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Investigation of the role of CD1d-restricted invariant NKT cells in experimental choroidal neovascularization ☆

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ABSTRACT

Choroidal neovascularization (CNV) is directly related to visual loss in age-related macular degeneration and other macular disorders. We have investigated the role of CD1d-restricted invariant natural killer T (NKT) cells in laser-induced experimental CNV. Quantitative real-time PCR detected increased expression of NKT cell-related genes (*Va14* and *CXCL16*) in whole eyes undergoing CNV, indicating local accumulation of NKT cells. We found a significant reduction of CNV and lower concentrations of vascular endothelial growth factor (VEGF) in ocular fluid in two different NKT cell-deficient mice, CD1d knockout (KO) and Jα18 KO mice. We also established *in vitro* co-cultures of retinal pigment epithelial cells and splenic NKT cells, and confirmed NKT cells could produce VEGF in the dish. Moreover, inoculating α-galactosylceramide, the ligand for NKT cells, into the vitreous cavity of C57BL/6 mice promoted CNV. We concluded that NKT cells play an important role in CNV as an inducer of VEGF.

Ocular neovascularization is responsible for the majority of cases of acquired blindness. There are two types of ocular neovascularization, which affect either the retina or the choroid. Retinal neovascularization is induced by hypoxia and occurs in diseases such as diabetic retinopathy and branch retinal vein occlusion. In contrast, choroidal neovascularization (CNV) results from abnormalities of Bruch's membrane and the retinal pigment epithelium (RPE) and is seen in patients with age-related macular degeneration (AMD), angioid streaks, high myopia, ocular histoplasmosis, and similar diseases. Most cases of CNV are induced by macular lesions, and it therefore directly causes severe loss of visual acuity in patients.

The exact cellular and molecular mechanisms that induce CNV remain to be elucidated. However, several recent reports demonstrated a role for the complement system, and particularly Factor H [1–3]. Patel et al. showed that elevated levels of autoantibodies against retinal antigens appeared in the sera of AMD patients [4]. In addition, we have shown a critical role for infiltrating macrophages and neutrophils in the eye, in the induction of experimental CNV [5]. Altogether, inflammatory process are clearly important in generating CNV.

NKT cells belong to a specialized population of lymphocytes that co-express the T cell receptor (TCR) αβ chains and NK markers [6] and are restricted by the MHC class I-like molecule, CD1d [7]. Since CD1d is also required for the development of NKT cells, CD1d knockout (KO) mice selectively lack these cells [8,9]. The majority of CD1d-restricted NKT cells are Vα14$^+$ and express a single invariant TCRα chain encoded by the Vα14Jα18 gene [7]. Jα18 KO mice have markedly reduced numbers of NKT cells in many organs [7]. Stimulated invariant NKT cells rapidly secrete large amounts of cytokines including IFN-γ and IL-4. Although the natural ligand which stimulates NKT cells remains to be identified, they have been reported to be stimulated by TCR ligation with the lipid antigen α-galactosylceramide (α-GalCer) presented by CD1d [10].

Our experimental goal was to confirm the role of invariant NKT cells in CNV and CNV-related diseases. Photocoagulation (PC)-induced CNV is a well-established animal model for investigating the mechanisms of human diseases [11]. We therefore induced experimental CNV in NKT-deficient mice and examined the cytokine profiles.

Materials and methods

Mice. Female 8- to 10-week-old mice were used in all experiments. C57BL/6 (B6) mice were obtained from SLC Japan (Shizuoka, Japan). CD1d KO mice were generated in the Transgenic Facility, Harvard Medical School (Boston, MA) and backcrossed to B6 mice for six generations. Jα18 KO mice (NKT KO mice) were generated

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at Chiba University (Chiba, Japan) and backcrossed eight times to B6 mice. All animals were housed in specific pathogen free conditions at Kyushu University. All animals were treated humanely and experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction and evaluation of CNV. CNV was induced by photocoagulation (PC) and evaluated as previously described [5]. Briefly, laser photocoagulation (wave length 532 nm, 0.1 s, spot size 75 μm, power 200 mW) around the disc of the retina was administered to burn the posterior pole of the retina. A week later, mice were anesthetized and perfused with 1 ml phosphate buffered saline containing 50 mg/ml fluorescent-labeled dextran (25,000 MW; Sigma, St. Louis, MO) and the eyes were removed. The entire retina was mounted on slide glasses. The total area of hyperfluorescence associated with each burn, corresponding to the total number of fibrovascular scars, was measured using MacScope (version 2.3; Mitani, Fukui, Japan).

Real-time reverse transcriptase (RT)-PCR. Total RNA was extracted from whole eyes except for conjunctiva. Three eyes were pooled to obtain enough amount of mRNA for analysis, 12 or 24 h after PC using Trizol (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Aliquots containing 1 or 2 mg total RNA were reverse-transcribed using 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. The reverse-transcribed cDNAs were then subjected to real-time PCR using SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) and a Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). The primers used were 5′-CTAAGCACAGCACGCTGCA CA-3′ and 5′-AGGTATGACAATCAGCTGAGTCCC-3′ for *Vα14*, 5′-T CCTTTTCTTGTTGGCGCTG-3′ and 5′-CAGCGACACTGCCCTGGT-3′ for *CXCL16*, 5′-CCCTTTTGGGCCTATGCAG-3′ and 5′-ATGCCTCGAAG AGTTTTGCAC-3′ for *CXCR6*, 5′-TTACTGCTGTACCTCCACC-3′ and 5′-ACAGGACGGCTTGAAGATG-3′ for *VEGF*, 5′-TGGAGTCACAGAA GGAGTGGCTAAG-3′ and 5′-TCTGACCACAGTGAGGAATGTCC AC-3′ for *IL-6*, and 5′-GATGACCCAGATCATGTTTGA-3′ and 5′-GGAGA GCATAGCCCTCGTAG-3′ for β-actin. All estimated mRNA values were normalized to β-actin mRNA levels. Each experiment was repeated at least twice and representative data are shown.

Vitreous cavity injection. Low dose (500 ng/ml, 1 μl) or high dose (2.5 μg/ml, 1 μl) α-GalCer (Kirin Brewery Co. Ltd., Japan) or vehicle (500 ng/ml, 1 μl) was injected into the vitreous cavity using fine, 32-ga needles (Cat. No. 0160832, Hamilton, Reno, NV) and 10-μl syringes (Cat. No. 80330, Hamilton). The tip of the needles penetrated the sclera, choroid and retina, to reach the vitreous cavity and maximum volumes of 2 μl per injection were introduced per eye. We ensured that antigen was injected into the vitreous cavity by carefully guiding the tip of the needle under the microscope, through the flattened cornea covered by a glass microscope slide. Inoculating 2 μl solution elevated the intraocular pressure sufficiently to completely seal the retinal incision without any bleeding or detachment.

In vitro culture system. RPE cells were prepared from eyes of B6 mice and incubated for about 10 days in DMEM supplemented with 20% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine, and 0.1 mM non-essential amino acids at 37 °C in 5% CO_2. T lymphocytes from the spleens of B6 or CD1d KO mice were enriched on columns of IMMULAN goat anti-mouse IgG-coated beads (Biotex Laboratories, Houston, TX). RPE cells were plated in six-well dishes (Collagen-Coated Microplate 6 Well with Lid Collagen Typel, IWAKI, Chiba, Japan) and incubated until almost confluent, and then the media was replaced to 2 ml of media containing about 2×10^6 T lymphocytes, and RPE cells and T lymphocytes were co-cultured with anti-CD1d antibody or control IgG for 6 h, when the culture supernatants were collected and used to measure cytokine and chemokine levels. Total RNA was also extracted from the floating T lymphocytes.

Luminex® assay. The concentrations of cytokines and chemokines were measured using a microbead-based ELISA system (Multiplex Ab Bead Kits; BioSource International, Camarillo, CA, USA) according to the manufacturer's directions with Luminex 100 (Luminex®, Austin, TX, USA). For *in vivo* experiments, eyes were enucleated from mice under deep anesthesia, conjunctival tissue was removed, and the remaining eye tissue was homogenized using a Biomasher (Nippi Inc., Tokyo, Japan). After centrifugation at 12,000*g* for 30 min, the supernatants were used in assays. For *in vitro* experiments, culture supernatants were used.

Statistics. Data were analyzed for significant differences between experimental groups using either ANOVA/Scheffe's test (more than three groups) or Student's *t*-test (two groups). *P* values $\leqslant 0.05$ were considered significant.

Results

Accumulation of NKT cells in the eye after laser treatment

First we measured the levels of invariant *Vα14* mRNA in the eye by using quantitative real-time PCR as previously reported [12]. Since Jiang et al. had reported that NKT cells are regulated by the chemokine *CXCL16* and its receptor *CXCR6*, which could be considered as new markers for NKT cells [13], we also measured local *CXCL16* expression. Total RNA was extracted from whole eyes of B6 mice, 24 h after laser treatment. *Vα14* and *CXCL16* mRNA were significantly elevated in eyes from laser-treated mice compared to untreated mice (Fig. 1), suggesting direct infiltration by invariant NKT cells in the eye in this model.

CNV in NKT cell-deficient mice

To determine whether the recruitment of NKT cells played a role in PC-induced CNV, we used two different NKT cell-deficient mice, and visualized the appearance of PC-induced CNV in choroidal flat mounts by fluorescent angiography. In contrast to wild-type mice, only a few hyperfluorescent areas of new vessel formation were observed in the KO mice, on day 7 after PC (Fig. 2A). The areas of CNV were shown to be statistically significantly smaller in the KO mice compared to wild-type mice (Fig. 2B). These observations suggested an important role for NKT cell infiltration in CNV development.

In vivo expression of angiogenic and/or inflammatory factors in NKT cell-deficient mice

Since NKT cells could mediate laser-induced angiogenesis, we reasoned that they probably synthesized or induced angiogenic and/or inflammatory factors. To identify candidate soluble factors that might be absent in NKT-deficient mice, we used microbead-based multi-protein analysis to compare B6 and CD1d KO mice after PC. Twelve hours after PC, ocular fluid samples from CD1d KO mice contained lower concentrations of angiogenic factors, such as VEGF and bFGF, than B6 mice, which was consistent with the reduced CNV in the KO mice. Interestingly, we found higher concentrations of IL-6 and keratinocyte-derived chemokine (KC), but lower concentrations of IL-12, IL-13, TNFα, IP-10, MIP-1α, and GM-CSF in CD1d KO than wild-type mice (data not shown). Among these soluble factors, we focused on VEGF and IL-6 expression. Consistent with the protein results, quantitative real-time PCR showed that mRNA expression was lower for *VEGF* and higher for *IL-6* in both NKT cell-deficient mice than in wild-type mice (Fig. 2C).

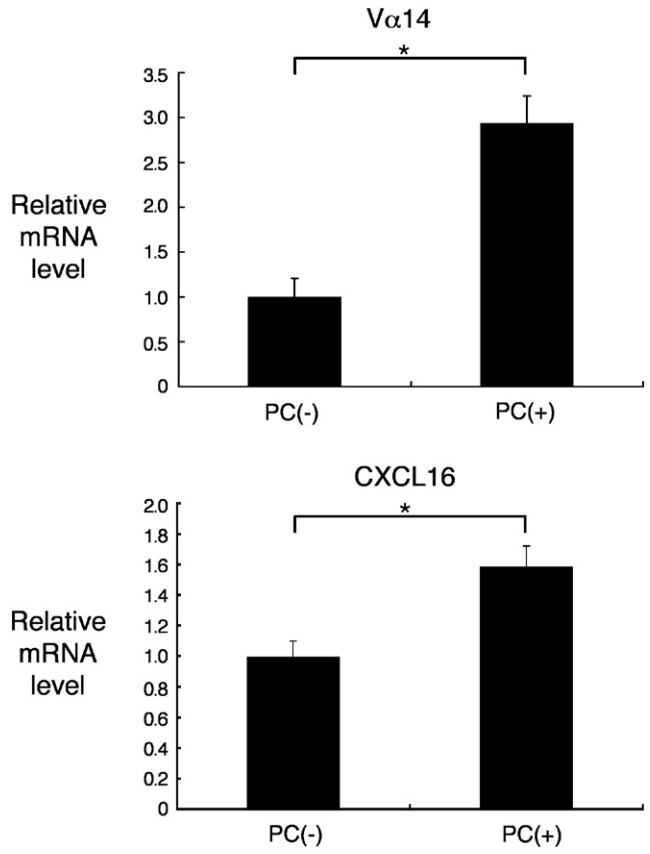

Fig. 1. Infiltration of NKT cells to the eye C57BL/6 mice were treated by laser and compared the expression of NKT cell marker (Vα14) and specific chemokine (CXCL16) with untreated mice. Twenty-four hours after laser, total RNA was extracted from the eyes and the amounts of *Vα14* and *CXCL16* mRNA were quantified by real-time RT-PCR and normalized by corresponding amounts of β-actin mRNA. Three eyes were pooled to obtain enough amount of mRNA ($n=3$, total nine eyes were used). The bars show means ± SD. The experiments were repeated twice with similar results.

Effect of anti-CD1d antibody or the absence of NKT cells on the expression of VEGF and IL-6 in vitro

We next investigated *in vitro* expression of VEGF and IL-6 in co-cultures of RPE cells and column-enriched splenic T lymphocytes (Fig. 3A). The reason why we planned to use *in vitro* system was to investigate the cellular source of VEGF, since *in vivo* system had clear limitation f41or this purpose. We selected RPE as the stimulator of NKT cells, because RPE are major antigen-presenting cells in chorio-retinal interface that is damaged in AMD. When cells from B6 mice were cultured with anti-CD1d antibody, to inhibit NKT cell activity, culture supernatants collected after 6 h (Fig. 3) contained less VEGF than those from control IgG-treated cultures, at both the protein level, measured by ELISA, and at the mRNA level, measured in extracts of the floating T lymphocytes (Fig. 3B and C). In contrast, no difference was seen in IL-6 expression between anti-CD1d antibody-treated cultures and controls (Fig. 3B and C). Consistent results were seen when we used T cells from CD1d KO mice in cultures (Fig. 3D). Blocking of NKT-CD1d interaction by the specific antibody or the absence of NKT cells *in vitro* result in the decrease of VEGF, but not IL-6.

α-GalCer treatment promoted CNV and VEGF production in vivo

Since our results implicated NKT cells in CNV, we also looked at the effect of activating NKT cells on this process. α-GalCer is known to bind to NKT cells and induce various immune responses [10]. We therefore inoculated α-GalCer directly into the vitreous cavity and looked at the effect on CNV. As expected, α-GalCer inoculation increased CNV in B6 mice at low dose (100 ng/ml) compared to vehicle-treated mice (Fig. 4A). We also evaluated the effect of α-GalCer on the production of VEGF. Total mRNA was extracted from eyes of B6 mice 12 h after PC + α-GalCer injection into the vitreous cavity, and the amounts of *VEGF* mRNA was quantified by real-time RT-PCR. *VEGF* expression was higher in low dose α-GalCer-treated mice than vehicle-treated mice (Fig. 4B).

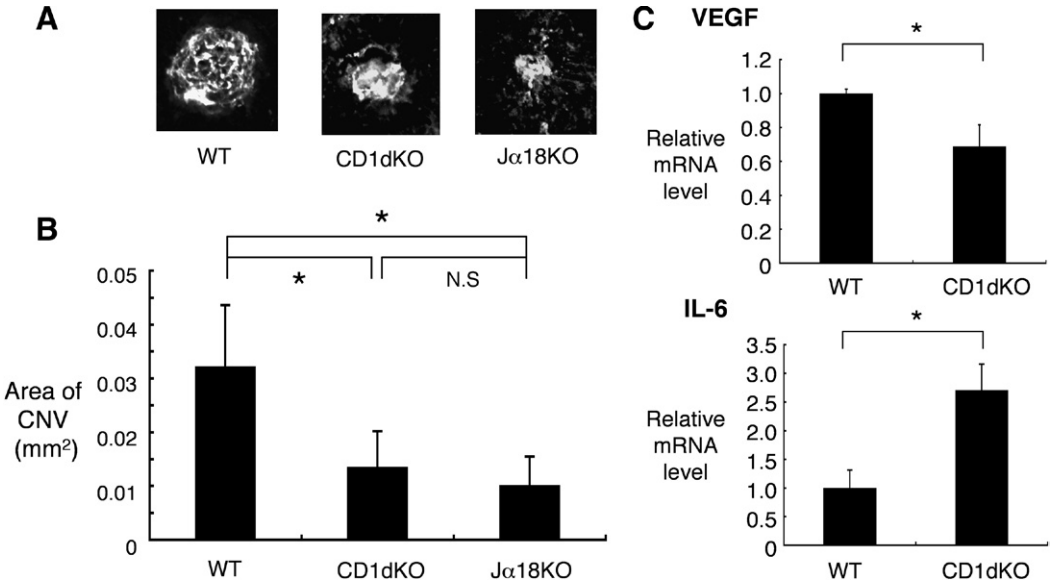

Fig. 2. CNV in NKT cell-deficient mice. C57BL/6 mice (WT), CD1d KO mice and Jα18 KO mice were treated by laser. The areas of CNV were compared between the three groups. The each experiments were repeated at least three times with similar results. (A) The representative CNV lesions of choroidal flat mount. Seven days after PC, the mice were perfused with fluorescein labeled dextran and choroidal flat mounts were made. CNV was detected as a hyperfluorescence vascular structure. (B) The area of CNV was compared among the three groups. The bars show means ± SD, $n=8$. (C) Twelve hours after PC, total RNA was extracted and the amounts of *VEGF* (upper panel) and *IL-6* mRNA (lower panel) was quantified by real-time RT-PCR and normalized by corresponding amounts of β-actin mRNA. The bars show means ± SD, $n=3$.

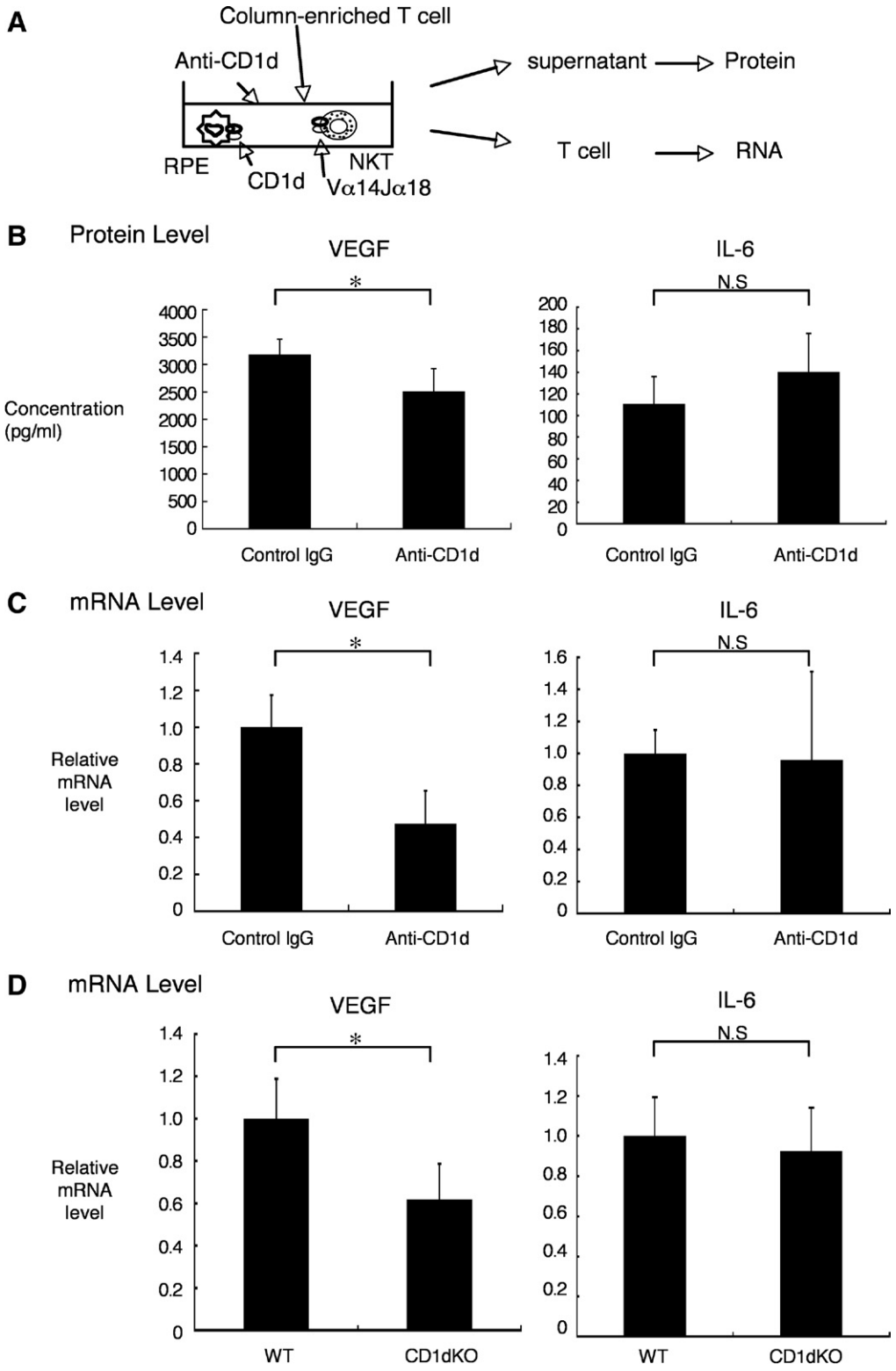

Fig. 3. *In vitro* expression of *VEGF* and *IL-6* from (adherent) T cells under treatment of anti-CD1d antibody. The each experiments were repeated more than twice with similar results. (A) RPE were prepared from eyes of C57BL/6 mice and incubated for about 10 days. After that, (adherent) RPE cells and (non-adherent) T lymphocytes, which were enriched from splenocytes of either C57BL/6 mice were co-cultured with anti-CD1d antibody or control IgG for 6 h. And then the culture supernatant was subjected to ELISA, and total RNA was extracted from floating T lymphocytes. Some experiments used splenic T cells from CD1d KO mice instead of using anti-CD1d antibody. (B) Culture supernatant was subjected to ELISA, and the concentration of VEGF and IL-6 was compared between two groups. The bars show means ± SD (triplicate wells). (C) Total RNA was extracted from floating T lymphocytes and the amounts of *VEGF* and *IL-6* mRNA was quantified by real-time RT-PCR and normalized by corresponding amounts of β-actin mRNA. The bars show means ± SD. Data represent the mean of three different well of samples. (D) The experiment used splenic T cells from CD1d KO mice instead of using anti-CD1d antibody.

Although 100 ng/ml range of α-GalCer must be the physiological dose, we unexpectedly found that higher dose of α-GalCer (500 ng/ml) reduced CNV (Fig. 4A) and VEGF (Fig. 4B). In fact, the dose-dependent different manner of response against α-GalCer has reported [14]. Since NKT cells can regulate immune response in multiple ways, we speculated that "high dose" α-GalCer might

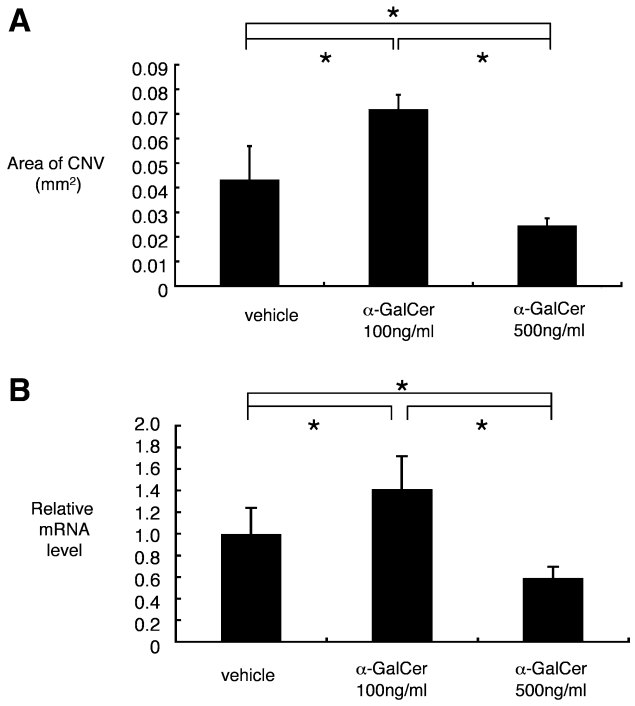

Fig. 4. *In vivo* α-GalCer treatment after laser. (A) C57BL/6 mice were treated by laser. Immediately after PC, 1 μl of vehicle (500 ng/ml) or α-GalCer (500 ng/ml or 2.5 mg/ml) was injected into vitreous cavity. Since the volume of vitreous fluid in mice is approximately 5 ml, the adjacent concentration of α-GalCer was around 100 ng/ml and 500 ng/ml, respectively. Seven days later, the mice were perfused with fluorescein labeled dextran and the eyes were removed to make choroidal flat mounts. CNV was detected as a hyperfluorescence vascular structure. The area of CNV was measured as described in Materials and methods. The bars show means ± SD, $n = 4$. The experiments were repeated at least three times with similar results. (B) Immediately after PC, 1 μl of vehicle or α-GalCer was injected into vitreous cavity. Twelve hours later, mice were sacrificed and their eyes were enucleated, and then eyes were homogenized and centrifuged. Total RNA was extracted from eyes (per three eyes) and the amounts of *VEGF* mRNA was quantified by real-time RT-PCR and normalized by corresponding amounts of β-actin mRNA. The bars show means ± SD. Data represent the mean of three independent sets of samples. The experiments were repeated twice with similar results.

activate NKT cells differently to produce some angiostatic factors and so may have therapeutic potential *in vivo*.

Discussion

Our data demonstrated for the first time that CD1d-restricted NKT cells play an important role in the formation of PC-induced CNV. After the type of insult reproduced by laser burns, NKT cells migrate to the eye and modulate the resulting inflammatory process associated with CNV.

Although NKT cells can directly produce VEGF *in vitro* (Fig. 3), it does not exclude possibility that other cells than NKT cells can also produce VEGF or somehow participate in CNV formation. In CNV, several reports have demonstrated RPE cells, microglia and infiltrating bone marrow-derived macrophages as sources of VEGF [15,16]. We currently postulate that NKT cells might also augment the production of VEGF by other cells in the eye rather than produce angiogenic factors themselves. In fact, NKT cells have been shown to activate macrophages either through a direct cell-to-cell or a cytokine mediated process [17].

We need to discuss about the diversity of effect that intravitreous α-GalCer can have on NKT cells. Its effect may depend upon the conditions, such as the dose and/or timing of inoculations [16]. Our data shows α-GalCer treatment *in vivo* enhanced CNV at "low dose" but reduced at "high dose". Since 100 ng/ml range of α-GalCer is the physiological dose, we speculate "high dose" α-GalCer may activate NKT cells differently to produce angiostatic factors. Our data also suggests that inoculating "high dose" α-GalCer into the vitreous cavity can have therapeutic potential. Controlled studies to evaluate the effects of different doses and times of administration of α-GalCer into the vitreous cavity will be needed to investigate its therapeutic use in CNV-related, sight-threatening diseases.

Although we found that NKT cell-deficient mice expressed low levels of the angiogenic factor VEGF, we found high levels of IL-6 expression compared to wild-type mice. Contradictory, several reports have shown that IL-6 promotes angiogenesis by inducing VEGF expression [18,19]. The mechanisms involved here are unclear, but Hatzi et al. have reported that IL-6 inhibited VEGF-induced corneal neovascularization [20]. Since IL-6 is known to be a multifunctional cytokine, it may have anti-angiogenic functions under some circumstances or at certain stages of inflammation. Another explanation is that NKT cells may promote VEGF induction by IL-6, and that the IL-6 overproduction in NKT cell-deficient mice was a response to the lower expression of VEGF in these mice. However, it is important to note that IL-6 concentrations were not raised in the co-cultures of RPE and NKT cells (Fig. 3). We speculate that the cellular source of IL-6 may be missing in this system, neither RPE nor NKT cells being the source, and we suggest that microglia and/or infiltrating macrophages in the eye may produce IL-6 *in vivo*. Further studies will be required to elucidate these mechanisms.

In summary, CD1d-restricted NKT cells, a type of innate immune cell, play an important role in PC-induced CNV. The therapeutic potential of modulating NKT cell activity with higher dose α-GalCer in sight-threatening eye disease warrants further research in this area.

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