# Table of Contents

**Declaration** iii
**Summary** iv
**Samevatting** vi
**Publications** viii
**Acknowledgments** ix
**Table of Contents** x
**List of Figures** xiii
**List of Tables** xvi
**Abbreviations** xvii

## *Chapter 1: Literature Review*

**1.1 Metals** 1
***1.1.1 Platinum*** 2
***1.1.2 Palladium*** 6
***1.1.3 Cobalt*** 8
***1.1.4 Vanadium*** 12

**1.2 Neutrophils** 17
***1.2.1 Neutrophil activation*** 17
*1.2.1.1 Neutrophil recruitment to inflammatory sites* 17
*1.2.1.2 Neutrophil phagocytosis and degranulation* 19
*1.2.1.3 Respiratory burst and the NADPH oxidase system* 21
*1.2.1.4 Calcium homeostasis in activated neutrophils* 24
***1.2.2 Neutrophil apoptosis*** 25
***1.2.3 Interleukin-8 (IL-8)*** 26

**1.3 C5a** 28
***1.3.1 Introduction*** 28
***1.3.2 C5a as anaphylatoxin*** 28
***1.3.3 C5a as chemoattractant*** 28
***1.3.4 C5a actions on neutrophils, monocytes and macrophages*** 29

**1.4 Pneumolysin** 29
***1.4.1 Introduction*** 29
***1.4.2 Origin of pneumolysin*** 30
***1.4.3 Structure of pneumolysin*** 30
***1.4.4 Biological effects of pneumolysin*** 30

**1.5 Nuclear Factor-Kappa B** 31
***1.5.1 Introduction*** 31
***1.5.2 NF-κB proteins*** 32
***1.5.3 The IκB proteins*** 32

***1.5.4 Activation of NF-κB*** 35
*1.5.4.1 Activating signals* 35
*1.5.4.2 IκB-kinase (IKK)* 35
*1.5.4.3 Degradation of IκB* 38
*1.5.4.4 Receptors* 38
*1.5.4.5 Transcription of NK-κB mediated genes* 40
***1.5.5 NF-κB and disease*** 40
***1.5.6 NF-κB and apoptosis*** 43
***1.5.7 NF-κB in neutrophils*** 45

## Chapter 2: Vanadium Promotes Hydroxyl Radical Formation by Activated Human Neutrophils

**2.1 Introduction** 47

**2.2 Materials and Methods** 49
***2.2.1 Chemicals and reagents*** 49
***2.2.2 Neutrophils*** 49
***2.2.3 Superoxide anion production*** 50
***2.2.4 Electron spin resonance (ESR) spectroscopy*** 50
***2.2.5 Spectrofluorimetric detection of hydroxyl radical*** 52
***2.2.6 Myeloperoxidase (MPO) activity*** 53
***2.2.7 Cellular ATP levels*** 54
***2.2.8 Expression and statistical analysis of results*** 54

**2.3 Results** 54
***2.3.1 Hydroxyl radical generation in cell-free systems*** 54
***2.3.2 Superoxide production by activated neutrophils*** 61
***2.3.3 Hydroxyl radical production by activated neutrophils*** 62
***2.3.4 Activity of myeloperoxidase*** 65
***2.3.5 Cellular ATP levels*** 65

**2.4 Discussion** 69

## Chapter 3: Activation of Nuclear Factor-Kappa B (NF-κB) in Human Neutrophils

**3.1 Introduction** 73

**3.2 Materials and Methods** 76
***3.2.1 Chemicals and reagents*** 76
***3.2.2 Neutrophils*** 76
***3.2.3 NK-κB activation*** 77
***3.2.4 Interleukin-8*** 78
*3.2.4.1 Production of IL-8* 78
*3.2.4.2 Immunological reactivity of IL-8* 78

***3.2.5 Phosphorylated proteins*** 79
***3.2.6 Expression and statistical analysis of results*** 80

**3.3 Results** 81
***3.3.1 NF-κB activation*** 81
*3.3.1.1 NF-κB activation in pneumolysin-treated neutrophils* 81
*3.3.1.2 Effects of the metals on NF-κB activation* 81
***3.3.2 IL-8*** 85
*3.3.2.1 Production of IL-8* 85
*3.3.2.2 Immunological reactivity of IL-8* 88
***3.3.3 Phosphorylated proteins*** 89
***3.3.4 Cellular ATP levels*** 89

**3.4 Discussion** 93

## *Chapter 4: Palladium Attenuates the Proinflammatory Interactions of C5a, Interleukin-8 and Pneumolysin with Human Neutrophils*

**4.1 Introduction** 96

**4.2 Materials and Methods** 97
***4.2.1 Chemicals and reagents*** 97
***4.2.2 Exposure of C5a, IL-8, and pneumolysin on the metals*** 98
***4.2.3 Neutrophils*** 98
***4.2.4 Spectrofluorimetric measurement of cytosolic $Ca^{2+}$*** 98
***4.2.5 Assay of neutrophil migration*** 99
***4.2.6 NF-κB activation*** 99
***4.2.7 Expression and statistical analysis of results*** 100

**4.3 Results** 100
***4.3.1 Effects of prior exposure to the metals on C5a-, IL-8, and pneumolysin-mediated alterations in neutrophil cytosolic $Ca^{2+}$ concentrations*** 100
***4.3.2 Effects of $Pd^{2+}$ on the leukotactic activity of C5a and IL-8*** 106
***4.3.3 Activation of NF-κB*** 106

**4.4 Discussion** 111

## *Chapter 5: Discussion* 114

## *Chapter 6: References* 117

# List of Figures

***Page***

**Figure 1.1:**
South Africa' role in world mineral reserves, 2003; South Africa's role in world mineral production, 2003. 3

**Figure 1.2:**
Major uses of cobalt. 10

**Figure 1.3:**
Vanadium reserves, 2003; Vanadium production, 2003. 14

**Figure 1.4:**
Structure of NF-κB and IκB. 33

**Figure 1.5:**
Structure of NF-κB bound to DNA 34

**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. 36

**Figure 1.7:**
Different strategies of NF-κB activation by viruses. 37

**Figure 2.1:**
ESR spectra following the addition of 100 μM hydrogen peroxide to 20 mM DMPO in HBSS only (a) and DMPO + 25 μM vanadium in the +2 (b), +3( c), +4 (d), and +5 (e) oxidation states in the absence or presence of 20mM sodium benzoate. 56

**Figure 2.2:**
ESR spectra following the addition of 100 μM hydrogen peroxide to 20 mM DMPO in HBSS only (a) and DMPO + vanadium in the +4 valence state at concentrations of 1.5 μ M (b), 6.25 μM (c), and 25 μM (d). 57

**Figure 2.3:**
The effects of addition of 100 μM hydrogen peroxide to vanadium in the +2, +3, +4, and +5 valence states (all at 25 μM) in the absence and presence of 20 mM sodium benzoate on formation of hydroxyl radical, using the spectrofluorimetric procedure. 59

**Figure 2.4:**
Effects of addition of vanadium in the +2, +3, +4, and +5 valence states (all at 100 μM) to xanthine/xanthine oxidase superoxide/hydrogen peroxide-generating system on formation of hydroxyl radical, using the spectro- 60

fluorimetric procedure. The effects of vanadium in the +5 valence state were measured in the absence and presence of 100 μM NADPH.

**Figure 2.5:** 63
ESR spectra of unstimulated neutrophils + 20 mM DMPO (a) and those of FMLP (1 μM)-activated neutrophils in the absence of vanadium (b) and in the presence of vanadium in the +2 (c), +3 (d), +4 (e), and +5 (f) valence states in the absence and presence of sodium azide (760 μM).

**Figure 2.6:** 64
ESR spectra of unstimulated neutrophils + 20 mM DMPO (a) and those of PMA (25 ng/ml)-activated neutrophils in the absence of vanadium (b) and in the presence of vanadium in the +2 (c), +3 (d), +4 (e), and +5 (f) valence states in the absence and presence of sodium azide (760 μM).

**Figure 2.7:** 66
Results of three experiments showing the paired ESR spectra of PMA (25 ng/ml)-activated neutrophils + 20 mM DMPO, treated with 25 μM vanadium in the +4 valence state in the absence (left) and presence (right) of 20 mM sodium benzoate.

**Figure 2.8:** 67
Effects of vanadium in the +2, +3, +4, and +5 valence states (25 μM) on FMLP-activated MPO-mediated autoiodination of intact neutrophils.

**Figure 2.9:** 68
Effects of vanadium in the +2, +3, +4, and +5 valence states (25 μM) on the oxidation of guaiacol by a cell-free system containing purified MPO and $H_2O_2$ (metal compounds at 25 and 100 μM).

**Figure 3.1:** 82
Phosphor-image showing the effects of pneumolysin on nuclear translocation of NF-κB in human neutrophils.

**Figure 3.2:** 83
Phosphor-image showing the effects of pneumolysin (Ply 8.37 and 41.75 ng/ml) on nuclear translocation of NF-κB in human neutrophils in the absence and presence of 10 mM EGTA, or 10 μg/ml docosahexaenoic acid (DHA).

**Figure 3.3:** 86
Effects of exposure to $Co^{2+}$, $Pd^{2+}$, $Pt^{4+}$, $V^{3+}$, $V^{4+}$, and $V^{5+}$ on the production of IL-8 (measured in cell-free supernatants) by human neutrophils.

**Figure 3.4:** 87
Effects of exposure to $Co^{2+}$, $Pd^{2+}$, $Pt^{4+}$, $V^{3+}$, $V^{4+}$, and $V^{5+}$ on the total (extracellular and intracellular) production of IL-8 by human neutrophils.

**Figure 3.5:** 90
Concentrations of phosphorylated proteins in the cytosol of unstimulated human neutrophils in comparison to the cell-free, phosphorylation-free background system, which is referred to in the result section as the negative control system.

**Figure 4.1:** 102
Effect of exposure to $Pd^{2+}$ (25 μM) on the $Ca^{2+}$-mobilizing interactions of C5a with neutrophils shown as fura-2 fluorescence traces of two representative experiments (5 in the series).

**Figure 4.2:** 103
Effect of exposure to $Pd^{2+}$ (25 μM) on the $Ca^{2+}$-mobilizing interactions of IL-8 with neutrophils is shown as the fura-2 fluorescence traces of 2 representative experiments (5 in the series).

**Figure 4.3:** 104
Effect of exposure to $Pd^{2+}$ (25 μM) on the $Ca^{2+}$-mobilizing interactions of pneumolysin with neutrophils is shown as the fura-2 fluorescence traces of 2 representative experiments (5 in the series).

**Figure 4.4:** 107
Effects of exposure to varying concentrations of $Pd^{2+}$ (6.25 – 25 μM) on the $Ca^{2+}$-mobilizing interactions of IL-8 (added as denoted by the arrow ↓) with neutrophils are shown as the fura-2 fluorescence traces of a single representative experiment (3 in the series).

**Figure 4.5:** 108
Effect of exposure to various concentrations of $Pd^{2+}$ (6.25 - 25μM) on the $Ca^{2+}$- mobilizing interactions of pneumolysin (added as denoted by the arrow ↓) with neutrophils are shown as the fura-2 fluorescence traces of a single representative experiment (3 in the series).

**Figure 4.6:** 109
Effect of exposure to $Pd^{2+}$ (25 μM) on the chemotactic activities of C5a and IL-8 for neutrophils.

**Figure 4.7:** 110
Phosphor-image showing the effect of untreated and $Pd^{2+}$-treated pneumolysin (Ply 20 ng/ml, $Pd^{2+}$ 25 μM) on nuclear translocation of NF-κB in neutrophils.

# List of Tables

*Page*

**Table 1.1:**
Vanadium and common vanadium compounds. 13

**Table 2.1:** 58
Comparison of the peak intensities of the DMPO-OH spectra generated following the addition of varying concentrations of vanadium (1.5, 6.25, 25 μM) in the +2, +3, and +4 valence states to DMPO (20 mM) and hydrogen peroxide (100 μM).

**Table 2.2:** 61
Effects of vanadium (in the four oxidation states) on FMLP- and PMA-activated superoxide production by neutrophils.

**Table 3.1:** 79
Lysates provided with the Bio-Plex assays

**Table 3.2:** 84
Mean values ± SEM of the densities, expressed as counts/mm$^2$, of nuclear factor-κB translocated nuclear proteins as determined by the Personal Molecular Imager FX and software from BIO-RAD Laboratories.

**Table 3.3:** 85
Effects of metals on the activation of NF-κB in neutrophils.

**Table 3.4:** 88
Effects of pre-treatment with $Co^{2+}$, $Pd^{2+}$, $Pt^{4+}$, $V^{2+}$, $V^{3+}$, $V^{4+}$, and $V^{5+}$ (all at 25μM) on the immunological reactivity of IL-8.

**Table 3.5:** 91
Phosphorylation of cytosolic proteins, involved in signalling pathways, in unexposed and metal-exposed human neutrophils.

**Table 3.6:** 92
Phosphorylation of the cytosolic transcription factors STAT3 and ATF2 in metal-treated and metal-untreated neutrophils.

**Table 4.1:** 105
Effects of pre-treatment with $Co^{2+}$, $Pd^{2+}$, $Pt^{4+}$, $V^{2+}$, $V^{3+}$, $V^{4+}$, and $V^{5+}$ (all at 25 μM) on the $Ca^{2+}$-mobilizing interactions of C5a, IL-8 and pneumolysin with neutrophils.

# Abbreviations

| | |
|---|---|
| Akt | alternative name for PKB |
| AP-1 | activator protein-1 |
| APC | antigen presenting cell |
| ATF | activating transcription factor |
| ATP | adenosine 5-triphosphate |
| BAL | bronchoalveolar lavage |
| BPI | bactericidal/permeability increasing protein |
| C | complement |
| CD | cluster of differentiation |
| CIAP | cellular inhibitors of apoptosis |
| COX | cyclooxygenase |
| CRC | colorectal cancer |
| DAG | diacylglycerol |
| DHA | docosahexaenoic acid |
| DISC | death-inducing signalling complex |
| DMPO | 5,5-dimethyl-1-pyrroline *n-oxide* |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DPI | diphenylene iodonium chloride |
| EBV | Epstein-Barr virus |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| EGTA | ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| EMSA | electrophoretic mobility shift assay |
| ER | endoplasmic reticulum |
| ERK | extracellular regulated kinase |
| ESR | electron spin resonance spectroscopy |
| FADD | fas-associated death domain |
| FMLP | N-formyl-L-methionyl-L-leucyl-L-phenylalanine |
| G-CSF | granulocyte colony-stimulating factor |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| gp120 | viral envelope glycoprotein |

| | |
|---|---|
| gp350 | viral envelope glycoprotein |
| GSK-3β | glycogen synthase kinase-3β |
| HA | influenza hemagglutinin |
| HAGG | heat-aggregated IgG |
| HeLa | cell line of human cancer cells |
| HLA | human leukocyte antigen |
| $H_2O_2$ | hydrogen peroxide |
| HBSS | Hanks' balanced salt solution |
| HBV | hepatitis B virus |
| HCl | hydrochloric acid |
| HCV | hepatitis C virus |
| HEK | human embryonic kidney cell line |
| HIV | human immunodeficiency virus |
| HOCl | hypochlorous acid |
| HTLV | human T-cell leukaemia virus |
| ICAM | intercellular adhesion molecule |
| IFN | interferon |
| IKAP | IKK-associated protein |
| IkB | inhibitory kappa B |
| IKK | IκB kinase |
| IL | interleukin |
| $IP_3$ | inositol-1,4,5-triphosphate |
| IRAK | IL-1 receptor-associated kinase |
| IRS | insulin receptor substrate |
| JNK | C-jun-amino-terminal kinase |
| LECL | Lucigenin-enhanced chemiluminescence |
| LPS | lipopolysaccharides |
| MAPK | mitogen-activated protein kinase |
| MBL | mannan-binding lectin |
| MEKK1 | mitogen-activated protein kinase/extracellular signal-regulated kinase |
| MIP-2 | macrophage inflammatory protein-2 |
| MPO | myeloperoxidase |
| MyD88 | myeloid differentiation primary response gene |
| mRNA | messenger ribonucleic acid |

| | |
|---|---|
| NADPH | nicotinamide adenine dinucleotide phosphate, reduced |
| NEMO | NF-κB essential modulator |
| NF-κB | nuclear factor kappa B |
| NIK | NF-κB-inducing kinase |
| NLS | nuclear localization sequence |
| $O_2$ | oxygen |
| $OH^-$ | hydroxyl radical |
| PAF | platelet-activating factor |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate-buffered saline |
| PI3-K | phosphatidylinositol 3-kinase |
| PKA | protein kinase A |
| PKB | protein kinase B |
| PKC | protein kinase C |
| PKR | double-stranded RNA-dependent protein kinase |
| $PLA_2$ | phospholipase $A_2$ |
| Ply | pneumolysin |
| PMA | phorbol 12- myristate 13-acetate |
| PMNL | polymorphonuclear leukocytes |
| PTK | protein-tyrosine kinase |
| PTP | protein-tyrosine phosphatase |
| PR3 | proteinase3 |
| RAST | radio allergro sorbent test |
| RHR | Rel homology region |
| RIP | receptor-interacting protein |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| RRV | rotavirus |
| SEM | standard error of the mean |
| SOD | superoxide dismutase |
| STAT | signal transducer and activator of transcription |
| TAK1 | transforming growth factor-β-activated kinase |
| Tat | transactivating protein |

| | |
|---|---|
| TCA | trichloracetic acid |
| TGF | transforming growth factor |
| TIR | Toll-IL-1receptor homology domain |
| TLR | Toll-like receptor |
| TNF | tumour necrosis factor |
| TNFR | TNF-receptor |
| TOLLIP | Toll-interacting protein |
| TRADD | TNFR-associated death domain |
| TRAF | TNF-receptor-associated factors |
| TRAIL | TNF-related apoptosis-inducing ligand |
| VP4 | viral capsid protein |

# 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-κB. 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, $NH_2$ 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).

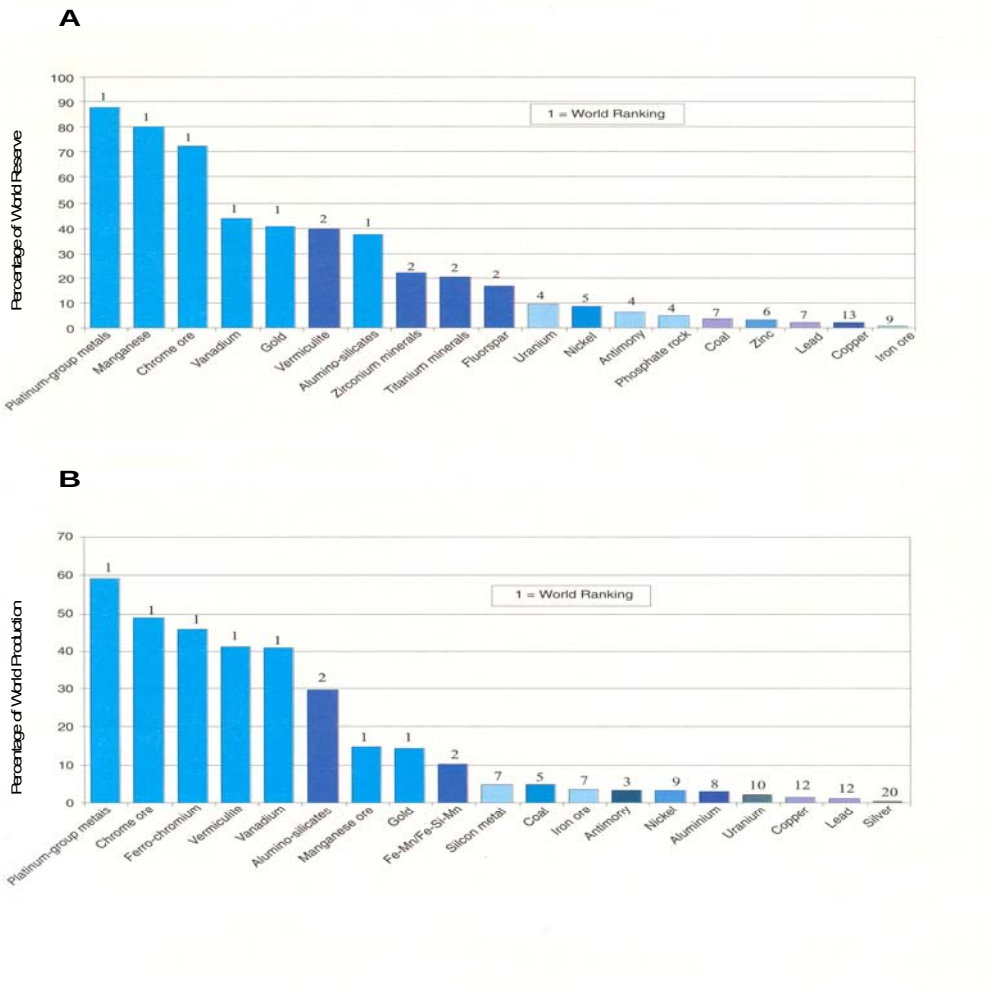
### *1.1.1 Platinum*

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.lanal.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 (Hostỳnek *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-$\gamma$, TNF-$\alpha$ 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 carboloy 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).

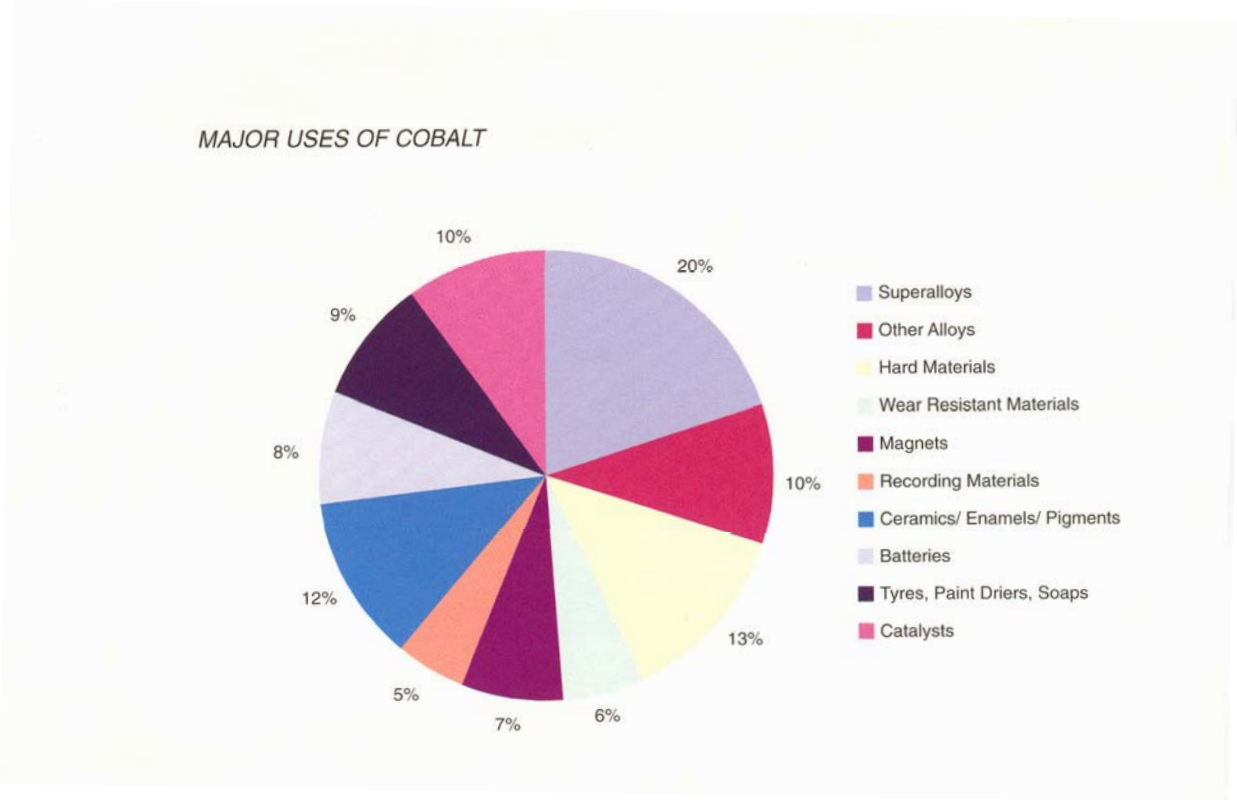



**Figure 1.2**

(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 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$\beta$, Il-6 and TNF-$\alpha$, as well as DNA binding of NF-$\kappa$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+/- 1.5 $\mu$g/L versus 0.3 +/-0.1 $\mu$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-$\alpha$ 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<br>Divanadium pentoxide<br>Vanadium oxide<br>Vanadic acid | $V_2O_5$ |
| Vanadyl sulphate | Vanadium oxysulphate<br>Vanadium oxide sulphate<br>Vanadium oxosulphate | $VOSO_4$ |
| Sodium metavanadate | Vanadic acid monosodium salt. | $NaVO_3$ |
| Sodium orthovanadate | Vanadic (II) acid trisodium salt.<br>Sodium vanadate.<br>Sodium vanadate oxide.<br>Trisodium orhtovanadate. | $Na_2VO_4$ |
| Ammonium metavanadate | Ammonium vanadate.<br>Vanadic acid ammonium salt | $NH_4VO_3$ |

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).

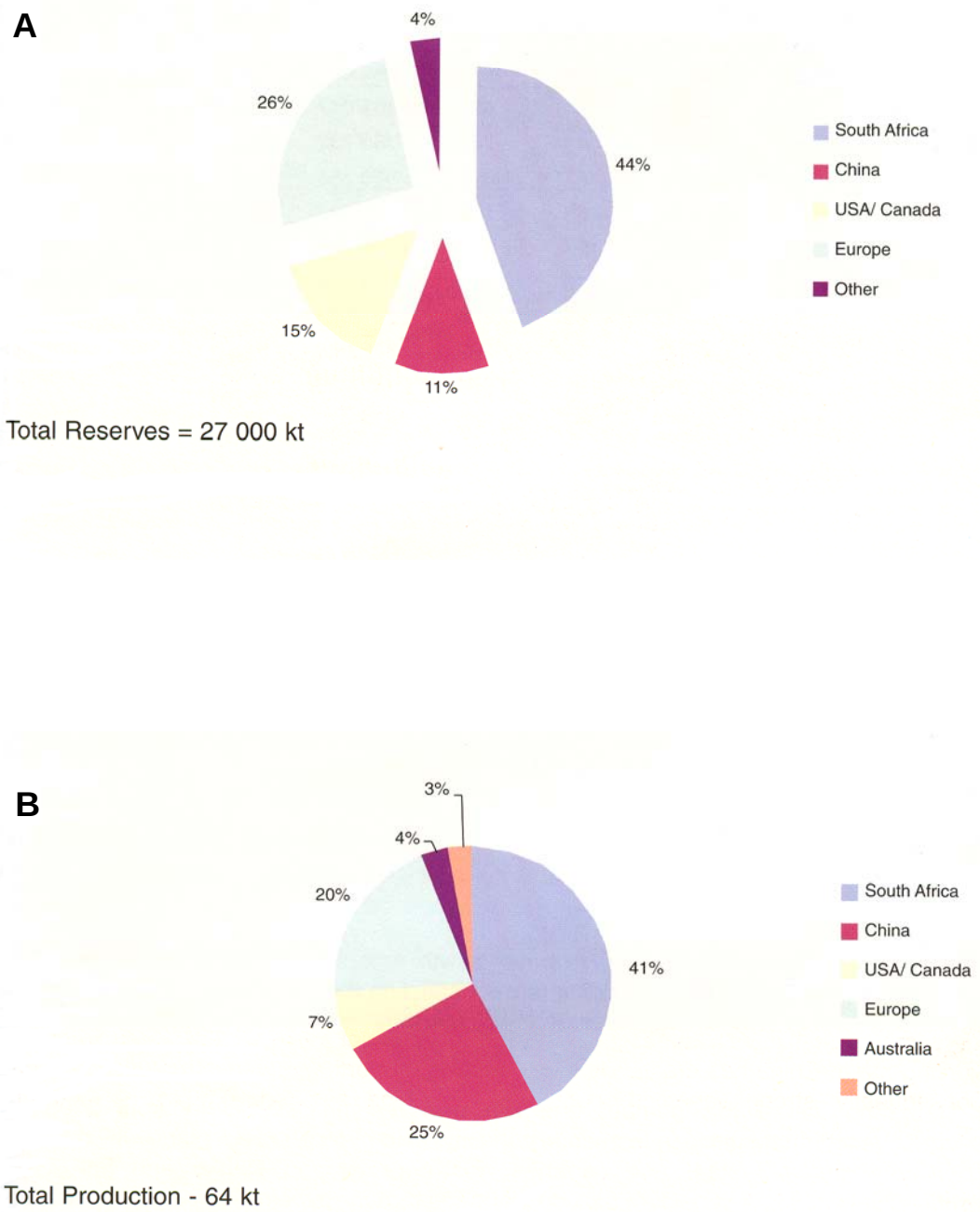



**Figure 1.3**

**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 μg/g.

Examples of the vanadium concentrations of some foods:

Black pepper, dill and mushrooms: 0.05 – 2 μgV/g.

Parsley: 1.8 μgV/g.

Shellfish and spinach: 0.5 – 0.8 μgV/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/aiq/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/aiq/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). Pervandanate 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 IκB-α which subsequently resulted in NF-κB DNA binding. Pervandanate did not induce serine phosphorylation of IκB-α, 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 $\beta_2$ integrin family. They are composed of variable $\alpha$-subunits (CD11a, CD11b and CD11c) and a common β-subunit (CD18). Two important $\beta_2$ 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 $\beta_2$ 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-$\alpha$ and IL-1$\beta$ 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 (Belaaouaj *et al*, 1998).

- Secretory phospholipase $A_2$ ($PLA_2$)

  Protein with potent bactericidal activity. $PLA_2$ 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 ($O_2^{-\cdot}$).

$$NADPH + 2\ O_2 \rightarrow NADP^+ + 2O_2^{\cdot -} + 2H^+$$

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

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

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 ($OH^{\bullet}$) 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).

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

*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

$$H_2O_2 + O_2^{-\bullet} \rightarrow O_2 + OH^- + OH^{\bullet}$$

*MPO-catalyzed reaction*:

Hypochlorous acid generated by MPO can react with superoxide to form $OH^{\cdot}$ according to the reaction

$$H_2O_2 + Cl^- \rightarrow HOCL + OH^-$$

$$HOCL + O_2^{-\bullet} \rightarrow OH^{\bullet} + O_2 + Cl^-$$

(Klebanoff, 1999)

Hydroxyl radical is one of the most powerful oxidants and may therefore contribute to the toxic activity of phagocytes. $OH^{\cdot}$ 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 $b_{558}$. Translocation of the cytoplasmic components to the membrane and their association with cyt $b_{558}$ render the complex functional. The cytochrome then transfers electrons from NADPH to $O_2$ to create superoxide in the reaction mentioned above.

*Cytochrome $b_{558}$*

Cyt $b_{558}$ 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 vescicles 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 $O_2$.

*$p47^{phox}$*

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 were it associates with cyt $b_{558}$.

*$p67^{phox}$ 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 $O_2$.

*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 $Ca^{2+}$ is present at very low levels (≈ 100nM). After receptor-mediated activation, there is an abrupt and short-lived increase in cytosolic free $Ca^{2+}$. This increase in the cytosolic $Ca^{2+}$ concentration peaks at 10 – 20 seconds, lasts for several minutes and is a prerequisite for the initiation of pro-inflammatory activities of neutrophils. These $Ca^{2+}$-dependent functions include activation of $\beta_2$-integrins and adhesion to vascular endothelium, activation of NADPH oxidase and subsequently superoxide production, degranulation, activation of phospholipase $A_2$ 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 $Ca^{2+}$ 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 $Ca^{2+}$ 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 $\beta_2$-integrins. The other site is located in the perinuclear space and is mobilized by chemoattractants, like FMLP, C5a, leukotriene $B_4$, 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 ($IP_3$). $IP_3$ interacts with $Ca^{2+}$ 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 $Ca^{2+}$ homeostasis is essential to prevent $Ca^{2+}$ 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-κB 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 $Ca^{2+}$, 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-Pocidalo *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, $CX_3C$ and C, on the basis of the relative positions of the first two cysteine residues. CXC and $CX_3C$ chemokines are distinguished by the presence of one in CXC and three in $CX_3C$ 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 $NH_2$ terminus before the first cysteine. This classification correlates with functional differences. $ELR^+$ CXC chemokines bind the receptors $CXCR_1$ and $CXCR_2$ 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 $NH_2$ 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-$\alpha$. 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 κB (NF-κB) 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 $PLA_2$ 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 $E_2$ and leukotriene $B_4$ 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-$\alpha$ and IL1-$\beta$ (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 cilial 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-$\kappa$B, first identified in 1986 by Sen and Baltimore in B-cells, was described as a nuclear factor bound to the immunoglobulin $\kappa$ light chain gene enhancer, and was therefore given the name 'nuclear factor-$\kappa$B'. But soon it was understood that this was a ubiquitous transcription factor present in virtually all cells. NF-$\kappa$B is present in the cytoplasm of nonstimulated cells, where it is bound and controlled by a family

of inhibitory proteins, the IκBs. Upon stimulation, subsequent phosphorylation, ubiquitination and proteolytic degradation of IκB, NF-κB 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 IκB, which then leads to inhibition of further DNA binding, as the affinity of NF-κB to Iκ-B is greater than to DNA (Baeuerle & Henkel, 1994; Baeuerle & Baltimore, 1996; Baldwin, 1996; Karin & Ben-Neriah, 2000).

### *1.5.2 NF-κB proteins*

In its active DNA binding form, NF-κB is a heterogeneous collection of dimers, composed of various combinations of members of the NF-κB/Rel family, which is characterized by the presence of the Rel homology domain. Five mammalian Rel proteins have been identified, NF-κB1 (p50 and its precursor p105), NF-κB2 (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 IκB proteins. It also contains the nuclear localization sequence (NLS). Different NF-κB 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-κB, 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 IκB proteins*

The IκB family includes IκBα, IκBβ, IκBγ, IκBε, Blc-3, the precursors of NF-κB1 and NF-κB2, and the *Drosophila* protein, Cactus. The most important regulators of mammalian NF-κB are IκBα, IκBβ and IκBε. All IkBs 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-κB1 and Nf-κB2 respectively, it can be concluded that these subunits are intramolecular IκBs (Baldwin, 1996; Karin & Ben-Neriah, 2000).

**Figure 1.4**

Structure of NF-κB and IκB.

(Huxford *et al,* 1998)

**Figure 1.5**

Structure of NF-κB bound to DNA

(Huxford *et al,* 1998)

### *1.5.4. Activation of NF-κB*

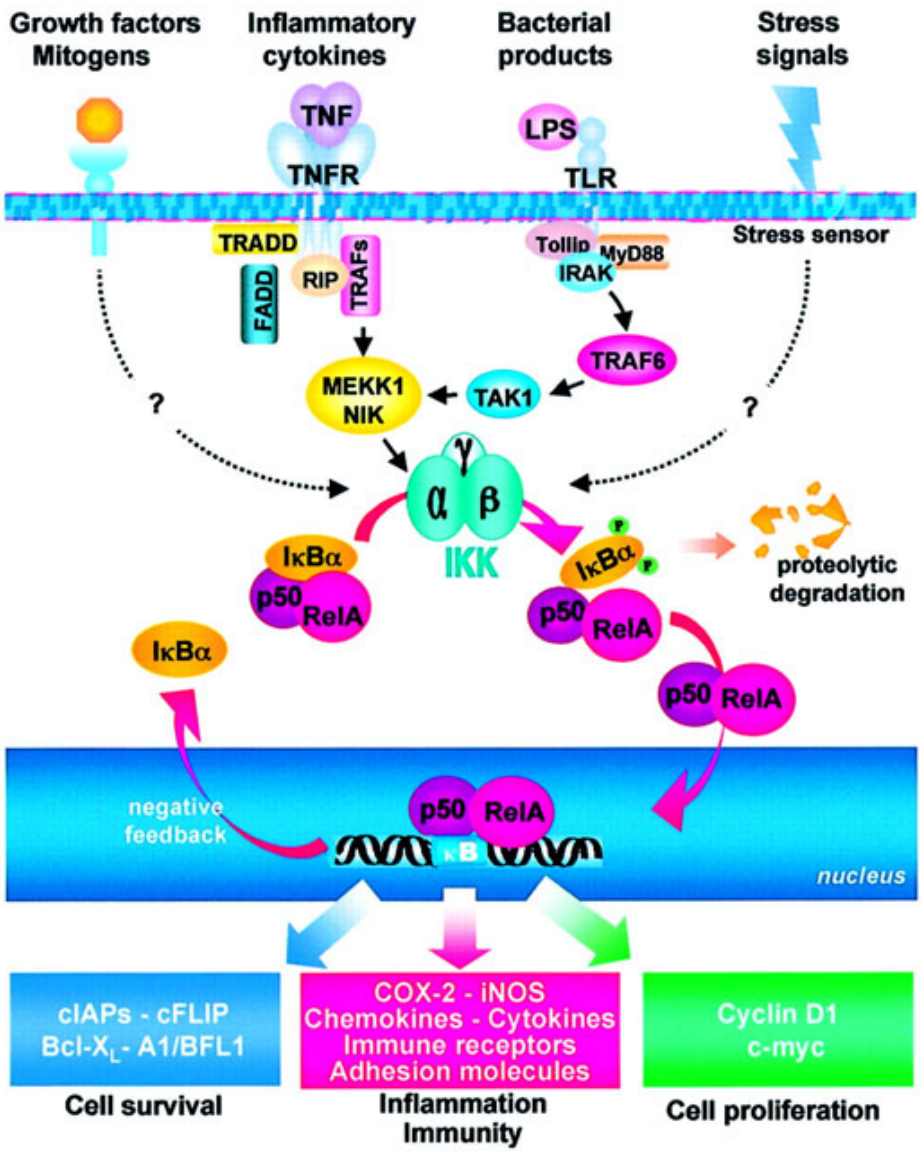
*1.5.4.1 Activating signals*

NF-κB 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-κB, and intracellular redox levels have been shown to regulate NF-κB 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 IκB-kinase (IKK)*

The signalling pathways induced by different stimuli and ligand binding to different receptors vary, but the key to NF-κB activation is phosphorylation and activation of IκB kinase (IKK). IKK is a large (>700 kDa) multicomponent enzyme complex containing two catalytic kinase subunits IKKα and IKKβ, which exist as a heterodimer. This enzyme complex also comprises two additional proteins, IKKγ and IKAP. IKKγ, which is also referred to as NEMO (NF-κB 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 IKKα and IKKβ. There are clear and distinct roles for IKKα and IKKβ. IKKβ is responsible for the phosphorylation of IκB in response to proinflammatory cytokines, such as TNF-α and IL-1. IKKα responds to yet unknown morphogenic signals and is crucial for NF-κB activation during embryonic development of the skin and skeletal system. IKKα and IKKβ are both activated by phosphorylation on specific serine residues. These are serines 176 and 180 in IKKα and serines 177 and 181 in IKKβ (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).

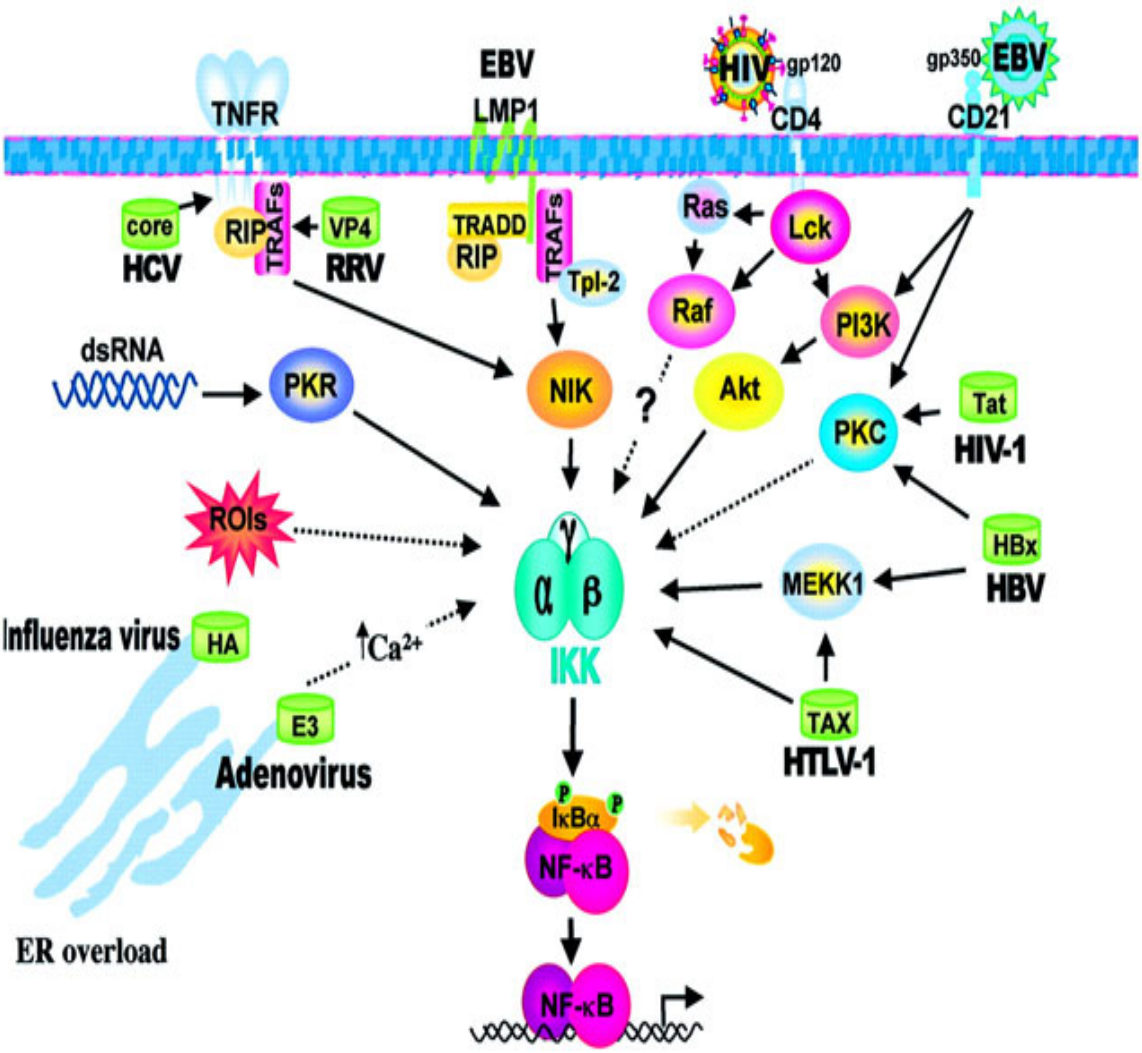



**Figure 1.7**

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

*1.5.4.3 Degradation of IκB*

Once IKK has been activated rapid degradation of IκB is induced. Three different pathways have been identified leading to either degradation or dissociation of IκB. The prototypical pathway being the phosphorylation of IκB on serines 32 and 36 in IKKα, serines 19 and 23 in IKKβ and serines 18 and 22 in IKKε. This phosphorylation leads to polyubiquitinylation, which targets the protein for rapid degradation by the 26S proteasome. This degradation exposes the NLS of NF-κB 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 IKKα at Tyrosine-42. This phosphorylation does not lead to proteosomal degradation, but rather induces dissociation of the inhibitor from NF-κB.

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-κB 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-κB 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-κB (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 IκB (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-κB 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-κB 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-κB 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-κB 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-κB, 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-κB and disease*

NF-κB 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-κB, 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-κB. However NF-κB also exerts an anti-viral innate immune response. Human parainfluenza virus type 3 is a mildly cytopathic virus that induces NF-κB early in the course of infection, but is converted to a virulent virus when NF-κB activation is inhibited. Similarly, human respiratory syncytial virus, a highly cytopathic virus, is converted to a mildly cytopathic virus after NF-κB induction before virus replication (Bose *et al*, 2003).

NF-κB 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-κB 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-κB 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-κB1 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 $ser^{312}$ 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-κB 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-κB is implicated in multiple stages of the carcinogenic process and has been validated as a prominent cancer drug target. Hyperactivation of NF-κB 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-1β, IL-6 and the NF-κB subunit p65 (Maihöfner *et al*, 2003). Aberrant function of transforming growth factor-β1 (TGF-β1), a growth inhibitor of prostate epithelial cells, has been implicated in prostate cancer. Serum TGF-β1 levels are elevated in patients with prostate cancer and are further increased in patients with metastatic carcinoma. TGF-β1 activates IL-6, which has been implicated in the malignant progression of prostate cancer via multiple signalling pathways including NF-κB (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-κB (Chen *et al*, 2005).

Activation of NF-κB 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-κB 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-κB activation in B-lymphocytes. In the alternative pathway the NF-κB2/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-κB 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-κB translocation, more severe organ dysfunction and mortality. Caspase-1 is the activating enzyme for the proinflammatory cytokines IL-1β 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-κB plays an important role in the ability of the P2X7R to activate caspase-1 (Kahlenberg *et al*, 2005).

### *1.5.6 NF-κB and apoptosis*

NF-κB has been reported to possess both anti-apoptotic and pro-apoptotic properties. Genes, whose transcription is mediated by NF-κB, 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-κB. The ability of NF-κB 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-κB 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-κB 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-κB 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 (iNKT) it was found that anti-apoptotic signals relayed by NF-κB 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-κB 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 $Ca^{2+}$-independent protein kinase (PKC) and the consequent nuclear translocation of NF-κB, which is responsible for FasL mRNA up-regulation. FasL is responsible for the induction of CD8 T-cell apoptosis (Contini *et al*, 2005).

NF-κB 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-κB in neutrophils

Dimers of p50NF-κB1, p65RelA, and c-Rel are present in human neutrophils, and these complexes are associated with IκB-α in resting cells. Following stimulation of neutrophils with LPS, TNF-α or FMLP, NF-κB was found to translocate to the nucleus, but binding to DNA was soon terminated because of fast *de novo* synthesis of IκB. It was therefore concluded that transcription factors or IκB itself must be already present in the nucleus of these cells (McDonald *et al*, 1997). Vollebregt *et al* (1998) found NF-κB activation in neutrophils within 10 minutes after phagocytosis of *Staphylococcus aureus*. The increase of NF-κB nuclear translocation was modest compared to the background and it was speculated that NF-κB 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-κB 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 IκB both in the cytoplasm and the nucleus (Castro-Alcaraz *et al*, 2002). Further research indicated that in human neutrophils, the sustained activation of NF-κB is regulated by continuous phosphorylation and degradation of nuclear IκB (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-κB, 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-κB activation in human neutrophils was described recently. Interaction of neutrophils with fibronectin via $\beta_2$-integrins provides a strong co-stimulatory signal for NF-κB activation. This allows cytokines such as GM-CSF and IL-8, which do not activate NF-κB 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-κB as mentioned above. However, in a recent study it is reported that $H_2O_2$ did not activate NF-κB in bone marrow derived neutrophils, but rather inhibited LPS- or TNFα-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-κB/Rel proteins and IκB-α in the nucleus. In these cells, IKKα, IKKβ and IKKγ also partially localize to the nucleus where they associate with chromatin, which suggest a potential role in gene regulation (Ear *et al*, 2005).

# Chapter 2

# Vanadium Promotes Hydroxyl Radical Formation By Activated Human Neutrophils

**Hypothesis**

Vanadium in its different valence states augments the production of reactive oxidants by activated human neutrophils.

**Aim**

The aim of the experiments described in this chapter was to identify the effects of vanadium in the +2, +3, +4, and +5 valence states on superoxide generation, myeloperoxidase (MPO) activity, and hydroxyl radical formation by activated human neutrophils in vitro, using lucigenin-enhanced chemiluminescence (LECL), autoiodination, and electron spin resonance spectroscopy.

## 2.1 Introduction

Vanadium is a ubiquitous, naturally occurring, transition metal which is found in high concentrations in the earth's crust, oceans, soil and fossil fuels (Barceloux, 1999b). Metallic vanadium does not exist in nature, but rather as vanadium compounds in oxidation states ranging from -1 to +5, the most common valences being +3, +4 and +5, with quadrivalent salts being the most stable (Barceloux, 1999b). Vanadium is an industrially important metal, which is used primarily in the manufacture of corrosion-resistant metal alloys. Consequently, those who mine and refine vanadium, as well as those involved in the manufacture of metal alloys, especially stainless steel, have high levels of occupational exposure to this metal (Barceloux, 1999b; Vanadium, 2000). Other high-risk occupations include those employed in oil-fired electricity power stations, as well as in the petrochemical industry, because of exposure to vanadium emissions into the atmosphere during the combustion of petroleum, coal and heavy oils (Barceloux, 1999b; Vanadium, 2000; Vanadium, 2001). Adverse health effects associated with excessive exposure to vanadium in the workplace include rhinitis, wheezing, nasal haemorrhage, conjunctivitis, cough, sore throat, and chest pain, all of which are usually transient (Vanadium, 2001), although persistent bronchial hyperresponsiveness and asthma have also been described (Irsigler *et al*, 1999).

Environmental pollution due to vanadium is a potential health threat, albeit of uncertain magnitude (Barceloux, 1999b; Vanadium, 2000; Vanadium, 2001). In the

environmental health setting, vanadium in the atmosphere originates predominantly from the combustion of heavy fuel oils, the metal being a major constituent of residual oil fly ash, which is both persistent and respirable (Van Klaveren & Nemery, 1999). Power plants and other industries that burn heavy oil are the primary offenders, while the contribution of vehicle exhaust fumes is slight because of the low vanadium levels in refined petroleum products. Cigarettes contain vanadium, which is present in both cigarette smoke and ash (Adachi *et al*, 1998) at concentrations comparable with those of iron (Mussalo-Rauhammaa *et al*, 1986).

In both the occupational and environmental settings, exposure to vanadium in the +4 and +5 valence states predominates (Vanadium, 2000) with interconversion between these two valence states occurring in biological systems (Mukherjee *et al*, 2004). Adverse health effects are likely to occur both from metal-induced toxicity and irritant pro-inflammatory effects. In the case of direct toxicity, vanadium compounds are well-recognized inhibitors of different ATPases, particularly $Na^+$, $K^+$-ATPase (Sabbioni *et al*, 1991; Mukherjee *et al*, 2004), while the pro-inflammatory activities result from the pro-oxidative activation of several transcription factors, including NFκB, JNK and AP-1, which cooperate to activate genes encoding pro-inflammatory cytokines following exposure of macrophages and T-lymphocytes to the metal *in vitro* (Chen *et al*, 1999; Huang *et al*, 2001). Moreover, inhalation or intracheal instillation of residual oil fly ash, or its major constituent vanadyl sulphate, have been reported to result in up-regulation of genes encoding pro-inflammatory cytokines in rat pulmonary tissue (Chong *et al*, 2000; Nadadur *et al*, 2002).

The neutrophil, a prototype inflammatory cell which is poorly responsive to conventional anti-inflammatory chemotherapy (Thomson *et al*, 2004; Tintinger *et al*, 2005), is mobilized to the airways following inhalation of toxic gases and particles, and is the probable perpetrator of inflammation-related airway damage and dysfunction associated with inhalation of these agents (Bassett *et al*, 2000; Saldiva *et al*, 2002; Douwes *et al*, 2002). However, with a few exceptions (Zhang *et al*, 2001; Wang *et al*, 2003), relatively little is known about the potential of vanadium to alter the pro-inflammatory activities of these cells. In the current study, the effects of vanadium on the potentially harmful, prooxidative activities of human neutrophils

were investigated, with emphasis on the formation of hydroxyl radical, one of the most potent and toxic oxidants in biological systems (Cheng *et al*, 2002).

## 2.2 Materials and Methods

### *2.2.1 Chemicals and reagents*

Vanadium was used in the +2, +3, +4 and +5 valence states as vanadium (II) chloride, vanadium (III) chloride, vanadyl sulphate hydrate and sodium metavanadate with respective molecular weights of 121.85, 157.30, 163.00 and 121.93, all of which were purchased from Sigma-Aldrich (St Louis, MO, USA). According to the certificates of analysis provided by the manufacturer for the batches of each agent used in the current study the respective vanadium contents of each compound were 39.50, 31.40, 20.71 and 41.10% (titration with $KMnO_4$). These were dissolved in distilled water to give stock solutions of 10 mM for each agent, and used in the various assays described below at concentrations of 1-25 μM. Unless indicated, all other chemicals and reagents were also purchased from Sigma-Aldrich (St Louis, MO,USA).

### *2.2.2 Neutrophils*

Purified neutrophils were prepared from heparinized (5 units of preservative-free heparin/ml) venous blood of healthy, non-smoking adult human volunteers and separated from mononuclear leukocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics) cushions at 400 g for 25 minutes at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 minutes. The neutrophils, which were routinely of high purity (>90%) and viability (>95%) were resuspended to $1x10^7$/ml in PBS and held on ice until used.

### *2.2.3 Superoxide anion production*

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg & Ferber, 1984). Neutrophils ($1x10^6$/ml, final) were pre-incubated for 10 minutes in 800 μl indicator-free Hanks' balanced salt solution (HBSS, pH 7.4, 1.25 mM $CaCl_2$) containing 0.2 mM lucigenin. Following pre-incubation, 100 μl HBSS or vanadium (1.5-25 μM) in the +2, +3, +4 and +5 valence states followed immediately by addition of either 100 μl HBSS, or 100 μl of the synthetic chemoattractant N-formyl-L-leucyl-L-phenylalanine (FMLP, 1 μM final) or the phorbol ester, phorbol 12-myristate 13-acetate (PMA, 25 ng/ml), were added to control and experimental systems respectively and LECL responses recorded using an LKB Wallac 1251 chemiluminometer (Turku, Finland). LECL readings were integrated for 11 second intervals and recorded as mV x $seconds^{-1}$ ($mV.s^{-1}$).

The stock concentrations of FMLP and PMA were 0.5 mM and 1 mg/ml in dimethylsulphoxide (DMSO) and ethanol respectively, giving final concentrations of each solvent in the assays of reactive oxidant generation (superoxide anion, hydroxyl radical generation, MPO-mediated auto-iodination) of 0.02% (DMSO) and 0.0025% (ethanol).

### *2.2.4 Electron spin resonance (ESR) spectroscopy*

A spin-trapping procedure was used to investigate the effects of vanadium in the +2, +3, +4 and +5 valence states on hydroxyl radical production using either a cell-free system with added hydrogen peroxide, or activated neutrophils. In the case of the cell-free system, the various vanadium compounds at a final concentration range of 1.5-25 μM were added to hydrogen peroxide (100 μM, final) in HBSS containing the hydroxyl radical spin trap, 1-pyroline N-oxide (DMPO, 20 mM final), in a final reaction volume of 1 ml, incubated at 37°C/5 minutes, and then analyzed for the DMPO-OH adduct using a Bruker EMX ESR analyzer (Bruker Biospin GmbH Rheinstetten, Germany), with the following parameter settings: receiver gain = 5.64 x $10^5$; modulation amplitude = 2 Gauss; sweep width = 80 Gauss; sweep time = 41.94 ms; power = 25 mW. These experiments were performed in the presence and

absence of the hydroxyl radical scavenger, sodium benzoate (20 mM, final). The results are presented as either the spectra for individual experiments, or as the peak-to-peak intensities in arbitrary units.

In the case of cell-containing systems, neutrophils were preincubated for 10 min at 37°C in the presence and absence of the myeloperoxidase inhibitor, sodium azide at a final concentration of 760 μM (50 μg/ml), followed by addition in rapid succession of DMPO (20 mM, final), the vanadium compounds (1.5 - 25 μM) and either FMLP (1 μM), or an equivalent volume of HBSS to unstimulated control systems. When PMA (25 ng/ml) was used to activate the neutrophils, DMPO and vanadium were added to the cells 5 minutes after PMA. The tubes, which contained 2 x $10^6$ neutrophils in a final volume of 2 ml HBSS, were incubated for either 2 minutes or 10 minutes at 37°C for FMLP- and PMA-activated systems respectively, followed by measurement of DMPO-OH. Superoxide dismutase (SOD, from bovine erythrocytes, 100 milliunits/ml final) was included in PMA-treated systems to protect the DMPO-OH adduct from superoxide during exposure of neutrophils to this potent activator of NADPH oxidase (Samuni *et al*, 1988).

To confirm the involvement of activated neutrophils in vanadium-mediated formation of hydroxyl radical, DMPO-OH adduct formation was measured using: i) a neutrophil-free system which contained all the other components of the assay, including SOD; and ii) a system in which varying concentrations of neutrophils (0.25, 1 and 4 x $10^6$/ml, final) were activated with FMLP (1 μM) in the presence of vanadium (25 μM) in the +4 valence state.

In additional experiments, the effects of the NADPH oxidase and protein kinase C (PKC) inhibitors, diphenylene iodonium chloride (DPI, 10 μM final) and GF109203X (5 μM final) respectively, as well as those of the hydroxyl radical scavenger, sodium benzoate (20 mM, final), and catalase (from bovine liver, 500 units/ml, final) on hydroxyl radical formation by vanadium-treated neutrophils were also investigated.

### *2.2.5 Spectrofluorimetric detection of hydroxyl radical*

As an alternative to ESR spectroscopy with DMPO, a spectrofluorimetric procedure based on the formation of catechol from salicyclate was used to detect hydroxyl radical formation in cell-free systems (Liu *et al*, 1997). The advantage of using this method is that it eliminates the complicating effects of superoxide on DMPO-OH, and can therefore be used to measure the possible involvement of superoxide in the conversion of vanadium from the +5 to the +4 valence states as described previously (Shi & Dalal, 1993; Zhang *et al*, 2001; Wang *et al*, 2003). In my experience, this method is suitable for detection of hydroxyl radical in cell-free systems, but not in cell-containing systems.

To investigate the potential of superoxide to reduce vanadium from the +5 to the +4 valence states, sodium metavanadate (100 μM) was added to an xanthine (0.9 mM, final) xanthine oxidase (66.6 mU/ml, final) superoxide/hydrogen peroxide-generating system with and without SOD (100 milliunits/ml, final) 5 minutes after initiation of the reaction and incubated for a further 15 minutes at room temperature in a final reaction volume of 2 ml HBSS containing 5 mM sodium salicyclate in the presence and absence of 100 μM NADPH (Liu *et al*, 1997). On completion of incubation, catechol was extracted from the incubation mixtures by addition of an equal volume of ethyl acetate. The ethyl acetate-extractable component was evaporated to dryness under a stream of nitrogen and reconstituted in 2 ml of borate buffer (0.3 M, pH 11.3) and 1 ml of a 1% aqueous solution of 2-cyanoacetamide as described previously (Liu *et al*, 1997). The tubes were then placed in a boiling water bath for 10 minutes, cooled immediately and fluorescence intensity recorded using a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 447 and 508 nm respectively (Liu *et al*, 1997). The effects of vanadium in the +2, +3 and +4 valence states on hydroxyl radical formation by the xanthine/xanthine oxidase system ± SOD was also investigated. Detection of hydroxyl radical using the xanthine/xanthine oxidase system required vanadium concentrations of 100 μM.

Positive control systems included vanadium (25 μM) in the +2, +3 and +4 valence states with added hydrogen peroxide (100 μM) in the absence and presence of sodium benzoate (20 mM), while systems consisting of xanthine/xanthine oxidase only, hydrogen peroxide only, sodium metavanadate + hydrogen peroxide, or the metals only served as negative controls.

### *2.2.6 Myeloperoxidase (MPO) activity*

Auto-iodination of neutrophils was used to measure the activity of MPO following activation of neutrophils with FMLP (1 μM, final) in the absence and presence of vanadium, with the metal in its various valence states being used at a fixed, final concentration of 25 μM. Briefly, neutrophils (1 x $10^6$) were preincubated for 10 minutes at 37°C in 0.8 ml HBSS containing 1 μCi of iodine-125 (as $Na^{125}I$, specific activity 17.4 Ci/mg, Perkin Elmer Life and Analytical Sciences, Boston, MA) and 25 nanomoles of cold carrier NaI. Following preincubation, 100 μl HBSS or vanadium (fixed, final concentration of 25 μM), followed immediately by addition of either 100 μl of HBSS (unstimulated systems), or 100 μl FMLP (1 μM, final) were added to each tube. The tubes, which contained a total volume of 1 ml, were incubated for 10 min at 37°C, after which the reactions were terminated and neutrophil proteins precipitated by addition of 3 ml 20% trichloracetic acid (TCA), after which they were pelleted by centrifugation and washed twice with TCA. The levels of radioactivity in the pellets were measured, and the results expressed as nmoles $^{125}I/10^7$ neutrophils.

The effects of the metals on the activity of purified MPO were investigated using a colorimetric procedure based on the oxidation of guaiacol. Reaction systems contained MPO (from human leukocytes, Sigma), guaiacol, and hydrogen peroxide at fixed final concentrations of 50 milliunits/ml, 4 mM and 5 mM respectively, with and without the metals, which were used at fixed final concentrations of 25 and 100 μM. The final volume in each well of a microtiter plate was 200 μl (all reactants in HBSS) and reactions were initiated by the addition of guaiacol/hydrogen peroxide, and MPO-mediated oxidation of guaiacol monitored at a wavelength of 450 nm in a microplate spectrophotometer.

### *2.2.7 Cellular ATP levels*

To determine the effects of the test vanadium compounds (25 μM) on neutrophil viability, intracellular ATP concentrations were measured in cell lysates (1 x $10^6$ cells/ml) following exposure to the metals for up to 6 hours at 37°C using a luciferin/luciferase procedure (Holmsen *et al*, 1972).

### *2.2.8 Expression and statistical analysis of results*

With the exception of the results of the ESR spectroscopy experiments, some of which are shown as the spectra from individual experiments, the results of the other investigations are expressed as the mean values ± SEM. Statistical analysis of data was performed by using the Mann-Whitney U-test, and ANOVA where appropriate. A computer-based software system (Graph Pad Prism® 4, Graph Pad Software Inc. San Diego, CA) was used for analysis and *P* value of <0.05 was taken as significant. In the case of ESR results, relative peak intensity values were used to calculate levels of statistical significance.

## 2.3 Results

### *2.3.1 Hydroxyl radical generation in cell-free systems*

The effects of vanadium in its various valence states in combination with hydrogen peroxide on hydroxyl radical formation using the ESR/DMPO system are shown in Figure 2.1 (page 56). Addition of hydrogen peroxide to vanadium in the +2, +3 and +4, but not the +5, valence states at all the concentrations tested (1.5-25 μM, the highest concentration shown in Figure 2.1 (page 56), resulted in an intense ESR spectrum consisting of the 1:2:2:1 quartet characteristic of the DMPO-OH adduct, compatible with hydroxyl radical formation. Inclusion of 20 mM benzoate markedly attenuated the formation of the DMPO-OH adduct. In the case of vanadium in the +2, +3 and +4 valence states, a clear dose response relationship was evident as can

be seen in Figure 2.2 (page 57), which depicts the ESR spectra generated following addition of vanadyl sulphate at concentrations of 1.5, 6.25 and 25 μM to 100 μM $H_2O_2$ in the presence of 20 mM DMPO, and in Table 2.1 (page 58), which shows the comparable data for the metal in the +2, +3 and +4 valence states, which indicate that the metal in the +3 valence state is most effective.

At the highest concentration of vanadyl sulphate (25 μM) shown in Figure 2.2 (page 57), it may seem that an additional spin adduct (s), possibly carbon-centred, is evident. Although the existence of such c-centred radicals is possible (Dikalov & Mason, 2001; Romero *et al*, 2003), their identity is not known. Moreover, the fact that the DMPO-OH radical signal increases to a greater degree than the peaks for these possible c-centred radicals, together with the benzoate data shown in Figure 2.1 (page 56), supports the primary involvement of the DMPO-OH radical.

Using the spectrofluorimetric procedure for the detection of hydroxyl radical, addition of vanadium in the +2, +3 and +4 valence states to hydrogen peroxide in the presence of sodium salicyclate resulted in the formation of catechol, compatible with the generation of salicylate-reactive hydroxyl radical, as shown in Figure 2.3 (page 59). Again, vanadium in the +5 valence state was unreactive. This procedure was also used to probe the possible involvement of superoxide in the conversion of vanadium from the +5 to the +4 valence states, in preference to the ESR/DMPO procedure because of the sensitivity of the DMPO-OH adduct to superoxide (Samuni *et al*, 1988). These results, which are shown in Figure 2.4 (page 60), demonstrate formation of hydroxyl radical on exposure of vanadium in the +2, +3 and +4 valence states to the xanthine/xanthine oxidase superoxide/hydrogen peroxide-generating system. However, formation of hydroxyl radical was not detected in the system containing vanadium in the +5 valence state, either in the absence or the presence of NADPH.

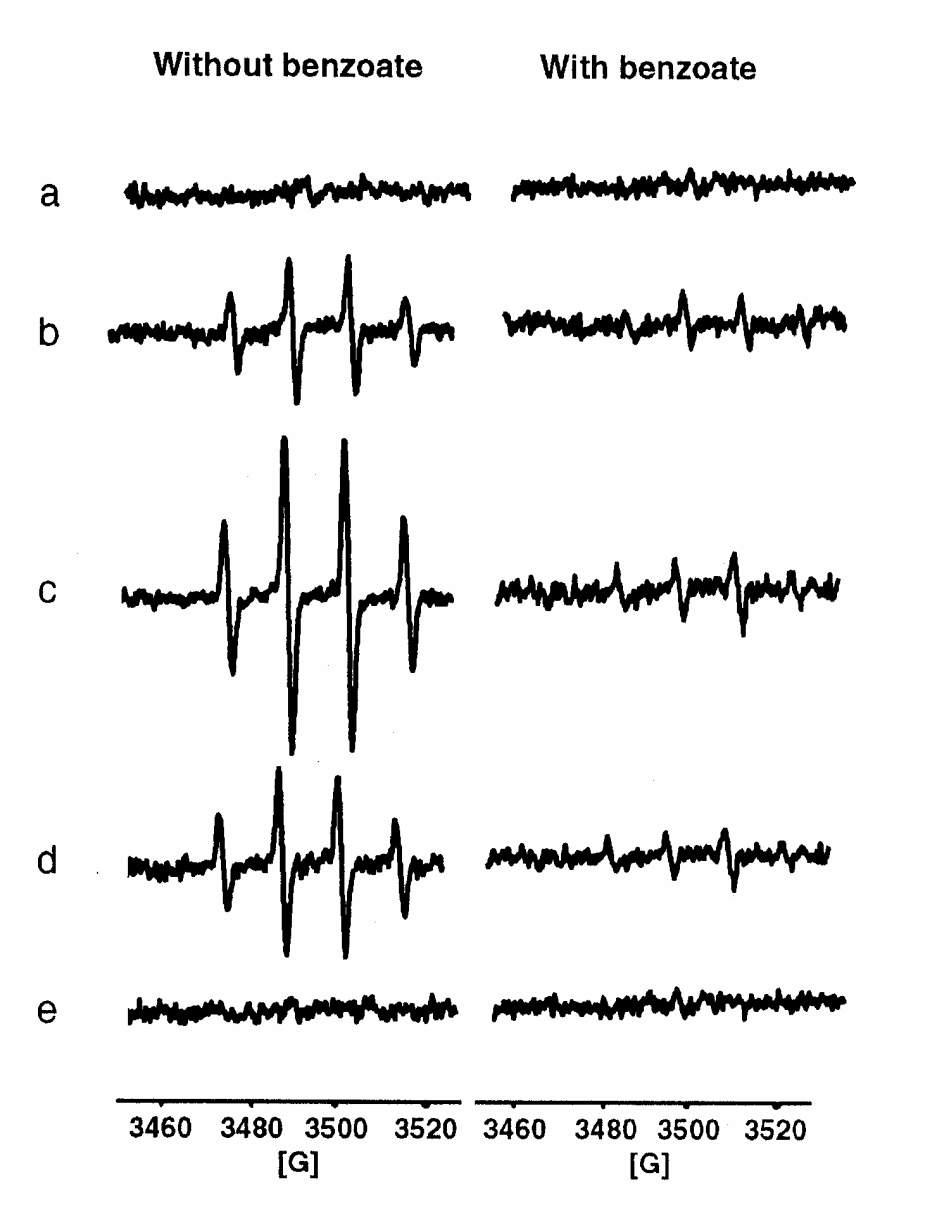
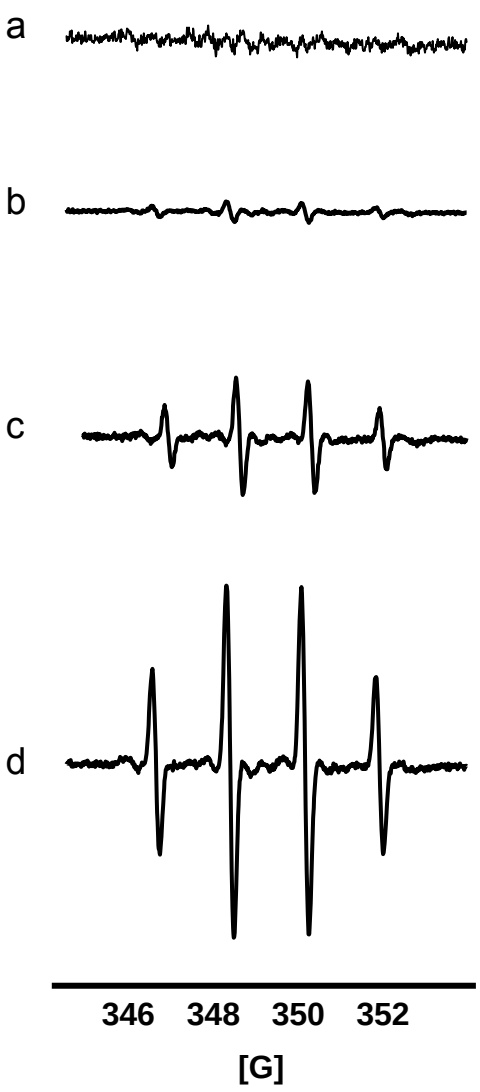



**Figure 2.1**

ESR spectra following the addition of 100 μM hydrogen peroxide to 20 mM DMPO in HBSS only (a) and DMPO + 25 μM vanadium in the +2 (b), +3 (c), +4 (d), and +5 (e) oxidation states in the absence or presence of 20 mM sodium benzoate. Spectra are representative of four separate experiments.




## Figure 2.2

ESR spectra following the addition of 100 μM hydrogen peroxide to 20 mM DMPO in HBSS only (a) and DMPO + vanadium in the +4 valence state at concentrations of 1.5 μM (b), 6.25 μM (c), and 25 μM (d). Spectra are representative of two separate experiments.

## Table 2.1

Comparison of the peak intensities of the DMPO-OH spectra generated following the addition of varying concentrations of vanadium (1.5, 6.25, 25 μM) in the +2, +3, and +4 valence states to DMPO (20 mM) and hydrogen peroxide (100 μM).

| | **Peak intensities observed** | | |
|---|---|---|---|
| | **1.5 μM** | **6.25 μM** | **25 μM** |
| $V^{2+}$ | 19,214 ± 1293 | 41,460 ± 627 | 102,058 ± 5134 |
| $V^{3+}$ | 29,302 ± 6173 | 60,717 ± 888 | 161,417 ± 7506 |
| $V^{4+}$ | 19,031 ± 4555 | 42,097 ± 2966 | 121,583 ± 3126 |

Results of three separate experiments presented as the mean peak-to-peak intensity values in arbitrary units ± SE. The mean background value for the metal-free, control system (hydrogen peroxide + DMSO) was 8024 ± 488.

Importantly, inclusion of SOD did not affect the conversion of salicylate to catechol by xanthine/xanthine oxidase in the presence of vanadium in the +2, +3 and +4 valence states. The values for systems containing xanthine/xanthine oxidase without vanadium (100 μM) and in the presence of the metal in the +2, +3, +4 and +5 valence states in the absence of SOD were 11 ± 0.5, 121 ± 8.5, 191 ± 3, 98 ± 3.5, and 11.5 ± 0.5 fluorescence intensity units respectively; the corresponding values in the presence of SOD were 13 ± 1, 120 ± 12.5, 185 ± 0.5, 124 ± 3.5 and 11.5 ± 0.5 respectively (data from 2 determinations ± SEM). These results confirm that vanadium (+2, +3, and +4) interacts with hydrogen peroxide, not superoxide, to generate hydroxyl radical. These results are shown in Figures 2.3 and 2.4 (page 59 and 60, respectively).

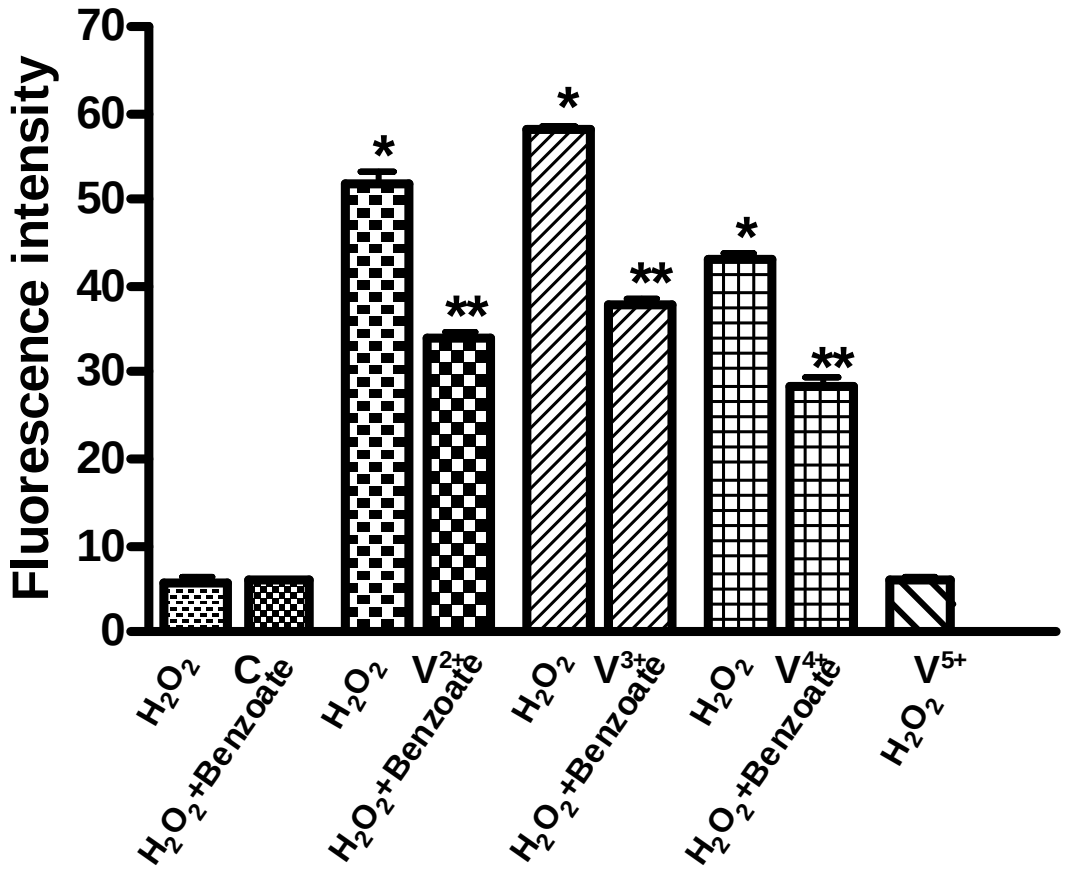



**Figure 2.3**

The effects of addition of 100 μM hydrogen peroxide to vanadium in the +2, +3, +4, and +5 valence states (all at 25 μM) in the absence and presence of 20 mM sodium benzoate on formation of hydroxyl radical, using the spectrofluorimetric procedure. The results are expressed as the mean values of six different experiments ± SE; ***p*<0.05 for comparison of the vanadium-treated systems with the vanadium-free systems and ****p*<0.05 for comparison of matched systems without and with sodium benzoate.

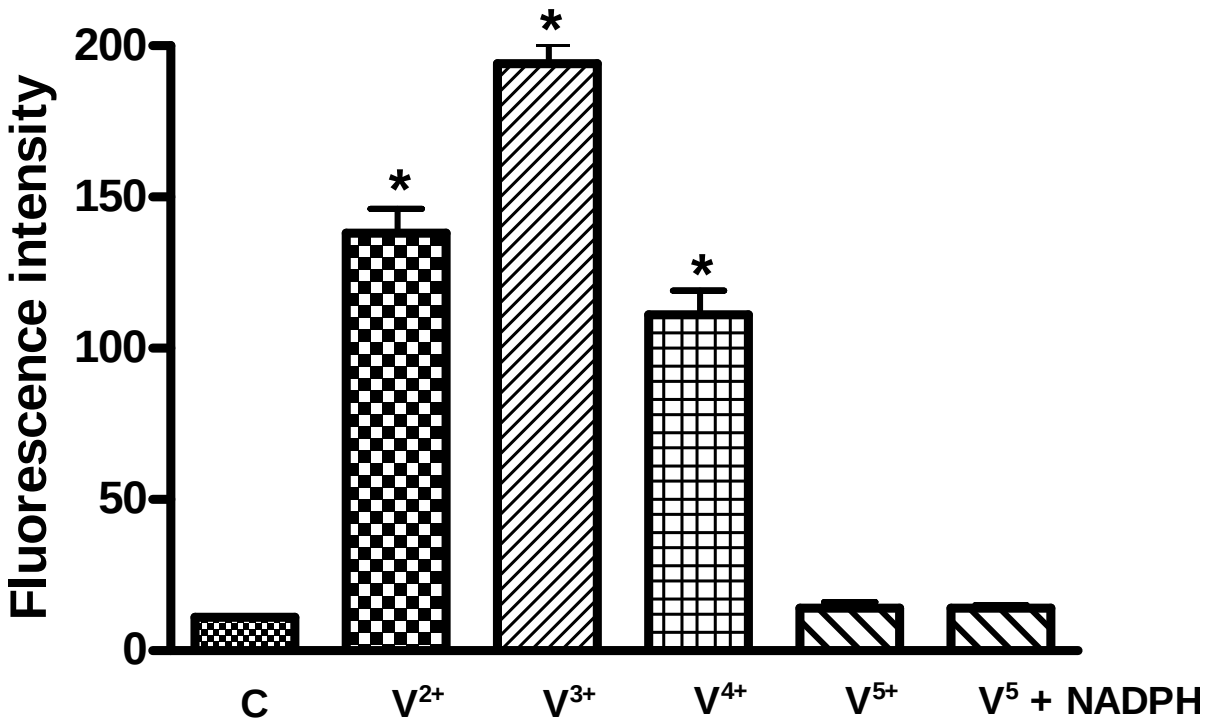



**Figure 2. 4**

Effects of addition of vanadium in the +2, +3, +4, and +5 valence states (all at 100 μM) to a xanthine/xanthine oxidase superoxide/hydrogen peroxide-generating system on formation of hydroxyl radical, using the spectrofluorimetric procedure. The effects of vanadium in the +5 valence state were measured in the absence and presence of 100 μM NADPH. The results are expressed as the mean values of three to six different experiments ± SE; ***p* <0.05 for comparison with the vanadium-free control system.

### *2.3.2 Superoxide production by activated neutrophils*

These results are shown in Table 2.2. Vanadium in the four different valence states tested, and at all concentrations used (1.5-25 μM, only data for the highest concentration shown), did not affect the lucigenin-enhanced chemiluminescence (LECL) responses of neutrophils activated with either FMLP or PMA.

No significant responses above basal level (lucigenin only) were observed in cell-free systems following addition of $H_2O_2$ (100 μM) alone, or $H_2O_2$ + vanadium (25 μM for each of the 4 valence states) to lucigenin, compatible with lack of reactivity of hydroxyl radical with lucigenin (Yildiz & Demiryurek, 1998; Myhre *et al*, 2003).

## Table 2.2

Effects of vanadium (in the four oxidation states) on FMLP- and PMA-activated superoxide production by neutrophils.

| System | PMA-activated LECL (mV/s) | FMLP-activated LECL (mV/s) |
|---|---|---|
| Control | 2513 ± 134 | 1143 ± 157 |
| $V^{2+}$ | 2491 ± 136 | 1057 ± 48 |
| $V^{3+}$ | 2565 ± 142 | 1249 ± 222 |
| $V^{4+}$ | 2477 ± 137 | 1104 ± 50 |
| $V^{5+}$ | 2585 ± 125 | 1161 ± 89 |

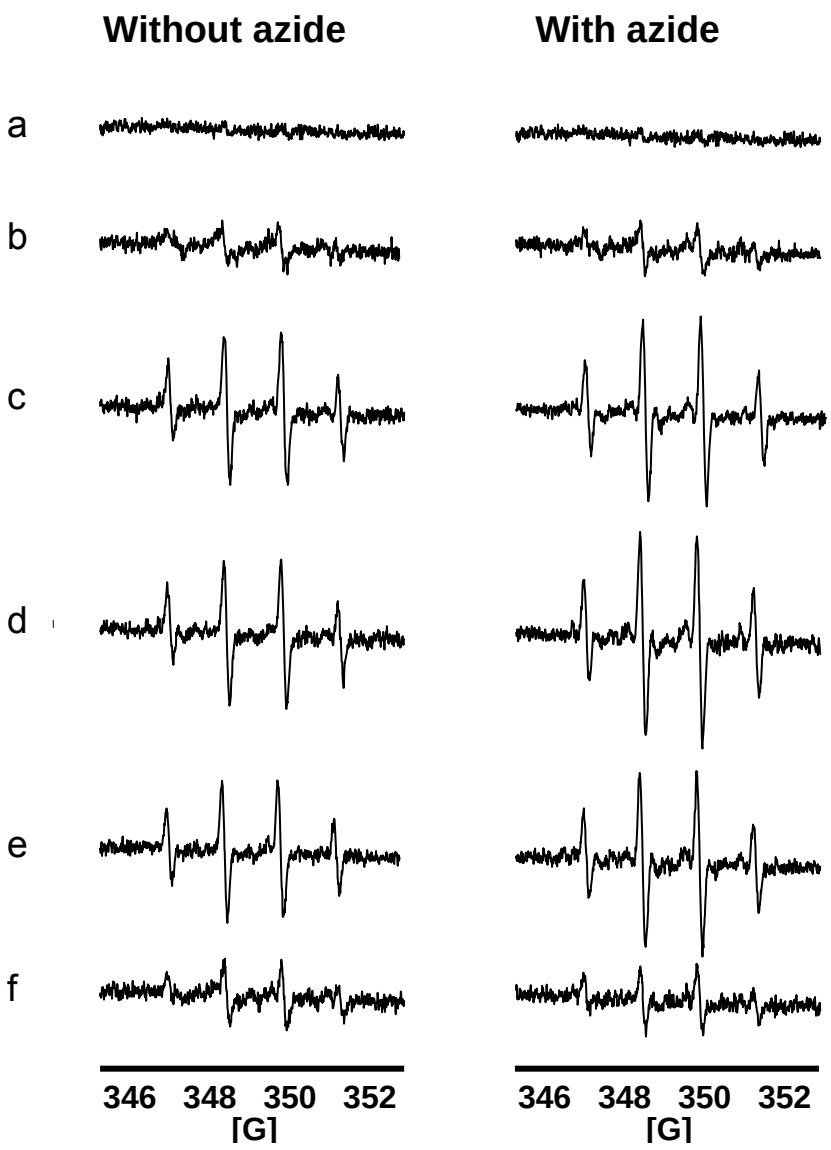
Results are expressed as the mean peak values ± SE of 3 – 11 experiments measured at around 40 seconds and 4 minutes for FMLP- and PMA-activated neutrophils, respectively. The corresponding values for unstimulated cells were 287 ± 33 and 230 ± 4 mV/s, respectively.

### *2.3.3 Hydroxyl radical production by activated neutrophils*

The effects of the various vanadium compounds on the generation of hydroxyl radical by neutrophils activated with FMLP in the presence and absence of the MPO inhibitor, sodium azide, are shown in Figure 2.5 (page 63). Hydroxyl radical formation was detected following exposure of neutrophils to FMLP, which was of similar magnitude in both the absence and presence of sodium azide. Inclusion of vanadium in the +2, +3 and +4 valence states significantly ($p < .05$) increased the formation of hydroxyl radical by FMLP-activated neutrophils, with additional and significant ($p < .05$) augmentation observed in the presence of sodium azide (Figure 2.5, page 63). However, vanadium in the +5 valence state did not affect the magnitude of hydroxyl radical formation by neutrophils in either the absence or the presence of sodium azide (Figure 2.5, page 63).

Importantly, the magnitudes of hydroxyl radical formation by FMLP-activated neutrophils treated with 25 μM vanadium in the +4 oxidation state (in the absence of azide) shown in Figure 2.5 (page 63), were considerably attenuated and augmented when the neutrophil concentration of 1 x $10^6$/ml was decreased or increased to 0.25 and 4 x $10^6$/ml respectively. The corresponding mean percentages of the control system (with 1 x $10^6$/ml neutrophils) being 6% and 353% respectively.

The effects of vanadium in all four valence states on the formation of hydroxyl radical by neutrophils activated with PMA are shown in Figure 2.6 (page 64) (all systems shown contained 100 mU/ml SOD because no spectra were detected in the absence of the enzyme). Slight formation of hydroxyl radical was detected in the vanadium-free, PMA-activated control systems, in both the absence and the presence of sodium azide. Addition of vanadium in the +2, +3 and +4, but not the +5, valence states at all concentrations tested (1.5 - 25 μM, only data for the highest concentration shown) was accompanied by significantly ($p <.05$) increased DMPO-OH signals, with additional and significant ($p <.05$) enhancement in the presence of sodium azide.




## Figure 2.5

ESR spectra of unstimulated neutrophils + 20 mM DMPO (a) and those of FMLP (1 μM)-activated neutrophils in the absence of vanadium (b) and in the presence of vanadium in the +2 (c), +3 (d), +4 (e), and +5 (f) valence states in the absence and presence of sodium azide (760 μM). Spectra are representative of three separate experiments, each with triplicate determinations for each system.

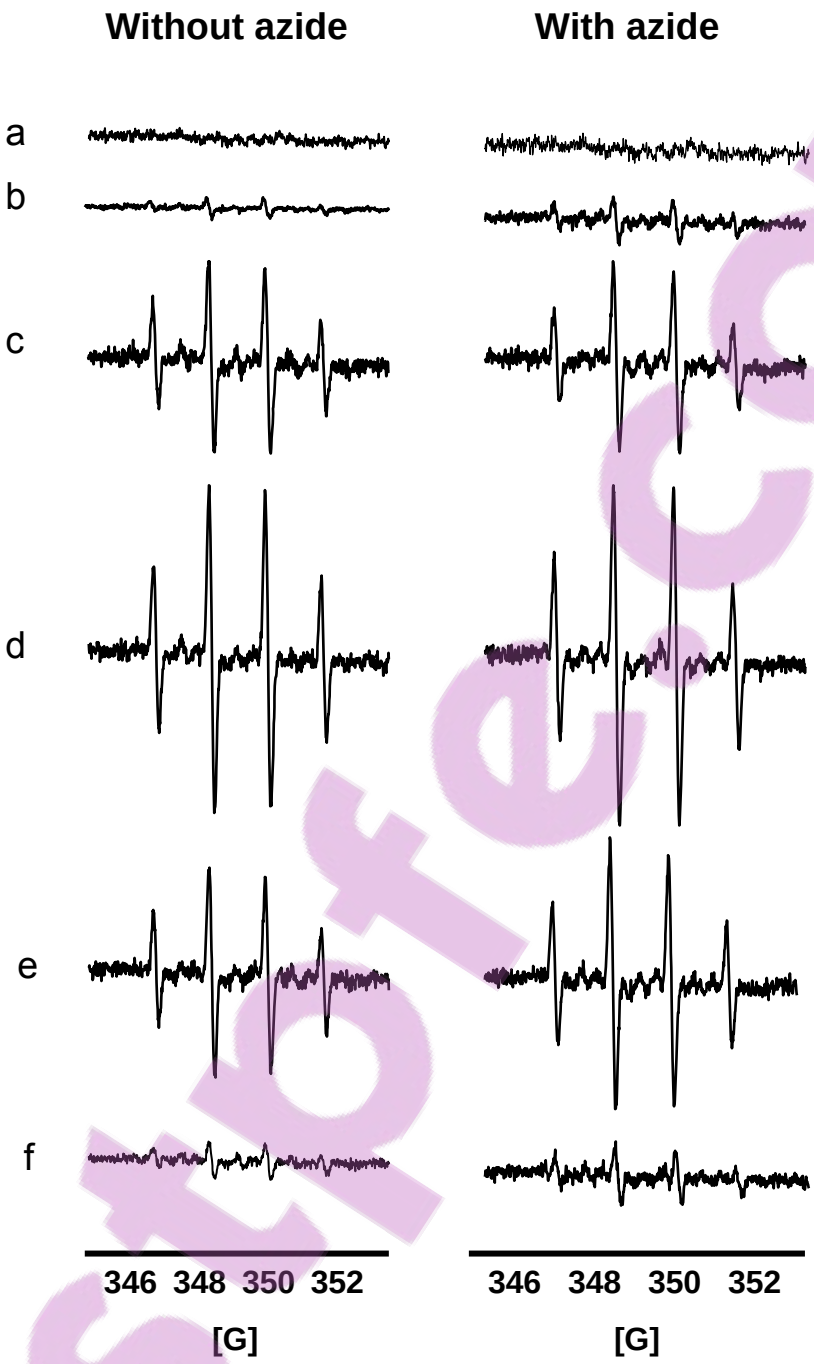



**Figure 2.6**

ESR spectra of unstimulated neutrophils + 20 mM DMPO (a) and those of PMA (25 ng/ml)-activated neutrophils in the absence of vanadium (b) and in the presence of vanadium in the +2 (c), +3 (d), +4 (e), and +5 (f) valence states in the absence and presence of sodium azide (760 μM). Spectra are representative of three separate experiments, each with triplicate determinations for each system.

As shown in Figure 2.7 (page 66), sodium benzoate partially neutralized hydroxyl radical generation by neutrophils activated with PMA in the presence of vanadium in the +4 valence state (with SOD, without sodium azide), while DPI (inhibitor of NADPH oxidase), GF109203X (inhibitor of protein kinase C) and catalase completely attenuated formation of the DMPO-OH adduct with no spectra observed.

In cell-free control systems containing all components of the assay system (except neutrophils) formation of the DMPO-OH adduct was not detected.

### *2.3.4 Activity of myeloperoxidase*

The effects of the various vanadium compounds (all at 25 μM) on MPO-mediated auto-iodination of FMLP-activated neutrophils, as well as on the oxidation of guaiacol by purified MPO (compounds at 25 and 100 μM) are shown in Figures 2.8 and 2.9 (pages 67 and 68, respectively). Inclusion of 25 μM vanadium in the +2, +3 and +4 valence states, but not the +5 valence state, significantly ($p$ <.05) decreased FMLP-mediated auto-iodination of neutrophils, as well as the oxidation of guaiacol by purified MPO.

### *2.3.5 Cellular ATP levels*

Exposure of neutrophils to the various vanadium compounds (25 μM) for up to 6 hours at 37°C did not affect cellular ATP levels, demonstrating lack of cytotoxicity of the metal at the concentrations used in the various experiments described in the current study. The values for control cells and those exposed to vanadium in the +2, +3, +4 and +5 valence states for 6 hours at 37°C were 26.5 ± 5.5, 25.3 ± 5.3, 26.4 ± 5.3, 28.5 ± 2.8 and 22.9 ± 3.2 nmoles ATP/$10^7$ cells respectively.

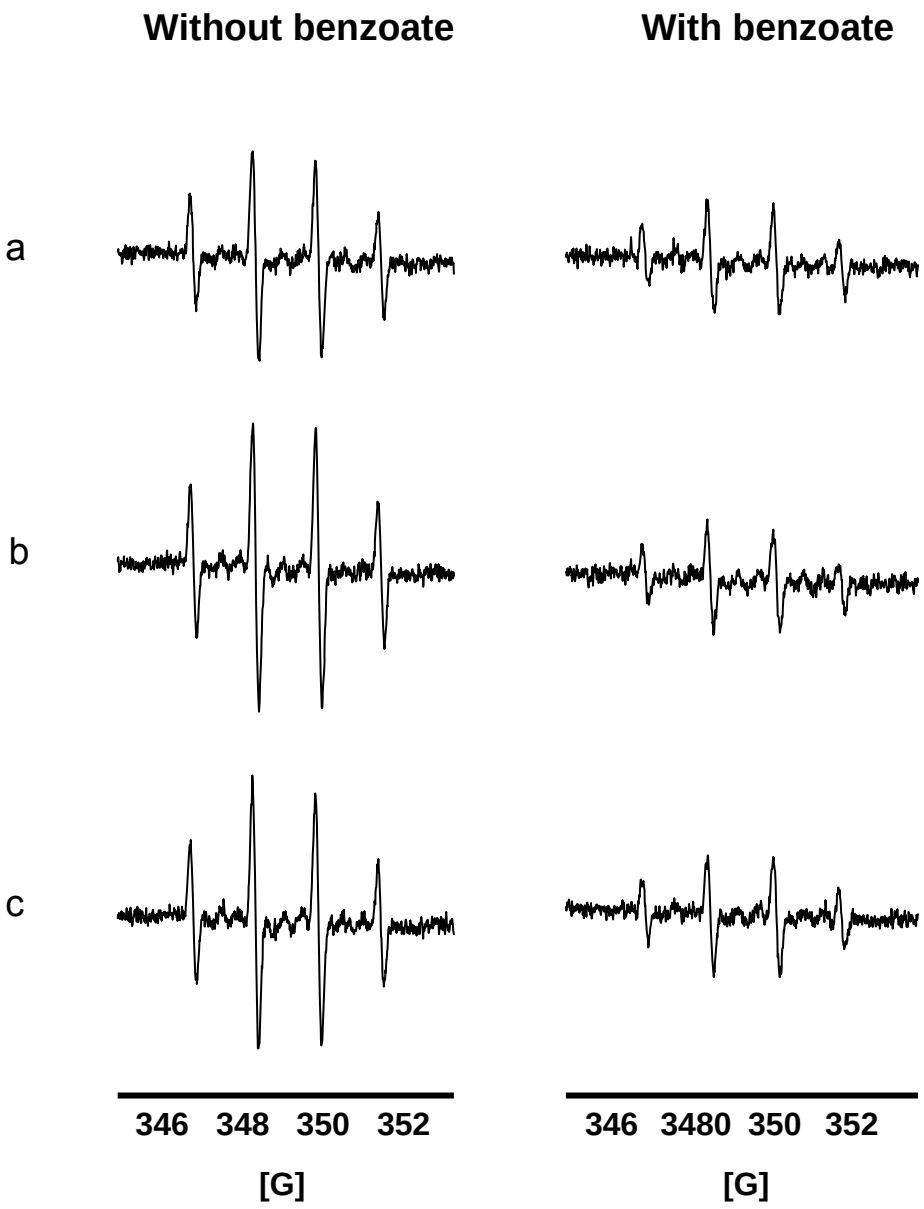



**Figure 2.7**

Results from three experiments showing the paired ESR spectra of PMA (25 ng/ml)-activated neutrophils + 20 mM DMPO, treated with 25 μM vanadium in the +4 valence state in the absence (left) and presence (right) of 20 mM sodium benzoate.

.

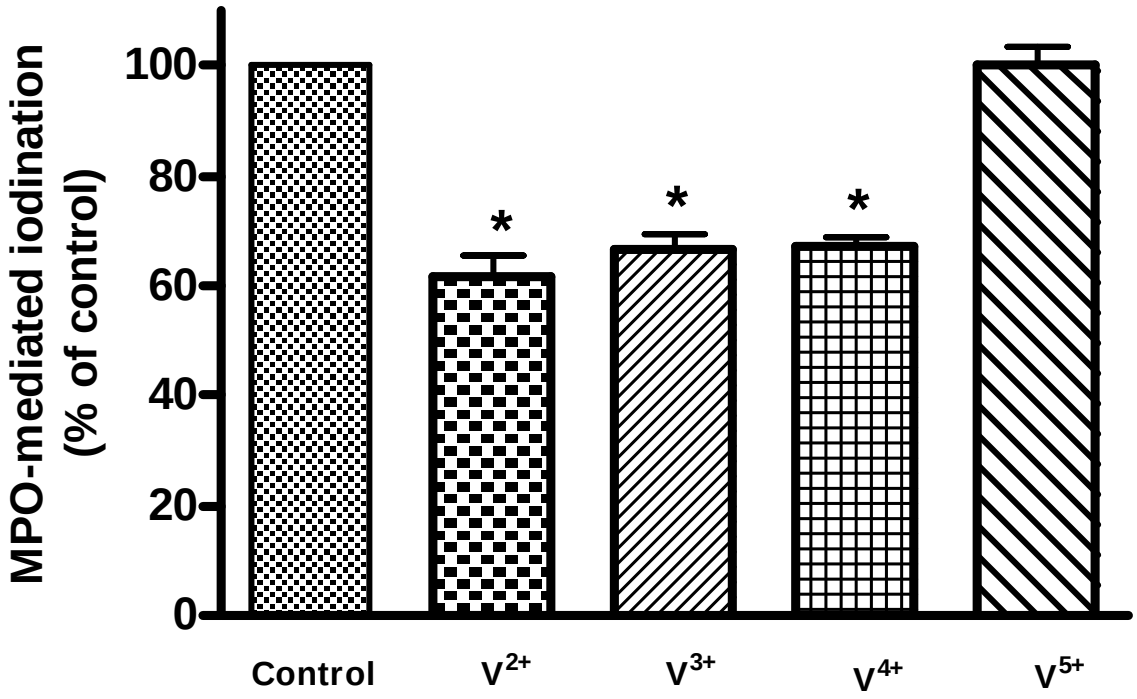



**Figure 2.8**

Effects of vanadium in the +2, +3, +4, and +5 valence states (25μM) on FMLP-activated MPO-mediated autoiodination of intact neutrophils. The results of three to six experiments with four to five replicates for each system are presented as the mean percentage of the corresponding metal-free control system ± SEM.

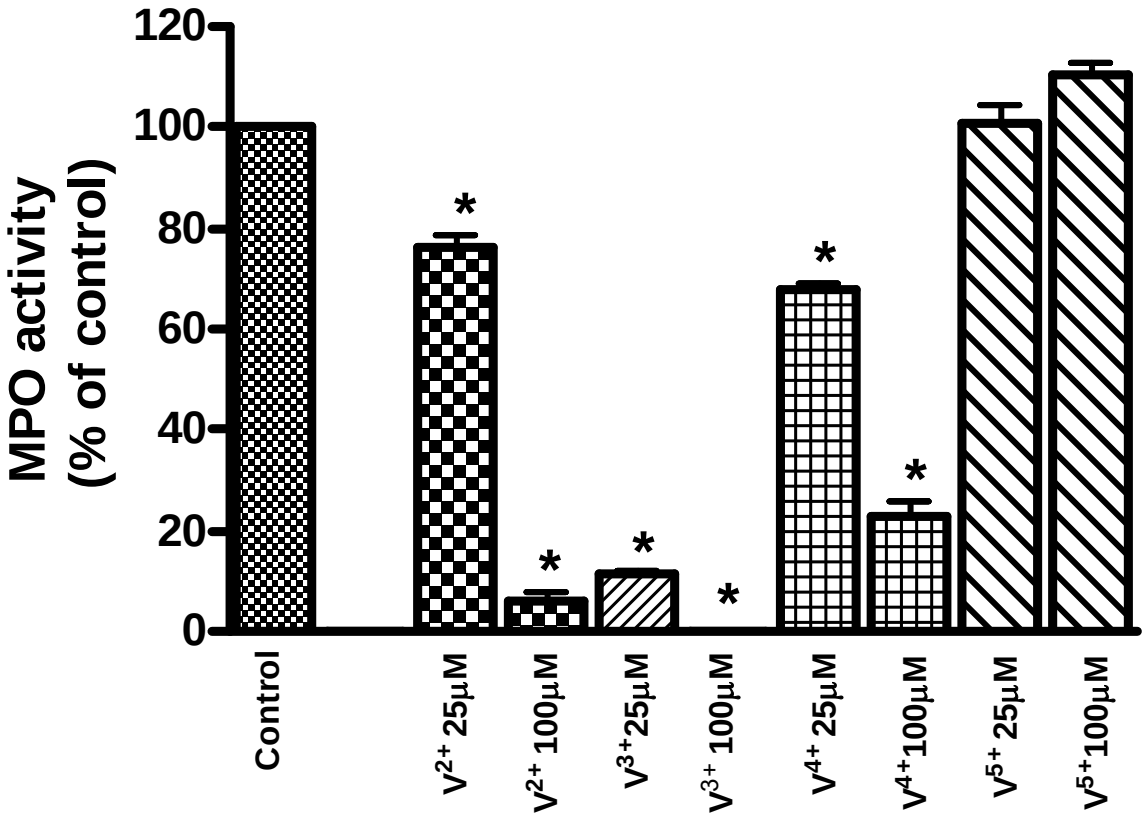



**Figure 2.9**

Effects of vanadium in the +2, +3, +4, and +5 valence states (25 μM) on the oxidation of guaiacol by a cell-free system containing purified MPO and $H_2O_2$ (metal compounds at 25 and 100 μM). The results of three to six experiments with four to five replicates for each system are presented as the mean percentage of the corresponding metal-free control systems ± SEM. * $p < 0.05$

## 2.4 Discussion

The pathophysiological relevance of hydroxyl radical production by human neutrophils has been questioned, largely because of regulatory mechanisms which restrict the availability of heavy metals, particularly iron, required to participate in the Haber-Weiss and/or Fenton reactions, as well as the efficiency of MPO in removing hydrogen peroxide (Samuni *et al*, 1988; Cohen *et al*, 1988; Britigan *et al*, 1989; Britigan *et al*, 1990). Nevertheless, situations may arise in which regulatory mechanisms are subverted, resulting in significant formation of hydroxyl radical by activated neutrophils (Winterbourn, 1986; Miller & Britigan, 1995; Andersen *et al*, 2003). The results of the current study demonstrate that exposure of neutrophils to vanadium favours hydroxyl radical formation by these cells.

Activation of neutrophils with the chemoattractant, FMLP, was accompanied by detectable, albeit modest formation of hydroxyl radical. Inclusion of vanadium in the +2, +3 and +4, but not the +5 valence states, resulted in significant augmentation of hydroxyl radical formation by FMLP-activated neutrophils. Detection of vanadium-mediated catalysis of hydroxyl radical formation by FMLP-activated neutrophils did not require inclusion of superoxide dismutase, while addition of the MPO inhibitor, sodium azide, caused a further increment in hydroxyl radical formation in vanadium (+2, +3, or +4)-treated systems. Apart from underscoring the prooxidative interactions of vanadium (+2, +3 and +4) with human neutrophils, these observations demonstrate that the levels of superoxide generated during the relatively brief activation of NADPH oxidase following exposure of the cells to FMLP are insufficient to inactivate the DMPO-OH spin adduct, while the metal appears to compete efficiently with MPO for hydrogen peroxide. The augmentative effects of inclusion of sodium azide, although modest, were nevertheless statistically significant, and are probably due to increased availability of hydrogen peroxide to interact with vanadium in a Fenton-type reaction. Because MPO negatively modulates superoxide generation by activated phagocytes (Locksley *et al*, 1983), increased generation of hydrogen peroxide by these cells may also contribute to the augmentative reaction of sodium azide.

In the case of vanadium-free control cells exposed to PMA, an extremely potent activator of NADPH oxidase, low-level formation of hydroxyl radical was

detected, which was dependent of the inclusion of superoxide dismutase. However, treatment of PMA-activated cells with vanadium in the +2, +3 and +4, but not the +5, valence states resulted in substantial formation of hydroxyl radical, detection of which was strictly dependent on inclusion of superoxide dismutase and was potentiated by sodium azide. Importantly, the stimulatory effects of vanadium (+2, +3, +4) on hydroxyl radical formation by activated neutrophils were attenuated by diphenylene iodonium chloride, GF 109203X and sodium benzoate, inhibitors of NADPH oxidase and protein kinase C, and a scavenger of hydroxyl radical respectively, as well as by catalase. The complete attenuation of hydroxyl radical formation observed when vanadium (+4)-treated, activated neutrophils were exposed to catalase, indicates that formation of hydroxyl radical is an exclusively extracellular event.

Using both ESR and spectrofluorimetric procedures, similar effects of vanadium on hydroxyl radical formation were also observed using a cell-free system consisting of the metal and added hydrogen peroxide. In agreement with previous reports (Keller *et al*, 1989; Carmichael, 1990), these observations are compatible with a Fenton-type mechanism, whereby vanadium (in the +2, +3 and +4 valence states) reduces hydrogen peroxide with consequent formation of hydroxyl radical. Using the spectrofluorimetric system, which is not prone to interference by superoxide, unlike the ESR/DMPO system, hydroxyl radical formation was also detected following the addition of vanadium (+2, +3, +4) to a xanthine/xanthine oxidase superoxide- and hydrogen peroxide-generating system. However, vanadium in the +5 valence state was completely unreactive, even in the presence of added NADPH. Taken together with the results of experiments using FMLP-activated neutrophils (without added superoxide dismutase), these results suggest that over the relatively short time course of these experiments, and at the concentrations of vanadium, cells and enzyme/substrate used, that superoxide, in both the presence and absence of NADPH, does not convert the metal from the +5 to the hydrogen peroxide-reactive +4 valence state.

While in agreement with several previous reports (Liochev *et al*, 1989a; Liochev *et al*, 1989b; Liochev *et al*, 1991), these observations that vanadium in the +5 valence state is not converted by superoxide-dependent mechanisms to the +4 valence state are at variance with other reports in which an alveolar cell line (Zhang

*et al*, 2001), and murine bronchoalveolar lavage cells, consisting predominantly of macrophages (Wang *et al*, 2003), or a cell-free system consisting of xanthine/xanthine oxidase + NADPH (Shi & Dalal, 1993) were used. These differences may reflect the different cell types used in the various studies, as well as the concentrations of vanadium (+5) which were higher than those used in the current study (Shi & Dalal, 1993; Zhang *et al*, 2001; Wang *et al*, 2003). The exact mechanisms by which vanadium is transformed from the +5 to the +4 valence states *in vivo* remain to be established, but may involve reduction by glutathione and/or NADPH-dependent flavoenzymes intracellularly (Barceloux, 1991b; Shi & Dalal, 1991).

Although vanadium in the +5 valence state did not promote the formation of hydroxyl radical by activated neutrophils, reagent hydrogen peroxide, or an enzymatic superoxide/hydrogen peroxide system, several alternative mechanisms exist by which vanadate may function as an occupational and environmental toxin. These are i) by conversion to the +4 valence state *in vivo*, favouring formation of hydroxyl radical; ii) via inhibition of plasma membrane ATPases, particularly $Na^+$, $K^+$-ATPase (Sabbioni *et al*, 1991), iii) by activating transcription factors and synthesis of pro-inflammatory cytokines (Chen *et al*, 1999; Chong *et al*, 2000; Huang *et al*, 2001; Nadadur *et al*, 2002), and iv) by inhibition of tyrosine phosphatases (Grinstein *et al*, 1990; Trudel *et al*, 1991; Zhao *et al*, 1996; Samet *et al*, 1997), which in the case of phagocytic cells, results in prolonged activation of NADPH oxidase. With respect to neutrophils, this latter effect requires permeabilization of the cells (Grinstein *et al*, 1990; Trudel *et al*, 1991) which explains why it was not observed in the current study.

Interestingly, vanadium in the +2, +3 and +4 valence states, but not the +5 valence state, decreased the activity of MPO both in intact neutrophils and in a cell-free system containing purified enzyme. Although the mechanisms by which vanadium in these oxidation states decreases the activity of MPO have not been identified, competition between the metal and the enzyme for hydrogen peroxide appears to be the most probable mechanism. Effective competition of the metal with MPO may also explain why hydroxyl radical formation by FMLP-activated neutrophils was detected in the absence of the MPO inhibitor, sodium azide.

The concentrations of vanadium used in the current study, which ranged from 0.2 - 4 μg/ml (1.5 - 25 μM), are comparable with those encountered in both the environmental and occupational settings. In 1973, the median level of vanadium in ambient air was reported to be 0.62 $\mu g/m^3$ in American cities with a high level of consumption of fossil fuels, while in localities in close proximity to metallurgical industries, or industries producing vanadium metal or compounds, atmospheric concentrations of the metal were reported to be around 1 $\mu g/m^3$ and several $\mu g/m^3$ respectively (reviewed in Barceloux, 1999b). Boiler cleaners are exposed to extremely high levels of vanadium oxides, which may reach concentrations of up to 500 $mg/m^3$ in dusts (Barceloux, 1999b).

Hydroxyl radical is one of the most damaging and reactive free radicals generated in biological systems (Cheng *et al*, 2002). However, its formation by phagocytes via Haber-Weiss/Fenton mechanisms *in vivo* is stringently controlled, primarily by binding proteins which limit the availability of heavy metals, especially copper and iron, as well as removal of hydrogen peroxide by MPO. The results of the current study have demonstrated a mechanism whereby exposure to vanadium enables these anti-oxidative mechanisms to be subverted, favouring hydroxyl radical formation by activated neutrophils. This is achieved by a Fenton reaction assisted by effective competition of the metal with MPO for hydrogen peroxide. Although the relevance of these findings to the pathogenesis of respiratory symptoms and dysfunction which may accompany environmental and occupational exposure to vanadium remain to be established, they do suggest that individuals with preexisting airways inflammation such as cigarette smokers, asthmatics, and those with chronic obstructive pulmonary disorders may be at highest risk for vanadium toxicity. This might also be the case in cardiovascular disease, as formation of the artherosclerotic plaques is augmented if LDL is oxidised. Only oxidised LDL can be taken up by macrophages, subsequently leading tp the formation of the plaque (Steinberg *et al*, 1989). Any chronic inflammatory condition exposes the individual to increased eactive oxidant species, including hydrogen peroxide, which through the catalytical properties of vanadium, can than be converted to the highly toxic hydroxyl radical.

# Chapter 3

# Activation of Nuclear Factor-Kappa B (NF-κB) in Human Neutrophils

**Hypothesis**

Cobalt, palladium, platinum, and vanadium initiate the signalling pathway leading to the activation of cytosolic kinases and other transcription factors responsible for the subsequent phosphorylation and degradation of IκB, the nuclear translocation of NF-κB, and the transcription of inflammatory genes introducing the production of IL-8.

**Aim**

The aim was to investigate the effects of the metals on the phosphorylation of cytosolic proteins, the nuclear translocation of NF-κB, and the production of IL-8 in human neutrophils, using a Bio-Plex suspension array system and an electrophoretic mobility shift assay (EMSA).

## 3.1 Introduction

NF-κB, in unison with several other transcription factors, plays an important role in inflammatory processes, which can be beneficial as well as detrimental to the host. Controlled activation of NF-κB is necessary for effective host defences, while increased or prolonged activation contributes to tissue damage in various pathological conditions. In its inactivated form, NF-κB, coupled to the inhibitory protein IκB, is present in the cytosol from where it translocates into the nucleus, following stimulation of the cell, with subsequent phosphorylation and degradation of IκB (Karin & Ben-Neriah, 2000). Different signalling pathways have been identified which vary according to the type of cell, as well as the stimulus and its corresponding receptor (Deva *et al*, 2003; Jiang *et al*, 2003; Sandor *et al*, 2003; Santoro *et al*, 2003; Asehnoune *et al*, 2004). After co-operative binding of several transcription factors, gene transcription is induced. In 1999 more than 150 different stimuli were already recognized which induced NF-κB activation, while active NF-κB was found to promote the expression of as many target genes (Pahl, 1999).

Activation of NF-κB follows not only exposure to bacteria and viruses and their proteins, but also occurs in response to different stress situations. The active NF-κB

transcription factor promotes the expression of many target genes, leading to the transcription of proteins participating in host immune and inflammatory responses (Holloway *et al,* 2001). These proteins include different cytokines and chemokines, as well as receptors required for immune recognition, such as MHC molecules, proteins involved in antigen presentation and receptors required for neutrophil adhesion and transmigration across the blood vessel wall (Pahl, 1999). Many viruses have NF-κB binding sites in their viral promoters, and low levels of NF-κB activation probably contribute to the mechanism by which some viruses, such as EBV, HSV, CMV and HIV-1, sustain chronic infection (Santoro *et al*, 2003).

Many activators of NF-κB have been described which are not bacterial or viral pathogens, and NF-κB can generally be understood to be a regulator of stress responses. NF-κB activity is induced during various pathophysiological stress conditions such as ischemia/reperfusion, liver regeneration and hemorrhagic shock. Physical stress in the form of irradiation, as well as oxidative stress to cells also induce NF-κB and, a large variety of stress response genes are in turn activated by NF-κB (Pahl, 1999).

NF-κB is also activated by both environmental stresses, such as heavy metals or cigarette smoke, and by therapeutic drugs (Pahl, 1999). Several studies have investigated the effects of metals on the activation and translocation of NF-κB in different cell lines. Goebeler *et al* (1995) found a strong increase in NF-κB DNA binding after exposing human umbilical vein epithelial cells (HUVEC) to nickel and cobalt chloride, which was confirmed by Sultana *et al* (1999) who also found increased expression of VCAM-1 and activation of MAP kinase. When alveolar epithelial (A549) cells were exposed to particulate air pollution matter/diameter 10 microns ($PM_{10}$) consisting of ultrafine particles in association with transition metals and endotoxins, increased activation of NF-κB was found (Jiménez *et al*, 2000). In macrophages, cobalt, as well as chromium ions, induced TNF-α secretion and cytotoxicity, while in synoviocytes DNA binding activity of NF-κB, as well as cytokine production was upregulated (Catelas *et al,* 2003; Niki *et al*, 2003). Exposure of monocytes to palladium caused a significant increase in activated NF-κB (Lewis *et al*, 2003); in another study, platinum salts were found to enhance cytokine release, while

palladium salts inhibited the release of IFN-γ, -TNF-α and -IL-5 by PHA-activated peripheral blood mononuclear cells (PBMC) (Boscolo *et al*, 2004) in contrast to the study by Lewis *et al* (2003).

Although pro-oxidative interactions between heavy metals and neutrophils have been described, little is known about the effect which heavy metals may have on the degradation of IκB and activation of NF-κB in human neutrophils. For example, cobalt was reported to increase the ROS generating capacity of neutrophils, as well as the serum opsonic activity (Ono *et al*, 1994). More recently, platinum and palladium were found to enhance the reactivity of superoxide anion generated by human neutrophils *in vitro* (Theron *et al*, 2004), while cobalt was found to potentiate the reactivity of neutrophil-derived $H_2O_2$ (Ramafi *et al*, 2004). As described in chapter 2 of this thesis, vanadium promotes hydroxyl radical formation by activated neutrophils. As mentioned in the literature review, oxidative stress plays a general role in the induction of signalling pathways leading to phosphorylation and degradation of IκB. In the case of the neutrophil, the involvement of ROS in facilitating nuclear translocation of NF-κB and subsequent production of the proinflammatory cytokines TNF-α, MIP-2, and IL-1β was described following activation of Toll-like receptor 4 (Asehnoune *et al*, 2004).

The research described in this chapter consists of two distinct, but related phases. The first of these was focussed on developing an electrophoretic mobility shift assay (EMSA) for detection of nuclear translocation of NF-κB proteins in neutrophils, which necessitated identification of a reliable, positive control system. This was achieved by using the pneumococcal, pore-forming cytotoxin, pneumolysin, which causes a sustained influx of extracellular $Ca^{2+}$ into these cells which is associated with, and appears to be a prerequisite for toxin-activated synthesis of IL-8 (Cockeran *et al,* 2001a; Cockeran *et al*, 2002b). The relationship between sustained increases in cytosolic $Ca^{2+}$, activation of NF-κB and synthesis of IL-8 is well-recognized (Kuhns & Gallin, 1995; Kuhns *et al*, 1998; Dolmetsch *et al*, 1997). The involvement of extracellular $Ca^{2+}$ in pneumolysin-mediated activation of NF-κB in neutrophils was probed by addition of EGTA, a chelator of extracellular $Ca^{2+}$, to the cells, and by pretreatment of the cells with the omega-3 polyunsaturated fatty acid

docosahexaenoic acid (DHA). The latter agent antagonizes influx of $Ca^{2+}$ via interference with various types of $Ca^{2+}$ channels (Wang *et al,* 2003; Honen *et al,* 2003) and DHA-mediated inhibition of NF-κB has been observed in LPS-activated macrophages (Komatsu *et al*, 2003; Lee *et al,* 2003; Park *et al*, 2004).

Having developed the NF-κB assay for neutrophils, the objectives of phase 2 were to investigate the effects of the metals on: i) activation and translocation of NF-κB in neutrophils, as well as production of IL-8; and ii) cytosolic signalling cascades which converge on NF-κB (ERK1&2, EGFR, IκB, JNK, p38MAPK), as well as on activation of other transcription factors which cooperate with NF-κB to optimize gene transcription ( STAT3, ATF-2).

## 3.2 Materials and methods

### *3.2.1 Chemicals and reagents*

Cobalt(II) chloride hexahydrate ($Co^{2}$), platinic chloride [hydrogen hexachloroplatinate (IV) ($Pt^{4+}$), palladium (II) chloride ($Pd^{2+}$), vanadium (II) chloride ($V^{2+}$), vanadium (III) chloride ($V^{3+}$), vanadyl sulphate hydrate ($V^{4+}$) and sodium metavanadate (V+5) were purchased from Sigma-Aldrich (St Louis, MO, USA). These were dissolved in distilled water to give stock concentrations of 10mM and used in the assays described below at concentrations of 25 μM for each metal. Unless otherwise indicated all other chemicals and reagents were also obtained from Sigma-Aldrich (St Louis, MO, USA). Recombinant IL-8 was also purchased from Sigma-Aldrich, while recombinant pneumolysin, prepared as described previously (Saunders *et al*, 1989), was kindly provided by Prof. T.J. Mitchell, Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, UK.

### *3.2.2 Neutrophils*

Purified human neutrophils were prepared as described in 2.2.2

### *3.2.3 NF-κB activation*

For these investigations, neutrophils were suspended in RPMI 1640 tissue culture medium (Highveld Biological, RSA) supplemented with 0.5% human serum albumin (HSA). Following 10 minutes of preincubation at 37°C, pneumolysin (8.37 and 41.75 ng/ml), metal (25 μM) or an equal volume of RPMI 1640 (control system) was added to the cells which were then incubated for 15 or 30 minutes at 37°C. The final volume in each tube was 1 ml containing $5x10^6$ cells. Following incubation, detection of NF-κB nuclear translocation was determined as described previously (Staal *et al*, 1995; Jimenez *et al*, 2000), with slight modifications. Briefly, cells were harvested and resuspended in 0.4 ml buffer (10 mM HEPES / 10mM KCl / 2 mM $MgCl_2$ / 1 mM DTT / 0.1 mM EDTA / 0.2 mM NaF / 0.2 mM $Na_3VO_4$) supplemented with the protease inhibitors 1 mg/L leupeptin and 0.4 mM PMSF, and placed on ice. After 15 minutes,
25 μl 10% Igepal CA-630 was added and the cells vortexed for 15 seconds and pelleted by centrifugation. Pellets containing the nuclear proteins were resuspended in buffer (50 mM HEPES / 50 mM KCl / 300 mM NaCl / 0.1 mM EDTA / 1 mM DTT / 10% glycerol / 0.2 mM NaF / 0.2 mM $Na_3VO_4$) supplemented with 0.1 mM PMSF and incubated on ice on a rotating platform for 20 minutes. After centrifugation for 5 minutes at 4°C, supernatants were collected and protein determinations performed.

For the electrophoretic mobility shift assay (EMSA), 7 μg of nuclear protein extract was incubated with $^{32}$P-radiolabelled NF-κB-specific oligonucleotide (Amersham Biosciences UK Ltd, Amersham, UK) for 20 minutes at room temperature. Binding of NF-κB nuclear proteins to the oligonucleotide results in a retardation ("shift") of the electromobility on a 5% nondenaturating polyacrylamide gel. These shifts were visualized by phosphor-imaging using the Personal Molecular Imager FX and software from BIO-RAD Laboratories, Inc. Specificity of NF-κB DNA binding was ascertained by competition with excess unlabelled oligonucleotide, resulting in disappearance of NF-κB complexes, and results are shown as either the mean percentage counts/$mm^2$ of the pneumolysin/metal-free control system, or as the complete phosphor-images for representative experiments.

Additional experiments were performed to investigate the effects of the following on pneumolysin-mediated activation of NF-κB in neutrophils: i) inclusion of the extracellular $Ca^{2+}$-chelating agent EGTA (10 mM, final) in the cell-suspending medium; ii) the effects of pretreatment of the cells for 5 minutes with the omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA, 5 and 10 μg/ml, final).

### *3.2.4 Interleukin-8*

#### *3.2.4.1 Production of IL-8*

To determine if exposure to metals would lead to increased synthesis of IL-8, neutrophils were preincubated for 10 minutes at 37°C in HSA (0.5%) supplemented RPMI 1640, followed by the addition of the different metals at a concentration of 25 μM. The tubes, containing $2x10^6$ cells in a final volume of 1 ml, were then incubated for 6hours at 37°C. Total and extracellular IL-8 was measured using the Bio-Plex suspension array system (see below), with the beads coated with anti IL-8 antibody. Total IL-8 was measured in the lysates of neutrophils that had been treated with 0.01% lysophosphatidylcholine, followed by centrifugation at 300g for 5 minutes to remove cellular debris, while extracellular cytokine was measured in cell-free supernatants, from which the cells had been removed by centrifugation.

#### *3.2.4.2 Immunological reactivity of IL-8*

To determine if exposure to metals would cause any changes in the immunological reactivity of IL-8 (human recombinant IL-8, Sigma-Aldrich), the cytokine, at a concentration of 1.25 μg/ml, was exposed to the metals (concentration 25 μM) for 3 minutes, after which the mixture was diluted 1:100 with HBSS (final concentration of IL-8, 12.5 ng/ml) and the concentration of IL-8 was determined in all systems, with unexposed IL-8 as control, using the Bio-Plex suspension array system.

### *3.2.5 Phosphorylated proteins*

Cell lysates were prepared according to the instruction manual using the reagents supplied for phosphoprotein determination in the Bio-Plex cell lysis kit. Neutrophils at a concentration of $2x10^6$/ml were preincubated for 10 minutes at 37°C. After exposure to the metals for 3 minutes, the reaction was stopped by adding ice-cold wash buffer. Lysate solution was added and after freeze-thawing the lysate once, the samples were centrifuged at 4,500rpm for 20 minutes at 4°C. Supernatants were collected and phosphorylated proteins determined using the Bio-Plex suspension array system, the beads being coated with antibodies directed against the phosphorylated proteins of interest. A combination of phosphorylated-p38 MAPK, -IκBα, -JNK, -ERK1&2 (all of which are proteins implicated in the signalling pathways leading to the activation of NF-κB), STAT3, and ATF2 (transcription factors cooperating with NF-κB) was used. In this assay, the positive controls are extracts of HeLa cells, activated with specific activators for each enzyme/transcription factor (phosphoprotein), while untreated HeLa cells function as the background and/or negative control, as shown in table 3.1 (page 79). The optimal exposure time of the cells to the metals was established by exposing the neutrophils to $Co^{2+}$, $Pt^{4+}$, $V^{3+}$, and $V^{5+}$ for 1, 3, 5 and 10 minutes followed by determination of the concentration of phosphorylated proteins.

**Table 3.1**

Lysates provided with the Bio-Plex assays:

| Lysate | Phospho-specific target |
|---|---|
| EGF-Treated HEK 293 | ERK1, ERK2, ERK1&2 |
| EGF-Treated HeLa | EGFR |
| IFN-α-Treated HeLa | STAT3 |
| TNF-α-Treated HeLa | IκB-α |
| UV-Treated HEK293 | ATF-2, JNK, p38 MAPK |
| Untreated HeLa | Background/ negative control |

The Bio-Plex array system utilizes the Luminex$^{R}$xMAP$^{R}$Multiplex technology to permit simultaneous detection and quantitation of up to 100 different analytes in a single microplate well. The system uses a liquid suspension array of 5.5 μm beads, each internally dyed with different ratios of two spectrally distinct fluorophores to assign it a unique spectral address. Each set of beads is conjugated with a different capture molecule, which binds to a target analyte. In the case of IL-8 and the phosphorylated proteins, the capture molecules are antibodies. This is followed by binding with biotinylated detection antibody and a reporter molecule, streptavidin-PE. The plate is then drawn into the Bio-Plex array reader, and precision fluidics align the beads in a single file through a flow cell where two lasers excite the beads individually. The red classification laser excites the dyes in each bead, identifying its spectral address, while the green reporter laser excites the reporter molecule associated with the bead, which allows quantitation of the captured analyte. Bio-Plex Manager software records the fluorescent signals simultaneously for each bead, translating the signals into data. Cytokine-results are expressed as pg/ml, while phosphorylation of target proteins is expressed according to fluorescence intensity.

### *3.2.6 Expression and statistical analysis of results*

The results of each series of experiments are presented as the mean values+/- standard errors of the mean (SEM), with the exception of some of the experiments for the determination of NF-κB nuclear translocation, were the actual images of the gels are shown. Statistical analysis of data was performed by using the Mann-Whitney *U*-test, and ANOVA where appropriate. A computer based software system (Graph Pad Prism®, Graph Pad Software Inc., San Diego, CA) was used for analysis and *P* value of <0.05 was taken as significant.

## *3.3* Results

### *3.3.1 NF-κB activation*

*3.3.1.1 NF-κB activation in pneumolysin-treated neutrophils*

Exposure of neutrophils to pneumolysin caused a time- and dosed-related activation of NF-κB, which was attenuated by inclusion of the $Ca^{2+}$-chelating agent, EGTA in the cell-suspending medium or by pretreatment of the cells with docosahexaenoic acid (DHA). Images of gels are shown in Figures 3.1 and 3.2 (pages 82 and 83). The mean values of the density, expressed as counts/$mm^2$ are shown in Table 3.2 (page 84). These results clearly demonstrate, that pneumolysin causes activation of NF-κB in human neutrophils and that the system developed is suitable for the measurement of nuclear translocation of NF-κB proteins in these cells.

*3.3.1.2 Effects of the metals on NF-κB activation*

Exposure of neutrophils to the metals did not result in a meaningful increase in nuclear NF-κB proteins. The values of the densities, determined from 5 different experiments, expressed as counts/$mm^2$, are shown in Table 3.3 (page85).

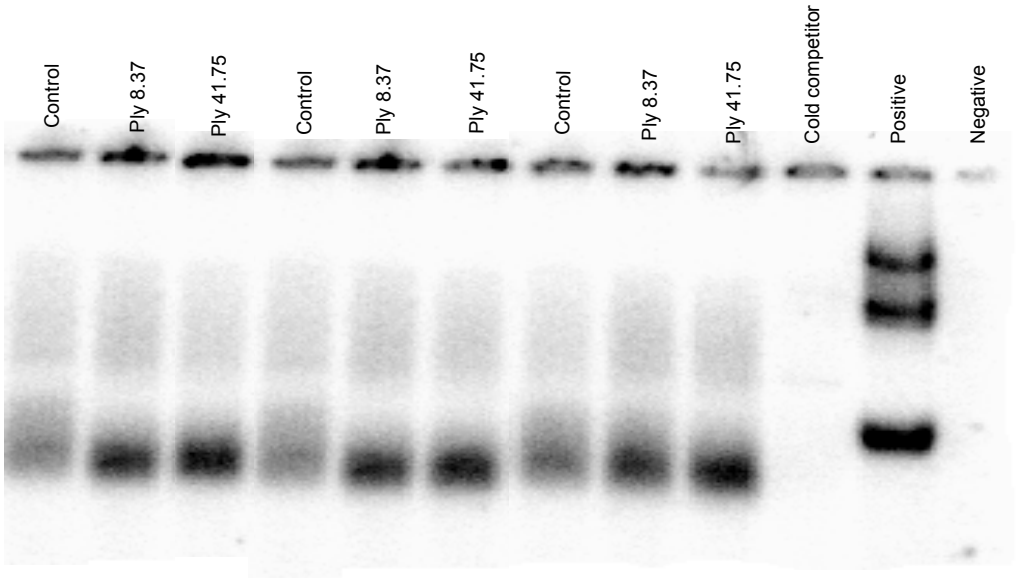



**Figure 3.1**

Phosphor-image showing the effects of pneumolysin on nuclear translocation of NF-κB in human neutrophils. The cells are from a single donor with triplicate determinations for each system (pneumolysin free control system, and systems treated with the toxin at concentrations of 8.37 ng/ml and 41.75 ng/ml).

Positive = HeLa cell nuclear extract

Negative = $H_2O$ without any nuclear protein

Control = Neutrophils not exposed to pneumolysin

Cold competitor = positive sample previously incubated with non-radiolabelled NF-κB specific oligonucleotide.

This image is representative of 4 different experiments.

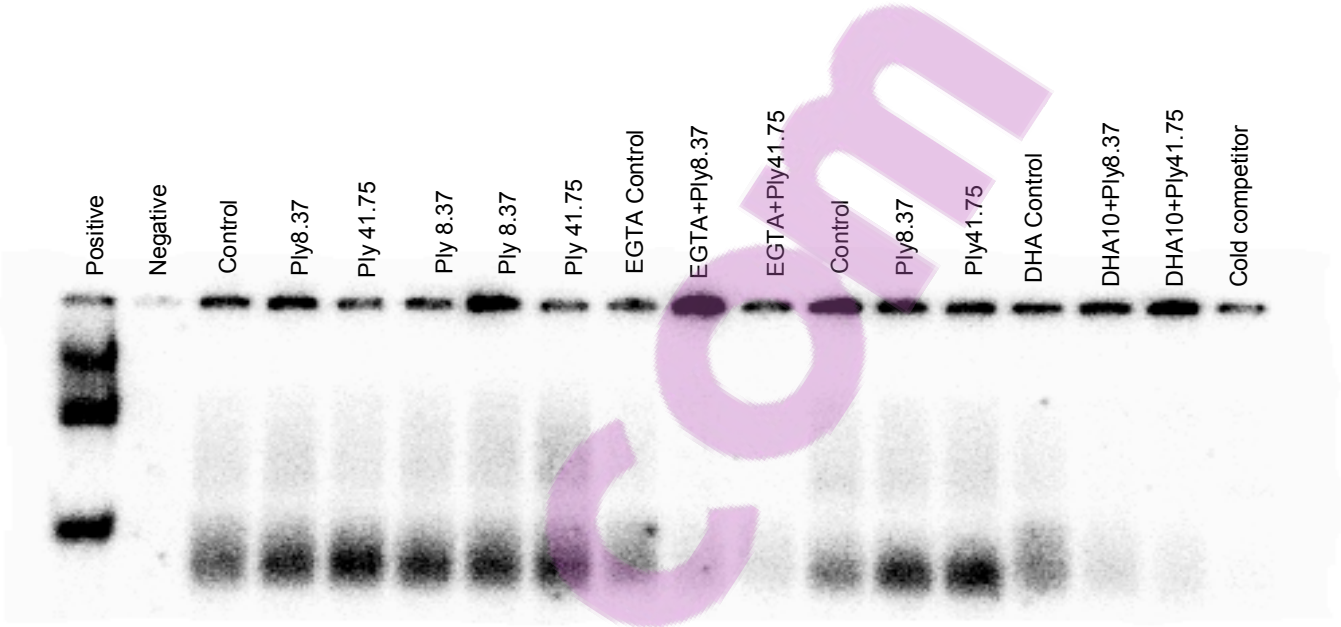



**Figure 3.2**
Phosphor-image showing the effects of pneumolysin (Ply 8.37 and 41.75 ng/ml) on nuclear translocation of NF-κB in human neutrophils in the absence and presence of 10 mM EGTA, or 10 μg/ml docosahexaenoic acid (DHA). The cells are from a single donor and the neutrophils were treated with pneumolysin for 15 min at 37°C in the absence and presence of EGTA or DHA and the nuclear extracts then analysed by electrophoretic mobility shift assay.
Positive = HeLa cell nuclear extract.
Negative = $H_2O$ without any nuclear protein
Control = Neutrophils not exposed to pneumolysin
Cold competitor = sample previously incubated with non-radiolabelled NF-κB-specific oligonucleotide.
This image is representative of 4 different experiments.

**Table 3.2**

Mean values + SEM of the densities, expressed as counts/mm$^2$, of nuclear factor-κB translocated nuclear proteins as determined by the Personal Molecular Imager FX and software from BIO-RAD Laboratories. These are the results of 4 different experiments.

| System | Density Counts/ mm$^2$ |
|---|---|
| Positive | 95 964 ± 2134 |
| Negative (no nuclear extract) | 12 619 ± 417 |
| Control (neutrophils alone) | 47 496 ± 2520 |
| Neutrophils exposed to pneumolysin 8.37 ng/ml | 71 652*± 2324 |
| Neutrophils exposed to pneumolysin 41.75 ng/ml | 80 305$^*$ ± 1266 |
| EGTA control | 49 782 ± 1936 |
| EGTA + pneumolysin 8.37 ng/ml | 23 273$^\Delta$ ± 1914 |
| EGTA + pneumolysin 41.75 ng/ml | 21 544$^\Delta$ ± 1580 |
| DHA control | 51 615 ± 1850 |
| DHA + pneumolysin 8.37 ng/ml | 22 020$^o$ ± 1906 |
| DHA + pneumolysin 41.75 ng/ml | 18 287$^o$ ± 1568 |
| Cold competitor | 11 035 ± 405 |

* $p < 0.05$ for comparison with the pneumolysin-free control system

Δ $p < 0.05$ for comparison with the corresponding pneumolysin- treated, EGTA-free system

o $p < 0.05$ for comparison with the corresponding pneumolysin-treated DHA-free system

## Table 3.3

Effects of the metals on the activation of NF-κB in neutrophils.

| Metal | NF-κB |
|---|---|
| $Co^{2+}$ | 101.22 ± 9.82* |
| $Pd^{2+}$ | 100.35 ± 13.7 |
| $Pt^{4+}$ | 116.58 ± 8.15 |
| $V^{3+}$ | 102.42 ± 11.03 |
| $V^{4+}$ | 111.69 ± 19.06 |
| $V^{5+}$ | 95.67 ± 17.90 |

* The results of 5 different experiments are expressed as the mean percentages ± SEM of the corresponding metal-free control systems. The absolute mean value ± SEM for the control system was 68 834 ± 10 624 counts/ $mm^2$.

### *3.3.2 IL-8*

*3.3.2.1 Production of IL-8*

Human neutrophils were incubated with the different metals for 6 hours at 37°C, which had been previously determined as the optimal time for IL-8 production (Cockeran *et al*, 2002). As shown in Figures 3.3 and 3.4 (pages 86 and 87), no significant changes in either total or extracellular concentrations of IL-8 were detected following exposure of neutrophils to any of the metals when compared to the control. These observations are in agreement with the failure of the metals to activate NF-κB.

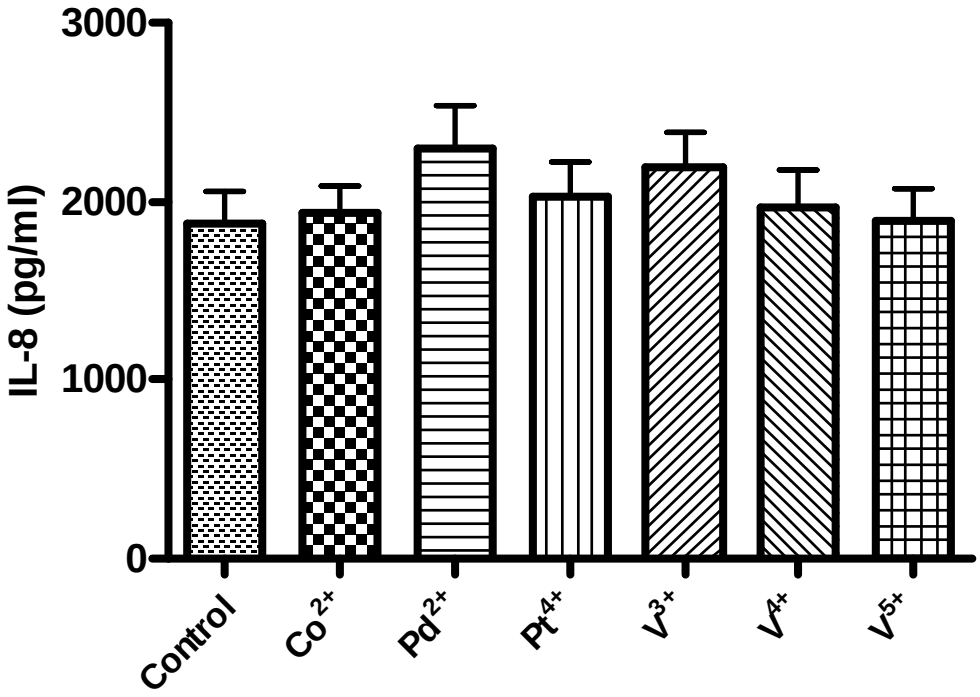



**Figure 3.3**

Effects of exposure to $Co^{2+}$, $Pd^{2+}$, $Pt^{4+}$, $V^{3+}$, $V^{4+}$, and $V^{5+}$ on the production of IL-8 (measured in cell-free supernatants) by human neutrophils. Results are expressed as the mean values ± SEM of 8 experiments.

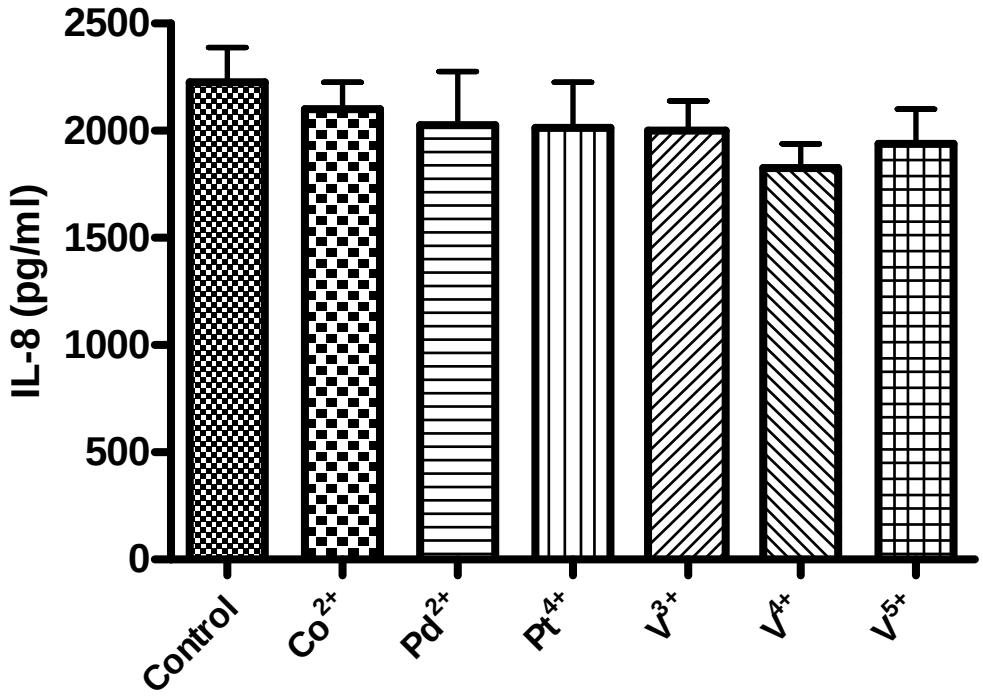



**Figure 3.4**

Effects of exposure to $Co^{2+}$, $Pd^{2+}$, $Pt^{4+}$, $V^{3+}$, $V^{4+}$, and $V^{5+}$ on the production of IL-8 by human neutrophils. The results are the total (extracellular and intracellular) concentrations of IL-8 and are the mean values ± SEM of 8 experiments.

*3.3.2.2 Immunological reactivity of IL-8*

As shown in Table 3.4, exposure of IL-8 (1.25 μg/ml) to the metals (25 μM) caused no significant change in the reactivity of the chemokine. This clearly demonstrates that the metals do not affect the immunoreactivity of IL-8 in the Bio-Plex assay system.

**Table 3.4**

Effects of pre-treatment with $Co^{2+}$, $Pd^{2+}$, $Pt^{4+}$, $V^{2+}$, $V^{3+}$, $V^{4+}$, and $V^{5+}$ (all at 25μM) on the immunological reactivity of IL-8.

| System | Mean concentration of IL-8 (pg/ml) |
|---|---|
| IL-8 only | 17 617 ± 2 753* |
| IL-8 + $Co^{2+}$ | 16 611 ± 1 920 |
| Il-8 + $Pd^{2+}$ | 14 883 ± 2 050 |
| IL-8 + $Pt^{4+}$ | 22 663 ± 1 710 |
| IL-8 + $V^{2+}$ | 19 465 ± 2 560 |
| IL-8 + $V^{3+}$ | 18 255 ± 1 8 62 |
| IL-8 + $V^{4+}$ | 24 938 ± 2 240 |
| IL-8 + $V^{5+}$ | 19 892 ± 1 790 |

The results of 5 experiments are expressed as the mean IL-8 concentrations ± SEM as determined with the Bio-Plex suspension array system.

***** Although used at a final concentration of 12 500 pg/ml, the values detected by the Bio-Plex assay system were somewhat higher (17 617 pg/ml in the control system). This may reflect a higher concentration of IL-8 than that reported by the manufacturer (Sigma) in the stock solution, and/or the high sensitivity of the Bio-Plex assay system.

### *3.3.3 Phosphorylated proteins*

Time course experiments determined an optimal exposure time of neutrophils to the metals of 3 minutes. Of the phosphoproteins tested, phosphorylated p38 MAPK and phosphorylated IκB-α were present at the highest concentrations in the cytoplasm (Figure 3.5, page 90). As shown in Tables 3.5 and 3.6 (pages 91 and 92) exposure of the cells to the metals was not accompanied by detectable alterations in the levels of the phosphorylated enzymes / transcription factors.

These findings again confirm that the metals do not, either directly by interaction with the cell membrane, nor indirectly by increasing oxidative stress, initiate the phosphorylation of proteins involved in the NF-κB signalling pathways, nor do they cause phosphorylation of other transcription factors which act in unison with NF-κB.

### *3.3.4 Cellular ATP levels*

The final concentration of each metal was 0.25μM, which was not cytotoxic to neutrophils as determined previously. The ATP levels of control neutrophils and those exposed to $Pd^{2+}$, $Pt^{4+}$ were 32 ± 1, 35 ± 5, and 30 ± 2 nmol/$10^7$ cells, respectively (Theron *et al,* 2004). Experiments with cobalt resulted in 47 ± 3 for control neutrophils and 47± 2 nmol ATP/$10^7$ cells for metal-exposed neutrophils (Ramafi *et al,* 2004).

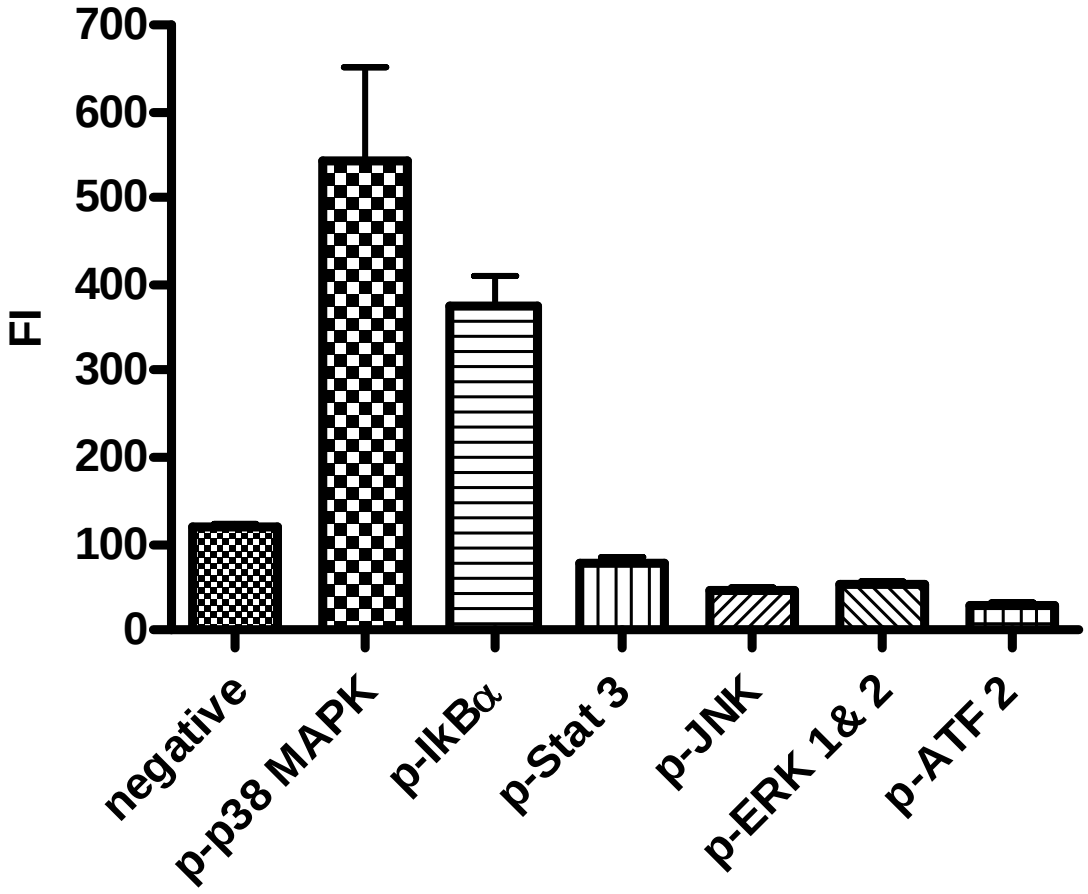



**Figure 3.5**

Concentrations of phosphorylated proteins in the cytosol of unstimulated human neutrophils in comparison to the cell-free, phosphorylation-free background system, which is referred to in the result section as the negative control system. The results are expressed as fluorescence intensity (FI) and are the mean values ± SEM from 4 different experiments.

**Table 3.5**

Phosphorylation of cytosolic proteins, involved in NF-κB signalling pathways, in unexposed and metal-exposed human neutrophils.

| System | p-p38MAPK FI | p-IκB FI | p-JNK FI | p-Erk 1&2 FI |
|---|---|---|---|---|
| Positive control | 2 052 ± 176 | 9 719 ± 555 | 2 072± 159 | 6 270 ± 149 |
| Negative control | 74 ± 2.1 | 198 ± 11 | 164 ± 45 | 250 ± 8.0 |
| Unstimulated neutrophils | 543 ± 107 | 375 ± 35 | 46 ± 3.7 | 54 ± 1.2 |
| Neutrophils exposed to $Co^{2+}$ | 545 ± 50 | 386 ± 52 | 39 ± 3.6 | 52 ± 4.0 |
| Neutrophils exposed to $Pd^{2+}$ | 435 ± 37 | 374 ± 48 | 46 ± 2.7 | 56 ± 1.7 |
| Neutrophils exposed to $Pt^{4+}$ | 416 ± 47 | 364 ± 37 | 40 ± 0.4 | 54 ± 2.9 |
| Neutrophils exposed to $V^{3+}$ | 496 ± 36 | 350 ± 31 | 37 ± 1.1 | 53 ± 3.6 |
| Neutrophils exposed to $V^{5+}$ | 353 ± 67 | 311 ± 26 | 38 ± 3.1 | 55 ± 3.9 |

The results are expressed as fluorescence intensity (FI), and are the mean values ± SEM of four different experiments.

**Table 3.6**

Phosphorylation of the cytosolic transcription factors STAT3 and ATF2 in metal-treated and metal-untreated neutrophils.

| System | p-STAT 3 FI | p-ATF 2 FI |
|---|---|---|
| Positive control | 1 757 ± 138 | 1 394 ± 202 |
| Negative control | 120 ± 3.0 | 249 ± 34 |
| Unstimulated neutrophils | 79 ± 7.3 | 29 ± 2.7 |
| Neutrophils exposed to $Co^{2+}$ | 72 ± 1.6 | 26 ± 2.0 |
| Neutrophils exposed to $Pd^{2+}$ | 73 ± 3.1 | 26 ± 1.1 |
| Neutrophils exposed to $Pt^{4+}$ | 72 ± 1.7 | 26 ± 1.0 |
| Neutrophils exposed to $V^{3+}$ | 67 ± 0.3 | 26 ± 2.6 |
| Neutrophils exposed to $V^{5+}$ | 76 ± 3.3 | 22 ± 0.5 |

The results are expressed as fluorescence intensity (FI), and are the mean values ± SEM from 4 different experiments.

## 3.4 Discussion

The first phase of the laboratory research described in this chapter was focussed on the development of an electrophoretic mobility shift assay (EMSA) for the detection of nuclear translocation of NF-κB in isolated human neutrophils. Based on previous reports that sustained elevations of cytosolic $Ca^{2+}$ result in efficient activation of NF-κB in various cell types, including neutrophils (Dolmetsch *et al,* 1997; Bekay *et al*, 2003) the pore-forming pneumococcal toxin, pneumolysin, which causes influx of $Ca^{2+}$ into neutrophils (Cockeran *et al.* 2001b), was identified as being a potential activator of NF-κB in these cells. The use of pneumolysin presented two advantages. Firstly, the pneumococcal toxin has been a research focus of the Department of Immunology, University of Pretoria, for several years, not only because of its involvement in the immunopathogenesis of invasive pneumococcal disease, but also because it represents an attractive target for drug and vaccine design. Secondly, the effects of pneumolysin on NF-κB activation in neutrophils have not been described previously, and thirdly, the effects of the various heavy metals ($Co^{2+}$, $Pd^{2+}$, $Pt^{4+}$, $V^{2+-5+}$) on the biological activities of pneumolysin represents a component of the studies presented in Chapter 4.

Exposure of neutrophils to low, sub-cytolytic, pathologically-relevant concentrations of pneumolysin (Spreer *et al.* 2003) resulted in significant, dose-related activation of NF-κB as determined by EMSA. The involvement of $Ca^{2+}$ influx in pneumolysin-mediated activation of NF-κB was supported by observations that nuclear translocation of the transcription factor in toxin-activated cells was attenuated by inclusion of the $Ca^{2+}$-chelating agent, EGTA, in the cell-suspending medium, or by treatment of the cells with docosahexaenoic acid. Although not included in this chapter, treatment of the cells with the NADPH oxidase inhibitor, diphenylene iodonium chloride (5 μM) did not affect pneumolysin-mediated activation of NF-κB, seemingly excluding the involvement of reactive oxidant species in this process. Likewise, it has been described that the activation of NF-κB which accompanies exposure of neutrophils to opsonized *Staphylococcus aureus* was not dependent on

oxidants generated during immune adherence/phagocytosis (Vollebregt *et al.* 1998).

The second phase of the study was focussed on the ability of the metals to: i) initiate activation/translocation of NF-κB in neutrophils, as well as synthesis of IL-8; ii) activate cytosolic signalling pathways which activate NF-κB; and iii) activate other transcription factors which interact with NF-κB to optimize gene transcription. However, none of the metals induced phosphorylation and translocation of NF-κB, which was supported by observations that the metals also failed to affect the production of IL-8 by these cells. Not surprisingly, in view of the NF-κB and IL-8 results, none of the metals affected either the activities of the various phospho-enzymes involved in cytosolic signalling cascades which converge on NF-κB, or the other transcription factors which co-operate with NF-κB to promote optimal gene transcription.

The failure of the various test heavy metals to activate NF-κB in neutrophils contrasts with their ability to activate the transcription factor in other cell types, including epithelial cells (Goebeler *et al.* 1995; Sultana *et al.* 1955; Jiménez *et al.* 2000), macrophages (Catelas *et al.* 2003), synoviocytes (Niki *et al.* 2003), and monocytes (Lewis *et al.* 2003). These differences may be related to the relatively short exposure times of the cells to the metals used in the current study during which no cytotoxicity was evident, according to the observed absence of effects of the metals in cellular ATP levels. Possibly, with longer exposure times, activation of NF-κB may have been detectable. If this were to occur, however, it is likely to be secondary to cytotoxicity, as opposed to a rapidly occurring activation of NF-κB.

The laboratory research presented in this chapter was designed with the specific objective of investigating the effects of a single exposure of isolated human neutrophils to the individual metals in specific oxidation states over a relatively short time course on the activation of NF-κB. Such a study design is clearly necessary to identify the possible pro-inflammatory

interactions of the individual metals with the cells. I do concede, however, that this experimental design may not be representative of the pathophysiological setting in which cells of the innate immune system in the airways, including neutrophils, may undergo sustained exposure to the metals over a prolonged time course. Furthermore, in this scenario it is highly improbable that exposure to a single metal will occur, but rather to several metals in combination with other environmental/occupational toxins such as gases, particulate material, and bacterial endotoxins, as well as components of cigarette smoke. In addition, the behaviour of neutrophils in the airways may be affected indirectly as a consequence of exposure to chemokines/cytokines, as well as other activators, released from different cell types, such as epithelial cells, on exposure to the test metals.

Irrespective of these possible limitations, the results presented in the current chapter clearly demonstrate that exposure of isolated neutrophils to the test metals is not accompanied by activation of NF-κB or synthesis of the proinflammatory chemokine, IL-8.

# Chapter 4

# Palladium Attenuates the Pro-Inflammatory Interactions of C5a, Interleukin-8 and Pneumolysin with Human Neutrophils

**Hypothesis**

Cobalt, palladium, platinum and vanadium might influence the ability, either by enhancment or by inhibition, of neutrophil chemoattractants to activate human neutrophils *in vitro.*

**Aim**

The aim was to investigate the effect of the four metals on the ability of the chemoattractants C5a and IL-8, as well as the pneumococcal toxin, pneumolysin, to activate human neutrophils *in vitro.* Neutrophil activation was determined according to the magnitude of the increase of cytosolic $Ca^{2+}$ concentrations using a spectrofluorimetric procedure, as well as by a chemotaxis assay using modified Boyden chambers.

## 4.1 Introduction

Occupational and possibly environmental exposure to heavy metals is often associated with an increased frequency of respiratory symptoms, including rhinitis, wheezing, dyspnoea, nasal haemorrhage, conjunctivitis, cough and sore throat, all of which are usually transient (Hughes, 1980; Goering, 1992; Ballach, 1997; Barceloux, 1999; Van Klaveren & Nemery, 1999; Merget & Rosner, 2001; Kielhorn *et al,* 2002; Brock & Stopford, 2003; Linna *et al*, 2003). However, persistent bronchial hyperresponsiveness, asthma and small airways dysfunction have also been described (*Swennen et al*, 1993; Niezborala & Garnier, 1996; Irsigler *et al*, 1999; Mapp *et al,* 1999). These adverse effects of heavy metals on the airways result not only from immunological sensitization (Calverley *et al,* 1995; Raulf-Heimsoth *et al*, 2000), but also as a result of irritant interactions with airway epithelium, resulting in the production of pro-inflammatory cytokines (Shishodia *et al*, 1997; Wang *et al*, 2003; Ramafi *et al,* 2004; Theron *et al,* 2004).

While their pro-allergenic and pro-irritant actions are relatively well characterized, almost nothing is known about the possible adverse effects of heavy metals on innate host defenses operative against commonly-encountered microbial pathogens. In the current study, the effects of four heavy metals of industrial and

environmental significance, *viz* cobalt, palladium, platinum and vanadium on the biological activities of C5a and interleukin-8 (IL-8), two key neutrophil-mobilizing chemoattractants generated by cells of the innate immune system, have been investigated. In addition, the effects of these metals on the neutrophil-activating potential of the cholesterol binding, pore-forming toxin, pneumolysin, which is produced by *Streptococcus pneumoniae* (Andrew *et al,* 2000), one of the major human pathogens, and one of the most common causes of community-acquired pneumonia, otitis media, sinusitis, and meningitis (Cockeran *et al,* 2003), have also been investigated. Notwithstanding its ability to activate synthesis of pro-inflammatory cytokines via interactions with Toll-like receptor 4 on inflammatory cells (Malley *et al*, 2003), pneumolysin also initiates the generation of C5a and IL-8 as a consequence of its complement-activating and pore-forming properties respectively (Mitchell & Andrew, 2000; Cockeran *et al*, 2002a; Van Rossum *et al*, 2005; Ratner *et al*, 2006).

## 4.2 Materials and Methods

### *4.2.1 Chemicals and reagents*

Cobalt (II) chloride hexahydrate ($Co^{2+}$), platinic chloride hydrogen hexachloroplatinate (IV) ($Pt^{4+}$), palladium (II) chloride ($Pd^{2+}$), vanadium (II) chloride ($V^{2+}$), vanadium (III) chloride ($V^{3+}$), vanadyl sulphate hydrate ($V^{4+}$) and sodium metavanadate ($V^{5+}$) were purchased from Sigma-Aldrich (St Louis, Mo, USA). These were dissolved in distilled water to give stock concentrations of 10mM and used in the assays described below at a maximum concentration of 25 µM for each metal.

Recombinant human C5a and IL-8 were also purchased from Sigma-Aldrich, while recombinant pneumolysin, prepared as described previously (Saunders *et al,* 1989), was kindly provided by Prof. T.J. Mitchell, Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, UK. C5a, IL-8 and pneumolysin were reconstituted in indicator-free Hanks' balanced salt solution (HBSS, pH 7.4, 1.25 mM $CaCl_2$) to stock concentrations of 4, 2.5 and 4.2 µg/ml respectively.

### *4.2.2 Exposure of C5a, IL-8, and pneumolysin to the metals*

C5a, IL-8, and pneumolysin at fixed concentrations of 2, 1.25, and 2.1 μg/ml respectively (in the $Ca^{2+}$ experiments) and at concentrations of 1 μg/ml (in the chemotaxis experiments) were coincubated with each metal at a fixed concentration of 25 μM for 1 min at 37ºC after which the various activators were added either directly to neutrophils, or to the lower compartments of modified Boyden chambers, and evaluated for their abilities to elevate cytosolic $Ca^{2+}$, or to induce a chemotactic response respectively. In these assays, the final concentrations of C5a, IL-8, and pneumolysin were 25 ng/ml, 12.5 ng/ml and 20 ng/ml respectively ($Ca^{2+}$ experiments) and 100 ng/ml (chemotaxis experiments), which represents a 1:100, 1:10 dilution respectively of each agent, while the final concentrations of each metal was 0.25 μM, which is not cytotoxic according to measurement of ATP levels of neutrophils exposed to the metals for 30 minutesat 37° C, as determined previously (Theron *et al,* 2004, Ramafi *et al,* 2004).

### *4.2.3 Neutrophils*

Purified human neutrophils were prepared as described in 2.2.2.

### *4.2.4 Spectrofluorimetric measurement of cytosolic $Ca^{2+}$*

Fura-2/AM was used as the fluorescent, $Ca^{2+}$-sensitive indicator for these experiments (Grynkiewicz *et al,* 1985). Neutrophils (1 x $10^7$/ml) were incubated with fura-2/AM (2 μM) for 25 minutes at 37ºC in PBS, washed and resuspended in HBSS. The fura-2-loaded cells (1 x $10^6$/ml) were then preincubated for 10 minutes at 37ºC after which they were transferred to disposable reaction cuvettes, which were maintained at 37ºC in a Perkin Elmer, LS 45, luminescence spectrometer with excitation and emission wavelengths set at 340 and 500 nm, respectively. After a stable baseline was obtained (± 1 minute), the neutrophils were activated by addition of the metal-treated or -untreated (control) chemoattractants or pneumolysin as described above and alterations in cytosolic $Ca^{2+}$ concentrations monitored over a 5

minute time course. Cytosolic $Ca^{2+}$ concentrations were calculated as described previously (Grynkiewicz *et al*, 1985).

### *4.2.5 Assay of neutrophil migration*

For these investigations neutrophils were suspended to a concentration of 3 x $10^6$/ml in HBSS supplemented with 0.1% bovine serum albumin (BSA). Modified Boyden chambers in which the upper (cell) and lower (chemoattractant) chambers were separated by a 5 μM pore–size membrane filter (Sartorius-membrane filter, Göttingen, West Germany) were used to assess neutrophil migration (Anderson *et al,* 1984). Cell suspensions (200 μl containing 0.6 x $10^5$ neutrophils) were added to the upper chamber, while 1 ml of the metal-treated or –untreated chemoattractant (C5a or IL-8 at final concentrations of 100 ng/ml in BSA-supplemented HBSS) was added to the lower chamber. The chemoattractants were omitted from random migration systems i.e. BSA-supplemented HBSS only in the lower compartment of the Boyden chamber. The fully-assembled chambers were then incubated for 45 – 60 minutes at 37ºC after which the filters were detached, fixed, stained and cleared, and the results expressed as the number of cells which had completely traversed the filter per microscope high-powered field (cells/HPF) as an average of triplicate filters for each system for each experiment.

### *4.2.6 NF-κB activation*

Extraction of nuclear proteins and EMSA was performed as described in Chapter 3, section 3.2.2. Neutrophils were exposed to either untreated or $Pd^{2+}$ (25 μM)-treated pneumolysin (2 ng/ml). Pretreatment with palladium was 1 minute, after which the pneumolysin ± $Pd^{2+}$ was diluted 100-fold followed by addition to the neutrophils, which were incubated for 25 minutes at 37º. The final, residual concentration of $Pd^{2+}$ in the assay system was 0.25 μM, which did not affect the biological activity of pneumolysin.

### *4.2.7 Expression and statistical analysis of results*

The results of each series of experiments are presented as the mean values +/- standard errors of the means (SEM), with the exception of some of the spectrofluorimetric determinations of cytosolic $Ca^{2+}$ for which the traces are also shown. Statistical analysis of data was performed by using the Mann-Whitney *U*-test, and ANOVA where appropriate. A computer-based software system (Graph Pad Prism® 4, San Diego, CA) was used for analysis and *P* value of <0.05 was taken as significant.

## 4.3 Results

### *4.3.1 Effects of prior exposure to the metals on C5a-, IL-8, and pneumolysin-mediated alterations in neutrophil cytosolic $Ca^{2+}$ concentrations*

These results are shown in Figures 4.1-4.3 (pages 102 - 104) and Table 4.1 (page 105). In control, metal-free systems, exposure of neutrophils to C5a and IL-8 was accompanied by the typical, abruptly-occurring increase in cytosolic $Ca^{2+}$ which attained maximum values within 10 - 20 seconds and declined steadily thereafter, returning to basal/or close-to-basal values after about 3 minutes and 2 minutes in the case of C5a and IL-8 respectively, (Figures 4.1 and 4.2, pages 102 and 103). The peak response coincides with chemoattractant-mediated mobilization of $Ca^{2+}$ from neutrophil intracellular stores, while the subsequent rate of decline in cytosolic $Ca^{2+}$ reflect the balance between efficacy $Ca^{2+}$ clearance systems and store-operated influx of the cation (Tintinger *et al,* 2005). As shown in Figure 4.3, (page 104), exposure of neutrophils to pneumolysin was accompanied by an increase in cytosolic $Ca^{2+}$ which was evident after a lag phase of about 1 minute, rising at a slower rate than that initiated by the chemoattractants, and reaching comparable peak values which were sustained over the time course of the experiments. In this setting, increased cytosolic $Ca^{2+}$ results from the pore-forming interactions of pneumolysin with neutrophils, with resultant influx of $Ca^{2+}$ (Cockeran *et al,* 2002a).

Prior exposure of C5a, IL-8, or pneumolysin to $Pd^{2+}$ (25μM), but not to any of the other metals, resulted in significant attenuation of the $Ca^{2+}$-mobilizing interactions of the chemoattractants, particularly C5a, and the toxin with the cells (Figures 4.1-4.3, pages 102 – 104, and Table 4.1, page 105).

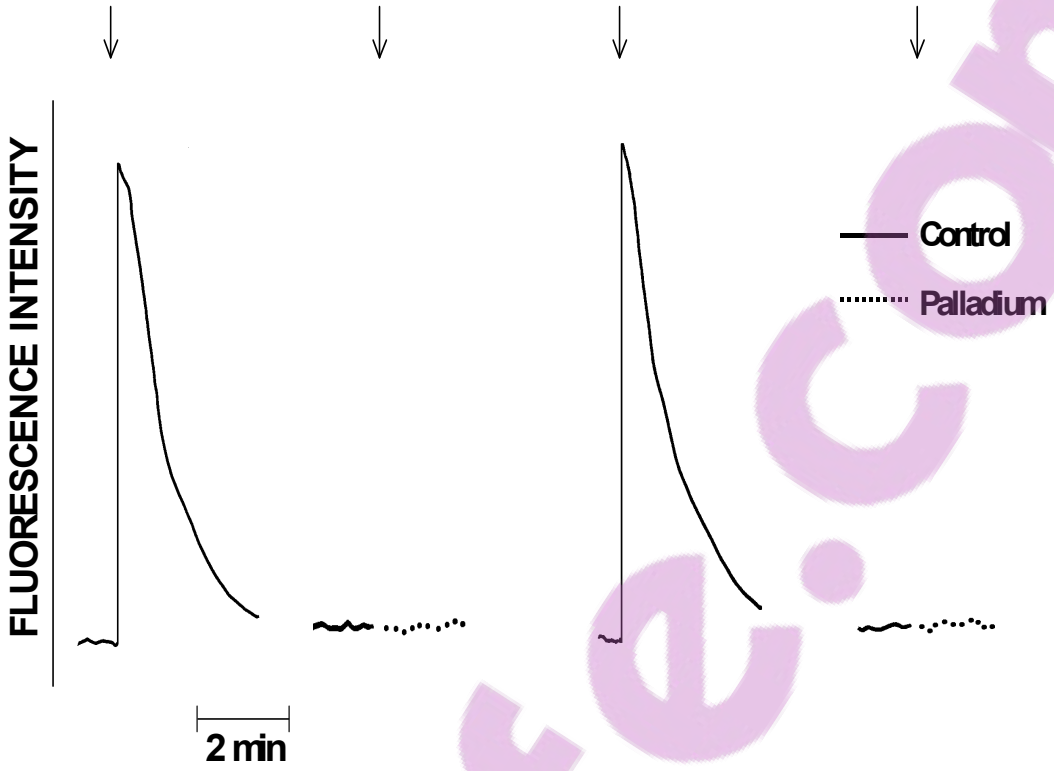
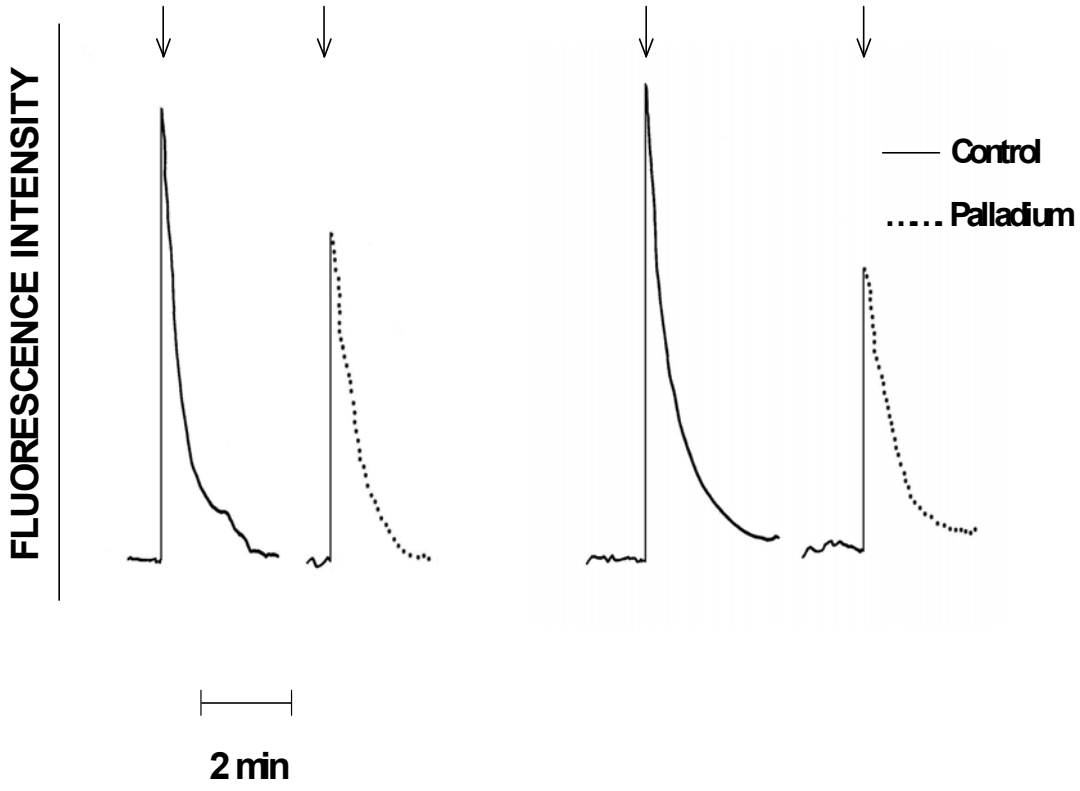


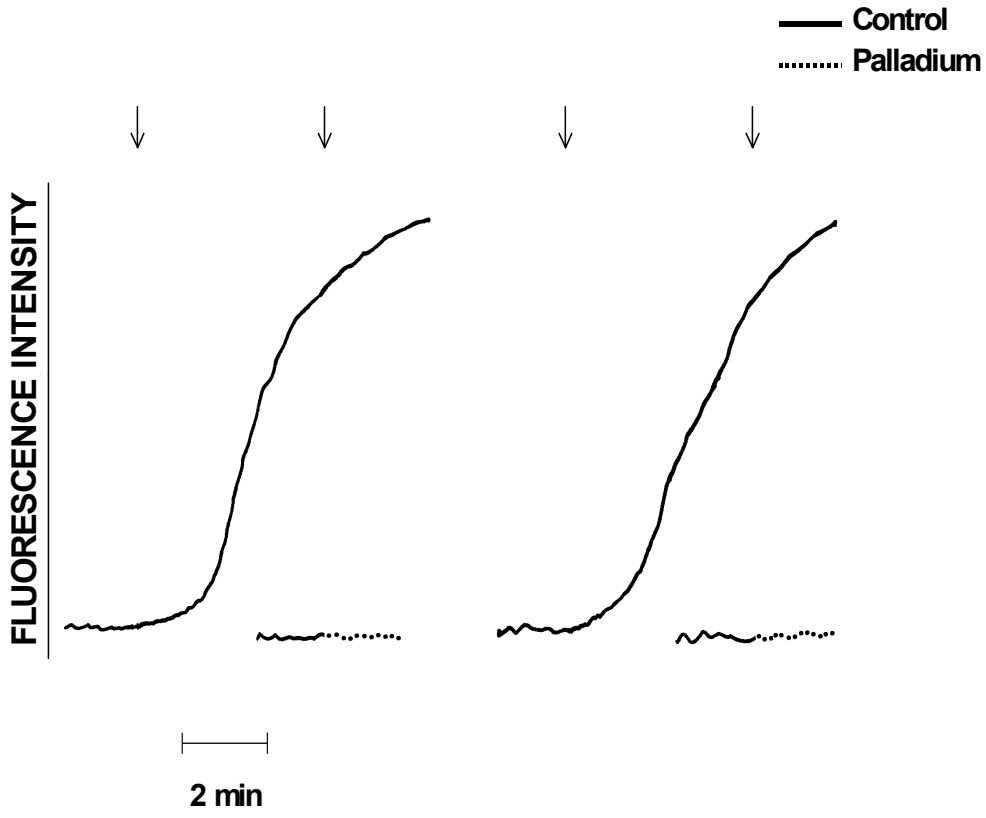

**Figure 4.1**

Effect of exposure to $Pd^{2+}$ (25 μM) on the $Ca^{2+}$-mobilizing interactions of C5a with neutrophils shown as fura-2 fluorescence traces of two representative experiments (5 in the series). The responses of cells exposed to untreated, or to $Pd^{2+}$-treated C5a (added as denoted by the arrow ↓) are shown on the left and right sides of each pair of traces respectively.




## Figure 4.2

Effect of exposure to $Pd^{2+}$ (25 μM) on the $Ca^{2+}$ -mobilizing interactions of IL-8 with neutrophils is shown as the fura-2 fluorescence traces of 2 representative experiments (5 in the series). The responses of the cells exposed to untreated, or to $Pd^{2+}$-treated IL-8 (added as denoted by the arrow ↓) are shown on the left and right sides of each pair of traces respectively.




## Figure 4.3

Effect of exposure to $Pd^{2+}$ (25 μM) on the $Ca^{2+}$ - mobilizing interactions of pneumolysin with neutrophils is shown as the fura-2 fluorescence traces of 2 representative experiments (5 in the series). The responses of the cells exposed to untreated, or to $Pd^{2+}$-treated pneumolysin (added as denoted by the arrow ↓) are shown on the left and right sides of each pair of traces respectively.

**Table 4.1**

Effects of pre-treatment with $Co^{2+}$, $Pd^{2+}$, $Pt^{4+}$, $V^{2+}$, $V^{3+}$, $V^{4+}$, and $V^{5+}$ (all at 25 μM) on the $Ca^{2+}$-mobilizing interactions of C5a, IL-8 and pneumolysin with neutrophils.

| System | Peak neutrophil cytosolic $Ca^{2+}$ value (nM) |
|---|---|
| C5a only (control) | 577 ± 24 |
| C5a + $Co^{2+}$ | 564 ± 9 |
| C5a + $Pd^{2+}$ | 129 ± 8* |
| C5a + $Pt^{4+}$ | 565 ± 15 |
| C5a + $V^{2+}$ | 554 ± 17 |
| C5a + $V^{3+}$ | 566 ± 6 |
| C5a + $V^{4+}$ | 587 ± 9 |
| C5a + $V^{5}$ | 554 ± 20 |
| IL-8 only (control) | 449 ± 40 |
| IL-8 + $Co^{2+}$ | 462 ± 18 |
| IL-8 + $Pd^{2+}$ | 303 ± 23* |
| IL-8 + $Pt^{4+}$ | 464 ± 14 |
| IL-8 + $V^{2+}$ | 463 ± 12 |
| IL-8 + $V^{3+}$ | 411 ± 6 |
| IL-8 + $V^{4+}$ | 385 ± 33 |
| IL-8 + $V^{5+}$ | 386 ± 31 |
| Pneumolysin only (control) | 457 ± 31 |
| Pneumolysin + $Co^{2+}$ | 413 ± 14 |
| Pneumolysin + $Pd^{2+}$ | 127 ± 6* |
| Pneumolysin + $Pt^{4+}$ | 456 ± 18 |
| Pneumolysin + $V^{2+}$ | 439 ± 16 |
| Pneumolysin + $V^{3+}$ | 456 ± 12 |
| Pneumolysin + $V^{4+}$ | 439 ± 19 |
| Pneumolysin + $V^{5+}$ | 447 ± 19 |

The results of 5 experiments are expressed as the mean peak neutrophil cytosolic $Ca^{2+}$ concentrations ± SEM recorded immediately after the addition of C5a or IL-8 to the cells, or at approximately 1 min after the addition of pneumolysin. The mean cytosolic $Ca^{2+}$ concentration for resting cells, recorded prior to the addition of the chemoattractants/pneumolysin, was 121± 7.1 nM.

* $p < 0.05$

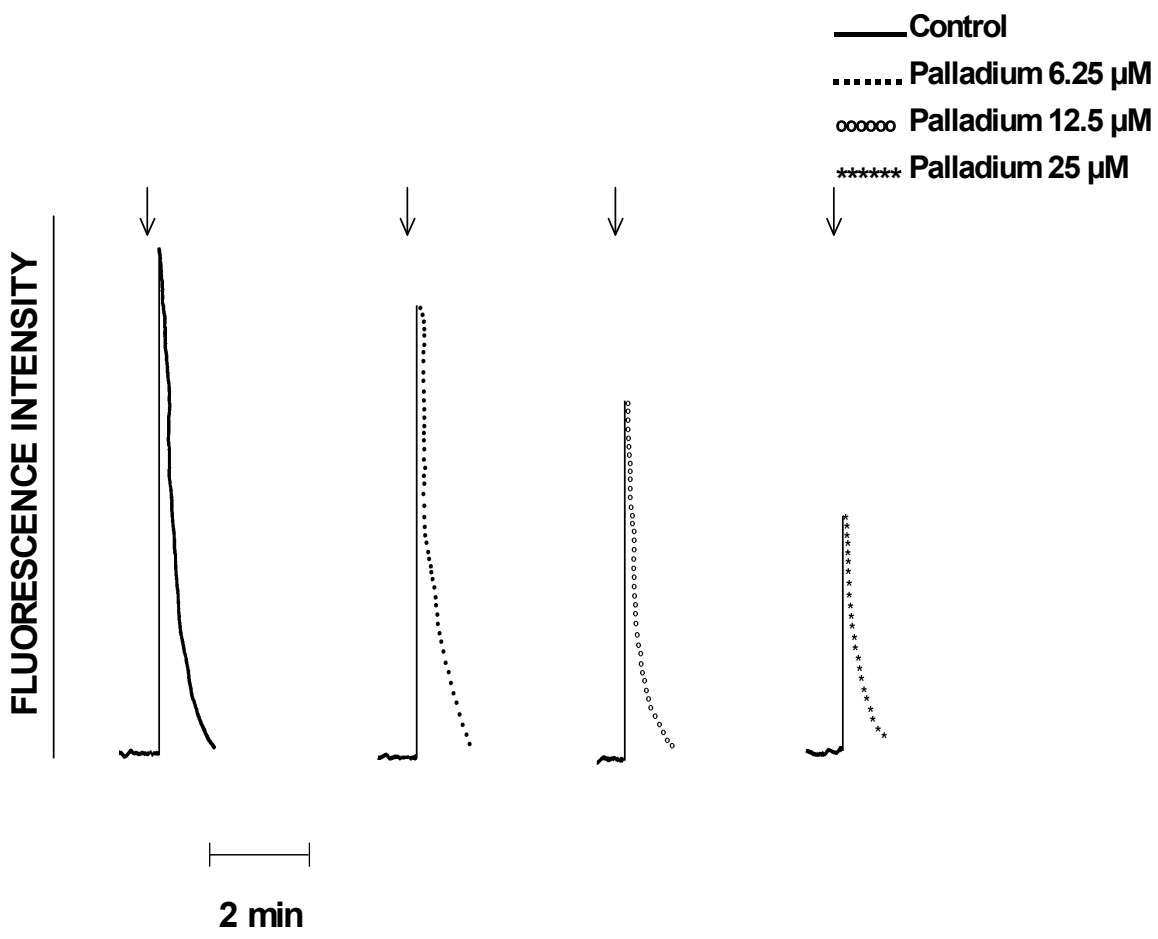
The effects of exposure of IL-8 or pneumolysin to $Pd^{2+}$ at concentrations ranging from 6.25 μM – 25 μM, are shown in Figures 4.4 and 4.5 (pages 107 and 108). The inhibitory effects of the metal on the reactivity of the chemoattractants and pneumolysin with neutrophils were detected at concentrations of 2, 1.25, and 2.1 μg/ml in the case of C5a, IL-8 and pneumolysin respectively.

### *4.3.2 Effects of $Pd^{2+}$ on the leukotactic activity of C5a and IL-8*

The effects of brief, prior exposure to 25 μM $Pd^{2+}$ on the chemotactic activities of C5a and IL-8 for neutrophils are shown in Figure 4.6 (page 109). Exposure of the chemoattractants to the metals was accompanied by a significant decrease, especially in the case of C5a, in chemotactic activity.

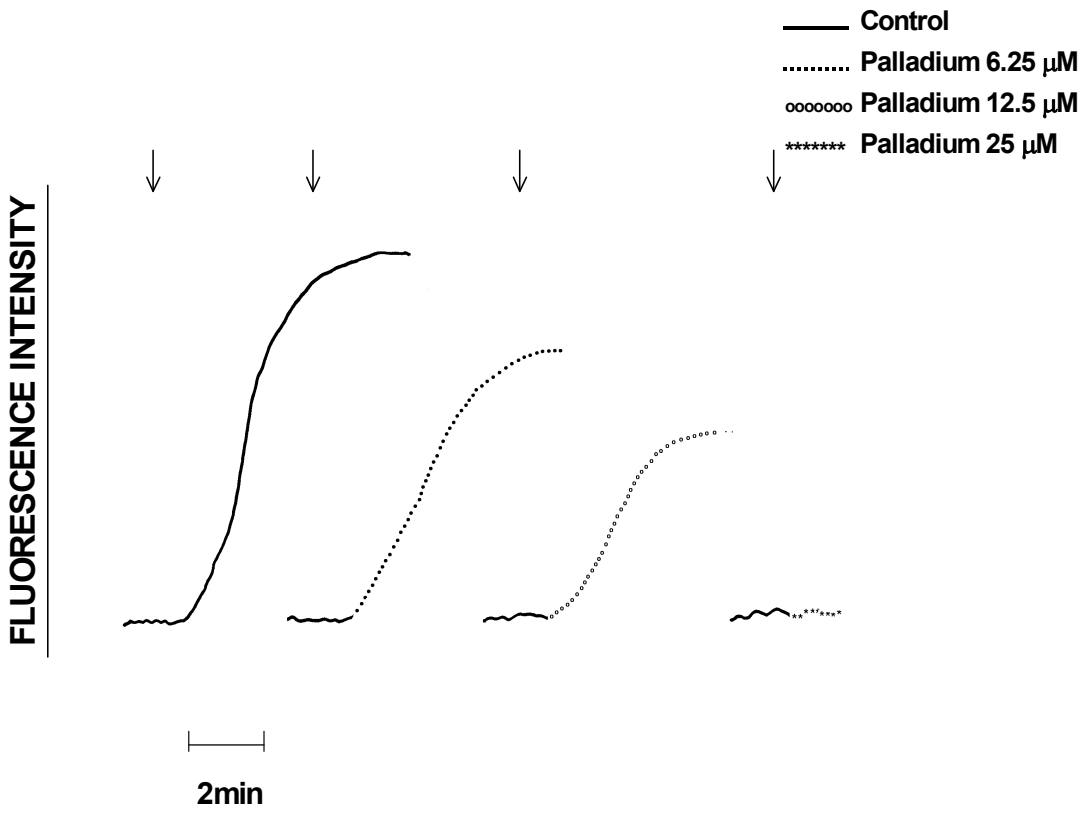
### *4.3.3 Activation of NF-κB*

The effects of pre-treatment of pneumolysin with $Pd^{2+}$ (25 μM) on the pore-forming, $Ca^{2+}$-dependent activation of NF-κB in neutrophils are shown in Figure 4.7 (page 110), which includes both the image of the gel, as well as the density of activation for each experimental system (counts/$mm^2$). Exposure of pneumolysin to $Pd^{2+}$ completely attenuated the ability of the toxin to activate NF-κB in neutrophils, probably as a consequence of failure of $Pd^{2+}$-treated pneumolysin to activate $Ca^{2+}$ influx.




## Figure 4.4

Effects of exposure to varying concentrations of $Pd^{2+}$ (6.25 – 25 µM) on the $Ca^{2+}$-mobilizing interactions of IL- 8 (added as denoted by the arrow ↓) with neutrophils are shown as the fura-2 fluorescence traces of a single representative experiment (3 in the series).




## Figure 4.5

Effect of exposure to various concentrations of $Pd^{2+}$ (6.25 – 25 μM) on the $Ca^{2+}$-mobilizing interactions of pneumolysin (added as denoted by the arrow ↓) with neutrophils are shown as the fura-2 fluorescence traces of a single representative experiment (3 in the series).

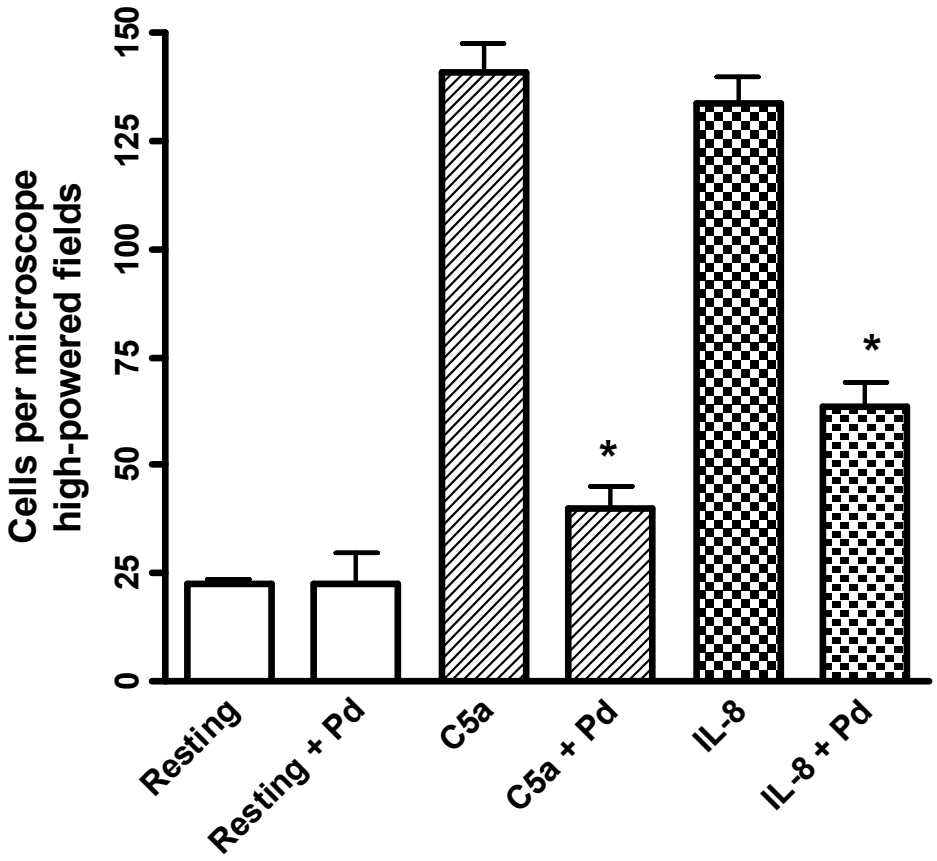



**Figure 4.6**

Effect of exposure to $Pd^{2+}$ (25 μM) on the chemotactic activities of C5a and IL-8 for neutrophils. The results of 3 experiments (with 6 replicates for each system) are expressed as the mean values (cells/microscope high-powered field) ± SEM.

* $p < 0.05$

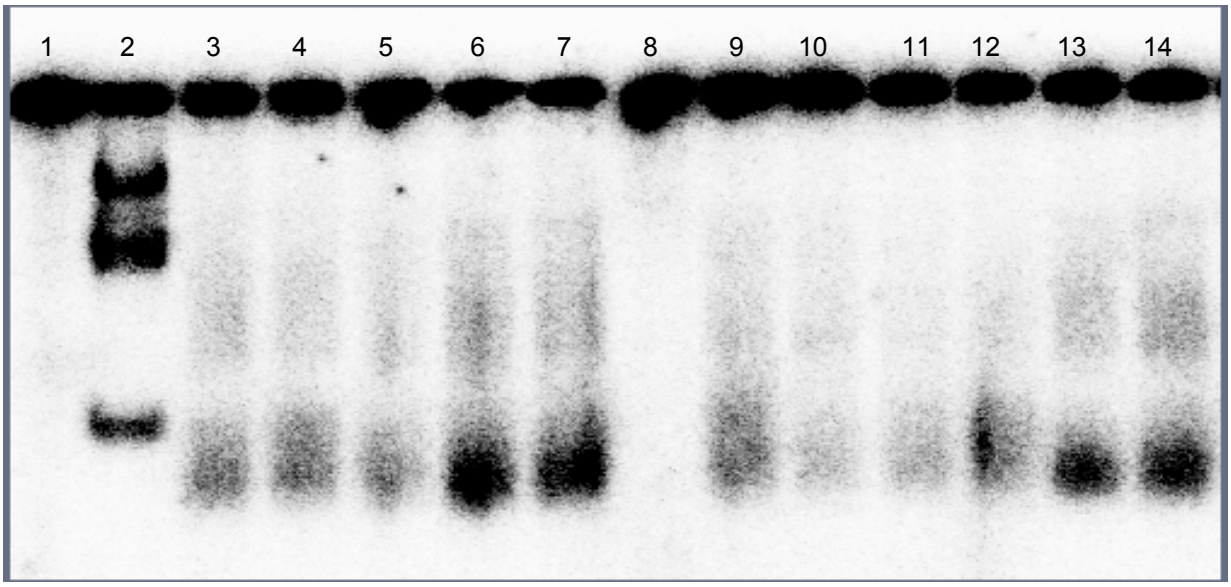



**Figure 4.7**
Phosphor-image showing the effect of untreated and $Pd^{2+}$-treated pneumolysin (Ply 20 ng/ml, $Pd^{2+}$ 25 μM) on nuclear translocation of NF-κB in neutrophils.

| | | **Density (counts/mm$^2$)** |
|---|---|---|
| 1. | negative | 12 037 |
| 2. | HeLa cell nuclear extract | 159 067 |
| 3. | control (unexposed neutrophils) | 66 744 |
| 4. | neutrophils exposed to $Pd^{2+}$-pretreated Ply | 71 159 |
| 5. | neutrophils exposed to $Pd^{2+}$ - pretreated Ply | 60 598 |
| 6. | neutrophils exposed to untreated Ply | 109 337 |
| 7. | neutrophils exposed to untreated Ply | 107 832 |
| 8. | cold competitor | 14 335 |
| 9. | control (unexposed neutrophils) | 66 644 |
| 10. | neutrophils exposed to $Pd^{2+}$ - pretreated Ply | 43 863 |
| 11. | neutrophils exposed to $Pd^{2+}$ - pretreated Ply | 49 752 |
| 12. | control ( unexposed neutrophils) | 68 741 |
| 13. | neutrophils exposed to untreated Ply | 97 381 |
| 14. | neutrophils exposed to untreated Ply | 94 031 |

## 4.4 Discussion

The results of the current study have demonstrated that $Pd^{2+}$, but not $Co^{2+}$, $Pt^{4+}$, or V in the various oxidation states tested, attenuate the neutrophil activating/mobilizing properties of the chemoattractants, C5a and IL-8, both of which are critical components of innate host defence. C5a is generated following activation of the alternative and mannan lectin-binding pathways of complement activation during innate host defence, and is a potent chemoattractant for neutrophils, monocytes and macrophages (Hopken *et al,* 1996). IL-8, which possesses selective chemotactic activity for neutrophils, is produced by epithelial cells, monocytes, macrophages, and several other cell types, including neutrophils themselves, following interaction of pattern recognition molecules on these cells with microbial pathogens (Haselmayer *et al,* 2006).

In addition to inactivating the two chemoattractants, $Pd^{2+}$ also neutralized the pore-forming interactions of the pneumococcal toxin, pneumolysin, with neutrophils, with resultant attenuation of $Ca^{2+}$ influx. Pneumolysin belongs to the family of cholesterol binding, pore-forming, microbial toxins, which are produced by many different bacterial pathogens (Andrew *et al,* 2000). Depending on the local density of toxin-producing bacteria in the airways, pneumolysin, which is produced by almost all clinical isolates of the pneumococcus, may either promote or prevent pneumococcal infection. In the case of the latter, exposure to small numbers of pneumococci in the airways results in the production of low, subcytolytic concentrations of pneumolysin, which induce production of IL-8 by airway epithelium by a mechanism dependent on $Ca^{2+}$ influx and activation of p38 mitogen-activated protein kinase and nuclear factor-κB (Ratner *et al,* 2006); in this setting the consequent influx of neutrophils is protective, resulting in clearance of *Streptococcus pneumoniae* from the airways (Van Rossum *et al,* 2005; Ratner *et al,* 2006).

NF-κB is activated following exposure of neutrophils to pneumolysin, which is accompanied by synthesis of IL-8. The results of experiments

described in this chapter have demonstrated that exposure of pneumolysin to $Pd^{2+}$ attenuates the activation of NF-κB by the toxin, which may further compromise host innate immunity to *Streptococcus pneumoniae.*

The first cells to come into contact with invading microorganisms are the airway macrophages and the lung epithelium. Pneumococci invade the lung epithelium and pulmonary endothelial cells through the action of pneumolysin, which slows ciliary beating of the epithelial cells, causing injury to alveolar epithelial cells and pulmonary artery endothelial cells, resulting in disruption of alveolus-capillary barrier. In addition to these direct effects, pneumolysin also induces the production of proinflammatory mediators, such as nitric oxide, COX-2, TNF-α, IL-1, and IL-6 production in macrophages (Zysk *et al*, 2001). As demonstrated in the current study palladium inhibits the pore-forming ability of pneumolysin; these mediators would therefore not be available to initiate the proinflammatory process necessary for the elimination of the invading microorganism. In addition the abilty of chemoattractants, like IL-8 and C5a, to attract neutrophils to the site of infection is compromised and microorganisms can multiply.

These observations demonstrate that several key mediators of inflammation, of both host and bacterial origin, which act in concert to initiate a protective, neutrophil-mediated response against a commonly-encountered and frequently life-threatening microbial pathogen, *Streptococcus pneumoniae,* are inactivated by $Pd^{2+}$. Given that C5a and IL-8 are of fundamental importance in host defence, while cholesterol-binding, pore-forming toxins are produced by many different microbial pathogens, exposure to $Pd^{2+}$ may broadly favour microbial persistence in the airways.

The mechanism by which exposure to $Pd^{2+}$, but not to any of the other metals tested, attenuates the protective, biological activities of C5a, IL-8, and pneumolysin remains to be established. Interaction with protein sulphydryls does not appear to be implicated, because all 4 test metals possess comparable activity in this respect (data not included). Interestingly, $Pd^{2+}$ has

been reported to acquire protease activity following binding to histidine and methionine residues, causing cleavage of the proximal, upstream peptide bond (Milovic & Kostic, 2002a; Milovic & Kostic, 2002b; Milovic & Kostic, 2003). Although somewhat speculative, such a non-specific, proteolytic mechanism would explain the susceptibility of C5a, IL-8, and pneumolysin to $Pd^{2+}$.

Notwithstanding occupational exposure to $Pd^{2+}$ during extraction, concentration, refining and separation of platinum group metals, other sources of exposure to $Pd^{2+}$ include atmospheric emissions from automobile catalytic converters, and corrosion of dental alloys (Drasch *et al,* 2000). In the current study, $Pd^{2+}$-mediated inactivation of C5a, IL-8 and pneumolysin was detected at concentrations of the metal as low as 6.25 μM, which equates to 1.11 μg/ml. This is likely to be considerably lower than concentrations of the metal which may be encountered in refineries, while a maximum load of 70.5 μg/day has been detected in saliva of subjects with $Pd^{2+}$-containing dental restorations (Drasch *et al,* 2000). Concentrations of $Pd^{2+}$ in roadside dust adjacent to motorways have been estimated at around 70 μg/kg (Jarvis *et al,* 2001).

In conclusion, the current study has documented interference with innate host defences as being a possible health risk of exposure to $Pd^{2+}$. While the implications, if any, of these findings for environmental/occupational health remain to be established, the potential of heavy metals to compromise innate host defence mechanisms represents an emerging field of heavy metal toxicity (Klein-Patel *et al,* 2006).

# Chapter 5

# Conclusion

## Conclusions

Adverse respiratory health effects are associated with occupational and/or environmental exposure to heavy metals (Hughes, 1980; Goering 1992; Vanadium, 2001). Neutrophils are mobilized to the airways following inhalation of toxic gases and particles, and are the probable perpetrators of inflammation-related airway damage (Bassett *et al,* 2000; Douwes *et al*; 2002; Saldiva *et al*, 2002). Cobalt, palladium, platinum and vanadium are all metals of environmental and occupational significance, particularly in South Africa, and the laboratory research presented in this thesis was undertaken with the primary objective of identifying possible pro-oxidative and pro-inflammatory interactions of these metals with human neutrophils *in vitro,* using sophisticated procedures, such as electron spin resonance spectroscopy, electrophoretic mobility shift assay combined with phosphor-scanning, and the Bio-Plex suspension array system. The major conclusions of the study are as follows:

- Inclusion of vanadium in the +2, +3, and +4, but not in the +5 valence states to activated human neutrophils promotes the formation of hydroxyl radical, one of the most reactive and damaging free radicals in biological systems (Cheng *et al*, 2002). In the physiological setting, exposure to $Fe^{2+}$ presents the highest risk of hydroxyl radical toxicity via the Fenton reaction; however, this is stringently controlled *in vivo* by iron-binding proteins, which limit the availability of free iron. Exposure to $V^{2+}$, $V^{3+}$ and $V^{4+}$, as is likely to occur in the environmental setting and occupational setting in particular, is likely to pose the potential threat of hydroxyl radical toxicity.

- Exposure of neutrophils to $Co^{2+}$, $Pd^{2+}$, $Pt^{2+}$ or $V^{2+-5+}$ was not accompanied by activation of NF-κB or synthesis of IL-8, which was underscored by the failure of the metals to activate cytosolic signalling mechanisms involved in activation of NF-κB, as well as lack of effects on other transcription factors which cooperate with NF-κB in the activation of target genes.

- Palladium, but not cobalt, platinum, or vanadium, attenuates the neutrophil-activating and -mobilizing properties of the chemoattractants, C5a and IL-8, both

of which are critical components of the host innate immune response. C5a is generated during complement activation and is a potent chemoattractant for neutrophils, monocytes and macrophages (Hopken *et al*, 1996). IL-8 is produced by epithelial cells, monocytes, macrophages and neutrophils and possesses selective chemotactic activity for neutrophils. This represents a previously undocumented mechanism by which exposure to a heavy metal may compromise innate host defences.

- Palladium also neutralizes the pore-forming action of the pneumococcal toxin, pneumolysin, resulting in attenuation of toxin-mediated $Ca^{2+}$ influx, with consequent attenuation of nuclear translocation of NF-κB proteins and production of IL-8. Because many different bacterial pathogens produce cholesterol-binding, pore-forming toxins (Andrew *et al*, 2000), exposure to palladium may favour microbial persistence in the airways, possibly predisposing to pneumococcal infection.

During occupational, as well as environmental, exposure to the metals, inhalation occurs over an extended period and the first cells to come into contact with the inhaled matter are alveolar macrophages and respiratory epithelium. It has been demonstrated that production of GM-CSF, IL-6, IL-1β, TNF-α, IL-8, and MCP-1 by alveolar macrophages is increased after exposure to particulate air pollution matter (Goto *et al,* 2004). Furthermore, exposure to air pollution causes a systemic inflammatory response, subsequently leading to bone marrow stimulation and the release of granulocytes into the circulation (Terashima *et al,*1997, Tan *et al,* 2000) . Functional studies showed that these immature granulocytes are less deformable and less chemotactic, and migrate less efficiently to the sites of inflammation (Van Eeden *et al*, 1997; Van Eeden *et al*, 1999). However, neutrophils will eventually reach the airways at sites of inflammation and come into contact with the inhaled particulate matter containing the metals. Apart from contact between phagocytes and inhaled pollutants within the respiratory tract, neutrophils might also encounter metals in the circulation from which they are transported by blood proteins to various tissues (Vanadium, 2001).

Taken together with previous published findings (Theron *et al,* 2004; Ramafi *et al,* 2004), the results of the current studies appear to demonstrate that the primary consequence of the interaction of $Co^{2+}$, $Pd^{2+}$, $Pt^{2+}$ and $V^{2+-4+}$ with human neutrophils is to increase the reactivity, as opposed to the generation of reactive oxidant species generated by these cells, presumably by functioning as catalysts of oxidation/reduction reactions. This clearly presents the potential threat of oxidant-mediated toxicity and carcinogenesis. Unlike the other test metals, $Pd^{2+}$ may compromise innate host defences by inactivating host- and bacterial-derived proteins with neutrophil activating/ mobilizing properties.

# Chapter 6

# References

Adachi A, Asai K, Koyama Y, Matsumoto Y, Kobayashi T. Vanadium content of cigarettes. *Bulletin of Environmental Contamination and Toxicology.* 1998; **61**:276-280.

Adami G, Smarelli D, Martinelli B, Acquavita A, Reisenhofer E. Cobalt blood levels after total hip replacement (THR): a new follow-up study in Trieste (Italy). *Annali de Chimica*. 2003; **93**:1-10.

Agius RM, Nee J, McGovern B, Robertson A. Structure activity hypotheses in occupational asthma caused by low molecular weight substances. *Annals of Occupational Hygiene*. 1991; **35**:129-137.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature*. 2001; **413**:732-738.

Andela VB, Gingold BI, Souza MD, O'Keefe RJ, Puzas EJ, Schwartz EM, Rosier RN. Clinical relevance of increased retinoid and cAMP transcriptional programs in tumor cells rendered non-malignant by dominant negative inhibition of NFκB. *Cancer Letters*. 2003; **194**:37-43.

Andersen JM, Myhre O, Aarnes H, Vestad TA, Fonnum F. Identification of the hydroxyl radical and other reactive oxygen species in human neutrophil granulocytes exposed to a fragment of the amyloid beta peptide. *Free Radical Research*. 2003; **37**:269-279.

Anderson MT, Staal FJT, Gitler C, Herzenberg LA, Herzenberg LA. Separation of oxidant-initiated and redox-regulated steps in the NF-κB signal transduction pathway. *Proceedings of the National Academy of Science, USA.* 1994; **91**:11527-11531.

Anderson R, Lukey PT, Naudé SPE, Jooné G. Benoxaprofen: A pro-oxidant anti-inflammatory drug. *Agents and Actions.* 1984; **14**:238-246.

Anderson R, Steel HC, Tintinger GR. Inositol 1,4,5-triphosphate-mediated shuttling between intracellular stores and the cytosol contributes to the sustained elevation in cytosolic calcium in FMLP-activated human neutrophils. *Biochemical Pharmacology*. 2005; **69**:1567-1575.

Anderson R, Visser SS, Ramafi G, Theron AJ. Accelerated resequestration of cytosolic calcium and suppression of pro-inflammatory activities of human neutrophils by CGS 21680 *in vitro*. *British Journal of Pharmacology.* 2000; **130**:717-724.

Andrew PW, Mitchell TJ, Morgan P, Gilbert TJC. Relationship of structure to function in pneumolysin. In: Tomasz A (ed). *Streptococcus pneumoniae: Molecular Biology & Mechanisms of Disease.* New York: Mary Ann Liebert Inc. 2000; 271-286.

Aruoma OI. Free radicals, oxidants and antioxidants: trend towards the year 2000 and beyond. In: Aruoma OI, Haliwall B (eds). *Molecular Biology of Free Radicals in Human Diseases*. 1998; 15.

Asehnoune K, Strassheim D, Mitra S, Kim JY, Abraham E. Involvement of reactive oxygen species in toll-like receptor 4-dependant activation of NF-κB. *Journal of Immunology*. 2004; **172**:2522-2529.

Ayub K, Hallett MB. $Ca^{2+}$ influx shutdown during neutrophil apoptosis: importance and possible mechanism. *Immunology*. 2004; **111**:8-11.

Baetu TM, Kwon H, Sharma S, Grandvaux N, Hiscott J. Disruption of NF-kB signalling reveals a novel role for NF-κB in the regulation of TNF-related apoptosis-inducing ligand expression. *Journal of Immunology* 2001; **167**:3164-3173.

Baeuerle PA, Baltimore D. NF-κB: Ten years after. *Cell*. 1996; **87**:13-20.

Baeuerle PA, Henkel T. Function and Activation of NF-κB in the Immune System. *Annual Review of Immunology*. 1994; **12**:141-179.

Baker DB, Gann PH, Brooks SM, Gallagher J, Bernstein IL. Cross-sectional study of platinum salts sensitization among precious metals refinery workers. *American Journal of Industrial Medicine.* 1990; **18**:653-664.

Balachandran P, Hollingshead SK, Paton J, Briles DE. The autolytic enzyme LytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin. *Journal of Bacteriology*. 2001; **183**:3108-3116.

Baldwin AS. The NF-κB and IκB proteins: New discoveries and insights. *Annual Review of Immunology*. 1996; **14**:649-681.

Ballach HJ. Ozone and heavy metals from automobile catalytic converters. *Environmental and Scientific Pollution Research*. 1997; **4**:131-239.

Barceloux DG. Cobalt. *Journal of Clinical Toxicology*. 1999a; **37**:201-206.

Barceloux DG. Vanadium. *Journal of Clinical Toxicology*. 1999b; **37**:265-278.

Barnes PJ, Karin M. Nuclear factor-κB – a pivotal transcription factor in chronic inflammatory diseases. *New England Journal of Medicine.* 1997; **336**:1066-1071.

Bassett DJ, Elbon-Copp C, Ishii Y, Barrachlough-Mitchell H, Yang H. Lung tissue neutrophil content as a determinant of ozone-induced injury. *Journal of Toxicology and Environmental Health Part A*. 2000; **60**:513-520.

Bekay RE, Avarez M, Monteseirin J, Alba G, Chacón P, Vega A, Martin-Nieto J, Jiménez J, Pintado E, Bedoya FJ, Sobrino F. Oxidative stress is a critical mediator of the

angiotensin II signal in human neutrophils: involvement of mitogen-activated protein kinase, calcineurin, and the transcription factor NF-κB. *Blood.* 2003; **102**:662-671.

Belaaouaj A, McCarthy R, Baumann M, Gao Z, Ley TJ, Abraham SN, Shapiro SD. Mice lacking neutrophil elastase reveal impaired host defense against gram-negative bacterial sepsis. *Nature Medicine.* 1998; **4**:615-618.

Benton G. Degranulation. In: Gallin JI, Snyderman R (eds). *Inflammation: Basic Principles and Clinical Correlates*. Raven Press, Philadelphia, 2002; 703-719.

Bergman A, Svedberg U, Nilsson E. Contact urticaria with anaphylactic reactions caused by occupational exposure to iridium salt. *Contact Dermatitis*. 1995; **32**:14-17.

Biagini RE, Moorman WJ, Lewis TR, Bernstein IL. Ozone enhancement of platinum asthma in a primate model. *American Review of Respiratory Disease*. 1986; **134**:719-725.

Blackwell TS, Blackwell TR, Christman JW. Impaired activation of nuclear factor-κB in endotoxin-tolerant rats is associated with down-regulation of chemokine gene expression and expression and inhibition of neutrophilic lung inflammation. *Journal of Immunology*. 1997; **158**:5934-5940.

Blackwell TS, Blackwell TR, Holden EP, Christman BW, Christman JW. In vivo antioxidant treatment suppresses nuclear factor-kappa B activation and neutrophilic lung inflammation. *Journal of Immunology*. 1996; **157**:1630-1637.

Bolm-Audorff U, Bienfait HG, Burkhard J, Bury AH, Merget R, Pressel G, Schultze-Werninghaus G. Prevalence of respiratory allergy in a platinum refinery. *International Archives of Occupational and Environmental Health*. 1992; **64**:257-260.

Boscolo P, Giampaolo LD, Reale M, Castellani ML, Volpe AR, Carmignani M, Ponti J, Paganelli R, Sabbioni E, Conti P, Giocchino MD. Different effects of Platinum, Palladium, and Rhodium salts on lymphocyte proliferation and cytokine release. *Annals of Clinical and Laboratory Science*. 2004; **34**:299-306.

Bose RN. Biomolecular targets for platinum antitumor drugs. *Mini Review in Medical Chemistry.* 2002; **2**:103-111.

Bose S, Kar N, Maitra R, DiDonato JA, Banerjee AK. Temporal activation of NF-κB regulates an interferon-independent innate antiviral response against cytoplasmic RNA viruses. *Proceedings of the National Academy of Sciences of the USA.* 2003; **100**:10890-10895.

Bowie A, O'Neill LAJ. Oxidative stress and nuclear factor-κB activation. *Biochemical Pharmacology*. 2000; **59**:13-23.

Britigan BE, Coffman TJ, Buettner GR. Spin trapping evidence for the lack of significant hydroxyl radical production during the respiration burst of human phagocytes using a spin adduct resistant to superoxide-mediated destruction. *Journal of Biological Chemistry*. 1990; **265**:2650-2656.

Brittigan BE, Hassett DJ, Rosen GM, Hamill DR, Cohen MS. Neutrophil degranulation inhibits potential hydroxyl-radical formation. Relative impact of myeloperoxidase and lactoferrin release on hydroxyl-radical production by iron-supplemented neutrophils assessed by spin-trapping techniques. *Biochemical Journal*. 1989; **264**:447-455.

Brock T, Stopford W. Bioaccessibility of metals in human health risk assessment: Evaluating risk from exposure to cobalt compounds. *Journal of Environmental Monitoring*. 2003; **5**:71N-76N.

Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF. Neutrophils in development of multiple organ failure in sepsis. *The Lancet*. 2006; **368**:157-169.

Burg ND, Phillinger MH. Short analytical review. The neutrophil: Function and regulation in innate humoral Immunity. *Clinical Immunology*. 2001; **99**:7-17.

Calverley AE, Rees D, Dowdeswell RJ. Allergy to complex salts of platinum in refinery workers: prospective evaluation of IgE and Phadiotop status. *Clinical and Experimental Allergy*. 1997; **29**:703-711.

Calverley AE, Rees D, Dowdeswell RJ, Linnett PJ, Kielkowski D. Platinum salt sensitivity in refinery workers: incidence and effects of smoking and exposure. *Occupational and Environmental Medicine*. 1995; **52**:661-666.

Carmichael AJ. Reaction of vanadyl with hydrogen peroxide. An ESR and spin trapping study. *Free Radical Research Communications*. 1990; **10**:37-45.

Cassatella MA. Neutrophil-derived proteins: Selling cytokines by the pound. *Advances in Immunology.* 1999; **73**:369-509.

Castro-Alcaraz S, Miskolci V, Kalasapudi B, Davidson D, Vancurova I. NF-κB regulation in human neutrophils by nuclear IκBα: Correlation to apoptosis. *Journal of Immunology*. 2002; **169**:3947-3953.

Catelas I, Petit A, Zukor DJ, Antoniou J, Huk OL. TNF-α secretion and macrophage mortality induced by cobalt and chromium ions in vitro - qualitative analysis of apoptosis. *Biomaterials*. 2003; **24**:383-391.

Chen F, Demers LM, Vallyathan V, Ding M, Lu Y, Castranova V, Shi X. Vanadate induction of NF-κB involves IκB kinase β and SAPK/ERK kinase 1 in macrophages. *Journal of Biological Chemistry*. 1999; **274**:20307-20312.

Chen X, Kandasamy K, Srivastava RK. Differential roles of RelA (p65) and c-Rel subunits of nuclear factor κB in tumor necrosis factor-related apoptosis-inducing ligand signalling. *Cancer Research*. 2003; **63**:1059-1066.

Chen X-M, O'Hara SP, Nelson JB, Splinter PL, Small AJ, Tietz PS, Limper AH, LaRusso NF. Multiple TLRs are expressed in human cholangiocytes and mediate host epithelial defense responses to *Cryptosporidium parvum* via activation of NF-κB. *Journal of Immunology.* 2005; **175**:7447-7456.

Cheng FC, Jen JF, Tsai TH. Hydroxyl radical in living systems and its separation methods. *Journal of Chromatography*: *B, Analytical Technology in Biomedical and Life Sciences*. 2002; **781**:481-496.

Chong IW, Shi MM, Love JA, Christiani DC, Paulauskis JD. Regulation of chemokine mRNA expression in a rat model of vanadium-induced pulmonary inflammation. *Inflammation*. 2000; **24**:505-517.

Christensen JM, Poulsen OM. A 1982-1992 surveillance programme on Danish pottery painters. Biological levels and health effects following exposure to soluble or insoluble cobalt compounds in cobalt blue dyes. *Science of the Total Environment*. 1994; **150**:201-207.

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

Cockeran R, Anderson A, Feldman C. Pneumolysin in the immunopathogenesis and treatment of pneumococcal disease. *Expert Review in Anti-Infective Therapy*. 2003; **1**:231-239.

Cockeran R, Anderson R, Feldman C. The role of pneumolysin in the pathogenesis of *Streptococcus pneumoniae* infection. *Current Opinion in Infectious Diseases*. 2002a; **15**:235-239.

Cockeran R, Durandt C, Feldman C, Mitchell TJ, Anderson R. Pneumolysin activates the synthesis and release of Interleukin-8 by human neutrophils in vitro. *Journal of Infectious Diseases*. 2002b; **186**:562-565.

Cockeran R, Steel HC, Mitchell TJ, Feldman C, Anderson R. Pneumolysin potentiates production of prostaglandin $E_2$ and leukotriene $B_4$ by human neutrophils. *Infection and Immunity.* 2001a; **69**:3494-3496.

Cockeran R, Theron AJ, Steel HC, Matlola NM, Mitchell TJ, Feldman C, Anderson R. Proinflammatory interactions of pneumolysin with human neutrophils. *Journal of Infectious Diseases* 2001b; **183**:604-611.

Coeshott C, Ohnemus C, Pilyavskaya A, Ross S, Wieczorek M, Kroona H, Leimer AH, Cheronis J. Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated or purified proteinase 3. *Proceedings of National Academy of Science USA*. 1999; **96**:6261-6266.

Cohen MD, McManus TP, Yang Z, Qu Q, Schlesinger RB, Zelikoff JT. Vanadium affects macrophage interferon-$\gamma$-binding and –inducible responses. *Toxicology and Applied Pharmacology*. 1996; **138**:110-120.

Cohen MS, Britigan BE, Hassett DJ, Rosen GM. Phagocytes, $O_2$ reduction, and hydroxyl radical. *Review of Infectious Diseases.* 1988; **10**:1088-1096.

Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood.* 1992; **80**:2012-2020.

Contini P, Ghio M, Merlo A, Poggi A, Indiveri F, Puppo F. Apoptosis of antigen-specific T lymphocytes upon the engagement of CD8 by soluble HLA class I molecules is Fas ligand/Fas mediated: Evidence for the involvement of $p56^{lck}$, calcium calmodulin kinase II, and calcium-independent protein kinase C signaling pathways and for NF-κB and NF-AT nuclear translocation. *Journal of Immunology.* 2005; **175**:7244-7254.

Cooper NR. Biology of the complement system. In: Gallin JI, Snyderman R (eds). *Inflammation. Basic principles and clinical correlates*. 1999; 281-315.

Cromwell O, Pepys J, Parish WE, Hughes EG. Specific IgE antibodies to platinum salts in sensitized workers. *Clinical Allergy*. 1979; **9**:109-117.

Daenen M, Rogiers P, Van de Walle C, Rochette F, Demedts M, Nemery B. Occupational asthma caused by palladium. *European Respiratory Journal*. 1999; **13**:213-216.

Dahlgren C, Karlsson A. Respiratory burst in human neutrophils. *Journal of Immunological Methods*. 1999; **232**:3-14.

Deva R, Shankaranarayanan P, Ciccoli R, Nigam S. Candida albicans induces selectively transcriptional activation of cyclooxygenase-2 in HeLa cells: pivotal role of Toll-like receptors, p38 mitogen-activated protein kinase, and NF-κB. *Journal of Immunology*. 2003; **171**:3047-3055.

Dikalov SI, Mason RP. Spin trapping of polyunsaturated fatty acid-derived peroxyl radicals: reassignment to alkoxyl radical adducts. *Free Radical and Biological Medicine*. 2001; **30**:187-197.

Dolmetsch RE, Lewis RS, Goodnow GG, Healy JI. Differential activation of transcriptional factors induced by $Ca^{2+}$ response amplitude and duration. *Nature*. 1997; **386**:855-858.

Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eospinophilic asthma; importance and possible mechanisms. *Thorax*. 2002; **57**:643-648.

Drasch G, Muss C, Roider G. Gold and palladium burden from dental restoration metals. *Journal of Trace Elements and Medical Biology*. 2000; **41**:71-75.

Ear T, Cloutier A, McDonald PP. Constitutive nuclear expression of the IκB kinase complex and its activation in human neutrophils. *Journal of Immunology*. 2005; **175**:1834-1842.

Ellson CD, Gobert-Gosse S, Anderson KE, Davidson K, Erdjument-Bromage H, Tempst P, Thuring JW, Cooper MA, Lim ZY, Holmes AB, Gaffney PRJ, Coadwell J, Chilvers ER, Hawkins PT, Stephens LR. Ptdlns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of $p40^{phox}$. *Nature Cell Biology*. 2001; **3**:679-682.

Feldman C, Mitchell TJ, Andrew PW, Boulnois GJ, Read RC, Todd HC, Cole PJ, Wilson R. The effect of *Streptococcus pneumoniae* pneumolysin on human respiratory epithelium in vitro. *Microbial Pathogenesis*. 1990; **9**:275-284.

Fibbe WE, Pruijt JF, van Kooyk Y, Figdor CG, Opdenakker G, Willemze R. The role of metalloproteinases and adhesion molecules in interleukin-8-induced stem-cell mobilization. *Seminars in Hematology*. 2000; **37**:19-24.

Flohé L, Brigelius-Flohé R, Saliou C, Traber MG, Packer L. Redox regulation of NF-κB activation. *Free Radical Biology and Medicine.* 1997; **22**:1115-1126.

Fu X, Kao JL, Bergt C, Kassim SY, Huq NP, d'Avignon A, Parks WC, Mecham RP, Heinecke JW. Oxidative cross-linking of tryptophan to glycine restrains matrix metalloproteinase activity: specific structural motifs control protein oxidation. *Journal of Biological Chemistry.* 2004; **279**:6209-6212.

Gagliardo R, Chanez P, Mathieu M, Bruno A, Costanzo G, Gougat C, Vachier I, Bousquet J, Bonsignore G, Vignolia AM. Persistant activation of nuclear factor-κB signalling pathway in severe uncontrolled asthma. *American Journal of Respiratory and Critical Care Medicine*. 2003; **168**:1190-1198.

Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J. Serine phosphorylation of insulin receptor substrate 1 by inhibitor-κB kinase complex. *Journal of Biological Chemistry*. 2002; **277**:48115-48121.

Geiszt M, Kapus A, Nemet K, Farkas L, Ligeti E. Regulation of capacitative $Ca^{2+}$ influx in human neutrophil granulocytes. Alterations in chronic granulomatous disease. *Journal of Biological Chemistry*. 1997; **272**:26471-26478.

Goebeler M, Roth J, Brocker EB, Sorg C, Schulze-Osthoff K. Activation of nuclear factor- kappa B and gene expression in human endothelial cells by the common haptens nickel and cobalt. *Journal of Immunology*. 1995; **155**:2459-2467.

Goering PL. Platinum and related metals: palladium, iridium, osmium, rhodium and ruthenium. In: Sullivan JB and Krieger GR (eds). *Clinical Principles of Environmental Health.* Baltimore: Lippincott, Williams & Wilkins , 1992; 874-881.

Goto Y, Ishii H, Hogg JC, Shih C, Yatera K, Vincent R, van Eeden S. Particulate matter air pollution stimulates monocytes release from bone marrow. *American Journal of Respiratory Critical Care Medicine,* 2004; **170**: 891-897.

Gougerot-Pocidalo MA, el Benna J, Elbim C, Chollet-Martin S, Dang MC. Regulation of human neutrophil oxidative burst by pro- and anti-inflammatory cytokines. *Journal de la Societe de Biologie*. 2002; **196**:37-46.

Grabowski GM, Paulauskis JD, Godleski JJ. Mediating phosphorylation events in the vanadium-induced respiratory burst of alveolar macrophages. *Toxicology and Applied Pharmacology*. 1999; **156**:170-178.

Grinstein S, Furuya W, Lu DJ, Mills GB. Vanadate stimulates oxygen consumption and tyrosine phosphorylation in electro-permeabilized human neutrophils. *Journal of Biological Chemistry.* 1990; **265**:318-327.

Grynkiewicz G, Poenie M, Tsien RY. A new generation of $Ca^{2+}$ indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry.* 1985; **260**:3440-3550.

Hackel H, Miller K, Elsner P, Burg G. Unusual combined sensitization to palladium and other metals. *Contact Dermatitis*. 1991; **24**:131-157.

Harding AJ. Cobalt. In: Robinson I (ed). *SAMI* (*South Africas Mineral Industry*). *Nonferrous metals and minerals*. Department of Minerals and Energy. 2004; 90-94.

Haselmayer P, Tenzer S, Kwon BS, Jung G, Schild H, Radsak MP. Herpes virus entry mediator synergizes with Toll-like receptor mediated neutrophil inflammatory responses. *Immunology.* 2006; **119**:404-411.

Henderson LM, Chappell JB. NADPH oxidase of neutrophils. *Biochemica et Biophysica acta*. 1996; **1273**:87-107.

Henriksen PA, Hitt M, Xing Z, Wang J, Haslett C, Riemersma RA, Webb DJ, Kotelevtsev YV, Sallenave JM. Adenoviral gene delivery of elafin and secretory leukocyte protease

inhibitor attenuates NF-κB-dependant inflammatory responses of human endothelial cells and macrophages to atherogenic stimuli. *Journal of Immunology*. 2004; **172**:4535-4544.

Hoch RC, Schraufstätter IU, Cochrane CG. In vivo, in vitro, and molecular aspects of interleukin-8 and the interleukin-8 receptors. *Journal of Laboratory and Clinical Medicine*. 1996; **128**:134-145.

Holloway AF, Rao S, Shannon MF. Regulation of cytokine gene transcription in the immune system. *Molecular Immunology*. 2001; **38**:567-580.

Holmsen HE, Storm E, Day HJ. Determination of ATP and ADP in blood platelets: a modification of the firefly luciferase assay for plasma. *Analytical Biochemistry.* 1972; **46**:481-501.

Honen BN, Saint DA, Laver DR. Suppression of calcium sparks in rat ventricular myocytes and inhibition of sheep cardiac RyR channels by EPA, DHA and oleic acid. *Journal of Membrane Biology*. 2003; **196**:95-103.

Hopken UE, Lu B, Gerard NP, Gerard C. The C5a chemoattractant receptor mediates mucosal defence to infection. *Nature.* 1996; **383**:86-89.

Hostỳnek JJ, Hinz RS, Lorence CR, Price M, Guy RH. Metals and the skin. *Critical Review in Toxicology*. 1993; **23**:171-235.

Houldsworth S, Andrew PW, Mitchell TJ. Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes. *Infection and Immunity*. 1994; **62**:1501-1503.

Huang C, Ding M, Li J, Leonard SS, Rojanasakul Y, Castranova V, Vallyathan V, Ju G, Shi X. Vanadium – induced nuclear factor of activated T cells activation through hydrogen peroxide. *Journal of Biological Chemistry*. 2001; **276**:22397-22403.

Huang YC, Wu W, Ghio AJ, Carter JD, Silbajoris R, Devlin RB, San JM. Activation of EGF receptor mediates pulmonary vasoconstriction induced by residual oil fly ash. *Experimental Lung Research*. 2002; **28**:19-28.

Hughes EJ. Medical surveillance of platinum refinery workers. *Journal of the Society of Occupational Medicine*. 1980; **30**:27-30.

Huxford T, Huang DB, Malek S, Ghosh G. The crystal structure of the IkappaBalpha/NF-kappaB complex reveals mechanisms of NF-kappaB inactivation. *Cell*. 1998; **95:**759-770.

Irsigler GB, Visser PJ, Spangenberg PAL. Asthma and Chemical Bronchitis in Vanadium Plant Workers. *American Journal of Industrial Medicine*. 1999; **35**:366-374.

Janssen YMW, Sen CK. Nuclear factor κB activity in response to oxidants and antioxidants. *Methods in Enzymology*. 1999; **300**:363-374.

Jarvis KE, Parry SJ, Piper JM. Temporal and spectral studies of autocatalyst-derived platinum, rhodium, and palladium and selected vehicle-derived trace elements in the environment. *Environmental and Scientific Technology.* 2001; **35**:1031-1036.

Jiang Z, Zamanian-Daryoush M, Nie H, Silva AM, Williams BRG, Li X. Poly(dI.dC)-induced Toll-like receptor 3 (TLR3)-mediated activation of NF-κB and MAP kinase is through an interleukin-1 receptor-associated kinase (IRAK)-independent pathway employing the signalling components TLR-3-TRAF6-TAK1-TAB2-PKR. *Journal of Biological Chemistry*. 2003; **278**:16713-16719.

Jiménez LA, Thompson J, Brown DA, Rahman I, Antonicelli F, Duffin R, Drost EM, Hay RT, Donaldson K, MacNee W. Activation of NF-κB by $PM_{10}$ occurs via an iron-mediated mechanism in the absence of IκB Degradation. *Toxicology and Applied Pharmacology* 2000; **166**:101-110.

Jones MR, Simms BT, Lupa MM, Kogan MS, Mizgerd JP. Lung NF-κB activation and neutrophil recruitment require IL-1 and TNF receptor signalling during pneumococcal pneumonia. *Journal of Immunology.* 2005; **175**:7530-7535.

Jung KS, Park HS. Evidence of neutrophil activation in occupational asthma. *Respirology*. 1999; **4**:303-306.

Kahlenberg JM, Lundberg KC, Kertesy SB, Qu Y, Dubyak GR. Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF-κB-driven protein synthesis. *Journal of Immunology*. 2005; **175**:7611-7622.

Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: The control of NF-κB activity. *Annual Review of Immunology*. 2000; **18**:621-663.

Keel M, Ungethum U, Steckholzer U, Niederer E, Hartung T, Trentz O, Ertel W. Interleukin-10 counter-regulates proinflammatory cytokine-induced inhibition of neutrophil apoptosis during severe sepsis. *Blood*. 1997; **90**:3356-3363.

Keller RJ, Coulombe RA Jr, Sharma RP, Grover TA, Piette LH. Importance of hydroxyl radical in the vanadium-stimulated oxidation of NADH. *Free Radical Biology and Medicine*. 1989; **6**:15-22.

Kettritz R, Choi M, Rolle S, Wellner M, Luft FC. Integrins and cytokines activate nuclear transcription factor-κB in human neutrophils. *Journal of Biological Chemistry*. 2004; **23**:2657-2665.

Kielhorn J, Melber C, Keller D, Mangelsdorf I. Palladium - a review of exposure and effects to human health. *International Journal of Hygiene and Environmental Health*. 2002; **205**:417-432.

Klebanoff SJ. Oxygen metabolites from phagocytes. In: Gallin JI, Snyderman R (eds). *Inflammation. Basic principles and clinical correlates*. 1999; 721-768.

Klein-Patel ME, Diamond G, Boniotto M, Saad S, Ryan LK. Inhibition of beta-defensin gene expression in airway epithelial cells by low doses of residual oil fly ash is mediated by vanadium. *Toxicological Sciences*. 2006; **92**:115-125.

Komatsu W, Ishihara K, Murata M, Saito H, Shinohara K. Docosahexaenoic acid suppresses nitric oxide production and inducible nitric oxide synthase expression in interferon-gamma plus lipopolysaccharide-stimulated murine macrophages by inhibiting the oxidative stress. *Free Radical Biology and Medicine* 2003; **34**:1006-1016.

Krejsa CM, Nadler SG, Esselsteyn JM, Kavanagh TJ, Ledbetter JA, Schieven GL. Role of oxidative stress in the action of vanadium phosphotyrosine phosphatase inhibitors. *American Society for Biochemistry and Molecular Biology, Inc*. 1997; **272**:11541-11549.

Kuhns DB, Gallin JI. Increased cell-associated IL-8 in human exudative and A23187-treated peripheral blood in neutrophils. *Journal of Immunology.* 1995; **154**:6556-6562.

Kuhns DB, Young HA, Gallin EK, Gallin JI. $Ca^{2+}$-dependent production and release of IL-8 in human neutrophils. *Journal of Immunology.* 1998; **161**:4332-4339.

Kweyama N. Vanadium. In: Robinson I (ed). *SAMI (South Africas Mineral Industry).* Departement of Minerals and Energy. 2004; 142-145.

La Grutta S, Gagliardo R, Mirabella F, Pajno GB, Bonsignore G, Bousquet J, Bellia V, Vignola AM. Clinical and biological heterogeneity in children with moderate asthma. *American Journal of Respiratory and Critical Care Medicine*. 2003; **167**:1490-1495.

Lamhamedi-Cherradi S-E, Zheng S, Hilliard BA, Xu L, Sun J, Alsheadat S, Liou H-C, Chen YH. Transcriptional regulation of type I diabetes by NF-κB. *Journal of Immunology*. 2003; **171**:4886-4892.

Lau D, Mollnau H, Eiserich JP, Freeman BA, Daiber A, Gehling UM, Brümmer J, Rudolph V, Münzel T, Heitzer T, Meinertz T, Baldus S. Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proceedings of the National Academy of Sciences of the USA*. 2005; **102**:431-436.

Lauwerys R, Lison D. Health risks associated with cobalt exposure - An overview. *Science of the Total Environment*. 1994; **150**:1-6.

Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA. Possible new role for NF-κB in the resolution of inflammation. *Nature Medicine*. 2001; **7**:1291-1297.

Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore N, Hwang DH. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidyl-inositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. *Journal of Biological Chemistry.* 2003; **278**:37041-51.

Leonard S, Gannett PM, Rojanasakul Y, Schwegler-Berry D, Catsranova V, Vallyathan V, Shi X. Cobalt-mediated generation of reactive oxygen species and its possible mechanism. *Journal of Inorganic Biochemistry*. 1998; **70**:239-244.

Levene GM, Calnan CD. Platinum sensitivity: treatment by specific hyposensitization. *Clinical Allergy*. 1971; **1**:75-82.

Lewis JB, Randol TM, Lockwood PE, Wataha JC. Effect of subtoxic concentrations of metal ions on NFκB activation in THP-1 human monocytes. *Journal of Biomedical Materials Research*. 2003; **64A**:217-224.

Linna A, Oksa P, Palmroos P, Roto P, Laippala P, Uitti J. Respiratory health of cobalt production workers. *American Journal of Industrial Medicine*. 2003; **44**:124-132.

Liochev S, Ivanecha E. Vanadyl causes hydroxyl radical mediated degradation of ribose. *Free Radical Research Communictions.* 1991; **14**:334-342.

Liochev S, Ivancheva E, Fridovich I. Effects of vanadate on the oxidation of NADH by xanthine oxidase. *Archives in Biochemistry and Biophysics*. 1989b; **269**:188-193.

Liochev SI, Fridovich I. Hydoxyl radical is not a significant intermediate in the vanadate-stimulated oxidation of NAD(P)H by $O_2$. *Archives in Biochemistry and Biophysics*. 1989a; **275**:40-43.

Liu HC, Chang WH, Lin FH, Lu KH, Tsuang YH, Sun JS. Cytokine and prostaglandin E2 release from leucocytes in response to metal ions derived from different prosthetic materials: an in vitro study. *Artificial Organs*. 1999; **23**:1099-1106.

Liu TZ, Lin TF, Chiu DTY, Tsai KJ, Stern A. Palladium or Platinum exacerbates hydroxyl radical mediated DNA damage. *Free Radical Biology and Medicine*. 1997; **23**:155-166.

Locksley RM, Wilson CB, Klebanoff SJ. Increased respiratory burst in myeloperoxidase-deficient monocytes. *Blood.* 1983; **62**:902-909.

Maihöfner C, Charalambous PM, Bhambra U, Lightfoot T, Geisslinger G, Gooderham NJ. Expression of cyclooxygenase-2 parallels expression of interleukin-1beta, interleukin-6 and NF-kappaB in human colorectal cancer. *Carcinogenesis*. 2003; **24**:665-671.

Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, Kurt-Jones E, Paton SC, Wessels MR, Golenbock DT. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proceedings of the National Academy of Science, USA.* 2003; **100**:1966-1971.

Mapp C, Boschetto P, Miotto D, De Rosa E, Fabbri LM. Mechanism of occupational asthma. *Annals of Allergy and Asthma Immunology*. 1999; **83**:645-664.

Massey D. Acute and Chronic Inflammation. In: Kumar V, Cotran RS, Robbins SL (eds). *Robbins' Basic Pathology*, (6th Edition). 1997; 25-46.

Matute-Bello G, Liles WC, Radella F, Steinberg KP, Ruzinski JT, Jonas M, Chi E, Hudson LD, Martin TP. Neutrophil apoptosis in the acute respiratory distress syndrome. *American Journal of Respiratory Critical Care Medicine*. 1997; **156**:1969-1977.

McDonald PP, Bald A, Cassatella MA. Activation of the NF-κB pathway by inflammatory stimuli in human neutrophils. *Blood*. 1997; **89**:3421-3433.

Mercurio F, Murray BW, Shevchenko A, Bennett BL, Young DB, Li JW, Pascual G, Motiwala A, Zhu H, Mann M, Manning AM. IkappaB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. *Molecular Cell Biology*. 1999; **19**:1526-1538.

Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennet BL, Li J, Youn DB, Barbosa M, Mann M, Manning A, Rao A. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science*. 1997; **278**:818-819.

Merget R, Caspari C, Kulzer R, Breistadt R, Rueckmann A, Schultze-Werninghaus G. The sequence of symptoms, sensitization and bronchial hyperresponsiveness in early

occupational asthma due to platinum salts. *International Archives of Allergy and Immunology*. 1995; **107**:406-407.

Merget R, Rosner G. Evaluation of the health risk of platinum group metals emitted from automotive catalytic converters. *Scientific Total Environment.* 2001; **270**:165-173.

Merget R, Schultze-Werninghaus G, Bode F, Bergmann EM, Zachgo W, Meier-Sydow J. Quantitative skin prick and bronchial provocation test with platinum salt. *British Journal of Industrial Medicine*. 1991; **48**:830-837.

Merget R, Schultze-Werninghaus G, Muthorst T, Friedrich W, Meier-Sydow J. Asthma due to the complex salts of platinum - a cross-sectional survey of workers in a platinum refinery. *Clinical Allergy*. 1988; **18**:569-580.

Midwinter RG, Visser MC, Winterbourn CC. Hypochlorous acid stimulation of the mitogen-activated protein kinase pathway enhances cell survival. *Archives of Biochemistry and Biophysics*. 2001; **394**:13-20.

Miller RA, Britigan BE. Protease-cleaved iron-transferrin augments oxidant-mediated endothelial cell injury via hydroxyl radical formation. *Journal of Clinical Investigation*. 1995; **95**:2491-2500.

Milovic NM, Kostic NM. Interplay of terminal amino groups and coordinating side chains in directing regioselective cleavage of natural peptides and proteins with palladium (II) complexes. *Inorganic Chemistry.* 2002a; **41**:7053-7063.

Milovic NM, Kostic NM. Palladium (II) complexes, as synthetic peptidases, regioselectively cleave the second peptide bond “upstream” from methionine and histidine side chains. *Journal of American Chemistry Society.* 2002b; **124**:4759-4769.

Milovic NM, Kostic NM. Palladium (II) complex as a sequence-specific peptidase: hydrolytic cleavage under mild conditions of X-Pro peptide bonds in X-Pro-Met and X-Pro-His segments. *Journal of American Chemistry Society.* 2003; **125**:781-788.

Minkenberg I, Ferber E. Lucigenin-dependent chemiluminescence as a new assay for NADPH-oxidase activity in particulate fractions of human polymorphonuclear leukocytes. *Journal of Immunological Methods*. 1984; **71**:61-67.

Miskolci V, Castro-Alcaraz S, Nguyen P, Vancura A, Davidson D, Vancurova I. Okadaic acid induces sustained activation of NFκB and degradation of the nuclear IκBα in human neutrophils. *Archives in Biochemical Biophysics*. 2003; **417**:44-52.

Mitchell TJ, Andrew PW. Biological properties of pneumolysin. In: Tomasz A (ed). *Streptococcus pneumoniae*: *Molecular Biology and Mechanism of Disease*. New York: Mary Ann Liebert Inc. 2000; p 279-286.

Mitchell TJ, Andrew PW. Biological properties of pneumolysin. *Microbial Drug Resistance*. 1997; **3**:279-286.

Mizgerd JP, Lupa MM, Kogan MS, Warren HB, Kobzik L, Topulos GP. Nuclear factor-κB p50 limits inflammation and prevents lung injury during *Escherichia coli* pneumonia. *American Journal of Respiratory and Critical Care Medicine*. 2003; **168**:810-817.

Monteseirin J, Bonilla I, Camacho MJ, Conde J, Sobrino F. IgE-dependent release of myeloperoxidase by neutrophils from allergic patients. *Clinical and Experimental Allergy*. 2000; **31**:889-892.

Monteseirin J, Bonilla I, Camacho J, Conde J, Sobrino F. Elevated secretion of myeloperoxidase by neutrophils from asthmatic patients: the effect of immunotherapy. *Journal of Allergy and Clinical Immunology*. 2001; **107**:623-626.

Mufson MA. Streptococcus pneumoniae. In: Mandell GL, Douglas Jr. RG, Bennett JE (eds). *Principles and Practice of Infectious Diseases*. Churchill Livingston Inc., New York. NY. 1990; 1539-1550.

Mukaida N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. *International Journal of Hematology*. 2000; **72**:391-398.

Mukaida N. Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. *American Journal of Physiology - Lung Cellular and Molecular Physiology*. 2003; **284**:L566-L577.

Mukherjee B, Patra B, Mahapatra S, Banerjee P, Tiwari A, Chatterjee M. Vanadium – an element of atypical biological significance. *Toxicology Letters*. 2004; **150**:135-143.

Mukhopadhyay A, Manna SK, Aggarwal BB. Pervanadate-induced nuclear factor-kappaB activation requires tyrosine phosphorylation and degradation of IkappaB-alpha. Comparison with tumor necrosis factor-alpha. *Journal of Biological Chemistry*. 2000; **275**:8549-8555.

Musher DM, Breiman RF, Tomasz A. *Streptococcus pneumoniae*: At the threshold of the 21st century. In: Tomasz A (ed). *Streptococcus pneumoniae: Molecular Biology and Mechanism of Disease*. Mary Ann Liebert Inc. Publishers. 2000; 485-491.

Mussalo-Rauhammaa HH, Salmela SS, Leppanen A, Pyysalo H. Cigarettes as a source of trace and heavy metals and pesticides in man. *Archives of Environmental Health*. 1986; **41**:49-55.

Mwape P, Roberts M, Mokwena E, Tjatjie T. South Africas Mineral Industry, General Review. In: Robinson I (ed). SAMI (*South Africas Mineral Industry*). Departement of Minerals and Energy. 2004; 1-16.

Myhre O, Anderson JM, Aarnes H, Fonnum F. Evaluarion of the probes 2',7'-dichlorofluorescein diacetate, luminol and lucigenin as indicators of reactive oxygen species formation. *Biochemical Pharmacology.* 2003; **65**:1575-1582.

Nadadur SS, Kodavanti UP. Altered gene expression profiles of rat lung in response to an emission particulate and its metal constituents. *Journal of Toxicology and Environmental Health*. 2002; **65**:1333-1350.

Niezborala M, Garnier R. Allergy to complex platinum salts: a historical prospective cohort study. *Occupational and Environmental Medicine*. 1996; **53**:252-257.

Niki Y, Matsumoto H, Suda Y, Otani T, Fujikawa K, Toyama Y, Hisamori N, Nozue A. Metal ions induce bone-resorbing cytokine production through the redox pathway in synoviocytes and bone marrow macrophages. *Biomaterials*. 2003; **24**:1447-1457.

Ohmae T, Hirata Y, Maeda S, Shibata W, Yanai A, Ogura K, Yoshida H, Kawabe T, Omata M. *Helicobacter pylori* activates NF-κB via the alternative pathway in B lymphocytes. *Journal of Immunology.* 2005; **175**:7162-7169.

Oishi K, Sonoda F, Kobayashi S, Iwagaki A, Nagatake T, Matsushima K, Matsumoto K. Role of interleukin-8 (IL-8) and an inhibitory effect of erythromycin on IL-8 release in the airways of patients with chronic airway diseases. *Infection and Immunity.* 1994; **62**:4145-4152.

Ono Y, Nakaji S, Sugawara K, Kumae T. Effects of metals (silver, nickel, cobalt and chromium) on the reactive oxygen species generating capacity of human neutrophils and on the serum opsonic activity. *Nippon Eiseigaku Zasshi*. 1994; **49**:645-653.

Oommen J, Steel HC, Theron AJ, Anderson R. Investigation into the relationship between calyculin A-mediated potentiation of NADPH oxidase activity and inhibition of

store-operated uptake of calcium by human neutrophils. *Biochemical Pharmacology.* 2004; **68**:1721-1728.

Ørbaek P. Allergy to the complex salts of platinum. *Scandinavian Journal of Work, and Environmental Health*. 1982; **8**:141-145.

Ortolan E, Tibaldi EV, Ferranti B, Lavagno L, Garbarino G, Notaro R, Luzzatto L, Malavasi F, Funaro A. CD157 plays a pivotal role in neutrophil transendothelial migration. *Blood*. 2006; **108**:4214-4222.

Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene*. 1999; **18**:6853-6866.

Palladium. http://www.platinuminfo.net/palladium200.html

Palladium. http://www.pearl1.lanl.gov/periodic/elements/46.html

Pandey SK, Chiasson JL, Srivastava AK. Vanadium salts stimulate mitogen-activated protein (MAP) kinases and ribosomal S6 kinases. *Molecular Cell Biochemistry*. 1995; **153**:69-78.

Park HS, Jung HY, Park EY, Kim J, Lee WJ, Bae YS. Cutting Edge: Direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-κB. *Journal of Immunology*. 2004; **173**:3589-3593.

Park JI, Lee MG, Cho K, Park BJ, Chae KS, Buyn DS, Ruy BK, Park YK, Chi SG. Transforming growth factor-beta activates interleukin-6 expression in prostate cancer cells through the synergistic collaboration of the Smad2, p38-NK-kappaB, JNK, and Ras signaling pathways. *Oncogene*. 2003; **22**:4314-4332.

Pastva A, Estell K, Schoeb TR, Atkinson TP, Schwiebert LM. Aerobic exercise attenuates airway inflammatory responses in a mouse model of atopic asthma. *Journal of Immunology*. 2004; **172**: 4520-4526.

Paton JC, Ferrante A. Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. *Infection and Immunity*. 1983; **41**:1212-1216.

Pease JE, Sabroe I. The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. *American Journal of Respiratory Medicine*. 2002; **1**:19-25.

Pierce LM, Alessandrini F, Godleski JJ, Paulauskis JD. Vanadium-induced chemokine mRNA expression and pulmonary inflammation. *Toxicology and Applied Pharmacology*. 1996; **138**:1-11.

Platinum. http://www.pearl1.lanl.gov/periodic/elements/78.html.

Platinum. http://www.inchem.org/documents/ehc/ehc/ehc125.htm.

Poynter ME, Irvin CG, Janssen-Heininger YMW. Rapid activation of nuclear factor-κB in airway epithelium in a murine model of allergic airway inflammation. *American Journal of Pathology*. 2002; **160**:1325-1334.

Pullerits R, Bokarewa M, Johnsson I-M, Verdrengh M, Tarkowski A. Extracellular cytochrome *c*, a mitochondrial apoptosis-related protein, induces arthritis. *Rheumatology*. 2005; **44**:32-39.

Rahman I. Regulation of nuclear factor-κB, activator protein-1, and glutathione levels by tumor necrosis factor-α and dexamethasone in alveolar epithelial cells. *Biochemical Pharmacology.* 2000; **60**:1041-1049.

Ramafi GJ, Theron AJ, Anderson R. Pro-oxidative interactions of Cobalt with human neutrophils. *Inhalation Toxicology*. 2004; **16**:649-655.

Ratner AJ, Hippe KR, Aguila JL, Bender MH, Nelson AL, Weiser JN. Epithelial cells are sensitive detectors of bacterial pore-forming toxins. *Journal of Biological Chemistry.* 2006; **281**:12994-12998.

Raulf-Heimsoth M, Merget R, Rihs HP, Föhring M, Liebers V, Gellert B, Schultze-Werninghaus G, Baur X. T-cell receptor repertoire expression in workers with occupational asthma due to platinum salt. *European Respiratory Journal*. 2000; **16**:871-878.

Robinson JM, Badwey JA. The NADPH oxidase complex of phagocytic leukocytes: a biochemical and cytochemical view. *Histochemistry*. 1995; **103**:163-180.

Romero N, Radi R, Linares E, Augusto O, Detweiler CD, Mason RP, Denicola A. Reaction of human hemoglobin with peroxynitrite: isomerization to nitrate and secondary formation of protein radicals. *Journal of Biological Chemistry*. 2003; **278**:44049-44057.

Sabbioni E, Pozzi G, Pintar A, Cassella L, Garattini S. Cellular retention, cytotoxicity and morphological transformation by vanadium (IV) and vanadium (V) in BALB/3T3 cell lines. *Carcinogenesis*. 1991; **12**:47-52.

Saldiva PH, Clarke RW, Coull BA, Stearns RC, Lawrence J, Murthy GG, Diaz E, Koutrakis P, Suh H, Tsuda A, Godleski JJ. Lung inflammation induced by concentrated ambient air particles is related to particle composition. *American Journal of Respiratory Critical Care Medicine*. 2002; **165**:1610-1617.

Samet JM, Graves LM, Quay J, Dailey LA, Devlin RB, Ghio AJ, Wu W, Bromberg PA, Reed W. Activation of MAPKs in human bronchial epithelial cells exposed to metals. *American Journal of Physiology*. 1998; **275**:L551-L558.

Samet JM, Stonehuerner J, Reed W, Devlin RB, Dailey LA, Kennedy TP, Bromberg PA, Ghio AJ. Disruption of protein tyrosine phosphate homeostasis in bronchial epithelial cells exposed to oil fly ash. *American Journal of Physiology – Lung Cellular and Molecular Physiology*. 1997; **272**:L426-432.

Samuni A, Black CD, Krishna CM, Malech HL, Bernstein EF, Russo R. Hydroxyl radical production by stimulated neutrophils reappraised. *Journal of Biological Chemistry*. 1988; **263**:13797-13801.

Sandor F, Latz E, Re F, Mandell L, Repik G, Golenbock DT, Espevik T, Kurt-Jones EA, Finberg RW. Importance of extra- and intracellular domains of TLR1 and TLR2 in NFκB signaling. *Journal of Cellular Biology*. 2003; **162**:1099-1110.

Santoro GM, Rossi A, Amici C. NF-κB and virus infection: who controls whom. *The EMBO* (*European Molecular Biology Organization) Journal*. 2003; **22**:2552-2560.

Santucci B, Valenzano C, de Rocco M, Cristaudo A. Platinum in the environment: frequency of reactions to platinum-group elements in patients with dermatitis and urticaria. *Contact Dermatitis*. 2000; **43**:333-338.

Sasai M, Oshiumi H, Matsumoto M, Inoue N, Fujita F, Nakanishi M, Seya T. Cutting edge: NF-κB-activating kinase-associated protein 1 participates in TLF3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. *Journal of Immunology.* 2005; **174**:27-30.

Saunders FK, Mitchell TJ, Walker JA, Andrew PW, Boulnois EJ. Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae* does not require a thiol group for *in vitro* activity. *Infection and Immunity*. 1989; **57**:2547-2552.

Savarino L, Granchi D, Ciapetti G, Stea S, Donati ME, Zinghi G, Fontanesi G, Rotini R, Montanaro L. Effects of metal ions on white blood cells of patients with failed total joint arthroplasties. *Journal of Biomedical Materials Research*. 1999; **47**:543-550.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tamaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. *The EMBO* (European Molecular Biology Organization) *Journal* 1998; **17**:1675-1687.

Schins RP, Polat D, Begerow J, Turfeld M, Becker A, Borm PJ. Platinum levels in nasal lavage fluid as a biomarker for traffic related exposure and inflammation in children. *Science of the Total Environment*. 2004; **334-335:**447-455.

Schmidt KN, Traenckner EB-M, Meier B, Baeuerle PA. Induction of oxidative stress by okadaic acid is required for activation of transcription factor NF-κB. *Journal of Biological Chemistry*. 1995; **270**:27136-27142.

Schoonbroodt S, Legrand-Poels S, Best-Belpomme M, Piette J. Activation of the NF-kappaB transcription factor in a T-lymphocytic cell line by hypochlorous acid. *Journal of Biochemistry*. 1997; **321**:777-785.

Segal AW, Abo A. The biochemical basis of the NADPH oxidase of phagocytes. *Trends in Biochemical Sciences.* 1993; **18**:44-48.

Shainkin-Kestenbaum R, Caruso C, Berlyne GM. Vanadium and oxygen free radicals: inhibition of superoxide dismutase and enhancement of hydroxydopamine oxidation. *Elements in Medicine*. 1991; **8**:6-10.

Shi X, Dalal NS. Vanadate-mediated hydroxyl radical generation from superoxide radical in the presence of NADH: Haber-Weiss vs Fenton Mechanism. *Archives of Biochemistry and Biophysics*. 1993; **307**:336-341.

Shishodia S, Shrivastava A, Sodhi A. Cisplatin-induced activation of bone-marrow derived macrophages requires protein tyrosine phosphorylation. *International Journal of Immunopharmacology.* 1997; **19**:683-690.

Sonis ST. The biological role for nuclear factor-kappaB in disease and its potential involvement in mucosal injury associated with anti-neoplastic therapy. *Critical Review of Oral Biological Medicine*. 2002; **13**:380-389.

Spreer A, Kerstan H, Bottcher T, Gerber J, Siemer A, Zysk G, Mitchell TJ, Eiffert H, Nau R. Reduced release of pneumolysin by Streptococcus pneumoniae in vitro and in vivo after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone. *Antimicrobial agents in Chemotherapy*. 2003; **47**(8):2649-2654.

Staal FJT, Anderson MT, Staal GEJ, Herzenberg LA, Gitler C, Herzenberg LA. Redox regulation of signal transduction: Tyrosine phosphorylation and calcium influx. *Proceedings of the National Academy of Science, USA*. 1994; **91**:3619-3622.

Staal FJT, Anderson MT, Herzenberg LA. Redox regulation of activation of NF-κB transcription factor complex: effects of N-acetylcysteine. *Methods in Enzymology*. 1995; **252**:168-174.

Staal FJT, Roederer M, Herzenberg LA, Herzenberg LA. Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. *Proceedings of the National Academy of Science, USA.* 1990; **87**:9943-9947.

Stanic AK, Bezbradica JS, Park J-J, Matsuki N, Mora AL, Van Kaer L, Boothby MR, Joyce S. NK-κB controls cell fate specification, survival, and molecular differentiation of immunoregulatory natural T lymphocytes. *Journal of Immunology*. 2004; **172**:2265-2273.

Stejskal J, Stejskal Vera DM. The role of metals in autoimmunity and the link to neuroendocrinology. *Neuroendocrinology Letters*. 1999; **20**:351-364.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witzum J. Beyond Cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *The new England Journal of Medicine.* 1989; **320**: 915-924.

Strassheim D, Asehnoune K, Park JS, Kim JY, He Q, Richter D, Mitra S, Arcaroli J, Kuhn K, Abraham E. Modulation of bone marrow-derived neutrophil signaling by $H_2O_2$: disparate effects on kinases, NF-κB, and cytokine expression. *American Journal of Physiology and Cell Physiology*. 2004; **286**:C683-C692.

Sultana C, Shen Y, Johnson C, Kalra VK. Cobalt chloride-induced signalling in endothelium leading to the augmented adherence of sickle red blood cells and transendothelial migration of monocyte-like HL-60 cells is blocked by PAF-receptor antagonist. *Journal of Cell Physiology*. 1999; **179** (1):67-78.

Suzuki YJ, Mizuno M, Packer L. Signal transduction for nuclear factor-kappa B activation. Proposed location of antioxidant-inhibitable step. *Journal of Immunology.* 1994; **153**:5008-5015.

Suzuki YJ, Packer L. Redox regulation of DNA-protein interactions by biothiols. *Methods in Enzymology*. 1995; **252**:175-186.

Swennen B, Buchet JP, Stanescu D, Lison D, Lauwerys R. Epidemiological survey of workers exposed to cobalt oxides, cobalt salts, and cobalt metal. *British Journal of Industrial Medicine*. 1993; **50:**835-842.

Szulakowski P, Crowther AJL, Jiménez LA, Donaldson K, Mayer R, Leonard TB, MacNee W, Drost EM. The effect of smoking on the transcriptional regulation of lung inflammation in patients with chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine.* 2006; **174**:41-50.

Tan WC, Qiu D, Liam BL, Ng TP, Lee SH, van Eeden SF, D;Yachkova Y, Hogg JC. The human bone marrow response to acute air pollution caused by forest fires. *American Journal of Respiratory Critical Care Medicine.* 2000; **161**: 1213-1217.

Theron AJ, Ramafi GJ, Feldman C, Grimmer H, Visser SS, Anderson R. Effects of Platinum and Palladium ions on the production and reactivity of neutrophil-derived reactive oxygen species. *Free Radical Biology and Medicine*. 2004; **11**:1408-1417.

Thomson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. *European Respiratory Journal*. 2004; **24**:822-833.

Terashima T, Wiggs B, English D, Hogg JC, van Eeden SF. Phagocytosis of small carbon particles (PM10) by alveolar macrophages stimulates the release of polymorphonuclear leukocytes from bone marrow. *American Journal of Respiratory Critical Care Medicine.* 1997; **155**: 1441-1447.

Tintinger GR, Steel HC, Anderson R. Taming the neutrophil: Calcium clearance and influx mechanisms as novel targets for pharmacological control. *Clinical and Experimental Immunology.* 2005; **141**:191-200.

Toya T, Fukuda K, Takaya M, Arito H. Lung lesions induced by intratracheal instillation of vanadium pentoxide powder in rats. *Industrial Health*. 2001; **39**:8-15.

Tran QK, Ohashi K, Watanabe H. Calcium signalling in endothelial cells. *Cardiovascular Research.* 2000; **48**:13-22.

Trudel S, Paquet MR, Grinstein S. Mechanism of vanadate-induced activation of tyrosine phosphorylation and the respiratory burst in HL60 cells. *Biochemical Journal*. 1991; **276**:611-619.

Tsujimura H, Tamura T, Kong HJ, Nishiyama A, Ishii KJ, Klinman DM, Ozato K. Toll-like receptor 9 signaling activates NF-κB through IFN regulatory factor-8/IFN consensus sequence binding protein in dendritic cells. *Journal of Immunology.* 2004; **172**:6820-6827.

Vanadium. Air Quality Guidelines – second edition. WHO Regional Office for Europe, Copenhagen, Denmark. 2000; Chapter 6.12: 1-9.
Accessed on: http://www.euro.who.int/document/aiq/6.12vanadium.pdf.

Vanadium. Environment Agency Pollution Inventory Data report: Trends and Analysis. 2001; 73-74. Accessed on: http://www.environment-agency.gov.uk

Van den Berg R, Haenen GRMM, van den Berg H, Bast A. Transcription factor NF-κB as a potential biomarker for oxidative stress. *British Journal of Nutrition*. 2001; **86**:S121-S127.

Van Eeden SF, Kitagawa Y, Klut ME, Lawrence E, Hogg JC. Polymorphonuclear leukocytes released from the bone marrow preferentially sequester in lung microvessels. *Microcirculation.* 1997; **4**: 369-380.

Van Eeden SF, Kitagawa Y, Sato Y, Hogg JC. Polymorphonuclear leukocytes released from the bone marrow and acute lung injury. *Chest.* 1999; **116**: 43S-46S.

Van Eeden SF, Terashima T. Interleukin-8 (IL-8) and the release of leukocytes from the bone marrow. *Leukemia and Lymphoma*. 2000; **37**:259-271.

Van Klaveren RJ, Nemery B. Role of reactive oxygen species in occupational and environmental obstructive pulmonary diseases. *Current Opinion in Pulmonary Medicine.* 1999; **5**:118-128.

Van Rossum AM, Lysenko ES, Weiser JN. Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. *Infection and Immunity.* 2005; **73**:7718-7726.

Vile GF, Rothwell LA, Kettle AJ. Hypochlorous acid activates the tumor suppressor protein p53 in cultured human skin fibroblasts. *Archives of Biochemistry and Biophysics*. 1998; **359**:51-56.

Vollebregt M, Hampton MB, Winterbourn CC. Activation of NF-κB in human neutrophils during phagocytosis of bacteria independently of oxidant generation. *FEBS Letters*. 1998; **432**:40-44.

Wang L, Medan D, Mercer R, Overmiller D, Leonard S, Castranova V, Shi X, Ding M, Huang C, Rojanasakul Y. Vanadium-induced apoptosis and pulmonary inflammation in mice: Role of reactive oxygen species. *Journal of Cellular Physiology.* 2003; **195**:99-107.

Wang X, Zhao X, Mao ZY, Wang XM, Liu ZL. Neuroprotective effect of docosahexaenoic acid on glutamate-induced cytotoxicity in rat hippocampal cultures. *Neuroreport*. 2003; **14**:2457-2461.

Ward C, Chilvers ER, Lawson MF, Pryde JG, Fujihara S, Farrow SN, Haslett C, Rossi A. NF-κB activation is a critical regulator of human granulocyte apoptosis in vitro. *Journal of Biological Chemistry*. 1999; **274**:4309-4318.

Ward C, Walker A, Dransfield I, Haslett C, Rossi AG. Regulation of granulocyte apoptosis by NF-κB. *Biochemical Society Transactions*. 2004; **32**:465-467.

Weiss J, Inada M, Elsbach P, Crowl RM. Structural determinants of the action against *Escherichia coli* of a human inflammatory fluid phospholipase A2 in concert with polymorphonuclear leukocytes. *Journal of Biological Chemistry*. 1994; **269**:26331-26337.

Winterbourn CC. Myeloperoxidase as an effective inhibitor of hydroxyl radical formation. Implications for the oxidative reactions of neutrophils. *Journal of Clinical Investigation*. 1986; **78**:545-550.

Yang J, Marden JJ, Fan C, Sanlioglu S, Weiss RM, Ritchie TC, Davisson RL, Engelhardt JF. Genetic redox preconditioning differentially modulates AP-1 and NF-κB responses following cardiac ischemia/reperfusion injury and protects against necrosis and apoptosis. *Molecular Therapy*. 2003; **7**:341-353.

Yang K-Y, Arcaroli J, Kupfner J, Pitts TM, Park JS, Strasshiem D, Perng R-P, Abraham E. Involvement of phosphatidylinositol 3-kinase γ in neutrophil apoptosis. *Cellular Signalling*. 2003; **15**:225-233.

Yildiz G, Demiryurek AT. Ferrous iron-induced luminol chemiluminescence: a method for hydroxyl radical study. *Journal of Pharmacology and Toxicology Methods*. 1998; **39**:179-184.

Zamanian-Daryoush M, Mogensen TH, DiDonato JA, Williams BRG. NF-κB activation by double- stranded- RNA-activated protein kinase (PKR) is mediated through NF-κB-inducing kinase and IκB kinase. *Molecular and Cell Biology*. 2000; **20**:1278-1290.

Zhang Z, Huang C, Li J, Leonard S, Lanciotti R, Butterworth L, Shi X. Vanadate-induced cell growth regulation and the role of reactive oxygen species. *Archives in Biochemistry and Biophysics*. 2001; **392**:311-320.

Zhao Z, Tan Z, Diltz CD, You M, Fischer EH. Activation of mitogen-activated protein (MAP) kinase pathway by pervanadate, a potent inhibitor of tyrosine phosphatases. *Journal of Biological Chemistry*. 1996; **271**:22251-22255.

Zysk G, Schneider-Wald BK, Hwang JH, Bejo L, Kim KS, Mitchell T, Hakenbeck R, Heinz HP. Pneumolysin is the main inducer of cytotoxicity to brain microvascular endothelial cells caused by *Streptococcus pneumoniae. Infection and Immunity*. 2000; **69**: 845-852.