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B Cell-Derived Vascular Endothelial Growth Factor A Promotes Lymphangiogenesis and High Endothelial Venule Expansion in Lymph Nodes

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Vascular endothelial growth factor A (VEGF-A) is a prominent growth factor for both angiogenesis and lymphangiogenesis. Recent studies have shown the importance of VEGF-A in enhancing the growth of lymphatic endothelial cells in lymph nodes (LNs) and the migration of dendritic cells into LNs. VEGF-A is produced in inflamed tissues and/or in draining LNs, where B cells are a possible source of this growth factor. To study the effect of B cell-derived VEGF-A, we created transgenic mice (CD19Cre/hVEGF-Afl) that express human VEGF-A specifically in B cells. We found that the human VEGF-A produced by B cells not only induced lymphangiogenesis in LNs, but also induced the expansion of LNs and the development of high endothelial venules. Contrary to our expectation, we observed a significant decrease in the Ag-specific Ab production postimmunization with OVA and in the proinflammatory cytokine production postinoculation with LPS in these mice. Our findings suggest immunomodulatory effects of VEGF-A: B cell-derived VEGF-A promotes both lymphangiogenesis and angiogenesis within LNs, but then suppresses certain aspects of the ensuing immune responses. The Journal of Immunology, 2010, 184: 4819–4826.

Host defense against infection requires the integrated function of both the innate and the adaptive immune systems. Innate immune responses, which represent the front line of the immune system, are elicited by a variety of cell types, including granulocytes, macrophages, mast cells, NK cells, and dendritic cells (DCs). DCs are the professional APCs that form the bridge between innate and adaptive immune responses (1). DCs process material from invading pathogens and damaged tissues, which results in the upregulation of CCR7. Expression of CCR7 allows the DCs to enter draining lymphatic vessels that express the CCR7 ligands CCL21 and CCL19 (2). On reaching the draining lymph nodes (LNs), the DCs interact with T and B cells, thus inducing adaptive immune responses.

Lymphatic vessels are essential for transporting tissue fluid, extravasated plasma proteins, and cells back to the blood circulation (3). Lymphatic vessels contribute to the immune surveillance of the body by transporting Ag-bearing DCs from peripheral tissues to the regional LNs, where they present Ags to lymphocytes. Congenital or acquired dysfunction of lymphatic vessels leads to chronic swelling, adipose degeneration, immune dysfunction, and susceptibility to infection (3). Lymphatic vessels are not simply inert drainage ducts; rather, they are actively involved in many physiologic and pathologic processes. For example, remodeling of lymphatic vessels by tumor-derived lymphangiogenic factors actively promotes cancer metastasis (4–6). Lymphatic vessels are also remodeled in various inflammatory conditions (7), and these remodeled vessels promote inflammation (8–10). Recent studies have revealed that lymphatic vessel growth (lymphangiogenesis) is regulated by vascular endothelial growth factor (VEGF)-C and -D via their receptor, VEGFR-3 (10, 11). In addition, VEGF-A and its receptor, VEGFR-2, also play an important role in lymphangiogenesis, especially in the enlargement of lymphatic vessels (6, 12, 13). During inflammatory conditions, remodeling of lymphatic vessels occurs not only in inflamed peripheral tissues, but also in the regional LNs. Expansion of lymphatic vessels within LNs is important because it enhances the mobilization of DCs to the draining LNs (14). Expansion of lymphatic vessels within LNs can be locally controlled by lymphangiogenic factors released within the LNs (14, 15) or remotely controlled by factors released in the peripheral tissues (16). In the former case, this process depends upon the
presence of B cells within the LNs (14, 15). B cells in inflamed LNs express VEGF-A and can be stimulated to secrete VEGF-A in vitro (14), suggesting the involvement of B cell-derived VEGF-A in lymphangiogenesis and DC mobilization. However, the exact role of B cell-derived VEGF-A in vivo is still unknown.

In this study, we investigated the effect of B cell-derived VEGF-A in vivo using CD19Cre/hVEGF-A mice that express human VEGF-A (hVEGF-A) specifically in B cells. We found that these mice had enlarged LNs, with expanded lymphatic vessels and increased high endothelial venules (HEVs), even when they were not immunized. To the best of our knowledge, this is the first study describing the effect of B cell-derived VEGF-A in vivo.

**Materials and Methods**

**Mice**

Mice were kept under environmentally controlled pathogen-free conditions (light from 7:00 to 19:00; water, and standard, rodent diet ad libitum; 23 °C; 55% humidity). Mice of C57BL/6N background were used to generate the transgenic (Tg) mice. Mice heterozygous for Cre recombinase inserted into the CD19 locus (CD19Cre mice) (17) were kindly provided by Dr. Ursula Lichtenberg, Institute for Genetics, University of Cologne, Cologne, Germany. Animal experiments were performed in accordance with the guidelines of the Frontier Science Research Center, Kagoshima University, Kagoshima, Japan. All efforts were taken to minimize the number of animals used and their suffering.

**Establishment of CD19Cre/hVEGF-A mice**

The plasmid construct, containing human VEGF-A flanked by second loxp site (p-hVEGF-A5), is shown in Fig. 1A. To construct the p-hVEGF-A5, the lacZ gene in pCE7-17 (18) was replaced by the 576 bp cDNA encoding lacZ in the proper orientation (CD19Cre/hVEGF-A mice). The resulting DNA construct (p-hVEGF-A5) contains a CMV enhancer/chicken β-actin promoter (CAT), an enhanced green fluorescent protein (EGFP)/chloramphenicol acetyltransferase (CAT) sandwiched between two loxP sites and human VEGF-A flanked by loxP.

The 5.4-kb SpeI fragment containing the hVEGF-A transgene was removed from the p-hVEGF-A vector and microinjected into the pronuclei of the fertilized eggs of C57BL/6N mice (18). The Tg founder (F0) mice (termed hVEGF-A mice) were identified by EGFP fluorescent blood nuclei of the fertilized eggs of C57BL/6N mice (18). The Tg founder (F0) mice (termed hVEGF-A mice) were identified by EGFP fluorescent blood nuclei of the fertilized eggs of C57BL/6N mice (18). The presence of the hVEGF-A transgene was confirmed by PCR. All numbers used and their suffering.

**Methods**

**In vivo assay for Cre-mediated DNA recombination**

In the absence of Cre, hVEGF-A expression is prevented by the intervening transcriptional EGFP/CAT sequence flanked by loxP sites. Cre-mediated DNA recombination results in the removal of the EGFP/CAT sequence, followed by hVEGF-A expression (Fig. 1Ai, Supplementary Fig. 1A). Genomic DNA was isolated and amplified using PCR primers Chis	extsuperscript{9} (5’-GGG GCC GTT CCG CTG TCG TGT GC) and Vesp	extsuperscript{9} (5’-TCA CCG CCT CGG TGT GTC CAT TCT GCA AGT-3), which recognize sequences of chicken β-actin promoter and hVEGF-A, respectively. In the case of hVEGF-A mice, a 3.2-kb fragment including the EGFP/CAT and hVEGF-A was amplified, whereas Cre-mediated recombination in CD19Cre/hVEGF-A mice resulted in the amplification of a 788-bp PCR product (Fig. 1Aii).

**LPS challenge**

CD19Cre/hVEGF-A	extsuperscript{5} mice (13 to 14 wk, *n* = 5) were injected i.p. with LPS (1 mg/kg, *Escherichia coli* 055:B5; Sigma-Aldrich, St. Louis, MO) in sterile saline prior to cytokine analysis. Blood samples were collected 8 h later by cardiac puncture. Serum was isolated from the blood samples and stored at −80 °C until required.

**Histopathological examination**

**H&E staining**

Spleen and LN specimens were fixed for 24 h in 10% formaldehyde neutral buffer solution, embedded in paraffin wax, and sectioned (5–10 μm). Sections were stained with H&E.

**Immunohistochemical staining**

For immunohistochemistry, paraffin sections were heated in a microwave oven for 20 min, dewaxed in xylene, and rehydrated through a graded series of ethanol solutions. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in absolute methanol for 15 min at room temperature. Ag epitopes were heat-retrieved in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). Samples were then incubated overnight at 4 °C with primary Abs: rabbit polyclonal anti-vascular endothelial hyaluronan receptor-1 (LYVE-1) (1:500 dilution; Upstate Biotechnology, Temecula, CA), rabbit polyclonal anti-mouse PECAM-1 (M-20) (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rat monoclonal anti-mouse CD45RB/220 (clone RA3-6B2, rat IgG2K, 1:50 dilution; BD Biosciences, San Jose, CA), and rat monoclonal anti-CD3 (clone CD3-12, IgGl, 1/1000 dilution; Acris Antibodies, Hiddenhausen, Germany). Primary Abs were diluted using 1% BSA in PBS containing 0.01% Tween. The incubation with the secondary Ab was carried out for 1 h using Histofine simple stain mouse MAX-PO (rabbit) or Histofine simple stain mouse MAX-PO (rat) (Nichirei, Tokyo, Japan) at room temperature. Peroxidase activity was visualized using 3,3'-diaminobenzidine (DakoCytomation, Carpinteria, CA), and the slides were lightly counterstained with Lillie-Meyer’s hematoxylin (Wako, Osaka, Japan).

Photographs were taken using a Zeiss Axioshot microscope with an AxioCam MRc5 camera equipped with AxioVision Release 4.6 software (Carl Zeiss, Oberkochen, Germany).

**VEGF-A ELISA**

Human and mouse VEGF-A level was measured in spleen homogenates and serum obtained from 13–19-wk-old CD19Cre/hVEGF-A	extsuperscript{5} and CD19Cre mice using Human or Mouse VEGF Immunoassay (Quantikine; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions (*n* = 6).

**Measurement of inflammatory cytokines**

TNF-α, IFN-γ, IL-2, IL-4, and IL-5 levels were determined using a mouse Th1/Th2 Cytokine Kit (BD Biosciences). IL-1β, IL-6, and IL-10 were determined using Mouse IL-1β, Mouse IL-6, and Mouse IL-10 ELISA kits, respectively (BioSource International, Camarillo, CA). IL-9 was determined using a Mouse IL-9 ELISA Kit (RayBiotech, Norcross, GA).

**Total RNA isolation and quantitative RT-PCR analysis**

Total RNA was extracted from spleen, LNs, and isolated B220+ and B220− cells using a total RNA isolation kit (RNAqueous, Ambion, Austin, TX). Total RNA was quantified spectrophotometrically. Total RNA (2 μg) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed using the TaqMan Gene Expression assay (Applied Biosystems). Reactions were run in 96-well plates in an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Data collection and analysis was performed using SDSv1.4 software (Applied Biosystems), after which data were exported and further analyzed. Data were normalized based on the expression levels of GAPDH. Absence of contaminating genomic DNA was confirmed by RT-PCR of the RNA samples.

**Isolation of B220+ B cells**

Single-cell suspensions were prepared from the spleens of 16–49-wk-old CD19Cre/hVEGF-A	extsuperscript{5} and CD19Cre mice by dissociation of the isolated tissues with glass slides in MACS separation buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) and passage through a 30-μm nylon mesh. B cells were enriched by positive selection using CD45RB (B220) microbeads and an AutoMACS Magnetic cell Sorter according to the manufacturer’s instructions (Miltenyi Biotec).

**Flow cytometry**

Blood was collected from sevofrane-anesthetized mice by cardiac puncture and was anticoagulated with sodium citrate. Single-cell suspensions from spleen and LNs were prepared as described above. Nonspecific binding was blocked by incubation with an Fc-blocking Ab (10 μg/ml; BD Biosciences). Samples were then stained with mAbs to mouse CD4, CD8, CD19 (Beckman Coulter, Miami, FL), and the remaining cells were fixed. Flow Count Beads (Beckman Coulter, Fullerton, CA) were added to the samples for quantitation. Cells were analyzed using an Epics XL flow cytometer (Beckman Coulter, Miami, FL).
OVA sensitization and challenge followed by determination of serum Ab concentration

For sensitization, 10–12-wk-old CD19Cre/hVEGF-A$^0$ and CD19Cre mice were injected s.c. on day 0 with 50 μg OVA (grade V; Sigma-Aldrich) adsorbed on 100 μg (100 μl) of CFA (Sigma-Aldrich) and again on day 10 with 25 μg adsorbed on 100 μl incomplete Freund’s adjuvant. Mice were challenged intranasally with 25 μg OVA in PBS on days 21, 22, and 23. Mice were killed on day 25. Serum and spleen homogenates were obtained for measurement of OVA-specific IgG1 and IgE Abs by ELISA using a mouse OVA-IgG1 and IgE kit (Shibayagi, Gunma, Japan) according to the manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed using the Student t test, Mann-Whitney’s U test, or Tukey-Kramer test through all of the experimental procedures. p values of *p < 0.05 and **p < 0.01 were considered significant.

Results

Generation of Tg mice expressing hVEGF-A specifically in B cells

We generated mice overexpressing hVEGF-A in B cells by crossing loxP flanked (floxed) EGFP mice (hVEGF-A$^0$) onto mice expressing Cre under the control of the B cell-specific CD19 promoter (CD19Cre) (Fig. 1Ai). Prerecombination, the loxP flanked EGFP/CAT hybrid sequence was expressed under the control of the CAG promoter, whereas the hVEGF-A gene was silent. Cre-mediated recombination resulted in the deletion of the EGFP/CAT sequence and subsequent expression of the hVEGF-A gene. This resulted in CD19$^{Cre}$/hVEGF-A$^0$ Tg mice. The loxP sequence, followed by VEGF-A sequence (Supplemental Fig. 1A) and the appearance of a 788-bp PCR product in the DNA from B220$^+$ cells of CD19$^{Cre}$/hVEGF-A$^0$ mice (Fig. 1Aii), confirmed successful recombination.

Because the EGFP gene is deleted from the B cells of hVEGF-A$^0$ mice during recombination to create CD19$^{Cre}$/hVEGF-A$^0$ mice, we measured the EGFP expression in CD19$^+$ B cells using flow cytometry (Fig. 1Bi). In CD19$^{Cre}$/hVEGF-A$^0$ mice, ~80% of the CD19$^+$ B cells were EGFP negative. This percentage was similar in CD19$^+$ B cells from spleen, LN, and blood samples (Fig. 1Bii). Flow cytometry using Abs against CD19, CD8, and CD4 showed that EGFP had been selectively deleted from the CD19$^+$ B cells in the CD19$^{Cre}$/hVEGF-A$^0$ mice (Supplemental Fig. 1B).

The expression of hVEGF-A mRNA was observed in spleen and LN samples from CD19$^{Cre}$/hVEGF-A$^0$ mice, but not in samples from of CD19$^{Cre}$ mice (Fig. 1C). The expression of mouse VEGF-A (CD19$^{Cre}$) (Fig. 1Ai). Prerecombination, the loxP flanked EGFP/CAT hybrid sequence was expressed under the control of the CAG promoter, whereas the hVEGF-A gene was silent. Cre-mediated recombination resulted in the deletion of the EGFP/CAT sequence and subsequent expression of the hVEGF-A gene. This resulted in CD19$^{Cre}$/hVEGF-A$^0$ Tg mice. The loxP sequence, followed by VEGF-A sequence (Supplemental Fig. 1A) and the appearance of a 788-bp PCR product in the DNA from B220$^+$ cells of CD19$^{Cre}$/hVEGF-A$^0$ mice (Fig. 1Aii), confirmed successful recombination.

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![FIGURE 1. DNA constructs and Cre-mediated recombination event. Ai, Schematic of the target plasmid (p-hVEGF-A$^0$) and its recombinated form are shown. LoxP sites are shown as triangles. The small arrows (Chi5' and Veg3') indicate the positions and directions of the primers used for PCR. Aii, Identification of Cre-mediated DNA recombination product by PCR amplification of DNA isolated from spleen of hVEGF-A$^0$ mice (lane 1), B220$^+$ cells of CD19$^{Cre}$/hVEGF-A$^0$ mice (lane 2), and B220$^+$ cells of CD19$^{Cre}$/hVEGF-A$^0$ mice (lane 3). The 788-bp fragment represents the successfully recombinated DNA. M indicates PCR marker. Bi, Flow cytometry analysis of B cells from the spleen of 19–22-wk-old hVEGF-A$^0$, CD19$^{Cre}$, and CD19$^{Cre}$/hVEGF-A$^0$ mice. Cells were stained with Abs to CD19. Percentages refer to EGFP-positive and -negative cells among CD19$^+$ cell population. Bii, Recombination ratio of hVEGF-A in the CD19$^+$ cells from spleen, LN, and blood samples. The ratio was defined as the percentage of EGFP-negative cells in the total CD19$^+$ cell population. C, hVEGF-A mRNA expression in spleen of CD19$^{Cre}$/hVEGF-A$^0$ and CD19$^{Cre}$ mice analyzed by quantitative RT-PCR (n = 6). D, hVEGF-A mRNA expression in B220$^+$ and B220$^-$ cells of CD19$^{Cre}$/hVEGF-A$^0$ (n = 4) and CD19$^{Cre}$ (n = 5) mice analyzed by quantitative RT-PCR. E, hVEGF-A protein expression in spleen homogenates and serum from CD19$^{Cre}$/hVEGF-A$^0$ and CD19$^{Cre}$ mice (n = 6). F, mVEGF-A protein expression in spleen homogenates from CD19$^{Cre}$/hVEGF-A$^0$ and CD19$^{Cre}$ mice. Data in A–F are representative of at least two independent experiments. **p < 0.01. ND, not detected.](http://www.jimmunol.org/Downloadedfrom)
(mVEGF-A) mRNA was similar in both the CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> and CD19<sup>cre</sup> mice (Supplemental Fig. 2A). The expression of mVEGF-D mRNA was also similar in both (Supplemental Fig. 2C), but mVEGF-C mRNA expression was significantly increased in CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice (Supplemental Fig. 2B), which is in agreement with previous studies (6).

To examine whether hVEGF-A mRNA was specifically expressed by the B cells, we isolated B220<sup>+</sup> cells from the spleens of CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> and CD19<sup>cre</sup> mice. B220<sup>+</sup> cells, but not B220<sup>−</sup> cells, expressed mCD19 mRNA, indicating the successful isolation of B cells (data not shown). In this experimental condition, B220<sup>+</sup> cells, but not B220<sup>−</sup> cells, expressed hVEGF-A mRNA (Fig. 1D), confirming the specific expression of hVEGF-A mRNA in B cells. The expression of mVEGF-A mRNA was not significantly different in either the B220<sup>+</sup> or the B220<sup>−</sup> cells of both CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> and CD19<sup>cre</sup> mice (data not shown).

Next, we examined hVEGF-A protein levels in the CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice. hVEGF-A was detected in spleen homogenates of CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice, but was not present in their serum (Fig. 1E), suggesting that hVEGF-A is localized to lymphoid organs and does not spread through the systemic circulation in CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice. No hVEGF-A was detected in either the spleen homogenates or the serum from the CD19<sup>cre</sup> mice. mVEGF-A protein levels in spleen homogenates were similar in CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> and CD19<sup>cre</sup> mice (Fig. 1F), and the total amount of VEGF-A (hVEGF-A plus mVEGF-A) in spleen homogenates was 3- to 4-fold higher in CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice.

The CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice appeared grossly normal in weight and life span, and peripheral blood cell levels were comparable to that of CD19<sup>cre</sup> mice. CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup>, CD19<sup>cre</sup>, hVEGF-A<sup>fl</sup>, and WT mice were born at the expected Mendelian ratio, but we noticed a slight reduction in the Mendelian distribution of hVEGF-A<sup>fl</sup> mice. The ratio of CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup>;CD19<sup>Cre</sup>;hVEGF-A<sup>fl</sup>;WT mice was 32.41%;24.83%;19.31%;23.45%, respectively, from a total of 145 mice. These findings further support the idea that hVEGF-A protein in CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice is localized to lymphoid organs and does not spread through the systemic circulation, as mice expressing systemic VEGF-A develop widespread tissue edema and die within days (20).

**Increased lymphangiogenesis and angiogenesis in LNs of CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice**

At around 14 wk, the LNs of CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice were significantly larger than those of CD19<sup>cre</sup> mice of the same age (Fig. 2Ai, Supplemental Fig. 3A). Quantitative FACS analysis of LN cell suspensions revealed significantly increased numbers of B and T cells in the LNs of CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice (Supplemental Fig. 3B). The LNs from CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice had an apparent reddish color, which may be due to the increased vascularization around LNs (Fig. 2Aii). Immunohistochemical analysis of the LNs showed an increase in both lymphangiogenesis and angiogenesis. LYVE-1<sup>+</sup> lymphatic vessels and PECAM-1<sup>+</sup> blood vessels were increased in the LNs of CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice compared with CD19<sup>cre</sup> mice (Fig. 2B). Higher magnification showed an increase in the number of HEVs within the LNs of the CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice that were stained for PECAM-1 (Fig. 3A, 3B). These findings suggest that B cell-derived VEGF-A promotes LN hypertrophy, lymphangiogenesis, and HEV expansion.

**Enlargement and disorganization of spleens in CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice**

On gross anatomical examination, we observed splenomegaly in the CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice (Fig. 4A), which developed from the age of 14 wk. Histological analysis of the spleens from CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice revealed a severe distortion of the microscopic structure, even in mice that were younger than 14 wk old, and this distortion was seen in both the red and white pulp areas (Fig. 4B). In addition, sinusoidal dilations were observed in the CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice. The spleens from CD19<sup>cre</sup> mice showed a normal structure. The distribution of T and B cells was similar in both CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> and CD19<sup>cre</sup> mice (Supplemental Fig. 4) despite the distortion of splenic structure present in the CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice. The number of CD8<sup>+</sup> T cells was significantly decreased in the spleens of the CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice, whereas that of CD19<sup>+</sup> B cells was similar in CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> and CD19<sup>cre</sup> mice (Supplemental Fig. 5).

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**FIGURE 2.** Lymphangiogenesis and angiogenesis in CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> mice. A, Weight of LNs taken from 19- to 22-wk-old CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> (n = 9) and CD19<sup>cre</sup> (n = 8) mice. Both individual and mean weights are presented. Data obtained from five independent experiments. Aii, The gross macroscopic structure of the ileocolic LNs of CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> and CD19<sup>cre</sup> mice. Scale bar, 2.5 mm. Arrowheads indicate the LNs. Representative images from five experiments are shown. B, Immunohistochemical analysis of expression of LYVE-1 and PECAM-1 in the ileocolic LNs of age- and sex-matched CD19<sup>Cre</sup>/hVEGF-A<sup>fl</sup> and CD19<sup>cre</sup> mice. Serial sections were taken and stained for LYVE-1 and PECAM-1 as indicated in the figure. Data are representative of three independent experiments. Scale bar, 500 μm. Original magnification ×25. *p < 0.05.
Decreased adaptive immune responses in CD19Cre/hVEGF-Afl mice

Next, we examined whether B cell-derived VEGF-A accelerated or suppressed the immune response in CD19Cre/hVEGF-Afl mice. To examine the adaptive immune response, we challenged CD19Cre/hVEGF-Afl and CD19Cre mice with OVA and then measured OVA-specific IgG1 levels. OVA-specific IgG1 levels were significantly lower in the serum of CD19Cre/hVEGF-Afl mice compared with CD19Cre mice (Fig. 5A). The OVA-specific IgG1 levels were also lower in spleen homogenates from CD19Cre/hVEGF-Afl mice (Fig. 5B), whereas the spleens were significantly larger in these mice (Fig. 5C). The OVA-specific IgE levels were not significantly different (data not shown). These findings indicate that B cell-derived VEGF-A promotes splenomegaly, but can suppress the Ab production.

LPS tolerance in CD19Cre/hVEGF-Afl mice

We examined cytokine levels in CD19Cre/hVEGF-Afl and CD19Cre mice. As shown in Fig. 6, LPS challenge resulted in the induction of TNF-α, IFN-γ, IL-5, and IL-6 production in both CD19Cre/hVEGF-Afl and CD19Cre mice, but the levels of these cytokines were significantly lower in CD19Cre/hVEGF-Afl mice. IL-1b, IL-2, IL-4, and IL-10 levels were similar in CD19Cre/hVEGF-Afl and CD19Cre mice. The cytokine levels in mice injected with saline alone were very low and were not significantly different in CD19Cre/hVEGF-Afl and CD19Cre mice. These findings indicate that B cell-derived VEGF-A induces LPS tolerance in CD19Cre/hVEGF-Afl mice.

Discussion

Besides being an angiogenic factor, VEGF-A has recently been identified as a pivotal mediator of inflammation-induced LN lymphangiogenesis (14, 16). However, the precise role of VEGF-A–induced inflammatory lymphangiogenesis in the modulation of immune function remains unclear. Hosts utilize various components of the immune system to carefully maintain the delicate balance between promoting a proper immune response to invading pathogens and preventing an excessive immune response that can

FIGURE 3. Increased numbers of HEVs in the LNs of CD19Cre/hVEGF-Afl. A, PECAM-1 staining of serial sections of ileocolic LNS from CD19Cre/hVEGF-Afl and CD19Cre mice. Ai, Low-power view of HEVs. Scale bar, 200 μm. Original magnification ×50. Aii, High-power view of the inset shown in Ai showing the characteristics of high endothelial cell structure. Data are representative of three independent experiments. Scale bar, 20 μm. Original magnification ×400. Bi, Number of HEVs in the LNs. Individual data and values are presented. **p < 0.01.

FIGURE 4. Enlargement and disorganization of spleens in CD19Cre/hVEGF-Afl mice. Ai, Gross macroscopic comparison of the spleens from age- and sex-matched CD19Cre/hVEGF-Afl and CD19Cre mice. Scale bar, 1 cm. Representative images from 10 experiments are shown. Aii, Spleen weights of 16–75-wk-old CD19/h-VEGF (n = 27) and CD19Cre (n = 16) mice. Individual data and mean values are presented. Data obtained from 10 independent experiments. B, H&E-stained sections of spleen from CD19Cre/hVEGF-Afl and CD19Cre mice. Representative images from 10 experiments are shown. Scale bar, 200 μm. Original magnification ×25. *p < 0.05.

FIGURE 5. Decreased adaptive immune responses in CD19Cre/hVEGF-Afl mice. OVA specific IgG1 levels in the serum (A) and the spleen (B) homogenates of 10–12-wk-old CD19Cre/hVEGF-Afl (n = 4) and CD19Cre (n = 4) mice postimmunization with OVA. C, Spleen weight (n = 4). Data are representative of two independent experiments. *p < 0.05; **p < 0.01.
lead to immunopathology (21). In this study, we have shown that B cell-derived VEGF-A might play a role in maintaining the balanced immune responses by orchestrating many aspects of the immune responses, including the expansion of lymphatic networks and the suppression of Ab production. Although our study does not reveal endogenous roles of B cell-derived VEGF-A or roles of other cell-derived VEGF-A, it provides an indication of the likely role of B cell-derived VEGF-A.

Lymphangiogenic roles of VEGF-A

A recent study suggested the involvement of B cell-derived VEGF-A in lymphangiogenesis and DC mobilization (14). In this study, we examined the role of B cell-derived VEGF-A in vivo using a Tg mouse model in which the B cells express hVEGF-A. We found that these mice had enlarged LNs, with expanded lymphatic vessels and increased HEVs, even when they were not immunized. These findings suggest that B cell-derived VEGF-A promotes lymphangiogenesis as well as angiogenesis in vivo.

VEGF-A induces lymphangiogenesis either directly or via upregulation of the lymphangiogenic factors VEGF-C and VEGF-D. Wirzenius et al. (13) reported that VEGF-A can directly promote lymphatic vessel enlargement via VEGFR-2 signaling. Crusiefen et al. (22) reported that inflammatory macrophages, in response to stimulation with VEGF-A, release VEGF-C/-D that contributes to lymphangiogenesis. We observed that the levels of VEGF-C mRNA (but not of VEGF-D mRNA) were increased in CD19Cre/hVEGF-Afl mice (Supplemental Fig. 2B, 2C), which is in agreement with a previous report showing that VEGF-A treatment upregulates VEGF-C expression in cultured endothelial cells (23). It is suggested that VEGF-A promotes lymphangiogenesis in CD19Cre/hVEGF-Afl mice either directly or via the upregulation of VEGF-C.

The cellular mechanisms of de novo lymphangiogenesis remain poorly defined and may involve the division of local pre-existing endothelial cells (24) or the incorporation of lymphatic endothelial progenitor cells of myeloid origin (25–27). We observed the accumulation of a CD11b + cell population in the LNs of our CD19Cre/hVEGF-Afl mice. CD11b + cells might play an important role in lymphangiogenesis either by secreting VEGF-C, which stimulates the division of pre-existing local lymphatic endothelial cells, (7) or by transdifferentiating and directly incorporating into the endothelial layer (25, 28).

Immunosuppressive roles of VEGF-A

Previous studies have indicated that lymphangiogenic responses lead to the increased migration of APCs to draining LNs, thereby boosting immune responses (14, 15, 29). Other studies have suggested that the growth of HEVs is associated with increased lymphocyte entry into the LNs, which, again, boosts the immune responses (30, 31). These findings led us to speculate that the increase in lymphangiogenesis and the growth of HEVs within LNs might stimulate...
immune responses in CD19Cre/hVEGF-Alox mice. However, VEGF-A is known to suppress both the development of T cells and the maturation of DCs (32–34), indicating that VEGF-A could suppress the immune response in CD19Cre/hVEGF-Alox mice. Therefore, we examined whether the immune response in CD19Cre/hVEGF-Alox mice was stimulated or suppressed by B cell-derived VEGF-A. We found a significant decrease in the Ag-specific Ab production post-inoculation with OVA in the pannlicrotic cytokine protein production postinoculation with LPS in these mice. Although the mechanisms underlying the immunosuppression in CD19Cre/hVEGF-Alox mice have not been elucidated, our data suggest that B cell-derived VEGF-A can suppress certain aspects of the immune responses.

**Immunomodulatory roles of VEGF-A**

VEGF-A can mediate negative as well as positive immunomodulatory roles, and we propose that VEGF-A can stimulate and later suppress certain features of the immune responses. Angiogenesis and lymphangiogenesis, mediated by VEGF-A, lead to the migration of immune cells into the LNs, thereby enhancing the immune response. VEGF-A can also enhance immune responses directly, in part through the activation of NF-kB and the induction of cytokines and chemokines (35–37). However, VEGF-A can also inhibit the development of T cells and the maturation of DCs and, in doing so, suppresses the immune response (32–34). Furthermore, VEGF-A plays a critical role in Ag clearance and resolution of inflammation (38).

One could speculate that VEGF-A might first promote the sensitization phase of the immune response and later help to limit the extent of the ensuing immune response and associated tissue pathology. This idea is consistent with the hypothesis that an important function of VEGF-A is to promote homeostasis. Further investigation is required to assess how, and under what circumstances, the immunomodulatory functions of VEGF-A can influence the magnitude of innate and adaptive immune responses.

In conclusion, this study shows the immunomodulatory effects of VEGF-A: B cell-derived VEGF-A promotes lymphangiogenesis and angiogenesis within LNs, but suppresses certain aspects of the ensuing immune responses.

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**Disclosures**

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