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

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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 method[3] 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 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).

Reagents and Monoclonal Antibodies

General methods for cell processing and stimulation have been described previously.[3] 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

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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 (FcγRIII), 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 ± 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).

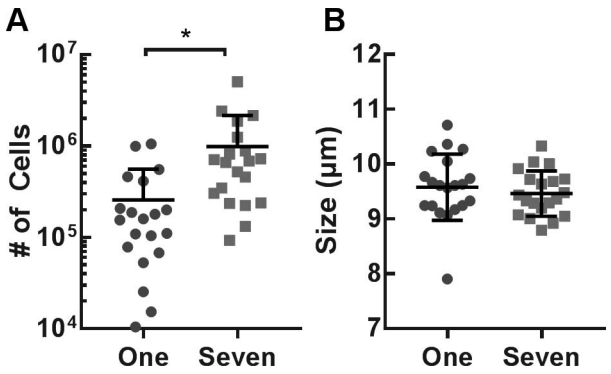

FIGURE 1. Total recovered cell count (**A**) and average cell size (**B**), as measured by the Moxi Z cell counter, following 1 and 7 hours of sleep. $^*P < 0.005$, $n = 20$ observations from 14 participants.

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 ± 0.60 µm and 9.46 ± 0.41 µm, 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

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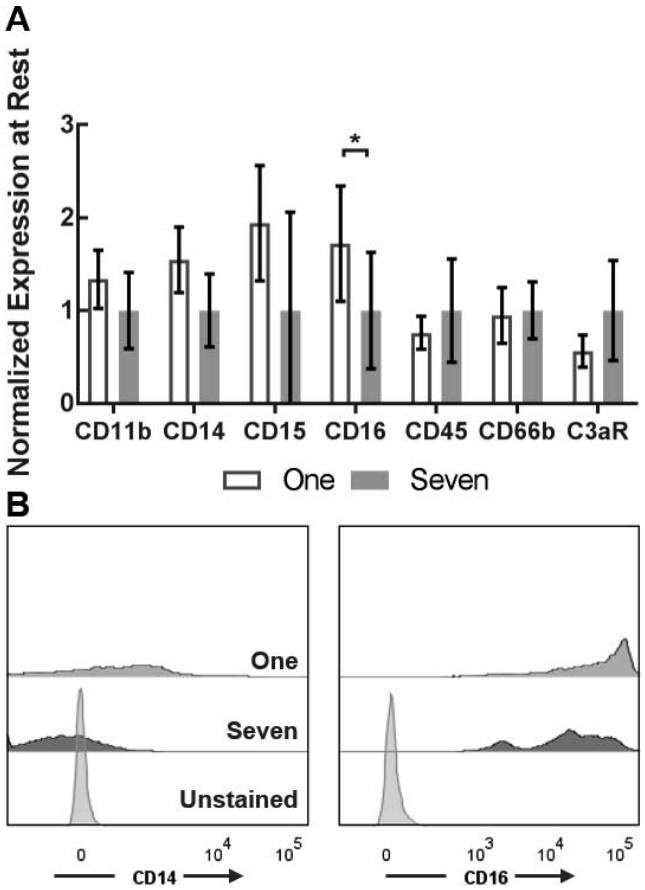

FIGURE 2. Expression of membrane receptors on closed eye tear neutrophils following 1 and 7 hours of sleep. (**A**) Membrane expression normalized to the mean of the expression for the 7-hour time point. All samples remained unstimulated (rest condition). (**B**) Representative spectra comparing 1 and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

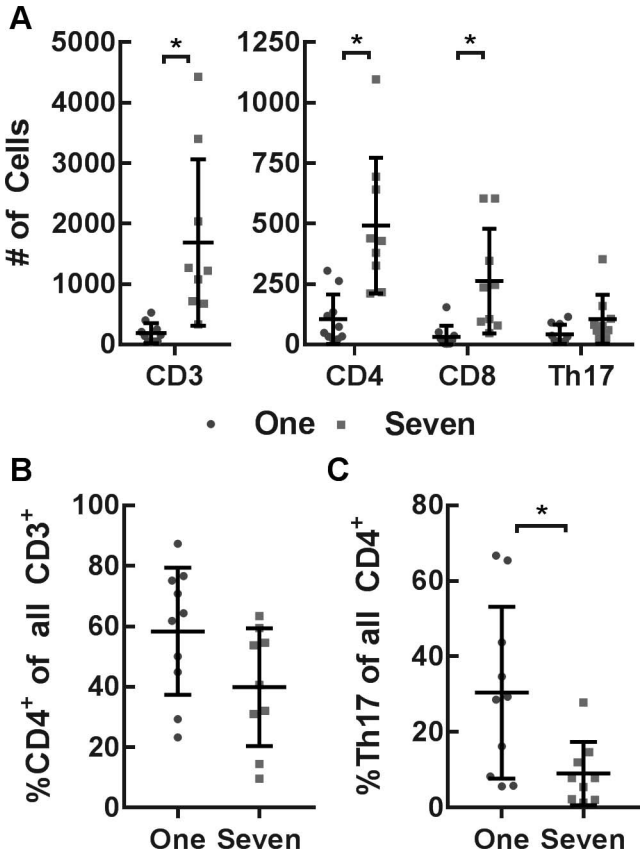

FIGURE 3. Analysis of the T-cell populations from the closed eye following 1 vs. 7 hours of sleep. (**A**) Total number of $CD3^+$ T cells, $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, and Th17 cells measured by flow cytometry. (**B**) Relative percentage of all $CD4^+$ T-helper cells out of all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

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 or regulatory in nature; these cells also may represent γδ 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 tract[33] 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 μL 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

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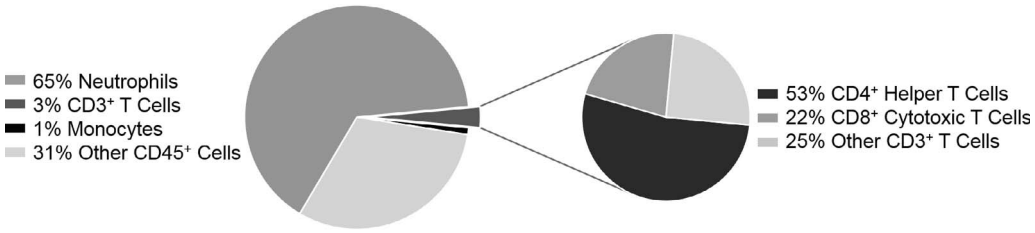

FIGURE 4. Breakdown of leukocytes from the normal eye at awakening. All cells measured are $CD45^+$ and represent live, viable, cells. Of the $CD4^+$ T cells, approximately 22% of this population are IL-17–producing T lymphocytes (Th17 cells). Other $CD45^+$ cells include cell types like B cells and 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.

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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 B_4, 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 stimulation[62] 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

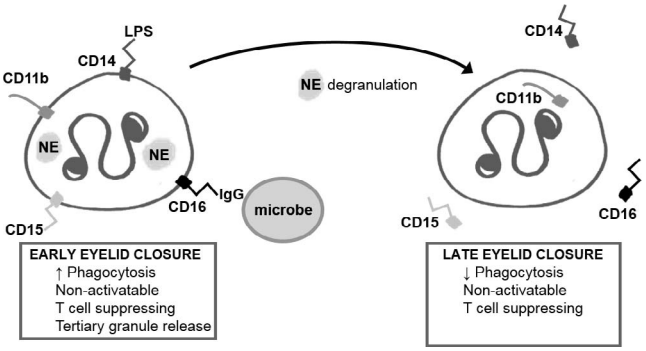

FIGURE 5. Neutrophil dynamics in the closed eye. Following eyelid closure, neutrophils are recruited in large numbers to the ocular surface. These neutrophils are hypothesized to be capable of phagocytosis, through the actions of CD14, which binds to LPS, and CD16, which binds to IgG on the surface of 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 T-helper 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

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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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References

1. McNab AA. The eye and sleep. *Clin Exp Ophthalmol*. 2005; 33:117–125.
2. Sack RA, Beaton A, Sathe S, Morris C, Willcox M, Bogart B. Towards a closed eye model of the pre-ocular tear layer. *Prog Retin Eye Res*. 2000;19:649–668.
3. Gorbet MB, Postnikoff CK, Williams S. The noninflammatory phenotype of neutrophils from the closed-eye environment: a flow cytometry analysis of receptor expression. *Invest Ophthalmol Vis Sci*. 2015;56:4582–4591.
4. Tan KO, Sack RA, Holden BA, Swarbrick HA. Temporal sequence of changes in tear film composition during sleep. *Curr Eye Res*. 1993;12:1001–1007.
5. Thakur A, Willcox MP, Stapleton F. The proinflammatory cytokines and arachidonic acid metabolites in human overnight tears: homeostatic mechanisms. *J Clin Immunol*. 1998;18:61–70.
6. Postnikoff CK, Nichols KK. Ocular surface homeostatic surveillance by Th17 cells in the closed eye. *Invest Ophthalmol Vis Sci*. 2016;57:3885–3885.
7. Pelletier M, Maggi L, Micheletti A, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood*. 2010; 115:335–343.
8. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol*. 2011;11:519–531.
9. Avunduk AM, Avunduk MC, Tekelioglu Y. Analysis of tears in patients with atopic keratoconjunctivitis, using flow cytometry. *Ophthalmic Res*. 1998;30:44–48.
10. Avunduk AM, Avunduk MC, Dayioglu YS, Çentinkaya K. Flow cytometry tear analysis in patients with chronic allergic conjunctivitis. *Jpn J Ophthalmol*. 1997;41:67–70.
11. Leonardi A, DeFranchis G, Zancanaro F, et al. Identification of local Th2 and Th0 lymphocytes in vernal conjunctivitis by cytokine flow cytometry. *Invest Ophthalmol Vis Sci*. 1999;40: 3036–3040.
12. Agnifili L, Mastropasqua R, Fasanella V, et al. In vivo confocal microscopy of conjunctiva-associated lymphoid tissue in healthy humans. *Invest Ophthalmol Vis Sci*. 2014;55:5254–5262.
13. Knop E, Knop N. The role of eye-associated lymphoid tissue in corneal immune protection. *J Anat*. 2005;206:271–285.
14. Dua HS, Gomes JAP, Jindal VK, et al. Mucosa specific lymphocytes in the human conjunctiva, corneoscleral limbus and lacrimal gland. *Curr Eye Res*. 1994;13:87–93.
15. Knop N, Knop E. Conjunctiva-associated lymphoid tissue in the human eye. *Invest Ophthalmol Vis Sci*. 2000;41:1270–1279.
16. Choi W, Li Z, Oh H-J, et al. Expression of CCR5 and its ligands CCL3, -4, and -5 in the tear film and ocular surface of patients with dry eye disease. *Curr Eye Res*. 2012;37:12–17.
17. Stern ME, Gao J, Schwalb TA, et al. Conjunctival T-cell subpopulations in Sjögren's and non-Sjögren's patients with dry eye. *Invest Ophthalmol Vis Sci*. 2002;43:2609–2614.
18. Perez VL, Pflugfelder SC, Zhang S, Shojaei A, Haque R. Lifitegrast, a novel integrin antagonist for treatment of dry eye disease. *Ocul Surf*. 2016;14:207–215.
19. Perfetto SP, Chattopadhyay PK, Lamoreaux L, et al. Amine-reactive dyes for dead cell discrimination in fixed samples. *Curr Protoc Cytom*. 2010;chapter 9;unit 9.34.
20. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem*. 1999;274:10689–10692.
21. Finkel TH, Pabst MJ, Suzuki H, et al. Priming of neutrophils and macrophages for enhanced release of superoxide anion by the calcium ionophore ionomycin. Implications for regulation of the respiratory burst. *J Biol Chem*. 1987;262: 12589–12596.
22. Böhmer RH, Trinkle LS, Staneck JL. Dose effects of LPS on neutrophils—in a whole blood flow cytometric assay of phagocytosis and oxidative burst. *Cytometry*. 1992;13:525–531.
23. Vachier I, Chanez P, Bonnans C, Godard P, Bousquet J, Chavis C. Endogenous anti-inflammatory mediators from arachidonate in human neutrophils. *Biochem Biophys Res Commun*. 2002;290:219–224.
24. Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012;122:327–336.
25. Koenig JA, Edwardson JM. Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol Sci*. 1997;18: 276–287.
26. Pillay J, Tak T, Kamp V, Koenderman L. Immune suppression by neutrophils and granulocytic myeloid-derived suppressor cells: similarities and differences. *Cell Mol Life Sci*. 2013;70: 3813–3827.
27. Lakschevitz FS, Hassanpour S, Rubin A, Fine N, Sun C, Glogauer M. Identification of neutrophil surface marker changes in health and inflammation using high-throughput screening flow cytometry. *Exp Cell Res*. 2016;342:200–209.
28. Maggi L, Santarlasci V, Capone M, et al. CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC. *Eur J Immunol*. 2010;40:2174–2181.
29. Cosmi L, De Palma R, Santarlasci V, et al. Human interleukin 17–producing cells originate from a $CD161^{+}CD4^{+}$ T cell precursor. *J Exp Med*. 2008;205:1903–1916.
30. Heine GH, Ortiz A, Massy ZA, et al. Monocyte subpopulations and cardiovascular risk in chronic kidney disease. *Nat Rev Nephrol*. 2012;8:362–369.
31. D'Acquisto F, Crompton T. $CD3^{+}CD4^{-}CD8^{-}$ (double negative) T cells: saviours or villains of the immune response? *Biochem Pharmacol*. 2011;82:333–340.
32. Voelkl S, Gary R, Mackensen A. Characterization of the immunoregulatory function of human TCR-$\alpha\beta^{+}$ $CD4^{-}CD8^{-}$ double-negative T cells. *Eur J Immunol*. 2011;41:739–748.

Investigative Ophthalmology & Visual Science

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33. Johansson M, Lycke N. A unique population of extrathymically derived αβTCR^{+}CD4^{-}CD8^{-} T cells with regulatory functions dominates the mouse female genital tract. *J Immunol*. 2003;170:1659–1666.
34. Martina MN, Bandapalle S, Rabb H, Hamad AR. Isolation of double negative αβ T cells from the kidney. *J Vis Exp*. 2014; 87:51192.
35. Feuk-Lagerstedt E, Jordan ET, Leffler H, Dahlgren C, Karlsson A. Identification of CD66a and CD66b as the major galectin-3 receptor candidates in human neutrophils. *J Immunol*. 1999; 163:5592–5598.
36. Dutzan N, Abusleme L, Bridgeman H, et al. On-going mechanical damage from mastication drives homeostatic Th17 cell responses at the oral barrier. *Immunity*. 2017;46: 133–147.
37. You I-C, Bian F, Volpe EA, de Paiva CS, Pflugfelder SC. Age-related conjunctival disease in the C57BL/6.NOD-Aec1Aec2 mouse model of Sjögren Syndrome develops independent of lacrimal dysfunction. *Invest Ophthalmol Vis Sci*. 2015;56: 2224–2233.
38. Postnikoff CK, Gorbet MB. The effect of closed eye tear film conditions on blood isolated neutrophils, in vitro [published online ahead of print March 21, 2017]. *Ocul Immunol Inflamm*. doi:10.1080/09273948.2017.1281423.
39. Fortunati E, Kazemier KM, Grutters JC, Koenderman L, Van den Bosch VJMM. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clin Exp Immunol*. 2009;155:559–566.
40. Kinhult J, Egesten A, Benson M, Uddman R, Cardell LO. Increased expression of surface activation markers on neutrophils following migration into the nasal lumen. *Clin Exp Allergy*. 2003;33:1141–1146.
41. Ssemaganda A, Kindinger L, Bergin P, et al. Characterization of neutrophil subsets in healthy human pregnancies. *PLoS One*. 2014;9:e85696.
42. Puga I, Cols M, Barra CM, et al. B-helper neutrophils stimulate immunoglobulin diversification and production in the marginal zone of the spleen. *Nat Immunol*. 2012;13:170–180.
43. Tirouvanziam R, Gernez Y, Conrad CK, et al. Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. *Proc Natl Acad Sci U S A*. 2008;105:4335–4339.
44. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood*. 1997;89:3503–3521.
45. Middelhoven PJ, Van Buul JD, Hordijk PL, Roos D. Different proteolytic mechanisms involved in FcγRIIIb shedding from human neutrophils. *Clin Exp Immunol*. 2001;125:169–175.
46. Sakata M, Sack RA, Sathe S, Holden B, Beaton AR. Polymorphonuclear leukocyte cells and elastase in tears. *Curr Eye Res*. 1997;16:810–819.
47. Dransfield I, Buckle AM, Savill JS, McDowall A, Haslett C, Hogg N. Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J Immunol*. 1994;153: 1254–1263.
48. Hart SP, Ross JA, Ross K, Haslett C, Dransfield I. Molecular characterization of the surface of apoptotic neutrophils: implications for functional downregulation and recognition by phagocytes. *Cell Death Differ*. 2000;7:493–503.
49. Heale J-P, Pollard AJ, Stokes RW, et al. Two distinct receptors mediate nonopsonic phagocytosis of different strains of *Pseudomonas aeruginosa*. *J Infect Dis*. 2001;183:1214–1220.
50. Nagarajan S, Chesla S, Cobern L, Anderson P, Zhu C, Selvaraj P. Ligand binding and phagocytosis by CD16 (Fc γ Receptor III) isoforms: phagocytic signaling by associated ζ and γ subunits in Chinese hamster ovary cells. *J Biol Chem*. 1995;270: 25762–25770.
51. Butcher SK, Chahal H, Nayak L, et al. Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans. *J Leukoc Biol*. 2001; 70:881–886.
52. Gorbet M, Willcox MDP. Interactions of tear-film neutrophils with clinical bacteria. *Invest Ophthalmol Vis Sci*. 2015;56: 4048–4048.
53. Remold-O'Donnell E, Parent D. Specific sensitivity of CD43 to neutrophil elastase. *Blood*. 1995;86:2395–2402.
54. Hallett MB, Lloyds D. Neutrophil priming: the cellular signals that say 'amber' but not 'green.' *Immunol Today*. 1995;16: 264–268.
55. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect*. 2003;5:1317–1327.
56. Naegelen I, Beaume N, Plançon S, Schenten V, Tschirhart EJ, Bréchard S. Regulation of neutrophil degranulation and cytokine secretion: a novel model approach based on linear fitting. *J Immunol Res*. 2015;2015:15.
57. Ling GS, Bennett J, Woollard KJ, et al. Integrin CD11b positively regulates TLR4-induced signalling pathways in dendritic cells but not in macrophages. *Nat Commun*. 2014;5:3039.
58. Mollinedo F, Nakajima M, Llorens A, et al. Major co-localization of the extracellular-matrix degradative enzymes heparanase and gelatinase in tertiary granules of human neutrophils. *Biochem J*. 1997;327:917–923.
59. Mobberley-Schuman PS, Weiss AA. Influence of CR3 (CD11b/CD18) expression on phagocytosis of *Bordetella pertussis* by human neutrophils. *Infect Immun*. 2005;73: 7317–7323.
60. Willcox MD, Morris CA, Thakur A, Sack RA, Wickson J, Boey W. Complement and complement regulatory proteins in human tears. *Invest Ophthalmol Vis Sci*. 1997;38:1–8.
61. Colditz IG, Movat HZ. Kinetics of neutrophil accumulation in acute inflammatory lesions induced by chemotaxins and chemotaxinigens. *J Immunol*. 1984;133:2169–2173.
62. Reutershan J, Basit A, Galkina EV, Ley K. Sequential recruitment of neutrophils into lung and bronchoalveolar lavage fluid in LPS-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol*. 2005;289:L807–L815.
63. Kim M-H, Liu W, Borjesson DL, et al. Dynamics of neutrophil infiltration during cutaneous wound healing and infection using fluorescence imaging. *J Invest Dermatol*. 2008;128: 1812–1820.
64. Li Z, Burns AR, Rumbaut RE, Smith CW. γδ T cells are necessary for platelet and neutrophil accumulation in limbal vessels and efficient epithelial repair after corneal abrasion. *Am J Pathol*. 2007;171:838–845.
65. Vidović A, Vidović Juras D, Vučićević Boras V, et al. Determination of leucocyte subsets in human saliva by flow cytometry. *Arch Oral Biol*. 2012;57:577–583.
66. Maes M, Stevens W, Scharpe S, et al. Seasonal variation in peripheral blood leukocyte subsets and in serum interleukin-6, and soluble interleukin-2 and-6 receptor concentrations in normal volunteers. *Experientia*. 1994;50:821–829.
67. Alexis N, Soukup J, Ghio A, Becker S. Sputum phagocytes from healthy individuals are functional and activated: a flow cytometric comparison with cells in bronchoalveolar lavage and peripheral blood. *Clin Immunol*. 2000;97:21–32.
68. Kalyan S, Kabelitz D. When neutrophils meet T cells: beginnings of a tumultuous relationship with underappreciated potential. *Eur J Immunol*. 2014;44:627–633.

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69. Matsushima H, Geng S, Lu R, et al. Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. *Blood*. 2013;121:1677–1689.

70. Abi Abdallah DS, Egan CE, Butcher BA, Denkers EY. Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation. *Int Immunol*. 2011;23:317–326.

71. Taylor PR, Roy S, Leal SM Jr, et al. Activation of neutrophils by autocrine IL-17A-IL-17RC interactions during fungal infection is regulated by IL-6, IL-23, RORγt and dectin-2. *Nat Immunol*. 2014;15:143–151.

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