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The Endothelial Cell-Produced Antiangiogenic Cytokine Vascular Endothelial Growth Inhibitor Induces Dendritic Cell Maturation

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Angiogenesis is an essential component of chronic inflammation that is linked to carcinogenesis. In this study, we report that human vascular endothelial growth inhibitor (VEGI, TNF superfamily 15), an endothelial cell-produced antiangiogenic cytokine, induces mouse dendritic cell (DC) maturation, a critical event in inflammation-initiated immunity. VEGI-stimulated bone marrow-derived immature DCs display early activation of maturation signaling molecules NF-κB, STAT3, p38, and JNK, and cytoskeleton reorganization and dendrite formation. The activation signals are partially inhibited by using a neutralizing Ab against death domain-containing receptor-3 (DR3) or a truncated form of DR3 consisting of the extracellular domain, indicating an involvement of DR3 in the transmission of VEGI activity. A VEGI isoform, TLI1A, does not induce similar activities under otherwise identical experimental conditions. Additionally, the cells reveal significantly enhanced expression of mature DC-specific marker CD83, secondary lymphoid tissue-directing chemokine receptor CCR7, the MHC class-II protein (MHC-II), and costimulatory molecules CD40, CD80, and CD86. Functionally, the cells exhibit decreased Ag endocytosis, increased cell surface distribution of MHC-II, and increased secretion of IL-12 and TNF. Moreover, VEGI-stimulated DCs are able to facilitate the differentiation of CD4+ naïve T cells in cocultures. These findings suggest that the anticancer activity of VEGI arises from coupling the inhibition of endothelial cell growth with the promotion of the adaptive immune mechanisms through the stimulation of DC maturation. The Journal of Immunology, 2007, 179: 3742–3751.

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acute inflammation in tissue wounds induces angiogenesis, and resolution of inflammation and completion of wound healing are accompanied by termination of angiogenesis. Angiogenesis is also an essential component of chronic inflammation, which is now widely recognized as a key event in cancer development (1–4). However, angiogenesis in malignant tumors is usually uncontrolled. It is in this light that a tumor is likened to a wound that will not heal (5). Significant progress has been made in understanding the points of control in angiogenesis (6, 7) and inflammation (8). It remains largely unclear, however, whether and how inflammation and angiogenesis are cross-regulated.

The vascular endothelium is a major secretory tissue. It has the capacity to produce a large variety of growth factors and cytokines (9, 10), including some of the TNF superfamily (TNFSF) members. The TNFSFs are important mediators of the immune functions or disorders such as inflammation, inhibition of viral replication and tumor growth, induction of transplant rejection and rheumatoid arthritis, and septic shock (11–14). Vascular endothelial growth inhibitor (VEGI) is a member of the TNFSF (TNFSF15) produced mostly by vascular endothelial cells (15–17). VEGI is readily detectable in the vasculature in normal tissues (15, 18). VEGI specifically inhibits the growth of endothelial cells in a cell cycle-dependent manner: it induces apoptosis to proliferating cells while enforcing a growth arrest in quiescent cells (19). Engineered overexpression of VEGI by cancer cells leads to inhibition of tumor growth in a variety of tumor models (16, 17). Systemic administration of rVEGI to tumor-bearing mice leads to inhibition of the growth of established tumors resulting from the eradication of the endothelial cells in the tumor vasculature (18). Dendritic cells (DCs) are sentinel cells and a central component of the innate immune system, which rapidly recognize, interrogate, and directly eliminate microbial pathogens and transformed cells, induce acute inflammation, and initiate, polarize, and regulate adaptive immune mechanisms (20). Thus, DCs mediate and regulate the critically important immune mechanisms that contain infection and cancer. DCs in the periphery exhibit an immature phenotype that includes a high capacity for Ag uptake, but low ability of Ag presentation and T cell activation. For a productive T cell priming, DCs must go through a maturation process to become fully activated (21). Maturation is, therefore, a critical control point in the initiation of immunity. This also accounts for the attractiveness of immunotherapeutic approaches that facilitate DC maturation for cancer treatment (22). DCs sense and respond to pathological events in tissues mainly through TLRs that recognize a wide variety of molecular patterns associated with pathogens and infected or transformed cells (23). The response is rapid and leads to DC maturation characterized by increased expression of MHC molecules and
the Ag-presenting machinery, costimulatory molecules, and lymph node-homing receptors, as well as secretion of major proinflammatory and immunoregulatory cytokines such as TNF and IL-12. The changed DCs, therefore, serve the two following main purposes: induction of inflammation at the periphery and, following their migration to lymph nodes, Ag presentation and regulation of adaptive immune responses.

DCs have been shown to regulate angiogenesis in cancers. Plasmacytoid DCs that induce angiogenesis in vivo are shown to be associated with tumors, whereas myeloid DCs that when derived in vitro suppress angiogenesis in vivo are absent from malignant ascites (24), although the mechanisms and molecules involved in these processes are unclear, except for the understanding that an array of growth factor, cytokines, and chemokines is involved and the balance of their functions is responsible. We found that the proinflammatory cytokine TNF up-regulates the production of VEGI by cultured HUVEC (15). This suggests that DCs through TNF could up-regulate VEGI secretion by endothelial cells in inflamed tissues. Because the TNFSF members TNF, CD40L, and RANKL are all known to mediate DC maturation (13, 25, 26), it is possible that VEGI as a member of the TNFSF when produced in inflamed tissues may also induce DC maturation and take part in the modulation of inflammation and promotion of adaptive immune mechanisms.

VEGI has been shown to play an important role in the responses of the immune system to inflammatory conditions. A VEGI isoform, TL1A, has been suggested as the ligand for death domain-containing receptor-3 (DR3, TNFRSF12, TNFRSF25) (27). Binding of TL1A to DR3 induces proliferation or activation signals, most likely through activation of NF-kB-mediated pathways. TL1A specifically induces secretion of IFN-γ by human T cells, suggesting that the TL1A-DR3 signaling system takes part in Th1-mediated responses. TL1A-DR3 interaction was shown to be of particular importance in chronic murine ileitis for the pathogenesis of chronic inflammatory conditions (28).

In the present study, we investigated the effect of VEGI on DC maturation. We show in this study that VEGI is able to facilitate the induction of conventional maturation of DCs, including early activation of key DC maturation signaling molecules, cytoskeleton reorganization, and dendrite formation, enhanced expression of maturation markers, as well as decreased Ag endocytosis, increased cell surface translocation of MHC-II, and facilitation of the secretion of IL-12 and TNF. We also determined the activation of CD4+ T cells by VEGI-stimulated DCs.

Materials and Methods

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Mice

Eight-week-old female C57BL/6 mice and OVA-specific TCR transgenic OT-2 mice were purchased from The Jackson Laboratory. Studies using the experimental animals were performed in accordance with protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Cell preparation

Mouse immature DCs were produced from lineage marker-negative bone marrow cells, which were obtained by flushing bone marrow cavities of femurs and tibias. The bone marrow cells were depleted of erythrocytes by hypotonic lysis and of lineage marker-positive cells by treatment with anti-CD4, anti-CD8, and anti-B220 Abs and rabbit complement. The cells were resuspended (0.5 × 10^6 cells/ml) in complete culture medium (CCM) consisting of RPMI 1640 medium supplemented with 0.1 mM nonessential amino acids, 2 mM sodium pyruvate, 1 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 20 mM HEPES buffer, 10% heat-inactivated FBS (all from Invitrogen Life Technologies), and 50 μM 2-ME (Bio-Rad), and cultured for 6 days in the presence of 15 and 7.5 ng/ml mouse GM-CSF and IL-4 (R&D Systems), respectively, as described (29). The cells were further purified by positive immunoselection using MACS CD11c isolation kit (Miltenyi Biotec). To generate mature DCs, the cells were cultured in CCM with the addition of 100 ng/ml LPS (R&D Systems) for 48 h. CD4 T cells were isolated from mechanically disrupted spleen of the OT.2 TCR transgenic mice. Naïve T cells were isolated using negative depletion of CD4+ T cell isolation kit (Miltenyi Biotec). Briefly, cells were magnetically labeled with anti-CD8a (Ly-2), CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119. Isolation of CD4+ T cells was achieved by depletion of the magnetically labeled cells.

Flow cytometry

For each sample, 1–3 × 10^6 cells were harvested and washed with 2 ml of FACS buffer (1% BSA and 0.05% sodium azide in PBS). The cells were collected by centrifugation, then resuspended in 100 μl of FACS buffer containing 0.5–1 μg of the indicated Ab, and dispensed in a minimum of 5 × 10^5 cells/sample, gently mixed, and incubated on ice for 30 min. The cells were washed with FACS buffer, centrifuged, and resuspended in 0.5 ml of FACS buffer, then placed in 12 × 75-mm Falcon tubes and analyzed within 1 h. Coulter FACS equipment and EXPO analysis software were used.

Cytokine secretion

Immature DCs generated from mouse bone marrow were harvested, re-plated (0.2 × 10^6 cells/ml), and cultured for 48 h, in the absence or presence of VEGI. Cell-free supernatants were then collected and analyzed for the presence of cytokines by ELISA.

OVA uptake

OVA processing assay was performed by using a self-quenched conjugate of OVA (DQ-OVA; Molecular Probes) that displayed fluorescence upon proteolytic degradation, as described (30). DCs were cultured in the absence or presence of VEGI for 3 days, then harvested and divided into two groups. Cells in the experimental group were incubated with 100 μg/ml DQ-OVA for 30 min at 37°C. Cells in the control group were treated identically, but incubated at 4°C. The number of cells with proteolytically processed OVA was determined by FACS.

Nuclear translocation of NF-κB

Translocation of NF-κB from cytoplasm to nucleus was analyzed by using Cellomics array scan, as described (31). DCs were plated on 96-well black plate and cultured in CCM in the absence or presence of VEGI, then fixed for 20 min at room temperature, and permeabilized in PBS containing 0.1% Triton X-100 for 5 min, washed with PBS, then blocked by incubation in 3% BSA for 30 min. NF-κB was labeled by adding mouse anti-p65 NF-κB Ab and Alexa 488 goat anti-mouse Ab. Nuclei were stained with Hoechst. The cells (3000 cells/well) were then subjected to Cellomics array scan (ArrayScan VTI HCS Reader; Cellomics). Images of the cells were taken and analyzed for fluorescence density in cytoplasm and nucleus.

Immunofluorescent staining of F-actin and vinculin

Structural features of DC cytoskeleton were analyzed by F-actin immunofluorescent staining, as described (32, 33). Immature DCs were plated on coverslips treated with or without VEGI-192 for indicated length of time.

Abs and reagents

Mouse and human rVEGI isoform TL1A, GM-CSF, IL-4, and LPS were purchased from R&D Systems. Hybridoma cells producing rat anti-mouse CD4 (clone GK1.5), CD8 (clone CT-CD8b), and CD45R/B220 (clone RA3-6B2) Abs were purchased from eBioscience. Low toxic rabbit complement. The cells were depleted of erythrocytes by hypotonic lysis and of lineage marker-positive cells by treatment with anti-CD4, anti-CD8, and anti-B220 Abs and rabbit complement. The cells were resuspended (0.5 × 10^6 cells/ml) in complete culture medium (CCM) consisting of RPMI 1640 medium supplemented with 0.1 mM nonessential amino acids, 2 mM sodium pyruvate, 1 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 20 mM HEPES buffer, 10% heat-inactivated FBS (all from Invitrogen Life Technologies), and 50 μM 2-ME (Bio-Rad), and cultured for 6 days in the presence of 15 and 7.5 ng/ml mouse GM-CSF and IL-4 (R&D Systems), respectively, as described (29). The cells were further purified by positive immunoselection using MACS CD11c isolation kit (Miltenyi Biotec). Briefly, cells were magnetically labeled with anti-CD8a (Ly-2), CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119. Isolation of CD4+ T cells was achieved by depletion of the magnetically labeled cells.

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Immunofluorescent staining of F-actin and vinculin

Structural features of DC cytoskeleton were analyzed by F-actin immunofluorescent staining, as described (32, 33). Immature DCs were plated on coverslips treated with or without VEGI-192 for indicated length of time.
FIGURE 1. VEGI-stimulated NF-κB translocation to nucleus. Mouse bone marrow-derived immature DCs are treated with VEGI at the indicated time intervals, fluorescent immunostained for NF-κB, and subjected to microscopic image analysis to determine subcellular distribution patterns of the fluorescent protein. A, Translocation of NF-κB (green) from cytoplasm seen in untreated cells to nucleus (blue) in VEGI-treated cells. Merged: superposition of the two images of the same cells. B, Quantitative image analysis of nuclear translocation of NF-κB. Nuclear fluorescence intensity is the average density of green pixels within the areas defined by the blue nuclear staining per cell. A total of 5000 cells/well was analyzed. The experiment was conducted in triplicate. Values are mean ± SD. C, VEGI-stimulated NF-κB translocation is abolished when VEGI protein is inactivated before use. Anti-VEGI: mAb against VEGI. Anti-DR3: mAb against DR3. Soluble DR3: preparation of DR3 extracellular domain. Boiled VEGI: VEGI preparation is subjected to boiling and centrifugation. Beads VEGI: VEGI is treated with Ni-conjugated magnetic beads.

The cells were then fixed in 4% paraformaldehyde for 20 min at room temperature, then permeabilized in PBS containing 0.1% Triton X-100 for 5 min, washed with PBS, and blocked with 3% BSA for 30 min. The cells were then incubated with Texas Red X-conjugated phalloidin (Molecular Probes) and FITC-conjugated anti-vinculin mAb (Sigma-Aldrich) for 30 min. The cells were then sealed with antifading agent for 24 h and analyzed with a fluorescent microscope (Nikon Eclipse E800). Alternatively, immature DCs and VEGI-192-induced DCs were replated on fibronectin-coated coverslips, incubated for 4 h to allow adherence, stained for F-actin by using Alexa Fluor 488-conjugated phalloidin (Molecular Probes), and similarly analyzed.

Western blotting analysis

Cells were subjected to lysis, and proteins in the cell homogenate were resolved by SDS-PAGE. The proteins were then transferred onto a Hybond-ECL nitrocellulose membrane, blocked with 5% nonfat dry milk powder in TBST buffer (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, and incubated overnight at 4°C with a primary Ab against the target protein. Washed with TBST, the membrane was incubated with an appropriate HRP-conjugated secondary Ab, washed with TBST, then developed with the ECL system (Amersham Biosciences).

Assays for DC and CD4+ T cell interaction in vitro

To determine DC-mediated T cell activation, mouse bone marrow-derived immature DCs were either untreated or treated with VEGI (72 h) or LPS (24 h), collected, washed with fresh medium, then cocultured on CD4+ T cells that had been pulse treated with 5 μg/ml OVA323–339 peptide. The DC-T cell ratio was 1:1. One volume of fresh culture medium was added every 1.5 days, and the cells were transferred into a larger container when necessary. Supernatant from each culture well was collected, frozen, and analyzed for IFN-γ and IL-2 by using specific ELISA kits (R&D Systems). The cells were collected, labeled with CD4 FITC and CD62L PE, and analyzed by FACS. CD4+ cells were gated to determine CD62L expression.

Results

VEGI activates intracellular signaling pathways critical for DC maturation

We determined the influence of VEGI on the activation of the transcription factor NF-κB, because NF-κB activation is critical to the process of DC maturation. We isolated DCs from the bone marrow of C57BL/6 mice, cultured the cells in the presence of GM-CSF and IL-4 for 7 days, then replaced GM-CSF and IL-4 with VEGI to determine whether there was any effect on NF-κB activation judged by enhanced nuclear translocation. By following its subcellular distribution pattern, we found that NF-κB was translocated from the cytoplasm to the nucleus upon the addition of VEGI to the culture medium (Fig. 1A). NF-κB concentration in the nucleus increased quickly and reached a plateau within 30 min of VEGI treatment (Fig. 1A). NF-κB activation is critical to the process of DC maturation. We isolated DCs from the bone marrow of C57BL/6 mice, cultured the cells in the presence of GM-CSF and IL-4 for 7 days, then replaced GM-CSF and IL-4 with VEGI to determine whether there was any effect on NF-κB activation judged by enhanced nuclear translocation. By following its subcellular distribution pattern, we found that NF-κB was translocated from the cytoplasm to the nucleus upon the addition of VEGI to the culture medium (Fig. 1A). NF-κB concentration in the nucleus increased quickly and reached a plateau within 30 min of VEGI treatment (Fig. 1B). To ensure that the observed activity was that of the rVEGI, we used three different methods to remove the VEGI protein before the use of the preparation. The VEGI preparation was either incubated with a mAb (3-12D; Ab-VEGI ratio was 10:1 by weight) against VEGI for 30 min, or boiled for 30 min and centrifuged to remove precipitation, or incubated with Ni-conjugated magnetic beads to remove VEGI because the rVEGI had a (His)6 tag at the N terminus. The VEGI preparation treated by any of these methods was no longer able to stimulate NF-κB translocation when VEGI was added to the culture medium (Fig. 1C).

These treatments caused a decrease of ~40–50% of the VEGI activity (Fig. 1C), suggesting that DR3 is responsible, at least partially, for the transmission of VEGI activity under these experimental conditions.
We also examined the activation of three MAPK, namely, ERK, JNK, and p38 MAPK, as well as a number of key signaling pathway components JAK/STAT and IκB/NF-κB (p105/p50) that are critical to DC maturation (Fig. 2). Western blotting analysis indicates that, whereas neither JAK nor STAT1 was affected, phosphorylation of STAT3 took place within 1 h of VEGI treatment. Additionally, MAPK p38 and JNK were both significantly phosphorylated within 1 h of VEGI stimulation. The activation of these signals exhibited dose dependence on VEGI (Fig. 2B). In contrast to phosphorylated STAT3 that was up-regulated, phosphorylation of STAT3 was down-regulated during the initial hours of VEGI treatment. As the level of STAT3 phosphorylation gradually diminished in 12 h, the level of activated ERK began to return to normal levels after 24 h (Fig. 2C). We also investigated the NF-κB signaling pathway, and found that phosphorylation of IκB-α took place within 5 min of VEGI treatment. Degradation of IκB-α was also observed within 30 min. Consistently, the NF-κB1 precursor protein p105 was proteolytically processed to yield the active form p50 within 1 h of VEGI treatment (Fig. 2D). These results indicate that VEGI acts directly on DCs to induce intracellular signals leading to maturation.

Additionally, we compared three different preparations of VEGI isoforms for their ability to stimulate DC maturation signals. These include rVEGI-192 produced in *Escherichia coli* (referred to as VEGI), rVEGI found in the conditioned medium of human breast cancer MDA-MB-231 cells transfected with VEGI-251 cDNA (referred to as CM VEGI-251), and TL1A, which is a truncated form of VEGI-251 consisting of aa residues 72–251.
STAT3 and NF-κB (Fig. 2E). In contrast, TL1A did not exhibit a similar activity at a concentration 5 times that reported for the stimulation of T cells (27). This difference may reflect structural or conformational variations that are likely to exist in VEGI-192, TL1A, and mammalian cell-secreted VEGI-251.

**VEGI promotes morphological changes in DCs characteristic of maturation**

We determined the morphological changes of mouse bone marrow-derived, immature DCs cultured in the presence of VEGI. The untreated control DCs remained floated in aggregates of various sizes, and only a few poorly polarized cells adhered to the base of the culture wells (Fig. 3A, inset). Within 8 h of VEGI treatment, however, DCs became adherent and many were polarized, forming filopodia and large leading-edge lamellipodia (Fig. 3Ab, inset). After 48 h in the presence of VEGI, a large percentage of DCs became rounded again, formed clumps, and exhibited multiple membrane ruffles and fine processes covering the surface of the cell (Fig. 3Ac, inset). Moreover, using Texas Red-conjugated phalloidin and FITC-conjugated anti-vinculin mAb to label F-actin and vinculin, an adaptor protein of cytoskeleton, we observed the presence of podosomes in the control immature DCs (Fig. 3Ba) and their VEGI-treated counterparts within 1 h of the treatment (Fig. 3Bb). F-actin forms a central core that is surrounded by adaptor proteins such as vinculin, shown as red dots and green circles in the images. VEGI-treated DCs began to exhibit a diminishing number of podosomes in 2 h, and the cytoskeleton exhibited a rearrangement such that the F-actin now became associated predominantly with the cell membrane (Fig. 3Bc). The VEGI-treated cells no longer had podosomes in 6 h (Fig. 3Bd). After 72 h, the control or VEGI-treated DCs were reseeded on fibronectin-coated coverslips, allowed to adhere, then examined for cytoskeleton arrangement by immunofluorescent staining. The control DCs adhered and exhibited a cytoskeleton with stress fibers across the entire diameter of the cell (Fig. 3Ca). In sharp contrast, the VEGI-treated DCs showed the absence of stress fibers, but instead the presence of numerous fine processes typical of mature DCs (Fig. 3Cb). These results indicate that VEGI treatment of bone marrow-derived, immature DCs caused morphological changes consistent with the maturation of DCs.

**VEGI induces an alteration of surface marker and cytokine production profiles typical of mature DCs**

As DCs become mature, they reveal an increased expression of the MHC-II, costimulatory molecules, and other specific markers on the cell surface. We analyzed the impact of VEGI on the expression of these markers. Immature DCs generated from mouse bone marrow were cultured in the presence of either GM-CSF and IL-4,
or VEGI alone. The presence of DC maturation markers on the cell surface was analyzed by flow cytometry. The VEGI-treated cells displayed a significantly enhanced expression of CD40, CD80, CD86, CD83, CCR7, and MHC-II (Fig. 4A). The stimulation effect of VEGI was dose dependent because the number of cells with each of the maturation markers substantially increased when the cells were treated with 2 U of VEGI instead of 1 U. LPS was used as a positive control because it is able to induce similar gene expression profiles in DCs. These results strongly support the view that VEGI stimulates the production of the key molecules or features associated with mature cell functions, such as a decreased capacity to take up Ags and an increased distribution of MHC-II on the cell surface. Bone marrow-derived immature DCs were cultured in the presence of various concentrations of VEGI for 72 h. A self-quenched fluorescent conjugate of OVA that would display intense green fluorescence upon proteolytic processing inside the cells was then added to the culture medium. Following the occurrence of the fluorescence, we found that VEGI-treated DCs were much less efficient in endocytosis of the Ag than were the immature DCs, evident from the decreased number of fluorescent OVA-associated cells identified by flow cytometry (Fig. 5A). Mature DCs generated by LPS treatment were used as a positive control in these experiments. A quantitative analysis of the flow cytometry data revealed a 40% decrease of OVA uptake by VEGI-treated DCs compared with their immature counterpart (Fig. 5B). A VEGI dose-dependent decrease of OVA uptake by DCs was observed, with ~40% decrease seen with DCs cultured in the presence of 2 U of VEGI (Fig. 5B). We further confirmed the changes of the Ag uptake capacity of the cells by determining the subcellular locations of the OVA molecules in comparison with those of the MHC-II molecules (Fig. 5C). The DCs were pulse labeled with the fluorescent OVA, then transferred to coverslips and incubated for 30 min at 37°C to allow adherence. Upon microscopic image analysis, we found that the internalized OVA molecules in untreated immature DCs were located in the cytosolic compartment, where the immunofluorescence-labeled MHC-II molecules were also present. In contrast, the amount of internalized OVA molecules in the cytosolic compartment was

**FIGURE 4.** VEGI induced expression of DC maturation markers and cytokines. A, Flow cytometric analysis of DC maturation markers. Mouse bone marrow-derived immature DCs are treated with VEGI (0, 1, or 2 U) for 72 h. LPS (100 ng/ml, 48 h) is used for comparison. Mature DC-specific markers CD40, CD80, CD83, CD86, CCR7, and MHC-II are labeled with fluorescence-conjugated Abs and analyzed by flow cytometry. Typical histograms of three independent experiments are shown. Black dotted line: untreated immature cells. Green line: cells treated with 1 U of VEGI (1 U). Red line: cells treated with 2 U of VEGI. Blue line: cells treated with LPS. B, VEGI-stimulated production of IL-12 and TNF in DCs. Mouse bone marrow-derived immature DCs are treated with VEGI at the indicated concentrations for 48 h. LPS (0–100 ng/ml) treatments are used for comparison. Cell-free medium is collected and analyzed by ELISA for IL-12 and TNF. *A*, Increasing concentrations of IL-12p70 (pg/ml) in the conditioned medium as a function of VEGI concentration. *B*, Increasing concentrations of TNF (pg/ml) in the conditioned medium as a function of VEGI concentration. All experiments were conducted in triplicate. Values are mean ± SD.

VEGI treatment of immature DCs leads to decreased Ag uptake and increased MHC-II translocation to cell surface

We then determined whether VEGI-stimulated DCs display characteristics associated with mature cell functions, such as a decreased capacity to take up Ags and an increased distribution of MHC-II on the cell surface. Bone marrow-derived immature DCs were cultured in the presence of various concentrations of VEGI for 72 h. A self-quenched fluorescent conjugate of OVA that would display intense green fluorescence upon proteolytic processing inside the cells was then added to the culture medium. Following the occurrence of the fluorescence, we found that VEGI-treated DCs were much less efficient in endocytosis of the Ag than were the immature DCs, evident from the decreased number of fluorescent OVA-associated cells identified by flow cytometry (Fig. 5A). Mature DCs generated by LPS treatment were used as a positive control in these experiments. A quantitative analysis of the flow cytometry data revealed a 40% decrease of OVA uptake by the LPS-induced mature DCs in comparison with their immature counterpart (Fig. 5B). A VEGI dose-dependent decrease of OVA uptake by DCs was observed, with ~40% decrease seen with DCs cultured in the presence of 2 U of VEGI (Fig. 5B). We further confirmed the changes of the Ag uptake capacity of the cells by determining the subcellular locations of the OVA molecules in comparison with those of the MHC-II molecules (Fig. 5C). The DCs were pulse labeled with the fluorescent OVA, then transferred to coverslips and incubated for 30 min at 37°C to allow adherence. Upon microscopic image analysis, we found that the internalized OVA molecules in untreated immature DCs were located in the cytosolic compartment, where the immunofluorescence-labeled MHC-II molecules were also present. In contrast, the amount of internalized OVA molecules in the cytosolic compartment was
nearly undetectable in VEGI-stimulated DCs and, at the same time, the MHC-II molecules were no longer found in the cytoplasm, but, instead, translocated to the cell surface. These data demonstrate that VEGI stimulation gives rise to DCs that are functionally mature.

Activation of CD4⁺ T cells by VEGI-stimulated DCs

To determine whether the VEGI-stimulated DCs were able to induce effective type 1 T cell response, which is a function of mature DCs, we isolated CD4⁺ T cells from OT.2 TCR transgenic mice.
and cocultured the cells with either immature DCs or VEGI-stimulated DCs at a 1:1 DC:T-cell ratio for 5 days. We measured the production of IFN-γ and IL-2, which are essential for type 1 immune response produced by activated T cells. We found that the production of IFN-γ by T cells was greatly up-regulated in cocultures with VEGI-stimulated DCs (Fig. 6Aa). There was a ~4-fold increase of IFN-γ production by the VEGI-treated cells as compared with that by the immature cells. We also found that IL-2 production by T cells in cocultures with the VEGI-stimulated DCs was ~3 times that by the control cells (Fig. 6Ab). Furthermore, we determined the expression of CD62L, the activated T cell marker, by CD4+ T cells at the end of the 5-day coculture period. Approximately 59% of CD4+ T cells in the VEGI-stimulated DC cocultures expressed CD62L. In comparison, CD62L-positive T cells were ~28% in cocultures with immature DCs (Fig. 6B). These data indicate that the VEGI-stimulated DCs are capable of facilitating the proliferation and differentiation of CD4+ naive Th cells.

Discussion
Uncontrolled angiogenesis is a critical element of chronic inflammation, which is now considered closely linked to carcinogenesis (1, 2, 4, 34). Chronic inflammation is characterized by sustained tissue damage, damage-induced cellular proliferation, and tissue repair (35). The endothelium is concerned in every aspect of these processes. Inflammatory conditions also induce DC maturation, which is a critical step in the initiation of immunity (20). Following Ag uptake, mature DCs migrate to lymph nodes, produce peptide-MHC complexes and other costimulatory molecules, and carry out the priming of CD4+ T cells and CD8+ CTL, the activation of B cells, and the initiation of an adaptive immune response (36). Because the overall aim of the immune responses is to control inflammation, it is interesting to find that VEGI, an antiangiogenic cytokine of the TNF-α superfamily, produces mostly by endothelial cells, may have a key role in both the termination of angiogenesis and facilitation of DC maturation.

That VEGI stimulates DC maturation is supported by the findings that VEGI regulates the activation of transcription factors STAT3 and NF-κB and the MAPKs ERK, JNK, and p38 in DCs. While ERK activation is reported to be associated with the inhibition of DC maturation (37), JNK is known to be necessary for the release of IL-12 and TNF from mature DCs (38), and p38 MAPK is involved in the allostimulatory function and expression of co-stimulatory and adhesion molecules (39). A number of recent studies highlighted the important role of STAT3 in DC differentiation, and that of JAK-STAT pathway in DC maturation (40–42). We found that p38, JNK, and STAT3 were activated within 1 h of VEGI treatment, and ERK phosphorylation was suppressed at the same time. As STAT3 phosphorylation dissipated within 4–8 h of VEGI treatment, ERK activation resumed, suggesting a balance of signal strengths between STAT3- and ERK-associated signaling pathways as reported in human DC maturation induced by TNF (43). There were findings that VEGI-induced intracellular signaling in DCs involving NF-κB is also important, because NF-κB is critical in the modulation of DC activation, Ag presentation, and cytokine responses (36, 44). We found that NF-κB1 (p105/p50), which was shown to be essential in regulating the MAPK pathway (45), was activated in DCs when stimulated with VEGI. Another NF-κB protein, p65, which together with p50 makes up the NF-κB transcription factor complex, underwent translocation from the cytoplasm to the nucleus within 2 h of VEGI stimulation. Taken together, changes in DC signaling pathways resulting from VEGI stimulation are consistent with a direct role of VEGI in promoting DC maturation.

Our data suggest that DR3 is involved in the transmission of VEGI activity in DC activation. The incomplete inhibition of VEGI activity that we observed by the DR3 Ab or soluble DR3 indicates that either these reagents cannot completely inhibit VEGI activity under the experimental conditions, or the DR3 is only partially responsible. These results may be associated with another observation that the VEGI isoform TL1A did not induce STAT3 and NF-κB activation in immature DCs, whereas VEGI-192 or the mammalian cell-produced VEGI-251 did under otherwise identical experimental conditions. This interesting result may be attributed to the fact that VEGI-192 and TL1A primary sequences are different in the segment that is likely to form the first β-strand of the tertiary structures of these proteins (15). The difference in DC stimulation activity between TL1A and the mammalian cell-processed VEGI-251, however, is not readily explained without an assessment of the protein structures regarding post-translational modifications because the two primary sequences are identical (27).

The notion that VEGI promotes DC maturation is also supported by our findings that VEGI induces morphological changes toward characteristics of mature DCs. Mouse DCs are immature when derived in vitro from their bone marrow precursors. They form clumps of cells that float in the culture wells. However, upon addition of maturation stimuli such as TNF and LPS, the cells rapidly adhere, develop polarity, and assemble actin-rich structures known as podosomes on the leading edge of the cell. As the maturation process progresses, DCs once again become rounded and devoid of podosomes (32). We documented similar changes that occurred when the bone marrow-derived DCs were cultured in the presence of VEGI. Additionally, the cytoskeletal component F-actin in VEGI-treated DCs displayed a rearrangement from a mainly cytosolic presence to a predominantly cell membrane association. The morphological changes in DCs by VEGI treatment closely resemble those arising from treatment with TNF, IL-1β, or LPS (46, 47).

Highly relevant to the function of mature DCs, IL-12 and TNF were found to be substantially up-regulated in DCs in response to VEGI stimulation. Interestingly, these cytokines play important roles in the modulation of both angiogenesis and inflammation. As a specific marker of functionally mature DCs, IL-12 is the main cytokine that regulates Th cell differentiation and T cell proliferation, and modulates the expression of other cytokines that coordinate the resulting immune response (48–50). IL-12 is considered a potent proinflammatory cytokine that possesses antitumor, antimetastatic, antiangiogenic properties, as well as the ability to elicit long-term antitumor immunity. Regarding angiogenesis modulation, IL-12 was reported to inhibit fibroblast growth factor-2-induced corneal neovascularization in mice (51). TNF is another interesting cytokine significantly up-regulated in VEGI-treated DCs. TNF with its wide ranging, sometimes seemingly contradictory functions is implicated in linking inflammation to tumor inhibition as well as carcinogenesis (52, 53). TNF under inflammatory conditions plays a critical role in both tissue destruction and damage recovery, maintenance of the reversibility of microenvironments, stimulation of cellular changes, and tissue remodeling. TNF may initiate an inflammatory cascade consisting of other inflammatory cytokines, chemokines, growth factors, and endothelial adhesion factors, recruiting a variety of activated cells at the site of tissue damage. One should be cautioned, however, that the markedly enhanced production of these factors, albeit characteristics of mature DCs, may be the result of an indirect effect of VEGI action, because the analysis of the DC conditioned medium was conducted 48 h after VEGI treatment, leaving a sufficient
length of time to subject the cells to respond to yet unidentified cytokines and chemokines primarily stimulated by VEGI.

With regard to Ag processing and presentation, mature DCs are functionally defined by a newly acquired ability to efficiently present Ags on the surface and a decreased capacity to take up Ags by endocytosis. We found that the VEGI-stimulated DCs were characterized by an enhanced expression of the mature DC-specific marker CD83 and the secondary lymphoid tissue-directing chemokine receptor CCR7, and the Ag-presenting machinery MHC-II and the costimulatory molecules CD40, CD80, and CD86. In addition, we studied the ability of the cells to translocate MHC-II to the cell surface in the same experiment in which we analyzed the capacity of the cells to take up Ags. MHC-II is essential for Ag processing in DCs by forming peptide-MHC-II complexes, which are then redistributed from the cytosolic compartment to the cell surface so as to allow interaction with TCRs to initiate T cell immunity. Immature DCs internalize Ags into late endosomes and lysosomes, where MHC-II molecules remain. As a result of VEGI treatment, the ability of the cells to internalize OVA declined substantially compared with that of the untreated immature DCs. Although the MHC-II molecules in the untreated cells were colocalized with internalized OVA in the cytosolic compartment, the MHC-II molecules in VEGI-stimulated DCs were translocated to the cell surface. Moreover, the VEGI-stimulated DCs are able to effectively prime CD4 T cells, as indicated by the increased growth rate of the CD4+ T cells cocultured with the VEGI-stimulated DCs, and the enhanced IFN-γ and IL-2 production as well as the up-regulated expression of the activated T cell marker CD69 by these cells. These results support our view that the VEGI-stimulated DCs changed from the Ag-uptaking phenotype typical of immature DCs to the Ag-presenting phenotype typical of mature DCs.

There is an intimate relationship between DCs and endothelial cells. Findings from studies on atherosclerosis revealed that B and T lymphocytes, macrophages, and DCs reside in the adventitia of normal, noninflamed mouse aortas before inflammation (54). Additionally, studies on giant cell arteritis show that a distinctive population of DCs localizes at the adventitia-medium border of normal, noninflamed mouse aortas before inflammation (54). Ad- ventitial inflammation and cancer development. It is conceivable that such proximity between DCs and endothelial cells allows close monitoring by DCs of blood-borne Ags and endothelium-derived cytokines and chemokines, including VEGI.

It is interesting to note that vascular endothelial growth factor (VEGF), which functions as a major angiogenic factor and inducer of inflammation (56), inhibits DC maturation (57, 58). This effect is the opposite of what we observed for VEGI, an antiangiogenic cytokine. It was found that the VEGF receptor VEGFR1 is the primary mediator of the VEGF inhibition of DC maturation (56). VEGFR1 signaling was sufficient to block NF-κB activation in bone marrow hemopoietic progenitor cells (59). VEGF was also reported to impair the functional ability of DCs through the Id pathways (60). Abs to VEGF enhance the efficacy of cancer immunotherapy by improving endogenous DC function (61). The findings that VEGF is able to simultaneously promote angiogenesis and inhibit DC maturation are consistent with our view that inhibition of angiogenesis and promotion of DC maturation could be coupled as the functions of one single protein factor such as VEGI.

Induction of DC maturation is the initial step in immune responses toward inflammatory stimuli. The goal of the immune responses is the resolution of inflammation. This goal is compatible with VEGI being an inhibitor of endothelial cell proliferation. Based on our experimental findings that VEGI can directly stimulate DC maturation, which is an essential component of host immunity against cancer development, we suggest that VEGI may play a central role in the interaction between the endothelium and the immune system to modulate angiogenesis and inflammation toward the suppression of carcinogenesis. It should be interesting to determine how VEGI activity is regulated by other inflammatory and angiogenic factors, and what are the consequences of VEGI action on the production of DCs of cytokines and chemokines that regulate other cellular components of the innate and adaptive immune system. Findings from these studies should provide needed insights into the link between inflammation and cancer development.

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**Disclosures**

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