

Modulation of adherent bovine neutrophil responses by extracellular matrix proteins
by Jessica James Davant Borgquist

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Molecular Biology
Montana State University
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Abstract:
The neutrophil inflammatory response can be profoundly altered by contact with extracellular matrix proteins (ECMs). We characterized functional responses (intracellular calcium, actin polymerization, degranulation, adhesion, and oxidative burst) of bovine neutrophils adhered to selected ECM proteins (collagen IV, fibronectin, laminin, thrombospondin, heparan sulfate proteoglycan [HSP]) in response to interleukin-8 (IL-8) and platelet-activating factor (PAF). Neutrophil adhesion to ECMs altered responses to PAF and IL-8, although some functions were more responsive to modulation. The most susceptible function was reactive oxygen species (ROS) production. ROS production in response to PMA and TNF-a was differentially supported by various ECMs, and PAF and IL-8 "priming" had strikingly different effects, depending on the ECM present. While both PAF and IL-8 inhibited TNF-a-induced ROS production in neutrophils adhered to collagen, fibronectin and laminin, PAF strongly enhanced ROS production in ESP-adherent cells. We next explored three potential mechanisms of signal integration that could contribute to the neutrophil's ROS response modulation by their environment. Changes in fibronectin adherent bovine neutrophil, integrin surface expression, NADPH-oxidase component recruitment to focal adhesions; and tyrosine phosphorylation patterns were considered as mechanisms behind IL-8 generated downregulation in the TNF-a-induced ROS production in fibronectin adherent cells. Integrin expression levels did not change significantly in response to the various stimuli. The accumulation of oxidase component gp91phox to focal adhesions increased after stimulation with TNF-a and co-stimulation with IL-8 caused a noticeable increase in the accumulation of gp91phox with vinculin in the focal adhesions. Therefore, at this point, no correlation can be drawn between modulation in ROS production of fibronectin adherent cells and integrin surface expression levels or NADPH-oxidase recruitment to focal adhesions. Tyrosine kinases could be a potential mechanism of signal integration in ECM adherent neutrophils. Results of this investigation show varied amounts of tyrosine phosphorylation with different treatments that correspond with a mechanism of downregulating ROS production in fibronectin adherent neutrophils. Even so, this study illustrates that neutrophils can integrate multiple stimuli, resulting in complex modulation of their functional response and provides valuable data towards characterization of the mechanism behind it.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style and consistency, and is ready for submission to the College of Graduate Studies.

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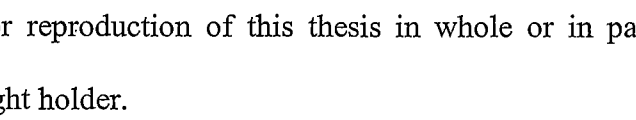
Signature 

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ABSTRACT

The neutrophil inflammatory response can be profoundly altered by contact with extracellular matrix proteins (ECMs). We characterized functional responses (intracellular calcium, actin polymerization, degranulation, adhesion, and oxidative burst) of bovine neutrophils adhered to selected ECM proteins (collagen IV, fibronectin, laminin, thrombospondin, heparan sulfate proteoglycan [HSP]) in response to interleukin-8 (IL-8) and platelet-activating factor (PAF). Neutrophil adhesion to ECMs altered responses to PAF and IL-8, although some functions were more responsive to modulation. The most susceptible function was reactive oxygen species (ROS) production. ROS production in response to PMA and TNF-a was differentially supported by various ECMs, and PAF and IL-8 "priming" had strikingly different effects, depending on the ECM present. While both PAF and IL-8 inhibited TNF-a-induced ROS production in neutrophils adhered to collagen, fibronectin and laminin, PAF strongly enhanced ROS production in HSP-adherent cells. We next explored three potential mechanisms of signal integration that could contribute to the neutrophil's ROS response modulation by their environment. Changes in fibronectin adherent bovine neutrophil, integrin surface expression, NADPH-oxidase component recruitment to focal adhesions; and tyrosine phosphorylation patterns were considered as mechanisms behind IL-8 generated downregulation in the TNF-α-induced ROS production in fibronectin adherent cells. Integrin expression levels did not change significantly in response to the various stimuli. The accumulation of oxidase component $gp91^{phox}$ to focal adhesions increased after stimulation with TNF-α and co-stimulation with IL-8 caused a noticeable increase in the accumulation of $gp91^{phox}$ with vinculin in the focal adhesions. Therefore, at this point, no correlation can be drawn between modulation in ROS production of fibronectin adherent cells and integrin surface expression levels or NADPH-oxidase recruitment to focal adhesions. Tyrosine kinases could be a potential mechanism of signal integration in ECM adherent neutrophils. Results of this investigation show varied amounts of tyrosine phosphorylation with different treatments that correspond with a mechanism of down-regulating ROS production in fibronectin adherent neutrophils. Even so, this study illustrates that neutrophils can integrate multiple stimuli, resulting in complex modulation of their functional response and provides valuable data towards characterization of the mechanism behind it.

CHAPTER 1

INTRODUCTION

Neutrophils

Neutrophils are granular white blood cells often referred to as the body's "first line of defense" against pathogenic invasion. They are essential in the host defense against bacterial and fungal pathogens (1). Neutrophils are present in all mammals; in humans between 40-70% of the circulating leukocytes are neutrophils (2), while in ruminants this figure is only 20-30% (3). Neutrophil lifespan is short, ranging between a few hours to a few days after entering the circulation. Even so, the neutrophil's contribution is far from trivial. At the first indication of infection, large numbers of these cells adhere to activated vascular endothelium, extravasate, and migrate towards the infection (4). Once at the site of infection, neutrophils phagocytose and intracellularly destroy most microbial pathogens they encounter. Through this rapid response, neutrophils contain and localize the infection, limiting its proliferation until other immune cells arrive. Without neutrophil activity, individuals are prone to recurrent and potentially overwhelming bacterial infections, even those caused by normal flora (5). Paradoxically, neutrophils are integral to the immune response, yet they are involved in the pathology of various inflammatory conditions (1,6). These cells contain devastatingly effective antimicrobial weapons with great destructive potential, which, if not tightly regulated, can damage surrounding cells and tissues. The mechanisms that control

neutrophil function are not entirely understood. Even less is known about the regulation of these functions in species other than humans. To address this discrepancy, my work focused on neutrophil regulation in cattle. Thus, understanding the functional regulation of neutrophils is important in developing therapy for inflammatory diseases aggravated by neutrophils without disabling their vital defenses.

Selected Neutrophil Functions

Adhesion and Migration

Part of what makes neutrophils so successful is their efficiency in detecting pathogens and moving to a site of microbial invasion. Neutrophils migrate out of the vasculature and into an adjacent site of inflammation hours before other leukocytes such as monocytes or lymphocytes arrive (4). Circulating neutrophils become exposed to chemoattractant gradients that originate at sites of damage or invasion in peripheral tissues (7). This results in one of the first steps of the acute inflammatory response: the localization of circulating neutrophils at sites of inflamed vascular endothelium. Receptors of the selectin family (including L-selectin, E-selectin and P-selectin) mediate loose, reversible tethering and rolling upon sialyl-Lewis^x moieties between cytokine-activated vascular endothelium and the neutrophil (7,8). This permits microenvironmental "sampling," in which the cell is rolling slow enough to interact with activating or chemotactic signals. At this point, the cells are surveying for chemical signals generated either by invading organisms or diffusing from a site of inflammation. Through receptors on their plasma membrane, neutrophils detect signals such as

chemokines [interleukin-8 (IL-8), interleukin-1 (IL-1), granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor-α (TNF-α)] (9), other inflammatory mediators [C5a, leukotriene B_4 (LTB_4), platelet activating factor (PAF)], bacterial products [lipopolysaccharide (LPS)] and other opsonized particles (10). In response to leukocyte chemoattractants, β_2 integrins on the cell surface undergo a conformational change, enabling tight adhesion between neutrophil integrins and Ig-like receptors on the vascular endothelium [e.g., intracellular adhesion molecules 1 and 2 (ICAM1 and ICAM2)] (6). This signal-dependent activation ensures that neutrophil integrins only mediate firm adhesion to endothelial cells at sites of tissue damage (9). Once firm adhesion has been established, it is rapidly followed by transmigration (diapedesis) in response to chemoattractant gradient(s) released from the site of injury. Neutrophils migrate (chemotaxis) through the extracellular matrix environment towards the source of the chemical signal, ultimately arriving at the site of inflammation. The process of neutrophil adhesion to vessel endothelium and migration into peripheral tissues is summarized in Figure 1.1.

Phagocytosis of Microbes

Once the neutrophils reach the source of the chemical gradient, they play a crucial role in host defense by destroying invading microorganisms (11). Neutrophils are known as professional phagocytes because they respond to foreign organisms by phagocytosing them (i.e., the pathogen is engulfed within a phagocytic vacuole, known as the phagosome, and destroyed by microbicidal agents and digestive enzymes). Phagocytosis

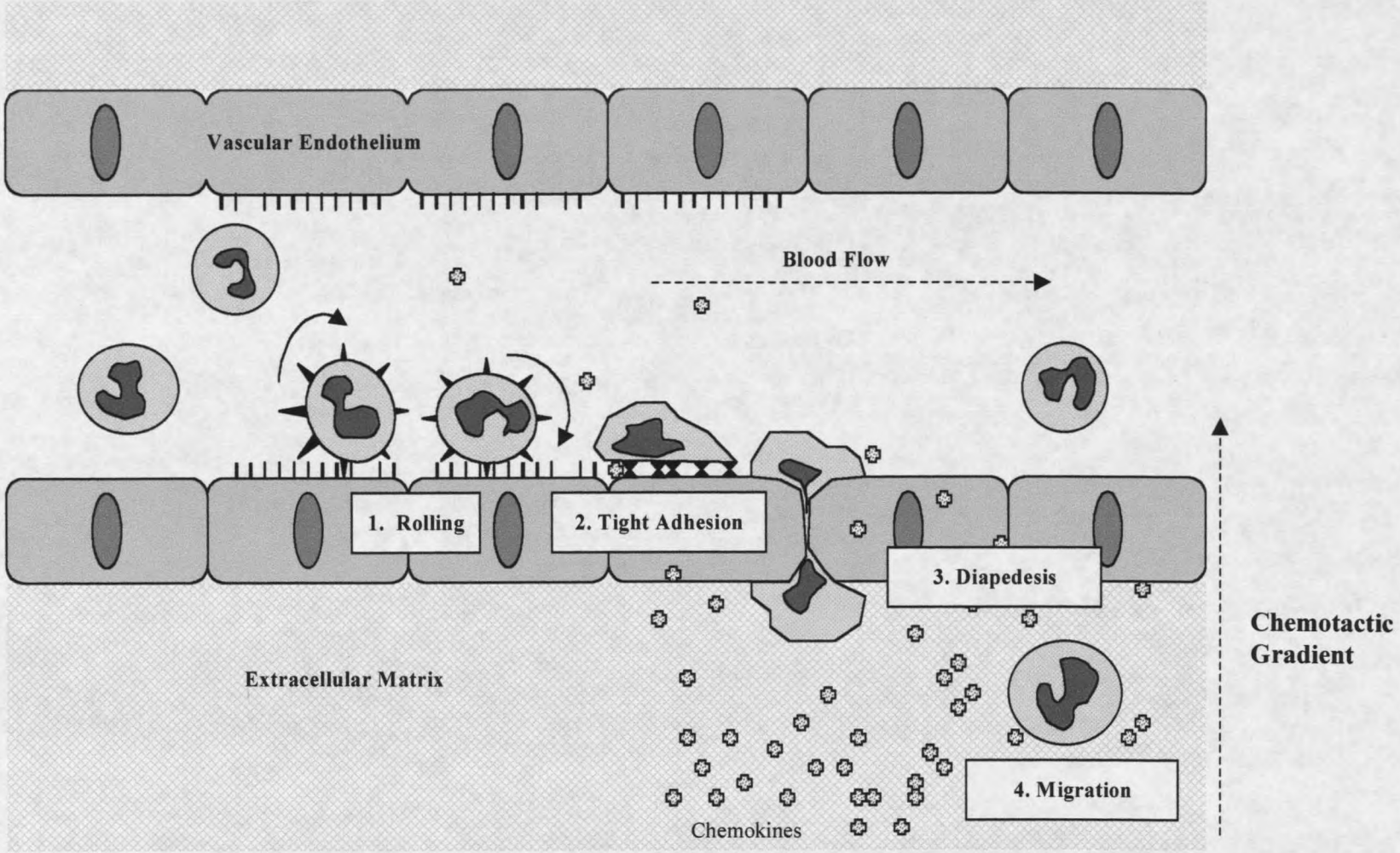

Figure 1.1: Schematic of neutrophil extravasation and migration towards a chemotactic gradient.

combines movement of cellular pseudopodia around a particle with engagement of receptors on the pseudopodia with particle ligands. Neutrophils depend on these receptors to determine which organism to ingest. Neutrophils can recognize some non-opsonized microbes by bacterial cell wall components, such as sugars or proteins (12); however, the most effective phagocytosis takes place in the presence of opsonins (13), and neutrophils express receptors to detect opsonized particles, coated with either complement proteins or antibodies. Activation of the complement system, a protein cascade that facilitates antigen clearance and amplifies inflammatory responses, generates diffusible products that coat antigen, making them easier targets for phagocytic cells (14). The most important opsonin in neutrophil phagocytosis is the complement protein C3b and its degradation product C3bi (13). A layer of these complement components on a particle is detected by the β_2 integrin, CD11b/CD18 (4), on the neutrophil surface and initiates phagocytosis. Another class of receptors mediates phagocytosis of antibody-coated particles. Immunoglobulin receptors FcγR I, II, and III mediate antibody-dependent phagocytosis by binding to the Fc region of antibodies (7) that coat the surface of previously encountered foreign particles. Engagement of particle ligands, such as complement components, antibodies or other surface molecules induces receptor-crosslinking, triggering a cascade of tyrosine kinase activity that sets phagocytosis in motion (7). As the cell receptors bind to the opsonins on the object, the cell slowly extends its pseudopodia, crawling along the object until it is entirely enclosed in a vacuole or phagosome. Once contained inside the cell, the phagosome fuses with granules containing microbicidal agents and degradative enzymes, which kill and digest the contents.

Secretion of Neutrophil Granule Contents

Heterogeneous cytoplasmic granules, the most notable structural feature of the neutrophil, are loaded with potent antimicrobial proteins. Using electron microscopy, spherical, elongated and rod- or dumbbell-shaped granules can be identified within the cytoplasm of bovine neutrophils (15). Neutrophil granule contents show vast heterogeneity; including degradative enzymes, antimicrobial peptides and membrane proteins. The release of these contents, termed degranulation, occurs in response to various stimuli, including cytokines, transmigration through an endothelial layer, and phagocytosis. Upon stimulation, granules can either fuse with the phagosome (releasing their contents within) or they can deliver their contents extracellularly by merging with plasma membrane (16). In addition to releasing their contents, the act of degranulating necessitates the fusion of the granule membrane with the cellular membrane, permitting delivery of membrane-bound proteins and new surface receptors to the cell (17). This can fundamentally change the neutrophil's ability to competently interact with the environment in response to stimuli (17).

The distribution and types of neutrophil granules varies between humans and cattle. In general the two species' granule contents display very similar biochemical properties (15). Bovine neutrophils have three main types of cytoplasmic granules: azurophil, specific and large. Both the peroxidase-positive azurophil granules and the lactoferrin-rich specific granules are found in relatively low numbers and contain proteins analogous to those found in granules of most animal species (15,18). Bovine neutrophils also contain a third class of granule that is not found in other species, the peroxidase-

negative large granules (15,18,19). Table 1.1. compares contents of human and bovine granules.

	Azurophil Granule Contents	Specific Granule Contents	Large Granule Contents
Bovine	Myeloperoxidase Acid Hydrolases β-galactosidase β-glucuronidase Neutral Acid Proteases Cathepsin G Elastase Serine proteases	Lactoferrin Vitamin B_{12}-Binding Protein	Lactoferrin Cationic Proteins Bactenecins
Human	Myeloperoxidase Acid Hydrolases Neutral Acid Proteases Cathepsins Elastase Serine proteases Defensins Bacterial Permeability-Increasing Protein Lysozyme	Lactoferrin Vitamin B_{12}-Binding Protein Lysozyme Collagenase Gelatinase Heparanase Cytochrome B_{558} Rap1, Rap2 ECM receptors Mac-1 (CD11b/CD18) TNF-receptor	

Table 1.1: Content comparison of bovine neutrophil and human neutrophil granules Table adapted from references (15,17,19,20).

Azurophil granule contents mainly contribute to the killing and digestion of phagocytosed material (17,21) by releasing hydrolytic enzymes, such as neutral serine proteases, elastase, cathepsin G and proteinase 3 into the phagosome (4,9,17). They also

contain cationic antimicrobial peptides, including defensins, bactericidal protein (BP), and bactericidal/permeability increasing protein (BPI). These agents provide activity against bacteria, yeast, tumor cells, and herpes virus (4,9,17). Myeloperoxidase (MPO), another enzyme stored within azurophil granules, is an integral part of the oxidative killing mechanism of neutrophils (4,17).

Specific granules act as reservoirs for adhesion molecules and cytokine receptors. Upon degranulation, specific granules fuse with the plasma membrane of the neutrophil. This changes the cell's ability to interact with its environment (17) and promotes the initiation of an inflammatory response (21,22). The specific granule membrane contains stores of integrins that are necessary for migration to sites of inflammation, including the CD11b chain of Mac-1, and receptors for ECM proteins such as fibronectin, vitronectin, thrombospondin, and laminin (17). In addition to adhesion molecules, cytokine receptors (such as the TNF-α receptor) are packaged in these granules as well as the majority of cytochrome b_{558} (the membrane component of the NADPH-oxidase) (16,17,23). These granules primarily regulate the surface expression of receptors, but they also contain antimicrobial proteins, such as vitamin B_{12}-binding protein and lactoferrin (10) and degradative enzymes, including collagenase, gelatinase, and lysozyme (9,17).

Bovine neutrophils contain another unique granule type, known as the large granule. These granules are more abundant and larger than azurophil or specific granules, therefore comprising most of the granule protein storage (19). Little is known about the contents of these granules and their role in neutrophil function. However, they do contain lactoferrin and appear to be excusive stores of oxygen independent anti-microbial agents, such as a group of cationic proteins that are the precursors to bactenecins (19). Unlike the

azurophil and specific granules, large granules do not contain lytic enzymes or peroxidases (19). It is believed that degranulation of the large granules parallels that of the specific granules in response to stimuli (19).

The release of these granule contents upon stimulation is a functional cornerstone of the neutrophil innate immune response. Cooperative granule fusion events coordinate the neutrophil's immune response by delivering surface receptors that fundamentally change the cell, transporting machinery necessary to produce reactive oxygen species in both the phagocytic vacuole and the extracellular environment, and releasing degradative enzymes and antimicrobial peptides necessary to kill and digest phagocytosed particles.

Oxygen-dependent killing

Oxygen-independent killing mechanisms such as cationic polypeptides and enzymes found in the neutrophil granules contribute to the antimicrobial potential of these powerful cells, but another vital contributor is the oxygen-dependent generation of reactive oxygen species. Neutrophils contain a unique oxidant-generating enzyme complex, the NADPH-oxidase. Through this enzyme, the neutrophil is capable of producing highly reactive oxygen radicals (10). The NADPH oxidase consists of a core of three plasma membrane associated enzymes and four cytosolic enzymes. The membrane-associated elements are Rap1A (a low-molecular weight GTPase) and flavocytochrome b_{558}, which a heterodimeric complex of small ($p22^{phox}$) and large ($gp91^{phox}$) subunits (phagocytic oxidase). Flavocytochrome b contains a flavin and two heme groups that serve in electron transfer (24). In resting cells, the membrane-bound flavocytochrome b is mainly found in specific granule membranes and in small amounts

on the plasma membrane (25), while the cytosolic oxidase components exist in the cytosol as a trimeric complex of p47 ^{phox}, p67 ^{phox}, and p40 ^{phox} (24). In addition to the cytosolic trimer, a cytosolic low-molecular weight GTPase (Rac) is required for activation of the NADPH-oxidase (26). A hypothetical model of the NADPH-oxidase assembly process is outlined in Figure 1.2.

Only through cell-surface receptor engagement (either by cytokines, opsonized particles or antibody-mediated receptor crosslinking (9) and subsequent kinase activity does the NADPH oxidase assemble and become active. During activation, the cytosolic components undergo a series of phosphorylation-dependent steps, resulting in association with the membrane complex. During this process, Rac becomes activated to facilitate transfer of cytosolic components to the membrane (26). Meanwhile, Rap1A becomes tightly associated with the cytochrome, functioning as a regulatory switch for superoxide formation (25,27).

NADPH oxidase assembly occurs within a few seconds of cellular activation, and electrons are rapidly transferred from the cytosol to O_2 on the external side of the membrane, reducing oxygen at the expense of NADPH. This reaction is often referred to as the "respiratory burst" because upon NADPH-oxidase activation the neutrophil increases its rate of oxygen consumption by almost 100x (10) (28). This reaction rapidly produces an accumulation of the free radical superoxide (O_2^-), either in the extracellular space or within a phagolysosome, as follows:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-.$$

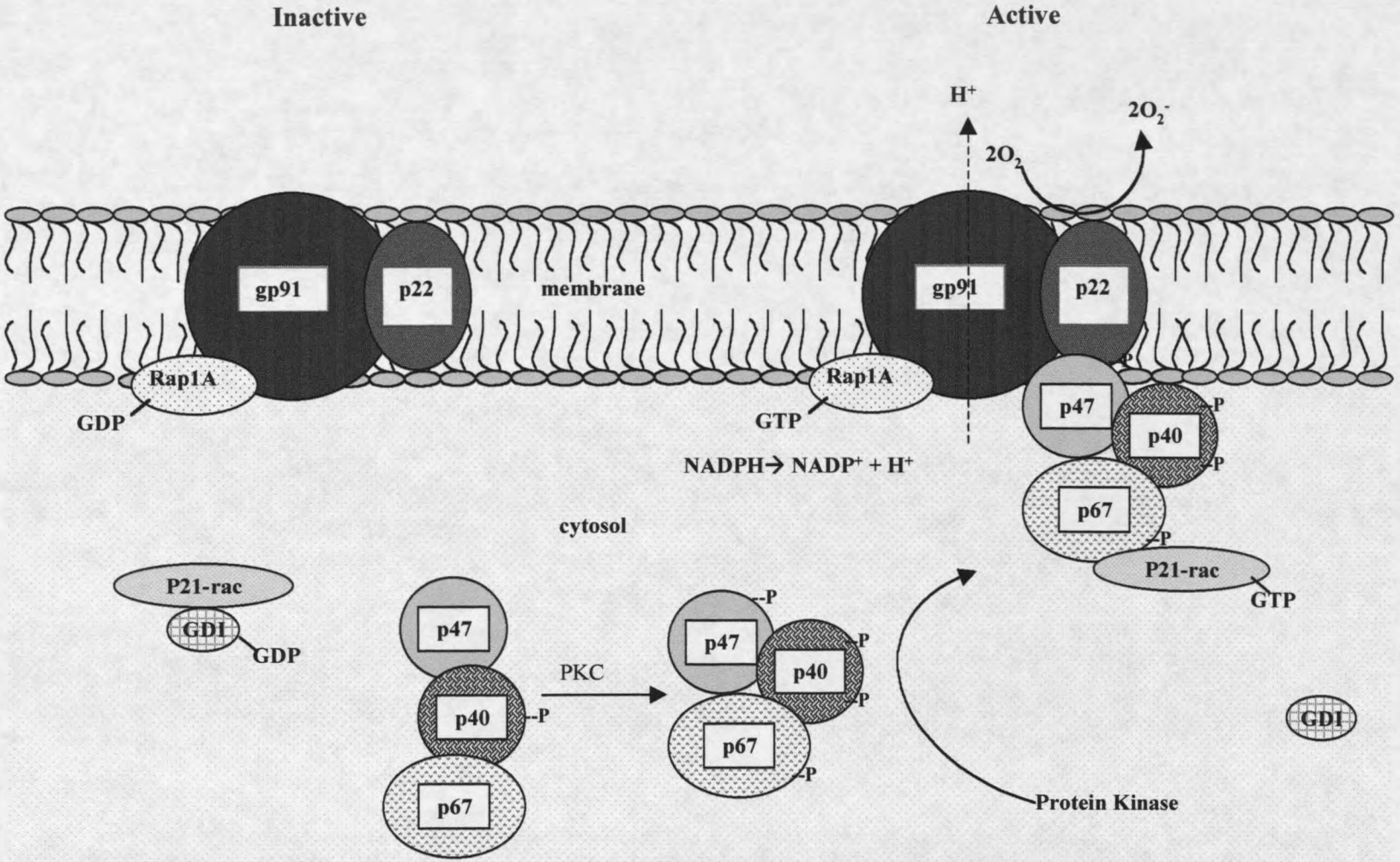

Figure 1.2: Hypothetical model of NADPH-oxidase assembly process. Figure adapted from reference 25.

Superoxide is a weak, unstable antimicrobial oxidant but it is a precursor to a cascade of highly potent oxidants. At high concentrations it rapidly dismutates into H_2O_2 as follows:

$$\cdot O_2^- + \cdot O_2^- + 2H_2^+ \rightarrow H_2O_2 + O_2.$$

At lower O_2^- concentrations, the enzyme superoxide dismutase (SOD) will catalyze the same reaction. H_2O_2 is subsequently converted into a variety of other reactive oxygen species. For example, H_2O_2 can in turn be reduced to form hydroxyl radical (OH^-). Hydrogen peroxide also serves as a substrate for myeloperoxidase (MPO) released from azurophil granules. MPO catalyzes the production of hypochlorous acid (HOCl, bleach) from H_2O_2:

$$H_2O_2 + Cl^- + H^+ \rightarrow H_2O + HOCl.$$

HOCl, one of the most lethal byproducts of NADPH-oxidase activation, can oxidize amino acids, nucleotides, and hemoproteins (7). Both H_2O_2 and HOCl inhibit glycolysis and the Kreb's cycle, in effect killing by ATP-depletion and subsequent cellular dysfunction and cytotoxicity (9). The combination of HOCl and H_2O_2 can also produce a more energetic form of oxygen, singlet oxygen ($^1O_2$), which attacks double bonds (26).

Another molecule that is important in the presence of superoxide is nitric oxide (NO). Nitric oxide synthase in vascular endothelial cells produce nitric oxide that rapidly diffuses out of the cells and combines with other free radicals. NO is not as toxic as O_2^- (29); however, if the two combine they produce peroxinitrite (^-OONO). Peroxynitrite can nitrate tyrosines in proteins, creating nitrotyrosine, and disrupting protein function (29). Peroxynitrite can also act as a priming agent for neutrophils, resulting in enhanced respiratory burst activity upon subsequent cellular stimulation and aggravating the

neutrophil response at sites of inflammation (30). The microbicidal actions of these reactive oxygen species are reviewed in Table 1.2.

ROS	Activites
O_2^-	--Weak, unstable molecule. Spontaneously reacts to form secondary metabolites (H_2O_2, HOCl, $OH^\cdot$, $^1O_2$, $ONOO^-$)
H_2O_2	--Reacts to form HOCl and $OH^\cdot$. Damages DNA, and may inactivate DNA repair enzymes. Penetrates cells and inhibits glycolysis and Kreb's cycle causing ATP-depletion and cellular dysfunction.
HOCl	-- Oxidizes bacterial proteins. Enhances some lysozymal enzyme activities. Penetrates cells and inhibits glycolysis and Kreb's cycle causing ATP-depletion and cellular dysfunction.
$OH^\cdot$	--Takes H^+ or adds double bonds to molecules, producing more free radicals. Reacts with lipids, damaging cell membranes, organelles and associated enzymes. Damages nucleic acids.
$^1O_2$	--Affinity for double bonds (ex. Polyunsaturated fatty acids)
$ONOO^-$	--Very stable molecule. Modifies protein tyrosines, forming nitrotyrosines. Mainly targets structural proteins, disrupting activities (ex. Nitrotyrosines on actin stops filament assembly). Oxidizes Iron/Sulfur centers, zinc fingers and protein thiols.
NO	-- Modest toxicity by itself. Often considered a "reactive nitrogen species" but reacts with O_2^- to form $ONOO^-$.

Table 1.2: Activity of reactive oxygen species. Table adapted from references (9,29,31).

With such potential toxicity, production of O_2^- by the NADPH oxidase must be highly regulated by many factors. As a protective measure, an inactive neutrophil segregates its oxidase components between the membrane and cytosol, keeping the

enzyme under spatial control until recruited for antimicrobial or proinflammatory purposes (26). For example, the majority of flavocytochrome b_{558} is found in the specific granule membranes, while very little is located in the plasma membrane (7,25). This type of spatial segregation allows the cell to regulate assembly of the oxidase proteins.

Defects in Neutrophil function

Defects in neutrophil function are characterized by enhanced susceptibility to infections. Leukocyte adhesion deficiency (LAD) or its analog in cattle, bovine leukocyte adhesion deficiency (BLAD) (5), is a hereditary deficiency in the β_2 integrin subunit CD18. LAD/BLAD neutrophils lack β_2 integrins and are unable to tightly adhere to the endothelium or efficiently transmigrate into adjacent tissue in response to infection (7,32). They also cannot bind C3bi, a complement opsonin, thereby limiting their ability to phagocytose foreign objects (5). Thus, LAD individuals do not benefit from neutrophils' protection, and they suffer from recurrent skin and mucosal infections. Most humans afflicted with LAD do not survive beyond the age of 10 (33).

Correct functioning of the innate immune system requires the NADPH oxidase. Rare genetic defects in the $p47^{phox}$, $p67^{phox}$, $p22^{phox}$, and $gp91^{phox}$ oxidase proteins cause chronic granulomatous disease (CGD) (7,11). As a result of these defects, CGD neutrophils are unable to generate the O_2^-. Without a functional NADPH oxidase, the body is unable to efficiently protect itself from certain types of infection. In CGD neutrophils, phagocytic activity is normal, but the cells are unable to kill some microbes,

and the unharmed contents of the phagosome can be released back into the body upon cell death (4). Like LAD patients, CGD patients suffer from recurring bacterial infections that can become fatal if untreated (7).

Granule dysfunction can also severely cripple the neutrophil's response to invading pathogens. For example, Chediak-Higashi syndrome is a rare autosomal recessive mutation in a gene involved in lysosomal trafficking (34). This disease affects all lysosomal granule-containing cells, including neutrophils (7). The factors that exist to limit the storage capacity of neutrophils and thereby, the size of intracellular granules, are absent in patients with Chediak-Higashi syndrome (17), and the giant granules indicative of this disease are reportedly derived from azurophil granules (35). Patients with this mutation have recurrent bacterial infections due to dysfunctional granule content release (34).

Balance of neutrophil activity

Neutrophils have devastating antimicrobial mechanisms (such as lactoferrin, degradive enzymes and reactive oxygen species), yet they have a reputation as indiscriminant killers unable to selectively inhibit their response (36). Indeed, the uncontrolled release of neutrophil antimicrobial weapons into the extracellular environment inadvertently damages bystander cells and tissues. Babior described neutrophil destruction in this way: "Unlike cytotoxic lymphocytes and the complement system, which destroy their targets with a drop of poison, professional phagocytes kill like Attila the Hun, deploying a battery of weapons that lay waste to both the target and

the nearby landscape with the subtlety of an artillery barrage." (36). With such capacity for damage, neutrophil recruitment and activation must be tightly regulated to provide an appropriate response proportional to the size of the infection (30).

Though some host tissue damage is inevitable during inflammation, little is known about why neutrophil response becomes unregulated and leads to inflammatory disease. Even with control mechanisms, neutrophil activity can become unmanageable resulting in persistent neutrophil extracellular release of reactive oxygen species (ROS) and degradative enzymes. If tissues are subject to activated neutrophils for extended periods of time, surrounding tissue damage will develop into chronic inflammation. Excessive neutrophil ROS levels are cytotoxic, immunosuppressive and carcinogenic to a broad range of eukaryotic cells, through oxidation of proteins, lipids, and nucleic acids (9).

There are a number of inflammatory diseases in which host tissue damage by neutrophils has been implicated. In humans, acute respiratory distress syndrome (ARDS) is manifest by an abnormally high accumulation of leukocyte sequestration in the lungs, development of granulomas and the leakage of fluids into alveoli as a result of damage to the endothelium of pulmonary capillaries (4). It is believed that the weakening of the endothelium is due, in part, to oxidant damage (31) resulting from activated neutrophils in the alveoli. Another inflammatory disease in which unregulated neutrophil activity leads to disease pathology is rheumatoid arthritis. The synovial tissue of peripheral joints becomes chronically inflamed. These regions are infiltrated with activated neutrophils, which are not normally found in healthy joints (30). Evidence shows that neutrophils in the surrounding fluids of arthritic joints have enhanced superoxide production, and these

tissues show signs of neutrophil-derived oxidant damage (31). The recurring presence of activated neutrophils has also been linked to disease in cattle. Mastitis, an inflammatory disease that affects milk productivity in cows, develops from a fervent neutrophil response to bacterial infection of the udder. Prolonged neutrophil activity maintains a chronic inflammatory state resulting in impairment of the udder (37) and decreased milk yield, ultimately causing significant economic loss (38). Pneumonic pasteurellosis, or shipping fever, in cattle is another bovine disease in which neutrophils are implicated. Shipping fever is characterized by pulmonary lesions subsequent to bacterial infection (39). In response, extensive infiltration of neutrophils and overzealous activity aggravate pulmonary damage, contributing to the pathogenesis of lung injury without effectively managing infection (37).

Ideally, a neutrophil response would be limited to result in destruction of the pathogen with the least amount of host damage. The self-limiting nature of the inflammatory response and the body's innate anti-oxidant defense systems provide intrinsic control mechanisms to avoid neutrophil-mediated host damage (9). Nevertheless, the role of neutrophil-mediated host injury in disease is significant, and the circumstances that generate detrimental neutrophil activity are not understood.

Environmental Regulation of Neutrophil Function

As described above, uncontrolled neutrophil responses such as degranulation or respiratory burst in an inappropriate context can be devastating. Fortunately in most cases, the neutrophil response is controlled and appropriate. The mechanisms by which

neutrophils achieve this measured response are diverse and not completely understood. One mechanism is to have specific receptors for bacterial molecules, such as LPS, and receptors for specific host proteins generated in response to infection, such as complement or antibodies. Another well-studied neutrophil modulatory mechanism is that of priming. Without directly activating the cell, priming agents shift the neutrophil into a "primed" or sensitized state so that subsequent stimulation results in a more rapid and enhanced functional response (for example ROS production) (30). Priming agents provide the ability to adjust the neutrophil's response to a stimulus that is commensurate with the need for protection (30). However, several studies have indicated that the response of neutrophils to agents such as bacterial compounds or cytokines is in itself highly variable. For example, respiratory burst kinetics (40), tyrosine kinase activity (41), and degranulation (42) in response to TNF-α vary between neutrophils in suspension and neutrophils adherent to physiological surfaces. IL-8 is chemotactic for bovine neutrophils *in vitro*, yet *in vivo* IL-8 did not attract neutrophils in a bovine mastitis study (43). In the same model, PAF was chemotactic *in vivo* but it is not considered to attract bovine neutrophils *in vitro* (43). Clearly, neutrophils are able to integrate multiple sources of stimulation and modulation before "deciding" on a response. It is not understood what agents interact to cause these complex responses. Obviously, neutrophils are exposed to many soluble agents that affect their behavior at any one time. Additionally, neutrophils are assessing their location via interactions with adjacent cells or extracellular matrix (ECM) proteins, and adjusting their response to be appropriate for any given environment.

Neutrophil responses such as phagocytosis or respiratory burst in an inappropriate context could be harmful. Evidence has shown that neutrophil functional responses vary depending on their environmental context, and environmental signals mediate the dynamics of the neutrophil activity, regulating the severity and location of a response.

Neutrophil and Extracellular Matrix Interactions

There is increasing interest in understanding the capacity of the ECM to modulate neutrophil function through adhesion-controlled cell responsiveness to soluble signals. The ECM is a structural matrix composed of a variety of macromolecules, including proteoglycans, multiple forms of collagen, elastins, and structural glycoproteins such as fibronectin, vitronectin, and laminin. These ECM components underlie both endothelial and epithelial cells and surround connective tissues (44), and the distribution of these proteins within the extracellular matrix is determined during development, tissue remodeling, and immune responses (44).

Neutrophils encounter ECM proteins as they leave the vasculature and at sites of tissue damage. Just as neutrophils are receiving activating signals, they are simultaneously adhering to and transversing the extracellular matrix. This provides an opportunity for transmission of signals that direct neutrophil functional responses. Indeed, the transition of neutrophils from the vasculature, adhering to and migrating across the ECM microenvironment and entering the site of inflammation induces changes in their effector functions (45).

Environmental regulation of the neutrophil inflammatory response can be profoundly altered by contact with ECM proteins. The ECM can also act as a reservoir for factors that affect cell proliferation, differentiation, activation, and migration, such as proteases inhibitors and cytokines (45). Studies have shown that neutrophils undergo a massive and prolonged respiratory burst in response to TNF-α only when they are in contact with ECM proteins via β_2 integrins CD18 (46,47). Contact with fibronectin primes human neutrophils for subsequent respiratory burst in response to PAF (48). Heparan sulfate has opposing effects on upon neutrophil function, enhancing responses to IL-8 (49) and inhibiting responses to PAF (50). Through interactions with these factors, the ECM microenvironment has a specialized role in providing signals that direct, either by promoting or diminishing neutrophil inflammatory responses, including oxidative burst, chemotaxis, and degranulation (44,45). This raises the question of whether individual ECM proteins have the capacity to modulate neutrophil responses towards stimulating agents such as TNF-α in unique ways.

Potential Signal Integration Mechanisms

There is considerable evidence that the neutrophil antimicrobial response depends on the incorporation of signaling information from both the external environment (e.g. extracellular matrix) and soluble stimulants (46,51,52). Neutrophil ROS production is particularly sensitive to modulation by combinations of ECM and cytokine signals. The integration of signals from extracellular matrix and cytokine signals could occur at any of

the numerous signaling steps within the cell. Three sites of integration that are potential mechanisms are: 1) Integrins; 2) Focal adhesions; and 3) Tyrosine kinase activity.

Integrins

The most likely candidates for cell-surface molecules that mediate the responses to ECM proteins are the integrins. Integrins are transmembrane heterodimeric proteins responsible for cell-cell and cell-matrix interactions (2). They are composed of an α-chain non-covalently linked to a β-chain. There are 17 different α-chain subunits and 8 β-chains (53). Integrins with common β-chains are classified as a family, forming the basis of integrin nomenclature. The numerous combinations of α- and β-chains result in more than 20 families of integrins (51,53). In addition, alternative splicing, and post-translational modification of the subunits provide integrins the necessary ligand diversity and specificity to coordinate countless cellular interactions (53).

Neutrophils express a variety of integrins, most prominently of the β_2-family; however, the levels of expression of neutrophil integrins are dramatically influenced by cellular exposure to proinflammatory agents (54). Integrin families are the neutrophil's "eyes," which means that engagement of a particular integrin tells the cell where it is and whether it is attached to another cell or to and ECM protein, for example. Generally, β_2 integrins bind to a broad range of surfaces including ICAMs on other cells and some ECM proteins. The dominant neutrophil integrin is the β_2 integrin, $\alpha_M\beta_2$ (a.k.a., CD11b/CD18, CR3, Mac-1), which is thought to adhere to fibronectin, fibrinogen, collagen, and to a lesser extent, laminin (55). β_2 integrins do not seem to show

discriminate binding to any one ECM (56,57). On the other hand, neutrophil β_1 integrins ($\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$) and β_3 integrins ($\alpha_v\beta_3$) are thought to bind distinct ECM components specifically (2,54,58,59). The broad specificity of β_2 integrins permits the cell to adhere to and migrate across the endothelial layer. The functional importance of β_1 and β_3 integrins is a little more controversial. It is believed that β_1 integrins mainly mediate direct interactions between the cell and the ECM (58), though $\alpha_4\beta_1$ integrins play a role in CD18 independent neutrophil migration across endothelium (60). Neutrophil migration in extravascular tissue is largely dependent upon the β_1 integrins (61). β_3 integrins have also been reported to mediate interaction with ECM (62). It is thought that β_3 integrins on neutrophils may provide a braking mechanism in migration through the matrix. It had been shown that blocking the β_3 integrins' ability to signal to the cell through phosphatidylinositol-3-kinase (PI3K) appears to stop neutrophil migration (62). Additionally, if the levels of phosphorylation of β_3 integrins are modified, their binding strength can be regulated (63).

These functions make integrins essential for neutrophil immunity. The importance of integrins in leukocyte function is demonstrated by LAD/BLAD. In LAD, defects in the β_2 chain of leukocyte integrins render the cells unable to adhere to endothelial cells lining the vessels, disabling their ability to migrate out of the vessel towards a chemotactic gradient (2,59). When these cells are unable to transmigrate out of the vessel, they fail to reach the site of infection and perform their protective functions.

Interestingly, even though integrins are crucial to neutrophil function, they have no direct intrinsic signaling capabilities. Integrins must communicate with the cell

through complex signaling cascades beginning with activation of leukocyte-specific tyrosine kinases (59). After integrin ligation, kinases from three families of kinases [Src, Syk, and focal adhesion kinase (FAK)] (54,59) associate with the cytoplasmic tail of the β-subunit, are activated and transport signals from the integrins into the cell. The mechanisms by which these kinases become activated through integrin signaling is unknown (54), but once activated, kinases trigger the localized accumulation and phosphorylation of a number of downstream substrates in several signal transduction pathways, including mitogen-activated protein kinase (MAPK), PI3K, and nuclear factor-κB (NFκB) (59). This signaling leads to many adhesion-dependent cellular events, regulated by engagement of both integrins and cytokine receptors, such as: rearrangement of actin cytoskeleton, locomotion, gene transcription, and activation of some cellular functions (54). Blocking phosphorylation with tyrosine kinase-specific inhibitors will knock out these integrin-dependent functions (54).

Neutrophils depend on integrin interactions for adhesion and subsequent migration out of high-endothelial venules (HEV), for navigation through the extracellular matrix to sites of infection. Additionally, neutrophil integrins have been shown to coordinate ligand-specific immune responses such as degranulation, phagocytosis, and oxidant production (51). In fact, the β_2-integrin adhesive interactions are believed to be a prerequisite for some functional responses, including the TNF-α-induced oxidative burst of adherent neutrophils (40,46,64). Neutrophils in suspension do not produce ROS in response to TNF-α; however, when neutrophils are adherent to certain ECM proteins, TNF-α becomes a potent direct stimulant of ROS production (40,65). ECM-adherent

neutrophils stimulated with inflammatory agents release granule constituents as well as ROS. This function has also been shown to be dependent upon β_2 integrins because the response is defective in LAD neutrophils and can be inhibited by anti-β_2 integrin antibodies (46). Like the β_2 integrins, engagement of β_3 integrins on human neutrophils has been shown to enhance the respiratory burst induced by TNF-α, as well as that induced by PMA, concanavalin-A, and fMLF (66).

Interestingly, not only can integrin ligand engagement affect cellular responses to soluble stimuli, but integrins are also capable of directing surface expression levels of other integrin classes. For example, Werr reported that simultaneously stimulating human neutrophils with fMLP and activating β_2-integrins through mAb crosslinking resulted in an up-regulation of β_1 integrins on the cell surface (67). Another study suggests that engagement of β_1 integrins on neutrophils results in a cross-talk signal leading to activation of the β_2 integrins, followed by β_2 integrin-mediated adhesion (68). This implies that one class of integrin is capable of signaling to and regulating expression of another, probably as a means of modulating adhesion and migration, but potentially also regulating other functional responses.

Focal adhesions

Anther structure that potentially mediates the integration of signals from ECM is sites of focal adhesions. Many enzymes, including NADPH-oxidase, are subject to activation and modulation via a number of signal transduction pathways. One way in which the regulation of these enzymes may be more tightly regulated is by colocalization

within the cell with elements of the relevant signal transduction pathways. This can occur by binding to scaffolding proteins (e.g., β-arrestin (69), Ste5 (70), or localization to membrane microdomains, such as lipid rafts (71) or focal adhesions (72-74). Sites of such localization provide interesting points to evaluate correlations between changes in enzyme localization or activation and functional changes. Focal adhesions are especially interesting with respect to adherent cells, as they are regions of the plasma membrane that act as an anchor point of the adhered cell (75). Integrins at sites of attachment to ECM, or other surface, mediate cellular adhesion on the outside of the cell and inside they associate with cytoskeletal proteins such as vinculin, paxillin, talin (76,77), which are anchored to bundles of actin microfilaments in the cytoskeleton. Figure 1.3 illustrates focal adhesion components. Through a series of signaling events, contraction of the actin-myosin cytoskeleton causes tight clustering of the integrins on the plasma membrane (75,77) forming the focal adhesions. These regions provide necessary scaffolding for many intracellular proteins to localize and assemble. Many of the proteins associated in focal adhesions are tyrosine kinases thought to be vital in integrin signaling pathways (75,77-79). In essence, focal adhesions are busy communication ports for intense signaling between the extracellular matrix and the cell (77). Signals pass from the matrix to cell in the process commonly referred to as outside-in signaling, in which the clustering of integrins induces a cascade of tyrosine phosphorylations and the downstream activation of many target molecules including small G-proteins (80). Focal adhesions also engage in inside-out signaling, where the cell appears to be able to induce re-organization of the extracellular matrix (75,80).

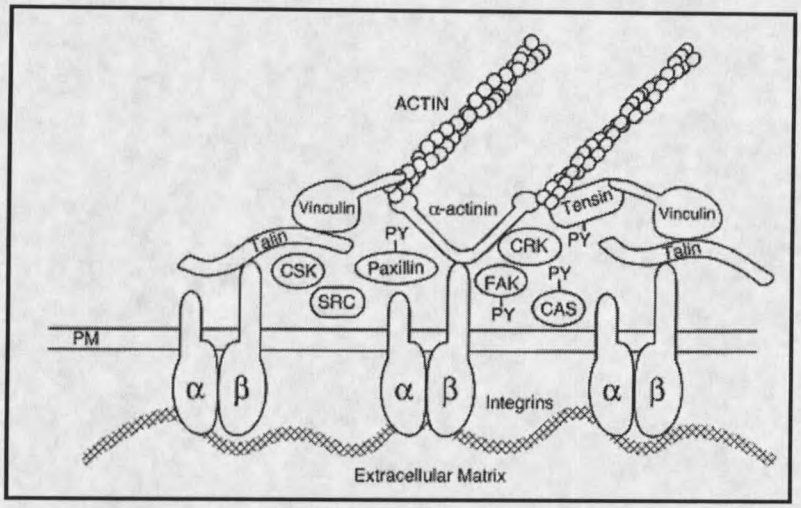

Figure 1.3. A model illustrating focal adhesion components linking sites of cell-ECM adhesion to the cytoskeleton. Figure adapted from reference 77.

It has been previously suggested that focal adhesions may provide a surface for the NADPH oxidase to assemble (81) or at least provide a site for accumulation of cytoskeletal components of the oxidase (41). This would associate formation of focal adhesions and regulation for oxidant production (51), but direct evidence has not yet been shown to support this idea. In order for the TNF-α-induced oxidative burst to occur, the neutrophil must have integrin engagement, most likely through interaction with ECM (40). Once adherent neutrophils are exposed to TNF-α, they undergo a rapid reorganization of their cytoskeleton and form focal adhesions (82) at sites of attachment to ECM. This reorganization is necessary for adhesion dependent-neutrophil respiratory burst in response to TNF-α (82) because disruption of the cytoskeleton inhibits the response (40). One report worth noting claims that adherent neutrophils only produce superoxide at points where the plasma membrane is in contact with an adherent surface (83). If this were the case, then it would certainly implicate focal adhesions, which are

major components of the adhesive interaction in the localization of NADPH oxidase. Thus these sites may provide the opportunity for necessary signaling molecules and enzymes such as the NADPH-oxidase to accumulate and assemble, directing responses to signals from ECM.

Tyrosine kinases

Another intracellular mechanism that has great potential as a site of signal integration is the activity of tyrosine kinases. Non-receptor tyrosine kinases are peripheral lipid-anchored enzymes that catalyze the ATP-dependent phosphorylation of tyrosines in a target protein. Phosphorylation induces conformational changes in the target protein (84), causing activation and initiating a signal transduction pathway within the cell. Unique sets of cellular proteins are often phosphorylated by tyrosine kinases in response to different stimuli (41). Ligand engagement or integrin clustering leads to rapid protein tyrosine phosphorylations in many cell types (77). Tyrosine phosphorylation is central in the signal transduction of neutrophil integrins (54,59,85). As described above, it is believed that integrins must associate with integrin-activated tyrosine kinases in order to communicate with the cell (77). In 1999 Lowell and Berton determined that three Src-tyrosine kinases, Hck, Lyn and Fgr, are critical in regulating integrin-mediated signaling in neutrophils (59). In the absence of these kinases, knock-out mice neutrophils have an impaired ability to spread on anti-integrin mAb-coated surfaces, to degranulate, and to produce ROS (59).

The tyrosine phosphorylation of target molecules is central to the signal transduction of neutrophil adhesion-dependent TNF-α-induced respiratory burst, as

blocking the activity of these kinases with inhibitors disables the response (41,86-88). Upon TNF-α stimulation of adherent neutrophils, several proteins undergo tyrosine phosphorylation (41); though, specific target molecules have not been identified that function to integrate signals from integrins and TNF-α receptors. However, one exciting discovery in human neutrophils is the identification of a proline-rich tyrosine kinase, pyk-2. Its phosphorylation is required for a cytokine-induced, adhesion-dependent respiratory burst (52). Through the phosphorylation of proteins such as pyk2, neutrophils may also regulate ROS production in response to environmental signals through tyrosine kinase signal transduction pathways; changes in respiratory burst may parallel variation in patterns of tyrosine phosphorylation.

Hypothesis

Based on the data outlined above, we hypothesized that adhesion to ECM proteins can modulate bovine neutrophil functional responses to soluble agonists.

Project Goals

To evaluate this hypothesis, we investigated the regulatory role the ECM environment has upon bovine neutrophil functional activity. Chapter 2 shows bovine neutrophils are capable of integrating multiple stimuli that results in complex modulation of their functional response. Several bovine functional responses (including intracellular calcium flux, actin polymerization, degranulation of azurophil and specific granules,

adhesion, and production of ROS) were characterized in response to IL-8 and PAF stimulation while the cells were simultaneously adherent to various ECM proteins. Neutrophil oxidative burst was the function most dramatically modified by the combination of adherence to ECM protein and cytokine stimulation. Chapter 3 explores potential mechanisms of this differential integration of extracellular signals. Three steps at which the integration of ECM contact and cytokine stimulation could be regulating the ROS production were evaluated, including tyrosine kinase activity, surface expression of integrins, and cytoskeletal regulation of NADPH-oxidase components within focal adhesions. Understanding neutrophil functional regulation and determining signal transduction behind it provides promise for therapy in diseases aggravated by neutrophil inflammatory damage without disabling vital neutrophil defenses.

CHAPTER 2

ADHESION TO EXTRACELLULAR MATRIX PROTEINS MODULATES BOVINE NEUTROPHIL RESPONSES TO INFLAMMATORY MEDIATORS

Introduction

Neutrophils play an essential role in the body's defense against bacterial and fungal pathogens (1,13). These phagocytic cells respond to the presence of a pathogen by migrating to its location, engulfing the pathogen, and releasing degradative enzymes and reactive oxygen species (ROS), which serve to contain and limit the infection. While the objective of this process is to remove infectious agents, foreign particles, and damaged tissue from the body, neutrophil-generated enzymes and ROS can also damage host tissues near the site of inflammation. Indeed, neutrophils have been reported to be involved in the tissue injury associated with a number of inflammatory diseases, including rheumatoid arthritis (89), ischemia-reperfusion injury (90), and adult respiratory distress syndrome (91) in humans, and pneumonic pasteurellosis (39) and mastitis in cattle (92).

Neutrophils must integrate a number of environmental signals, resulting in modulation of the type and amplitude of a given inflammatory response. For example, neutrophils in suspension typically exhibit different responses (e.g., respiratory burst kinetics) than neutrophils adherent to a physiological surface (40), and this modulation

seems to result from distinct signal transduction processes within the neutrophil (41,93). One facet of the cellular environment that is thought to contribute significantly to neutrophil responsiveness is the extracellular matrix (ECM). Neutrophils encounter ECM proteins as they exit the vasculature, particularly at sites of tissue damage, which would seem to be a logical location for regulatory cues. Indeed, it has been suggested that through interactions with cytokines and enzymes, ECM proteins play a specialized role in providing signals that coordinate all leukocyte behaviors, thereby regulating inflammation (44,45). Adhesion to ECM proteins is especially important in modulation of neutrophil function. Neutrophil functions such as chemotaxis (94) (95), degranulation (56,96), adhesion (97), and phagocytosis (98) all have been shown to be modulated by ECM proteins. Although most research into neutrophil/ECM interactions has focused on the necessity of ECM interactions to support the tumor necrosis factor-α (TNF-α)-induced oxidative burst (40), (65,99-101), it is clear that ECM proteins can have profound effects on neutrophil function in response to a number of inflammatory mediators.

Two inflammatory mediators that are implicated in regulating neutrophil function *in vivo* are interleukin-8 (IL-8) and platelet-activating factor (PAF). IL-8 is a proinflammatory cytokine produced by a wide range of cells, including monocytes, granulocytes, epithelial and endothelial cells (102). Most commonly known as a chemotactic factor for neutrophils, IL-8 has also been shown to activate, degranulate, and prime neutrophils for subsequent stimulation by other activators like TNF-α (103,104). PAF is a phospholipid produced by platelets, neutrophils, monocytes and endothelial

cells (105). Neutrophils exhibit a wide range of inflammatory responses to PAF, including changes in intracellular calcium levels, membrane potential, intracellular pH, and actin polymerization (106-108). Interestingly, IL-8 and PAF can elicit different responses in neutrophils, depending on the cellular environment. For example, in an *in vivo* model of mastitis in cattle, IL-8 does not seem to attract neutrophils, while IL-8 is strongly chemotactic for bovine neutrophils *in vitro* (43). Conversely, the same study suggested PAF was implicated in the recruitment of neutrophils *in vivo*, whereas it was not chemotactic for neutrophils *in vitro*. Currently, little is know about the mechanisms behind these differential responses; however, there is some evidence that ECM proteins can have complex modulating effects on neutrophil responses to these agents. For example, heparan sulfate has been reported to enhance neutrophil responses to IL-8 (49) but inhibit neutrophil responses to PAF (50). Clearly, further studies on the mechanisms involved in integration of environmental inputs will be essential to understand how neutrophils moderate their responses to provide the appropriate outcome to a given stimulus.

In the present study, we characterized functional responses of bovine neutrophils stimulated with IL-8 and PAF and determined if these responses were altered by interactions with a variety of relevant ECM proteins, including collagen IV, laminin, fibronectin, thrombospondin, and heparan sulfate proteoglycan (HSP). Our results indicate that interaction of neutrophils with extracellular matrix proteins can differentially modulate this cell's responses to both IL-8 and PAF. Cellular adhesive properties, F-actin polymerization, intracellular Ca^{2+} changes, and degranulation of neutrophils adherent to ECM proteins were distinct from those responses in cells adherent to plastic, and there

were also some subtle differences between individual ECMs. The neutrophil response most sensitive to modification by concurrent stimulation with IL-8 or PAF and adhesion to ECM proteins was the TNF-α-induced respiratory burst. The fact that different combinations of ECM protein and IL-8 or PAF can have totally opposite effects on this response suggests that further study is warranted into the signal transduction mechanisms involved in this message integration.

Materials and Methods

Materials

Fibronectin, thrombospondin, human recombinant interleukin-8 (IL-8), platelet-activating factor (PAF), and tumor necrosis factor-α (TNF-α) were from Calbiochem-Novabiochem (San Diego, CA). Fluo-3 AM, methylumbelliferyl phosphate (MUP) and BODIPY-phallacidin were from Molecular Probes (Eugene, OR). Lyso-PC was purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL). Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS) was from Gibco BRL (Grand Island, NY), while RPMI-1640 was purchased from BioWhittaker (Walkersville, MD). Fluoro-Nunc Module Microwell plates and Lab-tek Chambered Permanox Slide Systems were from Nalge Nunc International (Naperville, IL). All other reagents, including laminin, collagen type IV, heparin sulfate proteoglycan, phorbol myristate acetate (PMA), luminol (used at pH 9.0 in 0.2 M borate), and fatty acid free bovine serum albumin (BSA) were from Sigma Chemical Company (St. Louis, MO). IL-8 and PAF were diluted in DPBS containing 0.2% fatty acid free BSA. HEPES buffered saline

(HBS) was made with 20 mM HEPES (pH 7.4) with 125 mM NaCl, 5mM KCl, 0.62 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 6 mM glucose. Endotoxin-free water was used for all solutions to which cells were exposed.

Neutrophil Isolation

Blood from Holstein calves (6 and 18 months of age) was collected into tubes containing 5mM EDTA. Neutrophils were isolated by hypotonic lysis of red blood cells followed by separation from mononuclear cells on a two-step Histopaque gradient, as previously described (109). Neutrophils purified with this technique were 95% pure by flow cytometric analysis and Wright staining and were ≥98% viable, as determined by trypan blue exclusion.

Coating 96-well plates with ECM proteins

Ninety-six well microtiter plates were rinsed twice with DPBS and coated with 2 μg/ml extracellular matrix proteins. Briefly, fibronectin, laminin, thrombospondin, and heparin sulfate proteoglycan were diluted to 20 μg/ml in RPMI, whereas collagen IV was diluted to 20 μg/ml in sterile H_2O. The diluted proteins were then added to the wells and incubated at 37°C for 1 hour. The coated plates were rinsed three times in DPBS and finally with injectable grade water to remove any non-binding proteins or salts. Plates were wrapped in Parafilm and stored at 4°C for up to two weeks.

Measurement of neutrophil intracellular calcium flux

Changes in intracellular Ca^{2+} following treatment with either IL-8 or PAF were measured using the Ca^{2+}-sensitive probe, Fluo-3 AM (110). Isolated neutrophils were loaded with 3 μM Fluo-3 in the dark with rocking, at 24°C for 30 minutes. After washing in DPBS, $5x10^5$ cells (in 50 μl DPBS) were added to sets of ECM coated strips (one strip each of ECM-coated and uncoated wells) containing 150 μl HBS. Plates were incubated for 1 hour at 37°C. Using the Fluoroskan Ascent FL plate reader, the baseline level of fluorescence was measured for 50 seconds. Twenty μl of IL-8 ($1x10^{-8}$ M final concentration) or PAF ($1x10^{-7}$ M final concentration) were then injected into the wells, and the subsequent fluorescent change (reflecting the intracellular Ca^{2+} response) was recorded for 75 seconds (0.5-second intervals). Results are expressed as the maximum change in fluorescence upon addition of PAF or IL-8, with results pooled over 4 separate experiments.

Measurement of neutrophil filamentous actin polymerization

The relative dynamics of F-actin polymerization were visualized in adherent neutrophils using a fluorescently-labeled F-actin binding probe (phallacidin) and fluorescent microscopy. Permanox slide systems were coated with individual ECM proteins as described for the 96-well plates. After rinsing with DPBS, $2x10^6$ neutrophils in 200 μl DPBS containing 1 mM $CaCl_2$ were added to each chamber and incubated 1 hour at 37°C. The wells then received a treatment of either IL-8 ($1x10^{-8}$M final concentration) or PAF ($1x10^{-7}$M final concentration). After addition of the stimulus,

200 µl of 7.4% formaldehyde were added to fix individual wells of cells at 0 (no treatment), 30 or 120 seconds. After 15 minutes at room temperature, the chambers were rinsed twice with DPBS. The fixed cells were permeabilized and stained with $4.8x10^{-8}$ µM Bodipy-phallacidin and 6.4 µg/ml lysophosphatidylcholine of DPBS The chambers were incubated for 15 minutes at 37°C, rinsed, and examined with fluorescent microscopy. Images were recorded using a Spot digital camera (Diagnostic Instruments, Inc.). Image analysis was performed using a PC version of the NIH Image program (Scion Image). Our initial observations indicate that treatment with PAF or IL-8 caused a rapid distribution of polymerized actin at the periphery of the cells, but that there was some difference in the progression of this phenomenon, depending on the ECM being used. To provide a semi-quantitative assessment of this the following procedure was used. Three transects were made across each cell image (at 0°-180°, 90°-270°, and 150°-330°). The pixel intensity of the brightest point in the outer 10% of the cell (A) and the dimmest point in the inner 90% of the cell (B) was recorded for each transect. A ratio of these values was determined using the formula (A-B)/B. Values for the three transects for each cell were averaged. These values were determined for 5 cells in each image and averaged. Finally, the values for the same treatment (activator, ECM, and time) for three separate experiments were pooled.

Measurement of neutrophil degranulation

Degranulation of neutrophil specific and azurophil granules was determined in response to IL-8 or PAF. Neutrophils ($5 X 10^5$) in 50 µl DPBS were allowed to adhere to

ECM-coated wells containing 130 μL of HBS for 1hour at 37°C. Wells received IL-8 ($1x10^{-8}$M final concentration), PAF ($1x10^{-7}$M final concentration), or buffer control or a combination of "primary" (IL-8 or PAF) and secondary "activation" treatments of TNF-α (100ng/ml final concentration). All treatments were added as 20 μl from a 10x stock solution with the exception of TNF-α, which was added as 2 μl from a 100x solution. Cells treated with ionomycin (10 μM final concentration) were included as controls. Plates were incubated for 30 minutes at 37°C and then centrifuged at 4°C for 5 minutes with no brake at 350g. The supernatant was collected and used for the following assays. To determine specific granule degranulation, the amount of lactoferrin released was measured by single-antibody-antigen ELISA, exactly as described previously (111). Azurophil granule degranulation, as determined by myeloperoxidase activity, was initially measured using the substrates pyrogallol and H_2O_2, as previously described (112). However, no detectable release of myeloperoxidase was determined using this technique. To verify that no myeloperoxidase was present in the samples, and that assay sensitivity was not the limiting factor, a variation of luminol-enhanced chemiluminescence was also used. Since luminol chemiluminescence is dependent on myeloperoxidase (113), we incubated samples with excess endogenous oxidant (H_2O_2) and luminol to probe for myeloperoxidase activity. These assays were performed using 50 μl samples of supernatant fluids in a 96-well plate, with luminol and H_2O_2 provided by the Kierikegaard-Perry LumiGlo Chemiluminescent kit reagents, and chemiluminescent measurements were made using the Fluoroskan FL. Myeloperoxidase in the supernatant

fluids was also undetectable using this method, whereas measurable amounts were determined in supernatants from neutrophils treated with 10 μM ionomycin.

Measurement of neutrophil adhesion

The ability of neutrophils to form firm adherence with a substrate was determined using crystal violet staining. Briefly, isolated neutrophils (5 X 10^5) in 180 μl of buffer were applied to ECM-coated or uncoated wells and allowed to adhere for 1 hr at 37°C. Treatments of 10^{-8}M IL-8 or 10^{-7}M PAF 100 ng/ml PMA or 100ng/ml TNF-α were applied. Other wells were treated with combinations of IL-8 or PAF and TNF-α at the above concentrations. Plates were then incubated at 37°C for 30 minutes. The percentage of adhered cells was then determined exactly as described (114). Triplicate wells of untreated cells were fixed with formaldehyde before the first washing to estimate maximum adherence.

Measurement of neutrophil respiratory burst activity

The oxidative burst of adherent neutrophils was measured using luminol-enhanced chemiluminescence (28). ECM coated 96-well plate strips were rinsed with DPBS three times and $5x10^5$ neutrophils (in 50 μl RPMI) were added to each well. After incubation at 37°C for one hour to allow the neutrophils to adhere, each well was supplemented with 10 μl of luminol (150 μM final concentration). Two rounds of treatments were then applied to each well: first, a "priming" treatment consisting of IL-8 ($1x10^{-8}$M final concentration), or PAF ($1x10^{-7}$M final concentration) or buffer control;

second an "activation" treatment consisting of PMA (100ng/mL final), or TNF-α (100ng/ml final), or buffer control. All treatments were added as 20 μl from a 10x stock solution. The PMA was added last, and data collection began immediately after addition of the activating agent using a Fluoroscan Ascent FL microtiter plate reader (Labsystems, Helsinki, Finland) at 37°C with 30-second data intervals for 60 minutes. Results are expressed as total chemiluminescence measured over time (i.e., the area under the curve for a given time period).

Statistical analysis

One-way ANOVA, followed by Tukey's *post-hoc* testing was used to test for statistical significance among treatment groups, using Graph Pad Prism.

Results

ECM proteins block plastic-induced neutrophils activation

Because human neutrophils exhibit plastic-induced activation of ROS production when they adhere to plastic substrates (40,65,100), we determined to what extent this occurs in bovine neutrophils, to avoid any confounding of plastic and ECM protein effects. We found that bovine neutrophils also exhibit a period of ROS production as they adhere to plastic (Figure 2.1, upper panel), although this plastic-induced oxidative burst was typically over within 45 minutes of applying the cells, in contrast to a period of 60 minutes or more reported for human neutrophils (100). Coating the wells with ECM

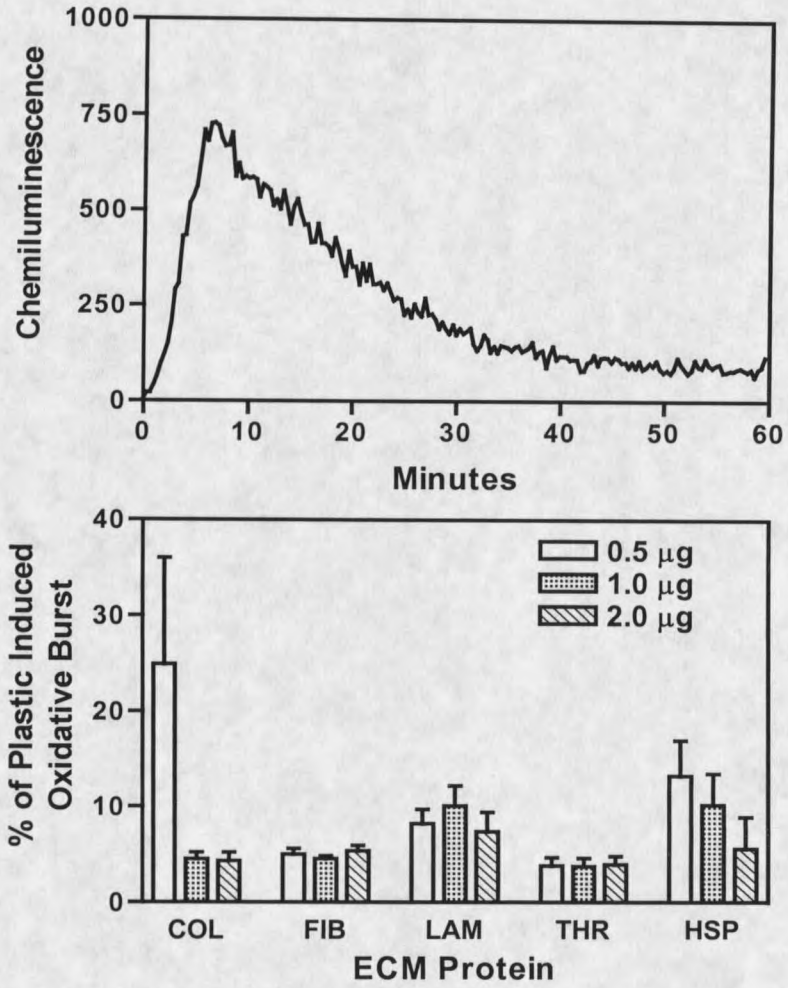

Figure 2.1: Suppression of plastic-induced activation of bovine neutrophils. Isolated neutrophils were applied to polystyrene wells containing RPMI and luminol at 37°C and chemiluminescence measurements were begun immediately and continued for 60 minutes. The upper panel shows a representative tracing of ROS production of cells on uncoated polystyrene. In the lower panel, wells were coated with the indicated amounts of ECM proteins prior to application of cells and measurements made as described above. Total ROS production (integrated chemiluminescence) of neutrophils is expressed as the percentage of that exhibited by neutrophils on uncoated polystyrene. Results are pooled from 5 separate experiments, n=6 for each bar (mean ± SEM). All values shown are significantly different from those measured in uncoated wells ($P \leq 0.01$).

proteins (at 0.5-2 μg/well) inhibited this response, although each ECM protein had a slightly different dose response (Figure 2.1, lower panel). Because 2 μg of any ECM protein reduced plastic-induced ROS production to 4-7% of that of non-coated wells, we routinely used this coating concentration of ECM coating to eliminate plastic activation of the neutrophils.

Intracellular Ca^{2+} flux of ECM-adherent neutrophils in response to PAF and IL-8

Since transient increases in intracellular Ca^{2+} concentration represents one of the earliest detectable responses of neutrophils to stimulation by IL-8 (104) or PAF (105,108), we determined whether adherence to ECM protein had differential effects on this response. Neutrophils adherent to any of the five ECM proteins tested (collagen IV, fibronectin, laminin, thrombospondin, or heparin sulfate proteoglycan) demonstrated a greater increase in intracellular Ca^{2+} after PAF treatment than did cells adherent to plastic alone (Figure 2.2). The Ca^{2+} response was very similar among all ECM groups; however, neutrophils adherent to laminin exhibited a slightly higher calcium response than that of many other ECM groups. As with PAF, IL-8 also induced a greater increase in intracellular Ca^{2+} in ECM-adherent neutrophils than in cells adhered to plastic. In fact, IL-8 induced Ca^{2+} increases in ECM-adherent cells was nearly twice that seen on plastic (Figure 2.2). Furthermore, the magnitude of the change in intracellular Ca^{2+} did not vary between any of the ECMs tested when IL-8 was used as the stimulus.

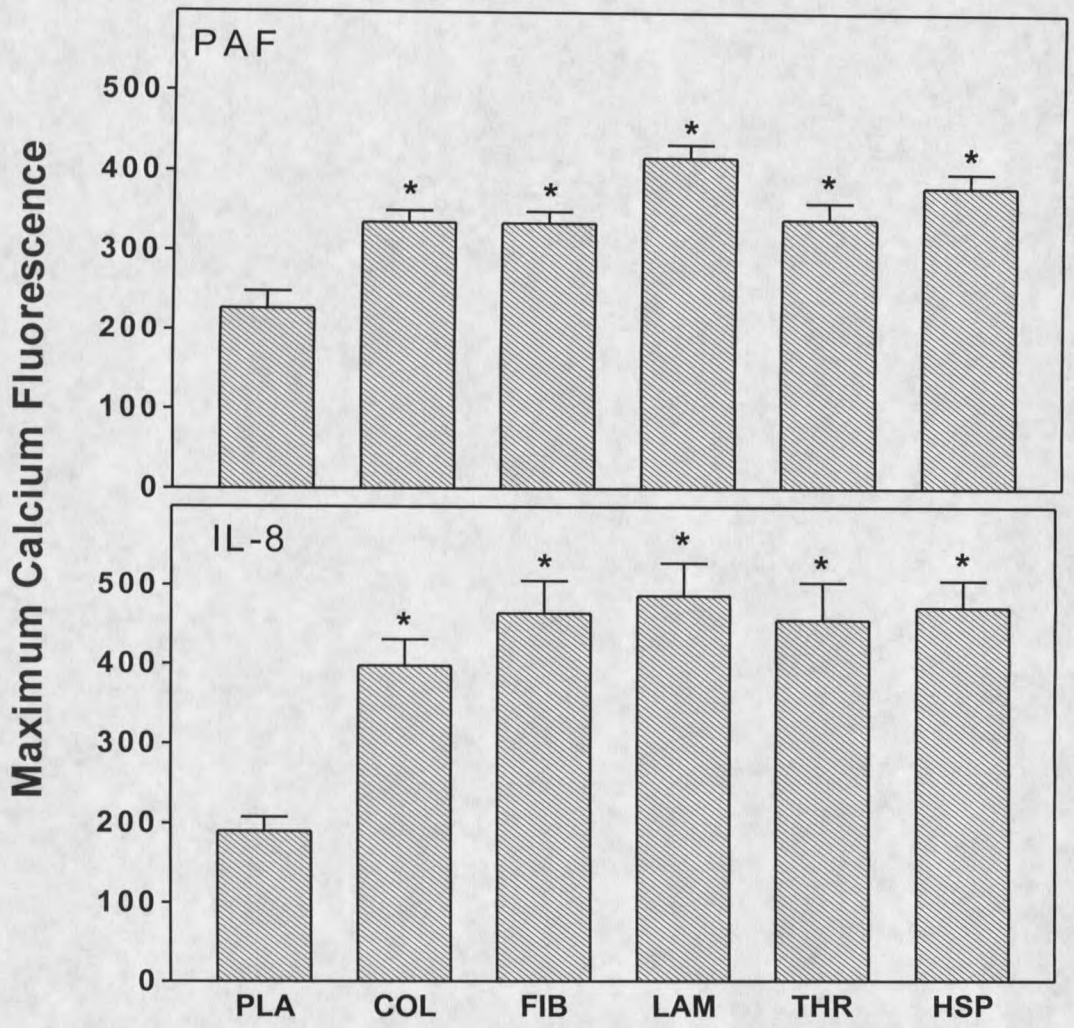

Figure 2.2: Intracellular calcium changes in ECM-adherent neutrophils. Isolated neutrophils were loaded with Fluo-3, adhered to the indicated ECM protein, or plastic, for 45-60 minutes at 37 °C, and stimulated with 10^{-7} M PAF (upper panel) or 10^{-8} M IL-8 (lower panel). Fluorescence was measured at 0.5-second intervals for 75 seconds, and the maximum fluorescence was determined as the peak fluorescence minus the pre-stimulus baseline fluorescence. Substrates are plastic (PLA), collagen type IV (COL), fibronectin (FIB), laminin (LAM), thrombospondin (THR), or heparan sulfate proteoglycan (HSP). The data are expressed as mean ± SEM of 4 pooled experiments, with 5 replicates on each ECM within each experiment. * Indicates a statistically significant difference compared to cells adhered to plastic ($P \leq 0.05$).

Neutrophil actin polymerization in ECM-adherent neutrophils treated with PAF and IL-8

Actin polymerization has been studied primarily with neutrophils in suspension (107,108,115,116), although one study demonstrated that IL-8 caused redistribution of F-actin in adherent neutrophils (117). Neutrophils adherent to uncoated plastic exhibited distinct spreading with visible lamellipodia, while polymerized F-actin appeared concentrated in punctate spots randomly scattered over the neutrophil (Figure 2.3). With stimulation by either IL-8 or PAF, there was little change in F-actin distribution on neutrophils adherent to plastic, except that there was possibly some tendency of F-actin to relocate to lamellipodia in IL-8-treated cells. F-actin distribution was much different in ECM-adherent cells. Untreated, ECM-adherent neutrophils were generally rounded with uniform, low intensity F-actin staining. Upon stimulation with either IL-8 or PAF, F-actin redistributed to the periphery of the cell, forming an intensely stained annulus or "doughnut" appearance in ECM-adherent neutrophils (Figure 2.3). This staining pattern was most visible at 30 seconds and began to disperse within the cell after two minutes, with the exception of an occasional intensely stained lamellipodium. Previous studies of ECM adhesion and actin polymerization indicated that the act of adhering to ECM proteins such as fibronectin or laminin caused initial actin depolymerization, followed by gradual repolymerization (118). Although these events may have occurred while cells were adhering to the wells, there were no detectable differences in baseline F-actin levels between neutrophils adherent to the various ECM proteins after this one-hour pre-incubation period.

Actin polymerization patterns varied slightly in appearance on the different ECMs depending on the stimuli. In an attempt to quantify these differences, we analyzed the

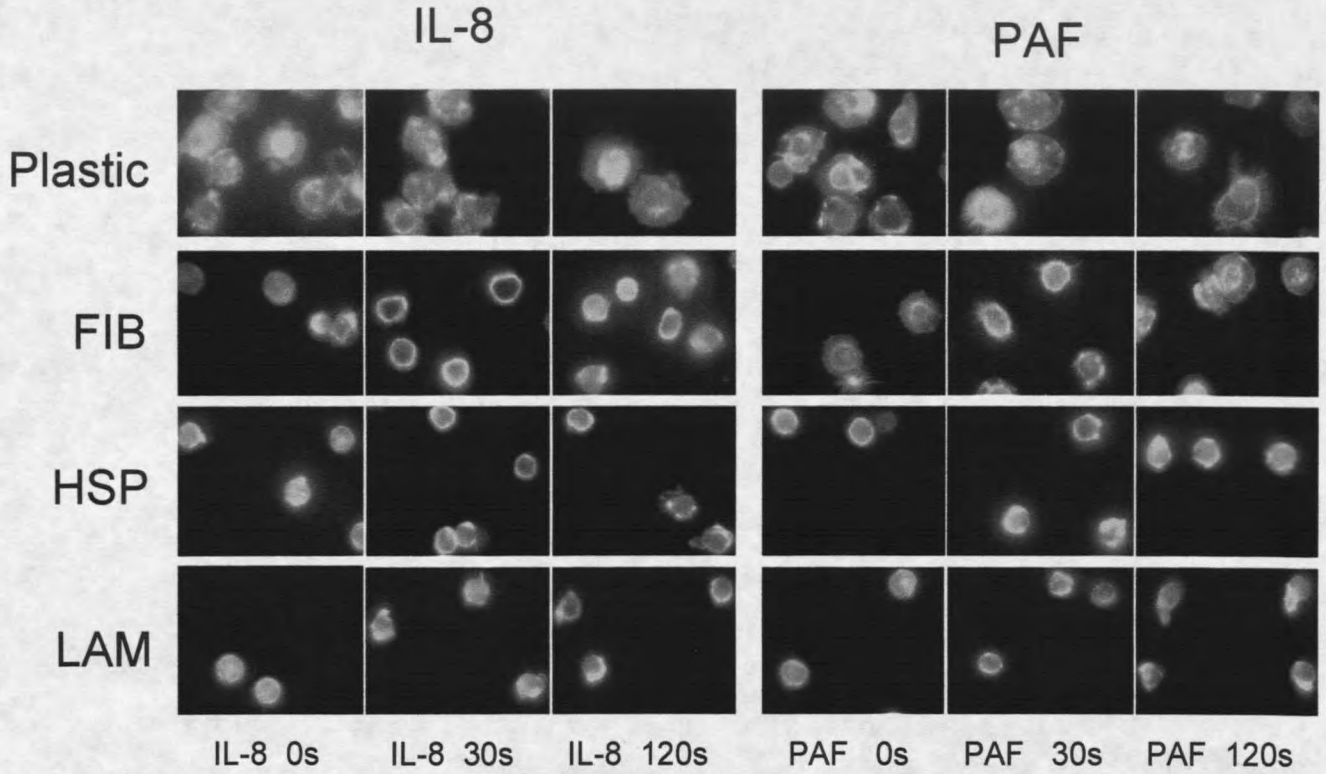

Figure 2.3: Actin polymerization in adherent bovine neutrophil stimulated with IL-8 ($1x10^{-8}$M) and PAF ($1x10^{-7}$M). Adhered neutrophils were fixed and stained with Bodipy-phallacidin, an intracellular stain for actin. Polymerization was recorded at 30s and 120s post-treatment. Representative results on plastic and three ECM proteins are sho9wn. Neutrophils adhered to thrombospondin and collagen are not shown; their F-actin polymerization was similar to the trends seen in fibronectin adherent cells. Data is representative of 3 (ranging from 3-6) experiments, n=3.

intensity of actin staining in the periphery of the cells, as compared to that in the center of the cells (Figure 2.4). This analysis confirmed our visual observations that peripheral F-actin distribution was high at the 30-second measurement, and declined to near baseline values at the two-minute measurement in most ECM adherent neutrophils (but not in neutrophils adherent to plastic). There were, however, some interesting exceptions. For example, neutrophils adherent to laminin exhibited a weak PAF-induced F-actin response at 30 seconds; however, F-actin peripheral intensity continued to increase at two minutes post-stimulation (Figure 2.4). Neutrophils adherent to HSP showed the strongest F-actin response, and this response was also sustained at the two-minute measurements. When IL-8 was used as the stimulus, a similar pattern of F-actin polymerization was observed in cells adherent to the various ECM tested, except that in this case, cells adherent to collagen had the most sustained peripheral actin staining (Figure 2.4).

Neutrophil degranulation induced by the combination of ECM adhesion and stimulation with IL-8 or PAF

PAF (106-108), IL-8 (111,119), and ECM adherence (95) have all been shown to influence neutrophil degranulation. Therefore we established whether bovine neutrophils differentially degranulate when adhered to ECM proteins. To trigger degranulation, adherent neutrophils were exposed to either PAF or IL-8. The release of two neutrophil granules, azurophil and specific, were estimated by determining the release of myeloperoxidase and lactoferrin, respectively.

PAF and IL-8 caused bovine neutrophils adherent to all the ECM proteins, but not plastic-adhered neutrophils, to release small amounts of lactoferrin, with the exception of

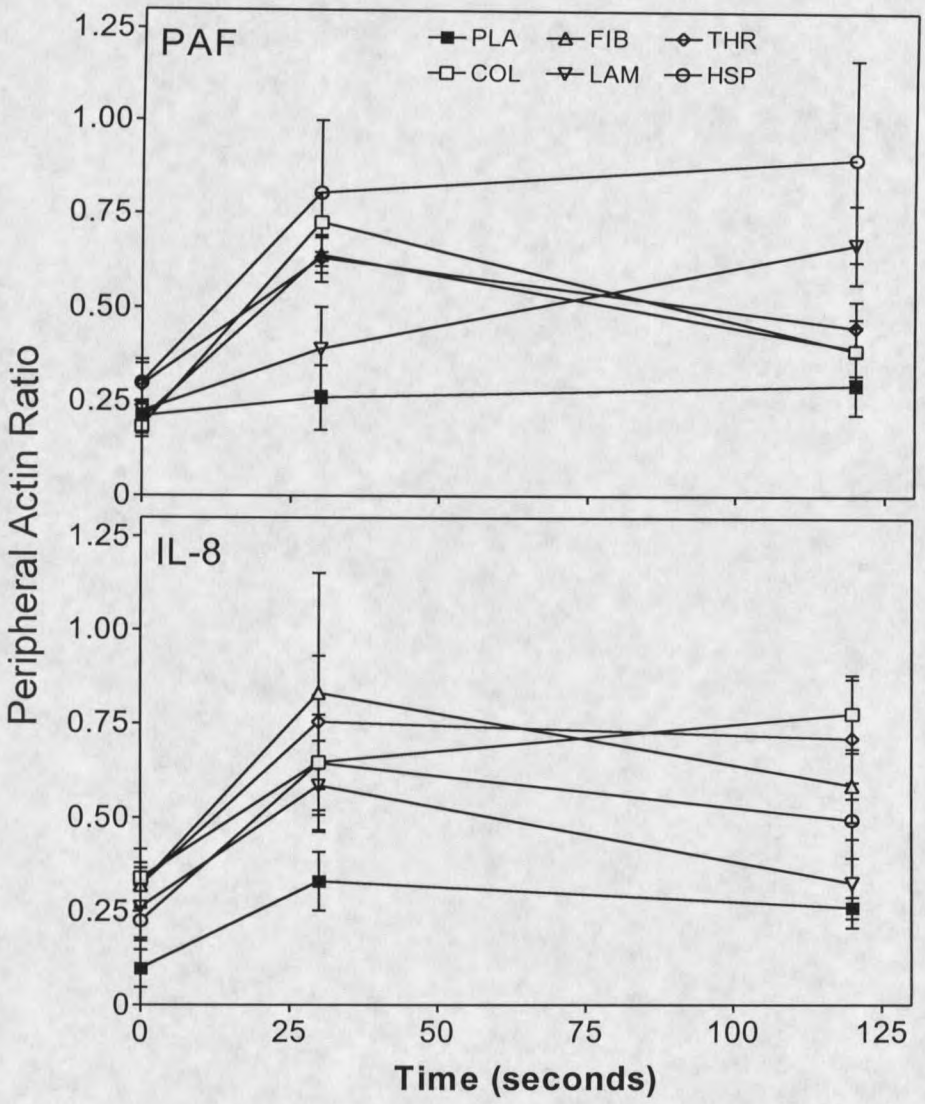

Figure 2.4: Analysis of actin polymerization in adherent neutrophils. Images of adherent neutrophils stained for intracellular actin were analyzed as described in Materials and Methods. Values represent a ratio of the increase in pixel intensity of the outer 10% of the neutrophil, as compared to the inner 90% of the same cell. Cells were adherent to the indicated ECM protein, and stained at the indicated times after 10^{-7}M PAF stimulation (A) or B, 10^{-8}M IL-8 stimulation (B) (see Fig. 2.3). The data are pooled from three separate experiments, with 15 data points per ECM protein, per experiment, expressed as the mean ± SEM.

IL-8 stimulated, fibronectin adherent neutrophils (Figure 2.5). However, based on the level of lactoferrin measured in wells of untreated, ECM-adherent neutrophils, it appears that simply the interaction of the neutrophils with the ECM (or plastic) during the adhesion period prior to stimulation caused some degranulation of specific granules. In fact, additional specific granule degranulation caused by IL-8 or PAF stimulation was usually only 20-50% of that induced by adhesion alone. Furthermore, the PAF and IL-8 induced lactoferrin release by adherent neutrophils was also small (10-35%) compared to that induced by stimulation with the calcium ionophore ionomycin (data not shown). Therefore, while adherence to these ECM proteins is permissive to specific granule release induced by PAF or IL-8, these combinations do not stimulate this response to the degree commonly seen in other experimental systems. No detectable myeloperoxidase was measured in any of our experiments using either spectrophotometric or luminescent detection. Thus, it is likely that none of the treatments used resulted in azurophil degranulation (data not shown).

Adhesive interactions of neutrophils with ECM proteins

Although most ECM proteins promote adhesion of neutrophils, there are also reports of differential adhesion of human neutrophils with different ECM proteins, as well as differential promotion of adherence to certain ECMs upon stimulation (97,120). We determined whether this was the case with bovine neutrophils, and if treatment with IL-8 or PAF affected these interactions. To determine if stimulation brought about preferential adhesion on ECM or plastic, the percentage of cells that exhibited firm adherence was established in response to treatment with PAF and IL-8, as well as to a

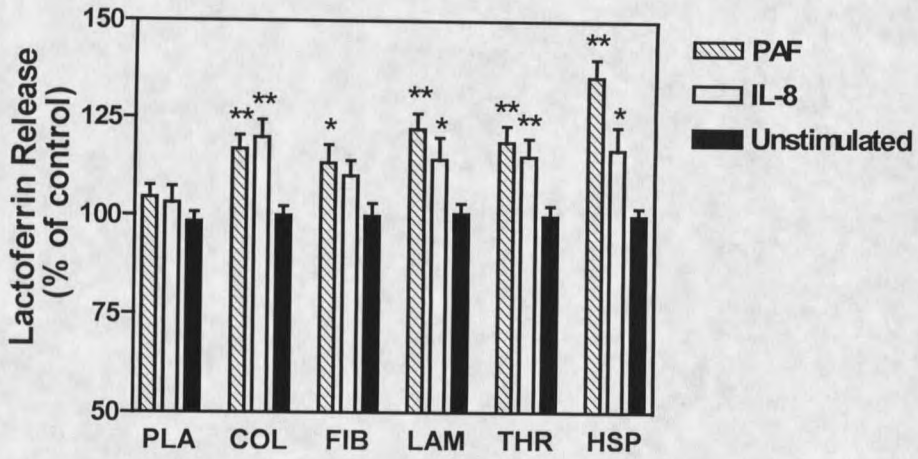

Figure 2.5: Release of lactoferrin by adherent neutrophils. Neutrophils were allowed to adhere to ECM-coated wells for 1 hr at 37°C, then stimulated for 30 minutes at 37 °C with 10^{-7}M PAF, 10^{-8}M IL-8, or buffer control. Plates were centrifuged, and supernatant lactoferrin was measured by ELISA. Results are expressed as the % of lactoferrin released by PAF or IL-8 stimulated adherent bovine neutrophils compared to unstimulated cells in the same experiment. Results are pooled from 5 independent experiments (mean±SEM), n=3 within each experiment. Statistically significant differences from unstimulated cells are indicated by * ($P \leq 0.05$) and ** ($P \leq 0.01$).

combination of each of these agents with TNF-α. For comparison purposes, neutrophils were treated with TNF-α, which has been shown to increase neutrophil adhesion to some substrates (65,96). Prior to any treatment, 60-70% of isolated neutrophils adhered to plastic, collagen, fibronectin and thrombospondin, and treatment with PAF or IL-8 induced little additional adhesion to these matrices (Figure 2.6). In contrast, only small numbers of untreated neutrophils (10-15%) adhered to laminin and HSP, and this was not enhanced much by PAF or IL-8 treatment (Figure 2.6). While TNF-α treatment of neutrophils did not enhance adhesion to fibronectin, collagen, or thrombospondin, it did induce a dramatic increase in neutrophil adhesion to laminin and HSP (Figure 2.6). When either PAF or IL-8 was added in combination with TNF-α, adhesion was not affected on

plastic, collagen, fibronectin or thrombospondin, compared to TNF-α alone. Interestingly, a combination of PAF or IL-8 with TNF-α caused decreased neutrophil adhesion to HSP, as compared to TNF-α treatment alone. PAF also decreased the neutrophil adhesion seen with TNF-α treated cells on laminin (Figure 2.6).

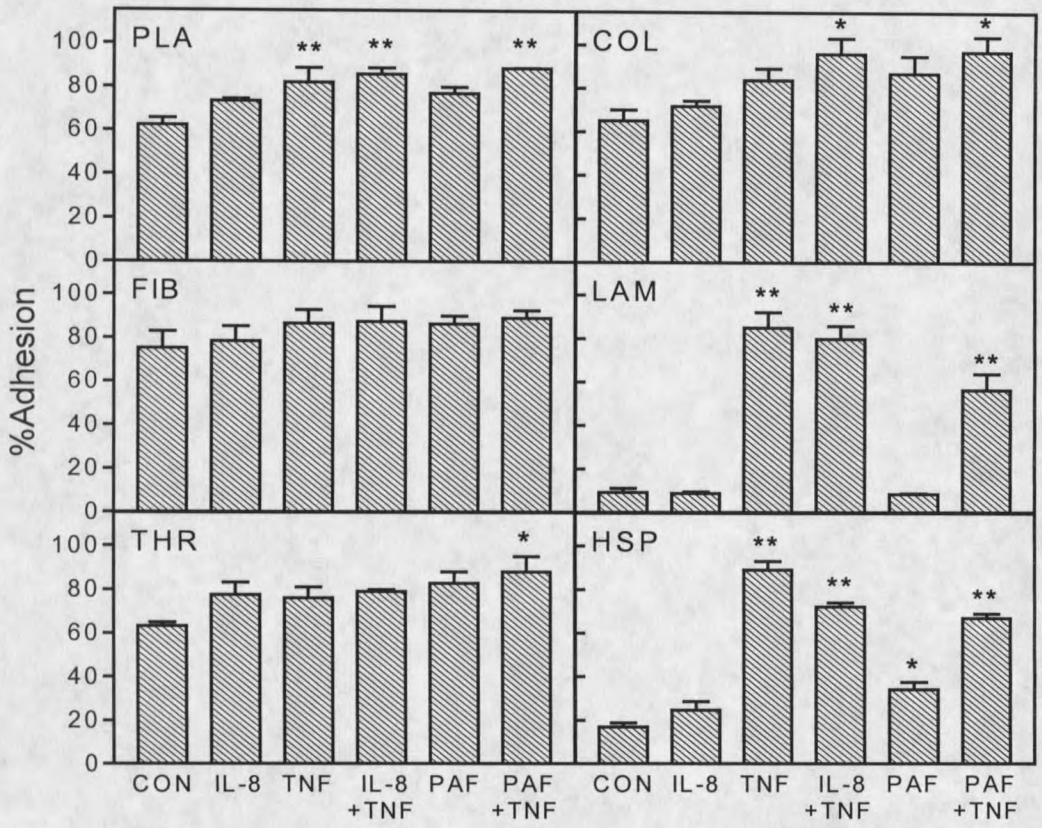

Figure 2.6. Adhesion of stimulated neutrophils to ECM proteins. Neutrophils were incubated in ECM-coated wells for 60 minutes at 37 °C, and then treated with 10^{-7}M PAF, 10^{-8}M IL-8, 100ng/ml TNF-α or a combination of PAF or IL-8 with TNF-α, as indicated. After 30 minutes at 37 °C, the wells were washed, fixed, and adherent cells were stained with crystal violet. The solubilized, stained cells were quantified using absorbance at 550 nm, and compared to wells where cells were fixed prior to washing (100% adherence). Results are expressed as the percent of cells adhered to each substrate (mean ± SEM). The data are representative of three independent experiments, n=3 for each ECM. *($P \leq 0.05$) or ** ($P \leq 0.01$) indicate a statistically significant difference compared to untreated cells on the same ECM.

ROS production in adherent neutrophils

ROS production in neutrophils is highly susceptible to modulation by factors such as ECM (65) and inflammatory agents like IL-8 (104,121,122) and PAF (106,123,124). Using luminol-enhanced chemiluminescence, we have found that ECMs and IL-8/PAF have complex, differential effects on the oxidative burst of adherent neutrophils. Because IL-8 and PAF are both considered priming agents for ROS production in neutrophils, we first examined whether this was the case in adherent bovine neutrophils. The stimulant used most commonly in human neutrophil priming studies is the chemoattractant peptide fMLF, although PMA is sometimes used (30,125,126). Since formyl peptides do not initiate responses in bovine neutrophils (127), we initially tested PMA. We observed a large discrepancy between various ECMs in their ability to support PMA-induced ROS production. While all ECM did support ROS production, collagen and thrombospondin were the most effective (Figure 2.7). The ability of IL-8 or PAF to prime ROS production in response to PMA was not substantial (data not shown). Although there were no differences in ROS production in primed and unprimed cells over the 1 hour measurement period, there did seem to be a slightly higher initial rate of ROS production in primed cells during the first 1-5 minutes after PMA stimulation.

Adherent human neutrophils also produce a strong oxidative burst in response to TNF-α (40,65). We confirmed this response in bovine neutrophils, and further show that ECM proteins had a differential ability to support TNF-α-induced ROS production (Figure 2.7). Interestingly, the pattern of ROS production induced by TNF-α was very different than that seen when PMA is used. For example laminin supported one of the strongest TNF-α -induced responses, but one of the lowest PMA-induced responses.

Additionally, cells adherent to either plastic or collagen exhibited a minimal TNF-α -induced ROS production, whereas these substrates supported maximal responses when activated by PMA.

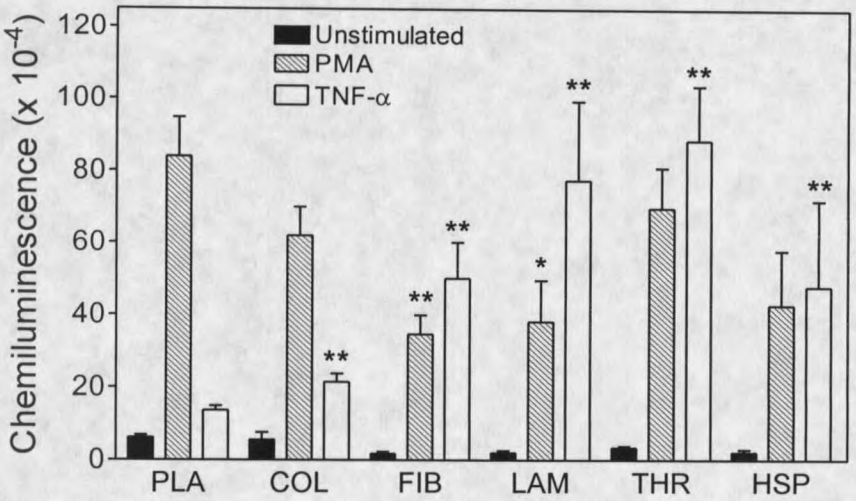

Figure 2.7: ROS production in adherent neutrophils. Isolated neutrophils were allowed to adhere to ECM coated wells for 60 minutes at 37 °C and then stimulated with 100ng/ml PMA, 100ng/ml TNF-α, or buffer control. Luminol-enhanced chemiluminescence was measured for 60 minutes, and values shown represent the integrated total chemiluminescence of the 60-minute measurement period. The data are means ± SEM of pooled experiments ranging from 6-20 experiments with each ECM, and n=3 replicates for each ECM in each experiment. *($P \leq 0.05$) or ** ($P \leq 0.01$) indicate a statistically significant difference compared to cells adhered to plastic and subjected to the same stimulation.

Despite their ability to act as priming agents for neutrophils in suspension, both PAF and IL-8 actually inhibited TNF-α -induced ROS production in neutrophils adherent to collagen, fibronectin, and laminin (Figure 2.8). In sharp contrast to the results observed with these ECM proteins, PAF and to a lesser extent IL-8, increase the TNF-α -induced ROS production in neutrophils adherent to HSP (Figure 2.8). Thus, it is apparent that

among all of the functional responses we analyzed, ROS production was most susceptible to differential modulation by ECM proteins and inflammatory agents.

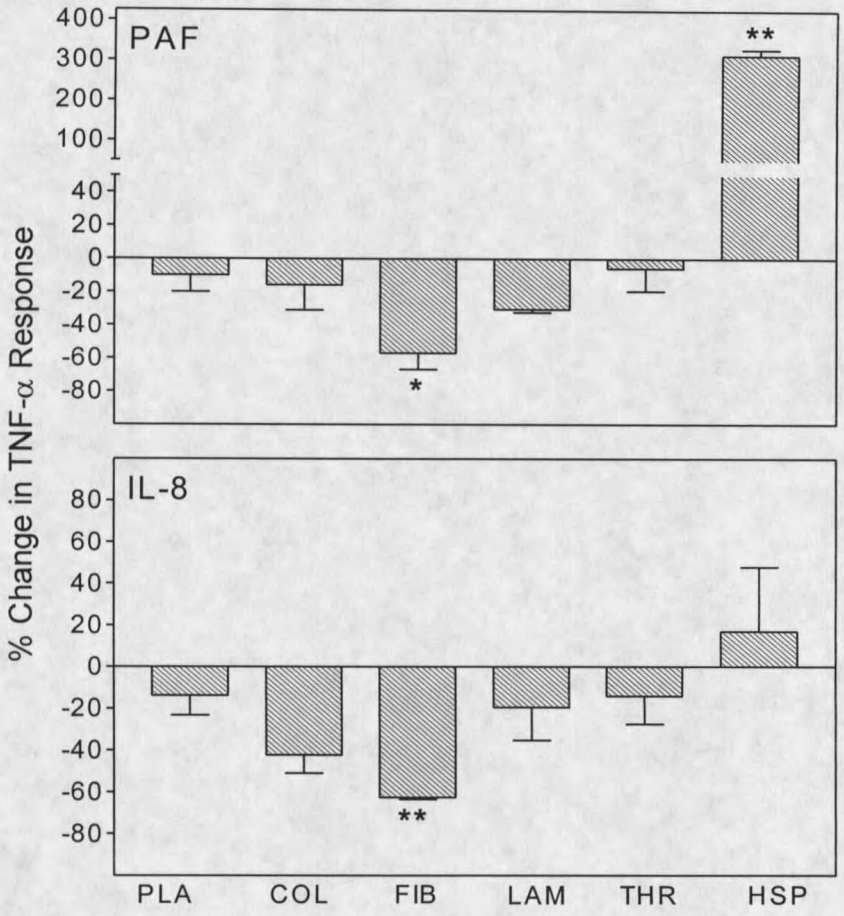

Figure 2.8: Effects of co-stimulation with IL-8 or PAF on the TNF-α -induced respiratory burst in adherent neutrophils. Neutrophils were allowed to adhere to ECM-coated wells for 60 minutes at 37 °C and then stimulated with 100ng/ml TNF-α or TNF-α combined with 10^{-7}M PAF (upper panel) or 10^{-8}M IL-8 (lower panel). Total chemiluminescence over a 60-minute measurement period was determined as in Fig. 3, and co-treatment (TNF-α wither either PAF or IL-8) values were converted to the percent of the average chemiluminescence of TNF-α alone treated cells, on the same ECM, in the same experiment. Results are expressed as mean ± SEM, and are pooled from 2-11 experiments on each ECM, with n=3 on each ECM, for each experiment. *($P \leq 0.05$) or ** ($P \leq 0.01$) indicate a statistically significant difference compared to cells adhered to plastic.

Discussion

Because neutrophils can have both positive and deleterious affects on host tissue, the hypothesis that ECM can modulate neutrophil responses, and allow for location-specific, and perhaps time-specific responses, has attracted some attention (95,98,128). We demonstrate here that ECM proteins are potent modulatory factors in the bovine neutrophil response to IL-8 and PAF, as well as to TNF-α. Moreover, we demonstrate that different ECM proteins induce differential responses in these cells, depending on the type of ECM protein and inflammatory agents present.

Clearly, ECMs have complex effects on neutrophil responses to both IL-8 and PAF, and, among the different ECMs, there are similarities and differences in their effects. Although we see, for example, that some responses of neutrophils adherent to collagen IV are lower than cells adhered to other ECMs, it would be a gross oversimplification to believe that one ECM enhances inflammatory responses to agents such as IL-8 and PAF, while another dampens those responses. Rather, the neutrophil appears to integrate the ECM interaction as one of multiple messages (including IL-8, PAF, etc.) to selectively modulate certain functional responses. The neutrophil response that is the most upstream among those we measured, i.e., changes in intracellular Ca^{2+} concentration, showed the least variability. This would suggest that under most conditions, the initial neutrophil responses to IL-8 and PAF are not differentially regulated. Other neutrophil responses that are more downstream, such as degranulation, actin polymerization, adhesion, and ROS production, demonstrated an increasingly more diverse set of responses, depending on the combination of ECM and stimulant. The

neutrophil function that exhibited the most differential response to ECM and IL-8 or PAF interactions was clearly the production of ROS. With neutrophils in suspension, IL-8, PAF, and even TNF-α are normally considered priming agents, that is, they potentiate ROS production to a second stimulus but are not themselves able to activate ROS production (30,125). However, when neutrophils are adherent to certain ECM proteins, TNF-α becomes a potent direct stimulant of ROS production (40,65). This TNF-α -induced ROS production occurs after a 15-30 minute lag period (40,65), is dependent on an intact actin cytoskeleton (40), and is believed to also be dependent on interactions of the CD11a/CD18b integrin with the substrate (46,64). Although very little is known about how priming agents such as IL-8 and PAF affect the TNF-α oxidative burst, there is one report that IL-8 actually decreases TNF-α -induced ROS production of fibronectin-adherent human neutrophils (47).

Our study with bovine neutrophils adhered to several ECMs demonstrates that TNF-α -induced ROS production is highly variable, depending on the type of ECM used, the presence of a priming agent such as IL-8 or PAF, and the interaction of both factors. This is most evident in the case where PAF co-stimulation can have totally opposite effects, depending on the type of ECM used: inhibitory when the ECM is fibronectin or laminin, enhancing when the ECM is HSP. Furthermore this effect seems to be separate from other functional responses that we measured. This is most notable with regard to adhesion. Although the TNF-α-induced oxidative burst is considered to be adhesion-dependent, there is no correlation between the effects we observed on firm adhesion and on ROS production. For example, PAF decreased TNF-α stimulated adhesion to both

laminin and HSP. However, ROS production in response to TNF-α decreased on laminin, but increased on HSP, when PAF was present.

Previous studies showing that human neutrophils adherent to ECM proteins responded to TNF-α with sustained ROS production led to speculation that neutrophil ROS production can be physiologically partitioned (i.e., they would respond after they had left the circulation and encountered high concentrations of ECM)(95). Indeed, the interstitial space is often considered to be the battleground where neutrophils and other host defense cells encounter microorganisms that have breeched the initial barrier of skin or epithelium (129). Although we confirmed that bovine neutrophils also exhibit high ROS production in the permissive company of ECM proteins, the heterogeneity with which different ECM proteins supported this response raises additional questions. One possibility to consider is whether differential responses to various ECM proteins reflect physiological partitioning on an even smaller scale. Distribution of ECM proteins in tissues is extremely complex, with heterogeneous topographic distribution of different proteins (130-134). For example, the basement membrane of some endothelia is predominately constructed of collagen type IV (131). Therefore, the relatively low TNF-α- induced ROS production observed when neutrophils were adherent to collagen type IV may represent a protective mechanism to preserve endothelial integrity. Conversely, the relatively high TNF-α-induced ROS production observed when neutrophils are adherent to fibronectin may be related to a different interstitial distribution of that ECM protein. Furthermore, local abundance of some ECM proteins can change during disease or inflammation (135-137), so neutrophil ROS production could be altered by these changes. These interpretations are complicated, however, by our observations of the

effects of PAF or IL-8 on TNF-α -induced ROS production. Although these agents are important for the recruitment of neutrophils to inflammatory sites, they also dampen ROS production when fibronectin, laminin, etc. are present. Thus, our results suggest the possibility that HSP interactions with neutrophils may have an overriding role in permitting ECM-dependent TNF-α-induced ROS production to proceed in the presence of IL-8 and/or PAF. However, the widespread distribution of HSP moieties, both in the extracellular matrix and on the surface of other cells, complicate any idea that different ECM-mediated responses represent a form of micro-scale physiological partitioning of neutrophil responses. Indeed, although our study shows strong, complex effects that ECM interactions can have on neutrophil functional responses, extrapolation of these results to *in vivo* situations should still be considered speculative. *In vivo*, neutrophils probably encounter multiple ECM moieties in a short time period, as well as multiple soluble pro- and anti-inflammatory agents. In this situation, many different types of signal integration could occur. Thus, further studies with more complex representations of the ECM are clearly needed to determine what these interactions may be *in vivo*.

On the other hand, the simplistic ECM interaction models used here would appear to be very useful in studying the mechanisms of interaction of signal transduction systems in neutrophils, and perhaps designing pharmacological agents that could differentially modulate neutrophil responses. For example, TNF-α -induced ROS production is initially dependent on the integration of two separate signals: from TNF-α, which probably involves multiple non-receptor tyrosine kinases, and from a cell surface ligand that interacts with the appropriate ECM. The simplest mechanistic explanation of differential responses to ECMs would involve each ECM interacting with the neutrophil

via unique cell-surface ligands. Because of their known interactions with both extracellular ECM proteins and the intracellular cytoskeleton, and their ability to modulate several signal transduction pathways, neutrophil integrins are the most attractive candidates as the necessary link (54,81). The question that comes to mind is whether there is sufficient specificity of integrin-ECM interaction to account for the differential response we see. The dominant neutrophil integrin is the β_2 integrin $\alpha_M\beta_2$ (CD11b/CD18, CR3, Mac-1), which has been implicated in adhesion to fibronectin, collagen, and to a lesser extent, laminin (46,56,57). Although β_2 integrin adhesive interactions are believed to be necessary for some functional responses, including the TNF-α induced oxidative burst of adherent neutrophils (98), they do not show discriminate binding to any one ECM. Additionally, some adhesion-dependent neutrophil responses have been shown to be CD11b/CD18 independent (97). $Beta_1$ integrins are also believed to mediate cell-ECM interactions. There is however, considerable controversy regarding which types of β_1 integrins are expressed on the surface of neutrophils. While evidence has been proposed for $\alpha_5\beta_1$ (138), $\alpha_2\beta_1$ (67), $\alpha_4\beta_1$ (68), $\alpha_6\beta_1$ (139) and $\alpha_9\beta_1$ (138) integrins on human neutrophils, there is no consensus among these studies. Whether β_1 integrins discriminate between different ECM ligands is unclear as well. For example, laminin is often cited as an ECM with specific integrin binding relationship with $\alpha_6\beta_1$ (140). However, laminin can also bind $\alpha_3\beta_1$, $\alpha_v\beta_3$, $\alpha_2\beta_1$ integrins, which are also reported to be on neutrophils (141).

Furthermore, other non-integrin cell surface molecules can bind laminin (142,143). The question of what cell surface receptor(s) mediates neutrophil/HSP

interactions is even less clear. Although HSP can interact with the $\alpha_M\beta_2$ integrin, it may also interact with a multitude of other molecules, including L-selectin, coagulation factors, enzymes, other ECM proteins, growth factors, and cytokines (144). Understanding these interactions is complicated by the fact that HSP-like molecules are contained within ECM domains and expressed on the surface of many cells (145). Research into HSP effects on cells has focused on their ability to potentiate growth factor actions (146), modulate other signaling molecules actions on cells (147), and little is known about any direct effects HSP may have on leukocytes. That our observations on HSP effects contradict both a published report on HSP actions on IL-8 induced calcium concentration changes (49), and a report on HSP induced inhibition of the neutrophil oxidative burst (50), suggest that this may be a very complex ECM/cell interaction. Therefore, although it might be appealing to look for straightforward, specific ECM/ligand interactions to explain these differential responses, the fact that each ECM can bind many integrin and non-integrin molecules (53) and some integrins can bind to more than one ECM (44), suggests that this view may be too simplistic.

In our experiments, although the outcome (ROS production) is likely the result of the integration of TNF-α and integrin signaling, both inhibition and enhancement of ROS production can occur as a result of a third, G-protein coupled receptor (GPCR) message, such as PAF. Furthermore, the effect of this third signal is in turn dependent on the type of the integrin (or other ECM binding protein) mediated signal. Indeed, research in various cell types has recently demonstrated how GPCR mediated messages integrate with other signals, such as MAP kinase cascades (148,149), Ras-dependent signaling

(150), receptor tyrosine kinases (74), and the focal adhesion related kinase Pyk2 (74,151). The latter studies showing the interactions of GPCR with focal adhesions also implicate integrins in these signaling interactions. Clearly, one or more integrative events are occurring in the relevant signal transduction pathways of neutrophils in our experimental system. It would be worthwhile to determine, for example, at which step a GPCR message acts to inhibit neutrophil ROS production, especially if other neutrophil responses are not hindered by this treatment. At this time we know very little about where in the signal transduction cascades these messages are integrating, although it is probably either downstream or on a divergent pathway from changes in intracellular calcium concentrations. If the actual steps where these messages are integrated could be elucidated, they could be attractive targets for new therapeutic agents that could potentially differentially regulate deleterious neutrophil responses (such as excessive ROS production), without inhibiting other important host defense functions.

CHAPTER 3

DEFINING A MECHANISM OF SIGNAL INTEGRATION DURING RESPIRATORY BURST MODULATION IN ADHERENT BOVINE NEUTROPHILS

Introduction

Neutrophil functional responses are modulated by their environment. In her 1999 paper, Fuortes comments, "adhesion-controlled cell responsiveness to soluble agonists has become a major paradigm in cell biology and immunology to explain how matrix and soluble signals combine to regulate cell behavior (52)." According to this type of paradigm, two or more signals must be integrated in order for a functional modulation to occur within a given cell. One, the neutrophil is informed of contact with extracellular matrix proteins in the surrounding tissue by the ligation of cell-surface molecules, such as integrins. The second message is conveyed by surface receptors for soluble signals, including inflammatory molecules such as cytokines. There is considerable evidence that the neutrophil antimicrobial response depends on the incorporation of signaling information from both the external environment (e.g. extracellular matrix) and soluble stimulants (46,51,52). For example, TNF-α, a broad cellular agonist, induces a respiratory burst in neutrophils adhered to a surface through engagement of β_2 integrins, but not in cells in suspension (46). The previous chapter demonstrated that ECM proteins to which neutrophils adhere are potent modulatory factors in the bovine neutrophil response to the

soluble stimuli IL-8 and PAF, as well as to TNF-α. Although the effects of the signal integration of soluble stimulus and adhesion to ECM proteins were seen in many functional responses, the most dramatic influence was on TNF-α-induced ROS production. The mechanism by which these environmental messages integrate and direct the neutrophil response is unclear. However, understanding the mechanisms that determine neutrophil responses to environmental cues could provide therapeutic means in neutrophil-aggravated tissue damage.

The integration of signals from extracellular matrix and cytokine signals could occur at any of the numerous signaling steps within the cell. In this chapter, three sites of integration were evaluated as potential mechanisms: 1) changes in β_1, β_2, or β_3 integrin surface expression; 2) differential recruitment of NADPH-oxidase components to focal adhesions; and 3) differential phosphorylation of proteins by tyrosine kinase activity. Each of these could potentially lend explanation to neutrophil functional response modulation seen in ECM adherent neutrophils. As a preliminary step towards defining a mechanism, the combination of both fibronectin-ligand engagement combined with stimulation by IL-8, TNF-α, or a combination of both IL-8 and TNF-α, were considered in each system. The results from each combination were compared to unstimulated, fibronectin-adherent cells and to the differential ROS production of neutrophils under the same conditions. Any parallels could indicate a mechanism of signal integration modulating respiratory burst in adherent neutrophils.

Cell-surface Integrin Expression

The most likely candidates for cell-surface molecules that mediate the responses to ECM proteins are the integrins. Integrins are essential for neutrophil immunity. Neutrophils depend on integrin interactions for adhesion and subsequent migration out of high-endothelial venules (HEV), for navigation through the extracellular matrix to sites of infection. Additionally, neutrophil integrins have been shown to coordinate ligand-specific immune responses such as degranulation, phagocytosis, and oxidant production (51). In fact, the β_2-integrin adhesive interactions are believed to be a prerequisite for some functional responses, including the TNF-α-induced oxidative burst of adherent neutrophils (40,46,64). Integrin classes are capable of signaling to and regulating expression of another, probably as a means of modulating adhesion and migration, but potentially also regulating other functional responses (67) (68). Integrins may permit or inhibit a stimulant-induced neutrophil response simply through their availability for ligand engagement. As a result, integrins are a site of environmental signal integration in neutrophils, and become likely candidates as mediators of functional responses that are dependent upon ECM proteins.

Integrins may permit or inhibit a stimulant-induced neutrophil response simply through their availability for ligand engagement. As a result, integrins are a site of environmental signal integration in neutrophils, and become likely candidates as mediators of functional responses that are dependent upon ECM proteins.

The TNF-α-induced respiratory burst is a straightforward model to look for effects integrins may have upon signal integration since it is dependent upon integrin

engagement. Functional regulation through integrins is implicit in the data shown in Chapter 2, where signals combined from both soluble stimulants and extracellular matrix modulated neutrophil inflammatory responses. One possibility worthy of consideration is that bovine neutrophils adherent to different ECM proteins engage different sets of integrins, those specific for the ECM encountered. This specific integrin-ligand engagement would provide a straightforward level of neutrophil regulation, in which unique subsets of integrins direct a differential response to a common soluble stimulus. This could explain, for example, how laminin-adherent neutrophils responded to TNF-α with strong production of ROS, whereas the respiratory burst of collagen-adherent cells was much less productive (Figure 2.7). This raises the question of whether integrin interactions are involved in the modulation of bovine neutrophil functional responses seen in Chapter 2. The neutrophil respiratory burst in response to TNF-α was dramatically inhibited by IL-8 co-stimulation when cells were adherent to fibronectin. It is not known if this functional modulation was occurring through integrin signaling; however, this would be evident through shifts in surface expression levels of selected integrins, since any integrin signaling depends on ligand engagement. Given that the TNF-α-stimulated respiratory burst is dependent on β_2-integrin engagement, perhaps IL-8 co-stimulation results in a relative change in the surface expression of three different cell surface integrin classes, β_1, β_2, and/or β_3, which correlates with the drop in ROS production. Looking for shifts in surface expression levels of ECM-specific integrins should provide valuable data towards determining whether integrin signaling is a potential mechanism of signal integration in neutrophils.

NADPH-oxidase component recruitment to focal adhesions

Many enzymes, including NADPH-oxidase, are subject to activation and modulation via a number of signal transduction pathways. One way in which the regulation of these enzymes may be more tightly regulated is by colocalization. Focal adhesions are regions of the plasma membrane that act as an anchor point of the adhered cell. These sites provide scaffolding for many intracellular proteins of relevant signal transduction pathways to localize and assemble. They are important points to look for correlations between changes in enzyme localization or activation and functional changes.

It has been previously suggested that focal adhesions may provide a surface for the NADPH oxidase to assemble (81) or at least provide a site for accumulation of cytoskeletal components of the oxidase (41). This would associate formation of focal adhesions and regulation for oxidant production (51), but direct evidence has not yet been shown to support this idea. Once adherent neutrophils are exposed to TNF-α, they undergo a rapid reorganization of their cytoskeleton and form focal adhesions (82) at sites of integrin attachment to ECM. One report worth noting claims that adherent neutrophils only produce superoxide at points where the plasma membrane is in contact with an adherent surface (83). If this were the case, then it would certainly implicate focal adhesions, which are major components of the adhesive interaction, in the localization of NADPH oxidase.

Since focal adhesions are sites of enzyme accumulation and regulation, and are an integral component of adhesive interactions between neutrophils and ECM proteins, focal adhesions may provide a site for NADPH oxidase assembly or accumulation. It is

plausible that the purpose of this accumulation is to regulate the adhesion-dependent oxidase activity through movement of its components into or out of the focal adhesions. Thus, an adhesion dependent-respiratory burst, such as TNF-α-induced burst, would correspond with the sequestration or liberation of oxidase proteins (whichever it may be) from the focal adhesions. Signals from inflammatory cytokines may then integrate at sites of adhesion, regulating ROS production by dictating changes in the availability of oxidase components through focal adhesions. This type of mechanism could be involved in the observed downregulation of the TNF-α-induced ROS production noted in ECM-adherent bovine neutrophils when IL-8 was present. Thus, as a potential site for functional regulation by environmental signals from both cytokines and ECM proteins, focal adhesions of fibronectin-adherent bovine neutrophils were examined for differential recruitment of NADPH-oxidase components.

Tyrosine Phosphorylation patterns

Another intracellular mechanism that has great potential as the site for signal integration is the activity of tyrosine kinases. Unique sets of cellular proteins are often phosphorylated by tyrosine kinases in response to different stimuli (41). Upon TNF-α stimulation of adherent neutrophils, several proteins undergo tyrosine phosphorylation (41). Since the ROS response in adherent neutrophils is dependent upon this tyrosine kinase activity, neutrophils may regulate ROS production in response to environmental signals through tyrosine kinase signal transduction pathways; changes in respiratory burst may parallel variation in patterns of tyrosine phosphorylation. In Chapter 2, the intensity of respiratory burst in response to common stimuli was moderated by adherence to

different ECM proteins. When adherent to collagen, fibronectin, and laminin, treatment of bovine neutrophils with either PAF or IL-8 inhibited TNF-α-induced ROS production. In contrast, PAF strongly enhanced ROS production in HSP-adherent cells. Since the ROS response in adherent neutrophils is dependent upon tyrosine kinase activity, any variations in patterns of tyrosine phosphorylation may indicate unique signal transduction pathways. To evaluate association between tyrosine kinase activity and magnitude of the respiratory burst in response to environmental signal integration, fibronectin-adherent bovine neutrophils were stimulated with IL-8, TNF-α or a combination of both and the tyrosine phosphorylation of resulting whole cell lysate was compared between treatments.

Materials and Methods

Materials

Fibronectin, human recombinant interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) were from Calbiochem-Novabiochem (San Diego, CA). Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS) was from Gibco BRL (Grand Island, NY). While, RPMI-1640 was purchased from BioWhittaker (Walkersville, MD). All other reagents were from Sigma Chemical Company (St. Louis, MO). IL-8 and TNF-α were diluted in DPBS containing 0.2% fatty acid free BSA. HEPES buffered saline (HBS) was made with 20 mM HEPES (pH 7.4) with 125 mM NaCl, 5mM KCl, 0.62 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 6 mM glucose. Endotoxin-free water was used for all solutions to which cells were exposed.

Cell-surface Integrin Expression

Bovine neutrophils were adhered to fibronectin-coated 24 well plates (from Falcon) for 60 minutes at 37°C. Then, the cells were treated for 30 minutes at 37°C with control buffer, $1x10^{-8}$M IL-8, 100ug/mL TNF-α (final concentrations) or a combination of both IL-8 and TNF-α. Next, they were lifted by 5-minute incubation at 37° C in a 0.5mM EDTA solution followed by gentle pipette-agitations. Resuspended cells were collected and then washed in DPBS-. Neutrophils were stained according to a standard protocol for FACS analysis (152). Briefly, cells were incubated with the primary antibody in the dark, on ice, for 30 minutes, then washed 2 times in 0.1% BSA in DPBS- before incubating in the secondary antibody for 30 minutes (in the dark, on ice). The excess secondary antibody was washed off in 0.1% BSA+DPBS- and the stained cells resuspended in the same buffer. The antibodies used to identify surface expression levels of the β_1, β_2, and β_3 integrins were: mouse anti-bovine CD29 (VMRD, clone FW-1-101) (25μg/ml), to mark the β_1 chain. MHM_{23} (DAKO) (25μg/ml), a mouse anti-human CD18, which we have shown to recognize bovine CD18 (153), and mouse anti-bovine CD41/61 (VMRD, clone CAPP2A) (25μg/ml), binds both chains of α_{IIb} β_3. The secondary antibody used was a goat anti-mouse IgG antibody conjugated to the fluorochrome FITC (Jackson ImmunoResearch, West Grove, PA). Neutrophil integrin expression levels were detected using a FACSCaliber or FACScan (Beckton-Dikinson, San Jose, CA). Unstained cells were used to set the FL1 baseline to a mean fluorescence less than 10. At least 10,000 cells were examined from each sample. The experiment was repeated three times, and n = 3 for each. Data is presented in two ways, first as changes in the average of mean

fluorescences for each integrin and secondly data was normalized to the percent of the average untreated control for each integrin class.

NADPH-oxidase component recruitment to focal adhesions

Focal adhesions of adherent bovine neutrophils were isolated using fibronectin-coated dynabeads (Dynal). Beads were coated according to the manufacturers instructions with 1 μg bovine fibronectin per $1x10^6$ beads. Any clumped beads were resuspended by mild sonication, and coated beads were stored for up to two weeks at 4°C. Bovine neutrophils were isolated as previously described (109). Cells were treated with either buffer control, TNF-α (100 μg/ml final concentration) or a combination of both TNF-α and IL-8 (100 μg/ml and $1x10^{-8}$ M final concentrations, respectively). Samples receiving both IL-8 and TNF-α were pre-treated for 30 minutes at 37°C with IL-8 followed by addition of TNF-α. The fibronectin-coated beads were immediately added to all neutrophil samples and allowed to adhere for 15 minutes under constant rotation at 37°C. Using a magnetic collection device (Dynal), cells adhered to beads were collected and lysed 10 minutes in cold lysis buffer (20 mM Hepes, 250 mM Sucrose, 10 mM Chaps, 25 μg/ml aprotinin, 25 μg/mL leupeptin, 1 mM PMSF, pH 7.5). Again, the magnet was used to separate focal adhesions and associated proteins complexed to the beads. Beads were washed 2x in cold $HBSS^-$, sonicated as necessary to break up aggregates, and resuspended in sample buffer. Heating 10 minutes at 65°C released the proteins from the beads. Focal adhesion protein samples were analyzed with whole neutrophil lysate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE) on 7%-18% polyacrylamide gels and transferred to nitrocellulose for Western blotting as previously described (154). Western blots were probed with mouse anti-human vinculin, mouse anti-actin, and an anti-$gp91^{phox}$ antibody that recognizes bovine $gp91^{phox}$ (109). Following staining with BioRad (Richmond, CA) horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody, the blots were developed with the West-Pico kit (Pierce, Rockford, IL) and images were recorded on x-ray film.

Tyrosine Phosphorylation Patterns

Isolated bovine neutrophils (1×10^7/ml) in 230 µl of RPMI were applied to fibronectin-coated wells (24 well plate) and allowed to adhere for 1 hr at 37°C. Treatments of 10^{-8}M IL-8, 100ng/ml TNF-α or both IL-8 and TNF-α in the same concentration were applied. After 30 minutes at 37°C, media was carefully drawn off and 200µl of ice cold lysis buffer was added to each well (100mM TRIS pH 7.4, 0.75% Triton X-100, 10mM EGTA, 2mM $MgCl_2$). The buffer also contained protease inhibitors (AEBSF, pepstatin A, E-64, bestatin, leupeptin and aprotinin) and phosphatase inhibitors (sodium vanadate, sodium molybdate, sodium tartrate, imidazole, microcystin LR, cantharidin and p-bromotetramisole). After 15 minutes on ice, all the material in each well was transferred to a microcentrifuge tube and centrifuged at 16,000g for 10 minutes at 4°C. Samples of the supernatant were analyzed for protein concentration using the Pierce BCA kit. Aliquots of the same supernatants were mixed with reducing sample buffer, and heated at 95°C for 4 minutes. Whole-cell tyrosine phosphorylation was compared in each sample by SDS-PAGE on polyacrylamide gels (identical amount of

protein were used from each sample). Western blots of the resulting protein separations were probed with 1ug/mL mouse anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology), followed by secondary horseradish peroxidase conjugated goat anti-mouse IgG (BioRad, Richmond, CA). The blots were developed with the West-Pico kit (Pierce, Rockford, IL) and images were recorded on x-ray film.

Statistical analysis

One-way ANOVA, followed by Tukey's *post-hoc* testing was used to test for statistical significance among treatment groups, using GraphPad Prism (La Jolla, CA).

Results

Cell-surface Integrin Expression

Integrins have been shown to coordinate the ligand-specific oxidant production in neutrophils (51). In Chapter 2, the addition of IL-8 inhibited the TNF-α -induced ROS production in neutrophils adherent to collagen, fibronectin, and laminin (Figure 2.8). Thus cytokine-induced shifts in surface expression levels of ECM-specific integrins could be a regulatory mechanism behind the modulation of neutrophil respiratory burst. Since the TNF-α-induced respiratory burst is dependent on β_2-integrin engagement, we evaluated if IL-8 co-stimulation resulted in a relative change in surface expression of three different cell surface integrin classes, β_1, β_2, and/or β_3, which may have correlated with the decreased ROS production. Fibronectin-adherent bovine neutrophils were

stimulated with IL-8, TNF-α, or a combination of both IL-8 and TNF-α and then stained for β_1, β_2, and β_3 integrins with monoclonal antibodies and the surface expression levels of each class of integrin was measured by flow cytometry.

Consistent with previous studies, bovine neutrophils adherent to fibronectin show moderate levels of β_1 integrins, β_2 integrins are the most widespread integrin on the cell surface, and expression of β_3 integrins is low (Figure 3.2). Treating the adherent cells with IL-8, TNF-α, or a combination of both IL-8 and TNF-α did not result in a significant change in cell-surface integrin expression in β_1, β_2 or β_3 (as indicated by mean fluorescence) integrins from untreated control expression levels. This holds true even when the data is normalized to account for changes in the mean fluorescence between experiments (Figure 3.3). Therefore, bovine neutrophils adherent to fibronectin express β_1, β_2 and β_3 integrins at expected levels, and the distribution is not significantly affected by exposure to cytokines IL-8 or TNF-α.

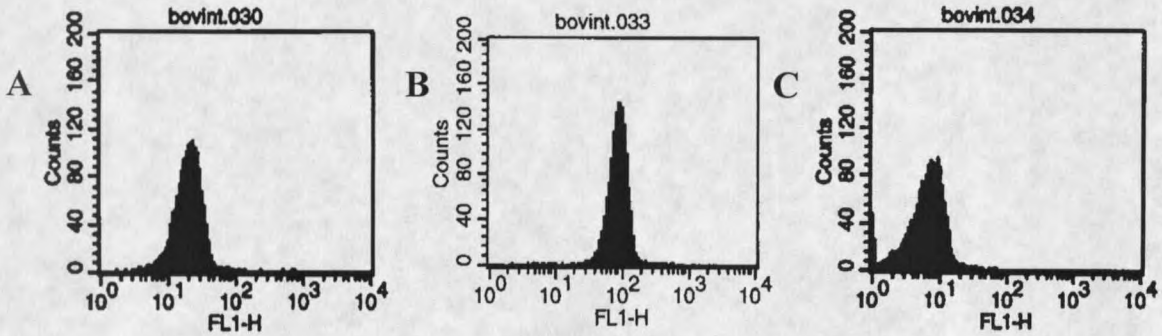

Figure 3.1: Flow Cytometric Analysis of Relative Integrin Surface Expression. Isolated neutrophils adhered to fibronectin-coated polystyrene wells for 1 hour at 37˚C. Cells were collected, stained with appropriate antibodies and analyzed by flow cytometry for FITC staining. Data is expressed as representative histograms of FITC staining levels seen in 3 separate experiments. Panel A shows cells stained for Cd29 (β_1) expression. In panel B cells were stained for Cd18 (β_2) expression, and panel C shows cells stained for Cd41/61 (β_3) expression.

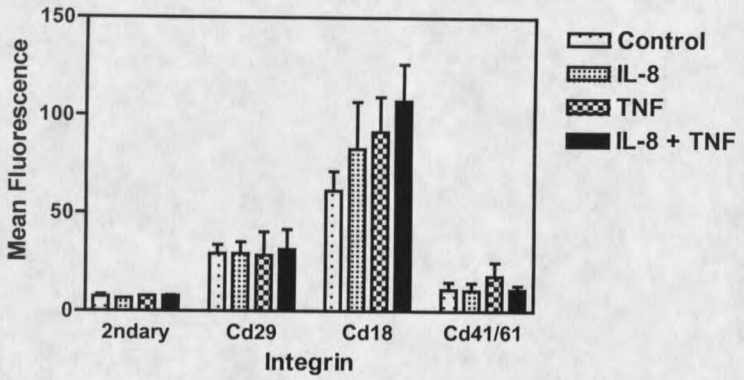

Figure 3.2: Effect of cytokine treatment on cell-surface integrin expression on fibronectin-adherent bovine neutrophils. Isolated neutrophils were allowed to adhere to fibronectin coated polystyrene wells for 1 hour at 37˚C. Cells were then treated with the for 30 minutes, 37˚ C, with control buffer, $1x10^{-8}$M IL-8, 100ug/mL TNF-α (final concentrations) or a combination of both IL-8 and TNF-α. Treated cells were collected and stained with appropriate antibodies and analyzed by flow cytometry for FITC staining. Data represents pooled mean fluorescence ± SEM of three experiments, n=3 for each. No significant differences between treatments and β_1, β_2 or β_3 integrin expression levels.

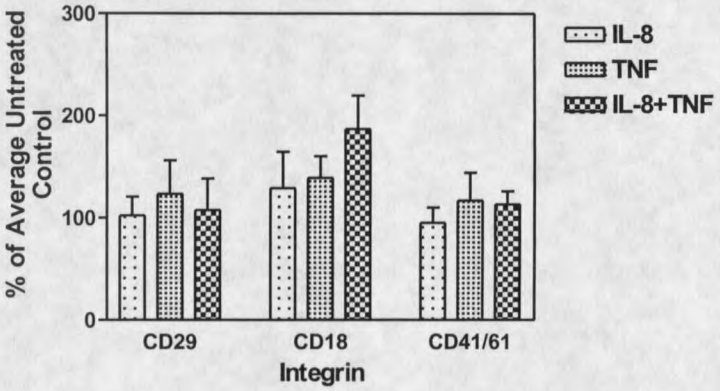

Figure 3.3: Percent cytokine treatment increased cell-surface integrin expression on fibronectin-adherent bovine neutrophils. Data in Figure 3.1 has been normalized to the average mean fluorescence of the untreated control (100%) ± SEM for each integrin class. Treatments did not result in any significant differences in β_1, β_2 or β_3 integrin expression levels.

NADPH-oxidase component recruitment to focal adhesions

Focal adhesions are sites of enzyme accumulation and regulation, and are integral in the adhesive interactions between neutrophils and ECM proteins. Therefore focal adhesions may provide a site for NADPH oxidase assembly or accumulation with the purpose of regulating adhesion-dependent oxidase activity through movement of its components into or out of the focal adhesions. We evaluated whether the IL-8-stimulated downregulation of the adhesion-dependent TNF-α-induced respiratory burst corresponded with sequestration or liberation of oxidase proteins from the focal adhesions. A correspondence could explain downregulation of TNF-α-induced ROS production noted in ECM-adherent bovine neutrophils when IL-8 was present. Focal adhesions from fibronectin-adherent bovine neutrophils were collected and examined for differential recruitment of NADPH-oxidase components under several stimulatory conditions including IL-8, TNF-α, and both IL-8 and TNF-α together. The accumulation of the NADPH oxidase membrane protein, $gp91^{phox}$, in focal adhesions was analyzed by Western blotting.

Western blots of fibronectin adherent bovine neutrophils were probed with monoclonal antibodies to vinculin, one of the most abundant focal adhesion proteins (77), to verify that focal adhesions had indeed been isolated with this method. Vinculin was present in equivalent concentrations in each of the samples and cytokine treatment did not affect its relative abundance (Figure 3.4). The presence of actin, another protein common at sites of focal adhesions, served as a secondary control for focal adhesion collection. Like vinculin, actin was detected in equal amounts in all samples, regardless of treatment (data not shown). The NADPH-oxidase membrane protein $gp91^{phox}$ was also

present in the extracted focal adhesion samples. Treatment of cells with TNF-α caused a distinct increase in the recruitment of $gp91^{phox}$ into focal adhesions above levels detected in non-treated cells or in cells treated with IL-8 alone (Figures 3.4 and 3.5). These findings correspond to the increase in ROS production seen in fibronectin-adherent cells treated with TNF-α (Figure 3.6). Neutrophils initially primed with IL-8 and then exposed to TNF-α did not display a reduction in $gp91^{phox}$ recruitment to focal adhesions (Figure 3.4) but perhaps an increase in $gp91^{phox}$ accumulation at these sites. This is in contrast to the inhibition of TNF-α-induced ROS production seen when cells were primed with IL-8 (Figure 3.6). Based on these experiments, treating cells with a combination of IL-8 and TNF-α does induce a reduction in ROS production, but it does not induce a parallel reduction in the accumulation of $gp91^{phox}$ to focal adhesions in fibronectin-adherent bovine neutrophils.

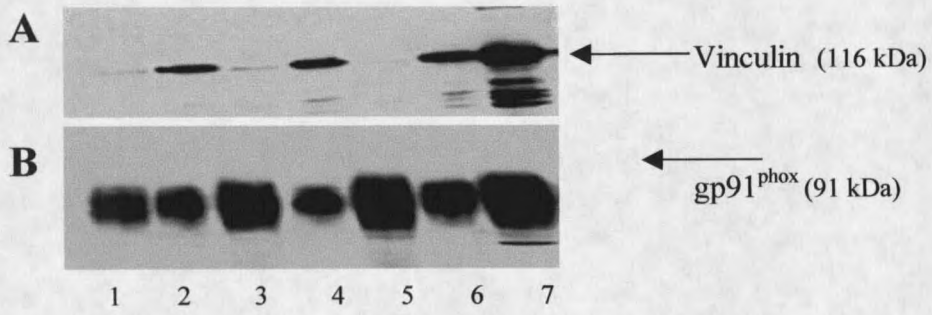

Figure 3.4: NADPH-oxidase gp91 recruitment to focal adhesions in fibronectin-adherent bovine neutrophils. Neutrophils, adherent to fibronectin coated magnetic beads, were lysed. Remaining proteins (adhered to beads) were isolated by magnetic separation. Equal amounts of protein samples were separated by SDS-PAGE and transferred to nitrocellulose. Western blots were probed with antibodies and stained as indicated. Panel A shows presence of vinculin at 116kDa. Panel B shows distribution of $gp91^{phox}$ at 91kDa. Lanes are defined as follows: 1-control beads, 2-control supernatant fluid, 3-TNF-α beads, 4- TNF-α supernatant fluid, 5-IL-8+TNF-α beads, 6-IL-8+TNF-α supernatant fluid, and 7-Lysate. Results are representative of 3 separate experiments.

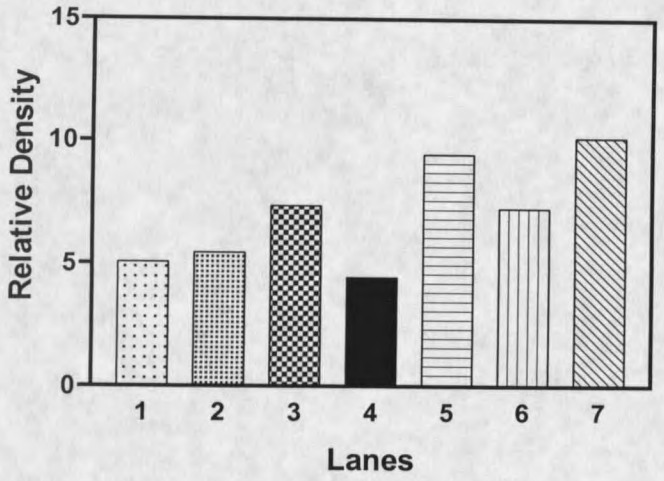

Figure 3.5: Density comparison of gp91 band in Figure 3.4. Western blot band intensities were calculated using AlphaImager software.

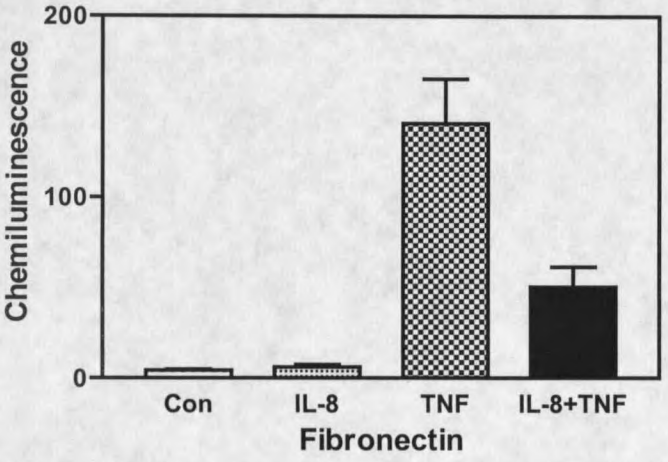

Figure 3.6: Effect of co-stimulation with IL-8 on the TNF-α -induced respiratory burst in fibronectin-adherent neutrophils. Graph represents selected data from Figure 2.7. Neutrophils were allowed to adhere to ECM-coated wells for 60 minutes at 37 °C and then stimulated with 10^{-8}M IL-8, 100ng/ml TNF-α or IL-8 and TNF-α combined. Luminol-enhanced chemiluminescence was measured for 60 minutes, and values shown represent the integrated total chemiluminescence of the 60-minute measurement period. The data are means ± SEM of pooled experiments ranging from >6 experiments, and n=3 replicates for each experiment.

Tyrosine Phosphorylation Patterns

Tyrosine kinase activity is necessary for both NADPH-oxidase activity and integrin signaling (41,86-88). Thus, tyrosine kinase activity has great potential for signal integration in adhesion-dependent ROS production. Therefore we established if overall patterns of tyrosine phosphorylation of cellular proteins varied in adherent bovine neutrophils stimulated to produce ROS by TNF-α. Lysates from fibronectin-adherent bovine neutrophils stimulated with IL-8, TNF-α, or a combination of both were probed for phosphoproteins by SDS-PAGE and Western blotting.

Examination of tyrosine phosphorylation patterns in fibronectin-adherent bovine neutrophils under different treatment conditions revealed differential levels of some phosphoproteins. Treatment of fibronectin-adherent bovine neutrophils with IL-8 caused noteable increases in phosphorylation of a group of proteins between 66 and 44 kDa (Figure 3.7). Treating adherent neutrophils with both IL-8 and TNF- α together further amplified the phosphorylation of these proteins (Figure 3.7). With better resolution, these changes can be distinguished in individual proteins bands at 50kDa and 58kDa (Figure 3.8). Treatment with IL-8 increased phosphorylation of proteins at 50 kDa and at 58kDa above levels seen in the control (Figure 3.7). Tumor Necrosis Factor-α decreased phosphorylation of proteins at 50kDa and 58kDa. Treating adherent neutrophils with both IL-8 and TNF- α together amplified the phosphorylation of both proteins (figures 3.7 and 3.8). Based on these experiments, treating cells with IL-8, TNF-α and a combination of both induces differential patterns of protein tyrosine phosphorylation in fibronectin adherent bovine neutrophils.

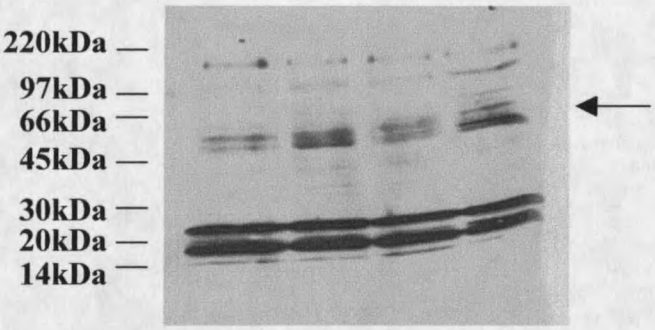

Figure 3.7: Phosphoproteins in fibronectin-adherent bovine neutrophils. Isolated neutrophils were allowed to adhere to fibronectin coated polystyrene wells for 1 hour at 37°C, lysed and proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blots were probed with mouse anti-phosphotyrosine antibody, 4G10 and stained as indicated. The lanes represent cytokine treatments as well as an untreated control. Differential levels of tyrosine phosphorylation were detected in of a group of proteins around 55kDa. The locations of prestained molecular weight markers and their relative standard molecular masses are indicated at the left. Results are representative of 3 separate experiments.

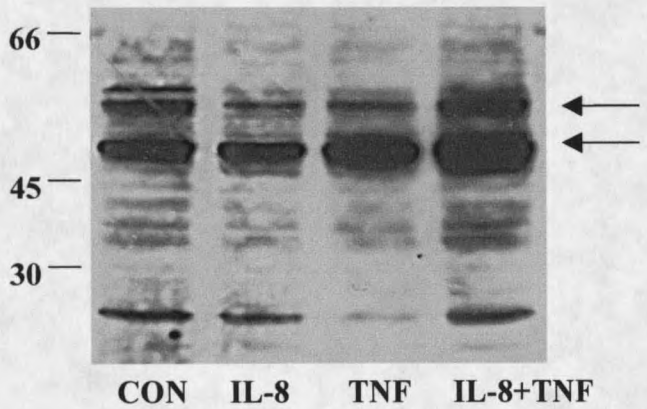

Figure 3.8: Expanded separation of phosphoproteins in figure 3.5. Western blot demonstrates changes in tyrosine phosphoproteins in bands in the 64-44 kDa range. Samples from experiment illustrated in figure 3.5 were separated on a lower (8%) percentage polyacrylamide gel. The lanes represent cytokine treatments as well as an untreated control. Differential levels of tyrosine phosphorylation were detected in of a group of proteins around 50kDa and 58kDa. The locations of prestained molecular weight markers and their relative standard molecular masses are indicated at the left.

Discussion

The integration of extracellular matrix and cytokine signals could occur at any of numerous signaling steps within the cell. The data presented in this chapter provides insight into three potential sites of signal integration, each of which could contribute to the neutrophil's ROS response modulation by their environment. Results from each line of investigation provide valuable data as a starting point for further investigation.

Integrins are a potential site of signal integration of soluble and environmental signal input for several reasons. We proposed that IL-8 co-treatment could result in a relative change in the surface expression in any of three different cell surface integrin classes, β_1, β_2, or β_3, perhaps accounting for the decrease in ROS production seen in the TNF-α-induced respiratory burst when IL-8 was added to the system.

Previous papers have shown that β_2 integrin engagement causes increased expression of the β_1 integrins and vice versa. In both cases the upregulated integrin was necessary for a functional response (67,68). The results did not show support for this hypothesis. Thus, it is unlikely that the change in ROS production after IL-8 and TNF-α co-stimulation is due to major changes in the levels of available β_1 or β_2 integrins. Finally in considering expression levels of β_3 integrins, we show very low expression of these integrins on bovine neutrophils, and expression levels of this integrin class were unaffected by treatments as well.

Although the TNF-α-induced burst is dependent upon integrins, from the data presented here it is unlikely that fluctuations in their expression levels are responsible for the decrease in ROS seen with IL-8 co-stimulation of fibronectin-adherent bovine

neutrophils. Neither the β_1 nor β_2 expression changed significantly in response to the various stimuli, and the β_3 integrins were not detectable at sufficient levels to make any conclusions about their expression. Therefore, at this point, no correlation can be drawn between ROS production and β_1, β_2, or β_3 integrin surface expression levels.

The possibility of changes in integrin expression being involved in the downregulation of TNF-α-induced ROS production cannot be ruled out entirely, however. Expression levels of β_2 integrins in general may not be affected in this system, but it would be interesting to consider changes among the different subtypes of β_2 integrins with the different stimulations. It may also be valuable to take into account the effect different ECMs or even combinations of several ECMs may have on integrin expression with different stimulations. Another consideration may be how the sequence of adhesion, then stimulation compares to concurrent adherence and stimulation (perhaps a more likely occurrence than in unison) effects integrins. Nonetheless, at this point there is no strong evidence to suggest that changes in the surface expression of different classes of integrins can modulate the downregulation of the TNF-α-induced respiratory burst, and likely the mechanism behind this downregulation lies in other intracellular system.

Because focal adhesions are areas of interaction of adherent cells with the extracellular matrix, and are also potentially sites where enzyme activity is regulated, we examined whether the degree of localization of the NADPH-oxidase into the focal adhesion correlated with the level of ROS production. Fibronectin-adherent bovine neutrophils showed an increase in the accumulation of oxidase component $gp91^{phox}$ to focal adhesions after stimulation with TNF-α. This is the first report of TNF-α inducing an increase in oxidase components to focal adhesions in adherent neutrophils. This result

provides intriguing preliminary support for a TNF-α-induced gathering of oxidase proteins at sites of focal adhesions as a mechanism for regulation of adhesion dependent respiratory burst in neutrophils.

Nevertheless, more evidence needs to be gathered in order to establish a direct relationship. First, the effects that TNF-α stimulation may have upon the accumulation/movement of other oxidase components needs to be determined. In the experiments performed here, the detection of bovine $p47^{phox}$ was inconclusive due to high non-specific background, and antibodies to the other bovine NADPH-oxidase components were not available. Secondly, the TNF-α induced recruitment of $gp91^{phox}$ and any other oxidase proteins to focal adhesions of neutrophils adherent to other ECM proteins could be determined. Recruitment could then be compared to the ROS production in cells adherent to each ECM considered. Since collagen supported very little TNF-α induced respiratory burst, one would predict a lower amount of $gp91^{phox}$ accumulation in focal adhesions of these cells. If these components are recruited to focal adhesions, an important question to consider is whether the oxidase is actually assembling or whether only some components are randomly accumulating at these sites. If only some oxidase components are accumulating at these sites, it is possible that focal adhesions are blocking oxidase activity by sequestering components. On the other hand, if the oxidase is assembling and is functional, perhaps focal adhesions are providing a surface for NADPH oxidase to assemble, as hypothesized by Calderwood (81), and spatially controlling ROS production to sites of adhesion as proposed by Vissers (83). Determining these answers would provide more basic evidence that would support a link

between environmental signal integration, NADPH-oxidase function and regulation at the focal adhesions.

If accumulation of the oxidase components in focal adhesions were a mechanism of ROS inhibition, the addition of soluble agonists known to downregulate the respiratory burst could potentially induce a movement of oxidase proteins out of focal adhesions. Although addition of IL-8 causes a downregulation in the TNF-α-induced ROS production in fibronectin adherent cells, co-stimulation with IL-8 did not cause a noticeable decrease in the accumulation of $gp91^{phox}$ with vinculin in the focal adhesions. Such a result would be expected if movement of the oxidase out of the focal adhesions were a mechanism of ROS inhibition with IL-8. This does not rule out the effects IL-8 may have on localization of other oxidase components to focal adhesions, or the possibility that other signaling components may be recruited/changed by IL-8 to alter oxidase activity.

Tyrosine kinase activity is necessary for both NADPH-oxidase activity and integrin signaling (41,86-88). Thus, tyrosine kinase activity has great potential for signal integration in adhesion dependent ROS production. In this system, we proposed that stimulating fibronectin-adherent bovine neutrophils with IL-8 and TNF-α and both Il-8 and TNF-α together would result in changes in the levels of phosphorylation of some proteins due to differential tyrosine kinase activity. Varying levels of tyrosine activity could correlate with differences in ROS production in ECM adherent neutrophils.

Our studies show that tyrosine kinases could be a potential mechanism of signal integration in ECM adherent neutrophils. Results of this investigation show varied amounts of tyrosine phosphorylation with different treatments. Treatment of fibronectin-

adherent bovine neutrophils with TNF-α decreased the phosphorylation of proteins at 50 kDa and 58 kDa, whereas IL-8 treatment increased their phosphorylation. Co-stimulation of neutrophils with IL-8 and TNF-α together resulted in the most dramatic increase in phosphorylation levels of these proteins. Increased phosphorylation of these proteins could be a mechanism of inhibiting a TNF-α-induced respiratory burst in fibronectin adherent bovine neutrophils.

The identity of these proteins is yet unknown, but the modifications seen justify further investigation into their identity and role in regulating neutrophil ROS production. Identifying any of these phosphoproteins would entail applying a broad selection of tyrosine kinase inhibitors to the system, hopefully knocking out both the phosphorylation of the desired protein and the resulting neutrophil ROS response. In addition to identifying affected proteins, it would be interesting to consider if their phosphorylation is altered when cells are adherent to other ECMs or stimulated with other agonists (e.g., PAF).

In conclusion, efforts at understanding how the expression patterns of integrins are regulated by soluble agonists, determining if focal adhesion sites can direct NADPH-oxidase activity, and identifying tyrosine kinase activities associated with adhesion-dependent ROS production have provided some information to explain how functions of neutrophils can be controlled by both extracellular matrix and cytokines. In addition to these, other regulatory mechanisms could be investigated for influence over adhesion dependent TNF-α-induced ROS production. Adhesion could direct differential granule release and subsequent fusion of proteins such as oxidase components or integrins with

plasma membrane could potentially regulate neutrophil response. The ability to pinpoint where such directive signal integration is occurring would provide potential therapeutic targets that would allow for tight regulation of neutrophil function with out knocking it out completely. This could diminish physical symptoms of neutrophil mediated disease without complete loss of neutrophil function that would lead to susceptibility to severe bacterial infections.

CHAPTER 4

CONCLUSION

Neutrophils are an essential component of the host defense system against bacterial and fungal pathogens. Yet, uncontrolled neutrophil antimicrobial activities can inadvertently damage nearby cells and tissues. Understanding the functional regulation of neutrophils is important in developing therapy for inflammatory diseases aggravated by neutrophils without disabling their vital defenses.

The goal of the research presented in this thesis is to contribute to the investigation of the regulatory role an environment has upon neutrophil activity. Efforts directed at modulating neutrophil function could provide therapeutic benefits, however it will require nearly complete understanding of the molecular processes that initiate and control neutrophil functional responses. The work contained in this thesis provides data that contribute to our understanding of these molecular processes.

We characterized functional responses of bovine neutrophils stimulated with IL-8 and PAF and determined if these responses were altered by interactions with a variety of relevant ECM proteins, including collagen IV, laminin, fibronectin, thrombospondin, and heparan sulfate proteoglycan (HSP). Our results indicate that interaction of neutrophils with extracellular matrix proteins can differentially modulate this cell's responses to both IL-8 and PAF. The neutrophil response most sensitive to modification by concurrent stimulation with IL-8 or PAF and adhesion to ECM proteins was the TNF-α-induced

respiratory burst. The fact that different combinations of ECM protein and IL-8 or PAF can have totally opposite effects on this response lead us to investigate the signal transduction mechanisms involved in the message integration regulating adhesion dependent NADPH-oxidase activity. Exploration of three potential mechanisms, integrin expression, enzyme regulation through focal adhesions and tyrosine kinase activity resulted in valuable preliminary data suggesting that each deserves further study. Understanding how the expression patterns of integrins are regulated by soluble agonists, determining if focal adhesion sites can direct NADPH-oxidase activity and identifying tyrosine kinase activities associated with adhesion-dependent ROS production may help explain how functions of neutrophils can be controlled by both extracellular matrix and cytokines.

These data contribute towards a more complete understanding of neutrophil regulation. A more clear appreciation for neutrophil regulation could provide a pathway for lessening destructive behaviors such as excessive ROS production without completely losing the respiratory burst. The ability to regulate this volatile neutrophil function through prediction of environments conducive to high ROS activity and inflammatory damage and developing methods of interrupting the signal transduction pathways that lead up to it could provide relief or prevent damage in patients suffering from neutrophil-mediated disease.

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