Interleukin-33 regulates tissue remodelling and inhibits angiogenesis in the eye

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Conflict of interest

The authors declare that they have no conflict of interest.

Abstract

Age-related macular degeneration (AMD) is the leading cause of central vision loss worldwide. Loss of retinal pigment epithelium (RPE) is a major pathological hallmark in AMD with or without pathological neovascularization. Although activation of the immune system is implicated in disease progression, pathological pathways remain diverse and unclear. Here, we report an unexpected protective role of a pro-inflammatory cytokine, interleukin-33 (IL-33) in ocular angiogenesis. IL-33 and its receptor (ST2) are expressed constitutively in human and murine retina and choroid. When RPE was activated, IL-33 expression was markedly elevated *in vitro*. We found that IL-33 regulated tissue remodelling by attenuating wound-healing responses, including reduction in migration of choroidal fibroblasts and retinal microvascular endothelial cells, and inhibition of collagen gel contraction. *In vivo*, local administration of recombinant IL-33 inhibited murine choroidal neovascularization (CNV) formation, a surrogate of human neovascular AMD, and this effect was ST2-dependent. Collectively, these data demonstrate IL-33 as a potential immunotherapy and distinguishes pathways for subverting AMD pathology.

Keywords: IL-33, AMD, RPE, angiogenesis, wound healing

Introduction

Age-related macular degeneration (AMD) is the leading cause of visual loss in developed countries, affecting 1 in 10 people older than 50 years (http://www.amdalliance.org). With a global increase in the aging population, the numbers suffering with this progressive disease are expected to increase in 2050 by 50% [1]. AMD is a progressive, polygenic and multifactorial disease leading to a clinico-pathological spectrum of cell loss and atrophy and neovascularisation. Although current anti-vascular endothelial growth factor (VEGF) therapies have revolutionized treatment for neovascular (n)AMD, we are treating late in disease, only after the insidious progression of degeneration has ensued. Tailoring therapies to treat early is desirable to overcome the potential amplification of degenerative pathways, including neovascularisation, and in such cases offer alternatives for those unresponsive to anti-VEGF agents [2].

Altered immune responses are recognized as integral to the pathogenesis of AMD [3]. The NLRP3 inflammasome has been reported to monitor cell stress through pattern recognition receptors (e.g. Toll-like receptors (TLRs)), activating inflammatory caspases [4]. The activation of the NLRP3 inflammasome is almost certainly a protective response initially, providing a rapid response to danger in order to preserve tissue function and integrity. The corollary is that inflammasome activation may also cause tissue damage. So why patients convert from early or intermediate AMD (clinically manifest as drusen and RPE changes) to advanced AMD (including choroidal neovascularization) is purported to be as a switch from the ageing homeostatic regulatory response [5] to a pathological inflammatory response. For example, recent studies have shown that the NLRP3-inflammasome can 'sense' drusen isolated from human AMD donor eyes which is capable of liberating active IL-1β and Interleukin (IL)-18 production. IL-18 however has been shown to protect against the development of choroidal neovascularization (CNV) [6].

Given the controversy over the role of inflammasome activation and the role for adjuvant immunotherapy in AMD treatment, we have investigated the role of the type 2 IL-1 family member, interleukin-33 (IL-33) [7], which is unique as is active without caspase-1 cleavage and does not require inflammasome activation for secretion and bioactivity [8]. IL-33 is released following cellular damage [9] and biomechanical overload [10], behaving as an 'alarmin' [11] that signals through the heterodimeric receptor consisting of ST2 and IL-1R accessory protein [12]. The membrane form of ST2 (IL1RL1) [13], is expressed on many immune cells [14, 15]. An alternative spliced soluble form (sST2) acts as a decoy receptor of IL-33 [16]. In experimental autoimmune uveitis (EAU), IL-33 attenuates disease severity [17]. However, IL-33 triggers an inflammatory response, recruiting monocytes, contributing to photoreceptor loss in a photoxic retinal model of degeneration [18] and this infers a pathogenic role of endogenous IL-33 and offers an *a priori* rationale for neutralizing IL-33 to reduce myeloid cell accumulation as a possible intervention.

On the basis of the emerging role of IL-33 in inflammatory disorders [19, 20] and the controversy over its role, we hypothesized that IL-33/ST2 signalling in absence of overt cell death regulates tissue responses in neovascular AMD disease pathology. We showed that IL-33 subverts angiogenesis, via direct inhibition of fibroblasts and endothelial cells that express high levels of ST2. We further showed that recombinant IL-33 protects against CNV development. Our data provides new insights into the regulation of angiogenesis by IL-33, and is contrary to previous data.

Materials and Methods

Cell Culture. The murine B6-RPE07, and human ARPE-19, cell lines were cultured in DMEM medium supplemented with 10% heat-inactivated fetal calf serum, 2% L-glutamine, 1 mM sodium pyruvate, 60 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. Primary human retinal and cerebral microvascular endothelial cells (HRMECs and HCMECs) were purchased from a commercial source (Innoprot, Derio-Bizkaia, Spain). HRMECs were isolated from healthy retinal tissue and were cryopreserved at passage one and delivered to our laboratory frozen. The vendor had characterized cells by immunofluorescent methods with antibodies directed against VWF/Factor VIII and CD31 (P-CAM) and by uptake of DiI-Ac-LDL. Human choroidal fibroblasts were purchased from SciencCell Research laboratories (Carlsbad, CA, USA), and cultured in fibroblast medium (SciencCell Research laboratories, Carlsbad, CA, USA).

Mice and **in vivo** *experimental procedures.* C57BL/6J mice were purchased from Charles River Laboratories, UK. ST2 knock-out (*St2-/-*) C57BL/6 mice (backcrossed for 10 generations) were kindly provided by C. Emanueli (School of Clinical Sciences, Bristol Heart Institute, University of Bristol), and were generated as described earlier [20]. IL-33 knock-out (*Il-33-/-*) mice were generated as described earlier [21]. *Il33-/-* mice were backcrossed for 6 generations to C57/Bl6J mice to remove the rd8 mutation. All mice were used at 8 –10 week of age and maintained in the animal house facilities of the University of Bristol, according to the Home Office Regulations. Treatment of the animals conformed to the Association for Research in Vision and Ophthalmology (ARVO) statement.

CNV was induced by laser photocoagulation in mice; four laser spots (power 200 mW, duration 75 ms, spot size 75 μm) were delivered to the posterior retina using an OculightSlx Krypton Red Laser system (IRIS Medical, Iridex, Mountain View, CA). Local administration of IL-33 (doses in 2 μl of normal saline) or vehicle control was performed by intravitreal injection using a 33-

gauge hypodermic needle. Optical coherence tomography (OCT) was performed using the Micron IV (Phoenix Research Laboratories, CA) to evaluate lesion size and retinal thickness.

Tissue preparation and immunofluorescence staining. To prepare RPE/Choroid whole-mounts, enucleated eyes were initially fixed in 2% PFA overnight. Eyes were dissected, and RPE/choroidal tissues were blocked and permeabilised in 5% BSA with 0.3% Triton X-100 in PBS for 2 h. For evaluation of CNV formation, the neovascular membrane was stained with biotin-conjugated isolectin B4 (IB4, Sigma-Aldrich, L2140; 1:100), followed by incubation with Rhodamine Red-X-labelled streptavidin (Jackson Immuno Research Laboratories, 016-290-084; 1:400). The CNV volume was measured using a series of Z-stack images (from the surface to the deepest focal plane) using Volocity® Image Analysis Software 6.0. TMR Red-dUTP TUNEL (Roche Diagnostics, Burgess Hill, UK) staining of DNA breaks was performed on 10μm cryosections of enucleated eyes from treated mice, according to the manufacturer's protocol.

Histology and immunohistochemistry. Eyes from treated mice were enucleated at various time points and cryosections prepared. For immunostaining, sections were fixed in acetone, and blocked in 5% donkey serum, 3% BSA and 0.3% Triton X-100 in PBS. Sections were incubated with primary antibodies; anti-IL-33 Nessy-1 (Enzo Life Sciences, ALX-804-840; 1:1000), ST2 (ProSci, 3363; 1:1000), Iba1 (Sigma, L2140; 1:100), IL1RAP (Abcam, ab8110; 1:1000), c-kit (Biolegend, 105816; 1:200), vimentin (Abcam, ab92547; 1:100) and tryptase (Bioworld, BS7353; 1:400) overnight at 4 °C. Sections were incubated with appropriate secondary antibody (Life Technologies, Paisley, UK) donkey anti-rabbit conjugated to AlexaFluor-488 or AlexaFluor-594, or donkey anti-rat AlexaFluor-594 (Life Technologies, at dilution 1:400) or goat anti-rabbit Cy2 (Abcam, Cambridge, UK) . DAPI (Vector laboratories, Peterborough, UK) was used to show nuclei in sections.

Human ocular samples. Healthy adult (age 18-65 years) human *post mortem* eyes from anonymous donors collected by Moorfields Eye Bank for corneal transplantation were fixed in 10% Formalin and embedded in paraffin wax. This work was ethically approved by the NHS Research Ethics Committee 10/H0106/57-14ETR41.

Recombinant proteins. Recombinant mouse IL-33 (ALX-522-101-C010, Enzo Life Sciences) and human IL-33 (ALX-522-098-C010, Enzo Life Sciences) were purchased from Enzo Life Sciences Ltd (Exeter, UK). The truncated form of human IL-33 was generated as described previously [22] and kindly gifted by Prof. Seamus Martin (Trinity College Dublin).

Proliferation and functional assays. Cell numbers were assessed using an MTT assay, with absorbance measured at 570 nm. For migration assays, cells were seeded at 1×10^4 cells per culture-insert and incubated overnight. Inserts were removed before addition of fresh medium with treatment or vehicle. Phase contrast images were taken at 0 h and 4 h post-treatment using a Wide-field microscope (Leica DM16000). The whole area of gaps was quantified by ImageJ 1.46r (National Institutes of Health, USA). For Boyden chamber assays, cells were cultured in EBM medium with treatment or vehicle for 24 h before the supernatant was transferred to the lower chamber of 24-well format Transwell (Corning, UK) for 16 h incubation. The membranes of the upper chamber were fixed with 4% PFA for 20 min followed by staining with DAPI for cell counting using confocal microscopy.

Cytokine measurements. IL-6 levels were measured with an ELISA and following the manufacturer's instructions (BD Biosciences, 550799, San Jose, CA, USA). For Western blots, cells lysates were separated by SDS-PAGE gel electrophoresis, and proteins transferred to a nitrocellulose membrane. Following blocking in 5% milk/TBS/Tween-20, the membrane was subjected to analysis using the anti-IL-33 Nessy-1 antibody (Enzo Life Sciences, ALX-804-840;

1:1000), the ST2 antibody (ProSci, 3363; 1:1000) and β-actin antibody (Cell Signaling, 4970; 1:1000).

Proteins were detected with HRP-conjugated polyclonal anti-mouse IgG and visualized using a chemiluminescent method (GE Healthcare Life Sciences). RNA was extracted using RNeasy Mini Kit (Qiagen, Hamburg, Germany) before cDNA synthesis using the ImProm-IITM Reverse Transcription System (Promega, Fitchburg, WI). cDNA was amplified using the Power SYBR® Green PCR Master Mix Reagent on a StepOne™ Applied Biosystems Real-Time PCR System. Relative expression was calculated using *Actb* and *RNA18S* as reference transcripts for mouse and human tissues, respectively.

Statistical analysis. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test or ANOVA as indicated for comparison between groups. *P* < 0.05 was considered significant.

Results

IL-33 expression is induced in human and murine RPE cells.

The retinal pigment epithelium (RPE) is central to retinal homeostasis. We first investigated the expression of IL-33 and ST2 in established human and murine RPE cell lines [23, 24]. Both human (ARPE-19) and mouse (B6RPE-07) RPE cells were stimulated with Toll-like receptor (TLR) agonists, including TLR3 and TLR4 agonists (poly(I:C) and LPS), to simulate activation of innate immune response and inflammasome [4]. Our data show that murine and human RPE cells constitutively expressed IL-33 and its cognate receptor ST2, at both mRNA and protein levels (Figure 1A-C). TLR-activation of RPE cells increased expression of *IL-33* and *ST2L (IL1RL1)* mRNA in a dose-dependent manner, although the effect of poly(I:C) was not as

profound as LPS stimulation (Figure 1A,B). The effect of TLR agonists on IL-33 and ST2 induction was confirmed at protein level by Western blot (Figure 1C). These results demonstrate that IL-33 expression is upregulated in the RPE cells following inflammatory stimulus.

IL-33 and its receptor ST2 are expressed in murine and human retina.

To establish expression patterns of IL-33 and ST2 *in vivo*, we examined normal eye tissues from naïve C57BL/6J mice. Immuno-staining of adult murine retina demonstrated that IL-33 was expressed by cells in the inner nuclear layer (INL) and RPE, with expression localized to the nuclei of Müller glial cells and retinal microvascular endothelial cells respectively (Figure 1D). In the developing mouse retina, there is a gradual but marked increase of IL-33 expression from post-natal day 4 to post-natal day 10; the ciliary body was similarly enriched (Supplementary Figure S1A-C).

Immunostaining of choroid from WT animals, demonstrated ST2 expression in RPE (phalloidin stained), as well as choroidal mast cells (c-kit+) and choroidal fibroblasts (vimentin +) (Figure 1E). Furthermore, immunohistochemical analysis of murine retina demonstrated that IL-1 receptor accessory protein (IL1RAP) was expressed by cells in the retina and RPE (Figure 1F). We next sought to determine whether similar expression profiles were present in healthy human ocular tissue. This revealed robust expression of IL-33, ST2 and IL1RAP in the nerve fibre layer of the inner retina and the RPE (Figure 1G). Furthermore, IL-33 is known to be stored within red blood cells [25] which may explain the strong IL-33 staining within choroidal vessels (Supplementary Figure S2).

Retinal endothelial cells are a direct target of IL-33.

Although IL-33 is expressed in human choroidal vascular endothelium, IL-33 is down-regulated at the onset of angiogenesis [26], suggesting a homeostatic role of IL-33 regulation of tissue vasculature. To investigate further, primary human retinal microvascular endothelial cells

(HRMECs) were cultured in the presence of IL-33. First, we found that HRMECs respond to IL-33, as evidenced by the increased production of downstream cytokine IL-6, as a marker of their activation, when cultured with IL-33 (Figure 2A). Furthermore, HRMECs express *ST2* at high levels, when compared to monocytes (Figure 2B), and soluble ST2 was present in the supernatant of HRMECs (Supplementary Figure S3A). More importantly, ST2 is almost completely absent in other tissue-derived endothelial cells, such as human cerebral microvascular endothelial cells (HCMECs) (Figure 2B). As shown in Figure 2A-B, HCMECs do not express IL-33 receptor and do not respond to IL-33.

Next, we investigated the effect of IL-33 on the migratory ability of HRMECs. Using a modified Boyden chamber, the chemotactic motility of HRMECs was inhibited when incubated with recombinant human IL-33, with the maximal effect at 100ng/mL, although proliferative responses remained unaltered (Figure 2C,D, Supplementary Figure S3B). The inhibitory effect elicited by IL-33 treatment was further demonstrated in a wound-healing assay, in which there was a significant decrease in the migratory ability of HRMECs (Figure 2E). We also assessed the production of matrix metalloproteinase (MMP)-9, and found that HRMECs produced significantly lower levels of *MMP9* in response to IL-33 stimulation (Figure 2F). Furthermore, there was a small but significant increase in tissue inhibitors of metalloproteinases-1 (*TIMP1*) and -2 (*TIMP2*) expression upon IL-33 treatment of HRMECs (Figure 2G). There was no change in the expression of urokinase-type plasminogen activator uPA (*PLAU*) or its receptor uPAR (*PLAUR*) (Figure 2H), as well as vascular endothelial growth factor receptor (VEGFR)-1 (*FLT1*) or VEGFR2 (*KDR*) in response to IL-33 (Supplementary Figure S3C,D,E).

IL-33 regulates the function of human choroidal fibroblasts.

Alteration in choroidal architecture including vascular changes and choroidal stromal thinning accompanying fibrosis, is associated with the RPE atrophy and photoreceptor death as hallmarks of AMD [27]. As choroidal fibroblasts express ST2, we wished to determine if IL-33 could

attenuate responses as observed with its anti-fibrotic and anti-hypertrophic effects in the myocardium [28]. Experiments using human choroidal fibroblasts, demonstrated that cellular migration was significantly inhibited when incubated with IL-33 (Figure 3A). Collagen gel contraction assays have been widely used to evaluate the interaction of fibroblasts with collagenous matrix and their ability to remodel this matrix [29-31]. Treatment of human choroidal fibroblasts with IL-33 significantly inhibited their ability to contract collagen gels by approximately 25% over the 12 h period examined, with no effect on their proliferation (Figure 3B,C). A number of factors including MMPs [32] are recognized to enhance the migration and contraction of three-dimensional collagen gels by fibroblasts. In response to IL-33, the human choroidal fibroblasts expressed significantly lower levels of both *MMP2* and *MMP9* (Figure 3D,E). Furthermore, we found an increase in *TIMP1* and *TIMP2* expression upon IL-33 stimulation of choroidal fibroblasts (Figure 3F), while there was no change in the expression of *uPA*(*PLAU*) or *uPAR*(*PLAUR*) (Figure 3G).

Intravitreal administration of IL-33 attenuates CNV.

To investigate a functional role of IL-33 we explored its action in angiogenesis in context of nAMD using the murine model of laser-induced choroidal neovascularisation (CNV). Groups of mice received an intravitreal injection of recombinant mouse IL-33 immediately following laser induced CNV. Seven days post laser exposure, *in vivo* optical coherence tomography (OCT) assessment and *ex vivo* isolectin-B4 (IB4) staining of RPE-choroidal flat mounts demonstrated that CNV volume was significantly reduced in eyes receiving IL-33 as compared to vehicle control (Figure 4A,B). Furthermore, the IB4-staining revealed that two lower doses of IL-33 (200pg/μl and 1ng/μl) were more effective at attenuating CNV development, while the higher dose of 5ng/μl of IL-33 had no significant effect. This effect is similar to what has been observed previously in a primate model of immunotherapy with AMD [33]. Intravitreal IL-33 at the dose of 200pg/μl decreased choroidal neovascular lesion (CNV) volume up to 50% (Figure

4B,C). This observation was manifest in reduced choroidal neovascular lesions and disease severity (sub-retinal fluid) in IL-33 treated eyes, as demonstrated in OCT images (Figure 4A). Furthermore, the reduction in clinical disease was accompanied by significantly decreased c-kit$^+$ cells, which demonstrated a potentially reduced population of endothelial progenitor cells in the lesions in IL-33 treated eyes compared with control eyes (Supplementary Figure S4A,B). Resident immune cells of the choroid include macrophages and mast cells [34]. In addition to the monocyte/macrophage cell infiltrate following laser treatment, we found tryptase-positive mast cells had infiltrated CNV lesions (Supplementary Figure S4C).

A recent report has suggested that endogenous release of IL-33 triggers recruitment of monocytes contributing to photoreceptor loss[18]. To examine this, RPE/choroidal tissue was collected at three days post laser exposure and intravitreal IL-33 injection, and immunostained for Iba1, a marker for both microglia and monocytes. In the laser-induced CNV model, macrophage numbers peak on day 3 following laser damage [35]. Our results show that Iba1+ cells accumulation is no different between vehicle and IL-33 treated eyes. Furthermore, a 500-fold increase to the therapeutic dose of IL-33 did not influence Iba1+ cell accumulation in RPE/choroid (Supplementary Figure S5A). Furthermore, monocyte recruitment was assessed via flow cytometric analysis of cell infiltrate from the retinas of mice receiving either the low (200pg/l) or high (100ng/l) doses of IL-33. IL-33 treatment did not lead to any significant increase of CD45$^+$/CD11b$^{+/}$Ly6C+ cells (Supplementary Figure S5B).

To examine the safety and tolerability of recombinant IL-33, groups of mice receiving varying doses of IL-33 were clinically monitored using OCT at 3, 5 & 7 days post-injection. Histological and OCT analyses of the retina at day 7, indicated that IL-33 administration did not alter retinal integrity and thickness, or result in cell death (Figure 4D,E, Supplementary Figure

S6A,B). Exogenous administration of recombinant IL-33 has no effect on retinal pigment epithelial (RPE) cell viability *in vitro* (Supplementary Figure S6C).

ST2 is required for IL-33 anti-angiogenic properties.

As exogenous IL-33 suppresses angiogenesis, we asked if endogenous IL-33 is protective. To this end we noted that choroidal lesion volume measurements were more pronounced in *Il33-/-* mice (Figure 4F). These results open a plausible explanation that endogenous IL-33 expression may regulate but although not significantly attenuate CNV development in this model. To confirm that IL-33 effect was direct, we noted that IL-33 treatment did not alter the severity of CNV development in *St2-/-* mice (Figure 4G).

A truncated form of human IL-33 is biologically active in mice.

As mast cells are activated by IL-33 [15], murine bone marrow-derived mast cells (BMMCs) were treated with mouse and human IL-33, and IL-6 and IL-1β mRNA levels were measured to compare their potency. We found that BMMCs respond similarly to both forms of IL-33 (Figure 5A), suggesting that human IL-33 is bioactive in mice.

In vivo, administration of recombinant human IL-33 reduced angiogenesis, with the lower dose of 200pg/µl being the most effective (Figure 5B,C). Treatment with human IL-33 had no effect on human RPE cell viability *in vitro* (Supplementary Figure S6D). Intravitreal administration did not induce cell death or alter retinal integrity or thickness (Figure 5D,E, Supplementary Figure S6E,F).

Discussion

Our data demonstrates a protective role for IL-33 in a murine model of injury-induced CNV. IL-33 is expressed in the human retina and RPE, and IL-33 and its cognate receptor are selectively expressed in primary human retinal microvascular endothelial cells. Local administration of recombinant IL-33 protected against development of CNV, supporting its immunomodulatory therapeutic potential for nAMD [6, 33, 36]. IL-33 suppression of CNV does not support a primary VEGF-dependency because VEGFR levels remained unchanged, but rather a direct inhibition of ST2-positive fibroblasts and endothelial cells.

Current anti-VEGF therapies arguably do not address major aspects of the underlying pathophysiology, such as circumventing inflammation and wound healing leading to angiogenesis. Furthermore, VEGF's role as a neuronal survival factor cannot be overlooked, underscored by the observation that in patients receiving long-term anti-VEGF therapy the neural retina can begin to degenerate [2]. These data and others' question the current development of therapeutic targets redressing either complement dysregulation or inhibiting the assumed negative impact of chronic low-grade inflammation such as inflammasome activation [37]. The identification of an anti-angiogenic role of IL-33 in CNV extends current understanding of the functional diversity of members of the IL-1 family in angiogenesis. For example, IL-1 receptor antagonist treatment significantly inhibits CNV, as there is a direct effect of IL-1β on choroidal endothelial cell proliferation [38]. On the other hand, IL-18 production following inflammasome activation suppresses CNV and down-regulates VEGFR signalling [36]. IL-33 treatments directly inhibit migration of retinal endothelial cells and choroidal fibroblasts without down-regulation of VEGFR and appear to be potent in reducing angiogenic responses. Conceivably, IL-33 may synergize with IL-18 and further amplify an anti-angiogenic response in both early and late phases of the disease. IL-33 is bioactive without requiring the NLRP3 inflammasome and caspase cleavage [8]. Inflammatory cues strongly induce expression

of endogenous IL-33 [39]. Indeed whether IL-33 is also liberated following intravitreal IL-4 administration directing a macrophage-mediated suppression of CNV warrants further investigation [40].

Supporting the diverse constitutive tissue expression of IL-33 [7, 41], the current data shows IL-33 and ST2 expression in human and murine RPE. Many RPE-derived molecules are central to regulating and maintaining local immune privilege in the eye [42]. To support a regulatory behaviour, IL-33 is constitutively expressed by cells in the inner nuclear layer (INL) of the retina, displaying eye-specific homeostatic expression similar to other's data [18]. Although IL-33 is released from Müller cells *in vitro* and *in vivo* after phototoxic stress [18], and the effect is detrimental, we show TLR-induced up-regulation of IL-33 in RPE cells without cell death. This likely represents an attempt to maintain homeostasis and tissue health. In support of our observation, Kakkar et al. have reported that IL-33 is localized simultaneously to both nuclear euchromatin and membrane-bound cytoplasmic vesicles, and demonstrated that mechanical strain induced IL-33 secretion in the absence of cellular necrosis in murine fibroblasts [10]. We speculate a similar dynamic inter-organelle trafficking of IL-33 in RPE under stress, which induces increased membrane-bound cytoplasmic vesicles and subsequent IL-33 release without cell death.

Endogenous intracellular IL-33 does not alter the development of angiogenesis in the laser-CNV model. The lack of effect with loss of endogenous IL-33 can be explained by the low level of its basal expression in normal tissue. We speculate that IL-33 acts as an in-built brake, and under stress conditions, as shown *in vitro* (Figure 1), IL-33 is upregulated, regulating angiogenesis. To support this notion, *exogenous* recombinant IL-33 suppresses CNV. Further, we found that the two lower doses of murine IL-33 were more efficient at reducing CNV development. We speculate that higher doses of IL-33 up-regulate ST2 expression, neutralizing IL-33 activity.

Such possibility is consistent with our *in vitro* data demonstrating IL-33-induced enhancement of the IL-33/ST2 axis in the RPE. Furthermore, we found no adverse effect of exogenous application of IL-33 as reported previously [18]. Injection of 500-fold higher than the therapeutic dose of recombinant IL-33 did not induce cell death or increased monocyte recruitment (Supplementary Figure S5).

IL-33 exhibits unique features and represents a guardian of barriers and a local alarmin [43]. In an intact barrier, cellular contacts provide the signals to produce full-length IL-33 'constitutively' but neither process nor release it. In these barrier cells, full-length IL-33 resides in the nucleus, where it controls gene expression and is possibly involved in maintaining the cells' resting state [43]. During injury, some of the barrier cells are destroyed and full-length IL-33 is liberated. Depending on the location of the barrier and the cause of perturbation, the bioactivity and effect of extracellular IL-33 will be different. Although triggers differ, the basic principles of alarming resident sentinel cells via IL-33 may yet apply. Extracellular IL-33 coordinates immune defence and repair mechanisms, and elucidating the local effects of IL-33 enables a rationally based approach to developing pharmacological intervention strategies to target the IL-33–ST2 axis. IL-33 is expressed in multiple endothelial cells [26, 28] and exogenous administration of IL-33 induces expression of adhesion molecules and inflammatory cytokines [44]. The presence of mast cells within CNV lesions infer the possibility of additional sources of IL-33 and ability to rapidly process IL-33 delivering enhanced biological activity, augmenting local immune responses [43]. On the other hand, IL-33 has anti-hypertrophic and anti-fibrotic effects [45]. Despite data indicating IL-33 promotes angiogenesis [46], we believe this is tissue context dependent, in that endothelial IL-33 receptor expression has tissue specificity. Human retinal endothelial cells express ST2 at high levels, compared to monocytes, and ST2 is almost completely absent in human brain endothelial cells (Figure 2B) and do not respond to IL-33 treatment. Cytokine pleiotropism is supported because IL-33 exacerbates

autoimmune collagen-induced arthritis via mast cell degranulation [20], but IL-33 elicits high levels of IL-5 and IL-13 [45], driving M2 macrophages and protecting against EAU [17] and atherosclerosis [45]. IL-33 prevents cognitive decline in mouse models of Alzheimer's disease [47] and inhibits atherosclerosis [45], and here IL-33 also directly inhibited choroidal fibroblast activity, implicating a potentially wider role clinically. These actions add to the increasing evidence and support for immunotherapeutic mediated adjuncts to aid the current treatment paradigm for patients suffering from nAMD [48].

Author contributions

ADD conceived the project and supervised all research. ST, DAC, JL, JW, PJG, EO and MC performed research. ST, DAC, SLD and MC analysed data. ST, DAC, SLD, MC and ADD wrote the manuscript.

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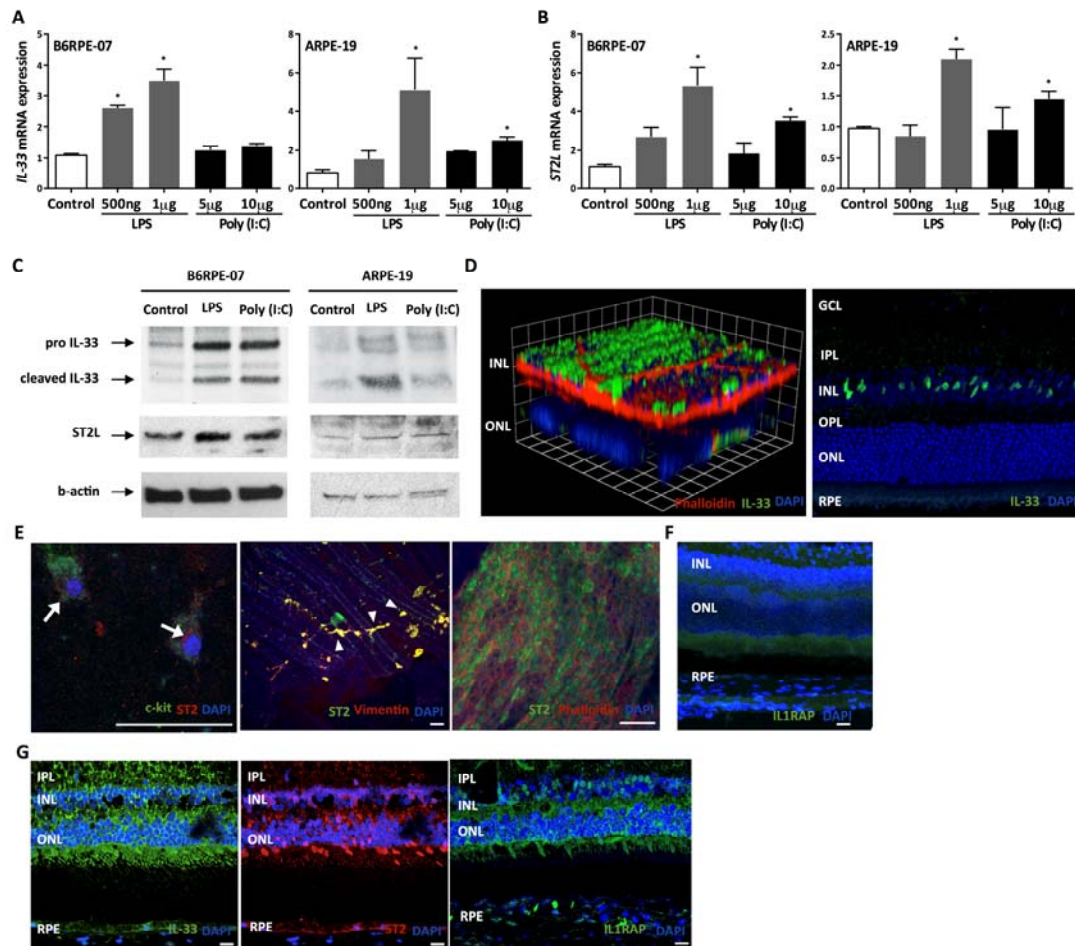
Figure Legends

Figure 1

Figure 1. IL-33 is expressed in murine and human retinas, and is induced in RPE. Expression of IL-33 (A) and its receptor, ST2L (B) mRNA expression in retinal pigment epithelium (RPE) treated with TLR agonists (LPS, poly(I:C)) as indicated for 24 h (n=4/group). (C) Western blot analysis of IL-33 and ST2 in mouse (B6RPE-07) and human (ARPE-19) RPE cells upon activation with TLR agonists. (D) Immunofluorescence staining showing expression of IL-33 in adult murine retina. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar, 100 μm, (E) ST2 expression in choroidal mast cells (arrows), choroidal fibroblasts (arrowheads) and RPE. (F) Immunofluorescence staining showing expression of IL-1 receptor accessory protein (IL1RAP) in murine retina. Scale bar, 100 μm, (G) Representative images of immunofluorescence staining

showing expression of IL-33, ST2 and IL1RAP in sections of human retina. Scale bar, 100 μm. Data are shown as mean ± SEM. Data are representative of at least three independent experiments with similar results. *$p < 0.05$, Statistical analysis was performed with Student's t-test.
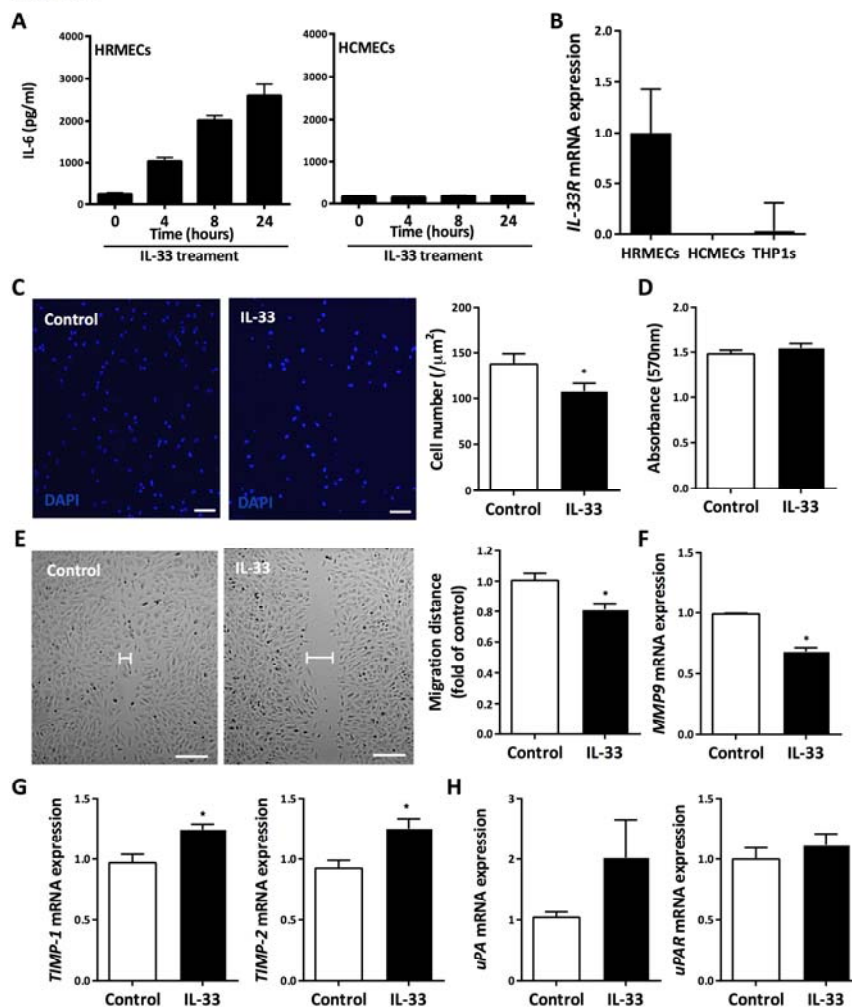

Figure 2. Retinal endothelial cells are a direct target of IL-33. (A) Production of IL-6 by HRMEC and HCMECs when treated with IL-33 for varying amounts of time, measured by ELISA (n=3/group). (B) ST2L mRNA expression in HRMECs and HCMECs and human myeloid cell line THP1 (n=3/group). (C) The Boyden chamber assay was performed and chemotactic activity was quantified by blind counting of the migrating cells on the lower surface of the membrane in 10 high-power microscope fields per chamber using a ×100 objective. IL-33

significantly inhibited the migratory ability of HRMECs (scale bar, 50 μm) (n=4/group). (D) MTT assay showed that IL-33 treatment had no effect on the proliferation of HRMECs (n=3/group). (E) IL-33 inhibited migration of HRMECs in a wound healing assay (n=5/group). Scale bar, 100 μm. (F) Matrix metalloproteinase-9 (*MMP9*) mRNA expression in HRMECs in response to IL-33 (n=3/group). (G) Tissue inhibitors of metalloproteinases-1 (*TIMP1*) and -2 (*TIMP2*) expression in HRMECs in response to IL-33 (n=3/group). (H) Urokinase-type plasminogen activator (*uPA*) and its receptor *uPAR* expression in HRMECs in response to IL-33 (n=3/group). Data are shown as mean ± SEM. Data are representative of at least two independent experiments with similar results. *p < 0.05, Statistical analysis was performed with Student's *t*-test.
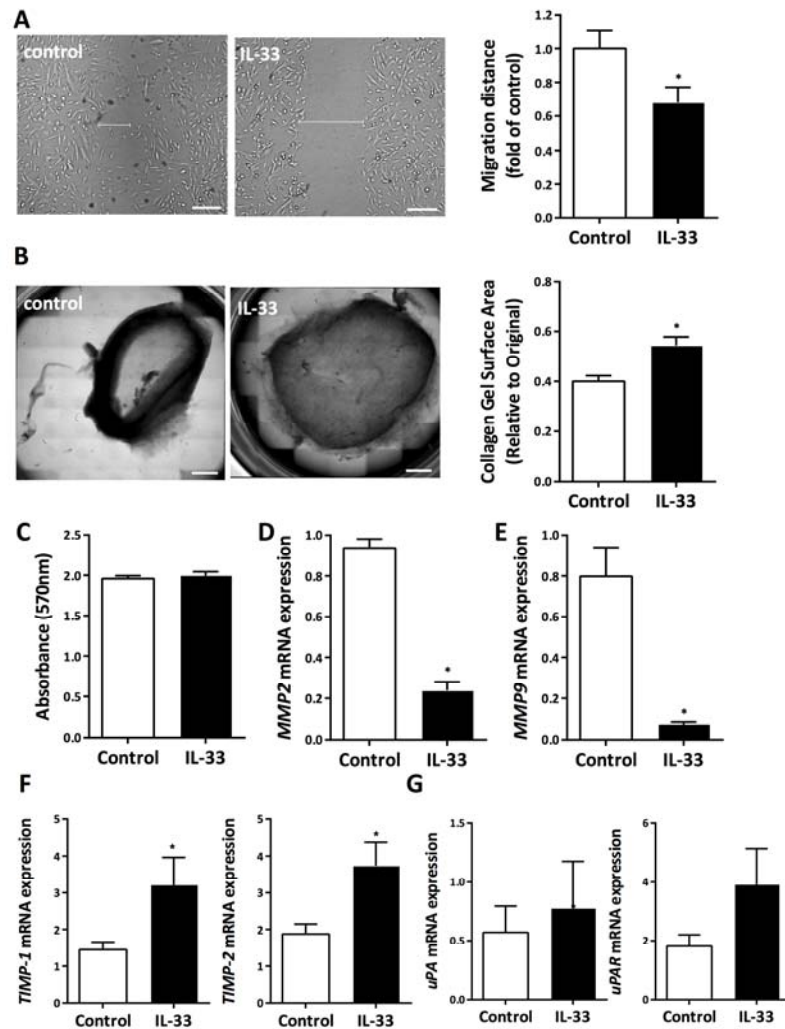
Figure 3. IL-33 regulates the function of human choroidal fibroblasts. IL-33 inhibited the ability of human choroidal fibroblasts to (A) migrate in a wound healing assay, and to (B) contract collagen gel (scale bar, 100 μm). (C) MTT assay showed that IL-33 treatment had no effect on their proliferation. (D) *MMP2* and (E) *MMP9* mRNA expression in choroidal fibroblasts upon treatment with IL-33. (F) *TIMP1* and *TIMP2* expression in choroidal fibroblasts in response to IL-33 (n=3/group). (G) *uPA* and its receptor *uPAR* expression in choroidal fibroblasts in response to IL-33 (n=3/group). Data are shown as mean ± SEM. Data are representative of three independent experiments with similar results. *$p < 0.05$, Statistical analysis was performed with Student's *t*-test.
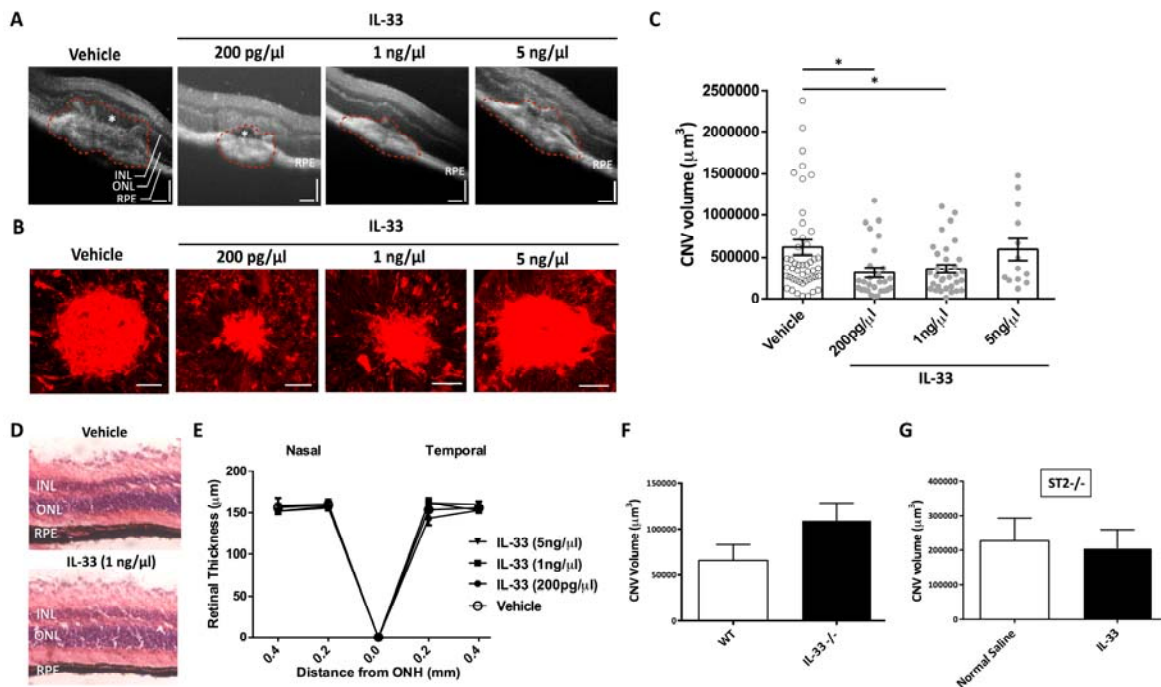
Figure 4. Intravitreal administration of IL-33 attenuates CNV in a ST2-dependent manner.

(A) Representative OCT images showing choroidal neovascular lesions in control and IL-33 treated eyes. INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retina pigment epithelium. The white asterisk denotes the sub-retinal fluid. Scale bar, 200 μm. (B) Fluorescence IB4-staining of choroidal neovascular (CNV) lesion in all treatment groups. Images are representative of CNV volume in each experimental group. Scale bar, 75 μm. (C) Quantitative analysis of volume of choroidal neovascular lesions showed that the two lower doses of IL-33 significantly attenuated CNV development. IL-33 did not affect the integrity and thickness of the retina as shown in histological sections (D) and OCT analysis (E) of eyes that had only intravitreal injection of IL-33. ONH, optic nerve head. Data representative of 2 measurements per retina with 4 eyes per group. (F) Il33-/- mice had more pronounced lesions but not to a statistically significant level. (G) IL-33 treatment did not affect the severity of CNV

development in *St2-/-* mice (n=10 eyes/group). Data are shown as mean ± SEM. Data are representative of at least three independent experiments with similar results. *$p < 0.05$. Statistical analysis was performed with ANOVA with *post hoc t*-test.
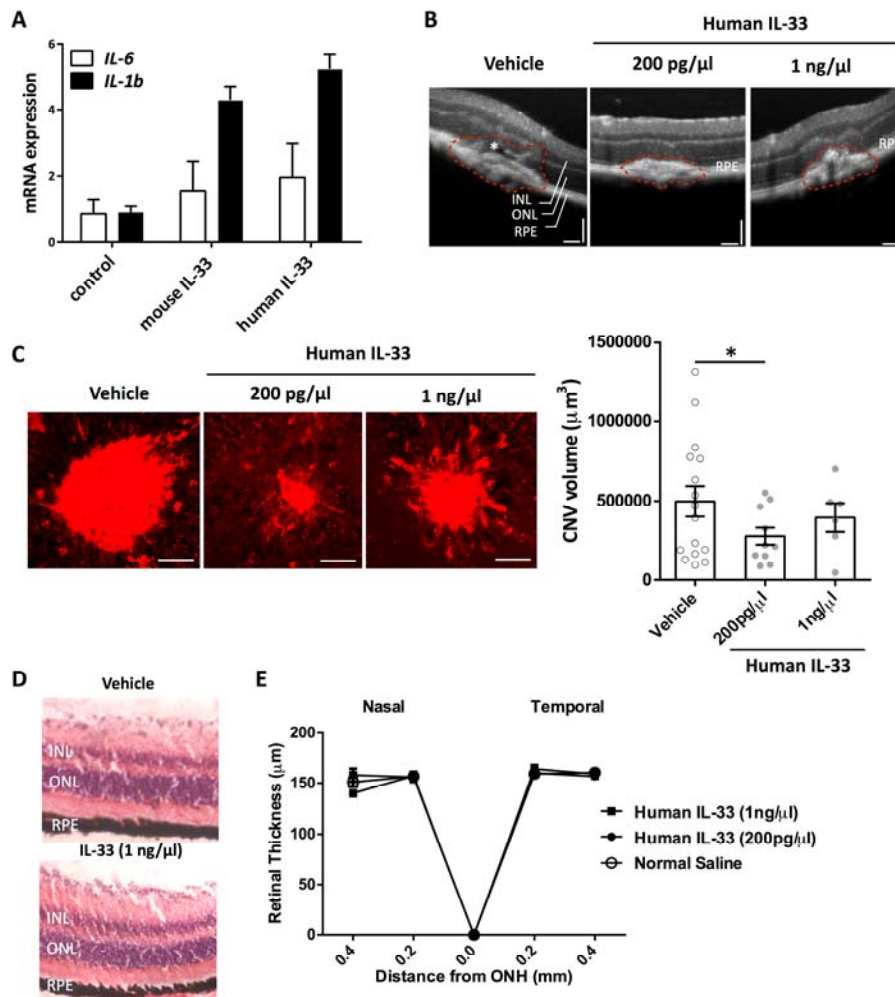

Figure 5. A truncated form of human IL-33 is biologically active in mice.

(A) We used a truncated form of human IL-33, which is bioactive in mice as shown by IL-6 and IL-1β mRNA levels produced by bone marrow-derived mast cells (BMMCs) upon treatment with mouse and human IL-33. (B) Representative images of OCT showing choroidal neovascular lesions in control and human IL-33 treated eyes. INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retina pigment epithelium. The white asterisk denotes the sub-retinal fluid. Scale bar, 200 μm. (C) Immunofluorescence IB4-staining and quantitative analysis of choroidal neovascular lesions in all treatment groups with normal saline or human IL-33.

Images are representative of CNV volume in each experimental group. Scale bar, 75 µm. (D) Representative images of histology in control and eyes treated only with intravitreal injections (n= 4 eyes/group). (E) The truncated form of human IL-33 did not affect the thickness of the retina as measured by OCT in eyes that had intravitreal injection of IL-33. ONH, optic nerve head. Data are representative of 2 measurements per retina with 4 eyes per group. Data are shown as mean ± SEM. Data are representative of at least two independent experiments with similar results. *$p < 0.05$ Statistical analysis was performed with ANOVA with *post hoc t*-test.