Corneal Expression of SLURP-1 by Age, Sex, Genetic Strain, and Ocular Surface Health

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Purpose. Although secreted Ly6/urokinase-type plasminogen activator receptor–related protein-1 (*Slurp1*) transcript is highly abundant in the mouse cornea, corresponding protein expression remains uncharacterized. Also, SLURP1 was undetected in previous tear proteomics studies, resulting in ambiguity about its baseline levels. Here, we examine mouse corneal Slurp1 expression in different sexes, age groups, strains, and health conditions, and quantify SLURP1 in human tears from healthy or inflamed ocular surfaces.

Methods. Expression of Slurp1 in embryonic day-13 (E13), E16, postnatal day-1 (PN1), PN10, PN20, and PN70 Balb/C, FVBN, C57Bl/6, and DBA/2J mouse corneas, $Klf4^{\Delta/\Delta CE}$ corneas with corneal epithelial–specific ablation of *Klf4*, migrating cells in wild-type corneal epithelial wound edge, and in corneas exposed to pathogen-associated molecular patterns (PAMPs) poly(I:C), zymosan-A, or Pam3Csk4 was examined by QPCR, immunoblots, and immunofluorescent staining. Human SLURP1 levels were quantified by ELISA in tears from 34 men and women aged 18 to 80 years.

Results. Expression of Slurp1, comparable in different strains and sexes, was low in E13, E16, PN1, and PN10 mouse corneas, and increased rapidly after eyelid opening in a Klf4-dependent manner. We found Slurp1 was downregulated in corneas exposed to PAMPs, and in migrating cells at the wound edge. Human SLURP1 expression, comparable in different sexes and age groups, was significantly decreased in tears from inflamed ocular surfaces (0.34%) than those from healthy individuals (0.77%).

Conclusions. These data describe the influence of age, sex, genetic background, and ocular surface health on mouse corneal expression of Slurp1, establish the baseline for human tear SLURP1 expression, and identify SLURP1 as a useful diagnostic and/or therapeutic target for inflammatory ocular surface disorders.

Keywords: Slurp1, cornea, ocular surface, inflammation

The secreted Ly6/urokinase-type plasminogen activator receptor–related protein-1 (SLURP1) is a member of the lymphocyte antigen (Ly6) superfamily characterized by the three-finger fold structure.[1] Mutations or deletions in *SLURP1* cause Mal de Meleda, an autosomal recessive palmoplantar hyperkeratotic disorder.[2–7] Structurally similar to the snake and frog cytotoxin α-bungarotoxin, SLURP1 acts as a ligand for the α7 subunit of the nicotinic acetylcholine receptors (α7nAchRs), regulating keratinocyte functions through cholinergic pathways.[6,8] Protein Slurp1 is involved in signal transduction, activation of the immune response, and cell adhesion, preventing tobacco nitrosamine-induced malignant transformation of oral cells.[9–13] Our previous studies revealed that Slurp1 serves as an immunomodulatory molecule at the ocular surface[14] by acting as a soluble scavenger of the uPAR ligand urokinase.[15]

Protein SLURP1 is widely expressed in a variety of cells including immune cells,[12] bronchial epithelial cells,[16] primary sensory neurons,[17] the skin, exocervix, gums, stomach, trachea and esophagus,[6] oral keratinocytes[10] and the cornea,[18] and secreted into bodily fluids such as plasma, saliva, sweat, urine, and tears.[3] Though *Slurp1* is one of the most abundant transcripts in both neonatal and the adult mouse corneas,[18,19] our understanding of its developmental expression patterns in different sexes and genetic strains remains incomplete. Several gene expression profiling studies suggested that *Slurp1* transcripts are downregulated in diverse proinflammatory conditions including asthmatic lungs,[20] corneal neovascularization,[21] Barrett's esophagus,[22,23] adenocarcinomas, malignant melanomas,[24] and esophageal and oral squamous cell carcinomas.[25,26] However, it is not clear if this high level of *Slurp1* transcripts in normal corneas and their sharp downregulation in proinflammatory conditions are reflected at the protein level as well. Moreover, previous proteomics studies have failed to identify SLURP1 protein in human tears, resulting in ambiguity about its expression there. In this study, we have attempted to fill these gaps by examining Slurp1 expression levels in mouse corneas from different sexes, age groups, and strains (Balb/C, FVBN, C57Bl/6, and DBA/2J); mouse corneas exposed to proinflammatory stimulants poly(I:C), zymosan-A, or Pam_3Csk_4;

and in human tears collected from adults with normal or inflamed ocular surfaces.

Materials and Methods

Breeding and Management of Mouse Strains

On postnatal days 10, 20, and 70, we procured Balb/C, C57/Bl6, DBA/2J, and FVBN strains of mice from Jackson Laboratories (Bar Harbor, ME, USA). Ternary transgenic (*Klf4*$^{LoxP/LoxP}$/*Krt12*$^{rtTA/rtTA}$/*Tet-O-Cre*) mice were generated by us on a mixed background by mating *Klf4*$^{LoxP/LoxP}$ with *Krt12*$^{rtTA/rtTA}$/*Tet-O-Cre* mice as described earlier.[27–30] Expression of Cre was induced in these ternary transgenic mice by doxycycline administered through intraperitoneal injections and drinking water to generate corneal epithelium-specific disruption of *Klf4* (*Klf4*$^{\Delta/\Delta CE}$). Ternary transgenic littermates injected with PBS and fed with sugar water served as the wild-type (WT) controls. All studies reported here were performed with mice maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of the University of Pittsburgh and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The data presented in this manuscript are representative of at least three independent experiments.

Intrastromal Injections of Pathogen-Associated Molecular Patterns (PAMPS)

Mice were anesthetized by intraperitoneal injection of a mix of ketamine and xylazine, and their eyes were further treated with a drop of topical anesthetic proparacaine. An intrastromal tunnel was then introduced using a 32-G beveled-edge needle, into which 2 μL different PAMPs (TLR2-ligands Pam_3CSK_4 [1 mg/mL] and Zymosan-A [1 mg/mL], and TLR3-ligand Poly(I:C) [5 mg/mL]) were injected using a Hamilton syringe fitted with a 33-G blunt-ended needle. Effect of different PAMPs on mouse corneal expression of Slurp1 was determined by quantitative PCR (QPCR) and immunofluorescent staining 48 hours after intrastromal injection.

Corneal Epithelial Debridement Wounds

Adult (PN70) mice of mixed genetic background were anesthetized by intraperitoneal (IP) injection of ketamine/xylazine and topical application of proparacaine as above. Minor epithelial scratch wounds were generated gently by six each of left-to-right and top-to-bottom strokes with a dulled 26 G needle. Larger, more severe wounds were generated by demarcating the central corneal 1.5-mm diameter area by trephine blades, and debriding it with an Alger brush. Mice were euthanized 6 hours later, and total RNA isolated from corneas was used for QPCR, or enucleated eyeballs were embedded in OCT, and 8-μm-thick cryosections from central corneas were probed by immunofluorescent staining with anti-Slurp1 antibody as described below.

Human Tear Collection and ELISAs

Tear samples were collected as described earlier,[31] following a protocol approved by the University of Pittsburgh Institutional Review Board (IRB) and the tenets of the Declaration of Helsinki. Thirty-four volunteers (aged 18–80 years) enrolled after being informed of the nature of the study and potential risks involved. Subjects with infectious keratitis, those undergoing any therapy for ocular surface disorders at the time of this study, or those wearing contact lenses were excluded from the study. Before collecting the tear samples, the subjects' ocular surface health status was determined as "normal" or "inflamed" based on case history and slit-lamp examination. Two minutes after instilling 0.5% proparacaine, tears were collected for 30 seconds from the lower cul-de-sac of the left eye using a 15-mm polyester fiber wick (Transorb Wick; Filtrona, Richmond, VA, USA). Each wick with tears was placed in a 0.5-mL Eppendorf tube at the bottom of which a hole was introduced with a 20-G needle, and the tube with the wick was then placed in a 1.5-mL Eppendorf tube for 70°C storage. Tears were subsequently eluted from wicks by incubating with 50 μL PBS on ice for 20 minutes, followed by centrifugation for 10 minutes at 12*g*. Protein concentration in eluted tears was quantified by bicinchoninic (BCA) assay with bovine serum albumin as a standard and the tear samples were stored at 70°C until further analysis.

For ELISA, high-binding capacity plates were coated in triplicate with increasing amounts of partially purified recombinant SLURP1 protein diluted in PBS for generating the standard curve. Adjacent wells in the same plate were coated in triplicate with 100 ng tear protein in PBS. After blocking for 1 hour with 4% milk in PBST at 37°C, anti-human SLURP1 antibody (2 μg/mL; Abnova, Taipei, Taiwan) was added and the plates incubated at 37°C for 2 hours. After washing four times each for 1 minute with PBST, horseradish peroxidase–conjugated anti-rabbit IgG (1:1000 dilution) was added and the plates incubated at 37°C for 1 hour. After washing five times with PBST, the bound antibody was quantified using peroxidase substrate tetramethylbenzidine and measuring absorbance at 450 nm using a plate reader (Biotek Synergy-II; Biotek, Winooski, VT, USA).

Isolation of RNA, Reverse Transcription, and QPCR

Relative expression of *Slurp1* transcripts was quantified by QPCR 6 hours after introducing epithelial scrape wounds by six left-to-right and top-to-bottom strokes of a dulled scalpel in anesthetized mouse corneas. Mouse corneal total RNA was isolated using purification columns (RNeasy; Qiagen, Germantown, MD, USA), 100 ng of which was used for cDNA synthesis with mouse Moloney leukemia virus reverse transcriptase (Promega, Madison, WI, USA). TaqMan gene expression assays were performed in triplicate in a thermocycler with prestandardized gene-specific probes using 18S rRNA or laminin as endogenous control (ABI StepOne Plus; Applied Biosystems, Foster City, CA). The results of QPCR were analyzed using StepOne software provided by the manufacturer (Applied Biosystems). At least three independent biological replicates were employed for each experimental condition tested.

Immunoblots

Equal amounts of total protein extracted using M-PER reagent and quantified by BCA method (Pierce Biotechnology, Rockford, IL, USA) were electrophoresed in SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting, and subjected to immunoblot analysis with 1:1000 dilution of rabbit anti-mouse Slurp1 antibody[17] or goat anti-human SLURP1 primary antibody, and 1:1000 dilution of horseradish peroxidase–coupled goat anti-rabbit IgG secondary antibody or donkey anti-goat IgG secondary antibody, respectively (Santa Cruz Biotechnology, Dallas, TX, USA). Immunoreactive bands were detected by chemiluminescence using Super Signal West Pico solutions (Pierce Biotechnology). Equal loading was confirmed by stripping and reprobing the blots with anti-actin antibody (Santa Cruz Biotechnology).

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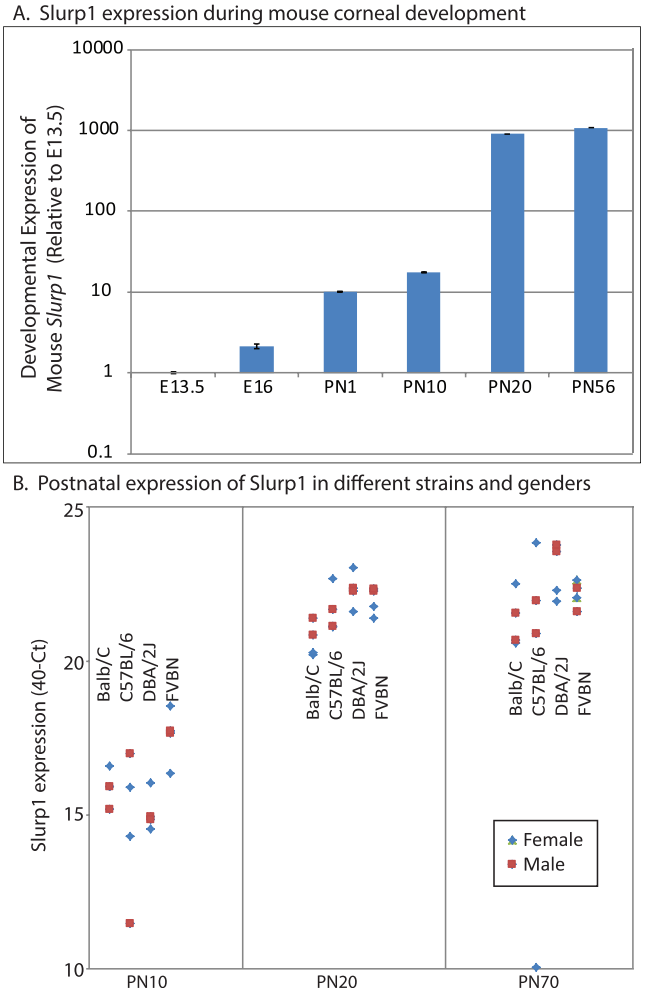

Figure 1. Developmental changes in *Slurp1* expression in mouse corneas. (**A**) Changes in *Slurp1* expression in developing mouse corneas (mixed background) measured by QPCR, relative to that in E13.5 corneas. (**B**) Comparison of *Slurp1* expression in PN10, PN20, and PN70 male and female mouse corneas from Balb/C, C57Bl/6, DBA/2J and FVBN genetic backgrounds by QPCR.

Immunofluorescent Staining

Cryosections (8-μm-thick) from OCT-embedded eyeballs were fixed in freshly prepared buffered 4% paraformaldehyde for 30 minutes, washed thrice for 5 minutes each with PBS (pH 7.4), permeabilized with 0.1% triton in PBS when necessary, followed by three washes of 5 minutes each with PBS, blocked with 10% goat or donkey serum in PBS containing 0.1% Tween-20 (PBST) for 1 hour at room temperature in a humidified chamber, washed twice with PBST for 5 minutes each, incubated with a 1:100 dilution of the rabbit anti-mouse Slurp1 primary antibody[17] for 2 hours at room temperature, washed thrice with PBST for 5 minutes each, incubated with appropriate secondary antibody (AlexaFluor 546–coupled goat anti-rabbit IgG, and AlexaFluor 488–coupled donkey anti-goat IgG; Molecular Probes, Carlsbad, CA, USA) at a 1:200 dilution for 1 hour at room temperature, washed thrice with PBST for 5 minutes each, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and cover-slipped using an aqueous mounting medium (Aqua-Poly/Mount; Polysciences, Warrington, PA, USA). After drying overnight, the coverslips were sealed with clear nail polish and the images collected using a confocal microscope (Olympus IX81; Olympus Corp., Tokyo, Japan).

Results

Slurp1 Expression During Mouse Corneal Embryonic Development and Postnatal Maturation

In order to describe the temporal changes in *Slurp1* expression during mouse corneal embryonic development and postnatal maturation, we first performed QPCR with total RNA from E13.5, E16, and PN1, PN10, PN20, and PN56 corneas. Expression of *Slurp1* was barely detectable in E13.5 corneas (Fig. 1A), increased gradually until eyelid opening, and was sharply increased between PN10 and PN20 when the eyelids opened (Fig. 1A). No significant difference was observed in *Slurp1* expression between PN20 and PN56 corneas (Fig. 1A). Additional QPCR reactions with PN10, PN20, and PN70 male and female Balb/C, C57Bl/6, DBA/2J and FVBN mouse corneas revealed that the post–eyelid opening increase in *Slurp1* expression is a common feature that is not influenced by the sex or genetic background of the mouse (Fig. 1B). Consistent with these results, immunofluorescent staining with anti-Slurp1 antibody revealed sharp increase in Slurp1 protein expression in the corneal epithelium between PN10 and PN21 and no significant difference between PN21 and PN70 across four genetic backgrounds and two sexes tested (Fig. 2).

Klf4 Regulates Post–Eyelid Opening Increase in Corneal Expression of Slurp1

Previously, we reported that the adult corneal Slurp1 expression is regulated by Klf4.[14] In order to determine if post–eyelid opening increase in *Slurp1* expression is Klf4-dependent, we measured *Klf4* and *Slurp1* levels in $Klf4^{\Delta/\Delta CE}$ mouse corneas which allow doxycycline-inducible spatiotemporally regulated corneal epithelium-specific ablation of *Klf4*.[27] While the WT corneas demonstrated significant increase in the expression of both *Klf4* and *Slurp1* between PN14 and PN16, $Klf4^{\Delta/\Delta CE}$ mouse corneas failed to do so (Fig. 3A) suggesting that post–eyelid opening increase in corneal expression of *Slurp1* is regulated by Klf4. Consistent with these results, immunoblots with PN20 mouse corneal lysates revealed abundant expression of Slurp1 in the WT but not the $Klf4^{\Delta/\Delta CE}$ mouse corneas (Fig. 3B). Immunofluorescent staining with anti-Slurp1 antibody revealed abundant expression of Slurp1 in the PN70 WT but not the $Klf4^{\Delta/\Delta CE}$ mouse corneal epithelium, confirming that Klf4 is required for post–eyelid opening increase in corneal expression of *Slurp1* (Fig. 3C).

Slurp1 Expression Is Suppressed in Response to Diverse PAMPs

Immunomodulatory molecule Slurp1 is abundantly expressed in healthy corneas and is rapidly downregulated in proinflammatory conditions.[14] In order to determine if Slurp1 downregulation is a common feature of corneal inflammation regardless of the nature of the insult, we quantified Slurp1 expression in corneas exposed to diverse PAMPs. We quantified Slurp1 by QPCR and immunofluorescent staining 48 hours after intrastromal injection of TLR2-ligands Pam_3CSK_4 and Zymosan-A, and TLR3-ligand Poly(I:C). Consistent with its role as an immunomodulatory switch, *Slurp1* expression was decreased in Pam_3CSK_4, Poly(I:C), and zymosan-A-injected mouse corneas (Fig. 4A). Levels of *Klf4* were not affected by these agents, suggesting that the decrease in *Slurp1* expression during acute infections is not mediated through Klf4 (Fig. 4A). Immunofluorescent staining with anti-Slurp1 antibody confirmed the decrease in Slurp1 expression in Pam_3CSK_4-, Poly(I:C)-, or zymosan-A-injected corneas. Hypercellularity of Pam_3CSK_4-, Poly(I:C)-, or zymosan-A–injected corneal stromas

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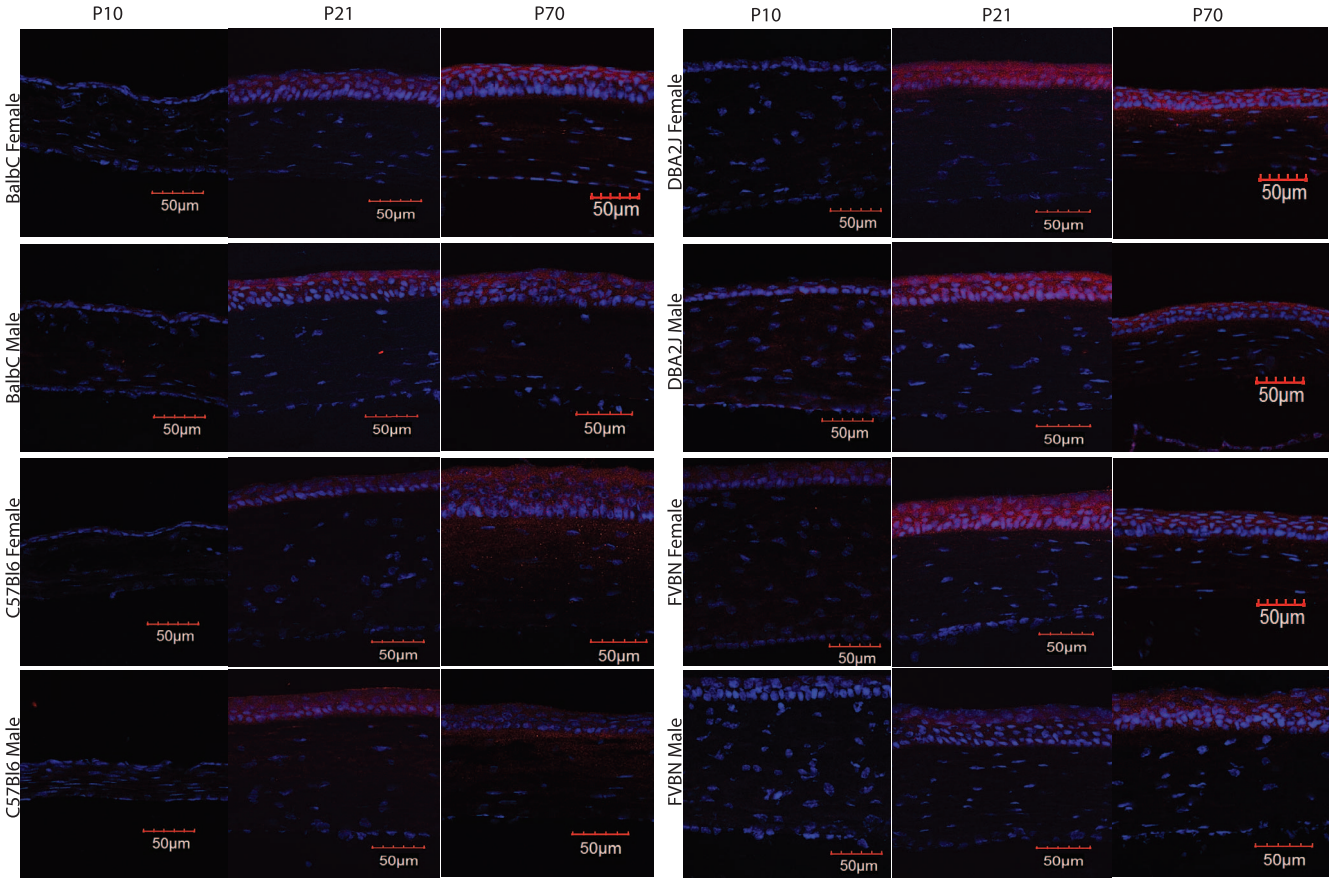

Figure 2. Mouse corneal expression of Slurp1. Immunofluorescent staining of cryosections from PN10, PN20, and PN70 male and female Balb/C, C57Bl/6, DBA/2J and FVBN mouse corneas with anti-Slurp1 antibody revealed comparable expression between different sexes and genetic backgrounds.

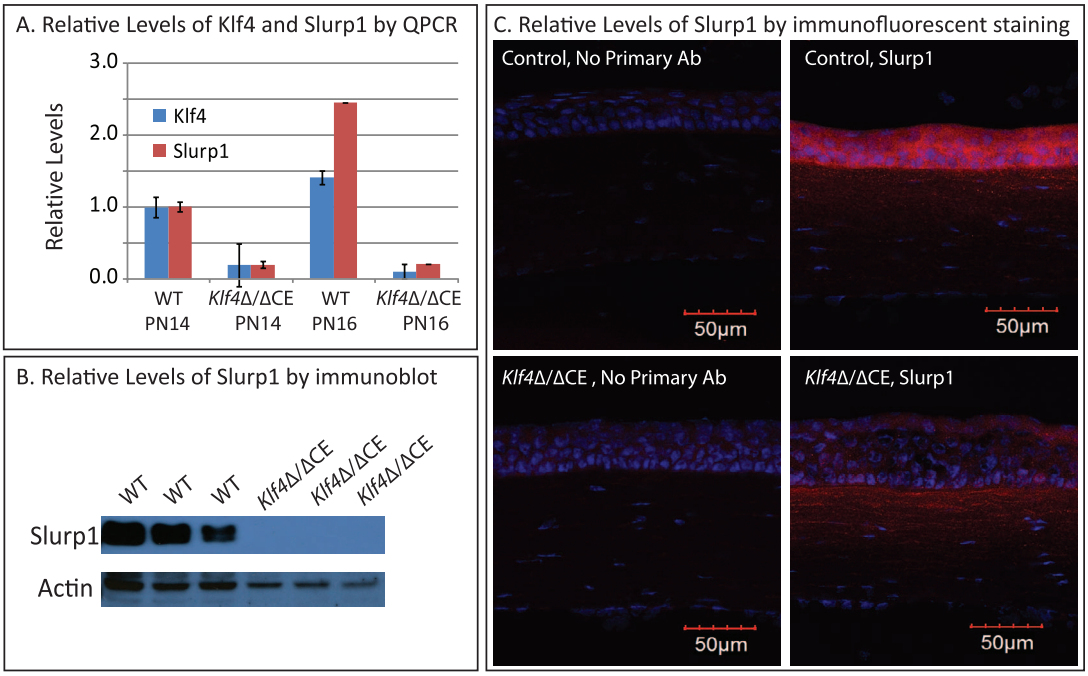

Figure 3. Post–eyelid opening increase in corneal expression of *Slurp1* is Klf4-dependent. (**A**) Relative levels of *Klf4* and *Slurp1* transcripts in PN14 and PN16 WT and $Klf4^{\Delta/\Delta CE}$ mouse corneas by QPCR. (**B**) Relative levels of Slurp1 protein in PN20 WT and $Klf4^{\Delta/\Delta CE}$ mouse corneas by immunoblot. (**C**) Relative levels of Slurp1 expression in PN70 WT and $Klf4^{\Delta/\Delta CE}$ mouse corneal epithelium by immunofluorescent staining.

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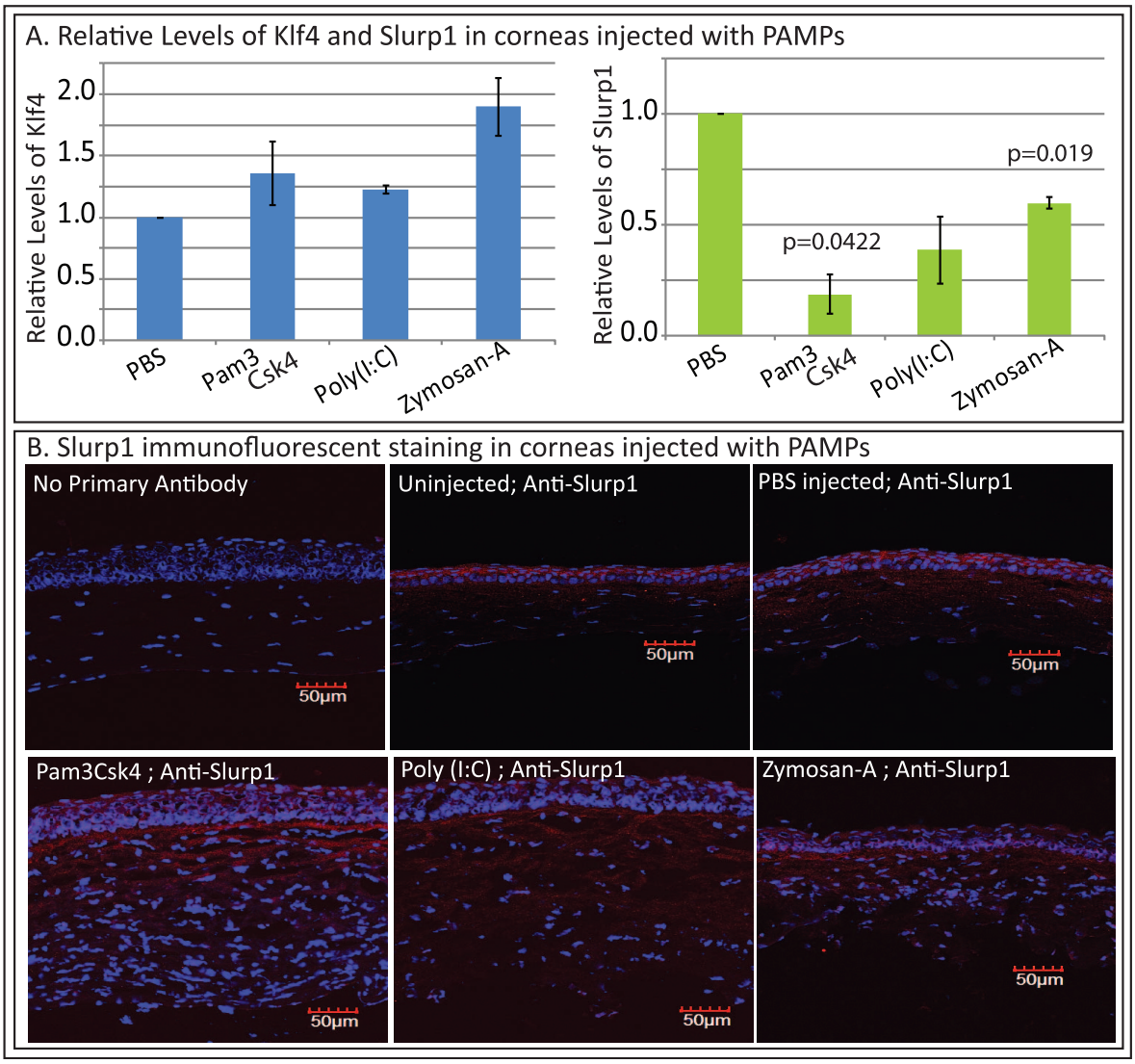

Figure 4. Slurp1 expression decreases in proinflammatory conditions in a Klf4-independent manner. (**A**) Quantitative PCR revealed that *Slurp1*, but not *Klf4*, levels are decreased in Pam_3Csk_4, Poly (I:C), or zymosan-A–injected mouse corneas compared with those injected with PBS. *P* values are shown where significant. (**B**) Expression of Slurp1 is decreased in mouse corneas 48 hours after intrastromal injections of different PAMPs compared with the uninjected or those injected with PBS, as determined by immunofluorescent staining with anti-Slurp1 antibody.

suggested influx of immune cells, confirming efficient generation of proinflammatory conditions by these PAMPs (Fig. 4B).

Slurp1 Expression Is Decreased in Migrating Cells at the Wound Edge

Considering the inhibitory effects of SLURP1 on migration of human corneal limbal epithelial (HCLE) cells,[15] mouse corneal stromal MK/T-1 cells,[15] and dermal keratinocytes,[32] we hypothesized that Slurp1 expression is suppressed at the corneal epithelial wound edge facilitating their rapid migration. Transcript levels of *Slurp1* did not vary significantly in corneas subjected to minor crisscrossing epithelial scratch wounds after 6 hours of wounding (Fig. 5A). However, immunofluorescent staining revealed decreased expression of Slurp1 in the migrating cells at the edge of a larger, 1.5-mm diameter epithelial debridement wound generated using an Alger brush at 6 hours post wounding, compared with the cells farther away from the wound, or the unwounded central corneal epithelium (Fig. 5B). Thus, Slurp1 expression was not altered in response to minor epithelial scratch wounds while it was decreased in migrating cells at the wound edges in severe epithelial debridement wounds. Taken together with our previous results,[15] these data confirm that Slurp1 expression is decreased in cells at the wound edge facilitating their rapid migration.

SLURP1 Expression in Human Tears

Previous studies have not established a baseline for SLURP1 expression in the human tears. Considering that a number of tear proteomics studies failed to identify SLURP1 protein in human tears it was not clear if human SLURP1 also is decreased in proinflammatory conditions. In order to overcome this ambiguity, we tested if SLURP1 expression in human tears is influenced by sex, and/or ocular surface health conditions. Tear samples were collected from 34 individuals using absorbent wicks following a University of Pittsburgh Institu-

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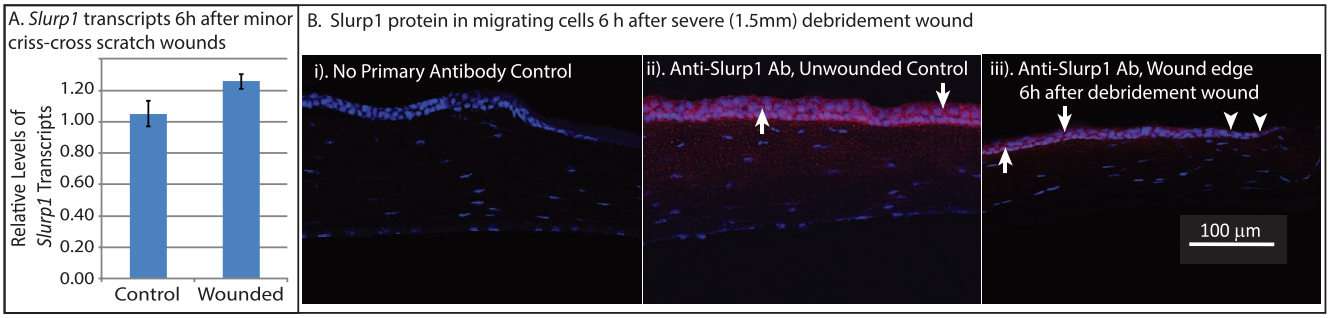

Figure 5. Expression of Slurp1 in wounded corneas. (**A**) Transcripts of *Slurp1* 6 hours after minor scratch wounds. Relative expression of *Slurp1* transcripts was quantified by QPCR 6 hours after introducing minor epithelial scrape wounds by six crisscross strokes of a dulled 26-G needle in anesthetized mouse corneas. (**B**) Expression of Slurp1 in migrating cells at the corneal epithelial wound edge 6 hours after severe debridement wound. Adult (PN70) mice were anesthetized, central corneal 1.5-mm-diameter area demarcated by trephine blades, and gently debrided with an Alger brush. After 6 hours, mice were euthanized and 8-µm-thick cryosections from central corneas were probed with anti-Slurp1 antibody. Immunofluorescent staining with anti-Slurp1 antibody revealed decreased expression of Slurp1 in the migrating cells at (**iii**) the wound edge (*arrowheads*), compared with (**iii**) the distal areas (*arrow*) or (**ii**) the unwounded control corneas. No primary antibody control is shown (**i**).

tional Review Board (IRB)–approved protocol as earlier.[31] In a blinded study, tear protein concentration was quantified by BCA method, equal amounts (10 µg) total protein were separated by SDS-PAGE, transferred to PVDF membranes, and probed with anti-human SLURP1 antibody. Immunoblots revealed that the SLURP1 expression is not influenced by sex and the tear samples from healthy ocular surface contained easily detectable levels of SLURP1 unlike those from inflamed ones where SLURP1 was undetectable, suggesting that SLURP1 is sharply decreased in inflamed ocular surfaces (Fig. 6A).

Next, we established standard curve-based ELISA to quantify the amount of SLURP1 in human tears. Plate-to-plate variability in ELISAs was overcome by the inclusion of internal standards (stepwise increments from 0.1 to 6.0 ng partially purified recombinant SLURP1 per well; Fig. 6B), which routinely resulted in standard curves with R^2 values exceeding 0.975 (Fig. 6B). Consistent with the immunoblots, ELISAs revealed that the tears from inflamed eyes contained significantly decreased amounts of SLURP1 (mean 0.34 ng/100 ng tear protein) compared with those from healthy individuals (mean

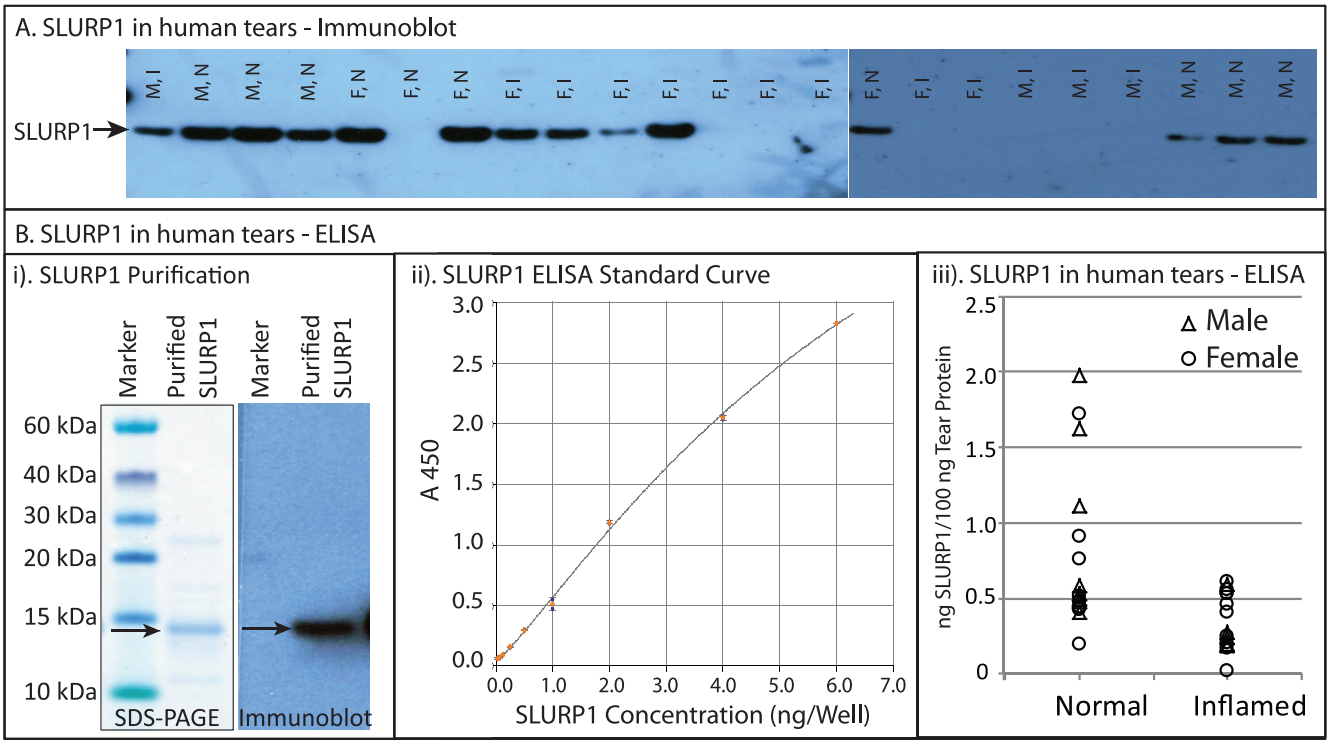

Figure 6. Expression of SLURP1 in human tears. (**A**) Immunoblots; equal amount (10 µg) tear protein from male and female subjects with healthy or inflamed ocular surface was separated by SDS-PAGE, transferred to PVDF membrane and subjected to immunoblot with anti-SLURP1 antibody. FI, female, inflamed ocular surface; FN, female, normal ocular surface; MN, male, normal ocular surface; MI, male, inflamed ocular surface. (**B**) Quantification of SLURP1 levels in human tears by ELISA. (**i**) We purified 6X His-tagged recombinant human SLURP1 by Ni-ion column chromatography, and (**ii**) used to generate the standard curve for ELISA. (**iii**) Expression of SLURP1 in adult human tears collected from normal or inflamed ocular surface was quantified by ELISA and presented as ng SLURP1/100 ng total tear protein.

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0.77 ng/100 ng tear protein; Fig. 6B). ELISAs also revealed that the human tear SLURP1 levels do not vary significantly between sexes (Fig. 6B).

Discussion

The cornea is considered both "immune privileged" and "immune competent," as it employs diverse pathways to suppress inflammation in response to mild insults, while retaining the ability to mount protective inflammation in response to severe insults.[33–35] The delicate balance between corneal immune privilege and competence is maintained by a variety of molecules and pathways.[33–46] Our previous work demonstrated that Slurp1 is a constitutive component of corneal immune privilege that inhibits leukocytic infiltration into the cornea in response to mild insults, and is rapidly downregulated when the cornea becomes infected, permitting protective inflammation to develop.[14] In this report, we: (1) provide a detailed description of the influence of age, sex, and genetic background on Slurp1 expression in the mouse cornea; (2) reveal that Slurp1 expression is decreased in response to different PAMPs and in migrating cells at the epithelial wound edge; (3) demonstrate that the post–eyelid opening increase in *Slurp1* expression is regulated by Klf4; (4) establish the baseline for SLURP1 concentration in human tears; and (5) demonstrate that the tears from inflamed human ocular surface contain decreased amounts of SLURP1. Together, these results establish SLURP1 as an important tear film component that needs to be further evaluated as a diagnostic marker and/or therapeutic target for inflammatory disorders of the ocular surface.

Tear fluid is a complex solution of hundreds of proteins, carbohydrates, lipids, and various ions.[47–50] Although advances in proteomics technologies have improved our understanding of the chemical composition of the tear fluid, large gaps remain in our knowledge. Recent proteomic analyses performed with tears from healthy ocular surfaces[48] or from patients with type-2 diabetes and dry eye syndrome,[51] Sjogren syndrome,[52] keratoconus[53] failed to detect SLURP1 in the tear fluid, likely due to its small size (~8 kDa) and relatively lower abundance (~0.5 ng/100 ng total tear protein). In contrast, lysozyme and lacritin, two of the major tear proteins, correspond to approximately 20% and 4% of the soluble proteins in human tears, respectively.[31] In addition, although SLURP1 is known to be secreted to tear film, it is possible that only a fraction of the protein is secreted, with a significant fraction remaining intracellular. Intense staining for SLURP1 in corneal epithelial cells is consistent with this possibility.[14]

Tear composition changes with age, and is influenced by sexes. For example, the expression of several tear proteins including PLA2G2A,[54] peroxidase,[55] lysozyme,[56] and lactoferrin[47,57] changes with age. Similarly, tear proteins lacritin, lipocalin, haptoglobin, mammoglobin B precursor, cystatin S precursor, and anti-α1 trypsinogen are expressed at a relatively higher level in female tears.[58] In contrast, our results suggest that SLURP1 expression is significantly influenced only by the ocular surface health condition, and not by the age or the sex. Considering that the human subjects' sample size employed in this study is relatively small, it would be necessary to perform a more extensive study with a larger sample size to draw firm conclusions on the lack of influence of age and sex on tear levels of SLURP1.

As Slurp1 transcript and protein levels depend on Klf4, go up with cellular differentiation, go down with inflammation, and are not influenced by the sex or the genetic background, *Slurp1* transcript levels appear to be predictive for the corresponding protein levels. However, high abundance of *Slurp1* transcripts in the adult mouse cornea[18,19] is not commensurately reflected at the corresponding protein level,[59–63] revealing a potential discord between relative abundance of Slurp1 transcript and protein. Whether this discord between Slurp1 transcript and protein levels is an outcome of relatively higher transcription and stability of *Slurp1* transcripts, and/or rapid turnover of Slurp1 protein remains to be determined.

In summary, this report coupled with our previous study[14] reveals that Slurp1 expression is decreased in proinflammatory conditions regardless of the cause for inflammation. Although the present study also revealed that SLURP1 expression is decreased in human tears from inflamed ocular surfaces, it did not consider the specific cause for inflammation. It would be important to do so in future studies involving a larger number of subjects with well-defined causes of ocular surface inflammation, to firmly establish the value of SLURP1 as a diagnostic marker for inflammatory disorders of the ocular surface. It would also be important to examine if the age of the subjects and/or the time of the day when the tears are collected has any influence on human tear SLURP1 levels, as demonstrated for other proteins.[64–66]

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