

Neutrophil and T-Cell Homeostasis in the Closed Eye

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PURPOSE. This study sought to examine the changes and phenotype of the tear neutrophil and T-cell populations between early eyelid closure and after a full night of sleep.

METHODS. Fourteen healthy participants were recruited and trained to wash the ocular surface with PBS for at-home self-collection of ocular surface and tear leukocytes following up to 1 hour of sleep and a full night of sleep (average 7 hours), on separate days. Cells were isolated, counted, and incubated with fluorescently labeled antibodies to identify neutrophils, monocytes, and T cells. For neutrophil analysis, samples were stimulated with lipopolysaccharide (LPS) or calcium ionophore (CaI) before antibody incubation. Flow cytometry was performed.

RESULTS. Following up to 1 hour of sleep, numerous leukocytes were collected ($2.6 \times 10^5 \pm 3.0 \times 10^5$ cells), although significantly ($P < 0.005$) more accumulated with 7 hours of sleep ($9.9 \times 10^5 \pm 1.2 \times 10^6$ cells). Neutrophils (65%), T cells (3%), and monocytes (1%) were identified as part of the closed eye leukocyte infiltration following 7 hours of sleep. Th17 cells represented 22% of the total CD4⁺ population at the 7-hour time point. Neutrophil phenotype changed with increasing sleep, with a downregulation of membrane receptors CD16, CD11b, CD14, and CD15, indicating a loss in the phagocytic capability of neutrophils.

CONCLUSIONS. Neutrophils begin accumulating in the closed eye conjunctival sac much earlier than previously demonstrated. The closed eye tears are also populated with T cells, including a subset of Th17 cells. The closed eye environment is more inflammatory than previously thought and is relevant to understanding ocular homeostasis.

Keywords: neutrophils, flow cytometry, T cells, closed eye, sleep

Eyelid closure is essential for maintenance of both ocular and diurnal homeostasis. At the level of the retina, eyelid closure is essential for circadian rhythm regulation through melatonin production.¹ Eyelid closure during sleep results in increases in proinflammatory cytokines, complement activation products, and matrix metalloproteinases in the tears within the conjunctival sac.² An additional hallmark of closed eye tears is the influx of hundreds of thousands of neutrophils onto the ocular surface and into the conjunctival sac, and prior studies have examined the role of these neutrophils and demonstrated that they have an aberrant phenotype compared with regular, blood-isolated neutrophils.³ Notably, the neutrophils of the closed eye are more activated, meaning that they have upregulated many of the cell surface receptors corresponding to inflammation, yet remain largely quiescent or nonresponsive when presented with an inflammatory stimulus. This implies that closed eye neutrophils have been primed and/or activated by a prior stimulus, and that they may have undergone some degranulation, as an example, but do not continue to degranulate on further stimulation. The present investigation sought to determine if neutrophil phenotype was consistent throughout eyelid closure, and thus required revisiting prior work to determine the time course for neutrophil recruitment to the ocular surface.

In early studies of tear neutrophils, the number of neutrophils reported from the closed eye was approximately 2000 to 8000 cells.^{4,5} Using our technique of adding additional volume with the ocular surface wash, we previously reported

that there are closer to 350,000 leukocytes recovered after a full night of sleep.³ Tan et al.⁴ performed a time course study on neutrophil recruitment in the closed eye, and observed that neutrophils were recruited in very low numbers (tens) between 0 and 3 hours of sleep, and only reached thousands of cells following 5 hours of sleep. Preliminary analyses performed with our improvements in cell collection demonstrated that up to 500,000 leukocytes could be recovered as early as 1 hour after eyelid closure.⁶ Given the quiescent, yet activated, nature of closed eye tear neutrophils following a full night (average 7 hours) of sleep, the goal of this project was to determine if neutrophil phenotype changes with increasing/prolonged eyelid closure, and thus we compared cellular phenotype following 1 and 7 hours of sleep.

Neutrophil recruitment is hypothesized to be driven by either trapped bacteria or lipopolysaccharide (LPS) in the closed eye tears, homeostatic mechanisms, or neurogenic mechanisms (potentially related to sleep).² Recruitment of neutrophils also may be coordinated through the actions of T cells, specifically IL-17 producing T-helper cells (Th17 cells).^{7,8} Specifically, IL-17 is known to stimulate epithelial cells to produce inflammatory mediators such as IL-8 (CXCL8), which in turn leads to recruitment of neutrophils.⁷ The presence of T cells in the closed eye leukocyte accumulation has yet to be elucidated, but T cells have been previously observed in open eye human tears,^{9–11} and this population of T cells has been shown to increase in vernal conjunctivitis,¹¹ allergic conjunctivitis,¹⁰ and atopic keratoconjunctivitis.⁹ It is



also known that CD3⁺ T cells populate the palpebral conjunctiva in the conjunctival-associated lymphoid tissue (CALT).^{12,13} This CALT region is proposed to play a pivotal role in closed eye regulation, as the overlying lid is primarily in contact with the lymphocyte-deficient cornea.¹³ There is also a small population of T cells and Th17 cells in bulbar conjunctiva,¹⁴⁻¹⁶ and biopsies of the bulbar conjunctiva show increased T-cell presence in dry eye disease and Sjögren's syndrome.^{16,17} Further, T cells, and specifically Th17 cells, are considered to be involved in dry eye pathogenesis at the ocular surface,^{13,14} which further highlights the importance of these cells in ocular surface immune regulation. Altogether, it is hypothesized that T cells, including Th17 cells, play a role in the closed eye leukocyte infiltration.

With the development of ocular therapeutics that focus on combatting inflammation,¹⁸ it is imperative to understand the role that closed eye leukocytes play in ocular surface homeostasis, given their presence in tears. The purpose of this investigation was 2-fold: to determine phenotypic and population changes in the closed eye neutrophil population, and to determine the relative presence of T cells in the closed eye leukocyte mélange, following 1 and 7 hours of sleep in a cohort of healthy human subjects.

METHODS

Subjects

The study was conducted in accordance with the tenets of the Declaration of Helsinki and received ethics clearance from the University of Alabama at Birmingham Institutional Review Board. A total of 14 healthy subjects were enrolled, with an average age of 34 ± 9 (range, 25–57), and subjects were evenly split male versus female.

Cell Collection

All participants were trained using a previously established method³ to self-collect their closed eye ocular surface and tear leukocytes using a polyethylene pipette containing sterile PBS. Briefly, on eye opening, the leukocytes are irrigated from the ocular surface and conjunctival sac, and include the resident tear film. This population of leukocytes is hereafter referred to as “tear leukocytes.”

Subjects collected tears following either up to 1 hour of sleep, or after a full night of sleep (7.33 ± 0.91 hours, range 5–9 hours). For simplicity, the former is referred to as the 1-hour time point and the latter is hereafter referred to as the 7-hour time point. An important caveat for the 1-hour time point is that there is no exact measure of length of sleep, although all subjects self-reported that they had fallen asleep by the time the alarm went off for the 1-hour collection. The presence of a diurnal effect on leukocyte recruitment has yet to be compared, but a 1-hour collection time point during nighttime sleep was used to compare with the 7-hour time point.

After awakening, participants were instructed to gently irrigate their ocular surface, with 5 mL PBS for each eye, with normal blinking, and the eye wash was collected in one sterile polypropylene tube (pooled sample, 10 mL). The 1-hour sample was collected following a normal evening bedtime. Collected samples were brought to the laboratory within 2 hours of each collection and were processed immediately. The cell collection was centrifuged at 270g and the supernatant was removed. Cells were counted, and average cell size was obtained using a Moxi Z automated cell counter (ORFLO, Hailey, ID, USA).

Reagents and Monoclonal Antibodies

General methods for cell processing and stimulation have been described previously.⁵ LPS from *Escherichia coli* serotype 0111:B4, calcium ionophore, and paraformaldehyde were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). PBS (pH 7.4) was acquired through Lonza (Allendale, NJ, USA). All other chemicals were of analytical reagent grade and were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Neutrophil Panel

Brilliant Violet (BV) 421-conjugated anti-CD11b, BV 510-conjugated anti-CD15, FITC-conjugated anti-CD66b, Peridinin-chlorophyll protein (PerCP)-Cy5.5-conjugated anti-CD14, R-phycoerythrin (PE)-conjugated anti-CD16, Allophycocyanin (APC)-conjugated anti-complement C3a receptor (C3aR), and APC-H7-conjugated anti-CD45 antibodies were all purchased from Becton Dickinson (BD) Biosciences (San Jose, CA, USA).

T-Cell Panel

BV 786-conjugated anti-CD127, BV 421-conjugated anti-CD196, Brilliant Ultraviolet (BUV) 395-conjugated anti-CD4, PerCP-Cy5.5-conjugated anti-CD8, APC-conjugated anti-CD3, APC-H7-conjugated anti-CD45, PE-conjugated anti-CD161, and Brilliant Blue (BB)-conjugated anti-CD25 antibodies were all purchased from BD Biosciences. To enhance the specificity of gating, a BV510-conjugated Fixable Viability Stain (FVS; BD Biosciences) was used to exclude dead cells.¹⁹

Cell Stimulation

To evaluate the activation state of neutrophils (i.e., whether neutrophils are quiescent, primed, or activated), the closed eye leukocytes were presented with two stimuli that are recognized to induce an inflammatory response in leukocytes, namely LPS and calcium ionophore (CaI). LPS presents a stimulation of neutrophils through toll-like receptor 4 (TLR4),²⁰ whereas calcium ionophore induces cellular activation through increases in cytosolic calcium ions.²¹ For LPS stimulation, cells were incubated in a final concentration of 6 µg/mL LPS in PBS, which should induce a significant stimulation.²² For CaI stimulation, cells were incubated in a final concentration of 2 µM CaI in PBS. A higher concentration (5 µM) with a shorter incubation time (5 minutes) has been shown to be sufficient to induce metabolite release by neutrophils.²³ A third aliquot was reserved for unstimulated samples that were left to rest. All samples were incubated following addition of stimulus for 30 minutes at room temperature. Importantly, T cells and other leukocytes were a part of the stimulation, although only membrane receptor analysis of the neutrophils was conducted.

Expression of Membrane Receptors on Neutrophils and T Cells

After incubation with stimulus, tear samples for neutrophil and monocyte phenotyping were transferred into tubes containing fluorescently labeled antibodies against CD11b, CD14, CD15, CD16, CD45, CD66b, and C3aR. The use of several markers to positively identify neutrophils is necessary in human work, as there is no single marker analogous to Ly6G in mice to identify neutrophils in humans.²⁴ Cells were incubated with antibodies for 30 minutes at room temperature, and were then washed twice by spinning down and

resuspending in 700 μL of PBS, before fixation in 2% paraformaldehyde.

For the tear samples for T-cell phenotyping, unstimulated tear collections were transferred into tubes containing fluorescently labeled antibodies against CD161 and CD196, and cells were incubated for 30 minutes at 37°C. This first incubation was performed to improve the specificity and sensitivity of measurement for these nonabundant receptors, given the temperature-dependence of membrane receptor internalization and subsequent recycling.²⁵ Following the first incubation, cells were then stained with FVS and fluorescently labeled antibodies against CD3, CD4, CD8, CD25, and CD45 for 30 minutes at room temperature. Cells were then washed twice in PBS, filtered using a 35- μm cell-strainer cap (Corning, Corning, NY, USA), and then fixed in 2% paraformaldehyde.

Flow Cytometry

All samples were acquired on a LSR II flow cytometer (BD Biosciences) within 8 hours of fixation using BD FACS Diva software, version 8.0.1 (BD Biosciences). Neutrophils were defined by stepwise exclusion of doublets and cell clumps, nongranulocytes or non-neutrophils, by using flow cytometric analyses (Supplementary Fig. S1). Specifically, neutrophils were specified as being CD45⁺ (leukocyte common antigen), with an appropriate forward scatter and side scatter profile associated with neutrophils. Doublets were also excluded to ensure only single cells were used for analysis. Identification of neutrophils in humans is complex, as no single membrane receptor can be used to identify neutrophils in humans.²⁶ Positive staining for CD11b (Mac-1), CD15 (Lewis X), and CD16 (Fc γ RIII), along with low staining for CD14 (LPS receptor common on monocytes/macrophages) can be used in combination to identify neutrophils.²⁶ A recent study of 374 clusters of differentiation (CD) antibodies on blood versus oral neutrophils, both in health and disease, suggested that all neutrophils, regardless of activation state or location, stain positively for CD11b, CD16, and CD66b (degranulation).²⁷ For this study, all the above markers were chosen to identify the neutrophils and characterize their activation state. The complement receptor for C3a (C3aR) was also used as a measure of complement activation.

Similarly, viable T cells were defined by stepwise exclusion of doublets, cell clumps, and dead cells; granulocytes or nonlymphocytes, and stained positively for CD3 (Supplementary Fig. S2). T cells were further broken down into CD4 (helper T cell) versus CD8 (cytotoxic T cell) lineage, and Th17 cells were gated following selection of CD4⁺ T cells using double-positive selection for CD161 and CD196 (CCR6). CD161 has been reported to be a marker of all human IL-17-producing T lymphocytes,^{28,29} and the addition of CD196 has shown improved specificity in selection of Th17 cells.²⁹ Appropriate compensation controls and fluorescence-minus one controls were used to determine gating strategies. Interdaily variations in flow cytometry acquisition were controlled for using the Application Settings feature in BD FACS Diva software. All data were analyzed post acquisition using FlowJo V10 (Ashland, OR, USA).

Statistical Analysis

All results are reported as means \pm SD. To evaluate the significance of differences in expression between 1 and 7 hours, nonparametric analysis was performed using the paired Wilcoxon signed rank test using Statistical Analysis Software (SAS, Inc., Cary, NC, USA).

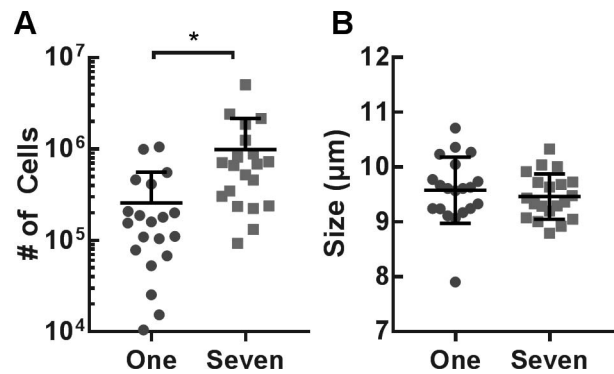


FIGURE 1. Total recovered cell count (A) and average cell size (B), as measured by the Moxi Z cell counter, following 1 and 7 hours of sleep. * $P < 0.005$, $n = 20$ observations from 14 participants.

RESULTS

Cell Count and Size

Following 7 hours of sleep, the average number of cells recovered from a pooled sample of left and right eyes was $9.88 \times 10^5 \pm 1.16 \times 10^6$ (Fig. 1). There were significantly fewer cells recovered at the 1-hour time point, with an average total recovery of $2.57 \times 10^5 \pm 3.00 \times 10^5$ cells ($P < 0.005$). Cell size, however, was unchanged between the 1- and 7-hour time points, with averages of $9.58 \pm 0.60 \mu\text{m}$ and $9.46 \pm 0.41 \mu\text{m}$, respectively ($P = 0.47$). Age-related differences were not investigated in this study given the relatively small sample size and small standard deviation of patients enrolled in the study. Sex-related differences in cell count were investigated, and on average, there were more neutrophils isolated from females both at 1 hour (1.6 times as many) and 7 hours (1.8 times as many); however, this did not achieve statistical significance at either time point ($P = 0.73$ at 1 hour, $P = 0.18$ at 7 hours). More detailed analyses of sex-related differences in neutrophil and T-cell expression were not investigated given the smaller sample size.

Tear Neutrophil Analysis

Phenotypic changes in the neutrophil population could be observed between 1 and 7 hours of sleep (Fig. 2A). Between 1 and 7 hours of sleep, it was observed that membrane receptor expression of CD14 was decreased ($P = 0.08$) and CD16 was significantly decreased ($P < 0.03$), as shown in Figure 2B. There were also decreases in expression of CD15 ($P = 0.05$) and CD11b ($P = 0.08$) between 1 and 7 hours of sleep. There was a slight increase in expression of C3aR, although this did not achieve statistical significance ($P = 0.31$). CD66b and CD45 remained mostly unchanged between 1 and 7 hours of sleep.

Stimulation with LPS or CaI resulted in no significant changes to tear neutrophil phenotype, and the activation ability remained unchanged between 1 and 7 hours (Supplementary Fig. S3).

Monocytes, as identified by CD14⁺ and CD16⁺ staining,³⁰ were minimally observed in the samples, and were approximately 1% of total recovered leukocytes, similar to prior reports.⁵

T-Cell Analysis

As with the neutrophil population, T cells also accumulate with eyelid closure (Fig. 3A). CD3⁺, and consequently CD4⁺, and CD8⁺ cell counts were all elevated between 1 and 7 hours of sleep ($P < 0.01$). There were more Th17 cells at the 7-hour

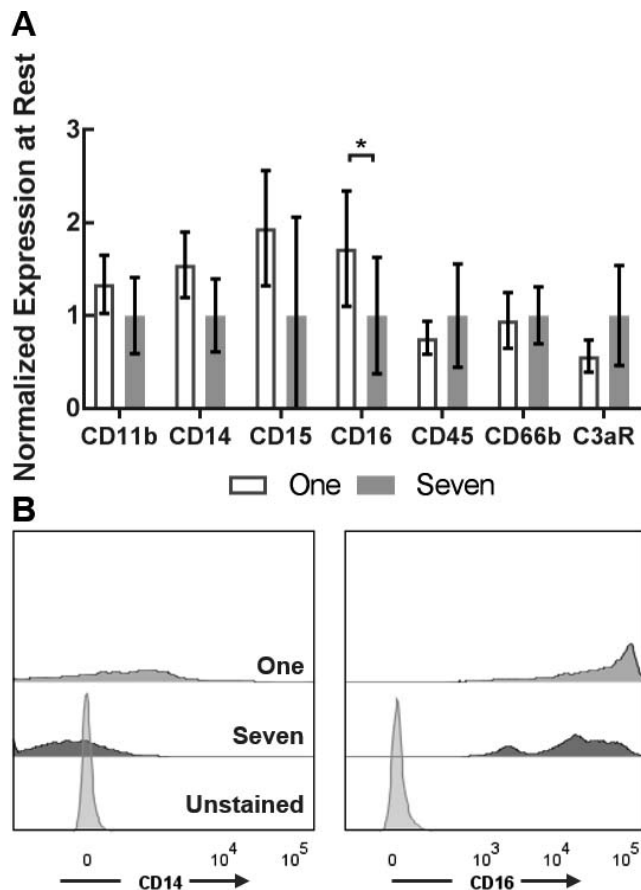


FIGURE 2. Expression of membrane receptors on closed eye tear neutrophils following 1 and 7 hours of sleep. (A) Membrane expression normalized to the mean of the expression for the 7-hour time point. All samples remained unstimulated (rest condition). (B) Representative spectra comparing 1 and 7 hours with both CD14 and CD16. * $P < 0.05$, differences in expression of CD11b, CD14, and CD15 all had $P < 0.1$, $n = 7$.

time point, but this did not reach significance ($P = 0.25$). The relative percentage of CD4⁺ cells out of all CD3⁺ T cells was also compared between the 1- and 7-hour time points to demonstrate that the CD4⁺/CD3⁺ ratio was slightly lower at the 7-hour time point; however, this did not reach statistical significance ($P = 0.16$, Fig. 3B). Similarly, there was no difference in the relative percentage of CD8⁺ T cells out of all CD3⁺ T cells between the two time points ($P = 0.43$). However, the relative percentage of Th17 cells out of all CD4⁺ T cells was significantly different between the 1- and 7-hour time points ($P < 0.03$, Fig. 4C), implying that Th17 cells are recruited or present early on after eyelid closure, but do not accumulate at the same rate as all other T cells by the 7-hour time point.

Altogether, the leukocyte composition in the eye, at awakening, is summarized in Figure 4. Following a full night of sleep, most of the leukocyte infiltrations are neutrophils, and there is a small percentage of CD3⁺ T cells, and an even smaller percentage of monocytes. B cells were not stained for, but there was a large population of CD3⁻ lymphocytes with appropriate forward scatter/side scatter characteristics that would suggest their presence. Natural killer (NK) cells likely constitute missing fractions in the current breakdown. Last, there is a population of CD3⁺ T cells that are CD4⁻ CD8⁻ and are known as double-negative T cells. Double-negative T cells may be involved with memory, and may be either pathogenic

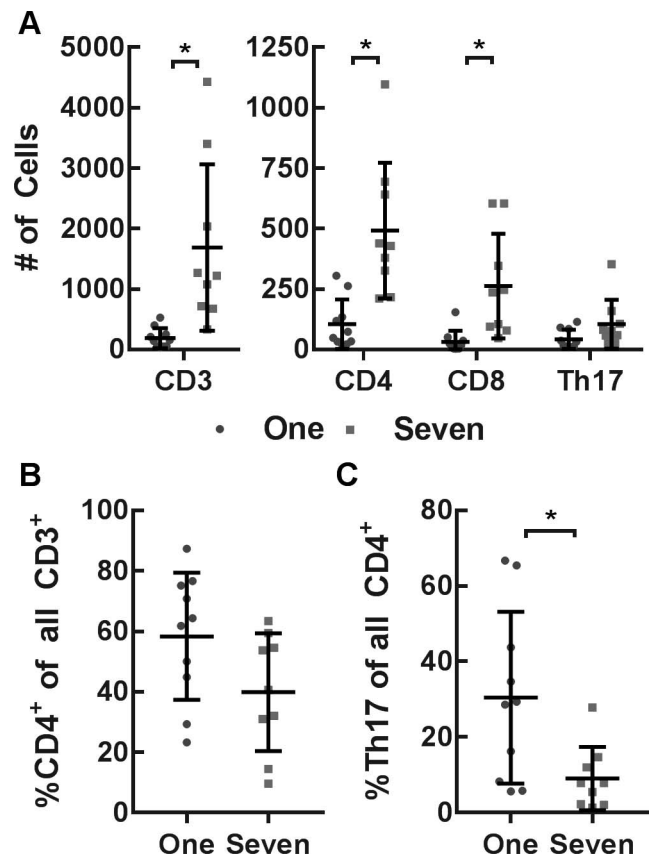


FIGURE 3. Analysis of the T-cell populations from the closed eye following 1 vs. 7 hours of sleep. (A) Total number of CD3⁺ T cells, CD4⁺ helper T cells, CD8⁺ cytotoxic T cells, and Th17 cells measured by flow cytometry. (B) Relative percentage of all CD4⁺ T-helper cells out of all CD3⁺ T cells. (C) Relative percentage of all Th17 cells out of all CD4⁺ T cells. * $P < 0.05$, $n = 9$.

or regulatory in nature; these cells also may represent $\gamma\delta$ T cells or NK T cells.³¹ Prior studies have shown that the normal physiological blood composition of double-negative T cells is approximately 1% to 5%.³² It has also been demonstrated that this population can be greatly elevated in certain healthy tissues and organs, such as the female genital tract³³ and the kidneys,³⁴ or in certain infections and diseases.³¹

DISCUSSION

As many as 1 million leukocytes may be recovered from the closed eye within 1 hour of sleep. This number is significantly larger than the previously reported value of tens of neutrophils, which was likely a result of 2 to 3 μ L microcapillary tear collection in contrast to our improved collection techniques.⁴ Our wash technique may remove any neutrophils that are adherent to mucins or the glycocalyx on the ocular surface. This adhesion could be possible through binding of galectin-3 to CD66b on the surface of the neutrophils.³⁵ Ultimately, this could be responsible for the increased yield observed with our technique versus the microcapillary collection. Altogether, the early leukocyte accumulation highlights that leukocyte recruitment is an active, and not passive process, given the short kinetics.

Following a full night of sleep, the average number of leukocytes increased in the closed eye, for an average of almost 1 million leukocytes. This number is larger than our previously reported average of 3.50×10^5 , but may arise from difference

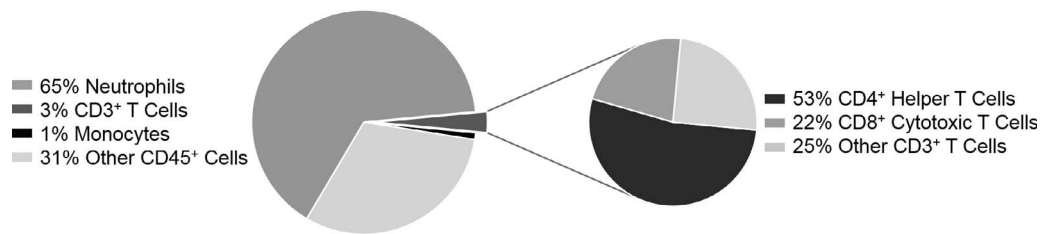


FIGURE 4. Breakdown of leukocytes from the normal eye at awakening. All cells measured are CD45⁺ and represent live, viable, cells. Of the CD4⁺ T cells, approximately 22% of this population are IL-17-producing T lymphocytes (Th17 cells). Other CD45⁺ cells include cell types like B cells and NK cells. Other CD3⁺ cells are predominantly double-negative T cells, which may have functions related to memory, pathogenicity, or regulation. Results are presented as averages and are representative of seven individuals

in counting methods (hemocytometer versus Moxi Z cell counter).³ Importantly, the previous value is still well within 1 SD of the current estimate. There is a large variability in the number of cells collected, which could be a result of the eye wash method itself, as different volumes are recovered from the washes. Sex and age also have not been controlled for, to determine if these factors affect cellular recovery, although anecdotal observations do not seem to support that either of those are major contributory factors. However, age is likely to be of importance, as Th17 cells increase in peripheral tissues with age,³⁶ and mouse models have demonstrated an increased population of Th17 cells in the lacrimal gland with age.³⁷

Our prior work demonstrated that closed eye tear neutrophils have increased expression of surface membrane receptors CD66b, CD11b, CD54, and have lost expression of L-selectin (CD62L),³ which are all consistent with neutrophil extravasation, or migration of neutrophils out of the bloodstream into tissue. Incubation of blood-isolated neutrophils following exposure to any permutation of artificial tear fluid, hypoxic conditions, or co-incubation with human corneal epithelial cells, in vitro, is also unable to replicate this phenotype, highlighting the importance of extravasation.³⁸ Although not directly compared with blood-isolated neutrophils, the tear neutrophils investigated in this study at the 7-hour time point demonstrated high positive staining for CD66b, CD11b, CD16, and CD15, which are consistent with our previous results,³ and also correlate with other aberrant neutrophil populations in healthy tissues, such as those in the mouth,²⁷ lung,³⁹ nose,⁴⁰ placenta,⁴¹ and spleen.⁴²

Our neutrophil results demonstrate a change in phenotype with increasing duration of eyelid closure, predominated by a downregulation of membrane receptors CD14 and CD16. Loss of CD14 and CD16 has been shown to correlate with increased CD63 and primary granule release.⁴³ Primary (azurophil) granule release results in the release of neutrophil elastase,⁴⁴ a potent serine protease, which is known to cleave CD16 from the surface of neutrophils.^{43,45} Neutrophil elastase is known to be upregulated in the closed eye, specifically as a result of neutrophil primary granule release.⁴⁶ CD16 also may be shed from neutrophils as they undergo apoptosis,^{47,48} along with CD15 and CD66b,⁴⁸ but our prior results demonstrated that fewer than 2% of the closed eye tear neutrophils were apoptotic, and stained negatively for Annexin V,³ so the observed CD16 membrane receptor downregulation is likely not mediated by apoptosis.

It has been suggested that the CD14 and CD16 downregulatory phenotype reduces the phagocytic capability of neutrophils,⁴³ as both CD14 and CD16 are phagocytic receptors on the surface of neutrophils. Although commonly thought of as a marker for monocytes and macrophages, CD14 is a glycoprotein that is also expressed on neutrophils, and binds LPS and is a receptor for phagocytosis of several microbial species.⁴⁹ CD16 is an FcγRIII receptor, which binds

the immunoglobulin molecule IgG connected to opsonized bacteria.⁵⁰ CD16 downregulation has been associated with decreased phagocytosis of pathogens by peripheral blood neutrophils in elderly individuals, potentially resulting in an increased risk of sepsis.⁵¹ Interestingly, it was shown that closed eye neutrophils after a full night of sleep have an impaired phagocytic ability.⁵² We hypothesize that neutrophils, as they arrive on the eye, may be more phagocytic, helping to clear pathogens that accumulate during the day, and as they remain on the ocular surface, they release their granule contents to effectively sterilize the closed eye tears. Future studies are required to better understand phagocytosis in the closed eye, and to examine primary granule release and CD63 surface expression on closed eye neutrophils with increasing sleep.

CD15 is also downregulated with eye closure, which is normally associated with neutrophil apoptosis, akin to CD16.⁴⁸ CD15 membrane receptor expression is reported to not be affected or cleaved by neutrophil elastase, nor are CD11b and CD45.⁵³ The precise mechanisms that result in CD15 downregulation in the closed eye remain unknown.

Neutrophil activation is a complex process that involves different, often sequential phases, of priming and activation, leading to different stages of degranulation.⁵⁴ Priming often occurs through stimulation of neutrophils by proinflammatory cytokines like IL-8, and this may simply lead to neutrophil entry into the tissue through release of secretory vesicles and gelatinase (tertiary) granules.^{55,56} Activation of neutrophils often requires a more potent stimulus, such as LPS, to release specific (secondary) granules and ultimately azurophilic (primary) granules.^{55,56} This process is oversimplified and there are many caveats, but generally, CD11b activation begins early in the priming process. CD11b pairs with CD18 to form the transmembrane receptor, part of the β2 integrin family, known as Mac-1 or complement receptor 3 (CR3).⁵⁷ CD11b is known to be expressed on gelatinase granules,⁵⁸ and the observed high CD11b membrane receptor expression at 1 hour may indicate that gelatinase granules have been released, facilitating the transfer of neutrophils into the tears. Its downregulation by the 7-hour time point may simply be explained by membrane receptor recycling into the neutrophil. However, each membrane receptor serves multifactorial roles, and CD11b is no exception. As CR3, the CD11b/CD18 complex is a receptor for phagocytosis of *Bordetella pertussis*.⁵⁹ CD11b also has demonstrated an important role for mediating TLR4 endocytosis in dendritic cells, in response to LPS stimulation.⁵⁷ Although the precise mechanism of CD11b internalization is not yet known, these results suggest that the phagocytic capability of closed eye tear neutrophils is altered between 1 and 7 hours of sleep. Future studies should investigate expression of TLR4 on the closed eye tear neutrophils to observe changes in expression with increasing sleep.

Importantly, we observe no upregulation in CD66b between 1 and 7 hours of sleep. CD66b is involved later in neutrophil activation and is a hallmark of degranulation of specific granules.⁵⁵ This implies that neutrophil activation is not increased with increasing sleep, and may challenge the notion that azurophilic granules are released, hence requiring additional studies examining the role of CD63 membrane receptor expression.

Our results indicate that there is a slight upregulation of C3aR with increasing sleep. The closed eye is known to have an increase in complement components, and that both the classic and alternative pathways are activated in the closed eye.^{2,4,60} The observed increase in C3aR may simply be a result of this complement activation, but a larger sample size is necessary to better understand these changes.

Our initial hypothesis was that the closed eye tear neutrophil response to inflammatory stimuli soon after eyelid closure, and therefore early in recruitment, would be measurably higher than a full 7 hours in the closed eye environment, as neutrophils at awakening do not respond to stimulus either in normal physiological conditions.³ However, this hypothesis was not confirmed. Both at 1 hour and 7 hours of sleep, neutrophils demonstrated minimal ability to respond to stimulation with LPS or calcium ionophore. The combined hypothesized pathway for neutrophil recruitment and activation in the closed eye is summarized in Figure 5.

Our results demonstrate that there are few monocytes in the closed eye, which is consistent with prior observations.⁵ This result is odd, given the high concentration of leukotriene B₄, which should induce chemotaxis of both neutrophils and monocytes.⁵

To our knowledge, this is the first report of T cells in the closed eye tears, although it is to be expected given the noted presence of T cells in the open eye.^{9,10} Like the neutrophil profile, T cells accumulate with increasing sleep. There is a population of both CD4⁺ helper T cells and CD8⁺ cytotoxic T cells in the closed eye tears, and Th17 cells also may be identified, as shown with double-positive staining for CD161 and CD196. Interestingly, the proportion of Th17 cells out of all CD4⁺ T cells following 1 hour of sleep was much greater than at 7 hours of sleep. Some subjects had more than 60% of all CD4⁺ T cells as Th17 cells, suggesting that they are recruited early on to be active in the closed eye. It is possible, however, that the recruitment of Th17 cells and neutrophils is cooperative, and that Th17 cells are not responsible for neutrophil recruitment given the short kinetics observed for neutrophil recruitment.

Neutrophil recruitment to a direct subcutaneous injection of chemokines may be as fast as an hour,⁶¹ but recruitment to peripheral sites usually involves a longer time course in inflammatory processes. Following LPS stimulation⁶² or wounding,⁶³ kinetics of neutrophil recruitment to peripheral sites usually takes approximately 4 hours for a significant response. In corneal wound healing, peak neutrophil recruitment occurs roughly 12 hours after abrasion, although some neutrophils are recruited as early as 6 hours.⁶⁴ Therefore, it is hypothesized that the closed eye tear neutrophils reside in a peri-ocular surface tissue for quick migratory ability.

It is feasible that neutrophils control and regulate the recruitment of T cells to the ocular surface. In blood, T cells represent approximately 26% of all leukocytes, whereas neutrophils represent approximately 60% of all leukocytes (monocytes contribute an additional 6% of cells).^{65,66} This ratio of T cells to neutrophils is drastically different at the ocular surface, where T cells represent only approximately 3% of the total recruited leukocytes. A similar imbalance in T cells to neutrophils has been reported in paraffin-stimulated human saliva, where approximately 96% of the total leukocyte

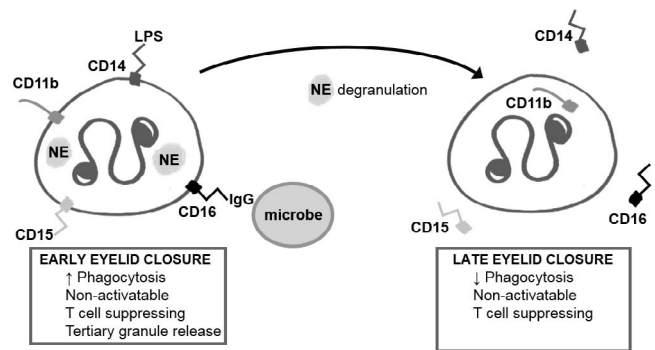


FIGURE 5. Neutrophil dynamics in the closed eye. Following eyelid closure, neutrophils are recruited in large numbers to the ocular surface. These neutrophils are hypothesized to be capable of phagocytosis, through the actions of CD14, which binds to LPS, and CD16, which binds to IgG on the surface of microbes. This is important for the clearance of microbes and pathogens that have accumulated in the eye throughout the day. Neutrophils also have high expression of CD11b, which implies that neutrophils have undergone tertiary granule release. As the neutrophils remain on the eye, they begin to release their primary granules, which contain a large amount of neutrophil elastase (NE). As a potent serine protease, neutrophil elastase cleaves surface membrane receptors CD14 and CD16, losing the potential to phagocytose. Reduction in CD11b expression also may imply a decrease in phagocytic capability. CD15 downregulation is poorly understood. Throughout eyelid closure, neutrophils do not appear to be activatable by LPS and CaI, and are hypothesized to suppress T-cell recruitment to the ocular surface.

accumulation are neutrophils, with monocytes and T cells each representing approximately 2% and 1% of the total accumulation, respectively.⁶⁵ Induced sputum also has a distinct imbalance, with 51% neutrophils and 2% total lymphocytes.⁶⁷ Interestingly, the ratio of CD4⁺ to CD8⁺ cells remained similar between the closed eye and blood, at 3.2 and 2.7,⁶⁶ respectively.

Therefore, it may be possible that the neutrophils suppress T-cell recruitment, which could be possible given recent research demonstrating the interactions between these two cell types.^{8,68} Both mouse and human neutrophils may act as antigen-presenting cells under certain conditions, through expression of the major histocompatibility complex type II, which could promote the differentiation of T cells.^{69,70} Neutrophils may recruit Th17 cells through the production of CCL2 and CCL20, which bind to CCR2 and CCR6, respectively.⁷ Neutrophils are capable of IL-17a production and are capable of self-stimulation through the IL-17 receptor IL-17RC, and this mechanism has been shown to be important in fungal killing at the ocular surface.⁷¹ Last, neutrophils can inhibit T-cell responses and perform immunosuppressive actions through proximal interactions involving Mac-1.²⁴ The exact mechanisms that drive the interaction of T cells and neutrophils in the closed eye tears have yet to be elucidated and warrant further study.

The results of our study suggest that the closed eye is a very dynamic cellular and inflammatory environment, with both neutrophils and T cells, even IL-17 producing Thelper cells, recruited to the ocular surface within 1 hour of sleep. Examination of neutrophil presence at an early time point following eye closure demonstrates neutrophil phenotype changes across 7 hours of sleep, suggesting that neutrophils are originally more phagocytic, but following primary granule degranulation and release of neutrophil elastase, neutrophils may lose some membrane receptor expression and be less activated. The drastic difference between neutrophils and T cells in the closed eye versus blood suggests that the closed eye

tear neutrophils could be T-cell suppressive, and may imply a new additional role for these neutrophils in ocular surface T-cell regulation. Altogether, the closed eye is an active inflammatory environment with numerous leukocytes that play a functional, although as yet understood, role in ocular surface homeostasis. Future studies are required to determine how and if these leukocytes are involved in ocular surface disease pathogenesis.

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