

DICER1/*Alu* RNA dysmetabolism induces Caspase-8–mediated cell death in age-related macular degeneration

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Geographic atrophy, an advanced form of age-related macular degeneration (AMD) characterized by death of the retinal pigmented epithelium (RPE), causes untreatable blindness in millions worldwide. The RPE of human eyes with geographic atrophy accumulates toxic *Alu* RNA in response to a deficit in the enzyme DICER1, which in turn leads to activation of the NLRP3 inflammasome and elaboration of IL-18. Despite these recent insights, it is still unclear how RPE cells die during the course of the disease. In this study, we implicate the involvement of Caspase-8 as a critical mediator of RPE degeneration. Here we show that DICER1 deficiency, *Alu* RNA accumulation, and IL-18 up-regulation lead to RPE cell death via activation of Caspase-8 through a Fas ligand-dependent mechanism. Coupled with our observation of increased Caspase-8 expression in the RPE of human eyes with geographic atrophy, our findings provide a rationale for targeting this apoptotic pathway in this disease.

macular degeneration | inflammasome | caspase

DICER1, encoded by the *DICER1* gene in humans, is a type III ribonuclease (RNase) best known for its role in processing precursor microRNAs (pre-miRNAs) into mature miRNAs that are involved in posttranscriptional gene regulation (1, 2). Our recent work highlighted another important role for DICER1 in healthy cell function: to process primary *Alu* RNA transcripts and prevent their cytotoxic accumulation in the retinal pigmented epithelium (RPE) (3–5). In humans with geographic atrophy, a form of untreatable age-related macular degeneration (AMD) that results in irreversible blindness, DICER1 protein levels in the RPE are reduced (3). DICER1 deficiency induces accumulation of cytotoxic *Alu* RNA, which in turn induces TLR-independent activation of the NLRP3 inflammasome (4).

NLRP3, a member of the NLR (nucleotide-binding domain, leucine-rich repeat containing, or NOD-like receptor) subfamily, is involved in the induction of the innate immune response and becomes activated when the cell is exposed to a variety of different agents, including pathogens, adenosine triphosphate, toxins, reactive oxygen species (ROS), and nucleic acids (6). Caspase-1 activation through cleavage of the Pro-Caspase-1 precursor is a known product of NLRP3 inflammasome activation (7) and typically is required for processing of pro-IL-1 β and pro-IL-18 to their active forms (8). This signaling pathway is an essential mediator of *Alu* RNA-induced RPE toxicity (4).

Although the role of Caspase-1 with respect to NLRP3 inflammasome activation is established, the identity of downstream signaling molecules mediating cell death remains largely unknown. Among these candidates is Caspase-8, which can mediate

Caspase-1 activation (9) and pro-IL-1 β processing (10). The NLRP3 inflammasome is most notably associated with cell death via pyroptosis, but previous work by our laboratory indicated that *Alu* RNA accumulation does not induce pyroptosis (4). Caspase-8 has been shown to play varying roles in different experimental systems; in systems deficient in Caspase-1, Caspase-8 is involved in inflammasome-mediated IL-1 β processing (9–11), whereas in other systems, Caspase-8 operates downstream of inflammasome activation and is instead important with respect to apoptosis (12). Because Caspase-8 can operate either upstream or downstream of inflammasome activation, we sought to test whether *Alu* RNA required Caspase-8 for cytokine processing or RPE cell death for its toxic effects. Here we present evidence that Caspase-8 is associated with RPE degeneration in human eyes with geographic atrophy and that it operates downstream of *Alu*

Significance

Geographic atrophy is a late stage of age-related macular degeneration (AMD) that causes blindness in millions worldwide characterized by death of the retinal pigmented epithelium (RPE). We previously reported that RPE death is due to a deficiency in the enzyme DICER1, which leads to accumulation of toxic *Alu* RNA. We also demonstrated that *Alu* RNA causes RPE death by activating an immune platform called the NLRP3 inflammasome. However, the precise mechanisms of RPE death in this disease remained unresolved. The present study indicates that *Alu* RNA induces RPE death by activating the enzyme Caspase-8 downstream of inflammasome activation and that blocking Caspase-8 rescues RPE degeneration. This implicates apoptosis as the cell death pathway responsible for *Alu* RNA cytotoxicity, and these findings provide new potential therapeutic targets for this disease.

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Conflict of interest statement: W.W.H. and the University of Florida have a financial interest in the use of adeno-associated virus therapies and own equity in a company (AGTC Inc.) that might, in the future, commercialize some aspects of this work.

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RNA-induced Caspase-1 activation to mediate nonpyroptotic cell death following NLRP3 inflammasome activation.

Results

Caspase-8 Is Activated in Human RPE Cells. We observed a marked increase in the abundance of total Caspase-8 protein in the RPE of human eyes with geographic atrophy compared with healthy, age-matched eyes (Fig. 1A). Further, primary human RPE cells treated with *Alu* RNA exhibited activated Caspase-8 protein (p10), which was undetectable in mock treated cells (Fig. 1B). We next interrogated whether conditional ablation of the *Dicer1* gene in the RPE of mice, which leads to *Alu* RNA accumulation, could induce Caspase-8 activation. Indeed, *Dicer1* floxed mice exposed to a subretinal injection of recombinant adeno-associated virus (rAAV) that encodes an RPE-specific Cre recombinase exhibited significantly greater Caspase-8 activation compared with controls (Fig. 1C). Activated Caspase-8 protein levels were also increased in the RPE/choroid of *Alu* RNA-treated wild-type mice (Fig. 1C).

Caspase-8 Blockade Protects the RPE Against *Alu* RNA Toxicity. DICER1 deficiency leads to an accumulation of *Alu* RNA (3, 4) that results in a loss of retinal function as measured by electroretinography (SI Appendix, Fig. S1), and we found there was greater Caspase-8 activation in wild-type mice treated with *Alu* RNA via subretinal injection (Fig. 1C). To determine whether Caspase-8 is necessary for *Alu* RNA-mediated RPE degeneration, we assessed whether delivery of *Alu*-expression vector (pAlu) or *Alu* RNA via subretinal injection induced RPE degeneration in Caspase-8 deficient systems. *Casp8^{fl/fl}* mice treated with subretinal injection of rAAV1-BEST1-Cre, which induced efficient, non-toxic Cre recombinase expression and Caspase-8 knockdown in the RPE, were protected from *Alu* RNA- and pAlu-induced RPE degeneration (Fig. 2A and SI Appendix, Figs. S2–S4). Additionally, exposure of human RPE cells to a Caspase-8

peptide inhibitor prevented *Alu* RNA- and pAlu-induced cell death (Fig. 2B and C). Corroborating these findings, intravitreal administration of the Caspase-8 peptide inhibitor prevented pAlu-induced degeneration in wild-type mice (Fig. 2C). Supporting the hypothesis that *Alu* RNA-induced cell death requires Caspase-8, retinal morphology (SI Appendix, Fig. S5) and electrical function (SI Appendix, Fig. S6) were preserved in mice treated with Caspase-8 peptide inhibitor and genetic ablation, respectively. Taken together, these data demonstrate that Caspase-8 is necessary for *Alu* RNA-induced RPE degeneration.

Caspase-8 Blockade Protects Against IL-18-Mediated Toxicity. Recently we demonstrated that *Alu* RNA-induced RPE degeneration in geographic atrophy is mediated through the activation of NLRP3 inflammasome and ensuing IL-18-driven signaling (4, 13). We next sought to determine whether Caspase-8 executes the IL-18-mediated RPE degeneration by injecting recombinant mature IL-18 into wild-type and Caspase-8 deficient systems. Subretinal injection of recombinant IL-18 increases Caspase-8 activation (Fig. 3A) and induces RPE degeneration in wild-type mice (Fig. 3B). *Casp8^{fl/fl}* mice treated with rAAV-BEST1-Cre are protected against IL-18-induced RPE degeneration (Fig. 3C). Collectively, these data indicate that Caspase-8 is a critical mediator of IL-18-induced RPE degeneration.

Fas and FasL Are Required for *Alu* RNA- and IL-18-Mediated RPE Toxicity. Caspase-8 activation requires upstream Fas ligand (FasL) binding to Fas receptor for cleavage of pro-Caspase-8 (14). Subretinal injection of *Alu* RNA causes up-regulation of both Fas and FasL (Fig. 4A), and intravitreal injection of recombinant mature IL-18 up-regulates both Fas and FasL in the RPE/choroid of wild-type mice (Fig. 4B). To determine whether increased Fas and FasL expression were involved in *Alu* RNA- and IL-18-induced RPE toxicity, we examined the susceptibility of *Fas^{-/-}* and *FasL^{-/-}* mice to RPE degeneration induced by *Alu* RNA or IL-18. Administration of *Alu* RNA, through subretinal injection of either *Alu* RNA or pAlu, did not induce RPE degeneration in either *Fas^{-/-}* or *FasL^{-/-}* mice (Fig. 4C and D). Similarly, IL-18-mediated RPE degeneration was blocked in *Fas^{-/-}* and *FasL^{-/-}* mice (Fig. 4E). We found that Caspase-1 activation in *Alu* RNA-treated human RPE cells was not reduced by exposure to the Caspase-8 inhibitor peptide (Fig. 4F), implying that Caspase-8 acted downstream of Caspase-1.

***Alu* RNA Induces RPE Cell Death via Apoptosis.** Previously, we showed that *Alu* RNA induced activation of Caspase-3 in human RPE cells (3), a critical executioner in apoptotic cell death. We now show that *Alu* RNA induces Caspase-3 activation in vivo in the RPE of wild-type mice and that Caspase-3 activation is blocked by a Caspase-8 inhibitor peptide (SI Appendix, Fig. S7). Taken together, these data suggest that Caspase-8 lies between Caspase-1 and Caspase-3 in the mechanistic stream of *Alu* RNA-induced cell death. In addition, we found that Necrostatin-1, an inhibitor of an alternate cell death pathway termed necroptosis, did not confer protection against *Alu* RNA-induced RPE degeneration (SI Appendix, Fig. S8). Overall our data suggest that *Alu* RNA/IL-18-induced RPE degeneration in geographic atrophy is mediated via activation of Caspase-8-induced apoptosis in a manner dependent on Fas and FasL signaling.

Discussion

Human eyes with geographic atrophy exhibit decreased DICER1 expression and greater *Alu* RNA accumulation in their RPE (3, 5), and *Alu* RNA accumulation triggers RPE cell death via the NLRP3 inflammasome (4, 13). NLRP3 inflammasome activity requires two distinct events for downstream cell death pathways: priming (the up-regulation of inflammasome components) and activation (assembly of active NLRP3 inflammasome complexes) (15). *Alu* RNA induces priming of the NLRP3 inflammasome

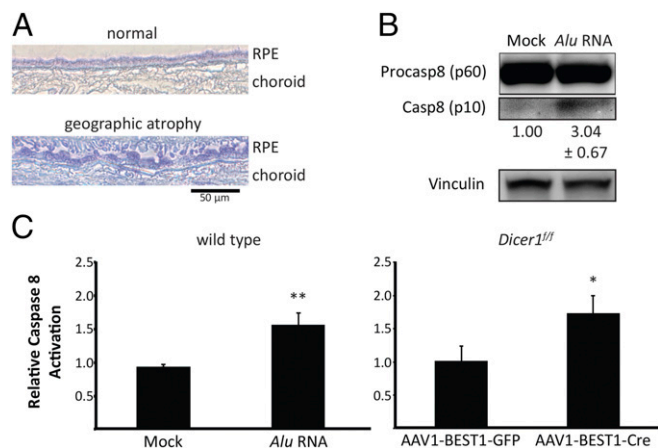


Fig. 1. Caspase-8 is up-regulated in the RPE of human eyes with geographic atrophy and mouse eyes with DICER1/*Alu* RNA dysmetabolism. (A) Caspase-8 (blue) abundance is increased in the RPE of human eyes with geographic atrophy compared with normal age-matched eyes. (B) Immunoblotting shows that *Alu* RNA activates Caspase-8 in cultured human RPE cells 24 h after treatment. Caspase-8 active p10 fragment is 3.04 ± 0.67 fold higher in abundance in *Alu* RNA-treated cells. Fold change in active Caspase-8 levels compared with Mock treatment, normalized to Vinculin, as determined by densitometry (mean ± SEM), are reported below their respective bands. (C) Caspase-8 activity assays of RPE/choroid lysates of AAV1-BEST1-Cre-treated *Dicer1^{fl/fl}* mice and *Alu* RNA-treated wild type mice reveal that Caspase-8 is activated by *Dicer1* knockdown or *Alu* RNA delivery ($n = 6$ independent experiments, * $P = 0.0004$ and ** $P < 0.0001$ by two-tailed Student t test). Images are representative of at least three independent experiments (A).

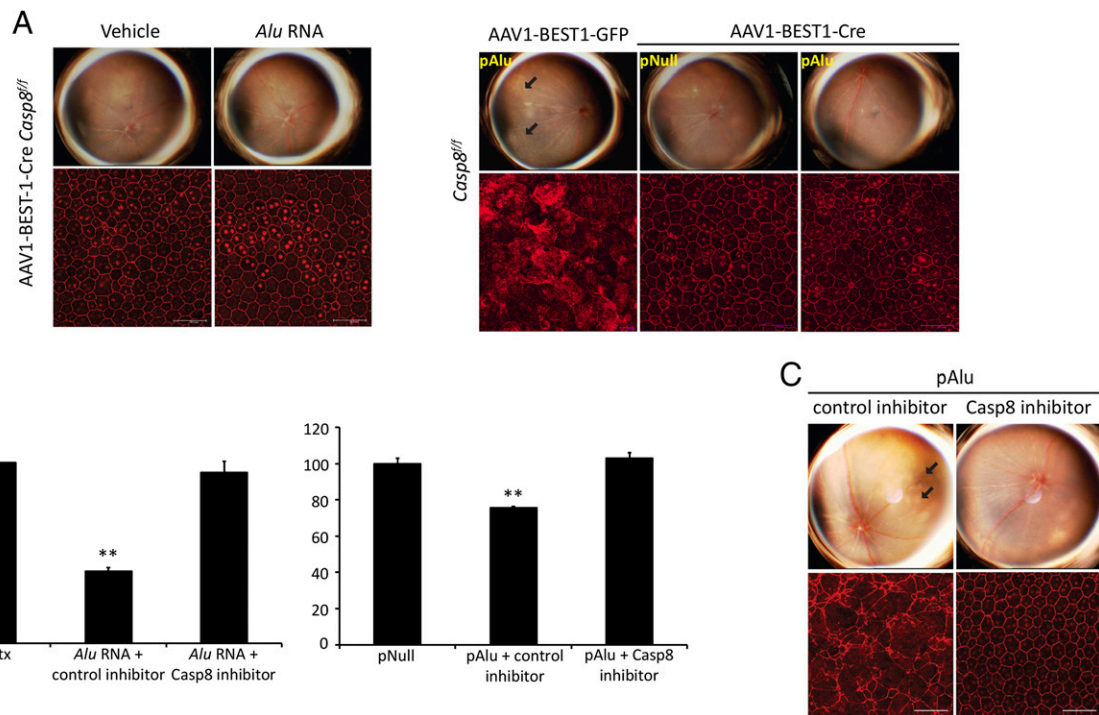


Fig. 2. Caspase-8 is required for *Alu* RNA-mediated RPE degeneration in mice. (A) Fundus photographs and ZO-1-stained (red) flat mounts show that AAV1-BEST1-Cre-treated *Casp8^{ff}* mice are protected against *Alu* RNA- and pAlu-induced RPE degeneration. (B) Administration of a Caspase-8 inhibitor peptide (Z-IETD-FMK), but not control peptide (Z-FA-FMK), prevents loss of cell viability in cultured human RPE cells either treated with synthetic *Alu* RNA or transfected with *Alu* RNA-expressing plasmid (pAlu). ** $P < 0.001$ by two-tailed Student *t* test. (C) Fundus photographs and ZO-1-stained (red) flat mounts show that Caspase-8 inhibitor peptide, but not control peptide, protects wild-type mice from *Alu* RNA-mediated RPE degeneration. $n = 10$, $P = 0.008$ by Fisher exact test. Representative images shown (A and C).

through mitochondrial ROS production and NF- κ B signaling (4, 13). NF- κ B signaling is triggered independently of TLR activation (4, 13), possibly through the action of a currently unidentified RNA sensor in the RPE. It is unclear how *Alu* RNA activates the NLRP3 inflammasome, but it is known that

Caspase-1 is activated via a P2X7-dependent pathway (13). Although Caspase-1 is required for *Alu* RNA-induced toxicity, it is not required for IL-18-induced RPE cell toxicity, suggesting that RPE cells can undergo inflammasome-triggered cell death independent of pyroptosis (4). The precise death signaling

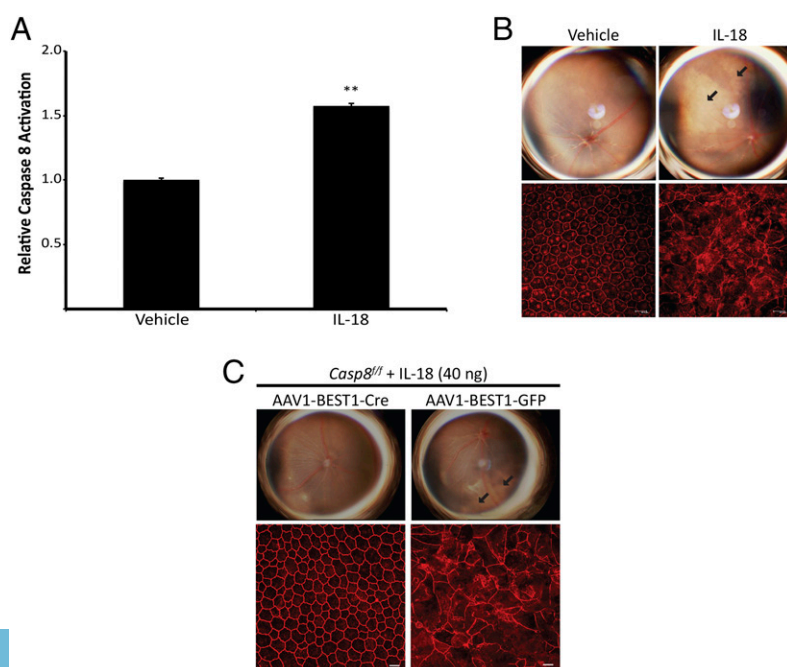


Fig. 3. Caspase-8 is required for IL-18-induced RPE degeneration in mice. (A) Caspase-8 activity assays of RPE/choroid lysates of mature IL-18 treated wild-type mouse eyes reveal that Caspase-8 is activated 3 d postinjection ($n = 3$ independent experiments, ** $P < 0.0001$ by two-tailed Student *t* test). (B) Fundus photographs and flat mounts stained with ZO-1 antibody (in red) show that subretinal administration of recombinant mature IL-18 induces RPE degeneration in wild-type mice. $n = 12$, $P = 0.002$ by Fisher exact test. (C) Fundus photographs and ZO-1-stained (red) flat mounts show that AAV1-BEST1-Cre-treated *Casp8^{ff}* mice are protected against IL-18-induced RPE degeneration. $n = 8$, $P = 0.03$ by Fisher exact test (C). Images are representative of at least three independent experiments (B and C).

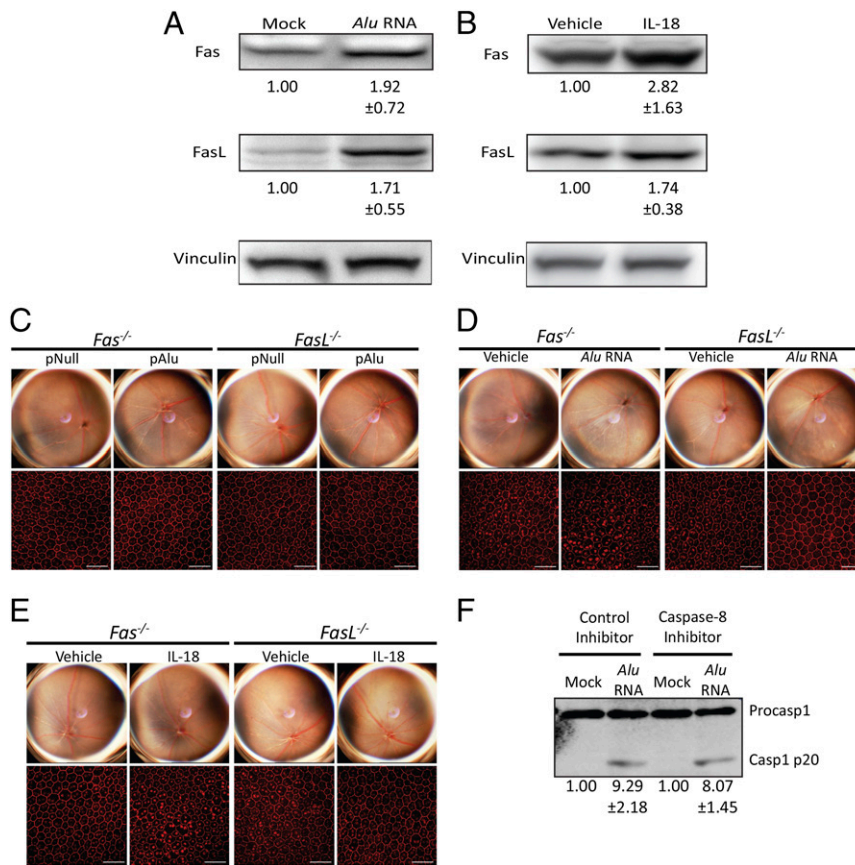


Fig. 4. Caspase-8 induces cell death via Fas and FasL. (A) Fas and FasL protein abundance in the RPE/choroid increases 3 d after subretinal injection of synthetic *Alu* RNA in wild-type mice as monitored by immunoblotting. Fas and FasL abundance is 1.92 ± 0.72 fold higher and 1.71 ± 0.55 higher in abundance in *Alu* RNA-injected mice, respectively. Fold change in Fas and FasL levels, compared with Mock treatment, normalized to Vinculin, as determined by densitometry (mean \pm SEM), are reported below their respective bands. (B) Immunoblotting reveals that Fas and FasL protein abundance in the RPE/choroid increases 2 d after subretinal injection of recombinant IL-18 in wild-type mice. Fas and FasL abundance is 2.82 ± 1.63 fold higher and 1.74 ± 0.38 higher in abundance in IL-18-injected mice, respectively. Fold change in Fas and FasL levels, compared with vehicle treatment, normalized to Vinculin, as determined by densitometry (mean \pm SEM), are reported below their respective bands. (C) Fundus photography and flat mounts stained with ZO-1 antibody (in red), show that *Fas*^{-/-} and *FasL*^{-/-} mice are protected against pAlu- (C) and *Alu* RNA-induced RPE degeneration (D). (E) Fundus photography and ZO-1-stained (red) flat mounts show that *Fas*^{-/-} and *FasL*^{-/-} mice are protected against IL-18-induced RPE degeneration. (F) The Caspase-8 inhibitory peptide does not impair *Alu* RNA-induced Caspase-1 activation in human RPE cells. Caspase-1 active p20 fragment is 9.29 ± 2.18 fold higher in abundance in control peptide inhibitor-treated cells and is 8.07 ± 1.45 higher in abundance in Caspase-8 inhibitor peptide-treated cells. Fold change in active Caspase-1 levels, compared with Mock treatment, normalized to Vinculin, as determined by densitometry (mean \pm SEM) are reported below their respective bands. $n = 8$, $P = 0.03$ by Fisher exact test. Representative images shown (C–F).

pathways invoked by DICER1/*Alu* RNA dysmetabolism had been ill-defined.

Because we found that *Alu* RNA toxicity was not dependent on pyroptosis for cell death, this finding suggested that another cellular pathway may mediate RPE cell death in geographic atrophy. IL-18 induces MyD88-dependent signaling (16) by extracellular binding of IL-18 to IL-18 receptor (17), and the resultant signaling can up-regulate both Fas and FasL (18) to enhance Fas signaling of apoptosis (19).

We and others have shown that a synthetic, high-molecular weight, fully complementary double-stranded RNA known as poly I:C induces RPE degeneration (20, 21). A recent report showed that poly I:C can trigger necroptosis in the RPE (22), which can be unleashed in the absence of Caspase-8 (23). However, we found that an inhibitor of necroptosis, which blocked poly I:C-induced RPE degeneration (22), did not prevent *Alu* RNA-induced RPE degeneration when used at the same dose. This difference between *Alu* RNA, an AMD-associated endogenous transcript with a complex RNA structure, and synthetic poly I:C could be due to the fact that the RNA sensor MAVS/IPS-1,

which is required for necroptosis (23), is activated by poly I:C (24) but not by *Alu* RNA (4).

Caspase-8 modulates innate immune responses in a variety of ways: Caspase-8 can be involved in IL-1 β and IL-18 processing independent of Caspase-1 activation by both AIM2-ASC (12) and MALT1-ASC (11) inflammasomes. Also, Caspase-8 can suppress NLRP3 inflammasome-dependent activation of IL-1 β and IL-18 (25). Because we have previously shown that *Alu* RNA induces IL-18 processing in a Caspase-1-dependent manner (4) and we demonstrate here that Caspase-1 activation occurs despite Caspase-8 inhibition (Fig. 4F), we conclude that Caspase-8 mediates *Alu* RNA toxicity downstream of IL-18. A summary of our findings concerning Caspase-8 in RPE cell death in the context of the known *Alu* RNA toxicity mechanistic pathway is shown in Fig. 5.

Recently, it was reported that IL-18 neutralization augments choroidal neovascularization (CNV) in a mouse laser injury model (26). However, a conglomeration of five laboratories determined that this finding was in fact due to glycerol, a proangiogenic excipient in the IL-18 neutralizing antibody preparation (27). Subsequently, it was reported that recombinant murine

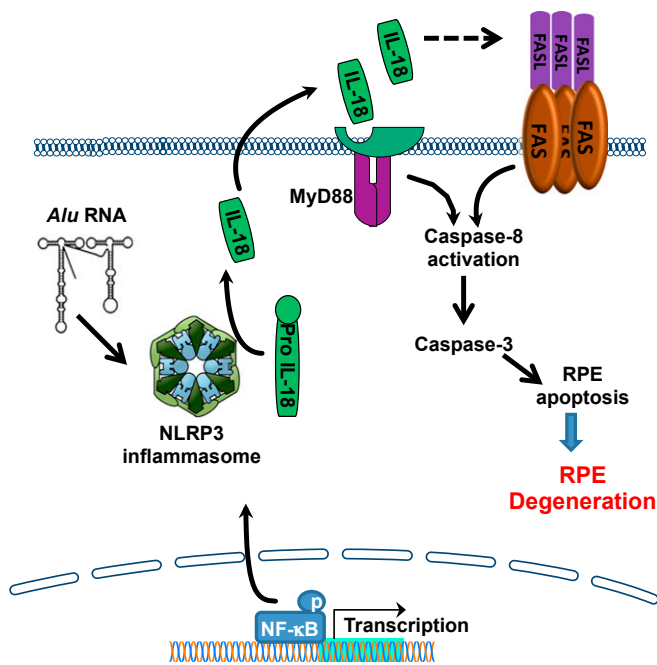


Fig. 5. *Alu* RNA induces Caspase-8-mediated cell death via the NLRP3 inflammasome. *Alu* RNA accumulation induces NF- κ B-induced priming of the NLRP3 inflammasome, which then activates to cleave Pro-Caspase-1 to its mature, active form, Caspase-1. Caspase-1 in turn processes Pro-IL-18 to its mature, active form, which is then secreted by the RPE. IL-18 signaling induces up-regulation of Fas and FasL via IL-18 receptor and MyD88 to activate Caspase-8-mediated RPE cell death via Caspase-3.

IL-18 reduced choroidal neovascularization in a laser injury model (28). However, a multicentered study failed to reproduce those data, finding instead that IL-18 had no effect on CNV over 5-log dose range, and instead that IL-18 induced RPE degeneration (27), which has been independently corroborated (29).

Given the findings of this study, Fas, FasL, and Caspase-8 emerge as potential therapeutic targets for atrophic AMD. There are currently no treatment strategies that are capable of slowing the irreversible loss of vision in atrophic AMD despite increased understanding of the role the innate immune system plays in its pathology and progression (30, 31). Apoptosis of various cell types in CNV has been associated with Fas and FasL expression (32). Future investigation of the role Caspase-8 plays in AMD pathogenesis could aid in the improvement of our understanding of a complex disease that affects an increasing proportion of the population as it ages and in the development of therapies that focus on either Caspase-8 itself, its activators, or downstream pathway components. Any therapies that target Fas, FasL, or Caspase-8 would require rigorous testing for safety and efficacy, however, given the widespread importance of these proteins in cellular pathways in tissues not affected by disease.

Materials and Methods

Detailed descriptions of the following procedures are available in *SI Appendix*: human tissue, immunoblotting, subretinal injection, RPE flat mounts, Caspase-8 inhibitor, protein isolation, and protein quantification.

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Immunohistochemistry. Donor eyes or ocular tissues from age-matched patients with geographic atrophy (GA) due to age-related macular degeneration (AMD) or patients without AMD were obtained from various eye banks upon securing informed consent from donors or their families. These diagnoses were confirmed by dilated ophthalmic examination before acquisition of the tissues or eyes or upon examination of the eye globes post mortem. The study followed the guidelines of the Declaration of Helsinki. Studies of these de-identified cadaver specimens were exempt from human subjects research approval requirements. All diseased eyes had central GA involving the fovea whereas all of the control eyes had no visible features of AMD.

Ocular tissue was obtained from age-matched patients with or without geographic atrophy. Human Caspase-8 protein was stained with antibody from Cell Signaling.

Cell Culture and Transient Transfection. Primary human fetal RPE cells were cultured in DMEM media (Cellgro) supplemented with 10% FBS at 37 °C.

Cell Viability. Cell viability was quantified using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assays (CellTiter 96 Aqueous One Solution Cell Proliferation Assay) according to the manufacturer's instructions (Promega).

Animals. All animal studies were approved by University of Kentucky Institutional Animal Care and Use Committee (IACUC) and performed according to their guidelines. C57BL/6J (wild type), *Dicer1^{fl/fl}*, *Fas^{-/-}*, and *FasL^{-/-}* mice were purchased from The Jackson Laboratory. *Casp8^{fl/fl}* mice have been previously described (33). Ablation of *Dicer1* and *Casp8* in those mice was performed using adeno-associated virus (AAV1) vector coding for Cre recombinase under the control of an RPE-specific promoter (*BEST1*).

Subretinal Injection. Plasmids (1 μ L volume), 2 μ g Caspase-8 inhibitor peptide, and 40 ng recombinant IL-18 were delivered to the subretinal space in mouse eyes using an Ito microsyringe (Ito Corporation).

In Vitro Transcription of *Alu* RNA. Synthetic *Alu* RNA was created using the AmpliScribe T7-Flash Transcription Kit (Epicentre) using the manufacturer's protocol.

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