REVIEW OF LITERATURE
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Blood, an aqueous solution of several substances, maintains its chemical composition constant within a fairly narrow margin. It comprises of three types of cells— the red cells or erythrocytes; the white cells or leukocytes; and the platelets or thrombocytes.

Erythrocytes or red cells are largely concerned with oxygen transport, the leukocytes play important role in defence against infections and tissue injury while thrombocytes or platelets are involved in maintaining the integrity of blood vessels and in the prevention of blood loss. Although, all erythrocytes and all thrombocytes appear virtually identical, two main types of leukocytes can be readily distinguished: they are, the granulocytes and the lymphocytes. In the granulocytes the cytoplasm is packed with enzyme-containing granules which are essential for the cells’ phagocytic function. In the lymphocytes the clear cytoplasm contains few, if any, granules.

The granulocytes are divided into two types according to the appearance of the nuclei. The most common cells have multiple lobes, they are the polymorphonuclear leukocytes (PMNLs) and are subdivided according to the colour of the granules into neutrophils, eosinophils and basophils. The other main type of granulocyte is the monocyte, which has a single round or lobulated nucleus; this cell has a much finer structure than the other granulocytes.

**ORIGIN AND LIFE SPAN OF NEUTROPHILS**

The human neutrophil has been recognized as the most abundant of the circulating phagocytes since its discovery by Ehrlich in 1879. Neutrophils play a critical role in host defense against a variety of microbial pathogens (Segal, 1989; Cross & Jones, 1991 and Morel et al., 1991). These rapidly moving, avidly phagocytic cells are essentially the first line of defense of mammalian organisms against the invading organisms.
The mechanisms of production and activation of neutrophils have been partially deciphered. In the normal adult human, the life span of PMNLs is spent in three compartments: bone marrow, blood and tissues. Myeloblasts in the bone marrow are the parent cells of neutrophils and monocytes. Bone marrow is the actual site of the important processes of proliferation and terminal maturation of neutrophil granulocytes (myeloblasts to mature PMNLs) (Fig 1).

**Fig 1:** Diagrammatic Representation of the Maturation of Polymorphonuclear Leucocytes (PMNs)

After the myelocyte stage, the cells become "end cells" (cells no longer capable of mitosis) and enter a large storage pool. About 5 days later, the mature neutrophils are released into the blood, where they circulate for about 10 hours.
Their fate after their migration to tissues is unknown, but they probably live only for 1-2 days (Klebanoff & Clark, 1978).

The bone marrow supplies $1.5 \times 10^9$ cells/kg/day (Golde, 1983). The neutrophils in human blood include the circulating neutrophil pool ($0.3 - 0.4 \times 10^9$ cells/kg) and marginating pool ($0.4 \times 10^9$ cells/kg). Neutrophils in the circulation have a short half life span of 6-8 hours. However, neutrophil number in the blood is maintained at 3000 to 6000 cells/mm³. The production of neutrophils may increase 10 folds during infections and is initially preceded by release of end stage neutrophils from a marrow pool, estimated to contain 90% of the total neutrophils (Craddock et al., 1956).

In vitro studies show that senescent neutrophils may undergo apoptosis and become engulfed alive by monocyte-derived macrophages. This process may serve to protect the neighbouring cells from proteolytic enzymes which may otherwise leak out from dying neutrophils (Savill et al., 1989).

**MORPHOLOGICAL AND STRUCTURAL FEATURES OF NEUTROPHILS**

The striking morphological features of the mature PMNLs are the multi-lobed nuclei, the abundance of cytoplasmic granules, the prominent deposits of particulate glycogen and the paucity of other cytoplasmic organelles. In mature neutrophils, the endoplasmic reticulum is scanty and neutrophil shows the presence of small mitochondria, few free ribosomes and a greatly diminished golgi complex. The cytosolic components are surrounded by a plasma membrane which is particularly active, as indicated by the requirements for cell movements (chemotaxis) and membrane invaginations (phagocytosis).

A normal neutrophil under the microscope appears as a colourless round cell with its diameter varying from 10-12 µm. Electron microscopy of unperturbed mature neutrophil, isolated from peripheral blood, shows the cell to be covered by a ruffled trilaminar plasma membrane. The plasma membrane consists of two layers of electron dense material separated by a layer of equal thickness, the total width
being 75 to 100 Å. The plasma membrane contains proteins involved in receptor signalling and those linking extracellular and intracellular processes. The plasma membrane becomes enriched in proteins from intracellular reservoirs when the cell is activated.

**CYTOPLASM**

The cytoplasm is rich in granules but scarce in mitochondria, endoplasmic reticulum and golgi complex, indicating that minimal synthesis of new cell components takes place in the mature cell. Distinct glycogen particles are present, possibly serving as energy reservoirs. Structures involved in cell locomotion (actin filaments) and in intracellular transport of granular contents (microtubules) are localized adjacent to the plasma membrane and organized in the interior part of the cell respectively.

**GRANULES**

Two major granule populations have been recognized -

(i) Azurophil granules
(ii) Specific granules

Both populations show variations in size and shape. The azurophil granules (which appear blue when stained with Wright’s stain) are larger and more dense than the specific granules, which, however, outnumber the azurophils in the mature PMNLs by 2-3 : 1. Granule number in resting neutrophils ranges between 1900-6300 per cell.

Azurophil granules are predominantly located in the outer region of the cells, whereas the specific granules tend to be placed more centrally (Bainton et al., 1971 and Schmid-Schonbein & Chien, 1988).
Both granule populations are sealed by a trilaminar membrane of similar structure as plasma membrane (Spitznagel et al., 1974).

Azurophil granules contain a wide range of hydrolytic enzymes involved in non-oxygen dependent microbicidal activity and degradation of substances (Table 1). These proteins are highly cationic and may be neutralized by chondroitin sulphate (present inside the granular matrix), thereby protecting the cell against enzymatic digestion. The protein profile of specific granules is markedly different and the constituents are engaged in the oxygen dependent microbicidal activity and interactions in the inflammatory process (Table 1).

NUCLEUS

The mature neutrophil is an 'end cell', incapable of cell division. Its nucleus is characteristically multi-lobed, with the nuclear lobes connected by thin strands of nuclear material. The chromatin is condensed and marginated and nucleoli are absent (Fig 2).

Fig 2 : Structure of Mature Neutrophil (showing large multilobed nuclei)
### Table 1: Neutrophil Granule Components

<table>
<thead>
<tr>
<th>Azurophil granules</th>
<th>Specific granules</th>
<th>Tertiary granules</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microbicidal enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>Lysozyme</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Collagenase</td>
<td>Tetranectin</td>
</tr>
<tr>
<td></td>
<td>Gelatinase</td>
<td>Gelatinase</td>
</tr>
<tr>
<td>Bactericidal permeability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increasing protein (BPI) or CAP 57</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Defensins</strong></td>
<td></td>
<td></td>
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<tr>
<td>Serprocidins</td>
<td></td>
<td></td>
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<tr>
<td>Elastase</td>
<td></td>
<td></td>
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<tr>
<td>Cathepsin G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteinase 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azurocidin or CAP37</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Acid Hydrolases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Glycerophosphatase</td>
<td>Lactoferrin</td>
<td></td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>Vitamin B₁₂-binding proteins</td>
<td></td>
</tr>
<tr>
<td>N-acetyl-B-glucosaminidase</td>
<td>Plasminogen activator</td>
<td></td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td>Histaminase</td>
<td></td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>β₂-microglobulin</td>
<td></td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Receptors</td>
<td>Receptors</td>
</tr>
<tr>
<td></td>
<td>- FMIP</td>
<td>- FcRIII</td>
</tr>
<tr>
<td></td>
<td>- CR3 (C₃bi)</td>
<td>- CRI</td>
</tr>
<tr>
<td></td>
<td>- Laminin</td>
<td>- CD IIB/CD 18</td>
</tr>
<tr>
<td></td>
<td>- CDII b/CD18</td>
<td>- Vitronectin</td>
</tr>
</tbody>
</table>
NEUTROPHIL FUNCTIONS

The mature PMNL is highly specialized for the performance of its primary function of phagocytosis and destruction of pathogens. Neutrophils have a critical role in host defense against a variety of microbial pathogens and hence act as first line of defense against invasion of the tissues by the foreign organisms mainly bacteria. Functional defects in neutrophils can lead to recurrent cutaneous abscesses, periodontitis, pneumonitis, osteomyelitis and occasionally life threatening sepsis. A significant reduction of neutrophil activity provokes life threatening fatal bacterial or fungal infections. These serious events are displayed clinically in patients suffering from disorders characterized by a reduced neutrophil function, mostly inherited or acquired disorders, and by a reduced production of mature, normal neutrophils as in leukemias or drug induced neutropenia.

GENERATION OF REACTIVE OXYGEN SPECIES (ROS) BY NEUTROPHILS

A list of active antimicrobial systems present in PMNLs is given for reference (Table 2).

Table 2: Antimicrobial System of the Polymorphonuclear Leukocyte

<table>
<thead>
<tr>
<th>Oxygen-dependent antimicrobial systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase - mediated (HOCl)</td>
</tr>
<tr>
<td>Myeloperoxidase - independent</td>
</tr>
<tr>
<td>H2O2</td>
</tr>
<tr>
<td>Superoxide anion (O2⁻)</td>
</tr>
<tr>
<td>Hydroxyl radical (OH⁻)</td>
</tr>
<tr>
<td>Singlet oxygen (O2⁻)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oxygen-induced antimicrobial systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequestration</td>
</tr>
<tr>
<td>Acid</td>
</tr>
<tr>
<td>Lysozyme</td>
</tr>
<tr>
<td>Lactoferrin</td>
</tr>
<tr>
<td>Granule cationic proteins</td>
</tr>
</tbody>
</table>
Efforts to define the pathology of neutrophil dysfunction have contributed to the delineation of the molecular basis of normal neutrophil function. It is now evident that neutrophils are equipped with a complex, highly integrated and sophisticated functional repertoire which enables the cell to confront microbes and play a heroic part in the first line of host defense.

The ultimate killing of microbes is executed by reactive oxygen species, oxidizing halogens and by proteins with antibiotic effects or proteolytic enzymes. The oxidative and non-oxygen dependent systems can act separately but the effect is fortified when unleashed concurrently (Borregaard, 1988; Weiss, 1989 and Spitznagel, 1990).

The respiratory burst is a special property of phagocytosis. During phagocytosis, neutrophils undergo an intracellular respiratory burst and their granule contents are released into the phagocytic vacuole. The respiratory burst is triggered by the activation of a latent plasma membrane multi-component, reduced nicotinamide adenine dinucleotide phosphate oxidase (EC 1.6.99.6; NADPH oxidase) that is coupled to a respiratory chain on the cell surface. This involves a series of cytosolic enzyme systems.

The NADPH oxidase system lies dormant in resting neutrophils, but is activated upon exposure to the appropriate stimuli. In unstimulated neutrophils three polypeptides of molecular masses 47 KDa (p47-phox), 67 KDa (p67-phox) and 22 KDa (rac-1, rac-2 or Krev-1) can be recovered from the cytosolic fraction. A cytochrome b$_{558}$ consisting of two polypeptides 91 KDa (gp 91-phox) and 22 KDa (p22-phox) is constitutively available in the plasma membrane. Upon appropriate stimulation of the mature neutrophil, the cytosolic components apparently migrate to the plasma membrane, associate with the cytochrome moieties and render the complex enzymatically active (Umeki, 1994).
Fig 3: Diagrammatic Representation of Respiratory Burst of Neutrophils
The NADPH oxidase activity is essential for the bactericidal function of neutrophils, and great effort has been made to establish the components and the mode of activation of this enzyme. Compared to other responses, the respiratory burst is subjected to most stringent control (Umeki, 1994 and Bastian & Hibbs, 1994), presumably because of the reactivity and consequent toxicity of its products. The respiratory burst is initiated by the binding of the agonist to its receptor. It is prevented by antagonists and is rapidly discontinued when agonist is displaced (Baggiolini & Kernen, 1992).

The first reaction of the respiratory burst is to catalyze an electron reduction of molecular oxygen yielding superoxide anion $O_2^-$ and NADP$^+$ was first reported by Babior et al., in 1973, (Fig 3). The NADP$^+$ augments the metabolism of the hexose monophosphate shunt, regenerating NADPH. Two molecules of $O_2^-$ in the presence of two molecules of $H^+$ are rapidly acted upon by superoxide dismutase to form hydrogen peroxide ($H_2O_2$). Superoxide may also interact with $H_2O_2$ to produce hydroxyl radicals (OH') in the presence of iron and copper (Borg & Siachiach, 1989). Hence, the system producing $O_2^-$ may also generate $H_2O_2$ and OH' (Halliwell & Gutteridge, 1986). Furthermore, myeloperoxidase (MPO) interacts with $H_2O_2$ and chloride anion ($Cl^-$) to form hypochlorite ion (OCl') (Odajima & Yamazaki, 1970). These intermediate products of the respiratory burst possess bactericidal activity and may also cause cell damage, if released into tissues.

FREE RADICAL SCAVENGERS

Several mechanisms to detoxify the products of respiratory burst are found to be present in the cells. Superoxide is acted on by superoxide dismutase (SOD) to form $H_2O_2$ and oxygen (Fridovich, 1975) and is more important by its catalytic location into two different subcellular sites, one is cytosolic SOD (Cu-Zn containing) and another is mitochondrial SOD (Mn containing). Yet another extracellular SOD has also been shown to exist (Marklund et al., 1982), this enzyme contains both copper and zinc. Extracellular SOD may function as a scavenger of
superoxide produced extracellularly as from neutrophils (Babior, 1978) or leaked from erythrocytes (Lynch & Fridovich, 1978).

SOD works in conjugation with $H_2O_2$ metabolizing enzyme, catalase (CAT, heme centered enzyme) and especially glutathione peroxidase (GPx) (Chance et al., 1979). Catalase specifically neutralizes $H_2O_2$, other peroxides are not decomposed by this enzyme. The subcellular distribution of catalase and GPx is complementary to each other, two third of GPx activity is located in cytosol and one third in mitochondria (Freeman & Crapo, 1982), whereas catalase activity is localized in cytosol in PMNLs (Evans & Rechcigi, 1967 and Michell et al., 1970).

Extracellular levels of both these enzymes are too low. GPx, a selenium containing enzyme catalyzes the decomposition of $H_2O_2$ and other organic peroxides in presence of reduced glutathione (GSH) and generates oxidized glutathione (GSSG). The oxidized glutathione is reduced in the presence of glutathione reductase (GR) and reduced pyridine nucleotide (Fig 3), at the expense of NADPH, which is provided by hexose monophosphate (HMP) shunt. These free radical scavenging enzymes are the first line of cellular defense against oxidative injury and are known as preventive antioxidants as they remove the reactants involved in initiation of free radical chain reaction (Buettner, 1993).

The non-enzymatic antioxidant, vitamin E, is a lipid soluble antioxidant present in all cellular membranes to maintain the cellular integrity by inhibiting lipid peroxidation and prevents intracellular propagation of free radicals. Other non-enzymatic antioxidants are beta-carotene, vitamin A, ascorbate (vitamin C) and sulphydryl groups which also quench residual free radicals. In addition to it, ascorbate and tocopherol function together to protect membrane lipid damage (Buettner, 1993). These antioxidants are able to inactivate the oxidising radicals directly and therefore are chain breaking antioxidants. The above mentioned antioxidants have been found to be present at detectable levels in rat neutrophils by Kumari et al., 1994.
Neutrophil degranulation has three important roles in host defense. Release of azurophilic granules containing MPO, cationic protein and acid hydrolases, can potentiate the digestive and microbicidal activities of phagocytes (Spitznagel & Shafer, 1985). Degranulation of secondary granules containing lysozyme (also found in azurophil granules), collagenase and lactoferrin helps to regulate inflammation (Sandberg & Smolen, 1988). In addition, degranulation of the tertiary granules is usually associated with translocation of fresh receptors for CRI, CR3, Formyl methionine leucine phenylalanine (FMLP), Laminin, NADPH oxidase and cytochrome b into the plasma membrane (Borregaard & Tauber, 1984 and Miller et al., 1987).

The major drawback of neutrophil phagocytic activity is that the cells do not 'choose' their targets. Instead, neutrophils operate guided by antibodies, (bacterial) chemoattractants, cytokines and complement components. The very same signals may be released in autoimmune or ischemic disorders, misleading the neutrophils into identifying host tissue as being "non-self" (Malech & Gatlin, 1987). Thus neutrophils which are meant for host defense may play a leading role in the pathology of non-infectious, inflammatory disorders (Table 3).

Table 3: Non-infectious Inflammatory Conditions with Neutrophil Involvement.

1) Gout 7) Ulcerative colitis 14) Reoxygenation injury following myocardial infarction
2) Asthma 8) Renal graft rejection
3) Psoriasis 9) Acute glomerulonephritis
4) Atherosclerosis 10) Adult respiratory distress syndrome
5) Immune Vasculitis 11) Chronic obstructive pulmonary diseases
6) Rheumatoid arthritis

Furthermore, oxygen radicals have also been reported to be involved both in carcinogenesis and in elimination of tumors (Cross et al., 1987).
DISORDERS DUE TO ABNORMALITIES IN NEUTROPHIL FUNCTIONS

Neutropenia is defined as a condition in which blood neutrophils count less than 1500/mm$^3$. Neutropenia can be caused by decreased bone marrow production, increased destruction by immune mechanisms, and increased clearance by the reticuloendothelium system. Malnutrition, infections, drugs, malignancy, and even metabolic disorders may cause neutropenia. The high incidence of infections in neutropenic disorders is primarily a consequence of the decreased number of circulating neutrophils. However, in some circumstances neutrophil dysfunction may be an additional risk factor.

Neutrophils from human immunodeficiency virus (HIV) infected children and adults are defective in their ability to generate reactive oxygen intermediates and this defect may make them more vulnerable to bacterial and fungal infections (Nielson et al., 1986; Murphy et al., 1988; Rolilides et al., 1991 and Chen et al., 1993).

The information available on the causes and types of neutrophil disorder, has been summarized in Table-4.

NEUTROPHIL INTERACTION WITH OTHER BLOOD CELLS

It has become evident in recent years that the response of either PMNLs or platelets to activating stimuli may be potentiated by the presence of the other cell type, and that each can release factors which directly activates the other.

Mutual augmentation of the activation of neutrophils or platelets has been most widely studied by measuring cell - cell aggregation. The presence of platelets increases granulocyte aggregation induced by chemotactic agents like FMLP and activated complement (Redl et al., 1983 and Boogaerts et al., 1986). Activation of neutrophils by FMLP can cause platelet aggregation directly or can increase the platelet response to aggregating agonists (De Gaetano et al., 1990; Del Maschio et al., 1990 and Zhou et al., 1992). In general, increased aggregation can be mediated by soluble factors released by the neutrophils or platelets, as well as by direct contact between the different cells.
<table>
<thead>
<tr>
<th>Disorder (Occurrence)</th>
<th>Molecular or Structural Defect</th>
<th>Primary Affected Neutrophil Function</th>
<th>Cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) C3bi receptor deficiency (rare)</td>
<td>Synthesis of the $\beta$-subunit of macrophage antigen-1</td>
<td>Adherence, Chemotaxis, Phagocytosis</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td></td>
<td>$\beta$-subunit of Lymphocyte function associated antigen-1, Glycoprotein 150,95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Specific granule deficiency (rare)</td>
<td>Development of specific granules and other granule proteins</td>
<td>Microbicidal activity, chemotaxis</td>
<td>Probably autosomal recessive</td>
</tr>
<tr>
<td>3) MPO deficiency (1:4000)</td>
<td>Production of myeloperoxidase</td>
<td>Altered oxidative microbicidal activity</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>4) $\alpha$-mannosidosis (rare)</td>
<td>Deficient synthesis of $\alpha$-mannosidase</td>
<td>Chemotaxis</td>
<td>Unknown</td>
</tr>
<tr>
<td>5) Chronic granulomatous disease (1:10^6)</td>
<td>Synthesis of b-cytochrome or flavoprotein or Synthesis of cytosolic factors p47, p67</td>
<td>Oxidative microbicidal activity or</td>
<td>65% X-Linked</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>35% autosomal recessive</td>
</tr>
</tbody>
</table>

Neutrophil cytotoxicity, oxidant production, lysozyme release and arachidonic acid metabolism are increased in the presence of platelets (Boogaerts et al., 1986; Dinerman et al., 1988; Del Maschio et al., 1989; Coeffier et al., 1990 and Parmantier & Borgeat et al., 1991), while activated neutrophils increased calcium mobilization and thromboxane B₂ release for platelets treated with PAF.

In other studies, superoxide production by neutrophils was decreased by resting platelets but increased by stimulated platelets (Moon et al., 1990). Spisani et al., in 1992 found that activated platelets released superoxide anions.

Nitric oxide released by neutrophils can reduce thrombin induced platelet aggregation (Faint et al., 1991). Furthermore, studies carried out by Salvemini et al., 1989 demonstrated that incubation of human platelets or mononuclear cells with washed platelets resulted in an inhibition of thrombin induced platelet aggregation which was dependent on the number of neutrophils added.

ROS generated from neutrophils have been well implicated to augment platelet aggregation. However, when free radical generation from neutrophils was measured in whole blood, platelets were found to inhibit the chemiluminescence response of neutrophils, indicating that platelets limit the oxidant production by neutrophils.

Dallegri et al., 1989 found that platelets had the potential to reduce neutrophil cytotoxicity to red cells by consuming the oxidants released by the neutrophils. Joseph et al., 1989 while studying the composition of the aggregate formed in whole blood with scanning electron microscope, observed that apart from platelets erythrocytes and leukocytes may contribute mechanically to the formation of "platelet" aggregates in vivo.

Neutrophils, are important entity of circulating blood which are assigned the crucial role of defending the body against pathogens. However, its role in
maintaining the vascular tone by releasing certain substances has also been identified.

Cardiovascular diseases, are increasing with increase in life expectancy and change in the life style. Thrombosis which may lead to stroke and heart attack have been now-a-days identified as one of the major causes of mortality and morbidity of the modern man.

Thrombosis, an undesirable form of hemostasis is a pathophysiological phenomenon. Recently, great interest has evinced in understanding the process of intravascular thrombus formation in intact non-traumatized arteries, veins and capillaries which may result in thrombosis.

The process of intravascular thrombosis is complex and requires the coordinated interaction of several elements. Thus, the interaction of platelets with polymorphonuclear leukocytes (PMNLs), red blood cells (RBCs) and plasma factor plays an important role in the initiation and regulation of thrombosis.

Platelet and neutrophils are brought into proximity in a variety of circumstances during thrombosis, hemostasis and the inflammatory response. Both cell types can modulate each others’ reactivity by virtue of physical contact per se and by releasing various factors which modulate the response of cell from which it is released and also the effector cell.

Thrombus formation occurs in vivo as a pathological consequence of interactions between blood hemostatic mechanisms and components of the injured vessel wall under variable flow conditions. Arterial thrombotic occlusion complicates atherosclerosis causing acute myocardial or cerebral infarction, whereas venous thromboembolic diseases complicates a variety of clinical settings generally characterized by blood stasis, activated blood factors and vascular dysfunction or damage. Vascular injury with activation of platelet and coagulation mechanisms under high shear flow produces arterial type thrombus rich is platelets but poor in
fibrin and red cells ("white" thrombus). In contrast the "red" thrombus seen in venous thromboembolic disease is rich in fibrin and red cells and relatively poor in platelets.

**COMPONENTS INVOLVED IN THROMBOSIS**

An understanding of the dynamic interplay between the components found to be present in the micro-environment of thrombus is a prerequisite for dealing with the process of thrombosis. Whether it is arterial or venous thrombus, the presence of neutrophils and platelets is always observed, though their ratio may vary. While the significance of platelet is well understood, the presence and physiological role of neutrophil in thrombus formation is still not clear. It is known that neutrophils generate oxygen free radicals, platelet activating factor (PAF) and several proteases which enhance thrombogenesis suggesting towards the pro-thrombogenic role of neutrophil. The possible components which have been identified till now have been summarized in Table 5.

Whenever a vascular injury occurs, the subendothelium is exposed to the circulating platelets which bind to the adherent surface forming a plug of aggregated platelets within few minutes (Bennett & Shattil, 1990). The platelet activation also takes place in thrombotic phenomenon (De Clerck & Janssen, 1990). Soon after platelet plug formation, the coagulation cascade is triggered off, fibrin strands trap other blood cells and thus coagulation occurs. Factors chemotactic for leukocytes are generated during platelet aggregation and the coagulation reactions (Weksler & Coupal, 1973).

Platelet - vessel, platelet - platelet and platelet - neutrophil interactions are the critical factors in the development of arterial and venous thrombosis.

Endothelial cells are an active non-thrombogenic surface, forming a continuous layer of cells and producing substances that maintain the hemostatic balance. The synthesis of tissue type plasminogen activator (t-PA) and its primary inhibitor, PAI-a, is a key function of endothelial cells in modulating the activity of the fibrinolytic system. Impairment of this system, causes increase in PAI levels
### Review of Literature

#### Table 5: Components Involved in Thrombogenesis

<table>
<thead>
<tr>
<th>Components involved</th>
<th>Mediators released</th>
<th>Effect on Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood Vessels</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Endothelial cells</td>
<td>- NO and PGI₂, Ecto ADPases, HODE - Platelet Activating Factor, Free radicals</td>
<td>Prevents adhesion and aggregations Proaggregatory</td>
</tr>
<tr>
<td>b) Subendothelium &amp; Vascular smooth muscle cells</td>
<td>- Collagen</td>
<td>Proaggregatory and adhesive</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Plasma</td>
<td>- Fibrinogen, vWF, Fibrinonectin etc. - ADP, Calcium, Serotonin, PAF, βTG, PGDF and TxA₂ - 12-HPETE and 12-HETE</td>
<td>Proaggregatory protein factors Proaggregatory Antiaggregatory</td>
</tr>
<tr>
<td>b) Platelets</td>
<td>- PAF and Proteases (Cathepsin G, Elastase) - Free radicals (ROS) - NO, EctoADPases - 13-HODE, EETs</td>
<td>Proaggregatory Antiaggregatory Antiadhesive Proaggregatory</td>
</tr>
<tr>
<td>c) PMNLs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d) Red Blood Cells (RBCs)</td>
<td>- Adenosine diphosphate(ADP)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EETs</th>
<th>Epoxyeicosatrienoic acid</th>
<th>PGI₂</th>
<th>Prostaglandins</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
<td>HODE</td>
<td>Hydroxy octa decadienoic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
<td>PGDF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
<td>βTG</td>
<td>β - Thromboglobulin</td>
</tr>
<tr>
<td>HPETE</td>
<td>12-L-Hydroperoxy-5,8,10, 14-eicosatetraenoic acid</td>
<td>HETE</td>
<td>12-L-Hydroxy-5,8,10 14-eicosatetraenoic acid</td>
</tr>
</tbody>
</table>

Or, by a reduced release of plasminogen activators from endothelial cells, may be related to the development of thrombosis in human patients (Juhan - Vague et al., 1987). On the other hand, the loss of functional or morphologic integrity of the vessel wall may result in evolution of atherosclerosis and development of thrombosis (Vane et al., 1990).
Activated PMNLs have been reported to impair the fibrinolytic system in cultured bovine endothelial cells (Gilboa et al., 1989). Pintucci et al., 1993, provided a new insight into the mechanisms by which activated PMNLs may promote thrombus formation. Their study suggested that t-PA activity was strongly depressed on cathepsin G treatment and PA-1 was released from cathepsin G-stimulated platelets and hence they support a potentially thrombogenic role of cathepsin G, which could impair the fibrinolytic potential of the endothelium.

**ROLE OF PLATELETS IN THROMBOSIS**

Platelets are small anucleated, discoid cells, which circulate in the blood of humans and animals (Bizzozero, 1882). Megakaryocytes in bone marrow are their parent cells, and following maturation, the cytoplasmic fragments of the polyploid megakaryocytes are pinched off and released as platelets into circulation.

Isolated platelets are colourless, anucleated and poorly refractile and discoid in shape. A platelet measures approximately 2 - 5 µm in diameter and 0.5 - 1.0 µm in thickness. Size and appearance are quite variable and are the function of their age (Karpatkin, 1969).

Platelets represent the first line of host defense when normal vessels are injured. Platelet adhesion to subendothelium, aggregation and further platelet recruitment, culminate in hemostatic plug formation. Although this process is well regulated at several levels, alterations in the regulation of this process may lead to intravascular thrombus formation resulting in serious medical problems.

Importance of platelets in the development of arterial thrombosis is well established (Mustard & Packham, 1970 and Fuster et al., 1985).

The primary role of platelet in coronary arterial occlusion has been documented in patients with coronary artery occlusion (Folts et al., 1976) as well as in animal models (Willerson et al., 1986 and Gold et al., 1988). Platelet aggregation plays a crucial role in the pathogenesis of thromboembolic cerebro-
vascular disease. Furthermore, the finding that thrombin plays an important role in platelet rich arterial thrombosis and that the thrombogenic stimulus is inhibited by selective thrombin inhibitor also indicates that platelets play a pivotal role in thrombogenesis (Jang Ik-Kyung et al., 1992).

Tohgi et al., 1991 suggested that platelet aggregation is reduced during the acute period due to the consumption of platelet during thrombogenesis but the remaining individual platelets are hyperactive. Platelet consumption during the acute period increases the infarct size.

**ROLE OF NEUTROPHILS IN THROMBOSIS**

During platelet aggregation and coagulation, several factors are generated which are chemotactic for leukocytes (Weksler & Coupal, 1973). This may account for the presence of white blood cells in the thrombi. Moreover, Henry, in 1965 demonstrated that arterial thrombi are composed not only of platelet aggregates but are also rich in neutrophils. Simultaneous association of neutrophils in hemostatic platelet plug and arterial thrombi suggest that neutrophils play a role in thrombosis (Selby et al., 1991). Recently, several studies have been taken up on interaction between platelets and PMNLs to understand their possible role in thrombogenesis (Marcus, 1990; Bazzoni et al., 1991 and Stewart, 1993).

Factors such as PAF, arachidonic acid metabolites and oxygen free radicals have been shown to be released from activated neutrophils. These mediators bring about platelet activation by various mechanisms which finally result in thrombogenesis (Cerletti et al., 1992).

Activated PMNLs act as potent agonists of blood platelet aggregation (Selak et al., 1988) and affect the integrity of blood vessels by endothelial cell injury (Harlan et al., 1981 and Evangelista et al., 1991).
Neutrophil adhesion to endothelial cells has also been shown to impair the protective effects of catalase and glutathione in preventing endothelial cell injury (Siflingerbirnboim & Malik, 1993).

**SIGNIFICANCE OF PLATELET - NEUTROPHIL INTERACTIONS IN THROMBOSIS**

Role of platelets in thrombogenesis is well documented but the presence of neutrophil in arterial as well as venous thrombus remains to be studied in detail. As neutrophils generate certain mediators which can modulate platelet function, neutrophils may play an instrumental role in the initiation and regulation of thrombosis.

Neutrophil activation may amplify the thrombotic process by releasing toxic oxygen free radicals and proteases which are able to damage the endothelial integrity (Ward, 1991). Platelet factor -4 (PF₄) and platelet derived growing factor (PDGF), the two proteins released upon platelet activation (Deuel et al., 1981 & 1982), as well as arachidonic acid metabolites of the 12-lipoxygenase pathway are chemotactic for leukocytes (Goetzl et al., 1977) and may favour neutrophil accumulation in vivo (Fretland et al., 1989). Platelets may amplify neutrophil recruitment by enhancing generation chemoattractant leukotriene (LT) B₄ from these cells (Maclouf et al., 1982). Adenine nucleotides and PDGF released upon platelet activation may stimulate neutrophil degranulation (Tzeng et al., 1984), phagocytosis (Sakamoto & Yokoya, 1991) and respiratory burst (Ward et al., 1988).

Besides activating the neutrophils, platelets may also dampen under certain circumstances, neutrophil responsiveness (Bazzoni et al., 1991). Unstimulated platelets may limit the effects of neutrophil activation by inhibiting oxygen radical production (Moon et al., 1990) and reducing their cytotoxic effects (Dallegrı et al., 1989). Moreover, substances released upon platelet activation such as transforming growth factor β (TGF-β) and "adherent inhibiting factors" (Gamble & Vardas, 1988 and Iwabuchi & Yamashita, 1990) inhibit the neutrophil adhesion to endothelial cells - one of the first events of inflammation and.
probably thrombosis. Soluble P-selectin also exerts a similar inhibitory effect. Furthermore, PDGF & soluble P-selectin (Wilson et al., 1987 and Wong et al., 1991) reduce the generation of $O_2^-$ by activated PMNLs.

Leukocytes may be important in the control of platelet aggregability because these cells can efficiently stimulate (Selak et al., 1988) as well as inhibit (Salvemini et al., 1989) platelet activation.

Recently, in vitro studies on platelet-neutrophil interaction have provided convincing biochemical and functional evidence for the thromboregulatory role of neutrophils and emphasize the multicellular aspect of hemostasis and thrombosis (Valles et al., 1993). Bednar et al., in 1991, provided evidence that in a stroke model, infarct size was reduced significantly in the neutropenic rabbits, suggesting important role of neutrophils in thrombogenesis. Several other groups also examined the role of neutrophils in myocardial reperfusion injury in vivo by neutrophil depletion (Romson et al., 1983; Mullane et al., 1984; Jolly et al., 1986 and Mitsos et al., 1986).

Transient neutropenia and abrupt complement activation in patients with acute myocardial infarction treated with streptokinase has been recently reported (Frangi et al., 1994).

**MODULATION OF PLATELET RESPONSE BY DIFFERENT MEDIATORS FROM NEUTROPHILS**

a) **REACTIVE OXYGEN SPECIES (ROS)**

Superoxide radicals are supposed to contribute to the myocardial reperfusion injury. Polymorphonuclear leukocytes have been discussed as a major post-ischemic $O_2^-$ source (Lucchesi & Mullane 1986). The role of reactive oxygen metabolites in myocardial ischemia reperfusion injury has been suggested by many workers (Braunwald & Kloner, 1985; Werns & Lucchesi, 1989; Richards et al., 1990 and Lehr et al., 1993).
Besides well documented evidences for neutrophil involvement in post-ischemic injuries, some reports have suggested the role of PMNLs in thrombus formation as well. The free radicals generated during neutrophil activation also serve as platelet activator. Free radical species modify both the adhesive and aggregatory response of platelets (Salvemini & Botting, 1993). Several neutrophil derived products such as myeloperoxidases, $\text{H}_2\text{O}_2$ and superoxide radicals reportedly induce platelet activation (Handin et al., 1977 and Clark & Klebanoff, 1980), hence free radicals can very specifically modulate the platelet function and play an important role in thrombogenesis and hemostasis.

b) PLATELET ACTIVATING FACTOR (PAF)

It is reported that PMNLs and platelets cooperate *in vitro* in PAF formation (Marcus et al., 1982 and Hirafuji & Shinoda, 1993). PAF is a lipid mediator produced by PMNLs which is responsible for platelet stimulating action (Chignard et al., 1987) and as it is a potent activator of both the cells, their interactions play a critical role in the pathogenesis of thrombosis (Hirafuji & Shinoda, 1991) and myocardial reperfusion injury (Raschke & Becker, 1995).

c) PROTEASES

Cathepsin G is another potent agonist for platelet activation which is released from the stimulated PMNLs (Bykowska et al., 1983 & Selak et al., 1988). Cathepsin G has been demonstrated to be an essential mediator of PMNL induced aggregation, serotonin release and TxA$_2$ production (Selak & Smith, 1990; Renesto & Chignard, 1991 and Evangelista et al., 1993).

Elastase is a serine protease and is known to attenuate the platelet aggregation induced by thrombin in absence of fibrinogen (Brower et al., 1985 and Wicki & Clemetson, 1985). It has been observed that thrombin binding sites were reduced following elastase treatment while GP IIb/IIIa binding sites were not degraded. The inhibitory effect of elastase on platelet aggregation have been related to proteolytic inactivation of high affinity thrombin receptors and clevage
of GP IIb on the platelet membranes (Brower et al., 1985). Selak, 1992 reported that neutrophil derived elastase potentiates platelet aggregation elicited by suboptimal concentrations of cathepsin G which was partially due to enhanced release of ADP and TxA₂.

d) ARACHIDONIC ACID METABOLITES:

Platelets and neutrophils are known to have active lipoxygenase and cyclooxygenase system which metabolize arachidonic acid (AA) into hydroperoxide derivatives and various types of prostaglandins. It is well accepted now that lipoxygenase system is the dominant metabolic pathway of AA in neutrophils. Evangelista et al., (1991) suggested that TxB₂ production during platelet - PMNL co-incubation is derived not only from platelet AA but also from the neutrophil AA pool.

Hernandez et al., (1993) have demonstrated that blockade of cyclooxygenase pathway in PMNLs had no effect either on platelet adhesion to the subendothelium or on TxB₂ levels, whereas inhibition of lipoxygenase pathway in PMNLs significantly increased the platelet thrombus formation and TxB₂ production. The amount of lipoxygenase products formed by human neutrophils are much more than cyclooxygenase products.

Buchanan et al., (1987) have suggested that platelet aggregation is regulated by lipoxygenase pathway catabolites as inhibition of lipoxygenase pathway by its inhibitor (nafazatrom) resulted in lowering of platelet aggregation.

It is well established now that there exists a transcellular co-operation in platelets and neutrophils to share arachidonic acid pool and their metabolites in the synthesis of mediator, which otherwise platelet or neutrophil alone can not synthesize. In addition, most of the metabolites formed from arachidonic acid in platelets and neutrophils modulate responses of each other.
e) NITRIC OXIDE (NO)

Neutrophils are known to release certain factors which are inhibitors of platelet activation. Recent work has demonstrated that human circulating cells inhibit platelet aggregation (McCall et al., 1989; Nicolini & Mehta, 1990 and Faint et al., 1991).

The inhibition was attributed to a factor quite similar to Endothelium derived relaxing factor (EDRF) (Rimele et al., 1988 and Salvemini et al., 1989) and its action is mediated by nitric oxide (NO). Recent discovery of nitric oxide (NO) has opened up a new area of biological research as NO is a free radical gas which is a potent vasorelaxant. PMNLs have also been found to release NO, therefore presence of PMNLs in thrombus can locally affect the availability of both constrictors as well as vasorelaxants, proaggregatory and antiaggregatory substances.

Nitric oxide is known to serve a variety of physiological functions such as mediating endothelium derived relaxation of vascular smooth muscle (Palmer et al., 1987 and Furchgott & Zawadzki, 1980) acting as a neuronal messenger (Garthwaite et al., 1988), cytotoxic moiety in macrophages (Babior, 1984 and Nathan & Hibbs, 1991) and as inhibitor of platelet aggregation and adhesion (Moncada et al., 1991), and variety of pathophysiological mechanisms (Gross & Wolin, 1995).

PMNLs and macrophages can synthesize NO from L-arginine (L-Arg) in presence of calcium independent, stereospecific nitric oxide synthase (NOS). L-Arg is converted to NO and citrulline through the enzyme NOS, which is of two main types, one is calcium dependent and the other is calcium independent (Moncada et al., 1991).

Molsidomine, a nitric oxide generator, is a potent vasodilator and an inhibitor of platelet adhesion and aggregation (Kukovetz et al., 1979 and Nishikawa et al., 1982). It has also been shown that NO releasing compounds such
as sodium nitroprusside and 3-morpholinosydnomine (SIN-1) protect the myocardium against ischemia and reperfusion induced damage (Masini et al., 1991 and Loskove & Frishman, 1995).

As discussed earlier PMNLs and macrophages when exposed to soluble or particulate stimuli, generate enormous amount of reaction oxygen species. Superoxide radicals and NO can react together to form peroxynitrite (ONO$\text{O}^-$, a relatively long lasting, strong oxidant) and hydroxyl radicals (Fig 4) (Ischiropoulos et al., 1992; Baggioiini & Wymann, 1990; Beckman et al., 1990 and Pryor & Squadrito, 1995). Peroxynitrite radical has been very recently shown to be far more toxic than NO and other ROS (Brunelli et al., 1995).

\[ \text{NO}^+ + \text{O}_2^- \rightarrow \text{ONO}^0 \rightarrow \text{ONO}_2^+ \]
\[ \text{Nitric} \quad \text{Superoxide} \quad \text{Peroxynitrite} \quad \text{Peroxynitrous} \]
\[ \text{oxide} \quad \text{radical} \quad \text{radical} \quad \text{acid} \]
\[ \text{ONO}_2^+ \rightarrow \text{OH}^- + \text{NO}_2^- \]
\[ \text{Hydroxyl} \quad \text{Nitrite} \quad \text{radical} \]
\[ \text{NO}_3^- \]
\[ \text{Nitrate} \]

Fig 4 : Representation of Reactions Occurring During NO and $O_2^-$ Interaction.

In mice and rats, NOS activity of macrophages can be induced by immunologic stimuli and their cytotoxic potential has been found to be proportional to their nitrite secreting capacity (Keller & Keist, 1989 and Stuehr et al., 1991).

**MODULATION OF NEUTROPHIL FUNCTIONS BY NITRIC OXIDE**

a) CHEMOTAXIS

Involvement of NO has been proposed by Kaplan et al., 1989 and Belenky et al., 1993, is an important PMNL function, the chemotaxis. Chemotaxis,
Review of Literature

from the vascular compartment leads to accumulation of PMNLs at site of microbial invasion. A variety of NO donors have been studied for their effect on chemotaxis in monocytes as well and few have been found to inhibit chemotaxis via a cyclic guanosyl monophosphate (cGMP) mediated mechanism which was tested by treating the cells with a cell permeable analogue of cGMP (Bath, 1993).

b) ADHESION

Leukocyte adhesion is mainly brought about by the glycoprotein CD11/CD18, as has been shown by the use of CD18 specific monoclonal antibodies IB4. Leukocyte adhesion to vascular endothelium is inhibited by endogenous nitric oxide. N\(^-\)Monomethyl L-arginine (L-NMMA) and N\(^-\)monomethyl L-arginine methyl ester (L-NAME), the two nitric oxide synthase inhibitors increased the leukocyte adhesion more than 15 folds. Incubation of isolated cat neutrophils with L-NMMA resulted in direct upregulation of CD11/CD18 (Kubes et al., 1991).

The L-NAME induced adhesion was inhibited by L-arginine but not by D-arginine (Kubes et al., 1991). Furthermore, Ma et al., 1993 provided evidence that PMNLs adherence to the coronary endothelium follows the decrease in basal release of endothelium derived NO after myocardial ischemia/reperfusion. Using cat neutrophils, they observed that myocardial ischemia without reperfusion did not increase the PMNL adherence to endothelial cells. However, the same was significantly increased after 20 mins of reperfusion (state where the NO production is decreased) and incubation of the arterial segments with L-arginine significantly attenuated the neutrophil adherence. The effects of L-arginine were stereospecific, as D-arginine did not attenuate the adherence, indicating the role of NO in controlling neutrophil adherence. Interaction of circulating leukocytes with the vascular endothelium is regulated by the balance of proadhesive and antiadhesive factors. Decreasing antiadhesive factors or increasing proadhesive factors would disturb the net balance and result in an increase in neutrophil adherence.

In the non-ischemic reperfused normal vascular endothelium, inhibiting NO production by L-NAME could promote the interaction of CD11/CD18 on the
PMNL surface with its major ligand, ICAM-1 (Intra cellular adhesive molecule) which is constitutively expressed on the unstimulated endothelium at low level of activity. During the early period of reperfusion, both adhesive molecules (ie CD11/CD18 on PMNL and ICAM-1 on endothelial cells) can be upregulated and NO production is significantly decreased.

The increase in proadhesive factors together with decrease in antiadhesive factors can promote PMNLs adherence.

NO mainly brings about its modulations of neutrophil adherence via cGMP. Lefer et al., (1993) have reported that NO donors can protect the reperfusion injury in cats by inhibiting the neutrophil-endothelium interaction.

As discussed earlier, the critical early event after reperfusion is endothelial dysfunction characterized by reduced release of EDRF/NO. Endothelial dysfunction leads to adherence of PMNLs to it. A sydnonimine NO donor (C87-3754), attenuated myocardial necrosis, and improved the survival of animals (Lefer et al., 1993). The NO donor reduced the PMNL adherence to endothelium and decreased the superoxide release from them, thereby reducing the oxidative damage to vasculature.

Yet another NO donor SPM-5185 has also been shown to reduce myocardial necrosis and neutrophil accumulation in an acute model of canine myocardial injury and reperfusion, probably due to inhibitory effects of NO donor on neutrophil adherence to coronary endothelium (Lefer et al., 1993).

On the basis of these research findings many authors have proposed that NO is an important endogenous modulator of leukocyte adhesion (Kubes et al., 1991; May et al., 1991; Ma et al., 1993 and Gaboury et al., 1993).

c) AGGREGATION

Involvement of NO has also been shown in PMNLs aggregation (McCall et al., 1988), neutrophil mediated tissue damage (Mulligan et al., 1991) and adhesion to endothelium (Kubes et al., 1991 and Niu et al., 1994).
Bhardwaj et al., 1988 have suggested that carbachol induced EDRF/NO release can inhibit calcium ionophore (A-23187) induced leukocyte aggregation *in vitro*. Further studies on the effect of NO on aggregation of rabbit polymorphonuclear leukocytes *in vivo* have shown that the effect of NO can be potentiated by superoxide dismutase. The attenuation of neutrophil aggregation by NO is mediated via activation of guanylate cyclase, as it could be augmented by selective cGMP phosphodiesterase inhibitor, M and B 22948 (McCall et al., 1988).

Neutrophil activation involves a coordinated sequence of events such as adherence, chemotaxis and degranulation, all of which have been shown to be regulated by NO.

**INTERACTION OF PMNLs DERIVED NITRIC OXIDE AND SUPEROXIDE RADICALS**

Role of NO in PMNLs is not well understood. Endogenous addition of NO to human neutrophils *in vitro* has been found to inhibit O$_2^-$ production (Clancy et al., 1992). On the other hand Rubanyi et al., in 1991, have reported that NO released from human PMNLs performs the cytoprotective function.

Dikshit et al., in 1993 demonstrated that circulating rat PMNLs release NO that was potentiated following thrombosis, where as Wang et al., (1993) reported that PMNLs do not release NO and by adding the extracellular arginine (the NO precursor), there was no synthesis of peroxynitrite radicals. Extracellular addition of L-arginine also resulted in inhibited O$_2^-$ release from circulating neutrophils (Weidemann et al., 1993).

The role of PMNL derived NO and its interaction with superoxide radicals on PMNLs function remains controversial and is yet to be studied in detail.