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The 10q26 risk haplotype of age-related macular degeneration aggravates subretinal inflammation by impairing monocyte elimination

Fanny Beguier[1*], Michael Housset[1*], Christophe Roubeix[1*], Sebastien Augustin[1], Yvrick Zagar[1], Caroline Nous[1], Thibaud Mathis[1], Chiara Eandi[3], Mustapha Benchaboune[5], Adèle Drame-Maigné[1], Wassila Carpentier[1], Solenne Chardonnet[2], Sara Touhami[1], Guillaume Blot[1], Jean Baptiste Conart[1], Hugo Charles-Messance[1], Anaïs Potey[1], Jean-François Girmens[5], Michel Paques[5], Fréderic Blond[1], Thierry Leveillard[1], Elod Koertvely[6], Jerome E Roger[4], José-Alain Sahel[1,5], Przemyslaw Sapieha[7], Cécile Delarasse[1], Xavier Guillonneau[1], Florian Sennlaub[1†]

[1]Sorbonne Université, INSERM, CNRS, Institut de la Vision, 17 rue Moreau, F-75012 Paris, France.

[2]Sorbonne Université, INSERM, UMS 37 PASS, Plateforme Post-génomique de la Pitié-Salpêtrière, P3S, F-75013 Paris, France

[3]University of Torino, Department of Surgical Science, Torino, Italy

[4]Paris-Saclay Institute of Neuroscience, CERTO-Retina France, CNRS, Univ Paris Sud, Université Paris-Saclay, F-91405 Orsay

[5]CHNO des Quinze-Vingts, DHU Sight Restore, INSERM-DGOS CIC 1423, 28 rue de Charenton, F-75012 Paris, France.

[6]Roche Pharma Research and Early Development, Roche Innovation Center Basel, 124 Grenzacherstrasse, 4070, Basel, Switzerland

[7]Department of Ophthalmology, Maisonneuve-Rosemont Hospital Research Centre, University of Montreal, Quebec, Canada.

*these authors have contributed equally

† Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact: Dr Florian Sennlaub, Inserm, UMR_S 968, Institut de la Vision, Paris, F-75012, France. Tel: (33) 1 53 46 26 93,

Email: florian.sennlaub@inserm.fr.

Summary

A minor haplotype of the 10q26 locus conveys the strongest genetic risk for age related macular degeneration (AMD), a blinding, neuroinflammatory disorder. Here we examined the mechanisms underlying this susceptibility. We found that monocytes from homozygous carriers of the 10q26 AMD-risk haplotype expressed high amounts the High-Temperature Requirement A Serine Peptidase 1 (HTRA1) and HTRA1 located to mononuclear phagocytes (MP) in donor eyes of non-carriers with AMD. Experimentally, HTRA1 induced the persistence of monocytes in the subretinal space and exacerbated pathogenic inflammation due to the hydrolysis of Thrombospondin 1 (TSP1). We observed that HTRA1 deactivated TSP1 by separating the two CD47-binding sites necessary for efficient CD47 activation. This HTRA1-induced inhibition of CD47 signaling induced the expression of pro-inflammatory osteopontin (OPN). Accordingly, OPN expression increased in early monocyte-derived macrophages in 10q26 risk carriers. In models of subretinal inflammation and AMD, OPN deletion or pharmacological inhibition reversed HTRA1-induced pathogenic MP persistence. Our findings argue for the therapeutic potential of CD47 agonists and OPN inhibitors for the treatment of AMD.

Keywords

age related macular degeneration (AMD); 10q26, mononuclear phagocytes (MP), neuro-inflammation, High-Temperature Requirement A Serine Peptidase 1 (HTRA1), Thrombospondin 1 (TSP1), CD47, osteopontin (OPN)

Introduction

Age-related Macular Degeneration (AMD) is a common (Wong et al., 2014), highly heritable, neuroinflammatory disorder characterized by subretinal deposits (drusen) in its early form and choroidal neovascularisation (wet AMD) or an extending lesion of the outer central retina (geographic atrophy) in its late form (Sarks, 1976). A variant of the Complement Factor H (CFH) and a minor haplotype (mH) of the chromosome 10q26 account for most of the genetic risk of AMD (Fritsche et al., 2014). The AMD-associated mH differs from the common non-risk haplotype (cH) by several linked Single Nucleotide Polymorphisms (SNPs) in a non-coding DNA sequence (Liao et al., 2017), and increases the risk for both early and late forms of AMD up to ~10 fold in homozygote mH (mH/mH) carriers (Chen et al., 2009; Rivera et al., 2005). These non-coding SNPs are located within the *Age-Related Maculopathy Susceptibility 2* (*ARMS2*) gene and the promoter of *High-Temperature Requirement A Serine Peptidase 1* (*HTRA1)*, and are in close proximity of *Pleckstrin Homology domain-containing family A member 1* (*PLEKHA1*).

Small studies of three to six mH/mH-carriers proposed that the mH is associated with increased transcription of the adjacent *HTRA1* gene in lymphocytes and the retinal pigment epithelium (RPE) (An et al., 2010; Yang et al., 2006), which was not confirmed by others (Chowers et al., 2008; Kanda et al., 2010). Independently of the haplotype, HTRA1 concentrations are increased in the aqueous humor of wet AMD patients (Tosi et al., 2017), HTRA1 deposits in drusen (Yang et al., 2006), and over-expression of HTRA1 in mice and fish induces an AMD-like phenotype (Iejima et al., 2015; Jones et al., 2011; Oura et al., 2018), suggesting a role of HTRA1 in AMD pathogenesis. HTRA1 is also elevated in the plasma of subjects with age-related frailty (Lorenzi et al., 2016), Alzheimer's disease (Grau et al., 2005), and arthritis, where it is strongly expressed by activated, infiltrating macrophages (Hou et al., 2014; Milner et al., 2008). HTRA1 is a protease of the trypsin family of serine proteases that hydrolyzes many extracellular matrix (ECM) proteins, including thrombospondin 1 (TSP1) (Chen et al., 2018; Lin et al., 2018), and inactivates transforming growth factor β (TGFβ) (Clausen et al., 2011). The importance and functional consequences of the hydrolysis of these proteins in AMD are unknown.

Under physiological conditions, the subretinal space is immunosuppressive and devoid of mononuclear phagocytes (MPs) (Guillonneau et al., 2017), a family of cells that include monocytes, and infiltrating and resident macrophages. A common feature of early and both advanced forms of AMD is the activation of MPs in the inner retina and the chronic

accumulation of subretinal MPs. Contrary to inherited retinal degeneration, the infiltrate is not only composed of displaced microglial cells (the resident Mφ of the retina) (O'Koren et al., 2019), but also of macrophages derived from infiltrating monocytes (Guillonneau et al., 2017; Sennlaub et al., 2013). MPs play a critical role in neovascularization and photoreceptor degeneration that characterize late AMD (Guillonneau et al., 2017).

The homeostatic elimination of infiltrating MPs is dependent on TSP1-mediated activation of the CD47 receptor and is inhibited by CFH, in particular the AMD-associated CFH(H402) variant (Calippe et al., 2017). Each monomer of the homotrimeric TSP1 contains two Valine-Valine-Methionine (VVM) sequences in its C-terminal domain that each interacts with a CD47 receptor. The efficient activation of CD47 by TSP1 necessitates the presence of both VVM sites of TSP1 (McDonald et al., 2003), but can be mimicked by the TSP1-derived 4N1K peptide (KRFYVVMWKK) (Martinez-Torres et al., 2015), at 50-fold higher molar concentrations (McDonald et al., 2003).

Here we examined the mechanisms whereby the risk 10q26 haplotype promotes AMD. We found that monocytes carrying this haplotype overexpress HTRA1. Overexpression of HTRA1, through the proteolysis of TSP1 and inhibition of CD47 signaling, induced osteopontin (OPN, also known as secreted phosphoprotein 1, SPP1), which in turn inhibited the homeostatic elimination of macrophages from the subretinal space and mediated HTRA1-induced pathogenic inflammation. Our study provides insight into the cellular mechanisms underlying the 10q26 AMD-risk haplotype of AMD, and argues for the use of CD47 agonists and OPN inhibitors for the treatment of AMD.

Results

Monocytes of homozygous carriers of the 10q26 risk-haplotype express increased HTRA1 and HTRA1 locates to mononuclear phagocytes in AMD donor tissues.

The AMD-risk mH of 10q26 is defined by a set of neighboring non-coding SNPs located within the *ARMS2* gene and *HTRA1* promoter, in close proximity of *PLEKHA1*. The mH has been proposed to be associated with increased expression of *HTRA1* in lymphocytes and decreased *ARMS2* in monocytes (Micklisch et al., 2017; Yang et al., 2006).

Our analysis of DNase I hypersensitive sites using the Encyclopedia of DNA Elements (ENCODE) database, revealed that open chromatin regions of the *HTRA1* promoter were most prominent in $CD14^+$monocytes compared to $CD4^+$T and RPE cells. An open region of *ARMS2* was observed in RPE but not in $CD4^+$T cells and $CD14^+$Mos, and open chromatin regions in the *PLEKHA1* promoter were similar in the three cell types (Figure 1A). Accordingly, *HTRA1* mRNA, detected by RT-PCR, was several hundred folds more transcribed in blood $CD14^+$monocytes compared to lymphocytes (Figure 1B) used in previous studies (Yang et al., 2006).

Taking advantage of our previous GWAS study (Fritsche et al., 2013) that included 10 SNP markers of the 10q26 haplotype, we re-invited 18 homozygous cH/cH- and 18 mH/mH-carriers and constituted a collection of mRNA extracted from their sorted $CD14^+$blood monocytes. None of the patients displayed one of the rare, incomplete haplotypes. The two study groups were similar in age (~80 years), gender distribution, composed of 50% subjects with late AMD and 50% subjects with no signs of AMD (to match a possible disease effect), and equal in the CFH402H variant frequency (Figure S1A). *PLEKHA1* mRNA levels in mH/mH monocytes were slightly lower compared to cH/cH donors (Figure 1C). *ARMS2* mRNA was not detected in either group using commercially validated and published primers (Micklisch et al., 2017) (>50 PCR cycles) (Figure 1D). But, mH/mH monocytes showed two-fold higher levels of *HTRA1* mRNA (Figure 1E) and secreted more HTRA1 into 24h supernatants (Figure 1F) compared to cH/cH monocytes.

Next, we analyzed the localization of HTRA1 in HTRA1-IBA1 double-immuno-labeled retinal flat-mounts and HTRA1 mono-labeled corresponding RPE flat-mounts (no double labeling because of autofluorescence) in four patients with known wet AMD and visible large drusen on dissection and four age-matched control donor eyes (all donors cH/cH).

Representative confocal images of the central inner retina show diffuse, low-level staining of HTRA1 (green) of the healthy retina and distinctly HTRA1-positive IBA1^{+} (red) monocytes in the inner retinal vessels (Figure 1G and S1E), confirming the mRNA expression in leukocytes above. The central inner retina of wet AMD tissues additionally displayed many IBA1^{+}HTRA1^{+}MPs (Figure 1H and S1E). Subretinally, the HTRA1 staining appeared increased in RPE with numerous subretinal HTRA1^{+}cells on the apical side of the autofluorescent RPE (orange) in AMD- (Fig. 1H), but not in control-samples (Figure 1G and S1F). Micrographs of the subretinal aspect of the double-labeled retinal flat-mounts demonstrate that HTRA1 again localized to IBA1^{+}MPs (Figure 1H insets). Taken together, these data show that MPs in AMD, which are in part derived from monocytes (Sennlaub et al., 2013), express HTRA1 and that the AMD-risk haplotype increases the expression of HTRA1 at least in monocytes.

HTRA1 hydrolyzes Thrombospondin-1 and separates its two CD47-binding sites.

We recently demonstrated that TSP1 as a ligand of CD47 is a crucial mediator for the homeostatic elimination of pathogenic subretinal MPs (Calippe et al., 2017). Gel electrophoretic separation and silver nitrate protein staining of co-incubated recombinant TSP1- and HTRA1- proteins, confirmed previous reports (Chen et al., 2018; Lin et al., 2018) that TSP1 is a substrate for HTRA1. The 170 kDa TSP1 monomer full-length band disappeared HTRA1-dose dependently (Figure 2A). Two major bands above 100 kDa, were detectable with the lowest HTRA1 concentration, and four weaker bands only appeared with higher HTRA1 concentration (2 bands in the 90 kDa range and 2 bands in the 30 kDa range).

To identify potential HTRA1 cleavage sites in TSP1, samples of TSP1 and TSP1 hydrolyzed with the highest HTRA1 concentration were submitted to tryptic digestion prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS). Cleavage sites of HTRA1 have never been reported distal to Arginine and Lysine (Eigenbrot et al., 2012) amino acids, the two exclusive cleavage sites of trypsin, making it unlikely that HTRA1 hydrolysis occurs at tryptic sites. We reproducibly identified eight peptides with non-tryptic cleavage at one of its ends (semi-tryptic peptide) with high identification scores in both complementary search engines, PEAKS and Mascot (Figure 2B and S2A). These peptides were found in tryptic-digestion of HTRA1-hydrolyzed TSP1 fragments only, but absent from TSP1 preparations without HTRA1. Three HTRA1 cleavage sites were located in the N-terminal domain, one in TSP1's vWFC domain and four in its C-terminal domain (Figure 2C and S2B). Western blotting of HTRA1-induced TSP1 fragments with an antibody raised against the first fourteen

N-terminal TSP1 amino acids (Figure 2D), demonstrates that the two major fragments (>100 kDa), and two minor cleavage products, still contained their N terminal extremity, revealing an HTRA1 hydrolysis at their C-terminal domain.

To determine which two of the four C-terminal cleavage sites identified by mass spectrometry led to the two major TSP1 fragments, we generated a recombinant protein of TSP1's C-terminus (rCter) from amino acid 834 to 1170, baring a FLAG-epitope at its N-terminus (Figure S2C). The expected sizes of HTRA1-digested flagged rCter fragments (cut distal to AA positions 1142, 1090, 1043 and 998) were 38 kDa, 32 kDa, 27 kDa and 21,8 kDa respectively (Figure 2E). Electrophoretic migration followed by silver nitrate stain of proteins shows that HTRA1 hydrolyzes Cter into a ~30kDa, a ~25kDa, and a ~10kDa band (Figure 2F), suggesting positions 1043 and 1090 as cleavage sites, with the un-tagged C-terminus fragment explaining the ~10kDa band. A western blot using an anti-FLAG antibody accurately identifyed a 32kDa and a 27kDa fragment containing the N-terminal FLAG (Figure 2G) confirming HTRA1 predominantly cleaves TSP1 after V1043 and V1090, in accordance with HTRA1's preferential hydrolysis after valine amino acids (Chamberland et al., 2009; Eigenbrot et al., 2012). These results demonstrated that HTRA1 preferentially cleaves TSP1 between its two VVM motifs, which should severely hamper TSP1's ability to dimerize CD47 receptors.

We next mono- or co-transfected COS-7 cells with plasmids coding for a V5 tagged- and Myc tagged- human CD47 receptors that are detectable with highly specific anti-tag-antibodies by immunohistochemistry (Figure 2H). The incubation of co-transfected cells with TSP1 greatly increased CD47-V5/CD47-Myc complex formation visualized by proximity ligation assay (PLA, red dots). HTRA1 cleavage of TSP1 reduced its ability to form these complexes (Figure I and J), in accordance with a separation of the two CD47-binding VVM motifs.

In summary, HTRA1 predominantly hydrolyzes TSP1 monomers at two cleavage sites, which separate its two CD47-binding VVM motifs of its C-terminal domain and reduces its ability to form CD47 complexes.

HTRA1-induced Thrombospondin-1 hydrolysis prevents CD47-dependent monocyte elimination.

TSP1 activation of CD47 expressed on subretinal MPs triggers their RPE-induced elimination (Calippe et al., 2017), likely acting as a sensitizing factor to cell death induction by RPE factors such as FASL (Levy et al., 2015; Manna et al., 2005; Quesada et al., 2005).

Quantification of co-cultures of confluent porcine primary RPE with human blood monocytes that mimic subretinal monocyte elimination (Mathis et al., 2017), immune-stained for myeloid transcription factor PU.1 and RPE transcription factor OTX2, revealed that the addition of HTRA1 (5µg/ml) reduced RPE-induced $PU.1^{+}$monocyte elimination (Figure 3A and B). HTRA1-exposure had no effect on $OTX2^{+}$RPE cell numbers, or on $PU.1^{+}$monocyte numbers in monoculture (Figure 3B). Similarly to HTRA1 exposure, function-blocking antibodies of TSP1 or CD47, but not CD36 (a TSP1 receptor necessary for TGF-ß activation (Yehualaeshet et al., 1999)), inhibited monocyte elimination in 48h co-cultures (Figure 3C). Inhibition of TGF-ß receptor signaling, shown to mediate effects of HTRA1 (Clausen et al., 2011), had no influence on monocyte elimination in this model (Figure S3A). Addition of HTRA1 to the function-blocking TSP1 antibody only marginally further increased monocyte survival (Figure 3C). When we pre-incubated the RPE or the monocytes with HTRA1 for 2hrs prior to co-culture, only the pre-incubation of the monocytes led to increased survival (Figure 3D). In addition, co-cultures of CFSE-prestained monocytes from wildtype and knockout mice with primary RPE demonstrated that deficiency in either TSP1 or CD47 (but not CD36) in monocytes was sufficient to increase monocyte survival similarly to HTRA1 exposed wildtype monocytes (Figure 3E). As with TSP1-blocking antibodies, the addition of HTRA1 to *Thbs1*$^{-/-}$ monocytes only increased monocytes by 25% compared to the 150% increase of wildtype Mos. These results suggested that TSP1 is important in HTRA1s promotion of monocyte-survival.

To evaluate if the effect of HTRA1 was due to TSP1 degradation, we pre-incubated monocytes with HTRA1 for 30min, removed HTRA1 and incubated the cells with either TSP1 or the rCter protein for 1h, prior to co-culturing for 48hrs. The HTRA1 pre-incubation induced increase in monocyte survival could be completely reversed by 200nM TSP1 and significantly reduced by 200nM Cter (Figure F). CD47 activation by TSP1 can be mimicked by the 4N1K peptide (KRFYVVMWKK) (Martinez-Torres et al., 2015) that is derived from the first VVM site of TSP1 and does not contain a HTRA1 cleavage site (Figure S2B). Compared to the control peptide, 4N1K antagonized the HTRA1-induced increased monocyte

survival in co-cultures partially at 20µM and completely at 50µM (Figure 3G). The inhibition occurred however at much higher molar concentrations compared to TSP1 and the Cter (200nM) that both contain two VVM sites in tandem. Hence we linked two 4N1K peptides with an eight-glycine spacer to produce a 4N1K bi-peptide. The bi-peptide partially reversed the effect of HTRA1 on monocytes at 200nM and completely inhibited it at 1µM in the culture, at 50-fold lower concentrations than the single 4N1K peptide. A control bi-peptide had no effect and an alternative bi-peptide using a polyethylene glycol spacer (NH2-$(CH_2CH_2O)_7$-CH_2CH_2COOH) gave similar results (Figure S3B).

In vivo, HTRA1 increased the 24h survival of CFSE-stained mouse monocytes after injection to the immunosuppressive subretinal space, which normally induces their quick elimination (Levy et al., 2015) (quantified on RPE and retinal flatmounts, Figure 3H). TSP1 addition promoted subretinal MP elimination, but TSP1 lost its immunosuppressive effect in the presence of HTRA1. Importantly, the HTRA1-induced monocyte clearance deficit was completely reversed by the co-administration of the 4N1K bi-peptide compared to control (Figure 3H). Similar effects were observed using freshly extracted brain microglial cells (Figure S3C). Conversely, HTRA1 had no effect on adoptively transferred $CD47^{-/-}$monocyte that resisted subretinal elimination similarly to HTRA1-treated wildtype monocytes and the elimination of $CD47^{-/-}$monocyte could not be accelerated by the 4N1K bi-peptide confirming the specificity of the peptide (Figure 3I). Furthermore, subretinal IBA-1^{+}MP accumulation (quantified on RPE and retinal flatmounts) in *Thsb1*$^{-/-}$-mice, provoked by a 4-day-light challenge calibrated to induce MP infiltration in inflammation prone *Thsb1*$^{-/-}$- but not in wildtype-mice (Calippe et al., 2017), was strongly repressed two days after subretinal injection of TSP1 compared to PBS (Figure 3I). Subretinal administration of HTRA1 in light-challenged $Thsb1^{-/-}$-mice did not further increase the infiltrate, but again fully reversed TSP1's immunosuppressive effect. The CD47-activating 4N1K bi-peptide strongly induced MP elimination in the presence of HTRA1 (Figure 3J).

Collectively, the observations that HTRA1 blocked the anti-inflammatory effect of TSP1, that HTRA1 had a much reduced effect in the absence of TSP1 or CD47, that the 4N1K bi-peptide completely reversed the effect of HTRA1 at much lower concentrations than the mono-peptide but only in the presence of CD47, strongly suggests that HTRA1 inhibits monocyte and MP elimination predominantly by separating the two CD47-binding sites of TSP1, inhibiting CD47 signaling.

HTRA1 induced inhibition of CD47 signaling promotes osteopontin secretion

To identify potential HTRA1-induced downstream mediators of increased MP survival that were inhibited by CD47 signaling, we sequenced the transcriptome of early human monocyte-derived macrophages (cH/cH monocytes cultured for 6hrs). Compared to PBS controls, exogenous HTRA1 induced 1699 protein coding transcripts (Figure 4A, Table S1). Interestingly, 1267 of these mRNAs (74%) were repressed by simultaneous activation of CD47 (bi4N1K), suggesting that the inhibition of CD47 signaling participates in the induction of the majority of HTRA1-induced transcripts in early monocyte-derived macrophages. A comparison of the TPMs (transcripts per million) of the ten transcripts that were induced at least 16 fold (4 log2) by HTRA1 (HTRA1 versus control) and most repressed by 2,5µM bi4N1K revealed that colony stimulating factor 1 (*CSF1*) and osteopontin (*OPN*), were among the most regulated mRNAs (Figure 4B, S4A). While CSF1 mainly increases the life span of resident macrophages (Hume and MacDonald, 2012; Lenzo et al., 2012), OPN promotes macrophage survival in inflammatory conditions (Bruemmer et al., 2003), including in the subretinal space (Fujita et al., 2011), making it a good candidate for a CD47-regulated MP survival factor. ELISA quantifications of supernatants from 24h human monocyte-derived macrophages confirmed that HTRA1 robustly induced OPN secretion, which was strongly, but not entirely, repressed by the bi4N1K peptide (Figure 4C). In accordance with a CD47-dependent mechanism, cultured $CD47^{-/-}$ bone marrow-monocytes (BMM) expressed *OPN* mRNA at higher levels compared to wildtype cells in 6h BMM-derived macrophages (quantitative RT-PCRs, Figure 4D). Importantly, at 18h of macrophage differentiation, although the strain–dependent difference was reduced, the addition of HTRA1 to the culture induced a major four-fold increase of *Opn* transcription in wildtype cells compared to a 1.5 fold increase in $CD47^{-/-}$ macrophages, demonstrating the important role of CD47 in HTRA1-dependent *Opn* induction (Figure 4D). During un-stimulated, early human macrophage differentiation of blood monocytes (cH/cH), quantitative RT-PCRs revealed a transient induction of *TSP1* (from 3 to 16h), followed by the induction of *HTRA1*, which preceded *OPN* induction (Figure 4E, each relative to the expression in fresh monocytes). Inhibition of HTRA1 by the inhibitor DPMFKLboroV (Truebestein et al., 2011) (that did not affect cell survival in culture, data not shown) dose-dependently reduced the subsequent OPN secretion in cH/cH macrophages at 72h (Figure 4F), showing the importance of endogenous HTRA1 in the induction of OPN. Furthermore, in our human study groups (Figure 1) we demonstrated a significant positive correlation between HTRA1 transcription in fresh monocytes and *OPN* transcription at 24h of culture (Figure 4G). Finally, mH/mH-monocytes, expressing higher

levels of HTRA1 (Figure 1), also transcribed and secreted significantly more OPN at 24h of culture compared to cH/cH -controls (Figure 4H and I) without affecting the secretion of other proteins typically secreted by MPs such as Apolipoprotein E (Figure S4B). The AMD associated CFH(H402) variant was not associated with differences in OPN expression (Figure S4C), in accordance with previously reported low CFH expression in monocyte and the observation that CFH(H402) inhibits the subretinal elimination of microglial cells, but not monocytes, more potently than the common CFH(Y402) (Calippe et al., 2017). A multiplexed ELISA analysis of the supernatants also revealed that mH/mH monocyte-derived macrophages secreted more CCL2 compared to cH/cH-controls (Figure 4SD), likely contributing to increased inflammation. Its transcript was also induced by recombinant HTRA1 in our transcriptome analysis, but not reduced by bi4N1K, pointing to a TSP1 independent mechanism.

Taken together, our data suggests that HTRA1 induces OPN in part due to the inhibition of CD47 signaling, and demonstrates the importance of HTRA1 in the induction of OPN in MPs, and the role of CD47 activation in its repression.

Inhibition of osteopontin reverses HTRA1-induced MP survival.

We next evaluated whether the HTRA1-induced increase in MP survival was dependent on OPN induction. Quantification of PU.1 (green), OTX.2 (red) immune-stained monocyte/RPE co-cultures revealed that a function-blocking anti-human OPN antibody reversed the HTRA1-induced increase of surviving PU.1^{+}monocytes at 48h compared to the control antibody (Figure 5A). In mice, adoptively transferred CFSE-stained C57BL6/J- and *Opn*$^{-/-}$-monocytes were eliminated similarly 24h after subretinal injection, but contrary to wildtype monocytes, HTRA1 did not increase the number of Opn$^{-/-}$ monocytes and a function blocking anti-mouse OPN antibody fully prevented the HTRA1-induced increase in wildtype monocytes (Figure 5B). The same antibody injected subretinally to light challenged *Thbs1*$^{-/-}$-mice reduced the number of IBA1^{+} MPs at 48h compared to the control IgG (Figure 5C).

In laser-induced subretinal inflammation and choroidal neovascularization (CNV), the classic model of wet AMD, HTRA1 again increased the number of MPs surrounding the lesions and increased CNV in wildtype-mice, quantified 10 days after the injury on flatmounts stained for IBA-1^{+}MPs (green) and CD102^{+}vascular endothelial cells (red, Figure 5D and E). HTRA1 was injected intravitreally at day four post-injury (peak MP accumulation, which are in part monocyte-derived macrophages (Figure S5)) and at day seven (resolution phase (Lavalette et al., 2011)). HTRA1 had no effect on the increased inflammation and CNV

observed in *Thbs1*$^{-/-}$-mice, confirming that the pro-inflammatory effect of HTRA1 during the resolution phase is mainly dependent on the presence of TSP1. TSP1, injected on the same days, strongly reduced the inflammation and CNV in particular in *Thbs1*$^{-/-}$-mice, but was inefficient when co-injected with HTRA1. *Opn*$^{-/-}$-mice displayed slightly reduced subretinal MP infiltration and HTRA1 injections only led to a minor increase of the subretinal MP population and CNV size in *Opn*$^{-/-}$-mice compared to wildtype mice (Figure 5D and E). Finally, the HTRA1-induced increase in subretinal MP accumulation, as well as the increase in CNV formation, was efficiently reduced by the function blocking OPN antibody (Fig. 5 D and E). These data highlight the involvement of OPN in HTRA1-mediated MP survival.

DISCUSSION

Chronic inflammation and accumulation of MPs characterizes all forms of AMD. Here we examined how a genetic predisposing factor, the 10q26 risk haplotype, promotes destructive inflammation in AMD. We found that circulating monocytes, which infiltrate the subretinal space in AMD alongside displaced resident macrophages (Sennlaub et al., 2013), expressed high amounts of HTRA1, in accordance with open chromatin sites in the HTRA1 promoter region in monocytes. Our analysis of monocytes from 18 common haplotype carriers (cH/cH) and 18 mH/mH carriers, who are at ~10 fold increased risk of developing late AMD (Chen et al., 2009; Rivera et al., 2005), revealed an association between the mH haplotype and increased *HTRA1* mRNA expression in monocytes and HTRA1 secretion in early monocyte-derived macrophages. mH showed little influence on *PLEKHA1* expression, while *ARMS2* mRNA was not detected in monocytes. Our findings establish the association of mH with increased HTRA1 in monocytes, a relevant cell type in AMD. The mH might have a similar effect on RPE and other MPs such as microglial cells, as an increase was observed in a number of other tissues analyzed in the GTEx database (~10 mH/mH- and ~90 cH/cH-carriers) (Liao et al., 2017). Independently of the haplotype, HTRA1 located to activated MPs in AMD donor eyes, akin to arthritis (Hou et al., 2014; Milner et al., 2008), which might explain the increased aqueous humor HTRA1 concentrations in AMD patients in general (Tosi et al., 2017).

Functionally, HTRA1 lowered the susceptibility of MPs to be eliminated by immunosuppressive signals from the RPE in (i) a co-culture model with human and murine monocytes, (ii) subretinal adoptive transfer in mice, and (iii) mouse models of laser- and light-induced subretinal inflammation *in vivo*. This effect of HTRA1 on MP elimination was largely dependent on the presence of TSP1, an inhibitor of MP accumulation and CNV

(Calippe et al., 2017; Ng et al., 2009; Wang et al., 2012), as HTRA1 had little effect on *Thbs1*$^{-/-}$- and *CD47*$^{-/-}$-mice. The fact that the CD47 agonist peptides were sufficient to completely antagonize the HTRA1-induced increased monocytes survival strongly suggested that TSP1 degradation leading to the incapacity to activate CD47 is the predominant mechanism by which HTRA1 disrupts subretinal immune-suppression.

Indeed, our analysis of HTRA1-hydrolyzed TSP1 fragments, from full length TSP1 and its C terminal domaine, localized the two major cleavage sites between the two CD47-binding VVM motifs. HTRA1 hydrolysis of TSP1 reduced its ability to form CD47-CD47 complexes, which are important for CD47 activation: TSP1 lacking one of the VVM sites (McDonald et al., 2003) and TSP-2 with only one VVM site (Isenberg et al., 2009) do not efficiently activate CD47. Indeed, our 4N1K bi-peptides were 50-fold more efficient than mono-peptides to antagonize the effect of HTRA1 in the culture, confirming that the separation of the VVM sites by HTRA1 is likely sufficient to dramatically reduce TSP1s ability to activate CD47. Interestingly, the well described HTRA1-induced inhibition of TGF-ß signaling (Clausen et al., 2011) did not play a role in HTRA1-induced subretinal MP accumulation in accordance with the observation that HTRA1 and TGF-ß both promote CNV (Wang et al., 2017).

Mechanistically, we show that HTRA1 induced OPN, a pro-inflammatory cytokine that promotes macrophage survival (Bruemmer et al., 2003; Lund et al., 2009) and is increased in AMD donor tissue (Newman et al., 2012). Inhibition of endogenous HTRA1 reduced OPN secretion from macrophages, and the OPN induction by exogenous HTRA1 was to a large part repressed by CD47 activation in human macrophages. In mice, HTRA1-induced *Opn* expression in macrophages was much reduced in CD47$^{-/-}$ cells compared to wildtype. Together these results suggested that TSP1 degradation and loss of CD47 signaling plays a major role in OPN induction in macrophages. Experimentally, OPN was necessary for HTRA1s pro-inflammatory effect as *Opn* -deletion and inhibition blocked HTRA1-induced MP persistence. Clinically, macrophages from 10q26 AMD-risk mH/mH carriers also secreted higher amounts of OPN compared to cH/cH macrophages, suggesting that the risk-haplotype associated *HTRA1* increase is sufficient to promote OPN secretion.

Taken together our study shows that the increased HTRA1 expression in monocytes and monocyte-derived macrophages, associated with the AMD-risk mH of the 10q26 locus, hydrolyzes TSP1's C-terminal domain, compromising its ability to activate CD47 and repress

the MP-survival factor OPN. Increased HTRA1 and OPN expression in MPs of mH 10q26 locus carriers thereby promote a stronger inflammatory reaction and might in our evolutionary past have increased fitness towards infectious diseases, which could explain its high frequency today. In AMD, the stronger pathogenic inflammatory reaction might explain the indiscriminate association of the mH with all disease forms (Fritsche et al., 2013) as subretinal inflammation is observed in early and both late forms of AMD (Guillonneau et al., 2017). Interestingly, the AMD-associated CFH(H402) also inhibits CD47 and promotes pathogenic subretinal inflammation (Calippe et al., 2017), which might be the reason for both variants to be similarly associated with all forms of AMD (Fritsche et al., 2013). Current anti-inflammatory therapies, such as steroids and non-steroidal anti-inflammatory drugs (NSAID) paradoxically stimulate pro-inflammatory HTRA1 expression (Spugnini et al., 2006; van de Garde et al., 2014), which might account for their lack of therapeutic effect in AMD.

Our findings describe a comprehensive mechanism of how the predominant 10q26 AMD-risk haplotype promotes AMD pathogenesis. Our study provides rational for targeting HTRA1 and OPN or pharmacological activation of CD47 to induce the resolution of subretinal chronic inflammation that leads to irreversible blindness in AMD.

Limitations of Study

In our study of changes associated with the 10q26 AMD-risk haplotype, we focused on the evaluation of monocytes, which infiltrate the subretinal space in AMD alongside displaced resident macrophages (Sennlaub et al., 2013). Future studies will show whether the AMD-risk haplotype also affects gene expression in other immune cells implicated in AMD pathogenesis such as resident choroidal macrophages, microglial cells, or mast cells. It is also not yet clear to what degree endogenous HTRA1 hydrolyses TSP1 inside the monocyte-derived cells or mainly after secretion of both molecules. This question might have important therapeutic implications as non-cell penetrating HTRA1 inhibitors will not affect intracellular TSP1 degradation. On a similar note, our evaluation of the effect of HTRA1 was mainly based on the short-term addition of exogenous HTRA1. Hereafter, using transgenic over-expression in animal models, it will be important to evaluate the consequences of chronic HTRA1 over-expression in monocytes and other mononuclear phagocytes and to compare the effect to over-expression in non-immune cells such as RPE and photoreceptors, which produces an AMD-like phenotype (Iejima et al., 2015; Jones et al., 2011; Oura et al., 2018).

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Author contributions:

Conceptualization, F.S., Fa.Be., M.H., and C.R.; Investigation, Fa.Be., M.H., C.R., S.A., Y.Z., C.N., T.M., C.E., M.B., A.D-M., W.C., S.C., S.T., G.B., JB.C., H.C-M., A.P., JF.G., M.P., Fr.Bl., T.L., JE.R., C.D., X.G., F.S.; Writing – Original Draft, F.S., Fa.Be., M.H., and C.R., C.D., X.G. and P.S.; Writing- Review & Editing, F.S., Funding Acquisition, F.S., JA.S. ; Resources, E.K. ; Supervision, F.S., and X.G.

Declaration of interests:

The authors declare no competing interests.

Figures and figure legends

Fig. 1: Monocytes of homozygous carriers of the 10q26 risk-haplotype express increased levels of HTRA1 and HTRA1 is strongly expressed in retinal mononuclear phagocytes in AMD.

(A) Diagram of the intron–exon structures of *PLEKHA1*, *ARMS2*, and *HTRA1* genes of the chromosome 10q26 region from the Reference Sequence Collection (RefSeq), the corresponding open chromatin sites, representing the highest DNase I hypersensitive sites by the darkest stripes, of $CD14^+$monocytes, $CD4^+$T cells and RPE extracted from the ENCODE database. Schematic representation of the location of the AMD-risk defining single nucleotide polymorphisms (SNPs, indicated by the thick red line) and an expanded non linear representation of the four haplotype markers used to identify the common (cH) and the minor (mH) AMD-risk haplotype.

(B-E) Quantitative RT-PCR of the indicated mRNA normalized with the average of *ACTB*-, *G6PDH*-, *GAPDH*-, *HPRT1*-, *RPS26*-, and *RPL37A*- mRNA of fresh sorted blood lymphocytes (B) and $CD14^+$monocytes (B-E) of all 36 study participants (B) and cH/cH- and mH/mH- donors (C-E, n=18/group; Students t-test, *p = 0.0233; Mann–Whitney U-test, *p = 0.0337)

(F) HTRA1 protein concentrations of supernatants (ELISA) of 24h monocyte-cultures from cH/cH- and mH/mH- donors (n=18/group; Students t-test, *p = 0.0348; Mann–Whitney U-test, *p = 0.0416).

(G and H) Representative confocal images of central flatmounts of HTRA1 (green)/IBA-(red)-double-immuno-labeled retina and single HTRA1 (green)- immuno-labeled RPE/choroid from control- (G) and AMD- cH/cH-donor eyes (H). The location of the image planes is indicated in the cartoons.

Data are represented as mean ± SEM. CD4+T: CD4-expressing T lymphocytes; Mo: $CD14^+$monocyte; RPE: retinal pigment epithelium; L: lymphocyte; chr10: chromosome 10; cH/cH: homozygous common haplotype carriers; mH/mH: homozygous minor AMD-risk haplotype carriers; MC: microglial cell; MP mononuclear phagocyte. Scale=100µm

Fig. 2: HTRA1 hydrolyzes Thrombospondin-1 and separates its two CD47-binding sites.

(A) Silver nitrate stained polyacrylamide gel after electrophoretic migration of recombinant TSP1, recombinant HTRA1, or 12h HTRA1-incubated TSP1 of indicated quantities. TSP1 fragments (arrowheads).

(B) Peptide sequences with non-tryptic cleavage sites (red double slash) identified consistently and exclusively in HTRA1-digested TSP1 condition in three different analysis of two digestion preparations by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and identified in two complementary search engines (PEAKS and Mascot).

(C) Schematic representation of the native TSP1 protein: N terminal domain (Nter); Von Willebrand factor domain (square); Type-1, -2 and -3 domains, C terminal domain (Cter), Valine-Valine-Methionine motifs (VVM) and regions of interaction with indicated ligands. Positions of potential HTRA1-induced cleavage sites identified by mass spectrometry are indicated (dashed lines).

(D) Western blot of 12h-incubated TSP1 alone or with HTRA1 using an antibody raised against TSP1 N-terminal fourteen first amino acids. The four lower-weight bands identified after incubation with HTRA1 are indicated (arrow head) with the corresponding predicted schematic representation of TSP1 N-terminal cleavage product (right cartoons).

(E) Schematic representation of the recombinant TSP1 C-terminal domain with a FLAG-epitope at its N-terminus (Cter) and its theoretical N-terminal cleavage products after HTRA1 digestion.

(F) Silver nitrate stained polyacrylamide gel after electrophoretic migration of Cter, HTRA1, or their mixture after 12h-incubation (37°C) in 50µL of a buffer optimized for HTRA1 activity at the indicated quantities. HTRA1 hydrolyzes Cter into fragments detectable at lower-weight bands (arrowheads).

(G) Western blot of 12h-incubated Cter alone or with HTRA1 at the indicated quantities using an anti-FLAG antibody. Flagged N-terminal cleavage products of Cter by HTRA1 hydrolysis are visible at lower-weight bands (arrowheads).

(H-J) V5- and Myc -immunohistochemistry (H) and -proximity ligation assay (I) counterstained with Hoechst nuclear stain of mono- or co-transfected COS-7 cells with plasmids coding for a V5 tagged- and Myc tagged- human CD47 receptor. V5-Myc

complexes were revealed by PLA (red dots) of co-transfected cells, cells incubated with TSP1, or HTRA1-digested TSP1 for 10 min prior to fixation. The PLA fluorescence intensity was quantified (J); the experiment was repeated twice with similar results; (one-way ANOVA/Bonferroni test: CTL versus TSP1 p =0,0002; TSP1 versus HTRA1digTSP1 p < 0.0001).

Data are represented as mean ± SEM. Nter: N terminal domain Cter: C terminal domain; LAP-TGFb: Latency-associated peptide transforming growth factor beta; TSP1: thrombospondin 1; HTRA1: High-Temperature Requirement A Serine Peptidase 1; AA: amino acid. Scale bar: 50µm

Fig. 3: HTRA1-induced Thrombospondin-1 hydrolysis prevents CD47-dependent monocyte elimination.

(A-I) black lines and black-border columns represent conditions without, and red lines and red-border columns represent conditions with exogenous HTRA1 (*in vitro*: 5µg/ml; *in vivo*: injection solution containing 100µg/ml). Dotted horizontal lines indicate the level of the PBS and HTRA1 conditions.

(A) Representative pictures (overview and magnification) of PU-1 (myeloid nuclear marker, green) and OTX2 (RPE nuclear marker, red) double-labeled co-cultures of primary porcine RPE and human cH/cH-CD14^{+} blood monocytes (48h), incubated without (upper panels) or with HTRA1 (lower panels).

(B) Corresponding quantification of PU1^{+} monocyte-density in monoculture (left panel, continuous lines) or RPE/ human monocyte co-culture (left panel, dotted lines), and OTX2^{+}RPE cell-density in co-culture (right panel) after the indicated times (n = 6/group; one-way ANOVA/Bonferroni test: RPE+monocytes versus monocytes*$p < 0.0001$; RPE+monocytes+HTRA1 versus RPE+monocytes†$p < 0.0001$).

(C) Monocyte counts in 48h RPE/ human cH/cH-CD14^{+}monocyte co-cultures with control- or function-blocking- antibodies as indicated (10µg/ml), normalized for the number of monocytes in the HTRA1 condition (Mann-Whitney: monocytes versus monocytes+HTRA1 *p=0.004; cIgG versus aCD47 †p=0.0286; ‡p=0.0286; cIgG versus aCD36 and aTSP1 versus aTSP1+HTRA1 not significantly different)

(D) Monocyte counts in RPE/ human cH/cH-CD14^{+}monocyte co-cultures with PBS or HTRA1 (normalized for the number of monocytes in the HTRA1 condition). RPE or monocytes were preincubated for 2h with PBS or HTRA1 prior to a 48h co-culture without HTRA1 (Mann-Whitney: without versus with HTRA1 *p=0.0079; 2h monocyte PBS versus 2h monocyte HTRA1 †p=0.0079; 2h RPE HTRA1 versus non HTRA1 CTL not significantly different)

(E) Counts of CFSE-labeled mouse monocytes co-cultured during 48h with RPE cells. Monocytes, were sorted from bone marrow of C57BL6/J mice (WT) or the indicated knockout mouse strains, and cultured with rHTRA1 or PBS (Mann-Whitney: PBS (WT) *versus* rHTRA1 (WT) *p=0,0286; PBS (WT) *versus* PBS (CD47$^{-/-}$) †p=0.0286; PBS (WT) *versus* PBS (*Thbs1*$^{-/-}$) ‡p=0.0286; PBS (*Thbs1*$^{-/-}$) *versus* rHTRA1 (*Thbs1*$^{-/-}$) ‡p=0,0206)

(F) Monocyte counts in RPE / human cH/cH-CD14^{+}monocyte co-cultures in which the monocytes were incubated for 30 min with PBS or HTRA1, followed by a 60min incubation with PBS, TSP1, or recombinant C terminal domain of TSP1 (Cter; 200nM), before 48h co-culture without additional treatment (Mann-Whitney: 30'PBS/60'PBS versus 30'HTRA1/60'PBS *p=0,0079; 30'HTRA1/60'PBS versus 30'HTRA1/60'TSP1 †p=0.0079; 30'HTRA1/60'PBS versus 30'HTRA1/60'rCter ‡p=0.0159)

(G) Monocyte counts in 48h RPE/ human cH/cH-CD14^{+}monocyte co-cultures with PBS or HTRA1 treated with the indicated concentration of the control peptide, with 4N1K (a peptide derived from the Nter-proximal VVM site of TSP1) or a 4N1K bipeptide that we designed by linking two 4N1K peptides with an eight glycine spacer (Mann-Whitney: *p 4N1K-8G-4N1K and †p 4N1K versus CtrPep at the same concentrations: 200nM* p=0.0028, 1 to 50µM* and 20 to 50 µM† p<0.0001)

(H) Representative micrographs of RPE flatmounts and quantification of subretinally adoptively transferred CFSE-stained bone-marrow monocytes, 24h after the injection in PBS with or without HTRA1 containing in addition TSP1 (80nM), the control-, or 4N1K-bipeptide (150µM) as indicated. (Mann-Whitney: TSP1 versus PBS *p = 0,0315; HTRA1+TSP versus TSP1 †p<0,0001; HTRA1 versus PBS ‡p=0.0002; bi4N1K versus CtrBiPep $^{\$}$p=0,0044)

(I) Representative micrographs of RPE flatmounts and quantification of subretinally adoptively transferred CFSE-stained C57BL6/J and CD47$^{-/-}$ bone-marrow monocytes, 24h after the injection in PBS with or without HTRA1 containing in the control-, or 4N1K-bipeptide (150µM) as indicated. (Mann-Whitney: CD47$^{-/-}$ PBS versus C57/BL6/J PBS *p =0,0143, CD47$^{-/-}$ PBS versus CD47$^{-/-}$ HTRA1 and CD47$^{-/-}$ bi4N1K versus CtrBiPep not significant)

(J) Representative micrographs of IBA-1-stained RPE flatmounts and quantification of IBA1^{+}MPs of *Thbs1*$^{-/-}$ mice, exposed for four days to 4500 lux of green light (which induces subretinal MP accumulation in these mice) sacrificed at d6 after a subretinal injection at d4 with 4µl of PBS with or without HTRA1 containing in addition TSP1 (80nM), the control-, or 4N1K- bipeptide (150µM) as indicated. (Mann-Whitney: TSP1 versus PBS *p < 0,0001; HTRA1+rTSP versus TSP1 †p<0,0001; HTRA1 versus PBS ‡p=0.139; bi4N1K versus CtrBiPep $^{\$}$p=0,0005)

Data are represented as mean ± SEM. IBA-1: ionized calcium-binding adapter molecule 1; PU-1: transcription factor PU-1; OTX2: orthodenticle homeobox 2; Mo: monocyte; RPE: retinal pigment epithelial cell; cIgG: control immunoglobulin; aCD36: function-blocking CD36 antibody; aCD47: function-blocking CD47 antibody; aTSP1: function-blocking TSP1 antibody; CFSE: carboxyfluorescein diacetate succinimidyl ester; Thbs1: thrombospondin 1 gene; CtrPep: Control peptide; CtrBiPep: control bi-peptide; n indicated in each column except for B (n=6 per group) n=number of independent wells or injected eyes; Scale bar= 50µm.

Fig. 4: HTRA1 induced inhibition of CD47 signaling promotes osteopontin secretion

(A) Scatter dot blot of the 2202 protein coding mRNAs with a TPM (transcript per million) greater than 25 in the HTRA1-group and a FDR (false discovery rate smaller than 0,01) regulated by HTRA1in 6h human cH/cH- $CD14^+$monocyte culture. The transcripts are plotted according to their log2-fold induction by HTRA1 (X-axis) and their log2-fold repression by 2,5µM bi4N1K in the presence of HTRA1 (Y-axis). The number of transcripts of each quadrant, HTRA1-induced transcripts (red) and transcripts repressed by bi4N1K in the presence of HTRA1 (blue) are indicated. The ten dots representing transcripts that were induced at least 16 fold (4 log2) by HTRA1 (HTRA1 versus control) and most repressed by 2,5µM bi4N1K are indicated and listed.

(B) TPMs of the ten top regulated mRNAs in human monocytes cultured for 6h with PBS, HTRA1 and HTRA1 and 2,5M bi4N1K peptide. (n=3 per group, p values see Figure S4A).

(C) Osteopontin concentrations in supernatants (ELISA) from 24h human cH/cH- $CD14^+$monocyte-derived macrophages cultured with PBS, HTRA1 and the peptides (2,5µM) as indicated. (n indicated in column; Mann-Whitney: PBS versus HTRA1 *p <0,0001; bi4N1K versus CtrBiPep $^{\$}$p=0,0095)

(D) Quantitative RT-PCR of S26mRNA-normalized *Opn*- mRNA expression in 6h and 18h C57BL6/J and *Cd47*$^{-/-}$ bone-marrow monocyte-derived macrophages stimulated with HTRA1 as indicated (n=4; Mann-Whitney: C57BL6/J versus *Cd47*$^{-/-}$ *p=0.0002; non- versus HTRA1-stimulated C57BL6/J and *Cd47*$^{-/-}$cells $^{\$}$p and †p =0,0286)

(E) Quantitative RT-PCR of *TSP1*-, *HTRA1*-, and *OPN*- mRNA of fresh sorted human cH/cH- $CD14^+$ monocytes of at the indicated time points (n=5/group; relative expression to 0h).

(F) ELISA quantification of OPN in supernatants of 72h human cH/cH- monocyte-derived macrophages cultures treated with the indicated concentrations of the HTRA1 inhibitor DPMFKLboroV (n=5; Mann–Whitney U-test versus control, *p <0,0001)

(G) Linear regression of the correlation of *HTRA1*- mRNA (X-axis) of fresh, sorted $CD14^+$ monocytes with their *OPN* mRNA expression in 24h monocyte-derived macrophages (Y-axis) from all study participants (n=36; three data points are outside the graph to better visualize the majority of points; dotted lines represent 95% confidence bands of the continuous regression line; slope 1,36+/-0,4235; P=0,0029 deviant fom zero; R^2=23%)

(G) Quantitative RT-PCR of normalized *OPN*- mRNA of 24h monocyte-derived macrophages of cH/cH- and mH/mH- donors (n=18/group; Students t-test, *p = 0.0155; Mann–Whitney U-test, *p = 0.0218)

(H) ELISA quantification of OPN and APOE concentrations of supernatants of 24h human monocyte cultures from cH/cH- and mH/mH- donors (n=17/group; Students t-test, *p = 0.0244; Mann–Whitney U-test, *p = 0.0477).

Data are represented as mean ± SEM. TPM: transcripts per million; CSF: colony stimulating factor; MMP: matrix metallopeptidase; TMEM: transmembrane protein; RGS16: regulator of G protein signaling 16; SPRY2: sprouty RTK signaling antagonist 2; DPYSL3: dihydropyrimidinase like 3; NR4A2: nuclear receptor subfamily 4 group A member 2; OPN: osteopontin; TSP1: thrombospondin; bi4N1K: bipeptide formed by two 4N1K peptides linked by an eight glycine spacer; CtrBiPep: control bi-peptide; cH/cH: homozygous common haplotype carriers; mH/mH: minor AMD-risk haplotype carriers; CTL: healthy control subject; AMD: age-related macular degeneration patient.

Fig. 5: Inhibition of osteopontin reverses HTRA1-induced MP survival.

(A) Representative micrographs of PU-1 (myeloid nuclear marker, green) and OTX2 (RPE nuclear marker, red) labeled cells after human monocytes were co-cultured for 48h with primary RPE cells with or without exogenous HTRA1 (5µg/ml) in the culture medium in addition to the anti human OPN-blocking antibody (aOPN; 50µg/ml) or the control IgG (cIgG; 50µg/ml) as indicated and quantification of $PU1^+$monocyte cell density at 48h after the co-culture in the indicated condition (aOPN; 50µg/ml) and quantification of $PU1^+$monocytes at 48h after the co-culture of the indicated groups. (Mann-Whitney: aOPN versus cIgG *p =0,0025)

(B) Representative micrographs and cell density quantification of subretinal $CFSE^+$ mouse C56BL6/J- and $Opn^{-/-}$ -monocytes, 24h after subretinal adoptive transfer to wildtype recipients in PBS or PBS with HTRA1 (100µg/ml), additionally to the anti human OPN-blocking antibody (aOPN; 10µg/ml) or the control IgG (cIgG; 10ug/ml) as indicated (Mann-Whitney: HTRA1 $Opn^{-/-}$ versus HTRA1 C57BL6/J: $^{€}p<0,0001$; aOPN versus cIgG *p <0,0001).

(C) Representative pictures of IBA-1-stained RPE flatmounts MPs of *Thbs1*$^{-/-}$ mice, exposed for 4d to 4500 lux of green light sacrificed at d6 after subretinal injection at d4 of 4µl of PBS containing HTRA1 (100µg/ml) with either cIgG or the anti-mouse OPN-blocking antibody (aOPN; 10µg/ml) and quantification of $IBA1^+$MPs of the indicated groups (Mann-Whitney: HTRA1+aOPN versus HTRA1+cIgG *p<0,0001).

(D and E) Representative micrographs of RPE flatmounts of CD102 (red) and IBA-1 (green) immunohistochemistry 10 days after laser injury (D). The mice were intravitreally injected at d4 and d7 with 2µl of PBS or PBS containing HTRA1 (100µg/ml) in addition to the anti-mouse OPN-blocking antibody (10µg/ml, aOPN) or the control IgG (10µg/ml, cIgG). Quantification of subretinal IBA-1^+ MPs situated on the RPE were counted at a distance of 0-500µm from $CD102^+$ CNV (D) and quantification of the $CD102^+$ area (E) of *Thbs1*$^{-/-}$ -, *Opn*$^{-/-}$ - ,and wildtype- C57BL6/J mice injected with the indicated compounds following the same protocol (Mann Whitney; PBS $Thbs^{-/-}$ versus PBS C57BL6/J (D)‡p=0.0002, (E)‡p=0.0160 ; TSP1 $Thbs^{-/-}$ versus PBS $Thbs^{-/-}$ (D)$^{\$}$p=0,0028, (E)$^{\$}$p=0,0007; PBS *Opn*$^{-/-}$ versus PBS C57BlL6/J (D)$^{\%}$p=0,003, (E)$^{\%}$p=0,0413; PBS *Opn*$^{-/-}$ versus HTRA1 *Opn*$^{-/-}$ (D)$^{€}$p=0,1294, (E)$^{€}$p=0,0939; HTRA1 C57BL6/J versus PBS C57BL6/J (D and E)*p<0,0001; aOPN versus cIgG (D)†p <0,0001, (E)†p <0,0001) .

Data are represented as mean ± SEM. *Thbs 1*: thrombospondin 1 gene; TSP1: thrombospondin 1; *Opn*: Osteopontin gene; cIgG: control IgG; aOPN: anti-osteopontin antibody. n=number of eyes. Scale bar: 20µm.

STAR Methods

RESOURCE AVAIBILITY

Lead Contact:

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Florian Sennlaub (florian.sennlaub@inserm.fr).

Materials availability and Data and Code Availability

This study did not generate new unique reagents. The peptide sequences of all peptides are indicated and can be produced by any peptide producing company. The study also did not make use of any unpublished custom code, software, or algorithm that is central to supporting the main claims of the paper

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals:

Wildtype (C57BL/6J) mice, $Opn^{-/-}$- and $Thbs1^{-/-}$-mice were obtained from Charles River and the Jackson laboratories respectively. All mice used in this study were male of two- to three-months old, weighted approximatively 25mg and were rd8 mutation free, as this mutation can lead to an AMD-like phenotype (Mattapallil et al., 2012). Male mice were used to eliminate the influence of the reproductive cycle on neovascularization. The mice were kept to the indicated ages under specific pathogen-free condition in a 12 hr/12 hr light/dark (100 lux) cycle with no additional cover in the cage and with water and normal chow diet available *ad libitum*. All experimental protocols and procedures were approved by the French Ministry of higher Education, Research and Innovation (laser-induced CNV model and intravitreal injections APAFIS#2218-2015090416008740, laser-induced CNV and light-challenge models on $Thbs1^{-/-}$ mice APAFIS#19412-2019022215422220, subretinal adoptive transfer of monocytes N°APAFIS#20132-201904041225363 and laser-induced CNV on $Opn^{-/-}$ mice N°APAFIS#22513-2019101817209742).

Primary cells:

Human Peripheral Blood Mononuclear Cells (PBMCs)

In accordance with the Declaration of Helsinki, all volunteers provided written and informed consent for the human monocyte expression studies, which was approved by the Direction Générale pour la Recherche et l'Innovation of the Ministère de l'Enseignement et

de la Recherche (Dossier n°14.007) and by the Commission Nationale de l'Informatique et des Libertés (N/Ref.: IFP/MKE/AR144088).

Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated from subjects with previously documented 10q26 haplotype status, as they participated in the Parisian branch of an AMD-GWAS study (1129 participants, 107 mH/mH-carriers, 412 mH/cH-, and 610 cH/cH-carriers) (Fritsche et al., 2013). Eighteen mH/mH-study participants of the Parisian region positively answered to our invitation for an ophthalmological re-examination and a blood sampling. The group was composed of 9 control subjects (absence of large drusen and late AMD) and 9 patients with neovascular AMD. They were homozygous-risk-nucleotide-carriers of all the markers of the 10q26 AMD-associated haplotype block included in our GWAS analysis (rs10490924, rs3750846, rs3750847, rs3750848, rs11200638, rs3793917, rs1049331, rs2293870, rs2284665, and rs932275) that define the common 10q26 risk haplotype (Liao et al., 2017). None of the patients displayed one of the rare, incomplete haplotype blocks. A group of 18 age-, and sex-matched homozygous cH/cH- carriers composed of 9 control subjects and 9 wet AMD patients (sFig. 1) was recruited in parallel. PBMCs from healthy cH/cH male donors from 25 to 50 years old were used served for all other experiments.

Briefly, human peripheral mononuclear cells from blood were obtained after a Ficoll (GE Healthcare) gradient centrifugation. $CD14^+$monocytes were isolated by negative selection using the EasySep Human Monocyte Enrichment Cocktail (StemCell Technologies) and cultivated in X-Vivo15 (Lonza) at 37°C and 5% CO_2.

Primary porcine RPE

Primary porcine RPE cells were isolated as previously described (Mathis et al., 2017). Porcine eyes were bought at a local slaughterhouse (Guy Harang, Houdan, France) in agreement with the local regulatory department and the slaughterhouse veterinarians. Porcine eyes were obtained 2 or 3 hr after enucleation in CO_2-independent serum (Thermo Fisher Scientific). Eyes were cleaned for muscles and immersed few minutes in an antiseptic solution (Pursept-A Xpress, Merz Hygiene GmbH). Anterior segment of the bulb was removed, as well as lens, vitreous, and retina. Each eyecup was washed 2 times with PBS (Thermo Fisher Scientific) and incubated for 1 hr at 37 °C with 0.25% trypsin–EDTA (Thermo Fisher Scientific). RPE cells were pipetted off the choroid and re-suspended in Dulbecco's modified Eagle's serum (DMEM, Thermo Fisher Scientific) supplemented with

20% fetal calf serum (FCS, Thermo Fisher Scientific) and 1% antibiotics penicillin/streptomycin (PS). Purified cells were then seeded on a 60mm Petri dish in DMEM-FCS20%-PS1% and incubated with a controlled atmosphere at 5% CO_2 at 37 °C. The culture medium was changed 24 h after the seeding. When cells were confluent, 0.05% trypsin–EDTA was added for 5 min at 37 °C to detach the RPE cells. They were finally seeded on 96-well culture plate (Corning) at the concentration of 75 000 cells/well in DMEM-FCS20%-PS1%. Confluence state was obtained after 3 days in culture at 37 °C, 5% of CO_2.

Mice bone marrow monocytes (BMM)

Mice bone marrow monocytes (BMM) were harvested from 2- to 3-month-old male C57BL/6, Thbs1$^{-/-}$, CD36$^{-/-}$ or CD47$^{-/-}$ mice killed by CO_2 inhalation. BMM were flushed from femurs and tibiae with PBS containing 5% fetal bovine serum. BMM were negatively selected by magnetic sorting following the protocol suggested by the manufacturer (EasySep Mouse Monocyte Enrichment Kit; Stemcell Technologies). Briefly, the mouse monocyte enrichment mixture is designed to enrich mouse monocytes from mouse bone marrow by depletion of T-cells, B-cells, NK cells, dendritic cells, progenitors, granulocytes, and red blood cells using a combination of biotinylated monoclonal antibodies directed against these cell-surface antigens. Unwanted cells were specifically labeled with dextran-coated magnetic particles using biotinylated antibodies against cell-surface antigens expressed on the unwanted cells. Magnetically labeled cells were then separated from unlabeled target cells by using a magnet. The purity of the BMM was assayed by flow cytometry using CD11b, LY6C, and LY6G markers. The CD11b+ LY6C+ LY6G− (BMM) cell content of the enriched cells ranges from 85 to 95%. The rest of the enriched cells were CD11b+ LY6G+ cells (neutrophils).

Human donor eyes:

Human eyes were obtained from donors giving written consent from the Minnesota Lions Eye Bank. For this study, four control maculae from four subjects and four patients with known history of wet AMD and visible large drusen on dissection were used. Samples were immersed in a 4% paraformaldehyde solution and stored at -80°C.

METHOD DETAILS

Search for open chromatin and transcription factor binding sites

The Encyclopedia of DNA Elements (ENCODE) database was used to search for DNase I sensitive sites within the human genome; this database has results from more than 125 cell types, including primary cultures of RPE, T lymphocytes, and monocytes.

Human blood monocyte isolation and culture, RNA extraction, RT-PCR and Enzyme-linked immunosorbent assay (ELISA) and Milliplex magnetic bead analysis

Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated from subjects described above. Briefly, human peripheral mononuclear cells from blood were obtained after a Ficoll (GE Healthcare) gradient centrifugation. $CD14^+$monocytes were isolated by negative selection using the EasySep Human Monocyte Enrichment Cocktail (StemCell Technologies). A part of the obtained purified monocytes were seeded in 48 well plates at 200 000 cells/well and cultivated for 24h in X-Vivo15 (Lonza) at 37°C and 5% CO_2. HTRA1 and OPN concentrations were determined in the medium using HTRA1- (E-EL-H0423, Elabscience) and OPN- ELISA Kit (DOST00, R&D Systems). APOE, C3, α2 microglobulin and CCL2 were determined using magnetic bead panels (Milliplex MAP, Millipore,). Total RNA were isolated with Nucleospin RNAII (Macherey Nagel) from the remaining isolated monocytes and the Ficoll gradient containing the lymphocyte fraction. Single-strand cDNA was synthesized with 1µg of RNA pretreated with DNase amplification grade, using oligo-dT as primer and superscript II reverse transcriptase (Thermo Fisher Scientific). For real-time PCR, 1/100 of cDNA was incubated with the polymerase and the appropriate amounts of nucleotides (TaqMan Gene Expression Master Mix, Applied Biosystems; Power SYBR Green PCR Master Mix, Applied Biosystems). qPCR were realized with the StepOne Plus real-time PCR system (Applied Biosystems) using the following parameters: 45 cycles of 15s at 95°C, 45s at 60°C. Results were normalized with expression of six housekeeping genes: GAPDH, RPS26, HPRT1, G6PDH, ACTB and RPL37A.

In a separate set of experiments, purified monocytes from healthy male cH/cH donors were seeded in 96 well plates at 150 000 cells/well and cultivated for the indicated times in X-Vivo15 (Lonza) at 37°C and 5% CO_2. They were incubated in the different experiments as indicated with PBS (control), 80nM of recombinant TSP1 (Carrier Free, 3074-TH-050, R&D), 5µg/ml of recombinant HTRA1 (Carrier Free, 2916-SE-020, R&D), rTSP1 with rHTRA1, rHTRA1 with 2,5µM of the bi4N1K peptide or rHTRA1with 2,5µM of the control bi-peptide

(see co-culture treatments), or the HTRA1 inhibitor DPMFKLboroV (Roche). RNA was extracted at 3, 6, h for real-time PCR analysis (normalized with GAPDH and RPS26) and supernatants of 24h and 72h culture were collected for OPN ELISA quantifications.

Gene expression analysis

For whole transcriptome analysis, healthy volunteer $CD14^+$monocytes were prepared as described above, seeded in 24 well plate at 500 000 cells/well in X-Vivo15 medium and stimulated for 6h with PBS, recombinant HTRA1 (5µg/ml; R&D) or rHTRA1 and bi4N1K peptide (2,5µM). After cell lysis, RNA was extracted using the Qiagen RNA Mini Kit with RNase (ribonuclease)–free DNase (deoxyribonuclease) I digestion. RNA quality and quantity were evaluated using BioAnalyzer 2100 with the RNA 6000 Nano Kit (Agilent Technologies). RNA sequencing libraries were constructed from 1 µg of total RNA using a modified TruSeq RNA Sample preparation kit protocol. Pass-filtered reads were mapped using STAR v2.6.1c and aligned to human reference genome GRCh38.92 (Dobin et al., 2013). The count table of the gene features was obtained using FeatureCounts (Liao et al., 2014). Normalization, differential expression analysis and TPM (transcript per million) values were computed using EdgeR (Liao et al., 2014). Protein coding mRNAs with greater than 25 in the HTRA1-group and a false discovery rate <0,01 were selected.

Donor eyes and immunohistochemistry

Immunohistochemistry was performed on RPE/choroid/sclera and retinal pieces from healthy and AMD donors dexcribed above. Samples were immersed in a 4% paraformaldehyde solution and stored at -80°C. Tissues were thawed at room temperature, rinsed with PBS and post-fixed 5 minutes in ice cold acetone 100% solution. Samples were then treated with primary antibodies: anti-HTRA1 antibody (1:100, ab38611, Abcam) and anti-IBA1 antibody (1:100, ab5076, Abcam) in PBS containing 0.1% Triton X-100 for one day at room temperature with gentle rocking. After few washes in PBS, samples were incubated for 5 hours at room temperature with appropriate Alexa Fluor® conjugated secondary antibodies (1:500) in 0.1% Triton solution and were counterstained with Hoechst (1:1000, 33258, Thermo Fisher Scientific). Preparations were rinsed, mounted on glass slides with Fluoromount aqueous mounting medium (Sigma-Aldrich). Acquisitions were made with an Olympus FV1000 (Olympus, Rungis, France) confocal microscope.

Synthesis of recombinant TSP1 Cter domain (rCter)

We designed a protein encompassing human TSP1 C-terminal sequence from Asp834 until Pro1169 fused to a HAVT20 leader peptide amino acid sequence followed by a Flag tag at the N-terminus of the TSP1 C terminal domain (Supplemental Figure 2D). The recombinant protein was produced and purified in human embryonic kidney cells by GTP Technology (Labège, France).

In vitro digestion of recombinant TSP1 and its Cterminal domain by HTRA1

HTRA1-digested recombinant TSP1 and Cter domain was prepared by mixing 1,5 µg of recombinant human TSP1 (Carrier Free, 3074-TH-050, R&D; rCter see above) with 50 to 880 ng of recombinant human HTRA1 (Carrier Free, 2916-SE-020, R&D) in 50µL of digestion buffer (50mM Tris HCl pH8.0; 150nM NaCl,) for 12 hr at 37°C. For controls, TSP1 (1,5µg), rCter (1,5µg) or HTRA1 (880ng) were incubated alone in the same conditions.

Silver nitrate staining and Western blot fragment analysis

Samples were diluted in 4X Laemmli buffer and heated at 95°C for 5-10 min before loading on the NuPAGE 4-12 % Bis-Tris gel. Each gel was loaded with PageRuler Prestained protein ladder (Thermo Fisher Scientific). Gels were either processed for silver nitrate staining or transferred to nitrocellulose membranes (0,2 µm, GE Healthcare) by BioRad blotting system for Western Blotting according to manufacturer's protocol. Immunoblots were blocked with 5% skimmed milk powder in TBS 1X 0,2% Tween, then probed with the following antibodies at a 1:500 dilution in the same buffer: Goat anti-Thrombospondin N-ter (ab111230, Abcam), Mouse anti-FLAG® M2 (F3165, Sigma Aldrich). Secondary antibodies, donkey anti-goat HRP (705-035-003, Jackson ImmunoResearch), Horse anti-mouse HRP (PI2000, Vector Laboratories), were applied at a dilution of 1/5 000 in blocking buffer. Chemoluminescence was performed using Pierce ECL-plus (32132, Thermo Fisher Scientific) and the ChemiDoc Imaging System (Biorad). Putative molecular mass of the fragments resulting from HTRA1 digestion was calculated using the ExPASy Compute pI/Mw bioinformatics tool.

Mass spectrometry

Tryptic digestion

Proteins were submitted to reduction by incubation with 5 mM dithiotreitol in 50 mM ammonium bicarbonate (AmBic) for 30 min at 37°C, then alkylation with 15 mM

iodoacetamide in 50 mM AmBic for 30 min, RT. Tryptic digestion was performed with a protein/enzyme ratio of 25/1 at 37°C overnight in 50 mM AmBic.

Mass spectrometry analysis

Two replicates of *in vitro* digestion of TSP1 (1,5µg) by HTRA1 (880ng) and TSP1 alone conditions were processed by LC-MS/MS. The second replicate was processed twice for LC-MS/MS. Peptide mixtures were supplemented with formic acid to a final concentration of 0.1% and analyzed on a U3000 nanoLC (Thermo) coupled to an HCTultra ion trap (Bruker). Peptides were concentrated and desalted for 5 min on a precolumm RP-C18 (5 mm, 300 µm i.d., 100 Å, Thermo) with a mobile phase A (2 % ACN/0.1 % formic acid) at a flow rate of 20 µL/min then separated on an analytical column RP-C18 (15 cm, 75 µm i.d., 100 Å, Dionex) at a flow rate of 300 nL/min. Elution gradient was run from 2% to 10 % of solvent B (95% ACN/0.1 % formic acid) in 10 min then 10% to 35% B in 60 min and 35% to 50% B in 10 min. The ion trap was used in the positive mode with the selection of 8 precursors from each MS spectrum for fragmentation by collision induced dissociation (CID). Capillary voltage was set at 2 kV, Full scan spectra were acquired in the mass range 250 to 1600 m/z and MS/MS spectra were acquired from 100 to 2800 m/z with singly charged ions exclusion, a dynamic exclusion of 30 sec and an isolation width of 4 Da. ICC smart target was set to 250'000 and the target mass to 622 m/z.

Protein identification

Raw data obtained by LC-MS/MS were processed using Data Analysis 3.4 (Bruker). Mgf files were generated with a maximum of 5000 compounds with a signal intensity threshold of 100'000 (AU) and spectra deconvolution. Protein identification was performed with ProteinScape 2.1 (Bruker) using Mascot 2.2.07 with SwissProt database (01/04/15), Homos sapiens taxonomy (20203 entries). Trypsin was selected as the enzyme with 2 missed cleavages. Carbamidomethylation of Cys was set as a fixed modification and oxidation of Met as variable modifications; MS tolerance and MS/MS tolerance were set at 0.5 Da. A p value < 0.05 was required for peptide validation (Supp Table 2). In addition, analyses were performed using semi-trypsin as the enzyme using the same parameters (Supp Table 1). LC-MS/MS data were also processed with the search engine PEAKS (PEAKS Studio 8.5) with the same parameters extended to 3 missed cleavages and acetylation (N-ter) as a variable modification. Semi-tryptic peptides were considered real if identified by both search engines (Mascot with a p value < 0.05 and PEAKS with an FDR < 1%) in all HTRA1-digested TSP1 experiments (2 replicates and the 2nd MS processing) and absent from the TSP1 alone condition. The mass

spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2019) with the dataset identifier PXD020546

CD47V5 CD47Myc proximity ligation assay

COS-7 cells, plated into a 24-well plate coated with Poly-L-Lysine, were mono- and co-transfected with plasmids coding for human CD47 with a C-terminal V5 or Myc flag using Lipofectamine 2000 reagent (Life). After 48 hours of transfection, mono- (immunohistochemistry) and co-transfected cells (immunohistochemistry and proximity ligation assay; PLA) were rinsed with PBS, fixed 10 minutes in 4% paraformaldehyde solution, rinsed, permeabilized and blocked by incubating cells 10minutes in 0,2% gelatin in PBS containing 0,25% Triton X-100 (PBS-GT). Cells were incubated overnight with mouse monoclonal anti-V5 Tag (Invitrogen, R960-25; 1/2000) and rabbit polyclonal anti-myc Tag (Abcam, ab9106; 1/2000) antibodies at 4°C under gentle agitation in the same solution. For immunohistochemistry, mono- and co-transfected cells were then incubated with donkey anti-rabbit-Alexa-488, anti-mouse-Alexa-594 secondary antibodies, and Hoechst (Thermo Fisher Scientific) 2 hours at room temperature. For Duolink® PLA assays anti-rabbit and anti-mouse oligonucleotides-labeled secondary antibodies (PLA probes) were incubated, followed by a ligase and polymerase reaction following manufacturer's instructions to amplify the signal. For the PLA experiments cells were starved for 2h and incubated 5 min with DMEM, recombinant TSP1 (R&D Systems) in DMEM 10µg/ml, or HTRA1-digested TSP1 in DMEM (10µg/ml previously incubated 16h with HTRA1) prior to fixation. Images were taken on an Olympus FLUOVIEW FV1000 confocal laser-scanning microscope or a fluorescence microscope (DM5500, Leica) to quantify the PLA fluorescence intensity per cell using MetaMorph (Molecular Devices).

RPE Monocyte Co-culture (Mathis et al., 2017)

Porcine RPE cells and mouse bone marrow monocytes (BMM) were obtained as described above. The day before experiment, RPE cells were serum-starved. A total of 100 000 freshly purified human monocytes from a healthy donor purified as described above, or 50 000 mouse BMM were added to confluent RPE cells in High-Glucose DMEM- 1% Penicillin and Streptomycin. Cells were incubated with human recombinant HTRA1 (5µg/mL, R&D Systems), a control peptide (KRFYGGMWKK), the 4N1K CD47-activating peptide (KRFYVVMWKK), double peptides where two 4N1K were linked by a 8 glycine spacer or a $(CH2CH2O)_7$ polyethylene glycol spacer, a control bi-peptide

(KVRMKFYVWKGGGGGGGGKVRMKFYVWK) (Genepep), human recombinant TSP1 (80nM, R&D Systems) or blocking antibodies : a CD36 blocking antibody (10µg/ml, Ab17044, Abcam), a CD47 blocking antibody (10µg/ml, Ab3283, Abcam), a TSP1 blocking antibody (5µg/ml, MA5-13390, Thermo Fisher Scientific), function blocking anti-human OPN (10µg/ml, AF1433, R&D). and a mouse anti-IgG isotype control (5µg/ml, MAB002, R&D Systems). Co-cultures were incubated at 37°C and 5% CO_2 for 48 hr. At the end of co-culture, cells were washed with PBS and fixed in a 4% paraformaldehyde solution for 10 min. Fixed cells were washed twice in PBS and incubated for 2 min in a permeabilization solution (freshly prepared 0.1% triton and 0.1% sodium citrate in PBS). Cells were blocked for 1 hr in PBS and triton 0.1% containing 5% horse serum (Thermo Fisher Scientific) and incubated overnight at 4 °C with the primary antibodies: polyclonal rabbit anti-human PU.1 (1:200, PA5-17505, Thermo Fisher Scientific) and polyclonal goat anti-human OTX2 (1:500, AF1979, R&D Systems) diluted in PBS triton 0.1% and 1% horse serum. Washed cells were then incubated for 1 hr at room temperature with appropriate Alexa Fluor® conjugated secondary antibodies (1:500) in 0.1% Triton solution and were counterstained with Hoechst (1:1000, 33258, Thermo Fisher Scientific). The cells were washed and acquisitions were made with a fluorescent microscope (Arrayscan VTI HCS Reader, Thermo Fisher Scientific). Twenty-five fields per well were analyzed and recorded by Arrayscan software (HCS iDev Cell Analysis Software, Thermo Fisher Scientific). Results are percentage of the number of PU.1 or OTX2 positive cells and normalized with the HTRA1 treated condition.

QPCR on bone marrow-derived Monocyte (BMM) mono-culture

50 000 mice BMM were seeded on 96-well plate in High-Glucose DMEM 1% Penicillin and Streptomycin. After 18 hours incubation, total RNA was prepared with NucleoSpin RNA Plus XS (Macherey-Nagel) and cDNA was synthesized with QuantiTect Reverse Transcription Kit (Qiagen). For real time PCR, 1/100 of cDNA was used per reaction using PowerSYBR green PCR mix and StepOne Plus apparatus and software (Applied Biosystems). *SPP1* gene (Opn) to S26 ratios were determined from three independent assays by the 2−DCt method. PCR conditions: PCR cycles: 15 s at 95°C, 30 s at 60°C, 30 s at 72°C.

Subretinal adoptive mononuclear phagocyte transfer and clearance (Calippe et al., 2017; Levy et al., 2015)

BMMs were sorted as described above, labeled in 10µM CFSE (Thermo Fisher Scientific), washed and resuspended in PBS. 12000 cells (in 4µL) were injected in the subretinal space of anesthetized WT male mice (10-14 weeks old) using glass microcapillaries

(Eppendorf) and a microinjector as previously described (Levy et al., 2015). A hole was pierced with the glass capillary prior to the injection in order to avoid intra-ocular pressure increase and to allow retinal detachment with 4µl of solution. The subretinal injection was verified by fundoscopy. In specific experiments, the cells were co-injected with recombinant human TSP1 (10 mg/mL), recombinant human HTRA1 (100µg/ml), or both, the control bi peptide, or the bi-4N1K CD47-activating peptide (150 µM), control IgG or function blocking anti-mouse OPN (10µg/ml). Eyes were enucleated after 24 hours, fixed 30 minutes in PFA 4% and counterstained with Hoechst nuclear stain. Eyes with hemorrhages were discarded. CFSE+ cells in the subretinal space were quantified on flatmounts on the RPE side of the retina and on the apical side of the RPE.

In vivo laser-injury (Lavalette et al., 2011) and light-challenge experiments (Hu et al., 2015; Levy et al., 2015)

Laser-coagulations were performed on male mice (Vitra Laser, 532 nm, 450 mW, 50 ms, and 250 mm), which were intravitreally injected (days 4 and 7) using glass capillaries (Eppendorf) and a microinjector, with 1 µL of PBS, recombinant human TSP1 (10 mg/mL), recombinant human HTRA1 (100µg/ml), or both, the control IgG or function blocking anti-mouse OPN (10µg/ml) and sacrificed at day 10.

For the light-challenge model, two- to three-months old male Thbs1$^{-/-}$-mice were adapted to darkness for 6 hours, pupils dilated daily and exposed to green LED light (starting at 2AM, 4500 Lux, JP Vezon Equipements) for 4 days, injected subretinally (as for adoptive transfer experiments) with 4µl of the indicated solutions of PBS, recombinant human TSP1 (10 mg/mL), recombinant human HTRA1 (100µg/ml), or both, the control bi peptide, or the 4N1K CD47-activating peptide (150µM), control IgG or function blocking anti-mouse OPN (10µg/ml) and subsequently kept for 2days in cyclic 12h/12h normal facility conditions as previously described (Sennlaub et al., 2013).

After fixation in a 4% paraformaldehyde solution, the retinas and choroids were incubated with anti-IBA-1 (1:400, 019-19741, Wako) and anti-CD102 (for laser-experiments only; 1:200, 553325, BD Pharmingen) in PBS containing 0.1% Triton X-100 for 12h at room temperature with gentle rocking. After few washes in PBS, samples were incubated for 2 hr at room temperature with appropriate Alexa Fluor® conjugated secondary antibodies (1:500) in PBS solution and were counterstained with Hoechst (1:1000, 33258, Thermo Fisher Scientific). Preparations were rinsed, mounted on glass slides with Fluoromount aqueous

mounting medium (Sigma-Aldrich). Preparations were observed under a fluorescence microscope (DM5500, Leica) and the surface covered by CD102+CNV was measured on photographs and the average CNV size was calculated; IBA-1+ MPs on the RPE were counted in a diameter of 500µm around the CD102+ neovascularizations; IBA-1+ cells were counted on whole RPE/choroidal flat-mounts and on the outer segment side of the retina for the light-challenge model.

QUANTIFICATION AND STATISTICAL ANALYSIS

Gene expression analysis:

Pass-filtered reads of whole transcriptome were mapped using STAR v2.6.1c and aligned to human reference genome GRCh38.92 (Dobin et al., 2013). The count table of the gene features was obtained using FeatureCounts (Liao et al., 2014). Normalization, differential expression analysis and TPM (fragments per kilobase of exon per million fragments mapped) values were computed using EdgeR (Liao et al., 2014). Protein coding mRNAs with greater than 10 TPMs in the HTRA1 group and an false discovery rate <0,01 were selected.

Analyze of the CD47V5 CD47Myc proximity ligation assay:

Images were taken on an Olympus FLUOVIEW FV1000 confocal laser-scanning microscope or a fluorescence microscope (DM5500, Leica) to quantify the PLA fluorescence intensity per cell using MetaMorph (Molecular Devices).

Quantification of RPE/Monocyte Co-culture:

Acquisitions were made with a fluorescent microscope (Arrayscan VTI HCS Reader, Thermo Fisher Scientific). Twenty-five fields per well were analyzed and recorded by Arrayscan software (HCS iDev Cell Analysis Software, Thermo Fisher Scientific). Results are rxpressed as percentages of the number of PU.1 or OTX2 positive cells and normalized with the HTRA1 treated condition.

Quantification in laser-injury and light-challenge model:

The surface covered by CD102+CNV was measured on photographs and the average CNV size was calculated; IBA-1+ MPs on the RPE were counted in a diameter of 500µm around the CD102+ neovascularizations; IBA-1+ cells were counted on whole RPE/choroidal flat-mounts and on the outer segment side of the retina for the light-challege model.

STATISTICAL ANALYSIS

GraphPad Prism8 (GraphPad Software) was used for data analysis and graphic representation. All values are reported as mean ± SEM. Experiments were analyzed with the nonparametric Student t-test and/or Mann–Whitney U-test, as indicated in the legends. $P < 0.05$ was considered statistically significant. The n and P-values are indicated in the figures and the figure legends.

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