


HIGHLIGHTED ARTICLE

Frontline Science: Aggregated neutrophil extracellular traps prevent inflammation on the neutrophil-rich ocular surface

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Abstract

Eye rheum is a physiological discharge, which accumulates at the medial angle of the healthy eye soon after opening in the morning. Microscopic evaluation of eye rheum revealed the presence of viable neutrophils, bacteria, epithelial cells, and particles, aggregated by neutrophil extracellular traps. We observed that in the evening, during eye closure, high C5a recruited neutrophils to the tear film and activated them. In this hypoxic area rich in CO₂, neutrophils fight microbial aggressors by degranulation. Immediately after eye opening, the microenvironment of the ocular surface changes, the milieu gets normoxic, and loss of CO₂ induces subtle alkalization of tear film. These conditions favored the formation of neutrophil extracellular traps (NETs) that initially covers the ocular surface and tend to aggregate by eyelid blinking. These aggregated neutrophil extracellular traps (aggNETs) are known as eye rheum and contain several viable neutrophils, epithelial cells, dust particles, and crystals packed together by NETs. Similar to aggNETs induced by monosodium urate crystals, the eye rheum shows a robust proteolytic activity that degraded inflammatory mediators before clinically overt inflammation occur. Finally, the eye rheum passively floats with the tear flow to the medial angle of the eye for disposal. We conclude that the aggNETs-based eye rheum promotes cleaning of the ocular surface and ameliorates the inflammation on the neutrophil-rich ocular surfaces.

KEYWORDS

degranulation, eye rheum, inflammation, NETs, neutrophils, resolution, serine protease

Abbreviations: aggNETs, aggregated neutrophil extracellular traps; CG, cathepsin G; CitH3, citrullinated histone H3; e.c., extracellular; H&E, hematoxylin and eosin; MPO, myeloperoxidase; MSU, monosodium urate; NE, neutrophil elastase; NET, neutrophil

extracellular trap; PI, propidium iodide; PMN, polymorphonuclear leukocytes; ROS, reactive oxygen species; SEM, scanning electron microscopy; sIgA, secretory IgA; TF, tear fluid.

1 | INTRODUCTION

The ocular surface is constantly exposed to various environmental Ags and pathogenic microorganisms. The ocular innate defense system is composed of anatomical and physiological components. The former include the physical barriers of orbital bones and eyelids, whereas the latter comprise cytokines, complement proteins, tear proteins, epithelia, and keratinocytes. Blinking action of eyelids maintains constant fresh flow of tear film over the ocular surfaces. Tear film proteins like lysozymes, lactoferrin, lipocalin, secretory IgA (sIgA), and protease inhibitors form part of the open eyes tear film, which possess an anti-inflammatory, inert nature.¹ Absence of eyelid blinking action during overnight eye closure leads to scarcity of these tear film proteins on the ocular surfaces. Prolonged eye closure promotes corneal hypoxia and acidosis, and increases epithelial permeability and edema.¹ The closed eye situation reportedly reflects a subclinical inflammation mediated by humoral factors and neutrophils.^{2,3}

Neutrophils are the first immune cells that migrate to the site of infection or tissue damage and serve as first line of immunological defense. Neutrophils canonically eliminate microorganisms by phagocytosis, generation of reactive oxygen species (ROS), and release of (bactericidal) granular proteins (degranulation). Neutrophils also externalize DNA fibers decorated with cytoplasmic and granular proteins.⁴ These structures are referred to as neutrophil extracellular traps (NETs). The primary function of NETs is to entrap and kill microorganisms.⁵ NET formation is a regulated physiological process induced by various endogenous and exogenous stimuli (pathogens and its products, monosodium urate (MSU) crystals, platelet-activating factor, IL-1 β , TNF- α , immune complexes, and apoptotic cells).^{4–6} Chemicals such as PMA and Ionomycin are widely used to induce in vitro NET formation.⁴ Recently, a high ratio of bicarbonate to CO₂ and moderately alkaline pH were identified to make neutrophils more prone to form NETs.⁷

NETs exert proinflammatory actions that contribute to the development of innate and adaptive immune responses during the early phases of infection. Later, the accumulation and aggregation of NETs actively prevent the dissemination of the inflammatory stimuli because they degrade inflammatory mediators. Chronic inflammatory responses to necrotic tissue are prevented by NETs that form barriers between the necrotic areas and the healthy surrounding tissues.^{8,9} Neutrophil-driven inflammations are often terminated by proteolysis of cytokines and chemokines by NET-born serine proteases limiting the inflammatory focus.^{10–12} However, chronic and pathologic accumulation of non-cleared NETs reportedly contributes to the perpetuation of autoimmune responses and autoinflammatory tissue damage.^{13–16}

The presence of neutrophils and NETs in ocular inflammatory diseases like dry eye¹⁷ and allergy¹⁸ has been reported. It has also been reported that the neutrophils play a significant role in ocular wound healing.¹⁹ In the absence of disease or infection, the closed eye condition during sleep also leads to neutrophil infiltration.^{1,2} In this report, we have investigated the physiological role of neutrophils and NETs on healthy ocular surface. The ocular flowing discharge (eye rheum) contains dust, foreign particles, bacteria, mucus, dying and dead epithelial cells, and immune cells. Eye rheum is formed after overnight sleep or

after prolonged eye closure, which supports a continuous infiltration by neutrophils of the eye surfaces.² These eye rheum components are embedded in a matrix built by aggregated NETs (aggNETs). We demonstrate the coordinated sequence of events of neutrophil activity during eye closure including infiltration, degranulation, and NETs formation.

2 | MATERIALS AND METHODS

2.1 | Chemicals and antibodies

Primary Abs for neutrophil elastase (NE ab68672, Abcam, Cambridge, UK), myeloperoxidase (MPO ab9535, Abcam, Cambridge, UK), and citrullinated histone H3 (citH3 ab5103, Abcam, Cambridge, UK) were used for immunohistochemistry. APC anti-human CD45 (368512, BioLegend, San Diego, USA), Pacific blue anti-human CD16 (558122, BD Biosciences, Allschwil, Switzerland), APC-eFluor780 anti-human CD3 (47-0038-42, eBioscience, San Diego, USA), FITC anti-human CD19 (555412, BD Biosciences, Allschwil, Switzerland), and PerCp anti-human HLA-DR (347402, BD Biosciences, Allschwil, Switzerland) were used to identify different leukocyte populations. FITC anti-human CD11a (301206, BioLegend, San Diego, USA), FITC anti-human CD11b activated (301403, BioLegend, San Diego, USA), FITC anti-human CD43 (555475, BD Biosciences, Allschwil, Switzerland), PE anti-human CD54 (555511, BD Biosciences, Allschwil, Switzerland), FITC anti-human CD62L (304838, BioLegend, San Diego, USA), FITC anti-human CD66b (0531, Immunotech, Praga, Czech Republic), and FITC anti-human CD89 (555686, BD Biosciences, San Diego, USA) were used for cell surface marker analysis. fMLP (F3506, Sigma, St. Louis-MO, USA), Human recombinant Complement C5a protein (10604-HNAE, Sino biologicals, Wayne, USA), PMA (P1585, Sigma, Darmstadt, Germany), and LPS from *Klebsiella pneumoniae* (L6143, Sigma, Darmstadt, Germany) were used.

2.2 | Eye rheum sample collection

Eye rheum and tear film cells were collected from healthy subjects after at least 6 h and no more than 8 h of sleep by wash with 20 ml of sterile saline (0.9% NaCl). To isolate eye rheum-derived cells, eye rheum was treated with 50 μ g/ml of DNase (Roche Merck, Darmstadt, Germany) in PBS (Thermo Fisher Scientific, Rockford, USA) with 1 mM calcium and 6 mM magnesium at 37°C for 20 min.

2.3 | Tear sample collection

Tears were obtained from normal healthy donors who were trained to observe aseptic techniques. The subject placed a Schirmer strip on the inner side of lower eyelid for 5 min. The Schirmer strips were then removed and placed in sterile 2-ml centrifuge tubes (Eppendorf, Eppendorf, Germany) and stored at -80°C until processed. Proteins from Schirmer strips were extracted as described previously.²⁰

2.4 | Immunohistochemistry

Eye rheum preparations were stained for NE, MPO, and citH3 as described previously.⁷ Stained eye rheum preparations were analyzed

with Leica SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany). Images were deconvolved with Huygens Professional version 17.10 Scientific Volume Imaging, The Netherlands, <http://svi.nl>) and further constructed with Imaris version 9.1 (Bitplane AG, Zürich, Switzerland).

2.5 | Scanning electron microscopy

Eye rheum preparations were fixed and dehydrated for scanning electron microscopy (SEM) as described previously.¹⁰ Dehydrated samples were sputter-coated with Au (Emitech K575X, Quorum Technologies, Laughton, England), 2×15 mA, 60 s. Samples were then examined using a Tescan Lyra3 field emitting SEM at 5 kV accelerating voltage.

2.6 | Cytochemical stainings of eye rheum

Eye rheum smears were prepared on glass slides. These slides were stained with Gram staining kit (Sigma, Darmstadt, Germany). Samples were also stained with hematoxylin and eosin (H&E). Samples were analyzed on a BZ-X710 fluorescence microscope (Keyence, Neu-Isenburg, Germany).

2.7 | Protein analyses

Lysates of eye rheum, human blood neutrophils, and PMA-induced NETs from blood-derived neutrophils were prepared in Laemmli buffer, and proteins were separated using 10% Tris-Tricine gel electrophoresis. Excised protein bands were prepared and proteins were identified by MALDI-TOF-mass spectrometry as described previously.²¹ Cytokines were analyzed using LEGENDplex™ bead-based immunoassay technology (BioLegend, San Diego, USA). Complement C5a was quantified using sandwich ELISA technique (R&D systems, Abingdon, UK).

2.8 | NE and cathepsin G activity

Eye rheum was incubated with fluorogenic NE substrate MeOSuc-AAPV-AMC (sc-201163, Santa Cruz Biotechnology, Heidelberg, Germany) in the presence and absence of NE inhibitor Sivelestat (S7198, Sigma, Darmstadt, Germany) and NE activity was measured at excitation 360 nm and emission 465 nm. Cathepsin G (CG) activity was determined by incubating with colorimetric CG substrate N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (S7388, Sigma, Darmstadt, Germany) in the presence and absence of CG inhibitor (ab142181, Abcam, Cambridge, UK). Absorbance at 405 nm with reference at 620 nm was recorded.

2.9 | Functional assays of human neutrophils

2.9.1 | Quantification of NET formation

Human blood neutrophils were isolated as described previously.⁷ Sytox Green (2.5 μ M) containing artificial tear buffer (supplementary material) was preincubated at 37°C and 5% CO₂ to achieve the required pH. Isolated blood neutrophils at concentration of 1.5×10^5 were added to artificial tear buffer in 96 well plates (Greiner Bio-One, Frickenhausen, Germany). DNA externalization (NET formation) was analyzed on an Infinite® 200 PRO plate reader (Tecan, Crailsheim,

Germany) at 37°C and 5% CO₂ with excitation at 485 nm and emission was detected at 535 nm.

To study the kinetics of NET formation by alkaline pH, isolated human blood neutrophils were added in Sytox green containing tear buffer with 9 mM sodium bicarbonate at 37°C and 5% CO₂, which resulted in pH 7.2 and followed by fluorescence measurement for 30 min. Then predetermined amount of bicarbonate (28 mM) was added to achieve pH 7.6 and measurement was resumed for additional 10 min.

2.9.2 | Neutrophil degranulation assay

RPMI (Roswell Park Memorial Institute) 1640 medium (Thermo Fisher Scientific, Rockford, USA) with 10% FCS was preincubated under hypoxia (2%) and normoxia (ambient air) conditions. Neutrophils (25×10^6 cells/ml) were incubated in equilibrated RPMI with C5a (250 pg/ml), PMA (10 ng/ml), or fMLP (200 nM) under hypoxia and normoxia for 4 h. Plates were centrifuged and supernatants were incubated with MPO substrate solution containing 10% 3-3',5,5'-tetramethylbenzidine and 0.02% H₂O₂ (BioLegend, San Diego, USA) at room temperature for 5 min. The reaction was stopped with H₂SO₄ (25%) and the absorbance at 450 nm with 620 nm as reference wavelength was recorded.

2.9.3 | Measurement of ROS

Isolated blood neutrophils and eye rheum derived cells were loaded with dihydrorhodamine 123 (3 μ g/ml, Molecular Probes, Oregon, USA) for 20 min at 37°C. Cells were then stained with Pacific blue anti-human CD16 Ab. ROS production was measured after stimulation with PMA (10 ng/ml) for 15 min at 37°C using Gallios™ flow cytometer (Beckman Coulter, Krefeld, Germany).

2.9.4 | Phagocytosis assay

For the quantification of phagocytosis, Fluoresbrite® YG beads (1 μ m; Polysciences, Hirschberg an der Strasse, Germany) were coated with 2 mg/ml human immunoglobulin (Grifols GAMUNEX®, Barcelona, Spain) by passive adsorption for 18 h in 50 mM 2-(N-morpholino)ethanesulfonic acid buffer at pH 6.1. These beads were opsonized with autologous serum in Hank's Balanced Salt Solution (Thermo Fisher Scientific, Rockford, USA) for 30 min. Beads were then added to eye rheum derived cells and isolated blood neutrophils in Opti-MEM™ Reduced Serum Medium (Thermo Fisher Scientific, Rockford, USA) for 1 h at 37 and 4°C and stained with APC anti-human CD15 Ab (Biolegend, San Diego, USA). Next, samples were measured by flow cytometry (Gallios™, Beckman Coulter, Krefeld, Germany).

2.10 | Statistical data analysis

Results are represented as a mean of indicated replicates/experiments. Statistical data analysis was performed using the GraphPad Prism 6 software (San Diego, USA). Flow cytometry data were analyzed using Kaluza 1.5 software (Beckman Coulter, Krefeld, Germany). For multiple comparisons among groups, either ANOVA with Turkey's or Kruskal-Wallis test with Dunn's correction was employed accordingly.

Student's *t*-test was performed for comparisons between 2 groups. A *P* value ≤ 0.05 was considered statistically significant.

2.11 | Ethics statement

All analyses of human material were performed in accordance to the institutional guidelines and with the approval of the ethical committee of the University Hospital Erlangen (permit # 243_15 B). Written informed consent was given by each donor in accordance with the Declaration of Helsinki.

3 | RESULTS AND DISCUSSION

3.1 | Eye rheum is a form of aggNET agglomerating particulate matter, viable neutrophils, and dead epithelial cells

Eye rheum is a discharge, which accumulates independent of a disease at the medial angle of the eye. Eye rheum preparations were stained with H&E and microscopic observation at lower magnification showed a mixture of aggregated cells. Higher magnification revealed the presence of hematoxylin positive extracellular (e.c.) fibers and cells with multilobulated nuclei entrapped along with dying or dead epithelial cells (Fig. 1A). Eye rheum preparations showed e.c. DNA fibers (stained by Sytox Blue; Fig. 1B–D). We, therefore, conclude that eye rheum is abundant in e.c. DNA traps and polymorphonuclear leukocytes (PMN). Immunocytochemistry showed colocalization of e.c. DNA traps and neutrophil granular proteins like NE and MPO (Fig. 1B–C). These stainings together with the typical nuclear morphology of the intact cells indicate that eye rheum consists of abundant NETs with bound neutrophils (Fig. 1B–D). Immunodetection of citH3 along with e.c. DNA in eye rheum confirmed the presence of NETs in eye rheum (Fig. 1D). Analyses by SEM of eye rheum showed aggregated cells, fibrillar structures, and entrapped particles and crystals (Fig. 1E). The presence of entrapped bacteria in the NETs was confirmed by Gram staining of eye rheum (Fig. 1F). All these microscopic observations provide evidence that eye rheum is composed of neutrophils and NETs and may contribute to the collection and removal of endogenous and exogenous waste from ocular surfaces.⁵

To attest the presence of e.c. DNA in these macro aggregates, we treated healthy eye rheum with the enzyme DNase-1. This enzyme degraded e.c. DNA and yielded a single cell suspension of cells, many of them impermeable to the small cationic dye propidium iodide (PI; Fig. 2A).²² Throughout this manuscript, this cell suspension will be referred to as eye rheum-derived cells.

We measured NE activity of eye rheum preparations in the presence and absence of DNase-1 using a specific fluorogenic NE-substrate.²³ All preparations showed robust NE activity, which was further increased with treatment of DNase-1 (Fig. 2B). This underlines the presence of e.c. DNA decorated with NE in eye rheum and suggests that NETs are one of the components of the eye rheum.⁷

For the immunophenotyping of eye rheum-derived cells, we employed an Ab panel composed of anti-CD45, anti-CD16, anti-CD19, anti-CD3, and anti-HLA-DR. Flow cytometry showed a significantly

higher amount of neutrophils (90%, $P < 0.001$) compared to other leukocyte populations (Fig. 2C). In order to check viability of eye rheum-derived CD16 positive neutrophils, we performed multiparametric cell death staining by using annexinA5-FITC, PI, and DiIC1(5), a mitochondrial potential sensitive dye.²² In this analysis, significantly higher percentages of viable neutrophils ($62.3 \pm 16.8\%$, $P < 0.001$) were observed when compared with apoptotic ($31.9 \pm 15.8\%$) and necrotic ($10.3 \pm 11.3\%$) cells (Fig. 2D). These data were confirmed by microscopic evaluation (Supplementary Fig. S1). The neutrophil-related proteins like MPO, NE, and CG and protein S100 were identified in eye rheum by performing gel electrophoresis and mass spectrometry (Figs. 2E and 1F). Postnikoff et al. recently reported that after 7 h of sleep, closed eyes were infiltrated by neutrophils, T cells, and monocytes.²⁴ The presence of viable neutrophils in tear films was also observed by trypan blue and PI exclusion in another study.²⁵ These findings along with our observations of eye rheum suggest that viable neutrophils patrol the closed eye and form NETs to expand their reach. This extended function of neutrophils and NETs helps in better collection of potential harmful particles/aggressors. This report presents compelling evidence that patrolling neutrophils form NETs on the surfaces of healthy eyes and collect potentially harmful particles for disposal. Our data are in line with previous findings that e.c. DNA, NETs, and neutrophils are abundantly present in the mucoid films of patients with dry eye disease.¹⁷ With our data, we follow the recommendation of the TFOS Subcommittee to study the PMN response in the closed eye tears.²⁶

To investigate the immunological role of eye rheum along the diurnal cycle, it is important to know open- and closed-eye tear film composition. In open-eye condition, protective tear fluid (TF) is maintained by the blinking action of the eyelids. It is thought that during blinking, microorganisms and potentially harmful inflammatory mediators are mechanically flushed via the lacrimal sac into the nasal cavity. The tear film is replenished by secretions of lacrimal and accessory glands. These tears contain fresh antimicrobial proteins like lysozyme, lactoferrin, and lipocalin. However, during eye closure, the absence of blinking reduces the turnover rate of TF and the concentration of antimicrobial proteins. Closed eye condition has a reduced oxygen and carbon dioxide exchange, which further leads to corneal hypoxia and edema. This hostile condition may favor corneal infections and pathological processes. Wilson and colleagues have also reported large number of neutrophils on the ocular surface during sleep.²⁷ Upon eye closure, tear flow does not cease completely but is maintained at low rates with constitutive secretion of sIgA.^{1,28} This suggests that neutrophils and sIgA may play a critical role in ocular defense during closed eye condition.

3.2 | Recruitment of neutrophils to the ocular surface

In order to identify the chemotactic factors responsible for neutrophil recruitment to the ocular surface, TF samples from normal healthy donor were collected at different time points. TF collected sequentially before sleep (evening TF), after sleep (morning TF), and around noon (noon TF) were analyzed for the presence of chemokine IL-8 and the anaphylatoxin C5a.²⁹

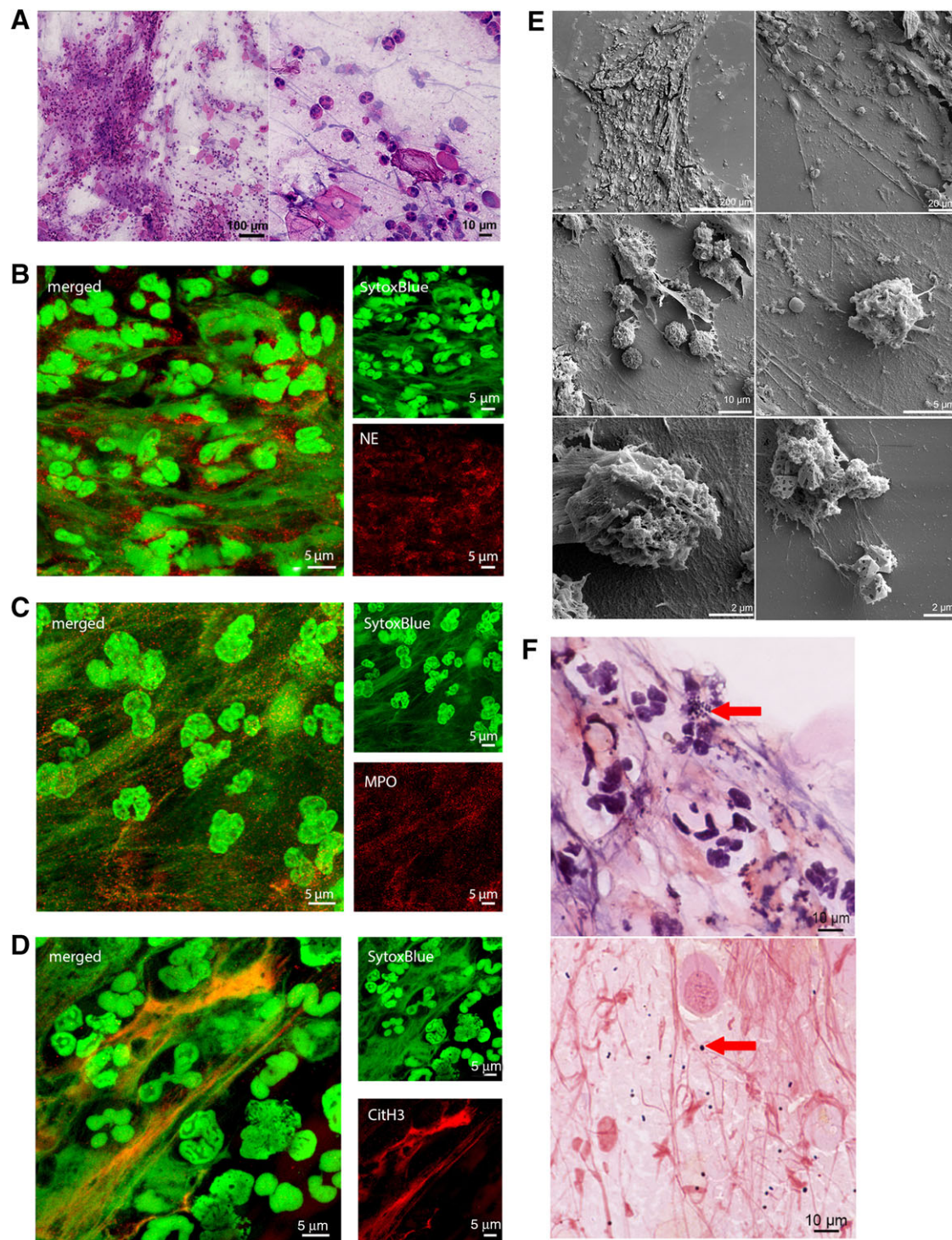


FIGURE 1 Eye rheum is composed of neutrophils, particulate matter, bacteria, and neutrophil extracellular DNA traps. (A) H&E staining of eye rheum showing extracellular (e.c.) DNA traps and polymorphonuclear and epithelial cells. (B–D) Confocal microscopy micrographs of eye rheum showing e.c. DNA stained with Sytox Blue and NETs specific marker proteins: (B) NE, (C) MPO, and (D) citH3. (E) SEM micrograph of eye rheum displaying NETs like structures, single cells, and calcium carbonate crystals. (F) Gram staining of eye rheum showing presence of trapped gram-positive microorganisms in the eye rheum

In the evening TF, C5a showed higher values ($P < 0.01$, Fig. 3B). This indicates that the complement system is activated in the TF before eye closure. This is in agreement with published data.³⁰ As C5a is a cleavage product of complement C5 by the action of the C5 convertase, the presence of this anaphylatoxin in the evening is most likely

due to the continuous exposure of the tear film to microorganisms and dust particles during the day. Notably, during night, the levels of C5a significantly increase further ($P < 0.001$, Fig. 3B). This can be explained by the fact that the levels of sIgA are increased in closed eyes^{1,28} and promote the activation of the alternative and lectin pathways of the

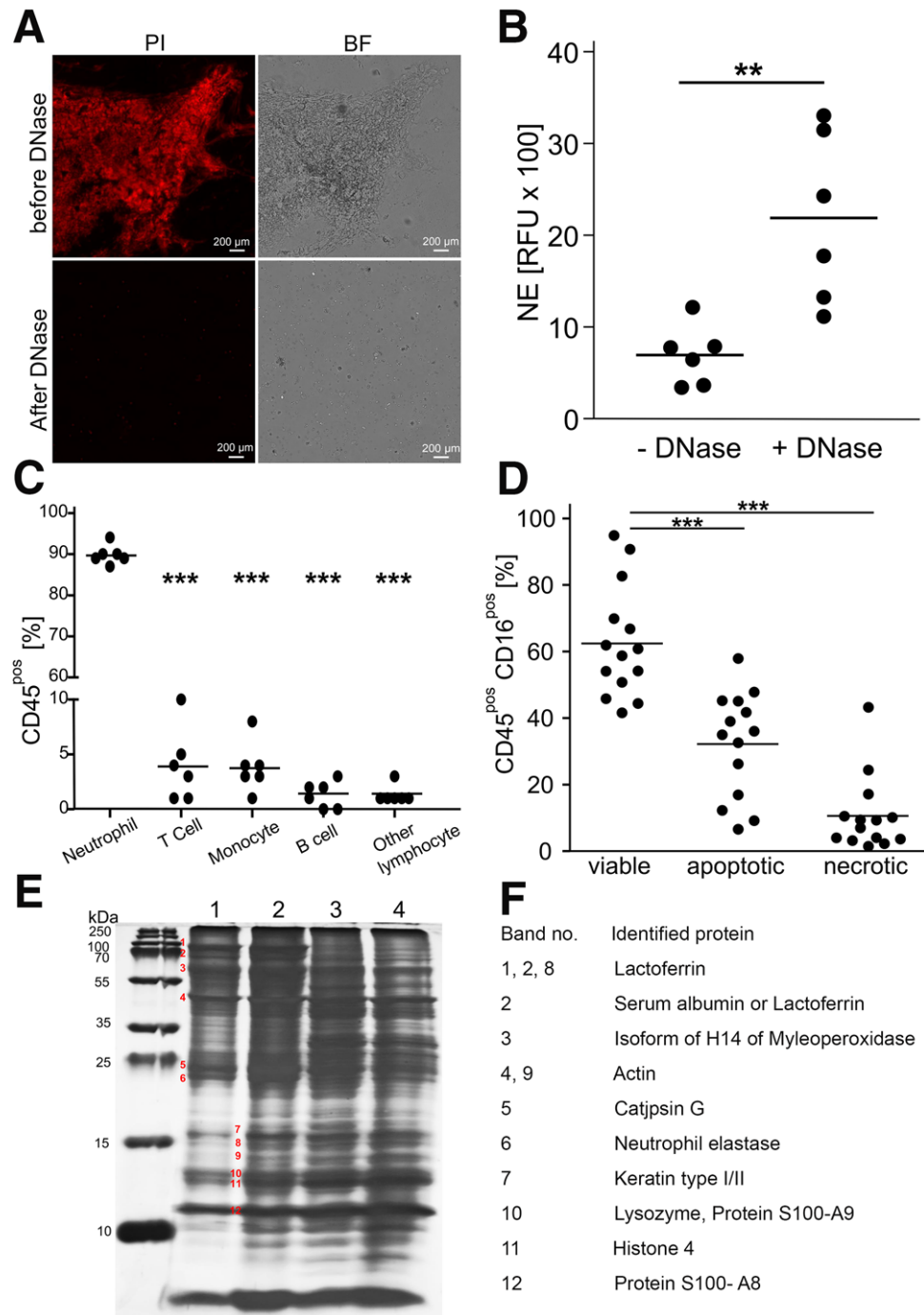


FIGURE 2 Presence of viable neutrophils and aggNETs in the eye rheum. (A) Microphotograph of eye rheum stained with PI, and e.c. DNA stained red degraded by DNase-1 and resulting in single cell suspension. (B) NE activity of the eye rheum in the presence and absence of DNase-1. Paired t-test was used (** $P < 0.01$, $n = 6$). (C) Immunophenotyping of eye rheum-derived CD45 positive cells after DNase treatment (** $P < 0.001$, $n = 6$). (D) Percent viability of CD16 positive eye rheum-derived cells assessed with flow cytometry (** $P < 0.001$, $n = 14$). (E) Separation of proteins from eye rheum (lanes 1 and 2), blood neutrophils (lane 3), and blood neutrophils-derived NETs (lane 4) using 10% Tris-Tricine gel electrophoresis. Numerically labeled identified protein bands are listed in (F). For multiple comparisons, Tukey's correction was performed (C and D)

complement cascade.^{1,28} Other authors have also monitored sequential changes of the chemotactic factors, plasmin, and vitronectin and complement C3c after eye closure concluding that the recruitment of neutrophils on ocular surfaces must be considered as a subclinical inflammation.² Hence, activated complement system most likely leads to recruitment of neutrophils on ocular surface after eye closure during sleep.

IL-8 dominated in the morning TF. Furthermore, it also showed the highest average concentration in the morning TF when compared with noon and night TF ($P < 0.01$, Fig. 3A). In vitro culture of LPS-stimulated corneal epithelial cells has shown release of IL-8 (Supplementary Fig. S2A). Human corneal epithelial cells secreted IL-8 at 24 h after the addition of *Pseudomonas*-derived LPS.³¹ Fungus induces higher levels of IL-8 mRNA expression and protein production in human corneal

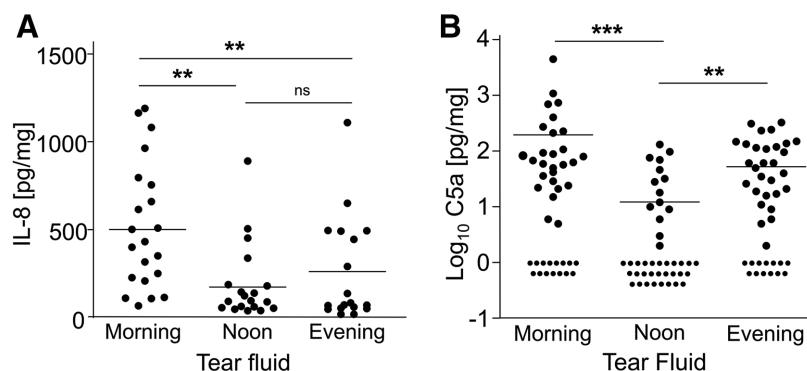


FIGURE 3 Neutrophils are recruited on the ocular surface by complement C5a and IL-8. (A and B) Quantification of (A) IL-8 $n = 9-11$ and (B) complement C5a $n = 21$ in eye TF from normal healthy donors, which were collected: before sleep (evening TF), after sleep (morning TF), and during day time (noon TF). Left and right eye of each donor were depicted in the graphs. Dunn's multiple comparison test was used to evaluate differences among means $**P < 0.01$, $***P < 0.001$

epithelial cells.³² It has been shown that chemokine IL-8 is synthesized and released by human corneal epithelial cells and keratocytes after stimulation with selected proinflammatory cytokines.³³ Hence, we suggest that during sleep, the absence of eyelid blinking action leads to retention of dust and bacteria on the ocular surface, which further contributes to IL-8 production from corneal epithelial cells and explains the elevated IL-8 levels in the morning TF. It has been previously shown in patients with dry eye disease that conjunctival exfoliated cells have an increased TLR9 and MyD88 gene expression and this lead to cytokine production and neutrophil recruitment on the ocular surface.¹⁷

In vitro culture of human blood neutrophils released IL-8 upon exposure to LPS from *Klebsiella pneumoniae* (Supplementary Fig. S2B). It has been reported that neutrophils release IL-8 in response to inflammatory stimulus such as bacterial LPS, IL-1 β , and TNF.^{34,35} An increased level of IL-8 is considered as the hallmark of neutrophilic infiltration, degranulation, and phagocytosis.³⁶⁻³⁹ We hypothesize that the neutrophils that have accumulated in the conjunctival space form NETs and concomitantly contribute to the IL-8 content found in TF after eye opening.

Taken together, we suggest that the activated complement system and LPS significantly contribute to the recruitment of neutrophils on the ocular surface. Interestingly, tears collected around noon had the lowest levels of IL-8 and C5a, most likely due to the continuous dilution of tear film by newly secreted fluid from the lacrimal glands and its outflow due to blinking (Fig. 3).

3.3 | Moonlighting functions of neutrophils during overnight eye closure

Surface marker expression on eye rheum-derived neutrophils and blood-derived neutrophils is summarized in Table 1. The integrins CD11a and CD11b activated as well as ICAM-1 did not differ, whereas leukosialin (CD43) and L-selectin (CD62-L) were lower in eye rheum-derived neutrophils. In contrast, CEACAM8 (CD66b) and the Fc α -receptor (CD89) were higher in eye rheum-derived neutrophils (Table 1).

Proteolytic cleavage by NE of CD43 on neutrophils has been observed after stimulation of phagocytosis or NADPH oxidase.^{40,41} Furthermore, proteolytic shedding of neutrophil CD43 has been directly correlated with neutrophil functions like spreading, aggregation, and ROS production.⁴² The phenotype of the eye rheum-derived

TABLE 1 Analyses of surface markers of eye rheum-derived and blood-derived neutrophils

	Eye rheum-derived neutrophils MFI	Blood-derived neutrophils MFI
CD11a	6.16 \pm 1.79	7.82 \pm 0.55
CD11b activated	15.84 \pm 3.55	13.21 \pm 1.12
CD43 (Leukosialin)	0.43 \pm 0.45***	13.94 \pm 2.01
CD54 (ICAM-1)	9.82 \pm 2.53	8.28 \pm 1.33
CD62L (L-selectin)	0.76 \pm 0.48***	18.05 \pm 2.05
CD66b (CEACAM8)	79.51 \pm 12.45***	7.82 \pm 0.43
CD89 (FCA-R)	23.44 \pm 4.69*	11.07 \pm 2.35

*** $p < 0.0001$, * $p < 0.01$

neutrophils indicates that these cells have been extravasated (low L-selectin,²⁵ are activated (low leukosialin⁴²) and have already partially degranulated (high CEACAM8,⁴³ Fig. 4A). Surprisingly, we did not observed up-regulation of integrins CD11a, CD11b, and ICAM-1 in eye rheum-derived neutrophils. This is in contrast to reports of extravasating neutrophils at other anatomical locations like in the mouse cremaster muscle⁴⁴ and in the bronchoalveolar fluid of smokers⁴⁵ and might be characteristic feature of eye rheum-derived neutrophils. The high expression of the Fc α R further suggests an increased responsiveness to IgA.⁴⁶

Next we performed functional assays with blood- and eye rheum-derived neutrophils. Figure 4A shows that the degranulation marker (CD66b) was already high in eye rheum-derived neutrophils and could not be further stimulated by the addition of fMLP. PMA-stimulated eye rheum-derived neutrophils produced ROS with a 90% reduced capacity as compared to blood-derived neutrophils (Fig. 4B). These data are supported by other investigators.²⁵ Eye rheum neutrophils also showed an 80% reduced uptake of IgG-opsonized fluorescent beads as compared to blood-derived neutrophils (Fig. 4C).

These findings suggest that eye rheum-derived neutrophils are viable and have reduced functional capabilities. On the basis of the results obtained from functional and cell surface marker expression analysis, we suggest that during prolonged eye closure, the infiltrating neutrophils that remain alive mainly perform degranulation in order to combat aggressors. The fact that they produce lower amounts of ROS than their blood-borne counterparts is protective for the ocular surface.

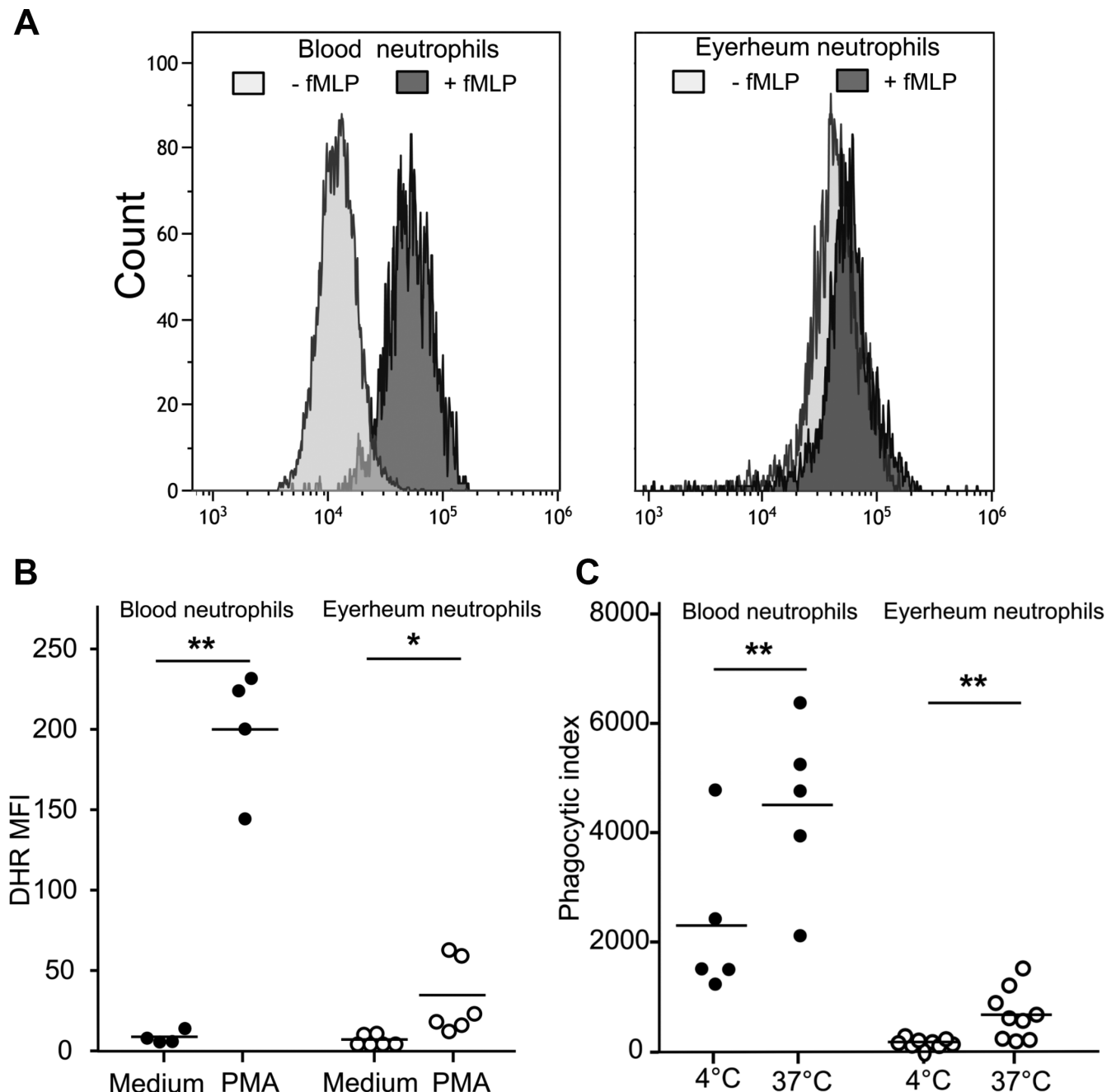


FIGURE 4 Functional characterization of eye rheum derived neutrophils. (A) CD66b expression on eye rheum-derived and blood-derived neutrophils after fMLP stimulation. (B, $n = 6$) ROS production and (C, $n = 9$) phagocytosis of opsonized beads by eye rheum-derived and blood-derived neutrophils. Paired t -test was performed (B and C); * $P < 0.5$, ** $P < 0.01$.

To test the effect of the environmental conditions of closed and open eye on neutrophils, we performed assays for degranulation and NET formation with blood-derived neutrophils in artificial tear buffer. Under hypoxic conditions (closed eye) but not in normoxia, neutrophils spontaneously degranulated (Fig. 5A, left 2 bars). Stimulation with C5a, fMLP, and LPS augmented degranulation especially under hypoxic conditions (Fig. 5A, right 6 bars). This suggests that the hypoxic microenvironment of closed eyes favors degranulation in neutrophils that have migrated to the ocular surface. This observation also explains the degranulated phenotype of eye rheum-derived neutrophils (Fig. 4A).

3.4 | The functional shift of neutrophils after eye opening

To investigate the mechanisms that induce NET formation after eye opening, we first tested the NET formation potential of C5a. As shown in Fig. 5B, C5a did not induce NET formation (Fig. 5B). Next, we investigated the effect of CO₂, bicarbonate, and pH on neutrophils. Figures 5C–E display that even a moderate increase in pH from 7.2 to 7.6 strongly promoted NET formation. Effect of moderately alkaline pH on NET formation was further augmented in the presence of LPS (Fig. 5C). Immediately after eye opening, the microenvironmental

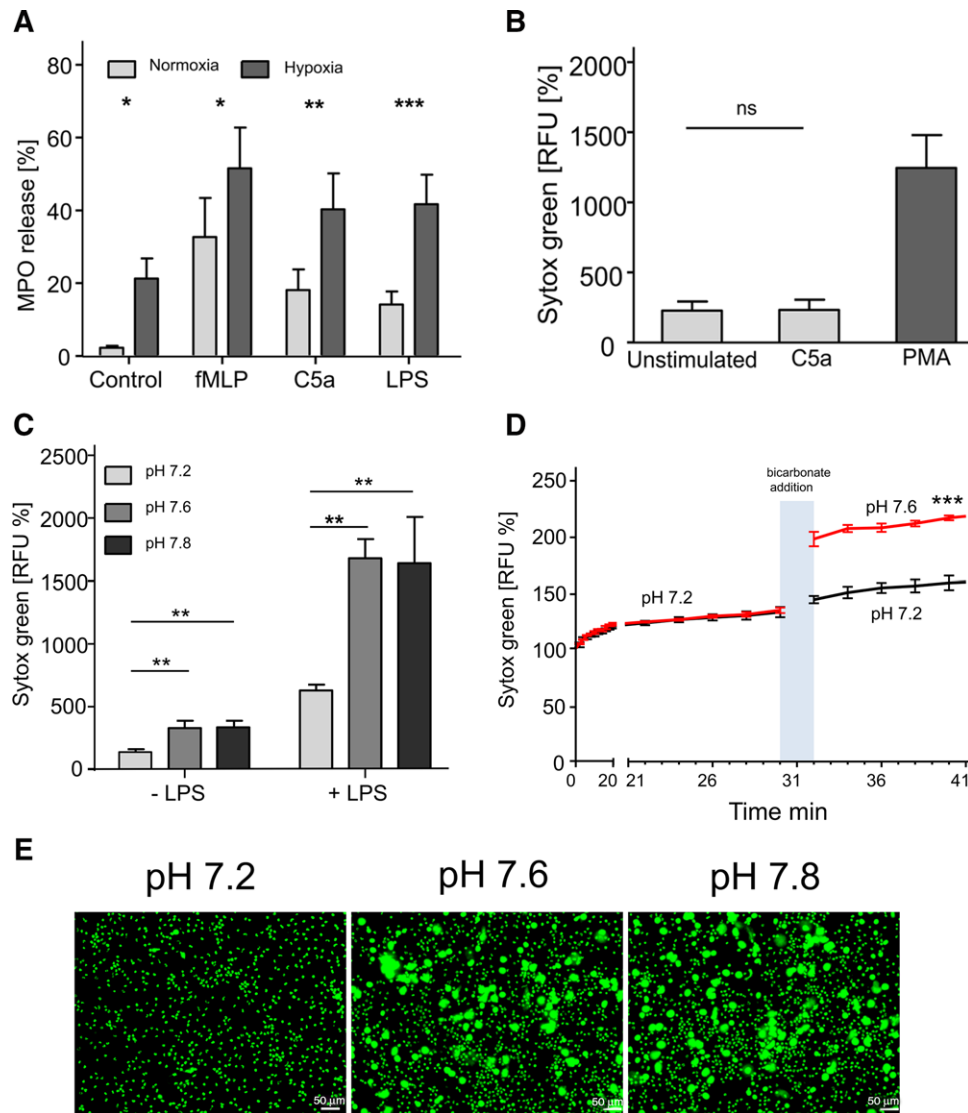


FIGURE 5 Mechanism of eye rheum formation. (A) Hypoxia (closed eye condition) supported neutrophil degranulation. (A) C5a, fMLP, and LPS significantly stimulated neutrophil degranulation ($n = 7$). Two way ANOVA was used; * $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$. (B) Quantification of DNA externalization by neutrophils (NET formation) stimulated with C5a and PMA ($n = 8$). (C) NET formation assay in the presence and absence of LPS in tear buffer at pH 7.2 (closed eye tear film), pH 7.6 and 7.8 (open eye tear film), $n = 3$. Tukey's multiple comparison test was used to evaluate differences among means, ** $P < 0.01$. (D) Extracellular pH induces rapid NET formation. Kinetics of NET formation in tear buffer at pH 7.2 (9 mM bicarbonate) and after addition of bicarbonate 0 mM (pH 7.2) and 2.8 mM (pH 7.6) $n = 3$. Paired t -test was used to evaluate differences among means, *** $P < 0.001$. (E) Fluorescence micrographs showing DNA externalization of neutrophils in (C). Cells stained for DNA with Sytox Green

conditions of the closed eye tear film change: the oxygen tension normalizes from 8 to 21%, CO_2 evaporates, and partial pressure of CO_2 drops from 40 mmHg to less than 5 mmHg.^{47,48} Concomitantly, the pH increases from 7.2 to 7.6. The alterations in e.c. pH have broadly influenced cellular and humoral immune functions.⁴⁹ We have studied the influence of the triangular relationship of bicarbonate, CO_2 , and pH on NET formation.⁷ In this study, we have found that a low pH and a high ratio of CO_2 to bicarbonate decreased the capacity of neutrophils to release NETs. However, high ratio of bicarbonate to CO_2 and a moderately alkaline pH increased capacity of neutrophils to form NETs.⁷ Similarly, we have seen that a moderately alkaline pH and high ratio of bicarbonate and CO_2 have increased the potential of NET-inducer stimuli like phorbol esters (PMA), Ionomycin, MSU,

and LPS to form NETs.⁷ Furthermore, we described the mechanism by which the alkaline environment leads to intracellular alkalization of neutrophils, which is further accompanied by additional influx of calcium into the cells. These increased levels of calcium activate neutrophil enzymes involved in NET formation.⁷

The intracellular pH of neutrophils is regulated by Na^+/H^+ , $\text{Cl}^-/\text{HCO}_3^-$ antiporters, and $\text{Na}^+/\text{HCO}_3^-$ cotransporters.^{50,51} Shimizu et al. have found a temporal correlation between the agonist-stimulated Cl^- efflux from neutrophils and intracellular alkalization following agonist stimulation.⁵² Furthermore, it was suggested that efflux of chloride is common phenomenon during neutrophil activation.⁵² Here we propose that a high e.c. concentration of bicarbonate can also result in a similar effect of neutrophil

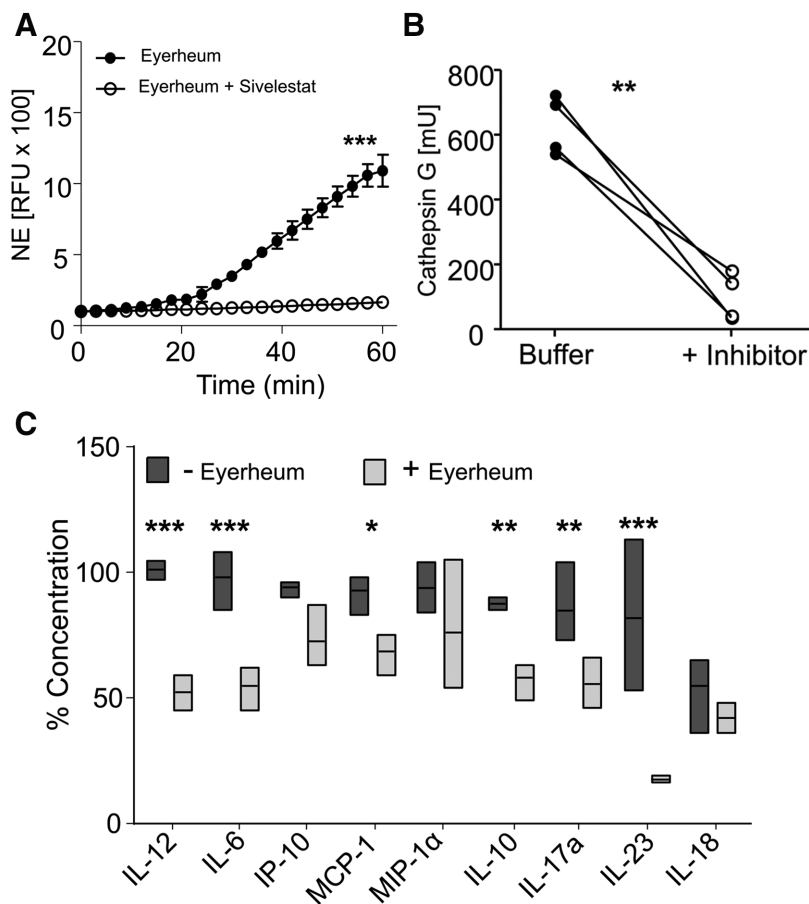


FIGURE 6 Proteolytically active aggNETs of eye rheum degrade cytokines and chemokines and prevent clinical ocular inflammation. (A and B) Eye rheum is proteolytically active; enzymatic activity of eye rheum for NE (A) and CG (B) was determined in the presence and absence of respective enzyme inhibitor ($n = 3-5$). Paired t-test was used to evaluate data. (C) Degradation of cytokines and chemokines by eye rheum was determined by using fluorescence bead based immunoassay ($n = 4-6$). Two way ANOVA was used to evaluate differences in mean, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

activation because of the presence of $\text{Cl}^-/\text{HCO}_3^-$ antiporters. This was further confirmed by the anion channel inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate inhibiting the bicarbonate-induced NET release in a time- and dose-dependent manner.⁷ The effect of pH on capacity of neutrophils to form NETs was later confirmed by other researchers.⁵³⁻⁵⁵ Furthermore, e.c. acidification prolongs the neutrophil survival, ROS production is inhibited, and phagocytosis is increased, but phagolysosomal killing is decreased.⁵⁶

On the basis of these observations, we consider that neutrophils are highly sensitive cells that execute different activation programs depending on the environment they encounter. On the one hand, the pH of closed eye tear film inhibits NETs formation, while hypoxia, high concentration of the anaphylatoxin C5a, and bacterial components lead to degranulation. On the other hand, open eye alkaline tear film and bacterial LPS promote NETs formation. Taken together, our findings lead us to propose a mechanism for eye rheum formation. Complement activation in closed eye tear film results in recruitment of neutrophils. Hypoxia and C5a in closed eye lead to neutrophil degranulation on the ocular surface. After eye opening, the pH of the tear film increased rapidly due to the loss of CO_2 , which favors bulky NET formation appearing as the viscous turbid layer on the ocular surface. Blinking action of eyelid aggregates this material to form complex eye rheum containing viable neutrophils, bacteria, and waste clumped together by NETs ready for discharge mostly at the inner angle of the eye.

3.5 | Eye rheum degrades inflammatory mediators by aggNET-borne serine proteases

Although the ocular surface is continuously infiltrated and patrolled by neutrophils, it usually does not show the canonical signs of inflammation. Because aggNETs reportedly degrade proinflammatory mediators through an intrinsic proteolytic activity,¹⁰ we analyzed the proteolytic potential of eye rheum. We observed robust NE activity in the eye rheum that was inhibited by the specific elastase inhibitor Sivelestat (Fig. 6A). This suggests that eye rheum has proteolytic potential similar to that reported for aggNETs induced by MSU crystals.^{57,58} CG is another serine protease in azurophilic granules of neutrophils. We robustly detected the activity of this enzyme in eye rheum samples employing a colorimetric substrate (Fig. 6B).

We then incubated eye rheum with a mixture of cytokines and chemokines and monitored their decay. The increased level of IL-8 in the presence of eye rheum can be attributed to their release from activated neutrophils,¹⁰ from aggNETs,⁵⁷ and from eye rheum (Supplementary Fig. S2C). IL-12, IL-6, IL-10, IL-17A, IL-23, and MCP-1 were strongly degraded by the proteolytic action of eye rheum (Fig. 5D). The proteolytic activity of neutrophil serine proteases reportedly served as regulator of inflammation by controlling signaling through modulation of chemokines, cytokines, and their cell surface receptors.⁵⁹ In a model of gout and MSU-induced inflammation, we have found that aggNETs resolved neutrophilic inflammation by degrading cytokines and chemokines and, thereby, prevent overshooting, uncontrolled neutrophil recruitment and activation.⁵⁷ The above described properties

of eye rheum highly resemble with those described for aggNETs generated after high density infiltration of neutrophils in other tissues and support a novel protective and inflammation resolving role of aggNETs on the ocular surface.

AUTHORSHIP

A.M., M.H., and L.M. planned, performed, and supervised most of the experiments and conducted data analysis; M.J.P., L.P., and F.D. performed protein analyses; G.L. performed mass spectrometry; A.G. performed confocal microscopy; L.K. and S.C. performed electron microscopy; C.M., V.K., B.H., F.B., A.J., C.S., and G.S. provided scientific input; A.M., M.H., L.M., and G.S. wrote the manuscript. All the authors read and approved the manuscript. B.H., M.H., and L.M. contributed equally to senior authorship.

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DISCLOSURE

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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