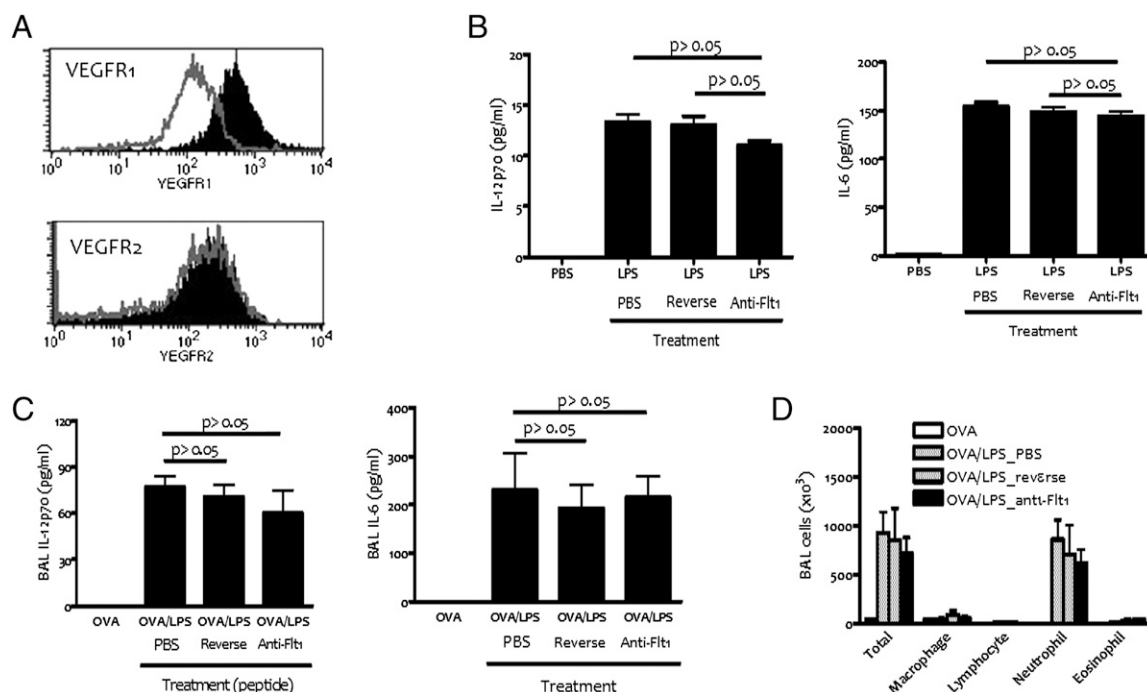


FIGURE 5. LPS-induced production of IL-12p70 and IL-6 does not depend on VEGFR-1 signaling. **A**, Expression of VEGFR-1 and VEGFR-2 on lung DCs isolated from allergen-free mice as determined by flow cytometry. **B**, Ex vivo production of IL-12p70 and IL-6 by lung CD11c⁺ DCs after stimulation with or without LPS (1 $\mu\text{g}/\text{ml}$) in the presence of a VEGFR-1 inhibitor (anti-Flt1 peptide, 100 μM), reverse peptide (100 μM), or PBS. For **C** and **D**, evaluation ($n = 5$ mice/group) was performed 24 h after sensitization three times on days 0, 1, and 2 with or without treatment of 200 $\mu\text{g}/\text{mouse}$ anti-Flt1 or reverse peptides. OVA indicates mice sensitized with OVA alone; OVA/LPS indicates mice sensitized with OVA plus LPS (10 μg). The levels of IL-12p70 and IL-6 in BAL fluids (**C**) and BAL cellularity (**D**) were evaluated after the sensitization, and these phenotypes were unaffected by the anti-Flt1 peptide treatment.

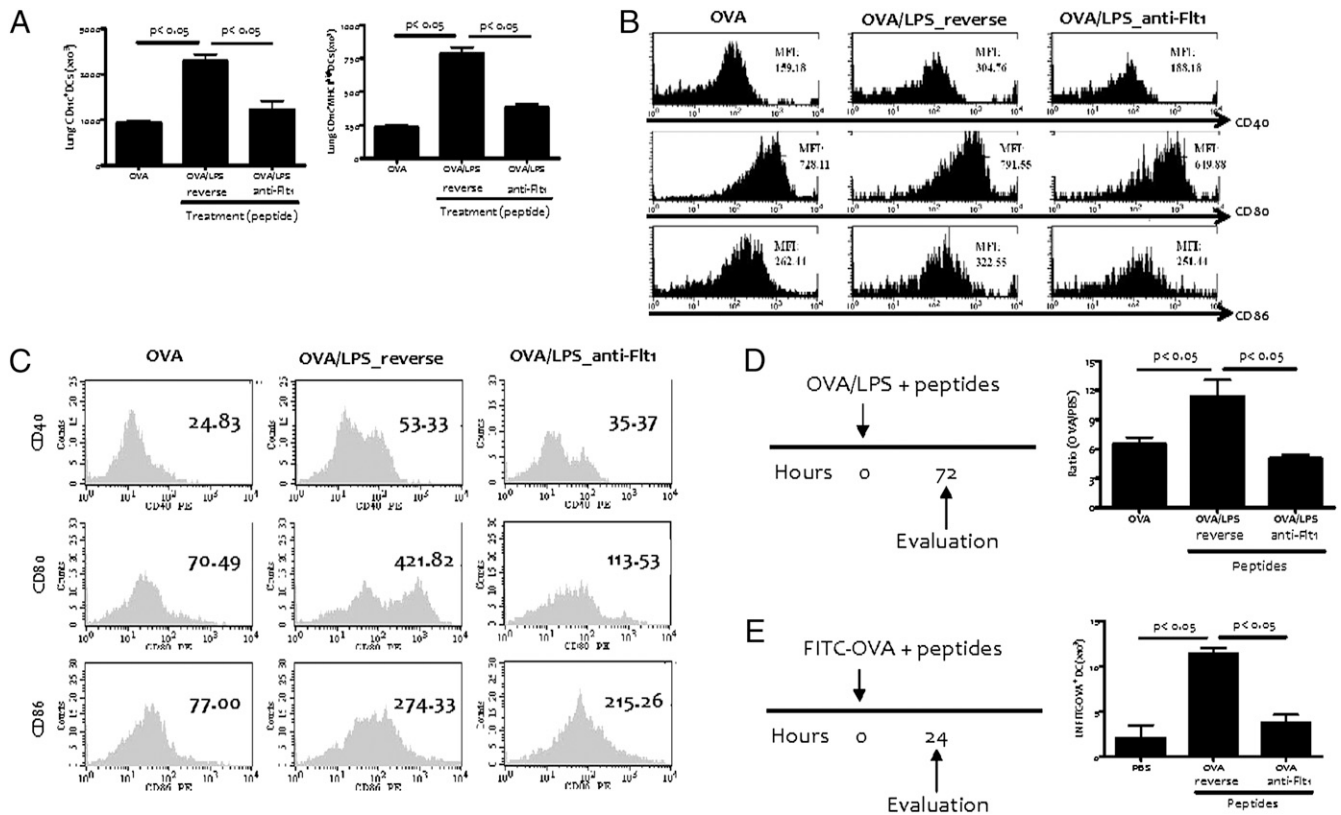


FIGURE 6. Maturation and migration of lung DCs and then T cell priming depend on VEGFR-1 signaling. For *A* and *B*, mice ($n = 5$ /group) were sensitized intranasally with OVA alone or OVA plus LPS ($10 \mu\text{g}$) on day 0 in the presence of anti-Flt or reverse peptides. *A*, Numbers of CD11c⁺ and CD11c⁺MHCII^{high} lung DCs isolated 24 h after sensitization. *B*, Expression of costimulatory molecules such as CD40 and CD80 on lung CD11c⁺ cells (DCs) isolated 24 h after sensitization. *C*, Expression of costimulatory molecules such as CD40, CD80, and CD86 on lung CD11c⁺CD11b⁺ cells (myeloid DCs) isolated 3 h after sensitization. Values indicate MFI. *D*, Allergen-specific proliferation of T cells in lung-draining LNs. For the T cell proliferation assay, mice ($n = 5$ /group) were sensitized intranasally with OVA alone or OVA plus LPS ($10 \mu\text{g}$) in the presence of anti-Flt1 peptide or the reverse peptide. Cells from LNs were collected 72 h after sensitization, and thymidine incorporation was measured with or without OVA ($100 \mu\text{g}/\text{ml}$) stimulation for 72 h. *E*, FITC-OVA ($60 \mu\text{l}$, $10 \text{ mg}/\text{ml}$) was applied intranasally to mice ($n = 5$ /group) treated with anti-Flt1 peptide or the reverse-sequenced peptide, and the number of FITC-OVA-containing DCs in lung-draining LNs was analyzed by flow cytometry. MFI, mean fluorescence intensity.

response to inflammatory stimulation, DCs increase the expression of CCR7, a receptor for CCR7 ligands such as secondary lymphoid tissue chemokine and EBI-1 ligand chemokine, which are produced by endothelial and stromal cells in secondary lymphoid organs (21). After Ag-loaded mature DCs migrate into LNs, they localize in the T cell zone within the LNs, encounter naive T cells, and induce the proliferation and activation of Ag-specific naive T cells (22). However, the role of VEGFR-mediated signaling in the migration of mature DCs into regional LNs and subsequent T cell priming is unknown. To perform a DC migration assay, we performed the migration of DCs into the regional LNs using OVA-FITC following the previous report (23). The present study showed that the migration of lung DCs harboring FITC-OVA was significantly inhibited by treatment of the VEGFR-1 inhibitor. However, this study has a limitation of not reflecting DC migration harboring LPS-containing allergens, because the present study used OVA rather than LPS-containing OVA. Furthermore, the present study showed that the proliferation of naive T cells in regional LNs when exposed to LPS-containing allergens depends mainly on VEGFR-1-mediated signaling. Taken together, these results suggest that VEGFR-1-mediated signaling enhances the migration of DCs for T cell priming.

There is mounting evidence that inflammatory mechanisms other than eosinophilic inflammation (noneosinophilic asthma) are also involved in the pathogenesis of asthma (4, 24). Our previous studies demonstrated that the noneosinophilic asthma phenotype

can be induced by mixed Th1 and Th17 cell responses, in the context of inhalation of LPS-containing allergens; furthermore, LPS-induced production of IL-12p70 and IL-6 during sensitization is vital for Th1 and Th17 polarization, respectively (4, 11). Until now, the exact role of VEGFR subtypes in the differentiation of Th1 or Th17 cells in the lung was unknown. The present study showed that the production of VEGF by airway exposure of LPS preceded the production of Th1- and Th17-polarizing cytokines IL-12p70 and IL-6, suggesting that VEGF is a key upstream mediator of Th1 or Th17 polarization. Interestingly, we found that the production of IL-6 and the subsequent development of the Th17 response depended on VEGFR-2-mediated signaling but not on VEGFR-1. In contrast, IL-12p70 production and the Th1 response did not depend on VEGFR-1 or VEGFR-2 signaling. It is well known that IFN- γ is a key inducer of the production of IL-12p70 (25). Taking the production of IFN- γ preceding IL-12p70 into consideration, these data suggest that IFN- γ and VEGF are key upstream mediators in the development of Th1 and Th17 polarization, respectively, in the context of inhalation of LPS-containing allergens.

In summary, airway exposure to LPS-containing allergens resulted in allergen-specific Th1 and Th17 responses and subsequent non-eosinophilic inflammation. LPS-induced innate immune responses, such as DC maturation and migration and polarizing cytokine production, modulate the development of allergen-specific adaptive immune responses. The present data indicate that T cell priming

depends on VEGFR-1-mediated signaling mainly via the maturation and migration of lung DCs, but that Th17 polarization depends on VEGFR-2-mediated signaling mainly via the production of Th17-polarizing cytokine IL-6.

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Disclosures

The authors have no financial conflicts of interest.

References

- Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258–2261.
- Michel, O. 2003. Role of lipopolysaccharide (LPS) in asthma and other pulmonary conditions. *J. Endotoxin Res.* 9: 293–300.
- Asher, M. I., S. Montefort, B. Björkstén, C. K. Lai, D. P. Strachan, S. K. Weiland, H. Williams; ISAAC Phase Three Study Group. 2006. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multi-country cross-sectional surveys. *Lancet* 368: 733–743.
- Kim, Y. K., S. Y. 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.
- Charan, N. B., E. M. Baile, and P. D. Paré. 1997. Bronchial vascular congestion and angiogenesis. *Eur. Respir. J.* 10: 1173–1180.
- Hoshino, M., M. Takahashi, and N. Aoike. 2001. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J. Allergy Clin. Immunol.* 107: 295–301.
- de Vries, C., J. A. Escobedo, H. Ueno, K. Houck, N. Ferrara, and L. T. Williams. 1992. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255: 989–991.
- Quinn, T. P., K. G. Peters, C. De Vries, N. Ferrara, and L. T. Williams. 1993. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc. Natl. Acad. Sci. USA* 90: 7533–7537.
- Voelkel, N. F., R. W. Vandivier, and R. M. Tuder. 2006. Vascular endothelial growth factor in the lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 290: L209–L221.
- Barleon, B., S. Sozzani, D. Zhou, H. A. Weich, A. Mantovani, and D. Marmé. 1996. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* 87: 3336–3343.
- 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.
- Bae, D. G., T. D. Kim, G. Li, W. H. Yoon, and C. B. Chae. 2005. Anti-flt1 peptide, a vascular endothelial growth factor receptor 1-specific hexapeptide, inhibits tumor growth and metastasis. *Clin. Cancer Res.* 11: 2651–2661.
- Vermaelen, K., and R. Pauwels. 2003. Accelerated airway dendritic cell maturation, trafficking, and elimination in a mouse model of asthma. *Am. J. Respir. Cell Mol. Biol.* 29: 405–409.
- Vermaelen, K. Y., I. Carro-Muino, B. N. Lambrecht, and R. A. Pauwels. 2000. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J. Exp. Med.* 193: 51–60.
- Lee, C. G., H. Link, P. Baluk, R. J. Homer, S. Chapoval, V. Bhandari, M. J. Kang, L. Cohn, Y. K. Kim, D. M. McDonald, and J. A. Elias. 2004. Vascular endothelial growth factor (VEGF) induces remodeling and enhances T_H2-mediated sensitization and inflammation in the lung. *Nat. Med.* 10: 1095–1103.
- Hahn, R. G. 2003. Endotoxin boosts the vascular endothelial growth factor (VEGF) in rabbits. *J. Endotoxin Res.* 9: 97–100.
- Kim, I., S. O. Moon, S. H. Kim, H. J. Kim, Y. S. Koh, and G. Y. Koh. 2001. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor- κ B activation in endothelial cells. *J. Biol. Chem.* 276: 7614–7620.
- Gabrilovich, D. I., H. L. Chen, K. R. Girgis, H. T. Cunningham, G. M. Meny, S. Nadaf, D. Kavanaugh, and D. P. Carbone. 1996. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat. Med.* 2: 1096–1103.
- Fricke, I., N. Mirza, J. Dupont, C. Lockhart, A. Jackson, J. H. Lee, J. A. Sosman, and D. I. Gabrilovich. 2007. Vascular endothelial growth factor-trap overcomes defects in dendritic cell differentiation but does not improve antigen-specific immune responses. *Clin. Cancer Res.* 13: 4840–4848.
- Mempel, T. R., S. E. Henrickson, and U. H. Von Andrian. 2004. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 427: 154–159.
- Cyster, J. G. 1999. Chemokines and cell migration in secondary lymphoid organs. *Science* 286: 2098–2102.
- Sallusto, F., and A. Lanzavecchia. 2000. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol. Rev.* 177: 134–140.
- Idzko, M., H. Hammad, M. van Nimwegen, M. Kool, T. Müller, T. Soullie, M. A. Willart, D. Hijdra, H. C. Hoogsteden, and B. N. Lambrecht. 2006. Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J. Clin. Invest.* 116: 2935–2944.
- Douwes, J., P. Gibson, J. Pekkanen, and N. Pearce. 2002. Non-eosinophilic asthma: importance and possible mechanisms. *Thorax* 57: 643–648.
- Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon- γ . *Annu. Rev. Immunol.* 15: 749–795.