**Differences between unstimulated and stimulated human male and female neutrophils in protein and phosphoprotein profiles**

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**Abbreviations**

ARDS - acute respiratory distress syndrome

CYFIP1 - Cytoplasmic FMR1-interacting protein 1

MPO - myeloperoxidase

NAP1 - Nck-associated protein 1

NE - neutrophil elastase

NETs - neutrophil extracellular traps

PAR2 - Protease activated receptor 2

RA - rheumatoid arthritis

RT - room temperature

SLIGKV - Ser-Leu-Ile-Gly-Lys-Val-NH2

SLE - systemic lupus erythematosis

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**Abstract**

Human males and females show differences in the incidence of neutrophil-associated diseases and differences in neutrophil responses such as a faster response to the chemorepellent SLIGKV in males. Little is known about the basis of sex-based differences in human neutrophils. We used mass spectrometry to identify proteins and phosphoproteins in unstimulated human neutrophils and in neutrophils incubated with the SLIGKV, a protease activated receptor 2 agonist. There were 132 proteins with higher levels in unstimulated male neutrophils; these proteins tended to be associated with RNA regulation, ribosome, and phosphoinositide signaling pathways, whereas 30 proteins with higher levels in unstimulated female neutrophils were associated with metabolic processes, proteosomes, and phosphatase regulatory proteins. Unstimulated male neutrophils had increased phosphorylation of 32 proteins compared to females. After exposure to SLIGKV, male neutrophils showed a faster response in terms of protein phosphorylation compared to female neutrophils. Male neutrophils have higher levels of proteins and higher phosphorylation of proteins associated with RNA processing and signaling pathways. Female neutrophils have higher levels of proteins associated with metabolism and proteolytic pathways. This suggests that male neutrophils might be more ready to adapt to a new environment, and female neutrophils might be more effective at responding to pathogens.

**Significance of the Study**

Some diseases are more common in females, and this sex difference may be due, in part, to sex differences in immune cells called neutrophils. However, little is known about the basis of sex-based differences in human neutrophils. To understand these differences, we examined proteins from neutrophils of healthy male and female humans. We find that the abundances of many proteins, and protein modification by the addition of a phosphate group (phosphorylation), are different between male and female neutrophils. The differences in protein levels and protein phosphorylation suggest that male neutrophils might be more ready to adapt to a new environment, and female neutrophils might be more effective at responding to pathogens. Male neutrophils have more phosphorylated proteins at 5 and 20 minutes after exposure to a compound that regulates neutrophil movement. These differences may contribute to the observed sex-based differences in neutrophil behavior and neutrophil-associated disease incidence and severity.

**1 INTRODUCTION**

Polymorphonuclear cells (neutrophils) are the most abundant circulating immune cell in humans, representing 50-70% of all leukocytes, are an important component of the innate immune system, and are part of the first line of defense against microorganisms [1, 2]. Neutrophils also have a role in tissue homeostasis, but aberrant activation and persistence can contribute to inflammation and the progression of some disease conditions [1, 2], including acute respiratory distress syndrome (ARDS) [3], rheumatoid arthritis (RA) [4], and many other disorders [1, 2, 5].

Sexual dimorphism in the mammalian immune system has been noted for decades [6, 7]. In general, women tend to have stronger innate and adaptive immune responses than men [8-10], including reduced rates of infection and an increased immune response to a variety of bacterial, viral, and parasitic infections [11-14] and some vaccines [15, 16]. However, women also have a higher incidence of autoimmune disorders compared to men [17, 18]. Some of these sex differences can be explained by hormonal differences [19, 20] or sex chromosome copy number [21], but there is much that is still unknown [22].

ARDS involves damage to the lungs triggering an influx of neutrophils into the lungs, and the neutrophils then activating, causing further damage to the lungs, and in a positive feedback loop the additional damage recruits more neutrophils [23]. We found that the peptide SLIGKV, a protease activated receptor 2 (PAR2) agonist, is a repellent for human neutrophils, and in a mouse model of ARDS, aspiration of SLIGKV inhibits the number of neutrophils in the lungs [24]. Surprisingly, compared to human female neutrophils, male neutrophils showed a faster response to SLIGKV [24, 25], and there were several differences between male and female neutrophils in the signal transduction pathway mediating chemorepulsion in response to SLIGKV [25].

Although studies on systemic lupus erythematosis (SLE) and Behçet’s uveitis patients found some neutrophil protein differences in healthy males and females, there was no systematic analysis of differences in total proteins in male and female neutrophils [26, 27]. Neutrophil phosphoproteomics analysis indicated differences between male and female neutrophils, but only with respect to specific neutrophil responses rather than a global analysis [28-30].

In this report, we describe sex-based differences in human neutrophil protein abundance and protein phosphorylation. In response to SLIGKV, we find that at 5 minutes there was increased phosphorylation of two proteins in male neutrophils, but no significantly increased phosphorylation of proteins in female neutrophils. These differences may contribute to the faster response time of male neutrophils to SLIGKV, and neutrophil-associated disease incidence and severity.

**2 MATERIAL AND METHODS**

**2.1 Ethics Statement**

Human venous blood was collected with the approval from the Texas A&M University Institutional Review Board (IRB2017-0792D) from healthy volunteers who gave written consent.

**2.2 Neutrophil isolation and culture**

Neutrophils were isolated at room temperature (RT) from blood collected directly into vacutainer tubes containing EDTA (#454209; Greiner Bio-One; Monroe, NC). Neutrophils were isolated by Polymorphprep (#1114683; Axis-Shield, Oslo, Norway) following the manufacturers’ instructions, except the centrifugation of gradients was done for 40 minutes [25, 31, 32]. After centrifugation, first the upper mononuclear band was removed and then a sterile wide-bore plastic pipette (#414004-005, VWR, Radnor, PA) was used to collect the lower neutrophil band. Neutrophils were then mixed with 10 mL PBS (#17-516F; Lonza, Walkersville, MD) and collected by centrifugation for 10 minutes at 300 x g at RT. Neutrophil cell pellets were resuspended in PBS and then recentrifuged. This process was repeated 3 more times. Neutrophils were then resuspended in RPMI-1640 (Lonza) with 2% BSA (Rockland Inc, Limerick, PA) (RPMI-BSA). To determine the purity of the neutrophil preparations, cell spots were prepared as described previously [33]. Cell spots were dried overnight, fixed in methanol for 10 minutes at RT, and then stained with Wright-Giemsa stain (Polysciences Inc., Warrington, PA) following the manufacturer’s instructions. At least 300 cells per donor were examined and quantified for cell type following the manufacturer’s instructions [32]. We never used the same donor twice for a given experiment. The age ranges for the donors were 18-44 years for males and 18-32 years for females. Cell preparations were 97.2 ± 0.3% neutrophils. The main contamination cell type was monocytes at 1.1 ± 0.2%, with basophils, eosinophils, and lymphocytes all < 0.6% (**Fig. S1**). The first step of the neutrophil isolation (layering on the Polymorphprep) began within 15 min of the blood collection. The isolation procedure was completed within 2 hours after the blood collection, and the neutrophils were used within 4 hours of blood collection.

**2.3 Proteomics, phosphoproteomics, and cell activation**

Proteomics was performed as described previously [25]. Briefly, 2 × 107 neutrophils in 1 mL of RPMI-BSA were collected by centrifugation at 500 × g for 3 minutes at 4oC. The supernatant was discarded, and cells were washed twice with 1 mL ice-cold PBS at 500 × g for 3 minutes at 4oC. The cell pellet was resuspended in 0.3 ml of ice-cold radioimmunoprecipitation assay buffer (RIPA buffer; #89900; Thermo Scientific, Rockford, IL) containing 10× Protease and Phosphatase Inhibitor Cocktail (78441, Thermo). A pipette was used to vigorously lyse the cells, and the lysate was then snap frozen in liquid nitrogen and stored at −80oC. In-gel protein preparation was performed as described on the University of Texas Southwestern Proteomics Core ([https://proteomics.swmed.edu/wordpress/?page\_id=553](about:blank)). Samples were sent to UTSW Proteomics Core by overnight delivery with ice packs. Samples were analyzed by Thermo Fusion Lumos standard gradient mass spectrometry. The proteins were analyzed using Proteome Discoverer 3.0 (Thermo) and searched using the human protein database from UniProt (www.uniprot.org) [34]. Raw and processed proteomic data was uploaded to MassIVE at the University of California at San Diego Center for Computational Mass Spectrometry

(<https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=002e367a56ef471da06a302861229930>) with accession number MSV000088857. For each donor, peptide counts were summed and then divided by the total counts for all peptides from that donor. Male and female values were compared to determine sex-based differential protein abundance.

Isolated neutrophils for SLIGKV activation and phosphoproteomics analysis were prepared as described above. For each condition, 5 x 106 cells were resuspended in 1 mL RPMI-BSA prewarmed to 37oC and then incubated in the presence or absence of 500 ng/ml SLIGKV-NH2 (#3010, Tocris-BioTechne, Minneapolis, MN; SLIGKV) at 37oC in a CO2 incubator as described previously [25]. After 5 minutes in the presence or absence of SLIGKV, and 20 minutes in the presence of SLIGKV, cells were placed on ice, tubes were filled with ice cold PBS, and then cells were collected by centrifugation at 500 x g for 5 minutes at 4oC. Cells were then resuspended in ice-cold PBS and recentrifuged. Cell pellets were then resuspended in 0.5 mL RIPA buffer containing 10 x protease and phosphatase inhibitors and incubated on ice for 10 minutes. Lysates were then clarified by centrifugation at 10,000 x g for 10 minutes at 4oC. Supernatants (soluble lysates) and pellets were separated and snap frozen in liquid nitrogen and stored at -80oC. Soluble lysate samples were sent to UTSW Proteomics Core by overnight delivery in dry ice.

At the UTSW Proteomics Core, soluble lysate samples were digested with trypsin and the peptides were analyzed at the using Tandem Mass Tag (TMT) quantitation with LC-MS/MS Orbitrap Eclipse mass spectrometry. An aliquot of each sample was run on the Orbitrap Eclipse for the total protein analysis (TMT system). The remaining material was processed using a two-step phosphopeptide enrichment protocol. Samples were first enriched using a High-Select TiO2 Phosphopeptide Enrichment kit (Thermo), and then the flowthrough was collected for secondary enrichment with High-Select Fe-NTA phosphopeptide enrichment columns (Thermo). Each of these steps enriches a different subset of phosphopeptides (with some overlap) leading to a more comprehensive coverage relative to using a single method. The phosphopeptides collected from each enrichment step were then combined and analyzed on the Orbitrap Fusion Lumos. The data were analyzed using Proteome Discoverer 3.0 (Thermo Scientific) using the human protein database from UniProt ([www.uniprot.org](http://www.uniprot.org)). Raw and processed proteomic and phosphoproteomics data from the Orbitrap Eclipse mass spectrometry dataset was uploaded to the MassIVE website at the UCSD Center for Computational Mass Spectrometry with accession number MSV000094295.

**2.4 Analysis of proteomics and phosphoproteomics data**

Differences in protein and phosphopeptide expression between males and females, and between unstimulated and SLIGKV stimulated cells were assessed using t-tests. Fold change in expression and t test values were ranked for volcano plot visualization. Gene ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was performed, and graphs were generated, using ShinyGO (v 0.8 using Ensembl Release 107) [35], and results were confirmed using g:Profiler (<https://biit.cs.ut.ee/gprofiler/gost>) and Metascape (<https://metascape.org/>). Groups were analyzed compared with the standard “all proteins” in the Homo sapiens database, and significance (p < 0.05) was determined by Fisher’s exact test with FDR correction. Terms were identified by comparing the list of differentially abundant proteins against the background list of all identified proteins in the proteomics results. Venn diagrams were generated using BioTools (<https://www.biotools.fr/misc/venny>).

**2.5 Whole cell lysate preparation and western blots**

Neutrophil whole cell lysates were prepared as previously described [25, 32] with the following modifications. A total of 2 x 106 neutrophils were resuspended in 0.1 ml of 1x SDS sample buffer with 2-ME with 10x protease and phosphatase inhibitor cocktail and pipetted vigorously to resuspend and lyse the cells, and heated for 5 minutes at 98°C. Cell lysates were analyzed by western blotting, as described previously [25, 36]. Briefly, samples were separated on 4-20% SDS-PAGE gels. Proteins were then transferred to polyvinylidene difluoride (PVDF, #88518, Thermo Scientific) membranes in Tris/glycine/SDS buffer containing 20% methanol. Western blots were blocked with TBS/0.1% Tween 20/5% nonfat milk protein for 1 hour at RT, then incubated overnight at 4◦C with antibodies. Western blots were stained with 2.3 µg/ml anti-CYFIP1 (NBP2-92695; Novus Biologicals, Littleton, CO), 0.05 µg/ml anti-NAP1 (NBP2-24727SS; Novus), or 0.1 µg/ml anti-GAPDH mouse mAb (60004-1-Ig; Proteintech, Rosemont, IL) following the manufacturer’s protocols. Blots were further incubated for 1 hour at RT with 500 pg/mL HRP-conjugated donkey F(ab’)2 anti-rabbit (#715-036-150; Jackson ImmunoResearch Labs, West Grove, PA) or anti-mouse (#711-036-152, Jackson ImmunoResearch) antibodies in TBS/0.1% Tween/5% nonfat milk protein for 30 minutes at RT. Bound antibodies were detected with an ECL Western blotting kit (Thermo). On each experiment day, neutrophils from one male and one female were collected. Western blot band intensities were quantified using Image Lab software (Bio-Rad) and normalized to each test sample’s GAPDH loading control, and the ratio for the female donor was normalizing to the ratio for the date-matched male donor.

**2.6 Fixed-cell microscopy**

Fixed-cell microscopy of unstimulated neutrophils was performed as previously described [25] with the exception that cells were incubated overnight at 4°C in a humid chamber with 4.7 µg/ml anti-CYFIP1 or 0.05 µg/ml anti-NAP1 in PBS/0.1% Tween 20. Immunofluorescence images were captured with a 40x objective using a Ti-Eclipse inverted fluorescence microscope (Nikon, Tokyo, Japan). Mean fluorescence intensity (MFI) of all neutrophils in a field of view (>10 cells per field of view with an average of five or more fields of view per antibody per donor) was quantified as described [25].

**2.7 Statistics**

Prism v7 (GraphPad Software Inc., San Diego, CA, USA) and Microsoft 365 Excel (Microsoft, Redmond, WA) were used for data analysis. Graphs were generated with Prism. Data are shown as mean ± SEM except where otherwise stated. To determine whether the mean difference between two groups was statistically significant, the Mann-Whitney test was used. Statistical significance was defined as p ˂ 0.05. For the volcano plots, one unpaired t test per row was calculated, without assuming consistent SD (the fewer assumptions option), with an uncorrected significance of p < 0.05. GO term groups were analyzed compared with the standard “all proteins” in the Homo sapiens database, and significance (p < 0.05) was determined by Fisher’s exact test with FDR correction.

**3 Results**

**3.1 Male and female neutrophils show differences in levels of some proteins**

To determine if the observed sex-based differences in neutrophil responses to multiple stimuli [7, 25, 26, 37, 38] are associated with differences in protein abundances, unstimulated neutrophils were analyzed by proteomics using Thermo Fusion Lumos gradient mass spectrometry, and this identified 2806 proteins. We also analyzed neutrophil proteins with TMT LC-MS/MS Orbitrap Eclipse mass spectrometry, and this detected 1,823 individual proteins, with 1,428 proteins identified in both the Lumos and TMT Orbitrap datasets (**Fig. 1A)**.

The most abundant proteins detected in the 1,428 proteins identified in both the Lumos and TMT Orbitrap datasets included myeloperoxidase (MPO), neutrophil elastase (NE), the neutrophil serine protease inhibitor SERPINB1, azurocidin (AZU1), the neutrophil gelatinase-associated lipocalin (LCN2), and S100A8 (**Fig. S2A and Table S1 Tabs 1-3)**. These are all proteins that are highly expressed in neutrophils [39, 40], and none of these were higher in males or females. GO term pathway analysis of the 1,428 proteins present in both datasets (**Fig. 1A**) identified proteins found in neutrophil granules (MPO, LYZ, CTSG, and LTF), and proteins involved with adhesion (RHOA, ACTN1, VIM, and EZR), and lysosomes and vacuoles (RAB2A, VPS18, and LAMP2) (**Fig. 1B**). Proteins expressed by monocytes such as CD14, CD32a, CD33 and CD58, by lymphocytes such as CD82, by NK cells such as CD16a, by platelets such as CD63, and by B cells and dendritic cells such as CD48/ SLAMF2, had either very low levels or were undetectable (**Fig. S2B)** [41-43] (Human Protein Atlas: www.Proteinatlas.org). Similar analysis of the proteins in just the Lumos or just the Orbitrap datasets also showed enrichment for neutrophil proteins and very little, if any, proteins associated with monocytes, lymphocytes, NK cells, platelets, B cells, or dendritic cells (**Table S1 Tabs 1-3**). These results are consistent with the cell counts (**Fig.** **S1**) indicating that the cell preparations were highly enriched for neutrophils.

In the Lumos dataset, 52 proteins had sex-based differences in protein abundance, with 48 proteins more abundant in male neutrophils and 4 proteins more abundant in female neutrophils (**Fig. 1C and Table S1 Tab 1**). In the TMT Orbitrap dataset, 112 proteins had sex-based differences in protein abundance, with 85 proteins more abundant in male neutrophils and 27 proteins more abundant in female neutrophils (**Fig. 1D**, **Table S1 Tab 2**, **and** **Fig. S2C**). Comparing the two proteomics sets, there was one protein that was higher in females in the Lumos set but lower in females in the TMT Orbitrap set, and this was excluded from further analysis. Proteins that were higher in one sex or the other in the Lumos dataset were either not present, or the data were not significant (generally because the peptide counts were low), in the TMT Orbitrap dataset, and vice versa. Combining the two proteomics datasets, there were 132 proteins more abundant in male neutrophils and 30 proteins more abundant in female neutrophils (**Table S1 Tab 3**).

KEGG and GO term pathway analysis of the 132 male enriched proteins (**Fig. 1E and Table S1 Tab 3**) identified 23 proteins involved with the spliceosome, nucleocytoplasmic transport, aminoacyl-tRNA biosynthesis, and the ribosome. These include 12 proteins involved with the spliceosome and nucleo-cytoplasmic transport (ALYREF, SNRNP200, LSM4, HNRNPA1, HNRNPC, HNRNPU, HSPA8, MAGOHB, SRSF4, SNRPA, TPR, and NUP93), 5 aminoacyl-tRNA synthetases (EPRS1, FARSA, HARS1, IARS1, and NARS1), and 6 ribosomal proteins (RPL6, RPL15, RPL23A, RPL36A, RPS3, and RPS15A). There were also 6 proteins involved with inositol phosphate metabolism and phosphatidylinositol signaling (INPP1, ALDH6A1, MTMR14, PIP4K2C, PPIP5K2, and DGKZ). The other male enriched proteins were in a variety of additional pathways (**Fig. 1E and Table S1 Tab 3**).

The 30 female enriched proteins (**Table S1 Tab 3**) were enriched for proteins involved in a variety of cytosolic metabolic processes (ALDH9A1, ACAT2, AHCY, and EPHX1), endosome/lysosome/proteosome proteolytic pathways (AHCY, EPHX1, TOLLIP, RAD23B, and GGA3), and serine/threonine phosphatase regulatory proteins (PPP1R3D and PPP2R2A).

In the Lumos dataset, cytoplasmic FMR1-interacting protein 1 (CYFIP1; UniProt Q7L576) is one of the 85 proteins that were more abundant in male neutrophils (**Table S1 Tabs 1 and 3**). In agreement with the proteomics results, CYFIP1 was more abundant in male neutrophils both by Western blots (**Fig. 2A**) and immunofluorescence staining (**Fig. 2B**). The proteomics indicated no sex-based differences in the abundance of Nck-associated protein 1 (NAP1; UniProt Q9Y2A7; also known as NAP125, NCKAP1, or HEM1) (**Table S1 Tab 1**), and this was also observed by Western blots and immunofluorescence (**Fig. 2D-F**).

To determine if the more rapid response of male neutrophils to the chemorepellent SLIGKV [25] corresponds to a more rapid change in protein levels, neutrophils were incubated with SLIGKV. After 5 or 20 minutes, only the protein phosphatase PPP1R3D showed a greater than 2-fold change in total protein levels, and this occurred in male neutrophils (**Fig. 3A-D**). We assessed if proteins that had a difference in protein abundance, irrespective of fold change, in males after 5 minutes incubation with SLIGKV (red dots in **Fig. 3A**) were also significantly changed in females after 5 minutes (red dots in **Fig. 3C**). Four proteins (AP2S1, RARS1, TIPRL, and IGBP1) were elevated in male compared to female cells (**Fig. 3E**). We also determined if the proteins that showed a significant change in levels in male neutrophils after 20 minutes incubation with SLIGKV (red dots in **Fig. 3B**) were also significantly changed in females after 20 minutes (red dots in **Fig. 3D**). Three proteins (NEDD9, PRKAG1, and ARHGAP27) were elevated in male compared to female cells (**Fig. 3F**). Together, the data indicate that SLIGKV affects levels of proteins in both male and female neutrophils within 5 minutes, but a comparison of the number of proteins with significantly changed levels (number of red dots in **Fig. 3A and 3C**) suggests that more proteins show changes in levels in male neutrophils. A similar effect was observed at 20 minutes (number of red dots in **Fig. 3B and D**).

**3.2 Male and female neutrophils show differences in protein phosphorylation**

To determine if the observed sex-based differences in proteins are also associated with differences in protein phosphorylation, neutrophil proteins were digested with trypsin, the phosphorylated peptides were purified, and these peptides were analyzed to identify phosphorylated proteins. There was no significant difference in the total number of phosphoproteins identified in male and female neutrophils (**Figs. S3A and S3B**). A total of 396 phosphoproteins were identified from male and female donors. GO term analysis of these phosphoproteins indicated enrichment for neutrophil and myeloid mediated immunity, including degranulation, activation, and exocytosis (**Fig. S3C**). The phosphoproteins included many common neutrophil proteins, such as MPO, S100A9, LTF, and AZU (**Table S1 Tab 4**). These phosphoproteins included 22 proteins encoded on the X chromosome including proteins involved in RNA processing (NKAP, HTATSF1, DKC1, RBMX2, MSN, MECP2, FLNA, and TMSB4X), and cellular activation (WAS, DKC1, MSN, MECP2, FLNA, NKAP, ELF4, SASH3, SH3KBP1, and PGRMC1). There were no Y chromosome-encoded phosphoproteins.

Of the 396 phosphoproteins identified in unstimulated neutrophils, 32 of the phosphoproteins had a significant and > 2-fold sex-based difference in phosphorylation, with all 32 phosphoproteins being more phosphorylated in male neutrophils (**Fig. 4A and Table S1 Tab 5**). The 32 phosphoproteins were enriched for proteins that inhibit transcription by RNA polymerase I and regulate RNA splicing (MACROH2A1, AHNAK, RALY, MFAP1, SRRM2, and CD2BP2), regulate protein localization and apoptotic signaling in mitochondria (BAD, NMT1, RPS3A, CALM3, and FLNA), and regulate neutrophil activation (MNDA, S100P, FTH1, PA2G4, and PSAP) (**Fig. 4B, Fig. S4A-S4B, and** **Table S1 Tab 6**). Of the 32 proteins with a sex-based difference in phosphorylation, 30 showed no significant sex-based difference in total protein abundance. Only 2 proteins with a sex-based difference in phosphorylation (EPRS1 and RALY) had a significant sex-based difference in total protein abundance; both showed increased phosphorylation in males, and increased abundance in males (TableS1 Tab3).

At 5 minutes, SLIGKV increased phosphorylation of TMC8 and NUP188 in male neutrophils, and SLIGKV did not significantly decrease phosphorylation of any detected proteins in males (**Fig. S5A, Fig. S4C-S4D**). There was no significant effect of SLIGKV on protein phosphorylation in female neutrophils at 5 minutes (**Fig. S5B**). SLIGKV did not significantly affect total protein levels of TMC8 and NUP188 at 5 minutes (**Fig. 3C-3D,** **Table S1 Tabs 1-2**)**.** TMC8 (also called EVER2) is a ion channel-like transmembrane protein associated with the ER and Golgi with higher expression in keratinocytes and immune cells including neutrophils (www.proteinatlas.org), and elevated levels of TMC8 are associated with increased numbers of immune cells in tumors [44]. Mutations in TMC8 are associated with Epidermodysplasia verruciformis [45]. NUP188 is a component of the nuclear pore complex (NPC), regulates chromosome segregation, and NUP188 mutations are associated with a variety of inherited genetic syndromes and cancers [46-49].

At 20 minutes, SLIGKV increased phosphorylation of HNRNPH1 in male neutrophils, did not significantly decrease phosphorylation of any detected proteins in males (**Fig. S5C, S4E**), and had no significant effect on protein phosphorylation in female neutrophils (**Fig. S5D**). SLIGKV did not significantly affect total protein levels of HNRNPH1 at 20 minutes (**Fig. 3C, 3D,** **Table S1 Tabs 1-2**). NHRNPH1 is an RNA binding protein that associates with pre-mRNAs in the nucleus and regulates mRNA processing and splicing [50]. The only protein showing higher phosphorylation in female neutrophils was PRUNE2, and the phosphorylation was only significantly higher at 20 minutes after SLIGKV exposure (**Fig. S4F**). There was no significant difference in total protein levels of PRUNE2 (**Fig. 3D,** **Table S1 Tab 1**). PRUNE2 (also called BMCC1), suppresses RHOA and AKT signaling, reducing cell migration and survival [51, 52]. It is unclear how phosphorylation of PRUNE2 affects its function. Together these data indicate that SLIGKV affects protein phosphorylation in male but not female neutrophils at 5 and 20 minutes, in agreement with the faster responses of male neutrophils to SLIGKV [25].

**4 Discussion**

We observed 132 proteins that were more abundant in unstimulated male neutrophils and 30 proteins were more abundant in unstimulated female neutrophils. In male neutrophils, many of the 132 upregulated proteins are involved with translation (tRNA biosynthesis, spliceosome regulation, and RNA and ribosome binding), inositol phosphate metabolism, and phosphatidylinositol signaling. CYFIP1 was more abundant in male neutrophils and interacts with translation initiation factor eIF4E [53], suggesting the intriguing possibility that changes in levels of CYFIP1 may cause sex-based differences in translation in neutrophils. CYFIP1 also regulates the actin cytoskeleton [54-57], suggesting that changes in levels of CYFIP1 may account for some of the observed sex-based differences in neutrophil chemorepulsion [25]. The 30 proteins with higher levels in female neutrophils were enriched for proteins present in granules, metabolic processes, and proteolytic pathways, but were generally not encoded by type I interferon stimulated genes. These data may help to explain observations that female neutrophils have a higher phagocytic activity and a more effective immune response to infection [5, 8-10]. In male neutrophils, there was an enrichment of proteins involved with translation, whereas female neutrophils were enriched for proteins involved with metabolic, proteolytic, and cytoskeletal pathways. These data may also help explain the observation that male neutrophils have an “immature” profile, suggesting recent release from the bone marrow and still undergoing differentiation with residual translation, whereas female neutrophils have a more mature profile and are primed for granule release and response to infections [8-10, 37, 38, 58].

We previously observed that male neutrophils have a more rapid response to the chemorepellent SLIGKV [25]. We found that there were 5 proteins that were elevated in male neutrophils at 5 minutes after incubation with SLIGKV, and no proteins elevated at 5 minutes in female neutrophils. PPP1R3D was enriched in unstimulated female neutrophils but showed a significant increase in protein levels in male neutrophils after 5 minutes with SLIGKV. PPP1R3D is a regulatory subunit of protein phosphatase 1, which regulates many cellular processes including cell polarization and migration [59, 60]. Four other proteins (AP2S1, TIPRL, IGBP1, and RARS1) were also elevated in male neutrophils at 5 minutes. AP2S1 is a component of the adaptor protein complex 2 which is involved with clathrin-dependent endocytosis [61], TIPRL in an inhibitor of the protein phosphatases 2A and 4 [62], IGBP1 binds the protein phosphatase PP2A and protects it from degradation [63], and Arginine-tRNA ligase (RARS1) is a tRNA synthetase involved in translation [64]. Besides translation, RARS1 is also involved in the arginylation of β-actin by arginyl-tRNA protein transferase 1 (ATE1) at the leading edge of migrating cells [65, 66]. Together, this suggests that the fast response to SLIGKV in male neutrophils may be due to effects on protein phosphorylation, endocytosis, and motility. The fast increase in levels of these proteins is difficult to explain by an increase in protein synthesis, so one possibility is that SLIGKV induces a very rapid inhibition of the degradation of these proteins in male but not female neutrophils.

After 20 minutes incubation with SLIGKV, three proteins (NEDD9, PRKAG1, and ARHGAP27) were elevated in male compared to female neutrophils, and no proteins were significantly elevated in female neutrophils. NEDD9 is an adaptor protein involved in adhesion and cell migration [67], PRKAG1 is a regulatory subunit of the AMP-activated protein kinase (AMPK), which not only regulates biosynthesis of fatty acid and cholesterol but also cell migration [68], and ARHGAP27 is a member of the RhoGAP protein family, which regulates cell motility [69]. ARHGAP27 is in a locus for susceptibility to SLE, which is more prevalent in females [70, 71]. These data suggest that although at 20 minutes, both male and female neutrophils move away from the chemorepellent SLIGKV[25], male neutrophils also upregulate proteins involved with cell motility.

The 32 proteins showing increased phosphorylation in male neutrophils include proteins that regulate processing of RNA (AHNAK, HNRNPH1, and RALY), proteins that transport molecules between the cytoplasm and nucleus (NUP188), and proteins such as calmodulins and actin binding proteins that regulate signaling and cell migration (CALM3, TMC8, and FLNA). Filamin-A (FLNA) is an X-chromosome encoded actin-binding protein that cross links actin and links membrane proteins to the cytoskeleton [72]. Phosphorylation of FLNA positively regulates cell migration in many cells, including neutrophils [73, 74]. Collectively, analysis of the 32 proteins indicates that compared to female neutrophils, male neutrophils have increased phosphorylation of proteins involved in RNA splicing, protein localization, the cytoskeleton, apoptotic signaling in mitochondria, and neutrophil activation.

After incubation with SLIGKV, TMC8 and NUP188 had increased phosphorylation in male neutrophils at 5 minutes, and HNRNPH1 had increased phosphorylation in male neutrophils at 20 minutes. The only protein showing higher phosphorylation in female neutrophils was PRUNE2, and the phosphorylation was only significantly higher at 20 minutes after SLIGKV exposure.

Our observation of sex-based differences in protein phosphorylation suggests that if phosphorylation is considered a general marker for cell activation, then our findings would help explain the observation that male neutrophils respond quicker to the chemorepellent SLIGKV [25]. The slower response time of female neutrophils to SLIGKV could also be due to the elevated levels of the protein phosphatases PPP1R3D and PPP2R2A, and phosphorylated PRUNE2 which suppresses RHOA and AKT signaling, thus reducing cell migration [5, 51, 52, 75]. Our data indicates the surprising finding that many of the sex-based differences in proteins and phosphoproteins are regulators of translation. As these proteins are associated with the translational pathway from spliceosome to ribosomes, it suggests that this is a fundamental process that is underappreciated in neutrophils, especially as it appears to be specific to neutrophils from males. Previous reports also indicate that male neutrophils have significant translation capacity, which may explain why male neutrophils are described as having an “immature” phenotype or possessing “phenotypic plasticity” [5, 76].

The sex-based differences in immune responses, where females have a stronger innate and adaptive immune response to infection but a higher incidence of autoimmune disorders, could in part be explained by our data as male neutrophils respond effectively to a chemorepulsive signal but neutrophils from females do not. In females, this could lead to the persistence of neutrophils at inflammatory sites, which during clearance of bacteria would be beneficial, but in an autoimmune infiltrate the accumulation of neutrophils could lead to a persistent and damaging immune response. An intriguing possibility is that therapies that affect neutrophil biology may need to be modified for male or female patients [8, 9, 77, 78].

**5 Associated Data**

Proteomic data has been uploaded to MassIVE at UCSD Center for Computational Mass Spectrometry with accession numbers MSV000088857 and MSV000094295.

**Figure Legends**

**Figure 1. Comparison of proteomics from 2 independent datasets. A)** Comparison of individual proteins identified by Lumos and TMT-Orbitrap mass spectrometry. The Lumos dataset has 5 male and 5 female donors, and the TMT-Orbitrap dataset has a different cohort of 3 male and 3 female donors. **B)** Gene ontology analysis of the 1428 proteins identified in both datasets indicates protein enrichment related to primary and secretory granules, and lysosomal proteins. Volcano plots from **C)** Lumos and **D)** Orbitrap datasets showing the fold change (Log2) and p-value (-Log10) comparing the proteomes from male and female donors. Proteins are marked in red have p values <0.05 (-Log10 >1.3), with those proteins having more than a two-fold change (Log2 <-1 or >1) indicated with gene ID. **E)** GO term KEGG analysis of combined proteins enriched in male proteins.

**Figure 2. CYFIP1 is enriched in male neutrophils.** Unstimulated male and female neutrophil lysates were analyzed by western blots to quantify levels of **A)** CYFIP1, or **D)** NAP1. Bars and error bars are mean ± SEM from four males and four females. Unstimulated neutrophils were fixed, permeabilized, and stained for F-actin (red), and either **B)** CYFIP1, or **E)** NAP1 (green). Blue is DAPI staining of DNA. Bars are 10 µm. **C and F**) Quantitation of the mean fluorescence intensity (green) in **B** and **E)**, respectively, normalized to the average mean fluorescence of each experiment’s male (one male and one female were used for each individual experiment) for each antibody. Images and quantitation are representative of three (NAP1) or four (CYFIP1) independent experiments. \* indicates p < 0.05 (Mann-Whitney *U* test).

**Figure 3. Comparison of proteomics from unstimulated and SLIGKV-stimulated neutrophils.** Volcano plots showing the fold change (Log2) and p value (-Log10) comparing significant differences in total protein abundance in **A)** unstimulated versus 5 minutes stimulated male cells, **B)** unstimulated and 5 minute stimulated female cells, **C)** unstimulated and 20 minute stimulated male cells, and **D)** unstimulated and 20 minute stimulated SLIGKV stimulated female cells. Proteins are marked in red have p values <0.05 (-Log10 >1.3), with those proteins having more than a two-fold change (Log2 <-1 or >1) indicated with gene ID. **E)** Volcano plot showing proteins with significant difference in abundance after 5 minutes with SLIGKV in male cells (red dots in **A**) versus females after 5 minutes (red dots in **C**). **F)** Volcano plot showing proteins with significant differences in abundance after 20 minutes with SLIGKV in male cells (red dots in **B**) versus females after 20 minutes (red dots in **D**).

**Figure 4. Comparison of phosphoproteomics from unstimulated and SLIGKV-stimulated neutrophils. A)** Volcano plot comparing phosphoproteins from unstimulated male and female neutrophils. Proteins having p values <0.05 (-Log10 >1.3) and more than a two-fold change (Log2 <-1 or >1) are indicated with gene ID and marked in red. **B)** GO term analysis of phosphoproteins enriched in unstimulatedmale cells.

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**Conflict of interest statement**

The authors have declared no conflict of interest.

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**Authors’ Contributions**

D.P. and K.M.C. designed, performed, analyzed experiments, and wrote the paper. S.A.K. performed experiments. R.H.G. designed experiments and wrote the paper.

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