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Abstract :

The pro-inflammatory cytokine interleukin 1β (IL-1β) has been shown to promote angiogenesis. It can have a neurotoxic or neuroprotective effect. Here we have studied the expression of IL-1β *in vivo* and the effect of the IL-1 receptor antagonist (IL-1Ra) on choroidal neovascularization and retinal degeneration. IL-1β expression significantly increased after laser injury (rt-PCR) in C57Bl6 mice, in the C57Bl6 *Cx3cr1*$^{-/-}$ model of age-related macular degeneration (ELISA) and in albino Wistar rats and albino BALB *Cx3cr1*$^{+/+}$ and $^{-/-}$ mice (ELISA) after light-injury. IL-1β was localized to Ly6G positive, Iba1 negative infiltrating neutrophils in laser-induced CNV as determined by immunohistochemistry. IL-1Ra treatment significantly inhibited CNV, but did not affect Iba1 positive macrophage recruitment to the injury site. IL-1β significantly increased endothelial cell outgrowth in aortic ring assay independently of VEGF, suggesting a direct effect of IL-1β on choroidal endothelial cell proliferation. Inhibition of IL-1β in light- and laser-induced RD models did not alter photoreceptor degeneration in Wistar rats, C57Bl6 mice or retinal degeneration prone *Cx3cr1*$^{-/-}$ mice. Our results suggest that IL-1β inhibition might represent a valuable and safe alternative to VEGF inhibition in the control of CNV in the context of concomitant photoreceptor degeneration as observed in AMD.

Introduction :

The IL-1 family of cytokines plays a key role in the initiation of acute inflammatory responses [1]. Interleukin 1 beta (IL-1β) interacts with IL-1 receptor I (IL-1RI), composed of IL-1R and IL-1R accessory protein (IL-1RacP) subunits. IL-1 receptor antagonist (IL-1Ra) competes with IL-1β for its binding site [1]. IL-1β is a potent inflammatory mediator with chemotactic [2] and angiogenic [3, 4] properties. It is a neurotoxic mediator in ischemic brain

injury [5] but can attenuate glutamate neurotoxicity in the retina [6] and protect against light-induced or hereditary photoreceptor degeneration [7, 8].

Age-related macular degeneration (AMD) is the leading cause of vision loss in elderly people in industrialized countries [9]. Its most prominent pathological features are photoreceptor degeneration and choroidal neovascularization (CNV) [10]. In AMD, IL-1β is secreted by RPE cells and CD68 positive cells in choroidal neovascular membranes [11] and is therefore a possible proangiogenic and neuroprotective or neurotoxic mediator in AMD.

IL-1Ra is clinically used to treat juvenile idiopathic arthritis [12], it has been shown to inhibit neurotoxicity in ischemia in animal models [13]. Intravitreal human IL-1Ra injections have been shown to efficiently inhibit CNV in a rat model [14]. To analyze the possible implication of IL-1β in CNV and retinal degeneration, we studied the expression of IL-1β in laser-induced CNV in mice and in light-induced retinal degeneration in rats and mice. We localized IL-1β expression by immunohistochemistry and inhibited IL-1β activity with IL-1Ra supplementation.

Materials and Methods

Animals

10 week old Wistar rats and C57B6j wild type mice were purchased from the Janvier breeding center (Le Genest-St-Isle, France). *Cx3cr1*$^{-/-}$ C57B6j mice were backcrossed for six generations into the BALB/c background (Janvier) to obtain the *Cx3cr1*$^{-/-}$ BALB/c strain and kept in specific pathogen-free conditions with food and water available *ad libitum* and housed in a 12/12 hr light/dark (100-500 lux) cycle. Animal experiments were approved by the Institutional Animal Care and Use Committee.

Laser coagulation

10-week-old C57Bl6 mice were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Their pupils were fully dilated with 1% tropicamide. Coverslips positioned on the mouse cornea were used as a contact glass. 4 laser coagulations were performed 4 to 5 disc diameters away from the papillae with an Argon laser (532 nm) mounted on a slit lamp (400 mW, 50 ms and 50 µm). Mice were treated with daily subcutaneous injections of PBS or human recombinant IL-1Ra (Kineret, Biovitrum, Stockholm, Sweden) at 1mg/d/kg until sacrifice.

Choroidal flatmounts, Immunohistochemistry and CNV quantifications

Eyes were enucleated, fixed in 4% PFA for 15 minutes at room temperature and sectioned at the limbus; the cornea and lens were discarded (for CNV quantification, mice were perfused with fluorescein dextran 10^6 before enucleation). Retinal and RPE/choroidal flatmounts were stained according to previously described standard immunohistochemical procedures [15]. The primary antibodies and lectins used were Bandeirae simplicifolia lectin (Sigma Aldrich, Saint Quentin Fallavier, France), goat anti-mouse IL-1β (R&D, Lille, France), rabbit polyclonal anti-IL-1RI (Santa Cruz, Heildeberg, France), rabbit polyclonal anti-Iba1 (Wako, Neuss, Germany), rat anti-mouse Ly6G (Miltenyi Biotec, Paris, France), and goat anti-human Collagen IV (R&D, Lille, France). The corresponding Alexa secondary antibodies (Molecular Probes, Leiden, the Netherlands) were used to reveal the primary antibodies, and flatmounts were counterstained with 4-6-diamino-2-phenylindole (DAPI). The choroids and retinas were radially incised, flatmounted, and viewed with the same fluorescence microscope. Flatmounts were viewed with a fluorescence microscope (DM5500B, Leica, Nanterre, France). All immunostaining was repeated at least 3 times, and stains omitting the primary antibody served as negative controls. Ly6G positive and Iba1 positive cells were counted on stained whole RPE/choroidal flatmounts up to the ciliary body and on the outer segment side of the retina.

The surface covered by perfused CNV was measured on photographs of fluorescein dextran 10^6 perfused choroidal flatmounts with ImageJ analysis software (surface covered by fluorescein staining). The average CNV size was calculated per eye.

Microvascular Sprouting from Aortic Explants

Aortae from adult C57BL6 were cut into 1-mm-thick rings and covered with 30 µL of Matrigel (BD Biosciences, Le Pont de Claix, France) in 24-well tissue cultures plates. Aortic rings were cultured for 3 days in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Cergy Pontoise, France) containing 10% FCS, 1% penicillin/streptomycin, and 0.2% fungizone. Explants were exposed to IL-1β (5ng/mL; R&D system, Lille, France), IL-1Ra (10mg/mL) and VEGFR1s (150ng/mL; R&D) from day 3 to day 7 of culture. Photographs of individual explants were taken every day and the surface covered by the aortic ring and the vascular sprouts was measured daily from day 3 to 7. The surface of each individual aortic ring and pre-incubation sprouts at day 3 was subtracted from the surface at day 4 to calculate the vascular sprouting that occurred in the presence of the ligands and controls.

Reverse Transcription and Real Time Polymerase Chain Reaction (RT PCR)

Total RNA was isolated with NucleoSpin RNA II Kit (Macherey-Nagel, Hoerdt, France). Single-stranded cDNA was synthesized from total RNA (pre-treated with DNaseI amplification grade) using oligodT as primer and superscript reverse transcriptase (Invitrogen, Cergy Pontoise, France). Subsequent real-time polymerase chain reaction (RT-PCR) was performed using cDNA, qPCR SuperMix-UDG Platinum SYBR Green (Invitrogen), and the following primers (0.5 pmol/µl): mm actin sense: 5'-AAGGCCAACCGTGAAAAGAT-3'; mm actin antisense : 5'- GTGGTACGACCAGAGGCATAC-3'; mm IL-1β sense : 5'-CATGGAATCCGTGTCTTCCT-3'; mm IL-1β antisense : 5'-GAGCTGTCTGCTCATTCACG-3'; mm VEGF sense : 5'-GTGAGCCAGGCTGCAGGAAG-3'; mm VEGF antisense : 5'-

GAATGCGTCTGCCGGAGTCT-3'; rn actin sense : 5'-AAAGAAAGGGTGTAAAACGCAG-3'; rn actin antisense : 5'-AAAGACCTCTATGCCAACACAG-3'; rn IL-1β sense : 5'-GGAACCCGTGTCTTCCTAAA-3'; rn IL-1β antisense : 5'-CTGACTTGGCAGAGGACAAA-3'

PCR reactions were performed in 40 cycles of 15 s at 95°C, 45 s at 60°C. Product was not generated in control reactions in which reverse transcriptase was omitted during cDNA synthesis.

Light-Induced Degeneration

10-week-old rats and 8-week-old Cx3cr1$^{-/-}$ mice were were adapted to darkness for 12h and pupils were fully dilated with 1% Atropin (Novartis, Rueil Malmaison, France). Animals were then exposed to green LED light (4500 Lux) for 12 hours and subsequently kept in cyclic 12h/12h normal animal facility conditions. Control and light-exposed rats and mice were treated with daily subcutaneous injections of PBS or IL-1Ra (Kineret, Biovitrum, Stockholm, Sweden) at 1mg/d/kg until sacrifice and a volume of 5 (rats) or 2 µL (mice) of IL-1Ra at 150 mg/mL was injected intravitreally at day 0 and day 3 after illumination.

Protein Analysis

Eyes were enucleated, sectioned at the limbus and the cornea and lens were discarded. The complex retina/RPE/choroid/sclera was placed in 150 µL PBS 1x supplemented with a protease inhibitor cocktail (Calbiochem, Fontenay-sous-Bois, France) followed by homogenization with a plastic pestle. The lysate were cleared of debris by centrifugation at 2000 rcf for 10 minutes at 4°C. Total protein content of supernatant was determined by commercial assay (Bradford kit, Eragny-sur-Oise, France). Supernatant IL-1β level was determined with a sandwich enzyme-linked immunoabsorbent assay (ELISA), according to

the manufacturer's instructions (R&D Systems, Lille, France) and normalized for total protein.

Histology

For histology, eyes were fixed in 0.5% glutaraldehyde, 4% PFA PBS for 2 hours, dehydrated and mounted in historesin. 5-µm oriented sections, crossing the centre of the laser injury (CNV model) or inferior pole, optic nerve and superior pole (light-induced model), were cut and stained with toluidin blue. Rows of nuclei in the outer nuclear layer were counted at different distances from the injury site (CNV model) or optic nerve (light-induced model).

Statistical Analysis

Graph Pad Prism 5 (GraphPad Software) was used for data analysis and graphic representation. All values are reported as mean ± SEM. Statistical analysis was performed by one-way and two-way analysis of variance followed by post-hoc Bonferroni's test for comparison among means. $P < 0.05$ was considered statistically significant.

Results

IL-1β is induced in laser-induced neovascularization and localizes to the injury site.

IL-1β is known for its proangiogenic properties [3, 4]. It is secreted by CD68 positive cells in choroidal neovascular membranes in AMD [11]. Its inhibition has recently been shown to prevent laser-induced subretinal neovascularization in rats, but its induction, expression pattern, and impact on inflammation in the laser model has not been analyzed [14, 16]. We first analyzed IL-1β expression at different time points in laser-induced CNV in the retina (Fig. 1A) and choroid (Fig. 1B) of C57Bl6 mice. A strong induction of IL-1β mRNA is observed as early as 1h in the retina (55fold) and at 6h (35fold) and 20 hours (18fold) in the choroid. To a

lesser extent, a tendency for vascular endothelial growth factor (VEGF) mRNA induction was detected in the retina at 1h (Fig. 1C) but not in the choroid (Fig. 1D; control n=4, 1h n=8, 6h n=7, 20h n=5, 72h n=4 retinas/choroids). To identify IL-1β expressing cells, we performed immunohistochemistry of IL-1β (green staining Fig. 1 E, I and M), the neutrophil marker Ly6G (Fig. 1 F, J and N) and the monocyte/macrophage marker Iba1 (Fig. 1 G, K and O) on choroidal/RPE flatmounts of uninjured control eyes (Fig. 1E-H) and 10h (Fig. 1 I-L) and 72h (Fig. 1M-P) after laser impact. Uninjured control eyes did not stain positive for IL-1β, Ly6G or Iba1 (Fig. 1 E-H). At 10h after the injury small IL-1β positive cells are visible at the site of impact (Fig 1I) (n=4 eyes). Triple labeling at 10h using the neutrophil marker Ly6G (red fluorescence Fig.1J) and the monocyte/macrophage marker Iba1 (blue fluorescence Fig. 1K), reveals that the IL-1β positive cells are Ly6G positive and Iba1 negative (Fig. 1L merge). The vast majority of detected IL-1β positive cells were Ly6G positive. 72 hours after the impact IL-1β (Fig. 1M) and Ly6G (Fig. 1N) was faint and diffuse, while numerous Iba1 positive cells were detected at the injury site (blue staining Fig.1 O, P merge). Immunohistochemistry at 10h and 72h omitting the primary antibodies resulted in no staining (data not shown) (n=4 eyes per time point).

IL-1Ra inhibits choroidal neovascularization independently of macrophage recruitment.

To evaluate the impact of IL-1β induction on CNV we treated one group of experimental animals with subcutaneous saline injections (n=4 mice) and one group with subcutaneous injections of 1mg/kg/day recombinant human IL-1Ra (n=5 mice). At day 14 after laser injury the CNV was visualized on isolectin-stained (red staining), fluorescein dextran perfused (green/yellow staining) RPE/choroidal flatmounts of saline (Fig. 2A) and Il-1Ra treated mice (Fig. 2B). Mature, perfused CNV (presenting a tubular structure with a lumen) was quantified as fluorescein positive surface (Fig. 2C). IL-1Ra treated animals showed a 60% reduction in

choroidal neovascularization. Initial lesion size at 10h after laser impact, identified by the loss of the RPE cell layer, was quantified on choroidal/RPE flatmounts of saline and Il-1Ra treated mice and showed no significant difference (Fig. 2D), excluding a possible initial difference of the laser injury in the two groups.

IL-1β has chemotactic properties and can alter macrophage recruitment to the granulomateous tissue that forms after injury [2]. It might thereby indirectly influence angiogenesis. To test this hypothesis, we visualized neutrophils and macrophages/microglial cells 10h (Fig. 2 E and F) and 72h (Fig. 2 H and I) after laser injury, on Ly6G (red staining) and Iba1 (green staining) stained RPE/choroidal flatmounts of saline (Fig. 2 E and H; n=3 mice) and Il-1Ra treated (Fig. 2F and I; n=3 mice) mice. Neutrophil (Fig.2 G) and macrophage/microglial cell (Fig. 2J) prevalence were quantified. Ly6G positive neutrophils were significantly recruited to the injury site, reaching their maximum at 10h. Neutrophil recruitment was stronger in IL-1Ra treated animals (Fig. 2G; n=3-4 mice per time point). IL-1Ra treatment did not alter Iba1 positive macrophage/microglial cell recruitment, which reached the maximum in both saline and IL-1Ra treated groups at 4d (Fig. 2 J).

IL-1β induces vascular sprouting in aortic rings.

IL-1β exerts its activity by acting on the IL-1 receptor I (IL-1RI) [1]. It induces angiogenesis in the cornea *in vivo* [3], IL6 expression [17], and microparticle release from vascular endothelial cells *in vitro* [18]. IL-1RI is expressed on numerous cell types, notably it is constitutively expressed on vascular endothelium [17]. IL-1RI immunohistochemistry on choroidal/RPE flatmounts of C57Bl6 mice 5d after the injury reveals IL-1RI positive staining around the lesion site (Fig 3A) (n=3 mice). Double labeling using the vascular endothelial cell marker collagen IV (red fluorescence Fig. 3B) shows that IL-1RI is expressed in the vascular endothelium surrounding the lesion and in some unidentified cells (Fig. 3C merge).

To test the effect of IL-1β on vascular sprouting we used the aortic ring assay. Photographs at 4d, 24 hours after ligand incubation, show the vascular sprouting of PBS (Fig. 3D), IL-1β (Fig. 3E), IL-1Ra (Fig. 3F), or IL-1β and IL-1Ra incubated aortic rings (Fig. 3G). Measurements of the area covered by the vascular sprouts revealed a significant increase of growth in IL-1β exposed aortic rings after 24 hours of IL-1β exposure at 4d that was inhibited by simultaneous IL-1Ra incubation (Fig. 3H). Vascular sprouting remained significantly increased up to 6d (72 hours of ligand exposure) before aortic rings started dying (data not shown). Simultaneous incubation of IL-1β with the soluble VEGFR1 did not significantly inhibit the effect of IL-1β incubation alone (Fig. 3I).

IL-1Ra does not affect photoreceptor survival laser and light-induced injury.

In AMD, CNV develops in the context of photoreceptor degeneration. IL-1β supplementation has been shown to inhibit photoreceptor degeneration in rat models of light-induced degeneration [7] and protect photoreceptor degeneration observed in Royal College of Surgeons rats [8]. IL-1β inhibition might have a deleterious effect on retinal degeneration. Laser-injury used to induce CNV also induces a local inflammatory reaction and retinal degeneration in the vicinity of the laser impact. To evaluate whether IL-1Ra influences the laser-induced photoreceptor degeneration, we treated laser-injured C57Bl6 mice with daily subcutaneous injections of saline (n=3 mice) or IL-1Ra (n=4 mice) and prepared histological sections crossing the lesion site at d14 (Fig. 4A and B respectively). Quantification of rows of nuclei at increasing distances from the injury site revealed no difference in photoreceptor cell loss in the vicinity of the laser impact (Fig. 3C).

To analyze the effect of IL-1β inhibition in a non-neovascular context, we exposed Wistar rats to light-induced retinal degeneration and analyzed IL-1β expression by RT-PCR in eye cups (Fig. 4D; control n=4 eyes, 1h n=5 eyes, 12h n=5 eyes). IL-1β transcript was significantly

induced (x5) in the eye at 12 hours after the onset of light-induced stress. To evaluate the influence of the endogenously-produced IL-1β on photoreceptor survival we injected Wistar rats with either PBS (n=4 rats) or IL-1Ra (n=6 rats) (subcutaneously 1mg/kg/day and intravitrealy and 5µL of a 150 mg/mL solution at d1 and d3 to ensure penetration of a possibly intact blood retinal barrier in the eye). Control animals that were raised under normal light conditions revealed no signs of toxicity at this dosage (data not shown). At 7d, histology of control (Fig. 4E) light-exposed PBS treated (Fig. 4F) and light-exposed IL-1Ra treated rats (Fig. 4G) revealed significant thinning of the outer nuclear layer (ONL) in both groups. Quantification of rows of nuclei at different distances from the optic nerve measured from the inferior pole to the superior pole revealed no differences in photoreceptor cell loss throughout the retina (Fig. 4H).

IL-1Ra does not affect photoreceptor survival in degeneration prone CX3CR1$^{-/-}$ mice.

We have previously shown, that the T280M polymorphism of *CX3CR1* leads to dysfunctional CX3CR1 and is associated with AMD. Furthermore, *Cx3cr1*$^{-/-}$ mice develop spontaneous photoreceptor degeneration by the age of 18 months and exaggerated photoreceptor degeneration in light-induced models [19, 20]. Here, we analyzed the expression and impact of IL-1β in the *Cx3cr1*$^{-/-}$ model of AMD. First, we analyzed IL-1β expression in C57Bl6 *Cx3cr1*$^{+/+}$ and C57Bl6 *Cx3cr1*$^{-/-}$ mice at 3 and 18 months of age (Fig. 5A n=4 eyes/group). IL-1β protein was significantly increased in 18-month-old C57Bl6 *Cx3cr1*$^{-/-}$ mice compared to *Cx3cr1*$^{+/+}$ mice. Similarly, 4500lux light-exposed BALB *Cx3cr1*$^{+/+}$ and BALB *Cx3cr1*$^{-/-}$ animals revealed a significant increase of IL-1β protein 24h after onset of the light injury compared to normal light-raised *Cx3cr1*$^{+/+}$ and *Cx3cr1*$^{-/-}$ mice (Fig. 5B; n=4 eyes/group). Interestingly, the basal level of IL-1β expression was more important in young C57BL6 mice than in BALB mice. The increase of IL-1β protein levels in light-induced-injury in BALB *Cx3cr1*$^{+/+}$ and *Cx3cr1*$^{-/-}$ mice were not different from each other and comparable to 18-month-

old C57Bl6 *Cx3cr1*$^{-/-}$ mice. To evaluate if IL-1β plays a role in photoreceptor degeneration in the context of CX3CR1 deficiency, we exposed two-month-old BALB *Cx3cr1*$^{-/-}$ mice before significant spontaneous degeneration was observed to light-induced degeneration [19] (Fig. 5C). PBS (n=4 mice) or IL-1Ra (n=6 mice) were injected subcutaneously 1mg/kg/day and intravitrealy (2µL of a 150 mg/mL solution) at d1 and d3 to ensure penetration of a possibly intact blood retinal barrier in the eye. Saline-treated controls (n=6 mice; Fig. 5D) degenerated similarly to IL-1Ra treated *Cx3cr1*$^{-/-}$ mice (Fig. 5E; n=6 mice) and no significant difference could be observed throughout the retina (Fig. 5F). Control *Cx3cr1*$^{-/-}$ animals that were raised under normal light conditions and treated with intravitreal and subcutaneous PBS or IL-1Ra revealed no signs of toxicity at this dosage (Fig. 5G).

Discussion

We demonstrate that IL-1β expression rapidly increases in laser- and light-induced injury. The expression in laser-induced injury shows rapid induction that lasts for about two days (Fig. 1). Immunohistochemistry at 10h localizes IL-1β mainly to infiltrating neutrophils, as demonstrated by a positive Ly6G co-staining (specific for neutrophils and not expressed on monocytes [21]) and negative Iba1 co-staining (specifically expressed on macrophages and microglial cells [22]) (Fig. 1). The identity of IL-1β expressing cells was further confirmed by their multilobulated nuclei (data not shown) and is corroborated by the IL-1β expression kinetics that coincide with early neutrophil recruitment in CNV [23]. The majority of IL-1β expressing cells in our experiments were Ly6G positive neutrophils and the contribution of Iba1 positive macrophages/microglia was negligible. Nevertheless, occasional IL-1β staining was also detected in RPE cells adjacent to the laser injury. Activated polymorphonuclear neutrophils have been shown to express IL-1β [24] and our data shows that these cells are the predominant source of IL-1β after laser injury in the mouse. IL-1β is expressed in CD68 positive cells in human neovascular membranes which were interpreted to be macrophages [11].

Although macrophages can express CD68, it has recently been shown that the main CD68 expressing cells in the injured central nervous system are neutrophils [25]. It is possible that neutrophils participate in IL-1β in AMD. Studies using more specific markers of neutrophils and macrophages are needed to determine the cell types expressing IL-1β in the human disease.

IL-1β inhibition by recombinant IL-1Ra significantly reduced the development of subretinal neovascularization in the mouse model of laser-induced subretinal neovascularization (Fig. 2). Our results corroborate an earlier report that IL-1Ra inhibits neovascularization in a rat model [14, 16].

Neutrophils and macrophages promote CNV [23, 26-28]; IL-1Ra has been shown to inhibit macrophage recruitment to the atherosclerotic plaque in a mouse model [2]. We therefore tested the influence of IL-1Ra treatment on neutrophil and macrophage recruitment to the laser-injury site as a possible mechanism of CNV inhibition (Fig. 2). Interestingly, IL-1Ra treatment significantly increased the number of Ly6G positive neutrophils at 10h, suggesting a possible IL-1β dependent negative feedback in neutrophil recruitment. No inhibition of macrophage/microglial cell recruitment was observed in IL-1Ra treated mice after laser injury. Therefore IL-1Ra-dependent inhibition of CNV is unlikely to be mediated by an inhibitory effect on the macrophage recruitment.

IL-1β exerts its activity by acting on the IL-1 receptor I (IL-1RI) [1]. Vascular endothelium, which constitutively expresses IL-1RI [17], releases IL6 [17] and microparticles [18] upon IL-1β stimulation. We show that collagen IV expressing vascular endothelial cells in CNV express IL-1RI *in vivo* (Fig. 3). To evaluate a direct effect of IL-1β on vascular sprouting independently of inflammatory recruitment, we submitted aortic rings to IL-1β *in vitro* (Fig. 3). We show that IL-1β significantly induces vascular sprouting of aortic rings after 24 hours

of IL-1β exposure. This difference was maintained for up to 72 hours of IL-1β incubation. A previous study has shown an inhibitory effect of IL-1β on vascular sprouting in the aortic ring assay at ten days [29]. In our study, aortic rings became unviable after 7 days in culture and we could therefore not corroborate or contradict these findings. IL-1β has been suggested to induce angiogenesis via a sustained VEGF induction in fibroblasts [30]. In our *in vivo* experiments we did not detect a sustained VEGF induction following the IL-1β induction in laser-injured eyes. Furthermore, vascular sprouting of aortic rings simultaneously incubated with IL-1β and the soluble VEGFR1 did not differ from rings incubated only with IL-1β, suggesting that the proangiogenic effect is independent of VEGF. Previous studies have shown that IL-1β directly induces IL-6 [17] and microparticles release from the vascular endothelium [31] and that IL-1β inhibitor CK112 and CK116 inhibit HUVEC cell proliferation [16]. The proangiogenic effect of IL-1β we observed *in vivo* and *in vitro* might therefore be a direct effect of endothelial cell proliferation.

In AMD, subretinal neovascularization occurs simultaneously with retinal degeneration. To make anti-IL-1β therapy a viable option, the influence of IL-1β on photoreceptors has to be evaluated *in vivo*. Previous reports have shown that IL-1β develops neurotoxic properties in the brain [5, 13, 32]. However in the retina IL-1β was shown to inhibit ganglion cell death [6] and IL-1β substitution saved photoreceptors in light-induced models [7] and genetic retinal degeneration [8]. Here we show that IL-1β is significantly induced in the light-induced model in albino rats (Fig. 4D) and in albino mice (Fig. 5B). To test the effect of IL-1β inhibition on retinal degeneration, we treated the laser-injured C57Bl6 mice and light-exposed rats with IL-1Ra at dosages that were sufficient to significantly inhibit CNV in mice (see above) and rats [14]. Interestingly, IL-1Ra at a dose that significantly inhibits CNV does not exacerbate photoreceptor degeneration *in vivo* (Fig. 4).

We have previously shown that a dysfunctional CX3CR1 is associated with AMD. *Cx3cr1*$^{-/-}$ mice develop spontaneous subretinal macrophage accumulation that is associated with photoreceptor degeneration by the age of 18 months [19]. Similarly, these mice develop exaggerated photoreceptor degeneration in light-induced models [20]. We analyzed the expression and impact of IL-1β in the *Cx3cr1*$^{-/-}$ model of AMD. IL-1β significantly increases with age in *Cx3cr1*$^{-/-}$ animals. This increase coincides with the observed subretinal inflammation in these animals [19] and might reflect IL-1β produced by subretinal inflammatory cells. In light-induced injury BALB *Cx3cr1*$^{+/+}$ and *Cx3cr1*$^{-/-}$ mice, IL-1β was rapidly induced, similar to the rat and to laser-induced CNV. As shown in the mouse CNV model and rat models (Fig. 4), IL-1Ra substitution in control and light-exposed *Cx3cr1*$^{-/-}$ mice in dosages sufficient to significantly inhibit CNV had no deleterious effect on photoreceptor survival (Fig. 5). Although exogenous IL-1β substitution has been shown to protect photoreceptor degeneration in a light-induced model [7], its inhibition does not exacerbate degeneration in wild type mice (CNV model) or light-exposed rats, nor in degeneration-prone *Cx3cr1*$^{-/-}$ mice. The apparent contradiction of IL-1β mediated photoreceptor protection and the absence of an exacerbating effect of IL-1Ra might be explained by different IL-1β concentrations. While the neuroprotective effect of LaVail et al. [7] and Whiteley et al. [8] are found at concentrations of 0,5-5μg of IL-1β per eye, the maximum IL-1β concentration we measured with age- and light-induced injury correspond to approximately 50 pg/eye. The endogeneously-produced amount of IL-1β might therefore be well below neuroprotective concentrations and therefore have no effect on photoreceptor survival.

In summary, IL-1β is induced by laser- and light-induced injury. We confirm that IL-1Ra substitution efficiently inhibits the development of CNV in the mouse and show that it has no deleterious effect on photoreceptor survival. Taken together, our data suggests that IL-1Ra is a safe alternative for the treatment of neovascular AMD.

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Figure Legends:

Fig.1 : **IL-1β expression in laser-induced neovascularization.**

IL-1β rtPCR in retina (A) and choroid (B) and VEGF rtPCR in retina (C) and choroid (D) at different time points in laser-induced CNV in the eye cup of C57Bl6 mice (control n=4 eyes, 1h n=8 eyes, 6h n=7 eyes, 20h n=5 eyes, 72h n=4 eyes; one way Anova * p=0,03 for A and B). Representative micrographs of immunohistochemistry of uninjured (E-H), 10h (I-L), and 72h (M-P) laser-injured choroid/RPE flatmounts (n=4 mice per time point). IL-1β staining (green staining E, I and M), Ly6G staining (red staining F, J and N), Iba1 staining (blue stainig G, K and O) merged photographs (H, L and P). Scale bar E to P = 50μm. arrow heads: Iba1 positive cells.

Fig.2: **IL-1Ra inhibits choroidal neovascularization independently of macrophage recruitment.**

Photographs of isolectin (red) stained and fluorescein dextran perfused (green) RPE/choroidal flatmounts of PBS treated (A; n=4) and Il-1Ra treated (B; n=5) C57Bl6 mice 14 days after laser injury. Quantification of fluorescein positive area (C; Mann Whitney *p < 0.0001). Quantification of RPE lesion size of PBS (n=4 mice) and Il-1Ra (n=4 mice) treated mice at

10h after laser-impact (D). Representative photographs of Ly6G (red) and Iba1 (green) stained RPE/choroidal flatmounts of the border of the injured area of saline (E and H; n=3 mice/group) and Il-1Ra treated (F and I; n=3mice/group) mice 10h (E and F) and 72h (H and I) after laser injury. Quantification of Ly6G (G) and Iba1 (J) positive cells / impact at different time points (F; Mann Whitney *p= 0,14). Scale bar A, B, E, F, H and I = 50μm

Fig. 3: **IL-1β induces vascular sprouting in aortic rings**

Confocal microscopy image of IL-1RI (green staining A) and Collagen IV immunohistochemistry (red staining B, merge C) of the laser-injury site at d5 (n=3 mice) of C57Bl6 mice. Photographs of aortic rings at day 4, 24 hours after incubation with control (D), IL-1β (E), IL-1Ra (F) or IL-1β and IL-1Ra (G). (H) Measurements of the area of vascular sprouting in the presence of control (n=6), IL-1β (n=12), IL-1Ra (n=6) or IL-1β and IL-1Ra (n=6). (I) Measurements of the area of vascular sprouting in the presence of control (n=6), IL-1β (n=12), soluble VEGFR1 (n=5) (VEGFR1s) or IL-1β and VEGFR1s (n=6). *p < 0.05 one way Anova. Scale bar: A-C = 50μm; D-G = 350μm

Fig. 4: **IL-1 and light and laser-induced injury**

Histology of the photoreceptor cell layer at 100μm of the laser injury in PBS (A n=3 mice) and IL-1Ra (B n=4 mice) treated C57Bl6 mice. Quantification of number of rows of photoreceptor nuclei at increasing distance from the injury site in PBS and IL-1Ra treated animals (C). rtPCR of IL-1β expression in Wistar eye cup mRNA extracts (D; control n=4, 1h n=5, 12h n=5). Normal light-raised Wistar rats (E); light injured PBS treated (F; n=4) and light-injured IL-1Ra treated (G; n=6) eyes. Quantification of number of rows of photoreceptor nuclei at different distances from the optic nerve measured on oriented sections, crossing the inferior pole, optic nerve and superior pole (H). Scale bar : A, B = 50μm, E-G= 20μm. ONL:

outer nuclear layer; INL: inner nuclear layer; RPE: retinal pigment epithelium; CHO: Choroid.

Fig.5: **IL-1β and *Cx3cr1*$^{-/-}$ AMD model and light-induced injury *Cx3cr1*$^{-/-}$mice**

IL-1β Elisa of eye cups of 3 and 18 month old C57Bl6 *Cx3cr1*$^{+/+}$ and C57Bl6 *Cx3cr1*$^{-/-}$ animals (A; n=5 to 6). IL-1β Elisa of eye cups of control and light exposed 8 week old BALB *Cx3cr1*$^{+/+}$ (wildtype) and BALB *Cx3cr1*$^{-/-}$ eye cups (B; n=4 eyes per group). Normal light raised BALB *Cx3cr1*$^{-/-}$ (C); light-injured PBS treated BALB *Cx3cr1*$^{-/-}$ (D; n=4) and light-injured IL-1Ra treated BALB *Cx3cr1*$^{-/-}$ (E; n=6) eyes. Quantification of number of rows of photoreceptor nuclei at different distances from the optic nerve on oriented sections of PBS and IL-1Ra treated light-injured 2 months old BALB *Cx3cr1*$^{-/-}$ mice (F). Quantification of number of rows of photoreceptor nuclei at different distances from the optic nerve on oriented sections of PBS and IL-1Ra treated control BALB *Cx3cr1*$^{-/-}$ mice (G). Scale bar = 25μm. ONL: outer nuclear layer; INL: inner nuclear layer; RPE: retinal pigment epithelium; RGC: retinal ganglion cells.

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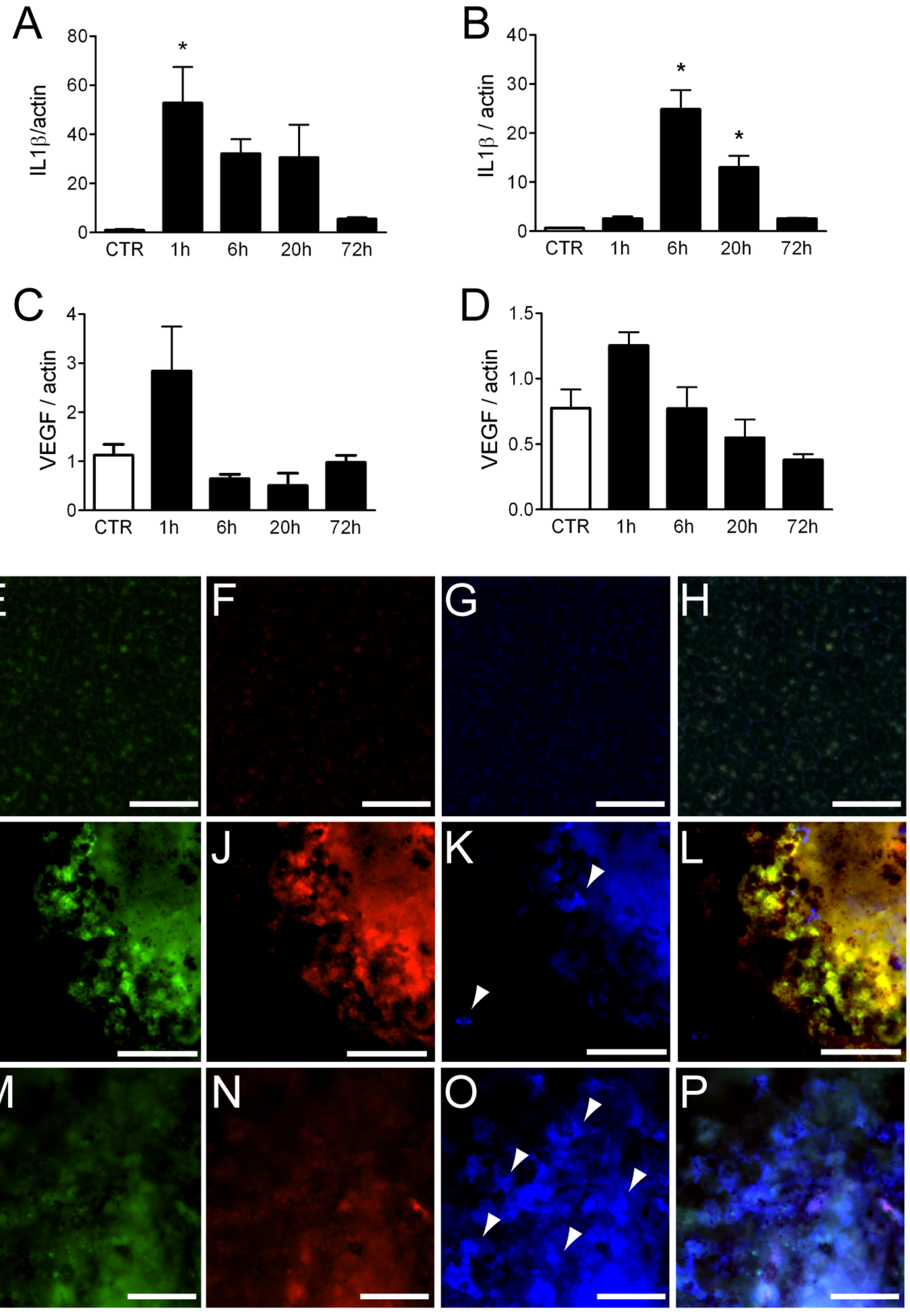
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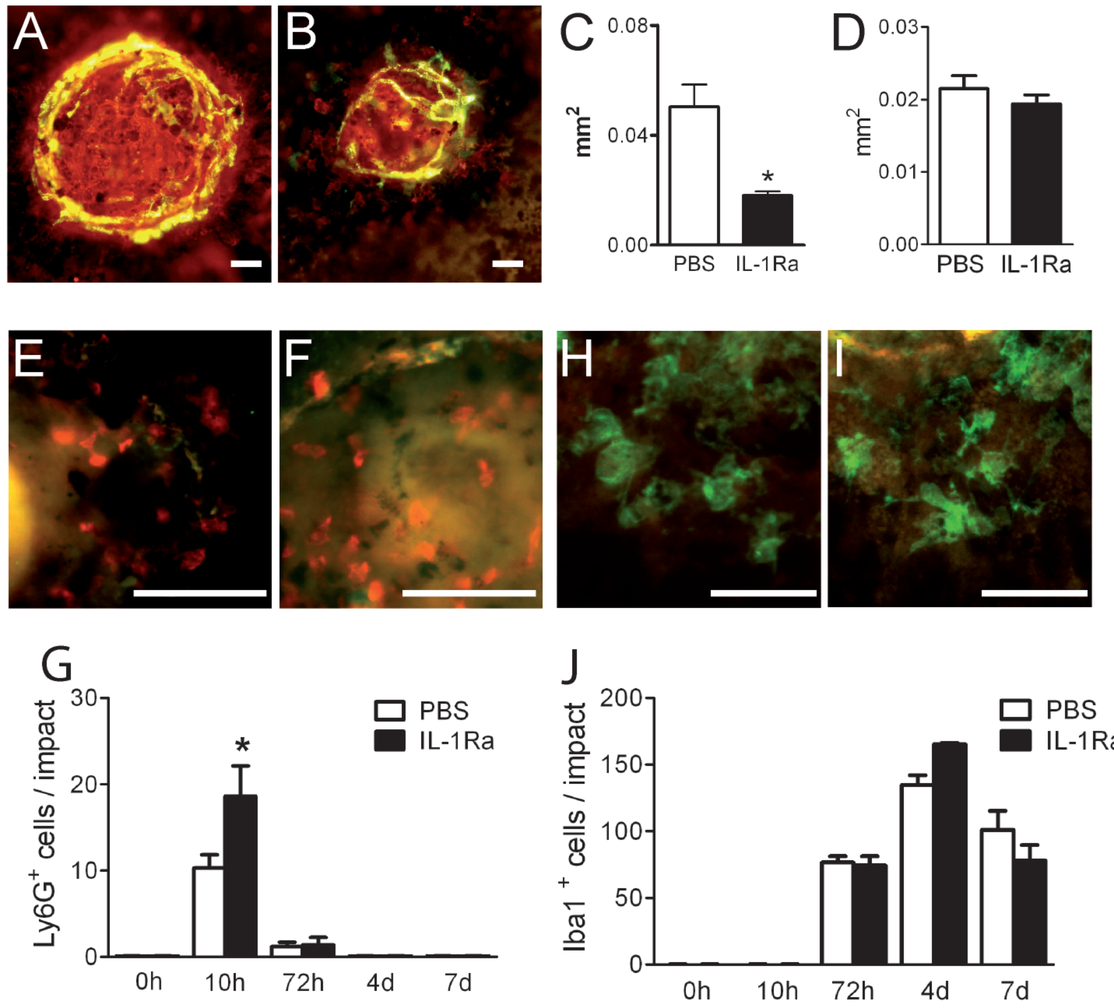
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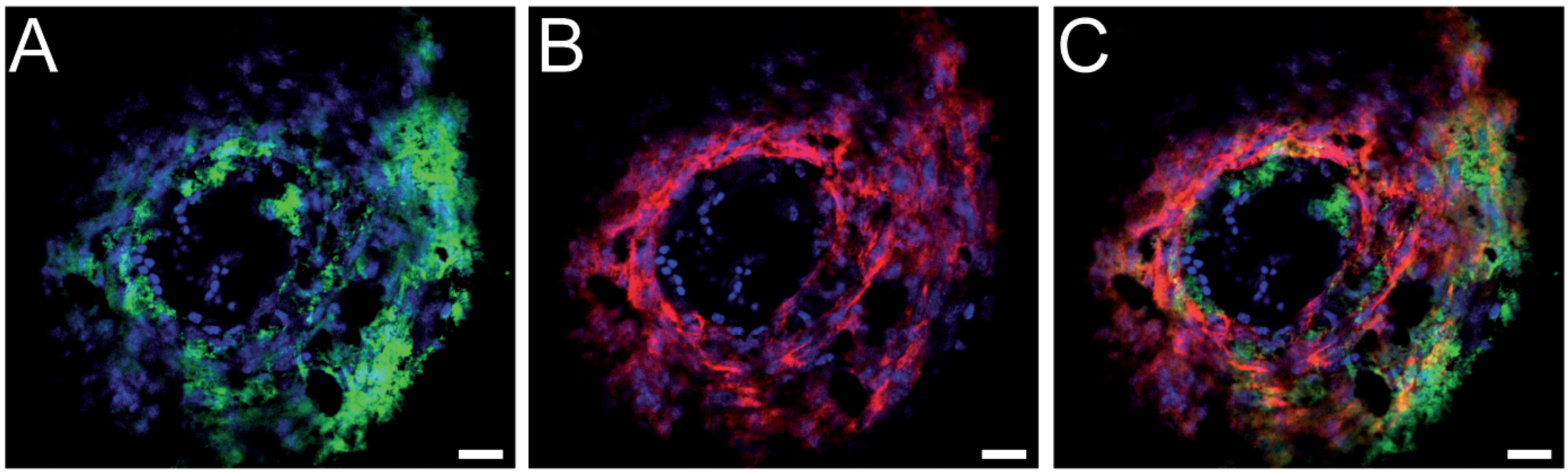
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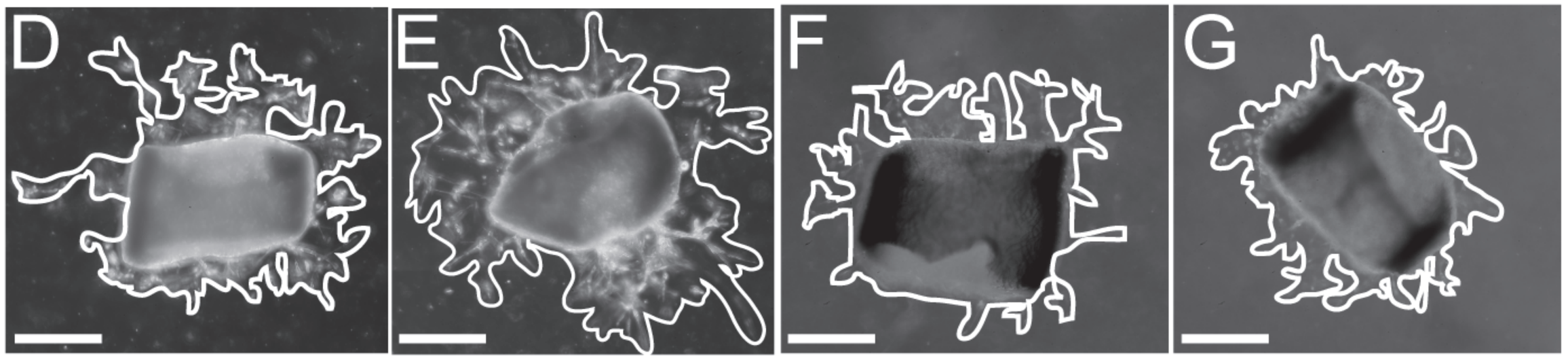
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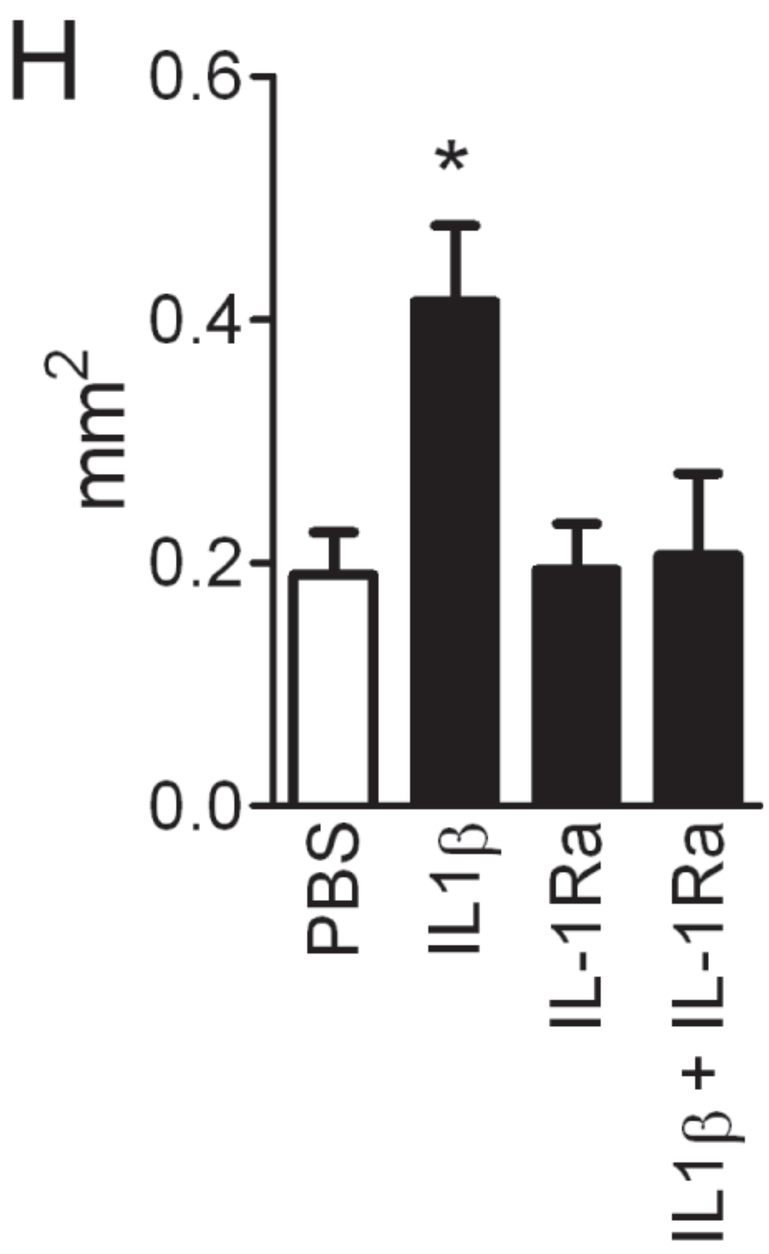
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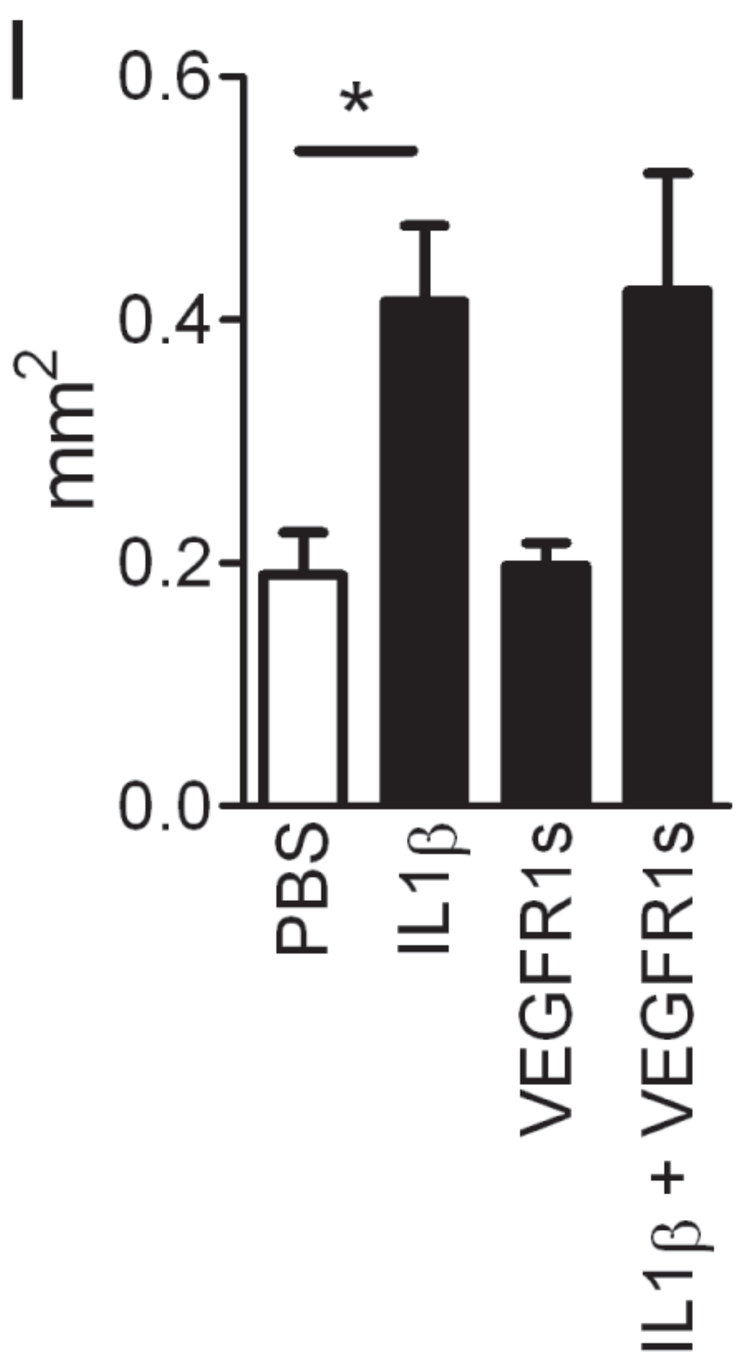
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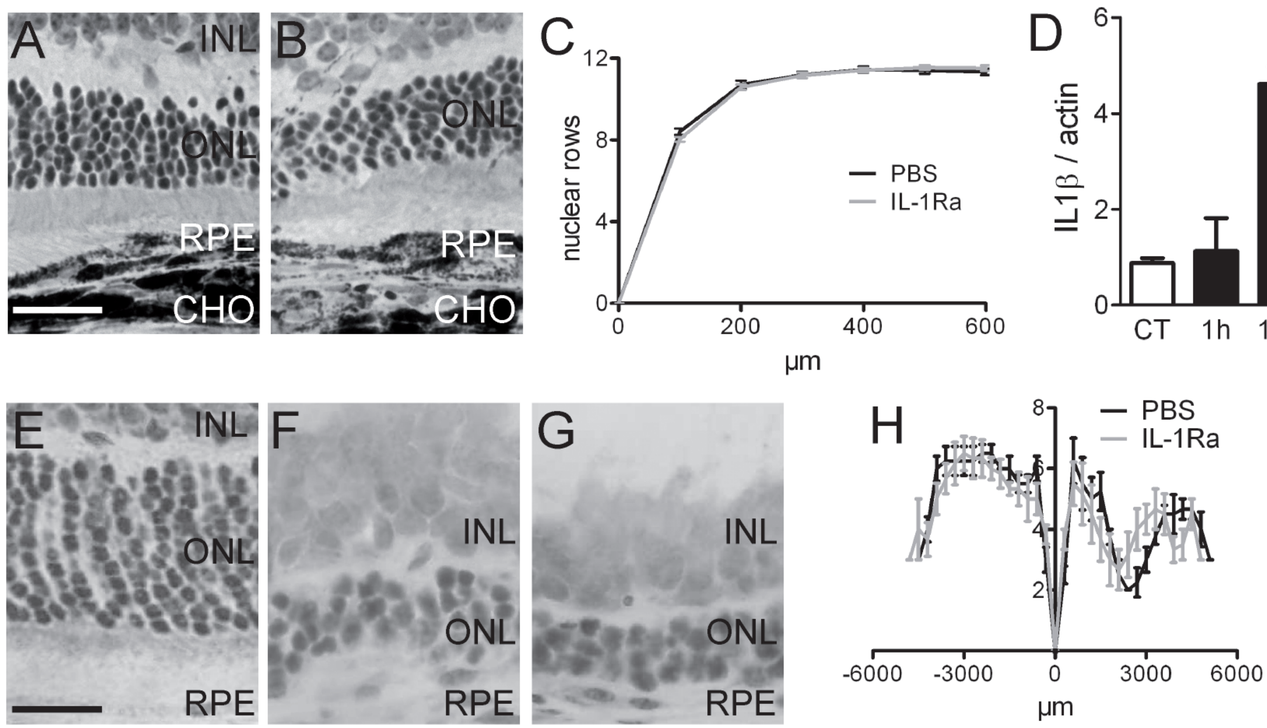
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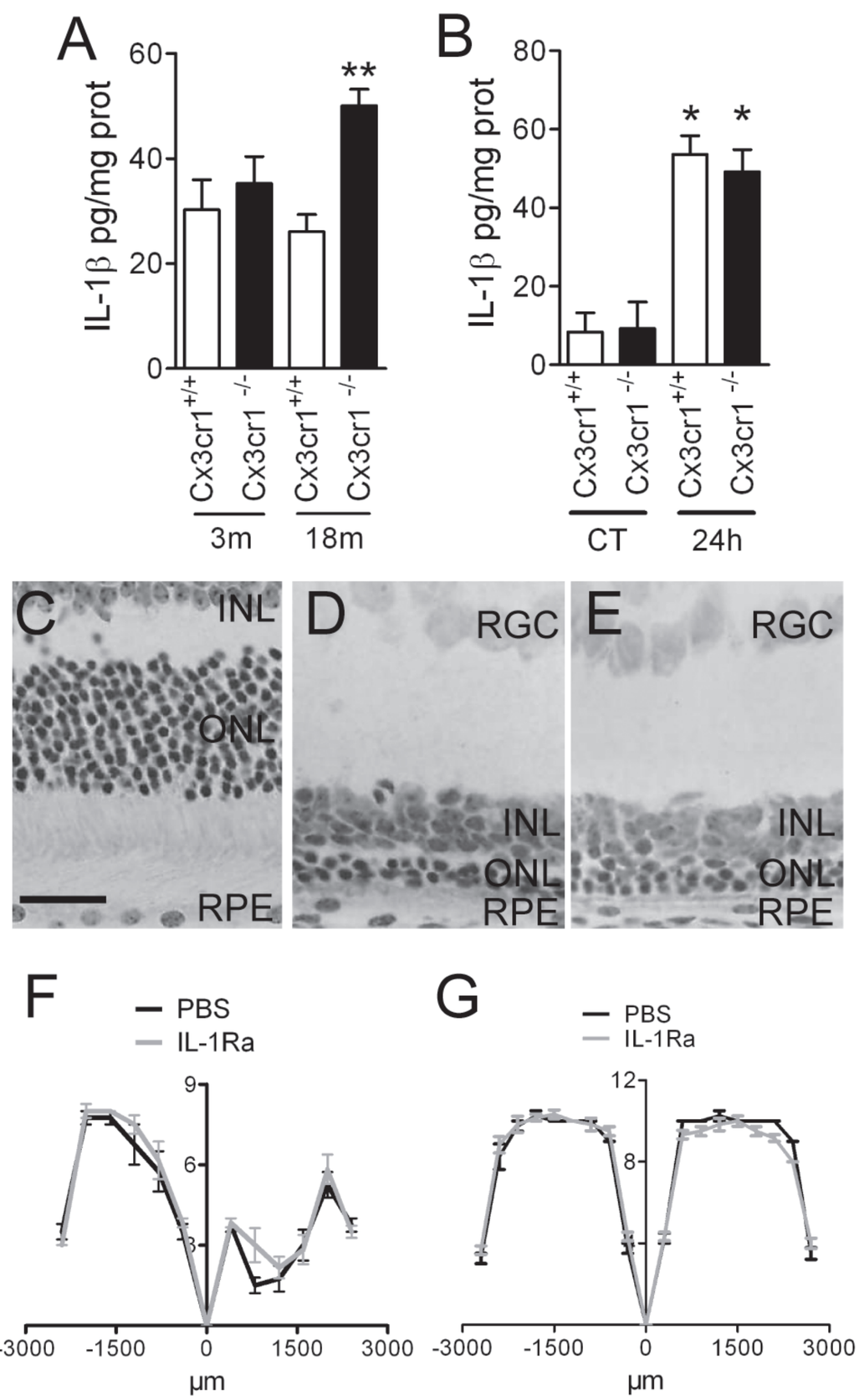
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Figure 5