Dendritic Cells Augment Choroidal Neovascularization

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PURPOSE. Dendritic cells (DCs) are innate immune cells that have recently been shown to support angiogenesis in tumors, endometriosis, and lymph nodes. A major cause of legal blindness is wet age-related macular degeneration (wet ARMD), wherein abnormal blood vessels grow under the retina, an abnormality also referred to as choroidal neovascularization (CNV). The purpose of the present study was to investigate the role of DCs in the development of CNV.

METHODS. Laser photocoagulation was used to induce CNV in C57BL/6j mice. The authors analyzed CNV lesions for the presence of DCs using flow cytometry and immunostaining at designated times. They also analyzed the effects of intravenous DC transplantation on CNV development by measuring the lesion area using confocal microscopy 1 week after laser injury.

RESULTS. The authors analyzed CNV lesions for the presence of DCs by flow cytometry and observed that CD11c+ major histocompatibility complex (MHC) class II+ DCs transiently infiltrated the CNV lesions, reaching a peak at 2 to 4 days after laser injury. These DCs were mostly immature (CD11c- MCHCIlow) and expressed vascular endothelial growth factor receptor 2. Immunostaining of laser-induced CNV lesions confirmed that DCs are located at the sites of newly formed blood vessels. Intravenously injected DCs incorporated into the CNV lesions. However, only immature DCs enhanced CNV size.

CONCLUSIONS. These results suggest a role for DCs in promoting angiogenesis and lesion growth in laser-induced CNV. The present data suggest that DCs may represent potential cellular targets for therapeutic intervention in wet ARMD. (Invest Ophthalmol Vis Sci. 2008;49:3666–3670) DOI:10.1167/iovs.07-1640

A ge-related macular degeneration (ARMD) is the leading cause of irreversible vision loss among the elderly in most industrialized nations.1 Severe vision loss in ARMD is commonly caused by choroidal neovascularization (CNV), wherein new vessels from the choroid invade the subretinal space through Bruch membrane. This results in the formation of fibrovascular tissue that has been shown to contain vascular endothelial cells, retinal pigment epithelial cells, fibroblasts, and macrophages.2 Bleeding and vascular leakage from the immature vessels in the proliferative tissue damages retinal function. Much is unknown about the molecular and cellular mechanisms of CNV. It has been hypothesized, however, that inflammation and immune activation play a role in the pathogenesis of this disease.3 Including macrophage infiltration1–7 and cytokine secretion,6–8 similar to the pathologic angiogenesis seen in tumors.

Dendritic cells (DCs) are innate immune cells of hematopoietic origin that have a pivotal role in the initiation of the adaptive immune response.9 Although DCs have been detected in normal uvea,10,11 inflamed retina,12 and drusen deposits,13,14 to date there have been no reports of their presence in CNV. Aside from their role in immune function, DCs were recently implicated in the support of angiogenesis in tumors and lymph node expansion during an immune response.15–17 Furthermore, it has recently been shown in our laboratory that DCs support angiogenesis and lesion growth in endometriosis, another angiogenic disease.18,19 Laser-induced CNV is a well-established animal model of CNV that is useful for investigating the mechanisms of the corresponding human disease.19–21 We hypothesized that DCs may play a role in the pathogenesis of CNV similar to their role in endometriosis and tumor growth. To this end, we analyzed CNV lesions for the presence of DCs and analyzed the effects of DC transplantation on CNV development. Our results indicate that in a laser-induced model of CNV, DCs transiently infiltrate the lesion and contribute to its growth.

MATERIALS AND METHODS

Induction of CNV

Laser-induced CNV was generated by a technique described previously, with some modifications.21 C57BL/6j mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized by intraperitoneal administration of tribromoethanol (240 mg/kg). A mixture of 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydrin P; Santen Pharmaceutical, Osaka, Japan) was applied to both eyes to dilate the pupils. Lesions were induced by a diode-pumped solid state laser (0.1 second; spot size, 50 μm; power 150 mW) around the optic nerve through a slit lamp delivery system with the use of a photoacoagulator (GYC2000; Nidek, Osaka, Japan) while a handheld cover slide was used as a contact lens. Only lesions in which a subretinal bubble or focal serous detachment of the retina developed were used for the experiments. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Flow Cytometry

Single cells were prepared from mouse CNV lesions. To collect a sufficient number of ocular-infiltrating cells for flow cytometry, 30 separate burns were delivered to the mouse eyes, similar to the panretinal photocoagulation procedure in humans. At designated times, mice were humanely killed, the eyes were enucleated, and the anterior segment (cornea, iris, and lens) was removed. The posterior segment of each eye, including the sclera, choroid, and retina, was disrupted with scissors and then incubated with 2.8 U/mL enzymes (Liberase Blendzymes; Roche Indianapolis, IN) at 37°C for 45 minutes. Digested
tissue was then filtered through a 40-μM cell strainer and resuspended in FACS buffer (PBS, 1% BSA, 5 mM EDTA/0.05% sodium azide). Immunostaining was performed in the presence of rat anti-mouse Fc gamma RIII/II receptor (CD16/32; PharMingen, San Diego, CA) by incubating the cells for 30 minutes on ice with monoclonal antibodies: anti-major histocompatibility complex (MHC) class II (I-A/I-E), fluorescein isothiocyanate (FITC), anti–CD11c mAb allophycocyanin (APC; eBioscience, San Diego, CA), and anti-VEGFR2 mAb phycoerythrin (PE; PharMingen). Flow cytometry was performed (FACSCalibur and CellQuest software; BD Biosciences, San Jose, CA). Dendritic cells (PE; PharMingen) were defined as CD11c+ MHCII+ cells. Preparations from two eyes were pooled to obtain a sufficient number of viable cells for a flow cytometric analysis in this process. Four eyes (two individual pools) were examined per group.

**Choroidal Flatmount Preparation and Immunohistochemistry**

To evaluate the size of the CNV lesions, four burns were performed per eye, with a space left around the optic disc. Seven days after laser burns, mice were humanely killed, and the eyes were removed and fixed in 4% paraformaldehyde for 60 minutes. The cornea and lens were removed, and the entire retina was carefully dissected from the eye cup. Radial cuts (average, eight) were made from the edge of the eye cup to the equator and then washed with cold buffer (0.3% Tween 20 in PBS). Blood vessels were labeled using a 1:200 dilution of isoelectric IB4 conjugated with dye (Alexa Fluor 488; Invitrogen-Molecular Probes, Eugene, OR). The eyecup was flatmounted in Aquamount and Immunostaining was performed in the presence of rat anti–mouse Fc gamma RIII/II receptor (CD16/32; PharMingen, San Diego, CA) by incubating the cells for 30 minutes on ice with monoclonal antibodies: anti-major histocompatibility complex (MHC) class II (I-A/I-E), fluorescein isothiocyanate (FITC), anti–CD11c mAb allophycocyanin (APC; eBioscience, San Diego, CA), and anti-VEGFR2 mAb phycoerythrin (PE; PharMingen). Flow cytometry was performed (FACSCalibur and CellQuest software; BD Biosciences, San Jose, CA). Dendritic cells (PE; PharMingen) were defined as CD11c+ MHCII+ cells. Preparations from two eyes were pooled to obtain a sufficient number of viable cells for a flow cytometric analysis in this process. Four eyes (two individual pools) were examined per group.

**Bone Marrow–Derived Dendritic Cell Cultures and Transplantation**

Bone marrow–derived dendritic cells (BMDCs) were prepared as described. Briefly, mice were humanely killed, and bone marrow was extracted from femurs and tibias by flushing the shaft with 5 mL RPMI-1640. Cells were then cultured in nonadhesive Petri dishes at a density of 1 × 10^6 cells/mL in the following medium: RPMI-1640, 10% FCS, 5 × 10^-5 M 2-mercaptoethanol, penicillin/streptomycin (all from Invitrogen, Carlsbad, CA) containing 10 ng/mL murine recombinant granulocyte macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ). The medium was replenished every 2 days, and the loosely adherent DCs were collected at the designated times and used for further studies. For BMDC maturation, the cells were treated overnight with 1 μg/mL LPS (L2880; Sigma-Aldrich, St. Louis, MO).

For transplantation into laser-injured mice, BMDCs were cultured at for at least 9 days. BMDCs (1.0 × 10^6 cells/mouse) were injected intravenously to mice at the indicated times. In some experiments, DCs were labeled before transplantation with 2 μM chloromethylbenz-amido (CM-Dil; Molecular Probes Inc., Eugene, OR) for 5 minutes at 37°C and then for an additional 15 minutes at 4°C. Five mice were used per group. After 7 days, the mice were killed and the lesions were measured and analyzed.

**Statistical Analysis**

The CNV area was measured and presented as mean ± SD for each group. Unpaired two-tailed Student’s t-test was performed when comparing lesion sizes. P < 0.05 was considered statistically significant.

**RESULTS**

**Dendritic Cell Infiltration of CNV Lesions**

We prepared single-cell suspensions of the posterior segment of laser-injured eyes and analyzed them for the presence of DCs (at the designated time intervals; Fig. 1). Dendritic cells were defined as FSChigh SSClow cells (gating not shown) expressing CD11c and MHCII surface molecules. Without laser injury, ocular-infiltrating CD11c+ MHCII+ DCs were rarely detected (day 0, 0.43%). However, after laser injury, they were clearly detected (days 1–7). The number of infiltrating DCs increased to a peak at 2 to 4 days (14%–16%) and gradually decreased thereafter (day 7, 6.52%; Figs. 1A, 1B).

Because we observed a peak in DC infiltration after 4 days, we further analyzed the CNV lesions by immunohistochemistry for the presence of DCs. We prepared flatmounts of the choroids and stained them with anti–CD11c antibody. As demonstrated in Figure 2, DCs were observed infiltrating the laser lesion of CNV (Fig. 2C).

**FIGURE 1.** Time course of DC infiltration after laser injury. At the designated times, single-cell preparations of the CNV lesions were prepared and analyzed for the presence of DCs by FACS. (A) DC population (CD11c+ MHCII+) at days 0, 1, 2, 3, 4, and 7 after laser injury. (B) Percentage of the DC population (CD11c+ MHCII+) in CNV from inflammatory cell infiltration. (C) Immature (MHCIIlow) and mature (MHCIIhigh) DCs in the axillary lymph nodes.

**VEGFR2-Expressing Immature DCs in CNV**

The DC population of a noninflamed lymph node is mostly immature (MHCIIlow; Fig. 1C). Similarly, DCs infiltrating CNV lesions also express low levels of MHCII, indicating an immature phenotype (Fig. 1A). Because VEGFR2 expression on DCs was shown to be critical for their proangiogenic phenotype,17,19 we analyzed its expression on DCs infiltrating CNV lesions (Fig. 3A). CD11c+ (40.2%) infiltrating DCs (gating in Fig. 3B) expressed VEGFR2, whereas only 12.7% of lymph node DCs expressed this receptor.

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Enhancement of CNV Growth by Transplanted DCs

We next sought to determine the function of DCs in CNV. To this end, we grew BMDCs by culturing bone marrow cells in the presence of GM-CSF. We used cultured cells derived from day 9 onward, yielding a population of more than 90% CD11c⁺/H11001 DCs that were mostly of immature phenotype (i.e., MHCII low; Fig. 4A). We injected fluorescence-labeled BMDCs and looked for their presence in CNV lesions using confocal microscopy. We incubated transplanted BMDCs with the vital stain chloromethylbenzamido (CM-DiI) before injection to label them. Starting immediately after laser, 1 × 10⁶ CM-DiI⁺ BMDCs were injected intravenously every 48 hours (0, 2, 4 days). In the mouse experimental CNV model, neovascularization can be detected within 7 days after laser burn. To study the effect of DC infiltration on the development of CNV, we evaluated this early phase of CNV using the flatmount technique, as described previously. On day 7, we stained the CNVs and observed that CM-DiI⁺ BMDCs incorporated into the CNVs and that CNVs were larger after the incorporation of CM-DiI⁺ BMDCs than after injection of vehicle (Figs. 4E–G).

To investigate whether the maturity state of the DCs determined its effect on angiogenesis, we transplanted mature and immature DCs in our mouse model of CNV. Starting immediately after laser injury, 1 × 10⁶ mature DCs, immature DCs, or PBS were injected intravenously every 48 hours (0, 2, 4 days). On day 7, mice were humanely killed, and the choroid was removed and analyzed (Figs. 5A, 5B). Immature DC injection led to an approximately twofold increase in CNV size (P < 0.001) compared with PBS injection, whereas mature DCs injection did not increase CNV size.

DISCUSSION

Laser-induced CNV is a well-established animal model of wet ARMD that is useful for investigating possible mechanisms of this disease. Laser-induced CNV is a well-established animal model of wet ARMD that is useful for investigating possible mechanisms of this disease. The disruption of the retinal pigment epithelium layer and Bruch membrane leads to the infiltration of inflammatory cells at the site of laser injury. In this study, we demonstrated for the first time that DCs play a pivotal role in the angiogenesis found in laser-induced CNV. We have shown that in the absence of injury, DCs are not present in significant numbers in the retina and choroid. Laser injury, however, induces the infiltration of CD11c⁺ MHCII⁺ DCs transiently into the lesions, reaching a peak at 2 to 4 days. Although the

![Figure 2](image1.png)  Localization of DCs in laser-induced CNV. Four days after laser injury, choroidal flatmounts were prepared and DCs were stained with anti-CD11c antibody and visualized by fluorescence microscopy (B, green). This staining colocalized with laser lesions as observed by regular light microscopy (B). Note the faint background of green signal in laser lesions stained with control IgG (A). Scale bar, 50 μm.

![Figure 3](image2.png)  Dendritic cells infiltrating CNV express VEGFR2. Three days after laser injury, single-cell preparations of CNV lesions or axillary lymph nodes were prepared and analyzed. (A) VEGFR2 expression on CD11c⁺ DCs. (B) CD11c⁺ FSChigh DCs were gated.
DCs are essential for lymph node angiogenesis. Taken together, previous reports pointing to the abundance of DCs at sites of angiogenesis suggest that these cells may serve as key players in angiogenesis. We have now shown that DCs infiltrating CNV also express VEGFR2. This receptor is expressed to a lesser extent in lymph node DCs. This finding, similar to our findings in endometriosis, is in agreement with that of Fernandez-Pujol et al., who have shown that DCs converted to endothelial-like cells in the presence of VEGF and that in this process they acquired endothelial markers such as VEGFR2. This observation suggests that DCs infiltrating CNV lesions acquire responsiveness to VEGF, which may in turn lead to acquiring a role of supporting angiogenesis.

Two reports have suggested that large drusen, a known risk factor for the development of ARMD, contain a central core originating from a choroidal monocyte. It is tempting to speculate that this cell may represent a dendritic cell precursor and may function as an inducer of CNV. We show that the infiltration of DCs peaks on day 2 to 4 after laser injury. Interestingly, it has been reported that 7 days after laser injury, CNV increases dramatically. Together, these results suggest an association between the early appearance of DCs after laser injury and the subsequent rapid angiogenesis.

We next tried to determine whether DCs play a functional role in CNV. We cultured murine bone marrow cells in the presence of GM-CSF, yielding CD11c+ BMDCs. We then injected CM-Dil-labeled DCs into mice after laser injury and showed that CM-Dil-labeled DCs incorporated into the lesions. Importantly, DC-injected mice grew large CNV lesions, whereas the control mice grew small CNV lesions. A similar incorporation of injected labeled BMDC and their angiogenesis promoting effect was observed by Webster et al. in lymph node angiogenesis and by us in a surgical model of endometriosis. Moreover, studies performed by Conejo-Garcia et al. and by us show DC incorporation and promotion of tumor growth after DC transplantation. We have now demonstrated that the injection of immature DCs, but not mature DCs, increased the size of CNV. These findings are in accordance with our previous observations that immature DCs, not mature DCs, enhance endothelial cell migration in vitro. This supports a prominent role for immature DCs in angiogenesis.

Previous studies have shown a contribution of macrophages to angiogenesis. These studies used the CNV laser model described here and then systemically depleted phagocytic cells by injection of clodronate encapsulated in liposomes. Because the systemic depletion of phagocytes decreased the size of CNV, the authors concluded that macrophages promote CNV. As a possible mechanism, Tsuchi et al. demonstrated that ocular-infiltrating macrophages are a potential source of various angiogenic factors, such as VEGF, in the development of CNV. However, the possibility that clodronate encapsulated in liposomes also caused a depletion of DCs has not been ruled out and warrants further study.

Recent data from several groups suggest that innate immune mechanisms and localized choroidal inflammation may generally contribute to the pathogenesis of wet ARMD. In addition to macrophages and DCs, other proposed innate immune mechanisms involved in the pathogenesis of wet ARMD include injurious stimuli (oxidants or infectious agents) and amplification cascades (such as complement, mediator systems, and cytokines). For example, Anderson et al. have identified complement and immune complexes in association with nodular drusen, known risk factors for wet ARMD. There is a possibility that immune complexes serve as stimuli for the recruitment of macrophages and DCs in human ARMD.

In conclusion, the pathogenesis of wet ARMD remains unresolved; however, our results indicate a role for DCs in promoting angiogenesis and lesion growth in laser-induced CNV. Given that the infiltration of DCs promotes increased CNV, therapies designed to inhibit DC infiltration or function should be studied as possible treatments for wet ARMD.

**Acknowledgments**

The authors thank Kristin Johnson for help in the preparation of digital art.

**References**


