

The Phenotype of Tear Neutrophils and Their Role in Ocular Homeostasis and Inflammation

by

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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

Introduction: Neutrophils (polymorphonuclear neutrophils, PMNs) are part of the innate immune system with potent killing mechanisms against pathogens. Although the anterior segment of the eye has a unique characteristic, immune privilege, a significant influx of PMNs has been detected after a prolonged eye closure at night, such as sleep. However, the functions and roles of tear PMNs in ocular homeostasis and complications remain largely unknown. The overall objectives of this thesis were to examine and compare the phenotype and functions of tear PMNs from healthy participants and participants with ocular allergy.

Methods: Participants used a gentle eye wash method to collect tear PMNs from the ocular surface by washing their eyes with sterile phosphate buffer saline (PBS).

- *Study 1:* Tear PMNs were either isolated from the eyewash by using the MACS-Column or EasySep cell separation system or reconstituted by centrifugation. Stimulated (phorbol-12-myristate-13-acetate (PMA), lipopolysaccharides (LPS) or N-Formylmethionyl-leucyl-phenylalanine (fMLP)) and unstimulated ROS production by both isolated and non-isolated tear PMNs were measured using luminol-enhanced chemiluminescence for 60 min.
- *Study 2:* Tear PMNs were incubated with different medium conditions, Hank's Balanced Salt Solution (HBSS) only, HBSS with lactoferrin, lysozyme, mucin, albumin, and IgG (ATS), and ATS without lactoferrin and lysozyme (MAI). Unstimulated or PMA-stimulated ROS production by tear PMNs was measured using luminol-enhanced chemiluminescence and flow cytometry with DCFH-DA.
- *Study 3:* Tear leukocytes were collected at four different time points, after 2-hr and 7-hr of sleep at night, after 2-hr sleep during the day, and towards the end of the day (around 5 pm). After stimulation with fMLP, changes in the degranulation (lactoferrin, CD66b, CD63) and cell aging state (CD184) of tear PMNs were measured via flow cytometry. Neutrophil extracellular traps (NETs) were quantified by flow cytometry and visualized by microscopy following staining with myeloperoxidase, citrullinated histones, and CD15.
- *Study 4:* Tear PMNs were collected from participants suffering from ocular allergy on two consecutive days, after a full night of sleep in the morning on Day 1 and at the end of the day

around 4:30 pm on Day 2. Tear PMNs were either activated with fMLP or left unstimulated, followed by staining with antibodies against degranulation markers (CD66b and CD63), adhesion markers (CD11b and CD54), eosinophil marker (CD193), aging markers (CD184 and CD62L), as well as with the fluorescent probe DCFH-DA. The intensity of fluorescence was measured via flow cytometry.

Results:

- *Study 1:* Tear PMNs have a high level of constitutive/spontaneous ROS production even in the absence of any stimulus. However, tear PMNs failed to appropriately respond to LPS and fMLP, although they were able to produce ROS in response to PMA. Higher ROS generation was observed in isolated tear PMNs which may be caused by priming from the magnetic bead cell separation system.
- *Study 2:* The presence of tear proteins significantly reduced the unstimulated and PMA-stimulated ROS production by tear PMNs in HBSS and ATS. However, the findings on ROS production by PMA-stimulated PMNs incubated in MAI were different between the flow cytometry and chemiluminescence, suggesting that lactoferrin and lysozyme may have a greater impact on extracellular ROS production.
- *Study 3:* Significantly more cells were collected from the nighttime compared to the daytime. 2hr EC night tear PMNs were less degranulated than 7hr EC night tear PMNs and possessed a higher activation ratio in response to fMLP. Furthermore, 7hr EC night tear PMNs exhibited hypersegmented nuclei and were prone to aggregation, when compared to 2hr EC night tear PMNs, suggesting an aged and activated phenotype. A significantly increased number of NETs were present in 7hr nocturnal closed-eye tear samples.
- *Study 4:* There were significantly more tear PMNs collected from individuals with ocular allergy compared to healthy participants upon awakening. Tear PMNs from ocular allergy participants exhibited a less activated phenotype but a higher activation potential in response to fMLP compared to healthy participants, which was correlated with their younger maturation state. However, no significant difference in the production of ROS by tear PMNs between these two groups was observed.

Conclusion: Tear PMNs become more aged and activated with increasing eye closure time at night, which can potentially aid in ocular surface surveillance. The presence of tear proteins may limit the ROS release by tear PMNs, thereby protecting the ocular environment from potential damage. However, in individuals suffering from ocular allergy, tear PMNs may be constantly recruited to the ocular surface, contributing to symptoms of ocular allergy and development of ocular complications associated with inflammation. This thesis identified some of the functionalities and potential roles of tear PMNs in maintaining ocular homeostasis and regulating inflammation. While further investigation is needed to comprehensively characterize the underlying mechanisms of tear PMNs involved in ocular inflammation, the current results may support the development of new therapeutical strategies to reduce ocular surface inflammation.

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Dedication

To my grandpa.

I consider myself incredibly fortunate to have such a loving and supportive grandpa.

I miss you so much.

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List of Abbreviations

APC: antigen-presenting cells

ARDS: Acute respiratory distress syndrome

ATS: Artificial tear solution

AUC: Area under the curve

BMAL1: Brain and muscle arnt-like 1

CCL: C-C motif chemokine ligand

CGD: Chronic granulomatous disease

CL: Chemiluminescence

CNS: Central nervous system

CRYs: CRYPTOCHROMEs

CXCR: C-X-C motif chemokine receptor

DAMPs: Damage-associated molecular patterns

DCFHDA: 2',7'Dichlorofluorescein diacetate

DED: Dry eye disease

DMEM: Dulbecco's modified eagle media

eDNA: Extracellular deoxyribonucleic acid

ELISA: Enzyme-linked immunosorbent assay

FBS: Fetal bovine serum

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

GPCR: G-protein-coupled receptor

HBSS: Hanks's balanced salt solution

hCECs: Human corneal epithelial cells

HO $\cdot$: Hydroxyl radical

HOCl: Hypochlorous acid

H₂O₂: Hydrogen peroxide

HPA: Hypothalamus-Pituitary-Adrenal axis

H3Cit: Citrullinated histones

ICAM-1: Intercellular adhesion molecule-1

IFN- α : Interferon- α

IL-3: Interleukin-3

IL-8: Interleukin-8

ipRGCs: Intrinsically photoreceptive retinal ganglion cells

KC: Keratoconus

LDNs: Low-density neutrophils

LPR: Late-phase reaction

LPS: Lipopolysaccharides

LTB₄: Leukotriene B₄

Mac-1: Macrophage-1 antigen

MAI: ATS but no lactoferrin and lysozyme (ATS with only mucin, albumin, and IgG)

MAPK: Mitogen-activated-protein kinase

MFI: Mean fluorescent intensity

MGD: Meibomian gland dysfunction

MK: Microbial keratitis

MMP: metalloproteinase

MPO: Myeloperoxidase

NADPH oxidase: Nicotinamide adenine dinucleotide phosphate oxidase

NDGs: Normal-density granulocytes

NE: Neutrophil elastase

NETs: Neutrophil extracellular traps

NK: Natural Killer cells

NLRs: Neutrophil-to-lymphocyte ratio

O²⁻: Superoxide anion

OA: Ocular allergy

PA: Phosphatidic acid

PAD4: Peptidyl-arginine deaminase 4

PAMPs: Pathogen-associated molecular patterns

PBS: Phosphate-buffered saline

PCD: Programmed cell death

PERs: PERIODs

PKC: Protein kinase C

PLA2: Phospholipase A2

PLC: Phospholipase C

PLD: Phospholipase D

PI3K: Phosphatidylinositol 3-kinase

PMA: Phorbol-12-myristate-13-acetate

PMN: Polymorphonuclear neutrophils

PRRs: Pattern-recognition receptors

RNA: Ribonucleic acid ROS: Reactive oxygen species

ROS: Reactive oxygen species

rTEM: Transendothelial cell migration

SAC: Seasonal allergic conjunctivitis

SCN: Suprachiasmatic nucleus

SD: Standard deviation

sIgA: Secretory immunoglobulin A

SLE: Systemic lupus erythematosus

SOD: Superoxide dismutase

STMR: Seven transmembrane-spanning receptor

S1PRs: Sphingosine-1-phosphate receptors

TFOS: The tear film and ocular surface society

TNF- α : Tumor necrosis factor α

TLRs: Toll-like receptors

2hr EC night samples: Samples collected after 2 hours eye closure of sleep at night

2hr EC day samples: Samples collected after 2 hours eye closure of sleep during the day

7hr EC night samples: Samples collected after 7 hours eye closure of sleep at night

Chapter 1. Thesis Introduction

1.1 Introduction

Neutrophils (polymorphonuclear neutrophils, PMNs) are the most abundant leukocytes in blood and the first cells to arrive at sites of inflammation and infection. They carry out their functions through various mechanisms, such as degranulation, respiratory burst, release of neutrophil extracellular traps (NETs), and phagocytosis [1]. Despite being a potent component of the innate immune system, PMNs have often been ignored or oversimplified in terms of their functions, subtypes, and roles in both physiological and pathological conditions. Besides their direct killing mechanisms against pathogens, PMNs are capable of regulating the responses of other immune cells via the release of pro-inflammatory mediators and surface receptors. For instance, PMNs can attract and activate T lymphocytes to inflammatory sites and can travel to lymph nodes to activate adaptive immune cells by secreting cytokines or functioning as antigen presenting cells (APCs), as reviewed in [2]. These findings suggest that PMNs are not merely simple phagocytes but rather active modulators of the immune system. Furthermore, a growing body of evidence reveals that PMNs exhibit different phenotypes under normal and abnormal physiological conditions, such as the B-helper phenotype of PMNs in the murine spleen [3] and the low-density PMNs (LDNs) in the blood of patients with systemic lupus erythematosus [4]. Hence, the heterogeneity of PMN functions and phenotypes may highlight the complexity of these cells and the need to better understand the role of PMNs in both normal and pathological scenarios.

The eye is a complex sensory organ responsible for vision, constantly exposed to a variety of external threats, including microbes and allergens. The incidence of immune-mediated damage to ocular structures, such as the cornea, is relatively low under normal physiological conditions due to the suppression of functions of immune cells. However, recent studies in healthy eyes have shown that following sleep, hundreds of thousands of leukocytes, with the main population being PMNs [5–8], can be harvested from the ocular surface by using a gentle eyewash method which has been utilized since 2013 [6]. In these studies and ongoing research, the PMNs that can be found on the ocular surface are being referred to as *tear-film* or *tear* PMNs to accurately reflect where they are being observed and collected (i.e., these cells are not in the corneal or conjunctival tissues) [5–8]. Several research groups, including our own, have now shown that a substantial number of activated PMNs can be consistently and reproducibly collected from the ocular surface after a full night of sleep, irrespective of whether the participants performed self-collection at home, or the collection is done by clinicians after an overnight sleep at the clinic. The gentle eye wash method developed by Gorbet et al has been adopted by several research groups across the world for cell collection purposes with minor modifications [5–14]. The origin of tear PMNs and other leukocytes present in the

eyewash is yet to be confirmed, although two hypotheses have been proposed: they either extravasate from the blood vessels of the ocular surface or migrate from the lacrimal glands [6,15]. The increase of chemoattractants, such as Interleukin-8 (IL-8), leukotriene B₄ (LTB₄), and C5a, in closed-eye tears may support the former hypothesis [8,16]. However, the link between increased cytokine levels and recruitment of tear PMNs to the ocular surface during the night remains largely unexplored due to limited research in this area. Furthermore, the infiltration of tear PMNs to the ocular environment has been shown to display a peak in the early morning and a trough at night [10], which may suggest that their presence is linked to a circadian rhythm.

Tear PMNs collected after sleep exhibit signs of activation and have also been shown to be quiescent *in vitro*, meaning they cannot alter their expression of membrane receptors in response to stimuli [6]. The lack of response might be due to immune paralysis (ie, they have exhausted their potential of activation while in the closed-eye environment [7]) or it may be due to a different phenotype altogether. Tear PMNs collected from healthy eyes after sleep do not display signs of apoptosis when characterized with caspase and propidium iodide, and they also spontaneously release reactive oxygen species in the absence of any stimulus *in vitro* [14]. With the presence of potent inflammatory cells such as PMNs on the ocular surface, a balance between anti- and pro-inflammatory responses must exist to protect against pathogens while preventing the overactivation of PMNs, which may lead to host tissue damage, and subsequently preserving ocular homeostasis. As the first cells to arrive at sites of infection or inflammation, the potential significance of PMNs in the pathogenesis of ocular diseases, such as dry eye disease and ocular allergy, cannot be ignored. Recent findings reported an enhanced degranulation of tear PMNs isolated from people with dry eye disease [9], further supporting that tear PMNs may contribute to the development of ocular diseases. However, the knowledge of the role of tear PMNs in ocular inflammation remains limited and it is difficult to interpret results in the absence of a good understanding of the functions of tear PMNs in ocular homeostasis. Thus, the overall objectives of my Ph.D. thesis were to:

- 1) investigate the oxidative and degranulation properties of tear PMNs in healthy eyes (Chapters 4 and 6);
- 2) examine the impact of tear proteins on tear PMNs collected from healthy eyes (Chapter 5);
- 3) assess the phenotype of tear PMNs in ocular allergy (Chapter 7).

1.2 Thesis Objectives and Hypotheses

In Chapter 2, the tear film and what is currently known about tear PMNs are presented. Chapter 3 provides a review of the current knowledge of the interactions between the circadian rhythm and the

immune system. Chapter 4 presents the study of the differential ROS production by tear PMNs and the impact of isolation methods on the phenotype of tear PMNs. In Chapter 5, the investigation of the impact of tear proteins on the oxidative burst of tear PMNs is presented. Chapter 6 reports on the impact of time of collection on the number and phenotype of tear PMNs and on the correlation between tear PMN numbers and chemoattractants in the tear film. The phenotype and potential role of tear PMNs in patients with ocular allergy are examined in Chapter 7. Finally, Chapter 8 provides a summary of conclusions of the studies presented in this thesis as well as recommendations for future research. Each experimental study (Chapters 4 to 7) had its own specific objectives and hypotheses, which are presented below.

Study 1. The Differential Reactive Oxygen Species Production of Tear Neutrophils in Response to Various Stimuli In Vitro (Chapter 4)

Hypotheses:

- 1) Tear PMNs are the main contributors to ROS production in the eyewash.
- 2) Tear PMNs produce ROS in a stimulus-dependent manner.
- 3) The isolation process affects ROS production in tear PMNs.

Objectives:

- 1) Determine the ability of tear PMNs to produce ROS.
- 2) Assess the impact of isolating tear PMNs from the eyewash on ROS production.

Study 2: The Impact of Tear Proteins on The Oxidative Burst in Tear Neutrophils (Chapter 5)

Hypothesis:

The presence of tear proteins, specifically lactoferrin and lysozyme, reduces ROS production in tear PMNs.

Objective:

Investigate the impact of tear proteins on the production of reactive oxygen species (ROS) by tear PMNs, using two different methods, luminol-enhanced chemiluminescence and fluorescence-derived DCFH-DA (flow cytometry).

Study 3: Investigation of the Circadian Rhythmic Recruitment of Tear Neutrophils to the Ocular Surface and Their Phenotypes (Chapter 6)

Hypotheses:

- 1) Short-time eye closure results in lower degranulation than prolonged eye closure.
- 2) Eye-closure during the day (i.e., a 2hr nap) results in leukocyte accumulation in the tear film.
- 3) The number of tear PMNs collected correlates to the concentration of chemoattractants (IL-8 and C5a).

Objectives:

- 1) Characterize the phenotype of tear PMNs collected at different time points.
- 2) Assess the concentration of chemoattractants in tears and their correlation with PMN number.

Study 4: Characterization of the Phenotype of Tear Leukocytes in Ocular Allergy and Their Potential Impacts on Ocular Surface Inflammation (Chapter 7)

Hypotheses:

- 1) Tear PMNs collected from individuals suffering from ocular allergy are more activated than tear PMNs collected from healthy eyes.
- 2) Higher number of tear PMNs are present on the ocular surface of allergy participants compared to non-allergy participants

Objective:

- 1) Investigate the phenotype of tear leukocytes in seasonal ocular allergy, in terms of their surface receptors expression and oxidative burst.
- 2) Measure the concentration of cytokines or pro-inflammatory mediators in tears.

Chapter 2. The phenotype of tear PMNs and their role in ocular homeostasis and inflammation

2.1 Introduction

The immune system is a crucial defensive mechanism in most living organisms that protects against foreign bodies and pathogens and defense is coordinated between the innate and adaptive immune systems. There are two elements in immunity, the cellular (innate and adaptive immune cells) and the humoral (immunoglobulins antibodies) elements [1]. Innate immune cells are the first to respond to inflammation or infection, whereas adaptive immune cells transmigrate and become activated later. The differences between innate and adaptive immune cells lie in their gene rearrangement abilities for producing antibodies and expressing surface receptors, specificity to particular types of pathogens, and capabilities to possess immunologic memory. Additionally, the innate immune system reacts faster than the adaptive immune system. There are several different types of innate and adaptive immune cells, as depicted in Figure 2-1. Macrophages are tissue-resident phagocytes that are derived from monocytes. Granulocytes, including basophils, neutrophils, and eosinophils, have granules in their cytoplasm, which contain various antimicrobial substances, and are also able to engulf and digest pathogens. Neutrophils are the most abundant leukocyte population in the bloodstream and are the first responder cells to fight against pathogens and foreign bodies. Eosinophils and basophils play important roles in both allergic reactions and parasitic infections. Although natural killer cells are derived from lymphoid cells, they are part of innate immune cells and can directly induce apoptosis of targeted cells. Dendritic cells serve as antigen-presenting cells (APC), acting as the connectors between the innate and adaptive immune systems. B cells are capable of secreting antibodies to effectively kill pathogens. T cells can be classified into two major groups, CD 4⁺ T cells (T_H helper cells) and CD 8⁺ (T_C cytotoxic cells), while T_H helper cells can be further divided into various effector T cell subsets once activated, such as T_H 17.

This thesis only focused on the functions and mechanisms of neutrophils and therefore will not discuss the functions of other immune cells, as this is beyond the scope of this thesis.

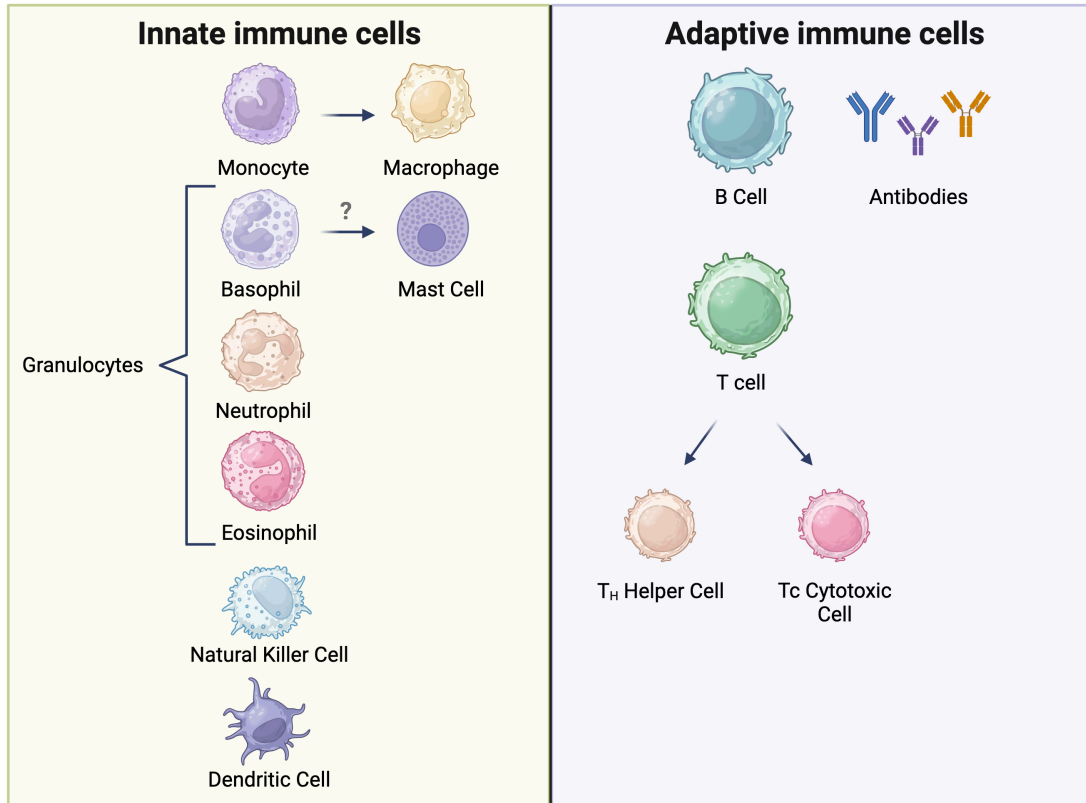


Figure 2-1. Schematic overview of innate and adaptive immune cells. Monocytes, granulocytes, natural killer cells, and dendritic cells are innate immune cells, while lymphocytes are adaptive immune cells. Dendritic cells act as a connector between innate and adaptive immune system. Created with BioRender.

A significant number of neutrophils (polymorphonuclear neutrophils, PMNs) can be harvested from the ocular surface after prolonged eye closure at night (such as during sleep), even in the absence of a wound or infection. These PMNs have been referred to as tear-film or tear neutrophils (tear PMNs) to reflect their location [2–8]. In 2015, it was demonstrated that large numbers of tear PMNs could be collected noninvasively upon awakening by using a gentle eyewash performed at home [4]. The procedure, depicted in Figure 2-2, involves participants drawing a sterile saline solution (phosphate buffer solution) using a disposable dropper and placing it at the inner angle of the eye to wash their ocular surface. The run-off is collected into a 50mL conical tube placed at the eye's outer angle. Several groups are now using this collection protocol with minor modifications [6,8,9].

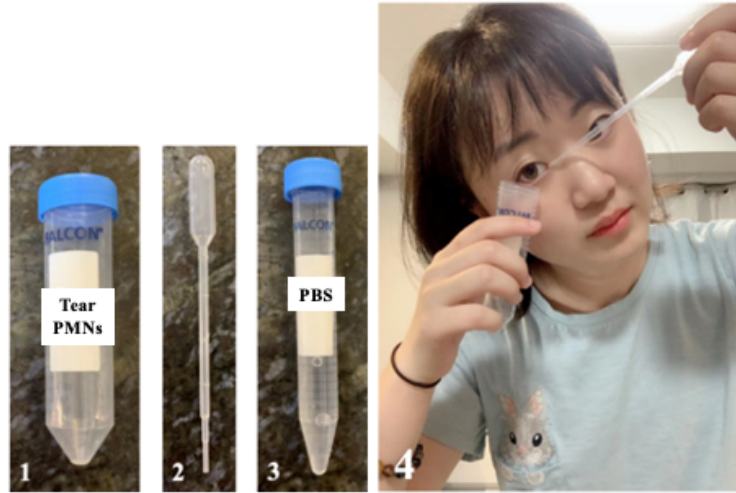


Figure 2-2. Tear PMN Collection Procedure.

Up to a million leukocytes can be harvested from healthy eyes, with an average of 61% of leukocytes being PMNs, 2.3% being lymphocytes, 0.6% being monocytes, and 25% being other CD45⁺ cells [6,10]. The phenotype of tear PMNs has also been reported to be affected by experimental procedures, such as centrifugation and long incubation time, so caution needs to be exercised during processing [5].

This chapter will provide a brief overview of chemotaxis and functions of circulating PMNs, followed by an in-depth discussion of current findings on tear PMNs and their role in ocular homeostasis and inflammation.

2.2 Chemotaxis and Transmigration Cascades of Neutrophils

As the most abundant type of leukocytes in circulation, polymorphonuclear neutrophils (PMNs) are the first cells to arrive at the infected or damaged sites. Compared to other leukocytes, PMNs can move 3 to 4 times faster with an average velocity of $19 \pm 6 \mu\text{m}/\text{min}$ *in vitro* (reviewed in [11]). To move rapidly (within minutes) and efficiently in a large number, PMNs have a specific way of sensing chemical concentration gradients, which guides them to the infected areas. This process is known as chemotaxis. The early signals to attract PMNs to local sites are the damage-associated molecular patterns (DAMPs) released from damaged or infected cells, such as the *N*-formylated peptides, DNA, and extracellular matrix components [12]. Although *N*-formylated peptides can be isolated from bacteria, they are also present in the cell mitochondria. These early signals are usually temporary, so they trigger damaged or infected cells to produce more prolonged and stable chemoattractants, such as chemokines and lipids, to further recruit

PMNs to the sites [12]. Meanwhile, the early-recruited PMNs release more chemoattractants, amplifying the recruitment of PMNs as well as the immune response, as shown in Figure 2-3.

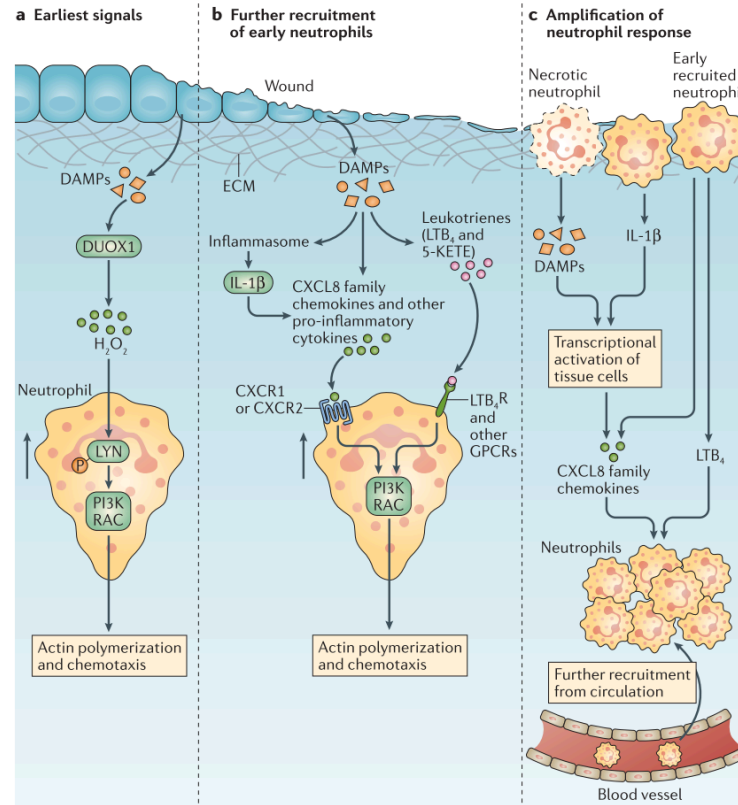


Figure 2-3. The timing sequence of PMN recruitment to the infected sites. A) the early release of DAMPs to recruit PMNs to the sites. B) DAMPs induce the release of chemokines and lipids from damaged cells. C) Early recruited PMNs further recruit more PMNs to the sites. Image reproduced with permission from de Oliveira S, Rosowski EE, Huttenlocher A. Neutrophil migration in infection and wound repair: going forward in reverse. *Nat Rev Immunol* 2016;16:378–91 [12].

Chemoattractants can be classified into four categories, *N*-formylated peptides (such as fMLP), complement anaphylatoxins (such as C5a), lipids (such as leukotriene B $_4$, LTB $_4$), and cytokines (such as IL-8), with the former two being “end-target” (more effective in attracting PMNs to the sites) and the latter two being the “intermediary-target.” As the potent chemotactic anaphylatoxin, C5a is the byproduct of the cleavage of C5 in the complement terminal pathway. C5a acts via its G protein-coupled receptors (GPCRs), C5aR1 and C5aR2, and can cause aggregation, degranulation, LTB $_4$ release, and superoxide generation by PMNs [13]. C3a is another important anaphylatoxin and has been shown to induce PMN aggregation.

morphological changes, and cell activation [14]. For example, C3a can trigger oxidative burst by PMNs, although it is not as potent as C5a [14]. However, C3a may not play a role in PMN chemotaxis [14]. IL-8, as one of the cytokines, transmits signals to PMNs through the GPCRs, C-X-C motif chemokine receptor 1 (CXCR1) and CXCR2. IL-8 is produced by many cells, including epithelial cells, monocytes, and PMNs. When endothelial cells are damaged, they release IL-8, which attracts and primes PMNs, thereby enhancing their adhesion and cellular responses [13].

The cascade of chemotaxis involves three steps, sensing the concentration gradient, polarization, and migration to the targeted sites. Chemoattractants relay or signal PMNs by interacting with the G protein-coupled receptors (GPCRs) on their surfaces. Then, PMNs process the information and undergo cytoskeleton rearrangement and morphological change, known as polarization. As a result, PMNs develop a leading edge or pseudopod at the front and a uropod at the rear [11,15], as shown in Figure 2-4A. Studies have shown that the pseudopod leads PMNs to move forward while the uropod at the rear pushes PMNs to move forward in a squeezing motion [11]. Both the pseudopod and uropod ensure that PMNs follow the concentration gradient and stay on the right path, and are also responsible for overcoming the shear force in blood [16]. This directed migration leads to the intraluminal crawling of PMNs. It has been shown that efficient crawling is crucial for PMNs to extravasate across the blood vessels, and it usually depends on the interaction between MAC-1 (also known as CD11b) on PMNs and ICAM-1 (Intercellular Adhesion Molecule-1, CD54) on endothelial cells [15]. PMNs transmigrate between the two endothelial cells primarily via the paracellular route, as illustrated in Figure 2-4B. It is also important to note that the nuclear and cell shape of PMNs deform during the extravasation [17]. A study by Gaines et al. reported that hypo-segmented (less than two lobules of nucleus) PMNs in mice exhibited impaired nucleus deformability and then extravasation [18], highlighting the importance of nuclear deformability in efficient transmigration.

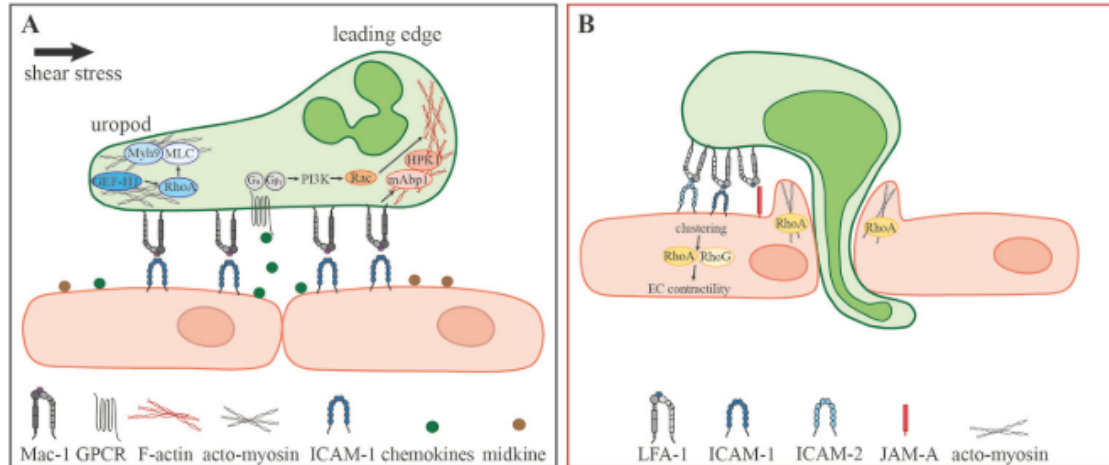


Figure 2-4. The cascades of PMN transmigration. A) the pseudopod or leading edge and uropod on PMNs after polarization. B) the deformation of the nuclear and cellular shape of PMNs during transmigration. LFA-1: lymphocyte function-associated antigen-1, ICAM: intercellular adhesion molecule, JAM-A: junctional adhesion molecule A. Image reproduced with permission from Salvermoser M, Begandt D, Alon R, Walzog B. Nuclear deformation during neutrophil migration at sites of inflammation. *Front Immunol* 2018;9:1–9 (open access article) [17].

Once PMNs extravasate from the blood vessels, they execute essential functions, such as phagocytosis, degranulation, oxidative burst, and releasing neutrophil extracellular traps (NETs), to eliminate pathogens and sanitize the tissues. After they perform their functions, aged PMNs will be phagocytosed by tissue-resident macrophages.

2.3 The Functions of Neutrophils

The functions of PMNs can be induced by various stimuli, such as bacteria-derived molecules (i.e., fMLP) and physiological inflammatory mediators (i.e., IL-8 and C5a). While necessary for maintaining host health, the functions of PMNs are double-edged, as they may cause severe damage if they are over-activated. PMNs have been shown to play a pathological role in several systemic autoimmune diseases, such as systemic lupus erythematosus (SLE) [19]. Therefore, precise regulation of PMN functions is necessary to limit the unexpected damage to host tissues and maintain homeostasis.

2.3.1 Degranulation

To respond to stimuli, degranulation happens amid the cellular responses [20], and involves the release of antimicrobial substances from different granules to the extracellular environment or the phagolysosome (formed by the fusion of a phagosome with lysosome). There are four types of granules in PMNs: the primary (azurophilic) granules, the secondary (specific) granules, the tertiary granules, and the secretory vesicles. Neutrophil elastase and myeloperoxidase (MPO) are present in primary granules [21], while CD63 is expressed on the surface of the primary granular membrane [22]. Lactoferrin is stored in secondary granules characterized by the surface expression of CD66b [21]. The tertiary granules and secretory vesicles contain the gelatinase (metalloproteinases (MMP), such as MMP9) and plasma proteins (such as serum albumin), respectively [21]. Lysozyme is found in all granules except for the secretory vesicles. Figure 2-5 further illustrates granular content and the membrane markers [23]. In response to stimulus, such as phorbol myristate acetate (PMA), the secretory vesicles are the first to be released, followed by gelatinase granules, specific granules, and a low level of azurophilic granules, as reported previously in [24]. Given different antimicrobial and bactericidal substances stored in the azurophilic and specific granules, the order of granule release may suggest a regulatory mechanism of PMN functions.

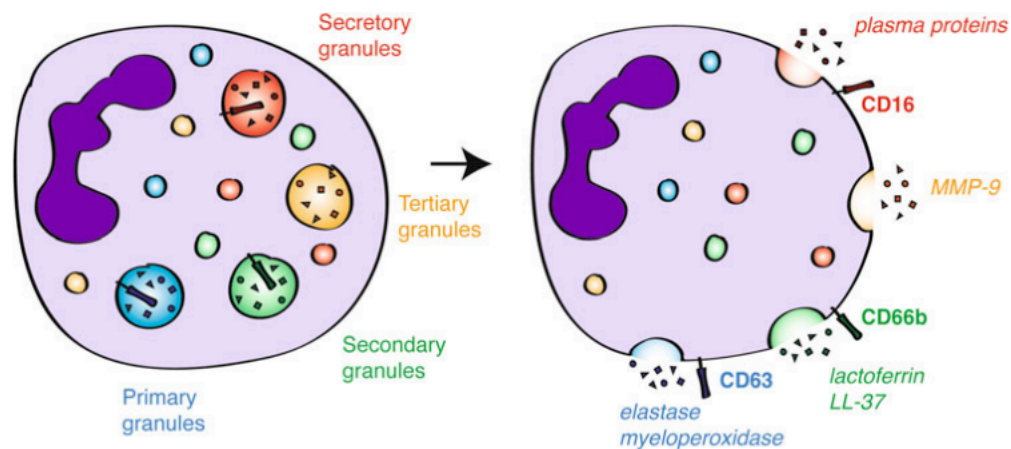


Figure 2-5. Schematic of degranulation process in PMNs. When neutrophils are activated, their granular membrane fuse with the cell membrane, leading to the release of granular contents to the extracellular environment as well as the upregulation of surface receptors on the cell surface. Image reproduced with permission from Eichelberger KR, Goldman WE. Human Neutrophil Isolation and Degranulation Responses to *Yersinia pestis* Infection. *Methods Mol. Biol.*, 2019, p. 197–209 [23].

Degranulation of PMNs can be assessed by either measuring the expression of surface receptors or the release of granular substances. Upon cell activation, the exocytosis of granules will cause an upregulation of granular-specific surface receptor expression on the PMN cell membrane, such as CD63 for azurophilic granules and CD66b for the specific granules. The standard method for measuring surface receptor expression on cells is flow cytometry (immunophenotyping). An advantage of flow cytometry is that it can quickly measure expression on thousands of cells in a short period of time [25]. Additionally, degranulation can be measured qualitatively using imaging flow cytometry which combines flow cytometry with microscopy, which provides information on granule fusion by determining the degree of colocalization of the degranulation markers [22].

Degranulation can be evaluated indirectly by measuring the release of granular substances, such as myeloperoxidase, in the physiological fluid such as blood, tears or even in vitro in the supernatant of cell culture experiments. This assessment can be carried out using techniques like enzyme-linked immunosorbent assay (ELISA), multi-plex assays (such as those from Meso Scale Discovery), and western blot. However, these techniques can be more complicated than flow cytometry and may result in a longer processing time.

2.3.2 Oxidative Burst

Oxidative burst or respiratory burst is another important mechanism for PMNs to kill pathogens and is oxygen dependent. Upon stimulation, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase reduces molecular oxygen to produce the superoxide anion (O_2^-), followed by the release of other reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [26]. Myeloperoxidase (MPO), stored in the azurophilic granules, catalyzes the reaction of H_2O_2 with Cl^- to produce HOCl, which is a potent antimicrobial and bactericidal substance [13], and H_2O_2 can further react with HOCl to produce a highly activated singlet oxygen $^1O_2$ [27]. NADPH oxidase is found on both the intracellular granular and cell plasma membranes, contributing to intracellular and extracellular ROS production, respectively. Intracellular ROS is crucial for killing pathogens phagocytosed by PMNs in the phagolysosome, while extracellular ROS plays a vital role in eliminating pathogens in an extracellular manner. ROS production also occurs in the absence of a pathogen or inflammatory stimulus as spontaneous ROS release was observed by circulating aged PMNs [28], suggesting a potential involvement of ROS in the mechanism of cell death. While ROS production is a defense mechanism, it may also have potentially detrimental effects on lipids, proteins, and DNA [27] and excessive ROS production can lead to severe damage to the host tissue. Additionally, the oxidative burst can promote further degranulation and NETs release [29]. Azurophilic granules contain protein complexes, azurosome, which include neutrophil elastase

and myeloperoxidase. These granular proteins are important in chromatin decondensation, a key step in NETosis as described in section 2.3.3. Research has reported that ROS can dissociate the azurosome, leading to the release of granular proteins into the cytosol [29].

The production of ROS can be measured using different techniques, such as chemiluminescence- and fluorescence-derived methods. Two common chemiluminescent probes used in the chemiluminescence assay are lucigenin and luminol, which can detect extracellular and intracellular ROS production, respectively [30]. While lucigenin detects mainly extracellular superoxide and is not cell-permeable, luminol can penetrate the cells and detect both extracellular and intracellular ROS, mainly superoxide, as shown in Figure 2-6. The use of superoxide dismutase (SOD) and catalase can deplete extracellular ROS. Chemiluminescence assays can provide kinetic measurements over time, but they require a high number of cells (5×10^5 cells) [31,32]. Thus, alternative techniques, such as fluorescence-derived methods, may be appropriate for measuring ROS production requiring a smaller number of cells (3.5×10^4 cells). Some fluorescent probes are sensitive to ROS and will be oxidized into fluorescent molecules when exposed to ROS. 2',7'-Dichlorofluorescein diacetate (DCFH-DA) is a commonly used fluorescent probe to assess ROS production. DCFH-DA can penetrate cells and be oxidized into fluorescent DCF once it binds to ROS, as illustrated in Figure 2-6. The fluorescent signal can then be captured using flow cytometry. However, this technique has certain limitations, such as its inability to differentiate between types of ROS as well as its difficulty in providing kinetic measurement.

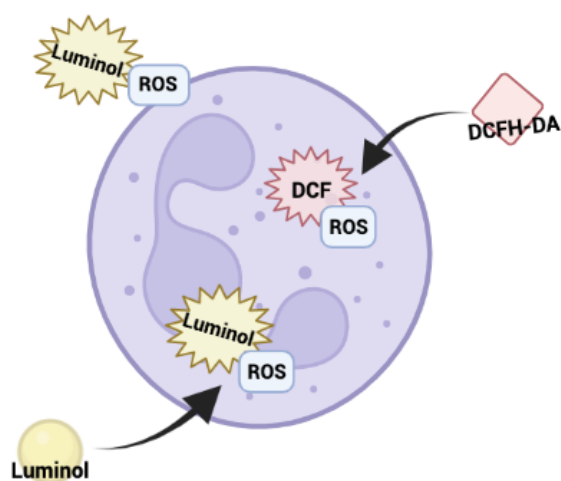


Figure 2-6. The mechanisms of luminol and DCFH-DA on ROS detection. Chemiluminescence using luminol as the detection probe measures both intra and extra-cellular ROS production, while flow cytometry using DCFH-DA as the fluorescent probe can only measure the intracellular ROS production. Created from BioRender.

2.3.3 Neutrophil Extracellular Traps

PMNs release NETs in response to various stimuli, such as chemical synthetic stimuli (PMA), bacteria-derived stimuli (LPS), and physiological stimuli (immune complexes and cytokines), in a process known as NETosis [29,33]. This process involves several steps, including chromatin decondensation, nuclear envelope breakdown, interactions between granular proteins and chromatin, and the release of chromatin in the extracellular environment [11]. Peptidyl-arginine deaminase 4 (PAD4) can catalyze the citrullination of histones, which is an essential prerequisite for chromatin decondensation [29]. Li et al. reported that PAD4 knockout mice failed to release NETs in response to PMA [34], further highlighting the importance of the PAD4 enzyme in initiating NETosis. Once PMNs release NETs, they can trap and kill pathogens directly through histones and PMN granular proteins, such as MPO, neutrophil elastase (NE), and lactoferrin [19], as illustrated in Figure 2-7. Stimuli can be used to trigger NETs release *in vitro*. A study by Sanne et al. reported that single or combined physiological stimuli, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), lipopolysaccharides (LPS), tumor necrosis factor (TNF), and N-formyl-methionyl-leucyl-phenylalanine (FMLF), were not enough to trigger the NETosis in PMNs, suggesting a tight regulation mechanism of PMN NETs release [35]. On the other hand, non-physiological stimuli, such as phorbol ester (e.g., PMA) and calcium ionophore, induce NETs release *in vitro* [35].

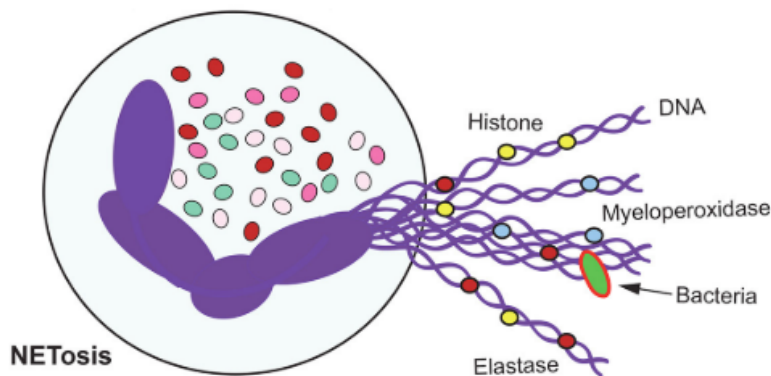


Figure 2-7. The production of NETs with DNA fibres, histones, and granular proteins. Once PMNs are activated, they release NETs, which are decorated with different granular proteins, such as myeloperoxidase and elastase, to the extracellular environment. Image reproduced with permission from Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Front Physiol 2018;9 (open access article) [36].

Two types of NETosis have been discovered, suicidal NETosis and vital NETosis, as depicted in Figure 2-8 [37]. Suicidal NETosis is a unique programmed cell death (PCD) [37]. In suicidal NETosis, the nuclear envelope is broken down, filling the cytoplasm with decondensed chromatin, leading to cell swelling and rupture of the cell membrane, ultimately resulting in cell death [11]. On the other hand, vital NETosis describes the exocytic release of DNA and granular proteins by PMNs without cell lysis, resulting in an enucleate PMN that still retains its cellular functions, such as chemotaxis, phagocytosis, and adherence [11]. Suicidal NETosis can be induced by chemical stimuli, such as PMA, over an extended period, whereas vital NETosis has been proposed to be triggered by microbe-related stimuli, such as LPS [38].

While NETosis shares some similarities with apoptosis, such as the morphological changes in the nucleus and cytoplasm, it does not activate caspases related to apoptosis [37].

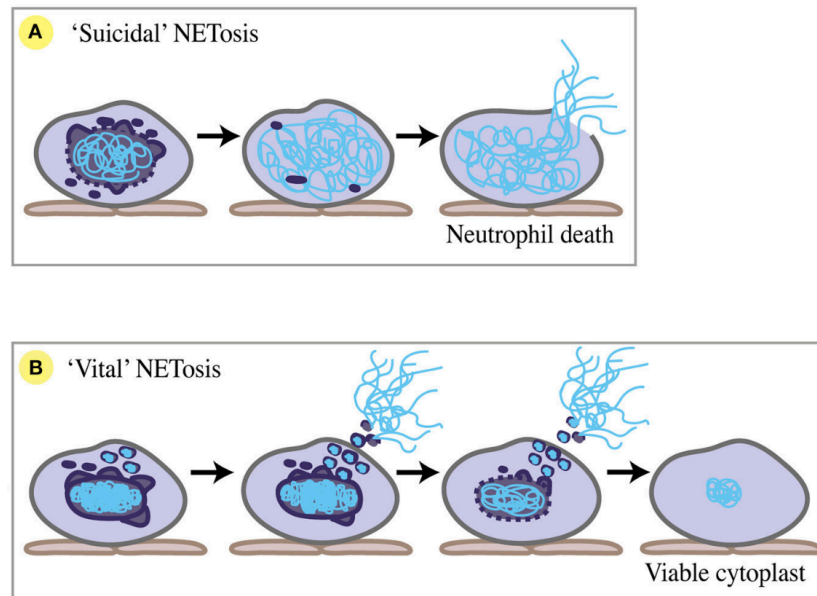


Figure 2-8. The changes in nuclear and cytoplasm of PMNs during a) suicidal NETosis and b) vital NETosis. Image reproduced with permission from Manley HR, Keightley MC. The Neutrophil Nucleus: An Important Influence on Neutrophil Migration and Function. *Front Immunol* 2018;9:1–18 (open access article) [11].

NETs can be assessed either qualitatively using microscopy or quantitatively using flow cytometry and spectrophotometry. NETs are composed of DNA filaments and granular proteins, thus research groups have used DAPI or SYTOXTM green to stain the extracellular DNA (eDNA), with the latter being a cell-impermeable dye [39,40]. However, these dyes can also stain eDNA from other sources and are not specific

to NETs. Therefore, immunofluorescence can be utilized to identify NETs more specifically with fluorescently labelled antibodies against granular proteins, including neutrophil elastase (NE) and MPO, as well as citrullinated histones (such as H3Cit), as shown in Figure 2-9. DAPI can also be combined with immunofluorescence staining to assist in visualizing NETs.

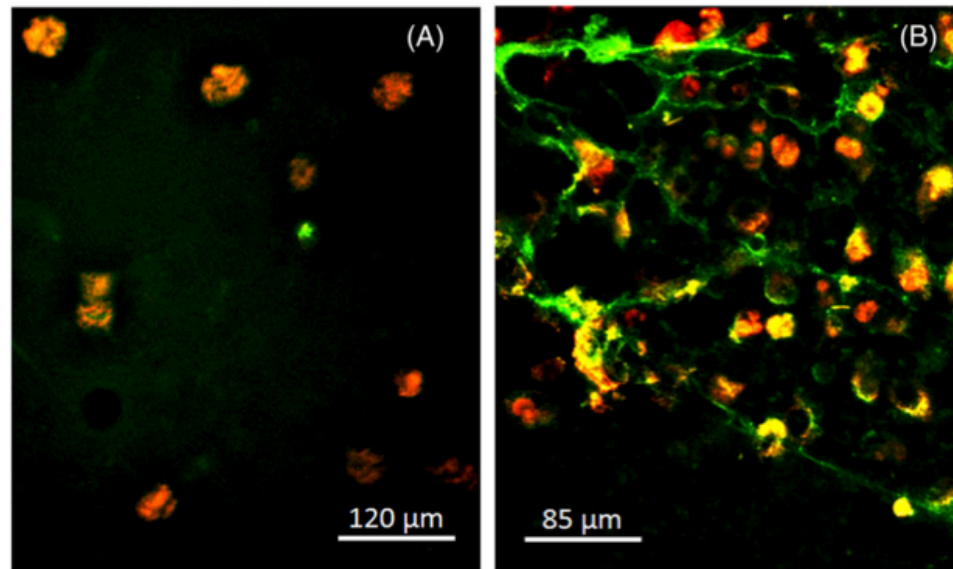


Figure 2-9. Immunofluorescence of NETs released by a) non-stimulated and b) ionomycin-stimulated PMNs. NETs were stained by anti-MPO antibodies (green) and anti-H3Cit antibodies (red). Image reproduced with permission from Pleskova SN, Gorshkova EN, Kriukov RN. Dynamics of formation and morphological features of neutrophil extracellular traps formed under the influence of opsonized *Staphylococcus aureus*. *J Mol Recognit* 2018;31:e2707 [41].

Although NETs can be visualized under microscopy, it is also important to quantify the amount of NETs as it can provide further insight into the extent of neutrophil activation and the degree of inflammation. One way to measure NETosis is through a flow cytometric-based technique that uses a combination of fluorescently conjugated antibodies against granular proteins, histones, or DNA [42,43]. One of the advantages of this technique is that it avoids contamination from other leukocytes by using a double-gating strategy to detect NETs released by PMNs in a mixture of cells. However, flow cytometry does not provide a complete picture of NETosis as it cannot measure the NETs released into physiological fluids or cell culture supernatants [44]; flow cytometry can only detect the expressions of markers, such as CitH3, MPO, and NE, on the cell membrane or in their cytoplasm.

NETs can also be quantified using the CitH3-DNA ELISA kit, targeting the complex of extracellular DNA and CitH3, which has high specificity and sensitivity for NETs [44]. Additionally, ELISA kits are available to measure the levels of complexes of extracellular DNA and granular proteins, such as MPO and NE [44]. However, using MPO-DNA ELISAs for *in vivo* measurements of NETosis has been an area of controversy, as it does not always correlate with other NETs markers [44]. While ELISA kits are easily accessible for researchers, one limitation of this method is the inconsistency or poor correlation between the *in vivo* and *in vitro* experiments, which makes it challenging to compare results among different studies [44].

2.3.4 The Interconnections between PMN Functions

PMN functions are interconnected and coordinated with each other. When PMNs engulf bacteria, the phagosome fuses with the lysosome, forming a phagolysosome which can trigger ROS release and degranulation to enhance their efficiency in pathogen killing [13]. This has been demonstrated in PMNs collected from patients with chronic granulomatous disease (CGD), where the mutant NADPH oxidase results in functional phagocytosis but an impaired ability to kill pathogens through oxidative burst [13,45]. Furthermore, the respiratory burst requires MPO, which is stored in the azurophilic granules, to catalyze the oxidation of HOCl. While NADPH-oxidase-derived ROS can promote the release of granules [46], they can also regulate degranulation to prevent excessive responses [47], indicating a negative feedback loop to avoid tissue damage. Both degranulation and respiratory burst are also essential for NET formation. A functional NADPH oxidase is crucial to induce NETosis as PMNs from CGD patients and PMNs from NADPH oxidase-knockout mice demonstrated an impaired NETs release, as reviewed in [33]. Although the oxidative burst is necessary to initiate NET formation [46], not all NETosis depends on it as the process of NETosis appears to be affected by the types of stimuli used *in vitro* [33]. For instance, calcium ionophore can activate NADPH oxidase and trigger NETosis in the presence and absence of NADPH oxidase inhibitor [48], suggesting that calcium ionophore-induced NETosis is independent of the oxidative burst. However, PMA-induced NETosis requires an active NADPH oxidase [48], revealing that the mechanisms underlying NETosis vary and depend on the stimuli. While many of these studies are characterizing some of mechanisms of NET formation *in vitro*, the stimuli used *in vitro* are “chemical” (for example PMA diffuses into the cell and activates protein kinase C directly) and not physiological stimuli, so the molecular mechanisms involved *in vivo* may differ. More research is needed to understand differences in observations and better identify physiological pathways.

Granular proteins, specifically MPO and NE, have been shown to play an essential role in chromatin decondensation during NETosis [33,46]. A study by Papayannopoulos et al. reported that NE

cleaves histones, which may initiate chromatin decondensation, while MPO binds the histones in a later phase, further promoting chromatin decondensation. This suggests a sequential mechanism of NE and MPO in the chromatin decondensation [46]. In contrast, lactoferrin, stored in specific granules and also found on NETs, can suppress the release and spread of NETs via electrostatic interactions, thus affecting the morphology of NETs and their ability to expand [39].

In conclusion, as described in this section, PMNs carry various functions as part of the host immune defense mechanism, with many functions being interconnected. PMN functions work together to increase the efficiency of pathogen killing, while they also have the capability to suppress each other to prevent excessive cellular responses and protect the host tissues from damage. These highlight the important role of PMNs in homeostasis.

2.4 Tear Film

The tear film, which is approximately 2-5.5 μm thick, is the outermost layer that covers the surface of the cornea and conjunctiva and helps to lubricate, nourish, and protect the ocular surface structures [49,50]. Maintaining the stability of the tear film is crucial for ocular health, as any disturbance in tear film stability can result in ocular diseases, including dry eye disease. Wolff [51] has presented the tear film as a triple-layer model, consisting of the innermost mucin layer, the middle aqueous layer, and the outermost lipid layer. However, the study by Cher [52] suggests that soluble mucins are spatially distributed, and their concentration decreases from the epithelium toward the lipid layer, so the aqueous and mucin layers may be better represented as a single muco-aqueous layer, which then leads to understanding the tear film more as a two-layered system, as shown in Figure 2-10. The lipid layer overlying the muco-aqueous layer is composed of meibum secreted from the meibomian glands [49]. This layer is shown to play an important role in reducing surface tension as well as preventing the evaporation of tears [49,53,54]. The muco-aqueous layer contains various molecules, such as lactoferrin, lysozyme, secretory immunoglobulin A (sIgA), and mucins.

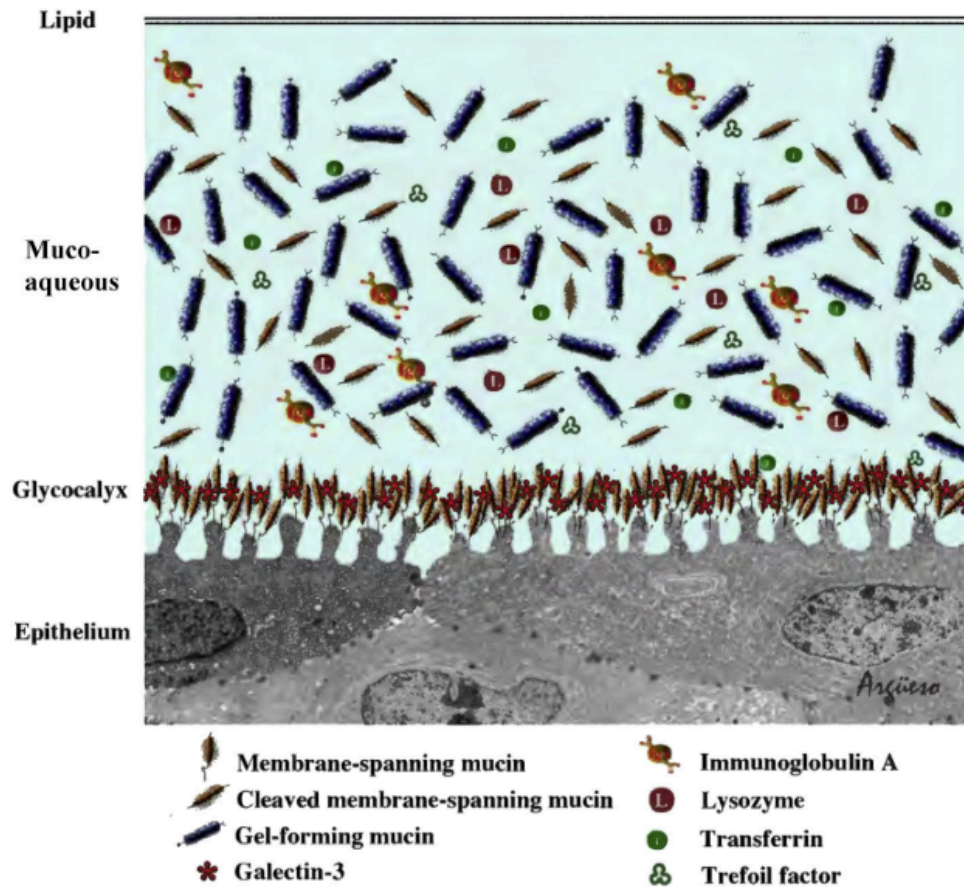


Figure 2-10. Tear film structure composed of muco-aqueous and lipid layers. Tear proteins, mucins and other components are distributed within the muco-aqueous layer, with the glycocalyx layer coating the ocular epithelium. Image from Willcox MDP, Argüeso P, Georgiev GA, Holopainen JM, Laurie GW, Millar TJ, et al. TFOS DEWS II Tear Film Report. *Ocul Surf* 2017;15:366–403 (open access article) [49].

Mucins, which are secreted mainly by goblet cells, can be classified into large gel-forming (such as MUC5AC and MUC2), transmembrane (such as MUC1 and MUC4), and soluble mucins (such as MUC7) [55]. The gel-forming mucins are responsible for maintaining the hydration of the ocular surface and preventing the binding of debris and pathogens to the ocular surface [49,56]. Those transmembrane mucins form a glycocalyx that acts as a protective barrier against the binding and entry of pathogens to the ocular epithelium [49,56]. Additionally, the hydrophilic nature of the glycocalyx layer allows it to spread the aqueous tear over the hydrophobic ocular epithelium [56]. Cantin et al. recently showed that airway mucins can impair the bacterial killing functions of PMNs by suppressing the oxidative burst and bactericidal ability of cationic proteins, such as NE, which highlights the anti-inflammatory role of mucin

on PMNs [57]. Interestingly, MUC5AC is the predominant mucin in the airways [57], which is also present in the tear film, raising the possibility of tear mucins modulating tear PMN functions.

The aqueous phase of the muco-aqueous layer, released by the lacrimal glands and accessory lacrimal glands (Krause and Wolfring) in the eyelids, contains several components, such as electrolytes and proteins, as mentioned previously [58]. Examples of the most abundant tear proteins are lysozyme, lactoferrin, and albumin [59], which are shown to have antimicrobial functions. Lysozyme makes up 20-30% of the total proteins in the basal and reflex tears [60]. It can kill bacteria directly by breaking down the peptidoglycan backbone in their cell walls through hydrolysis [61]. Furthermore, lysozyme can eliminate fungi by cleaving the chitodextrins in their cell walls [62]. Pathogens may release pathogen-associated molecular patterns (PAMP), such as the monomeric peptidoglycan, into the extracellular environment due to the increased permeability of their cell walls caused by lysozyme. This release can lead to the subsequent recruitment of innate immune cells, especially macrophages and neutrophils, which activates and enhances the immune response [63]. Furthermore, lysozyme has also been found to have anti-inflammatory effects on immune cells. For instance, lysozyme has been shown to suppress chemotaxis and superoxide generation of human neutrophils in response to N-formyl methionyl phenylalanine (fMLP) and zymosan [64], suggesting the ability of lysozyme to control inflammation.

Compared to other transferrin proteins, lactoferrin, which also accounts for 20-30% of total proteins in basal and reflex tears [60], has higher affinity binding to iron, which is an essential element for bacterial growth [65]. Except for its iron-dependent antimicrobial mechanism, lactoferrin can induce cell death of Gram-negative bacteria by binding to its receptors or interacting with LPS on bacterial surfaces [65]. It can also increase the permeability of the Gram-positive bacterial membrane through electrostatic interactions between the negatively-charged lipid layer of bacteria and the positively-charged lactoferrin [65]. Notably, lactoferrin receptors can be found on or within various types of cells, such as hepatocytes, mucosal epithelial cells, fibroblasts, macrophages, lymphocytes, monocytes, and neutrophils (as reviewed in [65]). Given the abundance of its receptors on immune cells, lactoferrin can modulate the immune response both positively (pro-inflammatory) and negatively (anti-inflammatory). For example, lactoferrin has been shown to promote the proliferation, differentiation, and activation of immune cells, potentially enhancing immune responses [65]. On the other hand, lactoferrin can inhibit or suppress the production of some pro-inflammatory cytokines by monocytes upon LPS stimulation, such as tumor necrosis factor α (TNF- α), interleukin 1 β (IL-1 β), and IL-6 [66]. Additionally, lactoferrin has been observed to inhibit LPS priming of neutrophils, an effect also seen with other transferrin proteins and albumin [67]. According to Baveye et al. [68], lactoferrin can decrease the production of ROS by inhibiting the binding of LPS to its receptor, L-selectin (CD62L).

As one of the major tear proteins, albumin may serve as a carrier of insoluble substances and assist in removing debris and pathogens along with blinking [49,69]. Shimmura et al. reported that the topical application of albumin in rabbit eyes accelerated wound healing and could improve symptoms in patients with dry eye disease [69]. This may be due to the anti-inflammatory and antioxidant effects of albumin [70,71], but this warrants further investigation. Additionally, albumin can block CD43 and suppress the mobilization of neutrophils and their production of hydrogen peroxide [71].

Studies have also reported differences in protein concentrations between closed-eye and open-eye basal tears. Closed-eye tears contained higher levels of lipocalin-1, IgA, and albumin, as reviewed in [50]. However, the concentrations of lysozyme and lactoferrin have not been consistent between studies, with one study reporting no difference between closed-eye and open-eye tears [72] and another showing higher levels of lactoferrin and lysozyme in closed-eye tears [73]. The increase in protein concentrations in closed-eye tears may be caused by two factors, an inflammatory-induced increase in the levels of proteins supported by the presence of PMNs in closed-eye tears, and the reduction of tear turn-over rate leading to the accumulation of proteins in the tear film.

In conclusion, lysozyme, lactoferrin, and albumin are the major tear proteins in the aqueous phase and possess pro and anti-inflammatory properties; the latter may aid in preventing unnecessary immune responses to protect the ocular structures. However, further investigation is needed to better understand their interactions with immune and ocular surface cells and the underlying mechanisms contributing to maintaining ocular homeostasis.

2.5 Recruitment of Tear Neutrophils to the Ocular Surface

The origin of tear PMNs has yet to be determined, and two hypotheses have been proposed [4,74]. The first hypothesis suggests that tear PMNs are brought to the ocular surface with the tear fluid from the lacrimal glands. The other is extravasation, whereby chemoattractants present in the ocular environment attract leukocytes from conjunctival blood vessels.

Some evidence may support the latter hypothesis. The concentrations of potent chemoattractants, including IL-8, C5a, and leukotriene B₄, were found to increase with increasing eye closure time at night [9,75]. Furthermore, the concentrations of IL-8 and C5a are significantly higher in tears collected at night compared to those collected from the open-eye tears and enhanced chemotaxis of blood-isolated PMNs has been observed when exposed to closed-eye tears collected after sleep compared to open-eye tears [75], suggesting that nighttime closed-eye tears can attract PMNs to the ocular surface. This is consistent with the observation that the number of PMNs collected in the morning after sleep is higher than collection

occurring during the daytime open-eye conditions [76]. Tear PMNs also exhibit a low level of CD62L (L-selectin) and a high level of CD11b (MAC-1) compared to the resting blood PMNs [4]. CD62L and CD11b are cell surface markers indicative of PMN activation and adhesion. During the process of transmigration, CD62L on the surface of PMNs interacts with its ligand on epithelial cells, followed by the binding of CD11b with its epithelial ligand, ICAM-1 (CD54), causing an upregulation of CD11b and a downregulation of CD62L on the surface of PMNs. Low CD62L and high CD11b may thus suggest that tear PMNs may extravasate from the blood vessels, although this phenotype is also a hallmark of neutrophil activation and tear PMNs have been found to activate in the closed-eye environment (see next section). Thus, while collectively high levels of chemoattractants observed in the closed-eye tears, along with the shedding of CD62L on tear PMNs, strongly suggest that tear PMNs found in the closed-eye environment have extravasated from the conjunctival blood vessels, no direct evidence exists that links the number of tear PMNs and chemoattractant levels found in tears.

2.6 The Role of Tear Neutrophils in Ocular Homeostasis

The phenotype of tear PMNs collected from the closed-eye environment has just started to be characterized. Compared to resting blood PMNs, unstimulated tear PMNs collected from the eye after sleep exhibit an activated phenotype [4–6,9], with changes in expression of several surface membrane receptors which are summarized in Table 2-1.

Table 2-1. CD markers commonly used to characterize the phenotype of PMNs.

CD marker – Antigen	Leukocyte function assessed
CD11b (Mac-1) [4–6,9,77,78]	Adhesive cell interaction, a marker for neutrophil activation
CD14 [4,6]	Binding receptor for LPS, a marker for phagocytosis
CD15 [4,6]	Mature neutrophils marker
CD16 [79–82]	Fc gamma type III receptor, a marker for degranulation and phagocytosis
CD32 [83–86]	Fc gamma type II receptor, a marker for phagocytosis
CD43 [9,71,87]	A marker for PMNs reactive oxygen species production and phagocytosis
CD45 [4–6,77,78]	Common leukocyte marker
CD54 (ICAM-1) [77,82]	Adhesion marker
CD55 [88]	Complement regulation marker
CD62L [4,6,77]	L-selectin
CD63 [88,89]	Marker for azurophil granules
CD66b [4–6,9,77,78]	Degranulation marker
CD88 [77,90]	Receptor for C5a ligand
CD170 [88,90]	Immunoregulation marker
CD177 [79,88,91]	Marker for regulating transmigration across the endothelium (adhesion marker)
CD181 [77,88]	Receptor for type I IL-8
CD182 [85,88,92]	Receptor for type II IL-8

As discussed above, tear PMNs show a higher baseline expression of CD11b and a lower expression of CD62L [4,5], which is similar to what has been observed on transmigrated PMNs found in other tissues, such as the lungs and mouth [77,88]. Additionally, tear PMNs express lower levels of CD16 and CD43 and higher levels of CD66b and CD63 [4–6,9]. CD16 is known to be shed/cleaved from the

surface of activated PMNs, a process mediated by metalloproteinases and serine proteinases, such as gelatinase B and neutrophil elastase [93]. Similar to CD16, CD43 was also shown to be cleaved by neutrophil elastase [87,94]. Thus, the downregulation of CD16 and CD43 observed on tear PMNs may suggest that azurophilic granules, where neutrophil elastase is stored, are being released by PMNs upon activation. Jin et al. examined the phenotype of tear PMNs after a full night of sleep and found that tear PMNs exhibited a 30-time lower level of CD16 compared to circulating blood PMNs [5]. Furthermore, CD16 expression on tear PMNs remained close to the background level and could not be modulated in response to IL-8 and PMA [5]. Mahajan et al. collected the eye discharge from the ocular surface of healthy participants and reported a background level of CD43 on tear PMNs, which was 32 times lower than blood PMNs [9]. These findings may indicate that tear PMNs have released azurophilic granules during the night. This hypothesis can be further supported by the observed upregulation of CD63 on tear PMNs collected from healthy participants in comparison to blood PMNs [95]. CD66b is present on the specific granules, and the elevated level of CD66b compared to blood PMNs may indicate the release of specific granules by tear PMNs [4,5,9,95]. Collectively, these findings suggest that tear PMNs in the closed-eye environment may undergo degranulation.

Tear PMNs have been reported to have a limited ability to upregulate key markers of activation upon stimulation *in vitro* [4–6], such as with PMA, LPS, fMLP and IL-8, suggesting an impaired phenotype of tear PMNs, which may be due to prior activation in the closed eye environment or to a different phenotype altogether. To better understand the phenotype observed with tear PMNs, experiments were performed by Postnikoff and Gorbett to expose blood PMNs to conditions similar to the closed-eye environment [96]. Results indicated that *in vitro* exposure to hypoxic conditions, human corneal epithelial cells, and artificial tear solution could not induce the non-inflammatory/refractory phenotype in blood PMNs, highlighting the complex nature leading to the impaired phenotype of tear PMNs collected after prolonged eye closure.

A recent study found that tear PMNs were capable of producing ROS in response to PMA but not to fMLP and LPS *in vitro*, suggesting a stimuli-dependent ROS production [10]. PMA enters cells directly to activate PMNs, while fMLP and LPS stimulate PMNs via binding to their receptors. Hence, it was hypothesized that the differential ROS production observed may be caused by the impairment of intracellular signalling pathways downstream of the surface receptors and upstream of the NADPH oxidases [10]. PMN activation is initiated through the binding of inflammatory molecules, including cytokines, chemotactic and chemical stimuli, and bacterial endotoxins, to their corresponding receptors, which triggers the downstream signalling pathways involving various protein kinases, such as protein kinase C (PKC), p42/44 (ERK) mitogen-activated protein kinase (MAPk), and p38 MAPK. Interestingly, ERK and p38

MAPK were found to be functional in tear PMNs, suggesting that the impaired ROS production was not linked to a dysfunction or a lack of availability of these molecules [10]. This study highlighted the need for further studies to assess the functionality of other signalling molecules in ROS production. Unstimulated tear PMNs were also found to constitutively produce ROS over time *in vitro* [10], similar to what has been observed in aged blood PMNs [28]. This would support the activation hypothesis and suggest that characterizing the maturation stage of tear PMNs would contribute valuable information. Noteworthy is also the fact that this spontaneous ROS generation by tear PMNs may shed light on their potential role in contributing to oxidative stress in several ocular complications, such as dry eye disease and keratitis [97].

NETs have been reported to be present in eye discharge, along with PMNs, dead epithelial cells, and entrapped bacteria [9]. As a critical killing mechanism of PMNs, NETs are efficient in trapping and killing pathogens through granular proteins and histones that are present in the NETs [98]. Thus, the discovery of NETs in eye discharge further supports that tear PMNs have been activated in the closed-eye environment.

Postnikoff et al. investigated cell number and phenotype of tear PMNs collected after 1-hour sleep and a full night of sleep (with an average sleep time of 7 hours; 7-hour sleep) [6]. Aside from the significant increase in cell number harvested after 7-hour sleep, they observed substantial downregulation in the expression of CD14 and CD16 with increasing eye closure time [6]. CD14 and CD16 are markers for phagocytosis [83,99], so the decrease in CD14 and CD16 levels may be associated with reduced phagocytic capability of PMNs, something which has not yet been fully investigated in tear PMNs. Reduced level in CD16 has been shown to be related to apoptosis [84], however it has previously been reported that tear PMNs are not apoptotic [4]. Additionally, the decrease in CD16 would be associated with degranulation of tear PMNs (CD16 is a marker for the release of azurophilic granules, which are released last [6]), which is contradicted by the fact that CD66b (a marker of degranulation) did not change between the two collection times. Therefore, while this study provided some insights in changes of activation markers over time of eye closure during the night, several questions remain unanswered around tear PMN activation in the closed eye environment.

2.7 The Role of Tear Neutrophils in Ocular Inflammation

As PMNs are innate immune response cells, their roles in other systemic inflammation, such as sepsis and acute respiratory distress syndrome (ARDS), have been extensively investigated but remain largely unknown in ocular surface inflammation. With the improved collection protocols that have been established recently [4], enough tear PMNs can be collected from the ocular surface, allowing for

subsequent phenotype-characterizing studies. This has led tear PMNs to be a new area of research in ocular surface inflammatory diseases, which is expanding further with the combination of high throughput methodologies such as 36-color flow cytometry [100] and mass cytometry [101].

2.7.1 Dry Eye Disease

Dry eye disease (DED) is a common ocular surface disease, which definition has evolved over time. The Tear Film and Ocular Surface Society (TFOS) and the Dry Eye Workshop (DEWS) II have recently provided a comprehensive definition of DED: “dry eye is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles” [102]. Research has shown that T cell-mediated inflammation may be a contributing factor to DED and plays an important role in its pathogenesis [103–105]. Additionally, the symptoms of dry eye patients improved significantly after taking treatments that aim to inhibit T cell activation and cytokine production, such as cyclosporine [106], which further emphasizes the etiological role of T cells in DED.

While the presence of PMNs had been reported on the ocular surface under both healthy and pathological conditions [4,107], the role of PMNs in DED remained largely unexplored until recent studies started to point towards a potential role of these innate immune cells. De Paiva et al. reported the elevated mRNA expression of inflammatory cytokines, including IL-17A, TNF- α , and IL-6, on the ocular surface of DED patients [108]. IL-17A, which is produced by Th17 cells, has been shown to enhance the production of multiple proinflammatory cytokines, such as the IL-8 and TNF- α , by epithelial cells and fibroblasts. Additionally, damaged corneal epithelial cells can release IL-1 β , leading to increased secretion of IL-6 and IL-8 [109]. These, in turn, could cause the recruitment of other leukocytes, such as PMNs, to the ocular surface [108]. Postnikoff *et al.* observed that individuals suffering from DED tend to have more tear PMNs than healthy individuals, although statistical significance was not achieved due to individual variations [76,95]. The higher cell number collected from DED patients matched with results reported by Nair et al. [8].

Patients suffering from DED exhibited elevated levels of oxidative stress biomarkers and an imbalance between pro- and anti-oxidants on the ocular surface [97]. This imbalance may cause a direct and/or indirect effect on the ocular structures, ultimately leading to the instability of the tear film. A low amount of ROS is essential for normal cellular physiological activities, such as signal transduction, proliferation, and pathogen elimination [110], but overproduction of ROS can lead to oxidative stress,

resulting in severe damage to the surrounding tissues. While oxidative stress can be induced by several sources, such as ultraviolet (UV) radiation [111], tear PMNs collected from healthy eyes after sleep produced ROS spontaneously without any stimuli [10], further highlighting the potential value in exploring the role of tear PMNs in DED.

Additionally, an excessive amount of extracellular DNA filaments (eDNAs) are observed on the ocular surface of severe DED patients [107,112] and has been positively correlated with the severity of DED symptoms [112]. Lipocalin and DNase are examples of nucleases that are naturally present in tears and help to prevent the accumulation of eDNAs on the ocular surface. An excessive amount of eDNAs may trigger the activation of the immune system and lead to ocular inflammation [107,112]. Both epithelial cells [107] and PMNs [112] can release eDNAs, with the former releasing it in a type of danger-associated response [107], while the latter releasing it as one of their killing mechanisms, known as NETs. In addition, hyperosmolar stress, one of the pathological mechanisms involved in DED, can induce the release of NETs by PMNs [112]. Recent research has demonstrated that the application of DNase eye drops can substantially relieve the symptoms in DED patients [113]. Taken together, these findings may suggest the pathological role of PMN-induced NETs in DED.

Compared to healthy individuals, tear PMNs collected from subjects with DED exhibited higher levels of CD63 and CD66b on their cell membrane, indicating that PMNs released more granular components to the extracellular environment, in this case, the ocular surface [95]. This can be further supported by elevated granular proteins, neutrophil elastase (NE, stored in primary granules) and MMP-9 (stored in secondary granules) in tears from DED patients, although no significant correlation between the increase in granular proteins in tears and upregulation of CD66b and CD63 in tear PMNs has been observed [95]. The elevated degranulation of tear PMNs in DED is further supported by Nair et al., who reported that DED patients showed a higher proportion of CD66b⁺ PMNs compared to the control group [8]. Interestingly, DED patients tend to have a higher neutrophil-lymphocyte ratio (NLR), approximately 64:1 upon awakening compared to 21:1 in healthy eyes [76], which parallels what has been observed in blood of DED patients [114,115]. NLR is a common and useful biomarker of systemic inflammation [115–117], and recent results reported elevated NLR values, derived from blood leukocyte count, in several ocular diseases, such as keratoconus [118] and optic neuritis [119].

Taken together, these findings suggest that there is an innate immune-mediated component involved in DED, with the excessive PMNs recruitment to the ocular surface potentially due to the release of cytokines, such as IL-17A and IL-1 β , either from other immune cells or damaged corneal epithelial cells. Tear PMNs may thus contribute to the pathogenesis of DED and future research will be needed to better understand the underlying pathways.

2.7.2 Ocular Allergy

Ocular allergy (OA) is a general term that includes two common diagnoses, seasonal and perennial allergic conjunctivitis (SAC and PAC, respectively) [120]. SAC occurs mainly in the spring and summer months due to the presence of airborne pollens, while PAC can manifest throughout the year when exposed to perennial allergens [120]. Itchy eyes, tearing, redness, and sensitivity to light are typical symptoms experienced by more than 30% of individuals in North America who suffer from seasonal allergies [121].

Acute allergic responses or hypersensitivity reactions are initiated by the interaction of IgE and allergens, which activate mast cells and stimulate the release of various pro-inflammatory mediators, such as histamines, leukotrienes, IL-4, and TNF- α [122–124]. These inflammatory mediators may further recruit other leukocytes to the ocular surface, such as eosinophils and neutrophils, exacerbating the allergic response and worsening symptoms. Several studies have reported an increased level of cytokines as well as the presence of leukocytes, such as eosinophils, PMNs, and lymphocytes, in the tears of individuals with OA [122–129]. Eosinophils, which are considered a hallmark of allergic responses [128], have been observed on the ocular surface of OA patients [130], but not in those that are free of OA [124,129]. While PMNs are present on the ocular surface of healthy individuals, their numbers were shown to increase in response to allergen challenges [127,129] as well as in OA patients [124]. However, these studies analysed very low cell numbers (with an average of less than 10 PMNs per participants) and large variations were observed, due to the cell collection technique and time of the day for the cell collection. The studies used capillary tubes [127], conjunctival scrapings [129], and snip biopsies [124], which limited the ability to collect sufficient number of cells and potentially making it difficult to assess phenotype or functionality of immune cells. Furthermore, as mentioned earlier, the number of PMNs peaks in the morning after sleep and reaches a trough during the day, suggesting that studies should consider collection at various time points.

The role of tear PMNs in OA remains poorly understood. A murine model of allergic eye disease demonstrated a significant presence of NETs [7], indicative of underlying neutrophil activation. While NETs have yet to be reported in the eyes of individuals suffering from ocular or nasal allergies (as no one has studied this yet), neutrophils have been found on their ocular surfaces [131,132]. These recent studies suggest that degranulation and NETs could be an important hallmark of tear PMNs in ocular allergy, and further research is necessary to investigate the role of tear PMNs in ocular inflammation.

2.7.3 Keratoconus

Keratoconus (KC) is an ocular disease characterized by a progressive thinning and distorted cornea, which may lead to various outcomes, including irregular astigmatism and decreased visual acuity. KC was

initially considered a non-inflammatory disease, but recent findings suggest that KC may be associated with ocular surface inflammation. Studies have shown elevated concentrations of various inflammatory factors, such as IL-6, TNF- α , and MMP9, in tears of patients with KC [133]. Additionally, D'Souza found that the number of tear neutrophils was lower in KC patients compared to healthy individuals, and while this did not achieve statistical significance, a higher proportion of tear PMNs were observed in KC patients compared to in healthy individuals [133]. Bhattacharya et al. also demonstrated that tear PMNs from KC patients exhibited a greater number of phagosomes compared to tear PMNs collected from healthy eyes [134]. These results point towards a potential role of PMNs in KC although more research is needed to further characterize the phenotype of tears PMNs in KC and how they may or may not contribute to the pathogenesis of KC and some of the inflammation observed in KC.

2.8 Conclusion

While extensive research has been conducted on PMNs in other tissues and blood, tear PMNs represent a novel avenue for investigation to better understand ocular homeostasis and ocular diseases. Tear PMNs collected upon awakening in the morning (after prolonged eye closure at night) exhibit signs of degranulation and activation, which also appear to increase in intensity in the diseased eye. While PMNs are potent effector cells, they may cause severe damage to host tissues if they are over-activated. The roles of tear PMNs in ocular homeostasis, specifically, ocular surface, and inflammation are not yet fully understood, and phenotypic changes may lead to the progression and severity of ocular inflammation. Given PMN functions are a double-edged sword, there must be a balance between anti- and pro-inflammatory responses to prevent the over-activation of tear PMNs and protect the ocular structures, which warrants further investigation.

Chapter 3. Effects of Circadian Rhythm on Immune Cells

3.1 Introduction

The rotation of the Earth around its axis causes variations in sunlight exposure and temperature, which can lead to the endogenous oscillations of biological and behavioural responses over a 24-hr period in almost all living organisms to better adapt to the environment, known as circadian rhythm [1,2]. Mammals have their master circadian pacemaker, the suprachiasmatic nucleus (SCN), in the anterior hypothalamus of the brain [3]. When light reaches the retina, a group of cells, termed the intrinsically photoreceptive retinal ganglion cells (ipRGCs), sense and transmit photic information to the SCN via the retinohypothalamic tract [4]. The SCN can also receive non-photoc information from disparate nerves [5]. All inputs converged and are integrated by the SCN, which will send the circadian information to other brain areas and peripheral tissues through neural inputs or the endocrine system [6].

Almost every peripheral tissue or cell possesses intrinsic clock mechanisms involving various transcription activators, such as CLOCK and the activators brain and muscle arnt-like 1 (BMAL1), and repressors, such as CRYPTOCHROMES (CRYs) and PERIODs (PERs), which form the transcription/translation feedback loops [4]. The CLOCK-BMAL1 complex supports the formation of the CRYs-PERs complex, which can enter the nucleus to inhibit the positive activators (the CLOCK-BMAL1 complex). However, if the CRYs-PERs complex level decreases in the nucleus, the synthesis of the CLOCK-BMAL1 complex will be re-initiated, and the feedback loop will be reset. The action of transcription/translation feedback loops can help set the phase of the circadian rhythm and induce the transcription of other clock-related genes [7]. Although the rhythmic cell responses and metabolic activities of peripheral tissues can be regulated by both intrinsic cellular clock mechanisms and the central clock SCN, the SCN is required and essential to synchronize and control the peripheral clocks over the long-term [4]. Thus, the circadian system comprises both the central nervous system, the SCN, and the autonomic circadian genes in peripheral cells or tissues.

The circadian rhythm regulates many physiological functions, such as cell proliferation, DNA repair, and immune responses [8]. The immune system is vital to defend against infections and other diseases and comprises organs (such as the spleen), blood cells, and proteins (such as antibodies). Depending on immune cell types, circulating leukocyte subpopulations show circadian variations in cell count, either peaking during the day or night (for a full review refer to [9]); for example, neutrophil (polymorphonuclear cells, PMNs) blood cell count peaks between 8 pm and 2 am in humans [7,10,11]. Additionally, most immune cells, except natural killer (NK) cells, have their maximal inflammatory activity and are more effective at engulfing pathogens at night [12]. Disruption of the circadian rhythm may increase

the incidence of systemic diseases, such as diabetes and cardiovascular diseases, and may cause individuals to be more susceptible to infections [7]. Additionally, symptoms of allergic diseases have been observed to follow a circadian pattern, with asthma symptoms worsening in the early morning or at night [13]. These findings highlight the impact of the circadian rhythm on the immune response. Although the autonomous cell clock and the SCN are required to work in coordination to relay the timing information to cells, how the circadian and immune systems communicate with each other is not well understood. In this chapter, the potential bidirectional communication mechanisms between the circadian system and the immune response, as well as the circadian regulation of different immune cells are discussed.

3.2 Bidirectional Communication Between Circadian System and Immune Responses

3.2.1 Modulation of the Immune System by the Circadian System

The excitatory and inhibitory SCN efferents can directly regulate the autonomic nervous system, which can deliver circadian information to the immune system through its sympathetic and parasympathetic nerve branches, as shown in Figure 3-1. These nerves innervate immune organs directly, such as the spleen, the lymph nodes, and the liver [5,6,9,14]. Norepinephrine released by sympathetic nerve terminals in the spleen has the ability to regulate the cellular activity of NK cells and macrophages [6]. Similar to the spleen, hepatic NK cells are also regulated by sympathetic inputs [5,6,15], suggesting the ability of the autonomic nervous system to modulate immune responses. In contrast, the binding of acetylcholine to its cholinergic receptors on macrophages can inhibit the release of cytokines, such as interleukin 1 (IL-1), suggesting an anti-inflammatory effect of the autonomic nervous system on immune cells [16].

Besides the direct innervations to the primary and secondary lymphoid organs, autonomic nerves also project to the glands, such as the pineal and adrenal glands [6], and this communication or pathway is also known as the Hypothalamus-Pituitary-Adrenal axis (HPA), as illustrated in Figure 3-1. Melatonin, the primary pineal hormone, reaches its peak concentration during nocturnal time and has been shown to have a circadian impact on immune cells [5,6]. Melatonin binding to its receptor on both the nuclear and cell plasma membranes of immune cells can modulate downstream cellular activities, such as inflammatory cytokine production and release [17]. Additionally, the high concentration of melatonin at night correlates with an increase in the T lymphocyte population in blood [6]. The role of melatonin has been further illustrated in a pinealectomy mouse model with reduced T-cell-related cytokines [18] and melatonin supplement restoring leukocyte proliferation in the bone marrow as well as its rhythmic pattern [19]. These findings suggest that melatonin plays an essential role in conveying circadian information to the immune

system, although Naidu et al. reported the potential involvement of other mediators in the circadian regulation of the immune response [20].

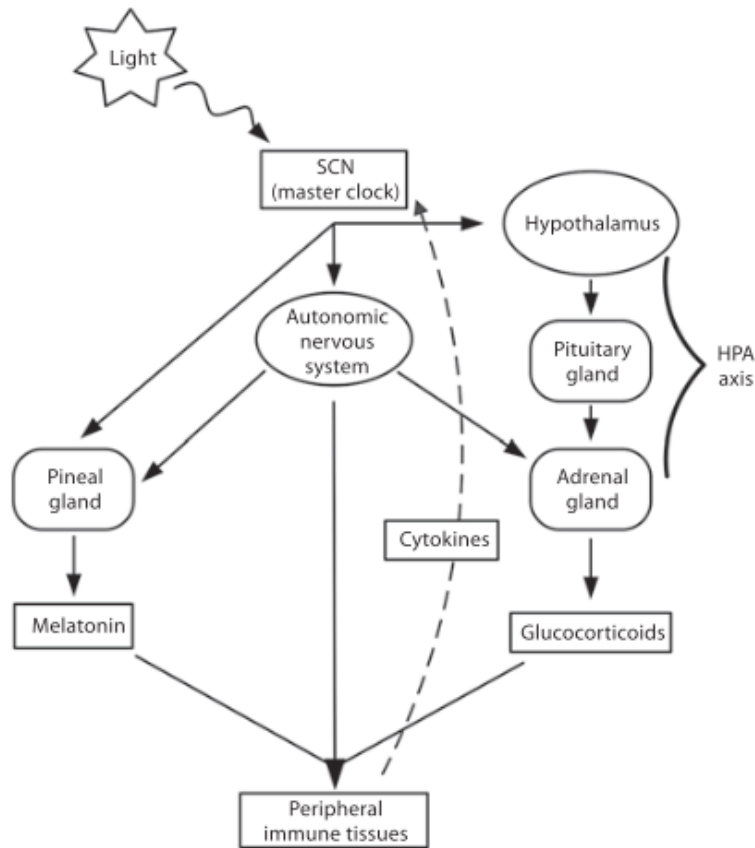


Figure 3-1. Schematic summary of the bidirectional communication between the circadian rhythm and the immune response [6]. Reprinted with permission from: Mavroudis PD, Scheff JD, Calvano SE, Androulakis IP. Systems Biology of Circadian-Immune Interactions. *J Innate Immun* 2013;5:153–62.

Glucocorticoids, such as cortisol in humans, are produced by adrenal glands and peak during the day, especially after sleep in the early morning [9]. Glucocorticoids can induce transcription of circadian genes by binding to receptors present on most peripheral cells, such as liver, heart, and kidney cells, to phase shift peripheral clocks [21]. Glucocorticoids have the capability to be the communicator between the SCN and peripheral tissues, although it is thought that glucocorticoids may not be the only conveyor signal [21]. Glucocorticoids play an essential role in transmitting rhythmic signals to immune cells [5,22] and have an anti-inflammatory effect [6], which is opposite to melatonin. Glucocorticoids can induce the upregulation of C-X-C motif chemokine receptor 4 (CXCR4, CD184) expression on T cells *in vitro* [23], which was also observed in naïve and central memory T cells by Dimitrov et al. [24]. These *in vitro* findings

may suggest that high concentration of glucocorticoids in the daytime aids the homing of T cells, specifically naïve and central memory T cells, to the bone marrow via CXCR4 to quiet the adaptive immune response. Furthermore, glucocorticoids affect rhythmic cytokine production, leukocyte distribution, cell proliferation, and apoptosis [5,6]. All in all, melatonin and glucocorticoids peaking during the night and in the early morning, respectively, are two crucial signalling molecules conveying circadian information from the central nervous system (CNS) to the immune system.

3.2.2 Modulation of the Circadian Rhythm by the Immune System

The section above discussed circadian regulation of the immune system by the autonomic nervous system and endocrine hormones, while the immune system may also regulate or modulate the circadian rhythm. Research has shown that the immune system is capable of signaling the CNS about injury/infection occurring in peripheral tissues [6,25,26], via the release of cytokines or the interactions between cytokines and nerves, such as IL-1 β and the vagus nerve, respectively [25]. Specifically, the release of IL-1 β by peripheral immune cells can induce the production of IL-1 β by glial cells in the brain via the release of neurotransmitters by the vagus nerve. The binding of IL-1 β on its receptor in the brain can then regulate physiological responses in the body, such as fever and peripheral glucose metabolism [25]. IL-1 β can also bind to receptors on paraganglia (a group of glomus cells that exhibits chemoreceptor functions), activating vagal fibres by releasing neurotransmitters and ultimately sending signals to the brain [25]. Cytokines produced by immune cells can thus affect hormone release, neural activity, and autonomic function of the CNS [27].

Injection of LPS, a bacterial endotoxin, into rats has been shown to disrupt the circadian rhythm of clock genes and locomotor activity [28]. The injection of LPS caused a significant increase in cytokine concentration, such as TNF- α , and transient suppression of *Clock* genes in the SCN and hepatic cells [29], suggesting that the immune system can affect the circadian rhythm and that inflammation may play a role in circadian disruption. However, a study in mice by Takahashi et al. [30] did not observe any changes in *Clock* genes after LPS injection, which may imply that differences may exist between animal models and other factors such as exposure time, times of measurements, and the concentration of LPS may also play an important role in the different responses observed. In many cases, cytokines have been reported to play a crucial role in the effect of LPS on circadian rhythms and *Clock* genes. TNF- α has been shown to affect some circadian *Clock* genes, and its concentration increased substantially in response to inflammatory stimuli [31]. Additionally, interferon- α (IFN- α) has been found to affect body temperature, locomotor

activity, and the mRNA expression of *Clock* genes in the SCN by binding to IFN- α receptors in the SCN [32].

Further research is needed to better understand the complex interactions between the immune system and circadian rhythm. This includes studying the specific cytokines and hormones involved, as well as the underlying molecular and cellular mechanisms.

3.3 Circadian Regulation of Immune Cells

3.3.1 Monocytes and Macrophages

Monocytes are phagocytic cells with antigen-presenting cell (APC) function, which can present the fragment of the phagocytosed pathogen on their cell membranes to communicate and activate other immune cells, especially T lymphocytes [33]. Monocytes constitute 5% to 10% of the total leukocytes and can differentiate into different subtypes. When monocytes transmigrate to local tissues, they differentiate into macrophages, specific tissue-resident cells. The autonomous clock mechanisms in macrophages and monocytes can regulate various downstream circadian-related genes, so most of their functions exhibit circadian rhythm, including their responses to LPS through the pattern-recognition receptors (PRRs), the release of cytokines, and their infiltration to the tissues [13].

PRRs can detect pathogen-associated molecular patterns (PAMPs) which can trigger downstream cellular activities, with toll-like receptors (TLRs) being one example. It has been shown that peritoneal macrophages isolated from mice at different time points respond to LPS in a time-of-day dependent manner [13,34]. Although TLR-4 is the receptor for LPS, its expression on cell membranes does not follow the circadian pattern. This may suggest that the time-of-day-dependent response to LPS by macrophages is likely due to the downstream signalling molecules which may be under the control of circadian rhythm [34]. While TLR-4 expression on macrophages is constitutive, TLR-9 expression, a receptor for bacterial or viral DNA and involved in regulating the inflammatory response during sepsis [13], shows a circadian variation.

Research showed that the secretion of TNF- α and IL-6 by LPS-stimulated macrophages follow the circadian rhythm [34,35], with the maximal concentration of IL-6 at the start of the active phase in mice [35]. The mechanism of this circadian regulation on cytokine release is hypothesized to be at the transcriptional or posttranscriptional level. As mentioned earlier, the autonomous transcription loops that regulate the clock pathways in the peripheral cells involve CLOCK and BMAL1 as the transcriptional activators and CRY1/CRY2 as the transcriptional repressors. Mice with a depletion of the *Bmal1* gene

(*arnt*) in macrophages, monocytes, and granulocytes failed to release the pro-inflammatory cytokine IL-6 in a circadian manner under LPS stimulation [35]. Additionally, macrophages isolated from *Clock* mutant mice were found to be less susceptible to release cytokines in response to LPS and *salmonella* [36]. However, there was an increase in the expression of cytokines produced by macrophages from *Cry1/Cry2* double knock-out mice [37]. Macrophages from *Clock* or *Bmal1* mutant mice were shown to have different cytokine release profiles from *Cry1/Cry2* mutant mice, which is expected as these genes play opposite roles in the peripheral clockwork.

C-C motif chemokine ligand 2 (CCL2) is a chemokine involved in the recruitment of monocytes to sites of infection or inflammation through the binding to the CCR2 receptor on the surface of monocytes. The expression of CCL2 is regulated by the circadian clock, which suggests that the recruitment of monocytes to peripheral tissues follows a circadian rhythm [13,38]. The mechanisms of the circadian regulation of CCL2 expression are proposed to be at the transcriptional level, which may involve the nuclear receptor REV-ERB α and the transcriptional activators, CLOCK and BMAL1 [35,38]. It has been shown that the depletion of *Bmal1* in myeloid cells eliminated the rhythmicity of CCL2 expression, leading to a dysregulation of monocytes migration to the peripheral tissues, which may subsequently cause an increase in susceptibility of mutant mice to the bacterial infection [39], suggesting the importance of circadian regulation in monocyte trafficking.

3.3.2 Neutrophils

Neutrophils make up about 50% to 70% of the circulating white blood cells and are the first cells to respond to infection and inflammation [33]. The egress of mature PMNs from the bone marrow into the blood and their clearance from circulation back to the bone marrow are shown to be regulated by C-X-C motif ligand 12 (CXCL12)/CXCR4 axis, which is under circadian control by the sympathetic nervous system [40]. Specifically, mature PMNs are retained in the bone marrow through the interaction between CXCR4 and its ligands, CXCL12, and their release into circulation occurs when the CXCL12 level decreases [40,41]. These findings suggest that CXCL12 is the primary retention signal for PMNs in the bone marrow. As PMNs age, the upregulation of CXCR4 leads to their migration to clearance sites where they are engulfed by resident macrophages [40,41]. Casanova-Acebes et al. identified and tracked the kinetics of aged PMNs in mice using the cell surface markers CD62L (L-selectin) and CXCR4 [42]. They found that the clearance of aged PMNs followed the circadian cycle [42], a finding that was also observed in human PMNs by Ella et al. [7]. Furthermore, rhythmic daily oscillations of CXCL12 and gene expression of CXCR4 in human blood PMNs were observed [7], which further suggests that the egress and clearance of PMNs follow the circadian cycle. However, the expression of the *Bmal1* protein in PMNs did not show

a circadian pattern, and only the expression of the *Per1* gene exhibited rhythmic changes, which was shown to be affected by the cortisol level [7]. This may suggest that the responses or kinetics of human peripheral PMNs may be controlled by systemic mechanisms rather than the intrinsic molecular clock [7]. In contrast, Adrover et al. reported rhythmic changes in the expressions of *Clock*-related genes, including *Arntl* (encoding Bmal1) and *Per2*, in mouse PMNs [43], which may control PMN aging. The discrepancy between these studies could be attributed to the different species, human versus mice. On the other hand, the capabilities of PMNs to release ROS and phagocytose microbes vary throughout the day [7,44], suggesting that the circadian rhythm affects not only the kinetics of PMNs mobilization but also the immune responses and cell functional activities of PMNs.

In healthy mice, the infiltration of PMNs into most peripheral tissues peaks at the resting period (equivalent to nighttime in humans), as depicted in Figure 3-2, such as in the lungs [45], displaying a rhythmic pattern. Gibbs et al. reported that PMNs recruitment to the lungs and their inflammatory effect against bacteria was associated with the chemokine CXCL5 that was produced by pulmonary epithelial cells in a circadian manner regulated by glucocorticoids and Bmal1 [46]. There is also a bidirectional effect between kidney disease and the circadian rhythm, whereby renal disease causes abnormal circadian rhythm and circadian disruption worsens kidney dysfunction [47]. Additionally, the pathological role of PMNs in kidney disease has been demonstrated [47], and pathogen clearance was enhanced at night due to an increased migration of PMNs to the kidney [40]. These findings may infer that the recruitment of PMNs to the kidney also follows a rhythmic pattern; however, this needs further elucidation.

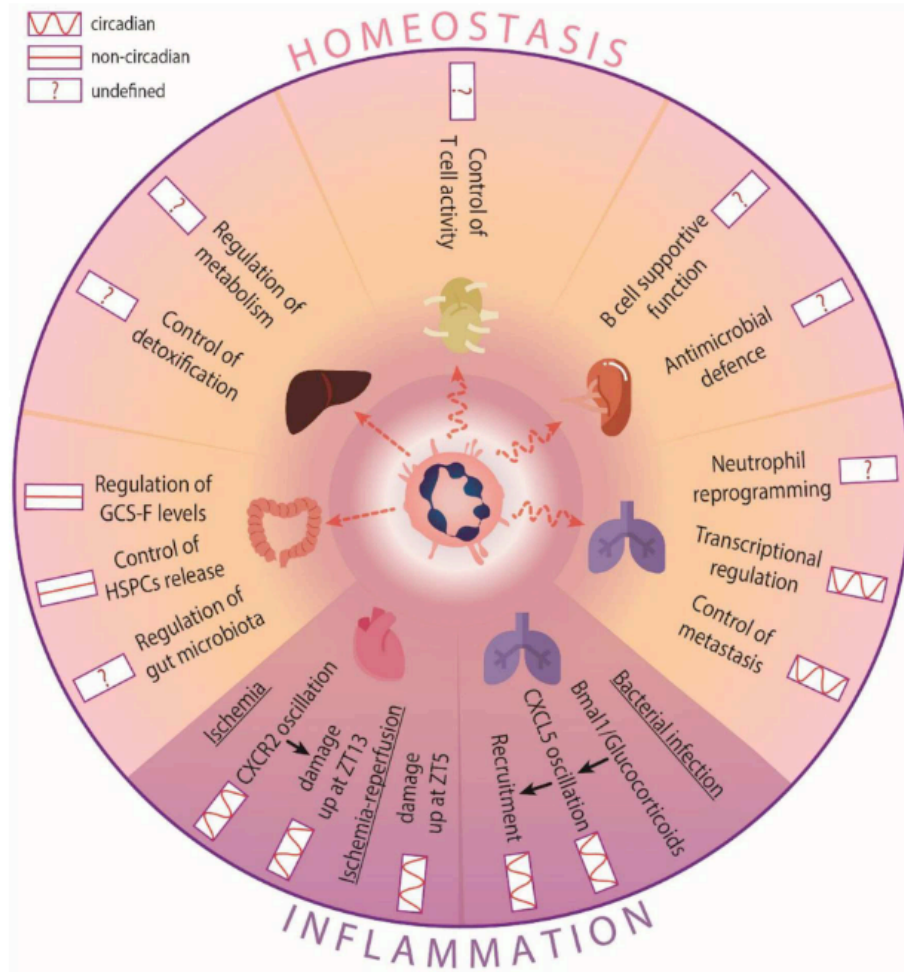


Figure 3-2. The circadian pattern of PMNs found in different tissues [41]. Under normal physiological conditions, the recruitment of PMNs to the lungs follows a circadian rhythm. However, this rhythmic pattern is not observed in the intestine nor in the liver and is also not well-established in other tissues due to limited knowledge. Furthermore, in the presence of inflammatory conditions in murine models, the oscillation in PMNs recruitment to the heart and lungs also exhibits a circadian rhythm. Image from Aroca-Crevillén A, Adrover JM, Hidalgo A. Circadian Features of Neutrophil Biology. *Front Immunol* 2020;11:1–9 (Open access article).

The recruitment of tear PMNs to the ocular environment shows signs of diurnal oscillation, with a peak at night [48,49]. The IL-8 concentration in the closed-eye tears was found to be highest upon awakening [50] and low during the day [49], indicating that IL-8 concentration may also follow the circadian cycle. The ocular surface is highly innervated by nerves, which could potentially signal the ocular epithelial cells to secrete IL-8 in a circadian manner, much like the secretion of CXCL5 by lung epithelial

cells [46]. However, future research is needed to investigate whether the rhythmic pattern of IL-8 secretion and changes in the phenotype of tear PMNs are mediated by systemic mechanisms, the central circadian pacemaker (the SCN), the intrinsic cellular clock, or both.

3.3.3 Natural Killer Cells

Natural killer (NK) cells are lymphoid cells but are considered part of the innate immune system [33]. NK cells distinguish normal from abnormal cells by recognizing self-MHC class I. Almost all normal cells express MHC class I on their cell membranes; however, the expression of MHC class I is suppressed on abnormal cells, such as tumor or cancer cells. Once the NK cells detect abnormal cells, they release pre-formed secretory granules containing cytokines and enzymes onto the targeted cells. INF- γ , granzyme B, and perforin are examples of the cytolytic substances that are released by immune cells, such as NK cells and cytotoxic T cells, to eliminate abnormal cells. By inducing apoptosis, NK cells help to eliminate abnormal cells and prevent their spread.

It has been shown by Arjona et al. that the mRNA expressions of interferon γ (INF- γ), granzyme B, perforin, and TNF- α that are released from NK cells followed the circadian pattern in the spleens of rats exposed to a light-dark cycle [51], which may indicate that the cytolytic factors and cytokines are regulated by the circadian rhythm. Additionally, TNF- α was shown to promote NK-cell-mediated killing [51], and enhanced NK cell activity during the dark period (active phase for rats), such as an increase in tumor clearance effectiveness, has been observed. These findings may suggest that the cytotoxic functions of NK cells are also regulated by the circadian rhythm, as demonstrated by the correlation between the maximum levels of NK cell responses and the highest levels of those cytokines during the dark period [52]. Furthermore, NK cells were found to express *Clock* genes in a circadian pattern [51]. The mRNA levels of *Per1* and *Per2* peaked during the light-dark transition, whereas the mRNA expressions of *Bmal1* and *Clock* reached their maximum around the early morning. Additionally, the knockdown of *Per2* in RNK 16 cells (NK cell line) decreased the levels of granzyme B and perforin proteins but did not affect the levels of INF- γ or TNF- α [13]. In contrast, Arjona and Sarkar reported that *Per2* mutant mice lost rhythmicity in the INF- γ mRNA expression and protein levels, whereas no alteration was detected in granzyme B, perforin, and TNF- α mRNA levels [53]. The inconsistency between the two studies may be due to the different models they used, as *Per2* mutant mice may still be capable of receiving timing information from the CNS.

While the impact of the intrinsic *clock* genes on the cellular responses of NK cells was discussed above, research showed that the CNS could also affect their activities through the sympathetic nervous system. Logan et al. exposed rats to repeated 6hr shifts of light-dark cycle and found that it disrupted the rhythmicity

of IFN- γ , granzyme B, and perforin mRNAs and proteins [54]. Furthermore, there was a decrease in NK cell cytotoxic activity during the dark phase, suggesting the ability of the CNS (e.g. the SCN) to control the functions of NK cells. Glucocorticoids, prolactin, melatonin, and catecholamines are all endocrine factors that are known to have circadian variation in their release, and they have been shown to play a role in modulating the function of NK cells [52]. However, it is yet to be determined if the NK cell functions are mainly controlled by their intrinsic *Clock* genes or the CNS or the coordination of both.

3.3.4 Lymphocytes

Lymphocytes comprise 20 to 40% of the circulating leukocytes in blood and 99% of the cells in the lymph nodes. T cells mature in the thymus and consist of various subtypes, including CD8⁺ (cytotoxic), CD4⁺ (helper), regulatory, and Th17 cells [55]. B cells, on the other hand, mature in the bone marrow and are capable of producing antibodies in response to foreign antigens [55]. Unlike innate immune cells, lymphocytes are able to rearrange their genes to produce a unique antigen receptor on the surface to specifically recognize and respond to a particular antigen [33]. Therefore, the rearrangement process in lymphocytes ensures diversity in the immune system, allowing it to respond to a wide range of pathogens effectively.

It has been widely investigated that changes in lymphocyte number follow a circadian pattern, with peaks at night and troughs in the morning (reviewed in [13]). Additionally, the reduced number of naïve T cells in the morning coincided with the increasing concentration of cortisol [9] as well as the upregulation of CXCR4 levels on the T lymphocytes [56]. The decrease in T-cell numbers in the morning, especially the naïve CD4⁺ and CD8⁺ T cells, was not observed when treating human participants with the cortisol blocker (metyrapone), indicating that the oscillation in lymphocytes may be regulated by the circadian rhythm-controlled hormones, such as cortisol and melatonin, as discussed in Section 3.2. Furthermore, metyrapone treatment also inhibited the upregulation of CXCR4 on T cells [56], which may suggest the critical role of CXCR4 in T-cell clearance to the bone marrow.

However, there is inconsistency among studies on the impact of circadian rhythm on the lymphocyte number in lymph nodes, which may be due to the species and experimental conditions (exposure to light and physical activity). Fortier et al. reported that the number of B, CD4⁺ and CD8⁺ (including the naïve, effector, and activated cells) and regulatory T cells did not display the circadian rhythm in the rat lymph nodes [57], while Druzd et al. found that lymphocyte counts in the lymph nodes of mice reached their maximum at night [58]. The latter research group also reported the circadian trafficking of lymphocytes to the lymph nodes [58]. Lymphocytes entered the lymph node at night and egressed from the

tissue during the day, and this process may be regulated through the expression of specific chemokine receptors and sphingosine-1-phosphate receptors (S1PRs) on the surface of the lymphocytes [58]. The chemokine receptor CCR7 is responsible for the recruitment of T cells to the lymph nodes, while S1PR1 controls lymphocyte egress from the lymph nodes into the circulation. The expression of these receptors is regulated under the circadian rhythm, with the expression of CCR7 being highest during the night, promoting lymphocyte entry into the lymph nodes, while S1PR1 expression is highest during the day, promoting lymphocyte egress from the lymph nodes [58]. Research showed the rhythmic expression of *Clock* genes, such as *Bmal1* and *Per2*, in lymph nodes, and the rhythmicity is lost in the *Clock* mutant mice [57]. However, one study showed that lymphocyte count could persist even when the SCN is experimentally inactivated by constant light exposure in rats [59], suggesting that *Clock* genes within immune cells may also contribute to regulating this rhythm.

Bollinger et al. showed that the production of IL-2, IL-4, IFN- γ , and the expression of CD40L in CD4⁺ T cells isolated from human male participants followed the circadian rhythm after phorbol myristate acetate (PMA) or ionomycin stimulation, with the lowest production or expression in the morning at 6 am [60]. Interestingly, when freshly isolated CD4⁺ T cells were cultured *in vitro* for 48 hours, the circadian production of IFN- γ and expression of CD40L still persisted [60], suggesting the important role of intrinsic clock genes in regulating the cellular functional responses. The rhythmic production of IFN- γ by T cells was further supported by Kirsch et al. that IFN- γ production was lowest in the morning after the stimulation with adenovirus antigens or *Staphylococcus aureus* enterotoxin B [61].

Moreover, it has been shown that more CD4⁺ T cells differentiated into Th17 cells when CD4⁺ T cells were collected during the day compared to the night, suggesting that T cell differentiation may also display the circadian rhythm [62]. The proliferation of T cells is essential in adaptive immune responses and is regulated by various factors, including cytokines, growth factors, and antigen stimulation. Recent research showed that T-cell proliferation in mice lymph nodes was more robust during the daytime compared to the nighttime, and the rhythmic proliferation of T cells was diminished in the *Clock* mutant mice [57], indicating that the clock genes may also control the T cell proliferation. It is also interesting to note that vaccination in the morning may double the antibody titre compared to the afternoon [63], and this may be associated with the increased proliferation of T cells in the lymph nodes during the day.

3.4 Conclusion

In conclusion, this chapter discussed the impact of the circadian rhythm on the immune response in terms of the underlying mechanisms and the circadian regulation of immune cells. It is well known that the symptoms of allergic or autoimmune diseases display a circadian rhythm, such as asthma patients experiencing more severe symptoms in the night or the early morning [64] and symptoms of rheumatoid arthritis being stronger in the early morning. These highlight the interplay between circadian rhythm and immune responses under normal physiological and pathological conditions. Thus, further investigation is needed to explore the role of circadian rhythm in immune response and how disruption or alteration in circadian rhythm may lead to the development of various diseases and disorders. This could help identify potential therapeutic targets for treating and preventing such conditions.

Chapter 4 The Differential Reactive Oxygen Species Production of Tear Neutrophils in Response to Various Stimuli In Vitro

This chapter is extracted from my published article, Jin Y, Dixon B, Jones L, Gorbet M. The Differential Reactive Oxygen Species Production of Tear Neutrophils in Response to Various Stimuli In Vitro. *Int J Mol Sci* 2021;22:12899. <https://doi.org/10.3390/ijms222312899>.

Part of the data was initially collected during my Master's degree as a pilot study with 9 participants. During my Ph.D., more participants were recruited to a total of 24 participants. Additional experiments were also conducted to investigate the impact of isolation on the phenotype and ROS production by tear PMNs and intracellular signalling molecules involved in ROS were also characterized.

4.1 Introduction

Polymorphonuclear neutrophils (PMNs), also known as neutrophils, are potent innate immune cells. PMNs play an essential role in protecting host tissues against foreign particles through oxidative bursts, degranulation, and phagocytosis, and possess many anti-inflammatory and pro-inflammatory responses. Like other immune cells, PMN populations have been shown to be heterogeneous and can exhibit phenotypes distinct from blood-circulating PMNs in different tissues (such as in the mouth [1], placenta [2], lungs [3], and the ocular surface [4]). A nocturnal-diurnal pattern has been observed in tear PMNs, the largest leukocyte population collected from the ocular surface. After prolonged eye closure, several thousand of PMNs can be collected from the ocular surface using a gentle wash, while collection during the day (open-eye conditions) often provides only a few hundred PMNs [4]. While this has not yet been fully elucidated, high levels of chemotactic molecules (e.g., interleukin-8 and complement products) detected in tears collected after sleep may explain the recruitment of PMNs at night [5]. Unlike blood PMNs, tear PMNs collected after more than 6 hr of sleep (ie closed-eye conditions) have upregulated expression of Mac-1 (CD11b, a cell adhesion and activation marker) and CD66b (a degranulation marker) and also exhibit downregulation of L-selectin, a molecule reported to be shed upon activation or extravasation [4,6]. These observations suggest that tear PMNs collected after sleep may have already been activated following their arrival on the ocular surface due to the presence of inflammatory cytokines and foreign particles such as bacteria [6]. This hypothesis has been further supported by the presence of neutrophil extracellular traps (NETs) in the eye discharge [7]. Additionally, tear PMNs have been reported to be unable to upregulate or downregulate cell activation membrane receptors in response to various

inflammatory stimuli *in vitro* [4,6]. However, there very little is currently known about their ability to generate reactive oxygen species (ROS), with only one study assessing intracellular ROS production using flow cytometry at one specific time point and pointing towards a potential exhaustion or non-inflammatory phenotype due to the lack of response [4].

Oxidative burst, a potent killing mechanism in PMNs, has been investigated extensively in blood PMNs. Although ROS produced during the oxidative burst can eliminate foreign particles, excessive ROS production may also cause severe damage to surrounding tissues [8]. During the oxidative burst, nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) reduces molecular oxygen to superoxide anion (O_2^-) upon activation, leading to an increase in oxygen consumption by PMNs [8,9]. NADPH oxidases found in PMN plasma membranes contribute to the extracellular production of superoxide anions, whereas NADPH oxidases in PMN granular membranes generate superoxide anions in an intracellular manner [10]. Complement fragment C5a, opsonized microorganisms, platelets activating factor (PAF), N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, a chemotactic peptide secreted by bacteria), lipopolysaccharides (LPS, fragments of gram-negative bacteria outer cell membranes), and phorbol-12-myristate-13-acetate (PMA) have been found to be efficient stimuli of the oxidative burst in PMNs [8]. The interaction between these stimuli and their corresponding receptors triggers a series of intracellular signalling pathways inducing different cell functional responses that have been well documented in the blood PMNs [11].

To gain a better understanding of the role and function of tear PMNs in ocular homeostasis, this study investigated their ability to produce ROS in response to fMLP and LPS, which act via G protein-coupled receptors (GPCRs) and toll-like receptors (TLRs), respectively, and PMA, which directly activates protein kinase C (PKC). ROS production was assessed by luminol-enhanced chemiluminescence, as it provides a kinetic measurement of ROS formation and can efficiently detect superoxide anions from both intra- and extracellular compartments [12,13]. ROS production in response to the stimulus was measured directly in the cell collection from the closed-eye environment as well as following isolation of the tear PMNs population from the cell collection. While PMNs represent the largest population in the cell collection from the closed-eye environment, other leukocytes as well as a few dead corneal epithelial cells and occasionally goblet cells are also present. The expression of cell membrane receptors associated with cell activation was also assessed by flow cytometry following PMN isolation from the eye wash collection.

4.2 Materials and Methods

4.2.1 Materials

Endotoxin-free reagents, medium and sterile polypropylene tubes were used in this study to avoid undesired activation of PMNs. LPS (*Escherichia coli* serotype 0111:B4), PMA, fMLP, Hanks's balanced salt solution (HBSS), luminol (5-amino-2,3-dihydroxy-1,4-phthalazinedione), and superoxide dismutase (SOD) were ordered from Sigma-Aldrich Co. (Oakville, ON, Canada). Phosphate-buffered saline (PBS) was purchased from Lonza (Allendale, NJ, USA). Dulbecco's modified eagle media (DMEM) was purchased from Life Technologies (Burlington, ON, Canada). The MiniMACS-column cell separation system was purchased from Miltenyi Biotec Inc (Auburn, CA, USA). EasySep™ Human CD15 positive selection kit for cell separation was purchased from STEMCELL technologies (Vancouver, BC, Canada). All fluorescently-labelled antibodies were purchased from BD Biosciences (Mississauga, ON, Canada)

4.2.2 Subjects

This study received ethics clearance from the University of Waterloo Human Research Ethics Committee (#30164; Waterloo, ON, Canada) and was conducted in accordance with the tenets of the Declaration of Helsinki. A total of twenty-four healthy non-lens wearer participants, 10 males and 14 females were recruited in this study, and ages ranged from 20 to 33. All participants provided written informed consent before cell collection.

4.2.3 Tear PMNs Collection and Separation

Participants were trained to collect their cells [4]. Upon awakening, using sterile PBS in a dropper, participants gently washed the ocular surface of each eye and collected the run-off in a sterile polypropylene tube. Participants were asked to bring their samples to the lab within two hours of collection.

Reconcentrated cell suspension (mixed tear PMNs): the collected eye wash was centrifuged at 280× g for 10 min, followed by resuspension in PBS.

MACS-column cell separation system: as per the manufacturer's instructions, the collected eye wash was incubated with CD15 antibody and conjugated with magnetic microbeads in PBS containing 0.5% BSA at 4°C for 20 min. The MiniMACS-column was placed in the magnetic field of the MACS separator and was rinsed with degassed PBS buffer containing 0.5% BSA three times. Then, the cell suspension was applied to the column. In this isolation procedure, unlabeled cells flowed through the

column, while the targeted PMNs (CD15⁺ cells) were retained on the column due to the strong magnetic field. Once the entire cell suspension had been applied to the column, the column was washed three times with degassed PBS buffer. The column was then removed from the separator, and a plunger was used to flush out the targeted cells. The cell suspension was reconcentrated by centrifugation at 280× g for 5 min.

EasySep cell separation system: as per the manufacturer's instructions, the collected eye wash was incubated with a CD15 positive selection cocktail for 30 min followed by another 30 min incubation with RapidSpheres™ that contained magnetic particles bound to CD15 antibodies. RoboSep™ buffer containing 2% FBS and 1 mM EDTA was added to dilute the cell suspensions. Then, the tube containing the cell suspension was placed into the magnetic separator for 10 min, followed by the removal of the supernatant, which was repeated one more time. The cell suspension was reconcentrated by centrifugation at 280× g for 5 min.

After the isolation and reconcentration, cell count and cell viability were assessed using a hemocytometer with Trypan Blue.

4.2.4 Blood PMNs Isolation

Blood was taken from four healthy participants who did not take any anti-inflammatory medication for at least 72 h. Due to the COVID-19 restrictions on research involving human participants, blood samples were not obtained from all participants who provided tear samples. Blood PMNs were isolated using a two-density gradient centrifugation process, using PolymorphPrep (Axis Shield PoC AS, Oslo, Norway) and Histopaque (Oakville, ON, Canada) as described previously [14]. Following isolation, blood PMNs were washed twice in DMEM/10% FBS with 5 mM EDTA and then underwent one last wash in PBS. A cell count was performed and cells were resuspended in PBS at ~10⁵ cells/mL.

4.2.5 Luminol-Enhanced Chemiluminescence

Chemiluminescence (CL) measurements were assessed on the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) using 96-well cell culture white microplates (Greiner bio-one, Frickenhausen, Germany). Cells were *stimulated* with PMA (2 μM), fMLP (1.5 μM), and LPS (2 μg/mL) [4], with or without SOD (250 U/mL) which converts the superoxide to either oxygen molecules or hydrogen peroxide. Each well was set to test one stimulus, and each had a total reaction volume of 200 μL containing 50,000 cells, 1 mM luminol, its corresponding stimulus, with or without SOD, and Hanks' balanced salt solution (HBSS) which was added to a final volume of 200 μL. Controls were included in each experiment and contained the same

concentration of reagents but no stimulus (i.e., HBSS was added to complete volume to 200 μ L). CL emissions were recorded at 37 °C for 60 min at 3 min intervals.

4.2.6 Antibody Staining and Flow Cytometry

Membrane receptor staining: cell suspensions were stained with fluorescently conjugated antibodies, CD11b, CD15, CD63, CD66b and CD45, for 20 min at room temperature in the dark. Then, cells were diluted with DMEM/10% FBS and were ready for flow cytometry.

Intracellular signalling molecules staining: after cells were stimulated with either 2 μ M PMA or 1.5 μ M fMLP, they were fixed with pre-warmed 4% formaldehyde at 37 °C for 10 min. Then, cells were pelleted by centrifugation, followed by two washes with perm/wash buffer (BD Bioscience) at 1200 rpm for 5 min. Cells were stained with anti-ERK, anti-p38 MAPK antibodies, and CD45 for 30 min in the dark at room temperature. After another wash with perm/wash buffer, cells were resuspended in DMEM/10% FBS and were ready for flow cytometry.

The PMN population was identified based on double gating the cells that were high in side-scattered light (granularity), low in forward-scatter light (size), and CD45⁺. All samples were acquired on a Becton Dickinson FACS Calibur flow cytometer (Mountain View, CA, USA) using CELLQuest software (Becton Dickinson, Mountain View, CA, USA). At least 2000 PMN events were acquired, and fluorescent values for each antibody (also known as Mean Fluorescent Intensities, MFI) were recorded for all samples.

4.2.7 Statistics

After analysis of the CL kinetic graphs and based on peak responses, statistical analysis was performed on the CL response at 0, 3, 6, 12, 24, 36, 48, and 60 min. The ratio of stimulated over control CL emissions ($CL_{\text{stimulated}}/CL_{\text{control}}$) was calculated and is reported as means $\pm$ standard deviations in the function of time. GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used to compute the rate and area under the curve (AUC). A Welch's *t*-test and paired *t*-test were performed using IBM SPSS software (IBM Canada Ltd., Markham, ON, Canada), and a *p*-value of less than 0.05 was required for statistical significance.

4.3 Results

The eye wash collected from the closed-eye environment upon awakening contained different cell populations. Flow cytometry was used to identify cell populations based on their properties of forward and sideward size scatter and CD45 expression. The following populations were identified: PMNs ($57.7 \pm 7.7\%$), lymphocytes ($1.5 \pm 1.2\%$), monocytes ($0.18 \pm 0.18\%$), other CD45⁺ cells ($20.2 \pm 5.0\%$), the remainder of the events being ocular surface cells and debris. Within the 20% of the CD45⁺ cell population, two subsets of the population, CD45^{low} (~5%) and CD45^{high} (up to 10%), could be observed: expression of CD11b and CD66b on these CD45⁺ cells strongly suggest that these cells are a subpopulation of PMNs with different size scatters (Figure 4-1a, b, d, e). Experiments were also performed with tear PMNs that had been isolated from the eye wash collection using CD15⁺ positive selection with the MiniMACS column-based cell separation system (Miltenyi Biotec Inc). PMNs in this purified fraction represented $88.6 \pm 7.9\%$ of the cell populations/events. When reporting and discussing results on ROS production, purified samples are referred as MACS-isolated tear PMNs, while the non-purified eye wash collection is referred to as mixed tear PMNs to reflect the presence of different cell populations in the eye wash while recognizing that PMNs represent the largest cell population and main contributor of ROS production of the cells present in the collection (epithelial cells, while able to generate ROS, are necrotic, and lymphocytes [15,16] and monocytes [17], which can also produce ROS, contributed to less than 2% of the collection).

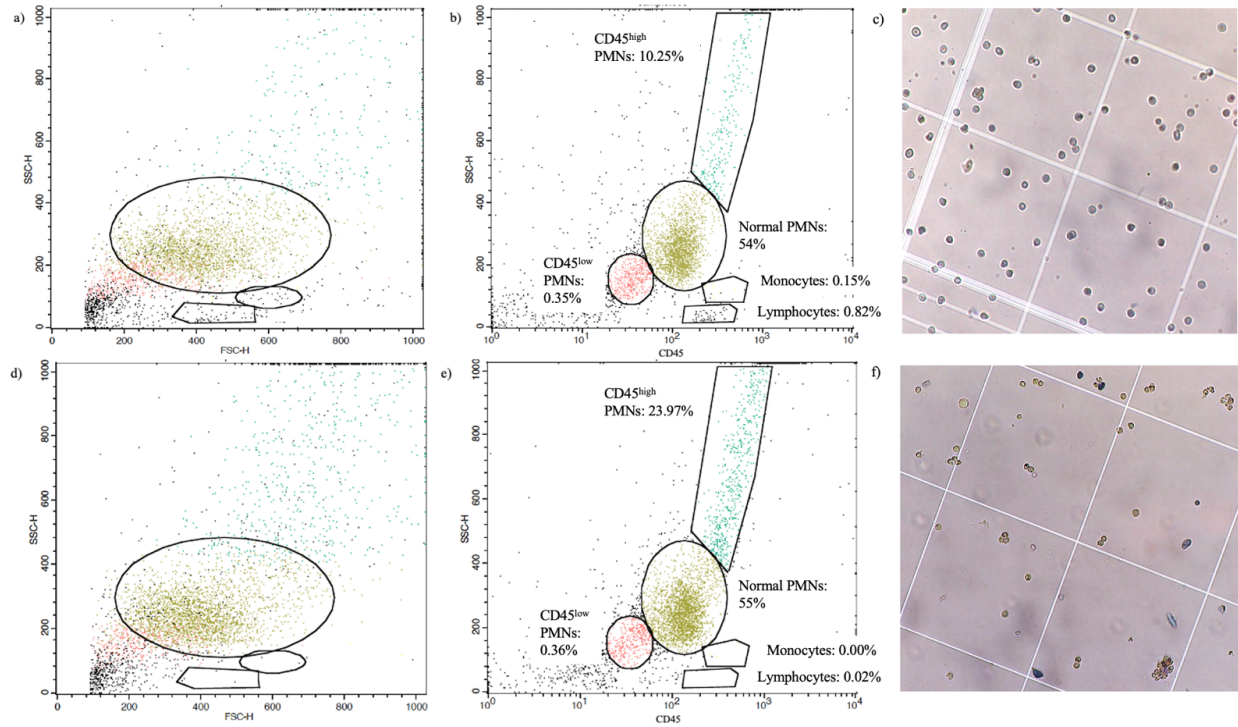


Figure 4-1. Identification of different cell populations using flow cytometry double-gating strategy in mixed tear PMNs (a–c) and MACS-isolated PMNs (d–f). (a,d) Side scatter versus forward scatter dot plots identifying leukocyte populations based on their size and cytoplasmic complexity. (b,e) Side scatter versus CD45 fluorescence (pan leukocyte marker) dot plot with CD45⁺ cells. Different cell populations have been identified along with their relative percentage. (c,f) Light microscopy images of mixed and MACS-isolated tear PMNs. Cell aggregates can be observed following isolation by the MACS column.

4.3.1 ROS Production in LPS-Stimulated PMNs

As shown in Figure 4-2, LPS did not induce an oxidative burst in either the mixed tear PMNs or the MACS-isolated tear PMNs (CL ratio being close to 1). As expected, blood PMNs produced ROS from 12 min onwards; ROS production was significantly different between blood and tear PMNs (isolated or non-isolated) after 30 min ($p \leq 0.037$).

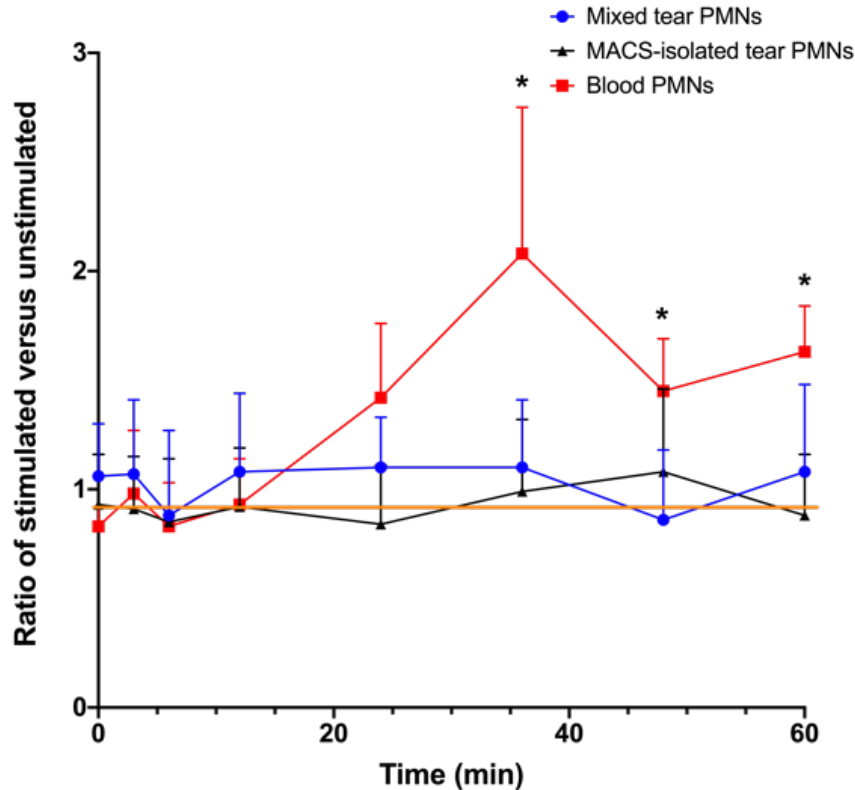


Figure 4-2. Changes in chemiluminescence (CL) of LPS-stimulated PMNs. Results are reported as ratio of LPS-stimulated PMNs CL emissions over control (unstimulated PMNs) CL emissions. A total of 50,000 cells were transferred to wells containing 1 mM luminol and HBSS followed by the addition of 2 $\mu\text{g/mL}$ LPS. Generation of ROS was measured over time using the Cytation 5 at 37 °C. The orange line indicates a ratio of 1, meaning no difference between stimulated and unstimulated CL values. Values are presented as means $\pm$ standard deviations; mixed tear PMNs (n = 10–12 from 10 participants), MACS-isolated tear PMNs (n = 6 from 6 participants), and blood PMNs (n = 5 from 4 participants). * Significantly different from mixed and MACS-isolated tear PMNs ($p \leq 0.037$).

The area under the curve (AUC) can be computed to reflect the quantity of ROS produced over 60 min. Both the absolute (for unstimulated and stimulated cells) and relative ROS production (using curves generated by calculating the ratio of stimulated versus unstimulated cells and activation ratio, as depicted in Figure 4-2) were calculated. As reported in Table 4-1, when considering absolute values, unstimulated mixed tear PMNs showed higher absolute ROS production as compared to both unstimulated and stimulated blood PMNs' ROS production. However, as shown by the AUC activation ratio values, LPS-stimulated tear PMNs produced relatively less ROS as they failed to generate ROS in response to LPS. No significant differences were observed between groups ($p > 0.05$).

Table 4-1. Absolute and relative ROS production in unstimulated and LPS-stimulated PMNs, as measured by AUC.

	Mixed Tear PMNs	MACS-Isolated Tear PMNs	Blood PMNs
AUC			
Unstimulated	10,803 ± 3810	6171 ± 4475	4384 ± 2275
AUC			
LPS-stimulated	11,048 ± 5075	5366 ± 4412	6659 ± 3262
AUC			
Activation ratio	18 ± 11	17 ± 12	39 ± 27

AUC (area under the curve) was computed using GraphPad Prism software. n = 12 from 10 participants for mixed tear PMNs, n = 6 from 6 participants for MACS-isolated tear PMNs, and n = 5 from 4 participants for blood PMNs; values are represented as means ± standard deviations.

4.3.2 ROS Production in fMLP-Stimulated PMNs

The kinetics of ROS production in fMLP-stimulated PMNs over 60 min are reported in Figure 4-3. At the 3 min time point, there was a transient peak of ROS production in fMLP-stimulated mixed and MACS-isolated tear PMNs. ROS production in tear PMNs at 3 min was significantly higher than that of blood PMNs ($p \leq 0.02$). The large CL ratio variations in the tear PMNs noted at 3 min may be due to individual differences in ROS production or the fact that the stimulus was added to the samples manually, which might have induced some variations in the time of the rapid response elicited by fMLP. As expected and previously reported by others [18], a second peak in ROS production was observed in blood PMNs around 24 min (ROS production was significantly different from tear PMNs, $p \leq 0.005$), while tear PMNs only showed one peak, at 3 min.

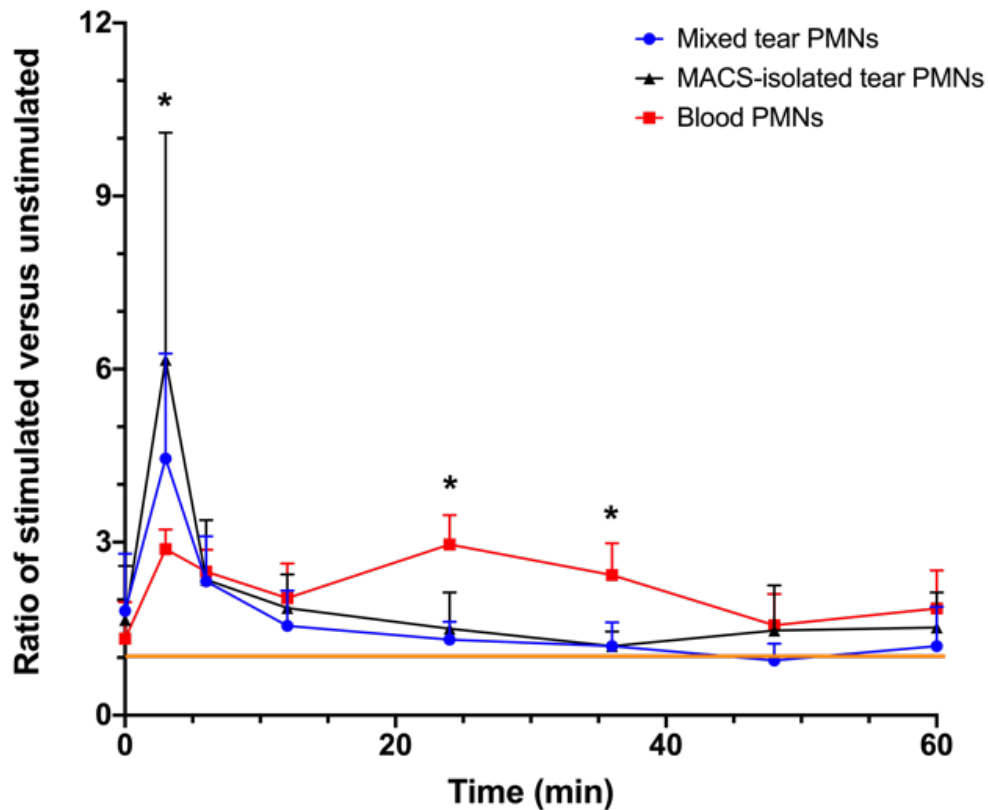


Figure 4-3 Changes in chemiluminescence (CL) of fMLP-stimulated PMNs. Results are reported as ratio of fMLP-stimulated CL emissions over control (unstimulated) sample CL emissions. A total of 50,000 cells were transferred to wells containing 1 mM luminol and HBSS, followed by the addition of 1.5 μ M fMLP. Generation of ROS was measured over time using the Cytation 5 at 37 °C. The orange line indicates a ratio of 1, meaning no difference between stimulated and unstimulated CL values. Values are presented as means $\pm$ standard deviations; mixed tear PMNs (n = 14-16 from 16 participants), MACS-isolated tear PMNs (n = 10 from 8 participants), and blood PMNs (n = 5 from 4 participants). * Significantly different from tear PMNs and blood PMNs ($p \leq 0.02$).

As seen with LPS stimulation, the absolute ROS production (AUC) was larger in tear PMNs compared to that of blood PMNs (Table 4-2). However, AUC values were similar for both unstimulated and stimulated (as one would expect, the small peak of production at 3 min did not significantly change AUC) tear PMNs, indicating that ROS could be produced, but tear PMNs were unable to mount a full oxidative response following fMLP stimulation.

Table 4-2. Absolute and relative ROS production in unstimulated and fMLP-stimulated PMNs, as measured by AUC.

	Mixed Tear PMNs	MACS-Isolated Tear PMNs	Blood PMNs
AUC			
Unstimulated	8943 ± 4389	5210 ± 4375	4384 ± 2276
fMLP-stimulated	10,489 ± 4475	7264 ± 5444	8529 ± 3296 [#]
Activation ratio	35 ± 14 *	50 ± 26 *	70 ± 15

AUC (area under the curve) were computed using GraphPad Prism Software. n = 16 from 15 participants for mixed tear PMNs, and n = 11 from 9 participants for MACS-isolated tear PMNs, and n = 5 from 4 participants for blood PMNs, values are represented as means ± standard deviations. * Significantly different from blood PMNs ($p \leq 0.01$). # Significantly different from unstimulated PMNs ($p \leq 0.005$, paired t-test).

The high and rapid generation of ROS within 3 min after exposure to fMLP has been previously observed in blood PMNs [19,20] and reported to be associated with extracellular production and superoxide anions [16,20]. Superoxide dismutase (SOD) is non-permeable and catalyzes the dismutation of superoxide into either hydrogen peroxide or molecular oxygen. As illustrated in Figure 4-4, the addition of SOD led to a significant decrease in the peak observed at 3 min in fMLP-stimulated mixed tear PMNs ($p = 0.001$). The average reduction in ROS production (AUC) in the presence of SOD was $74 \pm 13\%$ (n = 5). These results suggest that, similar to blood PMNs, initial ROS production in fMLP-stimulated tear PMNs is extracellular, and related to NADPH function on the plasma membrane.

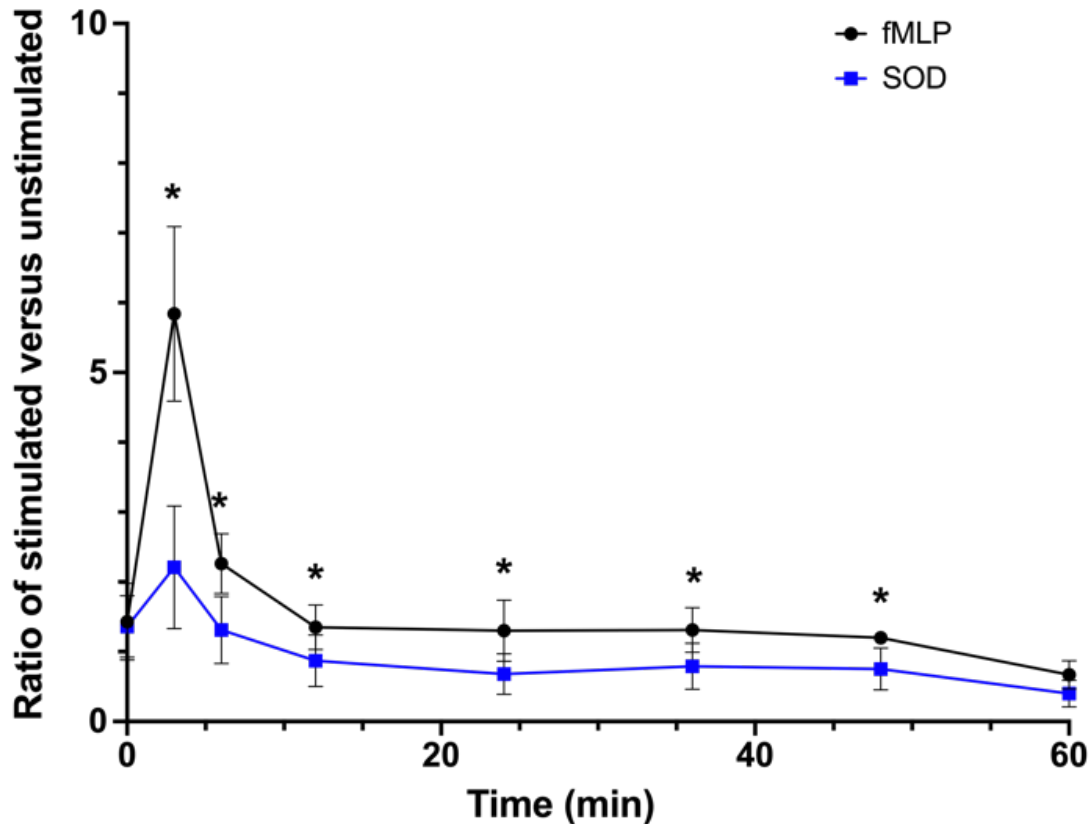


Figure 4-4. Changes in chemiluminescence of fMLP-stimulated mixed tear PMNs with and without the addition of superoxide dismutase (SOD). Results are reported as a ratio of fMLP-stimulated PMNs (with and without SOD) CL emissions over control (unstimulated PMNs) CL emissions. Values are presented as means $\pm$ standard deviations; mixed tear PMNs ($n = 5$). * Significantly different from the ratio of fMLP-stimulated mixed tear PMNs ($p \leq 0.029$, paired t-test).

4.3.3 ROS Production in PMA-Stimulated PMNs

ROS production in PMNs upon PMA stimulation is shown in Figure 4-5. Both PMA-stimulated mixed tear PMNs and blood PMNs generated ROS in a continuous manner, with similar trends in kinetics. Mixed tear PMNs had a sharp increase in ROS production around 9 min, and then the amount of ROS produced continuously declined, reaching a plateau around 27 min. There was no significant difference between ROS production of mixed tear PMNs and blood PMNs at all time points ($p \geq 0.085$) except at 0, 3 and 24 min ($p \leq 0.004$). Compared to the response to stimulation with fMLP and LPS, mixed tear PMNs showed a higher and prolonged production of ROS upon PMA stimulation.

As seen in Figure 4-5, large standard deviations in the ROS production of mixed tear PMNs were present due to large differences in individual responses. Two types of responder groups could be identified

based on the magnitude of the ratio: half of the participants were high responders (HR), with a ratio above 11 (ranging from 11.3 to 48.1) while the other half were low responders with a ratio below 11 (ranging from 4.9 to 9.8).

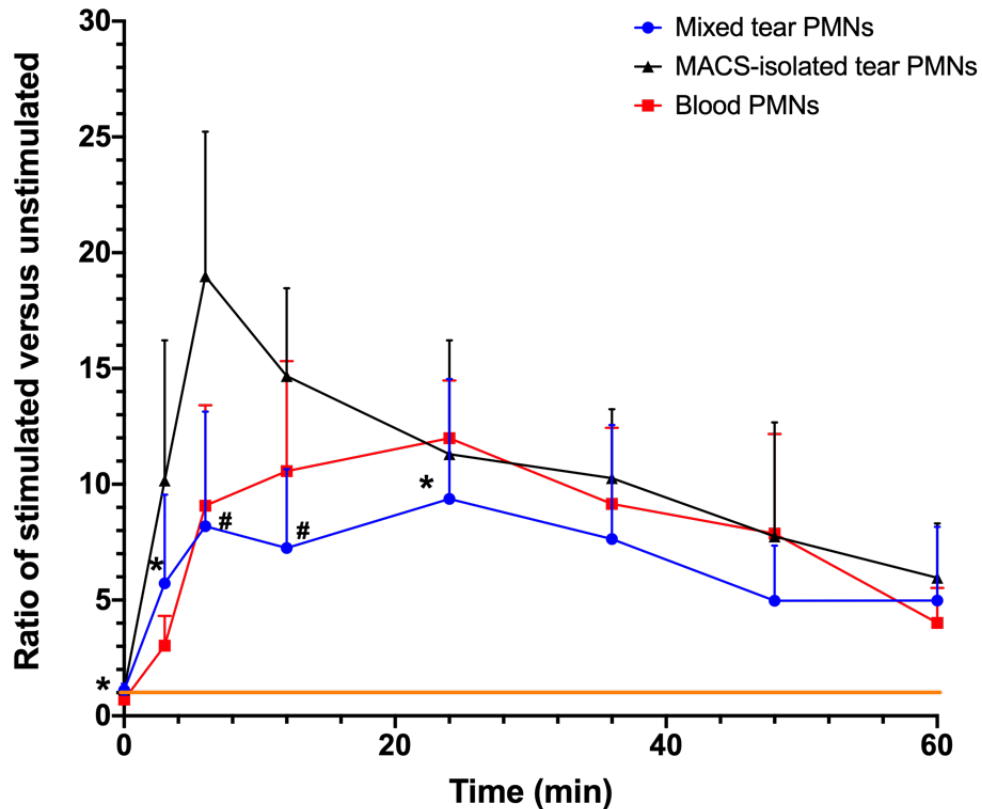


Figure 4-5. Changes in chemiluminescence (CL) of PMA-stimulated PMNs. Results are reported as the ratio of PMA-stimulated PMNs CL emissions over control (unstimulated PMNs) CL emissions. A total of 50,000 cells/well were transferred to wells containing 1 mM luminol and HBSS followed by the addition of 2 μ M PMA. The generation of ROS was measured over time using Cytation 5 at 37 °C. The orange line represents a ratio of 1, meaning no difference between stimulated and unstimulated CL values. Values are presented as means $\pm$ standard deviations; mixed tear PMNs (n = 21 from 18 participants), MACS-isolated tear PMNs (n = 10 from 7 participants), and blood PMNs (n = 7 from 4 participants). * Significantly different from the ratio of blood PMNs ($p \leq 0.04$); # significantly different from the ratio of MACS-isolated tear PMNs ($p \leq 0.001$).

ROS production induced by PMA stimulation in MACS-isolated tear PMNs led to significantly higher ratios ($p \leq 0.011$; Figure 4-5) with broader distribution (Figure 4-6) when compared to mixed tear PMNs.

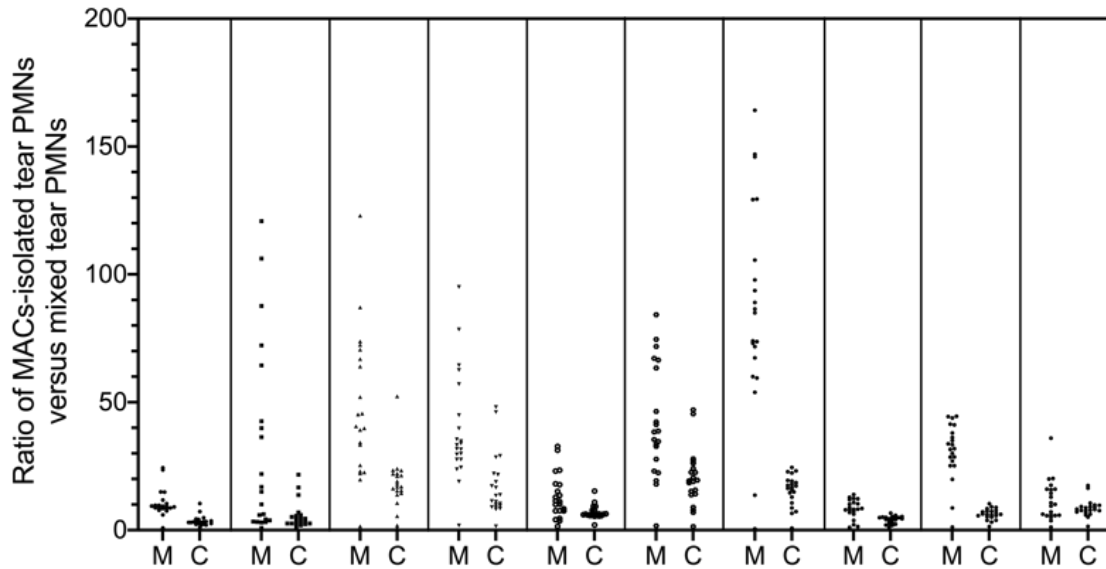


Figure 4-6. Comparison of ROS production by MACS-isolated tear PMNs and the non-isolated mixed tear PMNs. M: MACS-isolated tear PMNs; C: mixed tear PMNs from eye collection concentrated by centrifugation. Data points represented as the ratios of stimulated versus unstimulated CLU over 60 min at 3 min intervals. $n = 10$ from 10 different individuals, each presented in an individual column.

As shown in Table 4-3, as opposed to the results with LPS and fMLP, absolute ROS production (AUC-stimulated) with PMA stimulation significantly increased for both tear and blood PMNs. Despite the large standard deviations observed in the PMA-stimulated PMNs (Figure 4-5), the relative AUC (AUC for activation ratio curve) of MACS-isolated tear PMNs was significantly higher than that of mixed tear PMNs ($p = 0.016$), which further suggested that MACS-isolated tear PMNs were able to produce more ROS than non-isolated tear PMNs (i.e., mixed tear PMNs). Additionally, the slope between 0 and 15 min was calculated as an indication of the rate of ROS production, and the findings suggested that MACS-isolated tear PMNs could initiate a significantly more rapid ROS response than mixed tear PMNs ($p = 0.008$). The differences observed in ROS production between isolated and non-isolated tear PMNs suggested that tear PMNs may be either primed by the separation process or suppressed by the potential inhibitory/anti-inflammatory interaction with other cells or tear proteins present in the mixed tear PMNs samples.

Table 4-3. Absolute and relative ROS production in unstimulated and PMA-stimulated PMNs, as measured by AUC.

	Mixed Tear PMNs	MACS-Isolated Tear PMNs	Blood PMNs
AUC			
Unstimulated	8204 ± 4145	6384 ± 2256	4384 ± 2276
AUC			
PMA-stimulated	40,640 ± 13,529 [#]	50,819 ± 26,052 [#]	35758 ± 15365 [#]
AUC			
Activation ratio	552 ± 454 *	1910 ± 1604	473 ± 308 *
Slope	0.62 ± 0.71 *	3.49 ± 2.96	0.67 ± 0.35 *

AUC and slope of mixed tear PMNs and MACS-isolated PMNs were computed using GraphPad Prism software. n = 21 from 18 participants for mixed tear PMNs, n = 10 from 7 participants for MACS-isolated tear PMNs, and n = 7 from 4 participants for blood PMNs; values are represented as means ± standard deviations. * Significantly different from MACS-isolated tear PMNs, $p \leq 0.015$. [#] Significantly different from unstimulated PMNs ($p \leq 0.001$, paired t-test).

To determine if the higher ROS generation by MACS-isolated tear PMNs may be due to the potential priming of PMNs during the cell separation process, the phenotype of isolated tear PMNs was compared to non-isolated (i.e., mixed) tear PMNs using flow cytometry. Another cell separation system, EasySep cell separation, was also used to investigate if the difference observed was consistently seen in tear PMNs that were isolated by a microbeads magnetic system. As shown in Figure 4-7, MACS-isolated tear PMNs showed significantly higher expression of all selected markers of activation compared to non-isolated tear PMNs ($p \leq 0.042$), while only CD15, CD45, CD63, and CD66b were significantly higher on the EasySep-isolated tear PMNs ($p \leq 0.028$), suggesting a partial activation of cells by EasySep. The upregulation of the degranulation markers, CD66b and CD63, suggests that activation of tear PMNs may have occurred during the microbead magnetic separation, which would be further supported by the significant upregulation of the adhesion markers CD11b and CD54.

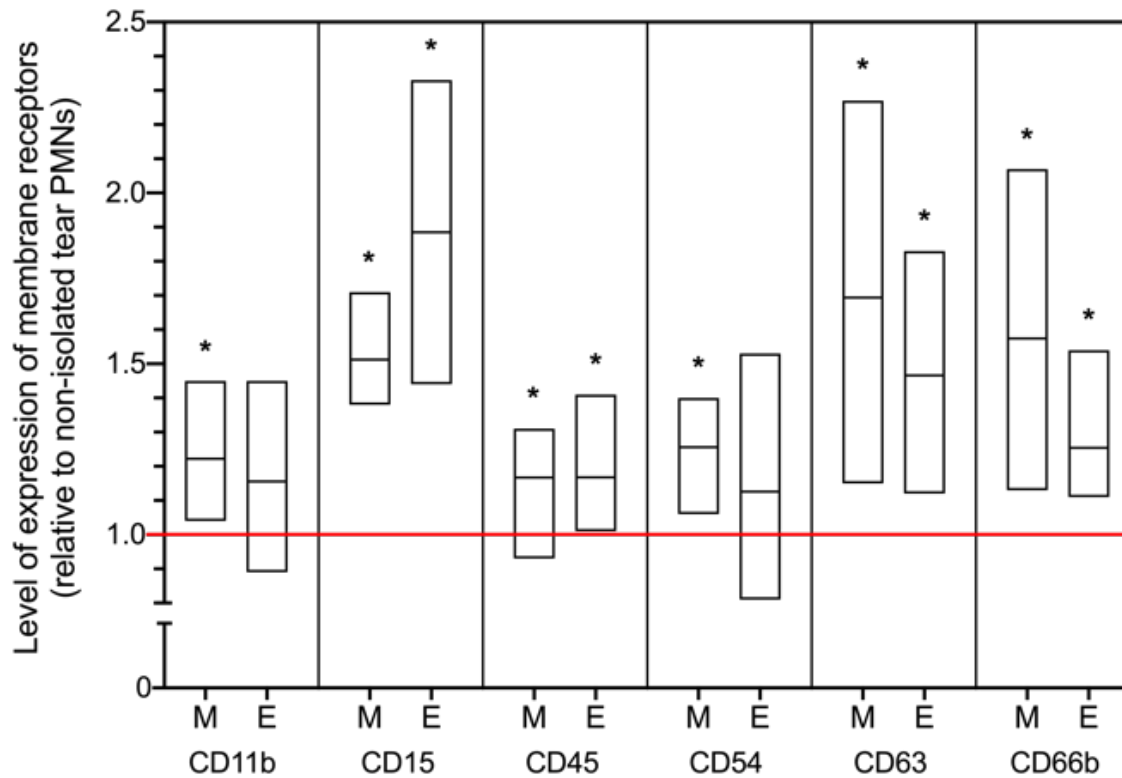


Figure 4-7. Comparison of the expression of membrane receptors on MACS-isolated or EasySep-isolated tear PMNs with mixed tear PMNs (non-isolated tear PMNs). M: MACS-column cell separation; E: EasySep cell separation. Once cells were collected from the ocular surface, tear PMNs were isolated either by MiniMACS column or the EasySep cell separation system. Samples from the non-isolated cell collection, concentrated by centrifugation, were used as controls. Cells were stained with fluorescently conjugated antibodies and analyzed by flow cytometry. Tear PMNs were identified by side scatter characteristics and CD45. The red line represents a ratio of 1, meaning no difference between the isolated tear PMNs and non-isolated mixed tear PMNs. Values are presented as means $\pm$ standard deviations. $n = 5$ from the same 5 participants for each cell separation system. * Significantly different from the non-isolated mixed tear PMNs ($p \leq 0.042$, paired t-test).

4.3.4 The Phosphorylation State of ERK and p38 MAPK

Since tear PMNs showed differential ROS production when treated with different stimuli, we hypothesized that the signaling molecules within their intracellular signaling pathways or/and the receptors on cell membranes may be impaired. Phosphorylation of p38 MAPK and ERK was assessed by flow cytometry, as these molecules have been reported to play a crucial role in PMA and fMLP-induced cell activation responses [21,22]. Blood PMNs and differentiated HL-60 cells (dHL-60), both neutrophil-like cells, were used as positive controls. As shown in Table 4-4, after stimulation with fMLP, tear PMNs were able to upregulate p38 MAPK and ERK, suggesting the active phosphorylated state of the signaling molecules. Collectively, these results suggest that the intracellular signaling molecules, at least ERK and p38 MAPK, are not depleted and functional in tear PMNs collected upon awakening.

Table 4-4. Changes in the expression of ERK and p38 MAPK on tear PMNs, blood PMNs and dHL-60 cells in response to fMLP and PMA.

	Mixed Tear PMNs		Blood PMNs		dHL-60 Cells	
	fMLP	PMA	fMLP	PMA	fMLP	PMA
ERK	1.59 ± 0.08	1.26 ± 0.08	1.30 ± 0.09	1.16 ± 0.04	1.28 ± 0.03	1.39 ± 0.20
p38 MAPK	1.53 ± 0.24	1.18 ± 0.05	1.41 ± 0.16	1.19 ± 0.16	1.33 ± 0.04	1.42 ± 0.08

Results are shown as the ratio of stimulated versus unstimulated cells; values are represented as means ± standard deviations. n = 3 for each blood PMN, mixed tear PMN, and dHL-60 cells.

4.4 Discussion

This study provides the kinetic measurements of ROS production during the oxidative (respiratory) burst in tear PMNs in response to LPS, fMLP and PMA using the chemiluminescence assay. The amount of ROS generated is directly correlated to the chemiluminescence (CL) when luminol reacts with ROS and emits a light [23]. The CL responses between the tear PMNs (from purified or non-purified cell collection) and the blood PMNs were compared to assess if tear PMNs have the ability to initiate an oxidative burst similar to that of blood PMNs. Each stimulus has different mechanisms of action, which likely explain the distinct CL responses observed in this study and provide further insights on the phenotype of tear PMNs collected after sleep. fMLP and LPS bind to PMNs cell membrane receptors, seven transmembrane

spanning GPCRs and TLRs, respectively, to induce various cell functional responses [8,24]. In contrast, PMA permeates into the cell and induces cell activation by directly binding to PKC [25].

fMLP acts via GPCRs to activate several intracellular signaling pathways involving MAPK (such as p38 MAPK and ERK), phospholipases, and PKC family to trigger various cell responses [8,11,25,26]. While a concentration of less than 10 nM of fMLP can only trigger migration, fMLP concentration larger than 100 nM can induce the oxidative burst [8]. In our study, fMLP at 1.5 μ M was able to induce a transient peak of ROS production in tear PMNs within the first three minutes, similarly to what had been observed in blood PMNs. Previous work demonstrated that extracellular ROS is produced by blood PMNs during the first five minutes after fMLP stimulation, while the later ROS/CL response is associated mostly with the intracellular production [18,22,27]. Additionally, it has also been reported that the initial ROS released upon activation by fMLP-stimulated blood PMNs is the superoxide anion [19,23]. Our results with and without SOD demonstrated that tear PMNs are able to produce extracellular ROS within the first three minutes of stimulation, indicating that the NADPH oxidase on their cell membranes is fully functional. Furthermore, this also suggests that binding of fMLP to its receptor was able to stimulate phospholipases (PLC and PLD) and produce extracellular ROS through the MAPK pathway and thus there is no potential impairment of fMLP binding to its receptor [11,28,29]. However, tear PMNs appear to be unable to generate the second peak, identified as the intra-cellular ROS response to fMLP [18,27]. The lack of, and therefore the inability of tear PMNs to produce intracellular ROS upon fMLP stimulation may suggest that some intracellular pathways leading to ROS production are dysfunctional or blocked. Under PMA-stimulated conditions, tear PMNs were able to produce ROS, indicating that the downstream PKC signaling pathway was functional, including NADPH oxidase. Thus, the impairment of intracellular ROS production under fMLP-stimulated conditions could be due to (1) alterations in the intracellular signaling pathways or transduction downstream of the fMLP receptors; and/or (2) a reduced number of substrates for phospholipases, which may lead to a shortage for the secondary messengers needed to activate downstream intracellular signaling molecules. The inability to generate ROS due to impaired receptors is unlikely, as discussed above. Based on our results, upon the stimulation of fMLP (and PMA), the expression of ERK and p38 MAPK was upregulated in tear PMNs to a similar extent observed in blood PMNs and dHL-60 cells, meaning that these intracellular signaling molecules could be phosphorylated, and thus that the pathways were functional. Other intracellular signaling molecules, such as phosphoinositide 3-kinase (PI3K) [17], involved in fMLP-induced oxidative bursts, also play key roles, and thus future research may be needed to further investigate the intracellular signaling pathways within tear PMNs in order to gain a better understanding on the regulation of their cellular response to an inflammatory stimulus.

In our study, LPS failed to induce ROS production in tear PMNs, with not even an initial 3 min burst of extracellular ROS. TLR-4 is the specific receptor for LPS, which activates the defensive

mechanisms of PMNs [30]. TLR-4 defects in PMNs are associated with impaired ability to initiate oxidative bursts in response to LPS [31]. The inability of tear PMNs (clearly observed in both the purified and non-purified PMNs eye wash collections) to produce ROS due to impaired receptor function needs to be further examined.

Under PMA stimulation, PMA diffuses into cells and binds to PKC leading to cell activation. Since PMA triggered an oxidative burst response in tear PMNs, this suggests that the intracellular signaling pathways downstream of PKC are fully functional in tear PMNs. Previous studies using dihydrorhodamine 123 (DHR-123) to measure oxidative burst via flow cytometry showed a limited ability of tear PMNs to generate ROS in response to PMA as compared to blood PMNs [4,7], which appears to contradict our current results. However, our current findings using luminol-enhanced chemiluminescence clearly indicate that ROS production in tear PMNs is time dependent. Flow cytometry can only measure ROS production in a definite point in time, which, depending on how this time point is chosen, may not be appropriate to infer the overall ability of ROS production by tear PMNs. Furthermore, while luminol-enhanced chemiluminescence detects both extra- and intracellular ROS generation [23], DHR-123 can only measure intracellular ROS production in flow cytometry [32]. PMA stimulates both extra-and intracellular ROS generation [10]. Thus, using DHR 123 and flow cytometry might underestimate ROS production unless ROS production is assessed at different time points.

As shown from computing AUC, unstimulated tear PMNs were found to produce more ROS compared to unstimulated blood PMNs. This constitutive or spontaneous ROS level was likely due to their state of activation and their age (having been in the closed-eye environment for around 7 h). Unstimulated aging blood PMNs have been reported to release their intracellular pool of ROS that has accumulated from previous activation over an incubation period *in vitro* [33]. ROS production in the absence of any stimuli has also been reported with the oral neutrophils of healthy individuals, which display signs of activation with high CD11b and CD66b and have higher constitutive ROS levels compared to unstimulated blood PMNs [34]. From receptor upregulation to the presence of NETs, tear PMNs present a phenotype of prior activation in the closed-eye environment [4,6,7]. Higher CL emissions from tear PMNs (29 ± 14 , 34 ± 21) compared to blood PMNs (17 ± 4 , 13 ± 6), respectively, at 0 and 3 min, in parallel with the higher constitutive ROS production provides further evidence of activation prior to their collection and of their mature stage in the life cycle of PMNs.

The collected eye wash not only contained PMNs, but also low numbers of monocytes and lymphocytes. As monocytes and lymphocytes are able to generate ROS, there was a need to further verify if the ROS measured in this study was produced mainly by tear PMNs. Thus, the MiniMACS-column cell separation system was used to purify tear PMNs. While the purification experiments with isolated tear PMNs confirmed our results with the non-purified cell collection, these experiments also highlighted some

interesting facts about the potential of these cells to be primed. Isolated tear PMNs produced higher ROS compared to non-purified/mixed tear PMNs. We hypothesized that this may either be due to the interactions between PMNs and other cells or tear proteins in the eye wash which may suppress their functions, or a priming effect caused by the separation process. By comparing the phenotypes of isolated tear PMNs with mixed tear PMNs (non-purified eye wash cell collection), an enhanced degranulation phenotype (CD66b⁺ and CD63⁺) for both MACS- and EasySep-isolated tear PMNs was observed, a clear indicator of activation that was further confirmed by the upregulation of CD11b and CD54, thus supporting a potential for priming. The activated phenotype on isolated-tear PMNs likely explains their higher ROS production, where elevated levels of CD66b have also been associated with enhanced oxidative responses [35]. Previous results showed that tear PMNs were nonresponsive to *in vitro* stimulation with fMLP, PMA, CaI, LPS and IL-8 [4,6,14], however, our current results suggest that tear PMNs still have the potential to be activated and/or primed for activation.

4.5 Conclusion

Although many questions remain to be answered around ROS production and its mechanisms in tear PMNs, the results of this study have potential translational outcomes around ocular inflammation and clinical research with tear PMNs. The impairment of intracellular ROS production identified with fMLP stimulation and the absence of response to LPS suggest that tear PMNs may be deficient in their ability to generate ROS intracellularly in the phagolysosome after the phagocytosis of bacteria. However, it is also possible that the tear PMNs' response to stimuli may differ at the end of the night (after prolonged eye closure, when these cells were collected) compared to when they first encounter potential pathogens, and thus further research is required before making a definite conclusion on their phagocytic functionality. While tear PMNs show evidence of activation in the closed-eye environment, stimulation with PMA and the priming/activation induced by the magnetic bead isolation process demonstrated that tear PMNs were not completely exhausted and were able to mount a potent oxidative burst response. This functional potential emphasizes how tear PMNs, the largest population of leukocytes present on the ocular surface under normal/healthy closed-eye conditions, can contribute to ocular defense/homeostasis or ocular inflammation depending on physiological conditions. Understanding how this potential may or may not be triggered during infection may help to develop better approaches to treatment for keratitis and reducing the risks of overnight lens wear. The high level of constitutive ROS production observed over time *in vitro*, a sign of their more mature stage (after residing in the closed-eye environment for several hours) also suggests that this ROS production took place in the ocular environment. Excessive ROS production or imbalance between oxidants and antioxidants can result in oxidative stress that may damage the host tissues [36]. In

healthy eyes, it would appear that this ROS production is part of the homeostatic balance, and the ocular environment is able to protect ocular cells from oxidative stress. Understanding how levels of constitutive ROS production in tear PMNs change in ocular surface diseases (such as dry eye [37]) may contribute a novel direction for therapeutic strategies to reduce ocular inflammation and damage. Given the potential role of tear PMNs in the innate immune response and in ocular inflammation, there is a strong interest and need for more clinical studies to further characterize their phenotypes and perform mechanistic studies that would inform the development of new therapeutic strategies or diagnostic tools [38]. Experimental protocols and the maturity of the collected tear PMNs will need to be carefully considered in result interpretations; our study highlights not only the differential tear PMNs response to the stimulation but also how an isolation process (performed in this case using magnetic beads) can affect tear PMNs and their responses.

Chapter 5 The Impact of Tear Proteins on the Oxidative Burst in Tear Neutrophils

5.1 Introduction

Polymorphonuclear neutrophils (PMNs) are part of innate immunity and play an important role in host defence against pathogens through multiple mechanisms, such as degranulation, phagocytosis, the release of neutrophil extracellular traps (NETs), and oxidative burst [1]. The release of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, is the hallmark of oxidative burst. These highly active ROS can directly damage microbial membranes and induce apoptosis. PMN functions are shown to be interconnected to each other, whereby they are capable of mutually reinforcing their killing mechanisms as well as suppressing each other to prevent any unexpected damage. However, in the event of over-activation, their functions become dysregulated and can result in severe damage to host tissues.

The concept of immune privilege describes the mechanisms by which immune responses are suppressed in certain locations to maintain immune homeostasis. The ocular surface is known as one of the immune privilege sites in the body. Although the ocular surface is exposed to dust and microorganisms consistently, the risk of having immune-mediated damage is low under normal physiological conditions. Hence, the fine coordination between the immune system and the eye allows for preserving the visual function and preventing immune-mediated tissue damage [2]. However, up to a million of PMNs can be collected after sleep at awakening by using the gentle eyewash [3]. These PMNs are found to be viable and not undergoing apoptosis [3], which suggests that they may participate in maintaining ocular health and homeostasis. Recent studies found that PMNs collected after a full night of sleep cannot be fully activated in response to a variety of stimuli, including phorbol 12-myristate 13-acetate (PMA), interleukin-8 (IL-8), lipopolysaccharides (LPS), N-Formylmethionine-leucyl-phenylalanine (fMLP), calcium ionophore, and zymosan [3–8]. This indicates that tear PMNs may possess a distinct phenotype from blood PMNs and may also suggest that tear PMNs have already been activated by the accumulated debris and microbes in the closed-eye environment. Furthermore, as reported in the previous chapter (also [7]), tear PMNs can spontaneously release ROS *in vitro* even in the absence of stimuli, providing further evidence of the activated phenotype of tear PMNs and also inferring the potential contribution of these cells to oxidative stress in the ocular environment. There must then be a balance between anti- and pro-inflammatory responses to prevent the overactivation of PMNs on the ocular surface and subsequently preserve ocular homeostasis. Tear proteins may be playing an important role in creating this balance, and thus to gain further knowledge on ROS production by tear PMNs, characterizing ROS in the presence of tear proteins would be relevant.

The muco-aqueous phase of the tear film contains several tear proteins that have anti-microbial and anti-inflammatory properties, such as lactoferrin and lysozyme. Lactoferrin, in particular, has a high affinity for iron which is an essential growth factor for bacteria, and it can also kill bacteria directly through its interaction with bacterial surfaces [9]. Lactoferrin has been shown to inhibit the production of various pro-inflammatory cytokines by monocytes [10] and suppress the release of NETs in blood PMNs [11], thus exerting an anti-inflammatory effect. Additionally, lactoferrin is capable of decreasing the generation of ROS in blood PMNs [12], highlighting its role in regulating the oxidative burst. Dry eye disease is a multifactorial condition that can be caused by several etiologies, including oxidative stress [13]. A decreased level of lactoferrin has been observed in tears from dry eye disease patients [14], which may highlight the potential association between lactoferrin and oxidative stress. Furthermore, lysozyme makes up 20-30% of the total proteins in the basal and reflex tears [15] and has bactericidal properties. It has been shown to suppress chemotaxis and superoxide generation of human blood neutrophils in response to N-formyl methionyl phenylalanine (fMLP) and zymosan [16]. As one of the contributors to oxidative stress, tear PMNs have not yet been investigated.

Therefore, this study aims to investigate the impact of tear proteins on the production of ROS by tear PMNs, which was assessed through two different methods, luminol-enhanced chemiluminescence and fluorescence-derived dichloro-dihydro-fluorescein diacetate (DCFH-DA). Our hypothesis was that the presence of tear proteins, especially lactoferrin and lysozyme, may reduce the production of ROS by tear PMNs, and as such contribute to maintaining ocular homeostasis.

5.2 Materials and Methods

5.2.1 Reagents and Antibodies

Luminol (5-amino-2,3-dihydroxy-1,4-phthalazinedione), PMA, Hank's Balanced Salt Solution (HBSS), dichloro-dihydro-fluorescein diacetate (DCFH-DA), human lactoferrin, human lysozyme, bovine mucin, human IgG, and human albumin were purchased from Sigma-Aldrich Co. (Oakville, Ontario, Canada). Phosphate-buffered saline (PBS) was purchased from Lonza (Allendale, New Jersey, USA). R-phycoerythrin-cytochrome 5 (PE-Cy5)-conjugated monoclonal antibody against CD45 was purchased from BD Biosciences (Mississauga, Ontario, Canada). Keratinocyte medium was ordered from ScienCell Research Laboratories (Carlsbad, California, US).

5.2.2 Subjects

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 (#43822; Waterloo, ON, Canada). A total of 10 participants (5 males and 5 females) were recruited, between 20 and 35 years old. Participants were free of any ocular diseases and were non-lens wearers.

5.2.3 Collection of Tear PMNs

This study consisted of two cell collections on different days, each of them occurred in the morning after a full night of sleep. Participants were trained to collect their cells before the actual cell collection using the gentle eyewash developed by Gorbet et al. [3]. Upon awakening, participants rinsed their eyes using a dropper containing sterile phosphate-buffered saline (PBS), allowing the run-off or eyewash to be collected in a sterile 50 mL polypropylene tube. Cell samples were delivered to the lab within two hours of time of collection. 8 ml of sterile saline solution (PBS) was given to the participants, and an eyewash average of 7 mL was returned, which represented 85% of the initially provided volume, suggesting minimal loss of eyewash. Cell suspensions were concentrated by centrifugation at $280\times$ g for 5 mins and were resuspended in PBS. Cell count and viability were performed using a hemocytometer with trypan blue.

5.2.4 Preparation of Artificial Tear Solution

Artificial tear solution (ATS) and modified ATS were used as cell incubation media. The components of ATS are listed in Table 5-1 and were either prepared in keratinocyte medium (KM) or Hank's balanced salt solution (HBSS) [17]. To investigate the impact of lysozyme and lactoferrin on the PMN oxidative burst, different combinations of tear proteins were used, which are ATS without lactoferrin and lysozyme (HBSS or KM with mucin, albumin and IgG only, referred to as MAI) and regular ATS.

Table 5-1. Components of artificial tear solution in Hank's balanced solution.

Components	Concentration (mg/mL)
Albumin	0.2
IgG	0.02
Lactoferrin	1.8
Lysozyme	1.9
Mucin (Bovine)	0.15

As measured in the previous study [6], the osmolarity of the ATS was 295 ± 1 mmol/kg, which is within the range of tear osmolarity.

5.2.5 Production of ROS as Measured by DCFH-DA

ROS production in tear PMNs was assessed using the fluorescent probe DCFH-DA through flow cytometry for both PMA-stimulated and unstimulated samples. DCFH-DA can readily diffuse into cells and be oxidized into fluorescent molecules, DCF, after reaction with ROS. This method is primarily used to measure intracellular ROS production, and it cannot differentiate between different types of ROS. A total of 35000 cells were added to each tube containing one of the three media (HBSS, ATS, and MAI). Then, cells were incubated with 48 μ M of DCFH-DA for 15 mins at 37°C, followed by incubation with CD45 with or without 40nM PMA for another 15 mins at 37°C. Cell samples were then diluted with PBS and analyzed immediately by flow cytometry.

Samples were acquired using CELLQuest Software (Becton Dickinson, Mountain View, California, USA) on a Becton Dickinson FACSCalibur flow cytometer (Mountain View, California, USA). A minimum of 2000 events were acquired, and fluorescent values were recorded as mean fluorescent Intensities (MFI) for all samples.

5.2.6 Production of ROS as Measured by Luminol-enhanced Chemiluminescence

Luminol is a permeable agent that can penetrate into cells to react with ROS, and it can thus detect both intra and extra-cellular ROS production. The assessment of chemiluminescence (CL) was conducted using the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek), which employed 96-well cell culture white microplates (Greiner bio-one, Frickenhausen, Germany). Cells were incubated with different media, HBSS

or KM, ATS, and MAI. To test the effects of different medium conditions, each well was assigned a specific medium and contained 50,000 cells, 1 mM luminol, and with or without PMA (40 nM). The total volume in each well was adjusted to 200 μ L using its corresponding media. The CL emissions were measured at 37°C at 3-minute intervals for 30 minutes.

5.2.7 Statistics

Results are presented as means $\pm$ standard deviations. The Shapiro-Wilk test was used to determine whether the data sets were normally distributed. One-way repeated measures ANOVA, Tukey Post Hoc, Friedman test, and Wilcoxon signed-rank test were used to calculate statistical significance. A p-value of less than 0.05 was considered for statistical significance and all the statistical analysis was performed with IBM SPSS Software (IBM Canada Ltd., Markham, Ontario, Canada). GraphPad Prism Software was used to compute the area under the curve (AUC) and plot all the figures (GraphPad Software, San Diego, California, USA).

5.3 Results

5.3.1 Preliminary Experiments with Keratinocyte Medium and Tear Proteins

Experiments were initially conducted using solutions prepared in keratinocyte medium (KM). As mentioned in 6.3.4, the KM solution containing all five tear proteins (lactoferrin, lysozyme, mucin, albumin, and IgG) is referred to as ATS, while another KM solution, referred to as MAI, only included mucin, albumin, and IgG. Additionally, two other conditions were tested: ATS-Lyso, which excluded lysozyme from the ATS, and ATS-LF, which excluded lactoferrin from ATS.

As shown in Figure 5-1, the presence of tear proteins led to a significant reduction in ROS production by unstimulated tear PMNs ($p \leq 0.023$). However, there was no significant difference observed between the media containing different tear proteins, indicating that all five tear proteins play an important role in reducing spontaneous ROS production by tear PMNs (ie, unstimulated conditions). When tear PMNs were stimulated with PMA, a significantly lower ROS release was detected in ATS ($p = 0.013$) and in ATS-Lyso ($p = 0.016$) compared to KM (control medium). Furthermore, ATS-Lyso exhibited significantly lower ROS release compared to MAI ($p = 0.017$). These results may suggest that the presence of lactoferrin played an essential in reducing PMA-stimulated ROS production by tear PMNs although no significant difference was detected between ATS-LF and ATS-Lyso, which warrants further research.

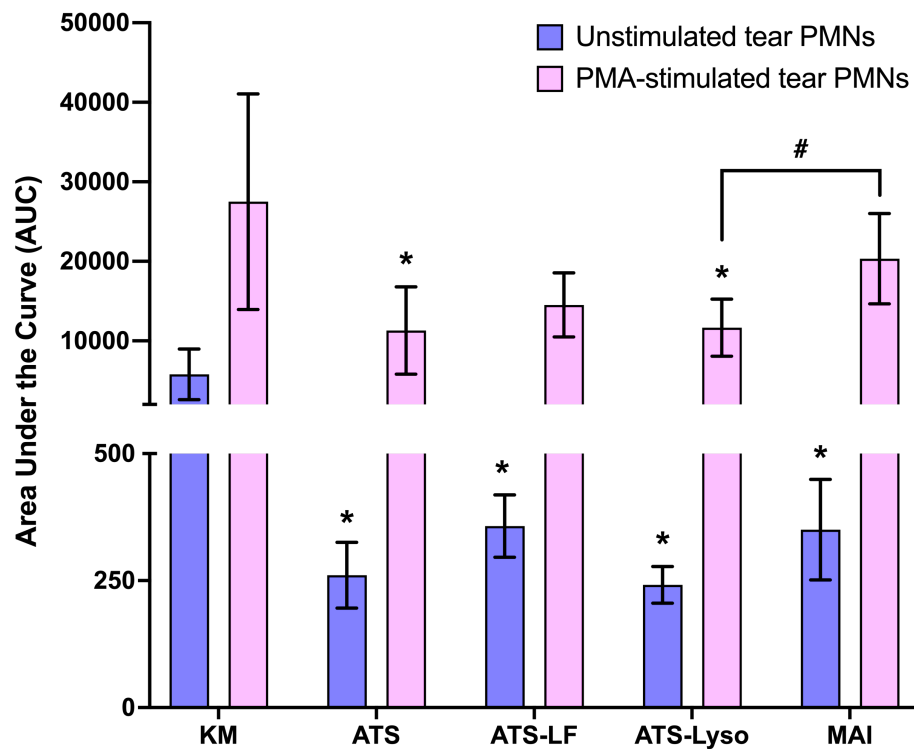


Figure 5-1. Changes in the ROS production of tear PMNs incubated in different KM solutions, as measured by luminol-enhanced chemiluminescence. Tear PMNs were incubated with luminol (1 mM) with and without PMA (40 nM) under different medium conditions. ROS production results are presented as area under the curve (AUC) mean $\pm$ standard deviations. $n = 4$ for MAI, 5 for ATS-LF and ATS-Lyso, 7 for KM and ATS, * significantly different from KM, $p \leq 0.016$. # Significantly different, $p = 0.017$. KM: keratinocyte medium (control), ATS: KM with tear proteins (lactoferrin, lysozyme, albumin, mucin, and IgG), ATS-LF: ATS with no lactoferrin, ATS-Lyso: ATS with no lysozyme, MAI: ATS with no lactoferrin and no lysozyme.

Then, the impact of the concentration of tear proteins on ROS production by tear PMNs was examined. As shown in Table 5-2, a larger ROS production by tear PMNs was observed with reduced tear protein concentration (30% ATS, i.e., all concentrations were 30% of what is listed in Table 5-1) compared to regular ATS (100% ATS, concentrations as listed in Table 5-1), suggesting that the effect of tear proteins on spontaneous ROS production was concentration dependent. However, no significant difference in ROS production in PMA-stimulated tear PMNs was detected between the 30% and 100% ATS.

Table 5-2. Changes in ROS production by tear PMNs incubated in KM and different concentrations of ATS.

	KM	30% ATS	100% ATS
Unstimulated AUC	5794 ± 3184	340 ± 82	261 ± 65
PMA-stimulated AUC	27498 ± 13546	14448 ± 2790	11302 ± 5483

ROS production results are presented as area under the curve (AUC) mean ± standard deviations. n = 4, KM: keratinocytes medium (control), ATS: KM with tear proteins (lactoferrin, lysozyme, albumin, mucin, and IgG). Percentage (30 or 100%) relates to the concentrations of tear proteins present in the ATS.

When measured by DCFH-DA and flow cytometry, a similar trend in reduced ROS production by unstimulated tear PMNs was observed, as depicted in Figure 5-2. However, under PMA stimulation, a different trend in ROS production of tear PMNs emerged. Specifically, a higher ROS production in stimulated tear PMNs incubated in MAI compared to KM was measured by DCFH-DA, whereas stimulated tear PMNs incubated in MAI exhibited a lower production of ROS compared to those incubated in KM when measured by chemiluminescence. More participants and experiments were needed to further investigate the ROS production measurements obtained from the two different methods. KM used in the preliminary experiments contained phenol red, which may affect the chemiluminescence and fluorescence measurements or signals [18]. Therefore, to minimize any potential confounding effects of phenol red on the results, a commonly used medium for testing ROS production by cells, Hanks' Balanced Salt Solution (HBSS), was employed [19,20] for the remainder of the experiments.

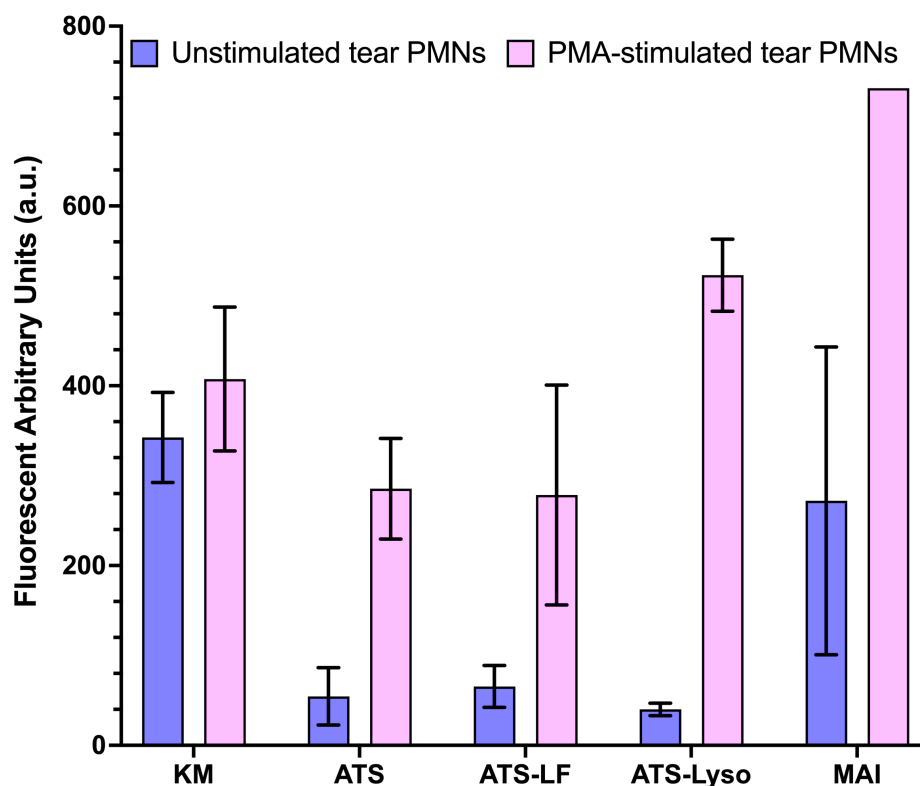


Figure 5-2. Changes in the ROS production by tear PMNs incubated in different KM solutions, as measured by DCFH-DA and flow cytometry. Tear PMNs were incubated with DCFH-DA (48 μ M), with and without PMA (40 nM), and CD45 under different medium conditions. Results are presented as mean $\pm$ standard deviations. $n = 2$ (no statistical analysis performed due to low sample number), KM: keratinocytes medium (control), ATS: KM with tear proteins (lactoferrin, lysozyme, albumin, mucin, and IgG), ATS-LF: ATS with no lactoferrin, ATS-Lyso: ATS with no lysozyme, MAI: ATS but no lactoferrin and lysozyme.

5.3.2 Production of ROS by Tear PMNs Incubated in Different Media as Measured by Luminol-enhanced Chemiluminescence

The changes in ROS production by non-stimulated tear PMNs, as assessed through luminol-enhanced chemiluminescence, followed a similar pattern to that detected using the DCFH-DA. The one-way repeated measures ANOVA indicated a statistically significant difference in ROS production by tear PMNs depending on the type of media used for incubation ($F = 29.03$, $p < 0.001$). As shown in Figure 5-3, tear PMNs incubated in HBSS exhibited a significantly higher level of ROS production compared to those incubated in ATS ($p = 0.001$) and MAI ($p < 0.001$). Moreover, the ROS level in MAI was significantly higher than that in ATS ($p = 0.019$). Based on the findings, the rank order for ROS production by unstimulated tear PMNs was HBSS, followed by MAI, and then ATS.

In contrast to the PMA-stimulated ROS production pattern observed with DCFH-DA, the luminol-enhanced chemiluminescence assay exhibited a distinct trend for PMA-stimulated tear PMNs. There was a statistically significant effect of tear protein contents in the media on ROS production by PMA-stimulated tear PMNs ($F = 15.47$, $p < 0.001$). The ROS levels in tear PMNs incubated with HBSS were significantly higher than those incubated with ATS ($p = 0.004$). Furthermore, tear PMNs in MAI exhibited a significantly higher level of ROS generation compared to those in ATS ($p = 0.006$). Although the mean ROS level produced by tear PMNs in HBSS was higher than in MAI (16937 ± 10588 and 12539 ± 3521 , respectively), this did not achieve statistical significance ($p = 0.113$). The findings demonstrate that the presence of tear proteins has a significant impact on ROS production by PMA-stimulated tear PMNs. However, it should be noted that the presence of lactoferrin and lysozyme appears to play a more important role in limiting ROS release by tear PMNs compared to albumin, mucin, and IgG, as the latter did not significantly reduce ROS production.

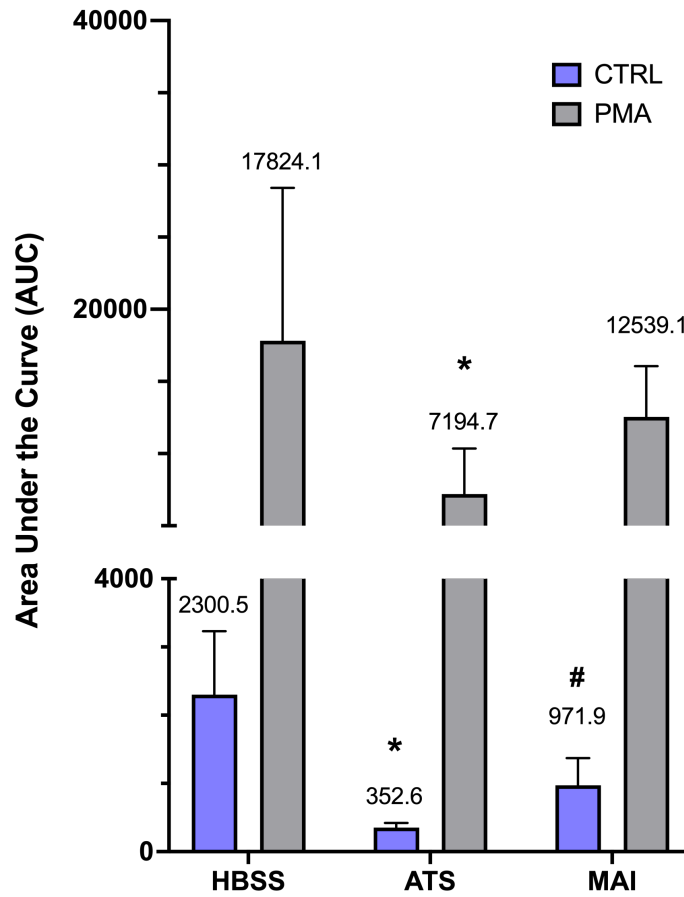


Figure 5-3. Changes in the ROS production by tear PMNs incubated in different medium conditions, as measured by chemiluminescence. Tear PMNs were incubated with luminol (1 mM) and with and without PMA (40 nM) under different medium conditions. ROS production results are presented as area under the curve (AUC) mean $\pm$ standard deviations. $n = 11$ for HBSS and ATS from 10 participants and one of them collected twice, and 7 for MAI. * Significantly different from HBSS and MAI ($p \leq 0.019$); # significantly different from HBSS ($p < 0.001$). HBSS: Hank's balanced salt solution (control), ATS: HBSS with tear proteins (lactoferrin, lysozyme, albumin, mucin, and IgG), MAI: ATS without lactoferrin and lysozyme.

5.3.3 Production of ROS by Tear PMNs Incubated in Different Media as Measured by DCFH-DA

There was a significant difference in the ROS production by tear PMNs incubated in different media ($F = 20.06$, $p < 0.001$). As shown in Figure 5-4, under the non-stimulated conditions, the amount of ROS produced by tear PMNs incubated in HBSS was significantly higher than those incubated in ATS ($p < 0.001$) and MAI ($p = 0.008$). Tear PMNs incubated in MAI also produced significantly higher ROS levels than in ATS ($p = 0.039$), suggesting that the presence of lactoferrin and lysozyme played a significant role in reducing constitutive ROS production. The unstimulated tear PMNs demonstrated a rank order of ROS production with the highest levels observed in HBSS, followed by MAI, and then ATS.

Furthermore, under PMA-stimulated conditions, there was a significant difference in ROS release by tear PMNs depending on the media used to incubate cells ($F = 8.68$, $p = 0.003$). As depicted in Figure 5-4, a significantly higher ROS production by tear PMNs was observed when incubated in HBSS compared to MAI ($p = 0.012$). The amount of ROS produced by tear PMNs incubated in MAI was significantly higher than those incubated in ATS ($p = 0.004$). However, there was no statistical significance in the ROS production by PMA-stimulated tear PMNs incubated in ATS and HBSS ($p = 0.323$). The order of ROS production by PMA-stimulated tear PMNs was MAI, as the highest, with then no significant difference between HBSS and ATS.

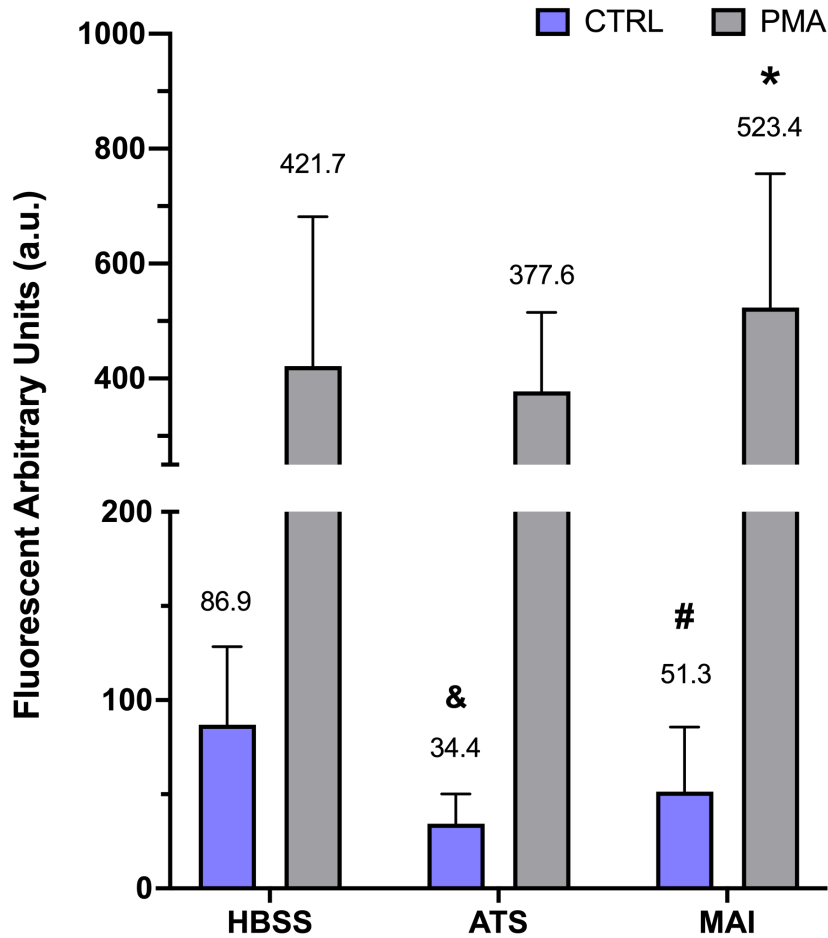


Figure 5-4. Changes in the ROS production by tear PMNs incubated in different HBSS media using DCFH-DA and flow cytometry. Tear PMNs were incubated with DCFH-DA (48 μ M), with and without PMA (40 nM), and CD45 under different medium conditions. Results are presented as mean $\pm$ standard deviations. $n = 11$; & Significant difference in unstimulated ROS production by tear PMNs from HBSS and MAI, $p \leq 0.039$. # Significant difference in unstimulated ROS production by tear PMNs from HBSS and ATS, $p \leq 0.039$. * Significant difference in PMA-stimulated ROS production by tear PMNs from HBSS and ATS, $p \leq 0.012$. HBSS: Hank's balanced salt solution (control), ATS: HBSS with tear proteins (lactoferrin, lysozyme, albumin, mucin, and IgG), MAI: ATS without lactoferrin and lysozyme.

Table 5-3. The rank order for ROS production by tear PMNs as measured by both DCFH-DA and Chemiluminescence.

		HBSS	ATS	MAI
DCFH-DA	Unstimulated	1	3	2
	PMA	2	3	1
CL	Unstimulated	1	3	2
	PMA	1	3	2

1: highest ROS production; CL: chemiluminescence; HBSS: Hank's balanced salt solution (control); ATS: HBSS with tear proteins (lactoferrin, lysozyme, albumin, mucin, and IgG); MAI: ATS without lactoferrin and lysozyme.

5.3.4 Confirming the Role of Tear Proteins in ROS Production

The substantial decrease in ROS production by tear PMNs when incubated with tear proteins suggests that the presence of tear proteins may inhibit ROS production by tear PMNs. However, it is important to consider the possibility of interaction between the tear proteins and probes (luminol and DCFH-DA) used in this study, as protocols for CL studies always use protein-free buffers. To address this concern, several experiments were conducted to confirm the observed reduction in ROS production was not the result of such interactions.

5.3.4.1 Incubation with Non-tear Proteins

Proteins that are not present in tears were used to evaluate their impact on ROS release by tear PMNs. Fibronectin, a common protein in blood, was first selected. However, the results were not consistent between the two methods, DCFH-DA versus luminol-enhanced chemiluminescence. As assessed by DCFH-DA, a 20% reduction in ROS generation was observed when tear PMNs were incubated with fibronectin (n = 3) as compared to a 60% reduction with ATS and a 46% reduction with MAI. In contrast, the degree of decrease in ROS generation was similar between fibronectin and ATS/MAI, as measured by luminol-enhanced chemiluminescence (n = 2). Fibronectin plays an important role in the wound-healing process and has been reported to interact with PMNs and ROS production [21], and thus the inconsistency between the results obtained by the two methods may be due fibronectin directly affecting ROS production in tear PMNs and to the different types of ROS measured. Specifically, the DCFH-DA method assessed the

intracellular ROS, while luminol-enhanced chemiluminescence measured the extracellular ROS. Therefore, these findings may indicate that the interaction between the tear proteins and probes is minimal.

Collagen was also used as one of the non-tear proteins in this study. However, collagen cannot be dissolved in a medium without acetic acid and the presence of acetic acid resulted in the probes precipitating, making it difficult to obtain reliable results.

5.3.4.2 Incubation with Heated ATS and MAI

In order to further evaluate the impact of proteins on the release of ROS by tear PMNs, tear proteins were denatured by heating at 90°C for 5 mins. If a decrease in ROS production was still observed following denaturation, it may suggest the reduction in ROS release with the presence of tear proteins may be due to the interaction between proteins and probes. Unfortunately, the ATS medium became cloudy, and proteins precipitated, leading to difficulties in accurately assessing ROS generation by tear PMNs using DCFH-DA and luminol-enhanced chemiluminescence.

Next, in order to reproduce the level of total tear protein concentration while avoiding potential precipitation, HBSS with 1% of fetal bovine serum (FBS) was heated to replace the denatured ATS experiment. If a reduction in ROS release was still observed with the addition of heated FBS, it may indicate that the observed decrease in ROS release with the presence of tear proteins may be due to the interaction between proteins and probes. However, a consistently lower signal, close to background level, was already observed for ROS production when cells were incubated with HBSS/1% FBS ($n = 3$), thus heat-inactivated FBS experiments did not provide any meaningful results. According to a published protocol on oxidative stress measurements, FBS tends to have an impact on the ROS release, causing it to drop to background levels [18], indicating that FBS is not a suitable medium for ROS measurement purposes.

5.3.4.3 Incubated with Human Tears

Tears collected from human participants were used to incubate with tear PMNs to validate the observed reduction in ROS release. The results showed a 90% reduction in unstimulated ROS levels and a 76% reduction in PMA-stimulated ROS release by tear PMNs incubated with tears when measured using luminol-enhanced chemiluminescence. This decrease was similar to the levels observed in tear PMNs incubated with ATS, as shown in Table 5-2. Interestingly, the presence of real human tears tended to delay the onset of ROS production by tear PMNs in response to PMA, but this needs to be further verified by recruiting more participants (see Appendix).

Table 5-4. The reduction in ROS release by tear PMNs incubated with different media, as measured by luminol-enhanced chemiluminescence.

	ATS	MAI	Human Tears
Unstimulated	82% $\pm$ 8%	59% $\pm$ 14%	90%
PMA-stimulated	52% $\pm$ 21%	11 % $\pm$ 6%	76%

The relative reduction in ROS release by tear PMNs incubated with different media compared to HBSS was calculated as a percentage. n = 10 for both ATS and MAI and n = 1 for human tears.

However, the assessment of ROS release by tear PMNs was unsuccessful by using the DCFH-DA method as no signal was detected, which could be due to the interaction between the tear components and the fluorescent probe, given the tears contain a diverse range of proteins, mucin, and lipids.

5.4 Discussion

This study evaluated the impact of tear proteins on ROS production and release by tear PMNs under unstimulated and PMA-stimulated conditions using luminol-enhanced chemiluminescence and DCFH-DA with flow cytometry. Regardless of the assay used, a significant decrease in unstimulated/spontaneous ROS production was observed in tear PMNs when incubated with mucin, albumin, and IgG (MAI). Although the mucin used in this study is sourced from the bovine submaxillary glands, it is commonly utilized to make artificial tear solutions [22]. Cantin et al. reported that airway mucins reduced the oxidative burst in human blood-isolated neutrophils in a concentration-dependent manner using a luminol-enhanced chemiluminescence [23]. The same assay used to measure the ROS production between Cantin et al. and our current study provides further evidence that the reduced ROS production is not due to the interaction with probe and tear proteins. Interestingly, one of the common mucins present in the airway, MUC5AC, is also found in tears [23]. Albumin is an iron-binding protein, although its ability to bind iron is weaker than the transferrin [24]. Iron has been shown to play an essential role in the process of ROS production, so albumin is considered to have antioxidant properties by sequestering iron ions [24]. PMNs express surface receptors for IgG, and the addition of pooled human IgG has recently been reported to suppress the release of neutrophil extracellular traps (NETs) by circulating blood PMNs [25], suggesting that IgG may play a role in suppressing PMN activation. Our study showed that mucin and albumin also have antioxidant and

anti-inflammatory properties with tear PMNs, and highlighted that the combination of mucin, albumin, and IgG present in tears can reduce ROS constitutive production in unstimulated tear PMNs, which may be one of the mechanisms protecting the ocular surface from oxidative damage.

The presence of lactoferrin and lysozyme along with mucin, albumin, and IgG (ATS) further lowered the amount of ROS produced by unstimulated tear PMNs, illustrating the combined anti-inflammatory effects of these tear proteins. Several studies have reported the oxidative inhibitory role of lactoferrin [12,26–28]. For instance, one study used lactoferrin-loaded contact lenses on human corneal epithelial cells to decrease oxidative stress [27]. However, the mechanisms by which lactoferrin decreases the amount of ROS have not yet been well established in neutrophils. Lactoferrin is an iron-binding protein and may act similarly to albumin by binding iron ions [26], which are essential for ROS generation, resulting in a decrease in ROS levels. Additionally, while this may not be a mechanism relevant to the current *in vitro* experiments, lactoferrin was shown to block the binding of LPS to one of its receptors, including L-selectin, by binding directly to LPS to suppress oxidative burst [12,26], so lactoferrin may inhibit ROS production by PMNs through direct binding to LPS. In the context of the ocular surface, where there is continuous exposure to bacteria and the external environment, lactoferrin in tears can then bind to LPS and prevent the overproduction of ROS by tear PMNs, ultimately reducing the risk of any unexpected damage to the ocular tissues. Furthermore, Francis et al. reported that lactoferrin, depending on its iron saturation state, can inhibit the spontaneous apoptosis of PMNs *in vitro* [29]. Tear PMNs collected upon awakening have been shown to be aged (see Chapter 6) but do not show signs of apoptosis [3]. Thus, this may suggest that lactoferrin present in the tear film helps to maintain the viability of tear PMNs while limiting their ROS production and killing mechanisms. However, the direct impact of lactoferrin on tear PMNs needs to be further investigated, given the large amount of lactoferrin that is also stored in the specific granules of PMNs.

Gordon et al. reported that human lysozyme, but not hen egg white lysozyme, could modulate the functions of PMNs, such as their chemotaxis toward chemoattractants and the oxidative burst [16]. Our findings are consistent with this, as a decrease in ROS release by unstimulated tear PMNs in the presence of lysozyme in ATS was observed. This study also investigated the addition of human lysozyme to PMNs 1 min before and after the stimulus; in the latter case, later addition reduced its inhibiting effect on ROS generation by PMNs, suggesting that the decrease in ROS release may be unlikely due to the quenching of the already formed ROS [16]. However, the exact mechanisms by which lysozyme modulates PMN function are still not fully understood but may involve interactions between lysozyme and the polysaccharide moieties on the PMN cell surface [16]. Further investigation is needed to fully elucidate the mechanisms of action of lysozyme on PMNs.

Although a similar trend was observed in unstimulated ROS production between the two methods, a slightly different trend was observed in PMA-stimulated ROS production. When using DCFH-DA and flow cytometry, the hierarchy of ROS production by PMA-stimulated tear PMNs was MAI > HBSS > ATS (no significant difference between HBSS and ATS), whereas it was HBSS > MAI > ATS (no significant difference between HBSS and MAI) as assessed by the luminol-enhanced chemiluminescence. The inconsistency between the two methods may be attributed to the different sources of ROS they measure, as luminol-enhanced chemiluminescence detects both intra- and extracellular ROS production, while DCFHDA measures intracellular ROS production only. The findings from the DCFH-DA method reveal that tear proteins, specifically lactoferrin and lysozyme, may not significantly impact intracellular PMA-stimulated ROS production, but they may help to limit the production of extracellular ROS, which is not surprising as lactoferrin and lysozyme cannot diffuse into cells. Additionally, although tear PMNs in MAI released a lower amount of ROS compared to those in HBSS, this did not achieve statistical significance, which further supports that lactoferrin and lysozyme may play a more essential role in limiting the stimulated extracellular ROS release by tear PMNs.

As discussed in Section 5.3.4., a series of experiments were conducted to confirm that the observed reduction in ROS release in tear PMNs incubated with tear proteins was not caused by any interaction between tear proteins and the probes. The results from these troubleshooting experiments suggest that reduced ROS production is unlikely due to any interaction between the tear proteins and probes. Furthermore, the distribution of cells incubated with tear proteins became more organized on the flow cytometric plots compared to those without tear proteins, which further indicates that tear proteins may have an impact on cells. Therefore, these findings collectively support the hypothesis of this study that tear proteins possess the ability to reduce the activation of tear PMNs and that the observed effect is not a result of the interaction between tear proteins and probes.

Further experiments are necessary to explore the underlying mechanisms of the action of tear proteins in reducing tear PMN activation. Albumin and lactoferrin can suppress the oxidative burst through their ability to bind to iron ions, and lactoferrin can also reduce the release of ROS by blocking the binding of LPS to its receptor. Lysozyme can decrease ROS production by PMNs through interaction with the cell surface. However, it remains to be determined whether tear proteins act directly on cells or indirectly on ROS. Additionally, the ATS used in this study did not contain IgA and lipocalin, which are two other major components of tear film proteins [14,30]. IgA has been recognized to play an important role in preventing adhesion of bacteria to the ocular surface, and it may also stimulate PMNs via binding to their receptors, which facilitates the killing of pathogens through oxidative burst and phagocytosis [31]. Lipocalin has been reported to be a major endonuclease in tears [30]. Given their high levels in tears and their potential role in

ocular surface inflammation, it would be relevant to modify our ATS formula to incorporate IgA and lipocalin, which would allow us to more comprehensively assess the impact of tear proteins on PMNs functions.

As one considers the closed-eye environment, the accumulation of microbes and debris, along with the presence of pro-inflammatory mediators, can trigger the recruitment and activation of tear PMNs, which may induce oxidative burst and potentially damage the surrounding tissues if overactivated. This draws the rationale of our study, which aimed to investigate whether there are substances in the closed-eye environment to limit the functions of tear PMNs. Our findings showed that the presence of lactoferrin and lysozyme reduced the production of extracellular ROS when tear PMNs are activated. Aged PMNs tend to spontaneously release ROS [7,32], and ROS are shown to increase apoptosis of PMNs [32,33]. Delaying the apoptosis of tear PMNs is important in the absence of macrophages or other phagocytic cells in the tear film, as the typical phagocytosis of apoptotic PMNs by tissue-resident macrophages helps to prevent the inflammatory responses that can occur. Therefore, lactoferrin may play an important role in delaying the apoptosis of tear PMNs in order to prevent unwanted inflammatory responses and then potential tissue damage. Since tear PMNs have already been activated and have aged in the closed-eye environment, they would release ROS spontaneously to the ocular environment if they were not well-controlled, which has been reported in an *in vitro* study [7]. Therefore, the presence of lactoferrin, lysozyme, mucin, albumin, and IgG may help to limit the spontaneous release of ROS by tear PMNs and prevent potential damage to the ocular structures.

5.5 Conclusion

Taken together, our findings may suggest that the presence of tear proteins, especially lactoferrin, lysozyme, mucin, and albumin, in the tear film contributes to “calming” tear PMNs, in terms of ROS production, to avoid any unexpected damage to ocular structures. It has been shown that oxidative stress in the ocular environment resulting from an excessive release of ROS may cause cellular oxidative damage, affecting cellular components such as cell membrane lipids, proteins, and nucleic acids, which can thus further lead to severe damage to ocular surface tissues [34,35]. Given that tear PMNs can release ROS, their contribution to oxidative stress cannot be neglected. Therefore, this study provides valuable insights into the role of tear proteins in regulating the innate immune response, particularly in the context of oxidative stress. The findings have important implications for the development of therapeutic interventions for ocular surface diseases that involve oxidative stress, such as dry eye. Understanding the role of tear proteins in suppressing the oxidative burst of tear PMNs, potentially limiting the risk of oxidative stress, can help

maintain the delicate balance necessary for ocular health as well as improve the management of ocular complications.

Chapter 6 Investigation of the Rhythmic Recruitment of Tear Neutrophils to the Ocular Surface and Their Phenotypes

6.1 Introduction

Neutrophils (polymorphonuclear neutrophils, PMNs) are the first white blood cell to arrive upon tissue infection or damage and can defend against pathogens through several functional activities, such as the oxidative burst, degranulation, and release of neutrophil extracellular traps (NETs) [1]. Previous studies showed that PMNs are recruited to peripheral tissues, such as the mouth [2], the lungs [3], and the ocular surface [4] under healthy conditions (i.e., in the absence of an inflammatory reaction). While the eye is considered an immune privilege site, a large number of leukocytes, with the main population being PMNs [5], can be collected from the ocular surface non-invasively using a gentle eye wash on waking, following prolonged eye-closure [4–9]. Only a few tear PMNs have been collected during the daytime [10,11], suggesting that infiltration of PMNs to the ocular surface may follow the circadian rhythm, although hypoxia caused by prolonged eye closure may also play a role in leukocyte recruitment [12].

The oscillations in physiological and behavioural activities enable light-sensitive organisms to anticipate and adapt to environmental changes, such as variations in light exposure and food, which creates the circadian rhythm in an approximately 24-hour cycle [13]. The suprachiasmatic nucleus (SCN) is the central pacemaker that can convey and synchronize timing or circadian information to peripheral tissues to regulate the physiological functions of the body, including metabolism, DNA repair, and the immune system [14]. Studies showed that depending on cell types, rhythmic changes in immune cell numbers in the circulation exist; for example, naïve CD4⁺ and CD8⁺ T lymphocytes peak at night [15,16], while natural killer cells [17] and effector T cells [16] peak during the day. Additionally, the circadian infiltration of immune cells to the peripheral tissues has been shown in several studies. The number of lymphocytes homing to lymph nodes peaks at night [18], while increased number of PMNs are recruited to the lungs [19] and the kidney [20]. Furthermore, the concentrations of proinflammatory mediators, such as interleukin-2 (IL-2), IL-6, IL-12, and tumour necrosis factor-alpha, have been reported to reach their maximum at night, which may play a role in attracting immune cells to the sites [21].

The guidance of PMNs by chemoattractants, such as complement anaphylatoxin C5a, IL-8, and leukotriene B₄ (LTB₄), to local sites, is known as chemotaxis. PMNs sense and follow the concentration gradient through the interaction of chemoattractants and their G-coupled protein receptors (GPCR) expressed on the cell surface, which induces the subsequent transmigration cascade in blood-circulating PMNs including rolling, arresting, crawling, and eventually migrating to the tissues [22]. Thakur et al. reported an increase in IL-8 and LTB₄ concentrations in the closed-eye tears with increasing eye closure

time [23]. Additionally, Mahajan et al. showed that C5a level peaked at awakening [7]. Taken together, such findings may suggest that the recruitment of tear PMNs to the closed-eye environment may be driven by the presence of chemoattractants in tears. However, research is needed to confirm the association between the recruitment of tear PMNs and chemoattractants across the day.

The high numbers of tear PMNs collected from healthy eyes upon waking are quiescent, exhibiting limited activation/pro-inflammatory response upon stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP), lipopolysaccharide (LPS), IL-8, calcium ionophore, and phorbol-12-myristate-13-acetate (PMA) *in vitro* [4,6,9], the first three being physiological stimuli while the latter two are chemical synthetic stimuli. It has thus been hypothesized that tear PMNs may have undergone activation while in the closed-eye environment, and become unresponsive to additional stimulation. NETs have gathered significant interest recently as a sign of neutrophil activation and are DNA filaments decorated with granular proteins, such as myeloperoxidase, neutrophil elastase, and lactoferrin that have been expelled from the neutrophils [24,25]. The presence of NETs found in the morning eye discharge [7] and the upregulation of degranulation markers on tear PMNs [4] support the concept of prior activation on the ocular surface during sleep (or closed eye conditions). Furthermore, Jin et al. reported a constitutive release of reactive oxygen species (ROS) of tear PMNs collected after nocturnal sleep [5]. This spontaneous ROS release has also been reported in unstimulated aging blood PMNs [26], which may suggest that tear PMNs are aged when collected after a nighttime sleep.

In the previous chapter (Chapter 5), we demonstrated the potential impact of tear proteins on ROS production by tear PMNs, so further investigation of the fundamental mechanisms of tear PMNs in the closed-eye environment, in relation to their recruitment and changes in their surface receptor expressions would contribute to gaining a better understanding of their role in maintaining ocular surface health. While recent studies have provided some insights on the phenotype of tear PMNs collected after sleep (prolonged eye closure at night) and potential activation, there is still limited knowledge on the difference between nighttime and daytime and the potential role of chemoattractants. Comparing the difference in cell population across time, tear PMNs number and change of phenotypes over time may provide further insights into the mechanisms of recruitment to the ocular surface and the role that tear PMNs play in patrolling and maintaining ocular homeostasis in the anterior segment of the eye. Using a gentle eye-wash method to collect cells from the ocular surface, the objectives of this study were thus to investigate the rhythmic infiltration of tear PMNs to the ocular surface and assess their phenotype, in terms of degranulation and maturation state, across different time points, 2-hr and 7-hr of sleep (closed-eye environment) at night, after 2-hr sleep (closed-eye environment) during mid-day, and towards the end of the day (open-eye, around 5 pm). CD66b, CD63, and lactoferrin were used to assess degranulation of tear

PMNs. CD66b and lactoferrin can be found on and in the specific (secondary) granules [27,28], while CD63 is expressed on the azurophilic (primary) granular membranes [29]. Upon cell activation, granular membranes fuse with the cell plasma membrane causing the upregulation of CD66 and CD63. The release of lactoferrin to the extracellular environment may cause part of lactoferrin to bind to the cell surface, leading to an elevated level of lactoferrin expression on PMNs. L-selectin (CD62L) and C-X-C chemokine receptor 4 (CXCR4 or CD184) were used to measure tear PMN maturation state [30].

6.2 Materials and Methods

6.2.1 Reagent and Monoclonal Antibodies

Fluorescein isothiocyanate (FITC)-conjugated antibodies against human CD11b (clone: ICRF44), CD62L, CD15, and CD66b (clone: G10F5), R-phycoerythrin (PE)-conjugated antibodies against human CD54 (clone: LB-2), CD184, and CD63 (clone: H5C6), and R-phycoerythrin- cytochrome 5 (PE-Cy5)-conjugated antibodies against CD45 (clone: HI30) and CD15 (clone: HI98) were purchased from Becton Dickinson Pharmingen (San Diego, CA, USA). IL-8 ELISA kit, FITC-conjugated anti-mouse IgG F(ab')₂ CF 488A, May-Grunwald's stain, DNase I, and fMLP were from Sigma-Aldrich Co. (Oakville, Ontario, Canada). FITC-conjugated antibody against myeloperoxidase, anti-histone H3 (citrulline R2 + R8 + R17) antibody, PE-conjugated anti-goat IgG, and C5a ELISA were purchased from Abcam (Waltham, Massachusetts, USA). Anti-lactoferrin antibody was purchased from Cedarlane (Burlington, Ontario, Canada). Anti-hCXCR1/IL8 RA antibody was purchased from R&D systems (Minneapolis, Minnesota, USA). 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 Subjects

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 (#43743; Waterloo, ON, Canada). A total of 23 non-contact lens wearers who were free of any ocular diseases were recruited, consisting of 10 males and 13 females, between the ages of 20 and 36 years old. An additional 7 participants were recruited to assess the expression of CD184 and IL-8Rs on tear PMNs collected at different time points.

6.2.3 Collection of Tear PMNs

Each participant was trained to collect cells using the gentle eyewash method previously described by Gorbet et al [4]. Participants collected the eyewash immediately upon awakening or after the 2hr sleep/eye closure period. Briefly, participants released saline solution onto their ocular surface using a sterile dropper placed at the inner angle of the eye. The run-off was collected into a sterile 50 mL polypropylene tube placed at the outer angle of the eye.

There were four different collection time points on four different days: three time points of prolonged eye closure (EC), after a full night of sleep (an average of 7hrs of sleep, 7hr EC night samples), after 2 hours of sleep at night (2hr EC night samples), after 2 hours of eye closure or sleep in the afternoon (2hr EC day samples); and one open-eye day collection towards the end of the day around 5 pm (end of day samples). The former two, full night and 2hr night, were considered nighttime collections, which were undertaken on two consecutive days, and the latter two, 2hr day and end of the day, were considered daytime collections. Participants collected the 7hr EC night samples upon awakening in the morning after a full night of sleep (average 7 hours), which were delivered to the lab within 2 hours. For 2hr EC night samples, participants collected the cells after the 2-hour sleep at night around 2 am, which were delivered to the lab in the early morning. Participants collected the 2hr EC day samples after closing their eyes for 2 hours in the afternoon (after 1 pm). For the end of day samples, participants collected the cells from the opened eyes in the late afternoon around 5 pm. All daytime collections were delivered to the lab within 2 hours of collection. For the 2hr night and 2hr day collections, it was difficult to have the exact length of sleep although participants reported that they fell asleep, and if they awoke within that 2hr time period, they kept their eyes closed until their alarm rang. 8 ml of sterile saline solution (PBS) was given to the participants, and an average eyewash of 7 mL was returned, which represented 85% of the initially provided volume, suggesting minimal loss of the eyewash.

All collected eyewash samples were centrifuged at 280×g for 5 minutes. The supernatants were kept for protein analysis, and the cell pellets were resuspended in PBS. Cell counts were performed using the MOXI Z automated cell counter (ORFLO, Hailey, Idaho, USA), gating on PMN population based on cell diameter, between 6.5 and 9 mm.

6.2.4 Protein Analysis

For nighttime collection samples, 1 mL of the eyewash supernatant was transferred to 1.5 mL Eppendorf tubes and stored at -80°C for future ELISA analysis. The supernatants of daytime collection samples were transferred to Amicon Ultra-15 centrifugal concentrators with 10,000 Dalton molecular

weight cutoff filters (Sigma, Ontario, Canada), followed by centrifugation at 3500×g for 20 minutes. The concentrates were transferred to 1.5 mL Eppendorf tubes and stored at -80°C until processing. The concentrations of IL-8 and complement C5a were measured using ELISA.

6.2.5 Cell Histological Staining

After incubation, cells were centrifuged onto a microscope glass slide using the StatSpin Cytofuge (HemoCue America, Brea, California, USA), at 1000×g for 2 minutes, followed by staining with May-Grünwald's stain. For all eyewash samples, a 100 mL aliquot of the concentrated eyewash (see 6.3.3) was added to the cytospin funnel and cells were directly centrifuged onto the glass slides, as shown in Figure 6-1a to 1c. Cells were then fixed with methanol and stained with May-Grünwald. Samples were examined using an Axiovert microscope (Nikon Instruments Inc., Melville, New York, USA) and pictures of the entire circular area of the cytospin, where the cells deposited, were taken at 10x magnification using a MS-2000-XY mechanical stage (Applied Scientific Instrument, Oregon, USA) and stitched using NIS elements-Advanced Research software package (Nikon Instruments Inc., Melville, New York, USA), as depicted in Figure 6-1d. The numbers of PMNs, corneal epithelial cells, and aggregates were then counted. In some experiments, cells from the 7hr EC night samples were incubated with 1mg/mL DNase I at 37°C and 5% CO₂ for 10 minutes prior to performing the cytospin.

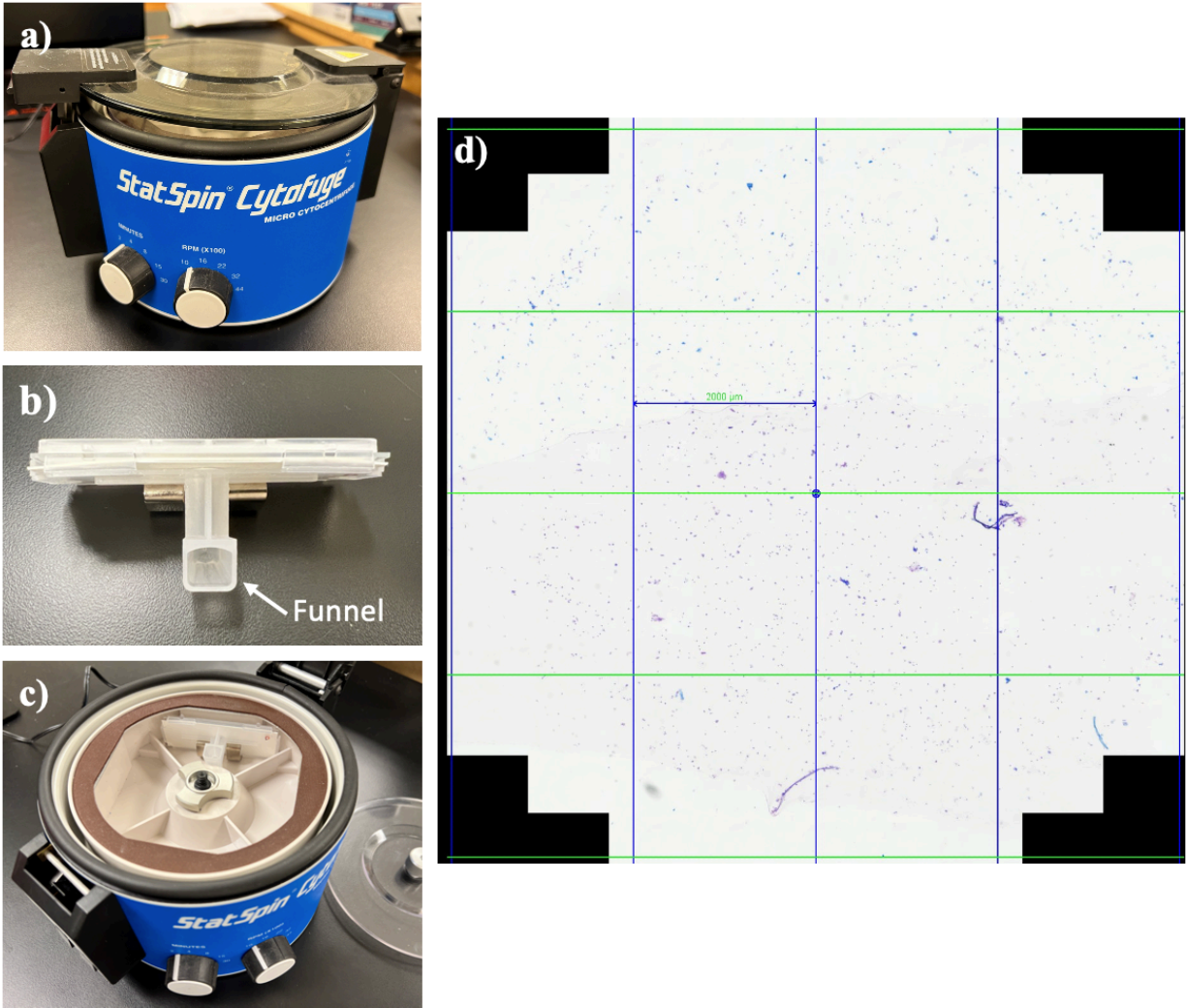


Figure 6-1. The procedure of cytopspin. a) StatSpin Cytofuge, b) The assembled cytopspin funnel with a glass slide and filter paper, c) cytopspin slide in the Cytofuge, d) picture of the circular area of the deposited cells onto the microscope slide following centrifugation and staining.

6.2.6 Cell Activation and Immunostaining

Due to the limited cell numbers obtained in the daytime collection, only nighttime collection samples were analysed. Cells were either stimulated with 1.5 μM fMLP for 15 minutes in the cell incubator (37°C, 5% CO_2) or not. The experimental procedure is summarized in Figure 6-2.

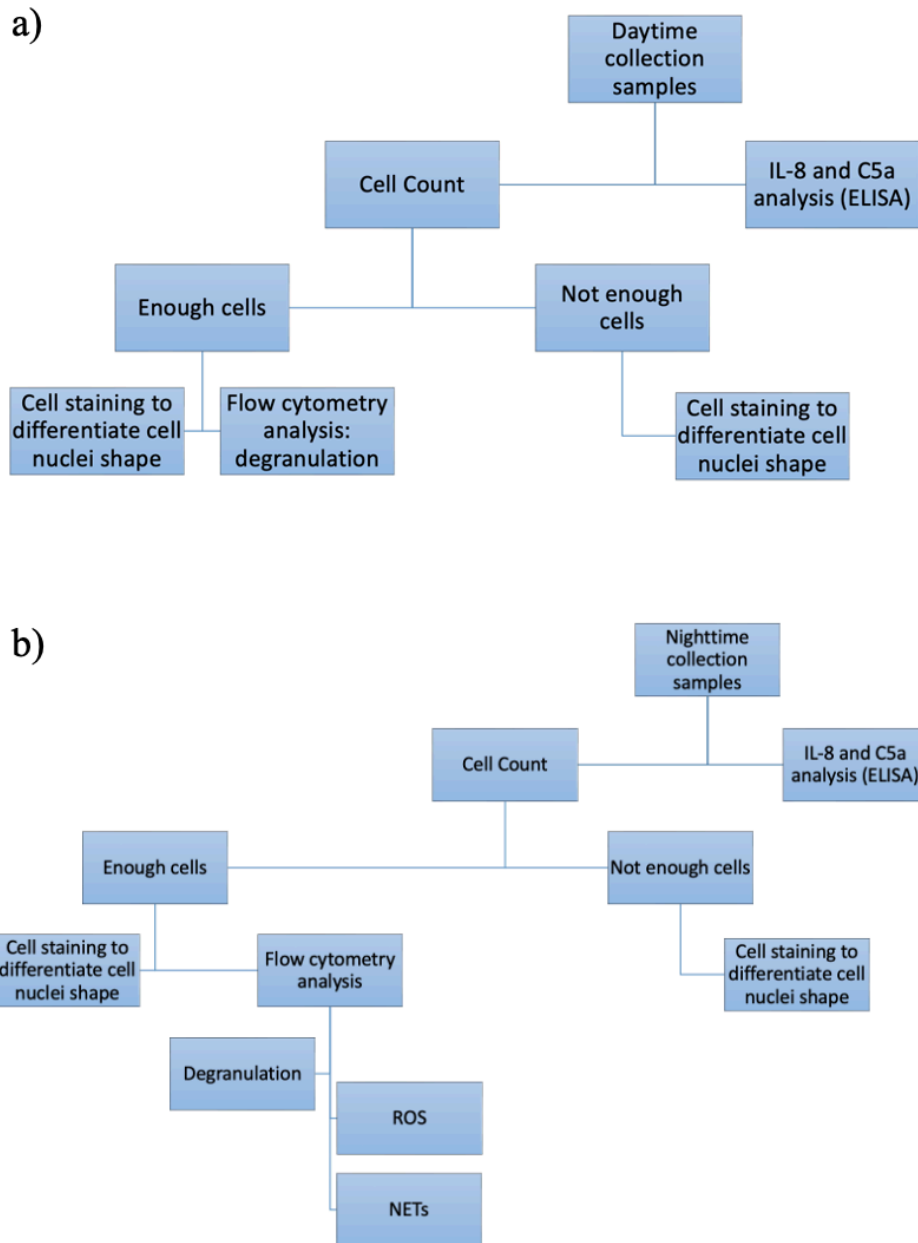


Figure 6-2. Summary of experimental procedures for daytime and nighttime cells.

Cells were stained with FITC-CD66b (a marker for specific granules), PE-CD63 (a marker for azurophilic granules), FITC-CD62L (L-selectin), PE-CD184 (a marker for cell maturation state), and PE-Cy-5-CD45 (pan leukocyte antibody) for 20 minutes in the dark at room temperature. Then, samples were diluted with DMEM/10% FBS and were ready for flow cytometry analysis.

To assess the degranulation of specific granules and NETs release, cells were stained with anti-lactoferrin antibody for 30 minutes at room temperature in the dark. Then, the cells were washed with DMEM/10% FBS at 280×g for 5 minutes, followed by staining with FITC-conjugated secondary antibody and PE-Cy5-CD45 for 30 minutes at room temperature in the dark. Cells were washed at 280×g for 5 minutes and were ready for flow cytometry analysis.

To identify NETs, samples were stained with anti-citrullinated-histones (H₃Cit) antibody for 30 minutes at room temperature in the dark, followed by the staining with PE-conjugated secondary antibody, FITC-anti-myeloperoxidase (MPO) antibody, and PE-Cy-5-CD15 for another 30 minutes at room temperature in the dark. Samples were diluted in DMEM/10%FBS and immediately analysed by flow cytometry.

6.2.7 Flow Cytometry

The PMNs population was identified based on double gating the cells that were high in side-scattered light (granularity), low in forward-scatter light (size), and CD45+ using a Becton Dickinson FACS Calibur flow cytometer (Mountain View, California, USA) and associated CELLQuest Software. At least 2000 PMNs events were acquired and mean fluorescent intensities (MFI) were recorded for all samples.

The release of NETs by tear PMNs was also analyzed through flow cytometry, as shown in Figure 6-3. Cells were not permeabilized and fixed, hence the surface expressions of MPO, and H₃Cit can be used to quantify the presence of NETs on tear PMNs. Following a previously published protocol for NETs via flow cytometry [31], CD15 was used to identify PMNs. Flow cytometry analysis is depicted in Figure 6-3 and shows the difference in the presence of NETs on tear PMNs collected at 2hr and 7hr of nighttime eye closure. As illustrated in the histogram plots (Figure 6-3c & d), the MPO and H₃Cit signals for 2hr EC tear PMNs were closer to the origin compared to 7hr EC tear PMNs, suggesting that 2hr EC tear PMNs released fewer NETs.

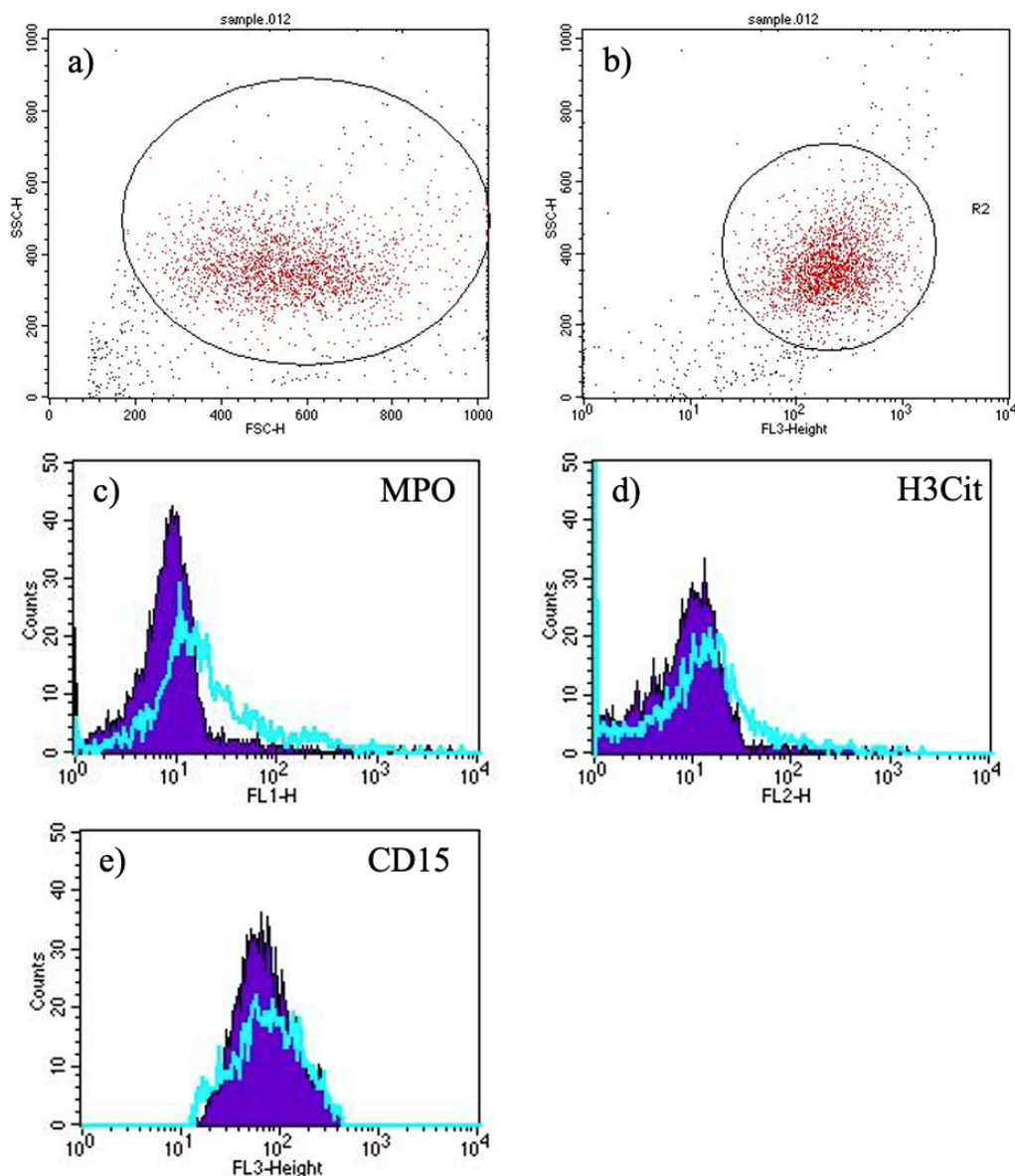


Figure 6-3. Measurement of NETs release by tear PMNs using flow cytometry analysis. Dot plots and histograms from 2hr (purple-filled) and 7hr (blue outlining) EC night tear PMNs. a) PMN population was identified based on their size (forward scatter, FSC) and cytoplasmic granularity (side scatter, SSC); b) side scatter versus CD15 (FL3) to identify the CD15 positive cells (i.e. neutrophils); c) the comparison of the MPO signals between 2hr and 7hr EC night tear PMNs on histogram plot; d) the comparison of the H3Cit (FL2) signals between 2hr and 7hr EC night tear PMNs on histogram plot; e) the comparison of the CD15 signals between 2hr and 7hr EC night tear PMNs on histogram plot.

6.2.8 Statistics

Results are presented as mean $\pm$ standard deviation. The paired t-test if normally distributed and Wilcoxon signed-rank test if not normally distributed were used to compute statistical significance. Correlations were evaluated through the Spearman correlation test. A p-value of less than 0.05 was required for statistical significance, and all the statistical analysis was performed via IBM SPSS Software (IBM Canada Ltd., Markham, Ontario, Canada). Outliers were identified by calculating the interquartile range (IQR) and were removed. All figures were plotted by using GraphPad Prism Software (GraphPad Software, San Diego, California, USA).

6.3 Results

6.3.1 Cell Count and Nuclei Shapes of Tear PMNs across Different Time Points

The increased presence of tear leukocytes in the eyewash depending on time of collection and eye closure is reported in Figure 6-4. The average number of leukocytes collected after 7 hours of eye closure at night (full night sleep) was 3.4×10^5 , which was the highest count among all time points. Significantly fewer leukocytes were collected after 2 hours of eye closure at night with an average cell count of 1.1×10^5 ($p < 0.001$), suggesting an increase in leukocyte recruitment with longer eye closure time at night. The numbers of leukocytes present in the nighttime collections were significantly higher than cell count in either daytime collection, with daytime collections yielding fewer than 4000 leukocytes on average. A significantly lower number of leukocytes were collected after 2 hours of eye closure during the day when compared to the 2 hours of eye closure at nighttime ($p = 0.001$). There was no significant difference between the 2 hours of eye closure during the day and the end of day samples, 3915 ± 2570 and 1959 ± 1691 , respectively ($p = 0.102$), suggesting that leukocytes were not recruited to the ocular surface during a 2hr eye closure period during the day.

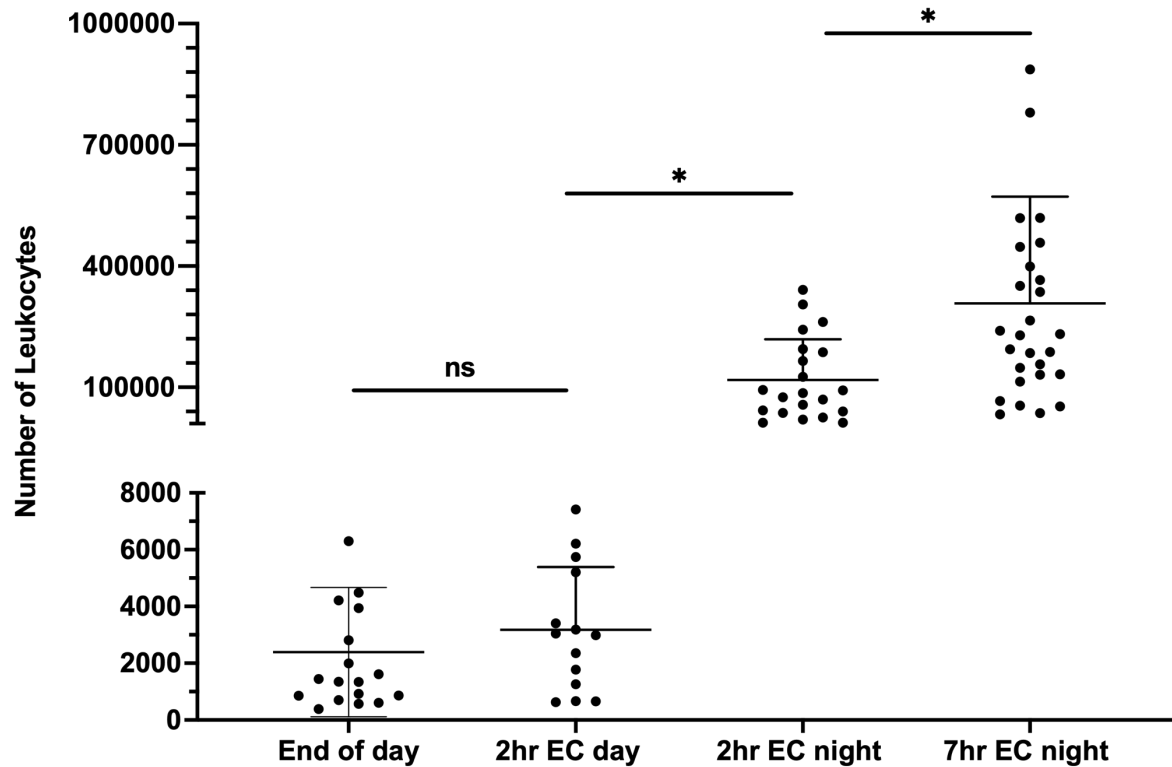


Figure 6-4. Number of leukocytes collected from the ocular surface at different time points. After the eyewash collections were centrifuged and resuspended in PBS, a cell count was performed using MOXI-Z automated cell counter. Values are presented as means $\pm$ standard deviations, $n = 19$ for end of the day, 14 for 2hr EC day, 21 for 2hr EC night, and 24 for 7hr EC night. Wilcoxon matched-pairs signed rank test was conducted to compare the paired data values between end of day and 2hr EC day, 2hr EC day and 2hr EC night, and 2hr EC night and 7hr EC night, ns: not statistically significant, *significantly different, $p < 0.001$. EC: eye closure.

Cell populations in the eyewash were further characterized by microscopy following a cytospin and May-Grunwald staining, as depicted in Figure 6-5. One of the hallmarks of fresh blood PMNs is their multi-lobed nuclei (2-4 lobules) [32], and nuclear morphology can provide information on cell maturation state. As shown in Figure 6-5c, some 7hr EC night tear PMNs displayed hyper-segmented nuclei (more than 4 lobes of nuclei). In comparison, the 2hr EC night tear PMNs exhibited hypo-segmented nuclei (Figure 6-5f). Most of the cells collected from daytime collections were ocular epithelial cells (Figure 6-5g & h), and only a few tear PMNs were harvested; these displayed normal nuclear morphology (Figure 6-5i), similar to fresh circulating blood PMNs.

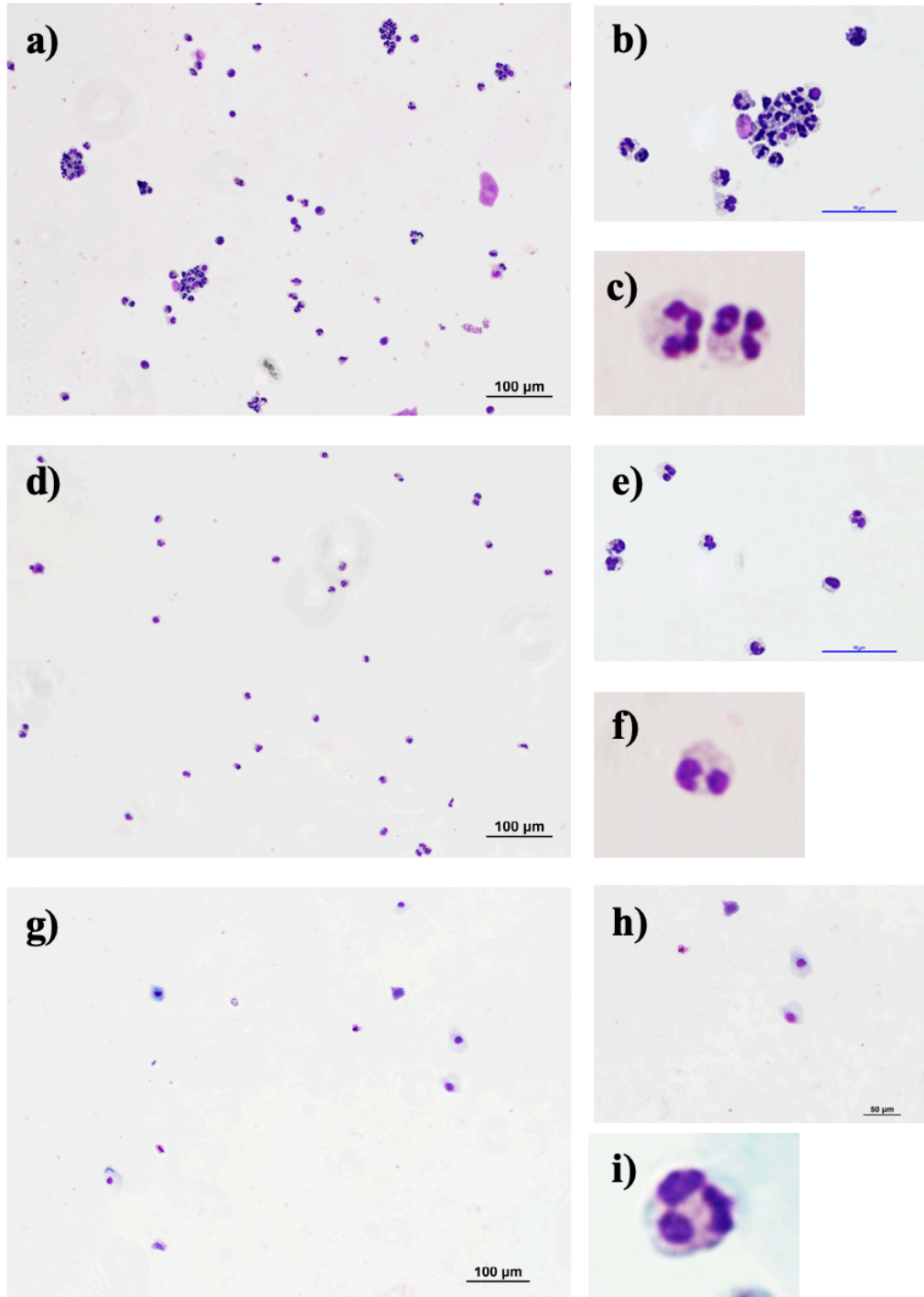


Figure 6-5. Analysis of the nucleus morphology of tear PMNs collected after a full night of sleep (a, b, c), after 2hr sleep at night (d, e, f), and during the day (g, h, i).

As illustrated in Figure 6-6, a significantly higher percentage (23%) of the 2hr EC night tear PMNs exhibited hyposegmented nuclei compared to the 7hr EC night tear PMNs ($p = 0.005$). Conversely, a significantly higher percentage (48%) of the 7hr EC night tear PMNs showed hyper-segmented nuclei compared to the 2hr EC night tear PMNs ($p = 0.024$).

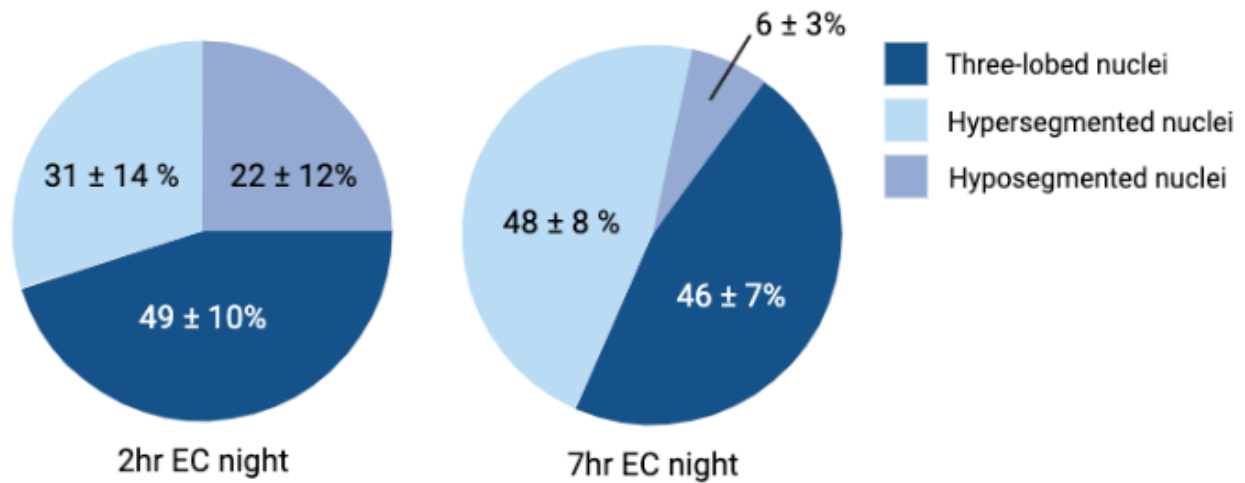


Figure 6-6. The percentage of a) 2hr EC night and b) 7hr EC night tear PMNs exhibiting three-lobed, hypersegmented, and hyposegmented nuclei.

It was also observed on the cytospin samples that 7hr EC night tear PMNs were more prone to aggregate (more than 3 cells clumped together), with significantly more aggregates present in the 7hr EC night collected samples compared to the 2hr EC night samples ($p < 0.001$), as shown in Figure 6-7a. The ratio of aggregates to tear PMN number further illustrates that the increase in aggregates was not due to the higher number of tear PMNs obtained after 7hr EC at night (Figure 6-7b).

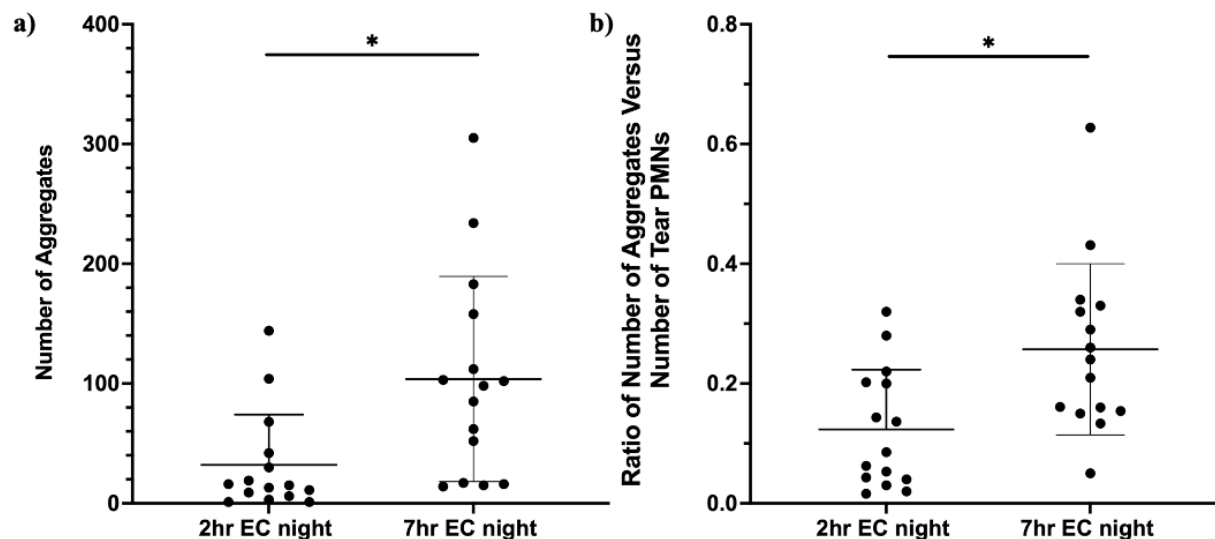


Figure 6-7. Number of leukocyte aggregates after nighttime collection. After the eyewashes were centrifuged and resuspended in PBS, cells were directly centrifuged on the glass slides by cytopspin and stained with May-Grunwald's stain. Pictures of the entire cytopspin area were taken and the numbers of aggregates and PMNs were counted. a) Number of aggregates in the 2hr and 7hr EC night samples; b) ratio of aggregates to tear PMN number. Values are presented as means $\pm$ standard deviations, $n=15$. EC: eye closure, *Significantly different, $p < 0.009$.

A small number of ocular epithelial cells were observed from the daytime collections with no significant difference between the two collections (2hr EC versus end of day) ($p = 0.572$), while significantly higher number of ocular epithelial cells were collected after a full night of sleep ($p < 0.006$), as shown in Figure 6-8. Although the number of ocular epithelial cells collected from 2hr EC night samples was higher than those collected from the 2hr EC day samples, this did not achieve statistical significance ($p = 0.135$). However, there were significantly more ocular epithelial cells collected from 7hr EC night samples compared to 2hr EC night samples ($p = 0.006$), suggesting that the increase in the number of ocular epithelial cells may be due to the duration of eye closure, leading to the accumulation of cells in the ocular environment.

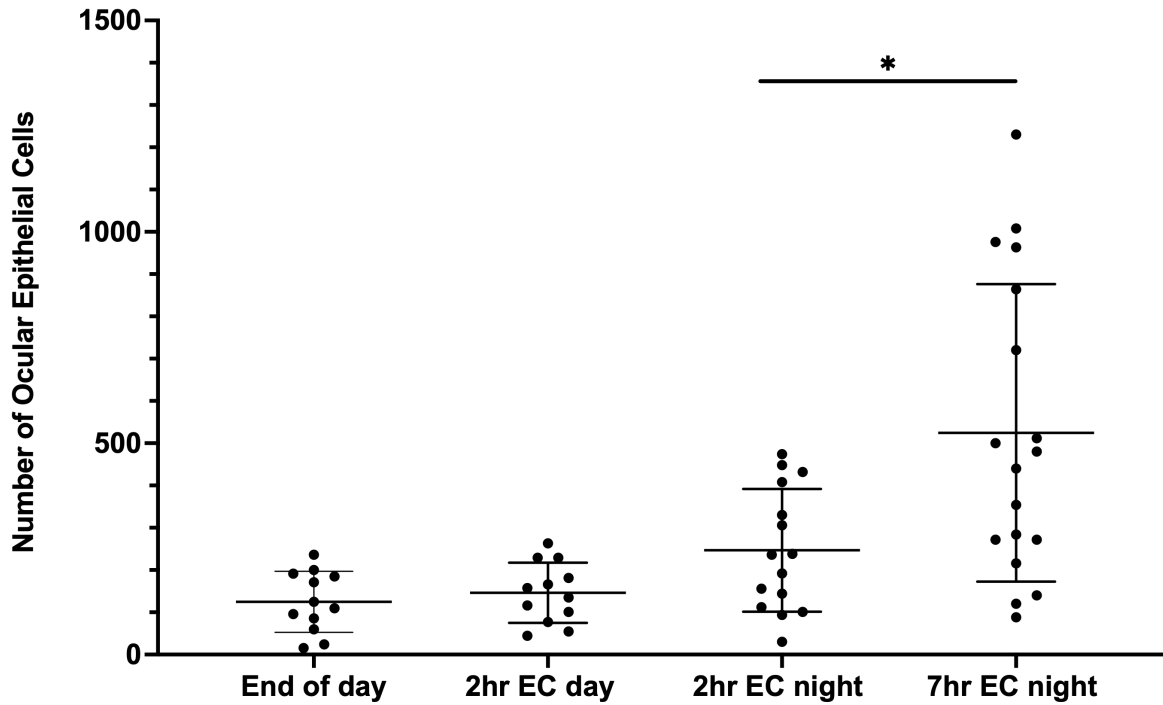


Figure 6-8. Number of ocular epithelial cells collected across different time points. After the eyewashes were centrifuged and resuspended in PBS, cells were directly centrifuged on the glass slides by cytopspin and stained with May-Grunwald's stain. Pictures of cytopspin area were taken, and the numbers of ocular epithelial cells were counted. Values are presented as means $\pm$ standard deviations, $n = 12$ for end of day and 2hr EC day samples, 15 for 2hr EC night samples, and 18 for 7hr EC night samples. Paired t-test was conducted to compare the paired data values between end of day and 2hr EC day, 2hr EC day and 2hr EC night, and 2hr EC night and 7hr EC night, *significantly different, $p = 0.006$. EC: eye closure.

6.3.2 The Correlation between Proinflammatory Mediators and Tear PMN Numbers

IL-8 and C5a have been shown to be present in the tear film and are chemoattractants, recruiting leukocytes to tissues [1]. The average concentration of IL-8 in 7hr EC night samples was significantly higher than in the 2hr EC night samples, as shown in Figure 6-9 ($p < 0.001$). No difference in IL-8 concentrations in the eyewash was observed between the two daytime collection samples ($p = 0.406$), with 32.9 ± 20.8 pg/mL for 2hr EC day and 28.9 ± 20.2 pg/mL for the end of day samples, while IL-8 concentration in the 2hr eye closure at night eyewash was significantly higher than in the eyewash collected after 2hr eye closure during the day ($p < 0.001$). This suggests that the increase in IL-8 concentration in 2hr EC night compared to 2hr EC day samples is unlikely due to “accumulation” of IL-8 in the closed-eye

environment and higher concentration at 2hr eye closure at night highlights the pro-inflammatory environment of the nocturnal closed-environment.

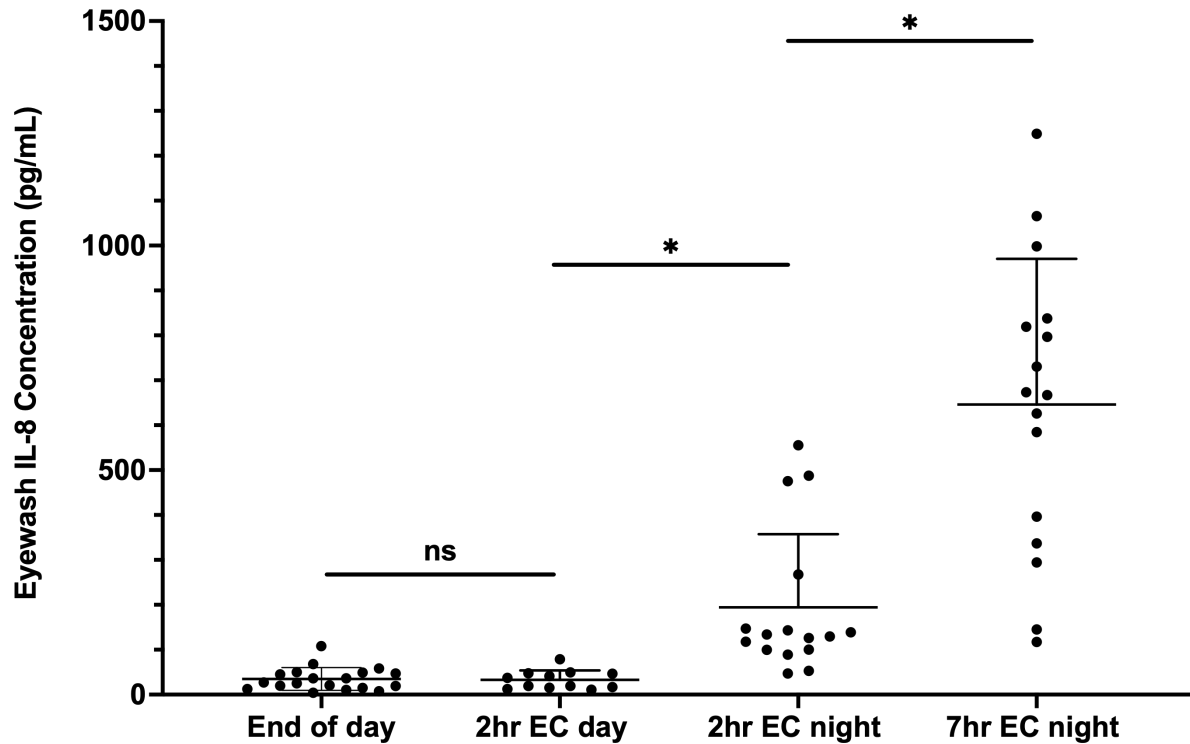


Figure 6-9. IL-8 concentration in the eyewash collected at different time points. The concentration of IL-8 in the supernatant of the eyewash was assessed by ELISA. Values are presented as means $\pm$ standard deviations, $n = 12$ for end of day and 2hr EC day samples, 17 for 2hr EC night, and 18 for 7hr EC night samples. Values have been corrected for concentration, as daytime supernatant samples were concentrated in the Amicon Ultra-15 centrifugal concentrator with 10,000 Dalton molecular weight cutoff filter. Wilcoxon matched-pairs signed rank test was conducted to compare the paired data values between end of day and 2hr EC day, 2hr EC day and 2hr EC night, and 2hr EC night and 7hr EC night, *significantly different, $p < 0.001$. EC: eye closure.

The changes in IL-8 concentrations across different time points followed a similar pattern to the PMN count. Data analysis identified a positive correlation between IL-8 concentration and PMN number collected after nighttime eye closure as well as during the daytime ($p < 0.014$, $r > 0.458$, Figure 6-10).

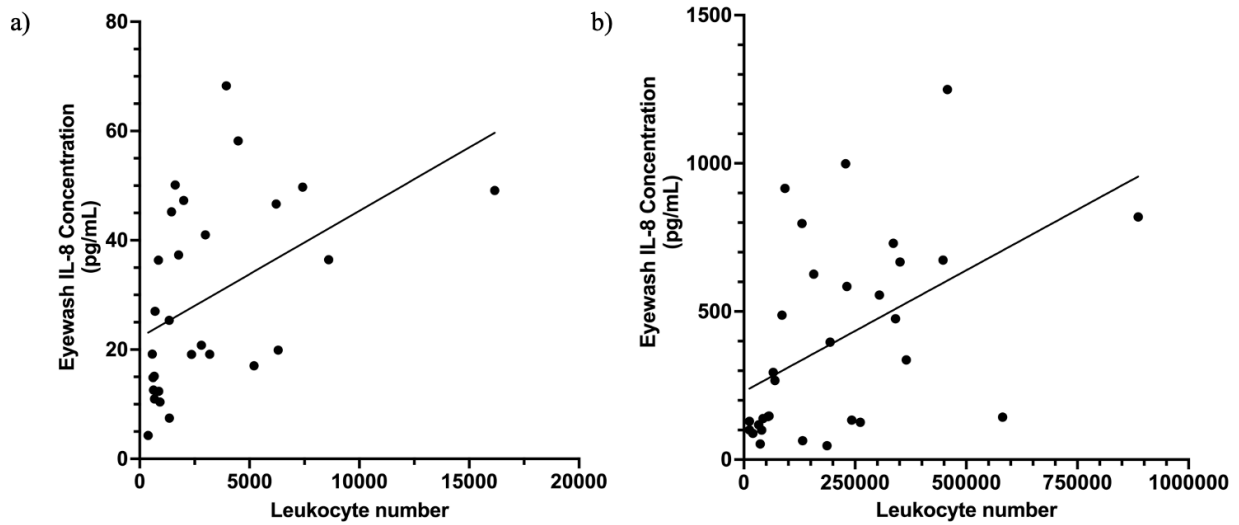


Figure 6-10. Correlation between eyewash IL-8 concentration and leukocyte number. a) during the daytime, $r = 0.458$, $p = 0.014$; b) after the nighttime eye closure, $r = 0.489$, $p = 0.005$.

As observed in Figure 6-11, there was a significant difference in the C5a concentration between the 7hr EC and 2hr EC night samples ($p = 0.005$), whereas C5a was barely detected in the daytime collection samples despite the eyewash concentration step. Additionally, the concentration of C5a in the 2hr EC night sample was significantly higher than the 2hr EC day sample ($p = 0.031$).

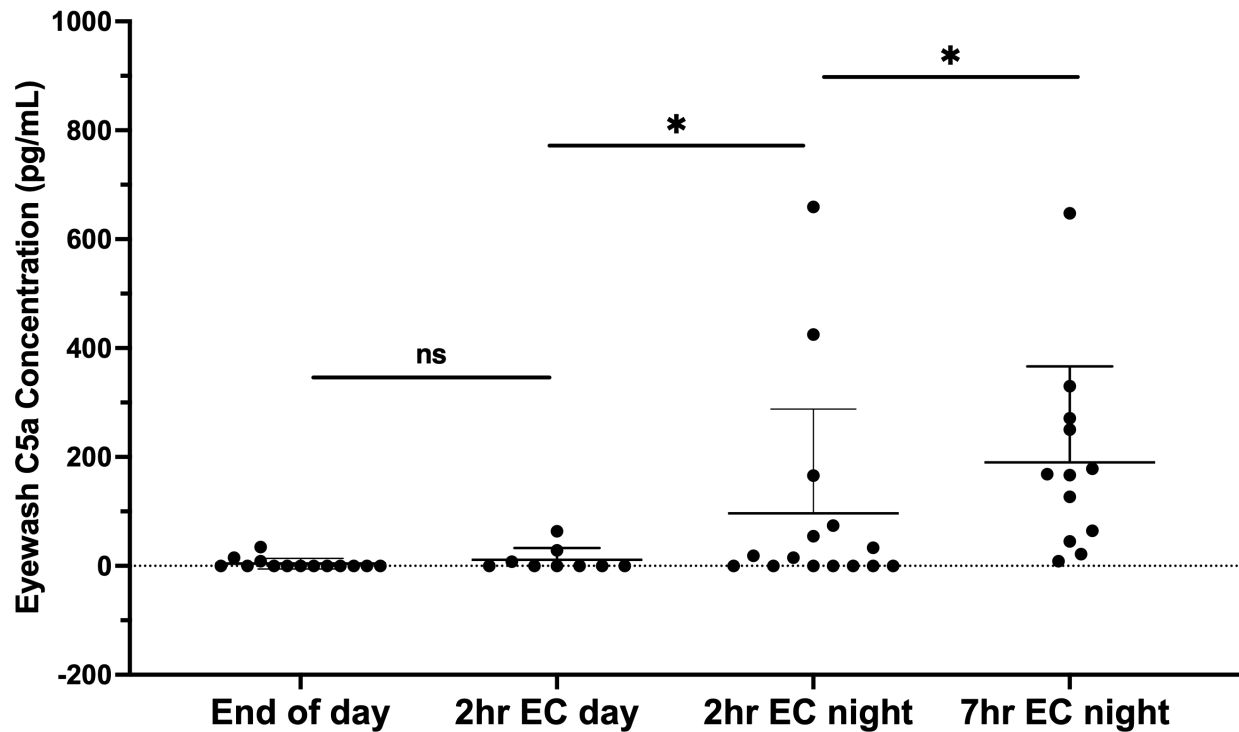


Figure 6-11. Concentration of eyewash C5a across different time points. The concentration of C5a in the supernatant of the eyewash was assessed by ELISA. Values are presented as means $\pm$ standard deviations, $n = 14$ for end of day sample, 9 for 2hr EC day sample, 15 for 2hr EC night sample, and 13 for 7hr EC night sample. Values have been corrected for concentration, as daytime supernatant samples were concentrated using Amicon Ultra-15 centrifugal concentrator with a 10,000 Dalton molecular weight cutoff filter. Wilcoxon matched-pairs signed rank test was conducted to compare the paired data values between end of day and 2hr EC day, 2hr EC day and 2hr EC night, and 2hr EC night and 7hr EC night, *significantly different, $p < 0.031$. EC: eye closure.

However, there was no correlation between leukocyte number and C5a concentration in the eyewash ($p = 0.984$, $r = 0.003$, Figure 6-12).

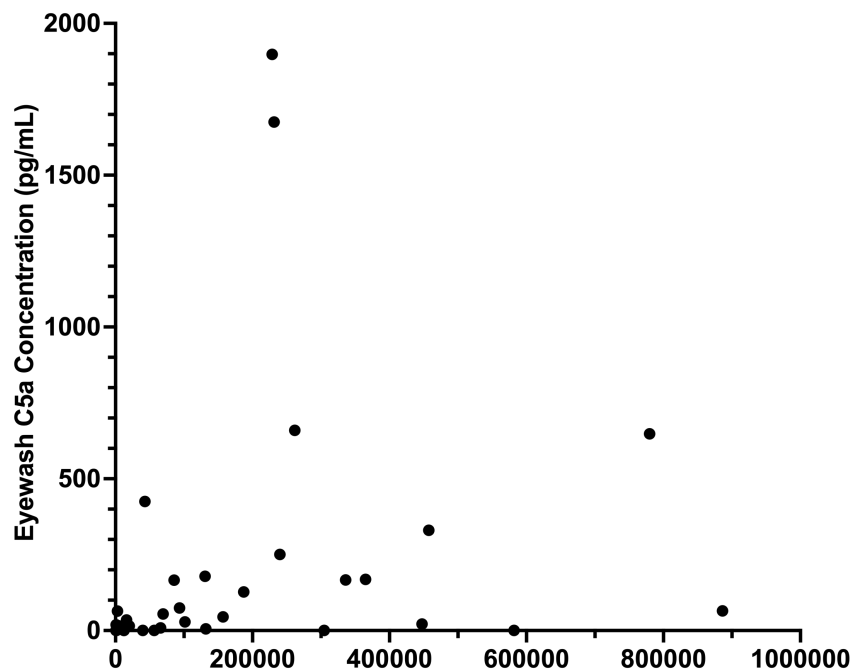


Figure 6-12. Lack of correlation between eyewash C5a concentration and leukocyte number. $r = 0.003$, $p = 0.984$.

6.3.3 PMN Phenotypes Collected from 2hr and 7hr EC Night Samples

The phenotypes of tear PMNs collected across different time points were analyzed by flow cytometry to examine the impact of collection time and eye closure. Due to the low PMN numbers from the daytime collections, only the phenotypes of 7hr EC and 2hr EC night tear PMNs were evaluated.

As shown in Table 6-1, a significantly higher expression of CD184, a marker for cell aging [33], was observed on 7hr EC night tear PMNs ($p = 0.008$), suggesting that tear PMNs collected after a full night of sleep were older or more aged than 2hr EC night tear PMNs, and corroborating the microscopy observations around nuclear morphology. As expected, no difference was observed in L-selectin expression (CD62L) between 7hr EC and 2hr EC night tear PMNs as L-selectin is known to be shed from the cell surface of PMNs during the transmigration process, resulting in background level or low fluorescent intensity of CD62L [34].

Table 6-1. Level of expressions of CD62L and CD184 on tear PMNs collected after 2hr and 7hr of sleep at night.

	Mean fluorescent intensity (MFI)	
	2hr EC night	7hr EC night
CD62L	8.4 ± 2.1	8.1 ± 1.4
CD184	46.0 ± 12.4	62.3 ± 14.6 *

n = 10 from 10 participants, EC: eye closure, *significantly different, $p = 0.008$.

Tear PMNs collected after a full night of sleep (7hr of eye closure) exhibited significantly higher expression of CD63 ($p = 0.018$), CD66b ($p = 0.015$), and lactoferrin ($p = 0.008$) compared to 2hr EC night tear PMNs (Figure 6-13). Furthermore, 7hr EC night tear PMNs showed a significantly lower expression of IL-8Rs than 2hr EC night tear PMNs ($p = 0.002$), indicating internalization of IL-8Rs that is associated with the phenotype of activated PMNs [35]. The change in the level of expression of surface receptors between tears PMNs collected at 2hr and 7hr EC night was also calculated and this ratio, which further illustrates increased tear PMNs activation with increased eye closure time is presented in Table 6-2.

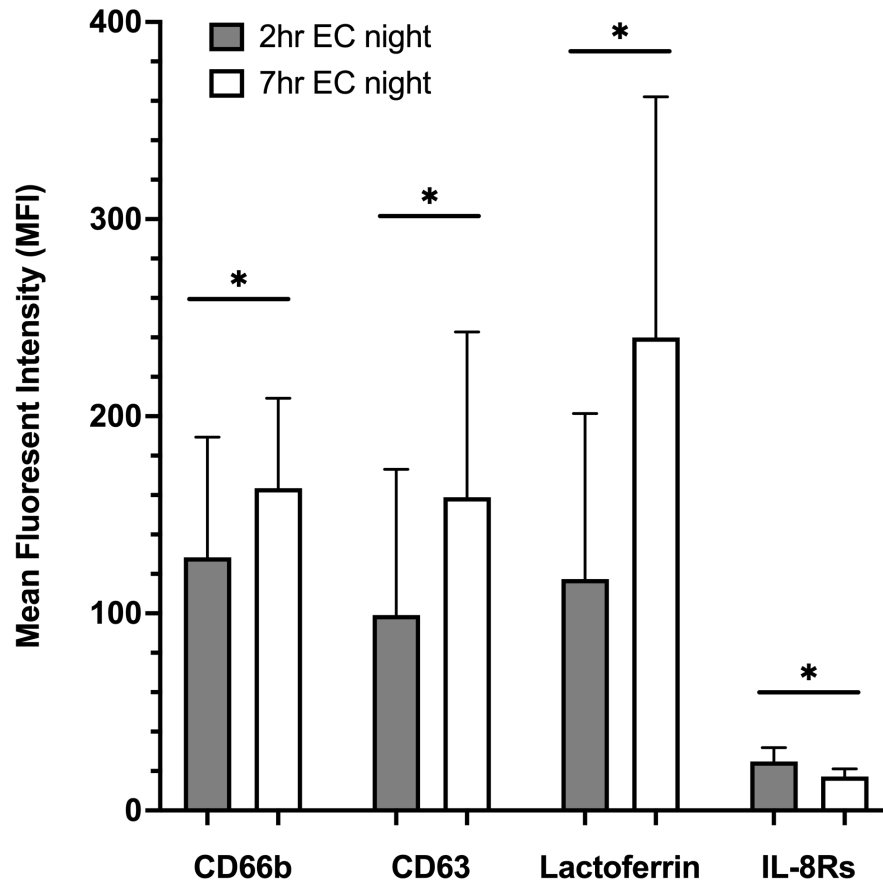


Figure 6-13. Changes in the expression levels of CD63, CD66b, IL-8Rs, and lactoferrin on 2hr EC night and 7hr EC night tear PMNs. Tear PMNs were stained with CD63, CD66b, anti-IL-8Rs, and anti-lactoferrin antibodies, and analysed by flow cytometry. Results are presented as mean $\pm$ standard deviations, $n = 7$ for lactoferrin, 9 for IL-8Rs, and 19 for CD63 and CD66b. EC: eye closure, *significant difference between the 2hr EC night and 7hr EC night tear PMNs ($p < 0.018$).

Table 6-2. Ratio of baseline expression of surface receptor between 2hr and 7hr EC night tear PMNs.

	CD Markers	Ratio of 7hr Vs 2hr EC Night Tear PMNs
Adhesion Marker	CD11b	1.07 ± 0.16
	CD54	1.42 ± 0.42
Degranulation Marker	CD66b	1.62 ± 0.61
	CD63	2.00 ± 0.84
	Lactoferrin	1.40 ± 0.53
Activation Marker	IL-8Rs	0.71 ± 0.12

Results are presented as mean ± standard deviations. n = 8 for CD11b and CD54, 19 for CD66b and CD63, 7 for lactoferrin, and 9 for IL-8Rs.

Additionally, in response to fMLP, 2hr EC night tear PMNs demonstrated a higher degree of degranulation compared to 7hr EC night tear PMNs ($p \leq 0.017$, Figure 6-14).

Taken together, these findings further suggest that 2hr EC night tear PMNs are younger than the 7hr EC night tear PMNs as they possess a higher activation potential (as shown in Figure 6-14) but lower baseline expressions of cell activation-related surface markers (Figure 6-13), which would concur with the cell aging results (Table 6-1).

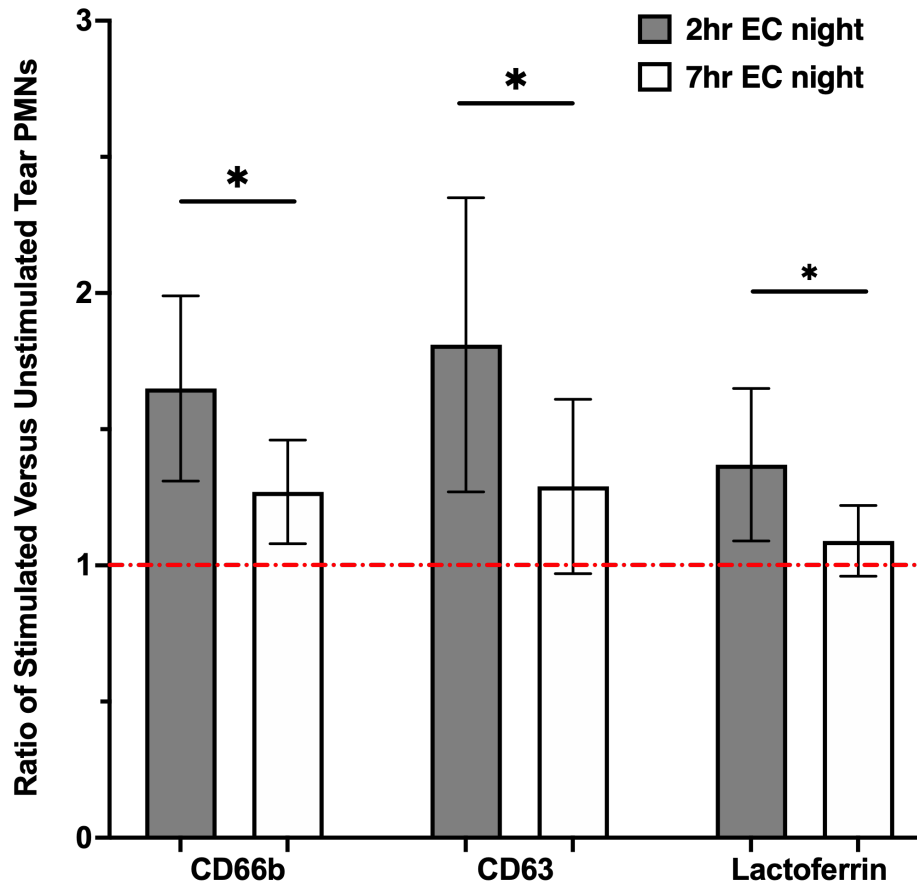


Figure 6-14. Changes in the expression levels of CD63, CD66b, IL-8Rs, and lactoferrin on 2hr EC night and 7hr EC night tear PMNs in response to fMLP. Tear PMNs were either stimulated with 1.5 μ M fMLP or not and were then stained with CD63, CD66b, anti-IL-8Rs, and anti-lactoferrin antibodies, and analysed by flow cytometry. Results are presented as mean $\pm$ standard deviations, and the dotted line represents a ratio of 1 (no difference between collection after 2hr and 7hr eye closure at night). $n = 8$ for lactoferrin, 14 for CD63 and CD66b, EC: eye closure, ns: not statistically significant, *significantly different ($p \leq 0.017$).

As both IL-8 and C5a are recognized to be pro-inflammatory mediators leading to PMN degranulation, a correlation between IL-8 and C5a concentration and tear PMN activation level was assessed. For both 7hr and 2hr EC night tear PMNs, IL-8 concentrations in the eyewash were not correlated with the expression of degranulation markers ($p > 0.138$) nor the activation ratio ($p > 0.121$) on collected tear PMNs, suggesting that while IL-8 appears to contribute to tear PMNs recruitment, other pro-inflammatory mediators play a role in tear PMN degranulation and exposure to IL-8 may not prime tear PMNs.

Although C5a concentrations did not correlate with the expressions of CD66b and CD63 on 7hr EC night tear PMNs ($p > 0.38$, $r > 0.24$), C5a concentration positively correlated with the expression of lactoferrin on 7hr EC night PMNs ($p < 0.001$, $r = 0.878$). C5a did not correlate with any of the activation ratios ($p > 0.3$, $-0.31 < r < -0.06$) on 7hr EC night tear PMNs.

Regarding 2hr EC night tear PMNs, C5a did not correlate with the baseline levels of CD66b ($p = 0.709$, $r = 0.291$), CD63 ($p = 0.603$, $r = -0.318$), and lactoferrin ($p = 0.496$, $r = -0.504$). However, there was a positive correlation between C5a concentration and the activation ratio of lactoferrin ($p = 0.004$, $r = 0.996$) despite no correlation with CD66b ($p = 0.776$, $r = -0.224$) and CD63 ($p = 0.340$, $r = -0.660$). Taken together, these results may suggest an interaction between C5a and lactoferrin expression on tear PMNs.

6.3.4 Neutrophil Extracellular Traps (NETs) and DNase

As shown in Table 6-3, tear PMNs collected after a full night of sleep showed significantly higher levels of myeloperoxidase (MPO) and citrullinated histones (H3Cit) than 2hr EC night tear PMNs ($p = 0.046$), suggesting that 7hr EC night tear PMNs released more NETs. Tear PMNs were found to tend to aggregate after being collected from a full night of sleep, as shown in Figure 6-5a. Since NETs are large structures that could trap and immobilize cells, the aggregation or cluster of cells observed in 7hr EC night samples on the cytospin may be due to the presence of NETs. DNase is shown to break or digest DNA molecules. Treating the 7hr EC night tear PMNs with DNase I resulted in a $73 \pm 12\%$ reduction in the number of aggregates ($p = 0.012$).

Table 6-3. Changes in the levels of MPO and H3Cit on 2hr and 7hr EC night tear PMNs.

	Mean fluorescent intensity (MFI)		Ratio of 7hr to 2hr EC night
	2hr EC night	7hr EC night	
MPO	12.7 ± 2.1	$23.0 \pm 12.5^*$	1.8 ± 1.0
H3Cit	8.7 ± 3.8	$20.2 \pm 9.7^*$	2.2 ± 1.7

Tear PMNs were stained with MPO and H3Cit, which were assessed through flow cytometry. Results are presented as mean $\pm$ standard deviations. $n = 6$, *significantly different from the 2hr night PMNs, $p = 0.046$.

Lactoferrin is also shown to decorate NETs [25,36], the presence of NETs in the eyewash samples was also identified by fluorescent microscopy with staining for lactoferrin, as shown in Figure 6-15.

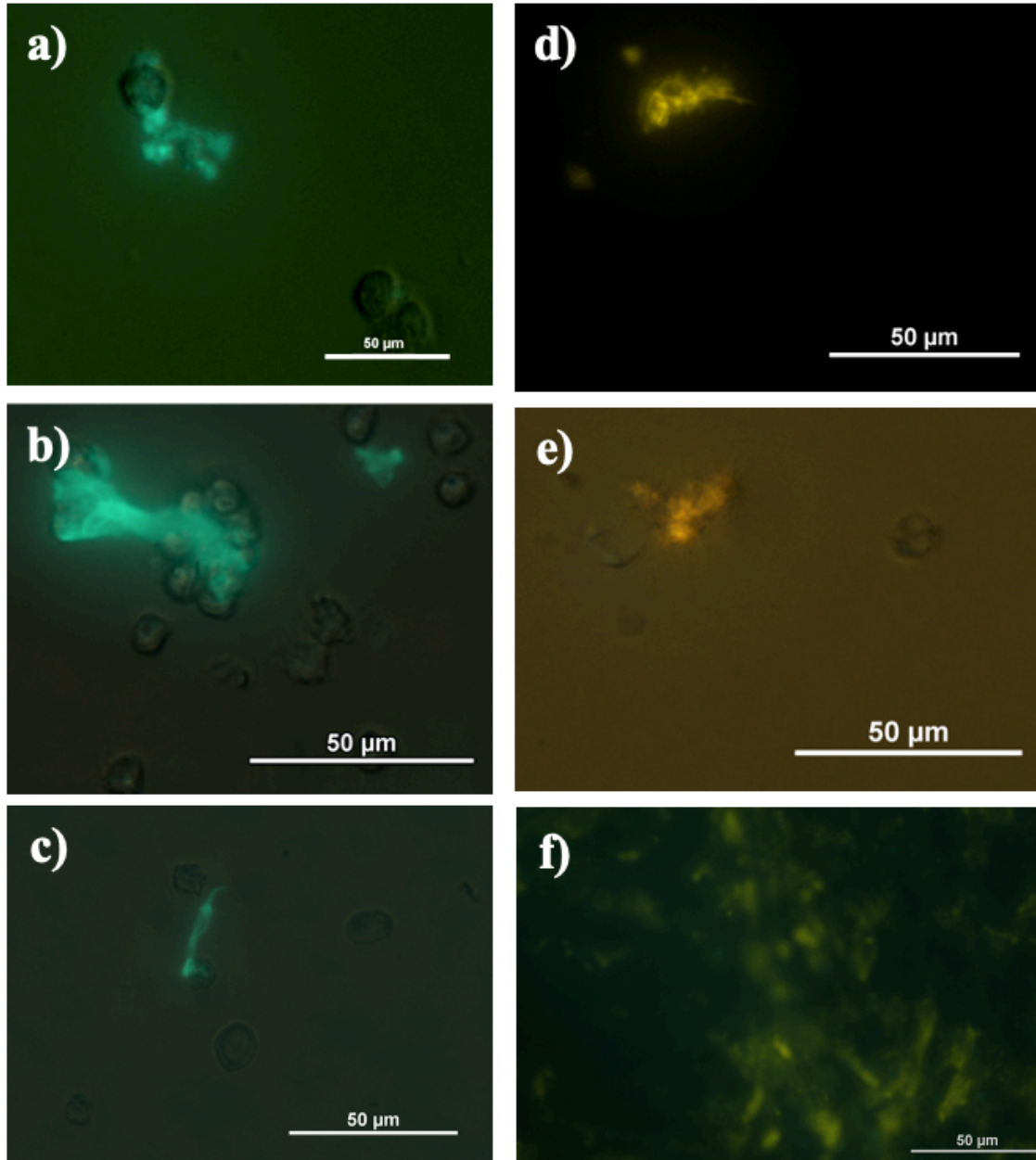


Figure 6-15. Cell aggregates entrapped in NETs as well as tear PMNs exhibiting NETs in tear samples collected at night with a-c) samples stained with antibody against lactoferrin and its FITC-conjugated secondary antibody, and d-f) samples stained with FITC-conjugated MPO and H3Cit using a PE-conjugated secondary antibody. f) Large cell aggregate entangled in NETs.

6.4 Discussion

This study investigated the rhythmic recruitment pattern of tear PMNs to the ocular surface and the changes in phenotypes across different time points. The average number of leukocytes collected in healthy eyes following sleep (prolonged eye closure at night) upon waking using the gentle eyewash has been shown to range from about 300,000 [4,37] to 80,000,000 [6,10]. The average leukocyte count in this study (330,000 cells) is in line with previously published work. The difference in cell count that can be observed across studies can likely be explained by the various methods used to both collect and count cells.

6.4.1 The Circadian Infiltration of Tear PMNs

Two hypotheses have been proposed regarding the origin of tear PMNs, whereby (a) they arise from the lacrimal glands and are washed away due to blinking during the day and then accumulate at night during prolonged eye closure with sleep [38], or (b) leukocytes extravasate from conjunctival blood vessels due to the presence of chemoattractants [4]. The fact that very few leukocytes were observed following 2hr eye closure during the day while over 100,000 leukocytes were collected after 2hr eye closure at night strongly supports the hypothesis that tear PMNs and other leukocytes found on the ocular surface during sleep/eye closure extravasate from the blood vessels in response to a chemoattractant. Noteworthy is that significantly higher numbers of leukocytes were observed after 7hr of eye closure compared to 2hr of eye closure at night, suggesting that there is continuous extravasation of leukocytes, which is likely related to the continuous presence of chemoattractants, such as IL-8 and C5a. The transmigration hypothesis is further supported by the level of L-selectin observed on tear PMNs. Gorbet et al reported that L-selectin (CD62L) is downmodulated on the surface of the tear PMNs [34], indicating a sign of PMNs transmigration and cell aging [39]. Our study corroborated prior observation of L-selectin downregulation on tear PMNs collected upon awakening but also showed that L-selectin was already downregulated on tear PMNs at 2hr of closed-eye at night, providing further evidence of leukocyte transmigration.

Our study highlighted the circadian recruitment of tear PMNs to the ocular surface with the infiltration of tear PMNs to the ocular surface peaking at night, which corroborated the findings of Tan et al. [11] and Postnikoff et al. [6,10]. However, Tan et al. had reported that only a few tear PMNs could be collected during the first 3 hours of sleep, which contradicts the observations by Postnikoff et al. [6] and our study. The inconsistency among results may be due to differences in cell collection methods, the former using capillary tubes, while the latter two research groups used a gentle eyewash method.

The circadian infiltration of PMNs has been observed in lungs and is believed to be regulated by the central circadian clock, the SCN, or/and the intrinsic autonomous transcription/translation loops

involving various transcription activators, such as Bmal1 participating in circadian gene regulation, present in peripheral cells [19,20]. One may wonder about the purpose of the circadian infiltration of PMNs to the tissues as well as the specific function of those circadian-infiltrated PMNs. In the lungs, the recruitment of PMNs may be associated with the presence of colonizing microbes through the expression of CXCL5 at night [40]. It has also been shown that more PMNs were recruited to the kidney at night to protect the kidney from fungal infection [20]. In *Arntl* (encoding Bmal1) mutant mice, there was a decreased immune response of PMNs against fungal or bacterial infections [41]. Taken together, these findings suggest that the infiltration of tear PMNs to the ocular surface at night may aim to eliminate pathogens which are accumulated from the day, ultimately enhancing the eye's defensive mechanisms. Alenezi et al. found that genes isolated from bulbar conjunctival cells upregulated in the early morning played a role in immune defence, cell turnover, and regulation of gene expression, while the genes that were upregulated in the evening were involved in signalling and mucin production [42], which further supports the defensive role of tear PMNs against pathogens at night.

6.4.2 The Relationship between Chemoattractants and Tear PMNs

Prior work by Thakur et al. demonstrated that blood-isolated PMNs showed enhanced chemotaxis in the presence of closed-eye tears collected after sleep compared to open-eye tears [23], highlighting that chemoattractants present in tears have the capability to attract PMNs to the ocular surface. The concentration of IL-8 in the eyewash peaked on waking in the morning (i.e., following a full night of sleep), which agrees with prior published studies [23,43]. An increase in IL-8 concentration with increasing eye closure time during the night was also observed in this study, corroborating results from Thakur et al. in tears (using capillary tubes) [23], which suggests that the eyewash method provides an effective way to sample the closed eye environment, whereby both tear proteins and cells can be assessed reliably.

IL-8 is known to be a potent chemoattractant that can attract leukocytes to local sites [44]. Our results identified a positive correlation between tear PMN number and IL-8 concentration, suggesting that IL-8 may contribute significantly to the recruitment of tear PMNs to the ocular surface. While C5a was present in the nocturnal eyewash, it was below a detectable level in the daytime, which is in agreement with observations by Thakur et al. in tears [23]. Pathogens are known to be strong activators of the complement pathways [1]. The presence of anaphylatoxin C5a, as one of the fragments generated upon the cleavage of C5, suggests the presence of foreign elements and pathogens activating complement during closed-eye conditions. During the day and in open-eye conditions, the eye blinking mechanism and the antimicrobial substances in tears, such as lactoferrin and lysozyme, are effective mechanisms to flush away debris and kill pathogens, reducing the potential for complement activation and inducing a pro-inflammatory

environment, that may damage the ocular surface. However, when people sleep at night, their eyes are closed, there is a lack of blinking, and the turnover rate of tear film decreases. To compensate, the ocular environment may require a variety of inflammatory mediators, which can recruit and activate immune cells to patrol the ocular surface. Although C5a can attract PMNs to the sites, our findings showed that C5a concentrations did not correlate with PMN numbers, which suggests that C5a may regulate other cell functional activities of tear PMNs.

No correlation was detected between IL-8 concentration and the baseline expression or the activation ratio of surface receptors on both 2hr EC night and 7hr EC night tear PMNs, which may suggest that the main function of IL-8 is to control the recruitment of tear PMNs to the ocular surface rather than priming or activating cells, and further highlighting that other inflammatory mediators in tears may regulate cell activation. The interaction between C5a and its receptors, C5aR1, can activate various PMNs functional activities, including cytokine release, oxidative burst, degranulation, and cytoskeleton reorganization [45,46]. Our data showed that C5a positively correlated with the baseline expression of lactoferrin on 7hr EC night tear PMNs but not the activation ratio of lactoferrin, which may be because 7hr EC night tear PMNs have aged, are exhausted and cannot be further activated. Additionally, there was a marginally positive correlation between the activation ratio of lactoferrin on 2hr night tear PMNs and C5a concentration. Thus, the above findings suggest the role of C5a in lactoferrin release by PMNs, which may include both priming and activation. Previous research has demonstrated that C5a may induce an increase in intracellular pH of blood PMNs, resulting in an enhanced degranulation as measured by increased release of myeloperoxidase and lactoferrin in supernatants [45]. However, Deriy et al. also reported that C5a cannot induce the upregulation of surface expression of lactoferrin on blood PMNs [47]. Despite the inconsistency between these studies in blood PMNs, the ability of C5a to induce degranulation in PMNs has been confirmed by several studies [1,45,48]. The impact of C5a on lactoferrin release in tear PMNs will need further investigation.

6.4.3 Activation and Maturation State of Tear PMNs During Prolonged Nighttime Eye Closure

Tear PMNs collected after 7hr of eye closure at night displayed hypersegmented nuclei, while 2hr of eye closure night tear PMNs exhibited hyposegmented nuclei. The hypersegmented nuclei morphology may indicate cell aging and activation [32,49,50]. However, there is limited data on the function or role of these hyposegmented nuclei PMNs. Chan et al. reported that in a normal neutrophil population, 10 to 30% of PMNs have two nuclear lobes, 40 to 50% of PMNs have three nuclear lobes (PMNs under steady state), 10 to 20% of PMNs have four nuclear lobes, and about 5% of PMNs have more than 5 nuclear lobes (highly

activated or aged PMNs) [51]. Our preliminary counts indicate that the percentages of three-lobed, hyposegmented (less than two nuclear lobes), and hypersegmented (more than four nuclear lobes) 2hr closed-eye night tear PMNs (Figure 6-6) were found to be similar to the percentages reported for blood PMNs by Chan et al [51]. However, a significantly lower percentage of hyposegmented PMNs and a higher percentage of hypersegmented PMNs were observed in the 7hr closed-eye night tear PMNs. Collectively, these findings suggest that PMNs exhibiting less than 2 nuclear lobes may represent the young or immature PMN population, but this hypothesis requires further investigation.

Several research groups including our own have hypothesized through indirect evidence that tear PMNs collected after a full night of sleep are being activated when they are in the closed-eye environment [5,6,34,43,52]. In the current study, 7hr closed-eye night tear PMNs exhibited higher levels of degranulation markers (CD66b, CD63 and lactoferrin) and NETs markers (H3Cit and MPO), and lower expression of IL-8Rs compared to the 2hr closed-eye night tear PMNs, providing direct evidence of activation/degranulation of tear PMNs in the nighttime closed-eye environment over prolonged residence time. Additionally, significantly more ocular epithelial cells were collected from the 7hr EC night samples compared to those from 2hr EC night samples. This may suggest that tear PMNs aid in the shedding of ocular epithelial cells from the ocular surface by releasing granular proteins, such as matrix metalloproteinase 9 (MMP-9) [53], further highlighting the potential role of tear PMNs in the regeneration of the ocular surface epithelium.

Although lactoferrin is stored inside the specific granules, it is also expressed on the surface of resting blood PMNs [47] and can be further upregulated upon stimulation [54]. Our preliminary findings indicate that the lactoferrin level on the surface of tear PMNs was substantially higher than on the surface of resting blood PMNs (Appendix Table 1), which further confirms the activated phenotype of tear PMNs. Lactoferrin is not only expressed on the surface of PMNs as a degranulation marker but is also expressed as one of the granular proteins on NETs [25,36]. Therefore, compared to the 2hr of eye closure night tear PMNs, the higher expression of lactoferrin on the surface of 7hr of eye closure night tear PMNs indicates that tear PMNs collected after a full night of sleep released more granules and NETs. It has been shown that lactoferrin has an anti-inflammatory effect, and Okubo et al. reported that lactoferrin could inhibit the release of NETs by PMNs without affecting their intracellular pathways, such as the generation of ROS and citrullination of histones [36]. Additionally, they showed that the presence of lactoferrin changed the morphology of NETs and one of the possible inhibitory mechanisms of lactoferrin may be to prevent the spread of NETs [36]. In our tear collection samples, an abnormal morphology of NETs, whereby NETs wrapped around together instead of spreading out like spider web, was also observed, which may have been caused by the presence of lactoferrin in tears. Additionally, It has also been reported that the binding of the anti-lactoferrin antibody to the lactoferrin on the surface of PMNs may promote PMN adherence and

superoxide generation [55], which reveals the inflammatory impact of lactoferrin on the activation of PMNs. Note that the anti-lactoferrin antibody belongs to the anti-neutrophil cytoplasmic antibodies (ANCA) which are autoantibodies produced by B cells against self-proteins, and are involved in many inflammatory diseases, such as SLE and rheumatoid arthritis [56]. These findings highlight the complex role that lactoferrin may play in the closed-eye environment. Further studies are needed to investigate the impact of lactoferrin in tears on ocular homeostasis and interactions with tear PMNs.

IL-8 mediates PMN cell activities, such as transmigration, by binding to its membrane-bound receptors (IL-8Rs), including CXCR1 and CXCR2 [57]. IL-8Rs were shown to be downregulated in response to LPS [58]. IL-8 may also induce the internalization of IL-8Rs, with CXCR1 and only partial CXCR2 being re-expressed on the cell membrane after a second stimulation [57]. Thus, activated PMNs may exhibit a reduced expression of IL-8Rs, so the lower IL-8Rs level on full-night tear PMNs suggests that they have already been activated. Interestingly, Pignatti et al. showed that the extent of downmodulation of IL-8Rs was greater in patients with asthma compared to healthy individuals [59], which suggests the potential role of IL-8Rs in pathological conditions and warrants future studies to investigate its function in ocular complications.

Compared to 7hr EC night tear PMNs, 2hr EC night tear PMNs were activated to a higher degree upon stimulation with fMLP, showing higher activation ratios of CD66b, CD63, and lactoferrin in 2hr EC night tear PMNs. Our previously published data indicated that fMLP failed to induce prolonged reactive oxygen species (ROS) production in tear PMNs when stimulated with fMLP, leading to a hypothesis that tear PMNs may not express fMLP receptors on the surface [5]. However, in this study, we demonstrated that fMLP receptors are expressed on both 7hr and 2hr EC night tear PMNs, so the inability to activate 7hr EC night tear PMNs is not due to the absence of fMLP receptors (data not shown) [5] and is thus more related to prior activation in the closed-eye environment. Our previous study also showed that tear PMNs released ROS spontaneously *in vitro* without any activation [5], which may be due to the release of intracellular ROS accumulated from prior stimulation. Furthermore, aged PMNs have been reported to exhibit enhanced capabilities in ROS production, adhesion, and phagocytosis [49] but possess a lower granule content and NET-forming capability compared to young or fresh PMNs [60], suggesting the differential impact of cell aging on PMNs functionalities.

Additionally, our results indicate that tear PMNs collected after a full night of sleep are aged (high expression of CD184), and as such full-night tear PMNs may have lost their capability to upregulate their degranulation makers upon further stimulation. The upregulation of CXCR4 (CD184) on the surface of PMNs has been found to be associated with the clearance of PMNs in the bone marrow, spleen, and liver [61–63], so CD184 is used as a cell aging marker. This diurnal cell aging was also reported in the blood

circulating PMNs by Adrover et al., which may be regulated by their intrinsic molecular clock [41] or the systemic mechanism [49]. Therefore, the circadian rhythm not only affects the recruitment of PMNs to the ocular surface but also has an impact on cell phenotypes.

6.5 Conclusion

Our study demonstrated that the infiltration of tear PMNs to the ocular surface in the closed-eye environment peaked at night under healthy conditions, suggesting a circadian rhythm of the recruitment of tear PMNs to the eyes. Decreased levels of L-selectin and increased concentration of IL-8 provided evidence to support the hypothesis of the recruitment of tear PMNs from the blood vessels is driven by the presence of IL-8. Tear PMNs become more activated and aged with increasing eye-closure time at night, given the upregulation of degranulation, NETs, and CXCR4 markers, and the lower expression of IL-8Rs. Furthermore, the presence of NETs in the eyewash may infer the potential pathological role of tear PMNs in ocular complications, for example, dry eye disease, if NETs were overproduced. Research showed that many diseases have a time-dependent severity of symptoms, such as rheumatoid arthritis [14], and asthma [64]. Although there is limited research on the variation in symptom severity across the day in ocular complications, our findings suggest that circadian regulation of tear PMNs may play a role in the pathogenesis of these ocular conditions. Thus, if the circadian responses or infiltration of tear PMNs could be controlled, it may help to relieve symptoms and reduce ocular inflammation.

Chapter 7 Pilot study on the phenotype of tear leukocytes in seasonal ocular allergy and their potential impacts on ocular surface inflammation

7.1 Introduction

Seasonal allergic conjunctivitis and perennial allergic conjunctivitis are the two most common types of ocular allergy with symptoms including itchy eyes, tearing, redness, and sensitivity to light [1–3]. Upon exposure to the antigen (such as dust mite or animal dander, pollen, etc), Th2 lymphocytes release cytokines that lead to the production of IgE by B lymphocytes [4–6]. The subsequent hypersensitivity allergic response involves the degranulation of conjunctival mast cells, which releases histamine and other inflammatory mediators upon IgE binding to their receptors, eventually leading to the recruitment of other leukocytes such as eosinophils, basophils, and neutrophils, thereby amplifying the allergic response and promoting ocular surface inflammation [2,5,6]. The presence of eosinophils on the ocular surface has been considered by some as a hallmark of an ocular allergic response [7–9]. Furthermore, tears from patients with ocular allergies have been reported to show changes in leukocyte population and elevated levels of cytokines, although leukocyte analysis has been limited by the low number of cells obtained using capillary tubes for tear sampling [2,7,10]. Most of these studies have focussed on cells and tear collection during the day, while increased levels of inflammatory cytokines are also present in allergic patients in the closed-eye tear film [9], which may lead to excessive recruitment of leukocytes and increased cell inflammatory responses, further worsening symptoms and increasing the risk of ocular surface damage. With seasonal allergies, leukocytes in the closed-eye environment may be exhibiting a higher inflammatory or activation state, creating a sub-inflammation environment on the ocular surface that persists throughout the day and further contributes to discomfort, which potentially leads to ocular complications, such as giant papillary conjunctivitis and conjunctival inflammation.

Research groups have primarily focused on the role of mast cells and eosinophils in ocular allergy. However, other types of leukocytes, such as neutrophils, may also play a role in the development and pathogenesis of ocular allergies. It has been reported that a large number of leukocytes, with over 60% of the cell population being polymorphonuclear neutrophils (PMNs), can be collected from the ocular surface after prolonged eye closure or sleep [11–13]. Recent research suggests that tear PMNs may migrate to the ocular surface from the conjunctival blood vessels and execute their cell functional responses [14], such as degranulation, reactive oxygen species (ROS) production, and release of neutrophil extracellular traps (NETs), to patrol and clean the ocular surface as part of ocular homeostasis. However, the functions of PMNs are a double-edged sword, and they may damage the host tissues if dysregulated and can play a

pathological role in diseases. For example, an excessive amount of NETs has been reported in patients with systemic lupus erythematosus (SLE) [15] and dry eye disease [16] as well as in the murine model of allergic eye disease [17], suggesting the potential pathological role of NETs (and hence PMNs) in ocular inflammation. Despite the influx of PMNs and potential inflammatory contributions in the closed-eye environment, there is limited knowledge about the specific role of PMNs in ocular allergy and no study has yet been conducted to compare the phenotype of tear PMNs between healthy and allergic eyes. Additionally, it would be valuable to determine whether tear PMNs isolated from allergic individuals are actively contributing to the inflammatory response and exacerbating symptoms. Such studies may provide new insights into the underlying pathogenesis of ocular allergy and may lead to the development of new treatment strategies that target tear PMNs specifically.

In the prior chapter (Chapter 6), we demonstrated the circadian infiltration of tear PMNs to the closed-eye environment as well as their aged and activated phenotype collected after sleep in the morning, suggesting the role of tear PMNs in patrolling the ocular surface and maintaining ocular homeostasis. Investigating tear PMNs phenotype and role in ocular surface inflammation, such as in the case of ocular allergy, would help identify differences that exist in this ocular environment and would provide further insights on their potential contributions to allergy symptoms and disturbance in homeostasis. A pilot study was thus conducted to investigate the phenotype of tear leukocytes in seasonal ocular allergy, in terms of their surface receptor expression related to cell activation and oxidative burst. The cell degranulation state was determined using CD66b and CD63, CD11b (Mac-1) and CD54 (Inter Cellular Adhesion Molecule 1, ICAM-1) assessed the expression of cell adhesion molecules, the cell maturation state was evaluated via CD184 and CD62L, and the presence of eosinophils was detected by CD16 and CD193. ROS was measured using dichloro-dihydro-fluorescein diacetate (DCFH-DA) and flow cytometry. The main hypotheses of this study were that a higher number of tear PMNs, exhibiting a higher level of activation, would be present on the ocular surface of allergy participants compared to non-allergy participants.

7.2 Methods

7.2.1 Reagents and Monoclonal Antibodies

Phorbol-12-myristate-13-acetate (PMA), Hank's Balanced Salt Solution (HBSS), and dichloro-dihydro-fluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich Co. (Oakville, Ontario, Canada). Phosphate-buffered saline (PBS) was purchased from Lonza (Allendale, New Jersey, USA). All antibodies were purchased from BD Biosciences (Mississauga, Ontario, Canada).

7.2.2 Subjects

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 (#44208; Waterloo, ON, Canada). 10 participants (3 males and 7 females), with ocular allergy and between the age of 20 and 53 years old (33.4 ± 11.5), were recruited. Their eligibility for the study was determined using the subjective symptom questionnaire, the MiniSQRL (see Appendix), which is a validated tool used to diagnose ocular allergies. Participants with ocular allergy were age- and gender-matched with 10 healthy participants (age: 31.0 ± 8.4 , 3 males and 7 females).

7.2.3 Study Visits and Cell Collection

The study consisted of 3 visits, one virtual training session and two cell collection visits. During the training session, participants were trained in the cell collection procedure, as described in Gorbett et al., 2015 [11]. In short, participants released the sterile saline solution onto the ocular surface and collected the run-off or eyewash into the 50 mL polypropylene tube. There were two cell collection visits, one was performed in the morning upon waking (closed-eye leukocytes), and the other one was scheduled on the next consecutive day during the daytime at the end of the afternoon (open-eye leukocytes). For the closed-eye collection, participants collected cells in the morning immediately after approximately 7 hours of sleep, and samples were dropped off in the morning after collection. For the open-eye collection, participants were asked to perform the daytime cell collection towards the end of the day (around 4-5 pm in the afternoon), and cell samples were delivered to the lab within two hours upon collection. 8 mL of sterile saline solution (PBS) was given to the participants, and an average of 7 mL of eyewash was returned, which was 85% of the initially provided volume and suggested minimal loss of eyewash.

All the collected eyewash samples were centrifuged at $280 \times g$ for 5 minutes, and the supernatants were stored at -80°C for further protein analysis. Once the cell pellet was resuspended in PBS, cell count was performed by using the MOXI-Z automated cell counter, gating on PMN population based on cell diameter, between 6.5 and 9 μm .

7.2.4 Cell Activation and Staining with Antibodies

Cells were either stimulated with $1.5 \mu\text{M}$ fMLP for 15 minutes in the cell incubator (37°C , 5% CO_2) or not. Cells were stained with the degranulation markers (CD66b and CD63), adhesion markers (CD11b and CD54), eosinophils marker (CD193), aging markers (CD184 and CD62L), CD16, and pan leukocyte

marker (CD45) for 20 mins in the dark at room temperature. Then, cells were diluted with DMEM/10% FBS and were ready for flow cytometry analysis.

7.2.5 ROS Production as Measured by DCFH-DA

The DCFH-DA method was used to measure the production of ROS by tear PMNs. Tear PMNs were divided into two groups, which were either stimulated with 40 nM of PMA or not. A different stimulus was used here to trigger the ROS release by tear PMNs as our previous work indicated that fMLP cannot induce a prolonged ROS release in tear PMNs [12]. A total of 35000 cells were added to each condition and were incubated with 48 μ M of DCFH-DA for 15 mins at 37°C in HBSS. Then, CD45 and PMA were added to the incubation tube for another 15 mins at 37°C. Cell samples were diluted with PBS and were analyzed through flow cytometry immediately.

7.2.6 Flow Cytometry

PMN population was identified using a double-gating strategy. All samples were acquired on a Becton Dickinson FACS Calibur flow cytometer (Mountain View, California, USA) using CELLQuest Software (Becton Dickinson, Mountain View, California, USA). At least 2000 PMNs events were acquired, and fluorescent values were recorded as mean fluorescent Intensities (MFI) for all samples.

7.2.7 Statistics

Results are presented as means $\pm$ standard deviations. Independent t-test and Mann-Whitney test were used to calculate the statistical significance. Correlations were assessed through Spearman correlations. Results were compared with age- and sex-matched healthy participants. A p-value of less than 0.05 was considered statistical significance and all the statistical analysis was performed via IBM SPSS Software (IBM Canada Ltd., Markham, Ontario, Canada). Outliers were identified by calculating the interquartile range (IQR) and were removed. GraphPad Prism Software was used to plot all the figures (GraphPad Software, San Diego, California, USA).

7.3 Results

7.3.1 Cell Count and Percentage of Leukocyte Populations

As shown in Figure 7-1, few tear leukocytes were collected in the afternoon, and there was no significant difference in open-eye leukocyte number collected from the ocular allergy participants compared to healthy individuals ($p = 0.178$). At awakening in the morning, more leukocytes were collected from individuals suffering from ocular allergy than from healthy participants, however, this did not achieve statistical significance ($p = 0.258$).

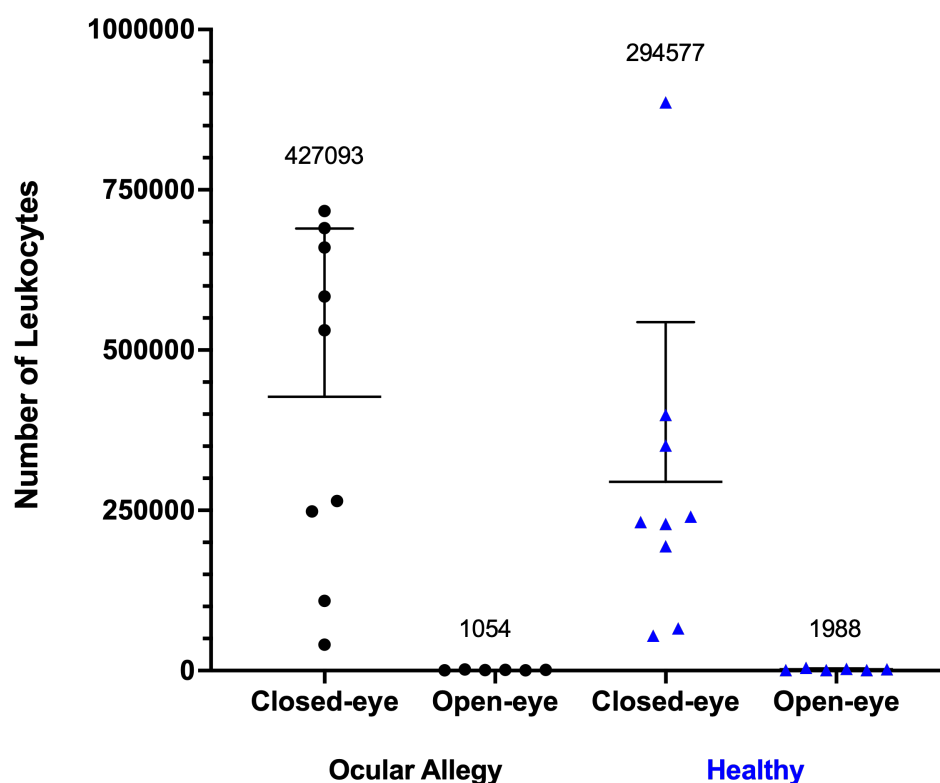


Figure 7-1. Comparison of leukocyte number between open-eye and closed-eye tears from ocular allergy and healthy participants. $n = 9$ for closed-eye cell count and 6 for open-eye cell count. The two tear collections were scheduled on two separate days, with one in the morning immediately after sleep and the other one in the afternoon. Results are represented as mean $\pm$ standard deviations. 4 outliers were removed based on the IQR calculation.

Leukocyte population (% eosinophils, neutrophils, monocytes, and lymphocytes) was identified using the double-gated strategy with CD45 as well as CD16 and CD193 by flow cytometry. Note that due

to the limited number of cells that were collected from the open eye time point, flow cytometry was only performed on samples collected from the closed-eye environment upon awakening.

Using flow cytometry analysis, as illustrated in Figure 7-2, ocular allergy participants were shown to have a significantly higher percentage of eosinophils compared to healthy individuals ($8.9 \pm 3.1\%$ versus $2.3 \pm 0.9\%$, $p = 0.001$), which provided further indication of the allergy status of these participants.

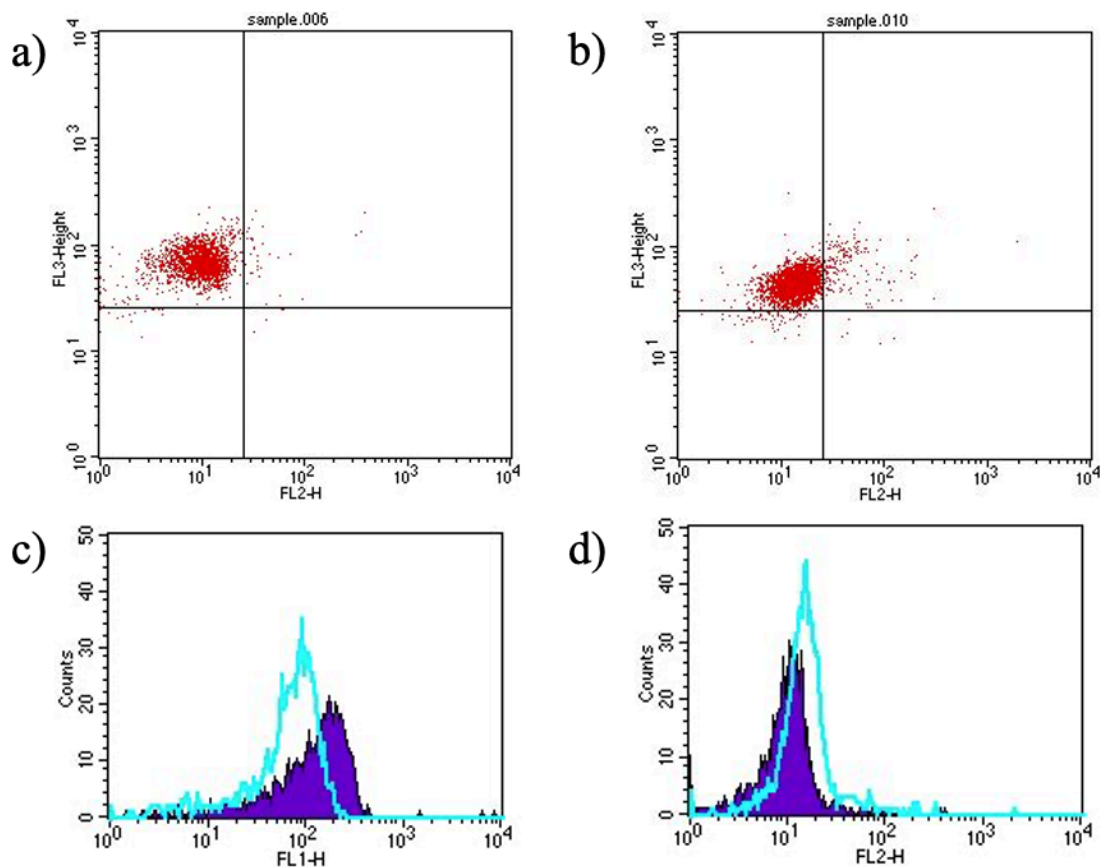


Figure 7-2. Identification of Eosinophils using CD16 and CD193 via flow cytometry analysis. Dot plots and histograms depict tear PMNs isolated from healthy participants (purple-filled) and ocular allergy patients (blue outlining). The eosinophil population was identified as $CD193^+$ (FL2) and $CD45^+$ (FL3). a & b) $CD45$ (FL3) versus $CD193$ (FL2) dot plots to identify eosinophils from neutrophils, c) a histogram plot comparing the $CD16$ (FL1) signals between tear PMNs from healthy participants and ocular allergy patients; d) a histogram plot comparing the $CD193$ (FL2) signals between tear PMNs isolated from healthy participants and ocular allergy patients.

The percentage of neutrophil and lymphocyte populations collected from the ocular surface of ocular allergy participants was similar to that from healthy individuals (see Table 7-1). The percentage of monocytes in the eyewash collected from ocular allergy participants was significantly higher than that from healthy participants ($p = 0.032$). The neutrophil-to-lymphocyte ratio (NLR) is a useful biomarker of systemic inflammation [18–20], and recent results reported elevated NLR values in ocular diseases, such as dry eye disease [20,21], keratoconus [22], and optic neuritis [23]. Interestingly, it was found that the NLR was higher in healthy participants compared to ocular allergy participants, although this did not achieve statistical significance ($p = 0.080$). This may indicate that a different etiology is involved in ocular allergy compared to other ocular diseases mentioned above. Since a large standard deviation was observed, more participants are needed to confirm this finding.

Table 7-1. Leukocyte subpopulations in tears collected from ocular allergy and healthy individuals.

	PMNs (%)	lymphocytes (%)	monocytes (%)	NLR
Ocular allergy	73.4 ± 8.2	2.7 ± 2.3	0.4 ± 0.3 *	64.7 ± 61.3
Healthy	72.6 ± 13.4	2.4 ± 1.3	0.1 ± 0.1	112.6 ± 91.2

Results are represented as mean ± standard deviations, n = 9 for ocular allergy and healthy participants.

* Significant difference between ocular allergy and healthy participants, $p = 0.032$. NLR: Neutrophil to Lymphocyte Ratio.

7.3.2 Changes in Surface Receptors between Healthy and Ocular Allergy Participants

The expression of surface receptors on tear PMNs was compared between ocular allergy and healthy participants. As shown in Figure 7-3, the levels of degranulation markers (CD66b and CD63) were significantly lower on the unstimulated tear PMNs from ocular allergy participants compared to those from healthy participants ($p < 0.001$), which surprisingly may suggest that tear PMNs from ocular allergy participants were less activated on the ocular surface than those from healthy eyes. There were no significant differences in the levels of CD11b and CD54 on tear PMNs between ocular allergy and healthy individuals ($p > 0.647$), which was somewhat expected as CD11b and CD54 expression is mostly upregulated upon transmigration and thus should not significantly change in the closed-eye environment between healthy and ocular allergy participants after transmigration.

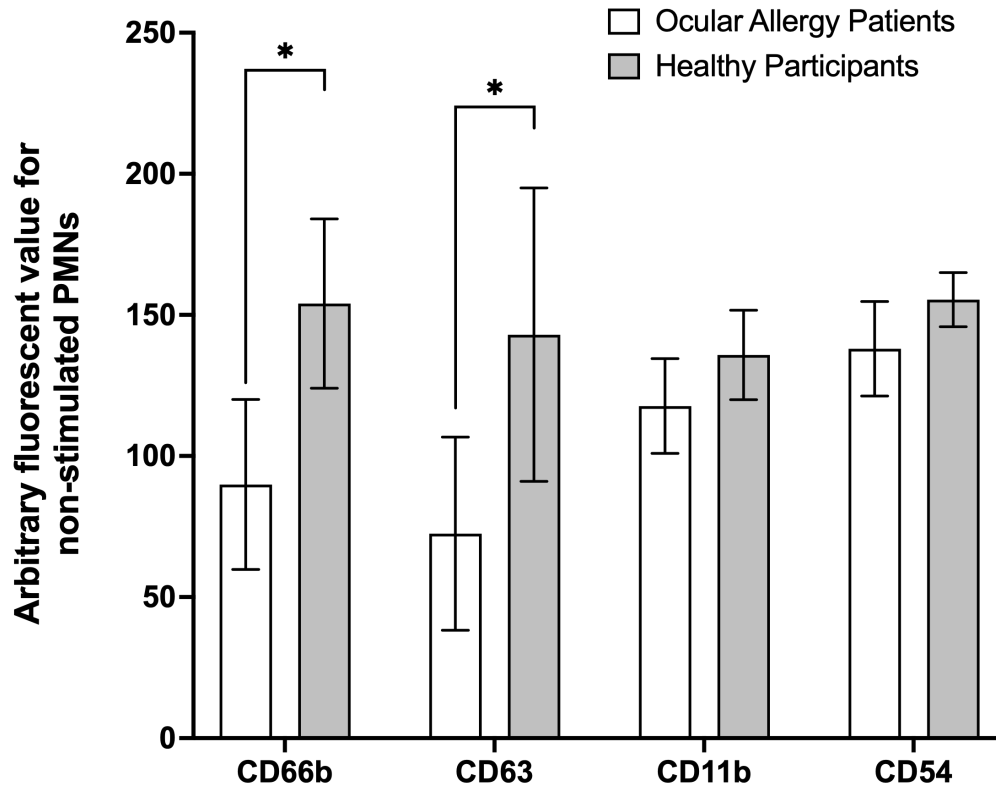


Figure 7-3. Level of expression of surface receptors on tear PMNs collected at awakening from ocular allergy and healthy individuals. Results are represented as mean $\pm$ standard deviations, $n = 10$ for ocular allergy patients and healthy participants. * Significantly different, $p < 0.001$.

In response to fMLP, tear PMNs from individuals with ocular allergy showed significantly higher activation levels of degranulation markers compared to those from healthy participants ($p < 0.001$), as shown in Figure 7-4. However, as expected and previously reported, neither CD11b nor CD54 was upregulated in response to fMLP [11].

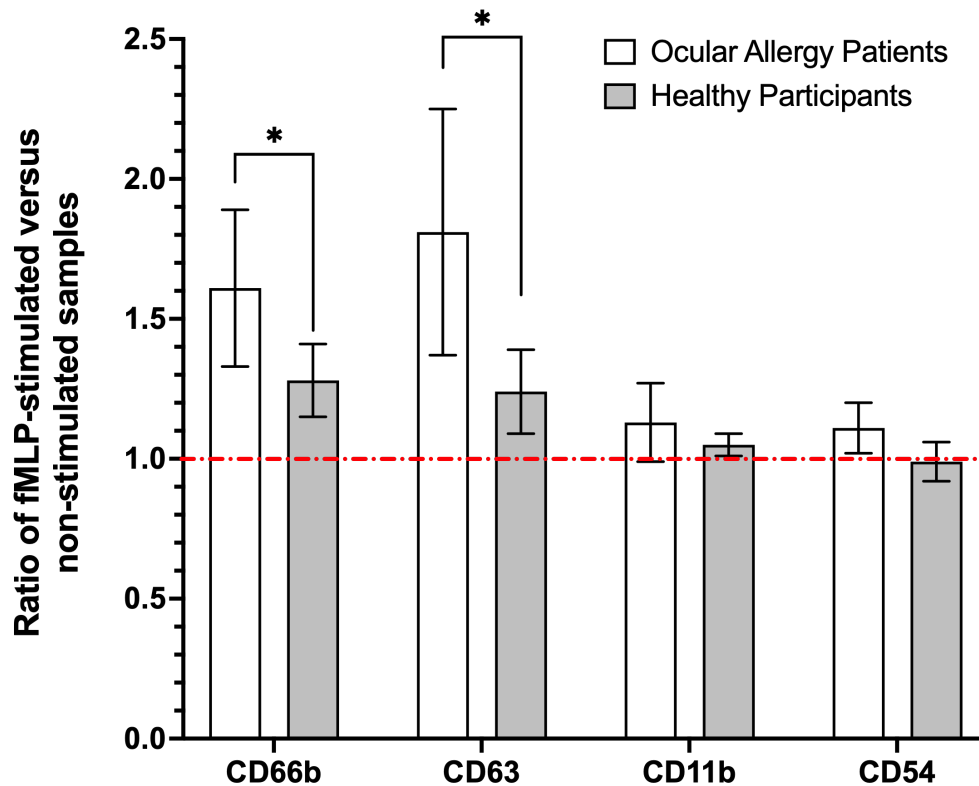


Figure 7-4. Changes in the expression of surface receptors on tear PMNs collected from ocular allergy and healthy individuals in response to fMLP. Results are represented as mean $\pm$ standard deviations, $n = 10$ for ocular allergy patients and healthy participants. * Significantly different, $p < 0.001$.

Further analysis was conducted where participants with ocular allergy were classified into three groups, mild (0-28), moderate (28-56), and severe (56-84), based on their scores obtained from the MiniRQLQ, and expression levels of CD66b, CD63, and CD184 and their activation ratio were plotted in Figure 7-5. While this analysis did not identify any statistical significance, it is interesting to note that the moderate group showed a lower baseline expression of CD66b and CD63 but a higher activation ratio of CD66b and CD63, as compared to the mild and severe groups. Additionally, the severe group showed a higher baseline expression of CD184 than the mild group, which suggests tear PMNs from individuals with severe allergy symptoms are more aged. More participants will need to be recruited to confirm these observations.

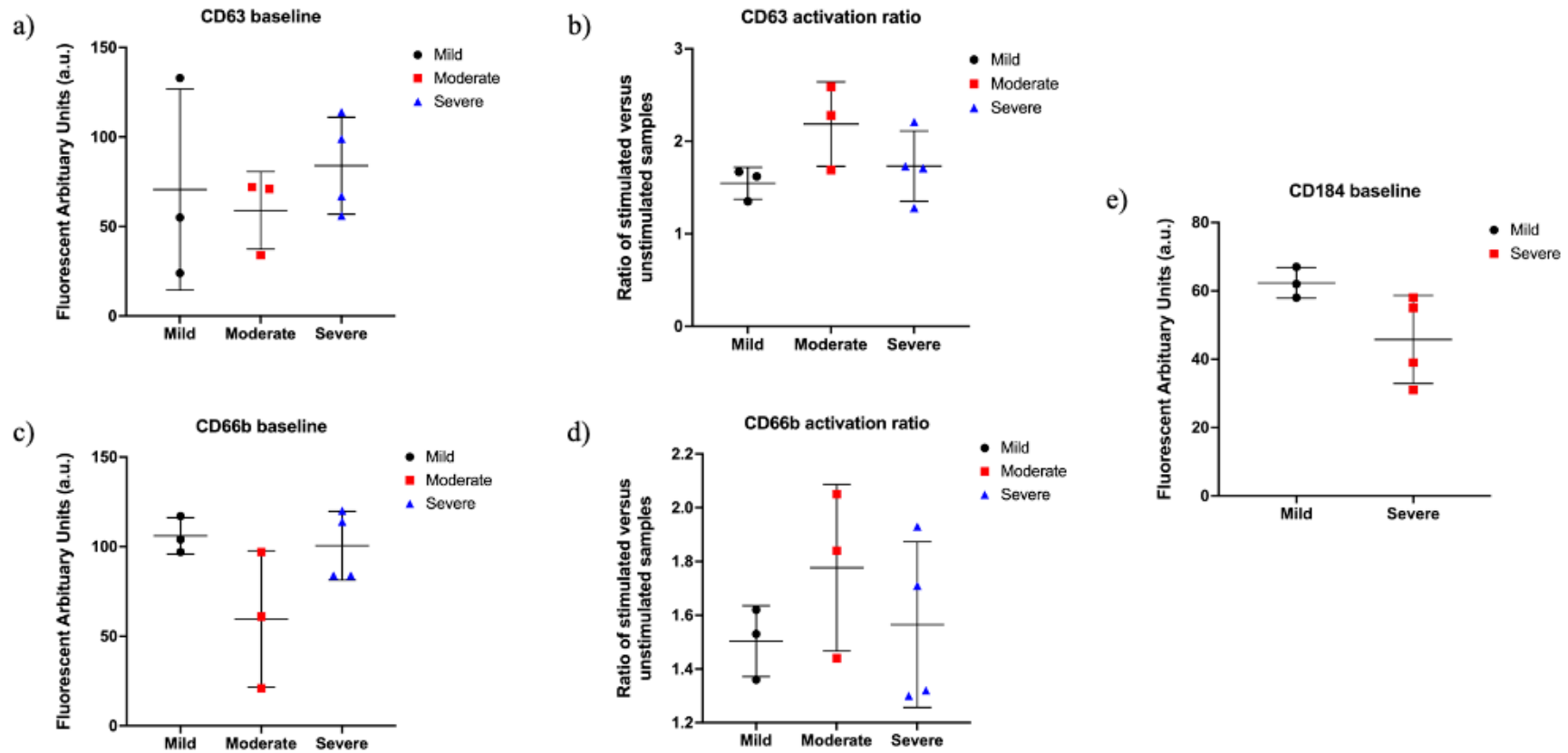


Figure 7-5. Expressions of CD66b, CD63, and CD184, and their activation ratio based on the severity of symptoms in people with ocular allergy. a) the baseline expression of CD63, b) the activation ratio of CD63, c) the baseline expression of CD66b, d) the activation ratio of CD66b, and e) the baseline expression of CD184. Results are represented as mean $\pm$ standard deviations. $n = 3$ for ocular allergy patients with mild symptoms (score: 0-28), $n = 3$ for ocular patients with moderate symptoms (score: 28-56), and $n = 4$ for ocular allergy patients with severe symptoms (score 56-84).

7.3.3 Correlation of the Activation to the Maturation State of Tear PMNs

Since tear PMNs collected from ocular allergy patients showed a less activated phenotype in comparison to healthy individuals, the maturation state of tear PMNs between these two groups was compared. The expression of CD184 on tear PMNs from ocular allergy patients was similar compared to healthy individuals, 60.6 ± 25.6 versus 62.8 ± 16.3 , respectively ($n = 8$).

Furthermore, a correlation analysis was conducted to investigate the potential relationship between the maturation and activation state of tear PMNs in ocular allergy participants. As seen in Figure 7-6, there were positive correlations between the CD184 level and CD66b expression ($r = 0.761$, $p = 0.040$) and CD63 expression ($r = 0.934$, $p = 0.006$). These results suggest that the maturation state of tear PMNs may be closely related to their activation state in patients with ocular allergy and provide further validation on the maturation and activation state that was reported in Chapter 5.

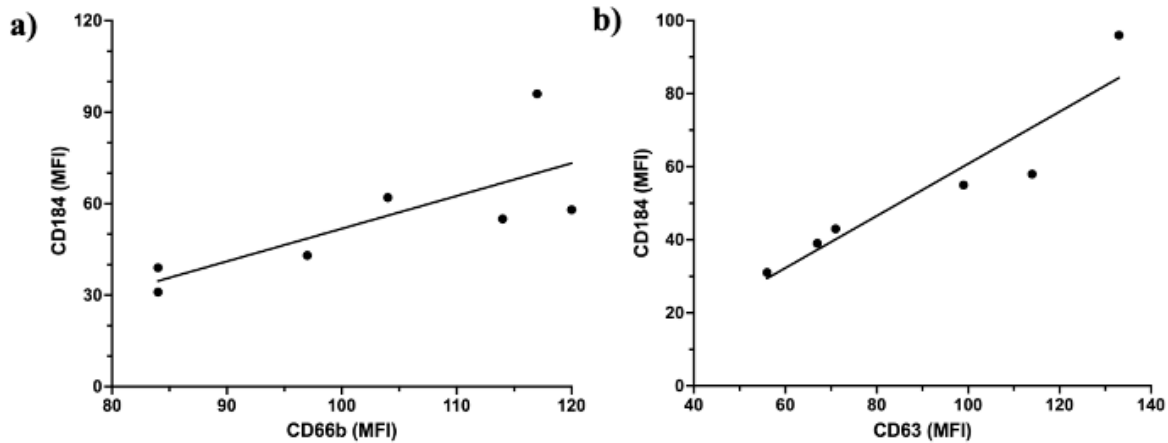


Figure 7-6. Correlations between the expression of aging and degranulation markers on tear PMNs. A) Correlation between the expression of CD66b and CD184. $r = 0.761$, $p = 0.040$. B) Correlation between the expression of CD63 and CD184. $r = 0.934$, $p = 0.006$.

7.3.4 ROS Production

The assessment of ROS production by tear PMNs was conducted on 9 participants with ocular allergy. However, it is important to note that during the experiment, the incubation medium for the first five participants was KM with phenol red, which was shown to have an impact on the fluorescence intensity [24]. Therefore, only the data points obtained from the experiments using HBSS as the incubation medium (rather than KM with phenol red) are discussed in this section ($n = 4$). Among the

four allergy participants, one participant exhibited a much higher baseline level compared to both healthy and allergy participants, although it doesn't seem to be correlating with other surface markers of activation. Additionally, the ocular allergy patients exhibited a higher activation ratio for ROS production in tear PMNs compared to healthy participants, 518 ± 168 versus 355 ± 220 , respectively, but this did not achieve statistical significance ($p = 0.285$). To further verify this observation, more participants will be needed.

7.4 Discussion

The role of eosinophils and mast cells in the pathogenesis of allergic diseases has been well studied, but based on our knowledge, there is limited data available on the potential contribution of PMNs in the development of allergic diseases, particularly in ocular disease. This study aims to fill this gap by investigating the phenotype of tear PMNs and their potential involvement in the progression and pathogenesis of ocular allergy.

The results showed a significantly higher number of tear leukocytes, and overall tear PMNs, collected from individuals with ocular allergy after sleep at awakening in comparison to those from healthy individuals. A higher number of PMNs has also been reported in the sputum of patients with asthma [25] and the blood of patients with rhinitis [26]. A significantly higher number of PMNs were also observed at 4 am in patients with nocturnal asthma compared to those whose symptoms did not worsen during the night [27]. Additionally, no significant difference in tear leukocyte number was observed during the daytime between healthy individuals and those with ocular allergy in our study. Our results may thus suggest that ocular allergy has a greater effect on recruitment of tear leukocytes in the closed-eye environment at night, but leukocyte recruitment does not increase significantly during the day.

Tear PMNs from healthy participants have been reported to express an upregulated expression of adhesion markers (CD11b, CD54), degranulation markers (CD66b and CD63), and complement activation markers (CD55 and C3aR), as well as higher constitutive production of ROS compared to blood PMNs [11–13,28], suggesting an activated phenotype of tear PMNs. Evidence from Chapter 6 indicates that tear PMNs from healthy individuals are being activated and age in the closed environment at night, which may explain their failure to further modulate their surface receptors upon stimulation [11,13,28]. However, contrary to our initial hypothesis, tear PMNs isolated from ocular allergy

participants were found to have lower levels of degranulation markers, CD66b and CD63, compared to healthy individuals. Tear PMNs from participants suffering from ocular allergy appear to be less activated and younger than those in healthy participants. The higher activation of tear PMNs from ocular allergy patients in response to fMLP corroborates this observation. While the difference in the maturity and age status (CD184) of tear PMNs between ocular allergy patients and healthy individuals did not reach statistical significance, the lower expression of CD184 on tear PMNs collected from ocular allergy participants provides also further evidence for this. In addition, the positive correlation observed between the expressions of CD184 and CD66b and CD63 may indicate that as the CD184 level increases, the expressions of CD66b and CD63 also increase, implying a potential association between the activation and maturity state of tear PMNs. Interestingly, the phenotype of tear PMNs collected upon awakening in ocular allergy patients is similar to that observed after 2 hours of sleep at night from healthy participants (Chapter 6). It then would appear that tear PMNs in ocular allergy patients exhibit a less activated and younger phenotype, which may be indicative of continuous recruitment to the ocular surface in ocular allergy patients. We had initially hypothesized that tear PMNs from individuals suffering from ocular allergies would exhibit higher levels of activation, due to a potentially more pro-inflammatory environment of the ocular surface. Postnikoff et al. and Nair et al. have previously reported higher levels of degranulation in tear PMNs collected from patients with dry eye disease [29,30]. This discrepancy in activation phenotype may be due to the different etiology between ocular allergy and dry eye disease, but further research will be needed to better understand the underlying mechanisms of the low activation of tear PMNs in ocular allergy. Furthermore, it has been shown that there are alterations in the concentration of tear proteins in ocular allergy patients, such as an increase in lactoferrin level [31,32] and a decrease in lysozyme level [31]. Given that tear proteins may have an impact on the functions of tear PMNs, as illustrated in Chapter 5, the alterations in tear protein concentrations may also exert an influence on the distinct phenotype of tear PMNs from ocular allergy patients compared to those from healthy controls.

Circulating blood PMNs isolated from asthmatic patients have been reported to show an upregulated CD11b level, which could be further elevated by fMLP stimulation *in vitro* [25]. Our study did not find any significant differences in CD11b and CD54 on tear PMNs between ocular allergy patients and healthy individuals. No significant changes were observed with fMLP. The lack of CD11b and CD54 upregulation in response to *in vitro* fMLP stimulation has been observed in prior studies with tear PMNs from healthy eyes [11]. Thus, our results with tear PMNs from ocular allergy participants

would further suggest that CD11b and CD54 expression levels relate more to the adhesion and transmigration process into the ocular environment [33,34], and may provide limited information in relation to activation level and potential for activation in tear PMNs studies. The differences in level of activation and response to fMLP that one may observe with circulating PMNs such as seen in [25] highlights the importance of using the appropriate PMN population when studying ocular complications, blood PMNs that have not extravasated into the tissue may still maintain the capability to upregulate CD11b, and thus response would not appropriately mimic what is observed in the ocular surface environment.

Mast cells are abundant in the peripheral cornea, limbus, and conjunctiva [35]. The allergic response is usually initiated by IgE binding to mast cells, which leads to the release of histamines and other proinflammatory mediators, causing the recruitment of immune cells to the site. The process of subsequent infiltration of immune cells is called the late-phase reaction (LPR). Therefore, many therapeutical strategies are focused on preventing the activation of mast cells or suppressing their release of proinflammatory mediators to minimize the exaggeration of the hyperreactive allergic responses. However, the presence of PMNs during the allergic LPR has been associated with more severe inflammation [6], as the cytokines and chemokines released by PMNs may attract other leukocytes to the sites and intensify the inflammatory response. This study classified participants into three groups based on their MiniRQLQs scores, and results revealed that tear PMNs may have a greater impact in patients with moderate ocular allergy compared to those with mild and severe ocular allergy. This contradicts the findings of PMNs in asthma patients, suggesting that other immune cells may play a more significant role in severe ocular allergy cases. It is possible that tear PMNs recruit or activate other potent immune cells, such as B cells and T cells, in severe allergy cases. However, it is important to note that the eligibility of participants for the study relied solely on self-reported symptoms, which may not accurately reflect the severity of ocular allergy. Therefore, it is necessary to confirm the presence and severity of ocular allergy by analyzing the concentration of pro-inflammatory mediators in tears, such as eotaxin, IL-4, IL-6 and IL-5 [36], which could provide a more reliable method of classifying patients based on the severity of their symptoms.

PMNs were reported to produce higher levels of ROS in atopic patients compared to nonatopic individuals, both with and without stimuli, such as fMLP, calcium ionophore, PMA, and zymosan [37]. Additionally, the level of ROS generated by PMNs can vary depending on the type of stimulus used. For example, platelet-activating factor (PAF) cannot induce a higher ROS release in allergic patients

compared to healthy participants [37]. However, the findings of our study show that there was no significant difference in unstimulated ROS production by tear PMNs between ocular allergy and healthy individuals. Based on the observation that one participant with severe ocular allergy expressed a high level of unstimulated ROS compared to the other two participants, it is possible that oxidative burst plays a role in the etiology of severe ocular allergy but not in moderate and mild cases. Given the anti-inflammatory effect of tear proteins presented in Chapter 5, it is possible that tear proteins, such as lactoferrin, lysozyme, and mucin, in the tear film may suppress the ROS release of tear PMNs in mild and moderate ocular allergy. However, this impact may be dysregulated in severe ocular allergy where there may be a higher pro-inflammatory environment and response. Further studies with larger sample sizes are needed to verify this observation.

Interestingly, a preliminary study recently performed in our lab revealed a difference in the expression and activation of lactoferrin between ocular allergy patients and healthy participants ($n = 3$). Specifically, the baseline level of lactoferrin on tear PMNs from allergy patients was lower than that in healthy individuals (46 ± 39 versus 309 ± 185), while the activation ratio of lactoferrin was higher in the former group (1.7 ± 0.1 versus 1.2 ± 0.2). These results show a similar trend with degranulation markers, CD66b and CD63, which is expected as lactoferrin is stored in the specific granules of PMNs and aligns with younger maturation state and low activated state. It has been shown that PMNs can release lactoferrin through an IgE-dependent mechanism, and the release of lactoferrin by PMNs was reported to be increased in patients with asthma [25]. The inconsistency may further highlight the difference between the ocular environment and other systemic locations.

It is interesting to observe the less activated phenotype of tear PMNs collected from ocular allergy participants compared to healthy participants, and the underlying mechanism leading to this phenotype remains poorly understood. Since tear PMNs from ocular allergy participants are less activated, it is possible that they may not be as functional as those collected from healthy participants. This may indicate that they cannot clean the ocular surface effectively, allowing pro-inflammatory foreign bodies to remain on the ocular surface and activate ocular surface cells and worsening symptoms. On the other hand, while lower expression of degranulation markers was observed on tear PMNs from participants suffering from ocular allergy at 7 hours relative to healthy participants, it was also observed that they were less mature and thus their initial (“resting”) level of expression of CD66b and CD63 may be much lower than that observed under healthy conditions. Degranulation and a pro-inflammatory response may thus still have occurred in the closed-eye environment. Further studies will

be needed to determine if the low level of degranulation markers is due to low activation or due to a lower initial resting expression.

7.5 Conclusion

The differences in the baseline expression of degranulation markers and activation potential levels may indicate a more readily activated phenotype of tear PMNs in ocular allergy patients compared to healthy participants, which suggests that these tear PMNs may not be able to contribute properly to ocular homeostasis and consequently may play a role in the development of symptoms. While our study highlighted differences in tear PMNs, only participants suffering from seasonal ocular allergy were recruited and the diagnosis of ocular allergy was based on self-reported symptoms. In the future, more participants recruited with a more objective measure/diagnosis of ocular allergy will allow us to further confirm the current findings, gain a deeper understanding of ocular inflammation, and may provide insights into the development of new therapeutical strategies.

Chapter 8. Conclusion and Future Work

8.1 Conclusions

Neutrophils have been considered a heterogeneous leukocyte population with critical functions in pathogen defence as well as the potential to contribute to the development of diseases. The significant influx of PMNs to the ocular surface during the night may further support the heterogeneity of the PMN population and also highlight their role in maintaining ocular health and homeostasis. The anterior ocular environment as the outermost compartment of the eye constantly exposed to microbes and debris may possess its own protective mechanisms against pathogens and partially suppress the functions of tear PMNs. However, there is limited research on the functionality of tear PMNs on the ocular surface and their interaction with the ocular environment. Therefore, my Ph.D. thesis focused on three aspects: 1) to investigate the activation and maturation state of tear PMNs *in vitro*; 2) to study the anti-inflammatory effect of tear proteins on tear PMNs; and 3) to examine the phenotype of tear PMNs in participants with ocular allergy.

In Chapter 4, we verified our hypotheses that tear PMNs were the main contributors to ROS production in the eyewash, produced ROS in a stimulus-dependent manner, and that the isolation process affected ROS production in tear PMNs. We demonstrated that PMA can trigger a prolonged ROS release in tear PMNs, while fMLP and LPS failed to induce oxidative burst in tear PMNs, showing a stimulus-dependent ROS production by tear PMNs. Furthermore, tear PMNs spontaneously released ROS in the absence of stimuli, which suggested that tear PMNs collected after sleep may be aged and which was later verified in the subsequent study. The use of magnetic beads for isolating/purifying tear PMNs resulted in a poor yield and the isolated tear PMNs produced higher levels of ROS as well as had upregulated levels of surface markers associated with activation when compared to non-isolated tear PMNs, highlighting the negative impacts of the isolation process.

The hypothesis for Chapter 5 was that the presence of tear proteins, especially lactoferrin and lysozyme, would reduce ROS production in tear PMNs. Our findings demonstrated that the presence of tear proteins, lactoferrin, lysozyme, mucin, albumin, and IgG, significantly inhibited the ROS production by tear PMNs, as measured by two methods, luminol-enhanced chemiluminescence and flow cytometry with DCFH-DA. Additionally, our results suggested that lactoferrin and lysozyme may have a greater impact on extracellular ROS release as opposed to intracellular ROS production, although the underlying mechanism needs to be further examined in future studies.

In Chapter 6, we aimed to verify three hypotheses: 1) Short-time eye closure results in lower degranulation than prolonged eye closure, 2) Eye closure during the day (i.e., a 2hr nap) results in leukocyte accumulation in the tear film, 3) The number of tear PMNs collected correlates to the concentration of chemoattractants (IL-8 and C5a). Our results revealed that tear PMNs collected after a full night of sleep exhibited higher levels of degranulation, NETs, and aging markers compared to tear PMNs collected after 2h of sleep at night, indicating that tear PMNs become more activated with increasing eye closure time at night. In addition, there was a significant difference in cell count between the 2hr eye closure during the day and at night, revealing that leukocytes did not accumulate in the closed eye during the day and providing evidence that tear PMNs may originate from the blood vessels rather than the lacrimal gland. Furthermore, compared to the daytime collections, there were significantly higher concentrations of cells, IL-8, and C5a obtained from nighttime collections, and a positive correlation between IL-8 and leukocyte number was observed. A nocturnal-diurnal pattern in leukocyte recruitment to the ocular surface, potentially driven by IL-8, was identified.

In Chapter 7, we assessed the phenotype of tear PMNs in participants with ocular allergy. Two hypotheses had been proposed: 1) Tear PMNs collected from individuals suffering from ocular allergy are more activated than tear PMNs collected from healthy eyes, and 2) higher number of tear PMNs are present on the ocular surface of allergy participants compared to non-allergy participants. While the second hypothesis was verified as significantly more tear PMNs were collected from ocular allergy participants compared to healthy individuals, tear PMNs from ocular allergy participants showed a significantly lower expression of degranulation and aging markers, suggesting that these cells were younger and less activated compared to tear PMNs collected from healthy eyes. Further studies are needed to gain a better understanding of the low activation level as well as a larger sample size, with an objective method to confirm ocular allergy, will be required to confirm the current findings.

All in all, this Ph.D. thesis contributed significant new knowledge on the phenotype and potential roles of tear PMNs in healthy and inflammatory conditions. The mechanism of tear PMNs in a normal/healthy physiological ocular environment is depicted in Figure 8-1 and characteristics of its phenotype identified in this thesis are illustrated in Figure 8-2.

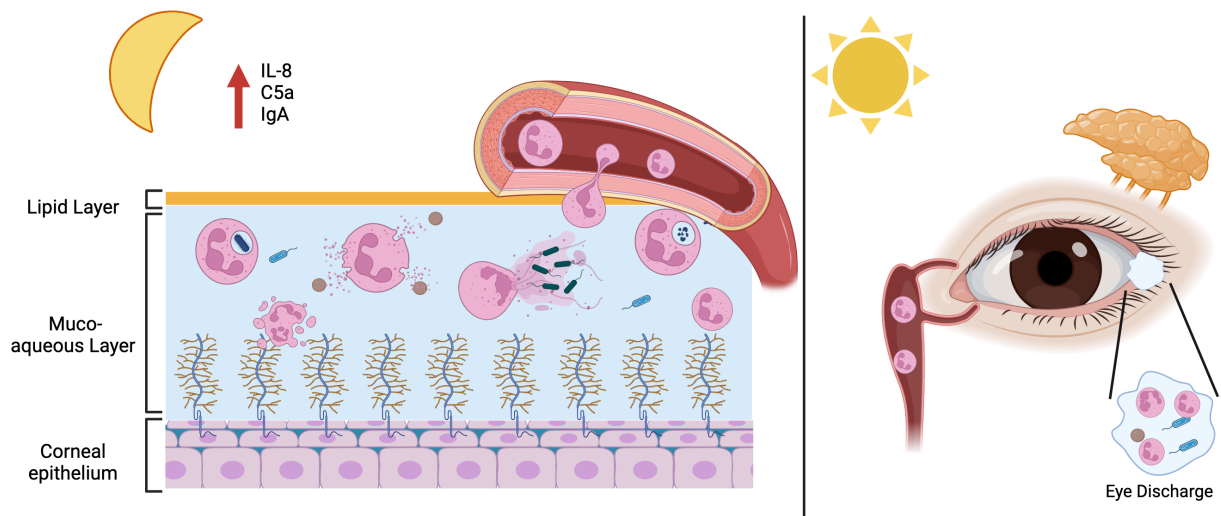


Figure 8-1. The kinetics and mechanisms of tear PMNs in the closed-eye environment. Created from BioRender.

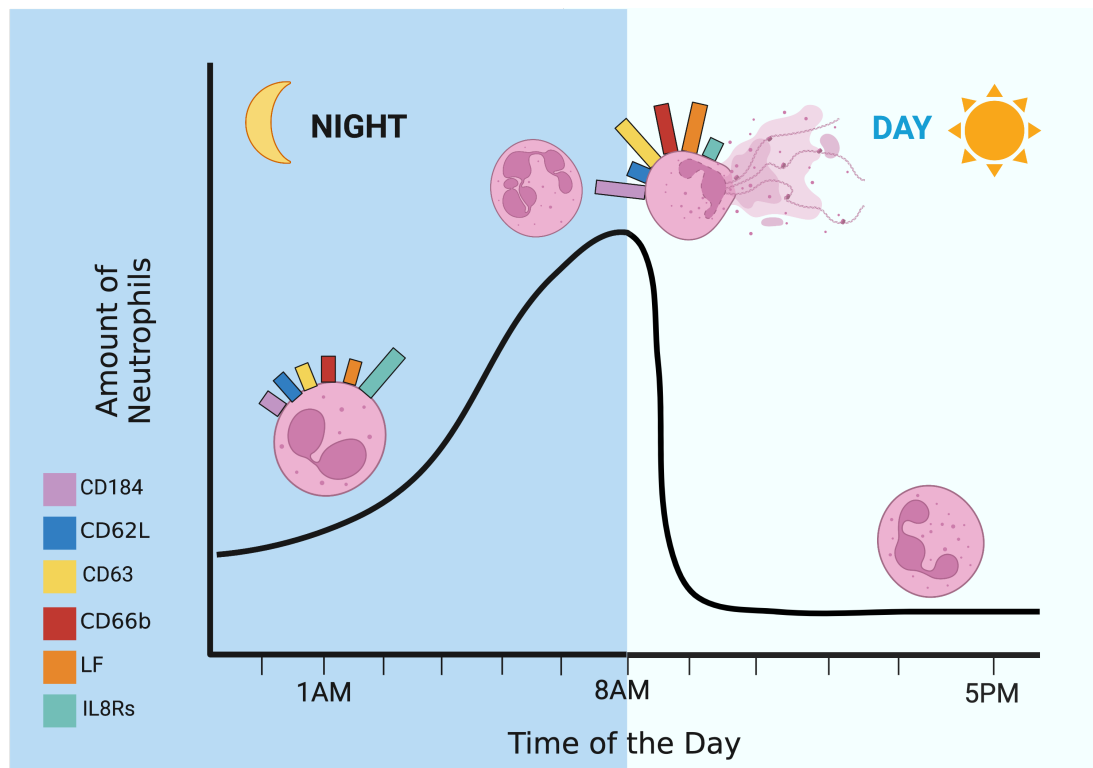


Figure 8-2. Changes in the phenotype of tear PMNs across the day. Created from BioRender.

Tear PMNs transmigrate from blood vessels to the ocular surface at night, in response to pro-inflammatory mediators and chemoattractants, including IL-8. The presence of accumulated microbes and debris can further activate tear PMNs, along with pro-inflammatory mediators. Tear proteins may play an important role in suppressing/dampening the oxidative burst of tear PMNs, thus preventing any unexpected immune-mediated damage to the ocular surface structures. In addition, tear proteins may help in delaying apoptosis of tear PMNs, which may further circumvent any unwanted inflammatory responses caused by apoptosis, given the limited number of macrophages in tears. Tear PMNs then age while residing in the closed-eye ocular environment rather than undergoing apoptosis. Tear proteins may also have an anti-inflammatory role on tear PMNs, leading to a para-inflammatory environment under closed-eye healthy conditions at night. In contrast, when acute inflammation is present, such as during ocular allergy, young tear PMNs may be recruited to the ocular environment constantly, and the anti-inflammatory effect of tear proteins may not be strong enough to resolve inflammation, potentially leading to exacerbation of the inflammatory response and worsening of symptoms. Overall, this thesis provides valuable insights into the dynamic interplay between tear PMNs and the ocular environment and sheds some light on ocular immunity, which may help in the development of novel therapeutical strategies for ocular complications.

8.2 Future Work

There are still many unknowns about the underlying mechanisms of tear PMNs, so future studies will be needed to gain a better understanding of their role in ocular homeostasis and inflammation.

8.2.1 The Crosstalk between the Ocular Epithelial Cells and Tear PMNs

In Chapter 5, we demonstrated that tear proteins may have the capability of suppressing the oxidative burst of tear PMNs, potentially alleviating oxidative stress in the ocular environment. However, there might be a crosstalk or feedback loop between the ocular epithelial cells and tear PMNs to further regulate the recruitment and activation of tear PMNs. Damaged or infected epithelial cells release damage-associated molecular patterns (DAMPs) to attract PMNs to the sites, inducing the release of cytokines, such as IL-8, to further recruit and activate PMNs. However, research showed that

inflamed or damaged epithelial cells may also express IL-10, an anti-inflammatory cytokine that can help to resolve inflammation [2]. An in vitro model whereby tear PMNs and ocular surface cells are incubated would provide valuable information on the crosstalk between these cells. For example, 2hr night tear PMNs could be incubated with ocular epithelial cells and lipopolysaccharides (LPS) for 5 hours and their phenotype and ROS production could be characterized in comparison to the control (tear PMNs without ocular epithelial cells).

8.2.2 Detection of IL-8 Secretion by Tear PMNs Using ELISpot

In Chapter 6, we identified a positive correlation between IL-8 concentration and PMN numbers. However, since PMNs have the capability to secrete IL-8, it is difficult to determine if higher IL-8 concentrations lead to an increase in PMN numbers, or if the presence of more tear PMNs in the closed-eye environment causes an elevated IL-8 concentration. To gain further insight into this observation, it may be interesting to examine whether tear PMNs collected after a full night of sleep are able to release IL-8. Enzyme-linked immunospot (ELISpot) assay could be used to accurately quantify the number of cells that secrete IL-8 both under unstimulated and stimulated conditions using two stimuli, fMLP and LPS [1].

8.2.3 Comparing Cytokine Concentrations in Tears and Eyewash

In Chapter 6, we measured the concentrations of IL-8 and C5a in the eyewash, which shed light on the underlying mechanisms of the transmigration of tear PMNs as well as the protective function of the ocular environment. However, due to the method of obtaining cytokine concentrations from the eyewash, it is difficult to compare with published articles that have detected cytokine concentrations in tears. Thus, it would be valuable to compare cytokine concentration in tears by using capillary tubes and eyewash collection from human participants to establish a method to correct or convert cytokine concentrations between eyewash and tears.

8.2.4 Circadian genes in tear PMNs

In Chapter 6, we reported that tear PMNs showed a circadian infiltration pattern in the closed-eye environment, with peak levels observed in the early morning and trough levels during the daytime. This circadian rhythm has also been found in PMNs at other locations of the body as well as in other immune cells, as discussed in Chapter 2. Despite there being controversy around the factors driving this circadian infiltration, it would be interesting to investigate if it is systemically driven or genetically driven. To explore this, DNA from tear PMNs can be extracted to determine if they expressed circadian genes, such as *clock*, *per2*, *bmal1*, and *per1*. Furthermore, extracting RNA from tear PMNs that are collected after 2 hours of sleep and a full night of sleep would allow to test the expression of the circadian genes and track the dynamics of cell activities over time. This will enable us to examine the differences between cells collected at different times at the transcriptional level and gain further insights into more reliable and appropriate therapeutical targets for ocular complications.

8.2.5 Comparing the Phenotype of PMNs in Blood and Tears from individuals with Ocular Allergy

In Chapter 7, we assessed the phenotype of tear PMNs in participants with ocular allergy and compared it with that of healthy individuals. We found tear PMNs from ocular allergy patients showed a less activated phenotype compared to healthy individuals. However, this finding contradicted the results of previous studies that examined sputum and blood PMNs collected from patients with asthma [3,4] as well as studies from chronic ocular diseases such as dry-eye and keratoconus [5,6]. Therefore, it would be recommended to investigate differences in phenotypes between circulating blood PMNs and tear PMNs from ocular allergy participants to gain a better understanding of ocular immunity. Additionally, it may help to determine if the ocular allergy is a localized response (in the eye) or part of the systemic immune response.

Furthermore, it is necessary to conduct experiments on tear PMNs collected after 2 hours of sleep at night from ocular allergy participants as an initial/baseline point. This data point can be used to compare with the phenotype of blood PMNs and 7hr tear PMNs. This will allow us to gain a better understanding of the phenotype of tear PMNs in ocular allergy and whether they become more activated while in the closed-eye environment.

8.2.6 Examine the Phenotype of Tear PMNs in People with Diabetic Retinopathy

While tear immune cells are being studied in dry-eye and keratoconus, other diseases such as diabetic retinopathy have not yet been considered. Research studies investigating the phenotype of immune cells in diabetic retinopathy have utilized either mice models or circulating blood PMNs from patients. As a next step, it would be valuable to examine the phenotype of tear PMNs in individuals with diabetic retinopathy to determine if there is a crosstalk between the front and the back of the eye. Given that the eye is highly innervated by nerves, it is plausible that there may be communication between the anterior and posterior segments of the eye. If this is the case, it could represent a new avenue for non-invasive and simple diagnosis or monitoring of disease progression in the posterior segment of the eye.

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Appendix Tables and Figures

Chapter 5

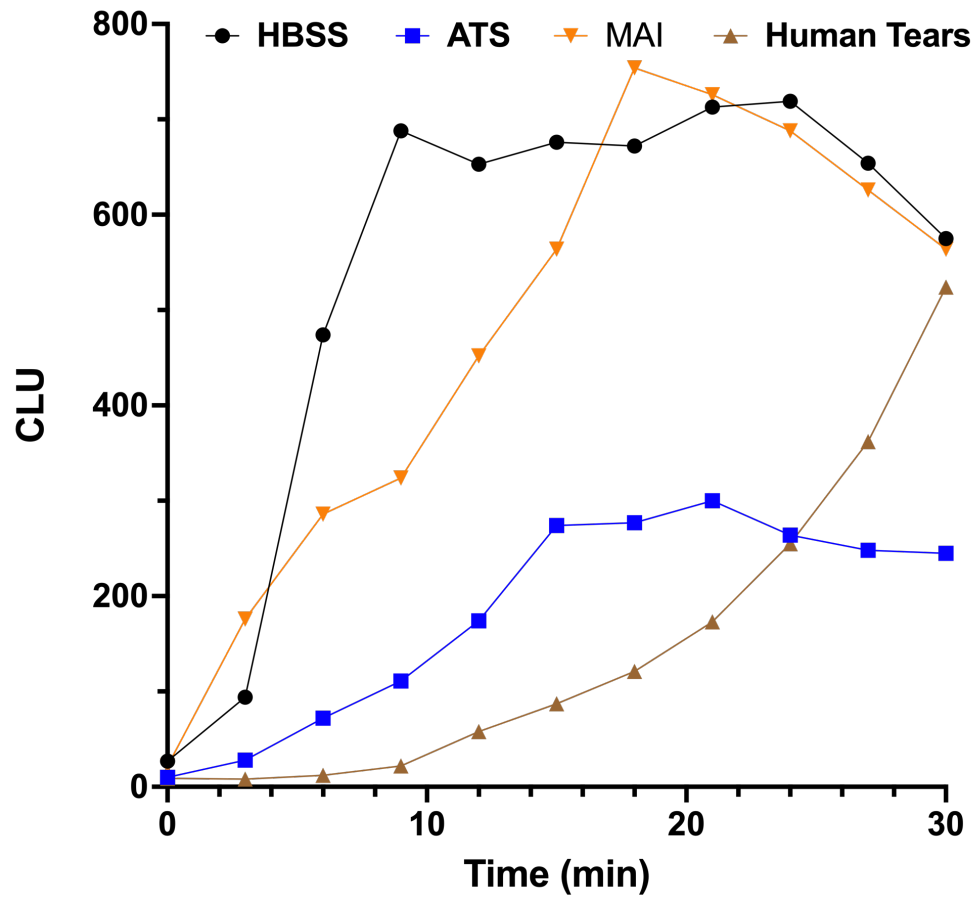


Figure 1. The chemiluminescence of PMA-stimulated ROS production by tear PMNs incubated in HBSS, ATS, MAI, and human tears. The presented results were from one participant and were obtained on the same day.

Chapter 6

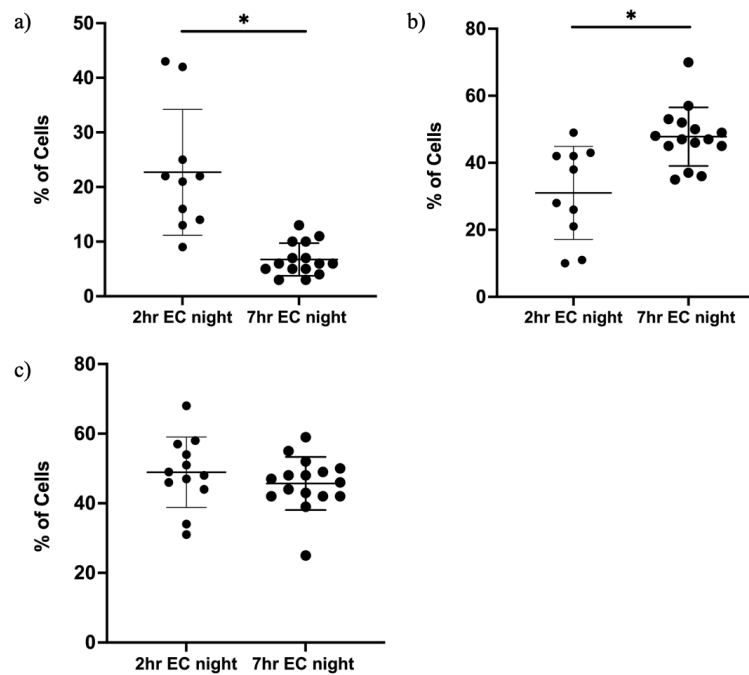


Figure 2. The percentages of a) hyposegmented, b) hypersegmented, and c) three lobed nuclei tear PMNs collected after 2hr and 7hr eye closures at night.

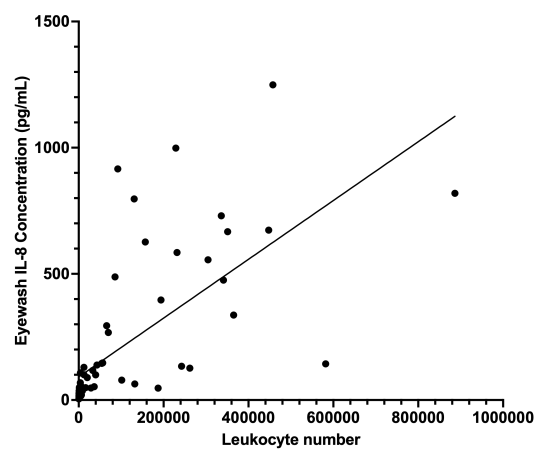


Figure 3. Correlation between eyewash IL-8 concentration and leukocyte number. $r = 0.675$, $p = 0.001$.

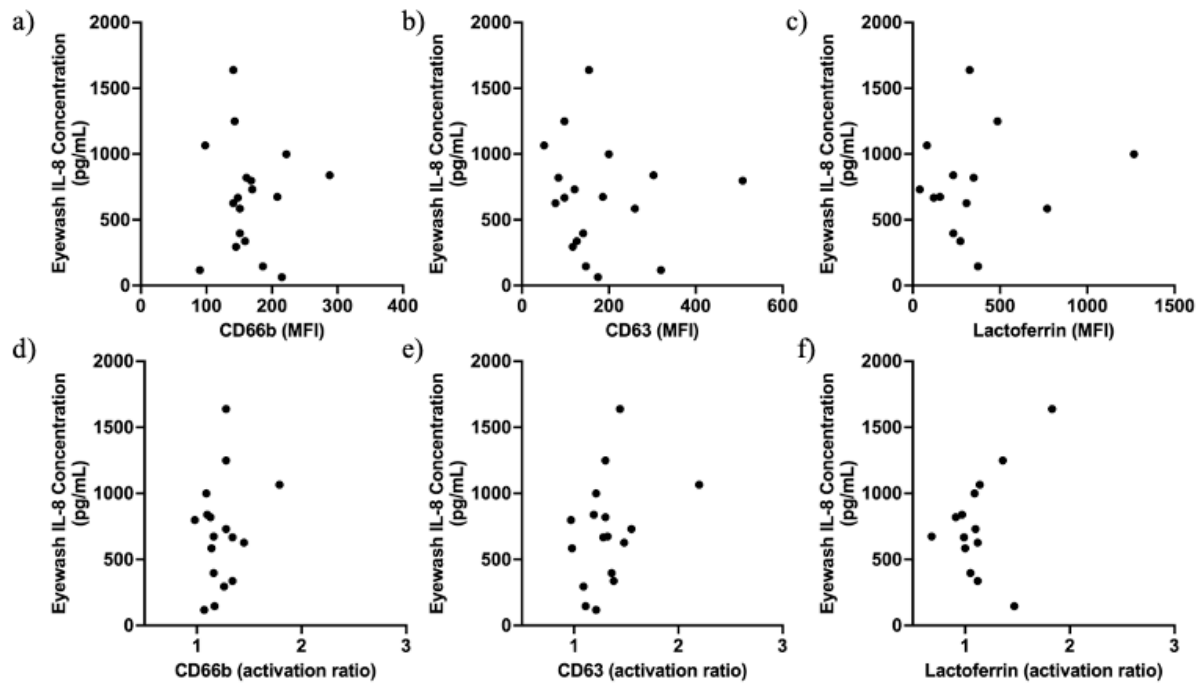


Figure 4. Correlations between IL-8 concentrations and a-c) baseline expressions of surface receptors and d-f) activation ratios on 7hr EC night tear PMNs. MFI: mean fluorescent intensity.

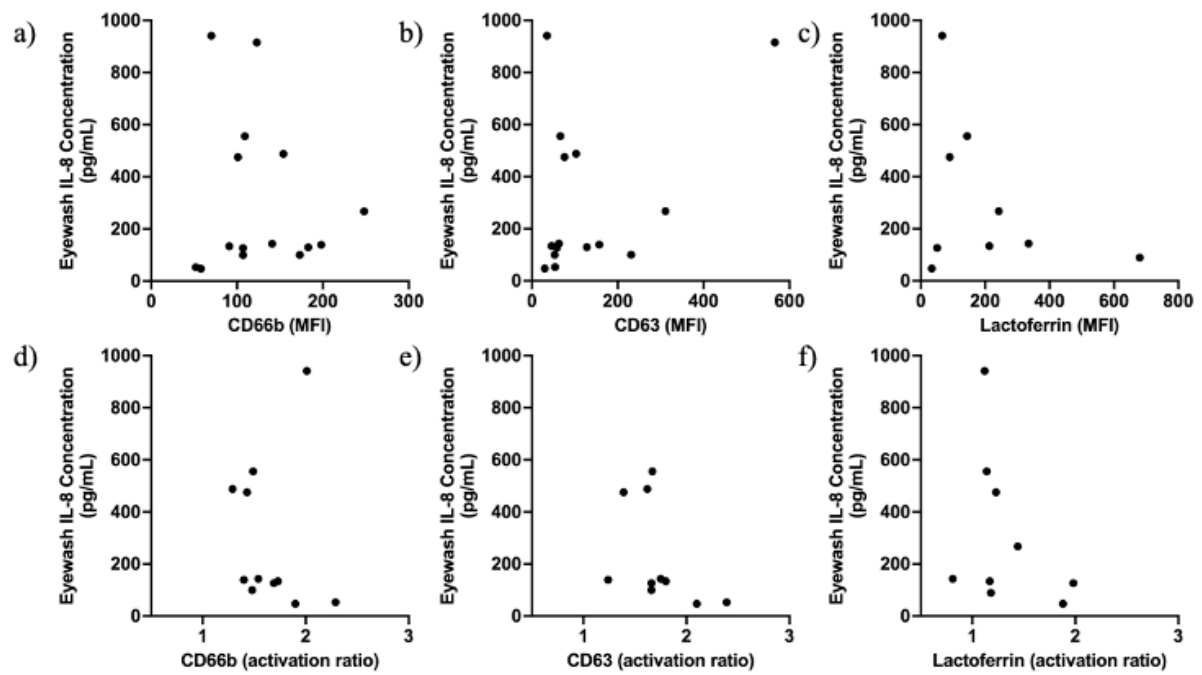


Figure 5. Correlations between IL-8 concentrations and a-c) baseline expressions of surface receptors and d-f) activation ratios on 2hr EC night tear PMNs. MFI: mean fluorescent intensity.

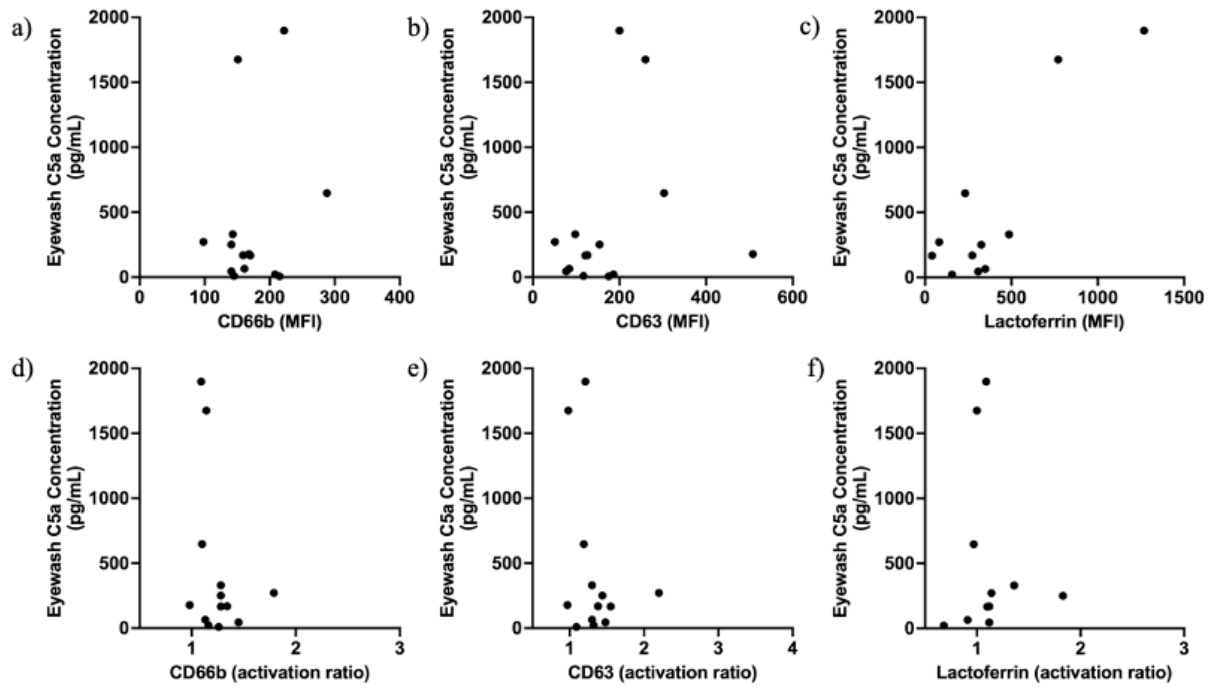


Figure 6. Correlations between C5a concentrations and a-c) baseline expressions of surface receptors and d-f) activation ratios on 7hr EC night tear PMNs. MFI: mean fluorescent intensity.

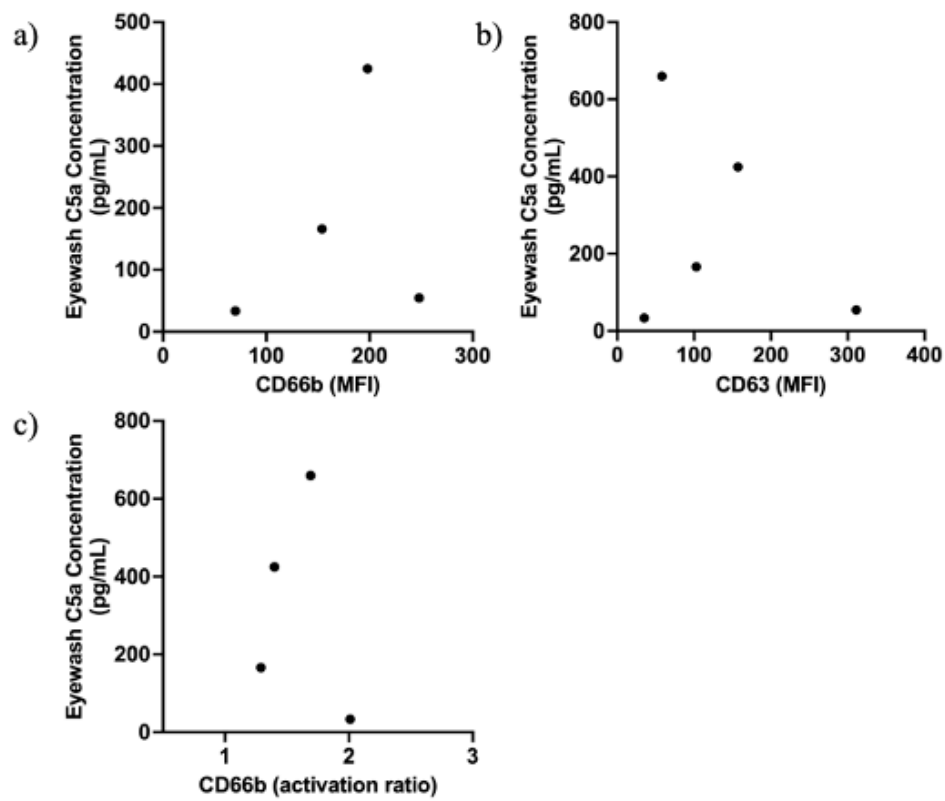


Figure 7. Correlations between C5a concentrations and a-c) baseline expressions of surface receptors and d-f) activation ratios on 2hr EC night tear PMNs. MFI: mean fluorescent intensity.

Table 1. The mean fluorescent values of lactoferrin expression on blood PMNs and 7hr EC and 2hr EC night tear PMNs.

	Unstimulated	fMLP stimulated	Ratio of stimulated VS unstimulated
Blood PMNs	40.8 ± 22.6	90.2 ± 66.2	2.2 ± 0.5
7hr EC night tear PMNs	285.5 ± 107.5	323.7 ± 199.6	1.2 ± 0.3
2hr EC night tear PMNs	120.0 ± 81.7	172.9 ± 105.0	1.4 ± 0.4

N= 5 for blood PMNs, n = 11 for 7hr EC night tear PMNs, and n = 8 for 2hr EC night tear PMNs.

Chapter 7

Appendix 1 Mini Rhinoconjunctivitis Quality of Life Questionnaire (MiniRQLQ)©

Please complete all questions by circling the number that best describes how troubled you have been during the last week as a result of your nose/eye symptoms.

	Not troubled	Hardly troubled at all	Somewhat troubled	Moderately troubled	Quite a bit troubled	Very troubled	Extremely troubled
<i>Activities</i>							
1. Regular activities at home and at work (your occupation or tasks that you have to do regularly around your home and/or garden)	0	1	2	3	4	5	6
2. Recreational activities (indoor and outdoor activities with friends and family, sports, social activities, hobbies)	0	1	2	3	4	5	6
3. Sleep (difficulties getting a good night's sleep and/or getting to sleep at night)	0	1	2	3	4	5	6
<i>Practical problems</i>							
4. Need to rub nose/eyes	0	1	2	3	4	5	6
5. Need to blow nose repeatedly	0	1	2	3	4	5	6
<i>Nose symptoms</i>							
6. Sneezing	0	1	2	3	4	5	6
7. Stuffy blocked nose	0	1	2	3	4	5	6
8. Runny nose	0	1	2	3	4	5	6
<i>Eye symptoms</i>							
9. Itchy eyes	0	1	2	3	4	5	6
10. Sore eyes	0	1	2	3	4	5	6
11. Watery eyes	0	1	2	3	4	5	6
<i>Other symptoms</i>							
12. Tiredness and/or fatigue	0	1	2	3	4	5	6
13. Thirst	0	1	2	3	4	5	6
14. Feeling irritable	0	1	2	3	4	5	6

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Figure 8. Mini Rhinoconjunctivitis Quality of Life Questionnaire (MiniRQLQ).