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Boston, MA 02114

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14. ABSTRACT

EYE INJURIES HAVE INCREASED IN WARFARE OVER THE PAST 200 YEARS. IN THE CURRENT CONFLICT THE EYE IS PARTICULARLY VULNERABLE TO BLAST INJURY AS WELL AS TO ENVIRONMENTAL STRESS FROM THE CURRENT THEATERS OF OPERATION. FOR CLEAR VISION, THE ENTIRE VISUAL AXIS (TEARS, CORNEA, AQUEOUS HUMOR, LENS, VITREOUS, RETINA, AND OPTIC NERVE) MUST BE TRANSPARENT, INTACT, AND HEALTHY AND EACH PART OF THIS AXIS FUNCTIONAL. EACH OF THESE STRUCTURES HAS ITS PARTICULAR VULNERABILITIES. IN THE PRESENT PROPOSAL WE HAVE DESIGNED MULTIPLE PROJECTS TO INVESTIGATE THE SPECIAL VULNERABILITIES OF THE CORNEA, RETINA, OPTIC NERVE, AND BRAIN TO MECHANICAL, LASER, INFECTIOUS, AND ENVIRONMENTAL TRAUMA AND TO DEVISE MEANS TO PREVENT, DIAGNOSE, AND REPAIR THE SEQUELAE OF THESE TRAUMAS.

15. SUBJECT TERMS

injury, laser injury, infection, angiogenesis, stem cells, dry eye, cornea, inflammation, traumatic brain injury, pain receptors, immune response

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FY08 - W91XWH-09-2-0091, Darlene A. Dartt, Ph.D.
October 24, 2011
Final Report, Revised Project
TITLE: The NALP3 Inflammasome: A Critical Receptor of Ocular Surface Inflammation

INTRODUCTION

This project aims to understand how specialized conjunctival cells, goblet cells, secrete mucins to protect the ocular surface shielding it from an array of potential pathogens. Mucin deficiency and reduced goblet cell number is evident in a number of serious ocular surface conditions, namely in alkali burns, thermal burns, Stevens- Johnson syndrome, neurotrophic keratitis, ocular cicatricial pemphigoid and dry eye syndromes increasing the chance of infection. Many of these pathogenic states including burns and trauma also occur in military conflicts. Such abnormalities can lead to serious visual impairment if not treated and treatment itself is difficult. The present proposal will study how bacteria induce goblet cell secretion and if this is accompanied by initiation of a protective inflammation using an intracellular group of enzymes known as the inflammasome. Activation of these enzymes produces a compound, interleukin 1-β that initiates a protective inflammatory response. Insight into the conjunctival response to infection would aid disease management in the form of therapies and preventative measures.

Conjunctival goblet cells are known to secrete soluble mucins into the tear film, playing a major role in the defense and regulation of the ocular surface. A reduced number of goblet cells and mucin deficiency is one of the hallmarks of a range of ocular surface diseases including, alkali burns, thermal burns, Stevens-Johnson syndrome, neurotrophic keratitis, ocular cicatricial pemphigoid and dry eye syndromes. In some diseases, overproduction of mucin owing to excessive goblet cell proliferation and/or secretion is due to the presence of activated T-cells or macrophages. If left untreated, mucin abnormalities could severely compromise the ocular surface and lead to serious visual impairment. Since treatment is expensive, of long duration, and often unsuccessful, managing these diseases presents a formidable problem.

The conjunctiva is continuously exposed to a plethora of bacterial flora that can trigger an innate immune response. To discriminate between self and pathogens, the eukaryotic host has evolved a series of pattern-recognition receptors (PRRs) that recognize highly specialized pathogen-associated molecular patterns (PAMPs), when PRRs are triggered by PAMPs, they activate a rapid innate immune response. The PRRs include (i) Toll-like receptors (TLRs), which are expressed on the cell surface and (ii) Nod-like receptors (NLRs), which are expressed in the cytoplasm. NALP3 is a member of the NLR family and was recently found to be constitutively expressed in the mucosal epithelium of the gut and genital tract and in several types of immune cells. Additional studies, using PCR, demonstrated constitutive expression of NALP3 mRNA in whole eye preparations, however, the cells that actually expressed NALP3 were not identified. NALP3 forms a protein complex with other components called the "***inflammasome***" that triggers the activation and secretion of IL-1β and IL-18, important early cytokines required to initiate an innate immune response. We recently identified, for the first time, constitutive expression of NALP3 and IL-1β in the conjunctival epithelium of normal patients. NALP3 is thought to be important in sensing pathogens and rapidly triggering an innate immune response. Interestingly, genetic mutations in NALP3 have been associated with several autoinflammatory disorders. Mutations within the NACHT domain of the NLR associated protein cryopyrin/NALP3 are associated with three autoinflammatory disorders characterized by skin rashes and prolonged episodes of fever in the absence of any apparent infection. These hereditary periodic fever syndromes are Muckle–Wells syndrome (MWS), familial cold autoinflammatory syndrome (FACS), and neonatal-onset multisystem inflammatory disease (NOMID), and they are collectively referred to as the cryopyrin/NALP3- associated periodic syndromes (CAPS). Functional studies revealed that the disease-associated cryopyrin/NALP3 mutations enhance caspase-1 activation and IL-1β secretion.

Upon NLR activation by bacteria, a potassium efflux from the cytosol occurs which results in the formation of reactive oxygen species (ROS) that may be formed due to phagocytosis of the microbes. ROS then initiate the formation of a complex known as the cryopyrin NALP3 inflammasome complex (also known as NLRP3). This activates the caspase 1 pathway, which leads to IL- 1β production. IL-1β participates in the generation of systemic and local responses to infection and in doing so initiates signaling cascades leading to the activation of nuclear factor κB (NFκB) and mitogen-activated protein kinases (MAPKs), which drive many processes

including inflammatory cytokine expression.

In summary the premise for this project proposal is based upon the following evidence: 1. A reduced goblet cell density and mucin secretion has been observed in dry eye patients and inflammation can lead to goblet cell apoptosis; 2. A reduced goblet cell density has been associated with increased bacterial population; 3. NALP3 is constitutively expressed in the conjunctival epithelium; 4. Bacteria induce the formation of the NALP3 inflammosome to produce IL-1β; 5. IL-1β is a potent inflammatory cytokine, which can lead to inflammation and is involved in dry eye syndromes; 6. Increasing the intracellular [Ca^{2+}] and activating MAPK are required for goblet cell secretion and inflammasome formation results in [Ca^{2+}] release and MAPK initiation.

BODY

Overall objective: We hypothesize that the NALP3 inflammasome is a critical first-line defense mechanism in the conjunctiva and quickly responds to pathogens by triggering protective innate immunity. Because the conjunctiva is exposed and vulnerable to pathogens, NALP3 is constitutively expressed in the epithelium in order to facilitate rapid inflammasome formation. However, in diseases associated with inflammation, the increased exposure to pathogens leads to chronic inflammasome/NALP3 activation in an effort to prevent bacterial keratitis, but a dangerous side effect of this protective mechanism is inflammation-induced damage to normal tissue.

Objectives:

1. Determine which components of the NALP3 inflammasome are constitutively expressed and/or induced in the stratified squamous epithelium and goblet cells of the conjunctiva
    a. Are the inflammasome components (NALP3, ASC, NACHT, Caspase-1) expressed in (i) primary conjunctival tissue and (ii) primary cultured goblet cells (via western blot (WB), immunohistochemistry (IHC), and RT-PCR)?
    b. If all components are not constitutively expressed can they be induced?

2. Determine if activation of inflammasomes leads to stimulation of goblet cell secretion.

Objective 1: Determine which components of the NALP3 inflammasome are constitutively expressed and/or induced in goblet cells of the rat conjunctiva

Sub-objective 1a. Are the inflammasome components (NALP3, ASC, Caspase-1) expressed and assembled in whole conjunctiva, cultured stratified squamous cells, and cultured goblet cells (via western blot (WB) and immunohistochemistry (IHC).

Conjunctiva was removed from sacrificed rats and either homogenized or used for cell culture. Conjunctival goblet cells were cultured using a method developed in our laboratory. Conjucntival tissue was finely minced into 1-mm^3 pieces and anchored onto either scored culture dishes or glass coverslips placed within six-well culture dishes. Cell medium used to culture goblet cells consisted exclusively of RPMI-1640 medium supplemented with 10% heat- inactivated fetal bovine serum, 2 mM L-glutamine, and 100µg/mL penicillin/streptomycin. To culture stratified squamous cells, DMEM was used. The cells grew from the conjunctival tissue plug for approximately 72 hr and the conjunctival plug removed. To obtain purified cells, cells were further isolated from other epithelial cells by scraping. Goblet cells were identified by the presence of numerous secretory granules within the cells. Stratified squamous cells were larger, wing-like cells with small nuclei. Goblet cells were identified by IHC using antibodies to cytokeratin 7 and MUC5AC. Stratified squamous cells were identified by positivity to cytokeratin 4 and MUC 1. Cells were grown on 6 well plates or on coverslips as per experimental requirement.

Goblet cells were grown on coverslips and stained for IHC with antibodies against NALP3, ASC, and Caspase-1. Cell protein was extracted from cells and analysed using western blotting analysis. We found that NALP3, ASC and Caspase 1 were present by IHC in human conjunctiva, rat and human goblet cells, and rat stratified squamous cells. Using WB all these components were detected in rat conjunctiva and goblet cells. We also detected pro-IL1β. Finally we used immunoprecipitation and discovered that the components of the NALP3 inflammasome were assembled. Thus all the components of the NALP3 inflammasome were constitutively

present and active in the conjunctiva and its cells in culture. However, mature IL-1β as measured by ELISA was not secreted.

Sub-objective 1b. If all components are not constitutively expressed can they be induced?

Even though all the components of the NALP3 were constitutively expressed they did not cause mature IL-1β secretion. We thus determined if in cultured rat goblet cells the amount of the inflammasome components, as well as and pro-and active IL-1β could be increased. We first investigated if the receptors needed for activation of the inflammasome were present. Using IHC we found that the toll-like receptor (TLR) 2 was present as were the purinergic receptors P2X4 and P2X7. The dogma for most cells is that two stimuli are necessary to activate the inflammasome to cause secretion of mature IL1β. We thus incubated cultured goblet cells with lipotechoic acid (LTA) bacterial lipoprotein, ATP, or the two together. We then measured the same components as in Aim1a. To determine if the receptors were functional we measured the increase in intracellular [Ca^{2+}] using the fluorescent dye fura-2. Addition of LTA, ATP, or ATP after LTA increased the intracellular [Ca^{2+}] suggesting that the receptors were active in the cultured goblet cells. We next measured the amount of the inflammasome components by WB after stimulation with LTA, ATP, or the combination. None of these stimuli increased the expression of the components of the inflammasome or caused secretion of mature IL-1β.

LTA is a lipoprotein produced by the bacteria *Staphlococcus aureus* (*S. aureus*), which is the type of bacteria associated with most of the infections of the anterior eye. As LTA was ineffective, we stimulated goblet cells with *S. aureus* RN6390 that contained toxins. We found that *S. aureus* increased the levels of each of the components of the NALP3 inflammasome, increased the level of pro-IL-1β, activated caspase-1, and caused secretion of mature IL-1β. This result suggested that the toxins produced by *S. aureus* in addition to the lipids were necessary for the activation of the inflammasome. In addition, only one stimulus was necessary to activate the inflammasome as the components were constitutively present and assembled.

Objective 2. Determine if activation of inflammasomes leads to stimulation of goblet cell secretion.

We found that *S. aureus* induced mucin secretion from cultured conjunctival goblet cells in a time-dependent manner.

Key Research Accomplishments

- Demonstrated that the components of the NALP3 inflammasome were present in human and rat conjunctiva, both in goblet cells and stratified squamous cells
- Found that toxin-containing *S. aureus* was necessary to activate the inflammasome, the lipoprotein component was not enough
- Showed that only one stimulus instead of two was sufficient to activate the inflammasome as the components were constitutively present and assembled
- Found that *S. aureus* stimulate conjunctival goblet cell mucin secretion

Reportable Outcomes

- Submitted a manuscript for publication based on this work
- Submitted an R01 grant to NIH, the National Eye Institute based on this work. Received a fundable score

Conclusion

We conclude that conjunctival cells, especially goblet cells, protect the eye from bacterial infection by activating the NALP3 inflammasome. In the conjunctiva toxin-containing *S. aureus* can activate the already synthesized and assembled NALP3 inflammasome to secrete IL-1β that attracts neutrophils and other immune cells to remove bacteria from the ocular surface. Conjunctival goblet cells can also secreted mucin to entrap and remove the bacteria.

Aim 2: Determine if activation of the NALP3 inflammasome lead to stimulation of goblet cell secretion.

Measurment of goblet cell mucin secretion
Goblet cells grown in 24-well plates will be analyzed for mucin secretion using an enzyme-linked lectin assay as described previously (6). Cells are grown in 24 well plates and stimulated with LTA, ATP or both. Supernatants are removed and analyzed for high molecular weight glycoproteins that indicate mucin secretion. Cells are scraped and homogenized and analyzed for protein to standardize secretion. Mucins are analyzed using the lectin UEA-1 linked to horseradish peroxidase. Peroxidase activty is measured by fluorescent spectrophotometry.

Cultured goblet cells will be stimulated with different concentrations of LTA, ATP or both and at increasing times from 0 to 8 hrs. Unstimulated cells will serve as negative controls. The cholinergic agonist carbachol will be used as the positive control.

Anticipated results:
We hypothesise that in healthy individuals, bacteria induce goblet cell secretion and this initiates a protective inflammatory response by activating the NALP3 inflammasome which in turn stimulates IL 1-b that starts a protective inflammatory response. However, in inflammatory diseases, burns, and trauma, the increased exposure to pathogens, due to reduced mucin secretion from goblet cells, leads to chronic inflammasome / NALP3 activation in an effort to prevent bacterial infection, but a dangerous side effect of this protective mechanism is inflammatory induced damage to normal tissue by IL-1b.

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BUDGET CATEGORY AND BREAKDOWN	**COST ($)**
Salary for 12 months: Grade 6/7, Spinal point 28 Basic salary National Insurance Superannuation	**34,501** 28,018 2,001 4,482
Consumables: Pipets, tips, cell culture plates, gloves, tubes Antibodies Human tissue culture reagents Western blot reagents RT-PCR reagents Primers Bacterial cell culture reagents	**18,000** 3,000 5,000 2,000 2,000 1,000 2,000 3,000
Travel to present results at ARVO	**2,000**
TOTAL:	**54,501**

Title: Role of *Staphylococcus aureus* in Activation of the NLRP3 Inflammasome in Human and Rat Conjunctival Goblet Cells

Running Title: Activation of the NLRP3 Inflammasome in the Conjunctiva

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Abstract

The conjunctiva is a moist mucosal membrane that is constantly exposed to an array of potential pathogens and triggers of inflammation. The NLRP3 protein is a Nod-like receptor that can sense pathogens or other triggers, and is highly expressed in wet mucosal membranes. NLRP3 is a member of the multi-protein complex termed the NLRP3 inflammasome that activates the caspase-1 pathway, inducing the secretion of biologically active IL-1β, a major initiator and promoter of inflammation. The purpose of this study is to investigate whether the NLRP3 inflammasome is present and constitutively assembled in human and rat cultured conjunctival goblet cells, and if pro IL-1β is constitutively expressed. We report that the receptors known to be involved in the priming and activation of the NLRP3 inflammasome, the purinergic receptors P2X4 and P2X7 and the bacterial Toll-like receptor 2 are present and functional in conjunctival goblet cells. *Staphylococcus aureus*, which activates the NLRP3 inflammasome, increased the expression of the inflammasome proteins NLRP3, ASC and pro- and mature caspase-1 in conjunctival goblet cells. The biologically active form of IL-1β was detected in goblet cell culture supernatants in response to *S. aureus,* which was reduced when the cells were treated with the caspase-1 inhibitor Z-YVAD. The NLRP3 inflammasome components were also identified in rat conjunctival tissue and rat conjunctival squamous epithelial cells. We conclude that the NLRP3 inflammasome promotes inflammation in the conjunctiva via the caspase-1 pathway and that goblet cells contribute to innate immunity in the conjunctiva by activation of the NLRP3 inflammasome.

Introduction

Inflammation is both the cause and consequence of most ocular surface conditions including bacterial infections, Stevens-Johnson syndrome, giant papillary conjunctivitis, seasonal allergic conjunctivitis, neurotrophic keratitis, ocular mucous membrane pemphigoid, and alkali and thermal burns. [1] The most common ocular surface disease related to inflammation is dry eye syndrome, and over 60 million people in the USA and Europe use some form of artificial tears to alleviate the symptoms.[1] Cyclosporine and corticosteroids are non-specific anti-inflammatory agents frequently used for treatment of ocular surface inflammation, but are associated with side effects and are often unsuccessful. [2] A need exists to understand the pathways that lead to inflammation, to provide a basis for development of new, more targeted therapies.

The cause of chronic ocular inflammation is unknown, but is thought to relate to desiccating stress,[2] hyperosmolarity,[3] pro-inflammatory cytokine release from surrounding tissue,[4] blinking abnormalities,[5] and pathogen infection.[6] Goblet cells have been shown to be important in defense against ocular surface infections via mucin secretion. [7, 8] In previous studies we found atypical bacteria on the ocular surface of patients with overt inflammation[6] and demonstrated that increased bacterial flora was associated with reduced conjunctival goblet cell density, a marker of dry eye syndrome and ocular surface inflammation.[1] We also found altered levels of goblet cell mucin secretion in severe dry eye patients.[3] It was therefore of interest to investigate the role of goblet cells in regulating inflammation induced by bacteria.

Being highly exposed to the environment and at the same time highly sensitive to the damaging effects of inflammation, the ocular surface requires a carefully balanced (this could be sophisticated or simple, and we don't have a priori evidence of either at this point in the paper) mechanism to initiate inflammation only when absolutely necessary. The innate immune system has evolved to discriminate pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) from 'self'. [9] The sensing of PAMPs and DAMPs is mediated by innate immune receptors, which include Toll-like receptors (TLRs) and nucleotide oligomerization domain-like receptors (NLRs). Activation of both TLRs found on the cell surface and NLRs found in the cytosol, induce the activation of host signalling pathways, which lead to innate and adaptive immune

responses.[9, 10] The NLR family is composed of 23 family members in humans. Most have a tripartite structure that consists of a variable amino terminal domain, a centrally located nucleotide-binding oligomerization domain (Nod) that mediates the formation of self-oligomers, and a carboxy-terminal leucine-rich repeat (LRR) that detects PAMPs or DAMPs [11].

A pioneering study to investigate molecular mechanisms controlling caspase-1 activity[12] led to the identification and characterization of the 'inflammasome', an elaborate multi-protein complex whose assembly and activation is responsible for the recruitment of caspase-1 that processes IL-1β to a mature and biologically active form. The NLRP (NACHT, LRR, and pyrin domain-containing protein) 3 inflammasome consists of the NLRP3 protein, which senses intracellular triggers resulting in oligomerization.[13] NLRP3 subsequently interacts with ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)), a central adaptor protein for the inflammasome, through homotypic interactions of the pyrin domain.[12] ASC then interacts with pro-caspase-1 through homotypic interactions of the CARD domain, resulting in cleavage and activation of caspase-1, which in turn cleaves pro-IL-1β to its active form.[12, 14, 15]

NLRP3 is activated by a plethora of stimuli such as endogenous molecules including urate crystals, adenosine trisphosphate (ATP), and particulate matter such as silica and asbestos.[16-18] Other activators include bacterial stimuli such as *S. aureus*, bacterial pore-forming toxins, bacterial RNA, and bacterial cell wall components lipopolysaccharide (LPS), lipoteichoic acid (LTA) and muramyl dipeptide (MDP).[17, 19-21] The mechanism of activation is not yet fully understood, but the processing of IL-1β via the inflammasome has been demonstrated to involve two pathways.[22] First, transcription of the pro-form of IL-1β is initiated by activation of the TLR induced NFκB pathway. IL-1β is then cleaved to produce the biologically active and secreted form by the activation of the caspase-1 pathway via inflammasome activation. The purinergic receptors (P2X4 and P2X7) have been shown to be involved in inflammasome activation, initiated by DAMPs such as ATP. [17, 23] . Purinergic receptor stimulation leads to potassium efflux from the cell, and subsequent generation of reactive oxygen species.[24]

It was previously reported that NLRP3 is highly expressed in wet mucosal membranes.[25] While past studies demonstrate that goblet cells are a critical component of the innate mediated defense against ocular surface infections via mucin secretion, the objectives of the present study were to: (1) determine whether NLRP3 is expressed in conjunctival tissue, and (2) determine whether goblet cells specifically express NLRP3 and contribute to innate mediated inflammation via secretion of IL-1β. In the studies presented herein, the conjunctival goblet cell cultures were challenged with the gram positive bacterium *S. aureus,* which is commonly associated with ocular surface infections [23,24] and is a specific activator of the NLRP3 inflammasome [15, 17-19].

Materials and Methods

Animals

Male Sprague Dawley rats weighing between 125 g and 150 g were obtained from Taconic Farms (Germantown, NY). Rats were anesthetized with CO_2 for 1 min, decapitated, and the nictitating membranes and fornix removed from both eyes. All experiments conformed to the U.S. Department of Agriculture Animal Welfare Act (2007), and were approved by the Schepens Eye Research Institute Animal Care and Use Committee.

Human material

The human eyes used for immunohistochemistry were obtained from the San Diego Eye bank. Human conjunctival tissue was obtained from patients during ocular surgery using a protocol that adhered to the tenets of the Declaration of Helsinki, and approved by the Schepens Eye Research Institute Human Studies Internal Review Board. The tissue, which was normally discarded during surgery, was donated by three patients and was placed in PBS solution containing penicillin-streptomycin (300 μg/ml, Lonza, Walkersville, MD).

Cell culture

Goblet cells from rat and human conjunctiva were grown in organ culture as described previously.[26-29] Pieces of minced tissue were placed in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine (Lonza, Walkersville, MD), and 100 mg/ml penicillin-streptomycin. For squamous epithelial cells, the medium contained equal volumes of RPMI 1640 plus keratinocyte serum free medium supplemented with 1% epidermal growth factor and 0.2% insulin (Lonza, Walkersville, MD). The tissue plug was removed after nodules of cells were observed. As described previously [26-29], cells were identified as goblet cells by the following characteristics: 1) morphology by light microscopy both bright-field and following histochemical staining with periodic acid–Schiff's reagent (indicates secretory product); 2) positive staining with the lectins *Ulex europaeus*

agglutinin type I (UEA-I), specific for rat, or *Helix pomatia* agglutinin (HPA) lectin, specific for human; and antibodies to MUC5AC (all three stain secretory product) and cytokeratin 7 (detects cell body); and 3) negative staining with antibody to cytokeratin 4. Squamous epithelial cells were identified by positive staining using an antibody against cytokeratin 4 (stains cell body) and by negative staining against cytokeratin 7.

Immunohistochemistry

For immunofluorescence microscopy of intact conjunctiva, eyes were enucleated with the lids intact and fixed in 4% formaldehyde in phosphate buffered saline (PBS, 145 mM NaCl, 7.3 mM Na_2HPO_4, and 2.7 mM NaH_2PO_4 (pH 7.2) overnight at 4°C. Eyes were embedded in paraffin. Sections (6 μm) were placed on slides and kept at -20°C until use. For immunohistochemistry, human sections were deparaffinized and antigen retrieval was used prior to staining for NLRP3. For immunohistochemistry of cultured cells, primary cells were grown on glass coverslips and then fixed in methanol before use for cytokeratin analysis and fixed in 4% paraformaldehyde for all other protein analyses. Antibodies used included: UEA-I conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich, St. Louis, MO), used at a dilution of 1:500; and HPA conjugated to FITC (Pierce, Rockford, IL), used at a dilution of 1:1000. Anti-UEA-I antibodies identify the goblet cell secretory product carbohydrate α-L fucose on terminal sugars present on mucins stored in secretory granules of goblet cells. Anti-HPA antibodies similarly identify L-galactosamine on terminal sugars of mucins. Other antibodies used were anti-human NLRP3 (1:50, catalog number 804-819-C100) and anti-rat caspase-1 (1:20) (Enzo Life Sciences, Plymouth, PA); anti-rat ASC (1:50) (Santa Cruz Biotechnology, CA, USA); anti-rabbit P2X4 or P2X7 (1:50) (Alomone Labs, Jerusalem, Israel); anti-rabbit TLR2 (1:100) (Santa Cruz Biotechnology, CA). DAPI was added to the mounting medium to identify cell nuclei. Secondary antibodies were conjugated to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) and used at a dilution of 1:300. Negative controls included use of the isotype controls for anti-rabbit (Santa Cruz Biotechnology) and anti-mouse (Millipore, MA, USA) antibodies.

Measurement of $[Ca^{2+}]_i$

Cultured goblet cells were seeded onto glass-bottom 35 mm petri dishes (MatTek, Ashland, MA) and allowed to attach overnight at 37°C. Cells were then incubated with 8 μM fura 2-AM (Invitrogen, Carlsbad, CA) for 1 h at 37°C, in buffer (119 mM NaCl, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$ supplemented with 10 mM HEPES, 5.5 mM glucose, 250 mM sulfinpyrazone, and 0.5% BSA) and then stimulated with ATP (0.1 μM- 5 mM). In separate experiments cells were pre-incubated with LTA (1 μg/ml or 10 μg/ml) for 5 h, loaded with fura-2 for 1 h, and then stimulated with ATP (5 mM). Fluorescent images of cells were recorded and analyzed with a digital fluorescence imaging system (InCyte Im2, Intracellular Imaging). Peak $[Ca^{2+}]_i$ was calculated by subtracting the basal values (before the addition of agonist) from the peak calcium value.

S. aureus (RN6390) *culture and challenge of rat conjunctival goblet cells*

S. aureus (RN6390) was cultured as previously described.[27] Briefly *S. aureus* was cultured at 37°C overnight with continuous agitation. This pre-culture was then diluted and grown at 37°C to an OD595 nm of 0.5 (early log phase). After centrifugation at 500 x g for 10 min, the supernatant was discarded and bacteria resuspended in RPMI-1640 medium with 1% FBS and antibiotics – give specifics of antibiotics – could they have killed the staph? Was that the intent? If so, the cells weren't infected but rather exposed to dead bacteria. Rat conjunctival goblet cells were seeded in 12 well culture plates (500 cells per well) in medium without antibiotics 24 h prior to infection, and some cultures were preincubated with the caspase-1 inhibitor Z-YVAD (10μM) for 30 min prior to infection with *S. aureus* (Biovision, California). *S. aureus* were added at a multiplicity of infection (MOI) of 20, 40 or 60, and incubated for 6 h at 37°C in 5% CO_2. Cultures were then treated for an additional 2 h with 5mM ATP or no additions. At 8 h cell culture supernatant was collected for analysis of IL-1β by ELISA (R&D Systems), according to the manufacturer's instructions. The ELISA detected IL-1β over a range of 1 pg/ml – 2500 pg/ml. In parallel, cells were lysed in RIPA buffer and protein collected for analysis by western blot. Cell viability assays using 0.05% (w/v) trypan blue (Sigma-Aldrich, St. Louis, MO) were performed in preliminary experiments to ascertain optimum time points to examine infection.

Western blotting and immunoprecipitation experiments

Pieces of rat conjunctiva, or goblet cells cultured in 6 or 12 well plates, were lysed in RIPA buffer. The lysate was centrifuged at 10,000 x g for 10 min at 4°C. Sample buffer (4X) was added to the lysate, and proteins separated by SDS-PAGE through 10% polyacrylamide, and transferred to nitrocellulose membrane to be processed for Western blot. RIPA used for Western blotting contained 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA. For immunoprecipitation, cell lysates (2 mg/ml) were incubated with either anti-NLRP3 (1:50), anti-caspase-1 (8 μg/ml), anti-mouse IgG, or anti rabbit IgG antibodies for 2 h at 4^{o}C with agitation. Then 20 μl of 50% (v/v) slurry of Protein A agarose beads were added and left at 4^{o}C overnight with agitation. The beads were washed three times with lysis buffer, then mixed with sample buffer and heated to 90^{o}C for 5 min. The same primary antibodies used for immunofluorescence experiments, were utilized for Western blots with the following dilutions: 1:1000 for NLRP3, 1:500 for ASC, and 1:500 for caspase-11:100 for P2X4 and P2X7, 1:200 for TLR2 and 5μg/ml for IL-1β (NCI Biological Resources Branch, Rockville, MD, USA), Anti-mouse secondary antibody conjugated to HRP was from Santa Cruz (Santa Cruz, CA) and was used at a dilution of 1:2000. Alternatively, anti-rabbit secondary antibody conjugated to HRP (Millipore, Billerica, MA) was used at a dilution of 1:5000. Immunoreactive bands were visualized by the Enhanced Chemiluminescence method (Thermo Scientific, Rockford, IL).

FLICA assay for active Caspase-1analysis

Active caspase-1 was detected using a fluorescent inhibitor of caspases (FLICA, Immunochemistry Technologies, Bloomington, MN, USA), according to the manufacturer's instructions. Cells were cultured, with or without *S. aureus* as previously described and 10 μl of a 30 x FLICA solution were added. The culture plates were covered with aluminium foil and incubated 1 h at 37 °C in 5% CO_2. Following incubation, the cells were washed with 2 ml of wash buffer, and then stained with Hoechst 33342 stain (0.5% v/v) according to manufacturer's instructions. Stained cells

were viewed on an inverted phase contrast microscope equipped for epifluorescence (Eclipse TE 300, Nikon, Tokyo, Japan; UV filter with excitation 490 nm, emission >520 nm for green fluorescence of caspase-1 positive cells, and excitation 365 nm, emission 480 nm for visualization of blue fluorescence from nuclear staining by Hoechst stain). The total number of nuclei in four (40X) fields of view was counted, and the number of cells staining green (indicative of active caspase-1) was expressed as a percentage of the total.

Statistical analysis

All results are representative of three independent experiments. Western blot results were expressed as the fold increase above basal. Results are presented as mean $\pm$ SEM. Data were analyzed by Student *t* test. A p value <0.05 was considered statistically significant.

Results

The NLRP3 inflammasome components NLRP3, ASC and caspase-1 are constitutively present in the human and rat conjunctiva, and rat cornea

Immunohistochemical analysis identified the NLRP3 protein in the human conjunctiva (Figure 1A). The NLRP3 inflammasome components NLRP3, ASC and caspase-1 were also identified in the rat conjunctiva and cornea (Figure 1B). The inflammasome components appeared to be most strongly expressed throughout the epithelium of the conjunctiva, whereas in the cornea, the staining pattern appeared to be stronger in the basolateral layer.

The NLRP3 inflammasome components (NLRP3, ASC, caspase-1) and pro-IL-1β are constitutively present in human and rat conjunctival goblet cells and stratified squamous epithelial cells

Primary cultures of human and rat conjunctival goblet cells were characterized using the goblet cell specific markers MUC5AC, HPA and cytokeratin 7 as described previously [27, 28, 30]. These cultures were negative for the stratified squamous epithelial cell marker cytokeratin 4 (data not shown). All NLRP3 inflammasome components NLRP3, ASC and caspase-1 were detected by immunofluorescence microscopy in both human and rat goblet cell cultures (Figure 2A), with each demonstrating a peri-nuclear staining pattern. The presence of the NLRP3 inflammasome components in rat goblet cell lysates was confirmed by Western blot (Figure 2B). Two isoforms were detected for NLRP3 at 50 kDa and 150 kDa. Caspase-1 was detected at 45 kDa and ASC at 24 kDa. We also detected the precursor form of IL-1β in rat goblet cell cultures at 31 kDa.

Rat stratified squamous epithelial cells were cultured and characterized using the specific marker, cytokeratin 4, and were negative for the goblet cell marker, cytokeratin 7 (Figure 3A). The NLRP3 inflammasome components with the same molecular weights as in goblet cells were also detected in rat stratified squamous epithelial cells (Figure 3B).

These results demonstrate that all three components of the NLRP3 inflammasome as well as pro IL-1β are constitutively present in both goblet and stratified squamous epithelial cells cultured from rat conjunctiva.

The NLRP3 inflammasome is constitutively assembled in rat conjunctiva and rat goblet cells

Immunoprecipitation was performed on rat conjunctival tissue or primary rat goblet cell cultures to determine if the NLRP3 inflammasome was constitutively expressed (i) as a pre-assembled complex, or (ii) as individual components. Homogenates were immunoprecipiated with antibodies against NLRP3 or caspase-1, and then analyzed by Western blot analysis. In cultured goblet cells (Figure 4A) and conjunctiva (Figure 4B), we found that NLRP3 was present in the anti-caspase-1 immunoprecipitate, and caspase-1 was present in the anti-NLRP3 precipitate. Therefore, at least a portion of the NLRP3 inflammasome appears to be constitutively assembled in the rat conjunctiva and primary goblet cell cultures.

The purinergic receptors P2X4 and P2X7 and the bacterial receptor TLR2 are present in rat conjunctival goblet cells

The purinergic receptors P2X4 and P2X7 are involved in inflammasome activation via binding of extracellular ATP,[23, 24] and TLR2 is critical in the priming of the inflammasome and activated by *S. aureu*s binding.[21] All three receptors are present in both goblet and stratified squamous cells of the rat conjunctiva (Figure 5A). These receptors are also expressed in conjunctival goblet cells as determined by perinuclear staining observed using immunfluorescence microscopy (Figure 5A) and confirmed by western blotting (Figure 5B). We also identified TLR 1, 4 and 6, and cluster of differentiation 14 (CD14) on rat goblet cells (data not shown).

Measurement of $[Ca^{2+}]_i$ was used to determine if the purinergic receptors (P2X4 and P2X7) were functional in rat goblet cells. Primary cultures of rat goblet cells were loaded with fura-2 and stimulated with ATP (0.1 μM to 5 mM). ATP increased $[Ca^{2+}]_i$ in

the goblet cells in a concentration dependent manner, with ATP from 10 μM to 5 mM increasing $[Ca^{2+}]_i$ to significantly higher levels than in untreated cells (Figure 6A and B).

Rat goblet cells were also stimulated with LTA, a component of the gram positive *S. aureus* cell wall and potent activator of TLR2. [31] Stimulation with LTA (1 μg/ml or 10 μg/ml) resulted in intracellular Ca^{2+} responses that were significantly higher than untreated goblet cells (Figures 6B and D). The average peak Ca^{2+} response of rat goblet cells to LTA (1μg/ml) was 93 nM as compared to 205 nM for ATP (5 mM). Goblet cell cultures were pre-incubated with LTA (1 μg/ml or 10 μg/ml) for 5 h prior to loading with fura-2 for 1 additional hour, and then stimulated with ATP (5 mM). Preincubation with LTA did not alter the intracellular Ca^{2+} response compared to ATP alone (Figure 6D).

These experiments show that the purinergic receptors P2X4 and P2X7 and TLR2 are present and functional in rat goblet cells. They also demonstrate that bacterial lipopolysaccarhide treatment does not alter the purinergic intracellular Ca^{2+} response.

S. aureus challenge with or without ATP increases NLRP3, ASC, Caspase-1and pro-IL-1β protein expression in rat conjunctival goblet cells

Rat goblet cell cultures were challenged with *S. aureus* (RN6390) at MOI 20, 40 or 60 over 24 h and cell viability determined using the trypan blue exclusion assay. These MOIs were chosen as they were shown to be effective in stimulating IL-1 β expression in human corneal epithelial cells in a previous study.[32] Over 80% of the cells remained viable at each MOI at 8 h before a rapid decline in cell viability (Figure 7). Therefore 8 h was chosen as the end point for subsequent experiments. Goblet cells incubated with *S. aureus,* with or without ATP (5 mM), resulted in significantly increased expression of NLRP3 and ASC compared to basal unstimulated cells (Figures 8A and B). ATP treatment alone had no significant effect on the expression of these three proteins. Notably, a double band was observed for the 50 kDa isoform of NLRP3 in rat goblet cells challenged with *S. aureus,* with or without ATP. This band was absent from untreated cells or cells treated only with ATP. Similar results were obtained for caspase-1 (Figure 9A). Goblet cells challenged with *S. aureus,* with or without ATP, also had significantly increased pro-IL-1β protein expression as compared to basal levels (Figure 9B). The level of pro-IL-1β protein expression between cells challenged only with *S. aureus*, and *S.*

aureus challenged with ATP, was not significantly different, suggesting that ATP has no effect on pro-IL-1β protein expression (Figure 9B).

S. aureus challenge, with or without ATP, leads to IL-1β secretion from rat conjunctival goblet cells dependent on caspase-1 activation

The active form of caspase-1 was detected in primary cultures of rat goblet cells challenged with *S. aureus,* with or without ATP (5 mM), as determined by the FLICA assay (Figure 10A). *S. aureus* challenge alone, ATP treatment alone, or the combination of *S. aureus* with ATP treatment, resulted in significant activation of caspase-1 as compared to untreated cells. The greatest amount of caspase-1 activation was observed in cultures that were treated with the highest MOI of *S. aureus* (MOI 60 [21%]), and this activation was enhanced when ATP was added to the cultures (36%) (Figure 10B). It is important to note that there was no significant difference in cell viability between MOI 20 and MOI 60 (Figure 7).

The level of IL-1β secretion from cultures in response to ATP treatment alone was not significantly different from untreated cells Figure 11), whereas ATP alone did significantly activate caspase-1 (Figure 10). Challenge of rat goblet cells with *S. aureus,* with or without ATP, resulted in a significant increase in IL-1β secretion as compared to untreated cells (Figure 11). Cells challenged with *S. aureus* at an MOI 60 resulted in significantly more IL-1β secretion than from cells challenged with *S. aureus* at MOI 20 (40 pg/ml and 24 pg/ml respectively). IL-1β secretion from goblet cells was slightly increased when ATP was added to *S. aureus* stimulated goblet cell cultures, compared to *S. aureus* alone (34 pg/ml for *S. aureus* MOI 20 + ATP compared to 24 pg/ml for *S. aureus* MOI 20 alone; and 55 pg/ml for *S. aureus* MOI 60 + ATP compared to 40 pg/ml for *S. aureus* MOI 60 alone). Activation of caspase-1 in the goblet cells challenged with *S. aureus,* with or without ATP, is in concordance with IL-1β levels that were detected in the goblet cell supernatants. However, the results do not concord when the cells were treated with ATP alone.

No significant difference was noted in IL-1β secretion between cultures treated with ATP alone, and cultures treated with ATP and the caspase-1 inhibitor Z-YVAD (Figure 11). The addition of Z-YVAD to the rat goblet cell cultures challenged with *S.*

aureus, with or without ATP, resulted in a significant decrease in IL-1β secretion with IL-1β secretion dropping from 54 pg/ml (no inhibitor present) to 12 pg/ml (inhibitor present) for cultures incubated with *S. aureus* MOI 60 and ATP (Figure 11). These results provide evidence that *S. aureus* activates the secretion of IL-1β via the caspase-1 pathway and suggests that although ATP alone activates the caspase-1 pathway, it does not activate the secretion of IL-1β in rat goblet cells.

Discussion

We found that the NLRP3 inflammasome is present in rat conjunctival goblet cells and can be activated by *S. aureus*. The NLRP3 inflammasome appears to contribute to inflammation in the conjunctiva by activating the secretion of IL-1β via the caspase-1 pathway. NLRP3 was previously reported to be highly expressed in wet mucosal epithelium within the cytoplasm of cells.[25] Kummer and colleagues (22) speculated (or concluded) that the occurrence (or abundance) of NLRP3 in such sites allows rapid sensing of invading pathogens or other danger signals, thereby triggering an innate immune response. The ocular surface is highly exposed to the environment, yet to maintain visual clarity, it is required to balance inflammation with immune privilege. Previous work demonstrated that mouse eyes express high levels of NLRP3 mRNA compared to other body tissues.[33] An additional study also reported NLRP3 mRNA expression in the whole eyes of mice challenged with LPS, but not in unchallenged eyes. NLRP3 mRNA was detected in human corneal epithelial cells, but was not detected at the protein level.[34] Our results show that NLRP3 protein, as well as the other constituent components of the NLRP3 inflammasome, are highly expressed in the conjunctival epithelium, and more specifically in conjunctival goblet and stratified squamous epithelial cells. Moreover, we show that not only are the components of the NLRP3 inflammasome present, but that the inflammasome is constitutively pre-assembled in goblet cells. This pre-assembly suggests that an initiation step that is usually required for response by immune cells and other tissues, is not required in the conjunctiva, which may be necessary to affect a rapid response in sites that are highly exposed to the environment. . We are unaware of other studies that have examined the assembly of the NLRP3 inflammasome in epithelial tissues.

In addition to the constitutive expression of NLRP3, we found that TLR2 is also expressed on the surface of rat goblet cells in culture, as were the purinergic receptors P2X4 and P2X7. Purinergic receptors are known to be activated by signals such as ATP, and TLRs by bacterial cell wall components, such as LTA. Both *S. aureus* and ATP are potent activators of the NLRP3 inflammasome, and we showed that ATP or LTA

stimulation of goblet cells increases $[Ca^{2+}]_i$. Thus TLR2, P2X4, and P2X7 are indeed functional in goblet cell cultures, and may play a role in the priming and activation of the NLRP3 inflammasome.

In this present study *S. aureus* challenge of rat goblet cells led to the activation of the NLRP3 inflammasome, demonstrated by increased expression of NLRP3, pro and active caspase-1, ASC, and the secretion of IL-1β into the culture supernatants. We found that ATP, in combination with *S. aureus,* significantly enhanced IL-1β secretion compared to *S. aureus* alone, but this only reached significance in cultures treated with the higher MOI (MOI 60) (p=0.05). This may be due to the amount of the pro form of IL-1β that is processed by the cell and made available for secretion, such that higher MOIs of *S. aureus* would result in more pro IL-1β being processed.. Western blots revealed that pro IL-1β is present in rat goblet cells constitutively, which suggests that a reserve is present and ready to become activated and released upon encounter with a danger trigger or pathogen. When the goblet cultures were stimulated with ATP alone, a small amount of IL-1β was secreted from the cultures into the supernatant, but this response failed to reach statistical significance when compared to untreated cells. Again this low response may be due to the limited amount of pro IL-1β available for processing. A recent study[35] reported that NFκB activation was required for both pro IL -1β and NLRP3 protein expression and that NLRP3 inflammasome activation was dependent on the level of NLRP3 expression. This study found that the NLRP3 inflammasome was only activated in mouse macrophages that were first primed with a TLR agonist to activate the NFκB pathway, leading to the expression of NLRP3 and subsequent activation of the inflammasome via a NLRP3 agonist such as ATP. The results from our study are in agreement, and provide evidence that although the inflammasome is constitutively assembled, and pro IL-1β is constitutively present in the conjunctiva, two signals are still required for IL-1β secretion. These signals are: 1) a *TLR2 agonist* such as LTA from *S. aureus* to activate the NFκB pathway, which leads to enhanced expression of pro IL-1β and NLRP3; and 2) a *NLRP3 inflammasome agonist* such as a toxin (*e.g. S. aureus* alpha toxin, (the RN6390 strain used in this study produces alpha toxin)) or a danger signal (*e.g.* ATP). Our study shows that *S. aureus* increases the synthesis of pro IL-1β and also increases the secretion of IL-1β. It remains uncertain if ATP enhances this secretion of IL-

1β in the conjunctiva after challenge with *S. aureus.* However, we propose that bacterial toxins, which have been previously shown to activate the inflammasome [17, 19, 21] may be responsible for the activation the NLRP3 inflammasome and hence secretion of IL-1β in rat goblet cells.

The specific mechanism of NLRP3 inflammasome activation is currently under study. Several stimuli are known to activate the inflammasome and it is not clear as yet if all stimuli use the same method of activation. Tschopp and colleagues [12] were the first to coin the term inflammasome, and later showed that it could be activated by bacterial muramyl dipeptide. Further research has demonstrated the activation of the NLRP3 inflammasome in response to bacteria such as *S. aureus*.[17, 19-21] Recent literature suggests that TLR agonists such as LTA first activate transcription of IL-1β via the NFκB pathway; however processing of IL-1β to the active form appears to be initiated by *S. aureus* toxins, such as alpha toxin, which activates the inflammasome through an unknown mechanism.[17, 19, 21] Our study confirms these findings. We demonstrated that *S. aureus* lead to an increase in pro-IL-1β protein expression and also to an increase in the expression of the NLRP3 inflammasome proteins. The *S. aureus* RN6390 strain used, which is known to produce toxins[32], also activated the caspase-1 pathway leading to IL-1β secretion from the cultures. Blocking of the caspase-1 pathway resulted in reducing the secretion of IL-1β. Thus, in conjunctival goblet cells, *S. aureus* is sufficient to activate both the NLRP3 inflammasome and IL-1β secretion.

NLRP3 was found in whole conjunctivas and primary cell cultures of conjunctival squamous epithelial cells and goblet cells in 150 kDa and 50 kDa forms. Different NRLP3 isoforms have been detected in immune cells including neutrophils, monocytes and dendritic cells.[25] The significance of the various isoforms is currently not known, but differences are thought to be related to the number of leucine rich repeats present in NLRP3.[36] Activation of the NLRP3 inflammasome in rat goblet cells by *S. aureus* resulted in the appearance of a double band at 50 kDa. The appearance of a double band has previously been observed for a 130 kDa NLRP3 isoform.[25] The double band suggests that the cellular responses to *S. aureus* somehow result in the post translational modification of NLRP3, and perhaps activation of caspase-1.

Our data indicate that the NLRP3 inflammasome plays a role in initiating/promoting inflammation in the conjunctiva by activating IL-1β. The molecular mechanisms of how NLRP3 recognizes activators of the inflammasome in the conjunctiva remain to be elucidated, as does the sequence of events leading to ocular surface inflammation via the NLRP3 inflammasome. The inflammasome has been associated with specialized forms of cell death, pyronecrosis [37] (caspase-1 independent) and pyroptosis,[38] which may occur in cases of exacerbated inflammation. Ocular surface inflammation is associated with a reduced goblet cell density.[7, 8] We therefore hypothesize that normally the NLRP3 inflammasome is constitutively expressed for pathogen/danger surveillance, and activates an inflammatory response when triggered in order to protect the cell. The NLRP3 inflammasome may play a pathophysiological role in chronic inflammatory states by inducing cell death when the acute inflammatory response fails. In the conjunctiva, subsequent goblet cell death may lead to lack of mucin on the ocular surface, which can enhance inflammation. [1] We conclude that the NLRP3 inflammasome is present and constitutively assembled in the conjunctiva. The NLRP3 inflammsome can be activated in goblet cell cultures by challenge with a strain of *S. aureus* that is known to produce toxins associated with NLRP3 inflammasome activation. With further research, the NLRP3 inflammasome may prove to be a valuable target in development of new and more specific therapies for ocular surface inflammation.

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Figure Legends

Figure 1: Human conjunctiva and rat conjunctiva and cornea constitutively express the NLRP3 inflammasome components. Human conjunctiva was analyzed by immunohistochemistry and NLRP3 was shown to be highly expressed in the epithelium as indicated by pink staining (A). Arrows indicate epithelial layer of conjunctiva; arrowheads indicate goblet cells. Isotype controls were negative. Rat conjunctiva or rat corneal sections were analyzed by immunofluorescence microscopy (B) and all three of the inflammasome components NLRP3, caspase-1, and ASC were identified as demonstrated by the red peri-nuclear staining. The green UEA staining indicates goblet cell secretory product, denoting the location of goblet cells in the conjunctiva. Arrows indicate goblet cells. Anti-mouse isotype controls (B) were negative as were the anti-rabbit isotype controls (not shown).

Figure 2: Constitutive expression of the NLRP3 inflammasome components in human and conjunctival goblet cells. Primary cultures of human or rat goblet cells were analyzed by immunofluorescence microscopy after staining with antibodies against inflammasome components NLRP3, caspase-1, and ASC (**A**). All three components were identified as indicated by the red peri-nuclear staining pattern. Anti-rabbit isotype controls (shown) and anti-mouse isotype controls (not shown) were negative. Results were confirmed by western blot analysis in lysates from rat goblet cells (**B**). Lanes 1-3 are samples from separate animals.

Figure 3: Constitutive expression of the NLRP3 inflammasome components in cultured rat conjunctival stratified squamous cells. Cultured stratified squamous cells were identified by morphology, expression of cytokeratin 4 and lack of expression of cytokeratin 7 (**A**). These cells were homogenized and subjected to western blot analysis for constitutive expression of inflammasome components NLRP3 and caspase-1, and pro-IL-1β (**B**). Lanes 1-3 in blots are samples from separate animals.

Figure 4: The NLRP3 inflammasome is constitutively assembled in the rat conjunctiva and cultured rat goblet cells. Lysates from rat conjunctiva (**A**) or cultured rat goblet cells (**B**) were immunoprecipitated with antibodies against caspase-1 (lane 1), NLRP3 (lane 3), anti-rabbit IgG, or anti-mouse IgG isotype control antibodies (lanes 2 and 4), and precipitates were analysed by western blot for presence of NLRP3 or caspase-1.

Figure 5: Purinergic receptors P2X4 and P2X7, and TLR2, are expressed in the rat conjunctiva and rat goblet cell cultures. All three receptors were identified by red immunoflourescent staining (**A**). UEA stains goblet cell secretory products green, allowing the identification of goblet cells within the conjunctiva. Anti-rabbit isotype controls were negative. Results for rat goblet cells were confirmed by western blot (**B**). Lanes 1-3 in B represent separate animals.

Figure 6: The purinergic receptors P2X4 and P2X7, and TLR2 are functional in cultured rat goblet cells. Rat goblet cells were loaded with fura-2 and then stimulated with ATP and intracellular calcium response measured. A typical trace from one experiment with ATP (5 mM, **A**) or lipoteichoic acid (LTA, 10 μg/ml, **B**) is representative of 3 animals. Representative photographs of a single field of cells after treatment with ATP or LTA, with warmer colors indicating intracellular Ca^{2+} increase, are shown as insets. Peak $[Ca^{2+}]_i$ calculated for ATP (0.1 μM - 5 mM) from 3 experiments, is shown in **C**. Peak $[Ca^{2+}]_i$ for goblet cells, also preincubated with LTA for 5 h and then loaded with fura-2 for 1 additional h before addition of ATP (5 mM), calculated from 3 experiments, is shown in **D**. Results are expressed as mean ± SEM. * indicates significance of $p<0.05$ from no addition (0).

Figure 7: Effect of *S. aureus* on Goblet Cell Viability. Cultured rat goblet cells were incubated with *S. aureus* at MOIs of 20, 40, and 60 for 0 - 24 h, and cell viability was determined by trypan pan blue exculsion. Data is mean ± SEM from 3 independent experiments.

.**Figure 8:** Effect of *S. aureus* on NLRP3 and ASC expression by cultured rat goblet cells. Cultured rat goblet cells were incubated with *S. aureus* (MOI 20 or 60) for 6 h. Some cultures were treated for an additional 2 h with ATP (5 mM). Cell lysates collected and analysed by western blot. Representative blots are shown in **A** and **C**. Blots were scanned, quantified, and means ± SEM from 3 independent experiments are shown in **B** and **D**. * indicates significance of $p < 0.05$ compared to no addition which was set to 1. Note that densitometry results for NLRP3 combine all three bands observed per lane.

Figure 9. Effect of *S. aureus* on Caspase- 1 and IL-1β protein expression. Cultured rat goblet cells were incubated with *S. aureus* (MOI 20 or 60) for 6 h. Some cultures were treated for an additional 2 h with ATP (5 mM). Cell lysates were collected and analyzed by western blot. Representative blots are shown in **A** and **C**. Blots were scanned and mean ± SEM from 3 independent experiments are shown in **B** and **D**. * indicates significance of $p < 0.05$ compared to no addition (basal) which was set to 1.

Figure 10: Active caspase-1 expression in rat goblet cells treated with *S. aureus* and ATP: Primary cultures of rat goblet cells were incubated with *S. aureus* (MOI 20 or 60) for 6 h. Some cultures were treated for an additional 2 h with ATP (5 mM). The FLICA reagent, which detects only active caspase-1, was added followed by the nuclear Hoescht stain and viewed by immunofluorescence microscopy. Representative micrographs are shown in **A**. The total number of nuclei in four fields of view and the number of cells with staining green (indicative of active caspase-1) were counted. Data is expressed as mean ± SEM from 3 independent experiments, and are shown in **B**. ***** indicates significance of $p < 0.05$ compared to no addition, which was set to 1. Magnification 40X.

Figure 11: Effect of Inhibition of Caspase-1 on IL-1β secretion in response to *S. aureus* and ATP: Primary cultures of rat goblet cells were treated with or without the caspase-1 inhibitor Z-YVAD for 1 h and then incubated with *S. aureus* (MOI 20 or 60) for 6 h. Some cultures were treated for an additional 2 h with ATP (5 mM). Culture supernatant was removed and analyzed for IL-1β by ELISA. Data is expressed as mean ± SEM from 3 independent experiments. ***** indicates significance of $p < 0.05$ compared to no addition.