



Chapter 1

Literature Review

Aims

The aims of this study were to investigate the direct and indirect interactions of cobalt, palladium, platinum and vanadium with human neutrophils *in vitro*, leading to either hyper-reactivity or under-reactivity of these cells, both of which have adverse health implications. With respect to the former, the pro-oxidative, pro-inflammatory potential of the metals was investigated by measuring their effects (vanadium only) on the generation of the highly toxic reactive oxidant, hydroxyl radical, by activated neutrophils, as well as effects (all of the metals) on the activation and translocation to the nucleus of the cytosolic transcription factor, NF- κ B. In the case of the latter, two strategies were used to investigate the indirect interactions of the metals with neutrophils. These were to characterize the effects of the metals on; i) the chemotactic/ Ca^{2+} -mobilizing functions of three key chemoattractants in innate host defences *viz* C5a, and IL-8; and ii) the interactions of the metals with pneumolysin, a toxin produced by *Streptococcus pneumoniae*, which also triggers innate, protective inflammatory reactions which prevent colonization with this microbial pathogen. *S. pneumoniae* is the most commonly encountered bacterial pathogen in community-acquired pneumonia, with particularly high mortality rates in the very young, the elderly, and those infected with HIV-1. In this setting, pneumolysin has been used as a prototype microbial activator of innate host defences.

Hypothesis

Cobalt, platinum, palladium and vanadium, heavy metals of both environmental and occupational significance, adversely affect, either directly or indirectly, or both, the functions of human neutrophils.

1.1 Metals

All of us are exposed environmentally and/or occupationally to metals. Metals can bind to sulfhydryl groups (SH) as well as to –OH, NH₂ and Cl groups in proteins, enzymes, co-enzymes and cell membranes. This binding can interfere with cellular processes, changing membrane charge, permeability and the antigenicity of

autologous structures (Stejskal & Stejskal, 1999). Metals are therefore potential toxins and have been implicated in the increased incidence of cardiopulmonary diseases in industrialised countries. Inhalation of metals and metal-containing compounds is associated with pulmonary inflammation and tissue injury. Occupational asthma, rhinitis, conjunctivitis and eczema are common amongst refinery workers in the platinum industry (Vanadium, 2001). Mining is South Africa's largest industry sector and South Africa is a world leader in respect of mineral reserves and production (Mwape *et al*, 2004). Figure 1.1 (page 3).

Because of the respiratory problems experienced by many platinum refinery workers, the platinum-group metals palladium and platinum were investigated in this study. Vanadium was included because South Africa dominates the world supply of this metal. Cobalt was chosen because of growing consumption and demand. Furthermore cobalt is a by-product of many platinum-group metal mines (Harding, 2004).

1.1.1 Platinum

Periodic table of elements:

Atomic number: 78

Atomic symbol: Pt

Group name: Precious metal or Platinum group metal

History:

Platinum was discovered in 1735 in Colombia, South America where the native Indians used it. The name "platinum" is derived from the Spanish word "platina" which means silver.

(Platinum. <http://www.pearl1.lanal.gov/periodic/elements/78.html>).

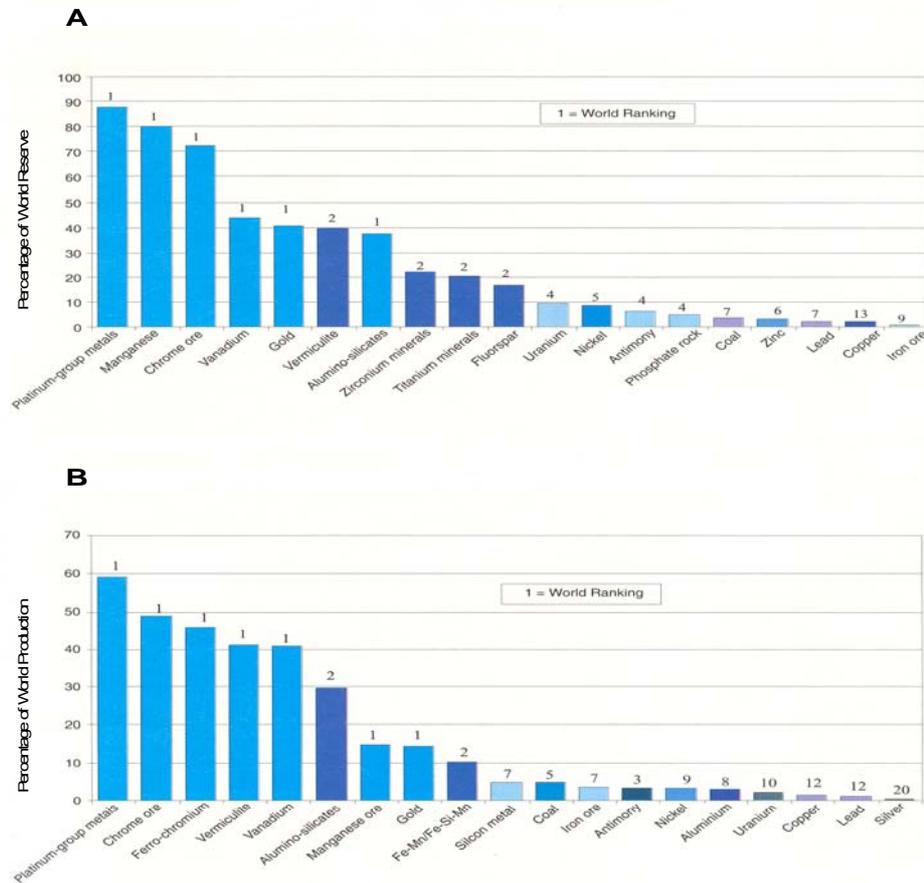


Figure 1.1

- A** South Africa's role in world mineral reserves, 2003
- B** South Africa's role in world mineral production, 2003
(Mwape *et al*, 2004)

Properties and sources:

Platinum is a silvery-white metal with exceptional catalytic properties. It exhibits high resistance to chemical corrosion over a wide temperature range. Platinum has a high melting-point and possesses high mechanical strength and good ductility.

It is found together with the other platinum group metals in the lithosphere or rocky crust of the earth at concentrations of about 0.001 – 0.005 mg/kg. Platinum is found either in the metallic form or in a number of mineral forms.

Economically important sources of platinum exist in South Africa and in the USSR. Smaller amounts are mined in the USA, Ethiopia, in the Philippines and in Colombia (Platinum. <http://www.pearl1.lanl.gov/periodic/elements/78.html>) (Platinum. <http://www.inchem.org/documents/ehc/ehc/ehc125.htm>).

Uses

- Catalysts in the automobile industry, in the production of sulphuric acid and in cracking petroleum products.
- Platinum-cobalt alloys have powerful magnetic properties.
- Resistance wires in high-temperature electric furnaces.
- Coating of missile nose cones and jet engine fuel nozzles, which must perform at high temperatures for long period of times.
- Jewellery.
- In dentistry and as anti-tumour drugs (cisplatin)
(Platinum. <http://www.pearl1.lanl.gov/periodic/elements/78.html>)
(Platinum. <http://www.inchem.org/documents/ehc/ehc/ehc125.htm>)
(Bose, 2002).

Platinum exposure is well known to constitute an occupational health hazard. Occupational sensitization to platinum causes both anaphylactoid, as well as delayed-type hypersensitivity reactions. Low molecular substances such as platinum salts can act as haptens and become antigenic by binding to human serum proteins (Agius *et al*, 1991). Other mechanisms involved include genetic predisposition, non-IgE immunologically-mediated responses and non-specific airway inflammation (Hostýnek *et al*, 1993; Mapp *et al*, 1999).

Platinum occurs in nature together with other group VIII metals of the periodic system, as well as with the sulphides of nickel, copper and iron. Refining of platinum from metal-rich ores is done by rigorous chemical processes involving sequential solubilization and precipitation. During the refining process, complex halogenated salts of platinum are always precipitated (Biagini *et al*, 1986). These complex platinum salts are used in the chemical, photographic and electroplating industries (Ørbaek, 1982). Exposure to the complex salts of platinum by inhalation or skin contact have resulted in allergic respiratory distress and skin symptoms such as itching, redness, contact dermatitis, urticaria, angioedema and chronic eczema. (Bergman *et al*, 1995). Respiratory symptoms consist of rhinitis, burning and itching of the eyes, cough, tightness in the throat and chest, and asthma (Levene & Calnan, 1971).

The sequence of events resulting in occupational asthma due to platinum salts was found to be: skin sensitization → symptoms → bronchial hyperresponsiveness (Merget *et al*, 1995). The latency period, from the first exposure to platinum salts to the occurrence of the first symptoms, usually varies between 3 months and 3 years. Generally symptoms worsen with increasing duration of exposure and do not always disappear when the subject is removed from exposure (Santucci *et al*, 2000). Cigarette smoking seems to be a risk factor, as smoking was definitely associated with the development of platinum salt sensitivity. Risk of sensitization was about eight times greater for smokers than non-smokers (Baker *et al*, 1990; Calverley *et al*, 1995). Smoking was also a significant predictive factor for both positive skin test and symptoms (Niezborala & Garnier, 1996).

Diagnosis of allergy to salts of platinum is based on a history of work-related symptoms and a positive skin-prick test with platinum salts. The skin-prick test is believed to be highly specific, but some workers with work-related symptoms have negative skin tests (Merget *et al*, 1991). A RAST was developed for the measurement of specific IgE to platinum chloride complexes (Cromwell *et al*, 1979), but subsequently was found not to be helpful in the diagnosis of platinum salt allergy (Merget *et al*, 1988). Total IgE and Phadiotop status were evaluated in a 24-month study in a South African primary platinum refinery and it was found that platinum salt sensitivity was associated with an increase in total IgE and conversion of Phadiotop status to positive (Calverley *et al*, 1997). In another study it was found that workers with work-related symptoms, as compared to other workers, had significantly more positive skin-prick test responses and higher total IgE and platinum specific IgE levels. They did not, however, show elevated circulating histamine concentrations. In the course of one week, a significant fall in lung function was recorded in a group of workers with work-related symptoms (Bolm-Audorff *et al*, 1992).

There is increasing evidence of neutrophil participation in asthma and the allergic process (Monteseirin *et al*, 2000; Monteseirin *et al*, 2001), but little is known about the interaction of platinum and other metals with neutrophils. In the nasal lavage of children living in areas of high-density traffic, a significant correlation was found between platinum and the number of neutrophils/ml as well as epithelial cells/ml (Schins *et al*, 2004). Addition of platinum to human neutrophils potentiated the reactivity of superoxide, resulting in a dose-related increase in lucigenin-enhanced chemiluminescence (Theron *et al*, 2004).

1.1.2 Palladium

Periodic table of elements

Atomic number: 46

Atomic symbol: Pd

Group name: Precious metal or Platinum group metal

History

Palladium was discovered in 1803 by the English chemist and physicist William Hyde Wollaston. Because of his fascination and interest in astronomy he named the metal after the newly discovered asteroid Pallas.

Properties and sources

Palladium is a steel-white metal which does not tarnish in air. It is found associated with platinum and other metals in deposits in the USSR, North and South America and Australia. It is also found within nickel-copper deposits in South Africa and USA.

(Palladium. <http://www.pearl1.lanl.gov/periodic/elements/46.html>)

(Palladium. <http://www.platinuminfo.net/palladium200.html>).

Uses

- Catalyst in the production of fine chemicals and pharmaceuticals and for hydrogenation and dehydrogenation reactions.
- In the jewellery industry as an alloying element used to whiten gold and to improve the working characteristics of platinum jewellery.
- Plating of electronic components: Used for electrode layers in mobile phones, digital cameras and other electronic devices.
- Automobile industry: palladium is used in catalytic converters, in which harmful exhaust gases are converted into harmless ones.
- As a substitute for silver in dentistry. Palladium is used as a “stiffener” in dental inlays and bridgework.
- In watch making.

(Palladium. <http://www.platinuminfo.net/palladium200.html>)

(Palladium. <http://www.pearl1.lanl.gov/periodic/elements/46.html>).

Increases of palladium in the environment have been shown in air and dust samples. Workers occupationally exposed to palladium include miners, dental technicians and chemical workers. The general population may come into contact with palladium mainly through mucosal contact with dental restorations and via emission from palladium catalysts. Low doses of palladium are sufficient to cause allergic reactions and people with known nickel allergy may be especially susceptible (Kielhorn *et al*, 2002).

The first case study of occupational asthma caused by palladium was reported in 1999. This was a previously healthy worker who developed rhinoconjunctivitis and asthma when exposed to the fumes of an electroplating bath containing palladium. Sensitization to palladium was documented by skin-prick test (Daenen *et al*, 1999).

The effects of platinum, palladium and rhodium on peripheral blood mononuclear cell (PBMC) proliferation as well as IFN- γ , TNF- α and IL-5 release were studied by Boscolo *et al* (2004). All metals had an inhibitory effect on the proliferation of PBMC, as well as on the production of the above-mentioned cytokines, with the palladium compounds being more immunotoxic than platinum or rhodium (Boscolo *et al*, 2004). Palladium is also known to cause allergic contact stomatitis in patients with dental prostheses. In many cases there are combined reactions to palladium and nickel and sometimes additionally to cobalt (Hackel *et al*, 1991).

1.1.3 Cobalt

Periodic table of elements

Atomic number: 27

Atomic symbol: Co

Group name: member of group VIII

History

Cobalt was discovered in 1735 by George Brandt, a Swedish chemist.

Properties and sources

Cobalt is a silver-white, lustrous, hard, brittle metal. Being similar in its physical properties to iron, it can be easily magnetized. Cobalt is usually found in association with other metals. The largest mine producer worldwide is Zambia, followed by the Democratic Republic of the Congo and Australia. In South Africa Cobalt is produced as a by-product of six platinum group metal mines and one nickel mine.

(Cobalt. <http://www.encyclopedia.com/printable.asp?url=/ssi/c1/cobalt.html>)

(Barceloux, 1999a).

Uses

- Pigments in the glass and ceramics industry: Cobalt yellow, green and blue.
- Invisible ink: Cobalt chloride is colourless in dilute solution when applied to paper. Upon heating it undergoes dehydration and turns blue, becoming colourless again when the heat is removed and water is taken up.
- Component of many alloys, like the high-speed steels carbonyl and stellite, from which very hard cutting tools are made; component of high temperature alloys used in jet engines.
- Component in some stainless steels.
- Trace element in fertilizers and additive in cattle and sheep feed. Cobalt prevents a disease called swayback and improves the quality of wool in sheep.
- In medicine as alloys used in dental and orthopaedic prostheses. Cobalt-60 is used in cancer radiotherapy. (See Figure 1.2, page 10)

(Cobalt. <http://www.encyclopedia.com/printable.asp?url=/ssi/c1/cobalt.html>).

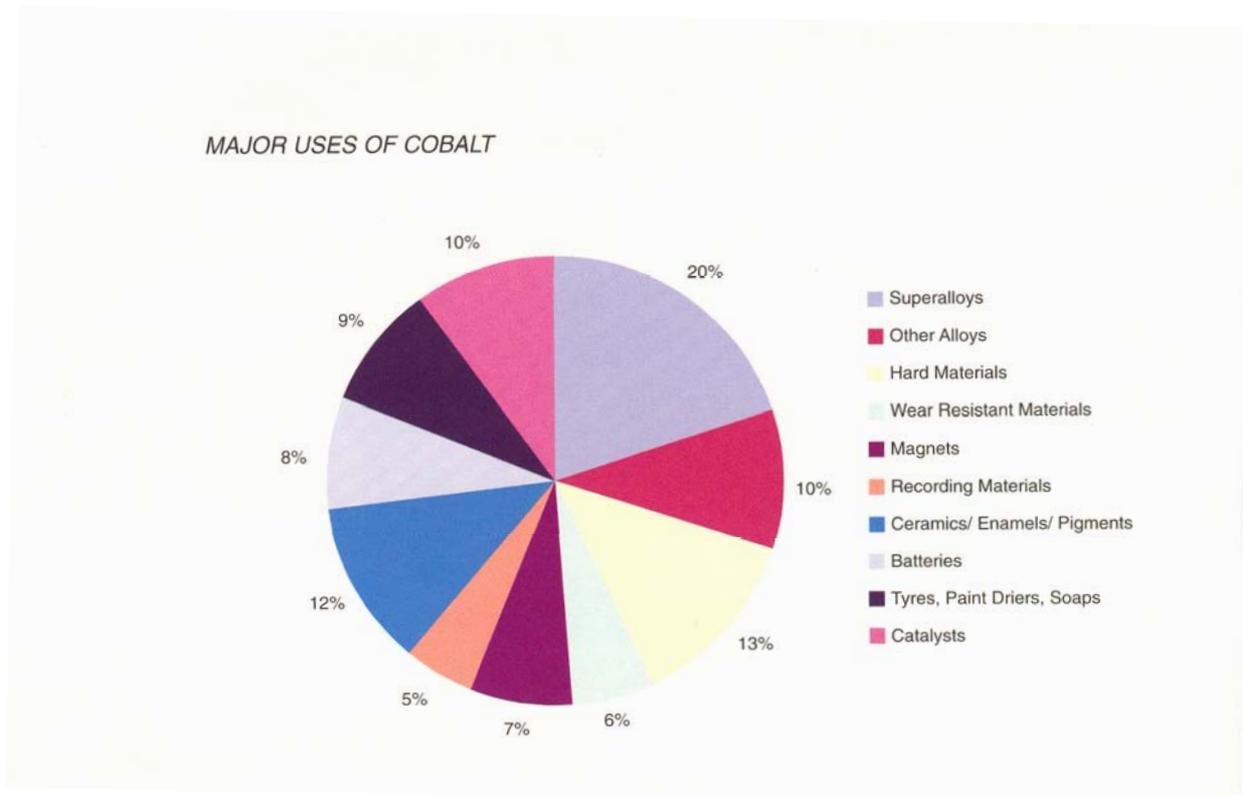


Figure 1.2

(Harding, 2004)

Several reports have confirmed that cobalt, when inhaled in the occupational setting may result in the development of bronchial asthma (Swennen *et al*, 1993; Christensen & Poulson, 1994; Lauwerys & Lison, 1994; Brock & Stopford, 2003; Linna *et al*, 2003). Cobalt caused direct induction of DNA damage, DNA-protein cross-linking, and sister-chromatid exchange (Leonard *et al*, 1998). Cobalt has also been reported to interfere with DNA repair processes. In animal studies cobalt has been found to have a carcinogenic effect. The mechanisms of cobalt-induced toxicity and carcinogenicity remain unclear, but it has been suggested that cobalt-mediated free radical reactions might be involved (Leonard *et al*, 1998). Ono *et al* (1994) reported that cobalt increased both the ROS generating capacity of neutrophils and the serum opsonic activity. Cobalt is present in dental and orthopaedic implants, and

total joint arthroplasty has become a common procedure. Complications are seen in greater numbers, with osteolysis and prosthetic loosening being one of the major problems. These problems have been attributed to a number of factors such as poor operative technique, infection, and biological reactions to constituents of the implants. The biological reactions may be mediated by three distinct mechanisms: i) hypersensitivity to metals; ii) foreign body reactions to particulate debris, also known as particle disease, which is mediated by macrophage phagocytosis; and iii) reactions to metal corrosion products, namely metal ions (Niki *et al*, 2003). Cytokines produced by leukocytes in the periprosthetic membranes surrounding joint replacements have also been implicated as causal agents. One study monitored the response of leukocytes to various metal ions and found that cobalt ions were the most potent stimulant for cytokine and prostaglandin secretion by leukocytes. Exposure of leukocytes to Co^{2+} ion increased the release of $\text{TNF-}\alpha$, IL-6 and prostaglandin E_2 (Liu *et al*, 1999).

Niki *et al* (2003) used synoviocytes and bone marrow macrophages to evaluate the biological reactions to metal ions potentially released from prosthetic implants. Cells were incubated with different metals (Ni^{2+} , Co^{2+} , Cr^{3+} and Fe^{2+}) and the following results were reported: The production of IL-1 β , IL-6 and $\text{TNF-}\alpha$, as well as DNA binding of NF- κ B was enhanced by all metals. This seemed to be mediated by ROS (Niki *et al*, 2003).

In another study it was found that in patients who had revision surgery for loosening of joint prostheses, serum cobalt levels were significantly higher than those of the control group. A significant decrease of leukocytes, myeloid cells, lymphocytes and CD16-expressing populations was found in these patients versus the controls (Savarino *et al*, 1999). The incidence of high serum levels of metal after prostheses surgery was later confirmed. Patients who had undergone total hip replacement had elevated levels of cobalt $4.1 \pm 1.5 \mu\text{g/L}$ versus $0.3 \pm 0.1 \mu\text{g/L}$ in the control group (Adami *et al*, 2003).

Catelas *et al* (2003) demonstrated that Co^{2+} induced a concentration- and time-dependent increase of TNF- α secretion in mouse macrophages. Cobalt also induced macrophage apoptosis at 24 hours (Catelas *et al*, 2003).

Finally it was demonstrated that Cobalt, as the soluble chloride salt, potentiates the luminol-enhanced chemiluminescence responses of activated neutrophils *in vitro*. This is an indication that cobalt potentiates the reactivity of neutrophil-derived oxidants which *in vivo*, may pose the risk of oxidant- and protease-mediated tissue injury (Ramafi *et al*, 2004).

1.1.4 Vanadium

Periodic table of elements

Atomic number: 23

Atomic symbol: V

Group name: member of group Va

(Mukherjee *et al*, 2004)

History

Vanadium was discovered in 1830 by Nils Sefstrom, a Swedish chemist, who named the metal after the Norse goddess Vanadis.

Properties and sources

Vanadium is a steel-grey, corrosion resistant metal, which exists in oxidation states from -1 to +5, the most common valences being +3, +4 and +5. The most common vanadium compounds are listed in Table 1.1 (page 13) (Barceloux, 1999b).

Table 1.1

Vanadium and Common Vanadium Compounds.

(Barceloux, 1999b)

	Synonyms	Chemical formula
Vanadium	Vanadium-51	V
Vanadium pentoxide	Vanadic anhydride Divanadium pentoxide Vanadium oxide Vanadic acid	V ₂ O ₅
Vanadyl sulphate	Vanadium oxysulphate Vanadium oxide sulphate Vanadium oxosulphate	VO ₂ SO ₄
Sodium metavanadate	Vanadic acid monosodium salt.	NaVO ₃
Sodium orthovanadate	Vanadic (II) acid trisodium salt. Sodium vanadate. Sodium vanadate oxide. Trisodium orthovanadate.	Na ₂ VO ₄
Ammonium metavanadate	Ammonium vanadate. Vanadic acid ammonium salt	NH ₄ VO ₃

Vanadium is a ubiquitous metal, its average concentration in the earth's crust being 150 µg/g. South Africa has the highest world vanadium reserves with 44%, and is also the leading producer with 41%. Vanadium export from South Africa was 52% in 2003. Other important Vanadium producers are China with 25% and Europe with 20% (Barceloux, 1999b; Kweyama, 2004). An overview on vanadium reserves and production is shown in Figure 1.3 (page 14).

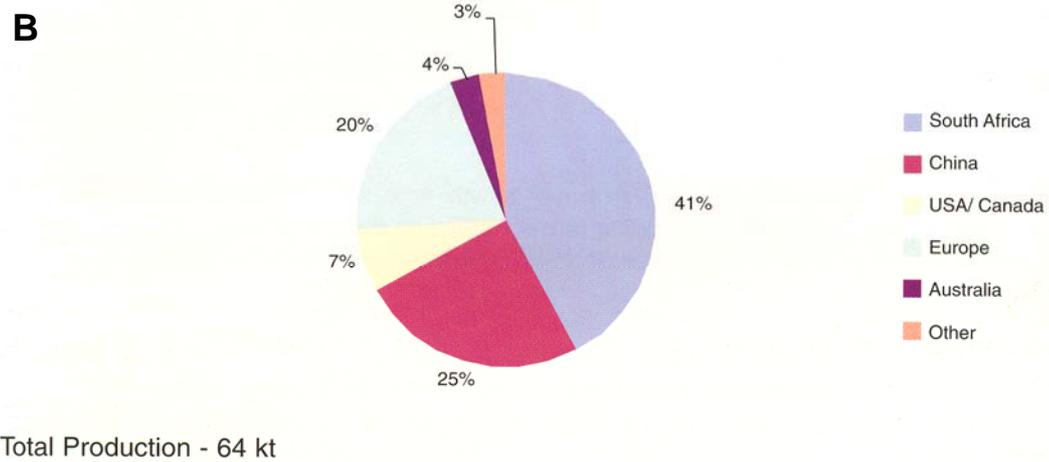
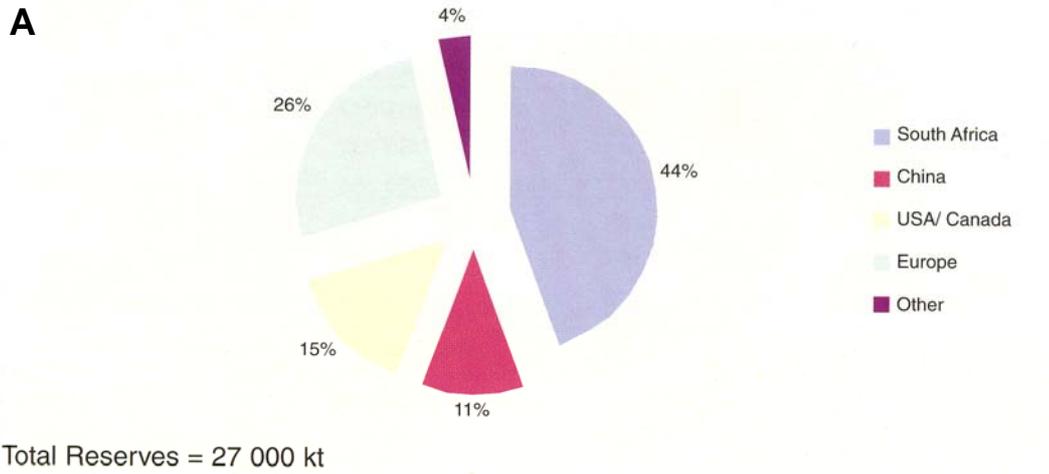


Figure 1.3

A Vanadium reserves 2003

B Vanadium production 2003 (Kweyama, 2004)

Vanadium is an essential trace element for normal human growth and nutrition, and most foods contain low concentrations of this metal. The daily requirement for vanadium is less than 10 $\mu\text{g/g}$.

Examples of the vanadium concentrations of some foods:

Black pepper, dill and mushrooms: 0.05 – 2 $\mu\text{gV/g}$.

Parsley: 1.8 $\mu\text{gV/g}$.

Shellfish and spinach: 0.5 – 0.8 $\mu\text{gV/g}$.

Fresh fruit, vegetables, cereals, liver, fats and oil: 1 – 10 ngV/g .

The processing of food tends to raise the concentration of vanadium.

Very high concentrations of vanadium are found in the non-edible mushroom *Amanita muscaria* (100 ppm V), as well as in tobacco smoke which contains 1 – 8 ppm V (Barceloux, 1999b).

(Vanadium. <http://www.euro.who.int/document/aig/6.12vanadium.pdf>)

Uses

- Production of steel and non-ferrous alloys
- Catalyst in the production of sulphuric acid and plastic, in petroleum cracking, purification of exhaust gases, and oxidation of ethanol.
- Manufacture of semi-conductors, photographic developers and colouring agents.
- Production of yellow pigments and ceramics.
- Addition of vanadium compounds improves the hardness and malleability of steel.
- Non-ferrous alloys containing vanadium are used in aircraft, space technology, and the atomic energy industry

(Barceloux, 1999b)

(Vanadium. <http://www.euro.who.int/document/aig/6.12vanadium.pdf>).

Occupational exposure to vanadium is common in petrochemical, mining, steel and utilities industries and results in toxic effects to the respiratory system (Irsigler *et*

al, 1999). Inflammation of the respiratory tract is possibly initiated by alveolar macrophages encountering vanadium-containing particles, with the subsequent release of proinflammatory cytokines (Grabowski *et al*, 1999). In a rat model, different vanadium compounds induced pulmonary inflammation. Significantly increased levels of mRNA for macrophage inflammatory protein-2 (MIP-2) were discovered in bronchoalveolar lavage (BAL) cells as early as 1 hour following exposure. Significant neutrophil influx was detected as early as 4 hours following the instillation of NaVO_3 and VOSO_4 but only 24 hours after exposure to V_2O_5 (Pierce *et al*, 1996).

Vanadium compounds have been found to inhibit superoxide dismutase (SOD) activity and to increase the production of reactive oxygen species by eukaryotic cells (Liochev *et al*, 1989a; Shainkin-Ketsenbaum *et al*, 1991; Trudel *et al*, 1991; Cohen *et al*, 1996; Krejsa *et al*, 1997; Wang *et al*, 2003).

Tyrosine phosphorylation of key cellular proteins is a crucial event in signal transduction pathways. The steady state of such phosphorylation is controlled by coordinate actions of protein-tyrosine kinases (PTKs) and phosphatases (PTPs). Pervandante is a powerful inhibitor of PTPs and therefore increases tyrosine phosphorylation and activation of mitogen-activated protein kinase (MAPK). This in association with a subsequent increase in IL-8 expression was observed in human bronchial epithelial cells (Samet *et al*, 1998). MAPK activation had been observed earlier in Chinese hamster ovary cells exposed to vanadium (Pandey *et al*, 1995) and in HeLa cells, an aggressively transformed cell line (Zhao *et al*, 1996). This correlated with the results of another study, in which it was demonstrated that pervanadate degraded $\text{I}\kappa\text{B}-\alpha$ which subsequently resulted in $\text{NF}-\kappa\text{B}$ DNA binding. Pervandante did not induce serine phosphorylation of $\text{I}\kappa\text{B}-\alpha$, but rather induced phosphorylation at tyrosine residue 42 (Mukhopadhyay *et al*, 2000).

Finally, in rats it was found that intratracheal instillation of V_2O_2 powder resulted in histopathological changes, such as desquamation and degeneration of swollen broncho-bronchiolar epithelium, hyperplasia of goblet cells, diffuse haemorrhage, effusions of fibrin and pulmonary oedema (Toya *et al*, 2001).

1.2 Neutrophils

All cellular elements of the blood arise from haematopoietic stem cells in the bone marrow. These pluripotent cells divide into two more specialized cells, a common lymphoid progenitor and a myeloid progenitor cell. The lymphoid progenitor gives rise to the T- and B-lymphocytes, the myeloid progenitor to basophils, eosinophils, monocytes and neutrophils. Basophils, eosinophils and neutrophils are known collectively as polymorphonuclear leukocytes. Neutrophils have a 14-day development period in the bone marrow and stay temporarily in a storage pool before being released into the blood. There they spend 12 – 14 hours in transit from a circulating pool into a marginating pool where they are in contact with the vessel walls. Thereafter, in the absence of any infection, neutrophils enter reticulo-endothelial organs, such as the liver, or even return to the bone marrow to undergo apoptosis (Brown *et al*, 2006).

Following penetration of the mechanical barriers of the host, neutrophils are the first line of defence against bacterial- and fungal infection. However, activated neutrophils can cause extensive harm to host cells and neutrophil-mediated tissue injury may contribute significantly to the pathogenesis of numerous diseases (Ayub & Hallett, 2004). Recently, it has been proposed that an inappropriate activation and positioning of neutrophils within the microvasculature contributes to multiple organ dysfunction and failure in sepsis (Brown *et al*, 2006).

1.2.1 Neutrophil activation

1.2.1.1 Neutrophil recruitment to inflammatory sites

Circulating neutrophils contact and transiently interact with endothelial cells, resulting in a rolling and release motion. This rolling is mediated by L-selectin. L-selectin on the surface of neutrophils permits interaction with endothelial cells, as well as with other neutrophils via the P-selectin glycoprotein ligand. Selectins also contribute to signalling. Interactions of neutrophils with P-selectin facilitate neutrophil degranulation, superoxide (O_2^-) production, and polarization in response to platelet-activating factor (PAF) and bacterial peptides such as formyl-methionyl-leucyl-phenylalanine (FMLP). Crosslinking of L-selectins on neutrophils primes the cells for

increased O_2^- production and calcium influx in response to chemoattractants and stimulates adhesion (Burg & Phillinger 2001).

Exposure of circulating neutrophils to chemoattractant gradients results in the conversion from neutrophil rolling to tight adhesion to endothelium. This step is mediated by cell surface molecules, specifically the members of the β_2 integrin family. They are composed of variable α -subunits (CD11a, CD11b and CD11c) and a common β -subunit (CD18). Two important β_2 integrins on the neutrophil are CD11a/CD18 (LAF-1) and CD11b/CD18 (MAC-1, CR3). Counterligands include the Ig superfamily members ICAM-1 and ICAM-2, as well as other ligands including fibrinogen, iC3b, heparin and factor X (Burg & Phillinger, 2001).

After firm adhesion via β_2 integrins, neutrophils transmigrate actively either between or directly through endothelial cells at endothelial junctions to extravascular sites of infection in a process known as diapedesis. The molecular details of this process are poorly characterized but appear to involve CD31 and JAMs 1,2,3. It was recently shown that CD157, a glycosylphosphatidylinositol-anchored ectoenzyme belonging to the NADase/ADP-ribosyl cyclase family plays a crucial role in neutrophil diapedesis. CD157 is constitutively expressed by endothelial cells with the highest density at intercellular junctions, and seems to be necessary for trans-endothelial – migration. CD157 is also expressed by neutrophils, while CD157-deficient neutrophils from patients with paroxysmal nocturnal haemoglobinuria are characterized by severely impaired diapedesis (Ortolan *et al*, 2006).

Following extravasation, neutrophils are attracted to the sites of tissue inflammation by chemotaxins. Both exogenous and endogenous substances can act as chemotactic agents. These include, 1) soluble bacterial products, like FMLP; 2) components of the complement system, particularly C5a; 3) products of the lipoxygenase pathway of arachidonic acid metabolism, particularly leukotriene B₄; and 4) cytokines, like IL-8 (Massey, 1997).

1.2.1.2 Neutrophil phagocytosis and degranulation

Phagocytosis consists of three distinct, but interrelated steps. 1) recognition and attachment of the particle to the ingesting neutrophil 2) engulfment with subsequent formation of the phagolysosome and degranulation and 3) killing of the ingested material. Recognition of micro-organisms is facilitated by coating them with serum proteins, called opsonins, which bind to specific receptors on the neutrophils. Most important opsonins are the Fc portion of the immunoglobulin G (IgG) and the C3b fragment of complement. Binding of the opsonized particle triggers engulfment. Pseudopods are extended around the particle forming the phagocytic vacuole. The membrane of the vacuole then fuses with the membrane of a lysosomal granule, resulting in discharge of the granule contents into the phagolysosome and degranulation of the neutrophil (Massey, 1997; Burg & Phillinger, 2001).

Phagocytosis results in the release of lysosomal enzymes not only within the phagolysosome but also potentially into the extracellular space with resulting cell injury and matrix degradation. Neutrophils contain a cytoplasm rich in different types of granules, namely “azurophilic” or “primary”, “specific” or “secondary”, “tertiary” or “gelatinase” and secretory vesicles. The cytoplasmic secretory granules contain proteinases, cytotoxic proteins and chelators. Neutrophil stimulation causes extracellular granule secretion in the following order: secretory vesicles, tertiary, specific and azurophilic (Henderson *et al*, 1996; Burg & Phillinger, 2001; Benton, 2002).

The following are examples of proteins released from the granules during neutrophil activation:

- Myeloperoxidase (MPO)

Contained within the azurophilic granules, MPO is a heme protein accounting for up to 5% of total cell protein. MPO catalyses the formation of hypochlorous acid, a potent oxidant with bactericidal activity.

MPO-derived oxidants are critically involved in the modulation of signalling pathways (Lau *et al*, 2005). For example: MPO-derived hypochlorous acid activates mitogen-activated protein (MAP) kinases (Midwinter *et al*, 2001), induces nuclear translocation of transcription factors (Schoonbroodt *et al*, 1997),

regulates cell growth by activating tumour suppressor proteins (Vile *et al*, 1998) and modulates the activity of metalloproteinases (Fu *et al*, 2004).

- Bactericidal/permeability increasing protein (BPI)
This protein is cytotoxic to many Gram-negative bacteria. Its N-terminal domain allows binding to LPS and its C-terminal mediates bacterial attachment to neutrophils. Binding of BPI causes an increase in the permeability of the outer membrane of Gram-negative bacteria.
- Defensins
Defensins are major components of azurophilic granules. They are present in phagocytic vacuoles at a concentration of 1 mg/ml and render target cell membranes more permeable.
- Proteinase 3 (PR3)
This enzyme is found in the azurophilic and in the secretory granules. PR3 has been shown to enhance activation of TNF- α and IL-1 β from LPS- stimulated cells. Therefore PR3 may play an important role in the amplification of inflammatory responses (Coeshott *et al*, 1999).
- Elastase
Potent serine protease which degrades an outer membrane protein in Gram-negative bacteria. Mice with an inactive elastase gene show impaired resistance against Gram-negative but not Gram-positive bacteria (Belaouaj *et al*, 1998).
- Secretory phospholipase A₂ (PLA₂)
Protein with potent bactericidal activity. PLA₂ synergizes with BPI for intracellular bacterial killing (Weiss *et al*, 1994).
- Metalloproteinases
These are calcium-requiring enzymes which are released in inactive pro-enzyme form. Collagenases degrade native collagen and enzymatic activity depends upon oxidation by HOCL. Gelatinase degrades denatured collagen and

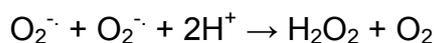
activation occurs by both oxidative and nonoxidative mechanisms (Burg & Phillinger, 2001).

1.2.1.3 Respiratory burst and the NADPH oxidase system

The ultimate aim of phagocytosis is to kill and degrade micro-organisms. This is accomplished largely by reactive oxygen species (ROS). During phagocytosis a sudden increase in oxygen consumption occurs concomitantly and has been termed “respiratory burst”. This increase in oxygen consumption is due to the activity of the NADPH oxidase which catalyzes the reaction which produces large amounts of superoxide ($O_2^{\cdot-}$).



Superoxide is rapidly converted to hydrogen peroxide by the enzyme superoxide dismutase.



The toxic effects of these oxidants are due in part to their ability to form more reactive oxygen species. H_2O_2 can lead by a number of further reactions to the generation of hydroxyl radical (OH^{\cdot}) and singlet oxygen. Myeloperoxidase converts H_2O_2 to hypochlorous acid (HOCl), the most bactericidal of oxidants produced by the neutrophils (Segal, 1993; Robinson & Badwey 1995; Henderson & Chappell 1996, Dahlgren & Karlsson, 1999).

Hydroxyl radical

Reduction of H_2O_2 resulting in the formation of hydroxyl radical results from several mechanisms, the most important being the “Fenton reaction”, the “Haber-Weiss reaction”, and MPO-catalyzed reactions.

Fenton reaction:

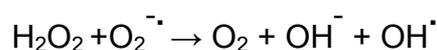
The formation of hydroxyl radical by the Fenton reaction requires stoichiometric amounts of Fe^{2+} and H_2O_2 . As the free iron concentration in biological

fluids is very low, the formation of hydroxyl radical by this mechanism is limited. An additional source of iron is the microorganism (Klebanoff, 1999).



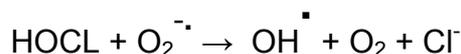
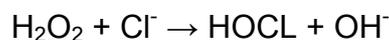
Haber-Weiss reaction:

When the iron concentration is limiting, ferric iron must first be reduced, which is done by superoxide. This iron-catalyzed interaction of hydrogen peroxide and superoxide is termed the Haber-Weiss reaction, or the superoxide-driven Fenton reaction. The overall reaction is as follows



MPO-catalyzed reaction:

Hypochlorous acid generated by MPO can react with superoxide to form OH^\bullet according to the reaction



(Klebanoff, 1999)

Hydroxyl radical is one of the most powerful oxidants and may therefore contribute to the toxic activity of phagocytes. OH^\bullet is a highly reactive oxygen-centred radical with a half life in cells of only 10^{-9} seconds. Hydroxyl radical attacks all proteins, DNA, polyunsaturated fatty acids in membranes and many other biological molecules (Aruoma, 1998).

The membrane-associated NADPH oxidase is dormant in resting cells but may be stimulated within 15 to 60 seconds by a wide variety of activators, including phorbol esters (PMA), heat-aggregated IgG (HAGG), unsaturated fatty acids and analogues of bacterial peptides (FMLP). Different stimuli activate different pathways.

The response to PMA is seen after about 25 seconds and lasts for many minutes and is calcium-independent. The response to FMLP occurs after a lag of 5 – 10 seconds, is lower in intensity and duration, and is calcium-dependent (Segal, 1993; Henderson & Chappell 1996).

In order for the NADPH oxidase to become activated, the different components have to be assembled at the plasma membrane. The cytosolic complex of the NADPH oxidase in resting neutrophils consists of p47^{phox}, p67^{phox}, p40^{phox} and rac-2. The membrane bound proteins are p22^{phox} and gp91^{phox}, the subunits of cytochrome b₅₅₈. Translocation of the cytoplasmic components to the membrane and their association with cyt b₅₅₈ render the complex functional. The cytochrome then transfers electrons from NADPH to O₂ to create superoxide in the reaction mentioned above.

Cytochrome b₅₅₈

Cyt b₅₅₈ is a membrane-bound flavohemoprotein and is named after its peak in infrared absorbance. Eighty five percent is found in the membranes of specific granules and secretory vesicles and 15% is located in the plasma membrane. The subunits p22^{phox} and gp91^{phox} closely interact with each other and are only separated by denaturation. The flavin group is critical for electron transport to O₂.

p47^{phox}

This protein is vital for oxidase function. Upon phosphorylation by protein kinase C (PKC) p47^{phox} interacts with the cytoskeleton and then moves to the plasma membrane where it associates with cyt b₅₅₈.

p67^{phox} and rac-2

p67^{phox} is phosphorylated by PKC and remains complexed with p47^{phox} after phosphorylation. p67^{phox} has an activation domain which is critical for NADPH oxidase function. p67^{phox} may regulate the electron transfer from NADPH to O₂.

Rac-2

Plays a critical role in the activation of the NADPH oxidase. In its inactive state rac-2 is complexed with rho-GDI. Activation dependent GTP-binding frees it from the

complex and allows its interaction with the N-terminal region of p67^{phox}, which is required for oxidase function (Segal 1993; Robinson & Badwey 1995; Henderson & Chappell 1996; Ellson *et al*, 2001; Burg & Phillinger 2001).

1.2.1.4 Calcium homeostasis in activated neutrophils

In unstimulated neutrophils cytosolic free Ca²⁺ is present at very low levels (≈ 100nM). After receptor-mediated activation, there is an abrupt and short-lived increase in cytosolic free Ca²⁺. This increase in the cytosolic Ca²⁺ concentration peaks at 10 – 20 seconds, lasts for several minutes and is a prerequisite for the initiation of pro-inflammatory activities of neutrophils. These Ca²⁺-dependent functions include activation of β₂-integrins and adhesion to vascular endothelium, activation of NADPH oxidase and subsequently superoxide production, degranulation, activation of phospholipase A₂ and activation of proinflammatory, cytosolic nuclear transcription factors, such as NF-κB. Nuclear transcription factors in turn activate the genes encoding the inflammatory cytokines IL-8 and TNF-α. The Ca²⁺ may originate exclusively from the intracellular stores, or from both intracellular and extracellular reservoirs and is dependent on the type of receptor-mediated stimulus (Anderson *et al*, 2000; Tintinger *et al*, 2005).

Intracellular Ca²⁺ is stored at different sites within the neutrophil. One store is located peripherally under the plasma membrane and seems to be involved in the activation of β₂-integrins. The other site is located in the perinuclear space and is mobilized by chemoattractants, like FMLP, C5a, leukotriene B₄, PAF and chemokines via leukocyte membrane receptors, which belong to the G-protein-coupled family of receptors. Binding to these receptors results in the activation of phospholipase C, which, through hydrolysis of phosphatidylinositol, produces inositol-1,4,5-triphosphate (IP₃). IP₃ interacts with Ca²⁺ mobilizing receptors on the intracellular storage vesicles, which in turn result in a rapid 5 to 10 fold increase in the cytosolic calcium concentration.

Following activation of neutrophils, restoration of Ca²⁺ homeostasis is essential to prevent Ca²⁺ overload and hyperactivity of the phagocytes. NADPH oxidase, the membrane-associated electron transporter of neutrophils fulfils an important anti –

inflammatory action by regulating the store-operated influx of Ca^{2+} through depolarization of the plasma membrane. The clearance of Ca^{2+} is then easily achieved by 2 adenosine triphosphate (ATP)-driven pumps. These are the plasma membrane Ca^{2+} -ATPase, which are a Ca^{2+} -efflux pump and the endo-membrane Ca^{2+} -ATPase which pumps Ca^{2+} back into the stores (Geiszt *et al*, 1997; Tran *et al*, 2000; Ayub & Hallett, 2004; Oommen *et al*, 2004; Tintinger *et al*, 2005; Anderson *et al*, 2005).

1.2.2 Neutrophil apoptosis

As neutrophils have the potential to inflict harm to host tissue, it is important that their activity is tightly regulated, especially in inflammation when large numbers of activated neutrophils may accumulate within one organ. These mechanisms are referred to as 'programmed cell death' or 'apoptosis'. Apoptotic neutrophils fragment to form 'apoptotic bodies', which can be phagocytosed by macrophages. Prior to this, signalling shutdown may limit the function of the neutrophil. Apoptotic neutrophils are non-functional; they are unable to move by chemotaxis, generate a respiratory burst or degranulate, and there is a clear down-regulation of cell surface receptors, preventing them from transducing signals. The rate of apoptosis may also be accelerated, as well as delayed. Delayed neutrophil apoptosis correlates with severity of clinical sepsis and multiple organ dysfunction (Keel *et al*, 1997; Matute-Bello *et al*, 1997). Experimentally, bacterial products and pro-inflammatory cytokines delay apoptosis (Colotta *et al*, 1992).

Accelerated apoptosis is triggered via the Fas receptor. Activation of this receptor results in the formation of the death-inducing signalling complex (DISC), which contains CD95, FADD and procaspase-8. Procaspase-8 is cleaved to form caspase-8, which in turn activates a cascade of protein-cleaving caspases (Scaffidi *et al*, 1998). Recent evidence points to Ca^{2+} signalling shutdown in fas-triggered apoptotic neutrophils with involvement of neutrophil mitochondria, resulting in non-energized mitochondria not being able to take up Ca^{2+} and therefore unable to signal Ca^{2+} influx, which would lead to non-functional neutrophils. It has been shown that loss of mitochondrial membrane potential is an early event in neutrophil apoptosis (Ayub & Hallett, 2004).

Another regulator of neutrophil apoptosis is phosphoinositide 3-kinase (PI3-K). Neutrophil apoptosis under basal, as well as LPS-stimulated conditions was increased in PI3-K^{-/-} mice. These mice had decreased amounts of activated Akt, phosphorylated CREB and NF- κ B nuclear translocation (Yang *et al*, 2003).

1.2.3 Interleukin-8 (IL-8)

Neutrophils are capable of generating many different cytokines, chemokines, growth factors and other proteins *in vitro* and *in vivo*. The production of individual cytokines by neutrophils is influenced to a great extent by the stimulatory conditions. The chemokine, IL-8, seems to play a critically important role in inflammatory processes. Several studies have identified an association of IL-8 with various acute and chronic inflammatory conditions including sepsis, psoriasis, rheumatoid arthritis, gout, severe trauma, pulmonary fibrosis, asthma, emphysema, pneumonia and adult respiratory distress syndrome (Hoch *et al*, 1996). When *Pseudomonas aeruginosa* was used as a stimulus, it was observed that the bacterium and its products induced IL-8 expression in airway epithelial cells and the recruitment of neutrophils into the airways (Oishi, *et al* 1994). IL-8 induces activation of G-protein coupled receptors which results in the activation of phospholipase C, which catalyzes the hydrolysis of membrane phosphoinositides to yield diacylglycerol (DAG) and IP₃, which in turn mobilizes the intracellular Ca²⁺, as mentioned above (Rahman, 2000). Significantly elevated levels of IL-8 and myeloperoxidase have been found in sputum of toluene diisocyanate-asthma and dust-asthma patients (Jung & Park 1999). Several studies have shown the potential role of IL-8 in haematopoiesis and trafficking of haematopoietic stem cells. Systemic administration of IL-8 induces rapid mobilization of progenitor cells from the bone marrow (Van Eeden & Terashima 2000; Fibbe *et al*, 2000). IL-8, as well as GM-CSF and TNF- α are also implicated in the regulation of neutrophil oxidative burst by modulating the activity of the NADPH oxidase through a priming (sensitizing) phenomenon. These cytokines induce a very weak oxidative response by neutrophils but strongly enhance neutrophil release of ROS on exposure to a second stimulus (Gougerot-Pocidallo *et al*, 2002).

As already mentioned IL-8 belongs to the group of chemokines. Chemokines represent a group of chemotactic cytokines whose importance in inflammatory

processes results from their ability to recruit leukocyte populations. Chemokines are low-molecular weight proteins with cysteines at well-conserved positions. Chemokines are divided into four subgroups, CXC, CC, CX₃C and C, on the basis of the relative positions of the first two cysteine residues. CXC and CX₃C chemokines are distinguished by the presence of one in CXC and three in CX₃C intervening amino acids, CC have the first two cysteines adjacent, while C chemokines possess only two cysteines, corresponding to the second and fourth in the other groups. IL-8 belongs to the group of CXC chemokines and is therefore also called CXCL8. CXC chemokines can be further classified into Glu-Leu-Arg (ELR)⁺ and ELR⁻ CXC, based on the presence or absence of ERL in the NH₂ terminus before the first cysteine. This classification correlates with functional differences. ELR⁺ CXC chemokines bind the receptors CXCR₁ and CXCR₂ with high affinity and have a potent chemotactic effect, particularly on neutrophils (Cassatella, 1999; Pease & Sabroe, 2002; Mukaida, 2000; Mukaida, 2003).

The DNA of CXCL8 encodes a 99 amino acid precursor protein, which is cleaved to yield mainly a 77- or 72-residue mature protein. Further processing at the NH₂ terminus yields different truncations, which are caused by the proteases released from CXCL8-secreting cells or accessory cells. These truncations contain 77, 72, 71, 70 or 69 amino acids, the two major forms being 77- and 72-amino acid forms, with a minor 69-amino acid protein. In vitro, fibroblasts and endothelial cells predominantly produce the 77-amino acid form, while leukocytes mainly secrete 72- or 69-amino acid forms. These three forms exhibit neutrophil chemotactic activities with distinct potencies: 69- > 72- > 77-amino acid form (Mukaida, 2003).

CXCL8 can be produced by leukocytic cells, like monocytes, T cells, neutrophils and natural killer cells and somatic cells like endothelial cells, fibroblasts and epithelial cells. IL-8 production is not constitutive, but inducible by proinflammatory cytokines such as IL-1 and TNF- α . IL-8 production can also be induced by bacteria (e.g. *Helicobacter pylori*, *Pseudomonas aeruginosa*), bacterial products (lipopolysaccharide, LPS) viruses (adenovirus, respiratory syncytial virus, cytomegalovirus, rhinovirus) and viral products (X protein of human hepatitis B, Tax protein of human T-cell-leukaemia virus type I). IL-8 can also be induced by environmental factors. CXCL8 production is regulated at the level of gene

transcription, through activation of the nuclear transcription factors Nuclear Transcription factor κ B (NF- κ B) and activator protein-1 (AP-1) (Mukaida, 2003).

1.3 C5a

1.3.1 Introduction

C5a is a 74-residue fragment of C5 and contains a single complex oligosaccharide moiety, which is important for the activities of the molecule. C5a is a pro-inflammatory polypeptide which is generated during complement activation. Complement (C) is the collective term for a series of 9 different circulating polypeptides, which are activated in a cascade-like fashion induced by 3 possible pathways. The “classical pathway”, activated by immune complexes containing either IgM or IgG, the “mannan-binding lectin (MBL) pathway” and the “alternative pathway.” The consequence of complement activation is the generation of the pro-inflammatory polypeptides C3a and C5a both of which are chemotactic and anaphylatoxic, as well as the opsonins C3b and C3bi, the bone marrow stimulator C3e and the cell wall attack complex (Cooper, 1999).

1.3.2 C5a as anaphylatoxin

C5a is the most potent anaphylatoxin generated during the activation of complement. It increases vascular permeability, contracts smooth muscle, and triggers the release of histamine from mast cells and basophils. Anaphylatoxic activity is inactivated by plasma-carboxypeptidase B, an enzyme which removes the carboxyl-terminal arginine residue of C5a (as well as C3a and C4a) thereby generating the “des Arg” forms of these peptides. Smooth muscle-contracting ability of C5a is rapidly inactivated, although C5a des Arg is able to induce permeability (Cooper, 1999).

1.3.3 C5a as chemoattractant

C5a is chemotactic for neutrophils, monocytes, and macrophages. Binding of C5a to the C5a receptor on human neutrophils and monocytes triggers the migration

of the cell to the complement-activating stimulus. C5a induces instantaneous changes in cell shape, transient microtubule assembly and fusion of lysosomal granules with each other and with the plasma membrane. Mice deficient in the C5a receptor were unable to clear intrapulmonary-instilled *Pseudomonas aeruginosa*, and, despite an increase in neutrophil influx, succumbed to pneumonia (Hopken *et al*, 1996).

1.3.4 C5a actions on neutrophils, monocytes and macrophages

Apart from its ability to trigger chemotaxis, C5a exhibits many more important actions on phagocytic cells. These actions include:

- Induced secretion of lysosomal enzymes and various mediators, including eicosanoids and platelet-activating factor (PAF).
- Augmented oxidative metabolism
- Increased adherence.
- Induction of monocyte and neutrophil aggregation.
- Augmented expression of C3 receptors, integrins, and other membrane proteins
- Induction of procoagulant activity

1.4 Pneumolysin

1.4.1 Introduction

Infections caused by *Streptococcus pneumoniae* are a major cause of morbidity and mortality. These infections include otitis media, community-acquired pneumonia, bacteremia and meningitis (Mufson, 1990). Mortality from pneumococcal diseases is high, ranging between 25% and 29% (Musher *et al.*, 2000). Pneumolysin, a toxin produced by *Streptococcus pneumoniae*, plays a major role in the virulence and pathogenesis of pneumococcal infections (Mitchell & Andrew, 2000).

1.4.2 Origin of pneumolysin

Pneumolysin is located in the cytoplasm of the pneumococcus. As this toxin lacks a N-terminal secretion signal sequence, it is generally believed that pneumolysin is released upon autolysin-induced autolysis of the pneumococcus in the late log phase of growth. However an autolysin-independent release of the toxin in the early log phase of bacterial growth has also been reported for several strains of the pneumococcus (Balachandran *et al.*, 2001).

1.4.3 Structure of pneumolysin

Pneumolysin, a member of the family of thiol-activated cytolysins, is a 35 kDa protein of 471 amino acids grouped into four domains. The first domain which contains the N-terminal region consists of negatively charged amino acids and possibly plays a role in the orientation of the molecule with respect to the membrane. Domain 1 and domain 3 are structurally associated with each other, but do not bind to the membrane. Domain 2 is a β -sheet structure and forms a junction between domains 1 and 4. Domain 4 is responsible for membrane binding (Cockeran *et al.*, 2002a)

1.4.4 Biological effects of pneumolysin

Pneumolysin is lytic for all eukaryotic cells that have cholesterol in their membrane. The toxin destabilises the membrane and renders it permeable through the formation of pores. Pore-forming activity involves a two-step pathway. The first step involves binding to membrane cholesterol and insertion of the toxin into the lipid bilayer. The second step involves lateral diffusion and assembly of a high molecular weight oligomeric structure, which represents a trans-membrane pore (Mitchell & Andrews, 1997; Mitchell & Andrews, 2000).

Low doses of pneumolysin (1 ng/ml) have been shown to inhibit the respiratory burst of human polymorphonuclear leukocytes (PMNL) and this was associated with reduced ability to take up and kill opsonized pneumococci. Chemotaxis and migration of PMNL were also inhibited (Paton & Ferrante, 1983).

In contrast, pneumolysin was found to alter the pro-inflammatory responses of human neutrophils by increasing the activity of PLA₂ and increasing the production of superoxide and the release of the primary granule enzyme, elastase (Cockeran *et al*, 2001b). Pneumolysin caused a calcium-dependent increase in the generation of the pro-inflammatory lipids prostaglandin E₂ and leukotriene B₄ by both resting and chemoattractant-activated human neutrophils *in vitro* (Cockeran *et al*, 2001a). Furthermore pneumolysin induces the synthesis and the release of IL-8 by human neutrophils (Cockeran *et al*, 2002b).

Pneumolysin is also capable of stimulating human monocytes to produce the pro-inflammatory cytokines TNF- α and IL1- β (Houldsworth *et al*, 1994).

Pneumolysin is able to activate the classical complement pathway in the absence of specific antibody (Mitchell & Andrew, 2000).

Pneumolysin has detrimental effects on ciliated epithelium. The toxin slows the ciliary beat of human nasal epithelium maintained in organ culture and disrupts the cells, which may reduce the ability to clear particles from the respiratory tract (Feldman *et al*, 1990).

Pneumolysin also seems to play a key role in sensorineural hearing loss, which is a common complication of pneumococcal meningitis (Mitchell & Andrew, 2000).

1.5 Nuclear factor-kappa B

1.5.1 Introduction

NF- κ B, first identified in 1986 by Sen and Baltimore in B-cells, was described as a nuclear factor bound to the immunoglobulin κ light chain gene enhancer, and was therefore given the name 'nuclear factor- κ B'. But soon it was understood that this was a ubiquitous transcription factor present in virtually all cells. NF- κ B is present in the cytoplasm of nonstimulated cells, where it is bound and controlled by a family

of inhibitory proteins, the I κ Bs. Upon stimulation, subsequent phosphorylation, ubiquitination and proteolytic degradation of I κ B, NF- κ B is freed to translocate into the nucleus, and, by binding to DNA, regulates the transcription of genes encoding inflammatory cytokines. One of the first genes transcribed is that encoding I κ B, which then leads to inhibition of further DNA binding, as the affinity of NF- κ B to I κ B is greater than to DNA (Baeuerle & Henkel, 1994; Baeuerle & Baltimore, 1996; Baldwin, 1996; Karin & Ben-Neriah, 2000).

1.5.2 NF- κ B proteins

In its active DNA binding form, NF- κ B is a heterogeneous collection of dimers, composed of various combinations of members of the NF- κ B/Rel family, which is characterized by the presence of the Rel homology domain. Five mammalian Rel proteins have been identified, NF- κ B1 (p50 and its precursor p105), NF- κ B2 (p52 and its precursor p100), c-Rel, RelA (p65) and RelB. Three Rel proteins were identified in *Drosophila melanogaster*, Dorsal, Dif and Relish. All of these proteins share a highly conserved 300 amino acid Rel homology region (RHR), composed of two immunoglobulin-like domains. The RHR is responsible for dimerization, DNA binding and interaction with the inhibitory I κ B proteins. It also contains the nuclear localization sequence (NLS). Different NF- κ B dimers exhibit different binding affinities, while binding site preferences have been identified for certain dimers. The dimer of p50 and RelA, which is considered the classic NF- κ B, binds to the sequence 5' GGGRNNYYCC 3', while the dimer RelA/c-Rel binds to the sequence 5' HGGARNYYCC 3' (H indicates A,C or T; R is purine; Y is pyrimidine, and N is any base) (Baldwin, 1996; Karin & Ben-Neriah, 2000). See Figures 1.4 and 1.5 (pages 33 and 34, respectively).

1.5.3 The I κ B proteins

The I κ B family includes I κ B α , I κ B β , I κ B γ , I κ B ϵ , Blc-3, the precursors of NF- κ B1 and NF- κ B2, and the *Drosophila* protein, Cactus. The most important regulators of mammalian NF- κ B are I κ B α , I κ B β and I κ B ϵ . All I κ Bs contain six or seven ankyrin

repeats, which mediate binding to the RHR. As there are also ankyrin repeats in the subunits p105 and p100 in Nf- κ B1 and Nf- κ B2 respectively, it can be concluded that these subunits are intramolecular I κ Bs (Baldwin, 1996; Karin & Ben-Neriah, 2000).

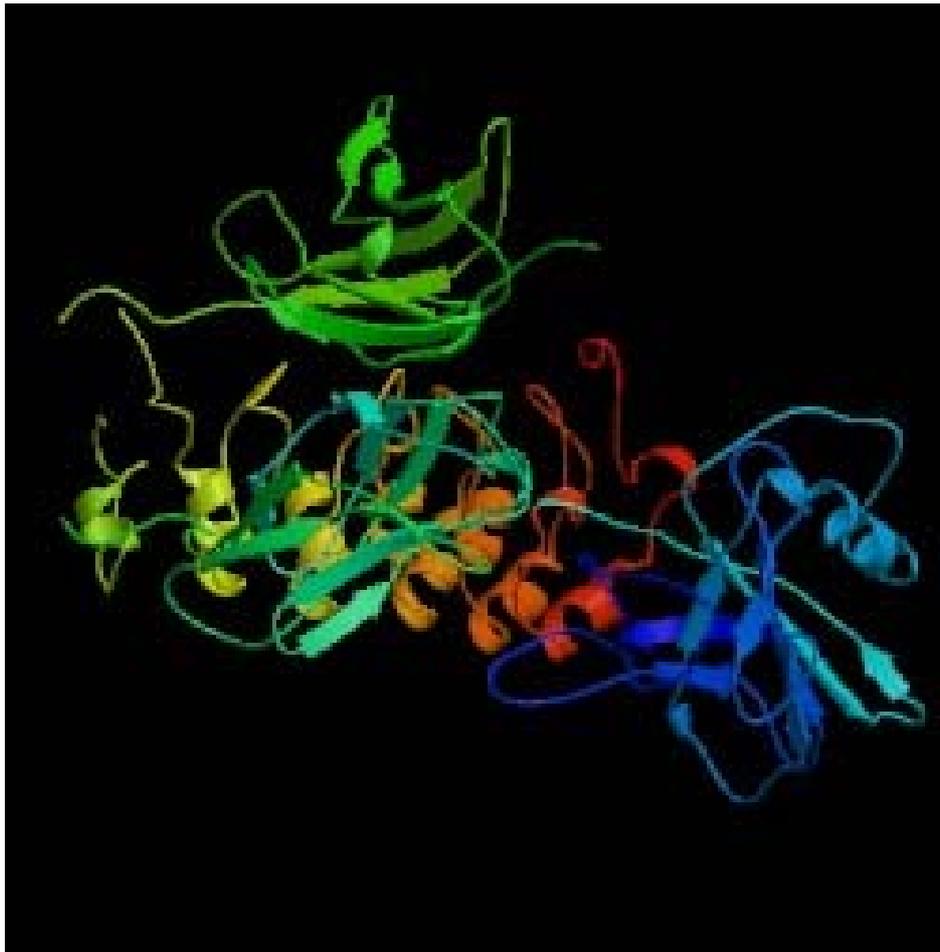


Figure 1.4

Structure of NF- κ B and I κ B.

(Huxford *et al*, 1998)



Figure 1.5

Structure of NF- κ B bound to DNA
(Huxford *et al*, 1998)

1.5.4. Activation of NF- κ B

1.5.4.1 Activating signals

NF- κ B is activated by a wide variety of different stimuli, including proinflammatory cytokines, T- and B-cell mitogens, bacteria and bacterial lipopolysaccharides (LPS), viruses, viral proteins, double stranded RNA, physical, chemical and oxidative stress (Pahl, 1999) as shown in Figures 1.6 and 1.7 (pages 36 and 37 respectively).

Many studies have focused on the importance of ROS and redox status in the activation of NF- κ B, and intracellular redox levels have been shown to regulate NF- κ B signal transduction triggered by a variety of stimuli (Anderson *et al*, 1994; Staal *et al*, 1994; Suzuki *et al*, 1994; Schmidt *et al*, 1995; Staal *et al*, 1995; Suzuki & Packer, 1995; Blackwell *et al*, 1996; Barnes & Karin, 1997; Flohé *et al*, 1997; Schoonbroodt *et al*, 1997; Janssen & Sen, 1999; Bowie & O'Neill, 2000; Rahman, 2000; van den Berg *et al*, 2001; Bekay *et al*, 2003; Yang *et al*, 2003; Asehnoune *et al*, 2004).

1.5.4.2 I κ B-kinase (IKK)

The signalling pathways induced by different stimuli and ligand binding to different receptors vary, but the key to NF- κ B activation is phosphorylation and activation of I κ B kinase (IKK). IKK is a large (>700 kDa) multicomponent enzyme complex containing two catalytic kinase subunits IKK α and IKK β , which exist as a heterodimer. This enzyme complex also comprises two additional proteins, IKK γ and IKAP. IKK γ , which is also referred to as NEMO (NF- κ B essential modulator), is essential for linking upstream signals to IKK. IKAP (IKK complex-associated protein) is a scaffolding protein required for the proper assembly of IKK and binds to both IKK α and IKK β . There are clear and distinct roles for IKK α and IKK β . IKK β is responsible for the phosphorylation of I κ B in response to proinflammatory cytokines, such as TNF- α and IL-1. IKK α responds to yet unknown morphogenic signals and is crucial for NF- κ B activation during embryonic development of the skin and skeletal system. IKK α and IKK β are both activated by phosphorylation on specific serine residues. These are serines 176 and 180 in IKK α and serines 177 and 181 in IKK β (Zamanian-Daryoush *et al*, 2000).

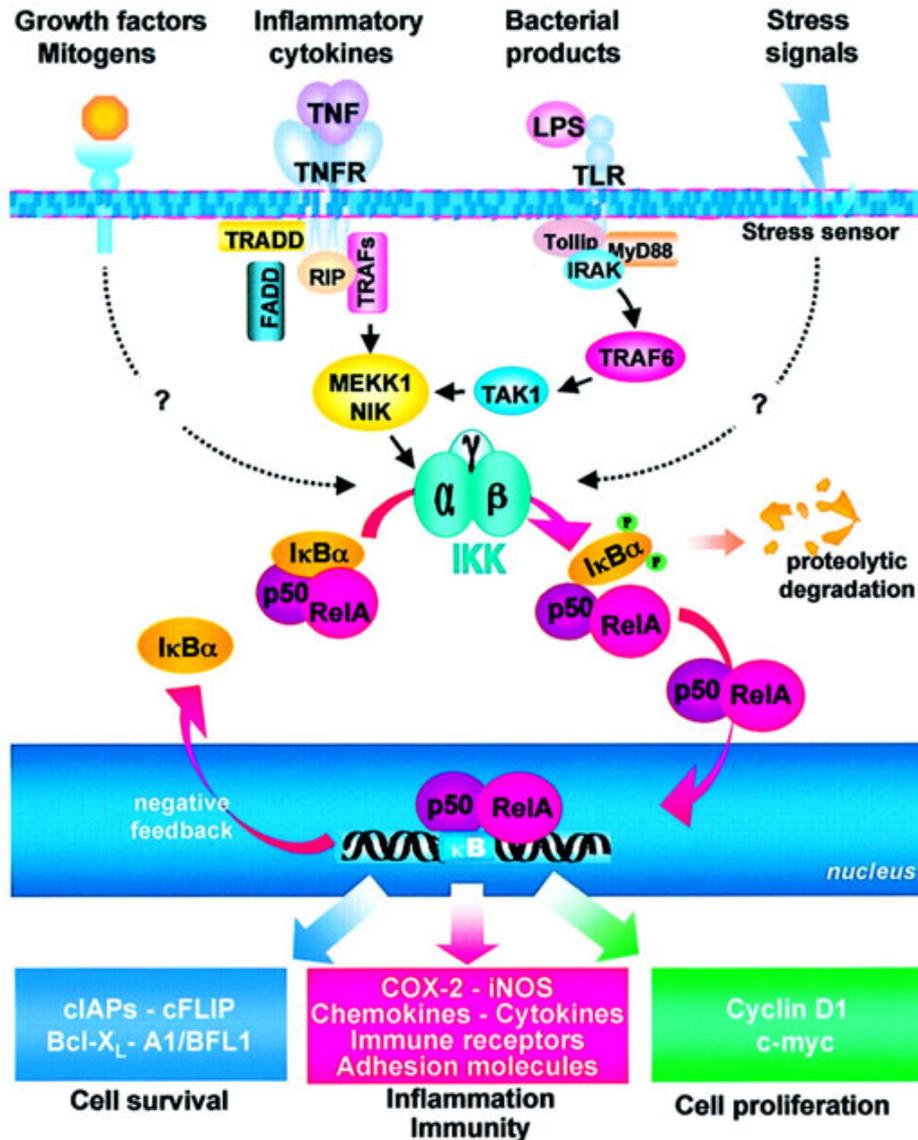


Figure 1.6

The NF-κB pathway. Stimulation by stress-inducing agents, or exposure to inflammatory cytokines, mitogens or bacterial and viral pathogens leads to the activation of signalling cascades (Santoro, 2003).

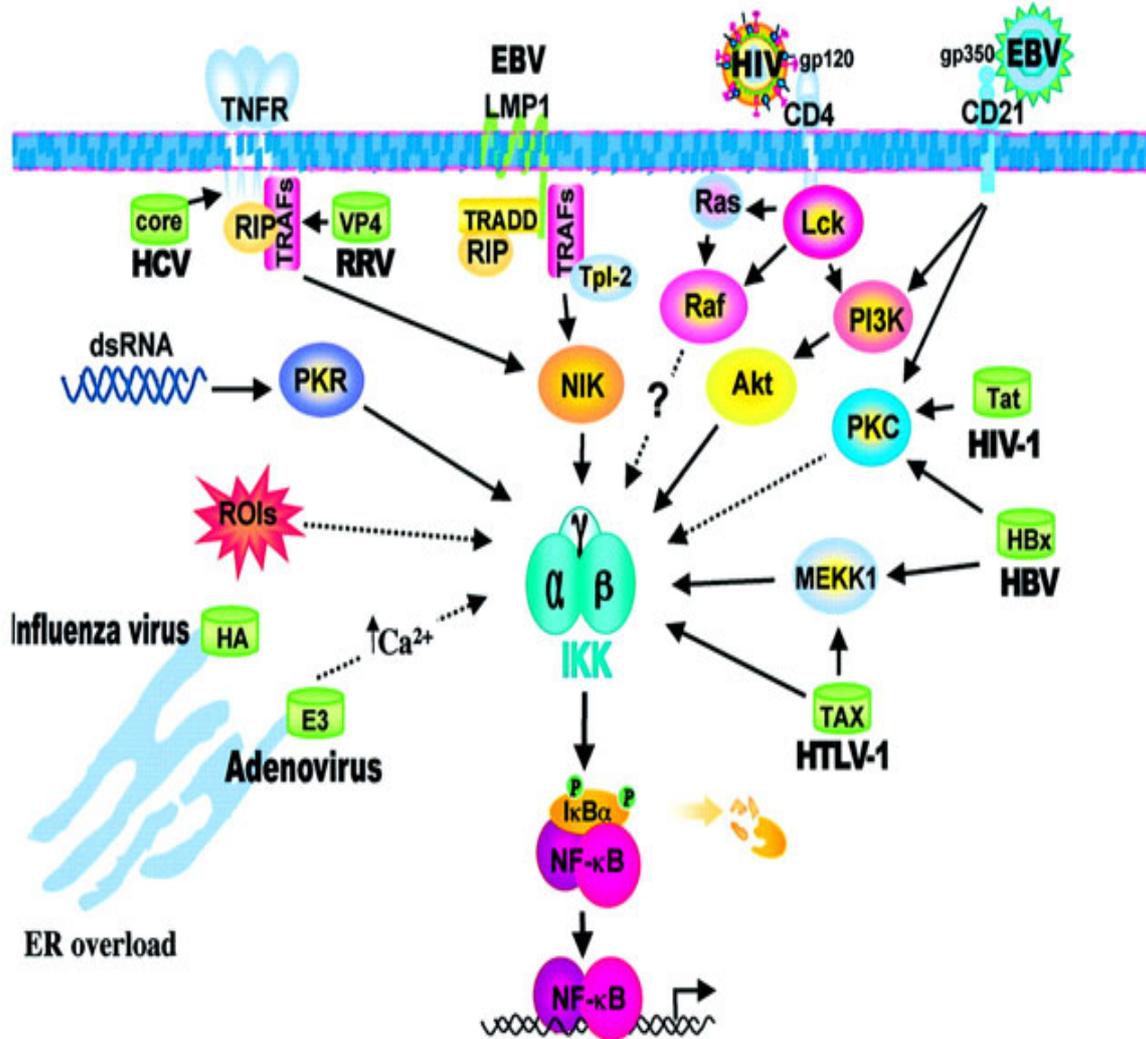


Figure 1.7

Different strategies of NF- κ B activation by viruses (Santoro, 2003).

1.5.4.3 Degradation of I κ B

Once IKK has been activated rapid degradation of I κ B is induced. Three different pathways have been identified leading to either degradation or dissociation of I κ B. The prototypical pathway being the phosphorylation of I κ B on serines 32 and 36 in IKK α , serines 19 and 23 in IKK β and serines 18 and 22 in IKK ϵ . This phosphorylation leads to polyubiquitinylation, which targets the protein for rapid degradation by the 26S proteasome. This degradation exposes the NLS of NF- κ B resulting in translocation into the nucleus and binding to the appropriate DNA sequence (Mercurio *et al*, 1997; Mercurio *et al*, 1999).

Two alternative pathways have been described, the second pathway being observed as a result of hypoxia or pervanadate treatment. This requires phosphorylation of IKK α at Tyrosine-42. This phosphorylation does not lead to proteosomal degradation, but rather induces dissociation of the inhibitor from NF- κ B.

The third pathway is initiated by short-wavelength UV radiation and results in ubiquitin-mediated proteosomal degradation. This process is not dependent on phosphorylation of serines 32 and 36 and is thus independent of IKK activity. In both of these alternative pathways, NF- κ B activation is considerably slower and weaker than in the classical pathway (Karin & Ben-Neriah, 2000; Zamanian-Daroush *et al*, 2000).

1.5.4.4 Receptors

Receptors are expressed on the cell surface and have to be activated by extracellular signals to initiate the signalling pathway which will lead to NF- κ B nuclear translocation, binding to DNA and finally transcription of cytokines, chemokines and other proteins which than can modulate the immune responses.

Receptors, which are activated by microbial products, include the Toll-like receptors (TLRs). TRLs consist of extracellular leucine-rich repeats and the cytoplasmic Toll-IL-1receptor (TIR) homology domain. To date 10 different human TLRs have been identified. TLRs are expressed on cells of the immune system, including monocytes, macrophages, neutrophils, dendritic cells and lymphocytes, but

their expression is also observed in other cells, including vascular endothelial cells, lung and intestinal epithelial cells, cardiac myocytes and adipocytes. TLR distinguish a wide variety of microbial ligands, including gram-positive and gram-negative bacteria, mycobacteria, viruses and fungi. The signalling pathways induced by TLRs vary, but mainly activate NF- κ B (Deva *et al*, 2003; Sandor *et al*, 2003; Sasai *et al*, 2005). TLRs interact with other different coreceptors as well as with each other. After binding of ligand to the receptor the cytosolic proteins MyD88 and Tollip are recruited to the cytosolic part of the receptor complex, which subsequently leads to the recruitment of IL-1 receptor-associated kinases (IRAK). IRAK are activated by phosphorylation and in turn initiate phosphorylation and activation of TRAF6. IRAK-TRAF6 leaves the receptor complex and interacts with TAK1, a member of the MAP kinase family. TAK1 activates MEKK1 and NIK and finally activates the IKK complex which leads to the degradation of I κ B (Jiang *et al*, 2003; Santoro *et al*, 2003; Tsujimura *et al*, 2004).

For TLR3, an IRAK-independent pathway was identified in response to dsRNA (Jiang *et al*, 2003). In contrast, Alexopoulou *et al* (2001) found that the TLR3-signalling pathway was also dependent on MyD88, IRAK, TRAF6 and Tollip .

Other results demonstrated that early events in the signalling pathway, which precede IRAK activation, are oxidant-dependent, and that ROS can modulate NF- κ B dependent transcription (Asehnoune *et al*, 2004).

Recently various reports have suggested that receptor-mediated ROS generation is coupled with Nox isozymes. Nox1, Nox3, Nox4 and Nox5 have been recently identified in non-phagocytic cells. They are novel homologues of p91^{phox} (Nox2) of NADPH oxidase in phagocytic cells. It has been found that direct interaction of TLR4 with Nox4 is involved in LPS-mediated ROS generation and NF- κ B activation (Park *et al*, 2004).

One of the best-understood signalling pathways is the one triggered by tumour necrosis factor- α (TNF- α), a proinflammatory cytokine. TNF- α signals by trimerizing

the TNF- α receptor1 (TNFR1), which via the adaptor protein TNFR-associated death domain (TRADD) recruits the proteins RIP and TRAF2 resulting again in the activation of MEKK1 and NIK, which then leads to the activation of IKK and NF- κ B translocation.

Other stimuli, such as stress factors, growth factors or mitogens all lead to the phosphorylation/activation of IKK by signalling pathways at present not identified (Santora *et al*, 2003).

1.5.4.5 Transcription of NF- κ B mediated genes

The controlled expression of cytokines is an essential component of an immune response, and uncontrolled transcription is associated with pathological conditions. The transcription of the relevant genes is cell-specific and depends on different families of transcription factors and enhancer proteins which have to act in unison to form so called 'enhanceosomes' on cytokine gene promoters. These complexes of transcription factors and enhancers, which bind to the promoters, differ in different cell types and lead therefore to the development of different cells and/or the production of a wide variety of cytokines, chemokines and other immune modulatory proteins. It is important to understand that NF- κ B, although playing a major role in immune responses, never acts alone and is dependent on other transcription factors and enhancer proteins (Holloway *et al*, 2001). In order for the chromatin to become accessible to the complexes of transcription proteins, deacetylation and acetylation of histones is also of importance. It has been found that there is some modification of the nucleosomal structure in response to smoking. The resulting imbalance between histone acetylation and deacetylation may contribute to the enhanced inflammation in smokers and susceptibility for the development of chronic obstructive pulmonary disease (Szulakowski *et al*, 2006).

1.5.5 NF- κ B and disease

NF- κ B has been implicated in many pathological conditions. Controlled activation is necessary for an effective immune response, but hyper- or prolonged activation contributes to tissue damage and pathological conditions in the host. HIV

and other viruses use NF- κ B, produced by the host, for replication (Staal *et al*, 1990). In contrast, it has been found that infection with respiratory viruses induces inflammatory cytokines by activation of p38 MAPK pathways with no significant involvement of NF- κ B. However NF- κ B also exerts an anti-viral innate immune response. Human parainfluenza virus type 3 is a mildly cytopathic virus that induces NF- κ B early in the course of infection, but is converted to a virulent virus when NF- κ B activation is inhibited. Similarly, human respiratory syncytial virus, a highly cytopathic virus, is converted to a mildly cytopathic virus after NF- κ B induction before virus replication (Bose *et al*, 2003).

NF- κ B seems to play an important role in asthma (Poyntner *et al*, 2002; La Grutta *et al*, 2003; Pastva *et al*, 2004). In asthma, granulocyte-macrophage colony-stimulating factor (GMSF) and IL-8 are over-expressed and down regulated by glucocorticoids through NF- κ B activity repression. However, high levels of these mediators are released in patients with severe asthma despite glucocorticoid treatment, which seems to be due to exaggerated NF- κ B activation (Gagliardo *et al*, 2003).

Type I diabetes is an inflammatory disease of the pancreatic island cells. The final outcome of the disease is the destruction of the insulin-producing β -cells. In a mouse model, it was observed that NF- κ B1 and c-Rel play distinct roles in the development of type I diabetes (Lamhamedi-Cherradi *et al*, 2003). In type 2 diabetes mellitus, insulin resistance plays a role and this may be mediated by phosphorylation of serine residues in insulin receptor substrate-1 (IRS-1). IRS-1 proved to be a substrate for IKK and it was suggested that phosphorylation of IRS-1 at ser³¹² by IKK may contribute to insulin resistance by activation of inflammatory pathways (Gao *et al*, 2002).

Atherosclerosis is a chronic inflammatory disease affecting arterial vessels. NF- κ B activity is increased within the intimal cells of human plaques, leading to the upregulation of the pro-inflammatory cytokines, IL-8 and TNF- α (Henriksen *et al*, 2004).

NF- κ B is implicated in multiple stages of the carcinogenic process and has been validated as a prominent cancer drug target. Hyperactivation of NF- κ B pathways have been implicated in the promotion of angiogenesis, invasive growth and metastasis (Andela *et al*, 2003). Elevated expression of cyclooxygenase-2 (COX-2) has been found in colorectal cancer (CRC) and this was paralleled by a significantly higher expression of IL-1 β , IL-6 and the NF- κ B subunit p65 (Maihöfner *et al*, 2003). Aberrant function of transforming growth factor- β 1 (TGF- β 1), a growth inhibitor of prostate epithelial cells, has been implicated in prostate cancer. Serum TGF- β 1 levels are elevated in patients with prostate cancer and are further increased in patients with metastatic carcinoma. TGF- β 1 activates IL-6, which has been implicated in the malignant progression of prostate cancer via multiple signalling pathways including NF- κ B (Park *et al*, 2003).

Inflammatory responses to infection must be precisely regulated to facilitate microbial killing, while limiting tissue damage to the host. In a mouse model, it was shown that deficiency of p50 resulted in increased expression of proinflammatory cytokines, exacerbated neutrophil recruitment and respiratory distress during pulmonary infection with *Escherichia coli*. P50 seems to protect the host by curbing inflammatory responses which prevent injury, and which are essential to survive pneumonia (Mizgerd *et al*, 2003). *Cryptosporidium parvum*, an intracellular parasite, is a common enteric pathogen in both immunocompetent and immunocompromised individuals. In cultured biliary epithelia (cholangiocytes), *Cryptosporidium parvum* infection induced the activation of IRAK-1, p-38 and NF- κ B (Chen *et al*, 2005).

Activation of NF- κ B has been reported in lung tissue and alveolar macrophages after exposure to endotoxin and this was related to the development of chemokine-mediated lung inflammation (Blackwell *et al*, 1996; Blackwell *et al*, 1997). Pulmonary inflammation is an essential component of host defence against *Streptococcus pneumoniae*. Important cytokines are TNF- α and IL-1, both potent activators of NF- κ B activation, which are rapidly induced upon microbial exposure. In mice deficient in TNF- α - and IL-1-dependent signalling pathways, bacterial clearance was decreased, neutrophil recruitment was impaired and there was a deficiency of chemokines KC and MIP-2 (Jones *et al*, 2005).

Helicobacter pylori causes various gastroduodenal diseases, including gastric lymphoma. It has been demonstrated that *Helicobacter pylori* activates the classical, as well as the alternative pathway, of NF- κ B activation in B-lymphocytes. In the alternative pathway the NF- κ B2/p100 precursor is cleaved and processed to p52 by phosphorylation of p100. This attenuates apoptosis and may contribute to malignancy (Ohmae *et al*, 2005).

Sepsis is a syndrome initiated by infection and is characterised by an overwhelming systemic inflammatory response. NF- κ B plays a central role in modulating the expression of proinflammatory cytokines and other mediators that contribute to organ dysfunction/ failure and death in sepsis. There exist two haplotypes of the IRAK-1 gene, and it has been found that the variant haplotype was associated with increased NF- κ B translocation, more severe organ dysfunction and mortality. Caspase-1 is the activating enzyme for the proinflammatory cytokines IL-1 β and IL-18, which play an important role in inflammatory disease, fever and septic shock. Caspase-1 is activated by stimulation of the P2X7 receptor, an ATP-gated ion channel, as well as during priming with LPS. It has been demonstrated that NF- κ B plays an important role in the ability of the P2X7R to activate caspase-1 (Kahlenberg *et al*, 2005).

1.5.6 NF- κ B and apoptosis

NF- κ B has been reported to possess both anti-apoptotic and pro-apoptotic properties. Genes, whose transcription is mediated by NF- κ B, include cellular inhibitors of apoptosis-1 (cIAP-1) and cIAP-2, and TNF-receptor-associated factors TRAF1 and TRAF2. Some members of the Bcl-2 gene family, which have anti-apoptotic properties, are also upregulated by NF- κ B. The ability of NF- κ B activation to block TNF- α induced apoptosis has been demonstrated in a wide range of cells, including fibrosarcoma, keratinocytes, endothelial cells, myeloid cells, chronic lymphocytic leukaemia, lymphoid cell lines, hepatocyte cell lines, melanoma cells, pancreatic cancer cells and head and neck squamous carcinoma (Sonis, 2002).

Leukocyte apoptosis and subsequent clearance by macrophages is important for the resolution of inflammation. NF- κ B activation in leukocytes recruited during the onset of inflammation is associated with pro-inflammatory gene expression, while activation during the resolution of inflammation is associated with the induction of apoptosis (Lawrence *et al*, 2001).

TNF-related apoptosis-inducing ligand (TRAIL) acts through membrane death receptors to induce apoptosis of activated T-lymphocytes and NF- κ B seems to play an essential role in the regulation of TRAIL (Baetu *et al*, 2001). It was later found that the dual function of NF- κ B as an inhibitor or activator of apoptosis depends on the relative levels of RelA and c-Rel subunits. RelA acts as a survival factor, while overexpression of c-Rel favours apoptosis (Chen *et al*, 2003).

In immunoregulatory T-lymphocytes (iT_H17) it was found that anti-apoptotic signals relayed by NF- κ B are critical regulators of cell fate specification and molecular differentiation (Stanic *et al*, 2004).

One of the key signalling molecules of apoptosis is cytochrome c. Normally located to the inner and outer mitochondrial membrane, it translocates to the cytosol and this efflux of cytochrome c, which also initiates NF- κ B activation, results in a cascade of caspase activation which leads to apoptotic cell death (Pullerits *et al*, 2005).

In EBV-specific T-lymphocytes it was found that CD8 engagement by HLA-class I molecules induces the activation of Ca²⁺-independent protein kinase (PKC) and the consequent nuclear translocation of NF- κ B, which is responsible for FasL mRNA up-regulation. FasL is responsible for the induction of CD8 T-cell apoptosis (Contini *et al*, 2005).

NF- κ B is also implicated in the regulation of both constitutive and stimulated apoptosis in human granulocytes (Ward *et al*, 1999; Ward *et al*, 2004).

1.5.7 *NF- κ B* in neutrophils

Dimers of p50NF- κ B1, p65RelA, and c-Rel are present in human neutrophils, and these complexes are associated with I κ B- α in resting cells. Following stimulation of neutrophils with LPS, TNF- α or FMLP, NF- κ B was found to translocate to the nucleus, but binding to DNA was soon terminated because of fast *de novo* synthesis of I κ B. It was therefore concluded that transcription factors or I κ B itself must be already present in the nucleus of these cells (McDonald *et al*, 1997). Vollebregt *et al* (1998) found NF- κ B activation in neutrophils within 10 minutes after phagocytosis of *Staphylococcus aureus*. The increase of NF- κ B nuclear translocation was modest compared to the background and it was speculated that NF- κ B might be constitutively present in circulating neutrophils (Vollebregt *et al*, 1998). This was later confirmed by Castro-Alcaraz *et al* (2002), who demonstrated that NF- κ B activity in human neutrophils is regulated by different mechanisms compared to other human cells. Stimulation of neutrophils with pro-inflammatory signals results in degradation of I κ B both in the cytoplasm and the nucleus (Castro-Alcaraz *et al*, 2002). Further research indicated that in human neutrophils, the sustained activation of NF- κ B is regulated by continuous phosphorylation and degradation of nuclear I κ B (Miskolci *et al*, 2003).

Although neutrophils from most patients with acute lung injury (ALI) were found to have increased levels of nuclear translocation of NF- κ B, there were some patients in which no increase could be observed when the neutrophils were stimulated with LPS. Interestingly, these so called non-responders needed significantly less ventilator support (Yang *et al*, 2003).

A novel mechanism for NF- κ B activation in human neutrophils was described recently. Interaction of neutrophils with fibronectin via β_2 -integrins provides a strong co-stimulatory signal for NF- κ B activation. This allows cytokines such as GM-CSF and IL-8, which do not activate NF- κ B in non-adherent cells in suspension, to trigger this pathway during adhesion. This effect might be important when neutrophils migrate through the extracellular matrix during inflammation (Kettritz *et al*, 2004).

ROS have been extensively linked to the activation of NF- κ B as mentioned above. However, in a recent study it is reported that H₂O₂ did not activate NF- κ B in bone marrow derived neutrophils, but rather inhibited LPS- or TNF α -induced nuclear translocation of this transcription factor. This indicates that oxidative stress also has negative regulatory effects on pro-inflammatory neutrophil pathways (Strassheim *et al*, 2004).

Finally new research has confirmed that neutrophils constitutively express NF- κ B/Rel proteins and I κ B- α in the nucleus. In these cells, IKK α , IKK β and IKK γ also partially localize to the nucleus where they associate with chromatin, which suggest a potential role in gene regulation (Ear *et al*, 2005).