Klf4 Regulates the Expression of Slurp1, Which Functions as an Immunomodulatory Peptide in the Mouse Cornea

Sudha Swamynathan,^{1,9} Kristine-Ann Buela,^{1,2,9} Paul Kinchington,^{1,3-5} Kira L. Lathrop,^{1,6} Hidemi Misawa,⁷ Robert L. Hendricks,^{1,3-5} and Shivalingappa K. Swamynathan^{1,5,8}

PURPOSE. The secreted Ly6/uPAR-related protein-1 (Slurp1), associated with the hyperkeratotic disorder mal de Meleda, is abundantly expressed in corneas. Here, we examine its corneal expression and functions.

METHODS. Gene expression was quantified by quantitative PCR (qPCR), immunoblots, and immunofluorescent staining. Effect of Kruppel-like factor 4 (Klf4) on *Slurp1* promoter was evaluated by chromatin immunoprecipitation (ChIP) and transient transfections. Adenoviral vectors were used to express Slurp1 in corneas. Leukocytic infiltration in bacterial lipopolysaccharide (LPS)-, herpes simplex virus type 1 (HSV-1)-, or adenovirus (serotype 5)-treated mouse corneas was characterized by flow cytometry.

RESULTS. Corneal expression of *Slurp1* increased sharply upon mouse eyelid opening, concurrent with the elevated expression of *Klf4*. *Slurp1* was significantly decreased in *Klf4* conditional null (*Klf4*CN) corneas that displayed elevated expression of cytokines and cytokine receptors, as well as neutrophil influx consistent with a proinflammatory environment. In additional models of corneal inflammation, *Slurp1* expression was abrogated within 24 hours of LPS injection or HSV-1 or adenoviral infection, accompanied by a predominantly neutrophilic infiltrate. Neutrophilic infiltration was enhanced in HSV-1-infected *Klf4*CN corneas lacking Slurp1. *SLURP1* promoter activity was stimulated by KLF4, suppressed by IL-4, IL-13, and TNF α , and unperturbed by IFN- γ . *Slurp1* downregulation and neutrophil influx were comparable in HSV-1-infected wild-type (WT) and *Ifng*^{-/-} mouse corneas. Mouse corneas infected with Slurp1-expressing adenoviral vectors displayed reduced signs of inflammation and restricted

neutrophilic infiltration compared with those infected with control vectors.

CONCLUSIONS. Klf4 regulates the expression of *Slurp1*, a key immunomodulatory peptide that is abundantly expressed in healthy corneas and is downregulated in proinflammatory conditions. (*Invest Ophthalmol Vis Sci.* 2012;53:8433-8446) DOI:10.1167/iovs.12-10759

The secreted Ly6/urokinase plasminogen activator receptor-related protein-1 (Slurp1) belongs to the Ly6/uPAR superfamily of proteins that participate in signal transduction, immune activation, and cell adhesion and are characterized by the presence of three-finger structure generated by five disulfide bridges.¹⁻³ It is expressed in a variety of cells including immune cells,⁴ bronchial epithelial cells,⁵ primary sensory neurons,⁶ skin, exocervix, gums, stomach, trachea and esophagus,⁷ oral keratinocytes,⁸ and cornea.⁹ In the skin, SLURP1 is abundantly expressed in the keratinocytes underlying the stratum corneum. *Slurp1* is one of the most abundant transcripts in neonatal and adult mouse corneas.⁹ Human *SLURP1* mRNA expression is regulated by retinoic acid, epidermal growth factor, and IFN- γ .⁷

As a secreted protein, Slurp1 is detected in many bodily fluids, including plasma, saliva, sweat, tears, and urine, and is considered a late marker of epidermal differentiation.¹⁰ Slurp1 is thought to fine-tune the physiologic regulation of keratinocyte functions through the cholinergic pathways, as it is structurally similar to snake and frog cytotoxin α -bungarotoxin and acts as a ligand for the $\alpha 7$ subunit of nicotinic acetylcholine receptors ($\alpha 7$ nAChRs).^{7,11} Slurp1 is involved in signal transduction, activation of the immune response, and cell adhesion, preventing tobacco nitrosamine-induced malignant transformation of oral cells.^{1,4,8,12,13} Mutations or deletions in *SLURP1* gene have been implicated in a rare autosomal recessive inflammatory disorder called mal de Meleda, characterized by diffuse palmoplantar keratoderma and transgressive keratosis with an onset in early infancy.¹⁴⁻¹⁸ We identified *Slurp1* as one of the most downregulated transcripts in the Kruppel-like factor 4 (*Klf4*) conditional null (*Klf4*CN) corneas that displayed several signs of inflammation, including epithelial fragility, loss of epithelial barrier function, and stromal edema.¹⁹⁻²¹ The ocular surface functions of Slurp1 have not been investigated in spite of its abundance in the cornea⁹ and its presence in tear film.¹⁰

The cornea is an immune-privileged tissue that tolerates mild insults without eliciting acute inflammatory response. A variety of molecules contribute to corneal immune privilege by suppressing inflammation in response to mild insults.²²⁻³⁵ A delicate balance exists between immune privilege and immune competence that allows protective inflammation to progress by rapidly downregulating such protective molecules in response to acute infections and injury. *Slurp1* expression is

From the ¹Department of Ophthalmology, ²Graduate Program in Immunology, ³Department of Immunology, ⁴Department of Microbiology and Molecular Genetics, ⁵McGowan Institute of Regenerative Medicine, ⁶Department of Bioengineering, and the ⁸Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, Pennsylvania; and the ⁷Department of Pharmacology, Faculty of Pharmacy, Keio University, Tokyo, Japan.

⁹These authors contributed equally to the work presented here.

Supported by NIH Grants K22 EY016875 (SKS), EY10359 (RLH), and P30 EY08098 (RLH); by unrestricted grants from Research to Prevent Blindness and the Eye and Ear Foundation of Pittsburgh; and by startup funds from the Department of Ophthalmology, University of Pittsburgh (SKS).

Submitted for publication August 13, 2012; revised October 22, 2012; accepted November 2, 2012.

Disclosure: S. Swamynathan, None; K.-A. Buela, None; P. Kinchington, None; K.L. Lathrop, None; H. Misawa, None; R.L. Hendricks, None; S.K. Swamynathan, None

Corresponding author: Shivalingappa K. Swamynathan, University of Pittsburgh School of Medicine, Eye and Ear Institute, 203 Lothrop Street, Room 1025, Pittsburgh, PA 15213; Swamynathansk@upmc.edu.

rapidly downregulated in several proinflammatory conditions including suture- or alkali burn-induced corneal neovascularization,³⁶ asthmatic lungs,³⁷ Barrett's esophagus, adenocarcinomas, malignant melanomas, cervical cancer, and oral squamous cell carcinomas (National Center for Biotechnology Information [NCBI] GEO accession numbers GSE23347, GDS1321, GDS3472, GDS1375, and GDS1584; <http://www.ncbi.nlm.nih.gov/gds>), suggesting an immunomodulatory role for Slurp1. Association of *SLURP1* with the inflammatory disorder mal de Meleda is consistent with such a role.^{7,10,11,13–18} Here, we explore the regulation of expression and functions of Slurp1 in the mouse cornea. Our results suggest that Slurp1 is a constitutively produced 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.

MATERIALS AND METHODS

Mice

*Klf4*CN mice were generated on a mixed background by mating *Klf4^{loxP/loxP}, Le-Cre^{-/-}* mice with *Klf4^{loxP/loxP}* mice to obtain roughly equal proportion of *Klf4^{loxP/loxP}, Le-Cre^{-/-}* (*Klf4*CN) and *Klf4^{loxP/loxP}* (wild-type control siblings) offspring as described earlier.²¹ Wild-type (WT) and IFN- γ knockout (GKO) BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were 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.

Lipopolysaccharide Injection, Herpes Simplex Virus Type 1 (HSV-1), or Adenoviral Infection of Mouse Corneas

Mice were anesthetized by intraperitoneal injection of 2.0 mg ketamine hydrochloride and 0.04 mg xylazine (Phoenix Scientific, St. Joseph, MO) in 0.2 mL Hanks' balanced salt solution (Cambrex, Charles City, IA). The corneas of anesthetized mice were abraded 10 times in a crisscross fashion followed by topical application of 3 μ L RPMI 1640 (Cambrex) alone (mock infected) or RPMI 1640 containing 1×10^5 plaque-forming units (PFU) of wild-type HSV-1 RE (HSV-1 infected).³⁸ Intrastromal injections of ultrapure bacterial lipopolysaccharide (20 μ g in 2 μ L sterile water/cornea; LPS; Sigma-Aldrich Co., St. Louis, MO) were performed using fine-tipped Hamilton syringes (Hamilton, Reno, NV) in tunnels generated by 32-gauge syringe needles.

Adenoviral vectors expressing Slurp1 were constructed in the Adeno-X Expression System (serotype 5; Clontech Laboratories, Mountain View, CA) and amplified in HEK293 cells; 2×10^6 PFU of Adv5-*Tet-Off* alone (control) or 10^6 PFU each of Adv5-*Tet-Off* and Adv5-*Slurp1* were used per abraded cornea in mice anesthetized as explained above. Slit-lamp biomicroscope images were collected from anesthetized mice using SL-130 slit lamp (Carl Zeiss Meditec, Dublin, CA) equipped with a digital camera (Canon Powershot G11; Canon USA, Lake Success, NY). Corneas were harvested at 4 days postinfection (DPI) and immersed in 1 $\times$ PBS-EDTA for 15 minutes at 37°C to remove overlying epithelium for isolating total RNA for quantitative PCR (qPCR). Stromal leukocytes were isolated, labeled, and characterized by flow cytometry as described below.

Isolation of Total RNA and qPCR

Corneas were excised from normal (noninfected) mice or at 6, 12, 24, or 48 hours after mock infection or HSV-1 corneal infection and soaked in RNAlater to preserve the RNA integrity. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). Unless otherwise

mentioned, Applied Biosystems (Foster City, CA) was the source for the reagents, equipment, and software for TaqMan gene expression quantitative real-time PCR assays (qPCR). qPCR assays with prestandardized gene-specific probes for different transcripts were performed in ABI StepOnePlus thermal cycler using 18S rRNA or laminin-B1 as endogenous control (to avoid skewing of the results due to the 18S rRNA from infiltrating immune cells in the inflamed corneas). Expression levels of different cytokines were quantified using the mouse cytokine PCR array following the protocol suggested by the manufacturer (SuperArray Bioscience, Frederick, MD).

Effect of Cytokines on *KLF4* and *SLURP1* Expression

cDNA was synthesized using 1 μ g total RNA isolated from human corneal-limbal epithelial (HCLE) cells³⁹ treated with IL-2 (1 ng/mL; R&D Systems, Minneapolis, MN), IL-4 (0.5 ng/mL; PeproTech, Rocky Hill, NJ), IL-13 (5 ng/mL; PeproTech), IFN- γ (1 ng/mL; Chemicon, Billerica, MA), or TNF α (0.2 ng/mL; Fisher Scientific, Pittsburgh, PA) for 2 days. qPCR was performed with *KLF4*- and *Slurp1*-specific probes and TaqMan reagents (Applied Biosystems, Carlsbad, CA) using 18S rRNA as endogenous control.

Immunoblots

Equal amounts of total protein extracted by homogenizing dissected corneas in urea lysis buffer (8.0 M urea, 0.08% Triton X-100, 0.2% SDS, 3% β -mercaptoethanol, and proteinase inhibitors) and quantified by the bicinchoninic acid method (Pierce, Rockford, IL) were electrophoresed in SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and subjected to immunoblot analysis. Rabbit anti-mouse Slurp1⁶ (1:1000 dilution) and goat anti-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) (1:1000 dilution) antibody were used as primary antibodies in phosphate buffered saline (PBS) with 0.1% Tween-20 (PBST). Horseradish peroxidase-coupled goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) (1:2000 dilution) or rabbit anti-goat IgG (Kirkegaard & Perry, Gaithersburg, MD) (1:5000 dilution) was used as secondary antibody. Immunoreactive bands were detected by chemiluminescence using Super Signal West Pico solutions (Pierce).

Immunofluorescent Staining of Corneal Cryosections

Cryosections (8–10 μ m thick) from optimal cutting temperature (OCT) compound-embedded mouse eyes or human central corneas were fixed in fresh 4% paraformaldehyde in PBS for 30 minutes, blocked for 1 hour at room temperature with 10% goat serum in PBST, washed twice with PBST for 5 minutes each, incubated overnight at 4°C with 1:500 dilution of rabbit anti-mouse Slurp1 primary antibody^{4,6} or 1:50 dilution of goat anti-human SLURP1 antibody (Santa Cruz Biotechnology Inc.), washed thrice with PBST for 10 minutes each, incubated with secondary antibody (Alexa Fluor 546-coupled goat anti-rabbit IgG or Alexa Fluor 488-coupled donkey anti-goat IgG; Molecular Probes, Carlsbad, CA) at a 1:500 dilution for 1 hour at room temperature, rinsed with PBS, incubated with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes, washed thrice with PBST for 5 minutes each, mounted with Aqua Polymount (Polysciences, Inc., Warrington, PA), and observed. Images were collected with an Olympus IX81 microscope and Olympus FluoView 1000 confocal system (both from Olympus, Center Valley, PA). All images presented within each composite figure were acquired under identical settings and processed in a similar manner using Adobe Photoshop and Illustrator (Adobe, Mountain View, CA).

Immunofluorescent Staining of Corneal Whole Mounts

Corneas were dissected, flattened by three radial incisions, washed three times for 15 minutes each in PBS with 4% fetal bovine serum

(FBS), and blocked in Fc Block (BD Pharmingen, San Jose, CA) for 20 minutes prior to incubation with FITC-conjugated anti-CD45 antibody (BD Pharmingen, San Jose, CA) overnight at 4°C. Corneas were then washed three times each for 30 minutes in PBS/4% FBS, fixed in 1% paraformaldehyde for 2 hours at 4°C, rinsed three times again for 30 minutes each in PBS/4% FBS, and mounted in Aqua-Poly/Mount (Polysciences, Inc.). Images were acquired on an Olympus FluoView 1000 confocal system with an Olympus IX81 microscope (both from Olympus). Stacks were imaged at 20× with numerical aperture (NA) of 0.85 and 60× (NA 1.42) and maximum intensity projections were imaged through the stromal portion of the stack.

Flow Cytometry

Corneas were excised 48 hours after mock or HSV-1 corneal infection and immersed in 1× PBS-EDTA for 15 minutes at 37°C to remove overlying epithelium. The corneal stroma was digested in collagenase type 1 (840 U/cornea; Sigma-Aldrich Co.) for 1 hour at 37°C. Single-cell suspensions were generated by trituration and filtered through a 40 µm cell strainer cap (BD Labware, Bedford, MA). Suspensions were incubated with anti-mouse CD16/CD32 (Fcγ III/II receptor, clone 2.4G2; BD Pharmingen, San Diego, CA) and then stained with fluorochrome-conjugated antibodies to various surface markers for 30 minutes at 4°C. The following antibodies and their corresponding isotypes were used for phenotypic analysis: PerCP-conjugated anti-CD45 (clone 30-F11; BD Pharmingen, San Diego, CA), APC-conjugated anti-Gr-1 (clone RB6-8C5; Caltag Laboratories, Burlingame, CA), and eFluor450-conjugated anti-CD11b (clone M1/70; eBioscience, San Diego, CA). After staining, cells were fixed with 1% paraformaldehyde and mixed with CountBright absolute counting beads (10,000 beads/sample; Invitrogen). Data were collected on a FACSAria cytometer and analyzed by FACSDiva software (BD Biosciences, San Jose, CA). A gate was established to stop acquiring events after collecting 80% of the beads. The absolute number of each cell type per cornea was determined by calculating the number of events shown in the gate, multiplied by a factor of 1.25.

Chromatin Immunoprecipitation (ChIP)

ChIP was based on the EZ-ChIP protocol (Upstate, Inc., Charlottesville, VA). Human corneal epithelial (HCE)⁴⁰ cell chromatin was cross linked with 1% paraformaldehyde, purified, sheared to 200 to 1000 bp fragment size by sonication, and immunoprecipitated with preimmune serum or anti-Klf4 antibody (Santa Cruz Biotechnology Inc.) and protein G sepharose. Following reversal of cross linking by heating overnight at 65°C in the presence of NaCl and purification of eluted DNA, *Slurp1* promoter fragments were detected by PCR with −396F (5′-TTTATCAGGC AGGCAGATAT AAAGC-3′) and +30R (5′-ATTCTT CAGT GCTCAGGAGC T-3′) primers.

Reporter Vectors, Cell Culture, and Promoter Activities

Reporter vectors were generated in pGL3Basic (Promega, Madison, WI) by cloning the *Slurp1* promoter fragments amplified using the following primers: −500F: 5′-ACA TCA GGT ACT CCC TCC T-3′, −150F: 5′-GGC CCC ACC CTG GGA TGG TAG GTG A-3′, and +30R: 5′-TCT TCA GTG CTC AGG AGC TAG GA-3′. Transient expression of Klf4 was achieved using cytomegalovirus (CMV) promoter in pCI-Klf4. Human keratinocyte National Collection of Type Cultures (NCTC) cells and SV40-transformed corneal epithelial (HCE) cells⁴⁰ were grown in six-well plates as described earlier¹⁹ and transfected with 0.5 µg reporter vector pSlurp1-Luc, 10 ng pRL-SV40 (Promega, for normalization of transfection efficiency), and 0.5 µg pCI or pCI-Klf4, using 3 µL FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). Transient knockdown of *KLF4* expression in HCE³⁹ cells was achieved using plasmids expressing anti-KLF4 small interfering RNA (siRNA) as described earlier (SuperArray Bioscience).¹⁹ HCE cells in six-well

plates were cotransfected with 1.0 µg control or anti-KLF4 siRNA plasmid, 0.5 µg reporter vector (−500/+27 bp *Slurp1*-Luc), and 15 ng pRL-SV40 (Promega, for normalization of transfection efficiency) using 4.5 µL Lipofectamine 2000 (Invitrogen). After 2 days of transfection, cells were lysed with 500 µL passive lysis buffer, and 50 µg protein in the supernatant was analyzed using a dual-luciferase assay kit (Promega) and a Synergy-II microplate luminometer (Biotek Instruments, Winooski, VT) as described earlier.¹⁹ Results from three independent experiments, normalized for transfection efficiency using the SV40 promoter-driven *Renilla* luciferase activity, were used to obtain mean promoter activities and standard deviations. Fold activation was determined by dividing mean promoter activity in the presence of pCI-Klf4 by the promoter activity in the absence of pCI-Klf4.

RESULTS

Corneal Expression of *Slurp1*

qPCRs revealed, and immunofluorescent staining confirmed, that *Slurp1* expression was increased by more than 15-fold between postnatal day 11 (PN11) and PN21 (Figs. 1A, 1B), suggesting a critical role for *Slurp1* in post-eyelid opening stages when the cornea is first exposed to the environment. Though *Slurp1* was detected in all layers of the cornea, its expression was much higher in the epithelium (Fig. 1B), consistent with the previous *in situ* hybridization data.⁹ The spatial distribution of *Slurp1* in human corneas was comparable to that in the mouse corneas, albeit with a little higher expression in the stroma, as demonstrated by immunofluorescent staining of human corneal sections from a healthy 52-year-old male organ donor (Fig. 1C). Together, these results reveal that *Slurp1* is expressed at high levels in mature mouse and human corneas, with comparable tissue distribution (Fig. 1).

Klf4 Binds and Upregulates *Slurp1* Promoter Activity

The increase in *Slurp1* expression during post-eyelid opening stages is concurrent with an increase in the expression of Klf4 (Fig. 2A), which plays critical roles in maturation and maintenance of the mouse ocular surface,^{19–21} raising the possibility that Klf4 regulates *Slurp1* expression. Consistent with this, the previous microarray data²⁰ revealed, and the current qPCR, immunoblots, and immunofluorescent staining confirmed, a significant decrease in *Slurp1* expression in Klf4CN compared with the WT corneas (Figs. 2B–D). Taken together, these results suggest that Klf4 regulates the sharp increase in post-eyelid opening expression of SLURP1.

In order to directly test if Klf4 regulates *Slurp1* promoter activity, we cotransfected the luciferase reporter vectors driven by mouse *Slurp1* promoter (−500/+27 bp or −150/+27 bp fragments; Fig. 3A) with increasing amounts of the empty control vector pCI or the expression vector pCI-Klf4 in HCE or skin keratinocyte (NCTC) cells. Activities of both −500/+27 bp and −150/+27 bp *Slurp1* promoter fragments were increased upon cotransfection with pCI-Klf4 (Figs. 3B, 3C), suggesting that the Klf4-responsive elements are located within the −150/+27 bp *Slurp1* proximal promoter. In addition, specific siRNA-mediated knockdown of *KLF4* resulted in reduced −500/+27 bp *Slurp1* promoter activity relative to that obtained with cotransfection of control siRNA-expressing plasmids in HCE cells (Fig. 3D). ChIP assays demonstrated that the −396/+30 bp SLURP1 promoter fragment is bound by Klf4 in HCE cells (Fig. 3E). Finally, examination of the SLURP1 promoter sequence revealed the presence of several potential KLF4-binding sites (GC-rich regions with a core sequence CACCC⁴¹)

FIGURE 1. Corneal expression of *Slurp1*. (A) qPCR demonstrating post-eyelid opening increase in *Slurp1* expression. *Slurp1* expression increases more than 15-fold between PN11 and PN21. (B) Immunofluorescent staining of PN11, PN21, and PN56 mouse corneas showing elevated expression of *Slurp1* (red) in corneal epithelium in post-eyelid opening stages. (C) Immunofluorescent staining demonstrating expression of SLURP1 (green) in human corneas. Postmortem corneal sections from a healthy 52-year-old male organ donor were used. Nuclei are stained with DAPI (blue), and corresponding no-antibody controls are shown in (B, C). Signals emanating from the Descemet's membrane (IC, iv) appear to be due to autofluorescence, as they were detected in controls with no primary antibody (IC, iii) as well. Scale bars: 25 μ m in (B) and 50 μ m in (C).

within the $-500/+27$ bp fragment, many clustered within the $-150/+27$ bp region (Fig. 3F), consistent with the stimulation of the $-150/+27$ bp proximal promoter activity by Klf4. Additional controls, including primers spanning the nearby regions of *Slurp1* gene not bound by Klf4 in ChIP assays, and direct examination of Klf4 binding by electrophoretic mobility shift assays will be required to precisely map the Klf4-responsive cis-elements in the *Slurp1* proximal promoter. Taken together, these results demonstrate that Klf4 binds and upregulates *Slurp1* proximal promoter activity.

Inflammatory Environment in *Klf4*CN Corneas

Considering that deletions or mutations in *SLURP1* cause the autosomal recessive inflammatory disorder mal de Meleda^{7,10,11,13–18} and also that *Slurp1* expression is decreased in diverse proinflammatory conditions (including suture- or alkali burn-induced corneal neovascularization³⁶ [NCBI GEO acces-

sion number GSE23347; <http://www.ncbi.nlm.nih.gov/gds>], asthmatic lungs,³⁷ Barrett's esophagus, adenocarcinomas, malignant melanomas, cervical cancer, and oral squamous cell carcinomas [NCBI GEO accession numbers GDS1321, GDS3472, GDS1375, and GDS1584; <http://www.ncbi.nlm.nih.gov/gds>]), we tested if downregulation of *Slurp1* is accompanied by proinflammatory conditions in *Klf4*CN corneas. qPCR revealed that the expression of *Ifng*, 19 different chemokines, 8 chemokine receptors, 5 interleukins, and 5 interleukin receptors is upregulated by more than 4-fold in the *Klf4*CN compared with the WT corneas (Tables 1 and 2), indicating a proinflammatory environment in *Klf4*CN corneas. Furthermore, *Klf4*CN corneas displayed a significantly higher density of bone marrow-derived CD45⁺ cells in comparison with the WT corneas (Fig. 4). While CD45⁺ cells were sparsely and evenly distributed throughout the WT corneal stromas, they were present in large numbers in discrete clusters in the

FIGURE 2. Downregulation of Slurp1 expression in the *Klf4CN* cornea. (A) Changes in *Klf4* expression during mouse corneal development. Absolute numbers of *Klf4* transcripts per nanogram total RNA were calculated using the standard curve method of qPCR with total RNA from mouse corneas at different stages of development. (B) *Slurp1* transcript levels in the WT and *Klf4CN* corneas measured by microarray²⁰ and qPCR. (C) Immunoblot with rabbit anti-mouse Slurp1 antibody detects a strongly reacting band at approximately 21 kDa in the WT, but not in *Klf4CN* corneal extracts (left panel). The blot was stripped of the primary antibody and reprobed with anti-actin antibody to ensure equal loading of protein (right panel). (D) Immunofluorescent staining with anti-Slurp1 antibody. Left panel, WT with no primary antibody; middle panel, WT with anti-Slurp1 antibody; right panel, *Klf4CN* with anti-Slurp1 antibody.

Klf4CN corneas (Fig. 4). Thus, *Klf4CN* corneas lacking Slurp1 exhibited a marked proinflammatory environment.

***Slurp1* Expression Is Reduced in Inflamed Corneas**

In order to determine if reduced expression of *Slurp1* is a common theme in inflamed corneas, we examined additional models of corneal inflammation including HSV-1 infection and bacterial LPS injection. *Slurp1* was sharply decreased 1 day after HSV-1 infection or LPS injection, while *Klf4* was not affected (Fig. 5). *Klf4* was only partially reduced after 2 days in HSV-1-infected corneas, suggesting that the rapid reduction in *Slurp1* levels within 24 hours of LPS or HSV-1 treatment is not due to inadequate *Klf4* levels (Fig. 5). Immunofluorescent staining confirmed the decreased expression of Slurp1 in HSV-1-infected mouse corneas at both 1 and 2 DPI, and revealed that much of the decrease occurs within the corneal epithelium (Fig. 5B). Along with a decrease in Slurp1 expression, a greater influx of cells stained with DAPI was also observed after HSV-1 infection. Taken together with the previous gene expression studies (NCBI GEO accession numbers GSE23347, GDS1321, GDS3472, GDS1375, and GDS1584; <http://www.ncbi.nlm.nih.gov/gds>), these results demonstrate that the decreased expression of *Slurp1* is a common theme in inflammation independent of the nature of insults, and suggest an immunomodulatory role for Slurp1.

Reduced Expression of *Slurp1* Is Associated with Increased Neutrophilic Infiltration

Flow cytometric analysis 2 days after mock infection revealed significantly ($P = 0.02$) higher frequency of CD45⁺ cells in the *Klf4CN* compared with the WT corneas (Figs. 6A, 6B). The *Klf4CN* corneal CD45⁺ cells were predominantly neutrophilic in nature (CD11b⁺ Gr-1⁺) compared with the predominantly macrophage phenotype (CD11b⁺ Gr-1⁻) of WT corneal CD45⁺ cells (Figs. 6C, 6D). At 2 DPI, both WT and *Klf4CN* corneas exhibited elevated infiltration of bone marrow-derived CD45⁺ cells composed predominantly of CD11b⁺ Gr-1⁺ neutrophils, the frequency of which was significantly higher ($P < 0.0001$) in the *Klf4CN* corneas (Fig. 6). Although it did not reach statistical significance, there was a trend toward higher absolute numbers of neutrophils in the infected *Klf4CN* corneas (Table 3). Thus, HSV-1 infection overcomes the barrier to neutrophilic infiltration into WT corneas, but does so more effectively in *Klf4CN* corneas lacking Slurp1 from the time of infection, consistent with an immunomodulatory role for Slurp1.

***SLURP1* Expression Is Inhibited by Proinflammatory Cytokines IL-4, IL-13, and TNF- α , but Not IFN- γ**

Considering (1) that IFN- γ and IL-13 suppress *Slurp1*,^{7,37} (2) that many inflammatory cytokines, chemokine ligands, and

FIGURE 3. Klf4 binds and stimulates *Slurp1* promoter activity. (A) Schematic representation of the reporter vectors used. (B, C) Relative promoter activities of different-sized *Slurp1* promoter fragments with increasing amounts (0, 100, or 500 ng) of cotransfected pCl-Klf4 in HCE (B) and NCTC (C) cells. (D) Effect of siRNA-mediated knockdown of *KLF4* on -500/+27 bp *Slurp1* promoter activity in HCLE cells. *Slurp1* promoter activity was reduced upon knockdown of *KLF4* expression by two different siRNAs relative to that obtained with cotransfection of control siRNA expressing plasmid. (E) Chromatin immunoprecipitation was performed using HCE cells and anti-KLF4 antibody. PCR-amplified *SLURP1* proximal promoter fragments from the input chromatin (lanes 1, 2) or immunoprecipitated chromatin (lanes 3, 4) are shown. Lane 3, mock immunoprecipitated with no antibody; lane 4, immunoprecipitated with anti-KLF4 antibody. (F) Nucleotide sequence of *SLURP1* proximal promoter. Potential KLF4-interacting elements are underlined. Transcription and translation start sites are indicated.

chemokine receptors are upregulated in *Klf4*CN corneas lacking *Slurp1* (Tables 1 and 2; Fig. 2), and (3) that *Slurp1* was downregulated in HSV-1-infected and LPS-injected corneas in the presence of normal levels of *Klf4* (Fig. 5), we predicted that inflammatory cytokines would selectively inhibit *Slurp1* production without affecting *Klf4*. Consistent with this, treatment of HCLE cells with IL-4, IL-13, and TNF- α suppressed *Slurp1* production, while IFN- γ did not affect *Slurp1* levels significantly (Fig. 7A). We further examined if elevated levels of IFN- γ play a role in downregulation of *Slurp1* in HSV-1-infected corneas by measuring the *Klf4* and *Slurp1* levels in abraded or HSV-1-infected WT and *Ifng*-/- (GKO) mice. At 2 DPI, both the extent of *Slurp1* downregulation and the nature of leukocytic infiltrate were comparable between the WT and

GKO corneas (Figs. 7B–D; Table 3), suggesting that IFN- γ is not involved in downregulation of *Slurp1* in HSV-1-infected corneas. Thus, we conclude that in proinflammatory conditions, *SLURP1* expression is inhibited by cytokines IL-4, IL-13, and TNF- α but not IFN- γ .

Slurp1 Restricts Neutrophilic Infiltrate in Adenovirus-Infected Corneas

In order to directly test the immunomodulatory role of *Slurp1*, we generated an adenoviral vector (serotype 5) expressing *Slurp1* under the control of CMV promoter (Adv5-*Slurp1*). WT BALB/c mouse corneas were abraded and infected with control

TABLE 1. Relative Expression Levels of Cytokines and Chemokine Ligands in *Klf4*CN Corneas

Cytokine/ Chemokine Ligand	Relative Expression in <i>Klf4</i> CN Cornea	Target Cells (Chemoattractant for)
<i>Il13</i>	19.126	
<i>Il4</i>	6.904	
<i>Il1f6</i>	5.380	
<i>Il3</i>	5.054	
<i>Il10</i>	5.342	
<i>Ifng</i>	5.089	
<i>Cxcl5</i>	12.707	Neutrophils
<i>Cxcl11</i>	9.563	Activated T cells
<i>Cxcl15</i>	8.923	Neutrophils
<i>Ccl12</i>	8.268	Peripheral blood monocytes
<i>Ccl19</i>	7.931	Dendritic cells
<i>Ccl11</i>	7.822	Eosinophils
<i>Cxcl10</i>	7.555	Macrophages, T cells, NK cells, dendritic cells
<i>Ccl1</i>	6.904	Monocytes, NK cells, immature B cells, dendritic cells
<i>Ccl6</i>	6.353	Macrophages, B cells, CD4 T cells, eosinophils
<i>Cxcl9</i>	6.309	Activated T cells, NK cells
<i>Ccl8</i>	5.969	Mast cells, eosinophils, basophils, monocytes, T cells, NK cells
<i>Ccl20</i>	5.726	Dendritic cells
<i>Ccl5</i>	5.531	T cells, eosinophils, basophils
<i>Cxcl13</i>	5.269	B cells
<i>Ccl17</i>	5.232	Activated T cells
<i>Ccl22</i>	4.985	Dendritic cells, NK cells, Th2 cells
<i>Ccl7</i>	4.882	Monocytes
<i>Cxcl12</i>	4.749	Lymphocytes
<i>Pf4</i>	4.250	Neutrophils, monocytes

Adv5-*Tet-Off* vector alone (2×10^6 PFU/cornea) or Adv5-*Slurp1* and Adv5-*Tet-Off* vectors (10^6 PFU each/cornea). Examination of the infected eyes at 4 and 10 DPI through a slit-lamp biomicroscope revealed signs of mild inflammation in corneas infected with Adv5-*Tet-Off* vector alone, while those infected with Adv5-*Slurp1* and Adv5-*Tet-Off* vectors remained normal (Fig. 8A). The corneas were harvested from these mice at 4 DPI, and *Slurp1* expression in epithelial cells was estimated by

TABLE 2. Relative Expression Levels of Chemokine Receptors in *Klf4*CN Corneas

Chemokine Receptor	Relative Expression in <i>Klf4</i> CN Cornea
<i>Ccr7</i>	15.322
<i>Il1r2</i>	14.395
<i>Ccr4</i>	8.325
<i>Il5ra</i>	8.325
<i>Ccr1</i>	7.001
<i>Ccr6</i>	6.487
<i>Ccr2</i>	5.726
<i>Il10ra</i>	5.342
<i>Ccr3</i>	5.342
<i>Cxcr5</i>	5.306
<i>Xcr1</i>	5.160
<i>Il8rb</i>	4.221
<i>Il2rg</i>	4.192
<i>Cd40lg</i>	12.884
<i>Itgb2</i>	11.856

FIGURE 4. Localization of CD45⁺ cells in the WT and *Klf4*CN corneas. Flat mounts of WT (A, C) and *Klf4*CN (B, D) corneas were stained with FITC-conjugated anti-CD45 antibody and examined by confocal microscopy. Representative stacked images of the central region of corneas are shown at 20 $\times$ (A, B; NA 0.85) and 60 $\times$ (C, D; NA 1.42) magnification. Note the relatively even distribution and lower density of CD45⁺ cells in the WT corneas compared with their higher density and clustering in *Klf4*CN corneas.

qPCR. In parallel, the nature of leukocytic infiltrate in corresponding corneal stromas was assessed by flow cytometry as described above. *Slurp1* expression in Adv5-*Tet-Off* virus-infected corneas was reduced to 17% of that in the abraded corneas and was partially restored in Adv5-*Tet-Off* and Adv5-*Slurp1* co-infected corneas, to 55% of that in the abraded corneas (Fig. 8B). The reduced expression of *Slurp1* in Adv5-*Tet-Off* virus-infected corneas was accompanied by significantly elevated neutrophilic infiltrate, compared with the small number of neutrophils identified in control abraded corneas expressing normal levels of *Slurp1* (Figs. 8C, 8D). Partial restoration of *Slurp1* expression in Adv5-*Slurp1* and Adv5-*Tet-Off* co-infected corneas significantly restricted the neutrophilic infiltrate (Fig. 8B). These results, taken together with those described above for HSV-1-infected, LPS-injected, and *Klf4*CN corneas, are consistent with an immunomodulatory role for *Slurp1*.

DISCUSSION

In this report, we demonstrate that *Slurp1* expression is (1) increased upon mouse eyelid opening when the cornea is first exposed to the environment; (2) decreased in inflamed *Klf4*CN corneas; (3) critically dependent on the transcription factor Klf4; (4) abrogated upon bacterial LPS injection, HSV-1, or adenoviral infection; (5) suppressed by proinflammatory cytokines IL-4, IL-13, and TNF- α ; and (6) capable of restricting neutrophilic infiltrate in adenovirus-infected corneas. Taken together, the results presented provide evidence that *Slurp1* is a key immunomodulatory molecule that contributes to corneal immune privilege by suppressing leukocyte infiltration in healthy corneas, and that is rapidly downregulated in acute

FIGURE 5. Expression of *Klf4* and *Slurp1* in HSV-1-infected (A, B) and (C) LPS-injected corneas. Control (mock infected or PBS injected), HSV-1-infected (A) or LPS-injected (C) WT mouse corneas were harvested at the indicated time after treatment. *Klf4* and *Slurp1* transcripts were quantified by qPCR. Bars indicate relative expression levels (mean $\pm$ SEM) of *Klf4* and *Slurp1* in HSV-1-infected (A), or LPS-injected (C) corneas. (B) Immunofluorescent staining with anti-Slurp1 antibody in mock- or HSV-1-infected mouse corneas at 1 and 2 days postscratching (DPS, Control) or postinfection (DPI). Nuclei are stained with DAPI (blue), and corresponding no-antibody controls are shown. Note that Slurp1 is abundantly expressed in control (iii, iv) and sharply decreased in HSV-1-infected corneal epithelia (v, vi) at both 1 and 2 DPI. Scale bars: 25 μ m.

inflammatory conditions to allow protective inflammation to develop.

Although our findings demonstrate the relationship between reduced Slurp1 and increased inflammation, we cannot state with certainty that the increased inflammation in the *Klf4*CN corneas is directly related to reduced Slurp1 expression, as *Klf4* modulates the expression of many genes, including *Slurp1*.^{19–21} Moreover, the absence of conjunctival goblet cells²¹ and the loss of corneal epithelial barrier function¹⁹ can also generate proinflammatory signals in the *Klf4*CN ocular surface. Additional experiments will be required to establish a direct causal relationship between reduced Slurp1 and increased inflammation in *Klf4*CN corneas.

The concept of corneal immune privilege arose from the antithetical nature of inflammation and essential corneal transparency.^{22–35} As the most anterior part of the eye, the cornea is constantly exposed to various biological, chemical, and physical insults. The requirement for its transparency to ensure proper vision mandates that the cornea be kept free of chronic inflammation in the presence of mild but constant insults. Corneal cells constitutively express a variety of molecules that function to inhibit important components of the inflammatory response. For instance, the avascular nature of the cornea is maintained in part by the constitutive production of soluble vascular endothelial growth factor (VEGF) receptor-1 (sVEGF-R1) and -3 (VEGF-R3), which inhibit the angiogenic activity of VEGF.^{23,29,30} The cornea constitutively expresses surface molecules such as Fas ligand and

programmed death ligand 1 (PD-L1), which can inhibit or kill infiltrating leukocytes,^{31–34,42} and secreted molecules such as transforming growth factor beta that potentially inhibit the function of a variety of inflammatory cells.³⁵ Our study adds Slurp1 to the list of such constitutively expressed molecules that inhibit inflammatory response in the cornea.

We propose that Slurp1 is a part of the machinery that prevents neutrophil infiltration into the normal cornea by one or both of the mechanisms depicted in Figure 9. The first scenario predicts that Slurp1, which is structurally similar to membrane-tethered uPAR required for neutrophil recruitment in response to bacterial infections,^{43–45} functions as a soluble scavenger of uPAR ligands and blocks their functions, analogous to the role of soluble VEGFR in blocking corneal angiogenesis²⁹ (Fig. 9A, i). Although uPAR-mediated neutrophil recruitment is independent of uPA,^{46,47} other activities of uPAR, such as its interaction with vitronectin and β 1-integrin and bacterial clearance, are dependent on uPA.^{47–50} The second scenario predicts that Slurp1, which shares the structural features of α -bungarotoxin and serves as a ligand for the α 7nAChRs,¹¹ suppresses the release of inflammatory mediators such as TNF- α from macrophages by enhancing α 7nAChR-mediated responses⁶ (Fig. 9A, ii). Normal mouse corneas possess a network of stromal macrophages and a sparse population of dendritic cells^{51–53}; these are likely to express α 7nAChR,⁵⁴ ligation of which results in inhibition of cytokine and chemokine release.¹ In either scenario, when the cornea needs to mount a rapid immune response to deal with

FIGURE 6. Leukocyte populations in mock- and HSV-1-infected corneas. Mock- or HSV-1-infected corneas from WT and *Klf4CN* littermates were excised at 2 DPI; the cells were dispersed with collagenase, stained with fluorochrome-conjugated antibodies specific for CD45, CD11b, and Gr-1, and analyzed by flow cytometry. **(A)** Representative dot plots illustrate gating on CD45⁺ cells. Percentage of CD45⁺ cells among stromal cells is shown within each dot plot. **(B)** The scatter plot shows the frequency of CD45⁺ cells in mock-infected and HSV-1-infected corneas. **(C)** Representative dot plots illustrate gating on CD11b⁺ Gr-1⁺ cells within a population gated on CD45⁺ cells. Percentage of Gr-1⁺ CD11b⁺ cells among stromal CD45⁺ cells is shown within the *upper right quadrant* of each dot plot. **(D)** The scatter plot shows the frequency of CD11b⁺ Gr-1⁺ cells among CD45⁺ cells in mock-infected and HSV-1-infected corneas.

TABLE 3. Enumeration of CD45⁺ and Gr-1⁺ CD11b⁺ Cells in Abraded and HSV-1-Infected WT and *Klf4CN* Corneas (Mixed Background) and WT and GKO Corneas (BALB/c)

Treatment	CD45 ⁺ Cells ± SEM	Gr-1 ⁺ CD11b ⁺ Cells ± SEM
Abraded and HSV-1-infected WT and <i>Klf4CN</i> corneas		
WT abraded	210 ± 65	41 ± 14
WT infected	1,350 ± 361	1,310 ± 356
<i>Klf4CN</i> abraded	455 ± 160 (<i>P</i> = 0.1938)	403 ± 157 (<i>P</i> = 0.0507)
<i>Klf4CN</i> infected	8,687 ± 3,793 (<i>P</i> = 0.0903)	8,529 ± 3,733 (<i>P</i> = 0.0904)
Abraded and HSV-1-infected WT and GKO corneas		
WT abraded	1,997 ± 394	445 ± 129
WT infected	12,283 ± 1,677	9,729 ± 2,730
GKO abraded	1,994 ± 355 (<i>P</i> = 0.9956)	686 ± 201 (<i>P</i> = 0.3425)
GKO infected	13,125 ± 2,443 (<i>P</i> = 0.7835)	11,096 ± 2,319 (<i>P</i> = 0.7127)

Average number of cells derived from three independent replicates is provided with standard error of mean (SEM). The *P* values provide a measure of statistical significance in comparison with the corresponding WT samples. The differences in CD45⁺ and Gr-1⁺ CD11b⁺ cell numbers in the WT abraded and infected corneas between the two experiments may be due to the different strains used, and/or different sizes of the cornea analyzed, to account for the smaller *Klf4CN* corneas.

FIGURE 7. IFN- γ is not required for *Slurp1* downregulation and neutrophilic infiltration into infected corneas. (A) *Slurp1* and *Klf4* transcripts in HCLE cells exposed to different cytokines. qPCR was performed with total RNA from HCLE cells exposed to different cytokines indicated for 2 days, with 18S rRNA as endogenous control. Mean data from three independent experiments are presented. Error bars represent standard error of mean (SEM). The *P* values were calculated using Student's *t*-test. (B) *Slurp1* and *Klf4* transcripts in mock- or HSV-1-infected BALB/c WT or GKO corneas at 2 DPI. Bars indicate the mean $\pm$ SEM fold change in *Slurp1* expression in mock- or HSV-1-infected corneas over that in control corneas. The *P* values were calculated using Student's *t*-test. (C, D) Cells in mock- or HSV-1-infected WT or GKO corneas were dispersed with collagenase, stained with fluorochrome-conjugated antibodies specific for CD45, CD11b, and Gr-1, and analyzed by flow cytometry. (C) The scatter plot shows the frequency of CD45 $^{+}$ cells in mock-infected and HSV-1-infected corneas. (D) The scatter plot shows the frequency of CD11b $^{+}$ and Gr-1 $^{+}$ double-positive cells among CD45 $^{+}$ cells in mock-infected and HSV-1-infected corneas. Cumulative data from five different abraded mock- or HSV-infected WT and GKO animals each are shown.

acute infections or severe chemical or physical insults, downregulation of *Slurp1* serves as a molecular switch facilitating further progression of inflammation (Fig. 9B). This role of *Slurp1* as a molecular switch regulating inflammation may not be limited to the cornea; it may serve a similar function in the other epithelia where it is abundantly expressed. The recent demonstration of IL-13-mediated suppression of *Slurp1* in inflamed asthmatic airway mucosal epithelium and the inflammation in mal de Meleda epidermis support such a possibility.^{7,37}

An important observation in this study is that the resident population of bone marrow-derived CD45 $^{+}$ cells is altered in *Klf4*CN corneas that lack *Slurp1*, consistent with the previous report on altered dendritic cell phenotype and distribution in inflamed corneas.⁵⁵ The resident leukocyte population in these corneas is not only larger, but is also phenotypically distinct from that found in normal corneas of WT mice. While WT corneal stromas contain mainly CD11b $^{+}$ Gr-1 $^{-}$ macrophages,

those of *Klf4*CN mice lacking *Slurp1* expression contain a population of CD11b $^{+}$ Gr-1 $^{+}$ cells, a phenotype characteristic of neutrophils. As eosinophils express CD11b and Gr-1,⁵⁶ a contribution of eosinophils to the leukocytic population in corneas of *Klf4*CN mice cannot be ruled out. The chemokine showing the greatest upregulation in noninfected *Klf4*CN corneas is CXCL5, a chemokine that is induced by IL-1 and TNF- α and is a potent chemoattractant for neutrophils. In addition, two of the three most upregulated chemokines in noninfected *Klf4*CN corneas relative to WT corneas are chemotactic for neutrophils (Table 1). The *Klf4*CN corneas also exhibited increased expression of the type 2 cytokines IL-4 and IL-13 and the chemokine CCL11, all known to attract and activate eosinophils. Considering that IL-4 and IL-13 inhibit *Slurp1* expression, it is conceivable that the reduced expression of *Slurp1* in *Klf4*CN mouse corneas reflects the cumulative effect of the absence of *Klf4* and elevated levels of IL-4 and IL-13.

FIGURE 8. Evidence for immunomodulatory role for SLURP1. WT BALB/c mouse corneas ($n = 4$) abraded and infected with either 2×10^6 PFU Adv5-Tet-Off vector alone (a, b, e, f) or 10^6 PFU each of Adv5-*Slurp1* and Adv5-Tet-Off vectors (c, d, g, h) were imaged at 4 and 10 DPI under normal (a, c, e, g) and slit-lamp (b, f, d, h) illumination (A). Signs of mild inflammation were observed in corneas infected with Adv5-Tet-Off vector alone, while those infected with Adv5-*Slurp1* and Adv5-Tet-Off vectors remained normal (A). Corneas harvested at 4 DPI were separated into epithelium and stroma + endothelium. Total RNA isolated from epithelial cells was used to quantify relative expression of *Slurp1* by qPCR (B). Stromal cells were isolated and stained with fluorochrome-conjugated antibodies specific for CD45, CD11b, and Gr-1 and analyzed by flow cytometry (C, D). Number of CD45⁺ (C) and CD11b⁺ Gr-1⁺ cells (D) was significantly reduced in Adv5-*Slurp1*-infected corneas compared with those infected with Adv5-Tet-Off vector (control) alone.

Mock infection of the WT corneas involving abrasion of the corneal epithelium did not reduce *Slurp1* expression. These corneas showed few if any cells expressing a neutrophil phenotype. HSV-1 infection of the mouse corneas resulted in a rapid infiltration of leukocytes consisting primarily of neutrophils, consistent with the previous reports.⁵⁷ Based on our data suggesting a role for *Slurp1* in inhibiting neutrophil infiltration of the cornea, we hypothesized that *Slurp1* expression would have to be downregulated to permit neutrophil infiltration into HSV-1-infected corneas. Consistent with this notion, corneal expression of *Slurp1* was abrogated by 24 hours after HSV-1 infection, and *Slurp1* downregulation was associated with a leukocytic infiltrate predominated by neutrophils. Interestingly, *Slurp1* downregulation by 24 hours after infection occurred in the presence of relatively unchanged levels of *Klf4*,

indicating that the early reduction in *Slurp1* was not due to a deficiency in *Klf4*. Also of interest is the fact that *Slurp1* was virtually abrogated even though only a portion of corneal epithelial cells were infected by the virus, suggesting that downregulation of *Slurp1* does not require direct infection of corneal epithelial cells. Instead, it seems likely that suppression of *Slurp1* expression is mediated by a soluble mediator induced by HSV-1 infection. IL-4, IL-13, and TNF α -mediated suppression of *Slurp1* expression without perturbation of *Klf4* levels is consistent with this likelihood. Even though IFN- γ is rapidly produced in the cornea following HSV-1 infection⁵⁸ and inhibits *Slurp1* expression in vitro,⁷ our study shows that IFN- γ is not involved in downregulation of *Slurp1* following corneal infection with HSV-1, which is most likely due to overlapping effects of other inflammatory cytokines. The mechanism of

FIGURE 9. Proposed model for the function of Slurp1 in the ocular surface. Slurp1 is a part of the machinery that suppresses inflammation at the ocular surface. (A) Under normal conditions or conditions of mild trauma, Klf4 supports constitutive high-level production of Slurp1 by the corneal epithelium. Slurp1 may (i) block uPAR function by competing for its ligands uPA, vitronectin (Vn), and integrins (Intg), and/or (ii) interact with membrane-bound nAChR on the surface of resident corneal cells such as macrophages and dendritic cells, potentiating the ability of acetylcholine (bound to nAChR) to block the release of intracellular TNF- α , maintaining the cornea in a noninflamed state. (B) Under severe trauma or microbial infection favoring inflammation, Slurp1 production is rapidly reduced by proinflammatory cytokines, facilitating (iii) uPAR-mediated extracellular matrix (ECM) degradation and/or (iv) the release of intracellular cytokines such as TNF- α , culminating in neutrophilic infiltration.

Slurp1 downregulation following HSV-1 corneal infection will require further study.

Mutations or deletions in *Slurp1* are associated with mal de Meleda, a rare autosomal recessive palmoplantar hyperkeratotic disorder in humans.^{7,10,13–18} Although diverse inflammatory keratodermas are often associated with ocular surface defects,^{59–61} no such defects have been described so far in mal de Meleda patients. This may be due to the relatively small number of mal de Meleda patients examined so far and may change with additional case reports. Alternatively, it may reflect the presence of only subtle defects in mal de Meleda patients' ocular surface that have eluded detection so far, and/or compensatory mechanisms in the human ocular surface that make up for the loss of SLURP1. In any case, the present report highlights a need for careful examination of the ocular surface in mal de Meleda patients.

In summary, we have demonstrated that Klf4 regulates the expression of Slurp1, a key immunomodulatory molecule that is abundantly expressed in the healthy cornea and is rapidly downregulated in proinflammatory conditions. Regulation of expression of Slurp1 adds yet another way in which Klf4 contributes to maintenance of the corneal homeostasis. Our

study identifies Slurp1 as a novel target for therapeutic intervention in managing corneal inflammatory disorders of diverse etiologies. Expression of Slurp1 in other tissues such as skin, oral mucosa, intestine, lung, and cervical epithelium suggests that our findings are widely applicable in other epithelia frequently exposed to similar insults as with the ocular surface.

Acknowledgments

We thank Nancy Zurowski, Steve Harvey, Kate Davoli, and Doreeswamy Kenchegowda for technical help.

References

- Grando SA. Basic and clinical aspects of non-neuronal acetylcholine: biological and clinical significance of non-canonical ligands of epithelial nicotinic acetylcholine receptors. *J Pharmacol Sci.* 2008;106:174–179.
- Mazar AP. Urokinase plasminogen activator receptor choreographs multiple ligand interactions: implications for tumor progression and therapy. *Clin Cancer Res.* 2008;14:5649–5655.

3. Adermann K, Wattler F, Wattler S, et al. Structural and phylogenetic characterization of human SLURP-1, the first secreted mammalian member of the Ly-6/uPAR protein superfamily. *Protein Sci.* 1999;8:810-819.
4. Moriwaki Y, Yoshikawa K, Fukuda H, Fujii YX, Misawa H, Kawashima K. Immune system expression of SLURP-1 and SLURP-2, two endogenous nicotinic acetylcholine receptor ligands. *Life Sci.* 2007;80:2365-2368.
5. Horiguchi K, Horiguchi S, Yamashita N, et al. Expression of SLURP-1, an endogenous alpha7 nicotinic acetylcholine receptor allosteric ligand, in murine bronchial epithelial cells. *J Neurosci Res.* 2009;87:2740-2747.
6. Moriwaki Y, Watanabe Y, Shinagawa T, et al. Primary sensory neuronal expression of SLURP-1, an endogenous nicotinic acetylcholine receptor ligand. *Neurosci Res.* 2009;64:403-412.
7. Mastrangeli R, Donini S, Kelton CA, et al. ARS Component B: structural characterization, tissue expression and regulation of the gene and protein (SLURP-1) associated with Mal de Meleda. *Eur J Dermatol.* 2003;13:560-570.
8. Arredondo J, Chernyavsky AI, Grando SA. SLURP-1 and -2 in normal, immortalized and malignant oral keratinocytes. *Life Sci.* 2007;80:2243-2247.
9. Norman B, Davis J, Piatigorsky J. Postnatal gene expression in the normal mouse cornea by SAGE. *Invest Ophthalmol Vis Sci.* 2004;45:429-440.
10. Favre B, Plantard L, Aeschbach L, et al. Slurp1 is a late marker of epidermal differentiation and is absent in Mal de Meleda. *J Invest Dermatol.* 2007;127:301-308.
11. Arredondo J, Chernyavsky AI, Webber RJ, Grando SA. Biological effects of SLURP-1 on human keratinocytes. *J Invest Dermatol.* 2005;125:1236-1241.
12. Arredondo J, Chernyavsky AI, Grando SA. Overexpression of SLURP-1 and -2 alleviates the tumorigenic action of tobacco-derived nitrosamine on immortalized oral epithelial cells. *Biochem Pharmacol.* 2007;74:1315-1319.
13. Chimienti F, Hogg RC, Plantard L, et al. Identification of SLURP-1 as an epidermal neuromodulator explains the clinical phenotype of Mal de Meleda. *Hum Mol Genet.* 2003;12:3017-3024.
14. Fischer J, Bouadjar B, Heilig R, et al. Mutations in the gene encoding SLURP-1 in Mal de Meleda. *Hum Mol Genet.* 2001;10:875-880.
15. Eckl KM, Stevens HP, Lestringant GG, et al. Mal de Meleda (MDM) caused by mutations in the gene for SLURP-1 in patients from Germany, Turkey, Palestine, and the United Arab Emirates. *Hum Genet.* 2003;112:50-56.
16. Hu G, Yildirim M, Baysal V, et al. A recurrent mutation in the ARS (component B) gene encoding SLURP-1 in Turkish families with mal de Meleda: evidence of a founder effect. *J Invest Dermatol.* 2003;120:967-969.
17. Marrakchi S, Audebert S, Bouadjar B, et al. Novel mutations in the gene encoding secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related protein-1 (SLURP-1) and description of five ancestral haplotypes in patients with Mal de Meleda. *J Invest Dermatol.* 2003;120:351-355.
18. Ward KM, Yerebakan O, Yilmaz E, Celebi JT. Identification of recurrent mutations in the ARS (component B) gene encoding SLURP-1 in two families with mal de Meleda. *J Invest Dermatol.* 2003;120:96-98.
19. Swamynathan S, Kenchegowda D, Piatigorsky J, Swamynathan SK. Regulation of corneal epithelial barrier function by Kruppel-like transcription factor 4. *Invest Ophthalmol Vis Sci.* 2011;52:1762-1769.
20. Swamynathan SK, Davis J, Piatigorsky J. Identification of candidate Klf4 target genes reveals the molecular basis of the diverse regulatory roles of Klf4 in the mouse cornea. *Invest Ophthalmol Vis Sci.* 2008;49:3360-3370.
21. Swamynathan SK, Katz JP, Kaestner KH, Ashery-Padan R, Crawford MA, Piatigorsky J. Conditional deletion of the mouse Klf4 gene results in corneal epithelial fragility, stromal edema, and loss of conjunctival goblet cells. *Mol Cell Biol.* 2007;27:182-194.
22. Niederkorn JY, Stein-Streilein J. History and physiology of immune privilege. *Ocul Immunol Inflamm.* 2010;18:19-23.
23. Azar DT. Corneal angiogenic privilege: angiogenic and antiangiogenic factors in corneal avascularity, vasculogenesis, and wound healing (an American Ophthalmological Society thesis). *Trans Am Ophthalmol Soc.* 2006;104:264-302.
24. Hazlett LD, Hendricks RL. Reviews for immune privilege in the year 2010: immune privilege and infection. *Ocul Immunol Inflamm.* 2010;18:237-243.
25. Barabino S, Chen Y, Chauhan S, Dana R. Ocular surface immunity: homeostatic mechanisms and their disruption in dry eye disease. *Prog Retin Eye Res.* 2012;31:271-285.
26. Clements JL, Dana R. Inflammatory corneal neovascularization: etiopathogenesis. *Semin Ophthalmol.* 2011;26:235-245.
27. Yamanaka O, Liu CY, Kao WW. Fibrosis in the anterior segments of the eye. *Endocr Metab Immune Disord Drug Targets.* 2010;10:331-335.
28. Gronert K. Resolution, the grail for healthy ocular inflammation. *Exp Eye Res.* 2010;91:478-485.
29. Ambati BK, Nozaki M, Singh N, et al. Corneal avascularity is due to soluble VEGF receptor-1. *Nature.* 2006;443:993-997.
30. Cursiefen C, Chen L, Saint-Geniez M, et al. Nonvascular VEGF receptor 3 expression by corneal epithelium maintains avascularity and vision. *Proc Natl Acad Sci U S A.* 2006;103:11405-11410.
31. Stuart PM, Pan F, Plambeck S, Ferguson TA. FasL-Fas interactions regulate neovascularization in the cornea. *Invest Ophthalmol Vis Sci.* 2003;44:93-98.
32. Morris JE, Zobell S, Yin XT, et al. Mice with mutations in Fas and Fas ligand demonstrate increased herpetic stromal keratitis following corneal infection with HSV-1. *J Immunol.* 2012;188:793-799.
33. Jin Y, Chauhan SK, El Annan J, Sage PT, Sharpe AH, Dana R. A novel function for programmed death ligand-1 regulation of angiogenesis. *Am J Pathol.* 2011;178:1922-1929.
34. El Annan J, Goyal S, Zhang Q, Freeman GJ, Sharpe AH, Dana R. Regulation of T-cell chemotaxis by programmed death-ligand 1 (PD-L1) in dry eye-associated corneal inflammation. *Invest Ophthalmol Vis Sci.* 2010;51:3418-3423.
35. Tandon A, Tovey JC, Sharma A, Gupta R, Mohan RR. Role of transforming growth factor Beta in corneal function, biology and pathology. *Curr Mol Med.* 2010;10:565-578.
36. Jia C, Zhu W, Ren S, Xi H, Li S, Wang Y. Comparison of genome-wide gene expression in suture- and alkali burn-induced murine corneal neovascularization. *Mol Vis.* 2011;17:2386-2399.
37. Narumoto O, Horiguchi K, Horiguchi S, et al. Down-regulation of secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related peptide-1 (SLURP-1), an endogenous allosteric alpha7 nicotinic acetylcholine receptor modulator, in murine and human asthmatic conditions. *Biochem Biophys Res Commun.* 2010;398:713-718.
38. Sheridan BS, Cherpes TL, Urban J, Kalinski P, Hendricks RL. Reevaluating the CD8 T-cell response to herpes simplex virus type 1: involvement of CD8 T cells reactive to subdominant epitopes. *J Virol.* 2009;83:2237-2245.
39. Gipson IK, Spurr-Michaud S, Argueso P, Tisdale A, Ng TF, Russo CL. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Invest Ophthalmol Vis Sci.* 2003;44:2496-2506.

40. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995;36:614-621.
41. Shields JM, Yang VW. Identification of the DNA sequence that interacts with the gut-enriched Kruppel-like factor. *Nucleic Acids Res*. 1998;26:796-802.
42. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677-704.
43. Gyetko MR, Sud S, Kendall T, Fuller JA, Newstead MW, Standiford TJ. Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary *Pseudomonas aeruginosa* infection. *J Immunol*. 2000;165:1513-1519.
44. Rijnveld AW, Levi M, Florquin S, Speelman P, Carmeliet P, van Der Poll T. Urokinase receptor is necessary for adequate host defense against pneumococcal pneumonia. *J Immunol*. 2002;168:3507-3511.
45. Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. *Nat Rev Mol Cell Biol*. 2010;11:23-36.
46. Gueller F, Rong S, Mengel M, et al. Renal urokinase-type plasminogen activator (uPA) receptor but not uPA deficiency strongly attenuates ischemia reperfusion injury and acute kidney allograft rejection. *J Immunol*. 2008;181:1179-1189.
47. Connolly BM, Choi EY, Gardsvoll H, et al. Selective abrogation of the uPA-uPAR interaction in vivo reveals a novel role in suppression of fibrin-associated inflammation. *Blood*. 2010;116:1593-1603.
48. Nguyen DH, Catling AD, Webb DJ, et al. Myosin light chain kinase functions downstream of Ras/ERK to promote migration of urokinase-type plasminogen activator-stimulated cells in an integrin-selective manner. *J Cell Biol*. 1999;146:149-164.
49. Sidenius N, Andolfo A, Fesce R, Blasi F. Urokinase regulates vitronectin binding by controlling urokinase receptor oligomerization. *J Biol Chem*. 2002;277:27982-27990.
50. Caiolfa VR, Zamai M, Malengo G, et al. Monomer dimer dynamics and distribution of GPI-anchored uPAR are determined by cell surface protein assemblies. *J Cell Biol*. 2007;179:1067-1082.
51. Brissette-Storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL. Identification of a novel macrophage population in the normal mouse corneal stroma. *Invest Ophthalmol Vis Sci*. 2002;43:2264-2271.
52. Knickelbein JE, Watkins SC, McMenamin PG, Hendricks RL. Stratification of antigen-presenting cells within the normal cornea. *Ophthalmol Eye Dis*. 2009;1:45-54.
53. Hamrah P, Pavan-Langston D, Dana R. Herpes simplex keratitis and dendritic cells at the crossroads: lessons from the past and a view into the future. *Int Ophthalmol Clin*. 2009;49:53-62.
54. Kawashima K, Yoshikawa K, Fujii YX, Moriwaki Y, Misawa H. Expression and function of genes encoding cholinergic components in murine immune cells. *Life Sci*. 2007;80:2314-2319.
55. Hamrah P, Liu Y, Zhang Q, Dana MR. Alterations in corneal stromal dendritic cell phenotype and distribution in inflammation. *Arch Ophthalmol*. 2003;121:1132-1140.
56. Rothenberg ME, Hogan SP. The eosinophil. *Annu Rev Immunol*. 2006;24:147-174.
57. Hendricks RL, Tumpey TM. Contribution of virus and immune factors to herpes simplex virus type I-induced corneal pathology. *Invest Ophthalmol Vis Sci*. 1990;31:1929-1939.
58. Hendricks RL, Tumpey TM, Finnegan A. IFN-gamma and IL-2 are protective in the skin but pathologic in the corneas of HSV-1-infected mice. *J Immunol*. 1992;149:3023-3028.
59. Messmer EM, Kenyon KR, Rittinger O, Janecke AR, Kampik A. Ocular manifestations of keratitis-ichthyosis-deafness (KID) syndrome. *Ophthalmology*. 2005;112:e1-e6.
60. Mohammad Ali M, Azarbaik M. A distinct type of palmoplantar keratoderma. *Pediatr Dermatol*. 2009;26:113-115.
61. Sonoda S, Uchino E, Sonoda KH, et al. Two patients with severe corneal disease in KID syndrome. *Am J Ophthalmol*. 2004;137:181-183.