Lumican Regulates Corneal Inflammatory Responses by Modulating Fas-Fas Ligand Signaling

Neeraj Vij,[1,2] *Luke Roberts,*[1] *Sarah Joyce,*[1] *and Shukti Chakravarti*[1,3,4]

Purpose. The authors have previously shown that apoptosis of stromal cells is downregulated in the lumican-null mouse and that this may be due to disruption of Fas-Fas ligand (FasL) signaling. The present study was undertaken to investigate the role of lumican in regulating Fas and its impact on inflammation and healing of corneal injuries.

Methods. Apoptosis was determined by measuring caspase-3/7 activity in corneal extracts. Protein and RNA levels of Fas were estimated by immunoblot analysis and RT-PCR, respectively. Circular and incisional stromal wounds were exposed to *Pseudomonas aeruginosa* LPS, and healing was assessed by (1) observing wound closure with fluorescence and bright-field microscopy, (2) histology to quantify inflammatory infiltrates by immunostaining for macrophages (F4/80) and neutrophils (NIMP-R14), (3) measuring myeloperoxidase (MPO) levels by ELISA to quantify neutrophils, and (4) measuring proinflammatory cytokines by ELISA.

Results. $Lum^{-/-}$-injured corneas showed significantly lower caspase-3/7 activity (apoptosis). $Lum^{-/-}$-wounded corneas showed delayed healing, reduced recruitment of macrophages and neutrophils, lower MPO levels, and no induction of the proinflammatory cytokines TNFα and IL1β. The Fas protein level, before and after wounding, was dramatically lower in $Lum^{-/-}$- compared with $Lum^{+/+}$-injured cornea. However, Fas mRNA levels were comparable in both genotypes, suggesting regulation of Fas at the protein level. Moreover, a solid-state binding assay and coimmunoprecipitation of FasL and lumican suggested binding of FasL to lumican.

Conclusions. The data suggest that lumican binds FasL and facilitates induction of Fas. Poor signaling through Fas-FasL in lumican deficiency leads to impaired induction of inflammatory cytokines and corneal healing. (*Invest Ophthalmol Vis Sci.* 2005;46:88–95) DOI:10.1167/iovs.04-0833

The cornea is a specialized, avascular, transparent, barrier connective tissue that provides 75% of the refractive power of the eye.[1] The bulk of the cornea is its stroma, comprising an extracellular matrix of intricately balanced amounts of hydrated fibrillar collagens and proteoglycans. Lumican, a member of the small leucine-rich proteoglycan (SLRP) gene family, is the most abundant keratan sulfate proteoglycan in the corneal stroma. Keratocan and mimecan/osteoglycin are two other keratan sulfates, and decorin is the major dermatan sulfate proteoglycan in the cornea.[2,3] Studies of the structure of the corneal stroma over the past several decades indicate a role for these proteoglycans in regulating the collagen fibril structure and interfibrillar spacing that are conducive to optimal vision. Studies focusing on the core proteins have demonstrated their binding to fibrillar collagen, limiting lateral growth of fibrils in vitro.[4] Consistent with the in vitro studies, a major functional consequence of a gene-targeted null mutation in lumican is the presence of thick and disorganized collagen fibrils and corneal opacity.[5,6] To maintain the cornea as an optically transparent barrier, a sophisticated process is in place to balance inflammation and immune privilege.[7] Our recent studies are beginning to support a key role for the lumican core protein in this process. It is conceivable that the other SLRP core proteins may have similar functions in the cornea.

Approximately 40 kDa in size, the SLRP core proteins contain 6 to 10 leucine-rich repeat (LRR) motifs that interact with collagens. These LRR motifs are biologically very active and are the likely site of interactions with a vast number of other proteins with currently unknown biological implications. There are several forms of under-glycosaminoglycanated lumican that gain prominence in newly synthesized corneal extra cellular matrix (ECM) and other noncorneal tissues where these novel core-protein interactions are likely to have additional functions.[8,9] Beyond regulating collagen architecture, lumican influences such cellular functions as injury-related epithelial-mesenchymal transition,[10] cell proliferation, and apoptosis.[11] In our earlier study, we detected a marked decrease in apoptosis in cultured cells and the corneal stroma of lumican-null mice. There was also a significant downregulation in the Fas receptor. Because Fas-Fas ligand (FasL) signaling is a major regulator of programmed cell death, we hypothesized that Fas-FasL–mediated apoptosis is downregulated in lumican deficiency, primarily due to disruption of the Fas signal-transduction pathway in which lumican serves a critical function.[11] The Fas-FasL pathway has a special significance in corneal immune privilege. Interaction between Fas^{+} lymphoid cells invading the stroma and FasL is believed to trigger their apoptosis and limit inflammation.[7] Recent studies have unraveled implications of Fas signaling beyond apoptosis: in inducing proinflammatory cytokines and in initiating and amplifying inflammatory responses.[12–16]

In light of these current findings on Fas, we hypothesize that lumican modulates inflammatory responses in corneal wound healing by regulating Fas-FasL signaling. In the current study, the healing of bacterial LPS-mediated corneal injury was remarkably delayed in the lumican-null mouse. This phenomenon is associated with scant recruitment of neutrophils and macrophages at the wound site and poor induction of inflammatory cytokines.

Materials and Methods

Animal

The mice were housed in a pathogen-free facility according to policies approved by the Animal Care Committee, Johns Hopkins University,

From the Departments of [1]Medicine, [3]Cell Biology, and [4]Ophthalmology, Johns Hopkins University, Baltimore, Maryland.

[2]Present affiliation: Department of Pediatrics, Johns Hopkins University, Baltimore, Maryland.

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Corresponding author: Shukti Chakravarti, Department of Medicine, Johns Hopkins University School of Medicine, Ross 935, 720 Rutland Avenue, Baltimore, MD 21205; schakra1@jhmi.edu.

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and were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Stromal Wounding and In Vivo Microscopy of Cornea

Mice were anesthetized by intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). In addition, topical proparacaine hydrochloride 0.5% (Bausch & Lomb Pharmaceuticals, Inc., Tampa, FL) was applied to each eye just before surgery. To examine inflammatory infiltrates in injured corneas by immunostaining, incisional wounds were made in the stroma with a 26-gauge needle. To measure apoptosis and induction of cytokines in injured corneas, circular stromal wounds were made using an Algerbrush II (0.5 mm; Alber Equipment Co., Lago Vista, TX). Wounded corneas (incisional and circular) were inoculated with 1 μL (10 μg/μL) *Pseudomonas aeruginosa* LPS with a syringe (Hamilton, Reno, NV). Fluorescein sodium benoxinate hydrochloride ophthalmic solution (Bausch & Lomb Pharmaceuticals, Inc.) was used to detect healing with a 4′,6′-diamino-2-phenylindole (DAPI) filter on a dissection microscope. In addition, wound size was observed by bright-field microscopy using an external light source on a dissection microscope at 0, 8, 24, 48, and 72 hours after wounding. Images were captured by a microscope equipped with a digital camper (Axiovert 135-N from Carl Zeiss Meditec, Dublin, CA; Quantix 1401 charge-coupled device camera from Photometrix, Tucson, AZ; IP Laboratory software ver. 3.9.1; Scanalytics, Inc.).

Assay for Caspase-3/7 Activity

Apoptosis in the cornea was measured with a homogeneous caspase-3/7 assay (Apo-ONE; Promega, Madison, WI). $Lum^{+/+}$ and $Lum^{-/-}$ corneal protein extracts (n = 3) were prepared in tissue protein extraction reagent (T-PER; Pierce, Rockford, IL), 24 hours after circular stromal wounding. Caspase-3/7 activity was measured after incubating corneal extracts on a 96-well plate with caspase-3/7 substrate at room temperature (300 rpm) for 18 hours. Fluorescence recorded at an excitation wavelength of 480 nm and an emission wavelength of 530 nm (Spectra Max Plus reader and SoftMaxPro ver.4.0 software, Molecular Devices, Sunnyvale, CA) ×1000, relative fluorescence units (RFLU), was expressed as caspase-3/7 activity.

Immunofluorescence Staining

Six-week-old $Lum^{+/+}$ and $Lum^{-/-}$ mice (n = 3 per genotype) were euthanatized with CO_2, followed by cervical dislocation. Whole eyes were fixed in 1 mL 10% neutral buffered formalin for 4 to 6 hours (Fisher Scientific, Pittsburgh, PA), embedded in paraffin, sectioned, and prepared for immunostaining, as described previously.[5,17] Macrophages and neutrophils were immunostained with the rat monoclonal F/480 (2 μg/mL) or NIMP-R14 (8 μg/mL) primary antibody (Abcam, Inc., Cambridge, UK), respectively, followed by a secondary goat anti-rat Alexa Fluor 568, 5 μg/mL (Molecular Probes, Eugene, OR) antibody. Negative controls consisted of identical treatments with the omission of the primary antibody. Hoechst dye, 1 μg/mL (Molecular Probes) was used for nuclear staining. The slides were then mounted (Vectashield; Vector Laboratories Inc., Burlingame, CA), and images were captured with the equipment described earlier, with appropriate filter settings for Texas red and DAPI. F/480- and NIMP-R14-positive cells were counted in 10 uniform fields (n = 3), and the average number of positive cells at each time point was calculated.

Cell Culture and Transfection

Primary cultures of corneal fibroblasts (CFs) were derived from $Lum^{+/+}$ and $Lum^{-/-}$ corneas and maintained as described previously.[11] Human lumican cDNA clone pSecTag2/rhlum was used for transfections.[11] $Lum^{+/+}$ and $Lum^{-/-}$ CFs were transfected in 100-mm dishes with 40 μg of the vector pSecTag2/rhlum or the mock vector (pSecTag2), using 2 M $CaCl_2$ (Invitrogen, Carlsbad, CA). After 48 hours of transfection, total protein extracts were prepared from transfected cells using M-PER, mammalian protein extraction reagent (Pierce).

RNA Isolation and RT-PCR

Total RNA was isolated from the CFs (TRIzol reagent; Invitrogen). β-Actin and Fas were amplified with the following primers: forward (F)-β-actin, gtgaaaagatgacccagatcat; reverse (R)-β-actin, gcttctctttgatgtcacgcacga; F-Fas, atgcacactctgcgatgaag and R-Fas, ttcagggtcatcctgtctcc.

Immunoblot Analysis and Immunoprecipitation

Total protein extracts were prepared from corneal fibroblasts and cornea (M-PER and T-PER; Pierce, respectively). Protein extracts were resolved by SDS-PAGE followed by immunoblot analysis with antibodies against actin, Fas, or FasL (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To examine the binding of lumican to FasL, we incubated two aliquots of 800 μg/mL total corneal protein extract with 50 μL of protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) for 3 hour at 4°C. After preclearing, 5 μL of 1 mg/mL mouse lumican antibody[6] was added to one tube, and 5 μL of preimmune serum was added to the other tube (negative control). Protein A/G agarose beads (50 μL) were added to each tube after 1 hour, followed by overnight incubation at 4°C. Beads were washed once with lysis buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.5% Triton X-100 and 10 μM phenylmethylsulfonyl fluoride [PMSF]) followed with two washes with PBS. The samples were suspended in Laemmli's sample buffer (30 μL), vortexed for 1 minute, centrifuged for 5 minutes at 10,000g, and boiled for 5 minutes. The supernatants and total corneal protein extracts (as a positive control) were then resolved by SDS-PAGE and transferred to a 0.2-μm pore size polyvinylidene difluoride (PVDF) membrane. FasL protein was detected with a FasL antibody recognizing the C-terminal region of FasL.

Solid State Binding Assay

Flat-bottomed microtiter plates were precoated with increasing concentrations of recombinant FasL (R&D Systems, Minneapolis, MN) or BSA (0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 μg/mL), and the remaining binding sites were blocked with 0.1% BSA. Recombinant FasL contains the extracellular region of human FasL (amino acid residues 132–279) fused to the signal peptide of human CD33 and six histidine residues. Recombinant human lumican (10 μg/mL), prepared as described earlier,[11] was incubated on immobilized Fas-L/BSA for 1 hour at 37°C in 5% CO_2 and then washed gently. The binding of lumican to FasL or BSA was detected with an anti-human lumican antibody and peroxidase-conjugated goat anti-rabbit IgG. The plates were washed and incubated in the dark with tetra-methylbenzidine substrate for 15 minutes. The reaction was stopped with 2.5 N sulfuric acid, and the plates read at 450 nm (Spectra Max Plus reader; Molecular Devices; and SoftMaxPro ver. 4.0 software). Mean blank absorbance was subtracted from each reading.

Myeloperoxidase Assay

To obtain a quantitative estimate of neutrophils in the cornea, a myeloperoxidase (MPO) sandwich ELISA assay[18] was performed (OxisResearch; Oxis Health Products, Inc., Portland, OR) on wounded and unwounded corneal extracts. $Lum^{+/+}$ and $Lum^{-/-}$ corneas (n = 5) were wounded and exposed to LPS; and, 24 hours after injury, corneas were dissected, weighed, and homogenized as described earlier. For the ELISA, antigen captured by a solid-phase monoclonal antibody was detected with a biotin-labeled goat polyclonal anti-MPO antibody and avidin-conjugated alkaline phosphatase followed by its substrate p-nitrophenyl phosphate (pNPP). The product (p-nitrophenol) was detected at 405 nm (Spectra Max Plus reader; Molecular Devices; SoftMaxPro ver. 4.0 software). Purified MPO (standard) was included as a positive control.

Cytokine Profiling

Corneas, unwounded or 24 hours after wounding, were collected from 6-week-old $Lum^{+/+}$ and $Lum^{-/-}$ mice and homogenized in 20 μL of extraction solution (T-PER; Pierce) per milligram of cornea. These corneal extracts were used for cytokine quantification in TNFα, IL-1β, and IL-6 solid-phase sandwich ELISAs, as specified by the manufacturer (BioSource International, Inc., Camarillo, CA). Standards and high and low cytokine controls were included. The mean blank reading was subtracted from each sample and control reading.

Statistical Analysis

Student's *t*-test (probabilities) was run for comparison of $Lum^{+/+}$ and $Lum^{-/-}$ samples, using one-tailed distribution between two samples with equal variance. A P value ≤ 0.05 was considered to have statistical significance.

Results

Apoptosis in Wild-Type and Lumican-Null Corneas

Stromal incisional wounds were generated in 6-week-old $Lum^{+/+}$ and $Lum^{-/-}$ mice to induce apoptosis, and caspase-3/7 activity was measured to assess it ($n = 5$ animals per genotype). Caspase-3/7 activity was significantly lower ($P < 0.001$) in the lumican-null corneas than in their wild-type counterparts 24 hours after wounding (Fig. 1). These results clearly indicate a downregulation of programmed cell death in the lumican-deficient injured cornea. The finding is consistent with our prior observation that lumican-null fibroblasts and corneal keratocytes have reduced apoptosis when compared with the wild-type.[11]

Wound Healing in Wild-Type and Lumican-Null Cornea

Apoptosis of stromal cells is a major early event in wound healing. Furthermore, during infection and injury, apoptosis of invading inflammatory cells is important in the inflammation and healing cascade. Therefore, to determine how reduced apoptosis affects corneal healing in the lumican-null mouse, we generated circular stromal wounds injected with *P. aeruginosa* LPS in five $Lum^{+/+}$ and $Lum^{-/-}$ mice, and observed the wound healing from 0 to 72 hours. One representative animal of each genotype is shown in Figure 2. Wild-type corneas healed faster and recovered completely by 72 hours. In contrast, the healing of lumican null corneas was delayed significantly. Starting with circular wounds of similar size, by 24 hours, the size of wounds in wild-type corneas was reduced to half their original diameters, whereas those in $Lum^{-/-}$ corneas remained unchanged (Fig. 2).

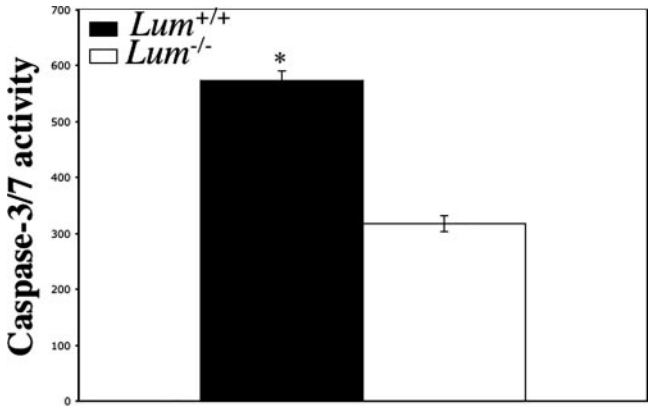

Figure 1. Lower caspase-3/7 activity indicating reduced apoptosis in $Lum^{-/-}$ corneal extracts. Caspase-3/7 activity was measured in $Lum^{+/+}$ and $Lum^{-/-}$ ($n = 3$) corneal extracts (CE), 24 hours after circular stromal wounds were generated. Caspase-3/7 activity was significantly lower ($P < 0.001$) in the $Lum^{-/-}$ mouse corneal extracts compared with wild type.

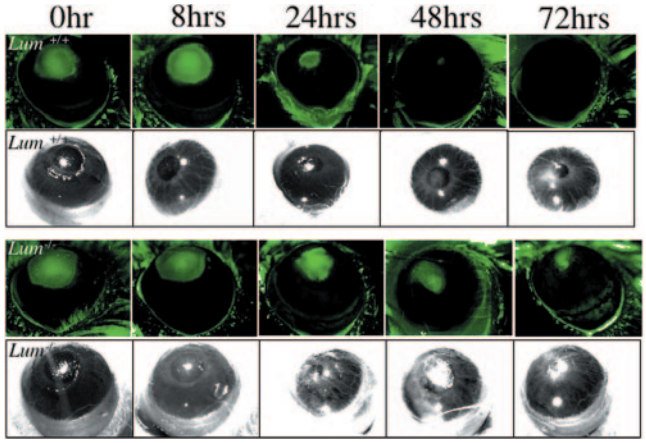

Figure 2. Corneal wound healing in wild-type and $Lum^{-/-}$ cornea. Wounded $Lum^{+/+}$ and $Lum^{-/-}$ corneas were exposed to LPS. The unwounded contralateral eye treated with saline served as a control. Fluorescein dye (*green*; *top*) was used to detect wound healing using a DAPI filter on a dissection microscope. Corneas were also checked by bright-field microscopy, using an external light source on a dissecting microscope (*bottom*). Wild-type corneas healed faster and recovered completely by 72 hours, whereas lumican-null corneas showed a significant delay in healing.

Macrophage and Neutrophil Infiltration in Wild-Type and Lumican-Null Corneas

To test the possibility that delayed wound healing in lumican-nulls is due to altered inflammatory responses, we examined the infiltration of macrophages and neutrophils in $Lum^{+/+}$ and $Lum^{-/-}$ corneas ($n = 3$), at 4, 8, and 24 hours after producing stromal incision wounds. Unwounded corneas were used as the control. Wounded and unwounded $Lum^{+/+}$ and $Lum^{-/-}$ corneal sections were immunostained for macrophages (F4/80) and neutrophils (NIMP-R14). We observed no significant difference in the number of macrophages and neutrophils in $Lum^{+/+}$ and $Lum^{-/-}$ corneas at 4 and 8 hours after injury (Figs. 3, 4). However, by 24 hours after injury, there was a significant ($P = 0.05$) increase in the number of macrophages in wild-type (43.67 $\pm$ 4.05) versus lumican-null (33.00 $\pm$ 3.21) corneas (Fig. 3). At this time point, the number of neutrophils in $Lum^{+/+}$ corneas (35.34 $\pm$ 2.85) was also significantly ($P < 0.05$) higher than that in injured lumican-null (23.67 $\pm$ 3.48) corneas (Fig. 4).

We next measured MPO levels in unwounded and wounded corneas to obtain a quantitative estimate of activated neutrophils. We found a significant ($P < 0.01$) quantitative increase in activated neutrophils in $Lum^{+/+}$ wounded (0.37 $\pm$ 0.06) corneas compared with unwounded (0.16 $\pm$ 0.02) corneas. In contrast, there was no significant difference in MPO levels between $Lum^{-/-}$ wounded and unwounded corneas (Fig. 5).

Proinflammatory Cytokine Levels in Wild-Type and Lumican-Null Corneas

To determine whether the observed difference in wound healing and inflammation in the $Lum^{+/+}$ and $Lum^{-/-}$ cornea is due to a difference in the induction of proinflammatory cytokines, we evaluated TNFα, IL-1β, and IL-6 levels by ELISAs in

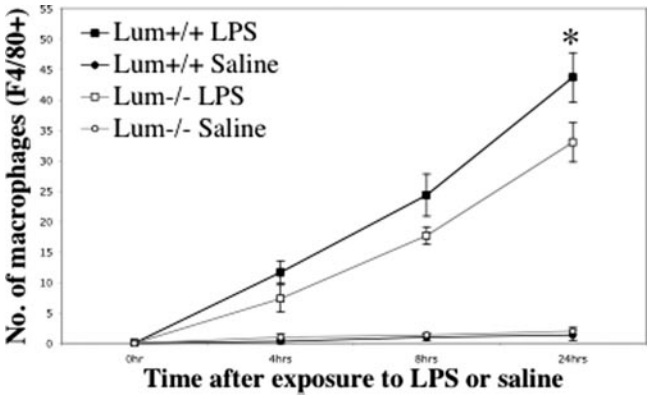

FIGURE 3. Macrophage infiltration of the corneal stroma after injury. $Lum^{+/+}$ and $Lum^{-/-}$ corneas were wounded by stromal incision and exposed to LPS. Unwounded corneas treated with saline were used as controls. Eyes were removed at 4, 8, and 24 hours after injury, sectioned, and immunostained with F/480 antibody. The number of macrophages in each section was determined by counting F/480 positive cells in 10 independent fields under 40× magnification. Lumican-null corneas showed significant reduction in macrophage infiltration compared with wild type, 24 hours after LPS wounding. Data are the mean ± SEM of measurements in three mice per group (*$P = 0.05$).

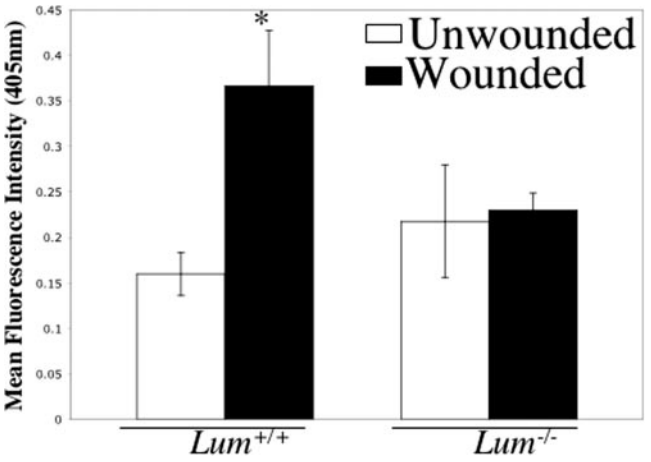

FIGURE 5. MPO assay. Wounded and unwounded $Lum^{+/+}$ and $Lum^{-/-}$ corneas ($n = 5$) were exposed to LPS and saline, respectively. After 24 hours, corneal protein extracts were used in an MPO assay. Wounded lumican-null corneas had significantly lower levels of MPO than did wild-type corneas. Moreover, wild-type corneas showed significant induction of MPO after stromal wounding, whereas lumican-null corneas showed no induction (*$P < 0.01$).

wounded corneas exposed to 10μg/mL of LPS. Unwounded corneas were used as the control. By 24 hours after wounding, there was a significant increase in the levels of TNFα (Fig. 6A), IL-1β (Fig. 6B), and IL-6 (Fig. 6C) in the wild-type corneas. In the $Lum^{-/-}$ wounded corneas, induction of TNFα (Fig. 6A) or IL-1β (Fig. 6B) was not significant, and increase in IL-6 was modest (Fig. 6C). Induction of TNFα and IL-1β are immune responses that seem to require lumican, and were disrupted in the $Lum^{-/-}$ mice.

Lumican Modulation of Fas-Mediated Signaling

Given that proinflammatory cytokines are not induced optimally in the wounded lumican-null cornea, the obvious question is what signaling mechanisms are affected by lumican.

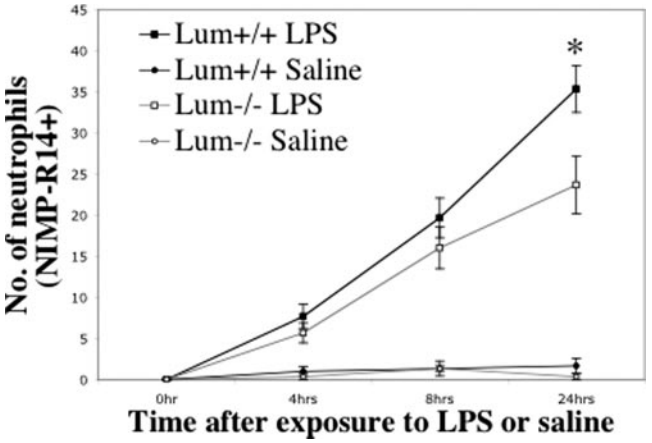

FIGURE 4. Neutrophil infiltration of the corneal stroma after injury. Wounded $Lum^{+/+}$ and $Lum^{-/-}$ corneas were exposed to LPS. The unwounded contralateral eye treated with saline served as a control. Eyes were removed, sectioned, and immunostained for neutrophils with NIMP-R14 antibody at 4, 8, and 24 hours after wounding. Neutrophils were counted in ten independent fields at 40× magnification. In the LPS-wounded series, at the 24-hour time point, the number of neutrophils in the $Lum^{+/+}$ corneas was significantly higher than in the $Lum^{-/-}$ corneas. Data are the mean ± SEM of measurements in three mice per group (*$P < 0.05$).

Based on our earlier study, we know that Fas is downregulated in the cornea and in fibroblast cultures of $Lum^{-/-}$ mice.[11] Moreover, Fas ligation on macrophages enhances an LPS-induced inflammatory response.[19] This suggests a connection between disrupted Fas signaling in $Lum^{-/-}$ corneas and reduced induction of the proinflammatory cytokines, TNFα and IL-1β. To test this connection, we assayed for Fas levels in $Lum^{+/+}$ and $Lum^{-/-}$ corneal fibroblasts (CFs) after treating cells with increasing doses of LPS. Fas levels increased in wild-type CFs, whereas it remained at basal levels in $Lum^{-/-}$ CFs (Fig. 7A). Similarly, in vivo, Fas remained at basal levels in wounded and LPS-exposed $Lum^{-/-}$ corneas but increased markedly on similar treatment of $Lum^{+/+}$ corneas (Fig. 7B). To confirm that the lack of Fas induction in lumican-nulls is indeed due to lumican deficiency and not an unforeseen change resulting from the targeting of the lumican gene, we determined the effect of the expression of recombinant lumican protein on Fas levels. Expression of recombinant lumican in $Lum^{-/-}$ CFs restored Fas to the basal level recorded in $Lum^{+/+}$ CFs, confirming a requirement of the lumican core protein in maintaining Fas levels. In wild-type CFs, expression of recombinant lumican also resulted in an increase in Fas. The mock control vector affected an increase in Fas as well (Fig. 7C). The latter may simply be a Fas-pathway–mediated cellular immune response to exogenous vector DNA in wild-type cells. With Fas signaling disrupted in $Lum^{-/-}$ cells, this response to the mock vector was not seen in the $Lum^{-/-}$ CFs.

To determine whether the differential induction of Fas in wild-type and $Lum^{-/-}$ mice occurs due to transcriptional regulation, we evaluated Fas mRNA levels in $Lum^{+/+}$ and $Lum^{-/-}$ corneas by RT-PCR. The results indicate comparable levels of Fas mRNA in both genotypes, ruling out its regulation at the RNA levels (Fig. 7D).

Lumican-FasL Binding

We have recently shown that FasL can induce Fas in $Lum^{+/+}$ mouse embryonic fibroblasts (MEFs). However, Fas was not induced in $Lum^{-/-}$ MEFs, indicating that a presence of lumican is obligatory for FasL-mediated induction of Fas.[11] We hypothesize that lumican binds to FasL and helps in its concentration and presentation to Fas. Indeed, immunoprecipita-

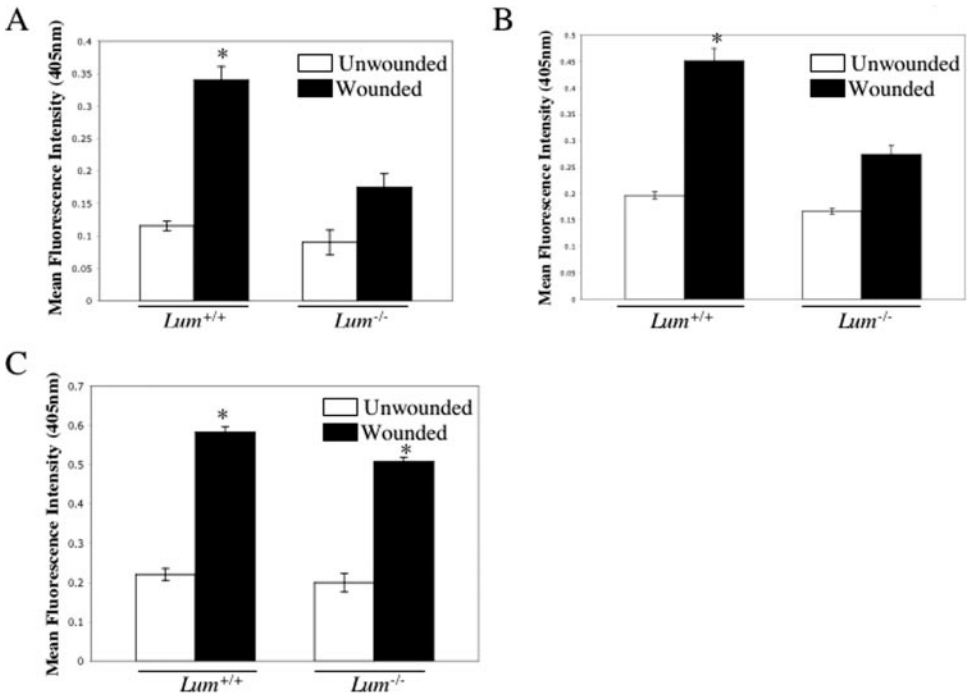

FIGURE 6. Cytokine levels in wild-type and lumican-null corneas. Wounded and unwounded $Lum^{+/+}$ and $Lum^{-/-}$ corneas ($n = 3$) were exposed to LPS and saline, respectively. After 24 hours, corneal proteins were extracted for cytokine ELISA. Wounded lumican-null corneas showed significantly lower induction of TNFα (**A**), IL-1β (**B**), and IL-6 (**C**) than did wild-type corneas ($^*P < 0.01$).

tion of lumican from corneal extracts, using an antibody against lumican, coimmunoprecipitated FasL (Fig. 8). We further confirmed the binding of lumican to FasL by a solid-state binding assay. Incubation of an excess of lumican in 96-wells coated with increasing amounts of immobilized soluble (s)FasL (0–12.8 μg/mL) resulted in an sFasL dose-dependent increase in binding of lumican, with no difference in binding to increasing doses of BSA control (Fig. 9).

DISCUSSION

In an earlier study, we detected increased cell proliferation and decreased apoptosis in lumican-null mouse corneas and cultured fibroblasts, establishing a direct role for lumican in suppressing proliferation and aiding apoptosis.[11] Cellular apoptosis and proliferation are key events during corneal wound healing,[20] which prompted us to investigate further the heal-

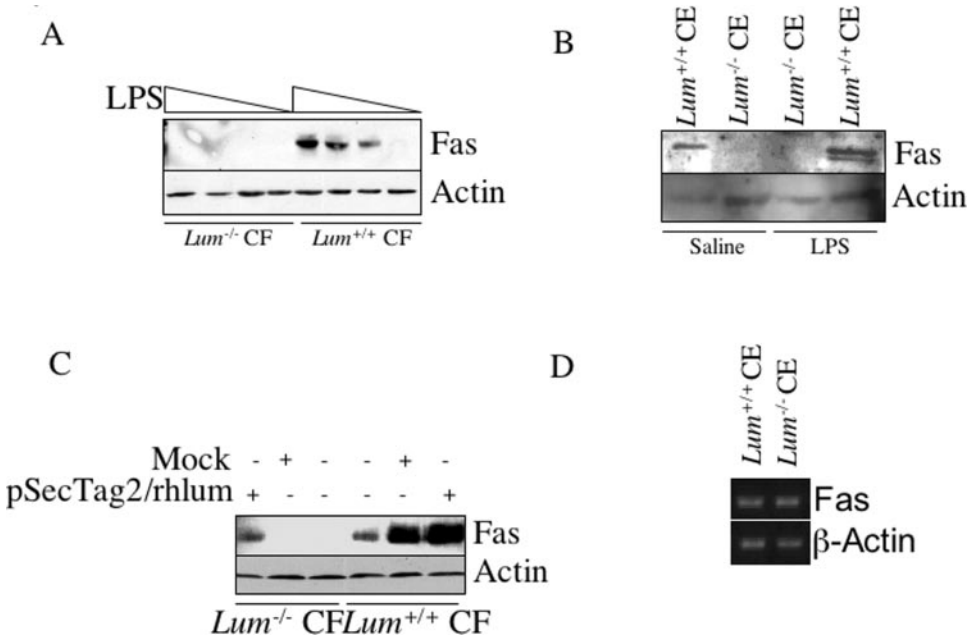

FIGURE 7. Lumican-modulated Fas-mediated signaling. (**A**) Treated with increasing concentrations of LPS (0–100 ng/mL) for 24 hours, CFs from $Lum^{+/+}$ but not $Lum^{-/-}$ mice showed an increase in Fas protein levels. Immunoblot analysis of actin indicated the equivalent loading of lanes. (**B**) Corneal wounds were exposed to LPS (10 μg), and total protein was extracted 24 hours later. Unwounded saline-treated corneas were extracted and used as the control. Immunoblot analysis for Fas showed increased levels in $Lum^{+/+}$ extracts after LPS wounding, with no change in the extremely low levels of Fas in $Lum^{-/-}$ extracts. Immunoblot analysis of actin indicates the equivalent loading of lanes. (**C**) $Lum^{-/-}$ CFs transfected with lumican expression vector (pSecTag2/rhlum) reactivated Fas expression. Immunoblot analysis of actin indicates the equivalent loading of lanes. (**D**) $Lum^{+/+}$ and $Lum^{-/-}$ corneas showed similar levels of Fas transcripts by RT-PCR. β-Actin was used as the internal control.

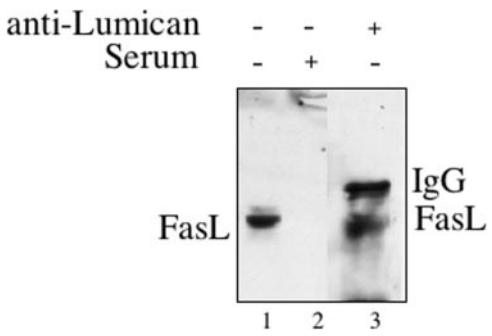

FIGURE 8. Coimmunoprecipitation of FasL with lumican antibody. Preimmune serum and anti-lumican immunoprecipitates were loaded in *lanes 2* and *3*, respectively. Total corneal protein extract was loaded in *lane 1* as a positive control. Immunoblot analysis was performed with FasL antibody. Lumican antibody coimmunoprecipitated FasL (*lane 3*) and lumican from corneal extracts (CEs), whereas serum did not (*lane 2*).

ing of stromal injury in lumican-null mice. Our present study indicates that whereas corneal wounds healed completely by 72 hours in wild-type animals, wounds failed to heal in lumican-null corneas during the same time frame. There was a marked difference in the recruitment of inflammatory cells, with the appearance of fewer macrophages and neutrophils in the stroma of injured $Lum^{-/-}$ corneas. Lower MPO levels in the lumican-null corneas further confirmed a lack of neutrophil activation in the absence of lumican. Furthermore, when measuring proinflammatory cytokines in corneal extracts before and after injury, we saw very little induction of TNFα and IL-1β in $Lum^{-/-}$ corneas after stromal injury. A low proinflammatory cytokine milieu in lumican-nulls may explain poor recruitment of inflammatory infiltrates at wound sites and a consequent delay in healing.

Our results clearly underscore a role for lumican in the interstitial ECM in establishing inflammation and healing of stromal injuries. The question, of course, is how lumican from the ECM communicates with cells to regulate inflammatory responses. An earlier finding that macrophages express a cell-surface receptor for lumican is certainly a piece of the puzzle and is consistent with our findings that lumican-null corneas fail to recruit wild-type levels of macrophages and neutrophils on injury.[21] However, little else is known about the nature of this interaction between lumican and macrophages/monocytes. Beyond poor recruitment of inflammatory cells, there is a broad proinflammatory cytokine deficiency. This suggests that additional steps have gone awry in establishing inflammation and repair in the injured $Lum^{-/-}$ cornea. From our earlier study we know that Fas is downregulated in lumican-null fibroblasts and in the cornea. This suggests cross talk between lumican and Fas-FasL signaling.[11] We speculate that this connection between Fas and lumican has a hand in the regulation of inflammatory responses.

Although Fas is best known for its involvement in regulation of apoptosis,[22] Fas ligation may contribute to nonapoptotic signaling, including NFκB activation,[23–25] early T-cell development,[26] and proliferation.[27] FasL, a death-inducing molecule and a natural ligand of Fas, is involved in homeostasis, self-tolerance, and immune privilege.[28] FasL also has proinflammatory functions,[29] and the ligation of Fas to circulating monocytes and tissue macrophages may induce proinflammatory cytokine responses that can induce and initiate inflammatory responses.[12] The Fas-induced monocyte/macrophage response may be a key event in innate immune responses contributing to the pathogenesis of a variety of clinically important inflammatory diseases. Fas-FasL interactions may contribute to disease pathogenesis and progression of chronic inflammation by a unique mechanism (FADD-MyD88 interaction), whereby Fas ligation promotes activation through IL-1R1 and TLR4 pathways.[19] Our findings showed that treatment of $Lum^{+/+}$ CFs with *P. aeruginosa* LPS resulted in the induction of Fas in wild-type but not in $Lum^{-/-}$ mice. In addition, LPS injected stromal wounds in wild-type, but not in lumican-null, corneas express Fas. These results suggest that lumican plays a key role in the Fas-FasL signaling involved in corneal injury and inflammatory intermediates required in the initial counteraction of injury and infection. Another study indicated transient expression of lumican in the wounded corneal epithelium, where it was considered to aid epithelial migration.[30] In fact, in the injured epithelium, lumican expression may primarily affect induction of proinflammatory cytokines rather than cellular migration.

To get a mechanistic insight in the regulation of Fas levels by lumican, we tested the possibility that Fas is regulated at the mRNA level. The RT-PCR results indicating equivalent mRNA levels eliminate the possibility that Fas mRNA is downregulated in the $Lum^{-/-}$ mouse cornea. Consequently, the increase in Fas levels must be due to its regulation at the protein level. In the $Lum^{-/-}$ mouse, FasL fails to elicit an increase in the receptor level, implying a lack of communication between the ligand and its receptor. We reasoned that lumican may be involved in concentrating and

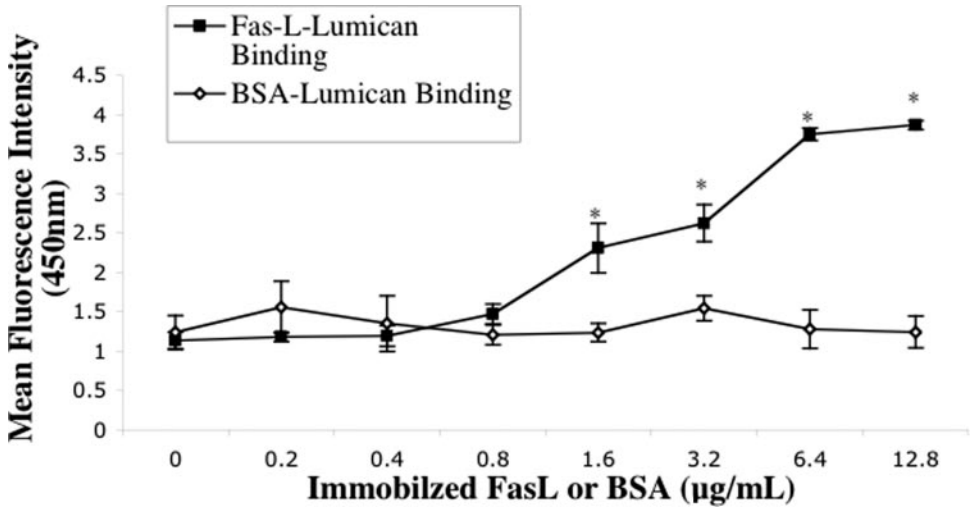

FIGURE 9. A solid-state binding assay showed binding of FasL to lumican. Recombinant lumican (10 μg/mL) was incubated with increasing concentrations of immobilized FasL or BSA (0–12.8 μg/mL) at 37°C. Recombinant lumican bound to FasL was detected with an anti-lumican antibody. The results show increased binding of lumican to increasing amounts of immobilized FasL but not to the BSA control (*$P < 0.01$).

presenting FasL to its receptor Fas for appropriate induction, and we further investigated the binding of lumican to FasL.[11] Coimmunoprecipitation of lumican and FasL and solid state binding assay provide evidence for lumican-FasL interactions. Released from the membrane bound form of FasL (mFasL) by a putative metalloproteinase, the capacity of sFasL to induce apoptosis is much lower than that of mFasL, which is believed to be the functional form.[31,32] sFasL, in contrast, reportedly induces tumor cell[33] and hepatocyte apoptosis.[34] This discrepancy could arise from disparate effects of sFasL in tissues, where it may reside bound to different ECM components retaining much of the functions attributed to mFasL.[35] Thus, fibronectin was reported to bind soluble FasL (sFasL), which retained effector T cells at sites of inflammation and later induced T-cell apoptosis, healing, and return of tissue homeostasis.[35,36] Binding of FasL to fibronectin was proposed to result essentially in the oligomerization of FasL, further enhancing its biological activity.[36] In the eye, FasL is expressed by the corneal epithelium and endothelium.[37] The sFasL released in stroma may be similarly bound by the lumican present in abundance in the corneal stroma. It is likely, in the corneal stroma, that lumican is largely instrumental in retaining sFasL and increasing its biological efficacy.

The functional implications of interactions between FasL and lumican and further induction of Fas in the cornea are not clear, given the complexity of the role played by FasL and Fas in promoting inflammation as well as counteracting it in the context of ocular immune privilege.[7] The eye is equipped with a distinct ability to restrain inflammatory activity, presumably by engaging FasL and Fas on inflammatory cells for their prompt apoptosis to preclude systemic response.[22,37,38] In recent studies, investigators of mFasL versus sFasL in inflammation and ocular immune privilege observed that only tumor cells expressing high levels of mFasL could override immune privilege and induce potent neutrophil-mediated inflammation, whereas, in non–immune-privileged sites, lower levels of mFasL could initiate inflammation.[28,29] The tissue environment in the corneal stroma, additional cytokines and growth factors that modify Fas-signal–mediated inflammatory processes, ECM components that modify localized concentration of FasL, and the availability of cytokines and growth factors may tip the balance toward inflammation or immune privilege.

In summary, the present study shows a major discord in cytokine induction and recruitment of inflammatory cells leading to impaired healing of corneal wounds in the lumican-null mouse. The underlying early defect in the lumican-null mouse may reside in inefficient signaling via Fas and its ligand, FasL, with further impact on other inflammatory cytokines. This mandates a close look at lumican as a potentially exciting therapeutic tool for tweaking these cell-signaling components. This study has unraveled a novel significant role for lumican in the presentation of immune/inflammatory signal to cells and facilitating inflammatory and healing responses in the cornea.

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