Chapter 1: Introduction and Literature Review

1.1 INTRODUCTION

Moxifloxacin is an antibiotic widely used as a bacteriostatic or bactericidal drug for the therapy of bacterial infections. Notwithstanding the direct interactions between antibiotics and bacteria, many antibiotics also interact, albeit indirectly, with the immune system. The immunomodulatory effects of antibiotics include alteration of phagocytosis, chemotaxis, endotoxin release, cytokine production, and hematopoietic recovery after immunosuppression. Moreover, some antibiotics can affect the life-span of immune and inflammatory cells through the induction or inhibition of apoptosis (Choi *et al.*, 2003). Such properties may have clinical significance for the modulation of the immune response of patients, especially those who are immunodeficient, and those with microbial infections that have harmful inflammatory effects (e.g. septic shock). Thus, antibiotics may play a dual role in infections, by having both direct antimicrobial effects, as well as indirect effects, which can be either beneficial or detrimental to the host response (Araujo *et al.,* 2002).

The increasing evidence for the involvement of immune system-derived cytokines and reactive oxygen species (ROS) in the severity of, or even death due to, infection indicates the importance of defining the immunomodulatory activity of antibiotics. It has become apparent that in the infected host, excessive or unrestricted activity of the immune response can be detrimental. Potentially harmful effects of T-cells and other leukocytes can be prevented by down-regulation of inflammatory responses. Lack of such control can result in tissue injury and death of the host. Among the various classes of antibiotics, fluoroquinolones exert immunomodulatory effects. These agents are widely used in clinical practice, and newer fluoroquinolones with enhanced potencies against microbial pathogens are continuously being developed (Ono *et al.,* 2000). Although moxifloxacin is considered to be a broad-spectrum fluoroquinolone due to its enhanced activity against both gram-positive and gram-negative bacteria (Weiss *et al,* 2004), relatively little is known about the effect of moxifloxacin on host defences, particularly innate protective mechanisms involving neutrophils.

This study has been designed to investigate the *in vitro* immunomodulatory effects of moxifloxacin, a synthetic methoxyfluoroquinolone with a broader antibacterial

spectrum than first, second and third generation fluoroquinolones, on the functions of human neutrophils and mononuclear leukocytes (MNL).

1.2 LITERATURE REVIEW

1.2.1 History of quinolones

Quinolones are entirely synthetic antibacterial drugs, with the first representative (nalidixic acid) having been synthesized in 1962. Their structures are based on the 4-oxo-1,4-dihydroquinolone skeleton (Levine *et al.*, 1998; Labro, 2000). They have evolved from agents used solely for the treatment of urinary tract infections to molecules with potent activity against a wide spectrum of significant bacterial pathogens, with resultant broad clinical utility. This evolutionary pattern has arisen through the development of new core and side-chain structures, with associated improvements in activity, pharmacokinetics and tolerability, and through the selection of molecules that remain useful and well tolerated (Ball, 2000).

All quinolones with antibacterial activity have a 4-quinolone nucleus with a nitrogen atom at position 1, a carboxyl group at position 3, and a ketone at position 4. The structure of the quinolones has developed along two parallel pathways: the naphthyridones (with the original naphthyridine core of nalidixic acid) and the fluoroquinolones, in which a carbon atom is substituted for nitrogen at position 8 of the naphthyridine nucleus. As mentioned above, the first member of the quinolone class of antibiotics was nalidixic acid, which has limited antibacterial activity against gram-positive bacteria (Owens *et al.,* 2000). Since then, thousands of compounds have been synthesized, of which the 6-fluorinated molecules (fluoroquinolones) represent a breakthrough in 4-quinolone research (Labro, 2000).

Recently, interest in the quinolone antibiotics has intensified following the publication of clinical and pre-clinical data confirming their potential for use in treatment of tuberculosis (TB) (Duncan and Barry, 2004). Data from quinolone studies suggest that these drugs may be used to shorten the duration of chemotherapy. In the development of fluoroquinolone containing “third-line” regimens, moxifloxacin was found to be superior to ofloxacin or levofloxacin, with sterilization being achieved in nine months (Veziris *et al.,* 2003; Duncan and Barry, 2004).

All fluoroquinolones have a fluorine substitution at the 6-position, which confers greater antibacterial potency and a broader spectrum of activity (Owens *et al.,* 2000;

Shalit *et al.,* 2002) than that of nalidixic acid and other nonfluorinated quinolones (e.g. cinoxacin, oxilinic acid). Addition of the fluorine and piperazine moiety at positions 6 and 7 respectively, substitution of carbon for nitrogen at position 8, and modification of the side chain at position 1 yielded the second generation agents ciprofloxacin and ofloxacin.

Other modifications to the fluoroquinolone structure yielded third generation agents (e.g. levofloxacin, sparfloxacin, grepafloxacin) with an improved antibacterial spectrum of activity, greater potency and an extended half-life. Recently a fourth generation of quinolones (e.g. gatifloxacin, moxifloxacin, trovafloxacin) with expanded coverage against anaerobes has been developed (Ball, 2000; Owens *et al.,* 2000). The methoxy group at position 8 of moxifloxacin and gatifloxavin theoretically may confer enhanced activity against resistant gram-positive bacteria and reduce development of resistance (Owens *et al.,* 2000).

1.2.2 Moxifloxacin structure and function

Moxifloxacin (1-cyclopropyl-7-(2, 8-diazabicyclo [4.3.0] nonane)-6-fluoro-8-methoxy-1,4-di-hydro-4-oxo-3-quiline carboxylic acid hydrochloride), is an 8-methoxy-1,quinolone (figure 1)and is considered a broad spectrum fluoroquinolone due to its enhanced activity against gram-positive and aerobic bacteria (Dalhoff *et al.,* 1998; Araujo *et al.,* 2002; Weiss *et al.,* 2004).

Moxifloxacin consists of a bicyclic aromatic core with a fluorine atom at the C-6 position, a methoxy group at position 8, an N-1 cyclopropyl group and an azobicycline group at the C-7-position. The C-8 methoxy group is thought to contribute to enhanced activity against gram-positive organisms, decreased development of resistance, (Kishii *et al.,* 2003), and to limit the potential for phototoxicity (Caeiro and Lannini, 2003). The bulky diazobicycline group at C-7 also contributes to its spectrum of activity and makes moxifloxacin a poor substrate for the active bacterial efflux pump (Owens *et al.,* 2000; Caeiro and Lannini, 2003). It has also been suggested that moxifloxacin has both inhibitory and stimulatory effects on the immune system, primarily affecting the production of several cytokines by both human and murine leukocytes (Weiss *et al.,* 2004).

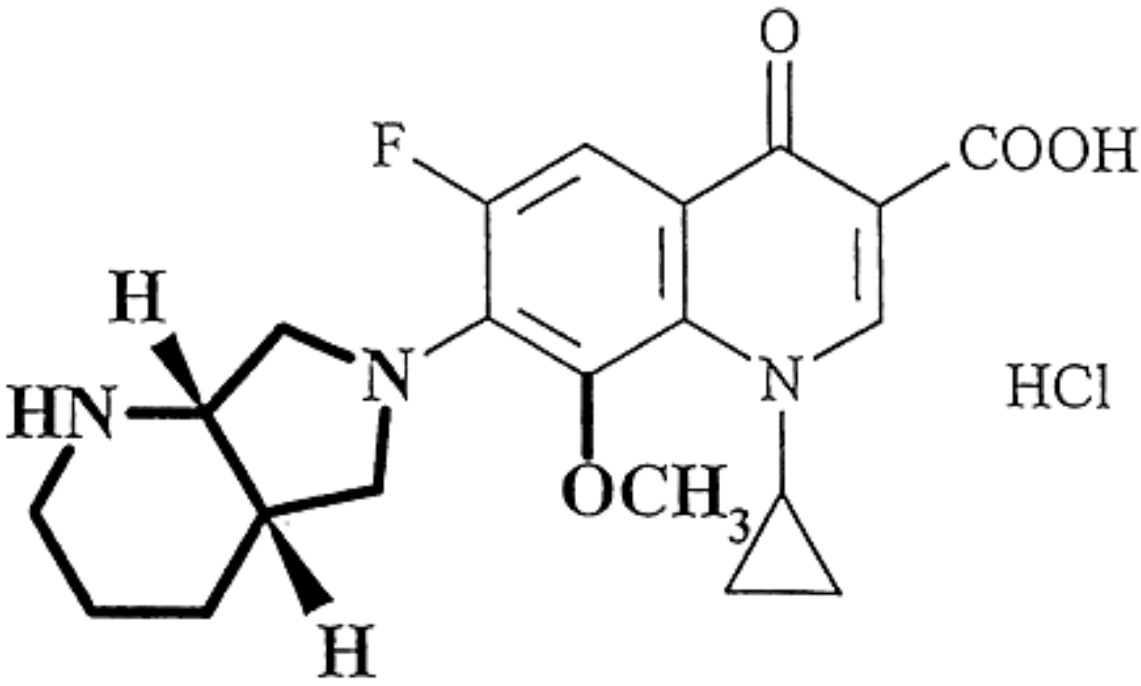

Fig. 1: Chemical structure of moxifloxacin (Source: Stass, *et al.*, 1999).

1.3 PHARMACOLOGY

Moxifloxacin is a broad-spectrum fluoroquinolone antibiotic. It is approved for the treatment of acute bacterial exacerbations of chronic bronchitis, acute bacterial sinusitis, and mild-to-moderate community-acquired pneumonia. It is usually administered by the oral route and is well absorbed from the gastrointestinal tract, although intravenous administration is also an option. Its absolute bioavailability is 90%. Approximately 50% of moxifloxacin is bound to serum proteins, independent of drug concentration. The volume of distribution of moxifloxacin ranges from 1.7 to 2.7 L/kg. It is widely distributed throughout the body, with tissue concentrations often exceeding plasma concentrations (Ball, 2000).

1.3.1 Metabolism

Approximately 52% of an oral or intravenous dose of moxifloxacin is metabolized via glucuronide and sulfate conjugation. Cytochrome P450 is not involved in moxifloxacin

metabolism and its activity is not affected by moxifloxacin (Caeiro and Lannini, 2003). The sulfates conjugate (M1) accounts for approximately 38% of the dose, and are eliminated primarily in the feces. Approximately 14% of an oral or intravenous dose is converted to a glucuronide conjugate (M2), which is excreted exclusively in urine. Peak plasma concentrations of M2 are approximately 40% of that of the parent drug, while plasma concentrations of M1 are generally less than 10% of that of moxifloxacin (Owens *et al.,* 2000).

1.3.2 Mechanism of action

Although the mechanism of action of the quinolones, including moxifloxacin, is not fully known, it differs from that of aminoglycosides, β-lactam antibiotics, macrolides and tetracyclines; therefore, microorganisms resistant to these classes of drugs may be susceptible to moxifloxacin and other quinolones (Owens *et al.,* 2000). Bacterial chromosomes require topoisomerase enzymes to maintain the function and configuration (topology) of the intricate DNA molecule (Owens *et al.,* 2000; Caeiro and Lannini, 2003). Type II topoisomerases (topoisomerase IV and DNA gyrase) characteristically alter DNA topology by introducing a transient double strand break in DNA, passing another duplex segment of DNA through the break (Stroman *et al.,* 2005), and relegating the broken ends. In general, type II enzymes are dyadic molecules that, in an ATP-dependent manner, catalyze the relaxation of supercoiled DNA, catenation and decatenation of DNA-rings, and knotting and unknotting of duplex DNA. These enzymes are found in all organisms comprising a family, which is structurally and evolutionarily conserved (Levine *et al.,* 1998).

Similar to other fluoroquinolone agents, moxifloxacin exhibits antimicrobial activity against susceptible bacteria through inhibition of the DNA gyrase (topoisomerase II) activity (Galley *et al.,* 2000; Bearden and Danziger, 2001), an essential bacterial enzyme required for DNA replication, transcription, repair and recombination (Pestova *et al.,* 2000). The drug is bactericidal during the stationary growth phase, as well as the logarithmic growth phase of certain bacteria. Moxifloxacin also inhibits topoisomerase IV, an enzyme structurally similar to DNA gyrase and essential for bacterial DNA replication. Topoisomerase IV may be the primary target of many quinolones in gram-positive bacteria (DNA gyrase appears to be the main target in gram-negative bacteria) (Owens *et al*., 2000; Stroman *et al.,* 2005). The enzymes are tetramers consisting of two

subunits, *GyrA* and *GyrB* in DNA gyrase, and *ParC* and *ParE* in topoisomerase IV (Caeiro and Lannini, 2003)

Levine and colleagues (1998) suggested that quinolones block DNA replication, not by depriving the cell of gyrase, but by converting gyrase to a poison of DNA replication (illustrated in figure 2). Topoisomerase poisons act by affecting the cleavage-relegation equilibrium (Marians and Hiasa, 1997; Caeiro and Lannini, 2003), effectively trapping the enzyme in a drug-DNA-enzyme ternary complex during the topoisomerization reaction in which the DNA gate is open. Ultimate denaturation of the enzyme therefore results in the generation of double strand breaks in the DNA (Levine *et al.,* 1998). Fluoroquinolone cytotoxicity is correlated with the appearance of double strand breaks (Drlica, 1999). These bactericidal drugs, which inhibit DNA gyrase, are highly active against *Mycobacterium tuberculosis*, including strains resistant to first line drugs (Duncan, 2003; Gosling *et al.,* 2003; Yoshimatsu *et al.,* 2002). Moxifloxacin has been shown to inhibit the growth of the main species of mycobacterium infecting humans (Ji *et al.,* 1998; Miyazaki *et al.,* 1999).

Although the formation of a drug-quinolone-topoisomerase ternary complex is critical for antimicrobial activity, these complexes are completely reversible, and the broken DNA strands can be relegated (Caeiro and Lannini, 2003). It has been proposed that the lesion must be fixed in some manner which will result in disruption of the complex, in order to generate the ultimate cytotoxic agent, the double strand break (Levine *et al.,* 1998; Drlica, 1999). A general scheme for intracellular quinolone action is sketched in Figure 1.2, page 10.

1.3.3 Resistance

Although quinolones are well tolerated and relatively safe, certain adverse effects are common to all agents in this antibiotic class. Quinolone resistance develops through four main mechanisms: 1) mutations in the target enzymes, which reduce the affinities of the fluoroquinolones for DNA gyrase or topoisomerase IV enzymes; 2) production of gyrase protection protein; 3) alterations in bacterial cell permeability; 4) drug efflux, preventing lethal levels of fluoroquinolones via decreased accumulation in the cytoplasm. The action of the efflux pump is dependent on the ability of the

fluoroquinolone to bind to the bacterial efflux protein, which expels it from the cell (Hooper, 1999).

Some fluoroquinolones, particularly moxifloxacin, are less affected by bacterial efflux mechanisms due to their bulky side chain moiety at position 7, which hinders export out of the cell (Caeiro and Lannini, 2003; Stroman *et al.,* 2005). Fluoroquinolone resistance usually develops in a step-wise fashion with initial mutation of the *ParC* subunit followed by mutation of the *GyrA* subunit (Wang, 1996; Fournier and Hooper, 1998; Caeiro and Lannini, 2003). Activity against single-step mutants may be an important characteristic of moxifloxacin, with the potential to limit higher levels of resistance development.

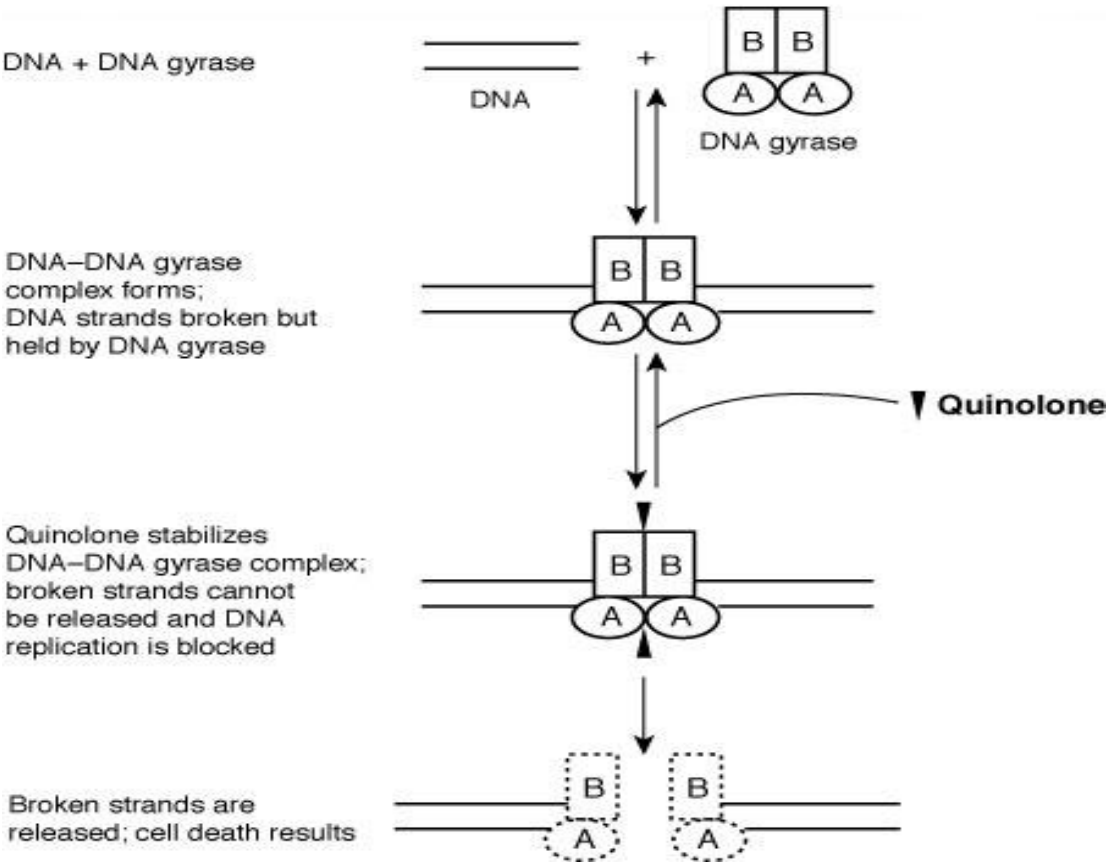

Fig. 1.2: Fluoroquinolones inhibit DNA synthesis by attaching to a complex of DNA gyrase or topoisomerase IV, blocking the attachment site to prevent replication, causing cell death (Adapted from: Drlica, 1999).

1.4 INFLAMMATION

Inflammation is a basic pathological mechanism that underlies a variety of diseases. The inflammatory reaction involves the complex interaction between inflammatory cells (neutrophils, lymphocytes, and monocytes/macrophages) and vascular cells (endothelial cells and smooth muscle cells). Multiple cytokines and growth factors are present at the site of inflammation, and each of these can potentially influence the nature of the inflammatory response. Endothelial cells and smooth muscle cells must integrate the signals generated by these multiple factors to effectively regulate the immunoinflammatory response through expression of adhesion molecules, cytokines, chemokines, matrix metalloproteinases, and growth factors (Tedgui and Mallat, 2001).

1.4.1 Leukocyte entry into sites of inflammation

During an infectious process, the recruitment and migration of leukocytes towards and inside a target tissue is crucial for resolving the infection and for re-establishment of homeostasis (Anderson, 1995; Cassatella, 1999). Both recruitment and migration are multi-step processes that depend on the nature and state of activation of the leukocyte generation of pro-inflammatory cytokines, expression of adhesion molecules, and extracellular matrix (ECM) components, and co-ordination of these events by a mosaic of chemoattractant molecules named chemokines (Shimizu and Shaw, 1991; Nathan and Sporn, 1991; Del Pozo *et al.,* 1995; Gilat *et al.,* 1996; Sallusto *et al.,* 2000; Gerard and Rollins, 2001).

At least four steps, with multiple signalling events at each step, control leukocyte emigration and contribute to its selectivity (figure 3). The attachment or tethering of circulating leukocytes to the vessel wall through labile adhesion permits leukocytes to roll in the direction of flow (step 1) and brings them into proximity with activating signals displayed on the endothelium. These signals (step 2) activate a second class of adhesion receptors, integrins, which firmly bind to immunoglobulin superfamily members inducibly expressed on the inflamed blood vessel (step 3). This results in an arrest of the rolling leukocyte. The arrested leukocyte is then sensitized by chemoattractant gradients, which originate in the nearby extravascular tissue, resulting in activation of integrins which is required for crossing the endothelial lining of the blood vessel and migration into tissue (step 4).

Because most integrins are unable to tether a circulating cell in shear flow, the initial tethering steps are obligatory for the recruitment of leukocytes at sites of extravasation. Binding of endothelial selectins (P-or E-selectin) or of the leukocyte selectin, L-selectin, a three member family of lectin adhesion molecules, to carbohydrate ligands expressed on leukocyte subsets or on specific endothelial surfaces, respectively, in shear flow, are the fastest cell-cell recognition events known in nature. Recently a unique leukocyte integrin member of the beta-1 integrin family, VLA-4, has been identified which is capable of supporting both tethering rolling and arrest on its endothelial ligand, VCAM-1, but is unable to interact in shear flow with its second ligand, the extracellular matrix protein, fibronectin. The ability of an integrin to participate in both labile rolling and firm adhesion depends on its state of activation, which is regulated by multiple cellular factors (Alon *et al.,* 2003; Steeber *et al.,* 1999).

Leukocyte interactions with vascular endothelium during inflammation depend on cascades of adhesion molecule engagement (Zen and Parkos, 2003), particularly during selectin-mediated leukocyte rolling. Leukocyte rolling is also facilitated by members of the integrin and immunoglobulin (Ig) super families. Specifically, leukocyte rolling velocities during inflammation are significantly increased in ICAM-1-deficient mice, with ICAM-1 expression required for optimal P- and L-selectin-mediated rolling. (Steeber *et al.,* 1999; Witko-Sarsat *et al.,* 2000).

In many cases, the loss of both L-selectin and ICAM-1 expression dramatically reduced leukocyte migration into sites of inflammation beyond that which was observed with loss of either receptor alone. In fact, the loss of both L-selectin and ICAM-1 effectively eliminated multiple chronic inflammatory responses in L-selectin/ICAM-$1^{-/-}$ mice. In contrast, the combined loss of L-selectin and ICAM-1 expression had minimal effects on the generation of Ag-specific T cell responses or humoral immunity. Thus, members of the selectin and Ig families function synergistically to mediate optimal leukocyte rolling and entry into tissues, which is essential for the generation of effective inflammatory responses *in vivo* (Steeber *et al.,* 1999).

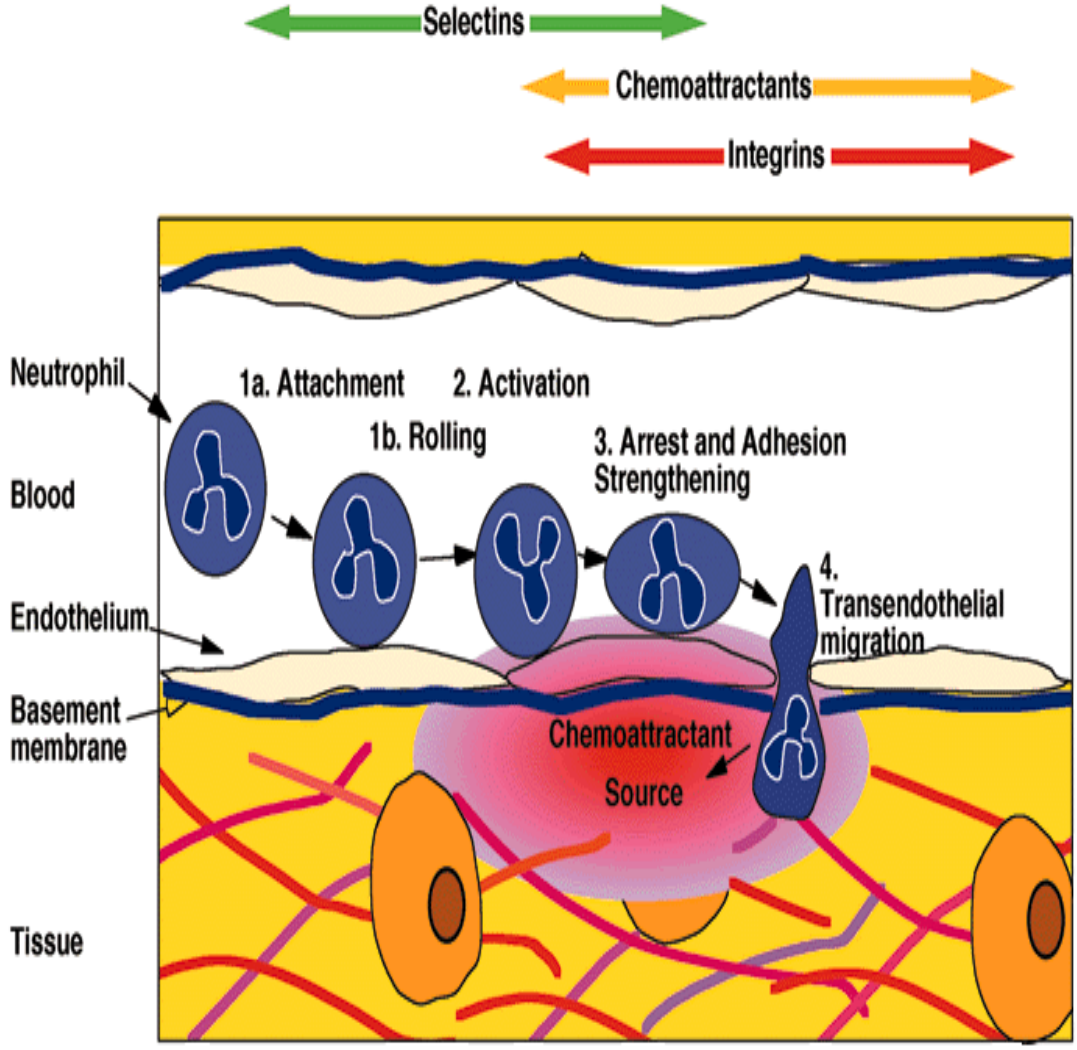

Fig. 3: The four-step model for leukocyte recruitment at sites of inflammation. Selectins, chemoattractants (or other activating signals) and integrins act sequentially with some overlap. Selectins mediate both leukocyte tethering and rolling (steps 1a and 1b). Alpha-4 integrins, can support the early steps of rolling, but still require further activation for their participation in firm leukocyte adhesion to endothelium together with other integrins (Source: Alon *et al.*, 2003).

1.5 NEUTROPHILS

Polymorphonuclear leukocytes (PMNLs) constitute the largest population of circulating leukocytes and the first line of cellular defense of mammalian organisms against invading microbes. They are not a homogeneous cell population since subpopulations exist in various stages from dormant to primed, to fully activated (Labro *et al*., 1993). Neutrophils evolve from pluripotent stem cells under the influence of cytokines and colony stimulating factors. Approximately 8 – 14 days are required for a cell to move through the sequence of 4 – 6 cell divisions and complete maturation. During this time the maturing cells can be released from the bone marrow into the blood under conditions of sufficient stress (Bainton, 1999).

Specific signals, including IL-1, IL-3, TNF-α, G-CSF, complement factors C3e, C5a and chemokines mobilize neutrophils from the bone marrow, which circulate in the unstimulated state. Under normal conditions, 90% of the neutrophil pool is in the bone marrow, 2 – 3% in circulation and the rest in the tissue. Up-regulation of the production of these signals during inflammatory stress increases the production and release of neutrophils from the bone marrow (Cooper, 1999).

The myeloblast is the first recognizable precursor cell and is followed by the promyelocyte, which is characterized by the appearance of lysosomal granules, known as primary or azurophil granules. The promyelocyte divides and differentiates into the myelocyte, which in addition to the azurophil, also contains secondary or specific granules. Following this stage, further division occurs and during the final stages of maturation, the cell passes through the metamyelocyte and the band phases. On maturation of the band form, the nucleus becomes lobulated, consisting of up to four segments.

The life-span of neutrophils is estimated to be between 24 – 48 hours, after which they undergo apoptosis (programmed cell death) and removal by the mononuclear phagocyte system, a process which is dependent on the interactions between phosphatidylserine translocated from the inner to the outer plasma membrane of the apoptotic neutrophil and the phosphatidylserine receptors on monocyte/macrophages. Prolongation of their life span occurs following migration of these cells out of circulation to sites of

inflammation and exposure to anti-apoptotic cytokines such as granulocyte/macrophage colony stimulating factor (GM-CSF) (Watson *et al.,* 1999).

1.5.1 Cytoplasmic granules

The most notable structural features of neutrophils are the abundant, heterogeneous cytoplasmic granules and the highly dynamic plasma membrane; these make this cell ideally suited to the performance of its primary function, which include, adherence to locally activated vascular endothelium, extravasation, migration to the site of infection and engulfment and intracellular destruction of invasive microbial pathogens (Anderson, 1995; Mayer-Scholl *et al.,* 2004).

Neutrophil granules consist of four different groups distinguished on the basis of protein content, size and density. These are primary (azurophil), secondary (specific) and tertiary (gelatinase) granules and secretory vesicles (Witko-Sarsat *et al.,* 2000). Primary and secondary granules are formed during the promyelocyte and myelocyte/metamyelocyte stages respectively, while tertiary granules develop at the metamyelocyte/band cell stage (Le Cabec *et al.,* 1996). Secretory vesicles are the last to appear (becoming evident) in band and segmented cells (Borregaard and Cowland, 1997). These different granule sub-types vary with respect to efficiency of mobilization during neutrophil activation.

In addition to functioning as a mobilisable reservoir of membrane constituents, these various neutrophil granules contain an array of proteases and antimicrobial peptides and polypeptides, which participate in the migratory and antimicrobial activity of neutrophils. The fact that several granule polypeptides are shared by different granules (e.g. lysozyme and protease 3), is indicative of overlap between these granules, which may represent a continuum, as opposed to clearly demarcated granule types (Witko-Sarsat *et al.,* 2000).

1.5.1.1 Primary granules

The major constituents of the primary granules are myeloperoxidase (MPO), an abundant component comprising about 5% of the total cellular protein, the antimicrobial enzyme, lysozyme, and at least 3 neutral serine proteinases, elastase, cathepsin G and proteinase 3 (Theilgaard-Mönch *et al.,* 2006). Unlike other granule

sub-types, azurophil granules do not function as reservoirs of membrane receptor and polypeptides, although CD63 and CD68 are present on their membranes, but the functional significance of this remains to be established (Cham *et al*., 1994). Myeloperoxidase and the defensins, also known as human neutrophil peptides (HNP-1 to HNP4), small cationic, broad-spectrum antimicrobial peptides that contain six cysteines in disulphide linkage, and are present in extremely high concentrations in primary granules (Witko-Sarta *et al.,* 2000).

Although azurophil granules have not been considered to act as reservoirs of membrane components involved in the migratory, phagocytic and oxidant-generating activities of neutrophils, the translocation of Sialyl Lewis-X from their membrane to the plasma membrane during activation of the cells with chemoattractants, suggests that this may not be the case (Suzuki *et al.,* 2000).

1.5.1.2 Secondary granules

Secondary granules outnumber primary granules by about 2:1. They are rapidly mobilized during cell migration, and, in addition to intragranule constituents, their membranes act as a reservoir for adhesion molecules, leukoattractant receptors and cytochrome b_{558}, thereby sustaining neutrophil activation and antimicrobial activities of the cell. Approximately 90% of the total cellular lysosome, vitamin B12- binding protein, the bacteriostatic iron binding protein, lactoferrin as well as the latent metallo-enzymes, collagenase and gelatinase are located in the secondary granules.

1.5.1.3 Tertiary and secretory granules

These granules act as a reservoir for the membrane polypeptides involved in neutrophil activation and function. Their major contribution to neutrophil function is thought to be achieved through extracellular release of gelatinase, which cleaves type IV (basement membrane) and V (interstitial tissues) collagen, thereby facilitating movement of the cells through basement membranes and into underlying tissues (Witko-Sarsat *et al.,* 2000). Secretory vesicles are thought to be endocytic in origin because they contain plasma proteins such as albumin. Interestingly, proteinase 3, a serine proteinase present in azurophil is also localized in the membrane of secretory granules, which are the most mobilizable granules of neutrophils.

1.5.2 Neutrophil elastase

Reactive oxygen species (ROS) and proteases are neutrophil-derived toxic molecules that have long been considered important in the pathophysiology of acute and chronic inflammation. Neutrophil elastase (NE) is a member of the chymotrypsin superfamily of serine proteases, being a 33-kDa enzyme with several isoforms that differ in their extent of glycosylation (Ohlsson and Olsson, 1974). NE is capable of degrading almost all extracellular matrix proteins, as well as a variety of key plasma proteins (Witko-Sarsat *et al.,* 2000). Under physiological conditions, NE is a powerful component of host defense and its activity is tightly regulated by endogenous protease inhibitors (Kawabata *et al.,* 2002). Upon activation, NE is rapidly released from the granules into the extracellular space with some portion remaining bound to the neutrophil plasma membrane (Owen *et al.,* 1997; Kawabata *et al.,* 2002).

1.5.3 Neutrophil elastase target molecules

The main intracellular physiological function of NE is the degradation of foreign organic molecules phagocytosed by neutrophils, whereas the main target for extracellular elastase is elastin (Kawabata *et al.,* 2002). Although NE has been defined as a protease that can degrade the elastin fibre, other proteases including protease 3, cathepsins, G, L and S, macrophage elastase, and gelatinase are able to degrade elastin as well (Lee and Downey, 2001; Kawabata *et al.,* 2002). NE is unique and recognizable as one of the most destructive enzymes because of its ability to degrade almost all extracellular matrix and key plasma proteins. In addition to elastin, NE is known to degrade the extracellular matrix proteins, collagen types I – IV, proteoglycan, fibronectin, platelet IIb/ IIIa receptor, complement receptor, thrombomodulin, and cadherins (Kawabata *et al.,* 2002).

With respect to plasma proteins, NE is able to cleave coagulation and complement factors, and immunoglobulin, as well as several proteases and protease inhibitors, leading to their activation or loss of function. Interestingly, NE-degraded fragments such as those derived from fibrin and laminin are known to be chemotactic for neutrophils. However, as opposed to the classical notion that the NE is a pro-inflammatory factor, recent studies suggest that NE is capable of degrading various pro-inflammatory cytokines such as IL-1, TNF (Owen *et al.,* 1997), IL-2 and IL-6 (Bank *et al.,* 1999). Kawabata and colleagues (2002) suggested that NE-induced release of

transforming growth factor-β, an anti-inflammatory cytokine, may be important in the remodelling of inflammation.

1.6 ANTIMICROBIAL MECHANISMS OF NEUTROPHILS

Among the elements of the human host defence armamentarium against microbial pathogens, neutrophils have a prominent role (Seguchi and Kobayashi, 2002). They are secretory cells that, on interaction with a wide array of stimuli, release microbicidal and pro-inflammatory agents into the extracellular milieu. In general, they utilize oxygen (O_2)-dependent and -independent mechanisms to eradicate microbial pathogens.

The oxygen-independent mechanisms encompass the contents of the three neutrophil granules: azurophil, specific and gelatinase granules, which contain characteristic proteases, antimicrobial proteins and peptides, and enzymes. Antimicrobial proteins such as defensins, bactericidal/permeability-increasing protein and the enzyme lysozyme, predominantly function by disrupting anionic bacterial surfaces, probably rendering bacteria more permeable. Proteases such as neutrophil elastase (NE) and cathepsin G (CG), degrade bacterial proteins, including virulence factors (Mayer-Scholl *et al.,* 2004). Roos and colleagues (2003) suggested that degranulation could be induced by a wide variety of stimuli that interact with distinct surface receptors and are able to elicit other neutrophil responses. Stimuli inducing degranulation include chemotactic factors (fMLP), chemokines (IL-8), FcγR ligands, and cytokines (G-CSF, GM-CSF, and TNF). The importance of the oxygen-independent defence mechanism is made clear in two rare inherited diseases, the Chediak-Higashi syndrome (neutrophils contain giant granules resulting from specific and azurophil-granule fusion) and specific granule deficiency.

Although neutrophils produce and release a variety of toxic agents directed toward microbial killing, those systems that depend on reactive products of oxygen metabolism are especially potent (Roos *et al.,* 2003). These agents are produced as a consequence of the respiratory burst, a series of events triggered by phagocytosis on exposure to certain inflammatory mediators, and featuring a dramatic increase in oxidative metabolism, with direct conversion of molecular oxygen to its univalent reduction product, the superoxide anion (O_2^-). Subsequent reactions lead to the formation of other

toxic species, including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$).

1.6.1 NADPH oxidase

The phagocyte NADPH oxidase (respiratory burst oxidase) is a membrane-associated enzyme complex that generates superoxide during the respiratory burst by catalyzing the transfer of electrons from NADPH to molecular oxygen (Kim and Dinauer, 2001; Roos *et al.,* 2003). The O_2^- generated by this enzyme serves as the starting material for the production of a vast array of reactive oxidants. These oxidants are used by phagocytes to kill invading microorganisms, but they also cause "collateral damage" to nearby tissues, such that their production has to be tightly regulated to ensure that they are only generated when and where required (Babior, 1999).

The active NADPH oxidase is made up of two membrane-bound components: gp91^{phox} and p22^{phox}, that together form the oxidase flavocytochrome *b* (cytochrome b_{558}) and at least three cytoplasmic subunits p40 ^{phox}, p47 ^{phox}, and p67 ^{phox} that translocate to the membrane upon cellular activation (figure 4). In addition to these five *phox* components, two small GTPases, Rap1A, which is located in membranes, as well as Rac, which is located in the cytoplasm in a dimeric complex with Rho-GDI (Guanine nucleotide Dissociation Inhibitor), both have been implicated in the regulation of the NADPH oxidase complex (Werner, 2004). Cytochrome b_{558} and p47 ^{phox} and p67 ^{phox} subunits are required for superoxide production, since a deficiency in either of these components results in chronic granulomatous disease (CGD), an inherited disorder characterized by absent phagocyte NADPH oxidase activity and recurrent bacterial and fungal infections (Kim and Dinauer, 2001; Kuribayashi *et al.,* 2002). Cytochrome b_{558} is the redox center of the enzyme and appears to be activated upon binding of the p47 ^{phox} and p67 ^{phox} subunits (Kim and Dinauer, 2001).

1.6.2 NADPH oxidase activation

Activation of NADPH oxidase through receptor-mediated signaling by chemoattractants involves modification of the GDP-binding state of G-proteins (DeLeo and Quinn, 1996), thus, the activating receptor catalyses exchange of GDP for GTP by both the G-protein α subunit and low molecular weight G-proteins of Ras, Rho and

ARF (ADP-ribosylation factor) families (Kuribayashi *et al.,* 2002). This process leads to serial activation of phospholipases (PL) C and D, and the generation of lipid second messengers. Several converging pathways (protein tyrosine kinases/phosphatidylinositol 3-kinase; Ras/Rho; PLD) activate the serine kinase Raf, which together with the serine/threonine kinase, protein kinase C (activated by PLC/PLD-derived diacylglycerol), activates MAP kinases, which in turn cause the phosphorylative activation of cytosolic PLA_2 (Alonso *et al.,* 1998).

With respect to activation of NADPH oxidase in neutrophils and other phagocytes, the key events are activation of kinases, particularly protein kinase C, and cytosolic PLA_2 (Shiose and Sumimoto, 2000). Phosphorylation of p47 ^{phox} at several serine residues in the SH3-containing C-terminal region alters the conformation of the polypeptide. This in turn leads to unmasking of SH3 domains enabling weak interaction of p47 ^{phox} with p22 ^{phox} (Segal *et al.,* 2000; Kuribayashi *et al.,* 2002), by binding to the proline-rich region (PRR) of p22^{phox}. However, this event alone is insufficient for efficient activation of NADPH oxidase, a second complementary/synergistic mechanism being required. This is provided by low concentrations of arachidonic acid, generated during cleavage of membrane phosphatidylcholine by cytosolic phospholipaseA_2 (PLA_2). Arachidonic acid maximizes the interactions of phosphorylated p47 ^{phox} with p22 ^{phox} resulting in complete activation of oxidase (Shiose and Sumimoto, 2000). However, a recent report has suggested that cytosolic PLA_2 is not involved in the activation of NADPH oxidase.

The component p47 ^{phox} in combination with p67 ^{phox}/ p40 ^{phox} interacts with p22^{phox} and initiates the electron-transporting activity of the oxidase; p47 ^{phox} and p67 ^{phox}, both which are essential for NADPH oxidase activation, have distinct roles in the regulation of electron flow in cytochrome b_{558}; p67 ^{phox} facilitates electron flow from NADPH to the flavin center resulting in the reduction of flavin adenine dinucleotide (FAD), while p47 ^{phox} is required for electron flow to proceed beyond the flavin center to the heme groups in cytochrome b_{558} and then to molecular oxygen. These events are summarized in figure 1.4. The final step in the electron transport chain occurs when oxygen accepts an electron and is converted to the superoxide radical:

$$2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$$

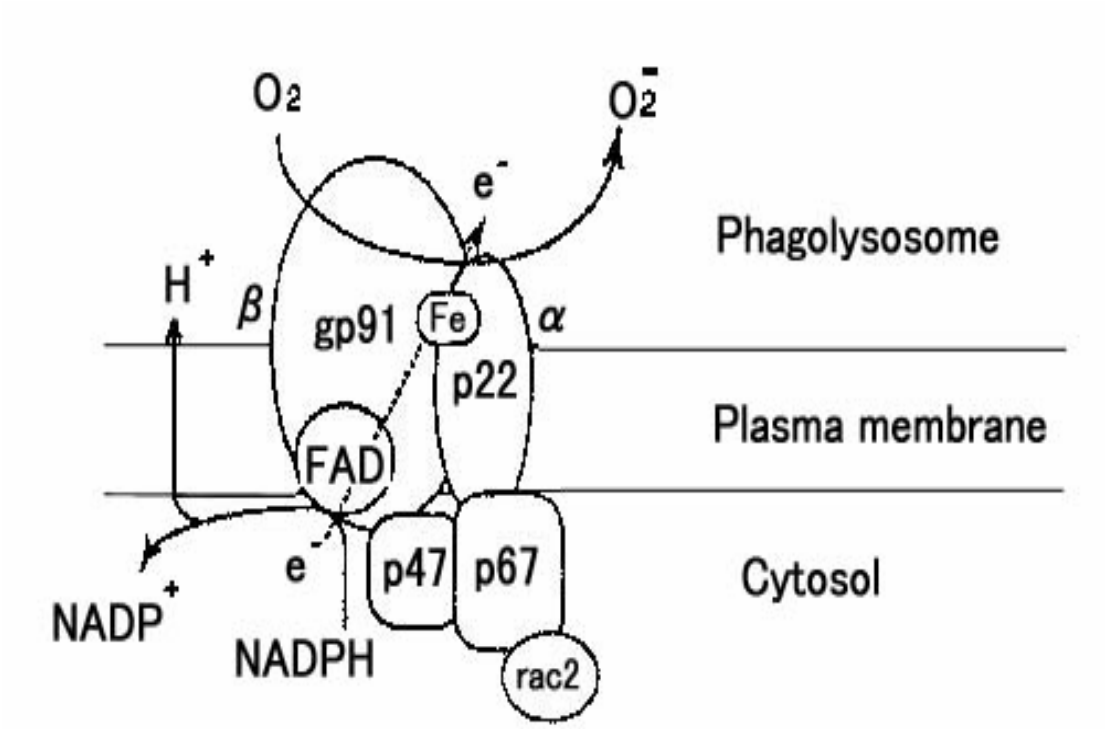

Fig. 1.4: Following stimulation by variety of stimuli, the cytosolic components of NADPH oxidase in the neutrophil become heavily phosphorylated and migrate to the membrane, where they associate with cytochrome b_{558} to assemble the active oxidase, which transfers electrons from the substrate to oxygen by means of its electron-carrying prosthetic group with resultant generation of $2O_2^{\cdot -}$.

1.7 Respiratory burst of human neutrophils

During the phagocytosis of microbial intruders, professional phagocytes of the innate immune system increase their oxygen consumption through the activation of NADPH-oxidase, resulting in the generation of superoxide anion $2O_2^{\cdot -}$. This oxygen-derived radical is a weak and unstable antimicrobial oxidant, but functions as precursor of a series of more potent microbial oxidants (Leto, 1999).

Superoxide is rapidly converted to the stable oxidant hydrogen peroxide (H_2O_2), either by spontaneous dismutation or by enzymatic transformation by superoxide dismutase (Dröge, 2002):

$$O_2^{\cdot -} : O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

The antimicrobial potential of H_2O_2 is dramatically potentiated by the granule enzyme, myeloperoxidase (MPO), which utilizes this oxidant to oxidize chloride to the extremely potent oxidizing agent hypochlorous acid (HOCl) (Roos *et al.,* 2003):

$$H_2O_2 + Cl \xrightarrow{MPO} H_2O + HOCl$$

Neutrophils transform H_2O_2/ $O_2^{\cdot -}$ to hydroxyl radical ($^{\cdot}OH$), the most potent oxidant known in biological systems by the iron-catalyzed Haber-Weiss reaction:

$$H_2O_2 + O_2^{\cdot -} \xrightarrow{Fe^{2+}/\ Fe^{3+}} {}^{\cdot}OH + OH^- + O_2$$

However, production of $^{\cdot}OH$ by neutrophils via this pathway has only been demonstrated *in vitro* in the presence of added iron. A transition metal-independent pathway of $^{\cdot}OH$ generation has been demonstrated in neutrophils which involves the interaction of $O_2^{\cdot -}$ and HOCl (Ramos *et al.,* 1992):

$$HOCl + O_2^{\cdot -} \longrightarrow {}^{\cdot}OH + O_2 + Cl^-$$

Neutrophils have also been reported to generate a significant amount of singlet oxygen [O_2 ($^1\Delta g$)], a highly reactive, diffusible and long-lived electronically excited state of molecular O_2. They do this by transforming up to 20% of O_2 consumed by NADPH Oxidase to [O_2 ($^1\Delta g$)] by a MPO-dependent pathway involving interaction of H_2O_2 and HOCl (Steinbeck *et al.,* 1992; Mayer-Scholl *et al.,* 2004):

$$H_2O_2 + OCl^- \longrightarrow O_2(^1\Delta g) + H_2O + Cl^-$$

These phagocyte-derived oxidants, acting directly, or through more stable intermediates, are powerful antimicrobial agents. They are indiscriminate, and if released extracellularly during hyperacute or chronic activation of phagocytes, they pose the potential threat of oxygen toxicity to bystander host cells and tissues in the vicinity of inflammatory reactions. In this regard they are cytotoxic for eukaryotic cells,

as well as being potential carcinogens, pro-proteolytic, pro-adhesive and immunosuppressive (Mayer-Scholl *et al.,* 2004).

1.8 NITRIC OXIDE SYNTHASE

Production of nitric oxide (NO) within phagocytes is an important component of host defence against microbial infections. Although NO *per se* is only weakly antimicrobial, its microbicidal activity is considerably enhanced by reaction with O_2^- to yield the highly reactive anion, peroxynitrite (Koppenol, 1998).

1.9 CALCIUM AND NEUTROPHIL ACTIVATION

Unraveling of the mechanisms by which activated neutrophils handle calcium (Ca^{2+}) has resulted in identification of strategies utilized by microbial pathogens to promote dysregulation of Ca^{2+} homeostasis, which in turn may contribute to excessive inflammatory responses, as well as to microbial virulence and persistence (Anderson *et al.,* 2002).

1.9.1 Calcium fluxes and restoration of Ca^{2+} homeostasis in activated neutrophils

During evolution cells have developed many sophisticated and uniquely tailored systems to effect efficient and fast spatio-temporal changes in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$). Thus, both the outer cell plasma membrane and that of intracellular organelles are equipped with highly specialized proteins, which regulate $[Ca^{2+}]_i$ through influx from the extracellular space and by active extrusion in the case of plasma membrane, and those which allow mobilization from, and uptake into intracellular stores such as the sarco-endoplasmic reticulum, mitochondria, Golgi apparatus, nucleus and acidic granules (Arredouani, 2004).

The increase in $[Ca^{2+}]_i$ is a powerful stimulus to cell activation. Transient elevations in cytosolic free Ca^{2+}, precede and are a prerequisite for the receptor-mediated activation of many neutrophil functions, including activation of β_2-integrin and adhesion to vascular endothelium, superoxide production through NADPH oxidase (Lucas *et al.,* 2003), degranulation, and activation of pro-inflammatory cytosolic nuclear transcription factors, including NF-kB (Dolmetsch *et al.,* 1997; Tintinger, *et al.,* 2005).

1.9.2 Ca^{2+} release from Stores

Calcium mobilization from stores during cellular responses to chemoattractants appears to be an essential mechanism for cellular activation (Bolotina, 2004; Oommen *et al.,* 2004). Intracellular Ca^{2+} in the neutrophils is reportedly stored in specialized storage vesicles known as calciosomes (Favre *et al.,* 1996; Corbett and Michalak, 2000; Balsinde and Balboa, 2005). However, since there are two distinct cellular locations of Ca^{2+} stores in neutrophils, these may have differential involvement in the activation of proinflammatory functions that utilize different molecular/biochemical mechanisms of Ca^{2+} mobilization (Pettit and Hallet, 1996; Steel and Anderson, 2002). One site is located peripherally under the plasma membrane and the other (probably calciosomes) is localized in the juxtanuclear space and is mobilized by the chemoatractant, N-formyl-L-leucyl-L-phenylalanine (fMLP) (Pettit and Hallet, 1996).

Occupation of neutrophil membrane receptors for the chemotactic tripeptide, fMLP, results in receptor-G-protein coupling with consequent activation of phospholipase C (PLC) and generation of inositol 1,4,5 triphosphate (IP_3) by hydrolysis of phosphatidylinositol 4,5-biphosphate (Alonso *et al.,* 1998; Patti and Banting, 2004). Once generated, inositol 1,4,5 triphosphate binds to an intracellular IP_3 receptor located on the surface of intracellular Ca^{2+} stores (endoplasmic reticulum), resulting in a rise in $[Ca^{2+}]_i$ (Machaca and Hartzell, 1999; Linn and Gafka, 2001). The phenomenon of calcium release via the IP_3 receptor is commonly termed IP_3-induced Ca^{2+} release (IP_3-ICR). These events are extremely rapid, occurring within less than 1 second after the ligand-receptor interaction (Arredouani, 2004). In the case of neutrophils the abrupt increase in cytosolic Ca^{2+} following exposure to fMLP, results exclusively from release of the cation from intracellular stores with little or no contribution at this early stage (within the first 30 – 60 sec.) from extracellular Ca^{2+} and results in an increase in the basal $[Ca^{2+}]_i$ from around 100 nM to ± 1 µM (Anderson and Goolam Mahomed, 1997; Geiszt *et al.,* 1997).

Extracellular Ca^{2+} influx is delayed, being detectable one minute after addition of fMLP and terminating around 5 minutes. This type of influx is a characteristic of store-operated Ca^{2+} influx (i.e. primarily involved in refilling of stores like ER, as opposed to contributing to activation of neutrophils) and is operative in a large variety of non-excitable cells, including neutrophils. Thus, the empty Ca^{2+} stores activate store-

operated Ca^{2+} channels (SOCCs) in the plasma membrane, which then allow Ca^{2+} ions to enter the cell. Ca^{2+} entry through this pathway is termed store-operated Ca^{2+} entry (SOCE), formerly known as capacitative Ca^{2+} entry (Machaca and Hartzell, 1999). When the Ca^{2+} stores are replete, the store-operated channels are closed, but once the stores discharge their contents, the store-operated channels open and Ca^{2+} ions enter the cell (Berridge *et al.,* 2000). The mechanism for coupling the stores to activation and deactivation of the store-operated channels are unknown (García-Sancho, 2000).

1.9.3 Restoration of Ca^{2+} homeostasis

Restoration of Ca^{2+} homeostasis in neutrophils is essential to prevent Ca^{2+} overload and hyperactivity of the cells (Corbett and Michalak, 2000; Anderson *et al.,* 2002). This is achieved by rapid clearance of cytosolic Ca^{2+} by efflux and is accomplished primarily through the action of the plasma membrane and endo-membrane Ca^{2+}-ATPases which are regulated by calmodulin and adenosine 3´, 5´ cyclic monophosphate (cyclic AMP)-dependent protein kinase (Steel and Anderson, 2002) respectively, while extracellular cation is excluded from the cells through the membrane depolarizing activity of NADPH oxidase.

Efficient Ca^{2+} clearance by these systems is greatly facilitated by the membrane depolarizing action of NADPH oxidase, which limits influx of extracellular Ca^{2+} (Rada *et al.*, 2003; Oommen *et al.,* 2004). The superoxide-generating NADPH oxidase undergoes Ca^{2+}-dependent activation during exposure of cells to chemoattractants, cytokines and opsonised agents. When activated, the oxidase causes the membrane potential to rise between +30 and +50 mV, reaching positive values (Schrenzel *et al.,* 1998; Jankowski and Grinstein, 1999). In the absence of any electrical effect, Ca^{2+} influx would occur when Ca^{2+} channels are open in the phagosomal and plasma membranes (Lundqvist-Gustafsson *et al.,* 2000).

However, when the oxidase is activated and the membrane potential reverses, the cell becomes more positive inside relative to the outside, so that the influx of positively charged ions, like Ca^{2+}, is impeded (Hallett, 2003). In other words, despite the Ca^{2+} channels being open, there would be no further net flux of Ca^{2+} into the cells. This has been shown with neutrophils from patients CGD, which are unable to activate NADPH oxidase with consequent failure of membrane depolarization. In CGD neutrophils, the

Ca^{2+} influx is exaggerated (Rada, *et al.*, 2003). Such cells are easily activated, hyper-responsive and prone to degranulate, all conditions that may lead to inappropriate activation and inflammatory disease. Thus, the activity of the neutrophil oxidase may be an important checkpoint for inflammatory conditions by restraining excessive Ca^{2+} influx and controlling neutrophil aggression. These events are summarized in Figure 1.5.

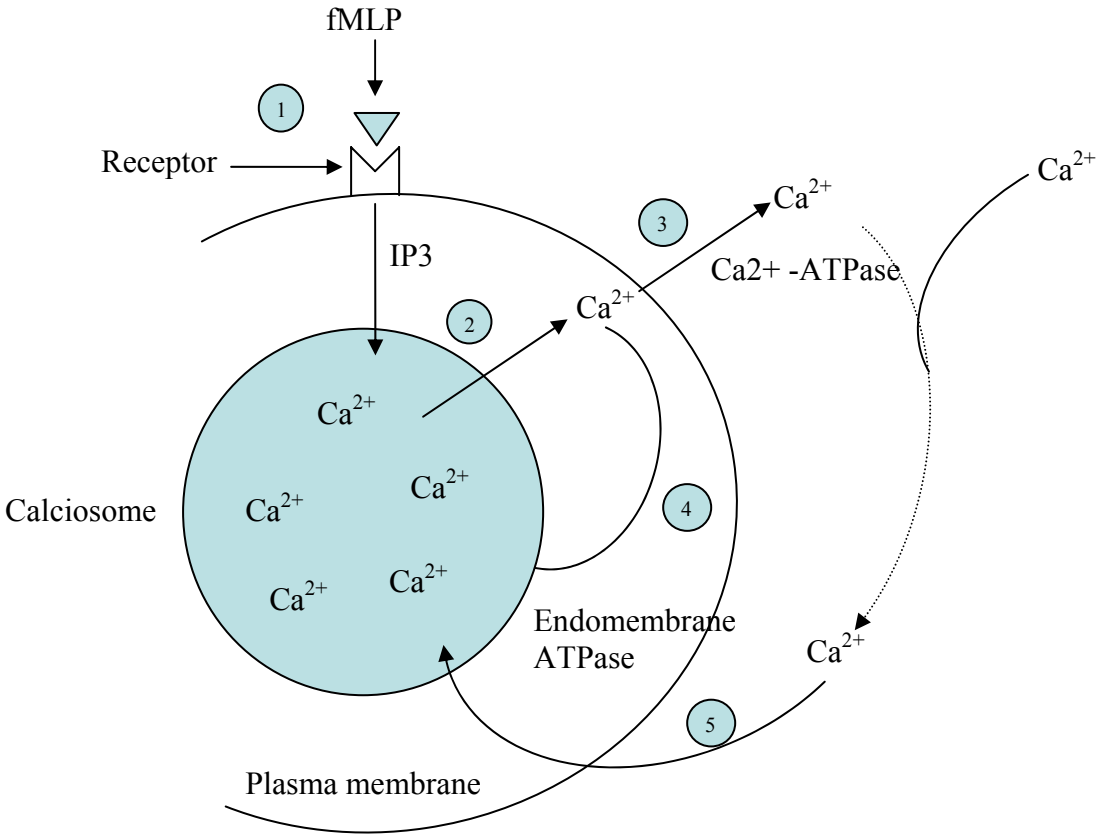

Fig. 1.5: Mechanisms of release of Ca^{2+} from intracellular stores, and clearance of cytosolic Ca^{2+} in fMLP-activated human neutrophils: 1) fMLP binds to its receptor and generates inositol triphosphate (IP3); 2) immediate release of Ca^{2+} from calciosomes into the cytosol 3) concomitant efflux of Ca^{2+} across the plasma membrane (Ca^{2+}-ATPase mediated); 4) early re-uptake of Ca^{2+} into calciosomes mediated by the endomembrane Ca^{2+}-ATPase; and 5) delayed (60 sec – 5 min) store-operated influx of extracellular Ca^{2+} to refill intracellular stores (Redrawn from: Tintinger et *al.*, 2005).

1.10 INTERACTIONS OF PORE-FORMING PNEUMOLYSIN WITH NEUTROPHILS

Pneumolysin, a membrane-damaging, pore-forming toxin produced by *Streptococcus pneumoniae* is considered to be intimately involved in the pathogenesis of infections caused by this microbial pathogens (Cockeran *et al.,* 2001). It is released during autolysis of bacteria, and has also been reported to disrupt Ca^{2+} homeostasis in neutrophils (Anderson *et al.,* 2002) by a non-cytolytic pore-forming mechanism, which results in influx of extracellular Ca^{2+} and flooding of the cytosol with the cation (Cockeran *et al.,* 2001).

In addition to being cytotoxic for eukaryotic cells, pneumolysin has been demonstrated to potentiate the pro-inflammatory activities of neutrophils and macrophages (Wellmer *et al*., 2002), and has also been reported to activate Ca^{2+}-dependent, cytosolic nuclear transcription factor in monocytes/macrophages, resulting in activation of the genes encoding IL-1, IL-6, TNF-α and inducible nitric oxide synthase (Anderson *et al.,* 2002). The pro-inflammatory potential of pneumolysin is supported by *in vitro* studies, in which treatment of phagocytes with this microbial toxin resulted in increased release of granule enzymes and proinflammatory cytokines (Cockeran *et al.,* 2002; 2003). Some of the pro-inflammatory effects of pneumolysin may also be due to cytolysis of various host cell types, including ciliated respiratory epithelium, and endothelial cells, as well as to activation of PL A_2 in endothelial cells.

1.11 LYMPHOCYTE DEVELOPMENT AND HETEROGENEITY

Lymphocytes under normal conditions make up about 20% to 35% of all white blood cells, but proliferate rapidly in the face of infection. There are two basic types of lymphocytes: the B lymphocytes and the T lymphocytes. B lymphocytes develop into plasma cells, which in turn produce highly specific antibodies against foreign antigens. Other B lymphocytes act as memory cells, ready for subsequent activation by the same organism. Some T lymphocytes kill invading cells directly, while others interact with different immune system cells, regulating the immune response (Abbas *et al.,* 1997).

In the initial stages of their development, lymphocytes do not produce surface receptors for antigens and are, therefore, unresponsive to antigens. As they mature, they begin to

express antigen receptors, become responsive to antigenic stimulation, and develop into different functional classes (Hale and Haynes, 1999). Lymphocytes consist of distinct subsets that are quite different in respect of their functions and protein products, even though they all appear morphologically similar. In humans, B lymphocytes, are so called because in birds they were first shown to mature in the Bursa of Fabricius. In humans, B lymphocytes mature in the bone marrow, and are the only cells capable of producing antibodies. The antigen receptors of B-cells are membrane-bound forms of antibodies. Interaction of antigen with these membrane antibody molecules initiates the sequence of B-cell activation, which culminates in the development of effector cells that actively secrete antibody molecules.

A second major class consists of T lymphocytes, whose precursors also arise in the bone marrow and then migrate to and mature in the thymus (thymus-derived). T lymphocytes are further subdivided into functionally distinct populations, the helper/inducer T-cells and the cytolytic/suppressor T-cells. The principal functions of the T lymphocytes are to regulate all immune responses to protein antigens and to serve as effector cells for the elimination of intracellular microbes. T-cells do not produce antibodies, they have specificity for antigens; they recognize only peptide antigens attached to proteins that are encoded by genes in the major histocompatibility complex (MHC) and expressed on the surface of other cells, and as a result, they recognize and respond to cell surface-associated but not soluble antigens (Katagiri *et al.,* 2002). Two major classes of T-cells are distinguished one from the other by the expression of surface markers CD4 and CD8. Helper cells are $CD4^+$ T-cells; cytotoxic cells are $CD8^+$ T-cells. CD4 and CD8 are surface glycoproteins, which function as adhesion molecules and T-cell "co-receptors" for antigen.

In addition to T and B lymphocytes up to 10% of the circulating lymphocyte population is composed of large granular lymphocytes known as natural killer (NK) cells (Hale and Haynes, 1999). NK cells were named for their ability to kill tumours in a non-MHC-restricted fashion without the need for prior activation by tumour antigens. NK cells arise in the bone marrow and share a common precursor with T lymphocytes. Although NK cells can develop within and traffic through the thymus, they do not rearrange or productively express a T-cell receptor (TCR), and they do not require the thymic microenvironment for maturation.

1.12 CD4 AND CD8 T-CELL FUNCTIONS

Although the CD4 and CD8 glycoproteins show no close sequence or structural similarities, they are nevertheless functionally homologous. CD4 and CD8 are expressed on two mutually exclusive subsets of mature T-cells. The CD4 molecule is expressed by cells (mainly helper T-cells [Th], but also a small subset of cytotoxic T-cells [Tc]) that interact with MHC class II antigen, whereas CD8 exhibits a reciprocal expression on cytotoxic T-cells that recognize class I antigens (Dofman and Germain, 2002).

CD4 and CD8 increase the intensity of cell-to-cell interactions by binding to monomorphic determinants on the appropriate MHC molecules of the target cell. They also have a regulatory function; their antibodies can synergize with anti-CD3 antibodies in T-cell activation because of their association with the T-cell specific tyrosine kinase $p56^{lck}$, which might bring $p56^{lck}$ into close proximity with the TCR complex, particularly its potential substrate the CD3ζ chain, during transient interactions of CD4 and CD8 with TCR-antigen/MHC complex (Hale and Hyners, 1999; Sewell *et al.,* 1999).

1.13 Helper T lymphocytes

$CD4^+$ T-cells can be subdivided into several populations using various operational and phenotypic parameters (Mosmann and Sad, 1996). At least two general classes of Th cells can be defined, based on their cytokine secretion profiles. In very broad terms, Th1 T-cells are considered to be responsible for cell-mediated effector mechanisms; they are characterized by production of interferon-γ (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-β (TNF-β), whereas Th2 T-cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, cytokines that play a greater role in the regulation of antibody production. Th1 and Th2 cells appear to differ quite markedly in their requirements for activation and growth. Th1 cells use IL-2 as their autocrine growth factor and respond weakly or not at all, to IL-4. Th2 cells produce and respond to IL-4, but will also proliferate strongly in response to IL-2. An important feature of Th1 and Th2 cells is the ability of one subset to regulate the activities of the other (Prabhakar *et al.,* 2004).

Th1 cells down-regulate antibody responses driven by Th2 cells. In addition, products of one subset can antagonize the activation of the other; IFN-γ inhibits the proliferative response of Th2 cells (driven by either IL-2 or IL-4), but has no effect on that of Th1 cells. Similarly, IL-10, originally called cytokine synthesis inhibitory factor (CSIF), inhibits the production of cytokines, particularly IFN-γ , by Th1 cells and inhibits their autocrine growth. The occurrence of such mutually inhibitory interactions of Th1 and Th2 cells helps to explain how the immune system can selectively trigger various effector mechanisms.

However, the divisions are not absolute and there is considerable overlap or redundancy in function between the Th cells that are assigned to the different subsets. Indeed, some researchers feel that the division of helper T cells into Th1 and Th2 subsets has been overplayed, and that in reality the situation is not so polarised, with many cells falling into the Th0 (intermediate) category (Hickling, 1998). Another subset of T-cells with immunosuppressive bystander effects, secretes T-cell growth factor-β as the dominant cytokine and has been designated Th3.

1.14 ANTIGEN PROCESSING AND PRESENTATION TO T-CELLS

Antigen processing and recognition is a key feature of the immune response to intracellular microorganisms and viruses (Kaufmann and Schaible, 2005). It has long been known that B-cells can recognize soluble antigens directly, but T-cells require the presence of an accessory cell population in order to be activated by antigens (Bachman and Kopf, 2002). The first evidence that an active processing step was required before antigen could be recognized by T-cells came from the studies by Ziegler and Unanué (1981) on macrophage presentation of *Listeria monocytogenes* antigens to polyclonal class II-restricted T-cells. They found that there was a lag period between the binding of antigen to the macrophage and detection of antigen recognition by T-cells. Macrophages rendered metabolically inactive by fixation with paraformaldehyde immediately after pulsing with antigen were not recognized by the T-cells, but were able to present *L. monocytogenes* antigens to specific T-cells if fixed after a lag period of 45 – 60 minutes. Similar results were subsequently obtained by other investigators using soluble protein rather than particulate bacterial antigens.

There are two major pathways of antigen processing (Figure 1.6) within the antigen-presenting cell (APC) and target cell (Hudrisier and Bongrand, 2002; Katagiri *et al.,* 2002). In the exogenous pathway, soluble proteins are taken up from the extracellular environment, generally by specialised or 'professional' APCs such as macrophages, B-cells or dendritic cells (DC). During processing, antigens are partially degraded and resulting peptide fragments are bound to MHC class II molecules. Peptide-MHC-class II complexes are brought to the cell surface of the APC for recognition by the TCRs of the $CD4^+$ T-cells (Hale and Haynes, 1999; Hudrisier and Bongrand, 2002).

As with CD8, the CD4 molecule functions as a co-receptor, increasing the strength of the interaction between the T-cell and the APC (Hickling, 1998). $CD4^+$ helper T-cells that are activated by the peptide-class II MHC complexes secrete cytokines such as IL-2 (IL-2, T-cell growth factor), which ultimately activate and promote the proliferation of T-cells (Blattman *et al.,* 2003; Driver, 2004), as well as other cells, including B-cells and macrophages to participate in antigen-specific immune responses.

Alternatively, antigens may be released within the cell as the result of infection by virus or other obligate intracellular pathogens or from alterations in normal cellular proteins generated by the tumour cells (Hale and Haynes, 1999). The endogenous pathway processes proteins that have been synthesized within the APC. In this pathway, proteins in the cytoplasm are cleaved by proteosomes (a proteolytic organelle) into peptide fragments of ~ 20 amino acids in length. These fragments are then transported into the lumen of the endoplasmic reticulum (ER) via the transporters associated with antigen processing (TAP) complex, where they encounter newly formed heavy-chain molecules of MHC class I and their associated b_2 microglobulin ($b_2$mg) light chains (Hudrisier and Bongrand, 2002; Al-Daccak *et al.,* 2004). The heavy chain, light chain and peptide form a trimeric complex, which is then transported to and expressed on the cell surface.

T-cells that express the CD8 cell-surface marker recognise antigens that are presented by MHC class I molecules, while those which express the CD4 cell-surface marker recognise antigens that are presented by MHC class II molecules (Hickling, 1998; Hale and Haynes, 1999; Dustin and Cooper, 2000; Kaufmann and Schaible, 2005). Cytolytic T-cells lyse or kill host cells that produce foreign antigens, such as cells infected by viruses and other intracellular microbes. Suppressor T-cells down-regulate B and T-

cells and thus modulate humoral and cell mediated immune responses (Dorfman and Germain, 2002).

This classical segregation of $CD4^+$ and $CD8^+$ T-cells is critical for control of bacterial and viral infections respectively, and was derived from the notion that intracellular bacteria typically survive inside the phagosomal compartments with access to the MHC II molecules, which are responsible for peptide presentation to $CD4^+$ T-cells. By contrast, viruses are newly generated by the protein-synthesis machinery within various host cells; which is the cytoplasm and the ER. Hence, viral peptides can be readily loaded onto MHC I molecules, which present them to $CD8^+$ T-cells. Accordingly, the intracellular habitat of the pathogen dictates the type of T-cell population responsible for protection. It is well known that not only $CD4^+$ T-cells, but also $CD8^+$ T-cells participate in acquired immunity to numerous intracellular bacteria. In fact, recent studies suggest that antigen from phagosome-processed bacteria might be presented to $CD8^+$ T-cells by a cross presentation pathway (Kaufmann and Schaible, 2005). These events are summarized in Figure 1.6.

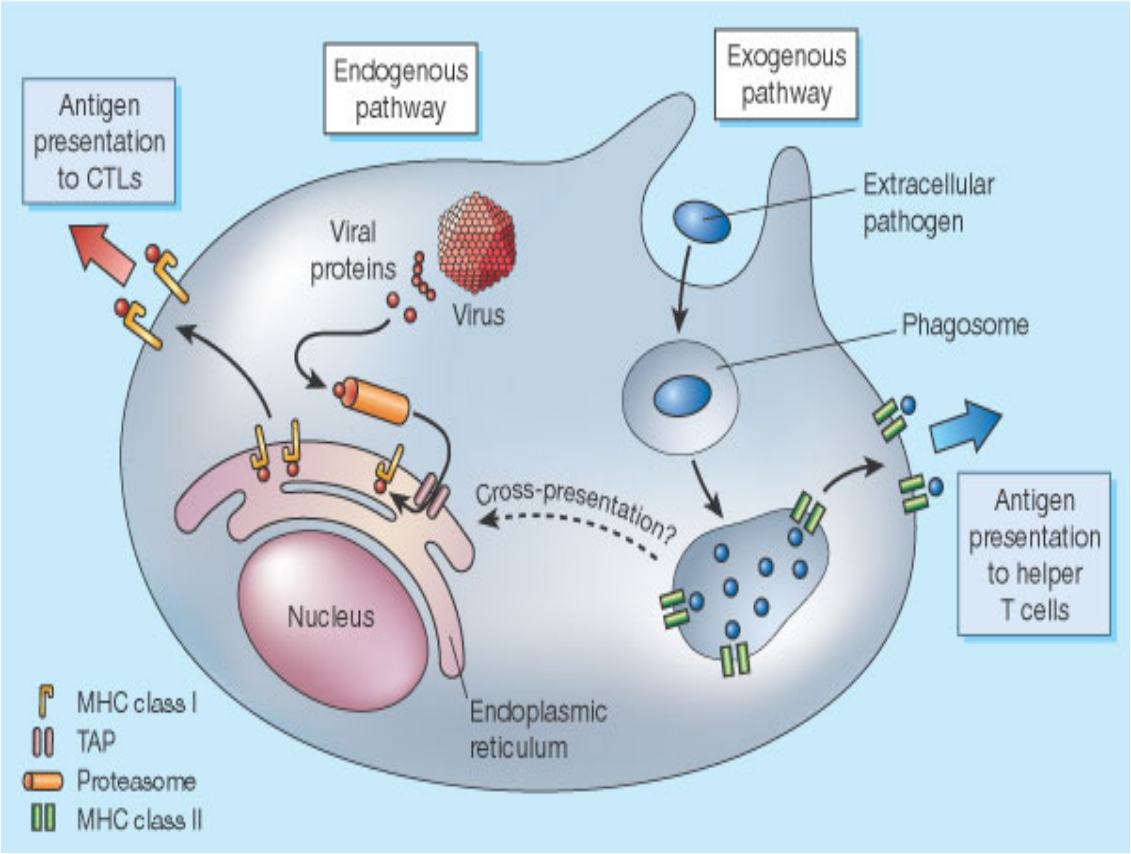

Fig. 1.6: Two major pathways of antigen processing and presentation exist. These are the endogenous MHC class I, and exogenous MHC class II pathways. Until recently the way in which antigen from phagosomal bacteria enter the MHC I pathway was unknown. These pathways are not mutually exclusive. Phagosome-processed bacteria are introduced to MHC I molecules by cross-presentation. (Source: http://www.vetmed.wsu.edu/research_vmp/itp/).

1.15 Antigen recognition by T-cells

Sensitivity, specificity, and context discrimination are three key properties of T-cell antigen recognition. T-cells recognize protein antigens in the form of peptide fragments that are presented at the cell surface by major histocompatibility complex (MHC) class I or MHC class II molecules (Lanzavecchia *et al.*, 1999; Pecht and Gakamsky, 2005). When the antigen-specific T-cell receptor (TCR) on the T-cell surface (specifically the α/β chains of the CD3 complex) interacts with the appropriate peptide–MHC complex, it triggers phosphorylation of the intracellular domains of the CD3 z (zeta) chains. Subsequently, the zeta-associated protein 70 (ZAP-70) binds to the phosphorylated zeta

chains, and is activated. Simultaneous co-ligation of CD4 (or CD8) with the MHC class II (or class I) molecule results in the phosphorylation of the *lck* kinases.

These events stimulate the activation of at least three intracellular signalling cascades. T-cell activation also requires a second co-stimulatory signal (such as the interaction between the cell markers CD28 on the T-cell, and CD80/CD86 on the antigen-presenting cell). This interaction also triggers several intracellular signalling pathways. Activation of T-cells can lead to cell division, cytokine secretion by the T-cell as well as expression of markers associated with the activated state. Alternatively, in the case of cytotoxic T lymphocytes (CTLs), interaction with antigen via the specific TCR leads to destruction of target cells (Hickling, 1998; Pecht and Gakamsky, 2005).

1.16 T-cell activation

Critical to the adaptive immune system is T-cell activation, which depends on the interaction of TCR with antigenic peptides bound to the MHC displayed on the surface of APC (Pahlavani, 1998; Hashemi *et al.,* 1999; Rachmilewitz and Lanzavecchia, 2002). T-cell activation presents a paradigm for both signal transduction and the orchestration of extracellular interactions that lead to the incredible sensitivity and specificity of antigen recognition (Shaw and Dustin, 1997; Lanzavecchia *et al.,* 1999). A characteristic feature of T-cell activation is that sustained TCR signalling is required for cytokine secretion and proliferation.

When finally activated, the TCR receptor complex transmits further signals via a cascade of signalling pathways involving phospholipase C-γ, calcium mobilization, protein kinase C (Trautmann and Valituti, 2003), calcium-dependent kinases and phosphatases, and mitogen-activated protein (MAP) kinase. These signals ultimately result in the activation of the nuclear factor of activated T lymphocytes (NFAT) family and other transcription factors, and the surface expression of activation markers on the plasma membrane.

The earliest surface marker is the CD69 molecule, which is expressed within a few hours of activation (Reddy *et al.,* 2004) and does not initially require new RNA or protein synthesis (Hashemi *et al.,* 1999). This is then followed by the expression of

CD71. The CD71 is the serum iron-transport protein that non-covalently associates with the TCRζ chain in the T-cells where it may play a role in signal transduction (Reddy *et al.,* 2004). Upon full activation of pathways necessary for gene expression, the receptor for IL-2 (CD25) is expressed on the plasma membrane. Surface expression of CD25 requires gene transcription beginning within 2 hrs after TCR stimulation (Hashemi *et al.,* 1999; Reddy *et al.,* 2004).

Although signalling responses occur within seconds of TCR triggering, the biochemical changes associated with TCR activation occur over a period of hours and may be subject to modification by other cellular regulation pathways. The approximate time course of TCR activation pathways is as follows: secretion of cytokines, 2 hours; initiation of DNA replication, 24 hours; cell division, 48 hours; and differentiation into effector cell, days. In the periphery during antigen-specific T-cell responses, most of the T-cells participating in the immune response are eliminated, but a subset of these cells survive and differentiate into long-lived memory cells.

1.17 T LYMPHOCYTE PROLIFERATION

Lymphocyte proliferation upon antigenic challenge plays an essential role in mounting an effective immune response. The large diversity of lymphocyte receptors means that there will be at least a few that can bind to any given foreign antigen. Because each lymphocyte has a different antigen receptor, the number of antigen-responsive cells is very small. To generate sufficient antigen-specific effector lymphocytes to fight an infection, a lymphocyte with appropriate receptor specificity must be activated to proliferate before its progeny finally differentiate into effector cells (Hunt *et al.,* 1999). Lymphocyte proliferation is initiated in the draining lymphoid tissues, where naïve lymphocytes and activated antigen presenting cells come together. On recognition of its specific antigen, a small lymphocyte stops migrating and becomes activated. One of the most rapid consequences of T lymphocyte activation through its antigen receptor is the *de novo* synthesis of IL-2. This is quickly followed by expression of high affinity IL-2 receptor, thus permitting rapid and selective expansion of effector T-cell populations activated by antigens (Abbas *et al.,* 1997; Ellery and Nicholls, 2002; Gaffen and Liu, 2004).

IL-2 is the principal mitogenic factor for activated T-cells, and delivers a proliferative signal through ligation of the IL-2 receptor. This proliferative signal is critically dependent upon cytoplasmic tyrosines on the β-chain of this receptor (IL-2Rβ) becoming phosphorylated in response to ligand (Lord *et al.,* 1998; Blattman *et al.,* 2003). IL-2 exerts its cellular effects through binding to specific cell surface receptors (Hunt *et al.,* 1999). The high affinity IL-2 receptor is a heterotrimeric complex consisting of α-, β-, and γ-subunits, the γc subunit being shared with the receptors of other T-cell mitogens, IL-4, IL-7, IL-9, and IL-15. The α-subunit is responsible for conferring high affinity cytokine binding, while the β- and γ-subunits recruit cytoplasmic molecules, thereby transducing the proliferative signal (Hunt *et al*., 1999; Driver, 2004).

Although other cytokines appear to be partially redundant with IL-2 in this regard, this cytokine is vital for determining the magnitude and duration of primary and memory immune responses. IL-2 also plays a central role in down regulating immune responses (Ellery and Nicholls, 2002). Its absence results in severe autoimmunity due to a failure to eliminate activated T-cells (Gaffen and Liu, 2004). Therefore, IL-2 is described as a growth and survival factor capable of inducing T-cell proliferation. This is supported by the *in vivo* studies of Chen and colleagues (2002), which show that elimination of IL-2 from proliferating T-cells can lead to cytokine withdrawal mediating cell death (Manjunath *et al*., 2001).

Following binding of IL-2 to IL-2R, the lymphoblast now begins to divide, normally duplicating two to four times every 24 hours for 3 - 5 days, so that one naive lymphocyte gives rise to a clone of around 1000 daughter cells of identical specificity, which differentiate into effector cells. After a naïve T lymphocyte has been activated, it takes 4 - 5 days before clonal expansion is completed and lymphocytes have differentiated into effector cells, and adaptive immune responses occur only after a delay of several days. Thus, specific recognition by clonally distributed receptors evolved as a late addition to existing innate effector mechanisms to produce an adaptive immune response. Induced lymphocyte proliferation is represented in Figure 1.7.

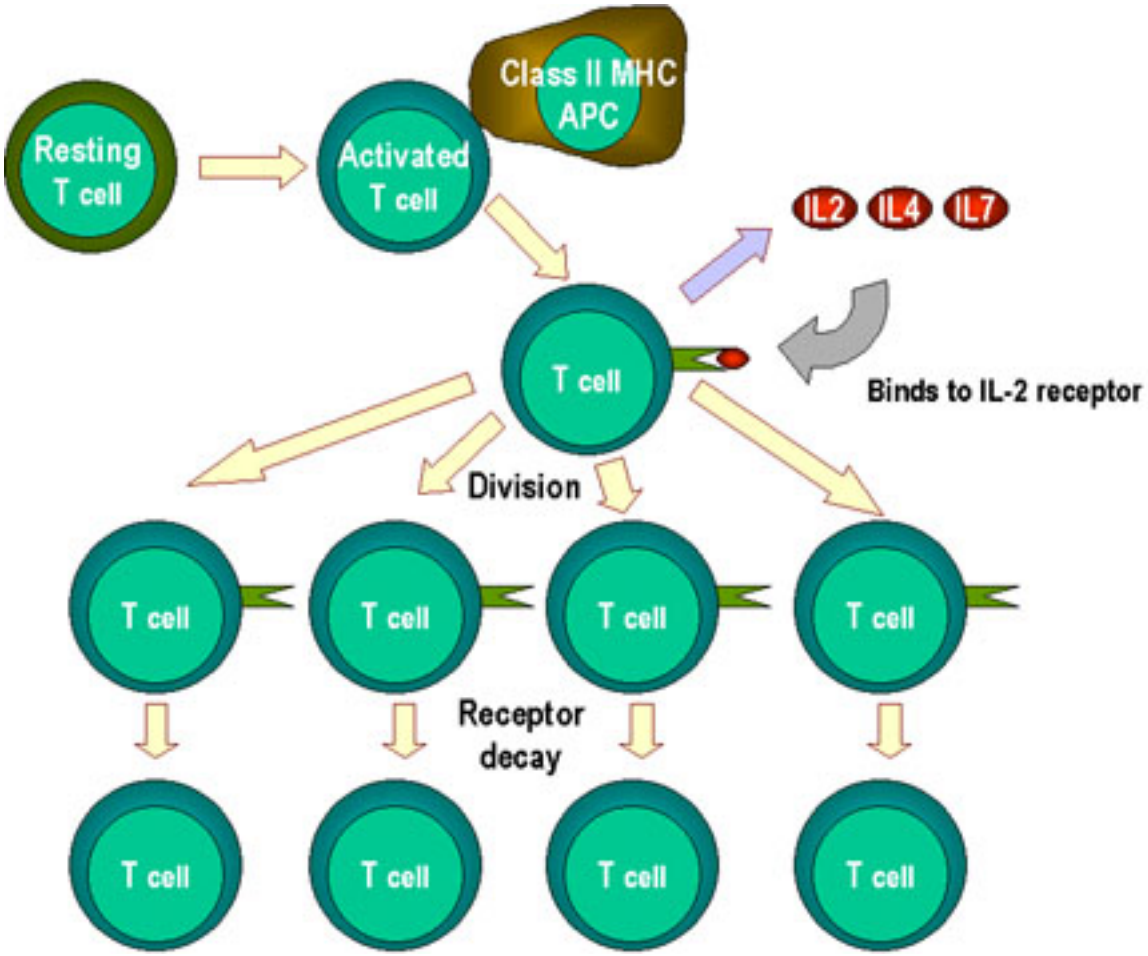

Fig 1.7: When T-cells are resting, they do not produce cytokines such as interleukins 2, 4 or 7, nor do they express large amounts of their receptors. Activation of T cells results in the formation of high affinity IL-2 receptors and induction of the synthesis and secretion of IL-2 and IL-4. These bind to their receptors and the T-cells proliferate into effector T-cells.

During an immune response, antigen-specific T-cells proliferate enormously and develop into effector T-cells capable of immediate effector functions, such as cytotoxicity and cytokine production (Butz and Bevan, 1998). Following a successful immune response, activated effector T-cells undergo large-scale apoptosis, presumably to maintain homeostasis in T-cell numbers (Manjunath *et al.,* 2001). However, some persist after the antigen has been eliminated and are known as memory cells, which ensure more rapid and effective responses on the second encounter with pathogen, thereby providing lasting protective immunity.

The measurement of the proliferation of lymphocytes that occurs following various stimuli (such as exposure to mitogenic agents, polyclonal stimuli or specific antigens) is a fundamental technique for assaying T-cell responses. However, simple enumeration of T-cells before and after such stimulation is laborious, and in most cases is not possible, because the cells that are responding represent only a small percentage of the total cell population at the start of the assay (Hickling, 1998).

1.18 IL-2 receptor structure and composition

As mentioned in section 1.17 page 33, the IL-2R complex is a heterotrimer composed of three distinct subunits, termed IL-2Rα (also known as CD25 or Tac), and is homologous to a similar affinity-modulating subunit in the IL-15 receptor complex (IL-15Rα), IL-2Rβ (p75, CD122; human chromosome 22) and IL-2Rγc (IL-2Rγ, p64, CD132; X chromosome), and these subunits work in concert to coordinate and drive specific signals (Nelson *et al.,* 1996; Chen *et al.,* 2002; Gaffen and Liu, 2004). The IL-2Rβ chain is shared with the IL-15 receptor, and γc is shared with the IL-4, IL-7, IL-9, and IL-15 receptors, all of which also deliver proliferative signals (Chen *et al.,* 2002). Alone neither IL-2Rβ nor γc bind IL-2 detectably, but the IL-2Rβ/γc complex comprises the intermediate affinity IL-2 receptor complex, and is capable of mediating the full spectrum of IL-2-dependent activities if exposed to IL-2 in sufficient quantities (Hunt *et al.,* 1999; Gaffen and Liu, 2004). IL-2Rβ and γc are members of the type I cytokine receptor superfamily, and activate a variety of signalling pathways common to this family (Nelson *et al.,* 1996).

Despite the inability of IL-2Rα to deliver intracellular signals (Ellery and Nicholls, 2002), its physiological significance should not be underrated. Mice with targeted deletions in IL-2Rα and humans with genetic deficiencies in this chain have clearly demonstrated that absence of IL-2α is functionally equivalent to absence of IL-2 (Sharfe *et al.,* 1997; Gaffen, 2001). When IL-2Rα is expressed on one cell, it can augment IL-2 signalling on an adjacent cell that expresses IL-2Rβ and γc but not IL-2Rα. For that reason, IL-2Rα plays a critical role in regulating responses to IL-2 by controlling the affinity of the IL-2R for ligand, even though this chain does not contribute directly to signal transduction due to the extremely short cytoplasmic tail. Resting T-cells express IL-2R in the form of β and γc chains, which bind with moderate

affinity, allowing T-cells to respond to very high concentrations of IL-2. Association of the α chain with the β and γ chains creates a receptor with much higher affinity for IL-2, allowing the cells to respond to very low concentrations of IL-2 (Janeway *et al.,* 2001; Ludányi *et al.,* 2004).

1.19 PROPERTIES AND FUNCTIONS OF CYTOKINES

The development of an effective immune response involves lymphoid cells, inflammatory cells, and haematopoietic cells. The complex interactions among these cells are mediated by a group of secreted, low-molecular-weight proteins that are collectively designated cytokines to denote their role in cell-to-cell communication (Prabhakar, *et al.,* 2004). They assist in regulating the development of immune effector cells, and some cytokines possess direct effector functions of their own. Just as hormones serve as messengers of the endocrine system, so cytokines serve as messengers for the immune system; however, unlike endocrine hormones, which exert their effects over large distances, cytokines generally act locally (Prabhakar *et al.,* 2004).

Unlike hormones, cytokines are not stored in glands as preformed molecules, but are rapidly synthesized and secreted by different cells, mostly after stimulation. Cytokines are pleiotropic in their biological activities and play pivotal roles in a variety of responses, including the immune response, haematopoiesis, neurogenesis, embryogenesis, and oncogenesis. They frequently affect the action of other cytokines in an additive, synergistic, or antagonistic manner. Cytokines have been classified on the basis of their biological response properties into pro-inflammatory (Th1 type) cytokines, for example, IL-1 (α and β), TNF-α, IL-8, IL-11, and IL-6 (Feghali and Wright, 1997; Nikolous *et al*., 1998), or anti-inflammatory (Th2 type) cytokines; IL-10, IL-4 and IL-13 (which is a series of immunoregulatory molecules that control the pro-inflammatory cytokine response) (Gimenes *et al.,* 2005) major properties of different human cytokines are listed in Table 1.1.

The net effect of any cytokine is dependent on the timing of cytokine release, the local milieu in which it acts, the presence of competing or synergistic elements, cytokine receptor density, and tissue responsiveness to each cytokine (Opal and Depalo, 2000).

Cytokines play an important role in the communication between cells of multicellular organisms. Besides their pleiotropic effects, which are often redundant, they exert their actions, which can be auto-, para- or endocrine, via specific cell-surface receptors on their target cells. They are key players in the regulation of the immune response, particularly during infections (Abbas *et al.,* 1997; Gouwy *et al.,* 2005).

1.19.1 Anti-inflammatory cytokines

The immune system is fighting a constant war against pathogens in its territory. This requires not only a potent arsenal for efficient control of pathogens, but also tight regulatory mechanisms in order to avoid excessive collateral damage (Bachmann and Kopf, 2002). Maintaining equilibrium is the daily challenge of the immune system. In order to counterbalance overshooting immune responses, T-cells and APCs secrete anti-inflammatory cytokines that are critical for maintaining a healthy balance between protection and immunopathology. Consequently, downregulation of inflammation is equally important in the host's inflammatory response as initiation inflammation. The failure to control inflammatory responses can lead to extensive tissue damage, thus, defective regulation of inflammation may contribute to the pathogenesis of many autoimmune diseases (Ben-Baruch, 2006). Mechanisms which downregulate inflammatory responses include apoptosis of inflammatory cells, production of inhibitors of activated complement components and production of cytokine receptor antagonists. The cytokines, IL-4, IL-10 and IL-13 are produced predominantly by T-cells and have intrinsic anti-inflammatory activities.

As shown in Table 1.1, acting alone or in concert, IL-4 and IL-10 decrease the production of the pro-inflammatory cytokines, IL-1, IL-6, IL-8, IL-12 and TNF-α (Opal and Depalo, 2000). IL-13 inhibits the production by lipopolysaccharide (LPS)-stimulated monocytes of IL-1, IL-6, IL-8, IL-10, IL-12, granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage inflammatory protein-1α (MIP-1α) and TNF-α, but upregulates IL-1Rα expression. IL-13 does not globally inhibit macrophage functions, but instead selectively inhibits cytotoxic and pro-inflammatory macrophage activities, since it increases the ability of macrophages to present antigens, resulting in an increased T-cell proliferative response.

CYTOKINE	CELL SOURCE	PRINCIPAL EFFECTS
IL-1 α and β	Macrophages and other APCs	Costimulation of APCs and T-cells, Phagocyte activation
IL-2	Activated Th1 cells, Tc cells, NK cells	Proliferation of activated T-cells, NK and Tc cell functions
IL-4	Th2 cells, mast cells	Class II MHC expression, Th2 and Tc-cell proliferation and functions, inhibition of monocyte functions
IL-6	Activated Th2 cells, APCs	Synergistic effects with IL-1 Or TNF to costimulate T-cells
IL-8	Macrophage and other somatic cells	Chemoattractant for neutrophils and T-cells
IL-10	Activated Th2, CD8 T-cells B-cells and macrophages	Inhibition of cytokine production by Th1 cells, promotion of B-cell proliferation, suppression of cellular immunity
IL12	B-cells and macrophages	Proliferation and function of activated Tc and NK cells, Th1 induction, promotion of cell-mediated immunity
TNF-α and -β	Activated macrophage and other stromatic cells	IL-1 like effects
INF-α and -β	Macrophages, neutrophils	Antiviral effects, up-regulation of class I MHC expression
INF-γ	Activated Th1 and NK cells	Promotion of cell-mediated immunity, induction of MHC class I and class II
G-CSF	Fibroblasts, monocytes	Promotion of granulocyte growth
GM-CSF	T lymphocytes, fibroblasts, endothelial cells, monocytes	Promotion of macrophage growth
MCP-1	Monocytes, basophils, mast cells	Macrophage activation, basophil degranulation (histamine release)
MIP-1α abd -β	T-cells, monocytes	Chemoattractant for monocytes and T lymphocytes, integrin expression by T-cells
TGF-β	Activated T-cells, platelets, macrophages	Suppression of cytokine production, antiproliferative for macrophages and lymphocytes

Table 1.1 Major properties of human interleukins and other immunoregulatory cytokines (Source: Janeway, *et al.,* 2001).

1.19.2 Chemokines

The recruitment of inflammatory and immune effector cells to sites of injury or infection is a hallmark of inflammation and an early event leading to the development of an immune response. This process is tightly regulated, in part by a network of locally released mediators, including chemokines, the largest family of cytokines, which form a complex system for the chemotactic activation of all types of leukocytes. These mediators control leukocyte trafficking during homeostatic migration, as well as during inflammation, and provide a linkage between innate and adaptive immunity (Rot and von Andrian, 2004). Together with adhesion molecules, such as integrins and selectins, chemokines and their receptors act primarily as part of a complex molecular network that facilitates the selective movement of specific cell types into, and out of tissues (Coelho *et al.*, 2005)

Production of chemokines is induced directly by exogenous irritants (bacterial and viral products), as well as by endogenous mediators (cytokines; such as IL-1, TNF-α and IFN-γ). Because they bind to specific cell surface receptors, chemokines can be considered second-order cytokines (Thelen, 2001). They appear to be less pleiotropic than first-order pro-inflammatory cytokines because they are not potent inducers of other cytokines and exhibit more specialized functions in inflammation and repair. In response to infection, cytokines and chemokines are produced simultaneously and interact in a complex network as either activators or inhibitors of inflammation (Gouwy *et al.*, 2005).

Receptor expression and generation of different chemokines form the basis for the selective recruitment of leukocytes and determine the composition of the inflammatory infiltrate. Traditionally, chemokines have four conserved cysteines that form two disulfide bonds (Cys1-Cys3 and Cys2-Cys4) C-X-C (where X is any amino acid) and C-C chemokines or α and β chemokines, are distinguished according to the position of the first two cysteines that are adjacent (CC) or separated by one amino acid (CXC) (Laing and Secombes., 2004). Most of the CXC chemokines are chemoattractants for neutrophils (and to some extent lymphocytes) but not monocytes, whereas CC chemokines appear to attract monocytes, granulocytes (except neutrophils) and

lymphocytes. Recently, the third C (γ) branch of these molecules has been discovered. The main chemokines are shown in Table 1.2 below.

Table 1.2: Common human chemokines

Family	Official name	Common name
CxC (α)	CxCL8	IL-8
	CxCL4	PF-4
	CxCL7	NAP-2
CC (β)	CCL2	MCP-1
	CCL3	MIP-1α
	CCL4	MIP-1β
	CCL5	RANTES
	CCL7	MCP-3
	CCL8	MCP-2
	CCL9	MIP-1γ
	CCL12	MCP-5
	CCL13	MCP-4
C (γ)	CL1	Lymphotactin-α
	CL2	Lymphotactin-β
Cx_3C (δ)	Cx_3CL1	Neurotactin/fractalkine

Source: Laing and Secombers, 2004.