Inflammasomes Induced by 7-Ketocholesterol and Other Stimuli in RPE and in Bone Marrow–Derived Cells Differ Markedly in Their Production of IL-1β and IL-18

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Purpose. The inflammatory process plays a major role in the pathogenesis of AMD, and recent data indicate the involvement of inflammasomes. Inflammasomes are intracellular structures that trigger inflammation by producing mature interleukin-(IL)-1β and IL-18. This study examined the capacity of 7-ketocholesterol (7KCh), an oxysterol that accumulates in the retinal pigmented epithelium (RPE) and choroid, to initiate inflammasome formation in RPE and bone marrow–derived cells.

Methods. Tested cells included fetal human RPE (fhRPE), human ARPE-19 cells, primary human brain microglia cells, and human THP-1 monocyte cells. 7-Ketocholesterol and other compounds were added to the cell cultures, and their stimulatory effects were determined by quantitative PCR and release of cytokines, measured by ELISA and Western blotting.

Results. 7-Ketocholesterol efficiently induced inflammasome formation by all primed cell populations, but secreted cytokine levels were higher in cultures of bone marrow–derived cells (microglia and THP-1 cells) than in RPE cultures. Interestingly, inflammasomes formed in cells of the two populations differed strikingly in their preferential production of the two cytokines. Thus, whereas bone marrow–derived cells produced levels of IL-1β that were higher than those of IL-18, the opposite was found with RPE cells, which secreted higher levels of IL-18. Importantly, Western blot analysis showed that IL-18, but not IL-1β, was expressed constitutively by RPE cells.

Conclusions. 7-Ketocholesterol efficiently stimulates inflammasome formation and is conceivably involved in the pathogenesis of AMD. In contrast to bone marrow–derived cells, RPE cells produced higher levels of IL-18 than IL-1β. Further, IL-18, a multifunctional cytokine, was expressed constitutively by RPE cells. These observations provide new information about stimuli and cells and their products assumed to be involved in the pathogenesis of AMD.

Keywords: bone marrow–derived cells, IL-1, IL-18, inflammasomes, RPE cells

In recent years, considerable evidence has been published indicating that inflammation plays a major role in the pathogenesis of age-related macular degeneration (AMD).[1–5] Particular attention has been given to data showing involvement of inflammasomes in the AMD pathogenic process. Inflammasomes are intracellular multimeric protein complexes that sense danger signals and generate mature interleukin (IL)-1β and IL-18, two potent proinflammatory cytokines.[6–8] Inflammasomes are made typically by myeloid cells, but several publications have demonstrated that retinal pigmented epithelium (RPE) cells are also capable of generating inflammasomes, when adequately activated. Stimuli for inflammasome generation by RPE and/or myeloid cells include drusen and components of these age-related structures,[9] A2E, the major hydrophobic component of RPE,[10] *Alu* RNA,[11,12] lysosomal activation,[13] and oxidative stress.[14]

7-Ketocholesterol (7KCh), a naturally occurring oxidized form of cholesterol, has been found associated with lipoprotein deposits in the choriocapillaris, Bruch's membrane, and RPE[15–17] and is found in atherosclerotic plaques.[18,19] 7-Ketocholesterol is highly toxic to vascular endothelial cells and smooth muscle cells and is also suspected of causing macrophages to transform into foam cells.[20] Our previous studies have provided evidence to show that, in addition, 7KCh drives inflammatory processes in RPE cells, as indicated by the enhanced expression of IL-6 and IL-8 via activation of NFκB.[16] In the present study, we compared the capacity of 7KCh to initiate inflammasome formation with that of the well-known inflammasome stimulators silica and cholesterol crystal preparations. To examine the potential involvement of 7KCh in AMD pathogenesis, we determined the capacity of this oxysterol to initiate inflammasome formation by RPE cells as well as by microglia and THP-1 cells, the two bone marrow–derived cell populations found in normal and

diseased retina. The major products of inflammasomes are IL-1β and IL-18, and measuring these two cytokines revealed profound differences between RPE- and bone marrow–derived cells in the preferential production of these two cytokines.

Materials and Methods

Reagents

7-Ketocholesterol (Steraloid, Inc., Newport, RI, USA) was complexed with hydroxypropyl-β-cyclodextrin (HPβCD; Sigma-Aldrich Corp., St. Louis, MO, USA) as previously described.[17] Cholesterol crystals were a gift from Alan Remaley (National Heart, Lung, and Blood Institute, NIH, Bethesda, MD, USA). Silica crystals were provided by US Silica (Frederick, MD, USA). Caspase-1 inhibitor Ac-YVAD was purchased from Millipore (Billerica, MA, USA). Cytochalasin D, adenosine triphosphate (ATP), and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich Corp. Anti-human IL-1β antibody was purchased from R&D Systems (Minneapolis, MN, USA). Anti-human IL-18 and anti-human-pro-IL-18 antibodies were purchased from MBL International (Woburn, MA, USA). Anti-human caspase-1 p20 antibody was purchased from Invitrogen-Life Technologies (Carlsbad, CA, USA). Recombinant human IL-1α was purchased from PeproTech (Rocky Hill, NJ, USA).

Cell Cultures

Primary cultures of fetal human RPE cells (fhRPE) were prepared from eyes of human fetuses.[21,22] Cells were grown in MEM (Sigma-Aldrich Corp.) supplemented with 5% fetal bovine serum (ThermoFisher Scientific, West Palm Beach, FL, USA), N2 supplement, glutamine penicillin (100 U/mL), streptomycin (100 μg/mL), and nonessential amino acids (Sigma-Aldrich Corp.). Fetal human RPE cell cultures at passage 1 were used in the present study.

ARPE-19 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium-F12 medium (DMEM-F12; Invitrogen-Life Technologies) containing 10% fetal bovine serum and antibiotics.

Microglial cells were derived from primary cultures of human brain microglia obtained from Clonexpress, Inc. (Gaithersburg, MD, USA). These cells were maintained in DMEM-F12 medium containing 5% fetal bovine serum, 10 ng/mL macrophage colony-stimulating factor (M-CSF; R&D Systems), and antibiotics. Cells of passage 1 were used in this study.

THP-1 cells, a monocyte cell line, were obtained from ATCC and maintained in RPMI-1640 medium (Mediatech, Manassas, VA, USA) containing 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and antibiotics.

Inflammasome Activation

Fetal human RPE or ARPE-19 cells were seeded in 24-well plates. When cells reached confluence, they were washed once in serum-free medium and then incubated for 6 hours with LPS at 20 μg/mL and with IL-1α at 25 ng/mL ("priming"). Then, stimulants were added for an additional 1 hour with ATP, or for 5 or 40 hours for other stimulants, as indicated. Microglial cells were seeded into 24-well plates and used for experimentation upon reaching approximately 5×10^4 cells per well. Cells were then washed once in serum-free medium and then incubated with LPS at 0.5 μg/mL and with IL-1α at 25 ng/mL for 6 hours. Tested inflammasome stimulants were added for an additional 40 hours, as indicated. THP-1 cells were seeded at 0.5×10^6 cells per well in 24-well plates. THP-1 cultures stimulated with 7KCh were primed with IL-1α (25 ng/mL) for 6 hours and then incubated with the stimulant for 20 hours. THP-1 cells stimulated with silica crystals were incubated with LPS at 0.5 μg/mL for 6 hours and then incubated with crystals for 20 hours. Caspase-1 inhibitor and cytochalasin D were added to the cultures, at 10 μM, along with the inflammasome stimulants after 6 hours of priming. The inhibitors remained in the cultures throughout the incubation period, as indicated.

ELISA

Culture supernatants were collected to measure the levels of IL-1β, IL-6, IL-8, or IL-18 at the indicated time points. All cytokine levels were quantified using ELISA kits (R&D Systems). Chemiluminescence ELISA kits were used for measuring IL-1β, IL-6, and IL-8 levels, whereas a colorimetric ELISA kit was used for IL-18 measurement.

Western Blotting

Cells were scraped from flasks or cell culture plates and pelleted. Pellets were homogenized, and lysates were collected. Cell lysates or concentrated cell culture supernatants were separated on NuPAGE *bis*-Tris 4%- to 12%-gradient gels (Invitrogen-Life Technologies) and transferred to polyvinylidene fluoride membrane (Invitrogen-Life Technologies). Membranes were blocked with 5% nonfat milk in TBS-Tween buffer for 2 hours at room temperature, followed by incubation with anti-human caspase-1 p20 antibody, anti-human IL-1β, anti-human IL-18 and anti-human pro-IL-18 antibodies overnight at 4°C. Membranes were incubated with the secondary antibody for 2 hours at room temperature. Signals were developed using an enhance chemiluminescent Western blot analysis system (ThermoFisher Scientific) and exposing the protein side of the membrane to X-ray film. Density of the immunoblot bands was assessed by densitometry, using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Quantitative (q)PCR

Total RNA was extracted from cultured cells with TRIzol (Invitrogen-Life Technologies). RNA (1 μg), SuperScript III reverse transcriptase (Invitrogen-Life Technologies), and random hexamer primer (Invitrogen-Life Technologies) were used for first-strand cDNA synthesis. Primer-probe sets for qPCR analysis of human NLRP3, IL-1β, IL-18, and β-actin (internal control) were purchased from Invitrogen-Life Technologies. Polymerase chain reaction parameters were those recommended for TaqMan Universal PCR Master Mix kit (Invitrogen-Life Technologies).

Statistical Analysis

One-way ANOVA associated with Tukey tests were performed for comparison of transcript levels or supernatant cytokine levels among groups. A P value of ≤ 0.05 was defined as statistically significant.

Results

7KCh Induces Generation of Active Caspase-1 in Human RPE Cultures

An early activity of inflammasomes is the cleavage of the precursor molecule of caspase-1 and generation of its enzymatically active forms, namely, P10 or P20. To examine the capacity of 7KCh to initiate inflammasome generation by human RPE cells, we added this oxysterol to cultures of ARPE-19 cells and determined by Western blotting the production of caspase-1 P20.

Before stimulation with 7KCh, the cultures were primed with LPS and IL-1α. Figure 1A shows that ARPE-19 cells incubated with 7KCh at 10 μM produce the active form of caspase-1, similarly to ATP, a known stimulant of inflammasomes.[23] Western blot data from three additional experiments are summarized by densitometry analysis in Figure 1B. The cell cultures in these experiments, stimulated by 7KCh at 6 μM, significantly increased production of the active form of caspase-1.

7KCh Stimulates Expression of NLRP3 and Pro-IL-1β in ARPE-19 Cells, Whereas Pro-IL-18 Is Expressed Constitutively

We further analyzed the capacity of 7KCh to initiate inflammasome formation by evaluating the transcript expression levels of NLRP3, a major component of the inflammasome and of pro-IL-1β and pro-IL-18. Transcript levels were determined by qPCR, and data collected from three repeated experiments are summarized in Figure 2A. Transcript levels of NLRP3 and IL-1β were increased by "priming" with LPS and IL-1α and were further elevated by 7KCh.

A different response pattern, however, was found with the pro-IL-18 transcript. This molecule was produced constitutively, and neither the priming process nor stimulation with 7KCh had measurable effects on the transcript levels of this cytokine.

The qPCR assay data concerning pro-IL-18 expression thus indicated that this procytokine is expressed constitutively by ARPE-19 cells, and we confirmed this observation by Western blotting with the cultured cell lysates. As shown in Figure 2B, ARPE-19 cells did not express pro-IL-1β when cultured with no additional stimulation but did so when primed with LPS and IL-1α. By contrast, these cells did express pro-IL-18 even when cultured with no stimulation, and incubation with the priming agents had no additional effect on the expression of this procytokine. Fetal human RPE cells resembled the ARPE-19 cells by constitutively expressing both pro-IL-18 and IL-18, but unlike ARPE-19 cells, failed to produce detectable pro-IL-1β after being stimulated with LPS and IL-1α. Fetal human RPE's poor capacity to produce IL-1β is in line with the only trace levels of this cytokine in the supernatants of these cells, discussed below (Fig. 3A).

Profiles of IL-1β and IL-18 Produced by Stimulated RPE Cells Are Different From Those Stimulated by Bone Marrow–Derived Cells

The major function of inflammasomes is the generation of mature IL-1β and IL-18, two prominent proinflammatory cytokines that are secreted by activated cells after proteolytic cleavage by caspase-1.[6–8] To measure the levels of IL-1β and IL-18 in the culture supernatants, we used the ELISA technique. Figure 3A presents the mean levels of the two cytokines, measured in repeated experiments, in supernatants of cultured fhRPE, ARPE-19, human brain microglia, and monocyte THP-1 cells. With the exception of THP-1, all cultures were primed with LPS and IL-1α before being stimulated by 7KCh. THP-1 cells are exceedingly sensitive to LPS (see below), and therefore, they were primed in these experiments by IL-1α alone. The hydrophobic 7KCh was added to all cultures complexed with HPβCD. Cells treated with HPβCD alone were found not to generate inflammasomes (data not shown). Importantly, striking differences were observed between the profiles of IL-1β and IL-18 levels in cultures of fhRPE and ARPE-19 cells and those of bone marrow–derived microglia and THP-1 cells. Whereas the two types of RPE cultures produced high levels of IL-18 but low (ARPE-19) or trace levels (fhRPE) of IL-1β, the opposite profile was observed with the two tested bone marrow–derived cells,

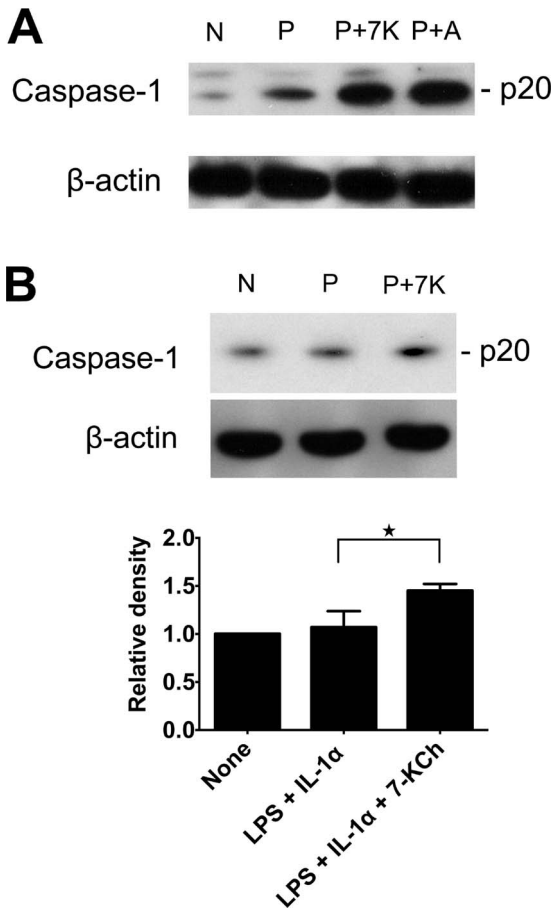

Figure 1. Mature caspase-1 (P20) is produced by RPE cells stimulated with 7KCh. (**A**) ARPE-19 cell cultures were primed for 6 hours with LPS and IL-1α and incubated with 7KCh (10 μM) for an additional 5 hours. Adenosine triphosphate (5 mM) was used as the positive control and was incubated with the cells for 1 hour after priming. (**B**) Similar experiments were repeated three times with 7KCh (6 μM), and Western blot data were summarized by densitometry. N, none; P, priming; 7K, 7KCh; A, ATP. $^*P < 0.05$.

which secreted high levels of IL-1β but relatively lower levels of IL-18. The reduction of the cytokine levels by the caspase-1 inhibitor confirmed the inflammasome origin of IL-1β and IL-18 (Fig. 3A). Cytokine levels secreted by THP-1 cultures were markedly low, because these cultures were used here primed with IL-1α but with no LPS (LPS stimulates excessive levels of IL-1β in THP-1 cells, which make it impossible to identify the additive stimulation by 7KCh).

Further evidence of the capacity of 7KCh to generate inflammasomes that preferentially produce IL-1β or IL-18 was provided by Western blot analysis of the culture supernatants. As shown in Figure 3B, IL-18 was detected in ARPE-19 cell cultures stimulated with 7KCh, whereas IL-1β was identified in similarly stimulated cultures of microglia. Cultures of THP-1 cells produced high levels of IL-1β but only when stimulated with LPS (Fig. 3B); IL-18 was not detected in the supernatant of these THP-1 cells (not shown).

Opposite Patterns of IL-1β and IL-18 Released by Cultured RPE or Bone Marrow–Derived Cells When Stimulated by Agents Other Than 7KCh

Next, we examined the patterns of IL-1β and IL-18 released by cultured RPE (ARPE-19) or bone marrow–derived cells (brain

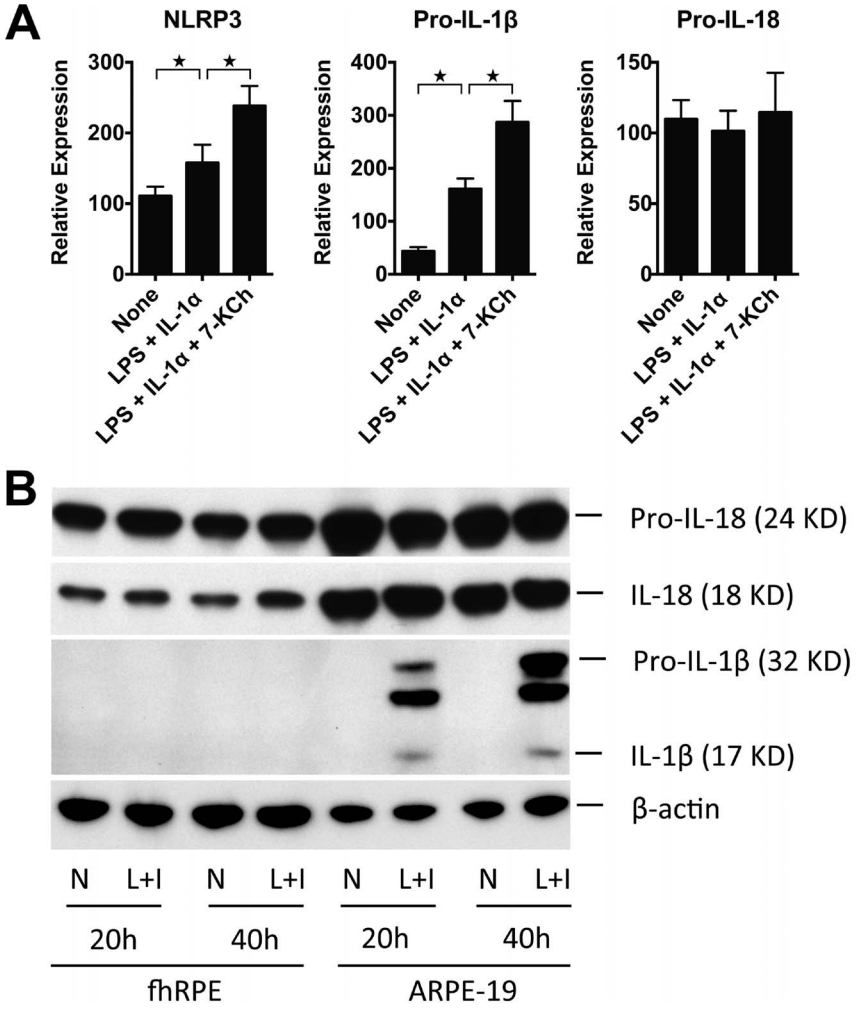

Figure 2. Interleukin-18 is expressed constitutively by RPE cells. (**A**) Quantitative PCR analysis of ARPE-19 cells were activated as indicated and tested for expression of NLRP3, pro-IL-1β, and pro-IL-18. Expression of NLRP3 and IL-1β transcripts was enhanced by priming with IL-1α plus LPS and additional stimulation with 7KCh (6 μM). In contrast, essentially no change was induced in IL-18 transcript expression by the stimulants, indicating constitutive expression. $N = 3$. $^*P < 0.05$. (**B**) Western blot analysis shows pro-IL-18 and mature IL-18 expression by unstimulated ("N") fetal human RPE (fhRPE) and ARPE-19 cells. In contrast, pro–IL-1β expression by ARPE-19 required priming (with LPS and IL-1α ["L+I"]). No IL-1β production was detected in fhRPE cultures, even after priming with LPS and IL-1α. Data from a representative experiment are shown; similar observations were seen in two additional experiments.

microglia) when stimulated by agents other than 7KCh. The agents included silica particles that mediate silicosis[24] and cholesterol crystals, components of atherosclerotic plaques.[25] As shown in Figure 4, these two tested agents resembled 7KCh by preferentially stimulating secretion of relatively high levels of IL-1β and low levels of IL-18 by microglia and THP-1, in contrast to ARPE-19 cells, which released higher levels of IL-18 than of IL-1β. THP-1 cells were primed in these experiments with LPS without IL-1α and responded with exceedingly high levels of secreted IL-1β. The stimulating capacity of silica or cholesterol crystals was suppressed by caspase-1 inhibitor or cytochalasin D, an inhibitor of actin polymerization that disrupts actin microfilaments.[26]

Priming of Cultured Cells Is Essential For Stimulation of Inflammasome Formation by 7KCh

To examine the capacity of 7KCh to activate inflammasome formation by RPE cells without priming, we added the oxysterol to ARPE-19 cultures and measured the levels of IL-1β and IL-18, as well as levels of IL-6 and IL-8, in the supernatants. Data of a representative experiment are shown in Figure 5 and show that IL-6 and IL-8 are expressed constitutively by these RPE cells and that the levels of both cytokines increased in cultures with 7KCh. In contrast, however, neither IL-1β nor IL-18 were detected in the supernatants of these cultures, suggesting that priming is essential for inflammasome formation in this in vitro system.

Discussion

Data recorded here show, for the first time, that 7KCh, an oxysterol that accumulates in the choriocapillaris, Bruch's membrane, and RPE[15–17] is a potent stimulator of inflammasome formation, and consequently, it causes release of two major proinflammatory cytokines, IL-1β and IL-18. 7-Ketocholesterol was found in the present study to stimulate inflammasome formation by human cells of the three types assumed to be involved in the inflammatory component of AMD pathogenic process, namely, RPE, microglia, and myeloid cells. It is noteworthy that, in addition to the commonly used ARPE-19 line cells,[9,10,13] we also tested the activity of hfRPE cells. The

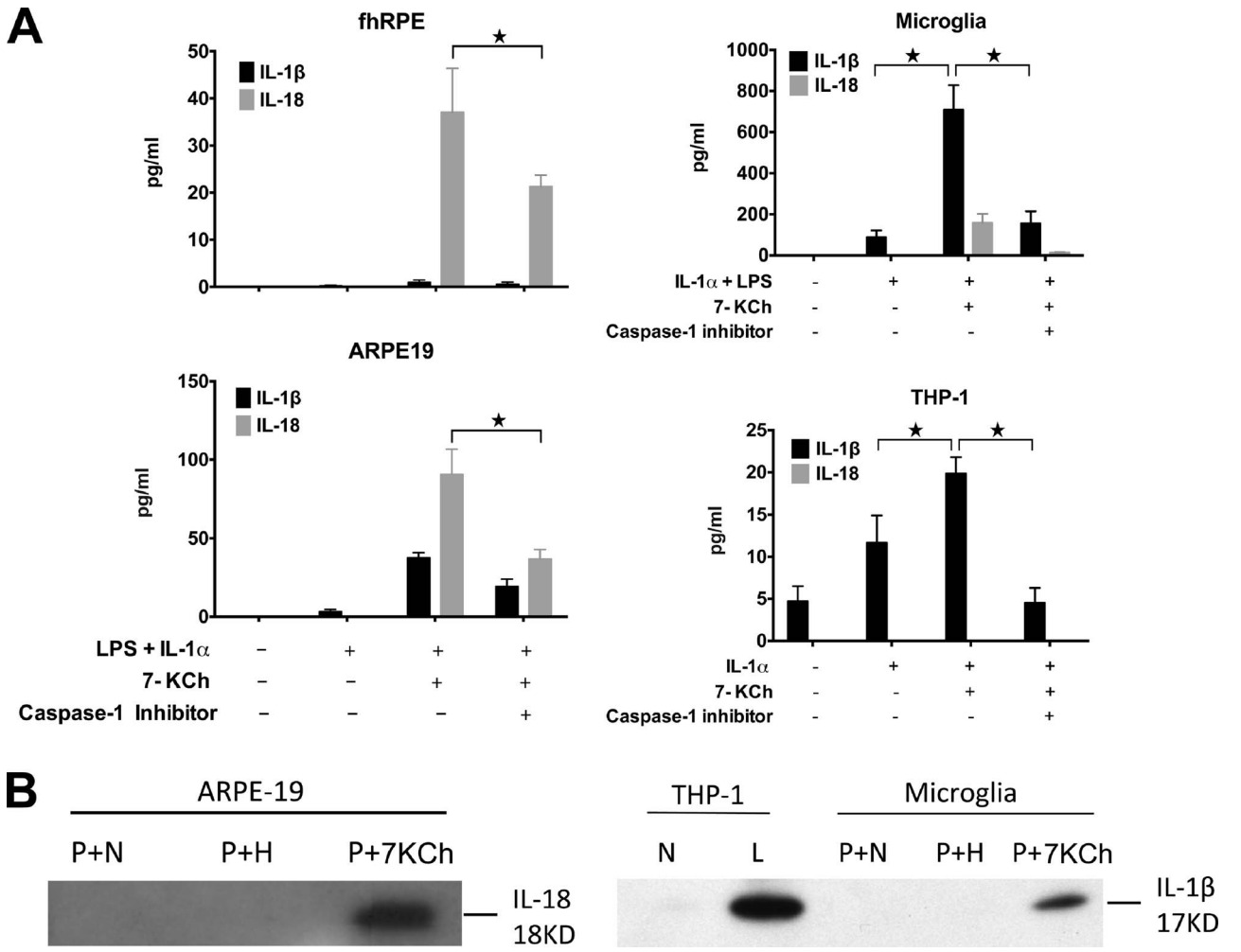

FIGURE 3. Opposite preferential production patterns of IL-1β and IL-18 by 7KCh-stimulated RPE and bone marrow–derived cells. (**A**) Levels of IL-1β and IL-18, measured by ELISA, in supernatants of fhRPE and ARPE-19 cultures or of microglia and THP-1 cultures are shown. Cultures were primed for 6 hours with IL-1α plus LPS or with IL-1α, as indicated, prior to stimulation with 7KCh (6 μM). Inflammasome involvement was examined by inhibition with caspase-1 inhibitor (at 10 μM). Values are means ± SEM. $N = 3$. (**B**) Western blot analyses of IL-1β and IL-18 production in supernatants of cultures of ARPE-19 or of THP-1 and microglia cells are shown. P, priming with LPS and IL-1α; N, none; L, LPS; H, HPβCD (carrier of 7KCh). $^*P < 0.05$.

primary cultured hfRPE cells resembled the ARPE-19 cells in their pattern of activity (Fig. 3). Unlike the tested RPE, brain microglia, and THP-1 cells, essentially no IL-1β or IL-18 was detected in cultures of human Müller cell line, stimulated for inflammasome production by the agents used for the other cell populations (Shi G, et al., unpublished data, 2014). This observation underscores the uniqueness of the non-bone marrow–derived RPE cells in their capacity to form inflammasomes.

Our data also show that priming the cell cultures is essential for stimulation of inflammasome formation by 7KCh (Fig. 5). This observation is in line with numerous publications (e.g., Ref. 27) in which inflammasome formation by cultured cells stimulated with agents such as silica or metallic nanoparticles required priming with LPS or similar agents. It may be assumed that inflammasomes in pathogenic processes such as silicosis are generated by stimulation with "endogenous priming" agents in addition to the silica particles.

The notion concerning the involvement of inflammasomes in the pathogenesis of AMD has been promoted by several recent studies that demonstrated inflammasome formation by RPE cells stimulated by drusen or drusen components.[9,10] Furthermore, Tarallo et al.[11] showed that the RPE of patients with geographic atrophy contained elevated levels of NLRP3 and IL-18, as well as increased activation of caspase-1 and MyD88. The retina is populated by microglia, as well as wandering myeloid cells (particularly in damaged retinas), and Doyle et al.[9] argued that these bone marrow–derived cells play the major role in forming inflammasomes in the affected retina in AMD. Our data support this point by showing the high levels of inflammasome-produced cytokines in cultures of microglia and THP-1 cells (Figs. 3, 4).

Analysis by qPCR of transcript expression levels of NLRP3, IL-1β, and IL-18 revealed, for the first time, that unlike the other two transcripts, IL-18 transcript is expressed constitutively by cultured ARPE-19 cells (Fig. 2A). Furthermore, the constitutive expression of pro–IL-18 and IL-18 by these cells was also shown by Western blotting of cell lysates of both ARPE-19 and fhRPE cells (Fig. 2B). These observations are in line with those of a study by Dinarello et al.[28] showing that IL-18, but not IL-1β, is constitutively expressed by human peripheral blood cells. The remarkable differences between the expression patterns of IL-1β and IL-18, the two major inflammasome products, is in line with the difference in their biological functions and

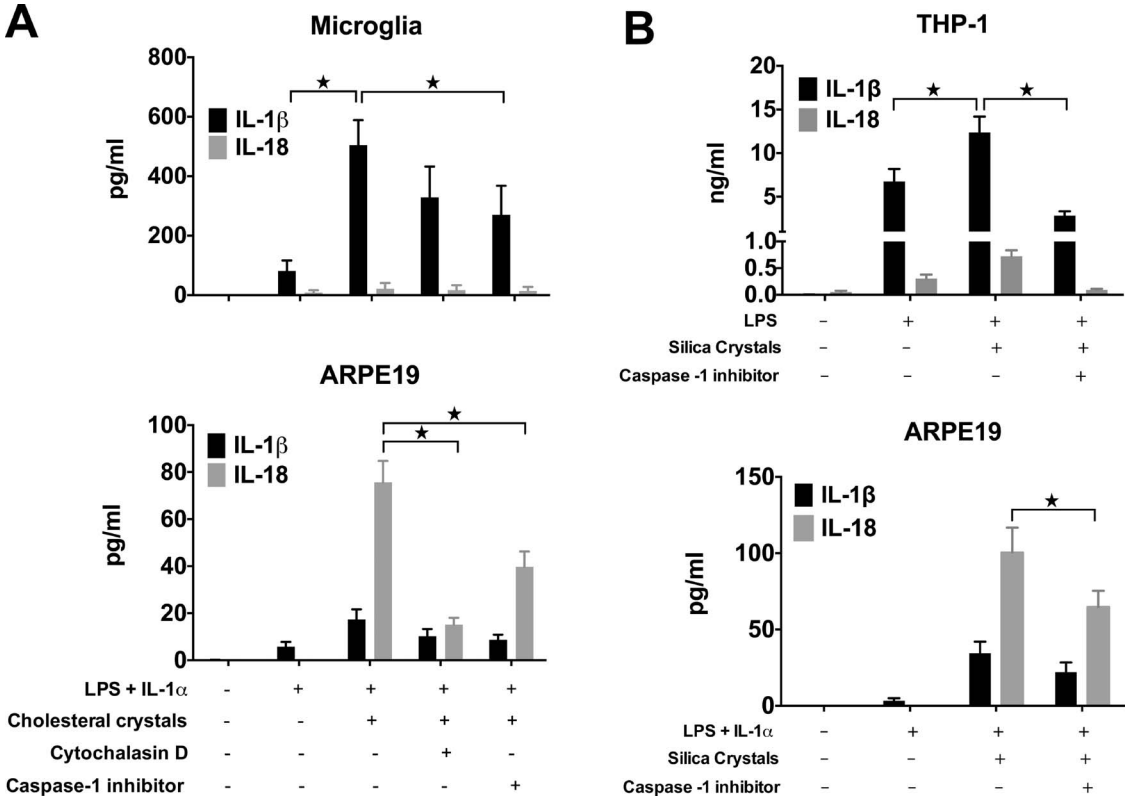

FIGURE 4. Opposite preferential production patterns are shown for IL-1β and IL-18 by RPE and bone marrow–derived cells stimulated by agents other than 7KCh. Cultures were set up, and cytokine levels were determined by ELISA as detailed in the legend for Figure 3. (**A**) Profiles are shown of cytokine production by microglia and ARPE-19 cells primed (with LPS plus IL-1α) and then stimulated with cholesterol crystals and inhibited by cytochalasin D (10 μM) or caspase-1 inhibitor (10 μM), as indicated. (**B**) Cytokine production patterns by THP-1 and ARPE-19 cells, primed and then stimulated by silica particles are shown. THP-1 cells were primed by LPS, with no IL-1α, due to the exceedingly high response of these cells to LPS. $N = 3$. $^*P < 0.05$.

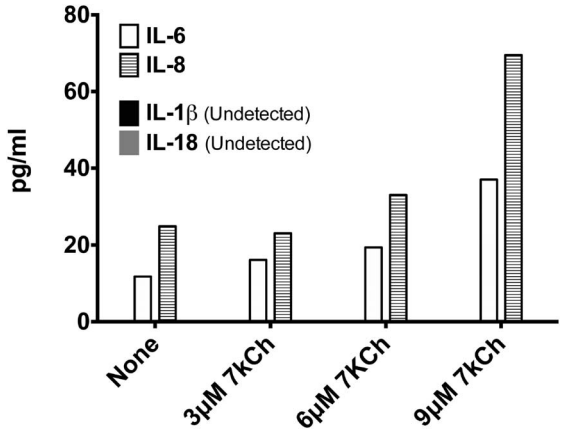

FIGURE 5. Priming of cultured cells was essential for stimulation of inflammasome formation by 7KCh. Cultures of ARPE-19 cells were incubated with 7KCh at the indicated concentration, with no priming, and supernatants were collected after 40 hours of incubation. Cytokine levels were determined by ELISA. Interleukin-6 and IL-8 were expressed constitutively by ARPE-19 cells, and their production was increased by 7KCh, whereas IL-1β and IL-18 were not detected in any of the supernatants. Representative result of three repeated experiments is shown.

indicates that additional investigations are required to further dissect their different roles in the inflammatory process of AMD.

The differences between IL-1β and IL-18 expression levels were further underscored in our study by the new observation that these two cytokines are preferentially expressed with opposite patterns by neural crest-derived and bone marrow–derived cells. Fetal human RPE and ARPE-19 cells preferentially express IL-18, whereas microglia and THP-1 cells produced much more IL-1β than IL-18 (Fig. 3). Our observations of these two cell populations are in line with those in the reports of Doyle et al.[9] and Tarralo et al.,[11] in which the preferential expression of IL-1β or IL-18 was observed in cultures of peripheral blood mononuclear cells[9] or RPE cells,[11] respectively. Our study, comparing concurrently the two different cell populations, made it possible for the first time to make the observation concerning the opposite preferential expression of IL-18 and IL-1β by RPE and bone marrow–derived cells, microglia, and THP-1 in this study.

The two studies cited above[9,11] focused on the involvement of IL-18 in the pathogenic process of AMD but reached two different conclusions. Tarallo et al.[11] reported the cytotoxic activity of IL-18 on RPE cells, whereas Doyle et al.[9] showed the capacity of IL-18 to inhibit the angiogenic process of "wet" AMD. Our finding that RPE cells constitutively expressed IL-18 could be interpreted to support the notion that IL-18 plays a role in protection against neovascularization in the retina. Indeed, two recent reports by Campochiaro et al.[29] and Doyle et al.[30] provide new evidence to show that IL-18 is antiangiogenic and inhibits the biological activity of VEGF.

More investigation is needed to shed light on the involvement of IL-18 and other cytokines in the complex process of AMD.

In summary, the present study demonstrates, for the first time, the potent capacity of 7KCh, an oxysterol that accumulates in the aged eye and induces inflammasome formation. This observation thus supports the notion that 7KCh may be involved in the pathogenic process of AMD. Another new observation made here is the opposite preferential production of IL-1β and IL-18 by bone marrow–derived and RPE cells, respectively. Both the cytokines and the two cell populations are assumed to be involved in the pathogenesis of AMD, and our data provide new information on this issue, which currently remains unresolved.[31,32]

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