The role of FPR2 signaling in the pathogenesis of bacterial keratitis

Thomas W. Carion, Matthew Greenwood, Karsten Gronert, Elizabeth A. Berger

Purpose: The formyl peptide receptor 2 (FPR2) is a promiscuous transmembrane protein belonging to the GPCR family. Ligands for FPR2 include pro-resolving lipids (LXA4, RvD1) and annexin 1. Pro-inflammatory serum amyloid A (SAA) and cathelicidin LL-37 also bind to FPR2. Depending on the ligand, different downstream signal transduction pathways can be activated. The role of FPR2 in disease pathogenesis of bacterial keratitis is the focus of the study described herein.

Methods: Bacterial keratitis was induced in resistant BALB/c and susceptible B6 mice using P. aeruginosa (PA) ATCC 19660 for in vivo studies. Peritoneal-derived PMN and macrophages were isolated from B6 and BALB/c mice, and then stimulated with PA for in vitro studies. Lipidomic analysis, real-time RT-PCR, Western Blot, and functional PMN and macrophage assays were used to assess differential FPR2 pathway activation between the two strains. Additionally, to further assess the role of FPR2 in vivo, WRW4 (FPR2 antagonist) was utilized to confirm the observed effects.

Results: mRNA transcript levels of FPR2 were increased in B6 vs BALB/c corneas and B6-derived PMNs and macrophages stimulated with PA. Significant increases in the pro-resolving intermediates for LXA4 and RvD1 (15-HETE and 17-HDHA) were observed in BALB/c corneas and B6-derived PMNs and macrophages stimulated with PA. Additionally, to further assess the role of FPR2 in vivo, WRW4 (FPR2 antagonist) was utilized to confirm the observed effects.

Conclusions: As the first study to explore the role of FPR2 in the pathogenesis of bacterial keratitis, our results suggest differential activation of the FPR2 pathway in BALB/c vs B6 mice. Thus, it appears a pro-inflammatory FPR2 interaction contributes to susceptibility observed in B6 mice; whereas pro-resolving molecule:FPR2 interaction in BALB/c, allows for proper PA clearance and a return to homeostasis. In addition, preliminary studies indicate the neuropeptide, VIP, not only increases mRNA levels of pro-resolving 12-/15-LOX but also anti-inflammatory p38 molecules, indicating the neuropeptide, VIP, not only increases mRNA levels of pro-resolving 12-/15-LOX but also anti-inflammatory p38 molecules, which could offer a therapeutic point of intervention for enhancing upstream and downstream pro-resolving FPR2 signal transduction.

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upregulation of KAMPs production. However, the proteolytic processing events of cytosolic K6A leading to KAMPs production are undefined. Here, we tested the hypothesis that the ubiquitin-proteasome pathway generates KAMPs through ubiquitination-coupled processing and degradation of cytosolic K6A.

**Methods:** Stratified telomerase-immortalized human corneal epithelial (hTCEpi) cells were pretreated with or without a specific 26S proteasome inhibitor (epoxomicin, 200nM) for 2 h, followed by 16 h incubation with purified *Staphylococcus aureus* lipoteichoic acid (LTA, 1 μg/ml). Cytosolic cell lysates were immunoblotted for KAMPs, as well as total and ubiquitinated K6A, or fractionated to isolate the KAMPs-enriched <10-kDa fractions. Antimicrobial activity of lysate fractions was determined against *P. aeruginosa* clinical isolate 6206 using viable counts after 3-h incubation. Mass spectrometry was used to identify endogenous ubiquitination sites on K6A.

**Results:** LTA stimulation concomitantly increased cytosolic K6A and KAMPs levels in stratified hTCEpi cells compared to untreated control. The <10-kDa lysate fraction of LTA-stimulated cells was potently bactericidal against *P. aeruginosa*. Epoxomicin treatment promoted accumulation of polyubiquitinated K6A in the LTA-stimulated cells but abolished the production of KAMPs. As such, the antimicrobial activity of the <10-kDa lysate fraction of epoxomicin-treated cells was significantly reduced (*P* = 0.006). Ubiquitination sites of K6A included the previously reported lysine-180, and two novel sites at lysine-194 and -204.

**Conclusions:** The data suggest that endogenous KAMPs are produced via ubiquitin-dependent proteasomal degradation of K6A. While proteasome processing is known to be involved in adaptive immunity, our data underscore the importance of ubiquitin-proteasome pathway in harnessing endogenous antimicrobial peptide production for epithelial innate defense. Further studies will determine the regulation of KAMPs production by specific ubiquitination sites and E3 ligases.

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**Thymic stromal lymphopoietin secreted by Aspergillus fumigatus-infected human corneal epithelial cells promotes a type 2 adaptive immune response**

**Xinyi Wu.** Ophthalmology, Shandong University, Jinan, China.

**Purpose:** To explore the potential role of TSLP in adaptive immunity of fungal keratitis.

**Methods:** Human corneal epithelial cells (HCECs) were stimulated with Aspergillus fumigatus hyphae (106 pieces/ml) with or without TSLP siRNA. Peripheral blood mononuclear cells (PBMCs) were co-cultured with HCECs in a transwell system for various periods. Then we collected PBMCs and detected proliferation and activation as well as Th2 differentiation by flow cytometry and quantitative RT-PCR. IgG and IgA levels in supernatants of PBMCs were measured by means of ELISA.

**Results:** Expression of TSLP was highly increased in HCECs stimulated with *A. fumigatus* hyphae. Aspergillus fumigatus-infected HCECs were capable of promoting human lymphocyte proliferation, and activating human CD4+ T cells, CD8+ T cells, and B cells by up-regulating expression of activation marker CD69. Importantly, Th2 differentiation of CD4+ T cells was induced during co-culture with *A. fumigatus*-infected HCECs in a transwell system. However, blockade of TSLP using siRNA prevented the proliferation and activation of lymphocytes as well as Th2 differentiation. We also detected an increased IgG level that was associated with TSLP.

**Conclusions:** These findings suggested that HCEC-derived TSLP has a key role in adaptive immune responses of fungal keratitis via skewing Th2 differentiation and promoting humoral immunity.

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**Conjunctival goblet cell produced secretory leukocyte peptidase inhibitor (SLPI) controls growth of Aspergillus fumigatus**

**Terry G. Coursey1, David B. Corry2, Stephen C. Pflugfelder1.** Ocular Surface Center, Ophthalmology, Baylor College of Medicine, Houston, TX; 2Department of Pathology & Immunology; The Biology of Inflammation Center, Baylor College of Medicine, Houston, TX.

**Purpose:** To determine the mechanisms that conjunctival goblet cells (GC) use to regulate fungal growth in the eye. The eye is constantly subjected to fungi as an exposed mucosal site. Surprisingly, fungi (of any kind) in healthy human eyes are rare. Very little is known about how the eye controls and prevents fungal growth in normal circumstances.

**Methods:** Primary conjunctival GC cultures were established from tissue explants of fornical conjunctiva dissected from female C57BL/6 mice. GC culture supernatants were harvested after 10 days of culture. 100 or 500 *A. fumigatus* conidia (fungal spores) were incubated with control medium (Keratinocyte serum-free media, KSFM) or GC conditioned KSFM supernatants for 24h. GC cultures were treated with protease from *Aspergillus oryzae* (PAO) (100 μg/mL) for 24h prior to harvesting conditioned supernatant. Fungal growth was quantified using cell-proliferation reagent WST-1 for the last two hours of the 24h incubation with GC supernatant or recombinant human (rh) SLPI (5 and 10 μM). Expression of SLPI in GC cultures was determined by qPCR.

**Results:** Incubation of conidia from *A. fumigatus* with supernatants from conjunctival GC culture supernatant inhibited fungal growth by 60% compared to control medium. Incubation with supernatant from cultures treated with PAO reduced fungal growth by 80%. Treatment of GC cultures with PAO increased mRNA expression of SLPI by 2.5-fold. GC cultures expressed SLPI 20-fold higher than corneal epithelial cell cultures. The addition of rhSLPI to conidia from *A. fumigatus* reduced fungal growth by 40%.

**Conclusions:** These studies suggest that conjunctival GC make antimicrobial products that inhibit fungal growth constitutively, and this GC function is augmented during exposure to fungal proteases. Production of SLPI by conjunctival GCs may be an important mechanism by which fungal growth is controlled in the eye. Understanding of the mechanisms employed by the eye to control fungal growth may lead to improved, less toxic treatments for fungal keratitis.

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Differential Alterations in Dendritic Cell Density and Morphology in Patients with Central and Peripheral Scleras from Herpes Simplex Keratitis: A Longitudinal In Vivo Confocal Microscopy Study

Hamid-Reza Moein1, 2, Rodrigo Müller1, 2, Deborah Pavan-Langston1, Bernardo M. Cavalcanti1, Clara Colon2, Debora Witkin3, Arsia Jamali4, Andrea Cruzat5, Pedram Hamrah6, 4. 1Department of Ophthalmology, Tufts Medical Center, Tufts University School of Medicine, Boston, MA; 2Boston Image Reading Center, New England Eye Center, Boston, MA; 3Ocular Surface Imaging Center, Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School, Boston, MA; 4Cornea Service, Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School, Boston, MA; 5Ocular Surface Imaging Center & Cornea Service, Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School, Boston, MA; 6Boston Image Reading Center & Cornea Service, New England Eye Center, Tufts Medical center, Tufts University School of Medicine, Boston, MA.

Purpose: To investigate the effect of corneal scar location in patients with herpes simplex keratitis (HSK) on longitudinal changes in dendritic cell (DC) density and morphology by in vivo confocal microscopy (IVCM).

Methods: This prospective, longitudinal, controlled study included 39 HSK patients with either central cornea scars (CCS, n=21) or peripheral cornea scars (PCS, n=18), as well as 16 age-matched normal reference controls. IVCM (HRT3/RCM) was applied to image the corneas centrally. Image J software was used to quantify DC density and to assess the DC area (cell size) and the DC field (span of a cell) in a masked fashion by two independent observers.

Results: Patients with HSK corneal scars demonstrated a significantly higher mean DC density (140 ± 141.8 cells/mm²) compared to controls (40.2 ± 28.6 cells/mm²; p=0.0003). In addition, mean DC area (70.3 ± 20.2 µm²) and DC field (106.9 ± 36.0 µm²) increased as compared to controls (48.2 ± 11.4 µm², and 69.8 ± 19.9 µm²; p<0.0001). Patients with CCS had significantly higher DC density (190.6 ± 163.7 cells/mm²) compared to patients with PCS (72.3 ± 61.5 cells/mm²; p=0.008) and controls. Mean DC area and field did not differ between CCS and PCS subgroups (71.7 ± 21.9 vs. 68.3 ± 18.2 and 108.2 ± 35.9 vs. 105.1 ± 37.4 µm²; p>0.05). There was a positive correlation between DC density and both DC area and field (r=0.31, p=0.01 for both). At follow-up visits (mean of 13.8 ± 3.5 months), mean DC density (119.5 ± 109.2 cells/mm²), DC area (68.9 ± 29.7 µm²), and DC field (107.3 ± 50.8 µm²) remained higher compared to controls (p=0.003, p=0.005, and p=0.003, respectively). DC density in CCS remained about two times higher than PCS at follow-up (147.5 ± 130.4 vs. 78.9 ± 51.6 cells/mm²; p=0.1). Mean DC area and field were not different in the CCS and PCS groups (66.3 ± 33.6 vs. 72.7 ± 24.4 and 100.73 ± 52.2 vs. 116.9 ± 50.2 µm²; p=0.05).

Conclusions: Patients with HSK demonstrate increased DC density, area, and field in affected eyes compared to controls at baseline and follow-up. Eyes with CCS had higher DC density compared with PCS even at 1-year follow-up. This study demonstrates differential alterations in corneal DC density, but not morphology, in patients with CCS vs. PCS due to HSK. We further demonstrate a novel methodology for quantification of DC parameters.

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Lack of neurokinin-1 receptor alters the homeostasis of ocular surface and causes an early development of herpes stromal keratitis

Subhash Gaddipatti1, 2, Pushpa Rao1, Andrew Jerome1, Bala Bharati Burugula2, 1, Susmit Suvas3, 1. 1Anatomy & Cell Biology, Wayne State University School of Medicine, Detroit, MI; 2Ophthalmology, Wayne State University School of Medicine, Detroit, MI.

Purpose: Neurokinin-1 receptor (NK1R) is the highest affinity receptor for substance P (SP), an eleven amino acid long neuropeptide present in the tear film and in sensory nerve fibers innervating the corneal epithelium. The purpose of this study is to determine the outcome of NK1R deficiency on ocular surface homeostasis and development of herpes stromal keratitis (HSK) in mouse model.

Methods: The corneal flat mounts from uninfected C57BL/6 (B6) and NKIR-/- mice were stained for dendritic cells, corneal nerves and adhesion proteins, and visualized under confocal microscope. Phenol red thread test was carried out to measure the volume of unstimulated tears in both groups of mice. BrdU single pulse labeling was carried out to determine the proliferative index of corneal epithelial cells. After ocular HSV-1 infection, the development of corneal opacity and angiogenesis was determined in both groups using slit lamp. Flow cytometry was carried out on draining lymph nodes (DLN) and corneal samples from infected groups of mice at different time-points post-infection.

Results: A significant decrease in the numbers of CD11c+ cells with dendritic processes was noted in the corneal epithelium of unmanipulated NKIR-/- than B6 mice. In addition, reduced corneal sub-epithelial nerve branching, sub-basal plexus leash numbers and patchy E-cadherin staining were detected in the corneal epithelium of NKIR-/- mice. BrdU labeling index for uninjured corneal epithelium was higher in NKIR-/- mice. NKIR-/- mice also showed significantly reduced volume of basal surface tear. Interestingly, naïve NKIR-/- mice showed a dramatic increase in the numbers of CD11c+ cells near the limbal region. After ocular HSV-1 infection, the numbers of CD11c+DCs infiltrating the infected corneas and then migrating to the DLN were significantly higher in NKIR-/- than B6 mice, when measured at early time-point after infection. This resulted into an early generation of larger numbers of IFN-g secreting virus specific CD4 T cells, which migrated to the inflamed corneas and caused an early development of severe HSK in NKIR-/- mice.

Conclusions: Together, our results showed the role of NK1R signaling in regulating corneal epithelial mitotic activity, adhesion, corneal nerve density, tear volume, homeostasis of corneal dendritic cells and the development of HSK.

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