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Leukocyte distribution in the open eye tears of normal and dry eye subjects

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Abstract

Purpose: Leukocytes accumulate in the eye with sleep, but little is known about the presence or absence of leukocytes in awake, open eye tears. This study sought to compare normal and dry eye subjects for daily variation in open eye leukocyte composition.

Materials and Methods: Ten normal subjects and nine dry eye subjects were enrolled. Subjects were trained for self-collection of tear leukocyte samples using an ocular surface wash with 5 mL of phosphate buffered saline per eye. Subjects performed washes at awakening, between 8 and 9 am, between 11 am and 12 pm, and between 4 pm and 5 pm on four separate days. Leukocytes were isolated from the wash and were counted with a cell counter before staining with an anti-CD45 antibody and viability stain. Stained leukocytes were then analyzed via flow cytometry. Side scatter characteristics were used to distinguish granulocytes from lymphocytes. Results were interpreted both by time of wash as well as time from awakening.

Results: At awakening, dry eye subjects had approximately twice as many recovered leukocytes and had a statistically significantly higher granulocyte-to-lymphocyte ratio as compared to normals. Leukocytes were rapidly cleared from the eye, with a significant decrease in leukocyte counts at the 8 am time point as compared to awakening. Leukocyte counts across all open eye time points appeared to be consistent, with no differences between normal and dry eye subjects.

Conclusions: There is a low level, constitutively expressed population of leukocytes in the open eye tears of normal and dry eye subjects. Higher levels of granulocytes in dry eye disease subjects warrants further investigation into this population of cells, and their role in homeostasis and dysregulation.

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Declaration of Interest

No authors have any potential conflicts of interest related to the research presented in this manuscript.

Keywords

closed eye; tears; dry eye disease; inflammation; leukocytes

Introduction

Every night during sleep, there is an influx of almost one million leukocytes to the ocular surface and conjunctival sac,^{1,2} and kinetics for clearance of these leukocytes from the ocular surface is currently unknown. Dry eye disease is known to have increased evaporation or reduced tear flow,^{3–5} and thus, the rate of clearance of leukocytes from the closed eye could be reduced in those with dry eye disease, and have increased residence time in the open eye. Overall, this investigation sought to determine the kinetics of granulocyte and lymphocyte reduction throughout the course of the day.

In the open eye, it has been previously demonstrated that there is a low level of leukocytes present in the normal asymptomatic eye.^{6,7} Specifically, neutrophils have been identified in tears, and it was hypothesized that these cells are constitutively secreted into the inferior fornix, though was not quantified, but found to be a low number and highly variable across subjects.⁶

Prior investigations have found 18,000 total cells following gentle scraping of the tarsal and bulbar conjunctiva in normal open eye human tears (200µL to 350µL collected volume via microcapillary tube) with an approximate percentage of 2.9% T cells.⁸ Given this difference in terms of overall cells collected versus yields from closed eye, it is hypothesized that there is clearance of leukocytes from the closed eye, and the basal number of open eye leukocytes is orders of magnitude less than at awakening.

Investigation of direct neutrophil involvement in dry eye disease has been limited, with one early study suggesting that neutrophils were elevated in closed eye tears of dry eye subjects.⁷ There has only been one explicit demonstration of neutrophil involvement in severe dry eye associated with Stevens Johnson Syndrome-Toxic Epidermal Necrolysis.⁹ Dry eye disease is defined as having an inflammatory component,¹⁰ but the role of neutrophils in dry eye disease pathogenesis is largely undefined. In a mouse model, systemic depletion of neutrophils with an anti-Ly6g antibody has been shown to increase the severity of dry eye disease.¹¹ There are also many pathognomonic signs of neutrophils in tears of individuals with dry eye disease or other ocular surface inflammatory disorders, such as elevated matrix metalloproteinases,^{12,13} neutrophil elastase,¹³ myeloperoxidase,^{12,13} and neutrophil extracellular traps.^{14,15} Matrix metalloproteinases, specifically, have been demonstrated to have a significant diurnal effect, with a strong attribution to diurnal changes in neutrophil recruitment.^{7,16}

T lymphocyte infiltration, specifically involvement of Th1 and Th17 cells, is thought to be involved in the pathogenesis of dry eye disease.^{17,18} There has been limited work to phenotype T cells from the human eye in dry eye disease, and most of the preliminary work in T cells in dry eye has been limited to assessments of conjunctival biopsies.^{19–21}

While neutrophils and T cells have been measured in open eye tears, investigation of the diurnal dynamics of these populations has yet to be performed, to our knowledge. This study sought to examine the changes in leukocytes at four different time points across the day, both in normals and in subjects with dry eye disease.

Materials and 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 19 subjects were enrolled: 10 normal subjects, and 9 subjects with dry eye disease.

Both normal and dry eye subjects were free of any prescription ocular medication use. Normal subjects had no prior diagnosis of dry eye disease and presented with no ocular abnormalities. Dry eye subjects had to present with a self-reported prior diagnosis of dry eye disease. The dry eye questionnaire 5 (DEQ-5) was administered to all subjects to evaluate symptoms.²²

Cell Collection

All participants were trained using a previously-established method^{1,2} to self-collect their tear leukocytes using a polyethylene pipette containing sterile phosphate buffered saline (PBS). Briefly, participants were instructed to gently irrigate their ocular surface, with 5mL of PBS for each eye, with normal blinking, and the eye wash was collected in one sterile polypropylene tube (pooled sample, 10 mL). Collected samples were then processed within two hours of collection. The cell collection was centrifuged at 270 g 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).

Visit Schedule

Subjects collected their tears at four different time points on four separate days to assess diurnal variation in numbers of tear leukocytes: at awakening, between 8 and 9 am, between 11 am and 12 pm, and between 4 and 5 pm. For simplicity, the awakening time point is referred to as the “closed eye” condition, and the 8 am, 11 am, and 4 pm time points are all considered to be “open eye” time points. All four collections were done within a two-week period for each individual subject. Collections were performed in random order, based upon subject availability. The collection performed at awakening was done immediately after awakening, following a full night of sleep (7.1 ± 1.0 hrs, range 6–10 hrs). Given that subjects woke up at different times, the time of awakening was recorded, to determine the amount of time the eyes had been open before tear collection.

Reagents and Monoclonal Antibodies

Phosphate buffered saline (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). Allophycocyanin-H7 (APC-H7)-conjugated anti-CD45

and BV510-conjugated fixable viability stain (FVS) were all purchased from Becton Dickinson (BD) Biosciences (San Jose, CA, USA).

Flow Cytometry

After cell counting with the Moxi Z cell counter, tear samples were transferred into tubes containing a fluorescently-labeled antibody against CD45 (leukocyte common antigen; pan-leukocyte marker) as well as a fixable viability stain. Cells were incubated for 30 minutes at room temperature in the dark, and were then washed twice by spinning down and resuspending in 700 μ L of PBS. Finally, cells were filtered using a 35 μ m cell-strainer cap (Corning, Corning, NY, USA) and were then fixed in 2% paraformaldehyde.

All samples were acquired on a BD LSR II flow cytometer (San Jose, CA, USA) within eight hours of fixation using BD FACS Diva software, version 8.0.1. Viable leukocytes were defined by stepwise exclusion of doublets and cell clumps, CD45 negative cells, and dead cells by using flow cytometric gating strategies (Supplemental Figure 1). Granulocytes and lymphocytes were specified by their appropriate side scatter characteristics; namely, granulocytes are distinguished as having a higher side scatter versus lymphocytes with a lower side scatter. Appropriate compensation controls were used to determine gating strategies. Inter-daily variations in flow cytometry acquisition were controlled for using the Application Settings feature in BD FACS Diva software. All data was analyzed post-acquisition using FlowJo V10 (Ashland, OR, USA).

Statistical Analysis

All results are reported as means $\pm$ standard deviation. To assess diurnal variation, results were expressed at each time point, as well as hours from awakening. Total numbers of leukocytes, granulocytes, and lymphocytes were transformed using the natural log scale to gain a normal distribution of these variables. Pearson correlation coefficients were used to examine the association between leukocyte measurements with the Moxi cell counter versus CD45+ cells measured by flow cytometry. Linear regression models using general estimating equations (GEE) were used to examine whether the association between each leukocyte type and time differed between those with and without dry eye disease. All data was analyzed using Statistical Analysis Software version 9.4 (SAS; Cary, NC, USA).

Results

Subjects

Comparison between the normal and dry eye cohorts is summarized in Table 1. The dry eye subjects, on average, were older, than their normal counterparts ($p = 0.03$). The percentage of males in each group was very similar ($p > 0.99$). The symptoms for the dry eye subjects were statistically more severe, with an average DEQ-5 score of 11.8 ± 1.6 , versus a score of 5.2 ± 1.0 for the normal group ($p = 0.002$). For reference, asymptomatic subjects are normally distinguished as having a DEQ-5 score of less than 6, with mild/moderate dry eye ranging from 6 to 12, and moderate/severe dry eye as greater than 12.²²

Leukocyte distributions by time of collection

Across all subjects, there was a sharp decline from the closed eye cell measurements versus the open eye measurements, as shown in Figure 1. In both normal and dry eye subjects, there were no differences between the 8 am, 11 am, and 4 pm time points in terms of total cell numbers ($p_{\text{normal}} = 0.56$, $p_{\text{dry eye}} = 0.44$), granulocytes ($p_{\text{normal}} = 0.47$, $p_{\text{dry eye}} = 0.36$), or lymphocytes ($p_{\text{normal}} = 0.31$, $p_{\text{dry eye}} = 0.51$).

On average, there were about twice as many leukocytes collected from dry eye subjects ($1.5 \times 10^6 \pm 1.7 \times 10^6$) versus normals ($7.3 \times 10^5 \pm 1.1 \times 10^6$) at awakening, however, this was not statistically significant ($p = 0.21$). Across the 8 am, 11 am, and 4 pm time points, there were no differences measured between the dry eye subjects and the normal subjects.

Given there were no differences between the 8 am, 11 am, and 4 pm time points, the average aggregated number of leukocytes, granulocytes, and lymphocytes in the open eye of a normal subject is 5878 ± 5761 cells, 575 ± 714 cells, and 123 ± 270 cells, respectively (Figure 2). In normal subjects, there were significantly more total cells in the closed eye versus the open eye ($p = 0.011$).

Dry eye subjects demonstrated a similar trend, with the closed eye yielding significantly more total cells versus the open eye ($p = 0.006$). The total aggregated number of leukocytes, granulocytes, and lymphocytes in the open eye of a subject with dry eye disease is 5057 ± 5367 cells, 348 ± 513 cells, and 51 ± 61 cells, respectively.

In terms of the open eye, there was no difference in yields between the normal and dry eye subjects in terms of total numbers of cells measured ($p = 0.47$).

As shown in Figure 3, the ratio of granulocytes to lymphocytes was also compared at all four time points measured, and in normal subjects, the ratio of granulocytes to lymphocytes did not change across all four time points ($p = 0.07$). However, at awakening, the ratio of granulocytes to lymphocytes was significantly higher in dry eye subjects (64.1 ± 54.6) versus normals (20.9 ± 14.6) ($p = 0.05$). In the open eye, the average ratio of granulocytes to lymphocytes was 9.89 ± 10.54 for normal subjects and 9.22 ± 8.77 for subjects with dry eye disease ($p = 0.83$). Interestingly, in the open eye, there were several subjects who had higher numbers of lymphocytes than granulocytes, as indicated by the grey shaded region in Figure 2.

Leukocyte distributions by time from awakening

Given that subjects all woke up at different times, analysis was also conducted between the numbers of leukocytes, granulocytes, and lymphocytes by the number of hours the subjects had been awake, prior to collection (Figure 4).

There were no differences in total open eye cell counts ($p = 0.15$), numbers of granulocytes ($p = 0.12$), or numbers of lymphocytes ($p = 0.43$), in normal individuals. Similarly, dry eye subjects had no differences in open eye cell counts ($p = 0.62$), numbers of granulocytes ($p = 0.81$), or numbers of lymphocytes ($p = 0.53$) by time of awakening. There was also no

difference between normal and dry eye cell counts ($p = 0.55$), numbers of granulocytes ($p = 0.21$), or numbers of lymphocytes ($p = 0.20$) over time.

Discussion

In our analysis, we measured a constitutively expressed, stable number of leukocytes in the open eye, and the distribution of these cells remains similar across all three time points. Total cell numbers, numbers of granulocytes, and numbers of lymphocytes appear to be relatively constant in the open eye in both normal individuals and individuals with dry eye disease. It has been previously hypothesized that inflammatory cells accumulate in the inferior fornix of the open eye,⁶ but our wash technique is not specific enough to determine where the inflammatory cells reside on the ocular surface. Importantly, there is a two- to three-order of magnitude decrease in the number of cells that are recovered from the eye within as early as an hour and a half from awakening. Clearance of leukocytes from the closed eye thus occurs rapidly in both normal subjects and in subjects with dry eye disease.

It was hypothesized that dry eye subjects would have greater numbers of leukocytes in their open eye tear film, or a reduced clearance rate from the ocular surface upon awakening, however, this was not supported by the data. Upon awakening, there are roughly twice as many leukocytes that are recovered from dry eye subjects, on average, which supports preliminary findings by Sack, et al, where greater numbers of neutrophils were observed in symptomatic subjects versus asymptomatic subjects.⁷ Given the small sample size and large variation in sample yields, it is important to verify this result in a larger cohort of subjects to see if this observation remains consistent.

Systemically, the neutrophil-to-lymphocyte ratio in normal subjects has been estimated to be 1.6–1.8:1, and in dry eye subjects, the systemic neutrophil-to-lymphocyte ratio is 2.6–2.8:1.^{23,24} In both of those studies, the dry eye cohort had a statistically significantly higher neutrophil-to-lymphocyte ratio. In this study, we observe that, at awakening, the granulocyte-to-lymphocyte ratio is, on average, 21:1 in normal subjects, and 64:1 in dry eye subjects. Even in the open eye, the granulocyte-to-lymphocyte ratio remains higher than that of blood, with an average of 9.6:1 across all points in the open eye. This suggests that both the open eye, and especially the closed eye, may either be suppressing T cell recruitment, or preferentially recruiting neutrophils. The statistically significantly increased granulocyte-to-lymphocyte ratio in dry eye subjects also points to neutrophils as being an important cell type in dry eye disease. Lastly, evidence supports that recruitment of leukocytes to the ocular surface is not a through passive diffusion from the ocular vasculature, given the drastically different ratios of cells from that of blood. Importantly, blood granulocyte-to-lymphocyte ratio was not measured in this study, so there is no comparison in ratios amongst the same group of participants.

In this study, we designated one of the flow cytometry gates as “granulocytes” as we did not specifically stain for neutrophil-specific membrane receptors. Given the side scatter characteristics of neutrophils, and our prior experience in working with these cells, we make the assumption that measured granulocytes are neutrophils,² but we cannot confirm this without assessment of additional surface membrane receptors.

This investigation used two different methods for cell counting, namely, the Moxi cell counter and flow cytometry. The Moxi is used to count total numbers of cells, whereas the flow cytometer is meant to identify specific populations of cells, such as granulocytes and leukocytes, and is better in comparing relative populations rather than identifying total counts. To assess the correlation between the two different measures for cell counts, we performed a statistical analysis to examine the relationship between number of leukocytes measured by flow cytometry versus the number of cells measured by the Moxi cell counter (Figure 5). The total leukocyte count, as measured by flow cytometry, was calculated as the sum of the granulocyte and lymphocyte fractions, to keep it consistent with the data presented previously. As expected, there was a strong and significant correlation between these two parameters both at awakening ($R^2 = 0.78$, $p < 0.001$) and from the open eye ($R^2 = 0.72$, $p < 0.001$). As can be seen from the illustration in Figure 1, the variation is smaller when there are more cells, due to the cell loss that occurs in processing for flow cytometry, it may be more susceptible to reaching thresholds for noise in the data.

The dry eye subjects that were recruited for this study were not assessed for any clinical signs. Entry criteria was limited to a prior diagnosis of dry eye disease, and indeed, the dry eye cohort had significantly worse symptoms as measured by the DEQ-5. While the dry eye group was older, on average, controlling for age in the analysis did not make any measurable changes to the statistical conclusions that were reached. As dry eye disease is highly multifactorial, there could be subsets of patients with more inflammatory dry eye disease than those measured here. As described by the DEQ-5, the dry eye subjects were moderate/severe, but were not highly severe, based on symptoms.²² It could be hypothesized that there may be dysregulation in the open eye leukocytes in very severe dry eye disease, given the prior observation of neutrophil clusters that may even be clinically visible in those subjects.¹⁵

Overall, we observed no difference among the open eye time points between the normal and dry eye subjects, which suggests that clinical stratification of subjects (eg. mild versus severe dry eye) would likely be uninformative with such low subject numbers. However, subsequent investigation of subjects with neuropathic pain versus a more inflammatory dry eye could yield interesting results. Investigation of differences in closed eye neutrophil accumulations between normal and dry eye subjects, with additional clinical metrics, will be the focus of subsequent investigations.

Recently, there has been evidence in the literature to point to neutrophils being involved in dry eye disease pathogenesis. In a mouse model, it has been demonstrated that depletion of neutrophils leads to increased severity of dry eye symptoms.¹¹ Neutrophils have also been casually implicated in dry eye disease, through the presumed role of neutrophil elastase in galectin-3 cleavage at the ocular surface in dry eye.²⁵

In severe dry eye, or conditions associated with dry eye like severe dry eye disease, the implications for neutrophil involvement has been much stronger. There is evidence that in severe dry eye disease, neutrophil extracellular traps (NETs) play a role in disease pathogenesis,¹⁵ and pharmaceuticals targeting these NETs may be beneficial for those patients.¹⁴ There is also a resident population of neutrophils that has been observed in

Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis (SJS-TEN) in conjunctival epithelium.⁹ Those signatures in SJS extend to the tear film, where there is increased presence of neutrophil degranulation products such as matrix metalloproteinase 8 (MMP8), MMP9, and myeloperoxidase.¹²

T cells have been previously observed in open eye human tears, and this population of T cells has been shown to increase in vernal conjunctivitis⁸ and allergic conjunctivitis.^{26,27} To our knowledge, this is the first time that lymphocytes have been identified in open and closed eye tears of dry eye subjects. We have previously demonstrated the presence and breakdown of T cells in closed eye tears in normal individuals.¹ Given the low cell counts observed, breakdown of lymphocytes was not performed in this analysis given the limited reliability of such a small sample. Future investigations may benefit from a more detailed, pooled analysis, of tear leukocytes, especially given the hypothesized involvement of T cells in dry eye pathogenesis.¹⁷

Closed eye leukocytes are rapidly cleared from the ocular surface, upon awakening, but the normal open eye has low-grade homeostatic inflammation, with a measurable presence of both granulocytes and lymphocytes. While there appears to be no major differences in the open eye of normal individuals versus individuals with dry eye disease, upon awakening, dry eye disease subjects have almost twice as many cells, with a drastically higher granulocyte-to-lymphocyte ratio. Future studies are required to determine how the phenotype of neutrophils changes in dry eye disease to better understand their role in ocular surface dysregulation, and to evaluate their role as disease biomarkers.

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Figure 1:

Differences from ocular surface washes in (a) total numbers of collected cells, (b) numbers of granulocytes, and (c) numbers of lymphocytes between awakening, 8 am, 11 am, and 4 pm time points. All four time points/collections were measured on four separate days. Cell numbers in (a) were measured by a Moxi cell counter, whereas granulocytes and lymphocytes were measured by flow cytometry as CD45+ cells with appropriate forward scatter and side scatter characteristics. Normal subjects (n=10) and dry eye subjects (n=9) were compared across all four time points.

Figure 2:

Differences in total cell numbers between the open and closed eye. Same data as reported in Figure 1, but all open eye time points have been collapsed into the same category. Normal subjects (n=10) and dry eye subjects (n=9).

Figure 3:

Difference in granulocyte-to-lymphocyte ratios from ocular surface washes between awakening, 8 am, 11 am, and 4 pm eye wash collections, between normal individuals (n=10) and subjects with dry eye disease (n=9). All four time points/collections were measured on four separate days. Cell counts for granulocytes and lymphocytes are both measured by appropriate forward scatter, side scatter, and CD45+ gating on a flow cytometer.

Figure 4:

Differences in (a) total leukocyte, as measured by a Moxi cell counter, (b) numbers of granulocytes, as measured by flow cytometry, and (c) numbers of lymphocytes, as measured by flow cytometry, from ocular surface washes conducted at different time points throughout the day. Same data as in Figure 1, but independent axis is expressed as hours from awakening instead of time of collection. Each collection from each individual was measured on a separate day.

Figure 5:

Correlation of numbers of cells measured by the Moxi cell counter versus the number of CD45+ leukocytes measured by flow cytometry.

Table 1:

Normal and dry eye cohort subject characteristics

	Normal	Dry Eye	P value
n	10	9	
Males	2 (20%)	1 (11%)	$p > 0.99$
Females	8 (80%)	8 (89%)	
Age (mean $\pm$ sd)	30.4 $\pm$ 3.0	45.3 $\pm$ 5.5	$p = 0.03$
(range)	24–56	28–64	
DEQ-5 (mean $\pm$ sd)	5.2 $\pm$ 1.0	11.8 $\pm$ 1.6	$p = 0.002$