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# **MECHANISMS CONTRIBUTING TO THE ENHANCED RESPIRATORY BURST OF NEUTROPHILS OBSERVED IN PERIODONTITIS**

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## Summary

Inflammatory periodontitis is a common chronic condition and is initiated by plaque biofilm. Several periodontopathogenic bacteria, such as *Porphyromonas gingivalis* (*Pg*) have been implicated in the aetiology of this disease, which causes destruction of the connective tissue and bone around the root area of the tooth. The invading bacteria trigger the release of cytokines leading to elevated numbers and activity of neutrophils. Neutrophils are white blood cells that play a pivotal role as a first line in host defense against invading pathogens and foreign materials. These cells possess a cell membrane bound enzyme, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, which is responsible for the production of reactive oxygen species (ROS), including superoxide and hydrogen peroxide, and has an important role in bacterial killing. NADPH oxidase consists of a catalytic subunit gp91<sup>phox</sup>, the regulatory subunits p22<sup>phox</sup>, p47<sup>phox</sup>, p40<sup>phox</sup>, p67<sup>phox</sup>, and the small guanosine triphosphatase enzyme, Rac1. Natural endogenous antioxidants [e.g. glutathione (GSH)] protect the host cells at inflammatory sites from the damaging effects of ROS. If ROS production exceeds antioxidant defences, oxidative stress ensues, which is associated with chronic inflammatory conditions. Peripheral blood neutrophil hyperactivity and hyper-reactivity is well documented in periodontitis, however, the underlying mechanisms for increased ROS production remain unclear. Therefore, the aim of this thesis is to investigate possible mechanisms that may contribute to neutrophil hyperactivity and hyper-reactivity. One possibility is the presence of a neutrophil priming factors within the peripheral circulation of periodontitis patients. To examine this possibility differentiated HL-60 cells and primary neutrophils were studied in the presence and absence of plasma from periodontitis patients. In independent experiments, plasma was depleted of IL-8, GM-CSF, interferon- $\alpha$ , immunoglobulins and albumin. This work demonstrated that plasma factors such as IL-8, GM-CSF, and interferon- $\alpha$  present during periodontitis may contribute towards the reported hyperactive neutrophil phenotype. Furthermore, this work demonstrated that products from *Pg* may regulate neutrophil accumulation at infected periodontal sites by promoting gingipain-dependent modification of IL-8-77 into a more biologically active chemokine. To elucidate whether the oxidatively stressed environment that neutrophils are exposed to in periodontitis could influence hyperactivity and hyper-reactivity, neutrophils were depleted of glutathione. This work showed that during oxidative stress, where cellular redox-levels have been altered, neutrophils exhibit an increased respiratory burst. In conclusion, this work highlights the multiple mechanisms that may contribute to neutrophil hyperactivity and hyper-reactivity including gingipain-modulated activity of IL-8 variants, the effect of host factors such as IL-8, GM-CSF, interferon- $\alpha$  on neutrophil priming and activation, and the shift of neutrophil GSH:GSSG ratio in favour of a more oxidised environment as observed in periodontitis.



## Abbreviations

Ab, antibody; APS, ammonium persulphate; ASMase, acid sphingomyelinase; ATRA, All-*trans* retinoic acid; BCA, bicinchoninic acid; BSA, bovine serum albumin; BSO, buthionine sulfoximine; Bt<sub>2</sub>cAMP, dibutyl cyclic adenosine 3':5'-monophosphate; CAPP, ceramide activated protein phosphatase; COX-2, cyclooxygenase-2; CGD, chronic granulomatous disease; CL, chemiluminescence; CRP, C-reactive protein; DAG, diacylglycerol; DCFDA, 2',7'-dichlorofluorescein diacetate; DIG, detergent insoluble glycolipid enriched membranes; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; DRM, detergent resistance membrane; DNA, deoxyribonucleic acid; GPI, glycosyl phosphatidylinositol; DTNB, 5,5-dithio-bis-(2-nitrobenzoic acid); EDTA, ethylene diamine tetraacetic acid; ERK, extracellular-signal-regulated-kinase; ESAM, endothelial cell-selective adhesion molecule; FAD, flavine adenine dinucleotide; FBS, foetal bovine serum; FcR, Fc receptor; FITC, fluorescein isothiocyanate; FS, forward scatter; fMLP, formyl-met-leu-phe; GCF, gingival crevicular fluid; GCSF, granulocyte colony stimulating factor; GEF, guanine nucleotide exchange factor; GEMs, glycolipid enriched membranes; GM-CSF, granulocyte macrophage colony stimulating factor; Gp, gingipain; GPCR, G-protein coupled receptor; GSH, glutathione; GSR, glutathione reductase; GSSG, oxidised glutathione; GTP, guanine triphosphate; HSPs, heat shock proteins; HL-60, human leukemic cell line-60; HOCl, hypochlorous acid; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HRP, horse radish peroxidase; ICAM, intracellular adhesion molecule; IFN- $\alpha/\gamma$ , interferon- $\alpha/\gamma$ ; IFNAR, type-1 interferon receptor; IgG, immunoglobulin G; IL-, interleukin-; IP, immunoprecipitation; IP<sub>3</sub> inositol 1,4,5-trisphosphate; ISGs, IFN-stimulated genes; IRF, Interferon regulatory factor; JAM, junctional adhesion molecule; JAK, Janus kinase; JE, junctional epithelium; LBP, LPS-binding protein; LC, liquid chromatography; LDL, low density lipoprotein; LPS, lipopolysaccharide; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; LR, lipid raft; MAPK, mitogen activated protein kinase; MdX, median X; M $\beta$ CD, methyl- $\beta$ -cyclodextrin; MCP, monocyte Chemotactic protein; MPO, myeloperoxidase; MS, mass spectroscopy; MW, molecular weight; NAC, N-acetyl cysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NETs, neutrophil extracellular traps; NO, nitric oxide; Nod, nucleotide-binding oligomerisation domain proteins; O<sub>2</sub>, oxygen molecule; O<sub>2</sub><sup>-</sup>, superoxide anion radical; OH $\cdot$ , hydroxyl radical; ONOO $\cdot$ , peroxynitrite; PAF, platelet activating factor; PAMPs, pathogen associated molecular patterns; PBS, phosphate buffered saline; PECAM, platelet endothelial cell

adhesion molecule; PEG, prostaglandins; PGNs, peptidoglycans; Pg, *Porphyromonas gingivalis*; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLA<sub>2</sub>, Phospholipase A<sub>2</sub>; PPR, pathogen recognition receptors; PRR, proline rich region; PSS, physiological salt solution; PVDF, polyvinylidene Fluoride; RLU, relative light units; ROS, reactive oxygen species; RNS, reactive nitrogen species; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SOD, superoxide dismutase; SS, side scatter; SSA, sulfo salicylic acid; STAT, signal transducer and activator of transcription; TEMED, N,N,N',N'-tetramethylethylenediamine; TCA, trichloro acetic acid; TIEFs, Triton-insoluble floating factors; TLR, toll-like receptor; TNF- $\alpha$ , tumor necrosis factor alpha; VCAM, vascular endothelial adhesion molecule; VVO vesiculo-vascular organelles; WB, western blotting.

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## **Chapter 1.0: General Introduction**

## **1.1 Inflammation**

Inflammation is phylogenetically the oldest defence mechanism which is initiated as the first line of protective response without a requirement for previous experience and recognition (Kindt et al., 2006). Inflammatory responses are the first group of organised reactions that arise against any damage or bacterial infection. The complex host systemic and local inflammatory responses are clinically characterised by basic symptoms such as redness, swelling, heat, pain, and result in loss of function. These symptoms are common in everyday life and have been reported as early as the first century AD by Celsus. During the inflammatory process, increased vascular permeability allows blood cells in the vasculature to infiltrate into the site of infection/injury. Importantly, inflammation is a local response which extends to involve many systems and organs such as the central nervous system, cardiovascular system (increased heart rate), liver (elevated plasma protein production), and endocrine systems (adrenal) all of which contribute to a host's dynamic response (Janeway et al., 2005, Kindt et al., 2006). Therefore an undesirable consequence of extensive inflammation is a systemic loss of homeostasis.

Inflammation is divided into acute and chronic inflammation in the literature (Janeway et al., 2005, Kindt et al., 2006). However, apart from some discrete differences, these two processes form a continuum. Acute inflammation develops over minutes or hours depending on the type and the severity of the tissue damage and generally lasts for hours to days (Janeway et al., 2005). The processes of acute inflammation are often initiated by resident cells such as macrophages, which preexist in all tissues and therefore provide a fast response to the stimulus (Chaudhuri and Sabroe, 2008). The acute inflammatory response is not a disease itself but is the host response that attempts to limit the extent of



the damage, counteract infection and promote healing and recovery of function. Activated macrophages release small molecule inflammatory mediators such as nitric oxide (NO), prostaglandins, leukotrienes, platelet-activating factor (PAF ) and the inflammatory chemokine and cytokine proteins such as TNF- $\alpha$  and IL-1 (Chaudhuri and Sabroe, 2008, Ley et al., 2007, Janeway et al., 2005). NO is a potent vasodilator that stimulates arteriolar dilatation (Eiserich et al., 1998), which leads to an increase blood flow to the area of the infection. Increased vascular permeability results from the action of prostaglandins (Birukova et al., 2007) enables increased movement of plasma and leukocytes from plasma into the injured tissue via diapedesis (section 1.1.2). These chemical mediators together with bacterial stimuli such as lipopolysaccharide (LPS) (Fokkema et al., 2002) further amplify the inflammatory response by stimulating recruited cells such as leukocytes and resident mast cells, endothelial cells and also epithelial cells to produce mediators including prostaglandins, leukotriene B<sub>4</sub> (LTB<sub>4</sub>), interferon- $\gamma$  (IFN- $\gamma$ ), cytokines and activated complement (Kindt et al., 2006). Acute inflammation exists to eliminate pathogens after invasion and also to clear accumulated leukocytes in the tissue to restore the host homeostasis. The restoration of tissue homeostasis following acute inflammation involves the production of late inflammatory lipid mediators such as eicosanoids; lipoxins, resolvins and protectins (Serhan and Savill, 2005, Serhan, 2007, Dyke, 2008). The activation of leukocyte signaling pathways via these mediators is suggested to promote the resolution of inflammation through a number of mechanisms. These include; limiting neutrophil chemotaxis (Serhan, 2007); activating monocyte phenotypes with non-superoxide generating activity; inducing neutrophil apoptosis; and stimulating the uptake of apoptotic neutrophils by macrophages (Serhan and Savill, 2005). However, the process of resolution is overcome by the progression of an acute response to a chronic response, which is evident in many chronic inflammatory diseases

such as chronic bowel disease, periodontitis (Dyke, 2008), atherosclerosis (Hansson et al., 2002), and rheumatoid arthritis (Griffiths, 2008).

Chronic inflammation associates with simultaneous healing and tissue destruction, which results in destruction of extra-cellular matrix, scarring, and fibrosis. Chronic inflammatory responses may last for days, months and even for years (Janeway et al., 2005). The processes of tissue damage and injury results from the activation of the acute response, but most infections, e.g. viral and fungal, can stimulate chronic inflammatory responses if the initiating infection is not removed. In the chronic response, a progressive shift in the type of cells that are present at the site of inflammation is observed. Briefly, polymorphonuclear leukocytes (neutrophils) are the first white blood cells to infiltrate into site of infection; these cells are usually observed within an hour of the initiating stimulus. Non-activated neutrophils have an average life span of 12 hours in the circulation and survive after migration into a tissue for 1-2 days (Witko-Sarsat et al., 2000). Having a short life span, the activity of neutrophils slowly diminishes, but their numbers are maintained at the site by new arrivals from the circulation; the systemic response to acute inflammation induces release of neutrophils from the bone marrow into the blood (Stevens et al., 2002).

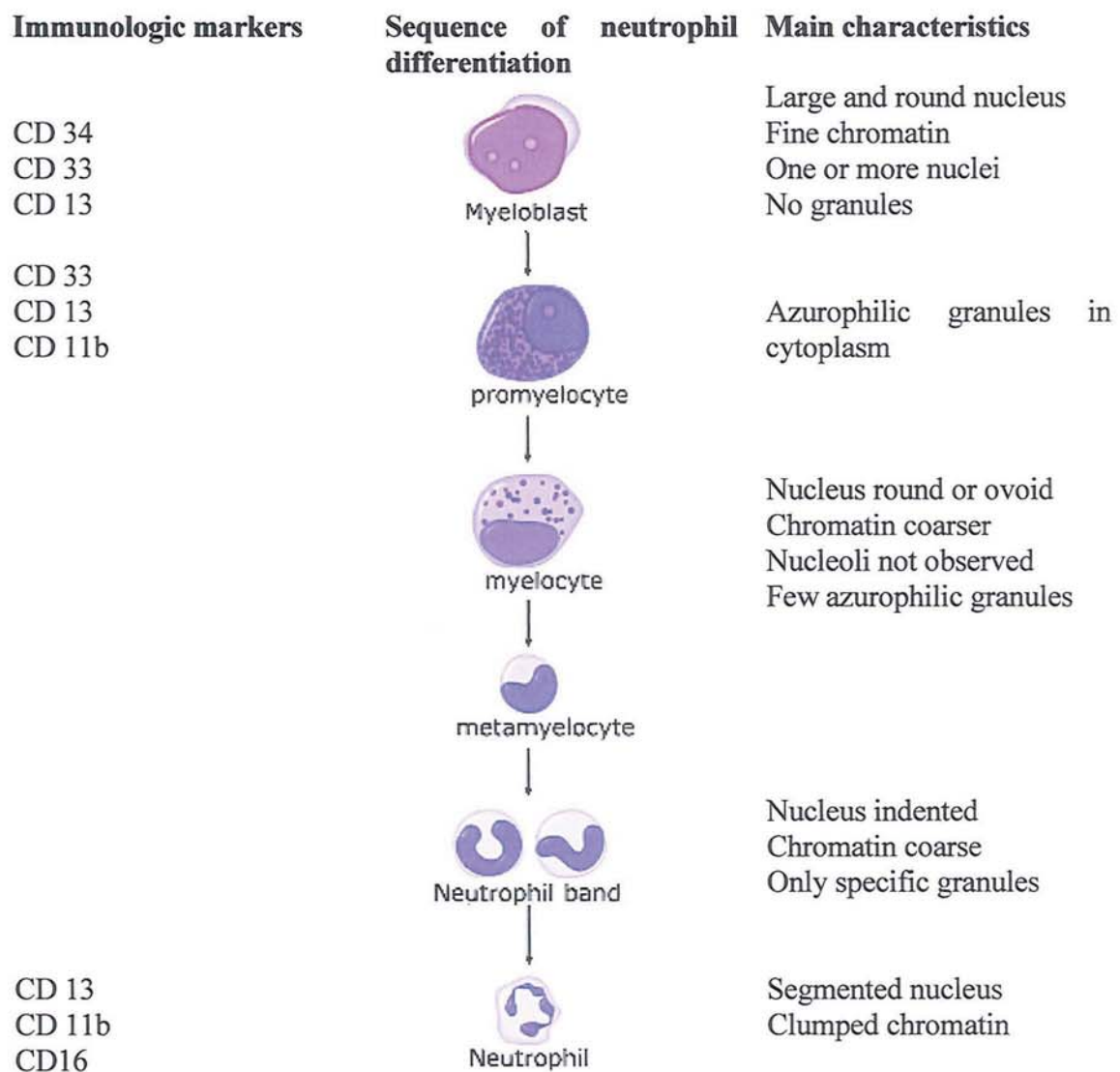
It has been reported that in the later stages of acute inflammation, increases in influx of the mononuclear leukocyte (monocytes/macrophages) population are observed at the site, thereby decreasing the neutrophil to macrophage ratio (Stevens et al., 2002). However, this generalised statement has been recently re-evaluated, where the existence of at least two subtypes of neutrophils have suggested (Connally, 2003). Degenerate neutrophils with condensed (pyknosis) and fragmented (karyorrhexis) nuclei are observed in cytological specimens of the acute disease condition, abdominocentesis, alongside

bacterial etiological agents (Connally, 2003). In contrast, inflammation with non-degenerative type neutrophilic phenotypes is observed in association with severe irritation away from the infection (Connally, 2003, Forsyth et al., 2007).

### **1.1.1 Myelopoiesis of neutrophils**

Neutrophils are the most abundant white blood cells comprising 50-60% of the total circulating leukocytes. Neutrophil development takes place in the bone marrow from hematopoietic stem cells derived from a common hematopoietic stem cell that includes erythroid, granulocytic, monocytic, and megakaryocytic lineages. Early stages of cell development from pluripotent stem cells are regulated by local bone marrow cytokines such as IL-7 and IL-3 (Barreda DR et al., 2004). During this period, cells undergo proliferation and differentiation. Later, cells differentiate under the influence of growth factors such as granulocyte colony stimulating factor (GCSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Barreda DR et al., 2004). During the process of maturation, cells pass through morphologically and physiologically distinct stages namely, myeloblast, promyeloblast, myelocyte, metamyelocyte, non-segmented (band) neutrophil, and segmented neutrophil (Figure 1.1). Neutrophils are fully matured when they released into the circulation (Witko-Sarsat et al., 2000).





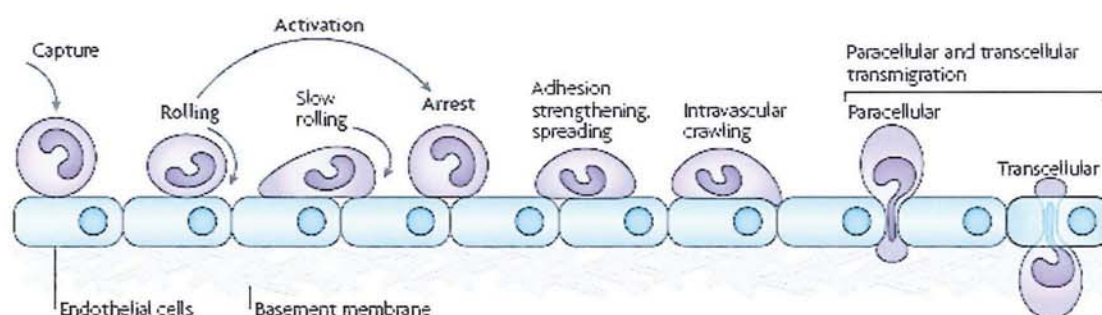
**Figure 1.1 Maturation sequence of myelocyte lineage into neutrophils.** Myelocyte lineage passes through morphologically distinct stages before resulting in a mature neutrophil. These morphological changes include the shape of the nucleus, the state of chromatin and type of the granules present. In histological and cytological preparations, neutrophil granules are stained neutral pink with hematoxylin and eosin stains. Stages of neutrophil differentiation can also be monitored by changes of immunologic markers, identified by monoclonal antibodies. However, this approach does not differentiate close stages as markers can overlap between two stages. Figure adapted from (June 2009): <http://en.wikipedia.org/wiki/Hematopoiesis>.

The matured neutrophils are easily recognisable by their unique morphology. The neutrophil cytosol is densely populated with azroophilic or primary and specific or secondary granules. Neutrophils in their mature form possess characteristic multilobular nuclei (3-5 lobes) with no nucleolus. Human bone marrow produces around  $10^{11}$  neutrophils per day and  $2.5-7.5 \times 10^9$  neutrophils per litre of blood are found circulating in healthy individuals. Neutrophils are abundant in the blood but not present in normal, healthy tissues. Neutrophils are partitioned in the blood between a circulating pool; present in large and small blood vessels and marginated pool; transiently arrested in narrow capillaries (Witko-Sarsat et al., 2000). Thus, cells of circulating and marginating pools can exchange with each other at any time.

### **1.1.2 Neutrophil adhesion and migration**

The recruitment of activated phagocytes to the sites of infection is one of the most important functions in innate immunity. Recruitment is mediated via chemotaxis and by cell-adhesion molecules that are induced on the surface of the local blood vessel endothelium. Local injury or infection results in production of inflammatory mediators such as prostaglandins,  $\text{LTB}_4$ ,  $\text{IFN-}\gamma/\alpha/\beta$ ,  $\text{TNF-}\alpha$ , IL-1, and IL-8 which act as chemoattractants to recruit neutrophils to the site of injury (Witko-Sarsat et al., 2000). This is further facilitated by vasodilation at the inflamed site that contributes to a slowing down of blood flow and thereby increasing leukocyte interaction with vascular endothelium. The migration of leukocytes out of blood vessels into tissues or lumen is known as extravasation. Neutrophil extravasation proceeds via a three step adhesion cascade; slow rolling, tight adhesion, diapedesis and migration through basement membrane (Figure 1.2) (Ley et al., 2007). The whole process is mediated through

adhesion receptors and their ligands presented on neutrophils and interacting endothelial cells. Several structural families of adhesion molecules have a role in leukocyte extravasation namely, selectins, integrins and the immunoglobulin superfamily of cell adhesion molecules (Ley et al., 2007, Witko-Sarsat et al., 2000).



**Figure 1.2 Schematic representation of different steps and methods involved in neutrophil chemotaxis through endothelial cells (Ley et al., 2007).** In the systemic blood flow neutrophils are either freely flowing or undergo reversible adhesion with adhesion molecules on the endothelium. During inflammation/injury, stimulated endothelium expresses increased level of adhesion molecules, which arrest neutrophils on the endothelium. Arrested neutrophils in the presence of the proinflammatory stimuli, make stronger attractions with the endothelium and generate a change of the cytoskeleton and increase the flexibility to change shape of the cell. These morphological changes facilitate their diapedesis and migration through the endothelium into the infected/injured sites.

The early stages of neutrophil interaction with endothelial cells are mediated by neutrophil expressed L-selectins and endothelial cell expressed P or E selectins. Selectins are membrane glycoprotein receptors that bind to specific carbohydrate ligands on cells. P and E selectins interact with the sulfated-sialyl-Lewis<sup>x</sup> moiety of glycosylated ligands on neutrophils. These interactions allow a loose but transient adhesion and rolling of leukocytes over the endothelium. Most neutrophils express L-selectins although these do not have high affinity for ligands when neutrophils are at rest. However, L-selectin binding capacity is rapidly and transiently up-regulated upon neutrophil activation



possibly through receptor dimerisation (Li et al., 1998). Expression of E- and P selectins on endothelial cells is driven by interaction with many agonists including macrophage-derived cytokines, especially TNF- $\alpha$  or interaction with bacterial LPS. Additionally, in response to LPS, endothelial cells up-regulate production of secretory IL-8 and also store the chemokine in Weibel-Palade bodies and release it upon further stimulation (Utgaard et al., 1998). On the other hand secreted TNF- $\alpha$  and IL-8 together with bacterial LPS or fMLP may activate neutrophils to degranulate or to produce a respiratory burst (see section 1.2.4). Changes in membrane structure during the activation process may contribute to altered receptor clustering (Zhang et al., 2007) and these changes may support a prolonged respiratory burst (section 1.3.4).

Binding of isolated neutrophils to E-selectin induces integrin activation through a p38 mitogen activated kinase (MAPK) dependent pathway for cell survival (Simon et al., 2000). Neutrophil integrins encounter intercellular adhesion molecules (ICAMs) on endothelial cells and form firm yet reversible attractions. This type of adhesion allows neutrophils to continue their rolling motion as well as to make more firm interactions between different types of integrins expressed on endothelial cells (Simon et al., 2000). Integrin-adhesion molecule interaction of neutrophils with endothelial cells appears to be exclusively dependent on  $\beta_2$  subfamily engagement of (CD11a, CD11b, CD11c/CD18) with ICAM-1 (Ley et al., 2007). *In vitro* and *in vivo* studies have demonstrated that neutrophil movement arrest on endothelial cells requires enhanced interaction with integrins i.e. integrins in the unstimulated state have low binding affinity for endothelial cells (Berton and Lowell, 1999, Simon et al., 2000, Ley et al., 2007). These interactions are facilitated by chemokines expressed and secreted by endothelial cells, and high affinity cytokine receptors on neutrophils. Chemokines that bind to G-protein coupled receptors (GPCRs) are activated and correspondingly the integrin expression is increased



within milliseconds (Ley et al., 2007). The up-regulation of ligand binding capacity or neutrophil integrins upon activation signals such as chemokines is referred to as inside-out signaling. The absence of neutrophil  $\beta_2$  integrin coupling with their corresponding endothelial ligands in the unstimulated state provides a mechanism for the control of neutrophil extravasation.

The final step of neutrophil extravasation includes transendothelial cell migration or diapedesis. Neutrophils are arrested on the endothelial cell surface and migrate through the blood vessel with minimal disruption to the complex structure of the vessel walls. Leukocytes migrate through the endothelial cell barrier rapidly (<2-5min) then pass through the basal membrane (>5-15min) (Ley et al., 2007). Transendothelial migration of neutrophils is traditionally described via a route of endothelial cell junctions. However, recent findings suggest that this can also be achieved by a transcellular pathway. Some endothelial junctional molecules actively mediate neutrophil transendothelial migration. These molecules include immunoglobulin superfamily members such as platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31), junctional adhesion molecule (JAM), and endothelial cell-selective adhesion molecule (ESAM) (Martin-Padura et al., 1998, Wegmann et al., 2006). Neutrophil migration through endothelial junctions is facilitated by loosening of tight junctions such as release of endothelial-expressed vascular endothelial cadherin (VE-cadherin) and secondly, the release of PECAM-1, JAM and ESAM. These interactions enable neutrophils to squeeze between endothelial junctions. Transcellular migration was reviewed recently (Ley et al., 2007) and reported to occur in thin parts of the endothelium. The process involves formation of intracellular channels with the aid of vesiculo-vascular organelles (VVO), through which neutrophils can migrate.

### 1.1.3 Pathogen recognition

Pathogens or non-self molecules are consistently found with small molecular patterns called pathogen associated molecular patterns (PAMPs) (Mogensen, 2009). The inflammatory neutrophils and monocytes have toll-like receptors that recognise PAMPs and enable them to identify and destroy invading microbes. The host has developed pathogen recognition receptors (PPRs) that are able to recognise specific PAMPs such as bacterial lipopolysaccharides (LPS), formyl-methionyl-leucyl-phenylalanine (fMLP) and fimbriae, peptidoglycans (PGNs), lipoteichoic acids (LTA) from gram positive bacteria, bacterial and fungal proteases, heat-shock proteins (HSPs) and toxins (Madianos et al., 2005).

These non-self signals are recognised mainly through toll-like receptors (TLRs), GPCRs or by nucleotide-binding oligomerization domain protein- (Nod) like receptors (NLRs) in host cells (Madianos et al., 2005). Receptor activation initiates the downstream signaling pathway, which stimulates several transcription factors such as NF-kappaB, receptor-interacting protein (IRF), MAPK, PI-3K and signal transducer and activator of transcription (STAT), that up regulate gene expression of many cytokines such as IL-6, IL-10, IL-12, and TNF- $\alpha$  (Makela et al., 2009). As a result, epithelial cells, mast cells, fibroblasts and macrophages secrete proinflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, IL-12 tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), IFN inducible protein-10 (IP-10), monocyte chemotactic protein-5 (MCP-5), and also increase cyclooxygenase-2 (COX-2) expression leading to elevated levels of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (Mogensen, 2009).

Cytokine and chemokine production is important to combat microbial pathogenesis in many ways. Primarily, chemokines form a concentration gradient from the site of

infection, which enhance the chemotaxis (Baggiolini, 1998, Silva et al., 2007). Second, some of these mediators, such as IL-1 $\beta$ , TNF- $\alpha$ , and histamine along with other bacterial factors activate host endothelial cells that create positive feedback reaction (Makela et al., 2009, Minami et al., 2007). Third, cytokines increase surface receptors on endothelial cells that are important in leukocyte extravasation (Sugiyama et al., 2002). Certain cytokines are responsible for up-regulation of specific cell surface receptors. For an example, TNF- $\alpha$  and histamine induce expression of P-selectin and E-selectin (Miki et al., 1996), whilst ICAM-1, -2, IL-8 receptor and MCP-1 are up-regulated on endothelial cells in the presence of TNF- $\alpha$ , and IL-1 $\beta$  (Chen et al., 2009).

## **1.2 Neutrophil microbial killing**

The ability of neutrophils to engulf and degrade microbes at the site of infection indicates a primary killing function. This microbicidal function is enabled through specific mechanisms that exist in neutrophils; with the recognition of targeted particles, neutrophils initiate degranulation, phagocytosis, and production of neutrophil extracellular nets (NETs) and each of these processes is described in the following sections.



### 1.2.1 Degranulation

A great majority of neutrophilic functions involve their ability to mobilise and release cytoplasmic granules and secretory vesicles. Biogenesis of these reservoirs occurs during the neutrophil differentiation and maturation pathway and therefore the cells are pre-prepared to release granule contents upon cell activation. Early work suggested that primary or azurophilic granules are the first to develop along with neutrophil differentiation and they contain myeloperoxidase, serine proteases, and antibiotic proteins (Fouret et al., 1989). The secondary granule population contains lactoferrin and collagenase, and later in development, granules containing gelatinases are produced. Secretory vesicles are produced in maturing neutrophils at a very late stage. However, the contents of different classes of granules may not absolutely discrete as proteinase 3 and serine proteases, originally found in azurophilic granules, have also been found in secretory vesicles (Witko-Sarsat et al., 1999). Upon exposure to inflammatory stimuli, neutrophil granules are exocytosed. Through this process, previously stored receptors retained in the endoplasmic reticulum or golgi vesicles are incorporated into the plasma membrane. Newly membrane-incorporated receptors such as  $\beta$ 2-integrin, fMLP receptor, CD35, CD66 on the neutrophil surface are necessary for the early phase of inflammatory response and pathogen recognition to direct the phagocytosis response (Borregaard and Cowland, 1997, Sengelov et al., 1994).

### 1.2.2 Phagocytosis

Phagocytosis is a special form of endocytosis, where neutrophils recognise pathogens or foreign particles through receptors such as PRRs and engulf them into the cell within a phagosome (Botelho et al., 2000). Neutrophil phagocytosis involves mainly two different receptor classes, FcγReceptors; FcγRIIIA (CD32) and FcγRIIIB (CD16) and complement receptors; CR1 (CD35) and CR3 (CD11b/CD18 integrin) (Witko-Sarsat et al., 2000). These two types of receptors trigger two different signaling pathways to initiate discrete phagocytic mechanisms. Fcγ-receptor mediated phagocytosis includes activation of Src family and Syk tyrosine kinases at the phagocytic cup followed by stimulation of inositol signaling pathway to produce IP<sub>3</sub>, which is responsible for the rise in the free cytosolic Ca<sup>2+</sup> concentration observed during Fcγ mediated phagocytosis (Botelho et al., 2000). Rac and Cdc42, members of the Rho family of small GTPases, are involved in early F-actin recruitment and producing membrane protrusions through cytoskeletal rearrangements form a phagocytic cup (Greenberg et al., 1996, Hackam et al., 1997, Green et al., 1996). Polymerisation of actin, together with the activation of the IP<sub>3</sub> sensitive myosin X drives the formation of fully closed phagosomes around the microbe (Lee et al., 2003). These membrane surrounded vesicles are internalised via a dynamin dependent pathway (Desjardins, 2003).

Immediately after its formation, the membrane of the phagosome is comparable to the cell membrane and its contents are mainly extracellular fluid. Soon after endocytosis, the phagosomes undergo series of changes both in membrane composition and its contents by a process known as maturation. Following formation, nascent vacuoles are targeted to fuse with early endosomes and lysosomes, which contain active lipases and proteases in

an extremely acidic environment  $\text{pH} \leq 5.5$  (Vieira et al., 2002) generated via the activity of vascular-type  $\text{H}^+$ -ATPases (Lukacs et al., 1990). These are then termed as phagolysosomes and possess a number of degradative properties, including a very low pH, hydrolytic enzymes, defensins and other bactericidal peptides, and the ability to generate the respiratory burst via active nicotinamide adenine dinucleotide phosphate-(NADPH) oxidase enzymatic complex.

### **1.2.3 Neutrophil extracellular traps**

Neutrophil extracellular traps (NETs) are another, recently identified mode of neutrophilic antimicrobial action (Brinkmann et al., 2004). NETs are composed of mixed chromatin and granule proteins and are produced from activated neutrophils (Brinkmann et al., 2004, Brinkmann and Zychlinsky, 2007). These web-like structures are suggested to trap gram positive and gram negative bacteria from spreading and microbicidal enzymes are then involved in bacterial killing (Brinkmann et al., 2004). The formation of NETs is the final step in a program of active cell death and is clearly distinguished from apoptosis and necrosis by morphological and molecular criteria (Fuchs et al., 2007). NETs forming neutrophils have been shown to have a disintegrated nuclear envelope and granule membranes in the absence of DNA fragmentation. However, the morphology of cytoplasm and organelles remained unchanged (Fuchs et al., 2007). Furthermore, apoptotic marker caspases were not reported to be up-regulated during NETs formation (Fuchs et al., 2007). A similar mechanism of action was recently observed in mast cells (von Köckritz-Blickwede et al., 2008). These were termed as extracellular structures formed by mast cells (MCETs) and found to compose of DNA, histones, tryptase, and the antimicrobial peptide LL-37 (von Köckritz-Blickwede et al., 2008). Disruption of these



structures significantly reduced antimicrobial activity of mast cells. Pathogen virulence factors against NETs are also reported in literature. Bacteria such as *Streptococcus pneumoniae* has been shown to produce DNases to degrade NETs (Buchanan et al., 2006) or produce capsules and modified lipoteichoic acid (LTA) as a protection mechanism in NETs (Wartha et al., 2007).

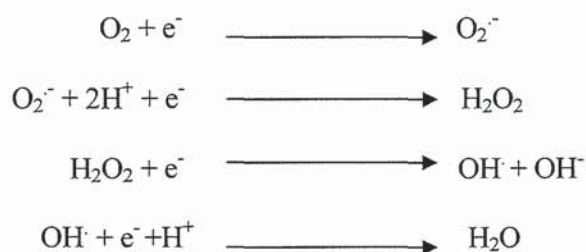
NET formation is mediated by ROS-dependent cell death mechanism (von Köckritz-Blickwede et al., 2008). It has shown that addition of hydrogen peroxide, PMA or inhibition of neutrophil catalases can promote NETs formation (Fuchs et al., 2007, von Köckritz-Blickwede et al., 2008). It is also observed that neutrophils from patients with CGD are unable to produce NETs, further highlighting the importance of ROS produced by activated NADPH oxidases.

#### **1.2.4 Neutrophil respiratory burst**

The consumption of oxygen during the generation of reactive oxygen species has been termed the respiratory burst or oxidative burst, although it is unrelated to cellular respiration or energy production. Even though a low level of rapid ROS generation is observed in many cell types such as epithelial and endothelial cells (Nisimoto et al., 2008, Zhang et al., 2007, Pendyala et al., 2009), neutrophils and macrophages are the major ROS producers via the respiratory burst. The oxygen molecule is a stable diradical, represented by  $\cdot\text{O}-\text{O}\cdot$  and has two parallel spin electrons. This makes it highly combustible and reactive due to the single electron in the outermost orbitals. During the process of the respiratory burst, the assembled membrane-bound enzyme NADPH



oxidase transfers one electron onto an oxygen molecule. As a result, the oxygen molecule carries an unpaired electron (free radical), which makes it highly reactive. Free radical ions rapidly and spontaneously react with other atoms or molecules in the vicinity. Half-lives of these species are reported to be very short ( $10^{-9}$ - $10^{-6}$  seconds) (Chapple, 1996). Therefore they initiate free radical chain reactions generating further radicals and radicalised ions until the process is terminated by formation of a stable species. Superoxide anion radical, generated as a result of transferring an electron, is ultimately reduced to water which is accompanied by a large amount of free energy release. During this process one electron is added in four stepwise reduction steps.

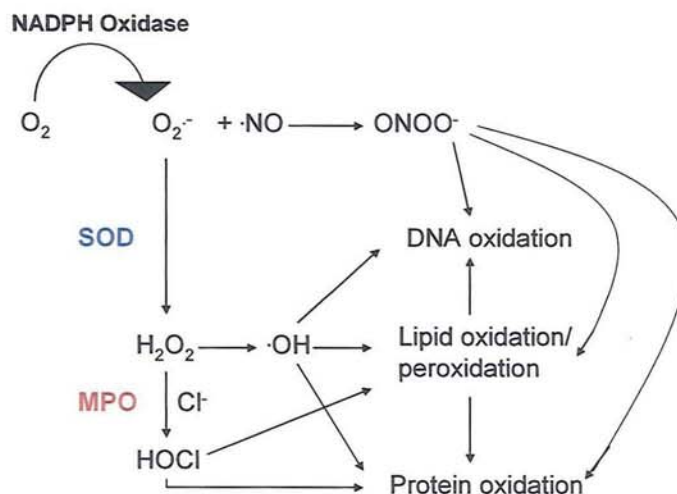


Neutrophils are found to produce large quantities of superoxide anion radicals. However, these radicals rapidly undergo dismutation with the aid of the superoxide dismutase enzyme. Therefore, cells generally retain only  $\mu\text{M}$  range of  $\text{O}_2^{\cdot -}$  in steady state conditions. The hydroxyl radical is extremely reactive and is usually depleted at the site of production by non-specific reaction with all biomolecules. Hydrogen peroxide on the other hand is a small lipophilic molecule that can permeate through membranes and therefore may act as an intracellular signaling molecule (Hancock, 1997, Hancock et al., 2001, Schröder and Eaton, 2008).

### 1.2.5 Other reactive metabolites

One of the principal enzymes released into phagosomes/extracellular space upon neutrophil activation is myeloperoxidase (MPO), a haem protein that plays an important role in the oxidative pathway. The secondary NADPH oxidase product, hydrogen peroxide, interacts with myeloperoxidase and a halide ion, predominantly chloride, to generate the potent antibacterial substance, hypochlorous acid (HOCl) (Figure 1.3). In addition to the antimicrobial effects, the MPO-HOCl system has been shown to oxidise low-density lipoprotein (LDL) (Winterbourn et al., 2000) and impact deleteriously on nitric oxide bioavailability (Eiserich et al., 1998).

Although different reactive oxygen and nitrogen species have been found to be produced in large quantities in activated neutrophils, their adverse effects on host can be limited by intracellular and extracellular antioxidants.



**Figure 1.3 Different oxidising species generated from superoxide and their effects on biomolecules.** Superoxide generated as a result of the neutrophil respiratory burst is involved in oxidising other intracellular or extracellular molecules. Superoxide dismutase (SOD) in the vicinity of the NADPH oxidase dismutates  $O_2^{\cdot-}$  into  $H_2O_2$ . Myeloperoxidase (MPO) in the presence of a halide ion (most commonly chloride) generates HOCl, which is a strong oxidizing molecule. Superoxide reacts with NO to yield the potent biological oxidizing and nitrating species peroxynitrite ( $ONOO^-$ ) and its conjugate acid, peroxynitrous acid ( $ONOOH$ ).

### 1.2.6 Cellular redox balance

Endogenous cellular antioxidants inactivate oxidant free radicals and protect aerobic cells from oxidant injury. Different antioxidants are present at wide range of concentrations in body fluid and tissues. Among these, glutathione (GSH) is the most abundant non-protein thiol in almost all aerobic species (Wang and Ballatori, 1998). Neutrophils have an intracellular GSH concentration of  $2\text{--}5\text{ nmol}/10^6$  cells (Naisbitt et al., 1999, Pirmohamed et al., 1996). However, extracellular GSH levels are 3–4 fold lower than intracellular



levels. Its main antioxidant activity consists of the detoxification of peroxides. In the presence of free radicals or peroxides, GSH is oxidised to glutathione disulfide (GSSG), which is rapidly reduced back to GSH with the aid of enzyme GSSG reductase at the expense of reduced NADPH (Griffiths et al., 1998, Cesaratto et al., 2004). The alteration of GSH and GSSG creates an intracellular redox system, which maintains high cellular GSH level under normal conditions, where GSSG reductase effectively maintains 98% of cellular GSH in its reduced state (as reviewed in Wang and Ballatori, 1998). This provides a safe intracellular environment for proteins and protects most macromolecules from oxidative damage.

In addition to scavenging ROS, GSH plays an important role in forming mixed GSH S-conjugates with wide range of electrophilic chemicals. GSH forms thioether bonds with leukotrienes, prostaglandins, dopa, dopamine, ascorbic acid, maleic acid and forms thioesters with cysteine, coenzyme A, and wide range of proteins (Wang and Ballatori, 1998). With other protein thiols, under oxidising conditions GSH may form mixed disulfides with proteins; this is called protein glutathiolation (Di Stefano et al., 2006, Brennan et al., 2006, Phillips et al., 2009). In principle, protein glutathiolation is a result of GSSG reacting with protein thiol or an oxidised protein cysteine reacting with GSH (Mieyal et al., 2008). Furthermore, proteins may be glutathiolated either by thiol/disulphide exchange between protein cysteines by direct oxidation or through the NO-mediated formation of S-nitrosogluthione (Phillips et al., 2009, Mieyal et al., 2008).

Protein glutathiolation is generally considered as deleterious as it may change physical and functional properties of the modified protein. Even though protein glutathiolation is another form of thiol oxidation induced directly by ROS and RNS, more recent studies recognise that this phenomenon is important to regulate the reactivity of some proteins (Cesaratto et al., 2004, Forman and Torres, 2001, Zhang et al., 2007, Phillips et al., 2009, West et al., 2006). These groups of proteins are termed as redox sensitive proteins and have one or more accessible redox sensitive cysteine residues in their structure. Proteomic studies suggest that even in the presence of a highly reducing intracellular milieu, some redox sensitive cysteine residues can undergo reversible oxidations to form disulphides and glutathiolated proteins (Camerini et al., 2005). Changes in cellular redox balance, by shifting GSH/GSSG ratio in the cellular milieu, are consistent with a more oxidizing environment (Hancock et al., 2001, Hancock et al., 2003).

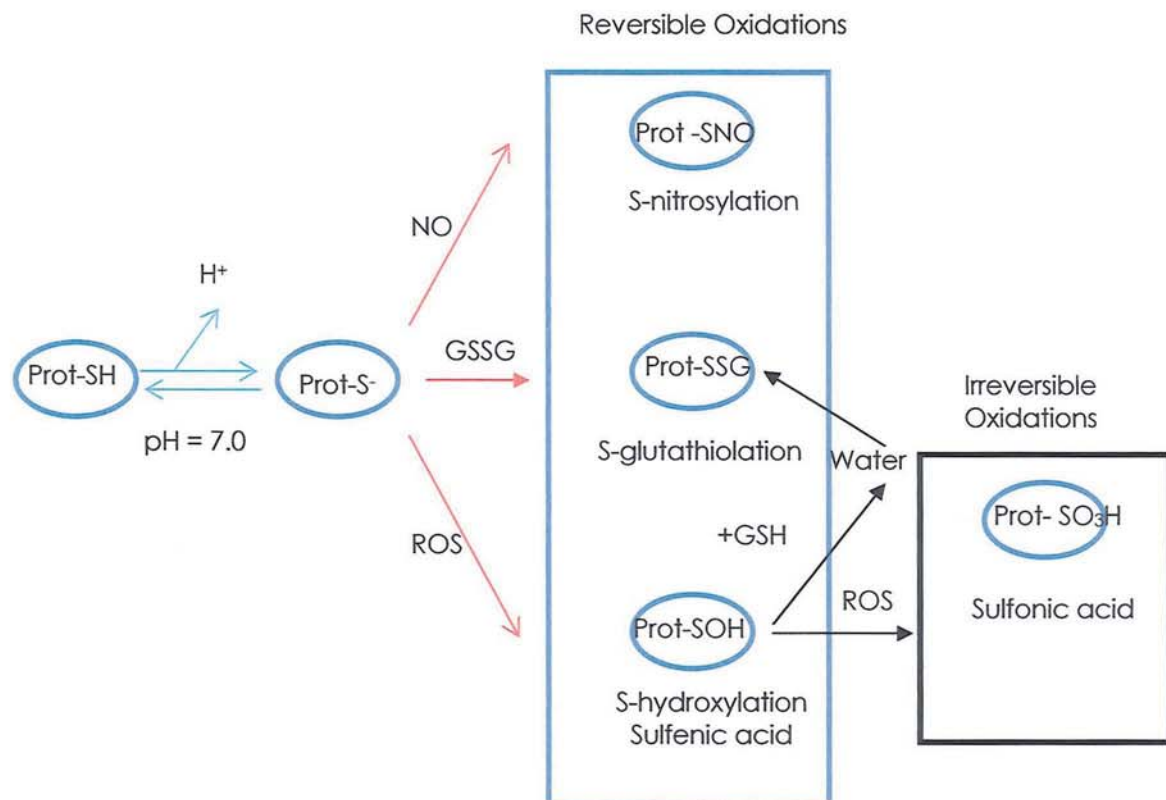
Protein cysteinyl thiols that undergo oxidative addition reactions give rise to several oxidation states including sulfenic (-SOH), sulfinic (-SO<sub>2</sub>H) and sulfonic (-SO<sub>3</sub>H) derivatives (Jacob et al., 2003). Biochemically, formation of cysteine sulfenic (Poole et al., 2004) and sulfinic acids (Woo et al., 2003) are reversible in the presence of reducing enzymes/agents such as thioredoxin (Trx), glutaredoxin (Grx) or GSH (Figure 1.4). Further oxidised state of sulfonic acids however, found to be irreversible modify the subjected protein.



**Figure 1.4: Reversible oxidation of cysteine thiol**

On the basis of high reactivity with reducing agents, the cysteine sulfenic acids have been identified as intermediates of biochemical reactions (Figure 1.5) that are readily reduced into their original thiol (Reddie and Carroll, 2008). In some proteins the formation of higher oxidation states of sulfinic or sulfonic acids could form irreversible interactions where the protein would lose its functionality by structural alterations (Jacob et al., 2003, Reddie and Carroll, 2008). It is evident that protein sulfenic acids have more biochemical importance than other forms of intermediates (Denu and Tanner, 1998, Poole et al., 2004). In this scenario, the generation of glutathiolated protein would be preferred in the environment of a protein sulfenic acid, where it reacts with GSH to form a mixed disulphide bond; the resulting disulphide is then relatively stable towards reduction by a second GSH (Jacob et al., 2004, Mieyal et al., 2008).





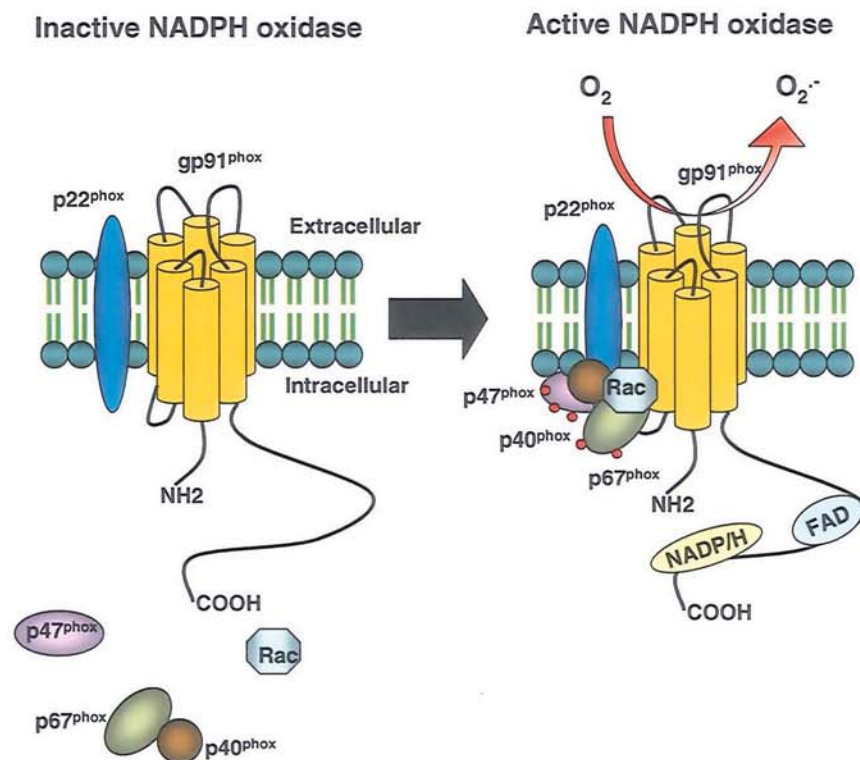
**Figure 1.5 Protein-thiol oxidation pathways and their contribution to redox signaling.** Oxidative and nitrosative stress can induce protein Prot-SH modifications that contribute to biological signaling pathways. Protein cysteinyl groups nitrosylate (S-nitrosylation), glutathiolate (S-glutathiolation) or hydroxylate (S-hydroxylation) by reacting with NO, GSSG and ROS respectively. The reversibility of these reactions permits proteins to act as redox switches in changing redox environment. However further oxidation of Prot-SOH into sulfonic acids is irreversible and may destroy protein biological function.

## 1.3 NADPH oxidase complex

### 1.3.1 Structure

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex is a membrane bound multi-subunit enzymatic complex originally discovered in neutrophils. Six homologues of the cytochrome subunit of the phagocyte NADPH oxidase have now been described: NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2 (Sheppard et al., 2005, Groemping and Rittinger, 2005). Together with the phagocyte NADPH oxidase itself (NOX2/gp91phox), the homologues are now referred to as the NOX family of NADPH oxidases (Pendyala et al., 2009). NADPH oxidase enzyme complex or its subunits have shown to present in many eukaryotic cells (Abid et al., 2007, Moulton et al., 1998, Nisimoto et al., 2008) including plants (Torres et al., 1998, Sumimoto, 2008). Phagocytic NADPH oxidase complex is composed of two membrane components; gp91<sup>phox</sup>, gp22<sup>phox</sup> also known as flavocytochrome b<sub>558</sub> and three cytosolic components; p47<sup>phox</sup>, p40<sup>phox</sup>, p67<sup>phox</sup>, and a low molecular weight G protein (either Rac 1 or Rac 2). The Racs are kept inactive by binding to a guanine nucleotide dissociation inhibitor, which prevents the exchange of guanine nucleotides from the Rac proteins (Schalk et al., 1996). It is well established that Rac-GTP is a component of the active oxidase complex, binding to both p67<sup>phox</sup> and also likely the flavocytochrome (Price et al., 2002).

The importance of the respiratory burst oxidase is illustrated by the inherited condition Chronic Granulomatous Disease (CGD) in which any one of the several components e.g  $p47^{\text{phox}}$ ,  $p67^{\text{phox}}$ ,  $gp91^{\text{phox}}$  or  $p22^{\text{phox}}$  of the respiratory burst oxidase is absent or defective (Bakri et al., 2009, Watanabe et al., 2006, Segal et al., 2000). Affected individuals suffer from recurrent, chronic and severe infections due to the inability of their neutrophils to kill microbes (Segal et al., 2000). The assembly NADPH oxidase in cell membranes is shown in figure 1.6.



**Figure 1.6 Schematic diagram of the NADPH oxidase under inactive and active conditions.** Phagocytic NADPH oxidase complex consist of membrane bound flavocytochrome b<sub>558</sub> and cytosolic components of  $p47^{\text{phox}}$ ,  $p67^{\text{phox}}$ ,  $p40^{\text{phox}}$  and small peptide rac. Activation of the oxidase involves the phosphorylation of one of the cytosolic components. The translocation of phosphorylated cytosolic subunits into the membrane results in the assembly of a fully functioning NADPH oxidase enzyme. It generates superoxide by transferring electrons from NADPH inside the cell across the membrane and coupling these to molecular oxygen to produce the superoxide anion radical.



Two NADPH subunits gp91<sup>phox</sup> (also called  $\beta$  subunit) and p22<sup>phox</sup> (also called  $\alpha$  subunit) form a heterodimeric flavocytochrome b<sub>558</sub> that constitutes the catalytic core of the enzyme, but exist in inactive state in the absence of other subunits. The gp91<sup>phox</sup> subunit has a molecular mass of 65.3kDa but is detected in polyacrylamide gels at approximately at 91kDa due to its high glycosylation pattern (Groemping and Rittinger, 2005). This subunit carries a binding site for flavine adenine dinucleotide (FAD) and NADPH at the cytosolic C terminal. In addition, gp91<sup>phox</sup> contains two haem groups in its membrane bound domain and together, the NADPH, FAD, and haem groups are responsible for transferring electrons to reduce O<sub>2</sub> to O<sub>2</sub><sup>-</sup> (Jones et al., 2000). The p22<sup>phox</sup> has a molecular mass of 21kDa and associates with gp91<sup>phox</sup> with 1:1 ratio. This small subunit has conserved proline rich (PXXP) motif and found to be mutated in CGD patients. This subunit may be involved in the interaction with cytosolic subunits (Groemping and Rittinger, 2005). In resting cells ~10% of flavocytochrome b<sub>558</sub> is contained within the plasma membrane, 5-15% in secretory vesicles, and the remainder (75-90%) is stored in the membrane of specific granules (DeLeo et al., 1998). Flavocytochrome b<sub>558</sub> is recruited to the membrane from cytosolic granules after stimulation.

Among the three aforementioned cytosolic subunits, p47<sup>phox</sup> is recognized as an essential subunit in neutrophils. The importance of this subunit was confirmed with the discovery of CGD where patients suffered p47<sup>phox</sup> deficiency. This subunit has a molecular mass of 44.7kDa and consists of a PX domain (phosphoinositide-binding structural domain), two adjacent SH3 domains, a region rich in arginine and lysine residues and proline rich region (PRR) (Groemping and Rittinger, 2005). p47<sup>phox</sup> consists of several phosphorylation sites and is the most phosphorylated subunit of the NADPH oxidase (Taura et al., 2009). In the resting state, the p47<sup>phox</sup> membrane binding domain is

intramolecularly masked through interactions between its PX domain and SH3 domain (Taura et al., 2009). Phosphorylation liberates the PX domain unmasking the SH3 domains to interact with other protein subunits of the NADPH oxidase complex (Yaffe, 2002). The subunit p67<sup>phox</sup> has a molecular mass of 59.8kDa (Groemping and Rittinger, 2005) and consist of two SH3 domains, which allow it to make interaction with other NADPH subunits. CGD also occurs in patients with deficiencies in p67<sup>phox</sup>. The p40<sup>phox</sup> has a molecular mass of 39kDa and consists of a PX, SH3 and a PBI domain (Phox and Bem 1). This subunit is found to co-immunoprecipitates with p47<sup>phox</sup> and p67<sup>phox</sup> (Tsunawaki et al., 1996). This protein probably functions as a shuttle partner, transporting p67<sup>phox</sup>, which does not contain PX domain, to the membrane.

### **1.3.2 Priming and activation**

Priming of the NADPH oxidase enzyme enhances the ability of the neutrophil to generate a higher burst to a second stimulus. However, priming agents are not directly involved in the process of NADPH oxidase complex activation. To date there have been a wide range of priming agents for NADPH oxidase that have been discovered (Table 1.1). These have varying efficiencies for priming and show different kinetics to manifest their effect. Some priming mediators such as platelet activating factor (PAF), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), C5a, and lysophosphatidylcholine (LPC) take a shorter time (3-5min) to prime, while others such as TNF- $\alpha$ , GM-CSF, IL-8 a take longer time (15-60min) to stimulate neutrophils (Sheppard et al., 2005, Groemping and Rittinger, 2005).

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<b>Cytokines</b>
IL-1 $\beta$
IL-6
IL-8
IL-18
TNF- $\alpha$
GM-CSF
GCSF
IFN- $\gamma$
<b>Lipid mediators</b>
Lysophosphatidylcholine
Arachinoid acid
Leukotrienes LTB1
Ceramide C2 and C6
PAF
PMA
<b>Bacterial</b>
LPS
Opsonised zymosan
<b>Other agents</b>
Angiotensin II
Homocysteine
C5a
Ca <sup>2+</sup>
Immunecomplexes

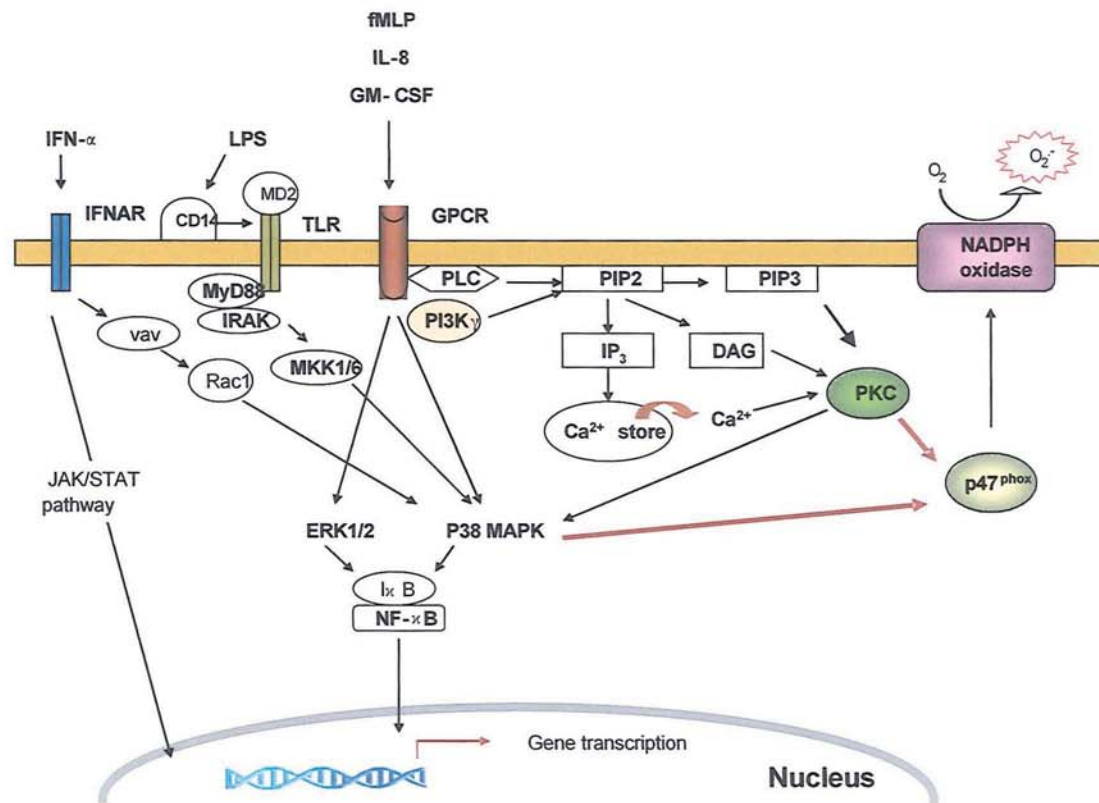
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**Table 1.1: Neutrophil priming agents**

Several studies have described the ability of priming agents to enhance neutrophil respiratory burst to a second stimulus (Green et al., 1996, Guichard et al., 2005, Silliman et al., 2003, Elbim et al., 1994, Matthews et al., 2007). Since NADPH oxidase is the primary site for neutrophil superoxide production, the interaction between different signal transduction pathways and activation of NADPH oxidase has been given great attention in the literature. As previously described, several individual priming mediators are recognised (Table 1.1) and these molecules signal through different receptor systems and may stimulate more than one signaling pathway. However, these ultimately trigger the assembly and activation of NADPH oxidase to generate the respiratory burst.



The exact mechanism by which each agent primes NADPH oxidase is not always fully explained; the involvement of components in activation of mitogen activated protein kinase (MAPK) cascades have been given repeated attention. It has been shown that phosphorylation and translocation of cytosolic subunits to the plasma membrane is a key event in NADPH oxidase activation. Priming agents may either stimulate neutrophils to produce low level of respiratory burst (Guichard et al., 2005) or may sometimes be unable to initiate the respiratory burst (Koenderman et al., 1989). However, in both cases the primed neutrophils elicit a much higher respiratory burst when subsequently challenged. Therefore, it has been suggested that priming agents may be involved in the partial phosphorylation of cytosolic components as detected in IL-8 primed neutrophils (Guichard et al., 2005) and TNF- $\alpha$  primed neutrophils (Dewas et al., 2003). Therefore, these agents may activate a series of kinases such as mitogen-activated protein kinases (Guichard et al., 2005), or may inhibit phosphatases such as CD45 (Fialkow et al., 1994, Fialkow et al., 2007). In this model, partial phosphorylation is suggested to facilitate the subsequent fMLP stimulated phosphorylation of the cytosolic components and their translocation to the membrane (Guichard et al., 2005). In addition to pathways involving activation of different kinases such as MAPK and ERK1/2, other mechanisms proposed to explain the mechanisms of priming include increased membrane expression of cytochrome  $b_{558}$  (DeLeo et al., 1998); membrane lipid raft organization (Li et al., 2007); increased phospholipase  $A_2$  (PLA $_2$ ) activation (Daniels et al., 1998); increased intracellular free  $Ca^{2+}$  concentration (Wozniak et al., 1993); and increased expression and cycling of triggering receptors such as fMLP receptors (Metzner et al., 1995). Intracellular signaling pathways stimulated by both host cytokines and bacterial products are summerised in figure 1.7.



**Figure 1.7: Model for linking cell surface receptors to signaling networks and their involvement in NADPH oxidase activation.** Pre-exposure of neutrophils to proinflammatory mediators such as IL-8, GM-CSF, and IFN- $\alpha$  or bacterial LPS strongly primes the generation of ROS induced by subsequent stimuli such as fMLP. The cellular mechanisms behind the stimulated cell surface receptors and the respiratory burst response are orchestrated through carefully controlled intracellular signaling molecules. Neutrophils recognise IL-8, GM-CSF and fMLP through GPCRs; CXCR, GM-CSF receptor and fMLP receptor respectively. Intracellular signaling in fMLP-stimulated human neutrophils is connected to three main pathways. The first pathway involves PLC (phospholipase C), PIP<sub>2</sub> (phosphoinositide 4,5-diphosphate) and its cleavage products IP<sub>3</sub> (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP<sub>3</sub> stimulate Ca<sup>2+</sup> mobilisation from intracellular calcium stores such as endoplasmic reticulum, which in turn stimulate PKC activity. DAG stimulates PKC activity by its phosphorylation. A central element of the second pathway is the PI3K (phosphoinositide 3-kinase). PI3K $\gamma$  isoform is recruited to the inner cell membrane, where they directly interact with GPCR to phosphorylate PIP<sub>2</sub> to form the second messenger PIP<sub>3</sub> (phosphoinositide 3,4,5-trisphosphate). GPCRs also activate MAPK and ERK1/2 pathway and thereby phosphorylate the NADPH oxidase subunit p47<sup>phox</sup>. Despite the similarities that exist in the signaling pathways, the level of respiratory burst exerted by CXC receptors and GMSCF receptors are not comparable.



This may be due to differential rate of phosphorylation or involvement of inhibitory mechanisms such as  $\beta$ -arrestin attenuated ERK1/2 phosphorylation (Ming et al., 2004) in IL-8 stimulation. Bacterial LPS is recognised by membrane bound TLR4 receptors (toll like receptor-4) with help of CD14. TLR-4 activates p38/MAPK pathway via the adaptor protein MyD88 then signals through IRAK (interleukin-1 receptor-associated kinase). Interferon- $\alpha$  is recognised through type I IFN receptor IFNAR. IFNs stimulate classical JAK/STAT (Janus kinase/ signal transducer and activator of transcription) pathway to transcribe ISGs (IFN-stimulated genes). Apart from this, IFN-activated JAKs regulate the phosphorylation of Vav or other GEFs (Guanine nucleotide exchange factors), resulting in the downstream activation of Rac1 that can regulate the signaling pathway of p38/MAPK.

### **1.3.2.1 Host cytokines as priming agents**

Extracellular priming agents signal through their specific receptors at the cell surface and often exhibit other regulatory functions in addition to priming. As an example, TNF- $\alpha$  up regulates expression of adhesion molecules, chemoattractant receptors, and heterotrimeric G proteins (McColl et al., 1990). Most of the priming agents (IL-8, PAF, or C5a) are recognised by G-protein coupled receptors on the neutrophil membranes (Holmes et al., 1991, Schroder et al., 1990, Green et al., 1996, Brown et al., 2004), whereas, TNF- $\alpha$  exerts its cellular effect by binding to two types of receptors, the p55 and p75 TNF receptor (Dewas et al., 2003). While these two receptors are linked to different signaling pathways, the p55 TNF receptor induces the phosphorylation of p47<sup>phox</sup> subunit (Dewas et al., 2003). Partial phosphorylation of p47<sup>phox</sup> is also observed in the GM-CSF-primed respiratory burst (Gainet et al., 1999). The engagement of GM-CSF with its receptor activates receptor-tyrosine kinase, PI3K, PLA<sub>2</sub> and MAPK signaling molecules. The involvement of p38 MAPK pathway in both TNF- $\alpha$  and GM-CSF stimulation has been reported in many studies (Waterman and Shaafi, 1995). However, it is noteworthy that up- or down-regulation of a signaling cascade also depends on different neutrophilic phenotypes. As an example, failure of TNF- $\alpha$  to stimulate ERK activation is related to



neutrophils in suspension (Waterman and Shaafi, 1995) while, TNF- $\alpha$  stimulated, increased extracellular signal-regulated kinase (ERK) stimulation is reported in the adherent phenotype (Rafiee et al., 1995). This may be a result of integrin outside-in signaling, where up-regulation of integrin clustering and their interaction with ICAMs-1-3 or extracellular matrix (ECM) proteins is reported to trigger an array of cell responses including stimulation of ERK (Berton and Lowell, 1999). GM-CSF generates a robust increase of ERK activity in human neutrophils (Waterman and Shaafi, 1995). It is now evident that p38 and ERK signaling pathways are involved in regulation of NADPH oxidase but not janus activated kinase (JNK) (Groemping and Rittinger, 2005, Benna et al., 1996, Abid et al., 2007, Guichard et al., 2005). Activated p38 MAPK also regulates distinct proinflammatory functions such as leukocyte adhesion, activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), and the synthesis of cytokines (Jinnouchi et al., 2005).

Proinflammatory cytokines, GM-CSF, TNF- $\alpha$  and IL-8 use similar signal transduction mechanisms. IL-8 is recognised through a CXC type G protein coupled receptor and receptor activation is reported to enhance p38 MAPK dependent phosphorylation and translocation of p47<sup>phox</sup> and p67<sup>phox</sup> to the membrane. Guichard and co-workers (2005) showed the IL-8 mediated kinetics and assembly of subunits of NADPH oxidase complex (Guichard et al., 2005). They provided the first evidence of the redistribution of NADPH oxidase into lipid raft microdomains upon IL-8 stimulation (Witko-Sarsat et al., 1999). IL-8 induce phosphorylation of NADPH oxidase subunits, which in turn induce the sequential assembly of NADPH oxidase complex, first engaging flavocytochrome b<sub>558</sub> with the lipid raft and then activating the sequential translocation of p47<sup>phox</sup>, rac2 and p67<sup>phox</sup> into the lipid raft and finally activating superoxide production.

Mobilisation of flavocytochrome  $b_{558}$  into the plasma membrane is also observed with another priming agent; granulocyte colony stimulating factor (GCSF) (Mansfield et al., 2002). Unlike GM-CSF, that primes NADPH oxidase through partial phosphorylation of  $p47^{\text{phox}}$ , GCSF does not affect phosphorylation or membrane translocation of cytosolic NADPH oxidase subunits (Mansfield et al., 2002). It has been suggested that GCSF primes the respiratory burst by increasing membrane fusion of flavocytochrome  $b_{558}$  containing gelatinase granules (Mansfield et al., 2002).

#### **1.3.2.2 Bacterial products as priming agents**

Bacterial lipopolysaccharide (LPS) is another pluripotent agonist that is found to prime superoxide production in neutrophils (Jinnouchi et al., 2005). LPS is presented to neutrophils in a complex with LPS-binding protein (LBP): a component of mammalian serum, and stimulates cells via the glycosylphosphatidyl-inositol anchored cell surface glycoprotein, CD14 (Hayashi et al., 1999). Priming of neutrophils with LPS via CD14 initiates a signal that results in the activation of MKK3 (MAP kinase kinase-3), which in turn phosphorylates and activates p38 MAPK (Ward et al., 2000). Similar to TNF- $\alpha$  and GM-CSF priming, LPS mediated activation of p38 MAPK may be involved in subsequent  $p47^{\text{phox}}$  phosphorylation. LPS also increase plasma membrane association of flavocytochrome  $b_{558}$  and enhances the assembly of cytosolic oxidase factors within the plasma membrane after stimulation with the secondary stimulus, fMLP (DeLeo et al., 1998, Jinnouchi et al., 2005). In resting cells, flavocytochrome  $b_{558}$  is associated with both plasma membrane and neutrophil specific granules, with approximately 20% present in tertiary granules (Mansfield et al., 2002). LPS priming induces the plasma membrane

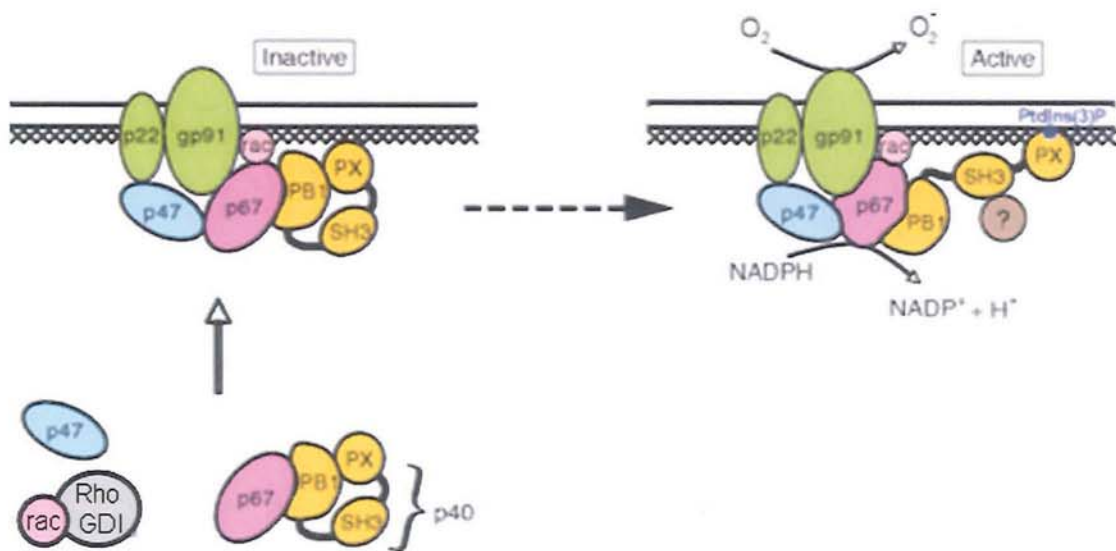
fusion of specific granules and thereby translocation of flavocytochrome  $b_{558}$  to the plasma membrane (Jinnouchi et al., 2005). However, translocation of flavocytochrome  $b_{558}$  is not the only determinant of the amount of superoxide produced (DeLeo et al., 1998, Ward et al., 2000). Partial phosphorylation of  $p47^{\text{phox}}$  and the reported translocation of NADPH subunits may be equally important in LPS priming.

### 1.3.3 Assembly of NADPH oxidase

Assembly of NADPH oxidase components within the plasma membrane has been shown to be a sequential and cooperative process. The first step of NADPH oxidase activation involves the phosphorylation of one cytosolic component. Translocation of the phosphorylated component onto the plasma membrane greatly facilitates the translocation of this next component. In this process phosphorylation of  $p47^{\text{phox}}$  by protein kinase C (PKC) and its subsequent association of other components acts as the key step for NADPH assembly and activation (Morozov et al., 1998, Pendyala et al., 2009). At the membrane  $p47^{\text{phox}}$  interacts with  $p67^{\text{phox}}$ ,  $p22^{\text{phox}}$  and cyt  $b_{558}$  (Pendyala et al., 2009). Using permeabilised human neutrophils in the presence of siRNA, it has been shown that the small GTPase protein Rac is important in activating neutrophil respiratory burst (Miyano et al., 2006). In resting cells, Rac is bound to an inactive cytosolic complex with Rho-GTP dissociation inhibitor (Rho-GDI). The second step in NADPH oxidase assembly is the binding of GTP to Rac, which leads to the dissociation of Rac from Rho-GDI, and its migration to the cell membrane independently to other phox proteins (Tamura et al., 1999). Rac interacts with the membrane through its geranyl lipid modification and associates with the N-terminal domain of  $p67^{\text{phox}}$ . The  $p40^{\text{phox}}$  protein



associates with p67<sup>phox</sup> through its PBI domain (Tsunawaki et al., 1996) and interacts with p47<sup>phox</sup> through its SH3 domain in the resting state (Figure 1.8). After activation, the p67<sup>phox</sup>-p47<sup>phox</sup>-p40<sup>phox</sup> complex is translocated to the membrane. The PX domain of p47<sup>phox</sup> and p40<sup>phox</sup> interacts with the F-actin-binding protein moesin at the membrane via phosphoinositol-mediated signaling (Wientjes et al., 2001). Recent studies have revealed that the PX domain of the p40<sup>phox</sup> subunit binds to phosphatidylinositol 3-phosphate (PtdIns(3)P) at the membrane also important for the activation of NADPH oxidase (Bissonnette et al., 2008). These authors propose a model where native p40<sup>phox</sup> exists in a closed form but uses PX domains to bind p67<sup>phox</sup> and transport it to the membrane: this allows interaction with (PtdIns(3)P) containing membranes, and activation of the burst.



**Figure 1.8: Model for the role of subunits in the activation of NADPH oxidase.** In the inactive state, p40<sup>phox</sup> is bound with p67<sup>phox</sup> through PBI-PBI domain-mediated interactions. Intramolecular association between the PBI domain with the PX domain maintain a “closed conformation” of p40<sup>phox</sup> in the resting neutrophils. Upon neutrophil activation, p67<sup>phox</sup>-p40<sup>phox</sup> complex and p47<sup>phox</sup> are translocated to the membrane. The association of p40<sup>phox</sup> PX domain with membrane PtdIns(3)P is important to activate NADPH oxidase. In stimulated neutrophils, in an independent mechanism, the small GTPase protein Rac also dissociate from its inhibitor, Rho-GDI and translocate to the membrane bound NADPH oxidase complex to make essential interactions with membrane and activation of NADPH oxidase. Figure adapted from (Bissonnette et al., 2008)

### 1.3.4 Membrane lipid raft organization

In eukaryotic cells, the plasma membrane and membranes from different organelles show differential lipid compositions. The choline containing lipids, phosphatidylcholine, sphingomyelin and glycosphingolipids are enriched on the external leaflet of the plasma membrane. In contrast, the cytoplasmic leaflet is enriched with the amine containing glycerophospholipids, phosphatidylethanolamine and phosphatidylserine. Other minor phospholipids associated with intracellular signaling roles, such as phosphatidic acid (Qiu et al.), phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>), are also enriched on the cytoplasmic facing side of the membrane (Daleke, 2003). Membrane lipid organization reflects a dynamic equilibrium of lipids that move across the bi-layer in both directions. Therefore, these membranes show an asymmetric distribution of lipid types across the membrane bi-layer. The amphipathic lipids in biological membranes are organised in a lateral dimension. Lipids provide a structural backbone punctuated by the proteins in a membrane. Phospholipids with one *cis*-unsaturated acyl fatty acids are not able to pack tightly as lipids with unsaturated acyl chains. Therefore, the differential packing of phospholipids creates lateral heterogeneity of the membrane.

Organisation of lipid molecules into ordered domains upon cell activation has been reported in a variety of mammalian cells such as endothelial cells (Li et al., 2007), epithelial cells (Pike, 2003), fibroblasts (Liu and Anderson, 1995), and neutrophils (Shao et al., 2003, Bissonnette et al., 2008, Vilhardt and Deurs, 2004). These domains arise from the dynamic assembly of sphingolipids and cholesterol. The close packing of saturated acyl chains and weak attractions of carbohydrate head groups of glycosphingolipids



permits localised formation of ordered lipid domains. Any spaces created by large and bulky sphingolipids are filled by cholesterol intercalating with hydrophobic lipid acyl chains. Cholesterol depletion assays using methyl- $\beta$ -cyclodextrin have revealed the importance of cholesterol for the structural integrity of these domains and also for several receptor signaling events. Uneven distribution of cholesterol and sphingomyelin is observed and organisation in ordered domains in the outer leaflet has been referred to as lipid raft formation (Pike, 2003).

Lipid rafts behave as liquid ordered assemblies within the liquid disordered surrounding of phospholipids and phosphatidylcholine molecules. However, individual lipids may move in and out of the rafts explaining why sphingolipid-cholesterol clustering is difficult to detect spectroscopically. Therefore, there is a debate as to the existence of lipid rafts (Cottingham, 2004). Although in continual change of lipid/protein composition, recent studies suggest the presence of these specialized regions within a variety of cell membranes (Day and Kenworthy, 2009). The most widely used assay for raft existence is based on their insolubility in cold non ionic detergent Triton X-100. Rafts are recognised by the acronyms DRM (detergent resistant membranes), DIGs (detergent insoluble glycolipid enriched membranes), GEMs (glycolipid enriched membranes), TIMs (Triton insoluble membranes) and TIFFs (Triton insoluble floating fractions). The traditional method of preparation of detergent resistant lipid rafts and subsequent flotation in a 5% to 30% linear sucrose density gradient, is reported to yield fairly consistent fractions that are enriched with cholesterol and raft marker proteins such as flotillin (Pike, 2003). However, some variations of lipid composition have been observed depending on physical handling and use of different detergents. As reviewed in Pike et al., varying levels of lipid



composition in detergent insoluble rafts and DRMs illustrate the importance of the method of extraction (Pike, 2003).

Proteins with at least one transmembrane domain, with a hydrophobic modification such as glycosyl phosphatidylinositol (GPI)-tail, or that are modified with saturated acyl chains, including myristoylated, and palmitoylated proteins, are enriched in rafts. Mammalian cells typically express 5-10 different GPI anchored proteins. Flotillin is an integral membrane protein that has been shown to be present in lipid rafts and these proteins may have a structural role in raft formation (Evans et al., 2003). Caveolae are another specialised form of lipid rafts and contain a significant enrichment in the protein caveolin (Sargiacomo et al., 1993), a marker protein for caveolae. Caveolae were first discovered as 50 to 100nm “flask shaped” invaginations of the plasma membrane (Yamada, 1955). These are found in the majority of cell types but are highly abundant in terminally differentiated cells such as adipocytes, endothelial cells, smooth muscle cells and fibroblasts (Lisanti et al., 1993). In contrast to early work that reported the absence of caveolin in neutrophil membrane (Sengeløv et al., 1998), more recent work using caveolin-1-null mice has shown the expression of this protein and its importance in activation, adhesion and transepithelial migration of neutrophils (Zemans and Downey, 2008, Hu et al., 2008). Quantitative proteomic analysis of lipid raft in HeLa cells by Foster and coworkers revealed that in addition to raft marker proteins, there are raft-associated and non-specific proteins present in lipid rafts (Foster et al., 2003). This supports the transient nature of rafts that recruit smaller lipid domains with different receptors/ protein associate into larger platforms. In this environment proteins are found to be selectively included or excluded depending on the function of the raft (Chamberlain et al., 2001, Mañes et al., 2003).

A wider role for lipid rafts is now proposed including signal transduction pathways, apoptosis, cell adhesion and migration, synaptic transmission, organisation of the cytoskeleton and protein sorting during exocytosis and endocytosis, cellular entry of wide range of viruses, bacteria and toxins (as reviewed in Munro, 2003, Pike, 2003). The formation of these lipid raft platform aggregates into different signaling molecules may support an amplification of transmembrane signaling (Pike, 2003). Similarly, using activated vascular endothelial cells suggest that NADPH oxidase assembly is supported by lipid rafts (Li et al., 2007). In addition, the synergy between actin filaments and membrane cholesterol that facilitates the protein co-clustering in lipid raft (Chichili and Rodgers, 2007) and together with the known association of p40<sup>phox</sup> with moesin, these findings suggest a cooperate role for rafts and the cytoskeleton in NADPH oxidase organisation and activation.

As discussed earlier, neutrophils are important cells in acute and chronic inflammation. Using membrane lipid raft depletion studies it has been shown that depletion of plasma membrane cholesterol prevents neutrophil chemoattractant- stimulated actin polymerization, cell polarization and formation of membrane microdomains (Pierini et al., 2003). In addition, the retention of Rac; an important component for NADPH oxidase activity in the membrane can also be inhibited by disrupting the lipid rafts (Pierini et al., 2003). These evidences suggest that lipid raft integrity is critical for the neutrophilic chemotactic and respiratory burst responses found in inflammation.

#### 1.4 Inflammatory diseases

Bacterial infection is a primary aetiologic driver of the host-mediated response of inflammation. An acute inflammatory response is initiated and resolved in a timely manner that prevents the tissue injury and also eliminate the infection. However, the inadequate resolution and failure to return tissue homeostasis results in neutrophil-mediated destruction and chronic inflammation (Dyke, 2008). As such, a timely and appropriate host response is necessary to prevent the progression into a chronic disease. The pathology of chronic inflammatory disease is associated with continuous infiltration of neutrophils to the site of infection, a potential for continuous contamination of bacterial products into the circulation, production of proinflammatory mediators such as IL-1, IL-8, TNF- $\alpha$ , IFNs, GM-CSF and their release into the systemic circulation.

Chronic inflammatory diseases are associated with the activity of neutrophils to a differing extent. Rheumatoid arthritis (RA), is a chronic systemic inflammatory disorder that principally affects joints, producing inflammatory synovitis, in which a large number of neutrophils ( $5 \times 10^4$  per  $\text{mm}^3$ ) are reported to accumulate in the synovial fluid (Ottonello et al., 2002, Harris, 1990). These neutrophils have also been shown to increase elastase release (Parsonage et al., 2008), have an increased respiratory burst (Griffiths and Lunec, 1988), increased production of potent effectors of cartilage destruction, such as serine and metalloproteases (Ottonello et al., 2002) and an increase neutrophil life span by delaying apoptosis in synovial fluids in the presence of inflammatory cytokines such as IL-17 (Parsonage et al., 2008). The association of excessively produced free radicals by neutrophils with wide range of other inflammatory diseases such as Coeliac disease (Stewart and Kerr, 1991), Crohn's disease (Maor et al., 2008), ulcerative colitis (Nielsen SE et al., 2004), and periodontitis (Matthews et al., 2007) have been reported.



#### **1.4 Inflammatory periodontitis**

Periodontitis is one of the most common inflammatory diseases that is initiated by the plaque biofilm; a diverse microbial community that is found on the tooth surface, and embedded in a matrix of polymers of bacterial and salivary origin (Marquis, 1995). Development of dental plaque associates with destruction of the tooth-supporting connective tissue structure; the periodontium and ultimately results in tooth loss. It has been estimated that 90% of the world wide population is affected by early gum disease and among these 15% of the population progress to severe periodontitis (Pihlstrom et al., 2005). The basic symptoms associated with periodontal diseases are therefore similar to normal inflammatory symptoms; red, swollen, inflamed gums, which can easily bleed upon brushing. Gingivitis is the mildest and most prevalent form of periodontal disease and can be reversed with effective oral hygiene. Untreated or poor oral hygiene increases the severity of the disease and inflammation penetrates deeper connective tissues, and this requires dental treatments.

Although the initial causative agent is a bacterial challenge, there are other factors such as systemic (Chapple, 1997), genetic (Townsend et al., 1998), environmental, and behavioural factors, which influence the progress of the disease. Therefore, periodontitis is commonly referred to as a multifactorial disease. Also, the destruction of periodontium has strong connection with smoking (Bergström, 2004), systemic diseases such as diabetes, cardiovascular disease, osteoporosis, HIV (Chapple and Hamburger, 2000) and other immuno-suppressed conditions. In general, periodontal diseases are common in both children and adolescents. Epidemiological studies on young individuals suggest that periodontal disease prevalence increases from the age group of 12-17 (Dibart, 1997). It is

also evident that older age groups more susceptible to periodontitis (Albandar and Rams, 2002).

Although gingivitis is globally and equally distributed, individuals in some parts of the world are more prone to develop the severe stages of periodontitis. For an example, a high prevalence is found in rural parts of Africa that might indicate low health care facility and social welfare systems (Albandar and Rams, 2002). Interestingly, individuals in developed countries with approachable healthcare facilities also develop severe periodontitis. This highlights the multifactorial involvement of the disease rather than simple oral hygiene.

#### **1.4.1 The periodontium**

The human periodontium, the tooth supporting structure, is a functional system of different tissues, including cementum, periodontal ligaments, alveolar bone and gingiva (Figure 1.9). The gingiva is the only visible periodontal tissue in a healthy mouth. It consists of three different tissues, junctional epithelium (JE), oral epithelium and lamina propria (connective tissue). JE is semi-permeable cell layer and allows diffusion of metabolic products of plaque bacteria such as toxins, chemotactic agents and antigens. Even in non-clinical conditions, the JE is transmigrated by neutrophils moving towards sulcus; the space between enamel and JE (Schluger 1990). Therefore, the JE plays a key role in maintenance of periodontal health. The JE is continuously renewed throughout the life of a healthy individual (Carranza 1996).



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**Figure 1.9: Schematic diagram of the periodontium.** The periodontium consists of gingiva, cementum, alveolar bone, and periodontal ligament. Gingiva is the only visible part of the periodontium. Periodontitis is initiated by the plaque biofilm and propagated by an abnormal inflammatory-immune response that causes periodontal tissue destruction. JE = junctional epithelium; OE = oral epithelium; GCF = gingival crevicular fluid; GBV = gingival blood vessels. Figure adapted from (Chapple and Hamburger, 2000) and <http://www.royzmandental.com/Services/periodontal.php>.

#### 1.4.2 Bacterial challenge in periodontitis

Gingivitis is an early but reversible stage of periodontitis. It is initiated from the colonisation of harmful and pathogenic bacteria around the tooth collar. Small food particles including salivary polypeptides and proteins retained in the oral cavity can easily deposit on a tooth or around the tooth even after brushing. These termed pellicles serve as primary ecological niches to gram positive oral bacteria such as *Actinomyces* species. The successive colonisation of bacterial species moves the plaque composition towards a gram negative, anaerobic and more mobile bacterial flora. It has been reported that depending on the severity of the disease condition, the microbial complexes can be varied



in the dental plaque (Table 1.2 ) (Socransky et al., 1998, Haffajee et al., 2008). Periodontal pathogens, similar to most other pathogens show an ability to successfully colonise, an ability to evade host defense mechanisms, and a capacity to produce substances that directly degrade host tissues (Amano, 2003).

Condition	Organism
Gingival health	<i>Streptococcus sanguis</i>
	<i>Actinomyces viscosus</i>
	<i>Streptococcus oralis</i>
Necrotising ulcerative gingivitis	<i>Treponema vincentii</i>
	<i>Fusobacterium nucleatum</i>
	<i>Prevotella intermedia</i>
	<i>Candida species</i>
Gingivitis	<i>Fusobacterium nucleatum</i>
	<i>Porphyromonas gingivalis</i>
Localised aggressive periodontitis	<i>Actinobacillus actinomycetemcomitans</i>
Generalised aggressive periodontitis	<i>Porphyromonas gingivalis</i>
Chronic periodontitis	<i>Porphyromonas gingivalis</i>
	<i>Bacteroides forsythus</i>
	<i>Fusobacterium nucleatum</i>
	<i>Campylobacter recta</i>
	<i>Eikenella corrodens</i>
	<i>Peptostreptococcus micros</i>
	<i>Prevotella intermedia</i>
	<i>Eubacterium species</i>
	<i>Spirochaete species</i>

**Table 1.2: Pathogenic microorganisms involve in different stages of periodontitis.** *Porphyromonas gingivalis* is the most common bacterium in any stage of periodontitis (Chapple and Gilbert, 2002).

The very irregular distribution of bacteria contributes to the varying histologic response in the adjacent soft tissue. Different host mechanisms are important; initially the sulcular and JE act as physical barriers against bacteria. However, being a living and non-keratinised cell layer, the JE is not an effective barrier against bacterial invasion. The washing effect of saliva and gingival crevice fluid (GCF) also helps to control and minimize bacterial colonisation. GCF contains antioxidants, protease inhibitors,  $\beta$ 2-macroglobulin, albumin, lipoproteins and fibrinogen, and is similar to serum indicating passive filtering system of the intact gingival tissues, which serves as a chemical challenge to microbes (Pauletto et al., 2000). Once this equilibrium is disturbed by pathogens in the JE, the host will mount inflammatory responses. During the early stages of inflammation, the neutrophils are the most important determinant of the innate response and are the focus of this thesis.

## **1.5 Neutrophils and periodontitis**

### **1.5.1 Neutrophil counts in periodontitis**

The number of leukocytes present in the circulation is a diagnostic tool for inflammation. Evidences from epidemiological studies indicate a relationship between leukocyte count and periodontitis (Table 1.3). The report by Kweider *et al* described for the first time that periodontal plasma has a higher leukocyte count compared with control subject plasma (Kweider et al., 1993). Subsequent studies, as reviewed in Loos (2005), confirmed that the peripheral blood of periodontal patients carries a higher level of leukocytes in the circulation, although this is not always statistically significant (Loos, 2005). In addition, these leukocyte numbers fall below the normal reference range ( $>10 \times 10^9$  cells/l) for leucocytosis (Loos, 2005). Moreover, surgical and/or non surgical periodontal therapy has

been shown to decrease the leukocyte count. High levels of leukocyte count are repeatedly observed in smokers with periodontitis and a modest increase of cell count is observed with non-smokers (Pauletto et al., 2000, Güntsch et al., 2006, Matthews et al., 2007). In the presence of a chronic oral bacterial infection, neutrophils are the first line of host defence. These cells contribute to 10% of the total leukocyte count and they are the main contributor to the increased systemic cell count. The persistent pathogenic stimuli at the periodontium may recruit more neutrophils along the concentration gradient of the stimulant or inflammatory mediators.

Reference	Patients		Controls	
	N	Mean $\pm$ SD	N	Mean $\pm$ SD
(Kweider et al., 1993)	50	8.7 $\pm$ 2.3	50	6.0 $\pm$ 1.3
(Jarnbring et al., 2000)	14	7.7 $\pm$ 2.1	14	7.3 $\pm$ 2.2
(Fredriksson et al., 1998)	17	7.0 $\pm$ 1.4	17	5.6 $\pm$ 1.1
(Loos et al., 2000)	107	6.5 $\pm$ 1.9	43	5.8 $\pm$ 1.4
(Fokkema et al., 2002)	19	6.1 $\pm$ 1.7	19	5.9 $\pm$ 1.3
(Lee et al., 2008)	150	4.22 $\pm$ 1.81	94	3.2 $\pm$ 0.91

**Table 1.3: Leukocyte counts in peripheral blood from periodontitis patients.** Case control and pilot studies that reported the total number of leukocytes ( $\times 10^9/l$ ) in peripheral blood of periodontitis patients and healthy controls (Loos, 2005).

### 1.5.2 Hyperactive and hyper-reactive neutrophils in periodontitis.

As previously described, neutrophils are critical components of periodontal inflammation that help to maintain oral defence against the constant bacterial challenge. The pathogenic role of neutrophils in mediating tissue destruction in periodontitis has been suggested in many previous studies (Chapple and Matthews, 2007, Chapple, 1997, Fredriksson et al., 1998, Fredriksson et al., 1999, Wright et al., 2008, Restaino et al., 2007, Asman et al.,



1985). Neutrophils isolated from periodontal patients are hyperactive with respect to generation of reactive oxygen species in the absence of the stimuli, as well as being hyper-reactive to a second stimulus with increased release of elastase (Gustafsson and Asman, 1996, Fredriksson et al., 1998, Matthews et al., 2007). The critical role of neutrophils to the pathology of periodontitis has also been demonstrated in rodent models (Yu et al., 2007, Gomes et al., 2009). Recent work using IL-17 receptor knock-out mice, showed reduced serum CXC chemokine levels and neutrophil infiltration in infected periodontal sites (Yu et al., 2007). While GCF dependent IL-17-induced life span extension of neutrophils is reported in RA (Parsonage G et al., 2008), this may be a possible mechanism for neutrophil survival and activation in periodontal pockets. Therefore, pathways involving the extension of increased neutrophil life span, their duration in periodontal tissues and factors increasing neutrophil numbers may be important in inducing the reported basal level hyperactive phenotype of neutrophils. These neutrophils could be further primed either with bacterial products such as LPS and fMLP or plaque induced host modulators (Matthews et al., 2007), such as IL-8, IFN- $\alpha$  and GM-CSF to generate an increased respiratory burst relative to matched controls with a subsequent stimulus.

Furthermore, some studies have suggested that the increased Fc $\gamma$  receptor induced respiratory burst of neutrophils is due to a constitutional effect of the disease rather than peripheral priming (Fredriksson et al., 2003). An inherited susceptibility for a relatively low prevalent (approximately 0.53% in adolescents aged 14-17 years in United states (Loe and Brown, 1991)) form of early-onset juvenile periodontitis is reported in different ethnic groups (Wilson and Kalmar, 1996). A possible relationship between periodontal diseases and Fc receptor polymorphisms is documented (Wilson and Kalmar, 1996, Loos et al., 2003, Kobayashi et al., 2007). However, some host mediators such as TNF- $\alpha$  and

LPS did not prime the respiratory burst of this hyperactive neutrophils (Gustafson et al., 1997, Matthews et al., 2007), indicating at least partially, a priming agent dependent neutrophil phenotype in juvenile periodontitis. However, as discussed in Matthews *et al.*, (2007), this needs further investigation from longitudinal studies investigating ROS responses of neutrophils pre- and post- therapy.

Since the neutrophil respiratory burst is mainly governed by the activation of the NADPH oxidase complex, the genetic regulation of phox gene expression in inflammatory diseases such as periodontitis (Matthews et al., 2007), type II diabetes (Adaikalakoteswari et al., 2006), chronic obstructive pulmonary disease (Noguera et al., 2001) has been investigated in previous studies. The involvement of increased p22<sup>phox</sup> gene expression related neutrophil hyperactivity is observed in type II diabetes (Adaikalakoteswari et al., 2006). However, p22<sup>phox</sup>, p47<sup>phox</sup> and p67<sup>phox</sup> mRNA levels remain unchanged between healthy control and patient neutrophils in contrast to the significant differences observed in the respiratory burst (Matthews et al., 2007). It is possible that post-transcriptional regulations such as controlling the mobilisation of secretory vesicles or post-translational modifications of phox subunits are more important in up-regulating the NADPH oxidase complex activity.

An increased level of pathogenic survival (Restaino et al., 2007), and sustained exposure to pathogenic products are also reported as causative agents for neutrophil hyper-responsiveness (Ishikawa et al., 1997, Restaino et al., 2007). Other possible explanations include; increased levels of neutrophil priming agents such as IL-8 (Gainet et al., 1999); IL-6 (Yumoto et al., 1999); increased susceptibility of the neutrophils to priming agents; or functional polymorphism of IL-1 and Fcγ receptors (Kobayashi et al., 2007) may be involved in this neutrophil phenotype.

The mechanism/s by which systemic neutrophils exhibit hyperactivity and hyper-reactivity in periodontitis is not fully explained. Therefore using a neutrophil model, this thesis aims to address underlying mechanisms of hyperactive/hyper-reactive neutrophil behaviour, particularly the respiratory burst, within the context of periodontitis.



## 1.6 Aims and objectives

### Aims

The purpose of this thesis is to examine the mechanisms that may contribute to the hyperactive and hyper-reactive neutrophilic phenotype that is observed in inflammatory periodontitis. To address this, three studies have been designed that investigate whether bacterial products from the perio-colonising microbe *P. gingivalis* can modulate the activity of the priming agents and chemotaxis of IL-8; whether plasma factors such as IL-8, GM-CSF and IFN- $\alpha$  present during periodontitis can promote neutrophil priming and activation; and the effects of excessive intracellular oxidative stress on extracellular superoxide production using dHL60 and primary neutrophils from healthy subjects as model systems.

### Specific objectives

- Develop and validate cellular systems/models for studying neutrophil function.
- Culture *P. gingivalis*, extract its principal enzymes gingipains, and investigate their effects on IL-8 bioactivity and biochemistry.
- Identify the nature of plasma factors present in periodontitis that promote neutrophil respiratory burst.
- Develop methods to investigate the assembly of NADPH oxidase in neutrophils at rest and under oxidative stress.

## **Chapter 2.0 Materials and Methods**

## 2.1 Materials

The human promyelocytic cell line HL60 was purchased from the European Collection of Cell Cultures (ECACC no 98070101). RPMI 1640 with Ultraglutamine 1, foetal bovine serum (FBS) and penicillin ( $1000 \text{ U ml}^{-1}$ )/ streptomycin ( $10,000 \mu\text{g ml}^{-1}$ ) were obtained from Lonza Wokingham Ltd (Berkshire, U.K.). *P.gingivalis* W83 was kindly provided by Dr. A. Roberts (School of Dentistry, University of Birmingham, U.K.). Recombinant IL-8 and endothelial derived recombinant IL-8 were purchased from R&D systems (Abingdon, U.K.). RCDL protein assay kit and Aurum serum protein mini kit were purchased from BioRad (Hercules, CA, USA) cell culture plastics and plasticware were from Appleton Woods (Birmingham, UK) or Greiner Bio One (Stonehouse, UK). Needles and syringes were purchased from Terumo Europe (Leuven, Belgium). Optilyse C and Isoton were purchased from Beckman Coulter (Miami, USA). Cholera toxin subunit B (recombinant) with Alexa fluor647<sup>®</sup> conjugate was from Molecular Probe (Invitrogen, Paisley, UK). All the reagents were obtained from Sigma Chemical Company (Poole, U.K.) and solvents from Fisher (Loughborough, U.K.) unless otherwise stated. Antibodies used in this study are summarised in Table 2.1.



Antibody	Application	Discription	Product code	Company
Anti-interleukin-8	Neutralisation	Mouse Monoclonal	MAB208	R&D Systems, UK
Anti-interleukin-8	WB	Rabbit Polyclonal	AHP781	AbD Serotec, UK
Anti-gp91-phox	WB	Rabbit Polyclonal	07-024	Millipore,CA,USA
Anti-p47-phox	WB,IP	Rabbit Polyclonal	07-001	Upstate, NY, USA
Anti-flottilin-1	WB	Rabbit Monoclonal	04-592	Millipore,CA,USA
Anti-IFN- $\alpha$	Neutralisation	Mouse Monoclonal	21100-2	R&D Systems, UK
Anti-GM-CSF	Neutralisation	Mouse Monoclonal	MAB215	R&D Systems, UK
IgG1 Negative Control:RPE	IF	Mouse Monoclonal	MCA928PE	AbD Serotec, UK
Anti-human CD14:RPE-Cy5	IF	Mouse Monoclonal	MCA1568C	AbD Serotec, UK

**Table 2.1: Antibodies used in this study.** WB; western blotting, IF; immunofluorescence. IP; immunoprecipitation.

## 2.2 Methods

### 2.2.1 HL60 cell culture

The human promyelocytic cell line, HL60 cells grow as single-cell suspension culture without tendency to clump or adhere to plastics or glass (Collins et al., 1977). HL60 cells were cultured in RPMI-1640 medium with ultra-glutamine (Gibco Ltd., Carlsbad, Calif.) supplemented with 20% heat inactivated foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), immediately after recovery from frozen stock and until they establish an actively growing culture. Immediately after recovery from the frozen stock, HL60 cells show slow doubling time of 72h and once it is established, the cell density is reported to double every 24h (Fleck et al., 2005). Actively growing HL60 cell cultures were maintained between  $2 \times 10^5 - 5 \times 10^5$  cells/ml in RPMI-1640 medium with ultra-glutamine supplemented with 10% FBS and 1% P/S. The cell cultures were at 37°C in a 5% CO<sub>2</sub>/ 95% air humidified atmosphere. The number of viable cells was determined by Trypan blue exclusion method using an improved Neubauer haemocytometer (Appleton Woods, UK). Cells were passaged when reached to the concentration between  $2 - 5 \times 10^6$ /ml. The cell concentration was not allowed to exceed  $5 \times 10^6$ /ml as the high cell density may favour the differentiation of HL60 cells into different sublines (Fischkoff and Rossi, 1990). All the experiments were performed between passage numbers 5-30 as high passage numbers (above 35) were reported to increase tolerance to differentiating agents (Grillier et al., 1997) and therefore loss the ability to differentiate (Romero-Steiner et al., 1997). During the process of HL60 cell differentiation, the cells were treated with 1% DMSO in RPMI medium supplemented with 1% P/S and 10% FBS and incubated at 37°C in a 5% CO<sub>2</sub>/ 95% air humidified atmosphere for 5 days.

### **2.2.2 Patient and control plasma samples**

Plasma samples from chronic periodontitis patients (n=6; age range=50-60yrs; four male; two female) were collected from patients attending periodontal clinic at Birmingham Dental Hospital. The plasmas from healthy matching controls (n=6 age range 47-62yrs) were collected from staff of the Birmingham Dental Hospital. Both patient and healthy plasma was prepared and kindly provided by Dr. H. Wright, Birmingham Dental Hospital. Further, plasma samples from chronic periodontitis (n=10; age range=45-60yrs; five male, five female) patients were kindly provided by Mrs. E.M. Allen, National University of Ireland Cork, Ireland. Age- and gender-matched periodontally healthy control individuals (n = 10; age range = 37–62 yrs) were recruited from staff of the Birmingham Dental Hospital. Chronic periodontitis was defined as previously described (Brock *et al.*, 2004). All study participants were systemically healthy and were never-smokers, did not use recreational drugs, and had no special dietary requirements. Ethical approval was granted by the South Birmingham Local Research Ethics Committee (LREC 5643) and donors gave their informed consent. Ethical approval for collection of blood from healthy volunteers has provided by Aston University and donors gave their informed consent.

### **2.2.3 Collection and isolation of peripheral blood neutrophils.**

Neutrophils were isolated as described by Matthews *et al.* using Percoll<sup>®</sup> density centrifugation (Matthews *et al.*, 2007). Percoll<sup>®</sup> consists of colloidal silica particles of 15-30nm diameter (23% w/w in water), which have been coated with polyvinylpyrrolidone (PVP) (as manufacturer's description, Sigma Chemical Company, Poole, U.K.). The



silica particles forms self generated gradients by centrifugation in 1.5M NaCl. The isolation of the cells of interest by Percoll gradient is based on the differences in size or buoyant density exists in a population of cells. The inert nature of Percoll® media prevent interfering with down-stream assays.

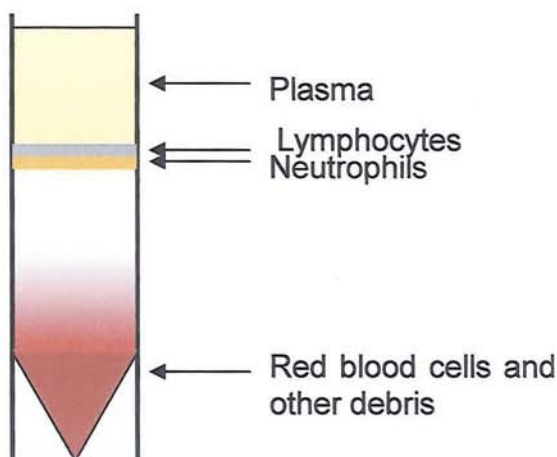
Venous blood was collected from systemically and periodontally healthy donors into 4% sodium citrate (weight/volume) in phosphate-buffered saline, with a citrate/blood ratio of 1:9. Two Percoll® densities were prepared (Table 2.1) and a discontinuous Percoll® gradient ( $\delta = 1.079:1.098$  g/ml) was prepared by layering equal volumes (8ml) of each density in a tube.

Reagent	Volume in ml for Percoll density 1.079 g/ml	Volume in ml for Percoll density 1.098 g/ml
Percoll	19.7	24.8
1.5M NaCl	3.5	3.5
Water	11.7	6.6

**Table 2.2: Reagents and volumes used to prepare percoll density  $\delta=1.079$  g/ml and  $\delta=1.098$  g/ml**

Whole blood (5ml) was carefully layered on top of the percoll gradient and centrifuged at 150g for 8 min followed by 400g for 8min at 4°C. The neutrophil layer (Figure 2.1) was carefully pipetted out into erythrocyte lysis solution (0.83% NH<sub>4</sub>Cl containing 1% KHCO<sub>3</sub>, 0.04% Na<sub>2</sub>EDTA.2H<sub>2</sub>O and 0.25% bovine serum albumin) to a volume ratio of 1:2 and incubated for 15 min with occasional mixing. Cells were then centrifuged at 400g for 5 min. Isolated cells were washed and re-suspended at  $1 \times 10^6$  cells/ml in physiological salt solution (PSS: 115 mM NaCl, 5 mM KCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM

glucose, 1 mM MgSO<sub>4</sub>, 1.25 mM CaCl<sub>2</sub>, 25 mM HEPES-Na supplemented with 0.1% bovine serum albumin at pH 7.4). Isolated cell viability was determined immediately prior to assay by trypan blue exclusion and was typically > 98%.



**Figure 2.1: Schematic representation of centrifuged whole blood in Percoll<sup>®</sup> density gradient**

#### **2.2.4 Chemiluminescence assay**

Chemiluminescence is the most sensitive method of superoxide detection (Afanas'ev et al., 2001), and is especially useful for the detection of low superoxide concentrations (Dobrian et al., 2001). The ionic and hydrophilic properties of lucigenin (10,10'-dimethyl-9,9'-biacridinium dinitrate) make it membrane impermeable, therefore it mainly detects O<sub>2</sub><sup>-</sup> produced as a result of the NADPH oxidase in the plasma membrane. This method is based on the reaction between lucigenin radicals, which are generated by the one electron reduction of lucigenin, and O<sub>2</sub><sup>-</sup> (Figure 2.2). The reaction produces an unstable dioxetane that decomposes with the generation of chemiluminescence.



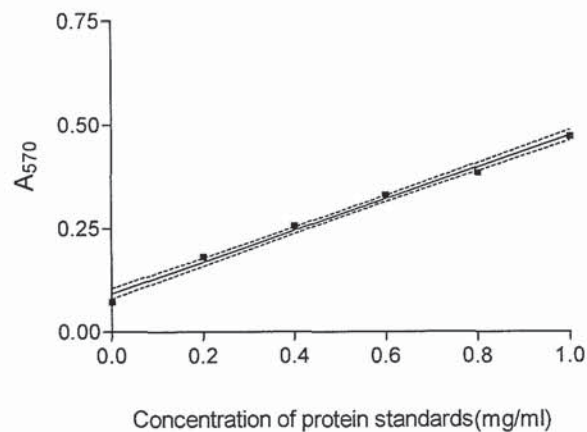


### 2.2.5 Flow cytometry

Flow cytometric analysis was performed on an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, USA). The XL system has capability to analyse up to four immunofluorescent colours within the specific wavelengths, for which it is equipped with four fluorescent channels FL1 (505-545nm), FL2 (560-590nm), FL3 (605-635nm), and FL4 (660-690nm). It uses a single air cooled argon laser as excitation beam. The flow cytometer can simultaneously measure and analyse multiple physical characteristics of single particles, i.e. cells. Generally, the flow cytometer can detect and analyse cell diameter ranging from 1-15  $\mu$ m. Briefly, the suspended cells are injected into a stream of sheath fluid within the flow chamber that maintains the hydrodynamic focusing of the sample in the sheath of fluid. Samples in the flow chamber are studied with the laser beam. The resulting light scattering and fluorescence is collected, filtered, and converted into electrical signals by the optical and electronics system that can be processed by the computer. Forward scatter with low angle scattered light is used as a relative measure of the particle size. Whereas, the side scatter represents relative granularity or internal complexity and relative fluorescence intensity of particles. Specific antigens on the surface or within permeabilised cells can be detected by labelling them with fluorescently-conjugated antibodies e.g. fluorescein isothiocyanate (FITC), R-Phycoerythrin-cyanine 5 (RPE-Cy5). In addition, endogenous metabolic reactions can be monitored by labelling the cells with non-fluorescent substrate and measuring the appearance of fluorescent product. Flow cytometry was used to compare and analyse dHL60 cell size, ROS production by 2',7'-dichlorofluorescein (DCF) oxidation, cell granularity and cell surface antigen and CD14 as described in chapter 3.

### 2.2.6 The bicinchoninic acid (BCA) assay

In order to calculate the total protein content of cells harvested after different treatments, the BCA assay was employed. The BCA method was adopted from Smith *et al.*, (Smith *et al.*, 1985). Unknown protein concentrations were determined from a standard curve prepared freshly for each experiment (Figure 2.3). The protein standards were prepared by diluting stock solution of 1mg/ml BSA standards with distilled water into 20%, 40%, 60%, 80%, and 100% v/v, where the final volume is 10 $\mu$ l and pipetted in to 96 well plates. Samples (10 $\mu$ l) with unknown protein content were also pipetted into the same plate. BCA reagent was prepared by adding bicinchoninic acid solution to copper sulphate II (50:1 v/v). BAC reagents were vortexed to mix and 200 $\mu$ l were pipetted into each well. The plate was incubated at 37°C for 30min and readings were taken at 570nm in a microplate reader (Dynex technologies, UK).



**Figure 2.3: A typical standard curve of BCA assay for analysis of protein content showing 95% confidence intervals.**

### 2.2.7 RCDC assay

After treatment, cell lysates for western blotting were prepared by adding warm laemmli buffer and sheared by passing them five times through a 21G needle. Cell lysates were then centrifuged at 1200×g for 5min to remove nuclei.

Total protein for western blot was assayed using the RCDC Protein Assay Kit (BioRad, Hercules, CA). The RCDC assay was carried out according to the manufacturer's instructions. Briefly, a concentration gradient (0, 0.25, 0.5, 1.0 and 2 mg/ml) of BSA standards were prepared in 1X Laemmli buffer and 5µl from each standards and samples were added into Eppendorf tubes in triplicates. Twenty five µl of RC reagent I is added to each tube vortexed and incubated for 1min at room temperature. RC reagent II (25µl) was then added into each tube, vortexed and centrifuged at 1200g for 3 min. The supernatant was drained by inverting the tubes and the pellet was allowed to dry for 10min. The reagent A mixed with reagent S to the ratio of 250:5µl. 25µl of the mixture was added into the pellet. Tubes were vortexed and incubated at room temperature for 10min until pellets were completely dissolved. At the end of the incubation contents were vortexed, 200µl of reagent B was added and vortexed immediately. Tubes were incubated at room temperature for 15min and dispensed into 96 well plates. The absorbance were measured at 630nm in MRX microplate reader (Dynex technologies, UK)



## 2.2.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

### 2.2.8.1 Reagents

Reagent	Description
Buffer 1 for Resolving gel	Tris-HCl (36.3g) was dissolved in 150ml water. SDS (0.8g) was added and dissolved. pH was adjusted to 8.8 and the solution was made up to 200ml
Buffer 2 for Stacking gel	Tris-HCl (6.06g) was dissolved in 80ml water. 0.4g SDS was added and dissolved. pH was adjusted to 6.8 and solution was made up to 100ml
Ammonium persulphate (10% w/v)	10% APS solution was aliquoted to 200 $\mu$ l and frozen at -20 <sup>0</sup> C
Running buffer	Tris-HCl (30g), glycine (144g) and SDS (10g) were dissolved in 900ml water. pH was adjusted to 8.3 and the solution was made up to 1 litre
Transfer buffer	Glycine (29g), SDS (1g) was dissolved in 2% methanol solution
TBS buffer (10X)	NaCl (120g) and Tris-HCl (60g) were dissolved in 900ml water. pH was adjusted to 7.5 and the solution was made up to 1 litre
TTBS Buffer	Tween 20 (0.5g) in 1 liter of TBS (1X)

**Table 2.3: Buffers and reagents required for SDS-PAGE and western blot**

### 2.2.8.2 Gel Electrophoresis

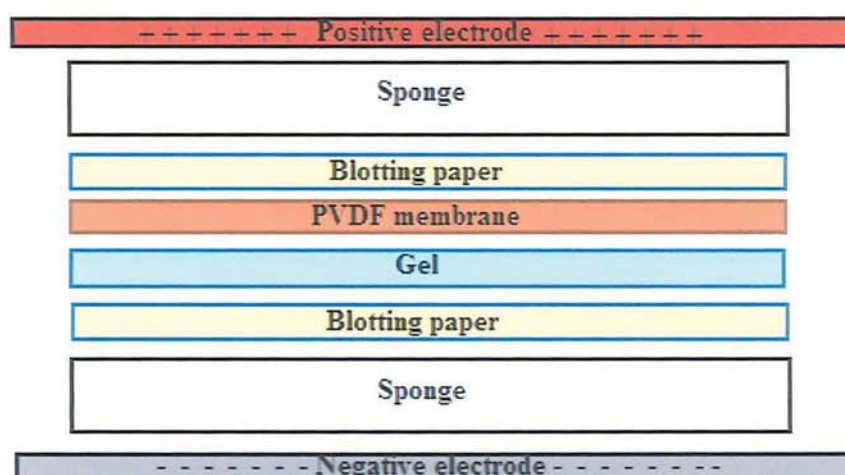
The samples were mixed 1:1 with Laemmli buffer (Sigma) and boiled to 95°C for 5min. Resolving and stacking gels were prepared in handcast Bio-Rad Mini Protean III system (Bio Rad Laboratories, Hercules, CA) where reagent concentrations are described in Table 2 for gels with differing crosslinks and pore sizes. Gels were left for 30 min to set. Samples (20µl) were loaded into the wells and electrophoresed at 150V for 1 hour and 30min.

Solution	Resolving gel concentration				Stacking gel
	7.5%	10%	12.5%	15%	
Resolving buffer	2.5ml	2.5ml	2.5ml	2.5ml	
Stacking buffer					1.5ml
Water	4.5ml	3.75ml	3ml	2.25ml	6ml
Acrylamide	2.5ml	3ml	4ml	5ml	2ml
APS	100µl	100µl	100µl	100µl	50µl
TEMED	20µl	20µl	20µl	20µl	15µl

**Table 2.4: Combinations of reagents required for different percentages of Acrylamide gels**

### 2.2.8.3 Western blot analysis

For subsequent protein detection of cell lysates using specific antibodies, proteins were transferred from gels to PVDF membranes. The PVDF membranes were cut to the size of the gel and prepared for protein transfer by soaking in 100% methanol for 10min followed by washing in transfer buffer for 5min. Proteins were then transferred to PVDF membrane in transfer buffer at 170mA, 150V for 2 hours (Figure 2.4).



**Figure 2.4: Schematic representation of sandwich assembly of wet transfer**

The membranes were blocked in 3% BSA for 2 hour or overnight. Western blots were then incubated with primary antibody followed by secondary antibody. The blot was washed with TTBS 15min  $\times$  4 times before adding each antibody. Western blots were developed and proteins were visualized using enhanced chemiluminescence reagent as described by manufacturer (ECL, Amersham Biosciences). The blot was then exposed to X-ray film (Amersham Biosciences) for 10sec-5min for the best image in the dark room and developed and fixed using developer (Sigma processing chemicals, Kodak<sup>®</sup>, polymax RT developer/replenisher) and fixer solutions (Sigma processing chemicals, Kodak<sup>®</sup>, Fixer).



### 2.2.9 Data analysis

Unless specified all data is presented as the mean  $\pm$  standard error of the mean (SEM) of at least three independent experiments, performed in triplicate. Statistical analysis was performed using a two tailed, unpaired student's t-test when comparing the difference between two group means. When comparing column means from more than two samples the one way analysis of variance followed by Tukeys Multiple Comparison test was used. A  $P < 0.05$  was considered as significant. A 95% confidence interval was used for the difference between all selected pairs of means in the t-test and the ANOVA/ Tukey's post test. Statistics were performed using the Graphpad Prism version 3.02 statistical software.

## **Chapter 3.0 Characterisation of promyelocytic HL60 cell line pre- and post- differentiation to a neutrophil-like phenotype**

### **3.1 Preface**

This chapter characterises differentiated HL60 cells as a model to resemble functional properties of normal peripheral blood neutrophils, including chemotaxis and ability to produce a respiratory burst. Promyelocytic HL60 cells were terminally differentiated using DMSO to neutrophil-like dHL60 cells, and they were characterised morphologically by size and shape and functionally with respect to  $O_2^-$  production, chemotaxis and  $Ca^{2+}$  mobilisation. The suitability of the dHL60 cell model to study the behaviour of neutrophils is discussed.

### 3.2 Introduction

Human peripheral blood polymorphonuclear neutrophils are terminally differentiated cells that represent about 60% of all circulating leukocytes. Phagocytes play an important role in both acute and chronic inflammation. Except for the main phagocytic functions of neutrophils such as chemotaxis, production of respiratory burst, degranulation, and formation of NETs, they also produce wide array of cytokines such as IL-8, in response to a physiological stimuli. Enhanced neutrophil activity with respect to the respiratory burst, chemotaxis and degranulation is often reported in chronic inflammatory diseases including chronic periodontitis. Although progress has been made in understanding the mechanisms behind this neutrophilic phenotype, using primary neutrophils often has limitations especially due their short life span (16-24 hours in circulation) and because they are refractory to transfection. Thus, the characterisation of a cell line that can be differentiated into neutrophilic phenotype and be further manipulated may provide a useful research tool to investigate the underlying mechanisms behind enhanced neutrophil activity in chronic inflammatory conditions.

Different human promyelocytic leukemia cell lines, HL-60, THP-1, NB-4 and PLB-985 have been extensively used in previous studies. Various approaches; dimethyl sulfoxide (DMSO) to differentiate HL-60 cells (Teufelhofer et al., 2003) and PLB-985 cells (Ear and McDonald, 2008, Ashkenazi et al., 2009); all-*trans* retinoic acid (ATRA) to differentiate HL60 cells (Sham et al., 1994), THP-1 cells and NB-4 cells (Oya-Ito et al., 2008); dibutyryl cyclic adenosine 3':5'-monophosphate (Bt<sub>2</sub>cAMP) to differentiate THP-1 cells (Ishibashi et al., 2006); N,N-dimethylformamide (DMF) to differentiate PLB-985 cells (Choi et al., 2006); and retinoic acid in combination with vitamin D3 and GCSF to



differentiate HL-60 and NB-4 cells (Fleck et al., 2003), have been employed to differentiate these cells into neutrophil-like cells. The level of resemblance to the neutrophilic phenotype after cell differentiation is found to be depend on the type of cells (Fleck et al., 2003), differentiating agent (Fleck et al., 2003) and other conditions such as concentration of the chemical used (Chang et al., 2006), supplements added (Fleck et al., 2003) and the time of exposure to the differentiating agents (Chang et al., 2006). Therefore, it is important to characterise and optimise the conditions required for the cell line of interest to differentiate into neutrophil-like cells.

Differentiated HL-60 cells have been extensively used to study the behaviour of primary neutrophils (Oya-Ito et al., 2008, Fleck et al., 2005, Brown et al., 2002, Fontana et al., 1981, Sham et al., 1994). The HL-60 cell line derived from the peripheral blood leukocytes of a female patient with leukemia and it was among the first long-term suspension cultures of human myeloid cells to be established (Fleck et al., 2005). In cell culture, these cells show round or ovoid morphology occasionally with pseudopods and a size range between 9-25 $\mu$ M in diameter (Collins et al., 1978, Fleck et al., 2005). They also exhibit typical promyelocyte characteristics of large round nuclei with two to four nucleoli. The cytoplasm is basophilic with prominent azurophilic granules (Fleck et al., 2005, Collins et al., 1978). It is reported that in cell culture, approximately 5-10% of undifferentiated cells appear to be mature myelocytes (Collins et al., 1979).

HL-60 cells can terminally differentiate into neutrophil-like cells by exposure of wide range of polar compounds including DMSO (Sham et al., 1994, Chang et al., 2006), DMF (Fontana et al., 1981) or ATRA (Ear and McDonald, 2008). However, exposing HL60 cells to vitamin D3 or PMA terminally differentiated into macrophages (Li et al., 2002).

When differentiated along the neutrophil cell line, they exhibit functional properties similar to granulocytes including chemotaxis (Collins et al., 1979, Sai et al., 2006), superoxide generation (Korchak and Kilpatrick, 2001, Oya-Ito et al., 2008), phagocytosis (Oya-Ito et al., 2008) and  $\text{Ca}^{2+}$  mobilisation to fMLP stimuli (Korchak et al., 2007). Differentiated HL60 cells also up-regulate several neutrophil receptors including CD11b/CD18 (Mac-1) (Huang et al., 2009, Chang et al., 2006), fMLP (Sham et al., 1994) and GPI-80 (glycosylphosphatidylinositol anchored 80kDa glycoprotein), which are involved in neutrophil adhesion and migration (Takeda et al., 2003). In contrast, differentiated neutrophil-like HL60 cells fails to show elastase release in response to fMLP stimulation (Korchak et al., 2007).

Due to the inconsistency of the methods used to differentiate HL60 cells into neutrophil-like HL60 cells, and also the differences observed within HL60 cell derived sublines (Fleck et al., 2005), this chapter focuses on the development of an in-house method to optimise and standardise conditions to provide reproducible yields of neutrophil-like cells suitable for use as effector cells in bioassays.

### 3.3 Materials and methods

#### 3.3.1 Chemiluminescence assay optimisation

DMSO differentiated or non-differentiated HL60 (dHL60 or HL60 respectively) cells were washed twice and resuspended in PBS containing 1% BSA. Briefly, to optimise, lucigenin dependant CL assay, dHL60 cell viability and level of respiratory burst produced in varying concentration of DMSO (0-1.75%), dHL60 cell viability on the duration of exposure to 1% DMSO (0-7days), concentration of lucigenin (0-1mM) and the effect of plastics on dHL60/primary neutrophil respiratory burst were evaluated experimentally.

Applying the optimised conditions for the use of lucigenin dependant CL for the detection of dHL60/primary neutrophils by CL assay was as follows.  $2 \times 10^5$  cells were treated with 1% DMSO in RPMI medium and incubated at 37°C in a 5% CO<sub>2</sub>/ 95% air humidified atmosphere for 5 days. Differentiated cells were washed with PBS twice and resuspended at  $1 \times 10^5$  cells. 100µM lucigenin were added to the preblocked (PBS containing 1% BSA, overnight, 4°C) white microwell plates (Greiner Bio-One Ltd, UK) or single tubes. The plate/tubes were then placed into the microplate reader/water bath and 100 µl of PBS buffer containing  $1 \times 10^5$  cells (dHL60 cells/isolated primary neutrophils) were then added to each well/tube and incubated for 30 min at 37°C. Light emission in relative light units (RLUs) was recorded to study baseline for 20min. Cells were stimulated with 1µM fMLP or 10nM PMA and stimulated respiratory burst was measured for further 30min. All the assays were performed (37°C) using Berthold microplate-luminometer (Orion II Berthold microplate-luminometer UK Ltd., Milton Keynes, Bucks, UK) unless otherwise stated.



### 3.3.2 Chemiluminescence assay for comparison of luminopors

PBS supplemented with 1% BSA, 3 mM luminol or 3mM isoluminol with 6U horseradish peroxidase or 100 $\mu$ M lucigenin were added to pre-blocked white microwell plates (Greiner Bio-One Ltd, UK). PBS buffer (100  $\mu$ l) containing dHL60 cells ( $5 \times 10^5$  cells) was then added to each well and incubated for 30 min at 37°C. RLU was recorded to study the baseline for 20min. At the end of pre-incubation period, cells were stimulated with 10 $\mu$ l of fMLP (1 $\mu$ M), 10nM PMA, 25 $\mu$ l of opsonized *Staphylococcus aureus* (NCTC 6571) (300 bacteria/neutrophil), *Fusobacterium nucleatum* suspension (ATCC 10953) (100 bacteria/neutrophil), *Escherichia coli* LPS (5  $\mu$ g/ml, equivalent to 50 ng/ $10^5$  cells) or PBS (unstimulated control) and the stimulated respiratory burst was measured for further 30min. Peak RLUs were determined for both pre- and post-stimulation incubation periods. All samples were run in triplicate and assays were performed using Berthold microplate-luminometer (Orion II Berthold microplate-luminometer UK Ltd., Milton Keynes, UK).

### 3.3.3 Flow cytometric assay for comparison of HL60 cell size and granularity

HL-60 cells ( $1 \times 10^6$ ) were harvested and washed twice with PBS. The cells were suspended in 1ml of PBS and were aspirated and analysed by EPICS® XL-MCL flow cytometer (Beckman-Coulter, Miami, USA). Viable cells were gated based on FS/SS results and data was collected for 10,000 events against forward scatter (FS). Median X (MdX) value FS as an indicator of cell size and SS as an indicator of cell granularity was compared for the cells grown in the presence or absence of DMSO.

### 3.3.4 Flow cytometric assay for cell surface CD14 analysis

dHL60 cells were washed twice in PBS and resuspended at a cell concentration of  $5 \times 10^5$  cells/ml. RPE-Cy5 labelled anti-CD14 monoclonal antibody (5 $\mu$ l; AbD Serotec, Oxford, UK) or 5 $\mu$ l of negative isotype was added to 1ml of cell suspension and incubated 30 min on ice in the dark. Whole-blood (100 $\mu$ l) was incubated with 5 $\mu$ l of RPE-Cy5 labelled anti-CD14 antibody or 5 $\mu$ l of negative isotype for 10min in dark in ice. Optilyse C (Immunotech, Marseille, France) solution (400 $\mu$ l) was added, vortexed and cells were incubated for further 20min in ice, in the dark. Blood was diluted by adding 500 $\mu$ l of PBS supplemented with 0.1% BSA. After incubation, neutrophils and HL60s were gated according to their scatter properties and were cytofluorometrically analyzed for surface CD14 expression. For each experiment, 30,000 events were counted and CD14 receptor expression was determined as the median fluorescence intensity.

### 3.3.5 Measurement of $[Ca^{2+}]_i$ mobilization in dHL60 cells

The method for measurement of  $[Ca]_i$  was adapted from *Brechard et al.*, (2005). Briefly,  $5 \times 10^5$  dHL60 cells were harvested and washed with PSS (physiological salt solution: 115mM NaCl, 5mM KCl, 1mM  $KH_2PO_4$ , 10mM glucose, 1mM  $MgSO_4$ , 1.25mM  $CaCl_2$ , 25mM Hepes-Na supplemented with 0.1% bovine serum albumin (BSA) at pH 7.4) twice, re-suspended in PSS (1ml) and incubated with 2.5 $\mu$ M Fura-2 acetoxymethyl ester (Fura-2/AM) at 37°C for 30min. Cells were centrifuged 1200g for 5min and the supernatant was discarded. Cell pellet was washed two times (1200g, 5min) with PSS to remove extracellular dye. dHL60 cells ( $5 \times 10^5$  cells) were re-suspended to the same cell density in

the absence or presence of 1.25mM  $\text{Ca}^{2+}$ . Fura-2 fluorescence was measured in kinetic mode (1 min time intervals) with shaking at 37°C in a spectrofluorometer (Molecular Probes, UK) at excitation wavelength of 340nm and 380nm, and emission wavelength of 510nm. A baseline level of  $[\text{Ca}^{2+}]_i$  was recorded for 2min before the agonist 1μM fMLP was added and emission monitored for 8min. An internal calibration was performed for each cell sample by addition of 10μM ionomycin (to measure  $\text{Ca}^{2+}$ -saturated fura-2) and 12.5mM EGTA (to measure  $\text{Ca}^{2+}$ -free fura-2).  $[\text{Ca}^{2+}]_i$  was calculated using a  $K_d$  of 224nM (Scanlon et al., 1987) for the reaction between fura-2 and  $\text{Ca}^{2+}$  (Palmer and Moore, 2000).

$$[\text{Ca}^{2+}] = K_d \left( \frac{R - R_{\min}}{R_{\max} - R} \right) \left( \frac{S_{f2}}{S_{b2}} \right)$$

$K_d$ = The dissociation constant between fura-2 and free  $\text{Ca}^{2+}$

$R$ = Fluorescence ratio defined as the fluorescence intensity induced by  $\lambda_1$  (excitation wavelength at 340nm) divided by the fluorescence intensity induced by  $\lambda_2$  (excitation wave length at 380nm)

$R_{\min}$ = $R$  recorded when  $[\text{Ca}^{2+}] = 0$  (in the presence of 12.5mM EGTA; where  $\text{Ca}^{2+}$ -free fura-2)

$R_{\max}$ =  $R$  recorded when  $[\text{Ca}^{2+}] \gg K_d$  (in the presence of 10μM ionomycin; where  $\text{Ca}^{2+}$ -saturated fura-2)

$S_{f2}$ = fluorescence intensity induced by  $\lambda_1$  (when  $\text{Ca}^{2+}$  is not bound to fura-2)

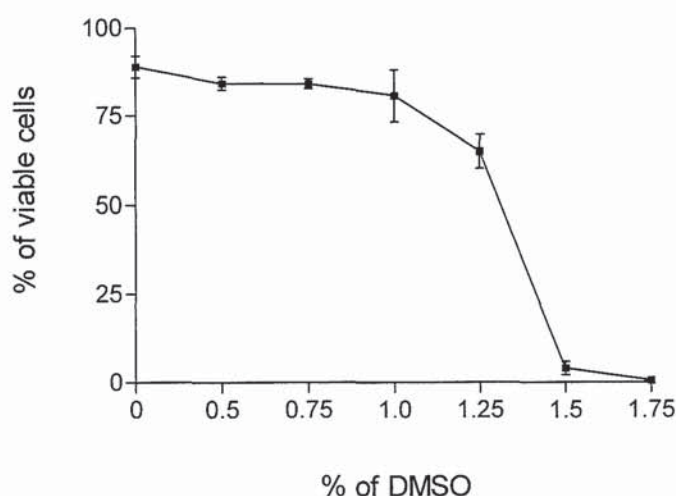
$S_{b2}$ = fluorescence intensity induced by  $\lambda_2$  (when fura-2 is fully bound with  $\text{Ca}^{2+}$ )



### 3.4 Results

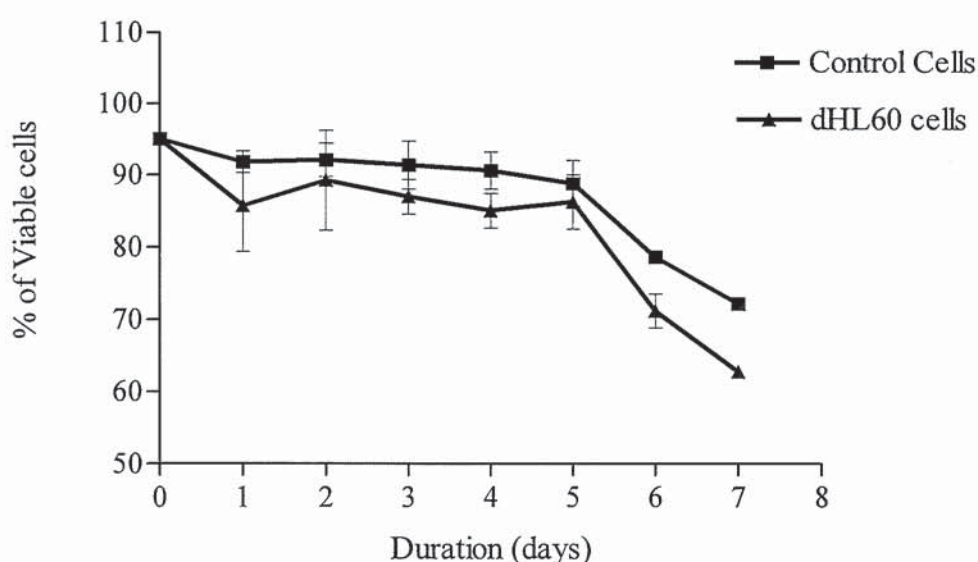
#### 3.4.1 Optimisation of conditions for DMSO-induced differentiation of HL 60 cells; effects on cell size and viability

In order to develop a reproducible model for investigating hyperactivity and hyper-reactivity in primary neutrophils, HL60 cells were differentiated in increasing concentrations of DMSO for five days. DMSO treatment caused a decrease in cell viability in a dose dependent manner (Figure 3.1A). Concentrations of DMSO of 1.5% and above induced a significant reduction of cell viability ( $P < 0.001$ ) as determined by Trypan blue exclusion, with fewer than 10% cells viable after five days in 1.5% DMSO.



**Figure 3.1A Effects of DMSO concentration on cell viability.**  $2 \times 10^5$  HL60 cells/ml were seeded on day 0 in varying DMSO concentrations and incubated at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$ / 95% air humidified atmosphere. Cell viability was measured on 5th day of incubation by Trypan blue exclusion, and results are expressed as mean  $\pm$  SEM of % of viable cells, where  $n=3$  independent experiments.

DMSO treatment (1%) significantly ( $p < 0.05$ ) decreased the original viable cell count after one day of incubation (Figure 3.1B). However, both differentiated and non-differentiated cell cultures maintained stable cell viable counts (90%) without passage for 5 days. On the 6<sup>th</sup> day of incubation, both HL60 and dHL60 cell viability was decreased to  $78\% \pm 0.11$  (mean  $\pm$  S.E.M) and  $71\% \pm 1.66$  respectively. This is a significant decrease in viability compared with initial cell viability ( $p < 0.01$ ).

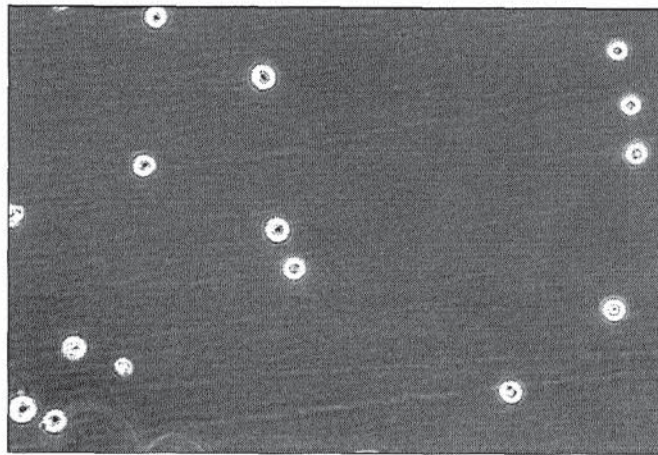


**Figure 3.1B: Effect of differentiation using DMSO on HL-60 cell viability over the time.** HL60 cells ( $2 \times 10^5/\text{ml}$ ) were seeded on day 0 in the presence or absence of DMSO (1%). Cell viability was measured each day by Trypan blue exclusion, and results are expressed as mean  $\pm$  SEM of % of viable cells, where  $n=3$  independent experiments.

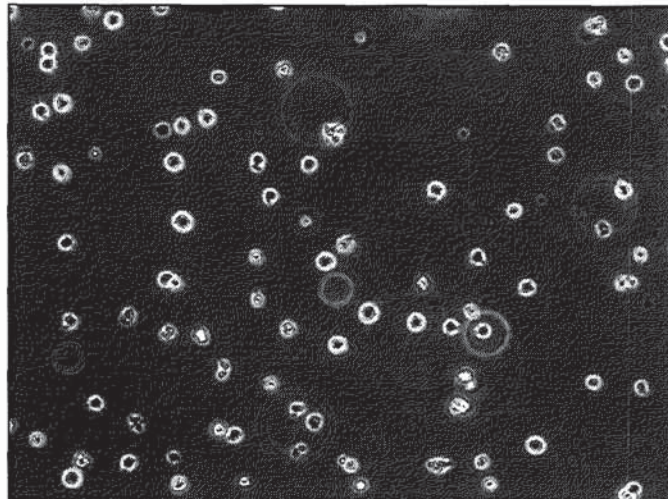
To investigate morphological changes after HL60 cell differentiation, the cells were observed under the microscope (Figure 3.2A and B) and cell size/granularity was analysed by flow cytometry. DMSO treatment (1%) for 5 days had a decreased cell size distribution compared with non-differentiated HL60 cells as shown in figure 3.2A and 3.2B. This was confirmed using flow cytometry, undifferentiated HL60 cells conformed to a median value of forward scatter (Mdx) size distribution of  $523.26 \pm 3.26$  (Mdx  $\pm$  SEM; Figure 3.3A). 1% DMSO-differentiated cells were of smaller size of  $373.33 \pm 14.19$  (Figure 3.3), confirming previous reports of a reduction in HL60 cell size after treatment with differentiating agents (Collins et al., 1978). However, the Mdx value for side scatter (Figure 3.3D), which is representative of cell granularity, not significantly changed when HL60 cells ( $247.7 \pm 5.21$ ) treated with 1% DMSO ( $278.6 \pm 12.67$ ).



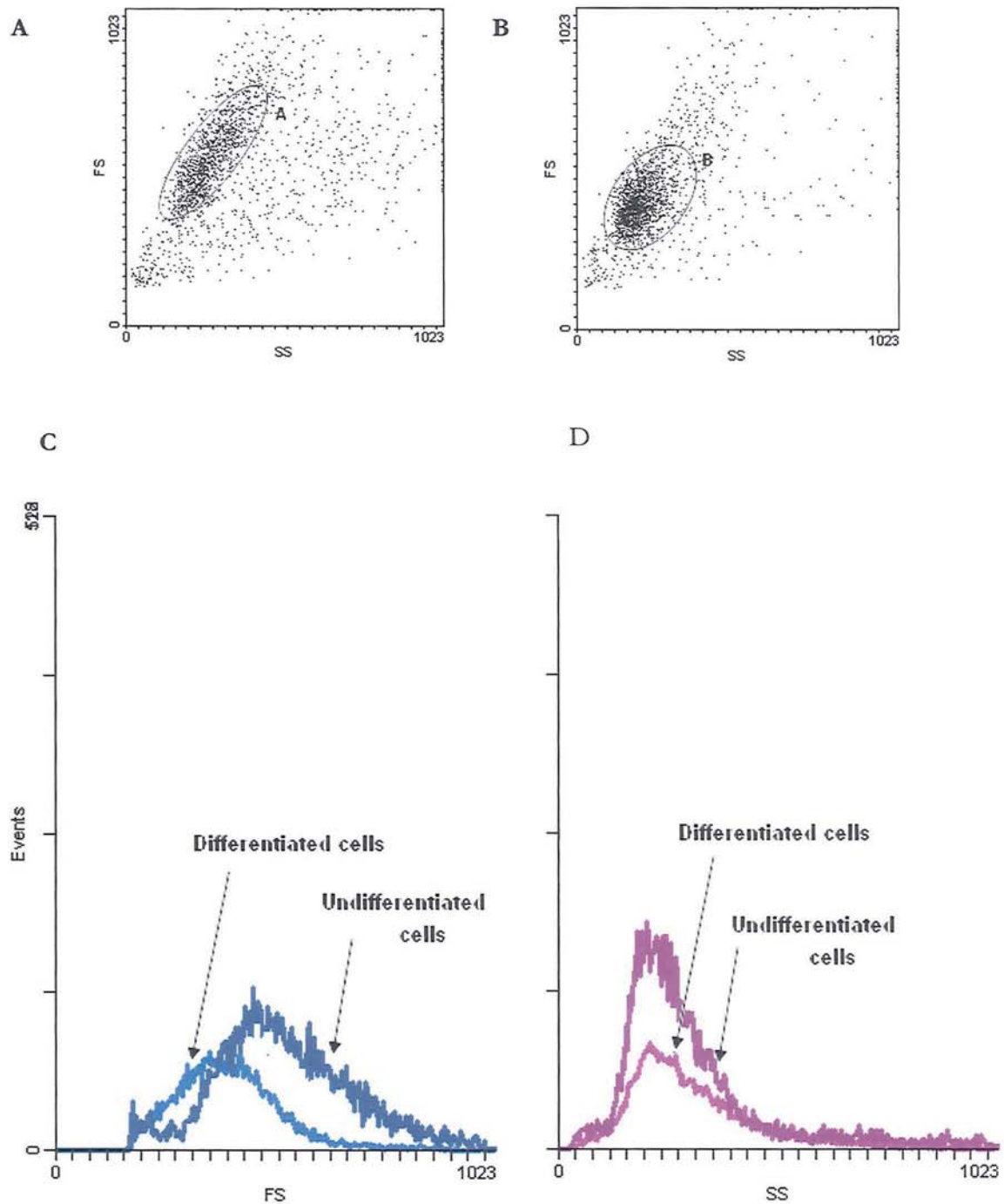
**A**



**B**



**Figure 3.2: Human promyelocytic cell line- HL60 cells before (A) and after (B) differentiation in 1% DMSO for 5 days.** Microscopy was performed using an inverted microscope at magnification of 20X (Zeiss Axiovert 200M, Carl Zeiss Ltd.UK). Differentiated cells are smaller and have an irregular shape compared to non-differentiated cells.



**Figure 3.3: Distribution of DMSO differentiated and non-differentiated HL-60 cell size.**  $1 \times 10^6$  HL-60 cells were harvested and washed twice with PBS. Suspended cells were aspirated and analysed by flow cytometry. Viable cells were gated based on FS/SS results (non-differentiated cells in -A and differentiated cells in -B) and data was collected for 10,000 events against forward scatter (FS). Median X (MdX) value FS as an indicator of cell size (C) and SS as an indicator for cell granularity (D) was compared for cells grown in the presence or absence of 1% DMSO.

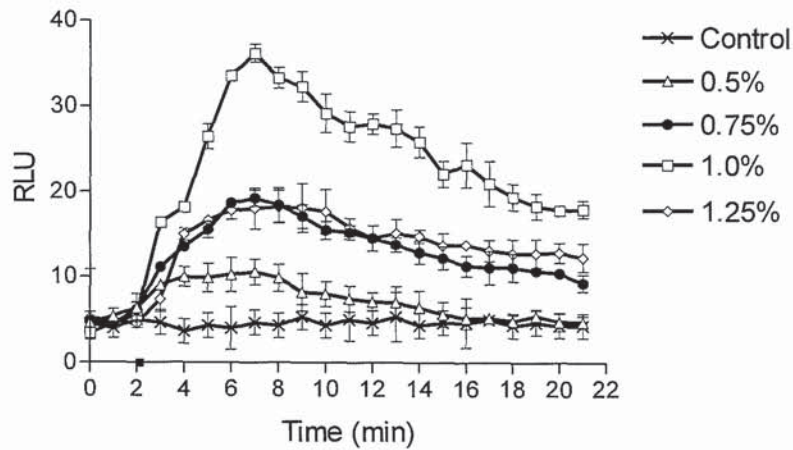
### **3.4.2 PMA induces a significant respiratory burst in DMSO differentiated HL60 cells and human neutrophils measured by lucigenin dependent chemiluminescence.**

To investigate the optimal concentration of DMSO for HL60 cell differentiation into a neutrophil with a superoxide anion radical producing phenotype, the  $O_2^-$  production was measured by  $O_2^-$  dependent lucigenin chemiluminescence following increasing concentrations of DMSO exposure for 5 days (Figure 3.4A) in single well Orbit Berthold luminometer (BioRad Co-operation, California, USA). dHL60 cells responded to PMA (10nM) by significant production of  $O_2^-$  ( $P<0.05$ ) after 1 min and the signal reached a maximum of  $36.22 \pm 0.61$  (RLU  $\pm$  S.E.M) within 4 min, whereas undifferentiated cells did not produce an enhanced  $O_2^-$  signal at rest or in response to PMA. 1% DMSO treated cells showed the maximum  $O_2^-$  production after stimulating with PMA.

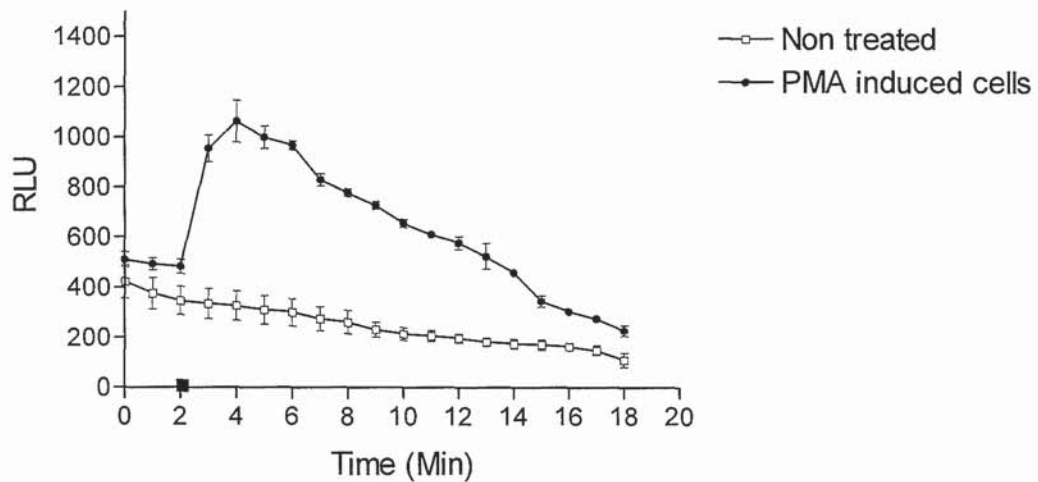
The respiratory burst of neutrophils isolated from healthy human volunteers was measured by repeating the same experimental conditions as for dHL60 cells (Figure 3.4B). Neutrophils show significant respiratory burst ( $P<0.001$ ) upon PMA (10nM) induction after 1min and the lucigenin chemiluminescence signal reached a maximum of  $1064 \pm 48.2$  (RLU  $\pm$  S.E.M) within 2min. After 6 min, rapid decay of chemiluminescence signal (indicative of lower superoxide production) was observed from neutrophils. Differentiation of HL60 cells with 1% DMSO gave the greatest PMA-dependent respiratory burst measured by lucigenin-dependent chemiluminescence without significant loss of viability and was used for all subsequent experiments.



A



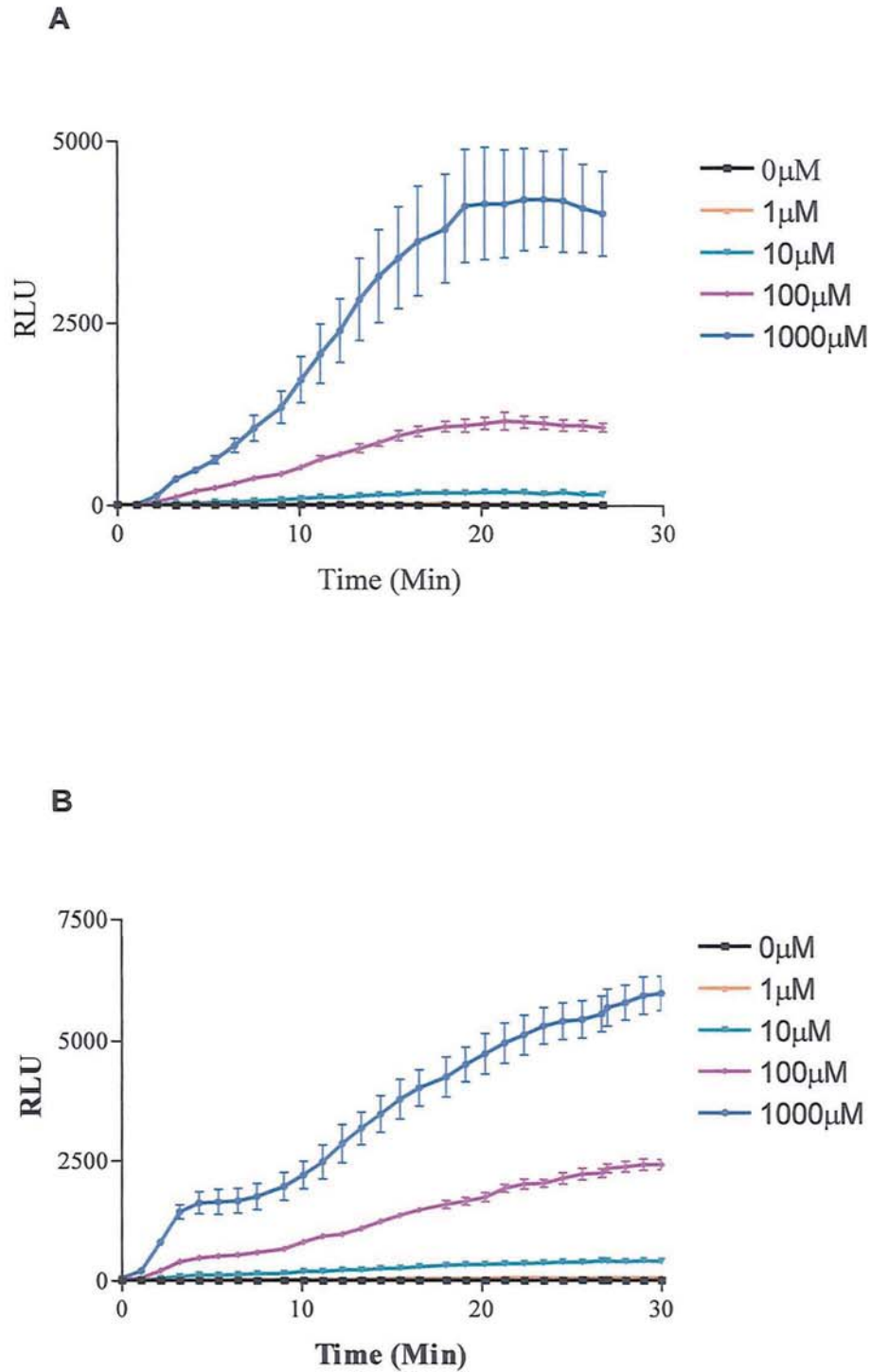
B



**Figure 3.4: PMA induces activation of the respiratory burst in differentiated HL60 cells and neutrophils.** (A)  $5 \times 10^5$  HL60 and dHL60 cells/ml were harvested and washed twice with PBS and (B)  $5 \times 10^5$  neutrophils were isolated using a Percoll® density gradient as described in the materials and methods. For both 3.4A and 3.4B,  $5 \times 10^5$  cells in 100  $\mu$ l of PBS were incubated with 100  $\mu$ M lucigenin at 37°C for 30min prior to stimulation of respiratory burst.  $O_2^-$  production was monitored as relative light unit (RLU) before and after stimulation (at 2min on X axis) with 50nM PMA. Results are expressed as mean  $\pm$  S.E.M, where n=3 independent experiments. The assay was performed in single well Orbit Berthold luminometer (BioRad Co-opreation, Calif., USA)

### **3.4.3 Optimisation of lucigenin concentration for use with dHL60 in the chemiluminescence assay**

The effect of lucigenin concentration on chemiluminescence assay was monitored in figure 3.5. 1% DMSO-differentiated (dHL60) cells were washed twice and resuspended in PBS containing 1% BSA, PBS and increasing concentrations (0 $\mu$ M, 1 $\mu$ M, 10 $\mu$ M, 100 $\mu$ M and 1mM) of lucigenin were added to independent wells of a 96 well white microplate (Greiner Bio-One Ltd, UK). The plate was then placed into the microplate reader (Orion II Berthold microplate-luminometer UK Ltd., Milton Keynes, UK). PBS buffer (100  $\mu$ l) containing dHL60 cells ( $1 \times 10^5$  cells) were then added to each well and incubated for 30 min at 37°C. After the incubation, light emission in relative light units (RLUs) was recorded to study baseline for 20min. Cells were stimulated with 10nM PMA and stimulated respiratory burst was recorded for further 30min. The relative light units measured were dependent on the concentration of lucigenin for both cell types used. Lucigenin (1mM) detected highest RLUs from neutrophils (6119 $\pm$ 359) and dHL60 cells (4210 $\pm$ 697) with maximum peak at 32min and 22min respectively. However, these readings were associated with higher variability than values obtained for neutrophils (2462 $\pm$ 52) and dHL60 cells (1147 $\pm$ 80) with 100 $\mu$ M lucigenin. Therefore 100 $\mu$ M lucigenin was used in future analysis.

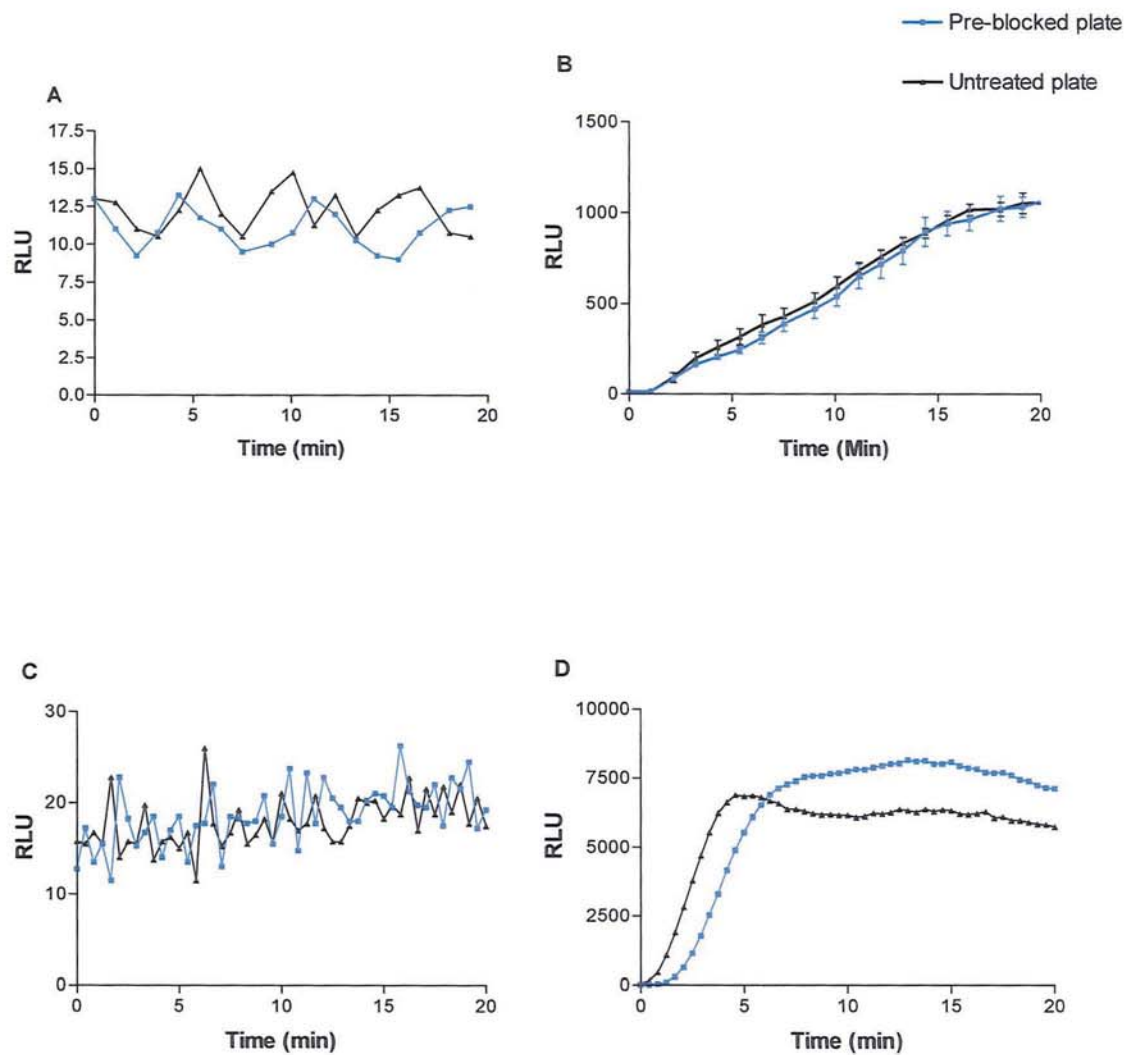


**Figure 3.5: The effect of lucigenin concentration on PMA stimulated respiratory burst of dHL60 cells and primary neutrophils.** Differentiated HL60 cells (A) or neutrophils (B) ( $5 \times 10^5$ ) were isolated as described in Methods 2.2.4 and incubated in white microwell plates with 0, 1, 10, 100 or 1000  $\mu$ M concentrations of lucigenin for 20min before stimulation with 10nM PMA. The stimulated respiratory burst was measured for 30min.



#### **3.4.4 The effect of pre-blocking white microplates on lucigenin-dependent chemiluminescent signals produced by PMA activated dHL60 cells and neutrophils.**

As neutrophils are particularly sensitive to activation by cell surface contact, white microplate wells were incubated with 200µl of PBS containing 1%BSA overnight at 4<sup>0</sup>C to investigate whether this would reduce non-specific activation of cells (Figure 3.6). 1% DMSO-differentiated HL60 cells and primary neutrophils were washed twice and resuspended in PBS containing 1% BSA. PBS and 100µM of lucigenin was added to cells which were incubated in pre-blocked and non-blocked white microplates (Greiner Bio-One Ltd, UK). Each plate was then placed into the microplate reader and the respiratory burst of dHL60 cells and neutrophils ( $1 \times 10^5$  cells) were measured as described previously. Peak RLUs were determined for both pre- and post-stimulation incubation period. Future CL experiments were performed with pre-blocked white microwell plates.

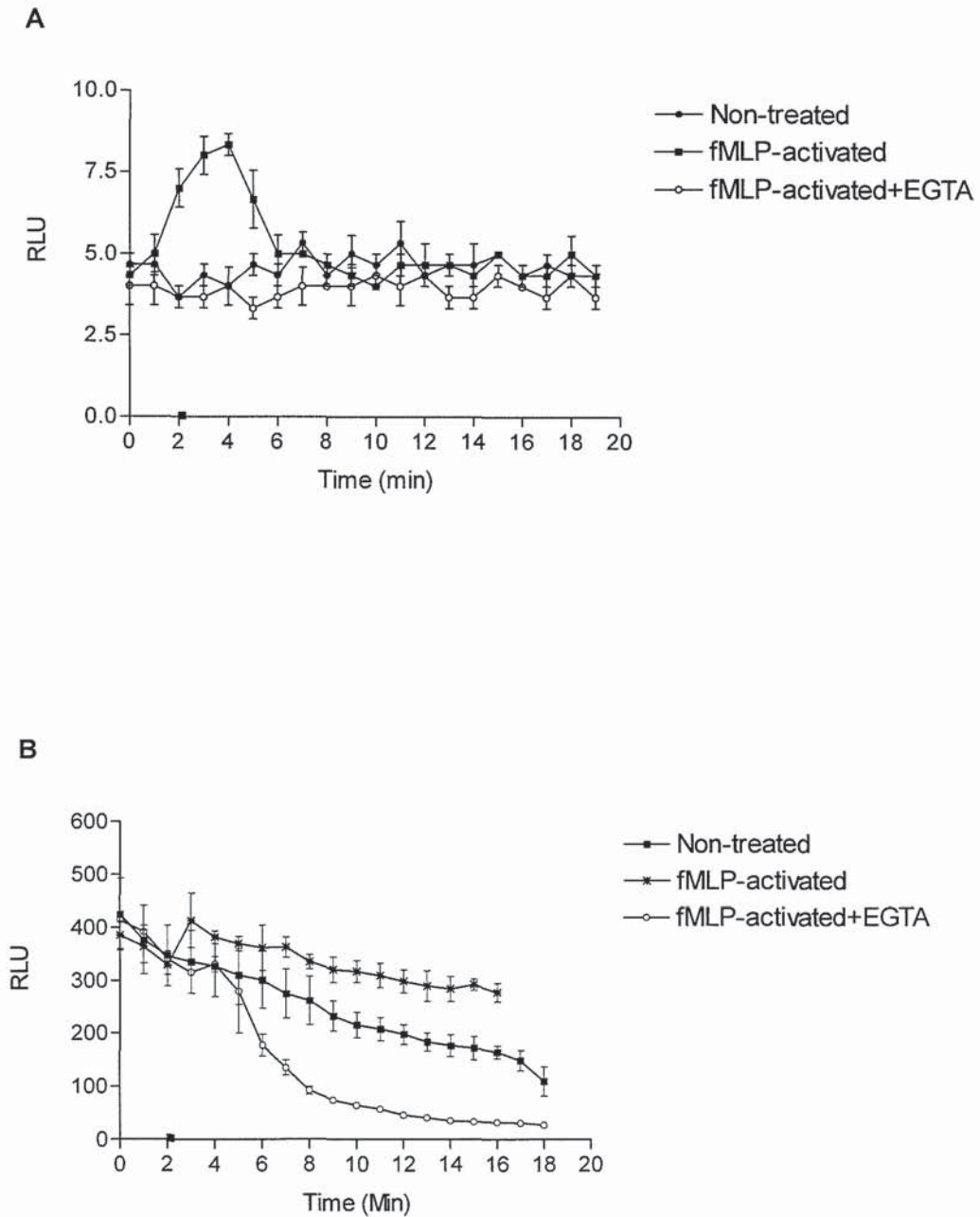


**Figure 3.6: Albumin-blocked microwell plates prevent an early respiratory burst activity of neutrophils in the presence of PMA.**  $1 \times 10^5$  cells of dHL60 cells (A and B) or neutrophils (C and D) were incubated in white micro well plates with or without pre-blocking as described in Method 3.3.1. Cells were incubated with  $100\mu\text{M}$  lucigenin at  $37^\circ\text{C}$  for 20min (A and C) before stimulating with  $10\text{nM}$  PMA. The stimulated respiratory burst was recorded for 20min (C and D).

### **3.4.5 Requirement for extracellular $[Ca]^{2+}$ for activation of the respiratory burst of dHL60 cells and neutrophils**

To investigate the association of calcium mobilization with the respiratory burst, the  $5 \times 10^5$  dHL60 cells (Figure 3.7A) or neutrophils (Figure 3.7B) were incubated in the presence (1.25mM) or absence of extracellular calcium. The dHL60 cells or neutrophils ( $5 \times 10^5$ ) were incubated in pre-blocked white microwell plates containing 100 $\mu$ M lucigenin and in the presence (1.25mM) or absence of calcium. After 30min pre-incubation period, cells were stimulated with 1 $\mu$ M fMLP. dHL60 cells produced significant respiratory burst of  $8.33 \pm 0.33$  (RLU  $\pm$  S.E.M) within 2min of fMLP stimuli ( $P < 0.01$ ). fMLP was unable to stimulate production of  $O_2^-$  in the presence of the  $Ca^{2+}$  chelator, EGTA. Neutrophils elicited a maximal respiratory burst of  $413.3 \pm 29.6$  (RLU  $\pm$  S.E.M) within 1min in the presence of  $Ca^{2+}$  and the respiratory burst signal was not induced in the absence of extracellular  $Ca^{2+}$ .

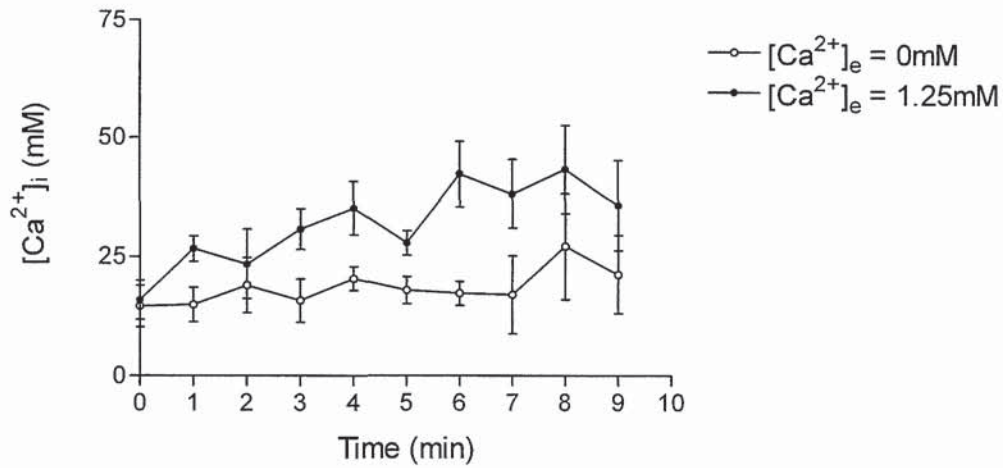




**Figure 3.7: dHL60 cells and neutrophils elicit a calcium-dependent respiratory burst with fMLP.**  $5 \times 10^5$  cells of dHL60 cells (A) or neutrophils (B) were stimulated with  $1 \mu\text{M}$  fMLP ( at 2 min on X axis) in the presence or in the absence of extra-cellular  $1.25\text{mM}$   $\text{Ca}^{2+}$ . The assay was performed in single well Orbit Berthold luminometer (BioRad Co-opreation, Calif., USA)

### 3.4.6 fMLP stimulated $[Ca^{2+}]_i$ mobilisation in dHL60 cells

To further analyse the dHL60 cell model as a suitable model for studying the intracellular signaling cascade, the mobilisation of  $Ca^{2+}$  in dHL60 cells was measured by the use of a double labelling fluorescent technique. In the presence of extracellular  $Ca^{2+}$  ( $[Ca^{2+}]_e$ ), 1 $\mu$ M fMLP stimulated dHL60 cells showed higher intracellular  $Ca^{2+}$  mobilisation compared with absence of extracellular  $Ca^{2+}$  (Figure 3.8).



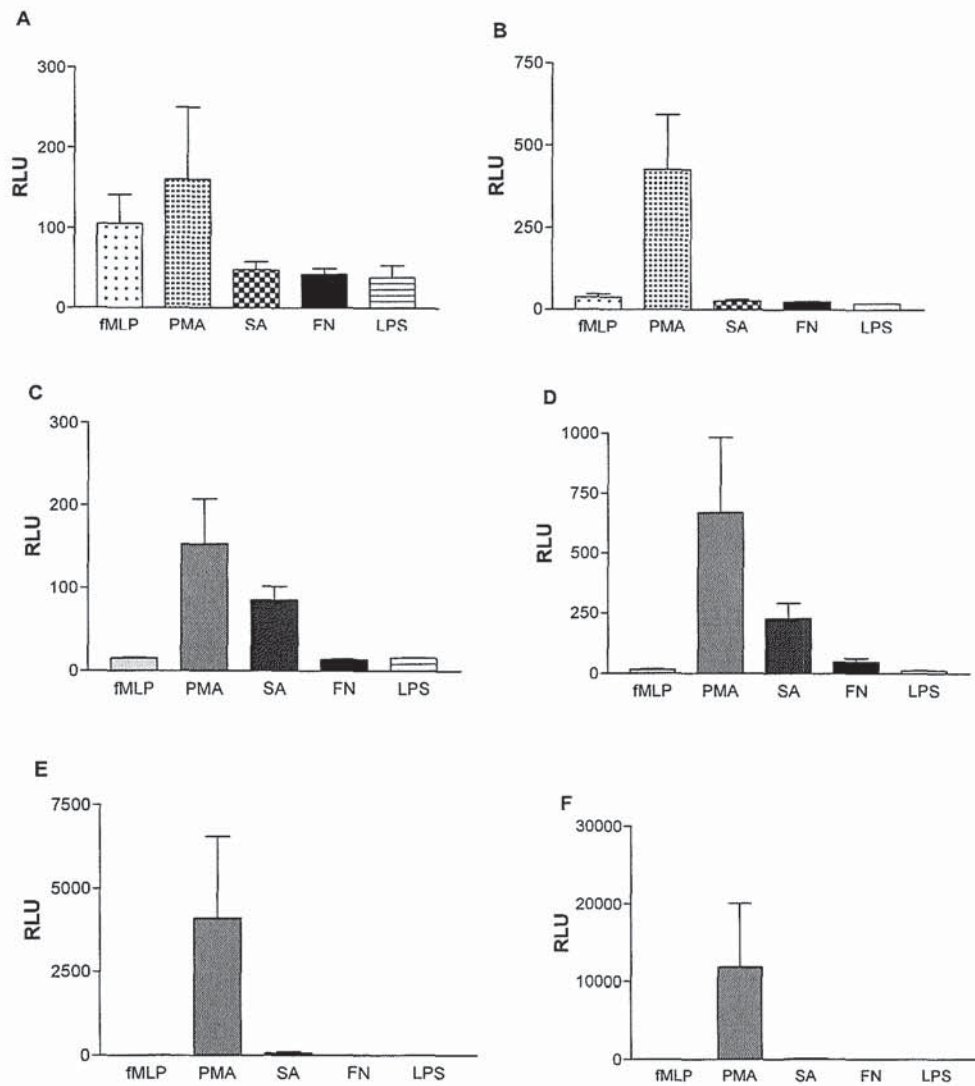
**Figure 3.8: Measurement of intracellular  $Ca^{2+}$  mobilisation.** dHL60 cells ( $5 \times 10^5$  /ml) in PSS were loaded with 2.5 $\mu$ M fura-2/AM for 30min at 37°C. Cells were centrifuged and washed to remove extracellular dye. Cells were re-suspended to the same cell density in the absence or presence of 1.25mM  $Ca^{2+}$ . Fura-2 fluorescence was measured in every minute by fluorometry at excitation wavelengths of 340 and 380nm, and emission wavelength of 510nm. Cells were stimulated at 2min after starting measurements. 10 $\mu$ M ionomycin used as a positive control and 12.5mM EGTA used as a negative control.

### **3.4.7 The comparison of respiratory burst of HL60 in luminol, isoluminol and lucigenin chemiluminescence agents.**

Experiments using  $5 \times 10^5$  cells per well were found to give a detectable CL signal with PMA and SA for lucigenin, luminol and isoluminol (Figure 3.9). Lucigenin detected ROS generated by all of the stimuli used (fMLP, PMA, SA, FN and LPS). Both fMLP and LPS induced the respiratory burst within 5min (Mean $\pm$ S.E.M;  $105.3 \pm 35.9$  and  $38.7 \pm 15.2$  respectively) but this was decreased with longer incubation ( $37.3 \pm 9.9$  and  $18.8 \pm 0.6$  respectively). PMA induced higher respiratory burst with longer exposure at 25min ( $425.6 \pm 167.4$ ) compared with short exposure at 5min ( $160.2 \pm 89.9$ ). SA-stimulated ROS detection peaked at 5min ( $47.83 \pm 10$ ) for lucigenin. FN-stimulated ROS production followed a similar pattern to SA, where lucigenin oxidation peaked at 5min ( $42 \pm 7.8$ ).

Luminol detected the highest levels of fMLP-stimulated ( $15.33 \pm 0.72$ ), SA-stimulated ( $227.6 \pm 65.12$ ) and FN-stimulated ( $49 \pm 14.78$ ) ROS at 5min and the chemiluminescence returned to basal level at 25min. The higher peak value for PMA-induced ROS was detected by luminol at 25min ( $668.6 \pm 316$ ). Isoluminol only detected fMLP-, PMA- and SA- stimulated ROS. fMLP-induced ROS detection by isoluminol peaked at 5min ( $12.9 \pm 3.8$ ). Peak PMA- and SA-induced ROS detected at 25 min ( $11838 \pm 8222$  and  $125.7 \pm 59.33$  respectively). Neither luminol nor isoluminol detected any LPS-stimulated ROS production at any time point. Results are combined from three independent experiments. However, between experiments a higher error associated with mean data values may be attributed to differential percentage of cell differentiation and also the level of passage number in each experiment (cells were used between passages 16-25).

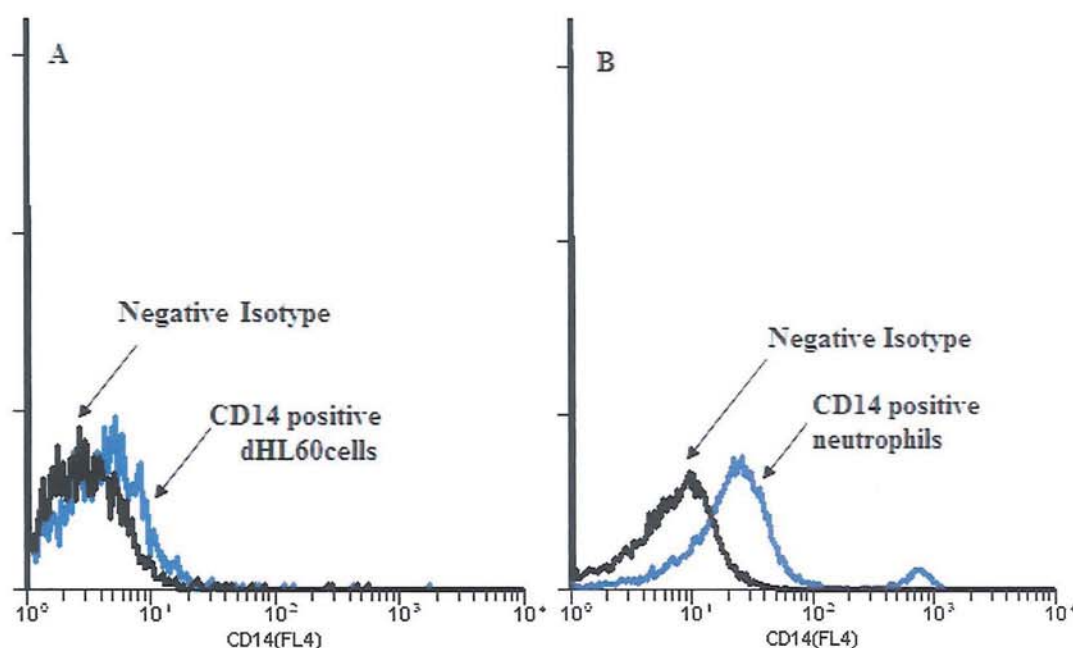




**Figure 3.9: Mean chemiluminescence induced by different agonists of the respiratory burst of dHL60 cells as detected by (A,B) lucigenin, (C,D) luminol and (E,F) isoluminol.** Differentiated HL60 cells were harvested and washed twice with PBS. Cells ( $5 \times 10^5$ ) were incubated with luminol (3 mmol/l) or isoluminol (3 mmol/l) with 6 U horseradish peroxidase or 100  $\mu$ M lucigenin in final volume of 190  $\mu$ l at 37°C for 20min prior to stimulation of respiratory burst. Cells were then stimulated with 1  $\mu$ M fMLP, 10nM PMA, opsonized *S. aureus* (300 bacteria/neutrophil), *F. nucleatum* suspension (100 bacteria/neutrophil) or *E. coli* LPS. RLU values at 5min (A, C, E) and 25min (B,D,F) after stimulation was tabulated. Results represent three independent experiments with quadruplets and are expressed as mean  $\pm$  S.E.M.

### 3.4.8 Flow cytometry for CD14 analysis

As LPS activation of inflammatory cells involves interaction between an LPS-binding protein and CD14, the expression of the CD14 antigen was investigated on dHL60 and primary neutrophils. For the assessment of dHL60 and neutrophilic CD14 antigen expression, isolated cells were stained with RPE-Cy5 labelled anti-CD14 monoclonal antibody in ice, in the dark. The expression of CD14 on resting neutrophils was analysed on peripheral blood neutrophils from 4 healthy controls (Figure 3.10). Neutrophils expressed 53.5% of CD14 compared with negative isotype 1%. However, CD14 expression was almost undetectable in dHL60 cells.



**Figure 3.10: Distribution of CD14 antigen on dHL60 and neutrophils.** dHL60 cells were isolated as described previously. Whole blood or dHL-60 cells were labelled with RPE-Cy5 labelled anti-CD14 antibody. Suspended dHL60 cells (A) or whole blood (pre-treated with Optilyse to fix leukocytes and lyse erythrocytes) (B) were aspirated and analysed by flow cytometry. CD14 positive cells were gated and data was collected for 30,000 events against forward scatter. Median X (MdX) value of CD14 positive peak was compared with negative isotype.

### 3.5 Discussion

The HL-60 cell line exhibits aggressive, malignant, proliferative potential *in vivo* but differentiates terminally when challenged *in vitro* (Chang et al., 2006). HL60 cells can differentiate towards monocytes, macrophages or into neutrophils after exposure to different chemical agents such as PMA, vitamin D3 (Breitman et al., 1980, Sham et al., 1994) and DMSO (Chang et al., 2006, Takeda et al., 2003) respectively. Consistent with other observations, this work has confirmed that HL-60 cells reproducibly differentiate into neutrophils when challenged with 1% DMSO for 5 days.

Compared with untreated HL60 cells, the rate of DMSO-treated cell replication was reduced and reached to a plateau on day 5, which implies terminal differentiation. Even though increasing the dose of DMSO may have increased the proportion of cells that become differentiated, DMSO is an organic solvent that exerts toxic effects on cells. Therefore, the toxicity of DMSO towards HL60 cells was determined. Of the cells allowed to grow in the culture medium containing varying concentrations of DMSO for 5 days, viability was decreased in a concentration dependent manner. Concentration of DMSO equal or exceeding 1.5% inhibited cell growth and induced cell death. DMSO may induce cell differentiation directly by affecting synthesis of DNA (Friend et al., 1971) or inducing transcription of genes responsible for cellular differentiation. It may also act indirectly by altering three-dimensional structures of proteins or by altering the composition of plasma membranes. Confirming the observations described in Collins *et al.*, (1978), the results in this chapter show that differentiated HL60 cells (dHL60) have a smaller cell size and decreased nuclear/cytoplasm ratio.



The maturation of dHL60 cells are evidenced by their ability to oxidise lucigenin through the respiratory burst following activation by PMA. The respiratory burst is a functional characteristic of peripheral blood polymorphonuclear leukocytes (PMN) raised against invading pathogens or danger signals. PMA is a chemical agent that is used to activate protein kinase C and the NADPH oxidase complex to initiate the respiratory burst of PMN (Teufelhofer et al., 2003, Choi et al., 2006). This study observed that dHL60 cells but not undifferentiated HL60 cells generate  $O_2^{\cdot -}$  in response to PMA and therefore exhibit functional characteristics related to PMN.

Respiratory burst produces  $O_2^{\cdot -}$  anions through the activity of NADPH oxidase. These act as precursor radicals for later developed ROS species (Figure 1.3). ROS are released into the surrounding medium or into a membrane enclosed subcellular organelle. The generation of these species can be measured by CL assays. In the literature, different chemiluminophores such as luminol, isoluminol and lucigenin have been used (Pavelkova and Kubala, 2004, Dyke et al., 2003, Ashkenazi et al., 2009) to measure ROS. These luminophores show specificity towards different ROS species; luminol reacts with both intracellular and extracellular HOCl,  $O_2^{\cdot -}$ ,  $OH^{\cdot}$ ; isoluminol reacts with extracellular HOCl,  $O_2^{\cdot -}$ ,  $OH^{\cdot}$  and lucigenin mainly reacts with extracellular  $O_2^{\cdot -}$  (Afanas'ev et al., 2001). Therefore granulocyte activators such as PMA, fMLP, SA, FN and LPS with different mechanisms of signal transduction have used to generate varying level of ROS detected by aforementioned luminophores. Lucigenin-enhanced CL was observed for all the activators used in this work at 5min with respect to luminol and isoluminol. These results suggested that in dHL60 cell model, the lucigenin CL method is suitable for respiratory burst analysis.

Cellular functional transition into a differentiated state was gradually increased with increasing concentration of DMSO up to 1% in the culture medium for 5 days. In contrast to other published studies, HL60 cells generated lower respiratory burst in DMSO concentrations at 1.25%. dHL60 cells also responded to a bacterial origin chemoattractant, fMLP by inducing the respiratory burst. However, the respiratory burst was prevented by removal of extracellular  $\text{Ca}^{2+}$ , which indicated the essential requirement for  $\text{Ca}^{2+}$ -mediated signaling following fMLP activation (Okajima et al., 1998).

Undifferentiated HL60 cells have high a proliferative potential, whereas during the process of differentiation, cells lose their capacity to divide while acquiring mature characteristics such as expression of CD11b (Chang et al., 2006). Despite its tremendous importance, the exact relationship between cell proliferation and differentiation still remains unanswered (Huang et al., 2009). PMA exposure for 3-5days induced the expression of CD14 and other macrophage markers such as TLR2 and TLR4 in HL60 cells (Esendagli et al., 2009), where as DMSO exposure for 3-5days induced the expression of CD11b (Chang et al., 2006). The differences between the expression of different types of cell surface maturation markers and cellular behaviour observed between different differentiating agents suggest that they regulate the cell maturation of HL60 cells by mechanisms that are at least partly independent (Drayson et al., 2001). Using mRNA array profiling methods, previous studies have shown that differentiating agents could initiate differentiation-related genes (IL-8, ICAM-1, GCSF, CD11b, macrophage inhibitory protein- $\alpha$ ) expression (Liu et al., 2000). Previous studies also shown that HL60 cells treated with a differentiating agent multiply and mature to >90% neutrophils with all-trans retinoic acid (Brown et al., 2002), and with DMSO (Fontana et al., 1981) or ~80% monocytes (with vitamin D<sub>3</sub> (Brown et al., 2002)).

HL60 cells treated with DMSO at 1% v/v in this study, did not support cell replication and induced a high percentage of cells differentiated along the neutrophilic pathway. Supporting previous observations, these cells exhibited neutrophilic properties of degranulation (Collins et al., 1979, Collins et al., 1978), cytoplasmic  $\text{Ca}^{2+}$  elevation (Okajima et al., 1998), generation of superoxide (Fontana et al., 1981) and chemotaxis (Fontana et al., 1980). Thus, a protocol has been optimised to produce a dHL60 cell model for use as a tool to understand neutrophil hyperactivity and hyper-reactivity as observed in chronic inflammatory diseases such as periodontitis.



## **Chapter 4.0 Gingipains from *Porphyromonas gingivalis* modulate respiratory burst and chemotactic properties of IL-8 variants**

### **4.1 Preface**

This chapter examines and identifies the consequences of a bacterial enzyme, gingipain from *Porphyromonas gingivalis*, on IL-8 activity. The oral anaerobic bacterium, *P. gingivalis*, which is strongly associated with active periodontitis, secretes degradative enzymes (including gingipains) and sheds proinflammatory mediators such as lipopolysaccharide (LPS), within the periodontal pocket/tissues. Gingipain-modulated chemokine activity and respiratory burst inducing activity of IL-8 was investigated in both the dHL60 cell model and primary neutrophils. A possible mechanism by which *P.gingivalis* regulates host neutrophil accumulation at the periodontium and modulate-neutrophil activity is presented.

## 4.2 Introduction

An important early step of the inflammatory response to bacterial infection is the recruitment of neutrophils which migrate from the vasculature into the site of infection. Periodontal diseases are a group of inflammatory disorders considered to represent an immunopathological response to a complex microflora that inhabits dental plaque. Sustained inflammation in periodontal pockets creates a progressing lesion of epithelial attachment to the tooth and then the migration of epithelial components down the tooth root, creating a cleft or pocket favourable for an abundant anaerobic microbial flora.

The oral anaerobic bacterium *P.gingivalis*, has been recognised as the primary pathogen in dental plaque and is strongly associated with active periodontitis (Amano, 2003). Although, *P.gingivalis* is widely distributed in various niches of the oral cavity, a substantial rise of bacterial counts is observed in the diseased sub-gingival environment compared with the sub-gingival area in healthy individuals (Socransky and Haffajee, 2005); this represents its effective adaptation to disease conditions. The presence of a capsular polysaccharide around the bacterium provides resistance to the host defense system. The pathogenicity of *P.gingivalis* is initiated by bacterial adherence to the junctional epithelium in the oral cavity followed by proliferation (Amano, 2003). Even though the bacterium can be eliminated by effective treatments it has a high probability of re-colonising in recurrent infections (Winkelhoff et al., 2002).

This organism possesses a number of pathogenic properties including the capacity to colonise, the presence of capsular polysaccharide and the ability to secrete a range of degradative proteinases (Amano, 2003). These virulent proteinases can hydrolyse a number of physiologically significant host proteins, including collagen, fibronectin, immunoglobins, complement factors C3, C4, C5 and B, lysozyme, iron-binding proteins,

plasma proteinase inhibitors, fibrin and fibrinogen which are key factors of the plasma coagulation cascade system. These proteases have been extensively studied for a number of functions associated with *P.gingivalis* virulence (Imamura, 2003, Pike et al., 1994). Since the protein targets of these enzymes are diverse, *P. gingivalis* challenge can modify most of the host connective tissue proteins, making a suitable environment for the bacterium to survive and proliferate. Further, the association of these proteases with adhesin/hemagglutinin creates a suitable attachment surface for bacterial fimbriae (Pike et al., 1994).

The *P.gingivalis* genome has been studied extensively in the past to determine the DNA sequence; the sequence of *P.gingivalis* W83 (<http://www.pgingivalis.org>) has been analysed and annotated. These studies have revealed that *P.gingivalis* produces two cell associated or extracellular cysteine proteases that are named as gingipains.

Gingipains are gene products of *rgpA* or *rgpB* (Arg-gingipain A & B) or *kgp* (Lys-gingipain) (Potempa et al., 1998). Extracellular or cell-associated gingipains with specificity for arginine peptide bonds are referred to as Arg-gingipain and gingipains with specificity for lysine peptide bonds are known as Lys-gingipain (Curtis et al., 2001). Gingipains with both specificities have not been reported. These enzymes are thiol proteases that require the presence of reducing agents such as cysteine, dithiothreitol, 2-mercaptoethanol or glutathione to express enzymatic activity (Rangarajan, 1997).

Some recent studies indicate that these proteases are capable of activating the prekallikren system, leading to the formation of the vasodilator bradykinin (Imamura, 2003). The prevention of blood clotting by gingipain-induced cleavage of clotting factor X, may explain partly the reason for easily bleeding gums in periodontitis. Gingipains are also reported to degrade host structural proteins directly or indirectly through alteration or



activation of host-derived proteases (Kuramitsu, 1998). For example, gingipains directly degrade collagen type I and type IV; the main structural proteins of matrix and induce expression of host matrix metalloproteases (MMP) or activate proenzymes (Kuramitsu, 1998). Furthermore, the pathogenic bacteria within the subgingival environment shed proinflammatory mediators such as lipopolysaccharide (LPS). LPS in turn triggers the secretion of chemokines/cytokines such as interleukin-1 $\beta$  (IL-1  $\beta$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), IL-6 and IL-8 (72-amino-acid variant) from resident inflammatory cells, which contribute to the initial inflammatory response.

IL-8, a small polypeptide with molecular weight of 8-10kD (Nakagawa et al., 1991), was originally isolated from monocytes (Baggiolini et al., 1989). Later studies have shown that IL-8 is also produced from wide range of cell types including fibroblasts (Myokai et al., 2004), epithelial cells (Saatian et al., 2006), keratinocytes (Jarnbring et al., 2000), lymphocytes (Prunet et al., 2006), endothelial cells (Utgaard et al., 1998) and neutrophils (Peng et al., 2007). IL-8 is a major chemokine with potent stimulatory effects on neutrophils including chemotaxis, degranulation, and cytoplasmic Ca<sup>2+</sup> elevation. In response to a stimulus, IL-8 is produced as a 99 amino acid long precursor polypeptide (Baggiolini et al., 1989), which is subsequently processed into a biologically active peptide (Figure 4.1). IL-8 varies in length from 79 amino acids through 77, 72, 71, 70 and 69 amino acid variants (Nourshargh et al., 1992). Although IL-8 is subject to variable processing at the N-terminus, the 72 amino acid long (IL-8-72) and 77 amino acid long (IL-8-77) peptides have been identified as the predominant variants. The major form of IL-8-72 has been extensively studied for its potent ability to prime neutrophils to stimulate the respiratory burst to a second stimulus such as fMLP (Nakagawa et al., 1991, Hubers et al., 1991).

10	20	30	40	50
MTSKLAVALL	AAFLISAALC	EGAVLPRSAK	ELRCQCIKTY	SKPFHPKFIK
60	70	80	90	100
ELRVIESGPH	CANTEIIVKL	SDGRELCCLDP	KENWVQRVVE	KFLKRAENS

**Figure 4.1 Amino acid sequence of human precursor IL-8.** Human IL-8 is translated as 99 amino acid long polypeptide and several post translational processing events at the N-terminal result in a variety of lengths of the molecule. Among the IL-8 variants, IL-8-77 (highlighted in yellow) and IL-8-72 isoforms are reported to be predominant (sequence from SwissProt database).

IL-8-77 is occasionally referred to as endothelial IL-8 because of its synthesis by endothelial cells stimulated with IL-1, LPS or TNF (Nourshargh et al., 1992, Hubers et al., 1991). It is also secreted by human dermal fibroblasts stimulated with IL-1 or TNF (Hubers et al., 1991). IL-8-77 is recognised as a less potent variant for neutrophil activation. It is less chemotactic and attenuates neutrophil adhesion to endothelial cell walls (Gimbrone et al., 1989). Recent reports have suggested that this long amino acid form is susceptible to cleavage by proteolysis into a biologically active form by several enzymes including plasmin, thrombin (Hebert et al., 1990), proteinase-3, and cathepsin L and cathepsin G (Ohashi et al., 2003, Padrines et al., 1994, Hebert et al., 1990). Neutrophils and monocytes are unlikely to produce plasmin and thrombin (Ohashi et al., 2003), and also levels of Cathepsin G and L (lysosomal cysteine proteases) secretion are low, therefore neutrophils are predicted to have a low capacity to degrade IL-8-77.



**Figure 4.2 Three dimensional structure of Interleukin-8 in solution as determined by Nuclear Magnetic resonance (Protein data base Entry -1IL8)** Three dimensional structure of human IL-8 contains two alpha helices and four beta turns. NMR and X-ray crystallography data indicate that quaternary structure of IL-8 exists as a non-covalently bound homodimer (Leong et al., 1997).

Chemokines such as IL-8 in general bind to specific receptors, which involves interaction between the ligand N-loop (Figure 4.2) on the chemokine and the receptor N-terminal domain residues. Therefore, the specificity between ligand and receptor is achieved by largest sequence difference in the ligand N-loop and receptor N-domain (Fernando et al., 2007). The interaction between IL-8 and its receptors is complex as more than one functional domain is involved and has been identified as essential. The N-terminal region of IL-8 has a characteristic N-terminal ELR sequence that bind to IL-8 receptors CXCR1 and CXCR2. Sequence analysis indicated that this N-terminal sequence is conserved, whereas N-loop residues are not, suggesting that the differences in binding may be due to binding of N-loop residues to the receptor N-domain (Fernando et al., 2007). However, as proven with synthetic analogues, the ELR sequence is necessary but not sufficient for high affinity binding and activities (Clark-Lewis et al., 1991) of IL-8. Therefore differential cleavage of the IL-8 N-terminal region could result in a change of receptor binding affinity and could change neutrophil responses in pathogenic environment.



It is suggested that IL-8-77 is susceptible to cleavage by gingipains, the main secreted cysteine proteases of *P.gingivalis* (Mikolajczyk-Pawlinska et al., 1998). In the event that gingipains may enhance activity of IL-8-77, this would present an additional mechanism of gingivalis pathogenicity, where the bacterium promotes neutrophil recruitment and further local tissue degradation. To date the effect of gingipains from *P.gingivalis* on IL-8 and subsequent chemotactic activity and respiratory burst activities has not been determined. Therefore the aim of this chapter is to investigate whether gingipains secreted from *P.gingivalis* can modify IL-8-77 chemotactic activity using neutrophil-like differentiated HL-60 cells and primary neutrophils.

### 4.3 Materials and methods

For cell culture, neutrophil isolation, respiratory burst assessment and BCA assay, please refer to general methods.

#### 4.3.1 Cultivation of *Porphyromonas gingivalis* Strain W83

*P.gingivalis* strain W83 was kindly provided by Dr. A. Roberts (School of Dentistry, University of Birmingham, UK). Culture purity was regularly checked by re-plating onto sheep blood agar plates. Cultures of *P.gingivalis* W83 were grown in 200ml of broth containing 6.0g of Trypticase Soy broth (Difco, Detroit, Mich), 2.0 g of yeast extract, supplemented with 1mg of hemin, 200mg of L-cysteine, 20mg of di-thiothreitol and 0.5mg of menadione (all from Sigma) under anaerobic conditions at 37°C in an anaerobic atmosphere of 5% H<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> for 48h.

#### 4.3.2 Isolation of Arg (R)-gingipain (gp) and Lys (K)-gingipain (gp)

Gingipains were isolated by the method described in (Yun *et al.*, 1999) with modifications. Briefly, *P.gingivalis* W83 cell-free culture supernatant was fractionated by centrifugation (4000g, 30min at 4°C). All steps were carried out at 4°C unless stated otherwise. Secreted proteins were extracted by acetone precipitation (chilled acetone was added 1:1 v/v ratio and incubated at 4°C for 30min) and the resultant protein pellet was redissolved in Tris buffer at pH 7.5 (0.05M Tris and 1mM CaCl<sub>2</sub>) by gentle mixing. Insoluble material was separated from supernatant by centrifugation (5000 ×g, 20min, and 4°C) and the supernatant was passed over a Mono Q fast-protein liquid chromatography

column (GE Healthcare) equilibrated with 0.05M Tris buffer pH 7.7 at a flow rate of 60ml/h<sup>-1</sup>. After being loaded and washed, the proteins were eluted from the column with 1M NaCl in 0.5M Tris buffer, pH 7.5. The eluant was subsequently loaded on to an arginine-Sepharose column (Amersham Biosci) previously equilibrated with Tris buffer at a flow rate of 60ml/h<sup>-1</sup>. The column was washed with 0.5 NaCl in the Tris buffer. Kgp was eluted with 0.75M L-lysine (pH 7.4). After re-equilibration, Rgp was eluted with 1M L-arginine (pH 7.4). Eluants were dialysed against Tris buffer overnight.

#### **4.3.3 Enzyme activity assays**

The amidolytic activities of the purified Rgp and Kgp were measured with the substrate N $\alpha$ -Benzoyl-L-arginine p-nitroanilide hydrochloride (L-BAPNA). 100 $\mu$ l of Rgp and Kgp fractions were incubated with L-BAPNA (final concentration of 1mM) in 100 $\mu$ l of 0.2M Tris-HCl, 0.1M NaCl, 5mM CaCl<sub>2</sub>, 10mM L-Cysteine in pH 7.6 at 37<sup>0</sup>C. After one-hour incubation, the reaction was stopped by addition of 10 $\mu$ l of glacial acetic acid (Potempaj *et al.*, 1998). The optical density was measured at 405nm for each fraction and the value of a control without proteinases was subtracted to give enzyme-dependent BAPNA cleavage in each fraction.

#### **4.3.4 Proteolytic degradation of IL-8 by purified gingipains**

Gingipains were activated in 0.2M tris HCl, 0.1M NaCl, 5mM CaCl<sub>2</sub>, 10mM Cysteine in pH 7.6, for 5min at 37<sup>0</sup>C. Pooled fractions for each gingipain were activated as described by Mikolajczyk-Pawlinska *et al.*, 1998. Rgp and Kgp were adjusted to equimolar concentrations in Tris buffer, pH 7.6. Activated gingipains (3 mM) were mixed with 1.65



$\mu$ M IL-8-77 or IL-8-72 in Tris buffer and incubated at 37°C for 30 min. Enzyme activity was terminated post-incubation by addition of 1  $\mu$ l of a protease inhibitor cocktail containing leupeptin hemisulphate.

#### **4.3.5 Western Blotting**

Gingipain treated IL-8 was prepared by heating the samples at 95°C for 10min in Laemmli buffer. The samples were resolved in 15% poly-acrylamide gels (SDS-PAGE); (see General Methods 2.2.8) in 1X running buffer (14g Tricine, 3g Tris HCl, 1g SDS in 1L, pH 8.3). Proteins were transferred to a PVDF membrane in transfer buffer (800ml water, 200ml methanol, 1g SDS, 29g tricine and 6g Tris HCl in 1l), and blocked for 2 hours at room temperature with 3% BSA in TTBS. After transfer to PVDF, IL-8 was detected using anti-human IL-8 rabbit polyclonal primary antibody (1:1000) in TTBS overnight at room temperature and the secondary polyclonal antibody horseradish peroxidase-conjugated goat anti-rabbit antibody (1:10000) in TTBS. Peroxidase-labelled proteins were visualised with the ECL system (Amersham Biosciences).

#### **4.3.6 Neutralization of IL-8-77 peptide chemotactic activity**

In order to investigate whether the enhanced biological activity of Kgp- or Rgp-treated IL-8 could be ascribed to the resultant formation of IL-8-72 rather than to the released peptides, neutralizing anti-human IL-8 antibody (5  $\mu$ g/ml), which recognises the whole molecule, was added to Kgp- or Rgp-treated IL-8-77 (8.25 nM) for 30 min at room temperature prior to addition of neutralized IL-8 to the lower chambers for chemotaxis experiments.

#### 4.3.7 Chemotaxis assay

dHL60 cell chemotaxis was assayed using 2µm pore size PVP-free polycarbonate filters in 96 multiwell chamber (Neuroprobe Inc.). dHL-60 cells ( $1 \times 10^5$ ) were washed and resuspended in PSS. Pre- and post-gingipain-treated IL-8 (8.25nM) samples and untreated gingipains were added to the lower wells of the chamber, the filter was fixed in place and upper wells were loaded with  $1 \times 10^5$  dHL-60 cells in 100µl of PSS. The chamber was placed in a 5% CO<sub>2</sub>/ 95% air humidified atmosphere at 37°C for 90 minutes. After chemotaxis, cell-containing PSS from the upper chamber was removed and top of the filter was washed with PSS. The microplate/filter assembly was centrifuged at 400×g for 10min. The filter was carefully removed and viable cell counts in the lower chamber were taken by flow cytometry (Coulter Epics XL) or by MTT assay.

#### 4.3.8 MTT assay

The MTT assay was used to count viable cells in the lower chamber of the chemotaxis chamber. Sample buffer (200µl) was carefully removed from the top layer without disturbing the cell pellet in the bottom. MTT (25µl) solution (5mg/ml in 0.01M PBS) was added to all wells including blanks. The plate was incubated for 4 hours in 37°C, 5% CO<sub>2</sub>. then the lysis buffer (100µl of 20% w/v SDS, in DMF(50%), dH<sub>2</sub>O(50%), pH 4.7 adjusted with 2.5% of 80% glacial acetic acid) was added to each well and the plates incubated for 16 hours at 37°C in a humidified 5% CO<sub>2</sub>/ 95% air incubator. The absorbance of the each well was read at 570nm in a microplate reader.

#### **4.3.9 Flow cytometric analysis of cell number**

100µl of dHL60 or neutrophils were diluted with 400µl of isoton. Suspended cells were aspirated and analysed by flow cytometry. Viable cells were gated based on forward and side scatter. After establishing flow, cell counts were collected for 10µl volume against forward scatter and for each sample, three counts were made.

#### **4.3.10 MS/MS analysis of Kgp- and Rgp-treated IL-8-77 and IL-8-72**

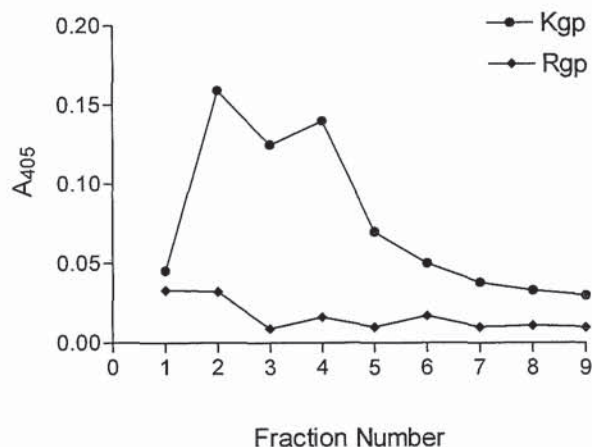
Kgp- or Rgp-treated IL-8-77 and IL-8-72 (0.14 pg/µl) were diluted in 50% methanol in water with acetic acid (1%) to enhance ionization and subjected to mass analysis after injection at 1 µl/min using a Thermo LXQ tandem mass spectrometer (MS/MS) in electrospray mode. The machine was externally mass-calibrated using peptides, caffeine, and Ultramark 1621 (ABCR GmbH & Co.). The acceleration voltage was set at 2 kV, and data were collected as the average total scan of 100 scans with the scan range set from 100 to 2,000  $m/z$  to search for fragmented IL-8-77 and IL-8-72. Charged molecular ions were subjected to collision-induced dissociation (CID) with helium gas, and the resulting tandem mass spectrometry (MS/MS) data were recorded for comparison against sequences from SWISSPROT predicted cleavage sites in IL-8.



## 4.4 Results

### 4.4.1 Amidolytic properties of Kgp and Rgp

Each of the gingipain fractions eluted from the arginine-sepharose column was subjected to an activity assay with 1mM L-BAPNA to confirm the enzymatic activity of gingipain. Fractions 2 to 4 of Kgp and fractions 1 and 2 of Rgp were found to yield L-BAPNA cleaved products (p-nitroaniline) measured as increases in OD at 405nm (Figure 4.3). Fraction 2-4 of Kgp were pooled and labelled as Kgp 1 and the rest of the fractions were pooled and labelled as Kgp 2 for BCA assay. Fractions 1 and 2 (Rgp 1) and the rest of the fractions (Rgp 2) were also pooled for the BCA assay.



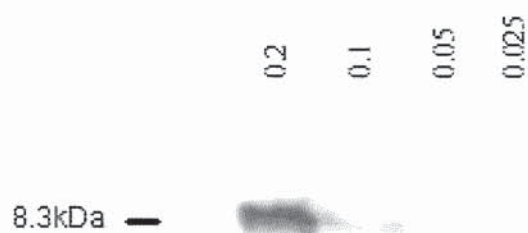
**Figure 4.3: Analysis of the amidolytic activities of purified Rgp and Kgp.** Secretory forms of Rgp and Kgp were isolated from *P.gingivalis* W83 and purified from culture supernatant as described in the materials and methods. Fractions of purified Rgp and Kgp treated with 1mM of chromogenic substrate L-BAPNA and generation of chromophore p-nitroaniline was measured at 405nm.

Protein content in each of the pooled gingipain fractions was measured by the BCA assay and results show at least 0.1µg/ml in each fraction (Table 4.1). Gingipain fractions with high enzyme content (Rgp1 with 0.321µg/ml and Kgp1 with 0.208µg/ml) were used for future experiments.

	Gingipain concentration (µg/ml)
<b>Rgp 1</b>	0.321
<b>Rgp 2</b>	0.156
<b>Kgp 1</b>	0.208
<b>Kgp 2</b>	0.191

**Table 4.1: Protein content in gingipain samples.** Protein content in each gingipain sample was determined using the BCA method against the standard curve of BSA.

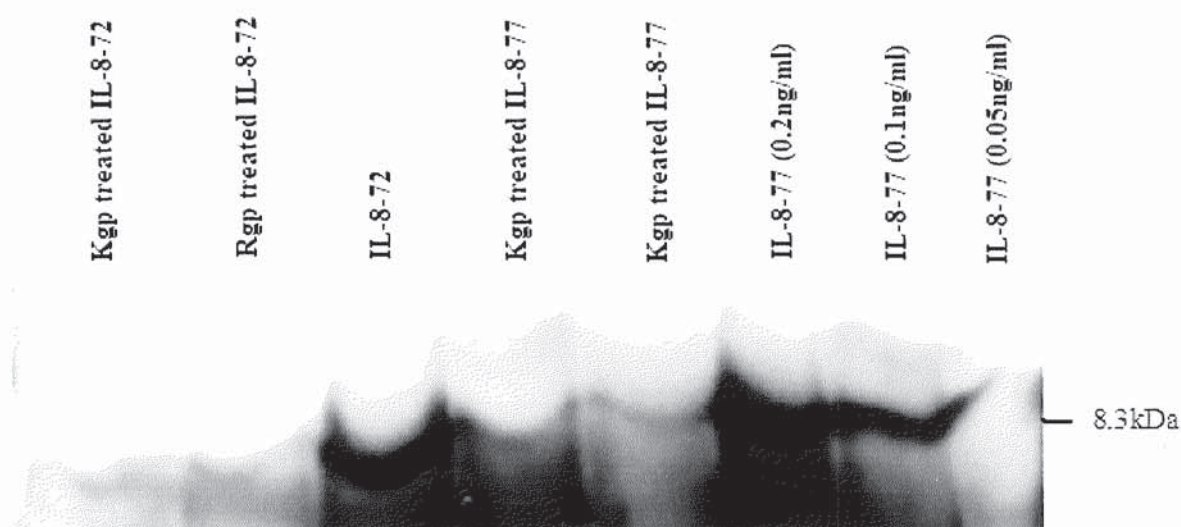
To analyse the concentration of IL-8 that should be used for western blotting, a dilution series of IL-8 was prepared and run in 15% SDS-PAGE gel. Figure 4.4 shows that 0.1ng/ml IL-8 gave an optimal signal to noise ratio. This concentration of IL-8 was subsequently used for western blotting experiments to examine gingipain induced IL-8 fragmentation.



**Figure 4.4: Effect of IL-8 concentration on western-blot visualization.** IL-8 dilutions of 0.025, 0.05, 0.1, 0.2 ng/ml were prepared by dilution in PBS. Protein samples (20 $\mu$ l) were then loaded into 15% SDS-PAGE gel and the gel was run 115V for 1 hour and 30min. Proteins were transferred to a PVDF membrane, blocked and labelled with primary and secondary antibodies as described in Materials and Methods. Proteins were visualized on a film using ECL detection kit.



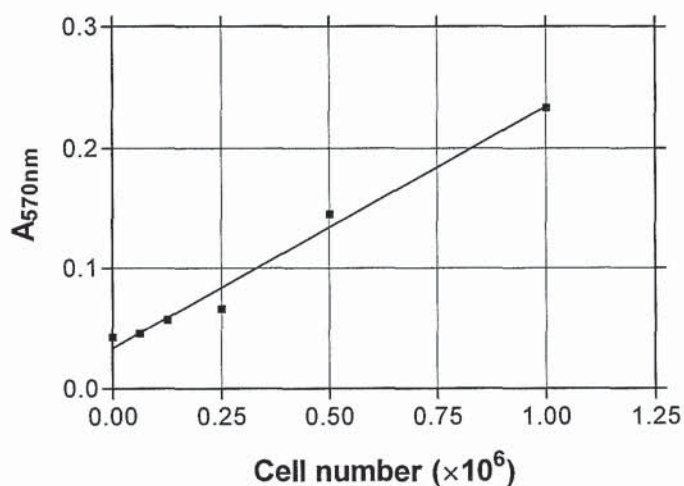
The effect of Rgp and Kgp on the electrophoretic mobility of IL-8 isoforms under SDS-PAGE was determined by western blotting (Figure 4.5). Gingipain treated IL-8 isoforms show a lower band intensity than untreated IL-8 at 8.3kDa, which indicates that the proteolytic activity of gingipains has degraded the mature IL-8 protein. However, the band intensity of gingipain treated IL-8-72 was lower than gingipain treated IL-8-77 isoform compared to non-gingipain treated controls. Kgp treated IL-8-77 also shows higher migration compared with non treated IL-8-77, which indicates truncation of 77 amino acid long peptide into a shorter form.



**Figure 4.5: Western blot analysis of Rgp and Kgp treated IL-8-77.** L-cysteine activated Rgp or Kgp was incubated with IL-8-77 and IL-8-72 (550:1 molar ratio) at 37°C for 30min and the reaction was terminated with the addition of leupeptin. Aliquots of IL-8 (0.2ng/ml) and gingipain treated IL-8 samples were resolved by 15% SDS-PAGE for western blot analysis with polyclonal antibodies against human IL-8 as described in materials and methods. The blot is representative of two independent experiments.

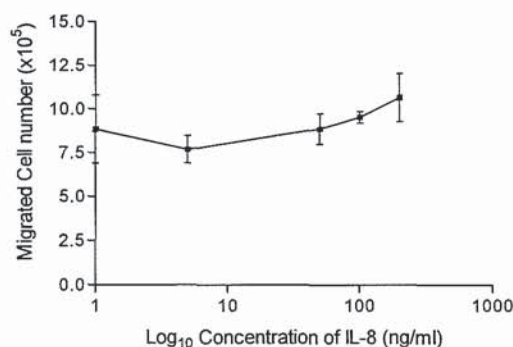
#### 4.4.2 Optimization of experimental conditions for chemotaxis assay.

To optimize conditions for assessment of chemotaxis, the concentration of IL-8, dHL60 cell count, pore size of the filters and duration of chemotaxis, dHL60 cell migration towards IL-8 was monitored. The number of migrated cells were determined by the MTT assay; yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. Therefore, this assay provides a measure of living cells in an experiment. Figure 4.6 shows the dose response relationship of cell number to absorbance at 570nm in colorimetric MTT assay.



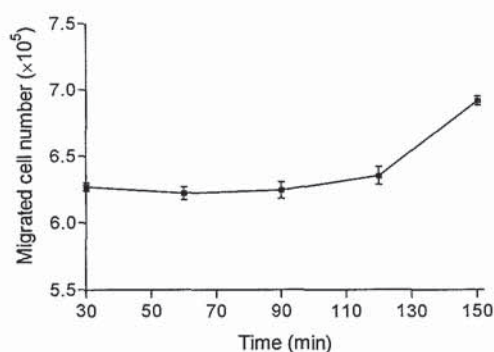
**Figure 4.6: Standard curve for MTT assay.** dHL60 cells (100 $\mu$ l of 0, 0.0625, 0.125, 0.25, 0.5 and 1 million/ml) were seeded in a 96 well plate. 25 $\mu$ l of MTT solution was added and incubated for 4 hours. Cells were further incubated for 16 hours after adding 100 $\mu$ l of lysis buffer. Absorbance was measured at 570nm. Mean absorbance expressed as  $\pm$ S.E.M, where n=3 independent experiments.

The effect of IL-8 concentration on dHL60 cell chemotaxis was investigated over 90min through 5 $\mu$ m pore size and >50ng/ml promoted IL-8 chemotaxis as shown in figure 4.7. However, high non-specific cell migration in the absence of IL-8 masked any effect of low concentrations of IL-8 (1ng/ml and 5ng/ml).



**Figure 4.7: Effect of IL-8 concentration on dHL60 chemotaxis.** Dilution series of IL-8-72 (1, 5, 50, 100 and 200 ng/ml) were prepared using PBS buffer. Chemotaxis of  $1 \times 10^6$  dHL60 cells towards IL-8-72 through 5 $\mu$ m filter was observed for 90min in multiwell chemotaxis chamber. Cell viable counts in the lower wells were taken by MTT assay.

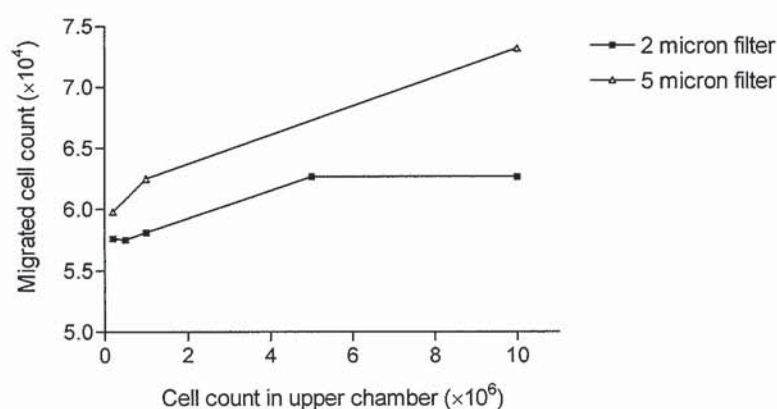
Figure 4.8 shows that the non-specific migration of cells was not changed by incubation in the chemotaxis chamber for up to two hours ( $6.36 \pm 0.07 \times 10^5$  cells), however, incubation for 150min increased the non-specific migration to ( $6.9 \pm 0.03 \times 10^5$  cells).



**Figure 4.8: The effect of incubation time on non-specific cell migration.** dHL60 cells ( $1 \times 10^6$  cells) were seeded in upper wells and non-specific cell migration through 5 $\mu$ m filter at 37°C was allowed. Cell migration was terminated at designated time points by removing cells from upper wells. Cell counts were taken by MTT assay.



The effect of pore size of the filter on non-specific cell migration was investigated using flow cytometry to count cells in order to improve accuracy of the measurement. As shown in figure 4.9, the filter with pore size 5 $\mu$ m allows high non-specific cell migration ( $6.25 \times 10^5 \pm 1.2$ ) at  $1 \times 10^6$  cells were added in the upper chamber compared with 2 $\mu$ m filter ( $5.81 \times 10^5 \pm 0.94$ ). Non-specific dHL60 cell migration through the 5 $\mu$ m filter was significantly increased ( $P < 0.001$ ) compared with 2 $\mu$ m filter in the presence of increasing cell numbers in the upper chamber.

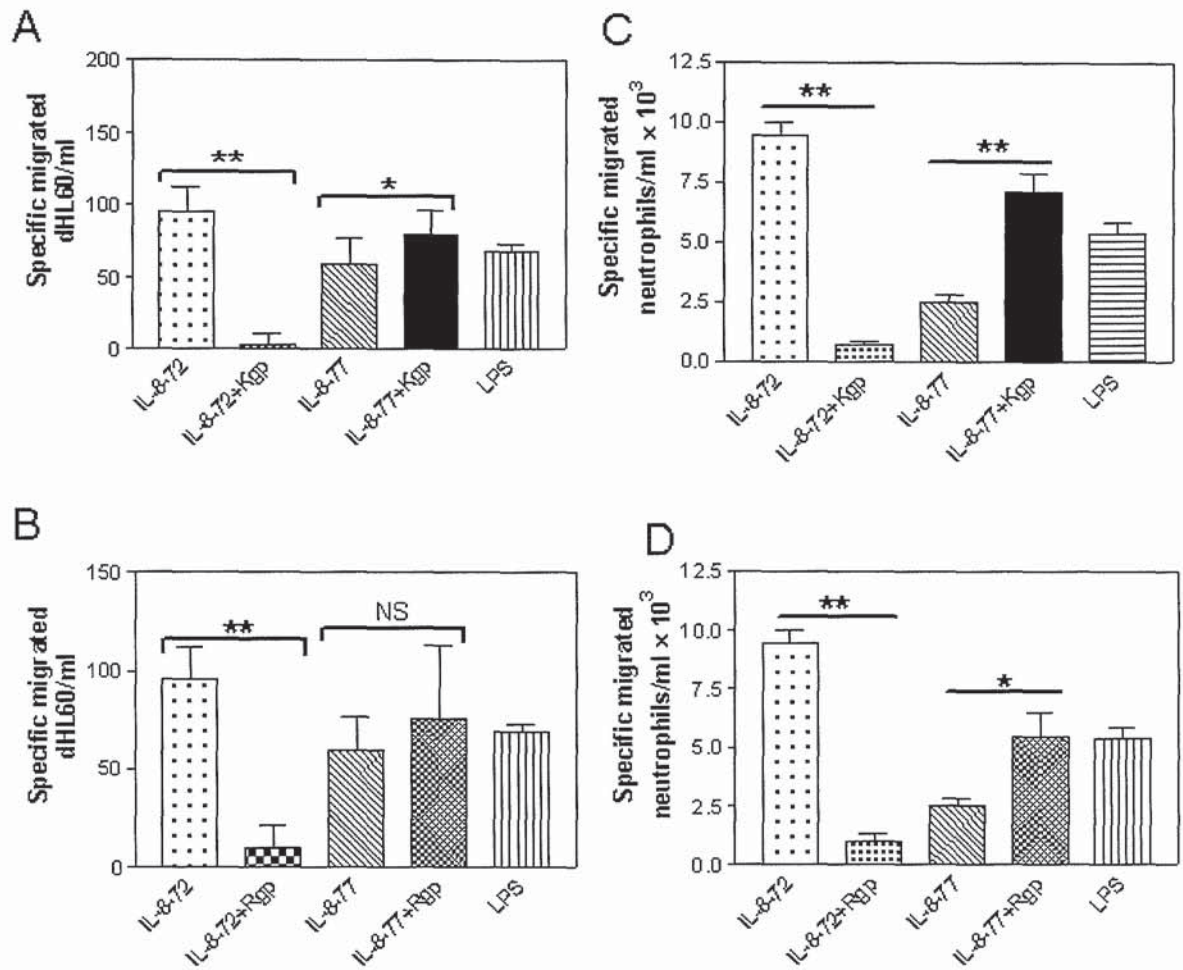


**Figure 4.9: Pore size affects non-specific migration of dHL60 cells.** The upper wells of the 96 well chemotaxis chamber were seeded with dHL60 cells over a concentration range from 0.1-10 million cells. Non-specific cell migration was observed over 90 mins using either 2 $\mu$ m or 5 $\mu$ m filter, and cell counts in lower wells were taken by MTT assay as described in Methods 4.3.8.

#### **4.4.3 Kgp and Rgp increase chemotactic properties of biologically inactive IL-8-77**

In order to determine the effects of Rgp and Kgp on the chemotactic properties of IL-8 isoforms, dHL60 cells/ primary neutrophils were allowed to undergo chemotaxis towards pre- or post-gingipain treated IL-8-72 and IL-8-77 isoforms. The effect of gingipain treatment on IL-8-dependent chemotaxis was corrected for chemotaxis toward either inactivated Rgp or inactivated Kgp, where migration was always lower toward gingipains than to LPS or IL-8 isoforms. The 72 amino acid long interleukin-8 isoform showed higher chemotactic activity than the epithelial cell derived IL-8-77 isoform. Both Rgp (Figure 4.10A) and Kgp (Figure 4.10B) treatments significantly decreased the chemotactic activity of IL-8-72 ( $P < 0.05$  and  $P < 0.01$  respectively). In contrast, Kgp treatment significantly increased IL-8-77 chemotactic properties ( $P < 0.05$ ).

In order to compare the behaviour of dHL60 cells with peripheral blood neutrophils, chemotaxis of primary neutrophils toward IL-8 isoforms was measured. Confirming the observations in dHL60 cells, both Kgp (Figure. 4.10A) and Rgp (Figure 4.10B) treatments significantly increased the chemotactic properties of IL-8-77 ( $P < 0.001$  and  $P < 0.01$ , respectively) towards primary neutrophils.

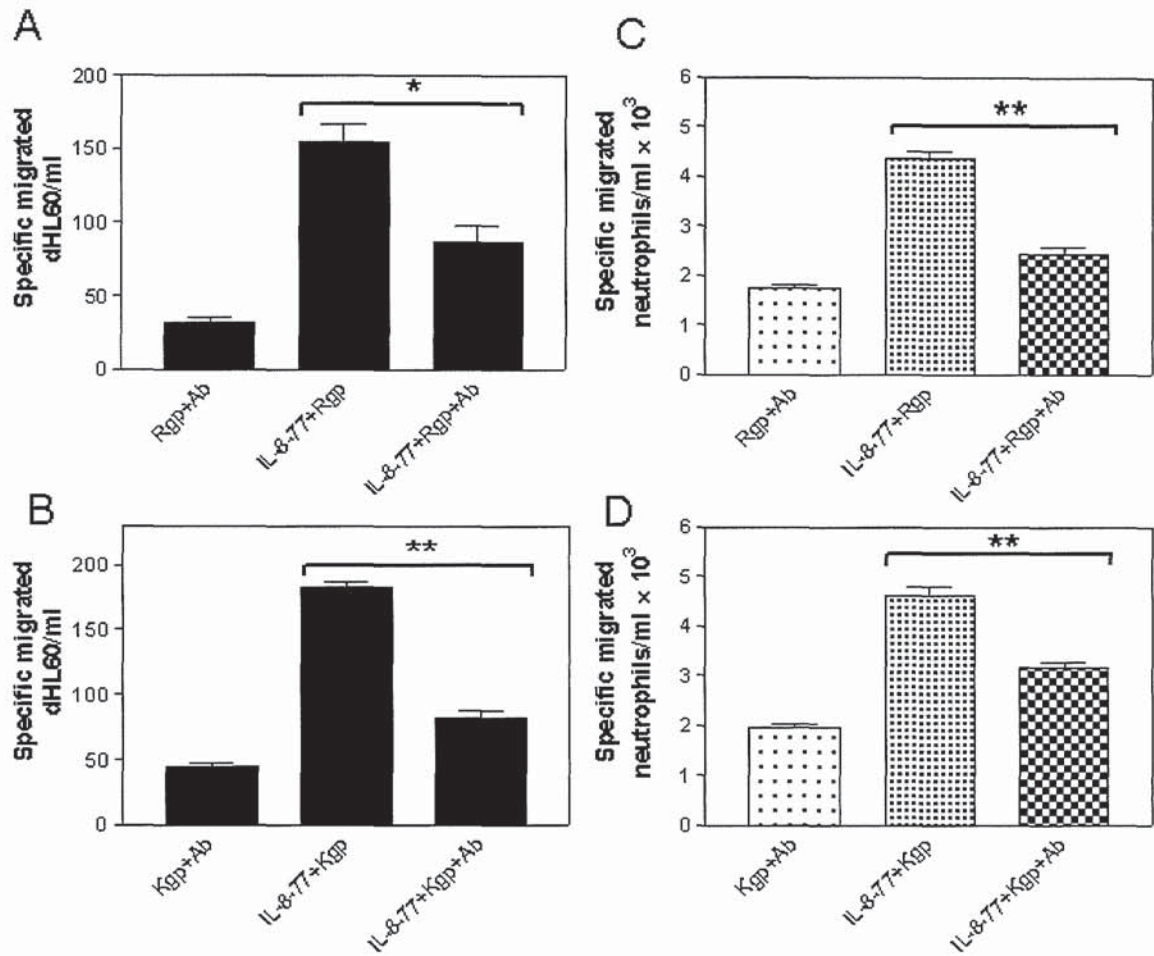


**Figure 4.10: Gingipain treatment increases chemotactic activity of IL-8-77 toward dHL60 cells and neutrophils.** IL-8 isoforms of 72- and 77-aa-long peptides (IL-8-72 and IL-8-77, respectively) were treated with 10 mM cysteine activated Kgp or Rgp for 30 min. Chemotaxis (corrected for background) of  $1 \times 10^5$  dHL60 cells through a  $2 \mu\text{m}$  filter toward Kgp (A)- and Rgp (B)-treated or untreated IL-8 isoforms was observed for 90 min in a multiwell chemotaxis chamber. Specific chemotaxis of  $1 \times 10^5$  primary neutrophils toward Kgp (C) - and Rgp (D)-treated IL-8 isoforms through a  $5 \mu\text{m}$  filter was observed for 90 min in a multiwell chemotaxis chamber. Cell counts in the lower wells were taken by flow cytometry and are expressed as mean cell number migrated standard error of the mean, where  $n=3$  independent experiments performed in triplicate. \*  $P < 0.01$ ; \*\*  $P < 0.001$ ; and NS, not significant by Tukey's multiple comparison test.



#### **4.4.4 N-terminally shortened peptide fragments of IL-8-77 account for the increased chemotactic activity.**

To investigate whether N-terminally shortened IL-8-77 peptides accounted for the observed chemotactic activity, gingipain-treated IL-8-77 was neutralized with anti-human IL-8 antibody prior to chemotaxis assay. Both antibody-neutralized Rgp-treated IL-8-77 (Figure 4.11A) and Kgp-treated IL-8-77 (Figure 4.11B) showed significantly decreased chemotactic activity toward dHL60 cells ( $P < 0.05$  and  $P < 0.01$ , respectively) compared with gingipain treated IL-8-77 in the absence of neutralizing antibody. Similarly, when the experiment was repeated using neutralized Kgp- or Rgp-treated IL-8-77 as a chemotaxin for primary neutrophils, again, the enhanced biological activity of gingipain-cleaved IL-8-77 was not evident (Figure 4.11C and D).

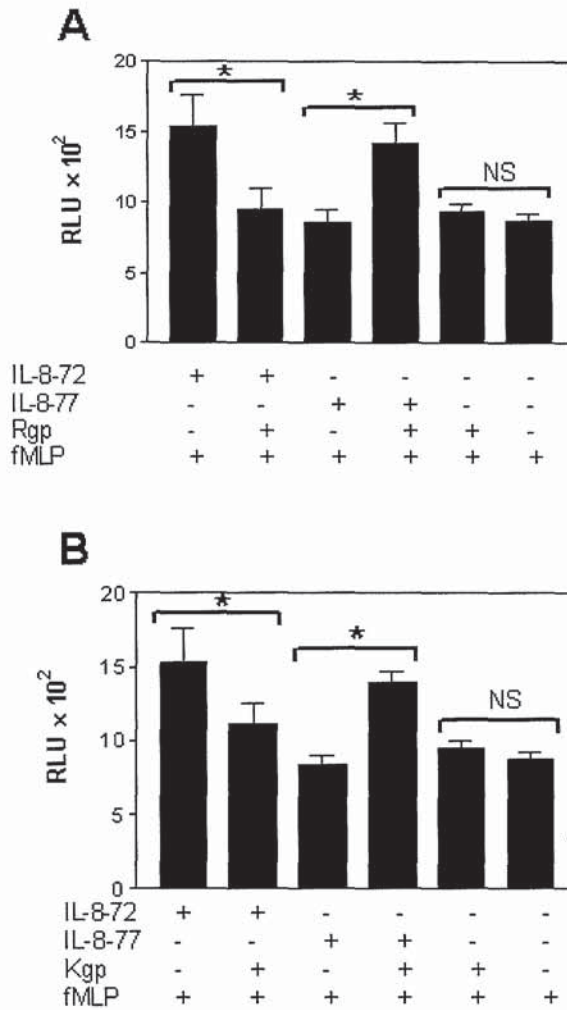


**Figure 4.11: Major IL-8 peptides released from gingipain treatment of IL-8-77 are responsible for enhanced chemotactic activity of dHL60 cells and primary neutrophils.** IL-8 isoforms of 72- and 77-amino acid-long peptides (IL-8-72 and IL-8-77, respectively) were treated with 10 mM cysteine-activated Kgp or Rgp for 30 min. After neutralization of gingipains, the resultant peptides were subsequently incubated with neutralizing anti-IL-8 antibody (Ab). Chemotaxis (corrected for background) of  $1 \times 10^5$  dHL60 cells through a 2- $\mu$ m filter toward Kgp-treated IL-8-77 and Kgp-treated IL-8-77 plus IL-8-neutralized isoforms (A) or Rgp-treated IL-8-77 and Rgp-treated IL-8-77 plus IL-8-neutralized isoforms (B) was observed for 90 min in a multiwell chemotaxis chamber. Specific chemotaxis of  $1 \times 10^5$  primary neutrophils toward Kgp-treated-IL-8-77 and Kgp-treated IL-8-77 plus IL-8-neutralized isoforms (C) and Rgp-treated-IL-8-77 and Rgp-treated IL-8-77 plus IL-8-neutralized isoforms (D) through a 5- $\mu$ m filter was observed for 90 min in a multiwell chemotaxis chamber. Cell counts in the lower wells were taken by flow cytometry and are expressed as mean cell number migrated standard error of the mean, where  $n = 3$  independent experiments performed in triplicate. \*,  $P < 0.05$ , \*\*,  $P < 0.001$  by Tukey's multiple-comparison test.

#### **4.4.5 Kgp and Rgp increase the priming effect of IL-8-77 for the respiratory burst in response to fMLP.**

The priming effect of IL-8 on the fMLP-induced respiratory burst was measured by lucigenin-dependent chemiluminescence using isolated peripheral blood neutrophils. IL-8-72 primed neutrophils for enhanced superoxide production after fMLP stimulation, whereas neither IL-8-77 nor isolated gingipains had any priming effect (Figure 4.12). However, both Rgp (Figure 4.12A)- and Kgp (Figure 4.12B)-treated IL-8-77 primed neutrophils for fMLP-induced superoxide production, demonstrating significantly increased superoxide generation compared with native IL-8-77 ( $P < 0.05$ ). In contrast, gingipain treatment decreased the priming activity of IL-8-72 ( $P < 0.05$ ) for fMLP-stimulated superoxide production.

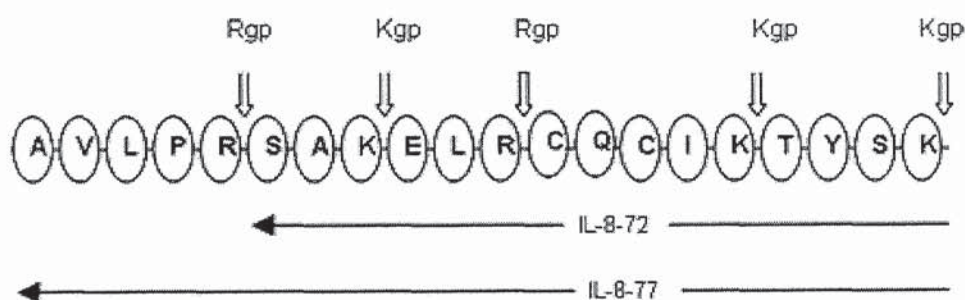




**Figure 4.12: Gingipain treatment enhances the priming effect of IL-8-77 on neutrophils.** Neutrophils ( $5 \times 10^5$ ) were primed with Rgp-treated or untreated 72- and 77-amino acid IL-8 isoforms (IL-8-72 and IL-8-77, respectively) (A) or Kgp-treated or untreated IL-8 isoforms (B) for 10 min prior to stimulation with 1  $\mu$ M fMLP. Mean peak (RLU  $\pm$  standard error of the mean; where  $n=3$  independent experiments performed in triplicates) chemiluminescence generated by neutrophils was recorded. Significant differences were calculated (\*,  $P < 0.05$ ) with Tukey's multiple-comparison test. NS, not significant.

#### 4.4.6 Kgp and Rgp specifically cleave the N terminus of IL-8-77

Rgp has two theoretical, amino peptidase cleavage sites for IL-8-77, and Kgp has three amino peptidase cleavage sites in IL-8-77 (Figure 4.13). At the N terminus of IL-8-72, Rgp has one theoretical cleavage site, while Kgp has three sites. To investigate the effects of gingipain activity on IL-8-77, released N-terminal peptides corresponding in mass to Rgp- and Kgp cleaved IL-8-77 isoforms were investigated by MS/MS with CID. Rgp treatments preferentially cleaved the N-terminus of IL-8-77, releasing peptides of  $m/z$  555.7 and 703.82 corresponding to residues 1 to 5 and 6 to 11 of the N-terminal region with the sequences reported in Table 4.2, to produce 72- and 66- amino acid long IL-8 peptides. Rgp treatment also released a peptide of  $m/z$  703.41 and sequence SAKELR from IL-8-72 to yield an IL-8-66 polypeptide. However, Kgp treatment of IL-8-77 released an 8- amino acid long polypeptide of  $m/z$  842.03 (AVLPRSAK) from the N-terminus, resulting in a 69- amino acid long IL-8 polypeptide. Kgp treatment of IL-8-72 released peptides of  $m/z$  305.18, 1135.4, and 498.5, resulting in 69-, 61-, and 57- amino acid long peptides, respectively.



**Figure 4.13 N-terminal amino acid sequence of IL-8 and possible Rgp and Kgp cleavage sites.** IL-8-77 has a peptide sequence extended by 5 amino acid relative to IL-8-72. Rgp has specific amino peptidase activity to R-X peptide bonds, and Kgp has specific amino peptidase activity to K-X peptide bonds.

IL-8 isoform and gingipain treatment	<i>m/z</i>	Amino acid position		Sequence
		Start	End	
IL-8-77				
Rgp	555.7020	1	5	AVLPR
	703.8212	6	11	SAKELR
Kgp	842.0352	1	8	AVLPRSAK
IL-8-72				
Rgp	703.4097	1	6	SAKELR
Kgp	305.1819	1	3	SAK
	1135.4	4	11	ELRCQCIK
	498.5	12	15	TYSK

**Table 4.2: Release of amino-terminal peptides by gingipain treatment of IL-8-77 and IL-8-72 as determined by MS/MS**



## 4.5 Discussion

This chapter has described an investigation into a possible mechanism by which *P.gingivalis* could manipulate IL-8 cytokine-mediated neutrophil chemotaxis using a dHL60 cell model and primary human neutrophils. This study has shown the successful isolation secreted forms of arginine and lysine specific gingipains from culture medium of *P. gingivalis* W83. Previous studies of inhibition and activation assays showed that gingipains have cysteine proteinase activity and have the unique property of stimulation of their amidolytic activity by amino acids and dipeptides, particularly glycyl-glycine (Curtis et al., 2001). This characteristic was successfully utilized to detect activity of gingipains against L-BAPNA as the substrate.

In developing a method to investigate the effects of gingipains on the chemotactic activity of IL-8, an MTT assay was initially used to optimise experimental conditions. However, the sensitivity of this assay was not high enough to differentiate subtle differences in cell migration in response IL-8 isoforms. The use of a flow cytometric method was therefore adopted to provide a clearer understanding of cellular response towards different chemoattractants. As detected by flow cytometry, only <0.1% cells showed specific migration towards IL-8 isoforms.

In culture, dHL60 cells are ovoid or round but occasionally express pseudopodia and are heterogeneous in size (9 to 25  $\mu\text{m}$  in diameter) (Fleck et al., 2005). These cells therefore easily migrate through 5  $\mu\text{m}$  filter and have shown increased non-specific migration. Use of smaller pore size (2 $\mu\text{m}$ ) filter greatly reduced the number of cells migrating through by

diffusion, but provided consistent results for specific cell migration in response to chemoattractants.

To investigate any proteolysis post-gingipain treatments, IL-8 isoforms were separated on SDS-PAGE gel. The tricine buffer system has advantages over tris-glycine buffers by allowing resolution of proteins with molecular weights as low as 2kDa, more appropriate for direct sequencing after transferring to PVDF as tricine does not interfere with sequencing and minimises the risk of protein modification by having a lower pH. Although loss of mature IL-8 isoforms and a shift in molecular weight is observed after gingipain treatments and by anti-IL-8 detection, a clear understanding of the molecular weight of smaller fragments could not be detected using this methodology. This may be due to lack of specificity of primary anti-IL-8 antibody towards smaller fragments or their low abundance to generate a detectable signal. Nevertheless, these studies confirmed an effect of gingipains on IL-8 mass and further investigation focused on any change in IL-8 function.

In contrast to biologically active IL-8-72, endothelial cells secrete a form of 77 amino acid long IL-8 (IL-8-77) peptide, which is known to be resistant to wide range of host proteinases. It has a low chemotactic activity (Gimbrone et al., 1989) and is less biologically active e.g. in priming for the respiratory burst than the IL-8-72 variant (Nourshargh et al., 1992). The results in this chapter show that gingipain increased the priming activity induced by IL-8-77 on fMLP-induced oxidative burst in primary neutrophils. In addition the results presented in this chapter demonstrate a significant increase in the chemotactic properties of IL-8-77 and a higher priming capability of IL-8-77 after incubation with L-cysteine-activated gingipains. Previous studies have also

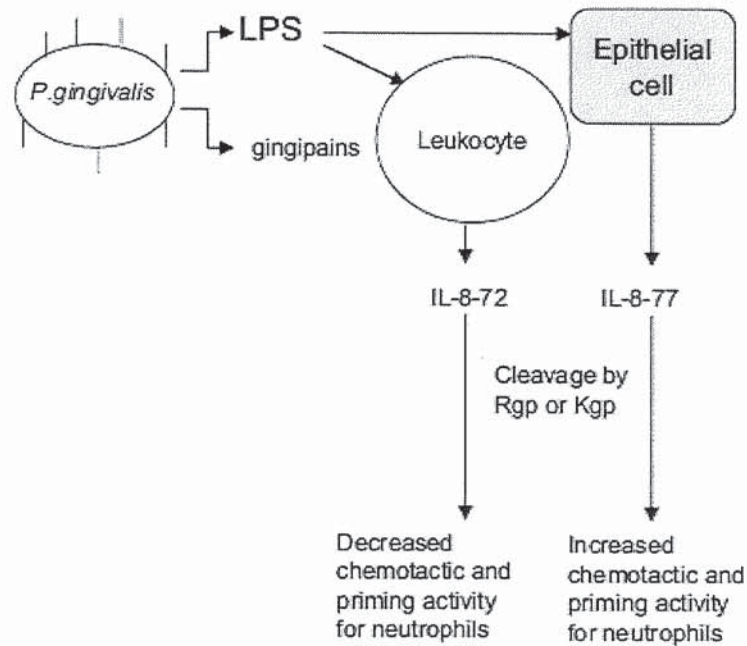
shown that these truncated products of IL-8-77 could significantly increase the ability of neutrophils to release elastase (Mikolajczyk-Pawlinska et al., 1998). Chemotactic properties and priming ability of these truncated forms were found to be 3-4-fold higher compared with non-treated IL-8-77. Given that reported concentrations of reduced glutathione/cysteine in gingival fluids are 1000-fold higher those of serum (Chapple et al., 2002), this may represent a physiologically relevant mechanism whereby gingipains contribute to neutrophil recruitment and activation at *P. gingivalis* infected sites. In contrast, the more biologically active form of IL-8-72 showed low chemotactic activity after treatment with both Rgp and Kgp. This hypothesis is supported by the findings that stimulation of inflammatory cells by *P.gingivalis* produces IL-8, which is subsequently degraded upon secretion (Zang et al., 1999).

The most important receptor binding regions of IL-8 lies in the N-terminal 20 residues of the IL-8 protein and these are the least ordered in solution (Clark-Lewis et al., 1995). The well ordered C-terminal of the peptide is not involved in receptor binding, but conserved amino acids and three dimensional structures must be crucial for its biological activity. Therefore the overall structure of IL-8 is important for its function as a chemokine and respiratory burst activation. The N-terminal amino acid sequence of IL-8-77 is AVLPRSAKELRCQCIKTYSK-. Rgp has theoretical specific amino-peptidase cleavage sites at Arg<sup>5</sup>-Ser<sup>6</sup> and Arg<sup>11</sup>-Cys<sup>12</sup> to yield peptides of 72 and 66 amino acid lengths. After Rgp treatment, cleavage products of IL-8-77 were detected by MS/MS of 5 and 6 amino acids, corresponding to the putative N-terminal cleavage sites. There was no evidence of low molecular weight peptides corresponding to cleavage at the putative C-terminal sites. It has been reported that Glu<sup>4</sup>-Leu<sup>5</sup>-Arg<sup>6</sup> of IL-8 are necessary for high affinity binding to IL-8 receptor; the data presented here show that an increase in activity of IL-8-77 is



observed after Rgp treatment which results in products of 72 and 66 amino acid length. Kgp, with its specificity for the Lys-X peptide bond, is predicted to cleave the IL-8 amino terminal sequence at Lys<sup>8</sup>-Glu<sup>9</sup>, and this product was observed in by MS/MS. The resultant 69 amino acid long form of IL-8 shows enhanced biological activity compared with IL-8-77 in these studies of chemotactic activity and respiratory burst priming. In contrast, the more biologically active form of IL-8-72 showed reduced chemotactic activity after treatment with both Rgp and Kgp. The presence of five amino acids at the N- terminus of IL-8-77 compared to IL-8-72 appears to modulate cleavage of the peptide by gingipains. IL-8-77 may have either altered three dimensional structures at the N-terminus or specific charge differences which contribute to change in altered accessibility and thus cleavage by gingipains.

Previous studies have shown that the capacity of gingipains to manipulate the host cytokine network is partly due to degradation of other cytokines such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (Calkins et al., 1998). Therefore, it is been suggested that the ability to inactivate cytokines by *P.gingivalis* in the early stages of pathogenesis is advantageous for the organism. Rgp has recently been shown to digest secretory leukocyte protease inhibitor released from neutrophils thus reducing the protective effect against bacterial pro-inflammatory molecules by which disease in periodontal tissues may be accelerated. In contrast, degradation of pro-inflammatory cytokines could diminish neutrophil chemotaxis towards infected periodontal sites by lowering inflammatory cytokine secretion. However, in periodontal patients, neutrophil recruitment to the gingival crevice is maintained despite the presence of gingipains.



**Figure 4.14: Schematic representation of gingipain-modulated IL-8 response.** *P. gingivalis* stimulates production of host proinflammatory mediators, including IL-8. (IL-8-77) secreted by host epithelial cells can be cleaved into more active, truncated forms. Collectively, these truncated forms may recruit more neutrophils to the site of infections and also prime their activation, which may contribute to the increased hyperactivity of neutrophils in periodontitis. Prolonged exposure to gingipains may trigger further degradation of IL-8-77 which may reduce chemotaxis and neutrophil priming, thus prolonging the inflammatory lesion.

It is probable that an alternative mechanism exists to promote neutrophil chemotaxis and activity at periodontitis sites which may involve the secretion of the longer form of IL-8-77 by non-immune cells. This *in vitro* study provides a possible mechanism for *P.gingivalis* manipulated neutrophil chemotaxis into periodontal pockets via activation of IL-8-77 as illustrated by figure 4.14. In conclusion, products from *P. gingivalis* may regulate host neutrophil accumulation at infected periodontal sites by stimulating the production of IL-8-77 by non-immune cells and promoting gingipain-dependent modification of IL-8-77 into a more biologically active chemokine which promotes neutrophil chemotaxis and priming.

## **Chapter 5.0 - The effect of periodontal plasma on neutrophil hyperactivity/hyper-reactivity**

### **5.1 Preface**

This chapter investigates any roles of specific inflammatory plasma factors in periodontal patient plasma with respect to reported systemic neutrophil hyperactivity and hyperreactivity in periodontal diseases. The effect of periodontal disease- and healthy-plasma isolated from the peripheral blood of individuals, were analysed in neutrophils isolated from healthy individuals. The differential responses of neutrophils to periodontitis and controls plasmas were compared by measuring the respiratory burst during priming by plasmas, and after stimulation in the presence of plasmas. The potential for circulating plasma factors to contribute to systemic neutrophil hyperactivity and hyper-reactivity in periodontitis is then discussed.



## 5.2 Introduction

Neutrophil priming has been implicated in the development of various inflammatory diseases (Partrick et al., 2000, Brown et al., 2004, Elbim et al., 1994). It is possible that neutrophil hyper-reactivity in periodontitis is due to increased levels of priming substances or increased susceptibility of the neutrophils to priming substances in patient plasma.

Agents such as fMLP and PMA can directly activate NADPH oxidase activity, however, early studies have shown that lower concentrations of these agonists can themselves act as priming agents in the absence of respiratory burst activation (Bender et al., 1983). A wide range of plasma factors such as pro-inflammatory cytokines (Gainet et al., 1999), acute phase proteins (Ebersole et al., 1997), soluble immune complexes (Lu et al., 1994, Wilton et al., 1992), activated complement (Abid et al., 2007), soluble adhesion molecules (Hayashi et al., 1996) or bacterial components (Ishikawa et al., 1997) are at elevated concentrations in periodontally-diseased individuals. A number of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1, GM-CSF and bacterial components such as LPS are examples of agonists that have been shown to induce neutrophil priming (Matthews et al., 2007).

The early understanding that periodontitis is an inflammatory disease which is localised to the marginal periodontium and that rarely has systemic implications in otherwise healthy individuals was overturned in the last 15 years (Kweider et al., 1993, Beck and Offenbacher, 2001, Loos et al., 2000, Ebersole et al., 1997). These studies have indicated that periodontal diseases have a strong correlation with systemic diseases such as

cardiovascular diseases, diabetes mellitus, respiratory infections, pre-term birth defects, stroke, arthritis and severe osteopenia. While a strong associative relationship exists between cardiovascular disease (CVD) and periodontitis (Loos et al., 2000), periodontitis has an independent influence on CVD; even after controlling for many potential confounding factors such as smoking, serum lipid concentrations and diabetic heart-attack, CVD patients still are at risk for predisposition to periodontitis than control subjects (Beck and Offenbacher, 2001). CVD is a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation, where locally produced periodontal inflammatory mediators may contribute to systemic inflammation.

The association between two physically discrete diseases may be established by secretory mediators. The peripheral circulation may play a vital role in this aspect by transporting inflammatory factors and other effector molecules from one part of the body to the other. It has been proposed that increased concentrations of mediators released in the inflamed periodontium could greatly enhance or induce disease conditions in another part of the body. This hypothesis is reviewed by Golub *et al.*, 2006 in their hypothetical “Two hit” model. This model is supported by evidence from both human case studies and animal models (Golub et al., 2006). As an example, the relationship between rheumatoid arthritis and the incidence of periodontitis was explained by systemic osteoporosis as a complication of arthritis, which may potentially accelerate the periodontal bone loss (Payne et al., 1999, Greenwald and Kirkwood, 1999); proinflammatory mediators produced in rheumatoid arthritis are found at elevated concentration in the circulation and have the potential to stimulate endothelial cells throughout the body including in the periodontium to produce matrix metalloproteinases (MMP), mediating connective tissue destruction and activating osteoclasts to destroy bone tissue. These authors argue that the

concentration of induced proinflammatory mediators in the circulation is insufficient to generate destructive effects by themselves but they act as initiators of inflammatory cascade at local sites.

Other studies have indicated that patients with severe periodontitis have increased serum levels of acute phase proteins such as C-reactive protein (CRP), hyper-fibrinogenemia, moderately increased haptoglobins and increased levels of cytokines and chemokines (Noack et al., 2001, Ebersole et al., 1997, Bircan et al., 2008). Furthermore, in periodontal patients, elevated serum CRP is associated with high level of infection with periodontal pathogens including *P.gingivalis* (Noack et al., 2001). CRP is a member of the class of acute phase serum proteins, including  $\alpha$ -2 macroglobulin, serum amyloid A, haptoglobins, fibrinogen, ferritin, serum amyloid P,  $\alpha$  1-acid glycoprotein and complement components C3, C4 which are produced and released during an inflammation. Bacterial infections that provoke pro-inflammatory cytokine production such as IL-6 and TNF- $\alpha$  frequently provide a strong stimulus for the systemic acute-phase response. Although most of acute phase reactants are produced by hepatocytes, some are synthesised by other cell types including monocytes, endothelial cells, fibroblasts and adipocytes. Increasing evidence for elevated level of CRP, haptoglobin and fibrinogen in periodontitis has reported by several groups (Noack et al., 2001, Ebersole et al., 2002). The extent of CRP levels in periodontitis patients are found to be dependent on the severity of the disease after adjusting factors that could elevate CRP level such as age, smoking, body mass index, triglycerides, and cholesterol (Noack et al., 2001). It is likely that persistent bacterial stimuli in the periodontium may increase proinflammatory cytokines systemically and contribute to elevated levels of CRP.



CRP has a direct proinflammatory effect on human endothelial cells via up regulation of ICAM-1, VCAM-1 and E-selectin (Yamazaki et al., 2005). CRP-induced monocyte activation and up-regulation of integrins (CD14 and CD11b) are reported in *ex vivo* studies in our group (Woollard et al., 2002, Woollard et al., 2005). These data suggest the importance of CRP on leukocyte activation and adhesion on to endothelial cells. Elevated levels of circulating CRP could also be involved indirectly in the development of atherosclerosis. Some intervention studies have investigated the periodontal treatment on CRP levels. It has been shown that successful periodontal treatment could reduce serum level of CRP (Yamazaki et al., 2005).

As reflected in most studies, plasma level of pro-inflammatory mediators in moderate periodontitis do not differ from healthy control subjects (Gońska et al., 2003, Yamazaki et al., 2005, Ide et al., 2003). However, increased TNF- $\alpha$  secretion has been observed in extensive periodontitis (Bretz et al., 2005). This suggests that plasma TNF- $\alpha$  level is associated with extent of the periodontal disease. The elevated TNF- $\alpha$  plasma levels are also likely to be associated with subclinical and clinical cardiovascular disease as observed in Health ABC cohort study (Cesari et al., 2003).

The positive correlation of cytokine levels and the extent of periodontitis are observed in many instances. The levels of IL-2 (Gońska et al., 2003), IL-4 (Gońska et al., 2003), IL-6 (Bretz et al., 2005, Yamazaki et al., 2005), IL-8, IL-10 (Havemose-Poulsen et al., 2005) and IL-1 receptor activator (Havemose-Poulsen et al., 2005) are reported to be higher in periodontal patients than healthy control subjects. Higher IL-8 levels are an important indicator for rapidly aggressive periodontitis (Gainet et al., 1999). The study of gingival biopsies has shown that the expression of macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ )

and interferon-gamma inducible protein 10 (IP-10) is associated with higher IFN- $\gamma$  expression and lower interleukin-10 expression in aggressive periodontal patients (Garlet et al., 2003). Also, gingival biopsy from chronic periodontitis patients had higher expression of monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR4. However, the extent of chemokines secreted during periodontitis does not exceed the threshold levels that would have profound systemic effects such as fever or severe overall illness (Loos, 2005).

Immunoglobulins are the major constituent in the adaptive host response. Five main classes, called IgG, IgA, IgM, IgD and IgE are produced by B-lymphocytes from the humoral immune response. Antibodies are induced following bacterial challenge, and other risk factors such as smoking and age can also influence antibody production in periodontitis. Three main periodontal pathogens namely, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Bacteroides forsythus* have been intensively studied in the literature regarding host antibody production (Ishikawa et al., 1997). It has been found that levels of different immunoglobulin and IgG subclasses are elevated according to type of the antigen and/or different periodontal conditions (Ishikawa et al., 1997, Trindade et al., 2008). As reported by Trindade and co workers, chronic periodontitis, aggressive periodontitis and patients with gingivitis have increased level of immunoglobulin and IgG subclasses production and reaction towards *P. gingivalis* compared with healthy controls (Trindade et al., 2008). Most often chronic periodontitis patients developed a higher *P.gingivalis* specific IgG level than patients at the active periodontal stage and after treatments. Prolonged IgG response is indicative of persistent immune activation and periodontal destruction. Higher serum *P.gingivalis* specific IgG1 levels were also observed with treated and maintained periodontal patients compared with

healthy controls (Sakai et al., 2001). *Actinobacillus actinomycetemcomitans* serotype-b specific IgG2 was found to be the predominant antibody that is elevated in non-smoking patients (Lu et al., 1994, Wilton et al., 1992). Although total IgG levels are not different between patients and controls, IgG1 and IgG2 subclasses were shown to be elevated in patients with periodontitis (Graswinckel et al., 2004).

The nature of the relationship between systemic diseases and oral disorders still remains to be established. In fact recent observational epidemiological studies suggest that most of the systemic diseases should not be considered as the direct cause of periodontal disease but rather as systemic promoting factors, able to produce environments suitable for local agents to induce gingivitis and periodontitis. In this regard, there is a need to investigate and compare plasma components that may have the ability to increase neutrophil activity in inflammatory periodontitis.



## **5.3 Materials and Methods**

### **5.3.1 Depletion of plasma albumin and immunoglobulin**

In order to investigate whether albumin, which may transport inflammatory lipids (Duarte et al., 2009) or immune complexes present in the plasma of periodontitis patients contribute to neutrophil hyperactivity and hyperreactivity in periodontitis, the lipid transporter albumin and immunoglobulins were depleted from healthy and periodontitis plasmas. Depleted plasmas were subsequently incubated with normal neutrophils and the respiratory burst activity measured in the absence and presence of fMLP.

Plasma albumin and immunoglobulin G were removed by the Aurum serum protein mini kit (Biorad, CA, USA) according to the manufacturer's instructions. Briefly, the Aurum serum protein column was placed in a 12 x 75 mm test tube and the resin was allowed to settle for 5min. The cap was removed and the tip from the bottom of the Aurum serum protein column was broken off. The residual buffer was allowed to drain from the column via gravity flow (approximately 2 min). Once the residual buffer was drained, the column was washed two times with binding buffer (1 ml). Each wash was allowed pass fully through the column and drain. After the last wash, the column was placed in an empty 2.0 ml collection tube and centrifuged for 20sec at 10,000 x g in a microcentrifuge to dry the resin bed and frit. The collection tube was discarded and yellow column tip was placed on the bottom of the column to stop any residual buffer flow from column. The column was placed in a clean 2ml collection tube labeled "unbound". In a separate 2ml collection tube, the sample to be purified was prepared by diluting 60µl of plasma or serum with 180µl of Aurum serum protein binding buffer. Plasma and binding buffer was

mixed by inverting tube several times. 200µl of the diluted serum sample was added to the top of the resin bed and the sample was allowed to penetrate column matrix. The column was gently vortexed and each step was repeated after 5 and 10 min. The column was allowed to sit for an additional 5 min, to give a total incubation time of 15 min. The yellow tip from the column was removed and the column returned to a 2ml collection tube. The column was centrifuged for 20 sec at 10,000 x g in a microcentrifuge, collecting the eluate in the “unbound” 2ml collection tube. The column and tube were removed from the centrifuge and the resin washed with 200µl of the binding buffer. The tube and column was centrifuged for 20 sec at 10,000 x g in a microcentrifuge, collecting the eluate in the same “unbound” tube; this contains the albumin- and IgG-depleted serum sample. Patient and control plasma samples were diluted 1:3 ratio in PBS. Ten µl of control/patient plasma before albumin/immunoglobulin depletion and after depletion was used to stimulate neutrophils.

### **5.3.2 Optimisation of neutralising antibody concentrations**

In order to investigate the contribution of specific cytokines to any priming effect of periodontal plasma on neutrophils from healthy donors, specific antibodies were used to neutralise the cytokines of interest. Initial experiments were undertaken to identify the concentration of antibody required to neutralise the respiratory burst-stimulating effects of periodontitis plasma.

Healthy control plasma samples were heat-inactivated by incubating at 60<sup>0</sup>C in a water bath for 10min on the day of analysis and allowed to cool to room temperature. One of the

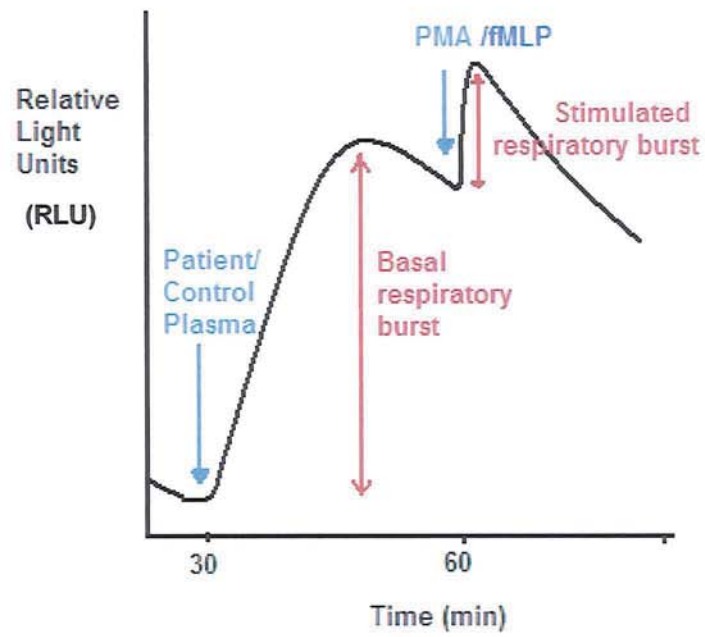
control plasmas was spiked with 10µl of 50nM IL-8, 1.25ng/ml GM-CSF (equivalent to 12.5 pg/10<sup>5</sup> cells), and 25 IU IFN-α and was used to investigate the optimal neutralisation concentration for IL-8, GM-CSF and IFN-α antibody. Heat-inactivated plasma samples (10µl) were treated with 20µg/ml, 10µg/ml or 5µg/ml of IL-8, GM-CSF or IFN-α antibody for 15min.

### **5.3.3 Neutralisation of plasma IL-8, GM-CSF and IFN-α and effect plasmas on lucigenin-dependent chemiluminescence**

Heat inactivated plasma samples were treated with 5µg/ml anti-IL-8 (1µg/ml for 10<sup>5</sup> cells), 5µg/ml anti-GM-CSF, or 5µg/ml anti-IFN-α antibody for 15min at room temperature in three independent experiments.

In order to determine plasma factors on the respiratory burst, lucigenin-dependent chemiluminescence from neutrophils was measured as described in general methods (see section 2.2.4). Luminescence was recorded during the last 10min pre-stimulation period to study baseline O<sub>2</sub><sup>-</sup> production from isolated neutrophils (Figure 5.1). Ten µl of immunoglobulin-depleted, antibody neutralised, or untreated plasma was added into microwells containing neutrophils. Pre-stimulation radical release was measured for 20min. Cells were then stimulated with 1µM fMLP and RLU measurements were taken for further 30min. The peak RLU values for pre- and post- stimulation are reported as mean relative light units RLU± SEM. All samples were run in triplicate, with paired patient and control samples analysed at the same time.



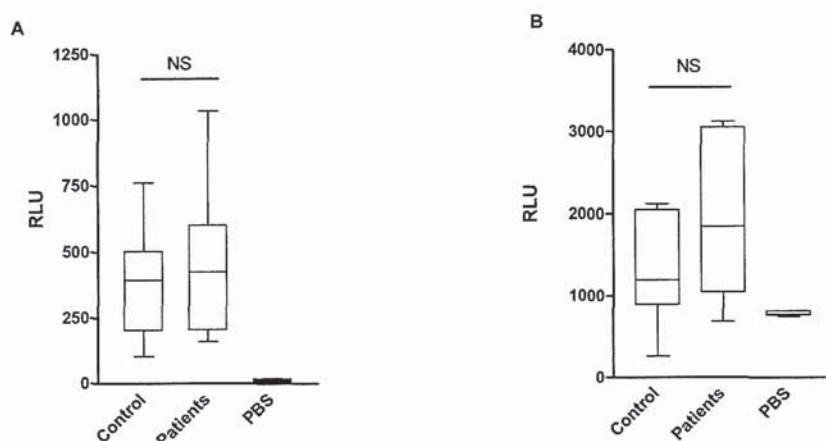


**Figure 5.1: Schematic representation of neutrophil respiratory burst in plasma and in the presence of second stimulus**

## 5.4 Results

### 5.4.1 Periodontal plasma exerts basal respiratory burst of dHL60 cells

The effect of plasma on dHL60 cell basal and PMA-stimulated respiratory burst is presented in Figure 5.2. dHL60 cells in the presence of plasma exhibited measurable levels of respiratory burst activity detected as lucigenin-chemiluminescence during the one hour assay period. The peak RLU emission was obtained at  $15.31 \pm 3.4$  min (mean  $\pm$  SEM). Before stimulation, dHL60 cells incubated with patients' plasma showed a trend to induce higher extracellular lucigenin-dependent chemiluminescence (mean  $\pm$  SEM;  $448.9 \pm 74.4$  RLU) compared with control plasmas (mean  $\pm$  SEM;  $360.5 \pm 60.23$  RLU). Stimulation with PMA enhanced the dHL60 respiratory burst of both controls (mean  $\pm$  SEM;  $1244 \pm 246.5$  RLU,  $P < 0.05$ ) and patients (mean  $\pm$  SEM;  $1906 \pm 381.8$  RLU,  $P < 0.01$ ). PBS treated dHL60 cells produce negligible amount of burst ( $11.14 \pm 2.2$ ), whereas post-PMA stimulation generated higher burst ( $780.7 \pm 10.32$ )

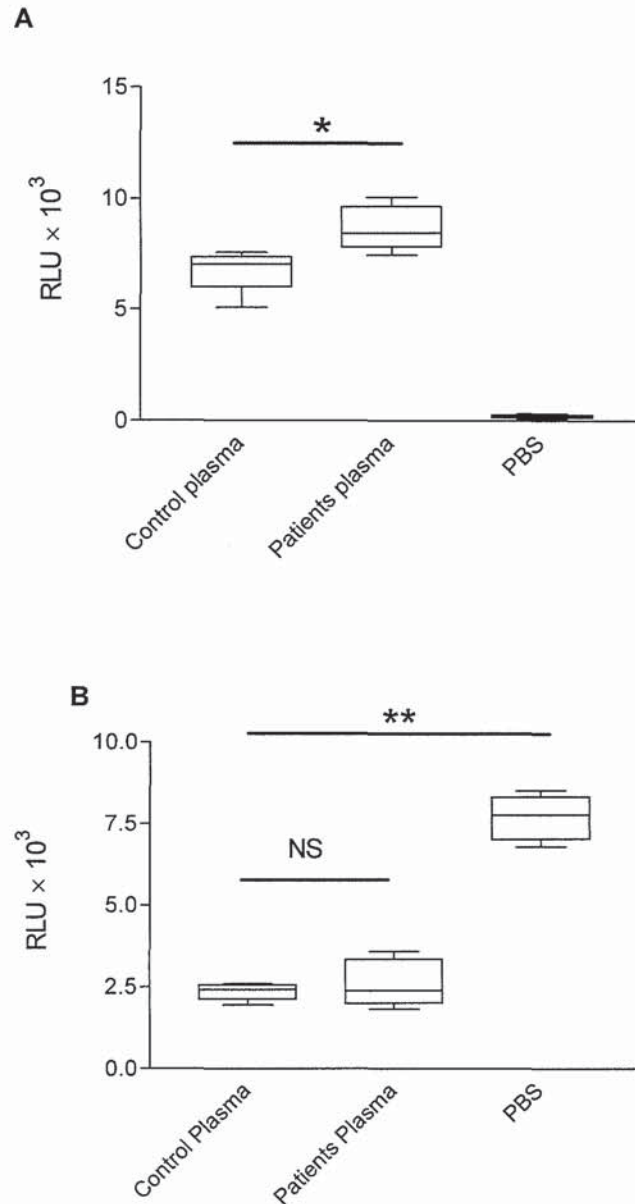


**Figure 5.2: Box-and-whiskers plots showing the effect of periodontally healthy and unhealthy peripheral blood plasma on the respiratory burst of dHL60 cells.** dHL60 cells ( $5 \times 10^5$ ) were washed twice in PBS and incubated in pre-blocked white microwells containing  $100\mu\text{M}$  lucigenin in PBS for 20min. Cells were then treated with  $100\mu\text{l}$  of patient ( $n=6$ ), healthy control plasma ( $n=6$ ) or PBS and RLU recording was immediately started. Chemiluminescence measurements were taken for 30 min before addition of  $10\text{nM}$  PMA (A). PMA stimulated respiratory burst was measured for further 30min (B). Peak RLU values for both pre- and post stimulations were plotted and Wilcoxon signed rank test was used for statistical comparison.

#### 5.4.2 Periodontal plasma elicits basal respiratory burst of neutrophils

To validate the results obtained from dHL60 cells, the same experiment was repeated with isolated peripheral blood neutrophils. Incubation of healthy, resting neutrophils ( $5 \times 10^5$ ) with patient or control plasma induced a basal respiratory burst (Figure 5.3A and B). As measured in the chemiluminescence assay, patient plasma induced significantly higher basal luminescence ( $8.65 \times 10^3 \pm 0.37$  RLU) than control plasma ( $6.88 \times 10^3 \pm 0.39$  RLU;  $P < 0.05$ ). However, PMA stimulation of neutrophils when co-incubated with periodontal plasma showed no difference ( $2.58 \times 10^3 \pm 0.26$  RLU), compared with age; sex matched healthy control plasma ( $2.37 \times 10^3 \pm 0.09$ ). PBS treated neutrophils showed low basal level burst ( $0.22 \times 10^3 \pm 0.03$ ) compared with post-PMA stimulated respiratory burst ( $7.23 \times 10^3 \pm 0.27$ ;  $P < 0.01$ ).

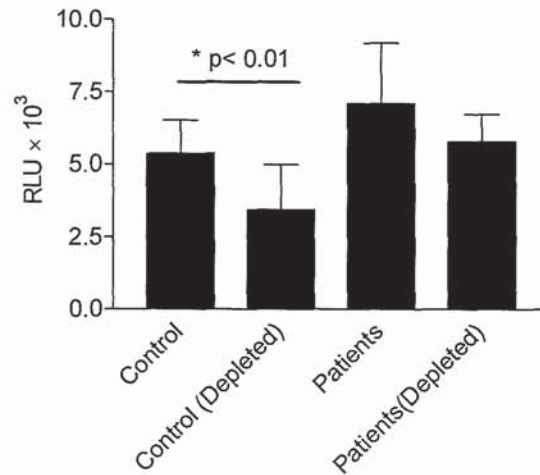




**Figure 5.3** Box-and-whiskers plots showing the effect of periodontally healthy and unhealthy peripheral blood plasma on respiratory burst of neutrophils. Neutrophils from healthy control individuals were isolated by Percoll density centrifugation as described in materials and methods. Neutrophils ( $5 \times 10^5$ ) were incubated in pre-blocked white microwells containing  $100\mu\text{M}$  lucigenin in PBS for 20min. Cells were then treated with  $100\mu\text{l}$  of patient ( $n=6$ ), age, sex matched healthy control plasma ( $n=6$ ) or PBS and RLU values were recorded immediately. Chemiluminescence measurements were taken for 30 min before addition of  $10\text{nM}$  PMA (A). The PMA stimulated respiratory burst was measured for further 30min (B). Peak values for both pre- and post stimulations were plotted and significant differences between controls and patients were calculated using paired t-test. \*  $P<0.05$ , \*\*  $P<0.01$ .

#### **5.4.3 The effect of plasma albumin and immunoglobulin depletion on neutrophil respiratory burst**

To investigate the effect of different plasma components on neutrophil hyperactivity and hyper-reactivity, depletion and inhibition approaches were undertaken. First, plasma was depleted of the main serum proteins albumin and immunoglobulin (IgG). Confirming previous results, as represented in figure 5.4, plasmas from periodontal patients elicit a higher respiratory burst ( $7.11 \times 10^3 \pm 2.09$  RLU) from healthy subject neutrophils compared with healthy control plasmas ( $5.38 \times 10^3 \pm 1.14$  RLU). Albumin and IgG depletion from control plasma resulted in a significant reduction of lucigenin-dependent luminescence peak value from neutrophils by more than 25% ( $P < 0.05$ ). However, the lucigenin-dependent chemiluminescence signal observed for neutrophils in the presence of patient plasma ( $7.11 \times 10^3 \pm 2.09$  RLU) was not significantly influenced by albumin and IgG depletion ( $5.79 \times 10^3 \pm 0.948$  RLU).

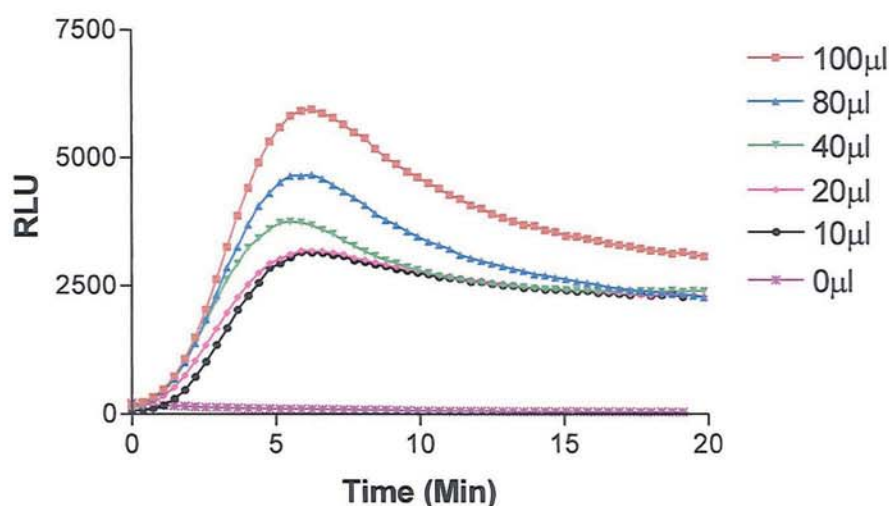


**Figure 5.4: Mean chemiluminescence generated by peripheral blood neutrophils pre- and post- depletion of plasma albumin and IgG.** Neutrophils from healthy control individuals were isolated by Percoll® density centrifugation as described in materials and methods. Neutrophils ( $5 \times 10^5$  cells) were incubated in pre-blocked white microwells containing 100µM lucigenin in PBS for 20min. Control and patient plasma depleted for albumin and IgG using Arum serum protein mini kit® (BioRad, California). Neutrophils were then treated with 30µl of patient plasma (n=3; with or without albumin/IgG depletion) or, age, sex matched healthy control plasma (n=3; with or without albumin/IgG depletion) with the immediate start of recording RLU values. Basal lucigenin-dependent chemiluminescence measurements were taken for 30 min.

#### 5.4.4 The effect of volume of plasma on neutrophil basal respiratory burst

To determine the effect of volume of plasma on neutrophil basal respiratory burst, the plasma was diluted in PBS supplemented with 1% BSA. Lucigenin-dependent basal chemiluminescence of neutrophils with plasma or without plasma was also compared (Figure 5.5). Even in the absence of a stimulus, healthy control plasma induced measurable CL as measured in RLU. Neutrophils incubated with 10µl plasma generated significantly higher lucigenin-dependent CL ( $3149 \pm 228.09$  RLU) compared with neutrophils in the absence of plasma ( $100.33 \pm 25.15$  RLU;  $P < 0.001$ ). Lucigenin dependent CL increased with plasma volume.



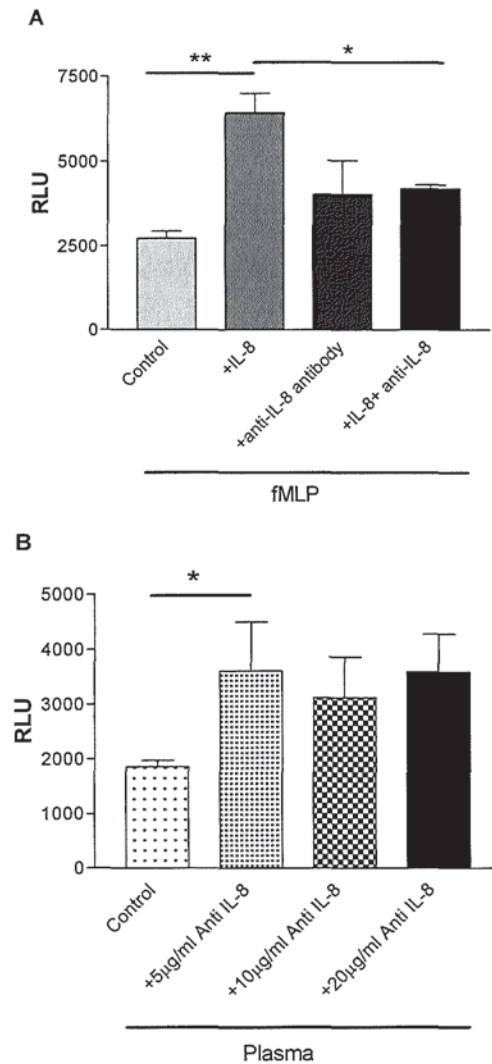


**Figure 5.5: The kinetic profiles of plasma-stimulated lucigenin-enhanced plasma stimulated chemiluminescence obtained with different volumes of plasma.** Neutrophils from healthy control individuals were isolated by Percoll® density centrifugation as described in materials and methods. Neutrophils ( $5 \times 10^5$ ) were incubated in pre-blocked white microwells containing 100µM lucigenin in PBS for 20min. Healthy control plasma (10, 20, 40, 80 or 100µl) was diluted to a total volume of 100µl in PBS supplemented with 1% BSA. After recording the baseline CL, neutrophils were stimulated with different concentrations of plasma and RLU values were started recording immediately and for 20min. Each data point represents the mean of three values obtained by measuring neutrophil burst in response to one healthy control plasma sample.

#### 5.4.5 IL-8 priming of fMLP-induced respiratory burst in the presence of neutralising antibody

To determine the optimal concentration of neutralising antibody required to inhibit effects of antigen-induced neutrophil activation, the respiratory burst of neutrophils was determined in the presence or absence of plasma and varying antibody dilutions. In the absence of plasma (Figure 5.6A), IL-8 primed, fMLP-stimulated neutrophils generated a significant respiratory burst ( $6435 \pm 235.8$  RLU) compared with non-stimulated neutrophils ( $2719 \pm 150.7$  RLU). IL-8 priming ability was significantly neutralised with the addition of 5µg/ml monoclonal anti-IL-8 antibody ( $P < 0.01$ ). However, the addition of neutralising

anti-IL-8 antibodies into plasma in the absence of heat inactivation of complement enhanced the basal respiratory burst of neutrophils by 40% (Figure 4.5 B). This enhancement was not dependent on anti-IL-8 antibody concentration.

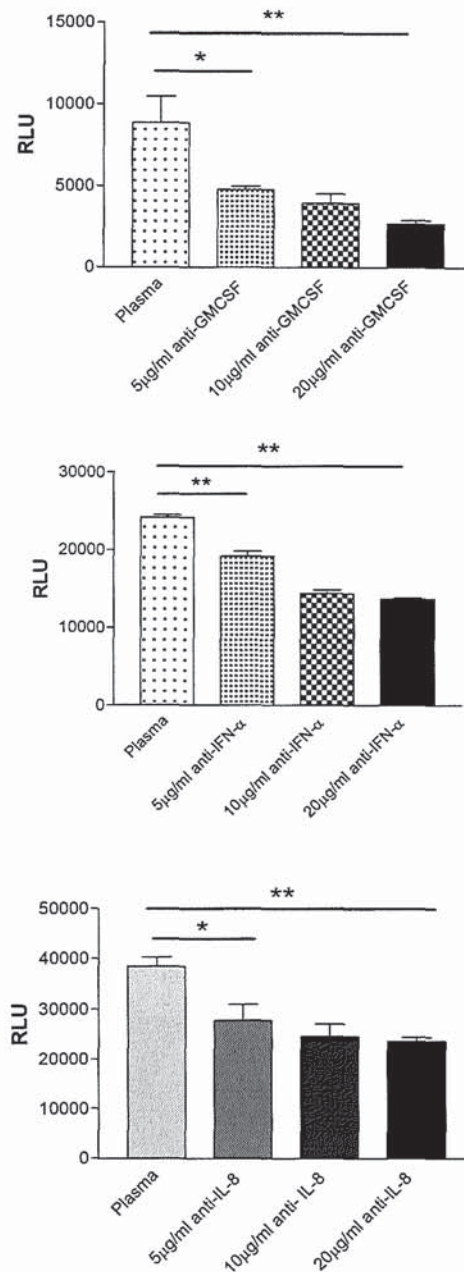


**Figure 5.6: The neutralising ability of anti IL-8 antibody in the presence or absence of plasma.** Neutrophils ( $5 \times 10^5$ ) in PBS were incubated in white microwells containing 100µM lucigenin. Cells were primed with 50nM IL-8 in the presence or absence of anti-IL-8 antibody (5µg/ml) and the plate is incubated at 37°C for 15 min before stimulation with 1µM fMLP (A). In a separate experiment, non-heat inactivated plasma was incubated with 5, 10 or 20µg/ml anti-IL-8 antibody for 15min at 37°C before adding to neutrophils in white microwells (B). The lucigenin-dependent chemiluminescence signal was monitored for 30min and peak values for basal respiratory burst are presented.

#### **5.4.6 The effect of antibody concentration on plasma neutralisation**

In order to eliminate the potential for complement activation in plasma following interaction between antibody and antigen, plasma was heat-inactivated for 15min at 57<sup>0</sup>C. Heat-inactivated plasma was examined for optimal anti-IL-8, anti-GM-CSF, and anti-IFN- $\alpha$  neutralising antibody concentration (Figure 5.7). In all three conditions, introduction of 5 $\mu$ g/ml antibody concentration lowered the ability of plasma to stimulate neutrophils to produce a basal respiratory burst (anti-IL-8: anti-GM-CSF: and anti-IFN- $\alpha$ ). The level of inhibition of the respiratory burst induced by heat-inactivated plasma in the presence of any of the three antibodies increased with the increasing antibody concentration.

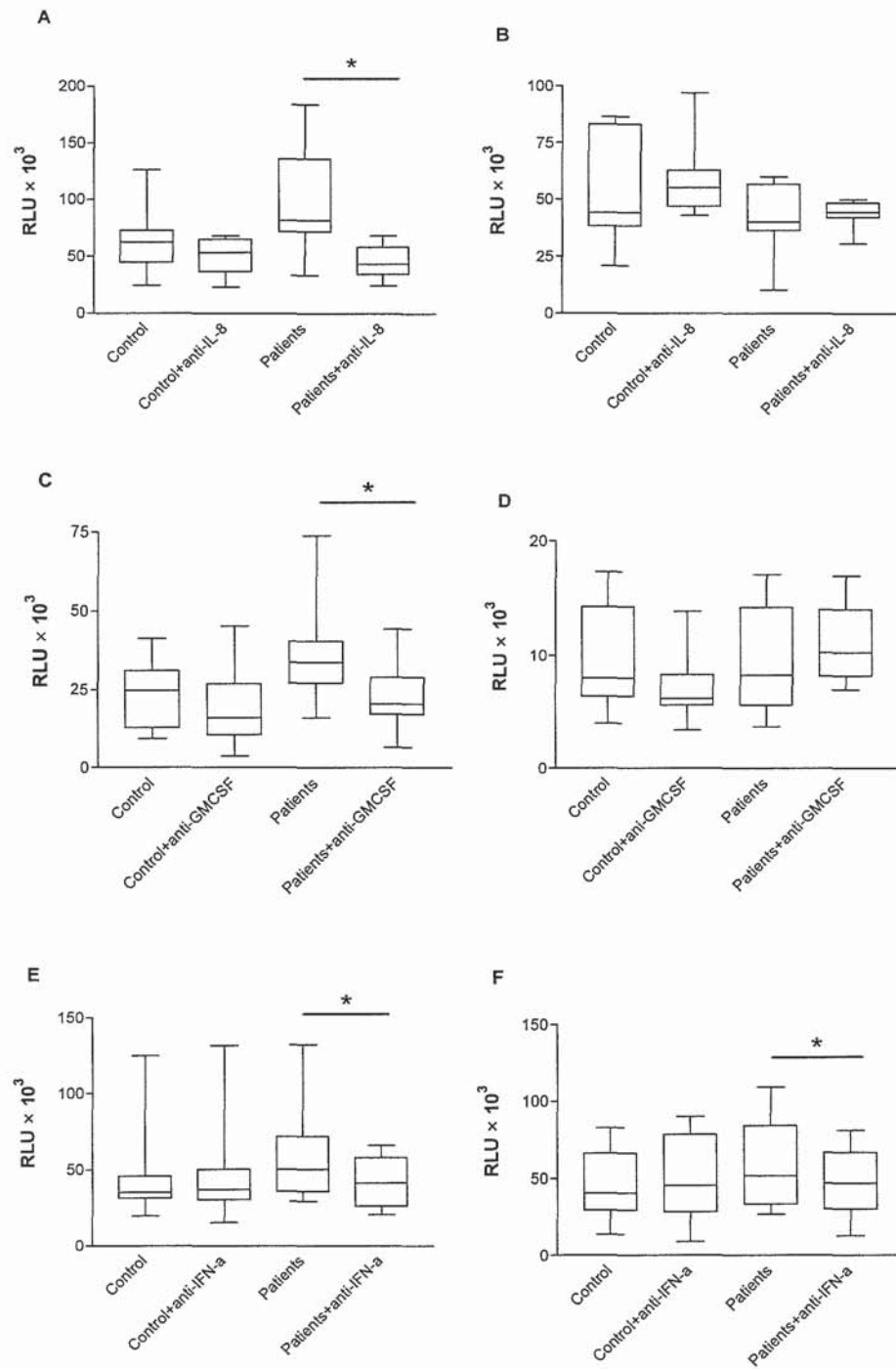




**Figure 5.7: The effect of neutralising antibody concentration on basal neutrophil respiratory burst.** Healthy control plasma (n=3) was heat inactivated by incubating at 57°C for 15 min in a water bath. Different concentrations of (5 μg/ml, 10 μg/ml, 20 μg/ml) neutralising antibodies against IL-8, GM-CSF or IFN-α were added into separate heat inactivated plasma samples and incubated at 37°C for 15 min. Neutrophils from healthy donors were isolated using Percoll® gradient as described in materials and methods (chapter 2). Neutrophils ( $5 \times 10^5$ ) were added into white microwell plates and incubated at 37°C for 20 min. Plasmas (10 μl) with or without neutralising antibodies were then added into neutrophils and CL was recorded immediately and for 30 min. Peak RLU values were plotted and statistical significant was calculated ANOVA analysis followed by Tukey's multiple comparison between columns. \*\* P<0.01, \*P<0.05.

#### **5.4.7 Neutralising IL-8, GM-CSF and IFN- $\alpha$ antibodies decreases neutrophil hyperactivity**

Confirming previous observations, in figure 5.8 healthy neutrophils isolated from three different periodontally healthy individuals and incubated in patient plasma showed a significantly higher respiratory burst than neutrophils incubated in healthy control plasma figure 5.8A and B for neutralising IL-8 (control  $60.96 \pm 8.4 \times 10^3$  RLU vs patients  $92.55 \pm 13.31 \times 10^3$  RLU); figure 5.8C for neutralising GM-CSF (control  $22.49 \pm 3.06 \times 10^3$  RLU vs patients  $35.08 \pm 4.59 \times 10^3$  RLU) and figure 5.8E and F for IFN- $\alpha$  (control  $42.6 \pm 8.6 \times 10^3$  RLU vs patient  $55.65 \pm 8.92 \times 10^3$  RLU). Both control- and patient-neutralised plasmas showed a decrease in basal neutrophil respiratory burst compared to non-neutralised plasma-treated neutrophils. However, the decrease was significant for patients' plasmas (IL-8 antibody-neutralised plasma  $44.92 \pm 4.02 \times 10^3$  RLU, GM-CSF antibody-neutralised plasma  $22.51 \pm 2.97 \times 10^3$  RLU and IFN- $\alpha$  antibody neutralised plasma  $39.9 \pm 4.9 \times 10^3$  RLU). After the 30min pre-stimulatory period, cells were stimulated with  $1 \mu\text{M}$  fMLP. Mean RLU values were not significantly different between control and patients plasmas or antibody-neutralised plasmas post-stimulation. The only significant decrease in respiratory burst post-fMLP stimulation was found for IFN- $\alpha$ -neutralised periodontitis plasma  $44.4 \pm 6.65 \times \text{RLU}$  compared to to non neutralised periodontitis plasma





**Figure 5.8 The effect of anti-IL-8, anti-GM-CSF and anti-IFN- $\alpha$  neutralising antibody on neutrophil respiratory burst.** Healthy control plasma (n=11) and patient plasma (n=11) was heat inactivated by incubating at 57°C for 15 min in a water bath. 5 $\mu$ g/ml of neutralising antibodies against IL-8 (A), GM-CSF (C) or IFN- $\alpha$  (E) were added into heat inactivated plasma samples in three independent experiments. Plasma samples in the presence or absence of antibodies were incubated at 37°C for 15 min. Neutrophils were isolated from three different periodontally healthy individuals using Percoll<sup>®</sup> gradient as described in materials and methods (chapter 2). Neutrophils (5 $\times$ 10<sup>5</sup>) were added into white microwell plates and incubated at 37°C for 20 min. Plasmas (10 $\mu$ l) with or without neutralising antibodies were then added to neutrophils and CL was recorded immediately and for 30 min. At the end of the incubation, cells were stimulated with 1 $\mu$ M fMLP and stimulated (anti-IL-8 treated (B), anti-GM-CSF treated (D), and anti-IFN- $\alpha$  treated(F)) respiratory burst was measured for further 20min. Peak RLU values were plotted and statistical significant was calculated by Wilcoxon signed rank test. \*P<0.05.

## 5.5 Discussion

Increased activity of neutrophils may contribute to destructive changes in inflammatory periodontitis (Chapple and Matthews, 2007). The relationship between the microbial insult of dental plaque and the inflammatory response of the host creates a complex environment resulting in increased levels of bacterial and host inflammatory factors in both gingival crevice fluid as well as in the systemic circulation (Matthews et al., 2007). It is possible that these mediators could prime or induce activity/reactivity of neutrophils in patients with periodontitis. In this sense, understanding the contributions of a variety of plasma components in periodontitis will provide valuable information about neutrophil respiratory burst activity regardless of whether the activity is due to a constitutional or secondary effect. In addition, the levels of systemic plasma factors are useful biomarkers for severity of periodontitis (Loos, 2005).

The hyperactivity of neutrophils from periodontitis patients is shown as increased chemotaxis, phagocytosis and increased production of ROS. Significant ROS generation by neutrophils in the absence of antioxidant defence (Chapple et al., 2007) is considered to cause local oxidative damage and contribute to local tissue damage. However, previous studies mainly based on analysis of peripheral blood neutrophils have demonstrated that the differences in neutrophil behaviour detected are systemic (McColl et al., 1990, Gustafsson and Asman, 1996, Fredriksson et al., 1999, Matthews et al., 2007). Aligning with the previous studies, this chapter investigated the production of ROS by peripheral blood neutrophils from healthy donors in the presence of plasma from healthy controls and periodontitis patients as measured by chemiluminescence. The phenomenon of neutrophil mediated oxidation of lucigenin to give a CL signal occurs when these cells are

challenged with bacterial LPS, fMLP or chemical agents such as PMA. Each CL curve that was derived from neutrophils challenged by either healthy control or patient plasma showed a typical time dependent pattern and produced a single peak with first order kinetic response (e.g. Figure 5.5). Primary consideration was given to peak CL counts, which reached at about 5-20min, as a measure of level of respiratory burst.

Plasma is a complex mixture of proteins, opsonins, immunoglobulins and their complexes, pro-inflammatory mediators and factors that vary according to inflammatory and immune status, age and gender (Graswinckel et al., 2004, Schreiber et al., 1982). The ability of plasma to activate the neutrophilic respiratory burst has been shown in many other studies (Fredriksson et al., 1999, Fredriksson et al., 1998, Gustafsson and Asman, 1996, Matthews et al., 2007). Confirming these observations, this chapter has shown that normal healthy plasma activated neutrophils to generate a measurable basal respiratory burst. Interestingly, plasma from patients with periodontitis induced a higher basal respiratory burst compared with control subjects both with dHL60 cells and peripheral neutrophils. This suggests the presence of more stimulatory factors in plasmas from patients with periodontitis. Therefore, it is possible that periodontitis causes systemic increases in inflammatory mediators, which increases risk for other systemic diseases including CVD.

The interesting questions are which factors are important for inducing the burst in neutrophils and whether levels of these are different between healthy and periodontitis patients? In search of answers for these questions, plasma was depleted of different factors independently. This is the first time that this approach been used to investigate possible factors that increase neutrophil respiratory burst in periodontitis.



Depletion of albumin and IgG from plasma significantly decreased the unstimulated neutrophil respiratory burst induced by control plasma but not by plasma from periodontal patients. Albumin is the most abundant plasma protein. As shown using *in vitro* studies, albumin does not participate directly in activating neutrophils (Liu et al., 1997) but it can act as a carrier protein to present other small molecular weight biomarkers (Mehta et al., 2003). Previous studies have demonstrated a change of plasma protein profile during inflammation and more likelihood of decreasing plasma albumin levels in experimental inflammation (Schreiber et al., 1982). Plasma albumin levels, however, are more likely to depend on the stage of the periodontal disease; whereas, lower plasma albumin levels are reported in aggressive periodontitis (Shi et al., 2008), juvenile periodontitis patient plasma albumin levels show no change (Asman et al., 1985). Moreover, Shi *et al.*, 2008 emphasised that the lower albumin-globulin ratio is more important and positively correlate with clinical parameters of periodontitis. Early findings from plasma analysis studies indicated increased concentrations of specific dental bacteria/bacterial products and serum immunoglobulins in patients with juvenile periodontitis (Lehner et al., 1974, Asman et al., 1985). In this sense, increased levels of globulins such as immunoglobulins in both types of juvenile and aggressive periodontitis may provide valuable information as disease biomarkers.

Increased concentrations of immunoglobulins such as IgM, IgG and immune complexes also have potential capacity to activate neutrophils (Asman et al., 1985). Soluble immune complexes interact with neutrophils via Fc $\gamma$  receptors, Fc $\gamma$ RII and Fc $\gamma$ RIII. Neutrophil priming slightly increases the surface level of Fc $\gamma$  receptors (Watson and Edwards, 1998). As discussed by Watson *et al.*, (1997), soluble immune complexes fail to activate the unprimed neutrophil respiratory burst. In unprimed cells, the soluble immune complexes

activate a transient increase in intracellular  $\text{Ca}^{2+}$  that is due to mobilisation of intracellular stores (Watson et al., 1997). Although, intracellular  $\text{Ca}^{2+}$  mobilisation is important, it is not sufficient to activate the NADPH oxidase complex (Watson and Edwards, 1998). Whereas, in primed neutrophils which have higher intracellular  $\text{Ca}^{2+}$  mobilisation, soluble immune complexes show enhanced NADPH activity. Patients' plasmas, which carry a large number of priming factors could prime neutrophils and induce high respiratory burst compared to healthy control plasmas even after depletion of albumin and immunoglobulins; other stimulators present in patient plasma might promote the respiratory burst, such that it is not inhibited after depletion compared to replete plasma.

Resting neutrophils did not produce lucigenin-dependent CL in the absence of plasma, but produced a plasma concentration gradient-dependent increase of basal respiratory burst with the addition of plasma. This indicated that priming or inducing factors in plasma are proportionally combining to activate neutrophils to produce superoxide detectable by lucigenin over the cell density and plasma volume added. It is important to work with physiologically relevant plasma volume to neutrophil ratio, as over exposure of neutrophils to priming factors in both control and patients plasma could mask the differences between them. After measuring basal respiratory burst with plasma, neutrophils were then activated with a second stimulus e.g. PMA or fMLP.

The PMA-activated lucigenin-dependent CL signals produced from neutrophils in the presence of plasma were significantly lower when compared to the PMA-activated lucigenin-dependent CL of isolated neutrophils in the absence of plasma. It could be suggested that the decrease of PMA-activated CL was due to the antioxidant capacity of

plasma. To combat ROS and to maintain cell and tissue homeostasis, plasma naturally contains a wide range of antioxidant molecules including ascorbic acid (vitamin C),  $\alpha$ -tocopherol (vitamin E), uric acid, glutathione, lipoic acid, ubiquinol and carotenes (Jackson et al., 2002). It has been found that inflammatory diseases are associated with enhanced production of ROS such as inflammatory lung diseases, CVD, rheumatoid arthritis and periodontitis. As reviewed in Chapple and Matthews (2007) studies investigating the total antioxidant capacity (TAOC) of plasmas have demonstrated a significantly lower TAOC in plasma from periodontitis patients compared to healthy controls.

Although PMA is a strong stimulator for neutrophil respiratory burst, no significant response to PMA was observed in the presence of plasma from both control and patient groups when compared to the presence of plasma alone. Activated neutrophils continuously recruit and recycle NADPH oxidase subunits (Li et al., 2008) into the plasma membrane until the burst reaches its peak. However, carefully controlled anti-inflammatory pathways are needed to inhibit NADPH activity and terminate the neutrophil respiratory burst. It has been shown that some oxidised phospholipids such as phosphatidylcholines, phosphatidylserines, and phosphatidylglycerol can inhibit NADPH oxidase activity and assembly without effecting phagocytosis (Bluml et al., 2008). This may explain the observation here of a lower response of neutrophils towards the second stimuli. Plasma contains a vast amount of stimulator that may induce a maximal respiratory burst in neutrophils. ROS production from this stimulation may oxidise phospholipids that act negatively on neutrophil respiratory burst. It is also possible that after this stimulation, the turnover for NADPH oxidase subunits may become limited for a



second stimulus. Thus, due to up-regulation of inhibitory pathways for NADPH oxidase assembly and activation the effect of second stimulus is greatly hindered.

The previous chapter described that gingipains secreted by the periodontal pathogen *P. gingivalis* could manipulate IL-8 primed neutrophil respiratory burst (Dias et al., 2008). In order to find whether IL-8 present in the plasma of patients with periodontitis could contribute to an enhanced respiratory burst, IL-8 was neutralised with anti-IL-8 neutralising antibody. This significantly reduced the basal respiratory burst produced by neutrophils in plasma from patients. There was no significant decrease in respiratory burst with IL-8-neutralised plasma from control subjects compared to non-depleted control plasma. This indicates that the presence of high activity of IL-8 in periodontitis plasmas, may contribute to the observed hyperactive phenotypes of neutrophils in periodontitis. Increased IL-8 production by neutrophils in rapidly progressive periodontitis is reported (Gainet et al., 1998). Further, this group have shown that plasma from rapidly progressive periodontitis patients have significantly higher IL-8 plasma levels than other types of periodontitis (Gainet et al., 1999). It is also well established that IL-8 strongly primes neutrophils to produce an enhanced respiratory burst in response to PMA and fMLP stimulus. However, the present results for neutrophils incubated in IL-8-neutralised plasma did not show a significant difference in respiratory burst with plasma from either control or patient groups after exposing to a subsequent stimulus of fMLP.

Recent data from collaborators at Birmingham Dental hospital have demonstrated that *in vitro* priming by GM-CSF enhanced baseline extracellular ROS production in peripheral neutrophils from perio-patients (Matthews et al., 2007). This observation is consistent with previous findings for GM-CSF up-regulation of neutrophil-mediated pathology

(Mansfield et al., 2002, Waterman and Shaafi, 1995). The work reported in this chapter, GM-CSF-neutralised patient plasma showed a significant decrease in neutrophil basal respiratory burst compared to healthy controls. Previous studies have demonstrated that up-regulation of GM-CSF gene expression occurs in an oral epithelial cell line after challenging with oral pathogens such as *P. gingivalis* (Prime et al., 1990). The results presented in this chapter, therefore may confirm the higher activity of GM-CSF in the plasma of patients with periodontitis. GM-CSF has similarities in its priming abilities to TNF- $\alpha$  and is reported to enhance the fMLP-induced oxidative burst (Elbim et al., 1994). The obtained here however, show no significant difference in plasma-induced neutrophil respiratory burst between patient and control groups after subsequent stimulation with fMLP.

The ability to enhance degranulation and the respiratory burst of neutrophils by IFNs has been reported in previous studies (e.g. Atzeni et al., 2002). The production of IFNs from a wide range of cell types is markedly increased during an infection (Koyama et al., 2008). Even though IFNs exhibit diverse biological functions, three main activities have been identified; anti-viral, anti-tumour and immunomodulatory effects. The production of IFN- $\alpha$  is a specific and reliable marker for viral infections. For this reason, little attention has been given in the literature to IFN- $\alpha$  activity in the inflammatory disease, periodontitis. As, IFN- $\gamma$  is strongly produced by activated T cells or natural killer cells but not by virus infected cells, it has been given greater attention in periodontitis research (Garlet et al., 2003, Silva et al., 2007). IFN- $\gamma$  levels have been shown to be higher in gingival tissues (Garlet et al., 2003), serum and gingival crevicular fluid from periodontitis patients (Go'rska et al., 2003). Given that type I IFNs (IFN $\alpha/\beta$ ) are associated with other biological activities such as controlling neutrophil life span, in addition to their main



functions as antiviral and autoimmune responses; they could potentially be involved in the reported hyperactivity of neutrophils in periodontitis. The results shown here support the concept of the higher activity of IFN- $\alpha$  in patient plasma as a contributor to neutrophil hyperactivity; the basal respiratory burst induced by patients periodontitis plasma is significantly reduced after neutralising with anti IFN- $\alpha$  antibody. Evidence also supports the concept that IFN- $\alpha$  could act as a priming agent, where it enhances the fMLP-, leukotriene B<sub>4</sub>- or influenza A virus-induced respiratory burst (Little et al., 1994). Confirming this hypothesis, the results in this chapter show that plasmas from patients induced a significantly increased fMLP-stimulated respiratory burst than healthy control subject plasmas in the absence of neutralising antibodies. Also, the high respiratory burst induced by patient plasma after fMLP stimulation was quenched significantly with the addition of neutralising anti-IFN- $\alpha$  antibodies. Further support for the importance of IFN- $\alpha$  in periodontitis comes from the observations that IFN- $\alpha$ -stimulated gene expression (ISG) is elevated in peripheral blood neutrophils from periodontitis patients (Wright et al., 2008). In comparison, LPS did not recapitulate the ISG that was observed *in vivo*. According to this study, the most likely IFNs to stimulate these genes are type I IFNs. This suggests that increased IFN- $\alpha$  level in patient plasma primes neutrophils to express more ISG, which in turn causes neutrophil maturation and activation.

Collectively, this chapter has investigated different priming factors in periodontal plasma and presented evidence that patient plasmas have higher activities of IL-8, GM-CSF and IFN- $\alpha$  for the increased respiratory burst compared with matching healthy controls. This effect was significantly reduced after neutralising with their corresponding antibodies. In addition, the role of plasma IFN- $\alpha$  as a pathophysiological priming agent may be important for the observed hyper-reactivity of periodontal neutrophils. The reported



ability of IL-8, GM-CSF and IFN- $\alpha$  to prime the oxidative burst of neutrophils combined with their elevated activities present in patients plasma provide support for their involvement in the pathogenesis of chronic periodontitis.

## **Chapter 6.0 – Lipid raft clustering and assembly of NADPH oxidase in oxidatively stressed dHL60 cells**

### **6.1 Preface**

Herein, a detailed review of the activity and assembly of NADPH oxidase in the membrane and into lipid rafts under oxidatively stressed conditions is provided. It is evident that cellular antioxidants can play a key role in modulating NADPH activity. This has been investigated using buthionine sulfoximine to manipulate the endogenous antioxidant, glutathione (GSH), in dHL60 cells. Total glutathione level together with GSH/GSSG ratio provided valuable information of how dHL60 redox balance influences respiratory burst and protein glutathiolation. Lipid raft analysis of p47<sup>phox</sup> was undertaken using sucrose density ultracentrifugation and immunofluorescence microscopy. The discussion addresses the issues of the shift of cellular GSH/ GSSG ratio in pathological conditions, such as periodontitis leading to protein glutathiolation and potential for constitutive activation of neutrophils.

## 6.2 Introduction

The antimicrobial efficiency of neutrophils depends on the generation of ROS by assembly and activation of the NADPH oxidase enzymatic complex and the release of antimicrobial proteins within the endosomes. As discussed in chapter 4, the activation of the respiratory burst through NADPH oxidase is triggered by numerous agonists presents in plasma. The generation of superoxide anion via NADPH oxidase is the first step for the production of a range of reactive oxygen and nitrogen species. Although, these  $O_2^{\cdot-}$  initiated cascades of reactions are important in clearing foreign pathogens, sustained NADPH oxidase activation results in adverse effects on the host. This is prevented by biological systems which include naturally occurring enzymes and low molecular weight molecules within the cells that prevent the uncontrolled formation of free radicals and activated oxygen species. Among these, glutathione plays a pivotal role as an intracellular antioxidant (Griffiths, 2000).

While redox regulation of expression is described for many proteins, in plants (Desikan et al., 2001, Neill et al., 2002) and other eukaryote (Griffiths, 2005) enzymes that undergo reversible thiol oxidation act as redox switches (Hancock, 2008). Acid sphingomyelinase (ASM) is an example for a redox-regulated enzyme (Schissel et al., 1998, Holopainen et al., 1998). ASM is a phosphodiesterase that hydrolyzes sphingomyelin to ceramide and phosphocholine. The importance of ASMase activity is evident from the ASMase defective Niemann-Pick disease, an autosomal recessive lipid storage disorder caused by loss-of-function mutations within the ASM gene (Qiu et al., 2003). Symptoms of the diseases are the accumulation of sphingomyelin in lysosomes. ASM is translated as a 75kDa precursor protein and stepwise processing results in a mature form of 70kDa



enzyme (Qiu et al., 2003). ASM displays an optimum activity at around pH 4.8. These enzymes have been further subclassified into the endosomal/lysosomal (L) ASM and secretory (S)  $\text{Zn}^{2+}$  dependent ASM. However, earlier findings support that both L- and S-ASMases are activated by  $\text{Zn}^{2+}$  (Schissel et al., 1998).

Redox regulation of ASM was described by Qiu *et al.*, (2003). A free thiol group modification in a protein may alter its structure and/or activity. As observed in cysteine proteases, a thiol group stabilises the inactivate conformation of ASMases forming hydrogen bonds with adjacent electron donors. In a specific sub-cellular environment such as lysosomes or in the extracellular environment, the enzymatic activity is initiated by deprotonating the thiol group and forming an intermediate thioester bond. The requirement of  $\text{Zn}^{2+}$  for ASM activation together with the acidic environment has been explained by the recombinant ASM “cysteine switch” activation mechanism (Qiu et al., 2003). During low activity, C-terminal unbridged free cysteine ( $\text{Cys}^{629}$ ) of the ASM coordinates with  $\text{Zn}^{2+}$  masking the nucleophilic activity of  $\text{Zn}^{2+}$ . In highly active states, the N-terminal residue of the enzyme containing  $\text{Cys}^{629}$  is either proteolytically cleaved (Springman et al., 1990) or may be involved in enzyme dimerisation or cysteine modification that permits the optimal structure for catalysis. The regulation of sphingomyelinase activity by the redox state of the cell is also reported for neutral sphingomyelinase (Martin et al., 2007). It has been reported that cellular GSH/GSSG ratio but not the total concentration of glutathione is important for the activation of neutral sphingomyelinase.

Activation of the ASM correlates with translocation of the enzyme from intracellular stores onto the extracellular leaflet of the cell membrane (Schissel et al., 1998). This

migration is also supported by fluorescence microscopy detection of sphingomyelinase and colocalisation with GPI gangliosides labelled with the  $\beta$ -subunit of cholera toxin (Li et al., 2007). ASM stimulation and activation *in vivo* and *in vitro* is well studied with ligands such as TNF- $\alpha$ , Fas or CD95, which also contribute to altered cellular redox balance (Li et al., 2007, Zhang et al., 2007).

ASM generates transient ceramide accumulation in acidic cellular compartments within minutes. Sphingomyelinase activity in the outer leaflet results in the breakdown of sphingomyelin into ceramide and choline. It has been shown that ceramide is incorporated to stabilise lipid rafts (LR). Studies with model membranes showed that natural ceramides, long chain ceramide or analogues of triglycerides are competitively incorporated in plasma membrane displacing the cholesterol (Megha and London, 2004). This ceramide-sterol displacement may have important consequences in lipid raft structure, such as removal of cholesterol binding proteins, which could affect the lipid raft function. It has been shown that a small amount of ceramide can flip-flop rapidly within the cytoplasmic leaflet and might trigger ordered domain formation by phosphatidylethanolamine and phosphatidylserine, thereby transmitting a signal through the membrane. Ceramide localisation within lipid rafts is important for their function in internalisation of pathogens (Grasse *et al.*, 2003), CD95 clustering, induction of apoptotic signals (Grasse, 2003), and as a second messenger (Liu and Anderson, 1995). Ceramide has been demonstrated to activate PKC, phospholipase A2 and CAPP (ceramide-activated protein phosphatase). Holopainen and coworkers have demonstrated that sphingomyelinase-induced ceramide formation could generate lipid microdomains in large unilamellar vesicles (Holopainen et al., 1998).

There is considerable evidence that ceramides induce lipid raft clustering into unusually large raft domains in plasma membrane called “ceramide-enriched membrane platforms” (Grassme et al., 2003, Li et al., 2007). Ceramide induced lipid raft clustering has been observed in several cell types including vascular endothelial cells and neutrophils (Megha and London, 2004, Liu and Anderson, 1995). It has been reported that the formation of ceramide enriched platforms brings different protein or receptor complexes together and will activate, facilitate or amplify signal transduction, pathogen recognition or cellular stimulation.

The contribution of lipid rafts to the efficient activation of NADPH oxidase has been investigated in neutrophils in several studies (Shao et al., 2003, David et al., 2005, Vilhardt and Deurs, 2004, Guichard et al., 2005). It was demonstrated that agonists such as TNF- $\alpha$ , fMLP, PMA, opsonised *S. aureus* or IL-8 (Guichard et al., 2005) induced intracellular signaling events may trigger the redistribution of the NADPH oxidase components into raft microdomains.

Agonist-induced aggregation of membrane bound gp91<sup>phox</sup> and translocation of cytosolic p47<sup>phox</sup> into lipid raft clusters, was shown to markedly increase the NADPH oxidase activity. It was hypothesised that lipid raft platforms with aggregated NADPH oxidase subunits form a number of lipid raft associated NADPH complexes, which results in increased production of O<sub>2</sub><sup>-</sup> by endothelial cells (Li et al., 2007). More recent studies have revealed that ceramide enriched platforms importantly participate in redox signaling of endothelial cells through NADPH oxidase. Aggregated oxidase complexes amplify the transmembrane signal, intracellular signaling events and functional responses of neutrophils. As demonstrated in Guichard and coworkers (Guichard et al., 2005), IL-8



induced the sequential preassembly and recruitment of NADPH oxidase components into lipid raft. This implies that lipid rafts have important regulatory functions in chemoattractant-mediated responses in neutrophils.

Activation of NADPH oxidase in response to IgG-opsonised *S. aureus* and fMLP is very rapid and occurs in seconds, suggesting a high efficiency of coupling of the receptors to the oxidase (Shao et al., 2003). In neutrophils FcγRIIIB (CD16), a GPI anchor protein, is the most abundant receptor for IgG-opsonised particles. It has been reported that FcγRIIIB together with FcγRIIA co-localise with lipid rafts. Although some controversies exist about the participation of LRs in the activation of NADPH oxidase in neutrophils through Fcγ receptors and the fMLP receptor (Katsumata et al., 2001), it is evident that Fcγ stimulation could enhance NADPH oxidase assembly and activation (Shao et al., 2003, David et al., 2005). It has been shown that IgG-opsonised *S. aureus* stimulation resulted in the recruitment of PKCδ to the lipid raft (Shao et al., 2003); PKC-δ regulates NADPH oxidase activation through p47<sup>phox</sup> phosphorylation. Previous studies using both neutrophils and HL60 cells, have demonstrated that NADPH oxidase subunits localise in cholesterol-rich lipid rafts (Vilhardt and Deurs, 2004). The involvement of lipid rafts in regulating neutrophil NADPH oxidase activity is suggested by using the raft disrupting agent methyl-β-cyclodextrin (MβCD). Based on cholesterol depletion studies with MβCD, it was suggested that association of cyt b<sub>558</sub> with lipid rafts was entirely cholesterol dependent (Vilhardt and Deurs, 2004).

Therefore, the aim of this chapter is to investigate whether the change of intracellular GSH:GSSG ratio has an effect on NADPH-oxidase activity measured by ability to produce O<sub>2</sub><sup>-</sup> or membrane assembly into lipid rafts.

## **6.3 Materials and methods**

### **6.3.1 Modulation of cellular thiol level**

HL60 cells were differentiated in 1% DMSO for five days. On the third (for 48 hours) or fourth (for 24 hours) day of differentiation cells were treated with 10 $\mu$ M buthione sulfoximine (10 $\mu$ M BSO/2 $\times$ 10<sup>5</sup> cells). To investigate whether increasing intracellular thiols could exert any effect on the BSO-modulated respiratory burst, BSO-treated cells were preincubated with the antioxidant, N-acetylcysteine (NAC). NAC was made up in serum free RPMI 1640 medium. dHL60 cells in the presence or absence of 10 $\mu$ M BSO (24h), were treated with 5mM NAC for 30min at 37<sup>0</sup>C. Cells were washed three times with PBS to remove extracellular NAC and resuspended in PBS. Chemiluminescence assay was carried out as described in general methods (chapter 2.2.4) with 10nM PMA or 1 $\mu$ M fMLP.

### **6.3.2 Preparation of cell lysate for GSH and GSSG assay**

Cells were analysed on the fifth day of differentiation. Differentiated control and BSO treated cells (5 $\times$ 10<sup>6</sup>) were centrifuged in 1.5ml eppendorf tube in a microcentrifuge for 6600  $\times$  g for 1.5 min. Supernatants were removed and the cell pellet was washed two times with 500 $\mu$ l of PBS by centrifugation at 6600  $\times$  g for 1.5 min. Supernatant PBS was removed as much as possible and the pellet was air dried for 5min. Sulfosalisilic acid (SSA; 3.33 $\mu$ l of 100% made up in distilled water) was then added to the cell pellet and, vortexed. The tubes were immediately centrifuged at 6600  $\times$ g for 1.5min. Stock buffer (96.6 $\mu$ l of 125mM sodium phosphate, 6.3mM disodium EDTA, pH 7.5) was then added

to each tube, vortexed and recentrifuged as above. Supernatants were collected into fresh tubes and analysed for GSH, GSSG or total protein (BCA assay) on the same day or immediately stored at  $-80^{\circ}\text{C}$  to use within one month.

### 6.3.3 GSH Assay

The total GSH levels (t-GSH) were assessed by the GSR-DTNB (glutathione reductase and 5,5 dithiobis-2-nitrobenzoic acid) recycling procedure, as described in previous studies (Gherghel et al., 2005). A standard curve was prepared from 0 to 2 nmol/well in 0.5-nanomole/well increments using 100mM GSH stock solution (0.2– 0.8  $\mu\text{l}$ ) as shown in Table 6.1.

Concentration ( $\mu\text{M}$ )	nmol/well	GSH ( $\mu\text{l}$ )	SSA ( $\mu\text{l}$ )	Water ( $\mu\text{l}$ )
0	0.0	0.0	33.3	966
20	0.5	0.2	33.3	966
40	1.0	0.4	33.3	966
60	1.5	0.6	33.3	966
80	2.0	0.8	33.3	966

**Table 6.1: Combinations of reagents required for the determination of intracellular total GSH level.** The final micromolar concentration ( $\mu\text{M}$ ) of GSH and its concentration per well (nmol/well) are listed in first two columns.



The standards contained the same final concentrations of SSA as for the samples (3.33%). To each well of a 96-well plate, 150µl of daily buffer (125mM Na<sub>2</sub>HPO<sub>4</sub>, 6.3mM Na<sub>2</sub>EDTA, pH 7.5), 50µl of DTNB solution (6mM DNTB in stock buffer), and 25µl of standards or samples were added in triplicate, and the plate was incubated at 37°C for 3 min. Finally, 25µl GSR (2U/ml) was added to the previous mixture and the plate was read at 410 nm using a 96-well plate reader (MRX microplate reader, Dynex).

#### 6.3.4 GSSG assay

The GSSG levels were assessed by the GSR-DTNB recycling assay. The GSSG standard curve was prepared from 0-0.25 nmol/well in 0.05nmol increments using 5mM GSSG stock solution (0.4-1.6µl) as detailed in table 6.2.

Concentration (µM)	nmol/well	GSSG (µl)	SSA (µl)	Water (µl)
0	0.0	0.0	33.3	966
2	0.05	0.4	33.3	966
4	0.10	0.8	33.3	966
6	0.15	1.2	33.3	966
8	0.20	1.6	33.3	966

**Table 6.2: Combination of reagents required for the determination of cellular oxidised GSH (GSSG) level.** The final micromolar concentration (µM) of GSSG and its concentration per well (nmol/well) are listed in first two columns.

Standards and samples (100µl) were transferred into separate centrifuge tubes and 2µl of 2-vinylpyridine (2-VP) was added to each tube. Contents were mixed well by pipetting and 2µl of triethanolamine (TEA) was added to adjust the pH of standards/samples to ~pH 7.5. The 96-well plate was prepared and analysed as described for the GSH assay.

### **6.3.5 Lipid raft isolation**

The common methods used to detect the association of membrane proteins, including NADPH oxidase subunits with lipid rafts include sucrose density gradient separation and fluorescence microscopy techniques. Previous studies have used antibody-mediated co-patching approaches of cell surface fluorescent labelled antigens flotillin, caveolin or ganglioside GM1 with cholera toxin B to demonstrate the co-localization of NADPH oxidase subunits in raft environment.

#### **6.3.5.1 Preparation of cell lysates for density gradient centrifugation**

dHL60 cells ( $1 \times 10^7$ ), which had been subjected to different treatments, were harvested and washed twice in ice-cold PBS. Cells were then lysed in 1ml of ice-cold MNE buffer (150mM NaCl, 2mM EDTA, 25mM MES, pH6.5) containing 1% Triton-100 on ice for 30min. Cell lysates were sheared by passing them 5 times through a 21G needle and final lysates were obtained by centrifuging at  $16,000 \times g$  in a microcentrifuge at 4°C for 5min to remove the nuclei and unsolubilized materials.

### **6.3.5.2 Sucrose gradient**

A 5-42.5% (w/v) sucrose density gradient was made in Beckman 14×89 mm Ultra-Clear™ tubes (Beckman Instruments, Inc., California, USA). The clear supernatant from HL60 cell lysates (1ml) was mixed 1:1 with 85% sucrose solution, and the total volume of 2ml was layered in the bottom of the tube followed by carefully layered 6mls of 30% and 3.5mls of 5% sucrose solution respectively to make a non-continuous sucrose gradient. The tube was then centrifuged at 200,000×g for 16 hours at 4°C in a SW41Ti Rotor (Beckman Coulter Optima L series, Palo Alto, California). Nine fractions (1ml) were collected sequentially from the top of the tube.

### **6.3.5.3 Protein precipitation and sample preparation**

Proteins in the 1ml fractions were precipitated with the addition of 100µl of 100% (w/v) trichloro acetic acid (TCA). As TCA is denser than the sample, slow, drop wise addition with occasional agitation was required to ensure its proper mixing with the sample. Samples in 10% TCA were then incubated on ice for 15 minutes. These samples were centrifuged at 14000×g for 5 minutes at 4°C. Excess TCA was washed off twice in ice-cold acetone. Precipitated protein was centrifuged at 14,000×g for 5 minutes at 4°C. Proteins were then dissolved in 135µl sample dissolving buffer (1X Laemmli buffer, 4M Urea, 0.2% ABF-14, 20% DMSO, where Laemmli buffer contains 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125M Tris-HCl, pH 6.8). Samples were heated 5min at 95°C before storage at -20°C.



#### **6.3.5.4 SDS-PAGE and western blot analysis of proteins in lipid rafts**

Equal volumes (15µl) of density gradient fractions from lysates per lane were resolved on a 12.5% SDS-PAGE gel as described in general methods. After transfer, PVDF membranes were blocked with 3% BSA and probed with gp91 antibody (1:1000 dilution) in 1% BSA, TTBS blocking buffer overnight at 4°C or with flotillin-1 antibody (1:1000 dilution) in 1% BSA, TTBS for 2 h at room temperature. After washing 15min × 4 times with TTBS, the membrane was probed with horse-radish peroxidase (HRP)-conjugated secondary antibody and protein was detected with an ECL reagent (Amersham).

#### **6.3.6 dHL60 cell treatment with *N, N*-biotinyl glutathione disulfide**

Intracellular protein glutathiolation sites were detected using the method described by P. Eaton and co-workers (Brennan et al., 2006). *N, N*-biotinyl glutathione disulfide (Biotin-GSSG) solution (50mM) was kindly gifted by Dr. P. Eaton (Kings College, London, UK). dHL60 cells were treated with 5mM biotin-GSSG or an equal volume of water (control) for 30 min. Following treatment cells were pelleted at 500 × g for 5 min, and the supernatant was aspirated. Cells were resuspended in PBS and reconstituted in 1X Laemmli buffer ( 63mM Tris HCl, 2mM NaP2O7, 5mM EDTA, 10% v/v glycerol, 2% SDS, 0.007% Bromophenol Blue, and 100 mM maleimide).

#### **6.3.7 SDS PAGE and western blot analysis of biotinylated proteins**

Biotin-GSSG labelled cell lysates (10 µl) in SDS sample buffer without a reducing agent were resolved by 10% SDS-PAGE as described in general methods. All SDS-PAGE gels were analyzed in duplicate with one set of samples supplemented with 10% 2-

mercaptomethanol to confirm that any *S*-glutathiolation signal detected was reversible. After electrophoresis samples were transferred to PVDF membranes. The membrane was blocked in 5% milk overnight and washed 15min, 4 times in TTBS. Western blots were incubated with streptavidin-HRP followed by ECL reagent (Amersham Biosciences).

### **6.3.8 p47 phox immunoprecipitation (IP)**

#### **6.3.8.1 Preparation of cell lysate for IP**

The dHL60 cells ( $1.0 \times 10^7$  cells) were harvested and washed twice in PBS to remove media. The cells were lysed in 200 $\mu$ l of RIPA buffer (150mM NaCl, 50mM Tris-HCl, pH 7.4 and 1% Triton X-100) containing freshly added 1% v/v protease inhibitor cocktail and incubated on rotating mixer for 30min at 4<sup>0</sup>C. The cell lysate was centrifuged at 12000 rpm for 20min at 4<sup>0</sup>C and supernatant was collected into fresh tube and stored at -80<sup>0</sup>C until use.

#### **6.3.8.2 Protein A-sepharose preparation**

Lyophilized protein A-sepharose was swelled by adding 4ml buffer A (20mM NaH<sub>2</sub>PO<sub>4</sub>, 150mM NaCl, pH 8) to 400mg powder and mixed on a rotating mixer for 1hr at room temperature. The sepharose beads were pelleted by centrifugation at 10000rpm for 2min at room temperature and supernatant was carefully removed. 4ml of 1% BSA in buffer A was added and mixed on a rotating mixer for 1hr at room temperature. Sepharose beads were pelleted by centrifugation (10000rpm, 2min) and washed twice in buffer A. Sepharose beads were resuspended in RIPA buffer to give 50% slurry (1.6ml buffer to 400mg initial powder). For long term storage, the supernatant was removed from beads and

they were resuspended in 5ml of ethanol in phosphate buffer (20mM NaH<sub>2</sub>PO<sub>4</sub>, 150mM NaCl, pH 8.0).

#### **6.3.8.3 Immunoprecipitation**

The total protein in each lysate was measured by the BCA assay as described in general methods (2.2.6). The cell lysate was diluted to 1µg/µl total cell protein in a microcentrifuge tube with PBS. 5µl of anti-p47-phox was added to 500µg of cell lysate. The reaction mixture was gently rocked at 4<sup>0</sup>C overnight. The immunocomplex was captured by adding 100µl of washed protein-A sepharose bead slurry. The reaction mixture was gently rocked at 4<sup>0</sup>C for 2 hours. The sepharose beads were collected by pulsing (5 seconds in the microcentrifuge at 14,000g) and the supernatant was drained off. The beads were washed three times with ice-cold PBS. The sepharose beads were then resuspended in 60µl 2X Laemmli sample buffer containing 100mM maleimide. The beads were boiled for 5 min and collected by centrifugation (5 seconds in the microcentrifuge at 14,000g). The supernatant was subjected to SDS-PAGE and western blot analysis.

#### **6.3.8.4 SDS PAGE and western blot analysis of p47phox**

Protein samples (20µl) were resolved by electrophoresis using 10% SDS-PAGE as described in general methods (2.2.8). After transfer to PVDF membranes were blocked with TTBS for 4hr and incubated with rabbit anti p47phox (1:50,000) antibody in TTBS, overnight. The membrane was washed for 15min× 8times with TTBS and incubated with anti rabbit goat secondary antibody (1:80,000) for 2 hours. The membrane was washed again 15min× 8 times before developing using ECL reagent.



#### **6.3.8.5 Coomassie staining**

SDS-PAGE gels were stained in coomassie blue staining solution (0.05% (w/v) coomassie brilliant blue R-250 was dissolved in 50% (v/v) methanol; 10% (v/v) acetic acid; and 40% distilled water) for 30min in a rotator. Gels were then destained in destaining solution (5% methanol, 7% acetic acid in 88% distilled water) at least 3 times, for one hour for each step, to remove excess background stain.

#### **6.3.9 In-gel protein digestion for Mass Spectrometry**

In order to identify predominant protein separated by immunoprecipitation, protein bands were digested within the gels. Electrophoresed protein bands separated on an SDS-Tris-Glycine gel were stained with coomassie blue and destained as described previously. Protein bands were then carefully cut with the aid of a clean razor blade, eliminating as much polyacrylamide as possible and placed in safe locked, PCR clean 1.5ml microcentrifuge tube (Eppendorf AG, Germany) that was prewashed twice with 50% acetonitrile/0.1% trifluoroacetic acid (TFA). The gel slices were destained twice with 0.2ml of 100mM  $\text{NH}_4\text{HCO}_3$ /50% acetonitrile for 45 minutes each treatment, at 37°C to remove Coomassie blue stain. The gel slices were then dehydrated for 5 minutes at room temperature in 100 $\mu\text{l}$  of 100% acetonitrile. At this point the gel slices were much smaller than their original size and were whitish or opaque in appearance. The gel slices were dried in a Speed Vac® concentrator for 10–15 minutes at room temperature to remove the acetonitrile. The gel slices were preincubated in a minimal volume (10–20 $\mu\text{l}$ ) of the trypsin solution (Trypsin Gold was resuspended at 1 $\mu\text{g}/\mu\text{l}$  in 50mM acetic acid, then diluted in 40mM  $\text{NH}_4\text{HCO}_3$ /10% acetonitrile to 20 $\mu\text{g}/\text{ml}$ ) at room temperature (not

exceeded 30°C) for 1 hour. The slices rehydrate during this time. An additional 10–20µl of trypsin was added as required and incubated for another hour at room temperature. Enough digestion buffer (40mM  $\text{NH}_4\text{HCO}_3$ /10% acetonitrile) was added to cover the gel slices and tightly capped tubes were incubated at 37°C overnight. The gel slices were then diluted with 150µl of MilliQ water for 10 minutes, with frequent vortex mixing. The liquid was removed and transferred to a new microcentrifuge tube. The gel slice digests were extracted twice, with 50µl of 50% acetonitrile/5% TFA (with mixing) for 60 minutes each time, at room temperature, all the extracts were pooled including washes with milliQ water. Extracts were then dried in a Speed Vac® concentrator at room temperature for 2–4 hours (do not exceed 30°C). The precipitate was dissolved in 100µl of 0.1% formic acid.

### 6.3.10 LC- MS/MS analysis of isolated proteins

A LC system (Finnigan, Thermoscientific, UK) consisting of a flow control valve, a vacuum degasser, a pump and an autosampler was used to deliver a mobile phase (solvent A: water with 0.1% formic acid, solvent B: acetonitrile, with 0.1% formic acid) at a flow-rate of 100 $\mu$ l/min. The chromatographic separations were achieved on a Biobasics C18 column (100 mm  $\times$  0.075mm; Integrafrit, Presearch, UK). The samples (5 $\mu$ l) were injected onto the LC–MS/MS system through an autosampler. This flow was split to achieve an elution flow rate approximately 300nl/min off the reverse phase column. The gradient was as follows: 0-5min, loading column; 5-44min 5%-40% buffer B; 44-45min, 40%-70% buffer B; 45-50min, 70% buffer B; 50-51min, 70%-0% buffer B; 51-65min 0% buffer B.

The mass spectra were obtained using a Finnigan LXQ linear ion trap mass spectrometer (Thermoscientific, UK) equipped with a nano-electrospray ionisation (ESI) source operating in positive ionisation (PI) mode. The data system was the Xcalibur<sup>®</sup> software version 2.0 SR2 (Thermoscientific, UK). The instrument was set to acquire m/z from 465 to 1600Da and analyse top three peaks for zoom scan and proceeded to fragmentation by collision induced dissociation (CID). MS/MS sequence identifications were obtained using the Bioworks software version 3.3. Those sequences with P value <0.05 were considered high-scoring identifications and sequence similarity compared against bovine and human databases using FASTA program.



### **6.3.11 Immunofluorescence**

#### **6.3.11.1 Cell fixation**

dHL60 cells were harvested and washed twice in cold PBS supplemented with 0.1% BSA by centrifugation at 400g for 5min. Cells were then resuspended at  $2 \times 10^6$  cells/ml in PBS with 0.1% BSA. Cell suspension (20 $\mu$ l) was placed onto the glass superfrost plus slide (Fisher Scientific) and spread into a thin smear using a glass coverslip. The cell smear was allowed to air dry for 10min and fixed by adding 50 $\mu$ l of ice cold 4% paraformaldehyde. The slides were incubated for 20min at 4°C, and then washed three times with PBS with 0.1% BSA to remove excess paraformaldehyde. The slides were then blocked in 5% milk in PBS for 10min at 37°C and washed three times with PBS supplemented with 0.1% BSA.

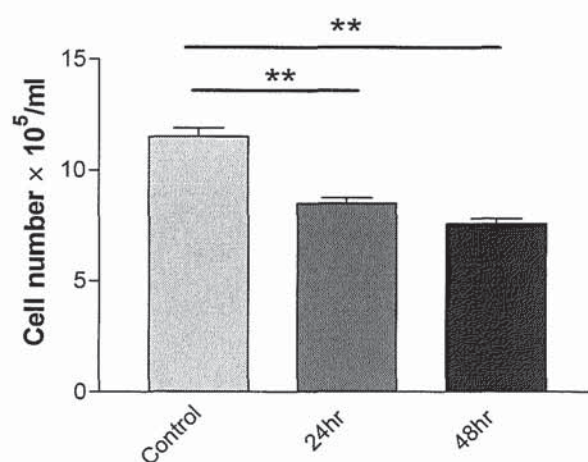
#### **6.3.11.2 Cell staining**

GM1 gangliosides, which are enriched in lipid rafts, were stained with FITC-labeled cholera toxin  $\beta$  subunit (CTX; 1 $\mu$ g/ml, 15min at room temperature, Sigma). dHL60 cells were washed in PBS three times. 20 $\mu$ l of Hoechst 33342 solution (1mg/ml in DMSO) was added to each slide, then incubated at room temperature for 30 min. Cells were then washed three times with PBS to remove excess stain. Cells were mounted on a glass slide with VECTASHIELD mounting media (Vector Laboratories) and staining was visualized using a conventional Zeiss fluorescence microscope.

## 6.4 Results

### 6.4.1 The effect of GSH synthetase inhibitor, buthionine sulfoximine, on dHL60 cell viability

To determine whether the intracellular GSH level is directly involved in cell viability, dHL60 cells were pre-treated with 10 $\mu$ M of BSO, a selective inhibitor of glutamylcysteine synthetase for 24 or 48hrs. dHL60 cell number was lower after incubation with BSO for 24 hrs ( $8.5 \times 10^5 \pm 0.26$ ; Mean $\pm$ SEM) as measured by a Trypan blue exclusion method (Figure 6.1). Cell number was further decreased when incubated with BSO for 48hrs ( $7.6 \times 10^5 \pm 0.24$ ) but the different between 24hr and 48 hr incubation was not significant.

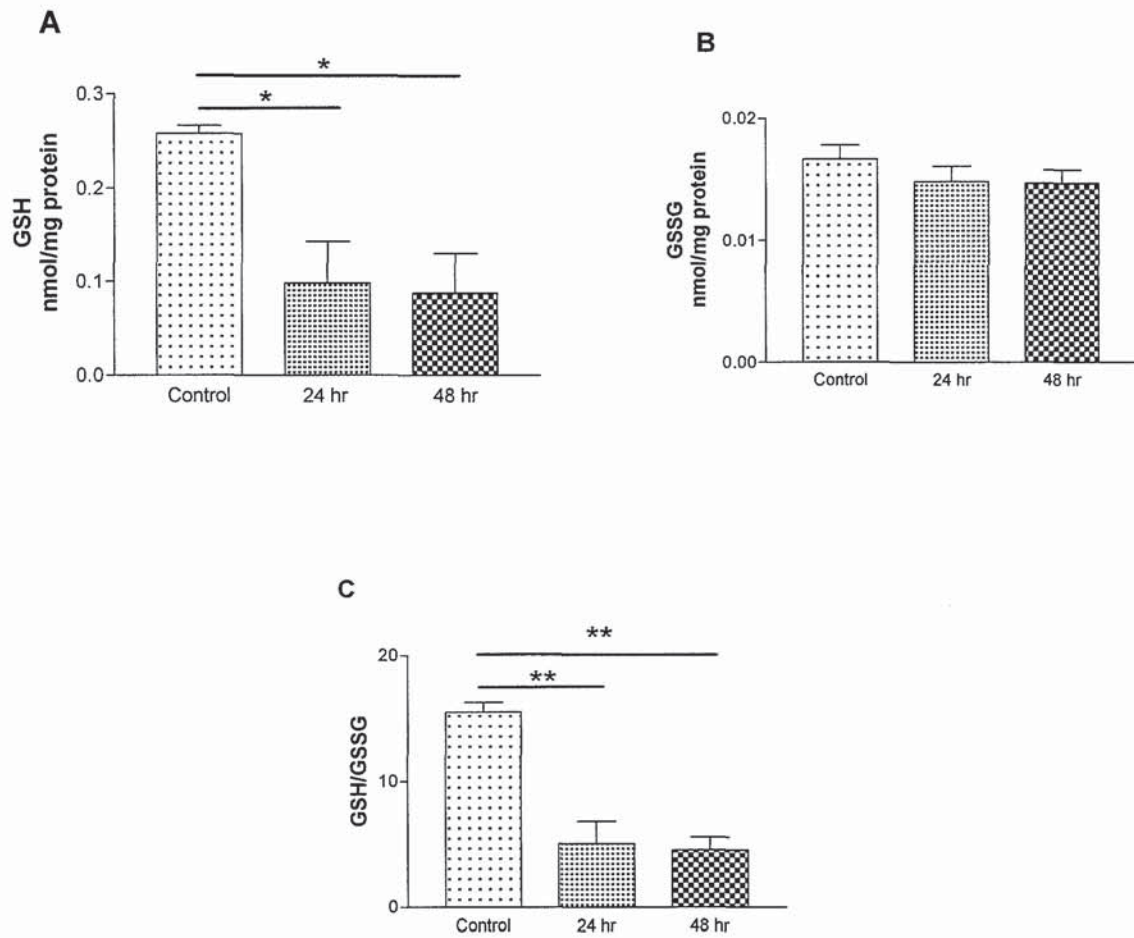


**Figure 6.1: BSO pre-incubation decreases dHL60 cell viability.** Cells (15 $\mu$ l) were transferred into an eppendorf tube with an equal volume of Trypan blue. Contents were mixed and transferred onto a haemocytometer for cell counting. Significant differences were calculated (\*\*P<0.001) with Anova analysis followed by Tukey's multiple-comparison test. Data is representative of three independent experiments.

#### 6.4.2 Effects of BSO on dHL60 total GSH level

As shown in figure 6.2, when the cells were incubated with BSO, the intracellular GSH concentration significantly decreased depending on the length of the incubation with the inhibitor ( $P < 0.01$ ). However, BSO treatment for 24 hrs ( $0.1 \pm 0.07$  nmol/total protein; mean  $\pm$  SEM) did not differ significantly to 48hr incubation ( $0.09 \pm 0.06$  nmol/total protein). dHL60 GSSG levels in BSO 24 hr were  $0.015 \pm 0.002$  and after 48hr were  $0.015 \pm 0.002$ . The levels of GSSG in BSO-treated cells remained close to non-treated cells ( $0.017 \pm 0.002$ ). As a result, intracellular GSH/GSSG ratio showed a significant decrease after 24 hr treatment and also 48 hr treatment compared with non-treated cells ( $P < 0.01$ ).

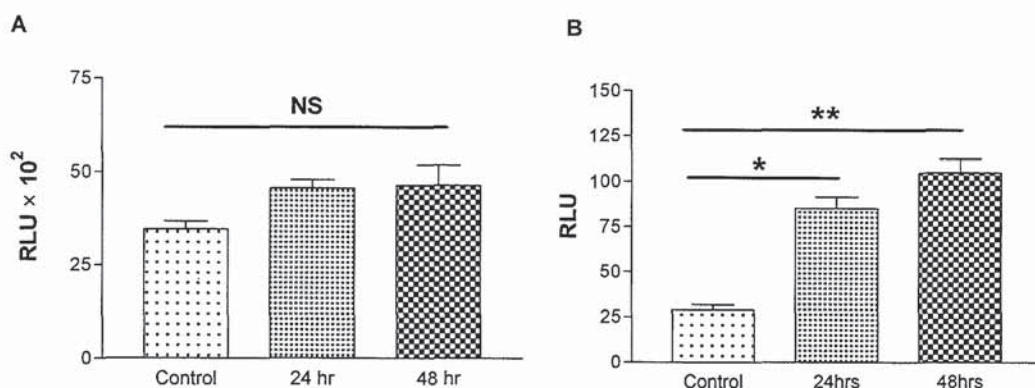




**Figure 6.2: BSO pre-incubation decreases GSH levels in dHL60 cells.** dHL60 cells ( $5 \times 10^6$ ) were harvested and washed twice in PBS by centrifugation at  $6600 \times g$  for 1.5 min.  $3.33 \mu\text{l}$  of 1% SSA was added to the dry pellet and the tubes were immediately centrifuged at  $6600 \times g$  for 1.5 min. Contents were dissolved in  $96.6 \mu\text{l}$  of stock buffer. Cell lysates were vortexed and centrifuged at  $6600 \times g$ . Supernatant was collected into a fresh tube and analysed for GSH and GSSG as described in methods. Significant differences were calculated (\*\* $P < 0.01$  and \*,  $P < 0.05$ ) with Tukey's multiple-comparison test.

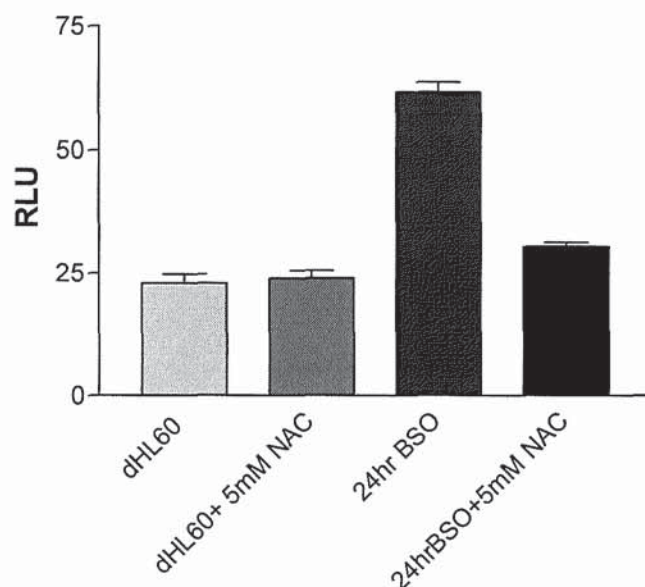
### 6.4.3 Effect of BSO on dHL60 respiratory burst

In order to investigate dHL60 cell respiratory burst in oxidatively stressed conditions, such as GSH depletion, cells were stimulated with either 10nM PMA (Figure 6.3A) or 1 $\mu$ M fMLP (Figure 6.3B). Both stimuli enhanced the lucigenin-sensitive respiratory burst in GSH-depleted dHL60 cells; while direct NADPH oxidase stimulation by PMA did not cause a significant increase in burst activity in either 24hr ( $43.42 \pm 2.2 \times 10^2$ ) or 48hr ( $41.04 \pm 5.4 \times 10^2$ ) BSO pre-treated dHL60 cells compared to controls, a significant increase in fMLP stimulated respiratory burst as measured by CL was observed after both 24 hr ( $85 \pm 6.5$ ) and 48hr ( $105 \pm 7.5$ ) BSO pre-treatment of dHL60 cells ( $P < 0.01$  and  $P < 0.001$  respectively).



**Figure 6.3: BSO pre-incubation increases dHL60 cell respiratory burst.** dHL60 cells were treated with 10 $\mu$ M BSO for 24 or 48 hrs. BSO pre-treated dHL60 cells ( $5 \times 10^5$ ) were washed two times with PBS to remove media and excess BSO. Cells were collected by centrifugation at  $1200 \times g$  for 5min. dHL60 cells and 100 $\mu$ M lucigenin was then added into pre-blocked white microwell plates and incubated at  $37^\circ\text{C}$  for 30min. Cells were then stimulated with 10nM PMA (A) 1 $\mu$ M fMLP (B) and CL recorded immediately for 20min. Mean peak (RLU  $\pm$  standard error of the mean) chemiluminescence generated by dHL60 cells was plotted. Data represent three independent experiments of three replicates. Significant differences were calculated (\*,  $P < 0.05$ ) with Tukey's multiple-comparison test. NS, not significant.

Chemical properties of the cysteinyl thiol of NAC include its redox activity, providing scavenger and antioxidant properties or thiol-disulfide exchange reactions with other thiol redox couples. In this study, the fMLP induced respiratory burst, which is enhanced in 24h BSO treated cells, was inhibited by NAC (Figure 6.4).

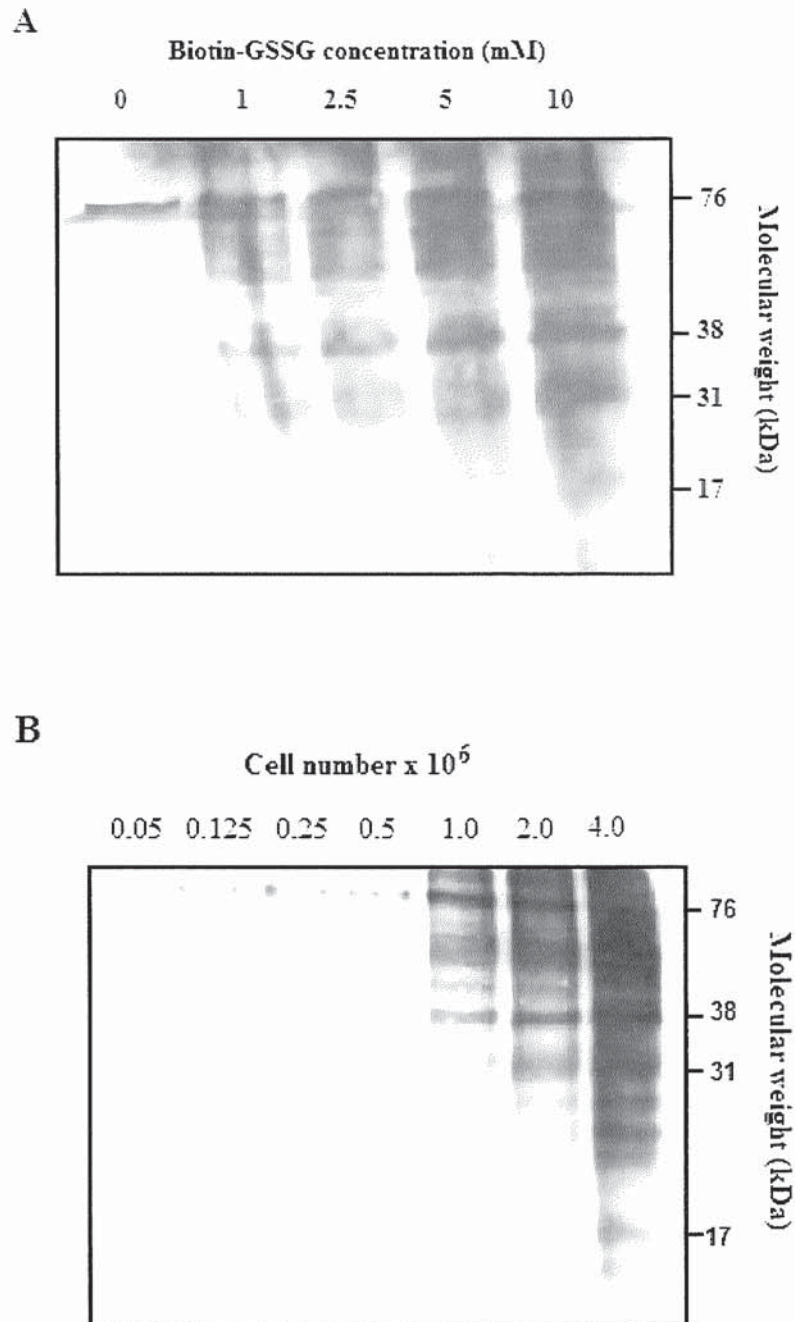


**Figure 6.4: The effect of N-acetylcysteine on dHL60 respiratory burst.** dHL60 cells were treated with 10 $\mu$ M BSO for 24h. dHL60 or BSO pre-treated cells ( $5 \times 10^5$  cells) were treated with 5mM NAC incubated at 37 $^{\circ}$ C for 30min. At the end of the incubation cells were washed three times in PBS. Chemiluminescence assay was performed as described in general methods (2.2.4) using 1 $\mu$ M fMLP as the stimulus. Data is representative of one experiment.



#### 6.4.4 Detection of S-glutathiolated proteins in dHL60 cells

Protein glutathiolation following oxidative stress in dHL60 cells was investigated by a method adapted from Eaton and co-workers (Brennan et al., 2006). Initial experiments were undertaken to establish optimal cell densities to visualise glutathiolation sites. Figure 6.5 shows western blots probed with streptavidin-HRP to detect *S*-glutathiolated proteins following biotin-GSSG treatment of dHL60 cells. Figure 6.5A shows the concentration-dependent *S*-glutathiolation of dHL60 proteins following treatment with a range of concentrations of biotin-GSSG (0–10 mM). According to the results, 5mM biotin-GSSG was selected for use in subsequent studies as this was the lowest concentration of the reagent to give standard labelling. Confirming previous observations, a prominent band was observed at 75 kDa regardless of biotin-GSSG treatments. As argued in Brennan et al 2006, this band may be used to represent equal loading of samples. Western blots probed with streptavidin-HRP detected biotin-GSSG labelled protein lysates from at least  $0.5 \times 10^6$  cells (Figure 5.3B). Cell numbers above  $0.5 \times 10^6$  resulted in a concentration dependent increase of the streptavidin reactive signal. On the basis of these results,  $2 \times 10^6$  cells were used in subsequent experiments.

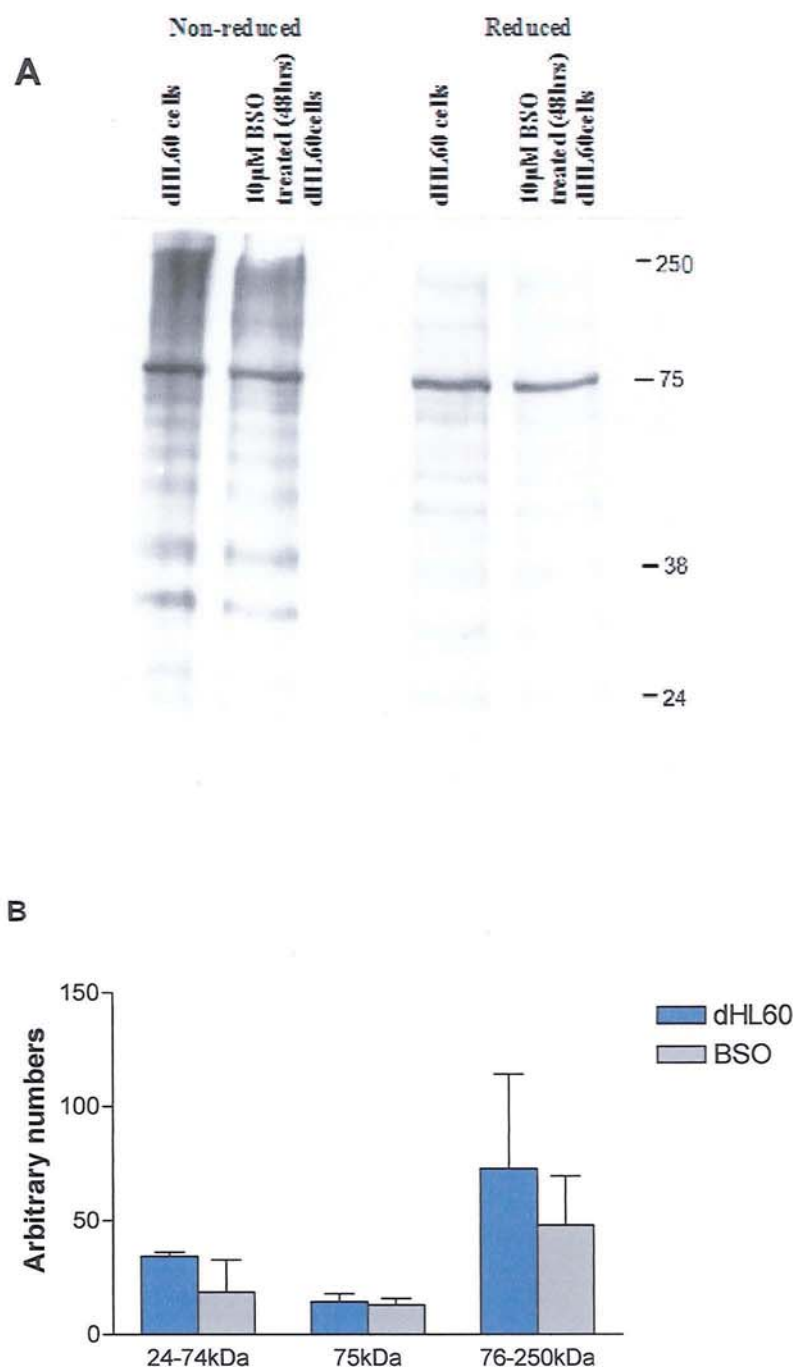


**Figure 6.5: Western blot probed with streptavidin-HRP to identify S-glutathiolated proteins in dHL60 cells following biotin-GSSG treatment.** dHL60 cells were washed two times in PBS. (A)  $2 \times 10^6$  Cells were treated with 0, 0.25, 1, 5 or 10mM biotin-GSSG. (B) 0.05, 0.125, 0.25, 0.5, 1, 2 or  $4 \times 10^6$  cells were treated with 5mM biotin-GSSG or an equal volume of water (control) and incubated at  $37^\circ\text{C}$  for 30 min. Following biotin-GSSG treatments cells were pelleted at  $500\times g$  for 5 min, and the supernatant was aspirated. Cells were resuspended in PBS and reconstituted in SDS sample buffer, free of reducing agents, containing 100 mM maleimide prior to analyse SDS-PAGE and western blotting using streptavidin peroxidase to detect biotin-glutathiolated proteins.

#### **6.4.5 Detection of S-glutathiolated proteins in BSO-treated dHL60 cells**

Under normal conditions and oxidatively stressed conditions, the extent of protein S-glutathiolation by biotin-GSSG treatment in dHL60 cells was investigated and results are displayed in figure 6.6. Control cells without BSO treatment showed a more intense banding pattern particularly proteins of high molecular weight as detected by biotin-GSSG labelling. When dHL60 cells were challenged by BSO for 48 hrs, the intensity of protein S-glutathiolation by biotin-GSSG treatment was decreased. This was confirmed by densitometric analysis (Figure 6.6B). Biotin-GSSG labelling was reversible when treated with 10%  $\beta$ -mercaptoethanol except for a prominent band at 75kDa that may represent an endogenously expressed, streptavidin-reactive protein, which may be used as an internal marker for equal loading.

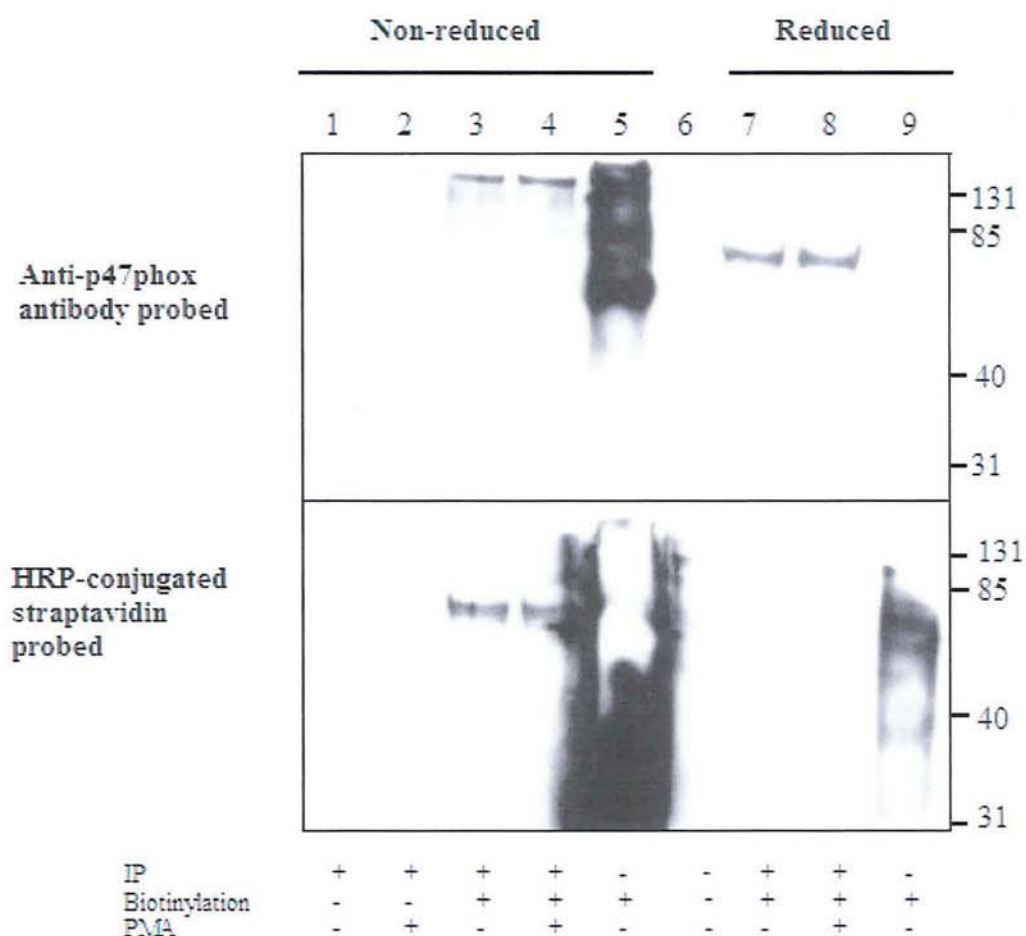




**Figure 6.6 Representative western blot of proteins from dHL60 cells labelled with biotin-GSSG either with or without BSO pre-treatment.** (A) Differentiating HL60 cells were treated with 10  $\mu$ M BSO 48hrs.  $2 \times 10^5$  cells were washed two times in PBS and incubated in 5mM biotin-GSSG for 30min. End of the incubation cells were collected and proteins were extracted as described in material and methods. Half of the volume in each sample was treated with 10%  $\beta$ -Mercaptoethanol. Reduced or non-reduced proteins were resolved in non-reducing 10% polyacrylamide gel. Proteins were transferred onto PVDF membrane and the membrane developed as described before. (B) The blot is representative of one of the two independent experiments. The density of protein bands were quantified from the western blot signals using the Quantity One 1-D analysis software program (BioRad laboratories, California, USA).

#### 6.4.6 Immunoprecipitation of p47<sup>phox</sup>

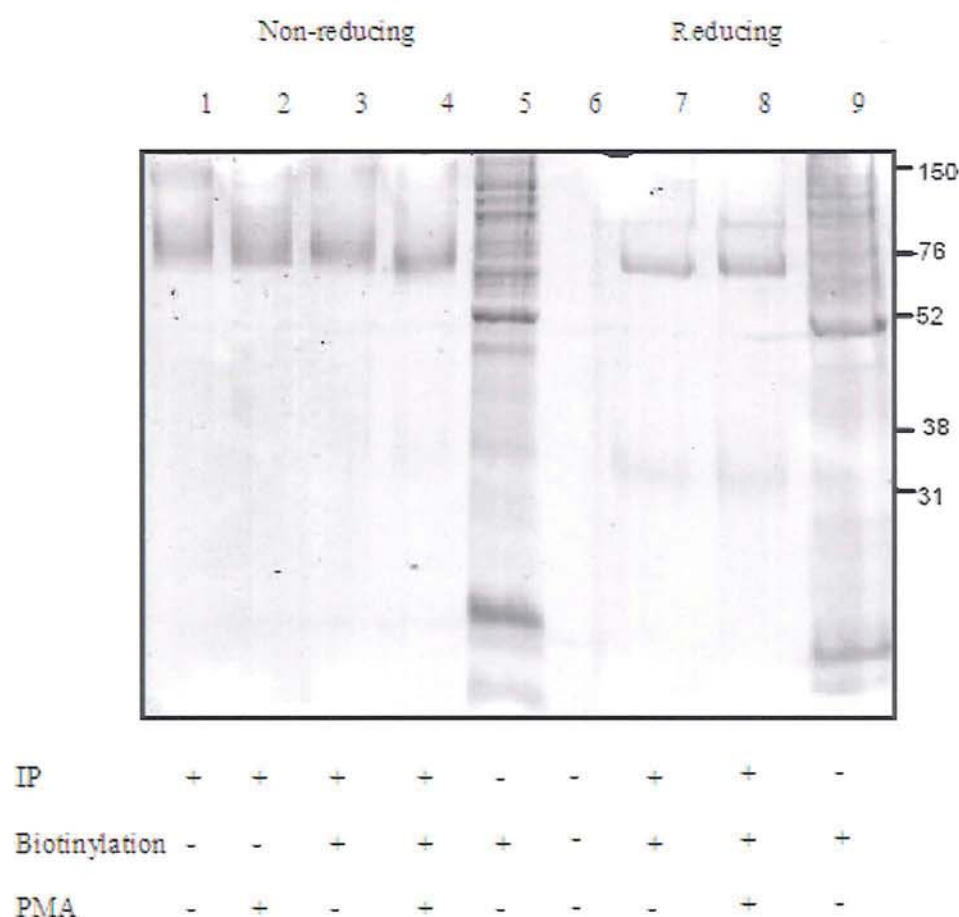
A large body of evidence suggests that p47<sup>phox</sup> phosphorylation is a key step for NADPH oxidase assembly and activation. Additionally, several oxidising or alkylating thiol reagents are found to modulate oxidase activity. The possibility that glutathiolation of the NADPH subunits particularly p47<sup>phox</sup> by oxidative stress generated during the respiratory burst was investigated. The effect of PMA stimulation on p47<sup>phox</sup> glutathiolation pattern was tested by stimulating cells with 10nM PMA for 20min. Specific glutathiolated protein biotinylation of dHL60 cells was followed by p47<sup>phox</sup> immunoprecipitation. Proteins were separated in SDS-PAGE gels and probed for p47<sup>phox</sup> using monoclonal anti-p47<sup>phox</sup> antibody (upper panel) or HRP-conjugated streptavidin (lower panel). The anti-p47<sup>phox</sup> antibody detected two high molecular weight (150kD) bands after affinity purification of biotinylated proteins and IP of p47<sup>phox</sup>; protein bands corresponding to 47kDa were absent in the non-reducing gel (lane 3 and 4 upper panel). However, after treating the biotinylated, IP protein extracts with  $\beta$ -mercaptoethanol, 65kDa bands were detected with anti-p47<sup>phox</sup> antibody (lane 7 and 8 upper panel). In both reducing and non reducing conditions, PMA stimulation did not considerably change the content of p47<sup>phox</sup> (lane 3 vs 4 and lane 7 vs 8). In non-reducing conditions, non-specific background staining from non-IP lysates was also visible for the whole cell lysate (lane 5 upper panel) but was absent in reducing conditions (lane 9 upper panel). After probing of blots with HRP-conjugated streptavidin (lower panel), two prominent bands were detected corresponding to 65kDa in non-reducing conditions (lane 3 and 4). After treating with  $\beta$ -mercaptoethanol, these two signals were not detected (lane 7 and 8). A strong signal for the whole cell lysate was detected before reducing with  $\beta$ -mercaptoethanol (lane 5). The weak signal in reducing conditions (lane 9) indicates reversibility of protein biotinylation.



**Figure 6.7: Representative western blot p47<sup>phox</sup> glutathiolation by biotin-GSSG in the presence or absence of PMA stimulation.** dHL60 cells ( $1.0 \times 10^7/\text{ml}$ ) were treated with 5mM biotin GSSG for 1 hour. In the last 20min of incubation, cells were stimulated with 10nM PMA. Cells were then harvested and washed twice in PBS to remove media. Cell lysates were prepared in RIPA buffer and immunoprecipitated for p47<sup>phox</sup> as described in materials and methods. Equal protein contents as determined by RCDC assay were loaded into each lane of 10% SDS-PAGE gel in the presence or absence of reducing agent (10%  $\beta$ -mercaptoethanol). Proteins were transferred into PVDF membrane and western blots were probed with anti-p47<sup>phox</sup> antibody (upper panel) or HRP conjugated straptavidin (lower panel). This is a representative blot from two independent experiments.



In order to identify specific protein bands, the same samples were resolved in SDS-PAGE and visualised by coomassie staining as shown in figure 6.8. Protein bands in non-reducing gels were detected at a molecular weight of around 65kDa and a high molecular weight around 131kDa (lanes 1-4). Whole cell lysates were loaded into lane 5 and lane 9. Under reducing conditions, protein bands were visualised with a molecular weight around 65kDa and lower molecular weight bands were detected around 32kDa. Major protein bands (65kDa) were visualised in both non-reducing and reducing sections of the gel (lanes 4 and lane 8 respectively); these were excised and protein digests were prepared for mass spectroscopy. Protein digests were prepared as described in materials and methods (6.3.9). The tryptic digests of proteins were separated by liquid chromatography and subject to mass analysis using a Thermo LXQ mass spectrometer. Peptide mass fingerprints were searched through the human and bovine databases. Both protein bands (65kDa) excised from lane 4 and 8 were identified as bovine serum albumin (accession number 30794280) after searching for protein sequence similarity against bovine and human databases using the FASTA program (table 6.3).



**Figure 6.8: Coomassie staining of immunoprecipitated proteins after SDS-PAGE gel.** dHL60 cells ( $1.0 \times 10^7/\text{ml}$ ) were treated with 5mM biotin GSSG for 1 hour. In the last 20min of incubation, cells were stimulated with 10nM PMA. Cells were then harvested and washed twice in PBS to remove media. Cell lysates were prepared in RIPA buffer and immunoprecipitated for p47<sup>phox</sup> as described in materials and methods. Equal protein contents as detected by RCDC assay were loaded into each lane of 10% SDS-PAGE gel.

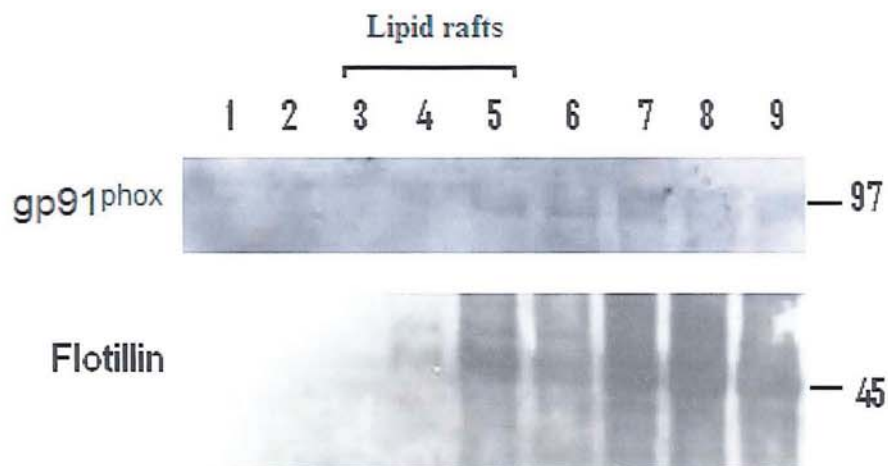
Lane 4 protein coverage		Lane 8 protein coverage	
Sequence	Amino acid residues in BSA	Sequence	Amino acid residues in BSA
LVNELTEFAK	66-75	LVNELTEFAK	66-75
QEPERNECFLSHKDDSPDLPK	118-138	KFWGK	156-160
NECFLSHKDDSPDLPK	123-138	YLYEIAR	161-167
YLYEIAR	161-167	GACLLPK	198-204
YNGVFQECCQAEDK	184-197	LVTDLTK	257-263
GACLLPK	198-204	ECCHGDLLECADDRADLAK	267-285
ALKAWSVAR	233-241	SHCIAEVEKDAIPENLPPLTADFAEDKD	310-340
AEFVEVTK	249-256	DDPHACYSTVFDK	387-399
LVTDLTK	257-263	LGEYGFQNALIVR	421-433
ECCHGDLLECADDR	267-280	KVPQVSTPTLVEVSR	437-451
ECCHGDLLECADDRADLAK	267-285	MPCTEDYLSLILNR	469-482
LKECCDKP LLEK	298-309	CCTESLVNR	499-507
EYEATLEECCA K	375-386	RPCFSALTPDETYVPK	508-523
HLVDEPQNLIK	402-412	AFDEKLFTFHADICTLPDTEK	524-544
LGEYGFQNALIVRYTRK	421-437	LFTFHADICTLPDTEK	529-544
KVPQVSTPTLVEVSR	437-451	QTALVELLK	549-557
VPQVSTPTLVEVSR	438-451	CCAADDKEACFAVEGPK	581-597
CCTKPESER	460-468	LVVSTQTALA	598-607
CCTESLVNR	499-507		
RPCFSALTPDETYVPK	508-523		
RPCFSALTPDETYVPKAFDEK	503-528		
CCAADDKEACFAVEGPK	581-597		
LVVSTQTALA	598-607		
N=23 peptides, 38.55% sequence coverage		N=18 peptides, 36.74% sequence coverage	

**Table 6.3: Amino acid sequences overlapped with bovine database as detected by MS/MS.**



#### 6.4.6 Lipid raft distribution on dHL60 cells

Lipid rafts were isolated from cold Triton X-100 lysates of dHL60 cells by sucrose gradient ultracentrifugation at 4°C. Fractions collected from the top of the gradients were analysed by western blots for the presence of gp91<sup>phox</sup> and of the raft marker flotillin-1 (Figure 6.9). The NADPH oxidase subunit gp91<sup>phox</sup> was detected in both low density fractions (fractions 3-5) and high density fractions indicating their distribution in both membranes and cytosol. Although lipid rafts were expected to separate in to low density fractions (fractions 3-5), the high background in the blot masked visualisation of flotillin distribution in the western-blot. Further experiments were undertaken to optimise western blotting conditions for gp91<sup>phox</sup> and flotillin.



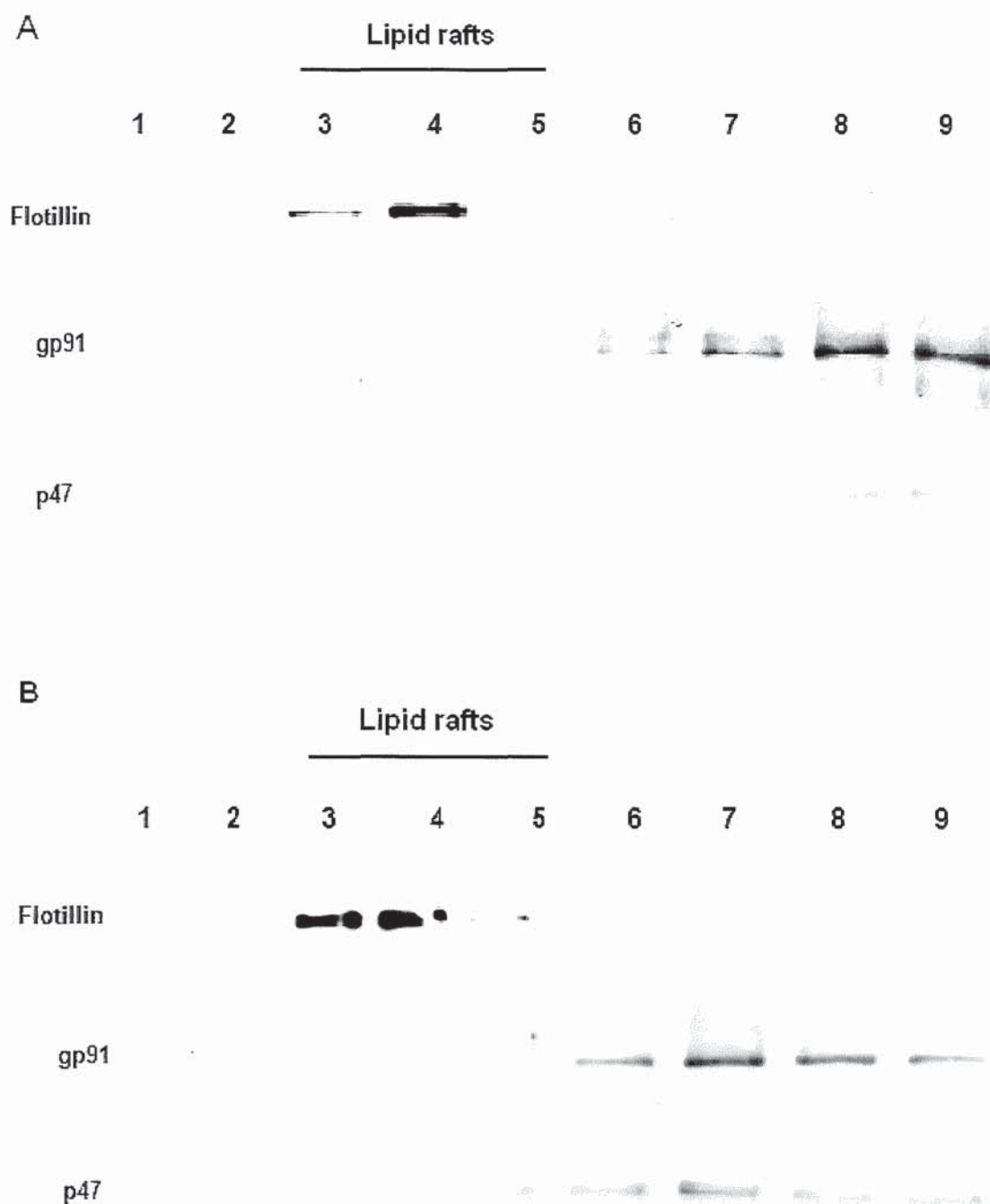
**Figure 6.9 Distribution of gp91<sup>phox</sup> and flotillin in rafts isolated from dHL60 cells.** dHL60 cells ( $1 \times 10^7$ ) were lysed in 1% Triton X-100 at 4°C, subjected to sucrose gradient 5%-40% ultracentrifugation and fractionated. Protein fractions were resolved by SDS-PAGE. Membranes were blocked in 5% milk in TTBS and immunoblots were probed with anti-gp91<sup>phox</sup> and anti-flotillin antibodies.

Priming of neutrophils has been previously reported to increase association of p47<sup>phox</sup> with lipid rafts (Jin et al., 2008, Guichard et al., 2005). To examine the involvement gp91<sup>phox</sup>, p47<sup>phox</sup> and flotillin distribution into raft fractions, dHL60 cells were treated with PMA, IL-8 with or without fMLP stimulation and BSO treatment (48hrs) followed by fMLP stimulation. Membrane fractionation experiments were performed to examine the effect of treatment on lipid rafts and NADPH oxidase subunit association, using a detergent extraction protocol with 1% Triton X-100. The protein content in each fraction was calculated by RCDC assay (table 6.4) and analysis the lysates prepared from BSO treatment and BSO+fMLP treatments showed any significant recovery. Therefore, proteins isolated from control, PMA, IL-8 and IL8+fMLP treatments were concentrated in a vacuum concentrator (concentrator 5301, eppendorf, Hamburg, Germany) prior to analysis. However, over the three independent experiments it was not possible to recover sufficient proteins, to detect the presence of flotillin, gp91<sup>phox</sup> or p47<sup>phox</sup> on any blot from each of these treatments. In BSO treated, unstimulated dHL60 cells (Figure 6.10A), gp91<sup>phox</sup> and p47<sup>phox</sup> are distributed in high density fractions (fractions 6-9). Flotillin-1, a protein highly enriched in lipid rafts, was detected in low density fractions (3-4). Experiments were subsequently performed to determine whether cellular oxidative stress together with stimulation affects the partitioning of gp91<sup>phox</sup> and p47<sup>phox</sup> within membrane fractions. When BSO challenged dHL60 cells stimulated with 1 $\mu$ M fMLP (Figure 6.10B), both gp91<sup>phox</sup> and p47<sup>phox</sup> proteins showed association with lower density fractions (from fractions 5-9) having overlap with flotillin found in lipid raft fractions (fraction 5). The most dense staining fractions for p47<sup>phox</sup> and gp91<sup>phox</sup> are 8-9 in figure 6.10A and fraction 7 in figure 6.10B.

Treatment	Protein concentration in each fraction (mg/ml)								
	1	2	3	4	5	6	7	8	9
Control	0.008	0.011	0.012	0.014	0.014	0.018	0.023	0.078	0.145
PMA	0.002	0.012	0.025	0.034	0.045	0.051	0.078	0.067	0.156
IL-8	0.001	0.012	0.011	0.018	0.015	0.012	0.047	0.045	0.098
IL-8+fMLP	0.006	0.009	0.010	0.014	0.017	0.015	0.034	0.098	0.167
BSO	0.115	0.118	0.241	0.245	0.256	0.261	0.362	0.789	0.981
BSO+fMLP	0.121	0.232	0.342	0.401	0.411	0.427	0.546	0.783	1.234

**Table 6.4 Protein concentrations determined in each fraction for different treatments.** Proteins in 1ml fractions were carefully removed from sucrose gradient were precipitated as described in materials and methods 6.3.5.2. Proteins were dissolved in 135 $\mu$ l sample dissolving buffer and 10 $\mu$ l was taken from each fraction for protein analysis by RCDC assay.

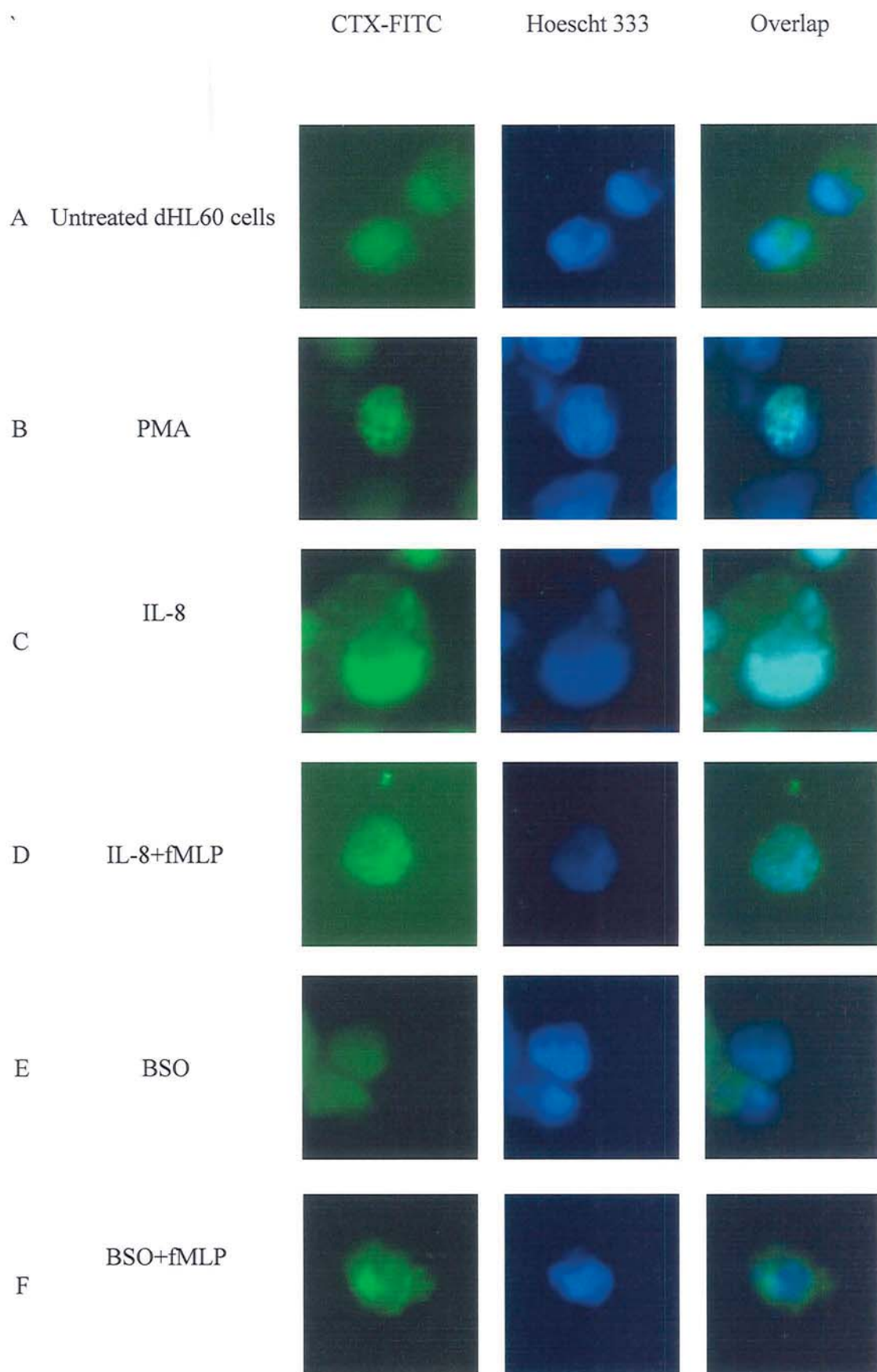




**Figure 6.10 Distribution of gp91phox and p47phox in detergent-extracted and fractionated membranes of BSO-treated dHL60 cells.** dHL60 cells treated with 10µM BSO for 48hrs. Unstimulated dHL60 cells (A) and 1µM fMLP stimulated (B) were extracted with 1% triton X-100 lysis buffer and applied to discontinuous sucrose gradients, as described in Materials and Methods. Proteins were extracted and immunoblot was analysed using indicated antibodies. Fractions 3 to 5 were designated as lipid rafts as indicated by the marker protein flotillin-1.

#### 6.4.7 Cell imaging

To examine whether different activating or oxidating treatments change the membrane lipid raft organisation to form redox-sensitive signaling platforms, treated dHL60 cells were labelled with FITC conjugated cholera toxin  $\beta$ -sub unit (FITC-CTX). Cellular nuclei were visualised using Hoechst 33342. Under normal conditions, untreated dHL60 cells exhibited evenly distributed lipid raft as detected by diffuse green fluorescence of FITC-CTX (Figure 6.11A). PMA stimulation increased discrete lipid raft formation as observed by increased green fluorescence patches with a more punctate pattern of staining (Figure 6.11B). After IL-8 stimulation, a slight increase in green patch formation is observed compared to control (Figure 6.11C). However, post-fMLP stimulation a more punctate pattern of staining of IL-8 primed cells is seen showing increased lipid raft formation indicated by the presence of intense green fluorescence patches and a lack of diffuse stain (Figure 5.8D). BSO treatment showed little effect on lipid raft formation (Figure 6.11E) and post-fMLP showed increased intensity of green patches and it is indicative of lipid raft re-distribution within the membrane (Figure 6.11F).





**Figure 6.11: Detection of agonist-induced CTX-FITC association indicative of GPI clustering in dHL60 membrane.** dHL60 cells were untreated (control) (A) or treated with 10nM PMA for 20min (B), 50ng/ml IL-8 for 15min (C) followed by 5min exposure to 1 $\mu$ M fMLP (D) or cells were treated with 10 $\mu$ M BSO (E) for 48hrs followed by 5min exposure to 1 $\mu$ M fMLP (F). Cells were washed in PBS two times and fixed in paraformaldehyde on a glass slide. Ganglioside GM1 was used as a raft marker and the nucleus was stained with Hoechst 33342. Microscopy was performed using an inverted microscope with fluorescence imaging at magnification of 63X (Zeiss Axiovert 200 M, Carl Zeiss Ltd. UK).

## 6.5 Discussion

Healthy cells generally maintain a reducing intracellular environment, relative to extracellular fluids. A main factor influencing the redox status of the cytosol is the concentration of glutathione (Hancock et al., 2003). It has been estimated that cultured cells maintained cytosolic GSH/GSSG redox potential of -260mV to -200mV compared with the more oxidised redox potential found in plasma, -140mV (Kemp et al., 2008). Data from *in vivo* studies show values in the range of -255 mV (liver) to -193 mV (red blood cells) (Kemp et al., 2008). Inflammation, ageing (Samiec et al., 1998) and also BSO treatments [+30mV at 100µM BSO; (Hansen et al., 2004)] are found to increase cytosolic GSH/GSSG-coupled redox potential. However, other redox couples such as thioredoxins and Cys/CySS are also important in determining the cellular redox environment. Therefore, it has been proposed that the redox environment can be represented by the equation; redox environment =  $\Sigma$  redox potential ( $E_h$ )  $\times$  [reduced species] (Hancock et al., 2004).

BSO has been widely used to induce GSH depletion due to its inhibitory effects on  $\gamma$ -glutamyl cysteine synthetase production, the rate limiting enzyme in GSH synthesis (Seçkin et al., 1997). BSO is preferred as a GSH depleting agent both in cell culture models (Di Stefano et al., 2006) and animal studies, since it does not appear to possess secondary toxicities specially in animal models (Seçkin et al., 1997). In pharmacology, BSO is used as a chemosensitising agent due to its ability to target tumor cells to apoptosis induced by standard chemotherapeutic agents.

Differentiated HL60 cells exposed to BSO for 24hrs showed a significantly depleted cellular glutathione pool. This level was not further altered upon prolonged (48hrs)

BSO exposure, whereas, the GSSG level was generally maintained in dHL60 cells with a decrease of 10% after treating with BSO for 24 or 48 hrs. This created a decrease in cellular GSH/GSSG level indicative of a more oxidised cellular environment. The DTNB-based glutathione recycling assay measures total (both GSH and GSSG) glutathione level. It has been postulated that the mechanism for total GSH depletion as measured in this assay could be due to export of oxidised glutathione into the extracellular space or formation of mixed disulfides with proteins (Phillips et al., 2002). This suggests that oxidative stress caused by BSO treatment in this study may have increased S-glutathiolation of the free protein thiols via disulphide exchange with GSSG.

An increased respiratory burst stimulated by PMA and fMLP was observed in glutathione depleted dHL60 cells. This increased respiratory burst was inhibited by the antioxidant, NAC when cells treated for 30min. This suggests that the increased production of ROS in BSO treated cells can be reversed by adding antioxidants such as NAC. Previous data presented in chapter 2 has shown that as a direct PKC stimulator, PMA generated a higher respiratory burst compared with fMLP stimulus. However, PMA did not produce significantly increased respiratory burst after BSO depletion compared with fMLP stimulus. It is widely believed that the signal transduction pathway in the activation of the NADPH oxidase by fMLP in neutrophils and dHL60 cells involves the G-protein coupled receptor activated phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to generate inositol 1,4,5-trisphosphate (IP<sub>3</sub>); a trigger for release of intracellular Ca<sup>2+</sup> stores, and diacylglycerol (DAG); an activator of DAG dependent PKC. Elevation of cytosolic Ca<sup>2+</sup> is essential for an optimal fMLP-induced respiratory burst (Brechard et al., 2009). Previous studies have shown that fMLP stimuli generate a rapid and transient (within 18 seconds of fMLP stimulus)



increase of cytoplasmic  $\text{Ca}^{2+}$  concentration (Korchak et al., 2001). Change of cellular calcium ion concentration is associated with rapid membrane polarisation and activation of NADPH oxidase (Rada et al., 2005). As a result, fMLP stimulates a rapid burst of ROS via NADPH oxidase. PMA directly stimulates PKC phosphorylation, which acts via a  $\text{Ca}^{2+}$  independent pathway to activate the NADPH oxidase complex. PKC also contains structural features that are susceptible to oxidative modification (Gopalakrishna and Jaken, 2000). When oxidised the autoinhibitory function of the regulatory domain of PKC is found to be compromised and PKC activity is stimulated (Gopalakrishna and Jaken, 2000). This may explain the trend for a higher respiratory burst observed in BSO-treated dHL60 cells with PMA stimulus.

In an environment where the cellular GSH/GSSG ratio is decreased, thiol sensitive proteins are more likely to become glutathiolated proteins (S-glutathiolation) by exchanging GSSG with free and accessible thiol groups in protein amino acids or to become oxidised to thiolate species. To investigate protein glutathiolation in dHL60 cells, a chemical derivatization method was adapted from Bernnan *et al.*, (2006). This method involved the use of biotinylated-GSSG, where two biotin groups have been coupled to the primary amino groups of GSSG. This approach of detection of s-glutathiolation sites has advantages over other types of immunochemical and radiolabel methods by its simplicity and ease of detection. Protein thiols that have been s-glutathiolated by GSSG-biotin will carry biotin tag with them and allow their detection via western blot methods, cellular localisation by fluorescence microscopy, and their purification using avidin-based techniques (Brennan et al., 2006). Using this method, live dHL60 cells were treated with biotin-GSSG, which crosses membranes into the cells and artificially creates an oxidative stressed environment. Cellular proteins with free accessible thiol groups in this environment exchange with biotin-GSSG disulfide

bonds to form biotin tagged S-glutathiolated proteins. dHL60 cells treated with BSO for 48hrs showed a reduced availability for biotin-GSSG-induced glutathiolation as detected by a less intense ECL signal (Figure 6.6). This indicates that BSO treatment reduced available glutathiolation sites for biotin-GSSG, possibly by increasing the protein thiol oxidation and these protein targets are broadly the same for biotin-GSSG. Therefore, BSO treatment prior to biotin-GSSG reduces the amount of proteins that become biotinylated. Brennan *et al.*, (2006) argue that this method of biotinylation is not suitable to identify protein glutathiolation in cellular stressed conditions due to low ECL signal detected for already glutathiolated proteins. In contrast, we have demonstrated the possibility of adopting this method to detect protein glutathiolation in BSO-treated dHL60 cells. Specific proteins that show loss of S-glutathiolation by biotin-GSSG after BSO treatment could be identified using follow up methods such as excising individual protein bands and analysing them by mass spectrometry after trypsin digestion.

Upregulation of the respiratory burst in response to a stimulus in oxidatively stressed dHL60 cells suggests that this environment favours NADPH oxidase activity. Since NADPH oxidase subunits are distributed between the cytosol, intracellular vesicle membranes and plasma membrane, the cytosolic components require an activation signal for phosphorylation and translocation to the membrane, which is may be followed by assembly into lipid rafts before generating superoxide radicals. Therefore the activity of NADPH oxidase could be regulated at one of the above mentioned steps. Cellular oxidative stress may either affect protein structure of susceptible individual NADPH oxidase subunits or may affect NADPH oxidase membrane assembly by altering lipid raft structure. Ageing is another example where cellular oxidative stress increases and there is altered redox balance (GSH:GSSG). Some studies on ageing have

shown that under oxidative stress, expression of major NADPH subunits p47<sup>phox</sup> and gp91<sup>phox</sup> are up-regulated (Li et al., 2005).

As the cytoplasmic NADPH oxidase subunit p47<sup>phox</sup>, has four cysteine residues (C-98, C-111, C-196, C-378) it may potentially be modified in the oxidised redox state of dHL60 cells in the presence of BSO, when the GSH pool is depleted. The human p47<sup>phox</sup> subunit has an approximate molecular weight of 47kDa. As a first step to address this hypothesis, dHL60 cells were stimulated with PMA, treated with biotin-GSSG and immunoprecipitation was undertaken. While no evidence of a 47kDa phox reactive protein was evident under immunoprecipitation conditions, high molecular weight bands were observed in non-reducing conditions and this may be explained by co-immunoprecipitation of other proteins along with the desired target of p47<sup>phox</sup>. These proteins may have formed inter-chain disulphide bonds in the oxidised cellular environment created by addition of biotin-GSSG (Figure 6.6; lanes 3 and 4). This is supported by their migration into lower molecular weight in the presence of reducing conditions (lanes 7 and 8).

Biotin-GSSG may form thiol exchange with available cysteine residues in p47<sup>phox</sup> which may remain uncomplexed of very low levels which are not detectable by anti-p47 and due to sensitivity of avidin signal this showed in the molecular weight band and around 60kDa in figure 6.7B, which was reversible in reducing conditions. However, in the high molecular weight protein complex seen in figure 6.7A, where all p47<sup>phox</sup> thiol residues are occupied to form inter-disulfide bonds, the biotin-GS-protein signal is not observed. Co-immunoprecipitation of p47<sup>phox</sup> with other cellular proteins such as protein kinase C (Korchak and Kilpatrick, 2001, Reeves et al., 1999), or IRAK-4 (interleukin-1 receptor-associated kinase-4) (Pacuelet et al., 2007) has been reported in



previous studies. Both of the above proteins phosphorylate p47<sup>phox</sup> and signal for membrane translocation. However, further experiments are required to prove the hypothesis that p47<sup>phox</sup> crosslinks with other proteins involved inter-disulfide bond formation.

The use of the same anti-p47<sup>phox</sup> antibody for IP and western blot detection may cause the antibody to compete with the same epitope and therefore could generate low signal in the latter method. Therefore, the use of different epitope targeted anti-p47<sup>phox</sup> antibodies for probing the immunoprecipitated bands is important. In order to identify different signals detected in western blots, the same protein samples were loaded on to an SDS-PAGE gel followed by coomassie blue staining. This revealed equivalent staining and one major band around molecular weight 65kDa. To identify this band, LC-MS/MS was undertaken. As detected by mass spectrometry, the protein bands related to molecular weight 65kDa are identified as bovine BSA (MW=69279). The sepharose beads were washed with 1% BSA during the IP protocol and this may have remained as impurities in the protein extract. This will also have influenced the total protein detection by RCDC assay and therefore, the loading total protein may contain only a fraction of IP proteins, which may be present below the sensitivity level of the coomassie stain. The use of more sensitive staining methods such as silver staining may allow detection lower levels of proteins in the gel.

Previous studies have shown that NADPH oxidase subunit redistribution into lipid rafts could initiate by different stimuli (Evans et al., 2003, Shao et al., 2003, Foster et al., 2003). Some priming agents such as IL-8 are shown to sequentially recruit NADPH oxidase subunits into lipid rafts (Guichard et al., 2005). A possible indirect method that could activate and enhance the neutrophil respiratory burst under a decreased redox

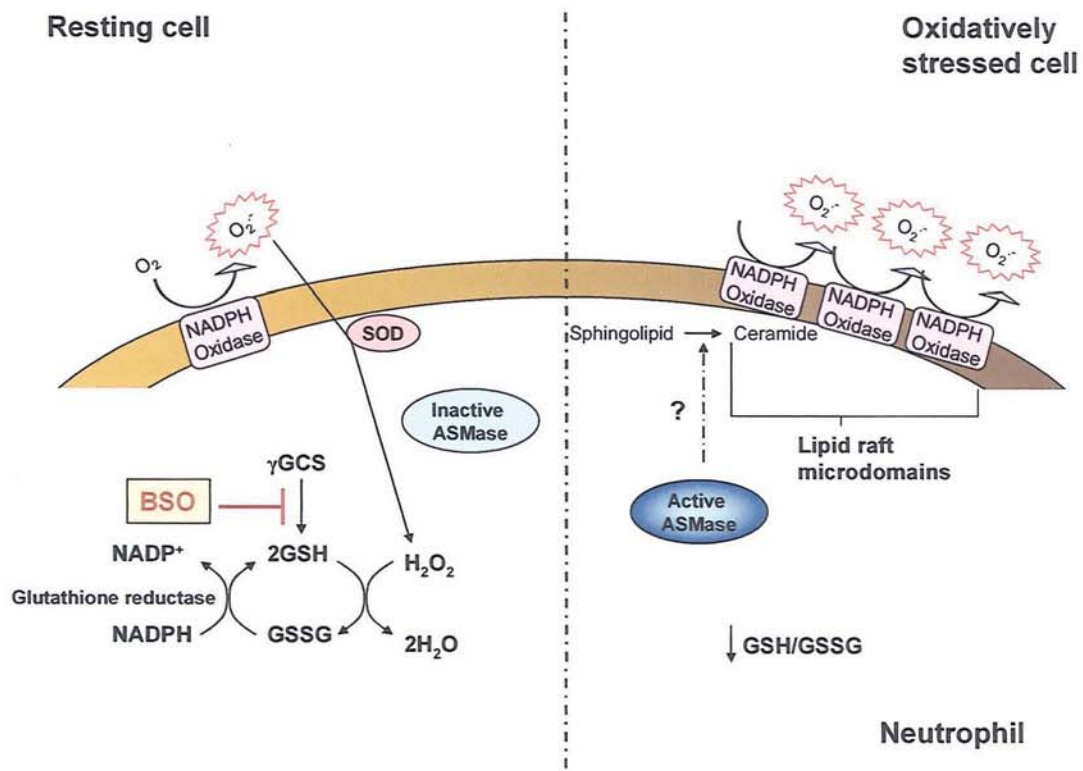
balance is via other regulatory enzymes that involve in cellular signaling. Phospholipases are enzymes that catalyse the hydrolysis of membrane phospholipids in mammalian cells. Among different phospholipase enzymes, a special attention has been given to SMases, mainly due to their activity in generating ceramides in membranes (Martin et al., 2007). More importantly, SMases are reported to be controlled by the cellular redox state. Evidence suggests that lower GSH levels in the cell potentially activate SMases (Martin et al., 2007). Ceramide-rich membranes form stable cellular platforms, where more NADPH oxidase cluster together prior to activation, as reviewed in Li *et al.*, (2007). In the past, the partial insolubility of neutrophil NADPH oxidase subunits in the non-ionic detergent Triton X-100 has been demonstrated (Vilhardt and Deurs, 2004). To investigate this phenomenon in dHL60 cells, the raft marker protein flotillin was associated with low density Triton X-100 insoluble fractions indicating that dHL60 cells indeed contain lipid rafts and these are characterised by the marker protein, flotillin. Distribution of NADPH subunits in lipid rafts was then analysed in the presence or absence of oxidatively stressed conditions. The fractionation of gp91<sup>phox</sup> and p47<sup>phox</sup> was analysed by western blotting. Previous investigators have shown a proportion of gp91<sup>phox</sup> was present in the raft fraction of resting neutrophils/HL60 cells (Vilhardt and Deurs, 2004, Shao et al., 2003). In contrast, in the work presented here, gp91<sup>phox</sup> subunit co-localisation did not localise with flotillin in BSO-treated resting dHL60 cells. Furthermore, in support of previous studies, the cytosolic NADPH subunit p47<sup>phox</sup> was not detected in low density membrane fractions. Upon activation of the cells with fMLP, the levels of NADPH oxidase components gp91<sup>phox</sup> and p47<sup>phox</sup> in lipid raft increased. However, these observations also need further validation with a positive control such as cells stimulated with PMA and control, where dHL60 cells are extracted in the absence of stimuli. As different subdomains may co-exist in lipid rafts, Triton X-100 extraction may exclude some of the protein associations with markers

such as flotillin when separated by sucrose density centrifugation. Therefore, the association of NADPH subunits with lipid rafts in oxidative stressed conditions was also investigated with the use of different a lipid raft marker, ganglioside GM1.

Therefore, corroboration of sucrose density gradient analysis was sought by using immunofluorescence to examine the effect of BSO on lipid raft formation in intact cells. In agreement with previous observations (Guichard et al., 2005), IL-8 slightly increased formation of lipid rafts as detected by formation of fluorescent detected membrane patches. Secondary fMLP stimulation, up-regulated lipid raft formation in these cells as detected by polarised membrane patches. Similar results were observed with the cells in oxidatively stressed environments created by introducing BSO. The dHL60 cells treated with BSO initiated lipid raft formation as detected by a slightly increased punctuate pattern of staining followed by more polarised staining with a secondary fMLP stimulus. This may explain the reason behind the higher respiratory burst produced by BSO-treated dHL60 cells than control cells, where partially organised lipid rafts would respond more strongly to a second stimulus such as fMLP. These results could be further validated using positive controls such as inducing lipid raft formation by addition of ASmases.



Others have shown that the involvement of lipid rafts in regulating NADPH membrane assembly could be severely curtailed by depleting the level of cholesterol in the plasma membranes using methyl- $\beta$ -cyclodextrin (M $\beta$ CD) (Guichard, Pedruzzi et al. 2005). The disruption of membrane integrity using M $\beta$ CD not only directly uncouple NADPH oxidase subunits but also effects down-stream signaling pathway with the membrane dependent initiation of tyrosine phosphorylation (Guichard, Pedruzzi et al. 2005). Therefore the effect of M $\beta$ CD on BSO-enhanced, fMLP-stimulated respiratory burst should be investigated.



**Figure 6.12: A possible mechanism for the upregulation of NADPH oxidase activity under oxidatively stressed conditions.** The pathway leading to the formation of GSH by the action of  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ GCS) is blocked by buthionine sulfoximine (BSO), inducing artificial stress condition in dHL60 cells. Decreased cellular GSH/GSSG ratio may activate redox sensitive enzymes such as ASMases. The break down of sphingolipids to ceramide and sphingosine by ASMases may favour lipid raft formation and thereby clustering active NADPH oxidase complexes in the membrane.

In summary these data suggest that GSH-depleted dHL60 cells, which resemble neutrophils in oxidatively stressed environments such as inflammatory sites, generate increased ROS (Figure 6.12). Increased NADPH oxidase activity of this neutrophilic phenotype appears to be regulated by cellular redox balance. A change of activity in cellular enzymes such as ASMases may promote lipid raft formation and thereby assembly of the NADPH oxidase enzyme.

## **Chapter 7.0- General discussion**



## 7.1 Discussion

Infection of host tissue by pathogenic bacteria and/or stimulation by microbial components/virulence factors triggers production of pro-inflammatory peptides that have the ability to activate and recruit neutrophil polymorphonuclear leucocytes along a concentration gradient. Patients with periodontitis show increased numbers of neutrophils within periodontal tissues and pockets relative to periodontally healthy subjects (Chapple, 1997). These neutrophils are also reported to have an increased respiratory burst and higher elastase release in the presence of stimuli in the systemic circulation and the inflamed periodontium than neutrophils isolated from a healthy individual. Recent work has demonstrated baseline hyper-activity of peripheral blood neutrophils, with respect to extracellular ROS in periodontitis patients relative to age and gender matched healthy controls. Such mechanisms when simultaneously active may contribute to a significant amount of the oxidative stress reported in periodontitis tissues and therefore, factors behind this neutrophil behaviour are important to elucidate (Panjamurthy et al, 2005).

The relationship between the microbial insult of dental plaque and the inflammatory response of the host creates a complex environment resulting increased levels of bacterial and host inflammatory factors in both gingival crevice fluid as well as in the systemic circulation (Matthews et al., 2007). It is possible that these mediators could prime or induce activity/reactivity of neutrophils in patients with periodontitis. One such hyperactive response that exists in systemic periodontitis neutrophils is an enhanced respiratory burst and an impaired redox balance; redox state is determined by the balance between ROS and the levels of cellular antioxidants particularly GSH. The hypothesis investigated here is that increased leakage of ROS affects the neutrophil redox balance and redox-regulated enzyme activity. This in turn may affect the

regulation of the neutrophil respiratory burst, thereby presenting a hyperactive phenotype.

Given their short life span, neutrophils are not amenable to most molecular biology/biochemistry methods and they are not always readily available in the numbers required in research laboratory conditions. Moreover, the variation that exists between individuals will significantly influence the data, unless a large sample number is used. Use of higher sample numbers is practically limited especially for method optimisation experiments. Therefore, the thesis has described the development of a versatile neutrophil-like cell model that is easy to maintain and provide reproducible results under different experimental conditions. This model has been used as a system for testing different conditions that neutrophils encounter in periodontitis.

The HL-60 cell line exhibits an aggressive, malignant and proliferative potential *in vivo* but can be induced to differentiate terminally when challenged *in vitro* (Chang et al., 2006); the human promyelocytic HL60 cells can differentiate towards monocytes-, macrophages- or into neutrophil-like cells after exposure to different chemical agents such as PMA, retinoic acid (Breitman et al., 1980, Sham et al., 1994) and DMSO (Chang et al., 2006, Takeda et al., 2003). Consistent with other observations, the work described in this thesis has confirmed that HL-60 cells can differentiate into neutrophil-like cells when challenged with 1% DMSO for 5 days. dHL60 cells exhibit the main neutrophil characteristic of respiratory burst stimulated by a chemical stimulus (PMA) or a bacterial derived stimulus (fMLP). However, they fail to reproduce the exact neutrophil phenotype; compared with primary neutrophils, dHL60 cells have low CXCR2 receptor expression (Sai et al., 2006), and this may account for the lower rate of cell migration towards IL-8. In addition, dHL60 cells express low number of CD14



receptors compared with primary neutrophils (chapter 2); hence dHL60 cells show low response to bacterial LPS. Collectively, dHL60 cells demonstrated a low level of priming and stimulating responses in comparison with primary neutrophils. This may be a result of low receptor expression or a lower rate of receptor turnover in dHL60 cells.

The increased neutrophil respiratory burst found in periodontitis may be responsible for the oxidatively stressed environment in the gingival cavity and gingival crevicular fluid (GCF) shown as a decreased GSH: GSSG in GCF (Chapple et al., 2002). Although, this mechanism of neutrophil action is present as a defence against aerobic/anaerobic bacteria in subgingival cavities, severe growth of dental plaque is always associated with periodontitis suggesting that the surviving organisms have developed survival mechanisms (Marquis, 1995). Therefore, periodontitis may involve both the direct cytotoxic and proteolytic effects of oral microorganisms and the indirect pathologic consequences of the host immune response to these microorganisms. Interaction of bacterial products and antigens of periodontal pathogens with host inflammatory cells results in the release of cytokines. *In vitro* studies have demonstrated that the proinflammatory cytokine, IL-8 is released by gingival fibroblasts, gingival epithelial cells, and endothelial cells, as well as leukocytes (Yumoto et al., 1999, Takigawa et al., 1994, Myokai et al., 2004). After cessation of tooth brushing, a rapid increase in the levels of IL-8 in CGF precedes the clinical signs of periodontitis (Zhang et al., 2002). Furthermore, high IL-8 plasma levels are reported in rapidly progressive periodontitis and neutrophils isolated from these patients do not show further enhanced the production of  $H_2O_2$  after priming by IL-8 (Gainet et al., 1999). The IL-8 released to the site of the infection has been shown to stimulate neutrophil chemotaxis, degranulation and respiratory burst. Using rodent experimental models of periodontitis it has been



shown that the expression of the chemokine KC (rodent analogue of human IL-8) in periodontal tissues, preferentially in junctional epithelium and their levels correlate with the neutrophil chemotaxis (Garlet et al., 2005). However, the levels of IL-8 do not necessarily demonstrate the severity or progression of the disease. As described in chapter 3, IL-8 is secreted as two main isoforms; IL-8-72 amino acid long peptide and IL-8-77 amino acid long peptide. Confirming other reports, the work described in this thesis and published in Dias *et al.*, (2008) has shown that the IL-8-77 isoform is biologically inactive in initiating neutrophil/dHL60 chemotaxis or priming. The biological activity of this isoform is increased upon *in vitro* digestion with the bacterial cysteine protease, gingipain. Gingipains are secreted by the anaerobic bacterium *P.gingivalis*, which has been identified as a common and successful host-adapted oral pathogen (Yilmaz, 2008), whereby it increases production of host cytokines including IL-8 (Mikolajczyk-Pawlinska et al., 1998, Yamamoto et al., 2006). The work in this thesis showed that the biologically active IL-8-72 isoform has a decreased priming and chemotactic ability after digestion with gingipains. Therefore, the activity rather than the level of IL-8 provides valuable information about IL-8 influence on neutrophils. Previous studies that have attempted to build a relationship between clinical parameters and cytokine profiles in serum samples from patients with chronic periodontitis may have been unsuccessful for this reason (Gońska et al., 2003). Higher abundance of active IL-8 in plasma from patients with periodontitis may prime systemic neutrophils to produce a higher burst to a second stimulus. Neutralising the active form of IL-8 with anti-IL-8 antibodies *in vitro* lowers the neutrophil basal respiratory burst (chapter 3 and 4). Furthermore, professional treatment of gingival inflammation is shown to decrease plasma IL-8 levels (Gainet et al., 1998). This indicates a positive relationship between periodontitis and IL-8 activity.

The cytokine, GM-CSF is produced by many cell types including epithelium and has been shown to prime neutrophils *in vitro* (Fuhler et al., 2004). The up-regulation of GM-CSF gene expression by gingival epithelial cells after exposing to oral pathogens such as *P.gingivalis*, *P.intermedia* and *F.nucleatum* has been previously reported (Prime et al., 1990, Sugiyama et al., 2002). Nevertheless, gingival crevicular fluid has been shown to have increased levels of GM-CSF in periodontitis patients (Ren et al., 2002). GM-CSF has also been shown to prime neutrophils from periodontal patients compared with neutrophils isolated from healthy individuals *in vitro* (Matthews et al., 2007). Based on its stimulatory effects on granulocyte differentiation, recombinant human GM-CSF has been used in the treatment of chemotherapy-induced bone marrow suppression in patients undergoing transplantation for the treatment of cancer (Calderwood et al., 1996). However, later studies revealed that patients receiving GM-CSF therapy are at increased risk to get inflammatory periodontitis (Baqui et al., 1998). This provides further supportive evidence for the role of GM-CSF and granulocytes in periodontitis. GM-CSF in the presence of the oral pathogen LPS is able to hyperactivate monocytes to release the cytokines; TNF- $\alpha$  and IL-1 $\beta$  (Baqui et al., 1999, Baqui et al., 1998). Circulating GM-CSF in plasma is reported to facilitate neutrophil adhesion, delay neutrophil apoptosis (Fanning et al., 1999) and prime neutrophils for increased phagocytosis and respiratory burst (Weisbart et al., 1987, Matthews et al., 2007). Therefore, it is possible that the interplay between GM-CSF and the oral pathogen LPS may contribute to hyperactive neutrophil phenotype. IFN $\gamma$  has been given a great deal of attention in the literature as a possible priming agent for periodontitis (Silva et al., 2007, Garlet et al., 2003) however, little research is done on the type I interferon; IFN- $\alpha$ . Recent studies have revealed that peripheral neutrophils isolated from patients with periodontitis expressed high levels of IFN- $\alpha$  stimulated genes and also a higher level of IFN- $\alpha$  in plasma (Wright et al., 2008). The importance of these findings for neutrophil



priming and activation were further confirmed in this thesis by experiments designed to deplete IFN- $\alpha$  level in plasma using neutralising antibodies.

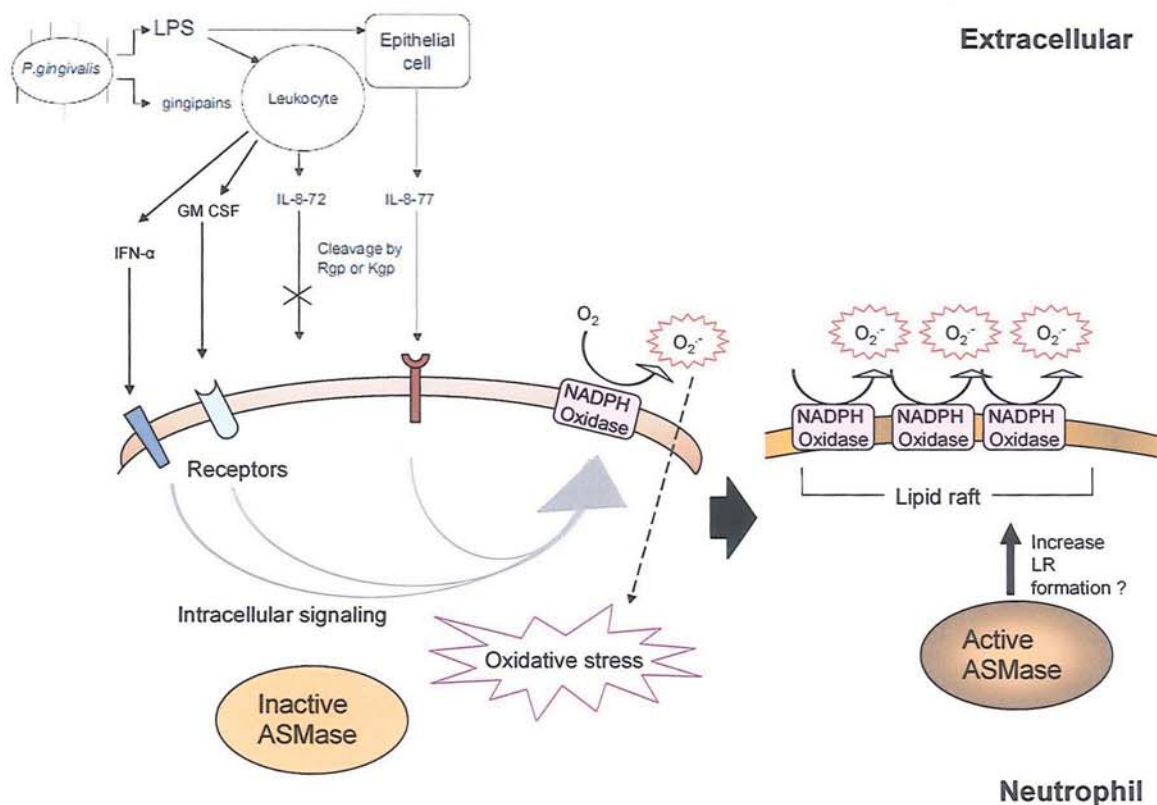
Increased systemic levels of active stimulators found in chronic inflammatory conditions such as periodontitis may constantly prime/activate neutrophils. This, at least partially, may explain the observed high basal level of ROS production in neutrophils isolated from patients with periodontitis. Increased ROS production in neutrophils also creates an intracellular oxidatively stressed environment demonstrated as in ageing and degenerative diseases (Kadenbach et al., 2009). It is well established that cells behave differentially in an oxidatively stressed environment due to altered intracellular signaling cascades (Forman and Torres, 2001, Hansen et al., 2004). The possibility of NADPH oxidase up-regulation as a result of oxidative stress was investigated in chapter 5. Low levels of intracellular ROS are quickly detoxified by various antioxidant enzymes such as SOD or small molecular weight scavengers such as GSH. Usually, the cellular GSH:GSSG ratio is carefully controlled to maintain a reducing environment in the cytoplasm and any change in the redox potential can be used as an index of the oxidative stress level in the cell. Therefore, the GSH/GSSG couple serves as a redox buffer in cells/plasma that maintains the redox state. Using BSO as a model system, a reduction of GSH/GSSG ratio was observed in oxidatively stressed dHL60 cells. As a chemical inhibitor, BSO inhibited glutathione synthesis to create an artificially stressed intracellular environment. However, pathophysiologically neutrophils may not encounter such inhibition directly, and therefore may encounter oxidative stress in different pathways such as increased cellular ROS levels (Kadenbach et al., 2009). Therefore it is possible that hyperactive neutrophils with increased ROS production found in periodontitis may have an oxidatively stressed cellular environment. Reduction of the GSH/GSSG level in dHL60 cells was associated with increased respiratory burst



measured by extracellular lucigenin. However, this data need further validation as the change in activity of ROS scavenging enzymes such as intracellular SOD could influence the outcome of the respiratory burst (Mumbengegwi et al., 2008).

Some protein thiols (-SH) are more sensitive to oxidation reactions, which may involve ROS, RNS or glutathione. These thiols also can interact with a variety of oxidants, to form in many cases a reversible covalent modification such as S-glutathiolation. The reversibility of oxidative modifications, allow proteins to act as molecular switches in a changing redox environment. PKC, the cysteine-rich domains of serine/threonine kinase, is thought to be one of the redox sensitive proteins that switched on by oxidative stress (Brechard et al., 2009). Therefore, PKC oxidation could enhance activity of NADPH oxidases in this setting. ASMases are another group of enzymes that are reported to be activated in oxidative stress and have been shown to have an indirect stimulatory effect on NADPH oxidase assembly and activation (Zhang et al., 2007). Activated ASMases induce lipid raft formation through ceramide synthesis (Figure 6.0). Holopainen and co-workers have demonstrated that ASMase-induced ceramide formation could generate lipid microdomains in large unilamellar vesicles (Holopainen et al., 1998). Ceramide has also been demonstrated to activate PKC, phospholipase A2 and CAPP (ceramide-activated protein phosphatase) (Holopainen et al., 1998). According to the literature, it is possible that the active ASMases could up-regulate NADPH oxidase assembly by forming lipid raft microdomains as well as inducing translocation of cytosolic subunits via PKC activation (Figure 6.0). Using lipid raft isolation and cell imaging techniques, this thesis has investigated the assembly of NADPH oxidase subunits, p47<sup>phox</sup> and gp91<sup>phox</sup> into lipid rafts under normal, stimulated and oxidatively stressed conditions. Oxidatively stressed dHL60 cells showed a low level of punctate membrane labelling followed by more polarised labelling as detected

by fluorescence imaging, indicating the initiation of lipid raft organisation in an oxidatively stressed environment. The hypothesis that ASMase induced NADPH oxidase assembly in this environment could be further analysed by adding ASmases into cells (positive control) or by using ASMase inhibitors such as desipramine, imipramine or SR33557 to block its action (negative control) followed by lipid raft analysis for the distribution of NADPH oxidase subunits.



**Figure 7.1 Schematic diagram representing the pathways that might lead to hyperactivity of neutrophils in periodontitis.**

Dental plaque is often removed scaling non-surgically to arrest and control progress of chronic periodontitis. Periodontal procedures are usually followed by antibiotics to prevent the propagation of disease causing bacteria. Typically, antibiotics are given for acute infections, and have little effect on prevention of periodontitis. However, antibiotics have been recommended for control of chronic periodontitis. Tetracycline antibiotics, which include tetracycline hydrochloride, doxycycline, and minocycline, not only have antibacterial actions but also act as anti-inflammatory drugs (Sapadin and Fleischmajer, 2006). Administration of doxycycline to chronic periodontitis patients has been found to reduce the collagenase and other matrix metalloproteinase activity in gingival and crevicular fluid (Lee et al., 2004). Some macrolide antibiotic such as roxithromycin (Oyama et al., 2007) or metronidazole in combination with



tetracycline or amoxicillin (Cionca et al., 2009) have also been shown to improve periodontal health. However, there is a growing bacterial resistance to many of these antibiotics such as roxithromycin and metronidazole, therefore limiting their use in periodontal disease (Ellison, 2009). Anti-inflammatory drugs are reported to work better for periodontitis than antibiotics (Oyama et al., 2007).

Several therapeutic strategies have been explored in other chronic inflammatory diseases such as rheumatoid arthritis to prevent neutrophil chemotaxis and activation (Johnson et al., 2004). One of the common method is to use inhibitors antagonists to block chemokine-chemokine receptor interactions, e.g CC receptor antagonist BX-471 (Johnson et al., 2004). The use of inhibitors for GPCR signaling pathway may prevent activation for more than one chemokine, and will be more effective to reduce neutrophil activation. The development of orally active small-molecule inhibitors of PI3K for the treatment of disorders with enhanced neutrophil responses have been investigated in previous studies on mouse models (Wetzker and Rommel, 2004, Stoyanova et al., 1997, Rommel et al., 2007). Treatment with small molecule inhibitor of PI3K; AS605240 are shown to reduce chronic inflammatory disease, glomerulonephritis (Barber et al., 2005). Therefore, knowledge of cytokine activity in periodontitis may be used to design effective therapeutic drugs for chronic inflammatory periodontitis.

## 7.2 Conclusion

The findings of this thesis suggest that dental pathogens such as *P.gingivalis* could modulate chemokine activity and thereby increase neutrophil priming and enhance chemotaxis into periodontal tissues. Periodontal pathogens may also stimulate host cells to produce cytokines such as GM-CSF and IFNs, and IL-8 which in turn prime/activate systemic neutrophil ROS production. Higher level of ROS also creates an intracellular oxidative stress environment, which in turn may have positive effects on up regulating NADPH oxidase assembly and activation.

## 7.3 Future work

- The NADPH oxidase, cytosolic subunit of p47<sup>phox</sup> may be associated/cross-linked with another protein/s at resting stage. This can be investigated with use of different antibodies for IP and WB followed by MS/MS analysis. Furthermore, the effect of BSO treatments on aforementioned associations could be analysed using the same methodology.
- Enhanced respiratory burst observed in dHL60 cells during the BSO induced oxidative stress needs to validate by measuring the activity of superoxide dismutase under the same conditions.
- Oxidatively stressed cells may increase NADPH oxidase activity due to activation of ASMases. This can be further investigated using positive (addition of ASMases) or negative (using inhibitors for ASMases) controls followed by lipid raft analysis for distribution of NADPH subunits and fluorescence microscopy.

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## **Chapter 9.0 Appendix**



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