Interaction of Tear Inflammatory Markers with Contact Lens Materials

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

Purpose: Biomaterials are natural or synthetic materials that come into contact with biological tissue. Contact lenses are the most commonly used biomaterials, being worn by an estimated 140 million people worldwide. While contact lens wear could be considered successful, up to 50% of patients discontinue contact lens wear, primarily due to the development of contact lens discomfort. Due to the interaction of contact lenses with the ocular surface, the ocular environment is of great interest when considering factors contributing to contact lens discomfort. One of these factors may be cytokines released by human corneal epithelial cells, which have the potential to initiate ocular inflammation. The purpose of the investigations presented in this thesis were to assess cytokine adhesion to various contact lens materials, as an excessive binding of cytokines to contact lenses may contribute to the pathology of contact lens discomfort.

Methods and Materials: Conventional hydrogels and silicone hydrogel contact lens materials were soaked in solutions containing proinflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α. These cytokines were either recombinant proteins derived from a standard, lyophilized blend, or obtained directly from human corneal epithelial cells. Over various time points, samples of the soaking solutions were collected and later analyzed using the Meso Scale Discovery platform. This platform evaluates biomarker concentrations via electrochemiluminescence and was used in these investigations to detect and measure the concentration of the cytokines of interest in the soaking solution. This served as an indirect measurement of the amounts of cytokines that had adhered to the contact lens materials.

Results: The presence of the cytokines of interest in the soaking solutions were detected using the Meso Scale Discovery platform and the amount of uptake onto the contact lens materials were quantified. Additional surface modifications to the contact lens materials mimicking the ocular surface of a clinically-worn contact lens were explored. Balafilcon A, comfilcon A, etafilcon A and omafilcon A were the only contact lens materials that appeared to exhibit some uptake of the cytokines of interest, although this amount varied between experiments and between the cytokines of interest. There was no uptake of IL-6 quantified on any of the contact lens materials investigated.

Discussion and Conclusion: To our knowledge, data does not exist in the literature for the quantification of IL-1β, IL-6, IL-8 and TNF-α uptake onto contact lens materials through the use of electrochemiluminescence. This work, in part, helped to address issues surrounding ocular inflammation and contributed to providing a better understanding of the role of inflammatory markers in contact lens discomfort.

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Dedication

For my loving family.

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List of Abbreviations

9-cisRA	9-*cis*-retinoic acid
AKC	Atopic keratoconjunctivitis
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
AP	Activating protein
ATRA	All-*trans*-retinoic acid
ATS	Artificial tear solution
B cell	B lymphocyte
BALT	Bronchus-associated lymphoid tissue
BCR	B-cell receptor
BK	Bacterial keratitis
bpy	2,2'-bipyridine
C/EBPβ	CCAAT/enhancer-binding protein beta
CALT	Conjunctiva-associated lymphoid tissue
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
cDC	Classical dendritic cell
CIE	Corneal infiltrative event
CL	Contact lens
CLD	Contact lens discomfort
CLDE	Contact lens-related dry eye
CRT	Corneal refractive therapy
CU	Corneal ulcers
CXCL	Chemokine (C-X-C motif) ligand
DES	Dry-eye syndrome
DME	Diabetic macular edema
E.g.	Exempli gratia
EALT	Eye-associated lymphoid tissue
ECL	Electrochemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FimH	Type 1 fimbrin D-mannose specific adhesion protein
FoxP3+	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GP	Glycoprotein

HCEC	Human corneal epithelial cell
HDPE	High-density polyethylene
HEV	High endothelial venule
I.e.	Id est; "in other words"
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
K_d	Dissociation constant
kDa	Kilodalton
LDALT	Lacrimal drainage-associated lymphoid tissue
LLOQ	Lower limit of quantification
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
M cell	Microfold cell
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
mg	Milligram
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mL	Milliliter
mM	Millimolar
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSD	Meso Scale Discovery (Meso Scale Diagnostics, LLC assay platform)
n	Sample size
NALT	Nasal-associated lymphoid tissue
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NK	Natural killer cell
NLR	Nucleotide-binding oligomerization (NOD)-like receptor
nM	Nanomolar
Ortho-K	Orthokeratology
p	Probability value
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PDR	Proliferative diabetic retinopathy
pg	Picogram
pH	Potential of hydrogen
pHEMA	Poly-2-hydroxyethyl methacrylate
pI	Isoelectric point

PMMA	Polymethyl methacrylate
Prox1	Prospero-related homeobox-1
PRR	Pattern recognition receptor
RAR	Retinoic acid receptor
RORγt	Retinoic acid receptor-related orphan receptor γt
RPM	Rotations per minute
RT-PCR	Real-time polymerase chain reaction
Ru	Ruthenium
RXR	Retinoid X receptor
SiHy	Silicone hydrogel
STAT3	Signal transducer and activator of transcription-3
T cell	T lymphocyte
TCR	T-cell receptor
TGF	Transforming growth factor
Th cell	T Helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TPA	Tripropylamine
T_{reg}	Regulatory T cell
TREM	Triggering receptor expressed on myeloid cells
μL	Microliter
ULOQ	Upper limit of quantification
μM	Micromolar
USAN	United States Adopted Name
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VKC	Vernal keratoconjunctivitis

Chapter 1: Introduction - Ocular Immunology

1.1 The Cornea

As the avascular outer tunic of the eye, the human cornea features many unique characteristics that allow it to serve as an important refractive structure.[1] The small diameter of the collagen fibrils of which the cornea is comprised, in addition to the regular arrangement of these fibers, allows the cornea to exist as a highly organized tissue.[1] This organization, in conjunction with the meticulous spacing of the collagen fibrils permitting destructive interference of adjacent light rays, prevent light scatter and contribute to the overall transparency of the cornea, a key requirement for the cornea's refractive ability.[1]

Histologically, when viewed anteriorly to posteriorly, the cornea is comprised of the following five layers: the corneal epithelium, Bowman's membrane, the corneal stroma, Descemet's membrane and the corneal endothelium (Figure 1-1).[1] Each layer contributes a respective role in the protection of the cornea and the maintenance of its organization and regularity.[1] This is extremely important as the integrity of the cornea can be otherwise compromised, leading to the deterioration of vision and an unhealthy ocular state.[1] As a result, there are numerous physical, mechanical and immunological defenses in place to aid in the maintenance and protection of this integrity, in addition to providing overall protection to all ocular structures and to the ocular surface.

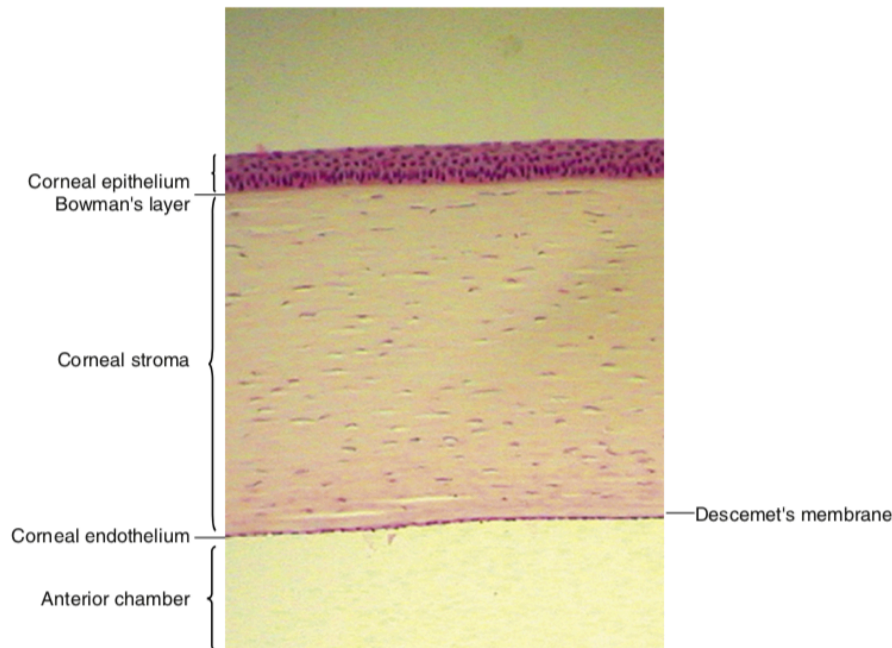

Figure 1-1: Histological Cross-Section of the Human Cornea Outlining its Five Distinctive Layers.[1] Figure Reprinted from Clinical Anatomy and Physiology of the Visual System, 3rd Edition (Remington, 2012), with Permission from Elsevier.

1.2 Ocular Defense Mechanisms

Uniquely, the eyes remain passively exposed to microorganisms and debris from the environment, yet in most cases (unless an infection results or there is damage to the ocular structures), the present defense mechanisms are sufficient to allow for quick recognition of foreign entities and subsequent elimination of any pathogens.[2,3] This protection to the eye also specifically helps to maintain the transparency of the cornea and supports vision preservation.[3] The eyes' defense mechanisms are a combination of physical, mechanical and immunological barriers.[3]

1.2.1 Physical and Mechanical Defenses

The simplest physical defenses are the bony orbit and eyelids, which act as physical barriers to protect the eyes from trauma that could weaken the integrity of the cornea.[3] Further, eyelashes are an example of a mechanical defense that protects the eyes from any debris and foreign particles.[4] Additionally, such debris and foreign particles, upon contact with the ocular surface, will be washed off by the aid of a blink reflex, another mechanical defense that continuously

spreads tears over the ocular surface.[2,3] This action helps to remove microbes from the ocular surface by washing them towards the ocular surface drainage system and prevent their settling onto the eye.[4]

1.2.2 Immunological Defense: Tears

The human tear film, playing a crucial role in the nourishment, protection and hydration of the ocular surface, including the cornea, is a bi-phasic structure consisting of a superficial lipid phase and an underlying aqueous-mucin phase.[5] The lipid phase, as the name implies, consists of lipids largely secreted by the meibomian glands within the tarsal plate of the eyelid, through their orifices just at the eyelid margin, and to a lesser extent, lipids contributed by the glands of Moll and Zeiss within the subcutaneous areolar tissue of the eyelid.[1,5] The lipid phase both stabilizes the tear film and prevents tear evaporation.[5,6] The aqueous-mucin phase, on the other hand, constitutes the bulk of the tear film and plays several important roles in the overall function of the tear film, including, but not limited to: protection of the corneal epithelium, facilitating the movement of oxygen and nutrients for nourishment of the avascular cornea, regulation of pH, elimination of toxins and debris, and providing an optically smooth surface for light refraction.[5,7] Within this phase, there are as many as 1400 unique proteins, in addition to the presence of peptides, electrolytes, small metabolites and mucins (glycosylated proteins), of either cleaved, membrane-spanning or gel-forming types.[5-7] These secretions are produced by two accessory glands (glands of Krause which exist in higher concentration near the fornix and glands of Wolfring within the tarsal plate of the eyelid), although recently, it has also been suggested that the secretions of the main lacrimal gland (which are similar to that of the accessory lacrimal glands), can also contribute to the aqueous-mucin phase of the tear film.[1,5] Epithelial cells of the cornea and conjunctiva may contribute small amounts of electrolytes, fluids and mucins to the tear film as well.[5,6] Mucins

specifically help to maintain ocular hydration by providing a wettable surface and contributing to the epithelial barrier to prevent pathogen binding.[6,8]

In addition to preventing corneal dryness and flushing away foreign particles and debris, certain constituents of the tear film also help to prevent the onset of infections.[3] This includes lysozymes, immunoglobulins (antibodies), and cytokines, all of which play an integral role in maintaining an immunological barrier for the ocular surface.[3] Lysozymes are enzymes capable of breaking down microbial cell walls.[4] Immunoglobulins (Ig) are plasma proteins produced during an immune response due to the presence of an antigen.[9] The antigen can be any foreign substance, such as a bacterium or a virus, to which an antibody will bind and destroy.[4] One important immunoglobulin found in the tear film is IgA, which will bind pathogens to prevent their adhesion to the corneal epithelium.[3] Further, IgA-secreting plasma cells also exist within the conjunctiva, lacrimal gland and lacrimal drainage tissue of the eyes, all three of which are involved in a key immunological defense of the eye known as eye-associated lymphoid tissue (EALT).[10-12]

1.2.3 Immunological Defense: Inflammatory Cells and Markers

Immunity is provided to the body in either a non-specific manner (innate immunity), or as a specific response (adaptive immunity).[9] Innate immunity is conserved through evolution and is present at birth, while adaptive immunity is acquired throughout an individual's lifetime as a result of the systemic immune system producing antibodies against new, "non-self" antigens (foreign entities), as they are encountered.[9] Antibodies are a class of proteins known as immunoglobulins, which initiate immune responses either by binding the pathogen or by recruiting other immune cells to destroy it instead.[9] The structure of an antibody consists of two identical heavy and light chains, each with a constant region determining its effector function, as well as a variable region

possessing an antigen-binding site.[9] The antigen, on the other hand, has an epitope region that is recognized by the antigen receptor of the antibody.[9]

At the forefront of both the innate and adaptive immune systems are leukocytes, or white blood cells.[9] Leukocytes develop from the bone marrow where most will also mature.[9] Upon maturation, these cells can circulate in the bloodstream, within the lymphatic system, or they can be transported to tissues where they will reside.[9] The lymphatic system is a specialized system of vessels that drain immune cells and extracellular fluids from tissues as "lymph", which eventually empties back into the bloodstream via the thoracic duct.[9] Prior to maturation, immune cells are naïve and inactivated, that is until they encounter an antigen and subsequent development into effector cells allows them to carry out a variety of effector mechanisms with molecular and cellular functions.[9] Within the bone marrow, pluripotent hematopoietic stem cells can give rise to common lymphoid progenitor cells or common myeloid progenitor cells.[9] The former are precursors to antigen-specific lymphocytes and innate cell lineages without antigen-specific receptors, while the latter are precursors to granulocyte/macrophage progenitor cells or megakaryocyte/erythrocyte progenitor cells.[9]

Arising from the common lymphoid progenitor cell are B lymphocytes (B cells), T lymphocytes (T cells), natural killer (NK) cells and innate lymphoid cells (ILCs).[9] Immature dendritic cells may also arise from a common lymphoid progenitor cell; however, the majority arise from a common myeloid progenitor cell instead.[9] B lymphocytes mature in the bone marrow where they originate, whereas T lymphocytes mature in the thymus, although they too originate in the bone marrow.[9] Both of these cells remain as naïve lymphocytes with little functionality until they encounter an antigen and differentiate into functional, effector lymphocytes.[9] This encounter depends on the interaction of the antigen with either B-cell receptors (BCRs), or T-cell receptors

(TCRs), on the surfaces of these cells.[9] Genetically, BCRs consist of the same genes that encode antibodies.[9] In fact, the membrane-bound form of an antibody, which is specific to each antigen, is the BCR on the surface of the B cell for that antigen.[9] Shortly after the antigen binds to its BCR, proliferation and differentiation of the B cell into its effector form ensues, producing plasma cells which secrete antibodies in order to target and destroy the antigen.[9] While TCRs are related to immunoglobulins, they are also distinct from BCRs due to their inability to independently recognize and bind antigens, but rather their dependence on major histocompatibility (MHC) molecules to present antigens to them.[9] MHC molecules are cell-surface glycoproteins and exist in two classes: MHC class I and MHC class II.[9] Similar to B cells, T cells will proliferate and differentiate into one of three classes of functional, effector T cells, upon binding of an antigen to the appropriate TCR on their cell surface.[9] This includes the cytotoxic T cells which kill pathogens or infected cells, T Helper (Th) cells which secrete cytokines to signal and activate other cells (such as B cells), to destroy or engulf pathogens, in addition to regulatory T cells (T_{reg}), which serve a protective function by limiting tissue damage through suppression of lymphocyte activity as required.[9] Cytotoxic T cells express CD8 protein which selectively recognizes MHC class I-bound peptides, while T Helper cells express CD4 protein which selectively recognizes MHC class II-bound peptides.[9] The majority of the cells within the body express MHC class I molecules, since these molecules commonly present peptides from viruses to CD8 T cells and viruses can infect any cell with a nucleus.[9] Conversely, only antigen-presenting cells such as dendritic cells, macrophages and B cells will express MHC class II molecules.[9]

Arising from the common myeloid progenitor are immature dendritic cells, granulocytes (neutrophils, eosinophils and basophils), mast cells and monocytes.[9] Immature dendritic cells will mature upon encountering a pathogen, while monocytes and mast cells complete their maturation

in tissues.[9] Upon maturation, monocytes will differentiate into either dendritic cells or macrophages.[9] The common myeloid progenitor also gives rise to megakaryocytes which produce platelets for blood clotting, as well as erythroblasts which mature into erythrocytes (red blood cells).[9]

Macrophages, granulocytes and dendritic cells are three types of phagocytic cells within the immune system.[9] Macrophages, the mature form of monocytes, exist in virtually all tissue types and are long-lived, engulfing and killing pathogens and producing other inflammatory mediators to in turn recruit additional immune cells.[9] In comparison, granulocytes are short-lived and possess granules within their cytoplasms, which store various degradative enzymes and toxic proteins.[9] Once these cells engulf a pathogen, they become activated and release the appropriate enzymes or proteins from their granules in order to effectively destroy it.[9] Of the granulocytes, neutrophils are the most abundant in comparison to eosinophils and basophils.[9] Eosinophils and basophils are also important for initiating allergic responses, in addition to mast cells, which also have granules consisting of histamine and various proteases.[9] Moreover, through a process called micropinocytosis, dendritic cells ingest or “drink” large amounts of particulate matter and extracellular fluid, and will degrade any pathogens which they take up in this way.[9] More importantly, however, dendritic cells, along with macrophages and neutrophils, are sensor cells and they play an important role in detecting an infection and initiating an immune response.[9]

Macrophages, neutrophils and dendritic cells express pattern recognition receptors (PRRs), that allow them to recognize simple molecules as well as pathogen-associated molecular patterns (PAMPs).[9] PAMPs are present in microorganisms and are distinct from the molecular patterns of the host’s cells.[9] PRRs include toll-like receptor (TLR) proteins and NOD-like receptor (NLR)

proteins, both of which will detect PAMPs from extracellular bacteria.[9] Activation of PRRs leads to the production of cytokines and chemokines which help to amplify the immune response.[9]

Cytokines are small, regulatory proteins that are secreted by cells during inflammation and can either stimulate or inhibit normal cell functions.[4,10,13] This includes activation or deactivation of cells involved in either the innate or adaptive immune system, in order to amplify or suppress an immune response.[4,10] Cytokines are "hormone-like", as they are involved in either autocrine or paracrine signaling, if they act on the cell that secreted them, or act on an adjacent or nearby cell, respectively.[4] For example, helper T cells can release interleukin-2 (IL-2), a cytokine that can both activate nearby immune cells or stimulate the same helper T cell that secreted it to proliferate, in turn secreting more IL-2 to strengthen the immune response.[4]

Interleukins (ILs), interferons (IFNs), chemokines and tumor necrosis factors (TNFs) are all various groups of cytokines.[9] Interleukins are cytokines that were initially thought to be secreted from one leukocyte to act on another, and though they are mainly secreted by subtypes of leukocytes such as macrophages and helper T cells, it is now known that other tissues in the body can also secrete and respond to interleukins.[3,14] Interferons are cytokines that are secreted by NK cells and lymphocytes.[4,10,14] Some IFNs, such as IFN-γ, activate macrophages, while other IFNs, such as IFN-α and IFN-β, have antiviral effects.[4,10,14] These cytokines will target virus-infected host cells and promote the production of antiviral proteins to prevent viral replication, in addition to inducing an antiviral state in neighbouring cells.[3,14] Chemokines are small cytokines of molecular weight 6-12 kDa that act as chemoattractants.[10,15] An example of a chemokine is IL-8 which is 6-8 kDa in size.[15] Chemokines induce the directional migration of leukocytes such as neutrophils to areas of inflammation via chemotaxis.[4,10] Tumor necrosis factors, mainly produced by macrophages, can also act on macrophages in addition to neutrophils, by promoting the

accumulation of these cells at the site of inflammation and stimulating them to kill the present microbes.[10,14] Further, TNFs also induce apoptosis within cells.[14]

In the human eye, cytokine-releasing cells exist within the conjunctiva, and resident macrophages exist in the corneal stroma; thus, a variety of interleukins are also found in the eye.[3] As mentioned, macrophages produce interleukins, some of which include IL-1α, IL-10, IL-12 and IL-18 in the eye.[10] IL-1α has also been found in corneal epithelial cells where it is stored and can be released if the cell membrane is damaged either due to infection or trauma.[3] Corneal keratocytes, specialized fibroblast cells of the cornea, synthesize IL-6 under the influence of IL-1α and TNF-α.[3] Further, TNF-α, which is present in the conjunctival epithelium, has shown to be an inducer of IL-1β, an interleukin which can upregulate IL-6 and IL-8 in corneal epithelial cells.[16,17]

The epithelial cells of both the cornea and the conjunctiva are known to secrete proteins and cytokines that are microbicidal and immunomodulatory.[18] Moreover, these cells also use toll-like receptor signaling systems to respond to pathogens and unusual molecular patterns that are associated with danger.[18] Detecting and distinguishing between beneficial and pathogenic microorganisms is a key factor in immunity.[19] Typically, microorganisms possess PAMPs and TLRs will bind conserved motifs on the PAMPs, to allow for the detection of pathogens and the subsequent activation of the cell to secrete inflammatory cytokines, along with other co-stimulatory molecules.[19,20] In addition to being expressed by corneal epithelial cells and conjunctival epithelial cells, keratocytes (fibroblasts), also present in the eye, express TLRs.[14,20]

During ocular allergy, typically upon initial exposure to an antigen, IgM is the first immunoglobulin to be produced from B cells and serve as antigen receptors.[4,10] If the antigen is encountered again, B cells will switch their production from IgM to IgG, a process that requires

gene rearrangement and is said to be controlled in part by lymphokines.[10] IL-4, in particular, is an example of a lymphokine that stimulates B cells to switch their production to either IgG or IgE.[10] IgG is the most abundant immunoglobulin and is also found in abundance within the eye.[4,10] IgG neutralizes toxins and enhances phagocytosis to protect against bacteria and viruses.[4] IL-4 regulates B cell development, and IL-5 is also said to play a role in inducing B cells to differentiate into IgM and IgG-secreting plasma cells.[10] Furthermore, IL-4 activates mast cells, while IL-5 is a regulator of eosinophils.[10] After the production of IgE, this immunoglobulin will immediately become bound to the surface of mast cells and subsequent binding of the antigen to IgE on the mast cell will stimulate degranulation and the release of histamines and cytokines.[10] Mast cells, in addition to releasing IL-4, will also release IL-6, IL-8, and TNF-α.[10] Eosinophils participate in allergic responses by releasing toxic enzymes contained within their granules when activated, causing inflammation.[14]

When epithelial cells are exposed to conditions that are desiccating, such as in dry eye disease, the secretion of chemokines CCL20, CXCL9, CXCL10 and CXCL11 from the epithelial cells will in turn help recruit Th1 and Th17 cells from circulation.[18] NK cells will also be activated and will release IFN-γ.[18] Th1 cells secrete IL-2 and IFN-γ.[3,10] Th17 cells produce IL-17, IL-21, and IL-22.[10]

Allergy cytokines include IL-4, IL-5, IL-10, IL-13 and IFN-γ.[10] IL-4 can stimulate naïve T cells to differentiate into Th2 cells, which are associated with disease downregulation or allergy initiation.[10,21] Th2 cells can secrete their own IL-4, and during allergic conjunctivitis, a Th2 response causes over-expression of this cytokine.[13,21] Th2 cells can also secrete IL-5, IL-10 and IL-13.[10] As previously noted, IL-4 can stimulate the differentiation of B cells into IgG or IgE-secreting plasma cells and also activate mast cells, while IL-5 can induce differentiation of B cells

into IgM or IgG-secreting plasma cells, and regulate eosinophils.[10,21] During chronic and severe ocular allergies such as vernal keratoconjunctivitis (VKC), an upregulation of IL-5 is observed, while an upregulation of IFN-γ, IL-10 and IL-13 is associated with atopic keratoconjunctivitis (AKC).[22] Recall, IFN-γ is produced by Th1 cells, which are associated with the initiation of disease, as well as NK cells.[3,10,18] IL-17, produced by Th17 cells, is another notable cytokine that has been identified in severe ocular allergies, in addition to other inflammatory diseases of the ocular surface including dry eye syndrome and uveitis.[22,23] Although traditionally it was believed that allergies were mediated by a Th2 response only, it is now also believed that Th1-mediated inflammatory responses are involved in severe ocular allergies and diseases.[22]

Anti-inflammatory cytokines include IL-4, IL-10, IL-13 and TGF-β.[24,25] All four of these cytokines can be produced by Th2 cells and although IL-4, IL-10 and IL-13 are also involved in allergies, as noted earlier, Th2 cells are not only associated with allergy initiation, but they are also associated with disease downregulation (or anti-inflammatory effects).[10] IL-4 can block the expression of proinflammatory cytokines IL-1, TNF-α, IL-6, IL-8, in addition to CCL3 (formerly MIP 1-α).[21,26] Moreover, it can also prevent macrophage-derived nitric oxide production, suppress parasite killing and block the cytotoxic activity of macrophages.[21] IL-10 is involved in the down-regulation of proinflammatory cytokine receptors and can block proinflammatory cytokine synthesis by monocytes or macrophages, thereby suppressing the expression of TNF-α, IL-6 and IL-1β.[21,24] Furthermore, IL-10 can inhibit Th1 cytokines such as IL-2 and IFN-γ.[21] Similarly to IL-4, IL-13 can also downregulate IL-1, IL-8, TNF-α and CCL3 synthesis.[21] Lastly, TGF-β can inhibit both Th1 cells and macrophages to block cytokine production, thereby suppressing IL-1, IL-2, IL-6 and TNF-α expression.[24]

Evidently, numerous immune cells are present within the eye. Apart from the existing immunoglobulins and macrophages which secrete interleukins and tumor necrosis factors, other subtypes of leukocytes, such as T cells and NK cells also exist within the eye, along with fibroblasts (keratocytes), epithelial cells, neutrophils and dendritic cells.[3,10,14,18] In fact, within the conjunctiva there is a substantial population of T cells, NK cells, and dendritic cells.[18] Neutrophils are also present and they play a protective role in preventing microorganisms from invading the corneal epithelium.[3] Further, they can move through the endothelial cells of the limbal vasculature via a process known as diapedesis, where they will adhere to vascular endothelial cell receptors.[3]

1.2.4 Immunological Defense: MALT, Introduction to EALT

Any internal surface of the body that is lined by a mucus-secreting epithelium is referred to as "mucosal tissue".[9] This epithelium will always act as the first line of defense against pathogens.[9] Within the body, mucosal surfaces include the respiratory tract, gastrointestinal tract, urogenital tract, the middle ear and exocrine glands such as the salivary and mammary glands.[9] When specifically considering ocular tissues, the conjunctiva and lacrimal gland are also classified as mucosal tissues.[9] Mucosal surfaces are unique in the sense that they are often in direct contact with the external world and therefore encounter a substantially greater diversity of antigens (and pathogens) more frequently, in comparison to other tissues within the body.[9] Thus, these surfaces present as a convenient route for antigens to enter the body.[9] To cope with this, all mucosal tissues are protected by a large and distinctive immune system known as the "mucosal immune system", which differs from the systemic immune system in many ways, as it must be specialized in order to combat this wide variety of antigens that may be encountered.[9]

Mucosa-associated lymphoid tissue (MALT), is a generic term that refers to the extensive lymphoid tissue system composed of lymphocytes, found at mucosal surfaces, which can exist

either in an organized manner or scattered within.[9] Under this term falls tissue-specific MALT including gut-associated lymphoid tissue (GALT) and recently identified eye-associated lymphoid tissue (EALT).[9,11] Nasal-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) exist habitually in some species such as mice, but are only present in adult humans during an infection.[9] It has been hypothesized that the mucosal immune system may have in fact been the first part of the vertebrate immune system to have evolved, due to the coevolution of vertebrates and commensal bacteria.[9] In fact, differences in GALT and lymphoid tissue of the systemic immune system appear to be determined very early on in life.[9] All mucosa-associated lymphoid organs are categorized as peripheral (secondary) lymphoid organs, along with the lymph nodes and the spleen.[9] Secondary lymphoid organs maintain mature, naïve lymphocytes and initiate adaptive immune responses.[9] On the contrary, primary lymphoid organs are where lymphocytes originate and these include the bone marrow and thymus.[9]

The mucosal immune system and the systemic immune system differ in many ways.[9] For example, while it is completely natural to find numerous effector cells (e.g. effector lymphocytes), scattered at mucosal surfaces, within healthy non-lymphoid tissue, this would indicate the presence of an infection.[9] Within the mucosal immune system, however, these effector cells may exist as a result of a local response to the harmless antigens that are present, such as gut commensal microorganisms.[9] Other interesting distinctions between the mucosal immune system and the systemic immune system is that the former includes the presence of microfold cells (M cells), retinoic acid-producing dendritic cells and large populations of macrophages with unique characteristics.[9]

Unique to the mucosal immune system, M cells are specialized epithelial cells involved in the uptake of antigens and the facilitation of antigen presentation by macrophages and dendritic

cells.[9] Within GALT, M cells are found in Peyer's patches of the small intestine, scattered among the traditional, non-specialized epithelial cells.[9] Where these epithelial cells are covered by a thick layer of mucus, M cells lack this glycocalyx and hence present as an easily exposed and convenient route for antigens to enter the gut lumen.[9] Some antigens, such as several types of bacteria, are readily recognized by a glycoprotein (GP2) on the surface of M cells, which binds bacterial FimH (adhesion) protein found within bacterial type 1 pilli.[9] This initiates transcytosis, a process whereby the antigen is transported from the cell interior to the basal cell membrane as a membrane-bound vesicle that is then released into the extracellular space.[9] Conveniently so, the basal cell membrane of the M cell is convoluted such that a pocket is formed which encircles T lymphocytes and B lymphocytes, in addition to creating a close vicinity where local contact between lymphocytes and myeloid cells (e.g. dendritic cells), is encouraged.[9] Once released into the extracellular space, local dendritic cells, as well as those recruited via chemotaxis, will bind the antigen and the antigen-dendritic cell complex will migrate to areas containing naïve, antigen-specific T lymphocytes.[9] The chemotaxis of the recruited dendritic cells occurs in response to the binding of CCL9 and CCL20 chemokines released from epithelial cells to their respective receptors (CCR1 and CCR6) on dendritic cells.[9] Collectively, the dendritic cells and the now primed T lymphocytes will activate B lymphocytes and initiate the release of IgA antibodies.[9] IgA is the principal antibody of the immune response for mucosal surfaces.[9]

While the presence of M cells within EALT remains controversial, with some research groups claiming successful identification of these cells within cadaveric human lacrimal sacs and within the conjunctiva of rabbits, felines, canines, and mice, other groups have reported no evidence of M cells within the conjunctiva of such animal models.[27] If present within the human conjunctiva, however, and within the lacrimal sac, initiation of an immune response within EALT,

or even within MALT of other mucosal surfaces other than the gut, will likely involve a similar process as outlined above.[9]

In 2001, Knop and Knop claimed to have found evidence for M cells within the lacrimal sac of human cadaveric tissue.[28] Using immunohistochemistry and electron microscopy, they observed flat, covering epithelial cells within follicle-associated epithelia, displaying a thin cytoplasm containing many small vesicles, characteristic of M cells within Peyer's patches.[28] In 2005, using complete conjunctival sacs from normal, young adult rabbits, Knop and Knop identified specialized epithelial cells with different cytoplasms than ordinary epithelial cells, using both transmission electron microscopy and scanning electron microscopy.[29] These M cells existed in follicle-associated epithelia, displayed lighter cytoplasms and active mRNA transcription as suggested by bright euchromatic nuclei and a prominent nucleolus, in addition to the presence of many vesicles for antigen transport, among mitochondria, ribosomes and few rough endoplasmic reticula.[29] In 2002 and later in 2011, with the use of similar methods, Giuliano and colleagues provided morphological evidence of M cells within the nictitating membranes of canines and felines, respectively.[30,31] In both animal models, the M cells did not display the typical dense brush border observed with ordinary epithelial cells and instead displayed pocket formation that enclosed T lymphocytes, B lymphocytes and macrophages.[30,31] Furthermore, in the investigation involving the canine model, the use of heat-killed *Staphylococcus aureus* bacteria showed selective attachment to M cell apical membranes and further provided evidence of transcytosis across the surfaces of these cells into the pockets where the bacteria was engulfed by the residing macrophages.[30] Finally, in 2010, Seo and colleagues found evidence for M cells using a murine model when investigating eye mucosa as a vaccine delivery route.[32] They found M cells with long and irregular microvilli similar to the rabbit model rather than the canine model, displaying the

ability to sample and internalize *Salmonella typhimurium* and *Yersinia pseudotuberculosis* bacteria.[32]

Another unique feature of mucosal immunity is the presence of retinoic acid-producing dendritic cells that induce tolerance within mucosal tissue.[9] Outside of mucosal tissue, dendritic cells do not produce retinoic acid, however, they do so within the mesenteric lymph nodes and the lamina propria and Peyer's patches of the small intestine.[9] Recently, retinoic acid-producing dendritic cells have also been identified within ocular tissue and are believed to play a role in ocular immune privilege.[33] Recalling the antigen-dendritic cell complex that forms as a result of antigen presentation to dendritic cells once transported across the cell by M cells, there is migration of this complex to areas containing naïve T lymphocytes, depending on where the antigen was first encountered.[9] This migration may be to T-cell dependent areas of Peyer's patches or to the mesenteric lymph nodes.[9] Typically, 5-10% of dendritic cells will migrate to the former on a daily basis.[9] Migration of dendritic cells occurs as a result of "licensing" – a process whereby chemokine receptor CCR7 is induced on the surface of immature dendritic cells, thereby activating them.[9] Under inflammatory conditions or during an infection, if naïve T lymphocytes are able to recognize an antigen presented by the dendritic cells, they will become "primed", in a process which involves replacement of CCR7 (which they also express), and L-selection adhesion molecule, with gut-homing molecules CCR9 and α_4:β_7.[9] In contrast, unsuccessful recognition of an antigen will result in the exit of T lymphocytes via efferent lymphatics into the bloodstream.[9] By expressing these gut-homing molecules, the regulatory T lymphocytes are able to exit mesenteric lymph nodes and return back to the gut, where they can help to fight invading organisms.[9] Additionally, under normal, non-inflammatory conditions, interaction of dendritic cells with naïve T lymphocytes in mesenteric lymph nodes will produce antigen-specific FoxP3^{+} T_{regs}.[9] These T_{regs} will still express

gut-homing molecules CCR9 and α_4:β_7, however, they will be homed back to the gut in order to suppress inflammation to harmless antigens or commensals.[9] Interestingly so, the production of T_{regs} and their ability to express these gut-homing molecules requires the production of retinoic acid by dendritic cells.[9] These dendritic cells express retinal dehydrogenase enzymes and can metabolize dietary vitamin A or vitamin A produced by stromal cells in mesenteric lymph nodes.[9]

Retinoic acid signaling plays several important roles in mammalian eye development and is required for interactions between the optic vesicle and lens placode, as well as for proper retinal and optic nerve development.[34] In 2011, Zhou and colleagues investigated the role of retinoic acid in immune privilege.[33] Due to its role in visual development, retinoic acid exists in abundance within the eye, particularly within ocular fluids such as the aqueous humor.[33] Aqueous humor is a fluid produced by the ciliary processes (pars plicata), of the ciliary body, which nourishes the avascular cornea and the lens by providing oxygen and glucose.[1] The presence of various neuropeptides within the aqueous humor may also induce an immunosuppressive property, as some studies in the literature had previously suggested that aqueous humor could inhibit ocular T lymphocytes from producing IFN-γ and instead convert these T lymphocytes to TGF-β-producing T_{regs}, though details of the mechanism remained largely unknown.[33] Through the use of a murine model, Zhou and colleagues highlighted the roles of aqueous humor and TGF-β in the production of $FoxP3^+$ $T_{regs.}$[33] Similarly to requiring retinoic acid for T_{reg} production within the gut, retinoic acid appeared to be essential for this pathway as well, though this investigation studied the effects of retinoic acid on T_{reg} production in the absence of dendritic cells.[33] Results from this study demonstrated that the presence of aqueous humor inhibited the proliferation of naïve T lymphocytes and prevented these cells from developing into effector cells.[33] Furthermore, IL-17 and IFN-γ secretions, which correlated to naïve T lymphocytes acquiring either Th17 or Th1

effector functions, respectively, were found to be inhibited by aqueous humor.[33] Thus, not only was Th17 and Th1 effector function blocked by the aqueous humor, but mRNA levels of IL-17A, IL-21, IL-22, IL-23R and Th-17-associated transcription factor RORγt (retinoic acid receptor-related orphan receptor γt), were drastically reduced.[33] Instead, T lymphocytes were converted to FoxP3^{+} T_{regs} rather than being committed to the Th17 or Th1 lineage, and they also demonstrated to be progressively more stable with additional rounds of aqueous humor stimulation.[33] Interestingly, Zhou and colleagues found that retinoic acid was required to enhance this process as there was substantial mRNA expression of retinoic receptor RARα in the presence of aqueous humor.[33] Moreover, neutralization of TGF-β to block its function in turn blocked the induction of this receptor, suggesting that TGF-β was required for upregulating RARα expression and indirectly enhancing the effects of the aqueous humor on T lymphocytes.[33]

In 2016, Ahadome and colleagues suggested evidence for retinoic acid-producing dendritic cells within ocular mucosa, similar to those found in the gut mucosa.[35] However, in this case, these dendritic cells induced fibrosis in a severe ocular allergy setting.[35] Fibrosis is a condition involving excessive collagen deposits and appears to be commonly present during allergic inflammation, such as atopic dermatitis and atopic keratoconjunctivitis when considering ocular allergy.[35] In the eye, fibrosis may lead to blindness depending on its severity.[35] Retinoic acid, among other retinoid or vitamin A metabolites, have shown involvement in fibrosis, in addition to promoting fibroblast growth and proliferation.[35] Also through the use of a murine model, this study demonstrated that two dendritic cell subsets, CD11b^{+} and cDC2, had increased activity of aldehyde dehydrogenase (ALDH) enzyme, which is required for the conversion of retinal to all-*trans*-retinoic acid (ATRA), or isomerized 9-*cis*-retinoic acid (9-CisRA).[35] Increased activity of ALDH resulted in fibrosis within the conjunctiva of the mouse model under allergy eye disease conditions, whereas depletion

of these dendritic cells inhibited ALDH and conversely played a protective role in preventing the development of fibrosis under such conditions.[35] Further, evaluation of two nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR), both of which are involved in retinoic acid signaling, revealed that inhibition of the latter prevented fibrosis, whereas inhibition of the former did not.[35] Additionally, when primary conjunctival fibroblasts were exposed to 9-CisRA, the results were fibrogenic, while exposure to 9-CisRA in the presence of an RXR inhibitor was not, suggesting that 9-CisRA is a high-affinity, fibrogenic ligand of RXR, that shows increased expression with increased ALDH activity under allergic eye disease conditions.[35]

Large populations of macrophages with unique characteristics is another aforementioned feature of mucosal immunity.[9] Within the lamina propria of a healthy intestine of the human body, the largest population of macrophages is found, however, these macrophages differ from those residing in other tissues.[9] First, while other macrophages are maintained locally, intestinal macrophages require constant replenishment from blood monocytes.[9] This may be in response to the high levels of commensal bacteria within the gut.[9] Second, rather than producing inflammatory cytokines or reactive oxygen/nitrogen species, they are anti-inflammatory cells that constitutively produce large amounts of IL-10.[9] Thirdly, they uniquely act as powerful scavengers that ingest or degrade antigens, while simultaneously limiting inflammation through maintenance of an antigen-specific tolerant environment that can sustain FoxP3^{+} T_{reg} cells as they migrate back from mesenteric lymph nodes.[9] While there appears to be little evidentiary support within the literature to confirm the presence of IL-10-producing macrophages within ocular mucosa that possess similar characteristics to those of the intestinal mucosa, the ocular mucosa does have other unique immune cells whose functions appear to be well-suited to the immune-privileged environment of the eye.[36,37] For example, in a study by Williams and colleagues in 2014, using human conjunctival

epithelial cells, it was found that the majority of the resident CD8 T lymphocytes evaluated were of $CD45RA^{-}$ $CCR7^{-}$ effector memory cells, and almost all of these cells expressed mucosal homing molecule α_{E}:β_{7}.[36] This is similar to the expression of gut homing molecule α_{4}:β_{7} on primed intestinal T lymphocytes.[9] Upon further investigation, it was also found that these conjunctival CD8 T lymphocytes constitutively expressed Granzyme B, a protease produced by CD8 T lymphocytes that induces apoptosis in target cells.[9,36] Moreover, these cells also had the ability to produce IFN-γ.[36] Williams and colleagues concluded that the expression of these molecules by CD8 T lymphocytes serves an immune surveillance function at the ocular surface, and may confirm that recruitment of T lymphocytes to the ocular mucosa is mechanistically similar to homing within the intestine.[9,36] Where IL-10 does appear to exist in abundance within the eye is in the cornea during inflammatory conditions.[37] Under normal circumstances, IL-10 is absent in the healthy cornea, however, its expression is highly upregulated within infiltrating corneal macrophages ($CD11^{+}$ cells) during inflammation.[37] Further, it appears that the secretion of IL-10 by these cells serves an anti-inflammatory but pro-lymphangiogenic function.[37]

Lymphangiogenesis is a term that describes the formation of new lymphatic vessels or secondary ingrowth of lymphatic vessels in tissue.[37,38] Lymphangiogenesis first occurs during embryogenesis and will rarely occur into adulthood unless it is inflammation-induced.[38] The process involves vascular endothelial growth factor receptor-3 (VEGFR-3), and transcription factor Prospero-related homeobox-1 (Prox1).[38] During embryogenesis, vascular endothelial growth factor-C (VEGF-C), or VEGF-D, will bind to VEGFR-3, which is expressed in lymphatic endothelial cells and is a key protein involved in lymphangiogenesis regulation.[38] VEGF-C and VEGF-D are produced in elevated amounts at sites of inflammation by various immune cells such as macrophages, dendritic cells, neutrophils and mast cells, and their binding to VEGFR-3 will

activate its signaling.[38] Additionally, the expression of VEGFR-3 in blood vascular endothelial cells has also been shown to be induced by Prox1 and is thereby another method of regulating lymphangiogenesis.[38] Experiments involving VEGFR-3 and Prox1 have shown that the two appear co-localized in lymphatic vessels, suggesting that a connection exists, and down-regulation of VEGFR-3 expression in lymphatic endothelial cells can be achieved through silencing of Prox1.[38] Additionally, there may also be involvement of transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), if induced through inflammatory stimuli.[38] Prox1 may become activated by NF-κB and the two may collectively activate the VEGFR-3 promotor, in order to increase expression of this receptor in lymphatic endothelial cells.[38] As a result, pre-existing lymphatic endothelial cells may become more responsive to the binding of VEGF-C and VEGF-D for activating VEGFR-3, resulting in more enhanced lymphangiogenesis.[38]

In tissues other than the eye, lymphangiogenesis is an important process during inflammation because lymphatic vessels act as a drainage system for immune cells and debris at the site of inflammation, allowing the termination of an ongoing inflammatory response.[37] Moreover, they allow for tissue pressure regulation and prevention of edema or chronic inflammation within tissues, which would otherwise occur if the vessels were to be blocked, as shown in recent studies.[37] As previously mentioned, however, the cornea is avascular, which means that it is also devoid of lymphatic vessels and several anti-lymphangiogenic mechanisms help to actively ensure this avascular state.[1,37] As a result, nourishment is provided to the cornea from the aqueous humor via diffusion and also through limbal capillaries (from the conjunctiva and episclera).[1] Thus, while lymphangiogenesis has physiological functions in other tissues, within the eye and specifically within the cornea, lymphangiogenesis is considered to be pathological.[37] Under normal conditions, expression of soluble forms of VEGFR-1, VEGFR-2 and VEGFR-3, in

addition to the expression of thrombospondin-1, constitute anti-(lymph)angiogenic factors within the corneal epithelium.[39] Soluble VEGFR-1 acts as a decoy receptor for VEGF-A to limit hemangiogenesis and soluble VEGFR-3 acts as a decoy receptor, binding VEGF-C and VEGF-D such that they cannot bind the membrane-bound form.[39] Soluble VEGFR-2 helps to prevent lymphatic invasion at the central cornea and thrombospondin-1 binds CD36 to regulate VEGF-C production by monocytes and macrophages.[39] In the cornea, if lymphangiogenesis occurs from pre-existing limbal lymphatic vessels, the mechanism is also mediated by VEGFR-3 and ligands VEGF-C and VEGF-D.[39] Alternatively, if the process occurs independent of limbal lymphatics, then $CD11b^+$ macrophages in the corneal stroma will express Prox1 and lymphangiogenic marker LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1).[39] Additionally, damaged corneal tissue produces proinflammatory cytokines TNF-α, IL-1 and chemokines CCL2 and CCL20, which will in turn recruit neutrophils and macrophages and promote lymphangiogenesis as they produce VEGF-C and VEGF-D.[39]

In 2015, with the use of a murine model to investigate the role of IL-10 in corneal lymphangiogenesis, Hos and colleagues found this cytokine to be indirectly involved in regulating lymphangiogenesis and reducing inflammation through macrophages.[37] When comparing healthy corneas with corneas where inflammation was induced through the placement of a corneal suture, IL-10 mRNA expression was upregulated during suture placement and declined two weeks post suture removal.[37] Additionally, IL-10 protein expression appeared co-localized with CD11b, which is expressed by macrophages, suggesting the presence of IL-10 in infiltrating corneal macrophages during inflammation.[37] *In vitro*, experiments whereby peritoneal macrophages were stimulated using recombinant IL-10 revealed through real-time polymerase chain reaction (RT-PCR) technique, significantly increased expression of this cytokine, indicating an autocrine effect

or a positive feedback loop.[37] Moreover, macrophage anti-inflammatory markers arginase-1 and CD163, in addition to LYVE-1 (lymphangiogenic marker) were also significantly upregulated.[37]

Analysis of VEGF-A, VEGF-C and VEGF-D following IL-10 stimulation revealed decreased expression of VEGF-A, significantly upregulated expression of VEGF-C and a low but unchanged expression of VEGF-D.[37] Note, VEGF-A is pro-hemangiogenic while VEGF-C and VEGF-D are pro-lymphangiogenic.[37] As it appeared that IL-10 resulted in macrophages with anti-inflammatory, anti-hemangiogenic but pro-lymphangiogenic phenotypes, possibly with the involvement of VEGF-C, further immunohistochemistry staining on inflamed corneas 2 weeks post suture placement showed co-localization of VEGF-C and LYVE-1.[37] Suture placement in a IL-10$^{-/-}$ (deficient) murine model, revealed the mRNA expression of VEGF-A to instead increase, while the mRNA expression of VEGF-C decreased.[37] IL-10$^{-/-}$ mice without suture placement had similar VEGF-A and VEGF-C mRNA expression levels as their wild-type littermates.[37] Morphometric analysis showed significantly reduced lymphangiogenesis in the inflamed corneas of IL-10$^{-/-}$ mice with no change in hemangiogenesis, consistent with the previous finding and suggesting that a loss of IL-10 reduced VEGF-C expression, leading to reduced lymphangiogenesis during inflammatory conditions.[37] Interestingly, when lymphatic endothelial cells were incubated with either VEGF-C, IL-10 or a combination thereof, cell proliferation appeared to increase in the presence of VEGF-C, however, RT-PCR demonstrated no changes in mRNA expression of VEGF-C or LYVE-1 upon incubation with IL-10.[37] This suggested that IL-10 did not directly affect proliferation of these cells or the expression of these two markers.[37] Rather, the effect of IL-10 appeared to be indirect through anti-inflammatory VEGF-C-secreting macrophages.[37] Further, IL-10$^{-/-}$ mice also showed significantly higher levels of IL-1β, TNF-α and CD11b^{+} in suture-induced inflamed corneas and these levels remained elevated following suture

removal.[37] Thus, it appeared that a deficiency in IL-10 lead to a more severe and persistent inflammatory response even post stimulus removal.[37] Upon conditional deletion of a gene (Stat3) in the myeloid cell lineage of mice, it was determined that treatment of isolated peritoneal macrophages from these mice with IL-10 was insufficient for upregulating VEGF-C or LYVE-1 mRNA expression, demonstrating a dependence for the Stat3 gene and the importance of macrophages in this process.[37] Corneal suture placement in these mice mimicked the phenotypes of IL-10$^{-/-}$ mice, as less evidence of lymphangiogenesis and higher presence of inflammatory CD11b^{+} cells was observed.[37] Local treatment with IL-10 in mice with conditional deletion of Stat3 resulted in higher egress of inflammatory cells and therefore faster resolution of corneal inflammation.[37] Overall, it appeared that the crucial but indirect role for IL-10 during inflammatory corneal conditions was to modulate lymphangiogenesis through macrophages and the upregulation of VEGF-C and LYVE-1 expression.[37]

1.2.5 Immunological Defense: Ocular Surface Immune System Anatomy, EALT

Within the eye, MALT is comprised of conjunctiva-associated lymphoid tissue (CALT) and lacrimal drainage-associated lymphoid tissue (LDALT), collectively referred to as eye-associated lymphoid tissue (EALT).[11] The lymphoid tissue is continuous from the lacrimal gland throughout the conjunctiva via excretory ducts (CALT) and into the lacrimal drainage system (LDALT).[11] CALT and LDALT and their associated structures (i.e., the lacrimal gland, the conjunctiva and the lacrimal drainage system), are connected via tears that flow over the ocular surface.[11] Additionally, specialized blood vessels known as high endothelial venules, or HEV, exist within the conjunctiva and the lacrimal drainage system, and allow for a regulated migration and exchange of lymphocytes between ocular tissue and other mucosal organs.[11] HEV provide a regulated inflow of these lymphocytes, while lymph vessels offer a regulated outflow.[11] Figure 1-2

below provides a visual representation of the EALT system.[12] Upon examination of the anatomy of the mucosal immune system that exists at the surface of the eye, it becomes increasingly evident that the ocular mucosa shares numerous immune cells, structures and features with extraocular mucosa located elsewhere in the body, such as in the gut.

First, the cornea is devoid of lymphoid cells; however, MHC class II-positive antigen-presenting Langerhan cells exist in the epithelium of the peripheral cornea.[12] Langerhan cells are a type of immature dendritic cells which are typically found within the skin, although they have also been identified to exist in the eye.[9,12] The conjunctiva, in contrast to the cornea, has many blood vessels, lymph vessels and high endothelial venules.[12] Lymphoid tissue within the conjunctiva consists of lymphocytes found in both the basal epithelial layer and lamina propria, in addition to IgA-producing plasma cells.[12] CD8 T lymphocytes are found in abundance within the conjunctiva basal epithelial layer, while CD4 T lymphocytes exist in abundance within the conjunctival lamina propria – a distribution similar to the mucosa located in the small intestine epithelia and lamina propria.[12] Numerous macrophages and dendritic cells also exist in the conjunctiva and similarly to the gut mucosa, there is evidence of lymphoid follicles composed mainly of B lymphocytes with parafollicular T lymphocytes present as well.[12] Of interest, is the age-dependent frequency of these lymphoid follicles within the conjunctiva, as it is estimated that roughly 40% of individuals in their mid-seventies will lose these follicles, while for the other 60%, the number of follicles may decrease to about 10 per conjunctival sac on average.[12]

Second, at the lacrimal gland, CD8 T lymphocytes predominate over CD4 T lymphocytes and IgA-producing plasma cells exist in abundance.[12] Further, while the lacrimal gland rarely appears to contain lymphoid follicles, it has been reported in the literature that T lymphocytes may distinctly form groups around intralobular ducts within the gland instead.[12] In contrast to the

lacrimal gland, but similarly to the conjunctiva, the lacrimal drainage system does contain lymphoid follicles among having lymphoid tissue as well.[12] The lacrimal drainage system is continuous with the conjunctiva and the lacrimal sac through the lacrimal puncta, the canaliculi and the nasolacrimal duct into the nose.[4] This system comprises a moist, mucous membrane with lymphoid tissue and follicles identified within the canaliculi and lacrimal sac as well.[12,28]

Lymphoid tissue protects the ocular surface by detecting antigens and producing effector cells to process and initiate an immune response.[11,27] Specialized M cells within the EALT system are responsible for phagocytosing an antigen and delivering it to antigen-presenting cells (such as dendritic cells).[12] The antigen will then be presented to the lymphoid follicles and subsequent activation of the lymphocytes within the follicles will occur.[11] The lymphocytes will proliferate and differentiate into effector cells of either B-lineage or T-lineage.[11] Typically, the center of lymphoid follicles is composed of B cells with a few T cells located there as well; however, T cells are mainly found to be parafollicular, surrounding the B cells instead (Figure 1-2).[12,40] Although the cornea is avascular and does not contain lymphoid cells under physiological conditions, the EALT system also plays a role in corneal immune protection based on its topographical location.[11,12] During eye closure, conjunctival lymphoid tissue is projected in such a way that it co-localizes with the cornea (Figure 1-3).[11]

Taken together, it is understood that although the ocular immune system possesses its own unique features, which must exist in order to protect the integrity of the cornea and to help preserve vision, there are also numerous features that are shared and conserved with the mucosal immune system elsewhere. Further, it is evident that the ocular mucosa plays an integral role in the protection of ocular tissues, which may have previously been underestimated or thought to have not existed at all.

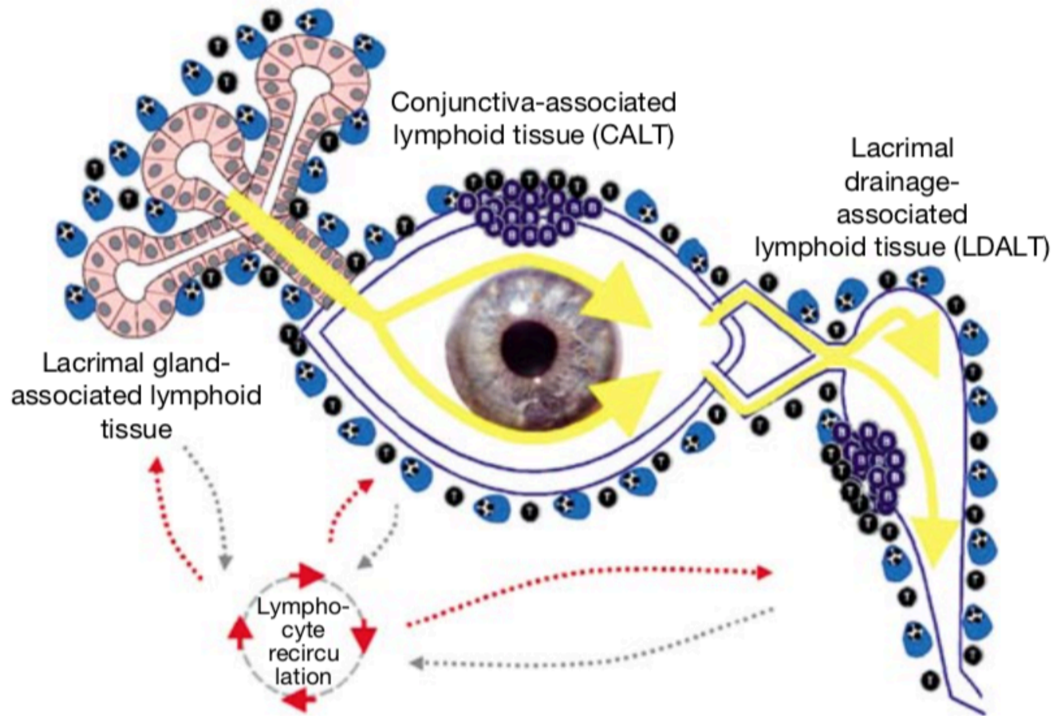

Figure 1-2: EALT (Blue Cells = Plasma Cells, Purple Cells = B Lymphocytes, Black Cells = T Lymphocytes).[12] Figure Reprinted from Anatomy and Immunology of the Ocular Surface (Knop & Knop, 2007), with Permission from Karger Publishers.

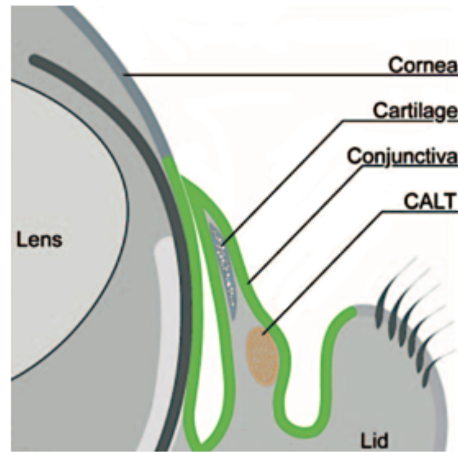

Figure 1-3: Topographical Location of CALT Within the Conjunctiva Relative to the Cornea.[40] Figure Modified and Reprinted from Experimental Induction and Three-Dimensional Two-Photon Imaging of Conjunctiva-Associated Lymphoid Tissue (Steven et al., 2008). Permission for Modification and Use Obtained from the Copyright Holder: Association for Research in Vision & Ophthalmology (ARVO).

Chapter 2: Introduction - Contact Lenses

2.1 Contact Lenses as Biomaterials

A biomaterial is defined as any natural or synthetic material that comes into contact with biological tissue.[41] By this definition, a contact lens (CL) is classified as a biomaterial due to its interaction with the cornea, conjunctiva and tear film upon placement onto the ocular surface. Generally, biomaterials are considered to be inert, although today, drugs that can slowly release from the biomaterial may be included in the device matrix.[41] Within the fields of ophthalmology and optometry, biomaterials are used for both vision preservation and restoration.[42] For example, intraocular lenses (artificial lens implants placed surgically into the eye), can replace a cataract (clouding lens) that is obstructing vision.[41,42] On the other hand, contact lenses are used for correcting vision[43], for cosmetic purposes[41] and with recent advancements, may have potential to be used as drug-delivering devices for treating ocular diseases and infections in the future.[43-47] Currently, contact lenses are estimated to be worn by 140 million people worldwide, rendering them the most commonly used biomaterial.[48] Contact lenses have evolved greatly over the past several decades and their properties continue to be developed upon today, with the goal of improving comfort for extended wear remaining at the forefront of their advancement.

2.2 A Brief Introduction to the History of Contact Lenses

Contact lenses first rose to popularity roughly 50 years ago upon the introduction of "soft" contact lens materials to the market.[49] These lenses, also termed "conventional hydrogels", consisted of poly-2-hydroxyethyl methacrylate (pHEMA) derivatives and offered slightly more oxygen permeability in comparison to the preceding "hard" contact lenses that were composed of polymethyl methacrylate (PMMA) polymer.[49,50] PMMA offers no oxygen permeability to the corneal surface and has very limited hydrophilicity.[50] In contrast, pHEMA is a relatively

hydrophilic polymer, yet the oxygen permeability of contact lenses of such material largely depends on their water content and remains relatively low due to the low solubility of oxygen in water.[51] As a result, where the use of PMMA-derived contact lenses results in hypoxic corneal conditions among other ocular health risks, pHEMA-derived contact lenses also fail to eliminate these health risks.[49,50] Fortunately, by 1999, the introduction of a new class of soft contact lens materials, termed "silicone hydrogels" or "SiHys", allowed for such hypoxic conditions to be overcome.[49] Interestingly, clinical studies have also shown reduced bulbar and limbal hyperemia in patients wearing SiHy lenses when compared to those who wear conventional hydrogels.[52] While SiHy lenses are hydrophobic in nature due to the incorporation of silicone, these lenses actually offer five to six times greater oxygen permeability to the corneal surface in comparison to conventional hydrogels, due to the high solubility of silicone in oxygen.[51,53,54] Furthermore, the formation of Si-O-Si linkages within siloxane macromers also allows for increased gas transmission.[53,54] In the case of SiHy lenses, their oxygen permeability depends largely on their silicone content rather than the water content of the lens.[51] Early generation SiHy lenses had surface treatments or an internal wetting agent incorporated into their bulk material in order to increase hydrophilicity, as these early "untreated", relatively hydrophobic lenses, would cause discomfort due to their poor wettability.[51] Such surface treatments, in addition to other characteristics of the lens (including water content and ionicity), are the basis for which the Food and Drug Administration (FDA) categorizes contact lens materials.[55]

2.3 Categorization of Contact Lenses

Currently, there are a total of five classes of contact lenses according to the FDA.[55] The first four groups classify conventional hydrogels based on total water content and ionicity (Table

2-1).[55] The fifth group subdivides silicone hydrogels into five subgroups based on total water content, ionicity and other characteristics (Table 2-2).[55]

Table 2-1: FDA Categorization of Conventional Hydrogels.[55]

FDA Group	Water Content	Ionicity
Group I	Low (<50%)	Nonionic (neutral charge)
Group II	High (>50%)	Nonionic (neutral charge)
Group III	Low (<50%)	Ionic (negative charge)
Group IV	High (>50%)	Ionic (negative charge)

Table 2-2: FDA Categorization of Group V Silicone Hydrogels into Subgroups.[55]

Subgroup	Characteristics
Group V-A	Low water content (<50%), nonionic, surface treated.
Group V-B1	Low water content (<50%), nonionic, contain hydrophilic monomers, no surface treatments.
Group V-B2	Low water content (<50%), nonionic, contain a semi-interpenetrating network, with no surface treatments.
Group V-C	High water content (>50%), nonionic.
Group V-D	Low water content (<50%) or high water content (>50%), ionic.

2.4 Contact Lens Discomfort

While contact lens wear is generally considered successful, clinical studies report that between 12% to 51% of contact lens wearers will discontinue CL use primarily as a result of experiencing contact lens discomfort (CLD).[56] CLD is a term encompassing the occurrence of adverse ocular sensations such as dryness, itchiness, scratchiness, watering and tiredness, whether continuous or episodic, that is experienced as a result of low compatibility between a contact lens and the ocular environment.[56] Visual disturbances such as blurry vision may or may not occur, however, CLD will often lead to decreased contact lens wear time and eventual discontinuation altogether.[56] Numerous factors can lead to CLD, whether these factors are due to the contact lens

characteristics themselves, such as water content or base curve, or environmental aspects such as age, gender, ocular health or air quality (Figure 2-1).[57] Although conventional hydrogels and SiHy lenses differ in their material composition and levels of oxygen permeability, the same level of CLD appears to exist with both of these classes of materials.[52]

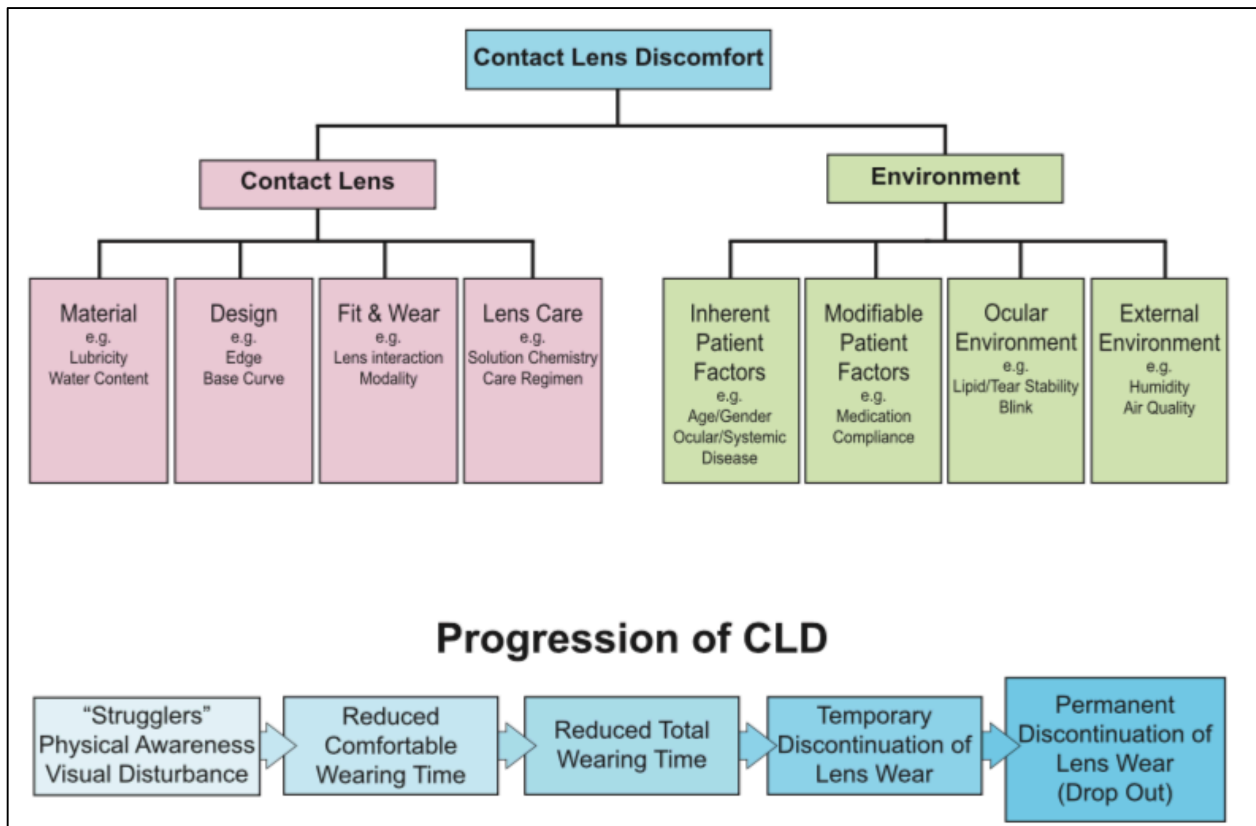

Figure 2-1: Contact Lens Discomfort Classification.[57] Figure Reprinted from The TFOS International Workshop on Contact Lens Discomfort: Report of the Definition and Classification Subcommittee (Nichols et al., 2013), with Permission from the Copyright Holder: Association for Research in Vision & Ophthalmology (ARVO).

One clinical study comparing a SiHy lens with three conventional hydrogels found no significant differences in ratings reported for dryness or comfort between the two classes of contact lens materials in either asymptomatic or symptomatic patients.[52] Instead, the study found higher levels of dryness reported by symptomatic patients, in addition to reports of reduced comfort, over the 7-hour study period in comparison to asymptomatic patients.[52] This result, however, was

consistent whether the symptomatic patients wore silicone or conventional hydrogels.[52] Previous studies have also presented similar results.[52]

When considering the factors leading to CLD, the ocular environment is of great interest due to the interaction of a contact lens with the ocular surface upon its placement onto the cornea. Though not directly a cause of CLD, the tear film, due to the components it is comprised of, may potentially play a role in discomfort.

2.5 Interaction of a Contact Lens with the Tear Film

As evident through sections 1.2.2 and 1.2.3, numerous proteins (including inflammatory markers), mucins and lipids are found on the ocular surface and circulating within tears. Craig and colleagues have effectively depicted the interaction of such molecules with a contact lens and with the tear film as it sits on the corneal epithelium (Figure 2-2).

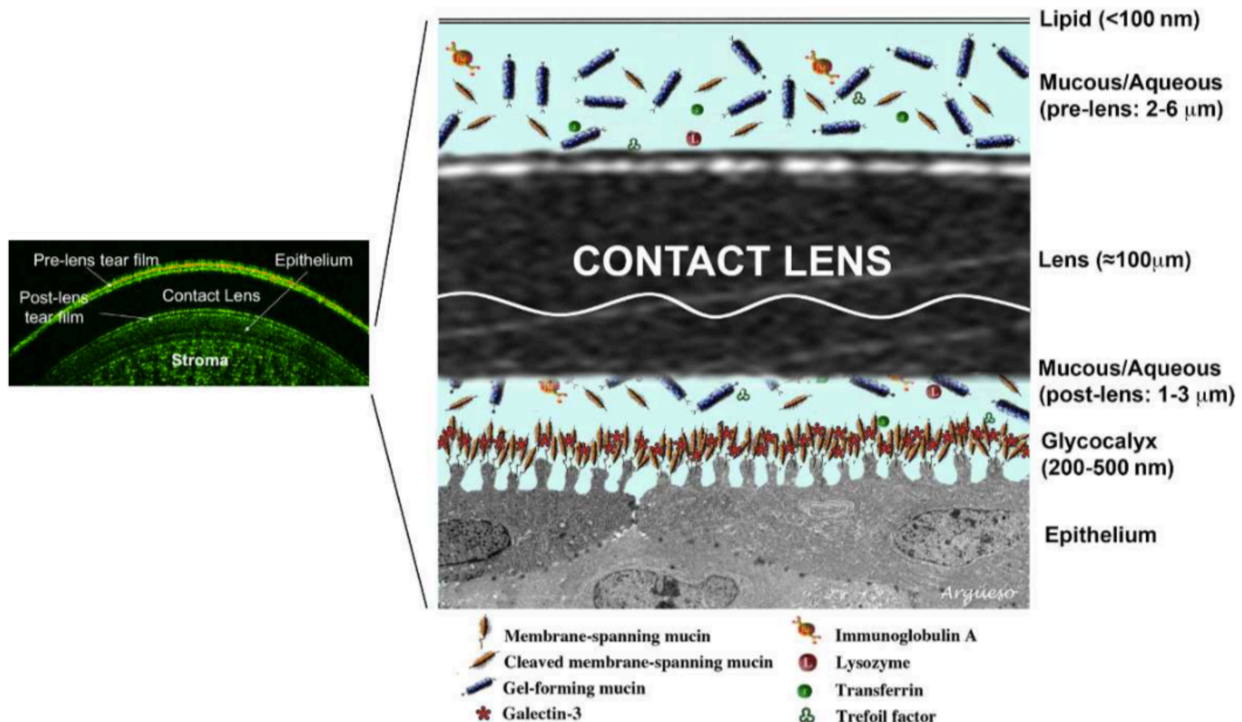

Figure 2-2: Comparative Schematic of an in situ NaFl Cross-Section of a CL on the Corneal Surface vs. Interaction of a CL in an Updated Tear Film Model.[5] Figure Reprinted from The TFOS International Workshop on Contact Lens Discomfort: Report of the Contact Lens Interactions with the Tear Film Subcommittee (Craig et al., 2013), with Permission from the Copyright Holder: Association for Research in Vision & Ophthalmology (ARVO).

Of interest in Figure 2-2 are the presence of cleaved membrane-spanning mucins and gel-forming mucins within the aqueous-mucin phase, which increase in concentration toward the corneal epithelium, as a heavy concentration of membrane-spanning mucins constitutes a glycocalyx.[5] Recalling the roughly 1400 unique proteins that also exist within this phase, one such protein outlined in Figure 2-2 is positively-charged lysozyme protein that has been shown in the literature to readily deposit on the surface of negatively charged contact lens materials such as conventional etafilcon A lenses.[5,7,58,59] Typically, SiHy lenses attract lipids more readily than they do proteins.[58] Additionally of note are the IgA proteins that exist within the aqueous-mucin phase, which as previously mentioned, are the principal class of antibodies that provide immunity to mucosal tissues such as the eyes.[5,9]

It is inevitable that these proteins, mucins and lipids will come into contact with a contact lens at the cornea-contact lens interface. Nonetheless, whether these molecules will actually deposit onto the surface of the lens or be taken up into its bulk material, depends largely on the chemical properties of the lens itself, such as its principal monomers or its ionicity (charge). The interaction (and subsequent deposition), of proteins and lipids onto contact lens materials appears to be well understood within the literature.[58-60] However, the deposition of inflammatory proteins such as cytokines, and any resulting consequences which may be caused as a result of their deposition, is much less clear and has attracted little interest to date.

Based on what is reported in the literature, it appears that if there is deposition of proteins or lipids onto the surface of a contact lens, whether that be onto a silicone or conventional hydrogel, this interaction may, in turn, result in CLD, ocular surface dryness, reduced visual acuity or potentially lead to ocular inflammation and disease.[56,59] Of interest is the underexplored potential

effects of the uptake and deposition of inflammatory markers onto these contact lens materials as well, and to what extent this may contribute to CLD or dry-eye syndrome.

2.6 Dry-eye syndrome

Intraocular immunology appears to be quite complicated, yet similar to the body, many of the same immune cells exist within ocular tissue and they secrete various cytokines. It can be said then, that a basal level of cytokines exists within the eye; however, these levels may be regulated differently based on ocular conditions and environmental conditions. For example, every night during sleep when the eyes are closed, a physiological but proinflammatory shift takes place on the ocular surface.[18] During the first hours of sleep, complement activation is increased in the tear film, and a significant influx of neutrophils later follows.[18] Complement is a defense system consisting of over 30 complement proteins that act in a cascade to collectively destroy microbes through cytolysis, phagocytosis, and inflammation.[4] Further, when the eyes are closed, and there is limited oxygen reaching the eye, otherwise described as hypoxic conditions, an increase in toll-like receptor expression in the epithelial cells of the conjunctiva is also observed.[18]

Dry-eye syndrome (DES) is an inflammatory disorder involving many cytokines and chemokines.[22] Extensive research has been conducted in an attempt to identify which cytokines and chemokines are involved in DES.[22] Studies have shown that there are elevated levels of IL-1α, IL-1β, IL-6, IL-8 and TNF-α in tears and conjunctival epithelium of DES patients.[16,22] This could, in part, be due to two major signaling pathways that exist in corneal and conjunctival epithelial cells, known as MAPK and NF-κB.[18] MAPKs, an acronym for mitogen-activated protein kinases, are a group of key signaling molecules that participate in a well-conserved signaling pathway cascade.[61] Tear hyperosmolarity, a universal feature of dry eye, in conjunction with other desiccating stresses on the ocular surface, will activate this signaling pathway, leading to the

secretion of IL-1β, IL-8, TNF-α and metalloproteinases.[18,61] Additionally, these proteins initiate a protein phosphorylation cascade that ultimately activates nuclear transcription factors including NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which will further stimulate the secretion of IL-8 and metalloproteinases as well.[18,61] As noted previously, TNF-α, a key initiator of ocular inflammation, has been shown to induce IL-1β, which also has high potential to initiate ocular inflammation through its significant upregulation of other proinflammatory cytokines including IL-6 and IL-8.[16,17] Suitably, IL-1β is described as a potent proinflammatory cytokine and furthermore, it can also stimulate helper T cells to differentiate into Th17, which as previously mentioned, secretes IL-17, IL-21 and IL-22.[10,15] IL-6 promotes neutrophil migration to areas of inflammation through the production of CXCL2 (previously known as MIP-2) and CCL3 (previously known as MIP 1-α).[26,62] Also, as noted earlier, IL-8 will induce directional migration of leukocytes (mainly neutrophils) to sites of inflammation.[4,10,15]

2.6.1 Contact Lens-Related Dry Eye

Contact lens-related dry eye (CLDE) is an issue that affects roughly 50% of all contact lens wearers.[63] It is already known that up to 51% of contact lens wearers may discontinue their use of contact lenses due to discomfort.[56] One symptom of contact lens discomfort is dryness and in some patients, CL wear alone can cause dry eye symptoms which have the potential to alter the tear film altogether.[56,63] As a result of the discomfort, reduced visual acuity may be experienced, compelling patients to either discontinue CL use or to decrease their lens wear time.[56,63] As mentioned, dry eye can lead to the activation of MAPK and NF-κB signaling pathways within corneal and conjunctival epithelial cells, leading to the secretion of several cytokines; thus, cytokines may also influence contact lens-related dry eye.[18,61] The deposition of proteins and lipids onto contact lens

materials may also be a contributing factor to CLDE, and this has been extensively studied in the literature.[6,8,59]

2.7 Protein and Lipid Deposition onto Contact Lens Materials

The deposition of proteins and lipids onto contact lenses depends largely on the lens chemistry (principle monomers), which influences its water content and ionicity.[55] One study investigating lysozyme deposition on contact lens materials found significantly more deposition of this protein onto etafilcon A (FDA group IV) in comparison to omafilcon A (FDA group II) and silicone hydrogels (FDA group V).[59] This occurs due to the principle monomer of etafilcon A, methacrylic acid, carrying a negative charge, which has an increased affinity for positively charged lysozyme at physiological pH.[59] Omafilcon A is zwitterionic due to its principle monomer phosphorylcholine and can largely resist protein deposition.[59] Another study investigating lipid and protein deposition on etafilcon A and polymacon A (FDA group I) lenses, found higher deposition of lipids on the latter in comparison to the former, however, etafilcon A had higher protein deposition than polymacon A.[60] Once again, the ionic charge of etafilcon A appeared to predominantly control protein deposition.[60] On the other hand, lipid deposition has been shown to be greater on nonionic lenses (such as polymacon A), in comparison to ionic lenses (such as etafilcon A).[60] It is proposed that silicone hydrogels readily attract lipids more so than they do proteins, however, any amount of protein that deposits onto silicone hydrogels, though less than that deposited on conventional hydrogels, is still an important consideration, as any denaturation of these proteins on the ocular surface can lead to inflammatory conditions.[58] A study involving two conventional hydrogels and four silicone hydrogel lenses found that the silicone hydrogel lenses studied had less lysozyme deposition compared to the conventional hydrogels studied.[58] Of the conventional hydrogels, etafilcon A had the highest lysozyme deposition at 1800 μg/lens.[58]

While the amount of protein deposition can vary among different silicone hydrogels, depending on the specific principle monomers, balafilcon A accumulated up to 44 μg/lens – the highest among the silicone hydrogels tested, yet small in comparison to the conventional hydrogels.[58]

2.8 Quantification of Inflammatory Markers within the Eye, Deposition onto Contact Lenses

In the literature, there exist many studies where various inflammatory markers have been quantified in the tear film of non-CL wearers and CL wearers, under both normal and diseased conditions. In 1998, a study by Nakamura and colleagues on 270 healthy, normal subjects who did not wear CLs, attempted to establish a baseline for the basal amounts of IL-1α, IL-1β, IL-6 and IL-8 present within tears.[64] Tear samples were collected, pooled and assayed using an ELISA (enzyme-linked immunosorbent assay).[64] A large range was reported from 10.9 $\pm$ 1.2 pg/mL to 731.4 $\pm$ 116.2 pg/mL (mean $\pm$ SD), for IL-1α and IL-8 concentrations.[64] IL-1β was quantified at 12.9 $\pm$ 2.3 pg/mL, while IL-6 levels were reported as 226.2 $\pm$ 29.6 pg/mL.[64]

In 2013, Wei and colleagues quantified IL-1β, IL-6, IFN-γ and TNF-α levels from the tears of normal subjects and CL-wearers assessed for symptoms of dry eye disease.[65] Tears were collected using microcapillary tubes, pooled and sampled with a Milliplex immunoassay using Luminex.[65] The cytokine concentrations reported (as mean $\pm$ SD), were higher in CL wearers (15.56 $\pm$ 12.94 pg/mL, 14.86 $\pm$ 0.19 pg/mL, 1.25 $\pm$ 3.69 pg/mL and 14.82 $\pm$ 15.8 pg/mL for IL-1β, IL-6, IFN-γ and TNF-α, respectively), in comparison to the non-CL wearer subjects (7.42 $\pm$ 5.62 pg/mL, 13.43 $\pm$ 8.74 pg/mL, 0.27 $\pm$ 0.88 pg/mL and 7.46 $\pm$ 8.74 pg/mL for IL-1β, IL-6, IFN-γ and TNF-α, respectively).[65] Previously in 2009, Massingale and colleagues had also analyzed the expression of IL-1β and TNF-α in the tears of dry eye syndrome patients (non-CL wearers) and healthy controls.[66] Utilizing a similar microcapillary method as Wei and colleagues to collect and pool tear samples, Massingale and colleagues used RT-PCR to analyze mRNA levels

of their cytokines of interest, in addition to a Multiplex bead immunoassay to quantify protein expression levels.[66] As reported by Wei and colleagues, Massingale and colleagues also found increased concentrations (reported as mean $\pm$ SD), of IL-1β, IL-6 and TNF-α (644.3 $\pm$ 148.8 pg/mL, 1625.7 $\pm$ 430.9 pg/mL and 435.7 $\pm$ 145.6 pg/mL, respectively), in comparison to healthy controls (436.3 $\pm$ 116.7 pg/mL, 632.3 $\pm$ 167.9 pg/mL and 250.6 $\pm$ 63.2 pg/mL, respectively).[66] Further, the same trend was also observed after quantifying levels of cytokines IL-2, IL-4, IL-5, IL-8, IL-10 and IFN-γ.[66] Interestingly, of all the cytokines assayed, the cytokine of highest concentration was IL-8, in both healthy controls (16791.4 $\pm$ 2841.2 pg/mL) and subjects with dry eye (48508.6 $\pm$ 9397.3 pg/mL).[66]

In 2014, Yamaguchi and colleagues investigated whether a correlation existed between human tear cytokine levels and patients suffering from bacterial keratitis (BK).[67] 28 healthy controls and 26 subjects with unilateral BK (affecting one eye only), were recruited and diluted tear samples were collected from both eyes of the healthy controls, as well as from the affected eye and unaffected eye of the BK subjects.[67] Tears were diluted as sterile saline was injected into the inferior conjunctival fornix using a micropipette.[67] The following cytokines were quantified using a Luminex microbeads immunoassay: IL-1Ra, IL-1β, IL-2, IL-5, IL-6, IL-7, IL-8, IL-10, IL-17a, fibroblast growth factor-2 (FGF-2), granulocyte/macrophage colony-stimulating factor (GM-CSF), chemokine ligand-2 (CCL-2) and triggering receptor expressed on myeloid cells-1 (TREM-1).[67] Interestingly, there were upregulated levels (mean $\pm$ SD) of IL-1β, IL-6 and IL-8 (66.6 $\pm$ 26.8 pg/mL, 7174 $\pm$ 2430 pg/mL and 810 $\pm$ 315 pg/mL, respectively), in the affected eye of BK subjects, in comparison to the eyes of the healthy controls (13.0 $\pm$ 4.0 pg/mL, 171.8 $\pm$ 32.1 pg/mL and 56.5 $\pm$ 33.8 pg/mL, respectively).[67] In contrast, CCL-2, IL-10 and IL-17A appeared to be upregulated in the unaffected, contralateral eye of the BK subjects (813 $\pm$ 478 pg/mL, 86.7 $\pm$

38.3 pg/mL and 3350 $\pm$ 881 pg/mL, respectively), in comparison to the eyes of the healthy controls (73.7 $\pm$ 25.3 pg/mL, 17.5 $\pm$ 4.9 pg/mL and 1350 $\pm$ 337 pg/mL, respectively).[67] Uniquely, TREM-1 was the only cytokine reported to be upregulated in both the affected and unaffected eyes of the BK subjects (551 $\pm$ 231 pg/mL) versus healthy controls (31.3 $\pm$ 12.4 pg/mL).[67]

Similarly, in 2015, Santacruz and colleagues also examined the expression of proinflammatory cytokines in the tears of subjects with either gram-positive or gram-negative, unilateral BK in comparison to healthy controls.[68] For both cases of BK, subjects had upregulated levels of IL-1β, IL-6 and IL-8 in the affected eye in comparison to the contralateral, unaffected eye, similar to what was reported by Yamaguchi and colleagues.[68] Levels of IL-1β, IL-6 and IL-8 were reported (mean $\pm$ SD) as 64 $\pm$ 40 pg/mL, 758 $\pm$ 1166 pg/mL and >2500 pg/mL, respectively, in the case of gram-positive BK subjects and 423 $\pm$ 240 pg/mL, 1596 $\pm$ 971 pg/mL and >2500 pg/mL, respectively, in the case of gram-negative BK subjects.[68] Levels reported in the unaffected eyes were 58 $\pm$ 89 pg/mL, 25 $\pm$ 17 pg/mL and 420 $\pm$ 377 pg/mL, respectively.[68] Tears were collected using sterile capillary tubes, while cytokines were quantified using a cytometric bead assay.[68] Likewise to what was reported by Massingale and colleagues, Santacruz and colleagues also observed IL-8 (of all cytokines tested), to exist in the highest concentration in tears of both normal controls and BK subjects.[68]

The presence of proinflammatory cytokines and matrix metalloproteinases (MMPs) in the tears of patients with noninfectious corneal ulcers was investigated by Sakimoto and colleagues in 2014.[69] MMPs are zinc-dependent enzymes that can degrade the extracellular matrix and cell surface proteins.[69] Subjects with noninfectious corneal ulcers within the peripheral cornea, as examined via a slit-lamp, were recruited in addition to normal subjects with no corneal ulcers, and the tears of all subjects were collected using Schirmer strips.[69] A multiplex bead immunoassay was

utilized to quantify concentrations of IL-1β, IL-6, TNF-α and MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10 and MMP-13.[69] IL-6 and TNF-α mean concentrations in the tears of the normal subjects were quantified (mean $\pm$ SD), as 17.5 $\pm$ 20.3 pg/mL and 8.8 $\pm$ 6.2 pg/mL, respectively.[69] IL-1β was undetectable in this control group.[69] In contrast, levels of IL-1β, IL-6 and TNF-α were upregulated in the tears of corneal ulcer patients and were reported as 377.6 $\pm$ 239.7 pg/mL, 1394 $\pm$ 1399 pg/mL and 52.6 $\pm$ 59.7 pg/mL, respectively.[69] Of the MMPs evaluated, while all appeared upregulated in the tears of the corneal ulcer group rather than the control group, only levels of MMP-8 and MMP-9 were statistically significant between the two groups.[69] MMP-8 levels were reported as 78.2 $\pm$ 19.5 pg/mL and 1523.5 $\pm$ 1035.1 pg/mL (mean $\pm$ SD) in the control and corneal ulcer groups, respectively, while MMP-9 levels were reported as 161.5 $\pm$ 19.4 pg/mL and 1608.5 $\pm$ 1051.5 pg/mL in the control and corneal ulcer groups, respectively.[69] Inflammatory cytokines can stimulate the production of MMP-8 from corneal epithelial cells under inflammatory conditions and the upregulation of MMP-9 can result in tissue destruction, leading to the formation of ulcers.[69] Thus, the high levels of proinflammatory cytokine expression and overexpression of MMP-8 and MMP-9 observed in the tears of the corneal ulcer group appeared to correspond to their condition, as compared to the control group with no signs of ulceration.[69]

To assess whether there were any changes to the levels of inflammatory mediators in the tears of individuals who routinely wore CLs, González-Pérez and colleagues in 2012 recruited subjects for a year-long study which evaluated the tears of CL wearers in comparison to non-CL wearers.[70] Subjects either wore monthly SiHy lenses (lotrafilcon A), or corneal refractive therapy rigid gas permeable lenses (Paragon CRT) on an overnight basis, while control subjects had never worn any CLs at all.[70] Corneal Refractive Therapy (CRT), also known as overnight orthokeratology (Ortho-K), is a procedure whereby reverse geometry contact lenses are worn

overnight to help flatten the curvature of the central cornea in a short-term attempt for reducing myopia.[70] Tear samples were collected using microcapillary tubes and later analyzed through ELISA assays for IL-6, IL-8 and MMP-9 concentrations.[70] Interestingly, no significant differences were found for expression levels of IL-6, IL-8 or MMP-9 between the tears of the SiHy and control groups.[70] Conversely, levels of all three of these proteins were significantly upregulated in the tears of the CRT group in comparison to the control group.[70] IL-6 and IL-8 levels were quantified as 4.7 $\pm$ 1.2 pg/mL and 935.3 $\pm$ 254.4 pg/mL, respectively, in the CRT group, 2.6 $\pm$ 0.6 pg/mL and 659.5 $\pm$ 105.1 pg/mL, respectively, in the SiHy group, and 2.2 $\pm$ 0.5 and 601.5 $\pm$ 72.7, respectively, in the control group (all reported as mean $\pm$ SD).[70] MMP-9 levels were reported in ng/mL (mean $\pm$ SD) as 74.3 $\pm$ 23.0 (CRT), 45.0 $\pm$ 7.8 (SiHy) and 39.2 $\pm$ 8.3 (control).[70]

In 2016, Chao and colleagues also investigated changes in tear cytokine concentrations following discontinuation of CL wear.[71] They recruited subjects who were habitual wearers of either SiHy or conventional CLs (wearing CLs for at least six hours per day, over five days per week, for at least one year).[71] Tears were collected and pooled from the lateral canthus of each eye using glass capillary tubes, once immediately after CL removal on visit 1, as well as on each subsequent visit 1-, 2-, 3-, 4- and 7 ($\pm$ 1) days-post CL removal.[71] Levels of IL-1β, IL-1RA, IL-6, IL-10, IL-12p70 and TNF-α were quantified using multiplex bead assays.[71] Chao and colleagues reported no significant differences in the expression of any of these cytokines in the tears of the subjects over the duration of the study post CL removal.[71]

Given that tears must inevitably interact with a CL at the ocular surface, the results from the studies by González-Pérez and colleagues and Chao and colleagues, suggesting that CL wear may not induce any significant changes in tear expression levels of the cytokines of interest, could also suggest that minimal interactions (or uptake), exists for these cytokines onto the CL materials

tested. Recently, however, in 2019, Chao and colleagues reported absorption of inflammatory mediators onto CL materials in an *in vitro* experiment, upon soaking lenses in individual and combined solutions of IL-8, IL-1RA or MMP-9.[72] This investigation was not a clinical study; rather, comfilcon A, omafilcon A, balafilcon A and etafilcon A were soaked in both 500 pg/mL and 100 pg/mL solutions of IL-8, IL-1RA and MMP-9 individually, as well as in 500 pg/mL and 100 pg/mL combined solutions of all three molecules.[72] 1:1 2% trifluoroacetic acid:acetonitrile was used to extract these inflammatory mediators from the surfaces of the lenses and both the extracted concentrations and residual concentrations still remaining on the lenses were quantified using ELISA assays.[72] The extracted concentrations revealed levels of absorption of each inflammatory mediator, while residual concentrations revealed whether or not the mediator remained tightly bound to the surface of the lens.[72]

In terms of individual solutions, for IL-8 there were no statistical differences in absorption between any of the soft lens materials; however, there were greater residual concentrations on omafilcon A (336 $\pm$ 25 pg/mL) rather than etafilcon A (106 $\pm$ 133 pg/mL), only for the 500 pg/mL solution.[72] At 500 pg/mL, omafilcon A also absorbed more MMP-9 (466 $\pm$ 9 pg/mL) than balafilcon A (437 $\pm$ 11 pg/mL) or etafilcon A (428 $\pm$ 13 pg/mL).[72] There were no statistical differences observed between any of the lenses at individual 100 pg/mL solutions of IL-8 or MMP-9.[72] As well, there were no statistical differences in individual IL-1RA concentrations, either at 500 pg/mL or 100 pg/mL, for any of the materials.[72] Furthermore, there was less residual concentration of MMP-9 on etafilcon A (128 $\pm$ 22 pg/mL), in comparison to omafilcon A (174 $\pm$ 3 pg/mL), comfilcon A (168 $\pm$ 34 pg/mL) and balafilcon A (186 $\pm$ 14 pg/mL) contact lens materials.[72] Analysis of the both 500 pg/mL and 100 pg/mL combined solutions revealed IL-8 to be absorbed in the highest concentration onto all of the materials, with etafilcon A demonstrating

the greatest absorption.[72] Residual concentrations of IL-8, however, in addition to IL-1RA and MMP-9 were minimal for all materials, including on etafilcon A, suggesting that these mediators did not remain firmly bound to the lens surfaces when combined together.[72]

As evident through the literature, there have been many studies investigating the levels of inflammatory mediators in the tears of patients who are either CL wearers, exhibit symptoms of dry eye or have inflammatory conditions as a result of an infection (bacterial keratitis), or some other health condition (corneal ulcers). Many of the investigations within the literature are based on clinical studies, where not only can discrepancies exist between the subjects themselves (patient variability), but discrepancies can also exist between the methods utilized to quantify these mediators.[73] For example, a study by Dionne and colleagues in 2016 which aimed to compare between two methods of tear inflammatory mediator analysis, Quantibody microarray and Luminex assay, found the former to detect more significant differences and more cytokines within its range of detection than the latter.[73] Furthermore, Dionne and colleagues suggest that differences in tear method collection (e.g., microcapillary tubes vs. Schirmer strips), whether or not the tears are diluted, how the tears are stored following collection, the time of collection, subtle differences in collection techniques by different clinicians, differences in assay protocols including the minimum amount of proteins recommended to be used and the limit of detection of the assays, are just some of the many discrepancies that can influence the results of a study.[73]

In addition to these factors, consideration may also be given to the half-life of the inflammatory markers resulting in some of the large discrepancies that is observed in the literature. For example, in the case of IL-1β, IL-6, IL-8 and TNF-α, the half-lives of these cytokines *in vivo* are reported in the literature as approximately 3-4 hours[74], 1 hour[75], < 4 hours[76], and 20 minutes[77], respectively. Thus, depending on the method of tear collection utilized to quantify tear cytokine

levels[73], or the amount of time in between collection and quantification[73], it is possible that degradation of the cytokines could occur and hence result in largely variable results to be reported by different researchers. Furthermore, in cases where it would appear that there is uptake of these cytokines onto biomaterials, if the cytokines begin to quickly degrade due to their half-lives, this degradation may interfere with the ability to properly quantify their values and therefore incorrectly present as uptake instead. *In vivo* half-life is highly impacted by the presence of proteases in the tear film due to their ability to degrade proteins.[9,69,78] The *in vitro* studies to be presented in this thesis did not incorporate proteases.

As evident from above and as outlined in Table 3-3 for cytokines IL-1β, IL-6, IL-8 and TNF-α, there is no standard level of cytokine expression that should be measured within tears, but rather a large range exists. As many of the studies in the literature are clinical studies, the aim of this thesis was to investigate the inherent properties of various CL materials solely through *in vitro* experiments, in order to determine whether these materials intrinsically allow for the uptake of cytokines, with no potential interference from patient variability, as in *in vivo* investigations.

Chapter 3: Introduction - Thesis Rationale

3.1 Objectives

It was important to understand whether conventional hydrogels and SiHy contact lens materials could uptake and deposit inflammatory proteins, namely cytokines, onto their lens surface, as this would provide tremendous insight into the interaction of inflammatory proteins with CL materials and its potential effects such as contact lens discomfort. The aim of this thesis was to investigate various types of CL materials to find one that exhibited substantial uptake of proinflammatory cytokines, acting as a reservoir for these inflammatory markers, in the context of inflammatory ocular conditions such as DES or allergies, where the CL could be utilized as an application to an over-reactive immune system. Depending on the amount of uptake and wear modality of the lens, the CL could aid in dampening the immune response (daily lens wear), without causing collateral damage to the ocular tissue, as the inflammatory markers would be removed from the ocular surface by the end of the day. In contrast, the CL could further facilitate an immune response (reusable lens wear), as the inflammatory markers linger on the lens surface and continue to interact with the ocular surface. The purpose of this work was to, in part, help address issues surrounding DES and CLD. To our knowledge, data in the literature does not exist where the uptake of cytokines onto conventional hydrogels and SiHy contact lens materials has been quantified through electrochemiluminescence and compared.

3.2 Cytokines of Interest

The cytokines of interest evaluated in this thesis were IL-1β, IL-6, IL-8, and TNF-α. These cytokines were chosen because elevated levels of all of these four cytokines have been observed in dry eye syndrome patients.[16,22] Furthermore, IL-1β is a potent proinflammatory cytokine that has the highest potential of initiating ocular inflammation.[16,22] It is induced by TNF-α and

upregulates IL-6 and IL-8.[16,17] Additionally, three of these cytokines, IL-1β, IL-8 and TNF-α are secreted by the two major signaling pathways, MAPK and NF-κB, that exist within corneal and conjunctival epithelial cells.[18,61] As previously described, there are other cytokines that are important to the development and maintenance of inflammation within the eye; however, the four cytokines chosen seemed to be more universally present in a variety of inflammatory conditions.

As mentioned above, IL-1β is a potent proinflammatory cytokine that is important for initiating a host-defense response in cases of infection or injury.[79] Of the other cytokines within the IL-1 family, IL-1β was chosen as it is widely studied in the literature and appears to be the best characterized cytokine of its family.[79] A variety of cells are capable of producing and secreting IL-1β, including the monocytes and macrophages of the immune system.[79] The production of IL-1β begins through a priming step that produces the inactive precursor, pro-IL-1β, upon recognition of PAMPs by PRRs on the surface of the appropriate cell.[79] As noted previously in section 1.2.3, macrophages, dendritic cells and neutrophils are sensor cells that express PRRs.[9] Following this step, interactions between a cytosolic PRR, an adapter molecule and pro-caspase-1, results in the formation of a multi-protein complex known as an inflammasome, which activates the protease caspase-1 that cleaves pro-IL-1β into its mature form (IL-1β).[79] IL-1β can secrete from the cell in a quick manner, however, the mechanism of its secretion does not follow a conventional route, as this cytokine lacks a signal sequence.[79] Typically, proteins are secreted through an endo-membrane system formed by the endoplasmic reticulum and Golgi apparatus.[79] This process involves recognition of the signal sequence by a signal recognition particle, translocation of the protein into the endoplasmic reticulum lumen and trafficking of the protein through the endoplasmic reticulum and Golgi apparatus until the extracellular destination is reached.[79] Lopez-Castejon and colleagues, however, suggest that due to lacking a signal sequence, IL-1β is instead secreted through various

routes that depend on both the strength of the inflammatory stimulus and the levels of extracellular IL-1β required to support an inflammatory response that is effective.[79] Lopez-Castejon and colleagues have recently proposed three mechanisms of IL-1β secretion, named, “Rescue and Direct”, “Protected Release” and “Terminal Release”.[79]

The first mechanism, “Rescue and Direct”, involves an appropriate secretion stimulus to redirect IL-1β localized to vesicles (and therefore subject to degradation), back into the extracellular space.[79] Since only a small fraction of IL-1β is typically localized to vesicles, this mechanism is proposed to occur either when there is little extracellular demand for IL-1β, or as a supplement to extracellular IL-1β that is secreted through other mechanisms.[79] The second mechanism, “Protected Release”, involves either the release of IL-1β from the shedding of plasma membrane microvesicles (100-600 nm), or secretion from exosomes (small vesicles, 50-80 nm).[79] IL-1β in microvesicles is bioactive and has been shown in the literature to release upon contact with cells expressing IL-1RI receptor.[79] Additionally, there is evidence in the literature to suggest that the microvesicles shed from dendritic cells contain IL-1β and caspase-1, and will release these proteins upon ATP stimulation, thereby providing a mechanism for eliciting an inflammatory response at target sites distant from the site of inflammation.[79] Secretion of IL-1β from exosomes also allows for signaling processes to occur at distant sites, as exosomes, in addition to shed microvesicles from antigen-presenting cells, have been shown to also contain MHC II molecules.[79] The third mechanism, “Terminal Release”, is proposed to occur only when there is extreme inflammatory stress and once the cell is committed to apoptosis.[79] In this case, large quantities of active IL-1β is rapidly secreted across the disintegrating plasma membrane.[79]

IL-6 is a pleiotrophic cytokine.[80] During inflammation, it is first synthesized within the local lesion but can quickly move through the bloodstream into the liver, where it can induce the

synthesis of various proteins including C-reactive protein, serum amyloid A, fibrinogen, haptoglobin and α1-antichymotrypsin, while lowering the production of fibronectin, albumin and transferrin molecules.[80] As IL-6 reaches the bone marrow, it induces the maturation of megakaryocytes, leading to thrombocytosis, as a result of the release of platelets.[80] IL-6 plays an important role in both the innate and adaptive immune systems.[80] In conjunction with TGF-β, IL-6 can promote the differentiation of naïve $CD4^+$ T cells into Th17 cells, while simultaneously inhibiting TGF-β-induced T_{reg} differentiation.[80] As noted previously, one cytokine produced by Th17 cells is IL-17, which has been identified in severe ocular allergies, in addition to other inflammatory diseases of the ocular surface including dry eye syndrome and uveitis.[22,23] Upregulation of this Th17/T_{reg} balance by IL-6 is known to disrupt immunological tolerance and lead to autoimmune or chronic inflammatory diseases.[80] In contrast, IL-6 also induces both the differentiation of naïve $CD8^+$ T cells into cytotoxic T cells and B cells into plasma cells that produce antibodies.[80] Recall, CD8 T cells selectively recognize MHC class I-bound peptides, which commonly present peptides from viruses to CD8 T cells, while plasma cells secrete antibodies that target and destroy an antigen.[9] The stimulation of NF-κB signaling pathway, which occurs upon recognition of PAMPs by PRRs, will enhance mRNA transcription of IL-6 (in addition to IL-1β and TNF-α).[80]

The transcription factor NF-κB is also an important element of IL-8 expression.[81] Typically, in an unstimulated cell, deacetylation of histones, active repression of NF-κB by NF-κB repressing factor, and the binding of octamer-1, allow the transcription of the IL-8 gene (*CXCL8*) to remain repressed.[81] NF-κB, activating protein (AP-1) and CCAAT/enhancer-binding protein beta (C/EBPβ), are binding sites of the IL-8 promoter element. In the cell cytoplasm, NF-κB is stored in an inactivated form due to the binding of IκBα and IκBβ inhibitory proteins.[81]

During stress, two IκB kinases known as IKKα/β and IKKγ/Nemo, will phosphorylate the IκB proteins and result in the activation of NF-κB.[81] The inhibitory proteins are quickly degraded and NF-κB will translocate to the cell nucleus where it can bind the *CXCL8* promoter.[81] AP-1 and C/EBPβ synergize with NF-κB to ensure optimum expression of *CXCL8*.[81]

Like IL-6, TNF-α is also a pleiotropic cytokine that is produced by a variety of different cell types, although the primary synthesizers of TNF-α are cells of the monocytic lineage.[82] TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) are the two transmembrane receptors through which TNF-α acts.[82] The former is expressed in all mammalian tissue in a constitutive manner, while the expression of the latter is highly regulated and tends to only be expressed in immune cells.[82] TNF-α has high affinity for both receptors, though its binding onto TNFR1 is an irreversible mechanism, while its binding onto TNFR2 involves rapid binding and dissociation.[82] In fact, in some cells, TNFR2 may be regarded as a ligand passer to TNFR1, which acts to increase local concentrations of TNF-α.[82] In response to an inflammatory signal, matrix metalloproteinases cleave TNFR1 and TNFR2 from the cell surface.[82] Most cellular responses to TNF-α are due to activation of TNFR1, including cytotoxicity, cell growth, upregulation of both adhesion and cytokine genes, as well as activation of NF-κB.[82] In contrast, while TNFR2 may also be important for cytotoxicity and NF-κB activation, activation of this receptor is mainly responsible for the proliferation of lymphoid cells.[82] Like IL-1β, TNF-α is also a potent proinflammatory cytokine that appears to also be an early mediator present in abundance during inflammation.[82] As outlined in Table 3-1 below, TNF-α can induce the production of IL-1β, among other proinflammatory cytokines and for this reason, it is regarded as a “master regulator”.[16,17,82] PRRs such as TLRs can induce macrophages to produce TNF-α, which can in turn further activate macrophages.[82] TNF-α

signalling is also important to processes such as cell proliferation, differentiation, survival and apoptosis.[82]

Table 3-1 briefly summarizes a few of the interactions existing between the cytokines of interest. Table 3-2 provides information in regard to their size, isoelectric point (pI), and other ocular conditions in which these cytokines may be upregulated. Isoelectric point refers to the pH of a solution where there is no net charge on the protein.[83] The surface of a protein will be predominately positively charged if the pI of the protein is above the pH of the solution (pI > pH).[83] In contrast, the surface of a protein will be predominately negatively charged if the pI of a protein is below the pH of the solution (pI < pH).[83]

Table 3-1: Interactions that Exist Between the Cytokines of Interest

Cytokine	Interactions
IL-1β	Potent proinflammatory cytokine with high potential to initiate ocular inflammation.[15,16] Induced by TNF-α and upregulates IL-6 and IL-8, in addition to stimulating helper T cells to produce Th17.[10,15-17]
IL-6	Upregulated by IL-1β.[16,17] Produces CXCL2 and CCL3 which promote neutrophil migration to the site of inflammation.[26,62]
IL-8	Upregulated by IL-1β.[16,17] Promotes directional migration of neutrophils to the site of inflammation.[4,10,15]
TNF-α	Key initiator in ocular inflammation and induces IL-1β.[16,17]

Table 3-2: Size (kDa) and Isoelectric Points of the Cytokines of Interest, Ocular Conditions They Pertain To

Cytokine	Size	Isoelectric Point (pI)	Ocular Conditions They Pertain To
IL-1β	17.5 kDa[84]	6.9 - 7.0[85]	Uveitis[16], proliferative diabetic retinopathy (PDR)[16], diabetic macular edema (DME)[16]
IL-6	21-28 kDa[15]	4.9[86], 5.0 - 6.0[87]	Dry eye syndrome[22], uveal melanoma[88], PDR, DME[16]
IL-8	6-8 kDa[15]	9.4[89]	Dry eye syndrome[22], uveal melanoma[88]
TNF-α	17 kDa[90]	5.0 - 7.0[91], 5.3[92]	Dry eye syndrome[22], uveal melanoma[88], PDR, DME[16]

The basal levels of these cytokines (pg/mL) observed in normal patients, as well as elevated levels during CL wear or inflammatory conditions, as reported in the literature, are summarized in Table 3-3 below. As evident from the discrepancy in the numbers, there seems to exist a large degree of variability in the measured levels, and a consistent or specific quantity of these cytokines does not appear to exist among different individuals. Given that this broad range exists in the literature, there is no single concentration for the cytokines of interest that is recognized as an ideal concentration to investigate for uptake onto contact lens materials. This does not, however, negate the objectives of this thesis and what was aimed to be measured. Thus, concentrations utilized for the uptake experiments presented in this thesis were chosen to represent all of low, moderate and high concentrations, ranging from as low as 2.36 pg/mL to as high as 10,047 pg/mL.

Additionally, it is important to note that individuals may have varying cytokine thresholds for what they deem to be “comfortable” and that while there exists a basal level of these cytokines within the eye, these basal levels may vary between an individual who wears contact lenses and another individual who does not.[64-70,93-95]

Table 3-3: Basal Levels of Cytokines (pg/mL) Reported in the Literature for Normal Patients versus Levels Reported for Contact Lens (CL) Wearers (including SiHys), and Patients with Dry Eye Syndrome (DES), Bacterial Keratitis (BK) or Corneal Ulcers (CU).

Cytokine	Basal Levels (pg/mL)	CL, DES, BK, CU Levels (pg/mL)
IL-1β	7.42±5.62[65], 12.9±2.3[64], 13.0±4.0[67], 58±89[68], 436.3±116.7[66]	15.56±12.94 (CL)[65], 66.6±26.8 (BK)[67], 377.6±239.7 (CU)[69], 664.3±148.8 (DES)[66]
IL-6	2.2±0.5[70], 13.43±8.74[65], 17.5±20.3[69], 25±17[68], 100-400[93], 171.8±32.1[67], 226.2±29.6[64], 632.3±167.9[66], 731.4 ± 116.2[94]	2.6±0.6 (SiHy CL)[70], 14.86±0.19 (CL)[65] 1394±1399 (CU)[69], 1625.7±430.9 (DES)[66], 7174±2430 (BK)[67]
IL-8	56.5±33.8[67], 420±377[68], 601.5±72.7[70], 731.4±116.2[64], 16,791.4±2,841.2[66]	14.82±15.8 (CL)[65], 659.5±105.1 (SiHy CL)[70], 810±315 (BK)[67], 48,508.6±9,397.3 (DES)[66]
TNF-α	7.46±8.74[65], 8.8±6.2[69], 14±7[68], 250.6±63.21[66]	52.6±59.7 (CU)[69], 435.7±145.6 (DES)[66]

3.3 The Contact Lenses of Interest and their Lens Chemistry

Overall, ten commercially-available contact lenses were utilized throughout the work presented in this thesis and among them, eight different types of soft lens materials were used collectively. The United States Adopted Name (USAN) of these materials are etafilcon A, omafilcon A, polymacon A (all conventional hydrogels), and balafilcon A, comfilcon A, delefilcon A and senofilcon A (all SiHy lenses). The proprietary names of the lenses used are: 1-Day Acuvue® Moist (etafilcon A), Acuvue® 2 (etafilcon A), Proclear® 1 Day (omafilcon A), SofLens® 38 (polymacon A), PureVision® (balafilcon A), Biofinity® (comfilcon A), Dailies Total1® (delefilcon A), Acuvue Oasys® 1-Day with HydraLuxe™ Technology (senofilcon A), Acuvue Oasys® 2-week with Hydraclear® Plus (senofilcon A) and Clariti™ 1 Day (somofilcon A). Full specifications for the contact lenses of interest are outlined in Table 3-4 below.

The contact lenses were chosen from three of the five FDA classes of contact lens materials. Three conventional hydrogels were chosen from FDA groups I, II and IV, covering those of low

water content/nonionic, high water content/nonionic and high water content/ionic properties (Table 2-1).[55] Further, these materials had previously been utilized in the literature for investigating protein and lipid deposition on contact lenses.[58-60] No contact lenses were chosen from FDA group III (conventional), as the only commercially-available contact lens of a low water content/ionic property is Metrosoft (deltafilcon A), which is not commonly used. Instead, for a low water content/ionic lens, SiHy material balafilcon A (group V) was chosen due to this lens already having shown protein deposition based on investigations within the literature.[55,58] Balafilcon A is within subgroup D of the SiHy lenses (FDA group V), and SiHy lenses from group V subgroups B-1, B-2 and C were chosen as well (Refer to Table 2-2 and Table 3-4).[55]

In addition to CLD, soft contact lens wear can also be accompanied by the risk of corneal infiltrative events (CIEs).[96] CIEs are inflammatory events of the cornea which include, but are not limited to: microbial keratitis, infiltrative keratitis (including asymptomatic infiltrative keratitis), or contact lens-induced acute red eye.[96] There is evidence within the literature suggesting that the use of reusable silicone hydrogel contact lens materials will increase the risk of CIEs by two-fold in comparison to lower oxygen permeability, poly-HEMA-based conventional hydrogel materials.[96,97] Furthermore, when comparing reusable lenses to daily disposable ones, some studies have reported up to a 12.5-fold increased risk of CIEs associated with the use of reusable lenses.[96,97] Thus, the use of daily disposable contact lenses could potentially reduce this risk, as demonstrated by one study which found a low rate of CIEs at 0.4% per year for daily disposable silicone hydrogels and 0% per year for daily disposable conventional hydrogels, while a rate of 3.3% to 10.7% was associated with reusable lens wear.[96]

With the above taken into consideration, as well as the greater risk associated with silicone hydrogel materials for CIEs, it was important to incorporate both reusable and daily disposable

silicone hydrogel lenses in the studies performed. Of the conventional lenses chosen, 1-Day Acuvue Moist and Proclear 1 Day are designed for daily disposable lens wear, while SofLens 38 is designed for reusable lens wear. Of the SiHy lenses chosen, Dailies Total1, Acuvue Oasys 1-Day with HydraLuxe Technology and Clariti 1 Day are designed for daily disposable lens wear, while PureVision and Biofinity are reusable lenses that are typically replaced after one month of use. It was important to have a wide variety of contact lens materials to not only maximize the number of different materials on which inflammatory marker uptake could be studied, but to also investigate uptake under different wear modalities. In fact, in order to investigate whether any differences existed between two lenses of the same material but different wear modalities (i.e., effect of thickness differences between daily wear or resuable wear contact lens materials), Acuvue 2 (conventional, etafilcon A material) and Acuvue Oasys 2-week with Hydraclear Plus (SiHy, senofilcon A material), were added for comparisons with 1-Day Acuvue Moist (conventional, etafilcon A material) and Acuvue Oasys 1-Day with HydraLuxe Technology (SiHy, senofilcon A material).

3.4 Hypothesis

Commercially-available contact lens materials were investigated for the continuous uptake of proinflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α. It was hypothesized that the lenses evaluated would exhibit deposition of these cytokines onto their surface, as measured through electrochemiluminescence (pg/mL), comparable to the deposition of other proteins as reported in the literature. The properties of all lenses utilized throughout this thesis are specified in Table 3-4.

Table 3-4: Specifications for Contact Lenses of Interest[55,59,98-100]

	Proprietary Name							
	1-Day Acuvue® Moist, Acuvue® 2	**Proclear® 1 Day**	**SofLens® 38**	**PureVision®**	**Biofinity®**	**Dailies Total1®**	**Acuvue Oasys® 1-Day with HydraLuxe™ Technology or Acuvue Oasys® 2-week with Hydraclear® Plus**	**Clariti™ 1 day**
USAN	Etafilcon A	Omafilcon A	Polymacon A	Balafilcon A	Comfilcon A	Delefilcon A	Senofilcon A	Somofilcon A
FDA Group	IV	II	I	V (D)	V (B-1)	V (C)	V (B-2)	V (C)
Water content	58%	60%	38%	36%	48%	Water gradient (33% core transitions to 80% at lens surface)	38%	56%
Iconicity	Ionic	Nonionic	Nonionic	Ionic	Nonionic	Nonionic	Nonionic	Nonionic
Principal Monomers*	HEMA+MA	HEMA+PC	pHEMA	NVA+TPVC+ NCVE+NVP+ PBVC	NVP, VMA, IBM, TAIC, M3U, FM0411M, HOB	Silicone hydrogel material core with non-silicone hydrogel surface	HEMA+mPDMS+DMA+ siloxane macromer +PVP+TEGDMA	Alkyl methacrylates, siloxane monomers, NVP
Diopter	-3.00	-3.00	-3.00	-3.00	-3.00	-3.00	-3.00	-3.00

*HEMA, (poly[2-hydroxyethyl methacrylate]); mPDMS, (monofunctional poly-dimethylsiloxane); DMA, (*N,N*-dimethylacrylamide); PVP, (polyvinyl pyrrolidone); TEGDMA, (tetraethyleneglycol dimethacrylate); MA, (methacrylic acid); PC, (phosphorylcholine); NVA, (*N*-vinyl aminobutyric acid); NVP, (*N*-vinyl pyrrolidone); VMA, (*N*-vinyl-*N*-methylacetamide); IBM (isobornyl methacrylate); TAIC, (1,3,5-triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione); M3U, (bis(methacryloyloxyethyl iminocarboxy ethyloxypropyl)-poly(dimethylsiloxane)-poly(trifluoropropylmethylsiloxane)-poly[met hoxy-poly(ethyleneglycol] propylmethylsiloxane)); FM0411M, (methacryloyloxyethyl iminocarboxyethyloxypropyl-poly(dimethylsiloxy)-butyldimethylsilane); HOB, (2-hydroxybutyl methacrylate); TPVC, (tris-(trimethylsiloxysilyl) propylvinyl carbamate); NCVE, (*N*-carboxyvinyl ester); PBVC, (poly[dimethysiloxy] di [silylbutanol] bis[vinyl carbamate]).

Chapter 4: Materials and Methods

4.1 Preface

This chapter will outline any materials and methods common to all experiments presented within this thesis. Any materials, methods, or concentrations specific to an individual experiment will be discussed where appropriate in the subsequent chapters.

4.2 List of Contact Lenses Investigated in All Experiments

The following is a list of all contact lenses utilized throughout the experiments in this thesis: 1-Day Acuvue® Moist (Johnson & Johnson, Jacksonville, FL); Acuvue® 2 (Johnson & Johnson, Jacksonville, FL); Acuvue Oasys® 1-Day with HydraLuxe™ Technology (Johnson & Johnson, Jacksonville, FL); Acuvue Oasys® 2-week with Hydraclear® Plus (Johnson & Johnson, Jacksonville, FL); Biofinity® (CooperVision, Lake Forest, CA); Clariti™ 1 Day (CooperVision, Lake Forest, CA); Dailies Total1® (Alcon, Fort Worth, TX); Proclear® 1 Day (CooperVision, Lake Forest, CA); PureVision® (Bausch + Lomb, Rochester, NY) and SofLens® 38 (Bausch + Lomb, Rochester, NY). The specifications of these lenses are reported in Table 3-4.

4.3 A Simplified, General Outline of the Experimental Design

For all experiments utilizing contact lenses, the lenses were first rinsed to remove any residual packaging solution. This involved two 20-minute soaks in phosphate-buffered saline (PBS) solution (Lonza BioWhittaker, Walkersville, MD), followed by a third soak overnight (between 12-24 hours). Each lens was placed in an individual well of a Costar 12-well polystyrene plate (Corning, Corning, NY) containing 4 mL of PBS per well and in between each soak, the existing PBS solution was pipetted out and replaced with fresh PBS solution. The plates were placed on a VWR Advanced 3500 orbital shaker (VWR International, Radnor, PA) to ensure a dynamic environment.

Following this, all contact lenses were dried on Fisherbrand lens paper (Fisher Scientific, Hampton, NH) and were placed, along with any other autoclaved, sterile membranes (if applicable), in a cytokine solution prepared from the Meso Scale Discovery (MSD) Proinflammatory Panel I Human Calibrator Blend in Diluent 2 (Meso Scale Diagnostics, LLC, Rockville, MD). Drying the contact lenses was a necessary step for preventing any excess liquid on the lens surfaces from interfering with the concentration of the prepared cytokine solution. The calibrator blends used were the same as that provided in the assay kit utilized for all experiments: The V-PLEX® Human Proinflammatory Panel II (4-plex) assay kit (Meso Scale Diagnostics, LLC, Rockville, MD), where the calibrator blend generates the standard curves for the cytokines of interest. Although the MSD® calibrator blend is a lyophilized blend of ten different cytokines (see section 4.4), of which only four were tested in the thesis experiments, it was utilized in order to better model ocular conditions, as a variety of different cytokines are found within the eye, and therefore the four cytokines of interest would not be found in isolation.

Samples were collected at desired time points and stored within 0.6 mL Axygen microtubes (Axygen, Inc, Union City, CA) in a -80℃ freezer until the concentration of the four cytokines of interest could be analyzed with the MSD assay. For cost purposes, samples were not analyzed until a total of thirty-seven samples were collected to fill an entire 96-well MSD assay plate (samples were run in duplicates – refer to Table 4-3). A general summary of the experimental design is provided in Figure 4-1 below.

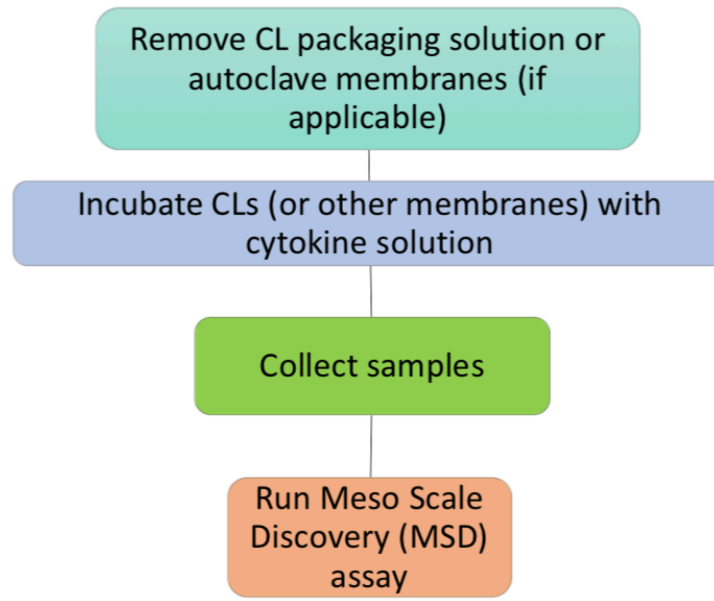

Figure 4-1: Experimental Design.

4.4 Components of the V-PLEX Human Proinflammatory Panel II (4-plex) Assay Kit

The Meso Scale Discovery MESO® QuickPlex Imager (Meso Scale Diagnostics, LLC, Rockville, MD) and commercially-available assay kit (V-PLEX Human Proinflammatory Panel II (4-plex) kit), were used as the basis for quantifying the uptake of cytokines onto biomaterials. This assay is specific to the quantification of the four cytokines of interest, IL-1β, IL-6, IL-8, and TNF-α, hence the name “4-plex”. There are seven components to this kit, as outlined below.

1. **Proinflammatory Panel 1 (human) Calibrator Blend:** A lyophilized blend of ten cytokines of known concentration used for generating a standard curve signal for each cytokine of interest. This signal is given as electrochemiluminescence (ECL) vs. picogram/milliliter (pg/mL). The ten cytokines within this blend are IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α. Depending on the specific lot of each calibrator blend supplied, there is a slight variation between the concentrations of each

cytokine once reconstituted, however, sample concentrations are provided in Table 4-1 to outline the general range of concentration for each cytokine.

2. **Proinflammatory Panel 1 (human) Controls 1, 2, 3:** A set of three lyophilized controls, containing a blend of cytokines at known concentrations (pg/mL). These controls are used for validating the generated standard curves. By preparing the controls and obtaining a concentration reading using the MESO QuickPlex® Imager, a percent recovery is calculated by the MSD software, which indicates the accuracy of the generated standard curves. Similar to the calibrator blend, there is slight variation in the concentrations of the cytokines within each reconstituted control; however, sample concentrations are provided in Table 4-2. Note that each succeeding control has lower concentrations than the preceding one.
3. **Diluent 2:** A proprietary PBS-based solution used for reconstituting the calibrator blend and the controls, as well as diluting the samples to be analyzed. According to Meso Scale Discovery Scientific Support, Diluent 2 also contains fetal bovine serum, bovine serum albumin (stabilizer), sodium chloride (salt), animal IgG, and small amounts of background lowering agents and blocking agents.
4. **Diluent 3:** A proprietary solution for preparing the detection antibody solution.
5. **Detection Antibodies:** Anti-human IL-1β, IL-6, IL-8 and TNF-α antibodies, each conjugated with an electrochemiluminescent label (MSD SULFO-TAG™ reagent).
6. **Wash Buffer:** Used to wash the plate prior to adding samples, antibodies and read buffer.
7. **Read Buffer:** Creates the proper chemical environment for reading the plate.

According to Meso Scale Discovery Scientific Support, while half-life studies have not been performed on the cytokines lyophilized in the MSD calibrator blend and MSD controls, appropriate tests have been conducted for these proteins to confirm that they do not significantly change before their expiration dates. Thus, all materials utilized for the experiments presented in this thesis were fresh materials used within their expiration date, and appropriate experimental controls were incorporated into the study design to account for any potential changes in activity over the study periods. Additionally, the MSD Diluent 2 that is utilized for reconstituting the MSD calibrator blend and the MSD controls, contains stabilizers such as bovine serum albumin. Serum albumin may aid in extending the stability of the cytokines.[101]

Table 4-1: Sample Concentrations (pg/mL) of Cytokines in MSD Lyophilized Calibrator Blend When Reconstituted in 1 mL Diluent 2, as Obtained from a Certificate of Analysis.

Cytokine	Concentration (pg/mL)
IFN-γ	1450
IL-1β	589
IL-2	1450
IL-4	250
IL-6	736
IL-8	574
IL-10	375
IL-12p70	514
IL-13	516
TNF-α	380

Table 4-2: Sample Concentrations (pg/mL) of Cytokines in MSD Lyophilized Controls 1, 2 and 3 When Reconstituted in 1 mL Diluent 2, as Obtained from a Certificate of Analysis.

	Concentration (pg/mL)		
Cytokine	**Reconstituted Control 1**	**Reconstituted Control 2**	**Reconstituted Control 3**
IFN-γ	682	155	52.3
IL-1β	215	50.4	11.0
IL-2	431	41.2	3.83
IL-4	101	20.1	2.88
IL-6	275	41.2	9.03
IL-8	223	36.9	5.91
IL-10	133	23.3	3.61
IL-12p70	168	34.0	6.12
IL-13	150	46.4	12.3
TNF-α	89.6	16.0	2.72

4.5 The Basis of the MSD Assay: Sandwich Immunoassays and Electrochemiluminescence

The Meso Scale Discovery assay utilizes the concepts of sandwich immunoassays and electrochemiluminescence to quantify the concentration of various cytokines in a solution. The specific protocol for the assay was followed as per the manufacturer's guidelines; however, there were three overall main steps to the assay that allowed the analytes within a sample to become sandwiched between a capture antibody and a detection antibody – hence the term "sandwich immunoassay".[102]

Each well of the 96-well MSD plate that comes standard in the assay kit has a working electrode – hence "electro-", which is pre-coated with ten capture antibodies on independent, well-defined spots.[102] Initially, prior to the addition of the samples to the wells, the plate is washed with the MSD wash buffer. Although this is not an essential step, it is recommended in order to ensure uniformity of results.[102] Upon the addition of the samples to the wells of the plate, the analytes bind the capture antibodies that are immobilized on the working electrode surface within each well.[102,103] During the second step, the plate is washed and a solution of the detection antibodies is

prepared using Diluent 3 and added to the wells of the plate.[102,103] These detection antibodies are conjugated with an electrochemiluminescent label, the MSD SULFO-TAG reagent, which also binds the analyte, completing the sandwich.[102,103] As the plate is washed for the third time and the MSD read buffer is added to the wells, the proper chemical environment, hence "chemi-", is created for the plate to be read, and the data to be analyzed, using the MESO QuickPlex Imager.[102,103]

The MSD read buffer contains tripropylamine (TPA) and the MSD SULFO-TAG reagent contains $Ru(bpy)_3^{2+}$, both of which become oxidized to $TPA^{\bullet+}$ and $Ru(bpy)_3^{3+}$, respectively.[103,104] $TPA^{\bullet+}$ then will be reduced to $TPA^{\bullet}$, which is a highly active radical, capable of reducing $Ru(bpy)_3^{3+}$ to excited state $^*Ru(bpy)_3^{2+}$.[102,103] $^*Ru(bpy)_3^{2+}$ will drop to a lower energy state and emit light, hence "luminescence", which can then be measured as an electrochemiluminescent signal (ECL signal), that is used to quantify the concentration of cytokines in solution.[103,104]

4.6 Validation of the MSD Assay and Creating the Plate Map

During each MSD assay, four individual standard curves are generated for the cytokines of interest using the lyophilized MSD calibrator blend ("Calibrator 1"). A sample standard curve for IL-8 is given in Figure 4-2 below, although the standard curves for the remaining cytokines also follow a similar trend. Calibrator 1 is the highest calibrator concentration and it is used to prepare seven subsequent calibrator solutions through 4-fold serial dilutions. "Calibrator 8" is the zero calibrator and only contains a volume of Diluent 2 (with no cytokines). The cytokine concentrations to be chosen for testing uptake onto contact lenses or other membranes must fall within the range of the standard curve for each cytokine in order to be quantifiable.

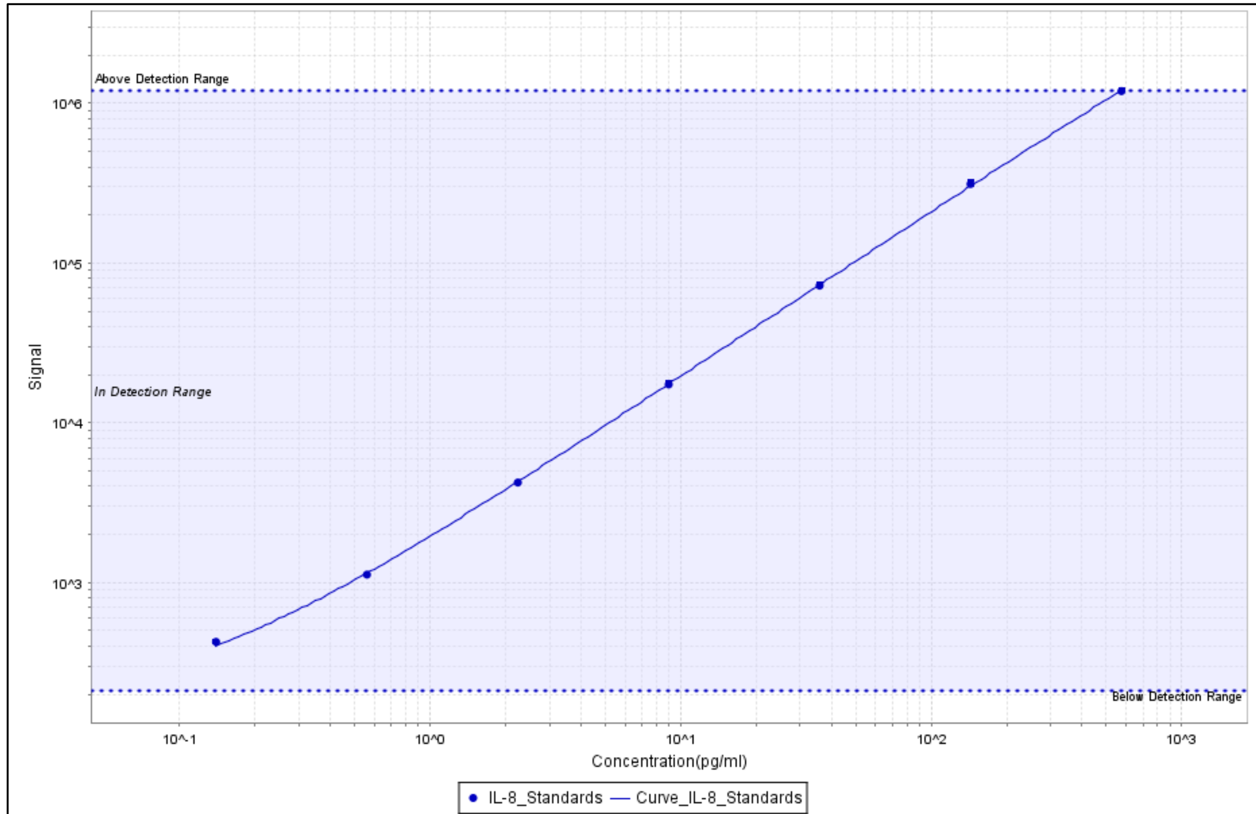

Figure 4-2: A Sample MSD Assay Standard Curve for IL-8, as Obtained from an Experiment.

The MSD controls are used to validate the standard curves generated. Each control has a known concentration of cytokines that are also measured by the MESO QuickPlex Imager. A percent recovery for each cytokine is calculated by the software, based on the cytokine concentrations measured from the controls and the cytokine concentrations measured from the standard curves generated with the calibrator solutions. In this way, the controls can provide insight into the accuracy of the generated standard curves, which in turn provides information in regard to the accuracy of the calculated cytokine concentrations in the unknowns (samples).

The eight calibrator solutions, three control solutions and samples are added to the wells of the MSD plate in duplicates, as outlined in Table 4-3. Up to thirty-seven samples can be measured at one time in order to fill the 96-well plate, however, this number varies depending on

if replicates are to be tested for a sample (e.g., n = 3 or n = 4). The MSD software calculates a cytokine concentration for each well and then averages the concentrations for duplicate wells to give a mean calculated concentration.

Table 4-3: Sample Plate Map for MSD Assay Plate Including Calibrators ("Cal"), Controls ("Cntrl") and Samples ("S"), Measured in Duplicates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cal 1	Cal 1	Cntrl 1	Cntrl 1	S6	S6	S14	S14	S22	S22	S30	S30
B	Cal 2	Cal 2	Cntrl 2	Cntrl 2	S7	S7	S15	S15	S23	S23	S31	S31
C	Cal 3	Cal 3	Cntrl 3	Cntrl 3	S8	S8	S16	S16	S24	S24	S32	S32
D	Cal 4	Cal 4	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
E	Cal 5	Cal 5	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
F	Cal 6	Cal 6	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
G	Cal 7	Cal 7	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
H	Cal 8	Cal 8	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37

4.7 The Sensitivity of the MSD Assay

While running an MSD assay, it is important to be considerate of the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) for each cytokine. These values are outlined in Table 4-4 below. Typically, samples are diluted 2-fold with Diluent 2 prior to being pipetted onto the plate, to ensure that the concentrations of the cytokines, though unknown, will fall within the LLOQ and ULOQ. The dilution factor is inputted into the MSD software such that it is taken into account for the concentration calculations. If the values calculated fall outside of the detection range, results cannot be assumed to be accurate and the assay would have to be repeated with further dilution of the samples.

Table 4-4: Lower Limit of Quantification (LLOQ) and Upper Limit of Quantification (ULOQ) in pg/mL for All Cytokines Included in the V-PLEX Human Proinflammatory Panel II (4-plex) Kit, as of 2018.[102]

Cytokine	LLOQ (pg/mL)	ULOQ (pg/mL)
IFN-γ	1.76	938
IL-1β	0.646	375
IL-2	0.890	938
IL-4	0.218	158
IL-6	0.633	488
IL-8	0.591	375
IL-10	0.298	233
IL-12p70	1.22	315
IL-13	4.21	353
TNF-α	0.690	248

4.8 Method of Data Analysis and Presentation

All data was normalized, graphed, and statistically analyzed using GraphPad Prism V6 software (GraphPad Software Inc., San Diego, CA). Details of the specific statistical analyses performed will be given in the results sections of subsequent chapters. Most of the data presented throughout the thesis is graphed as a percentage of uptake normalized to control values for each time point. The amount of uptake was determined by quantifying cytokine concentrations remaining in solution at specific time points for samples containing either a contact lens and/or another material (excluding controls), and normalizing these values to the cytokine concentrations measured for the control samples at that time point, which were set at 100%. Thus, the method of data collection is through the use of a subtractive assay, in that the amounts quoted relative to the 100% control reflect the values remaining in the solution, while any difference is surmised to have been taken up onto the surface of the materials of interest.

All data is graphed as mean $\pm$ SD of percent pg/mL in solution as normalized to controls. To interpret these graphs, a value of 100% would indicate no uptake, while any values falling

lower than 100% could indicate little to significant amounts of uptake, depending on the exact percentage and the statistics in comparison to the control. In certain instances, some sample values were calculated to be above 100% of the control, depending on the magnitude of variation that existed between the absolute values, as these differences translated to larger differences in the percent of control on a picogram scale (especially for smaller absolute values). For example, given a control value of 100 pg and a sample value of 101 pg, or a control value of 10 pg and a sample value of 10.1 pg, in both cases the sample values would be 101% (1% difference from the control that is set at 100%), even though there is a 1 pg difference in the case of the former and only a 0.1 pg difference in the case of the latter. In another example, if the average absolute value for a control is 15.21 pg and for a biomaterial is 15.94 pg, this is the difference of 0.73 pg (where a picogram is a factor of 10^{-12} of a gram), however, the percent of control for the biomaterial would be 104%, which is higher than the control that is set to 100%. This does not necessarily indicate that the biomaterials are eluting a component into the solution that is mimicking cytokines, or that there are errors in the experimental techniques.

Chapter 5: Method Optimization

5.1 A Survey of Different Biomaterials

5.1.1 Introduction: Pilot Study #1

Initially, it was unknown whether contact lenses would exhibit uptake of cytokines or how the experimental design could be optimized to ensure maximum uptake (if any). To our knowledge, no studies exist in the literature where the MSD assay has been utilized to investigate the inherent property of an unworn contact lens for cytokine uptake. As such, a variety of biomaterials, including contact lenses, were surveyed in a pilot study.

The pilot study investigated the uptake of cytokines IL-1β, IL-6, IL-8 and TNF-α onto silicone sheeting, cotton, gauze and filter paper membranes, in addition to etafilcon A, delefilcon A, omafilcon A and senofilcon A contact lens materials. Silicone sheeting was chosen due to the incorporation of silicone in SiHy lenses and in other synthetic biomaterials, such as the use of silicone rubber in cardiac pacemakers.[41,105] Cotton and gauze were chosen as they are utilized in a wide array of medical applications. Filter paper was chosen as it is slightly liquid absorbent, readily available, and has been used in the literature for spotting blood and later analyzing the dried blood sample for inflammatory markers.[106]

Senofilcon A (Acuvue Oasys 1-Day with HydraLuxe Technology), delefilcon A (Dailies Total1), etafilcon A (1-Day Acuvue Moist) and omafilcon A (Proclear 1 Day), were chosen as two SiHy and two conventional hydrogel materials, respectively, to be studied. The rationale behind choosing these materials was to utilize ones that had previously shown potential for uptake of Levofloxacin antibiotic in solution (data not shown). It was hypothesized that if these contact lens materials could exhibit drug uptake, then they could potentially uptake cytokines as well. Furthermore, evidence of protein deposition onto etafilcon A and omafilcon A was reported in the

literature, and since cytokines are small proteins, these materials were specifically incorporated in the pilot study.[59]

5.1.2 Materials and Methods: Pilot Study #1

The silicone sheeting (Specialty Manufacturing Inc., Saginaw, MI), Whatman filter paper (Sigma-Aldrich, St. Louis, MO) and Millipore filter paper (Sigma-Aldrich, St. Louis, MO), were cut into 10.5 mm pieces using a circular lens punch. The cotton was cut from the tips of Puritan 3-inch cotton tip applicators (Hardwood Products Company, Guilford, ME), and the gauze rolls (Wasip Ltd., Toronto, ON) were cut into 5 mm x 5 mm squares. All materials were autoclaved (except the contact lenses which come in sterile blister packs), and the contact lenses were soaked in PBS, as described in section 4.3. The cytokine solution was prepared from the MSD calibrator blend, as also described in section 4.3, except PBS was utilized rather than Diluent 2. The solution contained IL-1β, IL-6, IL-8 and TNF-α at concentrations of 19 pg/mL, 23 pg/mL, 18.5 pg/mL and 12 pg/mL, respectively. These concentrations were chosen from the approximate lower range of the values reported in the literature, as a dynamic, large range exists (Table 3-3). While reported concentrations of IL-8 in the literature are greater than 18.5 pg/mL, this value was obtained as a result of diluting the other three cytokines to desired concentrations within the lower range. Since all of these cytokines are lyophilized into a single pellet, diluting the concentration of one would inevitably dilute the concentration of others. Additionally, it was important to choose cytokine concentrations within the LLOQ and ULOQ of the MSD instrument (Table 4-4).

All materials were placed in wells of 24-well Greiner Cellstar polystyrene plates (Sigma-Aldrich, St. Louis, MO), and 1 mL of the prepared cytokine solution was added to each well. Control wells contained only 1 mL of the cytokine solution and no contact lenses or other membranes. The samples in each well were evaluated for cytokine uptake after 24 hours of

incubation at 37°C and 25 rpm (rotations per minute) in the Innova 4300 Incubator Shaker (New Brunswick Scientific, Edison, NJ), to simulate body temperature and to ensure a dynamic soaking environment.

5.1.3 Results: Pilot Study #1

After 24 hours, the mean cytokine concentrations obtained from the control wells, as quantified by the MSD, were 18 pg/mL, 15 pg/mL, 9 pg/mL, and 3.5 pg/mL for IL-1β, IL-6, IL-8, and TNF-α, respectively. Results are summarized in Figure 5-1 to Figure 5-4, and a summary of the statistical analyses are summarized in Figure 5-5 to Figure 5-8. Ordinary one-way ANOVAs with Tukey's Multiple Comparisons test were performed and differences were considered significant if $p < 0.05$.

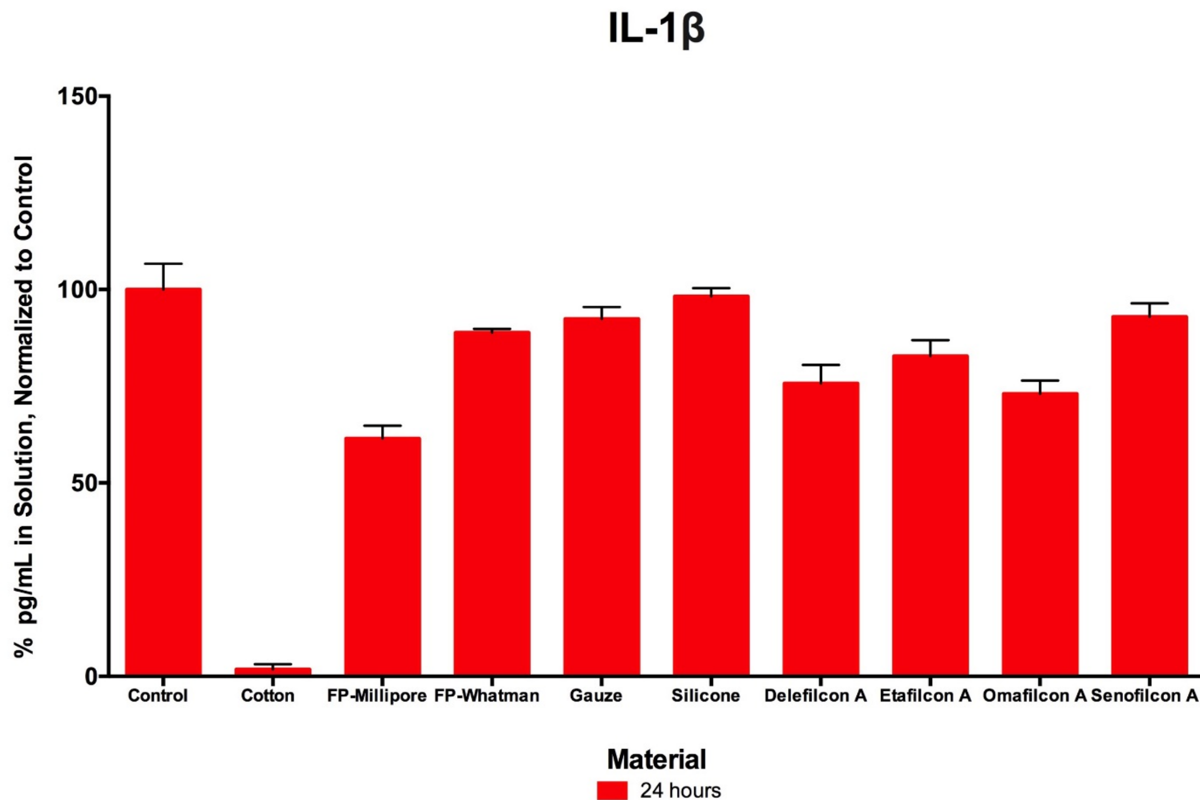

Figure 5-1: Percent pg/mL of IL-1β Remaining in Individual Solutions (n = 3) of Nine Different Biomaterials at 24 Hours.

Relative to the control after 24 hours (100%), there appeared to be little uptake of IL-1β (Figure 5-1) by gauze (92 $\pm$ 3 %) and silicone sheeting (98 $\pm$ 2 %) materials, as well as by senofilcon A (93 $\pm$ 3 %). There were no statistical differences ($p > 0.05$) between these materials and the control (Figure 5-5). In comparison, cotton (2 $\pm$ 1 %, adjusted P-value < 0.0001), Millipore filter paper (62 $\pm$ 3%, adjusted P-value < 0.0001) and Whatman filter paper (89 $\pm$ 1 %, adjusted P-value 0.0373) materials, as well as delefilcon A (76 $\pm$ 5 %, adjusted P-value < 0.0001), etafilcon A (83 $\pm$ 4 %, adjusted P-value 0.0005) and omafilcon A (73 $\pm$ 3%, adjusted P-value < 0.0001), exhibited greater uptake (Figure 5-1) and were all statistically different from the control after 24 hours (Figure 5-5).

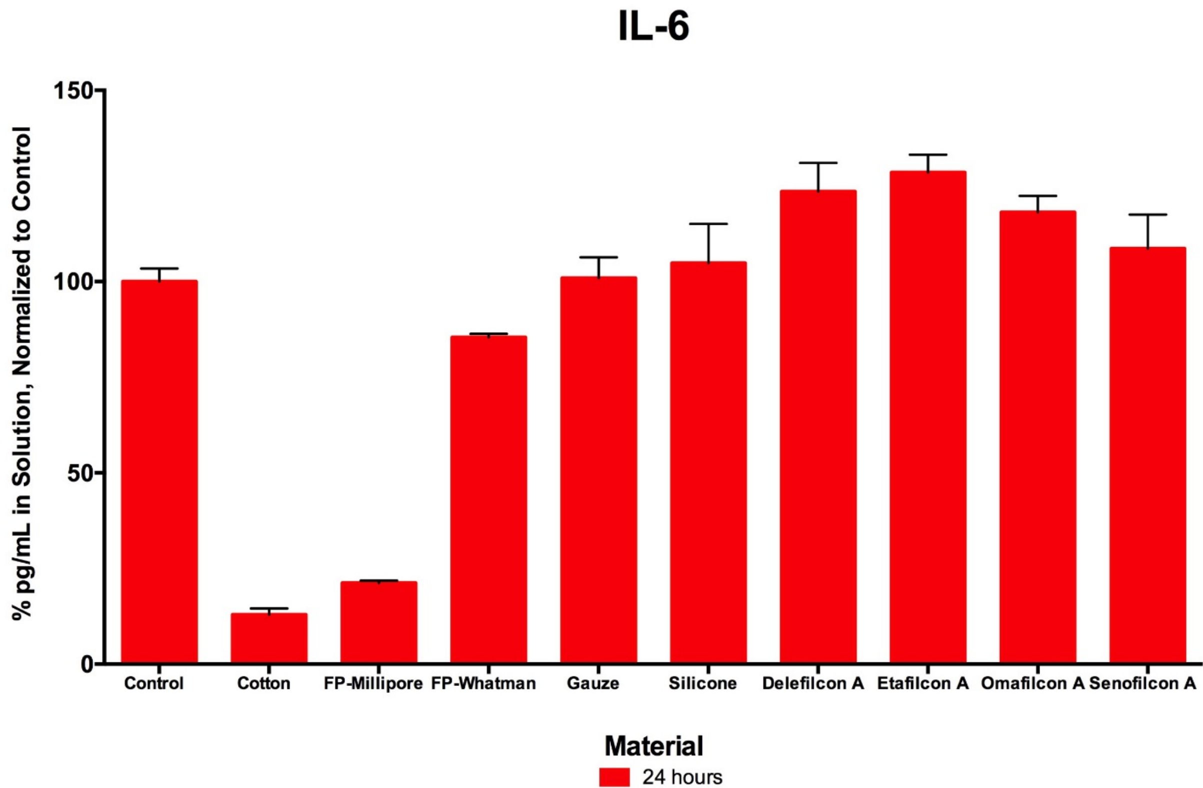

Figure 5-2: Percent pg/mL of IL-6 Remaining in Individual Solutions (n = 3) of Nine Different Biomaterials at 24 Hours.

Relative to the control after 24 hours (100%), there appeared to be little uptake of IL-6 (Figure 5-2) by Whatman filter paper (85 ± 0.9 %), gauze (100 ± 5 %) and silicone sheeting (105 ± 10 %) materials, as well as by senofilcon A (109 ± 9 %). There were no statistical differences ($p > 0.05$) between these materials and the control (Figure 5-6). Delefilcon A (123 ± 7 %, adjusted P-value 0.0019), etafilcon A (128 ± 5 %, adjusted P-value 0.0002) and omafilcon A (118 ± 4 %, adjusted P-value 0.0241), also did not exhibit uptake (Figure 5-2), even though they were statistically different from the control after 24 hours, due to having values above 100% of the control (Figure 5-6). Cotton (13 ± 2 %, adjusted P-value < 0.0001) and Millipore filter paper (21 ± 0.6 %, adjusted P-value < 0.0001) materials did exhibit great uptake (Figure 5-2) and were statistically different from the control after 24 hours (Figure 5-6).

Relative to the control after 24 hours (100%), there appeared to be little uptake of IL-8 (Figure 5-3) by silicone sheeting (81 ± 29 %) and delefilcon A (70 ± 15 %), omafilcon A (70 ± 1 %) and senofilcon A (70 ± 14 %). There were no statistical differences ($p > 0.05$) between these materials and the control (Figure 5-7). Cotton (13 ± 2 %, adjusted P-value < 0.0001), Millipore filter paper (40 ± 2%, adjusted P-value 0.0001), Whatman filter paper (50 ± 4 %, adjusted P-value 0.0009) and gauze (59 ± 3 %, adjusted P-value 0.0074) materials, as well as etafilcon A (58 ± 4 %, adjusted P-value 0.0065) exhibited greater uptake (Figure 5-3) and were all statistically different from the control after 24 hours (Figure 5-7).

Relative to the control after 24 hours (100%), there appeared to be little uptake of TNF-α (Figure 5-4) by Whatman filter paper (70 ± 10 %), gauze (86 ± 6 %) and silicone sheeting (87 ± 22 %) materials, as well as by delefilcon A (66 ± 30%), omafilcon A (76 ± 10 %) and senofilcon A (72 ± 1 %). There were no statistical differences ($p > 0.05$) between these materials and the control (Figure 5-8). In comparison, cotton (22 ± 2 %, adjusted P-value < 0.0001), Millipore filter

paper (38 ± 9 %, adjusted P-value 0.0007) and etafilcon A (54 ± 7 %, adjusted P-value 0.0157), exhibited greater uptake (Figure 5-4) and were all statistically different from the control after 24 hours (Figure 5-8).

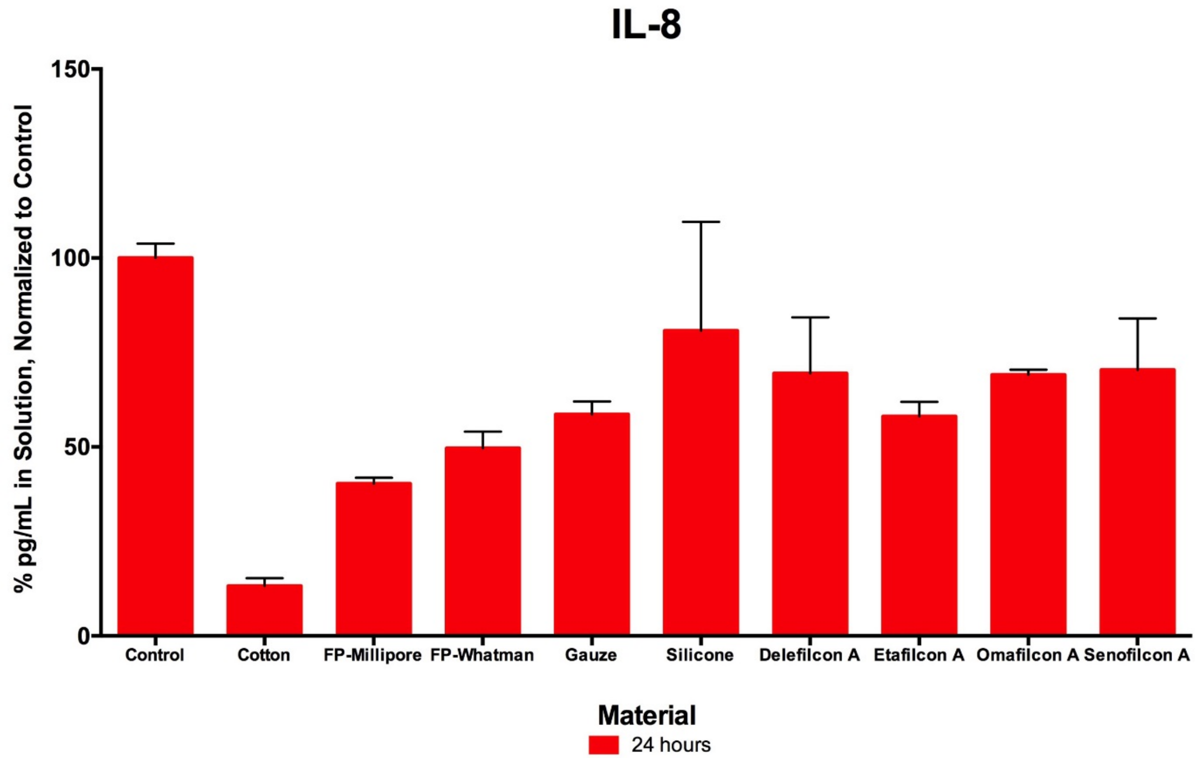

Figure 5-3: Percent pg/mL of IL-8 Remaining in Individual Solutions (n = 3) of Nine Different Biomaterials at 24 Hours.

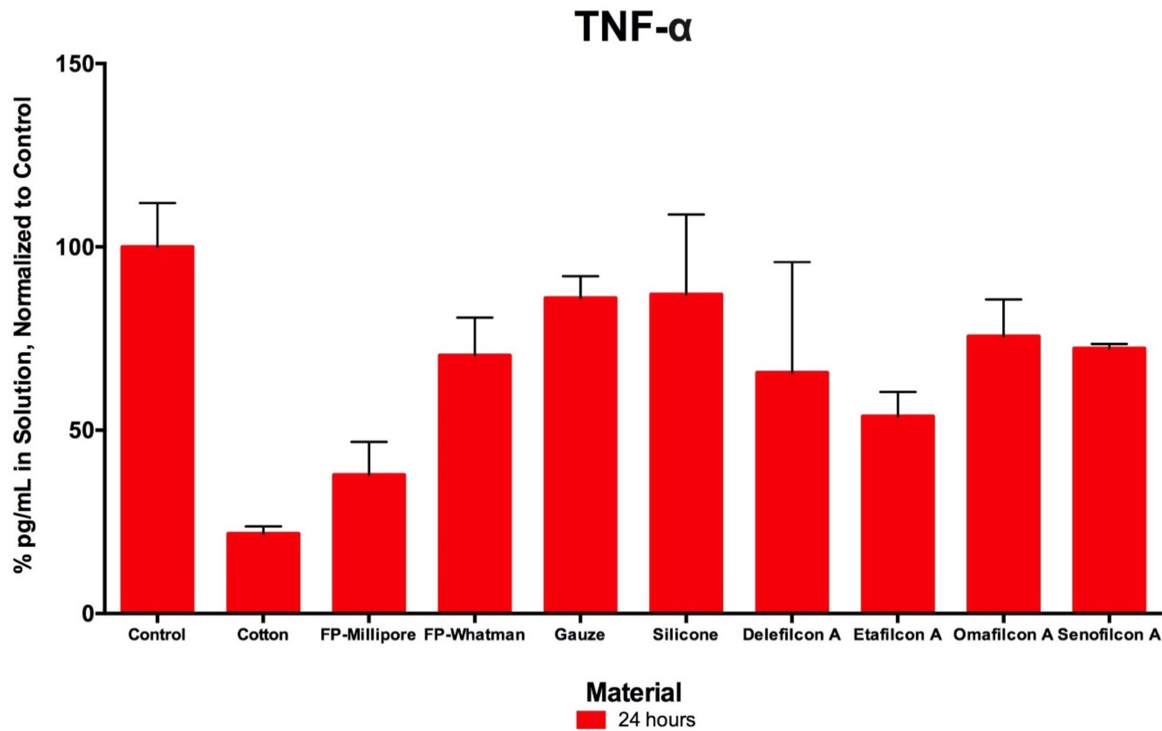

Figure 5-4: Percent pg/mL of TNF-α Remaining in Individual Solutions (n = 3) of Nine Different Biomaterials at 24 Hours.

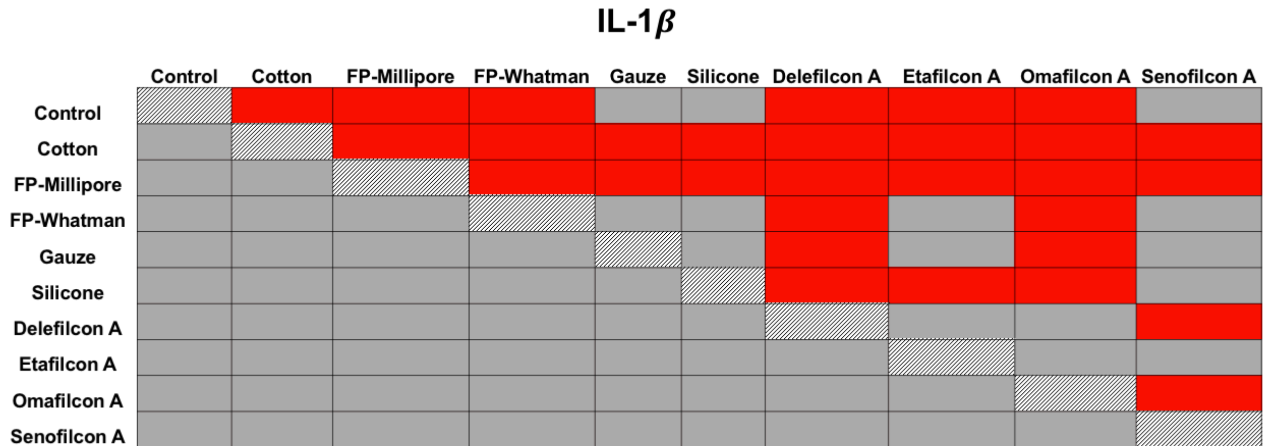

Figure 5-5: Results of Statistical Analyses (Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test), for the Data Presented in Figure 5-1: (Percent pg/mL of IL-1β Remaining in Individual Solutions (n = 3) of Nine Different Biomaterials at 24 Hours). Differences were Considered Significant if $p < 0.05$. "FP" = Filter Paper; Solid Red = Statistical Difference; Solid Grey = No Statistical Difference ($p > 0.05$).

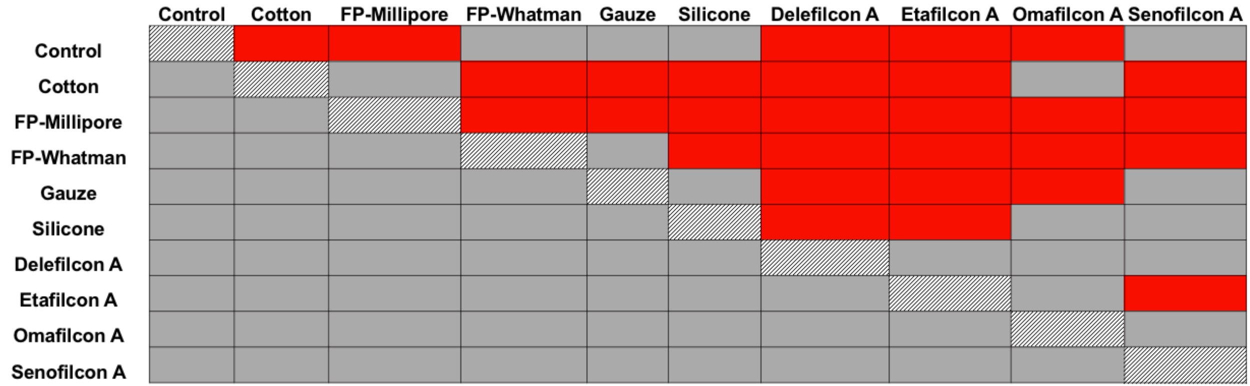

Figure 5-6: Results of Statistical Analyses (Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test), for the Data Presented in Figure 5-2: (Percent pg/mL of IL-6 Remaining in Individual Solutions (n = 3) of Nine Different Biomaterials at 24 Hours). Differences were Considered Significant if p < 0.05. "FP" = Filter Paper; Solid Red = Statistical Difference; Solid Grey = No Statistical Difference (p > 0.05).

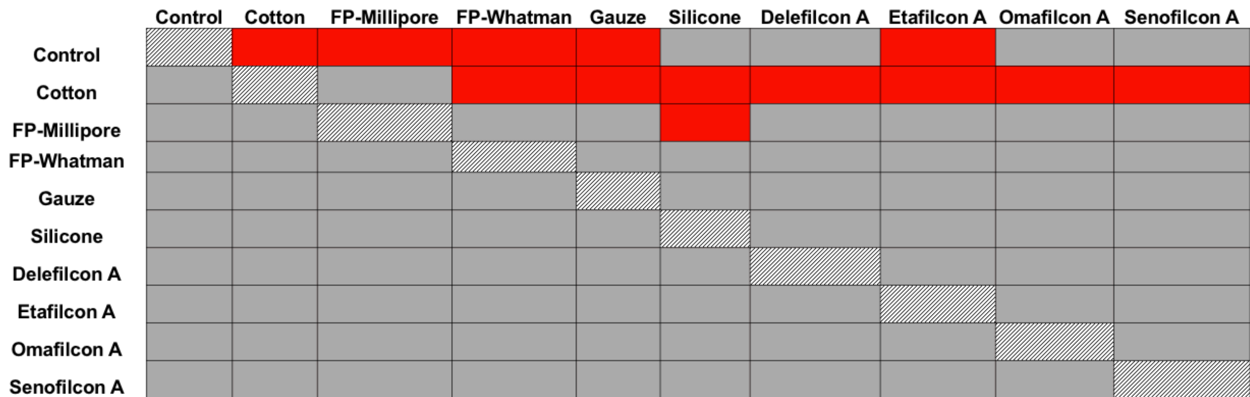

Figure 5-7: Results of Statistical Analyses (Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test) for the Data Presented in Figure 5-3: (Percent pg/mL of IL-8 Remaining in Individual Solutions (n = 3) of Nine Different Biomaterials at 24 Hours). Differences were Considered Significant if p < 0.05. "FP" = Filter Paper; Solid Red = Statistical Difference; Solid Grey = No Statistical Difference (p > 0.05).

TNF-α

	Control	Cotton	FP-Millipore	FP-Whatman	Gauze	Silicone	Delefilcon A	Etafilcon A	Omafilcon A	Senofilcon A
Control										
Cotton										
FP-Millipore										
FP-Whatman										
Gauze										
Silicone										
Delefilcon A										
Etafilcon A										
Omafilcon A										
Senofilcon A										

Figure 5-8: Results of Statistical Analyses (Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test) for the Data Presented in Figure 5-4: (Percent pg/mL of TNF-α Remaining in Individual Solutions ($n = 3$) of Nine Different Biomaterials at 24 Hours). Differences were Considered Significant if $p < 0.05$. "FP" = Filter Paper; Solid Red = Statistical Difference; Solid Grey = No Statistical Difference ($p > 0.05$).

5.1.4 Introduction: Pilot Study #2

The second pilot study aimed to investigate the uptake of IL-1β, IL-6, IL-8 and TNF-α by balafilcon A (PureVision), comfilcon A (Biofinity), polymacon A (SofLens 38) materials and Schirmer tear test strips (White Ophthalmics, Calgary, AB). Of the biomaterials tested in pilot study #1, cotton and silicone materials were incorporated into the study design of pilot study #2, along with etafilcon A (1-Day Acuvue Moist) and senofilcon A (Acuvue Oasys 1-Day with HydraLuxe Technology). Schirmer strips were investigated due to their clinical application and their use in the literature for collecting human tear samples to quantify cytokines or matrix metalloproteinases.[78]

Filter papers, gauze, delefilcon A and omafilcon A were not tested in the second pilot study. Delefilcon A contact lens material is unique due to having a water gradient that transitions from 33% at the silicone hydrogel core to 80% at the non-silicone hydrogel lens surface.[99] As a result of having both a silicone core and a non-silicone surface, this material was excluded from further studies, as it would complicate the investigations when attempting to determine whether SiHy or conventional hydrogel materials uptake cytokines differently. Polymacon A was added to contrast etafilcon A, both of which are conventional hydrogel materials, however, since etafilcon A has high water content and is ionic, polymacon A was chosen for its low water content and nonionic nature (Table 3-4).[55] Comfilcon A and balafilcon A were chosen from two different subgroups of silicone hydrogel lenses (Table 2-2).[55] They are both lenses of low water content, though comfilcon A is nonionic (FDA group V B-1), and balafilcon A is ionic (FDA group V D).[55] Senofilcon A contrasts balafilcon A in terms of ionicity and contrasts comfilcon A in terms of water content.[55] Additionally, during the first pilot study, all four contact lenses tested were daily disposables. Thus, the addition of SofLens 38 (polymacon A), Biofinity (comfilcon A) and

PureVision (balafilcon A), added variety for wear modality, as all three of these contact lenses can be worn on a reusable basis.

5.1.5 Materials and Methods: Pilot Study #2

The silicone sheeting was cut into 10.5 mm pieces using a circular lens punch, cotton was cut from the tips of Puritan 3-inch cotton tip applicators and the Schirmer tear test strips were cut into 0.5 cm x 1.0 cm rectangular pieces. All materials were autoclaved (except the Schirmer strips which are provided in sterile packaging, and the contact lenses which are provided in sterile blister packs), and the contact lenses were soaked in PBS, as described in section 4.3. The cytokine solution was prepared from the MSD calibrator blend, as also described in section 4.3 (except PBS was again utilized rather than Diluent 2), and contained IL-1β, IL-6, IL-8 and TNF-α at concentrations of 16 pg/mL, 20 pg/mL, 15 pg/mL and 10 pg/mL, respectively. Similar concentrations to the first pilot study were chosen, which also fell within the approximate lower range of the values reported in the literature (Table 3-3).

All materials were placed in wells of 24-well Greiner Cellstar polystyrene plates and 1 mL of the prepared cytokine solution was added to each well. Control wells contained only 1 mL of the cytokine solution and no contact lenses or other membranes. The samples in each well were evaluated for cytokine uptake after 24 hours of incubation at 32℃ and 25 rpm for a dynamic environment in the Innova 4300 Incubator Shaker (New Brunswick Scientific, Edison, NJ). In contrast to the first pilot study, in the second pilot study, the biomaterials were incubated at eye temperature rather than at body temperature, as the investigation mainly incorporated ophthalmic biomaterials, rather than equally investigating biomaterials used elsewhere in the body.

5.1.6 Results: Pilot Study #2

After 24 hours, the mean cytokine concentrations obtained from the control wells, as quantified by the MSD, were 14 pg/mL, 14 pg/mL, 10.5 pg/mL and 6 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. Results are summarized in Figure 5-9 to Figure 5-12, and a summary of the statistical analyses are summarized in Figure 5-13 to Figure 5-16. For IL-1β, IL-8 and TNF-α, Welch's ANOVAs with T2 Multiple Comparisons test were performed, while for IL-6, an ordinary one-way ANOVA with Tukey's Multiple Comparisons test was performed. Differences were considered significant if $p < 0.05$.

Relative to the control after 24 hours (100%), there appeared to be little uptake of IL-1β (Figure 5-9) by Schirmer tear test strips (96 $\pm$ 5 %) and silicone sheeting (99 $\pm$ 3 %) materials, as well as by etafilcon A (99 $\pm$ 3 %), polymacon A (92 $\pm$ 7 %) and senofilcon A (97 $\pm$ 3%). There were no statistical differences ($p > 0.05$) between these materials and the control (Figure 5-13). In comparison, cotton (24 $\pm$ 7 %, adjusted P-value < 0.0001), balafilcon A (92 $\pm$ 4 %, adjusted P-value 0.0165) and comfilcon A (89 $\pm$ 3 %, adjusted P-value 0.0004), exhibited greater uptake (Figure 5-9) and were all statistically different from the control after 24 hours (Figure 5-13).

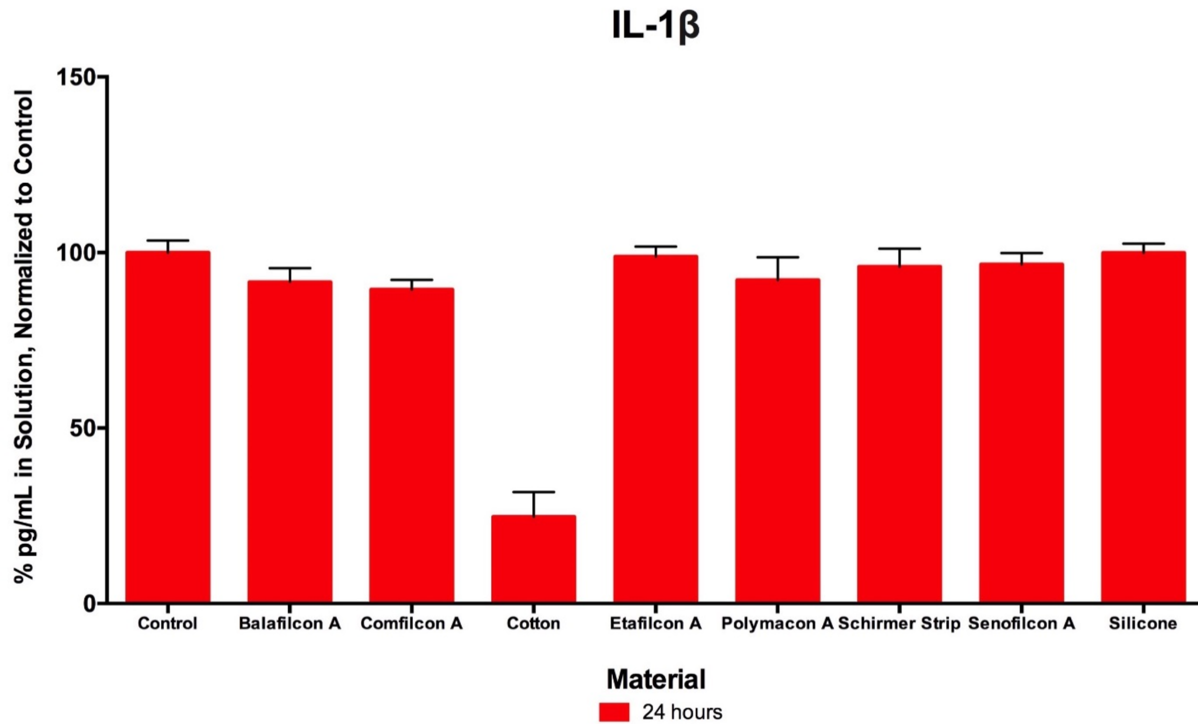

Figure 5-9: Percent pg/mL of IL-1β Remaining in Individual Solutions (n = 8) of Eight Different Biomaterials at 24 Hours.

Relative to the control after 24 hours (100%), there appeared to be little uptake of IL-6 (Figure 5-10) by silicone sheeting (99 ± 6 %) and balafilcon A (109 ± 7 %), comfilcon A (103 ± 5 %), polymacon A (109 ± 10 %) and senofilcon A (105 ± 4 %). There were no statistical differences ($p > 0.05$) between these materials and the control (Figure 5-14). Etafilcon A (116 ± 6%, adjusted P-value 0.0019) also did not exhibit uptake (Figure 5-10), even though it was statistically different from the control after 24 hours, due to having a value above 100% of the control (Figure 5-14). Cotton (14 ± 5 %, adjusted P-value < 0.0001) and Schirmer tear test strips (70 ± 12 %, adjusted P-value < 0.0001), exhibited greater uptake (Figure 5-10) and were statistically different from the control after 24 hours (Figure 5-14).

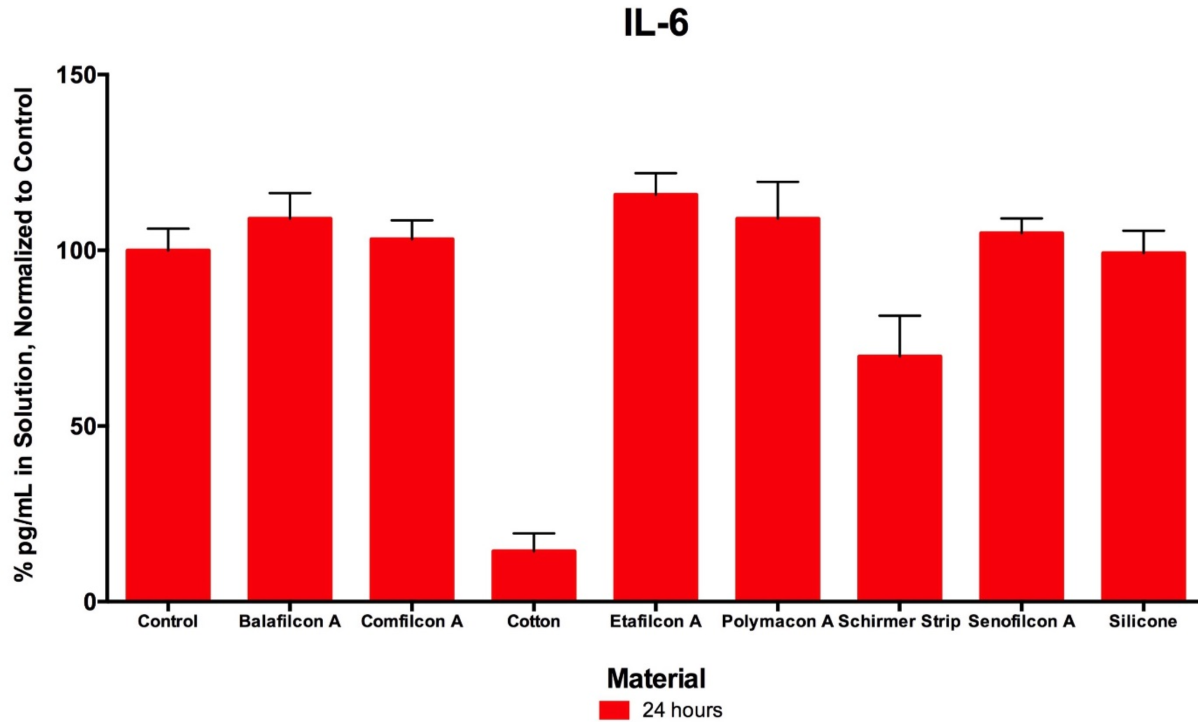

Figure 5-10: Percent pg/mL of IL-6 Remaining in Individual Solutions (n = 8) of Eight Different Biomaterials at 24 Hours.

Relative to the control after 24 hours (100%), there appeared to be little uptake of IL-8 (Figure 5-11) by etafilcon A (104 ± 5 %) and senofilcon A (96 ± 4 %), as there were no statistical differences ($p > 0.05$) between these materials and the control (Figure 5-15). Cotton (16 ± 3 %, adjusted P-value < 0.0001), Schirmer tear test strips (71 ± 9 %, adjusted P-value 0.0002) and silicone sheeting (81 ± 5 %, adjusted P-value 0.0004) materials, as well as balafilcon A (64 ± 19 %, adjusted P-value 0.0277), comfilcon A (61 ± 17 %, adjusted P-value 0.0068) and polymacon A (63 ± 17 %, adjusted P-value 0.0105), exhibited greater uptake (Figure 5-11) and were all statistically different from the control after 24 hours (Figure 5-15).

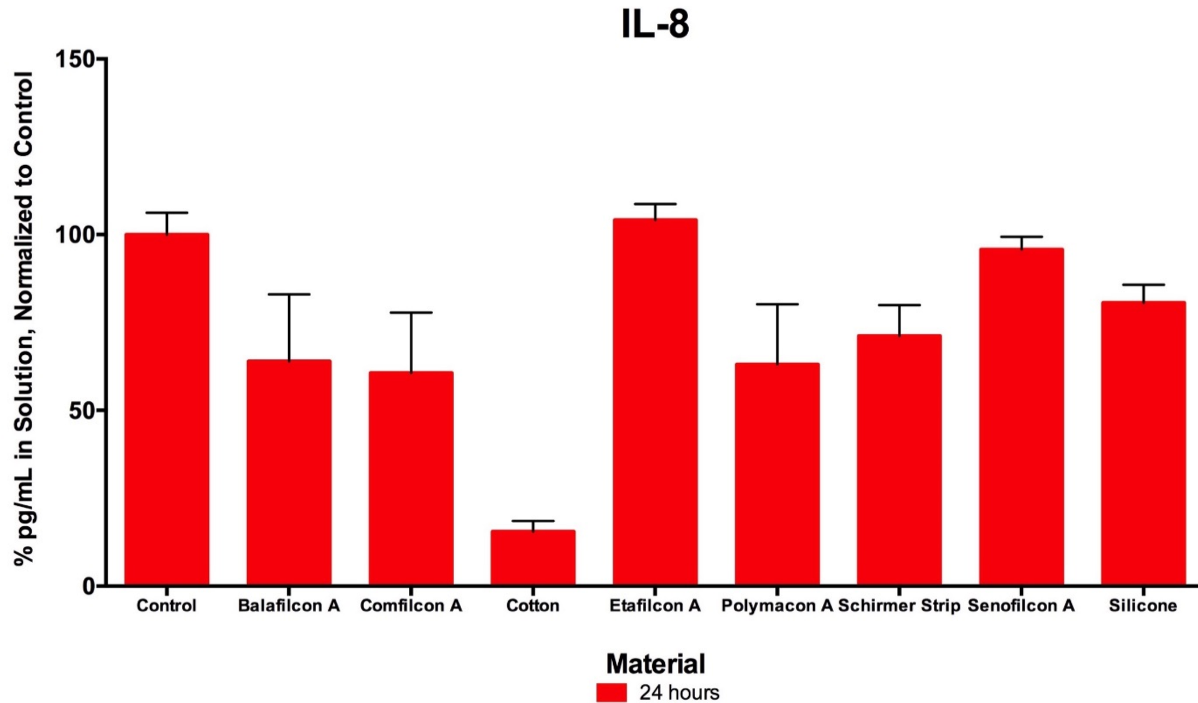

Figure 5-11: Percent pg/mL of IL-8 Remaining in Individual Solutions (n = 8) of Eight Different Biomaterials at 24 Hours.

Relative to the control after 24 hours (100%), there appeared to be little uptake of TNF-α (Figure 5-12) only by the silicone sheeting (86 $\pm$ 8 %), as there was no statistical difference ($p > 0.05$) observed between this material and the control (Figure 5-16). Cotton (14 $\pm$ 2 %, adjusted P-value < 0.0001) and Schirmer tear test strips (64 $\pm$ 10 %, adjusted P-value < 0.0001), along with balafilcon A (64 $\pm$ 17 %, adjusted P-value 0.0124), comfilcon A (50 $\pm$ 15 %, adjusted P-value 0.0002), etafilcon A (59 $\pm$ 7 %, adjusted P-value < 0.0001), polymacon A (58 $\pm$ 15 %, adjusted P-value 0.0011) and senofilcon A (82 $\pm$ 6 %, adjusted P-value 0.0035), all exhibited greater uptake (Figure 5-12) and were statistically different from the control after 24 hours (Figure 5-16).

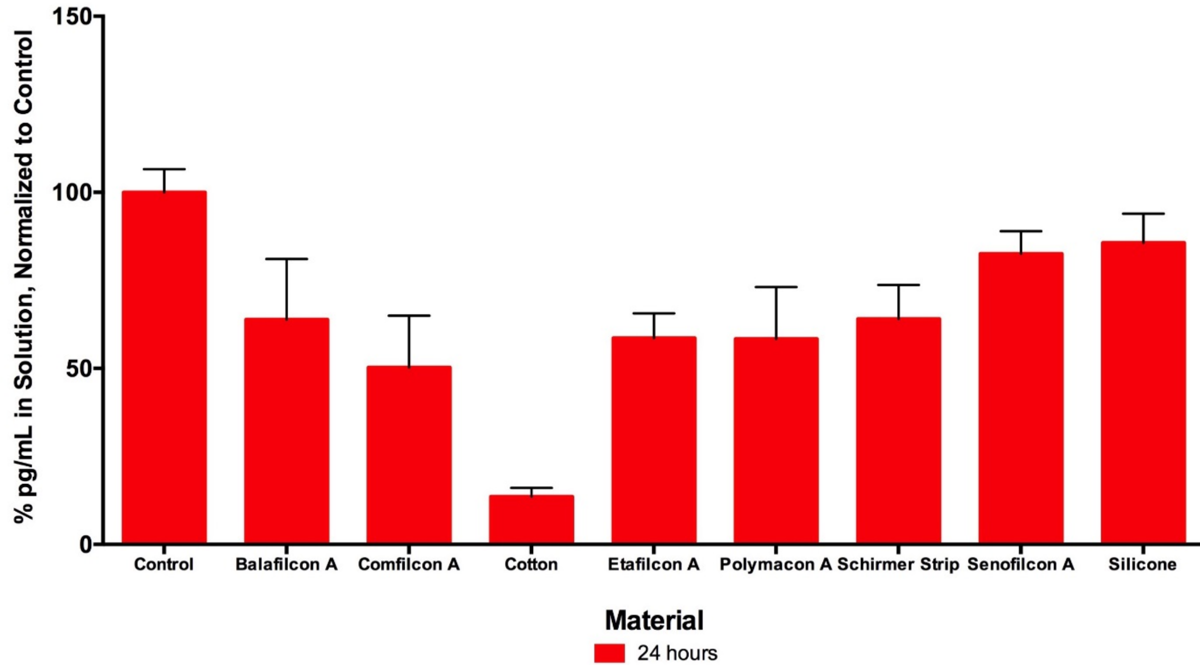

Figure 5-12: Percent pg/mL of TNF-α Remaining in Individual Solutions (n = 8) of Eight Different Biomaterials at 24 Hours.

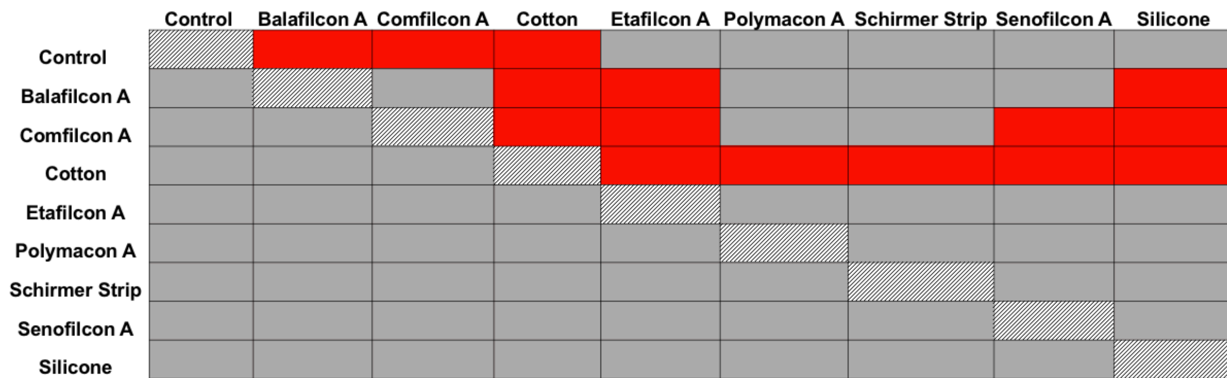

Figure 5-13: Results of Statistical Analyses (Welch's ANOVAs with T2 Multiple Comparisons Test), for the Data Presented in Figure 5-9: (Percent pg/mL of IL-1β Remaining in Individual Solutions (n = 8) of Eight Different Biomaterials at 24 Hours). Differences were Considered Significant if p < 0.05. Solid Red = Statistical Difference; Solid Grey = No Statistical Difference (p > 0.05).

IL-6

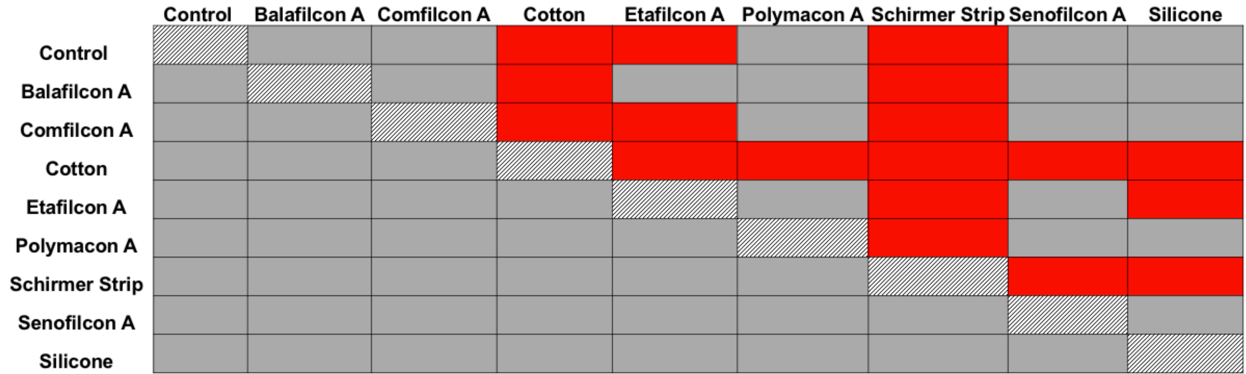

Figure 5-14: Results of Statistical Analyses (Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test), for the Data Presented in Figure 5-10: (Percent pg/mL of IL-6 Remaining in Individual Solutions (n = 8) of Eight Different Biomaterials at 24 Hours). Differences were Considered Significant if p < 0.05. Solid Red = Statistical Difference; Solid Grey = No Statistical Difference (p > 0.05).

IL-8

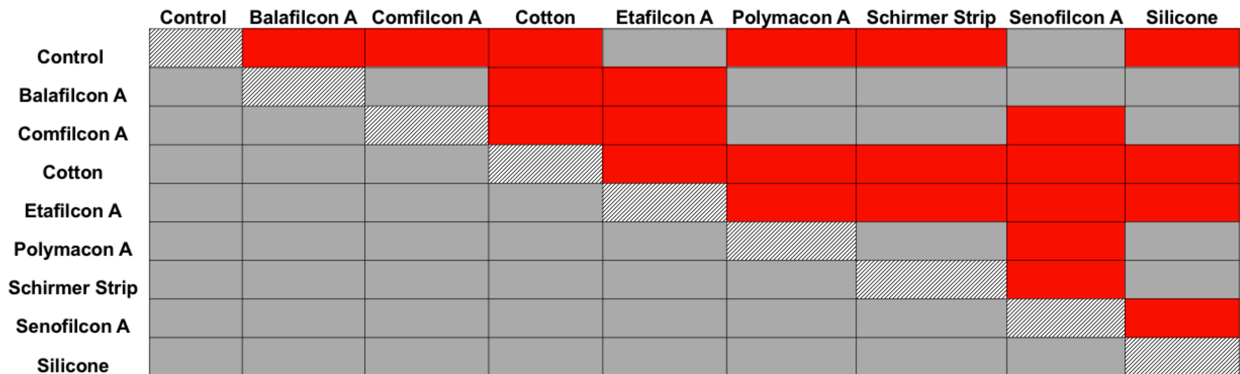

Figure 5-15:Results of Statistical Analyses (Welch's ANOVAs with T2 Multiple Comparisons Test), for the Data Presented in Figure 5-11: (Percent pg/mL of IL-8 Remaining in Individual Solutions (n = 8) of Eight Different Biomaterials at 24 hours). Differences were considered significant if p < 0.05. Solid Red = Statistical Difference; Solid Grey = No Statistical Difference (p > 0.05).

TNF-α

Figure 5-16: Results of Statistical Analyses (Welch's ANOVAs with T2 Multiple Comparisons Test), for the Data Presented in Figure 5-12: (Percent pg/mL of TNF-α Remaining in Individual Solutions (n = 8) of Eight Different Biomaterials at 24 Hours). Differences were Considered Significant if $p < 0.05$. Solid Red = Statistical Difference; Solid Grey = No Statistical Difference ($p > 0.05$).

5.1.7 Discussion: Pilot Study #1 and Pilot Study #2

In the first pilot study, silicone sheeting and senofilcon A (Acuvue Oasys 1-Day with HydraLuxe Technology), consistently exhibited no uptake of any of the four cytokines of interest, while cotton and Millipore filter paper exhibited uptake for all four cytokines (Figure 5-5 to Figure 5-8). In the second pilot study, silicone sheeting only exhibited uptake for IL-8 that was statistically different from the IL-8 control, while senofilcon A only exhibited uptake of TNF-α that was statistically different from the TNF-α control. Uptake by the other biomaterials in both pilot studies varied between the cytokines; however, balafilcon A and comfilcon A appeared to uptake higher amounts of IL-1β, IL-8 and TNF-α than IL-6. In both pilot studies, cotton consistently exhibited the highest amount of uptake for all cytokines; however, its ability to serve as an ideal positive control may be negated by its ability to also absorb a volume of the soaking solution. Although not tested in the second pilot study, Millipore filter paper also exhibited substantial uptake of all four cytokines, and since filter paper is much less liquid absorbent than cotton, it may therefore have served as a better positive control to validate the methodology used. Conversely, silicone

sheeting and perhaps senofilcon A, appeared to be good negative controls as materials that resisted cytokine uptake.

Of concern were the cytokine concentrations measured for the controls after 24 hours in both pilot studies. Recall, in pilot study #1, experimental control concentrations were 18 pg/mL, 15 pg/mL, 9 pg/mL and 3.5 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively, which were lower than the theoretical concentrations of 19 pg/mL, 23 pg/mL, 18.5 pg/mL and 12 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. In pilot study #2, experimental control concentrations were 14 pg/mL, 14 pg/mL, 10.5 pg/mL and 6 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively, which were also lower than the theoretical concentrations of 16 pg/mL, 20 pg/mL, 15 pg/mL and 10 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. This suggested either an unstable cytokine solution as a result of utilizing PBS rather than the MSD's Diluent 2, potential adhesion of the cytokines to the sides of the polystyrene wells, or cytokine denaturation over the 24-hour uptake period as a result of the incubation temperatures affecting protein stability. For this reason, definite comparisons could not be drawn between the contact lens materials and their ability to uptake cytokines. For example, concluding that one contact lens material could uptake one type of cytokine but not another would be inaccurate, as perhaps there was greater adhesion of that cytokine to the sides of the container, or more denaturation, making the contact lens material appear to have exhibited greater uptake, when in reality, the cytokine may not have been properly quantified by the MSD assay in its entirety as a result. Accordingly, the next set of experiments aimed to investigate any differences between PBS and Diluent 2, the material of the soaking container, and any effect of the incubation temperature on cytokine uptake. Furthermore, an investigation was also carried out to determine whether the results observed which indicated

greater than 100% pg/mL cytokines in solution, was at all due to any component of a contact lens eluting out into solution and then potentially mimicking cytokines.

5.2 Investigating Potential Signal Interference by Contact Lenses in the MSD Assay and Differences in Diluent 2 and PBS, High-Density Polyethylene and Polypropylene

5.2.1 Introduction: Pilot Study #3

Although Diluent 2 is a proprietary PBS-based solution according to Meso Scale Discovery, it also contains other constituents in its recipe, and it was therefore of interest to investigate whether creating the cytokine solution utilizing Diluent 2 or PBS would affect the stability of the cytokines over time. Additionally, rather than using polystyrene plates, it was of interest to investigate whether the use of polypropylene tubes or high-density polyethylene tubes would produce more favourable outcomes in terms of preventing potential adhesion of the cytokines to the sides of the container. High-density polyethylene tubes had previously been used in the laboratory for experiments involving protein extraction from contact lenses, whereas many of the items in the MSD assay kits were supplied in polypropylene tubes, suggesting a customary use of this material for experiments involving cytokines.

5.2.2 Materials and Methods: Pilot Study #3

An experiment was designed with 6 mL high-density polyethylene (HDPE) tubes (Fisher Scientific, Hampton, NH) and 5 mL conical-bottom polypropylene tubes (VWR, Radnor, PA), each containing 1 mL of cytokine solution prepared either in Diluent 2 or PBS (n = 3). Samples were collected from each tube after a half-hour time period and cytokine concentrations were quantified using the MSD assay. This time period was chosen as it was hypothesized that any adhesion to the sides of the materials could happen relatively quickly in the presence of a cytokine solution. The tubes were left at room temperature on the VWR Advanced 3500 orbital shaker to

ensure a dynamic environment, but were not incubated at either 32℃ or 37℃, as the investigation aimed to solely evaluate the materials without any potential influence of temperature.

5.2.3 Results: Pilot Study #3

The concentrations of the cytokines in the prepared solutions were 28.05 pg/mL, 37.5 pg/mL, 30.8 pg/mL and 17.3 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. Results are summarized in Figure 5-17 to Figure 5-20. In Diluent 2, the concentration of IL-1β following the half-hour uptake period was 26.98 pg/mL in HDPE and 25.84 pg/mL in polypropylene, while in PBS, the concentration of IL-1β was 28.73 pg/mL in HDPE and 25.15 pg/mL in polypropylene. Statistical analysis performed using an ordinary, one-way ANOVA with Tukey's Multiple Comparisons test, where differences were considered significant if $p < 0.05$, revealed Diluent 2 in HDPE to be statistically different from PBS in polypropylene (adjusted P-value 0.0460), as defined by the star (*) symbol, while PBS in HDPE was statistically different from both Diluent 2 in polypropylene (adjusted P-value 0.0039) and PBS in polypropylene (adjusted P-value 0.0010), as defined by the triangle (Δ) symbol.

In Diluent 2, concentrations of IL-6 following the half-hour uptake period were 31.5 pg/mL in HDPE and 32.5 pg/mL in polypropylene, while in PBS, concentrations of IL-6 were 34.1 pg/mL in HDPE and 32.3 pg/mL in polypropylene. Statistical analysis performed using an ordinary, one-way ANOVA with Tukey's Multiple Comparisons test, where differences were considered significant if $p < 0.05$, revealed no statistical differences between any of the experimental conditions for this cytokine.

In Diluent 2, the concentration of IL-8 following the half-hour uptake period was 28.0 pg/mL in both HDPE and in polypropylene, while in PBS, the concentration of IL-8 was 30.3 pg/mL in HDPE and 27.3 pg/mL in polypropylene. Statistical analysis performed using an

ordinary, one-way ANOVA with Tukey's Multiple Comparisons test, where differences were considered significant if $p < 0.05$, revealed no statistical differences between any of the experimental conditions for this cytokine.

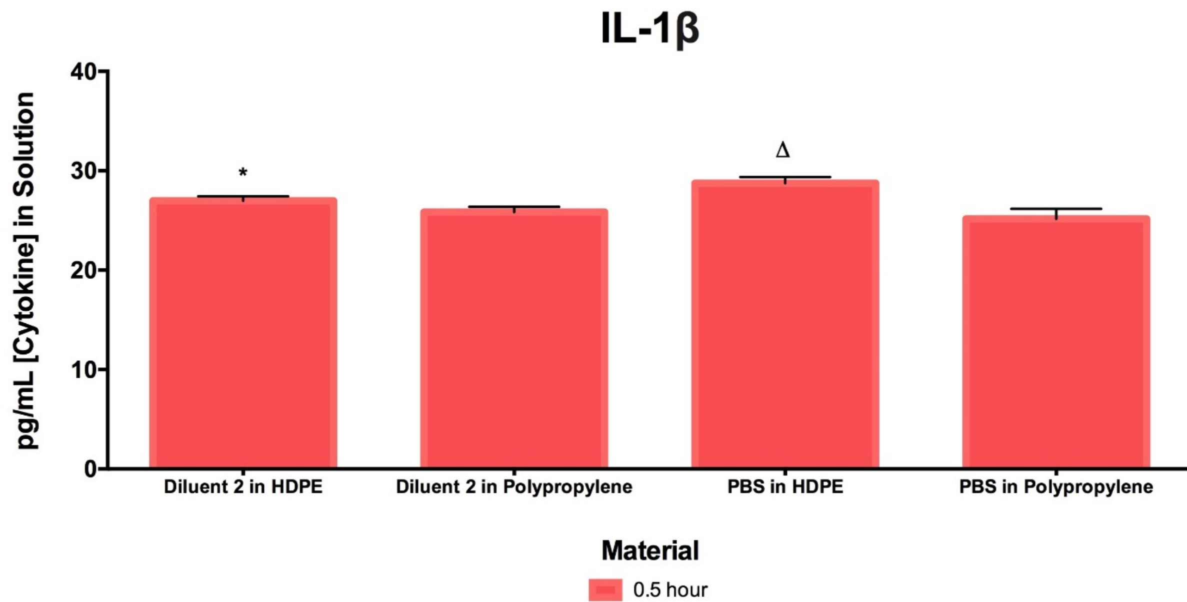

Figure 5-17: pg/mL of IL-1β Remaining in Individual Diluent 2 or PBS Cytokine Solutions (n = 3) in Either HDPE or Polypropylene at 0.5 hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. Diluent 2 in HDPE was Statistically Different from PBS in Polypropylene, as Defined by the Star (*) Symbol. PBS in HDPE was Statistically Different from both Diluent 2 in Polypropylene and PBS in Polypropylene, as Defined by the Triangle (Δ) Symbol.

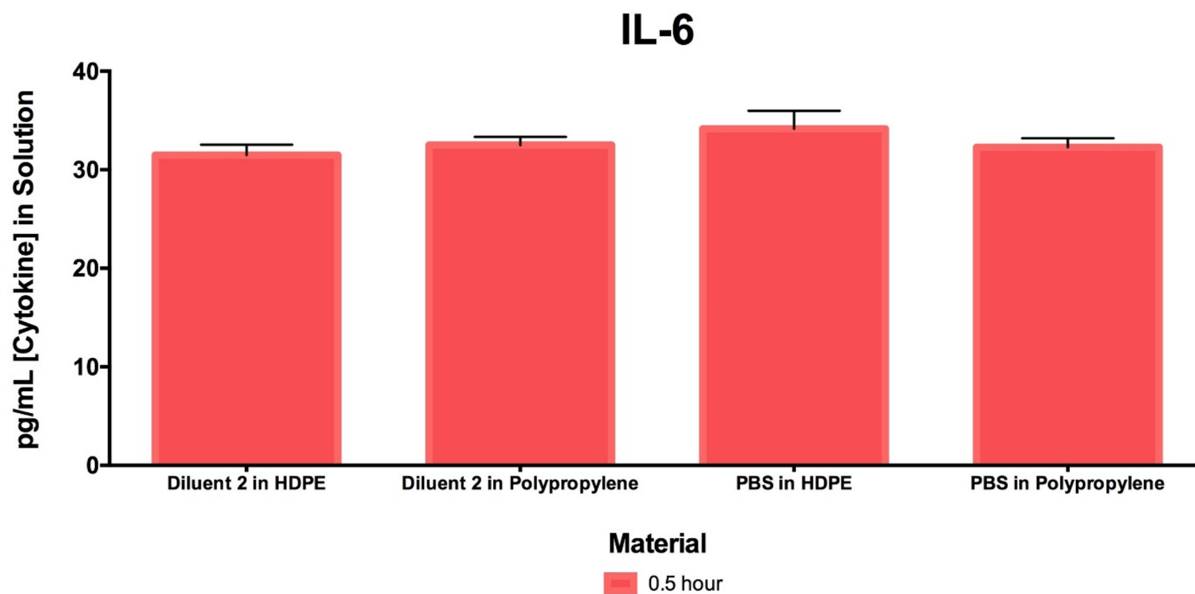

Figure 5-18: pg/mL of IL-6 Remaining in Individual Diluent 2 or PBS Cytokine Solutions (n = 3) in Either HDPE or Polypropylene at 0.5 hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

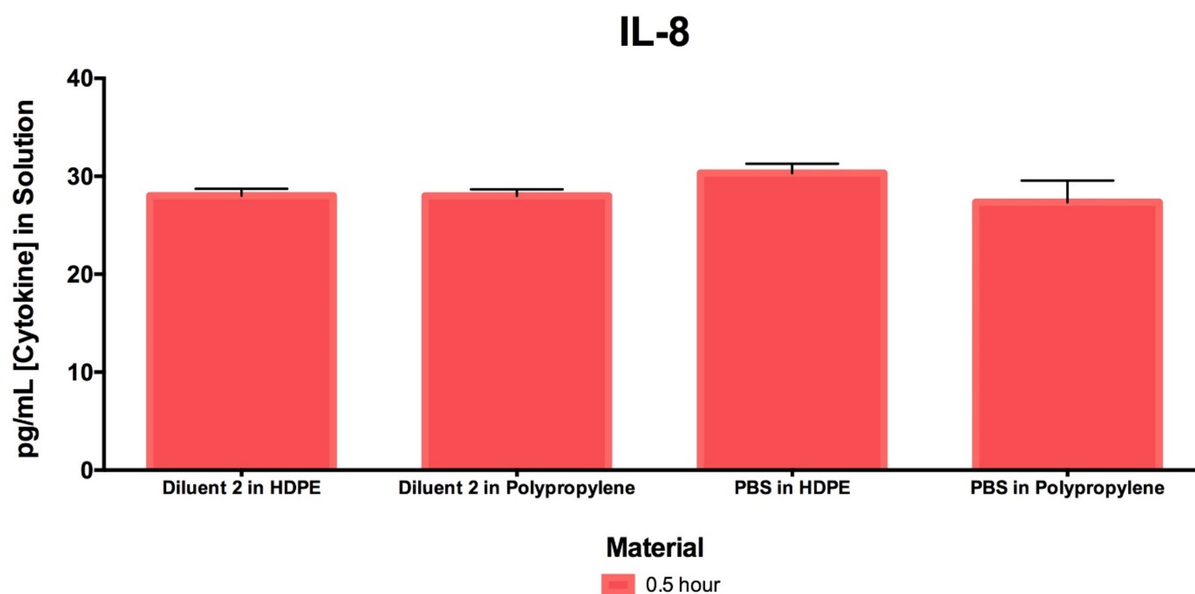

Figure 5-19: pg/mL of IL-8 Remaining in Individual Diluent 2 or PBS Cytokine Solutions (n = 3) in Either HDPE or Polypropylene at 0.5 hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

In Diluent 2, the concentration of TNF-α following the half-hour uptake period was 16.3 pg/mL in HDPE and 15.3 pg/mL in polypropylene, while in PBS, the concentration of IL-8 was 16.3 pg/mL in HDPE and 14.4 pg/mL in polypropylene. Statistical analysis performed using an ordinary, one-way ANOVA with Tukey's Multiple Comparisons test where differences were considered significant if $p < 0.05$, revealed no statistical differences between any of the experimental conditions for this cytokine.

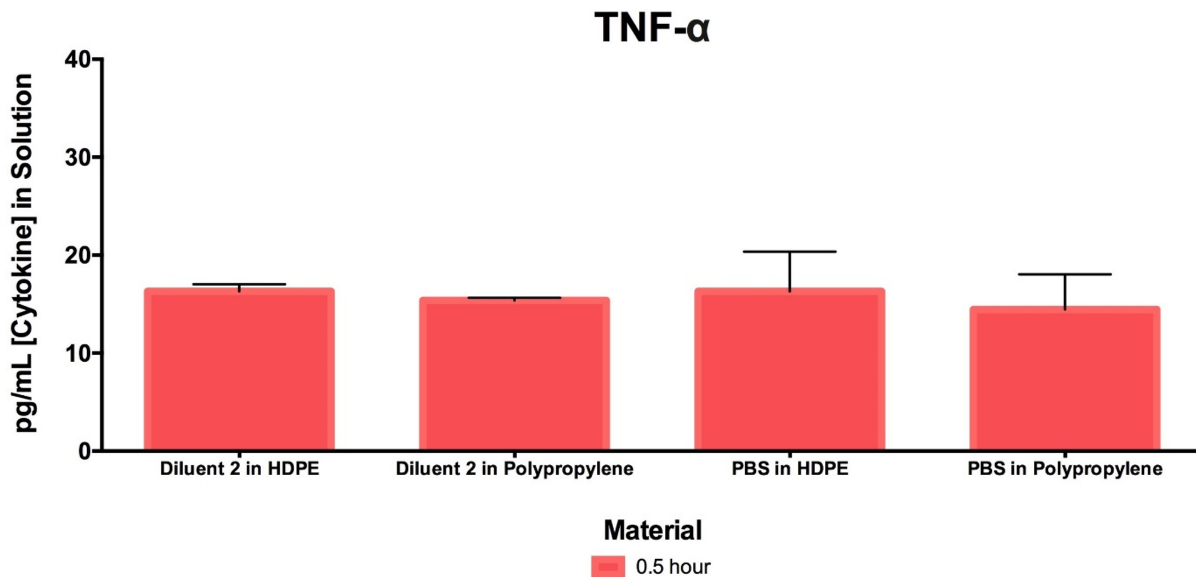

Figure 5-20: pg/mL of TNF-α Remaining in Individual Diluent 2 or PBS Cytokine Solutions (n = 3) in Either HDPE or Polypropylene at 0.5 hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

5.2.4 Discussion: Pilot Study #3

Based on observation from the graphs and the statistical analyses (which did not reveal any differences in most cases), there did not appear to be any great disparities between either the use of Diluent 2 versus PBS or the use of high-density polyethylene tubes versus polypropylene tubes. In all cases, the experimental control cytokine concentrations quantified did deviate to some extent from theoretical concentrations; however, the deviation was substantially less than what was

observed when polystyrene plates were utilized, where control concentrations of some cytokines were sometimes quantified as half the amount that was expected to exist in solution. Ultimately, polystyrene plates were no longer utilized, and polypropylene tubes were chosen as the material of choice.

5.2.5 Introduction: Pilot Study #4

The results of pilot studies #1 and #2, as described in sections 5.1.3 and 5.1.6 above, exhibited some cases where the percent pg/mL of cytokine remaining in solution after the uptake period exceeded 100% of the control value. These results were rather unusual, as it was expected that sample values would either indicate no uptake by having the same percent of cytokines remaining in solution as the control, or there would be some uptake, in which case the percent of cytokines remaining in solution would be lower than 100% of the control. Consequently, a fourth pilot study was carried out as an extension of pilot study #3 and aimed to determine whether there were any differences between soaking a contact lens in a cytokine solution prepared using Diluent 2 or PBS in polypropylene tubes. This was not evident from pilot studies #1 and #2 where cytokine solutions were prepared solely in PBS, and the potential influences of using polystyrene plates and incubating at body/eye temperatures may have affected results. Moreover, this was also not evident from pilot study #3, where only differences between materials and solutions were tested in the absence of any contact lens materials.

5.2.6 Materials and Methods: Pilot Study #4

A cytokine solution was prepared from the MSD calibrator blend, as described in section 4.3 using either Diluent 2 or PBS in polypropylene tubes (n = 6). There were two control conditions whereby the tubes contained either only the Diluent 2 or PBS cytokine solutions and no contact lens, in addition to two experimental conditions whereby the tubes contained either Diluent 2 or

PBS cytokine solutions with etafilcon A (1-Day Acuvue Moist). Samples were collected from each tube after a half-hour time period and cytokine concentrations were quantified using the MSD assay. Etafilcon A was the lens material of choice because this material, in particular, exceeded 100% of the control values in both pilot studies #1 and #2, in comparison to the other contact lens materials tested. Etafilcon A was soaked in PBS to remove packaging solution as described in section 4.3 and dried on lens paper prior to beginning the experiment. A half-hour time period was chosen as it was hypothesized that if any additional components on the lens surface were to elute out into solution, it could happen quickly within this time frame. Tubes were placed on the VWR Advanced 3500 orbital shaker during the duration of the study period in order to ensure a dynamic environment.

5.2.7 Results: Pilot Study #4

The concentrations of the cytokines prepared in the Diluent 2 solution were 28.6 pg/mL, 35.9 pg/mL, 30.2 pg/mL and 16.9 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. The concentrations of the cytokines prepared in the PBS solution were 30.7 pg/mL, 37.5 pg/mL, 34.0 pg/mL and 21.3 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. Results are summarized in Figure 5-21 to Figure 5-24. Theoretical concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.75 pg/mL, 38.25 pg/mL, 29.95 pg/mL and 18.4 pg/mL, respectively.

After a half-hour, there appeared to be no uptake of IL-1β (Figure 5-21) by etafilcon A in either Diluent 2 (98 ± 4 %) or in PBS (100 ± 3 %); no uptake of IL-6 (Figure 5-22) by etafilcon A in either Diluent 2 (102 ± 4 %) or in PBS (104 ± 4 %); no uptake of IL-8 (Figure 5-23) by etafilcon A in either Diluent 2 (100 ± 7 %) or in PBS (103 ± 2 %); and no uptake of TNF-α (Figure 5-24) by etafilcon A in either Diluent 2 (101 ± 6 %) or in PBS (103 ± 5 %). This was in comparison to each respective control for both Diluent 2 and PBS (100%). In each case, statistical analysis

with ordinary, one-way ANOVA and Tukey's Multiple Comparisons test revealed no significant differences between any of the experimental conditions (differences were considered significant if $p < 0.05$).

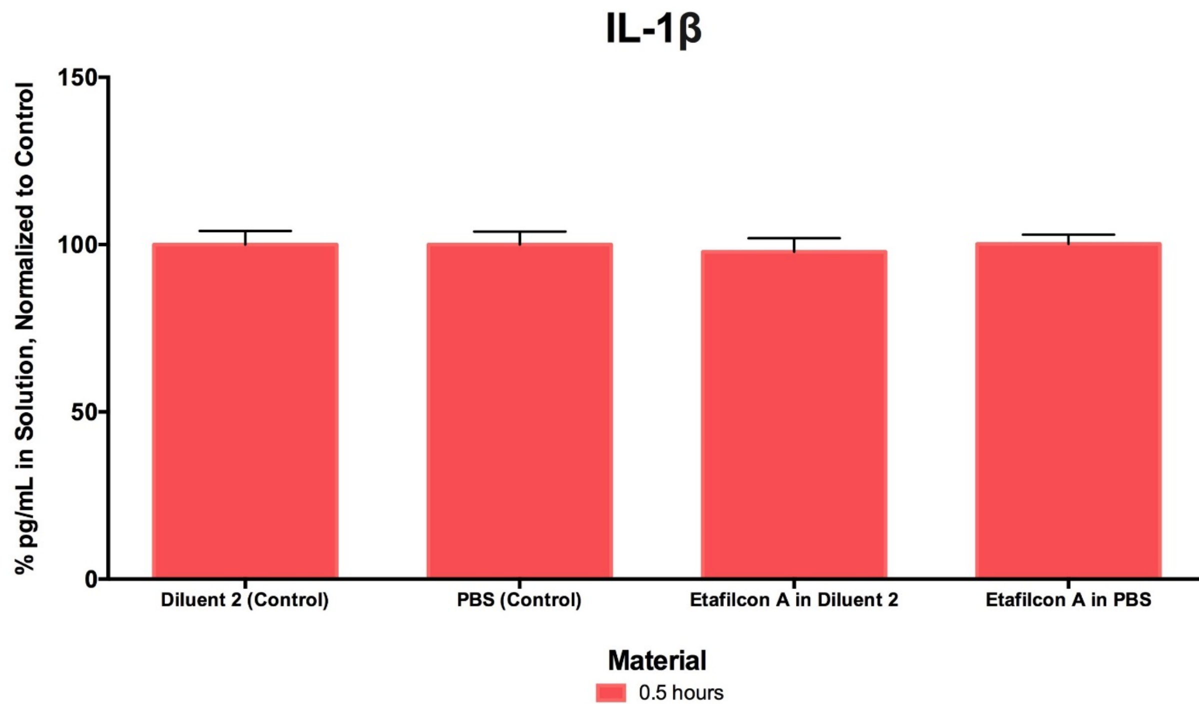

Figure 5-21: Percent pg/mL of IL-1β Remaining in Individual Diluent 2 or PBS Cytokine Solutions (n = 6) in Either HDPE or Polypropylene Tubes Containing Etafilcon A at 0.5 hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

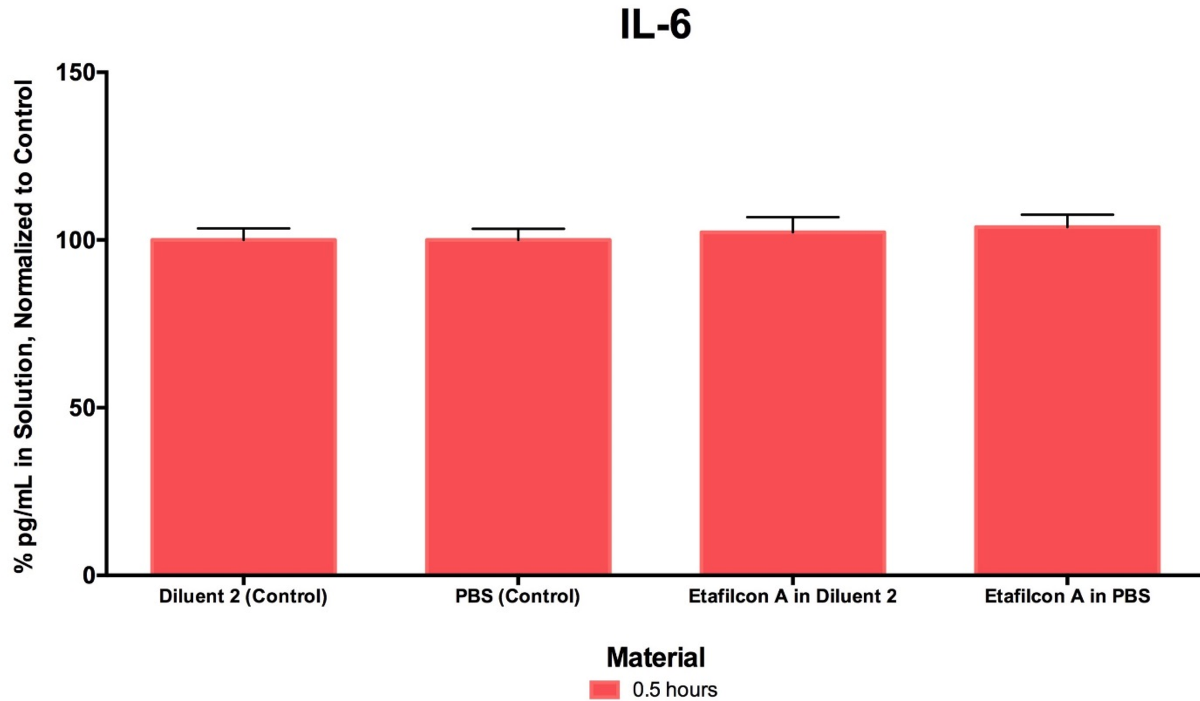

Figure 5-22: Percent pg/mL of IL-6 Remaining in Individual Diluent 2 or PBS Cytokine Solutions (n = 6) in Either HDPE or Polypropylene Tubes Containing Etafilcon A at 0.5 hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

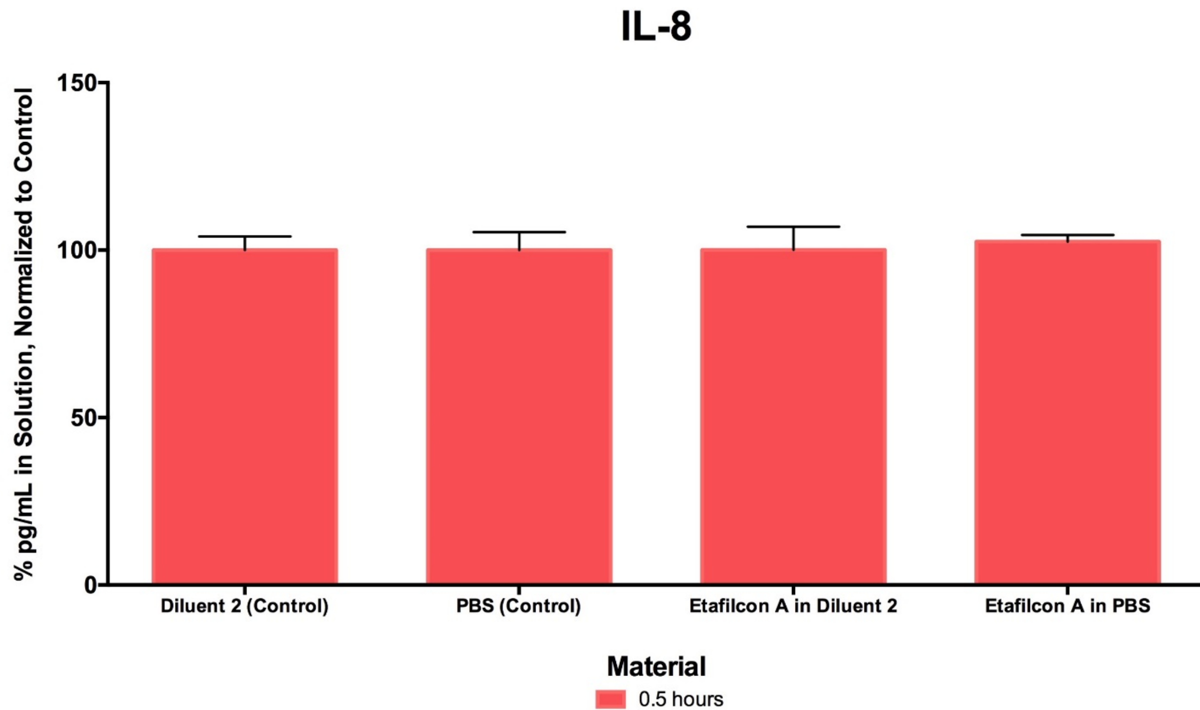

Figure 5-23: Percent pg/mL of IL-8 Remaining in Individual Diluent 2 or PBS Cytokine Solutions (n = 6) in Either HDPE or Polypropylene Tubes Containing Etafilcon A at 0.5 hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

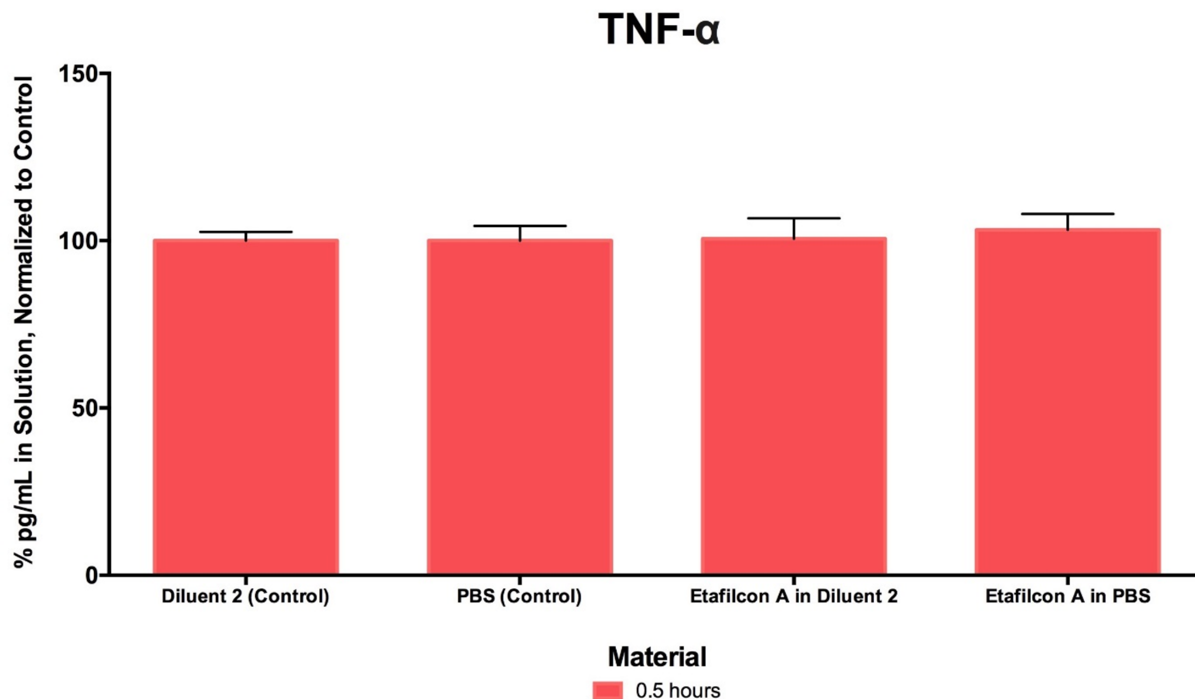

Figure 5-24: Percent pg/mL of TNF-α Remaining in Individual Diluent 2 or PBS Cytokine Solutions (n = 6) in Either HDPE or Polypropylene Tubes Containing Etafilcon A at 0.5 hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

5.2.8 Discussion: Pilot Study #4

Similar to the results obtained in pilot study #3, while there were no statistical differences ($p > 0.05$) between the controls and the experimental conditions in pilot study #4 and while the quantified control concentration of cytokines in either solution of Diluent 2 or PBS were very close in comparison, they did appear to resemble theoretical concentrations slightly more when prepared in Diluent 2 rather than when prepared in PBS. As a result of this and also to maintain consistency with the use of Diluent 2 when running the MSD assays, subsequent experiments utilized Diluent 2 rather than PBS to prepare cytokine solutions.

5.2.9 Discussion: Pilot Study #4 Continued - Additional Experiment

In pilot study #4, similar results were again observed as per pilot studies #1 and #2 for etafilcon A, where the percent pg/mL of cytokines in solution was greater than 100% of the control.

Accordingly, an additional experiment was carried out to determine whether there were any components on the surface of the contact lens materials, although unlikely, that could elute out into solution over the uptake period and interfere with the MSD signal or mimic cytokines such that it would be quantified through the MSD assay and result in higher concentrations of cytokines than expected.

Over a 24-hour period, with the exceptions of delefilcon A (Dailies Total1) and polymacon A (SofLens 38), any soft lens materials utilized in previous experiments, in addition to any soft lens materials expected to be utilized in future experiments, were placed in 1 mL of Diluent 2 in polypropylene tubes. The diluent 2 did not contain any cytokines. This included balafilcon A (PureVision), comfilcon A (Biofinity), etafilcon A (1-Day Acuvue Moist and Acuvue 2), omafilcon A (Proclear 1 Day), senofilcon A (Acuvue Oasys 1-Day with HydraLuxe Technology and Acuvue Oasys 2-week with Hydraclear Plus) and somofilcon A (Clariti 1 Day). As explained in section 5.1.4, delefilcon A would be excluded from future experiments, and polymacon A was also not investigated further, due to being an outdated material that is no longer commonly used.

Prior to beginning the experiment, all materials were soaked in PBS to remove the packaging solution and dried on lens paper, as described in section 4.3. Following the 24-hour time point, a sample was collected from each tube and analyzed using the MSD assay. Results are not shown because for all of the contact lenses tested, there was either minimal interference or no interference at all to be reported (in which case the signals were extremely low and below the MSD instrument's lower limit of quantification).

Given that cytokine concentrations in solution were sometimes quantified to be above 100% of the control regardless of the type of material used to soak the contact lenses, or whether Diluent 2 or PBS was used to prepare the cytokine solutions, and in addition to the fact that the

contact lenses on their own exhibited little to no interference with the MSD assay, it was reasonably concluded that this observation was due to the normal and expected slight variations existing between absolute values that translated into larger differences in percent of control on a picogram scale. Refer to section 4.8: "Method of Data Analysis and Presentation" for further explanation.

5.3 Investigating the Effect of Temperature on Cytokine Uptake (Pilot Study #5)

Pilot study #5 aimed to investigate whether temperature had any effect on cytokine stability over time, which was of interest given the results of pilot studies #1 and #2, where cytokine concentrations in the experimental controls deviated largely from theoretical concentrations.

5.3.1 Methods and Materials

One conventional hydrogel, etafilcon A (1-Day Acuvue Moist) and one SiHy, senofilcon A (Acuvue Oasys 2-week with Hydraclear Plus) were chosen. The lens materials were soaked in PBS to remove packaging solution as described in section 4.3, and dried on lens paper prior to being placed in polypropylene tubes containing 1 mL of a cytokine solution prepared from the MSD calibrator blend in Diluent 2. Control tubes had 1 mL of the cytokine solution with no contact lenses. All tubes were placed in the Innova 4300 Incubator Shaker at 32℃ (eye temperature) and 25 rpm. Samples were collected after 6 hours and later analyzed using the MSD assay. This time point was chosen as it was decided that future experiments would likely investigate cytokine uptake onto contact lenses for up to a maximum 6-hour soaking period, which is a reasonable wear time for either a daily disposable or reusable contact lens.

5.3.2 Results

Theoretical control concentrations were 28.75 pg/mL, 38.35 pg/mL, 29.95 pg/mL and 18.4 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. After 6 hours, experimental control concentrations were 21.12 pg/mL, 32.26 pg/mL, 22.0 pg/mL and 9.57 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. Also after 6 hours, there appeared to be no difference in uptake of IL-1β (Figure 5-25) between etafilcon A (103.7 $\pm$ 4 %) or senofilcon A (98 $\pm$ 3 %), in comparison to the control or in comparison to each other. This was also the case for the uptake of IL-6 (Figure 5-26) by etafilcon A (106 $\pm$ 5 %) and senofilcon A (104 $\pm$ 2 %); IL-8 (Figure 5-27) by etafilcon A (108 $\pm$ 7 %) or senofilcon A (103 $\pm$ 1 %); and for the uptake of TNF-α (Figure 5-28) by etafilcon A (105 $\pm$ 7 %) or senofilcon A (101 $\pm$ 4 %). Statistical analyses with ordinary, one-way ANOVAs and Tukey's Multiple Comparisons test revealed no significant differences between any of the experimental conditions (differences were considered significant if $p < 0.05$).

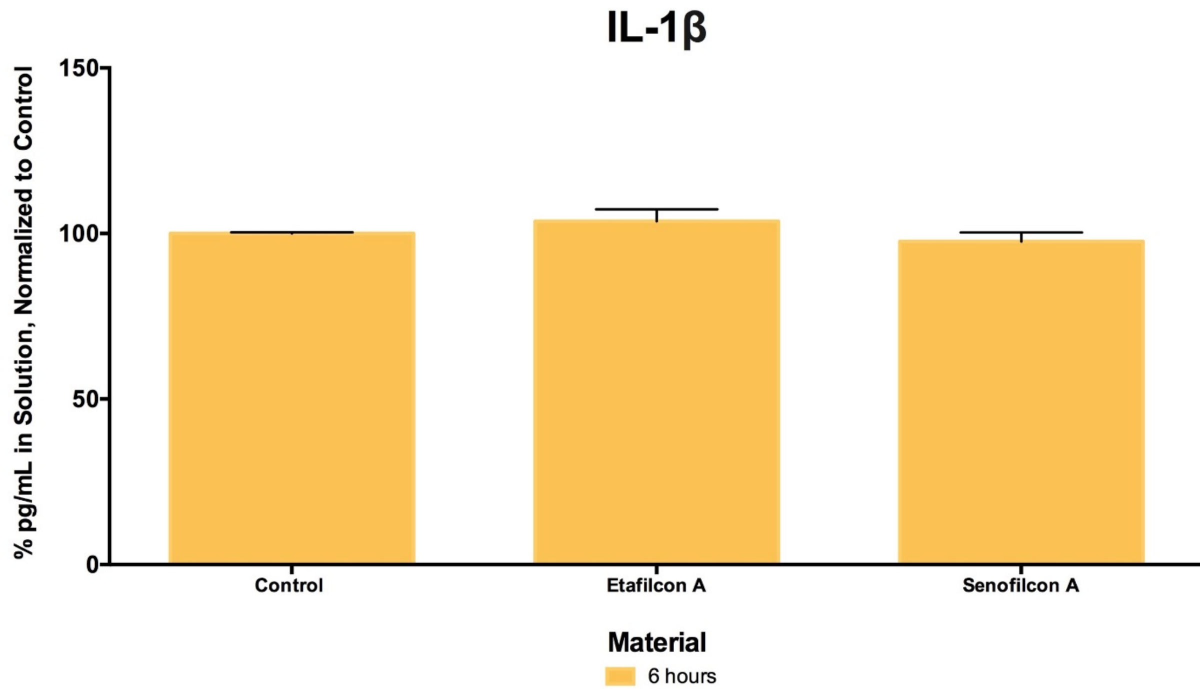

Figure 5-25: Percent pg/mL of IL-β Remaining in Cytokine Solutions Containing Either etafilcon A or senofilcon A (n = 3), at 6 hours of Incubation at 32℃. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

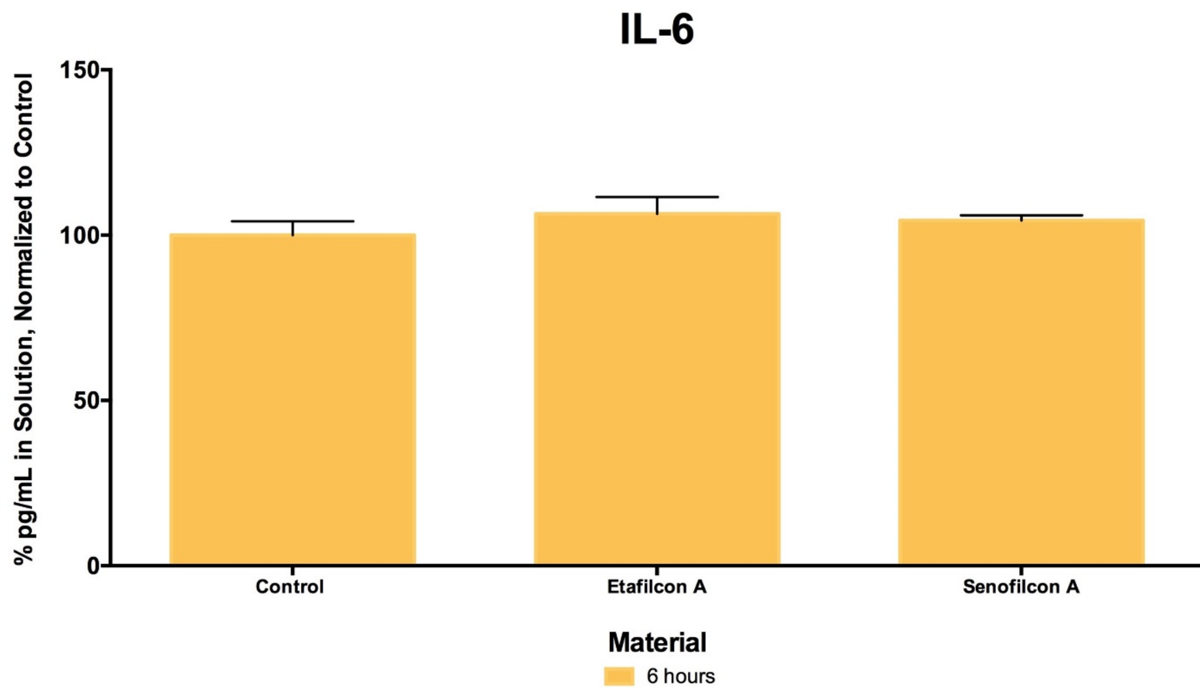

Figure 5-26: Percent pg/mL of IL-6 Remaining in Cytokine Solutions Containing Either etafilcon A or senofilcon A (n = 3), at 6 hours of Incubation at 32℃. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

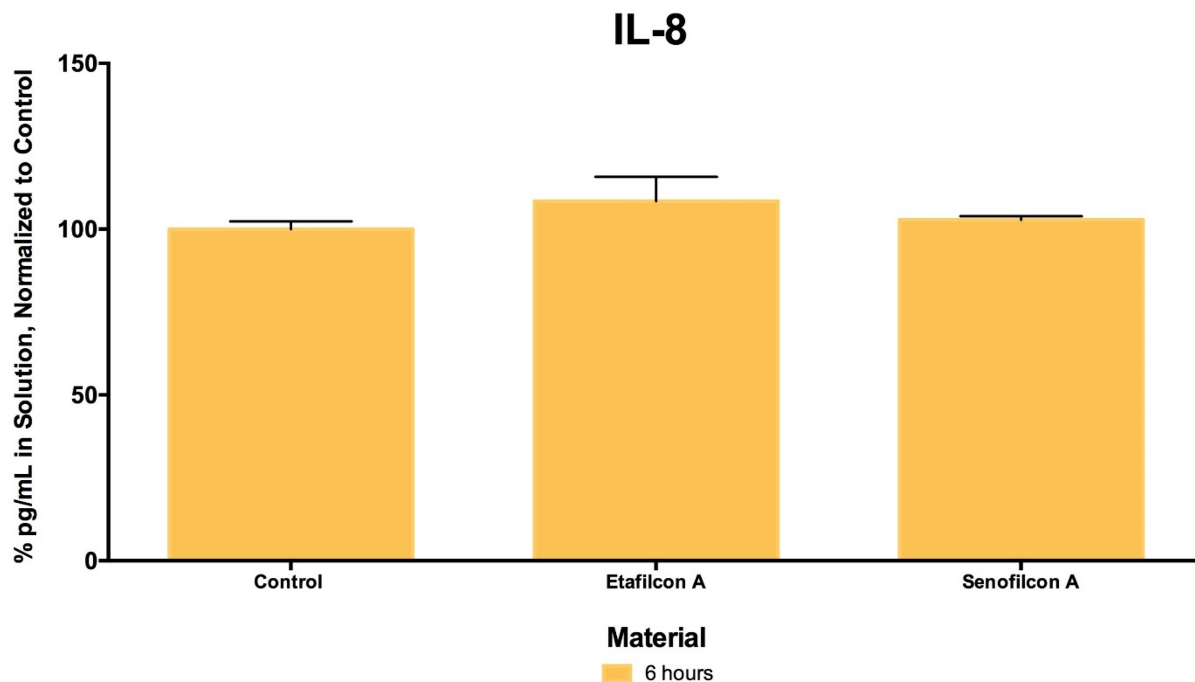

Figure 5-27: Percent pg/mL of IL-8 Remaining in Cytokine Solutions Containing Either etafilcon A or senofilcon A (n = 3), at 6 hours of Incubation at 32℃. Ordinary One-Way ANOVA with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

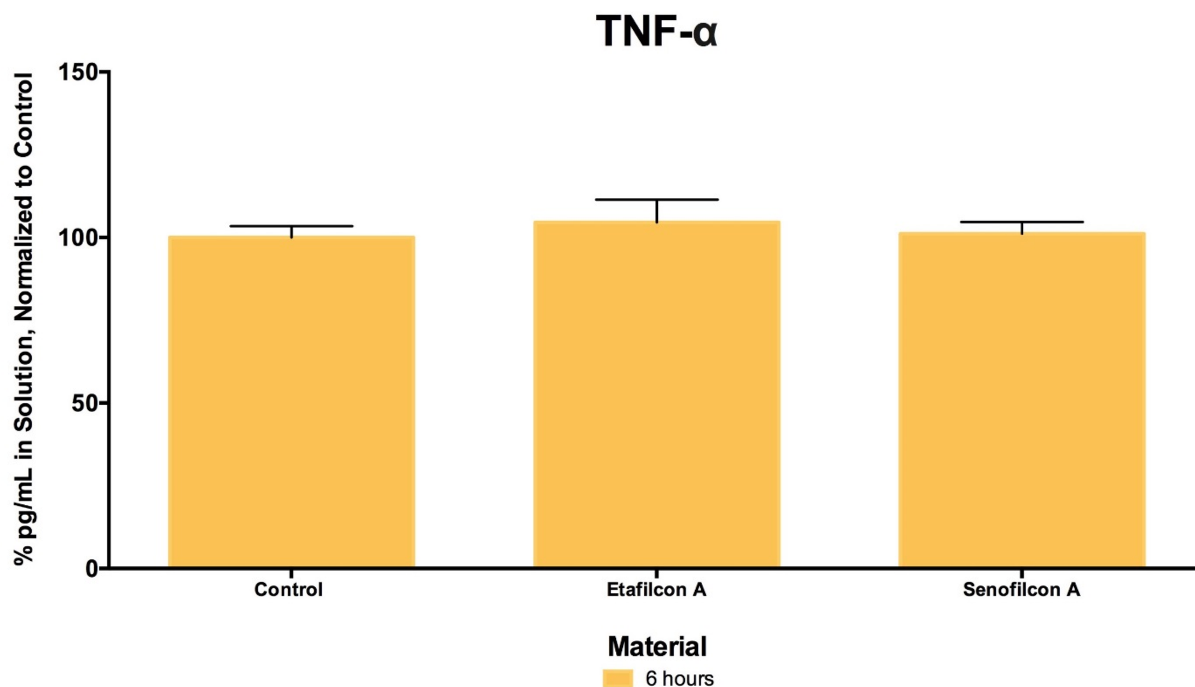

Figure 5-28: Percent pg/mL of TNF-α Remaining in Cytokine Solutions Containing Either etafilcon A or senofilcon A (n = 3), at 6 hours of Incubation at 32℃. Ordinary One-Way ANOVA with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

5.3.3 Discussion

While there were no statistical differences ($p > 0.05$) between the experimental controls and the samples to indicate cytokine uptake onto either etafilcon A or senofilcon A for any of the four cytokines of interest, the experimental control cytokine concentrations after 6 hours did appear to be somewhat lower than the theoretical cytokine concentrations. It appeared that the incubation temperature may have somewhat affected the stability of the cytokines over time, with some cytokines being more greatly affected than others (e.g., for TNF-α). Since incubating the contact lenses certainly did not enhance cytokine uptake, moving forward, all experiments were run at room temperature, and sample tubes were not incubated at eye temperature. While this may not have been representative of ocular conditions, the goal of this thesis was to investigate the inherent properties of contact lenses for cytokine uptake, and hence conditions that could interfere with this outcome were desirably eliminated. Future work could instead elaborate on the results of these experiments by incorporating additional parameters in a clinical setting, such as investigating the effect of ocular temperature on cytokine uptake, once the fundamental science is well understood.

Chapter 6: Uptake Using a Standard Blend of Cytokines

6.1 Preface

The experiments presented in this chapter explored eight different types of soft contact lens materials for the uptake of IL-1β, IL-6, IL-8 and TNF-α cytokines using a standard blend. The standard blend refers to the MSD calibrator blend, which contains ten cytokines at lot-specific concentrations. Utilizing a standard blend is advantageous as it has been prepared to MSD's specifications, has been validated for the MSD assay, and individual analyte concentrations have been tested and verified for quality control. Preparation of the standard blend followed the procedure described in section 4.3 using Diluent 2, which was concluded to be the solution of choice in Chapter 5: As also outlined in section 4.3, all contact lenses utilized were soaked in PBS to remove packaging solution and dried on Fisherbrand lens paper prior to beginning each investigation. All experiments presented in this chapter were performed at room temperature on the VWR Advanced 3500 orbital shaker to ensure a dynamic soaking environment.

6.2 Investigating Conventional Hydrogels for Cytokine Uptake Ability

6.2.1 Introduction

Of the contact lens materials listed in Table 3-4, etafilcon A and omafilcon A were the conventional hydrogels of interest studied for cytokine uptake. The other conventional hydrogel listed in Table 3-4 that was initially studied was polymacon A; however, as mentioned, this material ceased to be investigated further beyond the pilot studies. Etafilcon A and omafilcon A were of interest due to having been studied in the literature for protein deposition, specifically etafilcon A which had shown to deposit more positively-charged protein than omafilcon A, due to having an overall negative charge.[58-60]

6.2.2 Materials and Methods

1-Day Acuvue Moist (etafilcon A) and Proclear 1 Day (omafilcon A) contact lenses were placed in polypropylene tubes (n = 3) containing 1 mL of the prepared cytokine solution with theoretical IL-1β, IL-6, IL-8 and TNF-α concentrations of 28.05 pg/mL, 37.5 pg/mL, 30.8 pg/mL and 17.3 pg/mL, respectively. Control tubes contained 1 mL of the cytokine solution with no lenses. Experimental tubes contained the contact lenses which were soaked for 1 hour. Previously, pilot study #4 investigated uptake onto etafilcon A for only a half-hour time period (refer to section 5.2.7). Although pilot studies #1 and #2 did explore the omafilcon A material, the results from those studies were inconclusive due to the many potential influencing factors discussed throughout Chapter 5:, and thus omafilcon A lenses were also included in this study. At the 1-hour time point, 60 μL samples were collected from each tube and cytokine concentrations were quantified the same day using the MSD assay.

6.2.3 Results

After 1 hour, the experimental control concentrations of IL-1β, IL-6, IL-8 and TNF-α were quantified by the MSD assay as 26.33 pg/mL, 35.83 pg/mL, 29.32 pg/mL and 15.63 pg/mL, respectively. There appeared to be no difference in uptake after 1 hour for IL-1β (Figure 6-1) between etafilcon A (99 $\pm$ 7 %) or omafilcon A (102 $\pm$ 8 %), in comparison to the control (100%), or in comparison to each other. This was also the case for the uptake of IL-6 (Figure 6-2) by etafilcon A (101 $\pm$ 8 %) and omafilcon A (107 $\pm$ 5 %); IL-8 (Figure 6-3) by etafilcon A (99 $\pm$ 11 %) and omafilcon A (96 $\pm$ 5 %); and for the uptake of TNF-α (Figure 6-4) by etafilcon A (102 $\pm$ 8 %) and omafilcon A (100 $\pm$ 2 %). Statistical analyses with ordinary, one-way ANOVAs and Tukey's Multiple Comparisons test, where differences were considered significant if $p < 0.05$, revealed no significant differences between any of the experimental conditions.

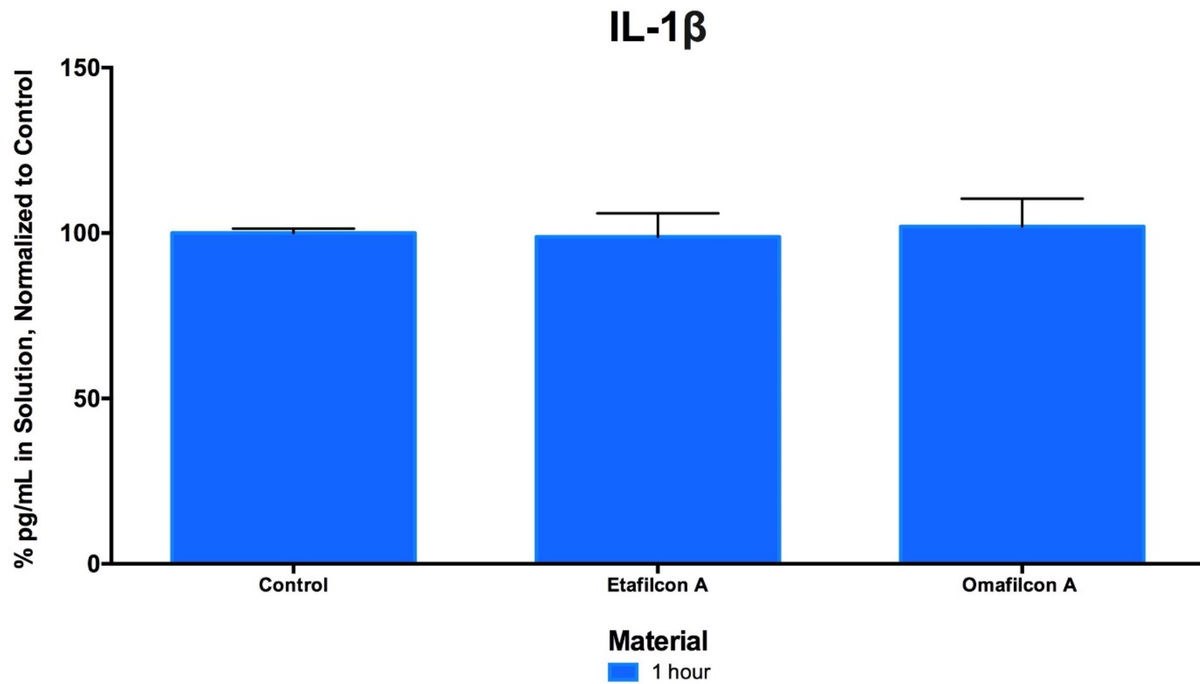

Figure 6-1: Percent pg/mL of IL-1β Remaining in Cytokine Solutions Containing Either etafilcon A or omafilcon A (n = 3) Contact Lens Materials at 1 Hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

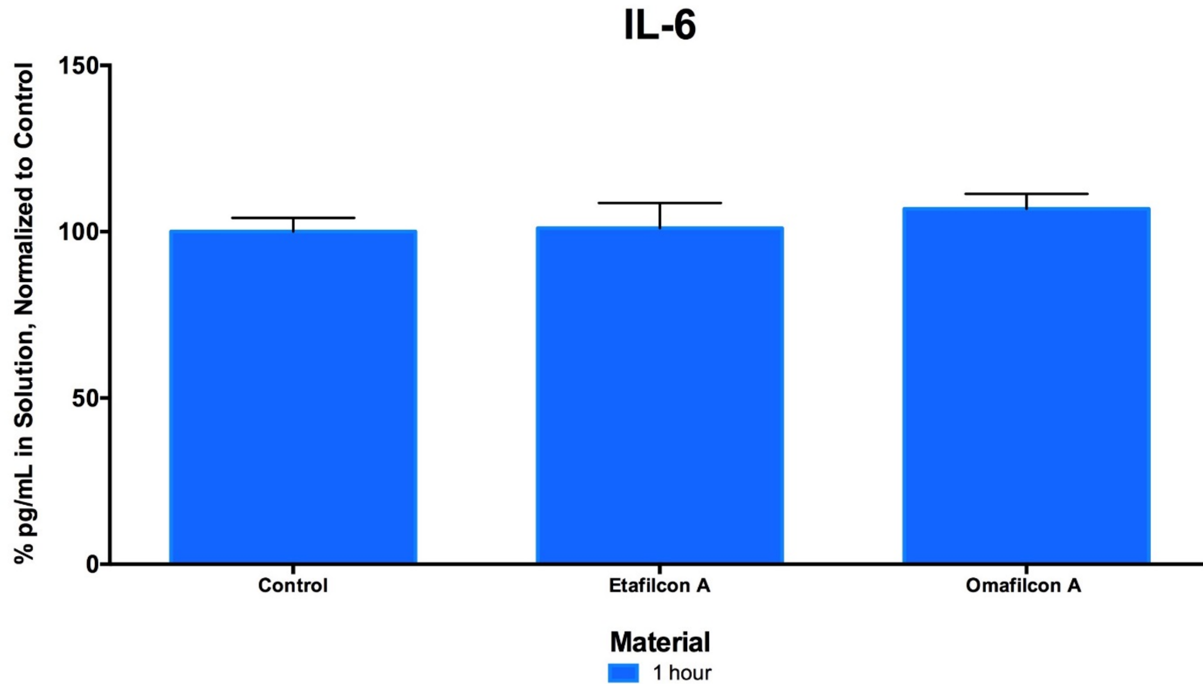

Figure 6-2: Percent pg/mL of IL-6 Remaining in Cytokine Solutions Containing Either etafilcon A or omafilcon A (n = 3) Contact Lens Materials at 1 Hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

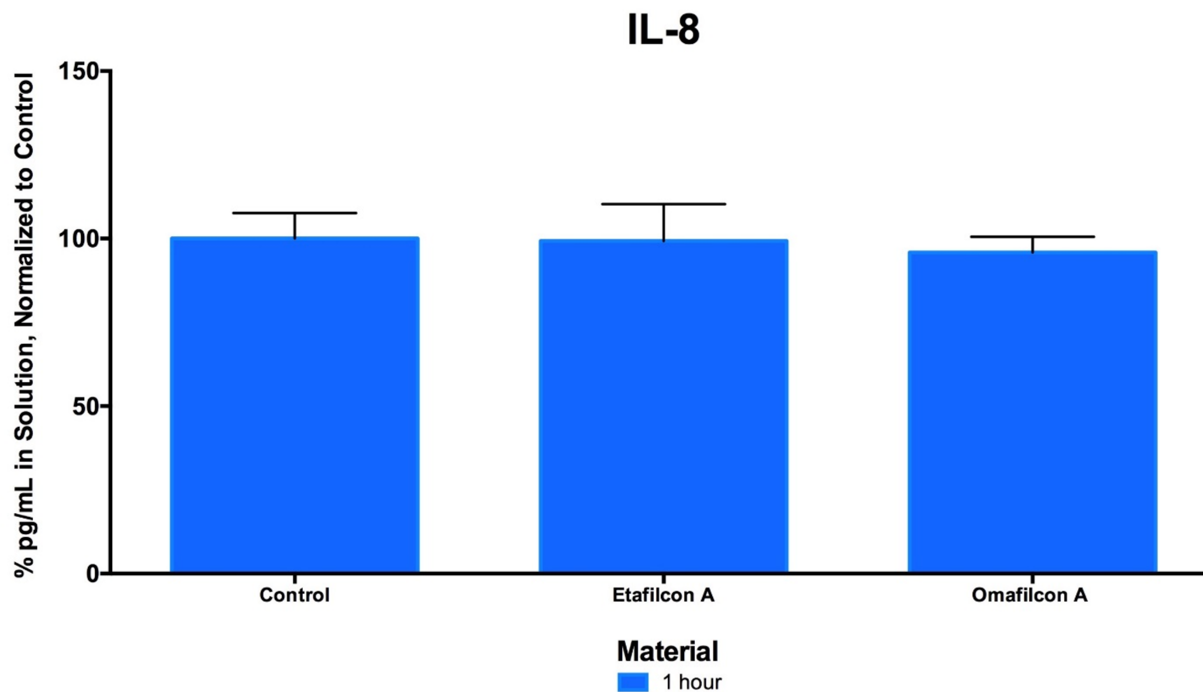

Figure 6-3: Percent pg/mL of IL-8 Remaining in Cytokine Solutions Containing Either etafilcon A or omafilcon A (n = 3) Contact Lens Materials at 1 Hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

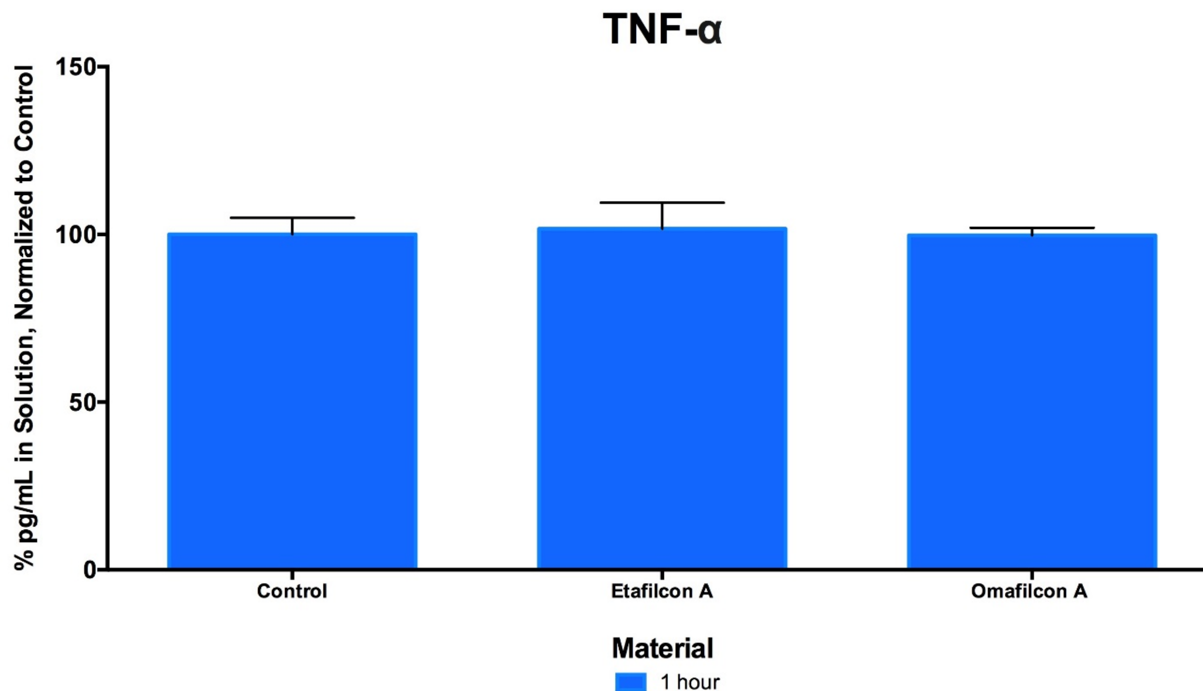

Figure 6-4: Percent pg/mL of TNF-α Remaining in Cytokine Solutions Containing Either etafilcon A or omafilcon A (n = 3) Contact Lens Materials at 1 Hour. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

6.2.4 Discussion

After 1 hour, etafilcon A and omafilcon A materials did not exhibit any uptake of the four cytokines of interest at the given concentrations. Comparing this to the results obtained in section 5.2.7, where the uptake of cytokines onto etafilcon A material was investigated during a half-hour time period as part of pilot study #4, it appeared that both of these time periods were inadequate for detecting uptake onto this material. Moreover, it appeared that the 1-hour time period was also insufficient for detecting uptake onto omafilcon A material.

Referring to Table 3-2, the isoelectric points (pI) of the cytokines of interest, as determined through physical characterizations, were stated in the literature to be approximately 6.9 – 7.0 (IL-1β)[85], 4.9[86] or 5.0 – 6.0 (IL-6)[87], 9.4[89] (IL-8) and 5.0 – 7.0[91] or 5.3[91,92] (TNF-α). According to Meso Scale Discovery Scientific Support, the pH of Diluent 2 is approximately 7.4 (slightly basic).

Since all of IL-1β, IL-6 and TNF-α have reported pI values falling below this pH, the surface of these cytokines should be predominately negatively charged in the standard blend soaking solution.[83] In contrast, IL-8 should have a predominately positively charged surface due to its pI value falling above the pH of the soaking solution.[83] Recall from Table 3-4 that etafilcon A is negatively charged overall and omafilcon A is zwitterionic due to having both a positive and negative charge (hence it is neutral).[59] If the surface charge of cytokines is a dominating factor influencing uptake, it would be expected then that etafilcon A would exhibit less attraction for the uptake of IL-1β, IL-6, or TNF-α, as a result of the contact lens material and cytokine having a similar charge. In comparison, etafilcon A would be more likely to uptake IL-8, as a result of an attraction between the opposing charges, although the results of this particular experiment did not indicate so. Omafilcon A may resist protein deposition in part due to its principal monomer phosphorylcholine, which provides an overall neutral charge and has been documented in the literature to aid in lowering protein adsorption to contact lenses of this material.[107,108]

6.3 Investigating Conventional Hydrogels with a SiHy for Cytokine Uptake Ability

6.3.1 Introduction – Experiment #1

As neither a half-hour soaking period for etafilcon A, nor a 1-hour soaking period for etafilcon A and omafilcon A, provided any indication of uptake for the four cytokines of interest onto the lens materials, a 6-hour time period was instead explored. In addition to utilizing the 1-Day Acuvue Moist (etafilcon A) and Proclear 1 Day (omafilcon A) contact lenses as in the prior experiment, Acuvue Oasys 2-week with Hydraclear Plus (senofilcon A), was also incorporated into the experimental design as a commonly used SiHy material to be investigated. Although etafilcon A and senofilcon A materials were previously studied at a 6-hour time point in pilot study #5, that study explored the effect of temperature (section 5.3.2) and due to the experimental control

concentrations differing vastly from theoretical controls (especially for IL-8 and TNF-α), the materials were again explored, this time with more stable controls and without incubation at 32℃.

6.3.2 Materials and Methods – Experiment #1

1-Day Acuvue Moist (etafilcon A), Proclear 1 Day (omafilcon A) and Acuvue Oasys 2-week with Hydraclear Plus (senofilcon A), were placed in polypropylene tubes (n = 3) containing 1 mL of a prepared cytokine solution. Control tubes contained 1 mL of the cytokine solution with no contact lenses. Theoretical concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.3 pg/mL, 35.6 pg/mL, 27.8 pg/mL and 18.45 pg/mL, respectively. At the 6-hour time point, 60 μL samples were collected from each tube, and cytokine concentrations were quantified the same day using the MSD assay.

6.3.3 Results – Experiment #1

Control solutions evaluated at 6 hours contained IL-1β, IL-6, IL-8 and TNF-α at concentrations of 26.42 pg/mL, 32.68 pg/mL, 24.76 pg/mL and 13.06 pg/mL, respectively. Statistical analyses were performed using ordinary, one-way ANOVAs with Tukey's Multiple Comparisons test. Differences were considered significant if $p < 0.05$. After 6 hours, there appeared to be no difference in uptake for IL-1β (Figure 6-5) between etafilcon A (95 $\pm$ 7 %) or senofilcon (95 $\pm$ 2 %), in comparison to the control (100%) or in comparison to each other. In contrast, there appeared to be some uptake of IL-1β (Figure 6-5) by omafilcon A (82 $\pm$ 2 %), as the material was statistically different from the control (adjusted P-value 0.0024), from etafilcon A (adjusted P-value 0.0185) and from senofilcon A (adjusted P-value 0.0198), as defined by the star (*) symbol.

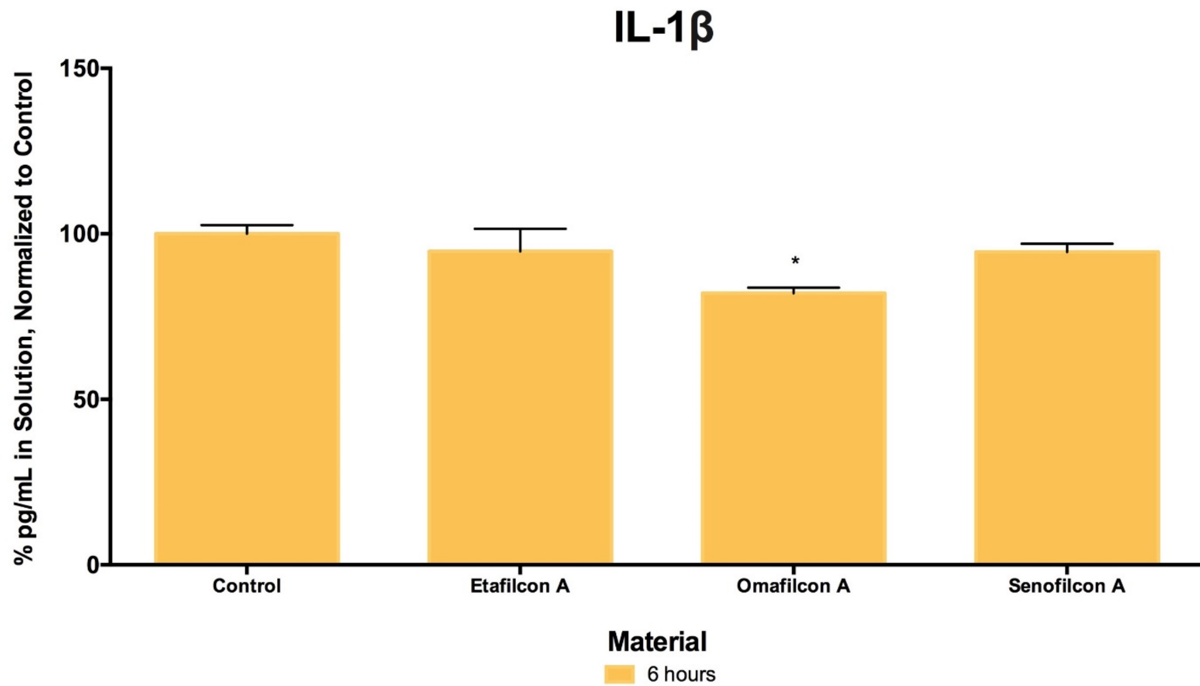

Figure 6-5: Percent pg/mL of IL-1β Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials at 6 Hours. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test Revealed omafilcon A to be Statistically Different from the Control, etafilcon A and senofilcon A, as Defined by the Star (*) Symbol.

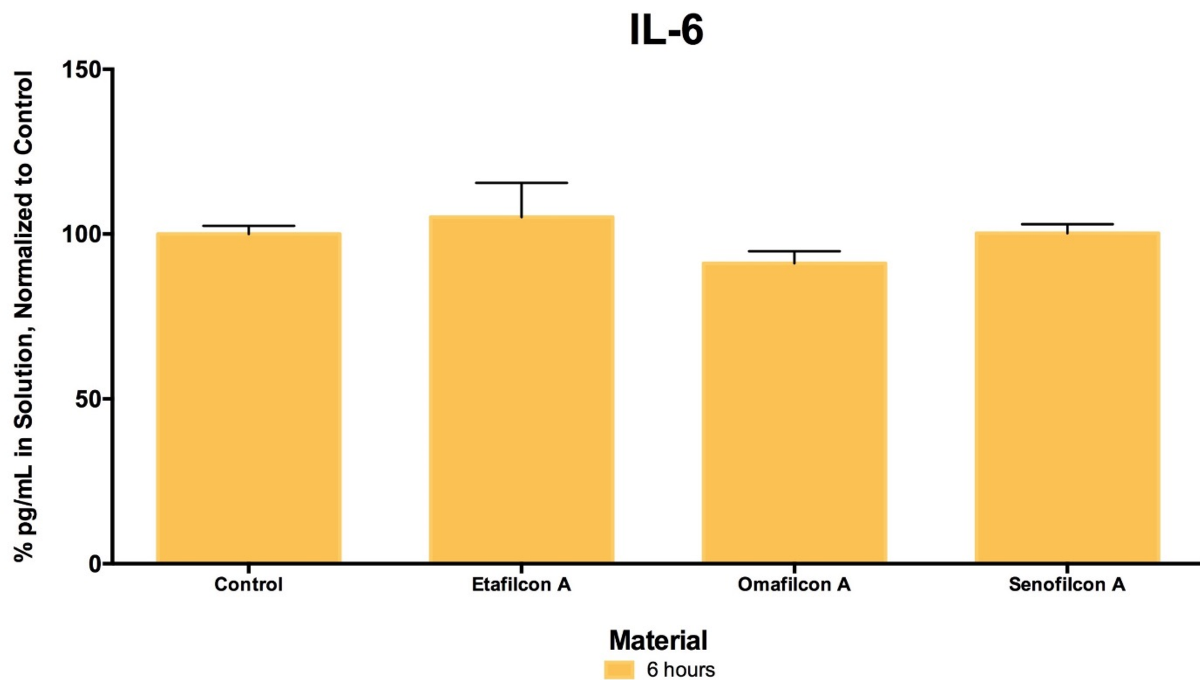

Figure 6-6: Percent pg/mL of IL-6 Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials at 6 Hours. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

There was little uptake of IL-6 (Figure 6-6) by etafilcon A (105 $\pm$ 10 %), omafilcon A (91 $\pm$ 4 %) and senofilcon A (100 $\pm$ 3 %). Likewise, there was little uptake of IL-8 (Figure 6-7) by etafilcon A (91 $\pm$ 17 %), omafilcon A (70 $\pm$ 10 %) and senofilcon A (99 $\pm$ 1 %), as well as of TNF-α (Figure 6-8) by etafilcon A (84 $\pm$ 20 %), omafilcon A (53 $\pm$ 12 %) and senofilcon A (97 $\pm$ 15 %). There were no statistical differences ($p > 0.05$) between the respective controls and the contact lens materials tested for any of these three cytokines of interest.

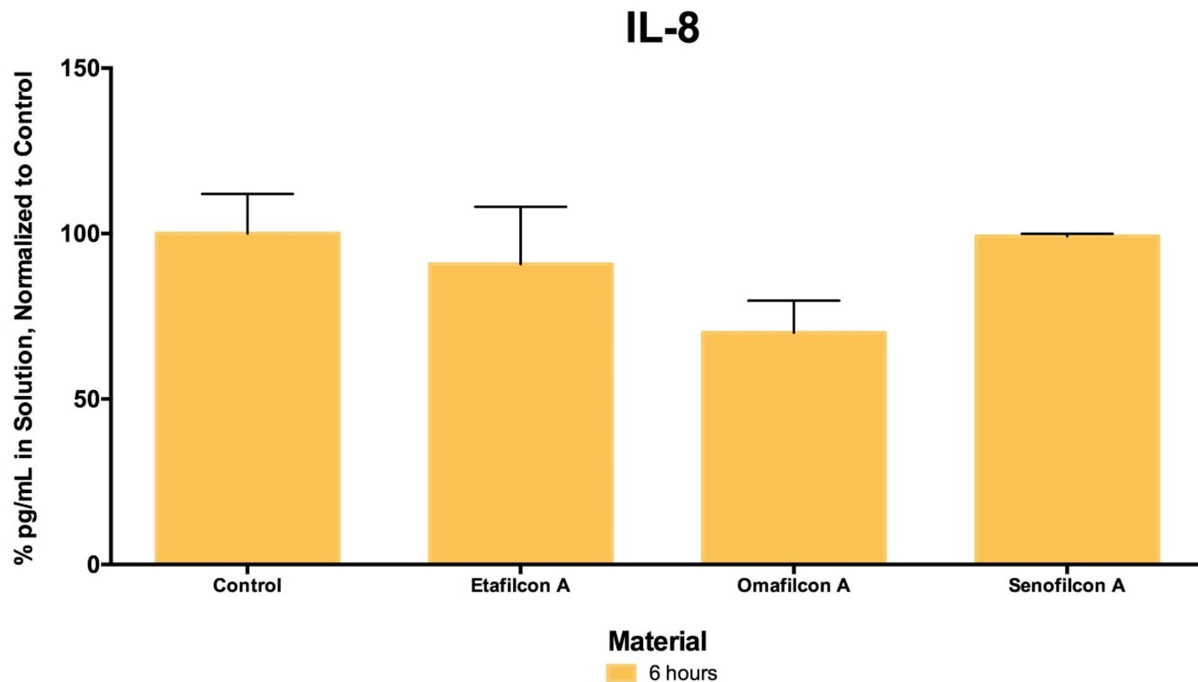

Figure 6-7: Percent pg/mL of IL-8 Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials at 6 Hours. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

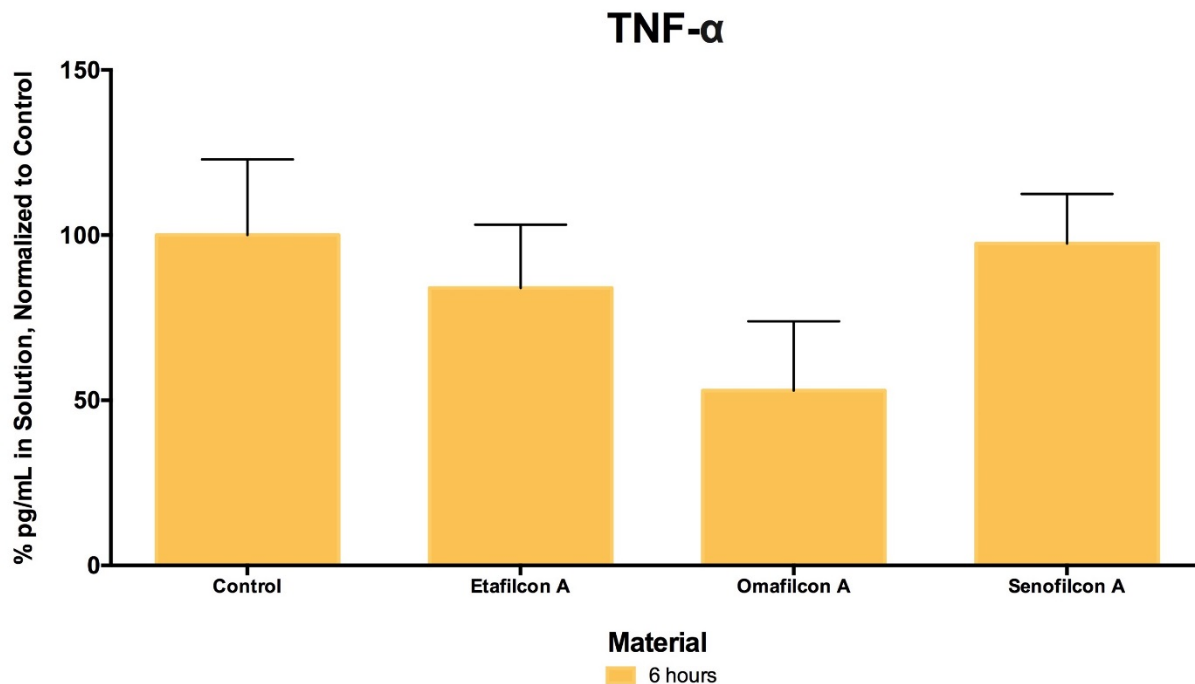

Figure 6-8: Percent pg/mL of TNF-α Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials at 6 Hours. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

6.3.4 Discussion – Experiment #1

While etafilcon A and senofilcon A did not exhibit any uptake of cytokines IL-6, IL-8 or TNF-α that were statistically different from the control or from each other after 6 hours, omafilcon A did appear to exhibit some uptake of IL-1β that was statistically different from the control and from both etafilcon A and senofilcon A materials after 6 hours. When comparing the differences between these three contact lens materials, between etafilcon A and omafilcon A there is a difference of ionicity, as the former is ionic and the latter is nonionic (Table 3-4), while they share the same high water content characteristic (58% for etafilcon A and 60% for omafilcon A).[59] Senofilcon A is a contact lens material of low water content (38%) and is also nonionic, similarly to omafilcon A, but in contrast to etafilcon A (Table 3-4).[59]

Based on the results of this experiment, etafilcon A and senofilcon A appeared to act similar to each other, though they differ in both their ionicity and water content characteristics.

Additionally, while omafilcon A shares similar characteristics with both etafilcon A (high water content) and with senofilcon A (nonionic), it appeared to act slightly differently from both materials, as it exhibited some uptake of IL-1β – a cytokine that should have a negatively-charged surface in the standard blend solution based on its pI.[59,85] Furthermore, it was interesting that omafilcon A had this interaction with IL-1β, but not with IL-6 or TNF-α, which should also have negatively-charged surfaces in the standard blend solution based on their pI.[86,87,91,92] As a result, a second experiment utilizing the same etafilcon A, omafilcon A and senofilcon A materials, aimed to explore the reproducibility of the results previously obtained.

6.3.5 Introduction – Experiment #2

To explore the reproducibility of the results obtained in sections 5.2.7 (where uptake onto etafilcon A material was investigated over a half-hour time period), 6.2.3 (where uptake onto etafilcon A and omafilcon A materials were investigated over a 1-hour time period) and 6.3.3 (where uptake onto etafilcon A, omafilcon A and senofilcon A materials were investigated over a 6-hour time period), a second experiment explored cytokine uptake onto all three of these contact lens materials over the same time periods of a half-hour, 1 hour and 6 hours. This allowed for the investigation of uptake onto omafilcon A and senofilcon A materials after a half-hour time point, at which they were not previously explored and in doing so, this would potentially provide insight into whether these materials may exhibit uptake earlier on during the soaking period. Likewise, senofilcon A had not yet been evaluated at 1 hour in the previous experiments either.

6.3.6 Materials and Methods – Experiment #2

1-Day Acuvue Moist (etafilcon A), Proclear 1 Day (omafilcon A) and Acuvue Oasys 2-week with Hydraclear Plus (senofilcon A), were placed in polypropylene tubes (n = 3) containing 1 mL of a prepared cytokine solution. Control tubes contained 1 mL of the cytokine solution with

no contact lenses. At each time point, 60 μL of the solution was pipetted out from each tube and stored in a -80℃ freezer to be analyzed by the MSD assay. Thus, half-hour, 1-hour, and 6-hour samples were collected from the same tubes containing the etafilcon A, omafilcon A, and senofilcon A materials. Between the 1-hour and 6-hour time points, there was 880 μL of the solution remaining in each tube, which was still a sufficient volume to cover the materials in their entirety. Theoretical concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.3 pg/mL, 35.6 pg/mL, 27.8 pg/mL and 18.45 pg/mL, respectively.

6.3.7 Results – Experiment #2

At a half-hour, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.92 pg/mL, 34.4 pg/mL, 29.3 pg/mL and 17.14 pg/mL, respectively. At 1 hour, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 31.68 pg/mL, 38.99 pg/mL, 31.88 pg/mL and 16.92 pg/mL, respectively. At 6 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 27.97 pg/mL, 35.92 pg/mL, 26.34 pg/mL and 11.87 pg/mL, respectively. Statistical analyses were performed using two-way, repeated measures ANOVAs with Tukey's Multiple Comparisons test, where differences were considered significant if $p < 0.05$.

At the half-hour time point, there was no uptake of any of the four cytokines of interest onto either etafilcon A, omafilcon A, or senofilcon A materials. Relative to the control (100%), the percent pg/mL remaining in solution at this time point was 103 $\pm$ 5 % (etafilcon A), 104 $\pm$ 3 % (omafilcon A) and 104 $\pm$ 2 % (senofilcon A) for IL-1β (Figure 6-9); 106 $\pm$ 3 % (etafilcon A), 110 $\pm$ 3 % (omafilcon A) and 102 $\pm$ 3 % (senofilcon A) for IL-6 (Figure 6-10); 102 $\pm$ 4 % (etafilcon A), 106 $\pm$ 3 % (omafilcon A) and 103 $\pm$ 4 % (senofilcon A) for IL-8 (Figure 6-11); and 97 $\pm$ 4 % (etafilcon A), 101 $\pm$ 4 % (omafilcon A) and 100 $\pm$ 4 % (senofilcon A) for TNF-α (Figure

6-12). There were no statistical differences ($p > 0.05$) between the control or the contact lens materials for any of the cytokines at this time point.

At the 1-hour time point, there was no uptake of IL-1β, IL-6 or IL-8 onto either etafilcon A, omafilcon A, or senofilcon A materials. Relative to the control (100%), the percent pg/mL remaining in solution at this time point was 93 $\pm$ 3 % (etafilcon A), 92 $\pm$ 3 % (omafilcon A) and 94 $\pm$ 3 % (senofilcon A) for IL-1β (Figure 6-9); 92 $\pm$ 6 % (etafilcon A), 98 $\pm$ 1 % (omafilcon A) and 95 $\pm$ 3 % (senofilcon A) for IL-6 (Figure 6-10); and 90 $\pm$ 3 % (etafilcon A), 90 $\pm$ 2 % (omafilcon A) and 92 $\pm$ 1 % (senofilcon A) for IL-8 (Figure 6-11). For TNF-α (Figure 6-12), the percent pg/mL remaining in solution after 1 hour was 86 $\pm$ 10 % (etafilcon A), 80 $\pm$ 6 % (omafilcon A) and 90 $\pm$ 3 % (senofilcon A). There was a statistical difference for both etafilcon A (adjusted P-value 0.0375) and omafilcon A (adjusted P-value 0.0026) relative to the control at this time point, as defined by the star (*) symbols, suggesting some uptake of TNF-α onto these materials.

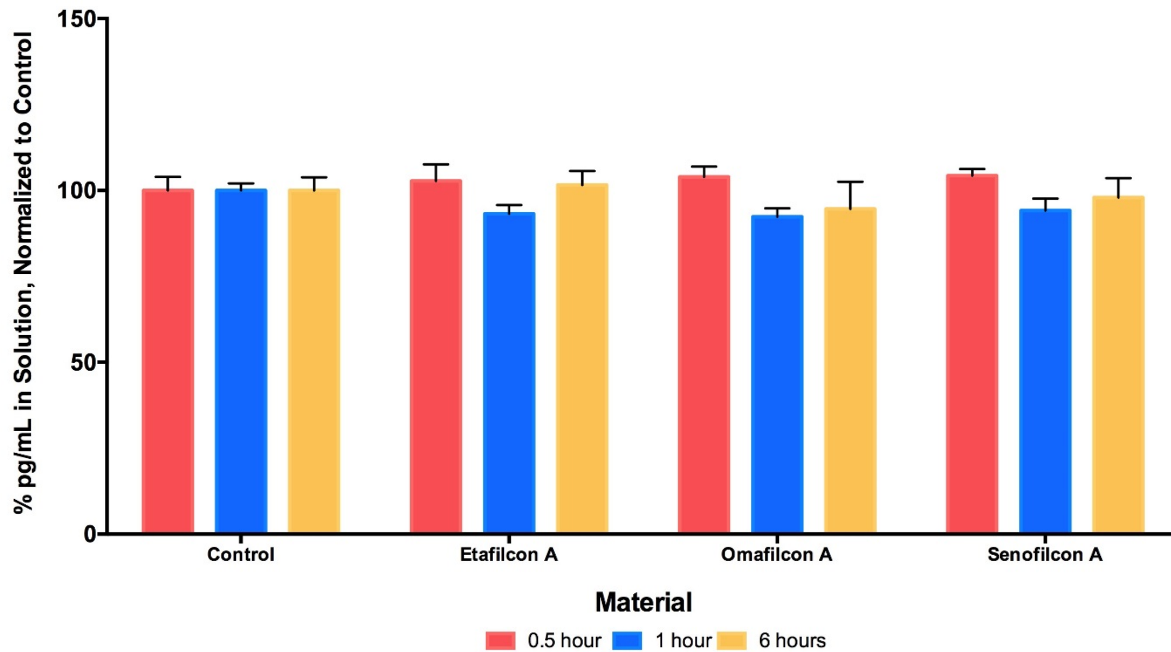

Figure 6-9: Percent pg/mL of IL-1β Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials, at Half-Hour, 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

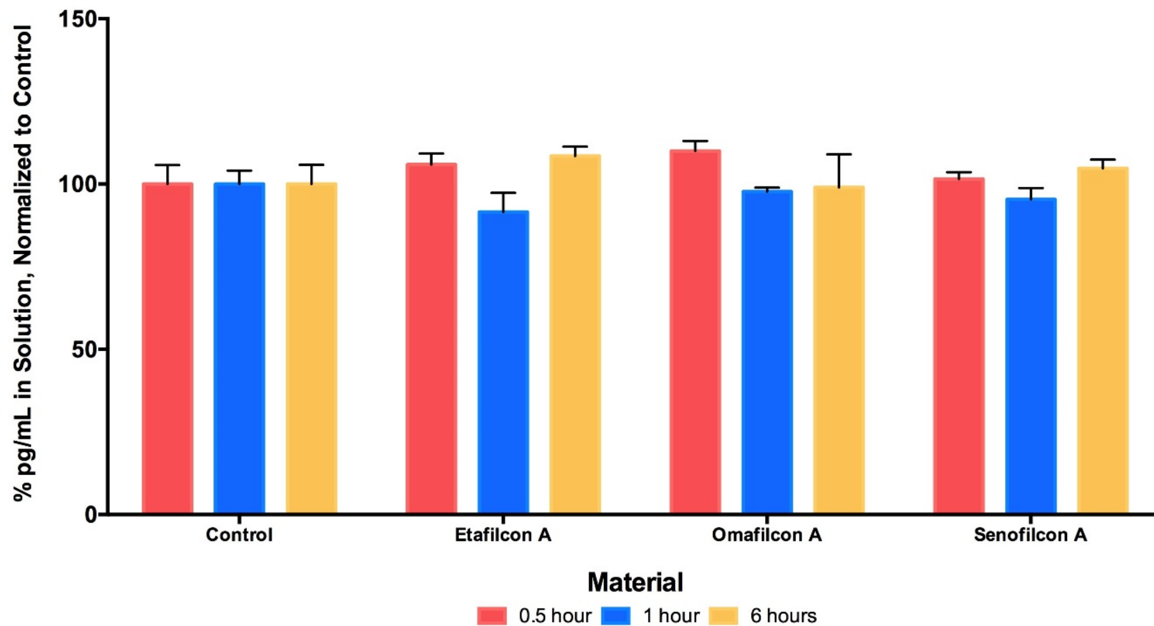

Figure 6-10: Percent pg/mL of IL-6 Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials, at Half-Hour, 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

At the 6-hour time point, there was no uptake of any of the four cytokines of interest onto either etafilcon A, omafilcon A, or senofilcon A materials. Relative to the control (100%), the percent pg/mL remaining in solution at this time point was 102 $\pm$ 4 % (etafilcon A), 95 $\pm$ 8 % (omafilcon A) and 98 $\pm$ 6 % (senofilcon A) for IL-1β (Figure 6-9); 109 $\pm$ 3 % (etafilcon A), 99 $\pm$ 10 % (omafilcon A) and 105 $\pm$ 3 % (senofilcon A) for IL-6 (Figure 6-10); 104 $\pm$ 4 % (etafilcon A), 93 $\pm$ 9 % (omafilcon A) and 101 $\pm$ 10 % (senofilcon A) for IL-8 (Figure 6-11); and 90 $\pm$ 11 % (etafilcon A), 90 $\pm$ 7 % (omafilcon A) and 90 $\pm$ 5% (senofilcon A) for TNF-α (Figure 6-12). There were no statistical differences ($p > 0.05$) between the control or the contact lens materials for any of the cytokines at this time point.

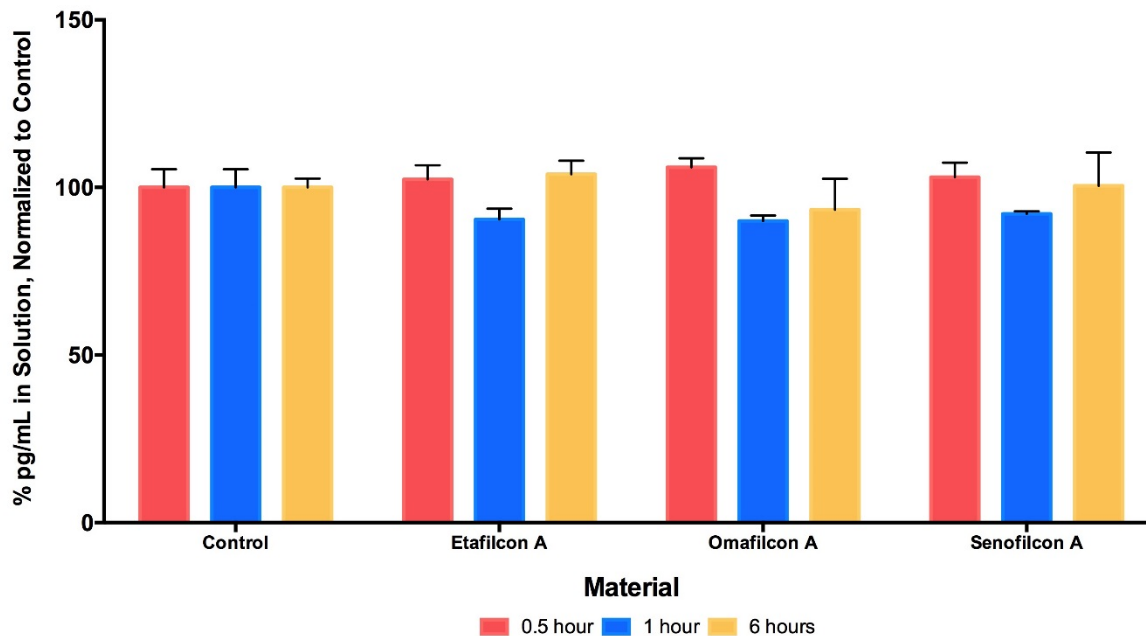

Figure 6-11: Percent pg/mL of IL-8 Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials, at Half-Hour, 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

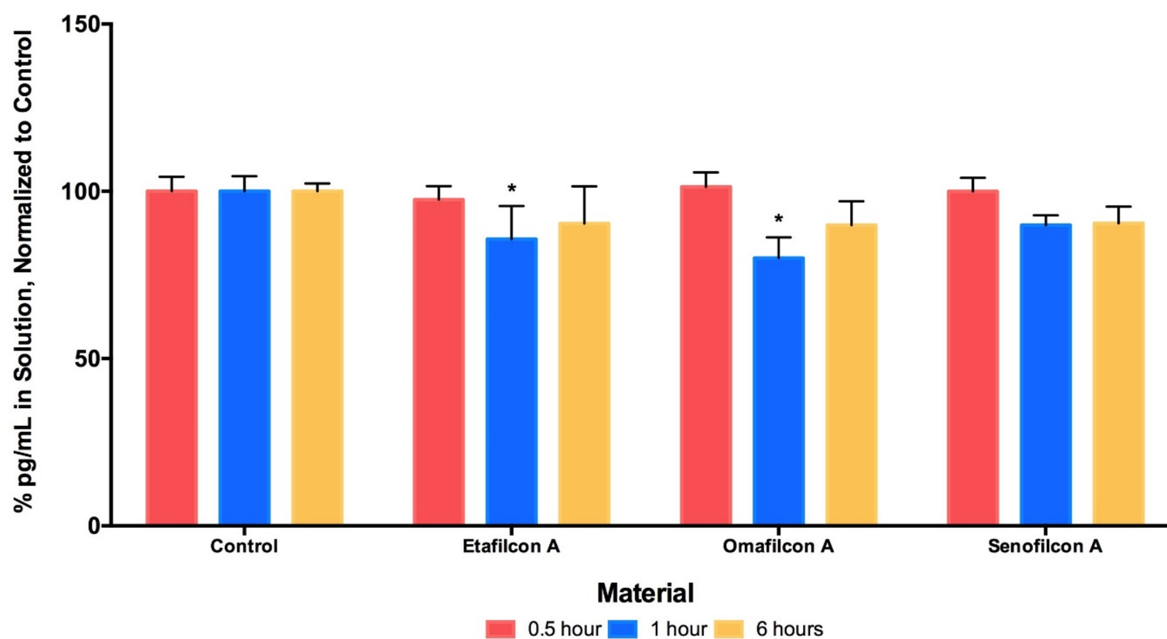

Figure 6-12: Percent pg/mL of TNF-α Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials, at Half-Hour, 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. At 1 hour, etafilcon A and omafilcon A were both Statistically Different from the Control at 1 Hour, as Defined by the Star Symbols (*).

6.3.8 Discussion – Experiment #2

In previous experiments, when uptake onto etafilcon A material after a half-hour time period was explored (section 5.2.7), in addition to uptake onto etafilcon A and omafilcon A after a 1-hour time period (section 6.2.3) and uptake onto etafilcon A, omafilcon A and senofilcon A after a 6-hour time period (section 6.3.3), there was no indication of uptake onto any of these materials at any of the three time points, except for IL-1β onto omafilcon A after 6 hours of soaking. In this experiment, cytokine uptake was collectively investigated at a half-hour, 1-hour, and 6-hour time points, which allowed uptake onto omafilcon A and senofilcon A materials to be further explored at time points which they were not investigated for previously.

Similar to the previous experiments, in this experiment there was also no indication of uptake onto any of the contact lens materials, except for TNF-α onto both etafilcon A and omafilcon A materials after 1 hour of soaking. At 6 hours, however, there was no indication of uptake onto these materials, as there was no statistical difference ($p > 0.05$) between the percent pg/mL remaining in the solution of the control, in comparison to the solutions containing etafilcon A or omafilcon A materials. It was interesting that omafilcon A appeared to be the common material in these experiments that exhibited slight uptake, in comparison to the other materials tested, even if the specific cytokine or the time point at which these results were observed differed among the experiments.

From the results of this experiment, however, it could not conclusively be said that the statistical difference observed for etafilcon A or omafilcon A was a true indication of uptake. Supporting this is the interesting observation where there did not appear to be any uptake onto these materials at the 6-hour time point, even though there appeared to be uptake at the 1-hour time point. A possible explanation for this, though unlikely, could be that the TNF-α cytokine was

loosely bound to the surface of these materials and was released back into the soaking solution sometime between the 1-hour and 6-hour time points. This would not, however, be indicative of true uptake for the cytokine onto the contact lens material. Another possible explanation for the fluctuation in measurement observed was hypothesized to be, in part, due to the 1 mL soaking volume, which could have complicated the measurement of small differences in cytokine concentrations on a picogram scale.

6.4 Reducing the Soaking Volume

6.4.1 Introduction

Considering a 1000 μL (1 mL) soaking volume, a 400 μL soaking volume and a 200 μL soaking volume, differences in cytokine uptake would likely be more easily detectable with a smaller soaking volume such as 200 μL. For example, if etafilcon A was to consistently absorb 10 pg of a given cytokine, then in a 1000 μL solution at 100 pg/mL concentration, where there would be 100 pg of that particular cytokine in solution, absorption of 10 pg would result in the remaining concentration in solution to be 90 pg/mL following uptake. In contrast, in a 400 μL solution at 100 pg/mL concentration, there would be 40 pg of the cytokine in solution, and absorption of 10 pg would result in the concentration remaining in solution to be 75 pg/mL. Detecting a 25 pg/mL difference (between 100 pg/mL and 75 pg/mL) by the MSD instrument, could likely be easier than detecting a 10 pg/mL difference (between 100 pg/mL and 90 pg/mL). Alternatively, if the soaking volume were to be reduced even further, such as in a 200 μL solution at 100 pg/mL concentration, there would be 20 pg of the cytokine in solution and absorption of 10 pg would result in the concentration remaining in solution to be 50 pg/mL. Detecting this 50 pg/mL difference (between 100 pg/mL and 50 pg/mL), could potentially be even easier in comparison to both the 25 pg/mL or 10 pg/mL difference.

The hypothesis above, however, only holds true under two assumptions: the initial concentration must remain constant and one contact lens material must absorb the same amount of a particular cytokine each time. Nonetheless, it was of interest to investigate the effect of reducing the soaking volume, in order to determine whether this would change how precisely cytokine uptake onto a contact lens material could be detected by the MSD instrument. Two experiments were run to investigate the uptake of cytokines onto etafilcon A, omafilcon A and senofilcon A contact lens materials, using both 400 μL and 200 μL soaking solution volumes.

6.4.2 Materials and Methods – Experiment #1

Given that the omafilcon A material had exhibited the greatest potential for uptake of cytokines, it was the first material chosen to explore initially for experiment #1 of the reduced soaking volume series. A cytokine standard blend solution was prepared, of which 200 μL was placed in plastic moulds (n = 3) designed specifically in size to fit contact lenses. Proclear 1 Day (omafilcon A) contact lenses were placed in the plastic moulds and another 200 μL of the cytokine solution was pipetted on top of the lens (total volume 400 μL). The lens moulds were then placed inside every other well of Corning 6-well polystyrene plates (Corning, Corning, NY). PBS was used to fill the in-between empty wells, in order to maintain a humid environment and to ensure that the cytokine solution would not evaporate over time. The plates were also wrapped in Parafilm wrapping film (Fisher Scientific, Hampton, NH). At 1-hour and 6-hour time points, 60 μL of each sample was collected and stored in a -80℃ freezer to later be analyzed with the MSD assay. Theoretical cytokine concentrations were 28.3 pg/mL, 35.6 pg/mL, 27.8 pg/mL and 18.45 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively.

6.4.3 Results – Experiment #1

An aliquot of the cytokine standard blend solution obtained approximately 5 minutes following preparation was later quantified by the MSD assay to have cytokine concentrations of 26.1 pg/mL, 35.5 pg/mL, 24.3 pg/mL and 11.4 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. At 1 hour, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 25.67 pg/mL, 38.32 pg/mL, 23.66 pg/mL and 10.36 pg/mL, respectively. At 6 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 26.66 pg/mL, 38.88 pg/mL, 24.26 pg/mL and 9.02 pg/mL, respectively.

Relative to the control (100%), there was no uptake of any of the four cytokines of interest onto omafilcon A material at 1 hour. The percent pg/mL remaining in solution was 98 $\pm$ 1 % for IL-1β (Figure 6-13); 102 $\pm$ 1 % for IL-6 (Figure 6-14); 103 $\pm$ 0.5 % for IL-8 (Figure 6-15); and 93 $\pm$ 5 % for TNF-α (Figure 6-16). Statistical analyses performed using two-way, repeated measures ANOVAs with Sidak's Multiple Comparisons test (where differences were considered significant if $p < 0.05$), revealed no statistical difference ($p > 0.05$) between omafilcon A and the control for any of the cytokines at this time point.

At 6 hours, there was a statistical difference between omafilcon A and the control (adjusted P-value 0.0454) for IL-1β, as defined by the star (*) symbol (Figure 6-13). The percent pg/mL of this cytokine remaining in solution relative to the control (100%) was 95 $\pm$ 3 %. In contrast, there were no statistical differences ($p > 0.05$) between omafilcon A and the controls for IL-6, IL-8, or TNF-α at this time point. Percent pg/mL remaining in solution at 6 hours was 102 $\pm$ 3 % for IL-6 (Figure 6-14); 96 $\pm$ 2 % for IL-8 (Figure 6-15); and 98 $\pm$ 10 % for TNF-α (Figure 6-16).

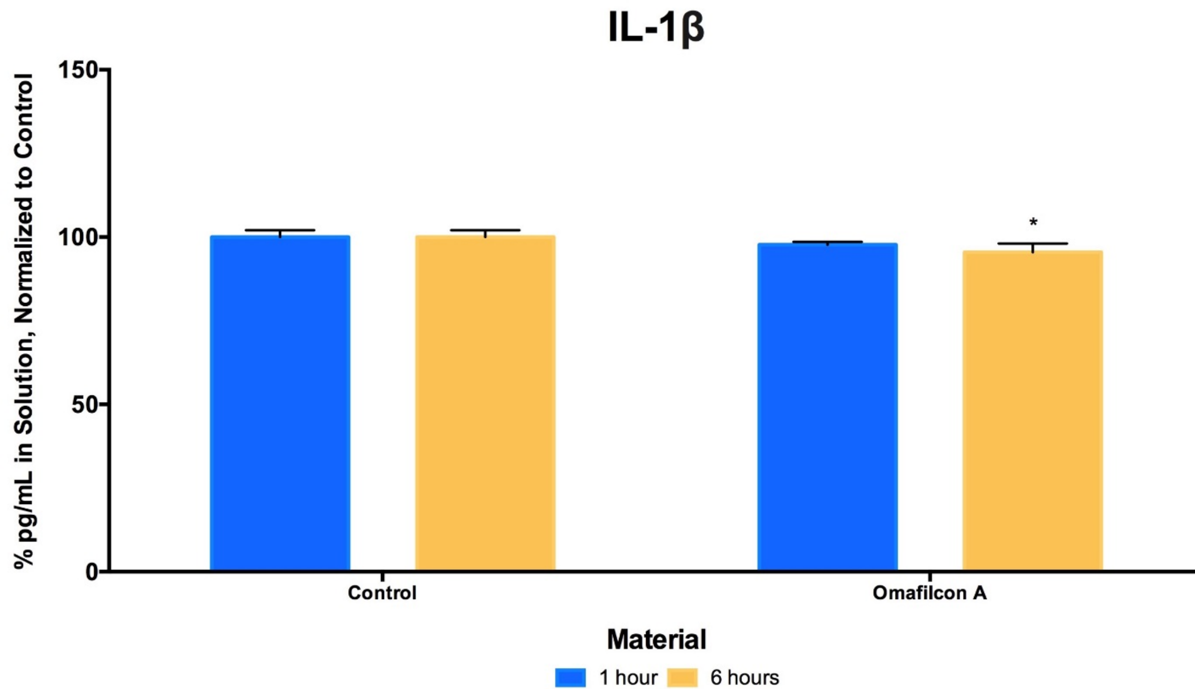

Figure 6-13: Percent pg/mL of IL-1β Remaining in Reduced Volumes of Cytokine Solutions Containing omafilcon A (n = 3) Contact Lens Material, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. Omafilcon A was Statistically Different from the 6-hour Control, as Defined by the Star (*) Symbol.

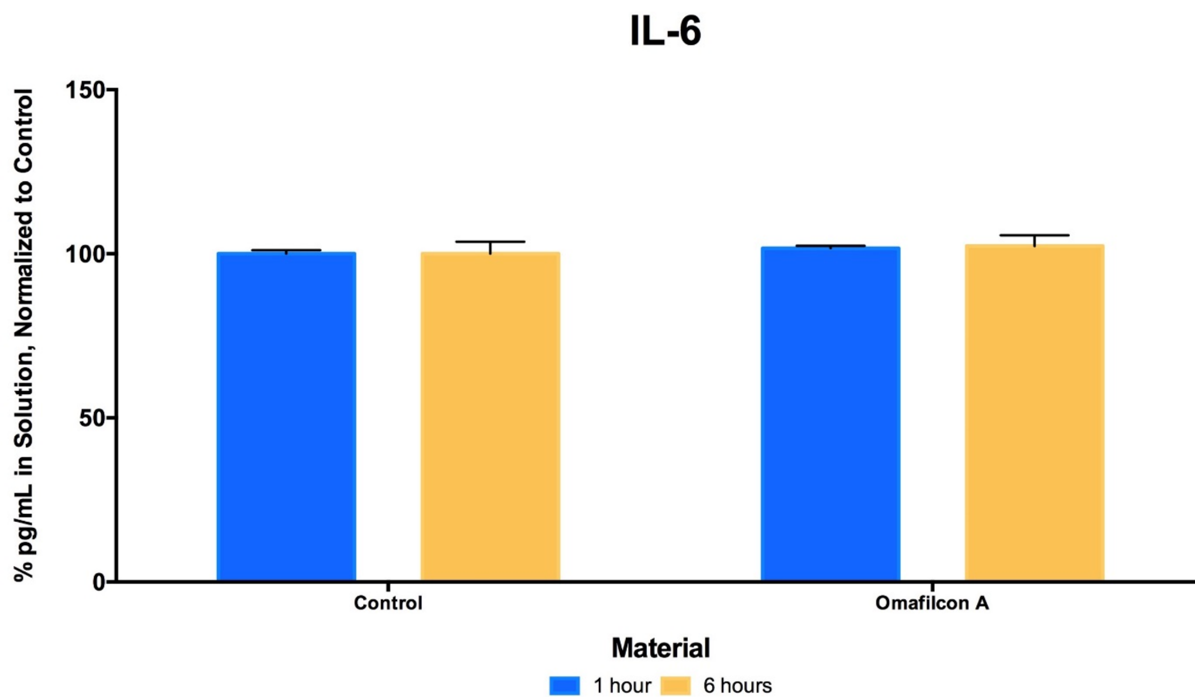

Figure 6-14: Percent pg/mL of IL-6 Remaining in Reduced Volumes of Cytokine Solutions Containing omafilcon A (n = 3) Contact Lens Material, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report ($p > 0.05$).

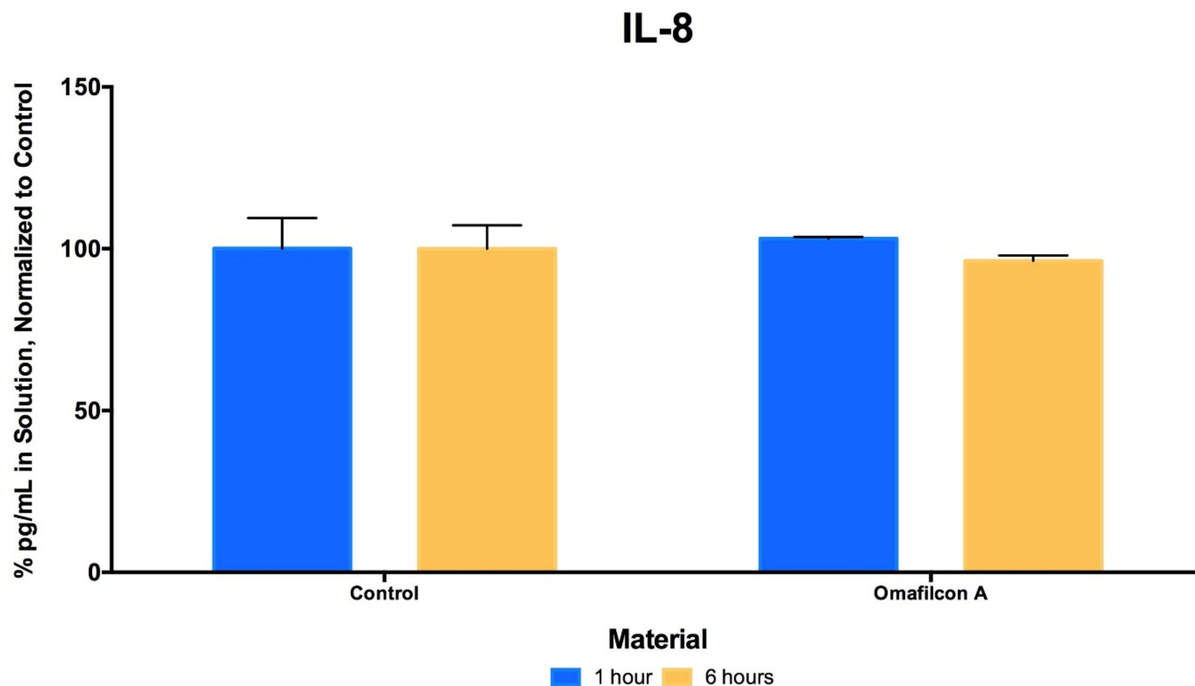

Figure 6-15: Percent pg/mL of IL-8 Remaining in Reduced Volumes of Cytokine Solutions Containing omafilcon A (n = 3) Contact Lens Material, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report ($p > 0.05$).

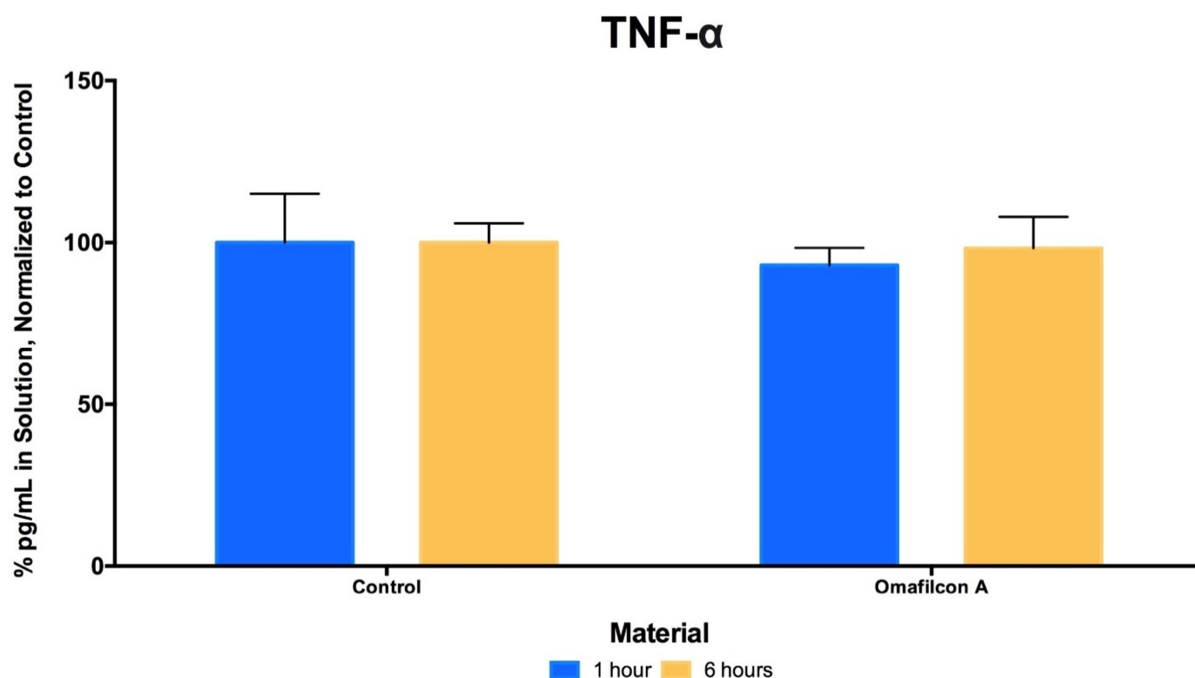

Figure 6-16: Percent pg/mL of TNF-α Remaining in Reduced Volumes of Cytokine Solutions Containing omafilcon A (n = 3) Contact Lens Material, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report ($p > 0.05$).

6.4.4 Materials and Methods – Experiment #2

The second "reduced soaking volume" experiment utilized polypropylene specimen cups (VWR, Radnor, PA) and sponges cut to sizes that would fit inside the cups, covering the majority of the empty space. The sponges were soaked in warm water and a lens mould was placed on top of each sponge. The purpose of utilizing sponges was to create a humid environment once the specimen cups were capped, in order to prevent evaporation of the soaking solution. 1-Day Acuvue Moist (etafilcon A) and Proclear 1 Day (omafilcon A) contact lenses were placed inside the moulds (n = 3) and 200 μL of a prepared cytokine standard blend solution was pipetted on the top surface of the lenses only. Separately, Acuvue Oasys 2-week with Hydraclear Plus (senofilcon A) contact lenses were also evaluated. The time points chosen for these experiments were 2 hours (rather than 1 hour) and 6 hours. At the specified time points, 60 μL of each sample was collected and stored in a -80℃ freezer to later be analyzed with the MSD assay.

6.4.5 Results – Experiment #2

For the experiment involving etafilcon A and omafilcon A materials, theoretical cytokine concentrations were 29.45 pg/mL, 36.8 pg/mL, 28.7 pg/mL and 19.0 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. An aliquot of the cytokine standard blend solution obtained approximately 5 minutes following preparation was later quantified by the MSD assay to have cytokine concentrations of IL-1β, IL-6, IL-8 and TNF-α at 28.62 pg/mL, 36.0 pg/mL, 30.03 pg/mL and 16.13 pg/mL, respectively. At 2 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.97 pg/mL, 35.03 pg/mL, 30.18 pg/mL and 15.17 pg/mL, respectively. At 6 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.64 pg/mL, 33.96 pg/mL, 29.41 pg/mL and 13.73 pg/mL, respectively.

Relative to the control (100%), there was no uptake of any of the four cytokines of interest onto either of the two contact lens materials at 2 hours or at 6 hours. At 2 hours, the percent pg/mL remaining in solution was 95 $\pm$ 2 % (etafilcon A) and 94 $\pm$ 4 % (omafilcon A) for IL-1β (Figure 6-17); 101 $\pm$ 1 % (etafilcon A) and 101 $\pm$ 4 % (omafilcon A) for IL-6 (Figure 6-18); 97 $\pm$ 2 % (etafilcon A) and 96 $\pm$ 6 % (omafilcon A) for IL-8 (Figure 6-19); and 97 $\pm$ 4 % (etafilcon A) and 94 $\pm$ 7 % (omafilcon A) for TNF-α (Figure 6-20). At 6 hours, the percent pg/mL remaining in solution was 95 $\pm$ 2 % (etafilcon A) and 93 $\pm$ 3 % (omafilcon A) for IL-1β (Figure 6-17); 101 $\pm$ 1 % (etafilcon A) and 101 $\pm$ 6 % (omafilcon A) for IL-6 (Figure 6-18); 97 $\pm$ 1 % (etafilcon A) and 97 $\pm$ 1 % (omafilcon A) for IL-8 (Figure 6-19); and 104 $\pm$ 2 % (etafilcon A) and 99 $\pm$ 5 % (omafilcon A) for TNF-α (Figure 6-20). Statistical analyses performed using two-way, repeated measures ANOVAs with Tukey's Multiple Comparisons test (where differences were considered significant if $p < 0.05$), revealed no statistical differences ($p > 0.05$) between the materials themselves, or between the materials and controls for these time points.

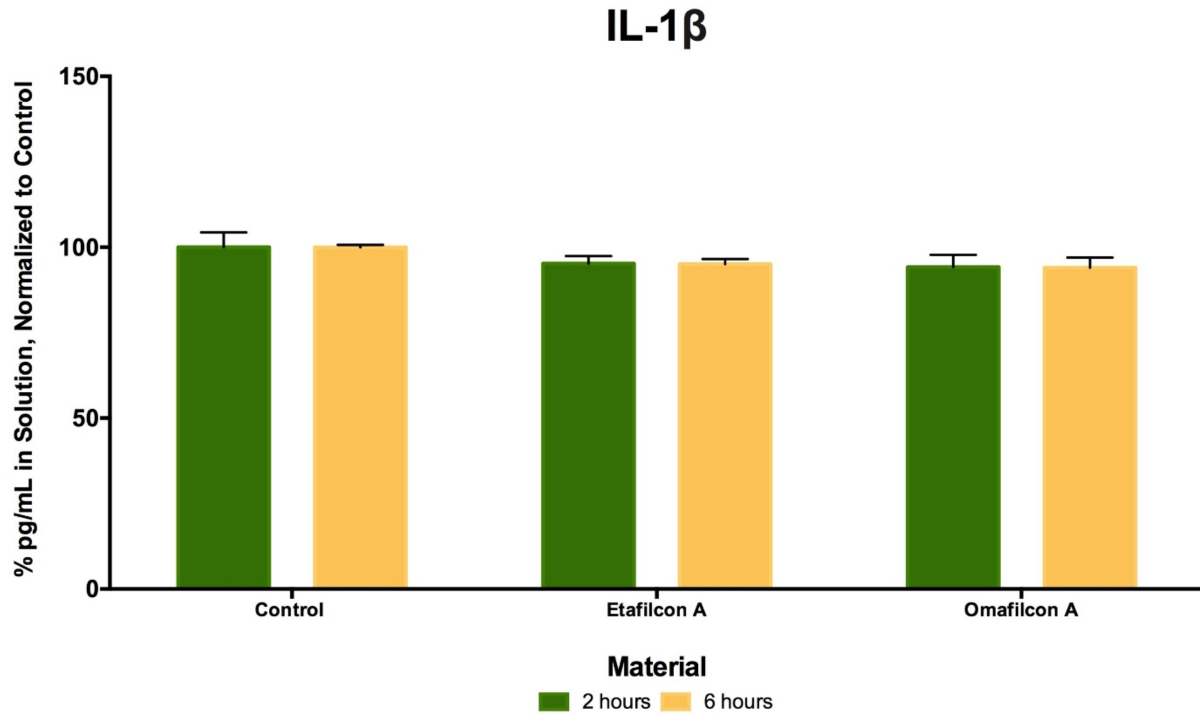

Figure 6-17: Percent pg/mL of IL-1β Remaining in Reduced Volumes of Cytokine Solutions Containing Either etafilcon A or omafilcon A (n = 3) Contact Lens Materials, at 2-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

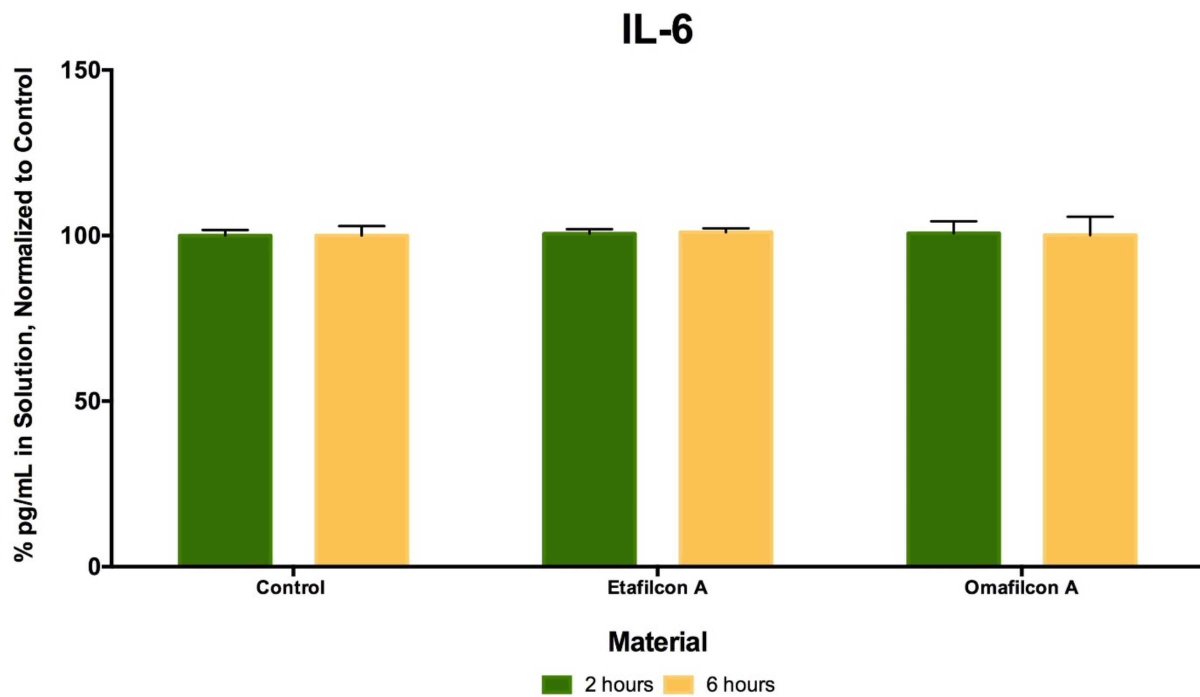

Figure 6-18: Percent pg/mL of IL-6 Remaining in Reduced Volumes of Cytokine Solutions Containing Either etafilcon A or omafilcon A (n = 3) Contact Lens Materials, at 2-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

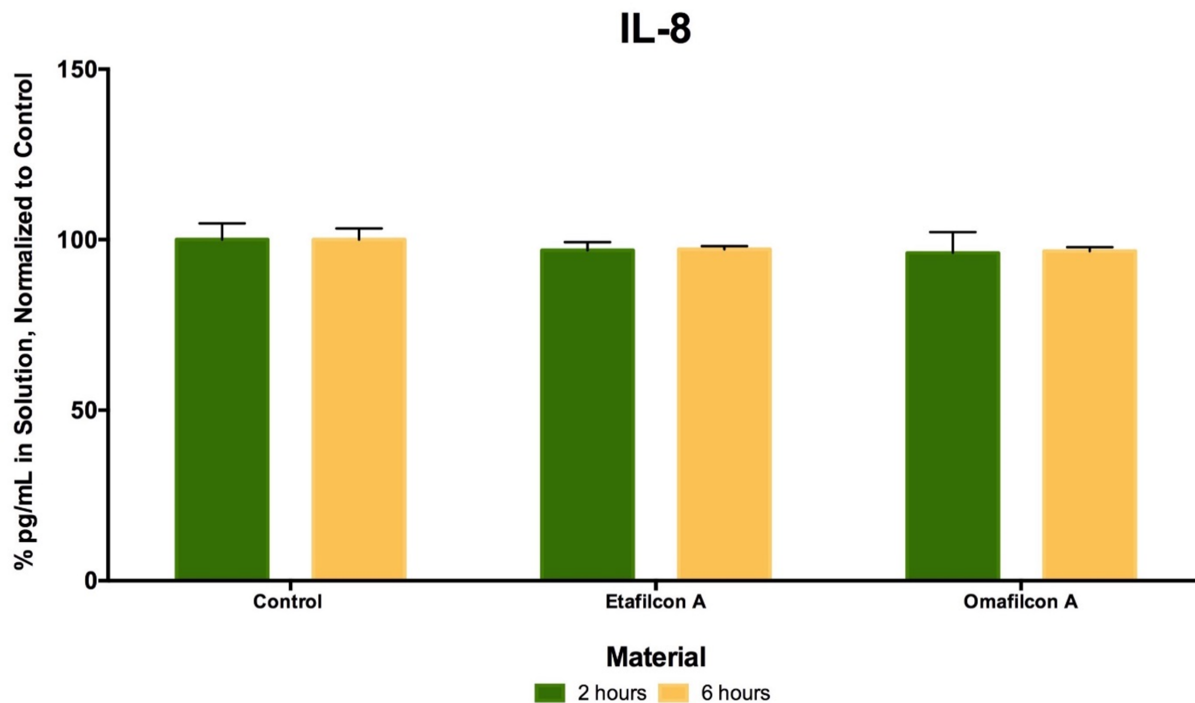

Figure 6-19: Percent pg/mL of IL-8 Remaining in Reduced Volumes of Cytokine Solutions Containing Either etafilcon A or omafilcon A (n = 3) Contact Lens Materials, at 2-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

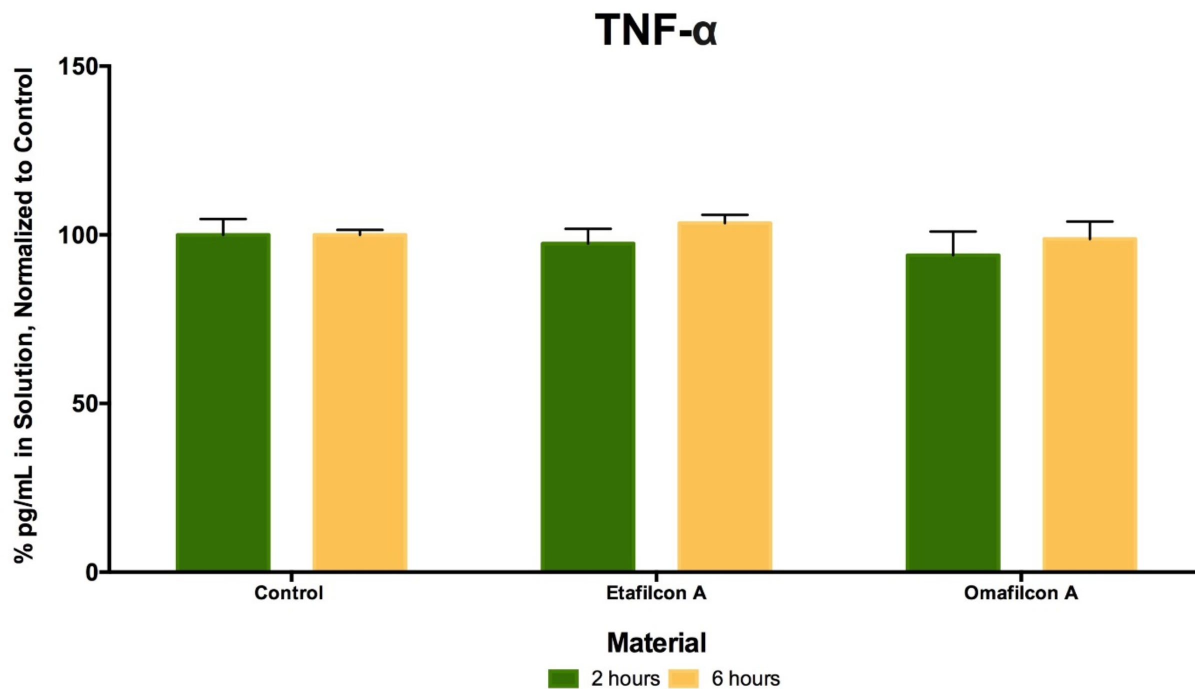

Figure 6-20: Percent pg/mL of TNF-α Remaining in Reduced Volumes of Cytokine Solutions Containing Either etafilcon A or omafilcon A (n = 3) Contact Lens Materials, at 2-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Tests were Performed. No Statistical Differences to Report (p > 0.05).

For the next experiment where the senofilcon A material was investigated only, theoretical cytokine concentrations were identical to the previous experiment with etafilcon A and omafilcon A, as the same calibrator blend was utilized to prepare the soaking solution. According to MSD specifications, reconstituted calibrator blends are stable through three freeze-thaw cycles. Theoretical concentrations were therefore 29.45 pg/mL, 36.8 pg/mL, 28.7 pg/mL and 19 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. An aliquot of the cytokine standard blend solution obtained approximately 5 minutes following preparation was later quantified by the MSD assay to have cytokine concentrations of IL-1β, IL-6, IL-8 and TNF-α at 23.59 pg/mL, 27.88 pg/mL, 23.76 pg/mL and 9.22 pg/mL, respectively. At 2 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 25.17 pg/mL, 30.06 pg/mL, 25.09 pg/mL and 9.18 pg/mL, respectively. At 6 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 24.81 pg/mL, 29.42 pg/mL, 25.32 pg/mL and 8.81 pg/mL, respectively. Although experimental concentrations deviated somewhat from the theoretical concentrations (perhaps as a result of the freeze-thaw), especially for TNF-α, this did not affect the results, as individual results were presented relative to the control for specific time points and hence any deviations were accounted for.

Relative to the control (100%), there was no uptake of any of the four cytokines of interest onto the senofilcon A material at 2 hours or at 6 hours. The percent pg/mL remaining in solution after 2 hours was 99 $\pm$ 3 % for IL-1β (Figure 6-21); 101 $\pm$ 4 % for IL-6 (Figure 6-22); 101 $\pm$ 2 % for IL-8 (Figure 6-23); and 102 $\pm$ 3 % for TNF-α (Figure 6-24). In contrast at 6 hours, the percent pg/mL remaining in solution was 97 $\pm$ 3 % for IL-1β (Figure 6-21); 102 $\pm$ 6 % for IL-6 (Figure 6-22); 100 $\pm$ 0.3 % for IL-8 (Figure 6-23); and 99 $\pm$ 3 % for TNF-α (Figure 6-24). Statistical analyses performed using two-way, repeated measures ANOVAs with Sidak's Multiple

Comparisons test (where differences were considered significant if $p < 0.05$), revealed no statistical differences ($p > 0.05$) between senofilcon A and the control for either time points.

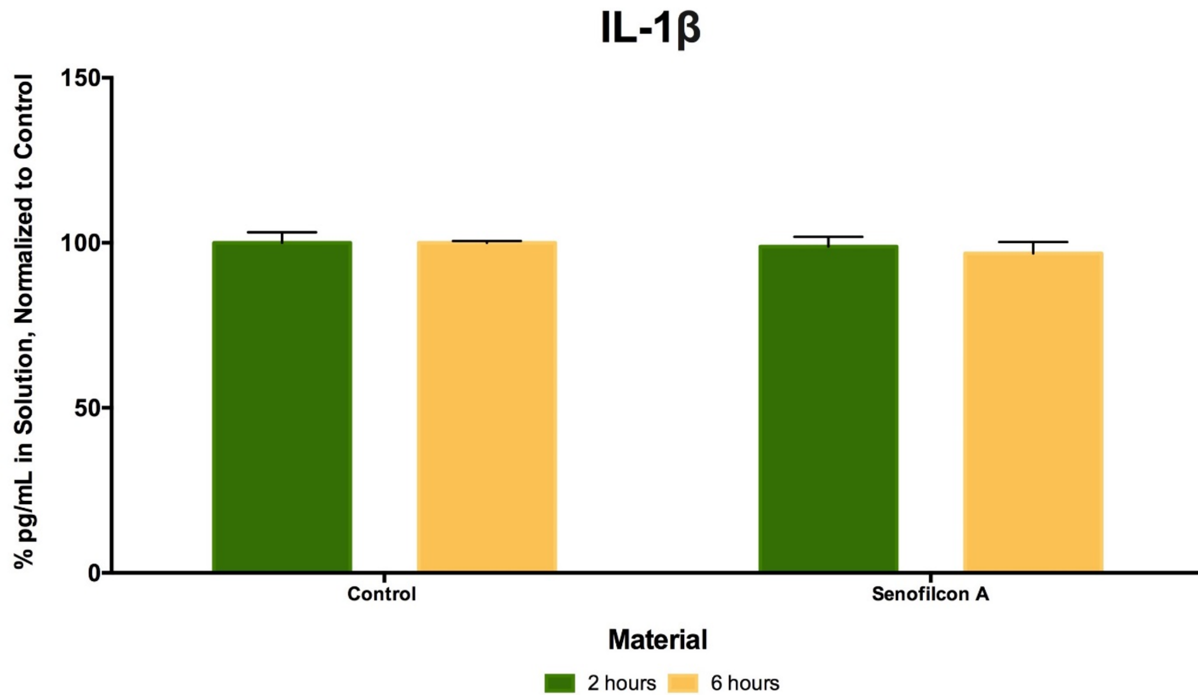

Figure 6-21: Percent pg/mL of IL-1β Remaining in Reduced Volumes of Cytokine Solutions Containing senofilcon A (n = 3) Contact Lens Material, at 2-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report (p > 0.05).

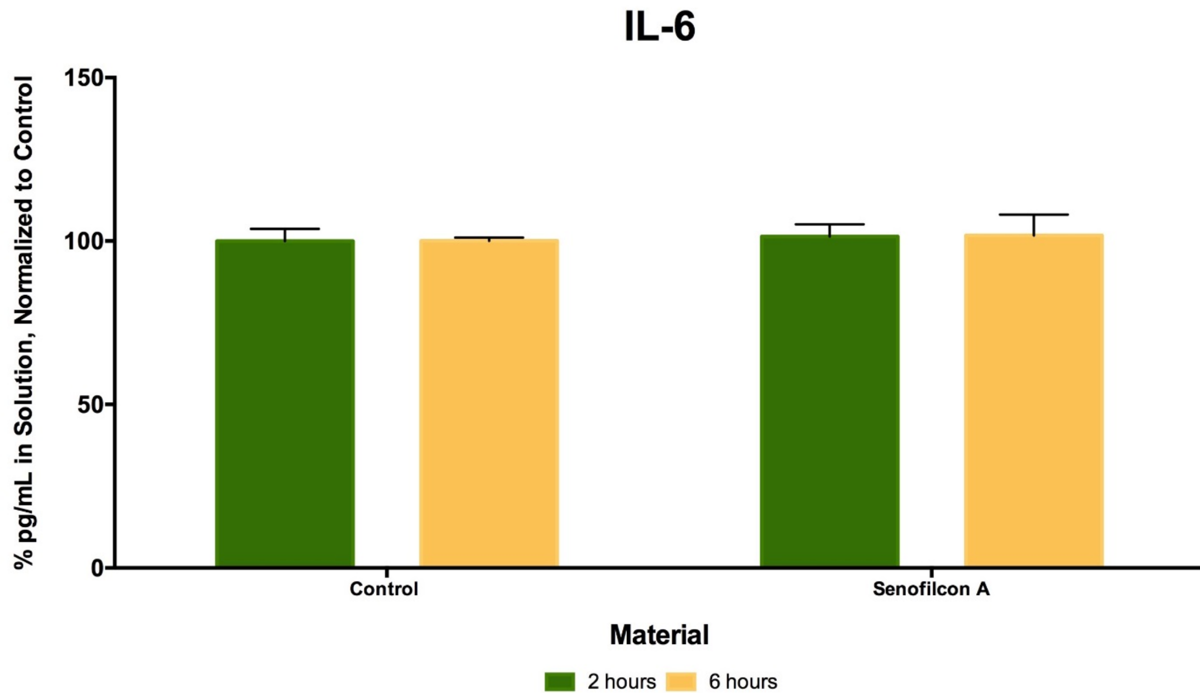

Figure 6-22: Percent pg/mL of IL-6 Remaining in Reduced Volumes of Cytokine Solutions Containing senofilcon A (n = 3) Contact Lens Material, at 2-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report (p > 0.05).

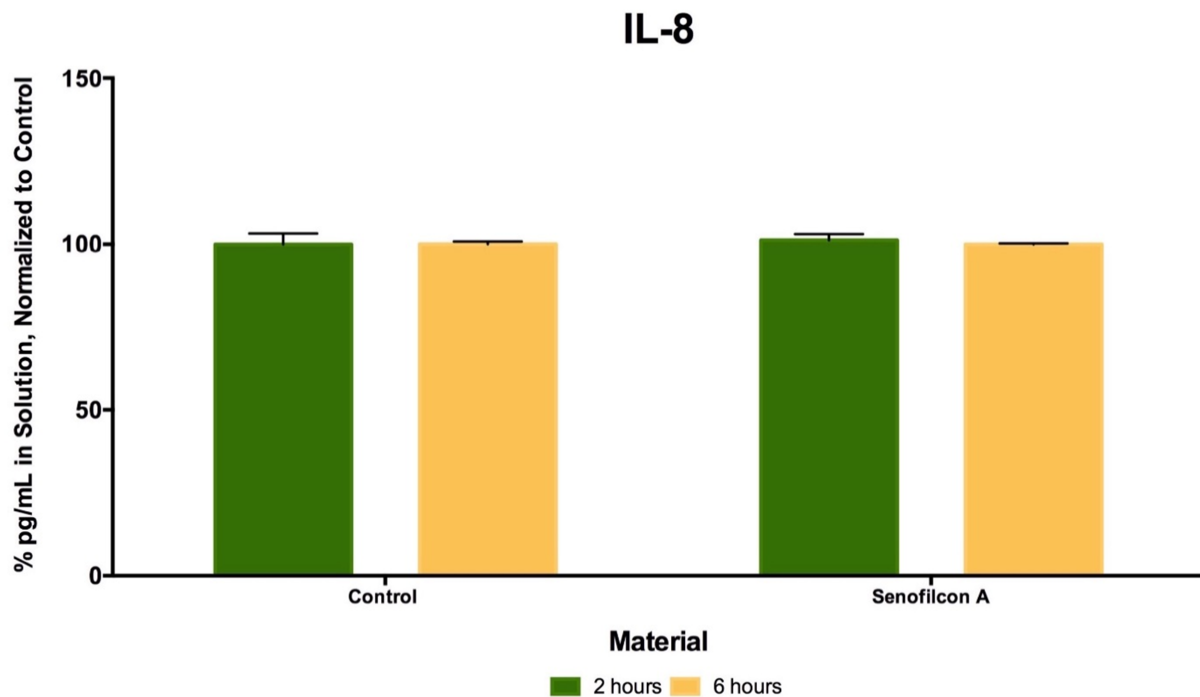

Figure 6-23: Percent pg/mL of IL-8 Remaining in Reduced Volumes of Cytokine Solutions Containing senofilcon A (n = 3) Contact Lens Material, at 2-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report (p > 0.05).

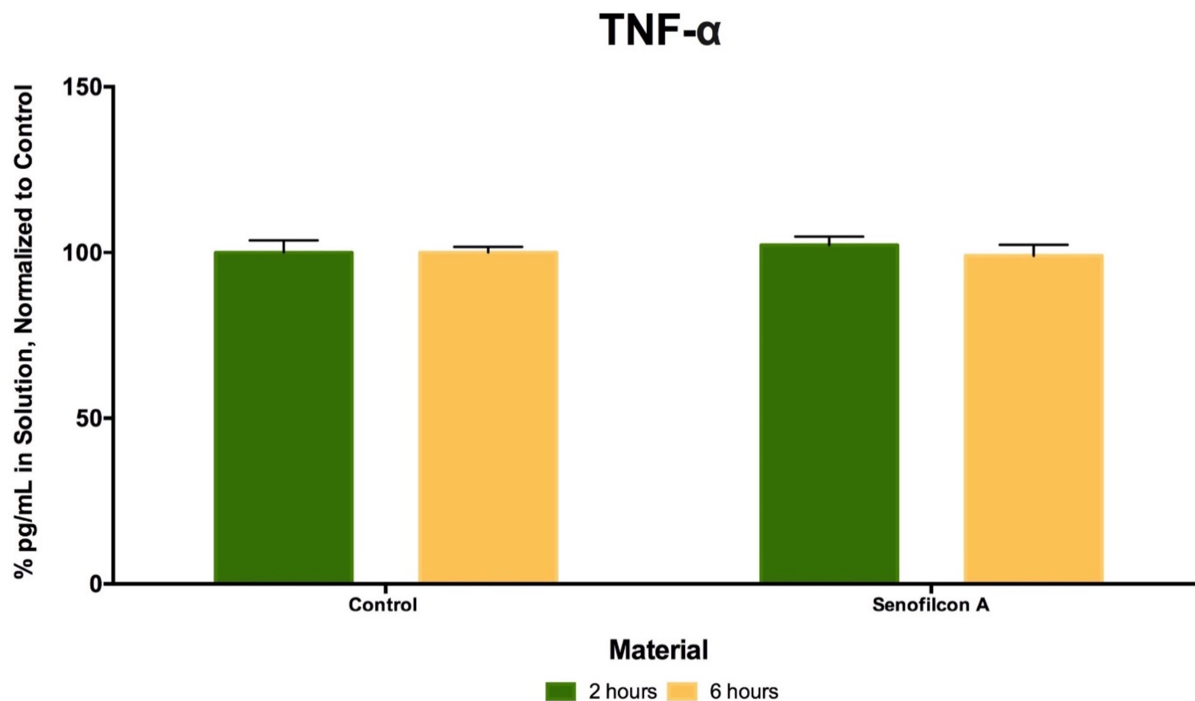

Figure 6-24: Percent pg/mL of TNF-α Remaining in Reduced Volumes of Cytokine Solutions Containing senofilcon A (n = 3) Contact Lens Material, at 2-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report ($p > 0.05$).

6.4.6 Discussion – Reduced Soaking Volume Experiments #1 and #2

Reducing the soaking volume from 1000 μL to 400 μL and a further reduction to 200 μL, did not appear to produce results that differed vastly from that which was already gathered through the previous studies. While no uptake was quantified from the cytokine solutions containing etafilcon A, omafilcon A and senofilcon A materials, even when soaked in smaller volumes, this did not necessarily negate the likelihood that reducing the soaking volume could facilitate quantification of uptake by the MSD instrument. While it is possible that reducing the soaking volume could improve the detection of uptake, as a result of the differences in the initial and final concentrations being larger and potentially more easily differentiated from each other on a picogram scale (as described in section 6.4.1), this would not be evident if the contact lens materials resist cytokine uptake in the first place.

Given the number of experiments performed thus far and the common result observed among them of little to no uptake of the cytokines of interest onto etafilcon A, omafilcon A or senofilcon A contact lens materials, it appeared more likely that these materials did not interact with the cytokines of interest in such a way that would allow for the cytokines to be taken up onto their surface over time, rather than the soaking volume or some other portion of the methodology preventing proper quantification of the cytokines in solution. However, the next set of experiments attempted to focus more closely on the interaction between cytokines and contact lens materials by better modelling ocular conditions, through the use of artificial tear solution (ATS), as a means to improve the interaction between the cytokines and the contact lens materials, and potentially result in more enhanced levels of uptake.

6.5 Investigating the Effects of Using Artificial Tear Solution on Cytokine Uptake by Contact Lenses

6.5.1 Introduction

The "artificial tear solution" (ATS) used in these studies closely mimics the human tear film as it is composed of many of the same constituents, including a complex salt solution of calcium chloride (0.5 mM), glucose (0.2 mM), hydrochloric acid (26.0 mM), potassium chloride (16.0 mM), potassium hydrogen carbonate (3.0 mM), sodium carbonate (12.0 mM), sodium chloride (90.0 mM), sodium citrate (1.5 mM), sodium phosphate dibasic (24.0 mM) and urea (1.2 mM); a 6-lipid stock of cholesterol (0.0018 mg/mL), cholesteryl oleate (0.024 mg/mL), oleic acid (0.0018 mg/mL), oleic acid methyl ester (0.012 mg/mL), phosphatidylcholine (0.0005 mg/mL) and triolein (0.016 mg/mL); in addition to bovine serum albumin (0.2 mg/mL), bovine colostrum lactoferrin (1.80 mg/mL), bovine immunoglobulin G (0.02 mg/mL), bovine submaxillary mucin (0.15 mg/mL) and hen egg lysozyme (1.90 mg/mL).[109] ATS can also share similar constituents to

that of Diluent 2, including bovine serum albumin, immunoglobulin G and sodium chloride, as well as a similar pH (typically between 7.2 – 7.6 for ATS versus 7.4 for Diluent 2).

The use of ATS in the following experiments aimed to more accurately model ocular conditions and when compared to the experiments performed in a Diluent 2 cytokine solution, could provide insight into whether there is a component in the human tear film that may potentially aid cytokine uptake onto the contact lens materials of interest. It was hypothesized that perhaps this component could somehow enhance cytokine uptake, which would not otherwise be evident through the *in vitro* experiments presented in this chapter thus far, where the cytokines were suspended in a solution of Diluent 2 alone. Utilizing ATS, however, could potentially allow for the exploration of a possible indirect route of cytokine uptake onto contact lens materials – one whereby the cytokines bind a component of the human tear film and it is this component that, along with the bound cytokines, would deposit onto the surface of the contact lens materials.

6.5.2 Materials and Methods – ATS Experiment #1

Two experiments were performed utilizing ATS. For both experiments, ATS was prepared in-house (protocol undisclosed), without bovine immunoglobulin G (for cost purposes), with a pH between 7.2 – 7.6 (typically 7.4) and the concentrations of all other constituents as outlined in section 6.5.1). In the first experiment, 1-Day Acuvue Moist (etafilcon A), Proclear 1 Day (omafilcon A) and Acuvue Oasys 2-week with Hydraclear Plus (senofilcon A), were soaked overnight (between 12-24 hours) in polypropylene tubes containing 1 mL ATS. This step followed the routine PBS soak to remove any contact lens packaging solution, as described in section 4.3. After the ATS soak, the lenses were placed in polypropylene tubes (n = 3) containing 1 mL of the cytokine standard blend solution (prepared in Diluent 2). The contact lenses were dried on Fisherbrand lens paper in between the PBS and ATS soaks, as well as prior to being transferred

from ATS to the standard blend solution. Alike to experiment #2 described in section 6.3.6, 60 μL of each sample was pipetted out from the tubes at the half-hour, 1-hour and 6-hour time points, and stored in a -80℃ freezer to later be analyzed by the MSD assay. Theoretical concentrations of IL-1β, IL-6, IL-8 and TNF-α were 26.0 pg/mL, 37.45 pg/mL, 27.3 pg/mL and 15.55 pg/mL, respectively.

6.5.3 Results – ATS Experiment #1

After a half-hour, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 27.77 pg/mL, 36.55 pg/mL, 27.90 pg/mL and 14.32 pg/mL, respectively. At 1 hour, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.98 pg/mL, 39.08 pg/mL, 28.19 pg/mL and 14.07 pg/mL, respectively. At 6 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 27.0 pg/mL, 38.21 pg/mL, 27.17 pg/mL and 11.2 pg/mL, respectively. Statistical analyses were performed using two-way, repeated measures ANOVAs with Tukey's Multiple Comparisons test, where differences were considered significant if $p < 0.05$. The results obtained in this experiment were quite similar to those obtained from the experiment outlined in section 6.3.7. These experiments were essentially identical, with the only difference being that this experiment involved an additional soaking step in ATS, prior to investigating uptake using the cytokine standard blend solution in Diluent 2.

Similar to the results outlined in section 6.3.7, at the half-hour time point, there was no uptake of any of the four cytokines of interest onto either etafilcon A, omafilcon A, or senofilcon A materials. Relative to the control (100%), the percent pg/mL remaining in solution at this time point was 99 $\pm$ 5 % (etafilcon A), 99 $\pm$ 0.5 % (omafilcon A) and 103 $\pm$ 4 % (senofilcon A) for IL-1β (Figure 6-25); 97 $\pm$ 6 % (etafilcon A), 103 $\pm$ 3 % (omafilcon A) and 102 $\pm$ 4 % (senofilcon A) for IL-6 (Figure 6-26); 95 $\pm$ 3 % (etafilcon A), 101 $\pm$ 3 % (omafilcon A) and 102 $\pm$ 2 %

(senofilcon A) for IL-8 (Figure 6-27); and 97 $\pm$ 6 % (etafilcon A), 96 $\pm$ 1 % (omafilcon A) and 102 $\pm$ 2 % (senofilcon A) for TNF-α (Figure 6-28). There were no statistical differences ($p > 0.05$) between the control or the materials for any of the cytokines at this time point.

At the 1-hour time point, there was no uptake of IL-1β, IL-6, or IL-8 onto either etafilcon A, omafilcon A, or senofilcon A materials. Relative to the control (100%), the percent pg/mL remaining in solution at this time point was 93 $\pm$ 5 % (etafilcon A), 93 $\pm$ 2 % (omafilcon A) and 97 $\pm$ 3 % (senofilcon A) for IL-1β (Figure 6-25); 96 $\pm$ 9 % (etafilcon A), 102 $\pm$ 1 % (omafilcon A) and 99 $\pm$ 6 % (senofilcon A) for IL-6 (Figure 6-26); and 101 $\pm$ 6 % (etafilcon A), 98 $\pm$ 2 % (omafilcon A) and 101 $\pm$ 5 % (senofilcon A) for IL-8 (Figure 6-27). For TNF-α (Figure 6-28), the percent pg/mL remaining in solution after 1 hour was 93 $\pm$ 7 % (etafilcon A), 85 $\pm$ 5 % (omafilcon A) and 101 $\pm$ 4 % (senofilcon A). There was a statistical difference for omafilcon A relative to the control (adjusted P-value 0.0388), suggesting potential uptake of TNF-α onto this material, as well as a statistical difference for omafilcon A relative to senofilcon A (adjusted P-value 0.0207) at this time point, both of which are defined by the star (*) symbol. This was similar to the result obtained for TNF-α in section 6.3.7 (Figure 6-12), where omafilcon A was also statistically different from the control at 1 hour.

At the 6-hour time point, there was no uptake of IL-1β, IL-6, or IL-8 onto either etafilcon A, omafilcon A, or senofilcon A materials. Relative to the control (100%), the percent pg/mL remaining in solution at this time point was 96 $\pm$ 4 % (etafilcon A), 95 $\pm$ 1 % (omafilcon A) and 101 $\pm$ 3 % (senofilcon A) for IL-1β (Figure 6-25); 98 $\pm$ 9 % (etafilcon A), 103 $\pm$ 3 % (omafilcon A) and 106 $\pm$ 3 % (senofilcon A) for IL-6 (Figure 6-26); and 93 $\pm$ 8 % (etafilcon A), 93 $\pm$ 3 % (omafilcon A) and 103 $\pm$ 7 % (senofilcon A) for IL-8 (Figure 6-27). For TNF-α (Figure 6-28), the percent pg/mL remaining in solution at this time point was 93 $\pm$ 4 % (etafilcon A), 85 $\pm$ 11 %

(omafilcon A) and 102 $\pm$ 12 % (senofilcon A). As defined by the star (*) symbol, there was a statistical difference for omafilcon A relative to the control (adjusted P-value 0.0364), suggesting potential uptake of TNF-α onto this material, and omafilcon A was also statistically different from senofilcon A (adjusted P-value 0.0150) at this time point. In this case, this result was not similar to that obtained for TNF-α in section 6.3.7 (Figure 6-12), where omafilcon A was not statistically different from the control at 6 hours.

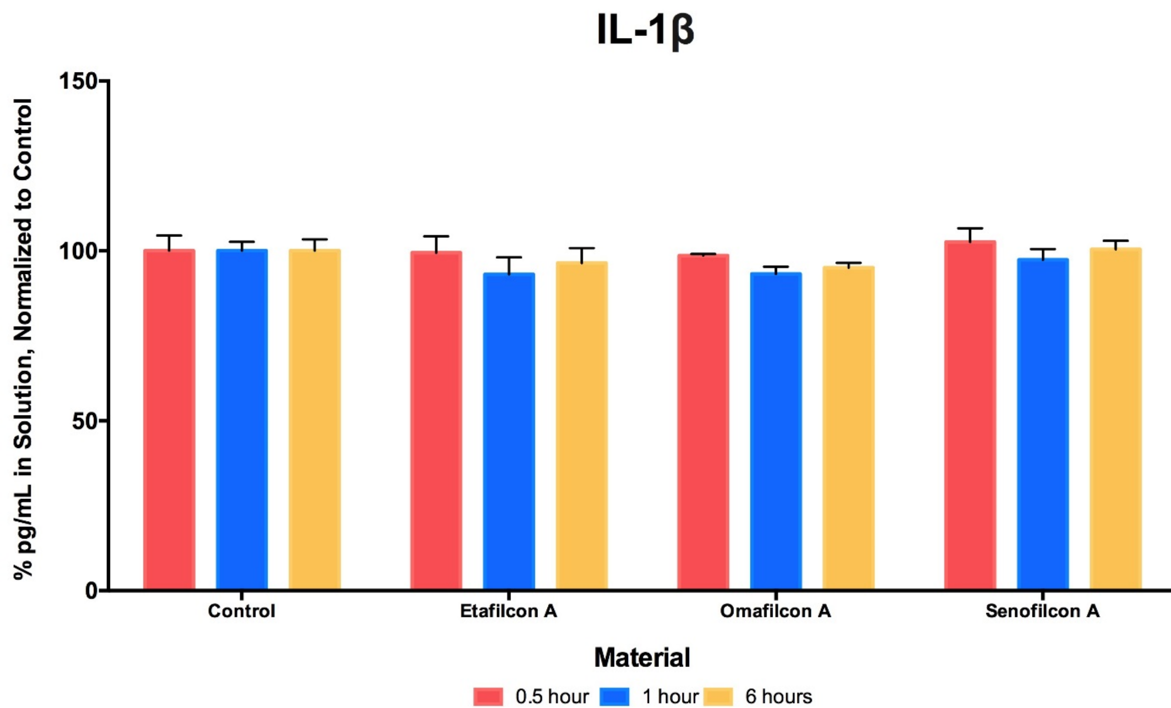

Figure 6-25: Percent pg/mL of IL-1β Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials Pre-Soaked in ATS, at Half-Hour, 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

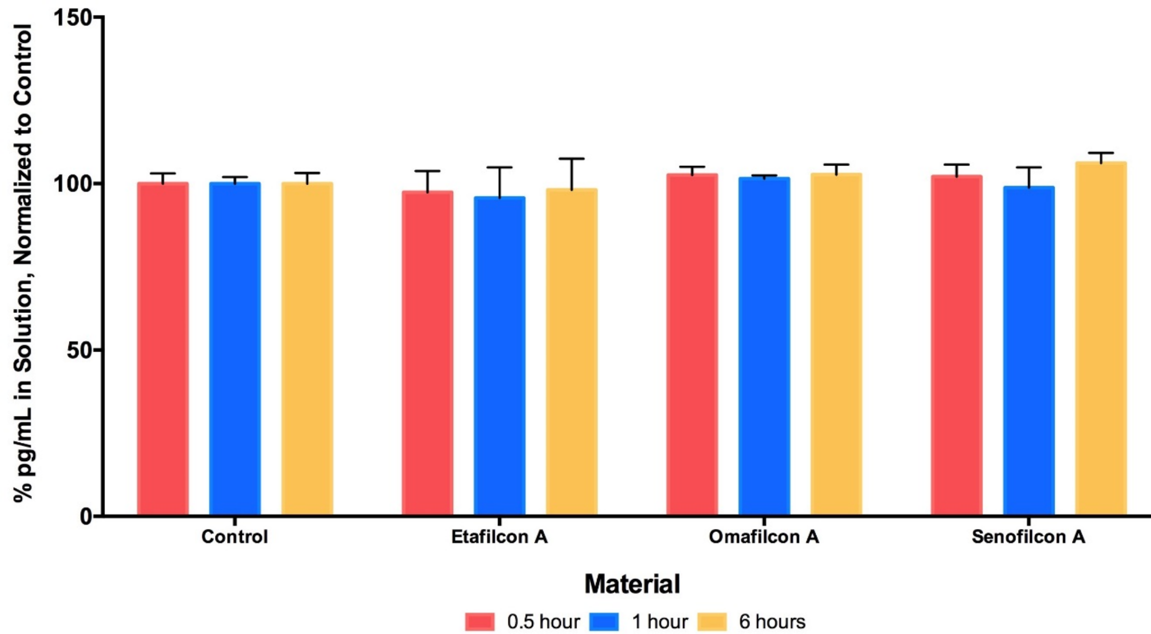

Figure 6-26: Percent pg/mL of IL-6 Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials Pre-Soaked in ATS, at Half-Hour, 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

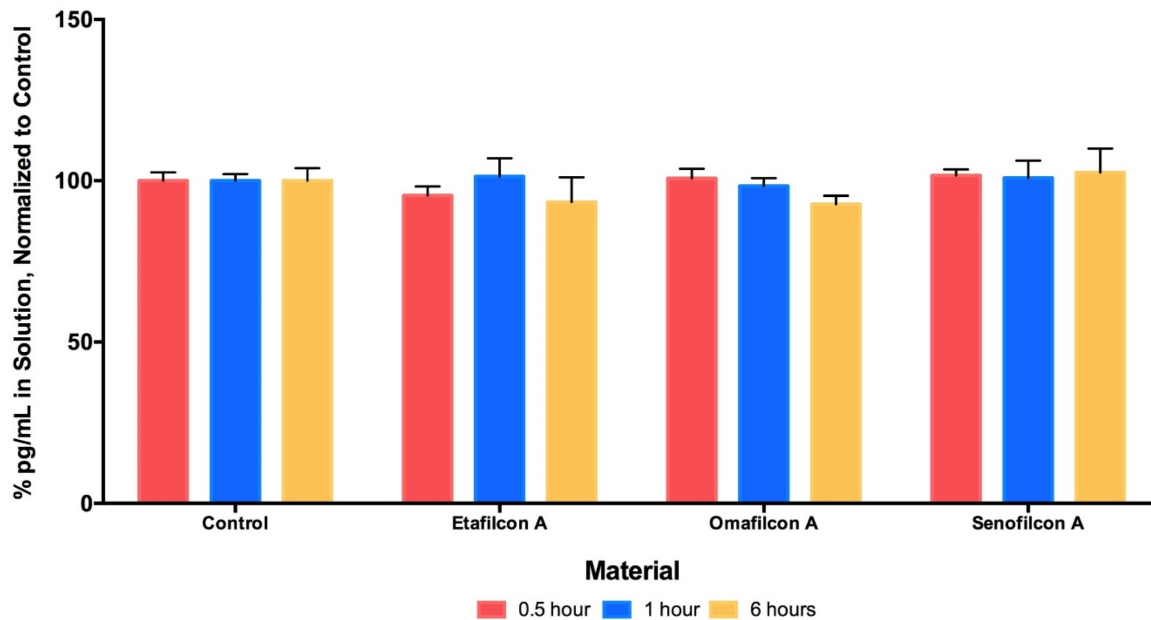

Figure 6-27: Percent pg/mL of IL-8 Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials Pre-Soaked in ATS, at Half-Hour, 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Tests were Performed. No Statistical Differences to Report ($p > 0.05$).

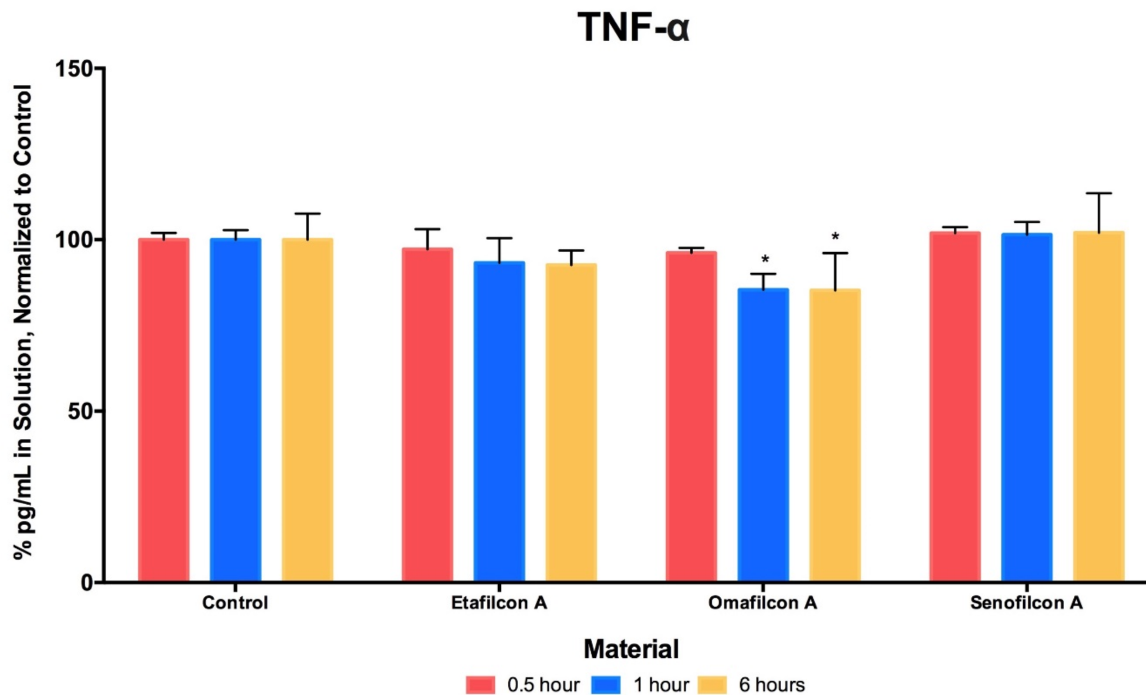

Figure 6-28: Percent pg/mL of TNF-α Remaining in Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials Pre Soaked in ATS, at Half-Hour, 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. At 1 hour and 6 hours, omafilcon A was Statistically Different from the Respective Controls and from senofilcon A, as Defined by the Star (*) Symbols.

6.5.4 Materials and Methods – ATS Experiment #2

In contrast to ATS experiment #1 described above, the second experiment was performed entirely in ATS. After soaking 1-Day Acuvue Moist (etafilcon A), Proclear 1 Day (omafilcon A) and Acuvue Oasys 2-week with Hydraclear Plus (senofilcon A) contact lenses in PBS and drying them on Fisherband lens paper, the lenses were transferred into polypropylene tubes (n = 3) containing 1 mL of a cytokine solution in ATS. This solution was prepared by reconstituting the lyophilized MSD calibrator blend in 1 mL (1000 μL) of Diluent 2, and then transferring 650 μL of the 1000 μL reconstituted solution into 12,350 μL of ATS, in order to provide the desired final volume of 13,000 μL for the experiment. The samples were evaluated for cytokine uptake after a 6-hour time period, when 60 μL was pipetted out from each tube and stored in a -80°C freezer to later be analyzed by the MSD assay. As per MSD's guidelines, it was necessary for the lyophilized

calibrator blend to be reconstituted in MSD Diluent 2 and therefore it could not be reconstituted in ATS. Apart from the 650 μL volume of Diluent 2 in the overall solution, the remaining 12,350 μL volume was ATS, whereas previous experiments would have entirely utilized Diluent 2 for the soaking solution.

6.5.5 Results – ATS Experiment #2

Theoretical concentrations of IL-1β, IL-6, IL-8 and TNF-α were 26.0 pg/mL, 37.45 pg/mL, 27.3 pg/mL and 15.55 pg/mL, respectively. An aliquot of the cytokine standard blend solution obtained approximately 5 minutes following preparation was later quantified by the MSD assay to have cytokine concentrations of 23.68 pg/mL, 37.91 pg/mL, 28.11 pg/mL and 14.43 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. At 6 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 19.34 pg/mL, 35.64 pg/mL, 30.68 pg/mL and 15.28 pg/mL, respectively. Statistical analyses were performed using ordinary one-way ANOVAs with Tukey's Multiple Comparisons test. Differences were considered significant if $p < 0.05$.

There did not appear to be any uptake of IL-1β (Figure 6-29) relative to the control (100%) for either etafilcon A (106 $\pm$ 2 %), omafilcon A (102 $\pm$ 1 %) or senofilcon A (97 $\pm$ 3 %). Etafilcon A was statistically different from the control (adjusted P-value 0.0496), as defined by the number (#) symbol, however, this was as a result of having a value greater than 100% of the control and was therefore not indicative of uptake. Additionally, senofilcon A was statistically different from etafilcon A (adjusted P-value 0.0040) and omafilcon A (adjusted P-value 0.0479), as defined by the star (*) symbol, although again, this did not indicate uptake, as neither of these materials were statistically different from the 6-hour control.

There was no uptake of IL-6 (Figure 6-30), IL-8 (Figure 6-31), or TNF-α (Figure 6-32) onto any of the contact lens materials tested either. Relative to the control (100%) for IL-6, the

percent pg/mL remaining in solution was 109 ± 4 % (etafilcon A), 110 ± 8 % (omafilcon A) and 108 ± 7 % (senofilcon A). Relative to the control (100%) for IL-8, the percent pg/mL remaining in solution was 102 ± 3 % (etafilcon A), 102 ± 3 % (omafilcon A) and 99 ± 7 % (senofilcon A). Lastly, relative to the control (100%) for TNF-α, the percent pg/mL remaining in solution was 99 ± 7 % (etafilcon A), 96 ± 2 % (omafilcon A) and 98 ± 4 % (senofilcon A). Statistical analyses did not reveal any differences between the control and any of these materials at 6 hours, or between the materials themselves.

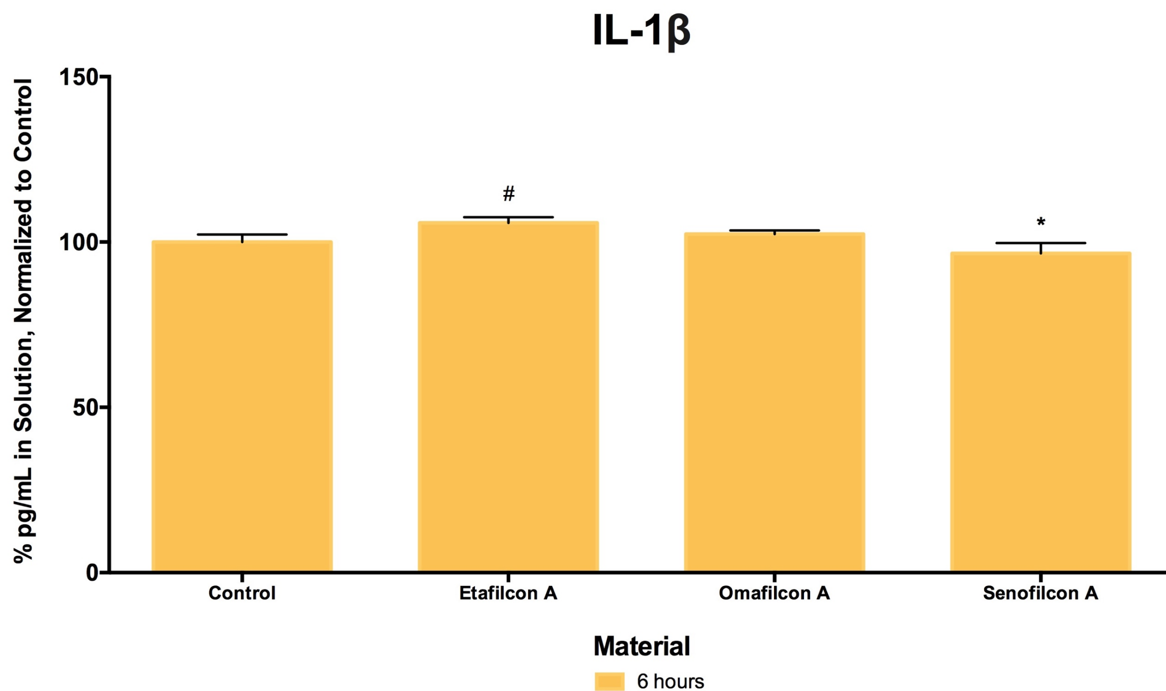

Figure 6-29: Percent pg/mL of IL-1β Remaining in ATS Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials at 6 Hours. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. Senofilcon A was Statistically Different from both etafilcon A and omafilcon A Only, as Defined by the Star (*) Symbol, but was Not Statistically Different from the Control. Etafilcon A was Statistically Different from the Control, as Defined by the Number (#) Symbol, Due to Having a Value Greater than 100% of the Control.

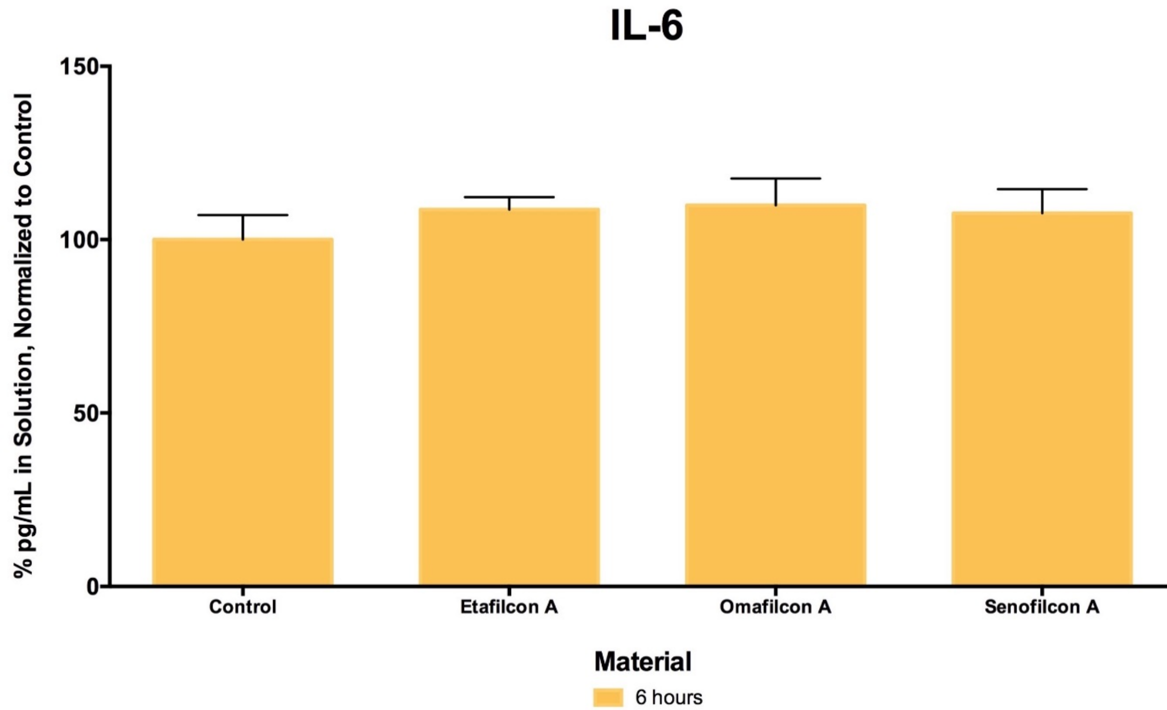

Figure 6-30: Percent pg/mL of IL-6 Remaining in ATS Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials at 6 Hours. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

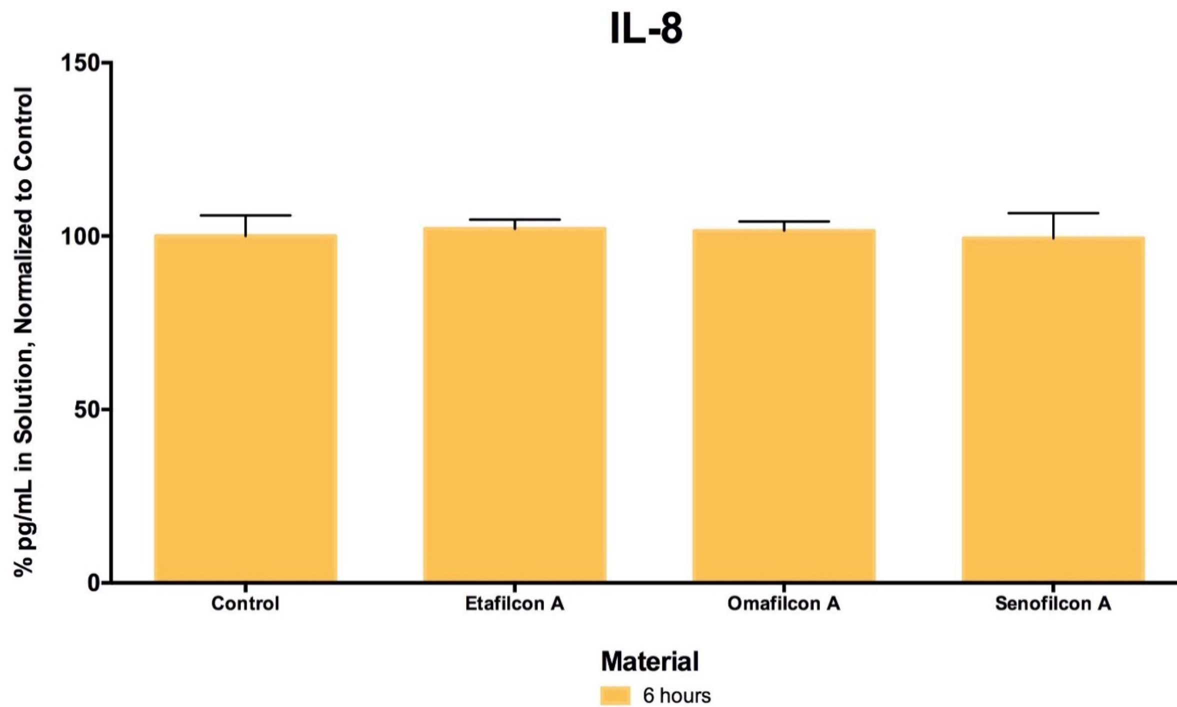

Figure 6-31: Percent pg/mL of IL-8 Remaining in ATS Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials at 6 Hours. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

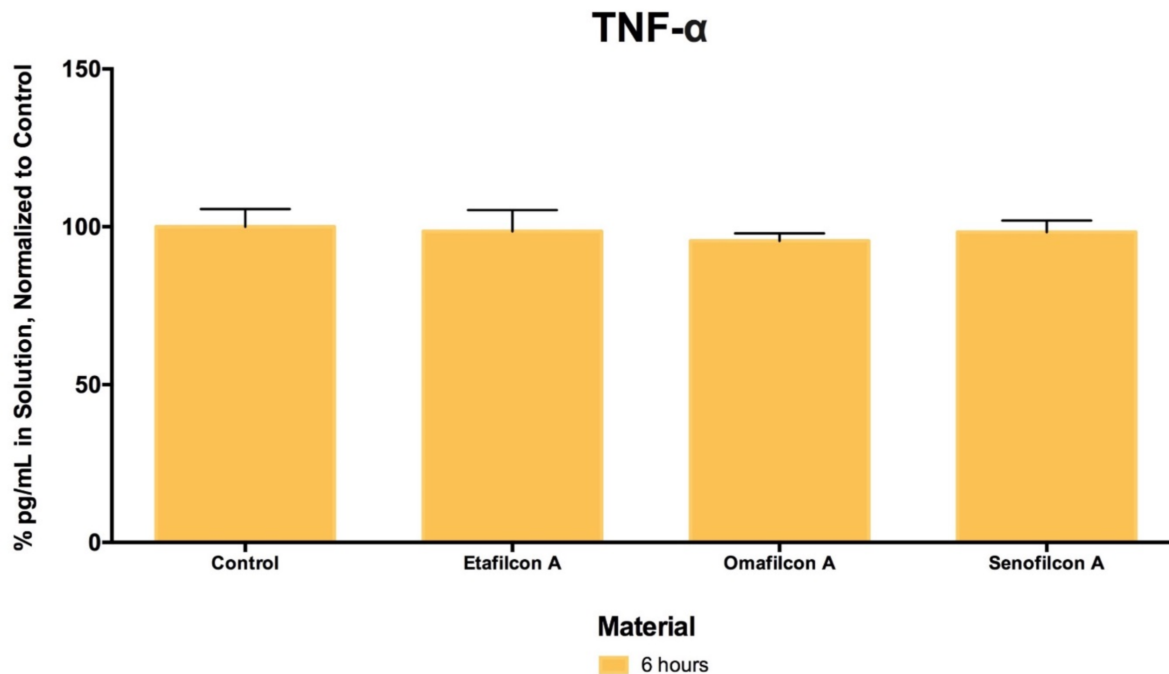

Figure 6-32: Percent pg/mL of TNF-α Remaining in ATS Cytokine Solutions Containing Either etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials at 6 Hours. Ordinary One-Way ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

6.5.6 Discussion – ATS Experiments #1 and #2

While there may have been some uptake onto omafilcon A contact lens material at 1 hour (85 ± 5 %) and at 6 hours (85 ± 11 %), in comparison to the respective controls, when this material was pre-soaked in ATS prior to being soaked in the cytokine solution in Diluent 2, this result was not observed for any of the other materials tested, regardless of if the materials were either pre-soaked in ATS or soaked entirely in an ATS solution containing cytokines. Thus, there did not appear to be much difference in cytokine uptake onto the contact lens materials under these experimental conditions, in comparison to when the materials were soaked in a Diluent 2 cytokine solution only. It is important to note that the ATS utilized in the presented experiments above did not include all components of the tear film (such as IgG, IgM or IgA), and that many different versions of ATS can be prepared. Since the results obtained from ATS experiments #1 and #2 were in accordance with previous results, however, this helped to support the use of Diluent 2 for

preparing cytokine solutions in subsequent experiments, and ATS was no longer utilized due to cost purposes.

6.6 Testing Additional Contact Lens Materials

6.6.1 Introduction

The experiments presented throughout Chapter 6 thus far explored cytokine uptake onto two high-water content conventional hydrogels differing in their ionicity. This was the ionic etafilcon A (1-Day Acuvue Moist) and nonionic omafilcon A (Proclear 1 Day) contact lens materials. Additionally, senofilcon A (Acuvue Oasys 2-week with Hydraclear Plus), a low water, nonionic SiHy, was explored for contrast. Of these, omafilcon A appeared to be the material that exhibited some amount of uptake, though this was not consistently evident throughout all of the experiments, but rather an occasional result that was observed for either IL-1β or TNF-α cytokines specifically.

To contrast the senofilcon A material, SiHy materials of high water content, nonionic property, i.e., somofilcon A (Clariti 1 Day) and low water content, nonionic property, i.e., comfilcon A (Biofinity) were also investigated, in addition to a low water content, but ionic SiHy material i.e., balafilcon A (PureVision). Studying uptake of the four cytokines of interest onto these additional materials would allow for further comparisons between somofilcon A and omafilcon A materials, which differ only in their class of material (SiHy vs. conventional hydrogel, respectively), but share both high water content and nonionic characteristics. In contrast, comparisons between somofilcon A and senofilcon A would also allow for an investigation of differences in uptake between the same class of soft contact lens material (SiHy), that share a nonionic character but differ in their water content levels. As well, comparisons between balafilcon A and etafilcon A could also be drawn for ionic materials of different classes (SiHy vs. conventional hydrogel, respectively) and different water content levels (low water content vs. high

water content, respectively). In addition, between comfilcon A and omafilcon A, comparisons could be drawn in regard to materials of different classes that share a nonionic character, but are of different water content levels as well.

6.6.2 Materials and Methods

In the first experiment, PureVision (balafilcon A) and Biofinity (comfilcon A) contact lenses were prepared as described in section 4.3 and placed in polypropylene tubes (n = 3) containing 1 mL of the cytokine standard blend solution. Control tubes contained only 1 mL of the cytokine solution. Samples were evaluated for cytokine uptake at 1-hour and 6-hour time points, when 60 μL was pipetted out from each tube and stored in a -80℃ freezer to later be analyzed by the MSD assay. In the second experiment, the same procedure was repeated for Clariti 1 Day (somofilcon A) contact lenses. Statistical analyses were performed using two-way, repeated measures ANOVAs with Tukey's Multiple Comparisons test. Differences were considered significant if $p < 0.05$.

6.6.3 Results – Balafilcon A and Comfilcon A

Theoretical cytokine concentrations were 28.05 pg/mL, 37.5 pg/mL, 30.8 pg/mL and 17.3 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. An aliquot of the cytokine standard blend solution obtained approximately 5 minutes following preparation was later quantified by the MSD assay to have cytokine concentrations of 27.30 pg/mL, 33.90 pg/mL, 30.23 pg/mL and 14.90 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. At 1 hour, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.51 pg/mL, 35.78 pg/mL, 31.50 pg/mL and 15.41 pg/mL, respectively. At 6 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.59 pg/mL, 35.90 pg/mL, 31.66 pg/mL and 13.26 pg/mL, respectively.

There appeared to be no uptake of IL-1β (Figure 6-33) relative to the control (100%) at 1 hour, as the percent pg/mL remaining in solution was 97 $\pm$ 1 % for balafilcon A and 97 $\pm$ 1 % for comfilcon A. In contrast at 6 hours, there did appear to be some uptake by the materials as the percent pg/mL remaining in solution was 90 $\pm$ 3 % for balafilcon A and 87 $\pm$ 2 % for comfilcon A, relative to the control (100%). There was a statistical difference between the 6-hour control and both balafilcon A (adjusted P-value < 0.0001) and comfilcon A (adjusted P-value < 0.0001), as defined by star (*) symbols.

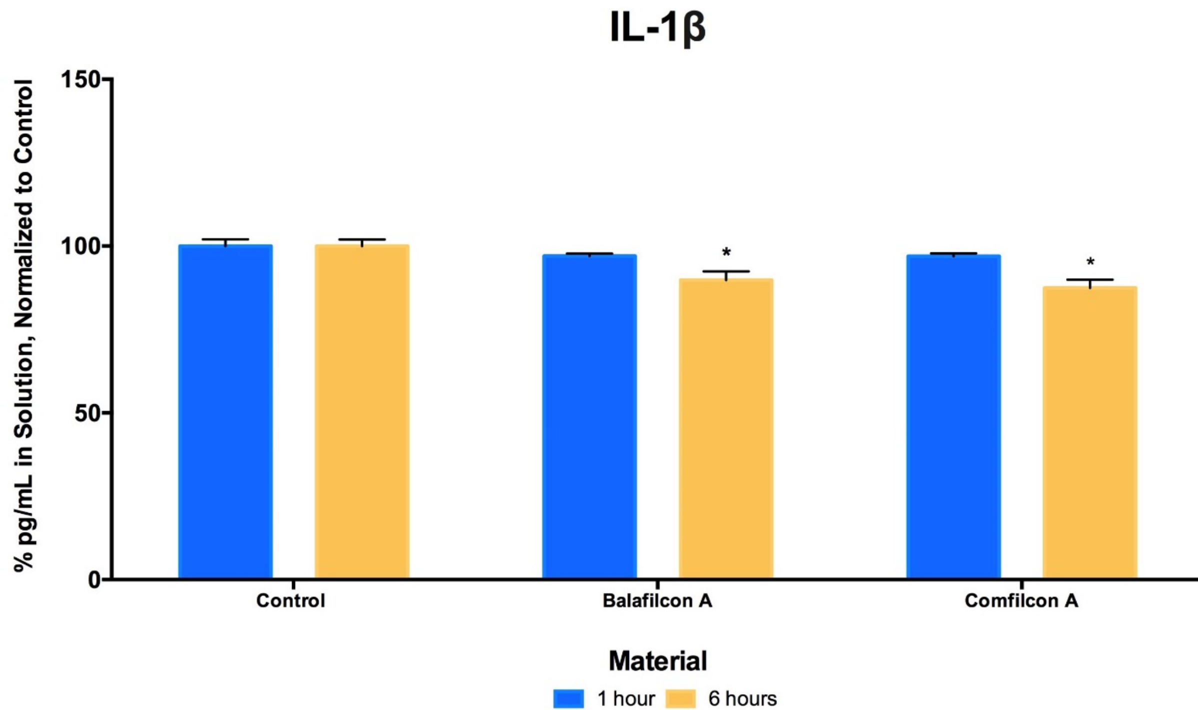

Figure 6-33: Percent pg/mL of IL-1β Remaining in Cytokine Solutions Containing Either balafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. At 6 Hours, balafilcon A and comfilcon A were Statistically Different from the 6-Hour Control, as Defined by the Star (*) Symbols.

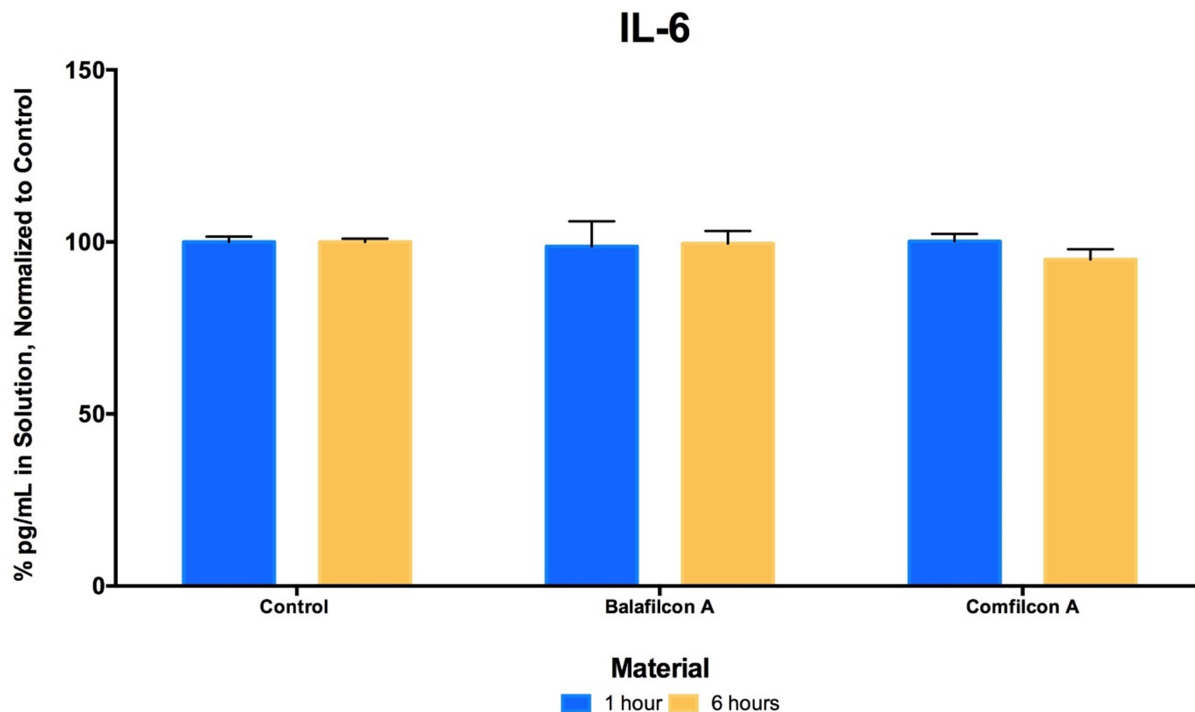

Figure 6-34: Percent pg/mL of IL-6 Remaining in Cytokine Solutions Containing Either balafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

For IL-6 (Figure 6-34), there did not appear to be any uptake by balafilcon A or comfilcon A at either 1 hour or 6 hours, as there were no statistical differences ($p > 0.05$) between these materials and the respective controls. Relative to the 1-hour control (100%), the percent pg/mL remaining in solution was 99 ± 7 % for balafilcon A and 100 ± 2 % for comfilcon A. In contrast, relative to the 6-hour control (100%), the percent pg/mL remaining in solution was 100 ± 4 % for balafilcon A and 95 ± 3 % for comfilcon A.

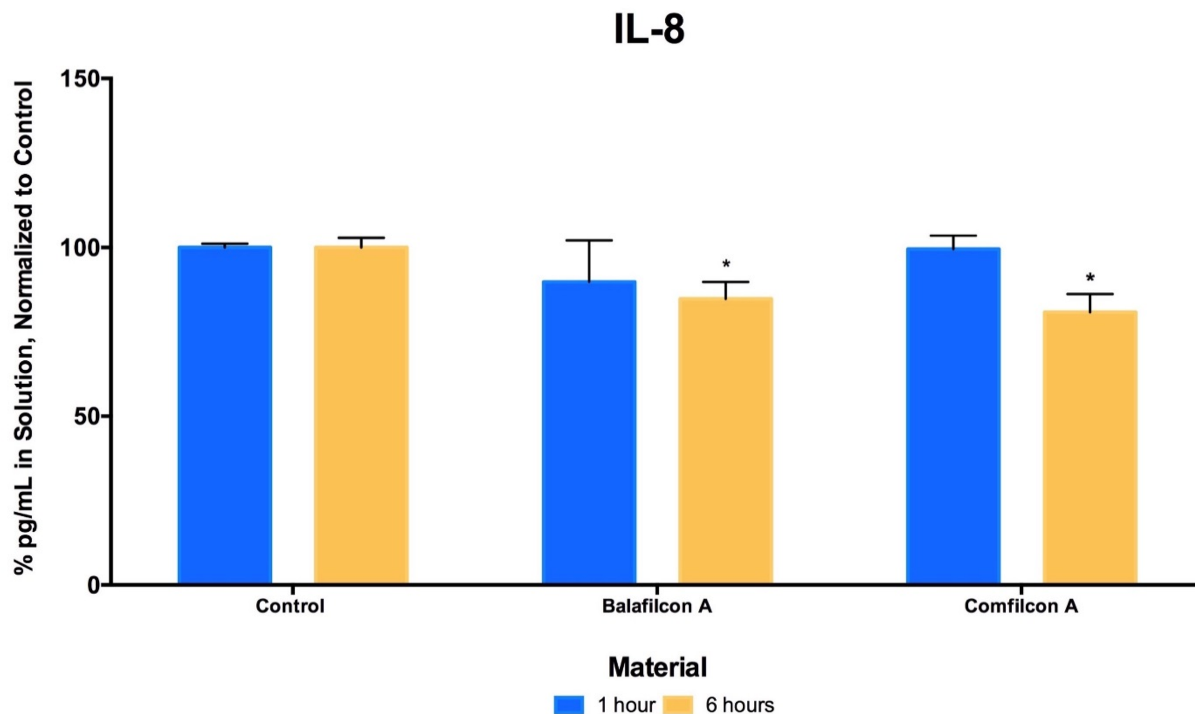

Figure 6-35: Percent pg/mL of IL-8 Remaining in Cytokine Solutions Containing Either balafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. At 6 Hours, balafilcon A and comfilcon A were Statistically Different from the 6-Hour Control, as Defined by the Star (*) Symbols.

There appeared to be no uptake of IL-8 (Figure 6-35) relative to the control (100%) at 1 hour, as the percent pg/mL remaining in solution was 90 $\pm$ 12 % for balafilcon A and 100 $\pm$ 4 % for comfilcon A. In contrast, at 6 hours, there did appear to be some uptake by the materials as the percent pg/mL remaining in solution was 85 $\pm$ 5 % for balafilcon A and 81 $\pm$ 5 % for comfilcon A, relative to the control (100%). There was a statistical difference between the 6-hour control and both balafilcon A (adjusted P-value 0.0269) and comfilcon A (adjusted P-value 0.0066), as defined by the star (*) symbols.

For TNF-α (Figure 6-36), there appeared to be no uptake relative to the control (100%) at 1 hour, as the percent pg/mL remaining in solution was 98 $\pm$ 23 % for balafilcon A and 88 $\pm$ 8 % for comfilcon A. In contrast, at 6 hours, there did appear to be some uptake by balafilcon A material, as the percent pg/mL remaining in solution was 75 $\pm$ 12 %. However, at this time point,

there was no uptake by comfilcon A, as the percent pg/mL remaining in solution was 80 ± 5 %. There was a statistical difference only between the 6-hour control and balafilcon A (adjusted P-value 0.0489), as defined by the star (*) symbol.

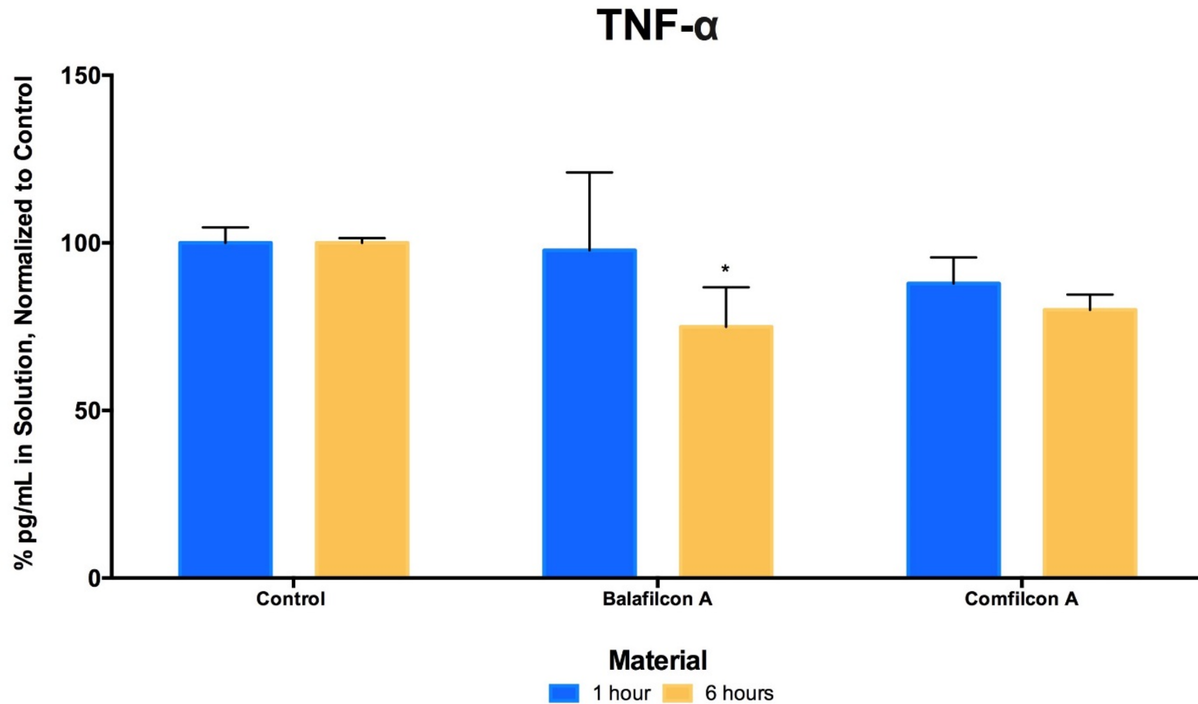

Figure 6-36: Percent pg/mL of TNF-α Remaining in Cytokine Solutions Containing Either balafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. At 6 Hours, balafilcon A and comfilcon A were Statistically Different from the 6-Hour Control, as Defined by the Star (*) Symbol.

6.6.4 Results – Somofilcon A

Theoretical cytokine concentrations were 28.7 pg/mL, 36.3 pg/mL, 27.75 pg/mL and 17.85 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. An aliquot of the cytokine standard blend solution obtained approximately 5 minutes following preparation was later quantified by the MSD assay to have cytokine concentrations of IL-1β, IL-6, IL-8 and TNF-α at 21.30 pg/mL, 35.36 pg/mL, 21.62 pg/mL and 10.59 pg/mL, respectively. At 1 hour, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 25.68 pg/mL, 35.11 pg/mL, 26.36 pg/mL and 13.38 pg/mL,

respectively. At 6 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 28.06 pg/mL, 34.61 pg/mL, 26.29 pg/mL and 11.70 pg/mL, respectively.

Relative to the control (100%), there was no uptake of any of the four cytokines of interest onto somofilcon A material at 1 hour or at 6 hours. The percent pg/mL remaining in solution after 1 hour was 98 $\pm$ 3 % for IL-1β (Figure 6-37); 94 $\pm$ 5 % for IL-6 (Figure 6-38); 89 $\pm$ 12 % for IL-8 (Figure 6-39); and 90 $\pm$ 15 % for TNF-α (Figure 6-40). In contrast, at 6 hours, the percent pg/mL remaining in solution was 98 $\pm$ 3 % for IL-1β (Figure 6-37); 99 $\pm$ 8 % for IL-6 (Figure 6-38); 91 $\pm$ 7 % for IL-8 (Figure 6-39); and 93 $\pm$ 7 % for TNF-α (Figure 6-40). Statistical analyses performed using two-way, repeated measures ANOVAs with Sidak's Multiple Comparisons test (where differences were considered significant if $p < 0.05$), revealed no statistical differences between somofilcon A and the control for either time points ($p > 0.05$).

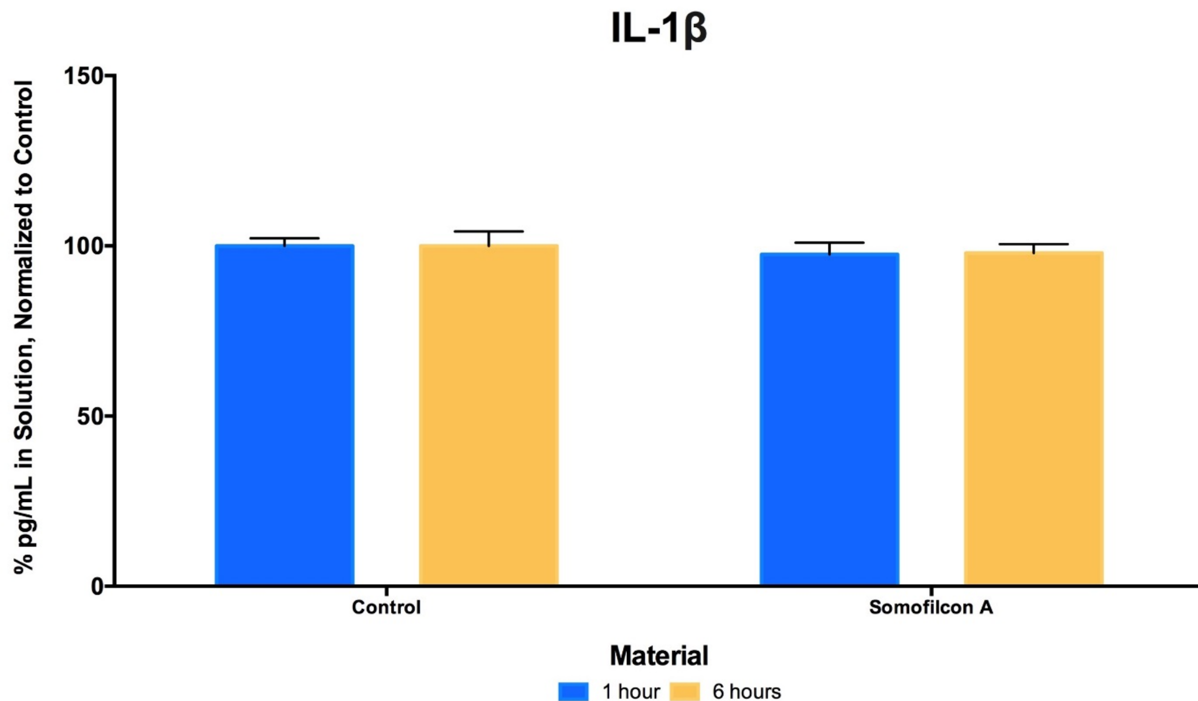

Figure 6-37: Percent pg/mL of IL-1β Remaining in Cytokine Solutions Containing somofilcon A (n = 3) Contact Lens Material, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report (p > 0.05).

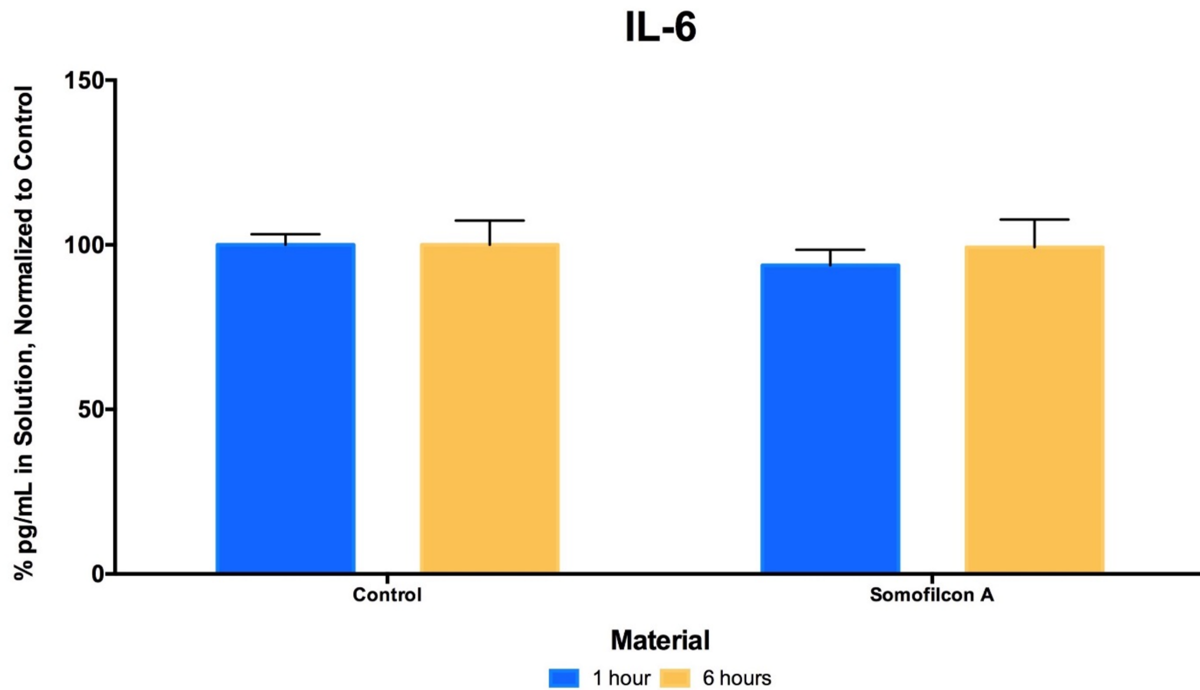

Figure 6-38: Percent pg/mL of IL-6 Remaining in Cytokine Solutions Containing somofilcon A (n = 3) Contact Lens Material, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report ($p > 0.05$).

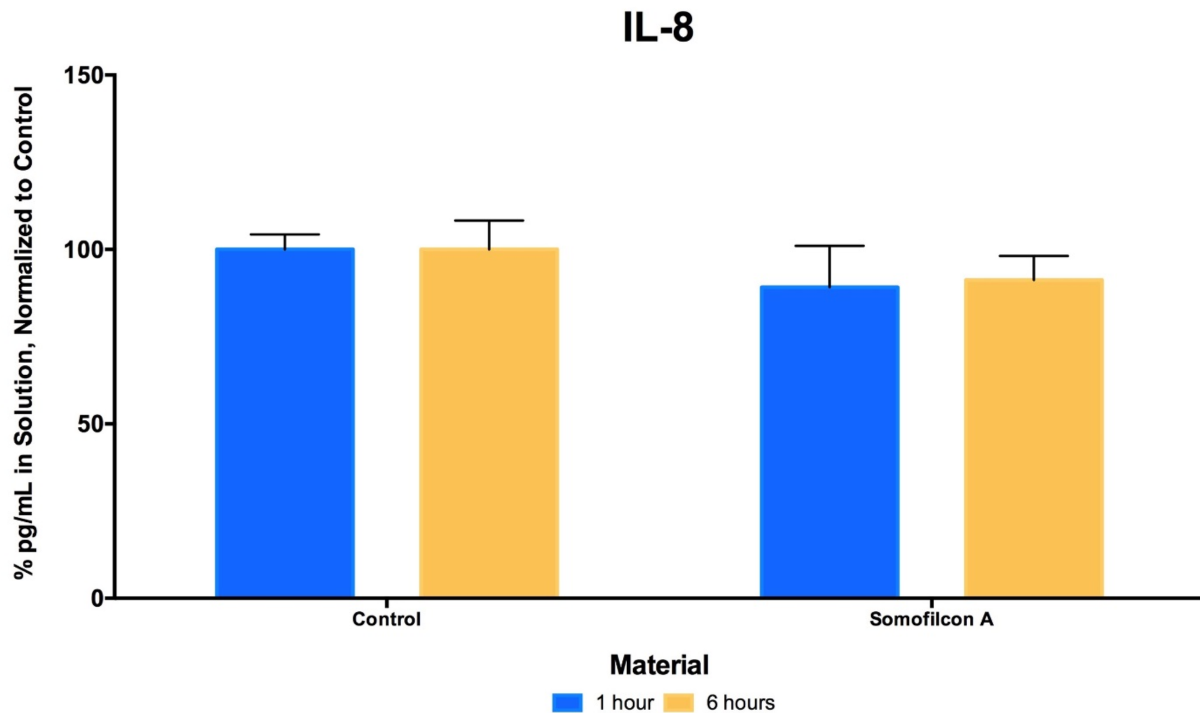

Figure 6-39: Percent pg/mL of IL-8 Remaining in Cytokine Solutions Containing somofilcon A (n = 3) Contact Lens Material, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report ($p > 0.05$).

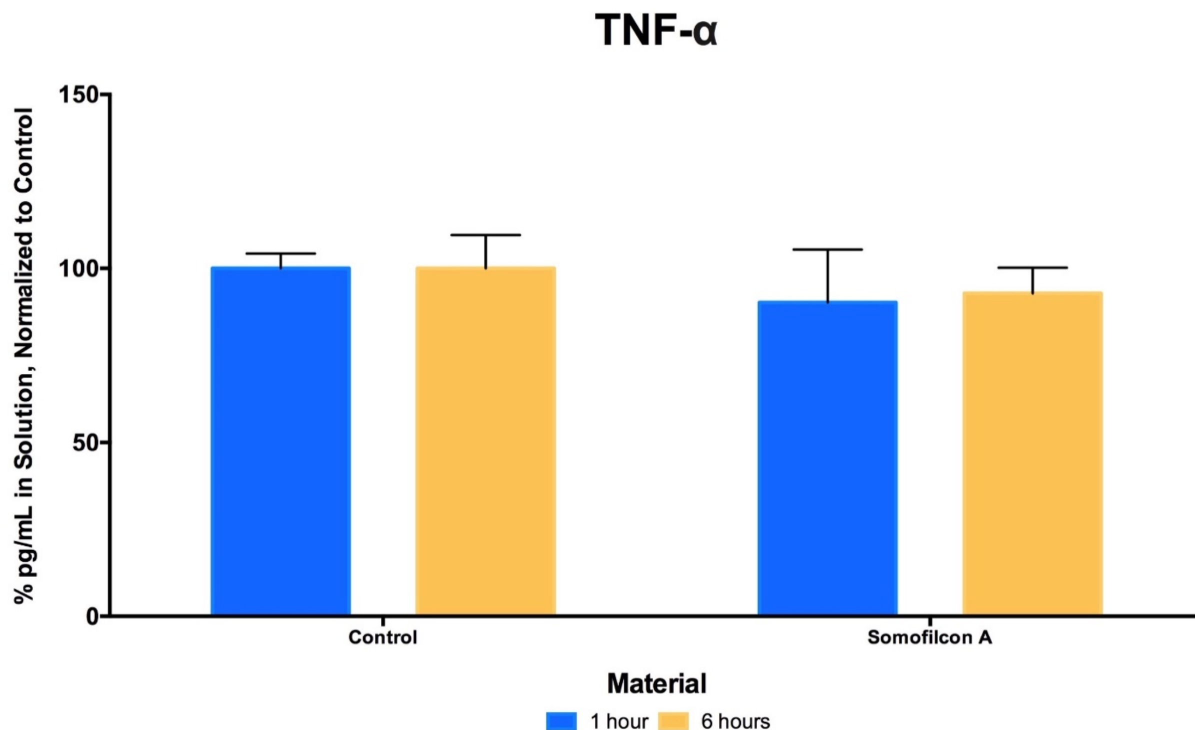

Figure 6-40: Percent pg/mL of TNF-α Remaining in Cytokine Solutions Containing somofilcon A (n = 3) Contact Lens Material, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Sidak's Multiple Comparisons Test was Performed. No Statistical Differences to Report ($p > 0.05$).

6.6.5 Discussion – Balafilcon A, Comfilcon A and Somofilcon A

Of the additional SiHy materials tested, somofilcon A did not exhibit any uptake of the cytokines of interest over the 6-hour study period; however, balafilcon A and comfilcon A materials did exhibit some uptake at 6 hours when compared to the control. Interestingly, balafilcon A and comfilcon A are both low water content materials that differ in their ionicity. Balafilcon A is ionic (negatively charged due to its principal monomer N-vinyl aminobutyric acid), while comfilcon A is nonionic.[58,100] Both of these materials exhibited some uptake of IL-1β and IL-8, while balafilcon A additionally exhibited some uptake of TNF-α, which comfilcon A did not. Recall, the surface of IL-1β and TNF-α should be predominately negatively charged based on their pI values and the pH of the standard blend soaking solution.[83,85,91,92] In contrast, the surface of IL-8 should be predominately positively charged.[83,89] Based on this, it appeared that the pI

values were not a significant factor that influenced cytokine uptake, as the results obtained were not in accordance with expected results should the pI values have influenced uptake. For example, a negatively charged material such as balafilcon A should have resisted uptake of negatively charged IL-1β and TNF-α in the standard blend solution.[58,59,83,85,91,92] While balafilcon A did uptake IL-8, which has a positively charged surface in the standard blend solution, it did not exhibit uptake of this cytokine in greater amounts than did comfilcon A, which is a nonionic material that would potentially have less attraction for a charged cytokine.[83] Moreover, in the previous experiments presented throughout Chapter 5: and Chapter 6:, etafilcon A did not exhibit uptake of positively charged IL-8 in the standard blend solution either, even though it is also a negatively charged material - akin to balafilcon A.[59,83,89]

Furthermore, when comparing the results of this experiment to previous experiments, there are some similarities between balafilcon A and comfilcon A in regard to the uptake of particular cytokines, however, parallels cannot be drawn between these materials in terms of what would be influencing this uptake that is observed. For example, balafilcon A, comfilcon A, and omafilcon A exhibited uptake of IL-1β; however, there was no single characteristic (e.g., same class of material, water content, or ionicity), that was collectively shared among all three of them.[58,59,100] Furthermore, balafilcon A and omafilcon A both exhibited uptake of TNF-α, yet they both also differ in their class of material, water content and ionicity as well.[58,59]

Although the following observations are not conclusive, perhaps the uptake of IL-8 could be dependent on the water content of a contact lens material, as both balafilcon A and comfilcon A did exhibit some uptake of IL-8 and they are both low water content materials.[58,100] Although, if this were definitely the case, then senofilcon A material should have also exhibited uptake of IL-8, as it is also a low water content SiHy material.[59] Furthermore, another observation is the

consistent lack of IL-6 uptake onto any of the materials tested in the experiments presented in Chapter 5: and Chapter 6:. This could perhaps be due to the size of IL-6, as it is one of the larger cytokines of the four cytokines of interest[15] (Table 3-2) and according to Meso Scale Discovery Scientific Support, its predicted size in the calibrator blend (which was used to prepare the cytokine solution), is 20.3 kDa, while the predicted sizes of IL-1β, IL-8 and TNF-α are 17 kDa, 8 kDa and 17.5 kDa, respectively. Given that the results of the experiments thus far remained inconclusive as to what exactly may or may not have influenced cytokine uptake onto the contact lens materials investigated, the subsequent experiment presented below explored whether utilizing higher concentrations of the cytokines of interest in the soaking solution could instead result in greater uptake onto the contact lens materials.

6.7 Testing Uptake Under Higher Cytokine Concentrations

6.7.1 Introduction

The experiments presented in Chapter 5: and Chapter 6: investigated uptake onto various contact lens materials at concentrations near the lower range reported in the literature (Table 3-3). These concentrations were representative of uptake under basal cytokine concentrations within the eye. The next experiment utilized a standard blend of higher cytokine concentrations to explore uptake under simulated inflammatory ocular conditions. Additionally, this was accompanied by an extended soaking period for up to 7 days. It was hypothesized that perhaps there would be more uptake by the contact lens materials if soaked at higher concentrations over a longer period of time.

Reusable contact lenses were chosen for this experiment due to the 7-day soaking period, as exploring uptake within this time would not be applicable to daily disposable contact lenses. One reusable contact lens was chosen from the conventional hydrogel class of materials and the other from the SiHy class. For the conventional hydrogel, etafilcon A material was chosen from one of the two conventional hydrogel materials that had previously been tested. While omafilcon

A appeared to exhibit more uptake than etafilcon A in the previous studies, Proclear 1 Day (omafilcon A) is a disposable contact lens and other omafilcon A material lenses were either multifocal or toric and were therefore not of interest to be utilized in the study. Acuvue 2, on the other hand, is a commonly used, reusable contact lens of etafilcon A material. For the SiHy, comfilcon A material (Biofinity) was chosen over balafilcon A (PureVision). Although both of these materials exhibited uptake for some of the cytokines when tested at lower concentrations, choosing comfilcon A better contrasted etafilcon A and allowed for investigations of differences in not only the class of material (conventional vs. SiHy), but also water content and ionicity. Comfilcon A is a low water content, nonionic material, while etafilcon A is a high water content, ionic material.[59,100]

6.7.2 Materials and Methods

Acuvue 2 (etafilcon A) and Biofinity (comfilcon A) contact lenses were prepared as described in section 4.3 and placed in polypropylene tubes (n = 3) containing 1 mL of a cytokine standard blend solution that was prepared by combining two MSD calibrator blends of the same lot number. Control tubes contained only 1 mL of this cytokine solution with no contact lenses. The polypropylene tubes were wrapped in parafilm to prevent evaporation over the 7-day soaking period. 60 μL samples were collected at 24 hours, 72 hours (3 days), and at the 7-day mark.

The cytokine soaking solution was not exchanged for fresh cytokine solution at the time points mentioned, as this would have introduced potential complications to the study that had not been investigated in the experiments thus far. In a clinical example, if there was uptake onto a reusable contact lens material, when the lens is removed from the eye at the end of the day, cleaned and placed into a care system in a contact lens case, it may be possible that any cytokines remaining on the surface of the lens could release back into the solution. This would then introduce questions

in regard to how tightly bound the cytokines are to the lenses and whether they can be washed off when the lenses are cleaned, in addition to questions about the level of release that could be expected should the cytokines not be washed off the surface of the lens, and also whether the cytokines could uptake back onto the lenses as they soak in the care system overnight and hence be reintroduced to the ocular surface when the same lens is worn again the next day. The experiments presented in this thesis did not focus on testing for cytokine release from contact lens materials, because many of the materials tested did not even exhibit an initial uptake of cytokines. Based on this, it was not expected that there would be much release from the lenses over the 7-day study period regardless.

6.7.3 Results

Theoretical cytokine concentrations were 106.97 pg/mL, 142.69 pg/mL, 118.94 pg/mL and 66.88 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. An aliquot of the cytokine standard blend solution obtained approximately 5 minutes following preparation was later quantified by the MSD assay to have cytokine concentrations of 92.86 pg/mL, 129.46 pg/mL, 92.93 pg/mL and 39.38 pg/mL for IL-1β, IL-6, IL-8 and TNF-α, respectively. At 24 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 94.33 pg/mL, 126.93 pg/mL, 108.88 pg/mL and 41.75 pg/mL, respectively. At 72 hours, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 96.15 pg/mL, 119.32 pg/mL, 112.28 pg/mL and 34.21 pg/mL, respectively. At 7 days, control concentrations of IL-1β, IL-6, IL-8 and TNF-α were 93.94 pg/mL, 113.06 pg/mL, 111.10 pg/mL and 28.59 pg/mL, respectively. Statistical analyses were performed using two-way, repeated measures ANOVAs with Tukey's Multiple Comparisons tests. Differences were considered significant if $p < 0.05$.

At 24 hours, there was no uptake of any of the four cytokines of interest onto either contact lens material tested. Relative to the control (100%), the percent pg/mL remaining in solution at this time was 103 $\pm$ 2 % (etafilcon A) and 103 $\pm$ 2 % (comfilcon A) for IL-1β (Figure 6-41); 101 $\pm$ 4 % (etafilcon A) and 98 $\pm$ 1 % (comfilcon A) for IL-6 (Figure 6-42); 103 $\pm$ 2 % (etafilcon A) and 104 $\pm$ 2 % (comfilcon A) for IL-8 (Figure 6-43); as well as 103 $\pm$ 1 % (etafilcon A) and 114 $\pm$ 2 % (comfilcon A) for TNF-α (Figure 6-44). Additionally, there were no statistical differences between the materials and the control at this time point ($p > 0.05$), with the exception of comfilcon A being statistically different from both the control (adjusted P-value 0.0056) and etafilcon A (adjusted P-value 0.0185) for TNF-α, as defined by the number (#) symbol. In both cases, however, this was as a result of the value of comfilcon A exceeding 100% of the control and was therefore not indicative of uptake.

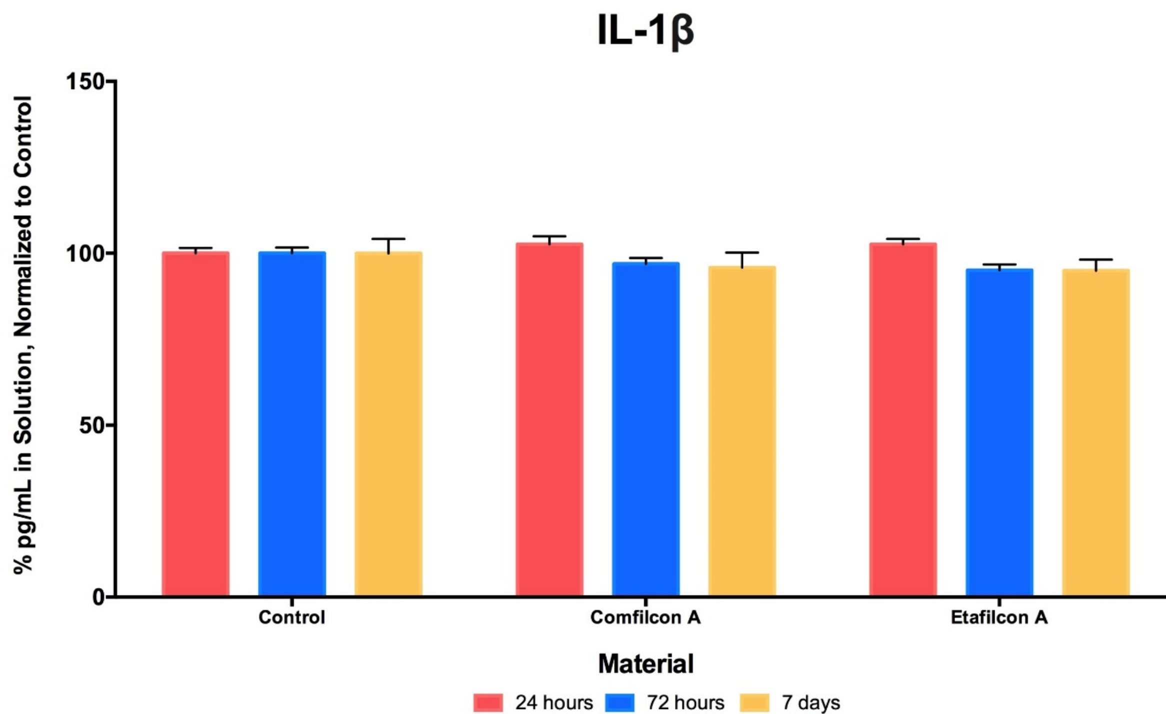

Figure 6-41: Percent pg/mL of IL-1β Remaining in Higher Concentration Cytokine Solutions Containing Either etafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 24-hours, 72-hours and 7-day Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

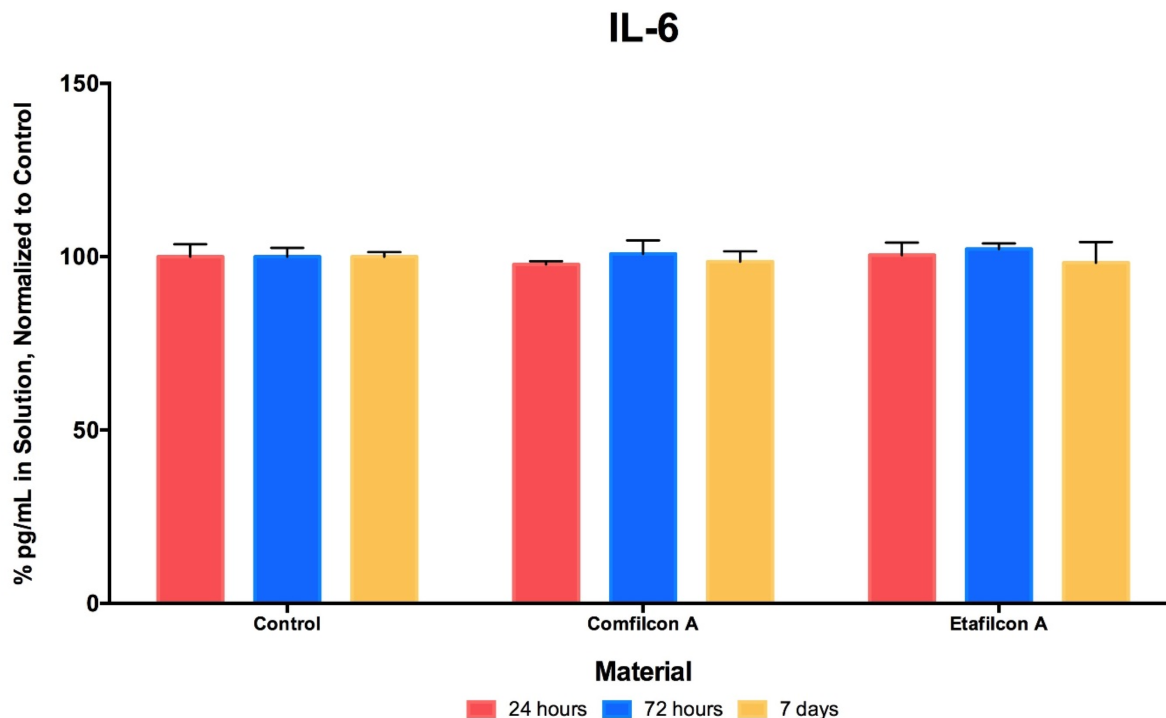

Figure 6-42: Percent pg/mL of IL-6 Remaining in Higher Concentration Cytokine Solutions Containing Either etafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 24-hours, 72-hours and 7-day Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

Likewise, at 72 hours, there was also no uptake of any of the four cytokines of interest onto either contact lens material tested. Relative to the control (100%), the percent pg/mL remaining in solution at this time was 95 $\pm$ 2 % (etafilcon A) and 97 $\pm$ 2 % (comfilcon A) for IL-1β (Figure 6-41); 102 $\pm$ 2 % (etafilcon A) and 101 $\pm$ 4 % (comfilcon A) for IL-6 (Figure 6-42); 100 $\pm$ 6 % (etafilcon A) and 98 $\pm$ 6 % (comfilcon A) for IL-8 (Figure 6-43); as well as 102 $\pm$ 3 % (etafilcon A) and 115 $\pm$ 3 % (comfilcon A) for TNF-α (Figure 6-44). Additionally, there were no statistical differences between the materials and the control at this time point ($p > 0.05$), except between comfilcon A and both the control (adjusted P-value 0.0036) and etafilcon A (adjusted P-value 0.0090) for TNF-α. However, again, this was due to the value of comfilcon A exceeding 100% of the control and was not indicative of uptake.

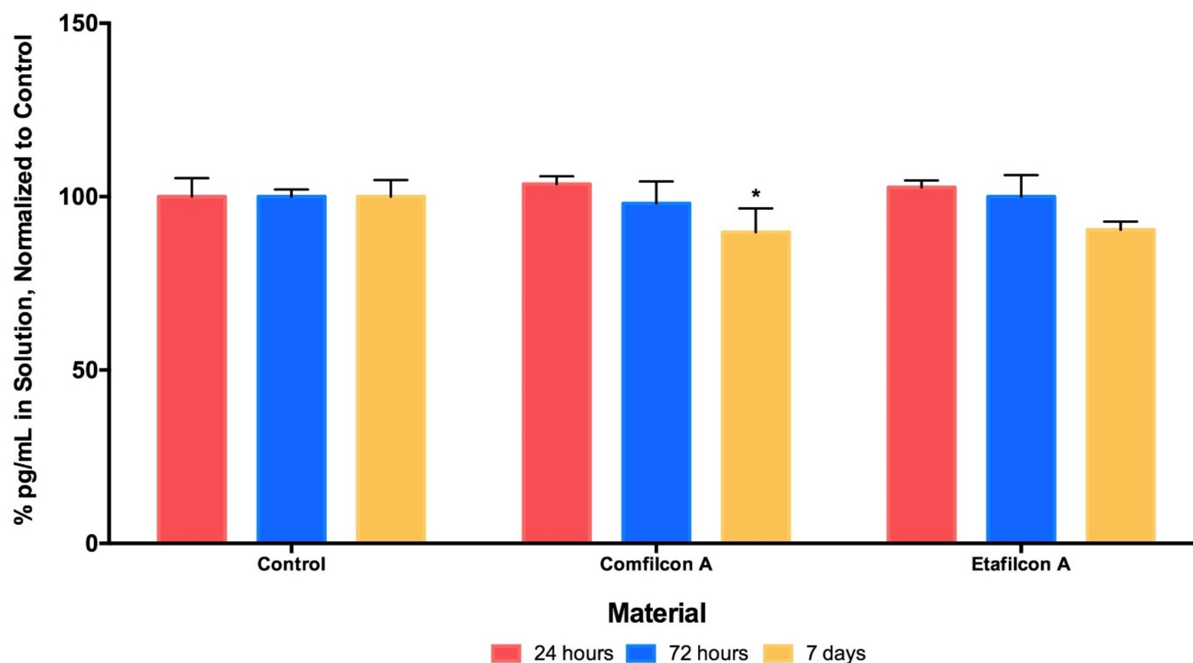

Figure 6-43: Percent pg/mL of IL-8 Remaining in Higher Concentration Cytokine Solutions Containing either etafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 24-hours, 72-hours, and 7-day Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. At 7 Days, comfilcon A was Statistically Different from the 7-Day Control, as Defined by the Star (*) Symbol.

At the 7-day mark, there was no uptake of IL-1β or TNF-α onto either contact lens material tested. Relative to the control (100%), the percent pg/mL remaining in solution at this time was 95 $\pm$ 3 % (etafilcon A) and 96 $\pm$ 4 % (comfilcon A) for IL-1β (Figure 6-41) and 98 $\pm$ 6 % (etafilcon A) and 99 $\pm$ 3 % (comfilcon A) for IL-6 (Figure 6-42). There were no statistical differences between these materials and the control ($p > 0.05$). For IL-8 (Figure 6-43), the percent pg/mL remaining in solution was 90 $\pm$ 2 % (etafilcon A) and 90 $\pm$ 7 % (comfilcon A). There was a statistical difference between comfilcon A and the control (adjusted P-value 0.0382), indicating uptake, as defined by the star (*) symbol; however, there was no statistical difference between etafilcon A and the control ($p > 0.05$). In contrast, there appeared to be some uptake of TNF-α (Figure 6-44) onto both materials, as the percent pg/mL remaining in solution was 90 $\pm$ 4 %

(etafilcon A) and 88 ± 10 % (comfilcon A). Moreover, there was a statistical difference between both etafilcon A and the control (adjusted P-value 0.0489), as defined by the triangle (Δ) symbol, as well as between comfilcon A and the control (adjusted P-value 0.0161), as defined by the star (*) symbol.

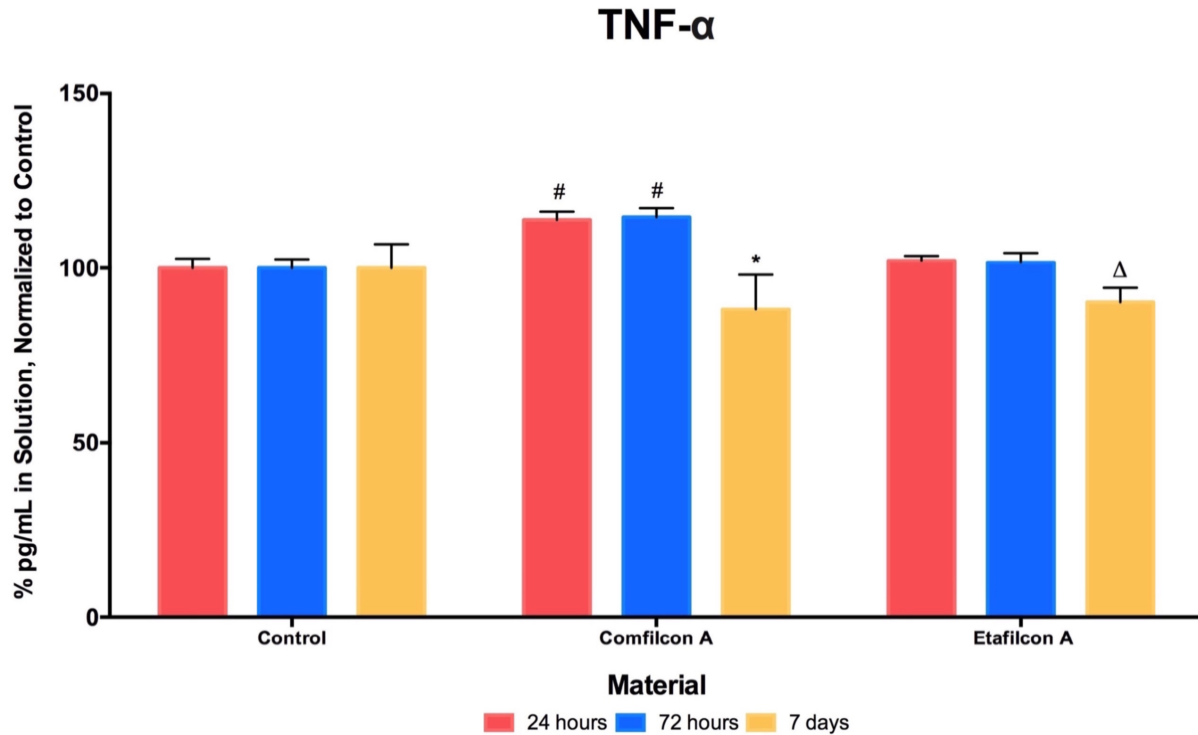

Figure 6-44: Percent pg/mL of TNF-α Remaining in Higher Concentration Cytokine Solutions Containing Either etafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 24-hours, 72-hours, and 7-day Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. At both 24 Hours and 72 Hours, comfilcon A was Statistically Different from the Control and etafilcon A at the Respective Time Point, as Defined by the Number (#) Symbol, Due to Having a Value Greater than 100% of the Respective Control. At 7 Days, comfilcon A was Statistically Different from the 7-Day Control, as Defined by the Star (*) Symbol and etafilcon A was also Statistically Different from the 7-Day Control, as Defined by the Triangle (Δ) Symbol.

6.7.4 Discussion

Consistent with the results obtained in section 6.6.3, comfilcon A again appeared to uptake IL-8 at the 6-hour time point. In contrast, there was also some evidence for uptake of TNF-α, contrary to the result obtained in section 6.6.3, where comfilcon A instead exhibited uptake of IL-1β. Interestingly, etafilcon A did not exhibit uptake of IL-8 and contrary to comfilcon A, it is a

high water content material.[59] Similar to balafilcon A and omafilcon A, etafilcon A did exhibit uptake of TNF-α, even though it is a negatively charged material and TNF-α should have a negatively charged surface in the standard blend solution.[83,91,92] Again, this uptake does not appear to be heavily influenced by the pI of the cytokine or the ionicity of the material, nor does it appear to be influenced by the water content of the material, since all three of these materials (balafilcon A, etafilcon A and omafilcon A), exhibited some uptake of TNF-α, yet two of these materials are of high water content, while the other is a low water content material.[58,59]

Finally, increasing the concentration of cytokines in solution did not significantly alter the levels of cytokine uptake onto the contact lens materials tested, even when the soaking period was increased to 7 days. As a result, the next chapter of the thesis aimed to utilize an entirely different source of cytokines, by investigating the uptake of IL-1β, IL-6, IL-8 and TNF-α produced directly from human corneal epithelial cells. Not only would this provide more clinical relevance through better modeling ocular conditions, as human corneal epithelial cells secrete cytokines in the eye[3,16,17], but this would also allow for an investigation of uptake using more freshly prepared cytokine solutions directly from human corneal epithelial cells cultured in the laboratory.

Chapter 7: Uptake Using Human Corneal Epithelial Cells Supernatant

7.1 Introduction

As discussed in section 1.1, the human cornea is comprised of five layers – one of which is the corneal epithelium.[1] The cells of the human corneal epithelium, termed, human corneal epithelial cells (HCECs), produce both a basal level of cytokines and an amplified level if damaged, in order to initiate the corneal wound healing (repair) process.[1,3,16,17] The experiments presented in Chapter 7: utilized cytokines sourced directly from cells of human corneas, rather than recombinant cytokines from the MSD calibrator blend. This allowed for the simulation of an *in vivo* model more closely depicting the interaction of a contact lens when placed onto the corneal epithelium during wear (Figure 2-2).

Cytokines produced by HCECs were collected from cell culture supernatants. Through an initial pilot study (data not presented), HCECs were tested for the production of basal amounts of the four cytokines of interest. In addition to producing IL-1β, IL-6, IL-8 and TNF-α, HCECs also produced IFN-γ, IL-2, IL-4, IL-10, IL-12p70 and IL-13. Other cytokines were not tested for, although it is possible that the cells did produce additional cytokines. Of the ten cytokines that were tested for, IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-12p70, IL-13 and TNF-α were produced at extremely low concentrations (all below 3 pg/mL), while higher concentrations of IL-6 and IL-8 were produced (around 85 pg/mL). Of note, however, was the variability that existed in cytokine concentrations produced by HCECs with each culture. This variability depended on many factors, including when the supernatant was collected (if it was collected from freshly cultured cells or older cells), how often the cell culture media was changed and the number of cells/mL grown per flask. Regardless, a similar trend was always observed whereby in the case of the four cytokines of interest, IL-1β and TNF-α were produced at significantly lower concentrations than IL-6 and

IL-8. Additionally, as a result of the variability, the concentrations of the cytokines in the collected cell culture supernatants were quantified by the MSD assay prior to their use as soaking solutions for the contact lenses.

7.2 Culturing Human Corneal Epithelial Cells

Human corneal epithelial cells (Millipore Sigma, Burlington, MA) were cultured under sterile conditions in Corning BioCoat Collagen 1-coated Flasks (Corning, Corning, NY), containing EpiGRO Human Ocular Epithelia (Millipore Sigma, Burlington, MA), which was supplemented, as per the manufacturer's guidelines, with 0.5 mL of 5 μg/mL Apo-Transferrin, 0.5 mL of 1.0 μM Epinephrine, 0.5 mL of 5 μg/mL Hydrocortisone Hemisuccinate, 0.5 mL of 5 μg/mL rh Insulin, 1 mL EpiFactor O (proprietary final concentration), 2 mL of 0.4 % EpiFactor P and 15 mL of 6 mM L-Glutamine (Millipore Sigma, Burlington, MA). The flasks containing the cells were incubated at 37℃ with 5% CO_2 and grown to 80% confluency. The cells were regularly observed using a microscope and the cell culture supernatant was replaced approximately every 2-3 days with fresh EpiGRO Human Ocular Epithelia Media containing supplements. The cell culture supernatants were stored in 15 mL polypropylene centrifuge tubes (Corning, Corning, NY) in a -80℃ freezer. 60 μL aliquots were separately stored in 0.6 mL Axygen microtubes (Axygen, Inc, Union City, CA) and later analyzed with the MSD assay to quantify cytokine concentrations prior to commencing each experiment.

7.3 Uptake of HCEC-derived IL-1β, IL-6, IL-8 and TNF-α onto Contact Lens Materials

7.3.1 Introduction

An investigation of HCEC-derived IL-1β, IL-6, IL-8 and TNF-α uptake onto contact lens materials was performed in two separate experiments. In the first experiment, levels of IL-1β and TNF-α uptake were quantified from solutions of two conventional hydrogels and three SiHys,

while in the second experiment, uptake levels of IL-6 and IL-8 were quantified in solutions containing the same contact lens materials. The four cytokines of interest were evaluated separately due to the differences in concentration that existed between them. Since IL-1β and TNF-α were generally produced at lower concentrations, while IL-6 and IL-8 were generally produced at higher concentrations, the supernatant solutions had to be diluted separately, such that the concentrations of the samples pipetted into each well of the 96-well MSD plate fell between both the lower and upper limits of quantification (Table 4-4). Thus, it was not possible to appropriately quantify IL-1β and TNF-α with IL-6 and IL-8 collectively, such that the concentrations of the first two did not fall below the lower limit of quantification, or that the concentrations of the second two did not fall above the upper limit of quantification.

For the conventional hydrogels, etafilcon A and omafilcon A were both chosen to be studied. Omafilcon A had previously shown some cytokine uptake throughout the experiments presented in sections 6.3.3, 6.3.7, 6.4.3 and 6.5.3, and while etafilcon A had exhibited less uptake than omafilcon A, it did exhibit some uptake in the experiment presented in section 6.3.7, as well as in section 6.7.3, when it was soaked in a higher concentration of TNF-α after 7 days. Since there was potential for obtaining high cytokine concentrations from HCECs, this contact lens material was incorporated into the study design for further investigation as well.

Of the silicone hydrogels tested thus far, balafilcon A, comfilcon A and senofilcon A were chosen. Balafilcon A and comfilcon A were chosen as they previously exhibited some cytokine uptake in the experiment presented in section 6.6.3 and likewise to etafilcon A, comfilcon A also exhibited uptake of TNF-α (as well as IL-8), after 7 days (section 6.7.3). Between senofilcon A and somofilcon A, although neither material exhibited any uptake of cytokines when the MSD calibrator blend solution was utilized, senofilcon A (due to being more commonly used), was

chosen to be investigated for any difference in its uptake ability in a solution of cytokines derived from HCECs.

7.3.2 Materials and Methods

Human corneal epithelial cells were cultured as described in section 7.2 and the cell culture supernatants were collected and quantified with the MSD assay. PureVision (balafilcon A), Biofinity (comfilcon A), 1-Day Acuvue Oasys (etafilcon A), Proclear 1 Day (omafilcon A) and Acuvue Oasys 2-week with Hydraclear Plus (senofilcon A), were prepared as described in section 4.3 and placed in polypropylene tubes ($n = 3$) containing 1 mL of the HCEC culture supernatant. Control tubes contained only 1 mL of the supernatant solution with no contact lenses. 60 μL samples were collected at 1 hour and again at 6 hours. Statistical analyses were performed using two-way, repeated measures ANOVAs with Tukey's Multiple Comparisons test. Differences were considered significant if $p < 0.05$.

7.3.3 Results – Experiment #1: IL-1β and TNF-α

The concentrations of IL-1β and TNF-α initially quantified from an aliquot of the cell culture supernatant of the batch of HCECs cultured for this experiment were 2.36 pg/mL and 4.96 pg/mL, respectively. While these concentrations were lower than those previously tested using the MSD calibrator blend, they were greater than that which was quantified from the HCEC culture supernatant tested in the pilot study (0.27 pg/mL and 0.77 pg/mL for IL-1β and TNF-α, respectively). Furthermore, this allowed for an investigation of uptake at a low concentration, which was different and unique from other experiments. On the day of the study, the mean cytokine concentrations in the 1-hour control solutions, as quantified by the MSD assay, were 2.73 pg/mL and 5.06 pg/mL for IL-1β and TNF-α, respectively, and in the 6-hour control solutions were 2.69 pg/mL and 4.50 pg/mL for IL-1β and TNF-α, respectively.

Relative to the control at 1 hour (100%), there was no uptake of IL-1β (Figure 7-1) onto either balafilcon A (102.4 ± 2 %), comfilcon A (100 ± 2 %), etafilcon A (99 ± 4 %), omafilcon A (99 ± 2 %) or senofilcon A (98 ± 1 %). Additionally, relative to the 6-hour control (100%), there was also no uptake of IL-1β (Figure 7-1) onto balafilcon A (95 ± 1 %), comfilcon A (96 ± 6 %), etafilcon A (92 ± 6 %), omafilcon A (95 ± 2 %) or senofilcon A (93 ± 3%). There were no statistical differences between the concentration of IL-1β remaining in solutions containing the contact lens materials of interest and the respective controls at 1 hour or at 6 hours ($p > 0.05$).

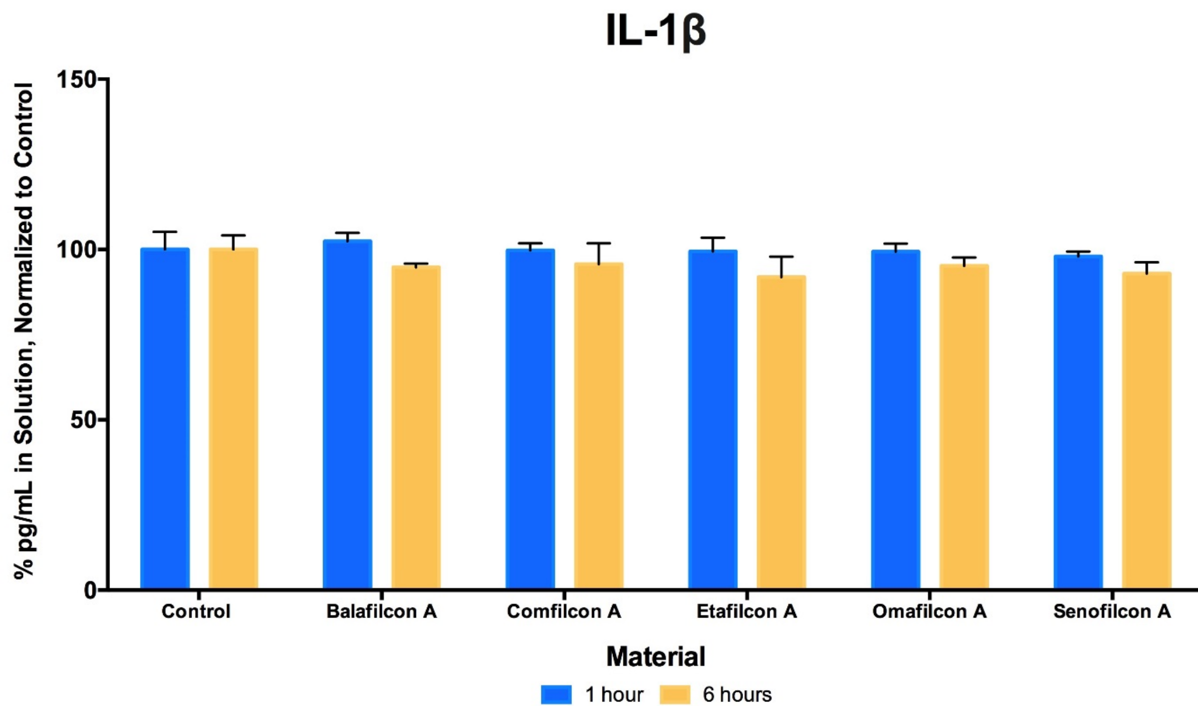

Figure 7-1: Percent pg/mL of IL-1β Remaining in HCEC-Derived Cytokine Solutions Containing either balafilcon A, comfilcon A, etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report (p > 0.05).

Relative to the control at 1 hour (100%), there was also no uptake of TNF-α (Figure 7-2) onto balafilcon A (106 ± 2 %), comfilcon A (91 ± 5 %), etafilcon A (97 ± 5%), omafilcon A (89 ± 6 %) or senofilcon A (91 ± 6 %). Moreover, the only statistical difference observed at this time

point was between balafilcon A and each of comfilcon A (adjusted P-value 0.0429), omafilcon A (0.0171) and senofilcon A (0.0302), as defined by the number (#) symbol. However, since balafilcon A was not statistically different from the respective control, there was no indication of uptake and hence the statistical differences were as a result of the value of balafilcon A exceeding 100% of the control.

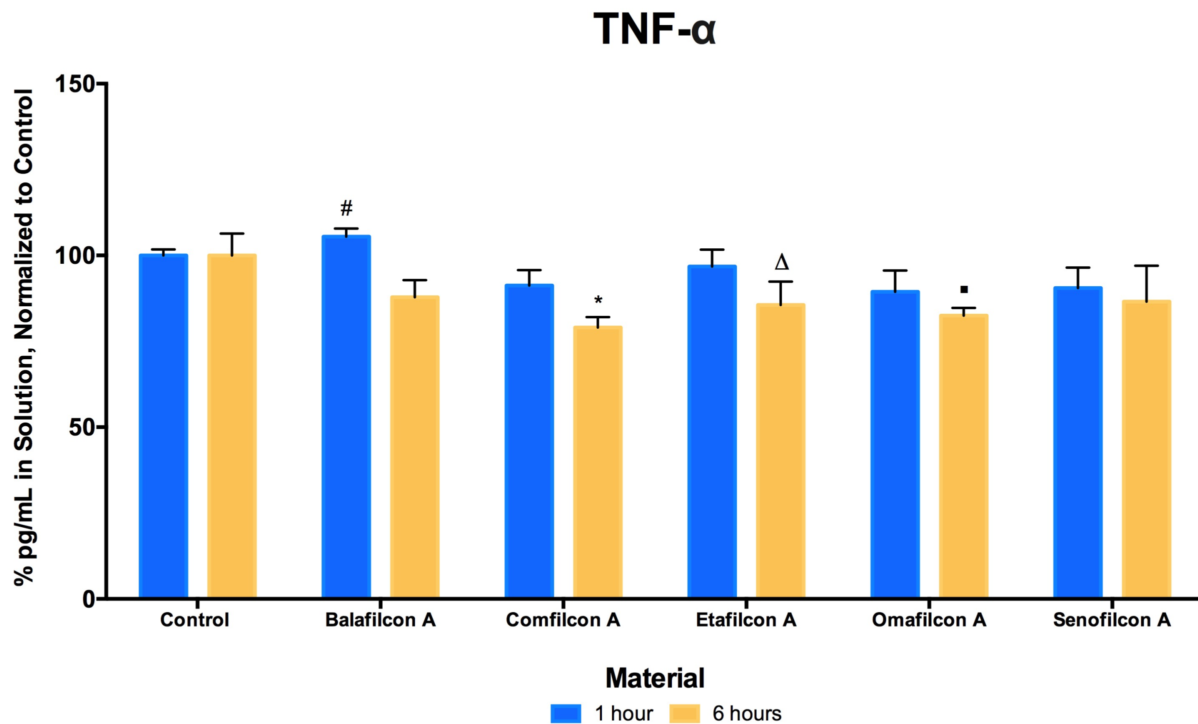

Figure 7-2: Percent pg/mL of TNF-α Remaining in HCEC-Derived Cytokine Solutions Containing either balafilcon A, comfilcon A, etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. At 1 Hour, balafilcon A was Statistically Different from Each of comfilcon A, omafilcon A and senofilcon A, as Defined by the Number (#) Symbol, Due to Having a Value Greater than 100% of the Control. At 6 Hours, comfilcon A, etafilcon A and omafilcon A were Each Statistically Different from the 6-Hour Control, as Defined by the Star (*), Triangle (Δ) and Square (■) Symbols, Respectively.

At 6 hours, there appeared to be some uptake of TNF-α (Figure 7-2) onto comfilcon A (79 $\pm$ 3 %), etafilcon A (86 $\pm$ 7 %) and omafilcon A (82 $\pm$ 2 %) relative to the control (100%). However, there was no uptake of this cytokine onto balafilcon A (88 $\pm$ 5 %) or senofilcon A (87 $\pm$ 11 %), as there were no statistical differences between these materials and the respective control

($p > 0.05$). In contrast, there was a statistical difference between the 6-hour control and each of comfilcon A (adjusted P-value 0.0012), etafilcon A (adjusted P-value 0.0395) and omafilcon A (adjusted P-value 0.0079), as defined by the star (*), triangle (Δ) and square (■) symbols, respectively.

7.3.4 Results – Experiment #2: IL-6 and IL-8

The concentrations of IL-6 and IL-8 initially quantified from an aliquot of the cell culture supernatant of the batch of HCECs cultured for this experiment were 808 pg/mL and 2438 pg/mL, respectively. These concentrations were appreciably higher than those previously obtained from the MSD calibrator blend, even when two calibrator blends were combined, as described in the experiment presented in section 6.7.3. These higher concentrations were obtained by extending the number of days between culture media changes and collecting supernatant from older cells. On the day of the study, the mean cytokine concentrations in the 1-hour control solutions, as quantified by the MSD assay, were 342 pg/mL and 1885 pg/mL for IL-6 and IL-8, respectively, and in the 6-hour control solutions were 356 pg/mL and 1995 pg/mL for IL-6 and IL-8, respectively.

Relative to the control at 1 hour (100%), there was no uptake of IL-6 (Figure 7-3) onto either balafilcon A (106 ± 7 %), comfilcon A (107 ± 2 %), etafilcon A (109 ± 4 %), omafilcon A (121 ± 7 %) or senofilcon A (115 ± 8 %). The only statistical difference observed at this time point was between omafilcon A and the respective control (adjusted P-value 0.0097), as defined by the number (#) symbol; however, this was due to the value of omafilcon A exceeding 100% of the control and was therefore not an indication of uptake.

Moreover, relative to the 6-hour control (100%), there was also no uptake of IL-6 (Figure 7-3) onto balafilcon A (95 ± 4 %), comfilcon A (102 ± 10 %), etafilcon A (98 ± 3 %), omafilcon A (104 ± 7 %) or senofilcon A (99 ± 6%). There were no statistical differences observed between

the concentrations of IL-6 in any of the solutions containing the contact lens materials of interest in comparison to the respective control ($p > 0.05$).

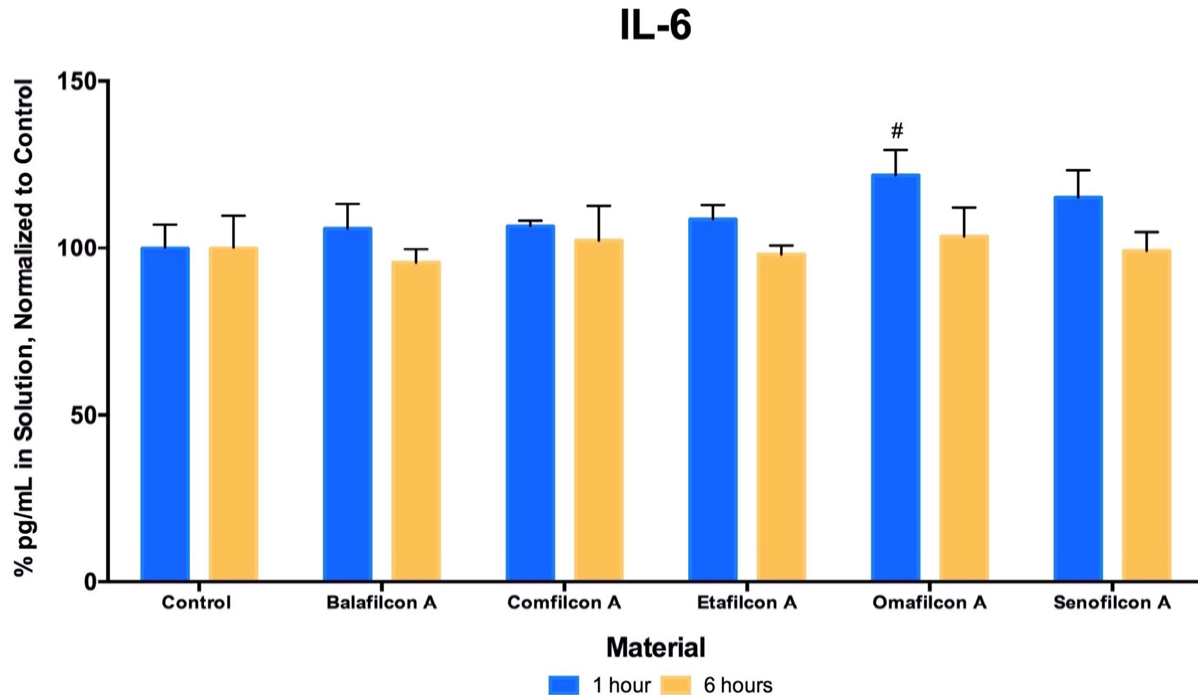

Figure 7-3: Percent pg/mL of IL-6 Remaining in HCEC-Derived Cytokine Solutions Containing either balafilcon A, comfilcon A, etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. At 1-Hour, omafilcon A was Statistically Different from the 1-Hour Control, as Defined by the Number (#) Symbol, Due to Having a Value Greater than 100% of the Control.

Relative to the control at 1 hour (100%), there was no uptake of IL-8 (Figure 7-4) onto either balafilcon A (108 ± 13 %), comfilcon A (105 ± 8 %), etafilcon A (109 ± 5 %), omafilcon A (113 ± 9 %) or senofilcon A (107 ± 3 %). Furthermore, relative to the 6-hour control (100%), there was also no uptake of IL-8 (Figure 7-4) onto balafilcon A (92 ± 2 %), comfilcon A (92 ± 15 %), etafilcon A (87 ± 5 %), omafilcon A (85 ± 8 %) or senofilcon A (81 ± 3%). There were no statistical differences observed between the concentrations of IL-8, at either the 1-hour or 6-hour time points, in any of the solutions containing the contact lens materials of interest ($p > 0.05$).

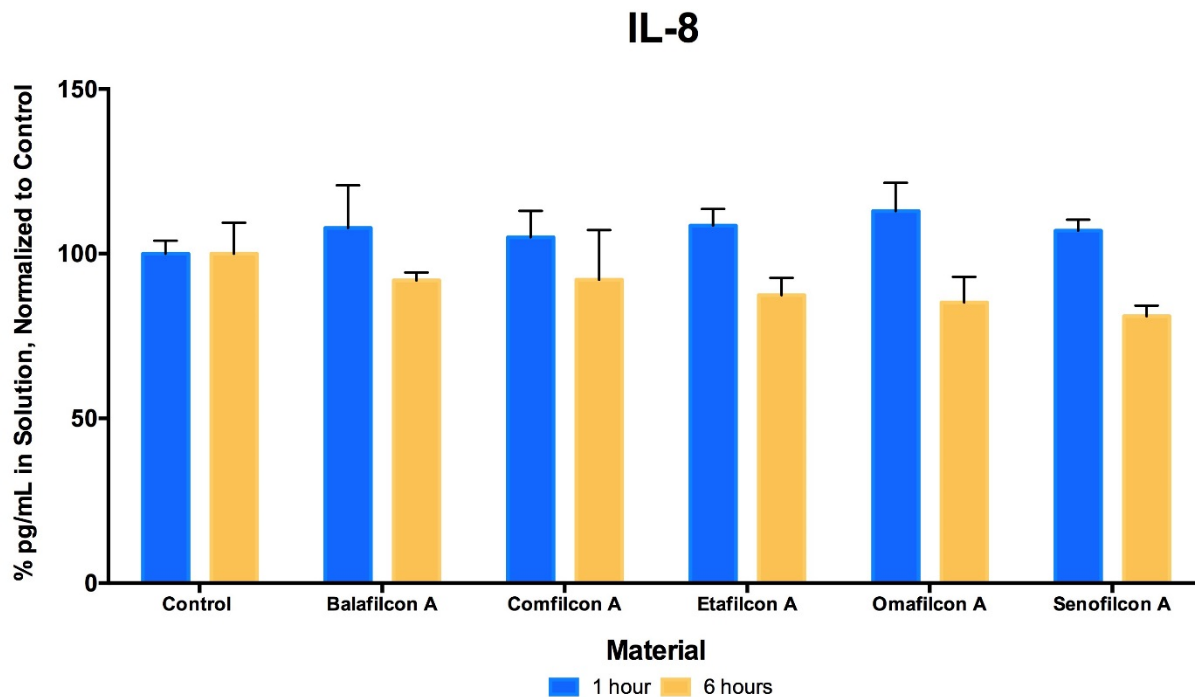

Figure 7-4: Percent pg/mL of IL-8 Remaining in HCEC-Derived Cytokine Solutions Containing either balafilcon A, comfilcon A, etafilcon A, omafilcon A, or senofilcon A (n = 3) Contact Lens Materials, at 1-Hour and 6-Hour Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test were Performed. No Statistical Differences to Report ($p > 0.05$).

7.3.5 Discussion – Experiments #1 and #2

Of the contact lens materials tested, only comfilcon A, etafilcon A and omafilcon A exhibited some uptake of TNF-α after 6 hours of soaking in the cytokine solution. Uptake was not observed for any of the other cytokines, by any other material. The results of these experiments were similar to the results of the experiments presented in sections 6.5.3 and 6.7.3, where uptake of TNF-α was observed after 6 hours onto omafilcon A when the material was pre-soaked in ATS, as well as onto comfilcon A and etafilcon A materials after a 7-day soaking period in a solution of higher cytokine concentration.

The pH of the HCEC culture supernatant was expected to be approximately 7.5 ± 0.2, as this was the pH of the EpiGRO Human Ocular Epithelia Media provided in its certificate of analysis. Thus, since the pH of the solution was similar to that of both Diluent 2 and ATS, similar

theoretical results were expected based on cytokine pI values, as explained in section 6.2.4. While TNF-α should have a predominately negatively-charged surface at this pH[83,91,92] and therefore have a less affinity for negatively-charged etafilcon A material[59], in this case, uptake of TNF-α onto etafilcon A was observed. Under many other experimental conditions presented throughout this thesis, however, this was certainly not a consistent observation. Additionally, there did not appear to be a consistent trend of interaction between the contact lens materials and the cytokines of interest to conclusively determine a single factor contributing to uptake.

7.4 Uptake of High Concentration HCEC-derived IL-6 and IL-8 onto Contact Lens Materials Over 7 Days

7.4.1 Introduction

The final experiment of the thesis repeated the experiment previously outlined in section 6.7, where one conventional hydrogel (etafilcon A) and one SiHy (comfilcon A) were investigated for cytokine uptake over a 7-day soaking period. In this case, the source of cytokines was from HCEC culture supernatant, rather than a combination of MSD calibrator blends. Additionally, the aim of this experiment was to investigate uptake at the highest possible cytokine concentration that could be obtained through a HCEC culture. As per section 6.7, reusable contact lenses were chosen for this experiment, as exploring uptake within a 7-day period would not be applicable to daily disposable contact lenses.

7.4.2 Materials and Methods

Human corneal epithelial cells were cultured as described in section 7.2 and the cell culture supernatants were collected and quantified with the MSD assay. Acuvue 2 (etafilcon A) and Biofinity (comfilcon A) contact lenses were prepared as described in section 4.3 and placed in polypropylene tubes (n = 3) containing 1 mL of a HCEC culture supernatant containing cytokines. Control tubes contained only 1 mL of the supernatant solution with no contact lenses. The

polypropylene tubes were wrapped in parafilm to prevent evaporation over the 7-day soaking period. 60 μL samples were collected at 24 hours, 72 hours (3 days) and at the 7-day mark. Statistical analyses were performed using two-way, repeated measures ANOVAs with Tukey's Multiple Comparisons test. Differences were considered significant if $p < 0.05$.

7.4.3 Results

The concentrations of IL-6 and IL-8 initially quantified from an aliquot of the cell culture supernatant of the batch of HCECs cultured for this experiment were 3613 pg/mL and 12,300 pg/mL, respectively. These concentrations were considerably higher than those previously tested using either the MSD calibrator blend or obtained from other HCEC culture supernatants. Furthermore, these concentrations represent the higher range of basal cytokine values (Table 3-3) reported in the literature.[66,68,70] On the day of the study, the mean cytokine concentrations in the 24-hour control solutions, as quantified by the MSD assay, were 2720 pg/mL and 9819 pg/mL for IL-6 and IL-8, respectively; in the 72-hour control solutions were 2656 pg/mL and 10,047 pg/mL for IL-6 and IL-8, respectively; and in the 7-day control solutions were 2288 pg/mL and 8520 pg/mL for IL-6 and IL-8, respectively. The high concentrations of these cytokines in this case were obtained through growing a larger number of cells/mL per flask, extending the number of days between culture media changes and collecting supernatant from older cells.

Given the high concentrations of IL-6 and IL-8, a 50-fold dilution factor was necessary when quantifying the concentrations of these cytokines by the MSD assay. Subsequently, the concentrations of IL-1β and TNF-α fell below the lower limit of detection of the MSD instrument, and therefore results for these cytokines are not reported. An initial quantification of the cell culture supernatant, however, revealed the concentrations of IL-1β and TNF-α to be roughly 1 pg/mL and 9 pg/mL. Evidently, a similar trend was observed as before, whereby IL-1β and TNF-α were

produced in lower concentrations by HCECs, in comparison to IL-6 and IL-8, which were produced at higher concentrations. Since previous experiments had already investigated the uptake of IL-1β and TNF-α at low concentrations, from both MSD calibrator blend and HCEC culture supernatant sources, results were not anticipated to be different and therefore it was not necessary to test the uptake of these particular cytokines again in this experiment.

Relative to the 24-hour control (100%), there was no uptake of IL-6 (Figure 7-5) onto either comfilcon A (108 $\pm$ 16 %) or etafilcon A (91 $\pm$ 2 %) and no uptake of IL-8 (Figure 7-6) onto comfilcon A (108 $\pm$ 9 %) or etafilcon A (96 $\pm$ 5 %). Furthermore, relative to the 72-hour control (100%), there was no uptake of IL-6 (Figure 7-5) onto either comfilcon A (97 $\pm$ 3 %) or etafilcon A (94 $\pm$ 7 %) and no uptake of IL-8 (Figure 7-6) onto comfilcon A (85 $\pm$ 10 %) or etafilcon (85 $\pm$ 7 %). There were no statistical differences observed at either time point between the respective control and either comfilcon A or etafilcon A material ($p > 0.05$).

Relative to the 7-day control (100%), there was also no uptake of IL-6 (Figure 7-5) onto comfilcon A (113 $\pm$ 11 %) or etafilcon A (102 $\pm$ 17 %). For IL-8 (Figure 7-6), there was no uptake onto comfilcon A (87 $\pm$ 6 %); however, there did appear to be some uptake onto etafilcon A (80 $\pm$ 5 %). While there were no statistical differences observed between the control and comfilcon A at this time point ($p > 0.05$), there was a statistical difference between etafilcon A and the control (adjusted P-value 0.0158), as defined by the star (*) symbol.

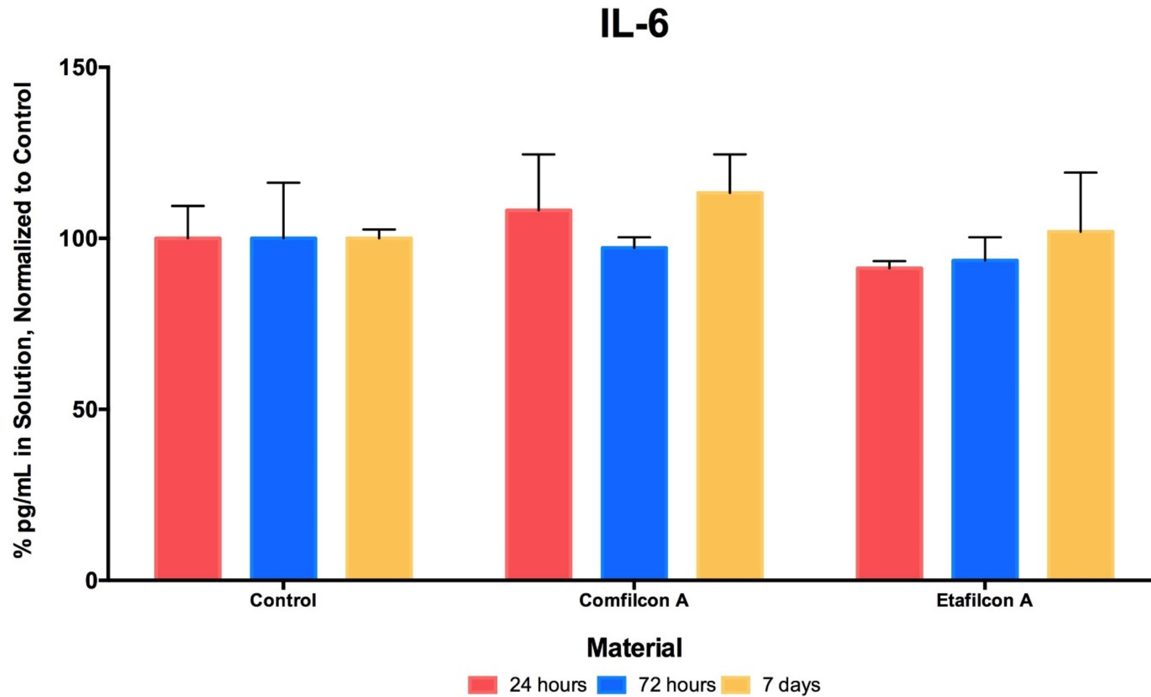

Figure 7-5: Percent pg/mL of IL-6 Remaining in Higher Concentrations of HCEC-Derived Cytokine Solutions, Containing either balafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 24-Hour, 72-Hour and 7-Day Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test Were Performed. No Statistical Differences to Report (p > 0.05).

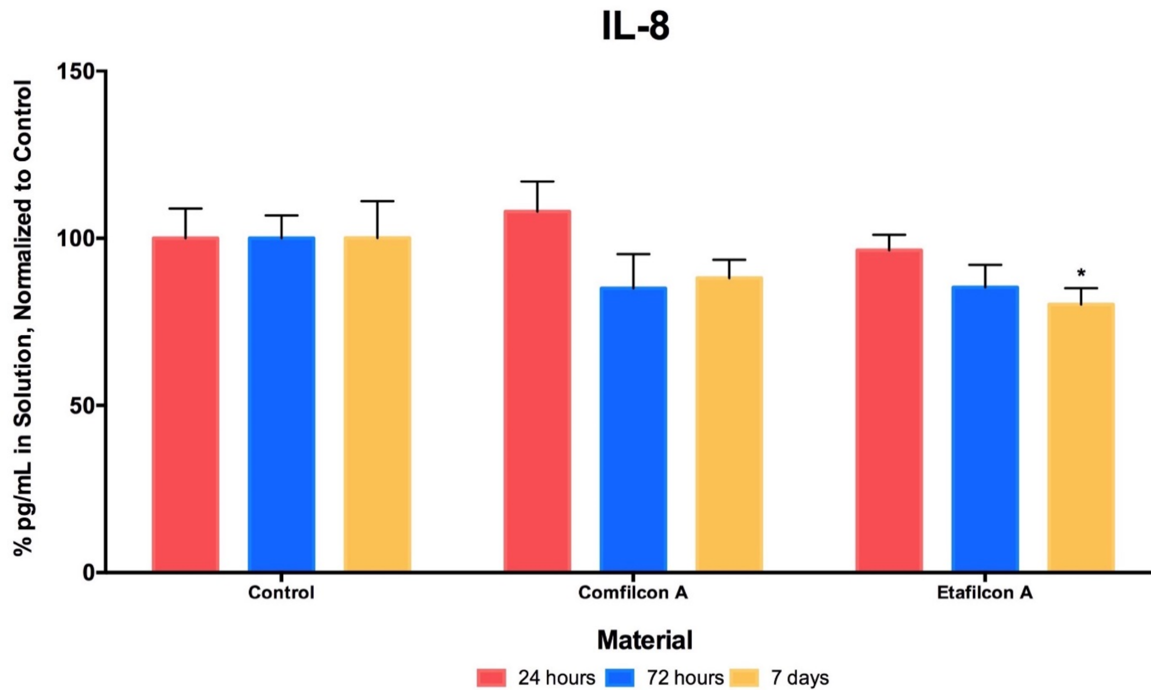

Figure 7-6: Percent pg/mL of IL-8 Remaining in Higher Concentrations of HCEC-Derived Cytokine Solutions, Containing either balafilcon A or comfilcon A (n = 3) Contact Lens Materials, at 24-Hour, 72-Hour and 7-Day Time Points. Two-way Repeated Measures ANOVAs with Tukey's Multiple Comparisons Test Were Performed. At 7 Days, etafilcon A was Statistically Different from the 7-Day Control, as Defined by the Star (*) Symbol.

7.4.4 Discussion

In this experiment, there only appeared to be some uptake of IL-8 onto etafilcon A at the 7-day time point. When this exact experiment was performed using MSD calibrator blends, there appeared to be some uptake of IL-8 onto comfilcon A (rather than etafilcon A), as well as uptake of TNF-α onto both comfilcon A and etafilcon A (6.7.3). In section 7.3.4, the concentrations of IL-6 and IL-8 tested were 342 pg/mL and 1885 pg/mL. These concentrations were the highest concentrations of cytokines that had been tested in comparison to all preceding experiments and were approximately 10 and 100 times larger than the concentrations of the cytokines obtained from a single MSD calibrator blend. In this experiment, even higher concentrations of IL-6 and IL-8 were tested (roughly 2000 pg/mL and 8500 pg/mL, respectively). Regardless of utilizing a very high concentration of cytokines in solution over a 7-day soaking period, there did not appear to be substantial amounts of uptake onto the contact lens materials. Moreover, even with the small apparent amount of uptake for etafilcon A, again, there appeared to be no identifiable trend for this uptake when compared to the results of an almost identical experiment.

Chapter 8: Conclusions and Future Work

8.1 A Summary

Based on the pilot studies performed, it appeared that cotton consistently exhibited the highest amount of uptake for all of the four cytokines of interest, however, due to its ability to also absorb a volume of the soaking solution, it may not have served as an ideal positive control. Hypothetically, if cotton were to absorb only liquid with no cytokines, the concentration of the cytokines in the soaking container would appear higher, as a result of a decrease in the soaking volume. In contrast, if cotton were to absorb both cytokines and some liquid (as in the pilot studies), while absorbing some of the soaking liquid (i.e., decreasing volume), will increase the concentration of cytokines in the soaking container, even if there is absorption of these cytokines, the amount of uptake cannot correctly be quantified since the initial concentration before uptake would not be representative of the true value due to this change in volume. In the pilot studies where cotton was investigated as a positive control, however, the appropriate experimental controls were employed, which contained only a volume of the cytokine solution with no cotton material. The cytokine concentrations quantified from the experimental controls were then regarded as the "initial concentrations", to which the concentrations from the solutions containing cotton were compared, in order to determine the amount of uptake over the specified time period. While Millipore filter paper also exhibited substantial uptake of all four cytokines and is much less liquid absorbent than cotton, thereby potentially serving as a better positive control, future experiments could place an emphasis on determining a biomaterial with more clinical relevance that could serve as a positive control, ideally one that can deposit cytokines with no absorption of liquid.

When considering the experiments outlined in Chapter 6:and Chapter 7:, of the contact lens materials tested in these two chapters (balafilcon A, comfilcon A, etafilcon A, omafilcon A, senofilcon A and somofilcon A), it was only balafilcon A, comfilcon A, etafilcon A and omafilcon A that exhibited any amount of cytokine uptake – though these amounts varied between experiments and between the cytokines of interest. Senofilcon A and somofilcon A did not exhibit any uptake of IL-1β, IL-6, IL-8 or TNF-α. Additionally, there was no uptake of IL-6 by any of the contact lens materials investigated.

Uptake of IL-1β was demonstrated by omafilcon A following a 6-hour soak in a MSD calibrator blend-derived cytokine solution (Figure 6-5) and following a 6-hour soak in a reduced volume MSD calibrator blend-derived cytokine solution (Figure 6-13). Both balafilcon A and comfilcon A also demonstrated uptake of IL-1β following a 6-hour soak in a MSD calibrator blend derived-cytokine solution (Figure 6-33). Uptake of IL-8 was demonstrated by balafilcon A and comfilcon A following a 6-hour soak in a MSD calibrator blend-derived cytokine solution (Figure 6-35); by comfilcon A following a 7-day soak in a MSD calibrator blend-derived cytokine solution of higher concentration (Figure 6-43); and also by etafilcon A following a 7-day soak in a HCEC-derived cytokine solution of very high concentration (Figure 7-6).

Uptake of TNF-α was demonstrated by etafilcon A and omafilcon A after a 1-hour soak in a MSD calibrator blend-derived cytokine solution (Figure 6-12), although this was likely not indicative of true uptake as no uptake was observed at the 6-hour time period. Additionally, it appeared that there was some uptake of TNF-α by omafilcon A following a 6-hour soak in a MSD calibrator blend derived-cytokine solution, after the material was pre-soaked in an ATS solution (Figure 6-28). Balafilcon A also exhibited uptake of TNF-α following a 6-hour soak in a MSD calibrator blend-derived cytokine solution (Figure 6-36). Comfilcon A and etafilcon A exhibited

uptake of TNF-α following a 7-day soak in a MSD calibrator blend-derived cytokine solution of higher concentration (Figure 6-44). Comfilcon A, etafilcon A, and omafilcon A exhibited uptake of TNF-α following a 6-hour soak in a HCEC-derived cytokine solution (Figure 7-2).

Interestingly, there were two conventional hydrogels (etafilcon A and omafilcon A) and two SiHys (balafilcon A and comfilcon A), which appeared to exhibit uptake of IL-1β, IL-8 and TNF-α. Of the conventional hydrogels, both were of high water content and one was ionic/negatively charged (etafilcon A), while the other was zwitterionic/neutral (omafilcon A).[59] Of the SiHys, in contrast, both were of low water content and similarly, one was ionic/negatively charged (balafilcon A), while the other was nonionic (comfilcon A).[59,100]

It did not appear that the isoelectric points of the cytokines or the ionicity of the contact lens materials greatly influenced cytokine uptake, as a trend did not seem to exist for uptake. For example, etafilcon A (negatively charged), exhibited some uptake of TNF-α (negatively charged surface based on its pI and the pH of the solution utilized), while other materials of nonionic nature also exhibited uptake of TNF-α.[59,83,91,92] Furthermore, in some experiments, etafilcon A did not exhibit uptake of TNF-α at all. Moreover, etafilcon A exhibited uptake of IL-8 (positively charged surface based on its pI and the pH of the solution utilized) in some experiments, while in other experiments, no uptake of IL-8 onto etafilcon A was observed.[59,83,89]

It also did not appear that the water content of the contact lens materials greatly influenced uptake. While it could be hypothesized (based on the results of the experiments presented in this thesis), that to demonstrate uptake of IL-1β, IL-8 or TNF-α, a high water content conventional hydrogel or a low water content silicone hydrogel contact lens material must be utilized, this would not be consistent with the observation that senofilcon A, a SiHy of low water content, did not exhibit cytokine uptake. Perhaps it could also be hypothesized that for conventional hydrogels,

having a high water content is enough to have some uptake of IL-1β, IL-8 or TNF-α, while for SiHys, a material of ionic nature is required. This would not, however, be consistent with the observation that comfilcon A, a nonionic material, did demonstrate some uptake of these cytokines.

Perhaps in the case of omafilcon A, the zwitterionic character is important for its interaction with cytokines. In that case, perhaps there is a certain pH of soaking solution that could give the surface of the zwitterion a certain charge, thereby making it more ideal for cytokine interaction. Alternatively, perhaps the structure of the cytokine or the contact lens is not uniform, and a purely positive or purely negative character is not required for uptake. Rather, perhaps different areas on the surface of the cytokine or contact lens may be more positive or more negative and result in different interactions than what may be expected. Moreover, it is possible that for some materials, the level of water content determines how hydrated the surface of the lens is and hence influences pore sizes in the contact lens material to which cytokines could potentially adhere.

Of the four cytokines of interest, it is of note that there was consistently no uptake of IL-6 demonstrated by any material. As hypothesized in section 6.6.5, this could possibly be due to the size of this cytokine, as it is one of the larger cytokines[15] of the four (Table 3-2), and also based on its predicted size in the MSD calibrator blend (20.3 kDa according to Meso Scale Discovery Scientific Support, while the predicted sizes of IL-1β, IL-8 and TNF-α are 17 kDa, 8 kDa and 17.5 kDa, respectively). Given that a molecular weight of 20.3 kDa is not substantially greater than 17 kDa or 17.5 kDa, however, it is possible that the oligomeric state of IL-6 may instead play a role in its ability to uptake onto the contact lens materials.[110]

Human IL-1β naturally occurs as a 17.5 kDa[84] monomer[9], while human TNF-α naturally occurs as a 17.5 kDa homotrimer.[111] In contrast, the oligomeric state of human IL-8 appears to be

concentration-dependent.[112,113] For example, it has been reported in the literature that this protein exists in a dimeric state at millimolar (mM) concentrations and exists in a monomeric state at nanomolar (nM) concentrations, with a dissociation constant (K_d) for dimerization reported as 10-20 μM.[112,113] As a result, while the molecular weight of monomeric IL-8 is reported between 6-8 kDa[15], the molecular size of the dimeric state can therefore be between 12-16 kDa. Supporting this, Kendrick and colleagues reported through their investigation that the molecular size of the wild-type IL-8 dimer was calculated as approximately 17 kDa.[113] The concentrations utilized throughout the experiments presented in this thesis were calculated to be less than 2 nM and thus, the IL-8 was predicted to be in its monomeric state accordingly.[112,113]

While human IL-6 naturally occurs as a 21-28 kDa[15] monomer[9,110], it has been reported in the literature that due to exhibiting an aggregation-prone nature, human IL-6 may also exist as a multimer composed of several aggregates ranging in molecular size.[110] Hence, it is possible that the IL-6 in the lyophilized MSD calibrator blend utilized for the experiments presented in this thesis, or even that obtained from a culture of human corneal epithelial cells, could have formed this multimeric complex through aggregation.[110] According to Meso Scale Discovery Scientific Support, the cytokines within the calibrator blend are biologically active and therefore exhibit the same oligomeric state as the endogenous cytokines. Thus, depending on the number of aggregates for IL-6, this would increase the overall molecular size of the multimer above its monomeric molecular size, which could have affected the ability of this protein to interact with the surface of the contact lens materials tested, such that deposition could not occur. Alternatively, it could be possible that the presence of the aggregates alone, irrespective of molecular size, may have hindered the protein's ability to deposit onto the contact lens materials.

It has been suggested in the literature that the very act of wearing a contact lens is intrinsically inflammatory.[95] This idea was presented in a paper by Nathan Efron, who explored this notion and concluded that hydrogel lens wear meets all five criteria outlining the classical and clinical definition of inflammation (i.e., heat, swelling, pain, redness and loss of function), in addition to all two criteria outlining a subclinical definition of inflammation (i.e., cellular reactions and biochemical reactions).[95] Similarly, silicone hydrogel wear met four of the total seven criteria (pain, loss of function, cellular reactions and biochemical reactions), and was therefore concluded to be inflammatory in nature as well.[95] Efron suggested that all contact lens wear, whether it be a hydrogel or silicone hydrogel contact lens material, induces a state of "para-inflammation", or a chronic, low-grade (non-damaging) inflammation on the ocular surface, close to that of the ocular surface's basal state, in order to restore the ocular surface's homeostasis.[95] As a result, Efron also suggested that during lens wear, the ocular surface has an "upregulated immune status", which in turn serves a protective function in that any antigens presented to the ocular surface can be more promptly dealt with due to the "heightened alert" state of the eye.[95]

In the body, and therefore in the eyes as well, there is always an immune response present to a certain degree, as a basal level of immune cells or inflammatory markers (such as cytokines), exist in both the blood and in the tears.[64-70,93,94,114] In the eye, this basal level of immune cells or inflammatory markers may exist as a result of the physiological but proinflammatory shift that takes place on the surface of the eye during sleep, when tear production is reduced and limited oxygen is able to reach the eyes as they are closed.[18] As mentioned earlier in section 2.6, complement activation is known to increase in the tear film during the first few hours of sleep and a significant influx of neutrophils later follows.[18] In addition, an increase in TLR expression in conjunctival epithelial cells has also been observed due to hypoxic ocular conditions during

sleep.[18] Based on the idea that contact lens wear can intrinsically be inflammatory, it could be possible that the basal level of cytokines on the ocular surface may be higher in a contact lens wearer, in comparison to an individual who does not wear contact lenses.[95] Similarly, different individuals may therefore have different threshold levels for comfort. Thus, it would not be appropriate to assume that contact lens discomfort can occur only as a result of large quantities of cytokines from the tear film depositing onto the contact lens material, such that the threshold for comfort is surpassed. Should a particular contact lens material exhibit uptake of cytokines, it may also be possible that contact lens discomfort could arise from having deposited the basal amounts of cytokines onto the lens material, such that not enough cytokines remain in the tears to maintain a standard, basal level of inflammatory markers that would otherwise exist.

A question then arises as to what would constitute an ideal contact lens and the answer to this question depends on the type of cytokine in question (i.e., proinflammatory or anti-inflammatory). Based on the results of the experiments presented in this thesis, since none of the contact lens materials investigated exhibited substantial amounts of cytokine uptake, it may be said that any of these contact lens materials could potentially be an ideal lens that would not cause discomfort from the deposition of inflammatory markers. This can only be said, however, for the four cytokines of interest and therefore, it cannot conclusively be said that these contact lens materials will not deposit other cytokines or inflammatory proteins, resulting in discomfort. In the case that both a reusable or daily disposable contact lens do exhibit uptake of proinflammatory cytokines, it would be more ideal for the daily disposable contact lens to deposit the cytokines, rather than the reusable contact lens, given that the daily disposable contact lens will be discarded at the end of the day and not worn again. In contrast, with a reusable contact lens, any deposited cytokines would likely denature once the contact lens is placed into the care system at the end of

the day, given that a large number of care systems utilize peroxide-based[96] storage solutions. The denatured cytokines, however, may remain adhered to the surface of the contact lens and once inserted back onto the eye the next day, may cause discomfort or irritation.[58] Additionally, there may also be some degree of bacteria, bacterial parts, or debris bound to contact lenses that can result in additional discomfort or irritation[96], and the degree of this discomfort or irritation may persist longer when utilizing a reusable contact lens, rather than a daily disposable contact lens. In this case, uptake of a proinflammatory cytokine by a daily disposable contact lens would be the most ideal, as it may aid in dampening an immune response in the context of inflammatory ocular conditions (such as DES or allergies), without causing collateral damage to the ocular tissue. The reusable contact lens would not be ideal due to the issues with protein denaturation as discussed above. In contrast, the uptake of an anti-inflammatory cytokine may not be ideal for either a daily disposable or reusable contact lens material. In the context of DES or allergies, it may be beneficial to have the presence of anti-inflammatory cytokines on the ocular surface to mitigate the immune response and therefore, having a contact lens material (of either wear modality), that could deposit anti-inflammatory cytokines such that they are removed from the ocular surface would not be ideal.

8.2 Future Work

While it appears that any amount of cytokine uptake observed by balafilcon A, comfilcon A, etafilcon A and omafilcon A contact lens materials was minor, perhaps these “minor” quantities of uptake are actually the maximum amounts of deposition of these cytokines onto the lens materials, given the small size and surface area of a contact lens. Additional experiments would therefore be required to determine the clinical relevance of this amount of uptake. For example, perhaps small quantities of uptake are sufficient to initiate local inflammation on the corneal surface and contribute to symptoms of contact lens discomfort. In the literature, while many

investigators have researched the levels of tear cytokines in both contact lens wearers and individuals who do not wear contact lenses, it is evident that large discrepancies exist in the levels quantified (refer to Table 3-3).[64-70,93,94] As explained in section 2.8, this may be due to the short half-life of each cytokine[74-77], resulting in quick degradation of the proteins that may falsely mimic uptake.

The assay utilized for the experiments presented in this thesis (apart from the MSD immunoassay), was an indirect measurement of cytokine uptake (subtractive assay), in that the amounts of uptake onto the contact lens materials quoted relative to the 100% controls, reflected the values remaining in the solutions over time. Thus, a limitation to this study design was that any differences between the control values and the values remaining in the solutions containing the contact lens materials of interest, were surmised to have been taken up onto the surface of that material. Thus, while the appropriate experimental controls were utilized, these differences (which were considered to be amounts of uptake), were not confirmed by a supplementary extraction assay. An assay that could provide clinical relevance while also serving as a more direct measurement of uptake can be utilized in future experiments. This includes thin slicing of the contact lens in conjunction with an ELISA assay, use of a confocal microscope to observe the biological effects of co-incubating contact lens materials soaked in a cytokine solution with human corneal epithelial cells, or co-incubation of contact lenses with a human macrophage cell line and an ELISpot assay to evaluate cytokine responses.

Given that there are more than 1400 unique proteins in the tear film[7] and the experiments presented in this thesis focused only on four types of inflammatory proteins (cytokines) present in the tear film, there exists a realm of possibility in regard to other types of inflammatory proteins that can be evaluated in the future for deposition onto contact lens materials. Some cytokines of

interest could include allergy cytokine IL-4 that is over-expressed by a Th2 response during allergic conjunctivitis, or another cytokine of the IL-1 family, such as IL-1α, which is produced by macrophages and also exists in the corneal epithelial cells where it is stored and released upon infection or trauma.[3,10,13,21] Ideally, any additional cytokines to be investigated should be one that exists at a high concentration in the eye, or one that is considered to be highly biologically active.

It is known that the use of silicone hydrogels is associated with two times the risk of inflammatory complications (corneal infiltrative events), in comparison to pHEMA-based hydrogels.[96] The results presented in this thesis did not appear to indicate that the uptake of proinflammatory cytokines IL-1β, IL-6, IL-8, or TNF-α, onto either balafilcon A, comfilcon A, senofilcon A or somofilcon A silicone hydrogel materials, result in the increased risk of inflammatory complications or discomfort that has been documented. Thus, the question still remains in regard to what factors may result in this statistic to be true. One hypothesis presented in the literature is that the hydrophobic nature of silicone hydrogels may attract more bacteria and a low water content may promote the adhesion of the bacteria to the lens surface.[96] Another hypothesis is that the low oxygen permeability of hydrogels may promote the adhesion of denatured proteins with retained antimicrobial efficacy to the lens, whereas silicone hydrogels would lack this efficacy as a result of having higher oxygen permeability.[96]

8.3 Conclusion

The inherent properties of the contact lens materials tested under these experimental conditions did not appear to exhibit any uptake of IL-6 and furthermore, did not appear to exhibit substantial uptake of IL-1β, IL-8, or TNF-α. These conditions included the use of a lyophilized standard blend of recombinant cytokines, or cytokines derived directly from human corneal epithelial cells; low, moderate or high cytokine concentrations; and with or without additional

surface modifications (i.e., ATS soak) to the contact lens materials, such that the ocular surface of a clinically-worn contact lens was mimicked. Thus, the inflammatory complications that have been reported in the literature with contact lens wear of SiHy or conventional hydrogel material,[52] which are thought to contribute to contact lens discomfort[51,56], may not be due to the interaction of these contact lens materials with IL-1β, IL-6, IL-8 or TNF-α inflammatory markers. As a result, it is possible that contact lens discomfort may be attributed to other factors such as biofilm formation on contact lens cases; differences in contact lens wettability; differential uptake of constituents in contact lens solutions onto contact lens materials; or the interaction of a contact lens with tear components that are not inflammatory markers. This work, in part, helped to address issues surrounding ocular inflammation and contributed to providing a better understanding of the role of inflammatory markers in contact lens discomfort.

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