Protocol development for reliable isolation of tear-film neutrophils and *in vitro* functionality testing.

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.

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Abstract

During prolonged eye closure, such as during sleep, leukocytes are recruited to the ocular surface, with polymorphonuclear neutrophils (PMNs) representing the largest population.

PMNs are essential inflammatory cells of the innate immune system, and possess several efficient killing mechanisms, such as phagocytosis and release of antimicrobial substances stored in granules, to protect the host tissues from invading pathogens. Tear-film neutrophils collected from the closed-eye environment have been shown to express high levels of degranulation and activation leukocyte markers (such as CD66b and Mac-1). As these cells may play a role in ocular inflammation and disease, it is important to determine their functionality. However, large variations in response and collection numbers have been observed previously. Limited knowledge also exists on the ability of tear film PMNs to respond to cytokines and mount an oxidative response. Thus, the objectives of this thesis were to develop a standard protocol to process tear-film PMNs to reliably conduct functionality tests *in vitro* and to assess the production of reactive oxygen species (ROS) in tear film PMNs. Specifically, 1) the sensitivity of tear-film PMNs to experimental procedures (fixation, centrifugation and incubation) was investigated in terms of expression of surface receptors via flow cytometry; 2) two different cell collection methods were evaluated, and changes in the expression of surface receptors of tear-film PMNs when exposed to interleukin-8 (IL-8) and phorbol 12-myristate 13-acetate (PMA) were examined through flow cytometry; and 3) the ability of tear-film PMNs to generate ROS was assessed

using luminol-enhanced chemiluminescence. The response of tear-film PMNs was also compared to blood-isolated PMNs.

Up to 20 participants were recruited in this research to perform cell collection and donate a small blood sample. A gentle eye wash method was used to collect cells from the closed-eye environment, whereby participants washed their eyes with sterile phosphate buffer saline (PBS) upon awaking at home, and collected the runoff into a sterile polypropylene tube, which was then delivered to the lab within two hours. The patch-OSCCA collection was also tested; participants slept at home and covered one of their eyes with a patch. When they woke up in the morning, they came to the lab directly with one eye covered, and cells were collected using the ocular surface cell collection apparatus (OSCCA), which gently irrigates the ocular surface with warm PBS and collects the solution and cells from a funnel into a centrifuge tube. Very few cells were obtained using the patch-OSCCA collection method and thus this method was not pursued further in this research.

When assessing the effect of experimental procedures and measuring cell activation upon stimulation with IL-8 and PMA (a PKC activator), changes in the expression of CD11b (activation and adhesion leukocyte membrane receptor), CD16 (degranulation and phagocytosis marker), CD66b (membrane receptor expressed upon cell degranulation), CD45 (leukocyte common antigen) and CD55 (complement activation marker) were characterized by flow cytometry. ROS production in stimulated (PMA, lipopolysaccharides (LPS) or N-Formylmethionyl-leucyl-phenylalanine (fMLP)) and unstimulated tear-film PMNs was measured using luminol-enhanced chemiluminescence (CL). In all experiments, blood-isolated PMNs were also used to allow for comparison in phenotype.

Fixation with paraformaldehyde (PFA) is an important step in flow cytometry. Fixing tear-film PMNs prior to the staining with antibodies resulted in a significant (5-fold or more) reduction in the expression of CD11b, CD16 and CD45 when compared to unfixed samples, while CD16 was the only receptor to undergo significant downregulation upon post-staining fixation. Furthermore, it was found that an additional centrifugation step prior to antibody incubation and long (4hr) incubation at 37^{o}C significantly altered the expression of membrane receptors with significant reduction in expression of CD11b, CD16 and CD55 when compared to control samples. Therefore, to preserve cell phenotype and cell integrity of tear film PMNs, any additional centrifugation and incubation step should be avoided and post fixation staining is recommended.

To gain a further understanding on the phenotype of tear-film PMNs, their ability to respond to IL-8, a cytokine present in the tear film of the closed-eye environment, was characterized via flow cytometry. The expression of surface receptors CD11b, CD16, CD55 and CD66b on tear-film PMNs remained relatively unchanged when exposed to IL-8, whereas some changes in the level of expression of surface receptors were observed in response to PMA but in a lower magnitude compared to blood-isolated PMNs.

The respiratory burst is one of the essential killing mechanisms for PMNs and is also related to phagocytosis. PMA stimulation was able to induce ROS production (as measured by chemiluminescence) in tear-film PMNs, and two distinct responder groups were observed, where the high responder group produced significantly more ROS than the low responders. LPS and fMLP failed to induce intracellular ROS production in tear-film PMNs although fMLP-stimulated tear-film PMNs generated ROS extracellularly in the first three minutes.

These results suggested that the signalling pathways downstream of PKC as well as the NADPH oxidase were functional but that intracellular signalling pathways upstream the PKC were impaired.

This thesis contributes new and important knowledge on tear film PMNs. We proved that the gentle eye wash method is currently the most effective at home collection method. In addition, our study demonstrated that experimental procedures can significantly affect the expression of membrane receptor expression on tear-film PMNs, and if these processing steps are not carefully considered, conclusions on the phenotype of tear-film PMNs can be severely impacted. Our findings also suggest that the lack of response to stimuli may be due to an impairment in the receptor-mediated intracellular signalling pathway and/or insufficient substrates within the cell. Finally, our study on respiratory burst identified two type of responders, which could have significant implications for microbial keratitis and contact lens-induced infiltrates.

This thesis highlights the potential role of tear film PMNs in ocular health and inflammation but more work is still needed to gain a better understanding of the phenotype of tear film neutrophils in the closed eye environment and their underlying mechanisms of activation.

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To my beloved grandmother,

Xiangqin Wang.

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List of Abbreviations

AA: Arachidonic acids

ARDS: Acute respiratory distress syndrome

AUC: Area under the curve

BAL: Bronchoalveolar lavage

CD: Cluster of differentiation

COPD: Chronic obstructive pulmonary disease

CP: Chronic periodontitis

DAG: Diacylglycerol

DAF: Decay-accelerating factor

DED: Dry eye disease

DMEM: Dulbecco's modified eagle media

DNA: Deoxyribonucleic acid

EW: Gentle eye wash method

fMLP: N-Formyl-L-methionyl-L-leucyl-L-phenylalanine

G-CSF: Granulocyte colony-stimulating factor

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GPCR: G-protein-coupled receptor

GPI: Glycosylphosphatidylinositol

CR3: Complement receptor

HBSS: Hanks's balanced salt solution

hCECs: Human corneal epithelial cells

HLDA workshop: Human leukocyte differentiation antigens workshop

$HO^{\cdot}$: Hydroxyl radical

HOCl: Hypochlorous acid

HR: High responders

H_2O_2: Hydrogen peroxide

IL-3: Interleukin-3

IL-8: Interleukin-8

IP_3: Inositol triphosphate

KM: Keratinocyte serum-free medium

LDGs: Low-density granulocytes

LPS: Lipopolysaccharides

LR: Low responders

Mac-1: Macrophage-1 antigen

MAPK: Mitogen-activated-protein kinase

MEK1: Mitogen-activated protein kinase kinase 1

MEKK-1: Mitogen-activated-protein kinase kinase kinase 1

MFI: Mean fluorescent intensities

MGD: Meibomian gland dysfunction

MK: Microbial keratitis

MPO: Myeloperoxidase

NADPH oxidase: Nicotinamide adenine dinucleotide phosphate oxidase

NDGs: Normal-density granulocytes

NETs: Neutrophil extracellular traps

O_2^-: Superoxide anion

OA: Ocular allergy

OSCCA: Ocular surface cell collection apparatus

PA: Phosphatidic acid

PAC: Perennial allergic conjunctivitis

PBS: Phosphate-buffered saline

PC: Patch-OSCCA collection method

PFA: Paraformaldehyde

PKC: Protein kinase C

PLA_2: Phospholipase A_2

PLC: Phospholipase C

PLD: Phospholipase D

PI3K: Phosphatidylinositol 3-kinase

PMA: Phorbol-12-myristate-13-acetate

PMN: Polymorphonuclear neutrophils

RNA: Ribonucleic acid

ROS: Reactive oxygen species

rTEM: Transendothelial cell migration

SAC: Seasonal allergic conjunctivitis

SD: Standard deviation

sIgA: Secretory immunoglobulin A

ST: Screening test

STMR: Seven transmembrane-spanning receptor

TNF-α: Tumor necrosis factor α

Chapter 1

Introduction

The anterior segment of the eye represents a unique physiological environment due to its specific characteristics linked to immune privilege[1,2]. Inflammatory cells are believed to be highly regulated because of the required balance between protection of the eye against pathogens and preservation of the integrity of the corneal epithelium[1,2]. In the context of an immune privileged-site, immune and inflammatory cells (lymphocytes, neutrophils, monocytes, etc.) are only to be introduced to the local environment when an infection or a wound is present. However, in the anterior eye, a significant influx of neutrophils, combined with an increase in complement activity, metalloproteinases and cytokines, have been detected in the closed-eye environment (during sleep) even in the absence of any wound or infection[1,3]. This neutrophil population is often referred to as "tear-film neutrophils" to reflect the local environment where their presence has been clearly identified[4]. Tear-film neutrophils were first discovered in 1989 by Wilson *et al*., where they observed that the number of leukocytes collected after sleep in the morning was significantly higher than in the afternoon[3]. The limited number of cells collected prevented assessing cell functionality. Thus, researchers, at that time, focused on comparing the numbers of tear-film neutrophils recovered between open-eye and closed-eye tears[3], and also between tears collected from lens-wearers and non-lens wearers, to determine if the presence of contact lenses altered the recruitment of tear-film neutrophils. The increase in cell numbers, chemokines and inflammatory mediators in the closed-eye environment have led researchers to believe that tear-film neutrophils are present to eliminate pathogens that may be "trapped" during closed

eye[5,6]. More efficient methods for ocular cell collection and characterization have renewed the interest on the role of tear-film neutrophils in ocular health.

Non-invasive methods used to collect cells from the ocular surface have evolved over the years and have been used to harvest not only neutrophils but also human corneal epithelial cells (hCECs). The "non-contact corneal irrigation chamber" and disposable glass micro-capillary tubes were used in 1986[7] and 1998[8], respectively, to collect cells, but less than 200 hCECs and 8000 leukocytes were usually obtained, preventing any cell phenotyping. The ocular surface cell collection apparatus (OSCCA), developed by Peterson *et al.* allowed researchers to harvest more cells from the ocular surface compared to previous methods[9]. While effective in cell collection, as it can collect over hundreds of thousands of neutrophils from the ocular surface[10], the cost involved with overnight studies limited the use of the OSCCA for collection of tear-film neutrophils, as participants were required to sleep at the clinic. A new method, using a gentle eye wash, was thus designed by Gorbet *et al.*, and allowed participants to collect tear-film neutrophils at home[1].

Collecting more cells from the closed-eye environment has enabled researchers to start characterizing the phenotype of tear-film neutrophils. The study by Gorbet *et al* indicated that tear-film neutrophils may have a non-inflammatory phenotype, as they were unable to respond to inflammatory stimuli through upregulation of membrane receptors commonly associated with activation[1]. Furthermore, Gorbet and Willcox showed that tear film neutrophils have an impaired ability to kill bacteria[11]. These findings suggest that the function of tear film neutrophils upon collection from the eyes after sleep may be diminished. This impaired function of tear-film neutrophils may be due to the presence of tear proteins, such as lactoferrin, or the hypoxic condition of the closed-eye environment. However,

efforts to mimic the conditions of the closed-eye environment failed to induce the same non-inflammatory phenotype in blood-isolated neutrophils, and further suggested the heterogeneity of neutrophil populations[4].

Tear-film neutrophils have been shown to express high level of degranulation and activation leukocyte markers (CD66b and Mac-1, respectively, which will be further explained in chapter 2) but low level of L-selectin[1,12]. Similar observations have been made on the phenotype of neutrophils that have migrated from the blood into tissues, such as the healthy lungs[13], the mouth[14] and the placenta[15]. Recent observations of tear-film neutrophils collected after various times of exposure to the closed-eye environment have also led Postnikoff *et al* to hypothesize that these cells have been already activated in their early recruitment to the ocular surface[12]. These recent studies on tear film-neutrophils thus tend to suggest a complex phenotype - with a yet undefined origin.

To characterize inflammatory phenotype of leukocytes, cells are activated *in vitro*. The stimuli used to characterize the response of tear-film neutrophils have been phorbol-12-myristate-13-acetate (PMA; a potent synthetic chemical activator of protein kinase C), lipopolysaccharides (LPS; a bacterial endotoxin), N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; a chemotactic peptide), and calcium ionophore[1,12,16]. However, these are not all physiological stimuli nor representative of other stimuli that tear-film neutrophils may encounter in the ocular environment. Cytokines, such as tumor necrosis factor α (TNF-α)[17], granulocyte-macrophage colony-stimulating factor (GM-CSF)[18], and interleukin-8 (IL-8)[19] have been recognized to activate blood-isolated neutrophils, however little is known on the response of tear-film neutrophils to these or other cytokines. Among these more physiological stimuli, IL-8 is a chemokine for neutrophils, leading to activation and

migration of blood leukocytes to infected sites through the actions of L-selectin and Mac-1[20,21], and high levels of IL-8 have been observed in the closed-eye environment[8]. Characterizing the response of tear-film neutrophils to IL-8 would provide information on their activation state in the closed-eye environment as well as contribute further evidence towards their phenotypic difference from blood leukocytes.

The respiratory burst, via the release of reactive oxygen species (ROS), is also part of the leukocyte inflammatory response and is one of the efficient ways for neutrophils to kill pathogens. The mechanisms that blood-isolated neutrophils used to generate ROS have been well characterized, and it is known that blood-isolated neutrophils used different intracellular signaling pathways to respond to various stimuli[22–24]. While the ocular surface is exposed to various pathogens, there is currently limited knowledge on ROS generation with tear-film neutrophils. Hume *et al.* have used blood-isolated neutrophils to characterize ROS generation upon exposure to ocular isolated bacteria, *Serratia marcescens*, to study the potential underlying mechanisms of microbial keratitis[25]. However, since recent research has indicated that blood-isolated and tear- film neutrophils exhibit different phenotypes, it may not be sensible to use blood-isolated neutrophils to assess mechanisms of inflammation from the ocular surface. Furthermore, using flow cytometry, the ability of tear-film neutrophils to generate ROS was reported to be significantly lower than blood-isolated neutrophils[16]. However, flow cytometry provides limited insights into the process and potential mechanisms of ROS generation by tear-film neutrophils. The extent and kinetics of ROS generation can be characterized by luminol-enhanced chemiluminescence and could contribute significant knowledge to the mechanisms of ROS generation in tear-film neutrophils and allow us to gain a better understanding of ocular inflammation.

As can be seen from the number of recent studies, tear-film neutrophils (both from the closed- and open-eye environment) are emerging as a new source of knowledge on inflammation at the ocular surface[1,12,16,26,27]. In addition, tear-film neutrophils may play a role in some ocular diseases, such as dry eye disease[12,28] and seasonal ocular allergy[29], but their interactions with other immune cells and their activation mechanisms need to be further examined. Different protocols have been used post-collection of neutrophils from the ocular surface, which may lead to variations in results and affect characterization of cell phenotype. Various collection protocols have also been used previously and no systemic study has yet been undertaken to investigate the sensitivity of tear-film neutrophils to experimental procedures[1,8,12,30,31]. This significantly affects our ability to reliably use these cells for the development of diagnostic or screening tools or as a potential source of cells for *in vitro* experimentation. Centrifugation, used to concentrate cells and to wash out any unwanted substances, and fixation, used to crosslink the fluorescent-conjugated antibodies to their membrane receptors, are usually involved in immunophenotyping experiments. The impact of preparation procedures on blood cells have been extensively investigated, but, based on our knowledge, this has not yet been studied with tear-film neutrophils. There is thus a need to investigate how cell collection and processing may affect cell phenotype and to develop standardized protocols that can be used to reliably collect and phenotype tear-film neutrophils.

The overarching hypothesis of this thesis is that tear-film neutrophils are sensitive to experimental procedures and exhibit a unique phenotype different from their counterpart blood leukocytes. The objectives of this thesis from the experimental procedure perspective were thus to compare two collection methods, using OSSCA and gentle eye wash, to

determine which could yield most cells and to assess the impact of centrifugation and fixation on the expression of membrane receptors on the collected tear-film neutrophils. From the perspective of further characterizing the phenotype of tear-film neutrophils, the objectives were to assess their response to cytokine IL-8 as well as their ability to generate ROS under various stimuli.

This thesis begins with an introduction on neutrophils, such as transmigration process and mechanisms for respiratory burst, in chapter 2, and provides more information on the heterogeneity of neutrophils, the ocular environment, and tear-film neutrophils in chapter 3. Chapter 4 reports on the sensitivity of tear-film neutrophils to experimental procedures involving centrifugation and fixation steps. Chapter 5 compares the two collection methods and presents the response of tear-film neutrophils to IL-8. Chapter 6 compares the ability of tear-film neutrophils and blood-isolated neutrophils in ROS generation upon stimulation using the luminol-enhanced chemiluminescence assay. Chapter 7 presents conclusions and recommendations for future work.

Chapter 2

Neutrophil Population and Selected Activation Pathways

2.1. Overview of neutrophils

Innate immunity is an older evolutionary mechanism protecting the host tissue against foreign particles, and involves many inflammatory cells, such as, granulocytes (also known as polymorphonuclear leukocytes), mast cells, and macrophages[1]. Neutrophils (polymorphonuclear neutrophils, PMNs) are one of the populations of granulocytes and are the first cells to arrive at infected sites[2]. The development of mature PMNs from hematopoietic stem cells in the bone marrow is a complex process involving multiple steps[2]. The entire process is highly regulated by the cooperation of various transcription factors, PU.1, and C/EBPα[3,4], and also hematopoietic growth factors, granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3), and granulocyte-macrophage colony factor (GM-CSF)[2]. In healthy individuals, there are approximately 10^{11} PMNs circulating in blood everyday[2]. A large number of mature PMNs can be found not only in the bone marrow, but also in the liver, spleen, and lungs, and these are referred to as marginated intravascular granulocyte pools[5]. However, the size of the pulmonary granulocyte pool is controversial, because blood needs to transit through the lungs to be oxygenated and thus margination can be difficult to assess; concentrated PMNs can be found in the healthy lungs[6] but in a small number[5].

Unlike other leukocytes population, such as macrophages and lymphoid cells[7], the neutrophil population, which represents approximately 60% of all white blood cells in bodies, is believed to be homologous, which means they do not hold a diversity in differentiation and activation, due to their short lifespan, their inability to transmigrate back to blood and their reduced transcriptional activity[8–10]. However, a recent experiment by

Pillay *et al.*, in which they traced PMNs in blood and bone[11,12], showed that PMNs can circulate in the blood for up to 5.4 days[12]. This challenged the traditional view of their lifespan, which is thought to be approximately 8 hours in humans[2,5]. In addition to this, using an *in vitro* flow model, Buckley *et al*. explored the fact that PMNs may be able to re-transmigrate back to blood[13]. These controversies around neutrophils draw our attention to their potential for heterogeneity, which needs to be further examined. Since many review papers exist on the phenotypic difference between PMNs in people affected by various diseases versus healthy individuals, we aimed to focus more on the heterogeneous neutrophil populations that can be found in different parts of the body, such as the lungs, the placenta, the mouth, and the eye. The information provided in this chapter provides some valuable background, as we aimed to characterize the phenotypes of tear-film PMNs and investigate their capability to induce the respiratory burst when exposed to different stimuli.

2.2. Flow cytometry and CD markers used to phenotype neutrophils

The cluster of differentiation (CD) is used to identify, discover, and organize different cell surface molecules present mainly in leukocytes[14]. It was developed by scientists in the human leukocyte differentiation antigens (HLDA) workshop held in Paris in 1985[14]. Apart from identifying or coding the cell surface molecules, the HLDA workshop also provided a forum for discussing the function and distribution of molecules and epitopes[15]. CD markers are utilized to identify and characterize leukocytes through the binding to their corresponding cell surface molecules, which act as receptors or ligands.

The phenotype of PMNs, describing the expression of receptors on their cell membranes, can be evaluated using (1) the immunophenotyping method[16] that is quantitative and can measure differences between samples, or (2) the immunocytochemistry method[17]

which allows visualizing targeted membrane receptors on the cell.

Flow cytometry (immunophenotyping) is the common method used to measure multiple characteristics of individual cells, including cell size, granularity or cytoplasmic complexity, deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), and membrane-bound and intracellular proteins[18]. It was largely used in this research to identify and assess PMNs activation, since when PMNs are stimulated, membrane receptors can either be upregulated or downregulated. Antibodies (or CD markers) conjugated with fluorescent molecules bind to their corresponding antigen and in this thesis, we focussed on selected membrane receptors of cell activation.

Acquisition of data through flow cytometry is depicted in Figure 1. Antibody-stained cells are aspirated into the flow cytometry microfluidics system and aligned into a single file line through laminar flow (step 1). Once in the flow cell, the cells are hit by a laser beam (step 2), the light will scatter based on the size and granularity of the cells (step 3) and the light will also excite any fluorescent molecules on cell membranes or inside the cell (step 4). Optic detectors will capture the light information (side scatter, forward scatter and fluorescent intensities) and this will be processed by the various electronic components and translated into quantitative values (step 5).

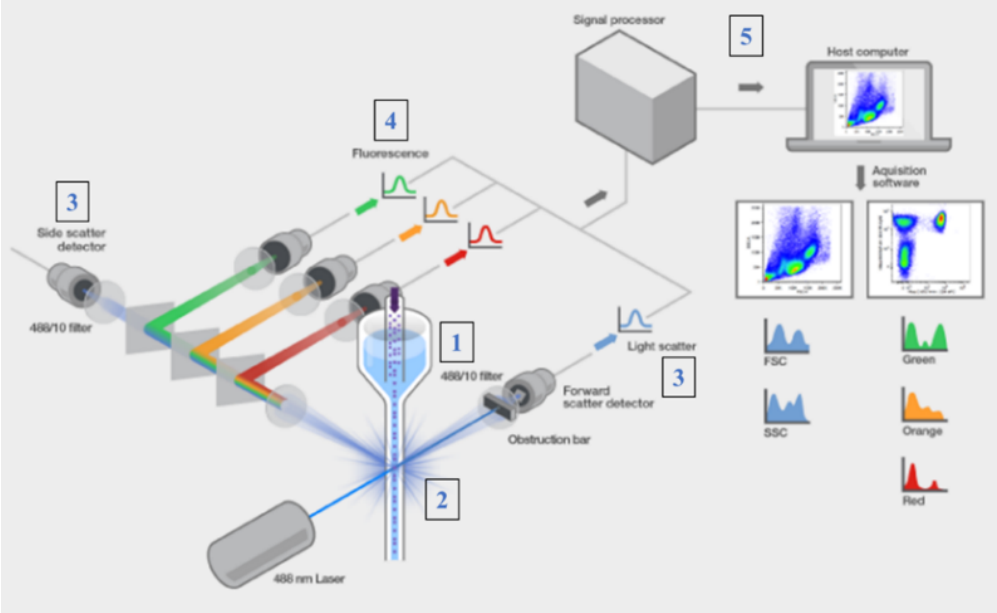

Figure 1: Flow cytometry. (1) sample intake with fluorescently labelled cells, (2) flow cell where laser illuminates cells one at a time, (3) detectors for forward and side scatter, (4) detectors for fluorescence, (5) data processing. Adapted from ThermoFisher/Invitrogen, www.thermofisher.ca

As neutrophils have a range of receptors for identification and activation, several markers need to be used to characterize neutrophil populations. It is well established that PMNs express CD11b, CD15, CD16, CD62L, and CD66b on their membranes[19–22]. CD markers commonly used to identify and characterize the phenotype of PMNs are summarized and listed in table 1.

In this thesis, we specifically examined the expressions of CD11b, CD16, CD45, CD55, and CD66b on tear-film PMNs. CD11b is an activation and adhesion marker and combines with CD18 to form the macrophage-1 antigen (Mac-1) or complement receptor (CR3), which is a member of the β_2 family of integrins[23]. CD11b plays a crucial role in PMNs transmigration from the blood vessel to the site of infection and also in mediating cell immune responses[24]. The upregulation of CD11b occurs early in the cell activation process and is used to determine the activation state of leukocytes[2]. CD16, a member of the Fc

gamma receptors for immunoglobulin, has been shown to be associated with the phagocytosis and degranulation of PMNs[25]. During PMNs activation, CD16 is shed off during neutrophil elastase release, and this reduction in CD16 expression has also been shown to be correlated with cell apoptosis[26,20]. CD55 is a glycosylphosphatidylinositol (GPI)-anchored decay-accelerating factor (DAF) and is one of the regulators of complement activation[27]. CD55 is mainly stored in secretory vesicles and has a low expression on resting PMNs in the circulation. It has been shown to be upregulated after activation and can help to differentiate between bacterial and viral infection[27]. CD66b is a degranulation marker, and its expression increases when cells are activated[23]. CD45 is a common leukocyte maker and is mainly used to identify leukocyte populations.

Table 1 - CD markers commonly used to characterize the phenotype of PMNs.

CD marker – Antigen	Leukocyte function assessed
CD11b (Mac-1)[11,19–22,28–31]	Adhesive cell interaction, a marker for neutrophil activation
CD14[19,20,32]	Binding receptor for LPS, a marker for phagocytosis
CD15[19,20,33]	Mature neutrophils marker
CD16[20–22,26,30,34–37]	Fc gamma type III receptor, a marker for degranulation and phagocytosis
CD170[21,30]	Immunoregulation marker
CD177[8,9,30]	Marker for regulating transmigration across the endothelium (adhesion marker)
CD181[21,22,34]	Receptor for type I IL-8
CD182[9,21,22,34]	Receptor for type II IL-8
CD32[21,22,26,36,38]	Fc gamma type II receptor, a marker for phagocytosis
CD43[39–42]	A marker for PMNs reactive oxygen species production and phagocytosis
CD45[11,19–22,28–31]	Common leukocyte marker
CD54 (ICAM-1)[13,22]	Adhesion marker
CD55[30]	Complement regulation marker
CD62L[19,20,22,38,43,44]	L-selectin
CD63[30,33]	Marker for azurophil granules
CD66b[11,19–22,28–31]	Degranulation marker
CD88[21,22]	Receptor for C5a ligand

2.3. Neutrophil recruitment

Recruitment of PMNs from blood to the peripheral tissue is caused by the presence of inflammatory mediators, such as histamine, chemokines, cytokines, and lipid mediators[11,31]. The process known as extravasation usually consists of multiple steps for PMNs migrating across the blood vessel walls, including tethering (capturing), rolling, adhesion, crawling, and

eventually transmigration (Figure 2)[11,31]. Integrins and selectins are the key molecules involved in these steps[11,31,43,45]. Upon stimulation by pathogens, endothelial cells lining the blood vessels upregulate the expression of selectins on their luminal surface[45]. Free-flowing PMNs can then be captured on the endothelium through the interaction between the leukocyte glycoproteins and their corresponding selectins (P- or E-selectin) on the endothelium[43,46]. Then, attached PMNs roll along with the blood vessel through the interactions between E-selectins and leukocyte glycoproteins[11,46]. This rolling step is not sufficient for PMNs to firmly attach to endothelial cells due to the shear force from blood flow[46]. The binding of the integrin Mac-1 (also known as CD11b) expressed by PMNs and immunoglobulin superfamily members (ICAM-1) expressed by endothelial cells help to mediate leukocyte arrest[43]. L-selectin (CD62L) expressed by PMNs play an essential role in secondary tethering, helping to bind free-flowing neutrophils to already adherent PMNs[47]. Next, PMNs roll along the blood vessel while maintaining adhesion to the endothelium, new bonds to the endothelium are formed as soon as the existing bonds are released during the crawling process[11]. This crawling step is dependent on ICAM-1 and Mac-1[48]. PMNs migrate to local tissue either through junctions between adjacent endothelial cells (paracellular) or through the body of the endothelial cell (transcellular), but paracellular transmigration is predominant[11,43,45]. Transmigration is associated with increased levels of intracellular Ca^{2+} and is supported by the binding of PMNs integrins and endothelium expressed ligands (ICAM-1 and VCAM-1) via re-arranging or reducing junctional molecules (such as VE-cadherin)[11,43,45], resulting in fewer barriers, which makes it easier for PMNs to migrate into the tissue.

The extravasation process significantly alters the PMNs expression of certain

membrane receptors. There is a drastic reduction in the expression of L-selectin (CD62L) as it is being shed during the interaction with the endothelial cells, but extravasated PMNs have a significant increase in $\beta 1$ and $\beta 2$ integrins (Mac-1) and recognition receptors (CXCR4)[49]. After transmigration, PMNs are programmed to undergo apoptosis after they execute their inflammatory functions in the tissue, and their fragments are phagocytosed by macrophages[9]. However, it has been shown that some PMNs can transmigrate from the peripheral tissue back to the blood vessels in zebrafish and mice, in a phenomenon called reverse transendothelial cell migration (rTEM)[13]. It is interesting to note that these rTEM PMNs now circulating back in blood exhibit a different phenotype from the normal circulating blood PMNs. rTEM PMNs have significantly higher expression in Mac-1 (β_2 integrins) but lower expression of L-selectin[13].

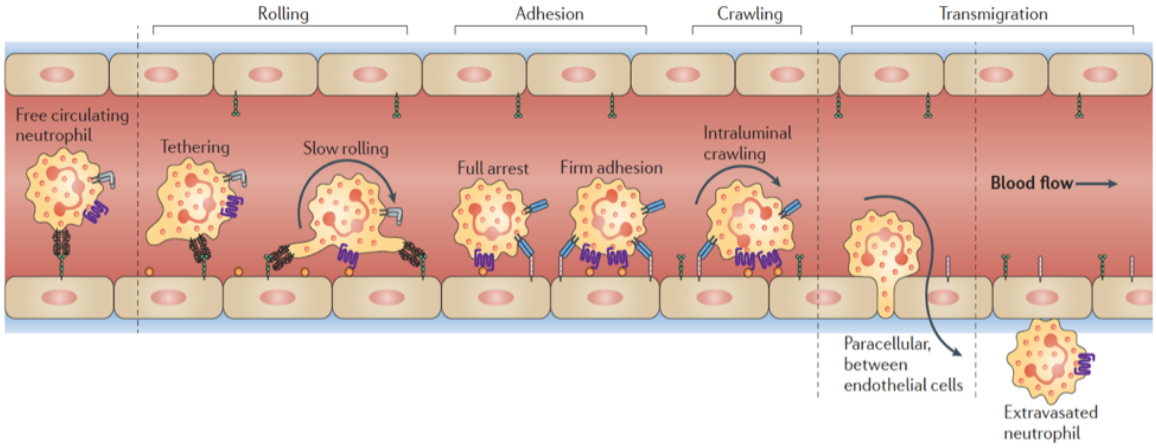

Figure 2 - Overview of neutrophil recruitment cascade for PMNs migrating from the blood vessels to the peripheral tissue [11]. Each step is mediated by the interaction between PMNs (integrins and leukocyte glycoprotein) and endothelial cells (selectins). PMNs transmigrate to local sites either via the paracellular way (through junctions between the adjacent endothelial cells), or via the transcellular way (through the body of the endothelial cells) which is not shown in this figure. Reprinted with permission from: Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. Nat. Rev. Immunol. 13, 159–175 (2013).

2.4. Intracellular signaling pathways in neutrophils

PMNs execute their functions mainly through the binding of the stimulus to its corresponding membrane receptor/ligand. Transmembrane signaling is crucial to control the intracellular events which direct the PMN functional responses. There is a wide range of stimuli that activate PMNs, such as cytokines (in this research, IL-8 was used), chemotactic stimuli (such as N-formyl-met-leu-phe (fMLP), used in this research), chemical stimuli (such as Phorbol 12-myristate 13-acetate (PMA), used herein), bacterial endotoxins (also known as lipopoly-saccharides (LPS)). In this section, the intracellular signaling pathways activated in response to stimulation of PMNs with fMLP and PMA are mainly discussed and are depicted in the schematic diagram in Figure 3.

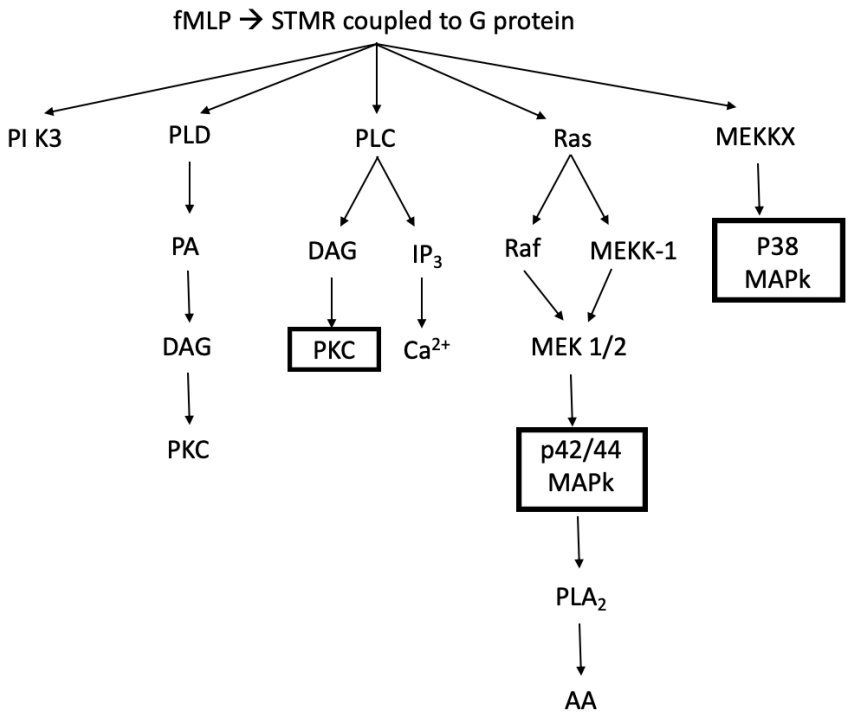

Figure 3 – Intracellular signaling pathways in response to stimulation of PMNs with fMLP and PMA. After fMLP binds to its receptor (G-coupled protein receptor), multiple pathways are activated. The black square represents the components that can be activated by PMA via the direct binding. STMR: seven transmembrane-spanning receptor; PLC: phospholipase C; DAG: diacylglycerol; IP_3: Inositol triphosphate; PKC: protein kinase C; MEKK-1: mitogen-activated-protein kinase kinase kinase 1; MEK1: mitogen-activated protein kinase kinase 1; MAPK: mitogen-activated-protein kinase; PLA_2: phospholipase A_2; AA; arachidonic acids; PLD: phospholipase D; PA: phosphatidic acid; PI3K: phosphatidylinositol 3-kinase.

fMLP receptor is a seven transmembrane-spanning and pertussis-toxin sensitive G-protein-coupled receptor (GPCR)[50]. Extensive studies show that the interaction between fMLP and the GPCR activates phospholipase C (PLC) and phospholipase D (PLD)[2,50–53]. Phospholipase A_2 (PLA_2) is activated by coupling to the receptor via a G protein, G_p[54]. The activation of PLD is mediated by the ADP-ribosylation factor and Rho proteins which are stimulated by G proteins[55]. PLC hydrolyzes phosphoinositide into two second messengers, diacylglycerol (DAG) and Inositol triphosphate (IP_3)[2,50]. DAG is a protein kinase C (PKC)

activator, whereas IP_3 regulates the mobilization of extra- and intracellular calcium[2,52]. PLD uses phosphatidylcholine as the substrate to generate the lipid messenger phosphatidic acid which can be further converted to DAG[2,56], which can activate PKC later on. There are two types of PKC which are conventional PKC requiring both DAG and Ca^{2+} to be activated, and nPKC only requires DAG[56]. Activated PKC has a variety of functions, for example, initiating the respiratory burst (see below for details) via the phosphorylation of the nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and upregulation of adhesion receptors such as Mac-1 (CD11b). In addition to the pathway involving PKC, several studies show that fMLP is able to stimulate Ras and Raf which eventually leads to the activation of p42/44 (ERK) mitogen activated protein kinase (MAPk)[50–52]. Activated MAPk phosphorylates phospholipase A_2 (PLA2)[52], causing the release of arachidonic acids (AA), is correlated with the generation of superoxide anion[2]. The general outcome of this MAPk pathway is to increase transcription through phosphorylation of transcription factors. Furthermore, fMLP activates p38 kinase which is one of the mitogen protein kinases[51]. This kinase cascade plays an essential role in altering PMNs responses, such as regulation of gene expressions[57], increased synthesis and release of cytokines, and release of superoxide anion[51]. Tyrosine kinase [2] and phosphatidylinositol 3-kinase (PI 3-kinase)[56] are the two other kinases that can be activated by fMLP to trigger cellular events, such as increase in chemotaxis.

The intracellular mechanisms for PMA is quite direct as PMA enters the cell directly and binds to PKC, which then upregulate CD11b/CD18 complex[58] and activate NADPH oxidase to induce the respiratory burst[59]. PMA is also able to phosphorylate and activate both p38 and p42/44 kinases[51]. Karlsson *et al*. showed that the intracellular oxidase response to PMA is dependent on PI 3-kinase which challenged the previous findings that PMA-

induced superoxide release is PI 3-kinase independent[59].

2.5. Respiratory Burst in neutrophils

Phagocytic cells, such as monocytes/ macrophages and PMNs, use the oxygen-dependent killing mechanism referred to as the respiratory burst to protect host tissues from invasion of foreign pathogens[60,61]. Molecules that are synthesized in this process and are involved in the oxygen-dependent killing mechanism are referred to as reactive oxygen species (ROS) and include superoxide anions (O_2^-), hypochlorous acid (HOCl) and hydroxyl radicals (HO^-). During phagocytosis, a significant increase in oxygen uptake in neutrophils and monocytes was observed by Sbarra and Karnovsky[62]. This increased consumption of oxygen is not due to respiration, but is related to an oxidase-reducing molecular oxygen to the superoxide anion, the primary product of respiratory burst[62, 2]. They also noted a positive correlation between oxygen consumption and concentration of particles available for engulfing/phagocytosis[62]. The ability of PMNs to generate ROS is not equivalent to their capability for phagocytosis[2]. Patients with chronic granulomatous disease can phagocytose microbes but cannot produce ROS[63]. Since some of the ROS are strong anti-microbial molecules, it may also cause damage to host tissues which can lead to many inflammatory diseases, for example, the contagious bovine pleuropneumonia (CBPP)[61,64]. Excessive production of ROS is not only harmful for tissues but also detrimental for cells, as it can result in T lymphocytes hyporesponsiveness[65].

Several enzymes cooperate together to generate the end products of the respiratory burst. First, an oxidase reduces molecular oxygen (O_2) to the superoxide anion (O_2^-) which initiates the respiratory burst[66]. Since NADPH is used as the electron donor in this reaction, the enzyme is known as NADPH oxidase. NADPH oxidase consists of a membrane bound

component which is the *b*-type cytochrome and four cytosolic proteins[61]. Cytosolic proteins contain the NADPH acting as the electron donor. The *b*-type cytochrome is embedded within both the plasma membranes (5%) and the membranes of intracellular granules and vesicles (95%)[67]. Once neutrophils are activated, cytosolic proteins translocate to the *b*-type cytochrome. Then, electrons are transferred from NADPH, in the cytosolic proteins, over the membranes and delivered to the molecular oxygen present sites, the phagosomes or the extracellular milieu, by *b*-type cytochrome[68]. NADPH oxidase catalyses the following reaction (equation 1) in which superoxide anion is the predominant product[61].

$$\mathrm{NADPH} + \mathrm{H}^+ + 2\mathrm{O}_2 \rightarrow \mathrm{NADPH}^+ + 2\mathrm{H}^+ + 2\mathrm{O}_2^- \quad (1)$$

Then, superoxide spontaneously dismutates to form hydrogen peroxide (equation 2), where superoxide dismutase catalyzes the reaction[2].

$$2\mathrm{O}_2^- + 2\mathrm{H}^+ \rightarrow \mathrm{O}_2 + \mathrm{H}_2\mathrm{O}_2 \quad (2)$$

Other more reactive ROS are further generated via the actions of nitric oxide synthase and myeloperoxidase (MPO)[61]. The overall generation of ROS is depicted in Figure 4.

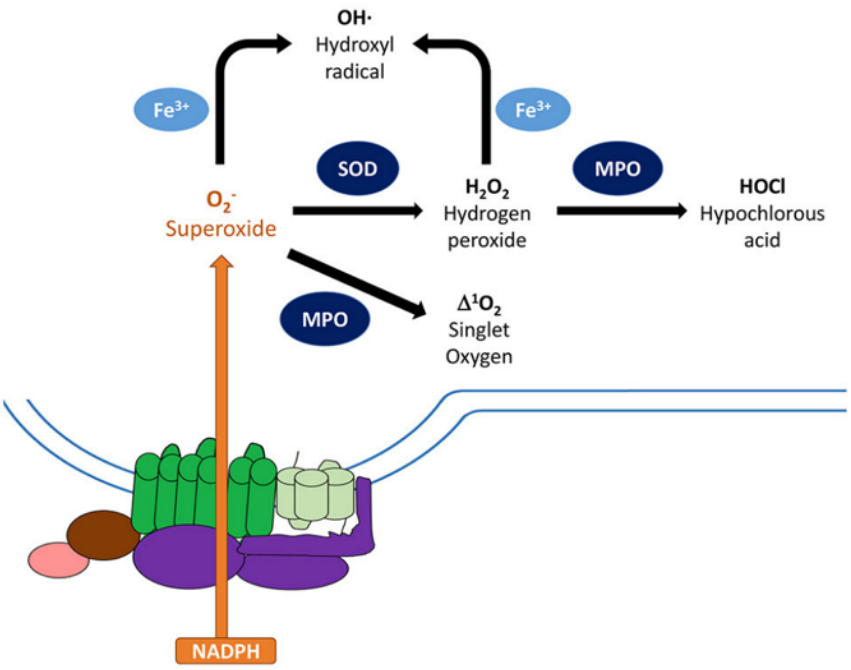

Figure 4 - The overall formation of ROS by a phagocytic cell [69]. Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are less reactive, while hypochlorous acid (HOCl) and OH radical are more reactive. © 2017 Nguyen, Green and Mecsas.

2.6. Conclusion

Following maturation in the bone marrow, PMNs are released into the circulation where they are distributed into two pools, the circulation pool and the marginated pool[2]. PMNs are short-lived cells, but their actual life-span in blood is currently controversial. The neutrophil population is heterogeneous, meaning PMNs exhibit distinct phenotypes under both pathological and normal physiological conditions. Immunopheynotyping via flow cytometry is the common method researchers often used to characterize PMNs phenotypes through the expression of surface receptors or antigens. Different intracellular signaling pathways, which involves multiple phospholipases and kinases, are used by PMNs upon exposure to stimuli in order to execute their cell functional responses, for example, the

respiratory burst. The hallmark of the respiratory bust is the substantial increase in the consumption of oxygen which is used to generate ROS. Several key enzymes have to work cooperatively to produce the strong antimicrobial agents.

Chapter 3

The Heterogeneity of Neutrophils and Tear-film Neutrophils

3.1. Introduction

Distinct phenotypes of PMNs have been detected in various pathological conditions, as well as in different tissues of healthy individuals. Thus, neutrophils don't exist as one type of cells but are now considered as heterogeneous neutrophil populations. Their heterogeneity in pathological conditions will be briefly illustrated by using sepsis as an example. In severe systemic inflammation induced by sepsis, the leading cause of death is the demonstration of immune suppression in patients, which makes them more susceptible to infections, despite the massive presence of circulating PMNs in blood[1]. This may suggest that the immune suppression observed in sepsis patients is due to an altered function in PMNs, where they exhibit an inability to kill pathogens, inhibit other immune cells (T cells)[2], and eventually suppress the immune response. In this chapter, we will focus mainly on the phenotypes of PMNs found in different tissues, the lung, the placenta, the mouth and the eye.

3.2. Neutrophils in the lung

PMNs can be detected in the lung alveoli under both physiological and pathological conditions[3,4]. Resting blood PMNs have a limited ability to respond to stimuli. The process, in which PMNs change to a more active state, is referred to as 'priming', whereby PMNs are exposed to low concentration of an inflammatory molecule[5], allowing PMNs to then have an amplified responses to pathogens[6,7]. There are many types of priming agents, including fragments secreted by microbes (LPS), chemokines (IL-8), and pro-inflammatory cytokines (tumor necrosis factor α)[7]. Paradoxically, primed PMNs can cause more damage to

endothelial cells than non-primed PMNs[8], which then leads to neutrophil-mediated tissue injury[9]. The invasion of primed PMNs, which are hyperactive, is probably one of the leading causes of acute respiratory distress syndrome (ARDS)[9]. Furthermore, lung damage has also been associated with remote organ dysfunction such as epithelial cell apoptosis in kidney[10,11]. As the lungs receive the entire cardiac output, it has been hypothesized that they may play a role in de-priming PMNs, and then release them back to the circulation in a quiescent state, in order to protect the host tissue from damage caused by primed PMNs[9,11].

There has been significant research in the area of neutrophils and lung inflammation to gain a better understanding of the underlying mechanisms of systemic inflammation. For pulmonary inflammation, cells can be obtained from bronchoalveolar lavage (BAL), whereby sterile saline solution (300 mL) is instilled into the lung in aliquots (25 mL) followed immediately by aspiration[3,12] . Sputum from lung tissues[13] can also be collected non-invasively. However, sputum contains mucus, cells and debris, making it harder to be analyzed by flow cytometry and requiring a precise gating strategy[14]. Upregulation or downregulation of specific membrane markers, such as CD11b and CD62L, respectively, has been used to assess the activation state of PMNs collected from patients with chronic pulmonary disease[3,4,15]. An increased number of PMNs extravasated in lung tissue have been observed in patients with chronic obstructive pulmonary disease (COPD). These PMNs exhibited high levels of CD11b/18 (Mac-1), suggesting that these PMNs have been primed[4].

It is also essential to examine the phenotype of PMNs after extravasation under non-pathological conditions to determine if the change in expression of membrane receptors is induced by inflammation in the tissue rather than the extravasation. When characterizing the phenotype of neutrophils before and after transmigration in healthy individuals and people

with disease, Fortunati *et al*. found that PMNs collected from lung tissues exhibited an activated phenotype (high CD11b expression and low CD62L expression) under inflammatory (patients with disease) and non-inflammatory conditions (healthy participants)[3]. The difference in neutrophil phenotype could only be identified following stimulation. Unlike the BAL PMNs of sarcoid patients, the expression of CD32 and CD11b on BAL neutrophils from healthy participants could not be further upregulated upon fMLP stimulation[3]. Altogether, these results suggest that the activated phenotype observed in lung PMNs is not due to the inflammation but rather a hallmark of extravasation to the lungs, and that, under healthy conditions, lung neutrophils have an impaired functionality, which may be due to a protective/suppressive property of the lungs[3,16]. Upon inflammation in the lungs, an imbalance may occur in the environment, leading the PMNs to be able to respond to stimuli, which will in turn lead to lung damage. These results emphasize how the environment may affect neutrophil phenotype and the importance of assessing neutrophil response to stimulation.

3.3. Neutrophils in the placenta

Many years ago, it was thought that pregnancy was associated with immune suppression, which caused both the mother and the fetus to be susceptible to infectious diseases[17]. However, the discovery of the presence of several types of immune cells in the human decidua (part of the endometrium)[17] led to a conclusion that the maternal immune system plays an important role in the fetal-maternal immune adjustment and protection against pathogens[17,18]. Neutrophils from term and preterm neonates also behave differently, whereby the latter showed lower capability of phagocytosis and lower amount of reactive oxygen species produced[19], suggesting that neutrophil phenotype may adapt to various

conditions.

The study of maternal, cord blood and placenta has allowed researchers to identify two neutrophil subsets with different phenotypes - low-density granulocytes (LDGs) and normal-density granulocytes (NDGs)[20]. The cells were isolated following various density-gradient centrifugation procedures. NDGs and LDGs phenotypes were characterized using flow cytometry with the following markers[20]:

- CD66b, upregulated on cell membrane upon degranulation,
- CD15, involved in stimulation of degranulation and respiratory burst,
- CD16, involved in degranulation as it is cleaved from the cell membrane when elastase is released from granules (downregulation upon release),
- CD63, upregulated on cell membrane following release of azurophilic granules.

When compared to NDGs, LDGs expressed higher levels of CD15, CD33, CD63 and CD66b as well as lower levels of CD16[20], suggesting that LDGs had undergone activation and degranulation (potentially as they were activating to protect the mother and foetus from pathogens). These results highlight that various phenotypes of neutrophils may co-exist and the significant potential of flow cytometry to identify if neutrophils have undergone activation *in vivo*.

3.4. Neutrophils in the mouth

To maintain an healthy oral environment, the presence of PMNs in the oral cavity is essential to eliminate ongoing exposure to bacteria[21]. It is well known that, upon exposure to bacteria, neutrophils can release reactive oxygen species (ROS) and enzymes and that these toxic substrates kill not only pathogens but also cause damage to host tissues[7,22]. The PMNs in the oral environment are thus exposed to a unique context, which is referred to as para-

inflammation, whereby PMNs have the potential to respond to low-grade foreign pathogens without clinical signs of inflammation and damage to the tissue[23]. On the other hand, chronic periodontitis (CP), which is induced by bacteria and biofilm formation, is a destructive inflammation causing irreversible damage to the periodontium and tooth-supporting tissue[22,23], and oral PMNs are believed to play a significant role in this disease.

PMNs from the oral cavities can be collected using a simple and non-invasive rinsing method, whereby participants rinse their mouth for 30 seconds with an isotonic sodium chloride solution (5 ml) and then expectorate into a tube[21,22]. When compared to blood-circulating PMNs, oral neutrophils are functionally different in terms of phagocytosis (evaluated using bright field microscopy to detect the presence of intracellular particles)[24], and also exhibit a lower ability to generate ROS upon stimulation (evaluated by flow cytometry)[22].

Not only has excessive PMNs recruitment been observed in severe or chronic periodontitis (CP) with a six-fold increase in PMNs counts compared to healthy controls[21,25], but two distinct phenotypes in oral PMNs, para- and pro-inflammatory, have also been identified in healthy individuals versus individuals with CP, respectively[23]. The pro-inflammatory PMNs found in the mouth of CP patients showed elevated degranulation, phagocytosis, ROS production, and neutrophil extracellular traps (NETs) release[23]. Furthermore, studies of ROS generation in oral neutrophils from individuals with refractory periodontitis also identified the existence of two groups: low (LR) and high responders (HR)[22]. HR showed a more substantial difference between resting and stimulated ROS levels compared to the difference in levels observed in LR, suggesting that HR hold a more significant activation potential than LR and such an enhanced response to stimuli may result

in more destructive tissue damage with periodontitis in HR versus LR[22].

Interestingly, para-inflammatory PMNs in the healthy oral cavity can be further divided into two subpopulations, para1 and para2, as defined by Fine *et al*[23]. Compared to para1, the para2 population is more activated, showing increased expression of activation markers (CD55 and CD63), and decreased expression of a phagocytosis marker (CD16)[23]. The two para-inflammatory populations appear to co-exist in the healthy oral cavity environment, with no tissue damage induced by the para2 (intermediate activated) population. In chronic periodontal diseases, only the PMNs present exhibit the pro-inflammatory phenotype, which result in the continuous damage to the periodontium. It is yet unclear what causes the shift in phenotype, but the presence of biofilm and the locally inflamed oral environment may provide molecular cues to oral PMNs in the mouth[23]. Studies of PMNs that have extravasated to the oral cavity in healthy or inflamed/infected environment not only highlight the complex nature of neutrophil phenotypes but also how the state of the local environment may induce one phenotype over another.

3.5. Neutrophils in the eye

3.5.1. Ocular Immune Privilege

The brain, the eye, the uterus, and the testis are all immune privileged sites in our body[26]. Immune privilege was thought to be "immune ignorance", which means completely blocking the entry of immune cells to the tissue[26]. However, this simplistic view of immune privilege poorly represents the dynamic mechanisms involved[27]. Shechter *et al* proposed that "the privilege of these organs resides not in their ability to block passive immune infiltration or to facilitate active immune tolerance or ignorance, but rather in the ability of the epithelium gate system to orchestrate active communication with the circulating immune

system"[26].

The eyes continuously encounter external threats, such as microbes, pollen and allergens, but the incidence of immune-mediated blindness is relatively rare due to immune privilege[27]. However, the presence of biomaterials, for example contact lenses, may challenge the immune privileged ocular environment and contribute to the development of adverse events, such as microbial keratitis and contact lens-associated corneal infiltrates[28]. Investigating neutrophils, the first responder innate immune cells, is therefore important to gain a better understanding on their contribution to ocular inflammation and interactions with ocular biomaterials.

3.5.2. Overview of the ocular environment

The eye is a sensory organ that is responsible for vision. It is enclosed by three layers of tissue, the innermost retina, the middle uveal tract, and the outermost sclera and cornea[29], as shown in Figure 5. Light rays enter the eye through a transparent tissue, the cornea, and the intensity of the light is adjusted by the iris muscles through contraction. Then, through a process called accommodation, the lens focuses the light rays on the retina which is embedded with photoreceptor cells, rods and cones[29]. The optic information is then sent to the brain via the optic nerves.

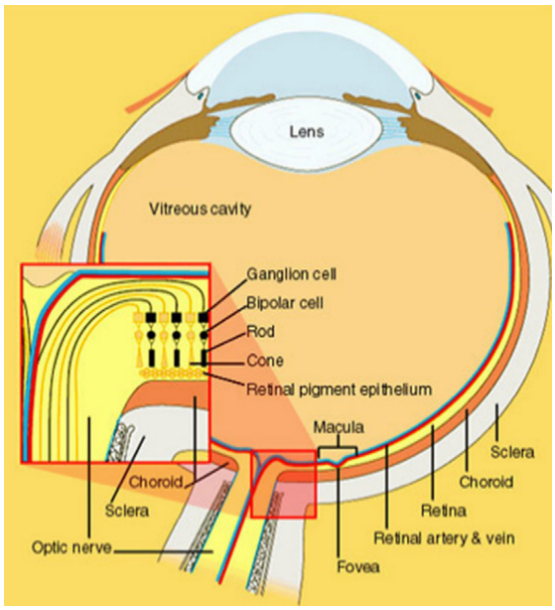

Figure 5 - Anatomy of the human eye[30]. Reprinted with permission from: Kels, B. D., Grzybowski, A. & Grant-Kels, J. M. Human ocular anatomy. Clin. Dermatol. 33, 140-146 (2015). © Elsevier 2015.

3.5.3. Tear film and its components

The human eye is directly exposed to the external environment (which contains various pathogens[31]), which may lead to eye diseases, such as microbial keratitis (MK). MK is one of the most common corneal infections caused by microorganism that could result in vision loss[32]. It is thus essential to have a structure, the tear film, acting as a barrier between the eyes and the outer environment[31] to reduce this occurrence. Components of the tear film are secreted by different epithelial and glandular tissues, such as the meibomian glands (the lipid layer), lacrimal glands (the aqueous layer), and the goblet cells within the conjunctiva (the mucin layer)[33]. The tear film was initially thought to have three layers, the innermost mucin layer, the middle aqueous layer and the outermost lipid layer[34], as shown in Figure 6a. This was further modified, with the lipid layer divided in the outermost non-polar phase exposed to the air, and the polar phase adjacent to the aqueous layer[35], as illustrated in Figure

6b. The mucin layer is in direct contact with the cornea, and forms a gel-like structure which helps to hold tears and provides a wet surface for blinking[31]. The aqueous layer lubricates the surface of the eye when blinking[31]. This layer contains aqueous contents secreted by the lacrimal gland and also numerous proteins, such as lysozyme and lactoferrin[31], which provide nutrition to the cornea and protect the eye against pathogens[31]. The polar phase keeps the ocular surface moist, while the non-polar phase acts as a barrier and also helps to relieve the surface tension[31,33,36].

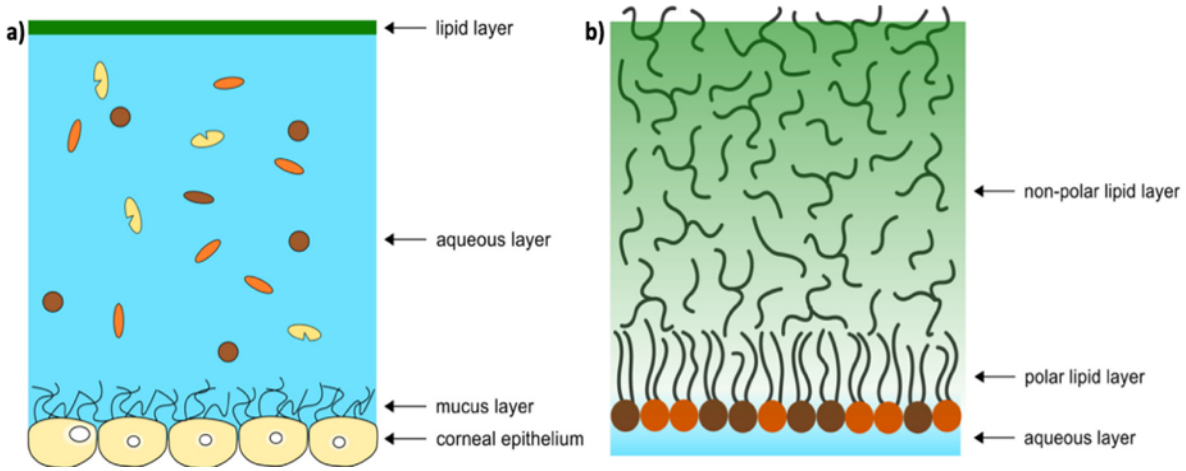

Figure 6 – a) An old view of three layered tear-film and b) the polar and non-polar phases within the lipid layer [31]. Reprinted with permission from: Cwiklik, L. Tear film lipid layer: A molecular level view. Biochim. Biophys. Acta - Biomembr. 1858, 2421–2430 (2016). © Elsevier 2016.

However, the layers of tear film have been revised. Instead of having the three border layers, it has been proposed that the tear film consists of the outermost non-polar lipid layer, the polar lipid layer, the aqueous-mucin layer, and a glycocalyx layer covering the cornea[35], as shown in Figure 7.

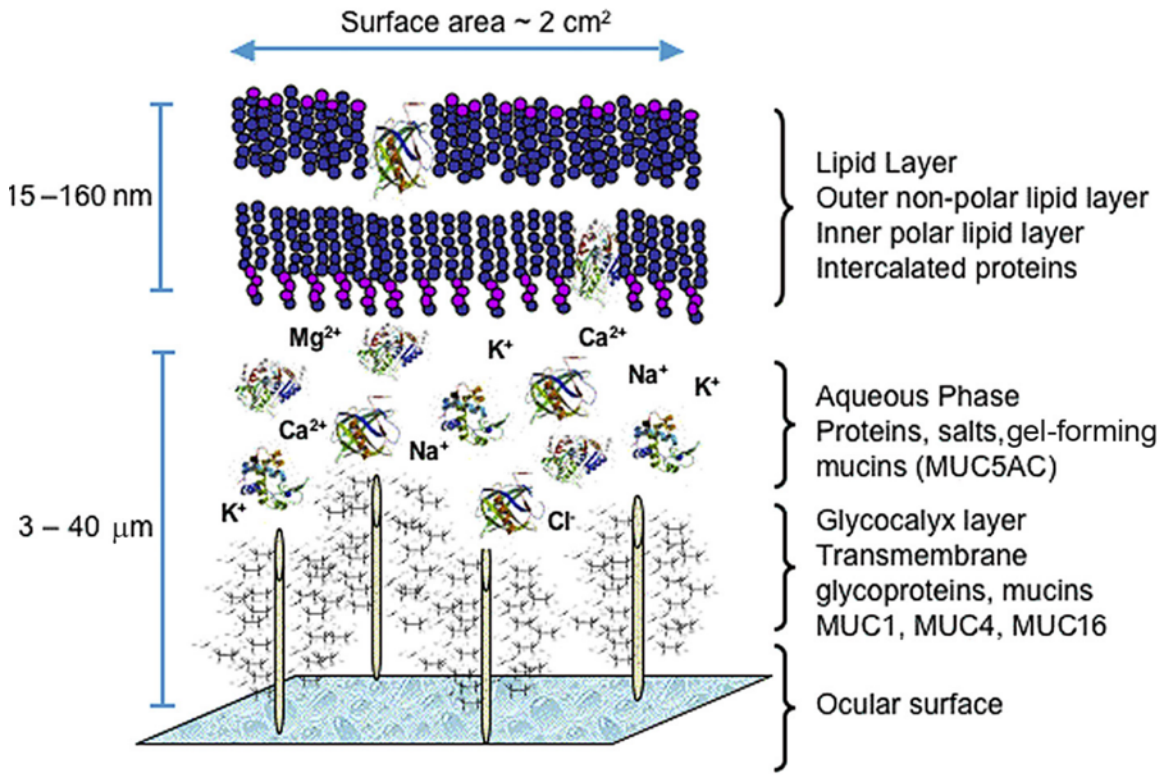

Figure 7 - The modern view of the tear film[37]. Reprinted with permission from: Green-Church, K. B., Butovich, I., Willcox, M., et al. The International Workshop on Meibomian Gland Dysfunction: Report of the Subcommittee on Tear Film Lipids and Lipid–Protein Interactions in Health and Disease. Invest Ophthalmology Vis Sci. 52, 1979 (2011). © Association of Research in Vision and Ophthalmology 2011.

To function optimally, tear film stability is important, or its disruption may result in dry eye. Evaporation rate has been shown to be one of the major factors affecting the stability of the tear film[38]. There is a strong correlation between the thickness of the lipid layer and the evaporation rate[38,39], which means "the thicker the lipid layer, the less evaporation and more stable the tear film"[33]. Therefore, the lipid layer plays a crucial role to maintain the normal function of the tear film[36].

Zhou *et al.* identified 1543 tear proteins in tears from healthy subjects[40], with lysozyme, lactoferrin, tear lipocalin and secretory immunoglobulin A (sIgA) being the main proteins found in the tear fluid[41,42]. Aside from the above listed proteins, tears contain

cytokines, which are small proteins that are important in the recruitment and activation of immune cells, as well as growth factors. Cytokines have been suggested to play an essential role in corneal homeostasis[43]. Research also showed that the components of the open-eye and closed-eye tears are significantly different from each other[44,45]. Closed-eye tears contain higher levels of albumin, fibronectin, complement proteins, sIgA and plasmin compared to open-eye tears[44–46], with sIgA representing up to 80% of the total protein content[45]. Furthermore, a large number of neutrophils have been observed in closed-eye tears[45].

3.5.4. Tear-film neutrophils

Neutrophils have been observed on the ocular surface in the absence of a wound or infection and have recently been termed "tear-film neutrophils" or "tear-film PMNs" to reflect their location[45,47,48]. Recent studies have collected tear-film neutrophils during the day time as well as following closed-eye conditions. While up to a few thousands PMNs can be collected from open-eye tears[49], hundreds of thousands to millions of PMNs have been collected after sleep (closed-eye conditions)[48].

The origin of tear-film PMNs has yet to be determined. Two hypotheses have been presented[40,48,50], one is extravasation, whereby chemotaxis leads to their migration to the ocular surface from conjunctival blood vessels; the other is that neutrophils are present in lacrimal glands, and are brought to the ocular surface with the tear fluid from the lacrimal glands. In the latter, the hypothesis is that in the closed environment, there is limited replenishment and blinking and thus tear-film PMNs accumulate. Chemokines play an essential role in the recruitment of PMNs to local tissues. Hence, the recruitment of tear-film PMNs to the ocular surface may be induced by the presence of specific cytokines (e.g., IL-8) and complement proteins (e.g., C5a), which have been shown to be present in the closed-eye

environment in large amounts[51]. The presence of IL-8 and C5a, chemoattractant factors for PMNs, was recently characterized in tear fluid (TF) samples collected at three time points, before (evening TF) and after sleep (morning TF), and around noon (noon TF)[52]. Concentrations of IL-8 and C5a in the morning TF (after sleep) were the highest compared to the noon TF[52]. Interestingly, tear fluid collected in the evening contained a high level of C5a, suggesting that the complement system is probably activated, which may due to the accumulation of microorganisms and dust particles, prior to eye closure[52]. Both human corneal epithelial cells (hCECs) and PMNs can secrete IL-8 in response to the presence of microorganisms and dust particles, and this may also contribute to the high levels of IL-8 observed in the morning TF[53,54]. Hence, the activation of complement pathway before eye closure may lead to the recruitment of PMNs, which could be further promoted by the increased level of IgA. IL-8 secreted by hCECs mediates PMNs migrating to the eye, and this recruitment may be further enhanced with the co-secretion of IL-8 by both hCECs and PMNs. Low levels of L-selectin were also observed on tear-film neutrophils collected after sleep, which may have been lost during extravasation[48]. As mentioned earlier, low expression of L-selectin has been observed in lung PMNs [3] and oral PMNs[23], which have already been proven to come from the blood vessels. Collectively, results from high levels of chemoattractant in evening TF and shedding of L-selection on collected tear-film PMNs strongly suggest that tear-film neutrophils present in the closed-eye environment have extravasated and come from the conjunctival blood vessels.

3.5.5. The non-inflammatory phenotype of tear-film PMNs

The phenotype of tear-film neutrophils collected from the closed-eye environment has just started to be characterized. Compared to blood circulating PMNs, tear-film PMNs

collected from the eyes after sleep have been shown to have a significantly higher expression of CD66b and lower expression of CD62L[48,52,55]. One research group reported that there is no difference in CD11b expression between tear-film and blood-isolated PMNs[52], whereas two other groups demonstrated that the level of CD11b is higher in tear-film PMNs[48,55]. In addition, tear-film PMNs are unable to upregulate key markers of activation upon stimulation, such as with phorbol 12-myristate 13-acetate (PMA), lipopolysaccharides (LPS) and N-Formylmethionyl-leucyl-phenylalanine (fMLP)[48,55], suggesting that they may have a non-inflammatory phenotype or may have been activated *in vivo* and are unable to respond to further stimulation. To better understand the phenotype observed with tear-film PMNs, experiments were performed by Postnikoff and Gorbet to expose blood-isolated PMNs to conditions similar to the closed-eye environment[56]. Results showed that *in vitro* exposure to hypoxic conditions, hCECs and artificial tear solution cannot induce the non-inflammatory phenotype in blood-isolated PMNs[56].

Postnikoff and Nichols hypothesized that tear-film PMNs have already been activated as they arrived at the ocular surface due to the reduced expression of CD14 and CD16[55]. CD14 and CD16 are markers for phagocytosis[57,58], so the decrease in expression is believed to be associated with the reduced phagocytic capability of PMNs. This observation appears to correlate with results indicating that PMNs recovered from the closed-eye tears may have an impaired phagocytic ability[59]. Although reduced levels in CD16 is also related to apoptosis[60], tear-film PMNs were not reported to be apoptotic[48]. CD43, which acts similarly to CD16, was used by Mahajan *et al.* to characterize the tear-film PMNs, and results showed that tear-film PMNs expressed low level of CD43[52]. This decrease in the expression of CD43 is associated with the cleavage by the neutrophil elastase after phagocytosis or

stimulated NADPH oxidase[61–63], indicating tear-film PMNs have already degranulated. In addition, eye discharge collected after sleep contains PMNs, NETs, dead epithelial cells and entrapped bacteria[52], suggesting that tear-film PMNs have the capability to eliminate pathogens when they are extravasated to the ocular surface by releasing NETs, phagocytosis and degranulation. Taken all together, those findings suggest that tear-film PMNs might have undergone activation during the sleep.

Interestingly, tear-film PMNs at both 1-hour and 7-hour time point showed no difference in their lack of ability to respond to inflammatory stimuli (LPS and CaI)[55], which would tend to suggest that their non-inflammatory phenotype is independent of their residence time in the ocular environment.

3.5.6. Tear-film PMNs and ocular surface diseases

As neutrophils are innate immune response cells, their role in other systematic inflammation, such as sepsis and acute respiratory distress syndrome (ARDS), has been largely investigated, but remains largely unknown in the ocular inflammation. Due to the improved collection protocols that have been established, enough tear-film PMNs can be collected from the ocular surface, allowing for the subsequent phenotype-characterizing experiments. This has led tear-film neutrophils to be a new area of research in ocular surface inflammatory diseases.

Dry eye disease is a common ocular complication which is caused by insufficient generation of tears or the quick evaporation rate of tears[39]. According to several findings, T cell-mediated inflammation is considered to be associated with dry eye disease (DED)[64,65]. However, the role of PMNs in DED remains undefined and several recent studies point towards a potential role of these innate immune response cells. Significantly more PMNs are

found in the tear samples of meibomian gland dysfunction (MGD) patients compared to healthy people, and a positive correlation exists between the number of PMNs and the severity of MGD[66]. Furthermore, an excessive amount of molecular components of NETs have been observed on the ocular surface of severe DED patients, and similarly to MGD, the amount of NETs has been positively correlated with the severity of DED[67]. The continuous formation of NETs by PMNs is believed to be induced by hyperosmolar stress, one of the core mechanisms of DED[67]. Due to the absence of nuclease, which is involved in the clearance of extracellular DNA, NETs accumulate on the ocular surface and may lead to the ocular inflammation observed in DED[67,68]. Postnikoff *et al.* also showed that twice as many PMNs are collected from the DED patients upon awakening (after sleep) compared to healthy individuals, but there is no difference in the numbers of PMNs in the open-eye tears[49]. People with DED also have a higher neutrophil-lymphocyte ratio, approximately 64:1 upon awakening compared to 21:1 in healthy eyes, suggesting that tear-film PMNs may affect T cells recruitment[49].

Ocular allergy (OA) is a general term that includes the two common diagnoses, seasonal and perennial allergic conjunctivitis (SAC and PAC, respectively)[69]. The underlying mechanism of SAC and PAC is mediated through IgE causing hypersensitivity reactions to allergens[69]. SAC occurs mainly in the spring and summer due to the presence of airborne pollens, whereas PAC can occur throughout the year when exposed to perennial allergens[69]. Itchy eyes, tearing, redness and sensitivity to light are common symptoms that affect over 30% of the North American population suffering from seasonal allergies[70]. Eosinophils, which release cytokines upon activation, are a hallmark of an allergic response[71] and have been observed on the ocular surface[72]. Several studies on seasonal ocular allergies

have shown an increased level of cytokines and also indicated the presence of leukocytes in tears[71,73–75]. However, no study has yet characterized the leukocyte population and its phenotype in the tear-film of individuals suffering from ocular allergies.

These recent findings and observations suggest that tear-film PMNs may have a role in ocular surface diseases. It is thus important to characterize their phenotype to enable future development of diagnostic and therapeutic strategies.

3.6. Conclusion

From the blood to the mouth and other tissues where neutrophils are found, it is evident that the neutrophil population is heterogeneous, and different phenotypes exist in both physiological and pathological conditions. Tear-film neutrophils represent a novel research avenue and there is currently limited knowledge as to their phenotype and origin. Tear-film neutrophils collected from the closed-eye environment appear to be non-inflammatory and the reduced expression in L-selectin suggest that they have extravasated from the conjunctival blood vessels. Similarly, to PMNs found in the lung, placenta and mouth, tear-film PMNs have a high expression of CD11b and CD66b but low expression of CD16 compared to blood-isolated PMNs, suggesting an already activated phenotype. However, unlike PMNs collected from the lung, placenta, and mouth, the involvement of tear-film PMNs in ocular diseases remain largely unknown as does ROS generation. There is a need for a better understanding of the mechanisms involved in impaired functionality and interaction with other immune cells. To support this need, it is also important to develop reliable protocols for collection and immunophenotyping and further characterize the phenotype of tear-film neutrophils in healthy individuals.

Chapter 4

Effects of Experimental Procedures on The Expression of Membrane Receptors on Tear-film Neutrophils

4.1. Introduction

Neutrophils (PMNs) are essential inflammatory cells of the innate immune system[1], and are the first cells to arrive at infected sites[2]. It is thought that, under normal physiological conditions, recruitment of immune cells to the ocular surface is restricted due to immune privilege[3,4]. However, despite the immune privilege conferred to the eye, a significant infiltration of neutrophils occurs on the ocular surface when eyes are closed for a prolonged time (such as during sleep)[4–6]; these cells are often referred to as tear-film neutrophils from the closed-eye environment[4]. As cells of the innate immune system with a significant role in the inflammatory response, their presence on the ocular surface has generated significant interest, since many of their roles and functions in the closed eye environment have yet to be discovered.

Neutrophils, which circulate in blood, have been well characterized, and recent research indicates that the neutrophil population is heterogeneous, as PMNs found in different types of tissues, such as the lungs[7], the mouth[8], the placenta[9] and the eye[4], have been shown to exhibit distinct phenotypes. To develop a better understanding of these neutrophil populations, functionality studies have been carried out to assess cell activation[4,10,11], which can effectively be characterized by flow cytometry via measuring the expression of cell membrane molecules[12,13]. Among others, CD11b, CD16, CD55 and CD66b are common membrane receptors used to characterize leukocyte activation and

phenotype. CD11b, also known as the macrophage-1 antigen (Mac-1) or complement receptor (CR3), plays a crucial role in PMNs transmigration and mediating immune/inflammatory response and adhesion[14]. The upregulation of CD11b occurs early in the cell activation process[2] and is often used as an indicator for cell activation and adhesion[15]. CD16, a member of the Fc gamma receptors for immunoglobulin, is shed from the cell upon neutrophil elastase release, and has been used as a phagocytosis and degranulation marker[16,17]. CD55 (decay-accelerating factor), a glycosylphosphatidylinositol (GPI)-anchored receptor and regulator of complement activation, is mainly stored in secretory vesicles and is upregulated on the cell membrane after activation[18]. CD66b is recognized to be a marker for degranulation, and its expression is known to increase upon activation[15].

In immunophenotyping experiments, fixation is often involved to preserve cell structure and avoid undesired cell activation and any artefactual changes. Fixation also contributes to preserving proteins, carbohydrate and other bio-active substances and prevents cell decomposition, putrefaction and autolysis[19,20]. Aldehydes, including paraformaldehyde (PFA) and formaldehyde[21], are the fixative agents most commonly used and preserve cell structures by combining the nitrogen base in one protein with an already formed hydroxymethyl group in another protein, and forms a crosslink methylene bridge (CH_2)[19]. PFA is most commonly used in flow cytometry and the fixation process of blood cells, especially lymphocytes, has been thoroughly investigated. There are two ways to fix cells, (1) adding PFA before the staining with antibodies (pre-fixed staining) and (2) adding PFA after the staining with antibodies (post-fixed staining). Fixation prior to staining has been shown to significantly decrease the expression of CD11b, CD18 and CD62L on blood leukocytes[22]. It has also been observed that expression of CD16 on blood-isolated PMNs

gradually decreases with increased time of post-fixation[23], suggesting that a fixation step can significantly affect the expression of the marker of activation being investigated. As heterogeneity exists in neutrophil population, it is not known if similar effects of PFA would be observed on the expression of membrane receptors on tear-film PMNs.

Neutrophils are also delicate and fragile cells that can be easily damaged by inappropriate experimental procedures[24]. For example, counterflow centrifugal elutriation, a protocol used to isolate PMNs, has been shown to cause an increased release of superoxide and granule contents when PMNs are activated[25]. Mechanical forces during centrifugation and resuspension as well as exposure to warm or cold temperature (37^{o}C versus 4 ^{o}C) result in changes in the expression of C3b receptors (CD35) and CR3 (CD11b) on PMNs[26,27]. Since tear-film PMNs have a distinct phenotype from blood-isolated PMNs, it is likely unreasonable to assume that tear-film PMNs will be impacted in the same manner as blood leukocytes. Experimental procedures have the potential to significantly affect results and have been largely unexplored with tear film PMNs. It is however a key component to ensure reproducibility and reliability of results. The objective of this study was thus to determine if experimental procedures, such as centrifugation and fixation, could alter the expression of cell activation membrane receptors. Using flow cytometry, the expression of CD11b, CD16, CD55, and CD66b on tear-film PMNs were characterized in unfixed samples and compared to pre-fixed stained (adding PFA before staining with antibodies) and post-fixed stained (adding PFA after staining with antibodies) and the centrifuged samples. Changes in the expression of CD45, the common leukocyte marker, were also assessed.

4.2. Materials and Methods

4.2.1. Reagents and Monoclonal Antibodies

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against human CD11b and CD66b, R- phycoerythrin (PE)-conjugated monoclonal antibodies against human CD55 and CD16 and R-phycoerythrin-cytochrome 5 (PE-Cy5)-conjugated monoclonal antibody against CD45 were purchased from Becton Dickinson Pharmingen (San Diego, CA, USA). Paraformaldehyde (PFA) was from Sigma-Aldrich Co. (Oakville, Ontario, Canada). Phosphate-buffered saline (PBS) was purchased from Lonza (Allendale, New Jersey, USA). Dulbecco's modified eagle media (DMEM) was purchased from Life Technologies (Burlington, Ontario, Canada). Keratinocyte serum-free medium (KM) was purchased from ScienCell (Carlsbad, California, USA).

4.2.2. Cell Collection

This study was conducted in accordance with the tenets of the Declaration of Helsinki and received ethics clearance from the University of Waterloo Human Research Ethics Committee (#30164; Waterloo, ON, Canada). A total of five healthy participants, with an age ranging from 23 to 30, were involved. Each participant provided one or more samples for each experiment.

Participants were provided with instructions and were trained to self-collect tear-film neutrophils after sleep using a polypropylene pipette containing sterile PBS. They slept at home, as usual. After 8 hours of sleep, upon awakening, participants gently washed their eyes with approximately 5 mL of PBS for each eye; the eye wash was collected in one sterile polypropylene tube (pooled sample) (Figure 8)[4]. After collection from both eyes, the tube

was placed in the provided storage container and delivered to the lab within two hours.

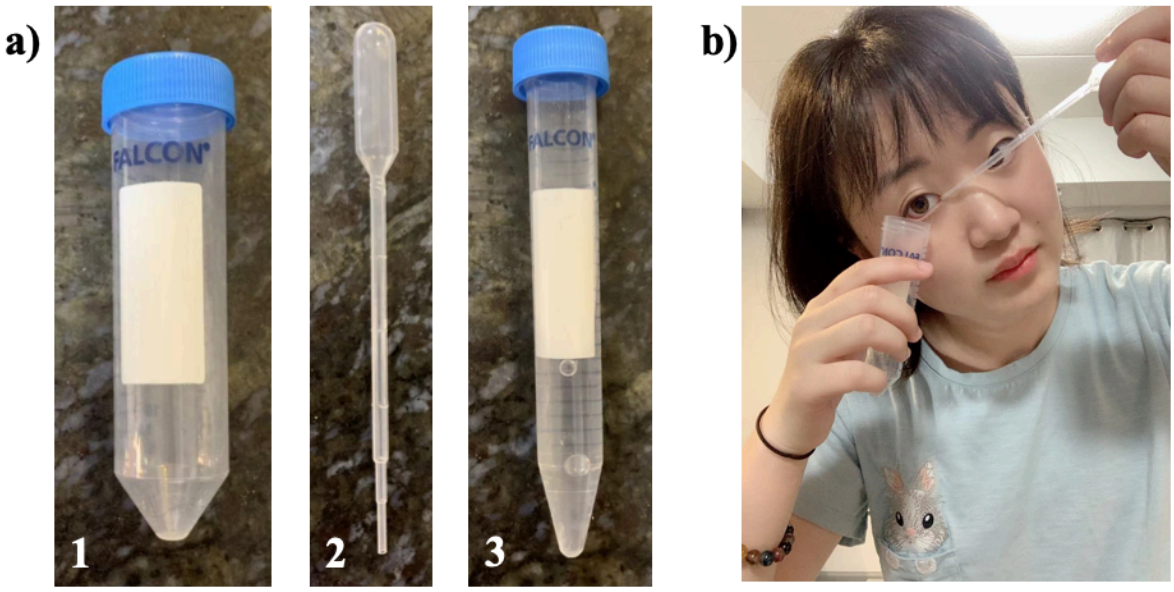

Figure 8 - Illustration of the at-home tear-film PMNs collection process. The required materials, 1) empty polypropylene tube, 2) disposable pipette sterile, and 3) phosphate buffered saline (PBS), are shown in (a). The overall demonstration of the collection procedure is shown in (b). It is recommended that the procedure be performed in front of the mirror. Participants used the polypropylene disposable pipette 2) to draw approximately 1 mL of PBS 3) and the pipette containing PBS was put at the inner angle of the eye with caution and as the pipette bulb is squeezed, PBS is slowly released and flows onto the ocular surface. The runoff was collected at the outer angle of the eye by using an empty polypropylene tube 1) Participants used approximately 5 mL of PBS per eye.

4.2.3. Experimental procedure

Once cell samples were delivered to the lab, they were centrifuged at 290xg for 10 min and resuspended in approximately100 µL of PBS. Cell count and viability were determined using trypan blue. Samples were diluted to a final cell concentration of 100,000 cells/mL in an artificial tear solution (keratocyte medium containing various tear film proteins), as detailed in Table 2.

Table 2 – Components of the artificial tear solution

Components	Mass (mg)
Albumin	2
IgG	0.2
Lactoferrin	18
Lysozyme	19
Mucin	1.5
KM	10mL

KM: keratocyte medium. KM is endotoxin-free, sterile and contains various salts and amino acids and has previously been used as a tear substitute for *in vitro* studies.

Our control samples were the cell suspension that was processed immediately for flow cytometry without any incubation and is referred to as the "time zero" samples (T0 samples). To determine the impact of re-exposure to physiological temperature, cell samples in polypropylene tubes were incubated for four hours in a cell incubator (37^{o}C, 5% CO_2). These samples are referred to as "4-hour incubation" samples (4h samples). Each T0 and 4h sample was also divided into centrifuged and non-centrifuged groups. Centrifuged groups were spun down at 280xg for 5 min and then re-suspended in PBS. The non-centrifuged group stayed on the bench without any involvement of centrifugation. Immediately following centrifugation, all samples were processed for flow cytometry.

4.2.4. Fixation

To avoid confounding factors of experimental procedures (centrifugation, exposure to change in temperature, etc), the impact of fixation on the expression of membrane receptors was assessed on T0 samples only. The effect of fixation was examined as follows: pre-fixed staining, post-fixed staining and no fixation.

For pre-fixed staining samples, a small aliquot of cell suspension was transferred into

different labelled tubes containing 1:1 DMEM/10% FBS and 2% PFA (1% final concentration) followed by incubation at 4ºC for 15 min, the common protocol for pre-fixation of samples[22,28]. After fixing, fluorescently-labelled antibodies against CD11b, CD16 CD45, CD55, and CD66b were added to corresponding tubes and samples were incubated for 20 min in the dark. Following incubation with antibodies, samples were diluted by adding DMEM/10% FBS and stored in the fridge until flow cytometry analysis the next day.

For post-fixation staining, the usual procedure for immunostaining was followed, whereby a small aliquot of cell suspension was incubated in tubes containing DMEM/10% FBS and fluorescently-labelled antibodies against CD11b, CD45, CD55, CD66b, or CD16. Samples were then incubated for 20 min at room temperature in the dark. At the end of incubation, samples were diluted with DMEM/10% FBS and fixed with PFA (1% final concentration). Samples were then stored in the fridge until flow cytometry analysis the next day.

For the unfixed samples, the procedure remained the same as the post-fixation samples except in the last step, PFA was replaced by DMEM/10% FBS. The unfixed samples were analysed by flow cytometry within 15 min.

The experimental procedure for the samples is illustrated in Figure 9. Note that due to the effect of pre-fixation (see results), all 4h samples were processed using the post-fixed staining method.

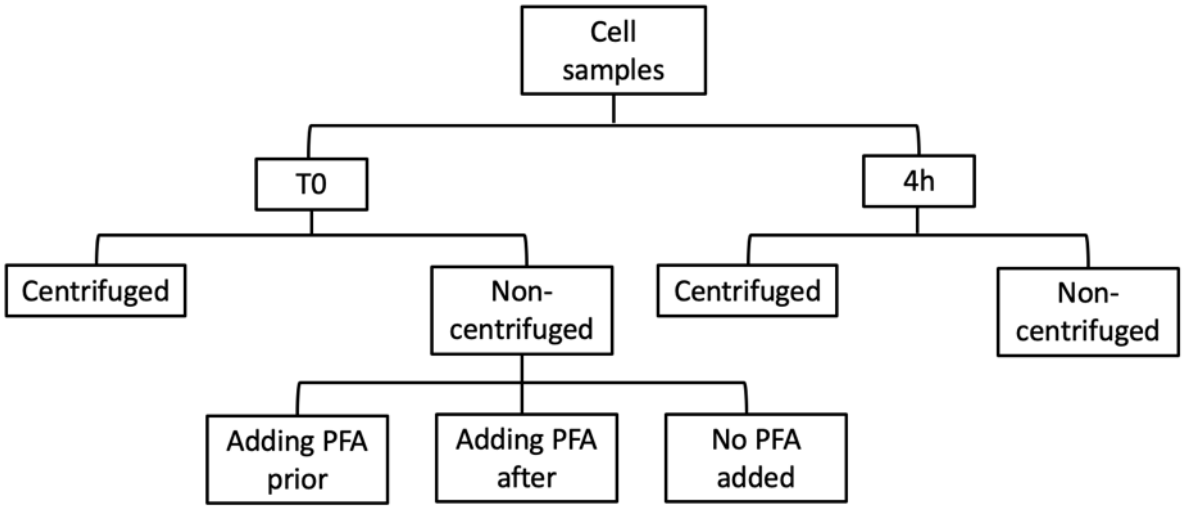

Figure 9 - Experimental flow chart. After cell collection, cells were divided into two groups, time zero samples were processed right after cell count (T0), and 4h samples were incubated for 4 hours at 37 ºC (4h). Cells from T0 and 4h groups were either centrifuged or not, and T0 non-centrifuged group was used to examine the impact of PFA. The rest of the groups, T0 centrifuged, 4h centrifuged, and 4h non-centrifuged were fixed after staining with antibodies.

4.2.5. Flow cytometry

All samples were acquired on a Becton Dickinson FACSCalibur flow cytometer (Mountain View, CA, USA) using CELLQuest Software (Becton Dickinson, Mountain View, CA, USA). At least 2000 PMNs events were acquired, and fluorescent intensities were recorded as arbitrary fluorescent units (AFU). The PMNs population was identified by doubled-gating on size and $CD45^+$ cells [4]. In order to compare expression of membrane receptors between different conditions, the ratio of fluorescent intensities of non-centrifuged and non-fixed samples (baseline) versus other conditions are presented.

4.2.6. Statistics

All results are reported as mean $\pm$ standard deviations. To assess the significant differences in the ratio of fluorescent intensities, paired sample t-test and Wilcoxon signed-

rank test were performed using IBM SPSS Software, and a p-value less than 0.05 was required for statistical difference.

4.3. Results

On average, the number of leukocytes collected by using the gentle eye wash method was 2.2 x10^6 (ranging from 1.1x10^6 to 9.4 x10^6 leukocytes) with less than 2% dead cells. The effect of fixation, centrifugation and incubation were assessed on the expression of CD11b, an activation and adhesion marker, CD16, a degranulation and phagocytosis marker, CD45, a common leukocyte marker, CD55, a complement activator marker, and CD66b, a degranulation marker, on tear-film PMNs.

4.3.1. The impact of fixation

As shown in Figure 10, pre-fixed and post-fixed staining affected the expression of activation markers on tear-film PMNs in different ways when compared to the non-fixed staining samples. When PFA was added prior to staining with antibodies, a significant decrease (78 to 98 % reduction) was observed in the expression of CD11b ($p < 0.001$), CD16 ($p = 0.008$), and CD45 ($p < 0.001$). Conversely, the expression of CD55 and CD66b remained relatively unchanged ($p \geq 0.84$). On the other hand, in the post-fixed staining group, a reduction of more than 50% was observed in the expression of CD16 ($p = 0.008$) and a small (14%) but statistically significant reduction in expression of CD11b ($p = 0.01$). The other markers, CD45, CD55 and CD66b, on post-fixed staining samples displayed some variations in expression but remained relatively unchanged ($p \geq 0.36$). The results indicated that exposing collected cells to PFA prior to staining with antibodies resulted in drastic reductions in CD11b, CD16 and CD45 expression and thus for the remainder of the study,

cells were fixed post staining.

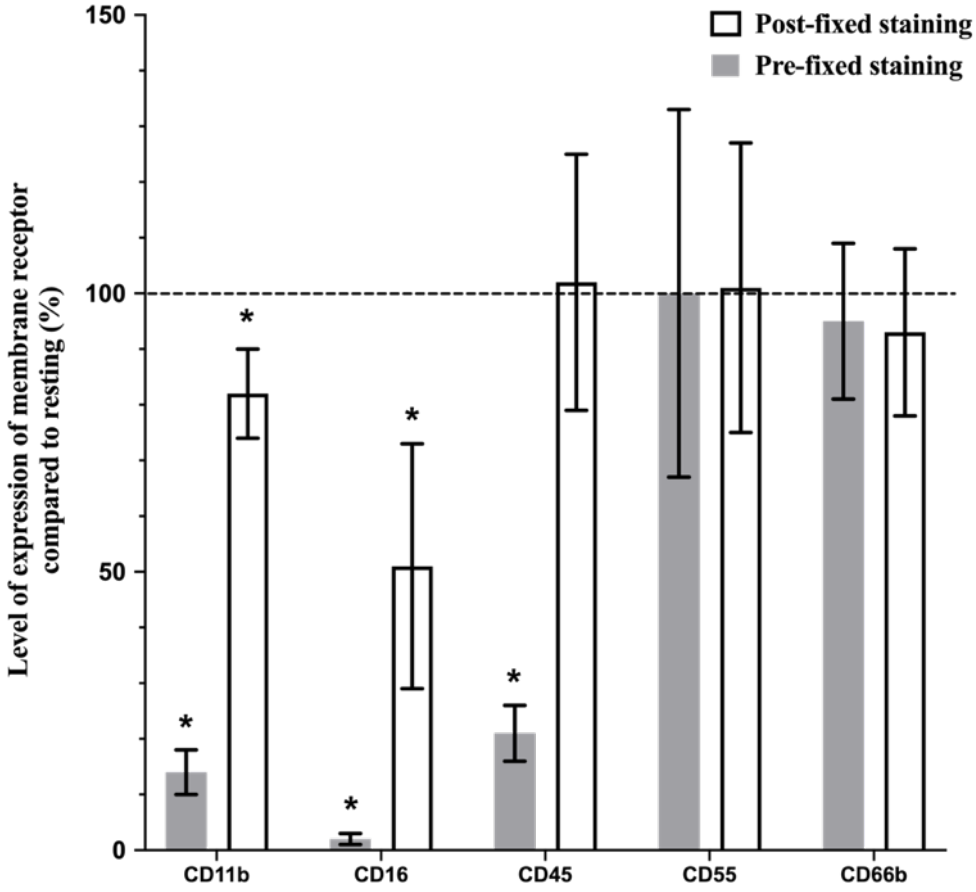

Figure 10 - Effect of PFA on the expression of cell activation surface markers on tear-film PMNs. Cells were either fixed with 2% PFA at 4°C for 15 min prior to staining with antibodies (pre-fixed staining) or stained with antibodies and then fixed with 2% PFA (post-fixed staining). Control samples were stained with antibodies and immediately analyzed by flow cytometry (non-fixed staining). All incubation with antibodies were performed at RT, in the dark, for 20 min. Fluorescence intensities for CD11b, CD16, CD45, CD66b, and CD55 were recorded as the arbitrary fluorescent units by flow cytometry and are expressed as a percentage of expression level compared to the non-fixed staining samples (resting samples). The dotted line represents the resting value. Mean ± SD, *significantly different from non-fixed samples, $p < 0.01$.

4.3.2. The impact of incubation temperature

To assess how exposure to physiological temperature following collection may activate tear-film PMNs, cells were exposed to a 4-hr incubation at 37°C, 5% CO_2 and expression of cell activation membrane receptors was evaluated by flow cytometry and is shown in Figure 11. When compared to T0 samples, exposing collected tear film PMNs to 37°C for 4 hours appeared to induce a mild state of cell activation, with a significant (p = 0.04) upregulation in CD11b (~ 30% increase) and CD45 (~ 50% increase) while expression of CD16 and CD66b decreased markedly; however, due large variations in response, the more than 30% reduction in CD16 and CD66b expressions were not statically significant (p = 0.09 and p = 0.06, respectively). The reduction in CD66b was also inconsistent with the lower expression of CD16, an indicator of degranulation that is cleaved upon degranulation; CD66b expression is expected to increase with release of granules. CD55 expression remained mostly unchanged (p = 0.78).

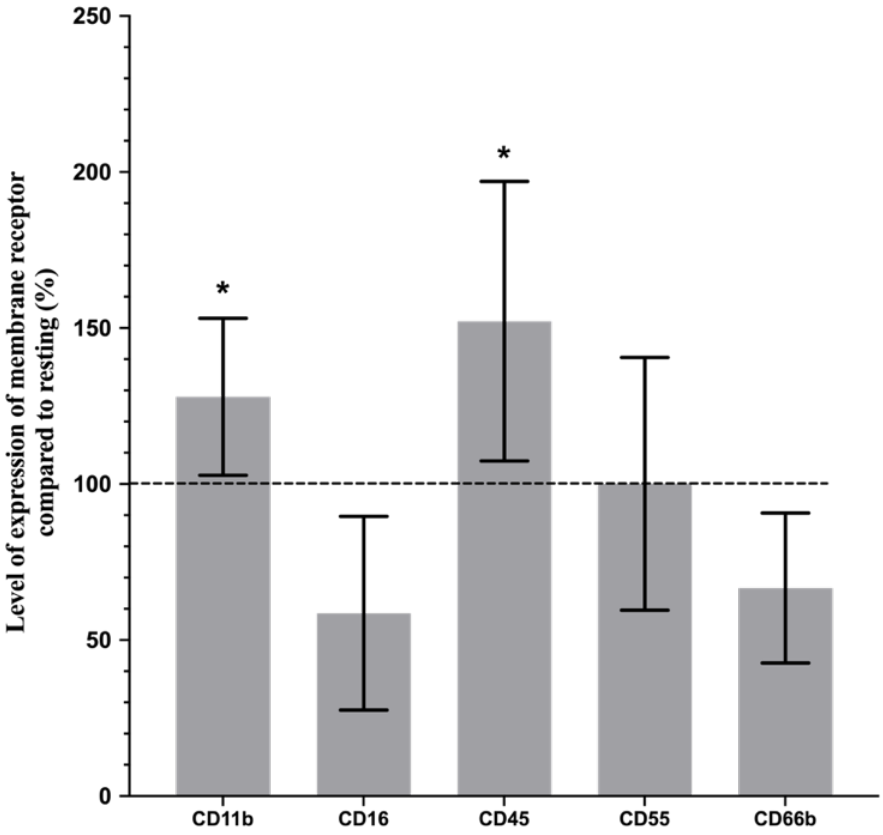

Figure 11 – Effect of exposure to 37°C for 4h on the expression of cell membrane activation markers on tear-film PMNs. Following collection, tear film PMNs were either processed for flow cytometry (antibody staining followed by PFA fixing) or incubated for 4hrs at 37°C, 5% CO_2. Following 4hr-incubation, cells were stained with antibodies and fixed with PFA. Fluorescence intensities for CD11b, CD16, CD45, CD66b, and CD55 were recorded as arbitrary fluorescent units by flow cytometry and are expressed as a percentage of expression level compared to the time zero samples (resting samples) (T0). The dotted line represents the resting value (T0). Mean ± SD, *significantly different from non-incubated samples, $p < 0.05$.

4.3.3. The impact of centrifugation

To determine the effect of centrifugation on the expression of cell membrane activation markers, tear-film PMNs from T0 and 4h-incubated groups were either centrifuged at 280xg (5 min) or not. Changes in the expression of cell activation membrane receptors are reported in Figure 12. For T0 samples, results indicated that an extra centrifugation step, which exposed cells to mechanical forces, caused some degranulation, as a significant decrease in the expression of CD16 ($p < 0.001$) and an increase in CD66b ($p = 0.16$) were observed. Reduction in the expression of CD45 ($p < 0.001$) and CD55 ($p = 0.07$) also occurred. The expression of CD11b was not affected by centrifugation ($p = 0.22$).

For the 4h samples, all changes observed with the time zero samples were further amplified after the 4hr incubation at 37^{o}C, confirming our above results that cells were affected by the 37^{o}C incubation (in section 4.3.2, we had identified signs that tear film PMNs may have undergone some degranulation) and that exposing cells to centrifugal mechanical forces induces further cell damage and degranulation. The expressions of CD11b ($p = 0.03$), CD16 ($p = 0.003$), CD45 ($p < 0.001$) and CD55 ($p = 0.004$) were significantly reduced while, as expected from a degranulation process, the levels of CD66b ($p < 0.001$) significantly increased.

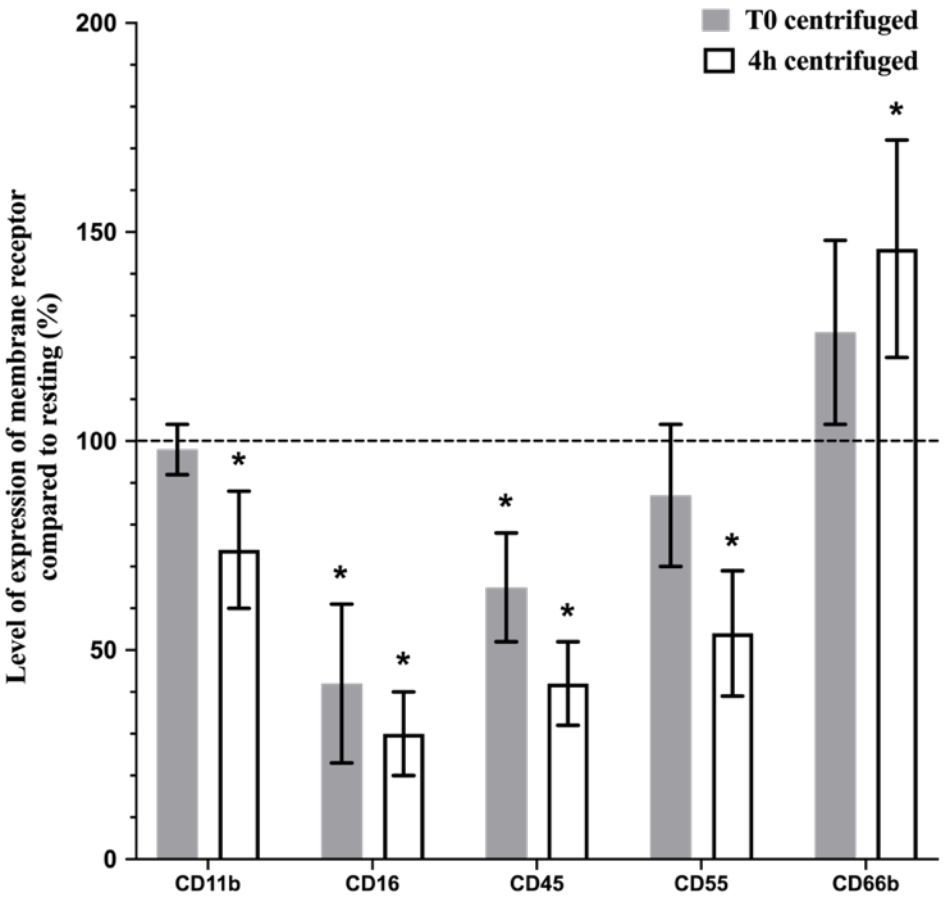

Figure 12 – Effect of centrifugation on the expression of cell membrane activation markers on tear-film PMNs. Following collection and concentration, tear film PMNs were either subjected to another centrifugation step or incubated for 4 hrs at 37°C – 5%CO_2 and then centrifuged. Samples were processed for flow cytometry (antibody staining followed by PFA fixing). Fluorescence intensities for CD11b, CD16, CD45, CD66b, and CD55 were recorded as arbitrary fluorescent units by flow cytometry and are expressed as percentage of expression level compared to the non-centrifuged time zero samples (resting samples). The dotted line represents the resting value. Mean ± SD, n = 8 – 10, *significantly different from resting, $p < 0.05$.

4.4. Discussion

As summarized in Table 3, our results identified how cell membrane activation receptors exhibit different sensitivity to experimental conditions (centrifugation, exposure to 37°C, fixation). For example, minimal changes occur in CD55 expression with fixation and 37°C incubation, while CD16 expression, a marker for degranulation, was the most sensitive to all experimental procedures tested leading to a significant decrease in expression. Post fixation was the most reliable fixation step, but an additional centrifugation step appeared to significantly affect membrane receptor expression (CD11b, CD66b). The level of CD45 remained unchanged only in post-fixed condition, however its changes in relation to experimental procedures are not as important as it is used mainly to identify leukocytes and not to assess leukocyte activation. The impact of experimental procedures on tear-film PMNs antigen expression is further discussed in details below.

Table 3 – Summary of changes in the expression of surface receptors on tear-film PMNs (processed versus unprocessed sample).

CD markers	Pre-fixed staining (%)	Post-fixed staining (%)	Centrifugation T0 ~ 4 hr sample (%)	Incubation 4hr-37°C (%)
CD11b	-85 ± 4 *	-14 ± 8 *	-(2 ~ 26) ± 14	+28 ± 25 *
CD16	-98 ± 1 *	-49 ± 22 *	-(58 ~ 70) ±19	-41 ± 31
CD45	-78 ± 5 *	+2 ± 23	-(36 ~ 60) ± 10	+52 ± 44 *
CD55	0 ± 33	-5 ± 26	-(13 ~ 46) ± 15	0 ± 40
CD66b	-4 ± 14	-1 ± 15	+(16 ~ 46) ± 26	-33 ± 24

Changes in expression of surface receptors on tear-film PMNs when exposed to experimental conditions: results obtained from different experiments are summarized. "+" represents an increase while "-" represents a reduction in expression. *Significantly different from rest values, $p < 0.05$.

To further demonstrate the effect of experimental procedures on expression, the fluorescent values of one participant are presented in Table 4. The low CD16 and high CD11b and CD66b suggest that tear-film PMNs may have already been degranulated and extravasated, indicating an activated phenotype of tear-film PMNs, corroborating previous studies[4].

Table 4 - Change in level of expression of receptors on tear film neutrophils following fixation.

CD markers	Non-fixed staining	Pre-fixed staining	Post-fixed staining
CD11b	527	72	422
CD16	67	2	23
CD45	171	33	163
CD55	273	305	226
CD66b	555	529	533

Values are from one participant and are representative from all experiments. Values are reported as arbitrary fluorescent units (AFU).

In flow cytometry, it is crucial to ensure that the preparation procedures before labelling with fluorochrome-conjugated antibodies exert a minimal effect on antigen expression. This study aimed to investigate the effect of pre-fixation and post-fixation staining and centrifugation on expression of membrane receptors on tear-film PMNs in order to optimize experimental procedures to preserve the expression of markers of activation as close as possible to the *in vivo* state by limiting *in vitro* changes. Any significant change induced by procedures would significant impact our interpretation of the results on the state of activation of tear-film PMNs.

Fixation, through the crosslinking of antigens and their antibodies, aims to preserve the binding structure for a prolonged time. However, research has shown that exposure of cells to fixative chemicals might cause damage to the expressions of function-associated

antigens[29], and an increase in autofluorescence[12]. According to our results, pre-fixed staining significantly decreased the expressions of CD11b, CD16 and CD45 on tear-film PMNs. Blood-isolated PMNs and hematopoietic cells have also shown a decreased the expression of CD11b with fixation prior to staining[12,28,29]. Fixation prior to staining may lead to a loss of antigenicity of the epitopes[30]. Aldehyde fixative reacts with amine groups and forms a methylene bridge to crosslink between proteins. During this process, the fixative may distort the tertiary structure of proteins[28], and consequently, cause the decrease in recognition of antibodies to their corresponding antigens. Depending on their structure, antigens may be affected differently which would explain why a pre-fixation step induced significant damage on CD11b, CD16 and CD45 but not on CD55 and CD66b. The changes induced by exposure to PFA prior to staining would have significant impact on our interpretation of results, ie low CD16 expression would indicate that tear film neutrophils had undergone extensive degranulation *in vivo*, although in this case, this is an artefact of how the cells were processed following collection.

A fixation step following staining significantly reduced the expression of CD16 and CD11b. Although the 14% downregulation of CD11b compared to the non-fixed sample was statistically significant, it may not carry biological significance and in this case would not significantly affect our interpretation of results in the context of cell activation. On the other hand, as with pre-fixed staining, CD16 warrants caution. A dramatic decrease in the expression of CD16 was previously reported by Stewart *et al*. after 24 h fixation on blood-isolated PMNs, and expression was also observed to further decrease between 24 and 96 h after fixation[23]. They also observed a small but significant reduction (12%) in CD11b after 24 h fixation[23], which is closed to the 14% reduction in CD11b we observed in our post-fixed

samples analysed around 24hrs. Interestingly, we also had observed a change in levels of expression with some of our activation markers depending on the time of storage after fixation post staining (results not shown), which is in agreement with Stewart *et al.*'s research on blood-isolated PMNs. Thus, to increase result reproducibility, all our samples were always analyzed around 24 h after fixation. The decrease in level of expression during 4°C storage after fixation may be due to internalization of surface receptors. Fixation tends to permeabilize cell membranes, resulting in a decrease in cell membrane integrity, which could eventually cause membrane receptors internalized[23,28]. The fact that not all receptors are affected the same way is not surprising, as differences exist in the stability of the antigen and antibody bond and the linkage between the antibody and its fluorochrome[31].

Tear-film PMNs incubated at 37°C for 4 hours showed a significant upregulation in CD11b and CD45, suggesting activation of tear film PMNs, which would be similar to previous observation in blood leukocytes[32]. This upregulation is noteworthy, as CD11b upregulation on tear-film PMNs has been shown to be minimal upon stimulation with fMLP, PMA, and LPS when collected after sleep[4], thus change in temperature may be a potential stress or stimulus to activate tear-film PMNs. However, not all results support the activation of tear-film PMNs during the 37°C incubation, as a decrease in CD66b was also observed (activation associated with degranulation would have resulted in an increase in CD66b expression). Since the downregulation of CD66b was also associated with lower levels of CD16, this may also be indicative of apoptosis[16]. Large variations in the level of change in expression between 0 and 4h incubation samples further suggested that tear-film PMNs may not remain stable at 4hr. Similar observations have been made with blood leukocytes where *in vitro* experiments tend to be limited to 2hr to preserve cell integrity[33].

Our study also showed that centrifugation resulted in a downregulation of all our selected cell membrane receptors except CD66b. Centrifugation is a necessary initial first step to concentrate cells, but following centrifugation steps are often performed to wash cells, re-concentrate cells, remove excess antibodies, etc. Since the centrifugation step was performed before immunostaining, the reduced expression cannot be explained by the antibodies being washed away. Internalization of surface receptors by tear-film PMNs due to damage cell membrane integrity induced by exposure to shear force of the centrifugation may be a possible explanation to the observed downregulation in receptor expression. This would be corroborated by the fact that centrifugation of the T0 samples had a lesser impact on the expression of surface receptors than in samples incubated for 4 hours: centrifugation may induce some damage to cell integrity, which is further exacerbated in the 4hr sample which may be undergoing activation and apoptosis (see above) due to the 4hr-incubation at 37^{o}C. Interestingly, previous work with blood isolated leukocytes have either reported an increase or no change following washing steps[34,35]. It is unclear why an upregulation in CD66b (hence potential degranulation) is observed when all the other markers are being downregulated.

A centrifugation step post staining (several centrifugation steps are often used post staining to wash samples and remove unbound antibodies) resulted in a decrease in CD11b expression and an increase in CD16, while CD45, CD55 and CD66b remained relatively unchanged (data shown), further highlighting the potential impact of centrifugation. Not only do our results emphasize the need to avoid extra centrifugation steps, it also highlights the complexity in the underlying mechanisms of cell activation and membrane receptor up and down regulation.

4.5. Conclusion

This study demonstrates that expression of membrane receptors on tear-film PMNs exhibit different sensitivity to fixation, centrifugation and incubation and thus caution should be exerted to ensure that experimental procedures will not affect marker of cell activation as this has the potential to significantly impact result interpretation. Due to the drastic decrease in expression of CD11b, CD16 and CD45, it is highly recommended to avoid exposing tear-film PMNs to fixative prior to immunostaining. If samples need to be fixed, our results suggest that post-fixation is preferred as post fixation induces minimal changes in activation markers, except with CD16. Any additional centrifugation step after the initial concentration/wash as well as long exposure to 37^{o}C (4hrs or more) of tear-film PMNs induce cell damage or activation and thus should be avoided unless appropriate controls are in place. From the markers of activation assessed in this study, CD16, a marker of degranulation, appeared to be most sensitive to experimental procedures and this may limit its use in functionality study of tear film PMNs. The study allowed us to gain a better understanding of how experimental procedures can affect the phenotype of tear film PMNs which will help in the development of better protocols to characterize tear film PMNs and assess their functionality *in vitro.*

Chapter 5

Comparison of Two Collection Methods and Investigation of the Response of Tear-film Neutrophils to IL-8

5.1. Introduction

Neutrophils (also known as polymorphonuclear neutrophils or PMNs) represent approximately 60% of the total leukocyte population and possess several efficient killing mechanisms, such as phagocytosis and release of antimicrobial substances stored in granules, to protect the host tissues from invading pathogens[1].

It is known that a large number of PMNs can be collected from the ocular surface, referred to as tear-film PMNs, after eyes are closed for a prolonged time[2,3]. Besides the presence of tear-film PMNs, closed-eye tear fluid is rich in proteins, such as cytokines, lysozyme, lactoferrin, secretory IgA and complement products, suggesting a state of sub-clinical inflammation[4]. Interestingly, tear-film PMNs have been reported to be quiescent upon stimulation and exhibit different expression of surface receptors associated with activation on their cell membranes in comparison to blood-isolated PMNs[2,5]. This suggests that tear-film PMNs may have already been primed and/or activated during their recruitment to the ocular surface[5,6]. Although their origin is not established firmly, the hypothesis that they originated from the conjunctival blood vessels and entered the ocular surface through extravasation is supported by the low expression of L-selectin and high level of Mac-1 (CD11b)[2]. There are still many unknowns about their interaction with other immune cells, their extensive presence in the nocturnal environment and their functionality, that need to be further investigated.

To investigate and characterize their phenotypes, it is essential to have a collection method that is non-invasive and able to consistently yield as many cells as possible for subsequent *in vitro* experiments. Non-invasive methods used to collect cells from the ocular surface have evolved over the years and have been used to harvest not only neutrophils but also human corneal epithelial cells (hCECs). The "non-contact corneal irrigation chamber" was used in 1986[7] to collect cells, but less than 200 hCECs and 8000 leukocytes were usually obtained, preventing any cell phenotyping. The ocular surface cell collection apparatus (OSCCA) developed by Peterson *et al.* allowed researchers to harvest more cells from the ocular surface compared to previous methods[8]. While effective in cell collection, as it can collect over hundreds of thousand neutrophils from the ocular surface[9], the cost involved with overnight studies limited the use of the OSCCA for collection of tear-film neutrophils, as participants were required to sleep at the clinic. A new method, using a gentle eye wash, was thus designed by Gorbet *et al.*, which allowed participants to collect tear-film neutrophils at home[2]. Each collection method has its drawbacks: there is an expensive participant remuneration involved in OSCCA studies due to the required overnight stay, whereas in the gentle eye method, inexperienced participants may lose cells due to the lack of proficiency in collection procedures. To address this cost issue, we aimed to prevent the overnight stay required by the OSCCA by evaluating the use of a patch to cover one of the eyes (patch-OSCCA collection method) and asking the participants to come to the lab early in the morning to perform cell collection using the OSCCA. The efficiency in collecting cells was compared between the two collection methods (patch-OSCCA and gentle eye wash) to determine which method would yield most cells, and if the phenotypes of tear-film PMNs collected from the two collection methods differed from each other.

PMNs express various types of surface receptors, such as cytokine receptors, G-protein coupled receptors, and Fc-receptors, which lead to different cell functional responses[10]. Hence, evaluation of the expression of surface receptors provides information on cell activation state. To characterize their immune response, tear-film PMNs are activated *in vitro* with various stimuli. The stimuli used to characterize the response of tear-film neutrophils have been phorbol-12-myristate-13-acetate (PMA; a potent synthetic chemical activator of protein kinase C), lipopolysaccharides (LPS; a bacterial endotoxin), N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; a chemotactic peptide), and calcium ionophore[2,5,6]. However, these are not all physiological stimuli nor are they representative of stimuli that tear-film neutrophils would typically encounter in the ocular environment. Cytokines, such as tumor necrosis factor α (TNF-α)[11], granulocyte-macrophage colony-stimulating factor (GM-CSF)[12] and interleukin-8 (IL-8)[13] have been recognized to activate blood-isolated neutrophils, however little is known on the response of tear-film neutrophils to cytokines. Among these more physiological stimuli, IL-8 is a chemokine for neutrophils, leading to activation and migration of blood leukocytes to infected sites through the actions of L-selectin and Mac-1[14,15] and high levels of IL-8 have been observed in the closed-eye environment[16]. Characterizing the response of tear-film neutrophils to IL-8 would provide information on their activation state in the closed-eye environment as well as contribute further evidence towards their phenotypic difference from blood leukocytes.

Our objectives were 1) to compare the number of cells collected between the patch-OSCCA collection and the gentle eye wash method; and 2) to investigate the response of tear-film PMNs to IL-8 and PMA, and compare it with that of blood-isolated PMNs. The response to stimuli of tear film PMNs from the closed eye environment was evaluated

through the expression of surface receptors, CD11b (an adhesion and activation marker), CD16 (a phagocytosis and degranulation marker), CD45 (a common leukocyte marker), CD55 (a complement activation marker) and CD66b (a degranulation marker).

5.2. Materials and Methods

5.2.1. Reagents

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against human CD11b and CD66b, R- phycoerythrin (PE)-conjugated monoclonal antibodies against human CD55 and CD16 and R-phycoerythrin-cytochrome 5 (PE-Cy5)-conjugated monoclonal antibody against CD45 were purchased from Becton Dickinson Pharmingen (San Diego, CA, USA). Paraformaldehyde (PFA), IL-8 human, Trypan Blue, Histopaque-1077 and PMA were from Sigma-Aldrich Co. (Oakville, Ontario, Canada). Phosphate-buffered saline (PBS) was purchased from Lonza (Allendale, New Jersey, USA). Dulbecco's modified eagle media (DMEM) was purchased from Life Technologies (Burlington, Ontario, Canada). The eye patch was ordered from Amazon Canada.

5.2.2. Study visits

The study received ethics clearance from the University of Waterloo Human Research Ethics Committee. A total of 14 participants were recruited into the study, and ocular health was verified through slit lamp observations, which were performed before and between the collections. Participants were trained on the home collection procedure a day before they actually performed it. Tear-film neutrophils were collected from each participant five times over a one-month period: three collections at home (day 1, day 2 and day 29) and two collections with the OSCCA took place in our laboratory (day 3 and day 30). The study

consisted of seven visits, including the two screening tests and five collections, as shown in Figure 13.

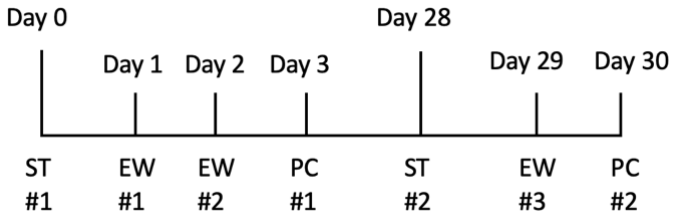

Figure 13 - Schematic diagram of the study visits. ST: screening test; EW: the gentle eye wash method; PC: the patch-OSCCA collection method.

5.2.3. Gentle eye wash method

Participants used a previously established collection method described elsewhere [2,5]. To ensure they were comfortable with the collection procedure, training took place on day 0. Briefly, 5 mL of sterile PBS was used to wash each eye, and the runoff was collected in a sterile polypropylene tube. The sample was delivered to the lab within two hours of collection. Cell suspension from each participant was centrifuged at 290xg for 10 min to recover the cell pellet which was then resuspended in PBS. Cell count and viability were determined under the microscope with Trypan Blue. Dilution to 100,000 cells/mL with PBS was performed if applicable.

5.2.4. Patch-OSSCA Collection

Participants were instructed on wearing the patch the night before the collection day. Patches were adjustable so as to firmly cover and fit the participant's eye (figure 14b). Participants were asked to cover one of their eyes with the provided patch just before going to sleep, and then they slept at their homes as usual. Once they woke up in the morning, they

were required to come to the lab between 9 and 10 am. In order to prevent cell loss, they were not allowed to take off the patches or wash their faces. Due to the potential associated risks, participants were not allowed to drive with one eye covered. After the eye patch was removed, tear-film PMNs were collected from the covered eye using the OSCCA (Figure 14a). Before the collection was performed, the funnel was rinsed with sterile distilled water, and sterile PBS was warmed up to approximately 35 ^{o}C. Any part that was in direct contact with the participant's skin was wiped with ethanol and all other materials (tubing, needle, etc) were autoclaved the day prior to collection. Once OSCCA preparation was complete, participants were asked to place their head securely on a head-rest and face down. Next, they were asked to hold their lower eyelid while the OSCCA operator held their upper eyelid. A warmed and sterile PBS sprayed gently over the ocular surface via a pump for approximately 30 seconds. This saline solution washed cells from the ocular surface, and the eye wash was collected into a sterile 15 ml polypropylene tube attached to the funnel. The suspension samples were centrifuged at 290xg for 10 min to recover the cell pellet, which was then resuspended in PBS. Cell count and viability were determined under the microscope with a hemocytometer and Trypan Blue.

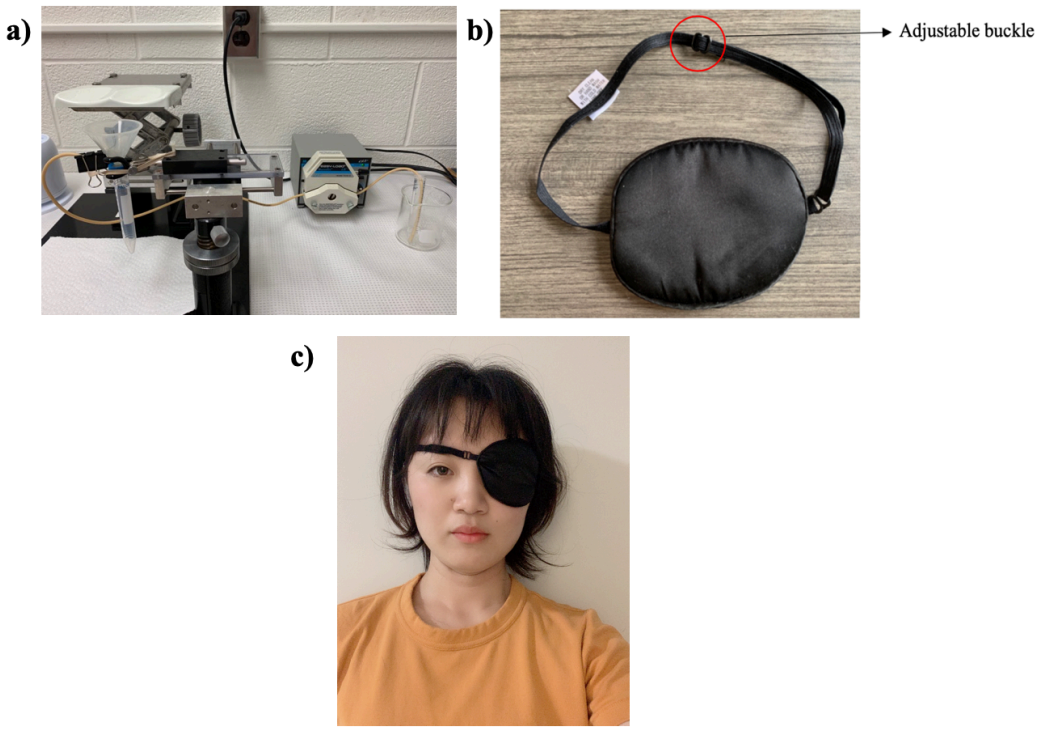

Figure 14 –OSCCA setup and the eye patch. a) the OSCCA; b) a patch with adjustable buckle; c) demonstration of wearing an eye patch.

5.2.5. Collection of blood-isolated PMNs

Peripheral blood was drawn from three medication-free and healthy participants and added to a sterile polypropylene tube containing 5 U/mL of heparin (blood collection happened on separate days for each participant). After centrifugation at 100xg for 10 min, platelet-rich plasma was removed, and density-gradient centrifugation using Histopaque and Polymorphprep (Axis Shield PoC AS, Oslo, Norway) was performed on the blood sample. The isolated PMNs were washed three times with the first two washes in DMEM/10% FBS with 5 mM EDTA (to prevent any leukocyte activation) and the last wash was performed in sterile PBS. The purified blood-isolated PMNs were counted under the microscope with the hemocytometer (Hausser Scientific, USA), and their viability was determined by Trypan

Blue. Dilution to 100,000 cells/mL was performed if applicable.

5.2.6. Cell stimulation and staining with fluorescent antibodies

Two stimuli were used in this study, PMA (2 μM, final concentration)[2], a protein kinase C (PKC) activator, and IL-8 (10 ng/mL, final concentration)[17]. Samples were divided into three aliquots, which were unstimulated, PMA-stimulated, and IL-8 stimulated samples. PMA stimulated samples were incubated for 20 min at room temperature[2] while IL-8 stimulated samples were incubated for 30 min at 37^{o}C[17].

After the incubation with stimuli, 25μl of cell suspension was transferred into corresponding labelled tubes containing antibodies against CD11b, CD16, CD45, CD55 and CD66b, and tubes were incubated for 20 min in the dark at room temperature. Finally, samples were diluted with DMEM/10% FBS and fixed with PFA (1%, final concentration). All samples were analysed by flow cytometry after 24 h.

5.2.7. Flow cytometry

The CELLQuest Software (Becton Dickinson, Mountain View, CA, USA) on a Becton Dickinson FACSCalibur flow cytometry (Mountain View, CA, USA) was used to assess the expression of surface receptors on leukocyte samples. At least 2000 PMNs events were acquired. Fluorescent values for each antibody (also known as Mean Fluorescent Intensity, MFI) were recorded for all samples. To compare the changes in activation of tear-film PMNs upon exposure to the various inflammatory stimuli, data is presented as the ratio of the fluorescent intensities of stimulated samples versus unstimulated samples.

5.2.8. Statistics

All results are reported as means ± standard deviations. Statistical analysis was

performed using a paired sample t-test, Wilcoxon signed-rank test or an independent t-test to evaluate if the difference of mean fluorescent intensities between unstimulated and stimulated samples was significant. IBM SPSS Software (IBM Canada Ltd., Markham, Ontario, CA) was used, and a p-value of less than 0.05 was required for statistical difference. All graphs were plotted by GraphPad Prism Software (GraphPad Software, San Diego, CA, USA).

5.3. Results

5.3.1. Patch-OSSCA collection versus home collection

As shown in Figure 15, the gentle eye wash method collected significantly more cells than the patch-OSCCA collection method ($p = 0.012$), with average cell counts of $2.4 \times 10^6 \pm 1.6 \times 10^6$ versus $1.1 \times 10^4 \pm 1.6 \times 10^4$, respectively.

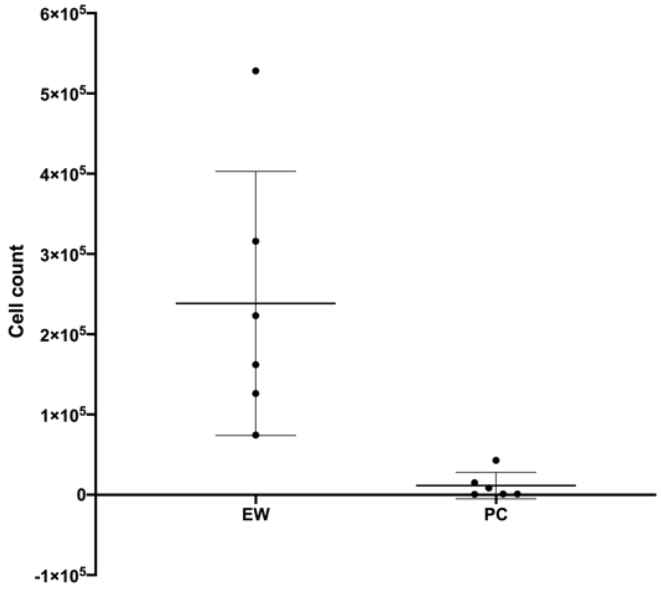

Figure 15 - The total cells collected using the home collection method with the gentle eye wash (EW) and the patch-OSCCA collection method (PC). Cell numbers were counted under the microscope with the hemocytometer and Trypan Blue. n=6, individual participants.

The number of cells collected by the patch-OSCCA collection method was too low to be analyzed via flow cytometry, so only cells obtained from the gentle eye wash method were used to assess change in expression of surface receptors following exposure to inflammatory stimulus. Due to the consistent low number of cells collected with the patch-OSCCA collection, this method of collection was abandoned for the following 8 participants, and the study visits were changed, as shown in Figure 16.

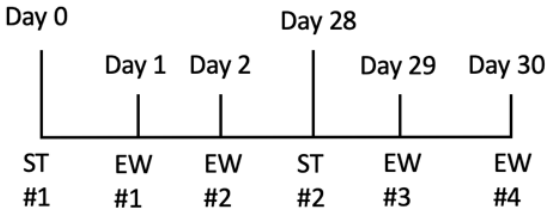

Figure 16 - Schematic diagram of the revised study visits. ST: screening test; EW: the gentle eye wash method.

The total number of cells collected using the gentle eye wash method among four different days, day 1, 2, 29 and 30, were compared. As illustrated in Figure 17, there was a significant difference between day 2 and 29 ($p = 0.03$), with the mean cell numbers of $3.1 \times 10^6 \pm 1.6 \times 10^6$ and $1.8 \times 10^6 \pm 1.4 \times 10^6$, respectively. The difference observed between the two days of collection is unlikely to be due to the lack of proficiency in collection procedures, because participants were instructed and practiced on day 0, and they did two collections before day 29. Furthermore, the volume of PBS they used to wash their eyes was recorded when they brought the samples, and for all cell collections, less than 1 mL of PBS was lost, indicating that participants performed the gentle eye wash method in an appropriate manner.

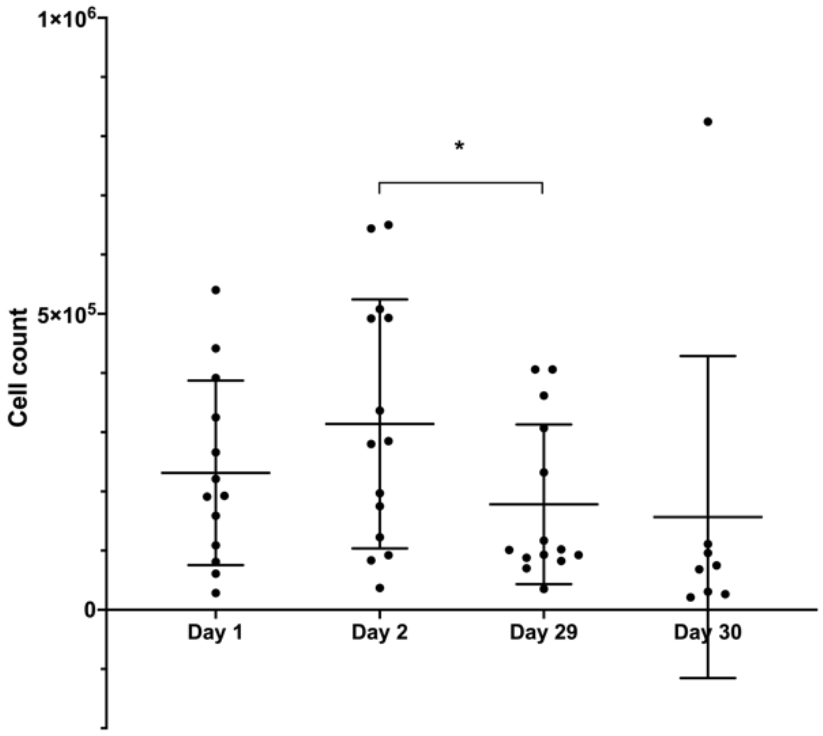

Figure 17 - Total cell numbers collected using the gentle eye wash method over a one-month period. Cell numbers were counted under the microscope with the hemocytometer and Trypan Blue. For each collection day, n=14, values are presented from 14 participants.

5.3.2. Tear-film and blood-isolated PMNs responses to IL-8 and PMA *in vitro*

After stimulation with IL-8, there was no change in surface receptor expression in tear-film PMNs as compared to unstimulated controls ($p = 0.12$), as shown in Figure 18a. While there were some changes in level of expression with PMA-stimulated samples, these did not reach statistical significance ($p \geq 0.1$) except for CD16 ($p = 0.003$), as illustrated in Figure 18b. This suggests that tear-film PMNs collected after sleep may be able to respond mildly to PMA but not to IL-8.

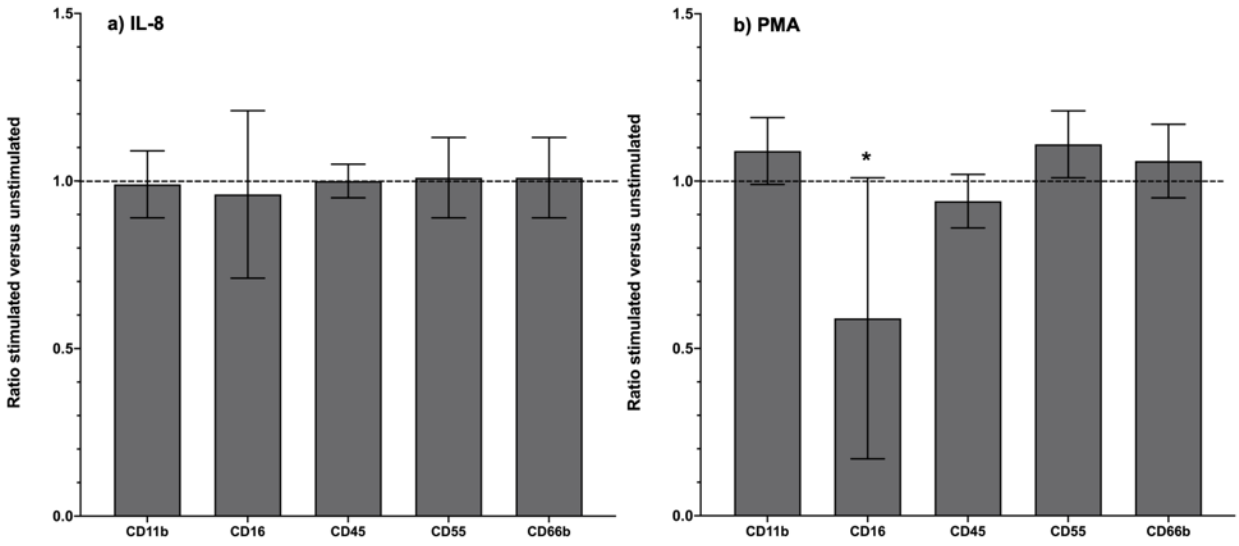

Figure 18 - The expression of surface receptors of tear-film PMNs after sleep following stimulation with a) IL-8 and b) PMA. Tear-film PMNs were stimulated with IL-8 (10 ng/mL) or PMA (2 μM), and expression of surface receptors of CD11b, CD16, CD45, CD55 and CD66b was assessed through flow cytometry. Data is expressed as the ratio between stimulated (IL-8 or PMA) and unstimulated samples. The dotted line represents the resting (unstimulated) value. Results are presented as means ± standard deviations from 13 participants (n = 21 for PMA and n = 30 for IL-8). * Significantly different from unstimulated controls ($p \leq 0.005$).

On the other hand, in blood-isolated PMNs, IL-8 induced a drastic increase in the expressions of CD11b, CD16 and CD45, while the levels of CD55 and CD66b remained relatively unchanged (Figure 19a). Following exposure to PMA, as expected, significant upregulation of CD11b and CD66b and downregulation of CD16 were observed, as shown in Figure 19b. The large deviations observed in CD11b and CD66b are due to high responders, a phenomenon often observed with PMA and these markers.

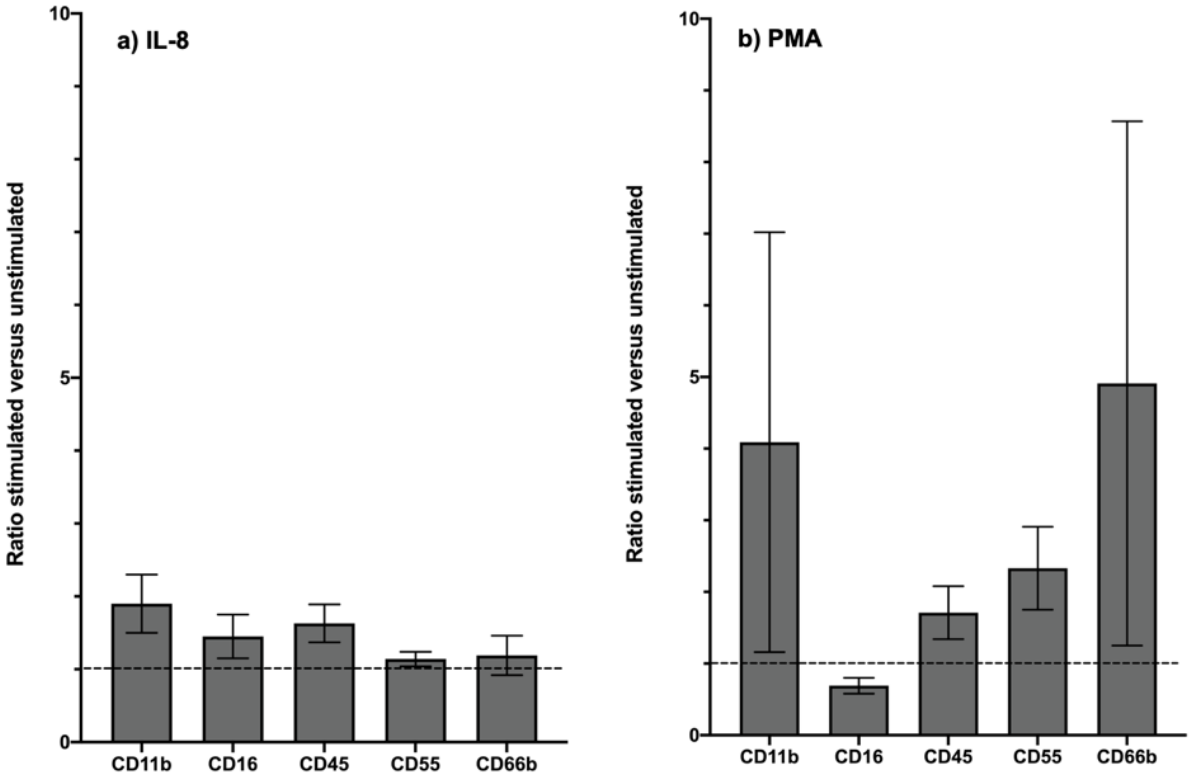

Figure 19 - The expression of surface receptors of blood-isolated PMNs following stimulation with a) IL-8 and b) PMA. Blood-isolated PMNs were stimulated with IL-8 (10 ng/mL) or PMA (2 μM), and expression of surface receptors of CD11b, CD16, CD45, CD55 and CD66b were assessed through flow cytometry. Data is expressed as the ratio between stimulated (IL-8 or PMA) and unstimulated samples. The dotted line represents the resting (unstimulated) value. Results are presented as means ± standard deviations from three participants (n = 3 for PMA and n = 4 for IL-8).

As shown in Figures 20 and 21, tear-film PMNs responded significantly differently from blood-isolated PMNs to stimulation with IL-8 and PMA. IL-8 induced upregulation of CD11b, CD16, CD45 and CD66b in blood-isolated PMNs was significantly higher than in tear-film PMNs ($p \leq 0.032$), while the upregulation in CD55 was not statistically significant ($p = 0.064$) (Figure 20). Similarly, PMA induced significant upregulation in CD11b, CD45, CD55 and CD66b in blood-isolated PMNs when compared to tear film PMNs ($p \leq 0.001$). PMA induced a similar downregulation in CD16 expression in both tear film and blood isolated PMNs. All in all, these results suggest that both IL-8 and PMA failed to activate

tear-film PMNs as compared to blood-isolated PMNs, further confirming previous results on the difference in phenotypes between blood and tear film PMNs.

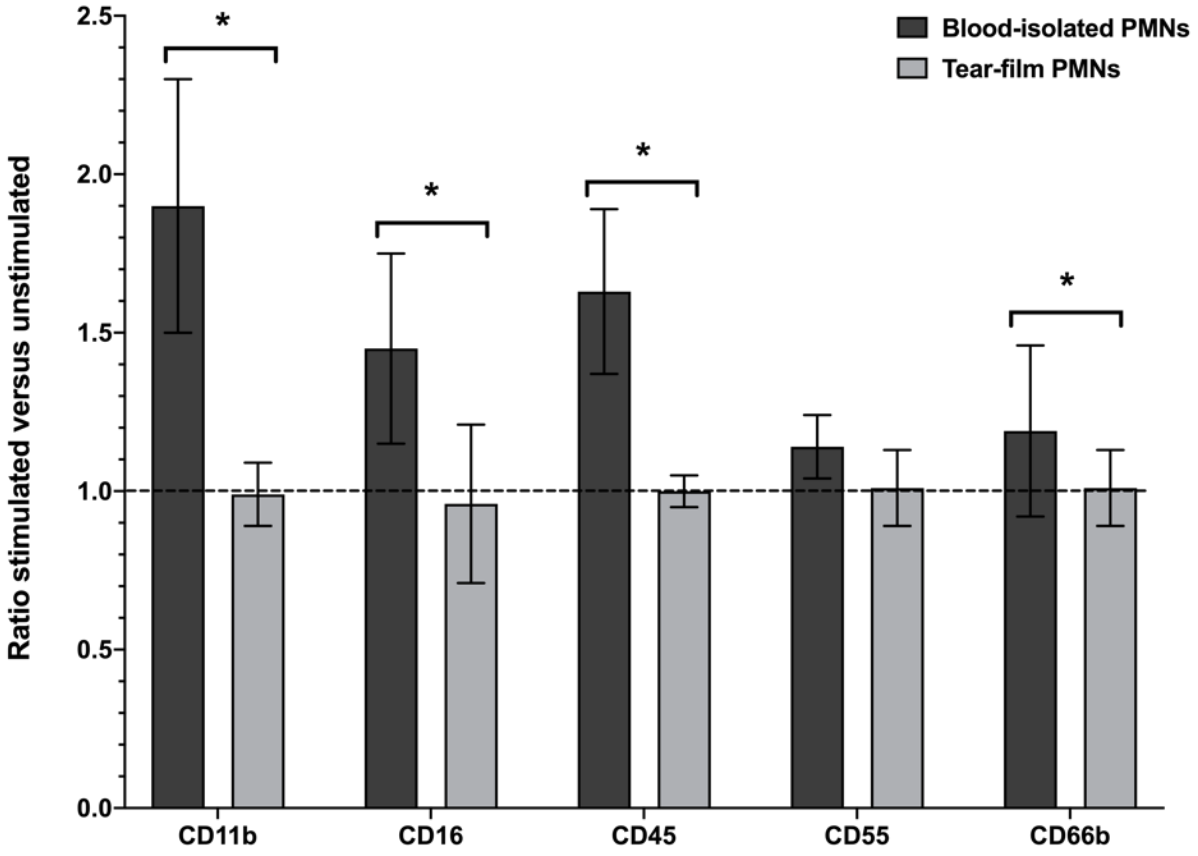

Figure 20 - The expressions of surface receptors of blood-isolated PMNs and tear-film PMNs following stimulation with IL-8. PMNs were stimulated with IL-8 (10 ng/mL), and their expression of surface receptor of CD11b, CD16, CD45, CD55 and CD66b was assessed through flow cytometry. Data is expressed as the ratio between stimulated (IL-8) and unstimulated samples. The dotted line represents the unstimulated value. Results are presented as means ± standard deviations from 13 participants collecting tear-film PMNs several times (n = 30) and 3 participants donating blood at least once (n=4). * Significantly different from blood-isolated PMNs ($p < 0.03$).

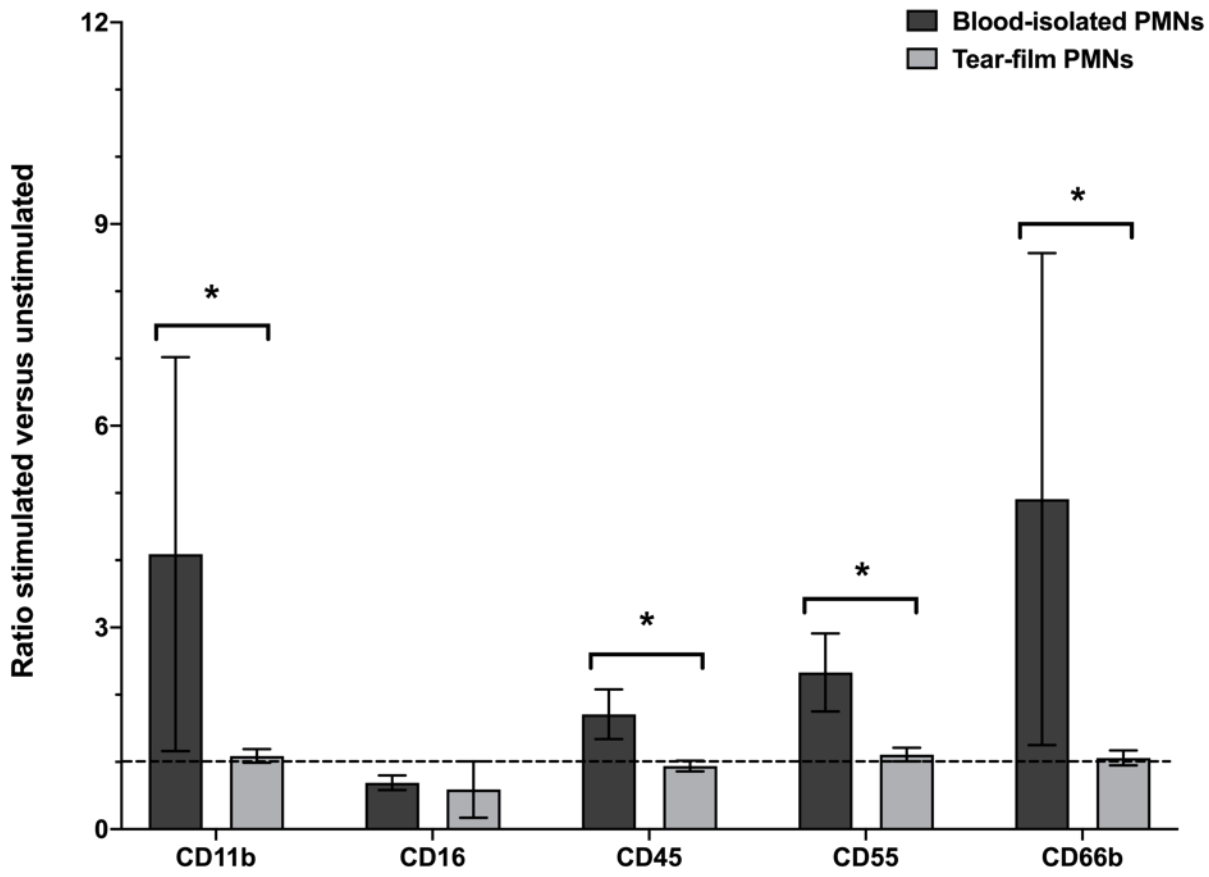

Figure 21 - The expression of surface receptors of blood-isolated PMNs and tear-film PMNs following stimulation with PMA. PMNs were stimulated with IL-8 (10 ng/mL), and their expression of surface receptor of CD11b, CD16, CD45, CD55 and CD66b was assessed through flow cytometry. Data is expressed as the ratio between stimulated (PMA) and unstimulated samples. The dotted line represents the unstimulated value. Results are presented as means ± standard deviations from 13 participants collecting tear-film PMNs several times (n = 21) and 3 participants donating blood for PMNs isolation (n = 3). * Significantly different from blood-isolated PMNs ($p \leq 0.001$).

To compare the phenotype of unstimulated (resting) tear-film and blood-isolated PMNs, the level of expression of the membrane receptors are presented as raw fluorescent values (also referred to as mean fluorescent intensities (MFI)) in Table 5. Note that all data was collected on the flow cytometer using the same voltage settings and thus any difference in fluorescence is due to a difference in level of expression on the cell membrane. As shown by the MFI values, the level of expression of CD11b, CD55 and CD66b on unstimulated

tear-film PMNs were significantly higher than that of blood-isolated PMNs, and CD16 expression was significantly lower in tear-film PMNs ($p < 0.001$). It is also important to highlight the high standard deviation observed in the expression of CD16 for tear film PMNs: with some participant's PMNs exhibiting the lowest fluorescent intensity of 3 while others have as high as 120 at the time of collection, indicating a significant range of CD16 expression across participants. We were unable to organize the participants into groups based on their CD16 fluorescent intensities of CD16, as the level of expression of CD16 changed between the various days of collection for the same participant.

Table 5 - Expression of selected membrane receptor on unstimulated tear-film and blood-isolated PMNs. Values are reported as mean fluorescent intensities (MFI).

CD markers	Tear-film PMNs	Blood-isolated PMNs
CD11b	358 ± 79*	48 ± 22
CD16	28 ± 31*	841 ± 198
CD45	71 ±14*	47 ± 17
CD55	154 ± 44*	55 ± 26
CD66b	297 ± 83*	35 ± 22

Results are presented as means ± SD from 13 participants collecting tear-film PMNs several times (n = 53) and 3 participants donating the blood-isolated PMNs (n = 6). * Significantly different from blood-isolated PMNs ($p < 0.001$).

5.3.3. Comparing the phenotype of tear-film PMNs between collection days for each participant

There was a two-week gap between repeated cell collections which divided the four cell collections into two parts: the first two took place on day 1 and 2, and the other two were performed on day 29 and 30. As there were not enough tear-film PMNs obtained on day 30 for most of the participants, only cells collected on day 1, 2 and 29 were used to compare their phenotypes to each other. As shown in Figure 22, there was no significant difference detected in the stimulation ratio for surface receptors of IL-8-stimulated tear-film PMNs among the three days ($p < 0.15$) except for CD55 where its expression on day 1 was significantly higher than that of day 29 ($p = 0.04$) with the mean ratio of 1.10 and 0.96, respectively. On the other hand, when tear film PMNs were exposed to PMA, only the expression of CD16 on day 2 was significantly lower than that on day 29 ($p = 0.01$) with a ratio of 0.39 and 0.55, respectively.

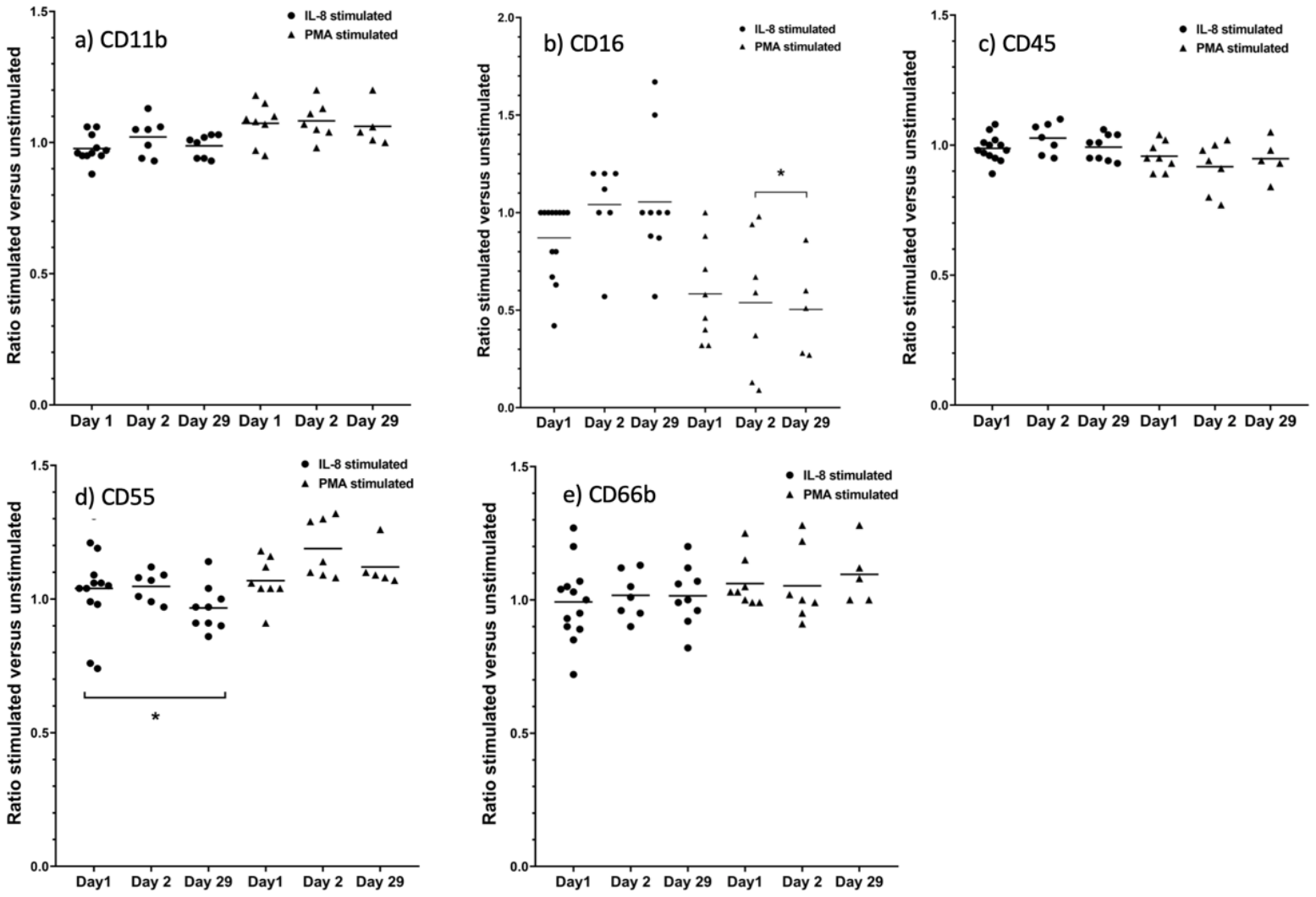

Figure 22 – Change in IL-8 and PMA stimulation of tear-film PMNs collected at the beginning and end of the month. a) CD11b; b) CD16; c) CD45; d) CD55; e) CD66b.

Figure 23 reports the level of expression of membrane receptors on unstimulated tear-film PMNs at day 1, 2 and 29. Except for CD55 and CD16, the level of expression of membrane receptors remained constant across the collection days. The expression of CD55 on day 1 was significantly higher than that on day 2, with a mean MFI of 176 and 143 (about 81% reduction on day2) ($p = 0.006$). Although the changes in level of expression of CD16 across the days did not reach statistical significance, as can be seen in Figure 11b, a spread in CD16 expression was observed not only across days but within single participant collection data, whereby on day 1, a MFI of 15 would be recorded and then 58 on day 2 or day 29.

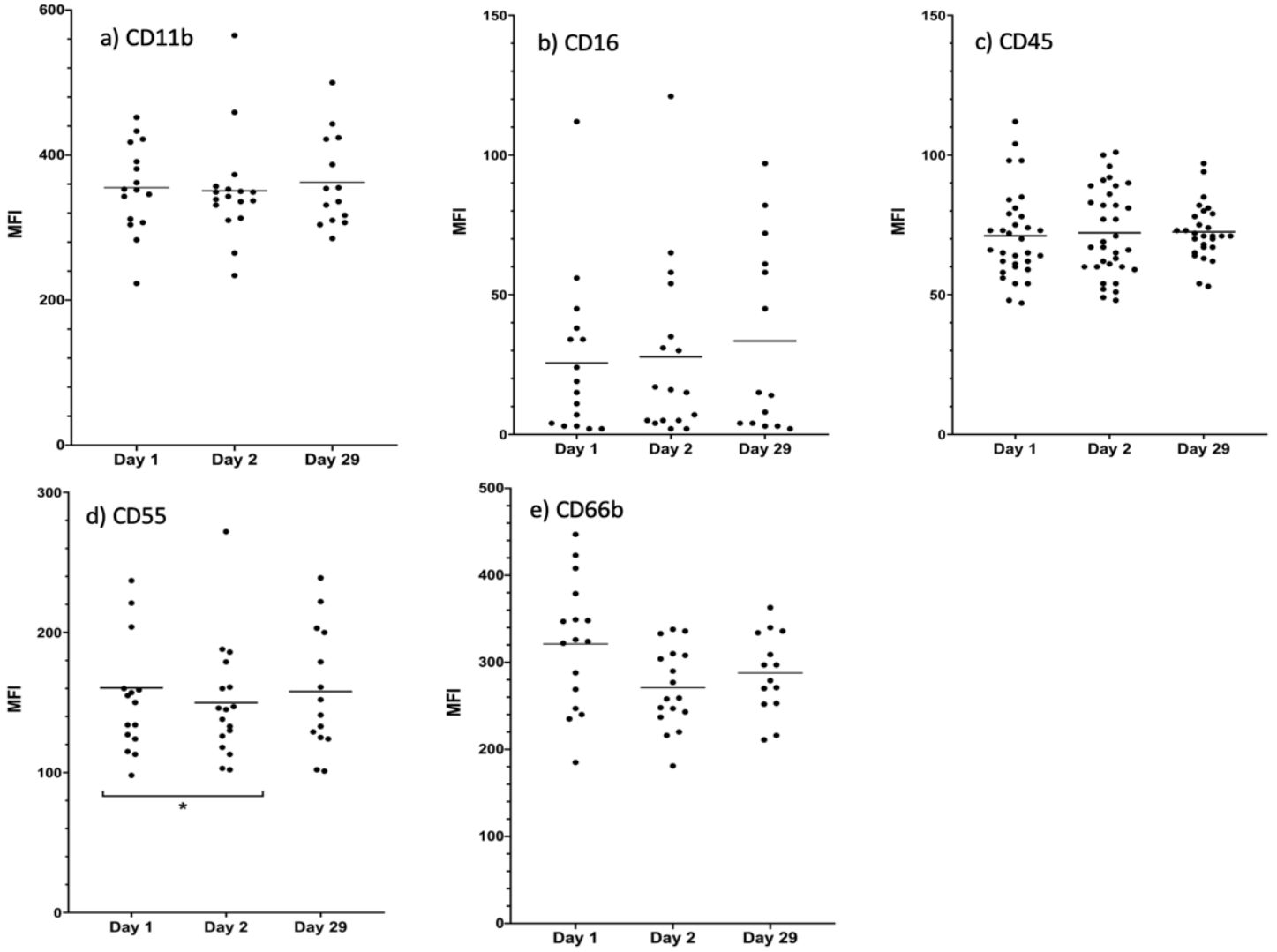

Figure 23 – Change in the level of unstimulated expression of membrane receptors of tear-film PMNs collected at the beginning and end of the month. A) CD11b; b) CD16; c) CD45; d) CD55; e) CD66b.

5.4. Discussion

An ideal non-invasive collection method should collect maximal cells without causing any artefactual changes. The gentle eye wash method was successfully designed to collect leukocytes from the ocular surface, and the total cells obtained have been reported to be significantly higher than the method using the non-contact corneal irrigation chamber[2,3]. However, the number of cells collected may be dependent on the participant's proficiency in collection procedures. Hence, a training session before the actual collection is usually recommended. In addition, large variations among expressions of surface receptors have been observed not only in our own results but also of others[2,5], which may be due to individual difference in the response or may also be due to the isolation or preparation procedures. The latter was addressed in the previous chapter. Thus, in this study, we aimed to determine which method could yield the most cells and also provide more reliable results. The gentle eye wash method collected 200 times more cells than the patch-OSCCA collection, indicating that the gentle eye wash method is the most effective method to collect tear-film PMNs at home so far. Unlike the gentle eye wash method that collects cells immediately upon awaking, the patch-OSCCA collection takes a much longer time, approximately 40-60min, as participants need to come to the lab to have the OSCCA cell collection procedure performed on their eye. Hence, during the time between home and the lab, participants will likely slightly open their eye unintentionally under the patch, resulting in significant cell loss.

While the gentle eye wash collected more cells, from our study, it appears that the number of cells collected with the gentle eye wash is not consistent on different days. Cell

counts were higher on day 2 compared to day 1. This may be because participants practiced their collection skill in day 1 and were thus more familiar on day 2. However, we cannot explain why there is a significant decrease in collection from day 2 to day 29 and a further decrease from day 29 to day 30. This reduction is unlikely due to the collection procedure, as by then the participants are familiar with the collection methods and the volume of cell suspension during cell collection remained consistent across all collection days. Weather (cold versus warm, etc) or other health related matters could also not account for the decrease/change observed. The possible factors contributing to poor collection may be more psychological conditions, whereby around the last collection days, the participants may be more careless in following the protocol of collecting immediately on awakening thinking that the study is coming to an end.

While differences were observed in the total number of cells collected among the various days for each participant, limited changes occurred in the level of expression of the membrane receptors on the collected cells, except for CD55 and CD16. In unstimulated tear-film PMNs, CD55 showed an 25% reduction on day 2 as compared to day 1. The different level of expression of CD55 between days may suggest that complement activation at the ocular surface may be highly variable. The changes observed in CD16 expression may be due to underlying closed-eye conditions, as a downregulation is correlated with elastase release. It remains unclear why CD16 and CD55 expression remains so variable, but the variability observed in our results with CD16 and CD55 expression would suggest that these membrane receptors may not be reliable indicator for future diagnostic and phenotyping purposes. Collectively, the intra difference (within a single subject) expression of CD11b,

CD45 and CD66b was relatively small, suggesting that these markers can be used more reliably and that only one cell collection would be necessary to identify cell phenotype.

IL-8 acts as a potent chemoattractant for PMNs and is able to induce various functions, such as regulating the transmigration from the vessels to the infected sites, respiratory burst, exocytosis and phagocytosis[18]. IL-8 has been shown to cause an increase in the expression of Mac-1 (CD11b/CD18) to promote adhesion of PMNs[14,15]. In our study, as expected, IL-8 induced cell activation in blood-isolated PMNs, as identified by CD11b upregulation. However, no change was observed in tear-film PMNs upon stimulation with IL-8. Almost 95% of Mac-1 is stored in cytoplasmic granules and can be rapidly mobilized to the PMNs cell surface during/after activation[1,19]. Hence, the impaired upregulation of CD11b of tear-film PMNs may be caused by the shortage of cytoplasmic stores, as tear-film PMNs already show a high level of CD11b expression (see Table 5). Our results suggest that tear-film PMNs may already be in an activated state and unable to respond further.

Both CD55 and CD66b levels on blood-isolated and tear-film PMNs remained similar to unstimulated samples, suggesting that IL-8 stimulation may have no impact on their expression. Research has shown that CD66b is less sensitive to cytokines compared to CD11b and CD62L[20]. In addition, the upregulation of CD55, which is known as decay-accelerating factor (DAF), is dose-dependent[19]; no increase in CD55 expression was observed with an IL-8 concentration of 4 ng/mL, whereas a significant upregulation of CD55 was detected with 400 ng/mL[19]. Hence, the lack of upregulation in CD66b and CD55 in this study may be due to the insensitivity to IL-8 and the low concentration of IL-8 used (10ng/ml), respectively.

Gorbet *et al.* showed that tear-film PMNs were unable to respond to fMLP[2]. The fMLP and IL-8 receptors (IL-8Rs) are members of the seven-transmembrane spanning receptor (STMR) coupled to a G protein[21], so IL-8 and fMLP may have some similarities in the signal transduction pathways[22]. Wu *et al.* demonstrated that the binding of IL-8 to G-coupled protein receptor leads to the dissociation of the subunits, which activates the phospholipase C (PLC) and eventually increases intracellular calcium ions[22]. The involvement of PLC, ras/raf, mitogen activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K) contribute to the IL-8 stimulated pathways[21], which are also involved in the fMLP-stimulated pathway[23]. Since their signal transduction pathways involve similar components, the inability for tear-film PMNs to respond to IL-8 may occur because of the impairment of compartments within the pathways shared with fMLP or/and the receptors.

Another possible factor contributing to the inability of tear-film PMNs to upregulate their surface receptors upon IL-8 stimulation may also be due to a lack of functionality in G-protein coupled-receptors.

Apart from the signal transduction pathway, downregulation of IL-8Rs may also cause the lack of response to IL-8 of tear-film PMNs[24]. Pignatti *et al.* showed that the numbers of IL-8Rs expressed on PMNs isolated from sputum (a "fluid" induced from the central airways[25]) collected from non-severe asthma patients were lower than on blood PMNs from healthy people[24]. They suggested that one of the possible causes was that exposure to IL-8 was inducing the internalization of IL-8Rs[24]. Studies have already demonstrated that the concentration of IL-8 increases during sleep[6,26]. Hence, the inability to elicit an

activation response from tear film PMNs may be due to the internalization of IL-8Rs when exposed to IL-8 in the closed-eye environment. More work is required to confirm such hypothesis as the expression of IL-8Rs on tear-film PMNs has not been investigated so far. Furthermore, as discussed above, the activation mechanisms for PMNs are complex, so further experiments will be required to understand the underlying mechanisms to fully explain the lack of response upon IL-8 stimulation.

Upon PMA stimulation, tear-film PMNs showed small magnitudes of changes in the expression of surface receptors in comparison to blood-isolated PMNs, except for CD16. The downregulation of CD16 upon activation has been shown to be associated with the cleavage by proteases, specifically neutrophil elastase[5,27]. Both tear-film and blood-isolated PMNs showed significant downregulation upon PMA-stimulation suggesting that release of primary granules containing elastase which can shed off the CD16 could occur in tear film PMNs upon PMA stimulation. It is important to note that the large variations observed in the ratio of CD16, especially in PMA-stimulated tear-film PMNs, was due to the fact that in some participants the unstimulated samples already had minimal CD16 expression and thus a further decrease below background was not possible. However, all in all, our results showed that CD16 was downregulated on tear-film PMNs stimulated by PMA, suggesting that some degranulation occurred in these cells upon exposure to PMA.

CD55 is present in secretory vesicles[19], which is one of the four storage granules in PMNs and has been shown to be quickly mobilized to the cell membranes after stimulation[28]. Therefore, the upregulation of CD55 is due to the release of secretory vesicles, which can be seen in both tear-film and blood-isolated PMNs after exposure to PMA. However, the

magnitude of CD55 upregulation is much lower in tear-film PMNs, suggesting that most of the secretory vesicles may have already been released by activated tear-film PMNs in the closed-eye environment[5]. PMA mimics the action of diacylglycerol (DAG) and directly binds and activates the protein kinase C (PKC). It has been shown that CD18 is phosphorylated during PKC activation[29]. Therefore, the mechanism of PMA upregulation of Mac-1 (CD11b/CD18) may occur via the activation of PKC. The lesser upregulation of CD55 with tear film PMNs correlates well with the lower magnitude of Mac-1 upregulation in tear-film PMNs as this can likely be explained by prior granules and secretory vesicle release in the closed eye environment.

As shown in Table 5, the level of expression of CD11b, CD55, and CD66b in resting tear-film PMNs were more than doubled that of unstimulated blood-isolated PMNs, while for CD16, it was approximately 20 times lower. All these markers are cell activation markers and an up or down regulation is indicative of an activated phenotype, and thus these results further contribute to the hypothesis that tear-film PMNs may have already been activated in the closed-eye environment. PMNs found in the mouth[30], placenta[31], and the lungs[32] show a similar pattern of expression of surface receptors.

5.5. Conclusion

In conclusion, the patch-OSCCA collection is not effective in collecting cells in comparison with the gentle eye wash method. While a high number of cells were collected with the gentle eye wash method, significant changes in the total number of cells collected were observed among the various days of cell collection for each participant. However, the

level of expression of cell activation membrane receptors on the collected cells remains relatively unchanged for each participant, except for CD16 and CD55 (whose expression is associated with the release of granules and vesicles). While variations in CD16 and CD55 expression might be an indication of what may have happened in the ocular environment during sleep, the significant changes that occur within a participant and across the population may make it difficult to use CD16 and CD55 for diagnostic purposes. Our results, however, suggest that one cell collection from a participant provides a reliable means of assessing the tear film PMNs phenotype with another marker of cell activation.

Our study also demonstrated that tear film PMNs collected from the closed eye environment were unable to up or down regulate cell activation membrane receptors upon IL-8 stimulation, suggesting an impairment that may be explained by prior exposure to IL-8 and activation in the closed-eye environment. However, the small response to PMA-stimulation was also observed suggesting that other potential mechanisms may be involved to lead to a quiescent state in tear film PMNs. Further investigations will be required to gain a better understanding on the phenotype and state of activation of tear film PMNs.

Chapter 6

Characterization of Respiratory Burst of Tear-film Neutrophils by Luminol-Enhanced Chemiluminescence

6.1. Introduction

Neutrophils (PMNs) comprise approximately half of the total circulating white blood cells, which are the hallmark of multi-lobed nuclei and cytoplasmic granules[1]. PMNs are one of the population of innate immune cells playing an essential role in protecting the host tissues against foreign particles, evoking multiple functional responses upon activation by stimuli. This includes the generation of reactive oxygen species (ROS), the release of antimicrobial substances from granules, and phagocytosis. PMNs, like other immune cells, are heterogeneous, meaning they exhibit distinct phenotypes in different tissues under normal physiological conditions. The eye represents a unique physiological environment due to its immune privilege[2], thus recruitment of immune cells to the ocular surface is restricted. PMNs isolated from the ocular surface exhibit an interesting nocturnal-diurnal pattern, as while few PMNs can be found on the ocular surface during the day in healthy eyes, hundreds of thousands of PMNs can be detected after eye closure for a prolonged time[3]. The underlying regulatory mechanism leading to this pattern has not yet been elucidated, although chemotactic molecules (IL-8 and complement products) are believed to play a role[4]. Two research groups have investigated the state of activation of tear film PMNs upon collection from the eyes after sleep by evaluating the expression of cell activation membrane receptors[3,5]. Unlike blood PMNs, tear-film PMNs expressed high levels of CD66b (a

degranulation marker) and CD11b (a cell adhesion and activation marker) but low levels of CD62L (L-selectin)[3,5], suggesting that they probably have already been activated on the ocular surface upon arrival through the extravasation or due to their prolonged presence in the closed eye environment[5]. A recent study has suggested that tear-film PMNs may have an impaired capability of phagocytosis[6] and are also unable to upregulate or downregulate cell activation membrane receptors in response to various inflammatory stimuli[3,5].

Respiratory burst, as one of their essential killing mechanisms, has been extensively investigated in blood PMNs. ROS generated during the respiratory burst can not only eliminate pathogens but can also cause severe damage to surrounding tissue if over-produced. An increase in oxygen consumption by PMNs has been detected during phagocytosis, as the activated nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) reduces the molecular oxygen to the superoxide anion (O_2^-)[1,7]. PMNs isolated from patients suffering from chronic granulomatous disease (CGD) can engulf microorganisms but cannot generate ROS, resulting in a failure in killing invading microorganism[8]. This suggests that the respiratory burst is crucial for PMNs to perform their functions. NADPH oxidases can be found in both PMNs plasma membrane, which contribute to the extracellular superoxide anions, and granule membranes, that release superoxide anions in an intracellular manner[9]. The superoxide anion is spontaneously converted to hydrogen peroxide and reacts with a chloride ion to form hypochlorous acid, a potent antimicrobial agent, under the catalyzation of myeloperoxidase[1]. The most efficient stimuli used to trigger the respiratory burst in PMNs are opsonized microorganisms, complement fragment C5a, N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP),

platelets activating factor (PAF), and phorbol-12-myristate-13-acetate (PMA)[1]. The interaction between these stimuli and their corresponding receptors activates a series of intracellular signalling pathways to induce different PMNs functional responses (except PMA, which acts directly on protein kinase C (PKC)).

Detection of ROS can be achieved by multiple methods, such as fluorescent and chemiluminescent probes, spectrophotometry, and chromatography[10]. Among these methods, luminol-enhanced chemiluminescence is easy to operate and also provides a kinetic measurement of ROS formation. Although the superoxide anion is able to spontaneously dismutate to hydrogen peroxide, hydrogen peroxide is relatively stable and cannot react with luminol to emit light[11]. Hence, this assay is efficient to detect superoxide anions from both intra- and extracellular compartments, as luminol is cell membrane permeable[10,12].

Although tear-film PMNs are shown to be non-inflammatory upon stimulation, it is worthwhile to investigate their ability to generate ROS and to compare this process with blood-isolated PMNs to determine if the two subsets of PMNs show similar patterns of the respiratory burst. Also, while Gorbet's research group has previously assessed the level of ROS produced by tear-film PMNs through flow cytometry, it can only provide endpoint values and cannot assess extra-cellular ROS generation. In this study, luminol-enhanced chemiluminescence was performed to characterize the kinetics of ROS production in tear-film PMNs. The stimuli selected to activate tear-film PMNs were fMLP, a chemotactic peptide secreted by bacteria, PMA, a chemical stimulus that can directly activate PKC, and lipopolysaccharides (LPS), fragments from the outer membrane of gram-negative bacteria. We hypothesized that tear-film PMNs generation of ROS in response to the selected stimuli

would be lower than that of blood-isolated PMNs.

6.2. Materials and Methods

6.2.1. Materials

Endotoxin-free reagents, medium and sterile tubes were used in this experiment to avoid undesired activation of PMNs. LPS (Escherichia coli serotype 0111:B4), PMA, fMLP, Hanks's balanced salt solution (HBSS) and luminol (5-amino-2,3-dihydroxy-1,4-phthalazinedione) were ordered from Sigma-Aldrich Co. (Oakville, Ontario, Canada). Phosphate-buffered saline (PBS) was purchased from Lonza (Allendale, New Jersey, USA). Dulbecco's modified eagle media (DMEM) was purchased from Life Technologies (Burlington, Ontario, Canada).

6.2.2. Blood-isolated PMNs isolation:

This study received ethics clearance from the University of Waterloo Human Research Ethics Committee. PMNs were isolated from fresh blood drawn from four healthy and asymptomatic participants, who did not take any anti-inflammatory medication for at least 72 hours. Around 10 ml of blood was collected from each participant and immediately added to a tube containing 5U/ml of heparin. Blood was then centrifuged at 100xg for 10 min, and platelet-rich plasma was removed. DMEM/10% FBS was added to dilute the blood in a ratio of 1:1 and 5 mM EDTA was added. Diluted blood was then transferred to the tube containing PolymorphPrep (Axis Shield PoC AS, Oslo, Norway) and Histopaque (Oakville, Ontario, Canada), resulting in a 1:1 ratio of diluted blood and the two density gradient media,

followed by centrifugation at 930xg for 25 min. PMNs (located in the bottom band) were carefully transferred into a new 15 mL polypropylene tube followed by three washes (at 280xg for 10 min) with the first two washes in DMEM/10% FBS with 5 mM EDTA and the last one in PBS. PMNs were resuspended in PBS, and cell count was performed.

6.2.3. Tear-film PMNs collection

Tear-film PMNs were collected after sleep from nine non-lens wearers without any ocular disease or complications, as described in a previous study[3]. Briefly, participants were trained to collect their cells via the gentle eye wash method, using sterile PBS to wash the ocular surface, and the run-off was collected in a sterile polypropylene tube. Samples were brought to the lab and the cell suspension was centrifuged at 290xg for 10 min, followed by the resuspension in PBS. Cell count and cell viability were assessed using the hemocytometer with Trypan Blue.

6.2.4. Luminol-enhanced chemiluminescence

Chemiluminescence (CL) measurements were evaluated on the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) using 96-Well Cell Culture White Microplates (Greiner bio-one, Frickenhausen, Germany). Each well was set to test one stimulus, and each had a total reaction volume of 200 μL containing 10^5 PMNs, 1 mM luminol, its corresponding stimulus (2 μM PMA, 1.5 μM fMLP and 2 μg/mL LPS) [3] and hanks's balanced salt solution (HBSS; components are listed in Table 6) which was added to 200µL. The controls were performed in each assay and contained the same concentration of reagents but no stimulus (ie HBSS was added to complete volume to 2200µL). To obtain kinetic

curves, CL emissions were recorded at 37 ºC for 120 minutes at 3-minute intervals (as show in Figure 24).

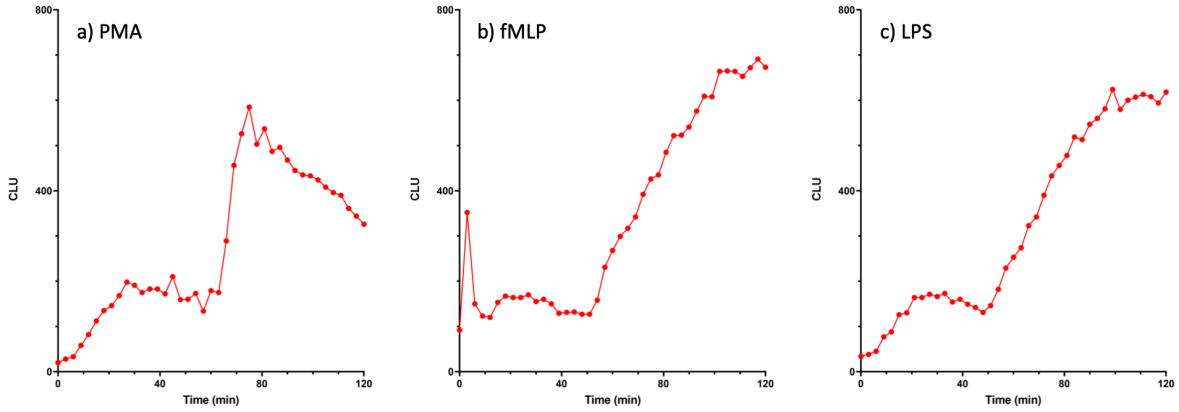

Figure 24 – Examples of chemiluminescence kinetic response recorded with tear film PMNs when exposed to a) PMA, b) fMLP and c) LPS. Values are recorded as CLU, chemiluminescent unit.

Table 6 – Components of HBSS [13].

Components	g/L
Calcium Chloride (anhydrous)	0.1396
Magnesium Sulfate (anhydrous)	0.09767
Potassium Chloride	0.4
Potassium Phosphate Monobasic (anhydrous)	0.06
Sodium Chloride	8.0
Sodium Phosphate Dibasic (anhydrous)	0.04788
D-Glucose	1.0

6.2.5. Statistics

After careful analysis of the CL kinetic graphs, the CL response at different time points was selected for statistical analysis, 0, 3, 6, 12, 24, 48, 60 min for fMLP, 0, 9, 27, 54,

60 min for PMA, and 0, 12, 21, 33, 45, 60 min for LPS. Changes in CL was expressed as the ratio of stimulated CL emissions to control CL emissions ($CL_{stimulated}$/ $CL_{control}$), which were then plotted over time. Results are reported as means ± standard deviations, except when the representative CL curves are presented. Kinetic curves and area under the curve (AUC) were computed by GraphPad Prism Software (GraphPad Software, San Diego, CA, USA). In order to assess the statistical significance, an independent t-test or Mann-Whitney test was performed using IBM SPSS Software (IBM Canada Ltd., Markham, Ontario, CA), and a p-value less than 0.05 was required for statistical difference.

6.3. Results

6.3.1. ROS production in fMLP-stimulated tear-film and blood-isolated PMNs

Respiratory burst was initiated by the addition of fMLP, and the generation of ROS was measured by chemiluminescence emissions. Changes in kinetics in ROS production of fMLP-stimulated tear-film and blood-isolated PMNs over 60 min are reported in Figure 25. Except at 0, 3 and 6 min, ROS production, as identified by emitted CL, by fMLP-stimulated tear-film PMNs, was close to background level, at 12-, 24-, 48- and 60-minute time points and was significantly lower than blood-isolated PMNs ($p \leq 0.007$). Tear-film PMNs ROS production showed a transient and sharp increase around the 3-minute time point, which was higher than blood-isolated PMNs but did not reach statistical significance ($p = 0.07$). Blood-isolated PMNs CL exhibited two peaks at 3 and 24 minutes, whereas tear-film PMNs only showed one peak at 3 minute and then continuously declined to the baseline level. This high generation of ROS within 3 minute after exposure to fMLP has been previously observed by

Zhu *et al*[11].

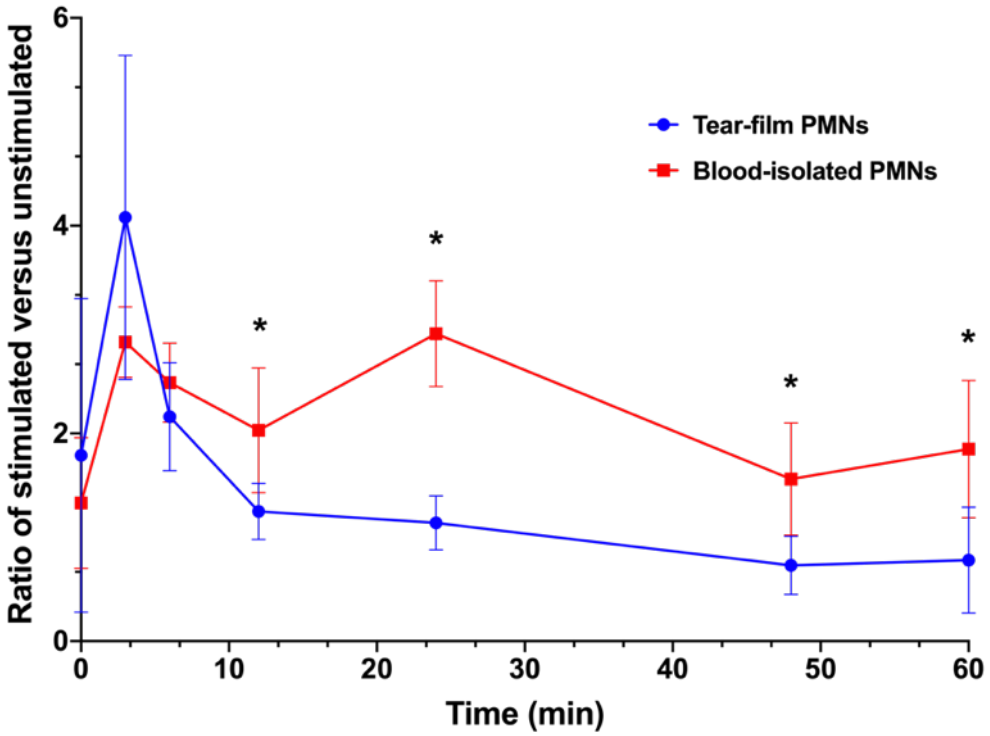

Figure 25 - Changes in chemiluminescence of tear-film and blood-isolated PMNs when exposed to fMLP. Results are reported as ratio of fMLP-stimulated CL emissions over control (unstimulated) sample CL emissions. Neutrophils (10^5 cells/well) were transferred to the well containing 1 mM luminol, 1.5 $\boldsymbol{\mu}$M fMLP and HBSS. Generation of ROS was kinetically measured using the Cytation 5 at 37°C, as described under Materials and Methods. Values are presented as means $\pm$ standard deviations from nine healthy participants collecting tear-film PMNs several times (n = 10) and four participants donating their blood for isolation of PMNs (n = 4). * Significantly different from unstimulated controls ($p \leq \mathbf{0.007}$).

6.3.2. ROS production of LPS-stimulated tear-film and blood-isolated PMNs

The response to LPS was also investigated and is reported in Figure 26. LPS could not induce a respiratory burst in tear-film PMNs, as shown by the CL ratio being close to 1,

indicating no difference in CL emissions between the stimulated and unstimulated samples. On the other hand, blood-isolated PMNs started to continuously produce ROS at 12 min. Starting at around 30 min of stimulation, production of ROS was significantly different between blood-isolated and tear-film PMNs ($p \leq 0.02$)

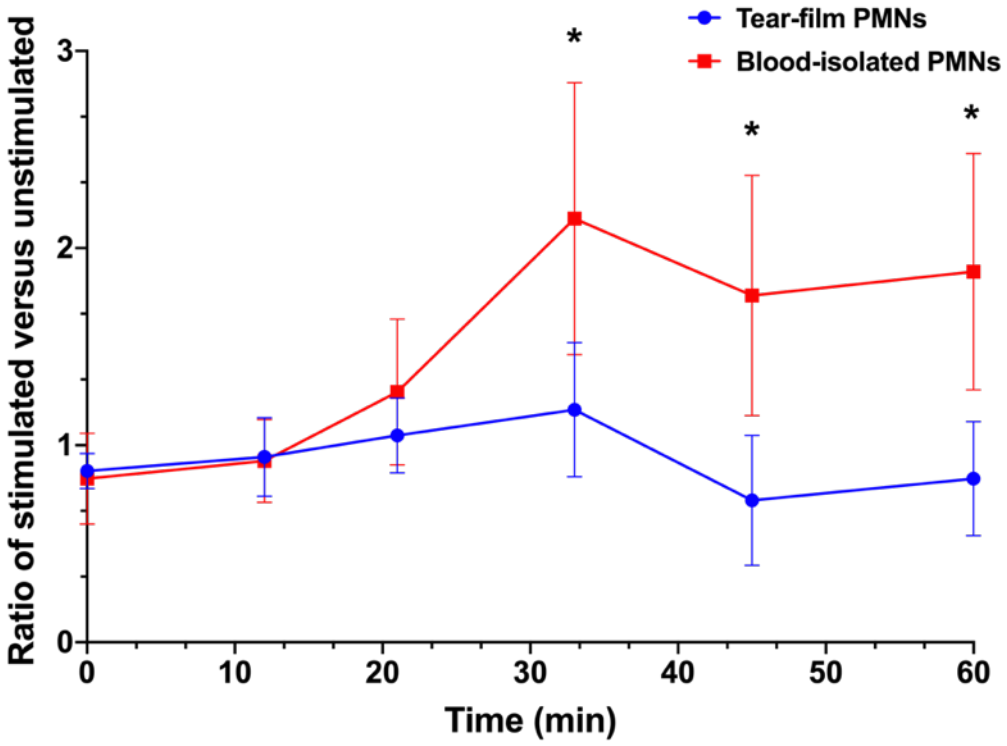

Figure 26 - Changes in chemiluminescence of tear-film and blood-isolated PMNs when exposed to LPS. Results are reported as ratio of LPS-stimulated CL emissions over control (unstimulated) CL emissions. Neutrophils (10^5 cells/well) were transferred to the well containing 1 mM luminol, 2 µg/mL of LPS and HBSS. Generation of ROS was kinetically measured using the Cytation 5 at 37°C, as described under Materials and Methods. Values are presented as means ± standard deviations. n = 6, tear film PMNs from different participants and n=4 for blood isolated PMNs from different blood donors * Significantly different from unstimulated controls ($p \leq 0.02$).

6.3.3. Production of ROS in PMA-stimulated tear-film PMNs and blood-isolated PMNs

To determine the responses of tear-film PMNs to other stimuli, PMA was selected, as a synthetic PKC activator that can directly bind to and activate PKC [1], whereas fMLP and LPS are receptor-dependent stimuli. Upon exposure to PMA, both tear-film and blood-isolated PMNs CL showed a peak of ROS generation and exhibited similar trend although the magnitudes were different, as shown in Figure 27. Blood-isolated PMNs CL had a sharp increase in ROS production at 9 min, and then CL continuously declined. On the other hand, tear-film PMNs CL reached their highest production level at 27 min, indicating a much slower ability to generate ROS upon PMA stimulation when compared to blood-isolated PMNs. Similar to blood-isolated PMNs, CL values gradually decreased afterwards. There was no significant difference between ROS production of tear-film and blood-isolated PMNs at all time points ($p \geq 0.22$) except at 0 min, which is further discussed in section *6.3.5*. Comparing the PMA-stimulated CL to fMLP- and LPS-stimulated CL, both tear-film and blood-isolated PMNs showed a higher and prolonged production of ROS.

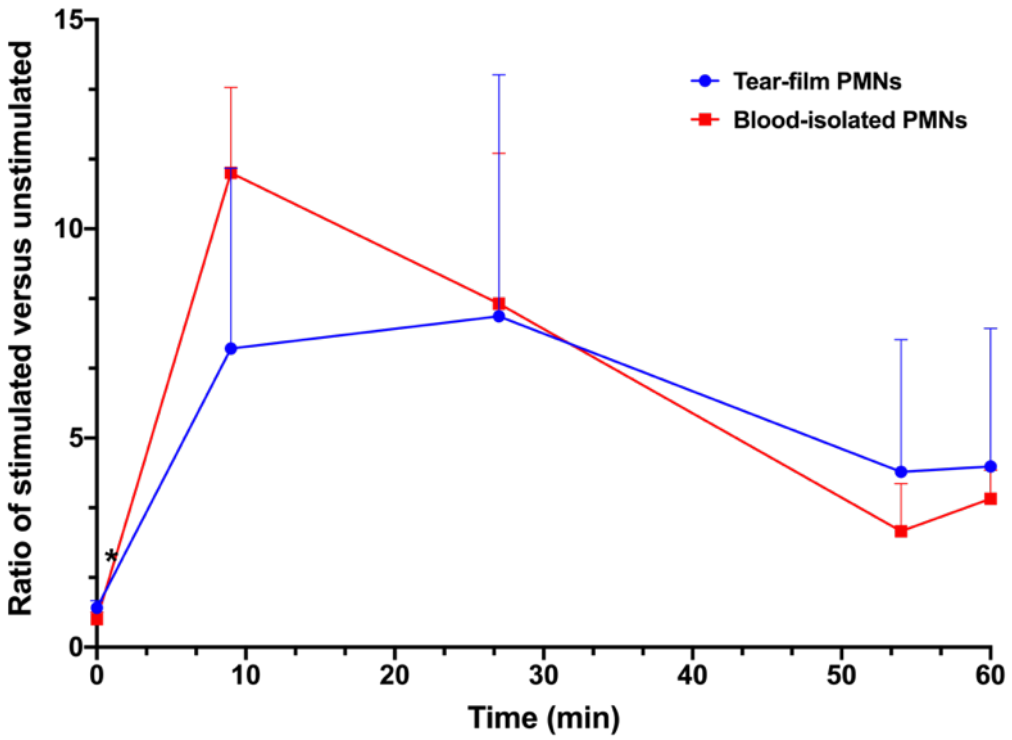

Figure 27 – Changes in chemiluminescence of tear-film and blood-isolated PMNs when exposed to PMA. Results are reported as ratio of PMA-stimulated CL emissions over control CL emissions. Neutrophils (10^5 cells/well) were transferred to the well containing 1 mM luminol, 2 µM PMA and HBSS. Generation of ROS was kinetically measured using the Cytation 5 at 37°C, as described under Materials and Methods. Values are presented as means ± standard deviations from eight healthy participants collecting tear-film PMNs several times (n = 10) and three participants donating their blood for isolation of PMNs (n = 3). * Significantly different from unstimulated controls ($p = 0.02$).

As seen in Figure 27, large standard deviations in ROS production of tear-film PMNs were present due to differences in individual responses. Two distinct groups of response could be observed, with some participants presenting greater changes in CL than others: two groups could be identified based on the magnitude of the ratio. Participants who had a ratio above 10 were classified as high responders (suggesting a high potential in producing ROS) while the others, with a ratio below 10, were classified as low responders. Individual results

are summarized in Table 7. An independent t-test confirmed that the means between the two groups of participants were significantly different ($p < 0.001$), further emphasizing how distinct these two groups were. It is interesting to note that the HR group was composed of female participants in their early twenties while the LR group consisted of both female and male in their late twenties, suggesting tear-film PMNs isolated from younger female participants may tend to be hyperactive. However, given our small sample size (n=8), this needs to be further examined.

As shown in Figure 28, once groupings were used, standard deviations evidently decreased, but most importantly the two distinct responses (LR and HR) in participants could be clearly observed.

Table 7 - Summary table of ROS generation potentials for high responders and low responders.

Participants Arbitrary ID#	Age	Sex	Highest ratio of $CL_{stimulated}/CL_{control}$
High responders *			
#1	25	F	17.12
#2	24	F	16.19
#3	24	F	19.68
#4	21	F	14.86
Mean			16.97
SEM			2.04
Low responders			
#5	29	F	5.79
#6	22	M	5.91
#7	32	F	4.38
#8	23	M	6.76
Mean			5.71
SEM			2.69

* significantly different from low responders, $p < 0.001$

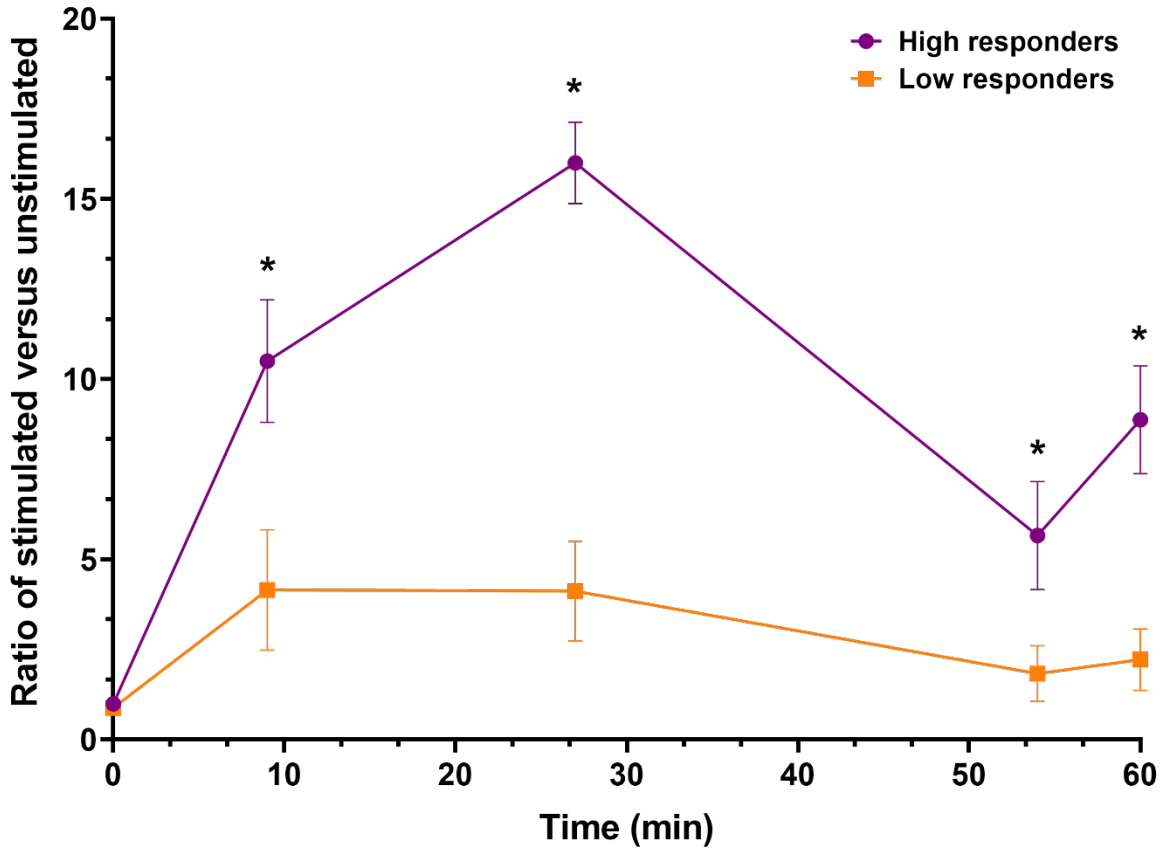

Figure 28 - Changes in CL of PMA-stimulated tear-film PMNs of high responders (HR) and low responders (LR). Mean and standard deviation were computed for each grouping at each time point (t = 0, 9, 27, 54, and 60 min) (n = 10, from 4 different participants in each group). * Significantly different from LR ($p \leq 0.02$).

6.3.4. Comparison of area under the curve and endpoints between tear-film and blood-isolated PMNs

The above kinetic CL curves (Figures 2 to 5) showed the real-time ROS production by tear-film and blood-isolated PMNs over 60 minutes. From such curves, the area under the curve (AUC) can be computed to reflect the quantity of ROS released from tear-film and blood-isolated PMNs after exposure to different stimuli, as shown in Figure 29a. The overall

ROS generation for blood-isolated PMNs were higher than that of tear-film PMNs; with AUCs for fMLP-stimulated ($p = 0.003$) and LPS-stimulated ($p = 0.031$) blood-isolated PMNs CL being significantly higher than tear-film PMNs CL. However, AUCs of PMA-stimulated tear-film and blood-isolated PMNs were not significantly different ($p = 0.184$). Since two responder groups were observed with PMA-stimulation, we also compared the AUC between the blood-isolated and tear-film PMNs based on the HR and LR groupings. Interestingly, there was no significant difference in the mean AUC between HR of blood-isolated and tear-film PMNs ($p = 0.17$), whereas the mean AUC between LR of blood-isolated and tear-film PMNs, 147 and 271, respectively, were significantly different ($p = 0.008$).

The chemiluminescence assay was run for 120 minutes; the CL values at the endpoints were recorded and computed as ratio as shown in Figure 29b. Changes in tear-film PMNs CLs stimulated by fMLP, LPS and PMA were significantly lower than in blood-isolated PMNs CLs at 120 minutes ($p \leq 0.03$).

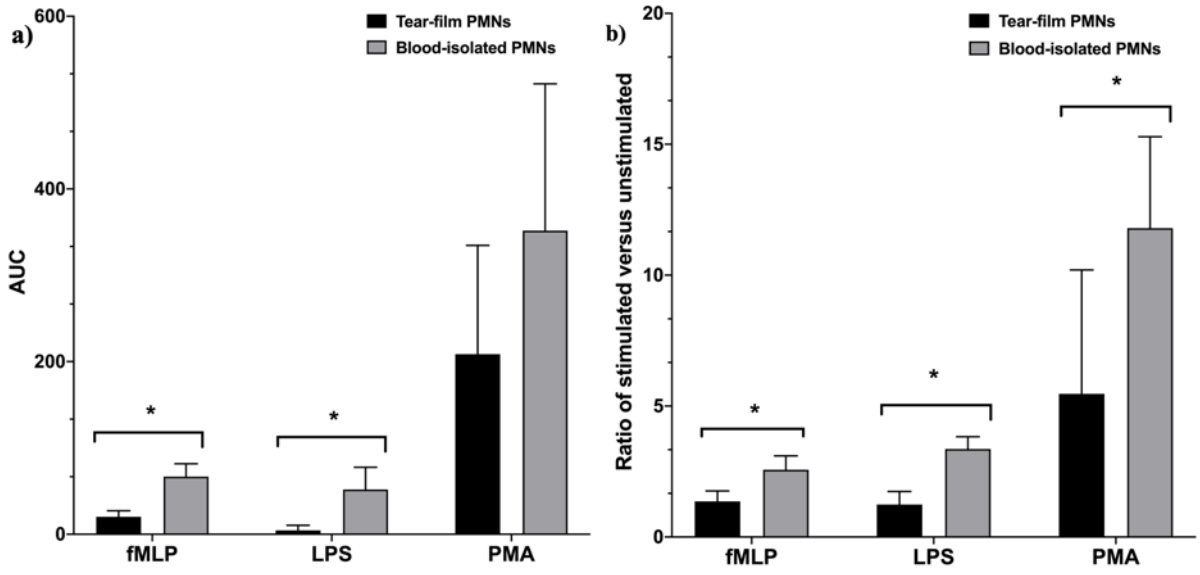

Figure 29 – Comparison of area under the curve (AUC) and the ratio of changes at the endpoint (t = 120 min) between tear-film and blood-isolated PMNs for all three stimuli. A) AUC computed by GraphPad Prism Software; B) changes in CL at 120 min. Values are presented as means ± standard deviations. * Significantly different from blood-isolated PMNs ($p < 0.05$).

6.3.5. CL values at time zero in unstimulated samples.

To determine if tear film PMNs collected from the closed eye environment may be in an activated state, the level of spontaneous ROS production in the unstimulated samples was compared to that of blood-isolated PMNs at early time points (0 and 3 minutes). As shown in Table 8, CL values of unstimulated tear film PMNs was significantly higher compared to blood-isolated PMNs, suggesting that tear film PMNs collected after sleep were in a state of activation and had been exposed to a pro-inflammatory environment.

Table 8 - ROS production in unstimulated tear film and blood-isolated PMNs

Time point	Tear film PMNs (CLU)	Blood-isolated PMNs (CLU)
0 min	38 ± 12*	20 ± 8
3 min	46 ± 20*	15 ± 6

CLU: chemiluminescence unit. Mean ± SD, n= 15, for tear film PMNs from 8 different participants and n=6, for blood-isolated PMNs from 4 different blood donors. *significantly different from blood isolated PMNs, $p < 0.01$

6.3.6. Participant with seasonal allergy

One participant with seasonal allergy was also unintentionally recruited during the study. Tear-film PMNs were collected and their ability to generate ROS after stimulation was assessed, as shown in Figure 30. The data of this participant was excluded from the above results but is presented as an interesting case study (n = 1), to provide some information on the potential effects of ocular allergy on ROS production in tear film PMNs. The overall tear-film PMNs CLs of the allergy participant was more than two times higher in comparison to healthy participants, indicating that the collected tear film PMNs were in a highly reactive and pro-inflammatory state. Apart from the fact that higher levels of inflammatory cytokines [14] have been observed in closed-eye tears of allergic participants, there is currently limited knowledge on how ocular allergy affects the leukocyte population and its phenotype. While this is but one participant, these preliminary results highlight how an inflammatory condition affects ROS production and how research in that area could contribute significant knowledge.

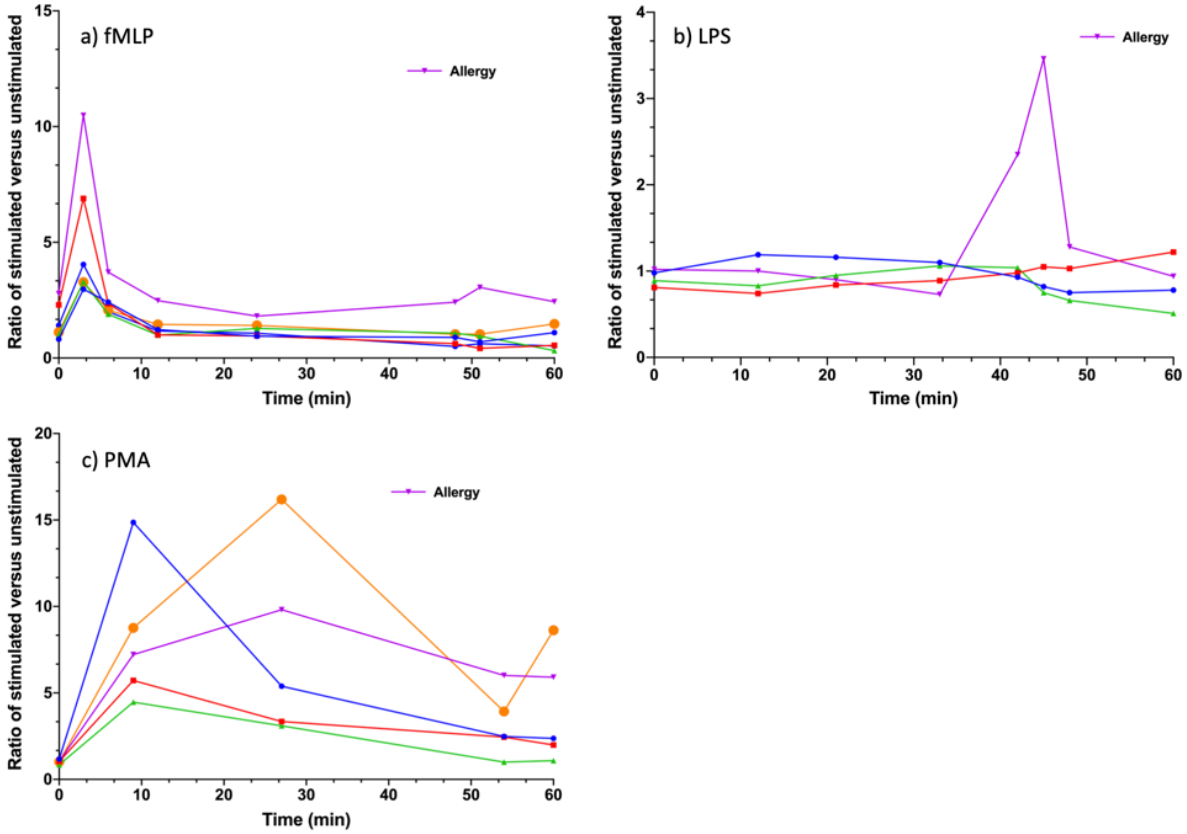

Figure 30 – Kinetic changes in CL of tear-film PMNs collected from the allergy participant over 60 min compared to asymptomatic/healthy participants. Results are reported as the ratio of stimulated CL emissions over control CL emissions, and each colored line represents one participant. The purple line identifies the ocular allergy symptomatic participant. A) fMLP-stimulated tear-film PMNs; B) LPS-stimulated tear-film PMNs; C) PMA-stimulated tear-film PMNs.

6.4. Discussion

Luminol reacts with ROS and releases energy in the form of light, chemiluminescence, thus the CL measurements are positively correlated with the amount of ROS generated[15]. This study measured the time-course changes in ROS produced during the oxidative burst in tear-film PMNs stimulated with fMLP, LPS and PMA. We also compared CL responses between tear-film and blood-isolated PMNs to determine if tear-film PMNs

have an impaired or reduced ability to initiate a respiratory burst. Both fMLP and LPS bind to the PMNs cell membrane via receptors, the seven transmembrane spanning G-protein linked receptors and toll-like receptors (TLR), respectively, to induce different cell functional responses[1,16]. On the other hand, PMA diffuses into cells and induces activation by binding directly to PKC[17]. These different actions of stimuli may result in the distinct CL responses observed.

fMLP acts via the pertussis-toxin G-coupled protein to activate several intracellular signalling pathways involving phospholipase A_2 (PLA_2), phospholipase C (PLC), mitogen-activated protein kinase (MAPk), and phosphatidylinositol 3-kinase (PI 3-k) to trigger various cell responses[1,17,18]. It has been shown that different functional responses are also associated with concentrations of stimuli. fMLP at less than 10 nM can only induce migration, while fMLP at a concentration greater than 100 nM can trigger the respiratory burst[1]. In our study, with a stimulus of 1.5μM of fMLP, both tear-film and blood-isolated PMNs exhibited a peak in ROS production within 3 minutes. Previous work demonstrated that extracellular ROS were released from PMNs during the first five minutes after stimulation, while the later CL response is associated mostly with intracellular production[19,20]. It is also known that the initial ROS released upon activation by fMLP is superoxide anion[11,15]. Our results thus suggest that tear film PMNs are able to produce extracellular superoxide indicating that the NADPH oxidase on their membrane is functional. fMLP uses various intracellular pathways to induce the respiratory burst and produce ROS intra and extracellularly (refer to figure 3 in chapter 2). A study has shown that fMLP-induced extracellular superoxide production is associated with the extracellular signal-

regulated kinase (ERK) and phospholipase D (PLD) and is dependent on the MAP kinase pathway[21,22]. A rapid and transient activation of MAPk within 1-2 min, which can last for up to 10 minutes, has also been observed in PMNs[23]. Hence, the peak and transient generation of ROS in tear film PMNs suggests that binding of fMLP to its receptor was able to stimulate PLD through the MAP kinase pathway and result in extracellular production of superoxide. The extracellular release of superoxide by fMLP-stimulated tear-film PMNs, however, will need to be further confirmed by using superoxide dismutase (SOD), a known ROS scavenger[11], as these studies have been performed in blood-isolated PMNs.

Blood-isolated PMNs reached a second CL peak around 25 minutes but tear-film PMNs failed to generate ROS again. The second peak has been associated with intracellular ROS production[19,20] and thus the inability of tear film PMNs to produce intracellular ROS suggests that some of intracellular pathways leading to ROS may be dysfunctional or blocked. Our results show that tear-film PMNs can generate ROS under PMA-stimulated condition so the pathway downstream PKC must be functional. If this is the case, then two hypotheses can be made around the impairment of intracellular ROS generation with fMLP: alterations in the downstream signalling/signal transduction from the fMLP receptors or reduced pool of intracellular signalling molecules. To produce extracellular ROS, the fMLP receptor must be functional as upon fMLP binding, a Gi signalling pathway is induced to activate PLD. Thus, the latter hypothesis may be more likely. The intracellular pathways of fMLP stimulation involve several phospholipases, for example, the PLC which hydrolyzes phosphatidylinositol into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), the latter can increase the intracellular Ca^{2+} concentration, and DAG serves as a PKC

activator[1,18]. The activation of these phospholipases is not only induced by fMLP but also other inflammatory molecules , such C5a and PAF[1]. The level of C5a increases dramatically during sleep[24] and thus C5a will bind to its receptor on tear film PMNs and lead to consumption of intracellular signalling molecules. Collectively, our results may thus suggest that most of the required substrates for phospholipases have probably already been hydrolyzed during sleep, and the lack of substrates affect the ability of phospholipases, resulting in an impaired ability to generate ROS using an intracellular pathway upstream of PKC. The results with LPS would support such a hypothesis, as upon binding to its receptor on the cell membrane, it activates similar intracellular signalling pathways. LPS was unable to trigger the respiratory burst in tear-film PMNs, suggesting that the intracellular mechanisms are affected. It is possible that the signal transduction from the LPS receptor is affected but it would be surprising to have the LPS receptor affected and not the fMLP while they have similar transmembrane receptor characteristics.

PMA was able to induce the respiratory burst in tear-film PMNs, suggesting that the mechanisms to generate ROS is functional in tear-film PMNs. However, two groups of participants were observed under PMA-stimulated conditions, the HR and the LR groups, where the HR produced significantly more ROS than the LR. Similar differences in the magnitude of ROS response have been observed in blood-isolated PMNs (including our own study, data not shown) and in PMNs collected from the oral cavities of patients previously diagnosed with refractory periodontitis[25]. The participants recruited in this study were healthy and free of any inflammatory or ocular diseases, suggesting that the higher ROS production in HR was not due to an underlying inflammatory condition. It has been shown

that leukocytes express a surface charge, and a lower surface charge is correlated with increased functional activity[26]. Hence, HR may possess more PMNs with a high electronegative charge that are sensitive and hyperactive to stimuli[26]. PMNs that have been primed also show an enhanced generation of ROS[1]. Pro-inflammatory cytokines are present in the closed-eye environment[4], which could prime the tear-film PMNs. It is thus possible that the higher response observed in the HR may be due to an increased concentration of inflammatory mediators in the closed-eye environment compared to that of the LR. It is currently impossible to determine which hypothesis explains the difference in ROS generation between LR and HR. This warrants further investigation, as the difference in LR and HR may result in a different ability to respond to bacteria invasion or the presence of a contact lens and contribute to microbial keratitis and sterile infiltrates.

In a previous study using dihydrorhodamine 123 to measure the respiratory burst via flow cytometry, it was reported that PMA-stimulated tear-film PMNs had a limited ability to produce ROS as compared to blood-isolated PMNs[3,24], an observation that our current results contradict. It is evident from our CL study that ROS production is time-dependent. Flow cytometry, however, can only assess ROS production at one endpoint, which may not be sufficient to infer the overall ability of ROS generation by tear-film PMNs depending on the time point chosen. In addition, dihydrorhodamine 123 in flow cytometry measures the intracellular ROS[27], whereas the luminol-enhanced chemiluminescence detects both the extra- and intracellular ROS generation[15]. PMA is recognized to stimulate both intra-and extracellular release of ROS[9], thus flow cytometry might underestimate ROS production. To activate cells, PMA needs to diffuse into the cell and then bind to PKC. The slower

responding time of tear-film PMNs might be due to difference in cell membrane properties, resulting in slower diffusion into the cells compared to blood-isolated PMNs, or there might also be a rate limiting factor in the intracellular pathway, whereby tear film PMNs may possess lower intracellular concentrations of one or more molecules involved in ROS generation. Such a hypothesis would be supported by the results with fMPL and LPS impaired ROS production as discussed above.

Our results also indicated that unstimulated tear film PMNs had a high level of ROS compared to blood isolated PMNs. The closed eye environment contains several inflammatory cytokines[4] and thus it is not surprising that tear film PMNs may have produced ROS intracellularly upon exposure to IL-8, C5a and other inflammatory mediators present. It is currently assumed that the ROS was intracellular as the presence of the intracellular pool of ROS would allow the tear film PMNs to kill the pathogens that have been phagocytosed and thus limit potential damage to the ocular surface from releasing ROS. Further investigation will be necessary to confirm that the measured CL was indeed from intracellular ROS. However previous flow cytometry results would suggest that this is the case[3].

6.5. Conclusions

Using the luminol-enhanced chemiluminescence assay, we demonstrated that tear-film PMNs collected from the closed-eye environment were able to produce ROS under certain conditions. ROS production was stimulus and time dependent, highlighting the importance of a kinetic study, especially with tear film PMNs, which have a different

phenotype from blood PMNs and thus may not be able to mount an oxidative response within the same time period. fMLP induced extracellular ROS in tear film PMNs, suggesting a functional NADPH oxidase, PLD and MAPk pathway. However, both fMLP and LPS failed to produce ROS intracellularly in tear film neutrophils, which implied that some of the intracellular signalling pathways of the tear-film PMNs may be impaired or the substrates for the phospholipases are insufficient. PMA triggered ROS production in tear-film PMNs, highlighting that any intracellular signalling impairment would then have to be upstream of the PKC pathway. Furthermore, our study identified two populations in their potential to generate ROS: the high responders (HR) and the low responders (LR), whereby the HRs produced significantly more ROS than the LRs upon PMA stimulation. Further work will be required to confirm this finding as it has important clinical implications for microbial keratitis and contact lens-induced infiltrates. The higher levels of initial ROS in unstimulated samples and failure to mount an oxidative response to LPS and fMLP also provide further evidence that tear film PMNs collected after sleep have undergone prior activation in the closed eye environment.

Chapter 7

Conclusions and Future Work

7.1. Conclusions

The presence in the closed-eye environment and the distinct phenotype of tear-film PMNs has drawn scientists' attention to their potential role in the ocular environment as well as in ocular inflammation. This thesis focused on two aspects, 1) investigating appropriate experimental procedures to process tear-film PMNs; and 2) assessing the inflammatory response of tear-film PMNs in terms of expression of surface receptors (when stimulated with IL-8 and PMA) and the production of reactive oxygen species (ROS) (when stimulated with fMLP, LPS and PMA).

We demonstrated that centrifugation, fixation, and incubation affected the expression of cell activation membrane receptors, CD11b, CD16, CD45, CD55 and CD66b differently. The observed changes may be due to the properties of the receptors and/or their interactions with the fluorochrome-labelled antibodies. Our results indicated that any additional centrifugation step following initial cell concentration and long incubation times should be avoided as they appeared to damage cells leading to receptor internalization. Post-fixation staining ought to be used with these cells as it led to minimal changes in the expression of surface receptors compared to unfixed samples. Our results may not generalize to all membrane receptors but highlight the importance of assessing the impact of experimental procedures on the expression of surface receptors before performing experiments with tear-film PMNs to ensure that reliable results are obtained.

We proved that the gentle eye wash method is currently the most effective in collecting tear-film PMNs from the ocular surface compared to the patch-OSCCA collection method. While the numbers of total cells collected using the gentle eye wash method were not consistent across the collection days (day 29 showed significantly fewer cells than day 2), our study indicated that participants could collect cells well within one day of training and that the expression of membrane receptors CD11b, CD45 and CD66b was consistent across collections. However, significant variabilities within a participant as well as within our population were observed with CD16 and CD55, indicating that CD16 and CD55 may not be reliable markers to use for diagnostic purposes.

We determined that tear-film PMNs were unable to alter their surface receptors when stimulated with IL-8, but that they had a limited potential to modulate their expression of surface receptors when exposed to PMA. A similar response trend was observed with the respiratory burst whereby tear-film PMNs could generate ROS in response to PMA, but not fMLP and LPS. Unlike PMA, IL-8, fMLP, and LPS stimulation act via a receptor transduction signalling process, thus our results suggest that the inability of tear-film PMNs to induce an inflammatory response is more related to dysfunctional receptors and/or the impairment of intracellular signalling pathways that are upstream of the PKC pathway.

In this research, we also identified that unstimulated tear-film PMNs displayed a high expression of CD11b, CD66b and CD55, low expression of CD16, and a high level of ROS which suggest that they have already been activated in the closed-eye environment.

Taken all together, this thesis contributed significant new knowledge to the characteristics of tear-film PMNs. Our study demonstrated that tear-film PMNs are sensitive

to preparation procedures, so any potential impact on the expression of surface receptors needs to be taken into consideration to increase the reproducibility and reliability of results. Furthermore, tear-film PMNs showed some functionality upon stimulation with PMA suggesting that biological factors, such as impaired intracellular signalling pathways, dysfunctional receptors or prolonged exposure to inflammatory mediators *in vivo*, is contributing to the reduced or limited inflammatory responses observed in tear-film PMNs collected from the closed-eye environment. Furthermore, among all the surface receptors, while interesting from an inflammatory perspective, CD16 may not be a reliable marker with tear-film PMNs due to its low level of expression, its hypersensitivity to experimental procedures, and its inconsistency in the expression across the collection days. While many questions remain to be answered, this thesis has allowed us to gain a better understanding of tear-film PMNs and will support future studies to investigate their phenotype and implication in ocular health and inflammation.

7.2. Future work

We presented some findings of the tear-film PMNs regarding the sensitivity to experimental procedures, the responses to IL-8, and the ability to generate ROS, but further research is needed to characterize the phenotype of tear film PMNs as well as conduct clinical studies to determine their role in ocular infection and inflammation. The first sections of the future work is related to phenotype the tear-film PMNs isolated from healthy people to study their functionality, origin and also the possible mechanisms leading to the noninflammatory phenotype (7.2.1 to 7.2.5) while the remainder focusses on investigation of

tear-film PMNs obtained from people with ocular inflammation and lens wearers.

7.2.1 Further characterization of tear-film PMNs phenotype – Granules in tear film PMNs

In Chapter 5, we demonstrated that PMA could slightly upregulate CD11b, CD55, and CD66b, and downregulate CD16, indicating tear-film PMNs have limited potential to respond to PMA. We hypothesized that the reduced ability for tear-film PMNs might due to the previous release of granules in the closed-eye environment because most of the CD11b is stored in primary granules and CD55 is stored in secretory vesicles. There is limited knowledge on the granule content of tear film PMNs as all the studies so far have indirectly measured granule release. There is thus a need to better characterize granules in tear film PMNs. Imaging flow cytometry presents a great opportunity to visualize the granules within the tear-film PMNs and assess heterogeneity within a population[1,2]. Granule staining combined with surface receptor staining would allow to gain significant insights to determine if the inability of tear-film PMNs to modulate their expression of surface receptors is due to the previous activation in the closed-eye environment and release of granules.

7.2.2 Further characterization of tear-film PMNs phenotype – Apoptosis

In addition, it has been shown that the activation of p38 kinases[3] and the downregulation of CD16[4] are associated with cell apoptosis. If cells are undergoing apoptosis, mechanisms of cell activation would be affected and thus may explain some of our results. While tear-film PMNs were found not to be apoptotic using caspase activation[5], further experiments should be performed to determine if they are at an early stage of

apoptosis when collected, with Annexin V, for example.

7.2.3 Further characterization of tear-film PMNs phenotype – Cytokine release

PMNs can secrete various proinflammatory cytokines, such as interleukin 8 (IL-8), tumour necrosis factor α (TNF- α), granulocyte-colony stimulating factor (G-CSF) and interleukin 6 (IL-6)[6], in response to stimulus. The primary function of these cytokines is to prime PMNs in order to amplify their inflammatory response and act as a feedback loop on cell activation. Our findings demonstrated that tear-film PMNs cannot generate ROS in response to LPS and fMLP, so it would be essential to test if tear-film PMNs can produce and release cytokines upon stimulation, as it would help to understand the impairment of ROS generation in tear-film PMNs.

7.2.4 Further characterization of tear-film PMNs phenotype – Inducing tear film PMNs phenotype in blood-isolated PMNs with IL-8 exposure

Combining our current results with recent work from Postnikoff & Nichols[7], there is strong evidence that tear film PMNs have been previously activated in the closed eye environment. Thus, when we assess their phenotype after collection, we are potentially looking at a second activation stimulus and then comparing their level of activation to blood-isolated PMNs, which may not be an appropriate reference. Previous work has also tried to reproduce the phenotype of tear film PMNs and demonstrated that exposure to hypoxic environment, human corneal epithelial cells and artificial tear solution could not induce the noninflammatory phenotype in blood-isolated PMNs[8]. The closed-eye environment has been shown to contain a high concentration of cytokines that can prime tear-film PMNs and may

significantly contribute to their distinct phenotype. This has yet to be further explored and would help us in better understanding the limited response observed with stimulus, such as LPS, fMLP and IL-8. Experiments where blood-isolated PMNs are primed/activated with cytokines, such as IL-8, and then activated with a stimulus, would be very valuable to determine if blood-isolated PMNs can become non-inflammatory/non-responsive as tear-film PMNs. This would provide significant insights on the mechanisms causing the non-responsive phenotype of tear-film PMNs.

7.2.5 Further characterization of tear-film PMNs phenotype – Using an *in vitro* model to characterize mechanisms involved in tear film PMNs phenotype

The upregulation of CD11b and downregulation of L-selectin is correlated with extravasation, indicating an activated state of cells. Recent work by Postnikoff & Gorbet[8] provided limited insights on how transmigration may affect tear film PMNs phenotype. It would thus be essential to mimic the transmigration of blood-isolated PMNs to verify if the later noninflammatory phenotype in tear-film PMNs is caused by transmigration and direct interaction with the corneal and conjunctival epithelium. Such a hypothesis could be tested using an *in vitro* cell culture model, as shown in Figure 31. Apart from this, it also allows us to study the interaction of the "extravasated" blood-isolated PMNs with the human corneal epithelial cells (hCECs) in order to have a better understanding of the ocular environment. The corneal epithelial cells could be substituted with conjunctival epithelial cells or a co-culture of cell type to gain further insights into mechanisms of activation.

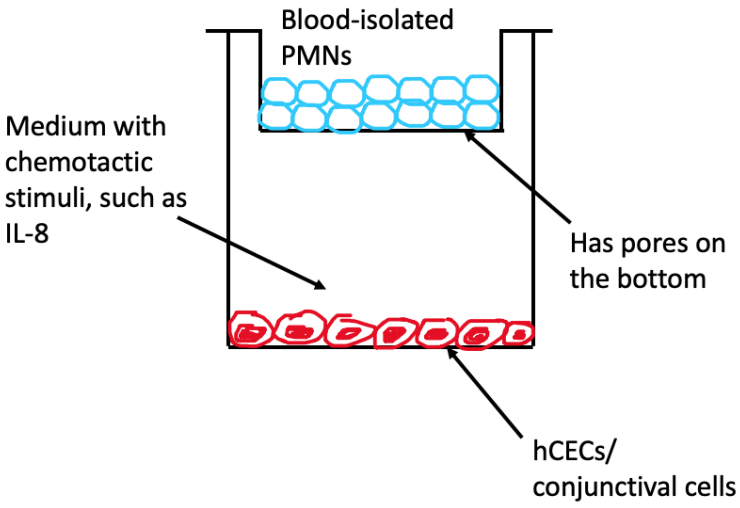

Figure 31 – An in vitro culture model to mimic the extravasation on blood-isolated PMNs.

7.2.6 The role of tear-film PMNs in seasonal allergy

It has been shown that at awakening, there were twice as many leukocytes collected from people with dry eye disease (DED) and that a higher ratio of granulocytes to lymphocytes exited in people with DED[9]. These results suggest that an inflammatory state may contribute to recruitment of PMNs. In chapter 6, we reported on one case of a seasonal allergy participant, showing that the tear-film PMNs isolated from the allergy person responded differently from the healthy participants. There is thus a need to further investigate the difference in phenotype between healthy people and people who suffer from seasonal allergies. This would provide valuable knowledge on how seasonal allergies impact the ocular environment and the presence of inflammatory cells. This may help in the development of therapies but would also have potential benefit as a tool to determine the

impact of therapeutic strategies.

7.2.7 The role of tear-film PMNs in lens-related ocular complications

In our study, we identified two responder groups upon stimulation with PMA: a high (HR) and low responders (LR). The difference in LR and HR may cause some individuals to be more susceptible to bacteria, resulting in the microbial keratitis (MK) or to damage the integrity of corneal epithelium, resulting in sterile infiltrates. Only 8 participants were involved in that specific experiment and thus it would be valuable to recruit more participants to determine their response. Not only should non-lens wearers be recruited but we should also assess the response of lens wearers. The study would have to be designed carefully as lens material would likely have an effect, but such a study would provide significant knowledge on ocular health and potential for complications in lens wearers versus non lens wearers.

7.2.8 The role of tear-film PMNs in lens-related ocular infections – *in vivo* clinical studies

While tear-film PMNs have been collected on the ocular surface in various inflammatory diseases such as dry eye[9] and meibomian gland dysfunction[10], no study has yet characterized their phenotype in lens-related infections. While our results are from a small sample size (n=8), the LR and HR groupings highlight that there could be some significant underlying factors that may contribute to risks of infections. Studies by Carnt *et al*[11,12] have focused on the role of cytokines in MK (IL-10 and IL-17) and while PMNs are key players in infections, little is known on their role and presence in human ocular infections. Being able

to further study tear film PMNs both from the closed-eye and open-eye environments in people affected by lens-related MK would have to the potential to contribute significant knowledge to the field of ocular health and contact lens-induced complications, similarly to what has been achieved in periodontal health and disease[2,13] in the past 10 years.

7.2.9 The role of tear-film PMNs in lens-related ocular infections – *in vitro* model

Wearing contact lenses during sleep increases the incidence of MK[14,15]. The presence of contact lenses and bacteria on the ocular surface, together with the hypoxic environment, and the need to protect the ocular surface without damaging its integrity leads to a set complex interaction for tear-film PMNs, which might affect their ability to respond to the bacteria. An *in vitro* model with tear-film PMNs, bacteria, a contact lens, and probably human corneal epithelial cells under the hypoxic condition may provide valuable information. However, based on our study, short incubation would be required, and this may limit the value of such a model. If we were able to induce the tear film phenotype with blood-isolated PMNs in 7.2.5 this would be the most useful model.

It would also be interesting to incubate bacteria with different types of lens materials to examine if there is a particular material that is preferential for bacteria binding and biofilm formation and determine how the presence of tear-film PMNs and blood-isolated PMNs and the release of their various mediators would affect the growth/stability of the biofilm. Our results clearly indicate that tear film PMNs are activated *in vivo* and thus it would be highly relevant to understand how such an activation contributes to fighting invading pathogens and how subsequent stimulus affect or not the outcomes.

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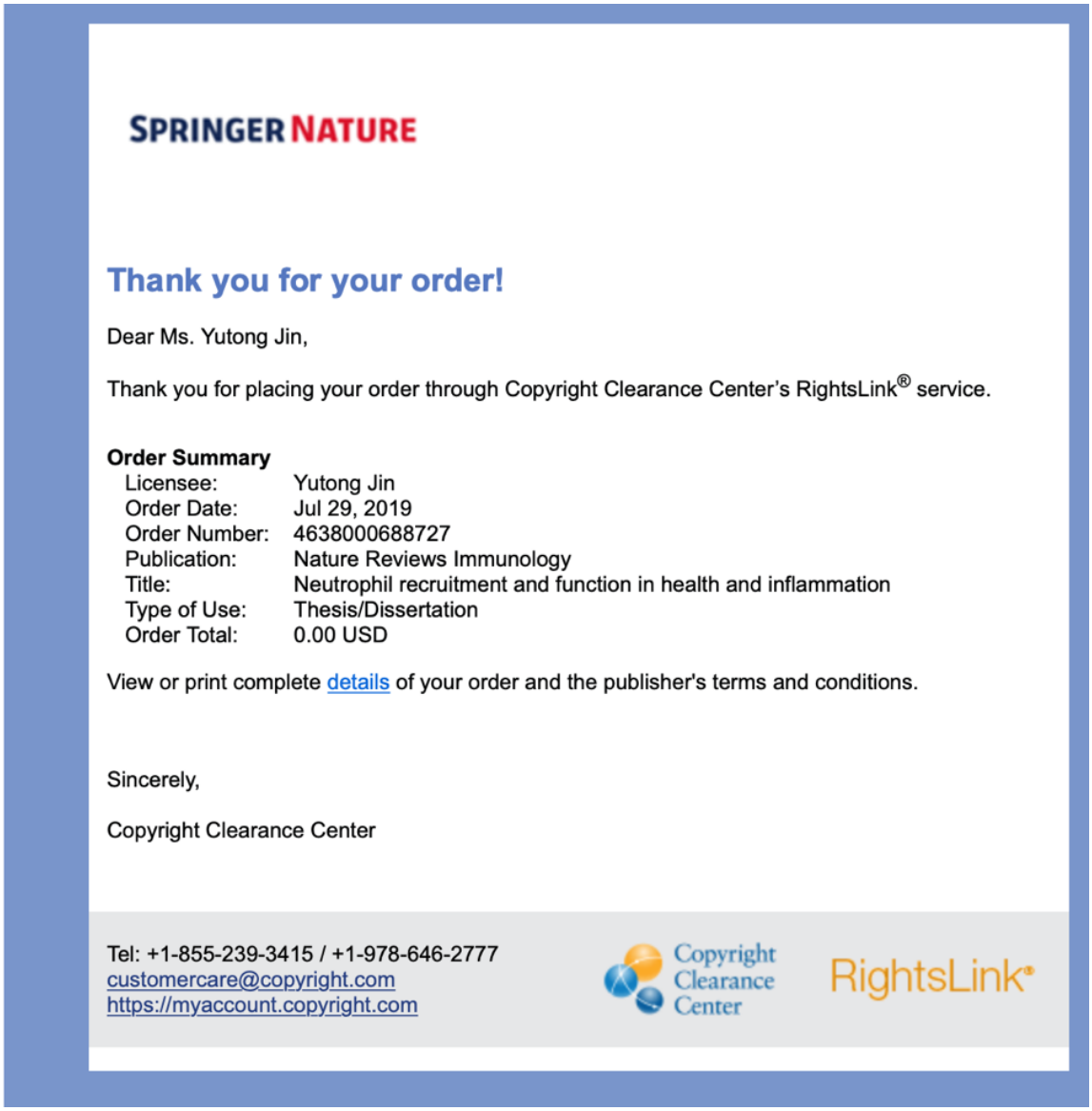

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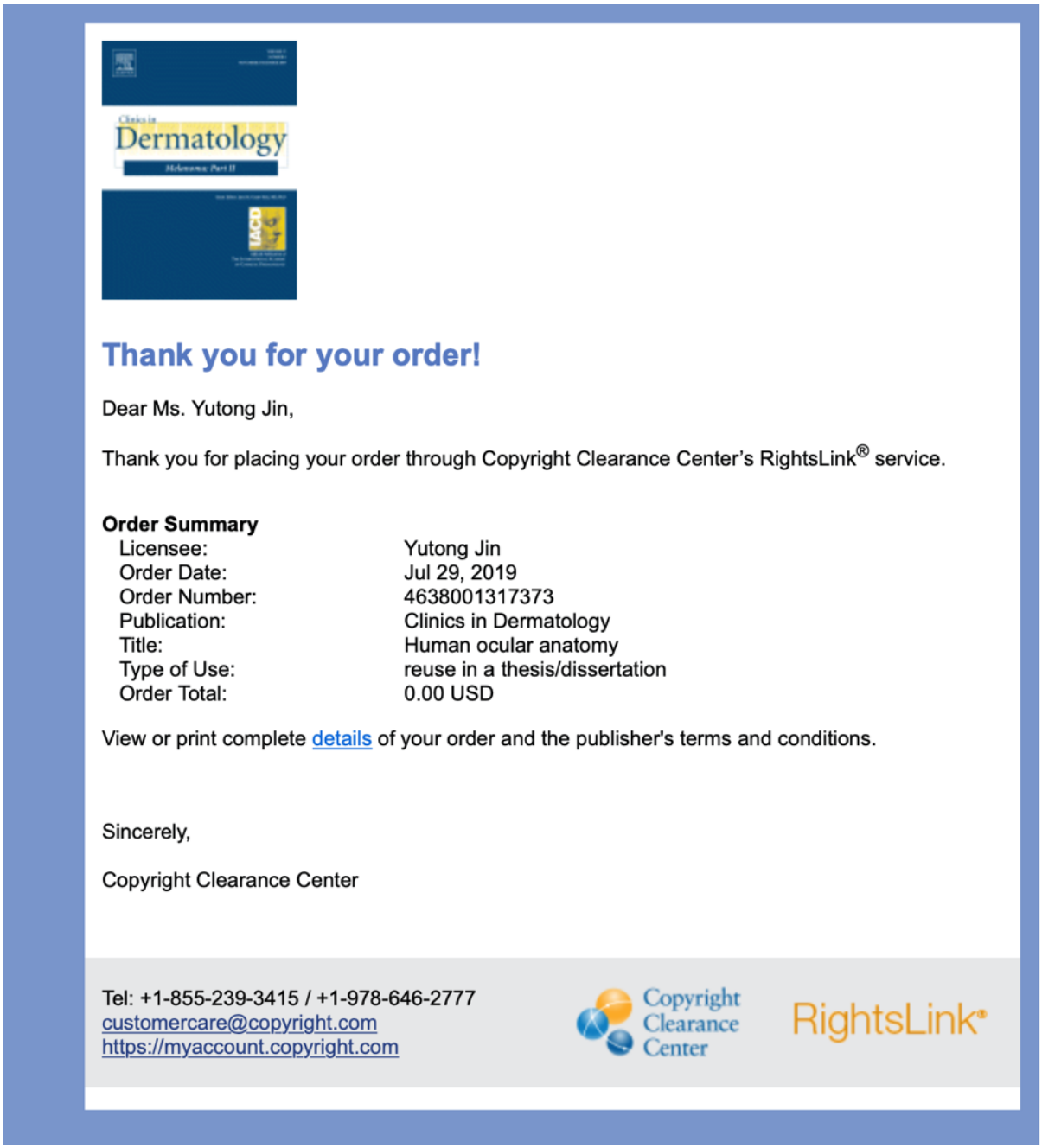
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Bibliography

Chapter 1 references

1. Gorbet, M., Postnikoff, C. & Williams, S. The noninflammatory phenotype of neutrophils from the closed-eye environment: A flow cytometry analysis of receptor expression. *Investig. Ophthalmol. Vis. Sci.* **56**, 4582–4591 (2015).
2. Keino, H., Horie, S. & Sugita, S. Immune Privilege and Eye-Derived T-Regulatory Cells. *J. Immunol. Res.* **2018**, 1–12 (2018).
3. Wilson, G., O'leary, D. & Holden, B. Cell content of tears following overnight wear of a contact lens. *Curr. Eye Res.* **8**, 329–335 (1989).
4. Postnikoff, C. & Gorbet, M. The Effect of Closed-Eye Tear Film Conditions on Blood-Isolated Neutrophils, In Vitro. *Ocul. Immunol. Inflamm.* **26**, 706–716 (2018).
5. Sack, R. A., Nunes, I., Beaton, A. & Morris, C. Host-defense mechanism of the ocular surfaces. *Biosci. Rep.* **21**, 463–480 (2001).
6. Tan, K. O., Sack, R. A., Holden, B. A. & Swarbrick, H. A. Temporal sequence of changes in tear film composition during sleep. *Curr. Eye Res.* **12**, 1001–1007 (1993).
7. Fullard, R. J. & Wilson, G. S. Investigation of sloughed corneal epithelial cells collected by non-invasive irrigation of the corneal surface. *Curr. Eye Res.* **5**, 847–856 (1986).
8. Thakur, A., Willcox, M. D. P. & Stapleton, F. The proinflammatory cytokines and arachidonic acid metabolites in human overnight tears: Homeostatic mechanisms. *J. Clin. Immunol.* **18**, 61–70 (1998).

9. Peterson, R. C., Gorbet, M., Cira, D., *et al.* Non-Invasive Collection and Examination of Human Corneal Epithelial Cells. *Optom. Vis. Sci.* **88**, 1317–1325 (2011).

10. Gorbet, M., Luensmann, D. & Jones, L. The response of tear film neutrophils to occasional overnight lens wear. *Investig. Ophthalmol. {&} Vis. Sci.* **54**, 2069 (2013).

11. Gorbet, M. & Willcox, M. D. P. Interactions of tear-film neutrophils with clinical bacteria. *Invest Ophthalmol Vis Sci.* **56**, 4048 (2015).

12. Postnikoff, C. K. & Nichols, K. K. Neutrophil and T-cell homeostasis in the closed eye. *Investig. Ophthalmol. Vis. Sci.* **58**, 6212–6220 (2017).

13. Fortunati, E., Kazemier, K. M., Grutters, J. C., Koenderman, L. & Van Den Bosch, V. J. M. M. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clin. Exp. Immunol.* **155**, 559–566 (2009).

14. Bender, J. S., Thang, H. & Glogauer, M. Novel rinse assay for the quantification of oral neutrophils and the monitoring of chronic periodontal disease. *J. Periodontal Res.* **41**, 214–220 (2006).

15. Ssemaganda, A., Kindinger, L., Bergin, P., *et al.* Characterization of Neutrophil Subsets in Healthy Human Pregnancies. *PLoS One* **9**, e85696 (2014).

16. Mahajan, A., Grüneboom, A., Petru, L., *et al.* Frontline Science: Aggregated neutrophil extracellular traps prevent inflammation on the neutrophil-rich ocular surface. *J. Leukoc. Biol.* **105**, 1087–1098 (2019).

17. Strieter, R. M., Kasahara, K., Allen, R. M., *et al.* Cytokine-induced neutrophil-derived interleukin-8. *Am. J. Pathol.* **141**, 397–407 (1992).

18. Dransfield, I., Buckle, A. M., Savill, J. S., *et al.* Neutrophil apoptosis is associated

with a reduction in CD16 (Fc gamma RIII) expression. *J. Immunol.* **153**, 1254–63 (1994).

19. Baggiolini, M., Walz, A. & Kunkel, S. L. Neutrophil-activating peptide-1/Interleukin 8, a Novel Cytokine That Activates Neutrophils. *J Clin Invest.* **84**, 1045–1049 (1989).
20. Nicholson, G. C., Tennant, R. C., Carpenter, D. C., *et al.* A novel flow cytometric assay of human whole blood neutrophil and monocyte CD11b levels: Upregulation by chemokines is related to receptor expression, comparison with neutrophil shape change, and effects of a chemokine receptor (CXCR2) antagonist. *Pulm. Pharmacol. Ther.* **20**, 52–59 (2007).
21. White, J. R., Lee, J. M., Young, P. R., *et al.* Identification of a Potent, Selective Non-peptide CXCR2 Antagonist That Inhibits Interleukin-8-induced Neutrophil Migration. *J. Biol. Chem.* **273**, 10095–10098 (1998).
22. Hellewell, P. G. & Williams, T. J. *Immunopharmacology of neutrophils (the handbook of immunopharmacology)*. (Academic Press, 1994).
23. Thompson, H. L., Shiroo, M. & Saklatvala, J. The chemotactic factor N - formylmethionyl-leucyl-phenylalanine activates microtubule-associated protein 2 (MAP) kinase and a MAP kinase kinase in polymorphonuclear leucocytes. *Biochem. J.* **290**, 483–488 (1993).
24. Worthen, G. S., Avdi, N., Buhl, A. M., Suzuki, N. & Johnsonv, G. L. FMLP Activates Ras and Raf in Human Neutrophils Potential Role in Activation of MAP Kinase. *J. Clin. Invest.* **94**, 815–823 (1994).
25. Hume, E. B. H., Stapleton, F. & Willcox, M. D. P. Evasion of cellular ocular defenses

by contact lens isolates of Serratia marcescens. *Eye Contact Lens* **29**, 108–12 (2003).

26. Postnikoff, C. K., Huisingh, C., McGwin, G. & Nichols, K. K. Leukocyte Distribution in the Open Eye Tears of Normal and Dry Eye Subjects. *Curr. Eye Res.* **43**, 1253–1259 (2018).

27. Reyes, N. J., Yu, C., Mathew, R., *et al.* Neutrophils cause obstruction of eyelid sebaceous glands in inflammatory eye disease in mice. *Sci. Transl. Med.* **10**, eaas9164 (2018).

28. Mun, C., Gulati, S., Tibrewal, S., *et al.* A Phase I/II Placebo-Controlled Randomized Pilot Clinical Trial of Recombinant Deoxyribonuclease (DNase) Eye Drops Use in Patients With Dry Eye Disease. *Transl. Vis. Sci. Technol.* **8**, 10 (2019).

29. Bonini, S., Ano, Bonini, S., *et al.* Allergen dose response and late symptoms in a human model of ocular allergy. *J. Allergy Clin. Immunol.* **86**, 869–876 (1990).

30. Flanagan, J. G., Stavropoulos, A., Luensmann, D., Postnikoff, C. & Gorbet, M. Comparison of the Cellular Response to Overnight Contact Lens Wear on the Bulbar Conjunctiva and Cornea. *Invest. Ophthalmol. Vis. Sci.* E-Abstract 5654 (2013).

31. Gorbet, M., Luensmann, D. & Jones, L. The response of tear film neutrophils to occasional overnight lens wear. *Invest. Ophthalmol. Vis. Sci.* E-Abstract 2069 (2013).

Chapter 2 references

1. Janeway, C. A. & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20**, 197–216 (2002).
2. Hellewell, P. G. & Williams, T. J. *Immunopharmacology of neutrophils (the handbook of immunopharmacology)*. (Academic Press, 1994).
3. Rankin, S. M. The bone marrow: a site of neutrophil clearance. *J. Leukoc. Biol.* **88**, 241–251 (2010).
4. Rosmarin, A. G., Yang, Z. & Resendes, K. K. Transcriptional regulation in myelopoiesis: Hematopoietic fate choice, myeloid differentiation, and leukemogenesis. *Exp. Hematol.* **33**, 131–143 (2005).
5. Summers, C., Rankin, S., Condliffe, A., *et al.* Neutrophil kinetics in health and disease. *Trends Immunol.* **31**, 318–324 (2010).
6. Hogg, J. Leukocyte Traffic in the Lung. *Annu. Rev. Physiol.* **57**, 97–114 (1995).
7. Lavin, Y., Winter, D., Blecher-Gonen, R., *et al.* Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment. *Cell* **159**, 1312–1326 (2014).
8. Silvestre-Roig, C., Hidalgo, A. & Soehnlein, O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. *Blood* **127**, 2173–2181 (2016).
9. Beyrau, M., Bodkin, J. V. & Nourshargh, S. Neutrophil heterogeneity in health and disease: A revitalized avenue in inflammation and immunity. *Open Biol.* **2**, (2012).
10. Ng, L. G., Ostuni, R. & Hidalgo, A. Heterogeneity of neutrophils. *Nat. Rev. Immunol.* **19**, 255–265 (2019).

11. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **13**, 159–175 (2013).

12. Pillay, J., den Braber, I., Vrisekoop, N., *et al.* In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood* **116**, 625–627 (2010).

13. Buckley, C. D., Ross, E. A., McGettrick, H. M., *et al.* Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J. Leukoc. Biol.* **79**, 303–311 (2005).

14. Zola, H. CD molecules 2005: human cell differentiation molecules. *Blood* **106**, 3123–3126 (2005).

15. Boumsell, L. The International Workshops and Conferences on Human Leukocyte Differentiation Antigens:Birth, current status and future. *Tissue Antigens* **48**, 238–241 (1996).

16. Mittag, A., Lenz, D., Gerstner, A. O. H., *et al.* Polychromatic (eight-color) slide-based cytometry for the phenotyping of leukocyte, NK, and NKT subsets. *Cytom. Part A* **65A**, 103–115 (2005).

17. Zhuang, H. & Matsunami, H. Evaluating cell-surface expression and measuring activation of mammalian odorant receptors in heterologous cells. *Nat. Protoc.* **3**, 1402–1413 (2008).

18. Brown, M. & Wittwer, C. Flow Cytometry: Principles and Clinical Applications in Hematology. *Clin. Chem.* **46**, 1221–1229 (2000).

19. Gorbet, M., Postnikoff, C. & Williams, S. The noninflammatory phenotype of neutrophils from the closed-eye environment: A flow cytometry analysis of receptor

expression. *Investig. Ophthalmol. Vis. Sci.* **56**, 4582–4591 (2015).

20. Postnikoff, C. K. & Nichols, K. K. Neutrophil and T-cell homeostasis in the closed eye. *Investig. Ophthalmol. Vis. Sci.* **58**, 6212–6220 (2017).

21. Lakschevitz, F. S., Hassanpour, S., Rubin, A., *et al.* Identification of neutrophil surface marker changes in health and inflammation using high-throughput screening flow cytometry. *Exp. Cell Res.* **342**, 200–209 (2016).

22. Fortunati, E., Kazemier, K. M., Grutters, J. C., Koenderman, L. & Van Den Bosch, V. J. M. M. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clin. Exp. Immunol.* **155**, 559–566 (2009).

23. Kujipers, T., Tool, A., van der Schoot, C., *et al.* Membrane surface antigen expression on neutrophils: A reappraisal of the use of surface markers for neutrophil activation. *Blood* **78**, 1105–1111 (1991).

24. Abdel-Salam, B. K. A. & Ebaid, H. Clinical immunology Expression of CD11b and CD18 on polymorphonuclear neutrophils stimulated with interleukin-2. *Cent. Eur. J. Immunol.* **2**, 209–215 (2014).

25. Wagner, C. & Hänsch, G. M. Genetic deficiency of CD16 , the low-affinity receptor for immunoglobulin G , has no impact on the functional capacity of polymorphonuclear neutrophils. *Eur. J. Clin. Invest.* **34**, 149–155 (2004).

26. Dransfield, I., Buckle, A. M., Savill, J. S., *et al.* Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J. Immunol.* **153**, 1254–63 (1994).

27. Nuutila, J., Jalava-Karvinen, P., Hohenthal, U., *et al.* Use of complement regulators,

CD35, CD46, CD55, and CD59, on leukocytes as markers for diagnosis of viral and bacterial infections. *Hum. Immunol.* **74**, 522–530 (2013).

28. Mann, B. S. & Chung, K. F. Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. *Respir. Res.* **7**, 59 (2006).

29. Yamagata, T., Sugiura, H., Yokoyama, T., *et al.* Overexpression of CD-11b and CXCR1 on Circulating Neutrophils. *Chest* **132**, 890–899 (2007).

30. Fine, N., Hassanpour, S., Borenstein, A., *et al.* Distinct Oral Neutrophil Subsets Define Health and Periodontal Disease States. *J. Dent. Res.* **95**, 931–938 (2016).

31. Mócsai, A., Walzog, B. & Lowell, C. A. Intracellular signalling during neutrophil recruitment. *Cardiovasc. Res.* **107**, 373–385 (2015).

32. Lay, J. C., Peden, D. B. & Alexis, N. E. Flow cytometry of sputum: assessing inflammation and immune response elements in the bronchial airways. *Inhal. Toxicol.* **23**, 392–406 (2011).

33. Ssemaganda, A., Kindinger, L., Bergin, P., *et al.* Characterization of Neutrophil Subsets in Healthy Human Pregnancies. *PLoS One* **9**, e85696 (2014).

34. Pillay, J., Ramakers, B. P., Kamp, V. M., *et al.* Functional heterogeneity and differential priming of circulating neutrophils in human experimental endotoxemia. *J. Leukoc. Biol.* **88**, 211–220 (2010).

35. Tosi, M. F. & Zakem, H. Surface expression of Fcγ receptor III (CD16) on chemoattractant- stimulated neutrophils is determined by both surface shedding and translocation from intracellular storage compartments. *J. Clin. Invest.* **90**, 462–470 (1992).

36. Bredius, R. G. M. & Medical, A. Role of neutrophil FcγRIIa (CD32) and FcγRIIIb (CD16) polymorphic forms in phagocytosis of human IgG1- and IgG3-opsonized bacteria and erythrocytes. *Transfus. Med. Rev.* **9**, 343 (2006).

37. Butcher, S. K., Chahal, H., Nayak, L., *et al.* Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans. *J. Leukoc. Biol.* **70**, 881–6 (2001).

38. Holt, P. G. & Jones, C. A. The development of the immune system during pregnancy and early life. *Allergy Eur. J. Allergy Clin. Immunol.* **55**, 688–697 (2000).

39. Mahajan, A., Grüneboom, A., Petru, L., *et al.* Frontline Science: Aggregated neutrophil extracellular traps prevent inflammation on the neutrophil-rich ocular surface. *J. Leukoc. Biol.* **105**, 1087–1098 (2019).

40. Remold-O'donnell, E. & Parent, D. Downregulation of Neutrophil CD43 by Opsonized Zymosan. *Blood* **85**, 337–342 (1995).

41. Remold-O'Donnell, E. & Parent, D. Specific sensitivity of CD43 to neutrophil elastase. *Blood* **86**, 2395–2402 (1995).

42. Nathan, C. F., Xie, Q. W., Halbwachs-Mecarelli, L. & Jin, W. W. Albumin Inhibits the Spreading of Neutrophils by Blocking the Shedding of CD43 (Sialophorin, Leukosialin). *J. Cell Biol.* **122**, 243–256 (1993).

43. Ley, K., Laudanna, C., Cybulsky, M. I. & Nourshargh, S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* **7**, 678–689 (2007).

44. Pillay, J., Kamp, V. M., van Hoffen, E., *et al.* A subset of neutrophils in human

systemic inflammation inhibits T cell responses through Mac-1. *J. Clin. Invest.* **122**, 327–336 (2012).

45. Nourshargh, S. & Alon, R. Leukocyte Migration into Inflamed Tissues. *Immunity* **41**, 694–707 (2014).

46. Herter, J. & Zarbock, A. Integrin Regulation during Leukocyte Recruitment. *J. Immunol.* **190**, 4451–4457 (2013).

47. Bargatze, R. F. Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. *J. Exp. Med.* **180**, 1785–1792 (1994).

48. Phillipson, M., Heit, B., Colarusso, P., *et al.* Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J. Exp. Med.* **203**, 2569–2575 (2006).

49. Nourshargh, S. & Marelli-Berg, F. M. Transmigration through venular walls: A key regulator of leukocyte phenotype and function. *Trends Immunol.* **26**, 157–165 (2005).

50. Worthen, G. S., Avdi, N., Buhl, A. M., Suzuki, N. & Johnsonv, G. L. FMLP Activates Ras and Raf in Human Neutrophils Potential Role in Activation of MAP Kinase. *J. Clin. Invest.* **94**, 815–823 (1994).

51. Zu, Y., Qi, J., Gilchrist, A., *et al.* p38 Mitogen-Activated Protein Kinase Activation Is Required for Human Neutrophil Function Triggered by TNF- α or FMLP Stimulation. *J Immunol* **160**, 1982–1989 (1998).

52. Nick, J. A., Avdi, N., Young, S. K., *et al.* Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. *J Clin*

Invest. **99**, 975–986 (1997).

53. Zhu, L., Castranova, V. & He, P. fMLP-stimulated neutrophils increase endothelial [Ca 2+] i and microvessel permeability in the absence of adhesion: role of reactive oxygen species. *Am. J. Physiol. Circ. Physiol.* **288**, H1331–H1338 (2005).

54. Cockcroft, S. Polyphosphoinositide phosphodiesterase: regulation by a novel guanine nucleotide binding protein, Gp. *Trends Biochem. Sci.* **12**, 75–78 (1987).

55. Fensome, A., Whatmore, J., Morgan, C., Jones, D. & Cockcroft, S. ADP-ribosylation factor and rho proteins mediate fMLP-dependent activation of phospholipase D in human neutrophils. *J. Biol. Chem.* **273**, 13157–13164 (1998).

56. Sato, T., Hongu, T., Sakamoto, M., Funakoshi, Y. & Kanaho, Y. Molecular Mechanisms of N-Formyl-Methionyl-Leucyl-Phenylalanine-Induced Superoxide Generation and Degranulation in Mouse Neutrophils: Phospholipase D Is Dispensable. *Mol. Cell. Biol.* **33**, 136–145 (2013).

57. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Dérijard, B. & Davis, R. J. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.* **16**, 1247–1255 (1996).

58. Zhang, J., Kaupke, C. J., Yousefi, S., Cesario, T. C. & Vaziri, N. D. Flow cytometric investigation of neutrophil activation pathways by n-formyl-Met-Leu-Phe and phorbol myristate acetate. *Biol. Cell* **84**, 147–153 (1995).

59. Karlsson, A., Nixon, J. B. & McPhail, L. C. Phorbol myristate acetate induces neutrophil NADPH-oxidase activity by two separate signal transduction pathways: dependent or independent of phosphatidylinositol 3-kinase. *J. Leukoc. Biol.* **67**, 396–

404 (2000).

60. Haugland, G. T., Jakobsen, R. A., Vestvik, N., *et al.* Phagocytosis and Respiratory Burst Activity in Lumpsucker (Cyclopterus lumpus L.) Leucocytes Analysed by Flow Cytometry. *PLoS One* **7**, e47909 (2012).

61. Dahlgren, C. & Karlsson, A. Respiratory burst in human neutrophils. *J. Immunol. Methods* **232**, 3–14 (1999).

62. Sbarra, A. J. & Karnovskyi, M. L. The Biochemical Basis of Phagocytosis I. The Biochemical Basis of Phagocytosis I. Metablolic Changes During the Ingestion of Particles by Polymorphonuclear Leukocytes. *J. Biol. Chem.* **234**, 1355–1362 (1959).

63. Holmes, B., Quie, P., Windhort, D. & Good, R. Fatal granulomatous disease of chilhood: An inborn abnormality of phagocytic function. *Lancet* **1**, 1225–1228 (1966).

64. Di Teodoro, G., Marruchella, G., Mosca, F., *et al.* Polymorphonuclear cells and reactive oxygen species in contagious bovine pleuropneumonia: New insight from in vitro investigations. *Vet. Immunol. Immunopathol.* **201**, 16–19 (2018).

65. Cemerski, S., Cantagrel, A., van Meerwijk, J. P. M. & Romagnoli, P. Reactive Oxygen Species Differentially Affect T Cell Receptor-signaling Pathways *. *J. Biol. Chem.* **277**, 19585–19593 (2002).

66. Mehrzad, J., Duchateau, L., Pyörälä, S. & Burvenich, C. Blood and Milk Neutrophil Chemiluminescence and Viability in Primiparous and Pluriparous Dairy Cows During Late Pregnancy, Around Parturition and Early Lactation. *J. Dairy Sci.* **85**, 3268–3276 (2002).

67. Borregaard, N., Heiple, J. ., Simons, E. R. & Clark, R. A. Subcellular localization of

the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J. Cell Biol.* **97**, 52 (1983).

68. Babior, B. M. NADPH-oxidase: an update. *Blood* **93**, 1464 (1999).

69. Nguyen, G. T., Green, E. R. & Mecsas, J. Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Front. Cell. Infect. Microbiol.* **7**, (2017).

Chapter 3 references

1. Beyrau, M., Bodkin, J. V. & Nourshargh, S. Neutrophil heterogeneity in health and disease: A revitalized avenue in inflammation and immunity. *Open Biol.* **2**, (2012).
2. Pillay, J., Kamp, V. M., van Hoffen, E., *et al.* A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J. Clin. Invest.* **122**, 327–336 (2012).
3. Fortunati, E., Kazemier, K. M., Grutters, J. C., Koenderman, L. & Van Den Bosch, V. J. M. M. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clin. Exp. Immunol.* **155**, 559–566 (2009).
4. Yamagata, T., Sugiura, H., Yokoyama, T., *et al.* Overexpression of CD-11b and CXCR1 on Circulating Neutrophils. *Chest* **132**, 890–899 (2007).
5. Yao, Y., Matsushima, H., Ohtola, J. A., *et al.* Neutrophil Priming Occurs in a Sequential Manner and Can Be Visualized in Living Animals by Monitoring IL-1β Promoter Activation. *J. Immunol.* **194**, 1211–1224 (2015).
6. Condliffe, A. M., Kitchen, E. & Chilvers, E. R. Neutrophil Priming: Pathophysiological Consequences and Underlying Mechanisms. *Clin. Sci.* **94**, 461–471 (1998).
7. Hellewell, P. G. & Williams, T. J. *Immunopharmacology of neutrophils (the handbook of immunopharmacology)*. (Academic Press, 1994).
8. Smedly, L. A., Tonnesen, M. G., Sandhaus, R. A., *et al.* Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase. *J. Clin. Invest.* **77**, 1233–1243 (1986).

9. Summers, C., Rankin, S., Condliffe, A., *et al.* Neutrophil kinetics in health and disease. *Trends Immunol.* **31**, 318–324 (2010).

10. Serpa Neto, A., Cardoso, S. O., Manetta, J. A., *et al.* Association Between Use of Lung-Protective Ventilation With Lower Tidal Volumes and Clinical Outcomes Among Patients Without Acute Respiratory Distress Syndrome. *JAMA* **308**, 1651 (2012).

11. Summers, C., Singh, N. R., White, J. F., *et al.* Pulmonary retention of primed neutrophils: a novel protective host response, which is impaired in the acute respiratory distress syndrome. *Thorax* **69**, 623–629 (2014).

12. Ziegenhagen, M. W., Rothe, M. E., Schlaak, M. & Müller-Quernheim, J. Bronchoalveolar and serological parameters reflecting the severity of sarcoidosis. *Eur. Respir. J.* **21**, 407–413 (2003).

13. Alexis, N. E., Hu, S.-C., Zeman, K., Alter, T. & Bennet, W. D. Induced Sputum Derives from the Central Airways. *Am. J. Respir. Crit. Care Med.* **164**, 1964–1970 (2001).

14. Lay, J. C., Peden, D. B. & Alexis, N. E. Flow cytometry of sputum: assessing inflammation and immune response elements in the bronchial airways. *Inhal. Toxicol.* **23**, 392–406 (2011).

15. Mann, B. S. & Chung, K. F. Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. *Respir. Res.* **7**, 59 (2006).

16. Morgan, D. J., Casulli, J., Chew, C., *et al.* Innate Immune Cell Suppression and the Link With Secondary Lung Bacterial Pneumonia. *Front. Immunol.* **9**, 2943 (2018).

17. Mor, G., Cardenas, I., Abrahams, V. & Guller, S. Inflammation and pregnancy: The role of the immune system at the implantation site. *Ann. N. Y. Acad. Sci.* **1221**, 80–87 (2011).

18. Mor, G. & Cardenas, I. The Immune System in Pregnancy: A Unique Complexity. *Am. J. Reprod. Immunol.* **63**, 425–433 (2010).

19. Carr, R. Neutrophil production and function in newborn infants. *Br. J. Haematol.* **110**, 18–28 (2000).

20. Ssemaganda, A., Kindinger, L., Bergin, P., *et al.* Characterization of Neutrophil Subsets in Healthy Human Pregnancies. *PLoS One* **9**, e85696 (2014).

21. Bender, J. S., Thang, H. & Glogauer, M. Novel rinse assay for the quantification of oral neutrophils and the monitoring of chronic periodontal disease. *J. Periodontal Res.* **41**, 214–220 (2006).

22. Aboodi, G. M., Goldberg, M. B. & Glogauer, M. Refractory Periodontitis Population Characterized by a Hyperactive Oral Neutrophil Phenotype. *J. Periodontol.* **82**, 726–733 (2011).

23. Fine, N., Hassanpour, S., Borenstein, A., *et al.* Distinct Oral Neutrophil Subsets Define Health and Periodontal Disease States. *J. Dent. Res.* **95**, 931–938 (2016).

24. Wilton, J. M., Renggli, H. H. & Lehner, T. A functional comparison of blood and gingival inflammatory polymorphonuclear leucocytes in man. *Clin.Exp.Immunol.* **27**, 152–158 (1977).

25. Raeste, A.-M. & Aura, A. Rate of migration of oral leukocytes in patients with periodontitis. *Eur. J. Oral Sci.* **86**, 43–51 (1978).

26. Shechter, R., London, A. & Schwartz, M. Orchestrated leukocyte recruitment to immune-privileged sites: Absolute barriers versus educational gates. *Nat. Rev. Immunol.* **13**, 206–218 (2013).

27. Niederkorn, J. Y. & Stein-Streilein, J. History and physiology of immune privilege. *Ocul. Immunol. Inflamm.* **18**, 19–23 (2010).

28. Stapleton, F., Keay, L., Edwards, K., *et al.* The Incidence of Contact Lens–Related Microbial Keratitis in Australia. *Ophthalmology* **115**, 1655–1662 (2008).

29. Purves, D., Augustine, G. J., Fitzpatrick, D., *et al. Neuroscience (3rd ed.)*. (Sunderland, MA, US: Sinauer Associates, 2004).

30. Kels, B. D., Grzybowski, A. & Grant-Kels, J. M. Human ocular anatomy. *Clin. Dermatol.* **33**, 140–146 (2015).

31. Cwiklik, L. Tear film lipid layer: A molecular level view. *Biochim. Biophys. Acta - Biomembr.* **1858**, 2421–2430 (2016).

32. Evans, D. J., McNamara, N. A. & Fleiszig, S. M. J. Life at the Front: Dissecting Bacterial-Host Interactions at the Ocular Surface. *Ocul. Surf.* **5**, 213–227 (2007).

33. Sweeney, D. F., Millar, T. J. & Raju, S. R. Tear film stability: A review. *Exp. Eye Res.* **117**, 28–38 (2013).

34. Holly, F. J. & Lemp, M. A. Tear physiology and dry eyes. *Surv. Ophthalmol.* **22**, 69–87 (1977).

35. Prydal, J. I., Arral, P., Woon, H. & Campbell, F. Study of human precorneal tear film thickness and structure using laser interferometry. *Investig. Ophthalmol. Vis. Sci.* **33**, 2006–2011 (1992).

36. Mcculley, J. P. & Shine, W. A compositional based model for the tear film lipid layer. *Trans. Am. Ophthalmol. Soc.* **95**, 79–88 (1997).

37. Green-Church, K. B., Butovich, I., Willcox, M., *et al.* The International Workshop on Meibomian Gland Dysfunction: Report of the Subcommittee on Tear Film Lipids and Lipid–Protein Interactions in Health and Disease. *Investig. Opthalmology Vis. Sci.* **52**, 1979 (2011).

38. Mathers, W. D. & Lane, J. A. Meibomian Gland Lipids, Evaporation, and Tear Film Stability. *Adv Exp Med Biol.* **438**, 349–360 (1998).

39. Craig, J. P. & Tomlinson, A. Importance of the Lipid Layer in Human Tear Film Stability and Evaporation. *Optom. Vis. Sci.* **74**, 8–13 (1997).

40. Zhou, L., Zhao, S. Z., Koh, S. K., *et al.* In-depth analysis of the human tear proteome. *J. Proteomics* **75**, 3877–3885 (2012).

41. Sack, R. A., Nunes, I., Beaton, A. & Morris, C. Host-defense mechanism of the ocular surfaces. *Biosci. Rep.* **21**, 463–480 (2001).

42. McDermott, A. M. Antimicrobial compounds in tears. *Exp. Eye Res.* **117**, 53–61 (2013).

43. Peterson, J. L., Phelps, E. D., Doll, M. A., Schaal, S. & Ceresa, B. P. The role of endogenous epidermal growth factor receptor ligands in mediating corneal epithelial homeostasis. *Investig. Ophthalmol. Vis. Sci.* **55**, 2870–2880 (2014).

44. Thakur, A., Willcox, M. D. P. & Stapleton, F. The proinflammatory cytokines and arachidonic acid metabolites in human overnight tears: Homeostatic mechanisms. *J. Clin. Immunol.* **18**, 61–70 (1998).

45. Wilson, G., O'leary, D. & Holden, B. Cell content of tears following overnight wear of a contact lens. *Curr. Eye Res.* **8**, 329–335 (1989).

46. Vannas, A., Sweeney, D. F., Holden, B. A., *et al.* Tear plasmin activity with contact lens wear. *Curr. Eye Res.* **11**, 243–251 (1995).

47. Tan, K. O., Sack, R. A., Holden, B. A. & Swarbrick, H. A. Temporal sequence of changes in tear film composition during sleep. *Curr. Eye Res.* **12**, 1001–1007 (1993).

48. Gorbet, M., Postnikoff, C. & Williams, S. The noninflammatory phenotype of neutrophils from the closed-eye environment: A flow cytometry analysis of receptor expression. *Investig. Ophthalmol. Vis. Sci.* **56**, 4582–4591 (2015).

49. Postnikoff, C. K., Huisingh, C., McGwin, G. & Nichols, K. K. Leukocyte Distribution in the Open Eye Tears of Normal and Dry Eye Subjects. *Curr. Eye Res.* **43**, 1253–1259 (2018).

50. Gao, Y., Min, K., Zhang, Y., *et al.* Female-Specific Downregulation of Tissue Polymorphonuclear Neutrophils Drives Impaired Regulatory T Cell and Amplified Effector T Cell Responses in Autoimmune Dry Eye Disease. *J. Immunol.* **195**, 3086–3099 (2015).

51. Thakur, A. & Willcox, M. D. P. Cytokine and lipid inflammatory mediator profile of human tears during contact lens associated inflammatory diseases. *Exp. Eye Res.* **67**, 9–19 (1998).

52. Mahajan, A., Grüneboom, A., Petru, L., *et al.* Frontline Science: Aggregated neutrophil extracellular traps prevent inflammation on the neutrophil-rich ocular surface. *J. Leukoc. Biol.* **105**, 1087–1098 (2019).

53. Strieter, R. M., Kasahara, K., Allen, R. M., *et al.* Cytokine-induced neutrophil-derived interleukin-8. *Am. J. Pathol.* **141**, 397–407 (1992).

54. Cubitt, C. L., Tang, Q., Monteiro, C. A., Lausch, R. N. & Oakes, J. E. IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes. *Investig. Ophthalmol. Vis. Sci.* **34**, 3199–3206 (1993).

55. Postnikoff, C. K. & Nichols, K. K. Neutrophil and T-cell homeostasis in the closed eye. *Investig. Ophthalmol. Vis. Sci.* **58**, 6212–6220 (2017).

56. Postnikoff, C. & Gorbet, M. The Effect of Closed-Eye Tear Film Conditions on Blood-Isolated Neutrophils, In Vitro. *Ocul. Immunol. Inflamm.* **26**, 706–716 (2018).

57. Butcher, S. K., Chahal, H., Nayak, L., *et al.* Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans. *J. Leukoc. Biol.* **70**, 881–6 (2001).

58. Bredius, R. G. M. & Medical, A. Role of neutrophil FcγRIIa (CD32) and FcγRIIIb (CD16) polymorphic forms in phagocytosis of human IgG1- and IgG3-opsonized bacteria and erythrocytes. *Transfus. Med. Rev.* **9**, 343 (2006).

59. Gorbet, M. & Willcox, M. D. P. Interactions of tear-film neutrophils with clinical bacteria. *Invest Ophthalmol Vis Sci.* **56**, 4048 (2015).

60. Dransfield, I., Buckle, A. M., Savill, J. S., *et al.* Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J. Immunol.* **153**, 1254–63 (1994).

61. Remold-O'donnell, E. & Parent, D. Downregulation of Neutrophil CD43 by Opsonized Zymosan. *Blood* **85**, 337–342 (1995).

62. Remold-O'Donnell, E. & Parent, D. Specific sensitivity of CD43 to neutrophil elastase. *Blood* **86**, 2395–2402 (1995).

63. Nathan, C. F., Xie, Q. W., Halbwachs-Mecarelli, L. & Jin, W. W. Albumin Inhibits the Spreading of Neutrophils by Blocking the Shedding of CD43 (Sialophorin, Leukosialin). *J. Cell Biol.* **122**, 243–256 (1993).

64. El Annan, J., Chauhan, S. K., Ecoiffier, T., *et al.* Characterization of Effector T Cells in Dry Eye Disease. *Investig. Opthalmology Vis. Sci.* **50**, 3802 (2009).

65. Stern, M. E., Gao, J., Schwalb, T. A., *et al.* Conjunctival T-Cell Subpopulations in Sjogren's and Non-Sjogren's Patients with Dry Eye. *Invest Ophthalmol Vis Sci* **43**, 2609–2614 (2002).

66. Reyes, N. J., Yu, C., Mathew, R., *et al.* Neutrophils cause obstruction of eyelid sebaceous glands in inflammatory eye disease in mice. *Sci. Transl. Med.* **10**, (2018).

67. Tibrewal, S., Ivanir, Y., Sarkar, J., *et al.* Hyperosmolar stress induces neutrophil extracellular trap formation: Implications for dry eye disease. *Investig. Ophthalmol. Vis. Sci.* **55**, 7961–7969 (2014).

68. Sonawane, S., Khanolkar, V., Namavari, A., *et al.* Ocular surface extracellular DNA and nuclease activity imbalance: A new paradigm for inflammation in dry eye disease. *Investig. Ophthalmol. Vis. Sci.* **53**, 8253–8263 (2012).

69. La Rosa, M., Lionetti, E., Reibaldi, M., *et al.* Allergic conjunctivitis: a comprehensive review of the literature. *Ital. J. Pediatr.* **39**, 18 (2013).

70. Geraldini, M., Neto, H. J. C., Riedi, C. A. & Rosário, N. A. Epidemiology of ocular allergy and co-morbidities in adolescents. *J. Pediatr. (Rio. J).* **89**, 354–360 (2013).

71. Arebro, J., Ekstedt, S., Hjalmarsson, E., *et al.* A possible role for neutrophils in allergic rhinitis revealed after cellular subclassification. *Sci. Rep.* **7**, 43568 (2017).

72. Leonardi, A. Allergy and allergic mediators in tears. *Exp. Eye Res.* **117**, 106–117 (2013).

73. Hosoki, K., Itazawa, T., Boldogh, I. & Sur, S. Neutrophil recruitment by allergens contribute to allergic sensitization and allergic inflammation. *Curr. Opin. Allergy Clin. Immunol.* **16**, 45–50 (2016).

74. Sack, R., Conradi, L., Beaton, A., *et al.* Antibody array characterization of inflammatory mediators in allergic and normal tears in the open and closed eye environments. *Exp. Eye Res.* **85**, 528–538 (2007).

75. Bonini, S., Bonini, S., Bucci, M. G., *et al.* Allergen dose response and late symptoms in a human model of ocular allergy. *J. Allergy Clin. Immunol.* **86**, 869–876 (1990).

Chapter 4 references

1. Gronert, K. Resolution, the grail for healthy ocular inflammation. *Exp. Eye Res.* **91**, 478–485 (2010).
2. Hellewell, P. G. & Williams, T. J. *Immunopharmacology of neutrophils (the handbook of immunopharmacology)*. (Academic Press, 1994).
3. Niederkorn, J. Y. & Stein-Streilein, J. History and physiology of immune privilege. *Ocul. Immunol. Inflamm.* **18**, 19–23 (2010).
4. Gorbet, M., Postnikoff, C. & Williams, S. The noninflammatory phenotype of neutrophils from the closed-eye environment: A flow cytometry analysis of receptor expression. *Investig. Ophthalmol. Vis. Sci.* **56**, 4582–4591 (2015).
5. Wilson, G., O'leary, D. & Holden, B. Cell content of tears following overnight wear of a contact lens. *Curr. Eye Res.* **8**, 329–335 (1989).
6. Tan, K. O., Sack, R. A., Holden, B. A. & Swarbrick, H. A. Temporal sequence of changes in tear film composition during sleep. *Curr. Eye Res.* **12**, 1001–1007 (1993).
7. Fortunati, E., Kazemier, K. M., Grutters, J. C., Koenderman, L. & Van Den Bosch, V. J. M. M. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clin. Exp. Immunol.* **155**, 559–566 (2009).
8. Bender, J. S., Thang, H. & Glogauer, M. Novel rinse assay for the quantification of oral neutrophils and the monitoring of chronic periodontal disease. *J. Periodontal Res.* **41**, 214–220 (2006).
9. Ssemaganda, A., Kindinger, L., Bergin, P., *et al.* Characterization of Neutrophil Subsets in Healthy Human Pregnancies. *PLoS One* **9**, e85696 (2014).

10. Postnikoff, C. K. & Nichols, K. K. Neutrophil and T-cell homeostasis in the closed eye. *Investig. Ophthalmol. Vis. Sci.* **58**, 6212–6220 (2017).

11. Mahajan, A., Grüneboom, A., Petru, L., *et al.* Frontline Science: Aggregated neutrophil extracellular traps prevent inflammation on the neutrophil-rich ocular surface. *J. Leukoc. Biol.* **105**, 1087–1098 (2019).

12. Lanier, L. L. & Warner, N. L. Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. *J. Immunol. Methods* **47**, 25–30 (1981).

13. Brown, M. & Wittwer, C. Flow Cytometry: Principles and Clinical Applications in Hematology. *Clin. Chem.* **46**, 1221–1229 (2000).

14. Abdel-Salam, B. K. A. & Ebaid, H. Clinical immunology Expression of CD11b and CD18 on polymorphonuclear neutrophils stimulated with interleukin-2. *Cent. Eur. J. Immunol.* **2**, 209–215 (2014).

15. Kujipers, T., Tool, A., van der Schoot, C., *et al.* Membrane surface antigen expression on neutrophils: A reappraisal of the use of surface markers for neutrophil activation. *Blood* **78**, 1105–1111 (1991).

16. Dransfield, I., Buckle, A. M., Savill, J. S., *et al.* Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J. Immunol.* **153**, 1254–63 (1994).

17. Wagner, C. & Hänsch, G. M. Genetic deficiency of CD16 , the low-affinity receptor for immunoglobulin G , has no impact on the functional capacity of polymorphonuclear neutrophils. *Eur. J. Clin. Invest.* **34**, 149–155 (2004).

18. Nuutila, J., Jalava-Karvinen, P., Hohenthal, U., *et al.* Use of complement regulators,

CD35, CD46, CD55, and CD59, on leukocytes as markers for diagnosis of viral and bacterial infections. *Hum. Immunol.* **74**, 522–530 (2013).

19. Thavarajah, R., Mudimbaimannar, V., Elizabeth, J., Rao, U. & Ranganathan, K. Chemical and physical basics of routine formaldehyde fixation. *J Oral Maxillofac Pathol.* **16**, 400–405 (2012).

20. Fox, C., Johnson, F., Whiting, J. & Roller, P. Formaldehyde Fixation. *J Histochem Cytochem.* **33**, 845–853 (1985).

21. Pollice, A. A., Philip Mccoy, J., Shackney, S. E., *et al.* Sequential paraformaldehyde and methanol fixation for simultaneous flow cytometric analysis of DNA, cell surface proteins, and intracellular proteins. *Cytometry* **13**, 432–444 (1992).

22. Macey, M. G. & McCarthy, D. A. Quantitation of adhesion molecules and other function-associated antigens on human peripheral blood leucocytes. *Cytometry* **14**, 898–908 (1993).

23. Stewart, J. C., Villasmil, M. L. & Frampton, M. W. Changes in fluorescence intensity of selected leukocyte surface markers following fixation. *Cytom. Part A* **71**, 379–385 (2007).

24. Glasser, L. & Fiederlein, R. L. The effect of various cell separation procedures on assays of neutrophil function. A critical appraisal. *Am. J. Clin. Pathol.* **93**, 662–669 (1990).

25. Berkow, R., Tzeng, D., Williams, L. & Baehner, R. The comparative responses of human polymorphonuclear leukocytes obtained by counterflow centrifugal elutriation and Ficoll-Hypaque density centrifugation. I. Resting volume, stimulus-induced

superoxide production, and primary and specific granule release. *J Lab Clin Med* **102**, 732–742 (1983).

26. Fearon, D. T. & Collins, L. A. Increased expression of C3b receptors on polymorphonuclear leukocytes induced by chemotactic factors and by purification procedures. *J. Immunol.* **130**, 370–5 (1983).

27. Porteu, F., Fischer, A., Descamps-Latscha, B. & Halbwachs-Mecarelli, L. Defective complement receptors (CR1 and CR3) on erythrocytes and leukocytes of factor I (C3b-inactivator) deficient patients. *Clin. Exp. Immunol.* **66**, 463–71 (1986).

28. Mecarthy, D. A., Macey, M. G., Cahill, M. R. & Newland, A. C. Effect of Fixation on Quantification of the Expression of Leucocyte Function-Associated Surface Antigens. **17**, 39–49 (1994).

29. Macey, M. G., Jiang, X. P., Veys, P., McCarthy, D. & Newland, A. C. Expression of functional antigens on neutrophils. *J. Immunol. Methods* **149**, 37–42 (1992).

30. Hamblin, A., Taylor, M., Bernhagen, J., *et al.* A method of preparing blood leucocytes for flow cytometry which prevents uoregulation of leucocyte integrins. *J. Immunol. Methods* **146**, 219–228 (1992).

31. Lal, R. B., Edison, L. J. & Chused, T. M. Fixation and Long-Term Storage of Human Lymphocytes for Surface Marker Analysis by Flow Cytometry. *Cytometry* **9**, 213–219 (1988).

32. Forsyth, D. K. & Levinsky, J. R. Preparative procedures of cooling and re-warming increase leukocyte integrin expression and function on neutrophils. *J. Immunol. Methods* **128**, 159–163 (1990).

33. Gorbet, M. B. & Sefton, M. V. Leukocyte activation and leukocyte procoagulant activities after blood contact with polystyrene and polyethylene glycol–immobilized polystyrene beads. *J. Lab. Clin. Med.* **137**, 345–355 (2001).

34. Macey, M. G., McCarthy, D. A., Vordermeier, S., Newland, A. C. & Brown, K. A. Effects of cell purification methods on CD11b and l-selectin expression as well as the adherence and activation of leucocytes. *J. Immunol. Methods* **181**, 211–219 (1995).

35. Kuijpers, T. W. Neutrophil migration across monolayers of cytokine-prestimulated endothelial cells: a role for platelet-activating factor and IL-8. *J. Cell Biol.* **117**, 565–572 (1992).

Chapter 5 references

1. Hellewell, P. G. & Williams, T. J. *Immunopharmacology of neutrophils (the handbook of immunopharmacology)*. (Academic Press, 1994).
2. Gorbet, M., Postnikoff, C. & Williams, S. The noninflammatory phenotype of neutrophils from the closed-eye environment: A flow cytometry analysis of receptor expression. *Investig. Ophthalmol. Vis. Sci.* **56**, 4582–4591 (2015).
3. Wilson, G., O'leary, D. & Holden, B. Cell content of tears following overnight wear of a contact lens. *Curr. Eye Res.* **8**, 329–335 (1989).
4. Tan, K. O., Sack, R. A., Holden, B. A. & Swarbrick, H. A. Temporal sequence of changes in tear film composition during sleep. *Curr. Eye Res.* **12**, 1001–1007 (1993).
5. Postnikoff, C. K. & Nichols, K. K. Neutrophil and T-cell homeostasis in the closed eye. *Investig. Ophthalmol. Vis. Sci.* **58**, 6212–6220 (2017).
6. Mahajan, A. *et al.* Frontline Science: Aggregated neutrophil extracellular traps prevent inflammation on the neutrophil-rich ocular surface. *J. Leukoc. Biol.* **105**, 1087–1098 (2019).
7. Fullard, R. J. & Wilson, G. S. Investigation of sloughed corneal epithelial cells collected by non-invasive irrigation of the corneal surface. *Curr. Eye Res.* **5**, 847–856 (1986).
8. Peterson, R. C. *et al.* Non-invasive collection and examination of human corneal epithelial cells. *Optom. Vis. Sci.* **88**, 1317–1325 (2011).
9. Gorbet, M., Luensmann, D. & Jones, L. The response of tear film neutrophils to occasional overnight lens wear. *Investig. Ophthalmol. {&} Vis. Sci.* **54**, 2069 (2013).

10. Futosi, K., Fodor, S. & Mócsai, A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int. Immunopharmacol.* **17**, 638–650 (2013).

11. Strieter, R. M. *et al.* Cytokine-induced neutrophil-derived interleukin-8. *Am. J. Pathol.* **141**, 397–407 (1992).

12. Dransfield, I. *et al.* Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J. Immunol.* **153**, 1254–63 (1994).

13. Baggiolini, M., Walz, A. & Kunkel, S. L. Neutrophil-activating peptide-1/Interleukin 8, a Novel Cytokine That Activates Neutrophils. *J Clin Invest.* **84**, 1045–1049 (1989).

14. Nicholson, G. C. *et al.* A novel flow cytometric assay of human whole blood neutrophil and monocyte CD11b levels: Upregulation by chemokines is related to receptor expression, comparison with neutrophil shape change, and effects of a chemokine receptor (CXCR2) antagonist. *Pulm. Pharmacol. Ther.* **20**, 52–59 (2007).

15. White, J. R. *et al.* Identification of a Potent, Selective Non-peptide CXCR2 Antagonist That Inhibits Interleukin-8-induced Neutrophil Migration. *J. Biol. Chem.* **273**, 10095–10098 (1998).

16. Thakur, A., Willcox, M. D. P. & Stapleton, F. The proinflammatory cytokines and arachidonic acid metabolites in human overnight tears: Homeostatic mechanisms. *J. Clin. Immunol.* **18**, 61–70 (1998).

17. Wozniak, A., Betts, W. H., Murphy, G. A., Queen, T. & Hospital, E. Interleukin-8 primes human neutrophils for enhanced. *Production* 608–615 (1993).

18. Altstaedt, J., Kirchner, H. & Rink, L. Cytokine production of neutrophils is limited to

interleukin-8. *Immunology* **89**, 563–568 (1996).

19. Videm, V. & Strand, E. Changes in Neutrophil Surface-Receptor Expression After Stimulation with FMLP, Endotoxin, Interleukin-8 and Activated Complement Compared to Degranulation. *Scand. J. Immunol.* **59**, 25–33 (2004).

20. Wittmann, S., Rothe, G., Schmitz, G. & Fröhlich, D. Cytokine upregulation of surface antigens correlates to the priming of the neutrophil oxidative burst response. *Cytom. Part A* **57A**, 53–62 (2004).

21. Knall, C. *et al.* Interleukin-8 Regulation of the Ras/Raf/Mitogen-activated Protein Kinase Pathway in Human Neutrophils. *J. Biol. Chem.* **271**, 2832–2838 (1996).

22. Wu, D., Larosa, G. J. & Simon, M. G Protein-Coupled Signal Transduction Pathways for Interleukin-8. *Science (80-.).* **261**, 101–103 (1993).

23. Worthen, G. S., Avdi, N., Buhl, A. M., Suzuki, N. & Johnsonv, G. L. FMLP Activates Ras and Raf in Human Neutrophils Potential Role in Activation of MAP Kinase. *J. Clin. Invest.* **94**, 815–823 (1994).

24. Pignatti, P. *et al.* Downmodulation of CXCL8/IL-8 receptors on neutrophils after recruitment in the airways. *J. Allergy Clin. Immunol.* **115**, 88–94 (2005).

25. Lay, J. C., Peden, D. B. & Alexis, N. E. Flow cytometry of sputum: assessing inflammation and immune response elements in the bronchial airways. *Inhal. Toxicol.* **23**, 392–406 (2011).

26. Thakur, A. & Willcox, M. D. P. Cytokine and lipid inflammatory mediator profile of human tears during contact lens associated inflammatory diseases. *Exp. Eye Res.* **67**, 9–19 (1998).

27. Middelhoven, P. J., Van Buul, J. D., Hordijk, P. L. & Roos, D. Different proteolytic mechanisms involved in FcgammaRIIIb shedding from human neutrophils. *Clin. Exp. Immunol.* **125**, 169–175 (2001).

28. Uriarte, S. M. *et al.* Comparison of Proteins Expressed on Secretory Vesicle Membranes and Plasma Membranes of Human Neutrophils. *J. Immunol.* **180**, 5575–5581 (2008).

29. Zhang, J., Kaupke, C. J., Yousefi, S., Cesario, T. C. & Vaziri, N. D. Flow cytometric investigation of neutrophil activation pathways by n-formyl-Met-Leu-Phe and phorbol myristate acetate. *Biol. Cell* **84**, 147–153 (1995).

30. Fine, N. *et al.* Distinct Oral Neutrophil Subsets Define Health and Periodontal Disease States. *J. Dent. Res.* **95**, 931–938 (2016).

31. Ssemaganda, A. *et al.* Characterization of Neutrophil Subsets in Healthy Human Pregnancies. *PLoS One* **9**, e85696 (2014).

32. Fortunati, E., Kazemier, K. M., Grutters, J. C., Koenderman, L. & Van Den Bosch, V. J. M. M. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clin. Exp. Immunol.* **155**, 559–566 (2009).

Chapter 6 references

1. Hellewell, P. G. & Williams, T. J. *Immunopharmacology of neutrophils (the handbook of immunopharmacology)*. (Academic Press, 1994).
2. Niederkorn, J. Y. & Stein-Streilein, J. History and physiology of immune privilege. *Ocul. Immunol. Inflamm.* **18**, 19–23 (2010).
3. Gorbet, M., Postnikoff, C. & Williams, S. The noninflammatory phenotype of neutrophils from the closed-eye environment: A flow cytometry analysis of receptor expression. *Investig. Ophthalmol. Vis. Sci.* **56**, 4582–4591 (2015).
4. Thakur, A., Willcox, M. D. P. & Stapleton, F. The proinflammatory cytokines and arachidonic acid metabolites in human overnight tears: Homeostatic mechanisms. *J. Clin. Immunol.* **18**, 61–70 (1998).
5. Postnikoff, C. K. & Nichols, K. K. Neutrophil and T-cell homeostasis in the closed eye. *Investig. Ophthalmol. Vis. Sci.* **58**, 6212–6220 (2017).
6. Gorbet, M. & Willcox, M. D. P. Interactions of tear-film neutrophils with clinical bacteria. *Invest Ophthalmol Vis Sci.* **56**, 4048 (2015).
7. Sbarra, A. J. & Karnovskyi, M. L. The Biochemical Basis of Phagocytosis I. Metablolic Changes During the Ingestion of Particles by Polymorphonuclear Leukocytes. *J. Biol. Chem.* **234**, 1355–1362 (1959).
8. Holmes, B., Quie, P., Windhort, D. & Good, R. Fatal granulomatous disease of chilhood: An inborn abnormality of phagocytic function. *Lancet* **1**, 1225–1228 (1966).
9. Karlsson, A., Nixon, J. B. & McPhail, L. C. Phorbol myristate acetate induces neutrophil NADPH-oxidase activity by two separate signal transduction pathways:

dependent or independent of phosphatidylinositol 3-kinase. *J. Leukoc. Biol.* **67**, 396–404 (2000).

10. Zhang, Y., Dai, M. & Yuan, Z. Methods for the detection of reactive oxygen species. *Anal. Methods* **10**, 4625–4638 (2018).
11. Zhu, L., Castranova, V. & He, P. fMLP-stimulated neutrophils increase endothelial [Ca 2+] i and microvessel permeability in the absence of adhesion: role of reactive oxygen species. *Am. J. Physiol. Circ. Physiol.* **288**, H1331–H1338 (2005).
12. Bedouhène, S., Moulti-Mati, F., Hurtado-Nedelec, M., My-Chan Dang, P. & El-Benna, J. Luminol-amplified chemiluminescence detects mainly superoxide anion produced by human neutrophils. *Am J Blood Res* **7**, 41–48 (2017).
13. Sigma-Aldrich. HANKS' BALANCED SALTS [HBSS] (H1387) - Product Information Sheet. *Sigma-Aldrich* (2007).
14. Sack, R., Conradi, L., Beaton, A., *et al.* Antibody array characterization of inflammatory mediators in allergic and normal tears in the open and closed eye environments. *Exp. Eye Res.* **85**, 528–538 (2007).
15. Dahlgren, C. & Karlsson, A. Respiratory burst in human neutrophils. *J. Immunol. Methods* **232**, 3–14 (1999).
16. Nick, J. A., Avdi, N., Young, S. K., *et al.* Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. *J Clin Invest.* **99**, 975–986 (1997).
17. Worthen, G. S., Avdi, N., Buhl, A. M., Suzuki, N. & Johnsonv, G. L. FMLP Activates Ras and Raf in Human Neutrophils Potential Role in Activation of MAP Kinase. *J.*

Clin. Invest. **94**, 815–823 (1994).

18. Sato, T., Hongu, T., Sakamoto, M., Funakoshi, Y. & Kanaho, Y. Molecular Mechanisms of N-Formyl-Methionyl-Leucyl-Phenylalanine-Induced Superoxide Generation and Degranulation in Mouse Neutrophils: Phospholipase D Is Dispensable. *Mol. Cell. Biol.* **33**, 136–145 (2013).
19. Müller, J., Petković, M., Schiller, J., *et al.* Effects of lysophospholipids on the generation of reactive oxygen species by fMLP- and PMA-stimulated human neutrophils. *Luminescence* **17**, 141–149 (2002).
20. Bender, J. G. & Van Epps, D. E. Analysis of the bimodal chemiluminescence pattern stimulated in human neutrophils by chemotactic factors. *Infect. Immun.* **41**, 1062–70 (1983).
21. Djerdjouri, B., Lenoir, M., Giroud, J.-P. & Périanin, A. Contribution of Mitogen-Activated Protein Kinase to Stimulation of Phospholipase D by the Chemotactic Peptide fMet-Leu-Phe in Human Neutrophils. *Biochem. Biophys. Res. Commun.* **264**, 371–375 (1999).
22. Orlic, T., Loomis, W. H., Shreve, A., Namiki, S. & Junger, W. G. Hypertonicity increases cAMP in PMN and blocks oxidative burst by PKA-dependent and -independent mechanisms. *Am. J. Physiol. Physiol.* **282**, C1261–C1269 (2002).
23. Thompson, H. L., Shiroo, M. & Saklatvala, J. The chemotactic factor N -formylmethionyl-leucyl-phenylalanine activates microtubule-associated protein 2 (MAP) kinase and a MAP kinase kinase in polymorphonuclear leucocytes. *Biochem. J.* **290**, 483–488 (1993).

24. Mahajan, A., Grüneboom, A., Petru, L., *et al.* Frontline Science: Aggregated neutrophil extracellular traps prevent inflammation on the neutrophil-rich ocular surface. *J. Leukoc. Biol.* **105**, 1087–1098 (2019).

25. Aboodi, G. M., Goldberg, M. B. & Glogauer, M. Refractory Periodontitis Population Characterized by a Hyperactive Oral Neutrophil Phenotype. *J. Periodontol.* **82**, 726–733 (2011).

26. Eggleton, P., Wang, L., Penhallow, J., Crawford, N. & Brown, K. A. Differences in oxidative response of subpopulations of neutrophils from healthy subjects and patients with rheumatoid arthritis. *Ann. Rheum. Dis.* **54**, 916–923 (1995).

27. Rothe, G. & Valet, G. Flow Cytometric Analysis of Respiratory Burst Activity in Phagocytes With Hydroethidine and 2′,7′-Dichlorofluorescin. *J. Leukoc. Biol.* **47**, 440–448 (1990).

Chapter 7 references

1. Björnsdottir, H., Welin, A., Dahlgren, C., Karlsson, A. & Bylund, J. Quantification of heterotypic granule fusion in human neutrophils by imaging flow cytometry. *Data Br.* **6**, 386–393 (2016).
2. Aboodi, G. M., Goldberg, M. B. & Glogauer, M. Refractory Periodontitis Population Characterized by a Hyperactive Oral Neutrophil Phenotype. *J. Periodontol.* **82**, 726–733 (2011).
3. Zu, Y., Qi, J., Gilchrist, A., *et al.* p38 Mitogen-Activated Protein Kinase Activation Is Required for Human Neutrophil Function Triggered by TNF- α or FMLP Stimulation. *J Immunol* **160**, 1982–1989 (1998).
4. Dransfield, I., Buckle, A. M., Savill, J. S., *et al.* Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J. Immunol.* **153**, 1254–63 (1994).
5. Gorbet, M., Postnikoff, C. & Williams, S. The noninflammatory phenotype of neutrophils from the closed-eye environment: A flow cytometry analysis of receptor expression. *Investig. Ophthalmol. Vis. Sci.* **56**, 4582–4591 (2015).
6. Tecchio, C., Micheletti, A. & Cassatella, M. A. Neutrophil-Derived Cytokines: Facts Beyond Expression. *Front. Immunol.* **5**, 1–7 (2014).
7. Postnikoff, C. K. & Nichols, K. K. Neutrophil and T-cell homeostasis in the closed eye. *Investig. Ophthalmol. Vis. Sci.* **58**, 6212–6220 (2017).
8. Postnikoff, C. & Gorbet, M. The Effect of Closed-Eye Tear Film Conditions on Blood-Isolated Neutrophils, In Vitro. *Ocul. Immunol. Inflamm.* **26**, 706–716 (2018).

9. Postnikoff, C. K., Huisingh, C., McGwin, G. & Nichols, K. K. Leukocyte Distribution in the Open Eye Tears of Normal and Dry Eye Subjects. *Curr. Eye Res.* **43**, 1253–1259 (2018).

10. Reyes, N. J., Yu, C., Mathew, R., *et al.* Neutrophils cause obstruction of eyelid sebaceous glands in inflammatory eye disease in mice. *Sci. Transl. Med.* **10**, eaas9164 (2018).

11. Carnt, N. A., Cipriani, V., Stapleton, F. J., Calder, V. & Willcox, M. D. Association study of single nucleotide polymorphisms in IL-10 and IL-17 genes with the severity of microbial keratitis. *Contact Lens Anterior Eye* 0–1 (2019). doi:10.1016/j.clae.2019.06.007

12. Carnt, N. A., Willcox, M. D. P., Hau, S., *et al.* Association of single nucleotide polymorphisms of interleukins-1β, -6, and -12B with contact lens keratitis susceptibility and severity. *Ophthalmology* **119**, 1320–1327 (2012).

13. Fine, N., Hassanpour, S., Borenstein, A., *et al.* Distinct Oral Neutrophil Subsets Define Health and Periodontal Disease States. *J. Dent. Res.* **95**, 931–938 (2016).

14. Stapleton, F., Keay, L., Edwards, K. & Holden, B. The Epidemiology of Microbial Keratitis With Silicone Hydrogel Contact Lenses. *Eye Contact Lens Sci. Clin. Pract.* **39**, 78–84 (2013).

15. Szczotka-Flynn, L. & Chalmers, R. Incidence and epidemiologic associations of corneal infiltrates with silicone hydrogel contact lenses. *Eye Contact Lens Sci. Clin. Pract.* **39**, 49–52 (2013).