Amniotic membrane conditioned medium (AMCM) reduces inflammatory response on human limbal myofibroblast, and the potential role of lumican

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Purpose: Viral infections such as herpetic keratitis (HSK) activate the innate immune response in the cornea triggering opacity and loss of vision. This condition is performed mainly by myofibroblasts that exacerbate secretion of inflammatory cytokines. Amniotic membrane transplantation (AMT) reduces ocular opacity and scarring inhibiting secretion of inflammatory cytokines and proliferation of myofibroblasts. We previously reported that the amniotic membrane (AM) favors an anti-inflammatory microenvironment inhibiting the secretion of inflammatory cytokines, expression of innate immune receptors, and translocation of nuclear NF-κB on human limbal myofibroblasts (HLMs). The aim of the present study was to determine whether the soluble factors of the AM decrease the immune response of HLMs stimulated with polyinosinic-polycytidylic acid sodium salt (poly I:C).
Methods: The AM was incubated in Dulbecco's modified eagle medium (DMEM)/F12, and the supernatant was collected to obtain amniotic membrane conditioned medium (AMCM). HLMs were isolated from cadaveric sclera-corneal rims. HLMs were cultured in DMEM/F12 or AMCM and stimulated or not with poly I:C (10 µg/ml) for 12 h to analyze synthesis of CCL2, CCL5, CXCL10, MDA5, RIG-1, and TLR3 or for 2 h to analyze translocation of nuclear NF-kB, IRF3, and IRF7. The proteins contained on AMCM were analyzed by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and the acquired peptide ions were analyzed with the Mascot program using both National Center for Biotechnology Information (NCBI) and expressed sequence tag (EST) databases.
Results: AMCM downregulated the mRNA levels of CCL2, CCL5, CXCL10, MDA5, RIG-1, and TLR3. In addition, AMCM decreased secretion of CCL2, CCL5, and CXCL10 and translocation of nuclear NF-κB. Interestingly, AMCM increased translocation of nuclear IRF3 and synthesis and secretion of type I IFN-β. We also identified small leucine-rich proteoglycan lumican in the AMCM. The administration of *rh*-lumican to poly I:C–stimulated HLMs reduced the mRNA levels of CCL2, CCL5, and CXCL10.
Conclusions: These results suggest that the AM can trigger an anti-inflammatory response on HLMs through soluble factors, and that lumican could play an important role in these effects.

The cornea is considered an immune privileged tissue due to control of the immune response that preserves corneal transparency. However, viral infections such as herpetic keratitis (HSK) induce exacerbation of the immune response that reduces optical clarity and visual acuity [1]. Viral recognition in the cornea is performed mainly by pattern recognition receptors (PRRs) expressed in epithelial cells [2]. After viral recognition, signaling pathways are triggered leading to activation of interferon regulatory factors (IRFs) and nuclear factor-κB (NF-κB). Translocation of nuclear IRFs induces

expression of type I interferons (IFNs); translocation of nuclear NF-κB stimulates expression of inflammatory cytokines [3]. The inflammatory microenvironment in corneal stroma promotes differentiation of quiescent keratocytes to fibroblasts and subsequently, to myofibroblasts [4]. Myofibroblasts play a key role in exacerbation of corneal opacity and inflammation. These cells increase the secretion of collagen and chemokines that induce corneal stroma disorganization and stimulate the recruitment of immune cells [5,6].

Cumulative evidence has shown that amniotic membrane transplantation (AMT) is beneficial in many ocular pathologies [7]. Currently, AMT is successfully used to treat HSK and persistent corneal ulcers due to its anti-inflammatory properties [8,9]. The amniotic membrane (AM) is the inner layer of the placenta that is in contact with the amniotic fluid and the fetus. It has been reported that the AM inhibits

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inflammation suppressing activation and inducing apoptosis of immune cells [10], downregulating synthesis and secretion of proinflammatory cytokines from peripheral blood mononuclear cells [11], and inhibiting innate immune receptors on human limbal myofibroblasts (HLMs) [12]. Additionally, the AM can inhibit TGF-β secretion and reduces differentiation of limbal myofibroblasts, allowing accurate corneal wound healing [13].

The AM is composed of a single epithelial cell layer, a thick basement membrane, and an avascular extracellular matrix [14]. Previous studies have described that the AM matrix possesses different bioactive compounds, such as proteoglycans, fibronectin, laminin, hyaluronic acid (HA), numerous growth factors, cytokines, chemokines, and metalloproteases, among many others, that are proposed to be involved in therapeutic effects of the AM [15-17]. Different studies have reported that the AM secretes soluble factors with immunosuppressive activities on innate and adaptive immune cells, such as neutrophils, macrophages, and T cells [18-20]. Furthermore, it was recently reported that AM conditioned medium (AMCM) inhibits proliferation and modulates miRNA expression in hepatocarcinoma cells [21].

We have described that the AM can inhibit synthesis and secretion of proinflammatory cytokines downregulating TLR-3, MDA-5, and RIG-1 through inhibition of translocation of nuclear NF-κB in polyinosinic-polycytidylic acid sodium salt (poly I:C)–stimulated HLMs [12]. Whether the activities described above regarding poly I:C–stimulated HLMs are due to soluble AM-secreted factors is still unknown. Therefore, the aim of the present study was to evaluate the effect of soluble factors from AMCM on poly I:C–stimulated HLMs and to identify a soluble factor responsible for the anti-inflammatory effect.

METHODS

Reagents: The study was approved by the Institutional Review Board of the Institute of Ophthalmology Conde de Valenciana. Fetal bovine serum (FBS), DMEM/F12, 0.25% trypsin-0.5% EDTA, dispase II, and collagenase II were purchased from Gibco (Waltham, MA). Twenty-four-well cell culture plates and cell culture flasks were obtained from Corning (Corning, NY). RNeasy Mini Kit and the Omniscript RT Kit were obtained from Qiagen (Hilden, Germany). KAPA2G Fast HotStart ReadyMix PCR kit was purchased from Roche (Basilea, Suiza). Polyinosinic-polycytidylic acid sodium salt (poly I:C) was obtained from Invivogen (San Diego, CA). Cytometric Bead Array (CBA) human chemokine Kit was purchased from BD Biosciences (San Diego, CA). Purified rabbit anti-NF-κB, anti-phospho-NF-κB (Ser

536), anti-IRF3, anti-phospho-IRF3 (Ser 386), anti-IRF7, and anti-phospho-IRF7 (Ser 477) antibodies were obtained from Abcam (Cambridge, England). AlexaFluor 488 conjugated-goat anti-rabbit antibody was obtained from Life Technologies (Carlsbad, CA). AmiconUltra-15 centrifugal filter devices were obtained from Millipore (Burlington, MA). The Human IFN-β Enzyme-Linked Immunosorbent Assay (ELISA) kit was obtained from PBL Assay Science (Piscataway, NJ). The Human Lumican DuoSet ELISA Kit (Minneapolis, MN) and recombinant human (*rh*)-lumican protein were purchased from R&D Systems (Minneapolis, MN). Vectashield-4′, 6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Burlingame, CA). Other reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Isolation of HLMs: The HLMs were isolated from cadaveric sclera-corneal rims as we previously described [12]. Briefly, the tissues were incubated with Dispase II (1.7 IU/ml) for 1 h at 37 °C to remove the epithelial cells. The remaining tissue was digested with Collagenase II (0.75 mg/ml) for 18 h at 37 °C. The cells were pooled out with centrifugation at 200 ×*g* for 5 min at 4 °C and cultured with DMEM/F12 supplemented with 10% FBS at 37 °C, with an atmosphere of 5% CO_2. All experiments were performed at two to four passages.

Preparation of AMCM: The AM was obtained after elective cesarean section with informed consent from the donor and was preserved at −80 °C until use as previously reported [7]. After thawing, the AM was de-epithelized by incubating with Dispase II (1.7 IU/ml) for 30 min at 37 °C; then, the epithelium was gently removed using a rubber policeman (denuded AM [dAM]). The complete AM de-epithelialization was corroborated in an inverted microscope. Fifty mg of denuded AM (dAM) were incubated in one ml of DMEM/F12 medium during 4 h at 37 °C. After the incubation period, the supernatant was collected diluted at 40% (v/v) with fresh medium and immediately used.

Cell culture conditions: The HLMs (1×10^5 cells per well) were seeded on a 24-well cell culture plate for 12 h in the presence of 10% FBS-supplemented medium to allow their adherence to the plate. Afterward, the cells were FBS starved for 12 h and stimulated or not with poly I:C (10 μg/ml) for 12 h in the presence or not of AMCM. The cytokines secreted from the HLMs exposed to different conditions were measured in the supernatants. Similarly, to analyze translocation of nuclear NF-κB, IRF3, and IRF7, the cells were adhered in poly-*L*-lysine coated coverslips for 12 h in the presence of 10% FBS-supplemented medium. Thereafter, the cells were FBS starved for 12 h and stimulated or not

with poly I:C (10 µg/ml) for 120 min in the presence or not of AMCM.

Identification of transcripts: The HLM exposed to different stimuli were harvested and total RNA was obtained using the RNeasy Mini Kit as indicated by the manufacturer. RNA quality and concentration were measured by the NanoDrop method (Thermo Scientific, Labtech, Uckfield, England). Reverse transcription was performed with 100 ng of RNA using the Omniscript RT Kit according to the manufacturer's instructions. Quantitative PCR was performed using KAPA2G Fast HotStart ReadyMix PCR kit and primers in a final volume of 20 µl according to the manufacturer's instructions. Specific mRNA levels were calculated after normalization of the results for each sample with those for β2-microglobulin (β2m) mRNA. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative differences in specific mRNA levels between groups. All the assays were performed three times in triplicate.

Measurement of chemokines: The supernatants from the HLMs exposed to different stimuli were collected to measure chemokine secretion using the CBA Human Chemokine Kit according to the manufacturer's instructions. The data were acquired in a BD FACSVerse flow cytometer, and analyses were performed using the BD FCAP Array v3.0 software. The results were based on a standard concentration curve and expressed as picograms per milliliter. All assays were performed three times in triplicate.

Immunofluorescence staining: HLMs seeded on coverslips were exposed to the stimuli above and used to identify the translocation of nuclear transcription factors, as well as to determine their phosphorylation form, with immunofluorescence staining assays. Briefly, the cells were fixed with 4% p-formaldehyde for 10 min at 4 °C and washed with PBS (1X; 120 mM NaCl, 20 mM KCl, 10 mM NaPO$_4$, 5 mM KPO$_4$, pH 7.4). Afterwards, the cells were incubated with blocking buffer (0.5% TritonX-100, 5% BSA in PBS) for 1 h at 4 °C. The cells were incubated with primary antibodies: NF-κB, phosphoNF-κB (pNF-κB), IRF3, phosphoIRF3 (pIRF3), IRF-7 or phosphoIRF7 (pIRF7) at 4°C overnight. After washing with PBS, the coverslips were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG for 2 h at 4 °C. Finally, the cells were washed three times with PBS, mounted with Vectashield-DAPI, observed with an ApoTome II microscope, and analyzed using Axiovison software (Carl Zeiss, Jena, Germany). The protein identification analyses were performed as previously described [22,23] with slight modifications. Briefly, the background signal was adjusted in all images assessed using the same magnification, identifying the nuclear and non-nuclear compartments, and the mean fluorescence intensity was achieved using ImageJ.

Quantification of IFN-β: The supernatants from the HLMs exposed to different stimuli were collected to measure secreted IFN-β using the human IFN-β ELISA kit following the manufacturer's instructions. Results were acquired with a Multiskan Ascent spectrophotometer (Thermo Fisher Scientific, Waltham, MA) at 450 nm wavelength. The assays were performed three times in triplicate.

Enzymatic treatment of AMCM: To determine whether the possible factors with bioactivity were proteins, AMCM was incubated or not with proteinase K (10 µg/ml) for 50 min at 37 °C. After protein digestion, the AMCM was boiled for 5 min to inactivate proteinase K and was used immediately.

Isolation of proteins: AMCM was concentrated using the Amicon centrifugal filter device, and the proteins were resolved in denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Briefly, 10 ml of AMCM was added to the filter device and centrifuged at 4,000 ×*g* for 30 min at 4 °C. Afterward, 200 µl of AMCM (50X concentrated) was obtained and resolved in a denaturing 4%–20% SDS–PAGE. Proteins were stained with Coomassie blue.

Identification of proteins: The proteins contained on AMCM were analyzed with mass spectrometry matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) as previously reported [24]. Briefly, the protein bands were removed from the SDS–PAGE with a sterile scalpel and washed with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate for 15 min twice to remove the Coomassie dye. After dehydration with 100% (v/v) acetonitrile for 10 min at 25 °C, the gel pieces were vacuum-dried and rehydrated with sequencing-grade modified trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate at 37 °C overnight. The in-gel tryptic digested samples were injected into an integrated nano-high performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry and electrospray ionization (HPLC-ESI-MS/MS) system (quadrupole/time of flight; Ultima API, Micromass, Manchester, UK). After dissolving with 0.1% formicacid, the samples were loaded into an analytical C18 capillary column connected online to the mass spectrometer. Instrumental operation, data acquisition, and analysis were performed under the full control of Mass-Lynx 4.0 (Micromass). The 1-s survey scans were run over the mass range of m/z 400 to 2000. A maximum of three concurrent MS/MS acquisitions were triggered for 2þ, 3þ, and 4þ charged precursor detection at an intensity above the predefined

threshold. The acquired peptide ions were analyzed with the Mascot program using the NCBI and EST databases.

Lumican quantification with ELISA: AMCM was used to measure lumican using the Human Lumican DuoSet ELISA kit, following the manufacturer's instructions. Results were obtained with a spectrophotometer at 450 nm wavelength. All assays were performed three times in triplicate.

rh-Lumican assays: The HLMs were cultured and exposed to different doses (2.5, 5.0, or 10 ng/ml, unless otherwise stated) of *rh*-lumican to analyze its effect on morphology of HLMs and synthesis of proinflammatory cytokines with inverted microscopy and transcripts analysis, respectively. All assays were performed three times in triplicate.

Statistical analysis: Data were assessed with one-way analysis of variance (ANOVA) followed by Bonferroni correction test. The data were expressed as mean ± standard error of the mean (SE), and p values of less than 0.05 were considered statistically significant. All statistics were performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA).

RESULTS

AMCM inhibited synthesis and secretion of chemokines and decreased synthesis of innate immune receptors in poly I:C–stimulated HLMs: In a previous report, we described that dAM inhibited the immune response of poly I:C–stimulated HLMs. However, it was not clear whether this effect was due to HLM-dAM contact or to the soluble factors released from dAM [12]. Consequently, poly I:C–stimulated HLMs were incubated in the presence of AMCM, and synthesis of the chemokines was evaluated. AMCM decreased RNA synthesis of CCL2, CCL5, and CXCL10. In addition, when the supernatants were obtained to measure the secretion of the chemokines, AMCM decreased the CCL2, CCL5, and CXCL10 protein secretion in poly I:C–stimulated HLMs. Additionally, AMCM decreased the expression of the transcripts from innate immune receptors MDA5, RIG-1, and TLR-3 in the poly I:C–stimulated HLMs (Figure 1).

AMCM inhibited translocation of nuclear NF-κB and p-NF-κB in poly I:C–stimulated HLMs: To determine whether the reduction in synthesis and secretion of inflammatory chemokines was associated with changes in activation of NF-κB, NF-κB phosphorylation and translocation of nuclear NF-κB were analyzed. As expected, poly I:C stimulation promoted translocation of nuclear NF-κB, while AMCM clearly inhibited translocation of nuclear NF-κB in poly I:C–stimulated HLMs. Moreover, AMCM decreased the presence of nuclear and cytoplasmic NF-κB and p-NF-κB molecules on poly I:C–stimulated HLMs (Figure 2).

AMCM increased translocation of nuclear IRF3 and synthesis and secretion of IFN-β in poly I:C–stimulated HLMs: To identify whether AMCM exerts an effect in the HLM antiviral response, phosphorylation and translocation of nuclear IRF3 and IRF7 transcription factors were achieved. Interestingly, AMCM increased phosphorylation and translocation of nuclear IRF3 on poly I:C–stimulated HLMs (Figure 3), and increased synthesis and secretion of IFN-β on poly I:C–stimulated HLMs. In contrast, the phosphorylation form and translocation of nuclear IRF7 were not modified by AMCM in the poly I:C–stimulated HLMs (Figure 4).

The proteinase K treatment on AMCM decreased inhibition of synthesis of chemokines in poly I:C–stimulated HLMs: We analyzed the activity of AMCM treated with proteinase K to evaluate the nature of soluble factors responsible for the anti-inflammatory response in poly I:C–stimulated HLMs. AMCM treated with proteinase K inhibited the capacity to decrease synthesis of CCL2, CCL5, and CXCL10 chemokines on poly I:C–stimulated HLMs (Figure 5). These results suggest that AMCM soluble factors with anti-inflammatory properties are partly protein.

Lumican was identified in AMCM: To identify the protein or proteins released from dAM, AMCM was 50 times concentrated and resolved in SDS–PAGE under reducing conditions. A strong band between 25 and 35 kDa was detected in comparison with AMCM treated with proteinase K (AMCM+PK, Figure 6). This band was isolated and analyzed with mass spectrometry, and the Mascot program identified one protein (Figure 6). This protein corresponded to small leucine-rich proteoglycan lumican with a 38 kDa core protein [25]. The presence of lumican in AMCM was corroborated with ELISA in which we determined a concentration of 10 ng/ml.

rh-Lumican decreased synthesis of the CCL2, CCL5, and CXCL10 in poly I:C–stimulated HLMs: To identify whether lumican exerts similar effects to those obtained with AMCM on HLMs, poly I:C–stimulated HLMs were incubated with different doses (1.0, 2.5, and 5.0 ng/ml) of *rh*-lumican. Interestingly, when the poly I:C–stimulated HLMs were cultured with *rh*-lumican, and RT–PCR assays were performed, there were significant reductions in synthesis of CCL2, CCL5, and CXCL10 in comparison with the poly I:C–stimulated HLMs without *rh*-lumican (Figure 7).

DISCUSSION

During corneal disruption, the inflammatory microenvironment in the ocular surface induces activation of fibroblasts exacerbating corneal opacity and scarring [26,27]. Corneal fibroblasts lead the immune response in the ocular surface
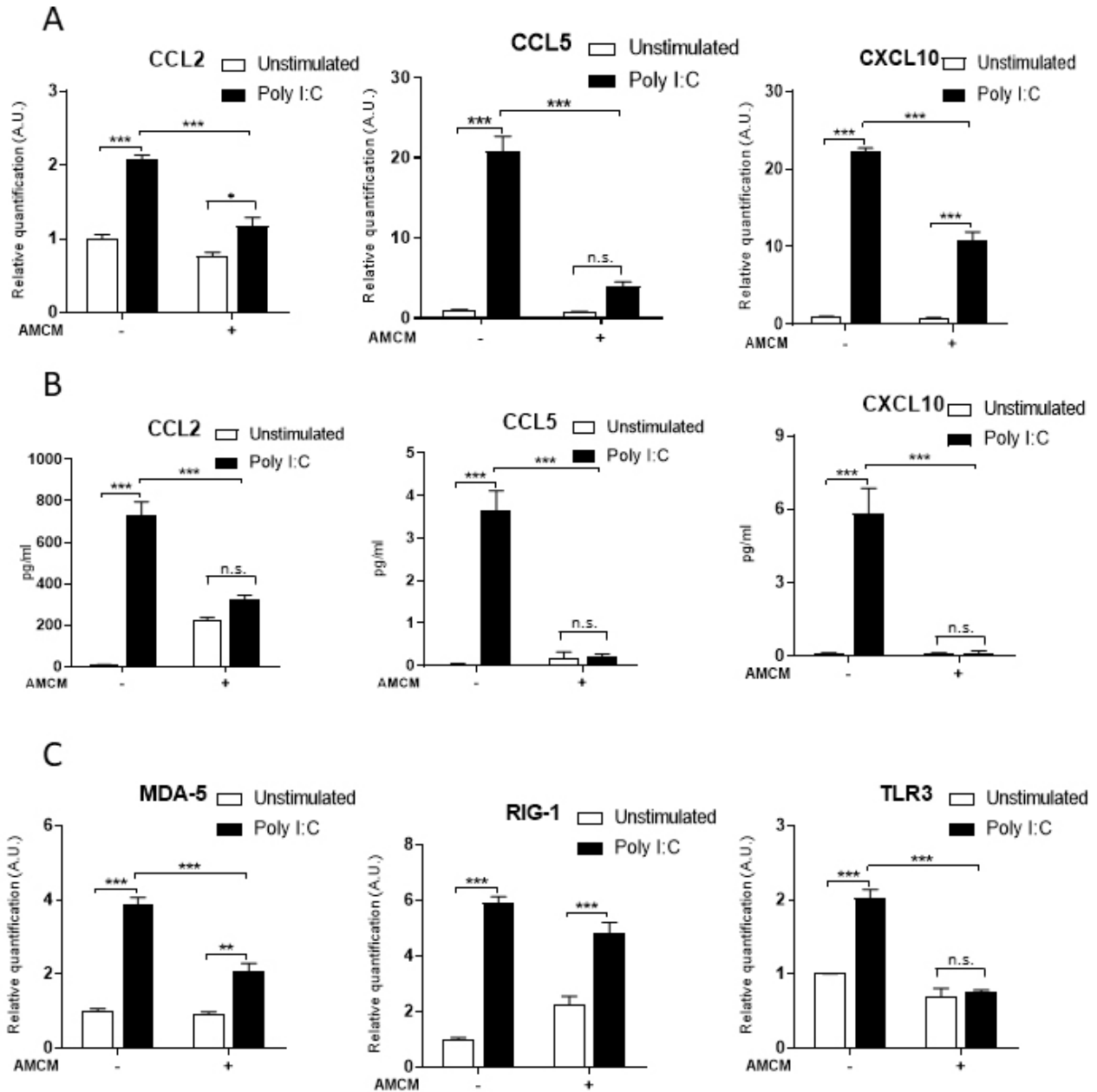
Figure 1. AMCM inhibited synthesis and secretion of chemokines and reduced innate immune receptors transcription in poly I:C–stimulated HLMs. **A**: As expected, polyinosinic-polycytidylic acid sodium salt (poly I:C) stimulation statistically significantly induced synthesis of the CCL2, CCL5, and CXCL10 chemokines, which was significantly inhibited by amniotic membrane conditioned medium (AMCM). **B**: Similarly, poly I:C stimulation statistically significantly induced secretion of CCL2, CCL5, and CXCL10 chemokines, which was statistically significantly inhibited by AMCM. **C**: Poly I:C stimulation statistically significantly induced synthesis of MDA5, RIG-1, and TLR3 innate immune receptors, which was statistically significantly inhibited by AMCM. Bars represent the mean ± standard error (SE); *$p<0.5$; **$p<0.01$; ***$p<0.001$; n.s.: no statistical difference. All assays were performed three times in triplicate.

through chemoattraction of immune cells and induction of inflammation. These conditions promote differentiation of these cells into myofibroblasts increasing proinflammatory cytokines and abnormal secretion of extracellular matrix proteins [5,28].

We previously reported that the AM exerts its beneficial properties modulating the innate immune response on HLMs [12]. Moreover, the AM induces an antiscarring environment, which is associated with reduction in fibrosis in the tissue

[10,29,30]. It has been suggested that AM soluble factors inhibit the TGF-β signaling pathway and α-SMA expression in corneal myofibroblasts decreasing their activity and proliferation [31,32], which favors correct corneal healing.

Several studies have suggested that the AM stroma release several soluble factors, such as IL-10, IL-1RA, and IαI, among many others, with the capacity to modulate the inflammatory response in the ocular surface F. In this report, we suggest that the soluble factors of the AM have
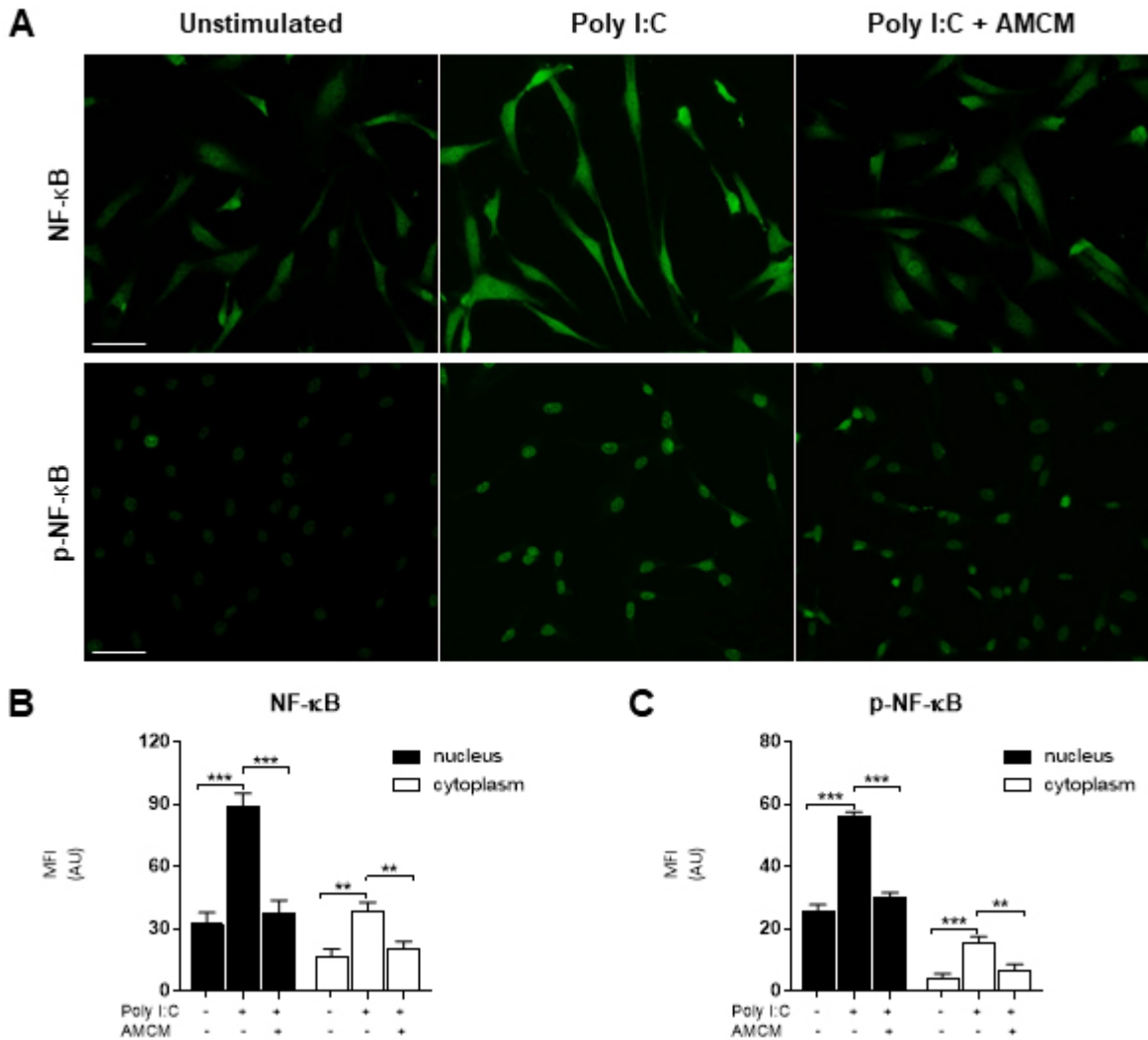

Figure 2. AMCM inhibited translocation of nuclear NF-κB and p-NF-κB in poly I:C–stimulated HLMs. **A**: Polyinosinic-polycytidylic acid sodium salt (poly I:C) induced translocation of nuclear NF-κB and p-NF-κB in human limbal myofibroblasts (HLMs), which was inhibited in the presence of amniotic membrane conditioned medium (AMCM). The medium fluorescence intensity (MFI) in each condition was achieved as described in the Materials and Methods section. Poly I:C statistically significantly increased the presence of nuclear and cytoplasmic NF-κB and p-NF-κB; these protein increases were statistically significantly abolished by AMCM in the cytoplasm and nucleus subcellular compartments in the poly I:C–stimulated HLMs (**B** and **C**). Bars represent the mean ± standard error (SE); ***p<0.001; **p<0.01. All assays were performed three times in triplicate. Scale bars = 50 µm.
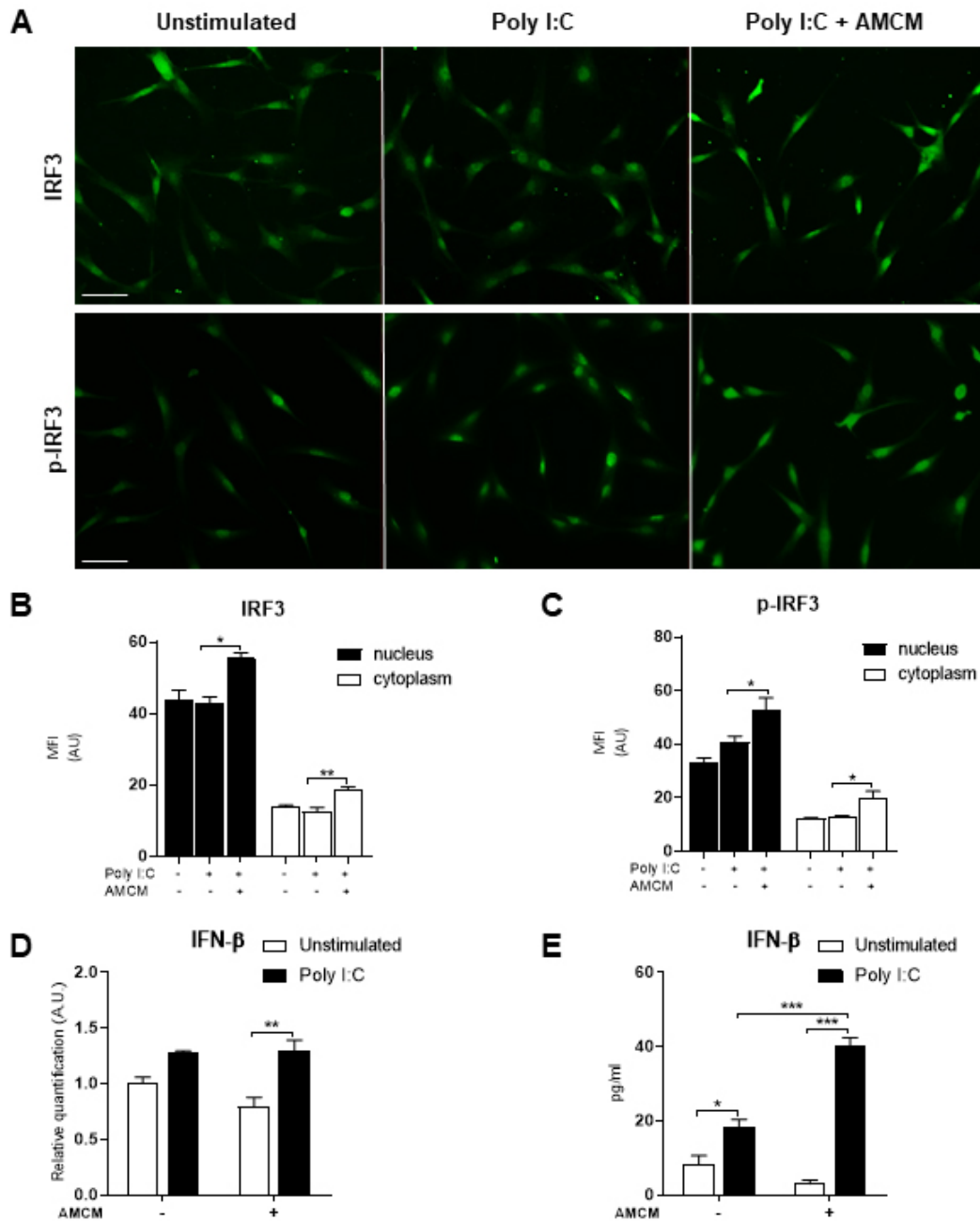
Figure 3. AMCM increased translocation of nuclear IRF3 and p-IRF3 and synthesis and secretion of IFN-β in poly I:C–stimulated HLMs. **A**: Amniotic membrane conditioned medium (AMCM) statistically significantly increased the presence of nuclear and cytoplasmic IRF3 and p-IRF3 in polyinosinic-polycytidylic acid sodium salt (poly I:C)–stimulated human limbal myofibroblasts (HLMs). The medium fluorescence intensity (MFI) in each condition was achieved as described in the Materials and Methods section. **B**: AMCM statistically significantly increased the presence of IRF3 in the nucleus and cytoplasm in poly I:C–stimulated HLMs. **C**: AMCM statistically significantly increased p-IRF3 in the nucleus and the cytoplasm in poly I:C–stimulated HLMs. Moreover, AMCM increased IFN-β synthesis (**D**) and secretion (**E**) in poly I:C–stimulated HLMs. Bars represent the mean ± standard error (SE); ***$p < 0.001$, **$p < 0.01$; *$p < 0.05$. All assays were performed three times in triplicate. Scale bars = 50 μm.
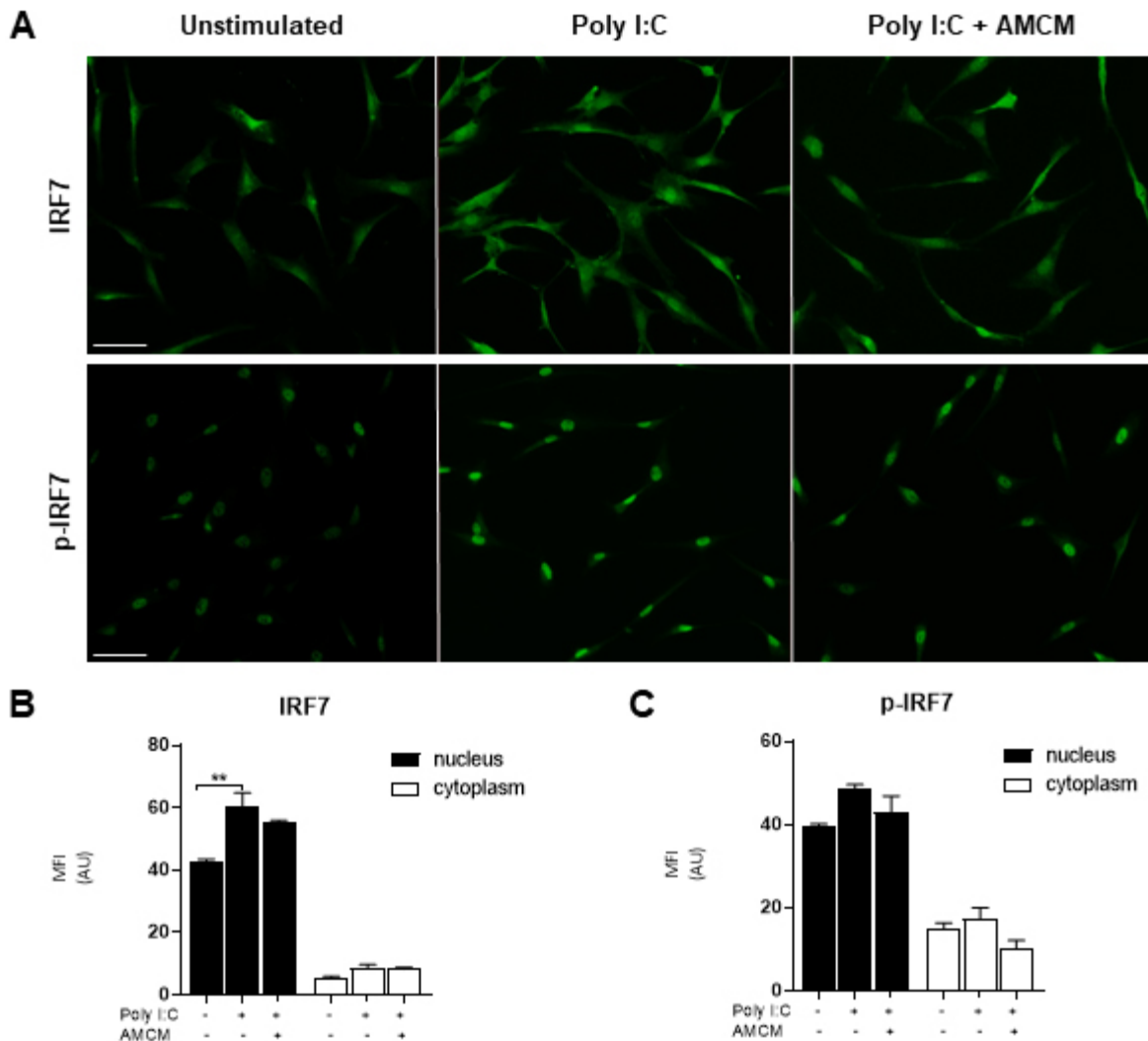
Figure 4. AMCM did not exert any effect on translocation of nuclear IRF7 or p-IRF7. **A**: Polyinosinic-polycytidylic acid sodium salt (poly I:C) statistically significantly induced translocation of nuclear p-IRF7; however, concomitant incubation with amniotic membrane conditioned medium (AMCM) did not exert any statistically significant change in translocation of nuclear IRF7 or p-IRF7 or the presence of cytoplasmic IRF7 or p-IRF7. The medium fluorescence intensity (MFI) in each condition was achieved as described in the Materials and Methods section. **B**: AMCM did not exert any change in the presence of IRF3 in the nucleus and the cytoplasm in poly I:C–stimulated human limbal myofibroblasts (HLMs). AMCM also statistically significantly increased p-IRF3 in the nucleus and the cytoplasm in the poly I:C–stimulated HLMs. Bars represent the mean ± standard error (SE); **$p<0.01$. All assays were performed three times in triplicate. Scale bars = 50 μm.

the ability to modulate the anti-inflammatory and antiviral response of HLMs. The synthetic analog of double-stranded RNA poly I:C increased the synthesis and secretion of the proinflammatory chemokines CCL2, CCL5, and CXCL10 in HLMs; however, when the cells were poly I:C stimulated in the presence of AMCM, the synthesis and secretion of these chemokines were abolished significantly. AMCM did not affect the cell morphology or induce any HLM detachment

(data not shown). Therefore, the decrease in synthesis and secretion of the chemokines observed was due to an immunomodulatory process [11] intrinsic to HLMs instead of the possible decrease in the cell number attached to the plate or an accelerated apoptosis. To understand inhibition of synthesis and secretion of these proinflammatory proteins, we investigated the dynamics of NF-κB. In this context, it is known that inhibition of translocation of nuclear NF-κB reduces
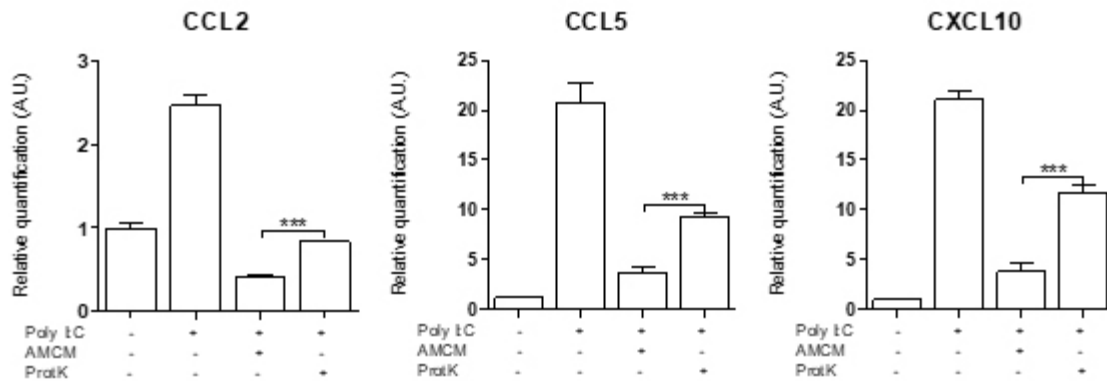
Figure 5. AMCM digested with proteinase K reverted inhibition of synthesis of chemokines in poly I:C–stimulated HLMs. Human limbal myofibroblasts (HLMs) stimulated with polyinosinic-polycytidylic acid sodium salt (poly I:C) and incubated with amniotic membrane conditioned medium (AMCM) statistically significantly decreased expression of CCL2, CCL5, and CXCL10; in contrast, proteinase K-treated AMCM (AMCM+PK) statistically significantly reverted inhibition of synthesis of CCL2, CCL5, and CXCL10. Bars represent the mean ± standard error (SE); ***p<0.001. All assays were performed three times in triplicate.

expression of proinflammatory cytokines, chemokines, and adhesion molecules in human corneal fibroblasts [33-35]. In this study, we showed that soluble factors of AMCM significantly reduced the presence of cytoplasmic and nuclear NF-κB as well as inhibited translocation of nuclear NF-κB in poly I:C–stimulated HLMs. These results indicate that inhibition of NF-κB activity decreased synthesis and secretion of CCL2, CCL5, and CXCL10, mediated by soluble factors contained in AMCM [36]. Similarly, AMCM was able to reduce synthesis of innate immune receptors TLR3, RIG-1, and MDA5; this result is in accordance with our preceding report [12], indicating that AMCM is able to reduce the innate immune response at the transcription level.

IRFs are transcription factors involved in expression of IFN-α/β and IFN-inducible genes that activate the antiviral response [37]. Interestingly, the soluble factors contained in AMCM significantly induced translocation of nuclear IRF3 and p-IRF3 which was associated with an increase in synthesis and secretion of IFN-β in poly I:C–stimulated HLMs, suggesting that soluble factors in AMCM increase the antiviral response of HLMs. This effect could be partially responsible for the beneficial outcomes of use of the AM for treatment of many ocular surface diseases, including HSK. It has been reported that the AM exerts an antimicrobial effect due to the presence of antimicrobial peptides [38-40]. Moreover, it has been suggested that the AM exerts an antiviral effect with the secretion of TNF-α, IFN-α, IFN-β, and IFN-γ constitutively presented in the membrane stroma [41];

however, the antiviral properties of the AM are still under study.

As described, the AM has the capability to release several soluble factors that could explain its anti-inflammatory, antiscarring, and antiviral properties [41,42]. In this study, we showed that treatment with proteinase K significantly reduces the anti-inflammatory effect of AMCM, which could indicate that the effect is due in part to proteins released from the AM. The mass spectrometry analysis and ELISA identified the proteoglycan lumican in AMCM [25]. Lumican is a small leucine-rich proteoglycan abundantly present in the stromal matrix of different tissues, such skeletal muscle, lung, liver, and cornea [43]. Interestingly, the proteome profile of the AM was described previously, and lumican was identified as one of the most abundant proteins in the AM stroma [44,45]. Moreover, lumican purified from the AM stromal matrix topically applied in mice with corneal de-epithelialization was able to ameliorate re-epithelialization inducing migration and proliferation of corneal cells [46]. In this context, in addition to the immunosuppressive activities exerted by lumican shown in the present study, it has been described that lumican directly binds to TGF-βR1 and promotes wound healing in corneal epithelial cells [47]. Thus, AM-derived lumican might possess dual activity in healing of ocular surface diseases.

In the present study, we showed that lumican reduced synthesis of chemokines such as CCL2, CCL5, and CXCL10 in poly I:C–stimulated HLMs. These results are consistent with other studies suggesting the anti-inflammatory role of
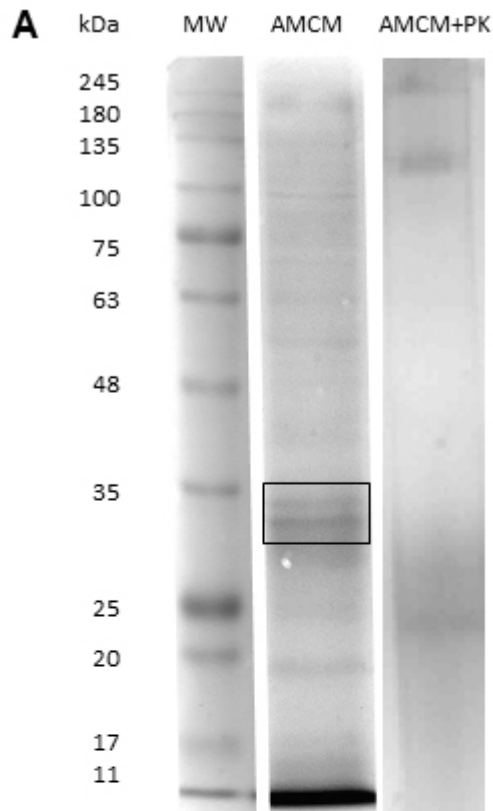
Figure 6. Identification of tryptic peptides from AMCM containing lumican. **A**: After concentration (50X), amniotic membrane conditioned medium (AMCM) and AMCM+PK (proteinase K) were resolved in a 4%–20% reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A protein between 25 and 35 kDa was selected. **B**: Mass spectrometry was performed which revealed that the protein was identified as lumican. An enzyme-linked immunosorbent assay (ELISA) showed that 10 ng/ml of protein were present in the AMCM.
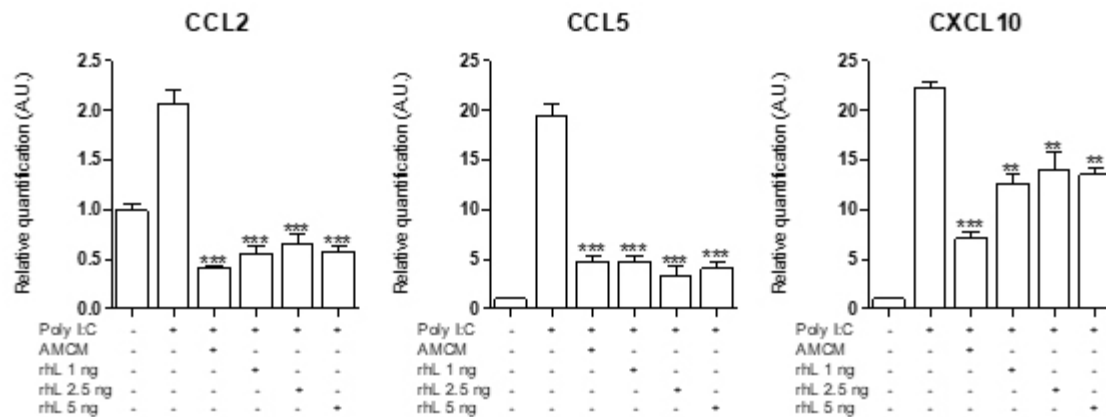
Figure 7. Lumican decreased synthesis of CCL2, CCL5, and CXCL10 in poly I:C–stimulated HLMs. *rh*-Lumican concomitantly incubated in polyinosinic-polycytidylic acid sodium salt (poly I:C)–stimulated human limbal myofibroblasts (HLMs) statistically significantly diminished the synthesis of mRNA of CCL2, CCL5, and CXCL10. Bars represent the mean ± standard error (SE); ***p<0.001; **p<0.01. All assays were performed three times in triplicate.

lumican in other models. The functions of lumican include regulation of corneal inflammatory responses by inducing apoptosis of inflammatory cells [48], inhibition of proliferation and migration of melanoma cells through alteration of actin filaments and focal adhesion complexes [49], inhibition of adhesion of cancer cells, and angiogenesis of umbilical venules by blocking adhesion of α2β1 integrins [50,51]. In accordance with those studies, we found that lumican modulates the inflammatory response of HLM; however, the signaling and molecular mechanisms involved are still unknown. Nevertheless, although we propose lumican as a soluble factor released from the AM that showed immunomodulatory properties, we cannot rule out the presence of multiple soluble factors secreted from the AM, which are still found even when the tissue is cultured, including great variability of cytokines and growth factors [52,53]. Therefore, lumican is partially responsible for these effects in in vitro conditions. Lee et al. previously reported that AMCM induced expression of insulin-like growth factor-1 in human corneal epithelial cells inducing their proliferation [54]; therefore, a similar effect of AMCM on HLMs cannot be discarded. Finally, it has been described that amniotic membrane suspension and amniotic membrane homogenate exert proliferation and migration activities on human corneal epithelial cells [55]; thus, AMCM could be beneficial in ocular cell surface diseases promoting corneal epithelialization and diminishing local inflammation process.

Taking all these data together, we can point out that lumican is an important factor of the AM that possesses anti-inflammatory properties. These in vitro findings correlate in part with the biologic properties of amniotic membrane transplantation. However, studies validating AM-secreted lumican as a potential molecule to treat ocular surface diseases are needed.

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