Acanthamoeba-Cytopathic Protein Induces Apoptosis and Proinflammatory Cytokines in Human Corneal Epithelial Cells by cPLA$_{2\alpha}$ Activation

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Purpose. We have shown that *Acanthamoeba* interacts with a mannosylated protein on corneal epithelial cells and stimulates trophozoites to secrete a mannose-induced 133 kDa protease (MIP-133), which facilitates corneal invasion and induces apoptosis. The mechanism of MIP-133–induced apoptosis is unknown. The aim of this study was to determine if MIP-133 induces apoptosis and proinflammatory cytokines/chemokines in human corneal epithelial (HCE) cells via the cytosolic phospholipase A$_{2\alpha}$ (cPLA$_{2\alpha}$) pathway.

Methods. HCE cells were incubated with or without MIP-133 at doses of 7.5, 15, and 50 μg/mL for 6, 12, and 24 hours. The effects of cPLA$_{2\alpha}$ inhibitors on cPLA$_{2\alpha}$, arachidonic acid (AA) release, and apoptosis were tested in vitro. Inhibition of cPLA$_{2\alpha}$ involved preincubating HCE cells for 1 hour with cPLA$_{2\alpha}$ inhibitors (10 μM methyl-arachidonyl fluorophosphonate [MAFP] or 20 μM arachidonyl trifluoromethyl ketone [AACOCF3]) with or without MIP-133 for 24 hours. Expression of cPLA$_{2\alpha}$ mRNA and enzyme was examined by RT-PCR and cPLA$_2$ activity assays, respectively. Apoptosis of corneal epithelial cells was determined by caspase-3 and DNA fragmentation assays. Expression of IL-8, IL-6, IL-1β, and IFN-γ was examined by RT-PCR and ELISA.

Results. MIP-133 induced significant cPLA$_{2\alpha}$ (approximately two to four times) and AA release (approximately six times) from corneal cells while cPLA$_{2\alpha}$ inhibitors significantly reduced cPLA$_{2\alpha}$ (approximately two to four times) and AA release (approximately three times) ($P < 0.05$). cPLA$_{2\alpha}$ inhibitors significantly inhibited MIP-133–induced DNA fragmentation approximately 7 to 12 times in HCE cells ($P < 0.05$). MIP-133 specifically activates cPLA$_{2\alpha}$ enzyme activity in HCE cells, which is blocked by preincubation with anti–MIP-133 antibody. In addition, MIP-133 induced significant IL-8, IL-6, IL-1β, and IFN-γ production, approximately two to three times ($P < 0.05$).

Conclusions. MIP-133 interacts with phospholipids on plasma membrane of HCE cells and activates cPLA$_{2\alpha}$. cPLA$_{2\alpha}$ is involved in apoptosis, AA release, and activation of proinflammatory cytokines/chemokines from HCE cells. cPLA$_{2\alpha}$ inhibitors may be a therapeutic target in *Acanthamoeba* keratitis. (*Invest Ophthalmol Vis Sci.* 2012;53:7973–7982) DOI:10.1167/iovs.12-10436

Acanthamoeba keratitis (AK) is a sight-threatening chronic inflammatory disease of the cornea caused by several species of free-living pathogenic amoebae.[1,2] Disease symptoms of AK include a ring-like corneal infiltrate, epithelial destruction, and disproportionately severe ocular pain. Topical or systemic treatment of AK with antibiotics, antifungals, and antivirals is often ineffective.[3–5] It has been shown that *Acanthamoeba* binds to the corneal surface by mannose-binding protein (MBP), which induces a cytopathic effect.[6,7] We have demonstrated that the binding of *Acanthamoeba* to corneal epithelial cells induces release of the mannose-induced 133 kDa protease (MIP-133). MIP-133 affects the subsequent steps in the pathogenic cascade of AK, including the cytopathic effects on the corneal epithelium and the stroma, penetration of the basement membrane, and the dissolution of the collagenous stroma.[1,8–10] MIP-133 protein was found to be effective at activating a caspase-3-dependent apoptosis pathway in corneal epithelial cells as well as in keratocytes.[1,8] We demonstrated that unlike "amoebapores," the *Entamoeba histolytica* cytolytic peptides, MIP-133 does not perforate the lipid bilayers to cause cell death.[1,11] How the MIP-133 protein interacts with the cell surface to cause apoptosis is still unknown. Recently, it has been demonstrated that *Pseudomonas aeruginosa* induces apoptosis in human lung fibroblasts and human conjunctiva epithelial cell lines through the activation of cytosolic phospholipase A$_2$ (cPLA$_2$) and arachidonic acid (AA) release via a contact-dependent mechanism.[12] It is known that MIP-133 induces apoptosis upon contact with corneal cells[1,8]; however, the cytopathic signaling involved with this interaction is unknown. We hypothesized that cPLA$_2$ is involved in apoptosis of corneal epithelial cells induced by MIP-133. PLA$_2$ enzymes are divided into four major families: platelet-activating factor acetylhydrolases (PAF-AHs); secreted PLA$_2$s (sPLA$_2$s); intracellular Ca^{2+}-independent PLA$_2$s (iPLA$_2$s); and cytosolic Ca^{2+}-dependent PLA$_2$s (cPLA$_2$s). cPLA$_2$s are classified into five subgroups, α through ζ.[13–15] cPLA$_{2\alpha}$ has been studied comprehensively because it is the only PLA$_2$ that exhibits specificity for hydrolysis of sn-2 AA from phospholipids for eicosanoid biosynthesis in response to a wide variety of extracellular stimuli,[16,17] and is regulated by phosphorylation and an increase in intracellular calcium.[13] Phosphorylation of cPLA$_{2\alpha}$ by mitogen-activated protein kinases (MAPKs) is required for cPLA$_{2\alpha}$-mediated release of AA in stimulated cells.[16,17] Previous studies demonstrated the dual role of PLA$_2$s in several eye diseases, which may be related to their enzymatic activities or to regulatory functions including signaling and protein–protein interactions.[18] AA is one of the biologically important free fatty acids released by cPLA$_{2\alpha}$,

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which subsequently converts to prostanoids and leukotrienes stimulating apoptosis through activation of the mitochondrial pathway. The release of AA by the activation of $cPLA_{2\alpha}$ in cells induced to undergo apoptosis is associated with loss of cell viability, caspase activation, and DNA fragmentation.[14] The present study addressed the role of MIP-133 in the induction of apoptosis and proinflammatory cytokines due to AA accumulation by the $cPLA_{2\alpha}$ pathway. Here, we demonstrate that MIP-133–induced apoptosis of human corneal epithelial (HCE) cells is associated with an increase in $cPLA_{2\alpha}$ activity and increases the levels of $cPLA_{2\alpha}$, AA, and proinflammatory cytokines/chemokines.

Materials and Methods

Amoebae and Human Cell Lines

Acanthamoeba castellanii (ATCC 30,868), isolated from a human cornea, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Amoebae were grown as axenic cultures in peptone yeast extract glucose at 35°C with constant agitation on a shaker incubator at 125 rpm.[19] Human telomerase-immortalized corneal epithelial (HCE) cells were a generous gift from James Jester (University of California-Irvine, Irvine, CA). HCE cells were cultured in keratinocyte medium (KGM-2 Bullet Kit; Lonza, Walkersville, MD) containing 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) at 37°C in a humidified 5% CO_2 atmosphere.

Mouse Corneal Epithelial Cell Cultures

Fas-deficient (B6.MRL-*Tnfrsf6*^{lpr}, H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Cell cultures were established from freshly dissected corneal explants as described previously[20] and cultured in minimum essential medium (MEM) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM MEM vitamins, and 1% penicillin–streptomycin–fungizone solution (BioWhittaker; Lonza) and 10% heat-inactivated FBS (HyClone Laboratories, Inc.).

MIP-133

The MIP-133 protein was isolated and characterized as reported previously,[21] and protein concentrations were determined using the bicinchoninic acid (BCA) protein assay.[22]

Caspase-3 Assay for Apoptosis

Corneal epithelial cells from Fas receptor–deficient mice (*lpr/lpr*) were cultured in 24-well plates at ~90% confluence and incubated with and without MIP-133 for 24 hours at a dose of 15 μg/mL. Cells were collected by centrifugation at 2000*g* for 10 minutes at 4°C and used for apoptosis assay. Briefly, cells were washed three times in Hanks' balanced salt solution (HBSS) and resuspended in cytofix/cytoperm solution (permeabilization buffer; BD Biosciences Pharmingen, San Diego, CA), then washed in Perm-Wash buffer (BD Biosciences Pharmingen). Cells were stained with phycoerythrin (PE)-labeled rabbit anti-caspase-3 antibody (BD Biosciences Pharmingen) for 20 minutes in the dark. Active anti-caspase-3 antibody detects the active form of the enzyme that occurs in apoptotic cells. The samples were analyzed by FACS Scan flow cytometry (BD Biosciences, Franklin Lakes, NJ). As a positive control of apoptosis, the cells were treated with 3 μg/mL staurosporine (Sigma Chemical Co., St. Louis, MO), and apoptosis was determined by caspase-3 assay. For each sample, 5000 to 10,000 ungated events were acquired and the results were analyzed with CellQuest Software (BD Biosciences, San Jose, CA). Isotype control IgG was used as a control antibody. The results were expressed as the percentage of cells that stained positively with anti-caspase-3 antibody.

HCE Cell Cultures and Treatment Experiments

HCE cells were cultured in 24-well plates at ~90% confluence in KGM-2 medium and incubated with or without MIP-133 at doses of 7.5, 15, and 50 μg/mL for 6, 12, and 24 hours. Inhibition of $cPLA_{2\alpha}$ involved preincubating HCE cells for 1 hour with $cPLA_{2\alpha}$ inhibitors (10 μM methyl-arachidonyl fluorophosphonate[12] [MAFP; Cayman Chemical Company, Ann Arbor, MI] or 20 μM arachidonyl trifluoromethyl ketone[12,23] [AACOCF3; Enzo Life Sciences, Inc., Farmingdale, NY]) or with the negative control compound arachidonyl methyl ketone (20 μM AACOCH3; BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), followed by incubation with or without 15 μg/mL MIP-133 for 24 hours. The inhibitors were dissolved in dimethyl sulfoxide (DMSO; Fisher BioReagents, Fair Lawn, NJ). HCE cells incubated with KGM-2 medium in each experiment without treatment with MIP-133 and $cPLA_{2\alpha}$ inhibitors served as control untreated group. Cells and supernatants were collected by centrifugation at 2000*g* for 10 minutes at 4°C.

Isolation of RNA and RT-PCR

HCE cells were collected from 24-well plates at the indicated times after treatments. Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration of total RNA and RNA integrity were determined by measurement of the absorbance at 260 nm and 280 nm and by agarose gel electrophoresis, respectively. cDNA was synthesized from 2 μg total RNA by RT-PCR using random primers (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). PCR was performed using AmpliTaq Gold PCR Master Mix (Applied Biosystems). The amplification profile included one cycle of initial denaturation at 94°C for 5 minutes, 40 cycles of denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, and subsequently, one cycle of final extension at 72°C for 10 minutes. The mRNA expression of each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control.

The oligonucleotide primers for $cPLA_{2\alpha}$, IL-8, IL-6, IL-1β, IFN-γ, and GAPDH were as follows:

$cPLA_{2\alpha}$ (450 bp):
5′-GAGTTTTGGGCGTTTCTGGT-3′ (sense)
5′-ACGGCAGGTTAAATGTGAGC-3′ (anti-sense)
IL-8 (289 bp):
5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ (sense)
5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′ (anti-sense)
IL-6 (200 bp):
5′-ATGAACTCCTTCTCCACAAGCGC-3′ (sense)
5′-GAAGAGCCCTCAGGCTGGACTG-3′ (anti-sense)
IL-β (204 bp):
5′-CCTGTGGCCTTGGGCCTCAA-3′ (sense)
5′-GGTGCTGATGTACCAGTTGGG-3′ (anti-sense)
IFN-γ (427 bp):
5′-GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC-3′ (sense)
5′-CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG-3′ (anti-sense)
GAPDH (450 bp):
5′-ACCACAGTCCATGCCATCAC-3′ (sense)
5′-TCCACCACCCTGTTGCTGTA-3′ (anti-sense)

All primers were verified by BLAST (Basic Local Alignment Search Tool; in the public domain, http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the National Center for Biotechnology Information database for specificity to the human genes of interest.

$cPLA_{2\alpha}$ Activity in Cell Lysates

HCE cells were collected from 24-well plates at the indicated times after treatments and then centrifuged at 2000*g* for 10 minutes at 4°C. The cell pellets were washed with phosphate-buffered saline (PBS) by

centrifugation at 2000 rpm for 5 minutes and then homogenized by Tissue-Tearor (Biospec Products, Inc., Bartlesville, OK) with its specific procedure in 1 mL cold buffer (i.e., 50 mM HEPES [Mediatech, Inc., Manassas, VA], pH 7.4, containing 1 mM EDTA [VWR International, LLC, West Chester, PA]). Cell lysates were collected by centrifugation at 10,000*g* for 15 minutes at 4°C. cPLA$_{2\alpha}$ activity was measured using the PLA$_2$ substrate arachidonoyl thiophosphatidylcholine according to the protocol recommended by the manufacturer (Cayman Chemical Company). The absorbance was measured at 414 nm in a microplate reader (Gen51.10; BioTek Instruments, Inc., Winooski, VT). cPLA$_{2\alpha}$ activity was expressed in nanomoles/milligram of protein/minute determined from the extinction coefficient of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) as instructed in the cPLA$_2$ assay kit (Cayman Chemical Company), and the protein content in the supernatants was quantified using the BCA protein assay.[22] Sensitivity of the activity assay was 3.5 to 42 nmol/min/mL without sample dilution.

Anti–MIP-133 Antibody Treatment

HCE cells were cultured in 24-well plates at ~90% confluence in KGM-2 medium. Inhibition of experiments involved preincubating HCE cells for 1 hour with either 1:75 diluted chicken anti–MIP-133 antiserum (Aves Labs, Inc., Tigard, OR) or chicken preimmune serum control followed by incubation with or without 15 μg/mL MIP-133 for 24 hours. Additional control wells contained untreated confluent cells. Following incubation, all wells were washed three times with their respective growth medium. Cells were collected from 24-well plates and then centrifuged at 2000*g* for 10 minutes at 4°C. The cell pellets were washed with PBS, and cPLA$_{2\alpha}$ activity was measured as described above.

AA Release Assay

HCE cells (1×10^5 cells/mL) were added to 24-well plates and then labeled for 18 hours with 0.05 μCi/mL [3H]arachidonic acid [5,6,8,9,11,12,14,15-3H(N)] (New England Nuclear, Boston, MA) as described previously.[12] Prior to stimulation, cells were washed with KGM-2 medium without serum. Cells were preincubated for 1 hour with cPLA$_{2\alpha}$ inhibitors (10 μM MAFP or 20 μM AACOCF3) and then incubated for 24 hours with or without 15 μg/mL MIP-133. To determine the release of extracellular AA, supernatants were collected after 24-hour incubation and centrifuged at 2000*g* for 5 minutes in a microcentrifuge to remove any cells that may have detached. The released radioactivity in the supernatants was evaluated by liquid scintillation counting. Maximum release values were obtained by quantitating the counts per minute (CPM) present in both the supernatants and the lysed cellular pellet remaining in the wells via addition of 0.5 mL 0.1% Triton X-100 in H_2O. The spontaneous release of AA from radiolabeled cells was determined by incubating the radiolabeled cells in medium alone. The percent specific release of radiolabel of extracellular AA release was calculated as described previously.[12]

Apoptosis Assessment by DNA Fragmentation Assay

Apoptosis was determined by Cellular DNA Fragmentation ELISA (Cell Death Detection ELISA^{Plus} kit; Roche Diagnostics GmbH, Mannheim, Germany) as described previously.[24] In brief, HCE cells (1×10^5 cells in 200 μL supplemented KGM-2 medium) were preincubated for 1 hour with or without cPLA$_{2\alpha}$ inhibitors (10 μM MAFP or 20 μM AACOCF3) or 20 μM AACOCH3 (a negative control compound) and then incubated for 24 hours with or without 15 μg/mL MIP-133. Cells were centrifuged for 10 minutes at 200*g*, and the cell pellets were incubated with lysis buffer supplied by the manufacturer for 30 minutes at room temperature. Twenty microliters of the supernatant (cytoplasmic fraction) was used in the Cellular DNA Fragmentation ELISA following the manufacturer's standard protocol. Absorbances were measured at 405 nm and 490 nm (reference wavelength) using a microplate reader (Gen51.10; BioTek Instruments, Inc.). Signals in the wells containing the substrate only were subtracted as background. The results were expressed in absorbance ($A_{405nm} - A_{490nm}$).

Table. Percent Apoptosis of Corneal Epithelial Cells of Fas Receptor–Deficient Mice

Groups	Mean ± SEM	*P* value*
Untreated corneal epithelial cells	7 ± 3	
MIP-133–treated corneal epithelial cells	67 ± 8	0.011
Staurosporine-treated corneal epithelial cells	82 ± 11	0.04

Corneal epithelial cells from Fas receptor–deficient mice (*lpr/lpr*) were cultured in 24-well plates (1×10^6 cells/well) with and without MIP-133 for 24 hours, and apoptosis was determined by caspase-3 assay. As a positive control of apoptosis, the cells were treated with 3 μg/mL staurosporine. The data are mean ± SEM of three independent experiments.

* *P* value < 0.05 by unpaired Student's *t*-test for treated corneal epithelial cells versus untreated corneal epithelial cells.

ELISA

Cytokines/chemokines (IL-8, IL-6, IL-1β, and IFN-γ) were quantified from cell supernatants using ELISA. Briefly, cell culture supernatants were collected at the indicated times after treatments and centrifuged to remove cell debris. Total protein concentrations of supernatants were determined by BCA protein assay.[22] Levels of IL-8, IL-6, IL-1β, and IFN-γ were determined using specific ELISA test kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Gen51.10; BioTek Instruments, Inc.). The minimum detectable levels of IL-8, IL-6, IL-1β, and IFN-γ by ELISA were 3.5 pg/mL, >0.70 pg/mL, >1 pg/mL, and >8.0 pg/mL, respectively. The results were expressed in pg/mg protein of IL-8, IL-6, IL-1β, and IFN-γ.

Statistics

All experiments were performed in triplicate, and results are presented as mean ± SEM. Differences between two groups were determined by unpaired Student's *t*-test. Differences between multiple groups were determined by two-way analysis of variance (ANOVA). In all analyses, $P < 0.05$ was considered statistically significant.

Results

MIP-133 Induces Apoptosis in Corneal Epithelial Cells of Fas Receptor–Deficient Mice (*lpr/lpr*)

We have shown that MIP-133 induces apoptosis in human and Chinese hamster corneal epithelial cells.[21] To determine the involvement of Fas/Fas ligand interactions in MIP-133–induced apoptosis in corneal cells, corneal epithelial cells of Fas receptor–deficient mice were treated with and without MIP-133, and apoptosis was detected by caspase-3 assay. MIP-133–treated corneal epithelial cells displayed a significant ($P < 0.05$) increase in apoptosis over the level in untreated cells. Staurosporine (positive control) induced a significant ($P < 0.05$) increase in apoptosis of the corneal epithelial cells (Table). These results indicate that MIP-133 induces apoptosis in corneal epithelial cells without involvement of Fas/Fas ligand interactions.

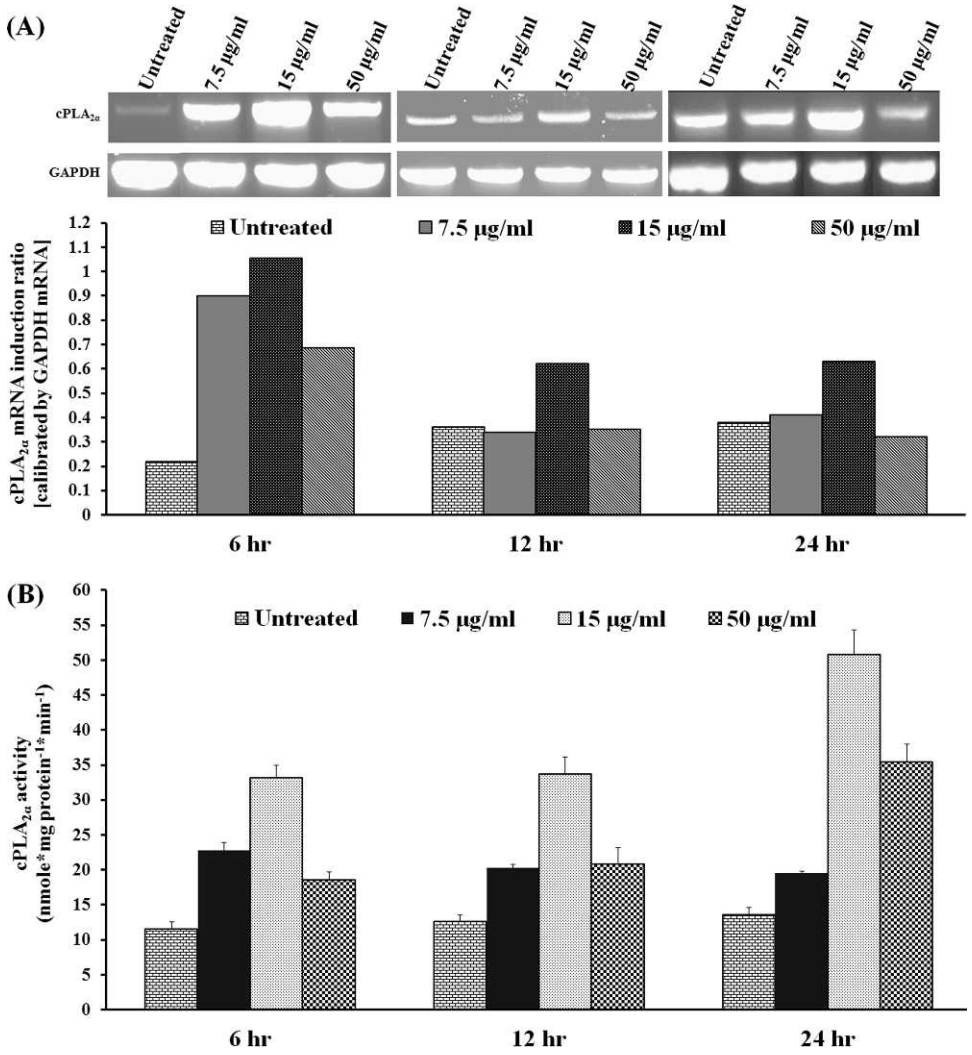

Figure 1. MIP-133 upregulates $cPLA_{2\alpha}$ in HCE cells. HCE cells were exposed to MIP-133 at doses of 7.5, 15, and 50 µg/mL for 6, 12, and 24 hours, and then total RNA was isolated for RT-PCR. The amount of mRNA expression was quantified by densitometry of bands in comparison to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (**A**). HCE cells were homogenized, and then cell lysates were used to perform $cPLA_{2\alpha}$ enzyme analysis (**B**). The data are mean ± SEM of three independent experiments. P values < 0.05 were obtained by ANOVA.

MIP-133 Upregulates $cPLA_{2\alpha}$ mRNA Expression and $cPLA_{2\alpha}$ Activity in HCE Cells

To determine if MIP-133 activates phospholipids on corneal epithelial cells, HCE cells were treated with different doses of MIP-133, and expression of $cPLA_{2\alpha}$ was detected by RT-PCR. MIP-133 upregulated $cPLA_{2\alpha}$ mRNA expression at doses of 7.5, 15, and 50 µg/mL after 6 hours of treatment. This upregulation was observed at the dose of 15 µg/mL at both 12 and 24 hours (Fig. 1A). $cPLA_{2\alpha}$ enzyme activity was determined in cell lysates. All three doses of MIP-133 significantly ($P < 0.05$) increased $cPLA_{2\alpha}$ activity at 6, 12, and 24 hours after incubation (Fig. 1B). These results indicate that MIP-133 induced upregulation of $cPLA_{2\alpha}$ at mRNA and protein levels.

$cPLA_{2\alpha}$ Upregulation by MIP-133 Is Diminished by $cPLA_{2\alpha}$ Inhibitors in HCE Cells

To determine if MIP-133 activates phospholipids in HCE cells via the $cPLA_{2\alpha}$ pathway, the effect of various $cPLA_{2\alpha}$ inhibitors on $cPLA_{2\alpha}$ activity was examined by enzyme assays. $cPLA_{2\alpha}$ activity increased significantly after MIP-133 treatment. Moreover, $cPLA_{2\alpha}$ inhibitors AACOCF3 and MAFP alone or in combination significantly inhibited MIP-133–induced $cPLA_{2\alpha}$ activity ($P < 0.05$). The inactive control compound AACOCH3 did not inhibit MIP-133–induced $cPLA_{2\alpha}$ activity (Fig. 2). These results indicate that MIP-133 activates $cPLA_{2\alpha}$ enzyme activity in HCE cells, which is blocked by preincubation with $cPLA_{2\alpha}$ inhibitors.

Anti–MIP-133 Antibody Attenuates $cPLA_{2\alpha}$ Activity Induced by MIP-133 in HCE Cells

To determine if MIP-133 activates phospholipids in HCE cells via the specific $cPLA_{2\alpha}$ pathway, the effect of chicken anti–MIP-133 antibody on $cPLA_{2\alpha}$ activity was examined by enzyme assays. Previously, it has been demonstrated that anti–MIP-133 antiserum (Aves Labs, Inc.) inhibits cytopathic effects (CPEs) against HCE cells in vitro, and its specific binding to the MIP-133 protein was revealed through Western blot analysis and ELISA.[1] We observed that $cPLA_{2\alpha}$ activity increased significantly after MIP-133 treatment ($P < 0.05$). Moreover, chicken anti–MIP-133 antiserum significantly inhibited MIP-133–induced $cPLA_{2\alpha}$ activity ($P < 0.05$). The chicken preimmune normal

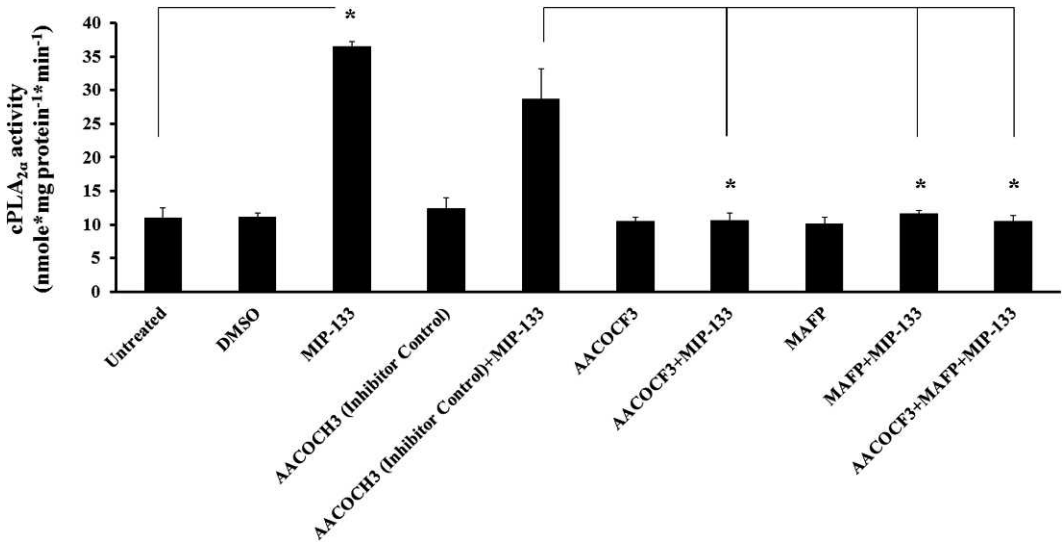

Figure 2. Effect of cPLA$_{2\alpha}$ inhibitors (AACOCF3 and MAFP) on MIP-133–induced cPLA$_{2\alpha}$ enzyme activity in HCE cells. HCE cells were preincubated for 1 hour with cPLA$_{2\alpha}$ inhibitors (10 μM MAFP or 20 μM AACOCF3) or an inactive negative control (20 μM AACOCH3), and then incubated with or without 15 μg/mL MIP-133 for 24 hours. Induction of cPLA$_{2\alpha}$ enzyme activity was examined using a cPLA$_2$ assay kit. The data are mean ± SEM of three independent experiments. *Asterisk* indicates P value < 0.05 by unpaired Student's t-test.

serum did not inhibit MIP-133–induced cPLA$_{2\alpha}$ activity (Fig. 3). These results indicate that MIP-133 specifically activates cPLA$_{2\alpha}$ enzyme activity in HCE cells, which is blocked by preincubation with chicken anti–MIP-133 antiserum.

MIP-133–Induced AA Release Is Diminished by cPLA$_{2\alpha}$ Inhibitors in HCE Cells

To determine whether cPLA$_{2\alpha}$ is involved in MIP-133–induced extracellular AA release from HCE cells, we tested the effect of various inhibitors on AA release. HCE cells were incubated with 15 μg/mL MIP-133, and AA release was detected after 24 hours of stimulation. cPLA$_{2\alpha}$ inhibitors AACOCF3 and MAFP significantly ($P < 0.05$) reduced AA release induced by MIP-133 from HCE cells (Fig. 4). The results suggest that cPLA$_{2\alpha}$ pathway is involved in AA release.

cPLA$_{2\alpha}$ Inhibitors Reduce MIP-133–Induced Apoptosis in HCE Cells

We aimed to gain insight into the functional role of AA release from HCE cells after MIP-133–induced cPLA$_{2\alpha}$ activity; therefore we studied apoptosis in HCE cells after stimulation with MIP-133. Treatment of HCE cells with MIP-133 induced significant ($P < 0.05$) apoptosis on HCE cells compared to untreated control cells (Fig. 5). cPLA$_{2\alpha}$ inhibitors AACOCF3 and MAFP significantly ($P < 0.05$) inhibited this MIP-133–induced apoptosis. In contrast, the inactive control compound AACOCH3 did not block apoptosis induced by MIP-133. The results indicate that cPLA$_{2\alpha}$ is involved in MIP-133–mediated apoptosis of HCE cells.

MIP-133 Upregulates Proinflammatory Cytokines in HCE Cells

We have shown that MIP-133 plays an important role in the pathogenicity of AK by inducing cytolysis of the corneal cells and stimulating the accumulation of polymorphonuclear neutrophils (PMN) in the cornea (Alizadeh H, et al. *IOVS* 2011;52:ARVO E-Abstract 5795). Several studies demonstrated the expression of proinflammatory cytokines in HCE cells after inflammatory stimuli.[25–35] We hypothesized that MIP-133 interacts with the corneal epithelial cells through activation of the cPLA$_{2\alpha}$ pathway, leading to the production of cytokines and chemokines and recruitment of neutrophils into the cornea. To determine the expression and production of cytokines in cultured corneal cells, cells and supernatants were collected at indicated times after MIP-133 stimulation. The cells and supernatants were subjected to RT-PCR and ELISA for detection of IL-8, IL-6, IL-1β, and IFN-γ mRNA (Fig. 6) and protein (Fig. 7). Unstimulated HCE cells constitutively expressed low levels of IL-8, IL-6, IL-1β, and IFN-γ mRNA. MIP-133 (7.5–50 μg/mL) significantly increased mRNA and protein expression ($P < 0.05$) of these cytokines at all three doses and all three time points (6, 12, and 24 hours).

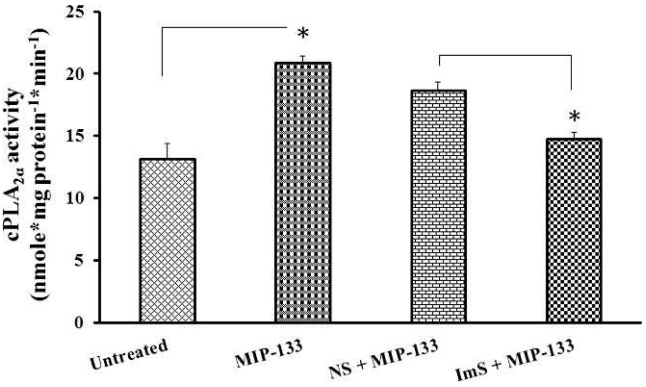

Figure 3. Effect of anti–MIP-133 antiserum (ImS) on MIP-133–induced cPLA$_{2\alpha}$ enzyme activity in HCE cells. HCE cells were preincubated for 1 hour with chicken anti–MIP-133 antiserum (ImS, 1:75 dilution) or chicken preimmune normal serum control (NS, 1:75 dilution), and then incubated with or without 15 μg/mL MIP-133 for 24 hours. Induction of cPLA$_{2\alpha}$ enzyme activity was examined using a cPLA$_2$ assay kit. The data are mean ± SEM of three independent experiments. *Asterisk* indicates P value < 0.05 by unpaired Student's t-test.

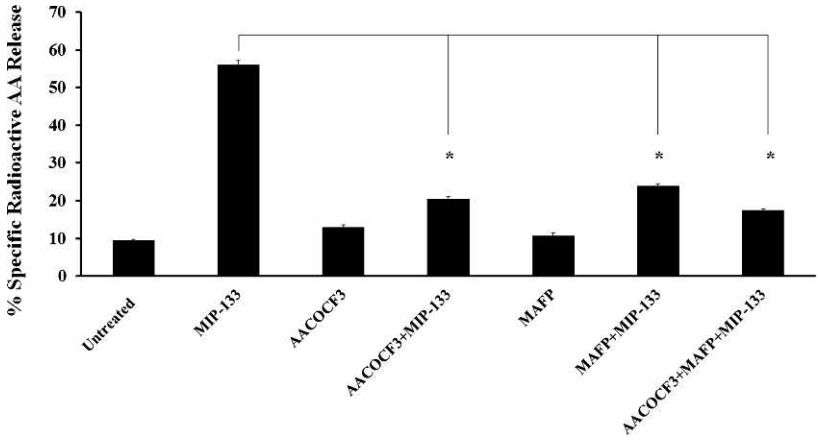

Figure 4. Effect of $cPLA_{2\alpha}$ inhibitors on MIP-133–induced AA release from HCE cells. The HCE cells were preincubated for 1 hour with $cPLA_{2\alpha}$ inhibitors (10 μM MAFP or 20 μM AACOCF3) and then incubated with or without 15 μg/mL MIP-133 for 24 hours. AA release by MIP-133 treatment was determined by liquid scintillation counting. The data are mean ± SEM of three independent experiments. *Asterisk* indicates P value < 0.05 by unpaired Student's t-test.

Proinflammatory Cytokine Induction by MIP-133 Is Diminished by $cPLA_{2\alpha}$ Inhibitors

To determine whether $cPLA_{2\alpha}$ is involved in MIP-133–induced proinflammatory cytokine release from HCE cells, the effect of various $cPLA_{2\alpha}$ inhibitors on the production of IL-8, IL-6, IL-1β, and IFN-γ was tested in vitro. HCE cells were preincubated with the $cPLA_{2\alpha}$ inhibitors MAFP or AACOCF3 and then incubated with 15 μg/mL MIP-133. IL-8, IL-6, IL-1β, and IFN-γ production was measured by ELISA 24 hours after MIP-133 stimulation. AACOCH3 was used as an inactive negative control. IL-8, IL-6, IL-1β, and IFN-γ production was increased significantly ($P < 0.05$) after MIP-133 stimulation (Fig. 8). MAFP and AACOCF3 significantly inhibited MIP-133–induced proinflammatory cytokine production ($P < 0.05$). However, the combination of MAFP and AACOCF3 did not have an additive effect, most likely because each inhibitor alone reduced cytokine expression to basal levels. These results indicate that $cPLA_{2\alpha}$ is involved in MIP-133–induced proinflammatory cytokine production in HCE cells.

Discussion

In the present study, MIP-133 induced apoptosis in corneal epithelial cells of Fas receptor–deficient mice (*lpr/lpr*), which

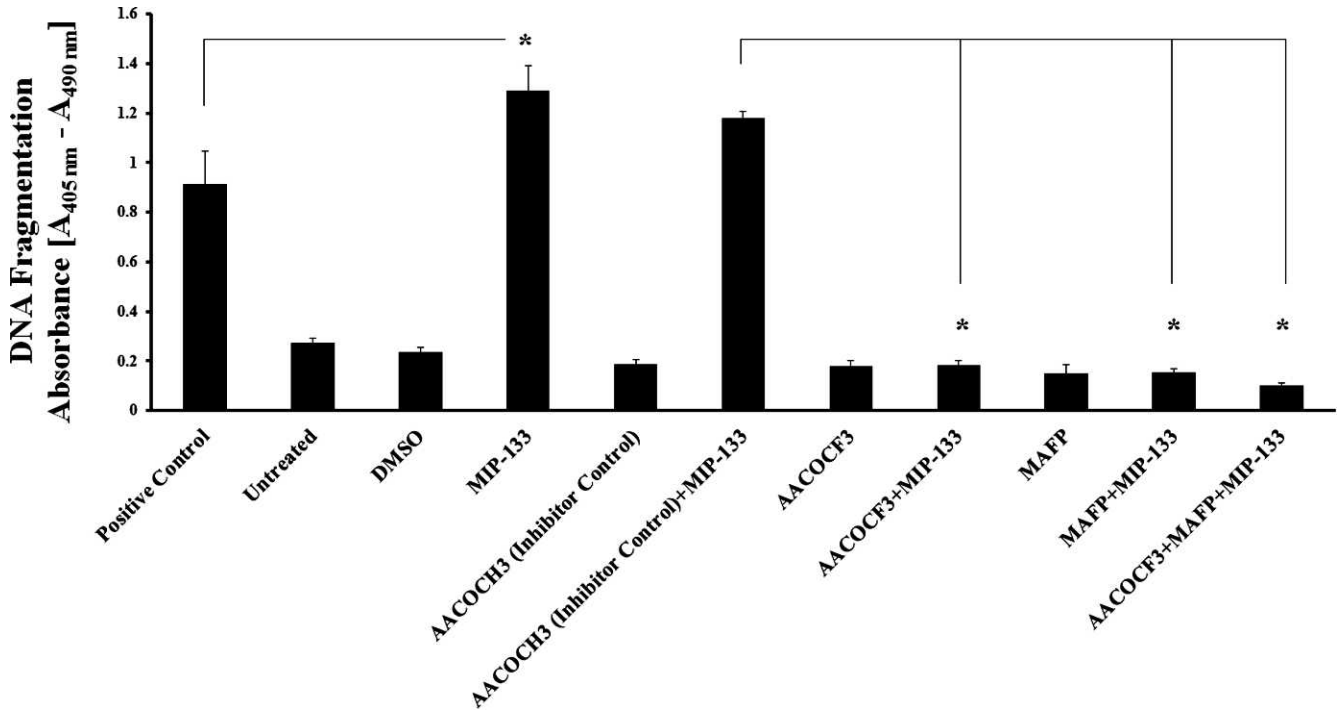

Figure 5. MIP-133–induced apoptosis is blocked by $cPLA_{2\alpha}$ inhibitors in HCE cells. HCE cells were preincubated for 1 hour with $cPLA_{2\alpha}$ inhibitors (10 μM MAFP or 20 μM AACOCF3) or an inactive negative control (20 μM AACOCH3), and then incubated with or without 15 μg/mL MIP-133 for 24 hours. Apoptosis was examined by Cell Death Detection ELISA^{Plus}. MIP-133–induced apoptosis was confirmed by a positive control provided in the apoptosis kit. The data are mean ± SEM of three independent experiments. *Asterisk* indicates P value < 0.05 by unpaired Student's t-test.

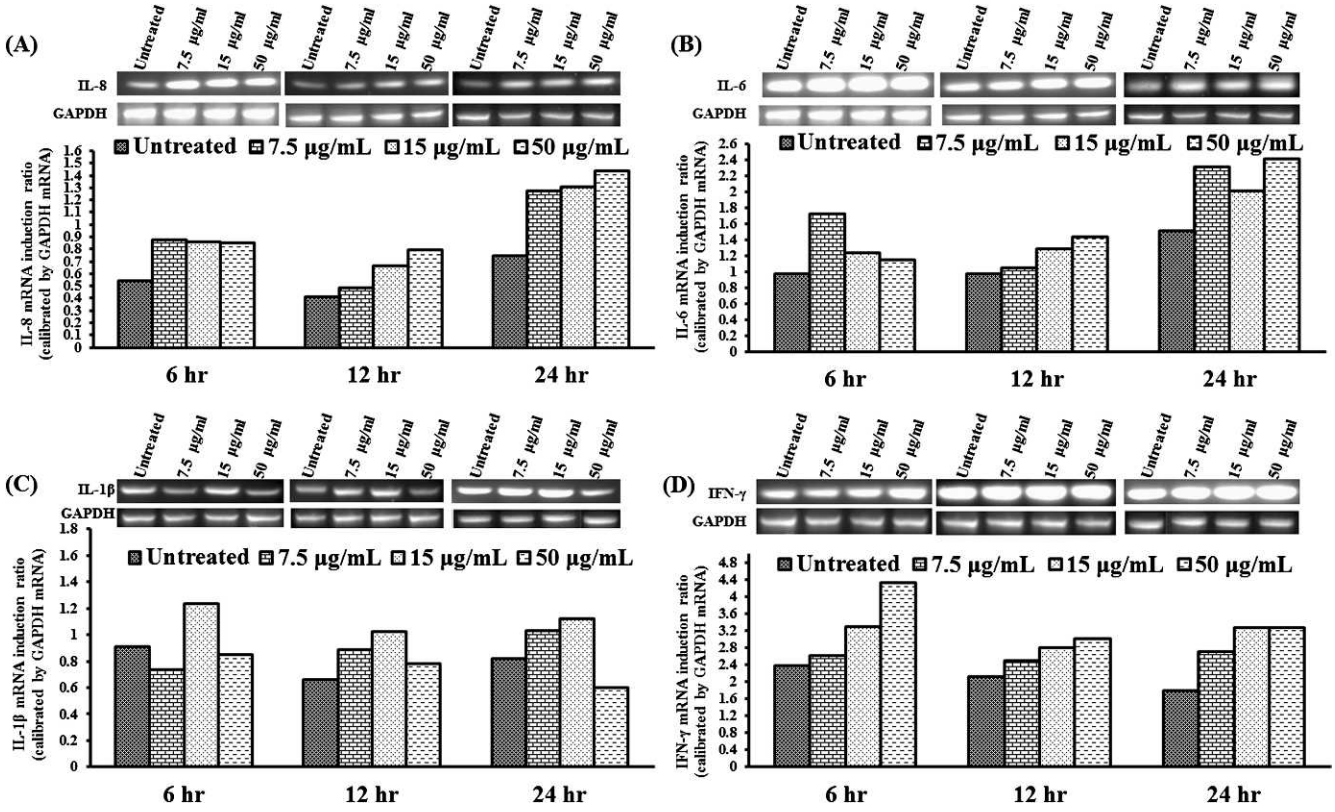

Figure 6. (**A**–**D**) MIP-133 upregulates IL-8 (**A**), IL-6 (**B**), IL-1β (**C**), and IFN-γ (**D**) mRNA in HCE cells. HCE cells were exposed to MIP-133 at doses of 7.5, 15, and 50 μg/mL for 6, 12, and 24 hours, and were then processed for total RNA isolation and RT-PCR analysis. The amount of mRNA expression was quantified by densitometry of bands in comparison to GAPDH. The experiment was repeated three times.

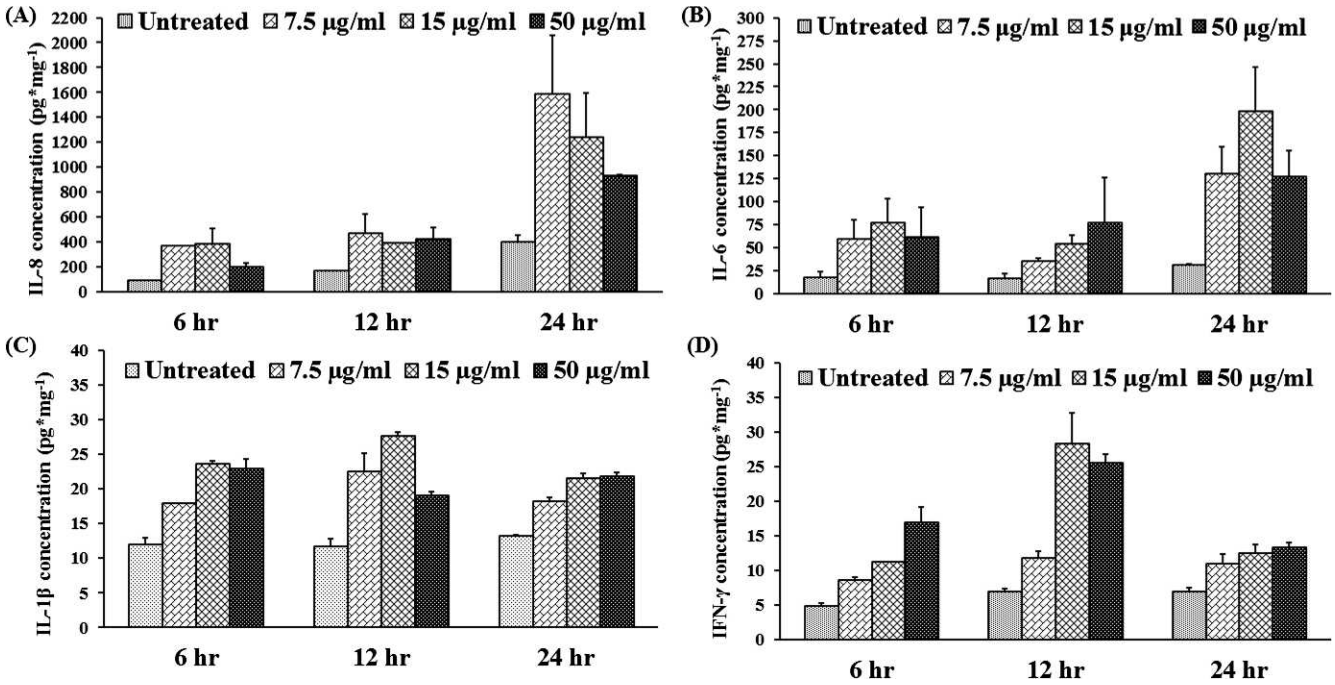

Figure 7. (**A**–**D**) MIP-133 induces IL-8 (**A**), IL-6 (**B**), IL-1β (**C**), and IFN-γ (**D**) protein expression in HCE cells. HCE cells were exposed to MIP-133 at doses of 7.5, 15, and 50 μg/mL for 6, 12, and 24 hours. Supernatants were collected from harvested cells and subjected to cytokine-specific ELISA. The data are mean ± SEM of three independent experiments. P values < 0.05 were obtained by two-way analysis of variance (ANOVA).

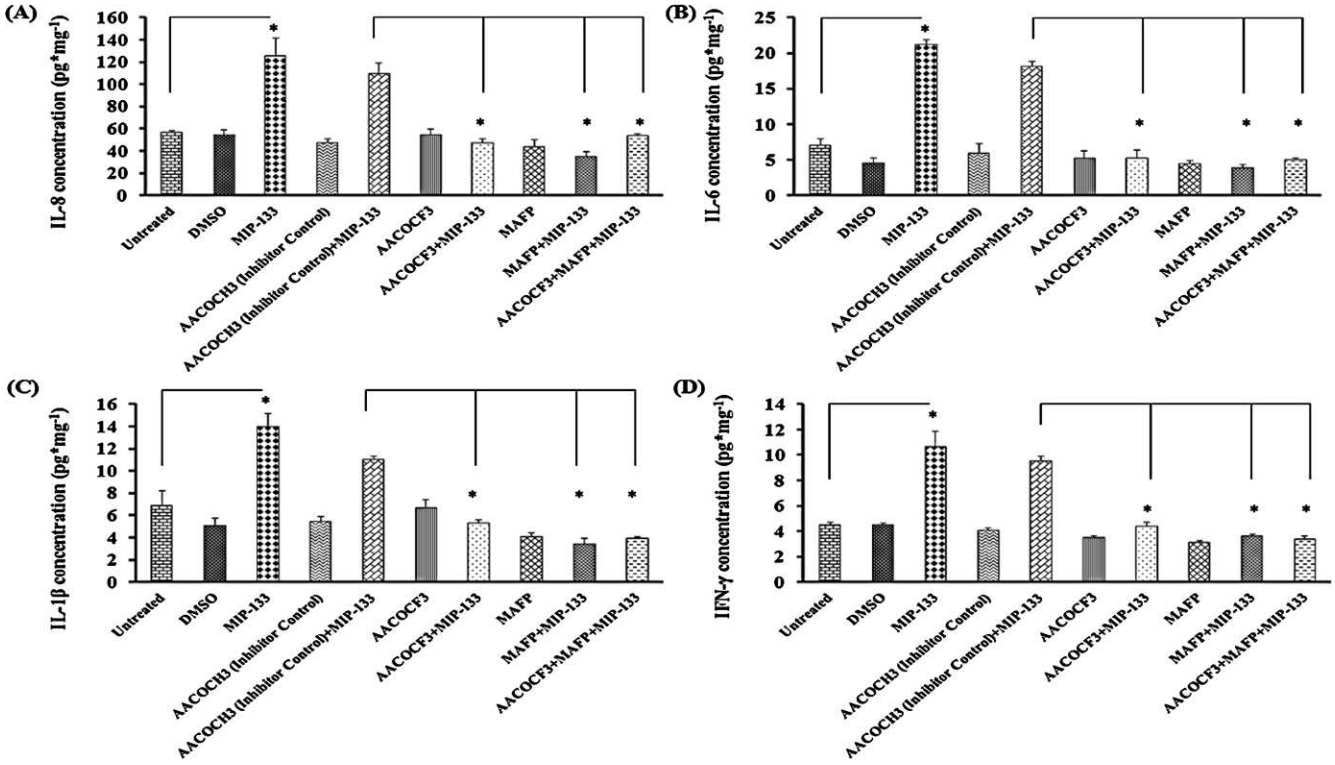

Figure 8. (**A–D**) MIP-133–induced upregulation of proinflammatory cytokines is blocked by $cPLA_{2\alpha}$ inhibitors in HCE cells. Inhibition of $cPLA_{2\alpha}$ involved preincubating HCE cells for 1 hour with $cPLA_{2\alpha}$ inhibitors (10 μM MAFP or 20 μM AACOCF3) or an inactive control (20 μM AACOCH3), then incubating with or without 15 μg/mL MIP-133 for 24 hours. Cytokine production was examined by ELISA. The data are mean ± SEM of three independent experiments. *Asterisk* indicates P value < 0.05 by unpaired Student's t-test.

suggests that Fas receptors are not involved in MIP-133–induced apoptosis. How the MIP-133 protein interacts with the cell surface to cause apoptosis is still unknown. We investigated the role of $cPLA_{2\alpha}$ in MIP-133–induced apoptosis of HCE cells. $cPLA_{2\alpha}$ is a family of lipolytic enzymes that hydrolyze membrane phospholipids and cause release of fatty acids from glycophospholipids, particularly AA,[13–15,17] a precursor of eicosanoids that are bioactive lipid molecules involved in numerous inflammatory processes.[15,16] Our results indicate that MIP-133 interacts with phospholipids on HCE cells to activate $cPLA_{2\alpha}$, induce AA release, and employ this $cPLA_{2\alpha}$ pathway to induce apoptosis of HCE cells. We have shown that specific inhibitors of $cPLA_{2\alpha}$ enzymes (AACOCF3 and MAFP) and chicken anti–MIP-133 antiserum block MIP-133 induction of $cPLA_{2\alpha}$ activity and inhibit MIP-133–mediated apoptosis of HCE cells. Although it is theoretically possible that induction of apoptosis and AA release is due to MIP-133 expression of $cPLA_{2\alpha}$ activity, no $cPLA_{2\alpha}$ activity was detected in our purified MIP-133 preparation (data not shown). Our previous findings of MIP-133 cytopathic activity suggested that MIP-133 is a serine protease.[9,36] $cPLA_{2\alpha}$ is activated by inflammation and oxidative stimuli by undergoing phosphorylation at amino acid residue serine-505 to become its soluble form, or by Ca^{2+} and MAPK phosphorylation to form p-$cPLA_{2\alpha}$, which translocates to the plasma membrane.[37–39] However, during apoptosis, the cell membrane's phospholipid asymmetry changes, and phosphatidylserine is exposed on the outer membrane.[40] Thus, our future targets on the basis of this study are to explore whether the $cPLA_{2\alpha}$ upregulation mechanism by MIP-133 serine protease is due to phosphorylation of MAPKs to induce the translocation of $cPLA_{2\alpha}$ in HCE cells, or due to the interaction of MIP-133 serine protease with phosphatidylserine on the outer membrane of HCE cells to induce $cPLA_{2\alpha}$.

Our current studies are largely in agreement with those of Kirschnek and Gulbins,[12] who reported a role for PLA_2 in *Pseudomonas aeruginosa*–induced apoptosis of conjunctiva epithelial cells and human lung fibroblast cell lines. In addition, *P. aeruginosa* produces several destructive proteins that associate with different ocular damage by the PLA_2 pathway and cause cell death.[41] In *P. aeruginosa* infection the induction of $cPLA_2$ leading to AA release, cytokine production, and apoptosis could be due to the spatial reorganization of PLA_2 and the metabolism of ceramide to ceramide-1-phosphate.[12,41]

We have shown that subconjunctival injection of the purified MIP-133 protein in Chinese hamsters induces epithelial ulceration, focal thickening, and PMN exocytosis. Stromal changes included lamellar connective tissue disruption, PMN infiltration, neovascularization, thickening, and edema (Alizadeh H, et al. *IOVS* 2011;52:ARVO E-Abstract 5795). These results indicate that MIP-133 plays an important role in the pathogenicity of AK by inducing cytolysis of the corneal cells and by stimulating the accumulation of PMN in the cornea. Our studies indicate that interaction of MIP-133 with the corneal epithelial cells induces a rapid immune response by the production of IL-8, IL-6, IFN-γ, and IL-1β, which can initiate an efficient host response to corneal infections. These results indicate a role of MIP-133 as a virulence protein, which may be responsible for PMN recruitment and inflammatory response to the corneal epithelium in vivo. The gene expression of proinflammatory cytokines and chemokines IL-6, IL-1β, TNF-α, and IL-8 also has been demonstrated in HCE cell lines challenged with *P. aeruginosa*.[26] We showed that the pretreatment of HCE cells with AACOCF3 and MAFP blocks the protein production of IL-8, IL-6, IL-1β, and IFN-γ. Collectively, these results indicate that activation of $cPLA_{2\alpha}$ induced by MIP-133 is responsible for the induction of

proinflammatory signaling in cornea. We found that the corneal epithelial cells express IFN-γ and that the level of this cytokine increased 6 hours after infection; however, it is not clear whether IFN-γ was produced in response to MIP-133 or by induction of other cytokines or chemokines. The expression of IFN-γ by corneal epithelial cells is somewhat surprising, since it is known that IFNs are involved in cell signaling and are produced by varieties of immune cells in response to pathogenic microorganisms.[41] Our results are in agreement with those of Ren et al.,[42] who demonstrated that corneal epithelial cells are capable of producing IFN-β, and the level of this cytokine increased significantly following *Acanthamoeba* infection in vitro. Ueta et al.[43] have shown that HCE produced IFN-β and that stimulation with poly(I:C) enhanced production of IFN-β in vitro. Rabbit corneal epithelial cells endogenously produce interferon, and its expression is increased in response to herpes simplex virus 1 (HSV-1) infection in vitro.[44] HCE cells initiated a potent antiviral response resulting in an increase of IFN-β mRNA expression. Poly(I:C) stimulation also upregulated mRNA expression of the antiviral chemokine IFN-γ inducible protein 10 (IP10).[45]

The cornea is a nonvascular tissue that is significantly different from other tissues with regard to function and biological response to various stimuli.[46] The activation of cPLA$_{2\alpha}$ may have both beneficial and detrimental inflammatory effects on the cornea, depending on the effectiveness and duration of the host inflammatory response. There is a critical balance between generating a successful inflammatory response to eliminate the microorganism and an excessive inflammatory response that can result in corneal scarring and blindness.[46] Understanding the molecular pathogenesis of MIP-133 that initiates proinflammatory cytokines and chemokines may permit the development of novel, specific therapies that can be delivered topically to prevent some of the destructive consequences of ocular infections.

In summary, MIP-133 is released from *A. castellanii* via interaction of corneal epithelial mannose with mannose receptors on the *A. castellanii* membrane. Our current results indicate that MIP-133 released by the interaction of mannose with the mannose receptors of *A. castellanii* cell membrane interacts with phospholipids on corneal epithelium and induces apoptosis and proinflammatory cytokines through cPLA$_{2\alpha}$ signaling. Identification of MIP-133–induced apoptotic signaling may facilitate the development of more effective therapeutic strategies in *Acanthamoeba* keratitis.

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