cGAS drives noncanonical-inflammasome activation in age-related macular degeneration

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Geographic atrophy is a blinding form of age-related macular degeneration characterized by retinal pigmented epithelium (RPE) death; the RPE also exhibits DICER1 deficiency, resultant accumulation of endogenous Alu-retroelement RNA, and NLRP3-inflammasome activation. How the inflammasome is activated in this untreatable disease is largely unknown. Here we demonstrate that RPE degeneration in human-cell-culture and mouse models is driven by a noncanonical-inflammasome pathway that activates caspase-4 (caspase-11 in mice) and caspase-1, and requires cyclic GMP-AMP synthase (cGAS)-dependent interferon-β production and gasdermin D–dependent interleukin-18 secretion. Decreased DICER1 levels or Alu-RNA accumulation triggers cytosolic escape of mitochondrial DNA, which engages cGAS. Moreover, caspase-4, gasdermin D, interferon-β, and cGAS levels were elevated in the RPE in human eyes with geographic atrophy. Collectively, these data highlight an unexpected role of cGAS in responding to mobile-element transcripts, reveal cGAS-driven interferon signaling as a conduit for mitochondrial-damage-induced inflammasome activation, expand the immune-sensing repertoire of cGAS and caspase-4 to noninfectious human disease, and identify new potential targets for treatment of a major cause of blindness.

Age-related macular degeneration (AMD) affects more than 180 million people1 and is the leading cause of blindness among elderly people worldwide. Geographic atrophy, an advanced form of AMD, is characterized by degeneration of the RPE, a monolayer of cells that provide trophic support to photoreceptors2,3. Levels of the RNase DICER1 are lower in the RPE in human eyes with geographic atrophy than in unaffected eyes, thus leading to accumulation of toxic Alu mobile-element RNA transcripts4; these Alu transcripts induce RPE cell death by activating the NLRP3 inflammasome5. Although NLRP3-inflammasome activation has been widely implicated in macular degeneration6–8, the mechanisms regulating the inflammasome in this disease remain elusive. Here we found that DICER1 deficit and Alu-RNA-driven RPE degeneration in mouse models of macular degeneration are mediated by caspase-4- and gasdermin D–dependent inflammasome activation.

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Unexpectedly, the activity of this noncanonical inflammasome was dependent on the activation of cGAS-driven type I interferon (IFN) signaling by cytosolic mitochondrial DNA (mtDNA).

RESULTS
Caspase-4 is activated in AMD
Caspase-4 (or caspase-11 in mice), which governs noncanonical-inflammasome activation, has recently been implicated in the immune response to exogenous pathogen-associated molecular patterns such as intracellular lipopolysaccharide (LPS)\(^9,^{10}\) and endogenously produced oxidized phospholipids (oxidized 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine (oxPAPC))\(^{11}\). We found that caspase-4 abundance in the RPE and choroid in human eyes with geographic atrophy was significantly higher than that in normal human eyes from aged subjects, as monitored by western blotting (Fig. 1a and Supplementary Fig. 1a). Introduction of in vitro–transcribed \(Alu\) RNA or plasmid-mediated enforced expression of \(Alu\) RNA (\(pAlu\)) induced and activated caspase-4 in primary human RPE cells, as
evidenced by increased abundance of p30 cleavage fragments (Fig. 1b and Supplementary Fig. 1b,c). Antisense-oligonucleotide-mediated knockdown of Dicer1 similarly induced caspase-4 activation in human RPE cells (Fig. 1b), and this effect was blocked by concomitant antisense-mediated inhibition of Alu RNA (Supplementary Fig. 1d). Caspase-11 activation was induced by subretinal injection of Alu RNA in wild-type (WT) C57BL/6J mice (Fig. 1c) and by Alu RNA transfection in primary RPE cells isolated from WT mice (Supplementary Fig. 1e). Collectively, these data indicate that caspase-4 is preferentially activated in human AMD and that dysregulation of Dicer1 and Alu RNA lead to caspase-4 activation in this condition.

Caspase-4 is required for Alu-RNA-induced RPE degeneration and inflammasome activation

We sought to determine whether caspase-4 is required for Alu-RNA-induced RPE degeneration, which was quantified by both binary grading and semiautomated cellular morphometry (Online Methods and Supplementary Fig. 2). Exogenous delivery or endogenous overexpression of Alu-RNA induced RPE degeneration in WT mice (Fig. 1d and Supplementary Fig. 1f). In contrast, neither Alu RNA nor pAAlu injected subretinally induced RPE degeneration in caspase-11-knockout mice (denoted Casp11−/−; official gene symbol Csfp4) (Fig. 1d and Supplementary Fig. 1f). 129S6 mice, which lack functional caspase-11 because of a passenger mutation12, were also resistant to RPE degeneration induced by Alu RNA or pAAlu (Supplementary Fig. 1g). Subretinal delivery of a cell-permeable, nonimmunogenic cholesterol-conjugated siRNA (17 + 2 nt in length)13,14 targeting Subretinal delivery of a cell-permeable, nonimmunogenic cholesterol-conjugated siRNA (17 + 2 nt in length)13,14 targeting Caspase-11- and caspase-1-dependent pyroptotic cell death can be executed by the pore-forming protein gasdermin D (encoded by Gsdmd)18–20. We found that Gsdmd−/− mice were resistant to Alu-RNA-induced RPE degeneration (Fig. 2a and Supplementary Fig. 5a,b). In agreement with the role of gasdermin D in noncanonical-inflammasome activation by intracellular LPS15, Alu-RNA-induced caspase-1 activation and IL-18 secretion were lower in Gsdmd−/− mouse RPE cells than in WT cells (Fig. 2b,c). However, caspase-11 activation in Gsdmd−/− mice was not impaired (Fig. 2d), suggesting that loss of caspase-1 activation in Gsdmd−/− mouse RPE cells was not due to an indirect effect of gasdermin D on caspase-11, and that, mechanistically, caspase-11 functions upstream of gasdermin D.

Gasdermin D is required for Alu-RNA-induced RPE degeneration and inflammasome activation

Caspase-11- and caspase-1-dependent pyroptotic cell death can be executed by the pore-forming protein gasdermin D (encoded by Gsdmd)18–20. We found that Gsdmd−/− mice were resistant to Alu-RNA-induced RPE degeneration (Fig. 2a and Supplementary Fig. 5a,b). In agreement with the role of gasdermin D in noncanonical-inflammasome activation by intracellular LPS15, Alu-RNA-induced caspase-1 activation and IL-18 secretion were lower in Gsdmd−/− mouse RPE cells than in WT cells (Fig. 2b,c). However, caspase-11 activation in Gsdmd−/− mice was not impaired (Fig. 2d), suggesting that loss of caspase-1 activation in Gsdmd−/− mouse RPE cells was not due to an indirect effect of gasdermin D on caspase-11, and that, mechanistically, caspase-11 functions upstream of gasdermin D.

Gasdermin D must be cleaved into a pore-forming p30 fragment in order to execute pyroptosis21. Interestingly, although gasdermin D was required for Alu-RNA-induced RPE degeneration and IL-18 secretion, we did not observe its cleavage into a p30 fragment in Alu-RNA-treated RPE cells in cell culture or in RPE cells in vivo (Fig. 2e); however, as previously reported15, intracellular LPS induced p30 cleavage in mouse bone marrow–derived macrophages (BMDMs) (Fig. 2e).

Next, we directly tested whether gasdermin D p30 cleavage was dispensable for the toxicity of Alu RNA by subjecting Gsdmd−/− mice to reconstitution with either WT gasdermin D (pGSDMD-WT) or a gasdermin D mutant (pGSDMD-D276A) unable to undergo cleavage into the pyroptotic p30 fragment19. Notably, expression of either WT or mutant gasdermin D in Gsdmd−/− mice restored susceptibility to Alu-induced RPE degeneration (Fig. 1i and Supplementary Fig. 3d), suggesting that both caspase-4/11 and caspase-1 are required for Alu toxicity.

Because we have previously demonstrated that PYCARD, an adaptor protein involved in inflammasome activation, and the purinoceptor P2X7 (encoded by P2rx7) are required for Alu toxicity5,16,17, we next assessed whether PYCARD and P2X7 might also be required for Alu-RNA-induced caspase-11 activation. Alu-RNA-induced activation of caspase-11 occurred at low levels in P2rx7−/− but not Pycard−/− mouse RPE cells, suggesting that caspase-11 is mechanistically downstream of P2X7 and upstream of PYCARD (Supplementary Fig. 3e,f). Collectively, these findings support a model of noncanonical-inflammasome activation by Alu RNA wherein caspase-4/11 functions upstream of caspase-1 activation.

Recent studies have implicated an endogenous lipid molecule, the oxidized phospholipid oxPAPC, in caspase-11-mediated noncanonical NLRP3-inflammasome activation11. To test whether Alu RNA promotes accumulation of this type of endogenous ligand, we extracted lipids from Alu-RNA-treated primary human RPE cells and used liquid chromatography–mass spectrometry to quantify the following products of 1-palmitoyl-2-arachidonoyl-3-phosphatidylcholine (PAPC): 1-palmitoyl-2-glutaryl-3-phosphatidylcholine (PGPC), 1-palmitoyl-2-(5-oxovaleryl)-3-phosphatidylcholine (POVPC), and 1-palmitoyl-2-hydroxy-3-phosphatidylcholine (lysoPC) (Supplementary Fig. 4a–c). Compared with control cells, Alu-RNA-treated human RPE cells exhibited a twofold increase in oxPAPC PGPC and lysoPC levels (Supplementary Fig. 4d) concomitantly with a trend toward lower precursor PAPC levels (Supplementary Fig. 4d). These results suggest an indirect mechanism of Alu-driven caspase-11 engagement, possibly via oxidized-phospholipid-derived damage-associated molecular patterns (DAMPs).
Alu-RNA-induced RPE degeneration (Fig. 2f), suggesting a nonapoptotic function of gásdémín D in this system.

Previously, we have demonstrated that Alu RNA induces activation of caspase-3 (refs. 4,22) as well as caspase-8, Fas, and FasL, and that this well-characterized pathway of apoptotic induction is critical for the RPE toxicity of Alu RNA. In addition, we and others have provided molecular evidence consistent with apoptosis in the RPE in human eyes with geographic atrophy4,23. To further clarify the role of apoptosis in Alu-RNA-induced cell death, we performed live-cell imaging of annexin V and propidium iodide (PI) staining in primary human RPE cells. Cells treated with Alu RNA developed plasma membrane blebs and displayed an annexin V+ PI+ staining pattern consistent with early apoptosis. After exhibiting several hours of annexin V positivity, cells frequently swelled and became PI positive, characteristics consistent with late apoptosis or secondary necrosis24 (Supplementary Fig. 6a,b). In vivo studies recapitulated these cell culture findings:
RPE flat mounts from Alu-RNA-exposed WT mice displayed predominantly annexin V+PI− cell death (Supplementary Fig. 7a). Alu-RNA treatment of RPE cells induced cleavage of caspase-3 and poly (ADP–ribose) polymerase 1 (PARP-1) (Supplementary Fig. 7b), further supporting induction of an apoptotic cell-death pathway. Along with our earlier demonstration that neither necrostatin-1, an inhibitor of primary necrosis, nor glycine, an inhibitor of pyroptosis25, blocks Alu-RNA-induced RPE degeneration26,27, these findings suggest that Alu RNA promotes cell death primarily via apoptosis rather than pyroptosis or necrosis in RPE cells.

Next, we explored the roles of IL-18 and gasdermin D in Alu-RNA-induced cell death. The resistance of Gsdmd−/− mice to Alu-RNA-induced RPE degeneration was overcome by administration of recombinant mature IL-18 or subretinal injection of an expression plasmid for mature IL-18, suggesting that this resistance was due to loss of IL-18 secretion (Fig. 2g). In support of this concept, Alu RNA did not induce secretion of IL-18 in Gsdmd−/− mouse RPE cells, and this effect was rescued by treatment of the cells with either pGSDMD-WT or the p30–cleavage-incompetent pGSDMD-D276A (Supplementary Fig. 5c). Additionally, whereas annexin V+ cells were not visible in RPE flat mounts for Alu-RNA-treated Gsdmd−/− mice, administration of recombinant mature IL-18 led to the appearance of numerous annexin V+PI− cells (Supplementary Fig. 8). These results, together with our previous demonstration that IL-18 neutralization or IL-18-receptor deficiency in mice blocks Alu-RNA toxicity in vivo5, suggest that gasdermin D is required for Alu-RNA-induced inflammasome activation and for RPE toxicity driven by IL-18-dependent apoptosis.

GSDMD mRNA abundance was higher in the RPE in human eyes with geographic atrophy than in eyes from unaffected age-matched controls (Fig. 2h). In contrast, we observed similar levels of transcripts of MIP-1α, IL-8, and IL-6 in geographic atrophy and normal specimens (Supplementary Fig. 5d), suggesting that global elevation of proinflammatory cytokines does not occur in geographic atrophy; instead, a more specific increase occurs in the expression of inflammasome-pathway genes. We also observed higher gasdermin D protein expression in the RPE in human eyes with geographic atrophy than in eyes from unaffected age-matched controls (Fig. 2i).

**Alu-RNA-induced noncanonical-inflammasome activation is driven by type I IFN signaling**

To interrogate the upstream regulation of caspase-4, we focused on IFN signaling, which is involved in activation of the caspase-11–driven noncanonical inflammasome10. Alu RNA did not induce RPE degeneration or caspase-11 activation in Ifnar1−/− mice or Ifnar1−/− mouse RPE cells (Fig. 3a,b), which are deficient in the type I IFN-α/β receptor (IFNAR). Treatment with recombinant IFN-β increased caspase-4 abundance in human RPE cells (Fig. 3c). Alu RNA induced secretion of IFN-β (Fig. 3d) and phosphorylation of IRF3 (Supplementary Fig. 9a), a transcription factor that induces production of IFN-β. Alu RNA also induced phosphorylation of STAT2 (Fig. 3e and Supplementary Fig. 9a,b), a signaling molecule activated by type I IFNs downstream of IFNAR. Alu-RNA-induced RPE degeneration was blocked by administration of an IFN-β-neutralizing antibody (Fig. 3f). Moreover, Alu RNA did not induce RPE degeneration in Ifr5−/− or Stat2−/− mice (Fig. 3g and Supplementary Fig. 9c, d), and induction of caspase-11 activation by Alu RNA was lower in Stat2−/− mouse RPE cells than in WT cells (Supplementary Fig. 9e). Human eyes with geographic atrophy, compared with eyes from unaffected age-matched controls, displayed pronounced IFN-β expression in the RPE (Fig. 3h,i). Together, these data suggest that Alu-RNA-induced RPE degeneration is dependent on regulation of the noncanonical inflammasome by type I IFN signaling.

cGAS-driven IFN signaling licenses noncanonical NLRP3 inflammasomes

We sought to identify the upstream activator of IRF3–driven IFN signaling induced by Alu RNA. We have previously shown that Alu-RNA-induced RPE degeneration is independent of several IRF3-activating signaling molecules, including various RNA sensors: TLR3, TLR4, TLR9, RIG-I, MDA5, MAVS, and TRIF5. However, cGAS (encoded by Mb21d1) has emerged as an innate immune sensor that can activate type I IFN signaling26. Additionally, cGAS has also been reported to have a role in broadly inhibiting several RNA viruses27.

We found that Alu RNA upregulated cGAS at the mRNA and protein levels in human RPE cells (Supplementary Fig. 10a,b). In contrast to WT mouse RPE cells, Alu RNA did not induce IFN-β (Fig. 4a), activate caspase-1 (Fig. 4b and Supplementary Fig. 10c) or caspase-11 (Fig. 4c), or induce IL-18 secretion (Fig. 4d) in Mb21d1−/− mouse RPE cells. In contrast, inflammasome activation by MSU crystals was unimpaired in Mb21d1−/− mouse RPE cells (Supplementary Fig. 3c). In human RPE cells, DICER1–knockdown-mediated IFN-β induction, STAT2 phosphorylation, and activation of caspase-4 and caspase-1 were all inhibited by knockdown of cGAS (Fig. 4e,f and Supplementary Fig. 10d,e). Moreover, Alu RNA did not induce RPE degeneration in Mb21d1−/− mice (Fig. 4g and Supplementary Fig. 10f,g). Additionally, reconstitution with mouse cGAS restored IFN-β induction in Mb21d1−/− mouse RPE cells and RPE degeneration in Mb21d1−/− mice (Fig. 4h, Supplementary Fig. 10h and Supplementary Fig. 11a). Moreover, the resistance of Mb21d1−/− mice to Alu-RNA-induced RPE degeneration was overcome by administration recombinant IFN-β or expression of IFN-β via subretinal plasmid transfection (Fig. 4i), suggesting that this resistance was indeed due to a lack of IFN signaling.

cGAS protein was more abundant in the RPE in human eyes with geographic atrophy than in eyes from unaffected age-matched controls (Fig. 5a). cGAS-driven IFN signaling can be transduced by the adaptor protein STING (encoded by Tmem173)28. Alu RNA did not induce IRF3 phosphorylation (Supplementary Fig. 11b,c) or activation of caspase-1 (Fig. 5b) or caspase-11 (Fig. 5c) in Tmem173−/− mouse RPE cells, nor did it induce RPE degeneration in Tmem173−/− mice (Fig. 5d), suggesting the involvement of the cGAS-STING signaling axis in this system. As in the case of Mb21d1−/− mice, the resistance of Tmem173−/− mice to Alu-RNA-induced RPE degeneration was overcome by administration of recombinant IFN-β or expression of IFN-β via subretinal plasmid transfection (Fig. 5e), results again suggesting a requirement for IFN signaling in Alu-RNA-induced RPE degeneration.

**Alu-driven cGAS activation is triggered by engagement with mtDNA**

cGAS is activated by cytosolic DNA but not by poly(I:C), a synthetic double-stranded RNA analog26. In agreement with the notion that cGAS does not recognize RNA directly, Alu RNA did not bind cGAS in an RNA immunoprecipitation assay (data not shown). Previous studies have implicated mitochondrial dysfunction in macular degeneration, including mtDNA damage, production of reactive oxygen species, and downregulation of proteins involved in mitochondrial energy production and trafficking25,28,29. Cytosolic escape of mitochondrial components, such as DNA and formyl peptides, has been shown to activate innate immune pathways, including cGAS30.
Both Alu RNA stimulation and DICER1 knockdown in human RPE cells increased the cytosolic abundance of mtDNA (Fig. 6a and Supplementary Fig. 12a,b). To examine whether Alu RNA triggers engagement of mtDNA by cGAS, we performed a pulldown assay to assess DNA–protein interaction in Mb21d1−/− immortalized mouse embryonic fibroblasts (MEFs) subjected to reconstitution with hemagglutinin (HA)-tagged cGAS30. Because these cells express HA-cGAS from a genomically integrated DNA sequence, they were expected to express HA-cGAS at levels similar to endogenous cGAS expression levels. We observed enrichment of mtDNA in cGAS immunoprecipitates.
**Figure 4** cGAS-driven signaling licenses the noncanonical inflammasome and degeneration of the RPE. (a) Relative abundance of *Ifnb1* mRNA in WT and *Mb21d1−/−* mouse RPE cells mock transfected or transfected with *Alu* RNA. Data are mean ± s.e.m.; *n* = 4 cell-culture replicates; *P = 0.0001*, two-tailed *t* test. (b) Immunoblots of pro-caspase-1 (Pro-Casp1) and the p20 cleavage product of caspase-1 (Casp1 p20) in WT and *Mb21d1−/−* mouse RPE cells transfected with *Alu* expression plasmid (pAlu) or empty-vector control (pNull). (c) Immunoblots of pro-caspase-11 (pro-Casp11) and the p30 cleavage product of caspase-1 (Casp1 p30) in WT and *Mb21d1−/−* mouse RPE cells transfected with *Alu* expression plasmid or empty-vector control. (d) IL-1β secretion by WT and *Mb21d1−/−* mouse RPE cells mock transfected or transfected with *Alu* RNA. Data are mean ± s.d.; *n* = 3 independent experiments; *P = 0.032*, two-tailed *t* test. (e) Relative abundance of *IFNB1* mRNA in control (shScramble) or cGAS shRNA-knockdown human RPE cells transfected with DICER1 or control (ctr) antisense oligonucleotides (AS). Data are mean ± s.e.m.; *n* = 3 cell-culture replicates; *P = 0.0002*, two-tailed *t* test. (f) Top, immunoblot of phosphorylated STAT2 (pSTAT2); pro-caspase-4 and caspase-4 p30; pro-caspase-1 and caspase-1 p20 in control (shScramble) or cGAS shRNA-knockdown human RPE cells mock transfected or transfected with *Alu* RNA. Bottom, immunoblot of cGAS demonstrating knockdown efficiency of cGAS shRNA. (g) Fundus photographs and immunofluorescence staining of zonula occludens-1 (ZO-1) on RPE flat mounts from eyes of WT (*n* = 6 eyes) and *Mb21d1−/−* (*n* = 8 eyes) mice subretinally injected with vehicle or *Alu* RNA. (h) Fundus photographs and immunofluorescence staining of ZO-1 on RPE flat mounts from eyes of *Mb21d1−/−* mice subretinally injected with *Alu* RNA (*n* = 7 eyes) and reconstituted with *in vivo*-transfected cGAS expression plasmid (pFlag-cGAS; *n* = 4 eyes) or control GFP expression plasmid (pFlag-GFP; *n* = 3 eyes). (i) Fundus photographs and immunofluorescence staining of ZO-1 on RPE flat mounts from eyes of *Mb21d1−/−* mice subretinally injected with *Alu* RNA and with recombinant IFN-β (*n* = 6 eyes), vehicle control (*n* = 6 eyes), IFN-β expression plasmid (pIFNB; *n* = 5 eyes), or empty-vector control (*n* = 5 eyes). For all immunoblots, cropped gel images of bands of interest of representative immunoblots from three independent experiments and densitometric analysis (mean (s.e.m.)) are shown. In g, h, and i, the degenerated retinal area is outlined by blue arrowheads in the fundus images; loss of regular hexagonal cellular boundaries in ZO-1-stained flat mounts is indicative of degenerated RPE. RPE degeneration was assessed through binary quantification (healthy (%)) and morphometric quantification (PM, polymegethism (mean (s.e.m.))) (Fisher’s exact test for binary; two-tailed *t* test for morphometry; *P < 0.05; *P < 0.01; **P < 0.001). For all micrographs, scale bars, 20 µm.

of *Alu*-RNA-stimulated but not mock- or poly(I:C)-stimulated cells (Fig. 6b and Supplementary Fig. 12c), suggesting that mtDNA in the cytosol engages cGAS. The positive-control transfected plasmid DNA was also enriched in the cGAS immunoprecipitate in this assay (Supplementary Fig. 12d). Additionally, subretinal delivery of mtDNA induced RPE degeneration in WT but not *Mb21d1−/−* mice (Supplementary Fig. 12e). Similarly, in cell-culture studies, mtDNA-induced *Ifnb* mRNA levels were lower in *Mb21d1−/−* than
in WT mouse RPE cells (Supplementary Fig. 12f). These data indicate that Alu-RNA-induced RPE degeneration is mediated via release of mtDNA into the cytosol, subsequent interaction of mtDNA with cGAS, and induction of IFN-β expression.

The mitochondrial permeability transition pore is required for Alu-driven mtDNA release

Under conditions of cellular stress, opening of the mitochondrial permeability transition pore (mPTP) leads to mitochondrial swelling, rupture, and release of mitochondrial contents into the cytosol. In cells lacking mitochondrial peptidyl-prolyl cis–trans isomerase F (PPIF, also known as cyclophilin D), a key enzyme involved in mPTP opening, mitochondria are resistant to swelling and the permeability transition. Using JC-1 and cobalt–calcein assays, we found that Alu RNA induced a decrease in the mitochondrial-membrane potential (ΔΨm), which is indicative of mPTP opening, in WT but not Ppif−/− mouse RPE cells, as determined by using the potential-sensitive fluorochrome JC-1 and assessing quenching of the calcein signal (Supplementary Fig. 12g-h). In addition, cyclosporin A, which inhibits mPTP opening via binding to PPIF, blocked Alu-RNA-induced mPTP opening in WT cells but did not alter ΔΨm or calcein signal intensity in Ppif−/− cells (Supplementary Fig. 12g-h). Collectively, these findings suggest that Alu RNA induces Ppif-dependent mPTP opening in RPE cells.

In agreement with an effect of Alu RNA on mPTP opening, Alu RNA triggered mtDNA release into the cytosol in WT but not Ppif−/− mouse RPE cells (Fig. 6c). Ppif−/− mice were protected against Alu-RNA-induced RPE degeneration (Fig. 6d), confirming the in vivo importance of mPTP in Alu toxicity; moreover, Alu-RNA-induced activation of caspase-1 and caspase-11 was lower in Ppif−/− mouse RPE cells than in WT cells (Fig. 6f). In human RPE cells lacking mitochondrial DNA (Rho0 ARPE19 cells), Alu RNA no longer activated caspase-4 (Fig. 6g), nor did it induce secretion of IL-18 (Fig. 6h) or IFN-β (Fig. 6i). Furthermore, the resistance of Ppif−/− mice to Alu-RNA-induced RPE degeneration was overcome by administration of recombinant IFN-β or expression of IFN-β via subretinal plasmid transfection (Fig. 6j). Collectively, these data support a model wherein mPTP-driven mitochondrial permeability mediates cytosolic release of mtDNA, which in turn promotes noncanonical-inflammation–mediated activation via engaging the cytosolic DNA sensor cGAS, which in turn induces IFN signaling.

Alu-driven RPE toxicity does not require macrophages or microglia

We focused on the RPE as the cellular locus of inflammasome activation because we have previously demonstrated that the various molecular abnormalities associated with geographic atrophy—DICER1 deficiency, Alu-RNA accumulation, and elevated

Figure 5 cGAS in geographic atrophy and RPE degeneration. (a) Immunolocalization of cGAS in the RPE in human geographic-atrophy eyes and age-matched unaffected controls. Representative images from control and dry AMD eyes are presented; n = 4 eyes per group. (b) Immunoblots of pro-caspase-1 and the p20 cleavage product of caspase-1 (Casp1 p20) in WT and Tmem173−/− mouse RPE cells transfected with Alu expression plasmid (pAlu) or empty-vector control plasmid (pNull). (c) Immunoblots of pro-caspase-11 and the p30 cleavage product of caspase-11 (Casp11 p30) in WT and Tmem173−/− mouse RPE cells mock transfected or transfected with Alu RNA. (d) Fundus photographs and immunofluorescence staining of zonula occludens-1 (ZO-1) on RPE flat mounts from eyes of WT (n = 6 eyes) and Tmem173−/− (n = 10 eyes) mice subretinally injected with vehicle (n = 7 eyes) or Alu RNA (n = 9 eyes). (e) Fundus photographs and immunofluorescence staining of ZO-1 on RPE flat mounts from eyes of Tmem173−/− mice subretinally coadministered Alu RNA with recombinant IFN-β (n = 4 eyes) or vehicle control (n = 4 eyes); or IFN-β expression plasmid (pIFNB, n = 4 eyes) or empty vector control (pNull; n = 4 eyes). For all immunoblots, cropped gel images of bands of interest of representative immunoblots from three independent experiments and densitometric analysis (mean (s.e.m.); n = 4 eyes per group) are shown. (f) In WT mouse RPE cells, the degenerated retinal area is outlined by blue arrowheads in the fundus images; loss of regular hexagonal cellular boundaries in ZO-1-stained flat mounts is indicative of degenerated RPE. RPE degeneration was assessed by binary quantification (healthy (%)) and morphometric quantification (PM, polymegethism (mean (s.e.m.))) (Fisher’s exact test for binary; two-tailed t test for morphometry; *P < 0.05; **P < 0.01; ***P < 0.001). For all micrographs, scale bars, 20 μm.
levels of NLRP3, PYCARD, cleaved caspase-1, and phosphorylated IAK1/4—are localized to the RPE layer of human eyes with geographic atrophy. Our current observations that elevated levels of cGAS, gadermin D, cleaved caspase-4, and IFN-β are similarly localized to the RPE layer in diseased eyes supports the notion that this cell layer is the locus of molecular perturbations in the noncanonical-inflammasome pathway.

However, recently reported observations of macrophages and microglia in the vicinity of pathology in human eyes with geographic atrophy call the functional involvement of these immune cells into RPE degeneration in mice. Using mouse chimeras, we have also demonstrated that RPE-cell-specific ablation of Myd88, the adaptor critical for IL-18-induced RPE cell death in this system, is sufficient to prevent Alu-RNA-induced RPE degeneration in mice. Using mouse chimeras, we have also...
demonstrated that ablation of Myd88 in circulating bone-marrow-derived cells does not prevent Alu-RNA-induced RPE degeneration. Nevertheless, given that macrophages and microglia are capable of inflammasome signaling, we studied their involvement more directly. We depleted macrophages by using clodronate liposomes and depleted microglia by administering tamoxifen to Cx3cr1CreER ROSA-DTA mice. Although these manipulations successfully depleted macrophages and microglia, respectively, neither type of depletion blocked Alu-RNA-induced RPE degeneration in vivo, providing direct evidence that these two cell populations are dispensable for RPE toxicity in this system (Supplementary Fig. 13).

Although these two cell types are apparently not required by Alu RNA to elicit RPE degeneration in mice, they might have subtle effects on disease pathology. In agreement with this possibility, we found that Alu RNA activated noncanonical inflammasomes in mouse BMDMs (Supplementary Fig. 14). Similarly to the observed effects in RPE cells, the ability of Alu RNA to induce caspase-1 activation was impaired in Casp1−/−, Mbx2id1−/−, and Gsdmd−/− BMDMs, as compared with WT BMDMs (Supplementary Fig. 14). Collectively, these findings suggest that cGAS-driven licensing of the noncanonical inflammasome by Alu RNA is not restricted to RPE cells.

**DISCUSSION**

Our data identify an unexpected role of the DNA sensor cGAS, the noncanonical caspase-4 inflammasome, and gasdermin D in mediating Alu-RNA-induced RPE cell death, both in mice in vivo and in human cell culture. Coupled with observations that cGAS, IFN-β, caspase-4, and gasdermin D are more abundant in the RPE in human eyes with geographic atrophy than in control unaffected eyes, our findings suggest the involvement of these proteins in the pathogenesis of this form of AMD. cGAS was originally recognized as a sensor of exogenous and endogenous cytosolic DNA that mediates IRF3-driven IFN signaling, and previous studies have demonstrated that the enzymatic activity of cGAS cannot be activated by an RNA stimulus. Nonetheless, cGAS has been reported to be critical for the antiviral response to multiple RNA viruses, although the mechanistic underpinnings of this effect are not fully understood. In this context, our work provides a new view of how endogenous RNAs can activate cGAS and raises the possibility that cGAS-driven antiviral immunity may involve Alu RNA, whose levels can be stimulated by viral infections, thereby leading to mitochondrial dysfunction.

Mitochondria have been increasingly implicated as gatekeepers of cell fate that have decisive roles in diverse cellular responses including apoptosis, autophagy, and innate immunity. Mitochondria can facilitate the innate immune response to infection and injury via release of mitochondrial components that are recognized as DAMPs by the cell’s innate immune components. Of note, mtDNA can activate the NLRP3 inflammasome by directly interacting with NLRP3 (ref. 40) or by amplifying the response to an initial trigger, such as ATP or reactive oxygen species; moreover, mtDNA can activate TLR9 on neutrophils, thereby triggering systemic lung and liver inflammation. In addition to engaging TLR9 and NLRP3 signaling, mtDNA has also recently been reported to be involved in the activation of cGAS signaling via cytosolic escape of mtDNA as a consequence of mitochondrial stress. We have previously demonstrated that TLR9 signaling is dispensable for Alu-RNA-induced RPE degeneration and that NLRP3-inflammasome priming is unaffected in mouse RPE cells lacking TLR9 (ref. 17). Instead, our present findings show that Alu-RNA-driven cytosolic mtDNA release leads to activation of both inflammasome and cGAS signaling pathways, thus highlighting the role of mitochondria as a signaling platform that integrates various cellular-stress cues into an innate immune signaling response in autoimmune and chronic inflammatory diseases.

Beyond its role in responding to infections, cGAS has been implicated in mouse models of autoimmune diseases and mouse tumor models. Our findings expand the functional repertoire of this innate immune sensor to chronic degenerative diseases. Activation of the noncanonical NLRP3 inflammasome by caspase-4/11 has been implicated in Gram-negative bacterial infection, sepsis, and antimicrobial defense at the mucosal surface. To our knowledge, our study provides the first reported example of caspase-4–driven noncanonical inflammasome activation in a noninfectious human disease. The involvement of caspase-4 and cGAS in other conditions such as systemic lupus erythematosus and diabetes mellitus, wherein Alu-RNA accumulation has been observed, bears future investigation. Activation of caspase-4 has been observed in conditions of endoplasmic reticulum stress. Given that several human diseases including Alzheimer’s disease and obesity-associated type 2 diabetes, both of which are driven by a hyperactive inflammasome, are associated with endoplasmic reticulum stress, it would also be worth exploring whether DICER1 deficiency and/or Alu-RNA-induced mitochondrial dysfunction and cGAS- and caspase-4–dependent inflammasome activation are linked to endoplasmic reticulum stress.

Gasdermin D has not, to our knowledge, previously been shown to be involved in a noninfectious human disease. Mechanistically, we found that gasdermin D functions downstream of caspase-11 activation and is required for Alu toxicity. The role of gasdermin D in this system appears not to be induction of pyroptosis, unlike its response to exogenous triggers such as intracellular LPS. Instead, gasdermin D supports Alu-RNA-induced RPE cell apoptosis by promoting IL-18 secretion; notably, we did not observe cleavage of gasdermin into its p30 fragment, a process required for its pyroptotic effect. Additional studies are required to determine the mechanisms that disengage the pore-forming function of gasdermin D from the inflammasome-activating function, i.e., caspase-1 activation and IL-18 secretion. The effects of noncanonical-inflammasome–dependent gasdermin D activation might be dictated by the activating trigger (for example, exogenous versus host) or the cell type. For instance, the only other endogenous molecule known to activate caspase-11, oxPAPC, also does not induce pyroptosis but instead triggers IL-1β release from dendritic cells.

The mechanisms underlying regulation of the NLRP3 inflammasome by caspase-11/4 have been elusive. Previously, we have demonstrated that Alu-RNA-induced RPE degeneration and NLRP3-inflammasome activation depend on NF-kB and P2X7 (refs. 16,17). In our current work, we found that Alu-RNA-induced caspase-11 activation levels were low in P2rx7−/− mouse RPE cells, suggesting that P2X7 is required for both caspase-1 and caspase-11 activation. Together, our observations suggest that Alu RNA–driven NLRP3-inflammasome activation requires both caspase-11/4 and P2X7. We also observed that Alu RNA induces oxPAPC synthesis, thus suggesting that Alu RNA might recruit other DAMPs that activate the noncanonical inflammasome and result in RPE cell toxicity. Earlier reports have implicated oxidized phospholipids in the pathophysiology of age-related macular degeneration; future studies to unravel the underlying molecular mechanisms should be performed.
Numerous groups using a variety of cell culture systems, animal models, and human-donor eyes have reported an important role of the inflammasome in AMD. Collectively, these studies suggest that the NLRP3 pathway is an important responder to a panoply of AMD-related molecular stressors and toxins in RPE cells, and inflammasome activation has attracted great interest as a therapeutic approach for AMD. Our identification of cGAS, IFN-β, caspase-4, and gasdermin D as critical mediators in inflammasome-driven RPE degeneration expands the array of therapeutic targets for AMD.

Although there is consensus that NLRP3-inflammasome activation is detrimental to RPE cell health and survival, similarly to the effects of NLRP3-inflammasome activation in other cell types, there is controversy over the role of this pathway in neovascular AMD. IL-18, a cytokine produced by NLRP3-inflammasome activation, has been reported to inhibit angiogenesis, and IL-18 neutralization has been found to increase angiogenesis in a laser-injury model of choroidal neovascularization. However, we and others have been unable to replicate this antiangiogenic effect of IL-18, and the promotion of angiogenesis by an antibody to IL-18 has also been found to be due to an excipient in its preparation. These conflicting data in neovascular AMD models do not, however, have any bearing on the conclusions from our current data and work by others indicating that inflammasome activation promotes RPE degeneration, a concept providing a mechanistic rationale for testing inflammasome inhibition in geographic atrophy.

In summary, this study has uncovered a contribution of endogenous-retroelement transcripts to disease via their induction of inflammasome-driven diseases. Our identification of cGAS, IFN-β, caspase-4, and gasdermin D as critical mediators in inflammasome-driven RPE degeneration expands the array of therapeutic targets for AMD.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

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**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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**ONLINE METHODS**

**Mice.** All animal experiments were approved by the University of Kentucky’s or the University of Virginia’s institutional review committee and were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. Male and female mice between 6 and 10 weeks of age were used in the study; WT C57BL/6j, Ppyf<sup>−/−</sup>, Prx7<sup>−/−</sup>, and Stat2<sup>−/−</sup> mice were purchased from The Jackson Laboratory. Gsdmd<sup>−/−</sup>, Pycard<sup>−/−</sup>, Casp1<sup>−/−</sup>, Casp1<sup>1/2</sup>, Casp1<sup>1/2</sup> (hCasp475) were as described elsewhere<sup>15</sup>, WT 129S6 mice (carrying an inactive passenger mutation in caspase-11) were purchased from The Jackson Laboratory. Mice, described elsewhere<sup>12,19,60</sup>, were a generous gift from M. Aguet. Ifr3<sup>−/−</sup> mice were a generous gift from T. Taniguchi via K. A. Fitzgerald (University of Massachusetts Medical School) on a C57BL/6 background using cryopreserved embryos obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM)<sup>83</sup>. Tmem173<sup>−/−</sup> mice were as previously described<sup>48</sup>. For all procedures, anesthesia was achieved by intraperitoneal injection of 100 mg/kg ketamine hydrochloride (Ft. Dodge Animal Health) and 10 mg/kg xylazine (Phoenix Scientific), and pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine (Alcon Laboratories).

**Fundus photography.** A TRC-50 IX camera (Topcon) linked to a digital imaging system (Sony) was used to obtain fundus photographs of dilated mouse eyes.

**Subretinal injection.** Subretinal injections (1 µl) in mice were performed with a Pico-Injector (PLI-100, Harvard Apparatus) or a 35-gauge needle (Ito Co.). In vivo transfection of plasmids expressing human caspase-4 (Casp1<sup>1/2</sup>−/−; hCasp475) were as described elsewhere<sup>15</sup>, WT 129S6 mice (carrying an inactive passenger mutation in caspase-11) were purchased from The Jackson Laboratory. Mice, described elsewhere<sup>12,19,60</sup>, were a generous gift from M. Aguet. Ifr3<sup>−/−</sup> mice were a generous gift from T. Taniguchi via K. A. Fitzgerald (University of Massachusetts Medical School) on a C57BL/6 background using cryopreserved embryos obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM)<sup>83</sup>. Tmem173<sup>−/−</sup> mice were as previously described<sup>48</sup>. For all procedures, anesthesia was achieved by intraperitoneal injection of 100 mg/kg ketamine hydrochloride (Ft. Dodge Animal Health) and 10 mg/kg xylazine (Phoenix Scientific), and pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine (Alcon Laboratories).

**Assessment of RPE degeneration.** Alu-mediated RPE degeneration was induced by exposing mice to Alu RNA, as previously described<sup>5,16,17,22</sup>. Seven days later, RPE health was assessed by fundus photography and immunofluorescence staining of zona occludens-1 (ZO-1) on RPE flat mounts (whole mount of posterior eye cup containing RPE and choroid layers). Mouse RPE and choroid flat mounts were fixed with 4% paraformaldehyde or 100% methanol, stained with rabbit polyclonal antibodies against mouse ZO-1 (1:100, Invitrogen, 339194), and visualized with Alexa-594 (Invitrogen). All images were obtained by microscopy (model SP-5, Leica, or Axio Observer Z1, Zeiss). Imaging was performed by an operator blinded to the group assignments.

**Quantification of RPE degeneration.** *Binary assignment.* Healthy RPE cells form a polygonal tessellation with a principally hexagonal ‘honeycomb’ formation. RPE degeneration was assessed on the basis of disruption of the uniformity of this polygonal sheet. Two independent raters who were blinded to the group assignments assessed RPE health according to the presence or absence of morphological disruption in RPE flat mounts. Both raters deemed 100% of images as gradable. Inter-rater reliability was measured by agreement on assignments, Pearson coefficient of determination, and Fleiss κ. Fisher’s exact test was used to determine statistical significance in the fraction of healthy RPE sheets across groups.

**Cellular morphometry.** Quantification of cellular morphometry for hexagonally packed cells was performed in a semiautomated fashion by three masked graders by adapting our previous analysis of corneal endothelial-cell density<sup>68</sup>. Because RPE cells when viewed *en face* typically exhibit a principally hexagonal morphology similar to that of the corneal endothelium, they readily lend themselves to a similar analysis strategy. We obtained measures of cell size, polyagomethism (coefficient of variation of cell size), and cell density. For this analysis, microscopy images of the RPE were captured and transmitted in deidentified fashion to the Doheny Image Reading & Research Lab (DIRRL). Images in which no cell borders could be seen were excluded from further analysis (1.8%). All images were rescaled to 304 x 446 pixels to permit importation into the Konan CellCheck software (ver. 4.0.1), a commercial US FDA-licensed software that has been used for registration clinical trials. RPE cell metrics were generated by three certified reading-center graders in an independent, masked fashion. Intergrader agreement was assessed for all three metrics by computing the multiple adjusted coefficient of determination. The previously published center method was used, which entails the user selecting the center of each identifiable cell in the image<sup>69–72</sup>. After the cell centers were defined, the software automatically generated the mean cell area, cell density, and polyagomethism values. By default, the Konan software assumes a scaling factor of 124 pixels per 100 µm. On the basis of the dimensions of the original RPE image (1,024 x 1,024 pixels, 0.21 µm/pixel), the Konan-provided values were converted to the actual physical values in micrometers.

RPE degeneration was quantified on the basis of ZO-1-stained flat-mount images through two strategies:

1. *Binary assignment (healthy versus unhealthy)*<sup>16,17,22</sup> by two masked raters (inter-rater agreement = 98.6%; Pearson ρ = 0.95, P < 0.0001; Fleiss κ = 0.97, P < 0.0001).

2. *Semiautomated cellular morphometry analysis* by three masked raters adapting our prior analysis of the planar architecture of the corneal endothelium<sup>68</sup>, which resembles the RPE in its polygonal tessellation. The inter-rater agreement was high for all three metrics (multiple adjusted ρ = 0.99 (cell size), 0.72 (polyagomethism, i.e., coefficient of variation of cell size), 0.99 (cell density)). For eyes treated with Alu RNA, Alus, and their respective controls, the inter-rater agreement on binary assignment was 100%, and the fraction of eyes classified as healthy was 100% for both control groups versus 0% for Alu RNA or Alu treatments (P < 0.0001 for both comparisons, Fisher’s exact test). All three morphometric features were significantly different between control treatments versus Alu RNA or Alu treatments (P < 0.0001, t test; Supplementary Fig. 3).

**Human tissue.** All studies on human tissue followed the guidelines of the Declaration of Helsinki. The study of deidentified tissue collected from deceased individuals and obtained from various eye banks in the United States was exempted from IRB review by the University of Virginia Institutional Review Board for Health Sciences Research, in accordance with US Health & Human Services human-subjects regulations. Donor eyes were obtained from patients with geographic atrophy, an advanced form of AMD, or age-matched patients without AMD were obtained from various eye banks. These diagnoses were confirmed through ophthalmic examination of dilated eyes before acquisition of the tissues or eyes or after examination of the eye globes post mortem. Enculeated donor eyes isolated within 6 h postmortem were immediately preserved in RNALater (Thermo Fisher). The neural retina was removed, and tissues comprising both macular RPE and choroidal tissue were snap frozen in liquid nitrogen. For eyes with geographic atrophy, RPE and choroidal tissue comprising both atrophic and normal areas were collected.

**Immunohistochemistry.** Human eyes fixed in 2–4% paraformaldehyde were prepared for immunohistochemistry and stained as previously described<sup>45</sup>. Briefly, immunohistochemical staining of fixed human eyes was performed with rabbit antibody against cGAS (0.1 µg/ml, Sigma-Aldrich, HPA031700) or IFN-β (0.2 µg/ml, Santa Cruz Biotechnology, sc-20107) and mouse antibody against gasdermin D (1.5 µg/ml, Abcam, ab577855). Rabbit or mouse IgG controls were used to ascertain the specificity of the staining. Biotin-conjugated
secondary antibodies, incubation with VECTASTAIN ABC reagent, and development with Vector Blue (Vector Laboratories, AK-5001) were used to detect the bound primary antibody. Slides were washed in PBS, then mounted in Vectamount (Vector Laboratories). All images were obtained with a Zeiss Axio Observer Z1 microscope.

**Real-time PCR.** Total RNA purified from cells with TRizol reagent (Invitrogen) according to the manufacturer’s recommendations was DNase treated and reverse transcribed with a QuantiTect Reverse Transcription kit (QIAGEN). The RT products (cDNA) were amplified by real-time quantitative PCR (Applied Biosystems 7900 HT Fast Real-Time PCR system) with Power SYBR green Master Mix. Relative gene expression was determined by the 2−ΔΔCt method, and 18S rRNA or GAPDH was used as an internal control. The following primers were designed and synthesized by Genlantis and purified with MEGAclear (Ambion), and its integrity was monitored by gel electrophoresis.

**Mitochondrial-DNA preparation.** Total DNA extracted from ARPE19 cells was used to PCR amplify mtDNA sequences, as previously described. The purified mtDNA PCR products were subterminally digested with 10% Neuroporter (Promega, E2311) with a standard transfection protocol. At 16 h after transfection, cell lysates were collected and analyzed.

**LPS transfection into BMDM cells.** Approximately 2 × 10⁶ BMDM cells were cultured overnight at 37 °C in a 6-cm dish. After 4–6 h of priming with 1 µg/ml Pam3CSK4 (Invivogen, tlr3), the cells were transfected with LPS (5 µg/ml final concentration, Invivogen, Irl-3pels, ultrapure) with Fugene HD (Promega, E2311) with a standard transfection protocol. At 16 h after transfection, cell lysates were collected and analyzed.

**Extraction of mitochondria-free cytosolic fractions.** Human and mouse RPE cells were either mock treated or stimulated with Alu RNA. At 24 h after AluRNA transfection or 48 h after transfection with scrambled or DICER1 AS oligonucleotides, the cells were harvested by trypsinization. Then 2 × 10⁶ cells were collected and lysed at 700 g for 10 min at 4 °C to completely remove nuclei or any unlysed cells. The resulting nucleocytoplasmic fraction was centrifuged at 13,000g for 15 min at 4 °C to pellet the mitochondria. The resulting supernatant was further centrifuged an additional six times at 13,000g for 15 min at 4 °C to remove all the mitochondria. The supernatant was then

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**Cell culture.** Primary mouse and human RPE cells were isolated as previously described. All cells were maintained at 37 °C in a 5% CO₂ environment. Mouse RPE cells were cultured in DMEM supplemented with 20% FBS and penicillin/streptomycin antibiotics. Human RPE cells were cultured in DMEM supplemented with 10% FBS and antibiotics. The human RPE cell line ARPE19 and ARPE19 cells lacking mitochondrial DNA (Rho⁰ ARPE19) were cultured as previously described. The Rho⁰ ARPE19 cells were maintained at 37 °C in 24 mN₂HCO₃, 10% FBS, 50 µg/ml uridine, 1 mM sodium pyruvate in DMEM-F12 (Gibco, 11320-033) containing pen/strep, Fungizone, and gentamicin. ARPE19 cells were maintained in DMEM-F12 containing pen/strep, Fungizone, and gentamicin. BMDM cells were cultured in DMEM with 10% FBS and 20% L929 supernatants. Mitochondria-free cytosolic fractions were collected at 16 h after transfection, and cell lysates were collected and analyzed.

**Synthesis of in vitro–transcribed Alu RNA.** T7 promoter–containing Alu expression plasmid was linearized and used for generating in vitro–transcribed Alu RNA with an AmpliScribe T7-Flash Transcription Kit (Epicerin), according to the manufacturer’s instructions. The resulting Alu RNA was DNase treated and purified with MEGAclear (Ambion), and the integrity was monitored by gel electrophoresis.

**Transfection.** Alu expression plasmid (pAlu) and empty-vector control (pNull) in vitro–transcribed Alu RNAs were transfected into human and mouse RPE cells with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. mtDNA prepared as described below was transfected into mouse RPE cells with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

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**LPS transfection into BMDM cells.** Approximately 2 × 10⁶ BMDM cells were cultured overnight at 37 °C in a 6-cm dish. After 4–6 h of priming with 1 µg/ml Pam3CSK4 (Invivogen, tlr3), the cells were transfected with LPS (5 µg/ml final concentration, Invivogen, Irl-3pels, ultrapure) with Fugene HD (Promega, E2311) with a standard transfection protocol. At 16 h after transfection, cell lysates were collected and analyzed.

**Extraction of mitochondria-free cytosolic fractions.** Human and mouse RPE cells were either mock treated or stimulated with Alu RNA. At 24 h after AluRNA transfection or 48 h after transfection with scrambled or DICER1 AS oligonucleotides, the cells were harvested by trypsinization. Then 2 × 10⁶ cells were collected and lysed at 700 g for 10 min at 4 °C to completely remove nuclei or any unlysed cells. The resulting nucleocytoplasmic fraction was centrifuged at 13,000 g for 15 min at 4 °C to pellet the mitochondria. The resulting supernatant was further centrifuged an additional six times at 13,000 g for 15 min at 4 °C to remove all the mitochondria. The supernatant was then...
tested for the absence of mitochondria by immunoblotsing for the mitochondrial marker protein VDAC and the cytosolic marker protein tubulin.

**Reconstitution experiment.** Mh2ild−/− mouse RPE cells were transfected with 2 μg cGAS expression plasmid26 or empty vector in a 60-mm dish at 70–80% confluency. At 24 h after transfection, cells were plated on six-well dishes. At 24 h after plating, cells were transfected with Alu RNA (50 pmol) or were mock transfected with Lipofectamine 2000. 18 h after Alu RNA transfection, cells were collected for RNA extraction to examine induction of Ifn-β mRNA. For caspase-11 reconstitution, Casp11−/− mouse RPE cells were transduced with control or caspase-11-expressing lentiviral particles. The transduced cells were allowed to rest for 3 d, and the cells were then plated in a 60-mm dish at 70–80% confluency. Control or caspase-11-reconstituted Casp11−/− cells were mock treated or stimulated with Alu RNA as described above, and activation of caspase-1 was assessed by western blotting. For the caspase-1 activity assay, Casp11−/− mouse RPE cells transfected with caspase-11 expression plasmid (pCasp11) or empty vector (pNull) were exposed to Alu RNA as described above, and caspase-1 activity was assessed with a CaspaLux 1-E1D2 kit (OncoImmunin, CPL1R1E-5). Quantification of the CaspaLux signal was performed by a blinded operator measuring the integrated density of fluorescence micrographs with ImageJ software (NIH) and normalizing to the number of cells.

**Lentiviral transduction.** Lentiviruses were produced either by the University of Kentucky Viral Production Core facilities or in house. Lentivirus vector plasmids expressing scrambled sequences or shRNA sequences targeting human caspase-4 (Sigma Aldrich, TRCN0000003511) and cGAS (Sigma Aldrich TRCN0000015001) were electroporated (MISSION shRNA, Sigma-Aldrich) and used to produce lentiviral particles. Human RPE cells at passage 3 were incubated with lentiviral particles at a multiplicity of infection (MOI) of 10 overnight in regular growth medium containing polybrene (4 µg/ml). On day 2, cells were washed, incubated in regular growth medium and allowed to rest for 24 h. Lentivirus-transduced cells were then cultured under puromycin (5 µg/ml) selection pressure for 5 d. Knockdown of the target proteins was determined by immunoblotting.

**Enzyme-linked immunosorbent assay (ELISA).** Secreted human and mouse IFN-β and IL-18 in the medium were detected with ELISA kits (mouse IFN-β, R&D Systems, 42400-1; mouse IL-18, R&D Systems, 7625; human IFN-β, R&D Systems, 4140; human IL-18, R&D systems, DY318-05) according to the manufacturer’s instructions. Primary mouse cells were cultured as described above. WT, Gsdmd−/−, Casp11−/−, or Mh2ild−/− mouse RPE cells were seeded at a density of 250,000 cells/well in 12-well plates. When the confluency reached 60–70%, cells were transfected with 20 pmol of in vitro–transcribed Alu RNA or mock transfected with Lipofectamine 2000 reagent (Life Technologies), according to the manufacturer’s protocol. Medium was collected to detect secreted cytokine content at 8 to 24 h after transfection. For examination of the induction of IL-18 secretion by MSU crystals (Invivogen tlr-mls), mouse RPE cells were primed with LPS (500 ng/ml) for 6 h and exposed to MSU (250 µg/ml) for 16 h, and medium was collected to detect secreted cytokines.

**Immunoprecipitation assays for cGAS–mtDNA interaction.** Immortalized cGAS−/− MEF cells subjected to reconstitution with HA-tagged mouse cGAS (HA-cGAS) were as previously described30. The interaction between mtDNA and cGAS was monitored with an Active Motif Immunoprecipitation Kit (Active Motif, ChIP-IT Express, 53008). Briefly mock transfected or plasmid DNA (pUC19)-transfected HA-cGAS-reconstituted cGAS−/− MEFs were fixed with 1% formaldehyde, per the manufacturer’s instructions. Control or caspase-11-expressing lentiviral particles. The transduced cells were allowed to rest for 3 d, and the cells were then plated in a 60-mm dish at 70–80% confluency. Control or caspase-11-reconstituted Casp11−/− cells were mock treated or stimulated with Alu RNA as described above, and activation of caspase-1 was assessed by western blotting. For the caspase-1 activity assay, Casp11−/− mouse RPE cells transfected with caspase-11 expression plasmid (pCasp11) or empty vector (pNull) were exposed to Alu RNA as described above, and caspase-1 activity was assessed with a CaspaLux 1-E1D2 kit (OncoImmunin, CPL1R1E-5). Quantification of the CaspaLux signal was performed by a blinded operator measuring the integrated density of fluorescence micrographs with ImageJ software (NIH) and normalizing to the number of cells.

**Determination of PAPC and oxDAPC by LC–MS.** MPT opening in WT and Ppyp−/− mouse RPE cells was monitored through the calcein-Co2+ technique80 with a Mitochondrial Permeability Transition Pore Assay Kit (Biovison, K239-100). Mitochondrial-membrane potential was evaluated with a JC-1–fluorescence-based MITO-ID Membrane Potential Cytotoxicity Kit (Enzo, ENZ-51019-KP02)31. MPT opening was inhibited by performing the above assays with medium containing cyclosporine A (10 µM). The assay was performed in a 96-well microtiter plate according to the manufacturer’s instructions.

**Live-cell Imaging.** 2 × 104 human RPE cells were plated in each well of an eight-well chambered slide (Thermo Scientific, 155411). At 18–24 h after plating, cells were transfected with 11.5 pmol in vitro–transcribed Alu RNA with Lipofectamine 2000 in culture medium supplemented with 2.5 mM CaCl2 and annexin V 488 (1:200, Invitrogen V13241), and PI (1:1,500, Invitrogen, P3566). Immediately after transfection, annexin V, PI, and DIC signals were acquired with a Nikon A1R confocal microscope equipped with an automated stage. Images were captured at 3-min intervals for a total duration of 50 h. Cells were maintained at 37 °C under 5% CO2 for the duration of the imaging study via a stage-top incubator.

**RPE flat-mount annexin V/PI staining.** Mouse RPE/chorioid flat mounts prepared in DMEM with 10% FBS were washed once with binding buffer and then incubated with Alexa Fluor 647–conjugated annexin V (Invitrogen, A23204) for 15 min. The annexin V–stained mouse RPE/choroid flat mounts were washed with cold PBS and trypsinized for 15 min. The annexin V–stained mouse RPE/choroid flat mounts were fixed in 2% paraformaldehyde for 30 min, stained with PI-containing RNase (Invitrogen) for 30 min, and mounted with ProLong Gold Antifade Mountant solution (Thermo Fisher).

**Microglia depletion.** Microglia were depleted by tamoxifen administration to Cx3CR1CreER, DTAtmlox mice, which express Cre-ER under control of a microglia-specific Cx3CR1 promoter and also contain a Foxo–stop–Fox diphtheria toxin subunit (DTA) gene cassette in the ROSA26 locus (DTAtmlox). Cx3CR1CreER, DTAtmlox mice were generated by breeding heterozygous Cx3cr1CreER mice with DTAtmlox mice (both mouse strains were generous gifts from W. T. Wong and L. Zhao, NIH). To deplete microglia, tamoxifen was administered to Cx3cr1CreER, DTAtmlox mice as previously described35. Briefly, adult 2–3-month-old TG
mice were administered with tamoxifen dissolved in corn oil (Sigma-Aldrich; 500 mg/kg dose of a 20 mg/ml solution) via oral gavage (on days ~2, 0, 5, 10, and 15). On day 11, Alu RNA was delivered via subretinal injection. Alu-RNA-induced RPE degeneration was assessed as described above. Microglial depletion was confirmed by staining retinal flat mounts for F4/80. Briefly, retinal flat mounts were prepared and fixed in 2% paraformaldehyde for 1 h, and stained with RPE-conjugated F4/80 (Bio-Rad, MCA497PET) and fluorescein-labeled Griffonia simplicifolia lectin isoelectin B4 (IB4, Vector Laboratories, FL-1201). All images were obtained with a Zeiss Axio Observer Z1 microscope.

**Macrophage depletion.** Depletion of macrophages was achieved via administering clodronate liposomes, which eliminates macrophages, in WT mice. Briefly, animals received 200 µl clodronate liposomes (Liposoma LIP-01) through the tail vein on days ~2 and day 0. Alu RNA or vehicle control were subretinally injected immediately after the day 0 tail-vein injection.

**Statistical analyses.** Real-time qPCR and ELISA data are expressed as means ± s.e.m. and were analyzed with Student’s t test. The binary readouts of RPE degeneration (i.e., the presence or absence of RPE degeneration on fundus and ZO-1-stained flat-mount images) were analyzed with Fisher’s exact test. Cell-morphometry data were assessed with Student’s t test. P values <0.05 were deemed statistically significant. Sample sizes were selected on the basis of power analysis (α = 5%; 1 – β = 80%, such that we were able to detect a minimum of 50% change, assuming a sample s.d. based on Bayesian inference. Outliers were assessed with Grubbs’ test. On the basis of this analysis, no outliers were detected, and no data were excluded. Fewer than 5% of retinal-injection-recipient tissues were excluded on the basis of predetermined exclusion criteria (including hemorrhage and animal death due to anesthesia complications) relating to the technical challenges of this delicate procedure.

**Life Sciences Reporting Summary.** Further information on experimental design is available in the Life Sciences Reporting Summary.

**Data availability.** The data sets generated during and/or analyzed during the current study are not publicly available, owing to commercialization of research findings, but are available from the corresponding author on reasonable request. Uncropped images of blots can be found in Supplementary Figure 16.

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Corrigendum: cGAS drives noncanonical-inflammasome activation in age-related macular degeneration

Nagaraj Kerur, Shinichi Fukuda, Daipayan Banerjee, Younghee Kim, Dongxu Fu, Ivana Apicella, Akhil Varshney, Reo Yasuma, Benjamin J Fowler, Elmira Baghdasaryan, Kenneth M Marion, Xiwen Huang, Tetsuhiro Yasuma, Yoshio Hirano, Vlad Serbulea, Meenakshi Ambati, Vidya L Ambati, Yuji Kajiwara, Kameshwari Ambati, Shuichiro Hirahara, Ana Bastos-Carvalho, Yuichiro Ogura, Hiroko Terasaki, Tetsuro Oshika, Kyung Bo Kim, David R Hinton, Norbert Leitinger, John C Cambier, Joseph D Buxbaum, M Cristina Kenney, S Michal Jazwinski, Hiroshi Nagai, Isao Hara, A Phillip West, Katherine A Fitzgerald, SriniVas R Sadda, Bradley D Gelfand & Jayakrishna Ambati

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In the version of this article initially published online, a micrograph in Figure 1d (WT, Vehicle) and a micrograph in Figure 2a (Gsdmd−/−, Alu RNA) are duplicates of two other micrographs and are incorrect. These errors do not affect the quantitative data reported or the conclusions of the article. These two micrographs have been replaced with the correct ones in the print, PDF and HTML versions of this article.
Experimental design

1. Sample size
    Describe how sample size was determined. Sample sizes were selected based on power analysis alpha=5%; 1–β = 80%, such that we could detect a minimum of 50% change assuming a sample SD based on Bayesian inference.

2. Data exclusions
    Describe any data exclusions. No data were excluded, however some animals (<5%) were excluded from the experiments due to technical challenges in the procedures such as hemorrhage and animal death due to anesthesia complications, etc.

3. Replication
    Describe whether the experimental findings were reliably reproduced. The experimental findings were reliably reproduced through repeated experiments.

4. Randomization
    Describe how samples/organisms/participants were allocated into experimental groups. For in vivo studies both male and female mice were randomly distributed into treatment groups within each genotype.

5. Blinding
    Describe whether the investigators were blinded to group allocation during data collection and/or analysis. To eliminate operator bias, the researchers performing the procedures and analyses were blinded to mouse genotype identity as well as the identity of the experimental groups.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
    For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

    n/a Confirmed

    □ ✓ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

    □ ☑ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

    □ ☑ A statement indicating how many times each experiment was replicated

    □ ☑ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

    □ ☑ A description of any assumptions or corrections, such as an adjustment for multiple comparisons

    □ ☑ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted

    □ ☑ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

    □ ☑ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study.

Microsoft Excel, Graphpad prism, LICOR image studio

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All unique materials used are available from standard commercial sources or from the authors upon request.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Sources and usage details of all the antibodies used in the study are described in the methods section.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

ARPE19 cells were obtained from ATCC

b. Describe the method of cell line authentication used.

STR Analysis

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines tested mycoplasma negative.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Both male and female mice (C57BL/6J background) between 6–10 weeks of age were used in the study. Additional detailed information is provided in methods section.

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Study did not involve human research participants.