2021

Glycoprotein 340 Expression in Dry Eye Disease and Ocular Surface Infection

Kwaku Antwi Osei
University Of Alabama At Birmingham

GLYCOPROTEIN 340 EXPRESSION IN DRY EYE DISEASE AND OCULAR SURFACE INFECTION

by

KWAKU ANTWI OSEI

JASON J. NICHOLS, COMMITTEE CHAIR
CHAMPION DEIVANAYAGAM
STEVEN J. PITTLER
MARK D.P. WILLCOX
HUI WU

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2021

GLYCOPROTEIN 340 EXPRESSION IN DRY EYE DISEASE AND OCULAR SURFACE INFECTION

KWAKU ANTWI OSEI

VISION SCIENCE

ABSTRACT

Glycoprotein 340 (Gp340) is a 340-kDa multi-domain pattern recognition receptor (PRR) belonging to the scavenger receptor cysteine-rich superfamily of proteins. On the ocular surface, Gp340 is expressed in the tear film, lacrimal gland, cornea, and conjunctiva. By their nature, PRRs detect pathogen-associated molecular patterns (PAMPs) on microbial organisms and damage-associated molecular patterns (DAMPs) released from injured, stressed, necrotic, and apoptotic cells. This, in turn, induces the expression of nuclear factor-κB- and the interferon regulatory factor-dependent expression proinflammatory cytokines and chemokines, eliciting both innate and adaptive immune response activation.

Thus, as a PRR, Gp340 has the potential to modulate microbial infection and inflammatory processes on the ocular surface. Consistent with this hypothesis, Gp340 expression in tear fluid has been shown to be dysregulated in dry eye, an ocular surface disease that is characterized by tear hyperosmolarity and chronic inflammation. In addition, Gp340 inhibits twitching motility of *P. aeruginosa* and promotes corneal epithelial wound healing. These findings suggest that Gp340 plays an important role in the pathophysiology of dry eye disease and could modulate events related to ocular surface infection.

The goals of this dissertation research were to (1) investigate the expression of Gp340 in human corneal epithelial cells (HCECs) under hyperosmolar stress, and its role in the pathophysiology of dry eye inflammation, (2) determine the effect of Gp340 on the adhesion of infectious *P. aeruginosa* and *S. aureus* strains to contact lens polymers, and (3) examine the expression pattern of Gp340 in HCECs infected with *P. aeruginosa* and *S. aureus*, two major microbial keratitis etiological agents.

Chapter 1 presents an overview of dry eye disease, Gp340, and Gp340's known roles in dry eye, infection, and contact lens-related adverse events. It highlights the justification and need for this research, specific aims, and hypotheses. Chapter 2 is an extensive review of the expression and roles of Gp340 in wet-surfaced mucosal immunity and on the ocular surface. Chapters 3 – 5 described studies on the three specific aims which tested the hypotheses of the proposed research. Summary and conclusions are provided in Chapter 6 with an outlook for future research.

Key words: glycoprotein 340, dry eye, hyperosmolar stress, inflammation, contact lens, infection

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure *Page*

INTRODUCTION

GLYCOPROTEIN 340 IN MUCOSAL IMMUNITY AND OCULAR SURFACE

HYPEROSMOLAR STRESS UPREGULATES GLYCOPROTEIN 340 AND THE COMPLEMENT FACTOR C1Q EXPRESSION IN HUMAN CORNEAL EPITHELIAL CELLS

GLYCOPROTEIN'S SCAVENGER RECEPTOR CYSTEINE-RICH DOMAIN PROMOTES ADHESION OF *STAPHYLOCOCCUS AUREUS AND PSEUDOMONAS AERUGINOSA* TO CONTACT LENS POLYMERS

GLYCOPROTEIN 340 EXPRESSION IN HUMAN CORNEAL EPITHELIAL CELLS EXPOSED TO *PSEUDOMONAS AERUGINOSA* AND *STAPHYLOCOCCUS AUREUS* INFECTION

LIST OF TABLES

Table *Page*

GLYCOPROTEIN'S SCAVENGER RECEPTOR CYSTEINE-RICH DOMAIN PROMOTES ADHESION OF *STAPHYLOCOCCUS AUREUS AND PSEUDOMONAS AERUGINOSA* TO CONTACT LENS POLYMERS

LIST OF ABBREVIATIONS

ADDE	aqueous deficient dry eye
APC	antigen-presenting cell
C1q	complement component 1q
CCL	C-C motif chemokine ligand
cGVHD	chronic graft versus host disease
CLDE	contact lens-related dry eye
CUB	C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein-1
CX3CL	C-X3-C motif chemokine ligand
CXCL	C-X-C motif chemokine ligand
DAMPS	damage-associated molecular patterns
DED	dry eye disease
DEW	Dry Eye Workshop
DMBT1	deleted in malignant brain tumor 1
DNA	deoxyribonucleic acid
EDE	evaporative dry eye
ELISA	enzyme-linked immunosorbent assay
Gp120	glycoprotein 120
Gp340	glycoprotein 340

HCEC	human corneal epithelial cell
HOS	hyperosmolar stress
IAV	influenza A virus
ICAM-1	intracellular adhesion molecule-1
IEC	intestinal epithelial cell
IFN	type 1 interferon
IL	interleukin
LFA-1	leukocyte functional antigen 1
MAPK	mitogen-activated protein kinase
MBL	mannose-binding lectin
MGD	meibomian gland dysfunction
MHC1	major histocompatibility complex 1
MMP9	matrix metalloproteinase 9
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor-kappa B
NLR	NOD-like receptor
NOD	nucleotide oligomerization domain
OSDI	ocular surface disease index
PA	*Pseudomonas aeruginosa*
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
RNA	ribonucleic acid

SA	Staphylococcus aureus
SID	SRCR interspersed domain
siRNA	small interfering RNA
SP-D	surfactant protein D
SRCR	scavenger receptor cysteine-rich
SRCRP2	SRCR peptide 2
SS	Sjogren's syndrome
SV40	simian virus 40
TFF	trefoil factor
TFOS	Tear Film and Ocular Surface
TLR	toll-like receptor
TNFα	tumor necrosis factor α
ZP	zona pellucida

CHAPTER 1 – INTRODUCTION

The Ocular Surface

The ocular surface consists of structures of the eye and its adnexa. These include the tear film, cornea, conjunctiva, eyelids, eyelashes, lacrimal glands, and meibomian glands.[1-3] The ocular surface is covered by a continuous sheet of epithelium that lines the cornea, the anterior globe and tarsi and extends to the mucocutaneous junctions of the lid margins.[2,3]

Tear film

Tears are produced by the main lacrimal gland and the accessory glands of *Krause* and *Wolfring* in the conjunctiva. The main lacrimal gland is innervated by both parasympathetic and sympathetic nerves.[4-6] These nerves are located in close proximity to acinar, ductal, and myoepithelial cells, and blood vessels.[4-6] Stimulation of the lacrimal gland and secretion occur via the cornea-trigeminal nerve-brain stem-facial nerve-lacrimal gland reflex arc.[6] This stimulation of the ocular surface activates the afferent corneal and conjunctival sensory nerves, which subsequently results in activation of the efferent parasympathetic and sympathetic nerves to stimulate secretions from the acinar and tubular cells in the lacrimal gland.[6,7] There are four types of tears, namely: basal, reflex, emotional, and closed-eye. The basal, reflex, and emotional tears are all produced by the lacrimal glands via the neural arc, whereas the basal tears are constitutively produced to coat the ocular surface, and in addition, the reflex type is produced when

either the ocular surface or the reflex arc is stimulated.[6] Tear turnover rate is approximately 16 ± 5%/min.[6]

The tear film consists of three indistinct layers. These are the lipid, muco-aqueous, and glycocalyx (Figure 1). A loss of homeostasis in tear production and biochemistry can results in a dysfunctional ocular surface.[1,2,6]

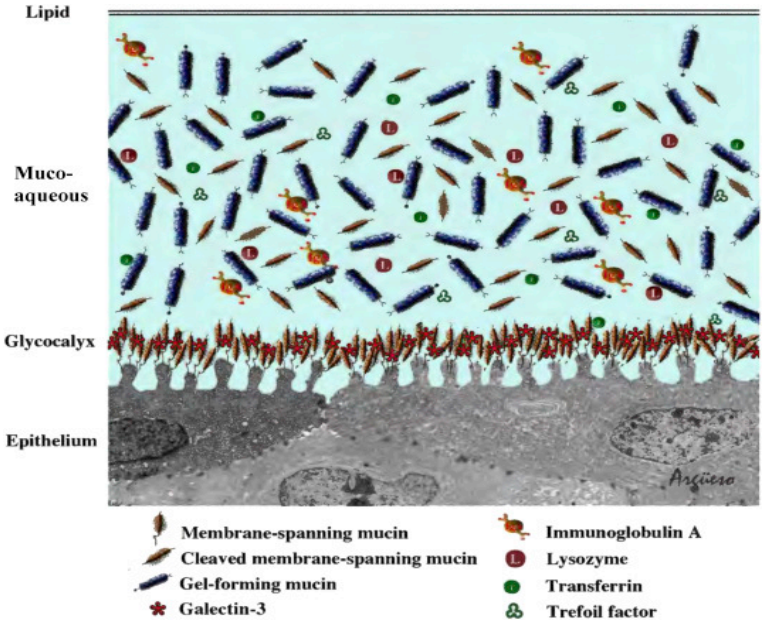

Figure 1. The structure of the precorneal tear film.
The tear film consists primarily of lipid and mucoaqueous layers linked to the ocular surface epithelium at the glycocalyx. The lipids are derived from meibum produced by the meibomian glands. The mucoaqueous layer consists of aqueous secretions from the main and accessory lacrimal glands, and gel-forming mucins produced by the conjunctival goblet cells. The glycocalyx is composed of transmembrane mucins such as MUC1, MUC4, MUC16.
Note: From "TFOS DEWS II Tear Film Report" by Willcox et al., 2017, Ocular Surface, 15:3, p. 380. Copyright 2017 by the Ocular Surface. Reprinted with permission.

Firstly, the lipid layer consists of polar and non-polar lipids derived from meibum that is secreted by the meibomian glands. Meibum is composed of approximately 95% non-polar lipids and 5% polar lipids.[6] The non-polar lipids in humans consist of 30 – 50 mol% of wax esters, 30 – 45 mol% of cholesterol esters, and about 2 mol% of

triglycerides.[3,6,8,9] The most abundant polar lipid in meibum secretion is the (O-acyl)-ω-hydroxy fatty acids, which constitute about 4 mol% of total meibum.[6,8] On the other hand, the 5% polar lipids are known to decrease the surface tension of the air-tear interface and enhance the spread of the lipid layer. The non-polar lipids are also known to increase the compressibility and stability of the lipid layer.

Secondly, the mucoaqueous layer contains ions and numerous proteins derived from the lacrimal gland, and gel-forming mucins that are secreted by the conjunctival goblet cells. Currently, approximately 1800 proteins have been identified in the tear film.[6] While over half of the proteins are intracellular or plasma membrane-associated, those that are differentially modulated in ocular surface diseases are largely extracellular in nature.[6] Most importantly, the ionic concentrations of the tear film constitute tear osmolarity.[2,6]

Thirdly, the glycocalyx is the boundary between the ocular surface epithelium and the tear film. It consists of several transmembrane glycoproteins including the membrane-associated mucins, MUC1, MUC4, MUC16.[2,6] The glycocalyx protects the ocular surface against mechanical and chemical damage, prevents pathogen entry, reduces friction during blinking, and maintains the hydrophilicity of the ocular surface.[2,6]

Conjunctiva

The conjunctiva is a mucous membrane composed of non-keratinizing squamous epithelium, intermixed with goblet cells, Langerhans' cells, and melanocytes.[2,10,11] It guards against microbial invasion of the ocular surface and plays a role in immune surveillance. The conjunctival epithelium consists of both tight and adherens junctions.[2]

These protect the ocular surface against shear stress and act as a barrier against the external environment. The conjunctival epithelial cells produce water, electrolytes, mucins, and functional proteins including aquaporins that are involved in water translocation between the conjunctiva and the tear film.[2,12,13]

The conjunctival goblet cells are plump, rounded cells scattered throughout the epithelium, and extending the entire thickness of the stratified epithelium to the apical surface.[2,10] They secrete the gel-forming mucin, MUC5AC, which has a high water-binding potential and hence convert the aqueous tears into a mucoaqueous gel that form the bulk of the tear volume and moisturize the ocular surface.[2,10] The mucin component of the mucoaqueous layer have other functional roles such as inhibiting microbial attachment to the epithelium and binding antimicrobial proteins and peptides.[2,14]

Cornea

The cornea is the anteriormost part of the eyeball, comprising about one-sixth of the total size of the eyeball. The cornea is horizontally oval, measuring approximately 11–12 mm horizontally and 9–11 mm vertically. The corneal horizontal diameter averages 11.71 ± 0.42 mm. The corneal diameter ranged from 11.04–12.50 in males and 10.7–12.58 in females. [15,16] The cornea is approximately 0.5 mm thick at the center and gradually increases in thickness toward the periphery.[17] The shape of the cornea is prolate; flatter in the periphery and steeper centrally, where the anterior curvature is 7.8 mm and posterior curvature is about 6.5 mm.[15]

The cornea is made up of both cellular and acellular components. The cellular components include the epithelial cells, keratocytes, and endothelial cells. The acellular

component includes collagen and glycosaminoglycans.[15] The human cornea consists of five layers, namely – epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium (Figure 2).

Epithelium. The epithelium is the first barrier to the outside environment. Embryologically, the corneal epithelium is derived from surface ectoderm between five and six weeks of gestation. It is composed of nonkeratinized, stratified squamous epithelium that is 4 to 6 cell layers thick, approximately 40 – 50 μm[17], and is characterized by extreme uniformity from limbus to limbus. The corneal epithelium has a close symbiotic relationship with the overlying tear film both anatomically and physiologically, where the gel-forming mucins produced by the conjunctival goblet cells interact with the corneal epithelial glycocalyx that allows the hydrophilic spread of tears on the ocular surface.[2,17] Positioned between basal epithelial cells and the stroma is the corneal epithelial basement membrane (BM). This is a specialized extracellular matrix that anchors the epithelial cells to the stroma and provides scaffolding during embryonic development, migration, and differentiation.[18] The BM is 40–60 nm in thickness and is made up of Type IV collagen and laminin secreted by basal cells.[15]

The Bowman's layer. The Bowman's layer is an acellular, non-regenerating layer beneath the epithelial basement membrane, approximately 8–12 μm in depth and decreases in thickness over time. It is made up of Type I and V collagen as well as proteoglycans.[15,18] The collagen fibrils are distributed such that their posterior surfaces merge with the anterior stroma, leaving a smooth anterior surface. [18,19] This Bowman's

layer contributes to maintaining the shape of the cornea and may also play a role in protecing the subepithelial nerve plexus.[18]

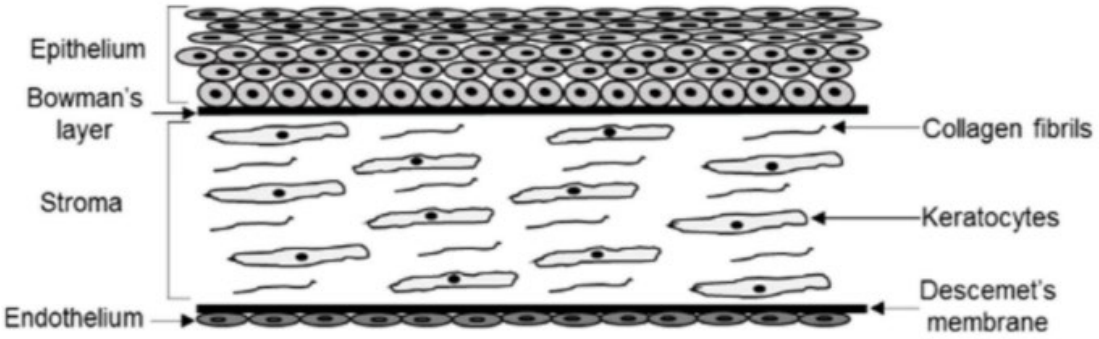

Figure 2. A cross-sectional schematic representation of the human cornea.
The corneal epithelium is highly regenerative and consists of non-keratinized, stratified squamous epithelium that is 4 – 6 cell layers thick, approximately 40 – 50 μm. The Bowman's layer is an acellular, non-regenerating layer composed primarily of Type I and V collagen as well as proteoglycans. Contributing about 90% of the corneal thickness, the stroma is about 500 μm thick and composed of keratocytes and extracellular matrix. The Descemet's membrane is essentially the basement membrane of the corneal endothelium, consisting banded and non-banded layers. The endothelium is a single polyhedral cell layer that maintains the cornea's state of deturgescence via ionic pumps.
Note: From "LCAT, ApoD, and ApoA1 Expression and Review of Cholesterol Deposition in the Cornea" by Flores et al., 2019, Biomolecules, 9:12, p. 785. Copyright 2017 by Biomolecules.

Stroma. The stroma is approximately 500 μm in thickness and represents an estimated 90% of the corneal anterior–posterior axis. The stroma provides structural support to the cornea and transparency by facilitating the passage of light through the collagen fibrils in a manner that prevents scattering. The stromal layer is made up of keratocytes and extracellular matrix (ECM). The keratocytes maintain the ECM environment by synthesizing collagen, glycosaminoglycans, and matrix metalloproteases, which are all crucial components in preserving stromal homeostasis.[17,18] The ECM is composed of Type I, III, V, VI collagens and glycosaminoglycans, water, and inorganic

salts.[15,18] These glycosaminoglycans in the stroma are predominantly composed of keratan sulfate, and to a much lower extent, chondroitin sulfate, and dermatan sulfate.

Descemet's membrane. The Descemet's membrane is the basement membrane of the corneal endothelium. It is relatively homogenous with a fine granular appearance. It is about about 3 μm thick in children and thickens to about 10 μm in adults. [17,18] This membrane is composed of two layers – an anterior banded layer, and a posterior non-banded layer. The banded layer is developed by collagen lamellae and proteoglycans and detected the fetal cornea as early as 12 weeks of gestation.[18,20] The collagen fibrils in this layer demonstrate a lattice-like configuration with periodic banding at 110 nm intervals. The non-banded layer is laid down by endothelial cells and thickens over decades.[18] The Descemet's membrane contributes to maintaining corneal dehydration.

Endothelium. The corneal endothelium consists of a single layer of flat, polygonal cells lining the posterior cornea. These cells are approximately 5 μm thick and 20 μm in diameter.[18] Endothelial cell density continues to change throughout life, from second to eighth decades of life.[15,17,21] Adjacent cells share lateral interdigitations and possess gap and tight junctions along their lateral borders. These lateral membranes have a high density of Na^{+}/K^{+-}ATPase pump sites.[17,22]. The basal side of the endothelium contains several hemidesmosomes that promote endothelial adhesion to the Descemet membrane. These endothelial cells keep the corneal stroma in a state of deturgescence (78% water content).[17,23,24] This dehydration is mediated by a pump–leak mechanism as

fluid moves from the corneal stroma down the osmotic gradient from a relatively hypo-osmotic stroma toward a relatively hypertonic aqueous humor.[17,24,25]

Dry Eye Disease: Definition and Classification

The definition of dry eye has evolved over the years. Based on a consensus from the NEI/Industry working group on Clinical Trials in Dry Eye, dry eye was first defined in 1995 as a disorder of the tear film resulting from tear deficiency or excessive tear evaporation which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort.[1,26] As understanding of the pathophysiology of dry eye improved, the Tear Film and Ocular Surface (TFOS) Dry Eye Workshop (DEWS), in 2007, redefined dry eye as a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface, and characterized by increased osmolarity of the tear film and inflammation of the ocular surface.[27] Realizing the need to recognize the multifactorial nature of dry eye and the fact tear film instability, hyperosmolarity, ocular surface inflammation and damage play crucial etiological roles, TFOS DEWS II, in 2017, provided a revised dry eye definition as "a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles."[1]

Based on underlying etiologies, dry eye disease is classified into three main types, namely, aqueous deficient dry eye (ADDE), evaporative dry eye (EDE), and mixed dry eye.[1,27-29] The aqueous deficient type arises from decreased tear volume due to deficient

lacrimal function (secretion or drainage).[2,27,28,30,31] The etiologies of ADDE include Sjögren's syndrome, intrinsic lacrimal gland deficiency, advanced age, inflammatory and other lacrimal gland infiltration, and lacrimal gland obstruction.[1,2,27] On the other hand, EDE is due to rapid evaporation of tears resulting from decreased quality of the tear lipid layer. It is mainly caused by diseases of the meibomian glands which include meibomian gland dysfunction (MGD), genetically determined meibomian gland diseases (ectrodactyly syndrome, Turner syndrome, meibomian agenesis and dystichiasis, anhydrotic ectodermal dysplasia, etc), and disorders of lid aperture, congruity, dynamics (example: Parkinson's Disease).[1,2,27,29] Mixed dry eye arises from the combined involvement of the lacrimal and meibomian glands, and it exhibits features of both ADDE and EDE.[1,2]

Evidence from epidemiological and clinical studies suggest that the preponderance of dry eye is evaporative in nature.[1,2,32] Either ADDE or EDE can occur without any signs of the other. However, as dry eye progresses, it is increasingly likely that characteristic signs of both ADDE and EDE will become evident.[1,33]

Epidemiology of Dry Eye Disease

Several studies have reported the prevalence rates for dry eye, and there exists a high variability in these reports.[32,34-49] Two main reasons account for this variability. Firstly, different dry eye definition criteria are used in epidemiological studies. While some studies define dry eye based only on symptoms, others rely on the results of dry eye diagnostic tests, or a combination of both.[32] Secondly, there exists heterogeneity of the

populations investigated in these studies.[32] These hinder the generalizability of the reported prevalence rates.

Studies profiling the prevalence of dry eye based on symptoms (symptomatic dry eye) define dry eye using criteria such as frequency of symptoms, self-reported diagnosis, and cut-off value of dry eye symptom questionnaires.[32] Based off symptoms, population-based studies in the Southeastern parts of Asia reported dry eye prevalence rates ranging from 20 to 52.4%.[35,36,38-40] In the United States, Spain, and the United Kingdom, prevalence rates of 14.5%[43] and 18.4,[37] and 20.8[44] respectively, have been reported. Two studies in Ghana also reported prevalence rates of 44.3 – 69.3% for symptomatic dry eye.[48,49] On the basis of clinical signs alone, a highly variable prevalence rates ranging from 5.8 – 77% have been reported.[32,35,37,39,45,50] Similarly, studies that defined dry eye on the basis of both symptoms and signs also reported variable prevalence ranging from 8.7 to 30%[32,37,44-46] Given that MGD is the most common etiology of dry eye,[2,32,51] the prevalence of MGD has also been investigated. Across different age, sex and race cohorts, prevalence rates ranging from 38 to 68% have been reported in adults over the age of 40 years.[32,36,37,40,41] In general, MGD appears to be more common in Asian population cohorts compared with Caucasian.[41,42]

The prevalence of dry eye generally has a significant linear association with age.[32,34] This association, however, is higher with diagnosis based on clinical signs compared with symptoms-based diagnosis.[32] To a large extent, there exists a positive association of dry eye with the female sex as majority of studies reported 1.33 – 1.74 times higher prevalence in women.[36-38,40,43,45-47] In general, the sex difference in dry eye prevalence is significant only when age is factored in.[32]

Tear Osmolarity and Pathophysiology of Ocular Surface Inflammation and Damage

Tear Osmolarity

Tear film osmolarity is an important variable in the pathophysiology of dry eye. It is a measure of the ionic concentration of the tear film. The bulk of tear osmolarity is contributed by the most abundant ions; sodium, potassium, and chloride.[52]In a healthy (non-dry) eye, tear osmolarity measured in the lower tear meniscus is considerably stable.[2,53] Thinning of the tear film due to evaporation during blink intervals leads to an increased tear osmolarity (hyperosmolarity).[2,54] Based on available data, tear osmolarity value of 302 ± 9.7 mOsm/L is considered physiological, with a modest variation between the right and left eyes (6.9 ± 5.9 mOsm/L).[2,55] The mean tear osmolarity values for mild – to – moderate dry eye , and severe dry eye have been reported as 315 ± 10 mOsm/L and 336 ± 022 mOsm/L, respectively.[2,56]

Mathematical modeling suggests that a slight osmolar differential exists between the tears and menisci such that the osmolarity of the tear film is higher than that of the menisci in the steady state.[2,57]. This suggests that measuring osmolarity at the tear meniscus underestimates the osmolarity of the tears overlying the ocular surface tissues.[2,57] This differential increases considerably in dry eye.[2] Hence, although the values for tear meniscus osmolarity measured clinically in dry eye are usually below 500 mOsm/L, it is likely that levels achieved at the ocular surface are much higher than this, especially at sites of tear film breakup. Consistent with this, Begley et al., suggested that local fluctuations in the tear film thickness induce hyperosmolarity “hot-spots” with significantly higher concentrations than the average tear value.[2,58-60] In a study investigating the link between increased tear osmolarity and tear instability, Liu et al.

observed that an osmolarity threshold of 450 mOsm/L was required for the induction of symptoms, while 800–900 mOsm/L was needed to mimic symptoms induced by tear film breakup.[61] This is significantly higher than that measured in the meniscus in dry eye patients.[2,61]

Ocular Surface Inflammation and Damage

Tear hyperosmolarity plays a critical factor in the pathogenesis of dry eye.[1,2] It arises from decreased lacrimal secretion or increased evaporation of the tear film. The increased tear osmolarity induces a desiccating stress which serves as a stimulus for immune response on the ocular surface.[2,62] Dry eye is characterized by both innate and adaptive immune responses.[2,63,64] These responses, arising from hyperosmolar stress contribute to ocular surface impairment through a cascade of damaging inflammatory response.[2] The response includes the recruitment of inflammatory cells to the ocular surface, which tend to become an additional source of inflammatory mediators.[2,63] The inflammatory response leads to decreased synthesis of glycocalyx mucins, apoptotic death of ocular surface epithelial cells, and loss of goblet cells.[2]

Tear hyperosmolar stress also induces corneal epithelial cell death through non-apoptotic processes. In fact, altered expression of glycocalyx mucins is considered a possible basis for ocular surface epithelial staining observed in dry eye as it impairs ocular surface wetting and can result in an early tear film breakup.[2] This can either exacerbate or initiate ocular surface hyperosmolarity, resulting in a vicious circle that perpetuates the disease.

Innate Immune Response in Dry Eye

The healthy, non-desiccated ocular surface has mechanisms to maintain its integrity. These include the presence of gel mucins in the tear film, the glycocalyx, the epithelium, and a host of antimicrobial proteins such as lactoferrin, lysozyme, lipocalin, and trefoil peptides, and surface molecules such as defensins.[2] These defense mechanisms, however, can be impaired when the ocular surface undergoes hyperosmolar stress.

Tear hyperosmolarity activates mitogen-activated protein kinases (MAPK), which subsequently activates the master regulator, NFκB, the translation of inflammatory cytokines, IL-1 (IL-1α; IL-1β), IL-6, and tumor necrosis factor-α (TNF-α).[2,65,66] These induce a cascade of downstream mediators and cellular signals that intensify the inflammatory immune response.[2] IL-1, IL-6, and TNF-α then result in an increased production of proteases, such as matrix metalloproteinase 9 (MMP9) from the corneal epithelial cells which can damage the corneal epithelial barrier.[2,67-69]

Two critical steps are involved in the amplification of the inflammatory immune response on the ocular surface. The first is the production of soluble and membrane-bound signals that recruit both innate and adaptive inflammatory to the site of inflammation.[2] They include chemokines and adhesion molecules. Chemokines generated during ocular surface inflammation include CCL3, CCL4, CCL5, CXCL9, CXCL10, and CX3CL1 that can bind macrophages, dendritic cells, neutrophils and activated T cells in which the respective chemokine receptors are up-regulated.[2,70] The other step is the expression of endothelial adhesion molecules such as the conjunctival and corneal epithelial-expressed intracellular adhesion molecule-1 (ICAM-1).[2] ICAM-1 binds

inflammatory cells expressing the ligand, integrin leukocyte functional antigen 1 (LFA-1) and causes rolling, transmigration, and activation at the site of migration.[2]

Evidence also suggests that pattern recognition receptor (PRR) activation is also involved in the innate inflammatory response in dry eye.[2,71-75] Stimulation of these receptors have been shown to correlate with an increased production of IL-1, IL-6, and TNF-α.[2,72,74]

Adaptive Immune Response in Dry Eye

A body of evidence suggests that adaptive immunity plays a role in the pathology of dry eye.[2,63,64] The adaptive immune response is initiated when antigens at the site of inflammation are processed and presented by professional antigen-presenting cells (APCs) that in turn migrate to regional lymphoid tissue to activate and expand antigen-specific effector T cells.[2] The antigens that initiate the adaptive response in dry eye is largely unknown. However, it is speculated that the expression of auto-antigens stimulates inflammatory epitheliopathy observed in Sjögren's syndrome.[2] The hypothesis that antigen presentation is the initiating step in the adaptive immune response is supported by a study that reported a correlation between accumulation of mature CD11c APCs and the activation of antigen-specific $CD4^+$ T cells in draining lymph nodes during desiccating stress, and the reduction of $CD4^+$ T cell infiltration in animals depleted of ocular surface macrophages and APCs.[2,64]

Dry Eye and Ocular Surface Infection

In dry eye, the quantitative reduction or qualitative alteration of the tear film results in the downregulation of certain proteins that have antimicrobial functions.[2,76] These proteins include lysozyme, lactoferrin, and immunoglobulins. Also, in dry eye, there is alteration in ocular surface mucins and chronic inflammation that can disrupt the ocular surface epithelial barrier and allows the entry of pathogenic microbes.[2,76] Based upon these, it is expected that dry eye would increase the susceptibility to infection.[76] However, existing literature is not supportive of an increased risk of infection in dry eye patients. In fact, the few studies that have suggested an association between dry eye and microbial infection had some confounding factors which preclude the establishment of a definite association between dry eye and infection. For instance, Jhanji et al. examined the microbiological and clinical profile of patients with microbial keratitis living in nursing homes and observed the presence of dry eye in 26% of patients.[77] However, 81% of these patients also had rheumatoid arthritis and were on concurrent use of steroid treatment.[77] Steroid use is associated with increased risk of infection due to its suppression of the immune system.[78] Also, while a study reported conjunctival *Chlamydia trachomatis* infection in approximately 40% of dry eye patients,[79] an earlier pilot study also showed that the successful treatment of chronic follicular conjunctivitis caused by chlamydia infection reduced dry eye.[80] This suggests that an ocular surface infection can predispose a person to dry eye, rather than dry eye increasing one's risk for infection.

The lack of strong evidence establishing an association between dry eye and ocular surface infection could partly be attributed to the beneficial effects of immune

response. In dry eye, hyperosmolar stress on the surface of the eye stimulates an inflammatory response and lead to the induction of immune cells.[2,70] These cells can mount a surveillance against invading pathogenic microbes. [81]

Pattern Recognition Receptors and Dry Eye Disease

As mentioned in an earlier section, available evidence suggests that PRRs play crucial roles in the pathophysiology of inflammation that characterize dry eye disease. A component of the immune system, PRRs are germ line-encoded proteins that recognize both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and stimulate innate immune response.[82-84] These PAMPs are molecules with conserved motifs on the surfaces of pathogenic microbes, and they include lipopolysaccharide, lipoteichoic acid, peptidoglycan, flagellin, unmethylated bacterial CpG DNA, fungal mannan, and yeast's zymosan.[82,85] The DAMPs, also known as danger-associated molecular patterns and alarmins, are endogenous molecules released by stressed, damaged or necrotic cells that act as endogenous danger signals to promote and exacerbate the inflammatory response.[86-88] Examples of DAMPs are high-mobility group box 1, S100 proteins, and heat shock proteins, nucleic acids (DNA, RNA), and purine metabolites including ATP and uric acid.[86,87,89] With a few exceptions, the sensing of PAMPs or DAMPs by PRRs upregulates the transcription of genes involved in inflammatory response.[90]

These genes encode proinflammatory cytokines, type I interferons (IFNs), chemokines and antimicrobial proteins, proteins involved in the modulation of PRR signaling, and other less-characterized proteins.[90] The inflammatory response is mediated

by proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6.[81,83,90] These cytokines are proteins with multiple effects, and they regulate the death of inflammatory cells and tissues, modify vascular endothelial permeability, recruit blood cells to inflamed tissues, and induce the production of acute-phase proteins.[90]

Toll-Like Receptors

Toll-like receptors (TLRs) are a class of PRRs expressed in innate immune cells such as dendritic cells and macrophages as well as non-immune cells such as fibroblast cells and epithelial cells. They are characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology domain.[90,91] Based on their localizations, TLRs are classified into two subfamilies; cell surface and intracellular. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, whereas intracellular TLRs are localized in the endosome and include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13.[71,92] The possible role of toll-like receptors in the dry eye inflammatory signaling has been the focus of many investigations.[71-75,93,94]

Spachidou et al. first observed TLR1-4 expression and upregulation of CD54, CD40 and MHC I in response to TLR activation in epithelial cells sampled from labial salivary gland biopsies.[93] Additionally, TLR1, 2 and 4 expressions were higher in the cells from Sjögren's syndrome (SS) patients. These findings were corroborated by Kawakami et al. who studied TLR expression in the labial salivary glands from SS patients and observed an increased expression of TLR2-3, and the adaptor molecule, MyD88.[94] This study also found that TLR agonists stimulated production of IL-6 and

expression of CD54 (ICAM-1) and thus hypothesized that TLR activation may contribute to the inflammatory responses in SS.

Given that severe dry eye characterizes SS, these findings suggest the possible involvement of TLR signaling in the pathophysiology of dry eye, and therefore became the basis for future investigations into the possible roles of TLR signaling in dry eye. He et al., thus, explored the potential participation of TLRs in dry eye inflammation by examining whether the TLR2-mediated nuclear factor-kappa B (NF-κB) signaling pathway contributes to the inflammatory process of dry eye associated with chronic graft versus host disease (cGVHD).[74] The study reported that TLR2 mRNA in the peripheral blood from patients with cGVHD-related dry eye increased significantly compared with the controls. In addition, TLR4 activation increased the translation of NF-κB, a downstream target of TLRs. This correlated with elevated levels of TNF-α mRNA and protein. More important, increased TLR2 level strongly correlated with dry eye symptoms and the amount of tear secretion.

Redfern and colleagues also investigated the expression and possible function of TLRs in dry eye inflammation using experimental dry eye model in mice.[72] They reported an increased expression of TLRs in the conjunctiva (TLR 2-4, 9), cornea (TLR 2, 3, 9), and lacrimal gland (TLR 2, 5). Similar trends were observed for these TLRs at the level of protein translation. It was also observed that treating cornea with TLR agonist resulted in corneal epithelial loss, a critical ocular sign of dry eye.

Seeking to understand TLRs' potential role in dry eye inflammation, Redfern et al. subsequently investigated the modulation of toll-like receptors in dry eye disease and dry eye-mimicking conditions.[73] Exposing primary human corneal epithelial cells

(HCECs), SV40 immortalized HCECs, and human conjunctival epithelial cells to hyperosmolar stress condition (400-500 mOsm/kg) for 24 hours, the authors observed in the SV40 HCECs, an upregulation of TLR4 (8.18 fold), downregulation of TLR9 (0.58 fold) at mRNA level. At the level of protein expression, there were reduced expressions of TLR4 and TLR9 by 67.7% and 72% respectively. TLR4 mRNA was also significantly upregulated by up to 9.70 and 3.36 folds in the primary HCECs and conjunctival cells, respectively. In response to desiccating stress, TLR4 and TLR5 mRNA transcription were significantly upregulated by 4.81 and 2.51-fold respectively, while TLR9 mRNA was downregulated by 0.86-fold in HCECs. A similar trend for TLR4 and TLR9 protein was observed. Among dry eye subjects, the authors also reported a downregulation of TLR9 mRNA transcription in the conjunctival epithelium.

Put together, these results suggest that TLR expression could play a role in ocular surface inflammation related to dry eye given that TLRs are involved in the inflammatory process in mucosal tissues and surfaces.

NOD-Like Receptors

Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are a specialized group of intracellular cystolic PRRs.[81] The recognition of PAMPs and damage-associated molecular patterns (DAMPs) from microbial structures or self-or environment-derived molecules leads to the induction of the innate immune response. NLRs act as scaffolding proteins that assemble signaling platforms that trigger nuclear factor-κB and mitogen-activated protein kinase signaling pathways and control the activation of inflammatory caspases.[95]

Among NLRs, the expression patterns of NOD-1 and NOD-2 in ocular surface inflammation have been well characterized. NOD-1 and NOD-2, upon detecting DAMPs and PAMPs, mediate proinflammatory responses through nuclear factor kappa B (NF-kB) and MAP kinase activation, leading to the production of proinflammatory cytokines. A few studies have examined NOD-like receptors' expression in dry eye and their possible functional roles in the dry eye inflammatory response.[96-98]

Kim et al. recently investigated the expression pattern of NOD-like receptors in dry eye and their correlation with clinical dry eye markers.[97] The study found that patients with SS-dry eye higher dry eye symptoms (ocular surface disease index, OSDI) and corneal fluorescein staining scores, and lower tear breakup time and Schirmer test scores compared with non-SS-DE patients. Compared with the control group, both the SS-DE and non-SS-DE groups showed higher levels of NOD-1 at both mRNA and protein levels. Similarly, there was an upregulation of IKKα and NF-kB at gene and protein levels. In dry eye subjects, the expression of NOD-1 correlated significantly with OSDI, Schirmer test score, and fluorescein staining. These findings suggest that NOD-1 receptor activation may play a role in initiating the inflammatory response in dry eye.

Other Pattern Recognition Receptors

Aside from TLRs and NOD-like receptors, other PPRs have been studied extensively in other mucosal tissues. These include the scavenger receptor class of PRRs consisting of proteins such as glycoprotein 340, stabilin-1, macrophage receptor with collagenous structure, lectin-like oxidized low-density lipoprotein receptor 1, CD36, lymphotoxin alpha.[99]

Glycoprotein 340. Glycoprotein 340 (Gp340) is a well-characterized member of the scavenger receptor cysteine-rich (SRCR) superfamily of proteins that is expressed in mucosal tissues like the ocular surface, gastrointestinal tract, brain, salivary gland, spleen, lung, and vaginal cavity.[100-103] It is also a normal component of mucosal fluids such as tears, breast milk, saliva, and vaginal fluid.[100,104-108] With a theoretical molecular weight of 340 kDa, the protein has extensive O- and N-linked posttranslational glycosylation that accounts for 25 – 40% of its mass.[105,109,110] Gp340 has been shown to be potentially involved in the pathophysiology of such wet-surfaced inflammatory conditions as nasal polyposis, inflammatory bowel disease, and Crohn's disease.[111,112] Given that the mechanisms of inflammation on wet-surfaced mucosae are similar, Gp340 has been hypothesized to be involved in the pathophysiology of ocular surface inflammatory diseases, including dry eye.[105]

Gp340 Dysregulation in Dry Eye Disease

Two studies have profiled the expression of Gp340 in the tears of subjects with dry eye. The first was a study by Nichols & Green-Church that analyzed tear film proteome using mass spectrometry to identify potential protein biomarkers for contact lens-related dry eye (CLDE).[113] Relative to normal (non-dry eye) subjects, the level of tear Gp340 in CLDE was found to be upregulated. In the second study, Perumal et al. conducted a proteomic analysis of tears of subjects with aqueous-deficient, evaporative, and the mixed types of dry eye.[114] Contrary to the earlier findings, [113] this study reported a downward expression of Gp340 in aqueous-deficient and the mixed dry eye forms.

The differences in the design of the two studies could partly account for the different regulation patterns reported by these two. Firstly, the two studies investigated different forms of dry eye. The dry eye subjects in Nichols & Green-Church's study were contact lens wearers while those investigated by Perumal et al. did not wear contact lens. Contact lens- and non-contact lens-related dry eye have some differences in their underlying pathophysiology given that contact lens wear, on its own, can alter the physiology and biochemistry of the tear film and enhances the risk of dry eye and inflammation on the ocular surface.[115-119] Secondly, the method of tear sampling differed between the two studies. While Nichols & Green-Church used microcapillary tubes for tear sampling, Perumal et al. employed Schirmer strips for tear collection. Different tear collection techniques can yield different concentrations of the same protein.[120,121]

Gp340 and Contact Lens-Related Adverse Events

As stated earlier, Gp340 can enhance the pathogenesis of microbial infections when it adsorbs or associates with a mucosal surface or tissue.[101,102,122,123] The deposition of tear proteins on contact lens promotes the adhesion of pathogenic bacteria to the lens and has been associated with the occurrence of certain contact lens-related adverse events such as contact lens discomfort, contact lens-related peripheral ulcer, contact lens-acute red eye, and microbial keratitis.[124-126] Gp340, in a previous study, was found to bind to soft contact lens materials such as lotrafilcon B.[127] Based off these facts, Osei et al. speculated that Gp340 can enhance contact lens-related adverse events such as microbial infection when it binds to a worn contact lens.[105]

Preliminary findings from a study by Osei et al. showed that the deposition of Gp340's first SRCR domain to contact soft contact lens polymers increased total adhesion of certain strains of *P. aeruginosa* (PA 6206, PA 6296) and *S. aureus* (SA 31 and SA 38) that are commonly associated with contact lens adverse events like microbial keratitis and contact lens-induced peripheral ulcer (Osei et al. IOVS 2019;60 ARVO E-abstract 6333).

Statement of the Problem and Justification of the Study

Dry eye affects 5-50% of the population worldwide and is a leading cause of outpatient visits.[32] It reduces workplace and non–job-related performances, and correlates with reduced quality of life.[128-130] The annual cost of dry eye management in the United States is estimated to be $3.84 billion.[131] Despite the frequent occurrence of dry eye, the pathophysiology underlying the inflammation that characterizes the disease continues to puzzle many ocular surface investigators. Given the unique role of PPRs in ocular surface inflammation, research into PRR signaling in dry eye has become imperative since it could lead to the development of novel therapeutic targets for the disease. Investigations targeting PRR signaling, however, have focused largely on the TLRs and NLRs[71-73,97,98,132] and have, to a larger extent, yielded equivocal results. This necessitates the need to target yet-to-explored ocular surface PRRs.

Among the non-TLR PRRs, Gp340 has been shown to be implicated in wet-surfaced mucosal inflammatory conditions such as nasal polyposis, ulcerative colitis and ethmoid sinusitis.[111,112] Also, Gp340 mRNA transcription in the intestinal epithelial cells (IECs) correlates with the severity of inflammation in inflammatory bowel disease.[111] In

addition, in the presence of the proinflammatory cytokine, TNF-α, the expression of Gp340 is activated in IECs.[133] Nichols & Green-Church reported an upregulation of Gp340 expression in the tears of subjects with contact lens-related dry eye.[113] Contact lens wear is a factor intricately linked to ocular surface inflammation.[116,117,134]

Taken together, these findings provide indirect evidence of the inflammatory signaling potential of Gp340. As a PRR, Gp340's activation could stimulate ocular surface inflammation and could be targeted in the therapeutic management of dry eye. Thus, the primary goal of the dissertation was to investigate the expression of Gp340 mRNA and protein under dry eye-related conditions and to elucidate Gp340's role in the pathophysiology of dry eye inflammation.

Aside from inflammation, Gp340 also modulates mucosal infection processes in a manner related to its conformation. In the soluble form, Gp340 is protective against oral HIV-1 infection and helps in the aggregation and clearance of oral microbial pathogens.[123] Thus when pathogenic microbes such as bacteria, virus and fungi invade the ocular surface, Gp340, as a PRR, could stimulate the recruitment of innate immune cells such as macrophages, neutrophils, and natural killer cells against the microbes. This is consistent with a study which found tear Gp340 to suppress the virulence of *P. aeruginosa* and promote corneal wound healing.[135,136] Investigating the regulation pattern of Gp340 in ocular surface infection is therefore crucial to exploring the protein as potential biomarker and therapeutic target for ocular surface infections.

Tear Gp340's potential protective role against infection on the ocular surface in general, however, is complicated by the fact the beneficial effect of Gp340 in infection appears to be reversed or lost when the protein adsorbs on/associates with a mucosal

surface or tissue.[102,105,123,137] Thus, although Gp340 could have a beneficial/antimicrobial effect on the ocular surface, the same cannot be said in the case of contact lens wear considering that the protein binds to contact lens polymers.[127,137]

On the account of the aforementioned dual roles of Gp340 in infection, the secondary goal of the dissertation was to determine the effect of Gp340 in bacterial adhesion to contact lens polymers and to preliminarily examine the expression pattern of Gp340 in ocular surface infection.

Specific Aims and Hypotheses

Specific Aim 1a

To examine Gp340 mRNA transcription and protein expression in human corneal epithelium in dry eye-associated hyperosmolar stress condition.

Hypothesis 1a

In dry eye, hyperosmolar stress can stimulate the expression of pattern recognition receptors (PRRs).[2,71-73] Gp340 is a PRR whose expression has been shown to be modulated in dry eye.[113,114] It is therefore hypothesized that Gp340 mRNA transcription and protein translation are upregulated in corneal epithelial cells under hyperosmolar stress.

Specific Aim 1b

To determine the effect of Gp340 expression on the production of the proinflammatory cytokines, IL-1β, IL-6, IL-8, and TNFα.

Hypothesis 1b

In dry eye, there is increased expression of the proinflammatory cytokines – IL-1β, IL-6, IL-8, and TNFα.[2] Since PRR activation triggers downstream inflammatory response, it is hypothesized that Gp340 expression increases the production of IL-1β, IL-6, IL-8, and TNFα.

Specific Aim 2

To determine the effect of Gp340's SRCR domain on bacterial adhesion to soft contact lens. The SRCR domain and the full-length Gp340 behave similarly relative to their interactions with bacteria.

Hypothesis 2

Gp340 is a normal component of tears that has been shown to bind to contact lens polymers.[100,127] Given that the immobilized form of Gp340 promotes microbial infection,[101,102,122,123,138] it is hypothesized that the adsorption of Gp340's SRCR domain on soft contact lens polymers potentiates the adhesion of infectious *P. aeruginosa* and *S. aureus* strains.

Specific Aim 3

To examine the regulation of Gp340 mRNA and protein in human corneal epithelial cells exposed to infectious *P. aeruginosa* and *S. aureus* strains.

Hypothesis 3

In the soluble conformation, Gp340 inhibits microbial infection.[123,135,136,139] Pattern recognition receptor expression is increased in infection as an immune defense mechanism. Given this, it is hypothesized that Gp340 expression is upregulated both at mRNA and protein levels in corneal epithelial cells challenged with infectious *P. aeruginosa* and *S. aureus* strains.

CHAPTER 2 – GLYCOPROTEIN 340 IN MUCOSAL IMMUNITY AND OCULAR SURFACE

by

KWAKU A. OSEI, CHAMPION DEIVANAYAGAM, JASON J. NICHOLS

The Ocular Surface

Format adapted [and errata corrected] for dissertation

ABSTRACT

Glycoprotein 340 (Gp340) is an innate immune receptor with well-defined roles in mucosal tissues. It is a normal component of mucosal fluids such as tears, breast milk, and saliva, and it is expressed in tissues such as the vagina, gastrointestinal tract, oral cavity, lung alveoli, and pancreas. In the eye, it is expressed in the lacrimal gland, cornea, conjunctiva, and retina. Investigations of the protein in wet-surfaced epithelia of the body show that the effects of Gp340 can be beneficial or harmful depending on the conformation in which it exists. In a fluid phase, Gp340 appears to be protective against mucosal infection, while in a surface-associated form it appears to promote infection. On the ocular surface, it is dysregulated in dry eye disease and inhibits twitching motility of *P. aeruginosa* in tears. This review discusses what is known about Gp340 in wet-surfaced mucosal epithelia and highlights the potential roles of the protein in ocular surface immunity, inflammation, and infections.

INTRODUCTION

Glycoprotein 340 (Gp340) is a 340-kDa extracellular protein which belongs to the scavenger receptor cysteine-rich (SRCR) superfamily of proteins. It is encoded by the "*deleted in malignant brain tumor-1*" (*DMBT1*) gene, located on human chromosome 10q26.13 [1]. Gp340 is also referred to as DMBT1 and salivary agglutinin because all three have identical protein core and are encoded by the same gene [2-6]. Being a glycoprotein, Gp340 is heavily glycosylated by post-translational modifications which accounts for 25-40% of the total molecular weight of the protein [2, 7].

Gp340 is a normal component of mucosal fluids such as tears, saliva, and breast milk [8-10]. It is expressed on the ocular surface and other mucosal epithelial tissues such as gastrointestinal tract, oral cavity, lung alveoli, and pancreas, as part of the innate immune system [1, 11-13]. Gp340 is also expressed in lower quantities in the brain, uterus, testis, and mammary glands [13]. As an innate immune receptor, Gp340 confers protection against pathogens on the ocular surface and other moist-surfaced epithelial surfaces where they are expressed. It is also involved in epithelial cell differentiation [14]. The effects of Gp340 may either be beneficial or harmful depending on the conformation in which the protein exists. In a fluid phase, Gp340 inhibits cariogenesis, HIV-1, and influenza A infections. However, in a surface-associated form, it promotes cariogenesis and HIV-1 infections. Gp340 also has both stimulatory and inhibitory effects on the complement system [15].

The purpose of this review is to describe the current state of our understanding of Gp340 on the ocular surface in relation to other mucosal tissues/surfaces, with emphasis on its roles and effects in normal physiology and altered states of the ocular surface.

OVERVIEW OF GP340

Human Gp340 exists in two variants, namely the secretory and cell-associated types [16, 17]. The secretory variant is expressed in tissues in the eye, lung, oral cavity, and breast whereas the cell-associated form is expressed on the vaginal and cervical epithelia [18, 19]. The secretory Gp340 exists in two conformations; the soluble (fluid-phase) and the immobilized forms [20]. The soluble form is found in tears, respiratory mucosal secretions, saliva, and break milk while the immobilized is found on the hydroxyapatite surface in the oral cavity [16, 20]. In the context of this review, the cell-associated variant and the immobilized form are classified as surface-associated conformation.

Gp340 contains four major well-defined domains: 1) the scavenger receptor cysteine-rich (SRCR) domain, 2) the SRCR interspersed domain (SID), 3) the C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein-1 (CUB) domain, and 4) the zona pellucida (ZP) domains (see Figure 1). The SRCR domain is found in a variety of secreted and cell-surface proteins and contains 100-110 amino acids. Two groups of SRCR domains have been characterized [21]. Group A SRCR domains contain six cysteine residues and are encoded by two exons, whilst group B SRCR domains consist of eight cysteine residues and are encoded by a single exon [22]. The SRCR domains of Gp340 are of the group B type [23]. There is variability in the number of SRCR domains in Gp340 due to alternative splicing occurring in both SRCR and SID regions at mRNA level which

results in mRNA transcripts of different sizes. The most extended transcript with 8 kb size consists of 7656 nucleotides and codes for Gp340 with 14 SRCR domains with the first 13 in tandem. The shortest mRNA transcript is of 6-kb size, consists of 5802 nucleotides and codes for Gp340 with only nine SRCR domains [19]. Fourteen N-linked glycosylation sites and several O-linked glycosylation sites exist in Gp340. These glycosylation sites are present in the SIDs, located between SRCR domains in tandem (except between the fourth and fifth SRCR domains). Following the SRCR domains and SIDs are two CUB domains separated by the 14th SRCR domain. The CUB domain is a 110-residue protein motif which consists of four conserved cysteine residues and exhibits a β-sandwich fold [24]. The CUB domains mediate the interaction of Gp340 with extracellular proteins [24]. At the C-terminus of the polypeptide is a single ZP domain which consists of 260 amino acids with 8 conserved cysteine residues. The ZP domain is primarily involved in oligomerization of Gp340 [25].

GP340 AND INNATE IMMUNITY

The innate immunity is an evolutionarily conserved system which functions as a first-line of defense against invading microbial pathogens and other potential untoward effects on the host [26]. It consists of nonspecific defense mechanisms that come into play immediately or within hours of the host's exposure to antigens. Gp340 is known to be an innate immune molecule with broad-spectrum antimicrobial functions. It is considered as a pattern recognition receptor (PRR), which identifies pathogens by engaging pathogen-associated molecular patterns (PAMPs) [27]. PAMPs are the conserved structures in microbial cells which are essential for the existence of microbes. PAMPs include

lipopolysaccharides on gram-negative bacteria, lipoteichoic acid on gram-positive bacteria, peptidoglycans, mannan, glucan, porins, flagellin, and bacterial RNA and DNA [28]. Recognition of PAMPs by PRRs, in turn, stimulates the innate immune system. Thus, as a PRR, Gp340 stimulates innate immunity.

Due to its ability to bind and agglutinate broad spectrum of microbes, Gp340 also plays a vital role in the innate protection against oral microbial infections [29, 30]. For instance, fluid-phase Gp340 has been implicated in the clearance of microorganisms from the oral cavity [31]. It achieves this by aggregating microbial species which in turn get washed down the acidic gut where they face extinction.

GP340 AND COMPLEMENT ACTIVATION

The complement system is an essential component of the innate immune response. It augments the opsonization of bacteria by antibodies and promotes the bactericidal functions of antibodies [26]. It thus complements the roles of antibodies and serves as a link between innate and adaptive immunity. The complement system is activated via three pathways: the classical pathway, activated by antibody or by direct binding of complement component C1q to the pathogen surface; the lectin pathway, triggered by mannose-binding lectin (MBL), a normal serum constituent that binds a mannose-containing carbohydrate on the surface of some class of bacteria; and the alternative pathway, triggered directly on pathogen surfaces [26]. All three pathways generate a critical enzymatic activity that, in turn, generates the effector molecules of the complement system. The main effects of complement activation are opsonization of pathogens, recruitment of inflammatory mediators, and direct killing of pathogens [26].

A study examining the effect of Gp340 on the complement system found Gp340 to modulate complement activation [15]. The effect of Gp340 on complement activation was investigated by incubating fluid-phase and surface-associated Gp340, each with serum and measuring the deposition of downstream complement factors. While surface-associated Gp340 activated the classical and lectin pathways, fluid-phase Gp340 inhibited the lectin pathway. The study further found fluid-phase Gp340 to inhibit the binding of MBL to *E. coli* and *C. albicans.* Given that the complement system is only activated when complement factors come into contact with antigen-antibody complex, foreign bodies, damaged tissues, or pathogens [32], perhaps the observed inhibition of complement activation against *E. coli* and *C. albicans* is due fluid-phase Gp340 binding to the microbial surfaces and preventing microbe-complement interactions.

Taken together, these data suggest surface-associated Gp340 and fluid-phase Gp340 have stimulatory and inhibitory effects on complement activation. It must be emphasized, however, that the beneficial and harmful effects of fluid-phase and surface-associated Gp340 in the pathogenesis of infections cannot be elucidated from the observed effects on complement activation. On the one hand, by inhibiting complement activation, fluid phase Gp340 appears to promote the pathogenesis of infections given that complement activation inhibits infection. On the other hand, the seeming downregulation of complement activation by fluid-phase DMBT1 could also imply the absence of infection in the presence of fluid-phase Gp340, given that complement may not be activated in the absence of infection.

GP340 AND MICROBIAL INTERACTIONS

A number of studies have reported interactions of Gp340 with bacteria [5, 23, 30, 33] and virus. A 16-mer peptide, SRCRP2 (QGRVEVLYRGSWGTVC), present in eight SRCR domains had been suggested as the only Gp340 peptide sequence which is capable of binding and agglutinating bacteria [23, 33]. However, this assertion has been questioned by a subsequent study [5] which examined the binding affinities of both SRCRP2 and the whole SRCR domain for the surface proteins, Antigen I/II of *S. mutans* and SspB of *S. gordonii* and found the SRCRP2 peptide to exhibit minimal bacterial aggregation compared with the full-length SRCR domain. The study found multiple adherence sites within a single SRCR domain, and the authors also speculate the non-specific nature of the adherence of SRCRP2 with Gp340.

Available evidence suggests that the interactions of Gp340 with microbial organisms may either be beneficial or harmful. For instance, a study by Rosenstiel et al. [30] found Gp340 to inhibit bacterial cytoinvasion in intestinal epithelial cells by *S. enterica*. At a concentration of 20 nM, recombinantly expressed Gp340 resulted in aggregation of enteroinvasive *S. enterica*. However, downregulation of Gp340 expression resulted in increased cytoinvasion. In addition, alveolar fluid–derived Gp340 has been shown to inhibit the infectivity of IAV, the causative organism of influenza [34]. The antiviral effect of Gp340 against IAV is proposed to be mediated by the interactions between the sialic acid-bearing glycosylation on Gp340 and the virus [34]. Aside from suppressing IAV, fluid-phase (saliva-derived) Gp340 binds and inhibits HIV-1 infectivity through the oral route [35]. In general, HIV-1 infectivity occurs via binding of the virus to a host cell's CD4 receptor, resulting in conformational changes in the viral glycoprotein

120 (Gp120) which permits interaction with chemokine receptors. By binding to Gp120, fluid-phase Gp340 inhibits infectivity of HIV-1 by blocking access of Gp120 to chemokine receptors [36].

Being a dual-faced molecule, Gp340 also appears to promote microbial infections. For instance, in the oral cavity, hydroxyapatite-adsorbed Gp340 enhances cariogenesis caused by *S. mutans* [16]. It achieves this by agglutinating *S. mutans* on tooth surfaces which allows bacterial colonization and subsequent biofilm formation; two crucial steps in the pathogenesis of microbial infections. A study by Purushotham & Deivanayagam [5] found SRCR domain as the component of Gp340 which directly interacts with Antigen I/II in the pathogenesis of caries disease. Also, Gp340 expressed on the vaginal epithelium has been shown to promote transmission of HIV-1 in the female vaginal tract. Gp340 achieves this by facilitating direct transcytosis of the virus from the apical to the basolateral side of vaginal epithelium by binding to Gp120 on the viral envelope [12, 37].

Taken together, it appears that while fluid-phase Gp340 inhibits, the surface-associated Gp340 promotes the pathogenesis of microbial infections. These seemingly contradictory observations are directly related to the structural conformations adopted by the fluid-phase and surface-associated Gp340.

Gp340 AND THE OCULAR SURFACE

Expression and Localization

Gp340 is a component of the precorneal tear fluid and ocular tissues [8, 9, 38, 39]. In the anterior segment, the *DMBT1* gene is expressed in the lacrimal gland, cornea, and conjunctiva tissues. Immunohistochemical analysis localized Gp340 in the secretory acini

of the main lacrimal gland, the basal epithelium of cornea, and the epithelium of conjunctiva [8]. The secretory acini account for about 85% of *DMBT1* gene expression on the ocular surface. Remarkably, the expression of *DMBT1* mRNA is significantly higher in the tear film than in saliva [8]. However, it is unknown if there is a correspondingly higher expression of the protein in tears compared to saliva. A mass spectrometric analysis has shown that O-linked glycosylation of tear film-derived Gp340 is mainly composed of branched, sialylated oligosaccharides with up to four sialic residues on them [9]. In the posterior segment, Gp340 has been detected at the leading edge of the capillary network in the developing retina. It is also localized in the retinal stalk cells contiguous to the endothelium [38].

Gp340 and Bacterial Aggregation

The effect of tear Gp340 on bacterial aggregation is yet to be directly investigated. However, Jumblatt et al. [8] examined the agglutinating properties of tears against bacteria in-vitro and found human tear fluid to aggregate the gram-positive bacteria, *S. aureus,* and *S. mutans* in a time- and dose-dependent manner [8]. It was proposed that tear film Gp340 may have agglutinating functions against bacteria after cell-associated Gp340 was detected in the isolated bacterial aggregates, through western blot analysis. It must be emphasized, however, that there could be additive aggregating effects from other tear antimicrobial peptides, such as lysozyme and lactoferrin, which are known to have bacterial aggregating functions [29, 40, 41]. Gp340, however, does not aggregate the gram-negative bacterium, *P. aeruginosa* [8, 42].

Gp340 and Dry Eye Disease

Fewer studies have suggested an association between dry eye disease (DED) and Gp340 expression pattern. DED is a "multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play significant etiological roles" [43]. Gp340 has been found to be one of the proteins dysregulated in DED [39, 44]. The patterns of dysregulation, however, have been equivocal. Nichols & Green-Church [39], in a study to profile tear film proteome of subjects with contact lens-related dry eye (CLDE) using mass spectrometry-based analysis, reported upregulation of Gp340 in CLDE. Quite remarkably, the level of Gp340 upregulation was higher than that of all other proteins in tears. In another study, Perumal et al. [44] investigated the regulation profiles of tear film proteins in subjects with aqueous-deficient dry eye (ADDE), evaporative dry eye (EDE) and a combination of the two (ADDE/EDE), using label-free quantification and targeted mass spectrometry techniques. The study found Gp340 as one of 79 proteins whose expressions were dysregulated in the tears of subjects with DED. However, contrary to the findings from Nichols & Green-Church's study [39], the authors reported downregulation of Gp340 in tears of subjects with ADDE and ADDE/EDE.

Though the findings of these two studies appear to contradict one another, it must be emphasized that the criteria for DED and tear sampling methods used in these studies differed. Whiles Nichols & Green-Church [39] used the microcapillary tear collection technique in their study, Perumal et al. [44] employed the Schirmer strip collection method. It has been previously demonstrated that microcapillary tube and Schirmer strip

collections, even in the same studies yield different concentrations of the same protein [45, 46]. The difference in tear collection technique thus may partly explain the difference in results between these two studies, and further research, employing both techniques at the same time, may help shed better light on these differing observations. The conflicting results could also be due to the fact that CLDE, ADDE, and ADDE/EDE have different underlying etiologies.

Gp340 and Ocular Surface Immunity/Infections

The ocular mucosal surface is immune privileged with antimicrobial mechanisms such as epithelial barrier, immune cells and antimicrobial peptides [47]. In spite of these protective and defensive mechanisms, the ocular surface is prone to microbial infections when these mechanisms are compromised. There appears to be a body of evidence to show tear Gp340 may inhibit the pathogenesis of bacterial keratitis, a major complication of contact lens wear [48, 49]. Bacterial keratitis is one of the reasons contact lens wearers drop out of lens wear [50]. It causes severe ocular pain and when left untreated, can cause severe loss of vision. *P. aeruginosa*, which is a gram-negative bacterium, is a common etiological agent of bacterial keratitis in contact lens wearers [51, 52].

A recent study [42] has identified Gp340 as a component of tear fluid which suppresses twitching motility in *P. aeruginosa*. Twitching motility is a type IV pilus-mediated movement in gram-negative bacteria which allows for translocation across a moist surface [53]. It plays vital roles in bacteria colonization of host surface by facilitating cell-surface adhesion and playing a role in biofilm formation [53, 54]. It is not surprising, therefore, that a study found twitching motility to enhance virulence of *P. aeruginosa* in a

murine model of keratitis by facilitating translocation of *P. aeruginosa* through corneal epithelial cells [36, 55]. In the study [42], tear fluid inhibited twitching motility in *P. aeruginosa* and as expected, Gp340-depleted tears lost its ability to inhibit twitching motility. Similarly, saliva-purified Gp340 also exhibited dose-dependent inhibition of twitching motility in *P. aeruginosa*. These observations show Gp340 is the component of tears with inhibits twitching motility.

To investigate the effect of Gp340 on *P. aeruginosa* traversal of multilayered human corneal epithelium, *P. aeruginosa* was added to the apical surface of corneal epithelial cells in Gp340 solution, phosphate buffered saline (PBS) or human tears. Both Gp340 and tears were found to significantly reduce *P. aeruginosa* traversal. To ascertain if suppression of twitching motility *in P. aeruginosa* confers protection against *P. aeruginosa* keratitis, injured mouse corneas were inoculated with *P. aeruginosa* in Gp340 or PBS. Gp340 was found to reduce overall disease severity. From these observations, tear Gp340 appears to inhibit ocular surface infections caused by *P. aeruginosa.* Given that Gp340 in tears is in the fluid-phase and inhibition of twitching motility is protective against *P. aeruginosa*, it appears fluid-phase Gp340 (in tears) inhibits microbial infections on the ocular surface, as it does in the oral cavity. This supports the assertion that fluid-phase Gp340 has beneficial effects.

A potential role of Gp340 in ocular surface infection could also be surmised from the seeming interaction of Gp340 with toll-like receptor 4 (TLR4). TLRs are a family of highly conserved glycoprotein PPRs that recognize conserved motifs on PAMPs on microbes and are expressed on a wide variety of cell types including epithelia, endothelia, antigen presenting cells and lymphocytes [56]. Dysregulation of TLR4 expression has been

associated with the ocular surface [56]. In fact, Huang et al. [57] reported that *P. aeruginosa* infection on the ocular surface results in an increased TLR4 expression on the cornea and TLR4 loss results in increased susceptibility to infection. Given that TLR4 is a PPR, it appears the upregulation of TLR4 in a *P. aeruginosa* infection is an adaptive immune response. Stimulation of TLR4 results in Gp340 upregulation and secretion [30]. In addition, TLR4 signaling regulates the direct binding of Gp340 to lipopolysaccharide, a PAMP on gram-negative bacteria. This binding has seemingly beneficial effects. In fact, the interaction of Gp340 with lipopolysaccharide has been shown to inhibit bacterial invasion of intestinal epithelial cells [30]. Given that Gp340 is also a PRR, there may be upregulation of GP340 in *P. aeruginosa* infection on the ocular surface as an immune response to modulate the infection. Gp340 expression on the ocular surface, thus, may be a potential biomarker for *P. aeruginosa* keratitis.

Gp340 and Ocular Surface Inflammation

The function of Gp340 in ocular surface inflammation is yet to be investigated. However, some potential roles can be proposed. Nichols & Green-Church, in their study found Gp340 to be upregulated in CLDE [39]. The reason for the observed upregulation is unknown, however, it may be in response to ocular surface inflammation resulting from CLDE, given that contact lens wear is a risk factor for ocular surface inflammation [58, 59]. There are similar reports of Gp340 upregulation in other wet-surfaced mucosal tissue inflammatory conditions such as nasal polyposis, Crohn's disease, and ulcerative colitis [60, 61].

The observed link between TLR4 and Gp340 regulation also suggests Gp340 may have a potential role in ocular surface inflammation. Current evidence suggests that TLR4 expression is involved in the inflammatory cascades in ocular surface inflammatory conditions such as DED and vernal keratoconjunctivitis [56, 62, 63]. A number of studies have also found upregulation of TLR4 in the lacrimal gland, corneal epithelium, and labial salivary gland in Sjögren's syndrome (SS) [64, 65]. Since Gp340 expression is downstream of TLR4, it is speculated that there will be upregulation of Gp340 in SS-related ocular surface inflammation.

Gp340's suggested expression pattern in ocular surface inflammation, however, is complicated by the seeming link between the expressions of Gp340 and the cytokine, interleukin 22 (IL-22). IL-22 has been shown to induce Gp340 expression in epithelial cells of the intestinal mucosa [66]. IL-22 has dual effects; protective and inflammatory, in modulating the responses of mucosal tissue during an immune response. It has been referred to as a "sheep in wolf's clothing" given these dual effects [67]. A recent study found lacrimal gland-derived IL-22 to be anti-inflammatory through suppression of IL-17 mediated ocular surface epitheliopathy and infiltration of Th17 cells and inhibition of DED induction [68]. Paradoxically, IL-22 has also been implicated in the development of psoriasis, an inflammatory disease [69]. These paradoxical effects of IL-22 on mucosal surface inflammation, thus makes it difficult to decipher the exact pattern of expression of Gp340 on the ocular surface.

Gp340's Proposed Interactions with Endogenous Proteins Associated with the Ocular Surface and in Tears

A body of evidence suggests Gp340 interacts with some endogenous proteins in tears and on the ocular surface. These interactions may modulate critical physiological processes on the ocular surface.

Gp340 and Trefoil Factors

Gp340 interacts with the trefoil factors, TFF2 and TFF3 [70]. TFFs are key modulators of epithelial restitution and are considered the key initiators of mucosal wound healing [71]. Gp340 is regarded as a putative receptor for TFF2 given the strong affinity between the two proteins [72, 73]. TFFs have been studied extensively on the ocular surface. In an experiment to investigate the role of TFFs in rabbit corneal wound healing in vitro, TFF2 (also called pancreatic spasmolytic polypeptide) and TFF3 were found to promote epithelial restitution [74]. Results from a subsequent study [75] to explore the importance of TFF3 in re-epithelialization also found a similar beneficial role TFFs in corneal wound healing. In the study, the rate of re-epithelialization of corneal wounds in TFF3 (+/+) mice was faster compared with TFF3 (-/-) mice. Upon further analysis, TFF3 expression was absent in intact cornea but was overexpressed after corneal injury. Taken together, it appears TFF3 is expressed in response to corneal injury to mediate the wound healing processes.

Though it is yet to be investigated, it appears the interaction of Gp340 with TFFs on the ocular surface will be beneficial. The seemingly beneficial effects could be inferred from the interaction between the two proteins in the gastrointestinal and biliary mucosa, given that the mechanism of ocular surface wound healing is similar to that of other

mucosae [74, 76]. In the gastrointestinal tract, the interaction of Gp340 with TFF2 has been shown to enhance the beneficial role TFF2 in intestinal epithelial restitution. It has also been shown that lack of expression of Gp340 and TFF increases the susceptibility to inflammatory bowel diseases. In the bile duct, TFF2/Gp340 complex is proposed to play a critical role in the cytoprotection and maintenance of biliary mucosa [77]. Taken together, these observations show Gp340 may interact with TFFs on the ocular surface to enhance regeneration of the corneal epithelium to maintain cornea homeostasis.

Gp340 and Galectin-3

Gp340 also interacts with galectin-3 [78]. Galectin-3 is a beta-galactoside-binding protein with defined roles in cell adhesion, cell activation, cell growth and apoptosis [79]. It regulates cell-cell and cell-matrix interactions and modulates wound re-epithelialization. It plays crucial immunological roles on the ocular surface. For instance, it interacts with transmembrane mucins to maintain the barrier function of the ocular surface epithelial glycocalyx [80]. In fact, binding of galectin-3 to transmembrane mucins has been shown to inhibit herpes simplex virus-1 infection on human cornea [81]. In a study to investigate the expression of galectin-3 in DED, an association was found between ocular surface epithelial dysfunction and degradation of galectin-3 in tears [82]. These observations highlight the critical roles galectin-3 plays in maintaining the integrity of the ocular surface. Surface plasmon resonance studies have found a strong binding affinity between galectin-3 and Gp340 [78, 83]. This interaction may modulate the physiological functions of either protein. As stated earlier, Gp340 is involved in epithelial cell differentiation, an important phase in wound healing process of mucosal tissues/surfaces [14, 84]. During epithelial cell

differentiation, Gp340 undergoes oligomerization, a process mediated by Gp340's interaction with galectin-3 [78].

The role of Gp340 in corneal wound healing has yet to be elucidated. However, given that galectin-3 helps maintain the ocular surface epithelial barrier function and promotes corneal wound healing *in vivo* [85], coupled with Gp340's critical role in epithelial cell differentiation, it appears Gp340 also has positive regulatory effects during corneal wound healing.

Gp340 and Lactoferrin

Available evidence also shows Gp340 interacts with lactoferrin [18, 86]. Lactoferrin is an iron-chelating bacteriostatic protein which has bacteriostatic effects on both gram-positive and gram-negative bacteria [87]. Its bacteriostatic activity is a function of its ability to chelate iron, an essential nutrient for bacterial growth. Iron is required for multiple bacterial physiological functions such as catalase, peroxidase, and cytochrome activities [88]. The interaction of Gp340 with lactoferrin affects some physiological activities of Gp340. For instance, a study to investigate Gp340's interaction with bovine milk lactoferrin found the interaction to inhibit Gp340-mediated aggregation of *S. mutans* [86, 89]. Though the effect of Gp340's interaction with lactoferrin on the ocular surface has yet to be investigated, it is proposed that the interaction will inhibit the aggregating functions of Gp340 in tears.

Gp340 and Gel-Forming Mucins

Gp340 also interacts with the gel-forming mucin, MUC5AC [77, 90]. Gel-forming mucins form the bulk of mucins [91]. On the ocular surface, gel-forming mucins act as a surfactant which allows the pre-ocular tear fluid to spread evenly on the ocular surface. They also remove ocular surface debris and hold antimicrobial peptides and other defense molecules on the ocular surfaced [92, 93]. Among gel-forming mucins, MUC5AC is the most abundant on the ocular surface and contributes significantly to the hydrophilicity of the tear film [92]. In the biliary epithelium, MUC5AC, after interacting with TFFs binds to Gp340 and results in increased viscosity of mucous gel [77]. Modulation in mucus viscosity may affect physiological activities of mucus on a mucosal surface. For instance, a marked increase in mucus viscosity has been proposed as a mechanism of gastric protection by gastric mucus [94]. It is proposed that, likewise, tear Gp340 interacts with MUC5AC on the ocular surface, resulting in increased viscosity of tears. Increased tear viscosity may modulate the barrier function of tears.

Gp340 and Surfactant Protein D

Surfactant protein D (SP-D) is an innate immune molecule expressed in the eye, lung, salivary gland, stomach, kidney, and breast [95]. It adheres to carbohydrate and lipid moieties on the surfaces of various pathogenic microorganisms and causes bacterial aggregation and facilitates microbial clearance [95, 96]. In the eye, SP-D expression is localized in the conjunctiva, corneal epithelium, and lacrimal glands [95, 97, 98]. SP-D plays protective roles on the ocular surface. For instance, it enhances the clearance of *S. aureus* and *P. aeruginosa* from the ocular surface and inhibits invasion of corneal epithelial

cells by *P. aeruginosa* [97, 99]. Additionally, there is increased expression of SP-D in human corneal epithelial cells exposed to *P. aeruginosa* or *A. fumigatus* spores, and in herpetic keratitis and corneal ulcer [96, 98]. The observed upregulation of SP-D is an immune response to microbial invasion or infection on the ocular surface [95]. Gp340 has been regarded as a putative receptor for SP-D given the strong affinity between the two molecules [2, 13]. Further investigations on the interaction between Gp340 and surfactant protein-D (SP-D) have shown a strong dependence on calcium, similar to the adherence observed with microbial surface proteins [5, 13, 16].

The interactions of Gp340 with SP-D in tears and on the ocular surface have yet to be investigated. However, it is proposed that the interaction of Gp340 with SP-D modulates the antibacterial effects of either protein and hence the overall antimicrobial effect of tears.

SUMMARY AND CONCLUSIONS

As discussed in this review, current evidence shows Gp340 to be an important endogenous protein in tears with diverse roles and effects. The ocular surface shares some similarities with wet-surfaced mucosal tissues such as oral cavity, vagina, and gut. The observed roles of Gp340 in infections of these wet-surfaced mucosae and its modulated expression in inflammation suggest Gp340 may play crucial roles in ocular surface infections and inflammation. The roles of the protein on the ocular surface are yet to be studied extensively, however, being a seemingly two-faced protein, Gp340 may either be protective against infections and inflammation or promote inflammation and microbial infections on the ocular surface. Thus, future studies on further exploration of the exact roles and effects of Gp340 in microbial infections and inflammatory conditions of the ocular surface are necessary.

Another aspect of tear Gp340 which needs detailed investigation is the proposed interactions of Gp340 with other endogenous proteins associated with the ocular surface and the tear film. Given that Gp340's interactions with endogenous proteins in other mucosal fluid and wet-surfaced mucosal epithelial tissues modulate the activities of these proteins, additional studies on Gp340's interactions with functional tear film proteins are warranted.

REFERENCES

[1] Mollenhauer J, Wiemann S, Scheurlen W, Korn B, Hayashi Y, Wilgenbus KK, et al. DMBT1, a new member of the SRCR superfamily, on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. Nat Genet. 1997;17:32-9.

[2] Holmskov U, Mollenhauer J, Madsen J, Vitved L, Gronlund J, Tornoe I, et al. Cloning of gp-340, a putative opsonin receptor for lung surfactant protein D. Proc Natl Acad Sci U S A. 1999;96:10794-9.

[3] Ligtenberg TJ, Bikker FJ, Groenink J, Tornoe I, Leth-Larsen R, Veerman EC, et al. Human salivary agglutinin binds to lung surfactant protein-D and is identical with scavenger receptor protein gp-340. Biochem J. 2001;359:243-8.

[4] Prakobphol A, Xu F, Hoang VM, Larsson T, Bergstrom J, Johansson I, et al. Salivary agglutinin, which binds Streptococcus mutans and Helicobacter pylori, is the lung scavenger receptor cysteine-rich protein gp-340. J Biol Chem. 2000;275:39860-6.

[5] Purushotham S, Deivanayagam C. The calcium-induced conformation and glycosylation of scavenger-rich cysteine repeat (SRCR) domains of glycoprotein 340 influence the high affinity interaction with antigen I/II homologs. J Biol Chem. 2014;289:21877-87.

[6] Martinez VG, Moestrup SK, Holmskov U, Mollenhauer J, Lozano F. The conserved scavenger receptor cysteine-rich superfamily in therapy and diagnosis. Pharmacol Rev. 2011;63:967-1000.

[7] Oho T, Yu H, Yamashita Y, Koga T. Binding of salivary glycoprotein-secretory immunoglobulin A complex to the surface protein antigen of Streptococcus mutans. Infect Immun. 1998;66:115-21.

[8] Jumblatt MM, Imbert Y, Young WW, Jr., Foulks GN, Steele PS, Demuth DR. Glycoprotein 340 in normal human ocular surface tissues and tear film. Infect Immun. 2006;74:4058-63.

[9] Schulz BL, Oxley D, Packer NH, Karlsson NG. Identification of two highly sialylated human tear-fluid DMBT1 isoforms: the major high-molecular-mass glycoproteins in human tears. Biochem J. 2002;366:511-20.

[10] Ronellenfitsch S, Weiss C, Frommhold D, Koch L, Mollenhauer J, Poeschl J, et al. High DMBT1 concentrations in breast milk correlate with increased risk of infection in preterm and term neonates. BMC Pediatr. 2012;12:157.

[11] Diegelmann J, Czamara D, Le Bras E, Zimmermann E, Olszak T, Bedynek A, et al. Intestinal DMBT1 expression is modulated by Crohn's disease-associated IL23R variants and by a DMBT1 variant which influences binding of the transcription factors CREB1 and ATF-2. PLoS One. 2013;8:e77773.

[12] Stoddard E, Cannon G, Ni H, Kariko K, Capodici J, Malamud D, et al. gp340 expressed on human genital epithelia binds HIV-1 envelope protein and facilitates viral transmission. J Immunol. 2007;179:3126-32.

[13] Holmskov U, Lawson P, Teisner B, Tornoe I, Willis AC, Morgan C, et al. Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein-D binding molecule. J Biol Chem. 1997;272:13743-9.

[14] Mollenhauer J, Herbertz S, Holmskov U, Tolnay M, Krebs I, Merlo A, et al. DMBT1 encodes a protein involved in the immune defense and in epithelial differentiation and is highly unstable in cancer. Cancer Res. 2000;60:1704-10.

[15] Reichhardt MP, Loimaranta V, Thiel S, Finne J, Meri S, Jarva H. The salivary scavenger and agglutinin binds MBL and regulates the lectin pathway of complement in solution and on surfaces. Front Immunol. 2012;3:205.

[16] Madsen J, Mollenhauer J, Holmskov U. Review: Gp-340/DMBT1 in mucosal innate immunity. Innate Immun. 2010;16:160-7.

[17] Reichhardt MP, Meri S. SALSA: A Regulator of the Early Steps of Complement Activation on Mucosal Surfaces. Front Immunol. 2016;7:85.

[18] Ligtenberg AJ, Karlsson NG, Veerman EC. Deleted in malignant brain tumors-1 protein (DMBT1): a pattern recognition receptor with multiple binding sites. Int J Mol Sci. 2010;11:5212-33.

[19] Mollenhauer J, End C, Renner M, Lyer S, Poustka A. DMBT1 as an archetypal link between infection, inflammation, and cancer. Inmunologia. 2007;26:17.

[20] Loimaranta V, Jakubovics NS, Hytonen J, Finne J, Jenkinson HF, Stromberg N. Fluid- or surface-phase human salivary scavenger protein gp340 exposes different bacterial recognition properties. Infect Immun. 2005;73:2245-52.

[21] Miro-Julia C, Rosello S, Martinez VG, Fink DR, Escoda-Ferran C, Padilla O, et al. Molecular and functional characterization of mouse S5D-SRCRB: a new group B member of the scavenger receptor cysteine-rich superfamily. J Immunol. 2011;186:2344-54.

[22] Sarrias MR, Gronlund J, Padilla O, Madsen J, Holmskov U, Lozano F. The Scavenger Receptor Cysteine-Rich (SRCR) domain: an ancient and highly conserved protein module of the innate immune system. Crit Rev Immunol. 2004;24:1-37.

[23] Bikker FJ, Ligtenberg AJ, End C, Renner M, Blaich S, Lyer S, et al. Bacteria binding by DMBT1/SAG/gp-340 is confined to the VEVLXXXXW motif in its scavenger receptor cysteine-rich domains. J Biol Chem. 2004;279:47699-703.

[24] Gaboriaud C, Gregory-Pauron L, Teillet F, Thielens NM, Bally I, Arlaud GJ. Structure and properties of the Ca(2+)-binding CUB domain, a widespread ligand-recognition unit involved in major biological functions. Biochem J. 2011;439:185-93.

[25] Jovine L, Qi H, Williams Z, Litscher E, Wassarman PM. The ZP domain is a conserved module for polymerization of extracellular proteins. Nat Cell Biol. 2002;4:457-61.

[26] Janeway C. Immunobiology 5 : the immune system in health and disease. 5th ed. New York: Garland Pub.; 2001.

[27] Suresh R, Mosser DM. Pattern recognition receptors in innate immunity, host defense, and immunopathology. Adv Physiol Educ. 2013;37:284-91.

[28] Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197-216.

[29] Golub EE, Cheruka J, Boosz B, Davis C, Malamud D. A comparison of bacterial aggregation induced by saliva, lysozyme, and zinc. Infect Immun. 1985;48:204-10.

[30] Rosenstiel P, Sina C, End C, Renner M, Lyer S, Till A, et al. Regulation of DMBT1 via NOD2 and TLR4 in intestinal epithelial cells modulates bacterial recognition and invasion. J Immunol. 2007;178:8203-11.

[31] Ericson T, Rundegren J. Characterization of a salivary agglutinin reacting with a serotype c strain of Streptococcus mutans. Eur J Biochem. 1983;133:255-61.

[32] Noris M, Remuzzi G. Overview of complement activation and regulation. Semin Nephrol. 2013;33:479-92.

[33] Bikker FJ, Ligtenberg AJ, Nazmi K, Veerman EC, van't Hof W, Bolscher JG, et al. Identification of the bacteria-binding peptide domain on salivary agglutinin (gp-340/DMBT1), a member of the scavenger receptor cysteine-rich superfamily. J Biol Chem. 2002;277:32109-15.

[34] Hartshorn KL, White MR, Mogues T, Ligtenberg T, Crouch E, Holmskov U. Lung and salivary scavenger receptor glycoprotein-340 contribute to the host defense against influenza A viruses. Am J Physiol Lung Cell Mol Physiol. 2003;285:L1066-76.

[35] Malamud D, Abrams WR, Barber CA, Weissman D, Rehtanz M, Golub E. Antiviral activities in human saliva. Adv Dent Res. 2011;23:34-7.

[36] Alarcon I, Evans DJ, Fleiszig SM. The role of twitching motility in Pseudomonas aeruginosa exit from and translocation of corneal epithelial cells. Invest Ophthalmol Vis Sci. 2009;50:2237-44.

[37] Stoddard E, Ni H, Cannon G, Zhou C, Kallenbach N, Malamud D, et al. gp340 promotes transcytosis of human immunodeficiency virus type 1 in genital tract-derived cell lines and primary endocervical tissue. J Virol. 2009;83:8596-603.

[38] Muller H, Hu J, Popp R, Schmidt MH, Muller-Decker K, Mollenhauer J, et al. Deleted in malignant brain tumors 1 is present in the vascular extracellular matrix and promotes angiogenesis. Arterioscler Thromb Vasc Biol. 2012;32:442-8.

[39] Nichols JJ, Green-Church KB. Mass spectrometry-based proteomic analyses in contact lens-related dry eye. Cornea. 2009;28:1109-17.

[40] Berlutti F, Ajello M, Bosso P, Morea C, Petrucca A, Antonini G, et al. Both lactoferrin and iron influence aggregation and biofilm formation in Streptococcus mutans. Biometals. 2004;17:271-8.

[41] Millar MR, Inglis T. Influence of lysozyme on aggregation of Staphylococcus aureus. J Clin Microbiol. 1987;25:1587-90.

[42] Li J, Metruccio MME, Evans DJ, Fleiszig SMJ. Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen Pseudomonas aeruginosa. PLoS Pathog. 2017;13:e1006392.

[43] Craig JP, Nichols KK, Akpek EK, Caffery B, Dua HS, Joo CK, et al. TFOS DEWS II Definition and Classification Report. Ocul Surf. 2017;15:276-83.

[44] Perumal N, Funke S, Pfeiffer N, Grus FH. Proteomics analysis of human tears from aqueous-deficient and evaporative dry eye patients. Sci Rep. 2016;6:29629.

[45] Ablamowicz AF, Nichols JJ. Concentrations of MUC16 and MUC5AC using three tear collection methods. Mol Vis. 2017;23:529-37.

[46] Farias E, Yasunaga KL, Peixoto RVR, Fonseca MP, Fontes W, Galera PD. Comparison of two methods of tear sampling for protein quantification by Bradford method. Pesq Vet Bras. 2013;33:4.

[47] Niederkorn JY, Kaplan HJ. Rationale for immune response and the eye. Chem Immunol Allergy. 2007;92:1-3.

[48] Eltis M. Contact-lens-related microbial keratitis: case report and review. J Optom. 2011;4:6.

[49] Stern GA. Contact lens associated bacterial keratitis: past, present, and future. CLAO J. 1998;24:52-6.

[50] Rumpakis J. New data on contact lens dropout: an international perspective. Review of Optometry. 2010.

[51] Musa F, Tailor R, Gao A, Hutley E, Rauz S, Scott RA. Contact lens-related microbial keratitis in deployed British military personnel. Br J Ophthalmol. 2010;94:988-93.

[52] Pachigolla G, Blomquist P, Cavanagh HD. Microbial keratitis pathogens and antibiotic susceptibilities: a 5-year review of cases at an urban county hospital in north Texas. Eye Contact Lens. 2007;33:45-9.

[53] Mattick JS. Type IV pili and twitching motility. Annu Rev Microbiol. 2002;56:289-314.

[54] Merz AJ, So M. Interactions of pathogenic neisseriae with epithelial cell membranes. Annu Rev Cell Dev Biol. 2000;16:423-57.

[55] Zolfaghar I, Evans DJ, Fleiszig SM. Twitching motility contributes to the role of pili in corneal infection caused by Pseudomonas aeruginosa. Infect Immun. 2003;71:5389-93.

[56] Redfern RL, McDermott AM. Toll-like receptors in ocular surface disease. Exp Eye Res. 2010;90:679-87.

[57] Huang X, Du W, McClellan SA, Barrett RP, Hazlett LD. TLR4 is required for host resistance in Pseudomonas aeruginosa keratitis. Invest Ophthalmol Vis Sci. 2006;47:4910-6.

[58] Robertson DM. The effects of silicone hydrogel lens wear on the corneal epithelium and risk for microbial keratitis. Eye Contact Lens. 2013;39:67-72.

[59] Thakur A, Willcox MD. Contact lens wear alters the production of certain inflammatory mediators in tears. Exp Eye Res. 2000;70:255-9.

[60] Liu Z, Kim J, Sypek JP, Wang IM, Horton H, Oppenheim FG, et al. Gene expression profiles in human nasal polyp tissues studied by means of DNA microarray. J Allergy Clin Immunol. 2004;114:783-90.

[61] Renner M, Bergmann G, Krebs I, End C, Lyer S, Hilberg F, et al. DMBT1 confers mucosal protection in vivo and a deletion variant is associated with Crohn's disease. Gastroenterology. 2007;133:1499-509.

[62] Lee HS, Hattori T, Park EY, Stevenson W, Chauhan SK, Dana R. Expression of toll-like receptor 4 contributes to corneal inflammation in experimental dry eye disease. Invest Ophthalmol Vis Sci. 2012;53:5632-40.

[63] Redfern RL, Barabino S, Baxter J, Lema C, McDermott AM. Dry eye modulates the expression of toll-like receptors on the ocular surface. Exp Eye Res. 2015;134:80-9.

[64] Christopherson PL, Smith J, Sosne G. Early corneal and lacrimal gland expression of inflammatory genes in a murine model of Sjogren's Syndrome. Invest Ophthalmol Vis Sci. 2005.

[65] Spachidou MP, Bourazopoulou E, Maratheftis CI, Kapsogeorgou EK, Moutsopoulos HM, Tzioufas AG, et al. Expression of functional Toll-like receptors by salivary gland epithelial cells: increased mRNA expression in cells derived from patients with primary Sjogren's syndrome. Clin Exp Immunol. 2007;147:497-503.

[66] Ambroziak AM, Szaflik J, Szaflik JP, Ambroziak M, Witkiewicz J, Skopinski P. Immunomodulation on the ocular surface: a review. Cent Eur J Immunol. 2016;41:195-208.

[67] Laurence A, O'Shea JJ, Watford WT. Interleukin-22: a sheep in wolf's clothing. Nat Med. 2008;14:247-9.

[68] Ji YW, Mittal SK, Hwang HS, Chang EJ, Lee JH, Seo Y, et al. Lacrimal gland-derived IL-22 regulates IL-17-mediated ocular mucosal inflammation. Mucosal Immunol. 2017;10:1202-10.

[69] Ma HL, Liang S, Li J, Napierata L, Brown T, Benoit S, et al. IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. J Clin Invest. 2008;118:597-607.

[70] Madsen J, Sorensen GL, Nielsen O, Tornoe I, Thim L, Fenger C, et al. A variant form of the human deleted in malignant brain tumor 1 (DMBT1) gene shows increased expression in inflammatory bowel diseases and interacts with dimeric trefoil factor 3 (TFF3). PLoS One. 2013;8:e64441.

[71] Taupin D, Podolsky DK. Trefoil factors: initiators of mucosal healing. Nat Rev Mol Cell Biol. 2003;4:721-32.

[72] Garay J, Piazuelo MB, Lopez-Carrillo L, Leal YA, Majumdar S, Li L, et al. Increased expression of deleted in malignant brain tumors (DMBT1) gene in precancerous gastric lesions: Findings from human and animal studies. Oncotarget. 2017;8:47076-89.

[73] Thim L, Mortz E. Isolation and characterization of putative trefoil peptide receptors. Regul Pept. 2000;90:61-8.

[74] Goke MN, Cook JR, Kunert KS, Fini ME, Gipson IK, Podolsky DK. Trefoil peptides promote restitution of wounded corneal epithelial cells. Exp Cell Res. 2001;264:337-44.

[75] Paulsen FP, Woon CW, Varoga D, Jansen A, Garreis F, Jager K, et al. Intestinal trefoil factor/TFF3 promotes re-epithelialization of corneal wounds. J Biol Chem. 2008;283:13418-27.

[76] Podolsky DK. Mucosal immunity and inflammation. V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. Am J Physiol. 1999;277:G495-9.

[77] Sasaki M, Tsuneyama K, Saito T, Kataoka H, Mollenhauer J, Poustka A, et al. Site-characteristic expression and induction of trefoil factor family 1, 2 and 3 and malignant brain tumor-1 in normal and diseased intrahepatic bile ducts relates to biliary pathophysiology. Liver Int. 2004;24:29-37.

[78] Vijayakumar S, Peng H, Schwartz GJ. Galectin-3 mediates oligomerization of secreted hensin using its carbohydrate-recognition domain. Am J Physiol Renal Physiol. 2013;305:F90-9.

[79] Dumic J, Dabelic S, Flogel M. Galectin-3: an open-ended story. Biochim Biophys Acta. 2006;1760:616-35.

[80] Mauris J, Mantelli F, Woodward AM, Cao Z, Bertozzi CR, Panjwani N, et al. Modulation of ocular surface glycocalyx barrier function by a galectin-3 N-terminal deletion mutant and membrane-anchored synthetic glycopolymers. PLoS One. 2013;8:e72304.

[81] Woodward AM, Mauris J, Argueso P. Binding of transmembrane mucins to galectin-3 limits herpesvirus 1 infection of human corneal keratinocytes. J Virol. 2013;87:5841-7.

[82] Uchino Y, Mauris J, Woodward AM, Dieckow J, Amparo F, Dana R, et al. Alteration of galectin-3 in tears of patients with dry eye disease. Am J Ophthalmol. 2015;159:1027-35 e3.

[83] Acton QA. Galectins - Advances in Research and Application. Atlanta: Scholarly Editions; 2012.

[84] Arwert EN, Hoste E, Watt FM. Epithelial stem cells, wound healing and cancer. Nat Rev Cancer. 2012;12:170-80.

[85] Yabuta C, Yano F, Fujii A, Shearer TR, Azuma M. Galectin-3 enhances epithelial cell adhesion and wound healing in rat cornea. Ophthalmic Res. 2014;51:96-103.

[86] Mitoma M, Oho T, Shimazaki Y, Koga T. Inhibitory effect of bovine milk lactoferrin on the interaction between a streptococcal surface protein antigen and human salivary agglutinin. J Biol Chem. 2001;276:18060-5.

[87] Oram JD, Reiter B. Inhibition of bacteria by lactoferrin and other iron-chelating agents. Biochim Biophys Acta. 1968;170:351-65.

[88] Rogers T. Immunopharmacology. Boca Raton: CRC Press; 1989.

[89] Oho T, Bikker FJ, Nieuw Amerongen AV, Groenink J. A peptide domain of bovine milk lactoferrin inhibits the interaction between streptococcal surface protein antigen and a salivary agglutinin peptide domain. Infect Immun. 2004;72:6181-4.

[90] Radicioni G, Cao R, Carpenter J, Ford AA, Wang T, Li L, et al. The innate immune properties of airway mucosal surfaces are regulated by dynamic interactions between mucins and interacting proteins: the mucin interactome. Mucosal Immunol. 2016;9:1442-54.

[91] Perez-Vilar J, Mabolo R. Gel-forming mucins. Notions from in vitro studies. Histol Histopathol. 2007;22:455-64.

[92] Ablamowicz AF, Nichols JJ. Ocular Surface Membrane-Associated Mucins. Ocul Surf. 2016;14:331-41.

[93] Gipson IK. Distribution of mucins at the ocular surface. Exp Eye Res. 2004;78:379-88.

[94] Elstein M, Parke DV. Mucus in health and disease. New York: Premium Press; 1976.

[95] Ujma S, Horsnell WG, Katz AA, Clark HW, Schafer G. Non-Pulmonary Immune Functions of Surfactant Proteins A and D. J Innate Immun. 2017;9:3-11.

[96] Zhang Z, Abdel-Razek O, Hawgood S, Wang G. Protective Role of Surfactant Protein D in Ocular Staphylococcus aureus Infection. PLoS One. 2015;10:e0138597.

[97] Ni M, Evans DJ, Hawgood S, Anders EM, Sack RA, Fleiszig SM. Surfactant protein D is present in human tear fluid and the cornea and inhibits epithelial cell invasion by Pseudomonas aeruginosa. Infect Immun. 2005;73:2147-56.

[98] Brauer L, Kindler C, Jager K, Sel S, Nolle B, Pleyer U, et al. Detection of surfactant proteins A and D in human tear fluid and the human lacrimal system. Invest Ophthalmol Vis Sci. 2007;48:3945-53.

[99] Mun JJ, Tam C, Kowbel D, Hawgood S, Barnett MJ, Evans DJ, et al. Clearance of Pseudomonas aeruginosa from a healthy ocular surface involves surfactant protein D and is compromised by bacterial elastase in a murine null-infection model. Infect Immun. 2009;77:2392-8.

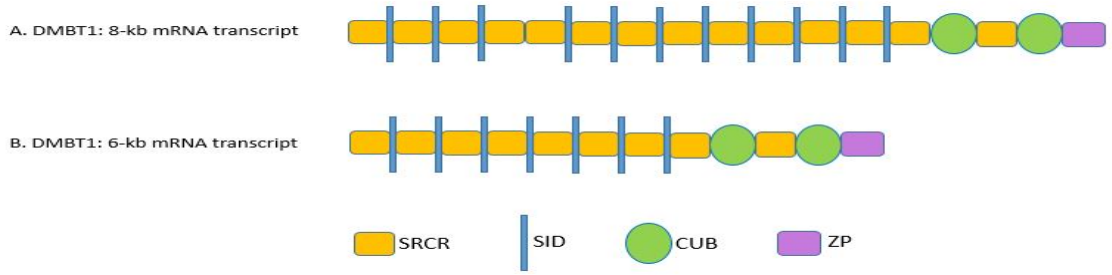

Figure 1. Schematic overview of the structural organization of Gp340. Gp340 contains up to 14 Group B SRCR domains. Interspersed between successive SRCRs are SIDs which are the sites for posttranslational glycosylation. There are 2 CUB domains which mediate Gp340's interactions with extracellular proteins. Located at the C-terminus is a single ZP domain which is primarily involved in Gp340 oligomerization. Alternative splicing results in mRNA transcripts which code for Gp340 with different number of SRCRs. The 8-kb transcript (A) codes for Gp340 with 14 SRCR domains, whilst the 6-kb transcript (B) codes for protein with nine SRCR domains.

CHAPTER 3 – HYPEROSMOLAR STRESS UPREGULATES GLYCOPROTEIN 340 AND THE COMPLEMENT FACTOR C1Q EXPRESSION IN HUMAN CORNEAL EPITHELIAL CELLS

by

KWAKU A. OSEI, CHAMPION DEIVANAYAGAM, JASON J. NICHOLS

In review at *Experimental Eye Research*

Format adapted for dissertation

ABSTRACT

Glycoprotein (Gp340) is a heavily glycosylated pattern recognition receptor expressed in the cornea, conjunctiva, and lacrimal gland. Previous investigations reported a dysregulation of Gp340 expression in the tears of dry eye subjects (Nichols and Green-Church, 2009; Perumal et al., 2016). Given that pattern recognition receptors are involved in dry eye inflammatory response, this study examined the expression pattern of Gp340 in human corneal epithelial cells (HCECs) exposed to hyperosmolar stress (HOS) and its possible role in proinflammatory cytokine production. To determine the effect of HOS on Gp340 expression, SV40-transformed (immortalized) HCECs were treated with either hyperosmolar (500 mOsmol/kg) or normoosmolar (285 mOsmol/kg) media. Gp340 mRNA transcription and protein expression levels were then determined by real-time RT-PCR and ELISA, respectively. Given that HOS stimulates inflammation, the mRNA and protein expression of four dry eye-associated proinflammatory cytokines – IL-1β, IL-6, IL-8, and TNFα, were also analyzed. In addition, the translation of the complement-activating proteins, C1q and mannan-binding lectin (MBL) were investigated since Gp340 activates the complement system. Finally, to examine the role of Gp340 in dry eye inflammatory signaling, Gp340 gene transcription in HCECs was transiently knocked down/silenced using *DMBT1*-specific siRNA and the concomitant effects on IL-1β, IL-8, TNFα, and C1q expression were determined. The study showed that Gp340 mRNA and protein expression significantly increased in HCECs exposed to HOS. There was a corresponding increase in IL-1β, IL-8, and TNFα mRNA and protein expression. For the complement proteins, HOS

increased C1q expression but had no effect on MBL. Knocking down Gp340 gene transcription, however, did not affect IL-1β, IL-8, TNFα, and C1q production. In conclusion, HOS exerts an independent influence on the regulation of Gp340 and the proinflammatory cytokines. More importantly, HOS increased both Gp340 and C1q expression in HCECs. This C1q upregulation is novel and suggests the involvement of the classical complement pathway in dry eye inflammatory response and warrants to be explored further.

INTRODUCTION

Dry eye disease (DED) is a multifactorial functional abnormality of the lacrimal and meibomian gland secretory systems, which is characterized by tear hyperosmolarity, ocular surface inflammation and damage, neurosensory abnormalities, and ocular symptoms (Craig et al., 2017). Dry eye affects 5-50% of the population worldwide and is a leading cause of outpatient visits and a major public health burden (Stapleton et al., 2017). In DED, hyperosmolar stress results in the release of damage associated molecular patterns like heat shock proteins, nucleic acids, high-mobility gene box 1, and extracellular matrix fragments, which in turn activate the expression of ocular surface pattern recognition receptors (PRRs) that trigger the expression of gene transcription factors and downstream inflammatory response (Bron et al., 2017; Lema et al., 2018). Investigations into the involvement of PRRs in dry eye inflammation have focused largely on the toll-like receptors (TLRs) (Bron et al., 2017; Lee et al., 2012; Redfern et al., 2015; Redfern et al., 2013; Reins et al., 2018; Simmons et al., 2016). While TLRs play a role in modulating ocular surface damage associated with DED, the diagnostic relevance of TLRs for DED is limited considering that TLR expression patterns vary considerably between the different ocular surface tissues and also differ with the underlying stimulus for hyperosmolar stress (Redfern et al., 2015; Redfern et al., 2013).

Among the less-studied ocular surface PRRs, glycoprotein 340 (Gp340) demonstrates a potential to be involved in the inflammatory response in DED (Nichols and Green-Church, 2009; Osei et al., 2018; Rosenstiel et al., 2007). Also known as “Deleted in

Malignant Brain Tumor 1" (DMBT1) and salivary agglutinin, Gp340 is a member of the scavenger receptor class of PRRs, and it is encoded by the *DMBT1* gene (Brown, 2006; Ligtenberg et al., 2010). This heavily glycosylated protein belongs to the scavenger receptor cysteine-rich (SRCR) superfamily of proteins and is composed of multiple (up to 14) SRCR domains, up to 11 SRCR-interspersed domains, two C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein-1 (CUB) domains, and a single zona pellucida (ZP) domain (see supplementary figure 1) (Ligtenberg et al., 2010; Osei et al., 2018). On the ocular surface, it is a component of tears expressed by the lacrimal gland secretory acini and the corneal and conjunctival epithelia (Jumblatt et al., 2006; Schulz et al., 2002). Tear Gp340 inhibits twitching motility of *P. aeruginosa* and in preliminary studies, the SRCR domain has demonstrated the potential to promote bacterial adhesion on contact lens polymers (Li et al., 2017a; Li et al., 2017b; Osei et al., 2019).

Nichols and Green-Church (2009) previously reported an elevated tear Gp340 level in DED related to contact lens wear, a factor which is intricately linked to ocular surface inflammation (Ramamoorthy et al., 2021; Robertson, 2013; Thakur and Willcox, 2000). Elsewhere in the body, Gp340 has been implicated in wet-surfaced mucosal inflammatory conditions such as nasal polyposis, ulcerative colitis, and ethmoid sinusitis (Abdelfattah et al., 2016; Liu et al., 2004; Renner et al., 2007). Rosenstiel et al. (2007) also showed the activation of Gp340 activation in response to TNFα inflammatory stimulus. Taken together, these findings present indirect evidence of the involvement of Gp340 in inflammation, and perhaps, it is directly involved in DED. Thus, the primary goals of this study were to examine Gp340 expression in human corneal epithelial cells under

hyperosmolar stress and to investigate the potential role of Gp340 in the production of proinflammatory cytokines associated with DED.

MATERIALS AND METHODS

Analysis of DMBT1 mRNA Transcription and Protein Expression in Human Corneal Epithelial Cells under Hyperosmolar Stress

Cell Culture and Hyperosmolar Stress Treatments

The methods used in previous studies (Redfern et al., 2015) were adapted for this study. Briefly, 1 x 10^6 HCECs were plated in triplicate in T-25 cm^2 tissue culture flasks containing complete DMEM/F12 media containing 5% fetal bovine serum, 0.5% DMSO, 10 ng/mL human EGF, and 5µg/mL insulin and allowed to grow for 48 h to reach 60-70% confluency. Following this the cells were washed two times with PBS and fresh basal DMEM/F12 media was added to the cells and allowed to incubate for 12 hours. Subsequently, the media was removed, and these cells were exposed to either hyperosmolar media (500 mOsmol/kg) or normosmolar media (285 mOsmol/kg). 500 mOsmol/kg osmolality was achieved by a stepwise titration of basal DMEM/F12 (285 mOsmol/kg) with 4.3 M NaCl stock solution while monitoring osmolality using a vapor pressure osmometer (VAPRO®) until it reached 500 mOsmol/kg. These cells were exposed to both hyperosmolar and normoosmolar media treatments for seven different time periods: 15 minutes, 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h. At the end of each experimental (exposure) time, the conditioned media was collected, aliquoted and stored at -80 °C for later protein analysis. Likewise, the cells were detached using TryPLE (Invitrogen, Carlsbad) and divided into equal halves and each was lysed in either RLT RNeasy® lysis buffer (Qiagen, Germantown, MD, USA) or RIPA buffer (Thermo Scientific, Carlsbad, CA, USA). The

respective lysates were centrifuged at 14,000 ×g and the supernatants were stored at -80 °C for later mRNA and protein analysis. Each of these experiments was repeated at least two times (n = 9 per each time point).

Primer Design

Primers specific to *DMBT1* and *18S RNA* (endogenous control) were designed using the National Center for Biotechnology Information's Primer-BLAST software (Ye et al., 2012). The primers used for *DMBT1* were GCAGGCAGACAATGACACCA (forward) and TGCAGCTGACGTGAATACGG (reverse) while CCGGACACGGACAGGATTGA (forward) and GCATGCCAGAGTCTCGTTCG (reverse) were the primers for *18S RNA*. These primers had 100% specificity to *DMBT1* and the *18S RNA.* The *DMBT1* and *18S RNA* primers yield 104- and 121-bp amplicons respectively.

Total RNA Isolation, cDNA Synthesis, Qualitative and Real-Time RT-PCR

Briefly, using the RNeasy® Plus Mini kit (Qiagen), total RNAs were isolated from the HCECs lysed in the RLT RNeasy® lysis buffer. The quality and quantity of the isolated RNA were determined using the NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was then synthesized from 500 ng isolated RNA using the High-Capacity RNA-to-cDNA™ Kit (Life Technology, Carlsbad, CA, USA) in a 20-µL reaction volume.

Using the predesigned *DMBT1*- and *18S RNA*-TaqMan™ gene expression assays (Life Technologies, DMBT1: Hs01069306_m1, 18S RNA: Hs99999901_s1), the mRNA

transcription of *DMBT1* and *18S RNA* were determined using 10 ng cDNA and TaqMan™ Fast Advanced Master Mix on the QuantStudio 3™ real-time PCR instrument (Applied Biosystems). Real-time PCR data was normalized to *18S RNA* and were analyzed with the Design and Analysis Software™ (version 1.5.1, Applied Biosystems). mRNA levels in the hyperosmolar media-treated cells were normalized to those in the control cells, and the results were expressed as fold change. These fold change differences were analyzed using the Mann-Whitney U test with $p < 0.05$ denoting statistical significance. Kruskal–Wallis H test with post hoc pairwise comparisons was used to determine the time dependence of hyperosmolar stress effect on *DMBT1* mRNA transcription.

To confirm real-time PCR data, gel-based RT-PCR was also performed on the 12 hour-treated samples using 20 ng cDNA, 1x DreamTaq Green PCR Master Mix, and 0.5 μM each of forward and reverse primers in a 50 μL reaction volume. At the end of the reactions, these PCR products were analyzed using 2% agarose gel electrophoresis.

Enzyme-Linked Immunosorbent Assay (ELISA)

Sandwich ELISA was used to determine the expression of Gp340 in the 12 hour-treated samples. The 12-hour time point was selected because while the effects of hyperosmolar stress on Gp340 mRNA transcription were highest at both the 12- and 24-hour time points, cells at the 12-hour point were in a more optimal morphological appearance. Prior to ELISA, the total protein content in all samples were determined by BCA assay. For the ELISA, Nunc MaxiSorp™ flat-bottom 96-well microtiter plate (Invitrogen) were coated with 100 μL of mouse anti-human Gp340 capture antibody (Invitrogen) at a concentration of 2.5 μg/mL and incubated for 18 hours at 4°C. These wells

were then aspirated and blocked for 2 hours at room temperature (RT) with 1% BSA in the PBST buffer. Between each of the steps described below, the wells were washed 3x times with PBST. (i) 100 µL of diluted samples were each added in duplicates to the wells and incubated at room temperature (RT) for 2 h. (ii) 100 µL of mouse anti-human Gp340 detection antibody (Invitrogen, 1:1000 dilution) was added to each well and incubated for 2 hours at RT. The capture and detection antibodies recognize different Gp340 epitopes. (iii) 100 µL HRP-conjugated secondary antibody was added and incubated for 2 hours. (iv) 100 µL of TMB solution (3,3′,5,5′-tetramethylbenzidine) was added to the wells and incubated at RT for 30 minutes, after which the reaction was quenched by adding 100 µL of 2M H_2SO_4 solution. The absorbances at 450 nm (OD_{450}) were then read on a microplate reader (BioTek® Synergy 2, Winooski, VT, USA) and adjusted for background noise. After determining the normality of the data, the differences in relative levels of Gp340 between the hyperosmolar stress and control samples were analyzed using the independent t test with $p < 0.05$ denoting statistical significance. The experimental design is summarized in Supplementary figure 2A.

Determination of the Correlation of Proinflammatory Cytokine Gene Transcription and Protein Expression with Gp340 mRNA and Protein Levels

Any potential inflammation-modulating effects of Gp340 would likely impact the expression of proinflammatory cytokines. On account of this, the levels of four proinflammatory cytokines whose expressions have been shown previously to be upregulated in a desiccated ocular surface epithelium, were investigated. These are TNFα, IL-1β, IL-6, and IL-8 (Bron et al., 2017).

Real-Time RT-PCR

The total isolated RNAs used in the previous *DMBT1*-related real-time PCR experiments were used to synthesize new cDNAs. Real-time RT-PCR was performed as described above using inventoried TaqMan™ gene expression assays (Life Technologies) designed for *TNFα* (Hs00174128_m1), *IL1-β* (Hs01555410_m1), *IL-6* (Hs00174131_m1), and *IL-8* (Hs00174103_m1). Only the 12 h and 24 h-treated samples were used for the analysis of the proinflammatory cytokine genes as the effects of hyperosmolar stress on Gp340 mRNA transcription were determined in the earlier section to be highest at these two time points.

ELISA

The 12 hour-treated samples (conditioned media), some of which were used in the Gp340 ELISA in the earlier section, were used in sandwich ELISA to examine the secretion of TNFα, IL1β, IL6, and IL8 from HCECs. The ELISA was performed using TNFα , IL1-β, IL-6, and IL-8 human matched antibody pairs (Invitrogen) and following the vendor's instructions. Briefly, the 96-well ELISA plate was coated with 100 µL of capture antibody per well and incubated for 18 h at 4 °C. The capture antibody concentrations were 2 µg/mL for TNFα and IL-1β, and 1 µg/mL for IL-6 and IL-8. The wells were then washed 3× with PBST here and between all the subsequent steps. (i) Wells were blocked with 0.5% BSA in PBST for 1 h at RT; (ii) the wells were aspirated and 100 µL of samples and standards (concentration: 15.6 – 1,000 pg/mL) were added in duplicate and incubated with the respective detection primary antibodies for 2 h at RT; (iii) wells were incubated with a 100 µL of streptavidin-HRP solution for 30 min at RT; (iv) wells were incubated with TMB

solution for 30 min and quenched with 2M H_2SO_4. The absorbances were then read at 450 nm (OD_{450}). The results were analyzed using the four-parameter logistic model in Gen 5™ analysis software (BioTek®, version 3.08). The data were normally distributed and therefore the student t test was used to determine the difference in cytokine expressions between the hyperosmolar stress and control treatments with $p < 0.05$ signifying statistical significance.

Determination of the Effects of DMBT1 Gene Knockdown on Proinflammatory Cytokine Gene Transcription and Protein Translation under Hyperosmolar Stress

Transient Knockdown of DMBT1 Gene Transcription

Following manufacturer's instructions, *DMBT1* gene transcription in HCECs was transiently knocked down by both forward and reverse transfection with Lipofectamine® RNAiMAX (Life Technologies) and *DMBT1*-targeting siRNA (Ambion® Silencer Select siRNA, ID: s4154). Cells transfected with non-targeting siRNA served as negative control. Briefly, for forward transfection, 2.5 x 10^5 cells were seeded in 12-well plates and cultured for 24 hours in complete DMEM/F12. Thereafter, the cells were transfected with 100 µL siRNA-lipofectamine complex (final concentration of siRNA = 20 nM) for 48 or 72 hours (n = 4 per transfection time for each siRNA). For reverse transfection, the cells were seeded and transfected at the same time (n = 4 per transfection time for each siRNA). In all transfection process, the cells were monitored closely for potential toxicity from the siRNA-lipofectamine complex.

At the end of each transfection time, the cells were harvested and divided into two. One half was lysed in RLT RNeasy® lysis buffer and the other was lysed in RIPA buffer and stored at -80 °C and later processed for real-time RT-PCR and ELISA to determine the

transfection efficiency. From real-time PCR analysis, the highest transfection efficiency was approximately 75% for forward transfection and approximately 90% for reverse transfection, each occurring at 48 hours post-transfection. At the protein level for reverse transfection, Gp340 expression in the *DMBT1* siRNA-transfected HCECs was 85% lower relative to the expression in the non-targeting siRNA-transfected cells. On account of these, the 48-hour reverse transfection method was chosen for all subsequent *DMBT1* gene knockdown studies.

Experimental Treatments

Briefly, 2.5×10^5 cells were seeded in 12-well plates and reverse-transfected in triplicate with either *DMBT1*-targeting siRNA or non-targeting siRNA for 48 h. Subsequently, the condition media was removed, and the cells were washed. The *DMBT1* siRNA-transfected cells were then treated with either 500 mOsmol/kg media or 285 mOsmol/kg for 12 hours. Similarly, the non-targeting siRNA-transfected cells were also exposed to 500 mOsmol/kg media or 285 mOsmol/kg for 12 hours. Afterward, the respective condition media were aliquoted and stored at -80 °C for future protein expression analysis. The cells were also harvested and stored at -80 °C for later mRNA transcription analysis. The experiments were repeated at least two more times (n = 9 per treatment per siRNA).

Real-Time RT-PCR and ELISA

Briefly, total RNAs were isolated and used to synthesize cDNAs that were subsequently used in real-time PCR to determine the transcription of *TNFα*, *IL-1β*, and *IL-*

8, using the inventoried Taqman assays described in the earlier section. TNFα , IL-1β, and IL-8 proteins were also analyzed using sandwich ELISA as described. The experimental design is summarized in Supplementary figure 2B.

Determination of the Effect of Gp340 on Complement Activation in Hyperosmolar Stress

Gp340 has been previously shown to stimulate the expression of the complement factors, C1q and mannan-binding lectin (MBL) which are the initiating molecules for the classical and lectin complement pathways, respectively (Boackle et al., 1993; Noris and Remuzzi, 2013; Osei et al., 2018; Reichhardt et al., 2012; Reichhardt and Meri, 2016). Given that complement activation could trigger inflammation signaling, we determined the expression of C1q and MBL in HCECs under hyperosmolar stress and the possible role of Gp340 in the expression.

Determining C1q and MBL Expression in HCECs under Hyperosmolar Stress

Briefly, 5.5×10^5 HCECs were seeded in 6-well plates and cultured in complete DMEM/F12 media for 24 hours. Afterwards, the media was removed, and the cells were exposed to triplicates of 1.5 mL of 500 mOsmol/kg basal media or 285 mOsmol/kg media (control) for 12 hours. Thereafter, the conditioned media were collected, and protease inhibitor was added before storage at -80 °C for C1q and MBL quantitation. The cells were lysed in 500 µL of cold RIPA lysis buffer containing protease inhibitor and the lysates were centrifuged at 14,000 × g for 15 minutes and the supernatants were stored at -80 °C for C1q and MBL protein analysis. The experiments were repeated two more times (n = 9 per treatment). The total protein content in the conditioned media and lysates were determined

by BCA assay. The levels of C1q and MBL in the conditioned media and lysates were determined using C1q and MBL sandwich ELISA kits (Invitrogen). In the sandwich ELISA, briefly, 100 µL of conditioned media and diluted lysates, along with the respective C1q or MBL standards at various concentrations (C1q: 0.9 – 60 ng/mL, MBL: 0.103 – 25 ng/mL) were loaded into 96-well microtiter plates pre-coated with either C1q or MBL primary antibody and incubated at RT for 2 hours under constant shaking. Afterwards, the wells were washed 4 times and 100 µL of biotin-conjugated C1q (1:100 dilution) or MBL antibody (1:80 dilution) were added and incubated at RT for 1 hour on a shaker. Subsequently, the wells were washed and incubated with 100 µL of streptavidin-HRP (1:100 dilution for C1q, 1:800 for MBL) for 1 hour at RT. Thereafter, the wells were washed incubated for 30 minutes with and 100 µL of TMB substrate at RT. After the addition of the stop solution, the absorbances at 450 nm (OD_{450}) were read and corrected for background noise. Both the C1q and MBL standard curves were generated and analyzed with the four-parameter logistic (4PL) model in GraphPad Prism. The concentrations C1q and MBL in the samples were interpolated from these curves. Independent t test was used to analyze the differences in C1q and MBL levels between normosmolar media-treated and hyperosmolar media-treated sample, after establishing the normality of the data. The experimental design is summarized in Supplementary figure 2A.

The Effect of Gp340 mRNA Knockdown on C1q Expression under Hyperosmolar Stress

Briefly, 2.5 x 10^5 HCECs were seeded in 12-well plates and reverse-transfected with either *DMBT1* siRNA or non-targeting siRNA, each in triplicate, for 48 hours as described in the earlier section. Subsequently, the growth media was removed, and the cells

were washed. *DMBT1* siRNA and control siRNA-transfected cells were both treated with 800 μL of 500 mOsmol/kg basal media for 12 hours. Thereafter, the conditioned media were removed, and the cells were washed and lysed for 5 minutes in cold RIPA buffer, and the lysates were then centrifuged, and the supernatants were collected and stored at -80 °C for C1q protein quantitation. The experiments were repeated at least two more times (n = 9 per siRNA type). C1q expression in the samples was determined using sandwich ELISA as described earlier. Data was analyzed using the student t test with $p < 0.05$ denoting a statistically significant difference in C1q levels between *DMBT1*- and control siRNA-transfected cells. The experimental design is summarized in Supplementary figure 2B.

RESULTS

The Effects of Hyperosmolar Stress Treatment on *DMBT1* mRNA Transcription and Gp340 Protein Expression in HCECs

Except the 15-minute and 1-hour treatments, HCECs exposed to 500 mOsmol/kg media had higher transcription of *DMBT1* compared to the cells treated with the normosmolar media (Mann-Whitney U test, $p < 0.01$, Figure 1A). The transcription of *DMBT1* was time-dependent whereby increasingly higher mRNA levels were observed up to the 24 h time point (Kruskal–Wallis H test with multiple pairwise comparisons). Gel-based (qualitative) RT-PCR on the 12 hour-treated samples confirmed the increased Gp340 mRNA expression with hyperosmolar stress treatment (Figure 1B). Likewise, for the 12-hour treatment samples, Gp340 protein expression was higher in the hyperosmolar-stressed cells compared to the control (t test, $p < 0.0001$).

The Effect of Hyperosmolar Stress on Proinflammatory Cytokine Gene and Protein Expression

In response to hyperosmolar stress, *TNFα* , *IL1-β,* and *IL-8* mRNA gene transcriptions increased compared to the control, at the two tested time periods, 12 and 24 hours (Mann Whitney U test, $p < 0.01$, Figure 2A). The *IL-6* gene transcription, however, reduced ($p < 0.05$). Qualitative RT-PCR of the 12 hour-treated samples confirmed the transcription patterns of the cytokines (Figure 2B). At protein level, TNFα (mean difference: 97.54 ± 25.30 pg/mL, $p = 0.002$), IL1-β (mean difference: 135.40 ± 40.66 pg/mL, $p = 0.008$), and IL-8 (mean difference: 57.38 ± 17.54 pg/mL, $p = 0.008$) expression

increased relative to the control at the 12-hour treatment time while IL-6 level (36.19 ± 28.21 pg/mL, p = 0.228) did not change significantly (t test, Figure 2C).

The Effect of *DMBT1* Gene Knockdown on TNFα , IL1-β, and IL-8 mRNA and Protein Levels

When *DMBT1* gene transcription in HCECs was transiently knocked down with *DMBT1* siRNA and the cells were exposed to hyperosmolar stress, the transcription levels of *TNFα* (p = 0.052)*, IL1-β,* (p = 0.098) and *IL-8* (p = 0.066) mRNA did not change (independent t test*,* Figure 3A – C). Similarly, at the protein level, TNFα (mean difference ± SEM: 29.51 ± 41.02, p = 0.48), IL1-β (mean difference: 14.18 ± 23.12, p = 0.55), and IL-8 (mean difference: 13.80 ± 12.95, p = 0.30) expression did not change (Figure 3D – F).

The Effect of Hyperosmolar Stress on C1q and MBL Expression in HCECs

Compared to the HCECs exposed to normosmolar media, the expression of the complement protein, C1q in the hyperosmolar media-treated cells (lysates) was higher (mean difference ± SEM: 39.89 ± 11.97 ng/mL, student t test, p = 0.03, Figure 4A). However, C1q could not be detected in the conditioned media samples. For MBL, no detectable levels were observed in both lysates and conditioned media for both hyperosmolar stress and control treatments.

The Effect of Gp340 Expression on C1q Expression

Silencing Gp340 mRNA in HCECs did not modulate C1q expression under hyperosmolar stress as there was no significant difference in C1q levels between the

DMBT1 siRNA- and control siRNA-transfected cells when both were treated with hyperosmolar media (mean difference ± SEM: 5.026 ± 6.737 ng/mL, test, p = 0.473, Figure 4B).

DISCUSSION

Dry eye is associated with a self-perpetuating inflammatory cycle. The core mechanism underlying dry eye inflammation is hyperosmolar stress that results from increased tear osmolarity (Bron et al., 2017). Pattern recognition receptors (PRRs) play a crucial role in dry eye inflammatory signaling. Gp340 is potential inflammation-modulating PRR, hence this study aimed primarily at examining the expression of Gp340 in human corneal epithelial cells (HCECs) under hyperosmolar stress and to determine if it affects the production of proinflammatory cytokine expression.

Under hyperosmolar stress condition, upregulation of both Gp340 mRNA transcription and protein expression was observed in HCECs (Figure 1). Nichols and Green-Church (2009) previously reported an upregulation of Gp340 in contact lens-related dry eye (CLDE). Another study by Perumal et al. (2016) however, showed a downregulation of Gp340 in the aqueous-deficient and the mixed (aqueous-deficient/evaporative) dry eye types using targeted mass spectrometry. While the difference in Gp340 expression patten in these two previous studies is counterintuitive, it could be attributed to the differences in the designs of the two studies. Firstly, while Nichols & Green-Church investigated dry eye associated with contact lens wear, the dry eye subjects in Perumal and colleagues' study were non-contact lens wearers. Contact lens- and non-contact lens-related dry eye have different underlying pathophysiology (Kojima, 2018; Ramamoorthy et al., 2021). In addition, different tear collection techniques were employed in the two studies. The microcapillary tear collection method was used in

Nichols & Green-Church's study while the Schirmer strip sampling technique was utilized by Perumal et al. These two tear sampling methods, even in the same study, could yield different concentrations of the same protein (Ablamowicz and Nichols, 2017; Farias et al., 2013). Thus, future studies employing these two collection techniques at the same time and including both primary dry eye and contact lens-related dry eye subjects would be helpful in clarifying the differing modulation patterns of dry eye. It must be noted that the current study differs from the two previous ones in certain respects. First, while the current study focused primarily on hyperosmolar stress, neither of the previous studies assessed tear osmolarity. In addition, the current study investigated Gp340 derived from only the corneal epithelium while the two studies analyzed total tear Gp340 derived from all sources – lacrimal gland, conjunctiva, and cornea.

Intuitively, if Gp340 is involved in dry eye inflammation, then an increased expression of Gp340 in hyperosmolar stress would be associated with an increased expression of proinflammatory cytokines that are upregulated in DED. As has been reported before (Bron et al., 2017; Massingale et al., 2009), three dry eye-associated proinflammatory cytokines, TNFα, IL1-β,and IL-8 were significantly upregulated at both the mRNA and protein levels (Figure 2). To determine if Gp340 is directly involved in the observed hyperosmolar stress-induced inflammation in HECEs, the effects of Gp340 expression on the levels of the three upregulated cytokines were investigated by silencing (knocking down) Gp340 mRNA transcription under hypothesis that downregulating Gp340 mRNA transcription would reduce proinflammatory cytokine production if Gp340 expression is involved in the inflammation process. However, when Gp340 gene transcription was knocked down and the cells were exposed to hyperosmolar stress

treatment, the levels of TNFα, IL1-β, and IL-8 did not change significantly (Figure 3). Summarily, these results suggest that Gp340 expression does not play a direct role in the signaling of dry eye inflammatory cytokines.

The complement system is another important component of innate immunity. It consists of soluble and membrane bound proteins that play crucial roles in the defense against infection and the modulation of immune and inflammatory responses (Janeway, 2001). The complement system can be activated via three routes: (i) the classical pathway which is triggered by antibody or binding of C1q to damage associated molecular patterns (DAMPs) or molecular patterns on pathogens, (ii) the lectin pathway, activated by MBL, and (iii) the alternative pathway which is activated spontaneously when the activated complement protein binds to a pathogen (Janeway, 2001). In previous studies, Gp340 have been shown to activate both the classical and lectin arms of the complement system (Reichhardt et al., 2012; Reichhardt and Meri, 2016). Given that complement activation can amplify inflammation (Noris and Remuzzi, 2013), it is hypothesized that any hyperosmolar stress-mediated upregulated Gp340 expression on the ocular surface could trigger complement activation and potentiate dry eye inflammation signaling. Therefore, the expression patterns of C1q and MBL in the corneal epithelium were examined to elucidate the possible role of Gp340 in hyperosmolar stress-induced complement regulation. The current studies show upregulation of C1q expression in the corneal epithelial cells exposed to hyperosmolar stress (Figure 4A). However, downregulating Gp340 mRNA transcription did not affect C1q translation (Figure 4B), an indication that Gp340 does not, on its own, activate the classical complement pathway in dry eye, and perhaps there are other factors involved.

In summary, this study has demonstrated that hyperosmolar stress on the ocular surface increases cornea-expressed Gp340 and the complement activating molecule, C1q. There are other studies that suggest a potential crosstalk between Gp340 expression and other pattern recognition receptors. In fact, Rosenstiel et al., demonstrated the regulation of Gp340 mRNA transcription through signaling of nucleotide-binding oligomerization domain 2 (NOD2) and TLR4 (Rosenstiel et al., 2007). Given that NOD2 and TLR4 activation are associated with dry eye-associated conditions (Lee et al., 2012; Redfern et al., 2013), future studies should explore the potential involvement of Gp340 in dry eye inflammation mediated by these other PRRs.

REFERENCES

Abdelfattah, M.M., Khattab, R.A., Mahran, M.H., Elborgy, E.S., 2016. Evaluation of patients with dry eye disease for conjunctival Chlamydia trachomatis and Ureaplasma urealyticum. Int J Ophthalmol 9, 1457-1465.

Ablamowicz, A.F., Nichols, J.J., 2017. Concentrations of MUC16 and MUC5AC using three tear collection methods. Mol Vis 23, 529-537.

Boackle, R.J., Connor, M.H., Vesely, J., 1993. High molecular weight non-immunoglobulin salivary agglutinins (NIA) bind C1Q globular heads and have the potential to activate the first complement component. Mol Immunol 30, 309-319.

Bron, A.J., de Paiva, C.S., Chauhan, S.K., Bonini, S., Gabison, E.E., Jain, S., Knop, E., Markoulli, M., Ogawa, Y., Perez, V., Uchino, Y., Yokoi, N., Zoukhri, D., Sullivan, D.A., 2017. TFOS DEWS II pathophysiology report. Ocul Surf 15, 438-510.

Brown, G.D., 2006. Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Immunol 6, 33-43.

Craig, J.P., Nichols, K.K., Akpek, E.K., Caffery, B., Dua, H.S., Joo, C.K., Liu, Z., Nelson, J.D., Nichols, J.J., Tsubota, K., Stapleton, F., 2017. TFOS DEWS II Definition and Classification Report. Ocul Surf 15, 276-283.

Farias, E., Yasunaga, K.L., Peixoto, R.V.R., Fonseca, M.P., Fontes, W., Galera, P.D., 2013. Comparison of two methods of tear sampling for protein quantification by Bradford method. Pesqui Vet Bras 33, 4.

Janeway, C., 2001. Immunobiology 5 : the immune system in health and disease, 5th ed. Garland Pub., New York.

Jumblatt, M.M., Imbert, Y., Young, W.W., Jr., Foulks, G.N., Steele, P.S., Demuth, D.R., 2006. Glycoprotein 340 in normal human ocular surface tissues and tear film. Infect Immun 74, 4058-4063.

Kojima, T., 2018. Contact Lens-Associated Dry Eye Disease: Recent Advances Worldwide and in Japan. Invest Ophthalmol Vis Sci 59, DES102-DES108.

Lee, H.S., Hattori, T., Park, E.Y., Stevenson, W., Chauhan, S.K., Dana, R., 2012. Expression of toll-like receptor 4 contributes to corneal inflammation in experimental dry eye disease. Invest Ophthalmol Vis Sci 53, 5632-5640.

Lema, C., Reins, R.Y., Redfern, R.L., 2018. High-Mobility Group Box 1 in Dry Eye Inflammation. Invest Ophthalmol Vis Sci 59, 1741-1750.

Li, J., Metruccio, M.M.E., Evans, D.J., Fleiszig, S.M.J., 2017a. Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen Pseudomonas aeruginosa. PLoS Pathog 13, e1006392.

Li, J., Metruccio, M.M.E., Smith, B.E., Evans, D.J., Fleiszig, S.M.J., 2017b. Correction: Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen Pseudomonas aeruginosa. PLoS Pathog 13, e1006612.

Ligtenberg, A.J., Karlsson, N.G., Veerman, E.C., 2010. Deleted in malignant brain tumors-1 protein (DMBT1): a pattern recognition receptor with multiple binding sites. Int J Mol Sci 11, 5212-5233.

Liu, Z., Kim, J., Sypek, J.P., Wang, I.M., Horton, H., Oppenheim, F.G., Bochner, B.S., 2004. Gene expression profiles in human nasal polyp tissues studied by means of DNA microarray. J Allergy Clin Immunol 114, 783-790.

Massingale, M.L., Li, X., Vallabhajosyula, M., Chen, D., Wei, Y., Asbell, P.A., 2009. Analysis of inflammatory cytokines in the tears of dry eye patients. Cornea 28, 1023-1027.

Nichols, J.J., Green-Church, K.B., 2009. Mass spectrometry-based proteomic analyses in contact lens-related dry eye. Cornea 28, 1109-1117.

Noris, M., Remuzzi, G., 2013. Overview of complement activation and regulation. Semin Nephrol 33, 479-492.

Osei, K.A., Deivanayagam, C., Nichols, J.J., 2018. Glycoprotein 340 in mucosal immunity and ocular surface. Ocul Surf 16, 282-288.

Osei, K.A., Deivanayagam, C., Nichols, J.J., 2019. The effect of glycoprotein 340's scavenger receptor cysteine-rich domain on bacterial adhesion on soft contact lens (abstract). Invest Ophthalmol Vis Sci 60, 6333.

Perumal, N., Funke, S., Pfeiffer, N., Grus, F.H., 2016. Proteomics analysis of human tears from aqueous-deficient and evaporative dry eye patients. Sci Rep 6, 29629.

Ramamoorthy, P., Khanal, S., J, J.N., 2021. Inflammatory proteins associated with contact lens-related dry eye. Cont Lens Anterior Eye, 101442.

Redfern, R.L., Barabino, S., Baxter, J., Lema, C., McDermott, A.M., 2015. Dry eye modulates the expression of toll-like receptors on the ocular surface. Exp Eye Res 134, 80-89.

Redfern, R.L., Patel, N., Hanlon, S., Farley, W., Gondo, M., Pflugfelder, S.C., McDermott, A.M., 2013. Toll-like receptor expression and activation in mice with experimental dry eye. Invest Ophthalmol Vis Sci 54, 1554-1563.

Reichhardt, M.P., Loimaranta, V., Thiel, S., Finne, J., Meri, S., Jarva, H., 2012. The salivary scavenger and agglutinin binds MBL and regulates the lectin pathway of complement in solution and on surfaces. Front Immunol 3, 205.

Reichhardt, M.P., Meri, S., 2016. SALSA: A Regulator of the Early Steps of Complement Activation on Mucosal Surfaces. Front Immunol 7, 85.

Reins, R.Y., Lema, C., Courson, J., Kunnen, C.M.E., Redfern, R.L., 2018. MyD88 Deficiency Protects Against Dry Eye-Induced Damage. Invest Ophthalmol Vis Sci 59, 2967-2976.

Renner, M., Bergmann, G., Krebs, I., End, C., Lyer, S., Hilberg, F., Helmke, B., Gassler, N., Autschbach, F., Bikker, F., Strobel-Freidekind, O., Gronert-Sum, S., Benner, A., Blaich, S., Wittig, R., Hudler, M., Ligtenberg, A.J., Madsen, J., Holmskov, U., Annese, V., Latiano, A., Schirmacher, P., Amerongen, A.V., D'Amato, M., Kioschis, P., Hafner, M., Poustka, A., Mollenhauer, J., 2007. DMBT1 confers mucosal protection in vivo and a deletion variant is associated with Crohn's disease. Gastroenterology 133, 1499-1509.

Robertson, D.M., 2013. The effects of silicone hydrogel lens wear on the corneal epithelium and risk for microbial keratitis. Eye Contact Lens 39, 67-72.

Rosenstiel, P., Sina, C., End, C., Renner, M., Lyer, S., Till, A., Hellmig, S., Nikolaus, S., Folsch, U.R., Helmke, B., Autschbach, F., Schirmacher, P., Kioschis, P., Hafner, M., Poustka, A., Mollenhauer, J., Schreiber, S., 2007. Regulation of DMBT1 via NOD2 and

TLR4 in intestinal epithelial cells modulates bacterial recognition and invasion. J Immunol 178, 8203-8211.

Schulz, B.L., Oxley, D., Packer, N.H., Karlsson, N.G., 2002. Identification of two highly sialylated human tear-fluid DMBT1 isoforms: the major high-molecular-mass glycoproteins in human tears. Biochem J 366, 511-520.

Simmons, K.T., Xiao, Y., Pflugfelder, S.C., de Paiva, C.S., 2016. Inflammatory Response to Lipopolysaccharide on the Ocular Surface in a Murine Dry Eye Model. Invest Ophthalmol Vis Sci 57, 2443-2451.

Stapleton, F., Alves, M., Bunya, V.Y., Jalbert, I., Lekhanont, K., Malet, F., Na, K.S., Schaumberg, D., Uchino, M., Vehof, J., Viso, E., Vitale, S., Jones, L., 2017. TFOS DEWS II Epidemiology Report. Ocul Surf 15, 334-365.

Thakur, A., Willcox, M.D., 2000. Contact lens wear alters the production of certain inflammatory mediators in tears. Exp Eye Res 70, 255-259.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13, 134.

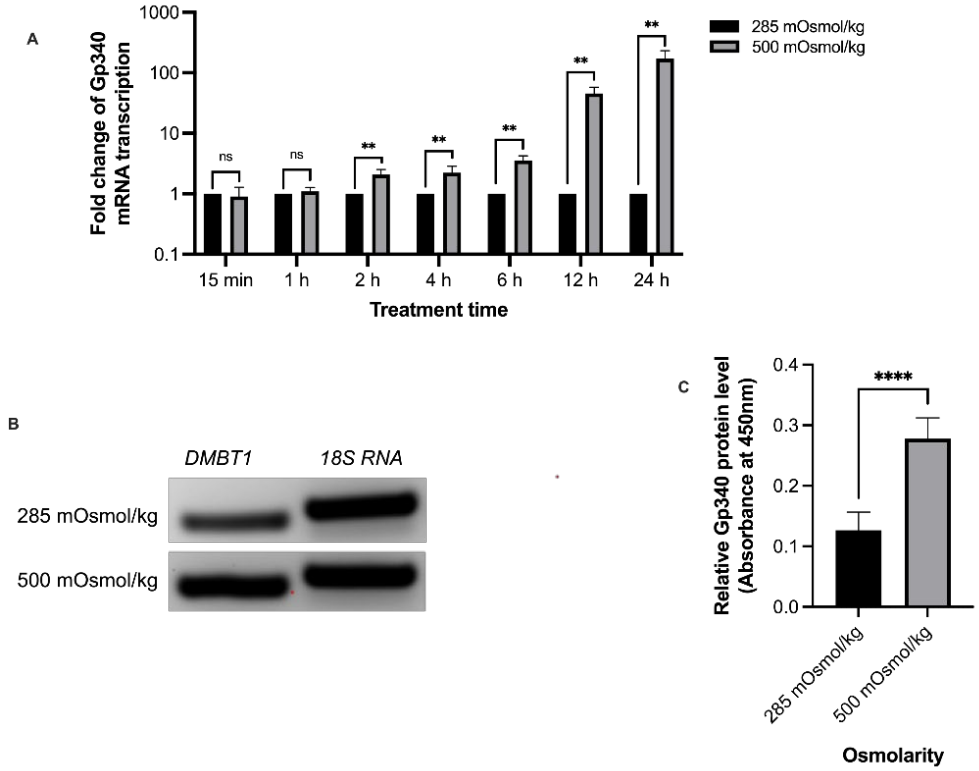

Figure 1. The effect of hyperosmolar stress on Gp340 mRNA and protein expression. To determine the effect of hyperosmolar stress on Gp340 expression, human corneal epithelial cells (HCECs) were treated with either 500 mOsmol/kg (hyperosmolar) or 285 mOsmol/kg (normoosmolar) medium and the effect on Gp340 mRNA and protein levels were determined by RT-PCR and ELISA. **A.** Hyperosmolar stress increased HCEC Gp340 mRNA levels from 2 h post-hyperosmolar media treatment ($p < 0.01$, Mann-Whitney U test). **B.** Gel-based RT-PCR with the 12-hour treated HCEC samples confirmed the upregulation of Gp340 mRNA transcription with hyperosmolar stress treatment. **C.** At protein level, hyperosmolar stress treatment also increased Gp340 translation in HCECs ($p < 0.0001$, independent t test). Error bars represent standard deviation. ** $p \leq 0.01$, **** $p \leq 0.0001$, ns $p > 0.05$

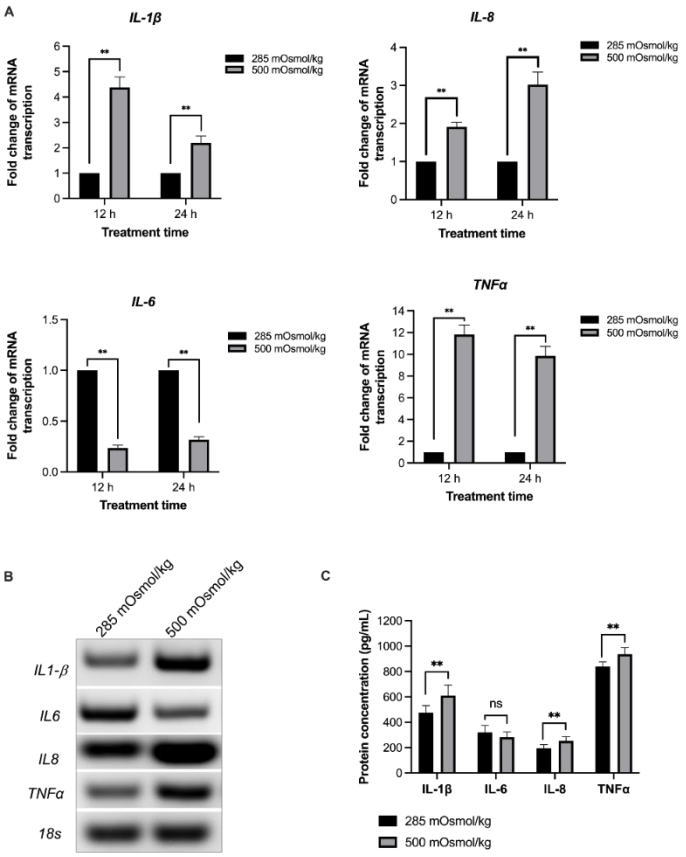

Figure 2. The effect of hyperosmolar stress on proinflammatory cytokine gene and protein expression in HCECs. To determine the effect of hyperosmolar stress on dry eye-associated proinflammatory cytokines, the levels of IL1β, IL6, IL8, and TNFα mRNA transcription and protein translation were determined by RT-PCR and ELISA. **A.** Real-time RT-PCR showed an upregulated *IL1-β, IL8*, and *TNFα,* and downregulated IL6 mRNA transcription in HCECs treated with hyperosmolar media for 12 h and 24 h ($p < 0.01$, Mann-Whitney U test). **B.** Gel-based RT-PCR on the 12-hour treated samples confirmed the modulation pattern of the four cytokines. **C.** At protein translation level, hyperosmolar stress increased IL1-β, IL8, and TNFα production ($p < 0.01$, Independent t test) but did not have a significant effect on IL6 production ($p > 0.05$). Error bars represent standard deviation. ** $p \leq 0.01$, ns $p > 0.05$

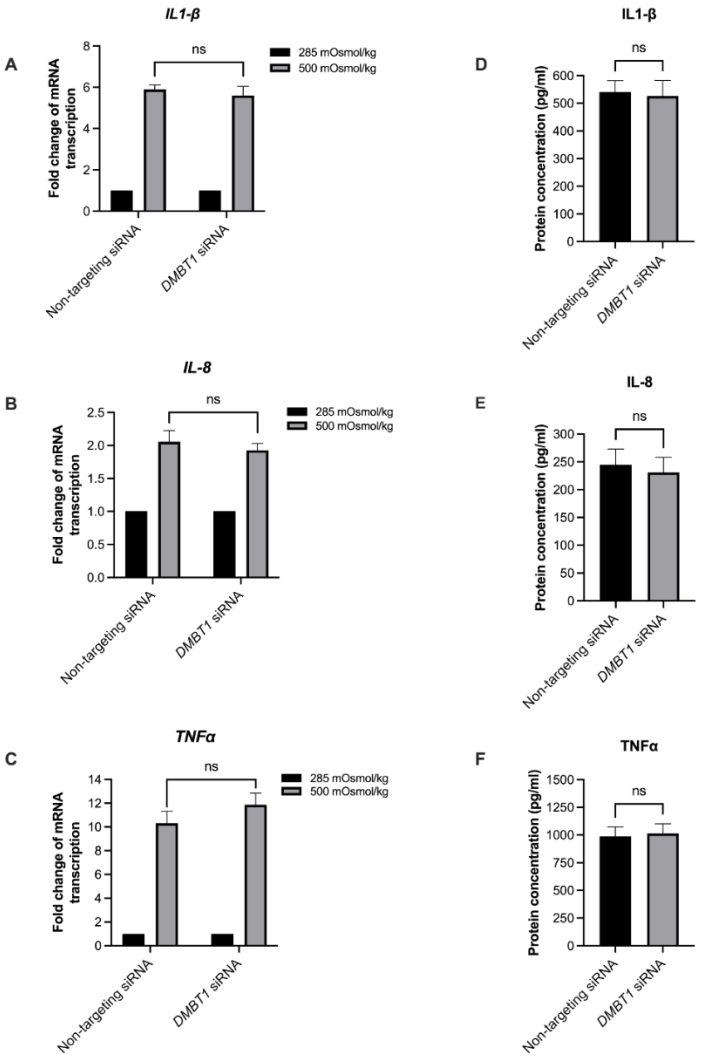

Figure 3. The effect of Gp340 expression on proinflammatory cytokine expression. HCECs were transiently transfected with either *DMBT1* siRNA or non-targeting siRNA and subsequently treated with hyperosmolar or normoosmolar media for 12 h. Thereafter, the levels of *IL1-β, IL8,* and *TNFα* mRNA and protein were determined using real time RT-PCR and ELISA. *DMBT1* gene silencing did not affect *IL1-β, IL8,* and *TNFα* gene transcription under hyperosmolar stress (A – C, $p > 0.05$, independent t test). Similarly, at protein level, *DMBT1* gene silencing did not affect IL1-β, IL8, and TNFα production (D – F, $p > 0.05$, independent t test). Error bars represent standard deviation. ns: $p > 0.05$

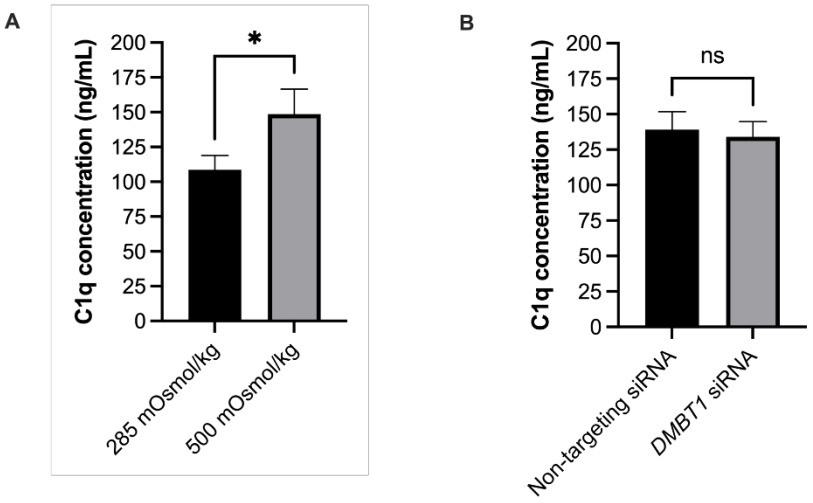

Figure 4. C1q expression in HCECs under hyperosmolar stress. A. Compared to the control (285 mOsmol/kg), C1q expression in the HCECs treated with 500 mOsmol/kg media was significantly higher ($p = 0.03$). **B.** However, when HCECs were transiently transfected with either *DMBT1* siRNA or non-targeting siRNA and subsequently treated with hyperosmolar or normoosmolar media, the expression level of C1q did not differ between the two transfecting siRNAs ($p = 0.473$). Error bars represent standard error of mean. * $p \leq 0.05$, ns $p > 0.05$

CHAPTER 4 – GLYCOPROTEIN 340'S SCAVENGER RECEPTOR CYSTEINE-RICH DOMAIN PROMOTES ADHESION OF *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA* TO CONTACT LENS POLYMERS

by

KWAKU A. OSEI, JOSHUA MEIHER, MANISHA PATEL, JASON J. NICHOLS, CHAMPION DEIVANAYAGAM

Accepted at *Infection and Immunity*

Format adapted for dissertation

ABSTRACT

Contact lenses are biomaterials worn on the eye to correct refractive errors. Bacterial adhesion and colonization of these lenses results in adverse events such as microbial keratitis. The adsorption of tear proteins to contact lens materials enhances bacterial adhesion. Glycoprotein 340 (Gp340), a tear component, is known to promote microbial colonization in the oral cavity, however, it has not been investigated in any contact lens-related adverse event. Therefore, this study examined the adsorption of Gp340 and its recombinantly expressed scavenger receptor cysteine rich (iSRCR$_1^{Gp340}$) domain on two common contact lens materials, etafilcon A and lotrafilcon B, and the concomitant effects on the adherence of clinical isolates of microbial keratitis causative agents, *Pseudomonas aeruginosa* (PA6206, PA6294), and *Staphylococcus aureus* (SA38, USA300). Across all strains and materials, iSRCR$_1^{Gp340}$ enhanced adherence of bacteria in a dose-dependent manner. However, $_i$SRCR$_1^{Gp340}$ did not modulate lysozyme's and lactoferrin's effects on bacterial adhesion to the contact lens. The Gp340 binding surface protein SraP significantly enhanced USA300 binding to iSRCR$_1^{Gp340}$-doped lenses. In addition, iSRCR$_1^{Gp340}$-coated surfaces had significantly diminished biofilms with the SraP mutant *(ΔSraP*), and with the Sortase A mutant *(ΔSrtA*), there was a further reduction in biofilms, indicating the likely involvement of additional surface proteins. Finally, the binding affinities between iSRCR$_1^{Gp340}$ and SraP were determined using surface plasmon resonance (SPR), where the complete SraP binding region displayed nanomolar affinity, whereas its smaller fragments adhered with micromolar affinities. This study concludes

that Gp340 and its SRCR domains play an important role in bacterial adhesion to the contact lens.

INTRODUCTION

Contact lens is a medical device worn on the surface of the eye to correct refractive errors, improve cosmesis, and to deliver ocular drugs. Despite its usefulness, contact lens wear is associated with adverse events such as microbial keratitis, contact lens-related acute red eye, contact lens peripheral ulcer, and infiltrative keratitis (1-3). These events are clinically relevant as they can result in significant vision loss and contact lens discomfort, and cause patients to drop out of lens wear (4). The adherence of bacteria to a worn lens is considered a primary factor in the development of these complications (1). During contact lens wear, proteins in the tear film deposit on the lenses and promote microbial adherence (1, 5). Tears contain more than a thousand proteins (6) that exert various functions such as anti-microbial defense, lubrication, wound healing and regulation of the inflammatory response (7). The adsorption of tear proteins and subsequent bacterial adhesion depend on several factors and key among these are the effective (net) charge of the protein, modality or period of lens wear, properties of the lens materials (such as water content, surface charge, and hydrophobicity), the surface characteristics of the adhering bacteria (1, 8-10).

Tear proteins such as lysozyme and lactoferrin promote adhesion of bacteria when they adsorb to contact lens materials (5, 11). However, this enhanced bacterial adhesion does not potentiate the risk of microbial keratitis as these proteins have antibacterial functions (12-14). Among the many tear proteins, glycoprotein 340 (Gp340) is known to promote microbial infections (15-18), it is a normal component of mucosal fluids such as tears, saliva and breast milk, and is also expressed in mucosal epithelial tissues such as the

gastrointestinal tract, oral cavity, lung alveoli, and pancreas (16, 19). Gp340, also known as salivary agglutinin and "deleted in malignant brain tumor 1" (DMBT1), is a 340-kDa innate immune protein that belongs to the scavenger receptor cysteine-rich (SRCR) superfamily (15, 16, 19). It contains multiple domains: SRCR, SRCR interspersed domain (SID), the C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein-1 (CUB), and the zona pellucida (ZP) (16, 20, 21). Functionally, Gp340 can either be beneficial or harmful depending on its form or conformation (15). In the solution form or soluble conformation, Gp340 aggregates and inhibits pathogenic microbes such as *Streptococcus mutans*, Influenza A and HIV-1 (15, 16). However, it promotes microbial attachments in dental caries and vaginal epithelial transcytosis of HIV-1 when it is surface-adsorbed (15-18).

On the ocular surface, Gp340 is a normal component of tears and the ocular surface tissues, lacrimal gland, cornea, and conjunctiva (22-24). Tear Gp340 has been shown to inhibit twitching motility of *P. aeruginosa* and promote corneal wound healing (25). While it was previously shown to bind to contact lens polymers (26), to date, the impact of contact lens-bound Gp340 in adverse events such as microbial keratitis has yet to be elucidated.

Given that the surface-adsorption of Gp340 can promote infections (17, 18), we hypothesized that the adsorption of Gp340 on a worn contact lens would promote bacterial adhesion and contribute to contact lens-related microbial keratitis. Therefore, this study aimed to examine the potential adsorption of Gp340 and its recombinantly expressed first SRCR domain (iSRCR$_1^{Gp340}$) on two common commercially available contact lens polymers, etafilcon A and lotrafilcon B, and the concomitant effect on the adhesion of the pathogenic bacteria, *P. aeruginosa* and *S. aureus*. In addition, this study explored the

components on the microbial surface that aid in bacterial binding to the lens surface through Gp340. Understanding such mechanisms by which tear protein binds to contact lens materials and how it enhances microbial infections and/or other adverse events is crucial toward developing therapeutical interventions.

In this study, we report that Gp340 and its recombinantly expressed scavenger receptor cysteine-rich (*i*SRCR$_1^{Gp340}$) domain adsorb onto etafilcon A and lotrafilcon B contact lens polymers. In addition, the clinical isolates of microbial keratitis causative agents *Pseudomonas aeruginosa* (PA6206, PA6294), and *Staphylococcus aureus* (SA8, USA300) displayed enhanced adherence in the presence of *i*SRCR$_1^{Gp340}$ in a dose-dependent manner. This study also determined that $_i$SRCR$_1^{Gp340}$ does not affect lysozyme's and lactoferrin's mediated bacterial adhesion to the contact lens. In addition, the interaction between *S. aureus* and *i*SRCR$_1^{Gp340}$ is predominantly mediated by the bacterial surface protein SraP. The SraP mutant *(ΔSraP*) and the Sortase A mutant *(ΔSrtA*) displayed a significant reduction in biofilm formation. Finally, using surface plasmon resonance (SPR) we quantified the nanomolar affinity interaction between *i*SRCR$_1^{Gp340}$ and SraP. This study concludes that, Gp340 and its SRCR domains play an important role in mediating bacterial adhesion to contact lens.

RESULTS

Gp340 and *i*SRCR$_1^{Gp340}$ Adsorb on Etafilcon A and Lotrafilcon B

To determine the effect of contact lens-adsorbed Gp340 and *i*SRCR$_1^{Gp340}$ on bacterial adhesion, we first assessed if Gp340 adsorbed on worn etafilcon A (more hydrophilic) and lotrafilcon B (more hydrophobic) lenses. We obtained 10 etafilcon A and 10 lotrafilcon B lenses from contact lens wearers, and using a dot blot assay, confirmed the adsorption of Gp340 to both lens materials. This established that tear secreted Gp340 does adsorb onto contact lens surfaces (Figure 1A). Using sandwich ELISA, we also determined the amount of tear Gp340 that adsorbs on the two lens polymers as 14.64 ng/lens and 22.49 ng/lens respectively for etafilcon A and lotrafilcon B. Subsequently, we investigated if the recombinantly expressed *i*SRCR$_1^{Gp340}$ would adsorb onto these lens polymers. Our results show that *i*SRCR$_1^{Gp340}$ adsorbed to all the lenses in a dose-dependent manner (Figure 1B, C, Kruskal-Wallis H test; $p = 0.017$ for etafilcon A, $p = 0.007$ for lotrafilcon B). More importantly, at 50, 500, and 5000 pg/µL coating concentrations, *i*SRCR$_1^{Gp340}$ adsorbed 25%, 34% and 22% higher to lotrafilcon B compared to etafilcon A.

*i*SRCR$_1^{Gp340}$ Promotes Bacterial Adhesion to Contact lens

Gp340 immobilized on the tooth surface and vaginal epithelial cells is known to promote infection (15-18). Through coating etafilcon A and lotrafilcon B lenses in *i*SRCR$_1^{Gp340}$ solution (50, 500, 5000 pg/µL) or PBS (control), and subsequently incubating with clinical isolates of microbial keratitis, SA38, USA300, PA6206, and PA6294, we

determined the effect of *i*SRCR$_1^{Gp340}$ on bacterial adhesion. Across all strains, lenses coated with 5000 pg/µL of *i*SRCR$_1^{Gp340}$ exhibited the highest amounts of adherent bacteria (Figure 2, Kruskal-Wallis H test with pairwise comparisons, $p < 0.05$). At 500 pg/µL, USA300 exhibited higher adherence to both etafilcon A and lotrafilcon B, whereas PA6294 showed higher adherence specifically to etafilcon A (Figure 2, Kruskal-Wallis H test with pairwise comparisons, $p < 0.05$). At 50 pg/µL there was no difference in adhesion across all strains ($p > 0.05$). Finally, irrespective of the lens type, at every *i*SRCR$_1^{Gp340}$ coating concentration *P. aeruginosa* strains adhered in much higher numbers compared to *S. aureus* strains (Figure 2).

Contact Lens-Adsorbed *i*SRCR$_1^{Gp340}$ Does Not Inhibit the Proliferating Ability of Adherent Bacteria

In the case of the lens adhered bacteria, only viable and culturable bacteria pose the threat of contact lens-related microbial keratitis. Through culturing and counting the colony forming units of contact lens-adherent bacteria, we determined the proliferating capacity of *i*SRCR$_1^{Gp340}$ – mediated adherent bacteria. Both etafilcon A and lotrafilcon B coated in 5000 pg/µL *i*SRCR$_1^{Gp340}$ displayed higher quantities of viable culturable bacteria (Figure 3, Kruskal-Wallis post-hoc pairwise comparisons, $p < 0.05$). At 500 pg/µL coating, only SA38 showed a significant difference in viable culturable bacteria with etafilcon A, whereas USA300, PA6206 and PA6294 did not. At 50 pg/µL there was no difference observed in the viable culturable adherent bacteria across all strains and materials (Figure 3, Kruskal-Wallis test with post-hoc pairwise comparisons, $p > 0.05$).

*i*SRCR$_1^{Gp340}$ Does Not Modulate Lysozyme's and Lactoferrin's Effects on Bacterial Adhesion to Contact Lens

The adsorption of lysozyme and lactoferrin on contact lens polymers enhances bacterial adhesion (1, 5, 11), and Gp340 interacts with these two abundant tear proteins (20, 27, 28). To determine if *i*SRCR$_1^{Gp340}$ modulates the lysozyme- and lactoferrin-mediated bacterial adhesion to the contact lens, the amounts of bacterial adhesion on lenses coated in *i*SRCR$_1^{Gp340}$ (500 pg/µL), lysozyme (2 µg/µL), lactoferrin (1.8 µg/µL), lysozyme + *i*SRCR$_1^{Gp340}$, and lactoferrin + *i*SRCR$_1^{Gp340}$ were compared. Across all strains and lens types, lysozyme- and lactoferrin-coated lenses had significantly higher bacterial adhesion compared with the lenses coated in *i*SRCR$_1^{Gp340}$ (Figure 4, One-way ANOVA with pairwise comparisons, $p > 0.05$). The addition of *i*SRCR$_1^{Gp340}$ to lysozyme and lactoferrin did not influence bacterial adhesion across all strains and lenses (Figure 4, One-way ANOVA with pairwise comparisons, $p > 0.05$).

SraP Adhesin is Involved in *S. aureus* Adhesion to *i*SRCR$_1^{Gp340}$-Coated Lens

The *S. aureus* surface adhesin SraP/SasA adheres to Gp340 (29, 30), hence we tested for its role in promoting bacterial adherence to lenses that were coated with *i*SRCR$_1^{Gp340}$. Sortase A covalently anchors bacterial surface proteins to the peptidoglycan cell wall using the LPxTG motif in gram-positive bacteria (31-34). The USA300 Sortase A mutant, therefore, will not display LPxTG-containing surface proteins and thus aids the study of *S. aureus* adhesion to *i*SRCR$_1^{Gp340}$-coated lenses in the presence/absence of these proteins. Here, lenses coated in either *i*SRCR$_1^{Gp340}$ (500 pg/µL) or PBS (control) were tested for adherence with wild-type USA300 (USA300 WT), the SraP mutant (USA300 ΔSraP), and the Sortase A mutant (USA300 ΔSrtA). Both USA300 WT and USA300 ΔSraP

adhered to *i*SRCR$_1^{Gp340}$-coated lenses in higher amounts compared to control lenses (Figure 5A, B; independent t test, $p < 0.05$), whereas the ΔSrtA adhered similarly to PBS and *i*SRCR$_1^{Gp340}$ -coated lenses. Upon normalizing bacterial adherence to the controls, the SraP mutant had significantly reduced binding compared to the wild-type (Figure 5C, D), an indication that SraP mediates *S aureus*' interaction with *i*SRCR$_1^{Gp340}$-coated lenses. Finally, the lowest adherence to lens surface was observed with the Sortase A mutant (Figure 5C, D), suggesting that additional surface adhesins are involved in *S aureus* adhesion to *i*SRCR$_1^{Gp340}$-coated lens.

SraP and Other Surface Proteins on *S. aureus* USA300 Mediate Biofilm Formation on $_i$SRCR$_1^{Gp340}$-Coated Surface

The results from the previous section showed that SraP facilitates the interaction between USA300 and $_i$SRCR$_1^{Gp340}$. To further confirm SraP's role, we analyzed biofilms formed by USA300 WT, USA300 ΔSraP, or USA300 ΔSrtA in $_i$SRCR$_1^{Gp340}$ -coated surfaces. Significant reductions in biofilms were observed with the SraP and Sortase A mutants compared to the USA300 WT (Figure 6, Kruskal-Wallis H test with pairwise comparisons, $p < 0.05$). However, between the two mutants, the Sortase A mutant had a higher reduction in biofilm, indicating that other surface adhesins of *S. aureus* bind to $_i$SRCR$_1^{Gp340}$, which agrees with our results from the lens studies.

SraP Binds to *i*SRCR$_1^{Gp340}$ with Nanomolar Affinity

We analyzed the kinetics of *i*SRCR$_1^{Gp340}$'s interaction with SraP using surface plasmon resonance (SPR) studies with the SraP constructs as analytes and *i*SRCR$_1^{Gp340}$ as ligand. The full-length binding region (BR) of SraP that consists of L-lectin, β-grasp fold

(β-GF), CDHL1 (cadherin-like) and CDHL2 domains (30), adhered with nanomolar affinity. The two truncated SraP fragments, namely L-lectin-β-GF-CDHL1 and L-lectin-β-GF-CDHL1-CDHL2 also adhered with micromolar affinity, two orders of magnitude lesser than the full-length BR region of SraP.

SraP Contributes to *S. aureus*' Adhesion to Patient-Worn Contact Lens

Having established that SraP promotes *S. aureus*' adherence to *i*SRCR$_1^{Gp340}$-coated lenses, we determined the role of SraP in *S. aureus*' adhesion to etafilcon A and lotrafilcon B lenses worn by patients. These lenses were incubated with USA300 WT, USA300 ΔSraP, and USA300 ΔSrtA mutants and the quantities of adherent bacteria were determined for all three strains. Significantly different amounts of adhesion were observed between the three USA300 strains across the two lens types (Figure 7A, B, One-Way ANOVA, $p < 0.05$). The ΔSraP mutant did not show statistically significant reduction in adhesion compared to the wild-type (etafilcon A: $p = 0.09$; lotrafilcon B: $p = 0.34$), however, the ΔSrtA mutant showed significant decrease in adherence to both etafilcon A ($p < 0.0001$) and lotrafilcon B ($p = 0.0001$) lenses. In relative terms, USA300 ΔSraP displayed 22% (etafilcon A) and 20% (lotrafilcon B) reduction, whereas USA300 ΔSrtA showed 68% (etafilcon A) and 74% (lotrafilcon B) reduction in bacterial adherence (Figure 7C, D).

DISCUSSION

Gp340 is a normal tear component expressed by the lacrimal gland, cornea, and conjunctiva (19). It has been previously shown to adsorb on contact lenses (26). However, whether this adsorption contributes to contact lens-related adverse events such as microbial adhesion or infection remains to be determined. With the hypothesis that Gp340 would bind to the contact lens and enhance the adhesion of bacteria through microbial surface proteins, this study aimed to investigate (a) the adsorption of Gp340 and its first SRCR domain (*i*SRCR$_1^{Gp340}$) on contact lens polymers; (b) the possible impact of *i*SRCR$_1^{Gp340}$ on bacterial adhesion and (c) the microbial proteins that mediate this interaction.

To begin with, the adsorption of Gp340 to patient-worn etafilcon A and lotrafilcon B were investigated, as any effect induced by *i*SRCR$_1^{Gp340}$ on bacterial adhesion would depend on the extent of Gp340 binding. Dot blot analysis of total tear protein extracted from worn lenses confirmed that Gp340 binds to both lens polymers (Figure 1), and the quantity of bound Gp340 was 14.64 ng/les for etafilcon A and lotrafilcon B was 22.49 ng/lens for lotrafilcon B. This finding laid the foundation for further assessments of *i*SRCR$_1^{Gp340}$ binding to lens material and the concomitant effects, if any, in contact lens-related adverse effects. Comparatively, *i*SRCR$_1^{Gp340}$ binding to lotrafilcon B was higher than that of etafilcon A. To explain this, we considered two major factors that are known to influence protein adsorption to biomaterials. These are (a) the surface properties of the material and (b) the effective charge of the protein that is determined by the isoelectric point (pI) and the pH of the medium containing the protein (35). The theoretical pI of

*i*SRCR$_1^{Gp340}$ is 5.96 (ExPASy server (36)) and so it would carry a net negative charge in PBS buffer whose pH is 7.4. Since etafilcon A and *i*SRCR$_1^{Gp340}$ both carry an anionic surface, their repulsions could have led to lower binding, whereas the non-ionic surface on lotrafilcon B results in higher deposition of *i*SRCR$_1^{Gp340}$on its surface. These observations are consistent with a previous study by Zhao et al., which reported a higher frequency of Gp340 deposition on lotrafilcon B (26).

Previously, investigating the influence of contact lens-adsorbed proteins on bacterial adhesion, Subbaraman et al. reported higher adhesion for *P. aeruginosa* strains compared with *S. aureus* (5). In subsequent studies, Subbaraman further reported the amounts of bound *P. aeruginosa* strains on etafilcon A to be higher (41-51 times) than *S. aureus*, whereas on lotrafilcon B, *P. aeruginosa* showed higher adhesion (26-85 times) compared to *S. aureus* (11). Similarly, Borazjani reported higher adhesion (185 times) of *P. aeruginosa* to etafilcon A compared to *S. aureus* (37). In this study, the adherence of bacteria mediated by *i*SRCR$_1^{Gp340}$ displayed a similar trend, where across all coating concentrations of *i*SRCR$_1^{Gp340}$ and lens polymers the *P. aeruginosa* strains adhered in higher quantities compared to *S. aureus* (Figure 2).

Four type III secretion system (T3SS) effector toxins, namely: ExoS, ExoT, ExoU, and ExoY, have been identified in *P. aeruginosa* but all four are rarely present in any given strain (38-40). While three are conserved, most strains carry either the *ExoS* or *ExoU* gene. The invasive strain, PA 6294, has *ExoS* and not *ExoU* (*ExoS*$^+$/*ExoU*$^-$). The cytotoxic strain, PA 6206, on the other hand, has *ExoU* but lacks *ExoS* (*ExoU*$^+$/*ExoS*$^-$). Despite this difference, in the current study, the two strains had similar adherence to both lens polymers under all conditions, suggesting that their adhesion to gp340-coated contact lens is

independent of the effector toxins, and is consistent with a previous observation by Shen et al. (41).

When bacteria adhere to the lens surface, the estimated total bacterial counts would normally include both viable and dead cells. Since viable adherent bacteria are the ones that could propagate and potentially become infectious, and among these, some may remain in a viable but non-culturable state (42), we also investigated the proliferating ability of the contact lens-bound bacteria mediated by *i*SRCR$_1^{Gp340}$. Our results show that *i*SRCR$_1^{Gp340}$ did not impact the adherent bacteria's ability to proliferate as the patterns of total adhesion and bacterial proliferation were similar (Figure 3).

Investigations into the role of two other major tear proteins, lactoferrin and lysozyme, showed that each enhanced bacterial adhesion more than *i*SRCR$_1^{Gp340}$ (Figure 4). This could partly be explained by the higher relative physiological concentrations of lactoferrin (1.8 μg/μL) and lysozyme (2 μg/μL) used in the study compared to *i*SRCR$_1^{Gp340}$ (500 pg/μL). At these higher concentrations, both lactoferrin and lysozyme would potentially adsorb on the lenses much more than *i*SRCR$_1^{Gp340}$ resulting in them displaying higher bacterial adhesion. While both lactoferrin and lysozyme have broad spectrum antibacterial actions (14, 43), and can adversely affect the ability of any bound bacteria to cause infection, the effect of Gp340 in infection is dependent on the conformation in which it exists. It is well known that Gp340 exists in two different conformations: soluble and immobilized (surface-associated/surface-bound) forms (15, 16, 19). In the soluble form, it inhibits cariogenesis, Influenza A and oral HIV-1 transmission (15). However, in the surface-bound conformation, it promotes cariogenesis and vaginal HIV transmission (15-18). Thus, the presence of contact lens surface bound Gp340/*i*SRCR$_1^{Gp340}$, even if limited,

could promote bacterial adherence and/or viability to propagate and increase the risk of infection. Thus, despite *i*SRCR$_1^{Gp340}$ showing comparatively lower counts of bacterial adhesion, the potential risk still exists for infection-enhancing effects.

Previously, Nichols & Green-Church in a study investigating the tear film proteome of contact lens wearers, reported an increased level of tear Gp340 in subjects with contact lens-related dry eye (23). With our studies conducted at physiological concentrations (500 pg/μL) in tears, *i*SRCR$_1^{Gp340}$ increased the adhesion of only two bacterial strains, PA6294 (etafilcon A) and USA300 (both polymers), but at an increased 10-fold higher concentration (5000 pg/μL), much higher adhesion was observed among all the strains (Figure 2). It is here we reason that deposition of Gp340 onto a contact lens surface will be accumulative, and perhaps much larger than the concentration in tears, which in turn would increase bacterial adherence to a worn contact lens and elevate the risk of contact lens-related complications such as microbial keratitis and peripheral ulcer. Further investigation into the potential effects of Gp340 in infection related to dry eye in future studies is needed as contact lens wear increases the risk of infection in dry eye disease (44).

As the third component of this study, the microbial component that mediates *S aureus*' adherence to *i*SRCR$_1^{Gp340}$ was evaluated. In *S. aureus*, among the CWA proteins, SraP mediates the adherence to Gp340 (29), and this is partly mediated by the glycosylation present on Gp340. The binding region (BR) of SraP at its N-terminus consists of an L-Lectin domain, followed by one β-grasp fold (β-GF) and two tandem cadherin-like (CDHL) modules (29). The L-lectin domain binds one Ca^{2+} ion and coordinates the adhesion to host cells by recognizing N-acetylneuraminic acid (Neu5Ac) of extracellular receptors (30). The CDHL domains also bind Ca^{2+} and together with the β-GF domain,

they project the L-lectin domain away from the bacterial cell surface (30, 31). The CDHL domains are known to promote cell-cell accumulation and biofilm formation (30, 31).

The ΔSraP mutant displayed diminished binding to the contact lenses that were coated with *i*SRCR$_1^{Gp340}$, and the Sortase A mutant (ΔSrtA) displayed even lower binding (Figure 5), thus indicating that there are other surface components on *S. aureus* that are involved in the interaction with Gp340. This was further confirmed with patient-worn lenses, where there was a >20% reduction for the ΔSraP mutant, and >65% reduction for ΔSrtA mutant strains (Figure 7). Similar observations were made in our biofilm studies, where both SraP and SrtA mutants showed reduced biofilm on the *i*SRCR$_1^{Gp340}$-coated surface, with higher reduction observed with SrtA mutant (Figure 6). Previous studies with *S aureus* ISP479C showed that SraP promoted biofilm formation (45), whereas *S. aureus* NCTC 8325 SraP mutant did not show a significantly decreased biofilm formation (30). Thus, SraP's role in biofilm formation was adjudged to be strain-dependent (30). In addition to SraP, several other surface proteins such as SasX, SdrC, FnBPA, FnBPB, and SasG are known to promote biofilm formation (31), but their roles in Gp340-driven biofilm formation have yet to be determined. Taken together, the mutant adhesion and biofilm studies clearly established that SraP and other CWA proteins mediate *S aureus*' interaction (adhesion and/or biofilm formation) on a Gp340-conditioned contact lens.

Through SPR studies, the interaction between *i*SRCR$_1^{Gp340}$ SraP binding region was determined to be at a nanomolar affinity level (Table 2, Figure S2). Studies with combination of domains indicate that the constructs encompassing L-lectin-β-GF-CDHL1 and β-GF-CDHL1-CDHL2 display micromolar affinity (Table 2, Figure S2), thus indicating cooperative elements are present within these domains that result in the

nanomolar affinity. This study has now established the nanomolar interaction between recombinant SraP's BR domain and *i*SRCR$_1^{Gp340}$. Gp340 is highly glycosylated (16, 20, 21, 24, 46, 47), and our biofilm and SPR binding studies confirm that the L-lectin module would interact with sialylated/glycosylated moieties on Gp340 (30). In addition, the homophilic interactions of CDHL domains in concert with L-lectin could further promote adhesion and biofilm formation.

The results observed with SraP confirm previous studies (29) and has also now identified potential means to develop inhibitors to this interaction. For this to happen, the mechanistic aspects of this interaction are to be determined through site-directed mutagenesis studies to establish the binding-site/motif/region on SraP. While we have some idea of the interacting partners between Gp340 and *S. aureus*, not much is known about the interacting partner with *P. aeruginosa*. Future studies could be aimed at the interaction between the minor pilins, FimU, PilV, PilW, PilX and PilE in *P. aeruginosa* (48), which we suspect could be involved in binding to Gp340. Once these interactions are mapped at the amino-acid level, the potential exists to selectively target pathogenic strains that are involved in Gp340-mediated contact-lens related microbial keratitis.

This study is the first to investigate the adsorption of Gp340 and its SRCR domain on a contact lens polymer and the potential effect on bacterial adhesion on the lens. This study has established that Gp340 and/or *i*SRCR$_1^{Gp340}$ (i) bind to both etafilcon A and lotrafilcon B polymers; (ii) mediate the adhesion of various strains of *S. aureus* and *P. aeruginosa* to these lenses; (iii) and this interaction is mainly mediated by the surface protein SraP of *S. aureus* which displays nanomolar affinity; and finally (iv) *S. aureus* has potentially other proteins on its surface that interact with Gp340.

MATERIALS AND METHODS

Contact Lens Polymers

A hydrogel polymer, etafilcon A (Acuvue 2®; Johnson & Johnson, Jacksonville, FL, USA) and a silicone hydrogel polymer, lotrafilcon B (Air Optix®; CIBA Vision, Duluth, GA, USA) were investigated in this study. Table 1 summarizes the characteristics of the lenses used in *in vitro* studies.

Expression and Purification of *i*SRCR$_1^{Gp340}$

*i*SRCR$_1^{Gp340}$ was recombinantly expressed using the Drosophila S2 expression system as previously described by Purushotham & Deivanayagam (21). Briefly, a synthesized sequence-optimized *DMBT1* gene was cloned in the recombinant expression vector, pMT/BiP/V5-HisA. The pMT/V5-HisA containing *i*SRCR$_1^{Gp340}$ was transfected stably into S2 cells using calcium phosphate transfection kit (Invitrogen, Inc.). These clones were expanded in selective growth media and copper sulfate (500 μM) was used to induce *i*SRCR$_1^{Gp340}$ expression. The growth media was dialyzed to remove the copper ions, centrifuged, and the supernatant was filtered and loaded onto a HisTrap™ affinity column for purification using an AKTA purifier (GE Healthcare Inc). Appropriate fractions containing *i*SRCR$_1^{Gp340}$ were pooled, dialyzed, and loaded onto a Mono Q® column for further purification. Purified fractions identified from SDS-PAGE gels were pooled and used for the *in vitro* *i*SRCR$_1^{Gp340}$ adsorption and bacterial adhesion studies.

Cloning of *S. aureus* SraP Constructs

The *SraP* gene was custom-synthesized (GeneArt), and subclones were developed from this gene. The three constructs, L-lectin-β-GF-CDHL1-CDHL2^{SraP} (245-751), L-lectin-β-GF-CDHL1^{SraP} (245-660), and β-GF-CDHL1-CDHL2^{SraP} (492-751) were designed with a C-terminal histidine tag utilizing the fast-digest restriction enzymes NcoI and XhoI (ThermoFisher, Inc.) for cloning into the pET23d vector (Novagen). The primers used for cloning are listed in Table S3. PCR amplification of the fragments was done using Phusion^{TM} DNA polymerase followed by digestion of both the PCR fragments and vector with appropriate enzymes. The products were ligated with T4 DNA ligase (NEB), transformed into *E. coli* DH5 cells and grown on LB-agar plates supplemented with ampicillin (50 μg/mL). Single colonies were grown in fresh 5 mL LB cultures, and the plasmids were harvested using the Mini-Prep kit (Zymo). DNA sequencing was carried out at the UAB Heflin Center, which confirmed the presence of the appropriate inserts in the pET23d vector. After confirmation, these plasmids were transformed into *E. coli* BL21(DE3) cells for protein expression.

Expression and Purification of the *S. aureus* SraP Binding Region

E. coli BL21(DE3) cells harboring the plasmids for each SraP construct were inoculated into a 20 mL starter Terrific Broth (TB) culture overnight at 37ºC. Next morning, these cultures were transferred into shaker flasks containing 1 L of TB, and cells were grown to an OD_{600} of 1.0, at which point they were induced with 1 mM IPTG for 5 hours at 30ºC, at which point they were supplemented with additional antibiotics and subsequently grown overnight at 18ºC. These cells were harvested by centrifugation at

5000 × g for 20 minutes using a Beckman Avanti JL-25 centrifuge, and the cell pellets were resuspended in nickel affinity column binding buffer (50 mM Tris, pH 8.0, 500 mM sodium chloride), augmented with a Complete EDTA-free protease inhibitor (Roche). *E. coli* cells were ruptured by sonication outfitted with a temperature sensor (Fisherbrand Sonicator) for a total of 5 minutes while maintaining a maximum temperature of 10ºC. These lysed cells were then centrifuged at 35,000 RPM for 1 hour using a Ti70 rotor, and the supernatant was collected and filtered through a 0.22 μm filter before being loaded onto a 20 mL HisPrep Nickel Column (GE Healthcare, Inc.). Using a first step gradient of 50 mM imidazole the non-specifically bound proteins were gently removed from the column, and thereafter the bound protein was eluted with a 50 – 300 mM imidazole gradient. The purity of the eluted samples was again visualized using an SDS-PAGE gel, and thereafter appropriate fractions were pooled together and dialyzed overnight into the Mono Q binding buffer (50 mM Tris, pH8.0, 50 mM sodium chloride, and 1 mM EDTA). The dialyzed sample was filtered and loaded onto a Mono Q column (GE Healthcare, Inc.), and the protein was eluted with a 0-400 mM NaCl gradient. The purest single banded fractions as identified by SDS-PAGE gels (Figure S1) were then pooled and concentrated under 55 psi nitrogen gas using an Amicon stirring concentrator. Protein concentration was measured using a modified method described elsewhere (49).

Surface Plasmon Resonance Studies

To determine the affinity coefficients between SraP and *i*SRCR$_1^{Gp340}$ surface plasmon resonance studies were performed. Serving as the ligand, *i*SRCR$_1^{Gp340}$ was immobilized on a CM5 chip using ethanolamine chemistry. Analytes, L-lectin-β-GF-

CDHL1-CDHL2, L-lectin-β-GF-CDHL1 and β-GF-CDHL1-CDHL2 were injected over $SRCR_1$ at various concentrations optimized for each analyte (serial dilutions within 0.250 - 32 μM), and dissociations were measured for 600 seconds following injections. The running buffer used for all analytes was 10 mM HEPES pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$. Each experiment was carried out in triplicate with a BIAcore2000 instrument. The sensorgrams were fitted using the BIAevaluation software, where both the residuals and chi^2 values were refined to convergence and the results are presented in Table 2 and Figure S2.

Determination of *i*$SRCR_1^{Gp340}$ Adsorption on Contact Lens

Ex Vivo Arm

This portion of the study was approved by the Institutional Review Board of the University of Alabama at Birmingham (UAB) and followed the tenets of the Declaration of Helsinki (50). Ten etafilcon A and 10 lotrafilcon B lenses worn for one month were obtained from 10 healthy, non-dry eye contact lens wearers (5 subjects per lens type). The total protein adsorbed to each lens was extracted using trifluoroacetic acid-acetonitrile buffer as described earlier (51, 52). These extracted samples were then pooled and concentrated. The presence of Gp340 in the extracted protein was then detected by dot blot assay after blotting equal amounts of total protein on PVDF membrane and probing with monoclonal primary antibody (Life Technologies, Carlsbad, CA, USA) and Alexa Fluor® 488-conjugated IgG secondary antibody (Life Technologies). Unworn etafilcon A and lotrafilcon B lenses were included as quality control.

The amount of tear Gp340 that adsorbs on the lens polymers was quantitated using a sandwich ELISA kit for Gp340 (MyBioSource, San Diego, CA, USA) and following manufacturer's recommendation. Briefly, the extracted samples and Gp340 standards (10 – 0 ng/mL) were added to the wells of microtiter plate (pre-coated with antGp340 antibody) and incubated for 1 hour at 37°C. After washing, anti-Gp340 antibody was added to the wells, followed by the addition of secondary detection antibody, all occurring at 37°C. Thereafter, substrate solution was added and incubated for 20 minutes after which a stop solution was added. The absorbances were then read at 450 nm and adjusted for background noise. Standard curves were generated with the four-parameter logistic model in the Gen 5™ analysis software (BioTek®, version 3.08) and used to interpolate the concentrations of Gp340 in the samples.

In Vitro Arm

To determine the adsorption of *i*SRCR$_1^{Gp340}$ on contact lens materials, unworn etafilcon A and lotrafilcon B lenses were placed in triplicate for 12 hours in 1.2 mL of histidine-tagged *i*SRCR$_1^{Gp340}$ at the following concentrations: 5000, 500 and 50 pg/µL at 37°C. Lenses (n = 3 lenses per polymer) were also placed in 1.2 mL of protein-free phosphate-buffered saline (PBS) to serve as control. Since Gp340 binding is calcium dependent (20) 1.5 mM Ca^{2+} was added to each solution. Subsequently, each lens was washed, blocked, and probed with a primary 6x-His Tag antibody (Life Technologies), followed by an Alexa Fluor® 488-conjugated IgG (Life Technologies). Four different areas of the lens surface were randomly visualized and imaged using fluorescence microscopy. Prior to imaging, the entire lens surface was scanned to confirm that there was uniform

binding of *i*SRCR$_1^{Gp340}$. Any lens that had tears or abrasions was excluded from the study. The observed fluorescence was considered a measure of *i*SRCR$_1^{Gp340}$-adsorption and was quantified using ImageJ densitometry analysis (53). The experiments were repeated two more times. Kruskal-Wallis H test with post-hoc pairwise comparisons was used to analyze the difference in the amount of bound *i*SRCR$_1^{Gp340}$ between the different coating *i*SRCR$_1^{Gp340}$ concentrations for both polymers. The ratio of adsorbed *i*SRCR$_1^{Gp340}$ between etafilcon A and lotrafilcon B was used to compare the amounts of *i*SRCR$_1^{Gp340}$ binding between the two lens types at each coating concentration.

Role of *i*SRCR$_1^{Gp340}$ on Bacterial Adhesion

Bacterial Culture

Two gram-positive pathogenic bacterial strains, *S. aureus* 38 (SA38) and methicillin-resistant *S. aureus* (MRSA) USA300, and two gram-negative strains, *P. aeruginosa* 6206 (PA6206) and *P. aeruginosa* 6294 (PA6294), were investigated in this study. They were selected because they are etiological agents for infections on the surface of the eye. Specifically, SA38, PA6206 (cytotoxic strain), and PA6294 (invasive strain) are clinical isolates from human microbial keratitis (5, 54) and were a gift from Dr. Mark Willcox (University of New South Wales, Sydney, Australia). USA300 is one of the most prevalent community-associated MRSA strains in the United States and a leading candidate in health-care associated keratitis (55-57). The method previously reported by Subbaraman et al. (5, 11) was adopted to prepare bacterial cultures from stocks stored at -80°C in 30% glycerol. Briefly, bacteria were grown overnight in Tryptone Soy Broth (TSB; Oxoid, Sydney, Australia) at 37°C for 18 hours. Bacterial cells were harvested by centrifugation

(Eppendorf 5810, Eppendorf AG, Hamburg, Germany) for 10 minutes (3000 rpm at 18°C) and resuspended in PBS, where the optical density of the bacterial suspension was adjusted to $OD_{660} = 0.3$.

Coating of Contact Lens and Bacterial Adhesion Assay

Firstly, both etafilcon A and lotrafilcon B lenses were coated in triplicate in 1.2 mL of $i\text{SRCR}_1^{\text{Gp340}}$ at three different concentrations: 5000, 500 and 50 pg/µL for 12 hours at 37°C (n = 3 lenses per coating concentration for each polymer). Lenses coated in protein-free PBS served as a negative control. The 500 pg/µL was selected because it falls within the physiological range of tear Gp340 concentration, which was determined through preliminary studies to be 620 pg/µL. Afterwards, the lens was rinsed with PBS to remove any unbound $i\text{SRCR}_1^{\text{Gp340}}$. Subsequently, the protocol previously employed by Subbaraman et al. (5) was adapted to determine the effect of $i\text{SRCR}_1^{\text{Gp340}}$ on bacterial adhesion. Briefly, each lens was placed in 1.2 mL of bacterial suspension at 37°C for 24 hours. Following this, the lens was rinsed three times in PBS for 30 seconds to remove loosely adhered bacteria. Thereafter, each lens was resuspended in 1.5 mL of PBS containing 200 µL of 0.25% trypsin-EDTA and vortexed for 1 minute with a magnetic stir bar to detach the adhered bacteria. This detachment strategy homogenizes the lens and removes more than 99.9% of adherent bacteria (5). The total bacterial adhesion on each lens was measured using the Bacterial viability assay kit (Abcam, Cambridge, MA, USA) and following manufacturer's protocol. The assay utilizes two highly specific fluorescent reagents, each staining either dead bacteria or both live and dead (total) bacteria. The experiments were performed three times for each bacterial strain. The amount of total

bacterial adhesion at the different coating *i*SRCR$_1^{Gp340}$ concentrations were compared using Kruskal-Wallis H test with a p-value less than 0.05 denoting statistical significance. Post hoc pairwise comparisons were done using the Bonferroni correction.

Role of Adsorbed *i*SRCR$_1^{Gp340}$ on the Proliferating Potential of Contact Lens-Adhered Bacteria

Briefly, 100 µl of detached bacterial suspension was diluted 1000-fold in the neutralizing broth (Difco Laboratories, Detroit, MI) and then serially diluted 1:10, 100, and 1000. Fifty microliters of these serially diluted samples were plated in triplicate on nutrient agar and incubated at 37°C for 16 hours. The number of colony-forming units (CFUs) on each plate was counted and accounting for the dilutions, the total CFUs per lens was estimated. The amounts of culturable, viable adherent bacteria at the different coating *i*SRCR$_1^{Gp340}$ concentrations were compared using Kruskal-Wallis H test with pairwise comparisons and Bonferroni adjustment.

Modulation of Lactoferrin's and Lysozyme's Effects on Bacterial Adhesion in the Presence of *i*SRCR$_1^{Gp340}$

Coating of Contact Lens and Bacterial Adhesion Assay

Based on previous studies and the established individual tear protein concentrations (58, 59), the following coating solutions were prepared and used for the study: (a) *i*SRCR$_1^{Gp340}$ (500 pg/µl), (b) lysozyme (2 µg/µl), (c) lactoferrin (1.8 µg/µL), (d) lysozyme + *i*SRCR$_1^{Gp340}$, and (e) lactoferrin + *i*SRCR$_1^{Gp340}$. Etafilcon A and lotrafilcon B lenses were placed in triplicate 1.2 mL of each solution for 12 hours at 37°C. Each lens was then rinsed three times with PBS to remove any unbound protein. Subsequently, each lens was placed

in 1.2 mL of bacterial suspension (OD_{660} = 0.3) and incubated at 37°C for 24 hours. Each lens was rinsed, and bound bacteria were detached as described in the previous section. The amount of total adherent bacteria was measured using the total/dead bacterial assay. The experiments were performed three times with each strain. Mann Whitney U test was used to compare the total bacterial adhesions between the *i*$SRCR_1^{Gp340}$ solution and each of the other coating solutions.

Role of SraP on Bacterial Adhesion to Contact Lens

Three different genotypes of *S. aureus* USA300, namely, the wild-type (USA300 WT), SraP mutant (USA300 ΔSraP), and Sortase A mutant (USA300 ΔSrtA) were acquired from the Center for Staphylococcal Research, University of Nebraska Medical Center, and used in this study. The mutant strains were created by transposon mutagenesis. Briefly, etafilcon A and lotrafilcon B lenses (n = 3 lenses per polymer type) were placed in 1.2 mL of $_i$SRCR$_1^{Gp340}$ solution (500 pg/µL) or PBS (control) as described in the previous sections. Cultures of USA300 WT, ΔSraP, and ΔSrtA were also prepared as described earlier. Each lens was incubated in 1.2 mL of bacterial suspension (OD_{660} = 0.3) for 24 hours at 37°C and subsequently rinsed, and adherent bacteria were detached as described in the previous sections. The amount of total adherent bacteria was measured using the total/dead bacterial assay. These experiments were repeated two more times for each polymer for all three USA300 strains. Independent t test was used to compare total bacterial adhesions between the *i*$SRCR_1^{Gp340}$-coated and control lenses for each USA300 genotype. To determine the effect of SraP on USA300 adhesion to *i*$SRCR_1^{Gp340}$-coated lens, the adhesion to *i*$SRCR_1^{Gp340}$-coated lens was first normalized to the control lens by dividing the

*i*SRCR$_1^{Gp340}$-coated lens fluorescence by the mean control lens fluorescence. Subsequently, One-way ANOVA with post hoc pairwise comparison was used to determine the differences in adhesion between USA300 WT, ΔSraP, and ΔSrtA strains.

The Impact of $_i$SRCR$_1^{Gp340}$/SraP Interaction on *S. aureus* Biofilm Formation

The USA300 WT, USA300 ΔSraP, and USA300 ΔSrtA strains were used in this study. Biofilm assay was performed using methods previously described with minor modifications (60). Briefly, frozen stocks of the *S. aureus* strains were inoculated in TSB and grown overnight at 37°C under 5% CO_2. Overnight cultures were then diluted into fresh 5 mL TSB media (1:100) and grown at 37°C under 5% CO_2 until the cultures reached an OD_{470} = 0.6. These growth phase cultures were further diluted 1:200 into fresh TSB containing 1% glucose, from which 200 µL was aliquoted into 96-well microtiter plate coated with 50 µg/mL $_i$SRCR$_1^{Gp340}$ and bacterial cells were grown for 24 hours at 37°C under 5% CO_2 under static conditions. TSB-only wells were included as wells. After the 24-hour incubation, bacteria growth in each well measured at OD_{470} and the cultures were gently removed, and non-adherent bacteria were removed by washing each well three times with 200 µL of PBS. Thereafter, the biofilms were fixed by heating the plate at 65°C for 1 hour and then stained with 150 µL of 0.1% (w/v) crystal violet for 5 minutes. Excess crystal violet stain was discarded, and the wells were washed to remove any residual dye and air-dried for 30 minutes at room temperature. Following this, 150 µL of 30% (v/v) glacial acetic acid was added to each well and incubated for 1 hour under constant shaking. The resulting biofilm was analyzed by measuring absorbance at 595 nm. Eight replicates of the assay for each USA300 strain were studied, and the experiments were performed

three times for each strain. The absorbance values were averaged to obtain the relative amount of biofilm formation. The absorbance values from negative control wells were subtracted from the positive wells and the resultant absorbance represented the amount of biofilm. Potential differences in bacterial growth rate were accounted by normalizing OD_{595} values to OD_{470} values. The differences in amount of biofilm formation between three USA300 strains were determined by analyzed $OD_{595/470}$ values using One-way ANOVA with post hoc pairwise comparisons.

The Role of SraP in *S. aureus* Adhesion to Contact Lens Worn on the Eye

This component of the study was approved by the UAB Institutional Review Board and followed the tenets of the Declaration of Helsinki (50). Briefly four etafilcon A and four lotrafilcon B lenses, each worn for 28 days, were obtained from healthy, habitual contact lens wearers. After rinsing in PBS, each lens was cut into four equal parts, and each was incubated with USA300 WT, USA300 ΔSraP, or USA300 ΔSrtA in TSB medium (OD_{600} = 0.1) for 12 hours at 37°C as described in the earlier section. One of the cut lenses was also incubated in bacteria-free TSB medium to serve as quality control. Afterwards, the lens was rinsed in PBS to remove unbound bacteria. To determine the amount of bacteria adhesion on the lens, the adherent bacteria were detached, serially diluted (1:100, 1:1000, 1:10000) and 50 – 100 µL was plated on TSB agar plates and incubated at 37°C for 14 hours. The quantities of CFUs per plates were enumerated and one-way ANOVA with post hoc pairwise comparisons was used to determine the difference in CFUs between the three USA300 strains. A p-value < 0.05 denoted statistical significance.

REFERENCES

1. Dutta D, Cole N, Willcox M. 2012. Factors influencing bacterial adhesion to contact lenses. Mol Vis 18:14-21.
2. Wu P, Stapleton F, Willcox MD. 2003. The causes of and cures for contact lens-induced peripheral ulcer. Eye Contact Lens 29:S63-6; discussion S83-4, S192-4.
3. Willcox MD, Holden BA. 2001. Contact lens related corneal infections. Biosci Rep 21:445-61.
4. Rumpakis J. 2010. New data on contact lens dropouts: An international perspective. Rev Optom 147:5.
5. Subbaraman LN, Borazjani R, Zhu H, Zhao Z, Jones L, Willcox MD. 2011. Influence of protein deposition on bacterial adhesion to contact lenses. Optom Vis Sci 88:959-66.
6. Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, Tanavde V, Li XR, Beuerman RW. 2012. In-depth analysis of the human tear proteome. J Proteomics 75:3877-85.
7. Kijlstra A, Kuizenga A. 1994. Analysis and function of the human tear proteins. Adv Exp Med Biol 350:299-308.
8. Jones L, Mann A, Evans K, Franklin V, Tighe B. 2000. An in vivo comparison of the kinetics of protein and lipid deposition on group II and group IV frequent-replacement contact lenses. Optom Vis Sci 77:503-10.
9. Minarik L, Rapp J. 1989. Protein deposits on individual hydrophilic contact lenses: effects of water and ionicity. CLAO J 15:185-8.

10. Fowler SA, Korb DR, Allansmith MR. 1985. Deposits on soft contact lenses of various water contents. CLAO J 11:124-7.

11. Subbaraman LN. 2009. Protein deposition and bacterial adhesion to conventional and silicone hydrogel contact lens materials. PhD. University of Waterloo, Waterloo, Ontario.

12. Gachon AM, Richard J, Dastugue B. 1982. Human tears: normal protein pattern and individual protein determinations in adults. Curr Eye Res 2:301-8.

13. Gachon AM, Verrelle P, Betail G, Dastugue B. 1979. Immunological and electrophoretic studies of human tear proteins. Exp Eye Res 29:539-53.

14. McDermott AM. 2013. Antimicrobial compounds in tears. Exp Eye Res 117:53-61.

15. Loimaranta V, Jakubovics NS, Hytonen J, Finne J, Jenkinson HF, Stromberg N. 2005. Fluid- or surface-phase human salivary scavenger protein gp340 exposes different bacterial recognition properties. Infect Immun 73:2245-52.

16. Madsen J, Mollenhauer J, Holmskov U. 2010. Review: Gp-340/DMBT1 in mucosal innate immunity. Innate Immun 16:160-7.

17. Stoddard E, Cannon G, Ni H, Kariko K, Capodici J, Malamud D, Weissman D. 2007. gp340 expressed on human genital epithelia binds HIV-1 envelope protein and facilitates viral transmission. J Immunol 179:3126-32.

18. Stoddard E, Ni H, Cannon G, Zhou C, Kallenbach N, Malamud D, Weissman D. 2009. gp340 promotes transcytosis of human immunodeficiency virus type 1 in genital tract-derived cell lines and primary endocervical tissue. J Virol 83:8596-603.

19. Osei KA, Deivanayagam C, Nichols JJ. 2018. Glycoprotein 340 in mucosal immunity and ocular surface. Ocul Surf 16:282-288.

20. Ligtenberg AJ, Karlsson NG, Veerman EC. 2010. Deleted in malignant brain tumors-1 protein (DMBT1): a pattern recognition receptor with multiple binding sites. Int J Mol Sci 11:5212-33.

21. Purushotham S, Deivanayagam C. 2013. Cloning, expression and purification of the SRCR domains of glycoprotein 340. Protein Expr Purif 90:67-73.

22. Jumblatt MM, Imbert Y, Young WW, Jr., Foulks GN, Steele PS, Demuth DR. 2006. Glycoprotein 340 in normal human ocular surface tissues and tear film. Infect Immun 74:4058-63.

23. Nichols JJ, Green-Church KB. 2009. Mass spectrometry-based proteomic analyses in contact lens-related dry eye. Cornea 28:1109-17.

24. Schulz BL, Oxley D, Packer NH, Karlsson NG. 2002. Identification of two highly sialylated human tear-fluid DMBT1 isoforms: the major high-molecular-mass glycoproteins in human tears. Biochem J 366:511-20.

25. Li J, Metruccio MME, Evans DJ, Fleiszig SMJ. 2017. Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen Pseudomonas aeruginosa. PLoS Pathog 13:e1006392.

26. Zhao Z, Wei X, Aliwarga Y, Carnt NA, Garrett Q, Willcox MD. 2008. Proteomic analysis of protein deposits on worn daily wear silicone hydrogel contact lenses. Mol Vis 14:2016-24.

27. Mitoma M, Oho T, Shimazaki Y, Koga T. 2001. Inhibitory effect of bovine milk lactoferrin on the interaction between a streptococcal surface protein antigen and human salivary agglutinin. J Biol Chem 276:18060-5.

28. Oho T, Bikker FJ, Nieuw Amerongen AV, Groenink J. 2004. A peptide domain of bovine milk lactoferrin inhibits the interaction between streptococcal surface protein antigen and a salivary agglutinin peptide domain. Infect Immun 72:6181-4.

29. Kukita K, Kawada-Matsuo M, Oho T, Nagatomo M, Oogai Y, Hashimoto M, Suda Y, Tanaka T, Komatsuzawa H. 2013. Staphylococcus aureus SasA is responsible for binding to the salivary agglutinin gp340, derived from human saliva. Infect Immun 81:1870-9.

30. Yang YH, Jiang YL, Zhang J, Wang L, Bai XH, Zhang SJ, Ren YM, Li N, Zhang YH, Zhang Z, Gong Q, Mei Y, Xue T, Zhang JR, Chen Y, Zhou CZ. 2014. Structural insights into SraP-mediated Staphylococcus aureus adhesion to host cells. PLoS Pathog 10:e1004169.

31. Foster TJ. 2019. Surface Proteins of Staphylococcus aureus. Microbiol Spectr 7.

32. Kumari P, Nath Y, Murty US, Ravichandiran V, Mohan U. 2020. Sortase A Mediated Bioconjugation of Common Epitopes Decreases Biofilm Formation in Staphylococcus aureus. Front Microbiol 11:1702.

33. Wojcik M, Vazquez Torres S, Quax WJ, Boersma YL. 2019. Sortase mutants with improved protein thermostability and enzymatic activity obtained by consensus design. Protein Eng Des Sel 32:555-564.

34. Mazmanian SK, Ton-That H, Schneewind O. 2001. Sortase-catalysed anchoring of surface proteins to the cell wall of Staphylococcus aureus. Mol Microbiol 40:1049-57.

35. Novák P, Havlíček V. 2016. Protein Extraction and Precipitation. *In* P C, J S (ed), Proteomic Profiling and Analytical Chemistry, 2 ed. Elsevier.

36. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. 2003. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 31:3784-8.

37. Borazjani RN, Levy B, Ahearn DG. 2004. Relative primary adhesion of Pseudomonas aeruginosa, Serratia marcescens and Staphylococcus aureus to HEMA-type contact lenses and an extended wear silicone hydrogel contact lens of high oxygen permeability. Cont Lens Anterior Eye 27:3-8.

38. Feltman H, Schulert G, Khan S, Jain M, Peterson L, Hauser AR. 2001. Prevalence of type III secretion genes in clinical and environmental isolates of Pseudomonas aeruginosa. Microbiology (Reading) 147:2659-2669.

39. Galle M, Carpentier I, Beyaert R. 2012. Structure and function of the Type III secretion system of Pseudomonas aeruginosa. Curr Protein Pept Sci 13:831-42.

40. Hauser AR. 2009. The type III secretion system of Pseudomonas aeruginosa: infection by injection. Nat Rev Microbiol 7:654-65.

41. Shen EP, Tsay RY, Chia JS, Wu S, Lee JW, Hu FR. 2012. The role of type III secretion system and lens material on adhesion of Pseudomonas aeruginosa to contact lenses. Invest Ophthalmol Vis Sci 53:6416-26.

42. Oliver JD. 2005. The viable but nonculturable state in bacteria. J Microbiol 43 Spec No:93-100.

43. Weinberg ED. 2001. Human lactoferrin: a novel therapeutic with broad spectrum potential. J Pharm Pharmacol 53:1303-10.

44. Narayanan S, Redfern RL, Miller WL, Nichols KK, McDermott AM. 2013. Dry eye disease and microbial keratitis: is there a connection? Ocul Surf 11:75-92.

45. Sanchez CJ, Shivshankar P, Stol K, Trakhtenbroit S, Sullam PM, Sauer K, Hermans PW, Orihuela CJ. 2010. The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that promotes bacterial aggregation in vivo and in biofilms. PLoS Pathog 6:e1001044.

46. Reichhardt MP, Meri S. 2016. SALSA: A Regulator of the Early Steps of Complement Activation on Mucosal Surfaces. Front Immunol 7:85.

47. Holmskov U, Mollenhauer J, Madsen J, Vitved L, Gronlund J, Tornoe I, Kliem A, Reid KB, Poustka A, Skjodt K. 1999. Cloning of gp-340, a putative opsonin receptor for lung surfactant protein D. Proc Natl Acad Sci U S A 96:10794-9.

48. Giltner CL, Habash M, Burrows LL. 2010. Pseudomonas aeruginosa minor pilins are incorporated into type IV pili. J Mol Biol 398:444-61.

49. Gill SC, von Hippel PH. 1989. Calculation of protein extinction coefficients from amino acid sequence data. Anal Biochem 182:319-26.

50. World Medical A. 2001. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. Bull World Health Organ 79:373-4.

51. Keith D, Hong B, Christensen M. 1997. A novel procedure for the extraction of protein deposits from soft hydrophilic contact lenses for analysis. Curr Eye Res 16:503-10.

52. Powell DR, Thangavelu M, Chandler HL, Nichols KK, Nichols JJ. 2010. Evaluation of extractants and precipitants in tear film proteomic analyses. Optom Vis Sci 87:854-60.

53. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671-5.

54. Vijay AK, Zhu H, Ozkan J, Wu D, Masoudi S, Bandara R, Borazjani RN, Willcox MD. 2012. Bacterial adhesion to unworn and worn silicone hydrogel lenses. Optom Vis Sci 89:1095-106.

55. Astley R, Miller FC, Mursalin MH, Coburn PS, Callegan MC. 2019. An Eye on Staphylococcus aureus Toxins: Roles in Ocular Damage and Inflammation. Toxins (Basel) 11.

56. David MZ, Daum RS. 2010. Community-associated methicillin-resistant Staphylococcus aureus: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev 23:616-87.

57. Peterson JC, Durkee H, Miller D, Maestre-Mesa J, Arboleda A, Aguilar MC, Relhan N, Flynn HW, Jr., Amescua G, Parel JM, Alfonso E. 2019. Molecular epidemiology and resistance profiles among healthcare- and community-associated Staphylococcus aureus keratitis isolates. Infect Drug Resist 12:831-843.

58. Tong L, Zhou XY, Jylha A, Aapola U, Liu DN, Koh SK, Tian D, Quah J, Uusitalo H, Beuerman RW, Zhou L. 2015. Quantitation of 47 human tear proteins using high

resolution multiple reaction monitoring (HR-MRM) based-mass spectrometry. J Proteomics 115:36-48.

59. Willcox MD. 2019. Tear film, contact lenses and tear biomarkers. Clin Exp Optom 102:350-363.

60. Lade H, Park JH, Chung SH, Kim IH, Kim JM, Joo HS, Kim JS. 2019. Biofilm Formation by Staphylococcus aureus Clinical Isolates is Differentially Affected by Glucose and Sodium Chloride Supplemented Culture Media. J Clin Med 8.

ACKNOWLEDGMENTS

Research was conducted with faculty development funds of CD (School of Medicine, UAB) and JN (School of Optometry, UAB). KO was supported by graduate student scholarship from the School of Optometry at UAB. The authors thank Dr. Mark Willcox of the University of New South Wales for providing the bacterial strains investigated in the study. Authors acknowledge the Heflin Genomic Core Facility and the Macromolecular Structural Core Facility in aiding with the research. Special appreciation also goes to Dr. Norbert Schormann of the University of Alabama at Birmingham for proofreading the manuscript.

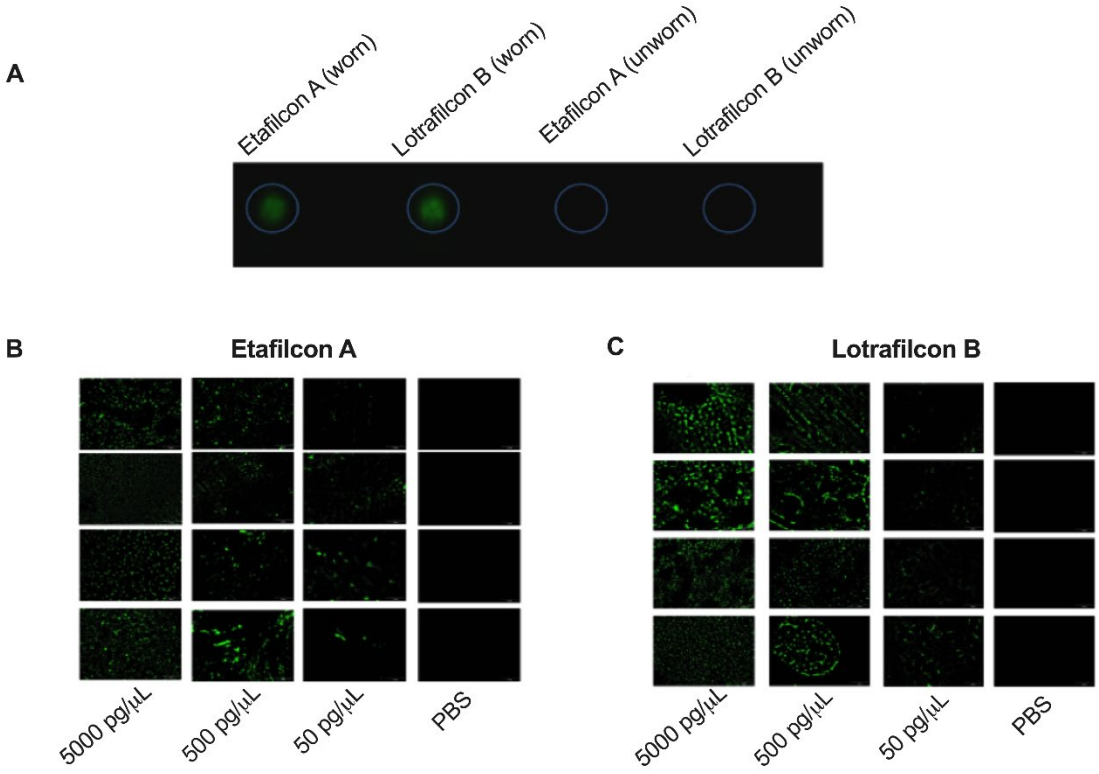

Figure 1. Gp340 and *i*SRCR$_1^{Gp340}$ adsorb on etafilcon A and lotrafilcon lenses.
Total tear protein was extracted from patient-worn etafilcon A and lotrafilcon B lenses and probed for Gp340 using dot blot assay. Gp340 was detected on both polymers (**A**). Unworn etafilcon A and lotrafilcon B lenses were also coated with *i*SRCR$_1^{Gp340}$ solutions of varying concentrations (0 – 5000 pg/uL) and *i*SRCR$_1^{Gp340}$ binding to the lenses was observed with fluorescence microscopy (**B, C**). Scale bar for the images in **B and C** is 100 μm.

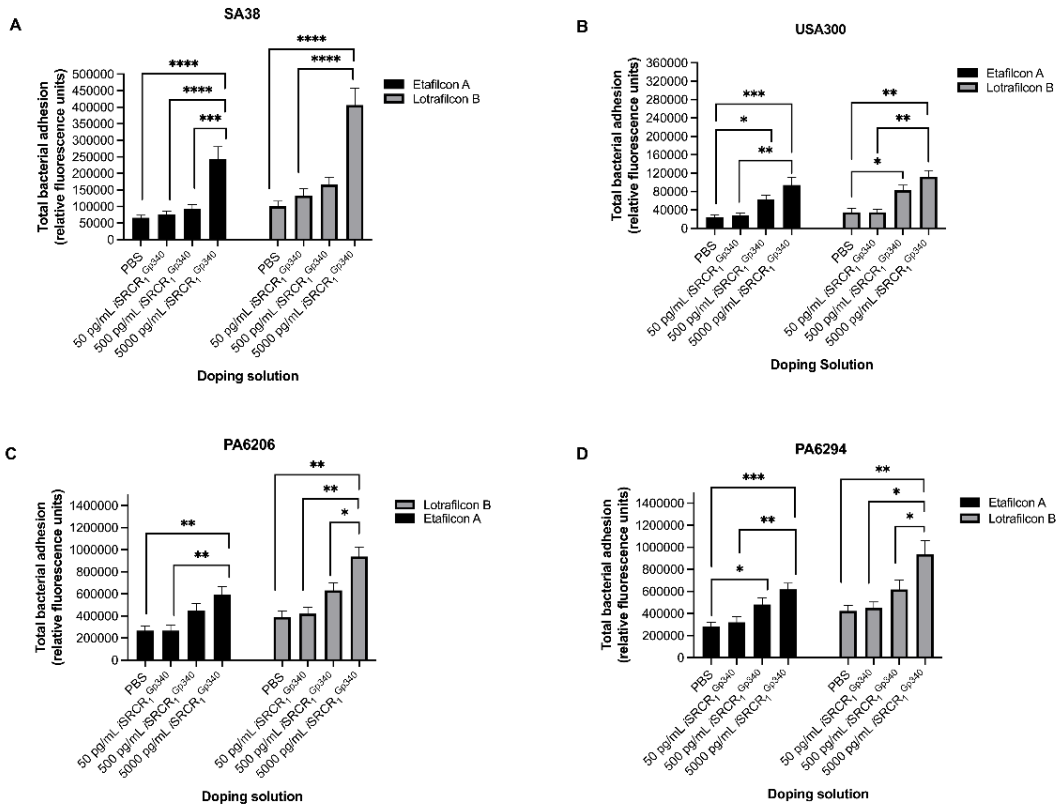

Figure 2. *i*SRCR$_1^{Gp340}$ enhances bacterial adhesion to etafilcon A and lotrafilcon B. Lenses were coated with *i*SRCR$_1^{Gp340}$ of varying concentrations (0-5000 pg/uL) for 12 hours and subsequently incubated with bacterial suspension consisting of SA38 (**A**), USA300 (**B**), PA6206 (**C**), or PA6294 (**D**) for 24 hours. The amount of total bacterial adhesion was determined using a live/dead bacterial assay kit. Significantly higher adhesion on *i*SRCR$_1^{Gp340}$ –coated lenses (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001). Error bars represent standard error.

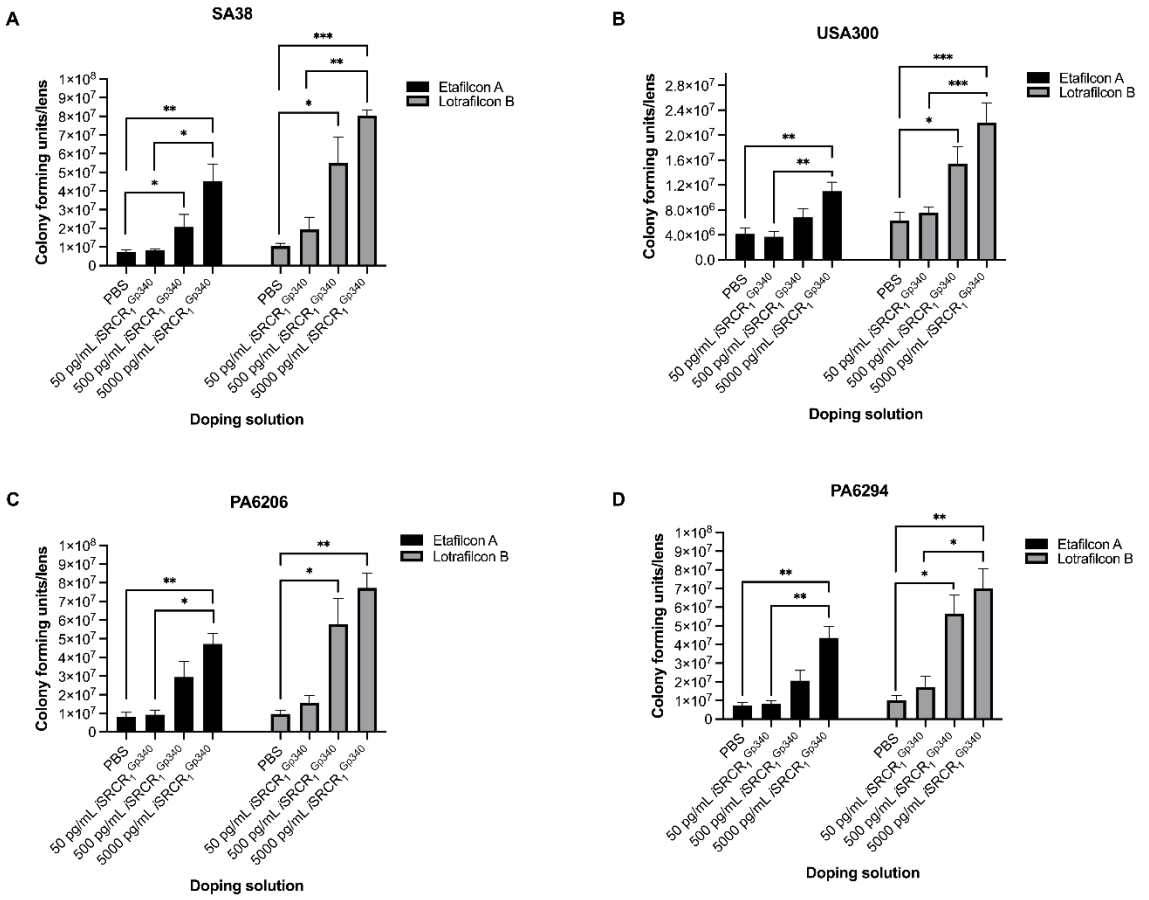

Figure 3. *i*SRCR$_1^{Gp340}$ does not affect the culturability of bacteria adherent on *i*SRCR$_1^{Gp340}$-coated lens. SA38 (**A**), USA300 (**B**), PA6206 (**C**), and PA6294 (**D**) were detached from the lens and plated on TSB agar plates and the amounts of colonies were enumerated. Significantly higher bacterial colonies observed on *i*SRCR$_1^{Gp340}$–coated lenses (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001). Error bars represent standard error.

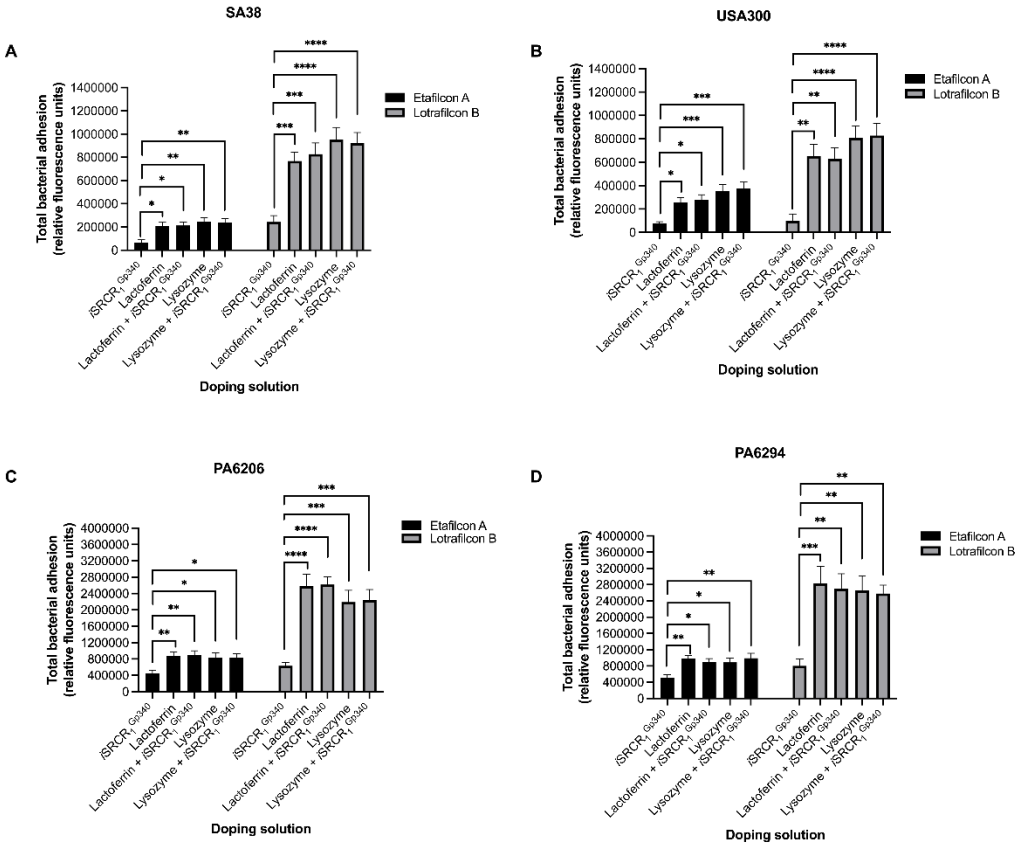

Figure 4. *i*SRCR$_1^{Gp340}$ does not modulate lactoferrin and lysozyme-mediated bacterial adhesion on etafilcon A and lotrafilcon B. Lenses were coated in solution containing *i*SRCR$_1^{Gp340}$ (500 pg/µL), lactoferrin (1.8 µg/µL), lactoferrin + *i*SRCR$_1^{Gp340}$ (1.8 µg/µL + 500 pg/µL), lysozyme (2 µg/µL) or lysozyme + *i*SRCR$_1^{Gp340}$ (2 µg/µL + 500 pg/µL) followed by incubation with bacterial suspension consisting of SA38 (A), USA300 (B), PA6206 (C), or PA6294 (D) for 24 hours. Significantly higher adhesion on lactoferrin and lysozyme–coated lenses (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001). Error bars represent standard error.

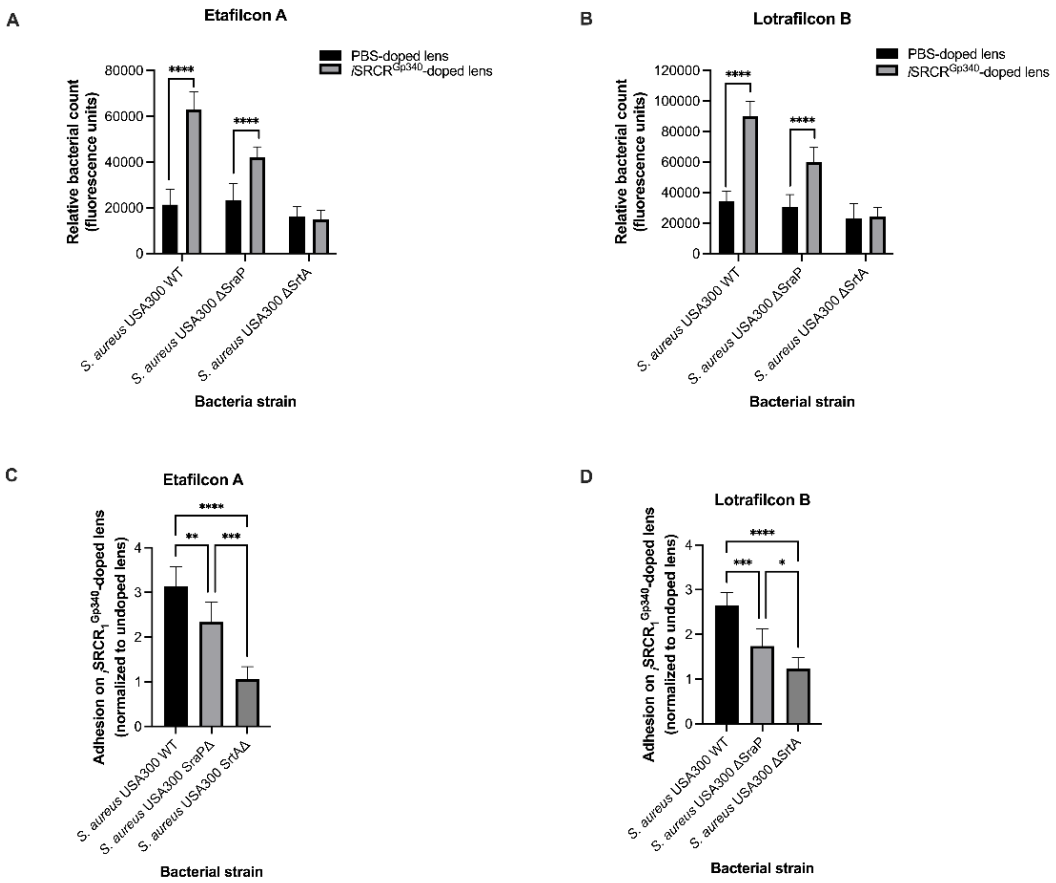

Figure 5. SraP plays significant role in USA 300 adhesion to *i*SRCR$_1^{Gp340}$-adsorbed etafilcon A and lotrafilcon B. Etafilcon A and lotrafilcon B lenses coated in a solution containing *i*SRCR$_1^{Gp340}$ (500 pg/µL) or PBS (control) were incubated with USA300 WT, USA300 ΔSraP, or USA300 ΔSrtA, and total bacterial adhesions were determined. Higher adhesion on *i*SRCR$_1^{Gp340}$–coated lenses compared to PBS-coated lenses, for USA300 WT and USA300 ΔSraP only (**A, B**). Significantly lower USA300 ΔSraP and USA300 ΔSrtA adhesion to *i*SRCR$_1^{Gp340}$-coated lens, compared with USA300 WT (**C, D**) (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001). Error bars represent standard deviation.

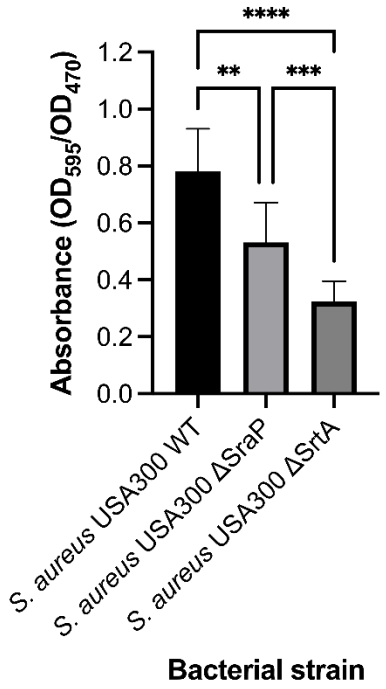

Figure 6. SraP mediates USA 300 biofilm formation on *i*SRCR$_1^{Gp340}$-coated surface. 96-well microtiter plate coated with 50 μg/mL *i*SRCR$_1^{Gp340}$ was incubated 24 hours with cultures containing USA300 WT, USA300 ΔSraP, or USA300 ΔSrtA and the relative sizes of biofilm were measured on a plate reader. Significantly smaller biofilm sizes were observed with USA300 ΔSraP and USA300 ΔSrtA compared to USA300 WT (**P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001). Error bars represent standard deviation.

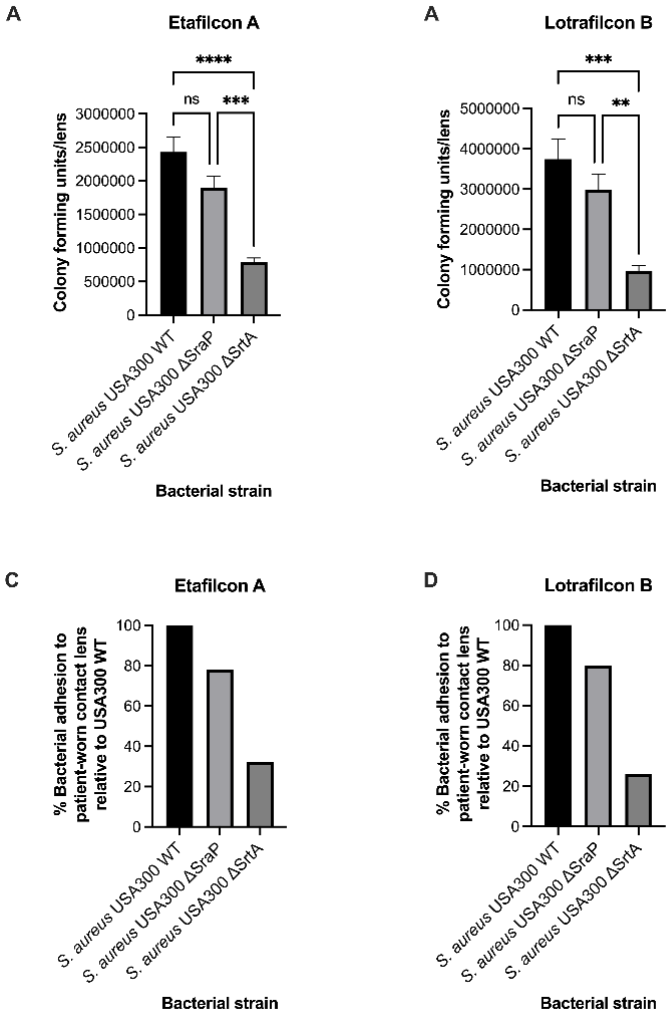

Figure 7. SraP and other CWA proteins contribute to USA300's adhesion to contact lens worn by patients. Patient-worn contact lenses were incubated with cultures containing USA300 WT, USA300 ΔSraP, or USA300 ΔSrtA. Adherent bacteria were detached and plated on TSB agar plates and the amounts of colonies were enumerated (**A, B**). Relative to USA300 WT (**C, D**), ΔSraP mutants displayed reduced by 21.86% (etafilcon A) and 20.21% (lotrafilcon B), and with ΔSraP reduced by 67.63% (etafilcon A) and 74.05% (lotrafilcon B). These results indicate other cell wall associated surface proteins on USA300 contribute to the adherence. (ns: no significant difference, **P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001). Error bars represent standard error.

Table 1. Characteristics of the lenses used in the in vitro arm of the studies.

	Etafilcon A (Acuvue 2)	**Lotrafilcon B (Air Optix)**
FDA group	IV	V
Diameter (mm)	14.0	14.2
Base curve (mm)	8.3	8.6
Water content (%)	58	33
Oxygen transmissibility (Dk/t at 35°C)	20	138
Surface treatment	None	25 nm plasma coating with high refractive index
Principal monomers	polyHEMA + MA	DMA + TRIS + siloxane

Table 2. SraP adherence to *i*SRCR$_1^{Gp340}$

Analyte	**Ligand**	**k_a (1/Ms)**	**k_d (1/s)**	**K_A (1/M)**	**K_D (M)**	**Chi^2**
L-lectin-β-GF-CDHL1-CDHL2^{SraP}	*i*SRCR$_1^{Gp340}$	4.4 x 10^4	1.04 x 10^{-3}	4.23 x 10^7	2.37 x 10^{-8}	4.48
L-lectin-β-GF-CDHL1^{SraP}	*i*SRCR$_1^{Gp340}$	1.24 x 10^3	2.34 x 10^{-3}	5.31 x 10^5	1.88 x 10^{-6}	3.28
β-GF-CDHL1-CDHL2^{SraP}	*i*SRCR$_1^{Gp340}$	159	4.37 x 10^{-4}	3.63 x 10^5	2.76 x 10^{-6}	6.09

SUPPLEMENTAL MATERIALS

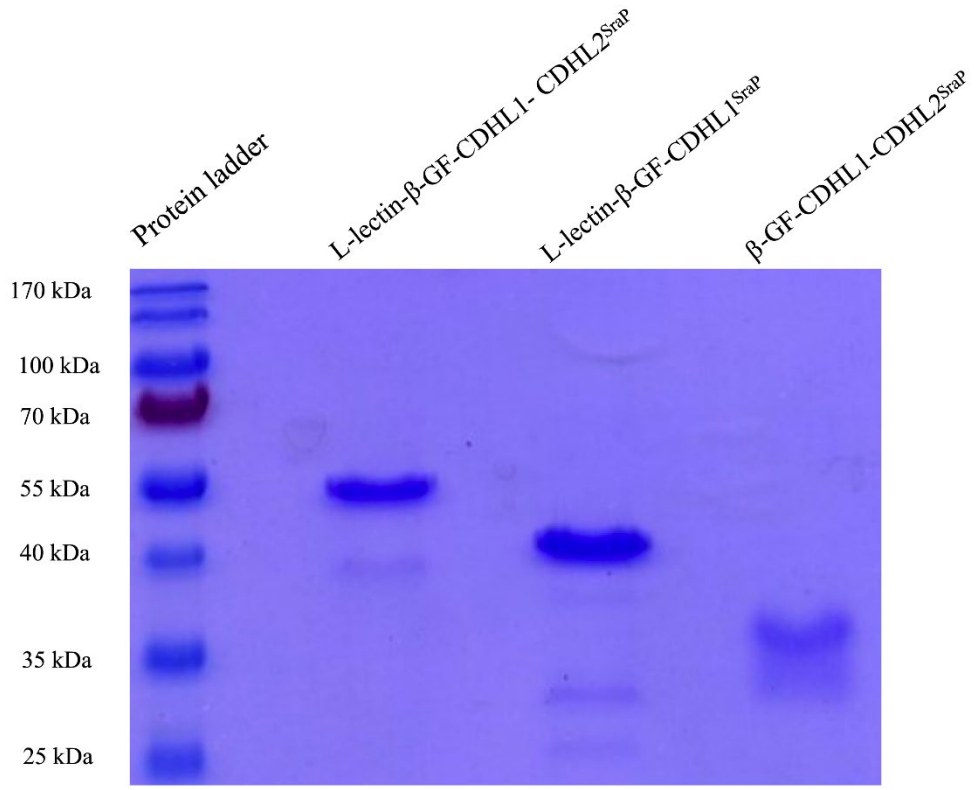

Figure S1. 12.5% SDS PAGE showing purified full-length SraP binding region and the smaller fragments.

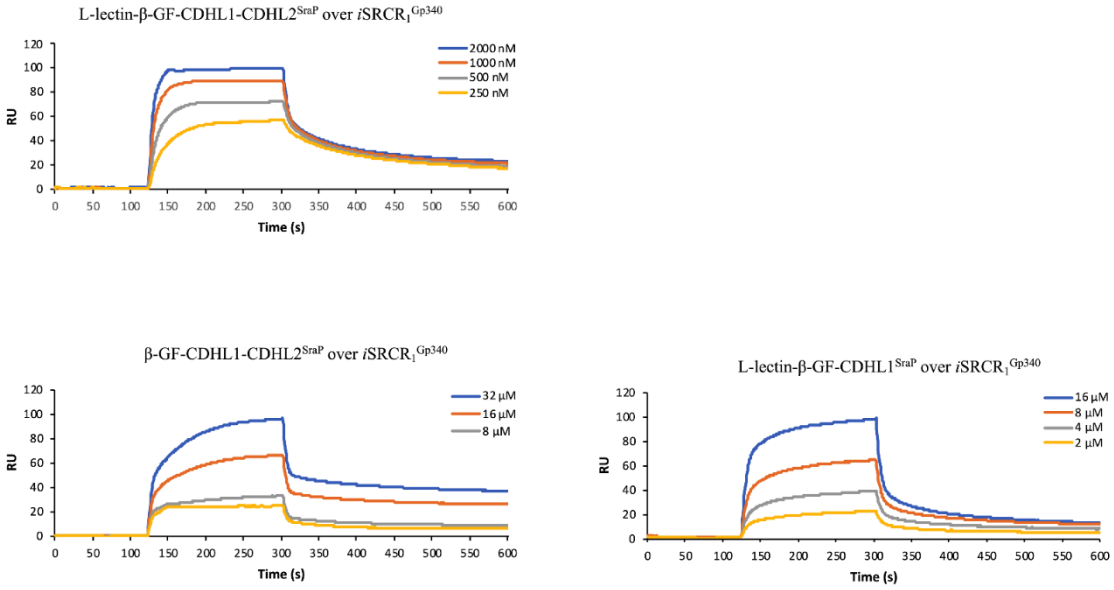

Figure S2. Sensorgrams for the interaction of SraP binding regions with *i*SRCR$_1^{Gp340}$. Serving as the ligand, *i*SRCR$_1^{Gp340}$ was immobilized on a CM5 chip using ethanolamine chemistry. Analytes, L-lectin-β-GF-CDHL1-CDHL2^{SraP}, L-lectin-β-GF-CDHL1^{SraP} and β-GF-CDHL1-CDHL2^{SraP} were injected over SRCR$_1$ at various concentrations optimized for each analyte (serial dilutions within 0.250 - 32 µM), and dissociations were measured for 600 seconds following injections. The sensorgrams were fitted using the BIAevaluation software.

Table S3. Primers used in cloning the SraP constructs.			
Construct	**Residues**	**Primers: Forward/Reverse**	**MW (Da)**
L-lectin-β-GF-CDHL1-CDHL2^{SraP}	517 (245-751)	ATATCCATGGGGTTTGCAAGC GCGCCTCGAGGTTACGGGT	54031
L-lectin-β-GF-CDHL1^{SraP}	426 (245-660)	ATATCCATGGGGTTTGCAAGC GCGCCTCGAGAACAACATT	40384
β-GF-CDHL1-CDHL2^{SraP}	270 (492-751)	ATATCCATGGGGACCGAATCA GCGCCTCGAGGTTACGGGT	16896

CHAPTER 5 – GLYCOPROTEIN 340 EXPRESSION IN HUMAN CORNEAL EPITHELIAL CELLS EXPOSED TO *PSEUDOMONAS AERUGINOSA* AND *STAPHYLOCOCCUS AUREUS* INFECTION

by

KWAKU A. OSEI, CHAMPION DEIVANAYAGAM, JASON J. NICHOLS

In preparation for *Cornea*

Format adapted for dissertation

INTRODUCTION

Glycoprotein 340, also known as salivary agglutinin is a 340 kDa heavily-glycosylated innate immunity factor that is expressed in mucosal surfaces and tissues.[1-7] It is encoded by the *deleted in malignant brain tumor 1 (DMBT1)* gene and as such it is also referred to as DMBT1.[1,3,8-11] It belongs to the scavenger receptor cysteine-rich (SRCR) superfamily and consists of 14 SRCR domains and 11 SRCR interspersed domains, two C1r/C1s, urchin embryonic growth factor bone morphogenetic protein-1 (CUB) and a single zona pellucida (ZP) domain.[2,3,5,7,12,13] Functionally, Gp340 has a dual face in infection depending on its conformation. In the soluble phase, it inhibits infections such as oral HIV and influenza A, while in the immobilized form, it promotes infections like vaginal HIV transmission and dental caries.[2,3,14]

On the surface of the eye, Gp340 is a normal component of the precorneal tear film secreted by the lacrimal gland, cornea, and conjunctiva.[15,16] Nichols & Green-Church[17] and Perumal et al.[18] have reported a dysregulation of Gp340 expression in the tears of patients with dry eye disease. However, the mechanisms underlying this observation have yet to be elucidated. While investigations on the functional effects and roles of Gp340 were focused on non-ocular surface-related tissues,[2-4,9,10,14,19,20] recently, the roles of Gp340 on the ocular surface have been the focus of investigations. Li et al., has demonstrated that Gp340 inhibits twitching motility in *Pseudomonas aeruginosa* and promotes wound healing in a murine keratitis model.[21,22] On the other hand, Osei et al., in preliminary studies, observed that the SRCR domain of Gp340 enhanced *Pseudomonas aeruginosa* and *Staphylococcus*

aureus adhesion to soft contact lens when it adsorbs onto the lens surface.[23] Put together, these findings show that Gp340 has the potential to modulate infection on the ocular surface, either positively or negatively. Establishing the regulation pattern of Gp340 gene transcription and protein translation in ocular surface tissues exposed to infection would enable understanding of Gp340's roles in an ocular surface infection.

This study, therefore, primarily aimed to profile both Gp340 mRNA transcription and protein expression in human corneal epithelial cells when exposed to ocular infectious bacterial strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

MATERIALS AND METHODS

Bacterial Strains

Two gram-positive bacterial strains, *S. aureus* 38 (SA 38) and methicillin-resistant *S. aureus* (MRSA) USA 300 and two gram-negative strains, *P. aeruginosa* 6206 (PA 6206) and *P. aeruginosa* 001 (PA 001), were investigated in this study. SA 38, PA 001, and PA 6206 which were isolates from human microbial keratitis were gifted by Dr. Mark Willcox Laboratory at the University of New South Wales, Sydney, Australia. USA 300 is the most prevalent community-associated MRSA strain in the United States and a leading cause of healthcare associated keratitis, a type of corneal infection that develops while a person is in a healthcare facility and receiving care for another condition.[24-26] The two *P. aeruginosa* strains are all cytotoxic in nature. All the strains were cultured in trypticase soy broth (TSB; Becton, Dickson and Company, Sparks, MD, USA) for 12 hours in a 5% CO_2 at 37 ºC. The cultures were diluted in basal DMEM/F12 medium to the appropriate concentrations and used in the subsequent infection studies.

Gp340 mRNA and Protein Expression in Corneal Infection

Human Corneal Epithelial Cell Culture and Infection Model

SV40-transformed human corneal epithelial cells (HCECs) were purchased from Riken Cell Bank, Ibaraki, Japan, and used in this study. These cells, in preliminary studies, have been shown to express the *DMBT1* gene. Briefly, 2.5×10^5 HCECs were seeded in quadruplicate in 12-well plate flasks at 5% CO_2 at 37 ºC in complete antibiotic-free

DMEM/F12 medium containing 5% fetal bovine serum, 0.5% DMSO, 10 ng/mL human EGF, and 5μg/mL and cultured for 24 hours. Subsequently, the conditioned media was removed, and cells were washed three times with PBS. The cells were then infected with 1 mL of bacterial suspension at a multiplicity of infection (MOI) of 50 and incubated for 2 hours. In parallel experiments, cells treated with 1 mL of bacteria-free medium served as control. Afterward, the conditioned media was removed, centrifuged at 12,000 ×g for 10 minutes and the supernatant stored at -80 ºC for future analysis. In two of these wells, HCECs were treated with RIPA buffer for 5 minutes, harvested and centrifuged at 14,000 ×g for 15 minutes, and the supernatants (lysates) were stored at -80 ºC for future protein analysis. For the other two wells, HCECs were detached by adding cell dissociation media (TrypLE, Invitrogen, Carlsbad, CA) for 5 minutes. The detached cells were centrifuged at 12,000 ×g for 10 minutes and the pellets were stored at -80 ºC for future mRNA analysis. The experiments were repeated a minimum of three times.

Primer Design

Primers for *DMBT1* and *18S RNA* (endogenous control) were designed using the National Center for Biotechnology Information's Primer-BLAST software.[27] The primers for *DMBT1* were GCAGGCAGACAATGACACCA (forward) and TGCAGCTGACGTGAATACGG (reverse) and that for *18S RNA* were CCGGACACGGACAGGATTGA (forward) and GCATGCCAGAGTCTCGTTCG (reverse) for *18s RNA*. These primers amplify a 104-bp *DMBT1* transcript and a 121-bp *18S RNA* transcript.

Total RNA Isolation, cDNA Synthesis, Real-Time RT-PCR, and Gel-Based RT-PCR

Briefly, the HCECs were lysed with 350 µL in RNeasy Plus lysis buffer (Qiagen, Germantown, MD, USA) and total RNAs were isolated using the RNeasy® Plus Micro kit (Qiagen). The quality and quantity of the isolated RNA were assessed using the NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized from the isolated RNA using the High-Capacity RNA-to-cDNA™ Kit (Life Technology, Carlsbad, CA, USA). Each 20 µL reaction volume contained 500 ng total RNA.

Real time RT-PCR was then performed on a QuantStudio 3™ real-time PCR instrument (Applied Biosystems) using *DMBT1*- and *18S RNA*-specific inventoried TaqMan™ gene expression assays (Life Technologies, *DMBT1*: Hs01069306_m1, *18s RNA*: Hs99999901_s1) and TaqMan™ Fast Advanced Master Mix (Life Technologies). Each RT-PCR reaction contained 10 ng of template cDNA. Real-time PCR data were analyzed with the Design and Analysis Software™ (version 1.5.1, Applied Biosystems). All data were first normalized to *18S RNA*. Gene expressions in the bacteria-infected HCECs were then normalized to the control cells and the results were expressed as fold change in Gp340 mRNA transcription. After establishing the non-normality of the data, the difference in Gp340 gene transcription between bacteria-infected and control HCECs was analyzed with the Mann-Whitney U test with $p < 0.05$ denoting statistical significance.

To confirm the real-time PCR results, qualitative (gel-based) RT-PCR was performed. This was done using 20 ng of template cDNA, 1× DreamTaq Green PCR Master Mix (Invitrogen), and 0.5 µM each of forward and reverse primers

in a 50-µL reaction volume. At the end of the reaction, the PCR products were examined on 2% agarose gel electrophoresis.

Enzyme-Linked Immunosorbent Assay (ELISA)

The total protein concentration in each sample was first determined using BCA assay. Sandwich ELISA was then employed to measure the relative levels of Gp340 protein in the bacteria-infected and control HCECs. Briefly, Nunc MaxiSorp™ 96-well microtiter plate (Invitrogen) was coated with 100 µL of rabbit anti-human Gp340 antibody (2.5 µg/mL) and incubated for 18 hours at 4°C. The wells were then aspirated and blocked for 2 hours at room temperature (RT) with 1% BSA in PBS-tween (PBST). These wells were washed four times with PBST. One hundred microliters of diluted lysates were added in duplicate to the wells and incubated for 2 hours at RT. After washing, 100 µL of mouse anti-human Gp340 antibody (1:10,000) was added to each well and incubated for 2 hours at RT. Subsequently, the wells were washed and 100 µL of goat anti-mouse HRP-conjugated secondary antibody (1:10,000) was added and incubated 2 hours. The wells were then washed and 100 µL of TMB solution (3,3′,5,5′-tetramethylbenzidine) was added for color development. After incubating the plate at RT for 30 minutes, the reaction was stopped with 100 µL of 2M H_2SO_4 solution and the absorbances were read at 450 nm (OD_{450}) on a microplate reader (BioTek® Synergy 2, Winooski, VT, USA) and adjusted for background noise. The difference in relative levels of Gp340 between the infected and control HCECs was determined by analyzing the OD_{450} values with the independent t test with $p < 0.05$ denoting statistical significance. The conditioned media samples were also analyzed in a similar manner.

Determination of Proinflammatory Cytokine Expression in HCECs Infected with Bacteria

Inflammation is a hallmark of corneal infection.[28-30] Hence, to confirm that exposing the HCECs to bacterial suspension elicited inflammatory response, the expression of four proinflammatory cytokines – TNFα, IL1β, IL6, and IL8, were determined by sandwich ELISA using matched antibody pairs (Invitrogen) and following vendor's instructions. Briefly, a microtiter plate was coated with 100 µL of capture antibody per well and incubated for 18 h at 4 °C. The capture antibody concentrations were 2 µg/mL for TNFα and IL-1β, and 1 µg/mL for IL-6 and IL-8. The wells were then washed with PBS-tween (PBST) and blocked with 1% BSA for 1 h at RT. Afterwards, the wells were aspirated and 100 µL of standards (1000 – 15.6 pg/mL) and conditioned media samples were added in duplicate and incubated with the respective detection primary antibodies for 2 h at RT. Thereafter, the wells were washed and incubated with a 100 mL of streptavidin-HRP solution (Invitrogen) for 30 min at RT. Finally, after washing, the wells incubated with TMB solution for 30 min and stopped with 2M H_2SO_4. The absorbances were then read at 450 nm and adjusted for background noise. Standard curves were generated with the four-parameter logistic model in the Gen 5™ analysis software (BioTek®, version 3.08) and used to interpolate the concentrations of the samples. The student t test was then used to determine the difference in cytokine expressions between the bacterial-infected and control HCECs with $p < 0.05$ signifying statistical significance.

Analysis of the Role of Gp340 Expression in Bacterial Adhesion to HCECs

Bacterial adhesion is the first step in the processes leading to infection as it leads to colonization, biofilm formation and subsequent infection.[31,32] In the preceding section,

Gp340 expression was found to be modulated in HCECs infected with PA 6206 (downregulation) and SA 38 (upregulation). Thus, these two strains were investigated further to determine if Gp340 plays any role in their adherence to HCECs.

Transient Knockdown of Gp340 mRNA

Gp340 mRNA transcription in HCECs was transiently knocked down by transfecting the cells with *DMBT1*-targeting siRNA (Ambion® Silencer Select siRNA, ID: s4154) and Lipofectamine® RNAiMAX (Life Technologies) and following manufacturer's recommendations as follows. Briefly, 2.5 x 10^5 cells were seeded in duplicate in 12-well tissue culture plates in complete DMEM/F12 medium in the presence of siRNA-lipofectamine complex (final concentration of siRNA = 20 nM) and cultured for 48 hours. Subsequently, the cells were washed three times and used in downstream studies. Transfecting the HCECs with the *DMBT1* siRNA reduced Gp340 mRNA transcription by at least 90%.

Bacterial Adhesion Assay

Briefly, the *DMBT1*- and control siRNA-transfected HCECs were both treated in triplicate with SA 38 bacterial suspension at MOI = 50 for 2 hours. Thereafter, the conditioned media was removed, centrifuged at 12,000 × g for 10 minutes, after the addition of protease inhibitor, to remove any debris, and stored at -80 °C for later protein analysis. The wells were washed times to detach any unbound bacteria, followed by cell lysis with 500 µL 1% triton x-100. The cell lysates were homogenized and serially diluted (1:100, 1000, 10000) and 100 µL each was plated on TSB agar plates. The plates were

cultured for 16 hours in a 5% CO_2, 37 ºC incubator. The quantity of bacteria on each plate was enumerated as colony forming units per mL (CFU/mL). The experiments were repeated at least two more times (n = 9 per siRNA type). The difference in CFU quantity between *DMBT1* and control siRNA-transfected cells was analyzed using the independent student t test, with statistical significance set at $p < 0.05$, after establishing the normality of data. The experiments were repeated using the PA 6206 strain.

Analysis of the Effect of Gp340 on Proinflammatory Cytokine Expression in SA 38 infection

In the preceding study, silencing Gp340 mRNA transcription resulted in an increased adhesion of SA 38 to HCECs. Given that corneal infection and by extension, bacterial adhesion modulate cytokine expression, the effect of Gp340 expression on cytokine expression in the SA 38-infected cells was investigated. The conditioned media obtained from the bacterial adhesion assay described above were analyzed by sandwich ELISA to quantitate the expression of TNFα, IL1β, IL6, and IL8. The sandwich ELISA for the four cytokines was performed as described in the earlier section. The student t test was used to determine the difference in cytokine expressions between the treatment conditions with $p < 0.05$ signifying statistical significance.

RESULTS

Gp340 and Proinflammatory Cytokine Expression in HCECs Challenged with Infectious *P. aeruginosa* and *S. aureus*

Real-time PCR analyses, confirmed by qualitative PCR (Figure 1A), showed that, compared to the respective controls, Gp340 mRNA transcription increased in SA 38-infected HCECs (fold change: 2.445) and reduced in PA 6206-infected cells (fold change: 0.102). However, no significant change in mRNA expression was observed in the cells exposed to USA 300 and PA 001. Similarly, Gp340 protein expression increased in SA 38-challenged HCECs and reduced in PA 6206-challenged cells, while there was no change in the cells infected with USA 300 and PA 001 (Figure 1B). Compared to the control, the expression all four proinflammatory cytokines, TNFα, IL1β, IL6, and IL8, were significantly higher in HCECs infected with each of the four bacterial strains ($P < 0.0001$, Figure 2)

The Effect of Gp340 mRNA Transcription Knockdown on SA 38 and PA 6206 Adhesion to HCECs

Compared to the control, SA 38's adhesion to Gp340-transfected HCECs was significantly higher ($P = 0.0034$, Figure 3). For PA 6206, however, there was no difference in adhesion between the Gp340 siRNA-transfected and control HCECs ($P = 0.4103$).

The Modulation of Proinflammatory Cytokine Expression by Gp340-Mediated Increased SA 38 Adhesion to HCECs

There was no difference in expression of TNFα, IL1-β, IL-6, and IL-8 between the *DMBT1* siRNA-transfected HCECs and the control cells (Figure 4), indicating that Gp340 does not modulate these set of proinflammatory cytokines in corneal epithelial cells exposed to the infectious agent, SA 38.

DISCUSSION

Gp340 is expressed in mucosal tissues and fluids that are susceptible to microbial infections. It displays dual characteristics in infection, where it can either inhibit or promote the process depending on the conformation in which it exists.[2,3,13,14,19] On the ocular surface, the protective effect of tear Gp340 has been established by Li et al.[21,22] In the study, Gp340 was determined to inhibit twitching motility, a flagella-independent form of translocation, in *Pseudomonas aeruginosa* and also promote corneal wound healing.[21,22] While the conformation of tear and ocular surface-expressed Gp340 has yet to be established, it is speculated that in the absence of contact lens wear, Gp340 derived from tears and ocular surface tissues exists in the soluble/fluid-phase conformation. The findings by Li et al.,[21,22] therefore, are consistent with Gp340's protective/beneficial role in infection.

The intact cornea is immune-privileged due to the presence of defense mechanisms such as epithelial barriers with tight junctions, sensory nerves that regulate tearing and blinking and precorneal tear films with lubricating functions and antimicrobial proteins such as lactoferrin, lysozyme, lipocalin, beta-lysin, defensin, and sensory nerves that regulate tearing and blinking.[33-35] Despite, the cornea is at a risk of microbial keratitis particularly when the defense mechanisms are impaired.[33,34,36] Given that both the tear film and cornea express Gp340, establishing the pattern of Gp340 regulation in corneal infection is a crucial step to elucidating the protein's role in microbial infections on the cornea. This study investigated *Pseudomonas aeruginosa* and *Staphylococcus aureus* because these

species, together with *Streptococcal pneumoniae,* account for 80% of bacterial corneal ulcers.[37]

Simulating corneal infection in vitro, the current study first determined if Gp340 mRNA transcription and protein expression are modulated in corneal infection. Of the four infectious bacterial strains investigated, Gp340 mRNA and protein levels were upregulated in SA 38 and downregulated in PA 6206 (Figure 1), indicating that Gp340 modulation pattern in corneal infection is strain-dependent. The infection model used in this study was cell culture-based and may not necessarily be reflective of in vivo (real world) infection. Given that a real-world infection stimulates inflammation, the expression patterns of four proinflammatory cytokines – IL-1β, IL-6, IL-8, and TNFα, which are known to be modulated in microbial keratitis[38,39] were examined under the hypothesis that exposing HCECs to infectious bacterial strains would induce an upregulation of IL-1β, IL-6, IL-8, and TNFα expression. As expected, the expression of all four cytokines were upregulated with all the fours bacterial strains investigated (Figure 2).

Having established that infecting HCECs with PA 6206 and SA 38 modulated Gp340 expression, these two strains were further examined for their role in microbial keratitis. Microbial adhesion is a very critical step in infection as it is the first step in colonization, invasion, and biofilm formation that ultimately leads to infection.[40,41] Given that the fluid-phased Gp340 is protective against infections, it was hypothesized that Gp340 secreted by corneal epithelial cells inhibits bacterial adhesion to HCECs. If true, then knocking down Gp340 gene transcription in HCECs would increase bacterial adhesion. Between the two strains, knocking down Gp340 expression increased adhesion of SA 38,

but had no effect on PA 6206 (Figure 3). The Gp340 gene knockdown, however, had no significant effect on the expression of the proinflammatory cytokines (Figure 4).

In conclusion, this study establishes that Gp340 expression in bacteria-challenged corneal epithelium is modulated based on the type of infectious bacterial strain. In addition, knocking down Gp340 gene expression increases the gram-positive SA 38 adhesion to corneal epithelial cells, and provides preliminary evidence of Gp340's protective effect against infections caused by certain bacterial strains.

REFERENCES

1. Mollenhauer J, End C, Renner M, Lyer S, Poustka A. DMBT1 as an archetypal link between infection, inflammation, and cancer. *Inmunologia.* 2007;26(4):17.
2. Loimaranta V, Jakubovics NS, Hytonen J, Finne J, Jenkinson HF, Stromberg N. Fluid- or surface-phase human salivary scavenger protein gp340 exposes different bacterial recognition properties. *Infect Immun.* 2005;73(4):2245-2252.
3. Madsen J, Mollenhauer J, Holmskov U. Review: Gp-340/DMBT1 in mucosal innate immunity. *Innate Immun.* 2010;16(3):160-167.
4. Reichhardt MP, Loimaranta V, Thiel S, Finne J, Meri S, Jarva H. The salivary scavenger and agglutinin binds MBL and regulates the lectin pathway of complement in solution and on surfaces. *Front Immunol.* 2012;3:205.
5. Purushotham S, Deivanayagam C. Cloning, expression and purification of the SRCR domains of glycoprotein 340. *Protein Expr Purif.* 2013;90(2):67-73.
6. Kukita K, Kawada-Matsuo M, Oho T, et al. Staphylococcus aureus SasA is responsible for binding to the salivary agglutinin gp340, derived from human saliva. *Infect Immun.* 2013;81(6):1870-1879.
7. Reichhardt MP, Meri S. SALSA: A Regulator of the Early Steps of Complement Activation on Mucosal Surfaces. *Front Immunol.* 2016;7:85.
8. Holmskov U, Mollenhauer J, Madsen J, et al. Cloning of gp-340, a putative opsonin receptor for lung surfactant protein D. *Proc Natl Acad Sci U S A.* 1999;96(19):10794-10799.

9. Renner M, Bergmann G, Krebs I, et al. DMBT1 confers mucosal protection in vivo and a deletion variant is associated with Crohn's disease. *Gastroenterology.* 2007;133(5):1499-1509.

10. Oho T, Nagata E. DMBT1 involvement in the human aortic endothelial cell response to Streptococcus mutans. *Mol Oral Microbiol.* 2019;34(3):108-117.

11. Bathum Nexoe A, Pedersen AA, von Huth S, Detlefsen S, Hansen PL, Holmskov U. Immunohistochemical Localization of Deleted in Malignant Brain Tumors 1 in Normal Human Tissues. *J Histochem Cytochem.* 2020;68(6):377-387.

12. Muller H, Hu J, Popp R, et al. Deleted in malignant brain tumors 1 is present in the vascular extracellular matrix and promotes angiogenesis. *Arterioscler Thromb Vasc Biol.* 2012;32(2):442-448.

13. Osei KA, Deivanayagam C, Nichols JJ. Glycoprotein 340 in mucosal immunity and ocular surface. *Ocul Surf.* 2018;16(3):282-288.

14. Stoddard E, Ni H, Cannon G, et al. gp340 promotes transcytosis of human immunodeficiency virus type 1 in genital tract-derived cell lines and primary endocervical tissue. *J Virol.* 2009;83(17):8596-8603.

15. Jumblatt MM, Imbert Y, Young WW, Jr., Foulks GN, Steele PS, Demuth DR. Glycoprotein 340 in normal human ocular surface tissues and tear film. *Infect Immun.* 2006;74(7):4058-4063.

16. Schulz BL, Oxley D, Packer NH, Karlsson NG. Identification of two highly sialylated human tear-fluid DMBT1 isoforms: the major high-molecular-mass glycoproteins in human tears. *Biochem J.* 2002;366(Pt 2):511-520.

17. Nichols JJ, Green-Church KB. Mass spectrometry-based proteomic analyses in contact lens-related dry eye. *Cornea.* 2009;28(10):1109-1117.

18. Perumal N, Funke S, Pfeiffer N, Grus FH. Proteomics analysis of human tears from aqueous-deficient and evaporative dry eye patients. *Sci Rep.* 2016;6:29629.

19. Stoddard E, Cannon G, Ni H, et al. gp340 expressed on human genital epithelia binds HIV-1 envelope protein and facilitates viral transmission. *J Immunol.* 2007;179(5):3126-3132.

20. Boks MA, Gunput ST, Kosten I, et al. The Human Glycoprotein Salivary Agglutinin Inhibits the Interaction of DC-SIGN and Langerin with Oral Micro-Organisms. *J Innate Immun.* 2016;8(4):350-361.

21. Li J, Metruccio MME, Smith BE, Evans DJ, Fleiszig SMJ. Correction: Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen Pseudomonas aeruginosa. *PLoS Pathog.* 2017;13(9):e1006612.

22. Li J, Metruccio MME, Evans DJ, Fleiszig SMJ. Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen Pseudomonas aeruginosa. *PLoS Pathog.* 2017;13(5):e1006392.

23. Osei KA, Deivanayagam C, Nichols JJ. The effect of Glycoprotein 340's scavenger receptor cysteine-rich domain on bacterial adhesion on soft contact lens. *Invest Ophthalmol Vis Sci.* 2019;60:ARVO E-Abstract 6333.

24. Peterson JC, Durkee H, Miller D, et al. Molecular epidemiology and resistance profiles among healthcare- and community-associated Staphylococcus aureus keratitis isolates. *Infect Drug Resist.* 2019;12:831-843.

25. Monegro AF, Muppidi V, Regunath H. Hospital Acquired Infections. In: *StatPearls.* Treasure Island (FL)2021.

26. Haque M, Sartelli M, McKimm J, Abu Bakar M. Health care-associated infections - an overview. *Infect Drug Resist.* 2018;11:2321-2333.

27. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics.* 2012;13:134.

28. Sloop GD, Moreau JM, Conerly LL, Dajcs JJ, O'Callaghan RJ. Acute inflammation of the eyelid and cornea in Staphylococcus keratitis in the rabbit. *Invest Ophthalmol Vis Sci.* 1999;40(2):385-391.

29. Kernacki KA, Berk RS. Characterization of the inflammatory response induced by corneal infection with Pseudomonas aeruginosa. *J Ocul Pharmacol.* 1994;10(1):281-288.

30. Singh P, Gupta A, Tripathy K. Keratitis. In: *StatPearls.* Treasure Island (FL)2021.

31. Dunne WM, Jr. Bacterial adhesion: seen any good biofilms lately? *Clin Microbiol Rev.* 2002;15(2):155-166.

32. Stones DH, Krachler AM. Against the tide: the role of bacterial adhesion in host colonization. *Biochem Soc Trans.* 2016;44(6):1571-1580.

33. Akpek EK, Gottsch JD. Immune defense at the ocular surface. *Eye (Lond).* 2003;17(8):949-956.

34. Narayanan S, Redfern RL, Miller WL, Nichols KK, McDermott AM. Dry eye disease and microbial keratitis: is there a connection? *Ocul Surf.* 2013;11(2):75-92.

35. Bolanos-Jimenez R, Navas A, Lopez-Lizarraga EP, et al. Ocular Surface as Barrier of Innate Immunity. *Open Ophthalmol J.* 2015;9:49-55.

36. Somabhai Katara R, Dhanjibhai Patel N, Sinha M. A clinical microbiological study of corneal ulcer patients at western Gujarat, India. *Acta Med Iran.* 2013;51(6):399-403.

37. Al-Mujaini A, Al-Kharusi N, Thakral A, Wali UK. Bacterial keratitis: perspective on epidemiology, clinico-pathogenesis, diagnosis and treatment. *Sultan Qaboos Univ Med J.* 2009;9(2):184-195.

38. Thakur A, Xue M, Stapleton F, Lloyd AR, Wakefield D, Willcox MD. Balance of pro- and anti-inflammatory cytokines correlates with outcome of acute experimental Pseudomonas aeruginosa keratitis. *Infect Immun.* 2002;70(4):2187-2197.

39. Chidambaram JD, Kannambath S, Srikanthi P, et al. Persistence of Innate Immune Pathways in Late Stage Human Bacterial and Fungal Keratitis: Results from a Comparative Transcriptome Analysis. *Front Cell Infect Microbiol.* 2017;7:193.

40. Klemm P, Vejborg RM, Hancock V. Prevention of bacterial adhesion. *Appl Microbiol Biotechnol.* 2010;88(2):451-459.

41. Pizarro-Cerda J, Cossart P. Bacterial adhesion and entry into host cells. *Cell.* 2006;124(4):715-727.

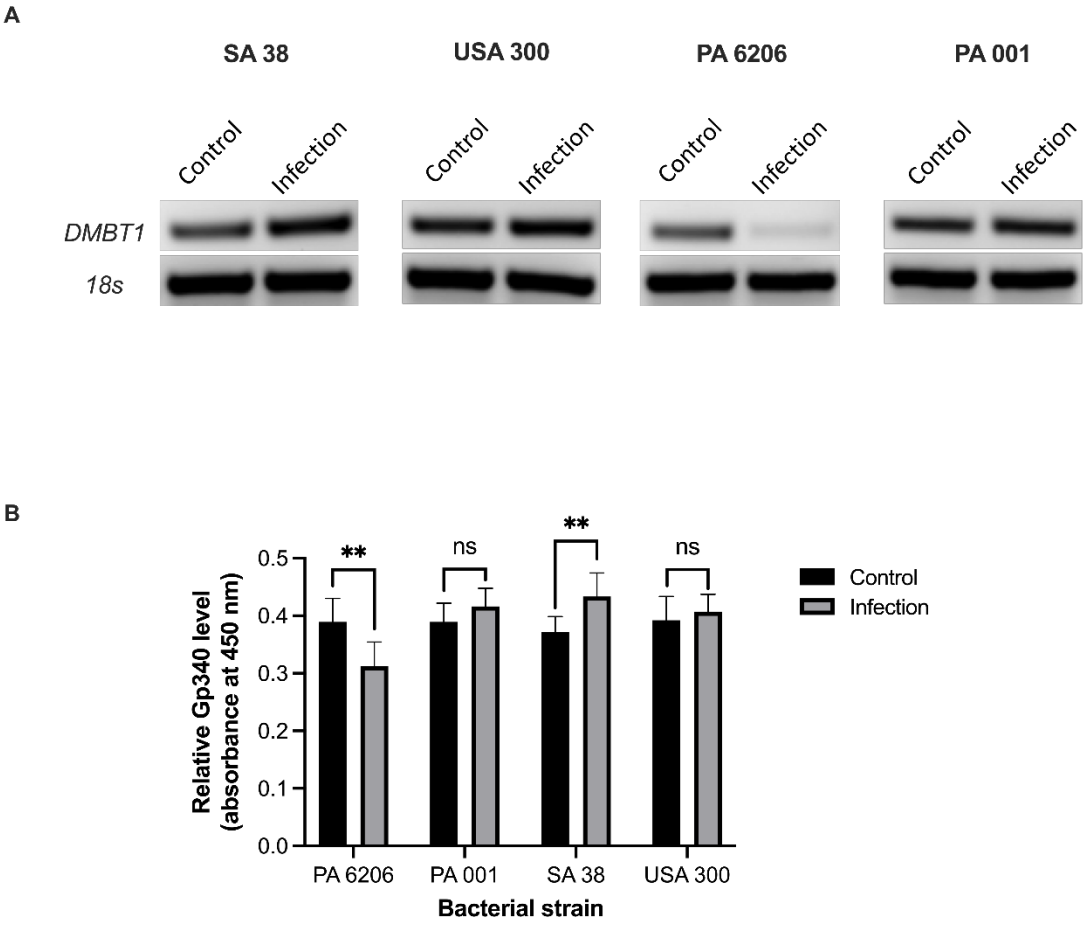

Figure 1. Determination of Gp340 mRNA and protein in HCECs infected with bacteria. Real-time RT-PCR results were confirmed by performing qualitative RT-PCR **(A)**. Gp340 protein expression was relatively quantitated by sandwich ELISA **(B)**. Gp340 mRNA transcription was upregulated in HCECs exposed to SA 38 infection and downregulated in HCECs infected with PA 6206 **(A)**. At protein level, Gp340 expression was upregulated and downregulated in HCECs challenged with SA 38 and PA 6206, respectively **(B)**. **$p \leq 0.01$, ns: $p > 0.05$. Error bars represent standard deviation.

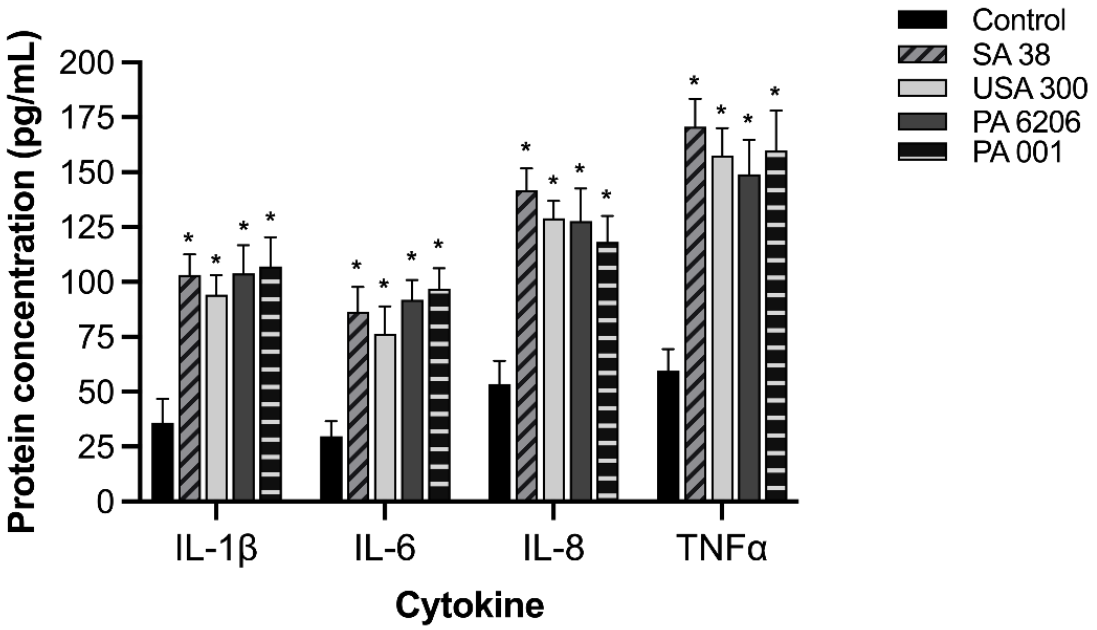

Figure 2. Determination of proinflammatory cytokine expression in HCECs infected with bacteria. The levels of IL-1β, IL-6, IL-8, and TNFα in the bacteria-treated and control HCECs were assayed using sandwich ELISA. The expressions of all the cytokines were upregulated with all four bacterial strains. *$p < 0.0001$. Error bars represent standard deviation.

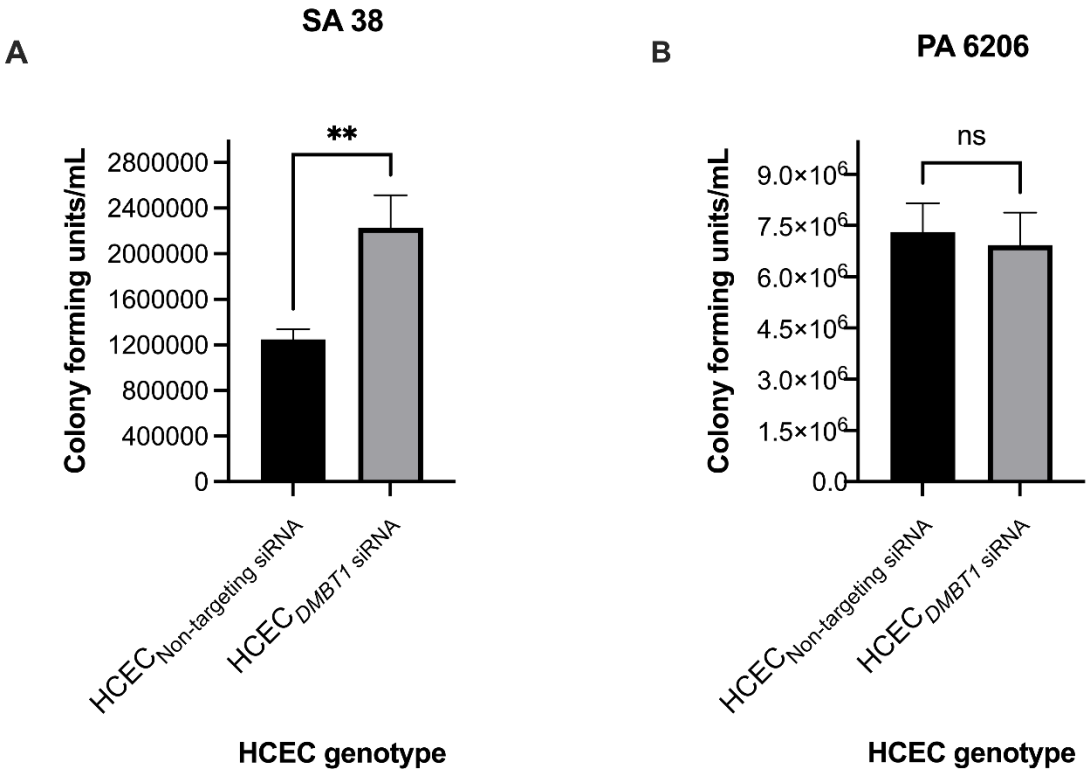

Figure 3. Determination of the effect of Gp340 expression on SA 38 and PA6206 adhesion to HCECs. HCECs were transfected with either *DMBT1*-targeting siRNA or non-targeting siRNA and subsequently infected with SA 38 and PA 6206. Bound bacteria were detached and plated on agar plates and the resulting colony forming units were enumerated. *DMBT1* gene knockdown resulted in a significantly higher SA 38 adhesion but had no effect on PA 6206 adhesion. **$p \leq 0.01$, ns: $p > 0.05$. Error bars represent standard error.

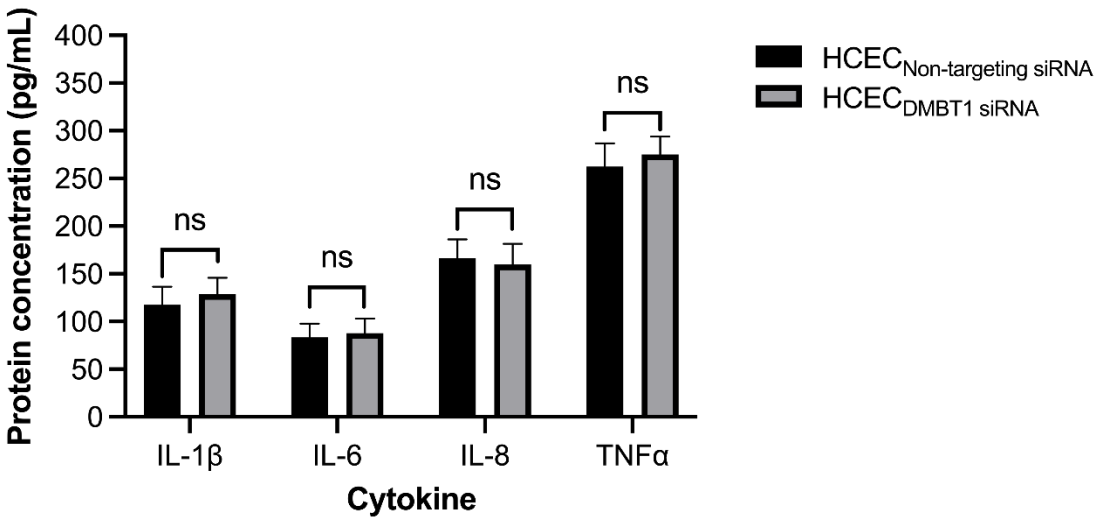

Figure 4. Examining the effect of *DMBT1* gene transcription on proinflammatory cytokine protein expression in HCECs infected with SA 38. HCECs were transfected with either *DMBT1*-targeting siRNA or non-targeting siRNA and subsequently infected with SA 38. Sandwich ELISA was used to quantitate IL-1β, IL-6, IL-8, and TNFα expression in the cells. *DMBT1* gene knockdown had no significant effect on the expression of all four cytokines. ns: $p > 0.05$. Error bars represent standard deviation.

CHAPTER 6 – SUMMARY AND CONCLUSIONS

Gp340, a pattern recognition receptor (PRR) is expressed/secreted in biological fluids and mucosal tissues with wet-surfaced epithelia.[99,101,102,105,122,123,139] In the eye, Gp340 is expressed in the precorneal tear film, lacrimal gland secretory acini and the epithelia of conjunctiva and the cornea.[100,106,113,114,127,136] PRRs play a crucial role in inflammatory response through the following sequence of events – (1) detection of DAMPs released by stressed, injured, apoptosed, or necrotic tissues, or PAMPs on surfaces of invading pathogens such as bacteria, fungi, and virus; (2) signaling downstream expression of inflammatory gene transcription factors and (3) subsequent expression of proinflammatory cytokines and chemokines as an immune response to protect the tissues.[2,88,99]

Dry eye is an ocular surface disease, characterized by tear hyperosmolarity and chronic inflammation that impair the ocular surface.[1,2,140,141] In dry eye, PRRs have been shown to play a role in the inflammatory process.[2,75,97,98] While previous studies have shown that Gp340 in the tear film is dysregulated in dry eye,[113,114] the mechanism underlying this modulation has yet to be elucidated. Understanding the mechanism that underscore Gp340's regulation in dry eye and its potential role in dry eye inflammation could lead to it being targeted in both the diagnosis and therapeutic management of dry eye.

Gp340 can have beneficial or infection-promoting effects based on the form in which it exists.[99,102,105,122,123,135,136] In its soluble/fluid-phase form, it inhibits infection

while in the immobilized/surface-adsorbed conformation, it promotes infection.[101,102,105,122,123,135,136,139] Tear Gp340 adsorbs/binds to worn contact lens polymers.[127] Thus, it was imperative to investigate the potential role of Gp340 in bacterial adhesion to contact lens as it could potentially lead to the design of small molecules to inhibit the adhesion and reduce the risk of contact lens adverse events. Given that Gp340, in its fluid-phase conformation confers innate protection against infection,[122,123,135,136] this dissertation also examined the expression of Gp340 in in vitro corneal infection and established its role in bacterial adhesion to corneal epithelial cells.

Specific Aim 1: Gp340 and Dry Eye

Chapter 3 of the dissertation profiled Gp340 expression in human corneal epithelial cells and elucidated its role in dry eye inflammatory signaling. This study confirmed that hyperosmolar stress upregulates Gp340 mRNA transcription and protein expression in human corneal epithelial cells, while also observing an increased production of three proinflammatory cytokines that are associated with dry eye – IL-1β, IL-8, and TNFα.[2,117,142] Since Gp340 is a PRR, the increased expression of Gp340 was expected to stimulate inflammation. To ascertain this, Gp340 mRNA was transiently knocked down and the effects on IL-1β, IL-8, and TNFα were determined. However, contrary to the hypothesis, reducing Gp340 expression in the corneal epithelial cells did not significantly affect the transcription and translation of these selected proinflammatory cytokines. This suggests that Gp340 and the proinflammatory cytokine expression in the corneal epithelium are independently regulated under hyperosmolar stress and further implying that Gp340 does not play a direct role in the inflammatory response in dry eye. An

implication of these studies is that the overexpression of Gp340 in hyperosmolar stress can be exploited as a potential biomarker in the diagnosis of dry eye. Another study that investigated inflammation in intestinal cells reported a cross-talk between Gp340 and two PRRs, TLR4 and NOD2.[133] These PRRs can trigger downstream mucosal inflammatory response. Thus, although Gp340 did not directly initiate the proinflammatory cytokine expression, it is possible the other PRRs such as NOD2 and TLR4 did. Therefore, future studies must investigate Gp340's role in dry eye inflammation with PRRs such as TLR4 and NOD2.

Among the three ocular surface tissues that are directly involved in the pathophysiology of dry eye and maintenance of ocular surface homeostasis; lacrimal gland, conjunctiva, and cornea,[2,73,75,98,142] the cornea has the least level of Gp340 mRNA transcription.[114] Replicating these studies with the lacrimal gland and conjunctival epithelial cells would therefore help provide a more complete picture of Gp340 regulation on the ocular surface in relation to dry eye-induced hyperosmolar stress. One aspect that was not assessed in the two previous studies that reported Gp340 dysregulation in the tears of dry eye patients[113,114] is tear osmolarity. The results of the two studies,[113,114] therefore, could not be directly compared with this current study. Future studies involving patients from all the different dry eye categories and with different levels of tear hyperosmolarity are warranted as they will provide more insight on the correlation between tear hyperosmolarity and tear Gp340 expression.

While not a direct focus of this study, this dissertation also presented an investigation on the expression of complement activating proteins in human corneal epithelial cells exposed to hyperosmolar stress. The observed upregulation of C1q, the

initiating molecule of the classical complement pathway,[143,144] is novel, and thus lays the ground for future studies into the role of complement system, particularly, the classical pathway, in the pathophysiology of dry eye.

Specific Aim 2: Gp340 and Contact-lens Related Bacteria Adhesion

The adsorption of tear film proteins on contact lens is linked to adverse contact lens events such as enhanced bacterial adhesion.[126,145] Given that Gp340 is among the proteins that adsorb on worn contact lens,[127] and its association with mucosal surfaces promotes infection-promoting processes,[101,102,105,123,146] Chapter 4 of the dissertation investigated the effect of Gp340 on *P. aeruginosa* and *S. aureus* on etafilcon A and lotrafilcon B. The overall hypothesis was that Gp340 adsorption on these lens polymers promotes bacterial adhesion. Through in vitro studies using recombinant SRCR domain of Gp340, $i\text{SRCR}_1^{\text{Gp340}}$,[138] it has been established that Gp340 enhances bacterial *P. aeruginosa* and *S. aureus* adhesion to the lens materials investigated.

The Chapter went further to establish that the cell wall-anchored (CWA) protein, SraP,[147-149] is a mediator of *S. aureus* adhesion to Gp340-bound contact lens. More importantly, it was shown that SraP in concert with other CWA proteins drives *S. aureus* adhesion to tear-conditioned (patient-worn) contact lens. Based on these results, small molecules targeting SraP and other adhesion-promoting CWA proteins such as SasX, SdrC, FnBPA, FnBPB, and SasG[148] can be designed to inhibit *S. aureus* adhesion to contact lens and reduce the risk of contact lens-related microbial infections.

While this study was able to focus on SraP/SasA and enumerate its role, future studies must focus on determining other surface factors on *S. aureus* that mediate

interaction with Gp340. Finally, similar studies must be initiated to identify the factors that are involved in the interactions between *P. aeruginosa* and Gp340. These could begin with known surface factors on *P. aeruginosa* such as minor pilins, FimU, PilV, PilW, PilX and PilE.[150]

Specific Aim 3: Gp340 Expression and Corneal Infection

Gp340's effect or role in infection is dual-phased in which it promotes infection-related processes when it associates with a surface and inhibits infection when in the fluid-phase.[122,123,135,136] Chapter 5 of this dissertation therefore profiled Gp340 mRNA transcription and protein expression in human corneal epithelial cells infected with strains of *P. aeruginosa* (PA 6206, PA 001) and *S. aureus* (SA 38, USA 300) that are known to cause microbial keratitis[145,151-153]. While USA 300 and PA 001 infection had no effect on Gp340 expression, infection with SA 38 and PA 6206 upregulated and downregulated Gp340 expression respectively, indicating that Gp340 expression in corneal infection is bacteria strain-dependent.

Given that Gp340 in the soluble phase is protective against infection,[105,122] it was hypothesized that bacterial adhesion to corneal epithelial cells with reduced Gp340 expression would be higher than adhesion to cells with normal Gp340 expression. To test this hypothesis, corneal epithelial cells transfected with either Gp340 siRNA (to knockdown/reduce Gp340 expression) or non-targeting siRNA were infected with SA 38 and PA 6206 and the amounts of adherent bacteria were compared between the transfecting siRNAs. The studies, however showed that the hypothesis can only be true for the gram-positive SA 38 since unlike the gram-negative PA 6206, the adhesion of SA 38 to cells

with knocked-down Gp340 gene transcription was significantly higher relative to the control cells. The observed increased SA 38 adhesion on corneal epithelial cells with downregulated Gp340 expression is consistent with the protective effect of Gp340 in the fluid-phase. It is worth noting that the infection model used in this study was non-contact lens-related. This means that any translated Gp340 will exist in the soluble conformation and suggests that in the absence of contact lens wear, tear Gp340 can be inhibit corneal infection caused by certain bacterial strains.

In summary, Chapter 5 has shown that Gp340's pattern of expression and anti-microbial effect in corneal infection depends on the type of infectious agent. Specifically, this study has shown that *S. aureus* 38 increased Gp340 expression, whereas *P. aeruginosa* 6206 downregulated it. More studies are required to determined why infective agents elicit such differential expression of Gp340, and more importantly, the factors that trigger such a response from the host. Finally, the beneficial role of Gp340 must be validated using in vivo infection models.

Conclusions

In conclusion, this dissertation has shown that: (a) hyperosmolar stress upregulates Gp340 along with the inflammatory cytokines, IL-1β, IL-8, and TNFα, and it is yet to be determined if Gp340 acts in concert with Toll-like and NOD-like receptors in triggering the expression of these cytokines; (b) Gp340/SRCR domain enhances bacterial adherence to contact lens polymers, and specifically, SraP of *S. aureus* mediates this interaction along with other yet-to-be determined surface proteins; and (c) Gp340 demonstrates a beneficial effects in an in vitro corneal infection model by reducing bacterial adherence to corneal

epithelial cells. In essence, the role of Gp340 in both infection and inflammation is intriguing and complex and must be further explored in future studies.

GENERAL LIST OF REFERENCES

1. Craig JP, Nichols KK, Akpek EK, et al. TFOS DEWS II Definition and Classification Report. *Ocul Surf.* 2017;15(3):276-283.
2. Bron AJ, de Paiva CS, Chauhan SK, et al. TFOS DEWS II pathophysiology report. *Ocul Surf.* 2017;15(3):438-510.
3. Cwiklik L. Tear film lipid layer: A molecular level view. *Biochim Biophys Acta.* 2016;1858(10):2421-2430.
4. Botelho SY, Hisada M, Fuenmayor N. Functional innervation of the lacrimal gland in the cat. Origin of secretomotor fibers in the lacrimal nerve. *Arch Ophthalmol.* 1966;76(4):581-588.
5. Sibony PA, Walcott B, McKeon C, Jakobiec FA. Vasoactive intestinal polypeptide and the innervation of the human lacrimal gland. *Arch Ophthalmol.* 1988;106(8):1085-1088.
6. Willcox MDP, Argueso P, Georgiev GA, et al. TFOS DEWS II Tear Film Report. *Ocul Surf.* 2017;15(3):366-403.
7. Botelho SY. Tears and the Lacrimal Gland. *Sci Am.* 1964;211:78-86.
8. Brown SH, Kunnen CM, Duchoslav E, et al. A comparison of patient matched meibum and tear lipidomes. *Invest Ophthalmol Vis Sci.* 2013;54(12):7417-7424.
9. Pucker AD, Nichols JJ. Analysis of meibum and tear lipids. *Ocul Surf.* 2012;10(4):230-250.

10. Gipson IK. Goblet cells of the conjunctiva: A review of recent findings. *Prog Retin Eye Res.* 2016;54:49-63.

11. Rodrigues MM, Rowden G, Hackett J, Bakos I. Langerhans cells in the normal conjunctiva and peripheral cornea of selected species. *Invest Ophthalmol Vis Sci.* 1981;21(5):759-765.

12. Levin MH, Verkman AS. Aquaporin-dependent water permeation at the mouse ocular surface: in vivo microfluorimetric measurements in cornea and conjunctiva. *Invest Ophthalmol Vis Sci.* 2004;45(12):4423-4432.

13. Schey KL, Wang Z, J LW, Qi Y. Aquaporins in the eye: expression, function, and roles in ocular disease. *Biochim Biophys Acta.* 2014;1840(5):1513-1523.

14. Gordon YJ, Romanowski EG, McDermott AM. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr Eye Res.* 2005;30(7):505-515.

15. Sridhar MS. Anatomy of cornea and ocular surface. *Indian J Ophthalmol.* 2018;66(2):190-194.

16. Rufer F, Schroder A, Erb C. White-to-white corneal diameter: normal values in healthy humans obtained with the Orbscan II topography system. *Cornea.* 2005;24(3):259-261.

17. DelMonte DW, Kim T. Anatomy and physiology of the cornea. *J Cataract Refract Surg.* 2011;37(3):588-598.

18. Eghrari AO, Riazuddin SA, Gottsch JD. Overview of the Cornea: Structure, Function, and Development. *Prog Mol Biol Transl Sci.* 2015;134:7-23.

19. Jacobsen IE, Jensen OA, Prause JU. Structure and composition of Bowman's membrane. Study by frozen resin cracking. *Acta Ophthalmol (Copenh).* 1984;62(1):39-53.

20. Murphy C, Alvarado J, Juster R. Prenatal and postnatal growth of the human Descemet's membrane. *Invest Ophthalmol Vis Sci.* 1984;25(12):1402-1415.

21. Yee RW, Matsuda M, Schultz RO, Edelhauser HF. Changes in the normal corneal endothelial cellular pattern as a function of age. *Curr Eye Res.* 1985;4(6):671-678.

22. Stiemke MM, Edelhauser HF, Geroski DH. The developing corneal endothelium: correlation of morphology, hydration and Na/K ATPase pump site density. *Curr Eye Res.* 1991;10(2):145-156.

23. Geroski DH, Matsuda M, Yee RW, Edelhauser HF. Pump function of the human corneal endothelium. Effects of age and cornea guttata. *Ophthalmology.* 1985;92(6):759-763.

24. Van den Bogerd B, Dhubhghaill SN, Koppen C, Tassignon MJ, Zakaria N. A review of the evidence for in vivo corneal endothelial regeneration. *Surv Ophthalmol.* 2018;63(2):149-165.

25. Srinivas SP. Dynamic regulation of barrier integrity of the corneal endothelium. *Optom Vis Sci.* 2010;87(4):E239-254.

26. Lemp MA. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. *CLAO J.* 1995;21(4):221-232.

27. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007). *Ocul Surf.* 2007;5(2):75-92.

28. Pflugfelder SC. Differential diagnosis of dry eye conditions. *Adv Dent Res.* 1996;10(1):9-12.

29. Mathers WD, Lane JA. Meibomian gland lipids, evaporation, and tear film stability. *Adv Exp Med Biol.* 1998;438:349-360.

30. Roberts DK. Keratoconjunctivitis sicca. *J Am Optom Assoc.* 1991;62(3):187-199.

31. Pflugfelder SC. Advances in the diagnosis and management of keratoconjunctivitis sicca. *Curr Opin Ophthalmol.* 1998;9(4):50-53.

32. Stapleton F, Alves M, Bunya VY, et al. TFOS DEWS II Epidemiology Report. *Ocul Surf.* 2017;15(3):334-365.

33. Shimazaki J, Goto E, Ono M, Shimmura S, Tsubota K. Meibomian gland dysfunction in patients with Sjogren syndrome. *Ophthalmology.* 1998;105(8):1485-1488.

34. The epidemiology of dry eye disease: report of the Epidemiology Subcommittee of the International Dry Eye WorkShop (2007). *Ocul Surf.* 2007;5(2):93-107.

35. Lu P, Chen X, Liu X, et al. Dry eye syndrome in elderly Tibetans at high altitude: a population-based study in China. *Cornea.* 2008;27(5):545-551.

36. Jie Y, Xu L, Wu YY, Jonas JB. Prevalence of dry eye among adult Chinese in the Beijing Eye Study. *Eye (Lond).* 2009;23(3):688-693.

37. Viso E, Rodriguez-Ares MT, Gude F. Prevalence of and associated factors for dry eye in a Spanish adult population (the Salnes Eye Study). *Ophthalmic Epidemiol.* 2009;16(1):15-21.

38. Tian YJ, Liu Y, Zou HD, et al. [Epidemiologic study of dry eye in populations equal or over 20 years old in Jiangning District of Shanghai]. *Zhonghua Yan Ke Za Zhi.* 2009;45(6):486-491.

39. Guo B, Lu P, Chen X, Zhang W, Chen R. Prevalence of dry eye disease in Mongolians at high altitude in China: the Henan eye study. *Ophthalmic Epidemiol.* 2010;17(4):234-241.

40. Han SB, Hyon JY, Woo SJ, Lee JJ, Kim TH, Kim KW. Prevalence of dry eye disease in an elderly Korean population. *Arch Ophthalmol.* 2011;129(5):633-638.

41. Viso E, Rodriguez-Ares MT, Abelenda D, Oubina B, Gude F. Prevalence of asymptomatic and symptomatic meibomian gland dysfunction in the general population of Spain. *Invest Ophthalmol Vis Sci.* 2012;53(6):2601-2606.

42. Siak JJ, Tong L, Wong WL, et al. Prevalence and risk factors of meibomian gland dysfunction: the Singapore Malay eye study. *Cornea.* 2012;31(11):1223-1228.

43. Paulsen AJ, Cruickshanks KJ, Fischer ME, et al. Dry eye in the beaver dam offspring study: prevalence, risk factors, and health-related quality of life. *Am J Ophthalmol.* 2014;157(4):799-806.

44. Vehof J, Kozareva D, Hysi PG, Hammond CJ. Prevalence and risk factors of dry eye disease in a British female cohort. *Br J Ophthalmol.* 2014;98(12):1712-1717.

45. Malet F, Le Goff M, Colin J, et al. Dry eye disease in French elderly subjects: the Alienor Study. *Acta Ophthalmol.* 2014;92(6):e429-436.

46. Hashemi H, Khabazkhoob M, Kheirkhah A, et al. Prevalence of dry eye syndrome in an adult population. *Clin Exp Ophthalmol.* 2014;42(3):242-248.

47. Tan LL, Morgan P, Cai ZQ, Straughan RA. Prevalence of and risk factors for symptomatic dry eye disease in Singapore. *Clin Exp Optom.* 2015;98(1):45-53.

48. Asiedu K, Kyei S, Boampong F, Ocansey S. Symptomatic Dry Eye and Its Associated Factors: A Study of University Undergraduate Students in Ghana. *Eye Contact Lens.* 2017;43(4):262-266.

49. Kobia-Acquah E, Ankamah-Lomotey S, Owusu E, et al. Prevalence and associated risk factors of symptomatic dry eye in Ghana: A cross-sectional population-based study. *Cont Lens Anterior Eye.* 2021:101404.

50. Uchino M, Dogru M, Yagi Y, et al. The features of dry eye disease in a Japanese elderly population. *Optom Vis Sci.* 2006;83(11):797-802.

51. Nichols KK, Foulks GN, Bron AJ, et al. The international workshop on meibomian gland dysfunction: executive summary. *Invest Ophthalmol Vis Sci.* 2011;52(4):1922-1929.

52. Gilbard JP, Rossi SR. Changes in tear ion concentrations in dry-eye disorders. *Adv Exp Med Biol.* 1994;350:529-533.

53. Lemp MA, Bron AJ, Baudouin C, et al. Tear osmolarity in the diagnosis and management of dry eye disease. *Am J Ophthalmol.* 2011;151(5):792-798 e791.

54. Nichols JJ, Mitchell GL, King-Smith PE. Thinning rate of the precorneal and prelens tear films. *Invest Ophthalmol Vis Sci.* 2005;46(7):2353-2361.

55. Tomlinson A, Khanal S, Ramaesh K, Diaper C, McFadyen A. Tear film osmolarity: determination of a referent for dry eye diagnosis. *Invest Ophthalmol Vis Sci.* 2006;47(10):4309-4315.

56. Sullivan BD, Whitmer D, Nichols KK, et al. An objective approach to dry eye disease severity. *Invest Ophthalmol Vis Sci.* 2010;51(12):6125-6130.

57. Gaffney EA, Tiffany JM, Yokoi N, Bron AJ. A mass and solute balance model for tear volume and osmolarity in the normal and the dry eye. *Prog Retin Eye Res.* 2010;29(1):59-78.

58. Harrison WW, Begley CG, Liu H, Chen M, Garcia M, Smith JA. Menisci and fullness of the blink in dry eye. *Optom Vis Sci.* 2008;85(8):706-714.

59. Begley CG, Himebaugh N, Renner D, et al. Tear breakup dynamics: a technique for quantifying tear film instability. *Optom Vis Sci.* 2006;83(1):15-21.

60. Liu H, Begley CG, Chalmers R, Wilson G, Srinivas SP, Wilkinson JA. Temporal progression and spatial repeatability of tear breakup. *Optom Vis Sci.* 2006;83(10):723-730.

61. Liu H, Begley C, Chen M, et al. A link between tear instability and hyperosmolarity in dry eye. *Invest Ophthalmol Vis Sci.* 2009;50(8):3671-3679.

62. Norn MS. Desiccation of the precorneal film. I. Corneal wetting-time. *Acta Ophthalmol (Copenh).* 1969;47(4):865-880.

63. Stevenson W, Chauhan SK, Dana R. Dry eye disease: an immune-mediated ocular surface disorder. *Arch Ophthalmol.* 2012;130(1):90-100.

64. Schaumburg CS, Siemasko KF, De Paiva CS, et al. Ocular surface APCs are necessary for autoreactive T cell-mediated experimental autoimmune lacrimal keratoconjunctivitis. *J Immunol.* 2011;187(7):3653-3662.

65. Luo L, Li DQ, Doshi A, Farley W, Corrales RM, Pflugfelder SC. Experimental dry eye stimulates production of inflammatory cytokines and MMP-9 and activates

MAPK signaling pathways on the ocular surface. *Invest Ophthalmol Vis Sci.* 2004;45(12):4293-4301.

66. Lan W, Petznick A, Heryati S, Rifada M, Tong L. Nuclear Factor-kappaB: central regulator in ocular surface inflammation and diseases. *Ocul Surf.* 2012;10(3):137-148.

67. Sobrin L, Liu Z, Monroy DC, et al. Regulation of MMP-9 activity in human tear fluid and corneal epithelial culture supernatant. *Invest Ophthalmol Vis Sci.* 2000;41(7):1703-1709.

68. Pflugfelder SC, Farley W, Luo L, et al. Matrix metalloproteinase-9 knockout confers resistance to corneal epithelial barrier disruption in experimental dry eye. *Am J Pathol.* 2005;166(1):61-71.

69. Chotikavanich S, de Paiva CS, Li de Q, et al. Production and activity of matrix metalloproteinase-9 on the ocular surface increase in dysfunctional tear syndrome. *Invest Ophthalmol Vis Sci.* 2009;50(7):3203-3209.

70. Pflugfelder SC, de Paiva CS. The Pathophysiology of Dry Eye Disease: What We Know and Future Directions for Research. *Ophthalmology.* 2017;124(11S):S4-S13.

71. Redfern RL, McDermott AM. Toll-like receptors in ocular surface disease. *Exp Eye Res.* 2010;90(6):679-687.

72. Redfern RL, Patel N, Hanlon S, et al. Toll-like receptor expression and activation in mice with experimental dry eye. *Invest Ophthalmol Vis Sci.* 2013;54(2):1554-1563.

73. Redfern RL, Barabino S, Baxter J, Lema C, McDermott AM. Dry eye modulates the expression of toll-like receptors on the ocular surface. *Exp Eye Res.* 2015;134:80-89.

74. He C, Lai P, Weng J, et al. Toll-like receptor 2-mediated NF-kappaB inflammatory responses in dry eye associated with cGVHD. *Mol Vis.* 2011;17:2605-2611.

75. Lee HS, Hattori T, Park EY, Stevenson W, Chauhan SK, Dana R. Expression of toll-like receptor 4 contributes to corneal inflammation in experimental dry eye disease. *Invest Ophthalmol Vis Sci.* 2012;53(9):5632-5640.

76. Narayanan S, Redfern RL, Miller WL, Nichols KK, McDermott AM. Dry eye disease and microbial keratitis: is there a connection? *Ocul Surf.* 2013;11(2):75-92.

77. Jhanji V, Constantinou M, Taylor HR, Vajpayee RB. Microbiological and clinical profiles of patients with microbial keratitis residing in nursing homes. *Br J Ophthalmol.* 2009;93(12):1639-1642.

78. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol.* 2011;335(1):2-13.

79. Abdelfattah MM, Khattab RA, Mahran MH, Elborgy ES. Evaluation of patients with dry eye disease for conjunctival Chlamydia trachomatis and Ureaplasma urealyticum. *Int J Ophthalmol.* 2016;9(10):1457-1465.

80. Krasny J, Hruba D, Netukova M, Kodat V, Tomasova BJ. [Chlamydia pneumoniae in the etiology of the keratoconjunctivitis sicca in adult patients (a pilot study)]. *Cesk Slov Oftalmol.* 2009;65(3):102-106.

81. Chaplin DD. Overview of the immune response. *J Allergy Clin Immunol.* 2010;125(2 Suppl 2):S3-23.

82. Amarante-Mendes GP, Adjemian S, Branco LM, Zanetti LC, Weinlich R, Bortoluci KR. Pattern Recognition Receptors and the Host Cell Death Molecular Machinery. *Front Immunol.* 2018;9:2379.

83. Suresh R, Mosser DM. Pattern recognition receptors in innate immunity, host defense, and immunopathology. *Adv Physiol Educ.* 2013;37(4):284-291.

84. Jang JH, Shin HW, Lee JM, Lee HW, Kim EC, Park SH. An Overview of Pathogen Recognition Receptors for Innate Immunity in Dental Pulp. *Mediators Inflamm.* 2015;2015:794143.

85. Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol.* 2002;20:197-216.

86. Srikrishna G, Freeze HH. Endogenous damage-associated molecular pattern molecules at the crossroads of inflammation and cancer. *Neoplasia.* 2009;11(7):615-628.

87. Roh JS, Sohn DH. Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune Netw.* 2018;18(4):e27.

88. Gong T, Liu L, Jiang W, Zhou R. DAMP-sensing receptors in sterile inflammation and inflammatory diseases. *Nat Rev Immunol.* 2020;20(2):95-112.

89. Lema C, Reins RY, Redfern RL. High-Mobility Group Box 1 in Dry Eye Inflammation. *Invest Ophthalmol Vis Sci.* 2018;59(5):1741-1750.

90. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell.* 2010;140(6):805-820.

91. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. *Front Immunol.* 2014;5:461.

92. Yamamoto M, Takeda K. Current views of toll-like receptor signaling pathways. *Gastroenterol Res Pract.* 2010;2010:240365.

93. Spachidou MP, Bourazopoulou E, Maratheftis CI, et al. Expression of functional Toll-like receptors by salivary gland epithelial cells: increased mRNA expression in cells derived from patients with primary Sjogren's syndrome. *Clin Exp Immunol.* 2007;147(3):497-503.

94. Kawakami A, Nakashima K, Tamai M, et al. Toll-like receptor in salivary glands from patients with Sjogren's syndrome: functional analysis by human salivary gland cell line. *J Rheumatol.* 2007;34(5):1019-1026.

95. Franchi L, Warner N, Viani K, Nunez G. Function of Nod-like receptors in microbial recognition and host defense. *Immunol Rev.* 2009;227(1):106-128.

96. Niu L, Zhang S, Wu J, Chen L, Wang Y. Upregulation of NLRP3 Inflammasome in the Tears and Ocular Surface of Dry Eye Patients. *PLoS One.* 2015;10(5):e0126277.

97. Kim YH, Li Z, Cui L, et al. Expression of Nod-like Receptors and Clinical Correlations in Patients With Dry Eye Disease. *Am J Ophthalmol.* 2019;200:150-160.

98. Li Y, Jin R, Li L, et al. Expression and Role of Nucleotide-Binding Oligomerization Domain 2 (NOD2) in the Ocular Surface of Murine Dry Eye. *Invest Ophthalmol Vis Sci.* 2019;60(7):2641-2649.

99. Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol.* 2006;6(1):33-43.

100. Jumblatt MM, Imbert Y, Young WW, Jr., Foulks GN, Steele PS, Demuth DR. Glycoprotein 340 in normal human ocular surface tissues and tear film. *Infect Immun.* 2006;74(7):4058-4063.

101. Stoddard E, Cannon G, Ni H, et al. gp340 expressed on human genital epithelia binds HIV-1 envelope protein and facilitates viral transmission. *J Immunol.* 2007;179(5):3126-3132.

102. Stoddard E, Ni H, Cannon G, et al. gp340 promotes transcytosis of human immunodeficiency virus type 1 in genital tract-derived cell lines and primary endocervical tissue. *J Virol.* 2009;83(17):8596-8603.

103. Bathum Nexoe A, Pedersen AA, von Huth S, Detlefsen S, Hansen PL, Holmskov U. Immunohistochemical Localization of Deleted in Malignant Brain Tumors 1 in Normal Human Tissues. *J Histochem Cytochem.* 2020;68(6):377-387.

104. Ronellenfitsch S, Weiss C, Frommhold D, et al. High DMBT1 concentrations in breast milk correlate with increased risk of infection in preterm and term neonates. *BMC Pediatr.* 2012;12:157.

105. Osei KA, Deivanayagam C, Nichols JJ. Glycoprotein 340 in mucosal immunity and ocular surface. *Ocul Surf.* 2018;16(3):282-288.

106. Schulz BL, Oxley D, Packer NH, Karlsson NG. Identification of two highly sialylated human tear-fluid DMBT1 isoforms: the major high-molecular-mass glycoproteins in human tears. *Biochem J.* 2002;366(Pt 2):511-520.

107. Boks MA, Gunput ST, Kosten I, et al. The Human Glycoprotein Salivary Agglutinin Inhibits the Interaction of DC-SIGN and Langerin with Oral Micro-Organisms. *J Innate Immun.* 2016;8(4):350-361.

108. Green-Church KB, Nichols KK, Kleinholz NM, Zhang L, Nichols JJ. Investigation of the human tear film proteome using multiple proteomic approaches. *Mol Vis.* 2008;14:456-470.

109. Oho T, Yu H, Yamashita Y, Koga T. Binding of salivary glycoprotein-secretory immunoglobulin A complex to the surface protein antigen of Streptococcus mutans. *Infect Immun.* 1998;66(1):115-121.

110. Holmskov U, Mollenhauer J, Madsen J, et al. Cloning of gp-340, a putative opsonin receptor for lung surfactant protein D. *Proc Natl Acad Sci U S A.* 1999;96(19):10794-10799.

111. Renner M, Bergmann G, Krebs I, et al. DMBT1 confers mucosal protection in vivo and a deletion variant is associated with Crohn's disease. *Gastroenterology.* 2007;133(5):1499-1509.

112. Liu Z, Kim J, Sypek JP, et al. Gene expression profiles in human nasal polyp tissues studied by means of DNA microarray. *J Allergy Clin Immunol.* 2004;114(4):783-790.

113. Nichols JJ, Green-Church KB. Mass spectrometry-based proteomic analyses in contact lens-related dry eye. *Cornea.* 2009;28(10):1109-1117.

114. Perumal N, Funke S, Pfeiffer N, Grus FH. Proteomics analysis of human tears from aqueous-deficient and evaporative dry eye patients. *Sci Rep.* 2016;6:29629.

115. Mann A, Tighe B. Contact lens interactions with the tear film. *Exp Eye Res.* 2013;117:88-98.

116. Thakur A, Willcox MD. Contact lens wear alters the production of certain inflammatory mediators in tears. *Exp Eye Res.* 2000;70(3):255-259.

117. Ramamoorthy P, Khanal S, J JN. Inflammatory proteins associated with contact lens-related dry eye. *Cont Lens Anterior Eye.* 2021:101442.

118. Giannaccare G, Blalock W, Fresina M, Vagge A, Versura P. Intolerant contact lens wearers exhibit ocular surface impairment despite 3 months wear discontinuation. *Graefes Arch Clin Exp Ophthalmol.* 2016;254(9):1825-1831.

119. Jansen ME, Begley CG, Himebaugh NH, Port NL. Effect of contact lens wear and a near task on tear film break-up. *Optom Vis Sci.* 2010;87(5):350-357.

120. Farias E, Yasunaga KL, Peixoto RVR, Fonseca MP, Fontes W, Galera PD. Comparison of two methods of tear sampling for protein quantification by Bradford method. *Pesqui Vet Bras.* 2013;33(2):4.

121. Ablamowicz AF, Nichols JJ. Concentrations of MUC16 and MUC5AC using three tear collection methods. *Mol Vis.* 2017;23:529-537.

122. Loimaranta V, Jakubovics NS, Hytonen J, Finne J, Jenkinson HF, Stromberg N. Fluid- or surface-phase human salivary scavenger protein gp340 exposes different bacterial recognition properties. *Infect Immun.* 2005;73(4):2245-2252.

123. Madsen J, Mollenhauer J, Holmskov U. Review: Gp-340/DMBT1 in mucosal innate immunity. *Innate Immun.* 2010;16(3):160-167.

124. Wu P, Stapleton F, Willcox MD. The causes of and cures for contact lens-induced peripheral ulcer. *Eye Contact Lens.* 2003;29(1 Suppl):S63-66; discussion S83-64, S192-194.

125. Willcox MD, Holden BA. Contact lens related corneal infections. *Biosci Rep.* 2001;21(4):445-461.

126. Dutta D, Cole N, Willcox M. Factors influencing bacterial adhesion to contact lenses. *Mol Vis.* 2012;18:14-21.

127. Zhao Z, Wei X, Aliwarga Y, Carnt NA, Garrett Q, Willcox MD. Proteomic analysis of protein deposits on worn daily wear silicone hydrogel contact lenses. *Mol Vis.* 2008;14:2016-2024.

128. Nichols KK, Bacharach J, Holland E, et al. Impact of Dry Eye Disease on Work Productivity, and Patients' Satisfaction With Over-the-Counter Dry Eye Treatments. *Invest Ophthalmol Vis Sci.* 2016;57(7):2975-2982.

129. Pouyeh B, Viteri E, Feuer W, et al. Impact of ocular surface symptoms on quality of life in a United States veterans affairs population. *Am J Ophthalmol.* 2012;153(6):1061-1066 e1063.

130. Uchino M, Schaumberg DA. Dry Eye Disease: Impact on Quality of Life and Vision. *Curr Ophthalmol Rep.* 2013;1(2):51-57.

131. Yu J, Asche CV, Fairchild CJ. The economic burden of dry eye disease in the United States: a decision tree analysis. *Cornea.* 2011;30(4):379-387.

132. Reins RY, Lema C, Courson J, Kunnen CME, Redfern RL. MyD88 Deficiency Protects Against Dry Eye-Induced Damage. *Invest Ophthalmol Vis Sci.* 2018;59(7):2967-2976.

133. Rosenstiel P, Sina C, End C, et al. Regulation of DMBT1 via NOD2 and TLR4 in intestinal epithelial cells modulates bacterial recognition and invasion. *J Immunol.* 2007;178(12):8203-8211.

134. Robertson DM. The effects of silicone hydrogel lens wear on the corneal epithelium and risk for microbial keratitis. *Eye Contact Lens.* 2013;39(1):67-72.

135. Li J, Metruccio MME, Smith BE, Evans DJ, Fleiszig SMJ. Correction: Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen Pseudomonas aeruginosa. *PLoS Pathog.* 2017;13(9):e1006612.

136. Li J, Metruccio MME, Evans DJ, Fleiszig SMJ. Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen Pseudomonas aeruginosa. *PLoS Pathog.* 2017;13(5):e1006392.

137. Osei KA, Deivanayagam C, Nichols JJ. The effect of glycoprotein 340's scavenger receptor cysteine-rich domain on bacterial adhesion on soft contact lens (abstract). *Invest Ophthalmol Vis Sci.* 2019;60(9):6333.

138. Purushotham S, Deivanayagam C. Cloning, expression and purification of the SRCR domains of glycoprotein 340. *Protein Expr Purif.* 2013;90(2):67-73.

139. Reichhardt MP, Meri S. SALSA: A Regulator of the Early Steps of Complement Activation on Mucosal Surfaces. *Front Immunol.* 2016;7:85.

140. Suzuki M, Massingale ML, Ye F, et al. Tear osmolarity as a biomarker for dry eye disease severity. *Invest Ophthalmol Vis Sci.* 2010;51(9):4557-4561.

141. Clouzeau C, Godefroy D, Riancho L, Rostene W, Baudouin C, Brignole-Baudouin F. Hyperosmolarity potentiates toxic effects of benzalkonium chloride on conjunctival epithelial cells in vitro. *Mol Vis.* 2012;18:851-863.

142. Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA. Analysis of inflammatory cytokines in the tears of dry eye patients. *Cornea.* 2009;28(9):1023-1027.

143. Noris M, Remuzzi G. Overview of complement activation and regulation. *Semin Nephrol.* 2013;33(6):479-492.

144. Boackle RJ, Connor MH, Vesely J. High molecular weight non-immunoglobulin salivary agglutinins (NIA) bind C1Q globular heads and have the potential to activate the first complement component. *Mol Immunol.* 1993;30(3):309-319.

145. Subbaraman LN, Borazjani R, Zhu H, Zhao Z, Jones L, Willcox MD. Influence of protein deposition on bacterial adhesion to contact lenses. *Optom Vis Sci.* 2011;88(8):959-966.

146. Ligtenberg AJ, Karlsson NG, Veerman EC. Deleted in malignant brain tumors-1 protein (DMBT1): a pattern recognition receptor with multiple binding sites. *Int J Mol Sci.* 2010;11(12):5212-5233.

147. Yang YH, Jiang YL, Zhang J, et al. Structural insights into SraP-mediated Staphylococcus aureus adhesion to host cells. *PLoS Pathog.* 2014;10(6):e1004169.

148. Foster TJ. Surface Proteins of Staphylococcus aureus. *Microbiol Spectr.* 2019;7(4).

149. Kukita K, Kawada-Matsuo M, Oho T, et al. Staphylococcus aureus SasA is responsible for binding to the salivary agglutinin gp340, derived from human saliva. *Infect Immun.* 2013;81(6):1870-1879.

150. Giltner CL, Habash M, Burrows LL. Pseudomonas aeruginosa minor pilins are incorporated into type IV pili. *J Mol Biol.* 2010;398(3):444-461.

151. Vijay AK, Zhu H, Ozkan J, et al. Bacterial adhesion to unworn and worn silicone hydrogel lenses. *Optom Vis Sci.* 2012;89(8):1095-1106.

152. David MZ, Daum RS. Community-associated methicillin-resistant Staphylococcus aureus: epidemiology and clinical consequences of an emerging epidemic. *Clin Microbiol Rev.* 2010;23(3):616-687.

153. Peterson JC, Durkee H, Miller D, et al. Molecular epidemiology and resistance profiles among healthcare- and community-associated Staphylococcus aureus keratitis isolates. *Infect Drug Resist.* 2019;12:831-843.