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The extracellular sialidase NEU3 primes neutrophils

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Abstract

Some extracellular glycoconjugates have sialic acid as the terminal sugar, and sialidases are enzymes that remove this sugar. Mammals have four sialidases, and can be elevated in inflammation and fibrosis. In this report, we show that incubation of human neutrophils with the extracellular human sialidase NEU3, but not NEU1, NEU2 or NEU4, induces human male and female neutrophils to change from a round to a more amoeboid morphology, causes the primed human neutrophil markers CD11b CD18, and CD66a to localize to the cell cortex, and decreases the localization of the unprimed human neutrophil markers CD43 and CD62-L at the cell cortex. NEU3, but not the other 3 sialidases, also causes human male and female neutrophils to increase their F-actin content. Human neutrophils treated with NEU3 show a decrease in cortical levels of Sambucus nigra lectin (SNA) staining and an increase in cortical levels of peanut agglutinin (PNA) staining, indicating a NEU3-induced desialylation. The inhibition of NEU3 by the NEU3 inhibitor 2-acetylpyridine attenuated the NEU3 effect on neutrophil morphology, indicating that the effect of NEU3 is dependent on its enzymatic activity. Together, these results indicate that NEU3 can prime human male and female neutrophils, and that NEU3 is a potential regulator of inflammation.

Graphical Abstract

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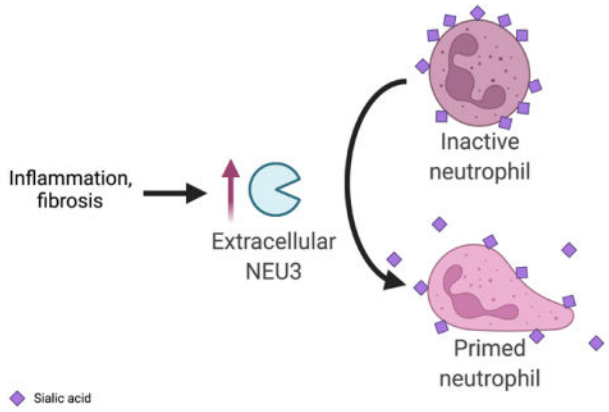

Introduction

An immune response is an essential process that allows the host organism to defend against pathogens [1–3]. Inflammation is a tissue pathological process for the purpose of resolving infection and starting tissue repair [4, 5]. The innate immune response includes cells that rapidly enter tissues after damage or inflammation, especially neutrophils [6–10]. Neutrophil recruitment into damaged or inflamed tissues involves rolling on, adherence to, and migration between endothelial cells [11]. Circulating neutrophils are quiescent and unprimed [12]. As neutrophils are exposed to local activating agents, such as N-formylmethionyl-leucyl-phenylalanine (fMLF), tumor necrosis factor alpha (TNF-α), and various chemokines [13–15], priming is initiated. The priming of neutrophils is characterized by increased levels and/or increased affinity of adherence molecules which allow them to attach to the endothelial cell lining of blood vessels [16]. The attachment of primed neutrophils to endothelial cells ensures maximum activation of neutrophils in a highly regulated manner [17, 18].

As neutrophils enter a site of inflammation, neutrophils begin to release neutrophil extracellular traps (NETs), reactive oxygen species, cytokines, and exosomes, resulting in fibroblast activation and formation of the extracellular matrix [19–23]. Many pulmonary disorders, such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and idiopathic pulmonary fibrosis (IPF), are characterized by an influx of neutrophils into the lung tissue [23–27], although for IPF the role of neutrophils is unclear [28].

Sialidases are enzymes that remove terminal sialic acids from glycoconjugates [29–31], and sialidases are upregulated in IPF [32, 33].There are four isoforms of sialidases in mammals, NEU1, NEU2, NEU3 and NEU4 [30], and of these, NEU3 is the only one that is primarily extracellular [34]. Upregulation of NEU3 is associated with multiple disorders including intestinal inflammation and colitis [35], neuroinflammation [36], periodontal disease [37] and lung fibrosis [32, 38]. NEU1, NEU2 and NEU3 are upregulated in fibrotic lesions in human and mouse lungs [32, 39, 40], but only NEU3 was detected in the lung fluid of mice with bleomycin- induced pulmonary fibrosis [32]. Mice lacking NEU3 (*Neu3*$^{-/-}$) had attenuated inflammation and fibrosis after bleomycin compared to wildtype mice [39]. The NEU3 inhibitors 2,3-didehydro-2-deoxy-*N*-acetyl-neuraminic acid (DANA),

Oseltamivir phosphate (Tamiflu), 2-acetyl pyridine (2AP), methyl picolinate (MP), and 4-Amino-1-methyl-2-piperidinecarboxylic acid (AMPCA) also reduced bleomycin-induced lung fibrosis in mice [32, 38].

As NEU3 is upregulated in inflammation and fibrosis [32, 38, 39, 41, 42], in this report we examined the effects of recombinant human sialidases on human neutrophils. We observed that NEU3 primes neutrophils and changes the levels of and/or localization of CD11b, CD18, CD43, CD62-L, and CD66a in neutrophils. This effect is attenuated in the presence of a NEU3 inhibitor, indicating that this effect is due to NEU3 activity.

Materials and Methods

Neutrophil treatment with sialidases and 2AP inhibition assays

Human venous blood was collected from healthy donors who signed written consent, and the procedure was approved by the Texas A&M University Institutional Review Board. Human neutrophils were isolated and prepared as previously described [43, 44]. The first step of the neutrophil isolation (cell dilution and layering on the Polymorphprep) began within 10 minutes of the blood draw. The isolation procedure was completed 90 minutes after the blood draw, and the human neutrophils were used within 4 hours of the blood draw. 96-well polystyrene plates (353072; BD Biosciences, Franklin Lakes, NJ) were coated with 10 μg/ml human plasma fibronectin (354008; Corning, Corning, NY) in PBS (#17–516F, Lonza, Walkersville, MD) for 1 h at 37°C in a humidified incubator with 5% CO_2. The plate was then washed three times with PBS (Lonza). 1 μl from a 200 ng/μl stock of NEU1 (TP300386; Origene; Rockville, MD), NEU2 (TP319858; Origene), NEU3 (TP316537; Origene), and NEU4 (TP303948; Origene) were each added to 0.1 ml of RPMI-1640 (Corning) to make a 2000 ng/ml solution of each. Serial dilutions of concentrations ranging from 2000 ng to 0.00002 ng for each sialidase were prepared and 100 μl of each sialidase concentration was added to each human fibronectin-coated well. Where indicated, NEU3 was incubated with or without 20 nM or 200 nM of 2AP (A302917, AmBeed, Arlington Heights, IL) for 30 minutes at 37°C in a humidified incubator with 5% CO_2. 100 μl of 2×10^5 neutrophils in RPMI-1640 (Corning) with 2% BSA (#BSA-50, Rockland, Limerick, PA) was added to each well and left to incubate for 40 minutes at 37°C in a humidified incubator with 5% CO_2. Differential Interference Contrast (DIC) images of live cells were taken with a 40x objective on a Nikon Ti2 Eclipse (Nikon USA; Melville, NY) microscope. Average circularity of human neutrophils was measured as described in [45] using FIJI image J software [46]. For each individual experiment, at least 40 cells were analyzed.

Sialidase-induced Interleukin-6 accumulation assays

IL-6 accumulation assays were performed using an IL-6 ELISA assay (#430501, BioLegend, San Diego, CA) as described previously [39, 44] with the exception that IL-6 levels were measured at 48 hours.

Activation maker localization and biotinylated lectin immunofluorescence assays

For the activation marker localization assay, human neutrophils were incubated with sialidases as described above for 40 minutes at 37°C in a humidified incubator with 5%

CO_2. 100 μl of the medium was gently removed, and 200 μl of 5% glutaraldehyde (#G-5882, SIGMA, St. Louis, MO) in 1x PBS was added to each well. The cells were fixed and stained as described in (Rijal et al. 2019) using a 1 μg/ml of anti-CD10 (#MCA1556GA, Serotec, Oxford, UK), anti-CD11b (#301302, BioLegend), anti-CD15 (#301902, BioLegend), anti-CD16b (#302002, BioLegend), anti CD18 (#302102,BioLegend), anti-CD43 (#555474, BD Biosciences), anti-CD62-L (#555542, BD Biosciences), or anti-CD66a (#MAB2244, R&D Systems, Minneapolis, MN). Secondary antibodies were 1:1000 Alexa fluor 488 Donkey Anti-Mouse IgG (#715-546-150, Jackson ImmunoResearch, West Grove, PA) and actin was stained with a 1:2000 dilution of Phalloidin-iFluor 555 Reagent (#ab176756, Abcam, Cambridge, UK). For the biotinylated lectins immunofluorescence, human neutrophils were incubated with NEU3 and fixed as described above. The human neutrophils were then blocked with 1x Carbo-Free Blocking Solution (#SP-50400, Vector) for 15 minutes then incubated in 1 μg/ml of primary biotinylated lectins Phaseolus vulgaris leucoagglutinin (PHA) (#B-1115, Vector), SNA (#B-1305, Vector) and PNA (#B-1075, Vector) in 1x Carbo-Free Blocking Solution (#SP-50400, Vector). Secondary antibodies were 1:1000 Alexa Fluor 647 conjugate (#S21374, Life Technologies, Eugene, OR) and actin was stained with a 1:2000 dilution of Phalloidin-iFluor 555 Reagent (#ab176756, Abcam, Cambridge, UK). Differential Interference Contrast (DIC) and fluorescence images of the cells were taken with a 40x objective on a Nikon Ti2 Eclipse microscope. Cytosolic and cortical localization of the activation markers were analyzed as described in [47] using FIJI image J software [46] and 40 randomly chosen cells per experiment were analyzed. Phalloidin staining was quantified as described in [48] using FIJI image J software [46] and 40 randomly chosen cells per experiment were analyzed.

Flow cytometry assay

Human neutrophils were analyzed by flow cytometry as described previously [49–52]. Briefly, unfixed live human neutrophils were incubated with 5 μg/ml primary antibodies diluted in PBS-BSA for 30 minutes on ice. Isotype-matched irrelevant mouse monoclonal antibodies (MG1–45, mouse IgG1; BioLegend), with the addition of anti-CD43 (#343202, BioLegend), at 5 μg/ml in PBS-BSA were used as controls. Human neutrophils were then washed twice in ice-cold PBS, before staining on ice for 30 minutes with 2 μg/ml Alexa 488-conjugated donkey $F(ab')_2$ anti-mouse IgG (Jackson ImmunoResearch) in PBS-BSA.

For flow cytometry to detect surface lectins, unfixed live human neutrophils were initially blocked with Streptavidin and Biotin using a Streptavidin/Biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA). The human neutrophils were then washed twice with cold PBS by centrifugation at 500 × g for 3 minutes. Human neutrophils were then blocked with 1x Carbo-Free Blocking Solution (#SP-50400, Vector) for 15 minutes then incubated in 1 μg/ml of primary biotinylated lectins PHA (#B-1115, Vector), SNA (#B-1305, Vector) and PNA (#B-1075, Vector) in 1x Carbo-Free Blocking Solution (#SP-50400, Vector). The human neutrophils were then washed twice ice-cold PBS before staining on ice for 30 minutes with 1 μg/ml streptavidin Alexa Fluor 647 conjugated secondary antibody (#S21374, Life Technologies) in 1x Carbo-Free Blocking Solution (#SP-50400, Vector).

Human neutrophils were analyzed by flow cytometry using a BD Accuri C6 Plus cytometer (BD Biosciences, Franklin Lakes, NJ). Human neutrophils were identified and gated by their size and granularity as exemplified by the E1 oval in Figure 4A or the P1 polygon in Figure 5A. At least 40,000 cells within the selected region were examined for each marker. This generated a new graph showing a histogram of fluorescence intensity on the x-axis and number of events (cells) on the y-axis. Using either FL1 (Alexa Fluor 488) or FL4 (Alexa Fluor 647) fluorescence channels, a linear marker gate was drawn to encompass 100% of the cells in this histogram. The fluorescence intensities of these events were then analyzed for the median fluorescence intensity (MFI) values by selecting the display tab and choosing the median statistics option. As all analysis was performed with a single fluorophore, no color compensation was required. For the identification of single cells in Figure 5, cells were assessed by forward scatter area and forward scatter height as described previously [49, 53]. Briefly, a dot plot was generated using forward scatter area on the x-axis and forward scatter height on the y-axis. A doublet will generate a signal that has approximately twice the FCS-Area of a single cell, but the FCS-Height will be approximately the same as a single cell. A gate as exemplified by the left polygon on the bottom row of Figure 5A was placed around those events (cells) at a 45 degree angle from the origin which encompasses single cells and excludes doublets that are labelled as aggregates in the right polygon in the bottom row of Figure 5A.

Western Blots

Analysis of lectin staining in whole cell lysates was performed as described in [32] with the exception that 1 μg/ ml biotinylated lectins PHA (#B-1115, Vector), SNA (#B-1305, Vector) and PNA (#B-1075, Vector) were used. Labelling was detected with streptavidin-HRP (#893975, R&D Systems, Minneapolis, MN) as described previously in [54]. Staining was detected with a SuperSignal West Pico PLUS Chemiluminescent Substrate for 10 min (Cat. #34087, Thermo). Images of the membrane were taken using a BioRad ChemiDoc XRS system and quantified using Image Lab software (BioRad).

Statistical analysis

Statistical analyses were done using Prism version 8.4.1 (GraphPad, San Diego, CA) for *t* tests and one-way or two-way ANOVA with appropriate posttests. Significance was defined as $p < 0.05$.

Results:

Human NEU3 alters human neutrophil morphology

Neutrophils circulating in the blood are in a quiescent non-adherent state, with a round morphology [17]. As pro-inflammatory signals are released into the blood, neutrophils are first primed, causing them to increase their cell adhesion and develop a more deformable phenotype so that they can leave the circulation and enter the tissue [37, 55–57]. As NEU3 is upregulated in inflammation and fibrosis [32, 38, 39, 41, 42], to test if NEU3 induces priming or activation of neutrophils, human neutrophils were incubated with recombinant human NEU1–4, and the live cells were imaged (Figure 1A). Male and female neutrophils treated with no sialidase, 10 nM 2AP, and 50 ng/ml of human NEU1, 2, or 4 showed

no significant changes in average circularity (Figure 1A, B, C, and E and Supplemental Figure 1). Both male and female neutrophils treated with 50 ng/ml of human NEU3 showed a crenulated phenotype and had a decreased average circularity (Figure 1A and C and Supplemental Figure 1). Both male and female neutrophils treated with NEU3 preincubated with either 20 nM or 200 nM of the NEU3 inhibitor 2AP [38] (to generate final 2AP concentrations of 10 and 100 nM in the presence of human neutrophils) had an appearance similar to controls and average circularity not significantly different from controls (Figure 1A and C and Supplemental Figure 1). These data suggest that human NEU3 alters both male and female neutrophil morphology, and that this effect is inhibited with a NEU3 inhibitor.

NEU1, NEU2 and NEU4 do not alter neutrophil morphology

To determine if other sialidases affect neutrophil morphology, human neutrophils were incubated with sialidases. At concentrations from 0.00001 to 1000 ng/ ml, NEU1, NEU2, NEU4, caused no discernable change in morphology or significant change in average circularity (Figure 2 and Supplementary Figure 2). Neutrophils treated with 100 or 1000 ng/ml of NEU3 showed a crenulated phenotype and decreased average circularity (Figure 2 and Supplementary Figure 2) compared to the control. We also observed that at high concentrations of NEU3, there were no differences in the response of male and female neutrophils (Supplemental Figure 3).

To test the hypothesis that the inability of NEU1, 2, and 4 to alter human neutrophil morphology was not due to these recombinant proteins lacking sialidase activity, we examined the ability of the sialidases to increase the extracellular accumulation of IL-6 by human peripheral blood mononuclear cells (PBMCs) [32]. As previously observed, NEU1, NEU2, NEU3 and NEU4 increased IL-6 extracellular accumulation (Supplementary Figure 4). This indicates NEU1, NEU2, NEU3 and NEU4 have enzymatic activity, and thus that some specific property of NEU3 in addition to its sialidase activity alters neutrophil morphology.

NEU3 changes the localization of neutrophil activation markers

Neutrophil priming is a rapid process that balances neutrophil activity and allows for the modulation of response at the inflammation site [58, 59]. Primed neutrophils undergo both phenotypic and functional alterations [17]. Primed neutrophils begin shedding surface selectins to alter their adhesion receptor pattern, undergoing inside-out signaling by promoting exocytosis, leading to the expression of adherence markers such as CD11b, CD35, and CD66a [58, 60, 61], which allow neutrophils to adhere to endothelial cells [17]. To determine if NEU1, NEU2, NEU3, or NEU4 induce the expression of activation markers on human neutrophils, human neutrophils were incubated in buffer or 100 ng/ml of NEU1, NEU2, NEU3, and NEU4. Cells were scored for the localization of activation markers either in the cytosol, at the cortex, or in both locations (Figure 3A). human neutrophils treated with NEU3 had increased expression of CD11b, CD18, and CD66a at the cell cortex and decreased expression in the cytosol (Figure 3C, F, I and Supplemental Figure 5). NEU3 treated human neutrophils also decreased localization of CD62L and CD43 at the cortex without a corresponding increase in the cytosol compared to the control (Figure 3G and

H and Supplemental Figure 5). Human neutrophils treated with NEU1, NEU2, or NEU4 showed no discernable changes in these markers (Figure 3 and Supplemental Figure 5). Compared to untreated controls, human neutrophils treated with all four sialidases showed no discernable changes in the localization of CD10, CD15, and CD16b (Figure 3 and Supplemental Figure 5). Compared to control, human neutrophils treated with NEU3 had increased levels of F-actin (Figure 3J and Supplemental Figure 5). NEU1, NEU2, and NEU3 did not increase F-actin levels in the human neutrophils (Figure 3J and Supplemental Figure 5). For all conditions, we observed no significant difference in the localization of the various markers in male and female neutrophils. These data suggest that NEU1, NEU2 and NEU4 do not regulate the localization of activation markers in human neutrophils, but NEU3 changes the localization of specific activation markers that may play a role in neutrophil adhesion and migration at inflammatory sites.

To confirm the increase of CD11b, CD18, and CD66a and the decrease of CD43 and CD62-L labeling on the human neutrophil surface after treatment with human NEU3, we performed flow cytometry. Unfixed live human neutrophils were incubated in buffer or 50 ng/ml of NEU3 then incubated with mouse monoclonal antibodies. Figure 4A represents the distribution of cells in the light scatter based on size (FSC-A) and side scatter/granularity (SSC-A). Compared to the control (absence of NEU3), human neutrophils treated with NEU3 had a significant increase of cell-surface CD11b, CD18, and CD66a and a significant decrease of cell-surface CD43, as measured by a sialidase sensitive antibody (1G10) [62] and a second mAb CD43 (10G7), as well as a significant decrease of cell-surface CD62-L (Figures 4C–I). There were no significant differences in the cell surface activation markers between male and female neutrophils after NEU3 treatment (Figures 4J and K). Previous studies have shown that anti-CD11b, anti-CD18, or anti-CD66a antibodies can induce aggregation of live unfixed neutrophils [117, 118]. Using flow cytometry and microscopy, we also observed that following incubation with NEU3, live unfixed human neutrophils showed increased aggregation when incubated with anti-CD11b, CD18, or CD66a antibodies and showed reduced aggregation when incubated with the anti-CD43 mAb 10G7 (Figure 5A–C). Since human NEU3 only primes neutrophils, human neutrophils treated with human NEU3 had a rounded morphology due to the removal of NEU3 and the continuous washing of the cells for antibody incubation (Figure 5C). There was no significant difference in homotypic aggregation of neutrophils between male and female neutrophils (Supplemental Figure 6).

NEU3 increases desialylated glycoconjugates on neutrophils

To determine if human NEU3 changes the level of sialylation on the surface of human neutrophils, we initially performed flow cytometry using PHA, which detects complex oligosaccharides containing galactose, *N*-acetylglucosamine, and mannose [63], SNA, which detects sialic acids on glycoconjugates, and PNA, which detects carbohydrates that are not sialylated [64]. We have previously shown that compared to control mouse lungs, fibrotic human and mouse lung showed less staining for sialylated glycoconjugates and increased staining for desialylated glycoconjugates [32]. Live human neutrophils were incubated in buffer or 50 ng/ml of human NEU3 then incubated with biotinylated lectin antibodies. The addition of biotinylated lectins to live human neutrophils caused the neutrophils to

aggregate, rendering the analysis inconclusive due to the loss of neutrophil population. We then performed immunofluorescence to determine changes in the levels of lectins in human neutrophils in both the cytosol and cortex. Live human neutrophils were incubated in buffer or 50 ng/ml of human NEU3 then fixed and stained for PHA, SNA and PNA (Figure 6). Human neutrophils treated with NEU3 showed a significant decrease in SNA staining and a significant increase of PNA staining at the cortex, compared to the control (Figure 6A, C, and D). There were no changes in the cytosolic levels of SNA and PNA (Figure 6A, C, and D). Compared to the control, there were no significant changes in the levels of PHA after human NEU3 treatment (Figure 6A and B). To confirm the changes in the levels of SNA and PNA staining of neutrophils after NEU3 treatment, we performed western blotting using whole cell lysates. Live human neutrophils were incubated in buffer or 50 ng/ml of human NEU3 and lysed. Samples were electrophoresed (Figure 7A), blotted and stained for PHA, SNA and PNA (Figure 7B–D). In both male and female neutrophils, there was a significant decrease in the levels of SNA staining and a significant increase in levels of PNA staining (Figure 7C–D and G–H). There were no changes in the levels of PHA staining (Figure 7B and F–H). Together, these data suggest that NEU3 desialylates glycoconjugates on human neutrophils.

Discussion

In this report, we observed that human NEU3 induces phenotypic changes, such as reduced circularity, associated with human neutrophil priming and that inhibition of human NEU3 with 10 nM and 100 nM of 2AP attenuates this effect. The total tidal volume of a mouse lung is approximately 0.18 – 0.2 ml [65–68]. We have previously found that at day 21, bronchoalveolar lavage of lungs from bleomycin- treated mice indicated that the lung fluid contained a total of ~15 ng of NEU3, compared to ~1.7 ng in control mice [39]. Assuming that the lung fluid is 10% of the lung volume, this would indicate that in a control mouse lung the NEU3 concentration in the lung fluid is ~1.7 ng/ (0.1 × 0.2 ml) = ~85 ng/ ml, while in a fibrotic bleomycin-treated and thus fibrotic lung the NEU3 concentration would be ~ 750 ng/ ml. We observed that human neutrophil priming is induced by treatment of human neutrophils with 50 – 1000 ng/ml human NEU3. Further assuming that the concentrations of NEU3 in human lung fluid are similar to the concentrations in mouse lung fluid, this indicates that physiological concentrations of NEU3 found in lung fluid induce human neutrophil priming. Human NEU1, NEU2, and NEU4 do not appear to prime human neutrophils or cause any phenotypic changes associated with human neutrophil priming. NEU3 increased the primed-neutrophil cell-surface markers CD11b, CD18 and CD66a, and decreased the quiescent neutrophil cell-surface markers CD43 and CD62-L. human neutrophils treated with NEU3 showed a significant decrease in cortical levels of SNA staining and a significant increase in cortical levels of PNA staining, indicating that the NEU3 treatment decreases sialylation on the human neutrophils. Together, these results suggest that physiological levels of extracellular NEU3 can prime human neutrophils, and that this effect is due to NEU3's sialidase activity.

Sialic acid residues are important for many immune cell receptors, and removal of these residues can either promote or inhibit immune cell function [69, 70]. Many pathogens also produce sialidases to inhibit immune responses [71, 72]. NEU3 can reduce LFA-1

(CD11a/CD18) adhesion to ICAM-1 in Jurkat but not peripheral blood mononuclear cells [73], and NEU3 increased adhesion of colon cancer cells to laminins, but decreased binding to fibronectin and collagen I and collagen IV [74]. In neutrophils, activation by fMLF leads to increased sialidase activity on the cell membrane [75], and *Clostridium perfringens* neuraminidase removal of sialic acids from CD11b/CD18 exposes activation epitopes that enhances adhesion [76]. Sialidase inhibitors can prevent immune cell migration into inflammatory lesions [32, 38, 39, 77–79]. Combined with the above observations, our results suggest that NEU3 removes cell-surface sialic acids to prime neutrophils.

Adhesion of primed neutrophils to the endothelial lining is important to induce outside-in signaling [80], allowing for the expression of adhesive markers on the surface of neutrophils [55, 81]. CD66 are glycoproteins that play a role in neutrophils adhesion to E-selectin on local endothelial cells through the presentation of CD15s epitope [82]. CD66 expression on neutrophils also promotes neutrophil adhesion to fibronectin through the expression of β2 integrin [83]. When neutrophils undergo priming after exposure to fMLF, CD66 expression initiates an increase in superoxidase production [83, 84]. We observed that when human neutrophils are treated with 50 ng/ml of human NEU3, CD66 localization increases at the cortex of the human neutrophils indicating the initiation of neutrophil adherence and priming. CD62-L is known as the tethering or rolling receptor [85]. Neutrophils that are primed or activated shed CD62-L and in turn, increase CD11b [86]. Neutrophils at infection sites had an upregulation in CD11b and reduced expression of CD62-L [87, 88]. We observed that when neutrophils are treated with 50 ng/ml of human NEU3, CD62-L expression at the cortex decreases with no increase in the cytosol indicating that upon neutrophil priming, CD62-L begins to shed. CD11b and CD18 are primarily expressed on a variety of immune cells, including neutrophils [89–91]. On neutrophils, CD11b and CD18 are noncovalently bound to form a functional heterodimer [92]. The cortical localization of this heterodimer is involved in regulating multiple leukocyte responses such as adhesion and migration [93]. We observed that on neutrophils treated with 50 ng/ml of human NEU3, CD11b and CD18 had increased localization at the cortex and decreased localization in the cytosol, indicating that NEU3 plays a role in regulating cell surface markers to indirectly promote neutrophil adhesion.

In patients with acute or chronic inflammatory conditions, CD10 is widely utilized to discriminate mature and immature neutrophil populations [94–97]. Although the function of CD15 is not well known, it has been observed to be expressed on mature neutrophils and is involved in multiple neutrophil functions such as cell-cell interactions, phagocytosis, and degranulation [98–103]. CD16b is specifically expressed on neutrophils (Zhang 2000). CD16b plays a role in neutrophil degranulation [104], phagocytosis [105], and calcium mobilization [106]. We observed that in human neutrophils treated with 50ng/ml of human NEU3, there was no change in the localization of CD10, CD15, or CD16b, indicating that these activation markers might be regulated at a later stage in human neutrophil priming or activation. CD43 is expressed by all leukocytes and in very rare cases, on colon carcinomas and on several non-hematopoietic cell lines [107, 108]. Similar to CD62-L, CD43 is shed from the membrane upon neutrophil activation or by TNF-α priming, and during adhesion and spreading [109–114]. When human neutrophils were treated with 50 ng/ml of human

NEU3, CD43 localization at the cortex decreased with no increase in the cytosol, suggesting that NEU3 induces shedding of CD43 on human neutrophils.

Neutrophil recruitment into damaged or inflamed tissues involves rolling on, adherence to, and migration between endothelial cells [11]. However, neutrophils can also bind to each other (homotypic aggregation) to amplify the recruitment process [115, 116]. We observed that following incubation with NEU3, neutrophils were more likely to form aggregates when incubated with anti-CD11b/CD18 (the integrin αMβ2; Mac-1; complement receptor 3) or anti-CD66a antibodies, and less likely to form aggregates when incubated with CD43 antibodies. Whether the antibodies to CD11b/CD18 and CD66a induce aggregation by mimicking the effect of these receptors' ligands, or induce aggregation due to increased affinity of FcγR, as desialylated FcγR have increased affinity for IgG [117, 118], is unclear. Whatever the mechanism, these data suggest that NEU3 (but not NEU1, NEU2, or NEU4) may promote neutrophil recruitment by removing sialic acid residues present on glycosylated membrane proteins.

Sialic acids are a common terminal residue of cell surface glycoconjugates [119]. Cell surface sialic acids serve a myriad of biological functions including cell adhesion, immune response and inflammation [120, 121]. The removal of sialic acids from cell surface ligands or receptors increases cell inflammatory responses [122–125]. Previous studies have shown that sialidases promote inflammation [125–131]. We have shown that mice have low levels of the sialidase NEU3 in their bronchoalveolar lavage fluid and aspiration of NEU3 into the lungs of mice caused inflammation and fibrosis at day 21 [132]. Fibrotic human and mouse lung showed less staining for sialylated glycoconjugates and increased staining for desialylated glycoconjugates [32]. We observed a decrease in SNA lectin staining and an increase in PNA lectin staining on human neutrophils treated with human NEU3. This suggests that NEU3 increases desialylation of glycoconjugates on the surface of human neutrophils, consequently priming human neutrophils.

The broad range of neutrophil responsiveness confers extensive functional flexibility, allowing neutrophils to respond rapidly and appropriately to varied and evolving threats throughout the body. Uncontrolled priming and activation of neutrophils may lead to multiple inflammatory disorders, such as COPD, arthritis, and IPF. The ability of NEU3 to induce neutrophil priming suggests that inhibiting NEU3 activity, or NEU3 upregulation, may be beneficial to patients with a variety of diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMPCA	4-Amino-1-methyl-2-piperidinecarboxylic acid
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disease
DANA	2,3-didehydro-2-deoxy-*N*-acetyl-neuraminic acid
F-actin	Filamentous actin
fMLF	N-formylmethionyl-leucyl-phenylalanine
IL-6	Interleukin 6
IPF	idiopathic pulmonary fibrosis
MP	Methyl picolinate
NETS	Neutrophil extracellular traps
NEU1–4	Neuraminidase / Sialidase 1–4
PBMCs	Peripheral blood mononuclear cells
PHA	Phaseolus vulgaris leucoagglutinin
PNA	peanut agglutinin
SNA	Sambucus nigra lectin
TNF-α	Tumor necrosis factor alpha
2-AP	2-acetyl pyridine

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TV Silva-Santos B Simon AK Sitnik KM Sozzani S Speiser DE Spidlen J Stahlberg A Stall AM Stanley N Stark R Stehle C Steinmetz T Stockinger H Takahama Y Takeda K Tan L Tarnok A Tiegs G Toldi G Tornack J Traggiai E Trebak M Tree TIM Trotter J Trowsdale J Tsoumakidou M Ulrich H Urbanczyk S van de Veen W van den Broek M van der Pol E Van Gassen S Van Isterdael G van Lier RAW Veldhoen M Vento-Asturias S Vieira P Voehringer D Volk HD von Borstel A von Volkmann K Waisman A Walker RV Wallace PK Wang SA Wang XM Ward MD Ward-Hartstonge KA Warnatz K Warnes G Warth S Waskow C Watson JV Watzl C Wegener L Weisenburger T Wiedemann A Wienands J Wilharm A Wilkinson RJ Willimsky G Wing JB Winkelmann R Winkler TH Wirz OF Wong A Wurst P Yang JHM Yang J Yazdanbakhsh M Yu L Yue A Zhang H Zhao Y Ziegler SM Zielinski C Zimmermann J and Zychlinsky A (2019) Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur J Immunol 49, 1457–1973. [PubMed: 31633216]

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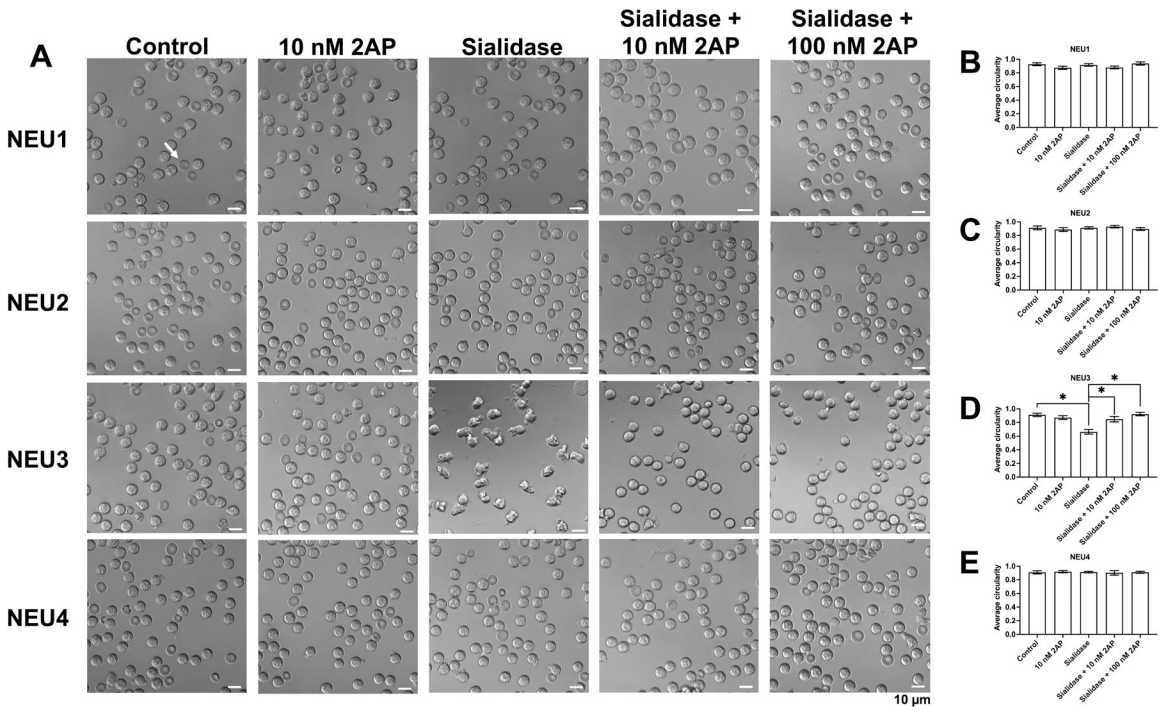

Figure 1. Human NEU3 alters human neutrophil morphology, and NEU3 inhibition attenuates this effect.

A) Live images of human neutrophils incubated in buffer, 10 nM 2AP, 50 ng/ml of sialidases (NEU1–4), or 50 ng/ml of sialidases (NEU1–4) with 100 nM or 10nM of 2AP. DIC images were taken after 40 minutes of incubation. Bar is 10 μm. The smaller toroidal objects (white arrow) are red blood cells. Images are representative of cells from 6 independent experiments (3 male and 3 female donors). Graphs shows average circularity of neutrophils treated with **B)** NEU1 **C)** NEU2 **D)** NEU3 or **E)** NEU4 with at least 50 randomly chosen cells examined for each condition in each experiment. Values are mean ± SEM, n=6. * Indicates $p < 0.05$ compared to control (unpaired t-tests, Welch's correction).

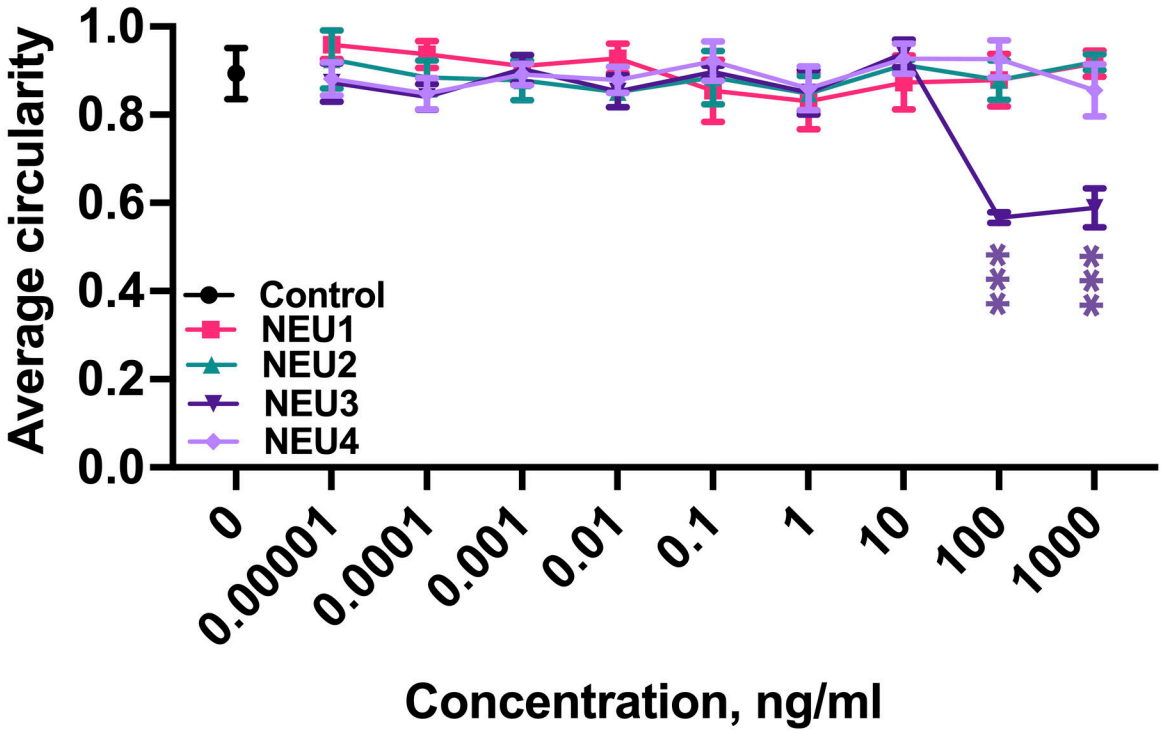

Figure 2. NEU1, NEU2, and NEU4 do not alter human neutrophil morphology.

Human neutrophils were incubated in the indicated concentrations of the indicated sialidases. Graphs show the average circularity after treatment with NEU1 (magenta), NEU2 (green), NEU3 (dark purple) and NEU4 (light purple). Values are mean ± SEM of the averages from 6 independent experiments (3 male and 3 female donors), with at least 50 randomly chosen cells examined for each condition in each experiment. *** indicates $p < 0.001$ comparing control to sialidases for each concentration (Unpaired t-tests, Welch's correction).

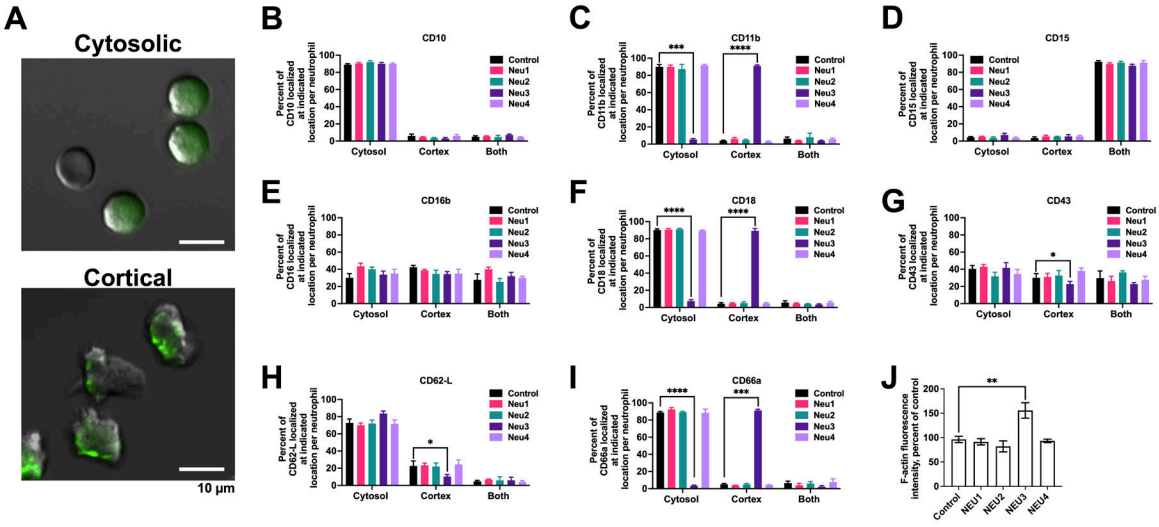

Figure 3. NEU3 changes the localization of some human neutrophil activation markers.

Human neutrophils were incubated in the absence (control) or presence (50 ng/ml) of the indicated sialidases. Graphs show the localization of (**A**) Example of cytosolic or cortical localization of CD18 in neutrophils after treatment without (top) or with (bottom) 50 ng/ml of human NEU3 for 30 minutes. Bar is 10 μm. (B) CD10, (**C**) CD11b, (**D**) CD15, (**E**) CD16b, (**F**) CD18, (**G**) CD43, (**H**) CD62L, or (**I**) CD66a in either the cytosol, cortex or both in neutrophils. (**J**) Graph shows phalloidin fluorescence staining (a marker for F-actin) intensity in human neutrophils after treatment with the indicated sialidases. 40 cells were analyzed for each individual experiment. Values are mean ± SEM of 6 independent experiments (3 male and 3 female donors). * Indicates $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$, **** $p < 0.0001$ compared to control (Unpaired t-tests, Welch's correction).

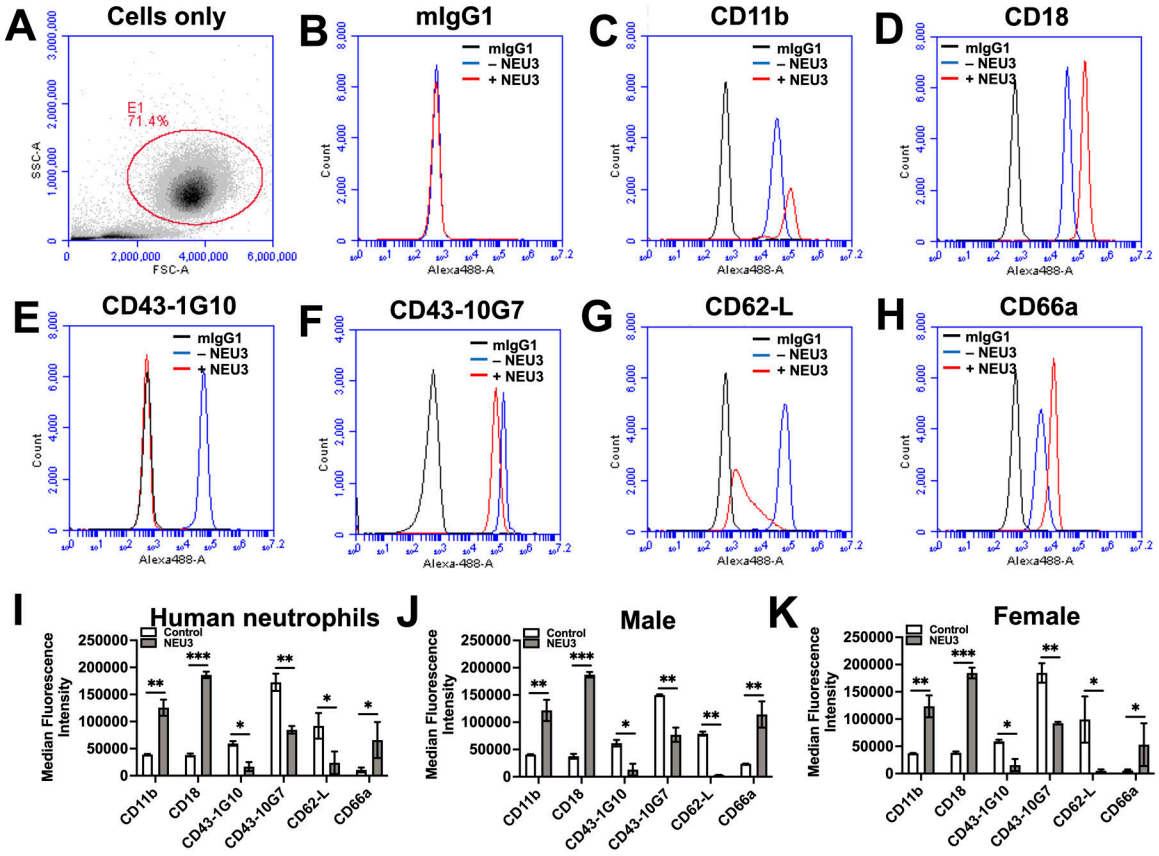

Figure 4. NEU3 changes human neutrophil cell surface activation markers.

Unfixed live human neutrophils were incubated in the absence (control) or presence of 50 ng/ml of NEU3 for 40 minutes and prepared for flow cytometry. (**A**) shows the forward scatter (X axis) and side scatter (Y axis), and the red line delineates the region used to gate neutrophils. Surface expression of proteins was measured relative to (**B**), an isotype-matched mouse IgG control (mIgG1). Neutrophils were stained with anti- (**C**) CD11b, (**D**) CD18, (**E**) CD43–1G10, (**F**) CD43 (10G7), (**G**) CD62-L and (**H**) CD66a antibodies. Black line indicates cells incubated with mIgG1 only, blue line indicates the absence of NEU3, and red line indicates the presence of NEU3. (**I**) Graph shows the median fluorescence intensity for each human neutrophil activation marker after treatment with and without NEU3. (**J**) Graph shows median fluorescence intensity for male neutrophils after treatment with and without NEU3. (**K**) Graph shows median fluorescence intensity for female neutrophils after treatment with and without NEU3. At least 40,000 cells were analyzed for each individual sample. Values are mean ± SEM of 6 independent experiments (3 male and 3 female donors). * indicates $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to control (Unpaired t-tests, Welch's correction).

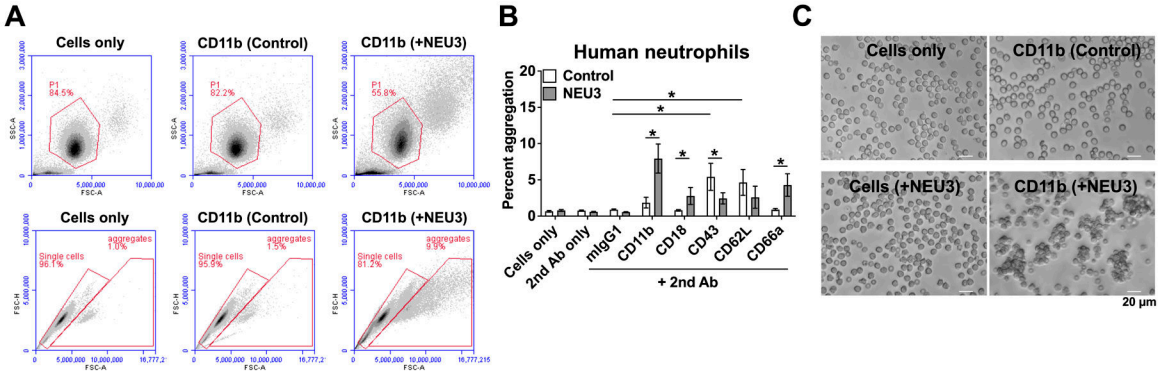

Figure 5. Homotypic cell aggregation of NEU3 treated neutrophils.

Human neutrophils were incubated in the absence (control) or presence of 50 ng/ml of NEU3. After 40 minutes, the cells were washed and incubated with the indicated antibodies for flow cytometry. Example of homotypic aggregation after treatment with NEU3 and labeling with CD11b (**A**) Top row - Cells were identified by their size (FSC-A) and granularity (SSC-A), and (**A**) bottom row – Cell aggregates were identified by FSC-A and forward scatter height (FSC-H). At least 40,000 cells were examined for each condition. Plots are representative of cells from 6 different donors. (**B**) Graph shows the percent aggregation of human neutrophils in each of the indicated conditions. Values are mean ± SEM from 6 donors (3 male and 3 female). (**C**) DIC images show human neutrophils treated with and without NEU3 (left column) and human neutrophils treated with and without NEU3 then incubated with CD11b (right column). Bar is 50 µm. The smaller toroidal objects are red blood cells. Images are representative of cells from 6 different donors.

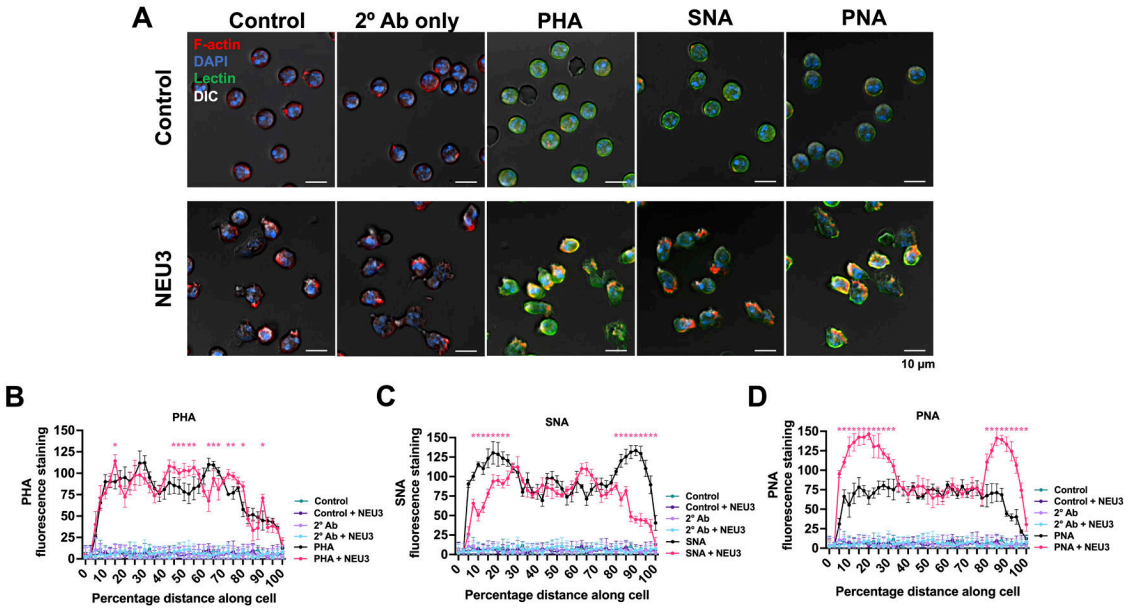

Figure 6. NEU3 changes levels of SNA and PNA staining at the cortex of human neutrophils.

Human neutrophils were incubated in the absence (control) or presence of 50 ng/ml of human NEU3. After 30 minutes, cells were fixed and stained for the indicated markers. (**A**) DIC and fluorescent images were taken with a 40x objective. Bar is 10 µm. Images are representative of 6 individual experiments (3 male and 3 female donors). Graphs show **(B)** PHA, **(C)** SNA and **(D)** PNA fluorescence staining, Values are mean ± SEM of the averages from 6 independent experiments (3 male and 3 female donors), with at least 50 cells examined for each condition in each experiment. * indicates $p < 0.05$ comparing the marker on cells with no added NEU3 to the marker on cells with NEU3 (t-tests).

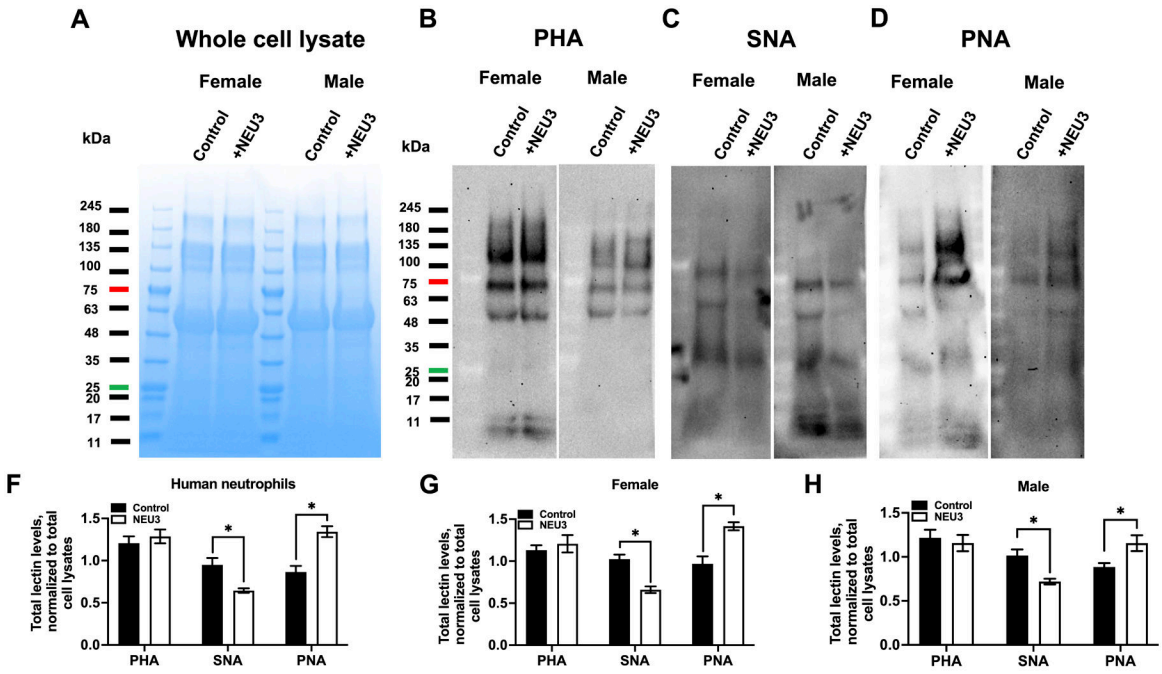

Figure 7. NEU3 decreases levels of SNA and increases levels of PNA staining in human neutrophils.

Human male and female neutrophils were incubated with or without 50 ng/ml of NEU3 for 30 minutes then lysed. (**A**) shows Coomassie staining of the samples after SDS-PAGE. Western blots of the lysed cells were stained for (**B**) PHA, (**C**) SNA and (**D**) PNA. Graphs represent (**F**) human neutrophils (**G**) female and (**H**) male total lectin levels normalized to total cell lysates. * indicates $p < 0.05$ (2-way ANOVA, Holm-Šídák test).