

Model Development for Corneal Inflammation and Biocompatibility

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The ocular surface presents a complex environment where the corneal epithelium has to interact with the tear film, the blink, and the many proteins, lipids, and signaling molecules that exist. As a contact lens is placed into this environment, homeostasis may be unbalanced, and it may affect the overall patient satisfaction with lenses. In order to study this effect at a cellular level, *in vitro* models offer the ability for cost-effective, tailored, and biologically-relevant methods to investigate material biocompatibility with the ocular surface.

The development of an *in vitro* curved, stratified, human corneal epithelium has been instrumental in allowing for a more detailed investigation into biocompatibility at the ocular surface. This advancement was followed by the creation of the tear replenishment system, which has incorporated the dynamic, fluidic effect of blinking and tear exchange, in combination with the *in vitro* epithelial model. While these models have been used to probe cytotoxicity from contact lenses soaked in benzalkonium chloride, contact lens and solution interactions had yet to be studied using these models.

Solution-induced corneal staining (SICS) is a clinical phenomenon that has led to the understanding that different lens and solution combinations interact differentially with the cornea. The first major goal of the thesis was to use *in vitro* models of the corneal epithelium to investigate SICS. It was determined that cell death is likely not responsible for SICS, but cell death may be implicated in disruption of the epithelial barrier which could evoke microbial susceptibility.

While the above models offer an advanced approach for the study of corneal biocompatibility, they lack incorporation of the innate immune system. Before an inflammatory component may be incorporated into an *in vitro* model, an improved understanding of the role of ocular surface immune cells is required. Hundreds of thousands of neutrophils are accumulated on the closed eye following sleep, and these neutrophils show a differential phenotype from blood-isolated neutrophils. The second main goal of the thesis demonstrated that incubation of blood-isolated neutrophils under closed eye conditions is not responsible for the drastic change in inflammatory phenotype.

Ultimately, this work has demonstrated the importance of *in vitro* models for the study of ocular phenomena and there is a great potential for the use of these models to better understand inflammation and biocompatibility of the cornea.

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To my Grandma, my Grandpa, my Baba, and my Uncle Sam

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Chapter 1

Introduction

A little aqueous humour.

Contact lenses are worn by over 140 million people worldwide [1], and while this constitutes a large market of wearers, contact lens adoption has a dropout rate of about 23% due to discomfort, dryness, and overall dissatisfaction with lenses [2]. There has been a lot of research to address this issue, and it may be attributed to lens design, multipurpose solution, or the innate biology that incites discomfort [3]. However, in terms of lenses, there has yet to be a material designed that is perfectly biocompatible: a material that can perform vision correction without inciting discomfort, or correlate to increased infection or inflammation with use. This thesis does not focus on comfort at all, but focuses on understanding the cellular basis of biocompatibility through the use of *in vitro* models. Through a better understanding of biocompatibility and inflammation at the ocular surface, we may in turn be able to design better lenses, which will provide fewer issues with biological integration, and will ultimately improve patient outcomes.

Our eyes provide us with arguably the most important sense of all: vision. As one of the most complex organs in the body, eyes have evolved to collect and transmit incoming light information to the brain. To do so, there are many structures and functions of both tissues and cells in order to preserve the ability of the eye to do its job. The eye is both offensive and defensive towards physical damage, infection, and inflammation. However, the eye is not without weakness: the eye may be subject to disease and infection just like every other organ in the human body. The normal homeostasis of the eye can become easily affected through its interaction with biomaterials and ophthalmic solutions, such as contact lenses and multipurpose disinfecting solutions, respectively. The purpose of this thesis was to develop cell culture models and conduct *in vitro* experiments in order to better evaluate biocompatibility at the ocular surface and to understand more about inflammation as it occurs in the eye.

Biocompatibility of a material may be loosely defined as the ability of a material to interact with a tissue and perform a specific function without adversely affecting the biology of interaction [4]. Therefore, a material that is biocompatible will seamlessly integrate into a biological environment without damage or stress of any cells without leading to inflammation or an immune response. To evaluate biocompatibility at the ocular surface, the metrics may be widely varied from *in vitro* cytotoxicity, microscopy, or immunohistochemistry; to *ex vivo* sample collection and analysis; or to *in vivo* microscopy.

In the development of new contact lenses and multipurpose solutions, *in vitro* studies are necessary to be undertaken, but are primarily used as a benchmark in order to demonstrate a lack of cytotoxicity. However, *in vitro* studies can provide us with much more information if we understand the limitations and abilities of our models. As an example, ocular surface health is examined *in vivo* solely through microscopic methods. Therefore, we are only able to visually inspect the eye to investigate abnormalities, or use one of a few biological stains that are approved dyes for human

exposure. This method only provides us with limited information on biocompatibility, and yet, it is considered the most important. *In vivo* studies may be performed on animals, but in the case of rabbits and mice, the shape of the eye and the frequency of blink are completely different, which offer their own limitations in understanding. However, with *in vitro* models, the capability exists to use human-derived tissues and study how they change at the cellular level. *In vitro* models are often much more cost-effective, practical, and customizable. If instead the focus shifts to what we can learn from *in vitro* models, instead of using them as a simple benchmark for cytotoxicity, we may solve many of the challenges that arise when products reach the clinical trial stage.

Silicone hydrogels were first introduced commercially in 1998 [5]. With the introduction of silicone hydrogels came the advent of multipurpose disinfecting solutions (MPS). MPS are used to clean and disinfect the lens in between lens wear. MPS contain biocides that are used to kill microbes, but most of these biocides may also be cytotoxic to the human cornea [6]. These components may be easily adsorbed into the lens and the contact lens may thus act as a drug delivery device with a prolonged release and potentially cause undue damage to the ocular surface [7]. It was first reported in 2002 that different lens and solution combinations may affect the health of the cornea, and that the unique combinations were more important than the individual lens or solution in question [8].

Our lab began by investigating these individual lens and solution interactions using monolayer cultures of human corneal epithelial cells. Monolayer models allowed for the discovery that borate-buffered solutions are more toxic than phosphate-buffered solutions [9] and for the investigation of lens and solution interactions using methodologies that are now commonplace [6]. The knowledge of monolayer cultures was then adapted to develop multilayered cultures [10]. In continuing to develop a comprehensive *in vitro* model to effectively mimic the *in vivo* situation, our lab also developed a tear replenishment system [11], which is a novel microfluidic device that effectively reproduces the

fluidic effect of blinking. This thesis explores some of the parameters that affect lens and solution biocompatibility by using different combinations of products, different kinds of models, and different lens parameters to provide improved understanding of how lenses and solutions interact at the ocular surface.

Assessment of *in vivo* lens and solution biocompatibility is generally performed using slit lamp biomicroscopy and a dye called sodium fluorescein. It has been shown that each lens and solution combination presents a different level of sodium fluorescein staining. This phenomenon is referred to as solution-induced corneal staining and is highlighted in Figure 1-1. However, much about this phenomenon has yet to be understood. Primarily, the mechanisms by which sodium fluorescein stains cells remained largely unknown for over 100 years. This thesis aims to investigate whether cell death can be correlated with sodium fluorescein staining and if it is a potential mechanism at work. Further, this thesis aims to reproduce solution-induced corneal staining using our *in vitro*, curved, stratified model.

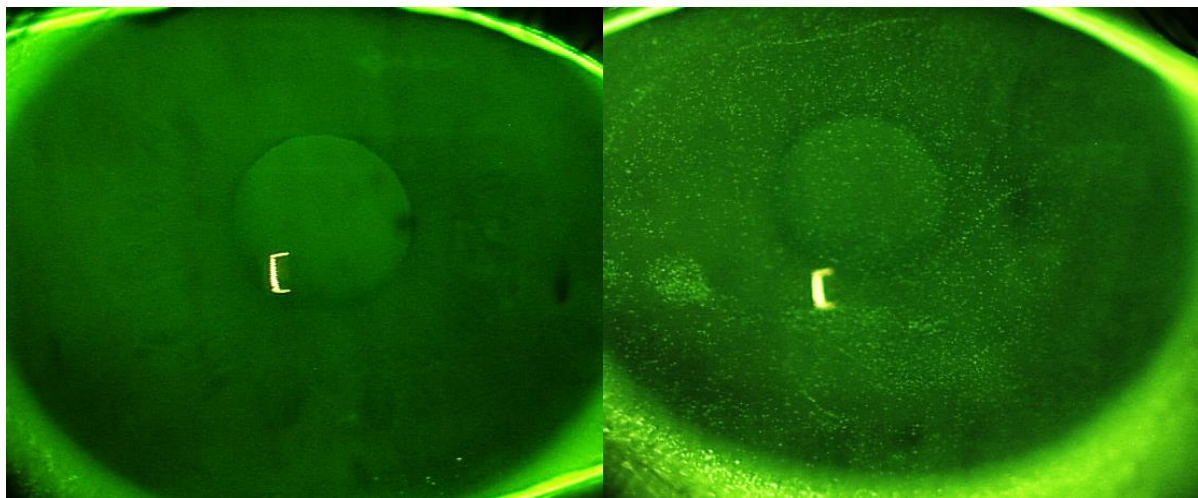


Figure 1-1: Two eyes stained with sodium fluorescein. The eye on the left shows no corneal staining, whereas the eye on the right shows classic micropunctate corneal staining. Pictures courtesy of the Centre for Contact Lens Research at the University of Waterloo.

One component that was missing from our current *in vitro* studies is the effect of infection and inflammation on the ocular surface. Infection, specifically in relation to contact lens wear, has been thoroughly investigated by other research groups [12-15] and is not the purpose of this thesis. The inflammatory system is very complicated and is actively maintained through interactions between white blood cells, epithelium, and all of the many proteins and lipids that constitute both the tissues and fluids of the eye. As the inflammatory system is in constant balance, this balance may be easily upset and may recruit inflammatory cells or cause the release of inflammatory mediators that could potentially damage the ocular surface.

One inflammatory cell of the ocular surface that has thus far been neglected is the tear-film neutrophil. When our eyes are closed for a prolonged period of time, the ocular surface becomes infiltrated with many polymorphonuclear neutrophils, and we call these cells tear-film neutrophils [16]. These cells were originally underestimated in their recruitment during sleep, and as a result, characterization was never fully performed on these cells. In the past, whole-blood-isolated neutrophils have been used as a mimic for tear-film neutrophils [17], but it has been shown that these cells do not act in the same way. This thesis tries to establish a tear-film neutrophil phenotype using blood-isolated neutrophils by incubating the cells under closed-eye conditions.

This thesis begins with a background introduction to the eye, biocompatibility, and contact lenses in Chapter 2 and continues with a background on inflammation at the ocular surface in Chapter 3. Chapter 4 presents studies using monolayer and multilayered models to examine the mechanisms of fluorescein staining and lens and solution interaction. Chapter 5 reports the work that has been done in trying to mimic the phenotype of tear-film neutrophils using blood-isolated neutrophils. Finally, Chapter 6 and Chapter 7 present the conclusions and recommendations, respectively.

Chapter 2

Ocular surface biocompatibility

The plusses and minuses of contact lens wear

2.1 The Eye

The eye is perhaps one of the most complex organs in the human body. Its purpose is to obtain visual information from the outside world and transmit that information to the brain. To do so, it is composed of many tissues with complex functions to collect, refract, and interpret incoming visual information. A schematic of the eye is shown in Figure 2-1. Incoming light first comes through the transparent cornea, followed by focusing through the lens, to arrive at the retina where light information is processed and transmitted to the brain via the optic nerve [18]. As a result, the cornea and lens must remain transparent as they account for the total refractory potential of the eye [18]. To detail the eye in terms of all of its many structures and functions requires many volumes of literature, so for the purpose of this thesis, the focus will be on the cornea, and specifically, on the ocular surface and the corneal epithelium.

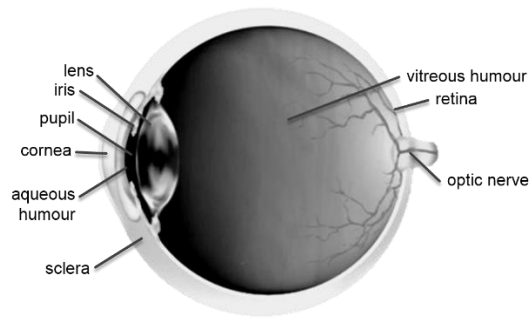


Figure 2-1: Anatomy of the eye: cross-section. Adapted from a drawing by Ben Stone.

2.2 The Cornea: Structure and Function

The cornea is primarily important for refraction and acts as both a mechanical and physiological barrier to the internal ocular machinery. More than two-thirds of the total refractive power of the eye is accounted for by the cornea alone [19]. The cornea is composed of six total layers, with three main cellular layers, as shown in Figure 2-2. The three main cellular layers of the cornea are: the endothelium, the stroma, and the epithelium. The acellular Bowman's layer lies between the stroma and the epithelium while Descemet's layer lies between the stroma and endothelium. It was recently reported that there is a pre-Descemet's layer, termed Dua's layer [20], but this claim may be improperly founded as it may just be part of the stroma [21]. The endothelium is comprised of a single layer of cells that serve as a barrier to the aqueous humour and ensure appropriate water content of the corneal stroma [19]. The stroma primarily contributes to structure and mechanical strength through the use of collagen networks [19]. The corneal epithelium also contributes to the structure of the cornea, but is most important as the first line of defense against many types of injury, trauma, and infection [22-24]. The epithelium has also been shown to be the primary barrier against transcorneal permeation [25].

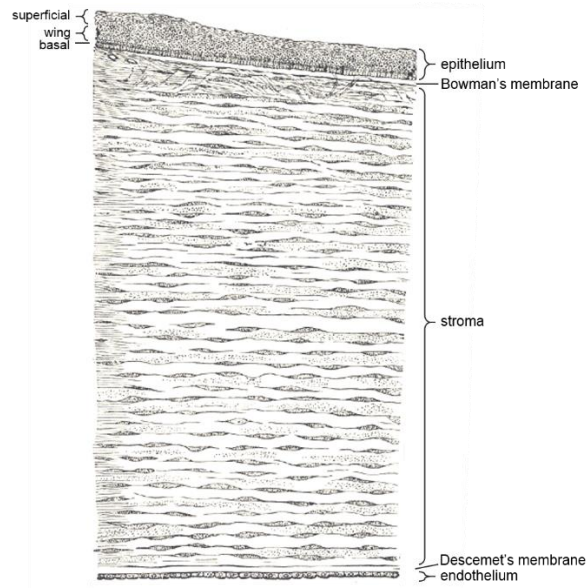


Figure 2-2: Cross-section of the human cornea. The breakdown of the layers of the human cornea is shown on the left. Dua's layer would be a pre-Descemet's layer that would sit between the stroma and Descemet's membrane [20]. The three layers of the human corneal epithelium are shown on the right. Histologic cross-section of the cornea obtained from [26].

From a lateral view, the ocular surface is composed of the conjunctiva, limbus, and cornea as illustrated in Figure 2-3. The conjunctiva contributes to the structure of the eye, and is mainly important in the production of goblet cell-secreted mucins. The conjunctiva is crucial in the immune response and does not have to remain transparent, which improves the diversity of its functionality. The limbus is identified as the region where the conjunctiva meets the cornea and is responsible for providing blood supply to the cornea. This area also contains corneal and conjunctival stem cells [27].

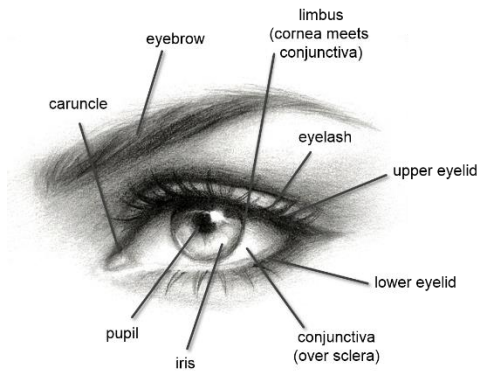


Figure 2-3: Anatomy of the eye: external. © G. DeCesaris, 2012. Used with permission.

The corneal epithelium is composed of one cell type, but can be further broken down into three types of cells (Figure 2-2). Columnar basal corneal epithelial cells sit as a monolayer on the basement membrane [28] which, as they differentiate, become more elongated and become wing cells [19]. Wing cells represent an intermediate stage of differentiation and are composed of two to three layers of cells [19]. The outmost layer of corneal epithelium is referred to as the superficial layer, where corneal epithelial cells are almost flat and contain desmosomes and tight junctions to impede passage of substances across the epithelium [19]. A more detailed outline of the function of the epithelium can be found in the next chapter.

2.3 Contact Lenses

Artificial lenses developed to be in contact with the ocular surface, i.e. contact lenses, have undergone a process of complicated development over the last 80 years. Starting in the 1940s, contact lenses were made out of polymethylmethacrylate (PMMA) and this technology continued through until the 1980s, seeing notable improvements such as the incorporation of silicon or fluorine to improve oxygen transmissibility [29]. The 1980s saw the arrival of poly(2-hydroxyethyl methacrylate) (polyHEMA) lenses, which formed the basis of conventional hydrogel contact lens materials [29]. Conventional polyHEMA lenses were an improvement upon the rigid gas permeable

(RGP) lenses, and remain on the market today in spite of a relatively low oxygen transmissibility. In the 1990s, silicone rubber was combined with conventional hydrogel technology to produce the first silicone hydrogel contact lenses [30]. Siloxane moieties were capable of greatly enhancing the oxygen delivery to the cornea [31]. However, this presented new challenges, as the silicone content decreased wettability and increased lipid interaction [30]. Therefore, surface modifications had to be developed to enhance the wettability of the polymer surface [30]. Examples of current conventional and silicone hydrogel lenses are shown in Table 2-1.

Table 2-1: Example of contact lenses and their material properties. Adapted from [32-34]

Proprietary Name	Acuvue 2	Acuvue Oasys	Biofinity	Focus Night & Day	Purevision
Manufacturer	Johnson and Johnson	Johnson and Johnson	Coopervision	CIBA Vision	Bausch & Lomb
US Adopted Name	Etafilcon A	Senofilcon A	Comfilcon A	Lotrafilcon A	Balafilcon A
Hydrogel Type	Conventional	Silicone	Silicone	Silicone	Silicone
FDA Group	IV	V (I)	V (I)	V (I)	V (III)
Water Content (%)	58	38	48	24	36
Oxygen Transmissibility (10^{-9})	20	147	128	140	99
Charge Principle	Charged	Neutral	Neutral	Neutral	Charged
Monomers	HEMA + MA	mPDMS + DMA + HEMA + Siloxane Monomer + TEGDMA + PVP	Undisclosed	DMA + TRIS + Siloxane Monomer	NVP + TPVC
Surface Treatment	None	None, internal wetting agent	None	25 nm plasma coating	Plasma Oxidation Process

DMA (N,N-dimethylacrylamide); HEMA (poly-2-hydroxyethyl methacrylate); mPDMS (monofunctional polydimethylsiloxane); NVP (N-vinyl pyrrolidone); PVP (polyvinyl pyrrolidone); TEGDMA (tetraethyleneglycol dimethacrylate); TPVC (tris-(trimethylsiloxysilyl) propylvinyl carbamate; TRIS (trimethylsiloxy silane).

2.4 Contact Lens Solution

Concurrent to the development of contact lenses was the development of contact lens solutions. Original contact lens solutions were primarily used for wetting and it was not until the 1970s that more sophisticated contact lens solutions were developed [35]. Contact lens solutions evolved into multipurpose disinfecting solutions (MPS) that are single solutions used to rinse, clean, and disinfect contact lenses. MPS contain a mix of disinfectants, surfactants, chelating agents, demulcents (lubricant), buffers, that work together to provide antimicrobial efficacy [35]. A summary of solutions currently available on the market are shown in Table 2-2.

Table 2-2: Example of common multipurpose disinfecting contact lens solutions. Adapted from [33,36]

Manufacturer	Brand (Abbreviation)	Disinfecting Agent	Buffer	Other Reported Agents (Surfactants, Chelating Agents)
Alcon	Opti-Free Replenish (OFR)	Polyquad® 0.001% Aldox® 0.0005%	Borate	Citrate; poloxamine (Tetronic 1304); non-anoyl ethylene-diaminetriacetic acid (C9-E3DA), propylene glycol
Bausch & Lomb	renu Fresh (renu)	PHMB 0.0001%	Borate	Sodium borate; hydroxyalkylphosphonate (Hydranate™); 0.1% EDTA; poloxamine (Tetronic 1107)
CIBA Vision	Clear Care (CC)	H ₂ O ₂ 3%	Phosphate	Sodium chloride; phosphoric acid; poloxamer (Pluronic 17R4)

2.5 Lens and Solution Interaction

Due to the highly porous nature of hydrogel materials, contact lenses may have the potential to uptake significant quantities of the components of multipurpose disinfecting solutions [37]. Given that MPS contain strong antimicrobial agents, it is possible that this extended release will potentially compromise the health of the cornea [7]. Therefore, there is a balance between the bactericidal efficacy, where the solution kills pathogens during overnight disinfection, and its biocompatibility, or

lack of cytotoxicity, towards the corneal epithelium. Kinetics of adsorption and desorption are known to be dependent on solution chemistry, as well as the lens composition and corresponding surface treatment [33,34,37].

2.6 Solution-Induced Corneal Staining

This effect of lens and solution interaction has been observed *in vivo* through the use of sodium fluorescein staining [8]. Sodium fluorescein staining was originally developed in the late 1800's as a method to observe changes in the overall health of the structure of the cornea. A more detailed history of the use of sodium fluorescein staining can be found in Chapter 4. In 2002, Jones *et al.* were the first to show that fluorescein staining could be observed *in vivo* following wear of specific lens and solution combinations [8]. This phenomenon has since been termed solution-induced corneal staining. Since that time, many research groups have investigated lens and solution interaction both *in vivo* [36,38] and *in vitro* [33,39,40]. A more detailed discussion of *in vitro* work in lens and solution biocompatibility can be found later in this chapter.

2.7 What is biocompatibility?

Traditionally, biocompatibility is thought of by a simple definition: “the ability of a material to perform without appropriate host response in a specific situation” [41]. This idea often gets conflated with cytotoxicity, which refers to the ability of a material to induce host cell death. There are many situations where a biomaterial may not be cytotoxic, but may indeed poorly integrate with the host, or lose its desired function upon integration. As a result, the following definition of biocompatibility was proposed [4]:

“Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in the specific situation, and optimising the clinically relevant performance of that therapy.”

In terms of development of new contact lenses or ophthalmic solutions, the FDA requires that researchers demonstrate that the material is not cytotoxic *in vitro* or *in vivo* through the agar overlay method or the ocular irritation assay (Draize test) [42]. The agar overlay method involves placing a contact lens on a bed of fibroblasts coated in agar, and measuring the radius of cell death extending from the contact lens [43]. Only recently has the FDA started to use this assay to investigate lens and solution interactions [42]. It is easy to assume that this *in vitro* method may be insufficient to show true biocompatibility of lens materials or of lens and solution interactions.

2.8 *In vitro* Ocular Toxicity Testing

The Draize test was developed as an ocular toxicity test in 1944 and involves the placement of test solutions on the eyes of living animals [44]. It became part of the United States Food and Drug Administration regulations in 1964 [45]. The Draize test has since come under much criticism in terms of its performance and reliability [45-47]. As the corneal epithelium represents the major barrier of the eye, many *in vitro* epithelial models have since been developed and proposed as alternatives to the *in vivo* Draize test. Since the 1960s, monolayer cell cultures have been developed using primary and immortalized corneal epithelial cell lines of rabbit and canine origin; and starting in the 1990s, the use of human-derived cells has become more popular [48,49]. Immortalization is the process that allows for continuous proliferation and a prolonged lifespan, and it may be achieved through methods such as: oncogenes, ectopic expression of the catalytic component of telomerase, or spontaneous establishment after serial cultivation of cells [45]. Cytotoxicity testing of ophthalmic solutions on corneal epithelial cells lines has been carried out on Madine-Darby Canine Kidney (MDCK) cells [50], human conjunctival epithelial cells [51], corneal-limbal epithelial cells [52], and human corneal epithelial cells (HCEC) [53-55].

Concurrent to the development of better cell lines was the improved understanding of multilayered cultures and the importance of the air-liquid interface. Multilayer models have since been grown

using many cell types and support membranes: immortalized human corneal epithelial cell lines CEPI 17 CL4 and SV40 immortalized cells have been grown on polyester/polycarbonate Transwell cell culture inserts [56-59] and human corneal limbal epithelial cells have been grown on polyethylene terephthalate (PET) membranes [52]. Gipson [60] and Robertson [61] have also recently developed multilayered cultures. Finally, tissue reconstruction for corneal transplantation has given rise to many different multilayered models with cells grown on human amniotic membrane or silk fibroin [62-64].

Commercially available models have also been developed, such as HCE by SkinEthic, EpiOcular by MatTek, and Clonetics by Lonza. The most popular of these *in vitro* models is the SkinEthic Human Corneal Epithelial model that was developed in 2003 by Nguyen *et al.* [65]. There has since been much use of the SkinEthic model to investigate the toxicity potential of ophthalmic solutions [66-72] including a pre-validation study by Van Goethem *et al.* in 2005 where the SkinEthic model was shown to be a useful alternative for the *in vivo* Draize test [73].

The majority of these *in vitro* models have primarily been used for toxicity testing of different ophthalmic solutions and for drug permeation studies [73-76]. Recently, our research group focused on the development of a curved, stratified model of the human corneal epithelium to be used to specifically study the interaction of contact lenses with a multilayered model [10]. The model is curved in order to mimic the ocular surface and to facilitate proper contact lens wear. This model has been used to demonstrate that it is sensitive to different concentrations of a known cytotoxic agent, benzalkonium chloride (BAK). Our model has also shown that a PBS-soaked balafilcon A lens is biocompatible, which is a similar result from previous monolayer *in vitro* studies [9].

One of the drawbacks with both monolayer and multilayered *in vitro* models so far is that they have all been used under static conditions. The eye is a very dynamic environment and the human eye holds approximately 7-30 μL of tears with a turnover rate of 0.5-2.2 $\mu\text{L}/\text{min}$ [77-79].

Spontaneous blinking happens at the rate of 6-15 times/min and helps to mechanically spread the tear

film evenly across the ocular surface and to remove any foreign objects in contact with the eye [79]. Our laboratory recently developed a tear replenishment system (TRS) to mimic these effects *in vitro* [11]. Importantly, we showed that the TRS did not damage the curved, stratified, epithelium. Our results also showed that there were mild differences in the cytotoxic responses of BAK-soaked lenses with and without tear replenishment [11]. It has been demonstrated in the intestine that three-dimensional culture models in conjunction with dynamic systems are much more useful for *in vitro* investigation [80].

There has thus far been minimal work to understand lens and solution effects *in vitro* [6,39], and only a few studies focused on the response of stratified epithelium to MPS have been conducted [81-83]. Chapter 4 presents the first study investigating lens and solution interactions using stratified human corneal epithelial cell cultures.

2.9 Flow Cytometry

Flow cytometry is a common analytical method used by our research group and methods in both Chapter 4 and Chapter 5 will demonstrate how it is used for practical purposes. Flow cytometry consists of a microfluidic system whereby cells are analyzed for their relative size, granularity, and fluorescence intensity. Cells travel single file to be illuminated by a laser beam, and based off of the diffracted (forward scatter) and reflected and refracted (side scatter) light, relative size and granularity can be obtained, respectively [84]. Fluorescent molecules within cells or fluorescent antibodies attached to cells may also be used to be excited and subsequently emit light. Collection optics are employed to capture light of a specific wavelength and measure light intensity. The events of each cell (forward scatter, side scatter, and fluorescent intensity) are counted and may be used as a quantitative metric.

The BD FACSCalibur system used by our research group is capable of detecting three wavelength ranges, as shown in Table 2-3.

Table 2-3: Description of the optics used by the BD FACSCalibur System. Filters used are either band pass (BP) or long pass (LP), meaning that they detect within a given wavelength range, or at longer wavelengths, respectively.

Detector	Filter	Colour	Fluorochrome
FL1	BP 530/30 nm	Green	FITC
FL2	BP 585/42 nm	Yellow/Orange	PE
FL3	LP 670 nm	Dark Red	PerCP, PerCP-Cy5.5

As a result, we may obtain a dot plot of side scatter versus forward scatter, which plots relative granularity versus relative size. This is especially important in our blood experiments, given that different blood cells have different size and granularity. This is highlighted in Figure 2-4.

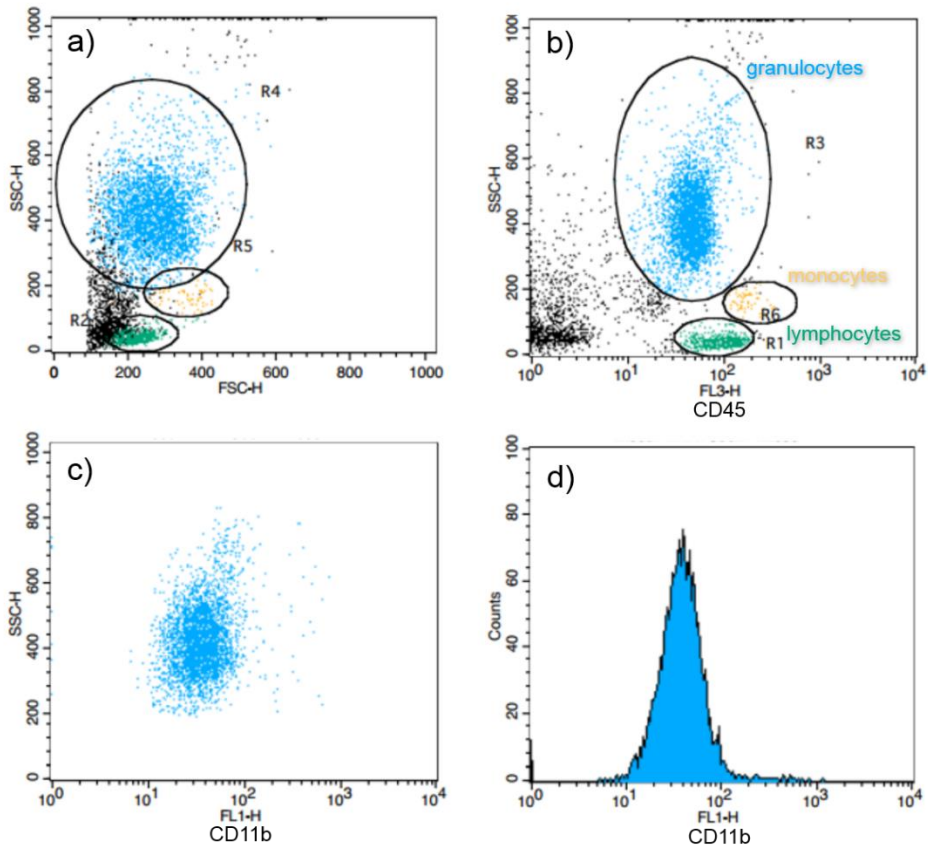


Figure 2-4: Flow cytometry analysis of blood-isolated leukocytes. The standard side scatter versus forward scatter plot is shown in a) which plots relative granularity versus relative size. b) plots side scatter versus fluorescence intensity of CD45, a pan-leukocyte marker. All leukocytes stain with CD45, and they can be differentiated based upon relative granularity [84,85]. Blue cells are the granulocytes, which consist of neutrophils, basophils, and eosinophils; yellow cells are monocytes; and green cells are the lymphocytes. c) shows a plot of side scatter versus fluorescence intensity for CD11b, a leukocyte activation marker, for only the granulocyte population. Finally, d) shows a plot of counts versus fluorescence intensity of CD11b and this data may be quantitated and used for statistical analysis.

Chapter 3

Inflammation and Immune Privilege at the Ocular Surface

Check your immune privilege.

Classically, immune privilege was considered as an “immunological ignorance” where tissue grafts could survive in regions of the body without immune rejection [86-88]. However, this definition has since broadened vastly as the cell and molecular environments that facilitate prolonged allograft survival are diverse and complex. Modern immune privilege is perhaps confounded with the notion that immune privileged sites are able to resolve inflammation without provoking typical leukocyte-mediated immune responses. As a result, an assumption may be made that immune privileged environments lack leukocytes, but in many cases, this is incorrect, and certain leukocytes may present an active tolerance.

Several organs in the body are immune privileged, including the brain, the eye, and the testes [89]. The eye is especially of interest, given the fact that the ocular surface has to contend with pathogens on a daily basis. It is also a unique organ given that many biomaterials interact with the eye, such as contact lenses, intraocular lenses, and glaucoma drainage devices. In spite of all of the adversity that

the eye faces, its tissues can often remain uncompromised. However, there are many cases where interaction with a biomaterial may exacerbate inflammation or be implicated in adverse events such as microbial keratitis [13] or posterior capsule opacification [90].

Overall, there has yet to be a complete understanding of the concert of immunological and inflammatory mechanisms of the eye, and further, how these mechanisms may affect interactions with biomaterials, such as contact lenses¹.

3.1 The Ocular Surface

The eye is responsible for 70% of the sensory information that is taken to the brain [91] and as a result, many immune mechanisms are in place to resist compromise of the overall biological system. Innate immunity of the eye begins with its unique anatomy, specifically the physical barriers of the bony orbit, the lid wiper, and the lashes. This defense continues to the ocular surface with the blink, tear film, and finally, to the epithelial cells of the cornea and conjunctiva. In order to protect from pathogen invasion or damage, a complex environment exists in the tear film, complete with many proteins, mucins, and even commensal bacteria, which act harmoniously to protect from pathogens at the ocular surface [92,93]. The cornea also serves as a physical barrier to pathogens, but is unique in the fact that it must remain transparent in order to preserve vision [18]. In preserving vision, the cornea is avascular and obtains its blood supply from the peripheral limbus [94]. The paramount structure in place is the corneal epithelium, which is rich with receptors and mechanisms to preserve vision by maintaining integrity and combatting local infection and inflammation. This delicate concert of structure and function is also aided by a ménage of inflammatory cells that patrol the ocular surface as part of both the innate and adaptive immune systems. As this system is in a constant effort to maintain balance, it is possible that this environment can become compromised either

¹ The above three paragraphs were co-written with Robert Pintwala as part of the introduction to a review paper on immune privilege of the anterior eye, yet to be submitted.

through auto-immune disorders or through microbial infection. With the introduction of a biomaterial into this environment, specifically a contact lens, it remains to be well understood how the machinery of the ocular surface is impacted.

3.2 Non-Leukocyte Mediated Innate Immunity

3.2.1 Proteins in the tear fluid

To date, over 1500 proteins have been found in human tear fluid [95]. Given the significant advances in mass spectrometry, we are now able to obtain very detailed resolution of the proteins that exist at the ocular surface [95,96]. Historically, the major proteins that have been of interest at the ocular surface include lactoferrin, lysozyme, lipocalin, and secretory immunoglobulin A (IgA), as these proteins represent the major portion of the tear proteome [95]. A summary of their properties may be found in Table 3-1.

Table 3-1: Description of main proteins of the tear film. Adapted from [92,93,97].

Name	% in Tear Fluid	Description	Origin
lysozyme	20-30%	-destabilizes cell wall of pathogens	lacrimal gland
lactoferrin	20-30%	-interferes with ability of pathogens to uptake iron	lacrimal gland
tear lipocalin	25%	-interferes with ability of pathogens to uptake iron -protect ocular surface from proteolytic damage	lacrimal gland
secretory IgA	diurnal variation	-promotes clearance of pathogens -resists bacterial adherence	plasma cells lacrimal gland

Among those 1500 proteins are many other proteins, including many anti-microbials and anti-virals. Some examples of anti-viral proteins in the tear fluid are lactate dehydrogenase, complement proteins, amylase, peroxidase, and interferons alpha and beta [98].

Perhaps most important in inflammation is the class of small proteins known as cytokines: specific proteins that are responsible for inflammatory and immune cell activation and recruitment, and are produced by all of the ocular surface cells such as the corneal and conjunctival epithelial cells, stromal cells, and resident antigen-presenting cells [99]. A subset of cytokines includes chemokines, which are usually used for recruitment and movement of inflammatory cells. Another relevant class of proteins in the tear film is growth factors, which induce many processes including re-epithelialization and neovascularization, and play an important role in corneal homeostasis [100]. It is important to note that these small molecules control the majority of cell-to-cell signaling and do not necessarily have to be in abundant quantities in order to have a significant effect on the inflammatory cascades present in the eye.

The tear film also contains a layer of mucins that are produced primarily by goblet cells in the conjunctiva [101]. Mucins are highly glycosylated proteins that are both secreted and membrane-associated [102,103]. They function to maintain tear stability and hydration of the ocular surface while providing a barrier to pathogens and particulates.

3.2.2 Lipid Mediators

While proteins have many functions within the body, lipids serve as an integral part of regulatory circuits in terms of initiating, perpetuating, and ultimately resolving² leukocyte-mediated inflammation [104,105]. A common lipid mediator family are the eicosanoids, which are derived from the polyunsaturated fatty acid arachidonic acid. Arachidonic acid is primarily catalyzed to form

² Resolution is a process of removal of neutrophils from sites of acute inflammation. This is particularly important as prolonged neutrophil residence (and stimulation) can result in tissue injury and even in systemic disease.

lipid mediators by one of two enzyme pathways: the cyclooxygenases or the lipoxygenases [106]. These lipid mediators are formed in response to stress, injury, or inflammatory stimuli.

Lipid mediators are particularly important at the ocular surface as they interact with the heme oxygenase enzyme system. Heme oxygenases are responsible for the catalysis of heme in order to produce antioxidants and carbon monoxide, which have essential cytoprotective functions [106]. The lipid circuits generate feedback in combination with the heme oxygenase system in order to amplify anti-inflammatory efforts [104,106]. Lipid mediators are also instrumental in wound healing responses in mice, and these circuits have a differential, sex-specific response [107].

3.2.3 Commensal bacteria

In normal, healthy, human corneas, there can often be found a population of bacteria that live either on the eyelid or the ocular surface. There does, however, seem to be a very large variation in the strains, types, and prevalence of ocular flora that has been identified depending both on the experimental techniques and the location where the studies were performed [12]. In spite of a constant or usual microbial burden at the ocular surface, the immune system has been able to develop mechanisms that regulate microbial inflammation. It has been suggested that the population of commensal bacteria is usually polyclonal, meaning that the ocular flora exists in a balanced state. However, when the population of bacteria becomes monoclonal, implying that one strain dominates, this is more likely when infection occurs, or conversely, during infection, one strain dominates the ocular surface [108]. Dysregulation of the immune system may lead to chronic blepharitis, conjunctivitis, and keratitis [108].

3.2.4 Epithelium

The human cornea is composed of three main cellular layers: the epithelium, the stroma, and the endothelium. The outermost of these layers is the epithelium, and its primary responsibility is to prevent pathogen invasion. As a result, the epithelium has many features such as tight junctions,

defensins, toll-like receptors, and ligand receptors within its arsenal in order to combat infection and inflammation.

Tight junctions are protein complexes in the superficial epithelial layer that serve as a physical barrier to pathogens. The tight junction complexes are composed of transmembrane proteins claudin and occludin, proteins of the membrane-associated guanylate kinase homologue family (ZO-1, ZO-2, and ZO-3), and actin filaments [109].

While the superficial epithelium serves as a physical barrier, it can also serve as a metabolic barrier. In order to maintain its immune privileged state, the corneal epithelium must also resist viruses. While there are many anti-viral mechanisms in place, perhaps one of the most interesting features is the turnover of the corneal epithelium. The outermost superficial corneal epithelial layer is terminally differentiated, and this creates a metabolic barrier, as viruses depend on replication and dissemination in metabolically active cells [98].

Defensins are a group of antimicrobial peptides at the ocular surface that can perform varied functions, primarily to disrupt microbial membranes [110]. The human corneal epithelium features many isoforms of human beta defensins and can produce inflammatory cytokines such as IL-1 α , IL-1 β , TNF α , IL-6, and IL-8 [108].

Toll-like receptors are expressed on many cell types, including the corneal epithelium, stroma, endothelium, antigen presenting cells, and lymphocytes [111]. Toll-like receptors are pattern recognition receptors that recognize pathogens via the pathogen associated molecular patterns (PAMPs) found on bacteria, viruses, fungi, and protozoa [108,111]. So far, there have been 10 TLRs identified, and all of these are expressed on ocular surface epithelia, though TLRs 7 and 8 are more rarely found [108,111]. Briefly, activation of the TLRs by PAMPs initiates innate and acquired immune responses through the release of cytokines and chemokines that recruit neutrophils and lymphocytes [111].

While TLRs are known for binding exogenous PAMPs, it is also possible that TLR activation may be caused by endogenous ligands, referred to as danger/damage associated molecular patterns (DAMPs). These DAMPs are often cytosolic proteins that are released following injury or necrosis [111,112]. Thus, the TLRs may act as a surveillance system for tissue injury as well as for infection. However, it is possible that endogenous activation of TLRs could lead to autoimmune disorders [112]. As TLRs may be triggered by both exogenous and endogenous ligands, the signaling pathways may be very complex and have been the subject of several reviews [108,111,113]

3.3 Leukocyte Mediated Innate Immunity

In response to infection, the cornea responds similarly to the rest of the body with the innate immune response of PMNs, natural killer cells, and macrophages, along with the adaptive immune response facilitated by dendritic cells and T cells [114]. Other research focuses on the subject of the detailed inflammatory response to wound healing in the cornea [115-117].

Of particular interest is how leukocytes aid in the maintenance of immune homeostasis at the cornea. Immune privilege easily conjures an image where inflammatory processes do not occur because inflammatory cells do not exist; however, it has since been shown that certain leukocytes may have developed an immune tolerance or ignorance [89]. The normal, healthy human cornea contains a resident population of antigen-presenting cells; namely, Langerhans cells, dendritic cells, and macrophages [118]. In a closed-eye environment (during sleep), large numbers of polymorphonuclear neutrophils (PMNs) are recruited to the ocular surface in a non-inflammatory state [92].

3.3.1 Antigen³-Presenting Cells

Within the cornea, there is a resident population of dendritic cells [118]. Around the periphery, these cells are major histocompatibility complex II (MHC II) positive, and can thus be thought of as mature dendritic cells. However, the central stroma contains immature dendritic cells that are MHC II negative. The maturity of a dendritic cell can be equated with how well the dendritic cell is able to recruit T-lymphocytes, where immature dendritic cells are strong at antigen capture, but weak at T-cell activation, and mature dendritic cells are weak at antigen capture, but strong at T-cell activation [118].

Langerhans cells are similar in structure and function to a dendritic cell, but are unique in that they are the only MHC II⁺ cells that exist in the corneal epithelium under non-inflamed conditions. These cells have been found in both the centre and the periphery of the epithelium and reside at the basal layer of epithelium [118]. Macrophages are the final type of antigen-presenting cell found in the cornea, and it has only been more recently that these cells have been discovered in the central stroma of mice [119]. It has been shown that resident macrophages are primarily responsible for neovascularization of the cornea [120].

Overall, it remains largely unknown how these antigen-presenting cells operate in a non-inflammatory state. It is also possible that these cells are meant to be quiescent in a non-inflammatory state, given that their role is to present antigens. However, without antigen presentation, it also remains a mystery as to how dendritic cells, macrophages, and Langerhans cells may be recruited to the cornea and ocular tissues [98].

³ An antigen is any molecule that promotes the formation of antibodies, and thus, initiates an immune response.

3.3.2 Tear-Film Neutrophils

Neutrophils are the most prominent of all immune cells in the body and are the first responders to inflammation. Neutrophils are known by their banded or multi-lobed nuclear structure, which is why they are also called polymorphonuclear leukocytes (PMNs) [121]. They contain an arsenal of inflammatory mediators, bactericidal agents, proteolytic enzymes and oxidative products with which to combat infection and inflammation [122]. They also have a short half-life and a high turnover rate [121].

When the eye is closed for a prolonged period of time, hundreds of thousands of PMNs are recruited to the ocular surface. This constitutes a very large population of PMNs, so much so that the concentration of PMNs in the closed-eye tear film can be equated to approximately 4 times the concentration of PMNs in the blood [16]. This influx of neutrophils that are collected immediately upon waking occurs in normal, healthy, individuals as a part of immune homeostasis, and will hereafter be referred to as tear-film neutrophils.

The tear-film neutrophils were first observed by Wilson *et al.* in 1989 [123]. In the late 90s' and early 2000s', Sack *et al.* investigated tear-film neutrophil recruitment, studying the variations in chemotactic and inflammatory mediators in the closed-eye environment, and the time course of PMN recruitment [92,124,125]. The result of the studies was that the tear-film neutrophils existed to phagocytose potential pathogens. No functional study was performed, and only cell numbers were recorded.

In the first study to characterize their phenotype [126], tear-film neutrophils demonstrate a significant difference from the classical inflammatory phenotype described above. In this study, tear-film neutrophils were tested in terms of their ability to activate. Neutrophils commonly exist in one of three inflammatory phenotypes: quiescent, primed, or activated [127]. Quiescent neutrophils are non-inflammatory, primed neutrophils have encountered a stimulus or environment that have changed

their ability to activate, and activated cells are those that release inflammatory mediators and oxidative products. Neutrophils that have been primed prior to activation mount a much larger inflammatory response as compared to neutrophils that were not primed. A common stimuli may be bacterial endotoxin, or lipopolysaccharide (LPS), which are fragments of cell walls from gram positive bacteria [128].

To investigate changes in neutrophil phenotype, it is common to examine expression of membrane receptors on neutrophils, which become increased (upregulated) or decreased (downregulated/internalized) depending on their function. A summary of important neutrophil membrane receptors is highlighted in Table 3-2.

Table 3-2: List of common antibodies used for investigation of neutrophil membrane receptor expression.

Cluster of Differentiation (CD) or Antibody	Leukocyte (Antigen) Function Assessed
CD11b	Important in cell adhesion and leukocyte activation, also known as the macrophage-1 antigen (Mac-1), binds to fibrinogen and iC3b
CD14	Binds lipopolysaccharide and pathogen-associated molecular patterns; typically used to differentiate neutrophils (CD14-) from monocytes (CD14+) [129,130]
CD16	FCγRIII receptor, shedding is implicated in cell death and may lead to resolution through nonphlogistic ⁴ phagocytosis by macrophages [131,132]
CD33	Myeloid antigen, expressed on immature granulocytes
CD45	Pan-leukocyte marker
CD54	Intracellular adhesion molecule (ICAM-1), upregulated on activated cells
CD62L	L-selectin, cellular adhesion molecule that is shed upon neutrophil transmigration
CD66b	Degranulation membrane marker, released upon activation
C3aR	Receptor for C3a, part of complement activation

⁴ Nonphlogistic phagocytosis refers to phagocytosis without the release of inflammatory cytokines.

The tear-film neutrophils from [16] were incubated with one of three inflammatory stimuli: lipopolysaccharide (LPS), bacterial endotoxin from the outer cell membrane of gram-negative bacteria; phorbol myristate acetate (PMA), a potent synthetic chemical and activator of protein kinase C; and N-formyl methionyl-leucyl-phenylalanine (fMLP), a chemotactic peptide. Following stimulus, tear-film neutrophils showed a lack of upregulation of inflammatory markers investigated: degranulation (CD66b), macrophage-1 antigen (CD11b), and intracellular adhesion molecule-1 (CD54). This was in contrast to the response observed with blood-isolated neutrophils, which under similar experimental conditions were able to mount the expected inflammatory response. Given these results, the role that tear-film neutrophils play and what effects they have on the ocular surface remain to be fully characterized.

Like the resident antigen presenting cells in the cornea, it is also not well understood how the tear-film PMNs are recruited to the ocular surface and where they come from. In response to inflammation, PMNs are recruited from limbal vasculature through the stroma [133]. This recruitment is complicated and requires extensive integrin signaling, selectin expression, and chemokine regulation that make it unlikely to be the source of hundreds of thousands of quiescent neutrophils.

Similar to toll-like receptor activation and commensal bacteria, neutrophils may also become systemically dysregulated [104]. Dysregulation of any of these processes may lead or contribute to epithelial, stromal, microbial, or fungal keratitis, allergic conjunctivitis, or autoimmune diseases such as dry eye or even age-related macular degeneration [98,104,111].

3.3.3 NETosis

Aside from the standard degranulation process that occurs in neutrophils, whereby neutrophils release toxic reactive oxygen species (ROS) and antimicrobial granular proteins [134], neutrophils are also capable of creating neutrophil extracellular traps (NETs). This process of neutrophil cell death is

distinct from apoptosis or necrosis [135]. NETs contain decondensed chromatin fibres, histones, and antimicrobial proteins [136]. These NETs serve to combat infection and inflammation through a process termed NETosis. However, improper regulation and clearance of NETs may have deleterious effects such as increased inflammation and this has been implicated in autoimmune disease pathogenesis [137]. NET components have been identified on the ocular surface and it has been suggested that dry eye disease pathogenesis may be affected by the dysregulation of NET clearance [138]. It has yet to be determined if tear-film neutrophils are capable of undergoing NETosis.

Returning to the concept of immune privilege, the main goal of the ocular surface machinery, ie. the proteins, epithelial cells, and inflammatory cells of the eye, is to preserve vision. A classical inflammatory response by neutrophils at the cornea would result in many cytolytic compounds being released on the cornea and would almost certainly compromise vision. It is possible that our immune system has evolved a special kind of phenotype for the tear-film neutrophil so that the ocular surface is able to combat inflammation without mounting a full inflammatory response. But how does this change in the presence of a biomaterial?

3.4 Biomaterials Interactions

The most common biomaterial that interacts with the ocular surface is the contact lens. Contact lenses are hydrogel polymers that are typically made from poly(2-hydroxyethylmethacrylate) (HEMA) or incorporate silicone rubber to create silicone hydrogels [29]. However, all of these polymers have various surface treatments or monomers (wetting agents) added in order to increase oxygen transmissibility to reduce corneal hypoxia [30].

In a classic model of biomaterial implantation into the body, the very first consideration that needs to be made is of protein adsorption to the biomaterial surface. Typical proteins that interact with biomaterials are albumin and fibrinogen, two main proteins in the blood. However, contact lenses have to interact with all of the 1543 proteins present in the tear film [95]. Protein adsorption depends

on lens material, surface charge, and the concentration, charge, and structure of the protein. Many studies have been performed to characterize protein adsorption on contact lenses, and to develop assays to image protein content both *in vitro* and *in vivo* (for a complete review of these studies, see [139]). Proteomics have also been used to investigate protein deposits following contact lens wear [140,141]. In spite of the wealth of research that has been conducted, there has yet to be any correlation between lens deposits and observable adverse effects on the cornea [141].

Contact lenses may greatly affect patient comfort, with roughly 23% of contact lens wearers discontinuing wear as a result of discomfort and dryness [2]. Decreased comfort could be a result of lens design, multipurpose solution, or changes in the ocular biology [3]. It has been shown that contact lens wear affects regular corneal homeostasis by impacting corneal epithelial cell turnover, the rate of cell exfoliation, and may result in corneal thinning [142].

As mentioned above, the eye may often be subject to commensal bacteria, and yet it is rarely compromised enough to result in microbial infection. Contact lens wearers, particularly while sleeping with a contact lens, are at an increased risk of microbial keratitis [13,143,144]. Between 1991 and 2006, a twofold increase in the occurrence of corneal infiltrative events (CIE)⁵ [145] was also observed with silicone hydrogel lenses as compared to conventional hydrogels [146]. However, silicone hydrogel contact lens wear comes in two different modalities; namely daily wear, where lenses are replaced daily; and extended wear, where lenses are worn continuously for up to a month. It was recently reported that a daily wear modality could significantly reduce CIE as compared to extended wear use [147], implying that the practical aspects of lens wear could also impact biocompatibility.

⁵ Corneal infiltrative events are clinically observed signs of inflammation that often correlate with patient symptoms of redness, irritation, or pain [60].

Most of the work that has been done on immune cell interaction with contact lenses has focused on the role of PMNs in ocular infection. Work that has been done so far has focused on the use of blood-isolated neutrophils, and these neutrophils may exacerbate infection as dead PMNs may promote biofilm formation on silicone hydrogel contact lenses [148,149]. Further, some strains of bacteria may not induce a respiratory burst in blood-isolated PMN, at all [149], though all strains affect cytokine release from blood-isolated PMNs [150]. It remains to be determined if tear-film neutrophils act in a similar capacity.

A brief investigation on tear-film neutrophils collected from contact lens wearers has shown a reduced expression of degranulation markers CD66b and CD33 when compared to non-lens wearers [151]. The same investigation also indicated that the tear-film neutrophils collected directly from the contact lens had a different phenotype from the tear-film neutrophils collected from the ocular surface, suggesting that tear-film neutrophils somehow respond to changes in their environment, such as the introduction of a silicone hydrogel contact lens. Internalization of the C3a receptor on tear-film neutrophils was also observed following overnight lens wear [152]. A pilot study by Sindt *et al.* showed that dendritic cell densities in the cornea are also affected by both contact lens wear and by specific multipurpose disinfecting solutions used, though the implications of this also have to be elucidated [153].

While these results have yet to be fully interpreted in the context of overall ocular inflammation, it can be demonstrated that a contact lens biomaterial significantly impacts the homeostasis of the eye in terms of its ability to preserve vision. Contact lens and materials science researchers have been consistently trying to develop contact lenses with increased oxygen permeability, and while their goals have been achieved, the overall biocompatibility, as defined by optimal comfort and an absence of inflammation or infection, has yet to show any improvement. Given our lack of understanding of immune privilege at the ocular surface, more thorough investigations into the inflammatory

mechanisms of the ocular surface will help us in solving contact lens related infection, discomfort, and inflammation.

Chapter 4

Reproduction of micropunctate corneal staining *in vitro*

Discovering that twinkle in your eye.

4.1 Introduction

Staining of the cornea following topical instillation of sodium fluorescein, referred to as fluorescein staining or corneal staining, was originally developed in the late 1800s as a method to observe changes in the morphology of the cornea, and specifically for the visual inspection of corneal epithelial abrasions in rabbits [154], followed by corneal epithelial lesions in humans [155,156]. Following sodium fluorescein instillation, clinicians evaluate the cornea using a slit-lamp biomicroscope and a cobalt blue light to induce fluorescence of the dye.

Sodium fluorescein has been used for the clinical evaluation of corneal epithelial abrasions or defects, tear break-up time, contact lens fitting, tear volume and clearance, aqueous humour flow and leakage, and corneal epithelial and endothelial permeability [157]. Sodium fluorescein is used to diagnose the cornea in response to chemical injury, mechanical insult, and dehydration [158]. It has

also been noted that sodium fluorescein staining occurs regularly in healthy individuals [159-162] as well as in patients with dry eye [163,164].

In 2002, Jones *et al.* observed that corneal staining may also arise as a result of an interaction between a contact lens and a multipurpose disinfecting solution (MPS) [8], a phenomenon that has since been termed solution-induced corneal staining (SICS). Andrasko *et al.* [36] and the IER staining matrix [38] have since dedicated much time and effort to catalogue the different responses as a result of all different lens and MPS combinations. Long before solution-induced corneal staining even existed, staining as a result of contact lens wear was stated to be a “danger signal” [159]. As a result, solution-induced corneal staining generally has a negative connotation and has even been plainly described as a manifestation of MPS cytotoxicity [165]. Solution-induced corneal staining is currently considered the *in vivo* metric of contact lens and MPS biocompatibility [7].

However, the overall significance of this phenomenon has been a topic of much debate [161,166-169], literally [170,171]. Without going into detail on the many arguments of the clinical manifestations of sodium fluorescein staining, it may suffice to say that a lot of argument on the impact of solution-induced corneal staining arises from a lack of knowledge on the fundamental biological processes at play. Specifically, the mechanisms of sodium fluorescein staining (let alone SICS) have yet to be fully elucidated.

Historically, sodium fluorescein staining was thought to occur under one of three different mechanisms: ingress, pooling, or dead cells [172]. Originally, given its ability to diffuse through the cornea, sodium fluorescein was presumed to be caught in the ingress around cells from a lack of tight junctions [156,157,173]. It was also postulated that fluorescein would pool in areas of epithelial defect as a result of shed cells [159]. Another mechanism postulated was that staining would occur in cells that are dead, dying, or desquamating as part of natural epithelial turnover [160,174].

Since, these historical mechanisms have mostly been disproven. Given that rinsing of the ocular surface does not change the overall pattern of fluorescein staining (or remove it), staining as a result of ingress and pooling is unlikely [175,176]. Further, micropunctate spots may be removed by impression cytology, which is not physically possible with any pooling mechanism [163,177]. Finally, it has been shown on numerous occasions that staining is intracellular [157,163,177] and that fluorescein-stained cells are alive [7,158,176,178,179]. However, while they are alive, it may be possible that the fluorescein-stained cells are undergoing some form of apoptosis [7,178,179].

Many cell types within the human body readily uptake sodium fluorescein [180] or rose bengal [181], and yet, the corneal epithelium does not consistently uptake either of these molecules. This implies that the cornea has a mechanism that blocks the entry into the cells. Diffusion is not alone responsible for fluorescein transport given that wing cells have been found to uptake sodium fluorescein in areas where superficial cells did not stain with the dye [163]. Recently, Bakkar *et al.* proved that fluorescein transport is mediated by active transport as there was a temperature dependence on uptake and release of sodium fluorescein [158]. Fluorescein transport may occur as an active process across tissues via monocarboxylic acid cotransporters [182,183]. These same transporters have been found in the corneal epithelium [184].

Lastly, sodium fluorescein may also be bound to or blocked by other components at the ocular surface such as mucins or PHMB [185], a molecule commonly found in ophthalmic solutions. Mucins are highly glycosylated glycoproteins that are both secreted and membrane-associated [102,103]. Together, they function to maintain tear stability and hydration of the ocular surface while providing a barrier to pathogens and particulates. Mucin defects could therefore increase sodium fluorescein permeability [83,163,177,181] and increased mucin mRNA expression has been previously correlated with increases in sodium fluorescein staining in atopic disease [186].

This current study presents an investigation of the mechanism of solution-induced corneal staining through the use of monolayer and multilayer *in vitro* human corneal epithelial models. Specifically, monolayer models were used to investigate sodium fluorescein uptake in correlation with cell death and a curved, stratified, corneal epithelial model was used to induce solution-induced corneal staining *in vitro* and correlate results with changes in cell viability and apoptosis. An outline of the experimental approach is shown in Figure 4-1.

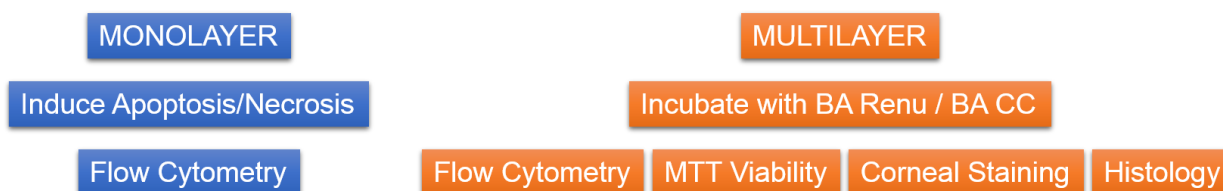


Figure 4-1: Outline of the experimental approach. BA Renu: renu-soaked balafilcon A lenses; BA CC: Clear Care-soaked balafilcon A lenses.

4.2 Materials and Methods

4.2.1 Reagents

Keratinocyte serum-free medium (KSFM), keratinocyte growth supplements (KGS), and penicillin/streptomycin (Pen/Strep) solution were purchased from ScienCell (Carlsbad, California, USA). Phosphate buffered saline (PBS) and Keratinocyte growth media (KGM-2) containing high calcium and the bullet kit were purchased from Lonza (Allendale, New Jersey, USA). Dulbecco's Modified Eagle Medium (DMEM), 1:1 DMEM in Ham's F12 nutrient medium, fetal bovine serum (FBS), and TrypLE Express were purchased from Life Technologies (Burlington, Ontario, Canada).

Propidium iodide (PI) and the FLICA caspase kits were purchased from ImmunoChemistry Technologies, LLC (Bloomington, Minnesota, USA). The FLICA kits use the fluorescent probes FAM-VAD-FMK (green) or SR-VAD-FMK (red) for caspase detection. FLICA is a fluorochrome-labeled inhibitor that binds to activated caspases in cells [187]. The FLICA inhibitor can penetrate

the cell and is noncytotoxic. Unbound FLICA molecules can be washed away; the fluorescent signal is a direct measure of the amount of active caspase that was present at the time the inhibitor was added.

4.2.2 Contact Lens and Ocular Solution

A daily-wear silicone hydrogel balafilcon A (BA; Bausch and Lomb, Rochester, New York, USA) was tested. Lenses were obtained in their original packaging from the manufacturer and had a curvature of 8.6 mm, diameter of 14 mm, and power of -3.00 dioptres. All lenses were used before their expiry date. Whole lenses were used and were not cut before placement on the cultures.

All contact lens solutions were sterile and came directly from the manufacturer's bottle. Two contact lens solutions were tested: namely the hydrogen peroxide-based solution Clear Care (Alcon; Fort Worth, Texas, USA) and the polyhexamethylene biguanide (PHMB)-based solution renu Fresh (Bausch & Lomb; Rochester, New York, USA). renu Fresh (Renu) contains Hydranate™ (hydroxyalkyl-phosphonate), boric acid, edetate disodium, poloxamine, sodium borate, sodium chloride and is preserved with polyaminopropyl biguanide (0.0001%) [35]. Clear Care (CC) contains 3% hydrogen peroxide, sodium chloride 0.79%, Pluronic 17R4, and uses a phosphate buffer system.

Contact lenses were removed from packaging under aseptic conditions and were rinsed in 3 mL of PBS before incubation with lens-cleaning solution. Lenses to be soaked in Clear Care (CC) were transferred to ASept Cups and were filled with Clear Care solution for disinfection. Renu-soaked contact lenses were soaked in 3 mL of renu Fresh in a 12 well polystyrene cell culture plate. Both renu- and CC-saturated lenses were soaked for 24 hours before application to the stratified cultures.

As a positive control of cytotoxicity [10], a sterile ophthalmic solution of benzalkonium chloride (BAK) was used from the commercially-available Moisture Eyes (ME; Bausch & Lomb; Rochester, New York, USA). ME has a BAK concentration of 0.01% w/v. ME is a proprietary solution that

contains other compounds such as surfactants. These surfactants have been shown to be biocompatible [10,188].

Staurosporine (STS) is an initiator of apoptosis [189]. Staurosporine isolated from *Streptomyces sp.* was obtained from Sigma Aldrich (Oakville, Ontario, Canada).

Fluorescein sodium ophthalmic solution (NaFl) was obtained as minims from Bausch & Lomb at a concentration of 2% w/v.

4.2.3 Monolayer Cell Culture

Telomerase-immortalized human corneal epithelial cells (hTCEpi) gifted from Dr. Danielle Robertson [61] were cultured in keratinocyte growth medium supplemented with the proprietary bullet kit; this medium may be referred to as KBM. Fresh medium was added every other day, and cells were grown to 90% confluency and were used before their twentieth passage. Adherent cells were removed using TrypLE Express dissociation solution. Cells were routinely inspected for any morphological changes.

Monolayers were prepared by seeding 1×10^5 cells in a 24-well polystyrene cell culture plate (Becton Dickinson, Mountain View, California) and were allowed to reach confluency for a full day after seeding.

After achievement of a monolayer of HTCEpi, cells were fed with a total of 500 μ L of solution. BAK-treated wells contained 1, 5, or 10% Moisture Eyes (0.0001, 0.0005, or 0.001% BAK) and STS-treated wells contained 0.1, 0.5, or 2 μ M STS. All dilutions were performed in KBM. All control wells received only KBM. Cells were incubated for 2 or 4 hours in a sterile humidified environment at 37°C and 5% CO₂.

Cells were then removed from the cell culture plates using TrypLE Express dissociation solution and were washed via centrifugation. Aliquots were then transferred to flow cytometry tubes. Tubes contained one of three mixtures: (a) 50 μ L of DMEM containing 0.5 μ L of propidium iodide (PI); (b)

50 μ L of 0.05% sodium fluorescein (NaFl); or (c) diluted FITC-VAD-FMK (pan-caspase inhibitor). PI-treated samples were immediately analyzed by flow cytometry. NaFl-stained samples were washed three times before analysis via flow cytometry. As per manufacturer's instructions [190], caspase samples were incubated for one hour at 37°C prior to washing and subsequent analysis. PI, NaFl, and caspase were all analyzed separately due to the broad emission spectra from NaFl and its signal overlap with both PI and caspase on our flow cytometer.

Modifications were made to the flow cytometry protocols for microscopy analysis of the samples. Simply, cells were left attached and washes were done in a cell culture plate. Cells were imaged using a Nikon Eclipse TS100 (Tokyo, Japan) fluorescent microscope and a Nikon DS camera.

4.2.4 Multilayer Cell Culture

HPV-immortalized human corneal epithelial cells (HCEC) gifted from Dr. May Griffith [49] were cultured in keratinocyte serum-free medium with keratinocyte growth supplements (bovine pituitary extract and recombinant epidermal growth factor) and Pen/Strep; this medium may be referred to as KSFM. Fresh medium was added every other day, and cells were grown to 90% confluency and were used before their twentieth passage. Adherent cells were removed using TrypLE Express dissociation solution. Cells were routinely assessed for any morphological changes.

Curved multilayers were prepared as previously described [10]. Briefly, 5×10^5 HCEC were seeded on deformed Millicell-HA (mixed cellulose esters, Millipore, Billerica, Massachusetts, USA) inserts with a 0.45 μ m pore size following a coating with collagen type I (Sciencell, Carlsbad, California, USA). Silicone Press-to-Seal sheets with adhesive (Life Technologies, Burlington, Ontario, Canada) were punched into rings and were placed prior to seeding on the curved filter. The curved multilayers were fed with KSFM on each of the basal and apical sides of the curve for the first seven days, with medium being exchanged every other day. After eight days, cell differentiation was induced by exposing the monolayer to an air-liquid interface. Cells were fed only on the basal side

with 2% FBS in 1:1 DMEM/F12 and medium was exchanged daily. The cells grew under these conditions for seven days prior to experimentation.

4.2.5 *In vitro* Onlay Model

Prepared stratified cultures were fed with KSFM on the basal side. Apically, KSFM was added to wet the surface and CC- or renu-soaked BA lenses were placed gently on the top of the multilayer, concave-side down, to fit the eye-shaped curve. An additional amount of KSFM was added on top of the lens to ensure moisture retention. Moisture Eyes was diluted to have a concentration of 0.01% or 0.005% BAK for MTT and staining experiments, respectively. Upon placement of the lenses, the stratified cultures were transferred to a humidified incubator for 2, 4, or 6 hours at 37°C and 5% CO₂.

Each plate of multilayers contains six wells. The six wells were divided into two sets of three containing a no solution control, a BA CC test, and a BA renu test. Each plate had two sets of three, which were run at different time points. Multiple plates were required for one replicate of the experiment.

4.2.6 MTT Assay

After incubation, lenses and medium were removed to quantify cellular viability using a modified MTT assay [10]. Thiazoyl blue tetrazolium bromide (0.5 mg/mL, MTT, Sigma Aldrich, Oakville, Ontario, Canada) was added to the apical and basal sides of the cell culture insert and was incubated for 3 hours at 37°C and 5% CO₂. The MTT solution was then removed and isopropanol was added to both the apical and basal sides of the inserts and plates were agitated for 2 hours. The solutions on the apical and basal sides were mixed and an aliquot was taken. Samples were read in a UV-Vis spectrophotometer for optical density at 595 nm with a reference at 650 nm. All results are expressed as relative viability compared to control cells; cells incubated in KSFM and in the absence of a contact lens (no lens control). Cellular metabolic activity determined from the mixed solution is reported in the results section.

4.2.7 Fluorescein Staining and Biomicroscopy

Following lens and solution incubation, lenses and medium were removed and 2 mL of fresh KSFM was added on the basal side of the inserts. 1 mL of 0.2% NaFl in KSFM was added to the top of the insert for 1 min. The fluorescein was then aspirated and cells were washed 3 times with PBS.

Curved multilayer inserts were mounted on a laboratory stand inside of a small polyvinylchloride holder with an opaque background to enhance contrast. The curved stratified epithelium was visualized with a Carl Zeiss slit lamp SL-130 (Oberkochen, Germany) with an attached Sony 3CCD colour video camera (Minato, Japan). To visualize the staining, a cobalt blue excitation filter was used with a yellow barrier filter to enhance contrast.

4.2.8 Cell Collection and Tissue Digestion

Digestion and flow cytometry on the curved, stratified, corneal epithelial model has been previously reported [10]. After lens incubation, the medium on the top of the well was used to wash the contact lens and the surface of the stratified culture. This population of cells and debris was collected and is referred to as the supernatant population. Upon removal of the supernatant population, DMEM/10% FBS was added to the supernatant cells and the samples were centrifuged. Supernatant cells were resuspended in DMEM/10% FBS and prepared for flow cytometry.

Once the supernatant population and the contact lens were removed, the multilayers were digested. 0.25% Trypsin-EDTA was added to the basal side of the insert and to the top of the insert. Cell dissociation buffer was then added to the top of the insert and cells were placed on a shaker at 120 rpm for 45 min in a 37°C and 5% CO₂ environment. After 45 minutes, FBS was added to the inserts and cells were briefly resuspended via pipetting. The cells were returned to the shaker for another 45 minutes. Once the cells were removed, the cell-containing media from the top of the filter was transferred to polypropylene tubes containing DMEM/10% FBS. Cells were then centrifuged and resuspended in fresh media. This population is referred to as the adherent population.

4.2.9 Flow Cytometry

After resuspension in DMEM/10% FBS, aliquots were transferred to flow cytometry tubes. Flow cytometry tubes contained FITC-VAD-FMK (pan-caspase inhibitor). As per kit instructions [190], caspase samples were then washed and propidium iodide (PI) was added. Samples were immediately read on a FACSVantage flow cytometer (Becton Dickinson, Mountain View, California, USA) using CELLQuest Software. At least 5000 events were collected per sample. Analysis was performed with CELLQuest post data acquisition.

4.2.10 Statistical Analysis

All results are reported as means \pm standard deviation. To evaluate the significances in monolayer necrosis and apoptosis, multilayer viability and caspase expression, and solution-induced corneal staining of the multilayers, an analysis of variance (ANOVA) was performed, followed by multiple pairwise comparisons using the Tukey's Honest Significant Difference test using Statistical Analysis Software (SAS, Cary, North Carolina, USA). Samples were compared to cells incubated without lens or solution (no lens control), as well as to cells exposed to a PBS-soaked BA lens (BA PBS control). Significant differences between compounds tested may also be reported. A p value of less than 0.05 was required for statistical significance. The number of experiments was equal to or greater than three and experiments were performed on different days.

4.3 Results

4.3.1 Monolayer Observations

As shown in Figure 4-2, BAK induced necrosis (as measured by PI) in a monolayer of hTCEpi. Cell death was also dependent on BAK concentration. STS induced necrosis and apoptosis (as measured by the pan-caspase inhibitor FAM-VAD-FMK) as illustrated in Figure 4-3. With increasing concentrations of staurosporine, there was a marked increase in the level of cell death

(apoptosis and necrosis) as compared to baseline. 0.5 and 2 μ M concentrations of STS induced significantly higher levels of caspase and PI staining at both 2 hours ($p < 0.03$) and 4 hours ($p < 0.007$).

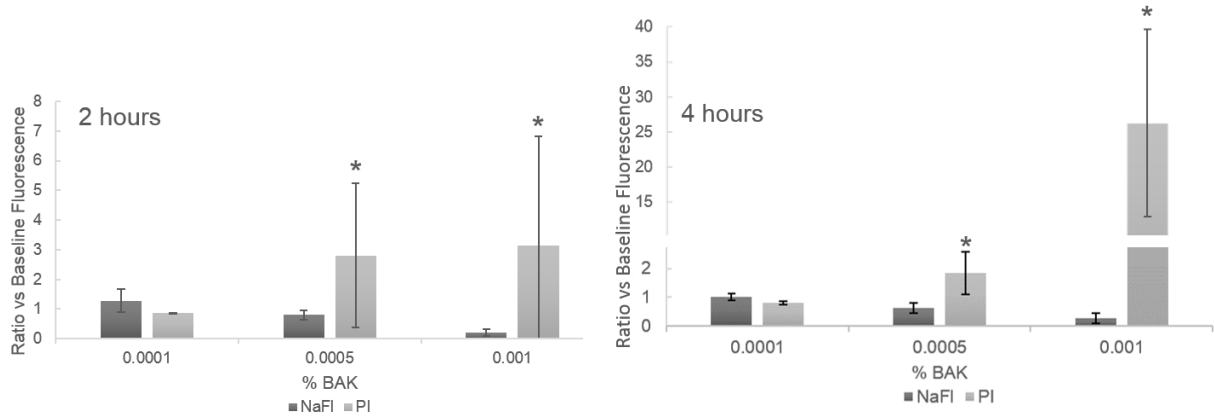


Figure 4-2: Effect of varying concentrations of BAK on necrosis and sodium fluorescein staining in monolayers of hTCEpi. Following exposure to 0.0001%, 0.0005%, and 0.001% BAK for 2 hours and 4 hours, monolayers were detached from a cell culture plate and processed for flow cytometry. Cells were stained with NaFl or PI and were read immediately on a flow cytometer. PI intercalates with DNA in membrane-compromised (necrotic) cells and fluoresces strongly in this capacity. n=3, *significantly different from NaFl ratios of fluorescence intensity, $p < 0.05$, BAK: benzalkonium chloride; NaFl: sodium fluorescein; PI: propidium iodide.

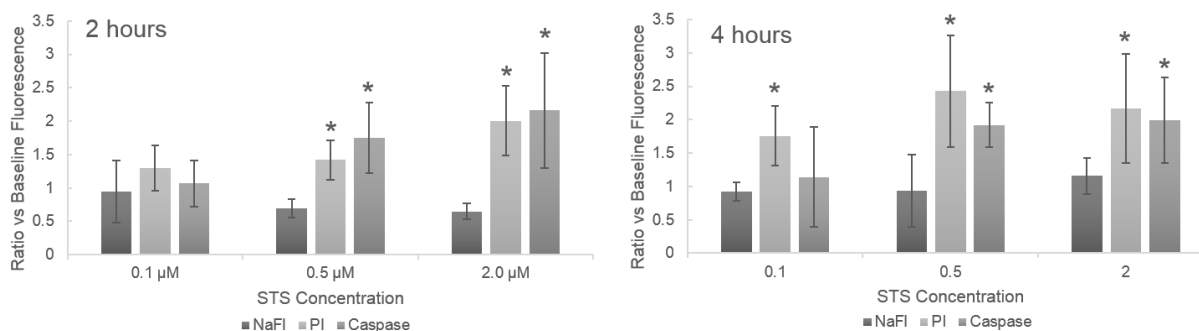


Figure 4-3: Effect of varying concentrations of STS on cell death and sodium fluorescein staining in monolayers of hTCEpi. Following exposure to 0.1 μM, 0.5 μM, or 2.0 μM STS for 2 hours and 4 hours, monolayers were detached from a cell culture plate and processed for flow cytometry. Cells were stained with NaFl, PI or a pan-caspase inhibitor and were read immediately on a flow cytometer. PI intercalates with DNA in membrane-compromised (necrotic) cells and fluoresces strongly in this capacity. In the presence of activated caspases within cells, a fluorescent signal is observed with the probe FAM-VAD-FMK: it is thus a marker of apoptosis. n=3, *significantly different from NaFl ratios of fluorescence intensity, p<0.05, NaFl: sodium fluorescein; PI; propidium iodide; STS: staurosporine.

In both BAK and STS treated samples, levels of sodium fluorescein staining intensity decreased with increasing cell death. Specifically, the proportion of cells that stained positive for sodium fluorescein actually decreased with increasing concentrations of staurosporine as illustrated in Figure 4-4.

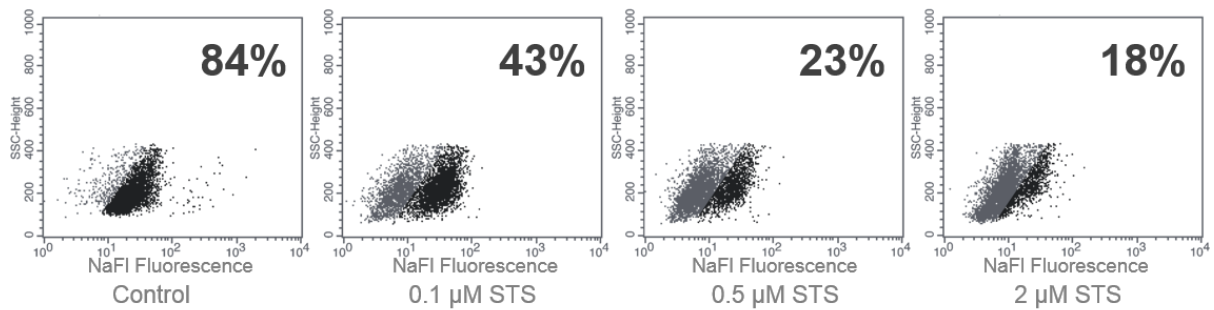


Figure 4-4: Flow cytometry dot plots showing percentages of cells stained positive with sodium fluorescein. Monolayers of hTCEpi were grown in polystyrene cell culture plates and were incubated with various concentrations of staurosporine (STS) for two hours. Control cells were incubated in the presence of cell culture media, alone. Cells were then detached and processed for flow cytometry. Black represents the population of sodium fluorescein-positive-stained cells while grey is for non-fluorescein-stained cells. With increasing concentrations of staurosporine, a decrease in the fluorescence intensity of sodium fluorescein staining can be observed. These plots are representative of three replicates.

To allow for staining of both fluorescein-stained cells and caspase-positive cells, SR-VAD-FMK was used in the microscopy experiments. SR-VAD-FMK fluoresces in the red spectrum, whereas FAM-VAD-FMK fluoresces in the green spectrum. Microscopy of cells stained with SR-VAD-FMK and sodium fluorescein, as illustrated in Figure 4-5, shows that normal, healthy epithelial cells all were capable of uptaking sodium fluorescein. Minimal caspase activity was detectable in the control samples. Alternatively, for the BAK-treated samples after a two hour incubation, cell death was prevalent. As highlighted in Figure 4-5, there was minimal overlap between the fluorescein-positive (green) and caspase-positive (red) cells.

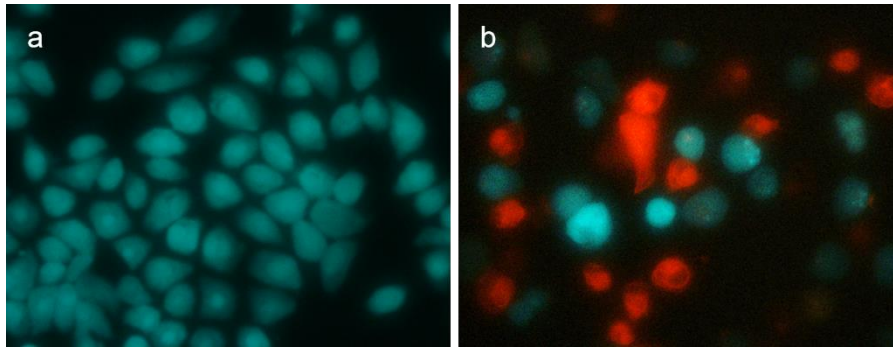


Figure 4-5: Fluorescent microscope images of (a) control and (b) BAK-treated samples after staining with sodium fluorescein (green) and SR-VAD-FMK, a pan-caspase inhibitor (red). hTCEpi cells were grown as a sub-confluent monolayer and were incubated for two hours in (a) cell culture medium or in (b) a solution of 0.001% BAK. Original images taken at 40x magnification.

4.3.2 Multilayer Observations

4.3.2.1 MTT Viability

As shown in Figure 4-6, no difference in viability was observed between BA PBS and the no lens control ($94\pm 5\%$ and $98\pm 10\%$ for 2 and 6 hours, respectively). Incubation with 0.01% BAK resulted in a drastic reduction in viability ($36\pm 6\%$ and $22\pm 5\%$ for 2 and 6 hours, respectively) as compared to the BA PBS control ($p < 0.001$). These results are in accordance with our previously published study [10].

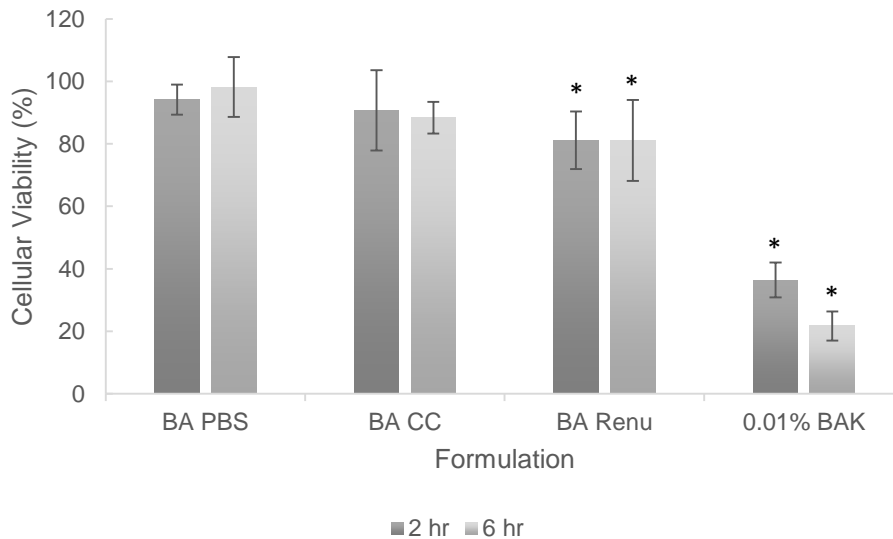


Figure 4-6: Effect of lens and solution combinations on cellular viability of curved stratified epithelium. Cells were incubated for 2 or 6 hours with a PBS-, CC-, or Renu-soaked balafilcon A contact lens. 0.01% BAK is used a positive cytotoxic control. Viability was measured by the MTT assay and is expressed as a percentage relative to cells grown in the absence of a contact lens or ophthalmic solution (control). n=4, *significantly different from BA PBS control, $p < 0.05$. BA: balafilcon A, BAK: benzalkonium chloride, CC: Clear Care, PBS: phosphate buffered saline.

The slightly reduced viability observed with BA CC was not statistically significantly different compared to the BA PBS control. While BA Renu had a lower viability compared to BA PBS, 78 ± 9 at 2 hours ($p < 0.04$) and 81 ± 13 at 6 hours ($p < 0.01$), the reduction in viability with BA Renu did not reach statistical significance when compared to BA CC.

4.3.2.2 Caspase Expression

BAK proved to be a strong positive control, with increases in caspase expression relative to control (Figure 4-7). No significant upregulation of caspases was observed with BA lenses soaked in PBS. Neither BA lenses soaked in Clear Care nor in Renu induced a statistically significant increase

in caspase expression when compared to a BA lens soaked in PBS. However, it should be noted that the supernatant portion of cells shows a mild upregulation with BA Renu (1.27 ± 0.5 and 1.28 ± 0.2 , 2 and 6 hours, respectively) versus BA CC (1.04 ± 0.2 and 0.98 ± 0.1 , 2 and 6 hours, respectively). The adherent population showed no upregulation of apoptosis in any of the lens and solution combinations.

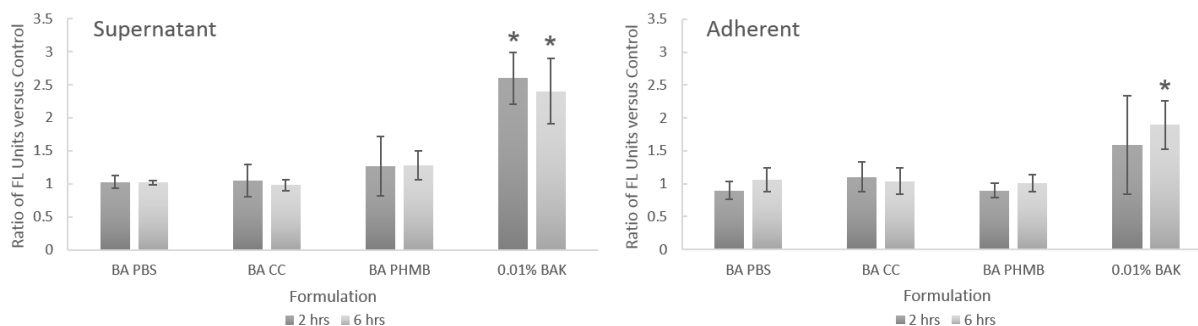


Figure 4-7: Effect of contact lens and solution interactions on caspase expression in the supernatant and adherent populations. Following exposure to stimuli after 2 or 6 hours, the curved, stratified cultures of human corneal epithelial cells were digested with trypsin and EDTA. Caspase expression was measured by flow cytometry and is expressed as a ratio relative to its expression on cells incubated in the absence of a contact lens or solution (control). n=3, *significantly different from the BA PBS control, p<0.05. BA: balafilcon A; BAK: benzalkonium chloride; CC: Clear Care; PBS: Phosphate buffered saline.

Caspase samples were also counterstained with propidium iodide to investigate changes in the necrotic population of cells, however, all values for lens and solution combinations remained similar to no lens controls (data not shown).

4.3.2.3 Corneal Staining

Following incubation with lens and solution combinations, the curved, stratified cultures were subjected to fluorescein staining and slit lamp biomicroscopy in a manner similar to clinical

examination. The curved, stratified epithelium was capable of presenting a micropunctate staining pattern and this is the first time, to our knowledge, that this has been replicated *in vitro*. Prior, slit lamp biomicroscopy has only been performed on monolayers [179].

There was variability in the amount of staining observed with the curved multilayer model (CML) as control wells that were treated solely with cell culture media occasionally expressed micropunctate staining. To better understand this phenomenon of variability in staining of our curved, stratified multilayers, we investigated how staining happens over time. As shown in Figure 4-8, formazan staining indicated a seemingly linear increase in surface coverage by the HCEC. However, sodium fluorescein staining of the curved model showed a drastically different pattern during growth of the multilayers (Figure 4-9).

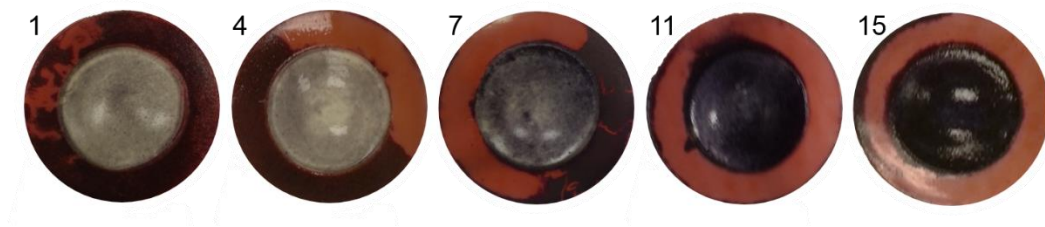


Figure 4-8: Growth of a curved stratified epithelium. Human corneal epithelial cells were grown for 15 days and were stained with MTT on days 1, 4, 7, 11, and 15 of growth. Formazan (purple) staining indicates viable cells.

Following one day after seeding, all cells uptake sodium fluorescein, which is consistent with our own monolayer observations, and with the literature, reproducibly showing that sub-confluent monolayers uptake sodium fluorescein [157,158,179]. However, as the monolayer becomes more confluent, this staining decreases in both intensity and distribution until Day 7 where there is no observable staining. It is worth noting the possibility that these cells still uptake sodium fluorescein, but lack the necessary contrast to observe staining. Cells are differentiated on Day 8, and on Day 9 (one day after differentiation), there is still no observable staining. On Day 11, intensity and

concentration of staining is the greatest that has been observed with our model. It is also important to note that staining on Day 11 could be observed by different cells on different focal points, implying that superficial layers of cells are not alone responsible for fluorescein staining at this time point.

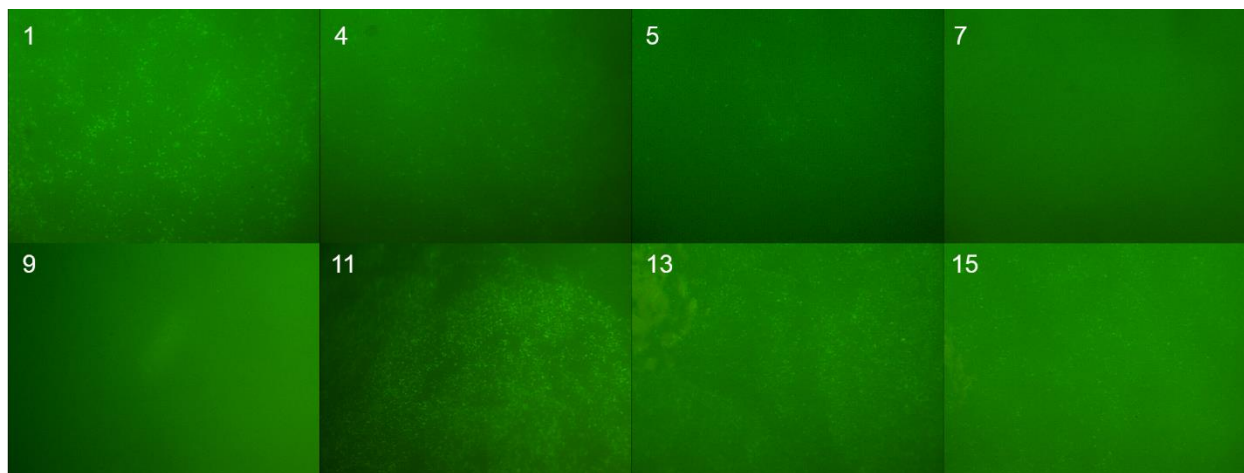


Figure 4-9: Slit lamp evaluation of a curved stratified epithelium during growth. Human corneal epithelial cells were grown for 15 days. Cells were stained with sodium fluorescein at days 1, 4, 5, 7, 9, 11, 13, and 15 and were imaged using a slit-lamp biomicroscope with a cobalt blue excitation filter and a yellow barrier filter to enhance contrast. An air-liquid interface was established on day 8. All images were taken at 32x magnification. This is representative of three different observations.

To determine if the CML was sensitive to solution-induced corneal staining, we first developed a staining score system as shown in Figure 4-10. A score of zero was given if there was no observable staining across the multilayer, a one was given if there was a minimal amount of corneal staining, a two was given if there was a decent amount of corneal staining, and a three was given if staining was prevalent across the multilayer. On occasion, some scores were hard to judge as staining may have been intensely localized to a portion of the multilayer, rather than been evenly distributed across the multilayer.

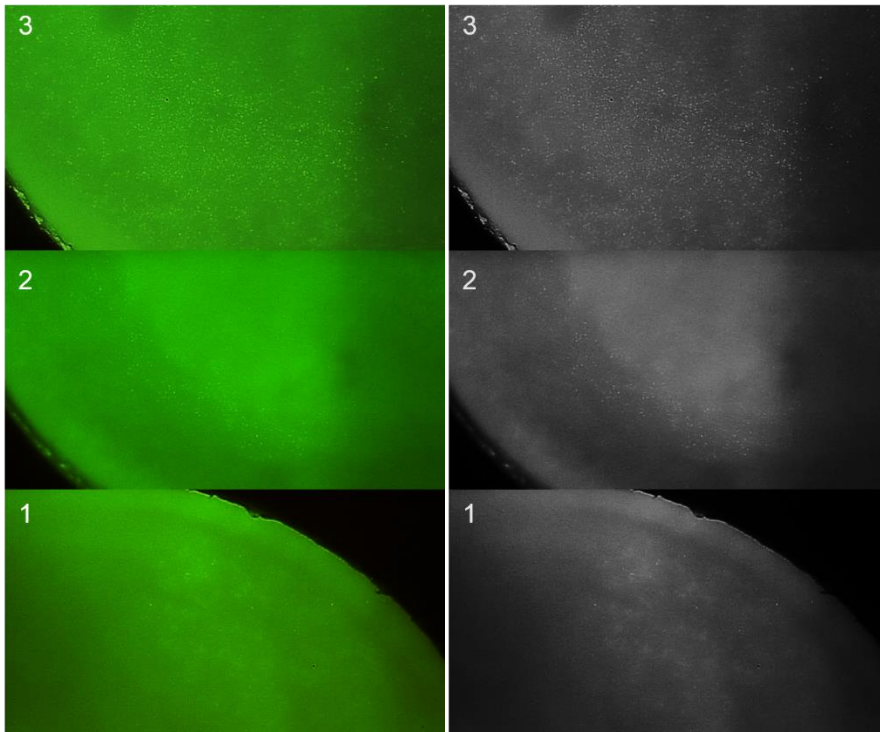


Figure 4-10: Examples of different staining scores of sodium-fluorescein stained curved stratified epithelium. Cells were incubated with lens and solution combinations and were then stained with sodium fluorescein and imaged using a slit-lamp biomicroscope with a cobalt blue excitation filter and a yellow barrier filter to enhance contrast. Original images on the left; contrast enhanced images on the right. All images taken at 8x.

Figure 4-11 shows the response of the CML to solution-induced corneal staining. Our model appears to be most sensitive at the 2 hour time point, where an almost statistically significant differences can be observed between BA Renu and BA CC ($p < 0.08$) and between BA Renu and a no-lens control ($p < 0.07$). By 4 hours and 6 hours, there are relatively no differences in the levels of staining between control, BA Renu, and BA CC.

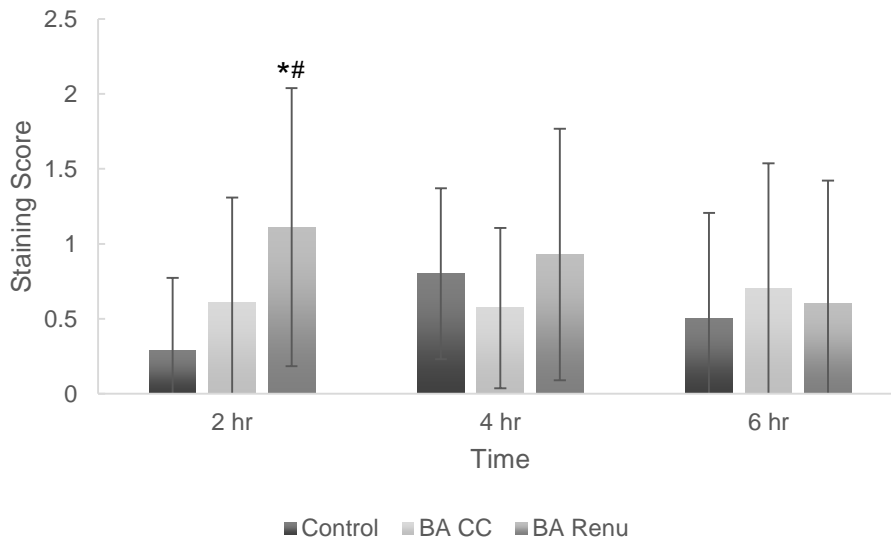


Figure 4-11: Relative levels of sodium fluorescein staining of the curved stratified epithelium. Cells were incubated for 2, 4, or 6 hours with different lens and solution combinations. Following lens incubation, lenses were removed and epithelium were stained with sodium fluorescein. Resulting staining patterns were given a score of 0-3 as described in Figure 4-10. n=5, * statistically different from no lens, no solution control (p<0.07), # statistically different from BA CC (p<0.08). BA: balafilcon A, CC: Clear Care.

4.4 Discussion

Our monolayer studies are in agreement with the work performed by Bakkar *et al.* [158] and by Bandamwar *et al.* [179]. These two studies have performed monolayer experiments to confirm two major observations: that fluorescein is localized intracellularly and that dead cells do not uptake sodium fluorescein. Intracellular staining was first proposed by Feenstra and Tseng in 1992 [157] and was more recently demonstrated by Glasgow's group [163,177]. Our research group has also observed intracellular staining by sodium fluorescein and we have previously confirmed that *ex vivo* collected corneal epithelial cells stained with fluorescein do not stain with propidium iodide [7,178]

The recent work of Bakkar has shown that active transport is responsible for uptake into L929 murine fibroblasts as there is a temperature-dependence upon both uptake and release of fluorescein by cells [158]. Bandamwar *et al.* performed a similar study, but arrived at a result that apoptotic cells are responsible for hyperfluorescent cells [179]. Our research group, in collaboration with the Centre for Contact Lens Research at the University of Waterloo, has also demonstrated that hyperfluorescence can be observed occasionally with apoptotic cells [178]. The present study does not strictly correlate with these results and may be a result of differences in experimental methods. Namely, samples in this work were analyzed via flow cytometry and were removed via trypsinization. Bandamwar *et al.* used a different cell line, different method of apoptosis induction, and a different marker of apoptosis [179]. It has been previously shown that FLICA markers of caspase activation (specifically FAM-VAD-FMK) may provide more information on early apoptosis whereas Annexin V may be more related to late apoptosis [191]. However, the difference in results likely comes from the difference of hyperfluorescence versus general fluorescein staining.

Monolayer *in vitro* models have much utility, but are limited in many ways because of their simplicity and hypersensitivity. Our current study also lacked a positive control that could effectively induce corneal staining or increase the levels of fluorescein staining. This is all the more complicated by the fact that cells in a monolayer already stain positive for NaFl (as illustrated in Figure 4-4). To date, no positive control for SICS has been found reliably *in vitro*, as BAK does not appear to induce a similar response.

In the development of an *in vitro* stratified model of sodium fluorescein staining, it was important to evaluate how fluorescein staining changes as a result of multilayer stratification. It has been shown in sub-confluent monolayers that uptake of sodium fluorescein is consistent and present among all cells, even across cell types [157,158,179]. In the stratified cultures used in our experiments, one day after seeding-while the cells are still in a monolayer-all cells displayed a similar, unilateral uptake of

sodium fluorescein. As the HCEC continue to form a confluent monolayer, the overall presence and intensity of fluorescein staining decreased even though more cells were present on the surface of the curve as demonstrated by formazan staining. By the time cultures were confluent, the level of staining was almost negligible. Thus, it is likely that there is some sort of reorganization of the multilayer that occurs during stratification which allows for the multilayer to become susceptible to stain with fluorescein. As medium was changed and an air-liquid interface was provided to promote stratification, no immediate changes were observed (Day 9), but by Day 11, there was a significant and intense level of fluorescein staining. It should also be noted that this staining occurred throughout different focal points, implying that more than the superficial layer stained with sodium fluorescein. As the multilayer completed its stratification (Day 15), this total level of staining was reduced to being unobservable. These progressive changes in sodium fluorescein staining suggest that as the cells stratify, they undergo a reorganization that allows for a susceptibility to sodium fluorescein uptake. Or, possibly, this reorganization allows for enhanced contrast of the human corneal epithelium.

Two contact lens and multipurpose solution combinations were tested in this study, namely BA Renu and BA CC. These solutions were compared to previously published controls of BA PBS and BAK which both performed as expected [10]. BA Renu is recognized to induce corneal staining *in vivo* [36] and has been used as a positive control of SICS in many studies [178,192,193]. Conversely, BA CC is known to be a suitable control as evidence shows that it stains much less frequently than other lens and solution combinations [36,38,165,192,194]. To our knowledge, this is the first time that staining has ever been induced by these lens-solution combinations *in vitro*.

Corneal staining has been shown to peak *in vivo* at around 1 hour after lens insertion and plateau until 2 hours before decreasing in intensity [192,195,196]. The CML shows a somewhat similar response with the highest level of staining for BA Renu at 2 hours, and generally lower at 4 and 6

hours. However, this result did not achieve statistical significance ($p=0.21$). BA CC showed almost no time dependence ($p=0.95$) which suggests that staining for BA CC may have been heavily influenced by model variability. While our control wells, incubated with cell culture media alone, also showed variable levels of staining, any cultures incubated with 0.005% BAK did not show any staining whatsoever, further confirming that BAK cannot be used as a control for SICS.

Flow cytometry of the multilayers confirm that BAK samples induce both apoptosis and necrosis in the cell population. Given the lack of a dynamic system and a tear dilution, such as blinking and tear exchange *in vivo*, the response of the stratified curved epithelium may likely be overestimated in this *in vitro* model, however, it serves as a good control to assess cell death. Across all lens and solution combinations, only BA Renu showed minimal upregulation of caspases at 2 hours. In both the monolayer and multilayer models, fluorescein staining did not correlate with a significant increase in cell death. In a stratified *in vitro* model, or even SICS *in vivo*, only a small percentage of the superficial layers of the epithelium stain with a micropunctate fluorescein pattern. It may therefore be possible that these cells are damaged, but in the context of digesting a tissue via flow cytometry, they would not be significant in an entire population of epithelial cells. Nevertheless, it may be possible that a slight upregulation of caspases or slight decrease in viability could provide enough of a destabilization of epithelial integrity to increase microbial susceptibility. It may be possible that incubation in a dynamic environment such as the tear replenishment system, which replicates the fluidic component of blinking *in vitro* [11], could affect solution release from lenses and their subsequent interaction with the corneal epithelium. This could help identify differences between lens and solution combinations, and requires further research.

While cell death has been one mechanism that has been implicated in sodium fluorescein staining, mucin disruption has been another. It has been previously reported by Gordon *et al.* that such a disruption in the mucin glycocalyx could allow for fluorescein staining to occur and thus put the

cornea at risk of infection [83]. In a monolayer study using human corneal limbal epithelial cells, the authors noted that rose bengal staining as a result of multipurpose solution exposure may lead to a loss of MUC16 and consequently have increased infection [83]. It has also been reported that exposure to MPS containing boric acid downregulate mucin expression *in vitro* and *in vivo* [197-199]. Rose bengal staining has been correlated with solution-induced corneal staining [193], and fluorescein staining has also been postulated to occur as a result of mucin loss [177]. In a recent study by Gorbet *et al.*, BA Renu was shown to have higher rates of shedding of corneal epithelial cells from the ocular surface as compared to BA CC [178]. With a limited sample size (8 participants), staining was also shown to be highest at 2 hours, and shedding was highest at 4 hours of lens wear – both were reduced at 6 hours. It has yet to be confirmed whether this change in shedding correlates with changes in mucin expression in the ocular surface epithelium.

Increased infection with staining has also been observed *in vivo*. 7% of fluorescein staining with lens wear coincides with both symptomatic and asymptomatic corneal infiltrative events [165], but SICS may not be causative of these events [200]. It was also recently reported there is no significant difference in comfort between BA Renu vs BA CC [201]. Susceptibility to infection could be a result of reduced barrier function, as it has been shown in a pilot clinical study that barrier function is reduced with lens and solution combinations that induce corneal staining [202].

Overall, fluorescein staining may not be a very sensitive metric of ocular health and of lens and solution biocompatibility. Uptake and release of sodium fluorescein by monocarboxylate transporters may be easily affected by pH [182], which could be affected either by multipurpose solution components [161] or by changes in physiological conditions in disease such as dry eye [203]. Intensity, prevalence, or resolution of corneal staining may be affected by humidity [160,204], punctum plugging [205,206], artificial tear substitutes [207-209], and smoking [210]. All of which could potentially affect the ocular surface mucin glycocalyx.

Over the last century, vital staining with sodium fluorescein and its derivatives, namely rose Bengal and lissamine green, has been able to provide us with a lot of information about changes to the health of the ocular surface. However, the aetiologies with which cells stain at a metabolic level may often be insufficient for diagnostic purposes. Apoptotic cells may have a hyperfluorescence, and yet dead cells do not fluoresce at all. Mucin disruption may be responsible for cells to uptake fluorescein, and yet a normal, healthy cornea, may exhibit cells that hyperfluoresce with fluorescein. It has been suggested that the fluorescein-labelled octadecyl ester (FODE) may be a better diagnostic stain given its specificity for tear lipocalin in mucin-depleted areas of the ocular surface [203]. Overall, there is a definite requirement by academia and industry for novel dyes with improved ability to diagnose dysregulation of health at the ocular surface.

4.5 Conclusions

Investigation of hyperfluorescent corneal epithelial cells may be a biomarker of susceptibility, however, monolayer and multilayer corneal epithelial cultures that indicate chronic damage and are prevalent with apoptotic and necrotic cell populations do not uptake sodium fluorescein.

The curved, stratified, corneal epithelium enabled the first reproduction of micropunctate corneal staining *in vitro*. BA Renu and BA CC are both biocompatible, though there are acute differences between the response of cultures to these different lens and solution combinations. While BA Renu shows signs of solution-induced corneal staining in our *in vitro* model, this was only associated with a slight downregulation in viability and a slight increase in apoptosis. This may indicate a loss of some of the barrier mechanism of the corneal epithelium, but further detailed investigation is required.

Fluorescein staining is responsible for the clinical finding of lens and solution interaction. However, given the ability of fluorescein staining to stain in healthy epithelium, and its uptake and release to be affected by different environmental and physiological parameters, there is a need for new dyes with greater specificity as probes of ocular surface health and disease.

As the development of novel ocular stains and the improvement of lens and solution interaction continues, our curved, stratified epithelial model has been shown to be sensitive under a wide array of parameters and may be a suitable *in vitro* model for the exploration of novel lenses, solutions, and stains.

Contributions to Chapter 4

Author	Concept / Design	Acquisition of Data	Analysis	Write Up / Publication
C. Postnikoff	Y	Y	Y	Y
C. Vigouroux	Y	Y		
V. Coindre	Y	Y		
L. Carcreff	Y	Y		
M. Gorbet	Y		Y	Y

Chapter 5

Inability to replicate tear-film neutrophil phenotype using blood-isolated neutrophils, *in vitro*

A story of blood, sweat, and tears.

5.1 Introduction

When the eye is closed for a prolonged period of time, there is an influx of neutrophils on to the ocular surface [92,123,125]. Following discussion with several investigators, these neutrophils have since been termed by our research group “tear-film neutrophils” [16]. These neutrophils have been shown to be alive, but are limited in their ability to respond to inflammatory stimuli. A study where we compared and contrasted the response of tear-film neutrophils and neutrophils isolated from whole blood, or “blood-isolated neutrophils” was recently completed [16]. The main results of the previous study are depicted by Figure 5-1, where it can easily be observed that receptor upregulation on tear-film neutrophils is negligible in comparison to blood-isolated neutrophils. The goal of the present

investigation was to determine if closed-eye environmental conditions could be used to induce a phenotypic change in blood-isolated neutrophils *in vitro*.

	Tear PMNs			Blood PMNs			
	LPS	PMA	fMLP	LPS	PMA	fMLP	
CD11b Activation, adhesion, fibrinogen binding, complement	■	■	■	↑↑	↑↑	↑↑	
CD45 Leukocyte common antigen	■	■	■	↑	■	↑	ND No Data
CD54 Intracellular adhesion molecule-1	■	■	■	↑	↑↑	■	■ 0.8-1.2
CD66b Degranulation, released upon activation	■	↑	■	↑↑	↑↑	↑↑	↑ 1.2-1.6
C3a Complement activation	■	ND	↑	■	↑	■	↑↑ 1.6+
DCF Reactive oxygen species	■	↑	ND	↑↑	↑↑	↑↑	

Figure 5-1: Comparison of baseline membrane receptor expression of tear-film neutrophils versus blood-isolated neutrophils. Symbols represent relative ratios as compared to unstimulated (rest) controls. All data reprinted from [16].

Our research group has focused on the development of *in vitro* models of biocompatibility at the ocular surface and specifically, we have created a 3D curved, stratified model of the human corneal epithelium [10], and also fabricated a tear replenishment system that mimics the fluidic effect of blinking on these 3D cultures [11]. While our curved multilayer (CML) model is capable of providing strong estimates of cytotoxicity, the model does not incorporate any inflammatory mechanisms that would occur *in vivo*. Ultimately, it is hoped that future models will include inflammatory cells, to provide better indications of biocompatibility (or lack thereof), but firstly, there needs to be an improved understanding of the role of tear-film neutrophils in ocular surface inflammation.

Neutrophils, or polymorphonuclear leukocytes (PMNs), were thought to be terminally differentiated cells that are incapable of altering their gene expression following differentiation and

maturation in the bone marrow [211]. This has been proven to be untrue as subsets within the neutrophil population have the ability to actively change their gene expression to respond to inflammation [212,213], and can even reverse transmigrate across the endothelium [214]. Heterogeneity in the neutrophil population is not a new concept as oral [215-217], airway [218], and nasal [219] neutrophils have all shown a refractory behavior.

Our previous study concluded that artificial tear solution had a small, but significant, effect on the activity of blood-isolated neutrophils [16]. The tear film contains many proteins that participate in the ocular surface inflammatory response [93]. Notably, lactoferrin is a potent anti-inflammatory protein [220], and it may be possible that extended incubation with anti-inflammatory proteins could affect the behavior of blood-isolated neutrophils.

The corneal epithelium has developed many mechanisms to withstand different types of compromise and infection through the use of receptor expression – such as toll-like receptors, mucin receptors – or release of soluble factors such as cytokines [221] or lipid autocoids [106]. It has been observed that lymphocytes adhere and show a differential expression when incubated with cultured human corneal epithelial cells (HCEC) [222], and it is possible that, for the preservation of vision, neutrophils adopt a quiescent phenotype with HCEC.

One of the main ways that the closed eye differs from the open eye is the level of oxygenation at the ocular surface. Oxygen is important for the maintenance of corneal integrity and homeostasis [223]. In open eye conditions, the cornea receives most of its oxygen from the atmosphere which has a partial pressure of 20.9% O₂ (sea level) [224], and the epithelium receives the oxygen through its diffusion in the tear film [225]. However, under closed eye conditions, the level of oxygenation at the ocular surface decreases significantly. Three different studies have reported values around 8%, with the first being Fatt and Bieber who observed a value of 7.4% O₂ in one subject, followed by Efron and Carney who reported an average of 7.7% O₂ in 12 subjects [226], and finally Holden and

Sweeney who found an average of 8.2% O₂ in 16 subjects [227]. The cornea still receives oxygen in closed eye conditions, both from the palpebral conjunctival blood vessels [94], and from eyelid flicker during sleep [225,228]. Oxygen deprivation has been shown to specifically affect neutrophils both *in vitro* and *in vivo* [229-231], with effects on neutrophil survival, expression of neutrophil membrane receptors, and release of cytokines and reactive oxygen species.

Three closed eye conditions were tested alone and in combination on blood-isolated neutrophils to determine if environmental conditions could alter expression of neutrophil membrane receptors and induce a tear-film neutrophil phenotype. Blood-isolated neutrophils were incubated for six hours with artificial tear solution, in a co-culture with human corneal epithelial cells, and under hypoxic (2% O₂) conditions. Changes in cell culture receptor expression were monitored using flow cytometry.

5.2 Materials and Methods

5.2.1 Reagents and Antibodies

LPS (*Escherichia coli* serotype 0111:B4), phorbol-12-myristate-13-acetate (PMA), N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), endotoxin-free water, ethylenediaminetetraacetic acid (EDTA), and paraformaldehyde was purchased from Sigma-Aldrich Co. (Oakville, Ontario, Canada).

Keratinocyte serum-free medium (KSFM), keratinocyte growth supplements (KGS), and penicillin/streptomycin (Pen/Strep) solution were purchased from ScienCell (Carlsbad, California, USA). Phosphate buffered saline (PBS) was acquired through Lonza (Allendale, New Jersey, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and TrypLE Express were purchased from Life Technologies (Burlington, Ontario, Canada). All other chemicals were of analytical reagent grade.

Fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against human CD11b and CD66b, R-phycoerythrin (PE) conjugated monoclonal antibodies against human C3aR and CD54,

and R-phycoerythrin-cytochrome 5 (PE-Cy5) conjugated monoclonal antibody against CD45 were purchased from Becton Dickinson Pharmingen (San Diego, California, USA).

The FLICA caspase kit was purchased from ImmunoChemistry Technologies, LLC (Bloomington, Minnesota, USA). The FLICA kit uses the fluorescent probe FAM-VAD-FMK for caspase detection. FLICA is a fluorochrome-labeled inhibitor that binds to activated caspases in the cells [187]. The FLICA inhibitor can penetrate the cell and is noncytotoxic. Unbound FLICA molecules can be washed away; the fluorescent signal is a direct measure of the amount of active caspase that was present at the time the inhibitor was added.

5.2.2 Peripheral Blood Neutrophil Isolation

This study was conducted in accordance with the tenets of the Declaration of Helsinki and received ethics clearance from the University of Waterloo ethics committee. 5 participants (2 male and 3 female) donated their blood for the purpose of these experiments.

Blood was drawn from healthy participants who were free of medication for at least 48 hours. Low molecular weight heparin (10 U/mL) was used as an anticoagulant. Platelet-rich plasma was first removed by centrifugation at 100g. Blood leukocytes were then isolated using density gradient centrifugation (Polymorphprep, Axis Shield PoC AS, Oslo, Norway) followed by three washes in serum-containing media and EDTA, and were ultimately resuspended in DMEM/10% FBS or an Artificial Tear Solution (ATS). The artificial tear solution was a solution of phosphate-buffered saline containing tear film proteins as detailed in Table 5-1.

Table 5-1: Components of the artificial tear solution. Adapted from [232].

Salt Component		Protein Component	
Sodium Chloride	3.0 mM	Bovine Albumin	32.0 mg/mL
Potassium Chloride	24.0 mM	Hen egg lysozyme	3.6 mg/mL
Sodium Citrate Monobasic	26.0 mM	Bovine submaxillary mucin	3.6 mg/mL
Glucose		Bovine colostrums lactoferrin	24.0 mg/mL
Urea		Bovine immunoglobulin G	48.0 mg/mL
Calcium Chloride	0.5 mM		
Sodium carbonate	12.0 mM		
Potassium hydrogen carbonate	3.0 mM		
Sodium phosphate dibasic	24.0 mM		
Hydrochloric acid (10M)	26.0 mM		
MilliQ Water			

5.2.3 6 Hour Incubation

To try and induce a tear-film phenotype in blood-isolated neutrophils, varying six-hour incubations were performed as shown in Figure 5-2. Incubation was performed in either ATS or DMEM/10% FBS, under either hypoxic or normoxic conditions, in either a polypropylene tube or in a cell culture plate on top of a monolayer of human corneal epithelial cells. Each batch of experiments was performed at the same time on the same day to test all conditions using the same blood-isolated neutrophils. Preliminary experiments were also performed with a polystyrene cell culture plate in absence of human corneal epithelial cells, but there was significant attachment and activation of the blood-isolated neutrophils, so further experimentation was discontinued.

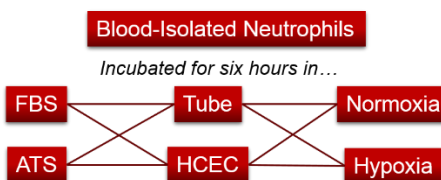


Figure 5-2: Protocol overview. Blood-isolated neutrophils were incubated for six hours in every combination of DMEM/10%FBS (FBS) or artificial tear solution (ATS), in a polypropylene tube or with a monolayer of human corneal epithelial cells, or in a normoxic or hypoxic condition.

Hypoxia

Hypoxia was induced through incubation of tubes and plates in a Billups-Rothenburg modular incubation chamber (Del Mar, California, USA). The sealed, air-tight chamber was flushed for 5 minutes with a gas mixture containing 5% CO₂, 2% O₂, and a balance of nitrogen. The chamber was then incubated at 37°C for 6 hours. Normoxic conditions were established through simple incubation in a humidified cell culture incubator at 37°C and 5% CO₂.

Cell Culture

HPV-immortalized human corneal epithelial cells (HCEC) gifted from Dr. May Griffith [49] were cultured in keratinocyte serum-free medium with keratinocyte growth supplements (bovine pituitary extract and recombinant epidermal growth factor) and Pen/Strep; this medium may be referred to as KSFM. Fresh medium was added every other day, and cells were grown to 90% confluency and were used before their twentieth passage. Adherent cells were removed using TrypLE Express dissociation solution. Cells were routinely observed for any morphological changes.

Monolayers were prepared by seeding 1×10^5 cells in a 24-well polystyrene cell culture plate (Becton Dickinson, Mountain View, California, USA) and were allowed to reach confluency for a full

day after seeding. After achievement of a monolayer of HCEC, cells were fed with a total of 500 μ L of DMEM/10%FBS or ATS immediately before the neutrophils were added.

5.2.4 Caspase Activity

After incubation, aliquots were transferred to flow cytometry tubes. Half of the tubes contained a dilute solution of staurosporine (5×10^{-10} mol/test) which is known to induce apoptosis [189]. As a positive control, staurosporine was used to “stimulate” the cells to ensure that the neutrophils were still capable of becoming apoptotic, and hence implying that they were not dead. Staurosporine-treated samples were incubated at 37°C for 30 minutes to induce cell death. Flow cytometry tubes contained FITC-VAD-FMK (pan-caspase inhibitor). As per kit instructions [190], caspase samples were incubated for one hour followed by three washes. Samples were immediately read on a flow cytometer.

5.2.5 Cell Stimulation

Following the 6 hour incubation of the blood-isolated neutrophils, the cells were then incubated with an inflammatory stimulus. Two stimuli, that are recognized to induce an inflammatory response in leukocytes, were used: lipopolysaccharide (10,000 EU/mL final concentration), also known as endotoxin [128]; and fMLP (16.7 nM final concentration), which induces a G-protein coupled receptor mediated physiological activation [233]. Samples were divided in aliquots (Rest, i.e. unstimulated; LPS-stimulated; fMLP-stimulated). Stimulation was performed at 37°C for 15 minutes (fMLP) and 30 minutes (LPS).

5.2.6 Expression of Membrane Receptors on Leukocytes

After incubation with stimulus, 30 μ L of cell suspension was transferred into tubes containing fluorescently-labelled antibodies against CD11b, CD45, CD54, CD66b, or C3aR and were incubated for 30 minutes at room temperature in the dark. At the end of the incubation, samples were diluted

and fixed with paraformaldehyde (1% final concentration). All samples were analyzed on the flow cytometer within 5 days.

5.2.7 Flow Cytometry

All samples were acquired on a Becton Dickinson FACSVantage flow cytometer (Mountain View, California) using CELLQuest Software. At least 5000 neutrophil events were acquired. Appropriate isotype controls were used with each experiment. Data analysis was performed using CELLQuest post data acquisition.

The following nomenclature is being used when reporting all results: neutrophils collected after sleep are referred to as “tear-film neutrophils”, neutrophils isolated from whole blood as “blood-isolated neutrophils”. To allow for comparisons between the different types of neutrophils, the mean fluorescence intensity in arbitrary units (AFU) as well as the ratio of the fluorescent intensities of rest (unstimulated) versus stimulated are presented.

5.2.8 Statistical Analysis

All subsequent data collected with blood-isolated neutrophils was collected from the same donors, within the same time period, and using the same settings, to allow for statistical comparison with the tear-film neutrophils of [16].

All results are reported as means \pm standard deviation. To evaluate the significance of the differences in the ratio of cell activation, an analysis of variance (ANOVA) was performed followed by multiple pairwise comparisons using the Tukey test. Analysis was performed using Statistical Analysis Software (SAS; Cary, North Carolina, USA) and a p value of less than 0.05 was required for statistical significance. For all experiments, the number of participants was equal to or greater than 3.

5.3 Results

Neutrophils were isolated from whole blood and were incubated in varied conditions to mimic some of the hallmarks of the closed eye environment: regular cell culture medium versus an artificial

tear solution; with or without a monolayer of human corneal epithelial cells; and in a normoxic (21% O₂) or hypoxic (2% O₂) environment.

After six hours of incubation in DMEM/10% FBS at 37°C and 5% CO₂, under normoxic conditions and in a polypropylene tube, blood-neutrophils remained capable of responding to inflammatory stimuli as shown in Figure 5-3. Following exposure to HCEC, blood-isolated neutrophils showed an increased expression of CD11b (cell-cell interaction) and CD66b (degranulation) in response to stimulation with LPS and fMLP. ATS changed the ability of blood-isolated neutrophils to activate as shown by a decrease in the response of CD11b and CD66b following stimulation with LPS. However, incubation in ATS showed an increase in the expression of CD11b and CD66b following stimulation with fMLP. Finally, incubation in hypoxic conditions showed negligible difference from incubation in normoxic conditions.

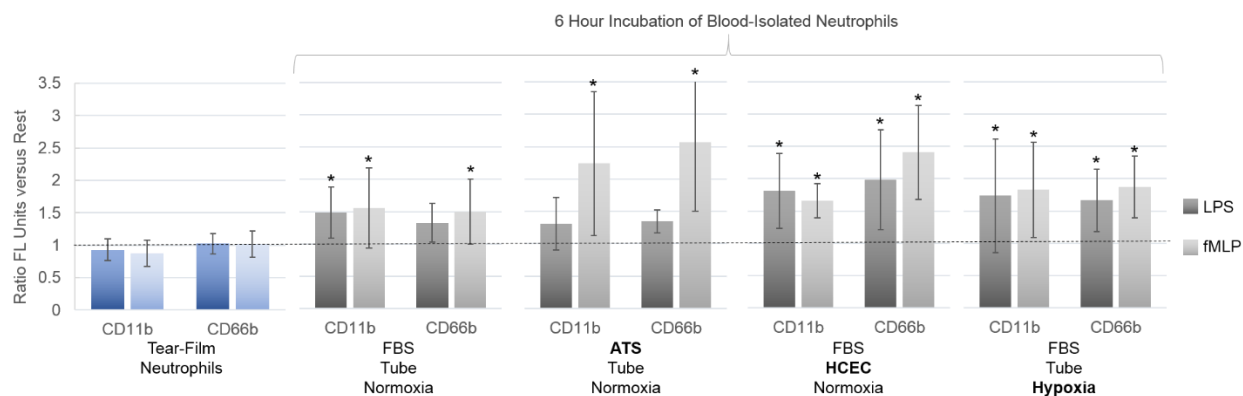


Figure 5-3: Comparison of tear-film neutrophil expression versus blood-isolated neutrophil expression following a 6 hour incubation under varied conditions. Neutrophils were isolated from whole blood using density gradient centrifugation and were incubated for six hours in different incubation media (DMEM/10%FBS vs ATS), with and without a monolayer of human corneal epithelial cells, and in a normoxic (21% O₂) or hypoxic (2% O₂) environment. Following incubation, cells were collected and were stimulated with fMLP or LPS. Neutrophils were assessed for membrane receptor expression of CD11b and CD66b using flow cytometry. All data is reported as ratios versus an unstimulated (rest) sample. The dotted line indicates the rest value normalized to 1. Tear-film neutrophil data is at baseline and is reprinted from [16]. n=3-5, *significantly different from rest values, p<0.05, FBS: fetal bovine serum; ATS: artificial tear solution; HCEC: human corneal epithelial cells; LPS: lipopolysaccharide; fMLP: n-formyl-methionyl-leuyl-phenylalanine.

Under closed-eye conditions for six hours, i.e. in an artificial tear solution, with exposure to human corneal epithelial cells, and in a hypoxic environment, the phenotype of blood-isolated neutrophils was unable to mimic the expression of tear-film neutrophils *in vitro* as shown in Figure

5-4. fMLP stimulus induced a dramatic increase in CD11b and CD66b receptor expression ($p < 0.002$) and LPS induced a higher, though statistically insignificant expression than tear-film neutrophils.

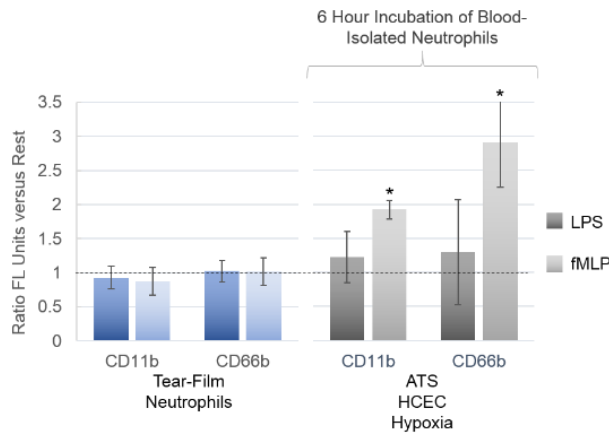


Figure 5-4: Comparison of tear-film neutrophil expression versus blood-isolated neutrophil expression following a 6 hour incubation in closed-eye conditions. Neutrophils were isolated from whole blood using density gradient centrifugation and were incubated for six hours in ATC, with a monolayer of human corneal epithelial cells, and in a hypoxic environment (2% O₂) to simulate closed-eye conditions in vitro. Following incubation, cells were collected and were stimulated with fMLP or LPS. Neutrophils were measured for membrane receptor expression of CD11b and CD66b using flow cytometry. All data is reported as ratios versus an unstimulated (rest) sample. The dotted line indicates the rest value normalized to 1. Tear-film neutrophil data is at baseline and is reprinted from [16]. n=3, *significantly different from rest values, $p < 0.05$, FBS: fetal bovine serum; ATC: artificial tear solution; HCEC: human corneal epithelial cells; LPS: lipopolysaccharide; fMLP: n-formyl-methionyl-leucyl-phenylalanine.

Blood-isolated neutrophils were tested for apoptosis (Figure 5-5) using the marker FAM-VAD-FMK which binds to activated caspases within cells and results in a strong fluorescence. Caspase levels remained low following a six hour incubation, and only a minimal increase in fluorescence intensity was observed when cells were incubated with HCEC, 19 ± 3 AFU versus 12 ± 3 without

HCEC ($p < 0.08$). All neutrophils were able to undergo apoptosis following incubation with staurosporine, indicating that, despite the 6 hour incubation under various conditions, the blood-isolated neutrophils remain alive and functional given the induction of the intracellular mechanisms of apoptosis.

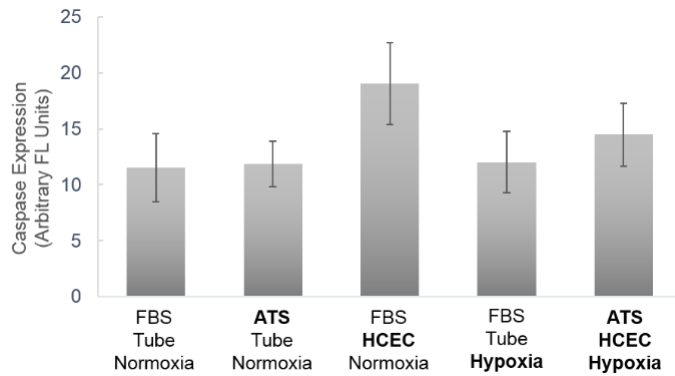


Figure 5-5: Relative levels of caspase expression of blood-isolated neutrophils following 6 hour incubation under various conditions. Neutrophils were isolated from whole blood using density gradient centrifugation and were incubated for six hours in different incubation media (DMEM/10%FBS vs ATS), with and without a monolayer of human corneal epithelial cells, and in a normoxic (21% O₂) or hypoxic (2% O₂) environment. Following incubation, cells were collected and processed for flow cytometry. Cells were stained with a pan-caspase inhibitor FAM-VAD-FMK which fluorescently binds to caspases within cells and is a marker of apoptosis. n=3, FBS: fetal bovine serum; ATS: artificial tear solution; HCEC: human corneal epithelial cells.

Across the rest of the markers tested, CD54 and C3aR showed upregulation amongst the blood samples as shown in Figure 5-6. CD45 remained mostly unchanged, as expected, given that it is a pan-leukocyte marker and less of a marker of neutrophil activation.

	6 Hour Incubation of Blood-Isolated Neutrophils												Ratio FL Units versus Rest
	Tear-Film Neutrophils		FBS Tube Normoxia		ATS Tube Normoxia		FBS HCEC Normoxia		FBS Tube Hypoxia		ATS HCEC Hypoxia		
	LPS	fMLP	LPS	fMLP	LPS	fMLP	LPS	fMLP	LPS	fMLP	LPS	fMLP	
CD11b <small>Activation, adhesion, fibrinogen binding, complement</small>	0.94	0.83	1.49*	1.56*	1.31	2.41*	1.84*	1.64*	1.78*	1.47*	1.22	1.96*	0.8 – 1.2 1.2 – 1.6 1.6+
CD45 <small>Leukocyte common antigen</small>	0.99	0.91	1.31	1.16	0.79	1.15	0.93	0.86	1.48*	1.3	0.85	1.43	
CD54 <small>Intracellular adhesion molecule-1</small>	0.95	1.13	1.37	1.51	1.78	3.21*	1.65	2.14*	1.34	1.98*	1.47	2.24*	
CD66b <small>Degranulation, released upon activation</small>	1.00	0.91	1.39	1.55*	1.37	2.69*	1.95*	2.42*	1.69*	1.85*	1.27	3.07*	
C3a <small>Complement activation</small>	1.07	1.49	1.59*	1.50	1.85*	2.17*	1.95*	1.87*	1.71*	1.59*	1.81*	1.81*	

All values are ratios compared to rest.

Figure 5-6: Comparison of tear-film neutrophil expression versus blood-isolated neutrophil expression following a 6 hour incubation under varied conditions (ratio). Neutrophils were isolated from whole blood using density gradient centrifugation and were incubated for six hours in different incubation media (DMEM/10%FBS vs ATS), with and without a monolayer of human corneal epithelial cells, and in a normoxic (21% O₂) or hypoxic (2% O₂) environment. Following incubation, cells were collected and were stimulated with fMLP or LPS. Neutrophils were measured for membrane receptor expression of CD11b, CD45, CD54, CD66b and C3aR using flow cytometry. All data is reported as ratios versus an unstimulated (rest) sample. Standard deviation is not included to improve readability, but is roughly 25% of the reported value. Tear-film neutrophil data is at baseline and is reprinted from [16]. n=3-5, *significantly different from rest values, p<0.05, FBS: fetal bovine serum; ATS: artificial tear solution; HCEC: human corneal epithelial cells; LPS: lipopolysaccharide; fMLP: n-formyl-methionyl-leuyl-phenylalanine.

While the ratio provides a good method to see the ability of neutrophils to upregulate surface receptors, it does not allow for a comparison between unstimulated controls. In Figure 5-7, the raw

fluorescent data shows that the overall level of expression in tear-film neutrophils is significantly higher as compared to the resting level of blood-isolated neutrophils after a six hour incubation, and is even higher than the activated level in terms of CD54 and CD66b.

		6 Hour Incubation of Blood-Isolated Neutrophils																				
		Tear-Film Neutrophils			FBS Tube Normoxia			FBS HCEC Normoxia			FBS Tube Hypoxia			ATS Tube Normoxia			ATS Tube Hypoxia			ATS HCEC Hypoxia		
		R	LPS	fMLP	R	LPS	fMLP	R	LPS	fMLP	R	LPS	fMLP	R	LPS	fMLP	R	LPS	fMLP	R	LPS	fMLP
CD11b	Activation, adhesion, fibrinogen binding, complement	73	70	69	40	54	56	62	115	101	38	62	65	34	42	72	37	43	73	52	62	99
CD45	Leukocyte common antigen	65	60	63	53	66	63	52	76	67	85	73	75	74	59	85	72	69	109	83	66	106
CD54	Intracellular adhesion molecule-1	42	40	44	13	17	19	12	17	22	30	47	61	13	19	32	15	21	26	27	37	56
CD66b	Degranulation, released upon activation	127	132	136	34	47	44	42	79	98	28	47	51	25	33	57	27	34	70	32	41	98
C3a	Complement activation	28	31	39	14	22	22	15	20	22	22	36	33	9	17	20	12	16	17	20	26	31

All values in raw fluorescent units.

Figure 5-7: Comparison of tear-film neutrophil expression versus blood-isolated neutrophil expression following a 6 hour incubation under varied conditions (raw). Neutrophils were isolated from whole blood using density gradient centrifugation and were incubated for six hours in different incubation media (DMEM/10%FBS vs ATS), with and without a monolayer of human corneal epithelial cells, and in a normoxic (21% O₂) or hypoxic (2% O₂) environment. Following incubation, cells were collected and were stimulated with fMLP or LPS. Neutrophils were measured for membrane receptor expression of CD11b, CD45, CD54, CD66b and C3aR using flow cytometry. All data is reported in raw fluorescent units. Standard deviation is not included to improve readability, but is roughly 25% of reported values. Tear-film neutrophil data is at baseline and is reprinted from [16]. n=3-5, *significantly different from rest values, p<0.05, FBS: fetal bovine serum; ATS: artificial tear solution; HCEC: human corneal epithelial cells; LPS: lipopolysaccharide; fMLP: n-formyl-methionyl-leuyl-phenylalanine.

5.4 Discussion

Blood-isolated neutrophils are well recognized to increase expression of CD11b and CD66b in response to LPS and fMLP [234-236]. CD11b is also known as macrophage-1 antigen (Mac-1) and is part of CD11b/CD18 β_2 integrin family that participates in leukocyte transmigration and phagocytosis [211]. CD66b is a marker of degranulation [236]. Increased expression of CD11b and CD66b as a response to stimulus is a sign of neutrophil activation. Overall, a six-hour incubation of blood-isolated neutrophils under various closed-eye conditions did not induce a tear-film neutrophil, or more simply, a non-inflammatory phenotype. However, each of the various incubation methods resulted in changes to the inflammatory potential of the blood-isolated neutrophils.

Artificial tear solution was capable of inducing small changes after a six-hour incubation, as was also found after a 30 minute incubation [16]. Noticeably, the overall receptor expression went down in unstimulated samples in ATS as compared to DMEM/10%FBS. Given the anti-inflammatory potential of lactoferrin [93], it is expected that ATS would decrease receptor expression of CD11b and CD66b. However, after incubation in ATS, the blood-isolated neutrophils were still capable of responding to LPS and fMLP, and even showed a more dramatic response to fMLP, suggesting that the experimental conditions actually primed⁶ the neutrophils [127].

Incubation of neutrophils under hypoxic conditions demonstrated a slight ability to prime the neutrophils given the marginally higher activation in response to LPS and fMLP. Hypoxic conditions did not appear to affect blood-isolated neutrophil expression following incubation in either ATS or FBS. Hypoxia has demonstrated an effect on neutrophil survival and on expression of neutrophil membrane receptors and release of cytokines and reactive oxygen species [230,231]. Notably, CD11b has shown a dramatic increase with hypoxia (0% O₂, 100% N₂) and subsequent stimulation

⁶ priming is the act of significantly augmenting membrane receptor expression given subsequent stimulation [41]

with LPS has also increased CD11b expression after 2 or 4 hours in hypoxic conditions [230]. Our results do not coincide with this same extent of activation, but it has been demonstrated in hypoxemia (low blood oxygen concentration) that expression of CD11b is unchanged under less severe oxygen deprivation [229]. Therefore, incubation at 2% O₂ may result in only a mild change in upregulation. The hypoxic challenge in the present investigation may also be severe as the closed-eye level of oxygen is around 8% [226,227]. Neutrophils also have a considerable decrease in apoptosis during incubation in a hypoxic environment [231], but the effects of this prolonged survival have yet to be interpreted on the tear-film neutrophil inflammatory potential. Overall, changes between hypoxic and normoxic conditions are observed with blood-isolated neutrophils, but neutrophils remain capable of upregulation following inflammatory stimuli post hypoxic challenge.

Exposure to human corneal epithelial cells resulted in a dramatic increase in the response of the blood-isolated neutrophils to inflammatory stimulus with an upregulation in CD11b and CD66b expression. The HCEC appears to have a strong ability to prime the blood-isolated PMNs. Interestingly, expression of CD11b and CD66b was also higher in the rest/unstimulated samples when compared to incubation in the absence of HCEC, suggesting that interactions of blood-isolated neutrophils with HCEC induce some activation. A brief exploratory study was conducted to visually inspect the response of blood-isolated and tear-film neutrophils in combination with human corneal epithelium. It was previously found that lymphocytes adhere to cultured human corneal epithelial cells [222], and a similar protocol was used to illustrate the ability of blood-isolated neutrophils to mount a significant inflammatory response when in contact with HCEC and in the presence of LPS (Figure 5-8b). Conversely, tear-film neutrophils remain visually quiescent while under the same conditions (Figure 5-8a).

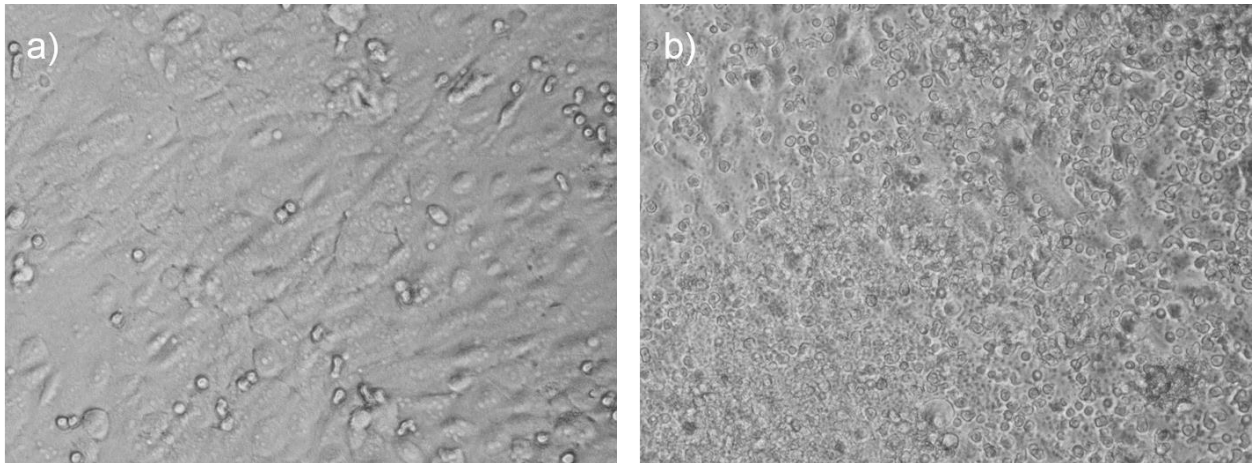


Figure 5-8: a) Tear-film and b) blood-isolated neutrophils on cultured human corneal epithelial cells in the presence of lipopolysaccharide. Note that the underlying, semi-transparent epithelium remains intact in a) and that blood-isolated neutrophils result in significant damage.

Further research will be needed to understand these differences and why blood-isolated neutrophils respond so strongly to the corneal epithelium. It is possible that the tear-film neutrophils do not interact with HCEC because they already extravasated through a tissue. Extravasated neutrophils have been shown to have higher levels of CD66b [237] and β -catenin, a receptor important in epithelial repair [238]. In order for neutrophils to enter tissue, they have to go through a complex process of activation, adhesion to endothelium, and ultimately endothelial transmigration [239]. This process results in numerous changes to the phenotype of neutrophils which may explain part of the strong differential effect observed in the interaction of the tear-film and blood-isolated neutrophils with human corneal epithelial cells. Furthermore, resident populations of neutrophils have been reported to exhibit refractory phenotypes in the peritoneum [240], lung [241], mouth [215], and nose [219]; and one strong commonality is that these neutrophils all have had to undergo the process of extravasation.

To further detail this refractory phenotype, airway neutrophils have been shown to not activate in the presence of LPS [218]. Following a bronchoalveolar lavage, neutrophils were found to have a high expression of CD11b, CD54, and CD66b, and also had a low expression of CD62L [242,243]. Nasal lavage fluid also contained neutrophils that have a similar expression, with higher CD11b and CD66b as compared to blood-isolated neutrophils [219]. It has been postulated that this phenotype of neutrophils is actually a unique kind of myeloid cell that can suppress T cell responses [212]. In patients induced with endotoxemia, a subset of circulating neutrophils can be isolated that has a similar high expression of CD11b, and a low expression of CD62L [212]; this phenotype has been shown both in bronchoalveolar neutrophils [242] and tear-film neutrophils [16]. Myeloid-derived suppressor cells are known to be a heterogeneous population of both monocytic and granulocytic cells that display an immunosuppressive phenotype [244,245]. It is therefore highly possible that in the immune-privileged environment of the closed eye, it is very advantageous to have a population of these cells present to mitigate the formation of a strong inflammatory response to any pathogens in the closed eye environment.

A similar neutrophil phenotype has also been found on the ocular surface in conjunction with Stevens-Johnson Syndrome (SJS)-Toxic Epidermal Necrolysis (TEN) and has been termed as infiltrative [129]. Interestingly, neutrophils isolated via impression cytology inversely correlate with the length of disease, meaning that in acute conditions, levels of neutrophils are highest, and decrease as the disease becomes more chronic [129]. SJS-TEN is an inflammatory condition that affects epithelium and is often found in patients with multiorgan failure. T-cell mediated cytotoxic response is thought to be implicated in SJS-TEN. In the light of the results of Williams' investigation, it may be possible that tear-film neutrophils, if they do indeed have a T cell immunosuppressive phenotype, could correlate with an increase in tissue damage as the disease progresses. Neutrophils have also been implicated in dry eye, which is another inflammatory disease [138].

Oral neutrophils have yet to be evaluated for their inflammatory phenotype, however, it has been shown that oral neutrophils differ from blood-isolated neutrophils given that they express T-cell receptors [215]. It has been hypothesized that the oral neutrophils offer a link between the innate and adaptive immune systems.

While the role of the tear-film neutrophil has yet to be elucidated, blood-isolated neutrophils have a different phenotype from their counterparts found at the ocular surface. Further, a non-inflammatory phenotype is not achieved through incubation of blood-isolated neutrophils in an artificial tear solution, in a hypoxic environment, or in co-culture with human corneal epithelial cells. There is a precedent for equating blood-isolated neutrophils with ocular surface neutrophils [17,149] or airway neutrophils [246], and this practice may affect the validity of the interpretation of experimental studies looking at ocular inflammation. The presence of neutrophils on the ocular surface is generally viewed as a negative phenomenon, and there has been increasing interest in developing anti-inflammatory pharmaceuticals to reduce neutrophil infiltrates. Given the pro- and anti-inflammatory roles of neutrophils [104] and the fact that we have yet to fully characterize and understand the purpose of these inflammatory cells at the ocular surface, such a strategy should be approached with caution.

5.5 Conclusions

After six hours of incubation, blood-isolated neutrophils remained viable as measured by a lack of apoptosis. Incubation in artificial tear solution had some effects on the resting phenotype, likely due to the anti-inflammatory potential of lactoferrin. The presence of human corneal epithelial cells induced upregulation of membrane receptors while incubation under hypoxic conditions alone had no effect. However, all three parameters primed the blood-isolated neutrophils, leading to increased activation with LPS and fMLP.

Tear-film neutrophils have a noticeable quiescent phenotype, whereas blood-isolated neutrophils both at baseline and after a six hour incubation under the various experimental conditions tested in this study underwent activation when stimulated.

This investigation was unsuccessful in recreating a non-inflammatory phenotype in blood-isolated neutrophils *in vitro*. Changes in environmental conditions such as those of the closed-eye environment were alone insufficient to induce significant changes in the ability of the blood-isolated neutrophils to respond to inflammation. Further investigation is required to determine if the process of extravasation contributes to the non-inflammatory phenotype of the tear-film neutrophils or if the presence of other tear-film components plays a role.

Contributions to Chapter 5

Author	Concept / Design	Acquisition of Data	Analysis	Write Up / Publication
C. Postnikoff	Y	Y	Y	Y
E. Kreinin		Y		
R. Pintwala		Y		
M. Gorbet	Y		Y	Y

Chapter 6

Conclusions

Do you see what eye did there?

The eye is one of the most complex organs in the human body, and its anterior surface has a balance of proteins, lipids, epithelial receptors, and inflammatory cells, which must all function harmoniously in order to avoid any compromise of vision. Introduction of contact lenses into this environment proves to affect ocular surface homeostasis with many contact lens wearers discontinuing use as a result of discomfort and dryness.

Many challenges remain in terms of developing a biocompatible contact lens. With significant improvements in oxygen delivery to the cornea, rates of comfort and of microbial infection remain a significant hurdle for manufacturers of both contact lenses and multipurpose disinfecting solutions. Two major opportunities that can provide researchers with capabilities to develop new and improved products are the development of better *in vitro* models and the improvement of the understanding of inflammation at the ocular surface. This thesis has focused on both of these areas.

The development of an *in vitro* curved, stratified, human corneal epithelial model (CML) has proved to mimic *in vivo* conditions. This model has thus far probed capability in discerning cytotoxicity of benzalkonium chloride, benzalkonium chloride released from contact lenses, and

lenses soaked in multipurpose disinfecting solution. The CML has also shown, for the first time, to replicate micropunctate patterns of corneal staining *in vitro*. Balafilcon A (BA) lenses soaked in renu Fresh (renu) are well known to induce corneal staining *in vivo*, and this result has affected clinician perception, given the negative attitudes towards corneal staining. renu-soaked BA lenses prove to be non-cytotoxic with the static CML model after 2 and 6 hours of incubation, though this lens and solution combination may not be as optimal as other solutions given a marginal decrease in viability and slight increase in apoptosis as compared to BA lenses soaked in the peroxide-based Clear Care solution.

It has been postulated that the major mechanism for hyperfluorescent corneal epithelial cells, as investigated by the instillation of sodium fluorescein, is a result of apoptotic cell populations. Our work has shown that apoptotic and necrotic cell populations do not result in a greater uptake of sodium fluorescein by cells in a monolayer model. Further, the multilayer model does not show a large increase in the numbers of caspase positive (apoptotic) cells as a result of incubation with BA renu, which is known to increase sodium fluorescein staining *in vivo*. However, a marginally higher apoptotic cell population could lead to a disruption of barrier function in the superficial epithelium, which could give rise to pathogen susceptibility. It is possible that the main barrier functions affected are those of the membrane-bound mucins.

While the CML and tear replenishment system (TRS) are great improvements for *in vitro* models, the *in vitro* models lack an inflammatory component. The epithelium is capable of dealing with some acute inflammatory issues, given its expression of tight junctions, toll-like receptors, and its ability to release soluble factors such as mucins and cytokines. However, leukocyte-mediated inflammation may be a very crucial part of ocular immunity, though it remains to be demonstrated how this actually happens at the ocular surface.

Every night when we sleep, our closed-eye tear film collects hundreds of thousands of neutrophils, and it is possible that these tear-film neutrophils are implicated in biofilm formation, in exacerbation of inflammation, or maybe the resolution of inflammation.

A common source for *in vitro* experiments with neutrophils is to isolate neutrophils from whole blood. Blood-isolated neutrophils, however, have a drastically different phenotype as compared to tear-film neutrophils. Blood-isolated neutrophils perform classically, releasing reactive oxygen species, cytolytic compounds in response to inflammatory stimuli, whereas tear-film neutrophils remain quiescent and non-inflammatory.

The closed-eye environment is very unique, in that the regular blink is removed and oxygen levels are subsequently greatly reduced. By mimicking the closed eye environment, namely incubating blood-isolated neutrophils in artificial tear solution, in a hypoxic environment, with human corneal epithelial cells, we hypothesized that one or some combination of these parameters would affect neutrophil performance *in vitro*. Following a six hour incubation under the different conditions tested, blood-isolated neutrophils remained able to respond to inflammatory stimuli, and may have actually been primed to respond more aggressively under the closed-eye conditions.

The specific contributions of this thesis have been twofold: namely, the establishment of an *in vitro* model of corneal staining, and the next step in determining the phenotype of the tear-film neutrophils. Solution-induced corneal staining and the overall observation of selective fluorescein entry into human corneal epithelium has yet to be deduced, but the use of a curved, stratified, corneal epithelial model may present a unique and novel opportunity to investigate corneal staining and lens and solution biocompatibility. The tear-film neutrophil has been shown to have a non-inflammatory phenotype and this work continues to highlight the refractory potential of tear-film neutrophils given that blood-isolated neutrophils do not adopt a similar phenotype through exposure to closed-eye conditions.

Overall, this body of work has presented many questions, but has further improved our understanding of *in vitro* models of the human corneal epithelium and inflammation as it occurs at the ocular surface. Our inferences would also have not been possible without the extensive use of flow cytometry, which has proved to be an excellent analytical method for investigation of changes in cell expression.

Chapter 7

Recommendations for Future Work

Putting a lid on it.

This thesis has provided more questions than answers, in terms of improving our understandings of fluorescein staining, lens and solution biocompatibility, and neutrophil-mediated inflammation at the ocular surface. There is thus a significant opportunity for future work through the development of improved cell culture models and the more rigorous investigation of cellular expression.

Sodium fluorescein staining has been used to investigate changes in the morphology of the ocular surface for over 100 years, and yet, there is still no consensus on how sodium fluorescein uptake by the human corneal epithelium occurs. That is not to say that there is no knowledge: sodium fluorescein uptake is likely an active transport process, and may occur by carboxylate transporters on the cells. However, the cornea is the only known organ in the body to show a differential uptake of sodium fluorescein. Future work is required to more specifically analyze the superficial epithelial response and more mechanistically determine if intact mucins block sodium fluorescein entry.

The cellular basis for sodium fluorescein staining may also have significant impacts on the understanding of solution-induced corneal staining (SICS), and may affect clinical practice, overall. Solution-induced corneal staining has been equated to solution toxicity, but our results show that lens

and solution combinations known to induce SICS do not correlate to a strong cytotoxic response in the corneal epithelium. SICS has been able to highlight a specific interaction between contact lenses and multipurpose disinfecting solutions, but more work is needed to characterize the differential effects of these lens and solution combinations on the ocular surface. Given the propensity of sodium fluorescein uptake to be affected by pH or other physiological changes, sodium fluorescein may not be the best metric for investigation lens and solution biocompatibility. One ocular surface dye that has been developed and may show improved potential is the fluorescein octadecyl ester (FODE) that has been shown to specifically bind to mucins. *in vitro* work is required to characterize this dye in context of the ocular environment before it reaches clinical practice.

The *in vitro*, curved, stratified human corneal epithelium (CML) in combination with the tear replenishment system (TRS) provides us with an ability to closely mimic the ocular environment. This *in vitro* system requires further characterization to better understand its limitations as a model, and to ensure that the epithelium has a structure and genetic profile that matches a human cornea. This system can also be further developed not only for testing of different materials, but also for growth of the CML, as a dynamic model may affect the health and overall growth of a robust epithelium. Further, this model can be adapted to incorporate diurnal and nocturnal variations in terms of changes in tear-replenishment and infiltration of tear-film neutrophils.

One of the limitations of the CML model of *in vitro* corneal staining is that experiments were performed under static conditions, implying that there was a lack of flow normally associated with tear exchange *in vivo*. By incubating the CML in the TRS, fluid exchange may affect release of MPS, which may subsequently affect biocompatibility at the ocular surface. Future work with the *in vitro* model of corneal staining should investigate if there are any changes in a dynamic environment.

Tear-film neutrophils have a markedly non-inflammatory phenotype, and blood-isolated neutrophils are a poor substitute given their completely different expression. Overall, the tear-film

neutrophils show a much higher level of activation as compared to blood-isolated neutrophils. These results are actually in accordance with a series of studies that have found a similar phenotype in the airway, lung and nose, which have all demonstrated different capabilities to interact with the adaptive immune system. It is therefore highly possible that in the immune-privileged environment of the closed eye, these neutrophils somehow act to mitigate the formation of a strong inflammatory response to any pathogens in the closed eye environment.

Another mechanism that requires further investigation is that of the tear-film neutrophils' ability to phagocytose or NETose. Phagocytosis is a typical property of neutrophils, as is the ability to release neutrophil extracellular traps (NETs). It has been shown that neutrophils may contribute to the pathogenesis of infection as a result of NETs at the ocular surface, and tear-film neutrophils may be somehow implicated.

Environmental conditions alone are unsubstantial in inducing a non-inflammatory phenotype in blood-isolated neutrophils. Future work should focus more on analysis of the tear-film neutrophil phenotype, and the literature may offer ideas on how to determine if these neutrophils are an ocular manifestation of a systemic neutrophil phenotype that is meant to suppress and control T-cell mediated inflammation.

Ultimately, the main goal of this body of work is to develop a complete *in vitro* model of the ocular surface: an environment that incorporates active epithelium, inflammatory cells, tear film proteins, and tear exchange to accurately mimic the host defense of the ocular surface. An ultimate *in vitro* model will allow for the development and testing of novel biomaterials and ophthalmic solutions, and may even provide us with a greater understanding of the biology of the eye.

Appendix A
**Development of a curved, stratified, in vitro model to assess ocular
biocompatibility**

Please see next page.



Development of a Curved, Stratified, *In Vitro* Model to Assess Ocular Biocompatibility

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Abstract

Purpose: To further improve *in vitro* models of the cornea, this study focused on the creation of a three-dimensional, stratified, curved epithelium; and the subsequent characterization and evaluation of its suitability as a model for biocompatibility testing.

Methods: Immortalized human corneal epithelial cells were grown to confluency on curved cellulose filters for seven days, and were then differentiated and stratified using an air-liquid interface for seven days before testing. Varying concentrations of a commercial ophthalmic solution containing benzalkonium chloride (BAK), a known cytotoxic agent, and two relevant ocular surfactants were tested on the model. A whole balafilcon A lens soaked in phosphate buffered saline (BA PBS) was also used to assess biocompatibility and verify the validity of the model. Viability assays as well as flow cytometry were performed on the cells to investigate changes in cell death and integrin expression.

Results: The reconstructed curved corneal epithelium was composed of 3–5 layers of cells. Increasing concentrations of BAK showed dose-dependent decreased cell viability and increased integrin expression and cell death. No significant change in viability was observed in the presence of the surfactants. As expected, the BA PBS combination appeared to be very biocompatible with no adverse change in cell viability or integrin expression.

Conclusions: The stratified, curved, epithelial model proved to be sensitive to distinct changes in cytotoxicity and is suitable for continued assessment for biocompatibility testing of contact lenses. Our results showed that flow cytometry can provide a quantitative measure of the cell response to biomaterials or cytotoxic compounds for both the supernatant and adherent cell populations. As a specifically designed *in vitro* model of the corneal epithelium, this quantitative model for biocompatibility at the ocular surface may help improve our understanding of cell-material interactions and reduce the use of animal testing.

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Competing Interests: The authors have read the journal's policy and the following conflicts have been identified. In the past 4 years, Gorbet has received funding from CIBA Vision (now known as Alcon) and from Vistakon/Johnson & Johnson. Wright is currently an employee from Alcon (formerly CIBA Vision). This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

The cornea is comprised of three main cellular layers: the epithelium, stroma, and endothelium. The corneal epithelium is the first line of defence against many types of injury, trauma, and infection and contributes to maintenance of transparency and rigidity of the cornea [1–3]. The epithelium has also been shown to be the primary barrier against transcorneal permeation [4]. As a result, to have simpler models than the entire cornea itself, many researchers have opted to develop alternative corneal epithelial models for the study of material interactions at the front of the eye.

Recently, *in vitro* ocular toxicity testing has experienced a major advancement with the development of multilayered corneal epithelial cultures. There has been much interest in developing

in vitro models that have the potential to replace the *in vivo* Draize test. The Draize test was developed as an ocular toxicity test in 1944 and involves the placement of test solutions on the eyes of living animals [5]. It became part of the United States Food and Drug Administration regulations in 1964 [6]. The Draize test has since come under much criticism in terms of its performance and reliability [6–8]. As the corneal epithelium represents the major barrier of the eye, many *in vitro* epithelial models have since been developed and proposed as alternatives to the *in vivo* Draize test. Since the 1960s, monolayer cell cultures have been developed using primary and immortalized corneal epithelial cell lines of rabbit and canine origin; and starting in the 1990s, the use of human-derived cells has become more popular [9,10].

Concurrent to the development of better cell lines was the improved understanding of multilayered cultures and the importance of the air-liquid interface. Combined, the research has led to many *in vitro* models of epithelial reconstruction [11–14] and the development of commercially available models such as HCE by SkinEthic, EpiOcular by MatTek, and Clonetics by Lonza. The majority of these *in vitro* models have been used for toxicity testing of different ophthalmic solutions and for drug permeation studies [15–18], though presently none of these models have been validated or accepted for regulatory purposes. On the other hand, little research has been performed with these *in vitro* models in the area of contact lens interactions, possibly due to their limited surface area that would require the manufacture of smaller lenses or the use of trephined lens segments.

As part of regulatory testing, contact lenses are rigorously tested for their interactions with the ocular surface. Beyond the initial qualification of the contact lens material, the overall biocompatibility of a multipurpose contact lens disinfecting solution (MPS) with the contact lens material also needs to be defined. It is recognized that contact lens materials uptake and release some components of MPS, which may in turn affect ocular cell response [19–21]. Most notably, lens-solution interactions have been shown to be of critical importance in patterns of solution induced corneal staining as seen in both the StainingGrid and the IER Matrix Study [22,23]. Current *in vitro* toxicological evaluations have been mostly conducted using a monolayer of cells to investigate the effects of different MPS [24–27] (see Choy *et al.* [27] for a review of these studies). In line with Powell's recent demonstration that lens material has an effect on uptake and release of biocides [19], our research group further showed that this difference in uptake and release can affect the response of corneal epithelial cells *in vitro* [28,29]. While monolayer cultures represent a rapid and simple model to investigate lens-solution interactions, these models are very sensitive as they solely consist of a single layer of cells and are static in nature. As a means to better characterize and understand lens-solution biocompatibility, we proposed the development of an *in vitro* reconstructed curved epithelium model that can better mimic interactions at the interface between the contact lens and corneal epithelial cells. To determine the sensitivity of the curved, stratified model, we chose to assess the response of our model to varying concentrations of benzalkonium chloride, a well-known cytotoxic agent [30], and compare that response to a silicone hydrogel lens soaked in phosphate buffered saline. As benzalkonium chloride is often found in a proprietary solution, common ophthalmic surfactants from the poloxamine family were also tested to ensure cytotoxicity was dependent on benzalkonium chloride.

To evaluate the response of the curved stratified epithelium, the MTT and luciferin-luciferase ADP/ATP assays were used to determine cellular metabolic activity and flow cytometry was performed to investigate changes in integrin expression and mechanism of cell death. To our knowledge, this current study reports the first *in vitro* stratified model developed specifically for studying contact lens-solution biocompatibility; this model may further prove useful for drug-delivery and cell-material interaction studies.

Methods

Reagents and Antibodies

Keratinocyte serum-free medium (KSFM), keratinocyte growth supplements (KGS), and penicillin/streptomycin (Pen/Strep) solution were purchased from ScienCell (Carlsbad, California). All other cell culture reagents, including Dulbecco's Minimum

Essential Medium (DMEM), 1:1 DMEM in Ham's F12 nutrient medium, fetal bovine serum (FBS), 0.25% Trypsin-EDTA, Hank's based cell dissociation buffer, and TrypLE Express were purchased from Life Technologies (Burlington, Ontario, Canada). Phosphate buffered saline (PBS) was purchased from Lonza (Allendale, New Jersey).

Monoclonal antibodies to β_1 integrin (CD29) and α_3 integrin (CD49c) were fluorescein isothiocyanate (FITC) and R-phycoerythrin conjugated, respectively and were purchased from Becton Dickinson (Mountain View, CA, USA). Propidium iodide (PI) and the FLICA caspase kit were purchased from ImmunoChemistry Technologies, LLC (Bloomington, MN, USA). The FLICA kit uses the fluorescent probe FAM-VAD-FMK for caspase detection. The ADP/ATP Bioluminescent Cell Viability Kit II was purchased from PromoCell GmbH (Heidelberg, Germany). All chemicals used to prepare paraformaldehyde and HEPES Tyrode Buffer were of analytical or reagent grade.

Contact Lens and Ocular Solution

A daily-wear silicone hydrogel balafilcon A (BA; Bausch & Lomb, Rochester, NY, USA) was tested. Lenses were obtained in their original packaging from the manufacturer and had a curvature of 8.6 mm, diameter of 14.0 mm, and power of -3.00 dioptres. All lenses were used before their expiry date. Whole lenses were used and were not cut before placement on the cultures.

Phosphate buffered saline was used as a negative control lens solution, which was previously shown to be biocompatible [31]. A sterile ophthalmic solution of benzalkonium chloride (BAK) was used as a positive control; the commercially available Moisture Eyes (ME; Bausch & Lomb, Rochester, NY, USA) has a BAK concentration of 0.01% w/v. Stratified cultures were exposed to undiluted and diluted Moisture Eyes at final BAK concentrations of 0.01% w/v (100% ME), 0.005% w/v (50% ME), and 0.002% w/v (20% ME).

As Moisture Eyes is a proprietary commercial source of BAK, it is possible that other ingredients within the solution may affect viability. To confirm that the cytotoxicity observed with our BAK source resulted mainly from BAK, two commonly used ocular surfactants from the poloxamine family, namely Tetronics 904 and 1304 (BASF, Ludwigshafen, Germany) were also investigated. Surfactants were tested at concentrations of 0.25% w/v and 1% w/v which are within a relevant range for ocular therapeutics [32,33].

In vitro Cell Culture

HPV-immortalized human corneal epithelial cells gifted from Dr. May Griffith [10] were cultured in keratinocyte serum-free medium with keratinocyte growth supplements (bovine pituitary extract and recombinant epidermal growth factor) and Pen/Strep. Fresh medium was added every other day, and cells were grown to 90% confluency and were used before their eighteenth passage. Higher passage numbers were avoided in order to reduce the genomic variations that have been observed in other immortalized epithelial cells [34]. Adherent cells were removed using TrypLE Express dissociation solution. Cells were routinely observed for any morphological changes.

Curved Multilayer Preparation

The curved multilayers were grown on a Millicell-HA (mixed cellulose esters, Millipore, Billerica, MA, USA) membrane with a 0.45 μm pore size. To deform the cell substrate with a curve that would mimic the curvature of the cornea, an aluminum mold/counter-mold was fabricated (Figure 1b). The 30 mm diameter

filters were first deformed using the custom-shaped curved die. Silicone Press-to-Seal sheets with adhesive (Life Technologies, Burlington, Ontario, Canada) were punched into rings (inner diameter of 15 mm and an outer diameter of 22.5 mm) using a laboratory bench press (Figure 1a). Once cut, the rings were disinfected with 70% ethanol and placed on top of the curved filters to reduce the cell culture surface area to the curved filter. The entire process is depicted in Figure 1 where the initial cellulose filter (Figure 1c) is deformed into a curved shape (Figure 1d) and finally, the ring is placed on top (Figure 1e). The assembled inserts were then UV sterilized. After sterilization, inserts were coated with collagen type I (0.05 mg/mL, 30 min at 37°C) and cells were seeded within 30 minutes of coating.

The curved inserts were seeded with 5×10^3 cells per insert. The curved multilayers were fed with KSEF on each of the basal and apical sides of the curve for the first seven days, with medium being exchanged every other day. After seven days, cell differentiation was induced by exposing the monolayer to an air-liquid interface. Cells were fed only on the basal side with 2% FBS in 1:1 DMEM/F12 and medium was exchanged daily. The cells grew under these conditions for seven days and were then ready for experimentation.

Histopathological Evaluation

Consecutive sections constituting the entirety of the specimens were processed routinely for microscopic examination: after initial fixation in 10% neutral buffered formalin, the cellulose filters were processed, embedded in paraffin, serially sectioned in 5- μ m-thick sections, and stained with hematoxylin and eosin (H&E). The histological slides were evaluated using bright field microscopy (Leica DM1000, Leica Microsystems Inc, Concord, ON).

In vitro Onlay Model

For the testing of solution soaked contact lenses, we adapted our monolayer onlay method [31] to be used in a stratified and curved epithelial model. Contact lenses were incubated in 3 mL of PBS for 24 hours in a tissue-culture-polystyrene well. Prepared stratified multilayers were fed with KSEF on the basal side. Apically, KSEF was added to wet the surface and PBS-soaked lenses were

placed gently on top of the multilayer, concave-side down, to fit the eye-shaped curve. An additional amount of KSEF was added on top of the lens to ensure moisture retention. For the BAK samples, 800 μ L, 400 μ L, and 160 μ L of BAK solution were added to the multilayers for the 0.01%, 0.005%, and 0.002% BAK dilutions, respectively. KSEF was added, where necessary, for a total apical volume of 800 μ L. Tetronecs 904 and 1304 were dissolved in PBS to a concentration of 2% w/v and were further diluted with KSEF to final concentrations of 0.25% and 1% on top of the stratified cultures. Control cells were fed with 800 μ L of KSEF. Upon placement of the lenses, surfactants, and BAK, the cells were returned to the humidified incubator at 37°C and 5% CO₂ for either 6 or 24 hours.

MTT Assay

After 6 and 24 hours incubation, lenses and medium were removed to quantify cellular viability using the MTT assay. The MTT assay was originally developed in 1983 by Mosmann et al. [35] based upon the reduction of tetrazolium salts as originally discovered by Altman [36,37]. The assay was then redeveloped for multilayers by Doucet in 1996 [38], adapted again by Van Goethem in 2006 to improve accuracy [15], and finally reworked by Pauly to further improve apical sensitivity in 2009 [39]. To allow the MTT assay to be used for our larger and curved stratified model, Pauly's protocol was further modified as follows. Briefly, thiazoyl blue tetrazolium bromide (0.5 mg/mL, MTT, Sigma Aldrich, Oakville, ON, Canada) was added to the apical and basal sides of the cell culture insert and was incubated for 3 hours 37°C and 5% CO₂. The MTT solution was then removed and isopropanol was added to both the apical and basal sides of the insert and plates were agitated for 2 hours. An aliquot was taken from both the apical and basal sides and the two remaining solutions in the apical and basal sides were then mixed together and a final aliquot of the mixed solution was taken. Samples were read in a UV-Vis spectrophotometer for optical density at 595 nm with a reference at 650 nm. All results are expressed as relative viability compared to control cells; cells incubated in KSEF and in the absence of a contact lens (no lens control). Cellular metabolic

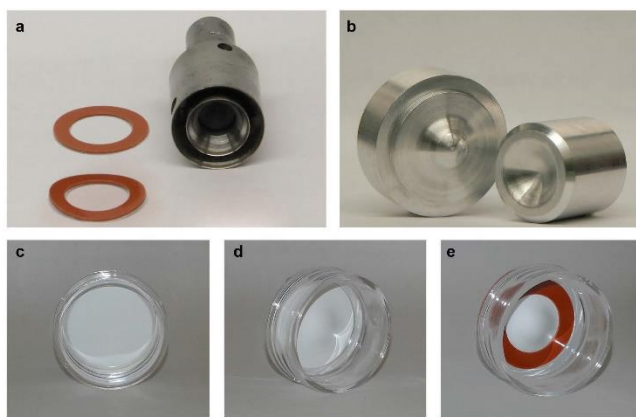


Figure 1. Necessary materials to build a curved cellulose insert for *in vitro* epithelial surface reconstruction. a) silicone rings and silicone ring die, b) top and bottom of eye form, c) cellulose insert before forming, d) final curved insert, e) final curved insert with one silicone ring. doi:10.1371/journal.pone.0096448.g001

activity determined from the mixed solution is reported in the results section.

Cell Collection and Tissue Digestion

After lens incubation, the media on top of the well was used to gently wash the contact lens and the surface of the stratified cell culture. This population of cells and debris was collected and is referred to as the **supernatant** population. Upon removal of the supernatant population, DMEM/10% FBS was added to the supernatant cells and the samples were centrifuged. Supernatant cells were resuspended in DMEM/10% FBS and prepared for flow cytometry (see below).

Once the supernatant population and the contact lens were removed, the multilayers were digested. 0.25% Trypsin-EDTA was added to the basal side of the insert and to the top of the insert. Cell dissociation buffer was then added to the top of the insert and the cells were placed on a shaker at 120 rpm for 45 minutes in a 37°C and 5% CO₂ environment. After 45 minutes, FBS was added to the inserts and cells were briefly resuspended via pipetting. The cells were returned to the shaker for another 45 minutes. Once the cells were removed, the cell-containing media from the top of the filter was transferred to polypropylene tubes containing DMEM/10% FBS. Cells were then centrifuged and resuspended in fresh media. This population is referred to as the **adherent** population.

Luciferin-luciferase ADP/ATP Assay

To further assess cellular viability, the Bioluminescent Cell Viability Kit II (ADP/ATP) was used for the determination of a viability ratio of ADP/ATP. The assay was performed as per manufacturer's instructions. Briefly, ATP monitoring enzyme and nucleotide releasing buffer were mixed to form a "Reaction Mix". 100 µL of the Reaction Mix was added to a 96-well plate. This sample was then read on a luminescence-capable spectrophotometer (FluoStar Optima, BLG Labtech, Ortenberg, Germany) to provide the background reading (Data A). Digested adherent cells were then diluted and 5 × 10³ cells per 10 µL of medium were added to the 96-well plate. This cell solution was then read on the luminometer, twice (Data B and C) to measure the amount of ATP within the cells. Finally, 1 µL of ADP converting enzyme was added and the cell solution was read for a final time (Data D). To obtain the ratio of ADP to ATP, the following calculation was used:

$$\frac{\text{DataD} - \text{DataC}}{\text{DataB} - \text{DataA}}$$

All results are expressed as ratios relative to control cells: cells incubated in KSFM in the absence of a contact lens (no lens control).

Flow Cytometry

After resuspension in DMEM/10% FBS, aliquots were transferred to flow cytometry tubes. Flow cytometry tubes contained one of two mixtures: (a) FITC-labeled anti-CD29 and PE-labeled anti-CD49; or (b) FITC-VAD-FMK (pan caspase). Integrins were incubated in the dark at room temperature for 30 minutes. Integrin samples were then diluted in buffer and paraformaldehyde fixative (1% final concentration) and were analyzed by flow cytometry within five days. As per kit instructions [40], caspase samples were incubated at 37°C for 60 minutes. The samples were then washed and propidium iodide (PI) was added.

All caspase samples were analyzed immediately on the flow cytometer.

Flow Cytometry Acquisition/Analysis

All integrin, caspase, and PI samples were acquired on a FACSVantage flow cytometer (Becton Dickinson, Mountain View, California) using CELLQuest Software. Appropriate isotype controls were used with each experiment. At least 5000 events were collected per sample. Analysis was also performed using FACSEXPRESS post data acquisition.

Statistical Analysis

All results are reported as means ± standard deviation. To evaluate the significance of the differences in cell viability and cell activation, an analysis of variance (ANOVA) was performed, followed by multiple pairwise comparisons using the Tukey's Honest Significant Difference using Statistical Analysis Software (SAS, Cary, NC, USA). Samples were compared to cells incubated without lens or solution (no lens control) as well as to cells exposed to a PBS-soaked BA lens (PBS-BA control). Significant differences between the compounds tested are also reported. A *p* value of less than 0.05 was required for statistical significance. The number of experiments was equal to or greater than three and experiments were performed on different days.

Results

While a cellulose insert can be curved and hold its shape, one of its drawbacks is that it is not transparent, and thus difficult to image. To illustrate how cells cover the entire curved surface, pictures were taken after incubation with the metabolic dye thiazoyl blue tetrazolium bromide (MTT) (Figure 2).

Histopathologic evaluation revealed squamous epithelial cells arranged in 3 to 5 defined, relatively uniform horizontal layers, firmly attached to the underlying cellulose filter (Figure 3).

Cellular Metabolic Activity

As shown in Figure 4, there was no difference in viability between the BA PBS (99 ± 10% and 102 ± 10% at 6 and 24 hours, respectively) and the no lens controls. All BAK concentrations significantly reduced viability when compared to the BA PBS control (*p* < 0.001). Exposure to 0.01% BAK resulted in the largest



Figure 2. Curved stratified epithelium following exposure to MTT. Surface coverage by formazan indicates well stratified and viable cells.

doi:10.1371/journal.pone.0096448.g002



Figure 3. Squamous epithelial cells arranged in layers, firmly attached to the underlying cellulose filters. H&E, original magnification 63X.
doi:10.1371/journal.pone.0096448.g003

reduction in viability with values of 16% and 12% at 6 hours and 24 hours, respectively. Time was not a significant factor ($p = 0.74$) suggesting that any of the tested concentration of BAK could be used as a positive control at either time point.

Both surfactants tested showed a negligible reduction in viability at 6 hours (Figure 5). Only exposure to 1% Tetricon 1304 for 24 hours resulted in a significant decrease in viability ($88 \pm 12\%$) when compared to the viability observed at 6 hours ($107 \pm 14\%$) ($p < 0.05$). However, this reduction in viability was not statistically significant when compared to the BA PBS control ($p = 0.14$).

ADP/ATP Viability Ratio

With the luciferase assay, when compared to controls (BA PBS and no lens), an increase in the ratio of ADP/ATP was observed with increasing concentrations of BAK, except in the case of 0.01% BAK (Table 1). It is important to note that due to the damage incurred from exposure to the most toxic concentration of BAK (0.01%), cells from this sample may have oversaturated the signal, which resulted in a calculated ADP/ATP ratio that appeared to be non-significantly different from the controls (Personal communication, 2014, Jacqueline White, PromoCell).

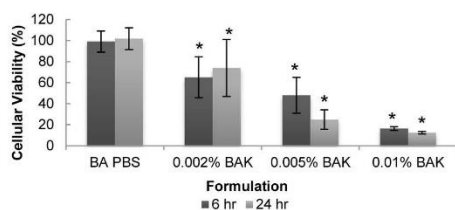


Figure 4. Effect of BAK and PBS-soaked lens on cellular viability of curved stratified epithelium. Cells were incubated for 6 and 24 hours with solutions of BAK or PBS-soaked contact lenses. Viability was measured by the MTT assay and is expressed as a percentage relative to cells grown in the absence of a contact lens (control). $n = 5$, *significantly different from BA PBS control, $p < 0.05$. BA PBS: PBS-soaked balafilcon A lens.
doi:10.1371/journal.pone.0096448.g004

Integrin Expression

To assess if exposure to BAK or to a PBS-soaked lens affected the cell adhesion phenotype, expression of the heterodimer $\alpha_3\beta_1$ was investigated via its two integrin subunits α_3 (CD49c) and β_1 (CD29).

Two distinct populations of cells were studied. The supernatant population corresponds to the cells that were sloughed off of the multilayer during and after lens incubation or BAK exposure, and the adherent population refers to the tissue that was digested via trypsinization.

In the adherent cell population (Figure 6), when compared to both a PBS-soaked lens and control (no lens, no solution), upregulation in CD49c expression was observed with increasing concentrations of BAK. Exposure to 0.01% BAK for both 6 and 24 hours resulted in a significant upregulation of CD49c expression when compared to all other samples ($p < 0.001$). At 24 hours, higher CD49c expression was also observed with 0.005% BAK ($p < 0.01$). While for all BAK samples, increases in CD49c expression appeared to occur between 6 hours and 24 hours, these changes were not statistically significant ($p > 0.2$).

CD29 expression on adherent cells, as shown in Figure 6, was also upregulated in the presence of BAK. Similar to CD49c expression, a significant upregulation could be observed following exposure to 0.01% BAK for 6 and 24 hours compared to BA PBS ($p < 0.001$). No change in CD29 expression was observed for BA PBS. There was also no effect of time on the expression of CD29.

For cells in the supernatant, as shown in Table 2, no difference in CD49c expression could be observed amongst any of the concentrations of BAK versus the BA PBS and no lens controls. Incubation time also had no effect. Regardless of treatment, there was also little difference in CD29 expression on cells from the supernatant. Some downregulation (albeit not significant) was observed at 6 hours for both 0.005% BAK and 0.01% BAK. However, at 24 hours, for all samples, a significant upregulation in CD29 expression was observed compared to 6 hours ($p < 0.05$).

Caspase Activation and Propidium Iodide Expression

To assess how the different conditions affected cell apoptosis and necrosis, caspase activation was detected by flow cytometry using the fluorescently-tagged pan caspase inhibitor FITC-VAD-FMK, which fluoresces most intensely in cells with active caspases. Dead cells were identified by propidium iodide staining.

For adherent cells, no difference in caspase activation was observed between BA PBS and the no lens control with ratios of 1.0 ± 0.1 and 0.99 ± 0.5 for 6 hours and 24 hours, respectively, as shown in Figure 7. The only formulation to induce significant caspase activation was 0.01% BAK at 6 hours ($p < 0.001$). Caspase activation in cells from the 0.01% BAK sample at 6 hours was also significantly higher compared to the 24 hour time point, with ratios of 1.7 ± 0.5 and 1.1 ± 0.2 , respectively.

For the supernatant cells, incubation with BA PBS led to caspase activation similar to the no lens control at both 6 hours and 24 hours (Figure 7). For 0.005% BAK and 0.01% BAK, a significant increase ($p < 0.001$) in the level of caspase activation was observed in the supernatant cells for both 6 and 24 hours.

The fluorescent intensities of caspase activation for the control show that, when compared to adherent cells, the supernatant cell population exhibited a significantly higher level of caspase activation ($p < 0.001$) at the 6 hour time point with values of 4.10 ± 1.0 and 8.48 ± 3.6 , respectively. At 24 hours, minimal difference in caspase expression was observed between adherent and supernatant cell populations for all samples.

In the adherent cell population, as expected, exposure to BAK led to an increase in cell death as shown by the increase in PI

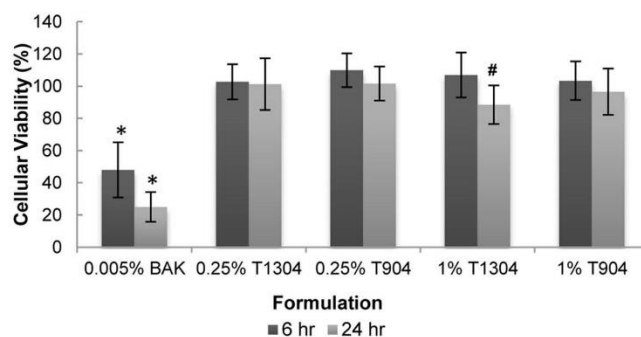


Figure 5. Effect of ocular surfactants on cellular viability of curved stratified epithelium. Cells were incubated for 6 and 24 hours with solutions of different poloxamine surfactants at different concentrations. Viability was measured by the MTT assay and is expressed as a percentage relative to cells grown in the absence of a contact lens (control). $n=5$, *significantly different from BA PBS control, #significantly different between 6 and 24 hours, $p<0.05$, T1304: Tetricon 1304, T904: Tetricon 904. doi:10.1371/journal.pone.0096448.g005

staining. At both 6 and 24 hours, PI staining in cells exposed to 0.01% BAK was significantly higher ($p<0.001$) compared to all other treatments (Figure 8).

In the supernatant cell population, following exposure to BAK, a difference in PI staining was present between the 6 and 24 hour time points (Figure 9). At 6 hours, 0.01% BAK was the only formulation that induced a significant level of cell death. At 24 hours, both 0.002% BAK and 0.005% BAK led to a significant increase in PI staining in supernatant cells ($p<0.05$). For cells exposed to 0.01% BAK, a reduction in PI staining was observed between 6 and 24 hours ($p<0.001$).

In comparing the adherent versus supernatant population of cells in our no lens control, levels of PI staining were found to be significantly higher in the supernatant population ($p<0.001$), as shown in Figure 9. The adherent population of cells appeared to have a very low necrotic population in control samples, but the overall profile changed dramatically in response to 0.01% BAK where there were significantly more PI positive cells. This is in agreement with the MTT viability data.

Discussion

In vitro Model

Many other multilayered epithelial models exist, with the most popular commercial model being the SkinEthic Human Corneal Epithelial model that was developed in 2003 by Nguyen *et al* [41]. There has since been much use of the SkinEthic model to investigate the toxicity potential of ophthalmic solutions [39,42–47] including a pre-validation study by Van Goethem *et al.* in 2006 where the SkinEthic model was shown to be a useful alternative for the *in vivo* Draize test [15].

The previously reported multilayered models have been grown using other cell types and support membranes: immortalized human corneal epithelial cell lines CIEPI 17 CL4 and SV40 immortalized cells have been grown on polyester/polycarbonate Transwell cell culture inserts [48–51] and human corneal limbal epithelial cells have been grown on polyethylene terephthalate (PET) membranes [12]. Gipson [13] and Robertson [11] have also recently developed multilayered cultures. Finally, tissue reconstruction for corneal transplantation has given rise to many different multilayered models with cells grown on human amniotic membrane or silk fibroin [52–54].

To the best of our knowledge, none of the prior models have been focused on the investigation of contact lens interactions on a

Table 1. Effect of surfactant, BAK, and PBS-lens exposure on the metabolic activity of corneal epithelial cells in a curved, stratified culture.

	Average	Stdev
BA PBS	1.03	0.65
1% T1304	1.32*	0.66
0.002% BAK	1.47*	0.38
0.005% BAK	2.69*	1.37
0.01% BAK	1.10	0.42

Following exposure to the stimuli for 24 hours, the stratified culture was digested and the cells were routinely processed for ADP/ATP expression using a luciferase assay. All values are expressed as a ratio relative to the ADP/ATP ratio on cells incubated with no lens or solution (no lens control).

$n=4$.

*significantly different from BA PBS control, $p<0.05$.

doi:10.1371/journal.pone.0096448.t001

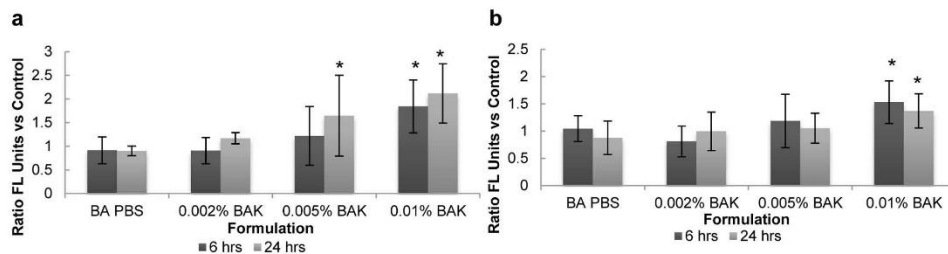


Figure 6. Effect of BAK exposure and PBS-soaked lens on (a) CD49c and (b) CD29 integrin expression. Following exposure to stimuli after 6 or 24 hours, the curved, stratified cultures of corneal epithelial cells were digested with trypsin and EDTA to form the adherent cell population. CD49c and CD29 expression was measured by flow cytometry and is expressed as a ratio relative to its expression on cells incubated with no lens or BAK (control). $n = 3$ to 5, *significantly different from PBS-soaked lens, $p < 0.05$. BA PBS: PBS-soaked balafilcon A lens. doi:10.1371/journal.pone.0096448.g006

curved multilayered epithelial substrate. A cellulose-based filter was chosen as the substrate upon which the cells would grow. These filters are capable of plastic deformation and are neither too rigid nor brittle for our purposes. Contact lenses also fit very well to the shape of the curve. The main disadvantage of using a cellulose support is its opacity, as this makes it very difficult to image. However, the MTT assay has shown to be a very useful indicator of cellular health as indicated by formazan coverage across the filter surface.

Two major implementations used in the development of the curved multilayered epithelium were the use of a collagen coating and the use of an air-liquid interface. The basement membrane, between the corneal stroma and epithelium, is a specialized extracellular matrix supporting the corneal epithelium. It is composed mainly of laminin, collagen type IV, and heparin sulphate proteoglycan (perlecan) [55]. Human corneal epithelial cells grown on collagen gels are known to secrete all of the components of the basement membrane, as well as collagen type VII: a main component of anchoring fibrils which help epithelial cells attach to the stroma [55]. The use of collagen as a matrix has been shown to support attachment, proliferation, and differentiation of corneal epithelial cells [50,56–58]. Collagen has also been shown to have a very low antigenicity [57].

Since the *in vivo* epithelium is exposed to air, it is a logical assumption that the main parameter affecting multilayer stratification and growth is the culturing of cells using an air-liquid interface. It has been shown that the air-liquid interface not only promotes proliferation, stratification and differentiation of corneal epithelial cells [56,59], but it is actually essential for growth of a properly stratified epithelium [60–62]. It is believed that the air-liquid interface promotes the shift of oxidative metabolism from the growth phase to the differentiation phase [60]. Further, it has been shown that an air-liquid interface encourages the deposition of type VII collagen and laminin [59]. The air-liquid interface thus provides a polarization effect that allows for proper stratification. Upon the introduction of an air-liquid interface, our HCEC were able to properly stratify on the curved cellulose filter.

Histological analysis confirmed that our cells were stratified to three to five layers. The curved multilayer model thus offers an *in vitro* model whereby cells can be exposed to an entire soaked lens as if the model “wears” the lens, providing a better approximation of material interaction as it occurs *in vivo*. It remains, however, limited in its ability to fully mimic the *in vivo* environment as it does not account for tear film interactions, blinking, and inflammation; all of which may affect material biocompatibility. This model also

does not account for other clinical implications of toxicity such as corneal edema, blisters, corneal opacity, and neovascularization [63]. In the current study, as a proof of concept and to ensure the reliability of the model, a PBS-soaked balafilcon A lens was used. As expected, the PBS-soaked lens proved to be very biocompatible with levels of cellular activity, integrin expression, and cell death, all comparable to a no lens control.

Previous work with stratified cultures has also focused on the viability and histology of the stratified corneal epithelium. To our knowledge, this is the first study that investigates differences in integrin expression and cell death in a stratified culture of corneal epithelial cells using flow cytometry. With the curved *in vitro* model, not only were the adherent cells that comprise the majority of the tissue characterized, but also the “shed” cells, which were collected from the culture media on the top of the filter prior to trypsinization. Digestion of *in vivo* corneal epithelial tissue for the purpose of flow cytometry has been done previously to study the necrotic population of cells [64]. *In vitro* stratified cultures have also been digested to examine the apoptotic response of corneal epithelium to UVB through colorimetric and fluorometric analyses [65].

Cellular Viability

As previously described, the MTT assay is based on the reduction of tetrazolium salts. Mitochondrial, cytosolic, and microsomal enzymes are all capable of performing the reduction of MTT [66]. MTT is endocytosed into the cells where it is reduced to formazan in the endosomal/lysosomal compartment [67]. The formazan then accumulates in blue cytoplasmic granules and is exocytosed as needle-like aggregates on the cell surface [67,68]. We therefore describe the MTT assay as a viability assay, which lends itself to a belief that it is a relative number of living cells to those that are dead. However, it may be more descriptive to refer to it as an assay of cellular activity. With increasing concentrations of BAK, cellular activity is greatly reduced at both 6 and 24 hours. Cells exposed to concentrations of 0.01% and 0.005% BAK sustained significant damage and continuous exposure to this dose of BAK prevented cell repair and growth. However, cells exposed to 0.002% BAK, while having a reduced viability at 6 hours, appeared to have a somewhat increased viability at 24 hours which would suggest that the stratified cells were able to repair themselves from the damage caused by BAK.

Our MTT viability results with BAK are in agreement with previously reported toxic mechanisms *in vitro*, primarily with the use of the SkinIt^h model in the work of Baudouin and Khob-

Table 2. Effect of BAK exposure and PBS-soaked lenses on cell integrin expression in a curved, stratified culture of corneal epithelial cells after 6 and 24 hours.

Supernatant	CD49c						CD29					
	Average			Stddev			Average			Stddev		
	6 hrs	24 hrs	6 hrs	24 hrs	6 hrs	24 hrs	6 hrs	24 hrs	6 hrs	24 hrs	6 hrs	24 hrs
BA PBS	0.92	1.06	0.43	0.09	0.43	0.09	0.99	1.30*	0.18	0.18	0.18	0.40
0.002% BAK	0.76	0.84	0.23	0.14	0.23	0.14	0.95	1.09*	0.29	0.29	0.29	0.15
0.005% BAK	0.99	0.72	0.57	0.40	0.57	0.40	0.78	1.00*	0.39	0.39	0.39	0.46
0.01% BAK	0.78	0.84	0.31	0.20	0.31	0.20	0.79	1.23*	0.31	0.31	0.31	0.32

Following exposure to stimuli, the surface of the stratified cultures were gently washed and this supernatant was collected and processed. CD49c and CD29 expression was measured by flow cytometry and is expressed as a ratio relative to expression on cells incubated with no lens or BAK (control). BA PBS: PBS-soaked balaifilicon A lens.
n = 3–5.
*Significantly different from 6 hour value, p < 0.05.
doi:10.1371/journal.pone.0096448.t002

Reiter [39,42,45–47]. Consistently, increasing concentration and time exposure to BAK is shown to have an increasingly negative effect on the health of corneal epithelial cells. However, our curved model appears to be slightly more sensitive to cytotoxicity than what has been shown using the SkinEthic model [39]: for similar levels of BAK exposure, approximately 20% to 60% viability was observed in our model compared to the SkinEthic model. These differences in viability may be due to differences in surface area, exposure time, BAK source, and other factors such as the different structure of the *in vitro* models.

The luciferase assay was originally developed using the biomimetic system found in fireflies [69]. Briefly, luciferase acts as an enzyme to catalyze the reaction between ATP and luciferin to form light: this light can then be measured using a luminometer. ADP can also be measured through its conversion to ATP and its subsequent reaction with luciferase. The ratio of ADP/ATP has since been shown to correlate with apoptosis and necrosis and to provide measures of cellular viability [70,71]. Geerling *et al* [72] previously used a luciferin-based assay to assess the cytotoxicity of BAK using a monolayer model of primary corneal epithelial cells. The assay showed to be more sensitive than scanning electron microscopy evaluation of morphological changes as well as the live/dead calcein/ethidium homodimer fluorescence assay. Our luciferase results further confirmed our cellular viability findings as measured by MTT, namely an increase in cell death with increasing concentrations of BAK, demonstrating that the luciferase assay may also be used to detect cytotoxicity in stratified cultures.

Poloxamine Surfactant Cytotoxicity

Poloxamines represent a family of nonionic amphiphilic surfactants and have been reported to be relatively nontoxic by BASF, their manufacturer [73]. Poloxamines are currently used in ophthalmology in contact lens cleaning solutions and eye drop formulations [32,33,74]. In our model, these surfactants show a negligible decrease in viability which is in accordance with recent *in vitro* and *ex vivo* corneal assays that demonstrated the safety of these surfactants for the ocular surface [75]. Poloxamers, similar to poloxamines but with a generally lower molecular weight, are also frequently used in ophthalmic preparations. While both families of surfactants have been shown previously to induce cytotoxicity *in vitro* [74], the study used a colony-forming assay of Chinese hamster lung fibroblasts V79 cells and a 6-day incubation. The long incubation time, the type of culture (isolated colonies versus stratified culture), and the cells used (V79), which may be more sensitive due to tissue and species variations, likely explain the difference in results. Overall, only 1% Tetronic 1304 showed a marginal decrease in viability ($88 \pm 1.2\%$ relative to control) as well as an increase in ADP/ATP relative to control, which may indicate some associated cell death. However, given the level of change observed compared to control, this concentration would likely not be considered cytotoxic.

Integrin Expression

Integrins are heterodimeric transmembrane glycoproteins that consist of an alpha and a beta chain, and are responsible for the interactions between epithelial cells and their extracellular matrix. Integrin chains α_3 (CD49c) and β_1 (CD29) form heterodimers that are known to have strong roles in epithelial adhesion and maintenance of cell-cell junctions; they have also been reported to play a role in cell spreading [28,76]. The heterodimer $\alpha_3\beta_1$ is a receptor for fibronectin, laminin 5, laminin 10, and all major components of the corneal basement membrane [76] and $\alpha_3\beta_1$ heterodimers are expressed in the basal or suprabasal layers of the

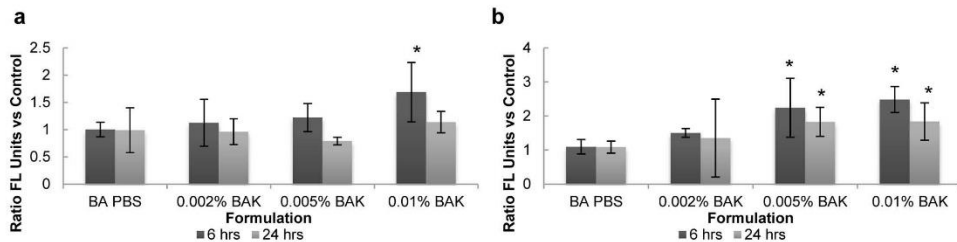


Figure 7. Effect of BAK exposure and PBS-soaked lens on caspase expression in the (a) adherent and (b) supernatant populations. Following exposure to stimuli after 6 or 24 hours, the curved, stratified cultures of corneal epithelial cells were digested with trypsin and EDTA. Caspase expression was measured by flow cytometry and is expressed as a ratio relative to its expression on cells incubated with no lens or BAK (control). $n = 3$ to 5, *significantly different from PBS-soaked lens, $p < 0.05$. BA PBS: PBS-soaked balafilcon A lens. doi:10.1371/journal.pone.0096448.g007

corneal epithelium [77,78]. Higher levels of $\alpha_3\beta_1$ are expressed on cells with higher proliferative properties [77,79,80]. It has also been reported that β_1 integrins can at least partially compensate for the loss of desmosomes and adherens junctions [77].

Integrin expression in supernatant cells appeared to be relatively consistent regardless of applied formulation and showed levels of expression similar to control cells. As the supernatant cells have been sloughed or worn off the multilayered tissue, it is likely that these cells no longer attempt to promote attachment. On the other hand, with increases in BAK concentration, the adherent cells showed a significant increase in CD49c (α_3) and CD29 (β_1) expression. This increase in integrin expression suggests that prolonged exposure to a cytotoxic stimulus such as BAK may lead the cells in the stratified tissue to enter a repair phase and consequently display a phenotype that increases their ability to proliferate and migrate.

Cell Death Mechanisms

Apoptosis is a programmed form of cell death and serves, among others, as a defense mechanism in the removal of damaged cells. Apoptosis is a highly regulated, programmed cell death pathway, mediated in part by the action of caspases [81]. Caspases are cysteine aspartate proteases that act as mediators for initiating cellular disassembly and are normally found in the inactive, pro-caspase form [82,83]. Initiation of apoptosis occurs upon activation of initiator caspases by intrinsic or extrinsic factors, which can subsequently activate downstream effector caspases.

Alternatively, necrosis is a form of dysregulated cellular death. Necrotic cells that have an intact nuclear membrane are stained by propidium iodide, which intercalates with DNA and provides a strong fluorescent signal in its bound form. It is also possible that cells positive for PI staining may be the result of an apoptotic death pathway.

Fluorescent levels of caspase and PI were much higher in the supernatant overall as compared to the adherent populations. This agrees with Pauly's previous findings that cell death was localized to the outer layers of the multilayer following exposure to BAK [84].

Similar trends in caspase expression were observed in both supernatant and adherent cells whereby increases in BAK concentration resulted in increased caspase activation at 6 hours; albeit by 24 hours, caspase levels were lower compared to 6 hours. This is likely due to the 24 hour time point being too late to observe caspase activation. Caspase activation is an initiator of apoptosis and activation may not be detectable at later time points depending on the type of death cycle the cells may have entered (secondary necrosis versus apoptosis). Interestingly, the response of the multilayer to a strong stimulus (0.01% BAK) was different from a weak stimulus (0.002% or 0.005% BAK), suggesting a dynamic cell death process within the stratified culture model. With the 0.01% BAK samples, the reduction in PI staining between 6 and 24 hours in the supernatant was paralleled by an increase in PI staining in the adherent population. Thus, prolonged exposure to BAK may cause cells in the supernatant to lose their nuclear membrane integrity and ability to stain with

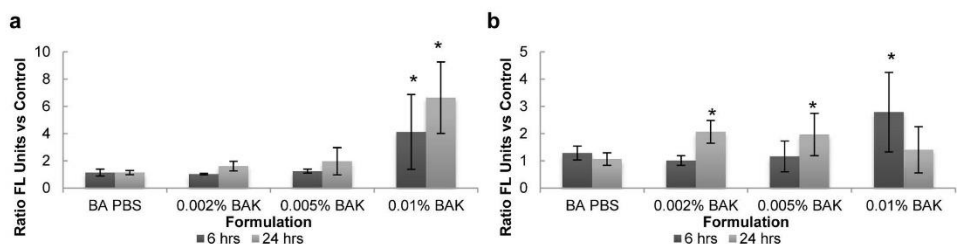


Figure 8. Effect of BAK exposure and PBS-soaked lens on PI staining in the (a) adherent and (b) supernatant populations. Following exposure to stimuli after 6 or 24 hours, the curved, stratified cultures of corneal epithelial cells were digested with Trypsin and EDTA. PI staining was measured by flow cytometry and is expressed as a ratio relative to its expression on cells incubated with no lens or BAK (control). $n = 3-5$, *significantly different from PBS-soaked lens, $p < 0.05$. doi:10.1371/journal.pone.0096448.g008

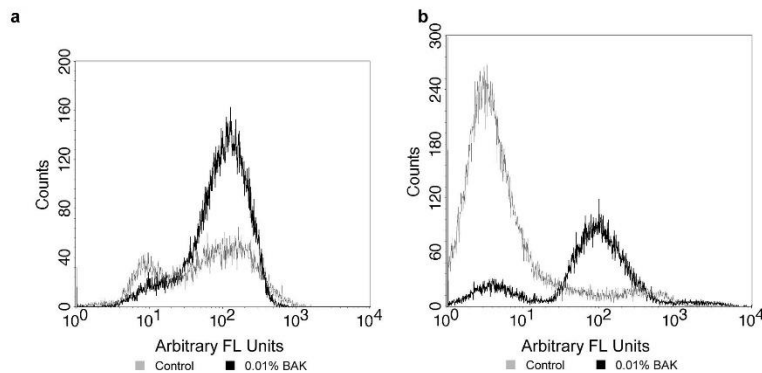


Figure 9. Cell death following BAK exposure as measured by PI staining. The histograms display PI staining in the supernatant (a) and adherent (b) cell population following 24 hour exposure to 0.01% BAK. The grey histogram represents cells from the no lens, no solution control; and the black histogram represents cells exposed to BAK. The positive peak for PI (>10 arbitrary fluorescent units) can be observed in both the control and BAK supernatant population, as cells slough off the stratified culture. A significant increase in cells staining positive for PI can be observed following BAK exposure.
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PI: these cells detached from the stratified culture and thus were potentially already damaged, making them more sensitive to cytolytic damage by BAK. Such a phenomenon does not appear to occur at lower BAK concentrations as an increase in PI staining in supernatant cells is observed for these treatments at 24 hours. The change in PI expression may be explained by the apoptotic cells in the supernatant population entering secondary necrosis, which has been previously observed [64,85]. The increase in PI staining at 24 hours for all BAK samples highlights the completion of the cell death cycle whereby a certain number of cells in the stratified culture were irreversibly damaged by exposure to BAK. The results from the 6 hour time point suggest that apoptosis played a role in the cell death observed at 24 hours. These results emphasize the importance of early time points when measuring cell death mechanisms. The PI staining results also correspond well with the observed reduction in viability as measured by MTT and the luciferase assay.

It is also worth discussing the overall lack of significant change observed with exposure to 0.002% BAK. While viability, as measured by MTT, decreased at both 6 and 24 hours, no significant upregulation of integrins or caspase expression occurred. PI staining increased in the supernatant cell population, but only after a 24 hour exposure. This was also noted for 0.002% BAK using the luciferase assay. This suggests that BAK may only be harmful at high concentrations: though cells may still be damaged, they may be able to withstand certain low concentrations of BAK, allowing the tissue to recover. These results are in accordance with the previous study by Pauly *et al.* [39] showing that cells exposed to lower concentrations of BAK did not see a continued decrease in reduction of viability following a 24 hour recovery period.

Conclusions

A healthy, reconstructed corneal epithelium in the shape of the regular human cornea *in vitro* was successfully grown. Our model appears to be well-stratified with three to five layers of cells. The overall suitability of our model for biocompatibility experiments

was verified by exposure to the well-known cytotoxic compound benzalkonium chloride, which is currently used as a preservative in commercially-available eyedrop formulations. Surfactant cytotoxicity was also found to not be implicated in the cytotoxicity of BAK.

To assess cellular viability and cell phenotype via flow cytometry, new protocols were developed for the stratified curved models. Both supernatant cells, which had been sloughed off of the multilayer, and adherent cells, which had remained adherent as part of the stratified culture, were characterized. Cells in the supernatant were found to be significantly more caspase and PI positive. A dynamic cell death mechanism was observed across the varying concentrations of BAK, leading to the hypothesis that many cells die through apoptosis. An increase in integrin expression was also noted with increasing concentrations of BAK implying that the cells were attempting to repair the damaged tissue. Examination of cell phenotype via flow cytometry proved to be a very useful quantitative method for detecting sensitive changes in our curved, stratified corneal epithelium.

Overall, our model was able to detect differences in cytotoxicity over time. Furthermore, exposure of the curved stratified culture to a PBS-soaked lens did not have a significant effect on cells, suggesting that our *in vitro* model is suitable to investigate contact lens/solution interactions.

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Author Contributions

Conceived and designed the experiments: CKP RP SW ANW MBG. Performed the experiments: CKP RP SW DII. Analyzed the data: CKP RP SW ANW DII MBG. Contributed reagents/materials/analysis tools: DII MBG. Wrote the paper: CKP MBG.

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Appendix B

The non-inflammatory phenotype of tear-film neutrophils

Please see next page.

The non-inflammatory phenotype of neutrophils from the closed-eye environment: a flow cytometry analysis of receptor expression

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Introduction

Neutrophils represent 40–60% of the leukocyte population ($3\text{--}5 \times 10^6$ neutrophils/ml) and, as cells of the innate immune system, are our first line of defense and play a critical role in inflammation and preventing infection.¹ While neutrophils circulate in blood, they are also found in tissue following extravasation in response to an inflammatory stimulus. Chemotactic molecules such as complement activation products and cytokines have been recognized to play a role in extravasation. In response to inflammatory stimulus (complement activation proteins, cytokines, chemokines, lipid mediators) and infection, neutrophils will undergo activation and release potent mediators such as inflammatory cytokines, metalloproteinases (which can degrade the extracellular matrix), lipoxygenase and cyclooxygenase enzymes (which can further generate both pro- or anti-inflammatory lipid mediators) and oxidative products.^{1, 2}

The anterior segment of the eye represents a unique environment due to its immune privilege. This environment is meant to protect the eye from the damage that inflammatory cells may cause upon activation.²⁻⁴ The presence of neutrophils in the anterior eye is thus highly regulated. In inflammatory diseases such as dry-eye⁵ and allergy,^{6, 7} neutrophils have been observed in the pre-corneal tear film and their relevance and contribution to disease is currently being investigated. Any wound to the cornea such as the ones induced by chemical burns or puncture also leads to the invasion of the cornea by neutrophils. During wound healing, neutrophils migrate into the tear film by leakage through the blood vessels and research has identified lumican as a key player in neutrophil extravasation to the cornea.^{8, 9} The presence of neutrophils contributes to the resolution of inflammation and ocular wound closure,^{2, 10, 11} and Wang *et al* recently demonstrated that lipid mediators can control the state of neutrophil activation inflammatory resolution in the mouse cornea.¹²

However, in the absence of any wound or infection, the closed-eye environment represents a unique condition in the eye where a significant influx of neutrophils regularly takes place: every day, during sleep, changes in tear composition with increases in complement activity, metalloproteinases and cytokines correlate with the recruitment of leukocytes to the ocular surface.¹³⁻¹⁵ Wilson *et al*¹⁴ first identified the presence of leukocytes in ocular cell collection upon awakening. Tan *et al*¹⁵ further reported that neutrophil recruitment, during sleep, appears to be intensified between 3 and 5 hours, directly following an increase in complement activation.

Ocular neutrophil recruitment by corneal infection, wounding or inflammation due to ocular diseases has seen extensive investigation in rabbit¹⁶⁻¹⁸ and mouse^{11, 12, 19, 20} models. However, these inflammatory conditions are significantly different from the closed-eye environment and thus mechanisms of cell migration/recruitment and activation likely differ all together. Furthermore, it is difficult to draw conclusions about the phenotype of closed-eye neutrophils in animal models given that these animals do not close their eyes.

This current study presents a simple and rapid method to collect tear-film neutrophils and was conducted to characterize the phenotype of closed-eye tear-film neutrophils. Their response to inflammatory stimuli was investigated and compared to that of blood-isolated neutrophils, as blood-isolated neutrophils have been commonly used to characterize interactions with ophthalmic biomaterials and/or ocular bacteria.^{21, 22} Cell viability was assessed following cell collection and flow cytometry was used to characterize cell activation through the expression of selected cell membrane receptors and oxidative products.²³

Materials and methods

Reagents and monoclonal antibodies

Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4, phorbol-12-myristate-13-acetate (PMA), N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), endotoxin-free water, ethylenediaminetetraacetic acid (EDTA), dichlorodihydro-fluorescein diacetate (DCF), Giemsa stain and paraformaldehyde were purchased from Sigma-Aldrich Co (Oakville, ON, Canada). Phosphate-buffered saline (PBS; pH 7.4) was acquired through Lonza (Allendale, NJ, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and Hoechst were purchased from Life Technologies (Burlington, ON, Canada). All other chemicals were of analytical reagent grade.

Fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against human CD11b and CD66b, R-phycoerythrin (PE) conjugated monoclonal antibodies against human C3aR and CD54 and R-phycoerythrin-cytochrome 5 (PE-Cy5) conjugated monoclonal antibody against CD45 were purchased from Becton Dickinson (San Diego, CA, USA). FITC conjugated annexin V was also from BD.

Tear-film Cell collection

Neutrophils were collected after sleep from healthy participants. This study was conducted in accordance with the tenets of the Declaration of Helsinki and received ethics clearance from the University of Waterloo Human Research Ethics Committee. Twelve healthy study participants, six female and six male, within the age range of 21 to 44-years, were involved. All participants were trained to self-collect their tear-film neutrophils using a polypropylene pipette containing sterile PBS. After 8 hours of sleep, participants were instructed to gently wash their eyes with

approximately 5 mL of PBS for each eye; the eye wash was collected in one sterile polypropylene tube (pooled sample). The collected samples were brought to the laboratory within 90 minutes of collection and processed immediately. Serum-containing medium (DMEM with 10% FBS, to be referred to as DMEM/FBS) was then added and the cell collection was centrifuged at 250g and cells were resuspended in a small volume of DMEM/FBS. In some experiments, cells were resuspended in artificial tear solution instead (ATS, see Table 1 for ATS composition).

Peripheral blood neutrophil isolation

Blood was drawn from the same healthy participants who were free of medication for at least 48 hours. Low molecular weight heparin (10 U/mL) was used as an anticoagulant. Platelet-rich plasma was first removed by centrifugation at 100g. Blood neutrophils were then isolated using the density gradient PolymorphPrep, washed three times in EDTA and serum-containing media, and were ultimately resuspended in DMEM/FBS.

For the experiments performed to assess the effect of tear proteins on cell activation, the last wash was performed in an excess of PBS to remove the serum proteins present in the resuspended blood-isolated neutrophils. After centrifugation, supernatant was aspirated and cells were resuspended in either ATS, DMEM/FBS, PBS, or PBS+Lactoferrin (LF).

Leukocyte viability

For blood-isolated leukocytes and tear-film cell collection, viability and cell count were determined using a haemocytometer following 1:1 dilution of a small aliquot in Trypan Blue. The fluorescent dye Hoechst was also used. Tear film neutrophils were incubated with Hoechst for 30 min at 37°C prior examination by fluorescence microscopy.

Viability of tear-film neutrophils and apoptosis were further characterized by flow cytometry using propidium iodide (PI) and the fluorescently-labelled pan-caspase inhibitor (FITC-VAD-FMK, Calbiochem, San Diego, California). Tear-film neutrophils were incubated with FITC-VAD-FMK for 1 hour at 37°C. Samples were washed and resuspended in wash buffer, before immediate analysis by flow cytometry.

Cell stimulation

Cells were allowed to “equilibrate” in medium for 30 minutes prior to incubation with stimulus. Three stimuli, that are recognized to induce an inflammatory response in leukocytes, were used: PMA (2µM final concentration), a protein kinase C activator; LPS (2µg/ml final concentration), also known as bacterial endotoxin; and fMLP (17nM, final concentration) which induces a G-protein coupled receptor mediated physiological activation. Samples were divided in aliquots (Rest, ie unstimulated; fMLP-stimulated; LPS-stimulated and PMA-stimulated). Stimulation was performed at 37°C for 15 min (fMLP-stimulated samples) and 30 minutes (LPS-stimulated samples). PMA-stimulated samples were incubated for 20 minutes at room temperature.

Expression of membrane receptors on leukocytes and oxidative response.

After incubation with stimulus, 30µL of cell suspension was transferred into tubes containing fluorescently labeled antibodies against C3aR, CD11b, CD45, CD54, CD66b (see Table 2) and incubated for another 30 minutes at room temperature in the dark. At the end of the incubation, samples were diluted and fixed with paraformaldehyde (1% final concentration). All samples were analyzed on the flow cytometer within 5 days.

The formation of reactive oxygen species in stimulated and unstimulated samples was characterized using the fluorescent probe dichlorodihydro-fluorescein diacetate (DCFH-DA).

DCFH-DA, a small non-polar, non-fluorescent molecule, diffuses into the cells and is oxidized by H₂O₂ to the fluorescent molecule DCF.²⁴ There is also evidence that nitric oxide may be able to convert DCFH to the fluorescent probe DCF²⁵ and thus DCF may assess more than just the production of reactive oxygen species. DCF was added to samples (48 μM final concentration). After 30-minute incubation at 37°C, samples were diluted and immediately analysed on the flow cytometer.

Flow cytometry

All samples were acquired on a Becton Dickinson FACSCalibur flow cytometer (Mountain View, CA, USA) using CELLQuest Software. At least 5000 events were acquired. Appropriate isotype controls were used with each experiment. Data analysis was performed using CELLQuest post data acquisition.

Due to their multi-lobed nucleus, neutrophils are also known as polymorphonuclear cells (PMN) and both terms are used interchangeably in the results and discussion. When reporting all results, the following nomenclature applies: neutrophils collected after sleep are referred to as “**tear-film PMNs**”, neutrophils isolated from blood as “**blood-isolated PMNs**”. To allow for comparisons between the different types of neutrophils, the mean fluorescence intensity in arbitrary units (MFI) as well as the ratio of the fluorescent intensities of rest (unstimulated sample) versus stimulated are presented.

Statistical analysis

All results are reported as means ± standard deviation. To evaluate the significance of the differences in the ratio of cell activation, an analysis of variance (ANOVA) was performed followed by multiple pairwise comparisons using the Tukey test. Analysis was performed using

Statistical Analysis Software (SAS, Cary, NC, USA) and a *p* value of less than 0.05 was required for statistical significance.

RESULTS

1) Characterization of tear-film neutrophils, cell count and viability.

After receiving the closed-eye cell collections, samples were processed immediately. A viable leukocyte cell count was performed using trypan blue exclusion and viability was determined to be 98%. Corneal epithelial cells, as identified by their cobblestone shape, were also observed during cell count but were not counted; in most cases, corneal epithelial cells were found to be dead (stained with trypan blue) (Figure 1). Based on the trypan blue count, total cell collection led to a leukocyte count of $3.78 \times 10^5 \pm 3.02 \times 10^5$ (ranging from 4.28×10^5 to 1.44×10^6 leukocytes).

Hoechst staining further confirmed the viability of tear-film neutrophils and the tri-lobed nucleus, which is a hallmark of these PMN cells (Figure 2). A Giemsa stain of a closed-eye cell collection is shown in Figure 3, which highlights the presence of neutrophils in the cell collection. Tear-film PMNs were found to have a smaller diameter than blood-isolated PMNs ($p < 0.03$), of $9.0 \pm 0.3 \mu\text{m}$ and $9.5 \pm 0.3 \mu\text{m}$ respectively, as measured by the Moxi Z automated cell counter (ORFLO; Hailey, ID, USA).

The leukocyte population in the closed-eye collection samples was identified using the pan-leukocyte marker CD45. As shown in Figure 4, regions were created around the PMN population (R1 and R3) and light scatter characteristics (proportional to cell size and granularity) as well as fluorescent values were obtained by gating on the PMN population only. The PMN population in the closed-eye cell collection was easily identifiable by its scatter characteristics (Figure 4a).

Lymphocytes and a few monocytes were also identified by flow cytometry. In all the collection performed, the PMN population represented $58 \pm 18\%$ of the total ocular cell collection. Consistent with the process of extravasation and the interaction of L-selectin with endothelial cells lining the blood vessels,^{26, 27} tear-film PMNs appeared to have shed L-selectin: L-selectin expression on tear-film neutrophils was close to background levels (Figure 5).

Flow cytometry was also used to further assess the viability of tear-film neutrophils (PI staining) and the presence of apoptotic cells was determined using FITC-FAD-FMK, a caspase inhibitor that binds to activated caspases in cells. Few leukocytes were observed to stain with PI and caspase, as illustrated in Figure 5, which further confirmed that the collected tear-film neutrophils were alive and did not display signs of apoptosis. Tear-film neutrophils also did not stain with Annexin V, another marker for apoptosis (data not shown).

2) Tear-film PMNs response to stimulus *in vitro*

All results that are presented refer to the double gated cell population that specifically identify PMNs.

a. Membrane receptor expression

To characterize tear-film PMNs, their response to various inflammatory stimuli was determined and then compared to blood-isolated PMNs. The response is expressed as the ratio between rest (unstimulated) versus stimulated samples to allow for comparisons between different sources of neutrophils and accounts for difference in baseline level of receptor expression. As illustrated in Figure 7, in response to LPS stimulation, all ratios remained around 1.0 indicating that the tear-film PMNs were unable to upregulate their expression of Mac-1, ICAM-1, CD66b or CD45. This response to LPS stimulation (or lack thereof) was significantly different from that of blood-

isolated PMNs, where a significant upregulation of receptors was observed. The lack of response of tear-film PMNs to stimulus was also observed with fMLP and PMA (Table 3). In contrast, blood-isolated neutrophils responded to stimulation in the expected manner as previously reported.^{23, 28} PMA, LPS and fMLP have been shown to upregulate CD11b, CD54 and CD66b. Upon a 30 min stimulation with PMA at 37°C, C3aR is generally internalized (i.e. a downregulation of C3aR expression is commonly observed).²⁹ However, no C3aR internalization occurred under the PMA-stimulation condition (20 min at 24°C).

A difference in the baseline level of receptor expression was also noted: the mean fluorescence values of membrane receptor expression are presented in Table 4. Significantly higher levels of expression of ICAM-1 and CD66b were observed on the unstimulated tear-film PMNs when compared to blood-isolated PMNs.

b. Oxidative response

DCF was used to assess oxidative products in neutrophils following stimulation. Significant variations in the generation of oxidative products were found, which resulted in a high standard deviation for both tear-film and blood-isolated neutrophils, an observation that has been shared by other researchers.³⁰ As shown in Table 3, tear-film neutrophils were able to mount an oxidative response following stimulus in a manner similar to blood-isolated neutrophils. The baseline level of oxidative products was also significantly higher with tear-film neutrophils when compared to blood-isolated neutrophils ($p < 0.04$).

3) The effect of an artificial tear solution on neutrophil response to stimuli

To determine if the response of tear-film neutrophils to different stimuli may be dependent on the presence of tear film proteins, activation was performed in an ATS containing appropriate

concentrations of proteins known to be present in the tear film, namely lactoferrin, lysozyme, mucin, IgG and albumin.¹⁵ Stimulating tear-film neutrophils in ATS resulted in a similar response, or lack thereof, previously observed in DMEM/FBS (Table 5). In ATS, blood-isolated PMNs were still able to mount an inflammatory response as shown by an activation ratio above 1.0 for all markers (Table 5). However, when compared to ratios observed with DMEM/FBS, a significant reduction in the ability of blood-isolated PMNs to upregulate the expression of CD11b and CD66b in response to LPS stimulation was identified ($p < 0.045$). On the other hand, isolated blood PMNs were able to mount a normal response to fMLP and PMA stimulation in the presence of ATS. Interestingly, the baseline levels of Mac-1 and CD66b expression were significantly reduced in ATS when compared to the levels observed in DMEM/FBS (see Table 4), with 42 ± 14 and 25 ± 10 for Mac-1 and CD66b expression respectively.

As DMEM/FBS is a complex salt and protein solution, to allow for better comparisons with stimulation in ATS, a series of stimulation experiments with physiological stimuli (LPS and fMLP) was performed using blood-isolated PMNs in PBS supplemented with various components (ATS is a PBS solution supplemented with tear film proteins). There was no significant difference between the level of response to stimulation between DMEM/FBS and PBS (data not shown), suggesting that the lower response to stimulation previously observed with ATS was due to the presence of the tear film proteins. The presence of lactoferrin in PBS resulted in a significant reduction of the activation ratio only with CD66b in response to LPS; upregulation of CD66b was also still higher in PBS+LF when compared to stimulation in ATS (Table 6). It is worth noting that a significant reduction in the baseline level of expression of CD66b were observed in the presence of lactoferrin, with 38 ± 11 and 23 ± 6 for PBS and PBS+LF respectively. While this highlights that lactoferrin has an anti-inflammatory role, these

results also suggest that other components of the ATS play a significant role in the reduced the inflammatory response observed with blood-isolated PMNs stimulated in ATS.

DISCUSSION

While it has been recognized that neutrophils invade the ocular surface during closed-eye conditions,^{14, 31, 32} there has been no study aimed at characterizing the phenotype of these cells. In a previous lens wear study by Wilson *et al*, between 630 and 22,000 leukocytes were collected in one eye (median for cell collection was 6500 leukocytes) following 8 hours of sleep.¹⁴ Tan *et al* also performed an ocular wash after sleep and reported $1,529 \pm 18$ and $6,583 \pm 2,354$ PMNs after 5 and 8 hours of sleep respectively.³² Using our collection protocol, we were able to collect a significantly higher numbers of PMNs; difference in materials used for the collection (polypropylene tubes, phosphate buffer),the centrifugation protocol and training of the study participants are likely responsible for the higher yield obtained in our study. Based on the numbers of PMNs collected and the volume of tears, the concentration of inflammatory cells at the ocular surface during closed-eye conditions may be approximated to be 3×10^7 cells/mL which is at least 5 times as concentrated as neutrophils in the blood. For such a high concentration of inflammatory cells to be present on a nightly basis, a balance needs to exist between protection of the eye against pathogens and preservation of the integrity of the corneal epithelium.

Current rabbit and mouse models have been able to contribute significant knowledge related to the presence and nature of neutrophils on the ocular surface during inflammation, wound healing³³ and infection.¹⁹ However, physiological differences such as protein concentrations¹⁷ and the lack of prolonged eyelid closure in animal models hinder our ability to extrapolate and perform studies related to the closed-eye environment in humans. While eyelid closure (through

suturing) mimics hypoxic conditions, the sutures may induce sub-inflammatory conditions and affect the normal recruitment. Costs, ethical issues and physiological differences^{17,18} with the human cornea and ocular environment make these animal models difficult to use to study closed-eye conditions and thus limited knowledge exists related to this “daily” presence of inflammatory cells on the ocular surface. We currently lack significant knowledge to understand what mechanisms regulate activation or lack thereof (see discussion below) of tear-film neutrophils in the closed-eye environment. Further investigations will be required to elucidate the complex balance of regulatory mechanisms that exists on the ocular surface.

While refractory behavior, whereby neutrophils respond distinctively from blood-isolated PMNs, has been observed previously in neutrophils extravasated to the lungs³⁴ and inflamed tissues,^{35, 36} our work is the first report of this observation with neutrophils from the ocular surface. A recent study by Baines et al³⁷ reported significant differences in level of cytokine synthesis in resting sputum neutrophils from asthmatics versus healthy participants as well as following response to LPS stimulation. Lakschevitz et al³⁸ also reported different cytokine and receptor expression profiles for oral neutrophils compared to circulating neutrophils. Extravasated neutrophils have been shown to have higher levels of CD66b³⁶ and β -catenin, a receptor important in epithelial repair.³⁹ Assuming neutrophils collected upon awakening extravasated from ocular blood vessels, the complex process of activation, adhesion to the endothelium, and ultimately endothelial transmigration may have contributed to the phenotype of tear-film neutrophils.²⁶

Changing the medium of incubation for tear-film neutrophils from DMEM/FBS to ATS did not result in any changes in the response to inflammatory stimuli, suggesting that the impaired response to stimulus was not due to the absence of a specific factor. On the other hand,

significant changes were observed in the response of blood-isolated neutrophils where a reduction in CD66b was observed when stimulation took place in ATS or in PBS+lactoferrin, suggesting that interactions with specific proteins in the tear film may contribute to inhibit degranulation (CD66b is a marker of degranulation). The concept of anti-inflammatory properties of tear film proteins is not new.^{40, 41} Among the many proteins of the tear film, numerous studies on lactoferrin have been published as it is present in both blood and tears and is also a secretory product of PMNs. Lactoferrin is known for its antimicrobial properties⁴² but is also recognized to have anti-inflammatory properties with leukocytes.^{43, 44} The role of lactoferrin in production of oxidative species appears to be more complex and dependent on experimental conditions. Gahr *et al* have shown that the presence of lactoferrin at 500µg/ml induced greater motility as well as release of superoxide in neutrophils.⁴⁵ However, lactoferrin at 50µg/ml inhibited LPS-induced production of reactive oxygen species.⁴⁶ Our results with ATS thus supports previous finding that lactoferrin at 1800µg/ml can reduce leukocyte response to inflammatory stimulus and that this inhibition is stimulus dependent. It is therefore possible that the anti-inflammatory phenotype of tear-film PMNs may be the result of prolonged exposure to lactoferrin and other anti-inflammatory compounds in the tear film. There is significant evidence that exposure to cytokines will result in phenotypic and functional changes in neutrophils in injured tissues; this mechanism is believed to relate to innate and adaptive immunity and further highlights the heterogeneity in the neutrophil population.⁴⁷

The non-inflammatory phenotype observed in the tear-film PMNs may seem in contradiction to the fact that ocular PMNs are also exposed to high levels of inflammatory mediators such as IL-8 and C3a. It is possible that exposure to high levels of C3a may also lead the refractory or non-inflammatory phenotype of PMNs in tears. Such an effect has been previously described with

cytokines⁴⁸ and C5a, which, similarly to C3a, is a potent chemoattractant and inflammatory mediator in neutrophils under normal conditions. Impaired neutrophil function in sepsis has been linked to prolonged exposure to C5a⁴⁹ and internalization of the C3aR has also been observed as a negative control mechanism.²⁹ Interestingly the baseline levels of C3aR expression on tear-film neutrophils did not significantly differ from blood-isolated neutrophils, suggesting that other control mechanisms may be responsible for the phenotype of tear-film neutrophils.

It is also possible that tear-film neutrophils have a distinct phenotype that is not induced by the closed-eye environment but is “inherited”. Several recent studies suggest that unique population of leukocytes exists in blood^{35,50} and in murine lacrimal glands (Gronert *et al*, ARVO 2014). Further investigations are required to determine the origin of the tear-film neutrophil phenotype, to understand how components of the tear film affect the neutrophil phenotype, and to investigate the potential of tear-film neutrophils to contribute to a pro- or anti-inflammatory response.

CONCLUSION

The current study highlights how neutrophils collected from the ocular surface following closed-eye conditions are alive and not apoptotic, but in a state where various inflammatory stimuli induce a limited cellular response, as measured by receptor expression and oxidative products. The response of tear-film neutrophils to physiological (fMLP, LPS) and chemical (PMA) stimuli is in contrast to that of blood-isolated neutrophils. The presence of tear-film proteins did not change the tear-film neutrophil response to stimulus, but did somewhat affect receptor expression and stimulus-induced response in blood isolated neutrophils. This suggests that the

non-inflammatory or refractory tear-film neutrophils phenotype may be induced in part by exposure to tear-film proteins. Due to their short life-span and limited DNA material, neutrophils have often been considered terminally differentiated with the assumption that minimal changes in gene expression and phenotype occur once they leave the bone marrow. Our study adds to the mounting evidence from the peritoneum⁵¹, lung⁵², mouth³⁸, nose⁵³, and ocular surface⁵⁰ that such concepts are ill-defined and blood-isolated neutrophils may not always represent an appropriate model to study mechanism of inflammation in extravasated neutrophils. Further investigations are required to fully phenotype the tear-film neutrophils from the closed-eye environment and determine the mechanisms leading to the observed refractory phenotype.

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Figure 1: Cell collection stained with trypan blue. Cells were collected immediately upon awakening. Many viable leukocytes and some dead epithelial cells (blue-stained cells) were present.

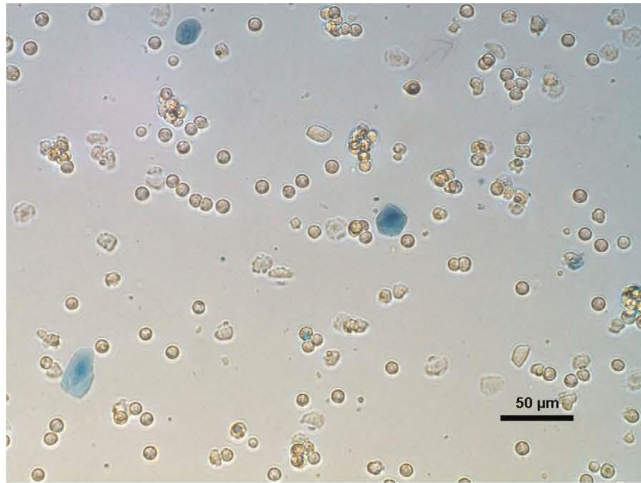


Figure 2: Cell collection stained with Hoechst. Cells were collected immediately upon awakening, stained with Hoechst and observed under an epifluorescence microscope. The tear-film PMNs show the typical multi-lobed nucleus.

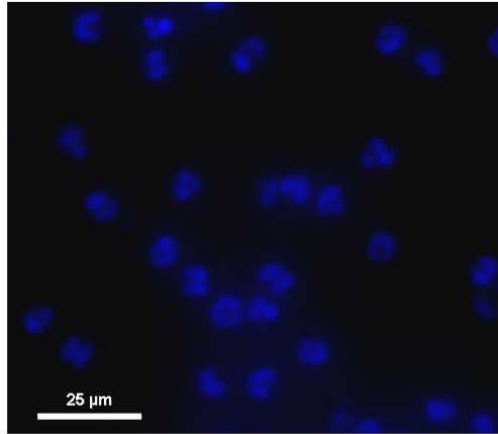


Figure 3: Giemsa stain of tear-film PMNs

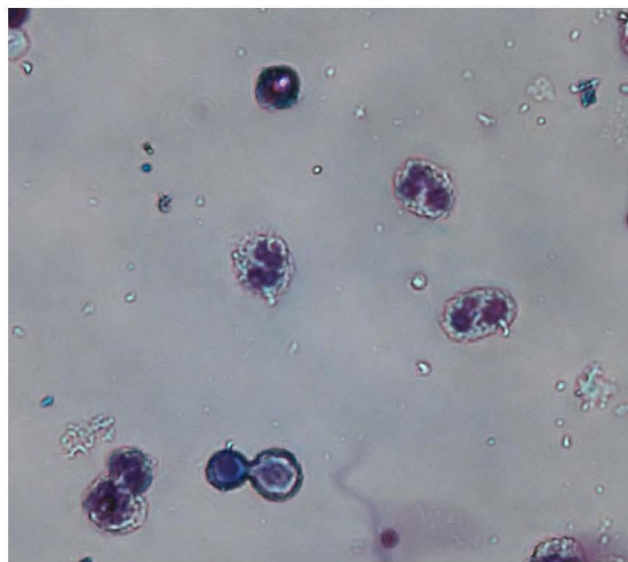


Figure 4: Flow cytometry analysis of caspase activity in tear-film PMNs (a) and BAK-treated tear-film PMNs (b). BAK was used as a positive control to induce apoptosis and necrosis. Minimal caspase activity (less than 2%) was observed in tear-film PMNs. A representative experiment (n=10) is depicted.

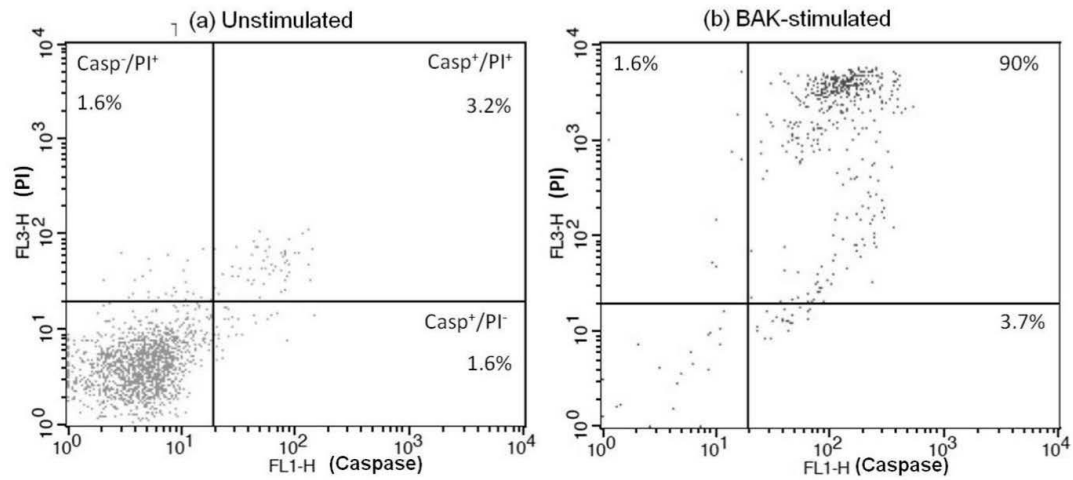


Figure 5: Example plots of flow cytometry analysis of tear-film neutrophils with side scatter to determine PMN cell size (a), Side scatter (SSC-H) versus CD45 fluorescence (FL3-H) dot plots to identify CD45 positive cells based on their granularity (b).

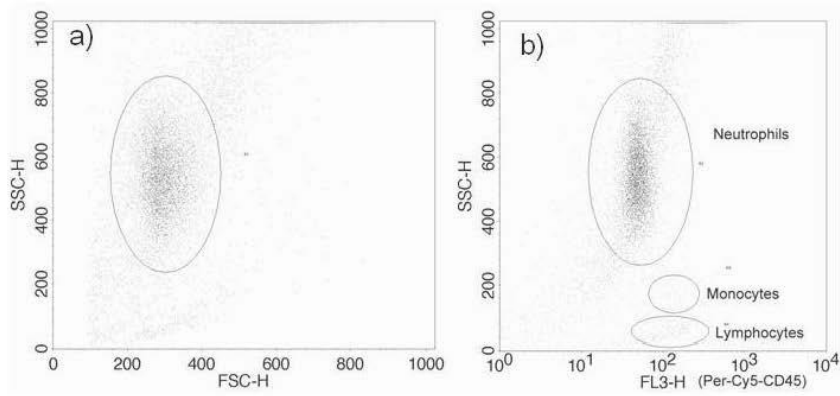


Figure 6: Fluorescent histogram for L-selectin expression on tear-film and blood isolated PMNs. Black-filled histogram: tear-film PMNs; grey-line histogram: blood-isolated PMNs. Shedding of L-selectin as shown by the significantly reduced expression of L-selectin is evident on tear-film PMNs.

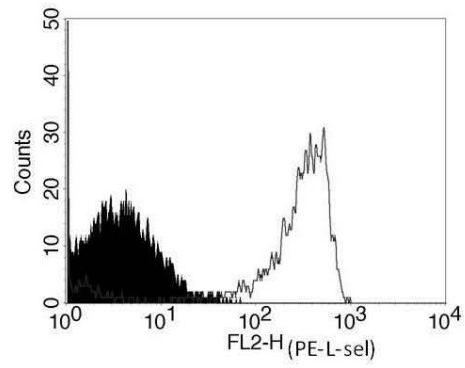


Figure 7: Receptor upregulation of tear-film neutrophils collected after sleep and blood isolated neutrophils following stimulation in DMEM/FBS. Fluorescence intensities were recorded by flow cytometry and are expressed as a ratio between unstimulated and LPS-stimulated samples. N=12, mean \pm SD. * significantly different from activation ratio of tear-film PMNs ($p < 0.001$)

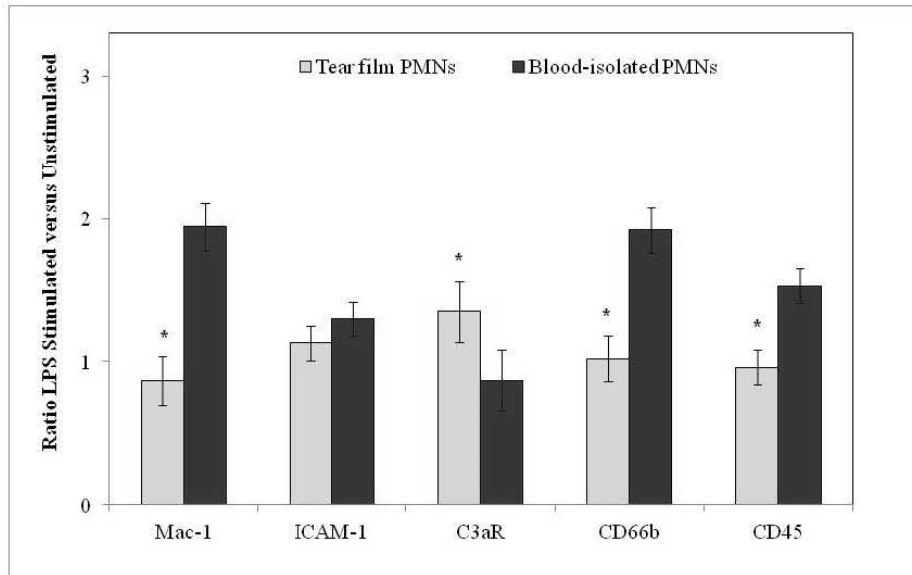


Table 1: Composition of Artificial Tear Solution⁵⁴

Salt Component (for 500mL)		Lipid Component	
Sodium Chloride	90.0 mM	Triglyceride	32.0 mg/mL
Potassium Chloride	16.0 mM	Sterol	3.6 mg/mL
Sodium Citrate Monobasic	1.5 mM	Fatty acid	3.6 mg/mL
Glucose	0.2 mM	Fatty ester	24.0 mg/mL
Urea	1.2 mM	Cholesteryl ester	48.0 mg/mL
Calcium Chloride	0.5 mM	Phospholipid	1.0 mg/mL
Sodium carbonate	12.0 mM	Protein Component	
Potassium hydrogen carbonate	3.0 mM	Bovine Albumin	0.20 mg/mL
Sodium phosphate dibasic	24.0 mM	Hen egg lysozyme	1.90 mg/mL
Hydrochloric acid (10M)	26.0 mM	Bovine submaxillary mucin	0.15 mg/mL
MilliQ Water		Bovine colostrums lactoferrin	1.80 mg/mL
		Bovine immunoglobulin G	0.02 mg/mL

Table 2: Antibodies and *stains* used in flow cytometry to characterize the phenotype of tear-film PMNs.

Antibody (CD) or fluorescent stain	Leukocyte (antigen) function assessed
CD11b	Adhesive cell interactions and marker of leukocyte activation, binds to fibrinogen, iC3b (also known as Mac-1)
CD45	PAN leukocyte marker
CD54	Intracellular adhesion molecule (ICAM-1), upregulated on activated cells
CD66b	Degranulation membrane marker, released upon activation
C3aR	Receptor for C3a
<i>PI</i>	Necrotic/dead cells
<i>Fluorochrome-VAD-FMK</i>	Apoptotic cells as identified by caspase activation

Table 3: **fMLP and PMA-induced tear-film and blood-isolated neutrophil activation.**

Fluorescence intensities were recorded and are expressed as a ratio between unstimulated and stimulated samples. N= 10 to12, mean \pm SD

		Tear-film PMNs	Blood-isolated PMNs
Mac-1	fMLP	0.87 \pm 0.21*	1.63 \pm 0.54
	PMA	0.93 \pm 0.15*	2.10 \pm 0.54
ICAM-1	fMLP	1.13 \pm 0.18	0.94 \pm 0.28
	PMA	1.12 \pm 0.31	1.81 \pm 0.81
C3aR	fMLP	1.35 \pm 0.29*	0.86 \pm 0.20
	PMA	1.26 \pm 0.33	1.52 \pm 0.99
CD66b	fMLP	1.02 \pm 0.20*	1.67 \pm 0.41
	PMA	1.33 \pm 0.50*	4.66 \pm 2.15
CD45	fMLP	0.96 \pm 0.15*	1.23 \pm 0.37
	PMA	0.82 \pm 0.14*	1.18 \pm 0.42
DCF	fMLP	0.99 \pm 0.13*	1.95 \pm 0.37
	PMA	2.45 \pm 1.95	2.37 \pm 2.54

* significantly different from level of activation observed with stimulated blood-isolated PMNs, $p < 0.02$

Table 4: Expression of membrane receptor on unstimulated tear-film and blood-isolated neutrophils (resting level). Raw fluorescence data are expressed as mean fluorescent intensities (MFI). N= 9 to 12, mean \pm SD

	Tear-film PMNs	Blood-isolated PMNs
	MFI	MFI
Mac-1	72 \pm 33	67 \pm 26
ICAM-1	50 \pm 29*	25 \pm 8
C3aR	28 \pm 12	30 \pm 10
CD66b	116 \pm 39*	45 \pm 16
CD45	65 \pm 31	46 \pm 17
DCF	1319 \pm 846*	649 \pm 461

* significantly different from blood-isolated PMNs, $p < 0.045$

Table 5: Response of tear-film and blood-isolated PMNs to LPS, fMLP and PMA

stimulation in artificial tear solution. Cells were resuspended in ATS containing several key tear-film proteins and stimulated with fMLP, LPS, and PMA. Activation was assessed by measuring level of fluorescent intensities and is expressed as a ratio between unstimulated (rest) and stimulated samples. No significant difference in receptor expression was observed between stimulated and unstimulated tear-film PMNs in ATS.

		Tear-film PMNs	Blood-isolated PMNs
Mac-1	LPS	1.01 ± 0.15	1.31 ± 0.36 [#]
	fMLP	1.02 ± 0.17*	1.95 ± 0.51
	PMA	1.04 ± 0.18*	1.86 ± 1.02
ICAM-1	LPS	1.06 ± 0.3	1.17 ± 0.44
	fMLP	1.38 ± 0.52	1.47 ± 0.66
	PMA	1.06 ± 0.11*	1.59 ± 1.00
CD66b	LPS	1.02 ± 0.10	1.48 ± 0.49 [#]
	fMLP	1.18 ± 0.19*	2.25 ± 0.78
	PMA	1.26 ± 0.35*	2.98 ± 1.45
CD45	LPS	1.05 ± 0.14	1.05 ± 0.27
	fMLP	0.99 ± 0.29*	1.42 ± 0.28
	PMA	0.85 ± 0.09	0.86 ± 0.16
DCF	LPS		1.03 ± 0.19
	fMLP		1.31 ± 0.49
	PMA		2.70 ± 1.99

* significantly different from ratio of activation observed with blood-isolated PMNs stimulated in ATS, $p < 0.04$

[#] significantly different from ratio of activation observed with blood-isolated PMN stimulated in DMEM/FBS $p < 0.04$

Table 6: Effect of stimulating blood-isolated PMNs in phosphate buffered saline (PBS) and PBS supplemented with lactoferrin.

		PBS (n=7)	PBS+LF (n=5)
Mac-1	LPS	2.06± 0.55 [#]	2.01 ± 0.51 [#]
	fMLP	1.96± 0.64	2.01 ± 0.58
ICAM-1	LPS	1.69 ±0.67	1.38 ± 0.24
	fMLP	1.53 ±0.95	1.40 ± 0.44
CD66b	LPS	2.45± 0.92 [#]	1.90 ± 0.39 ^{*#}
	fMLP	2.06 ±0.49 [#]	1.95 ± 0.59
C3aR	LPS	0.90 ±0.29	1.19 ± 0.20
	fMLP	0.99 ±0.28	1.26 ± 0.5
CD45	LPS	1.50± 0.35	1.30 ± 0.36
	fMLP	1.40± 0.15	1.40 ± 0.42

LF: lactoferrin

* significantly different from activation ratio observed with blood-isolated PMNs stimulated in PBS alone, $p < 0.05$

[#] significantly different from activation ratio observed with blood-isolated PMNs stimulated in ATS, $p < 0.045$

Appendix C

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Cameron Postnikoff <cpostnik@gmail.com>

Eye drawing

4 messages

Cameron Postnikoff <cpostnik@gmail.com>
To: ArtworkbyGabrielle@gmail.com

Thu, Aug 14, 2014 at 12:57 PM

Hi,

My name is Cameron Postnikoff and I am a graduate student at the University of Waterloo. I am wondering if I can use your image in my academic thesis? To do so, I require your copyright info.

I hope that I may obtain your permission. Please let me know as soon as possible.

Thanks!

Cameron Postnikoff

Gabrielle D <artworkbygabrielle@gmail.com>
To: Cameron Postnikoff <cpostnik@gmail.com>

Mon, Aug 18, 2014 at 5:22 PM

Hi Cameron,

This is just for your thesis correct, so just for school work non commercially?

If so, send me the photo you are referring to and I will give you the information on it.

Thanks for contacting!

Gabrielle

[Quoted text hidden]

Cameron Postnikoff <cpostnik@gmail.com>
To: Gabrielle D <artworkbygabrielle@gmail.com>

Mon, Aug 18, 2014 at 6:48 PM

Hi Gabrielle!

Yes, this is just for my thesis, it is not commercial.

I have edited the photo and added labeling to it, but the original drawing is yours, I promise. :)

Thanks so much!

Cameron

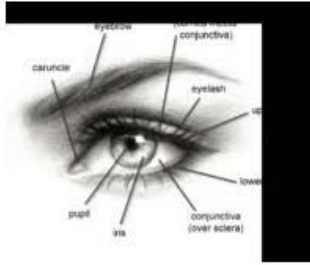
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eye gabriella.tif
260K

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9/18/2014

Gmail - Eye drawing



Gabrielle D <artworkbygabrielle@gmail.com>
To: Cameron Postnikoff <cpostnik@gmail.com>

Tue, Aug 19, 2014 at 11:30 PM

As long as it will not be published that is fine. Info is Gabrielle DeCesaris, Eye Drawing, 2012
[Quoted text hidden]

<https://mail.google.com/mail/u/0/?ui=2&ik=02d8f54c99&vi=pt&q=gabrielle&qs=true&e=arch=query&th=147d5742ba1ce48a&siml=147d5742ba1ce4...> 2/2

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