Suppression of Choroidal Neovascularization by Dendritic Cell Vaccination Targeting VEGFR2

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Purpose. To investigate whether the induction of cellular immunity against vascular endothelial growth factor receptor (VEGFR) 2 inhibits the development of choroidal neovascularization (CNV).

Methods. H-2Db-restricted peptide corresponding to amino acids 400 to 408 of VEGFR2 was used as an epitope peptide. Dendritic cells (DCs) were harvested from bone marrow progenitors of C57BL/6 mice. Six-week-old C57BL/6 mice received subcutaneous injections of the epitope peptide-pulsed mature DCs three times at 6-day intervals. After the third immunization, laser photocoagulation was performed to induce CNV. One week after photocoagulation, mice were killed to harvest the choroid and splenocytes. CNV volume was evaluated by volumetric measurements. To confirm the specific immunogenicity of the epitope peptides in C57BL/6 mice, CD8 T cells isolated from harvested splenocytes were restimulated to measure interferon (IFN)-γ and tumor necrosis factor (TNF)-α production through enzyme-linked immunospot assay and ELISA. To determine the T-cell subset responsible for the immunotherapy, mice were intraperitoneally injected with an anti-CD4 or anti-CD8 depletion antibody.

Results. CNV volume was significantly lower in mice immunized with the VEGFR2 epitope peptide than in those not immunized or immunized with a control peptide gp70. Cytokine assays showed the peptide-specific production of IFN-γ and TNF-α from the CD8 T cells in a dose-dependent manner. In vivo depletion of CD8, but not CD4, T cells significantly reversed the suppressive effect of the VEGFR2 peptide-pulsed DC vaccination on CNV to the level observed in nonimmunized or gp70-immunized animals.

Conclusions. These results indicate that the VEGFR2 peptide-specific induction of cellular immunity inhibits CNV through the cytotoxicity of CD8 T cells. Results of the present study suggested the possibility of DC vaccination targeting VEGFR2 as a novel therapeutic strategy for CNV. (*Invest Ophthalmol Vis Sci.* 2007;48:4795–4801) DOI:10.1167/iovs.07-0425

Age-related macular degeneration (AMD) is the most common cause of blindness in people older than 50 in the developed countries.[1] AMD is complicated by choroidal neovascularization (CNV), during which the choroidal new vessels invade the subretinal space through the Bruch membrane to form fibrovascular proliferative tissue containing vascular endothelial cells, fibroblasts, retinal pigment epithelial (RPE) cells, and various inflammatory cells.[2] Retinal neurons are irreversibly damaged by lipid leakage and bleeding from the immature new vessels in the CNV tissue. Although molecular and cellular mechanisms underlying CNV are not fully elucidated, vascular endothelial growth factor (VEGF) proved to be a critical angiogenic factor to promote CNV.[3–5] Additionally, increasing evidence has shown that CNV is associated with immunologic responses including complement activation[6,7] and macrophage infiltration.[8,9]

At present, there are two major therapeutic strategies for CNV, anti-VEGF therapy[10–12] and photodynamic therapy (PDT).[13–18] Various antiangiogenic therapies for targeting the VEGF-VEGF receptor (VEGFR) system have been investigated and established in the field of cancer research.[19–24] In the treatment of CNV as well, aptamer- and antibody-based VEGF blockers are clinically applied; however, their short-term effect on CNV requires repeated intraocular injections.[10,11] Moreover, although these drugs suppress vascular leakage and further enlargement of CNV, their effect on preexisting new vessels is thought to be limited. Injury to the choriocapillaris and abnormal new vessels from PDT leads to transient nonperfusion and inflammation, both of which induce VEGF expression from RPE cells in the treated area.[25] This adverse event is likely to cause the recurrence of CNV and subsequent repeated treatments. Therefore, these flaws of the current modalities have aroused further interest for the establishment of a novel therapeutic strategy for CNV.

As an immunologic approach to combat angiogenesis-dependent solid tumor, the regression of murine renal carcinoma was achieved by interleukin (IL)-12/pulse IL-2 combination therapy eliciting $CD8^+$ cytotoxic T lymphocyte (CTL)-mediated apoptosis of endothelial cells.[26] In addition, in the eye, our recent reports demonstrated $CD8^+$ CTL-mediated regression of physiologic and pathologic retinal new vessels.[27,28] Accordingly, we hypothesize a possible immunologic therapy for CNV by inducing CTL responses targeting a specific molecule highly expressed in CNV-associated endothelial cells. Of several candidate molecules, VEGFR2, which plays a pivotal role in endothelial cell proliferation and migration,[11,29,30] is upregulated during CNV.[31,32] In the present study, we deter-

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mined VEGFR2 as a target molecule for CTL-mediated apoptosis of endothelial cells in the CNV tissue. In tumor models, immunization with full-length cDNA or recombinant protein of VEGFR2 was reported to inhibit tumor growth and angiogenesis.[33,34] In particular, specific immunization with the major histocompatibility (MHC) class I-restricted epitope peptides of VEGFR2, which were recently identified in separate studies,[35,36] led to significant suppression of tumor angiogenesis. Here we examine for the first time whether experimental CNV is suppressed by inducing specific cellular immunity against VEGFR2 through vaccination with dendritic cells (DCs), professional antigen-presenting cells.

Materials and Methods

Animals

Male C57BL/6 mice (CLEA, Tokyo, Japan) at the age of 6 weeks were purchased and maintained in the specific pathogen-free Animal Facility of the Research Park, Keio University School of Medicine. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of CNV

Laser-induced CNV is a widely used animal model, reflecting the pathogenesis of inflammation-related CNV seen in human AMD. In this model, new vessels from the choroid invade the subretinal space after laser photocoagulation. Laser photocoagulation was performed around the optic nerve using a slit lamp delivery system (Novus spectra; Lumenis, Tokyo, Japan), as described previously.[8]

Quantification of Laser-Induced CNV

One week after laser photocoagulation, eyes were enucleated and fixed with 4% paraformaldehyde. Eyecups obtained by removing anterior segments were incubated with 0.5% fluorescein isothiocyanate-isolectin B4 (Vector, Burlingame, CA). CNV was visualized with blue argon laser wavelength (488 nm) using a scanning laser confocal microscope (FV1000; Olympus, Tokyo, Japan). Horizontal optical sections of CNV were obtained every 1-μm step from the surface to the deepest focal plane. The area of CNV-related fluorescence was measured by National Institutes of Health (NIH) imaging. The summation of whole fluorescent area was used as the volume of CNV, as described previously.[8]

Epitope Peptides

Major histocompatibility complex class I H-2Db-restricted peptide, corresponding to amino acids 400 to 408 of murine VEGFR2 (VILTNPISM; $VEGFR2_{400-408}$),[36] was used for an epitope peptide to induce cellular immunity specific for VEGFR2 in C57BL/6 mice. The gp70 peptide, the epitope sequence of p15E (KSPWFTTL; $p15E_{604-611}$),[37] served as a negative control for VEGFR2-specific immunotherapy and a positive control for cytokine assays because the gp70 peptide has potent immunogenicity in C57/BL6 mice. p15E is the envelope protein of an endogenous murine retrovirus of the Akv family found in the germline of C57BL/6 mice. We also used the epitope sequence of β-galactosidase (DAPIYTNV; $\beta\text{-gal}_{96-103}$)[38] as a negative control for cytokine assays to confirm the peptide specificity of T-cell responses. Peptides were synthesized and purified with high performance liquid chromatography (HPLC) by Sigma-Aldrich (St. Louis, MO).

Preparation of Mature DCs

Purified DCs were obtained using previously described methods[39–41] with slight modification. Briefly, marrow from tibias and femurs of C57BL/6 mice were harvested and then followed by DC enrichment with a magnetic cell sorting (MACS) kit (BD Biosciences PharMingen, San Jose, CA). The isolated precursors were cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/mL) to induce differentiation into DCs. After 1 week, OK432 and either of the epitope peptides ($VEGFR2_{400-408}$ or gp70) were added to the culture to induce DC maturation. OK432, a penicillin- and H_2O_2-killed lyophilized preparation of the Su strain of *Streptococcus pyogenes*,[42] was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). After 6 hours, generated mature DCs were harvested and used for subsequent vaccination. The maturation of DCs as $CD11c^+$ $CD40^+$ cells or $CD11c^+CD86^+$ cells was confirmed by flow cytometry using a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, Mountain View, CA) with software (CellQuest; Becton Dickinson; data not shown).

Vaccination with Peptide-Pulsed DCs

Mice were immunized as previously described[35,36,43] with slight modification. In brief, C57BL/6 mice underwent subcutaneous inoculation of 5×10^5 mature DCs and 200 μL vaccine mixture containing 100 μg epitope peptides ($VEGFR2_{400-408}$ or gp70) and 100 μL incomplete Freund adjuvant (IFA; Difco Laboratories, Detroit, MI) every 7 days for 3 weeks. After the third immunization, laser photocoagulation was performed to induce CNV. One week after laser photocoagulation, immunized mice were killed to harvest the choroid and splenocytes.

IFN-γ Enzyme-Linked Immunospot Assay

We determined interferon (IFN)-γ as an indicator to detect CTL responses in the assay. Naive splenocytes irradiated with 40 Gy served as stimulator cells. Using a MACS kit for $CD8a^+$ T-cell isolation (Miltenyi Biotech, Auburn, CA), CD8 T cells were isolated from the splenocytes excised from $VEGFR2_{400-408}$-immunized mice. Isolated CD8 T cells, subjected to 1-week in vitro restimulation with 10 μg/mL $VEGFR2_{400-408}$ and stimulator cells plus 10 U/mL IL-2, were used as effector cells.[36] In the 200-μL medium containing $VEGFR2_{400-408}$ at the dose of 0, 0.1, 1, or 10 μg/mL, 2×10^5 effector cells were cocultured overnight with 1×10^6 stimulator cells at 37°C in a 95% air-5% CO_2 atmosphere with a 96-well plate with a nitrocellulose base (Millititer HA; Millipore, Bedford, MA) coated with an anti-mouse IFN-γ capture antibody (clone R4–6A2; BD Biosciences PharMingen). Cells were removed by washing with phosphate-buffered saline (PBS), and the plates were overlaid with 1 μg/mL of a biotinylated anti-mouse IFN-γ detection antibody (clone XMG1.2; BD PharMingen) for 3 hours. The plates were washed again, and streptavidin-coupled alkaline phosphatase (Mabtech, Nacka Strand, Sweden) was added and incubated for 3 hours. After washing with PBS, a precipitating substrate for alkaline phosphatase was added, and the plates were incubated until spots emerged at the site of the responding cells. The number of spots was counted in a dissection microscope.

Enzyme-Linked Immunosorbent Assay for IFN-γ and TNF-α

To further confirm the peptide-specific T-cell responses, we examined the concentration of IFN-γ and tumor necrosis factor (TNF)-α that the effectors released in the medium. TNF-α and IFN-γ are regarded as indicators for CTL responses. After overnight coculture of the effector and stimulator cells, together with the epitope peptides ($VEGFR2_{400-408}$, gp70 or $\beta\text{-gal}_{96-103}$) as described, the supernatant was collected and the protein levels of IFN-γ and TNF-α were measured with mouse IFN-γ and TNF-α ELISA kits (Biosource, Camarillo, CA), respectively.

In Vivo Depletion of T-Cell Subsets

Immune cell subsets were depleted in vivo as previously described.[44,45] Mice were injected intraperitoneally with 5 mg/kg body weight of either an anti-CD4 or an anti-CD8 depletion antibody (clone GK1.5 or clone 53–6.72, respectively; eBioscience, San Diego, CA) or an isotype control antibody (BD Biosciences PharMingen) 1 day before laser photocoagulation. The depletion of T-cell subsets was confirmed by flow cytometry using splenocytes from immunized mice 7 days after treatment. The percentage of CD4 T cells ($CD3^+CD4^+$) or CD8 T cells

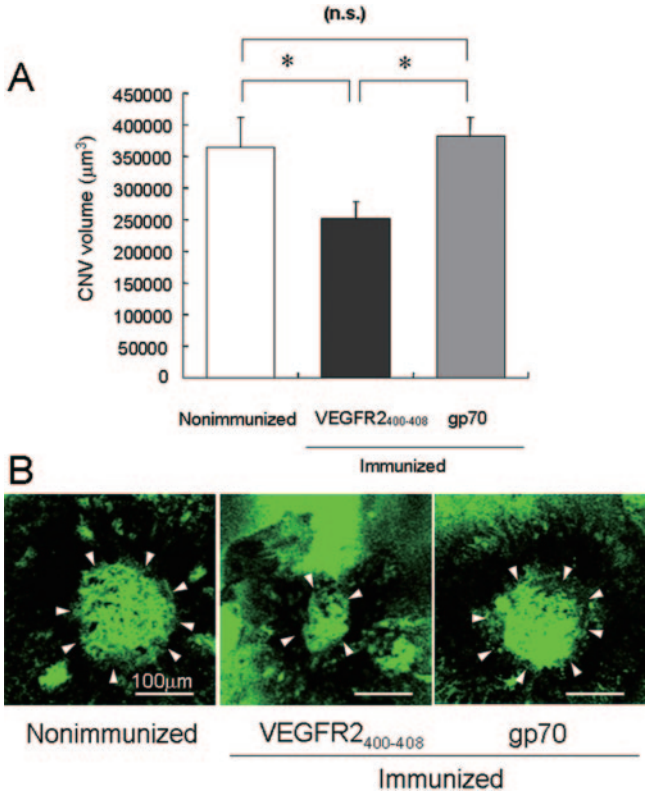

FIGURE 1. Suppression of CNV by vaccination with $VEGFR2_{400-408}$-, but not gp70-, pulsed DCs. Graph shows the volume of CNV (**A**). CNV volume was significantly reduced by immunization with $VEGFR2_{400-408}$. Flatmounted choroids from nonimmunized, $VEGFR2_{400-408}$-immunized, and gp70-immunized mice (**B**). *Arrowheads:* lectin-stained CNV. Scale bar, 100 μm. Results represent mean ± SE. $n = 24$, 34, and 38 for nonimmunized, $VEGFR2_{400-408}$-immunized, and gp70-immunized animals, respectively. $^*P < 0.01$ by Mann-Whitney test.

($CD3^+CD8^+$) was compared between anti-CD4 or anti-CD8 antibody-treated mice and control animals.

RESULTS

Suppression of CNV by Vaccination with $VEGFR2_{400-408}$-Pulsed DCs

The CNV volume was analyzed to evaluate the effect of vaccination with $VEGFR2_{400-408}$- or gp70-pulsed DCs on the development of CNV (Fig. 1). Interestingly, $VEGFR2_{400-408}$-immunized mice exhibited significant ($P < 0.01$) suppression of CNV (251,050 ± 153,970 μm^3) compared with nonimmunized (363,476 ± 237,641 μm^3) or gp70-immunized (381,898 ± 184,592 μm^3) controls. Importantly, there was no significant ($P > 0.05$) difference in the CNV volume between nonimmunized and gp70-immunized mice, suggesting that the observed suppression of CNV was attributable to the $VEGFR2_{400-408}$ peptide-specific immunologic responses.

Immunogenicity of $VEGFR2_{400-408}$ in C57BL/6 Mice

Because $VEGFR2_{400-408}$ is a self-antigen, we examined whether the vaccination-induced suppression of CNV (Fig. 1) resulted from specific T-cell responses. We evaluated antigen-specific cytokine release through two different assays (Figs. 2, 3). CD8 T lymphocytes harvested from immunized mice were used as effector cells in the assays. In our enzyme-linked immunospot assay to detect the number of IFN-γ–producing T cells (Fig. 2), $VEGFR2_{400-408}$-induced T-cell responses were observed in a dose-dependent manner in all five mice receiving vaccination with $VEGFR2_{400-408}$-pulsed DCs. Compared with nontreated levels, stimulation with the maximal dose in the assay (10 μg/mL) led to a 1.9- to 2.5- (average 2.2-) fold increase in the number of IFN-γ spots, showing that the T-cell responses were stable and equivalent in each individual receiving $VEGFR2_{400-408}$ immunization.

Peptide Specificity of T-Cell Responses

To further confirm the peptide specificity of T-cell responses, we used ELISA to examine the peptide-induced secretion of IFN-γ and TNF-α in the culture medium (Fig. 3). $VEGFR2_{400-408}$ application to CD8 T cells harvested from mice immunized with $VEGFR2_{400-408}$ led to significant ($P < 0.01$) production of IFN-γ and TNF-α in a dose-dependent manner (Figs. 3A, 3B). Similarly, a significant ($P < 0.01$) increase in the production of these cytokines in response to gp70 stimulation was detected in a dose-dependent manner with CD8 T cells from gp70-immunized mice (Figs. 3C, 3D), in which the CNV volume was not suppressed (Fig. 1). In contrast, $\beta\text{-gal}_{96-103}$ application at the maximal dose in the assay (10 μg/mL) did not significantly ($P > 0.05$) induce the production of these cytokines by CD8 T cells isolated from mice receiving $VEGFR2_{400-408}$ or gp70 immunization (data not shown).

Abrogation of Vaccination-Induced Suppression of CNV by CD8, but Not CD4, T-Cell Depletion

To confirm the role of CD8 T cells as effectors in the vaccination-induced suppression of CNV (Fig. 1), in vivo depletion of

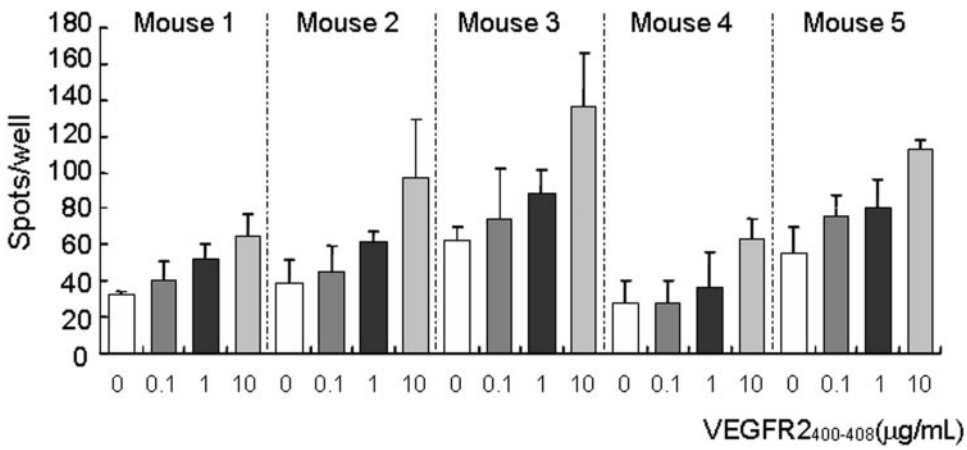

FIGURE 2. Dose-dependent increase in the number of IFN-γ spots in response to $VEGFR2_{400-408}$. All five mice examined showed similar T-cell responses of IFN-γ production in a dose-dependent manner. Results represent mean ± SD. $n = 3$ each.

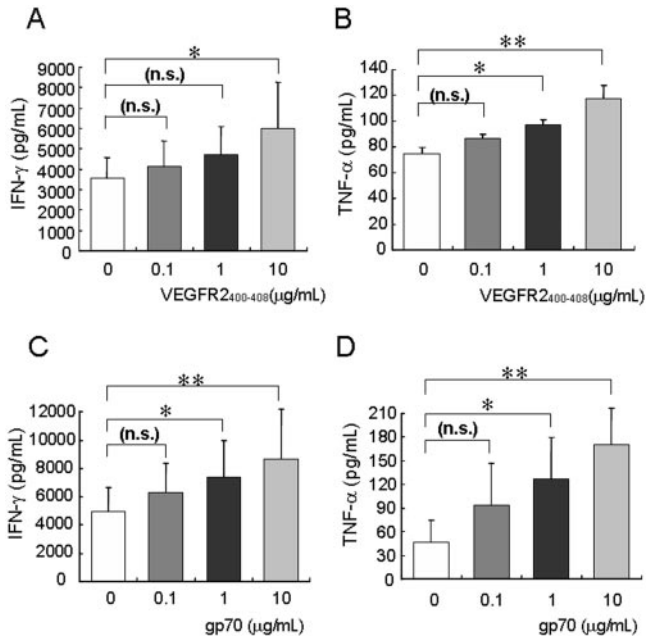

FIGURE 3. IFN-γ and TNF-α production by CD8 T cells stimulated with the epitope peptides. CD8 T cells from VEGFR2$_{400-408}$- (**A**, **B**) or gp70- (**C**, **D**) immunized mice released IFN-γ (**A**, **C**) and TNF-α (**B**, **D**) in a dose-dependent manner by stimulation with each corresponding peptide. Results represent mean ± SD. $n = 10$ each (**A**–**C**), and $n = 5$ each (**D**). $^{*}P < 0.05$, $^{**}P < 0.01$; Mann-Whitney test.

immune cell subsets using an anti-CD4 or an anti-CD8 antibody was performed on mice immunized with VEGFR2$_{400-408}$ (Fig. 4). Compared with treatment with an isotype control, antibody-based depletion of CD8, but not CD4, T cells significantly ($P < 0.01$) reversed the VEGFR2$_{400-408}$-induced reduction of CNV volume (Figs. 4A, 4B) to the level in nonimmunized mice (Fig. 1). In contrast, anti-CD8 antibody administration to nonimmunized mice did not alter CNV volume (data not shown), suggesting that the contribution of CD8 T cells was limited only when VEGFR2$_{400-408}$-specific cellular immunity was activated. The depletion of T cells in immunized mice was confirmed by flow cytometry 7 days after antibody treatment (Fig. 4C). Percentages of CD4 T cells among splenocytes were 27.8% with the isotype control and 3.4% with the anti-CD4 antibody. Similarly, the percentages of CD8 T cells were 12.7% with the isotype control and 2.0% with the anti-CD8 antibody.

DISCUSSION

Although increasing evidence has suggested that innate immunity regulates the development of CNV, the association of acquired immunity remains unclear. The present study reveals, to our knowledge for the first time, that the induction of peptide-specific cellular immunity targeting endothelial cells inhibits CNV generation (Fig. 1). We selected VEGFR2 as a target molecule on CNV-associated endothelial cells. Vaccination of DCs pulsed with the epitope peptide VEGFR2$_{400-408}$ elicited the CTL responses specific for the peptide (Figs. 2, 3). Additionally, CD8 T cells were shown as the major effectors in the immunologic treatment (Fig. 4). These data indicate the new concept of CTL-mediated specific immunotherapy for CNV.

In our peptide-pulsed DC vaccination, VEGFR2$_{400-408}$ immunization led to significant suppression of CNV compared with nonimmunized mice with CNV (Fig. 1). Although peptide-specific CTL responses were induced by VEGFR2$_{400-408}$ and gp70 (Figs. 2, 3), gp70 immunization did not reduce CNV volume (Fig. 1), suggesting that the suppressive effect observed in the present study resulted from VEGFR2$_{400-408}$-specific induction of cellular immunity. In a recent report[36] showing that VEGFR2$_{400-408}$ immunization inhibited tumor growth and angiogenesis, the adjuvant mixture of IFA, GM-CSF, and an anti-CD40 activating antibody was applied to enhance the specific immunoreaction. Because GM-CSF receptor signaling and CD40 ligation proved to be proangiogenic,[46–49] these were not used as the vaccine adjuvant in the present study. Our preliminary data revealing the negligible or minimal effect of VEGFR2$_{400-408}$ application with IFA only (data not shown), therefore, led us to determine the use of DCs, the most potent antigen-presenting cells,[40–43] which was considered a critical point for the successful induction of cellular immunity in the present study.

Peptide-specific CTL responses were confirmed with two cytokines and two cytokine assays (Figs. 2, 3), indicating that our peptide-pulsed DC vaccination broke the immunotolerance against the self-antigen VEGFR2$_{400-408}$. In these assays, IFN-γ and TNF-α were used as indicators for the peptide-specific activation of CTLs.[50] The direction of acquired immunity is regulated by the Th1/Th2 balance, in which Th1 and Th2 CD4 T cells promote cellular and hormonal immunity, respectively, through cytokines inhibitory to each other.[51,52]

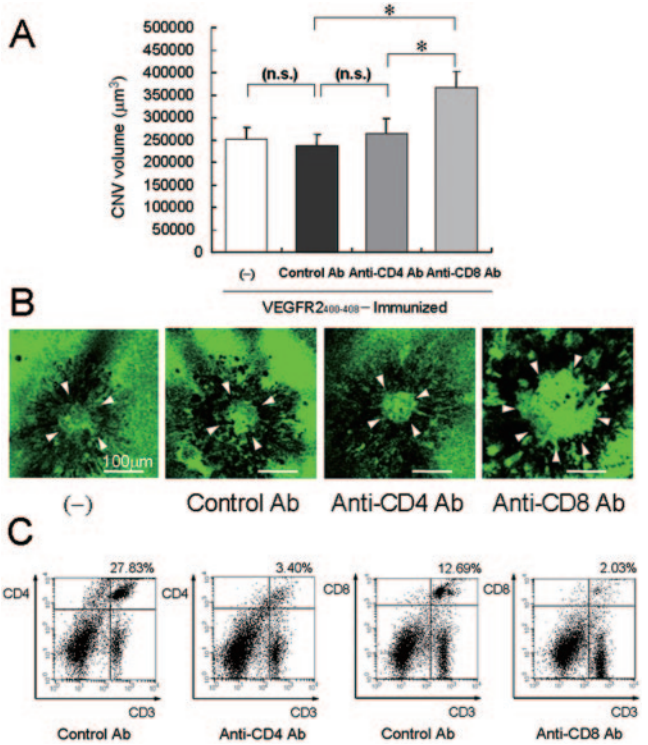

FIGURE 4. CD8, but not CD4, T cells as major effectors in CNV inhibition by vaccination with VEGFR2$_{400-408}$-pulsed DCs. Graph shows volume of CNV (**A**). Depletion of CD8, but not CD4, T cells diminished the suppressive effect of anti-VEGFR2$_{400-408}$ immunotherapy. Flatmounted choroids from VEGFR2$_{400-408}$- immunized mice receiving no antibody, a control antibody, an anti-CD4 antibody, and an anti-CD8 antibody (**B**). *Arrowheads*: lectin-stained CNV. Flow cytometric analyses showing the depletion of T cells in immunized mice by anti-CD4 and -CD8 antibodies (**C**). Representative data on the decreased ratio of the $CD3^{+}CD4^{+}$ cells (27.8%–3.4%) or $CD3^{+}$ $CD8^{+}$ cells (12.7%–2.0%) after antibody treatment. Scale bar, 100 μm. Results represent mean ± SE. $n = 34$, 35, 34, and 27 for mice receiving no antibody, control antibody, anti-CD4 antibody, and anti-CD8 antibody, respectively. $^{*}P < 0.01$ by Mann-Whitney test.

IFN-γ, produced by Th1 cells and CTLs, is one of the most important cytokines for the induction of cellular immunity. Moreover, IFN-γ was shown to induce endothelial cell apoptosis[53] and to contribute to CTL-mediated tumor rejection,[54] suggesting its role in the presently observed suppression of CNV. TNF-α is capable of activating cell survival and cell death.[55] TNF-α stimulates the nuclear factor-κB pathway, leading to cell proliferation, whereas endothelial cells undergo apoptosis through TNF-α–induced activation of caspase 8.[56] Accordingly, CTL-derived TNF-α is suggested to contribute to cytotoxicity, together with perforin and Fas ligand (FasL),[57] each of which triggers key distinct pathways responsible for CTL-mediated apoptosis. A recent report[58] showed, however, that TNF-α blockade led to significant suppression of CNV in the laser-induced model. At least in nonimmunized CNV mice without CTL induction or activation, macrophage-derived TNF-α, which stimulates RPE production of VEGF, may reasonably function as a proangiogenic factor.[5] FasL, the major cytokine in the TNF family responsible for CTL-mediated cytotoxicity, also proved to be produced by RPE cells[59] and macrophages[60] to regress CNV even without the induction of specific cellular immunity. Additionally, CNV-associated RPE cells were shown to secrete pigment epithelium-derived factor,[61–63] a potent inhibitor of angiogenesis. Together, the defense systems to combat CNV seem to be abundantly prepared in vivo, leading to time-dependent regression of CNV even in nonimmunized mice from 2 weeks after the induction (data not shown). In concert with these nonspecific systems, our DC vaccination-induced CTL activity might have enhanced or quickened the reduction of CNV, providing a significant implication as a new therapeutic strategy for CNV.

In vivo depletion experiments (Fig. 4) indicated CD8 T cells as the major effectors for the suppression of CNV in our immunotherapy. This is compatible with previous data showing $CD8^+$ CTLs as negative regulators of tumoral[26,33,34] and retinal[27,28] neovascularization. CD8 T cells from $VEGFR2_{400-408}$-immunized C57BL/6 mice were reported to kill MHC-matched H5V endothelial cells in the cytotoxic assays,[36] whereas $VEGFR2_{400-408}$ proved to be naturally processed and presented with MHC class I H-2Db in endothelial cells of C57BL/6 mice. Accordingly, the CD8 T cell-mediated suppression of CNV seen in the present study is thought to depend mainly on the specific CTL-endothelial cell interaction. On the other hand, CD8 depletion in nonimmunized mice, in agreement with previous data,[64] did not alter the CNV volume (data not shown), suggesting that CD8 T cells are limitedly operative for CNV when specific cellular immunity is induced by active immunization.

Considering immunotherapy in clinical practice, highly purified peptides for vaccination have several advantages over full-length proteins. Peptides are more easily synthesized and without the potential dangers of infection by recombinant viruses or exposure to exogenous allergens. Although DC vaccination is clinically useful,[65] the duration needed to prepare mature DCs may cause a problem when prompt intervention is required for progressive CNV. In contrast to anti-VEGF therapy requiring repeated injections, the sustained effect of immunotherapy may benefit patients with CNV because angiogenic activity lasts at least several months. Combined treatment would lead to additive efficacy because immunotherapy against activated endothelial cells highly expressing VEGFR2 is likely to differ from that of the two major established modalities—anti-VEGF therapy to inhibit vascular leakage and proliferation and PDT to occlude preexisting new vessels—in the effective phase.

Long-term attention should be paid, however, to systemic adverse effects that may be caused by the antiangiogenic action of VEGFR2-targeting CTLs. Additionally, VEGF signaling through VEGFR2, weakly expressed in normal vascular and nonvascular cells in various organs, including the eye, is suggested to play physiologic roles in cell survival and tissue maintenance.[66] CTLs induced by active immunization against VEGFR2 in the present study and previous studies[33–36] do not target functional VEGFR2 to block its downstream signaling but do induce apoptosis exclusively in endothelial cells that present the epitope peptide(s) naturally processed from VEGFR2 protein ($VEGFR2_{400-408}$ in the present study) with the MHC class I molecule. In previous data on mice receiving anti-VEGFR2 immunotherapy, vaccination of *Salmonella typhimurium* transfected with a VEGFR2-containing plasmid led to delayed wound healing and negligible impact on fertility.[33] In contrast, vaccination of DCs pulsed with VEGFR2 full-length protein did not affect wound healing.[34] Similarly, mice immunized with $VEGFR2_{400-408}$, which we used in the present study, exhibited no obvious adverse effects.[36] $VEGFR2_{400-408}$ immunization did not affect retinal vasculature or leukocyte recruitment in our study (data not shown). Minimal adverse effects observed in these series of immunotherapy suggest that the MHC-mediated presentation of the VEGFR2 epitope peptide(s) is preferentially limited to proliferating endothelial cells during tumor growth and CNV generation. However, delayed wound healing,[33] conceivably resulting from cytotoxicity for proliferating endothelial cells, has raised safety concerns as a potential adverse effect.

In summary, the present data are the first to show that CNV can be reduced by inducing cellular immunity specific for VEGFR2. These findings indicate the possibility of active immunization as a novel therapeutic strategy to inhibit CNV, a central abnormality of vision-threatening AMD.

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