# A Novel Pro-Angiogenic Function for Interferon-γ– Secreting Natural Killer Cells

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**PURPOSE.** To explore the function of natural killer (NK) cells in inflammatory angiogenesis in mice.

**METHODS.** To study ocular angiogenic responses we used the cornea BFGF micropellet and the laser-induced choroidal neovascularization (CNV) mouse models (C57BL/6). To deplete NK cells in these models, we injected an anti-NK1.1 antibody or an isotype antibody as a control. Corneas or choroids were immunohistochemically stained for blood vessels (CD31), macrophages (F4/80), or CNV (isolectin-IB4). Vascular endothelial growth factors (VEGF), IFN-γ, or TNF-α levels were measured by real-time quantitative PCR (qPCR) or flow cytometry. A coculture assay of macrophages, NK cells, and human umbilical vein endothelial cells (HUVECs) was analyzed morphometrically to examine the ability of NK cells to induce angiogenesis in vitro.

**RESULTS.** Our data demonstrate that in vivo depletion of NK cells leads to a significant reduction of corneal angiogenesis and CNV. Furthermore, NK cell depletion reduces macrophage infiltration into the cornea and mRNA expression levels of VEGF-A, VEGF-C, and VEGFR3 at day 7 after micropellet insertion. In the laser-induced CNV model, our data show that NK cell depletion leads to decreased areas of CNV and significantly reduced mRNA expression of VEGFs and IFN-γ in the choroid. An in vitro coculture assay shows an IFN-γ–dependent increase in VEGF expression levels, thereby increasing endothelial cell proliferation.

**CONCLUSIONS.** Our findings demonstrate a novel pro-angiogenic function for NK cells, indicating that IFN-γ–secreting NK cells can induce angiogenesis by promoting enhanced VEGF expression by macrophages.

Keywords: endothelial cells, neovascularization, NK cells, macrophages, interferons

Ocular angiogenesis, such as seen in choroidal neovascularization (CNV) in AMD and corneal neovascularization, is a principal cause of blinding disease. In these processes VEGF and BFGF increase the recruitment of monocytes, neutrophils, and macrophages to inflammatory sites.[1] Additionally, VEGF acts as a potent stimulator for proliferation, migration, and tube formation by endothelial cells,[2] and BFGF is known to potentiate cell adhesion molecule expression processes that can lead to matrix remodeling and VEGF over expression.[3,4]

The principal function of natural killer (NK) cells is their cytotoxicity to virally-infected cells and tumor cells.[5–7] In addition, recently, NK cells have been ascribed functions in physiological processes, such as induction of vascular growth, remodeling, and secretion of angiogenic factors.[8–11] However, the precise mechanisms by which NK cells induce inflammatory angiogenesis, especially in the eye, remain unclear.

In this study, we report novel angiogenic functions of NK cells using mouse models of ocular angiogenesis, as well as in vitro assays where we cocultured NK cells and macrophages with human umbilical vein endothelial cells (HUVECs). We demonstrate that IFN-γ–secreting NK cells enhance VEGF production by macrophages to induce angiogenesis at pathologically relevant sites.

## MATERIALS AND METHODS

### Corneal Micropocket Assay

The corneal micropocket assay was performed as described previously.[1,3] Briefly, micropockets containing 40 ng murine BFGF (R&D Systems, Minneapolis, MN, USA) were implanted into the middle stromal layer of C57BL/6 mice ($n = 14$/group; Stock Number: 000664; Jackson Laboratories, Chicago, IL, USA). The pellets were located 1.0-mm apart from the limbus in the temporal side, and tetracycline ophthalmic ointment was applied to the eye after pellet implantation. Seven mice received intravenous injections of 50 µg anti-NK1.1 (#108702; BioLegend, San Diego, CA, USA) or isotype control antibody

TABLE. List of Antibodies Used in This Study

| Antibody | Catalog Number | Company |
|---|---|---|
| Purified anti-mouse NK1.1 | #108702 | BioLegend |
| Purified Mouse IgG2a, κ isotype control antibody | #401502 | BioLegend |
| NK cell isolation kit | #130-096-892 | Miltenyi Biotec |
| LEAF-purified anti-mouse interferon (IFN)-γ antibody | #505811 | BioLegend |
| PE conjugated anti-mouse NK1.1 antibody | #108707 | BioLegend |
| FITC conjugated anti-mouse IFN-γ antibody | #505805 | BioLegend |
| PE Mouse IgG2a, κ isotype control antibody | #400211 | BioLegend |
| FITC conjugated anti-mouse IFN-γ antibody | #505805 | BioLegend |
| Anti-mouse IFN-γ antibody | #400405 | BioLegend |
| FITC anti-mouse CD49b antibody | #103503 | BioLegend |
| FITC Armenian Hamster IgG isotype control antibody | #400905 | BioLegend |
| FITC-conjugated anti-mouse CD31 antibody | #sc-18916 | Santa Cruz Biotechnology |
| Alexa-488–conjugated isolectin-IB4 | #I21411 | Invitrogen |
| APC conjugated anti-mouse CD3e | #17-0031-81 | eBioscience |
| APC conjugated Armenian Hamster IgG isotype control | #17-4888-81 | eBioscience |

(#401502, BioLegend) 2 days before, the day of, and 4 days following micropellet insertion. All antibodies used in this study are listed in the Table. All animal studies described herein were managed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under Institutional Animal Care and Use Committee approval of the Schepens Eye Research Institute.

## Laser-Induced Choroidal Neovascularization

Laser-induced CNV was performed in C57BL/6 mice as described previously.[4,12] Briefly, laser photocoagulation (Oculight-SLx; Iridex, Mountain View, CA, USA) was performed (wavelength: 810 nm; energy: 120 mW; duration: 100 ms; spot size: 100 μm) by a single individual. The appearance of a cavitation bubble indicated rupture of Bruch's membrane.

## Tube Formation Assay

Human umbilical vein endothelial cells (#C-015-5C; GIBCO; Life Technologies, Chicago, IL, USA) were maintained in EGM-2-bullet kits (Lonza, Inc., Houston, TX, USA) at 37°C in 5% $CO_2$. These assays were performed as described previously, with minor modifications.[13] Bottom and upper gel layer contained 80% type-I-collagen (Devro-Medical, San Jose, CA, USA), 0.02 N NaOH, 20 mM 2-(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mg/mL $NaHCO_3$, 0.5 μg/mL fibronectin, 0.5 μg/mL laminin, and 10.5 mg/mL RPMI-powder (Life Technologies). For the bottom gel layer, 200 μL of the mixture was added to a 48-well plate and incubated (37°C, 1 hour). Human umbilical vein endothelial cells ($4 \times 10^4$) were seeded, incubated overnight, then 100 μL gel mixture were added and incubated at 37°C for 1 hour. Magnetically sorted (NK cell isolation kit, #130-096-892; Miltenyi Biotec, Auburn, CA, USA) NK cells ($2 \times 10^4$) and/or thioglycollate-elicited macrophages ($2 \times 10^4$) were added in EGM containing 2% horse serum, 12 μg/mL bovine brain extract, and 40 ng/mL BFGF. Neutralizing LEAF-purified anti-mouse IFN-γ antibody (#505811; BioLegend) or isotype control (#400413; BioLegend) in 10 μg/mL was added. After 1 day, the entire field was photographed using SPOT software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and analyzed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The total length of all tubes within a field was measured in a masked fashion. Murine VEGF-A

levels in the supernatant were determined by ELISA (eBioscience, San Diego, CA, USA).

## Flow Cytometry

Cultured NK cells were first stained with a PE conjugated anti-NK1.1 antibody (cell surface; #108707; BioLegend) or isotype control antibody (#400211, BioLegend), then fixed and permeabilized, and finally intracellularly stained with a FITC conjugated anti–IFN-γ antibody (#505805; BioLegend) or isotype control antibody (#400405, BioLegend). To prove NK cell depletion on day 7, peripheral blood of anti-NK1.1 or isotype-treated mice was stained with PE-NK1.1 and FITC-CD49b (#103503, BioLegend). CD3+ cells were excluded (APC conjugated anti-mouse CD3e, #17-0031-81, eBioscience). Appropriate isotype-matched control antibodies (#400905, BioLegend) were used in the flow cytometry analyses. Stained cells were analyzed using a LSR II flow cytometer (Becton-Dickinson, Pittsburgh, PA, USA) and Summit v4.3 software (Dako, Pittsburgh, PA, USA).

## Immunohistochemistry

Corneal mounts were immunostained with a FITC-conjugated CD31 antibody (#sc-18916; Santa Cruz Biotechnology, Dallas, TX, USA) for epifluorescence microscopy (model E800, Nikon, Tokyo, Japan). Areas covered by blood vessels (CD31$^{hi}$) were calculated using ImageJ, as described before.[1]

To label CNV immunohistochemistry was performed on RPE/choroidal flat-mounts 10 days after laser injury using 0.5% Alexa-488–conjugated isolectin-IB4 (#I21411, Invitrogen, Eugene, OR, USA). The CNV volume was quantified using a confocal microscope (Leica TCS–SP5; Leica Microsystems, Wetzlar, Germany). Laser scars were scanned in total by Z-stack images of 1-μm intervals and the sum of the entire fluorescent area was measured by ImageJ, as described previously.[12]

## RT and Quantitative Real-Time PCR

Ribonucleic acid was isolated from cornea, conjunctiva, or the RPE/choroid complex using the RNeasy-kit (Qiagen, Hilden, Germany) and reverse transcribed using Superscript-III Kit (Life Technologies). Quantitative real-time PCR was performed using a Taq Man-Mastermix and preformulated primers (Life Technologies) for murine VEGF-A (Mm01281449_m1), VEGF-C (Mm00437310_m1), VEGF-D (Mm01131929_m1), VEGF-R2 (Mm01222421_m1), VEGF-R3 (Mm01292604_m1), IFN-γ

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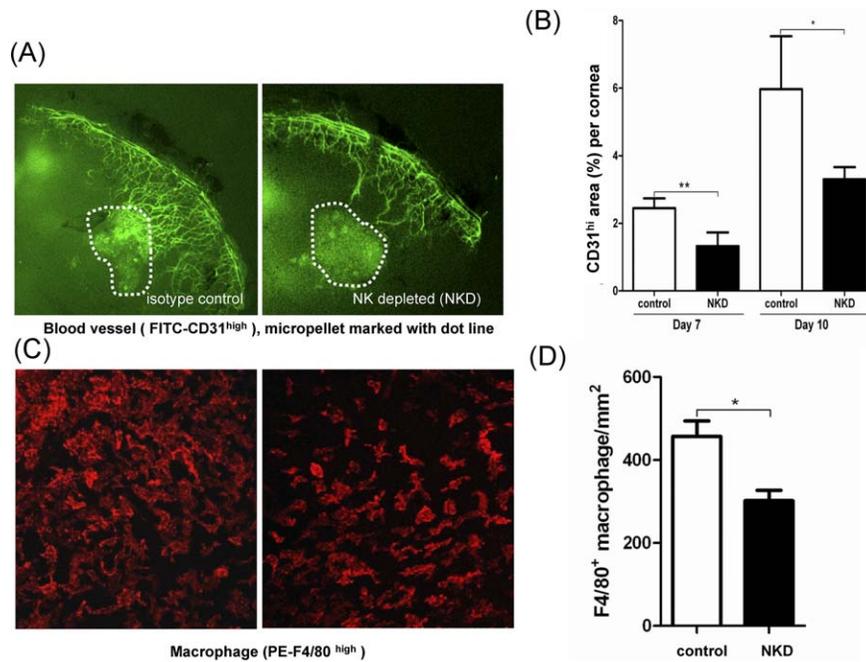
**FIGURE 1.** Natural killer cell depletion decreased vessel formation and macrophage infiltration in a BFGF micropellet model in vivo. Pellets containing 40 ng BFGF were implanted in corneal stroma of C57BL/6 mice. One-half of the mice were treated with NK1.1 and the other one-half with isotype antibody intravenously 2 days before pellet implantation, on the day of implantation, and at postoperative day 4 ($n = 14$–16 mice/group). (**A**, **C**) Corneas were harvested and immunochemically stained for CD31 (*green*; [**A**]) at days 7 and 10, or F4/80 (*red*; [**C**]) at day 7. (**B**, **D**) The area (%) covered by blood vessels (CD31$^{hi}$) was analyzed (**B**) and the numbers of macrophages (F4/80$^+$) were counted (**D**) in a blinded fashion. Data are the mean ± SEM of four mice/group at each time-point; *$P < 0.05$; **$P < 0.01$.

(Mm01168134_m1), TNF-α, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm99999915_gl). The *GAPDH* gene was used as the endogenous reference for each reaction. The results were analyzed by the comparative threshold cycle ($C_T$) method with LightCycler analysis software (Version 3; Roche, Atlanta, GA, USA) and the relative expression level of each sample was expressed as fold change from isotype control-treated group.

## Statistical Analyses

Data are expressed as the mean ± SEM of at least three trials. The significance of the difference between groups was analyzed with the two-tailed Student's *t*-test using Prism software (version 5.0; GraphPad, San Diego, CA, USA). Differences were considered significant when *P* was less than 0.05.
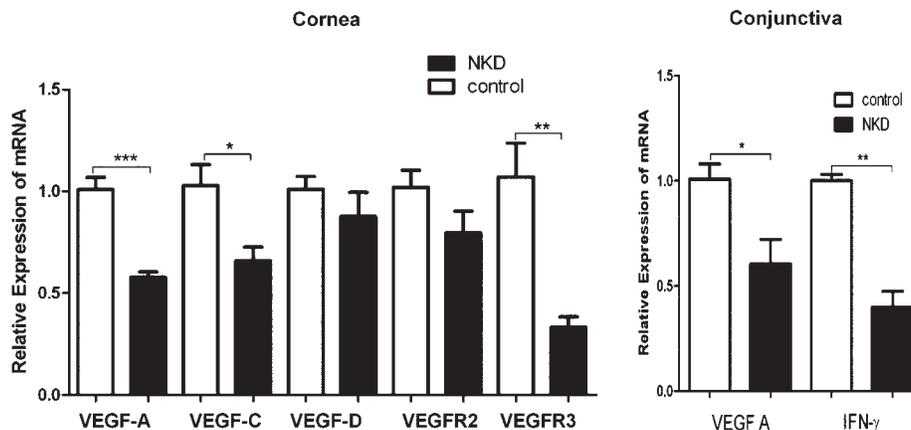


**FIGURE 2.** Natural killer cell depletion decreased mRNA expression of VEGFs in the corneas and conjunctivae of a BFGF micropellet model in vivo. At day 7 post pellet implantation, corneas or conjunctivae of NK depleted (NKD) or control mice were analyzed by real-time PCR for their mRNA expression of different VEGFs, VEGFRs, and IFN-γ. Expression levels of mRNAs were normalized to GAPDH levels as an internal control and then normalized to control corneas. Real-time PCR revealed that systemic NK cell depletion significantly decreased the mRNA expression of VEGF-A, VEGF-C, and VEGF-R3 in corneas, and VEGF-A and IFN-γ in conjunctivae as compared with isotype control antibody–treated mice ($n = 2$ corneas/group). Data is presented as the mean ± SEM of three to four experiments; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
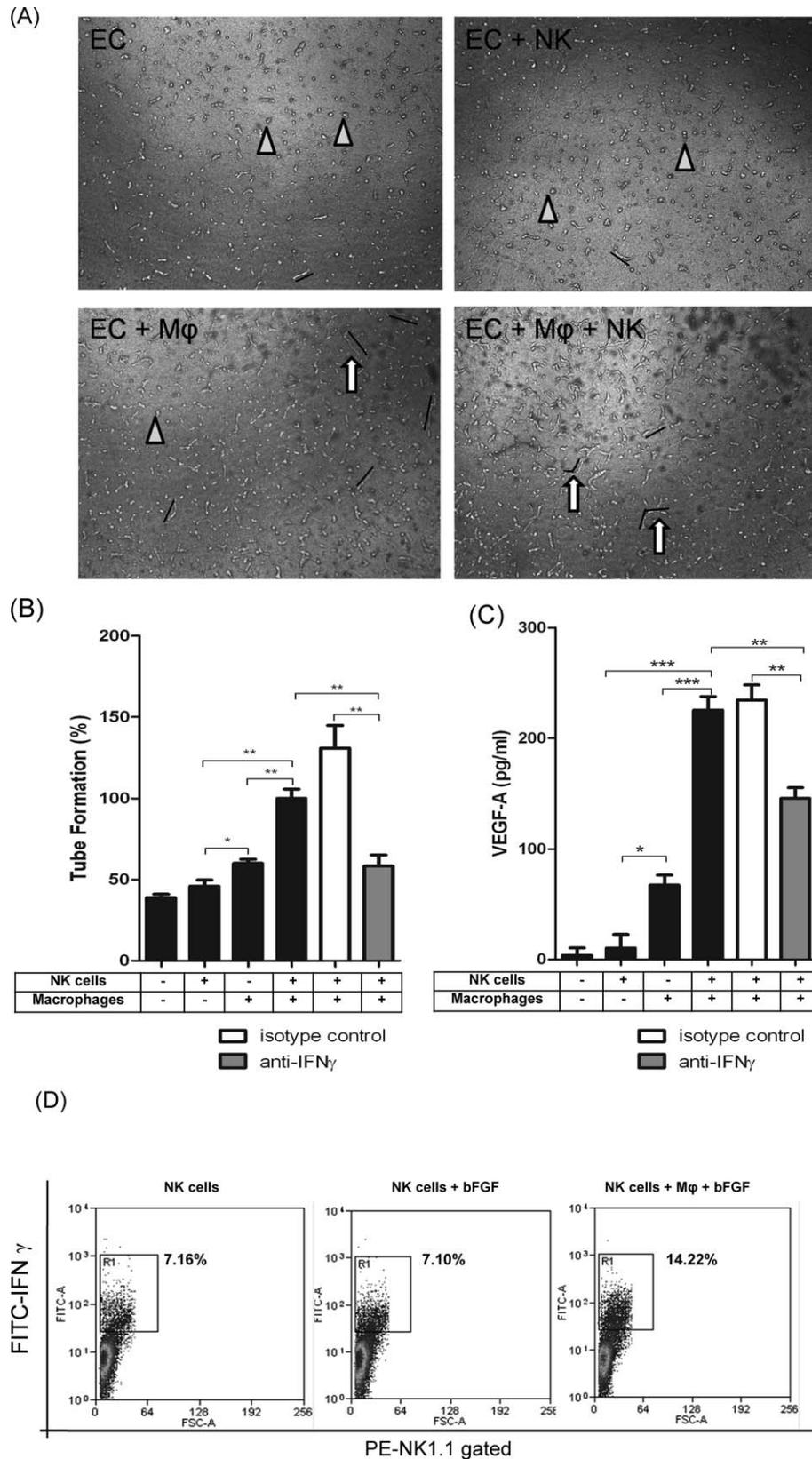
FIGURE 3. The presence of both NK cells and macrophages increased tube formation of HUVECs in vitro. (A) In a tube formation assay $4 \times 10^4$ HUVECs were added to the lower gel layer and incubated overnight. The next day, in a 48-well plate, $2 \times 10^4$ NK cells and/or $2 \times 10^4$ macrophages were plated with 40 ng BFGF on the upper gel layer, above the HUVECs, then incubated at 37°C overnight. Singular HUVECs are marked with an *arrowhead* and *black spots* show upper gel layer with macrophages or NK cells in some of the pictures. Human umbilical vein endothelial cells showed significantly spreading and growth, when cultured under the influence of macrophages and NK cells (marked with *arrow*, representative

measured tube length, as *black line*). (**B**) The entire field was photographed and analyzed for tube length using ImageJ. As indicated, 10 µg/mL IFN-γ neutralizing antibody or isotype control was added ($n = 3$/experiment, the experiment was repeated three times independently). (**C**) The VEGF-A levels were measured from the supernatant of the tube formation assay by ELISA ($n = 3$/experiment, the experiment was repeated three times independently). (**D**) Frequencies of IFN-γ+ NK cells were analyzed by flow cytometry after gating on NK1.1+ cells. Representative flow data from two trials per group. Data represent the mean ± SEM of three to four experiments; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

## RESULTS

### NK Cell Depletion Reduces Vessel Formation, Macrophage Infiltration, and mRNA VEGF Expression in a BFGF Micropellet Model In Vivo

The standard model of inducing inflammatory angiogenesis by placing BFGF pellets into mouse corneas was performed, and NK cells were systemically depleted using an anti-NK1.1 antibody. Successful depletion of NK cells was monitored by flow cytometry (Supplementary Fig. S1). Corneal angiogenesis was quantified by immunohistochemical staining of CD31, which showed a significant reduction of blood vessel formation in NK cell depleted mice at days 7 and 10 (Figs. 1A, 1B). Moreover, NK cell–depleted mice showed significantly reduced infiltration of macrophages into the cornea (Figs. 1C, 1D).

Further, we performed real-time qPCR to quantify mRNA levels of the proinflammatory cytokine IFN-γ as well as of ligands and receptors of the VEGF family, VEGF-A, -C, -D, -R2, and -R3 in the cornea and conjunctiva of NK cell–depleted and control mice with prior BFGF micropellet insertion. We detected reduced mRNA levels of VEGF-A, -C, and -R3 in corneas of NK cell–depleted mice and reduced mRNA levels of VEGF-A and IFN-γ in conjunctivae of NK cell–depleted mice compared with control mice (Fig. 2).

### NK Cells Induce Increased Tube Formation of HUVECs In Vitro

To investigate the direct effects of NK cells in inducing angiogenesis, we conducted an in vitro assay coculturing NK cells and macrophages with HUVECs in a sandwich-collagen matrix gel to prevent cell–cell contact between immune cells and endothelial cells, and measured HUVEC tube formation. Human umbilical vein endothelial cells cocultured with NK cells and macrophages showed a prominent increase in tube formation (Fig. 3A), which was inhibited in the presence of an IFN-γ–blocking antibody (Fig. 3B). Enzyme-linked immunosorbent assay analyses of supernatants from in vitro assays showed that coculturing of NK cells and macrophages led to increased levels of VEGF-A. Natural killer cells alone showed almost no competence to produce VEGF-A. Coculturing NK cells and macrophages in the presence of the IFN-γ–blocking antibody showed a significant decrease in VEGF-A secretion compared with controls (Fig. 3C). In addition, the population of IFN-γ–secreting NK cells was increased when cocultured with
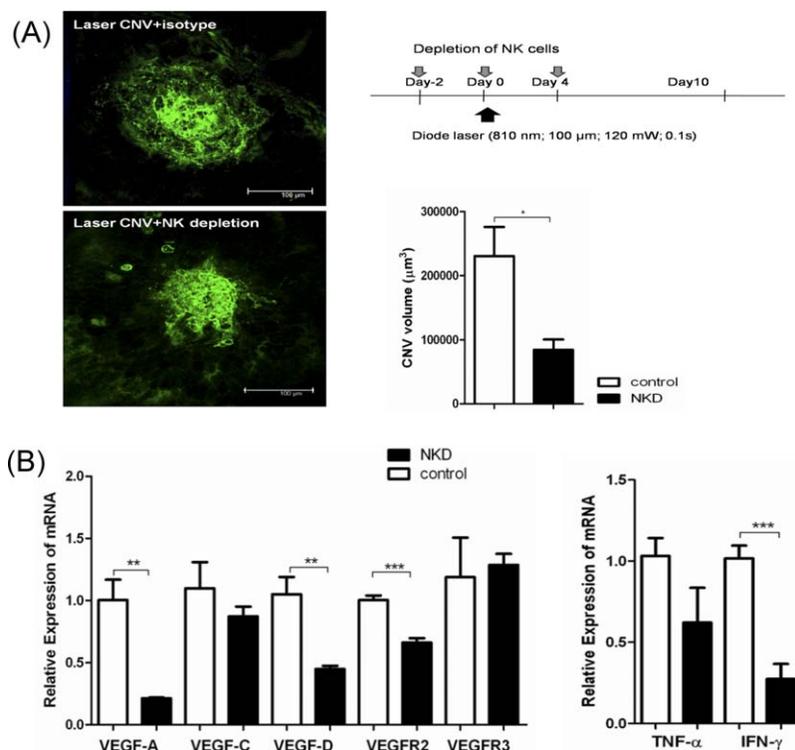


**FIGURE 4.** Depletion of NK cells decreased CNV and mRNA expression of VEGFs in a laser-induced CNV model. C57BL/6 mice were treated at Bruch's membranes by laser to induce CNV ($n = 7$ mice/group). Mice were treated with an anti-NK1.1 or isotype antibody intravenously 2 days before laser treatment, on the day of treatment, and on postoperative day 4. (**A**) Choroids were explanted and immunohistochemically stained for isolectin B4 at day 10. Choroidal neovascularization volume was analyzed in both groups. (**B**) Choroids of laser-treated mice (NKD or control) were harvested at day 10 and relative mRNA expression of different VEGFs, VEGFRs, IFN-γ, and TNF-α were measured by real-time PCR. Results are expressed as the relative amount of mRNA normalized to GAPDH. The results shown are the mean ± SEM values of three to four independent experiments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
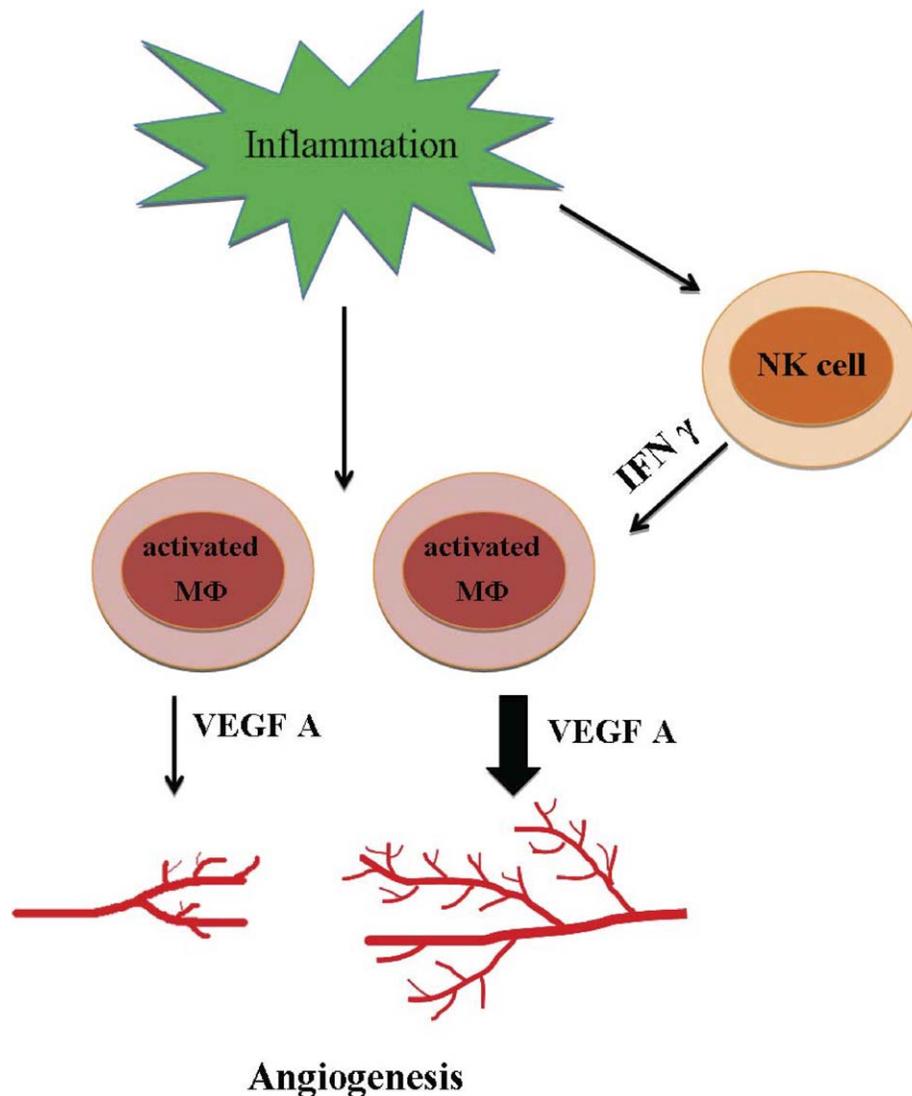
**FIGURE 5.** Conceptual figure summarizing the proposed mechanism of action of NK cells on angiogenesis through macrophage stimulation. Macrophages are not dependent on NK cells, per se, but NK cells are able to amplify the macrophage response through IFN-γ secretion, leading to enhanced macrophage recruitment and VEGF-A release, which can augment angiogenesis.[29]

macrophages, which might enhance VEGF-A production of macrophages reciprocally (Fig. 3D).

## NK Cell Depletion Reduced Neovascularization in a Murine Laser–Induced CNV Model

Next, we induced CNV by laser and investigated the effect of NK cell depletion on neovascularization. In the laser-induced CNV model, our data showed that NK cell depletion led to decreased areas of CNV (Fig. 4A) and significantly reduced mRNA expression of VEGF-A, VEGF-D, VEGFR2, and IFN-γ in the choroid of NK cell–depleted mice compared with isotype controls (Fig. 4B).

## DISCUSSION

In this study, we aimed to investigate the role of NK cells in ocular angiogenesis using two distinct mouse models as well as an in vitro culture system. Here, we demonstrate that (1) NK cell depletion leads to decreased corneal neovascularization in

vivo associated with reduced macrophage infiltration into the cornea and reduced mRNA levels of VEGF-A, -C, and -R3 in the cornea and conjunctiva, (2) NK cell depletion reduced CNV, and was associated with lower mRNA levels of VEGF-A, -D, and -R2 in the cornea, and (3) optimized HUVEC proliferation in vitro required both NK cells and macrophages, and was associated with elevated VEGF-A levels.

Natural killer cells are known to be important in ocular inflammatory diseases such as dry eye disease where NK cell depletion leads to inhibited maturation of antigen presenting cells.[14] While the role of macrophages in inducing inflammatory neovascularization through secretion of VEGF is well described,[1,15] the function of NK cells in angiogenesis is not well known. Our results show, for the first time, the importance of NK cells in the induction of angiogenesis: NK cell depletion inhibited blood vessel formation by reducing macrophage infiltration into the cornea after placing BFGF pellets. Basic fibroblast growth factor is known to be a potent angiogenesis inducer, including stimulation of endothelial proliferation and migration.[16]

Using an in vitro assay we found a prominent increase in tube formation of HUVECs and increased VEGF-A levels only when cocultured with both macrophages and NK cells together. Moreover, an IFN-γ–blocking antibody could inhibit tube formation and VEGF-A secretion. These results clearly indicate that IFN-γ–secreting NK cells can enhance VEGF-A production by macrophages, and seem to be necessary for full macrophage activation. This is in accord with the previous work by Xiong et al.[17] who emphasized the importance of IFN-γ to increase VEGF expression in IFN-γ/lipopolysaccharide (LPS)-double activated macrophages.

To date, few details are known about the pathogenesis of CNV in AMD,[18] although intravitreal administration of VEGF-A–neutralizing antibodies or VEGF trap are known to improve visual acuity in CNV patients.[15] Furthermore, macrophage depletion has been shown to reduce the size of laser-induced CNV associated with decreased VEGF levels[19] and a role of NK cells in the pathogenesis of AMD has been proposed[20]; however, the cytokines and pro-angiogenic molecules involved in this mechanism remain unknown. Thus, we investigated the effect of NK cell depletion in a murine model of CNV and detected decreased areas of CNV and reduced mRNA expression of VEGF-A, -D, -R2, and IFN-γ in the choroid of NK cell depleted mice. In tumor rejection studies, IFN-γ-dependent antiangiogenic and antitumoral effects have been shown.[21-24] On the other hand, pro-angiogenic effects of NK cells in nonsmall cell lung cancer has been identified lately,[25] but little is known about the angiogenic function of IFN-γ–secreting NK cells in an inflammatory environment. As NK cells are essential for immune defense of herpes simplex virus (HSV),[26,27] we can postulate that increased corneal angiogenesis associated with HSV, might be mediated by NK cell activation. Here, we demonstrate that the depletion of IFN-γ–secreting NK leads to decreased infiltration and activation of VEGF-secreting macrophages, which finally results in reduced laser injury–induced CNV.

It is known, that the anti-NK1.1 antibody depletes NK cells and natural killer T (NKT) cells. Others have selectively looked at NKT cells in CNV and found, similar to our results, that NKT cells augment the production of VEGF by other cells.[28] Here, we focused on the function of NK cells in angiogenesis and identified a direct effect of NK cells on macrophages and their VEGF production using two in vivo mouse models and an in vitro assay. Therefore, in the context of neovascularization, NK and NKT cells seem to work in a similar manner, but the distinct function of both cell populations in angiogenesis needs further investigation to better understand inflammatory angiogenesis.

In conclusion and summarized in Figure 5, these data suggest that IFN-γ–secreting NK cells promote angiogenesis by enhancing VEGF expression through macrophage activation, indicating a new mechanism for NK cells' interaction with macrophages in inducing angiogenesis, and opening new perspectives for the treatment of immune-mediated angiogenic conditions.

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