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Tear fluid neutrophils and neutrophil extracellular traps contribute to ocular graft versus host disease pathogenesis

Short title: Neutrophils and NETs in ocular GVHD patients

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Precis

Neutrophils and neutrophil extracellular traps (NETs) are abundant in the tear fluid of patients with ocular graft versus host disease and may contribute to severity of signs and symptoms of ocular surface disease. In these patients, neutrophils are hyperresponsive to stimuli that produce neutrophil extracellular traps.

ABSTRACT

Purpose: Ocular graft versus host disease (oGVHD) is a common complication in allogenic-hematopoietic stem cell transplant patients and presents as severe tear-deficient dry eye disease (DED). Previously, we demonstrated that neutrophil extracellular traps (NETs) are present on the ocular surface of tear-deficient DED patients. The purpose of the present investigation is to determine whether neutrophils and NETs in the tear fluid of oGVHD patients determine disease severity and to determine whether oGVHD patient neutrophils are abnormally responsive to stimuli that cause formation of NETs (NETosis).

Methods: Conjunctival washings from healthy subjects, pre-transplant, and definite oGVHD patients were collected and analyzed for extracellular DNA (eDNA), neutrophils, and epithelial cells. These data were correlated with the severity of signs and symptoms of DED. Isolated neutrophils from peripheral blood were stimulated, and NETosis amount was compared between groups.

Results: Patients with oGVHD who had an excess of neutrophils relative to epithelial cells in their tear fluid had significantly greater severity of ocular surface disease. eDNA, a structural component of NETs, was also present in higher amount in the tear fluid of these patients. The amount of eDNA in the tear fluid showed a significant positive correlation with the severity of patient-reported symptoms and signs of ocular surface disease. In oGVHD patients, peripheral blood neutrophils were hyperresponsive to NETosing stimuli.

Conclusions: These findings suggest that neutrophils and their extracellular products (NETs) may contribute to oGVHD pathology and make a case for investigating the clinical value of manipulating neutrophils and NETs to treat oGVHD.

Introduction

More than 50,000 hematopoietic stem cell transplant (HSCT) procedures are performed worldwide every year to treat hematological malignancies, and these numbers are increasing. It is estimated that ocular graft versus host disease (oGVHD) develops in approximately 38–50% of allogeneic HSCT recipients and usually occurs around one year following transplant. The signs and symptoms of oGVHD mimic other immunologically-mediated dry eye disease (DED) subtypes, and they severely impact quality of life. In the absence of a complete understanding of the pathogenesis of chronic oGVHD, treatment remains empirical.[1,2] Current understanding of the pathophysiological basis of oGVHD centers on destruction of lacrimal and meibomian glands by T-cell-mediated mechanisms leading to a tear-deficient DED with severe ocular surface disease. Based on our previous work that demonstrated the presence of neutrophil extracellular traps (NETs) on the ocular surface of tear-deficient DED, we hypothesize that neutrophils and their extracellular products may also contribute to ocular surface disease in oGVHD patients.

Neutrophils are recruited at low levels on the ocular surface[3] and are abundant in the tear film during ocular surface inflammation.[4] Neutrophils are key players in the host innate immune response and constitute the first line of defense.[4,5] Few discoveries in immunology have gained as much attention in the last decade as the discovery that neutrophils are able to release their extracellular DNA (eDNA) as a biologic spider web known as neutrophil extracellular traps (NETs) that can immobilize and chemotactically attract and kill pathogens. NETs are comprised of eDNA strands decorated with histones, neutrophil elastase, and cathelicidin peptides, and are formed in a process termed NETosis, which was first described by Brinkmann et al.[6] We have recently reported the role of NETs as possible sources of inflammation in tear-deficient DED, a

chronic inflammatory disorder of the ocular surface.[7,8] We reported that structural components of NETs (eDNA, neutrophil elastase, histones, and cathelicidin) are present on the ocular surface of patients with DED, along with a deficiency of tear fluid nucleases, and we showed that eDNA abundance was highest in patients with ocular Graft-vs-Host-Disease (GVHD).[8]

In this study, we evaluated the tear fluid of healthy subjects, pre-transplant patients, and oGVHD patients for the presence of neutrophils and eDNA, which we related to ocular surface disease severity. The amount of eDNA in tear fluid was used as a surrogate for NET abundance. We also determined whether neutrophils isolated from peripheral blood of patients with oGVHD produce NETs more vigorously than pre-transplant patients and healthy subjects. Our results show that oGVHD patients with abundant neutrophils in the tear fluid had more severe ocular surface disease. In addition, the neutrophils of these patients with severe ocular surface disease were hyperresponsive to NETosing stimuli, resulting in higher levels of eDNA in the tear fluid.

Materials and methods

Study population and clinical examination

Study approval was obtained from the Institutional Review Board of the University of Illinois at Chicago (UIC). Informed consent was obtained from all participants after the nature and possible consequences of the study were explained. Research was conducted in accordance with the requirements of the Health Insurance Portability and Accountability Act (HIPAA) and the tenets of the Declaration of Helsinki.

Demographic and health information including age, gender, and ethnicity was collected for each patient. All patients underwent a complete ocular surface examination by the same clinical team. Healthy subjects were age-matched volunteers without symptoms of ocular discomfort, prior history of DED, or current or past use of artificial tears or topical dry eye medication. Patients with pending bone marrow transplants were referred from the UIC Hematology Oncology division for baseline examination. After the transplant, ocular GVHD was diagnosed using the Chronic Ocular GVHD consensus scoring algorithm.[9] The parameters for diagnosis include: 1) Ocular Surface Disease Index (OSDI), 2) Schirmer's test score without anesthesia, 3) Corneal fluorescein staining, and 4) conjunctival injection. Severity scores 0, 1, 2, and 3 were assigned to OSDI, corneal fluorescein staining, and Schirmer's score. Conjunctival injection was scored 0, 1, and 2. The composite score was obtained by summing the subscores (maximum score=11). Patients diagnosed with definite oGVHD (score of ≥8 without systemic GVHD and ≥6 with

systemic GVHD) were enrolled in the study. Healthy subjects and pre-transplant patients were similarly evaluated and composite scores were calculated.

Matrix metalloproteinase 9 (MMP-9) test was performed using the InflammaDry kit (RPS Diagnostics, Sarasota, FL) according to the manufacturer's instructions. A positive result is indicated by the appearance of a red line in the result zone which indicates the presence of ≥40 ng/ml MMP-9. A positive test was scored as 1.0, and a negative test was scored as 0. Tear fluid osmolarity was measured using the TearLab Osmolarity Test (TearLab, San Diego, CA) according to the manufacturer's instructions. A measurement threshold of >300 mOsm/L was used to diagnose dry eye, whereas exact measurements were used for analysis. Ocular redness score (ORS) was calculated using Keratograph 5M (Oculus, Inc., Arlington, WA) according to the manufacturer's instruction. ORS is based on the area percentage ratio between the vessels (red) and the rest of the analyzed area (white). According to the manufacturer, the maximum ratio is 40%; therefore, the ORS that the machine generates range between 0.0 and 4.0. These measurements were used for analysis. Meibomian Gland imaging was performed using LipiView II (TearScience, Morrisville, NC) according to the manufacturer's instructions. The images were analyzed for meibomian gland truncation and drop-out and were scored (MGD score) on a scale ranging from 0–5 based on the extent of truncation and drop-out (MGD score 0: 0%; 1: <25%; 2: 25–50%; 3: 51–75%; 4: >75%; and 5: complete loss).

Tear fluid collection and analysis

Tear fluid was collected as described previously.[8] Briefly, 50 µL of artificial tear (Preservative Free Refresh Optive Sensitive, Allergan, Irvine, CA) was instilled into the lower fornix of the eye. The patient was instructed to perform ductions in all directions, and after 2 minutes the conjunctival washings were collected with a 5 µL glass microcapillary tube (Mirocaps, Drummond Scientific, Broomall, PA) and transferred to a sterile 0.2 mL microcentrifuge tube. The samples were stored on ice until further analysis.

Tear fluid extracellular DNA (eDNA) and protein concentration: eDNA was measured from the tear samples as described previously.[8] The tear fluid was mixed with a pipette, and 2 µL of tear fluid was then mixed with 98 µL of 1× TE buffer in a black 96-well plate. The λDNA was used as standard. PicoGreen dye (P11496, Thermo Fisher Scientific, Waltham, MA) was mixed with samples according to the manufacturer's instructions. The fluorescence intensity was measured with a Cytation 5 plate reader over the course of 20 minutes. The amount of eDNA was calculated based on the standard λDNA values and expressed as mean ± standard error of the mean (SEM; µg/mL).

Tear fluid cytology: With a pipette, 2 µL of tear fluid was mixed with 10 µL of balanced saline solution. To this mix, 12 µL of acridine orange/propidium iodide (CS2-016, Nexcelom Biosciences, Lawrence, MA) staining solution was added, and the number of viable and total cells were counted using Cellometer K2 (Nexcelom Bioscience). The results are expressed as mean number of cells/mL ± SEM. The tear fluid was smeared onto a silane-coated glass slide (Lab Scientific, #7801) and air dried. The cells were fixed with 4% paraformaldehyde for 20 min in phosphate-buffered saline at room temperature (RT) as described previously.[11] Primary

antibodies used are monoclonal mouse anti-human neutrophil elastase (dilution 1:100; Dako; Ref-M0752) and rabbit polyclonal keratin 14 (dilution 1:1000; Biolegend; #905301). The specificities of the primary antibodies have previously been validated.[7,8,9] Secondary antibodies used are donkey anti-mouse Alexa Fluor 594 IgG (dilution 1:1000; Jackson ImmunoResearch Lab; No. 715-585-150) and donkey anti-rabbit Alexa Fluor 488 IgG (dilution 1:1000; Jackson ImmunoResearch Lab; No. 711-546-152). The stained cells were analyzed using a Zeiss LSM 710 confocal microscope, and images were further processed with the Zeiss LSM Image Software. To characterize the cellular origin (donor or recipient) of the ocular surface eDNA, fluorescence in situ hybridization (FISH) was performed on a subset of specimens. Donor and recipient sex for patients were reviewed for donor-recipient sex mismatch. Cases in which a female patient received stem cell transplant from a male donor were identified. Vysis CEP X SpectrumOrange/Y SpectrumGreen Direct Labeled Fluorescent DNA Probe Kit (Abbott Laboratories. Des Plaines, IL), was used according to manufacturer instructions and hybridization signals were imaged.

Neutrophil isolation and analysis

For each patient, 16 mL of peripheral blood was collected in BD vacutainer sodium heparin tubes (BD Biosciences, # 367878) and immediately transferred to the lab for isolation of neutrophils.

Isolation of neutrophils: Neutrophils were isolated from peripheral blood using MACSxpress neutrophil isolation kit (Miltenyi Biotec, San Diego, CA, #130-104-434) according to the

manufacturer's instructions. Residual erythrocytes were removed by adding MACSxpress erythrocyte depletion reagent (MACSxpress erythrocyte depletion reagent kit, Miltenyi Biotech, #130-094-183). The cell pellet was resuspended with 3 mL of serum-free RPMI 1640 medium without phenol red (GIBCO, #11835-030). The purity of the neutrophils was evaluated by flow cytometry. After isolation, neutrophils were stained with CD15-PE (Clone VIMC; Miltenyi Biotec, #130-092-375) and CD16-APC (clone VEP13; Miltenyi Biotec, #130-091-246) antibodies (1:11 dilution) for 10 min and analyzed by flow cytometry (UIC core facility, Chicago, IL).

NETosis assay: NETosis was quantified from freshly isolated neutrophils using Sytox Green plate reader assay as described previously.[10] For the assay, cells were seeded at 20,000 cells/well in a 384-well black flat clear-bottom plate in the presence of 1 μM cell-impermeable nucleic acid stain Sytox Green (Thermo Fisher Scientific, #S7020) using a robotic pipetting system (epMotion507; Eppendorf North America, Hauppauge, NY). Neutrophils were stimulated with various doses of PMA (0, 1, 10 and 100 nM) and sodium chloride (0, 20, 40, 60 and 80 mM NaCl for hyperosmolar stress), and the kinetic fluorescence intensity was measured every 20 min over 12 h using Cytation5 imaging multi-mode reader (BioTek-U.S., Winooski, VT, USA) with a filter setting of 485 nm (excitation)/527 nm (emission). During the 12-h measurement, the plate reader was set at 37°C and supplied with 5% CO2. Unstimulated neutrophils were used as controls. For calculating total amount of NETosis during the 12 hour period (fluorescence intensity arbitrary units (AU) x hour), area under the curve (AUC) was determined using GraphPad prism 6 software (GraphPad Prism 6, GraphPad, Inc., La Jolla, CA). For imaging NETosis, neutrophils were stimulated with 1 nM PMA or 80 mM NaCl. Unstimulated cells were

used as controls. Neutrophils were mixed with 2.5 μM Vybrant DyeCycle Ruby (Life Technologies, #V10273), which is a membrane-permeable dye that binds dsDNA and serves as a nuclear stain, and 1 μM Sytox Green (Life Technologies, #S7020). Cells were imaged with the IncuCyte Zoom system (Essen Bioscience, Ann Arbor, MI).

Serum eDNA assay: We collected 5 mL of peripheral blood in serum blood collection tubes (BD Biosciences, #367815) and allowed it to stand at RT for 30 min. The sample was then centrifuged at 3000 rpm for 10 min at RT. The supernatant was transferred to a microcentrifuge tube, and the eDNA was measured using the PicoGreen dye kinetic assay as described above for tear fluid eDNA.

Statistical Analysis

Data was compiled using Microsoft Excel Office statistics software (Redmond, WA). For each patient in the pre-transplant and oGVHD groups, only one eye was included in the analysis. We chose the eye with worse ocular surface disease (worse total corneal and conjunctival staining score). In case of similar ocular surface disease in both eyes, we chose the eye (in descending order of priority) with higher tear fluid osmolarity, greater Schirmer's I tear deficiency, worse MGD score, and higher number of cells in tear fluid. The arithmetic means and standard errors of the means were calculated for all quantitative parameters. Univariate regression analysis was performed to calculate a correlation coefficient between each clinical marker and tear film eDNA concentration. Quantitative variables between different groups were compared using a Student’s t-test. $P<0.05$ was considered statistically significant. The results are expressed as mean ± SEM.

Results

Clinical features and NETs in oGVHD patients

Diagnosis of 'definite' ocular GVHD was made using the International Chronic Ocular GVHD Consensus Group classification.[9] Typical clinical features of patients with ocular GVHD include: 1) conjunctival injection (Figure 1A1), 2) conjunctival lissamine green staining (Figure 1A2), 3) corneal fluorescein staining (Figure 1A3), and 4) mucoid debris (Figure 1A4). NETs were present in the tear fluid of patients with oGVHD (Figure 1B1-B4). The mucoid debris was composed of numerous neutrophils and ocular surface epithelial cells (Figure 1C1-C4). In order to differentiate between donor and host origin of the neutrophils and eDNA, we performed FISH on cells from a patient receiving a stem cell transplant from a sex-mismatched donor (XY male donor, XX female host). Cells stained with DAPI (blue) showed that multilobed nuclei (neutrophils) had X (red) and Y (green) staining, confirming their donor origin (Figure 1D1-D2). The eDNA strands had XY staining (Figure 1D3), indicating that the eDNA and NETs in mucoid films of oGVHD patients are donor neutrophil-derived.

Our goal for this study was to compare the clinical features and tear fluid analysis in patients with oGVHD with those of pre-transplant patients to assess the role of neutrophils and their products (eDNA and NETs). First, we compared the demographic and clinical profiles of pre-transplant patients (n=20) with those of oGVHD patients (n=33). As a control, we also compared oGVHD patients to age-matched healthy subjects (n=10). The average age of the oGVHD patients (n=33; 50.7 ± 2.6; Table 1) was similar to that of the pre-transplant patients (n=20; 48.7

± 3.2; $p>0.05$) and healthy subjects (n=10, 54.2 ± 1.4) Although the pre-transplant and oGVHD patients were age-matched, we found significant differences in their clinical features. Symptom analysis using the OSDI revealed that patients with oGVHD had significantly greater symptoms of ocular discomfort (44.9 ± 4.8; Table 2) compared to pre-transplant patients (5.4 ± 2.6; $p<0.05$) and healthy subjects (0.62 ± 0.43). All of the clinical signs were also significantly worse in oGVHD patients compared to other groups (ORS, MGD score, Schirmer I, MMP-9 test, corneal staining, and conjunctival staining; Table 2). The composite score, which is an indicator for overall severity of the disease, was also significantly greater in oGVHD patients (7.5 ± 0.4) compared to the pre-transplant group (2.0 ± 0.4; $p<0.05$).

Extracellular DNA (eDNA) is elevated in oGVHD patients

NETs on the ocular surface contribute to the presence of extracellular DNA (eDNA) in tear fluid. We compared the level of eDNA between healthy, pre-transplant, and oGVHD patients using PicoGreen assay. The amount of eDNA was significantly higher in oGVHD patients (7.25 ± 1.86 μg/mL) compared to pre-transplant patients (1.85 ± 0.37; $p<0.05$) and healthy subjects (1.47 ± 0.16; $p<0.05$; Figure 2A). Serum eDNA was similar in all groups (Figure 2B). Next, we used univariate regression analysis to determine the correlation between tear fluid eDNA and clinical signs, symptoms, and results of tear film analysis (Table 3). The concentration of tear fluid eDNA showed a significant positive correlation with OSDI (r=0.45), ORS (r=0.45), corneal staining (r=0.35), and the composite score (r=0.42).

Tear fluid cells in ocular GVHD patients

Conjunctival washings were analyzed with acridine orange/propidium iodide (AO/PI) to identify live and dead cells (Figure 3A1-A3). Total tear fluid cells were significantly higher in oGVHD patients ($3.54e^{+005} \pm 1.11e^{+005}$) compared to pre-transplant patients ($5.85e^{+004} \pm 1.25e^{+004}$; $p<0.05$) and healthy subjects ($3.69e^{+004} \pm 4.70e^{+003}$; $p<0.05$; Figure 3B). The number of viable tear fluid cells was also significantly increased in oGVHD patients ($1.72e^{+005} \pm 5.86e^{+004}$) compared to pre-transplant patients ($1.91e^{+004} \pm 3.81e^{+003}$; $p<0.05$) and healthy subjects ($1.89e^{+004} \pm 2.35e^{+003}$; $p<0.05$; Figure 3C).

We immunostained tear fluid cells with antibodies specific for neutrophils (N) and epithelial cells (E) and found that patients with oGVHD either had an excess of neutrophils (N>E: 52.06 ± 17.23 neutrophils and 27.50 ± 11.53 epithelial cells per 20× field; n=16; Figure 4A-4B) or an excess of epithelial cells (E>N: 8.41 ± 3.52 neutrophils and 29.82 ± 12.25 epithelial cells per 20× field; n=17; Figure 4C) in their tear fluid. We compared the clinical features and tear fluid analysis of these two oGVHD subsets (N>E and E>N). Tear fluid eDNA was significantly higher in the N>E subset (6.74 ± 1.35) compared to the E>N subset (3.74 ± 0.64; $p<0.05$; Figure 4D). However, the serum eDNA amount was similar in both oGVHD subsets (Figure 4E). Patients in the N>E oGVHD subset had more severe disease as evidenced by a significantly higher composite score (8.50 ± 0.51) compared patients in the E>N oGVHD subset (6.65 ± 0.51, $p<0.05$; Table 4). The clinical signs that were significantly worse in N>E oGVHD subset include: 1) ORS, 2) corneal staining, and 3) MMP-9 test (Table 4).

Enhanced NETosis in oGVHD patients

We determined whether neutrophils in oGVHD patients are hyperresponsive to stimuli known to produce NETosis (PMA and hyperosmolarity). We used a negative selection technique to obtain >98% pure neutrophils from peripheral venous blood. We measured both onset of NETosis (time for more than 10% increase) and total NETosis over a 12-h period (by area under curve, AU hour) using a Sytox Green kinetic assay that detects eDNA released from neutrophils in response to the stimuli (PMA or hyperosmolarity). With graded PMA stimulation (1–100 nM) we observed increasing NETosis with greater PMA stimulation (Figure 5B-5D). We compared the data obtained with 1 nM PMA stimulation between oGVHD patients (n=10), pre-transplant patients (n=10), and healthy subjects (n=10). The amount of NETosis over 12 h was also significantly greater in oGVHD patients ($7.82e^{+007} \pm 4.84e^{+006}$ AU hour) compared to pre-transplant patients ($5.64e^{+007} \pm 6.23e^{+006}$ AU hour; $p<0.05$) and healthy subjects ($6.19e^{+007} \pm 3.43e^{+006}$ AU hour; $p<0.05$; Figure 5E). The onset of NETosis also occurred significantly earlier in oGVHD patients (170 ± 12.38 min) compared to pre-transplant patients (216 ± 10.67 min; $p<0.05$) and healthy subjects (234 ± 8.45 min; $p<0.05$; Figure 5F). With graded hyperosmolar stimulation (0–80 mM NaCl) we observed an increasing amount of NETosis with greater hyperosmolar stimulation (Figure 6A1-6A4, B-D). We compared the data obtained with 80 mM NaCl (420 mOsM) stimulation between oGVHD patients, pre-transplant patients, and healthy subjects. the amount of NETosis over 12 h was significantly greater in oGVHD patients ($4.83e^{+007} \pm 4.60e^{+006}$ AU hour) compared to pre-transplant patients ($3.14e^{+007} \pm 4.63e^{+006}$ AU hour; $p<0.05$) and healthy subjects ($3.15e^{+007} \pm 3.71e^{+006}$ AU hour; $p<0.05$; Figure 6E). However, the onset of NETosis was similar in all groups (Figure 6F).

Discussion

In the present study, we report several findings that increase our understanding of the role of neutrophils and their extracellular products in oGVHD pathogenesis. First, we found that patients with oGVHD who had an excess of neutrophils relative to epithelial cells in their tear fluid had more severe ocular surface disease. eDNA, a structural component of NETs, was also present in higher amount in the tear fluid of these patients. Second, the amount of eDNA in the tear fluid correlated with the severity of patient-reported symptoms and signs of ocular surface disease. Finally, in oGVHD patients, peripheral blood neutrophils were hyperresponsive to NETosing stimuli. Taken together, these findings suggest that neutrophils and their extracellular products (NETs) may contribute to oGVHD pathology and make a case for investigating the clinical value of manipulating neutrophils and NETs to treat oGVHD. Potential therapeutic strategies may include: 1) enhancing the clearance of NETs from the ocular surface using nucleases; 2) reducing the responsiveness of neutrophils to NETosing stimuli; and 3) reducing the egress of neutrophils onto the ocular surface.

The ocular surface epithelium undergoes continuous, dynamic turnover,[11,12] that is increased in tear-deficient dry eye.[18] Superficial corneal cells are shed into the precorneal tear film in a process that is regulated by apoptotic mechanisms.[19,20] Dead or dying cells recruit neutrophils by increasing expression of genes in the TLR9-MyD88 signaling pathway.[21-23] Neutrophil recruitment is linked inextricably to TLR9-MyD88 signaling. Once recruited, tear hyperosmolarity may simulate neutrophils to form NETs. NETosis is a unique neutrophil response in which nuclear DNA, histones, neutrophil elastase, and cathelicidin emerge from the cell in a spider's web-like structure.[24,25] Molecular stimulators of NETosis that are abundant in

343 tear fluid of oGVHD patients (e.g. IL8 and TNF-α) may also contribute to this process. In a
344 hyperosmolar milieu, the classical neutrophil-related innate defense mechanisms seem to be
345 compromised. [26-31] Viewed in this context, the release of NETs from neutrophils in response to
346 hyperosmolarity may compensate for the loss of classical neutrophil-related innate defense
347 mechanisms.
348
349 Our data show that peripheral blood neutrophils are hyperresponsive to NETosing stimuli in
350 oGVHD patients, and NETosis was significantly higher in oGVHD patients compared to healthy
351 or pre-transplant patients when induced with PMA or hyperosmolarity. Other blood cells are also
352 known to be hyperresponsive in chronic GVHD. B cells isolated from patients with chronic
353 GVHD have the capacity to respond more readily to BCR stimulation compared to B cells from
354 patients without disease.[32] In tear-deficient oGVHD patients, clearance of NETs from the ocular
355 surface may also be impaired due to absence of nuclease production by the lacrimal gland.
356 Increased production of NETs (due to hyperresponsive neutrophils in hyperosmolar tear fluid)
357 and reduced clearance (due to deficiency of tear fluid nucleases) may have contributed to
358 accumulation of NETs over the ocular surface leading to more severe disease in oGVHD
359 patients. NET components are known to have toxic effects on cells through multiple pathways.
360 For example, histones can cause direct cytotoxicity to epithelial cells[33] and are major mediators
361 of cell death in sepsis.[34] Cathelicidin peptide fragments can cause inflammation, erythema, and
362 telangiectasia, particularly in patients with rosacea.[35] Neutrophil elastase induces epithelial cell
363 apoptosis.[36] eDNA can also contribute to DED pathogenesis by re-entering a cell and binding
364 intracellular receptors to stimulate downstream signaling pathways.[37] This process can be aided
365 by cathelicidin, which binds eDNA and enhances its intracellular entry.[38] Once inside the cell,

366 the DNA binds TLR9 to stimulate signaling through MyD88, which initiates a signaling cascade
367 leading to an IFN-type I response. Type I IFNs (IFN-α/β) augment dendritic cell maturation and
368 activate the adaptive immune system. In this way, eDNA links the innate and adaptive immune
369 mechanisms.[39,40] Our data show that the composite score (measure of overall severity of
370 oGVHD), symptom analysis score, corneal staining and bulbar redness score (measure of ocular
371 surface disease), MMP-9 test (measure of inflammatory protein in tears), and eDNA amount
372 (measure of NETs in tears) were significantly greater in oGVHD patients who had an excess of
373 neutrophils in their tear fluid. Given the known toxic effects of the molecular components of
374 NETs, we hypothesize that excessive neutrophils in tear fluid of oGVHD patients may contribute
375 to the pathogenesis of ocular surface disease. In summary, our data implicate neutrophils and
376 their products (eDNA and NETs) in the pathogenesis of oGVHD.
377

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480

Table 1: Demographic data for pre-transplant and oGVHD patients

	Pre-transplant	**oGVHD**
Patients (n)	20	33
Age (average)	48.7	50.7
Male: Female	1.5:1	2.3:1

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Table 2: Clinical features of pre-transplant and oGVHD patients

	Pre-transplant	oGVHD	*P**
OSDI	5.4	44.9	<0.05
Ocular Redness Score	0.4	1.5	<0.05
Osmolarity (mOsM/L)	312.6	304.2	NS
MGD Score	1.0	2.1	<0.05
Schirmer I (mm/5 min)	19.4	3.9	<0.05
MMP9 test	0.2	0.9	<0.05
Corneal Staining	1.0	6.7	<0.05
Conjunctival Staining	0.7	5.1	<0.05
Composite score	2.0	7.5	<0.05

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Table 3: Correlation of tear fluid eDNA level (normalized, µg/mL) with clinical signs and symptoms.

	R value	***P****
OSDI	0.4531	<0.05
Ocular Redness Score	0.4509	<0.05
Osmolarity (mOsM/L)	-0.2217	NS
MGD Score	0.2097	NS
Schirmer I (mm/5 min)	-0.2874	<0.05
MMP9 test	0.2755	<0.05
Corneal Staining	0.3485	<0.05
Conjunctival Staining	0.1605	NS
Composite Score	0.4278	<0.05

488

Table 4: Comparison of clinical signs and symptoms in neutrophil excess (N>E) and epithelial excess (E>N) subsets of oGVHD patients.

	N > E (n=16)	**E > N (n=17)**	***P****
OSDI	51.5	38.7	NS
Ocular Redness Score	1.8	1.2	<0.05
Osmolarity (mOsM/L)	297.2	310.3	<0.05
MGD Score	2.0	2.1	NS
Schirmer I (mm/5 min)	2.5	5.2	NS
MMP9 test	1.0	0.8	<0.05
Corneal Staining	8.4	5.2	<0.05
Conjunctival Staining	5.4	4.8	NS
Composite Score	8.5	6.6	<0.05

489 N: Neutrophils; E: Epithelial Cells

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Figure legends:

Figure 1: Clinical features and NETs in a patient with oGVHD.

(A1-A4): (A1) Photograph showing ocular redness pattern of an eye. (A2) Conjunctival surface staining with lissamine green. (A3) Corneal surface staining with fluorescein staining. (A4) An oGVHD patient's eye showing mucoid debris. Inlet shows the enlarged view of the mucoid debris. (B1-B3): Tear fluid cells from an oGVHD patient immunostained with antibodies specific for: (B2) neutrophils (neutrophil elastase, red) and (B3) epithelial cells (keratin 14, green). The nuclear material was stained with DAPI (blue). (B4) Overlay image of B1-B3. Neutrophil elastase co-localized with extracellular DNA confirming the presence of NETs. C1-C4: Immunostaining of a mucoid film of an oGVHD patient showing: (C2) neutrophils (neutrophil elastase, red) and (C3) exfoliated conjunctival/corneal cells (keratin 14, green). (C1) Nuclei were stained with DAPI (blue). (C2) Neutrophils were stained with neutrophil elastase (red). (C4) Overlay image of C1-C3. (D1-D3): Using a DNA probe kit (red =X and green =Y) we performed fluorescence in situ hybridization (FISH) in a patient receiving a stem cell transplant from a sex-mismatched donor (XY male donor, XX female host). Cells with DAPI stained (blue) multilobed nuclei (neutrophils) had X (red) and Y (green) staining, confirming them being donor derived. (D1): Extracellular DNA strands also had XY staining (D3). Scale bars: (D1): 250 μm; (D2-D3): 10 μm.

Figure 2: Quantifications show tear and serum eDNA amount.

(A) Graph showing the amount eDNA in tear fluid of healthy subjects, pre-transplant, and oGVHD patients. Tear fluid eDNA in oGVHD patients was significantly higher than the other two groups. (B) Graph showing amount of eDNA in serum from healthy, pre-transplant, and

oGVHD groups. No significant difference was observed among these groups. For (A) and (B), *p<0.05.

Figure 3: Images and related quantifications show cell numbers from conjunctival washings.

(A1-A3) Acridine orange/propidium iodide (AO/PI) images of conjunctival washings. (A1) Brightfield image. (A2) Live cells stained with acridine orange (green). (A3) Dead cells stained with propidium iodide (red). (B) Graph showing the total number of cells from conjunctival washings. In oGVHD groups, total cell number was significantly increased compared to healthy and pre-transplant groups (n=20 eyes/healthy; n=20 eyes/pre-transplant and n=33 eyes/oGVHD group). (C) Graph showing the number of viable cells from conjunctival washings. (n=20 eyes/healthy; n=20 eyes/pre-transplant and n=33 eyes/oGVHD groups). For (B) and (C), *p<0.05.

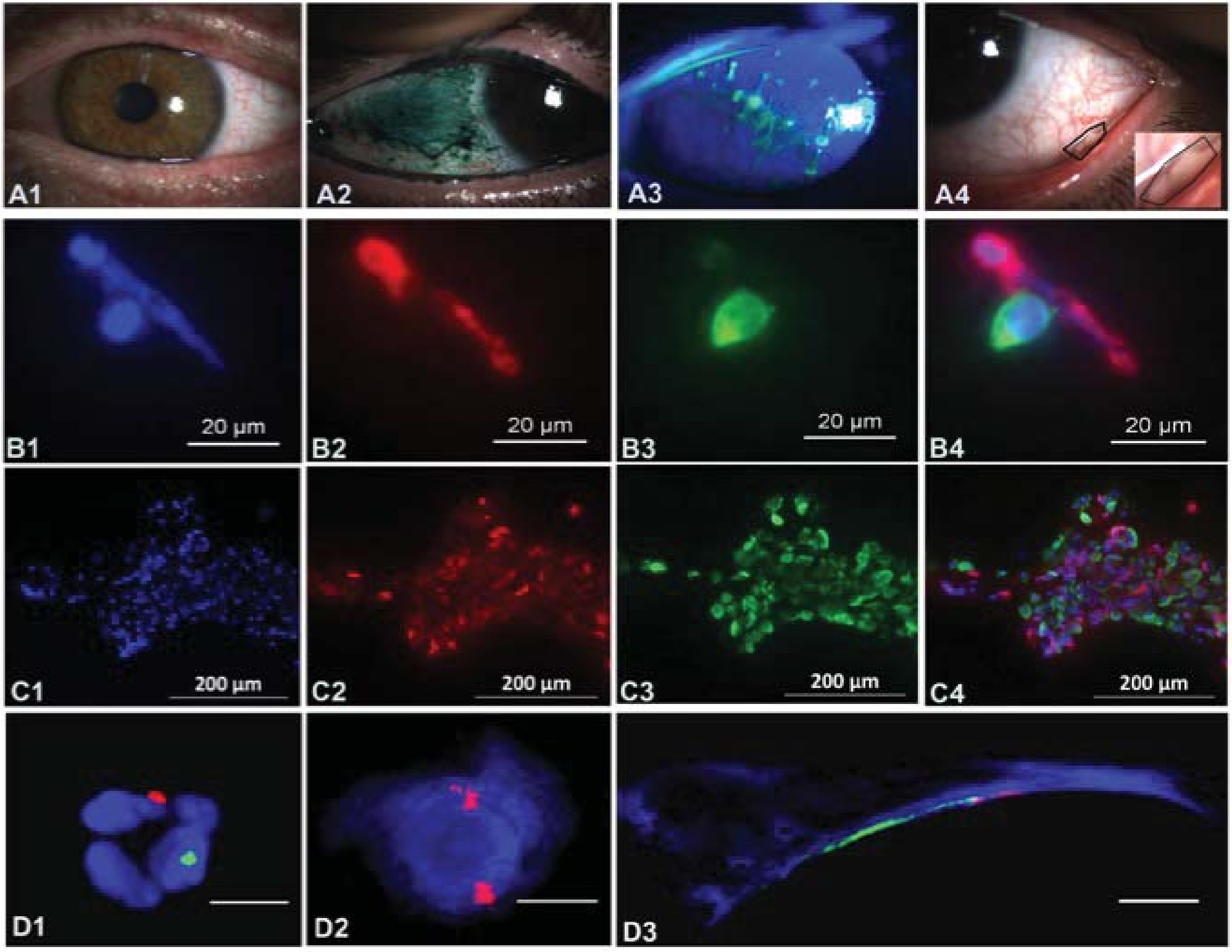
Figure 4: Images show neutrophils and epithelial cells from conjunctival washings, and quantifications relate cell type ratios to eDNA levels.

(A, B) Representative immunostaining images showing (A) neutrophil excess and (B) epithelial cells excess in two oGVHD subsets. (C) Graph showing the number of neutrophils and epithelial cells present in two oGVHD groups (n=16 eyes/N>E group; n=17 eyes/E>N group); ***p<0.0005. (D) Graph showing the tear eDNA of two oGVHD subsets. The tear eDNA was significantly higher in N>E group (n=16/N>E and n=17/E>N group); *p<0.05. (E) Graph showing the serum eDNA of two subsets of oGVHD patients.

Figure 5: Images and quantifications show NETosis stimulated by PMA.

(A) At left, flow cytometry data showing the purity of isolated neutrophils from peripheral blood. Neutrophils were stained with PE-CD15 and APC-CD16. At right, representative images showing NETosing neutrophils. Neutrophils were stimulated with 1 nM PMA and stained with Vybrant DyeCycle Ruby (red) stain and Sytox Green (green). Images were captured at 0, 2, 4 and 6 h post-stimulation. NETosing neutrophils are stained green due to compromised membrane integrity. The nuclei are stained red. (B-D): Representative kinetic curves showing NETosis induced with various doses of PMA as indicated for (B) healthy, (C) pre-transplant, and (D) oGVHD subjects. (E) Graph showing the amount of NETosis induced with 1 nM PMA. A significant increase in NETosis was observed in the oGVHD group (n=10/group). (F) Graph showing the onset of NETosis (min) from healthy, pre-transplant and oGVHD subjects. NETosis occurs early in oGVHD patients (n=10/group). For both (E) and (F), *$p<0.05$.

Figure 6: Images and quantifications show NETosis stimulated by hyperosmolar stress. (A1-A4): Representative images showing NETosing neutrophils. Neutrophils were stimulated with hyperosmolar stress (420 mOsM) and stained with Vybrant DyeCycle Ruby (red) stain and Sytox Green (green). Images were captured at 0, 4, 6 and 8 h. The NETosing neutrophils are stained green due to compromised membrane integrity. The nuclei are stained red. (B-D): Representative kinetic curve showing NETosis stimulated with various doses of NaCl as indicated for (B) healthy, (C) pre-transplant, and (D) oGVHD subjects. (E) Graph showing total amount of NETosis induced with hyperosmolar stress (420 mOsM; n=10 patients/group). (F) Graph showing the onset of NETosis (n=10 patients/group). *$p<0.05$


A1
A2
A3
A4
B1
20 µm
B2
20 µm
B3
20 µm
B4
20 µm
C1
200 µm
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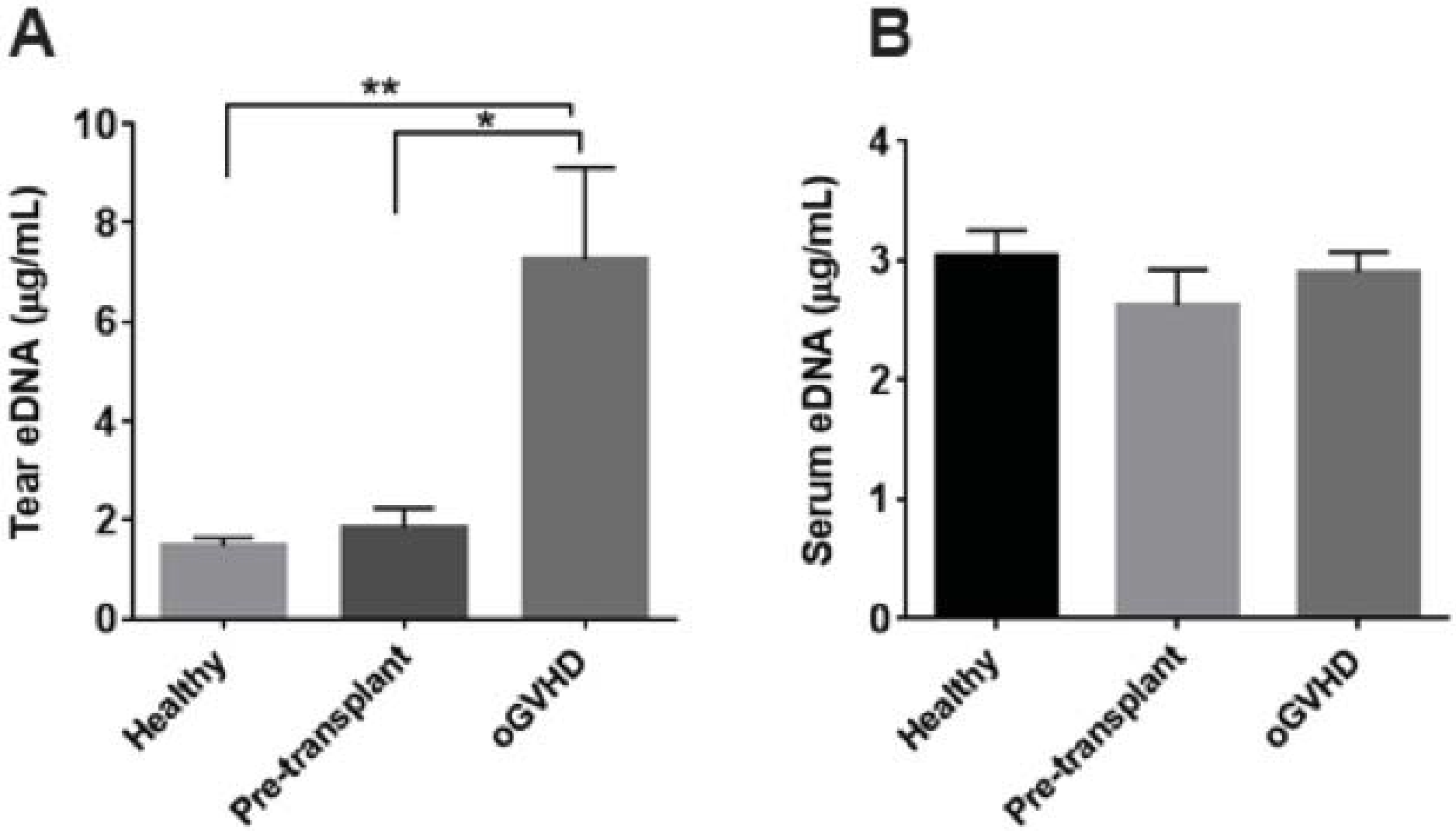
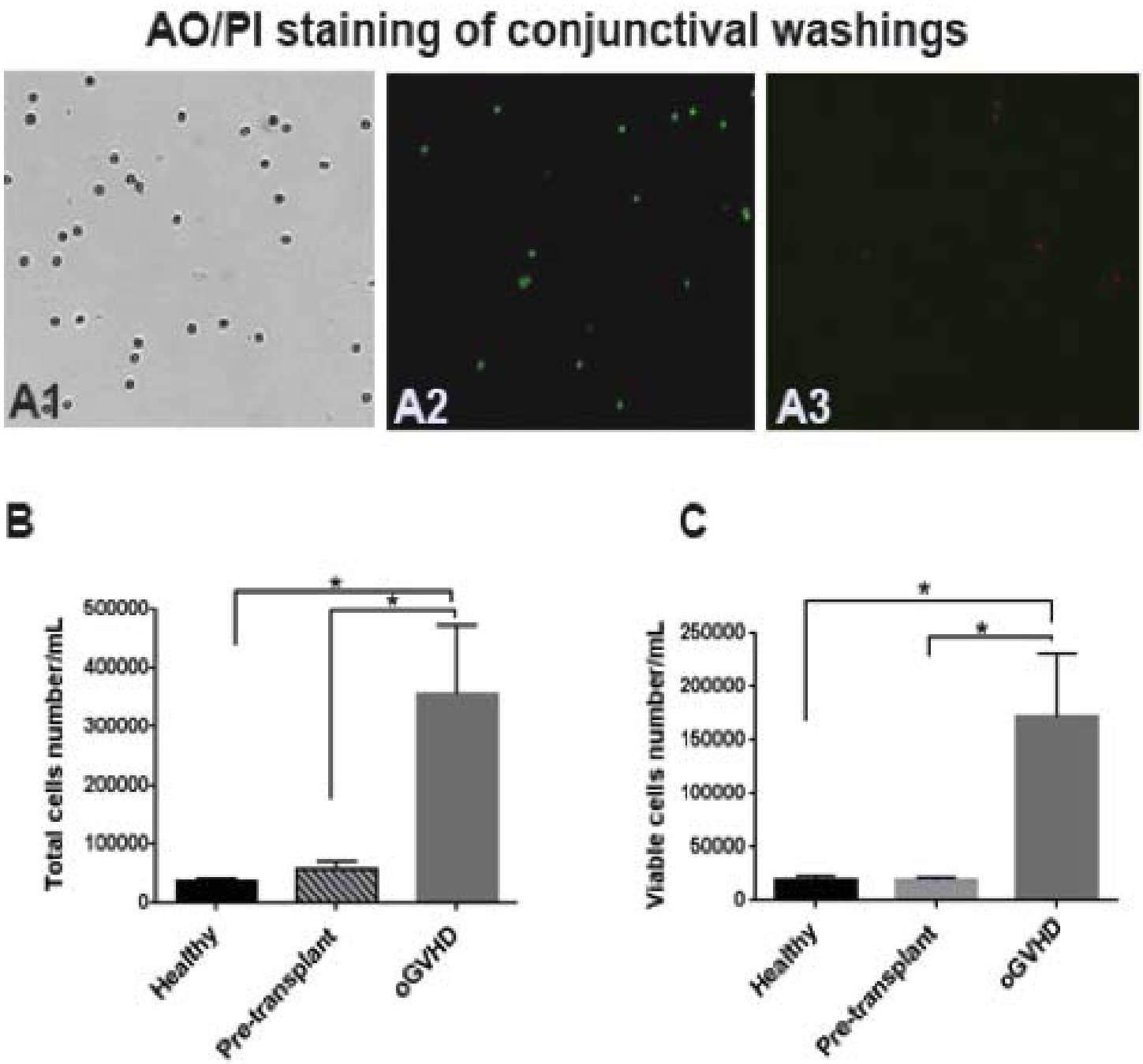
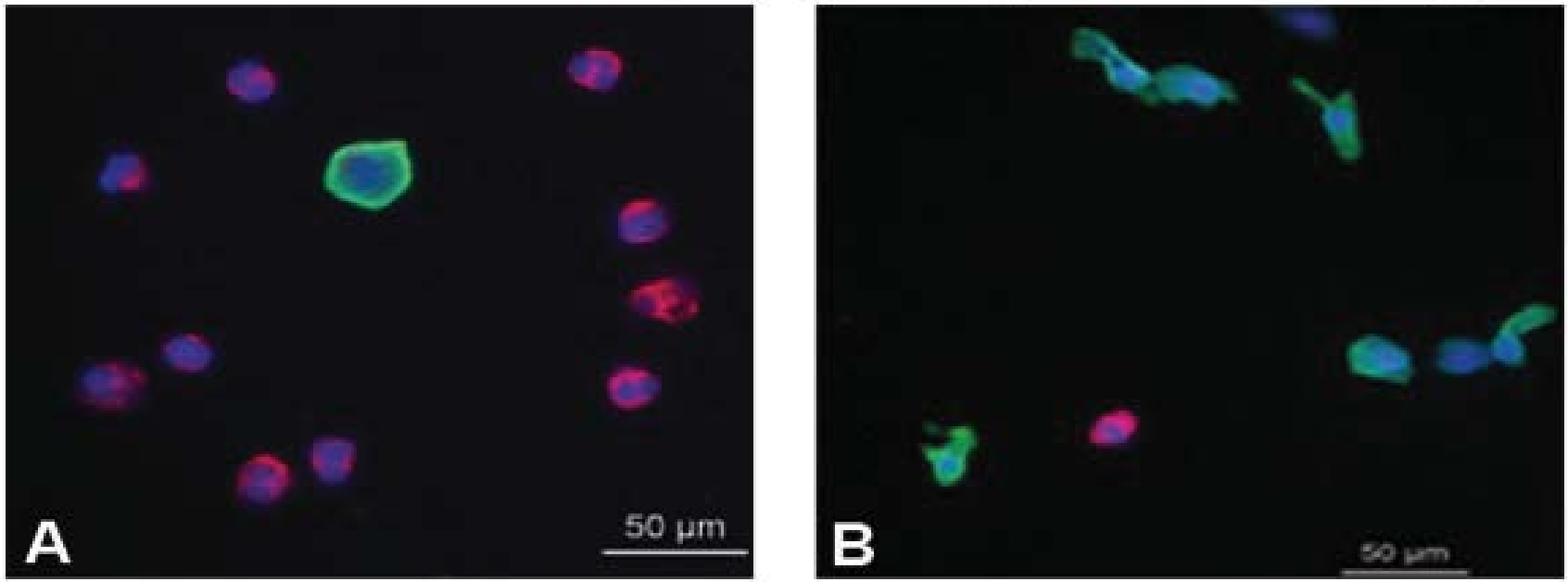
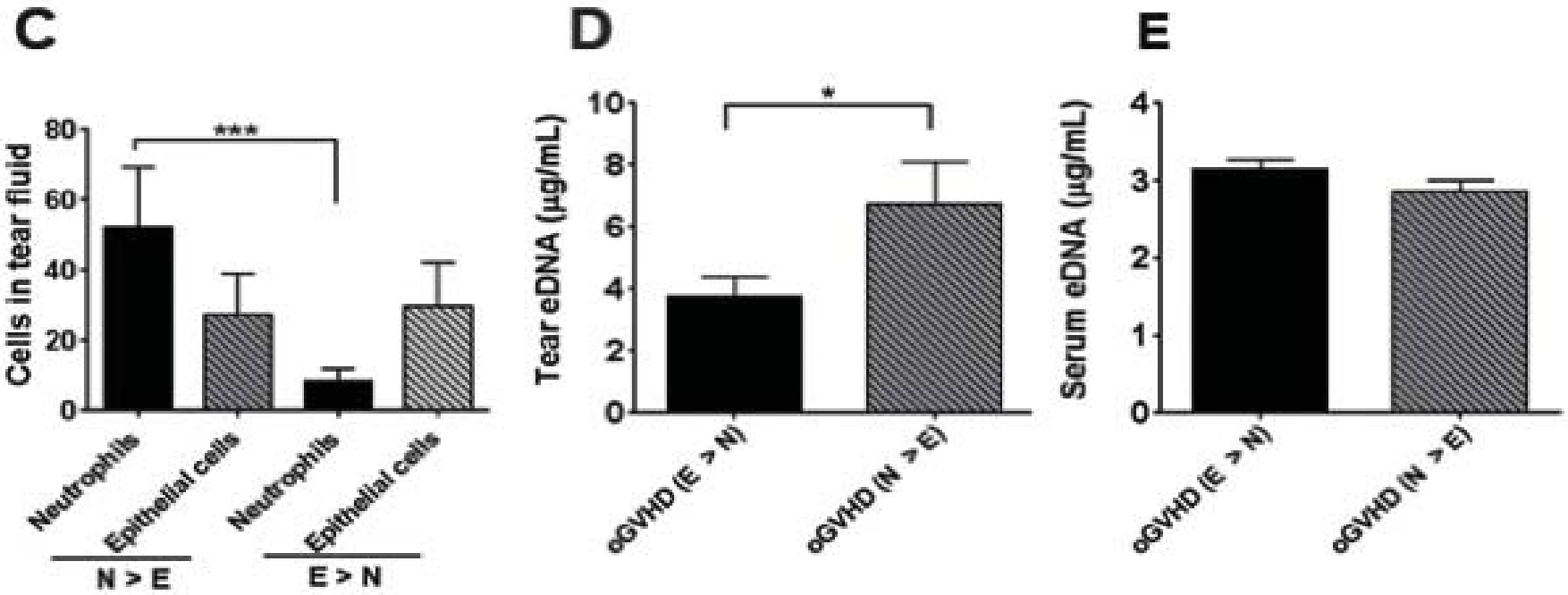
200 µm
C3
200 µm
C4
200 µm
D1
D2
D3

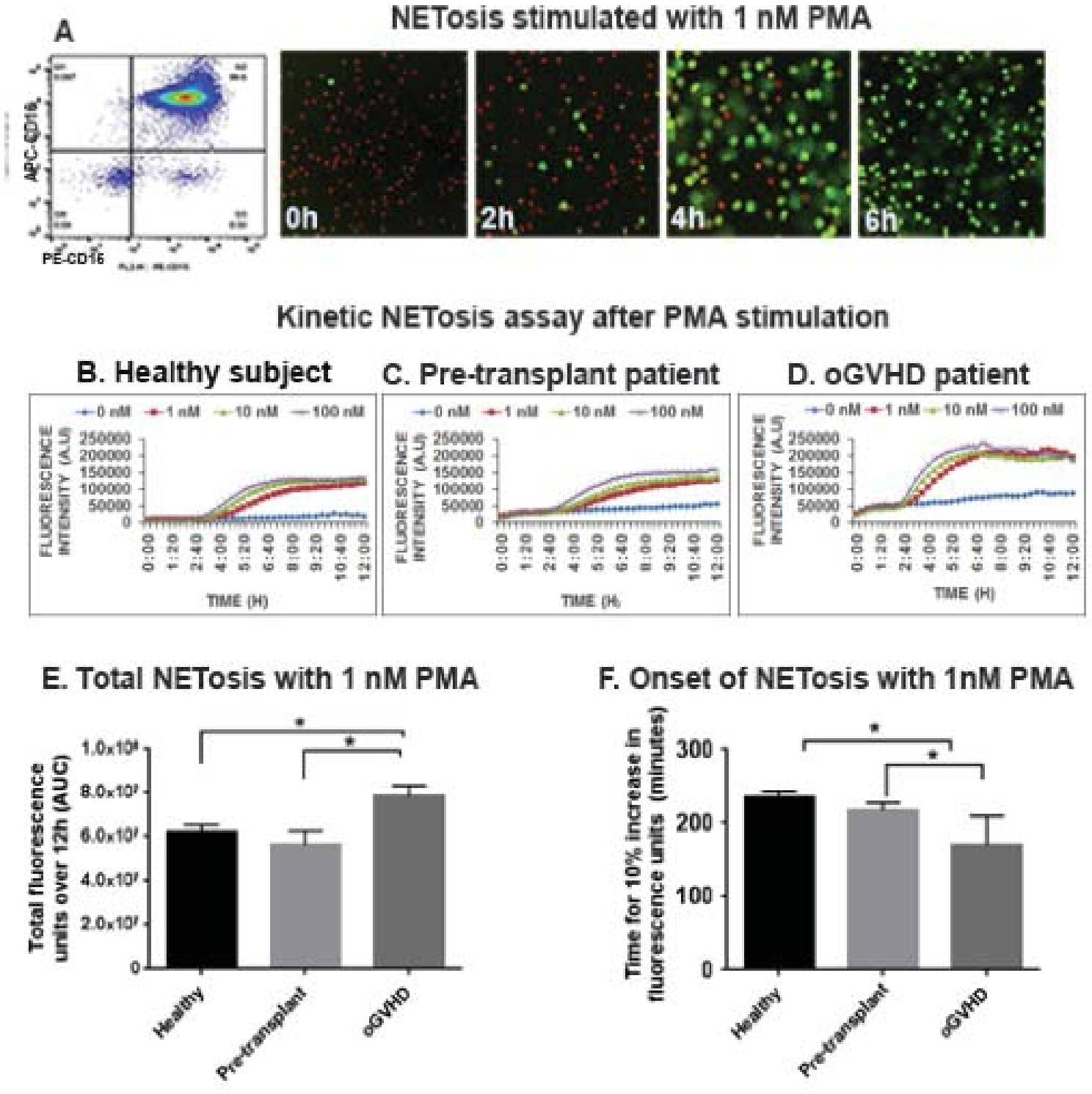

A
**
*
Tear eDNA (µg/mL)
10
8
6
4
2
0
Healthy
Pre-transplant
oGVHD
B
Serum eDNA (µg/mL)
4
3
2
1
0
Healthy
Pre-transplant
oGVHD


AO/PI staining of conjunctival washings
A1
A2
A3
B
Total cells number/mL
500000
400000
300000
200000
100000
0
*
*
Healthy
Pre-transplant
oGVHD
C
Viable cells number/mL
250000
200000
150000
100000
50000
0
*
*
Healthy
Pre-transplant
oGVHD

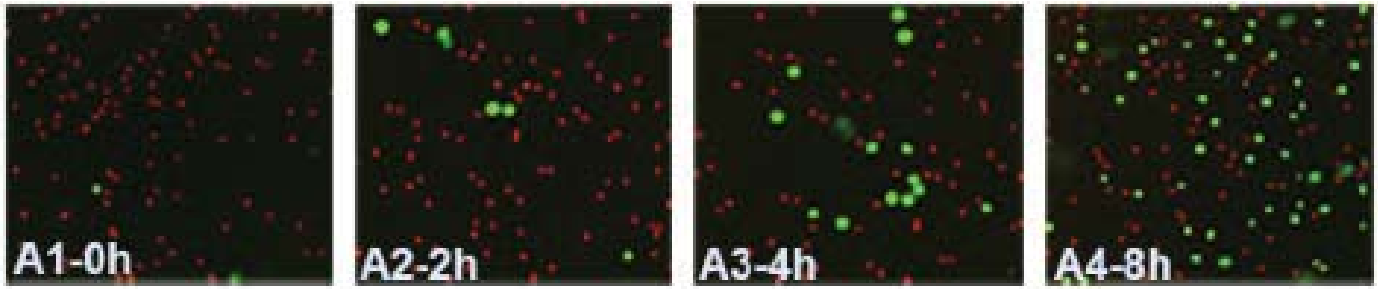
Immunofluorescence staining of conjunctival washings





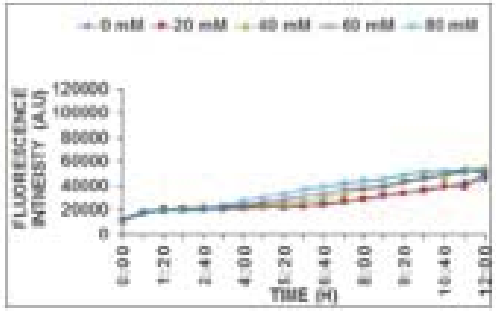

A
NETosis stimulated with 1 nM PMA
APC-CD16
PE-CD16
0h
2h
4h
6h
Kinetic NETosis assay after PMA stimulation
B. Healthy subject
C. Pre-transplant patient
D. oGVHD patient
0 nM
1 nM
10 nM
100 nM
FLUORESCENCE INTENSITY (A.U)
TIME (H)
E. Total NETosis with 1 nM PMA
Total fluorescence units over 12h (AUC)
F. Onset of NETosis with 1nM PMA
Time for 10% increase in fluorescence units (minutes)
Healthy
Pre-transplant
oGVHD

NETosis stimulated with hyperosmolar stress

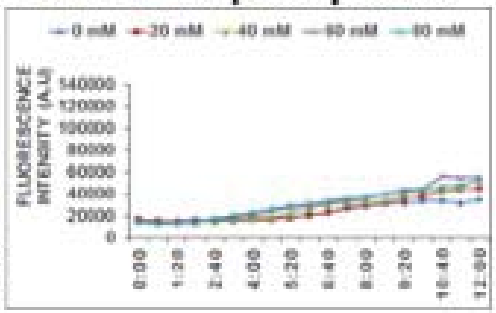


Kinetic NETosis assay after hyperosmolar stress

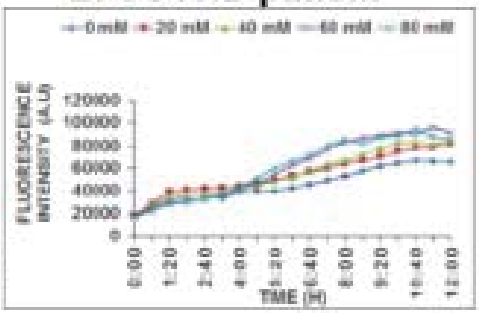
B. Healthy subject



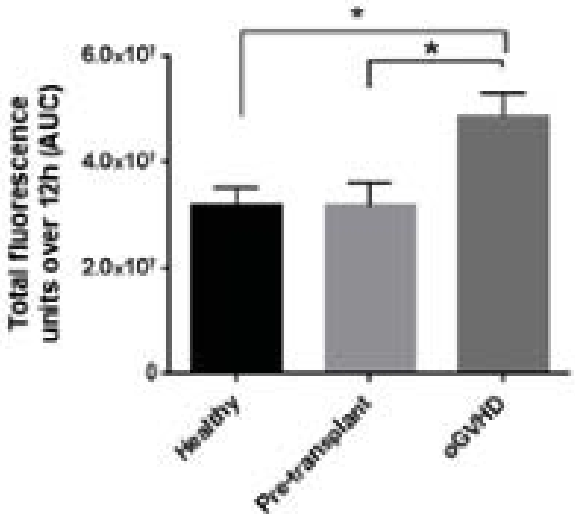
C. Pre-transplant patient



D. oGVHD patient



E. Total NETosis with 420 mOsM



F. Onset of NETosis with 420 mOsM