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Modulation of neutrophil inflammatory responses by iron oxide nanoparticles, DAMPs, and cytokines in health and psoriatic disease

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Modulation of neutrophil inflammatory responses by iron oxide nanoparticles, DAMPs, and cytokines in health and psoriatic disease

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List of Abbreviations:

ADAP - Adhesion and Degranulation-Promoting Adapter Protein

ARDS - Acute respiratory distress syndrome

Btk – Bruton's Tyrosine Kinase

CalDAG-GEFI - Calcium and Diacylglycerol-Regulated Guanine Nucleotide Exchange Factor I

CR3 - Complement Receptor 3

CRAC - Calcium Release Activated Calcium

DAG - Diacylglycerol

DAMP - Damage-Associated Molecular Pattern

EGF - Epidermal Growth Factor

ELISA - Enzyme-linked immunosorbent assay

ER - Endoplasmic Reticulum

ERK - Extracellular Signal-Regulated Kinase

FERM - 4.1/ezrin/radixin/moesin

GPCRs - G-Protein Coupled Receptors

GM-CSF - Granulocyte-macrophage Colony-stimulating Factor

GTPase - Guanosine Triphosphatase

IL - Interleukin

ILK - Integrin-Linked Kinase

IP3 - Inositol-1,4,5 Triphosphate

IP3R - IP3 Receptor

LFA-1 - Lymphocyte Function-Associated Antigen-1

Mac-1 - Macrophage-1 Antigen

MIDAS - Metal-Ion Dependent Adhesion Site

MRP8/14 - Myeloid-Related Protein 8/14

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NETosis - Neutrophil Extracellular Trap Formation

PBS- Phosphate-buffered saline

PH - Pleckstrin Homology

PI3K - Phosphoinositide 3-Kinase

PIP2 - Phosphatidylinositol 4,5-Bisphosphate

PIP3 - Phosphatidylinositol (3,4,5)-Trisphosphate

PKCα - Protein Kinase C Alpha

PSGL-1 - P-selectin Glycoprotein Ligand-1

PSI - Plexin-Semaphorin-Integrin

RA - Rheumatoid Arthritis

RAGE - Receptor for Advanced Glycation End Products

RIAM - Rap1-Interacting Adapter Molecule

ROS - Reactive Oxygen Species

SLex - Sialyl Lewis x

STIM1 - Stromal Interaction Molecule 1

SYK - Spleen Tyrosine Kinase

TGT - Tension Gauged Tethers

TLR4 - Toll-Like Receptor 4

TNF - Tumor Necrosis Factor

VCAM-1 - Vascular Cell Adhesion Protein 1

SPION - Superparamagnetic iron oxide nanoparticles

MNP - Magnetic nanoparticles

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Abstract

Neutrophils are vital for elimination of foreign particulates and pathogens, but their inappropriate activation in circulation can lead to tissue damage upon recruitment to sites of inflammation. Thus, neutrophil activation and recruitment is tightly regulated by the synergy between mechanical forces acting on neutrophil adhesion receptors and chemotactic stimulation mediated by inflammatory mediators. However, the synergy between outside-in signaling through selectins and integrins and inside-out signaling via G-protein coupled and Tumor necrosis factor receptors can be altered by foreign materials and chronic exposure to endogenous molecules that signal inflammatory responses such as Toll-like receptors during disease. In this thesis we explored how iron oxide nanoparticles and damage associated molecular patterns DAMPs alter the neutrophil's inflammatory response to chemokines and cytokines in freshly obtained blood samples from healthy individuals and patients with psoriasis.

Intravenous delivery of nanoparticles is increasingly being employed for diagnostics and therapeutics. Feraheme is an FDA approved intravenous delivered iron oxide nanoparticle for the treatment of iron deficiency anemia (IDA) in adult patients with chronic kidney failure, but research on its effect on the innate immune response of neutrophils during inflammation has not been reported.

Increasing interest in use of Feraheme for novel imaging techniques and therapeutics motivates the first objective of this dissertation; to study the immunomodulatory effects of Feraheme on neutrophil adhesive capacity and responses to GPCR agonists and function. In contrast to foreign nanoparticles, DAMPS and cytokines are produced endogenously to regulate inflammatory responses of immune cells, however during chronic inflammation continuous exposure to inflammatory stimuli can lead to aberrant neutrophil function. Psoriasis is a systemic inflammatory disease that is characterized by exuberant neutrophil recruitment into skin plaques where neutrophils are primed for production of ROS and release of extracellular traps that exacerbate inflammation. Here we explored the role of neutrophil activating cytokines IL-8 and TNF-α as well as DAMPs S100A8/A9 and LL37, known to be upregulated in psoriasis, to prime circulating neutrophils for enhanced β2-integrin-mediated recruitment and effector functions.

Exposure to the foreign iron oxide nanoparticles Feraheme had no effect on baseline levels of neutrophil adhesion marker expression, indicating no priming or activation. However, Feraheme treatment reduced neutrophil response to IL-8 mediated upregulation of membrane CD11b/CD18 receptors, activation of high affinity CD18, and shedding of CD62L. Feraheme uptake by neutrophils also inhibited N-formyl-Met-Leu-Phe (fMLP) induced reactive oxygen species (ROS) production. Preincubation of neutrophils with Feraheme greatly reduced capacity capture efficiency on a substrate presenting E-selectin and ICAM-1 in vascular mimetic flow channels. Lastly, Feraheme modulated calcium flux dynamics in neutrophils with accelerated clearance of cytosolic calcium following IL-8 induced flux. Ca^{2+} is a vital signaling molecule downstream of chemotactic signaling and integrin mechanosignaling. Thus, we conclude that inducing rapid sequestration of calcium is a likely mechanism by which Feraheme uptake inhibits neutrophil chemotactic activation and recruitment efficiency.

Psoriatic neutrophils in blood display a primed phenotype characterized by elevated β2-integrin receptor number and binding affinity that correlated with plasma calprotectin levels. Psoriatic neutrophils were exquisitely sensitive to ex vivo stimulation with TNF-α that elicited secretion of calprotectin and synergistically amplified CD18 activation. In accordance with increased β2-integrin activation, psoriatic neutrophils arrest on E-selectin and ICAM-1 twice as efficiently and had greater capacity for calcium flux and production of ROS than healthy neutrophils.

These studies highlight the contrasting effects that foreign and innate stimuli can have on modulating neutrophil responsiveness. We report that the Feraheme iron oxide nanoparticles limit neutrophil recruitment and inhibit inflammatory responses to GPCR simulation by accelerating clearance of calcium after stimuli induced flux. In contrast, psoriatic neutrophils were primed for increased adhesion and effector function that correlated with circulating levels of DAMPs and cytokines, particularly S100A8/A9 which is secreted more readily in response to TNF-α by psoriasis neutrophils compared to healthy control.

Chapter 1 Background and significance

1.1 Neutrophil recruitment cascade

Neutrophils are vital first responders from blood into sites of inflammation, but unbridled infiltration results in tissue damage and chronic inflammation. Thus, neutrophil vascular recruitment is tightly orchestrated by a multistep cascade incorporating mechanotransduced signals from adhesion receptors and chemical signals from inflammatory cytokines, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPS) (Kolaczkowska & Kubes, 2013; McDonald et al., 2010). Recruitment begins with neutrophil tethering to endothelial cells (EC) mediated by a family of carbohydrate-binding proteins known as selectins. The selectin family is comprised of the EC expressed E-selectin (CD62E), the leukocyte expressed L-selectin (CD62L) and the platelet and endothelial cell expressed P-selectin (CD62P) (Bevilacqua et al., 1991). Selectins adhere through recognition of the branched tetrasaccharide Sialyl Lewis x (SLex) which decorates selectins and their ligands (Cappenberg et al., 2022). Upon signs of inflammation, ECs rapidly express luminal E-selectin and P-selectin which bind to constitutively expressed neutrophil L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1), respectively, to initiate the process of neutrophil tethering under hydrodynamic shear flow.

These selectins form transient bonds whose association and dissociation under shear flow results in leukocyte rolling. Shear force applied on selectins opposing the flow of blood promotes the formation of selectin clusters on the cell membrane (Morikis et al., 2020). Mechanotransduction through selectin clusters combined with chemokines presented by ECs synergistically activate a signaling cascade that modulates the affinity of the neutrophil integrin, Lymphocyte function-associated antigen-1 (LFA-1). Activation and clustering of LFA-1

promotes bond formation with intracellular adhesion molecule (ICAM)-1 which decelerates neutrophils and subsequently mediates a transition from rolling to shear-resistant arrest (Lum et al., 2002; Yago et al., 2018). Chemotactic signaling synergizes with mechanotransduced integrin bond tension at sites of focal adhesion to activate a flux in cytosolic calcium that acts as a secondary messenger to drive F-actin-mediated neutrophil shape change and cell migration (Dixit et al., 2011). Finally, activated β2 integrin, Macrophage-1 antigen (Mac-1), promotes crawling along the endothelium towards gap junctions where neutrophils transmigrate out into sites of inflammation.

1.2 β2-integrin structure and affinity regulation

Integrins are comprised of a superfamily of heterodimeric proteins consisting of two non-covalently linked transmembrane α and β chains that promote adhesion and signal transduction. In mammals, there are 24 functionally distinct integrins formed by combinations of 18 α and 8 β subunits. The β2-integrins are a family of four adhesion receptors exclusively expressed in leukocytes made by pairing of the β2-chain (CD18) with one of four different α-chains (αL, αM, αX, and αD) (Bednarczyk et al., 2020). The β2-integrins are the most common integrin expressed by leukocytes and each of the four heterodimers exhibits unique and overlapping ligand binding and functional properties (Arnaout, 1990). The first β2-integrin to be discovered was LFA-1 which is composed of αL/ β2 (CD11a/CD18) heterodimers. LFA-1 binds to ICAM-1 through ICAM-5 as well as Junctional adhesion molecule -A (JAM-A) (Humphries et al., 2006). Mac-1, also known as complement receptor (CR3), consists of αM/ β2 (CD11b/CD18) and binds to a wide variety of ligands including ICAMs, complement fragment iC3b, and several extracellular matrix proteins (Arnaout, 2016). αXβ2 also known as CD11c/CD18, p150.95 or CR4 has similar homologies to Mac-1 and binds ICAM-1, ICAM-4, iC3b, vascular cell adhesion protein 1

(VCAM-1), and several extracellular matrix components. The most recently discovered β2-integrin αDβ2 (CD11d/CD18) also has very similar homologies to Mac-1 and binds ICAM-3, VCAM-1, and several extracellular matrix components.

The extracellular domain (ectodomain) of α-chains consists of a seven-blade β-propeller domain, an Ig-domain-like thigh domain, two calf domains, and a ligand binding inserted (I) domain that is inserted between the second and third blades of the β-propeller (Uotila et al., 2023).The I domain contains a metal-ion dependent adhesion site (MIDAS) domain that is modulated by divalent cations Mg^{2+}, Mn^{2+}, and Ca^{2+} (Lee et al., 1995)**.** The ectodomain of the β-subunit comprises seven domains with complex domain insertions: a β I domain that is homologous to the α I domain with insertion in the hybrid domain, a plexin-semaphorin-integrin (PSI) domain, four cysteine-rich epidermal growth factor (EGF) modules, and a beta-tail domain (βTD) domain (Kadry & Calderwood, 2020) (Figure 1.1).

In quiescent neutrophils, b2-integrins adopt a bent conformation with the helical α and β transmembrane domains associated non-covalently and the ligand binding I domain in a sequestered state resulting in low affinity for cognate ligands (Kim et al., 2011). During neutrophil rolling on E-selectin neutrophil L-selectin and PSGL-1 signal extension (E+) of the integrin ectodomain. However, the MIDAS domain remains obstructed resulting in an intermediate affinity for ligands which mediates slow rolling (Zhou et al., 2021). Subsequent signaling through selectin bonds and or chemokines is necessary to reveal the ligand binding MIDAS domain (H+). Both integrin extension and MIDAS exposure is required to achieve the fully competent high affinity ligand binding state (E+H+) which mediates firm arrest and subsequently signals calcium and downstream effector function (Schaff et al., 2008). In addition to the classical bent-closed (E-H-) conformation, extended-closed conformation (E+H-), and

extended-open conformation (E+H+), recent reports described a bent-open conformation (E-H+) that binds to ICAM-1 expressed on the neutrophil surface in cis rather than binding to ICAM-1 on an opposing cell in trans (Fan et al., 2016). The authors postulate that this conformation may limit recruitment as blocking neutrophil ICAM-1 and inhibiting this state led to increased recruitment. Activation to the E+H+ was reported to happen just as frequently from the E+H-, as the E-H+ conformation contradicting previous models of integrin activation that propose integrins transition from E-H- to E+H- and finally E+H+. The physiological relevance of the extended state remains unclear, and most studies have only focused on samples from healthy donors. In this thesis we explore the use of the extended state of cd18 as marker for inflammation in chronic disease.

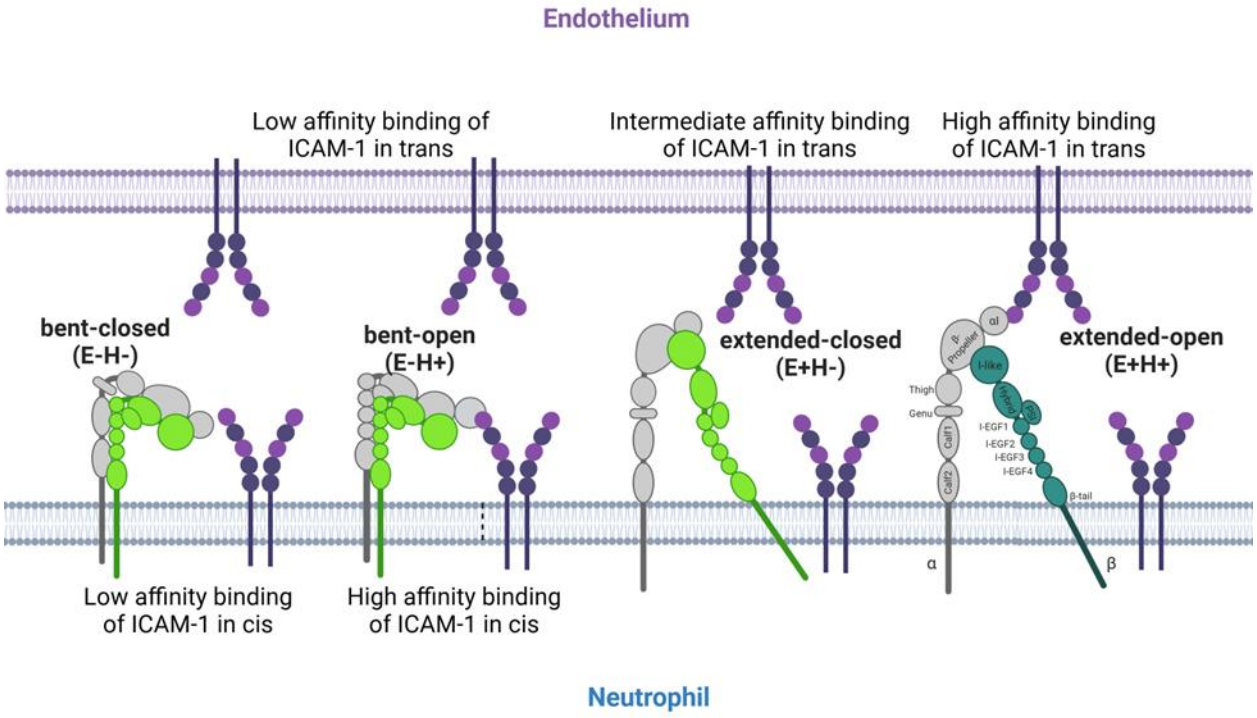

Figure 1.1: β2-integrin structure and affinity states. The bent-closed (E-H-) conformation has low affinity for ICAM-1 binding. The bent-open conformation (E-H+) binds to ICAM-1 expressed on the neutrophil surface in cis. The extended-closed conformation (E+H-) can bind to

ICAM-1 in trans with an intermediate affinity. The extended-open conformation (E+H+) binds to ICAM-1 in trans with high affinity and mediates firm arrest. Created with BioRender.com.

Shifts in integrin conformation are regulated by intracellular signaling cascades that promote recruitment and binding of the two cytosolic adaptors talin-1 and kindlin-3 to the β2-integrin tail (Wen et al., 2022). These two adaptor proteins contain 4.1/ezrin/radixin/moesin (FERM) domains with four subdomains (F0–3) (Moser et al., 2009). The F3 domains of talin-1 and kindlin-3 bind to two specific NPxF/Y domains on the β2-integrin cytoplasmic tails, with talin-1 binding the membrane proximal and kindlin-3 binding the membrane distal NPxF/Y domain (Calderwood et al., 2002; Moser et al., 2009). Knockout experiments in neutrophil-like HL-60 cells demonstrated that talin-1 is necessary for both extension and high affinity activation of β2-integrin, while kindlin-3 is only required for high affinity activation (Ye & Petrich, 2011).

Binding of talin-1 promotes the E+ conformation by inducing changes in the angle of the β2 transmembrane domain resulting in extension of the ectodomain (Sun et al., 2019). At baseline, talin-1 resides in the cytosol in an autoinhibited state with the C-terminal rod domain interacting with the FERM domain in the N-terminal head restraining talin-1 in a closed conformation (Goksoy et al., 2008). Talin-1 unfolding is regulated by two mechanisms involving activation of the small GTPase Rap1 (Wen et al., 2022). One mechanism, critical for integrin activation in leukocytes, involves formation of a complex between Rap1, Rap1-interacting adapter molecule (RIAM), and talin-1 (Lagarrigue et al., 2016).This process necessitates Src-dependent phosphorylation to disrupt an intermolecular interaction which conceals the binding site for the cell membrane protein phosphatidylinositol 4,5-bisphosphate (PIP2) contained within the pleckstrin homology (PH) domain of RIAM. Unveiling of this binding site promotes plasma membrane association and subsequent integrin activation (Yang et al., 2014). The second

mechanism entails a direct interaction between RAP-1 and talin-1, which leads to membrane association and integrin activation (Gingras et al., 2019). This latter pathway represents a universal activation mechanism present in all integrin classes and cell types and serves as the exclusive pathway for integrin activation in platelets (Lagarrigue et al., 2020). Mouse knockout studies reveal that while Rap1–RIAM–talin-1 is the dominant pathway, both pathways contribute to efficient neutrophil integrin activation and subsequent recruitment efficiency (Bromberger et al., 2021).

Kindlin-3 binding to the β2 tail results in disruption of a transmembrane association between the integrin tails necessary to achieve the high affinity conformation (Kondo et al., 2021). The signaling pathways and mechanisms that control recruitment of kindlin-3 to the cytosolic β tail have yet to be entirely elucidated. One mechanism by which kindlin-3 recruitment to the cell membrane is regulated is through Phosphoinositide 3-kinases (PI3Ks) activation. PI3K catalyzes conversion of PIP2 to phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5) P3) PIP3 which has higher affinity for the kindlin-3 PH domain inserted into the F2 subdomain (Ni et al., 2017). Kindlin-3–dependent integrin activation is also regulated by a requirement for post-translational phosphorylation mediated by protein kinase C alpha (PKCα) in an integrin-linked kinase (ILK)-dependent manner (Margraf et al., 2020). Recently, a study that reported the crystal structure of human full-length kindlin-3 revealed a novel homotrimer state that auto-inhibited binding to the integrin β2 cytoplasmic tail by occluding the integrin-binding pocket in the F3 subdomain. Whether this autoinhibited homotrimer occurs in vivo and plays a role in regulation of kindlin-3-mediated integrin activation is still to be confirmed (Bu et al., 2020) .

Integrin activation is synergistically signaled by selectin mechanotransduction and ligation of inflammatory mediators (Morikis & Simon, 2018). While high doses of chemokine alone can mediate integrin activation, low doses well below the K_D of chemokine occupancy typically do not signal activation of integrin and rely on synergy with selectin signaling (Yago et al., 2018). The striking cooperativity between selectin mechanosignaling and chemokine signaling is demonstrated by a 100-fold greater sensitivity to the chemokine IL-8 in neutrophils rolling on E-selectin, compared to those under no shear, resulting in a greatly amplified calcium flux which mediates integrin activation and arrest (Schaff et al., 2008).

Chemokine stimulation of G-protein coupled receptors (GPCRs) signals separation of G proteins, into Gα and Gβγ subunits triggering phospholipase C (PLC)-β and PI3Kγ activation (Lämmermann & Kastenmüller, 2019). Force acting on L-selectin and PSGL-1 promotes clustering and recruitment of FcRγ and DAP-12 into lipid rafts. Subsequent recruitment of Src family kinase FGR mediates phosphorylation of DAP12 and FcRγ, which recruits Syk (Zarbock et al., 2008). Downstream of Syk activation, SLP-76 and ADAP interaction signals activation of Bruton's Tyrosine kinase (Btk) (Block et al., 2012). Btk activates two distinct signaling pathways through activation of PLCpγ2 and PI3Kγ (Mueller et al., 2010).

Thus, PI3Kγ and PLC serve as common synergistic pathways downstream of selectin and GPCR. PI3Kγ catalyzes conversion of PiP2 to PiP3 promoting recruitment of important adaptor molecules kindlin-3 and Skap2, necessary for integrin transition from extension to high affinity (Boras et al., 2017). PLCs split PIP2 into diacylglycerol (DAG) and inositol-1,4,5 triphosphate (IP3), which signals downstream calcium flux and P38 activation. DAG and calcium flux then activate CalDAG-GEFI which along with P38 mediate Rap1 activation of talin-1 (Stadtmann et

al., 2011). DAG also regulates Rap1 activation through activation of PKC (Spitaler & Cantrell, 2004).

Recent studies have shown that ligation of PSGL-1 and L-selectin during rolling elicits release of MRP8/14 (S100A8/A9) which induce activation of Rap-1 and extension of β2-integrin in a Toll-like receptor (TLR)4 dependent manner (Morikis et al., 2017; Pruenster et al., 2015). Mouse experiments showed simultaneous blocking of MRP8/14 and GPCR signaling greatly inhibited neutrophil recruitment. Currently the signaling pathways both upstream and downstream of S100A8/A9 and their importance in mediating neutrophil integrin activation and recruitment are not entirely elucidated. However, increased circulating levels of S100A8/A9 have been linked to several chronic inflammatory diseases motivating work in this dissertation focused on examining the link between S100A8/A9 levels in psoriasis and integrin activation.

1.3 β2-integrin mechanosignaing of calcium flux and ROS

In addition to their paramount importance in mediating neutrophil recruitment from the vasculature, β2-integrins regulate several neutrophil effector functions vital for clearing infection and resolving inflammation. Focal clusters of high affinity integrin and their cytoplasmic adaptor proteins serve as a complex for mechanotransduction of calcium entry, kinase activation, and cytoskeletal reorganization necessary to achieve neutrophil migration and unleash effector functions including degranulation, phagocytosis, reactive oxygen species (ROS) production and NETosis (Bouti et al., 2021; Mayadas & Cullere, 2005). Here we will describe the role of β2-integrin signaling in mediating calcium flux and downstream production of ROS.

Baseline levels of calcium in quiescent neutrophils are low and elevations in cytosolic calcium regulate activation of intracellular signaling proteins that control neutrophil functions (Immler et al., 2018a). Elevations in cytosolic calcium levels in neutrophils is in part regulated

by an influx of calcium from the extracellular space through membrane channels in a process known as store-operated calcium entry (SOCE) (Immler et al., 2018b). SOCE in neutrophils can be initiated through several receptor pathways including engagement of GPCRs, Fcγ-receptors, β2-integrins, PSGL-1, and L-selectin (Futosi et al., 2013). The general signaling pathway mediating SOCE is regulated by activation of phospholipase C (PLC) isotypes. GPCRs trigger the activation of the PLCβ subfamily members 2 and 3, while Fcγ-receptors, L-selectin and PSGL-1, and β2-integrins predominantly activate PLCγ1 and PLCγ2, respectively. PLCs split phosphatidylinositol 4,5 PIP2 into DAG and IP3 (Xu & Jin, 2015). IP3 subsequently binds to IP3 receptor (IP3R) localized in the membrane of the endoplasmic reticulum (ER) triggering the release of ER-stored Ca^{2+} into the cytoplasm through nonselective Ca^{2+} channels (Davies-Cox et al., 2001). Depletion of Ca^{2+} levels in the ER are sensed by the ER transmembrane protein stromal interaction molecule 1 (STIM1) which precipitates colocalization of the ER with the calcium release activated calcium (CRAC) channel Orai1 on the neutrophil membrane (Demaurex & Saul, 2018). Stimulation of Orai1 leads to Ca^{2+} influx from the extracellular space and concomitant increase in intracellular cytosolic Ca^{2+} concentration (Schaff et al., 2010).

The integrin signaling complex shares homology with the selectin signaling pathway described earlier with phosphorylation of ITAM-containing proteins, DAP12 and FcRγ, by SFKs, resulting in the activation of SYK which signals activation of PI3K and PLCγ2. In addition to PLC-mediated calcium signaling, integrin mechanosignaling of calcium is regulated by kindlin-3-mediated integrin localization with membrane CRAC channel ORAI1 (Dixit et al., 2012). Recent studies have outlined the importance of kindlin-3 complex with receptor for activated C kinase 1 (RACK1) 1 in mediating ORAI1 clustering upon integrin tension buildup (Morikis et al., 2020). Bond tension across HA LFA-1 precipitates formation of a complex

between kindlin-3, and RACK1. Subsequently, after force build up, RACK1 diffuses away from the adhesion complex. RACK1 then interacts with ER membrane bound IP3R amplifying release of ER calcium store activating STIM-1. This results in STIM-1 mediated colocalization of ER to the cell membrane proximal to Orai1 promoting an influx of Ca^{2+} from the extracellular space.

Mechanical forces are critical in regulating integrin mediated Ca^{2+} transients. Recent experiments have elucidated the exquisite sensitivity of integrin to mechanical forces that regulate calcium signaling. On a recombinant ICAM-1 substrate, .25 dynes/cm2 shear stress was insufficient for signaling clustering of integrin and subsequent calcium flux suggesting the existence of a threshold level of force required for integrin mechanosignaling (Morikis et al., 2020). In this system, integrins responded to shear stresses over a narrow range from 0.5-2 dynes/cm^2 with increasing shear stress inducing more rapid integrin clustering and downstream calcium flux. Additionally, a system using novel DNA tension gauged tethers (TGT) that break at specified forces (i.e., 12, 33, and 54 pN) further demonstrated the sensitivity of integrin signaling to threshold force, with over 33 pN force required to induce a sustained calcium flux (Morikis et al., 2022). In experiments involving neutrophils adhered to physiological ligands under shear flow, the strength of bonds played a pivotal role in calcium signaling. Specifically, calcium signaling on ICAM-1 and JAM-A was primarily influenced by the LFA-1 receptor, whereas signaling on the receptor for advanced glycation end products (RAGE) was predominantly regulated by Mac-1. This corresponded to the relative bond strength of LFA-1 and Mac-1 to the respective ligands (Shu et al., 2020).

Neutrophils are professional killers and one of their most potent weapons is ROS production. ROS production occurs can occur intracellularly in the phagolysosome following phagocytosis of microbes and can be secreted into the extracellular space from the cell plasma

membrane. Neutrophils produce ROS via the multi-protein membrane-bound NADPH (Nicotinamide adenine dinucleotide phosphate-oxidase/Nox2) oxidase complex which converts oxygen to superoxide (Zeng et al., 2019). While release of ROS is important for combating microbes, its unbridled activation can result in tissue damage. Thus, ROS production is controlled by having the NADPH oxidase be composed of both membrane bound and cytosolic components that require cell activation to be phosphorylated and properly assembled (Sheppard et al., 2005). Flavocytochrome b558 (cytb558) is the membrane bound catalytic core that is a heterodimer of gp91phox and gp22phox and is found at low number in the plasma membrane of resting cells with reserves found in the membranes of secretory vesicles and specific granules. The cytosolic components of NADPH are p40phox, p47phox, and p67phox, which reside in the cytosol of resting cells and form a regulatory trimeric complex. This cytosolic complex then binds to p22phox on the cell membrane through interaction with p47phox (Lewis et al., 2010). Binding of the small GTPase protein Rac2 to p67phox is also essential for the electron transfer reaction of NADPH that catalyzes radical oxygen production(Diebold & Bokoch, 2001).The assembly and activation of the NADPH oxidase requires the activation of Rac2 via the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) and the phosphorylation of p47phox/p40phox/p67phox at multiple serine sites (El-Benna et al., 2016). Then Rac2 and the phosphorylated p47phox/p40phox/p67phox complex will translocate to the membrane to interact with cytb558, forming the NADPH oxidase complex.

β2-integrins are potent regulators of ROS production that promote formation and activation of NADPH oxidase. Integrin signaling induces recruitment and fusion of cytoplasmic granules to the plasma membrane upregulating cytb558 and promoting phosphorylation of the cytosolic phox units through transduction of Ca^{2+} flux, activation of PKC, PI3K, and PLC (Nguyen et al.,

2017). While integrin signaling alone can produce ROS, it is through the combination with a secondary stimulus that maximal production of ROS occurs (Nguyen et al., 2017). Most GPCR, Tumor necrosis factor (TNF) receptor or TLR-4 activators produce little to no ROS on their own, but through a process known as neutrophil priming, they are able to induce amplified ROS production in response to a secondary stimulus such as formyl peptide, Fc ligands, or integrin ligation. For example, in suspension picomolar of TNF-α are unable to induce strong ROS production, however, TNF-α induces much greater ROS production in adherent neutrophils in an integrin dependent manner (Mol et al., 2021). This adherence-dependent cooperative signaling activates PI3K and δ-PKC activation of p47phox (Kilpatrick et al., 2009). Thus, ROS production is keenly sensitive to mechanical forces acting on integrins. Neutrophils bound to ICAM-1-linked TGTs that rupture at 54pN were able to produce ROS while 12pN TGTs were unable to facilitate neutrophil ROS. This observation was directly linked to integrin mediated calcium flux as calcium inhibition resulted in abrogation of ROS (Morikis et al., 2022).

1.4 Neutrophil priming in acute and chronic inflammation

Unbridled activation of neutrophil effector functions can cause tissue damage and propagation of inflammation, necessitating careful regulation of the neutrophil activation state. Consequently, before neutrophils can unleash the full extent of their effector functions, a shift to an intermediate state with increased responsiveness to activation known as priming must occur (Condliffe et al., 1998). Priming of neutrophils is induced by exposure to low levels of one or more priming agents such as pro-inflammatory lipids, cytokines, DAMPs, and PAMPs. Once primed, neutrophils possess augmented effector functions (ROS production, degranulation, chemotaxis, and NETosis) in response to subsequent stimuli (Walker & Ward, 1992). While priming agents alone do not typically initiate strong effector function, they do signal phenotypic

and functional changes in neutrophils (Miralda et al., 2017). Priming agents will induce changes in adhesion receptor expression by, mediating L-selectin shedding, promoting activation of integrin, and inducing upregulation of integrin to the plasma membrane from intracellular vesicles. These changes result in increased adhesive capacity and consequently recruitment efficiency (Condliffe et al., 1996). Priming agents will also kickstart transcriptional activity that promotes neutrophil secretion of inflammatory mediators and prolonged lifetime (Wright et al., 2013). Priming will also result in phosphorylation of NADPH oxidase components vital for the amplified ROS production upon secondary stimulation (Elbim et al., 1994).

Acute inflammation is the initial response of the immune system to infection and damage, that is resolved in a short period of time as opposed to chronic inflammation which goes unresolved for months to years (Pahwa & Jialal, 2019). Neutrophils are crucial responders in acute inflammation that eliminate pathogens and clear cell debris. A temporal shift in populations of primed neutrophils is observed throughout the initiation and resolution of acute tissue inflammation. Neutrophil de-priming also occurs during resolution via anti-inflammatory mediators such as resolvins (Kurihara et al., 2013; Vogt et al., 2018). In the case of peritoneal insult, the small population of primed neutrophils in circulation at baseline are quickly recruited into the tissue. One hour after acute peritoneal insult, the majority of neutrophils in bone marrow, blood, and tissue are primed neutrophils. After resolution of inflammation, a return to baseline levels of primed neutrophils is observed (Fine et al., 2019). However, neutrophil priming and activation during acute inflammation such as severe burns and physical trauma can lead to damage not only to the affected tissue sites but to the endothelium, and lungs, and can induce multiple organ failure resulting in death (Adams et al., 2001; Mortaz et al., 2019) . Recently, neutrophil priming has been described to play a role in the pathogenesis of COVID-19, serving

as an independent biomarker for disease severity and adverse patient outcome (Dennison et al., 2021). Circulating neutrophils in COVID-19 displayed changes in adhesion markers characteristic of a primed phenotype and showed increased capacity for oxidative burst, NETosis, and phagocytosis (Masso-Silva et al., 2020). The specificity of priming in COVID-19 is demonstrated by the ability to distinguish SARS-CoV-2-induced acute respiratory distress syndrome (ARDS) from non-SARS-CoV-2-induced ARDS (Panda et al., 2022).

Failure to promote resolution of inflammation or dysregulation of the immune system can result in a chronic inflammatory state where constant release of inflammatory mediators perpetuates neutrophil priming and activation (Vogt et al., 2018). Consequently, primed neutrophils will not only damage through their effector functions, but also exacerbate inflammation by the secretion of factors including IL-1, IL-8, and TNF-α that promote further recruitment and activation of immune cells. Circulating primed neutrophils are reported in various chronic inflammatory diseases including lupus, rheumatoid arthritis (RA), and psoriasis (Denny et al., 2010; Sur Chowdhury et al., 2014; Teague et al., 2019). Psoriasis is a chronic inflammatory disease characterized by skin plaques with neutrophil-laden abscesses (Chiang et al., 2019). Neutrophils play a key role in psoriasis pathogenesis. Once recruited to the psoriatic plaque, primed neutrophils continue to secrete cytokines and chemokines to recruit additional immune cells from the circulation in a feedforward loop (Wang & Jin, 2020). Psoriatic neutrophils show changes in expression of adhesion markers CD62L, CD11b, CD66 that are characteristic of priming. This priming phenotype correlated with disease severity and was remedied with TNF inhibitors (Yamanaka et al., 2014). The mechanisms controlling psoriasis priming in vivo have not been fully established, however psoriatic keratinocytes in culture can

prime neutrophils for ROS production through secretion of IL-8, TNF-α, and Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Guérard et al., 2013).

1.5 Role of DAMPS and cytokines in neutrophil priming:

Neutrophil priming is a complex phenomenon in that specific combinations of priming agents and subsequent activating agents have differential effects on effector responses with no universal signaling pathway (Condliffe et al., 1998). Different priming agents will induce upregulation of overlapping and unique RNA expression add result in different phenotypic changes such as differences in adhesion marker expression (Wright et al., 2013). Upregulation of inflammatory cytokines and DAMPs is a hallmark of chronic inflammation. A thorough understanding of the priming agents associated with specific disease pathologies may provide key drug targets for resolution of systemic inflammation. In this dissertation we explore the role of cytokines (IL-8, TNF-α) and DAMPs (S100A8/A9, LL37) in neutrophil priming and inflammatory response in the chronic inflammatory disease psoriasis.

The pro-inflammatory cytokine TNF-α's capacity for neutrophil priming has been well documented (Vogt et al., 2018). TNF-α priming induces phosphorylation but not membrane recruitment of the NADPH oxidase component p47phox in a p38 dependent and calcium independent manner. However, when TNF-α is paired with integrin adhesion p47phox and p67phox are recruited to the plasma membrane following phosphorylation (Yan et al., 1995). The efficacy of anti-TNF-α antibody therapy highlights the central role of TNF-α in pathogenesis of several chronic inflammatory conditions including RA and psoriasis (Bradley, 2008) .

Treatment with TNF-α inhibiting antibodies reduces neutrophil priming-mediated changes in adhesive capacity in RA and in psoriasis (Dominical et al., 2011; Yamanaka et al., 2014). Since TNF-α can stimulate the release of neutrophil activators in resident cells, it is unknown if the

effects of TNF-α inhibitors are resultant from blocking TNF-α effects on neutrophils directly or indirectly in chronic inflammation.

IL-8 is the primary chemokine that mediates neutrophil recruitment and migration in a variety of acute inflammatory conditions (Harada et al., 1994). During inflammation, IL-8 is presented on the glycocalyx of endothelial cells and ligate neutrophil GPCRs synergizing with selectin signaling to rapidly induce activation of neutrophil integrin that mediate recruitment (Qu et al., 2021; Schaff et al., 2008). As a key inflammatory cytokine for neutrophil recruitment, IL-8 is strongly upregulated in various chronic inflammatory diseases where it serves as a biomarker of disease severity, including lung pathologies, arthritis, inflammatory bowel disease, and psoriasis (Russo et al., 2014). Low concentrations of IL-8 can prime neutrophils for amplified ROS production through phosphorylation and assembly of NADPH oxidase components in a calcium, phospholipase A2, BTK, and ERK dependent manner (Bréchard et al., 2005; Daniels et al., 1992; Guichard et al., 2005). Il-8 can also induce or prime NETosis and has been linked to increased capacity to release nets in chronic lymphocytic leukemia patients (Podaza et al., 2017). In COVID-19, IL-8 was demonstrated to drive a pro-thrombotic neutrophil phenotype with amplified degranulation, NETosis, and IL-8 release resulting in a positive feed-back loop exacerbating disease (Kaiser et al., 2021).

IL-8 has proved to be a reliable biomarker for psoriasis disease severity, with elevated levels in circulation and skin as well as elevated level of IL-8 mRNA in keratocytes (Arican et al., 2005; Gillitzer et al., 1991; P. Wu et al., 2017). TNF-α and IL-17 which are known biological targets in psoriasis promote IL-8 secretion from keratinocytes (Coimbra et al., 2012; Keijsers et al., 2014). In addition to mediating neutrophil recruitment, IL-8 also promotes keratinocyte proliferation, and angiogenesis exacerbating formation of psoriatic plaques (Heidenreich et al.,

2009). While a plethora of evidence supports a role for IL-8 in psoriasis pathogenesis, systemic treatment with a monoclonal antibody targeting IL-8 (ABX-IL8) showed only mild efficacy in phase 2 clinical trials, potentially due to limited penetration of the antibody into the upper epidermis (Pietrzak et al., 2008). However, a topical anti-IL-8 antibody treatment was shown to be effective for treatment of psoriasis and is approved in China (Tsai & Tsai, 2017).

DAMPS are a class of molecules that are released during tissue damage and cell death, or actively by secretion and during serve as a danger signal for inflammation (Murao et al., 2021). DAMPs are highly upregulated in various chronic inflammatory diseases modulating resident and immune cells through pattern recognition receptors including TLRs and cytoplasmic Nod-like receptors (Zindel & Kubes, 2020).

S100 proteins are a family of calcium binding proteins with intracellular and extracellular functionalities (Donato, 2001). S100A8 and S100A9 can form homodimers but preferentially form the heterodimer S100A8/A9 (Calprotectin, MRP8/14), a known antimicrobial protein that sequesters divalent cations such as iron necessary for bacterial respiration (Pruenster et al., 2016). S100A8/A9 and its subunits also have strong pro-inflammatory effects on neutrophils inducing degranulation, integrin activation, chemotaxis, and pro-inflammatory cytokine secretion (Pruenster et al., 2015; Scott et al., 2020). S100A8/A9 induces a primed neutrophil state characterized by increased adhesion, CD11b upregulation, shedding of CD62L, and increased ROS production in response to GPCR stimulation. S100A9 enhances the production of reactive oxygen species, IL-1, and IL-8 by human neutrophils following activation with monosodium urate crystals which induce gout. This was accompanied by potentiation of downstream and upstream signaling including the mobilization of intracellular calcium stores, tyrosine phosphorylation, phosphorylation of AKT and p38 by PKC, and increases in glycolysis

(Rousseau et al., 2017). A growing body of literature shows the reliability of S100A8/A9 as a biomarker of systemic and local inflammation in several inflammatory diseases including cardiovascular disease, inflammatory bowel disease, RA, psoriasis, and most recently COVID-19 (Cesaro et al., 2012; Mellett & Khader, 2022; Silvin et al., 2020; Sreejit et al., 2020). Elevation of S100A8/A9 in stratum corneum and serum correlates with psoriasis disease severity and reduction is observed after treatment and disease remission (Matsunaga et al., 2021). The S100A8/A9 inhibitor Paquinimod was observed to reduce severity in the Imiquimod-induced psoriasis mouse model, suggesting the potential of S100A8/A9 inhibition for psoriasis therapy (Khaleel & Zalzala, 2023).

The human cathelicidin LL37 is a small cationic peptide that binds to a variety of cell surface receptors modulating responses of various immune cells and keratinocytes (Verjans et al., 2016). LL37 is an antimicrobial protein that cleaves bacterial membrane, however high upregulation in skin is thought to play a role in pathology of several diseases including inflammatory bowel disease, ulcerative colitis, rheumatic diseases, and psoriasis (Fabisiak et al., 2016; Frasca & Lande, 2012). LL-37 is stored in neutrophil secondary granules and is released by both degranulation and during NETosis (Pahar et al., 2020). Binding of LL37 to CXCR2 was reported to induce calcium mobilization and migration of neutrophils (Zhang et al., 2009). Priming of neutrophils by LL-37 for influenza virus-mediated respiratory burst and NET formations is mediated by engaging formyl peptide receptor 2 in a calcium dependent manner (Tripathi et al., 2015). LL37 serves as a biomarker for psoriasis severity being upregulated in circulation and in skin of patients and reductions seen upon treatment of disease with Biologic therapy (Fuentes-Duculan et al., 2017; Lao et al., 2023). Complexing of LL37 with RNA has been thought to play a role in psoriasis pathogenesis by activation of dendritic cells and keratinocytes (Albanesi et al.,

2018; Takahashi & Yamasaki, 2020). Recently it was show that neutrophil NETS contain RNA that complex with LL37 and trigger TLR8/TLR13-mediated cytokine and NET release by PMNs in vitro and in vivo creating a self-propagating vicious cycle contributing to chronic inflammation in psoriasis (Herster et al., 2020).

1.6 Dissertation objectives

Neutrophils are designed for rapid response to both foreign matter and danger signals produced during inflammation and tissue damage. Neutrophil priming and activation are carefully regulated, and dysregulation can result in immunodeficiency or conversely exacerbated inflammation. A focus of the Simon lab is to elucidate how mechanical signaling through selectins and integrins and exposure to endogenous ligands and foreign therapeutics regulate neutrophil recruitment and effector functions in health and disease. This dissertation continues this tradition investigating how the iron oxide nanoparticle therapeutics and DAMPS and cytokines upregulated in psoriasis can modulate neutrophil mechanosignaling, calcium flux, and effector functions.

The use of nanoparticles in intravenous diagnostics and therapeutics has been increasing, necessitating research on the potential effects these foreign bodies may be having on neutrophil inflammatory response. Feraheme is an FDA approved iron oxide nanoparticle treatment for iron deficiency anemia. It's also increasingly being employed as a contrast agent for MRI based novel diagnostic imaging techniques. Previously our lab demonstrated the use of iron oxide nanoparticles for high frequency alternating magnetic field mediated thermal ablation of bacteria biofilms in wounds. However, the effect of these particles on innate immune cell function has not been adequately investigated. Thus, the first objective of this dissertation was to determine

how Feraheme, an FDA approved iron oxide nanoparticle treatment, affects neutrophil inflammatory responses.

Evidence of the relationship between circulating neutrophil activating DAMPS and cytokines with psoriasis disease severity, systemic inflammation, and cardiac and pulmonary comorbidities continues to grow. Understanding the mechanisms by which DAMPS and cytokines are mediating aberrant neutrophil activation and recruitment that exacerbates psoriatic disease may provide insight to more targeted approaches for psoriatic therapy. Thus, the second objective of this dissertation was to investigate the role of circulating cytokines and DAMPs in priming psoriatic neutrophils for enhanced adhesion and effector function.

Chapter 2 Neutrophil Inflammatory Response Is Downregulated by Uptake of Superparamagnetic Iron Oxide Nanoparticle Therapeutics

2.1 Introduction

Superparamagnetic iron oxide nanoparticles (SPION) are used as contrast agents for magnetic resonance imaging in the liver, central nervous system, gastrointestinal system, and in macrophages (Dadfar et al., 2019). SPION have also found clinical use as an FDA approved treatment of cancer (i.e., Nanotherm) and Iron deficiency anemia in patients with chronic kidney disease (i.e., Feraheme) (Dadfar et al., 2019). While SPION have been used for various clinical applications, a primary consideration for any biomaterial based therapeutic is the host immune response elicited by the interaction of the biomaterial with blood cells. The increased use of SPION has raised concerns over their safety, with several formulations showing cytotoxic and immunotoxic effects (Valdiglesias et al., 2016). This motivated the current study to examine the systemic effects of Feraheme on neutrophils in human blood.

The innate immune system is by design able to recognize and eliminate foreign pathogens and particulate matter through pattern recognition receptors and opsonin receptors that sound the alarm upon detection of danger signals (Gustafson et al., 2015). Opsonization involves the binding of molecules such as antibodies or proteins of the complement system that enhances recognition and removal via endocytosis and phagocytosis of foreign matter by innate immune cells (Owens & Peppas, 2006). As nanoparticles enter the blood stream, complement and other proteins immediately adsorb to the particle surface forming a corona (Barbero et al., 2017). Physiochemical properties of nanoparticles have a direct influence on the composition and formation of the protein corona in such a manner to impact particle-cell interactions (Szeto & Lavik, 2016).Induction of immune hypersensitivity reaction in patients prompted FDA to cancel

clinical use of the SPION Feridex and GastroMARK (Bobo et al., 2016; Wysowski et al., 2010). Further, studies have shown that SPION can induce oxidative stress and cell damage in vitro and in animal models (Gaharwar & R, 2015; X. Wu et al., 2010). Upon binding to the plasma membrane and endocytosis nanoparticles can induce a variety of functional responses in neutrophils such as reactive oxygen species (ROS) and chemokine production that vary with different SPION formulations. While some SPION can induce these immune responses, others exert an inhibitory effect, thereby highlighting composition as a key factor when considering nanoparticle use as a therapeutic (Soenen et al., 2011; Valdiglesias et al., 2016). Conventional wisdom is that nanoparticle clearance is mediated by macrophages, however, recent studies indicate that neutrophils also play a key role in particle clearance and this process has been exploited to deliver therapeutic nanoparticles to tumors for cancer therapy, (Jones et al., 2013; Naumenko et al., 2020). Current literature on the immune effects of SPION have largely focused on monocytes and macrophages. This has resulted in an incomplete understanding of the full scope of SPION immune effects on neutrophil function (Shah & Dobrovolskaia, 2018).

Neutrophils are the most common leukocyte in blood circulation and are essential first responders during inflammation. Thus, intravenous administration of nanoparticles is likely to result in frequent neutrophil-particle interactions. Capture of SPION onto the plasma membrane and subsequent endocytosis can perturb the inflammatory response and recruitment of neutrophils to sites of inflammation. Dysregulation of neutrophil activation and recruitment is implicated in various autoimmune and inflammatory diseases (Amulic et al., 2012). Failure of neutrophils to normally adhere to the endothelium and become activated can cause severe impairment of host defense against pathogens, while inappropriate levels of cell recruitment and activation leads to chronic inflammation and tissue damage (Del Fresno et al., 2018; Etzioni et

al., 1992). There are reports of the capacity for coated (Polyacrylic acid) and non-coated iron oxide nanoparticles and metal oxide nanoparticles TiO2, CeO2 and ZnO, to induce ROS production and activate neutrophil degranulation (Babin et al., 2013; Couto et al., 2014). Excessive release of neutrophil granules, which contain chemokine and integrin receptors, elastase, collagenase, and myeloperoxidase, along with overproduction of ROS can lead to inflammatory tissue injury (Lacy, 2006; Winterbourn et al., 2016).Uptake of gold nanoparticles, endoplasmic reticulum stress events and cleavage of cytoskeletal proteins in human neutrophils leading to apoptosis (Noël et al., 2016). Further, Nano and micro particles have been reported to inhibit the recruitment of neutrophils from the circulation to sites of inflammation in mice and to cause a reduction in neutrophil attachment to endothelial monolayers in vascular mimetic flow channels (Fromen et al., 2017; Kelley et al., 2019).The fine balance between the protective functions of neutrophils that maintain immune competence versus exuberant response that can result in tissue and organ damage has prompted the current studies on neutrophil activation and inflammatory recruitment in the presence of Feraheme. Intravenous infusion with Feraheme for delivery of complexed iron is FDA approved at a higher single dose than other products on the market (Macdougall et al., 2014). A typical treatment regime consists of two 510 mg doses separated by 5-8 days as compared to other products that require between 5-10 individual doses, thereby limiting the need for repeated infusion which typically lowers patient compliance. Feraheme also is approved for infusion at higher rates than other iron products with comparable safety (Coyne, 2009; Foster et al., 2013).The increased application of Feraheme in anemia patients who can receive frequent intravenous injections as a course of therapy highlight a need to understand the effects of SPION on neutrophils and their capacity to maintain normal immunosurveillance.

In the current study, we examined the effects of Feraheme on neutrophil degranulation and alterations in receptor expression and conformation on the plasma membrane. We hypothesized that neutrophil uptake of Feraheme in blood alters the normal process of adhesion receptor activation, shear stress resistant neutrophil rolling to arrest and subsequent shape change that precedes cell migration. Vascular mimetic flow channels were employed to assess the kinematics of neutrophil interactions on a substrate of recombinant E-selectin and ICAM-1 in an established model of endothelial inflammation. Feraheme in suspension inhibited neutrophil activation and degranulation induced by IL-8 stimulation, resulting in alteration in the expression of adhesion molecules necessary for the efficient recruitment on inflamed endothelium.

2.2 Materials and Methods

2.21Small molecules, antibodies, and other reagents

Monoclonal antibodies for flow cytometric detection of high affinity β2-integrin (mAb24), L-Selectin (Dreg-55, Dreg-56), CD11b (M170), CD18 (1B4), CD66b (G10F5) CD11a (HI111), PSGL-1 (PL-2, KPL-1), CXCR1(8F1/CXCR1), and CXCR2 (5E8/CXCR2) along with antibodies that block CD11b function (Mac-1 blocking, M1/70), fixation buffer, and IL-8 were purchased from Biolegend (San Diego, CA). Recombinant human ICAM-1-IgG and E-Selectin-IgG produced as Fc chimeric constructs were purchased from R&D Systems (Minneapolis, MN). Adenosine A2A receptor agonist CGS-21680, N-formyl-Met-Leu-Phe (fMLP), and reactive oxygen species indicator Dihydrorhodamine 123 were purchased from Millipore Sigma (Burlington, MA). Adenosine A2A receptor antagonist ZM 241385 was purchased from Tocris Bioscience (Minneapolis, MN). Feraheme (AMAG Pharmaceuticals, Waltham, MA) was purchased from the UC Davis Medical Center Pharmacy.

2.22 Human Neutrophil Isolation

Whole blood was obtained from healthy donors consented through a University of California, Davis institutional review board protocol #235586-9. Neutrophils were isolated from whole blood via negative enrichment using EasyStep® direct human neutrophil isolation kit purchased from StemCell Technologies as per manufacturers instruction (Cambridge, MA). Some studies employed a percoll gradient separation using Polymorphoprep® as per manufacturers instruction (Fresenius Kabi). Briefly, for gradient based isolation, whole blood is layered on an equal volume of Polymorphoprep solution and centrifuged at 760g for 30 min at 25°C. The neutrophil layer is collected and washed in Phosphate-buffered saline (PBS) before resuspension in HBSS buffer containing 0.1% human serum albumin without Ca^{2+} and Mg^{2+} and kept at $1x10^7$ cells/mL on ice after isolation and prior to experimentation. For EasyStep neutrophil isolation, whole blood was diluted 1:1 with PBS then 100 µL of isolation cocktail and magnetic RapidSpheres each were added and incubated for 5 minutes. Cells were then placed in the EasySep magnet for 5 minutes. Cells were poured into a new tube and treated for an additional 5 minutes with 100 µL of magnetic RapidSpheres and placed on the EasySep magnet for 5 minutes twice more. Cells were then spun down and resuspended in HBSS containing 0.1% human serum albumin and kept at $1x10^7$ cells/mL on ice prior to use. Cell purity was >90% as determined by Beckman cell coulter counter. Cell viability was ~99% as determined by flow cytometry detection of zombie violet fluorescent dye staining (Biolegend).

2.23 Flow cytometry detection of cell surface marker expression

Neutrophils (1×10^6 cells/ml) in HBSS buffer containing Ca^{2+} and Mg^{2+} at 1 mM were treated with IL-8 at a dose range of concentrations (0.01-100 nM) and Feraheme (1-6 mg/ml) for 5 min at 37°C before addition of antibody (1.5-5 µg/ml). Cells were stained with antibodies for

high affinity β2-integrin (mAb24), L-Selectin (Dreg-56), CD11b (M1/70), CD18 (1B4), CD11a (HI111), PSGL-1(KPL-1), CXCR1(8F1/CXCR1), and CXCR2 (5E8/CXCR2) for 20 min at 37°C before fixation with 1% paraformaldehyde at room temperature. After 2 washes with PBS cells were analyzed using the Attune NxT flow cytometer (Thermofisher). Neutrophils were gated by their characteristic forward scatter vs side scatter profile. Receptor expression in terms of sites/cell was determined by comparing the MFI of bound antibody to Quantum Simply Cellular beads (Bangs Laboratories, Inc., Fishers, IN) which contain five bead sets with increasing numbers of antibody binding sites on their surface. From this analysis, a linear relation between MFI and receptor expression was determined for each directly conjugated antibody bound to cells and the calibration bead set.

2.24 Microfluidic shear flow assay

Vascular mimetic flow chambers were utilized to record neutrophil rolling and arrest behavior on substrates of endothelial ligands under physiological shear stresses. Devices were prepared as described previously (Foster et al., 2013). Briefly, poly(dimethylsulfoxide) (PDMS) microfluidic flow chambers with a minimum feature size of 5 μm were produced by curing Sylgard 184 prepolymer (Dow Corning, Midland, MI, USA) over a patterned silicon wafer. Holes were punched into the PDMS for flow channel and vacuum port access and the device was reversibly vacuum sealed on a glass coverslip coated with E-selectin (1 μg/ml) alone or with ICAM-1 (1 μg/ml) in PBS for 60 min at room temperature. To limit non-specific adhesion of neutrophils, coverslips were treated with 1% casein for 10 min before washing with PBS and assembly of the microfluidic device.

Isolated neutrophils were treated with Ca^{2+} indicators 1 Fluo-4 AM or Rhod-2 AM (1 μg/mL) for 20 minutes at room temperature in the dark. Cells were spun down and resuspended

at $1x10^6$ cells/mL and were treated with Mac-1 blocking antibody M1/70 and incubated with or without MNP in HBSS buffer containing Ca^{2+} and Mg^{2+} for 20 min at 37°C. Neutrophils were then treated with 0.5 nM IL-8 or vehicle control and immediately loaded into an open 100 µL reservoir and were drawn through the channel by negative pressure produced by a syringe pump at a shear of 2 dynes/cm2.

Utilizing real time fluorescence microscopy, images of rolling neutrophils were acquired at 60 frames per minute for 4 minutes per field of view on an inverted microscope (Nikon) using a phase contrast 20x objective and recorded with 16-bit digital complementary metal oxide semiconductor (CMOS) camera (Andor ZYLA) connected to a PC (Dell) with NIS Elements imaging software (Nikon Instruments Inc.). Arrested neutrophils were defined as having a velocity less than 0.1 µm/second. Migrating neutrophils were identified by exhibition of a polarized shape defined as exceeding a length/width aspect ratio greater than 1.4 and phase dark contrast indicative of being outside the focal plane of rolling cells.

2.25 L-selectin and PSGL-1 clustering

Total internal reflection fluorescence (TIRF) microscopy and quantitative dynamic foot printing (qDF) were employed to record fluorescently tagged antibodies targeting L-selectin (non-blocking clone: DREG55) and PSGL-1(non-blocking clone: PL2). Cluster area and frequency during rolling of isolated neutrophils over E-selectin in the presence or absence of Feraheme was observed. A cluster was defined as an area of uniform fluorescence intensity two standard deviations above the mean fluorescence intensity of the cell. Additionally, a cluster was defined as having a surface area of 0.4 µm2 or greater.

2.26 Measurement of reactive oxygen species

Neutrophils (1×10^6 cells/ml) in HBSS buffer containing Ca^{2+} and Mg^{2+} at 1 mM were incubated with the Reactive oxygen species indicator Dihydrorhodamine 123 (2 µM) and treated with or without IL-8 (1 nM) and Feraheme (4 mg/ml) for 10 min at 37oC before addition of 1 µM fMLP. Cells were incubated with fMLP at 37°C for 5 min and then placed on ice to end reactions and reactive oxygen species was then quantified through flow cytometry.

2.27 Measurement of calcium flux

Isolated neutrophils were treated with the Ca^{2+} indicator Fluo-4 AM (1 µg/mL) for 20 minutes at room temperature in the dark. Cells were spun down and resuspended at $1x10^6$ cells/mL in HBSS buffer containing Ca^{2+} and Mg^{2+} at 1 mM and treated with or without the adenosine A2A receptor antagonist ZM 241385 (2.5 µM) for 5 min at 37C. Feraheme (4 mg/ml) and or adenosine A2a receptor agonist CGS 21680 (1 µM) were then added to the cell solutions for 10 min at 37°C. After incubation IL-8 (1nM) was added to cell solutions and calcium fluorescence time course was immediately read on the Facscan flow cytometer (BDbiosciences)

2.3 Feraheme inhibits neutrophil activation and degranulation stimulated by IL-8.

Stimulation with chemotactic factors activate within seconds neutrophil degranulation, which results from the fusion of granule membranes to the plasma membrane resulting in upregulation of additional CD11b/CD18 and CD66b receptors. Activation also results in a shift in the conformation of CD18 from constitutive low affinity conformation at rest to high affinity upon CXCR ligation, as well as the shedding of L-selectin receptors through the action of the metalloprotease ADAM17 (Lum et al., 2002; Miralda et al., 2017). We first assessed the capacity of different concentrations of Feraheme in suspension to alter neutrophil responses to chemotactic activation via CXCR1/2 in cells stimulated at the KD of IL-8 stimulation (~1 nM). Neutrophils were incubated at 370C in the presence and absence of Feraheme and IL-8 for 5

minutes before the addition of fluorescent antibodies for 20 min followed by cell fixation. The presence of Feraheme led to significant alterations in expression of CD62L, CD11b, and high affinity (HA) CD18 at each dose applied (1-6 mg/ml) (Figure 2.1). A dose dependent increase in the inhibition of degranulation was observed, as well as diminished activation of integrin and proteolytic cleavage of L-selectin. Since the effect of Feraheme was observed to plateau at 4 mg/ml, subsequent studies were performed using this concentration.

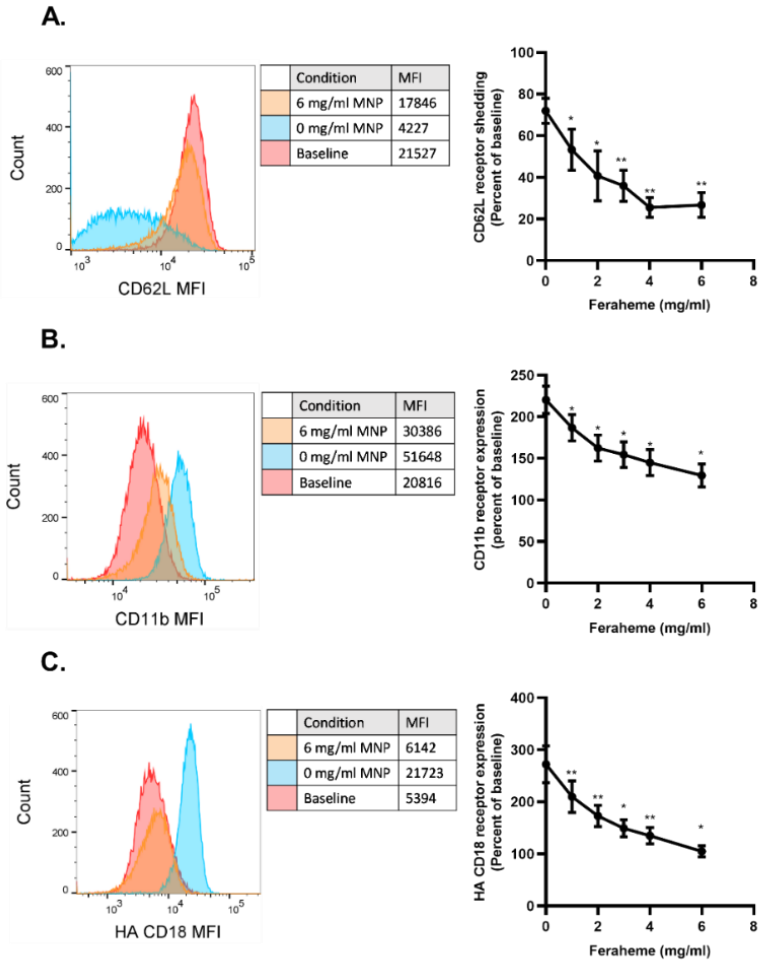

Figure 2.1: Effect of Feraheme concentration on integrin and selectin expression following chemotactic stimulation of neutrophils. Isolated human neutrophils were incubated with 1nM IL-8 and Feraheme magnetic nanoparticles (MNP) for 25 min and cell surface expression of (A) CD62L, (B) CD11b, and (C) HA CD18 was assessed by flow cytometry. Representative histograms depict fluorescent antibody detection for each adhesion receptor at baseline receptor

expression and following IL-8 stimulation in presence and absence of MNP. Bivariate data are presented as the percent shedding from unstimulated baseline mean ± SEM for CD62L and percent of unstimulated baseline expression mean ± SEM for CD11b and HA CD18 ($n \geq 4$ donors) with experimental replicates averaged for each donor. Paired T-test was performed comparing the average value at each concentration to the 0mg/ml Feraheme condition of the same donor * and ** denote p value $\leq .05$ and ≤ 0.01, respectively.

We next evaluated the effect of Feraheme on adhesion receptor expression over a dose range of chemotactic stimulation with IL-8. A typical sigmoidal dose dependent decrease in L-selectin expression followed addition of IL-8 between 0.1-100nM, which reached a maximum of ~90% loss at 5 nM IL-8 from baseline expression on quiescent neutrophils (Figure 2.2A). Upregulation in surface expression of CD11b and CD18 provided a sensitive measure of activation, increasing by 2-fold and 2.3-fold from baseline up to maximum expression at 10 nM IL-8 (Fig 2.2B-C). Another sensitive measure of neutrophil activation was provided by the increased binding of mAb24 that reports on the allosteric conversion of CD18 receptors from low to high affinity, which yielded a 3-fold increase in expression at maximum IL-8 stimulation (Fig 2.2B-D). It is noteworthy that the maximum extent of L-selectin shedding was lowered by Feraheme at 4 mg/ml compared with 0 mg/ml, as indicated by the elevated plateau at a dose of IL-8 > 5 nM from 10% up to 25% total receptor expression (Figure 2.2A). Moreover, the extent of upregulation of CD11b/CD18 and conversion of CD18 to the HA conformation was significantly inhibited, as indicated by a ~2-fold increase in the EC50 of IL-8 stimulation. This

resulted in a reduced sensitivity to activation that was significant at IL-8 doses of 0.5 and 1 nM for CD11b, total CD18, and HA CD18 (Figure 2.2B-D).

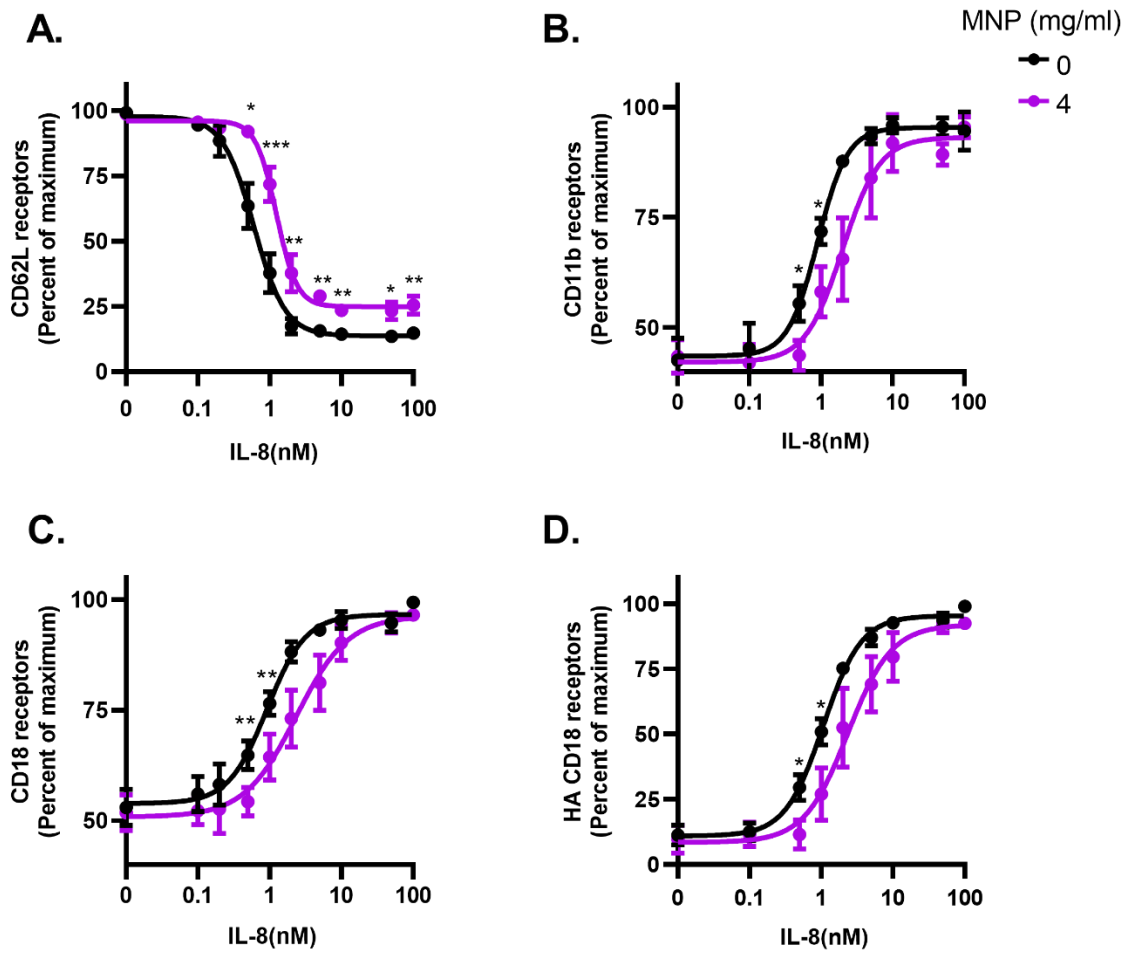

Figure 2.2: Feraheme alters adhesion receptor expression over a dose range in stimulation with IL-8. Isolated human neutrophils were incubated with IL-8 and Feraheme MNP for 25 min and cell surface expression of (A) CD62L, (B) CD11b, (C) CD18, and (D) HA CD18 was assessed by flow cytometry. The data are presented as the percent of maximum receptor expression mean ± SEM ($n \geq 5$ donors) with experimental replicates averaged for each donor. Paired T-tests were performed comparing the average of the 0 mg/ml to the 4mg/ml conditions for the same donor *, **, and *** denote p value $\leq .05$, ≤ 0.01, and ≤ 0.001, respectively.

A marker of the release of secondary granules is the increase in membrane expression of CD66b, which is transported to the plasma membrane along with CD11b/CD18 (Lacy, 2006). The presence of Feraheme elicited a significant inhibition in upregulation of CD66b, increasing

the EC50 of IL-8 stimulation by ~30% (Figure 2.3A). In contrast, CD11a and PSGL-1 adhesion receptors that are constitutively expressed on circulating neutrophils and typically do not register a change in receptor number in response to chemotactic stimulation, remained constant over the dose range of IL-8 stimulation in the presence of Feraheme (Figure 2.3B-C).

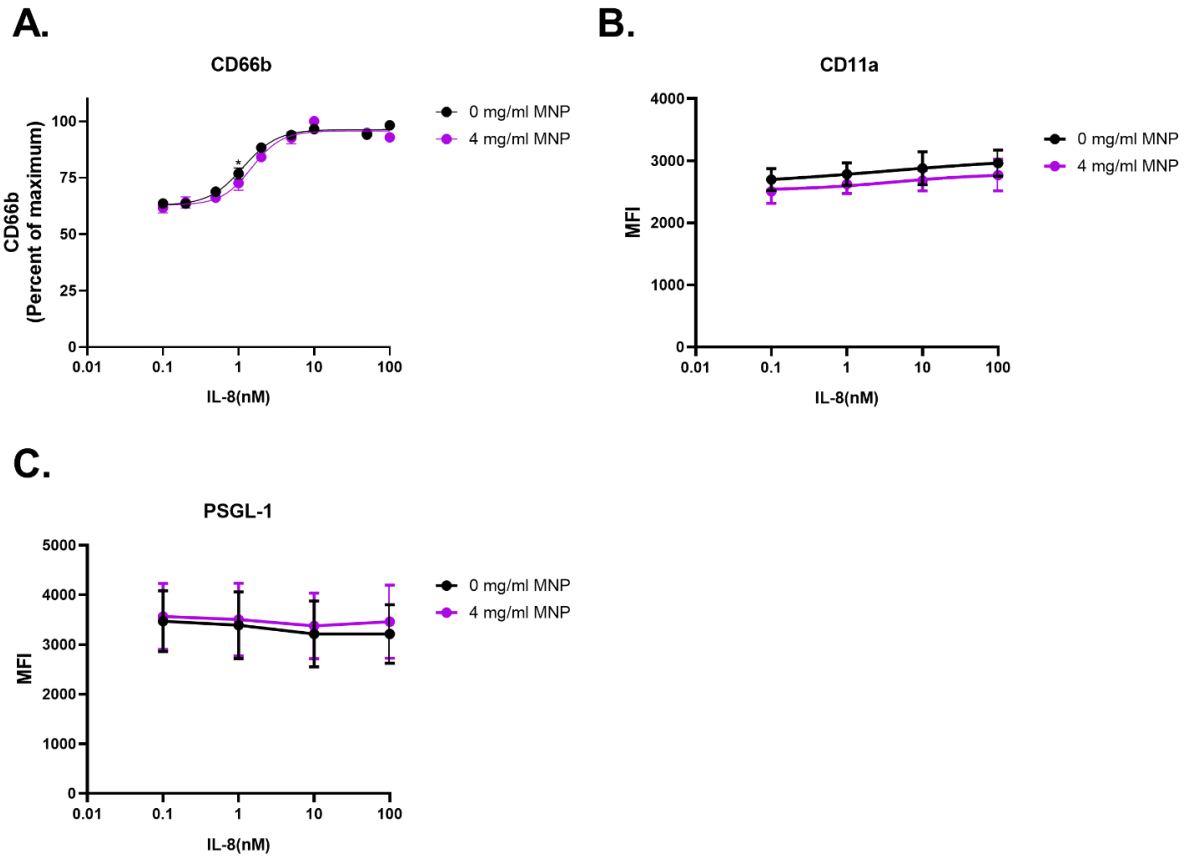

Figure 2.3: Feraheme does not inhibit antibody recognition of adhesion receptors. Isolated human neutrophils were incubated with IL-8 and Feraheme magnetic nanoparticles (MNP) for 25 min and cell surface expression of (A) CD66B, (B) CD11a, (C) PSGL-1 was assessed by flow cytometry. The data are presented in mean ± SEM ($n \geq 3$ donors) with experimental duplicates for each donor.

Neutrophils are reported to endocytose CXCR1/2 following ligation and signaling by IL-8 (Rose et al., 2004). This motivated experiments to determine if Feraheme altered the expression of CXCR1/2, consequently accounting for the diminished capacity to induce changes

in adhesion molecule expression following stimulation. Unexpectedly, incubating unstimulated neutrophils in suspension with Feraheme at 4 mg/ml elicited a ~26% increase in expression of CXCR1 and a ~15 % increase in CXCR2 (Figure 2.4). A significant amount of CXCR1 endocytosis following IL-8 simulation was not observed in the presence or absence of Feraheme (Figure 2.4A). In contrast, the small amount of CXCR2 endocytosis in response to IL-8 stimulation was not altered in the presence of Feraheme (Figure 2.4B). These results indicate that CXCR1/2 expression either remains constant or increases in the presence of Feraheme and IL-8 stimulation. Thus, it is unlikely that the mechanism left-shifting the IL-8 dose response for activation is diminished expression of chemotactic receptors. Feraheme is also not altering the endocytosis of CXCR2 receptors in cells treated with 1nM IL-8 indicating it is not interfering with IL-8 ligation of CXCR2. Thus, it is probable that Feraheme is leading to dramatically reduced responses to IL-8 stimulation by affecting intracellular signaling downstream of receptor ligation.

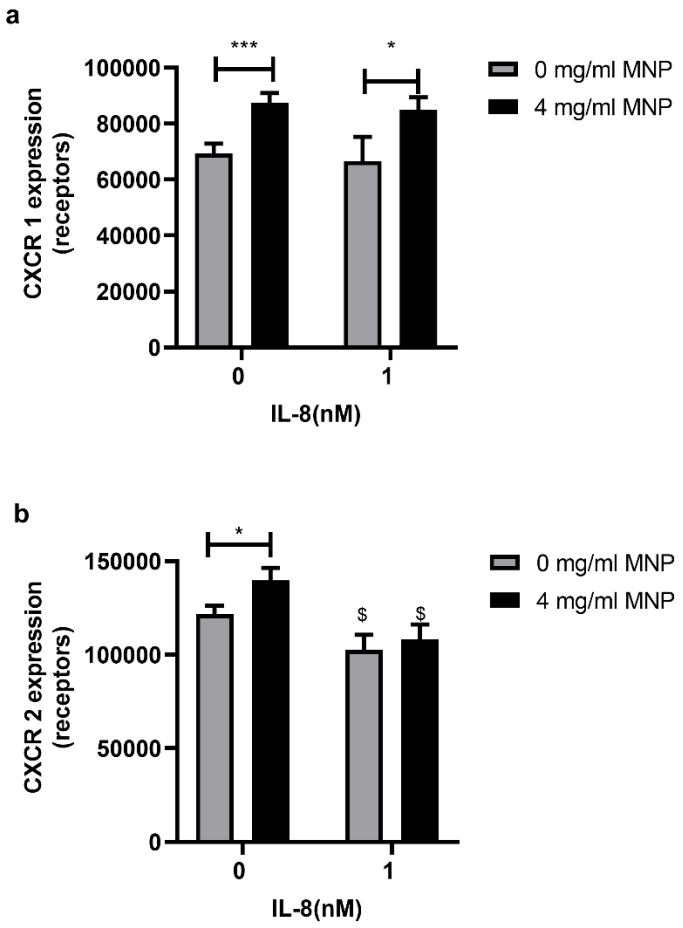

Figure 2.4: Feraheme alters CXCR expression in neutrophils. Isolated human neutrophils were incubated with IL-8 or vehicle and Feraheme for 25 min and cell surface expression of (A) CXCR1 and (B) CXCR2 was assessed by flow cytometry. The data are presented as mean ± SEM (n ≥4 donors) with experimental duplicates averaged for each donor. Paired T-tests were performed comparing the average value for the 4 mg/ml to the 0 mg/ml Feraheme conditions of the same donor * and *** denote p value ≤.05 and ≤ 0.001, respectively. Paired T-tests were performed comparing the average value for a condition to the 0 nM IL-8 condition of the same donor $ denotes p value ≤.05.

2.4 Feraheme inhibits the activation of ROS production in neutrophils.

To further explore the influence of Feraheme on neutrophil function, ROS production was quantified using flow cytometry to detect intracellular Dihydrorhodamine 123 fluorescence. Production of ROS is a key antimicrobial mechanism of neutrophils and has also been implicated in recruitment to sites of inflammation by increasing vascular permeability (Zhu & He, 2006).

The bacterial tripeptide fMLP is a potent chemotactic factor that binds to G-protein coupled Formyl peptide receptors expressed on neutrophils and activates the respiratory burst and production of ROS. Feraheme led to a significant 30% reduction of ROS production in neutrophils stimulated with 1 µM fMLP (Figure 2.5). Pre-treatment with IL-8 has previously been reported to prime neutrophils for enhanced fMLP induced ROS production through assembly of the NADPH oxidase components into lipid rafts on the plasma membrane (Guichard et al., 2005). Feraheme significantly reduced ROS production by 48% in cells primed with low dose IL-8 and stimulated with fMLP. While uptake of SPION other than Feraheme have been reported to induce ROS production, the presence of Feraheme did not lead to detectable ROS production in unstimulated neutrophils. This data indicates that Feraheme inhibits formyl peptide receptor signaling of ROS production, including increased ROS induced by IL-8 priming.

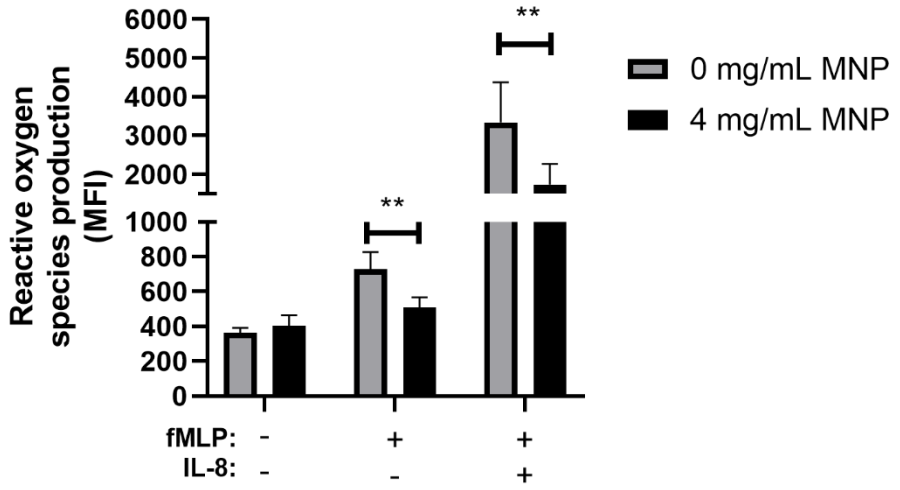

Figure 2.5: Feraheme inhibits ROS production in fMLP stimulated neutrophils. Isolated human neutrophils stained with 2µM DHR 123 and treated with or without Feraheme were preincubated with 1nM IL-8 or vehicle control for 10 min before addition of 1 µM fMLP or vehicle control for 5 min. The data are presented as mean ± SEM (n≥4 donors) with experimental duplicates averaged for each donor. Paired ratio T-test was performed comparing the average value for an experimental condition to the 0 mg/ml Feraheme condition of the same donor. ** denotes a p value ≤ 0.01.

2.5 Neutrophil recruitment on endothelial adhesion molecules is altered in the presence of Feraheme.

It has been reported that exposure to nanoparticles and microparticles reduced neutrophil recruitment to inflamed mesentery microvasculature in mice (Fromen et al., 2017). Moreover, polystyrene nanoparticles have been shown to reduce adhesion of human neutrophils to inflamed human umbilical vein endothelial cells in parallel plate flow chamber studies (Kelley et al., 2019). This motivated determination of the effect of Feraheme on the multistep process of recruitment in vitro using microfluidic flow channels (Fromen et al., 2017; Kelley et al., 2019; Schaff et al., 2007). Fluorescence microscopy was employed to image the dynamics of neutrophil rolling and arrest within vascular mimetic microfluidic channels containing substrates coated with E-selectin alone or in conjunction with ICAM-1. Neutrophil suspensions were incubated for 20 min with Feraheme at 1 mg/ml or 4 mg/ml and compared with vehicle control in response to stimulation with 0.5 nM IL-8 prior to perfusion through the flow channel. This dose of IL-8 corresponded with the lowest concentration of stimulus in which a significant inhibition of CD18 activation and L-selectin shedding was elicited by Feraheme. Capture and rolling on E-selectin is primarily mediated by recognition of sLex presenting ligands on L-selectin and PSGL-1 expressed on neutrophils. The velocity of neutrophil rolling on E-selectin at a venular shear stress of 2 dyne/cm2 increased ~37% in the presence of Feraheme at 4 mg/ml compared to vehicle alone, whereas no alteration in rolling velocity was detected at 1 mg/ml (Figure 2.6A-B). This increased rolling velocity was accompanied by a 40% increase in the variance of the mean velocity, which is consistent with an unsteady trajectory of neutrophils interacting with the substrate under shear flow. Neutrophils require ICAM-1 on the substrate to provide an anchor for activated integrins to bind in order to achieve shear resistant cell arrest

(Lum et al., 2002). Perfusion through flow channels derivatized with E-selectin and ICAM-1 were used to study Feraheme's effect on the kinematics of neutrophil arrest and transition to a migratory phenotype. In the absence of IL-8 chemotactic stimulation, Feraheme lead to a dose dependent increase in rolling velocity on E-selectin coated substrates, which increased by ~30 % and ~60% when neutrophils were exposed to 1 mg/ml and 4 mg/ml Feraheme, respectively (Figure 2.6C-D). Moreover, an ~80% increase in variance of mean velocity corresponded to treatment at 4 mg/ml Feraheme, while 1 mg/ml increased variance by 17%. Stimulation with IL-8 leads to the activation of HA CD18 that binds ICAM-1 and mediates deceleration to arrest (Lum et al., 2002).Consequently, stimulation with IL-8 on E-selectin/ICAM-1 coated substrates resulted in a 50% decrease in rolling velocity and within ~40 seconds most neutrophils exhibited a rapid transition to arrest (Figure 2.6C, F). Neutrophils treated with Feraheme at 4 mg/ml exhibited ~40% higher rolling velocity compared to the 1 mg/ml or vehicle control conditions (Figure 2.6D). In the absence of IL-8 stimulation, outside-in signaling via E-selectin binding and clustering of L-selectin is sufficient to activate ~40% of cells to convert from rolling to arrest that is supported by activation of HA CD18 to bind ICAM-1 (Morikis et al., 2017). Feraheme at 1 mg/ml and 4 mg/ml concentrations inhibited this L-selectin mediated signaling of arrest, as well as decreased transition to a migratory state from 12% of cells to <1% of cells assuming a polarized shape (Figure 2.6E). In contrast, in the presence of IL-8 stimulation, we did not detect significant inhibition in the frequency of neutrophils rolling to arrest in presence of Feraheme (Figure 2.6E). This is likely due to the fact that coupled with shear force mediated L-selectin signaling, even very low concentrations of IL-8 can lead to activation of HA CD18 receptors sufficient to mediate conversion from rolling to arrest (Schaff et al., 2008). However, in the presence of Feraheme, a significant increase was detected in the duration of rolling before the

onset of cell arrest for both IL-8 stimulated and unstimulated neutrophil suspensions. Increasing from 42.1 seconds to arrest in the 0 mg/ml condition to 55.1 and 91.9 seconds in the 1 mg/ml and 4 mg/ml conditions, respectively (Figure 2.6F). In IL-8 treated cells only the 4 mg/ml condition resulted in significantly increasing the time to arrest compared to the 0 mg/ml condition, from 20.0 to 34.7 seconds. We conclude that Feraheme exerts a significant effect on the kinematics of rolling to arrest on E-selectin and ICAM-1 in terms of increasing the velocity and signaling associated with HA CD18 mediated arrest.

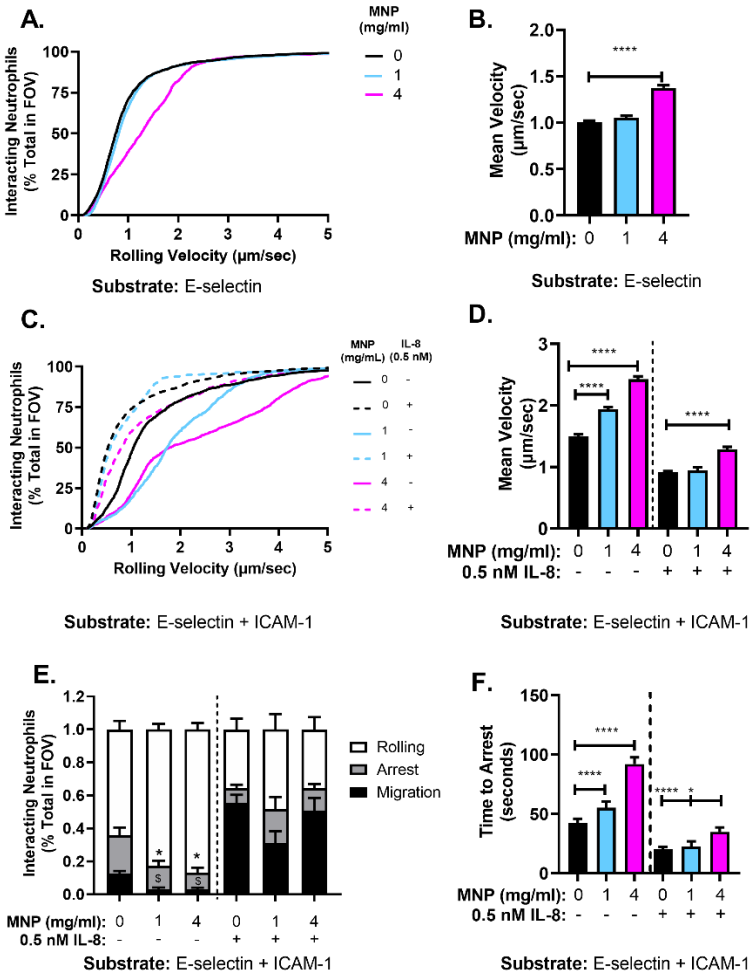

Figure 2.6: Feraheme alters the kinetics of neutrophil rolling and arrest in microfluidic flow channels. (A) Neutrophil rolling velocity over an E-selectin substrate treated with vehicle or Feraheme (1 mg/ml, 4 mg/ml). (B) Cumulative rolling velocities of neutrophils over an E-

selectin substrate treated with vehicle or Feraheme (1 mg/ml,4 mg/ml). (C) Mean neutrophil rolling velocity over an E-selectin+ ICAM-1 substrate treated with Feraheme (1mg/ml,4 mg/ml) and/or IL-8 (0.5 nM). (D) Cumulative rolling velocities of neutrophils over an E-selectin + ICAM-1 substrate treated with vehicle or Feraheme (1 mg/ml,4 mg/ml) and/or IL-8 (0.5 nM). (E) Neutrophil rolling to arrest and migration over an E-selectin+ ICAM-1 substrate treated with Feraheme (1 mg/ml,4 mg/ml) and/or IL-8 (0.5 nM). * and $ denote T-Test p-values of $\leq$.05 as compared to the 0 mg/ml MNP condition for arrest and migration, respectively. (F) Neutrophil time to arrest over an E-selectin+ ICAM-1 substrate treated with Feraheme (1 mg/ml,4 mg/ml) and/or IL-8 (0.5 nM). For B, D, and F, * and **** denote T-Test p-values of $\leq$.05 and $\leq$ 0.0001, respectively. Data in B, D, E, and F are presented in mean $\pm$ SEM ($n \geq 4$ donors) with at least 15 cells for each donor per condition.

2.6 Feraheme perturbs the process of E-selectin ligand clustering on rolling neutrophils.

E-selectin recognition of L-selectin and PSGL-1 under shear flow leads to receptor co-clustering and mechanotransduction of signals leading to HA CD18 binding to ICAM-1 (Morikis et al., 2017). To further examine how uptake of Feraheme effects the process of selectin mediated rolling and deceleration to arrest, total internal reflection fluorescence (TIRF) microscopy and quantitative dynamic footprinting (qDF) was performed to determine the L-selectin and PSGL-1 bond cluster formation during rolling on a substrate of E-selectin. Treatment with Feraheme resulted in an increase in L-selectin bond cluster area (i.e., lower density of L-selectin) and marginal changes in the frequency of cluster formation detected in the presence and absence of IL-8 stimulation (Figure 2.7A-B). Examining the average density of L-selectin receptors within clusters bound to E-selectin, a significant decrease was detected in the presence of IL-8 stimulation for 4 mg/ml compared to 0 mg/ml Feraheme (Figure 2.7C). In contrast, Feraheme did not affect E-selectin mediated formation of PSGL-1 cluster area and frequency nor PSGL-1 cluster density (Figure 2.7D-E). These data are consistent with the observed increase in rolling velocity and decreased outside-in signaling via E-selectin in the presence of Feraheme and indicate this effect may be a function of diminished L-selectin bond

formation despite the observed higher frequency and area of L-selectin within sites of adhesive contact.

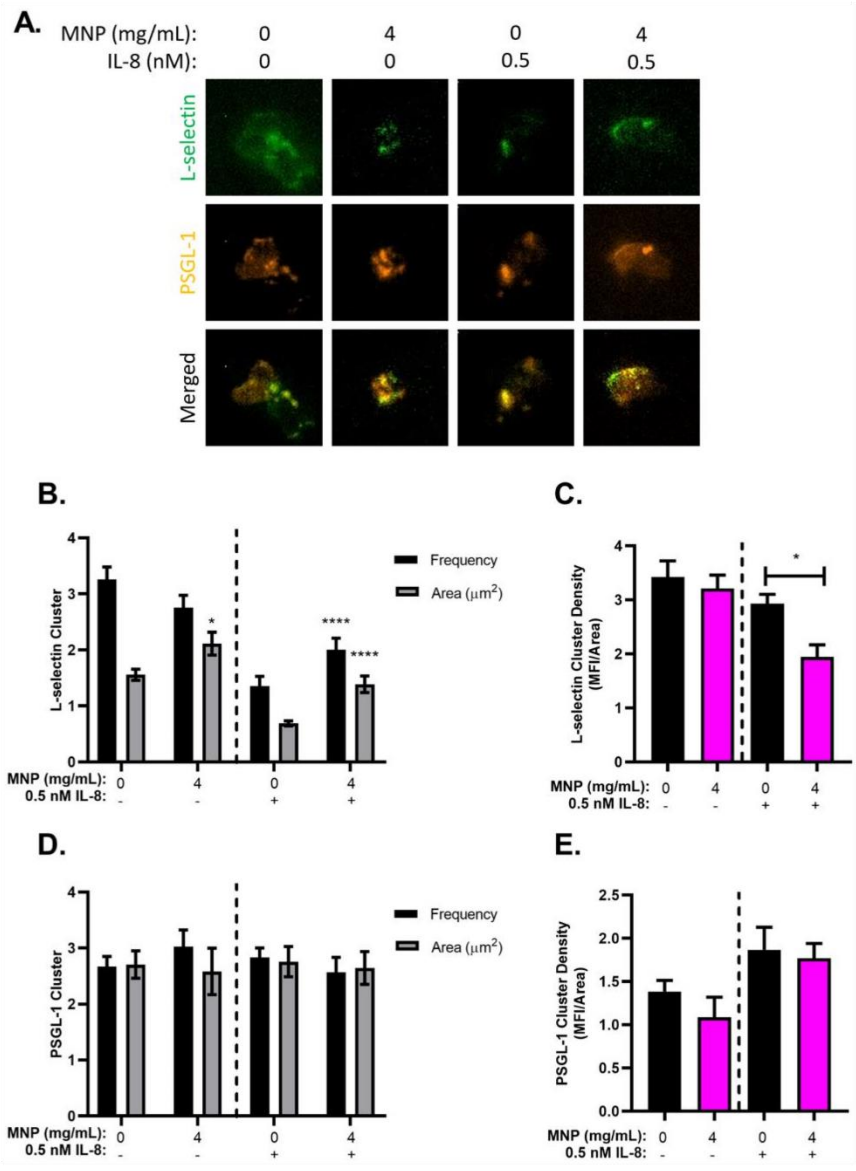

Figure 2.7: Feraheme antagonism of E-selectin ligand bond formation. (A) Neutrophil rolling on a substrate of E-selectin in the presence of vehicle control or Feraheme MNP (4 mg/ml) was dynamically imaged using qDF to detect L-selectin (AF488 anti-human DREG55) and PSGL-1 (PE anti-human PL-1) engagement in the plane of adhesive contact. (B) L-selectin and (C) PSGL-1 receptor cluster area and frequency were determined. (D) L-selectin and (E) PSGL-1 receptor density was determined and reported as mean ± SEM (n = 3 donors) with at least 10 cells for each donor per condition. * and **** denote T-test p-values of ≤.05 and ≤ 0.0001, respectively, compared with the 0 mg/ml condition.

2.7 Feraheme accelerates clearance of cytosolic calcium after flux.

Calcium serves as a secondary messenger downstream of GPCR signaling that mediate neutrophil inflammatory responses including degranulation, integrin activation and adhesion, shape change, and ROS production (Immler et al., 2018a). IL-8 ligation of CXCR leads to a release of endoplasmic reticular (ER) stored calcium into the cytosolic space which is subsequently sequestered by the ER calciosomes to replenish stores. High concentrations of extracellular adenosine which binds to the adenosine A2A receptor is known to dampen neutrophil inflammatory responses (Anderson et al., 2000). The mechanism as depicted in the schematic (Figure 2.8A) is initiated by ligand binding to the adenosine A2A receptor inducing disassociation and activation of the Gα subunit of the A2A receptor linked heterotrimeric G protein which then activates adenylyl cyclase. Adenylyl cyclase activation leads to production of cyclic AMP (cAMP), which in turn activates cAMP-dependent protein kinase (PKA) and leads to accelerated sequestration of calcium through PKA-activated endo-membrane Ca^{2+}-ATPases. To determine if Feraheme's inhibitory effects on intracellular signaling are mediated through accelerated clearance of IL-8 induced calcium flux, the time course of cytosolic calcium stimulated by IL-8 in Feraheme treated neutrophils was compared in the presence and absence of adenosine A2A receptor agonist CGS 21680 (CGS). Following IL-8 induced release of ER Ca^{2+}, Feraheme elicited an accelerated decrease in cytosolic calcium as compared to untreated cells. The level of accelerated Ca^{2+} clearance was equivalent and not additive with CGS (Figure 2.8B). The duration required for a decrease to 20% of the maximum Ca^{2+} flux elicited by IL-8 was 60 seconds in untreated cells compared to 36, 22, and 22 seconds for Feraheme, CGS, and Feraheme + CGS, respectively (Figure 2.8C). Neither Feraheme nor CGS alone reduced the

maximal Ca^{2+} flux elicited by IL-8, while added together they resulted in a slight reduction (Figure 2.8D).

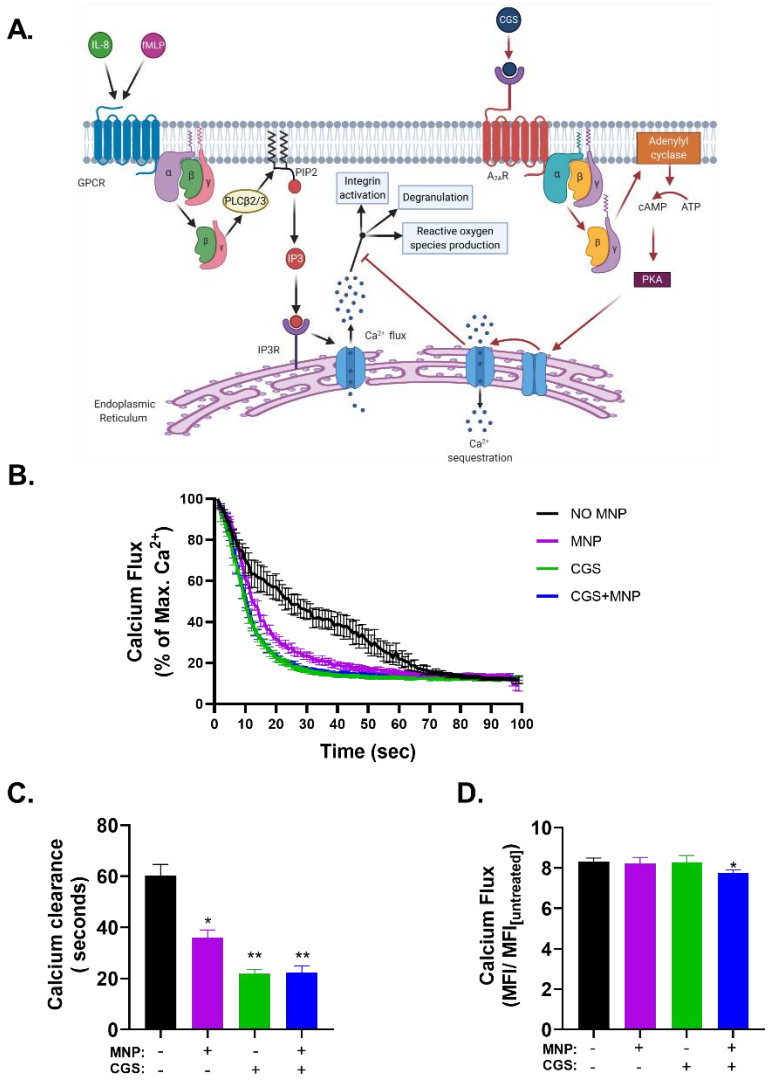

Figure 2.8: Feraheme MNP (4 mg/ml) accelerates clearance of cytosolic calcium following IL-8 stimulated calcium flux. (A) Diagram depicts the pathway of Ca^{2+} mediated neutrophil activation and antagonism by CGS 21680 via the Adenosine receptor. (B) Kinetics of cytosolic calcium clearance following maximal release due to 1 nM IL-8 at t=0 sec. (C) Duration for Ca^{2+} clearance to reach 20 percent of maximum value. (D) Fold change between maximal calcium flux and baseline level in untreated cells. Data are presented in mean ± SEM (n =3 donors) with experimental replicates for each donor. Paired T-tests were performed comparing experimental conditions to the NO MNP condition of the same donor *, and ** denote p value ≤.05, and ≤ 0.01, respectively.

To determine if Feraheme mediated inhibition acts through release and binding of extracellular adenosine, the clearance of calcium after IL-8 stimulated Ca^{2+} flux was compared to that activated by the adenosine A2A receptor agonist CGS 21680 (CGS) while blocking signaling via the A2A receptor with the adenosine A2A receptor antagonist ZM 241385 (ZM) as depicted in Figure 2.9A. As expected, in neutrophils preincubated with ZM the sequestration effect of CGS was completely abrogated. In contrast, Feraheme treatment remained effective in accelerating the decrease in cytosolic Ca^{2+} following IL-8 stimulation (Figure 2.9B). In untreated cells the duration to 20% of maximum Ca^{2+} flux was 65 seconds compared to 46, 63, and 42 seconds for Feraheme, CGS, and Feraheme+ CGS, respectively (Figure 2.9). All conditions registered the same maximal Ca^{2+} flux (Figure 2.9D).

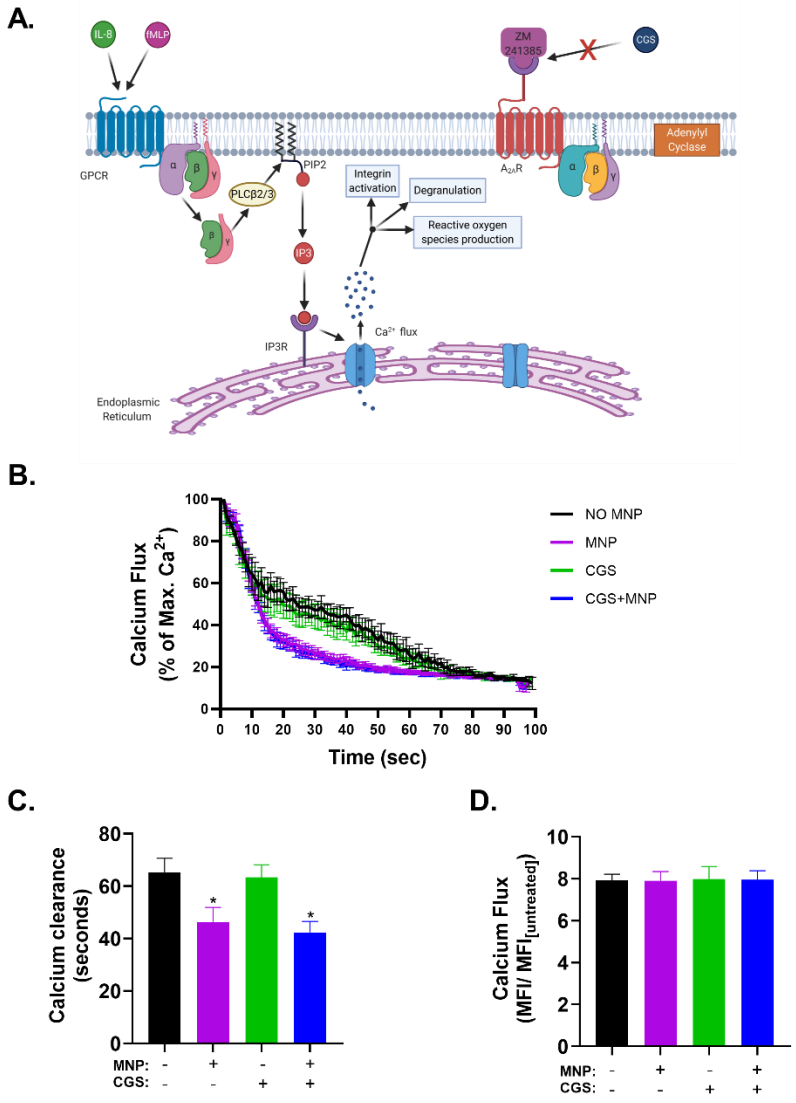

Figure 2.9: Feraheme MNP (4 mg/ml) accelerated clearance of IL-8 stimulated cytosolic Ca^{2+} is independent of the Adenosine A2A receptor. The Adenosine A2A receptor antagonist ZM (2.5 μM) was applied for 5 min in calcium buffer. (A) Diagram depicts calcium flux in cells preincubated with ZM before treatment with the Adenosine A2A receptor agonist CGS (B) Kinetics of cytosolic calcium resequestration from maximal flux induced by IL-8 (1 nM) at t=0 sec. (C) Time from maximal calcium flux until calcium levels reach 20 percent of max value. (D) Fold change between maximal calcium flux and untreated cells. Data are presented in mean ± SEM (n=3 donors) with experimental replicates for each donor. Paired T-tests were performed comparing experimental conditions to the NO MNP condition of the same donor. denotes a p value ≤.05.

2.8 Discussion.

Neutrophil recruitment and attachment to inflamed endothelium is mediated by a multistep process involving ligation and mechanosignaling from the outside-in through selectins and integrins following bond formation (Morikis & Simon, 2018). CXCR ligation of IL-8 by neutrophils interacting with inflamed endothelium under shear flow induce degranulation and the activation of CD18 to a high affinity state, as well as the rapid shedding of L-selectin (Lacy, 2006; Miralda et al., 2017). Here we demonstrated that, treating neutrophils in suspension over a dose range with Feraheme elicited reduced responsiveness to stimulation with 1nM IL-8 at each dose tested (1-6 mg/ml), resulting in increased inhibition of CD11b upregulation, HA CD18 activation, and L-selectin shedding with increasing particle concentration of Feraheme. Treating with a Feraheme dose of 4 mg/ml resulted in a consistent ~2-fold right shift in the EC50 of stimulation with IL-8 corresponding to diminished upregulation of CD11b/CD18, activation of HA CD18, and L-selectin shedding. Chemokine receptor expression in unstimulated cells was significantly elevated in the presence of Feraheme, but these changes in expression did not explain the inhibitory effects observed. Feraheme also did not alter endocytosis of CXCR2 in response to IL-8 signaling, suggesting that its mechanism of action is not through down regulation or interference with CXCR ligation. Feraheme also significantly reduced fMLP induced ROS production in the presence or absence of IL-8 priming. Taken together, we conclude that the inhibitory effects of Feraheme are not exclusive to CXCR1/2 signaling by IL-8, but include signaling through other GPCR namely, formyl peptide receptors.

Uptake of nanoscale poly(styrene) and liposomal particles by neutrophils was shown to not enhance apoptosis, activation, or cell death. Ingested nanoparticles were reported to reside in intracellular compartments that are retained during degranulation (Simon et al., 2000). Uncoated

SPION are reported to aggregate and induce the formation of neutrophil extracellular traps (NETs) that are extracellular fibers consisting of expelled DNA. This pro-inflammatory host response is mitigated by coating SPION with layers of dextran or human serum albumin (Bilyy et al., 2018). In our experiments Feraheme did not reduce cell viability, which exceeded 99%. Although we did not specifically test for netosis in our studies of neutrophil arrest and migration, no visible changes in cell morphology that is characteristic of netosis was observed. In-vivo studies have demonstrated that uptake of nanoparticles can diminish the efficiency of neutrophil recruitment to inflamed endothelium within the lungs of mice. However, the mechanism that may involve cell-particle uptake and competitive binding to adhesion receptors, which could interfere with chemotactic or adhesive ligand binding during recruitment remained elusive (Fromen et al., 2017; Kelley et al., 2019). Here we report that following 20 minutes of exposure to Feraheme in suspension, we observed diminished efficiency of neutrophil arrest and migration as measured in an established model of endothelial inflammation in vascular mimetic shear flow channels. We have recently reported that deceleration of neutrophils within the vascular mimetic substrate in microfluidic flow channels is mediated by as few as 200 HA CD18 binding ICAM-1 in order to transition from rolling to arrest (Morikis et al., 2017). In the absence of IL-8 stimulation and in the presence of Feraheme, a ~50% reduction was measured in the frequency of neutrophils rolling to arrest on E-selectin/ICAM-1 at both 1 and 4 mg/ml. Moreover, neutrophils rolled over twice the distance before activation and binding of HA CD18 integrin bonds formed stable arrest. Inhibition of neutrophil CD11b/CD18 mediated migration after arrest was also observed at 1 and 4 mg/ml Feraheme. Thus, Feraheme uptake in a concentration dependent manner alters both selectin and GPCR mediated signaling of HA CD18 necessary to transition from rolling on E-selectin to arrest on ICAM-1. It has been previously shown that L-

selectin catch-bonds, characterized by a prolonged bond lifetime as tensile force is increased, is necessary and sufficient to signal CD18 transition to a HA state (Morikis et al., 2017). We show that Feraheme reduced both L-selectin shedding in response to IL-8 and L-selectin clustering during rolling on E-selectin, but it had no effect on PSGL-1 expression. Noteworthy was the finding that cluster area of L-selectin bonds during rolling on E-selectin in the presence of shear stress and IL-8 stimulation was increased, while the density of bond formation within these clusters decreased. This indicates that Feraheme may alter L-selectin signaling, but not recognition by E-selectin on the surface of rolling neutrophils. This is consistent with the significant rise in the mean rolling velocity on E-selectin that was observed to increase with Feraheme concentration. This provides one potential explanation as to how Feraheme reduces the efficiency of the transition to cell arrest, in that it may alter the capacity of E-selectin/L-selectin to form catch-bonds. Increased availability of L-selectin to form bond clusters in the presence of Feraheme coincided with reduced density of bonds on E-selectin and also with a reduced frequency of cell arrest suggesting that the normal process of neutrophil deceleration at sites of inflammation may be perturbed. Reduced levels of L-selectin shedding may potentially contribute to the inhibition of arrest and polarization observed in Feraheme treated cells. Consistent with the latter observation are reports that L-selectin shedding has been reported to amplify integrin-mediated outside-in signaling–dependent processes, including neutrophil migration, production of ROS, and phagocytosis (Cappenberg et al., 2019). L-selectin shedding induces phosphorylation of phospholipase C (PLC)γ2, Akt, and Syk, critical regulators of intracellular calcium release that are important for E-selectin mediated slow rolling and CD18 integrin activation in neutrophils (Cappenberg et al., 2019; Mueller et al., 2010). Thus, inhibition of L-selectin shedding may also interfere with normal activation of HA CD18 during rolling on

E-selectin, despite the elevated expression of L-selectin on the surface. While it is likely that Feraheme is endocytosed by neutrophils in suspension, we cannot rule out that a fraction remain bound to the plasma membrane and sterically interfere with the recognition and mechanics of selectin bond formation. Due to optical limitations in real-time imaging of SPION bound to the plasma membrane at the nanoscale, we could not directly image whether Feraheme specifically bound to L-selectin receptors and sterically interfered with catch-bond formation associated with mechanotransduction of CD18 activation. What is clear is that Feraheme altered the efficiency of L-selectin's capacity to mechanosignal activation of HA CD18, but not necessarily its ability to bind E-selectin and form focal clusters.

To elucidate a possible mechanism for Feraheme inhibition of GPCR signaling the effects of Feraheme on calcium flux after IL-8 stimulation was recorded. Feraheme significantly accelerated the clearance of calcium after flux and this response was comparable to that of the adenosine A2A receptor agonist CGS 21680 known to inhibit degranulation and ROS production. The effect of Feraheme was not dependent on adenosine as treatment with the adenosine A2A receptor antagonist ZM 241385 did not perturb the acceleration of calcium clearance in response to Feraheme. Calcium signaling plays a key role in GPCR mediated degranulation, L-selectin shedding, integrin activation, and ROS production which are all inhibited by Feraheme. Calcium is also necessary for selectin mechanosignaling induced activation of HA LFA-1 and for integrin signaling of shape change and polarization after arrest (Morikis & Simon, 2018). Accelerated clearance of calcium could potentially be resulting in the decreased arrest and polarization observed in Feraheme treated cells by inhibiting selectin mechanosignaling. These experiments highlight a consistent effect of Feraheme uptake on neutrophil intracellular signaling, but the mechanism which may involve Feraheme steric

influence on normal cell surface receptor affinity or avidity in binding ligand needs to be confirmed in future studies. The non-additive inhibition in the presence of Feraheme and CGS could indicate a shared downstream mechanism for the accelerated clearance of Ca^{2+}. We propose that Feraheme may induce sequestration of calcium through activation of endo-membrane Ca^{2+}-ATPases rather than by efflux of calcium through activation of plasma membrane Ca^{2+}-pump, the proposed mechanism of action for CGS (Figure 2.10).

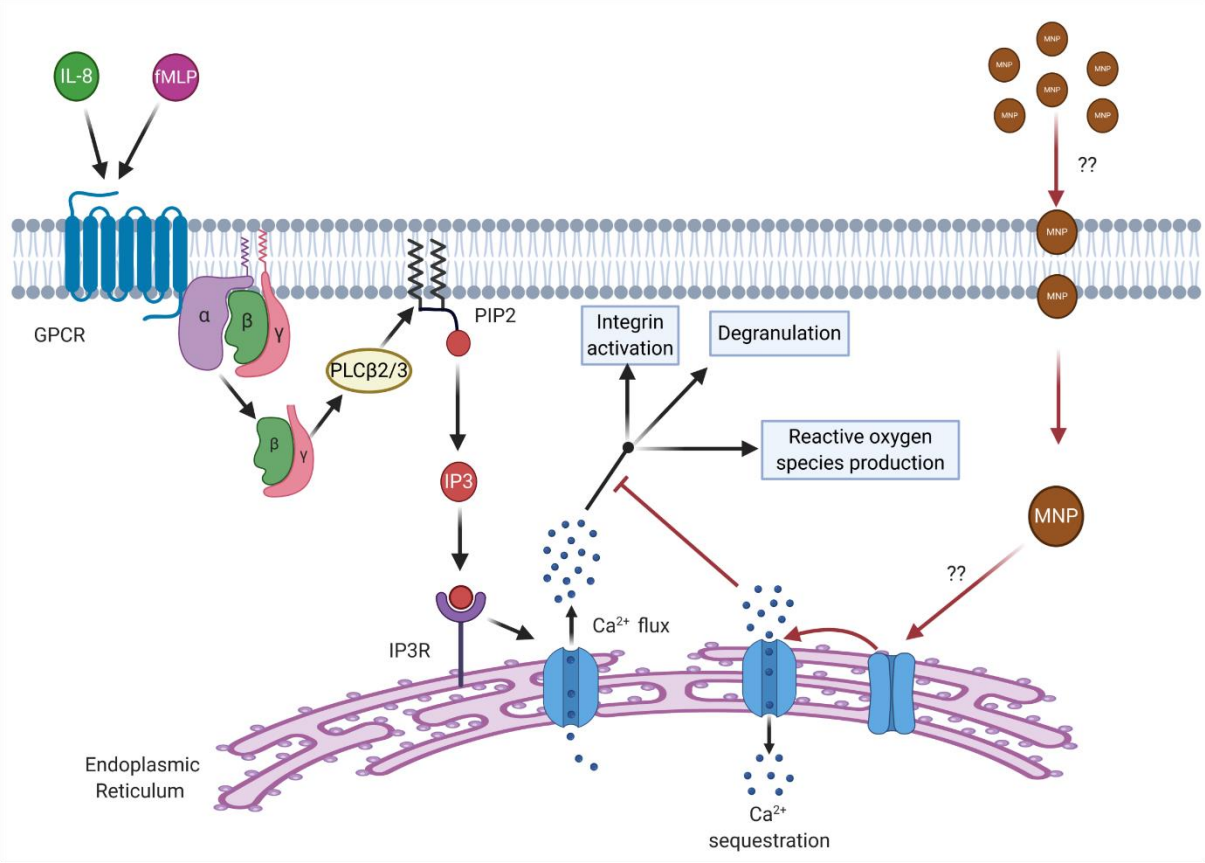

Figure 2.10: Schematic depicting the proposed mechanism of Ca^{2+} sequestration and inhibition of downstream response by Feraheme. Feraheme may inhibit neutrophil function through activation of endo-membrane Ca^{2+}-ATPases leading to accelerated clearance of cytosolic calcium inhibiting intracellular signaling. CXCR1/2 ligation by endogenous receptor ligands (IL-8, fMLP) leads to dissociation of Gα from Gβγ subunits of G proteins activating PLCβ2/3 which splits phosphatidylinositol 4,5 biphosphate (PIP2). PIP2 splits releasing inositol-1,4,5 triphosphate (IP3) that binds to IP3 receptor (IP3R) on the surface of the endoplasmic reticulum inducing calcium flux which signals for downstream effector functions (integrin

activation, degranulation, and reactive oxygen species production). We propose that Feraheme MNP are being endocytosed by neutrophils which through an unknown intermediary lead to activation of endo-membrane Ca^{2+}-ATPases that sequester calcium and inhibit calcium signaling of functional responses down stream of GPCR.

In summary, Feraheme exerted an immunosuppressive effect on neutrophil activation in response to IL-8 and fMLP, as well as outside-in signaling via E-selectin during neutrophil recruitment in shear flow. A potential mechanism may involve the observed Feraheme induced acceleration of calcium clearance resulting in a reduced capacity for cell signaling of immune responses. Further studies are warranted to elucidate the mechanism by which Feraheme induces accelerated calcium clearance, potentially though elevation of cAMP leading to downstream activation of endo-membrane Ca^{2+}-ATPases, and whether this is the primary mechanism for the inhibition of neutrophil inflammatory responses observed herein. Whether these effects are seen with other SPION formulations is also a relevant question to pursue. These, immunosuppressive effects could lead to impaired immune responses to infection or sterile tissue insult in patients treated with Feraheme. The current studies also point to the expanded use of SPION to downregulate the innate immune response as a therapy for ameliorating chronic inflammation and/or autoimmune diseases.

Chapter 3: Psoriatic Neutrophils are Primed in Circulation for Enhanced β_2-integrin Dependent Recruitment and Effector Function on E-selectin and ICAM-1.

3.1 Introduction

Psoriasis is an inflammatory disease that is initiated by pathogenic T cells within skin lesions that correlates with hyperproliferation of keratinocytes and the release of cytokines and damage associated molecular pattern (DAMP) molecules into the circulation (Wang & Jin, 2020). Exposure to DAMPs and low levels of cytokines and chemokines shift neutrophils from a quiescent to primed state characterized by increased sensitivity to subsequent stimulation that induces amplified adhesive and effector function at sites of tissue inflammation (Miralda et al., 2017). An early event in psoriatic plaque formation is the recruitment of primed neutrophils into the dermis where they degranulate, release reactive oxygen species (ROS), and produce neutrophil extracellular traps (NETs) enriched in DAMPs, which include cathelicidin (LL37) and calprotectin (S100A8/A9) that exacerbate skin inflammation (Shao et al., 2019).

In particular, calprotectin levels rise with neutrophil count in circulation that correlates with the intensity of plaque formation in psoriasis and cardiovascular disease comorbidity (Berg et al., 2022; Matsunaga et al., 2021). Calprotectin signals through toll-like receptor 4 (TLR4) to elicit an allosteric upshift of β2-integrins (CD11/CD18) from a bent to extended conformation with intermediate affinity to bind ICAM-1 on inflamed endothelium (Pruenster et al., 2015). Previously, we reported that ligation of L-selectin on neutrophils rolling on E-selectin elicits the secretion of calprotectin that primes them for enhanced recruitment efficiency on inflamed endothelium (Morikis et al., 2017). Here, we examined the importance of calprotectin in priming for enhanced β2-integrin-mediated neutrophil inflammatory response in psoriasis.

3.2 Materials and methods

3.21 Reagents

Recombinant human ICAM-1-Fc and E-selectin-Fc chimeric constructs were purchased from R&D Systems (Minneapolis, MN). Protein G-Biotin and Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (Burlington, MA). Fluorescently tagged monoclonal antibodies for flow cytometric detection of high affinity β2-integrin (mAb24), L-Selectin (Dreg-56), and CD11b (M1/70), along with unlabeled antibodies that block CD18 (IB4), CD11b (M1/70), and Fc receptor (TruStain FcX™), recombinant S100A8/A9, TNF-α, IL-8, and fixation buffer (4% paraformaldehyde) were purchased from Biolegend (San Diego, CA). Dihydrorhodamine 123 is a nonfluorescent reactive oxygen species (ROS) indicator that diffuses across membranes where it localizes in the mitochondria and exhibits green fluorescence. Fluorescent calcium indicator Fluo-4, NeutrAvidin Protein soluble and coated on black 96-well plates, Biotinylated Pierce™ Bovine serum albumin, and Invitrogen IL-8 Human ELISA Kit were all purchased from Thermo Fisher Scientific (Waltham, MA, United States). Small molecule glycomimetic pan-selectin antagonist Rivipansel (GMI-1070) was provided by GlycoMimetics Inc. (Rockville, MD). Human LL-37 ELISA Kit was purchased from Novus biologicals (Centennial, CO). KIM-127 producing hybridoma cell line was purchased from ATCC (Manassas, VA).

3.22 Patient demographics

Adults over the age of 18, who had been diagnosed with active plaque psoriasis by a dermatologist, were included in this study and consented through a University of California, Davis Institutional Review Board Protocol #1740034-5. Patients filled out a survey to self-report body surface area affected and prior/current treatment. Whole blood was obtained by

venipuncture and collected in 10 mL sodium heparin sterile tubes from psoriatic donors. Whole blood was similarly obtained from healthy donors consented through a University of California, Davis Institutional Review Board protocol (#235586-9).

Exclusion criteria included patients on biologics and patients with history of adverse reactions to venipuncture, hypotension, anaphylaxis anemia, hypotension, arrhythmia, bleeding diathesis, CNS, liver, kidney, or thyroid disease.

3.23 Human neutrophil isolation

Neutrophils were isolated from whole blood within 1 hour of collection using the EasySep™ direct human neutrophil isolation kit purchased (StemCell Technologies, Cambridge, MA) per manufacturer's instruction. Cells were spun down and resuspended in HBSS containing 0.1% human serum albumin without Ca^{2+} and Mg^{2+} (HBSS-/-). Neutrophil purity was >90% as determined on a Beckman Coulter Counter. Neutrophil suspensions at 1×10^7 cells/mL were kept on ice for no longer than 3 hours and subsequently rewarmed at 37ºC in diluted HBSS buffer containing 1 mM Ca^{2+} and Mg^{2+} and 0.1% human serum albumin (HBSS+/+) unless otherwise specified.

3.24 Flow Cytometry Detection of Cell Surface Marker Expression

For detection of baseline expression of adhesion receptors, whole blood was diluted 1:4 in HBSS+/+ and stained with 0.2 ug/mL of LDS-751, a cell-permeant nucleic acid stain that has a peak excitation at ~543 nm on dsDNA (Thermo Fisher) as well as antibodies for high affinity β2-integrin (mAb24), L-selectin (Dreg-56), CD11b (M1/70), and extended β2-integrin (KIM127) for 20 min at 37°C in a thermomixer at 400 rpm. Cells were than fixed with 1% fixation buffer for 20 min at room temperature. Cells were then analyzed on a Attune NxT flow cytometer

(Thermofisher). Leukocytes were identified by thresholding on LDS-751 fluorescence and gated based upon characteristic forward scatter vs side scatter profile. Experiments characterizing TNF-α and S100A8/A9 activation of isolated neutrophils were performed at $1x10^6$ cells/mL in HBSS+/+ using the same incubation times and antibody labeling conditions detailed above.

3.25 Elisa

Plasma was prepared by centrifuging heparinized blood for 10 min at 1000xg. Plasma was then aliquoted and stored at -80°C until the day of analysis and thawed on ice. Enzyme-linked immunosorbent assays (ELISA)s were performed according to manufactures instruction to quantify S100A8/A9, IL-8, and LL37 levels.

3.26 Calprotectin (S100A8/A9) secretion

Isolated neutrophils in suspension were treated with or without TNF-α (2pM or 1000pM) for 20 minutes at 37°C in a thermomixer at 400 rpm before centrifugation for 5 min at 500xg. Cell supernatant was collected and stored at -80°C until day of analysis using ELISA.

3.27 Glass coverslip functionalization

Glass coverslips were functionalized using a sequential incubation process with 3 washes of PBS between steps. Coverslips were first incubated with a 1 mg/ml solution of biotinylated BSA in PBS for 20 minutes. Next, they were treated with a 200 µg/ml solution of neutravidin in PBS for 20 minutes, followed by a 50 µg/ml solution of biotinylated protein G in PBS for 20 minutes. Coverslips were then incubated with a mixture of 1 µg/ml E-selectin and 1 µg/ml ICAM-1 overnight at 4°C. To minimize non-specific adhesion, the coverslips were then treated with 1% casein for 1 hour before washing with PBS and subsequent assembly of the microfluidic device.

3.28 Microfluidic Shear Flow Assay

Vascular mimetic flow chambers were utilized to record neutrophil rolling and arrest behavior on substrates of endothelial ligands under physiological shear stresses. Devices were fabricated as described previously (Morikis et al., 2022). Briefly, polydimethylsiloxane (PDMS) flow chambers with dimensions 60 µm × 2 mm × 8 mm (h × w × l) were produced by curing Sylgard 184 prepolymer (Dow Corning, Midland, MI, USA) over a patterned silicon wafer. Holes were punched into the PDMS for flow channel and vacuum port access and the device was reversibly vacuum sealed on a circular glass coverslips coated with E-selectin and ICAM-1.

Neutrophils at a concentration of 5×10^5 cells/ml were treated with CD11b and Fc blocking antibodies for 15 minutes at 37°C. Neutrophils were loaded into an open 100µl reservoir and drawn through the channel at a shear stress of 2 dynes/cm2 using a syringe pump. Rivipansel inhibition of E-selectin was performed by perfusing microfluidic channels during a 15-minute blocking step before neutrophil perfusion.

Neutrophil adhesion dynamics were analyzed on digital video recordings obtained using brightfield phase microscopy at 60 frames per minute for 4 minutes per field of view on an inverted microscope (Nikon 2000) using a phase contrast 20× objective and recorded with 16-bit digital complementary metal oxide semiconductor (CMOS) camera (Andor ZYLA) connected to a PC (Dell) with NIS Elements imaging software (Nikon Instruments Inc.). Arrested neutrophils were defined as those with velocity <0.1µm/s.

3.29 Cytosolic Ca^{2+} flux

Isolated neutrophils at 1×10^6 cells/mL were loaded with the Ca^{2+} indicator Fluo-4 AM (1 µg/mL) in HBSS -/- for 30 min at 37°C in the dark. Cell suspensions were then spun down and

resuspended at 1×10^7 cells/mL in HBSS-/- and incubated for 30 minutes in the dark at room temperature to allow cells to de-esterify. Cells were then sheared in microfluidic flow channels as described above. Fluorescence images were captured at the same frequency and on the same microscope setup as above. Fluorescence was quantified in individual cells that rolled to arrest using ImageJ software starting 10 seconds before cell arrest.

3.210 Reactive oxidant production

Neutravidin-coated 96-well plates were coated with ICAM-1-Fc (10 μg/ml) by coincubation with 100 μg/mL biotinylated Protein G in PBS for 2 hrs. Isolated neutrophils at a concentration of $1x10^6$ cells/mL were treated with or without CD18 blocking antibody- IB4 (50μg/ml) for 15 min at room temperature. Next, $2x10^5$ neutrophils were allowed to adhere to wells for 5 min with mechanical oscillation at 360 CPM before addition of PMA (10 ng/mL) and ROS indicator dihydrorhodamine 123 (2 μM). The ROS signal was then measured using a Synergy™ HT Multi-Mode Microplate Reader with peak levels reported at 120 minutes at 37°C.

3.211 Statistical analysis

GraphPad Prism, version 9.4.0, was used for all data analysis. Mann-Whitney U test was performed for comparing the nonparametric distributions of rolling to arrest time in Figure 2c. All other statistical comparisons were performed using Welch's t test.

	Psoriasis (n=14)	Healthy (n=10)
Age	61±14.2 (28-78 years)	33.2±15.0 (21-62 years)
Sex	M=4, F=10	M=5, F=5
Disease severity (% body surface area involvement)	Mild: 1-9% n= 9 Moderate: 10-29% n= 3 Severe: >30% n=2	NA

Table 3.1: Demographics of Psoriasis patients and Healthy control subjects

3.3 Psoriatic neutrophils are primed in circulation.

Fresh venous blood from psoriatic patients (Ps) and healthy controls (Hc) was collected under IRB protocol for functional studies (Table 3.1). Neutrophil priming through GPCR and TLR4 results in phenotypic changes in neutrophils that include upregulated Mac-1 (CD11b/CD18) resultant of degranulation and decreased levels of L-selectin (CD62L) resultant of shedding (Miralda et al., 2017). Psoriatic neutrophils in diluted whole blood exhibited a primed phenotype with Mac-1 upregulated 150% and L-selectin decreased 25% relative to healthy subjects (Figure 3.1a-b). Priming of neutrophils also increases neutrophil adhesion which is mediated by β2-integrins. A dual labeling scheme, with antibodies that detect the E+ conformation (KIM127) and the H+ conformation (mab24), was used to probe activation of CD18. Psoriatic neutrophils registered a ~200% upregulation in receptor expression of extended-intermediate state and a ~160% upshift of high affinity when compared to healthy neutrophils (Figure 3.1c-d).

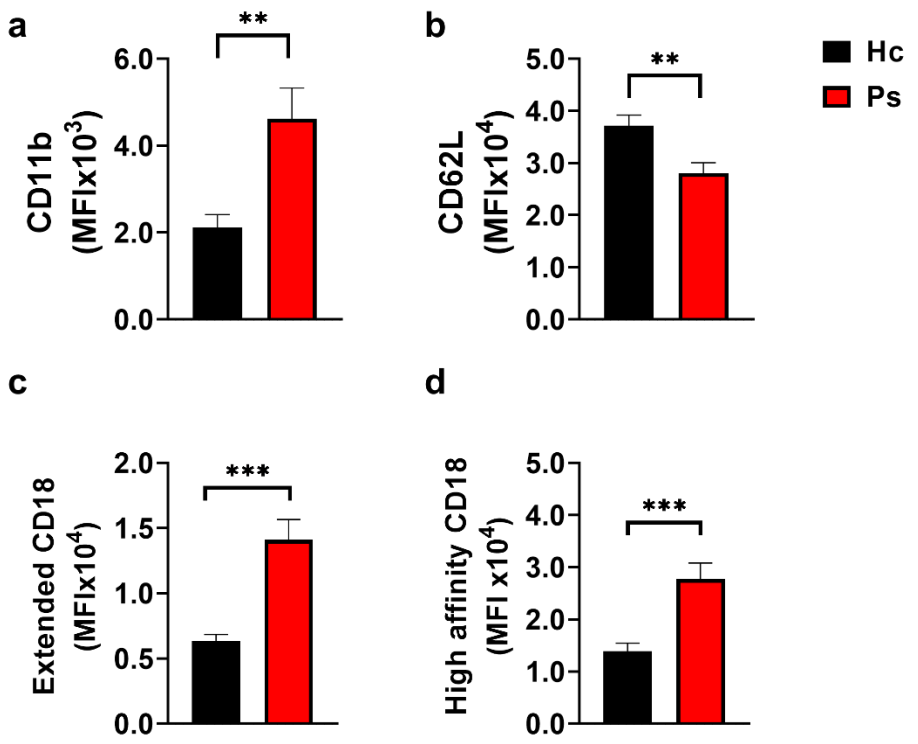

Figure 3.1: Psoriatic neutrophils displayed a primed phenotype with modified adhesion marker expression. Expression level of a) CD11b, b) CD62, c) extended-intermediate (KIM127, E+), d) and high affinity CD18 (mAb24, H+) in Ps (n=14) vs Hc (n=10) neutrophils as measured by flow cytometry in diluted blood. The results are presented as the mean ± SEM with significance between Ps and Hc samples denoted by **=$p < 0.01$ and ***=$p < 0.001$, respectively.

Flow cytometric analysis revealed that 79% of neutrophils from healthy samples occupied the (E-H-) quadrant, indicative that CD18 remained in the bent-quiescent state. In contrast, more than 50% of Ps neutrophils were present in (E-H+) and (E+H+) quadrants, indicative of adhesion competent high affinity CD18 (Figure 3.2a).

When comparing mild to moderate-severe plaque psoriasis patient neutrophils, there was a significant 51% higher expression of high affinity CD18 in moderate-severe patients. The extended conformation was 45% higher in moderate-severe patients compared to mild, but due to the small sample size there was not enough power to reach significance (Figure 3.2b). These

results demonstrate that psoriatic neutrophils present with a primed phenotype characterized by changes in adhesion marker expression important in the neutrophil recruitment cascade.

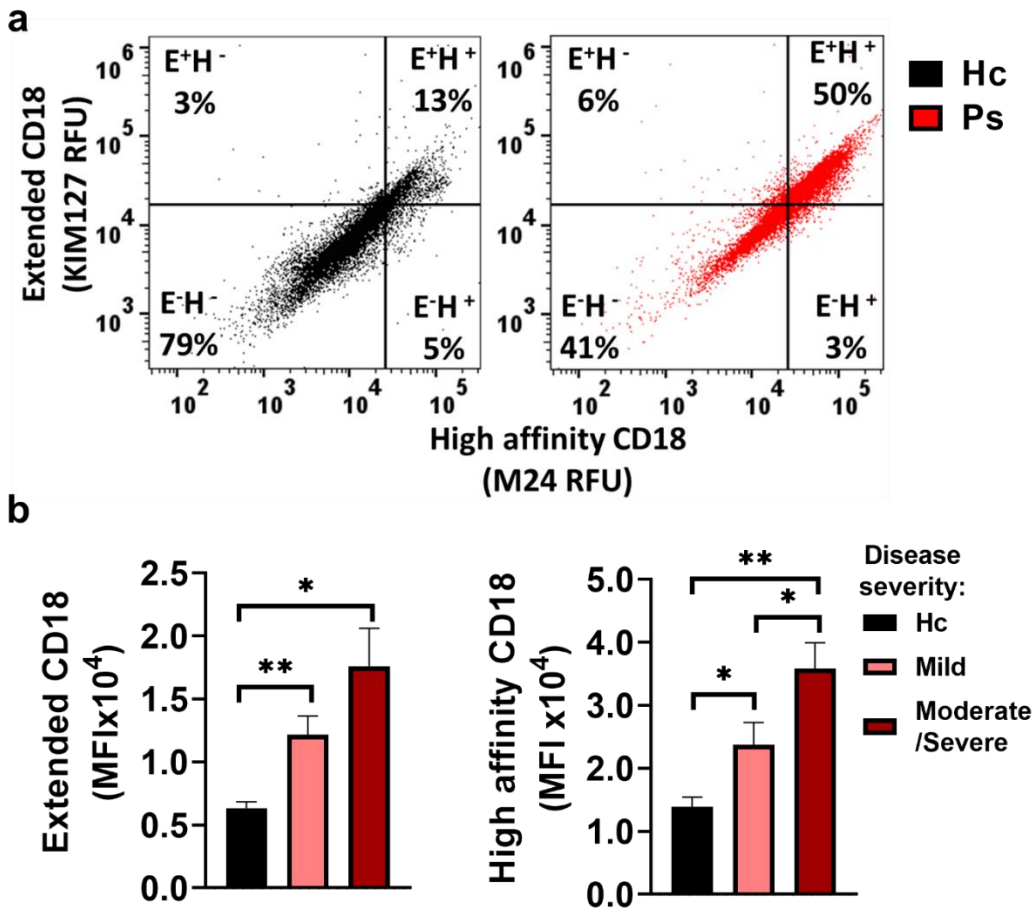

Figure 3.2: β2 integrins are activated in psoriasis. a) Scatter plots of fluorescence intensity of dual labeled neutrophils with positive gates for the fraction of neutrophils denoted E+ and H+ set at one standard deviation above the median for representative Hc and Ps samples (n= 5 each). b) Expression of extended-intermediate (KIM127, E+) and high affinity CD18 (mAb24, H+) in mild (n=9) and moderate-severe (n=5) Ps patients and Hc (n=10) as measured by flow cytometric analysis of dual labeled neutrophils in diluted whole blood. Data is presented as the mean ± SEM with significance between Ps and Hc samples denoted by ∗=$p < 0.05$ and **=$p < 0.01$, respectively.

3.4 Damp and cytokine biomarkers of neutrophil priming and activation

Having confirmed characteristic differences in receptor expression in psoriatic vs healthy neutrophils we next explored the role of neutrophil activating cytokines (IL-8) and DAMPs

(S100A8/A9, LL-37) in neutrophil priming. S100A8/A9 levels were 96% higher in psoriatic versus healthy plasma as determined by ELISA (Figure 3.3A). A significant positive correlation between increased circulating calprotectin levels and an upshift in extended CD18 was observed for psoriatic, but not healthy neutrophils (Figure 3.3b). Similarly, Il-8 was upregulated by ~92% in psoriatic vs healthy plasma (Figure 3.3c). The correlation between IL-8 levels and extended CD18 was slightly less than between S100A8/A9 and extended CD18 (Figure 3.3d). LL37 was also elevated 94% in psoriatic plasma compared to healthy, but unlike S100A8/A9 and IL-8, LL37 levels were not significantly correlated with the extent of CD18 activation (Figure 3.3e-f). These results suggest that S100A8/A9 and IL-8 may be priming neutrophils in circulation.

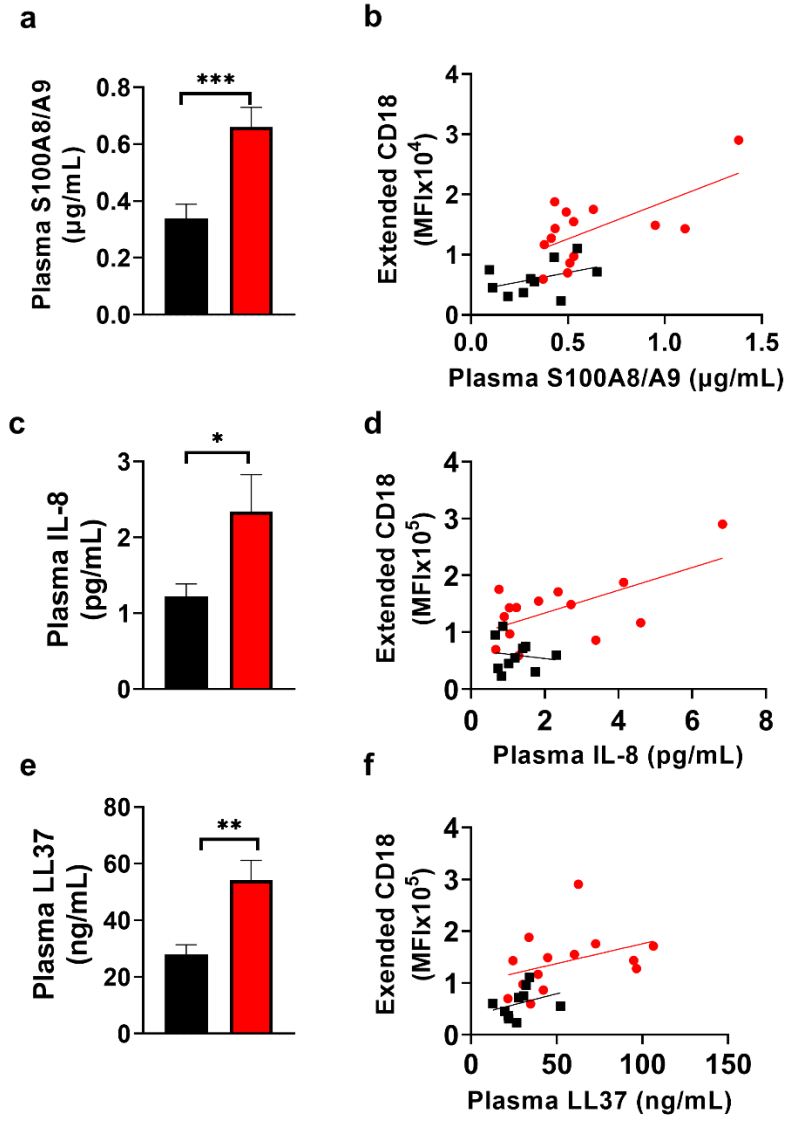

Figure 3.3: Plasma levels of DAMPs and cytokines correlate with integrin activation in psoriatic neutrophils. a) Plasma levels of S100A8/A9 in Ps (n=14) and Hc (n=10) detected by ELISA. b) Correlation plot of plasma S100A8/A9 versus extended-intermediate affinity CD18 expression detected in diluted whole blood from Ps (n=14) vs Hc (n=10), (R^2= .42, .16). c) Plasma levels of Il-8 in Ps versus Hc detected by ELISA. d) Plasma IL-8 versus extended-intermediate affinity CD18 expression detected in diluted whole blood from Ps versus Hc (R^2= .38 versus, .02), $p<0.05$. e) Plasma levels of LL37 detected by ELISA. f) Correlation of plasma LL37 versus extended-intermediate affinity CD18 expression detected in diluted whole blood. All data Ps (n=14) vs Hc (n=10), (R^2= .14 versus .10), no significance. Results for a, c, and e are presented as the mean ± SEM with significance between Ps and Hc samples denoted by $*=p < 0.05$, $**=p < 0.01$, and $***=p < 0.001$, respectively.

3.5 Psoriatic neutrophils are sensitized to TNF-α but not IL-8

A key characteristic of primed neutrophils is increased sensitivity to a subsequent stimulus. Since IL-8 is thought to play a role in the recruitment of neutrophils to psoriatic plaques we evaluated the sensitivity of psoriatic neutrophils to stimulation with IL-8. At a threshold dose of stimulation with IL-8 (0.1nM), psoriatic neutrophils exhibited significantly higher integrin activation than healthy controls, but the percent upregulation from baseline was <40% and was not significantly different between psoriatic and healthy (Figure 3.4a). In fact, over the dose range of IL-8, psoriatic neutrophils exhibited lowered sensitivity to IL-8 stimulation characterized by a right shifted dose response with a higher half maximal effective concentration (EC50).

Activated keratinocytes produced multiple inflammatory cytokines such as TNF-α, a well-known target in psoriasis (Grine et al., 2015). Since TNF-α is key to psoriasis pathogenesis and is known to stimulate neutrophil integrin activation, we tested the sensitivity of psoriatic neutrophils to TNF-α. Ps neutrophils were highly sensitive to TNF-α stimulation resulting in a left shift in the EC50 for high affinity CD18 activation (Figure 3.4b). The largest difference between psoriasis and healthy control neutrophil activationwas seen at a concentration of 2pM TNF-α. This dose induced a 94% increase in high affinity CD18 over baseline in psoriasis compared to a 52% in healthy control. Thus, our data demonstrates that psoriatic neutrophils are primed for increased sensitivity to TNF-α stimulation but not IL-8 stimulation of integrin activation.

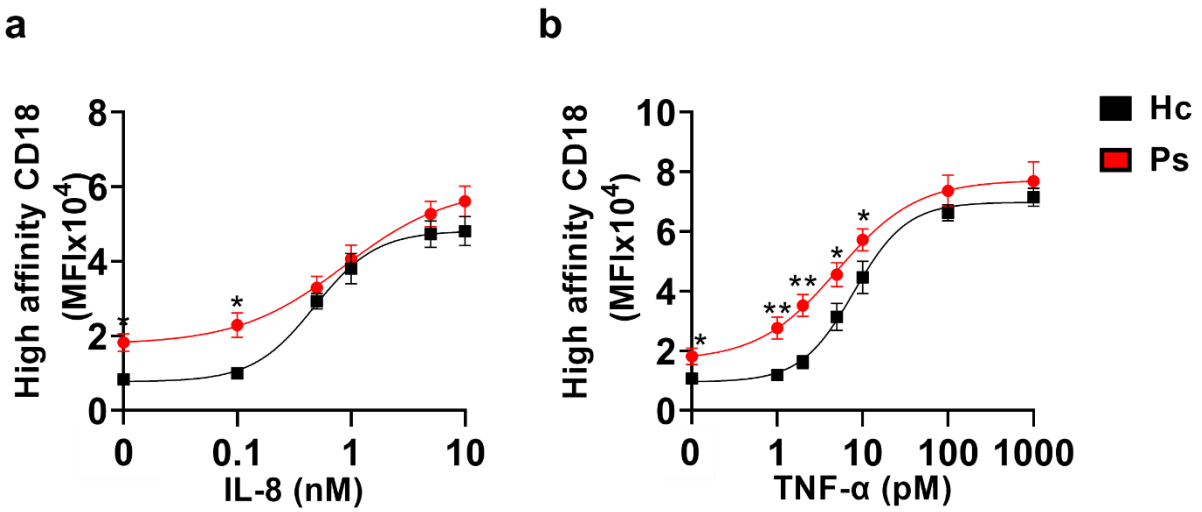

Figure 3.4: Psoriatic neutrophils are primed for increased sensitivity to TNF-α stimulation but not IL-8 stimulation. a) IL-8 stimulated expression of high affinity CD18 on neutrophils isolated from Ps (n=9) and Hc (n=5). b) TNF-α stimulated expression of high affinity CD18 on neutrophils isolated from Ps versus Hc (n=6 each). Asterisks denote statistical comparisons between Ps and Hc at the same concentrations of cytokine. Data is presented as the mean ± SEM with significance between Ps and Hc samples denoted by ∗=$p < 0.05$ and **=$p < 0.01$, respectively.

3.6 Elevated neutrophil S100A8/A9 secretion in psoriasis

Previous reports have demonstrated that TNF-α, but not IL-8, can stimulate secretion of S100A8/A9 (Tardif et al., 2015). Since psoriatic neutrophils exhibited amplified TNF-α mediated integrin activation we assessed their capacity for TNF-α induced secretion of S100A8/A9.

At baseline psoriatic neutrophils secreted significantly more S10A8/A9 than healthy controls and stimulation of psoriatic neutrophils with 2 pM TNF-α induced 128% more secretion of S100A8/A9 than healthy control neutrophils (Figure 3.5a). At 1μM TNF-α secretion in psoriatic neutrophils only slightly increased from 2pM. While 1μM TNF-α induced 61% more secretion of S100A8/A9 than 2pM in healthy neutrophils, the secretion of S100A8/A9 in healthy neutrophils at 1μM TNF-α was still significantly smaller than psoriatic neutrophils at the same

dose and was not significantly different from the secretion in psoriatic neutrophils at 2pM. This demonstrates much greater capacity of psoriatic neutrophils to secrete S100A8/A9.

To assess the role of calprotectin in priming Ps neutrophils for enhanced sensitivity to TNF-α, isolated neutrophils were treated with calprotectin and assessed for CD18 activation. Consistent with the level of CD18 activation in isolated Ps neutrophils, Hc neutrophils were primed in suspension by exogenous calprotectin (Fig 3.5b). In contrast, Ps neutrophils were refractory to CD18 activation with recombinant calprotectin above baseline but retained the high sensitivity to stimulation with TNF-α. Moreover, Hc neutrophils were synergistically activated by priming with calprotectin for enhanced stimulation by low dose TNF-α, whereas Ps neutrophils were refractory, suggesting that the latter are already primed in circulation for enhanced inflammatory response.

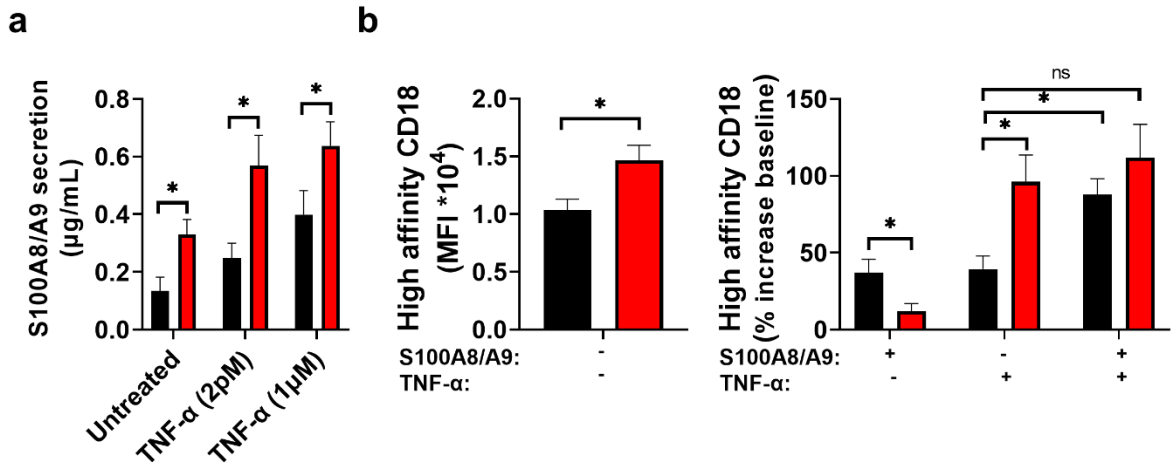

Figure 3.5: S100A8/A9 secretion is elevated in Ps neutrophils and primes TNF-α stimulated integrin activation. a) S100A8/A9 secretion in isolated neutrophil suspensions stimulated in the presence and absence of TNF-α, as noted (Ps n=5, Hc n=5). b) High affinity CD18 at baseline expressed as mean fluorescence intensity of mAb24 followed by the fractional increase from baseline MFI in isolated neutrophils incubated with recombinant S100A8/A9 (3µg/mL) in the presence and absence of TNF-α (2pM) stimulation (Ps versus Hc n=5 each). Data is presented as the mean ± SEM with significance denoted by $*=p < 0.05$.

3.7 Amplified neutrophil arrest efficiency in psoriasis

We next gauged the capacity for inflammatory recruitment and activation by digitally recording neutrophils perfused through vascular mimetic microfluidic channels coated with E-selectin and ICAM-1 (Figure 3.6a). Neutrophil rolling on E-selectin is supported by tension on L-selectin bond clusters that mechanosignal CD18 activation sufficient for transition to shear-resistant arrest via bond formation with ICAM-1 (Morikis et al., 2017).Consistent with the higher fraction of primed neutrophils expressing extended-intermediate affinity CD18, psoriatic neutrophils exhibited markedly slower average rolling velocity and arrested in half the time as healthy samples (Figure 3.6b-c). A direct correlation was observed between the fraction of neutrophils expressing high affinity CD18 and arrest efficiency, which averaged 60% for psoriatic compared with 30% for healthy subjects (Figure 3.6d).

To gauge the contribution of L-selectin mechanosignaling to the 1-fold higher arrest efficiency of psoriatic neutrophils, microfluidic channels were pretreated with Rivipansel, a glycomimetic antagonist that binds tightly to the lectin domain of E-selectin thereby inhibiting recognition of sLex on L-selectin (Morikis et al., 2017). Rivipansel abrogated arrest to background levels in healthy neutrophils, whereas arrest of psoriatic neutrophils was inhibited ~50% in a dose dependent manner (Figure 3.6e). The residual E-selectin/L-selectin independent arrest efficiency is consistent with the higher fraction of psoriatic neutrophils that express elevated levels of activated CD18. These data reveal that Ps neutrophils are primed for enhanced CD18 dependent arrest that is amplified by L-selectin mechanotransduction.

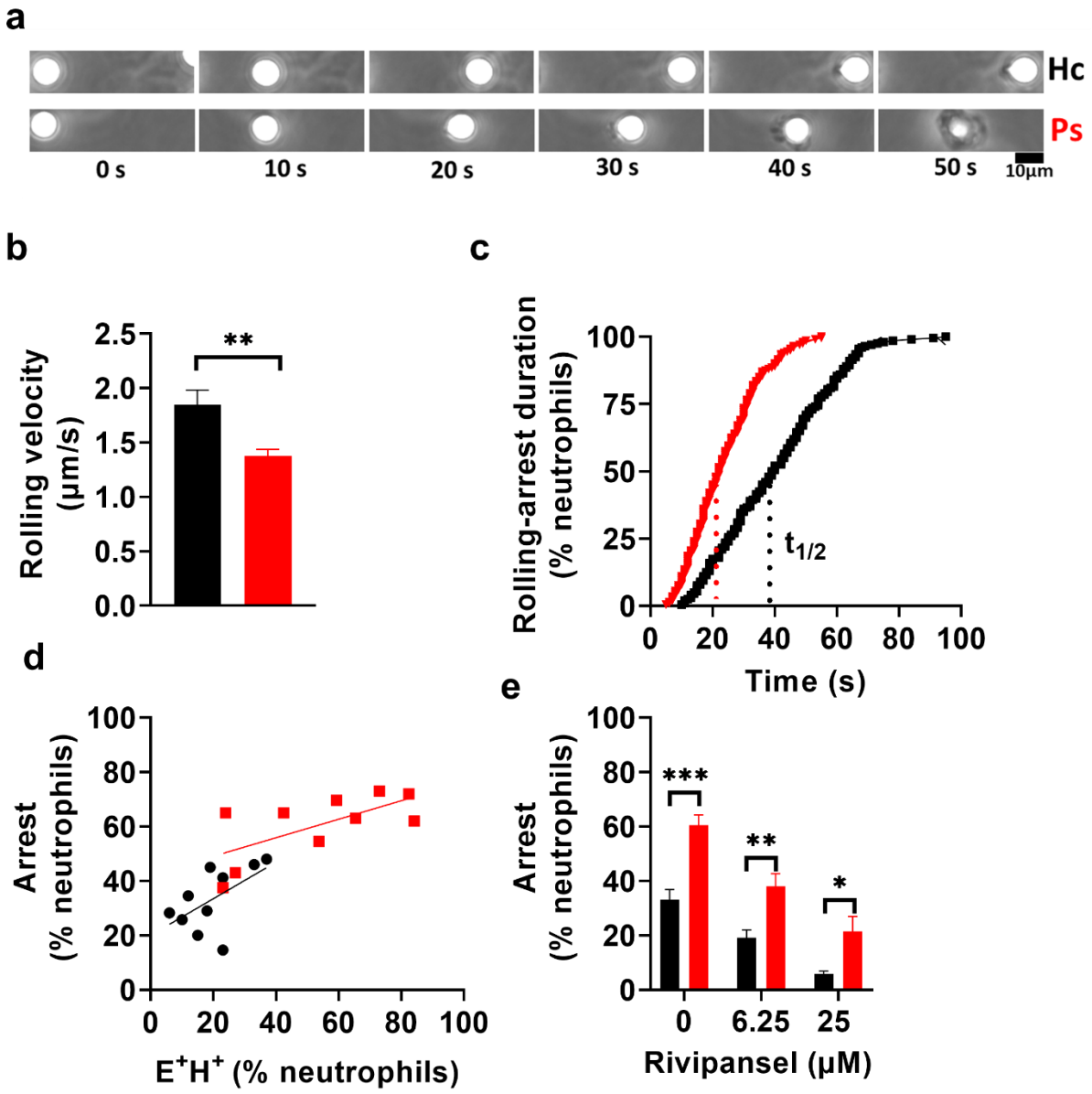

Figure 3.6: Psoriatic neutrophils recruit more efficiently than healthy neutrophils. a) Time-lapse series of brightfield images showing representative psoriatic (Ps) and healthy control (Hc) neutrophils rolling to arrest in microfluidic channels. b) Neutrophil rolling velocity (quantified from 10 cells from 2 fields of view from Ps n=10, Hc n=10 separate experiments. c) Cumulative plot of the duration from rolling to arrest. Dashed line denotes the median time for arrest Ps t1/2 ~ 22 sec and Hc t1/2 ~ 39, p<.001. d) Correlation of neutrophil fraction expressing activated CD18 (E+H+) versus arrest efficiency for Ps versus Hc (n=10 each) (R^2 = .44, .32), p<0.05. e) Neutrophil arrest efficiency at baseline and dose dependent inhibition by pretreatment with Rivipansel (Ps versus Hc n≥6 each per condition). Results for b and e are presented as the mean ± SEM with significance between Ps and Hc samples denoted by ∗=p < 0.05, **=p < 0.01, and ***=p < 0.001, respectively.

3.8 Psoriatic neutrophils are primed for mechanosignaling of Ca^{2+} flux and Ros production

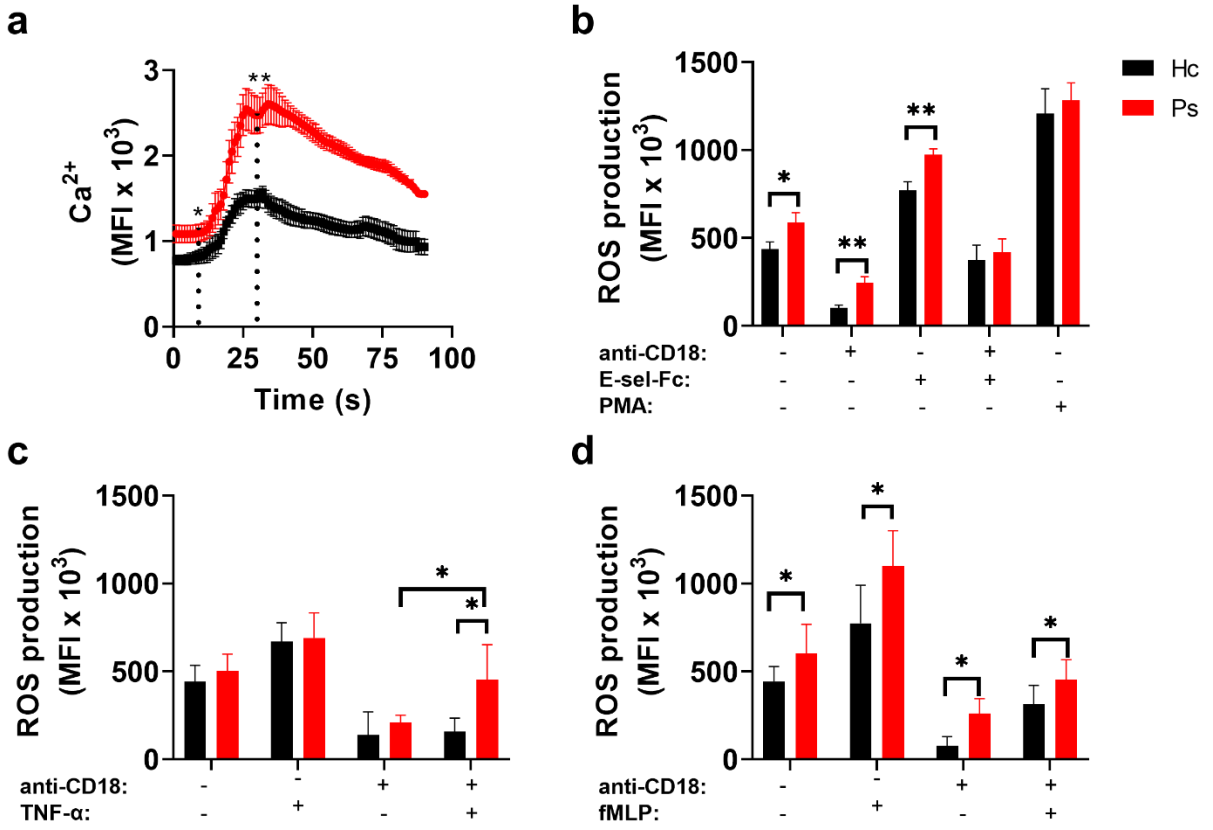

Figure 3.7: Psoriatic neutrophils are primed for mechanosignaling of Ca^{2+} flux and ROS production. a) Cytosolic Ca^{2+} flux during neutrophil rolling to arrest depicted (Ps versus Hc n=6 each). Statistical comparisons show significantly higher mean Fluo-4 fluorescence for Ps versus Hc at arrest-10 sec and peak flux- 30 sec. b) Ros production in neutrophils adhered to ICAM-1 coated wells at baseline with/out anti-CD18 (IB4) blocking and in presence of b) E-selectin-Fc (10μg/ml), c) TNF-α (500pM), d) and fMLP (1μM) stimulation (Ps n≥5 versus Hc n≥6 each per condition). Data is presented as the mean ± SEM with significance denoted by ∗=$p < 0.05$ and **=$p < 0.01$, respectively.

A faithful measure of neutrophil activation during rolling via E-selectin/L-selectin and arrest via β2-integrin/ICAM-1 bond formation is Ca^{2+} influx, which catalyzes the transition to

transendothelial migration (Schaff et al., 2010). Psoriatic neutrophils registered a higher baseline and more rapid rise to an elevated peak level of cytosolic Ca^{2+} (Fig 2e).

Exuberant ROS production following neutrophil recruitment exacerbates inflammation in skin lesions (Wroński et al., 2022). Additionally, increased calcium flux in primed neutrophils is known to amplify ROS production. This motivated assessing the contributions of selectin and integrin mechanosignaling on ROS production during neutrophil adhesion under shear stress.

At baseline, psoriatic neutrophils were 26% more adherent to ICAM-1 resulting in 30% more CD18 dependent ROS production (Fig 2g). Like arrest efficiency, E-selectin ligation of L-selectin doubled ROS production in both cohorts, however, psoriatic neutrophils retained a residual capacity to produce oxidant even when CD18/ICAM-1 mediated adhesion was blocked with antibody.

It has been previously reported that TNF-α is unable to induce strong ROS production in quiescent neutrophils in suspension. Expectedly, when blocking integrin mediated adhesion healthy neutrophils stimulated with TNF-α did not induce ROS production (Figure 2c). In contrast psoriatic neutrophils were able to produce large amounts of ROS when stimulated with TNF-α even in the absence of integrin mediated adhesion providing further evidence of priming in psoriatic neutrophils.

The bacterial tripeptide fMLP is a potent activator of ROS and is a quintessential second activator in priming studies. In the presence and absence of CD18 blocking, psoriatic neutrophil produced ~44% more ROS when stimulated with fMLP in comparison to healthy controls.

These results demonstrate that psoriatic neutrophils are primed for increase ROS production when stimulated with a variety of activators in the absence of adhesion. Adherence of

neutrophils through integrin resulted in even greater increases in ROS production and psoriatic neutrophils showed greater capacity to mechanosignal ROS production.

3.9 Discussion

In summary, psoriatic neutrophils in blood display a primed phenotype characterized by elevated β2-integrin receptor number and binding affinity that rose with calprotectin levels. Transition from rolling to arrest on E-selectin and ICAM-1 was twice as efficient for psoriatic neutrophils, which fluxed more Ca^{2+} and produced more ROS than healthy. Psoriatic neutrophils were exquisitely sensitive to ex vivo stimulation with TNF-α that elicited secretion of calprotectin and synergistically amplified CD18 activation. Given the large vascular surface area that perfuses the skin, we speculate that surveilling neutrophils rolling via E-selectin are a major contributor to elevated calprotectin in psoriatic circulation (Figure 3.8). In conclusion, measurable elevation in the capacity for neutrophil β2-integrin adhesion and effector function may provide a reliable biomarker to predict disease progression and the efficacy of biological therapy in controlling cutaneous inflammation in psoriasis.

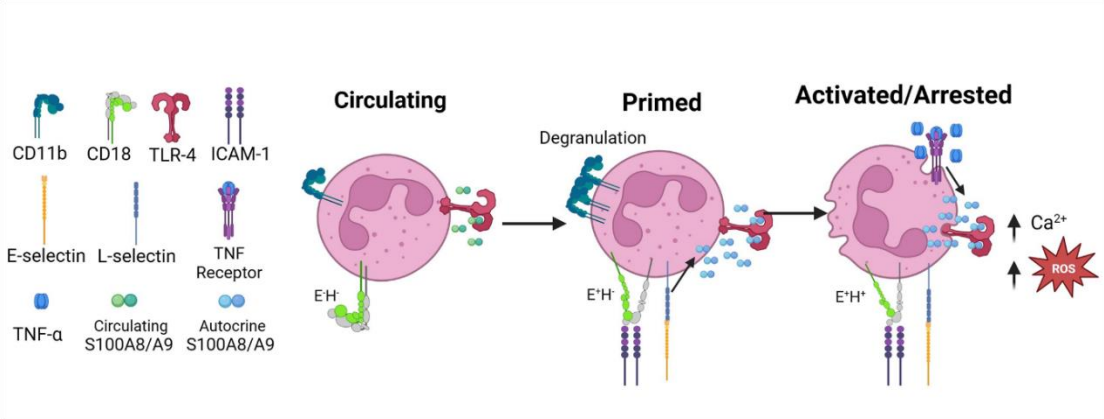

Figure 3.8: Psoriatic neutrophil priming schematic. Schematic depicting neutrophil priming through TLR4 detection of S100A8/A9 for enhanced E-selectin and β2-integrin dependent recruitment and amplified ROS production within inflamed tissue created with BioRender.com.

Chapter 4 Summary and future directions

The idea that neutrophils are merely homogenous automatous cells has shifted over time as it becomes increasingly clear that their inflammatory response is regulated by a complex interplay of synergistic signaling through a variety of adhesion receptors and pattern recognition receptors that bind endogenous and foreign ligands. Interaction with foreign materials and therapeutics as well as continued exposure to inflammatory agents during chronic inflammation can disturb the tight balance that limits neutrophil recruitment and function to avoid tissue damage. In this dissertation we examined how the iron oxide nanoparticle therapeutic Feraheme effected neutrophil recruitment and effector function. Similarly, we investigated the role that the DAMP S100A8/A9 and cytokines IL-8 and TNFa play in priming psoriatic neutrophils.

Feraheme exhibited immunosuppressive effects on human neutrophils. Feraheme inhibited IL-8 induced changes in neutrophil adhesion receptor expression, reduced neutrophil recruitment capacity, reduced ROS production, and led to rapid sequestration of calcium flux after IL-8 stimulation. Sustained elevations of calcium are vital for all the neutrophil inflammatory responses that were inhibited by Feraheme. Thus, we postulate that the rapid sequestration of calcium is the mechanism by which Feraheme inhibits neutrophil function. Feraheme is a widely used drug for IDA patients and off label uses for diagnostics and therapeutics has been the focus of considerable research. Due to the immunosuppressive effects observed in our research, examining whether Feraheme may impair immune responses to infection or sterile tissue insult in patients receiving novel Feraheme exposure regiments is advisable. Alternatively, Feraheme may have promise as an immunosuppressive therapy for chronic inflammation as has been shown in one study where topical application of Feraheme was used in a psoriatic mouse model.

In contrast to the inhibitory effects of Feraheme, we describe how circulating S100A8/A9 and IL-8 correlated with a primed neutrophil phenotype in psoriasis. Psoriatic neutrophils had increased integrin expression and activation levels as well as lower CD62L due to membrane shedding during degranulation. Psoriatic neutrophils also exhibited an exquisite sensitivity to TNFα mediated integrin activation that coincided with S100A8/A9 secretion. The increased integrin activation correlated with increased recruitment efficiency and increased mechaosignaling of calcium and ROS. Determining the relative importance of the variety of circulating DAMPs and cytokines priming neutrophils in psoriasis and other chronic inflammatory conditions could provide key targets for the development of novel anti-inflammatory treatments. The role of S100A8/A9 in psoriasis as a predictor of disease severity suggests that it may also play a primary role in disease progression. Our findings provide evidence that circulating S100A8/A9 is predictive of neutrophil integrin activation in psoriasis and indicates that neutrophils may be important contributors to the elevated circulating levels of S100A8/A9. Targeting of neutrophil release of S100A8/A9 potentially though inhibition of inflammasome activation could be a target for novel psoriatic therapeutics.

4.1: Future directions for elucidating the mechanism of Feraheme inhibition of neutrophil inflammatory responses

The rapid sequestration of calcium in neutrophils exposed to Feraheme is a novel mechanism of neutrophil inhibition by iron oxide nanoparticles and further investigation of the underlying pathways would be the next step for building on this research. The sequestration of calcium back into the ER after GPCR stimulated release of ER stores is mediated by adenylyl cyclase activation of cAMP production. Downstream cAMP-dependent protein kinase A (PKA) leads to accelerated sequestration of calcium through PKA-activated endo-membrane Ca^{2+}-ATPases. The

adenosine A2A receptor agonist CGS 21680 (CGS) induces rapid Ca^{2+} sequestration through activation of adenylyl cyclase and may serve as an important regulator of inflammation. The non-additive effects between Feraheme and CGS suggests a shared downstream mechanism for the accelerated clearance of Ca^{2+}. We propose that Feraheme may induce sequestration of calcium through activation of endo-membrane Ca^{2+}-ATPases rather than by efflux of calcium through activation of plasma membrane Ca^{2+}-pump, the proposed mechanism of action for CGS.

A logical next step would be to determine if incubation of neutrophils with Feraheme induces increases in the levels of cAMP or PKA. Additionally, experiments using pharmacological inhibitors of PKA could determine if Feraheme mediated inhibition is PKA dependent.

Contrary to our findings, others have reported pro-inflammatory effects of several iron oxide nanoparticle formulations on neutrophil function including polyacrylic acid-coated and poly(lactic-co-glycolic acid) /poly(ethylene glycol) coated particles (Couto et al., 2014). Thus, comparing the effects of Feraheme to other iron oxide nanoparticles with a variety of coatings and correlating this with neutrophil activation could elucidate the mechanism by which Feraheme results in diminished inflammation.

Chapter 4.2: Future directions for elucidating the intracellular signaling modalities that mediate neutrophil priming in psoriasis

One of the key findings of our work is that psoriatic neutrophils are primed for amplified response to TNFα mediated integrin activation and S100A8/A9 secretion. During completion of the studies in this dissertation the mechanisms controlling S100A8/A9 release from neutrophils was ill defined, but a recent study has elucidated an NLRP3 inflammasome complex mediated mechanism for secretion of S100A8/A9 from neutrophils upon ligation of E-selectin (Pruenster

et al., 2023).The authors showed that E-selectin engagement triggers two concomitant pathways: BTK dependent tyrosine phosphorylation of NLRP3 and potassium efflux via the voltage-gated potassium channel KV1.3 dependent Apoptosis-associated speck-like protein containing a CARD (ASC) oligomerization. Once activated ASC and NLRP3 form the NLRP3 inflammasome complex elicit Caspase 1 cleavage and downstream activation of pore-forming gasdermin D. Formation of Gasdermin D pores on the cell membrane enable cytosolic release of S100A8/A9 but does not result in cell death as pores are sealed by the endosomal sorting complexes required for transport III membrane repair machinery. Another study from this group shows that other NLRP3 activating agents also induce S100A8/A9 secretion through the NLRP3/gasdermin D axis (Jorch et al., 2023). TNF-α is a known NLPR-3 activator and studies have shown that psoriatic neutrophils are sensitized for NLRP3 activation by TNF-α. The results of these studies and our findings together point to a potential role of increased inflammasome activation mediated calprotectin release in amplifying neutrophil recruitment in psoriatic inflammation. Future studies to build on this work should focus on linking inflammasome activation, S100A8/A9 release, and integrin activation in psoriasis.

We expect that psoriatic neutrophils are more prone to inflammasome activation and pore formation that results in increased release of S100A8/A9 and downstream integrin activation. Studies that link inflammasome mediated pore formation with S100A8/A9 secretion and subsequent integrin activation via TLR4 signaling during rolling on E-selectin expressing skin microvasculature would greatly elucidate the mechanism for enhanced neutrophil recruitment in psoriasis.

Inhibitors of NLRP3 or potassium channels can be utilized to inhibit pore formation and elucidate the importance of S100A8/A9 release on the amplified recruitment efficiency observed

in psoriatic neutrophils. Another potential avenue for interrogating the role of S100A8/A9 in mediating neutrophil activation in chronic inflammation is using direct inhibitors of TLR4 signaling or small molecules that prevent S100A8/A9 binding to TLR4. Determining whether blocking of S100A8/A9 binding to TLR4 abrogates arrest to a greater extent in psoriasis than healthy neutrophils would point to a potential role in psoriasis pathogenesis.

Priming of neutrophils induces phenotypic changes not only in adhesion receptor expression, but in activation and complexing of intracellular signaling molecules associated with transcriptional activity. Our study reported a primed neutrophil phenotype in psoriasis that is correlated with S100A8/A9, but measurements of downstream signaling molecules that could clearly demonstrate activation of pathways through TLR4 in primed neutrophils are a key next step. The intracellular signaling downstream of S100A8/A9 is not well elucidated, but as it binds to the TLR4 receptor it likely shares some signaling pathways with LPS and other TLR4 ligands known to prime neutrophils for enhanced inflammatory response. Key targets for analysis would be differential activation of PKC, ERK1/2, JNK, and P38-MAPK which are upregulated to different extents during exposure to priming agents. Nuclear translocation of NF-κB and activation of the JAK/STAT pathway are also faithful markers of transcriptional pathways upregulated by TNFα and S100A8/A9.

Another approach for elucidating the priming phenotype associated with psoriasis would be using mRNA sequencing of transcriptional activation. Previous, studies using mRNA sequencing have illustrated differences in transcriptional activation in response to GM-CSF versus TNF-α (Wright et al., 2013). Comparing mRNA sequence of healthy neutrophils treated with priming agents prevalent in psoriasis with psoriatic neutrophils could provide reveal whether priming via S100A8/A9 and enhanced activation via TNFa is unique compared with other cytokines such as

IL-38 and IL-23 associated with psoriasis. Comparisons with atopic dermatitis, another inflammatory skin disease, could be used to discriminate disease-specific neutrophil phenotypes, as previously shown for COVID mediated ARDS (Panda et al., 2022).

Another novel aspect of this work is the demonstrated increased capacity for psoriatic neutrophils to mechanosignal calcium flux and amplified production of ROS. While priming is known to increase adhesive capacity of neutrophils there is limited research on how priming amplifies mechanosignaling and there are no current therapies designed to modulate mechanosignaling pathways while maintaining the necessary recruitment of neutrophils via selectin and integrin adhesion receptor function. Understanding the signaling pathways by which circulating DAMPs and cytokines in inflammation may mediate priming of mechanosignaling is a promising future direction and could provide a novel target to control inappropriate neutrophil recruitment to inflamed skin. Experiments should be focused on determining if psoriatic neutrophils exhibit a discernable pattern of priming starting from the complexing of adaptor proteins such as kindlin-3 at the integrin cytosolic tail. Further modulations in downstream phospholipases and kinases that promote calcium channel reorganization on the plasma membrane is of interest.

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