Investigation of the pro-oxidative and pro-inflammatory interactions of manganese with cells of the innate immune system

by

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DEDICATED TO MY HUSBAND PHASOANE AND CHILDREN: KOPANO, REBOTILE AND THATO AND IN MEMORY OF MY LATE PARENTS

Declaration

To my knowledge the work contained in this thesis is original and was undertaken by myself with occasional assistance as indicated in the acknowledgements. The interpretation and analysis of data were also my primary responsibilities.

It is being submitted for the degree of Doctor of Philosophy at the University of Pretoria. It has not been submitted previously for any degree or examination at any other university.

Signed:_____

Date:_____

SUMMARY

Manganese (Mn^{2+}) is an essential nutrient required in trace amounts for human health; however excessive exposure to the metal, predominantly reported in adults exposed occupationally via inhalation, has been associated with adverse central nervous system effects and respiratory symptoms. Pro-oxidative interactions with cells of the innate immune system may play a role in mediating these effects.

The aim of the current study was to investigate the pro-oxidative and pro-inflammatory interactions of Mn^{2+} with cells of the innate immune system, specifically neutrophils and monocyte-derived macrophages. The primary objectives of the current study were to investigate:i) the pro-oxidative interactions of $MnCl_2$ (1.56-100µM) with isolated human blood neutrophils stimulated with the chemoattractant, N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), or the phorbol ester, phorbol 12-myristate 13-acetate (PMA), according to effects on the generation of superoxide, hydroxyl radical, hypohalous acids and hydrogen peroxide;ii) the effects of $MnCl_2$ on the generation of the reactive oxygen species (ROS), superoxide, hydrogen peroxide and nitric oxide by PMA-activated human blood monocyte-derived macrophages;iii) the effects of $MnCl_2$ on the production of the pro-inflammatory cytokines, interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), granulocyte colony-stimulating factor (G-CSF), interferon gamma (IFNɣ), and tumor necrosis factor (TNF) by unstimulated and lipopolysaccharide (LPS)-activated monocyte-derived macrophages; and iv) the effects of $MnCl_2$ on signal transduction pathways involved in pro-inflammatory cytokine production. The generation of ROS was measured using a series of laboratory procedures designed to precisely characterize the pro-oxidative properties of $MnCl_2$. These included measurement of oxygen consumption, lucigenin/luminol-enhanced chemiluminescence, spectrofluorimetric detection of oxidation of 2,7-dichlorodihydrofluorescein, and radiometric assessment of myeloperoxidase-mediated protein iodination, while a spectrophotometric

method was used for measurement of nitric oxide (NO). In addition, several cell-free ROS-generating systems were used. The Bio-Plex Pro ™ assay kit was used to measure/ detect the production of pro-inflammatory cytokines, while inhibitors (NFκB activation inhibitor, a p38MAP kinase inhibitor and dithiothreitol) were used to probe the signal transduction pathways utilized by $MnCl_2$ to modulate the production of the pro-inflammatory cytokines by monocyte-derived macrophages.

The findings of the study demonstrated that treatment of neutrophils and macrophages with Mn^{2+} markedly increasedthe production of the ROS, H_2O_2 and HOCl in the setting of decreased reactivity of superoxide, while cellular O_2 consumption and generation of NO were unaffected. Taken together with the findings of experiments using cell-free ROS-generating systems, these observations are compatible with a mechanism whereby Mn^{2+},by acting as a superoxide dismutase mimetic, increases the formation of H_2O_2 by activated phagocytes.

Mn^{2+} also potentiated the production of pro-inflammatory cytokines by monocyte-derived macrophages, apparently by affecting H_2O_2-mediated redox-sensitive signaling pathways.

Importantly, this is the first study which has:
i) conclusively established that exposure of human neutrophils and macrophages to $MnCl_2$potentiates the generation of the relatively stable, cell-permeable, potentially damaging ROS, H_2O_2; and
ii) that H_2O_2, in turn, via activation of redox-regulated intracellular signaling mechanisms in macrophages, initiates and potentiates the generation of pro-inflammatory cytokines by the cells.

These findings not only provide a mechanism for Mn^{2+}-mediated toxicity, but also underscore the requirement for strictly controlled exposure to this metal in both

the occupational and environmental settings, as well as the potential for H_2O_2-neutralizing, anti-oxidative preventive and therapeutic strategies.

Keywords:manganese, reactive oxygen species, phagocytes, macrophages, neutrophils, innate immunity, heavy metals, cytokines, cellular signaling, nuclear factor kappa B.

Publications to date

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List of Abbreviations

ACGIH TLV	American Conference of Governmental Industrial Hygienists Threshold Limit Value
AIDS	Acquired immunodeficiency syndrome
As	arsenic
Bfgf	basic fibroblast growth factor
BALF	bronchoalveolar lavage fluid
BM	basement membrane
BPI	bactericidal/permeability increasing protein
BSA	bovine serum albumin
Cd	cadmium
CGD	chronic granulomatous disease
Cr	chromium
Cu	copper
DCs	dendritic cells
DNA	deoxyribonucleic acid
DPI	diphenylene iodonium chloride
DTT	dithiothreitol
EC	endothelial cells
EGF	epidermal growth factor
ERK	extracellular signal regulated kinase
ESADDI	Estimated Safe and Adequate Daily Dietary intake
FAD	flavin adenine dinucleotide
FMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
G-CSF	granulocyte colony-stimulating factor
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase

Hg	mercury
HIV	Human Immunodeficiency Virus
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
IFN	interferon
IgE	immunoglobulin E
IgG	immunoglobulin G
IL-1β	interleukin 1β
IL-8	interleukin 8
JNKs	c-jun-N-terminal kinases
LDL	low density lipoprotein
LFA-1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
LTB-4	leukotriene B4
Mac-1	β_2-integrin CD11b/CD18
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinases
MFF	metal fume fever
Mg	magnesium
MHC	major histocompatibility complex
MMP-8	matrix metalloproteinase-8
MMP-9	matrix metalloproteinase-9
MMT	methyl cyclopentadienyl manganese tricarbonyl
Mn	manganese
$MnCl_2$	manganese chloride
Mo	molybdenum
MPO	myeloperoxidase
MPS	mononuclear phagocyte system
MRI	magnetic resonance imaging
NAC	N-acetyl-L-cysteine
NADH	nicotinamide adenine dinucleotide (reduced)

NADPH	nicotinamide adenine dinucleotidephosphate (reduced)
NETs	neutrophil extracellular traps
NFκB	nuclear factor kappa B
Ni	nickel
NOS	nitric oxide synthase
O_2^-	superoxide
OEL	occupational exposure limits
$OH^·$	hydroxyl radical
PAF	platelet-activating factor
Pb	lead
Pd	palladium
PD	Parkinson's Disease
PDGF	platelet-derived growth factor
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear cell
PSGL-1	P-selectin glycoprotein ligand-1
Pt	platinum
RNS	Reactive nitrogen species
ROFA	residual oil fly ash
ROS	reactive oxygen species
Sb	antimony
SCF	supercritical fluid
SCN	thiocyanate
Se	selenium
SEM	scanning electron microscopy
SOD	superoxide dismutase
TCA	trichloroacetic acid
TEM	transendothelial migration
TGF	transforming growth factor

TNF	tumor necrosis factor
Zn	zinc

CHAPTER 1

LITERATURE REVIEW

1. INTRODUCTION

Humans are continuously exposed to low levels of potentially pro-inflammatory environmental toxicants, especially heavy metals. Chronic low level exposures to toxic metals can affect the health of populations.Of particular concern is that the consequences of interactions of heavy metals such as manganese (Mn) with several human systems especially the immune system, remain largely unknown. This study has therefore focused on exposure of cells of the innate immune system to Mn^{2+}, specifically the effects of the metal on the production of reactive oxygen species (ROS) by human neutrophils and macrophages, as well as on the production of pro-inflammatory cytokines by the latter cell type. Excessive and/or sustained increases in the production of ROS and pro-inflammatory cytokines have been implicated in the pathogenesis of several diseases such as cancer, autoimmune disorders, aging, acute respiratory distress syndrome, pulmonary emphysema and chronic bronchitis as well as cardiovascular and neurodegenerative diseases.[1]

1.1 Heavy metals

Khlifi and Hamza-Chaffai describe metals as natural elements that have beenextracted from the earth and harnessed for human industry forming a major category of globally-distributed pollutants[2].They are also known for their tendency to disperse widely around the environment and to accumulate in specific human tissues causing toxic effects even at low levels. Examples of metals which have the potential to be toxic to human beings as well as the environment include antimony (Sb), chromium (Cr), copper (Cu), lead (Pb), Mn, mercury (Hg), cadmium (Cd) and others.However some metals, Cu and iron (Fe) have been identified as being essential to life even though they display harmful effects with very high levels of exposure.[2,3]Metals such as Cd, Pb, arsenic (As), Cr, Nickel

(Ni) and Hg can also be classified asxenobiotics (those metals without a useful role in human physiology). Industrialized countries increasingly consume non-ferrous metals on a large-scale resulting from manufacturing and economic activities. Human exposure to metals such as As, Pb, Hg and Cd emanates from naturally-occurring and industrial sources, as well as cultural activities. Both high and cumulative low level exposures are detrimental to human health.[2] Freshwater systems are subject to metal pollution from domestic waste watereffluents (especially As, Cr, Cu, Mn and Ni), coal burning power plants (especially As, Hg and Se), iron and steel plants (Cr, Mo, Sb and Zn) and dumping of sewage sludge (As, Mn and Pb).[4]

Human health problems such as kidney, nervous system, pulmonary and liver injury inclusive of specific conditions such as Wilson's disease, asthma, and even death have been attributed to the presence of very high concentrations of Cd, Cu and Ni in living organisms.[5,6]Theron *et al.,* reported that respiratory symptoms including sneezing, rhinorrhea, cough, dyspnoea and bronchial asthma are associated with inhalation of platinum (Pt) salts during industrial processing of this heavy metal which triggers IgE-and cell-mediated immune responses.[7] Exposure to palladium (Pd) has also been identified as a health hazard with potential to compromise innate host immune defenses, a previously unrecognized potential health threat of environmental and/ or occupational exposure to this ubiquitous heavy metal.[8]

1.2 Manganese

There are various manganese compounds; however, the one used in this study was manganese chloride ($MnCl_2$) which is the most stable and abundant form.

1.2.1 Physical and Chemical Properties

Synonyms for Manganese: Manganesechloride, Manganese tetrahydrate, and Manganous chloride tetrahydrate
Atomic number: 25
Atomic symbol: Mn
Atomic weight: 54.938045
Chemical Formula: $MnCl_2$
Group in periodic table: 7
Period in periodic table: 4
Block in periodic table: d-block [9]

Mn is the twelfth most abundant element in the earth's crust and is naturally present in rocks, soil, water and food. It is an essential element for humans, animals and plants and is required for growth, development, and maintenance of health. There are inorganic and organic Mn compounds, with the inorganic forms being the most common in the environment.[10] Chemically it occurs in several species with different valence states (+2, +3, +4, +5, +6, +7). The key significance of Mn speciation in relation to drinking water is that both Mn (II) and Mn (VII) are soluble in water, while Mn (IV) is not. Mn is most stable in its +2 valence state, therefore most naturally occurring Mn is dissolved Mn (II).The next most common species is the particulate state of Mn (IV).[11]

1.2.2 Beneficial effects of manganese

Mn is a trace element and the Estimated Safe and Adequate Daily Dietary Intake (ESADDI) for adults is 2-5mg Mn/d.[12] It is necessary for a variety of metabolic functions including those involved in skeletal system development, energy metabolism, activation of certain enzymes, nervous, and immunological system and reproductive hormone function, and is an antioxidant that protects cells from damage due to free radicals. Mn also plays an essential role in regulation of cellular energy, bone and connective tissue growth and blood clotting. In the brain, Mn is an important cofactor for a variety of enzymes, including the antioxidant enzyme superoxide dismutase, as well as enzymes involved in neurotransmitter synthesis and metabolism.[10]Its deficiency may lead to negative effects such as skeletal abnormalities, brain damage, teratogenicity and abnormal metabolism of carbohydrates and lipids.[13]

1.2.3 Occurrence of manganese in the environment and occupational settings

Human exposures to exogenous, chemical genotoxicants include environmental pollution and hazardous working conditions.[11] Several reports have confirmed that health effects depend on lifetime and timing of exposure and the period of life when exposure occurs. Mn exposure for instance can start before birth from maternal inhalation and ingestion of foods that may contain high Mn concentrations from environmental pollution and can continue during childhood and adulthood from both environmental and occupational exposure.[14] Adulthood exposures are associated with industries such as metallurgy, petrochemistry, dye production, or occupations such as varnishing, asphalt laying and many others. Cr, Cd, Mn, Ni and Pb are of great importance because these

elements are largely used in the metal industry including welding and alloy smelter works.[11]

Several uses of Mn have been described. These include: iron and steel production, manufacture of dry cell batteries, production of potassium permanganate and other manganese chemicals, as an oxidizing agent in the production of hydroquinone, manufacture of glass, textile bleaching and as an oxidizing agent for electrode coating in welding rods, matches and fireworks and for tanning of leather. Organic compounds of Mn are present in the fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT), fungicides and in contrast agents used in magnetic resonance imaging (MRI). Furthermore, organic compounds of Pd and Mn used as anti-knock substances in fuel are emitted into the atmosphere in automobile exhaust fumes. Such environmental contamination of air, water, soil, and food poses a serious threat to all forms of life. The environmental burden of heavy metals has been rising substantially, due to smelter emissions and contamination of water sewage.[10,15]

1.2.4 Detrimental effects of manganese

The study of Mn as an environmental pollutant has gained importance due to the use of MMT, an organic material, as an anti-knock additive for gasoline in countries such as Canada, USA and Australia. It has been known that Mn, an essential element for humans and the fourth most widely used metal in the world, is a neurotoxic substance. But just as is the case with any chemical substance, the dose determines toxicity, therefore, Mn toxicity has primarily been observed in occupational and environmental settings where there is a potential for chronic exposure to high levels, or following the accidental ingestion of large quantities.[10] Mn may also exert its harmful effects in vulnerable subpopulations such as infants and children, the elderly, persons with an iron deficiency, persons with asymptomatic

pre-Parkinsonism or persons on parenteral nutrition or with congenital liver disease.[14,16-20]Within the construction industry, welding and associated processes are routinely performed by pipefitters, ironworkers, boilermakers and sheet metal workers. Other trades may also weld and perform thermal cutting of metals. This work often occurs in processing vessels, such as tanks or boilers or in other poorly ventilated settings. Safety control measures for health hazards, such as local exhaust ventilation, are rarely used on construction sites. Given the potential for excessive exposures to welding fumes, there is concern that workers may be at risk for manganism, Parkinson's Disease (PD) and/ or other preventable neurological disorders.[21]

The first scientific report on neurological effects resulting from exposure to Mn was published in 1837 by John Couper. Several symptoms such as muscle weakness, limb tremor, whispering speech, salivation, and a bent posture were identified in five men working in a Mn ore crushing plant in France. He called this collection of symptoms "manganese crusher's disease", which was later termed "manganism".Manganism is a neurological syndrome that resembles PD, but extensive evidence confirmed that Mn selectively damages different areas of the brain from those that are affected in PD.[10,22]A characteristic pathological difference between the two conditions is the presence of a kinetic tremor associated with extrapyramidal dysfunction in manganism, as opposed to the persistent resting tremor with idiopathic PD. Manganism presents in various stages; in early manganism patients display specific symptoms such as weakness, apathy, somnolence, headaches, and minor motor dysfunction. These symptoms are generally reversible depending on whether the exposure is reduced or eliminated. In the second stage (chronic exposure), patients display a clear disease progression resulting in a combination and potentially severe psychological disturbances such as memory loss, psychotic episodes, anxiety and hallucinations. Movement disorders such

as bradykinesia, dystonic postural abnormalities, gait disturbances, cock-walk, cogwheel rigidity, impaired motor coordination and in the worst cases, permanent and complete disability are associated with the late stage of the disease. Acute exposure to Mn results in the condition known as “metal fume fever” which is less incapacitating, where patients exhibit bronchial hyperresponsiveness, flu-like symptoms and motor deficits which can be alleviated upon removal of exposure.[21]

Various symptoms have been identified in welders exposed to Mn including, subclinical neuropsychological and neurophysiological effects such as insomnia, decreased motor function, decreased reaction time, reduced memory and concentration, mood changes, limb paresthesias, abnormalities in visual evoked potentials, reduced verbal learning and reduced cognitive flexibility.[10]

With regard to respiratory abnormalities, Saric and Piasek reported that individuals who are exposed to Mn through prolonged and high level occupational exposure are considered to be most at risk.[23] Thus, welders, miners and metal-plant workers have exhibited pulmonary distress, resulting in conditions such as pneumonia and bronchitis. The lung epithelium functions as a barrier to infection and its role in innate host defense is disrupted in the presence of Mn, as well as many other heavy metals.[23] In a study by Roth and Garrick, Mn was regarded as the second most potent metal after Cu with respect to metals which cause inflammation in the lung. This inflammation is thought to occur by means of cytokine release (interleukins) as opposed to being immunoglobulin E (IgE)-mediated.[24] Inflammatory changes in the lungs in cases of Mn-exposed individuals have been noted even at non-cytotoxic levels, implying that even modest doses of the metal are sufficient to affect lung physiology.[25]
The effect of Mn on the circulatory system in humans or animals has not been documented in detail. Mn has been reported to have a toxic effect on

cardiac cells and tissues isolated from animals *in vitro,* but this has not been reported in animals or humans in the *in vivo* setting.[26] Roth reported that cell damage can occur by a number of mechanisms with removal of damaged cells being achieved by either programmed cell death (apoptosis) or accidental cell death (necrosis). Metals have the ability to interfere with cellular activity resulting in cell lysis, cell phagocytosis or cell death.[27]Strong evidence has suggested that apoptosis contributes to Mn toxicity due to activation of cell death signaling pathways being triggered in cells treated with the metal.[27]

Early studies investigated the effects of Mn on embryogenesis using animal models. More complete investigations were performed in the late 1980s which concluded that Mn deficiency during the prenatal period would give rise to skeletal abnormalities, ataxia, reduced litter size and an increase in stillborns.[28] The effect of Mn in humans has been linked in a similar fashion to animal models, being associated with decreased fertility and increased foetal abnormalities.[26] Decreased fertility amongst Mn-exposed male workers has been previously suggested by Lauwerys *et al*. Such individuals have significantly fewer children than non-exposed individuals. However, this has not been reported in other studies.[29]

1.2.4.1 Mechanisms of Mn-mediated cellular toxicity

Flynn and Susi reported that elevated levels of Mn can result in toxic neurological effects, presumably through the mechanism of oxidative stress.[30] Experimental work suggests that Mn increases the production of free radicals measured as increased lipid peroxides, reduced glutathione, metallothionein, and other specific biomarkers derived from the actions of Mn on biogenic amines such as dopamine.[31] According to Zhang *et al.,*[32] it has been proposed that the mechanism of Mn toxicity is associated with mitochondrial dysfunction because Mn accumulates specifically within

mitochondria and adversely affects mitochondrial function both *in vivo* and *in vitro*. Through their investigations, effects of $MnCl_2$ exposure *in vivo* on mitochondrial function of liver and brain in Sprague-Dawley rats were observed. The findings indicated that Mn may promote free radical reactions and inhibit electron transfer. Manganese can accumulate in liver and brain mitochondria and disrupt calcium homeostasis.[32] Because Mn accumulates specifically in the mitochondrial matrix, there are three principle mechanisms whereby Mn might disrupt mitochondrial function:1) by inhibition of energy transduction; 2)by induction of mutation of the mitochondrial genome; and 3) through enhanced generation of free radicals. Intracellular Mn accumulates specifically in the mitochondrial matrix, probably using calcium transporters since Mn enhances the rate of calcium uptake into brain mitochondria and inhibits its efflux. It is known that inhibition of mitochondrial electron transfer enhances the generation of free radicals by electron transfer chain enzymes and that free radicals can induce mutations of the mitochondrial genome.[32]

According to Zhang *et al*., activated glial cells (microglia and astroglia) produce a variety of proinflammatory and neurotoxic factors that include cytokines, free radicals and lipid mediators that work in concert to induce and/or exacerbate neurodegeneration. Microglia are the primary source of extracellular ROS, such as superoxide anion, hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS), including nitric oxide (NO) to form the highly deleterious peroxynitrite intermediate. Hydrogen peroxide, on the other hand, can interact with free iron through the Fenton reaction to form the highly toxic hydroxyl free radical.[33]Zhang *et al* further reported that treatment of primary microglia with $MnCl_2$ leads to the increased production and release of oxygen free radicals. $MnCl_2$-induced ROS production by microglia may contribute to Mn neurotoxicity.In addition, Mn has been shown to augment: 1) astroglial expression of inducible NO synthase that is responsible for NO production; and 2) the expression of cyclooxygenase-2

that mediates the production of pro-inflammatory prostaglandins.[33]Unfortunately the mechanism of Mn-mediated neurotoxicity is poorly understood.[10]

1.3 South African perspective on the harmful effects of manganese

Although construction of a superb infrastructure and a vibrant economy are regarded as beneficial effects of metallic, non-metallic and industrial minerals in South Africa, detrimental effects such as environmental degradation and declining public health are associated with 100 years of mining and mineral processing.[34] Other factors influencing pollution of the country's soil and water resources have affected the food chain and consequently the health of the population and surrounding ecosystems. A factor that influences the extent of environmental pollution by Mn is the fact that South Africa currently accounts for about 80% of the world's identified Mn deposits. Again extensive karsting of the dolomites in the Griqualand west Supergroup in the Northern Cape Province, near Kuruman, has an influence in the formation and accumulation of substantial Mn deposits, which, along with the vast deposits in the Kalahari Manganese Field to the north around Hotazel, constitute the largest land-based repositories of Mn on earth.[34]Although other countries produce greater tonnages (e.g. China, Ukraine and other Commonwealth of Independent States), their metal content is low (23% Mn) compared to ores produced by South Africa (38-48% Mn), Gabon, Australia and Brazil.[35] According to the International Manganese Institute, China has been identified as the largest producer of Mn ore in both wet tonnages and in Mn content, producing 14 million wet metric tons (mt) and 2.8 million mt of Mn content. South Africa was found to be the second largest producer, producing 5.6 million wet mt and 2.5 million mt of Mn content.[36]

In the South African setting, a dispute was declared over compensation of workers (miners) suffering from Mn poisoning. Mn poisoning is arguably one of the lesser-known certifiable diseases in the mining industry caused by excessive or prolonged exposures to Mn dust or fumes. Its symptoms strongly resemble those of Parkinson's disease and it is as such frequently misdiagnosed.[37] Symptoms of chronic Mn poisoning include obesity, glucose intolerance, blood clotting, skin problems, lowered cholesterol levels, skeletal disorders, birth defects, changes in hair colour and neurological symptoms.[35]In addition, Spadavecchia reported on a South African smelter that was being investigated for suspected cases of manganism.[38]

Davies and Mundalamo again demonstrated that although the top ten leading underlying natural causes of death in South Africa have clinically related major causes, almost everyone is linked to some geo-environmental co-factor(s): tuberculosis to inhalation of silica dust; cerebro-vascular disease to heavy metal exposure, heart disease to insufficiency of magnesium (Mg) in drinking water supplies; diabetes mellitus to heavy metal exposure; chronic lower respiratory diseases to geogenic dust; and certain disorders involving the immune system to heavy metals and Se (HIV-AIDS). However, the health impacts of potentially harmful elements, especially toxic heavy metals, stemming from sulphide mineralization, mining and ore processing or mine waste disposal are generally less well-known, but are considered to be quite significant in determination of environmental disease ranking in the country.[34]

Heavy metal toxicity from environmental exposure may be directly or indirectly linked to many maladies including headaches, anger, irritability, depression, arthritis, asthma, chronic fatigue, diabetes, fibromyalgia, heart disease, arterial sclerosis, multiple sclerosis, Parkinson's disease, ulcers and many more. A specific form of geophagy which may involve Mn was

decribed in young cattle and sheep in restricted areas of the Barkly-West, Postmasburg and Vryburg Districts of the Northern Cape and North-West provinces of South Africa. It results in severe, subacute to chronic hepatitis and jaundice, with a high mortality rate in untreated cases. An association between the disease and high concentrations of Mn in soils was shown since all affected farms are situated on outcrops of the Reivilo Formation of the Campbell Rand Subgroup, which consists mainly of manganiferous dolomite.[34] Mn was also found to be toxic at high concentrations to fish, resulting in decreased liver glycogen levels and increased blood glucose levels.[39]

Myers *et al.*[40] reported that, there has been increasing interest in nervous system effects of occupational Mn exposures below the American Conference of Governmental Industrial Hygienists Threshold Limit Value (ACGIH TLV) of 0.2 mg/m^3 in total dust and in environmental exposures (respirable dust) at or above the United States Environmental Protection Agency reference concentration of 0.05 ug/m^3 sparked by increasing use of the organic Mn gasoline additive, MMT.As the exposure of mine-workers under modern industrial conditions to Mn is at the lower end of the range of occupational exposure, it was felt that early effects could have been ideally studied amongst South African Mn miners. A study about blue and white collar Mn mineworkers from South Africa was conducted with the aim of investigating the nervous system effects of medium-to-low occupational Mn exposures. Identified sites included underground mines and surface processing plants and offices. Respondents were exposed to 0.21 mg/m^3 manganese dust and analyses of results revealed that none of the symptoms or test results was associated with any measure of exposure, including blood Mn, after adjustments for confounders. The findings confirmed that Mn miners exposed on average across all occupations to MnO_2 at levels near the American Conference of Governmental industrial Hygienists Threshold Limit Value (ACGIH TLV) are unlikely to experience

the threat of subclinical neurotoxicity.[40] A number of factors could have accounted for these findings, including small numbers of exposed subjects and non-standard, and possibly insensitive neurobehavioral tests. In addition, one cannot discount the influence of primary preventative interventions such as occupational hygiene and environmental exposure assessment procedures, as well as secondary preventative measures such as occupational medical surveillance in relation to the early detection of potential neurotoxic effects in South African mineworkers, with important implications for permissible exposure limits in the workplace and general environments.[40]

In a South African study by Young *et al.*,[41] it was hypothesized that respirable Mn exposure is a more appropriate predictor of neurobehavioural effects than inhalable Mn and that there should be no observed effects at respirable dust levels below 0.1 mg/m^3.[41] After testing the hypothesis**,** none of the results were convincing except for motor function effects and the character of the exposure-response relationships.[41] These findings were found to be consistent with a theoretical perspective in which there are no convincing grounds for believing that respirable Mn should be a more sensitive metric for detecting adverse effects. Further studies are therefore necessary using respirable dust as the exposure metric in the evaluation of an appropriate Occupational Exposure Limits (OEL).[41]

1.4 Cells of innate immunity

1.4.1 Neutrophils

Neutrophil granulocytes are commonly known as neutrophils, the most abundant type of white blood cells in mammals and form an essential part of the innate immune system. They account for approximately 70% of all white blood cells (leukocytes). The stated normal range for human blood

counts varies between laboratories, but a neutrophil count of 2.5-7.5x10^9/l blood is a standard normal range. They also form part of the polymorphonuclear cell family (PMN) together with basophils and eosinophils.[42]

The name neutrophil, derives from staining characteristics on haematoxylin and eosin-stained histological or cytological preparations. Neutrophils are known to be different from other cells based on the following characteristics; i) they contain a nucleus consisting of 2-5 lobes; ii) they have an average diameter of 12-15 micrometers (μm) in peripheral blood smears and iii) their average half-life in the circulation is about 12 hours.[43] Activation of neutrophils results in adhesion (position themselves adjacent to the blood vessel endothelium), selectin-dependent capture followed by integrin-dependent adhesion, and finally migration into tissues where they survive for 1-2 days. The short lifetime characteristic of neutrophils is beneficial in exposing pathogens to other body's defenses. In addition to that, short life prevents damage of host tissues by neutrophil antimicrobial products. [43]

Neutrophils are highly motile and can produce and release cytokines as well. These characteristics enable them to play a key role as the first line of defence against invading pathogens. Neutrophils have three strategies for directly attacking micro-organisms: phagocytosis (ingestion), release of soluble antimicrobials (including ROS and granule proteins) and generation of neutrophil extracellular traps (NETs).[43]

1.4.1.1 Migration

Knowledge of the mechanism of leukocyte migration has expanded greatly in recent years.[44] According to Niggli,[45] chemotactic stimulation of neutrophils induces a sequence of events such as actin reorganization,

shape changes, development of polarity and reversible adhesion, culminating in directed migration in a gradient of stimulus (chemotaxis).[45] Neutrophils are an interesting model system for migration as they belong to the fastest moving mammalian cells known and they are also indispensable for host defense against pathogens. Directed migration along a chemical gradient of stimuli enables these cells to rapidly find the site of infection and destroy the invading bacteria.[45]The simple paradigm of rolling adhesion, tight adhesion and diapedesis still holds true; however, the number of variables involved in understanding the process has increased dramatically. Leukocyte migration from the vasculature occurs by a multistep process, dictated by the sequential activation of adhesive proteins and their ligands on both leukocytes and endothelial cells (EC). Monocytes, lymphocytes and neutrophils all migrate by these similar sequence-dependent mechanisms, but differ in their responses to chemotactic and inflammatory signals, particularly in their qualitative and quantitative expression of adhesion molecules.[44]

Activation of circulating neutrophils by an inflammatory stimulus results in migration of neutrophils out of the bloodstream at the site of inflammation by adhering to and then traversing the endothelial layer of the blood vessel wall.[46] There are at least two phases of adherence: the selectin-dependent phase and the integrin-dependent phase.[41] Glycoprotein adhesive molecules (L-selectin, P-selectin and E-selectin) found on both PMNs and ECs are responsible for capturing and removal of neutrophils from the flowing blood, and their rolling along the vessel wall. Rolling permits the leukocyte to probe the endothelium using L-selectin which is constitutively expressed on unstimulated leukocytes.[44] The best characterized ligand for L-selectin is CD34 expressed on platelets, lymphocytes and monocytes, which is found on ECs and binds selectively to L-selectin of lymphocytes. Mediation of rolling by ICAM-1 expressed on the ECs and leukocyte LFA-1

is normally in a low affinity state, but it increases with inflammation as two new selectins are expressed.[47]

The endothelium-bound selectins, P-selectin and E-selectin, facilitate neutrophil-EC adhesion.[44] As demonstrated by Niggli,[45] these selectins are expressed only when appropriate inflammatory stimuli are present. Exposure of ECs to inflammatory mediators such as complement products, ROS, or various cytokines result in P-selectin being mobilized to the cell surface where it can interact with its neutrophil counterpart, P-selectin glycoprotein ligand-1 (PSGL-1, CD162). P-selectin-PSGL-1 interaction is short-lived and reversible if additional adhesive events are not soon invoked. E-selectin supports rolling and tethering of neutrophils by maintaining neutrophil rolling after P-selectin has been down-regulated.[45] Rapid (with 30 sec), acute inflammatory responses involve P-selectin, but slower responses (3-6h) utilize E-selectin.[47]

The initiating signal for the next step of transendothelial migration (TEM) is either a receptor-mediated event in response to an inflammatory cytokine, or an event propagated from signals of activated selectins. These selectins invoke and mediate integrin expression pathways. Integrins in turn mediate cell-cell and cell-extracellular matrix adhesions.[45] The three leukocyte integrins are LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18 or CR3) and p150,95 (CD11c/CD18 or CR4). The integrin-dependent phase of adherence initiates diapedesis a process which promotes migration of leukocytes between the endothelial cells and egress through the capillary wall. In TEM, the stimulated endothelial cells release IL-8, GM-CSF and PAF, all of which cause the leukocyte to rapidly shed its L-selectin required for transendothelial egress.[46]
The neutrophil then expresses the leukocyte β2 integrins, Mac-1 (CR-3), and LFA-1 as a result of IL-8 and PAF stimulation. LFA-1 binds the immunoglobulin superfamily molecule, ICAM-1 (and ICAM-2) and is crucial

in mediating reversible leukocyte-endothelial adherence. In this state, the neutrophil begins to search for the boundaries between two endothelial cells together with CD31 which serves as a hemophilic glue interacting with another molecule of CD31.The β2-integrins Mac-1(CR3) and p150,95 (CR4) are also important in target binding. Mac-1 is found on granulocytes, monocytes, macrophages, large granular lymphocytes and some immature B-cells. The closely related p150,95 is associated almost exclusively with phagocytic myeloid cells. Mac-1 and p150,95 recognize iC3b and these are the two most important complement receptors for phagocytosis.[47]

Transmigration across the endothelial boundary does not require a chemotactic gradient. The leukocytes form a very close contact with the endothelial cell and finally accumulate briefly between the basement membrane and the endothelial cell before entering the connective tissues. The process the leukocyte uses to locate its target and subsequently migrate to the target location is known as chemotaxis (chemotactic stimulation of neutrophils induces a sequence of events such as actin reorganization, shape changes, development of polarity and reversible adhesion, culminating in directed migration in a gradient of stimulus). Mature neutrophils are capable of moving at a rate of 400 μm/h whereas immature neutrophils move more slowly at 60 μm/h.[47]

A family of receptors for bacterially derived or synthesized N-formyl polypeptides, PAF, leukotriene B4 (LTB4), and a variety of other chemokines and ligands for Toll-like receptors are classified as chemoattractant receptors expressed by neutrophils that facilitate migration of phagocytes after they leave the vascular compartments.[48]

Antibody may mediate phagocytic adhesion to targets by using surface receptors collectively known as Fc receptors. Fc refers to a portion of the antibody to which the receptor binds.[47]

1.4.1.2 Phagocytic activity and degranulation

Delivery of potent antimicrobial substances is associated with phagocytes. Regulatory mechanisms responsible for this process have been identified as phagocytosis, secretion (degranulation), apoptosis/cytolysis and the respiratory burst.[47] The ability of a neutrophil to interact with its surroundings depends on the complexity of its surface with myriads of folds, crevices and sites for interaction. Neutrophils and monocytes have several surface receptors responsible for interaction with opsonins (e.g Fc R-I, -II, and –III, and receptors for fragments of the third component of complement), facilitation of phagocytic movement and ingestion through pathways affecting cytoskeletal reorganization.[49]Each receptor type has a specific role to play. Opsonin receptors are known to increase the phagocytosis of immunoglobulin G (IgG) or complement coated bacteria. Scavenger receptors also bind to a large range of molecules on the surface of bacterial cells, while Toll-like receptors are responsible for binding to conserved molecular structures of pathogens releasing pro-inflammatory cytokines and chemokines.[50]

Upon exposure to chemical factors and ingestion of particles, the neutrophil undergoes metabolic and morphologic changes. Once a ligand binds to the neutrophil surface, a series of activities occurs, hyperpolarization and calcium fluxes are induced followed by a rise in cyclic AMP. As a result of these changes, chemoattractants polarize and orient attached neutrophils for movement and they acquire a characteristic asymmetric shape. They develop pseudopodia in the front of the cell containing the nucleus and cytoplasmic granules while the posterior forms a tail. Actin filaments and regulatory proteins are contained within the filament networks of pseudopodia and they are responsible for phagocytosis while a rise in calcium concentration permits neutrophil movement and directionality.[49]

The phagosome is formed by firm adherence of membrane to particles and within one minute it merges with cytosolic granules resulting in the formation of a phagolysosome (see Figure 1.1, Page 21). The bacterium is then subjected to an overwhelming array of killing mechanisms.[49-50]

The ability of phagocytes to protect cells against toxic substances such as bacteria is a critical physiological function of phagocytes.[49] Classification of phagocytes as secretory cells enable them to secrete potent substances into the phagosome, killing and digesting microbes, a process called phagolysosomal fusion. The activity of annexins (3 synexin-like cytosolic proteins) and intracellular calcium is critical to the process of phagolysosomal fusion.[47]

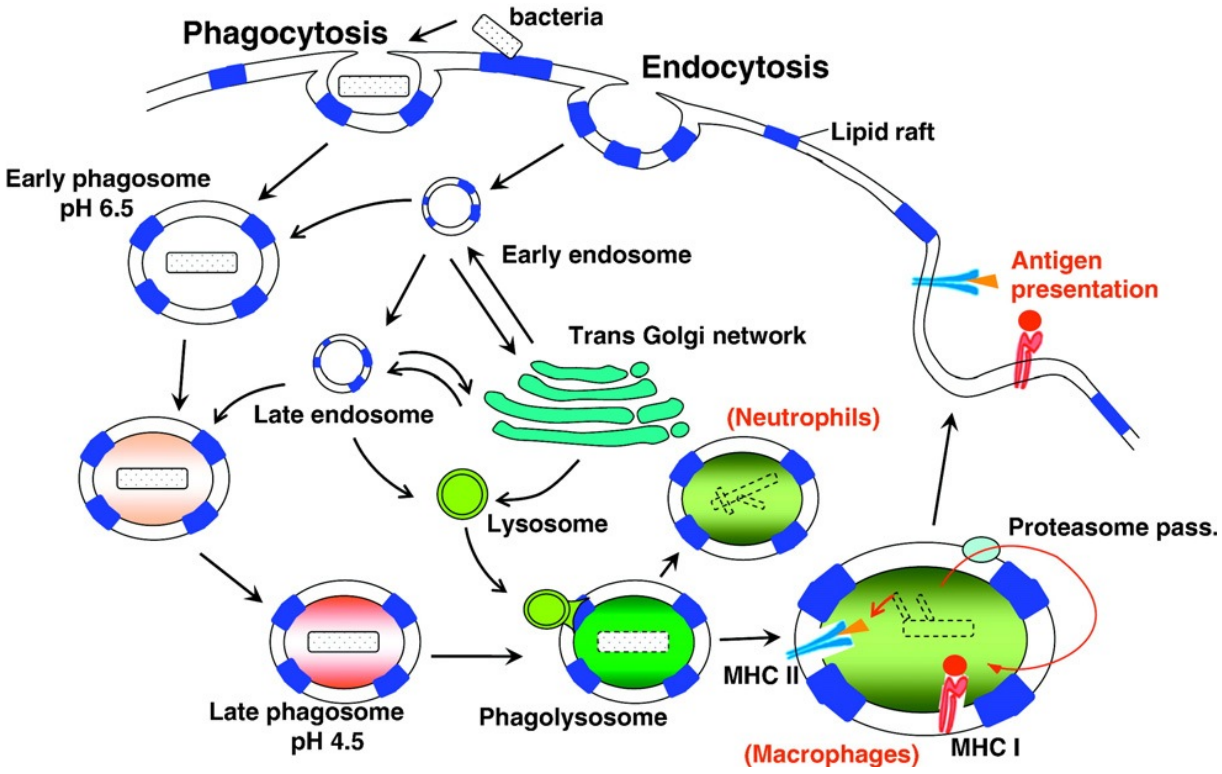


Figure 1.1:Schematic diagram of the engulfment process utilized by professional phagocytes and the role of membrane microdomains in this processs. Phagocytosis of large particles, such as bacteria, is a unique property of professional phagocytes whereas almost all eukaryotic cells can engulf small particles and fluids that are processed in the endosomal pathway. The phagosomal pathway undergoes interconnected maturation with early endosomes, late endosomes and lysosomes. Membrane microdomains may be converted into membrane platforms or act directly in many aspects of interactions between bacteria and PRRs, internalization of pathogens, induction of apoptosis, release of cytokines, and the formation of reactive oxygen species etc. Macrophages are antigen-presenting cells. Peptides generated by degradation in acidic phagolysosome are exported to the cytosol, ubiquitinated, processed by the proteasome, and transported back into the phagosome lumen where they are eventually loaded onto MHC-1 molecules. Then, peptide and MHC-I/II complex are exported to the cell surface. Permission granted by Elsevier (Yoshizaki *et al*, 2008).[51]

Mediators are contained in membrane-bound secretory granules or synthesized upon stimulation of receptors. They are proinflammatory in nature and released by neutrophils through the process of degranulation or exocytosis. The ability of neutrophils to subject a toxic substance to a diverse array of antimicrobial proteins and enzymes within the phagosomes is called oxygen-independent killing. Alternatively, they can produce ROS both inside and outside the cells, killing extracellular bacteria. The role of leukotriene B4 and IL-8 (chemoattractants) is to increase proinflammatory activities by recruiting additional leukocytes to the site of infection or inflammation.[52]

A number of granules contained in the neutrophils serve as key components in destroying microbial molecules and they are classified as: primary granules, also known as azurophilic granules, secondary granules, also known as specific granules, tertiary granules and secretory vesicles (see Table 1.1, Page 24).[52]This classification is due to the different roles they play at the site of infection. Movement of neutrophils from the blood-stream to the inflammatory site occurs concurrently with sequential release of granules. The primary granules serve as the main storage site of the most toxic mediators, while secretory vesicles are characterized by their immediate release when neutrophils bind to the endothelium. In response to neutrophil transmigration, tertiary granules are mobilized and the secondary and primary granules are released at the site of inflammation.[53]

The respiratory burst is primarily a key process in oxygen-dependent intracellular killing. It causes an increase in oxygen consumption resulting in production of reactive oxygen-containing molecules that are potentially antimicrobial. These ROS are toxic to both the invader and the cell itself, so they are generally confined to compartments inside the cell. Two processes are responsible for killing invading microbes by ROS; i) the first type is the oxygen-dependent production of superoxide, which is a

bactericidalsubstance and ii) the involvement of the activity of the enzyme myeloperoxidase present at high concentrations in the neutrophil primary granules.[50]

Table 1.1: Neutrophil granules and their major effectors of antimicrobial activity [52,54-56]

Type of granules	Protein released	Function
Primary/Azurophilic granules	MPO	Catalyzes the production of hypochlorite from chloride and hydrogen peroxide
	BPI	Has antibacterial activity against Gram-negative bacteria, enhances phagocytosis by acting like opsonin
	Defensins	Proteins that defend the body against a variety of bacteria, fungi and viruses
	Lysozyme	Enzyme that degrades peptidoglycans of Gram-positive bacteria
	Elastase and other proteases	Degrades an outer membrane protein in Gram-negative bacteria
Secondary/ Specific granules	Apolactoferrin	Binds iron, thereby depriving bacteria of the iron that is essential for cell growth
	Collagenase-2 (MMP-8)	Degrades collagen and thus augments movement of neutrophils through collagen matrices
Tertiary/ Gelatinase granules	Gelatinase B (MMP-9)	Functions as a regulator of neutrophil biology and to truncate IL-8 at the amino terminus into a tenfold more potent chemokine resulting in an important positive feedback loop for neutrophil activation and chemotaxis
Secretory vesicles	Secretory Phospholipase A2	Enzyme involved in the production of potent lipid mediators such as eicosanoids and lysophospholipids with antimicrobial activity.

1.4.1.3 Oxidative burst and the NADPH oxidase system

The “professional” phagocytes, such as neutrophils, eosinophils and macrophages, are known to exhibit “the respiratory burst” during phagocytosis. Increased consumption of oxygen during respiratory burst is specifically for the actual killing process and not for phagocytosis. The rare congenital condition of chronic granulomatous disease (CGD) is associated with an absent respiratory burst because of defects in gene coding for the cellular machinery which produces it.[53,54] Failure to mount effective defense against bacteria and fungi, resulting in severe and recurrent infections is symptomatic of this condition.[55]

Four enzymes: NADPH oxidase, superoxide dismutase (SOD), myeloperoxidase (MPO) and nitric oxide synthase (NOS) participate actively in the generation of ROS.[45] Neutrophil NADPH oxidase utilizes NADPH as its substrate and catalyzes the one-electron reduction of oxygen to superoxide:

$$NADPH + 2O_2 \rightarrow NADP^{+} + 2O_2^{-} + 2H^{+}$$ [57]

NADPH oxidase is described as a membrane-associated multi-subunit enzyme, primarily located in the plasma membranes of cells.[57]It is an importantsource of ROS and play a key role in the antimicrobial activity of the cells; however, additional sources may also contribute such as mitochondria.[58]The NADPH oxidase system was originally described as the main enzymatic source of ROS in phagocytes for the host defense system. However, recent evidence has demonstrated that the NADPH oxidase system is a major source of superoxide in non-phagocytic cells as well e.g. platelets. Although additional work has to be done pertaining to the biochemical characterization, regulation and structure of non-phagocytic NADPH oxidases since these aspects are not yet completely understood,

various cell types appear to functionally express this system.[59] Different cell types known to produceNADPH oxidases include all vascular cells, smooth muscle cells, fibroblasts, and resident monocytes/macrophage.[58]

Various components such as flavocytochrome b_{558} ($gp91^{phox}$) play a key role in NADPH oxidase activation since it contains binding sites for NADPH and FAD as well as two haem groups that are critical for transmembrane electron transport from NADPH to molecular O_2, to generate superoxide anion (O_2^-) and subsequently hydrogen peroxide (H_2O_2). The $gp91^{phox}$ protein operate in synergy with $p22^{phox}$, another membrane-bound protein and it can only be activated after recruitment and phosphorylation of 3 regulatory cytosolic proteins ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) and the GTPase, Rac1, which assemble with membrane-bound subunits to form a functional NADPH oxidase. Microbial infection or cell stimulation will then activate NADPH oxidase (see Figure 1.2, Page 28), phosphorylation of $p47^{phox}$ and migration of the entire cytosolic complex to the membrane where it binds to the cytochrome b_{558} to assemble the active oxidase.[60,61]The assembled oxidase is now able to transfer electrons from the substrate to oxygen by means of its electron-carrying prosthetic groups, its flavin and then its heme groups, thus activating the oxidase to produce superoxide (O_2^-).[61,62]Superoxide will then be contained in vesicles by internalized target will then be subjected to a lethal mixture of the phagocytes and undergoes rapid conversion into its successor products. The corrosive oxidants.[61]

Activation of NADPH oxidase emanates from various stimuli (physiological and non-physiological), with the products of phospholipase C-β (inositol-triphosphate and diacylglycerol), phospholipase D (phosphatidic acid, further degraded to diacylglycerol) and phospholipase A_2 (arachidonic acid and lysophosphatidic acid) acting as secondary messengers. Secondary messengers give rise to secondary events such as a rise in Ca^{2+} levels,

serine/threonine kinase activation, and tyrosine kinase activation. Dephosphorylation has been identified as one of the mechanisms associated with termination of electron transport.[63]

1.4.1.4 Neutrophil oxidants

ROS are the reactive forms of oxygen capable of reacting with a host of biologically important molecules and play a key role in many physiological and pathophysiological processes. They are classified into radicals such as superoxide (O_2^-) and hydroxyl radical ($OH^\cdot$) and non-radicals such as hydrogen peroxide (H_2O_2).[64]Reactive oxygen and nitrogen species have been identified as the major products of macrophages and neutrophils when exposed to chemoattractants and opsonized pathogens, as well as cytokines released by immunocompetent cells and may also be induced by other signaling processes.[49] Platelets also have the ability to produce ROS, however, the enzymatic sources of ROS in platelets are still to be investigated.[59] Microglia which form part of the central nervous system serve as the primary source of extracellular ROS including superoxide anion, hydrogen peroxide and reactive nitrogen species (RNS) as well as nitric oxide, the precursor of the highly toxic peroxynitrite intermediate.[33]
Being charged molecules, superoxide anions are not capable of crossing cellular membranes and they are normally confined to their site of production. This property of superoxide permits its oxidizing power to be easily compartmentalized, making it an ideal microbicidal molecule to kill invading pathogens within phagosomes of neutrophils and macrophages, without oxidative damage to the surrounding cells.

A further single electron reduction of superoxide produces H_2O_2 which is uncharged and can unlike O_2^-easily diffuse across cellular membranes through the process of dismutation:
$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$[63]

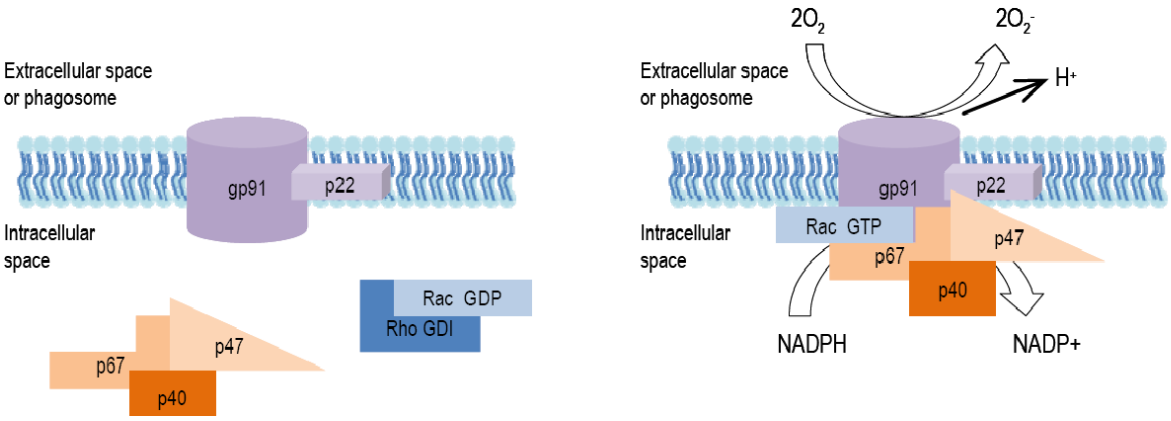


Figure 1.2: Schematic representation of resting and activated phagocyte NADPH oxidase. The gp91phox and p22phox components are associated at the plasma membrane and stabilize each other. NADPH oxidase activation is regulated by the organizer p47phox and activator p67phox subunits, and requires GTP-bound Rac. The cytosolic subunits p47phox, p67phox and p40phox are preassembled in the cytosol and upon activation translocate to the membrane, where gp91phox and p22phox are located. In resting cells, Rac is found in a GDP-bound state stabilized by RhoGDI. With stimulation, Rac translocates to the membrane independently of p47phox and p67phox. GTP-bound Rac interacts with p67phox. After the phagocyte cell is activated through the action of soluble chemoattractants and chemokines, or phagozitable particles, the stimulated NADPH oxidase enzyme catalyze the one-electron reduction of oxygen to produce superoxide anion by using NADPH as substrate. This enzyme uses electrons derived from intracellular NADPH to generate superoxide anion, which subsequently is transformed into other ROS. Permission granted by Carrera-Silva A (Carrera-Silva *et al*, 2011).[65]

Although both superoxide and hydrogen peroxide are classified as "reactive" oxygen species, there is a difference in terms of their biological targets. Superoxide prefers to interact with iron sulphur clusters, found on many transcription factors while H_2O_2 targets peroxidases and kinases.
The reaction between superoxide and H_2O_2 gives rise to the highly reactive hydroxyl radical ($OH^{\cdot}$) via the Haber-Weiss reaction:

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

In aqueous solutions this reaction takes place very slowly. However, the rate of hydroxyl radical formation is greatly enhanced in the presence of free transition metal ions (e.g Fe^{2+}, Cu^{2+}, known as the Fenton reaction):

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

Hydroxyl radicals are the most powerful oxidizing agents reacting with most biological molecules at near diffusion-limited rates. Conversely, superoxide can react directly with endothelium-derived nitric oxide ($NO^{\cdot}$) producing peroxynitrite ($ONOO^{-}$). It reacts similarly to hydroxyl radicals hence it is classified as an oxidizing agent. However, it has been implicated as a major mediator of lipid peroxidation and protein nitration in vascular disease:

$$O_2^{-} + NO^{\cdot} \rightarrow ONOO^{-}$$

Both oxidizing agents exhibit highly reactive properties to an extent that they often react with the first substrate they come into contact with.[66]

However, O_2^- and $H_2O_2$are less reactive, and cytoplasts, which are neutrophils from which the nuclei and cytoplasmic granules have been removed, phagocytose bacteria but fail to kill them. There must therefore be a factor in the granules that is released into the vacuole which enhances killing. This factor is myeloperoxidase (MPO), which converts H_2O_2 to toxic hypochlorous acid by oxidizing chloride:[59]

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

Superoxide anion also reacts with hypochlorous acid to yield free hydroxyl radical:

$$HOCl + O_2^- \rightarrow OH^{\cdot} + O_2 + Cl^-$$ [66]

Properties associated with MPO are: it is a tetrameric, heavily glycosylated haem-containing enzyme of mw 150 kDa. It is primarily contained in azurophilic granules of neutrophils and to a lesser extent in monocytes. Myeloperoxidase can be released to the outside of the cells by being secreted into the phagolysosomal compartment and by leakage before complete closure of the developing phagosome, or in response to stimulation by antibody/ complement-coated surfaces too large to be ingested. It accounts for 5% of the total neutrophil protein and targets compounds such as halides and the pseudohalide, thiocyanate (SCN^-), in combination with H_2O_2 to produce corresponding hypohalous acids.[59] Other substrates include, chloride, bromide, iodide, or thiocyanate with Cl^- being identified as the major and preferred halide.[59,67,68]Up to 80% of the H_2O_2 generated by $1x10^6$ maximally activated neutrophils is used to form 20-400µM HOCl an hour except for saliva which contains 10^3 M SCN^-. On the other hand, HOSCN is not as harmful as HOCl and therefore not identified as a toxicant. [59]Hypohalous acids are powerful reactive agents capable of destroying bacteria, endothelial cells, tumour cells and red cells.

[69]ConsequentlyHOCl has a role to play in the pathogenesis of neurodegenerative disorders such as: Parkinson's disease, Alzheimer's disease, cerebral ischemia and multiple sclerosis. It can further contribute to tissue destruction and cellular damage leading to cell death.[59]

Vitamin C, vitamin E, metal-sequestering proteins and specialized enzymes are identified as the most important compounds responsible for maintaining a balance between the level of ROS produced during normal cellular metabolism and the level of endogenous antioxidants. If a balance exists, oxidative damage of cells can be prevented. Superoxide dismutases (SOD) serve to remove damaging ROS from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen.[70]

Cellular functions such as survival, growth, metabolism and motility are attributed to redox signaling modulated by a regulated production of ROS. [62] ROS are known to be of biological significance since they participate in the host's defense mechanisms. In addition to that, production of ROS has been identified in non-phagocytic cells as a result of activation of receptor tyrosine kinases, signaling cascades, including activation by platelet-derived growth factor, basic fibroblast growth factor or epidermal growth factor. Well known inducers of ROS are the cytokines e.g tumour necrosis factor, ɣ-interferon and various interleukins. The fact that the response can be diminished by non-enzymatic and enzymatic antioxidants, explains the direct role played by ROS as second messenger molecules responsible for transducing the receptor-initiated signaling cascades that control diverse cellular events such as proliferation, apoptosis and inflammation.[71]

Conversely, ROS can react with a variety of cellular macromolecules such as lipids, proteins and DNA, leading to the disruption of cell membranes, inappropriate activation or inactivation of enzymes, and genetic

mutations.[57] An imbalance between the level of ROS produced during normal cellular metabolism and the level of endogenous antioxidants as a result of either an increase in ROS production, or decreased levels of antioxidants, produces a condition referred to as oxidative stress.[69]Oxidative stress therefore occurs when pro-oxidants overpower antioxidant capacity, leading to excessive production of ROS.[62]Oxidative stress has been implicated in a variety of pathological conditions including cardiovascular diseases, neurological disorders, lung pathologies and accelerated aging.[62,69]Airway diseases such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease involve increased oxidative stress, suggesting that reactive oxygen and nitrogen species may overwhelm antioxidant defenses. Extracellular superoxide dismutase is abundant in pulmonary tissues and protects the lung from increased oxidative stress. However, its role in asthma and other airway diseases has not been fully elucidated.[71]

1.4.2 Monocytes and Macrophages

Monocytes and neutrophils are produced from the same myeloid progenitor in the bone marrow. Monocyte cells constitute 5-10% of peripheral-blood leukocytes in humans. Mature monocytes in the peripheral circulation have a heterogeneous morphology manifested as differences in size, granule content and nuclear morphology. They have a short half-life which results in them having to circulate for several days before entering tissues, differentiating into and replenishing tissue macrophages.[72] They are also described as the oldest cell type of hematopoietic system.[73]

Stimuli associated with recruitment of monocytes include proinflammatory, metabolic and immune stimuli which induce the cells to differentiate into macrophages and dendritic cells (DCs). This differentiation results in cells which contribute to host defense, and tissue remodeling and repair.[72]

Infection promotes movement of circulating blood monocytes from the vasculature into the extravascular compartment. This movement is facilitated by various endogeneous and exogeneous factors. Once the monocytes become tissue-based, they mature into macrophages. However, they lose their ability to replicate. On the other hand, participation in inflammatory and immune responses is enhanced. Transcription factors are responsible for the differentiation process.[74]

Lipopolysaccharide (LPS) derived from Gram-negative bacteria is identified as a typical activator of the macrophage secretory response during which soluble inflammatory mediators such as tumor necrosis factor alpha (TNF-α) is secreted. Stimulation of macrophages with LPS *in vivo* and *in vitro* results in expression of TNF-α, classified as a major proinflammatory mediator for macrophages. It is not secreted from intracellular stores, but is rather synthesized *de novo* in response to an effective stimulus. Nuclear factor kB (NF-κB) is one of the key factors important in regulating TNF-α gene transcription.[74]The various subpopulations of macrophages work in synergy with DCs in tissue remodeling and repair. They play a critical role in both the initiation and recovery phases of scarring. The origin, activation state of the macrophage, and the microenvironment in which they reside are viewed as critical factors responsible for the macrophage response to injury. Macrophages possessing the alternative phenotype participate actively in wound healing and tissue remodeling since they secrete anti-inflammatory cytokines, necessary to promote angiogenesis. These macrophages are characterized by their heterogeneity and plasticity, which are reflected by their specialized function in the kidney and other organs.[74]

1.4.2.1 Different types of macrophages

According to Ricardo *et al.*,[73] recruitment of monocytes potentiated by cytokine stimulation results in differentiation of macrophages into classically activated (M1) or alternatively activated (M2). M2 macrophages further subdivide into M2a (upon exposure to IL-4 or IL-13), M2b (induced by immune complexes in combination with IL-1β or LPS), and M2c cells (following exposure to IL-10, TGF-β, or glucocorticoids). The plasticity and differentiation of macrophages into the M1 and M2 functional phenotypes therefore represent extremes of a continual spectrum of differential pathways. Activation of M1 macrophages by classical immune pathways involves an IFN-γ-dependent Th1-type response, while M1 polarization due to IFN-γ and LPS, or the cytokines TNF and GM-CSF, resulting in production of IL-12 and IL-23. Production of IL-12 and IL-23 is necessary for activation of Th1 and Th17 cells. Both Th1 and Th17 cells enhance recruitment to, and activation of phagocytes at sites of infection.[75]In the classical M1 pathway, activation by IFN-γ is crucial since together with a microbial trigger, it induces expression of MHC class II antigens and pro-inflammatory cytokines.[73]

Conversely, the alternate M2 macrophage pathway deactivates macrophages after exposure to Th2-type cytokines, which triggers immunoregulatory, immunosuppressive, and pro-tumoricidal settings. M2 cells induced by exposure to IL-4 and IL-13 (M2a) and deactivating cytokines such as IL-10 and TGF-β are responsible for suppressing immune responses and promoting tissue remodeling. Furthermore, M2 cells are capable of expressing the IL-1 receptor antagonist responsible for deactivating the pro-inflammatory cytokine IL-1, and activation via the mannose receptor, and release of chitinase 3-like 3. Macrophages do not remain in a single activation state. They can regress to a resting state and this characteristic is critical since they play an important role in

inflammatory response that determines tissue destruction or recovery. This is confirmed by the tendency of classically activated M1 macrophages to revert to the M2 activation state following phagocytosis of apoptotic cells.[73]

1.4.3 Production of pro-inflammatory cytokines and anti-inflammatory cytokines

Cytokines are small proteins with molecular weights ranging from 8 to 40 000 kDa. They were originally called lymphokines and monokines depending on their cellular sources. Macrophage/ monocyte-derived cytokines are classified according to their biological activities (see Table 1.2, Page 36).[76]

Stimulation of cells by a specific antigen, endotoxin, or other cytokines lead to secretion of these molecules. They are not antigen-specific in nature and they therefore bridge the innate and acquired immunity influencing the magnitude of inflammatory or immune responses. They act sequentially, synergistically, or antagonistically.[77] Cytokines are classified into pro-inflammatory cytokines which promote inflammation and the anti-inflammatory cytokines which are known to suppress the activity of pro-inflammatory cytokines. For example, IL-4, IL-10, and IL-13 are potent activators of B lymphocytes.

Table 1.2: **Major categories of monocyte/ macrophage-derived cytokines.**[77]

Cytokine	Actions
G-CSF	Stimulates neutrophil development and differentiation
GM-CSF (granulocyte macrophage colony stimulating factor)	Stimulates growth and differentiation of myelomonocytic lineage cells, particularly dendritic cells
IL-1α	Fever, T-cell activation, macrophage activation
IL-1β	Fever, T-cell activation, macrophage activation
IL-1RA(receptor antagonist)	Binds to, but does not trigger IL-1 receptor; acts as a natural antagonist of IL-1 function
IL-6	T- and B-cell growth and differentiation, acute phase protein production, fever
IL-8	Chemotactic factor that attracts neutrophils, basophils and T-cells, but not monocytes. Also involved in neutrophil activation
IL-10 (cytokine synthesis inhibitory factor)	Potent suppressant of macrophage functions
IL-12 (NK-cell stimulatory factor)	Activates NK cells, induces CD4 T-cell differentiation into T_H1-like cells
IL-18(IGIF,interferon-ɣ inducing factor)	Induces IFN-ɣ production by T cells and NK cells, promotes T_H1 induction
IL-19	Induces IL-6 and TNF-α expression by monocytes
IL-24	Inhibits tumor growth
IL-27	Induces IL-12R on T cells via T-bet induction
OSM (OM, oncostatin M)	Stimulates Kaposi's sarcoma cells, inhibits melanoma growth
TNF	Promotes inflammation, endothelial activation

However, IL-4, IL-10, and IL-13 are also potent anti-inflammatory agents.[76]They are anti-inflammatory cytokines by virtue of their ability to suppress the expression of genes which encode proinflammatory cytokines such as IL-1, TNF, and various chemokines. IFN-ɣ is considered a pro-inflammatory cytokine because it augments TNF activity and induces nitric oxide synthase. The concept of pro-inflammatory and anti-inflammatory cytokines is fundamental to cytokine biology and clinical medicine.[76]

The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response. The human immune response is regulated by cytokines acting in synergy with specific cytokine inhibitors and soluble cytokine receptors. Examples of major anti-inflammatory cytokines include the interleukin-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11 and IL-13 while pro-inflammatory cytokines include TNFα,IL-1, IL-6, IL-8 and G-CSF.[78,79]Pro-inflammatory cytokines recruit, alert, and activate neighbouring cells necessary for mounting an effective immune response.[79]

A balance between the effects of pro-inflammatory and anti-inflammatory cytokines contributes to the outcome of disease, whether in the short-term or long-term. This emanates from the fact that low concentrations of anti-inflammatory cytokines result in excess inflammation, while very high concentrations disrupt anti-microbial mechanisms. This is supported by an observation made in gene knockout in mice in which deletion of the IL-10 gene resulted in spontaneous development of fatal inflammatory bowel disease. Deletion of the TGF-β1 gene also resulted in a spontaneous inflammatory disease.[76,78] Cytokines deliver their signals via cell surface receptors and expression of receptor chains plays a critical role in receptor affinity for cytokines. For example, the IL-2 receptor consists of 3 chains: α, β and γ. Receptor affinity for IL-2 is high if all 3 chains are expressed, intermediate if only the β and γ chains are expressed, or low if only the α

chain is expressed. Lack of γ chain expression due to mutations or deletion is the basis for X-linked severe combined immunodeficiency.[78] IL-1 and TNF binding to target cells induces biochemical changes such as phosphorylation of multiple proteins and activation of phosphatases which can be observed within 5 minutes. Although the post receptor events of IL-1 and TNF are similar, their receptors are different. IL-1 induces several transcription factors and most of the biological effects of IL-1 take place in cells following nuclear translocation of nuclear factor (NF)-κB.[76]

1.4.4 Activation of NFκB

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is described as a transcription factor in B cells that binds to the enhancer element controlling immunoglobulin kappa light chain expression. In mammalian cells, the NFκB/ Rel family consists of five members: RelA (p65), c-Rel, RelB, NFκB1 (p50; p105), and NFκB2 (p52; p100). These proteins consist of a 300 amino acid sequence called the *REL* region. This region contains the dimerization, nuclear localization, and DNA-binding domains.[80]NFκB, like other transcription factors, responds to stimuli which include stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL and bacterial or viral antigens. Known inducers of NFκB activity are highly variable and include ROS, TNFα, IL-1β, LPS, isoproterenol, cocaine, and ionizing radiation.[80,81]According to Garcia *et al*., critical functions of NFκB involve regulation of the immediate early pathogen response, promotion of inflammation and regulation of cell proliferation and survival. [82]It has also been implicated in various diseases including rheumatoid arthritis (RA), atherosclerosis, multiple sclerosis and asthma via its stimulatory effects on chemokines, adhesion molecules, MMPs, cox-2, and inducible nitric oxide. In RA,NFκB is over-expressed in the inflamed synovium, recruits inflammatory cells and induces production of pro-inflammatory mediators like IL-1, IL-6, IL-8 and TNF.[83]NFκB inhibitors (IkB) are a family of

regulatory proteins responsible for maintaining a balance in production and inhibition of NFkB. They are expressed in the cytoplasm of virtually all cell types and they include IκBα, IκBβ, IκBa, and Bcl-3 family.[80,84]

Activation of NFκB is initiated by the signal-induced degradation of IκB proteins, see Figure 1.3, Page 40. This occurs primarily via activation of a kinase called IκB kinase (IKK). Structurally, IKK consists of a heterodimer of the catalytic IKK alpha and IKK beta subunits and a master regulatory protein termed NEMO (NK-kB essential modulator) or IKK gamma.[81]NFkB signaling pathways are classified into the classical NFκB signaling pathway (canonical NFκB pathway) and the alternative pathway (noncanonical pathway). In the classical pathway, IkB kinase (IKK) complex initiates phosphorylation of IκB proteins at specific sites equivalent to Ser 32 and Ser 36 of IκBα. Phosphorylation then triggers polyubiquitination at sites equivalent to Lys21 and Lys22 of IκBα and degradation by the 26S proteasome, resulting in the release of free NFκB dimmers.[80] With the degradation of IκB, the NFκB complex is then freed to enter the nucleus where it can “turn on” the expression of specific genes that have DNA-binding sites for NFκB in their promoter regions. Physiological changes associated with the activation of these genes by NFκB include an inflammatory or immune response, a cell survival response, or cellular proliferation. NFκB regulates its own repressor, IκBα by turning it on when necessary. IκBα then re-inhibits NFκB and, thus forms an auto feedback loop, which results in fluctuating levels of NFκB activity.[81] Although IKKα and IKKβ cooperate in IκB phosphorylation, these proteins differ in the signals that they mediate. The IKKβ component is essential for the signaling via the classical NFκB pathway. In the alternative pathway, IKKα homodimer phosphorylates NFκB2/ p100 at two C-terminal sites. This modification results in proteasomal degradation and production of p52.[80]

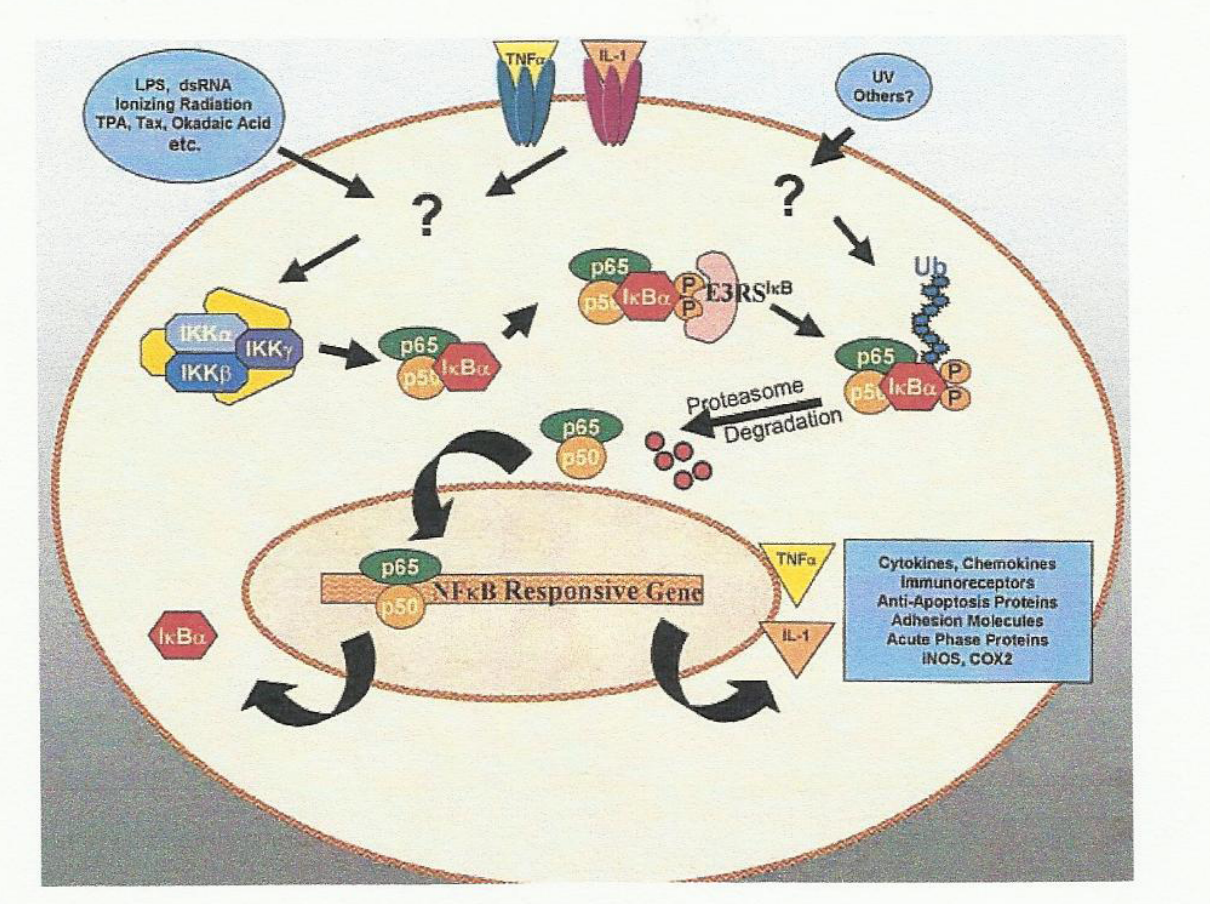


Figure 1.3: A schematic model of NFκB activation. Various stimuli, including the proinflammatory cytokines TNFα and IL-1, activate IKK. Once activated, IKK phosphorylates IκBα, leading to its recognition by E3RS^{IKB}, a receptor component of a SCF type E3, which results in the polyubiquitination of IκBα. This then targets IkBα for rapid degradation by the 26S proteasome. IκBα degradation exposes the nuclear localization sequence on NFκB resulting in its translocation to the nucleus. In the nucleus NFκB regulates transcription of target genes, including IkBα, which functions to terminate NFκB activity. Some of the NFκB target genes code for inflammatory mediators, such as TNFα and IL-1 and chemokines, which can lead to recruitment of additional cells to the inflammatory response. Permission granted by Karin M and Ben-Neriah Y (Karin and Ben-Neriah, 2000).[85]

In a study by Sigala *et al*, NFκB was found to be a transcription factor responsible for the increased expression of TNF, IL-2, IL-10, IL-1β, but not IL-6.[86]Although NFκB plays an essential and beneficial role in normal physiology, the up-regulation of this factor, as mentioned earlier, has been implicated in the pathogenesis of several diseases including inflammatory and rheumatic diseases.[87]

1.4.5 Redox regulation of cellular signaling

The ability of cells to communicate with each other and to respond to their environment relies on biological mechanisms that allow the information to travel from the cell surface to the nucleus.[88]Extracellular stimuli elicit a variety of responses, such as cell proliferation and differentiation, through the cellular signaling system. Binding of growth factors to the relevant receptor leads to the activation of receptor tyrosine kinases, which in turn stimulate downstream signaling systems such as mitogen-activated protein (MAP) kinases, phospholipase Cγ (PLCγ) and phosphatidylinositol 3-kinase. These events result in the activation of cytosolic transcription factors which translocate to the nucleus where they initiate target gene expression. Recent studies have revealed that signaling pathways are regulated by the intracellular redox state. Generation of ROS, such as H_2O_2, leads to the activation of protein tyrosine kinases followed by the stimulation of downstream signaling systems including MAP kinases and PLCγ. Not only are the cellular signaling pathways subjected to redox regulation, but also the signaling systems regulate the cellular redox state. When cells are activated by extracellular stimuli, they produce ROS, which in turn stimulate other cellular signaling pathways, indicating that ROS act as second messengers. Growing evidence has indicated that the cellular redox state plays an essential role not only in cell survival, but also in cellular signaling systems. The primary targets of redox regulation are

sulphydryl groups (RSH) on protein cysteine residues, which are easily oxidized to form a disulphide acid (RSO_2H) or sulphonic acid (RSO_3H). When cells are stimulated with ROS, signals are transferred through the same signaling pathways as those triggered by growth factors.[89]

1.5 Hypothesis

The hypothesis which forms the basis of this study is that exposure of isolated human neutrophils and monocyte-derived macrophages to Mn potentiates the production of ROS by both cell types, as well as the generation of pro-inflammatory cytokines by monocyte-derived macrophages. Alternatively, the pro-oxidative, pro-inflammatory activities of these cells are unaffected by Mn.

1.6 Objectives

The primary objectives of the laboratory research described are to investigate:

- The pro-oxidative interactions of $MnCl_2$ (1.56-100μM) with isolated human neutrophils stimulated with the chemoattractant, FMLP,or the phorbol ester, PMA, according to effects on the generation of superoxide, hydroxyl radical, hypohalous acids and hydrogen peroxide.

- The effects of $MnCl_2$ on the generation of superoxide, hydrogen peroxide and nitric oxide by PMA-activated monocyte-derived macrophages.

- The effects of $MnCl_2$on the production of the pro-inflammatory cytokines, IL-1β, IL-6, IL-8, G-CSF, IFNɣ, and TNF by unstimulated and lipopolysaccharide (LPS)-activated monocyte-derived macrophages.

- The signal transduction pathways involved in Mn^{2+}-mediated pro-inflammatory cytokine production

CHAPTER 2

MANGANESE PROMOTES INCREASED FORMATION OF HYDROGEN PEROXIDE BY ACTIVATED HUMAN MACROPHAGES AND NEUTROPHILS

2.1 Introduction

Although Mn^{2+} is classified as a trace element required for normal physiological functions, it can however display harmful effects in the occupational (and also environmental) setting in which individuals are chronically exposed to high levels of the metal. Mn^{2+} toxicity has been implicated in conditions such as manganism, a Parkinson-like neurological disorder, as well as respiratory conditions such as pneumonia, bronchitis and impaired pulmonary function.[90-93]

Occupational exposure can occur from the chronic inhalation of Mn^{2+}-containing fumes and dust associated with mining and ore-grinding, the ferromanganese, iron and steel industries, welding, and in dry-cell battery factories, as well as in the agricultural sector in workers using Mn^{2+}-based fungicides.[10,94-96]Environmental exposure may occur in those living in close proximity to mining industries where Mn-containing dust can be released into the atmosphere. Concern has also been raised about possible environmental exposure to Mn via the combustion of methylcyclopentadienyl manganese tricarbonyl (MMT), used as a fuel additive in some countries.[97]

Human polymorphonuclear leukocytes, predominantly neutrophils, as well as macrophages, are crucially involved in the innate host response to infection by phagocytosing, and killing microbial pathogens via an arsenal of toxic molecules such as proteolytic enzymes, ROS, and bacteriocidal proteins.[98,99]Although potent, these antimicrobial defenses are indiscriminate and may cause significant inflammation-mediated damage to bystander host tissues. Phagocyte-derived ROS are a group of chemically reactive molecules derived from molecular oxygen generated by the catalytic action of the multicomponent enzyme system, NADPH oxidase. The main members include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$) and hypohalous acids. The toxicity of H_2O_2 is enhanced by its reaction with free iron to form highly reactive $OH\cdot$ (Fenton reaction), or by the activity of myeloperoxidase (MPO). In

combination with H_2O_2, MPO can oxidize the halides to hypohalous acids such as hypochlorous acid, a highly reactive oxidizing agent. Up to 80% of the H_2O_2 generated by activated neutrophils is used to form 20–400μM HOCl per hour, depending on the potency of the stimulus and cell number.[100]

Exposure to Mn^{2+} in the environment with subsequent inhalation of particulate matter exposes individuals to both soluble and insoluble forms of Mn^{2+}. The bioaccessibility of Mn^{2+} in lung fluids has been investigated in animal models and found to be about 32 – 52% in fluids which closely mimic the lining fluids of the lungs. Therefore, it is likely that both soluble and insoluble metal components contribute to inhalation-mediated inflammatory responses.[101]Moreover, Mn^{2+} accumulation in the central nervous system following inhalation exposure is dependent on particulate solubility,[102,103] which suggests that the reactivity of the cation is retained during the translocation process. Metals such as Mn^{2+} induce monocytes/macrophages to release proinflammatory cytokines such as TNF-α, IL-1β and IL-8,[104] which are important in mediating transendothelial migration and chemotaxis of neutrophils. In keeping with these observations, metal extracts from welding fumes have been shown in a rat model to trigger a cytokine response and an influx of neutrophils into the animals' lungs with consequent lung injury.[104]Pulmonary cytotoxicity was confirmed by measuring lactate dehydrogenase and albumin concentrations in bronchoalveolar lavage fluid.[104]

Oxidative injury has also been implicated in the pathogenesis of Mn^{2+}-mediated toxicity.[105]However, the putative molecular/biochemical mechanisms, including possible interactions of the metal with the ROS-generating neutrophil, remain to be established. The primary aim of the current study was to investigate the effects of $MnCl_2$ on the generation of ROS by activated human neutrophils, as well as by human monocyte-derived macrophages in a more limited series of experiments.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

Manganese chloride ($MnCl_2$) was purchased from Sigma-Aldrich (St Louis, MO, USA), dissolved in distilled water to a stock concentration of 10mM, and used in the various assays described below at a final concentration range of 1-100µM. Unless indicated, all other chemicals and reagents were purchased from Sigma–Aldrich.

2.2.2 Neutrophil and monocyte preparation

Isolation procedures for neutrophils and monocytes, as well as the monocyte maturation procedure, were performed as described below. The monocyte maturation procedure was based on that developed by MP Motheo, an MSc student of the Department of Immunology, University of Pretoria.

2.2.2.1 Preparation of neutrophils

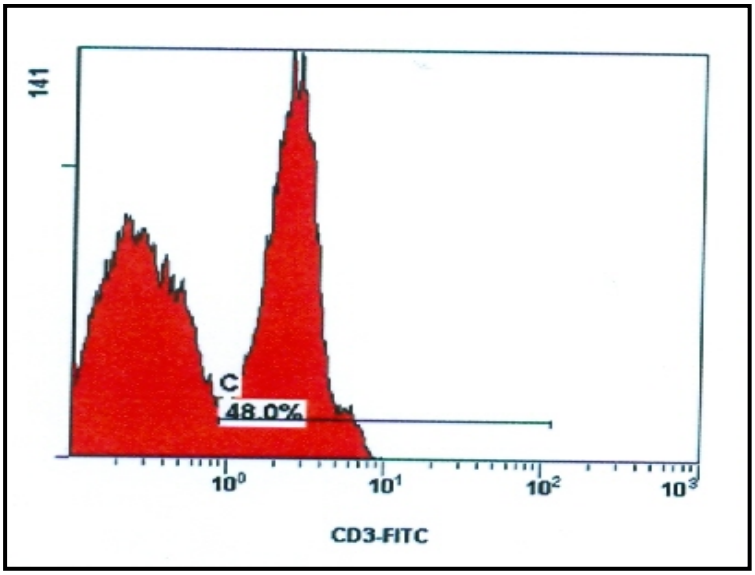
Permission to draw blood from healthy, non-smoking adult human volunteers was granted by the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria (Protocol number 43/2006, date of renewal: 29/6/2009). Subsequent to obtaining informed consent, neutrophils were prepared from heparinized (5 units of preservative-free heparin/ml) venous blood and separated from mononuclear leukocytes by centrifugation on Histopaque-1077 (Sigma-Aldrich) cushions at 400g for 25min at room temperature. The resultant cell pellet was suspended in phosphate-buffered saline (PBS, 0.15M, pH 7.4) and sedimented with 3% gelatin to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10min. Subsequent to another centrifugation step and after the supernatant fluid had been removed, the cells

were washed in PBS (150g for 10min). The neutrophil pellet was resuspended in PBS and the cells enumerated as follows: 50µl of the suspension was added to 0.45ml of leukocyte counting fluid, placed under the coverslip of a haemocytometer and the number of neutrophils counted microscopically. The neutrophils, which were routinely of high purity (>90%) and viability (>95%, propidium iodide exclusion), were resuspended to $1x10^7$/ml in PBS and held on ice until used.

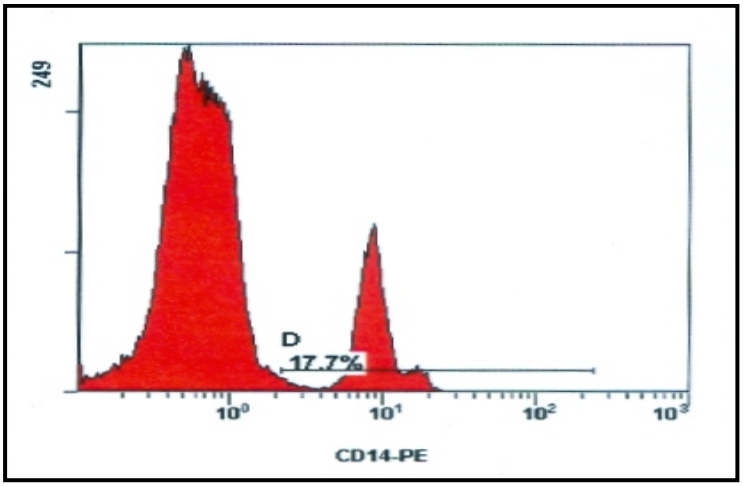
2.2.2.2 Monocyte/macrophage isolation and culture

Human venous blood was also used for the isolation of mononuclear cells (MNL). Following centrifugation of heparinized whole blood on Histopaque-1077 cushions as described above, the mononuclear leukocyte (MNL) fraction at the plasma/ Histopaque-1077 interface was aspirated, diluted 1/5 with PBS and the cells pelleted by centrifugation after which contaminating erythrocytes were removed by hypotonic lysis with ammonium chloride. Subsequent to another centrifugation step and discarding of the supernatant fluid, the cells were resuspended in sterile Hanks' balanced salt solution (HBSS, indicator-free, containing 1.25mM $CaCl_2$, pH 7.4, Highveld Biological, Johannesburg). The cell preparation was then analysed flow cytometrically using a Beckman Coulter FC500 Flow Cytometer using the following fluorochrome-labelled monoclonal antibodies (Beckman Coulter, Miami, FL, USA): CD3 (FITC), CD14 (PE), and CD19 (PE) for analysis and enumeration of total T cells, monocytes and B-cells, respectively. A representative analysis is shown in Figure 2.1 (A-C), Page 49-50. These histograms show that the MNL suspension for this particular MNL population consisted of 48% T cells (CD3), 17.7% monocytes (CD14) and 12.3% B-lymphocytes (CD19).

A



B



C

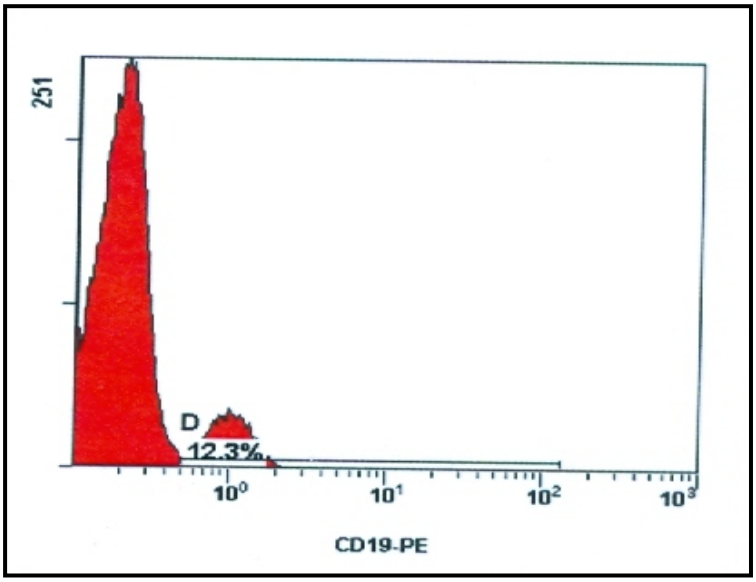
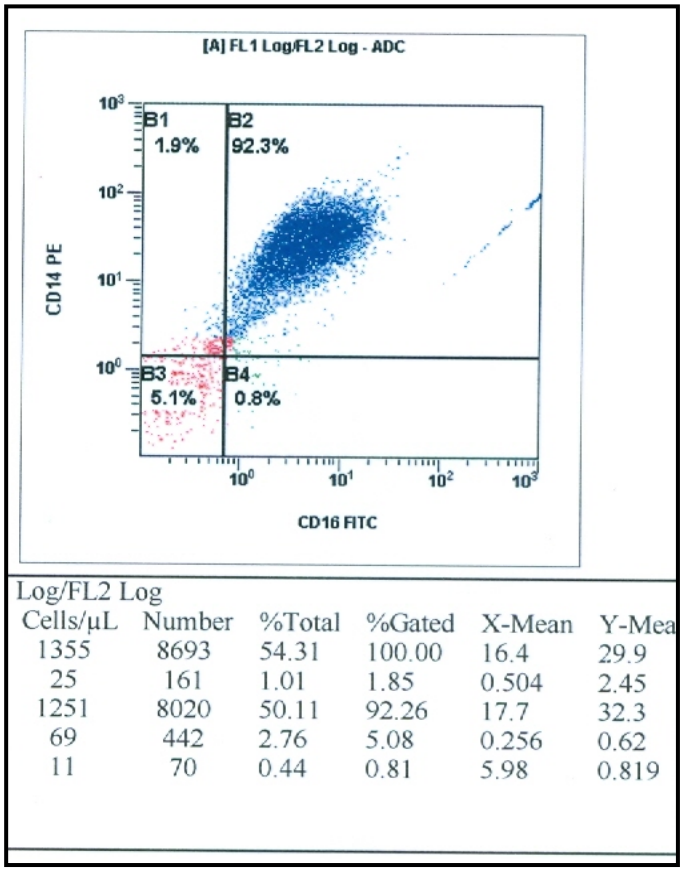


Figure 2.1(A-C): A representative, flow cytometric analysis of a preparation of mononuclear cells, isolated as described above, using monoclonal antibodies for CD3 (T-lymphocytes), CD14 (monocytes) and CD19 (B-lymphocytes) fluorochrome-labelled monoclonal antibodies.

Differential adherence to plastic was used to separate monocytes from other types of MNL and to promote their differentiation. MNL (30ml of a $3x10^7$ MNL/ml suspension in HBSS) was seeded onto sterile $75cm^3$ tissue culture flasks (Falcon, Becton Dickenson, Labware) and incubated for 2 hours at 37°C/ 5% CO_2 to promote adherence of monocytes. Following incubation, each flask was gently rinsed with 50ml of pre-warmed PBS to remove non-adherent cells. Thirty milliliters of tissue culture medium RPMI 1640 (Bio Whittaker, Walkersville, MD, USA) supplemented with, antibiotics (penicillin: streptomycin: amphotericin B, 0.1:0.25:0.1µg/ml) and 5% autologous serum were then added to each flask, which were then incubated for 7 days at 37°C/ 5% CO_2 as described previously. [106]

Following the 7-day incubation period, the tissue culture medium was discarded and each flask rinsed once with 10ml pre-warmed PBS, followed by addition of 10ml Ca^{2+}-free HBSS containing the Ca^{2+}-chelating agent ethylene glycol-bis (2-aminoethylene)-N,N,N,N-tetraceticacid (EGTA, 2mM, final) and the flasks placed on ice with gentle agitation every 10 minutes for at least 30 minutes, to promote detachment of the cells, which were then dislodged by scraping the surface of the flask with a sterile 1.8 x 25cm Cell Scraper (Adcock Ingram, Scientific Group). The cells were then pelleted by centrifugation, the supernatant discarded and the cell pellet resuspended in 3ml of Ca^{2+}-free HBSS containing 2mM EGTA. The cell suspension (350µl) was then analyzed flow cytometrically using the following combinations of fluorochrome-labelled monoclonal antibodies: CD14-PE/ CD16-FITC (monocytes/macrophages) and CD3-FITC/ CD19-PE (T-cells and B-cells). A representative analysis is shown in Figure 2.2, Page 52 in which it can be seen that 92.3% of the cells expressed both CD14 and CD16, having also become larger than monocytes. This is compatible with maturation of monocytes into macrophages. There was a very low

frequency of T-cells (CD3) and B-cells (CD19), results of which are not shown.



Log/FL2 Log

Cells/µL	Number	%Total	%Gated	X-Mean	Y-Mea
1355	8693	54.31	100.00	16.4	29.9
25	161	1.01	1.85	0.504	2.45
1251	8020	50.11	92.26	17.7	32.3
69	442	2.76	5.08	0.256	0.62
11	70	0.44	0.81	5.98	0.819

Figure 2.2: Data shown is for adherent cells co-expressing of CD14 and CD16. In this cell population, >90% of monocytes have matured into macrophages.

2.2.3 Preparation of macrophages for the morphological examination using scanning electron microscopy (SEM)

Mononuclear leukocytes (MNL) were cultured for 7 days. This was followed by scraping and washing with PBS as described before. Cells ($1x10^5$ /ml PBS) were mixed with 20µl of $CaCl_2$andcarefully layered on a round coverslip placed in a24-well plate. Cultures were then incubated for 2 hours at 37^oC/ 5% CO_2after which the monolayer was fixed with 2.5% gluteraldehyde in a 0.075M phosphate buffer at pH 7.4. After 60 minutes the fixative was removed and each well was washed three times (15 minutes each) with 0.075M phosphate buffer. Post-fixation was then done for 60 minutes in a 1% osmium tetraoxide (OsO_4) solution. After post-fixation each well was washed three times, (15 minutes each) with 0.075M phosphate buffer to remove any remaining osmium tetraoxide. Once the washing step was complete, a serial dehydration step was performed with ethanol at concentrations of: 30%, 50%, 70%, 90% and three times, 100%. Each dehydration step was performed for 15 minutes. The cover slips were then removed from the 24-well plates and dried, by means of CO_2 critical point drying procedures; samples were mounted, coated with gold and examined with a JEOL 840 scanning electron microscope.

A scanning electron micrograph of a typical monocyte-derived macrophage prepared by the above procedure is shown in Figure 2.3, Page 54 and clearly shows a mature phenotype with ruffled membrane and pseudopodia. This was also included in the dissertation of MSc student MP Motheo (Department of Immunology, University of Pretoria).

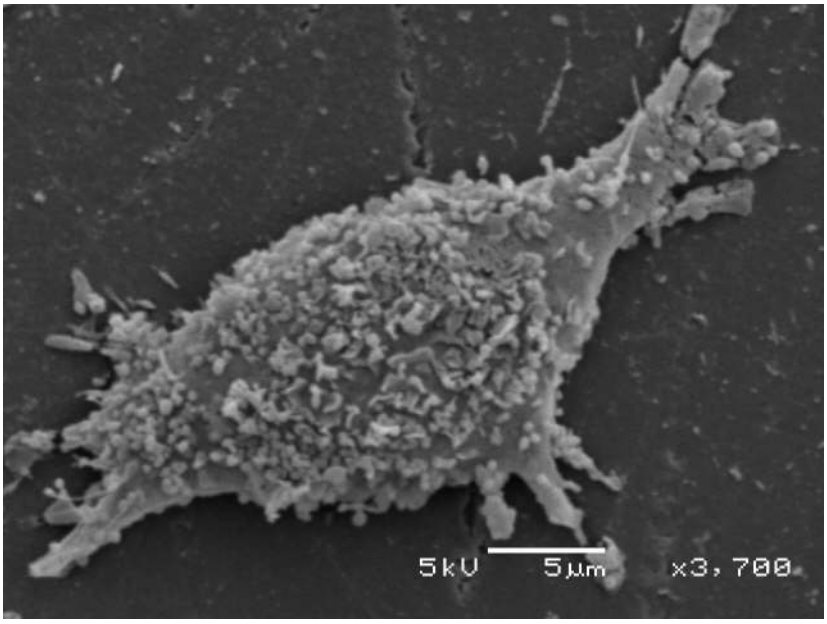


Figure 2.3: Scanning electron micrograph showing matured monocyte-derived macrophage.

The monocyte-derived macrophages were used in the assays of lucigenin chemiluminescence, intracellular H_2O_2 and NO production described below.

2.2.4 Measurement of the effects of Mn^{2+} on the generation and reactivities of ROS

When used in combination to measure the generation of ROS by activated neutrophils and cell-free systems, the assays shown in Table 2.1, Page 56 not only enable identification of the type of ROS, but also the mechanism involved *i.e.* increased production, conversion of one type of ROS to another, and/or ROS-scavenging activity.

Table 2.1: Strategies used to identify the effects of Mn on the generation of ROS by activated neutrophils and cell-free enzymatic systems, as well as on the reactivity of these oxidants

Assay	Application using:	
	Activated Neutrophils	Cell-free systems
Lucigenin-enhanced CL*	Primarily detects superoxide production	To detect superoxide scavenging activity using the xanthine/xanthine oxidase system
Luminol-enhanced CL	Primarily detects oxidants generated by the MPO/H_2O_2/halide system e.g HOCl (hypochlorous acid)	To monitor hydroxyl radical-scavenging activity using a glucose/glucose/vanadium(3^+) system
Dichlorofluorescein diacetate fluorescence	Detects intracellular hydrogen peroxide	-
MPO^+-mediated iodination	Detects hypohalous acids	To determine effects on MPO, as well as hypohalous acid-scavenging activity using a system with added MPO, ^{125}I, bovine serum albumin, and glucose/glucose oxidase to generate hydrogen peroxide
Oxygen Consumption	To measure effects on activation/activity of NADPH oxidase	To validate superoxide-scavenging activity using the xanthine/xanthine oxidase system

* CL= chemiluminescence; + MPO=myeloperoxidase

2.2.5 Lucigenin-enhanced chemiluminescence

Superoxide production was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence method.[107] Neutrophils ($1x10^6$/ml, final) were preincubated for 10min in 900µl indicator-free Hanks' balanced salt solution (HBSS, pH 7.4, 1.25mM $CaCl_2$) containing 0.2mM lucigenin in the presence and absence of $MnCl_2$ (0.1-100µM). The reaction mixtures were then stimulated with either the phorbol ester, PMA (phorbol 12-myristate 13-acetate; 25ng/ml final) or the synthetic chemoattractant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1µM final), and chemiluminescence responses recorded in an LKB Wallac chemiluminometer (Turku, Finland) for PMA-activated systems or a Lumac Biocounter 2010 for FMLP-stimulated systems. The results are expressed as the peak responses in mV/sec and relative light units for PMA- and FMLP-activated systems respectively. These peak responses occurred at 30-60sec (FMLP) and 5min (PMA).

The effects of $MnCl_2$ (50-400µM) on lucigenin-enhanced chemiluminescence were also determined using PMA-activated monocytes/macrophages ($3x\ 10^5$cells/ml) suspended in Ca^{2+}-replete HBSS. The set-up of these experiments was similar to that described above for neutrophils.

Additional experiments were undertaken to determine the potential of $MnCl_2$ to scavenge superoxide, using a cell-free, xanthine/xanthine oxidase superoxide-generating system. Reaction mixtures consisted of xanthine oxidase (64mU/ml), lucigenin (0.2mM) and xanthine (0.9mM) in the presence or absence of $MnCl_2$ (6.25-100µM) in a final volume of 1ml HBSS. Results are expressed as the peak chemiluminescence responses which occurred after approximately 14min.

2.2.6 Oxygen Consumption

This was measured using a three-channel oxygen electrode (Model DW1, Hansatech Ltd, King's Lynn, Norfolk, UK). Neutrophils ($2x10^6$) were preincubated for 10 minutes at 37°C in HBSS in the presence or absence of $MnCl_2$ (25μM) followed by activation of the cells with PMA (25ng/ml). PO2 was monitored for a further 10 minutes. Oxygen consumption was measured over the period when consumption was linear, which was 7minutes, and the results expressed as nmol O_2 consumed/min/$2x10^6$ cells.

2.2.7 Luminol-enhanced chemiluminescence

Luminol-(5-amino-2,3-dihydro-1,4-phthalazine dione)-enhanced CL, which detects hydroxyl radical,[108] was used in the following cell-free experiments to determine: i) the potential of $MnCl_2$ to generate hydroxyl radical from H_2O_2 by a Fenton type reaction; and ii) the effects of $MnCl_2$ on the generation of hydroxyl radical in a Fenton reaction involving the interaction of vanadium (III) chloride with H_2O_2.[109] In the case of the former, reaction systems contained luminol (0.1mM), glucose, (5mM in HBSS) and glucose oxidase (400mU/ml from *Aspergillus niger)* without and with $MnCl_2$ (25μM) in a final reaction volume of 1 ml. The latter system also contained luminol, glucose and glucose oxidase, as well as vanadium (III) chloride (25μM) without and with $MnCl_2$(25μM)and the hydroxyl scavenger, mannitol (20mM). In both experimental systems the reactions were initiated by the addition of glucose oxidase, and luminol-enhanced chemiluminescence measured using the LKB Wallac 1251 chemiluminometer as described above.

2.2.8 Intracellular H_2O_2 production

Intracellular H_2O_2 was measured using a 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA)-based spectrofluorimetric procedure.[110]This agent is hydrolyzed by cellular esterases to 2',7'-dichlorodihydrofluorescein which is oxidized to 2',7'-dichlorofluorescein primarily by H_2O_2. DCF-DA (1μM) was added to a reaction mixture containing $1x10^6$/ml neutrophils or $1x10^5$/ml monocyte-derived macrophages in the absence and presence of $MnCl_2$ (3-25μM) in a total volume of 3ml HBSS. The samples were then incubated for 10 minutes in a 37°C waterbath, then transferred to the thermoregulated cuvette holder of a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 500 and 530nm, respectively. Baseline fluorescence responses and those activated with FMLP (1μM) or PMA (25ng/ml) were then monitored for 10min. These experiments were performed in the presence and absence of the MPO inhibitors sodium azide (0.7mM) or 4-aminobenzoic acid hydrazide (ABAH, 50μM) to eliminate the complicating effects of oxidation by hypochlorous acid.[111,112] These results are shown as the traces of each experiment.

The validity of using DCF-DA as an oxidizable substrate for the spectrofluorimetric detection of H_2O_2 was confirmed in a series of preliminary experiments using a cell-free H_2O_2-generating system (glucose/glucose oxidase), which clearly demonstrated an intense increase in fluorescence intensity (data not shown).

2.2.9 MPO-mediated protein iodination

This was performed according to the method of Root and Stossel with minor modifications.[113]Neutrophils were preincubated for 10 min at 37°C in 900μl of HBSS containing 1μCi of iodine-125 (as $Na^{125}I$, 37MBq, Perkin Elmer Life and Analytical Sciences, Boston, MA, USA), 20μM cold carrier NaI and 2mg/ml of

bovine serum albumin (BSA) in the presence and absence of $MnCl_2$ (0.5-100μM). The cells were then activated by addition of either FMLP (1μM) or PMA (25ng/ml) and incubated for 10min at 37°C after which the protein in the reaction mixtures was precipitated by addition of 20% trichloroacetic acid (TCA) and the precipitates pelleted by centrifugation, followed by 3 more wash steps with TCA to remove unbound ^{125}I. The amount of protein-associated ^{125}Iin the precipitates was determined using a Perkin Elmer 2470 Automatic Gamma Counter and the results expressed as pmols ^{125}I per $2x10^6$ cells.

The following additional experiments were performed to determine: i) the effects of DPI (diphenyleneiodonium chloride-10μM), an inhibitor of NADPH oxidase, or sodium azide (0.7mM), on modulation of MPO-mediated iodination by $MnCl_2$(25μM);ii) the effects of $MnCl_2$ at a fixed concentration of 50μM on MPO-mediated protein iodination in a cell-free system containing 50mU/ml of purified MPO (from human leukocytes), $Na^{125}I$ (2μCi), 2mg/ml BSA and glucose (5mM in HBSS)/glucose oxidase (1.5U/ml) as a source of H_2O_2 in a final reaction volume of 1ml. Reactions were terminated and BSA precipitated after 10min of incubation and the protein precipitates processed and analysed as above.

2.2.10 MPO release

MPO was measured in the supernatants of neutrophils activated with FMLP (1μM)/cytochalasinB (1μM, added to enhance degranulation), or PMA (25ng/ml), in the absence and presence of 50μM $MnCl_2$. Neutrophils (2 $x10^6$/ml, final) in HBSS were pre-incubated for 10min at 37°C with $MnCl_2$ after which the cells were activated and the reaction mixtures incubated for a further 10min at 37°C. The tubes were then transferred to an ice-bath, followed by centrifugation at 400g for 5min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for MPO using a double-

antibody, capture ELISA procedure (Kamiya Biomedical Company, Seattle, WA, USA) and the results expressed as ng MPO/ml supernatant.

2.2.11 Nitric oxide production

For these experiments, monocyte-derived macrophages suspended in HBSS were added to the wells of micro-tissue culture plates (10^5 cells/well, final volume 200µl) and incubated for 2 hours at 37°C/ 5% CO_2 to promote adherence, after which the HBSS was replaced with serum-supplemented RPMI 1640 and the plates incubated overnight at 37°C/ 5% CO_2. The next day the RPMI 1640/ serum medium in each well was replaced with HBSS without and with $MnCl_2$ (100µM) followed 30 min later by the macrophage activator, bacterial lipopolysaccharide (from *Escherichia coli* 0127:B8, 1µg/well, final). The plates were then incubated for 24 hours at 37°C/ 5% CO_2 after which the cell-free supernatants were assayed spectrophotometrically for nitrite as a surrogate for NO using the Calbiochem Colorimetric Nitric Oxide Kit (Calbiochem - EMD4 Biosciences, San Diego, CA, USA). Using this procedure, nitrate, also a product of NO is converted to nitrite by the addition of nitrate reductase. Total nitrite is then determined spectrophotometrically using the Greiss reagent at a wavelength of 540nm and the results expressed as nmol nitrite/10^5 cells.

2.2.12 Cellular ATP levels

To determine the effects of $MnCl_2$ (100µM) on neutrophil viability, intracellular ATP concentrations were measured in cell lysates ($2x10^6$ cells/ml) following exposure of the cells to the metal for 10min using a luciferin/luciferase chemiluminescence procedure.[114]

2.2.13 Statistical analysis of data

With respect to organization of data in the "Results" section, these are grouped according to the assays mentioned in the "Materials and Methods" section. In the cases of MPO-mediated iodination and MPO release, only data for neutrophils are shown because of the absence of this enzyme in macrophages, while in the case of nitric oxide production only data for activated macrophages are shown.

The results of each series of experiments are presented as the mean values ± SEM, either as the absolute values or as mean percentages of the corresponding metal-free control systems, where *n* = the number of different donors used in each series of experiments. Levels of statistical significance were determined by comparing the absolute values for each metal-treated system with the corresponding values for the relevant metal-free control systems for each assay using the Wilcoxon matched-pairs signed-ranks test.

2.3 Results

2.3.1 Lucigenin-enhanced chemiluminescence

The effects of $MnCl_2$ on superoxide production by neutrophils activated with FMLP (1μM) or PMA (25ng/ml) are shown in Figure 2.4, Page 64. $MnCl_2$ at concentrations of 0.1-100μM and 1.5-100μM in the case of FMLP-and PMA-activated cells, respectively, caused significant dose-related inhibition of the lucigenin-enhanced CL responses. In the case of macrophages, inhibitory effects on chemiluminescence were only seen at higher concentrations of $MnCl_2$and the results are shown in Figure 2.5, Page 65.

The cell-free xanthine/xanthine oxidase superoxide-generating system was used to assess the superoxide-scavenging potential of $MnCl_2$. These results

are shown in Figure 2.6, Page 66, which demonstrate dose-dependent, statistically significant inhibition of chemiluminescence in the presence of the metal. (*P<0.05 for comparison of each concentration of the metal with the $MnCl_2$-free control system; data from 8 experiments).

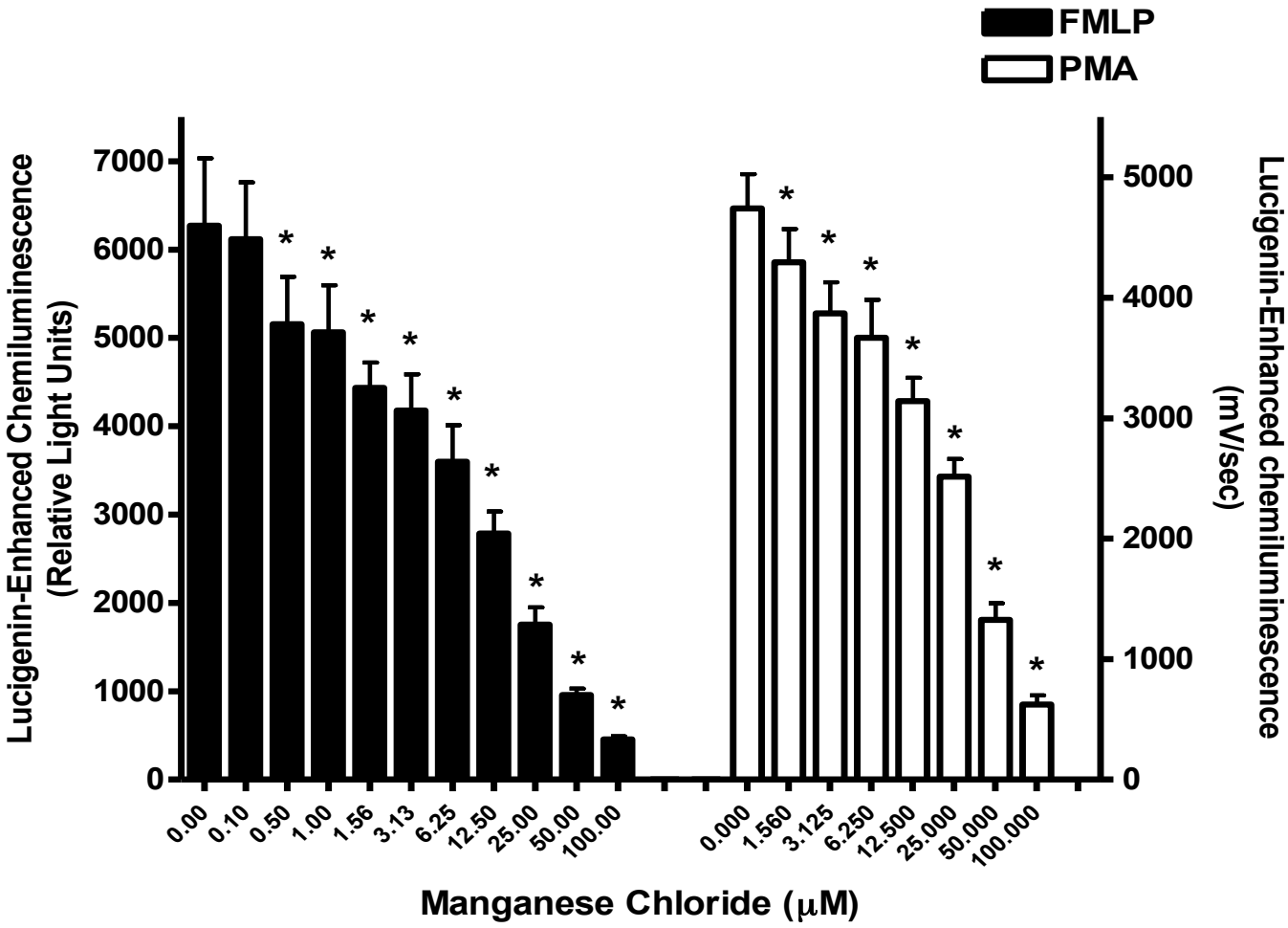


Figure 2.4

Effects of $MnCl_2$ (0.1-100μM) on the lucigenin-enhanced chemiluminescence responses of neutrophils activated by FMLP (1μM) and PMA (25ng/ml). The results are expressed as the mean peak chemiluminescence values in relative light units (FMLP systems) and mV/sec (PMA systems) ±SEM (n=5 with 2-5 replicates for each system). The absolute values for the unstimulated and FMLP-activated systems and those for the unstimulated and PMA-activated systems were 3453±1290 and 6272±763 relative light units, and 623±94 and 4742±284 mV/sec,respectively. *P<0.05 for comparison with the $MnCl_2$-free control system.

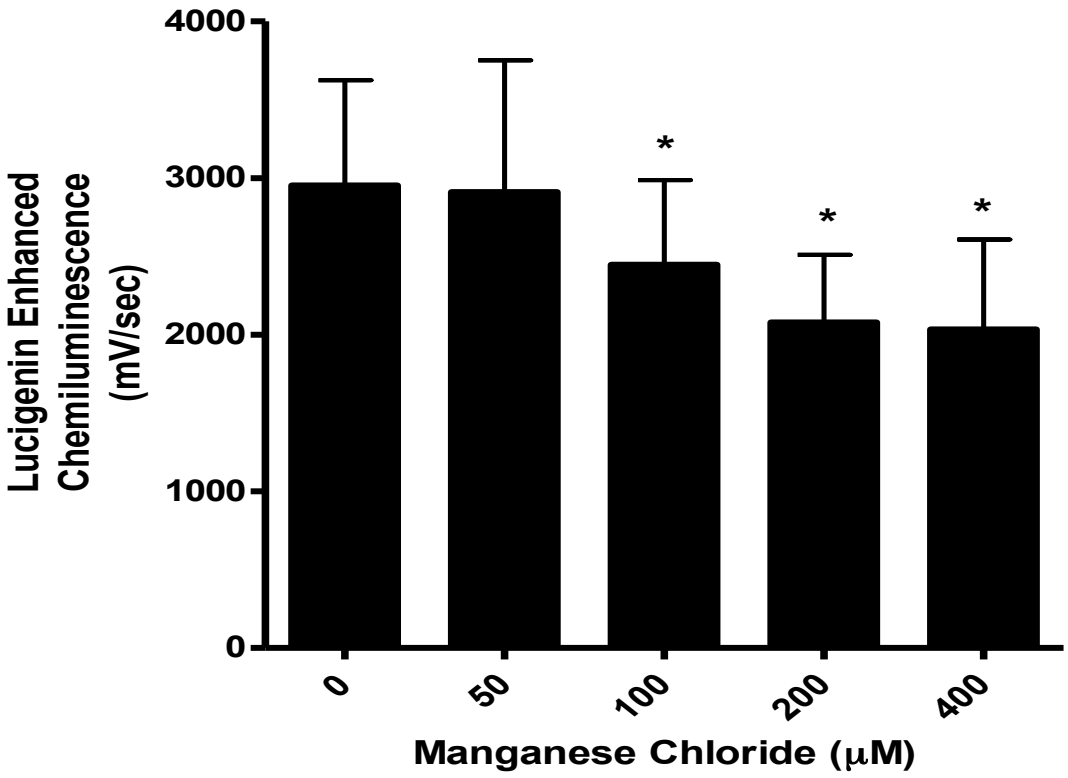


Figure 2.5

Effects of $MnCl_2$ (50-400μM) on the lucigenin-enhanced chemiluminescence responses of macrophages activated by PMA (25ng/ml). The results are expressed as the mean peak chemiluminescence values in mV/sec ±SEM (n=5). The absolute values for the unstimulated and PMA-activated systems were 557±177 and 2952±842 mV/sec, respectively. *P<0.05 for comparison with the $MnCl_2$-free control system.

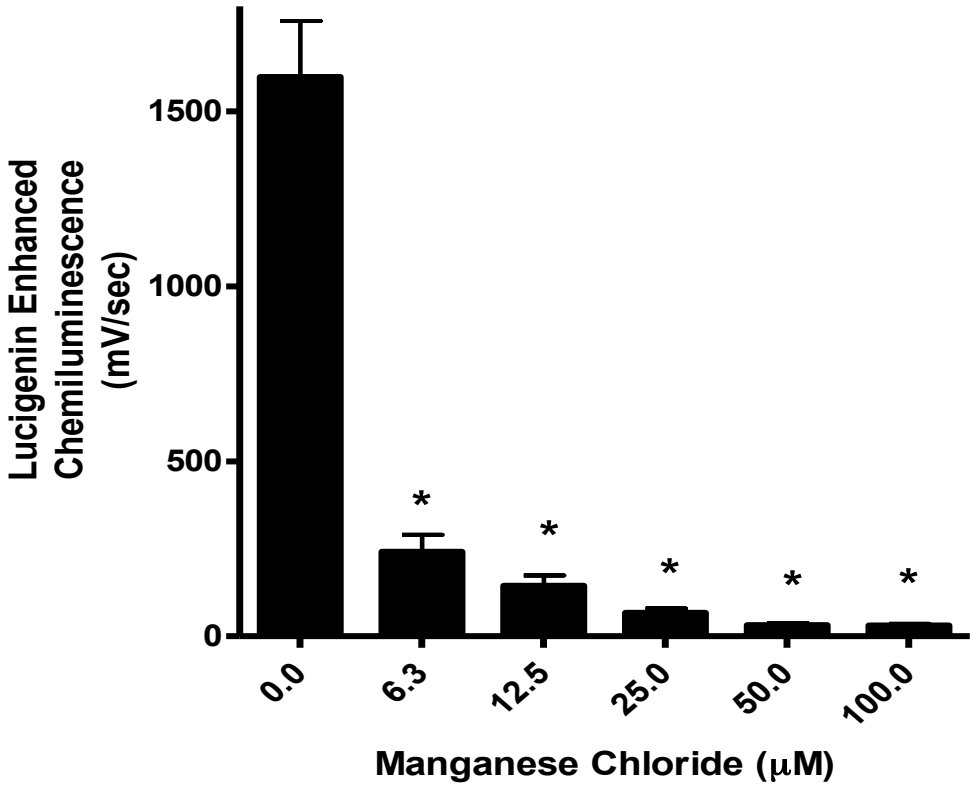


Figure 2.6

Effects of $MnCl_2$ (6.25-100μM) on the lucigenin-enhanced chemiluminescence responses of the cell-free, xanthine/xanthine oxidase superoxide-generating system. The results are expressed as the mean peak chemiluminescence values in mV/sec ± SEM (8 experiments). *P<0.05 for comparison with the $MnCl_2$-free control system.

2.3.2 Oxygen Consumption

Activation of neutrophils by PMA was accompanied by a marked increase in oxygen consumption by the cells that was sustained over a 5-10min period and unaffected by $MnCl_2$ (25µM). The results for the control PMA-stimulated systems and those treated with 25µM of $MnCl_2$ were 64±4 and 62±6 nmol O_2 consumed/min/2 x 10^6 cells, respectively (n=6). The corresponding values for unstimulated cells in the presence and absence of the metal were 21± 2 and 22±2 nmol/min/2x10^6 cells. These results clearly demonstrate that Mn^{2+} does not activate NADPH oxidase. Likewise oxygen consumption by the xanthine/xanthine oxidase system was unaffected by $MnCl_2$, excluding possible inhibitory effects on the oxidase. Results for oxygen consumption by the xanthine/xanthine oxidase control and $MnCl_2$ (25µM)-treated systems were 86±2 and 86±6 nmol O_2 consumed/min, respectively (data from 3 experiments).
Taken together these results are compatible with superoxide dismutase mimetic activity of $MnCl_2$which is clearly evident in both the neutrophil and xanthine oxidase systems.

2.3.3 Luminol-enhanced chemiluminescence

The cell-free, glucose/glucose oxidase, H_2O_2-producing, luminol-enhanced chemiluminescence system was used to assess the potential of $MnCl_2$ to: i) initiate hydroxyl radical generation via a Fenton-type reaction; and ii) to scavenge hydroxyl radical generated via a Fenton-type reaction (the interaction of vanadium (III) chloride (25µM) with H_2O_2). Luminol-enhanced chemiluminescence values for the control, glucose/glucose oxidase containing systems and those treated with 25µM $MnCl_2$ were neglible, being 11±2 and10 ± 3 mV/sec respectively, demonstrating lack of reactivity of H_2O_2 with luminol, as well as H_2O_2 with $MnCl_2$. Addition of vanadium III to the H_2O_2-producing systems, however, resulted in a significant increase in chemiluminescence with a peak response of 2142±235 mV/sec (P=0.008 for comparison with the vanadium-free

control system); inclusion of $MnCl_2$ significantly (P=0.008) attenuated the chemiluminescence signal generated by the vanadium III/H_2O_2 interaction which decreased to 175±36 mV/sec (data from 8 experiments). Importantly, the vanadium/H_2O_2 chemiluminescence signal was also attenuated by the traditional hydroxyl radical scavenger, mannitol (20mM). Luminol-enhanced CL values for the control, glucose/glucose oxidase systems and those treated with vanadium only or vanadium + mannitol were 37±7, 3107±411 and 1074±206 mV/sec, respectively (data from 5 experiments).

Taken together, these results demonstrate that under the experimental conditions used $MnCl_2$ does not interact with H_2O_2 to generate hydroxyl radical, but rather appears to function as a scavenger of this potent ROS.

2.3.4 Intracellular H_2O_2 production

The results shown in Figure 2.7, Page 69 are traces from one experiment which depicts the effects of $MnCl_2$ (3-25μM) on the DCF-DA fluorescence responses of FMLP (1μM) - and PMA (25ng/ml)-activated neutrophils in the presence of sodium azide. The results of additional experiments using the metal at a fixed concentration of 25μM are shown in Figures 2.8 and 2.9, Page 70-71. Treatment of the cells with $MnCl_2$ caused a dose-dependent increase in fluorescence intensity compatible with augmentation of intracellular H_2O_2 production by both FMLP and PMA-activated neutrophils. PMA-activated cells treated with $MnCl_2$ in the presence of ABAH showed similar trends (results not shown). As shown in Figure 2.10, Page 72, treatment of macrophages with 25μM $MnCl_2$ also resulted in significant augmentation of PMA-activated intracellular H_2O_2 production.

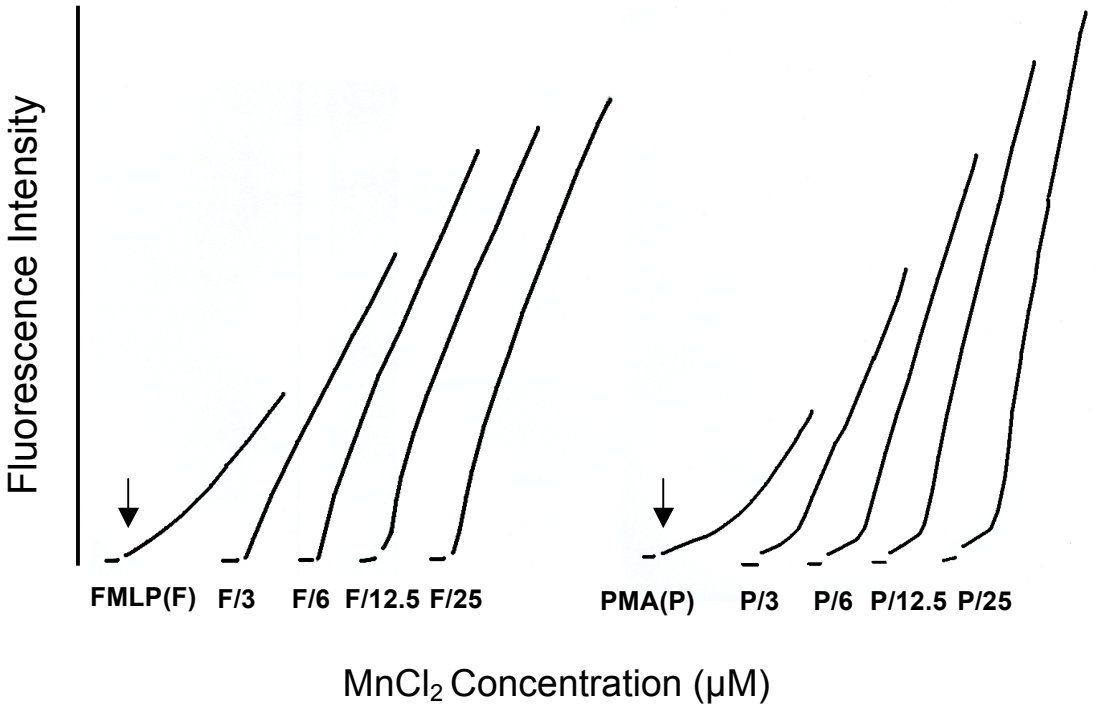


Figure 2.7

Traces from a single representative experiment (n=4) showing the effects of $MnCl_2$ at concentrations of 3-25µM on the dichlorofluorescein diacetate fluorescence responses of FMLP(1µM)-and PMA (25ng/ml)-activated neutrophils. FMLP and PMA were added as indicated (↓) after a stable baseline was obtained.

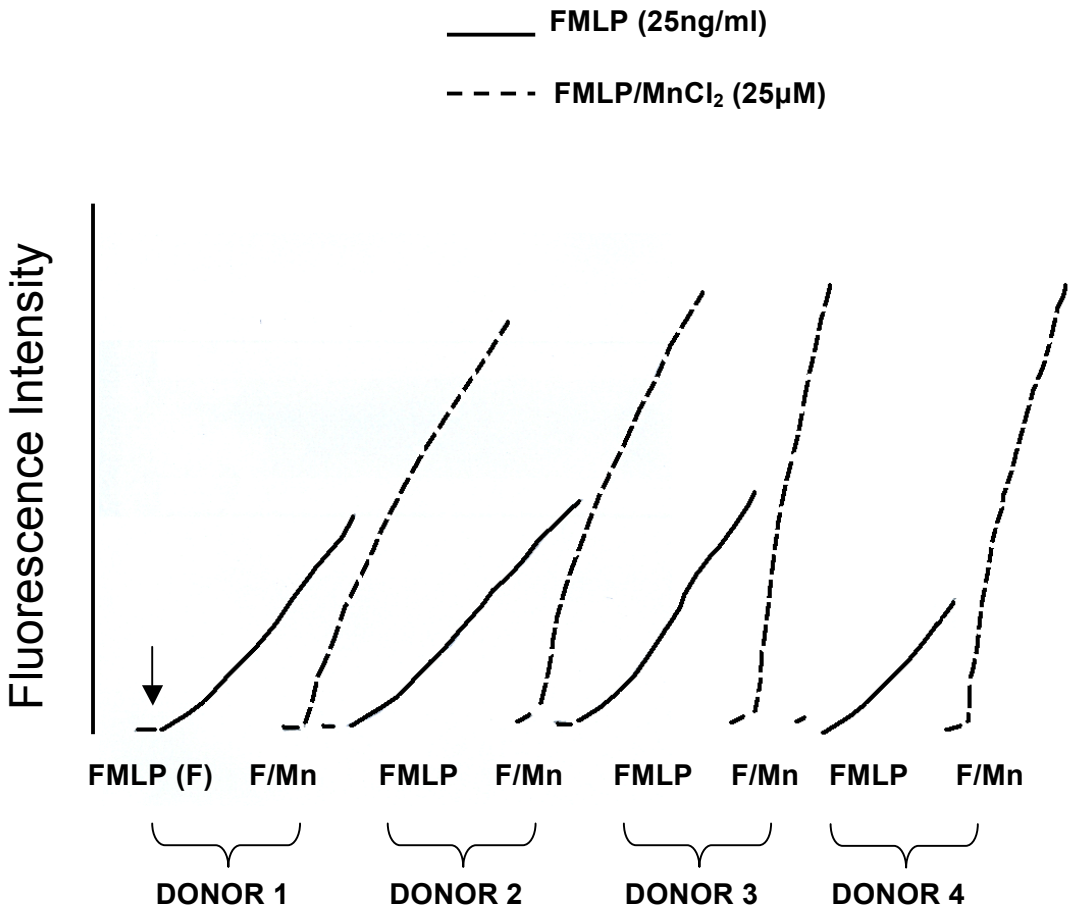


Figure 2.8

FMLP-activated dichlorofluorescein responses in neutrophils activated with FMLP (1µM) in the absence (—) and presence (----) of 25µM $MnCl_2$. FMLP was added as indicated (↓) after a stable baseline was obtained. These are traces obtained from neutrophils of 4 different donors.

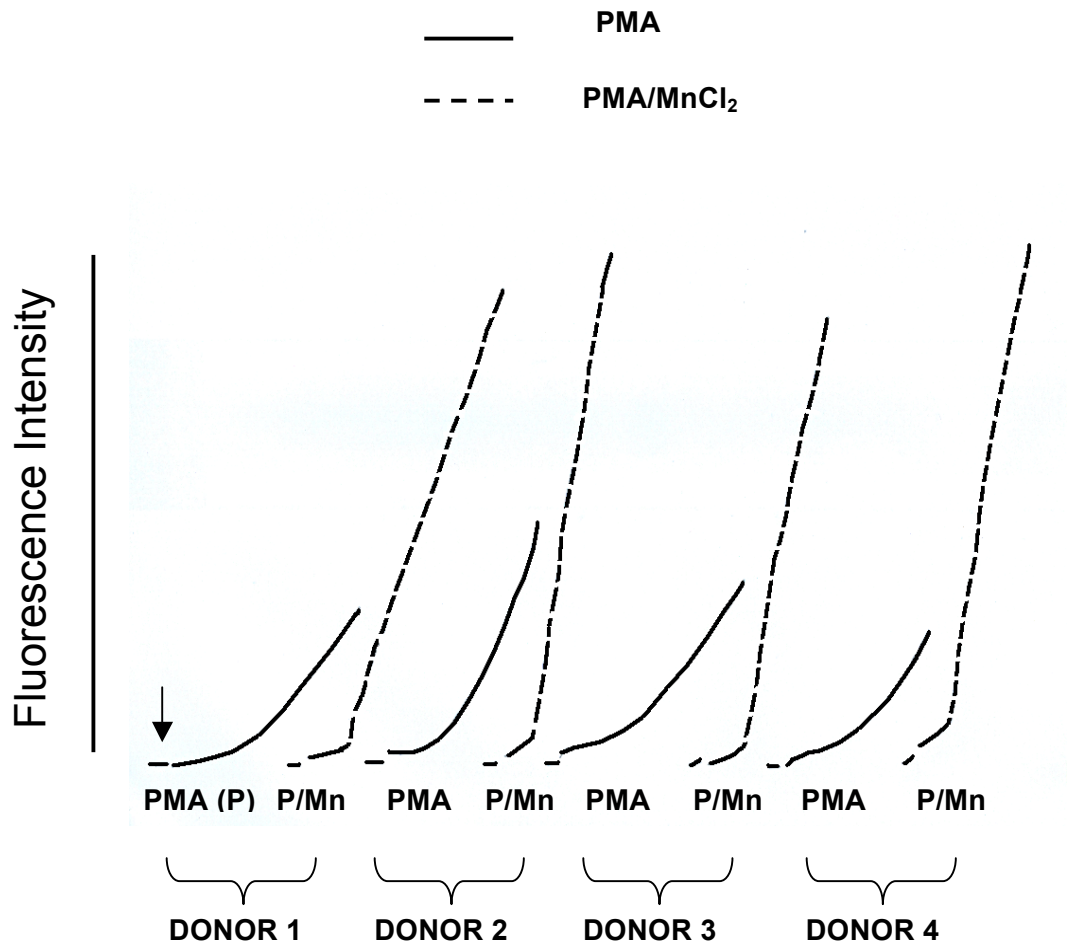


Figure 2.9

PMA-activated dichlorofluorescein responses in neutrophils activated with PMA (25µg/ml) in the absence (—) and presence (----) of 25µM $MnCl_2$. PMA was added as indicated (↓) after a stable baseline was obtained. These are traces obtained from neutrophils of 4 different donors.

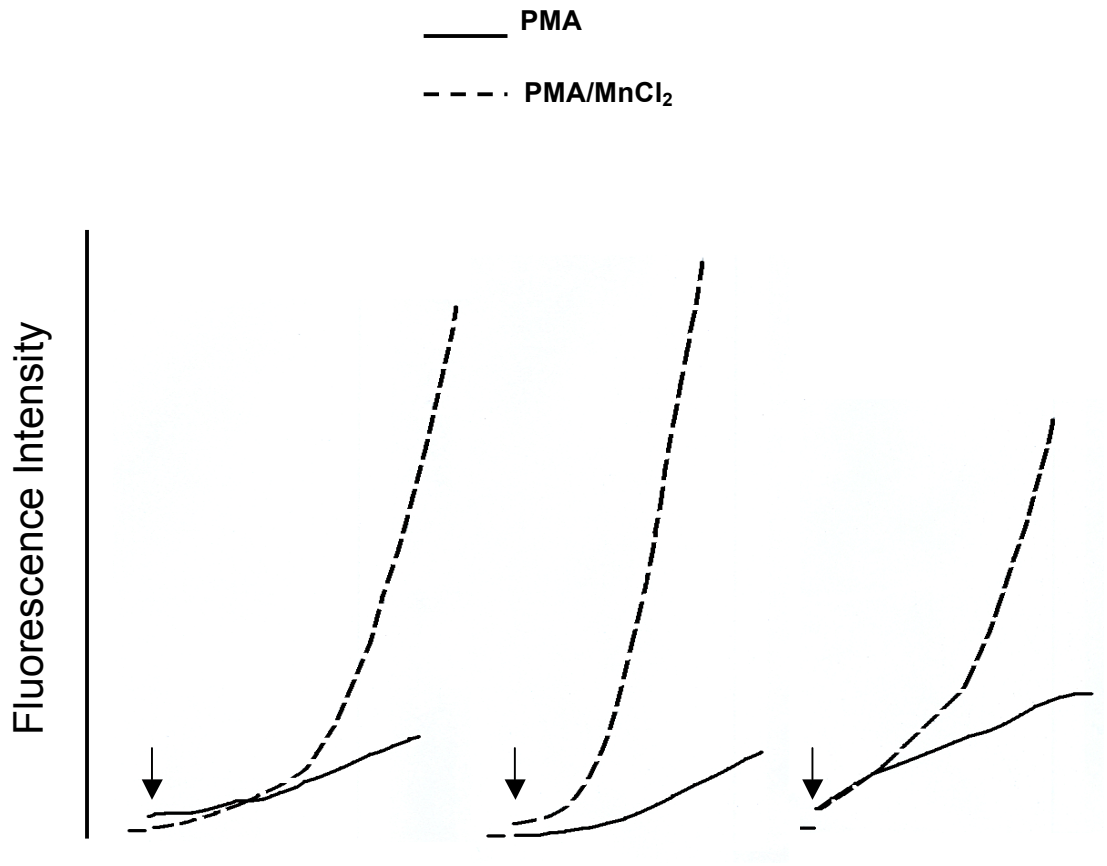


Figure 2.10

PMA-activated dichlorofluorescein responses in macrophages activated with PMA (25µg/ml) in the absence (—) and presence (----) of 25µM $MnCl_2$. PMA was added as indicated (↓) after a stable baseline was obtained. These are 3 typical traces obtained from neutrophils of 4 different donors.

2.3.5 MPO-mediated protein iodination

The effects of $MnCl_2$ (0.5-100μM) on the activity of the MPO/H_2O_2/halide system of FMLP- or PMA-activated neutrophils, are shown in Figure 2.11, Page 74. $MnCl_2$ at concentrations of 0.5μM and higher, caused significant, dose-related enhancement of neutrophil MPO-mediated iodination of BSA following activation of the cells with either FMLP or PMA. In the case of FMLP-activated cells, inclusion of $MnCl_2$ at 0.5-100μM resulted in 19-146% enhancement of MPO-mediated iodination, while with PMA 19-65% enhancement was noted with metal concentrations of 0.5-12.5μM, reaching a plateau thereafter.

The stimulatory effects of $MnCl_2$ (25μM) on FMLP-activated MPO-mediated protein iodination were significantly attenuated by inclusion of DPI (10μM) or sodium azide (1mM). The results for unstimulated cells, the FMLP-activated control system, and systems treated with $MnCl_2$ only, $MnCl_2$ + DPI, or $MnCl_2$ + sodium azide, were: 18±5, 214±39, 465±86, 0.13±0.07* and 141±49* pmol ^{125}I/2x10^6cells, respectively. (*P<0.05 for comparison with $MnCl_2$ only systems).

The effects of $MnCl_2$ (50μM) on the iodination of BSA by a cell-free MPO + glucose/glucose oxidase + ^{125}I system were also evaluated. $MnCl_2$ did not significantly affect protein iodination by the cell-free MPO/H_2O_2/^{125}I system. Values for the systems with and without $MnCl_2$ were 417±29, and 473±42 pmol ^{125}I/2mg BSA respectively, while the corresponding values for the background control systems (without glucose oxidase) were 94±9 and 86±5 pmol ^{125}I/2mg BSA.

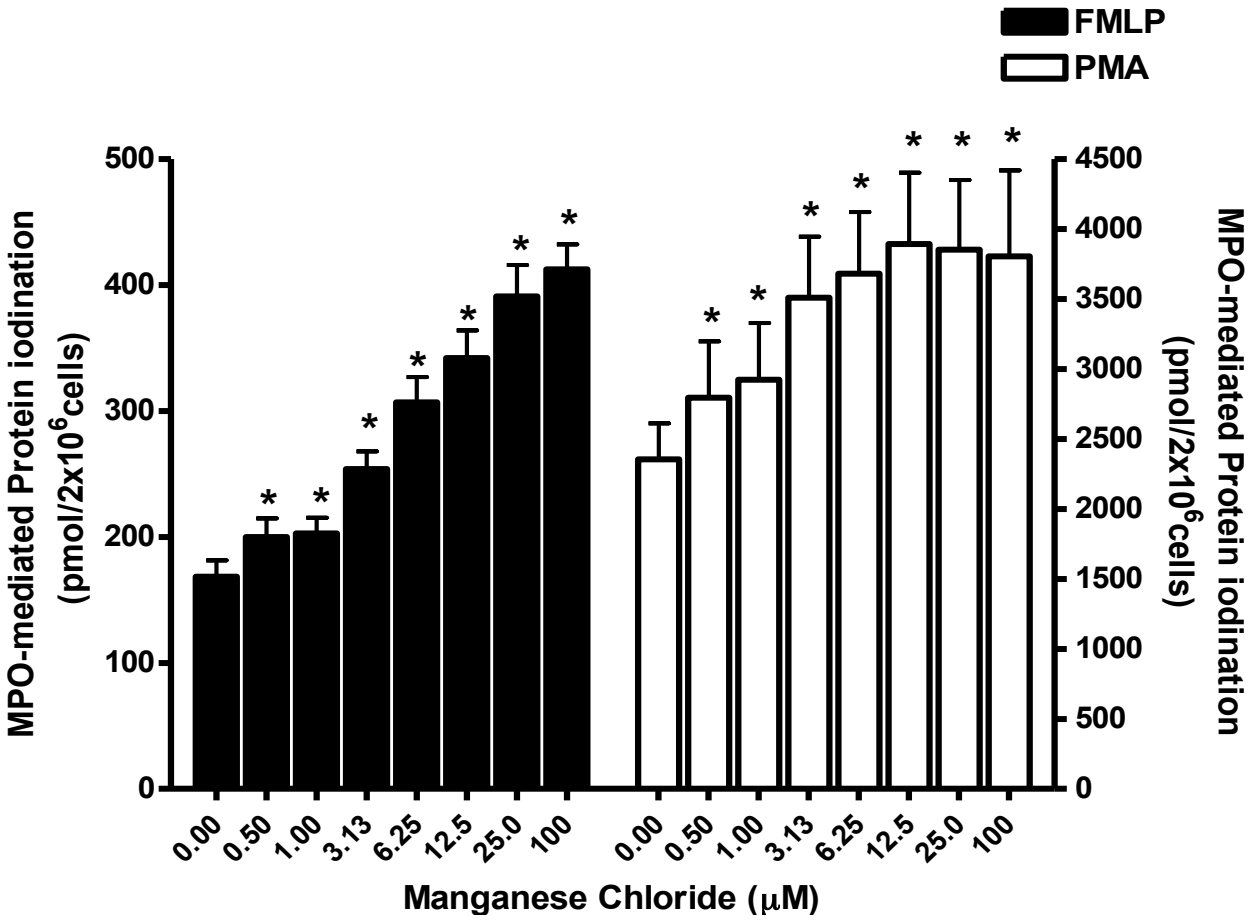


Figure 2.11

Effects of $MnCl_2$ (0.5-100μM) on MPO-mediated iodination of protein by neutrophils activated with FMLP (1μM) or PMA (25ng/ml). The results are expressed as the mean values in pmol $^{125}I/2x10^6$cells ± SEM (n=6 with triplicate values for each system). The absolute values for MPO-mediated iodination for unstimulated, FMLP- and PMA-activated systems were, 14±4, 168±13 and 2354±258 pmol $^{125}I/2x10^6$ cells, respectively. *P<0.05 for comparison with the $MnCl_2$-free control system.

2.3.6 MPO release

MPO release from neutrophils activated with FMLP/cytochalasin B (F/CB) or PMA was not significantly affected by $MnCl_2$. The results for the unstimulated cells, the F/CB-activated control system, and systems treated with 50μM $MnCl_2$were 146±17, 1243±133 and 1160±111ng/ml MPO, respectively. The corresponding results for the PMA-activated control system and those treated with 50μM $MnCl_2$ were 548±50 and 510±43 ng/ml MPO, respectively (n=6).

2.3.7 Nitric oxide production

The respective values for production of NO by control monocyte-derived macrophages and those treated with 100μM $MnCl_2$ were 6.2±0.8 and 6.2±0.8 nmols/10^5 cells, respectively. The corresponding values for LPS-activated control cells and those treated with 100μM of $MnCl_2$ were 9.6±0.8 and 6.6±0.8 nmols/10^5 cells (n=5 with 2 replicates for each system in each experiment; no significant differences were detected with respect to comparison of the control and $MnCl_2$-treated systems).

2.3.8 Cellular ATP levels

Exposure of neutrophils to $MnCl_2$ (100μM) for 10min did not significantly affect cellular ATP levels, demonstrating lack of cytotoxicity of the metal at the concentrations and experimental conditionsin which these cells were used. The values for control cells and those exposed to $MnCl_2$ at concentrations of 100μM were: 211±23 and 204±20 pmols ATP/2x10^7 cells, respectively (n=4, with 3-6 replicates for each system.) Similar results were found using a flow cytometric, propidium iodide dye exclusion procedure, the mean percentages viability for the control and metal-treated (50μM) systems being 99.4±0.1% and 99.5.±0.1%, respectively.

2.4 Discussion

The results of the current study have demonstrated that Mn^{2+} potentiates the production of H_2O_2 by human neutrophils and macrophages. Somewhat paradoxically, these pro-oxidative interactions of Mn^{2+} with human phagocytes are a consequence of the superoxide dismutase mimetic activity of the metal. While H_2O_2 *per se* may predispose to oxidant-mediated tissue damage, the toxicity of this ROS is enhanced via its transformation to hypohalous acids by neutrophils and monocytes.[1]

The SOD mimetic activity of Mn^{2+} was documented in an initial series of experiments in which co-incubation of activated human neutrophils and monocyte-derived macrophages with the metal resulted in dose-dependent inhibition of lucigenin-enhanced chemiluminescence. Relative to neutrophils, somewhat higher concentrations of Mn^{2+} were required to cause significant inhibition of the chemiluminescence responses of activated macrophages, possibly as a consequence of the absence of MPO in these cells, which was confirmed in the current study (not shown). MPO is a negative regulator of superoxide production by activated neutrophils and monocytes.[115,116]The following lines of evidence confirmed that Mn^{2+} neutralizes superoxide as opposed to being an inhibitor of its generation by activated phagocytes: i) similar effects to those observed with activated neutrophils and macrophages were observed using a cell-free xanthine/xanthine oxidase superoxide-generating system; ii) the metal did not affect oxygen utilization by either the phagocyte NADPH oxidase or xanthine oxidase; and iii) the production of NO, which in excess neutralizes superoxide,[117] was unaffected by treatment of macrophages with Mn^{2+}. These neutralizing interactions of Mn^{2+} with the superoxide anion have been described in several previous studies.[1,118-119] Although Mn^{2+} was also found to neutralize hydroxyl radical in a cell-free system, the relevance of this observation in the pathophysiological setting is doubtful, as the production of this

highly toxic ROS by phagocytes is stringently controlled by metal-binding proteins.

The effects of Mn^{2+}, at non-cytotoxic concentrations, on intracellular H_2O_2 concentrations in activated neutrophils and macrophages were investigated using DCF-DA, a fluorescent dye which emits light when oxidised by H_2O_2, and to a lesser extent with MPO-derived hypochlorous acid.[110,120] When using neutrophils, which contain high concentrations of MPO, these experiments were performed in the absence and presence of the MPO inhibitors, sodium azide and ABAH, to control for the complicating effects of oxidation of DCF by hypochlorous acid. Activation of DCF-DA-loaded neutrophils by FMLP or PMA resulted in marked increases in fluorescence intensity which were considerably greater in the presence of Mn^{2+}. Importantly, these effects were evident in the presence of sodium azide or ABAH, compatible with increased intracellular concentrations of H_2O_2. Similar effects were observed with PMA-activated monocyte-derived macrophages at concentrations of Mn^{2+} equivalent to those used in the neutrophil experiments, albeit in the absence of sodium azide/ABAH as these cells do not contain MPO.

Additional experiments were undertaken to investigate the effects of Mn^{2+} on the generation of hypohalous acids following activation of the neutrophils by FMLP or PMA. Inclusion of Mn^{2+} caused significant dose-related enhancement of the iodination of added protein, which was attenuated by the inclusion of inhibitors of NADPH oxidase or MPO. The following lines of evidence implicated increased formation of H_2O_2 via superoxide dismutase mimetic activity as the mechanism of Mn^{2+}-mediated increase in iodination of proteins by activated neutrophils: i) these effects of Mn^{2+} were not observed in a cell-free system consisting of purified human MPO,H_2O_2 and ^{125}I; and ii) there was no detectable increase in the release of MPO from activated neutrophils in the presence of Mn^{2+}.
With the exception of an earlier study byKlebanoff *et al*.,[1]the pro-oxidative interactions of Mn^{2+} with human phagocytes and their possible involvement in the

pathogenesis of occupation-related neurological and respiratory disorders are largely under-appreciated. Klebanoff and colleagues also concluded that Mn^{2+}, by acting as a superoxide dismutase mimetic, resulted in increased accumulation of H_2O_2 by activated phagocytes. However, these investigators used a scopoletin-based spectrofluorimetric procedure which does not distinguish between H_2O_2 and hypochlorous acid, as opposed to the DCF-DA-based method used in the current study. Notwithstanding efforts to exclude effects of Mn^{2+} on the release and activity of MPO, other important distinctions between the two studies include: i)the findings of the current study that Mn^{2+} does not affect the activity of NADPH oxidase, ii) the discerning of combinations of cell-based and cell-free ROS-generating systems to ensure accuracy of interpretation; and iii) most importantly, that the metal also interacts pro-oxidatively with human monocyte-derived macrophages.

Although SOD mimetics can be protective by scavenging superoxide radicals and attenuating oxidative stress,[121]these agents have the potential to induce tissue injury at higher concentrations. A bell-shaped dose-response curve exists for superoxide dismutases with the protective effect being lost above a threshold concentration.[122] Multiple mechanisms for the increase in tissue injury have been proposed, including an increase in H_2O_2 concentrations.[123]However, irrespective of the mechanism/s involved, SOD mimetics may exacerbate tissue damage.[124,125]

Blood levels of Mn^{2+} have been reported to range from 4-12µg/L (73-210 nmol/L) in healthy individuals[126], reaching up to 17.3µg/L in individuals occupationally exposed to high atmospheric levels of the metal.[127] Although somewhat lower than the threshold concentration of Mn^{2+} at which augmentation of intracellular H_2O_2 concentrations were observed in activated neutrophils/macrophages (0.5 µM=27.5 ng/ml Mn^{2+}), it is noteworthy that blood levels of the metal do not reflect those of cells and tissues, which are considerably higher.[127-129] Store-operated calcium channels, as well as other mechanisms operative at the blood-brain

barrier are likely to promote cellular uptake of Mn^{2+}.[130]However, the current study is potentially limited by an absence of data on the long-term effects of Mn^{2+} deposition within tissues. In humans, the respiratory tract represents the primary route of access of Mn^{2+}, predisposing in the occupational setting to the inflammatory airway disorders bronchitis and pneumonitis, as well as subacute bronchiolitis in experimentally-exposed rhesus monkeys.[131]Inhaled Mn^{2+} enters the bloodstream and accumulates in the central nervous system[103,104]where it interacts with tissue macrophages (microglial cells) to potentiate lipopolysaccharide/interferon-γ- induced TNF-α gene expression.[132]Activated glial cells release pro-inflammatory cytokines,[133]mediators and reactive oxidants,[132] which may injure adjacent neurons[134]and predispose to neurodegenerative disorders.[135]These pro-inflammatory effects of the metal appear to result from oxidative activation of the transcription factor NF kappa B, which is the topic of the following chapter.[133-136]These effects are summarized in Figure 2.12 below.

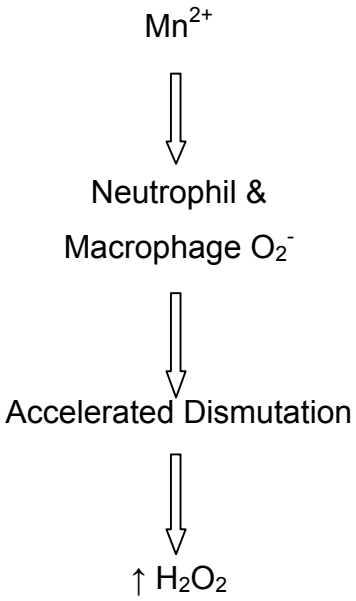


Figure 2.12: Exposure to Mn^{2+}potentiates H_2O_2 production by neutrophils and macrophages.

Phagocyte-derived ROS are potent cytotoxic and pro-inflammatory agents that directly oxidize critical protein sulfhydryls, iron-sulfur centers and haem moieties, and react with amines to form chloramines.[137] In addition, H_2O_2 is a well-recognised activator of intracellular signalling mechanisms, promoting oxidative activation of transcription factors such as nuclear factor κ-B and Ca^{2+} influx in various types of immune and inflammatory cells, creating a highly pro-inflammatory environment.[138-140]

In conclusion, Mn^{2+}, at concentrations which may be relevant in the setting of occupational exposure to the metal, increases the formation of the comparatively stable, cell-permeable ROS, H_2O_2, by activated neutrophils and macrophages. If not counteracted by endogeneous anti-oxidative defenses, the pro-oxidative interactions of the metal with phagocytes may contribute to the pathogenesis of Mn^{2+}-mediated respiratory and neurological disorders.

CHAPTER 3

OXIDATIVE ACTIVATION OF THE PRODUCTION OF PRO-INFLAMMATORY CYTOKINES BY MANGANESE-TREATED MONOCYTE-DERIVED MACROPHAGES

3.1 Introduction

The transcription factor NFκB has a central role in the expression of many cytokines, chemokines, and other mediators involved in acute inflammatory responses. Because oxidative stress and NFκB activation both have important roles in inflammation, the effects of ROS on NFκB have received considerable attention. NFκB was one of the first transcription factors shown to be activated by ROS.[141]It is evident that the production of ROS in Kupffer cells, which are the resident macrophages in liver, are responsible for the activation of NFκB and TNF-α production induced by peroxisome proliferators.[142]According to Forman and Torres, significant production of inflammatory mediators such as cytokines is induced by activation of MAP kinases due to oxidants which leads to activation of gene expression, coupled with activation of NFκB.[88]

Because relatively little is known about the pro-oxidative, pro-inflammatory interactions of Mn with human macrophages, especially in relation to the production of cytokine/chemokines, the primary aims of the current study were to investigate: i) the effects of $MnCl_2$ on pro-inflammatory cytokine production by human monocyte-derived macrophages; and ii) to identify possible relationships between Mn-mediated pro-oxidative activity, signal transduction, and pro-inflammatory cytokine production by these cells

3.2 Materials and Methods

3.2.1 Pro-inflammatory cytokine production

Monocyte-derived macrophages were isolated, matured and enumerated as described previously in 2.2.2.2(Page 48).After scraping, cells were analysed flow cytometrically and adjusted to a concentration of $1x10^5$/mland suspended in Ca^{2+}-free HBSS without calcium and added to the wells of micro-tissue culture plates (48 well) containing 20µl of 100mM $CaCl_2$. The final volume in each well

was 200µl ($CaCl_2$, cells and HBSS) containing $1x10^5$cells and the plates were incubated for 2 hours at 37°C/ 5% CO_2 to promote adherence. After incubation, the supernatant was removed and replaced with 500µl RPMI 1640 supplemented with antibiotics (penicillin; streptomycin; amphotericin B) and 5% autologous serum. Plates were incubated overnight at 37°C/ 5% CO_2. After 24 hours, the supernatants were discarded and the cells washed twice with 500µl of pre-warmed PBS. Serum (2.5%)-supplemented RPMI plus antibiotics was added to all wells. $MnCl_2$ at 12.5-100µM were added into all wells except control wells which received an equivalent volume of HBSS solvent and incubated for 30 min. Cells were then either left unstimulated or were treated with the macrophage activator, bacterial lipopolysaccharide (from *Escherichia coli* 0127:B8, 1ug/well, final) and incubated overnight at 37^0C/ 5% CO_2. After 24 hours the supernatants from each well were removed and frozen at -20^0C.

Analysis of the cytokines present in the supernatants was performed using the Bio-Plex Suspension Array System (Bio-Rad Laboratories, Inc. Hercules, Canada) and Bio-Plex Pro™ assay kit (Bio-Rad Laboratories, Inc). The Bio-Plex Pro™ assay kit is a magnetic bead-based multiplex assay designed to measure multiple cytokines simultaneously in different matrices. The assay kit used in the present study detected the following cytokines: IL-1β, IL-6, IL-8, G-CSF, IFN-ɣ and TNF. The procedure for the analysis of the cytokines was followed according to the manufacturers' specifications. A four-fold serial dilution was made of the premixed standards supplied with the assay kit (concentration range: 1.48i-77755.00 pg/ml). The supernatant was used undiluted. A 50µl volume of either standard or sample was then added to a 96-well plate containing the magnetic beads. An antibody directed against the desired cytokine is covalently coupled to the internally dyed beads. The plate was then incubated at room temperature on an orbital shaker (300 rpm) for 30 min in the dark. After incubation, the plate was washed three times using a Bio-Plex Pro wash station (Bio-Rad Laboratories, Inc). A biotinylated detection antibody (25µl) specific to an epitope different from that of the capture antibody was then added to the wells and the

plate was incubated for a further 30 min at room temperature on an orbital shaker (300 rpm) in the dark.

The plate was then washed a further three times using a Bio-Plex Pro-Wash Station followed by the addition of a streptavidin-phycoerythrin (streptavidin-PE) reporter complex (50µl) which then binds to the biotinylated detection antibodies on the bead surface. The plate was incubated again in the dark, at room temperature for 10 min on an orbital shaker (300 rpm). After a final 3 washes, a volume of 125µl of assay buffer was added to each well and the plate was shaken at 1100 rpm for 30 seconds to ensure that the beads were resuspended. The plate was then transferred to the Bio-Plex Suspension Array System (Bio-Rad Laboratories, Inc) and analyzed using the Bio-Plex Manager Software Version 4.1 (Bio-Rad Laboratories, Inc). The concentration of each cytokine (pg/ml) was calculated from a standard curve generated.

3.2.2 Effects of signal transduction inhibition on cytokine production by $MnCl_2$-treated macrophages

Additional investigations were performed to ascertain the signal transduction pathways utilized by $MnCl_2$ to activate the production of the proinflammatory cytokines. For this purpose, the following inhibitors were used: 1) NFκB activation inhibitor(1µM, Calbiochem); 2) the p38 MAP (mitogen activated protein) kinase inhibitor SB202190 (20µM, Sigma Aldrich);3) dithiothreitol (DTT, 5mM, Sigma Aldrich), a cell-penetrating scavenger of H_2O_2.

The experimental design was essentially as described above with the various inhibitors added to the cells 15 min prior to $MnCl_2$, which was used at a fixed, final concentration of 100µM. LPS was added as above and the supernatants analysed 24 hours later for 2 cytokines (IL-6 and IL-8, based on data from the initial series of experiments). Due to limitations on cell numbers, only LPS-

treated systems plus $MnCl_2$, with and without the inhibitors were used in this series of experiments.

3.2.3 Propidium iodide exclusion assay

To test for the potential cytotoxic effects of SB202190 (20 μM), NFκB activation inhibitor (1μM) and dithiothreitol (5μM), cell viability assays were performed on mononuclear cells ($2x10^6$/ml) using the flow cytometric propidium iodide exclusion assay following a 24 hour exposure of the cells to the test agents. The cells ($2x10^5$) were then incubated for 5 minutes with propidium iodide (DNA prepstain, Beckman Coulter, 50μg/ml). Cell viability was assessed flow cytometrically and data expressed as % viable cells.

3.3 Results

3.3.1 Production of pro-inflammatory cytokines by $MnCl_2$–treated macrophages

The effects of $MnCl_2$ on cytokine/ chemokine production by unstimulated and LPS-stimulated monocyte-derived macrophages are shown in Figures 3.1-3.6 (Pages 87-92). Exposure of the cells to the metal alone (in the absence of LPS) resulted in statistically significant increases in the production of IL-1β, IL-6, IL-8 , IFNɣ and TNF (all at 100μM $MnCl_2$with the exception of IL-6 for which the effects of 50μM also achieved statistical significance), but not G-CSF. Treatment of cells with LPS alone (in the absence of $MnCl_2$) caused moderate increases in the production of all the cytokines/ chemokines, most of which achieved statistical significance. In the case of monocyte-derived macrophages treated with the combination of $MnCl_2$ and LPS, the observed increases in the generation of IL-1β, IL-6, IL-8, IFNɣ and G-CSF were greater than those of cells exposed to the individual agents, achieving statistical significance in the cases of IL-6, IL-8 and IFNɣ when compared with the corresponding $MnCl_2$-free, LPS-

treated control systems. Although the combination of $MnCl_2$ and LPS treatment caused impressive, dose-related increases in production of IL-1β and G-CSF, these did not achieve statistical significance, probably as a consequence of the considerable variation in the magnitudes of the increases (these ranged from 0% to 374% and from 0% to 227% mean percentage increase in comparison with the corresponding LPS-treated control systems for IL-1β and G-CSF respectively). In the case of TNF, the values for the combination of LPS and $MnCl_2$ were comparable with those for the metal alone.

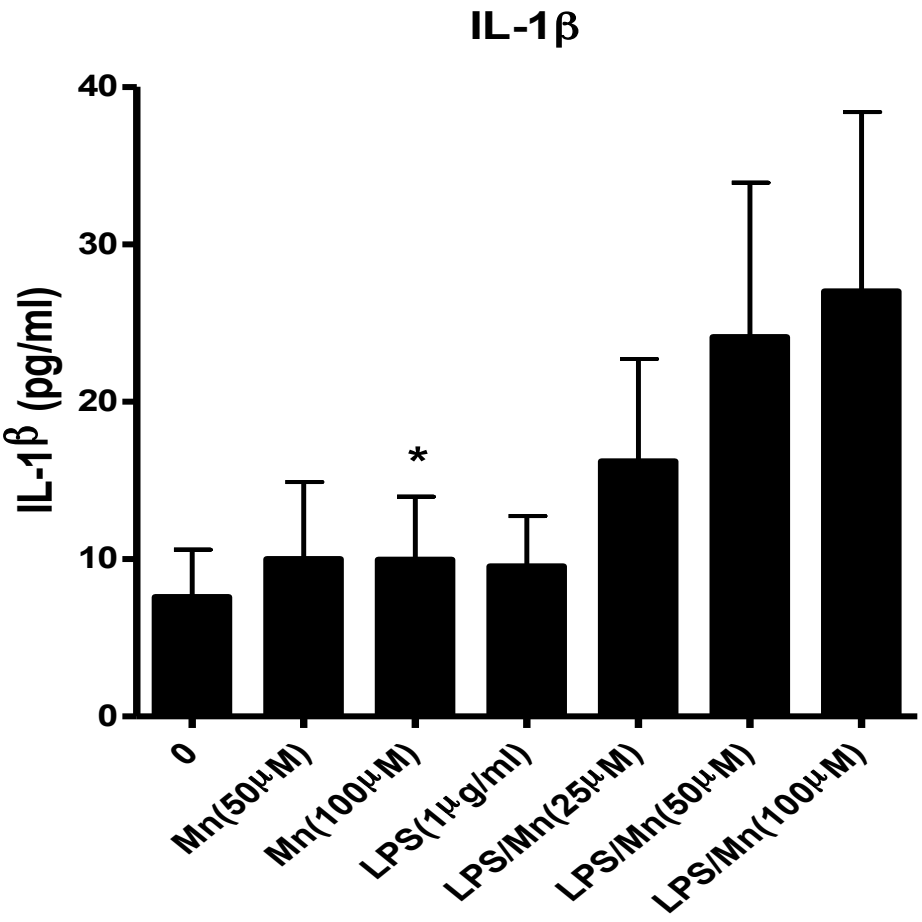


Figure 3.1

Effects of exposure to $MnCl_2$ (25-100μM) on the spontaneous production of IL-1β as well as on LPS-stimulated production of this cytokine by monocyte-derived macrophages. The results are expressed as the mean ±SEM for 6 experiments (n=6).

* P<0.05 for comparison with the manganese-and LPS-free control systems.

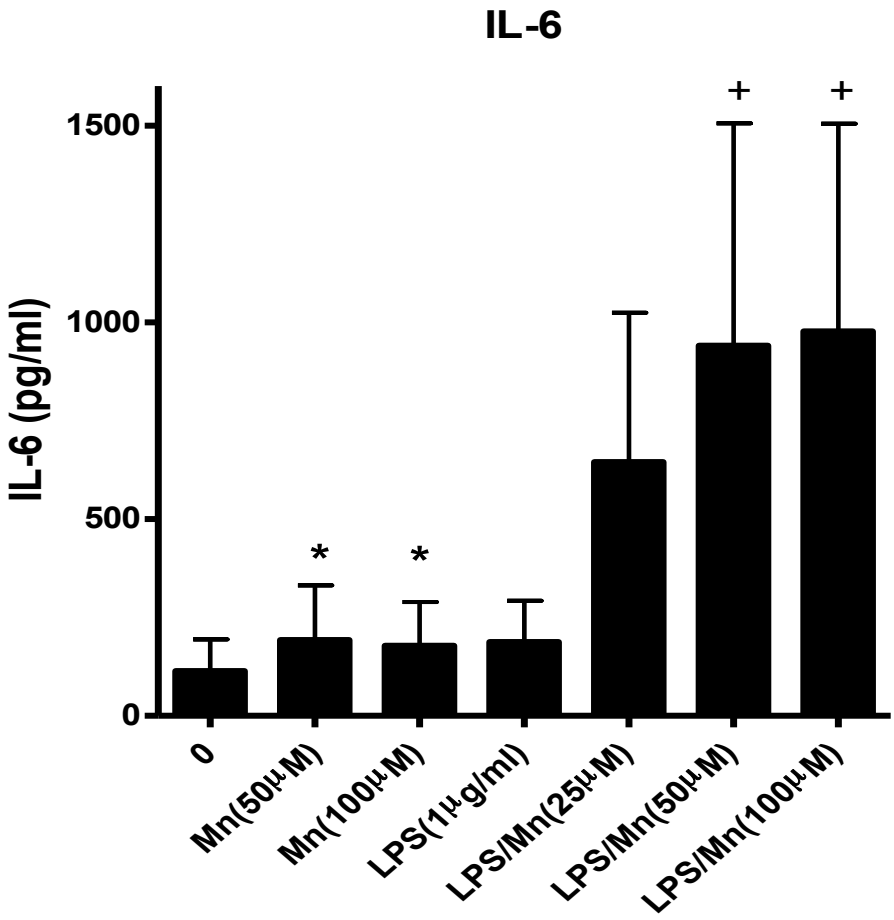


Figure 3.2

Effects of exposure to $MnCl_2$ (25-100µM) on the spontaneous production of IL-6 as well as on LPS-stimulated production of this cytokine by monocyte-derived macrophages. The results are expressed as the mean ± SEM for 6 experiments (n=6).

* P<0.05 for comparison with the manganese-and LPS-free control systems.

+ P<0.05 for comparison with the manganese-free, and LPS-treated control systems.

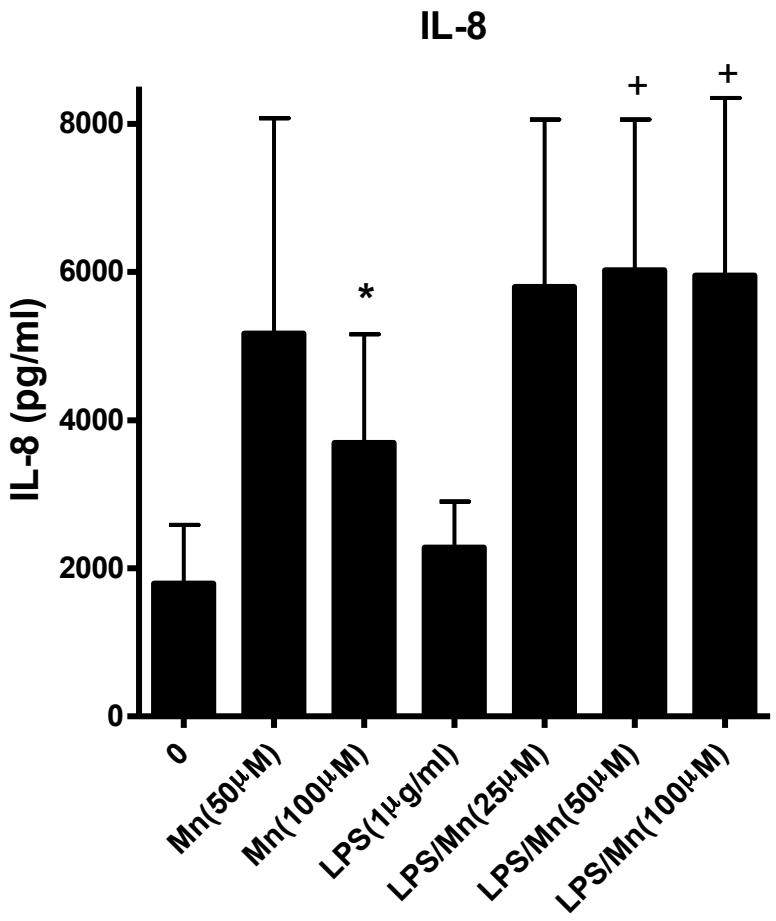


Figure 3.3

Effects of exposure to $MnCl_2$ (25-100µM) on the spontaneous production of IL-8, as well as on LPS-stimulated production of this cytokine, by monocyte-derived macrophages. The results are expressed as the mean ± SEM for 6 experiments (n=6).

* P<0.05 for comparison with the manganese-and LPS-free control systems.

\+ P<0.05 for comparison with the manganese-free and LPS-treated control systems.

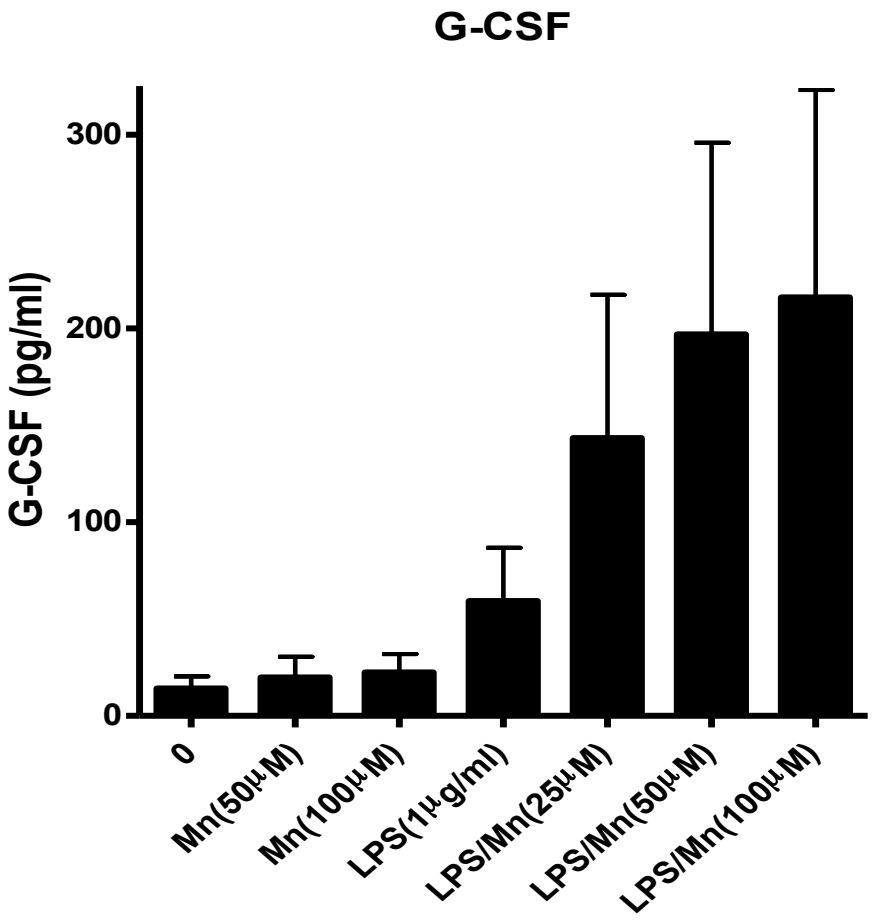


Figure 3.4

Effects of exposure to $MnCl_2$ (25-100µM) on the spontaneous production of G-CSF as well as on LPS-stimulated production of this cytokine by monocyte-derived macrophages. The results are expressed as mean ±SEM for 6 experiments (n=6).

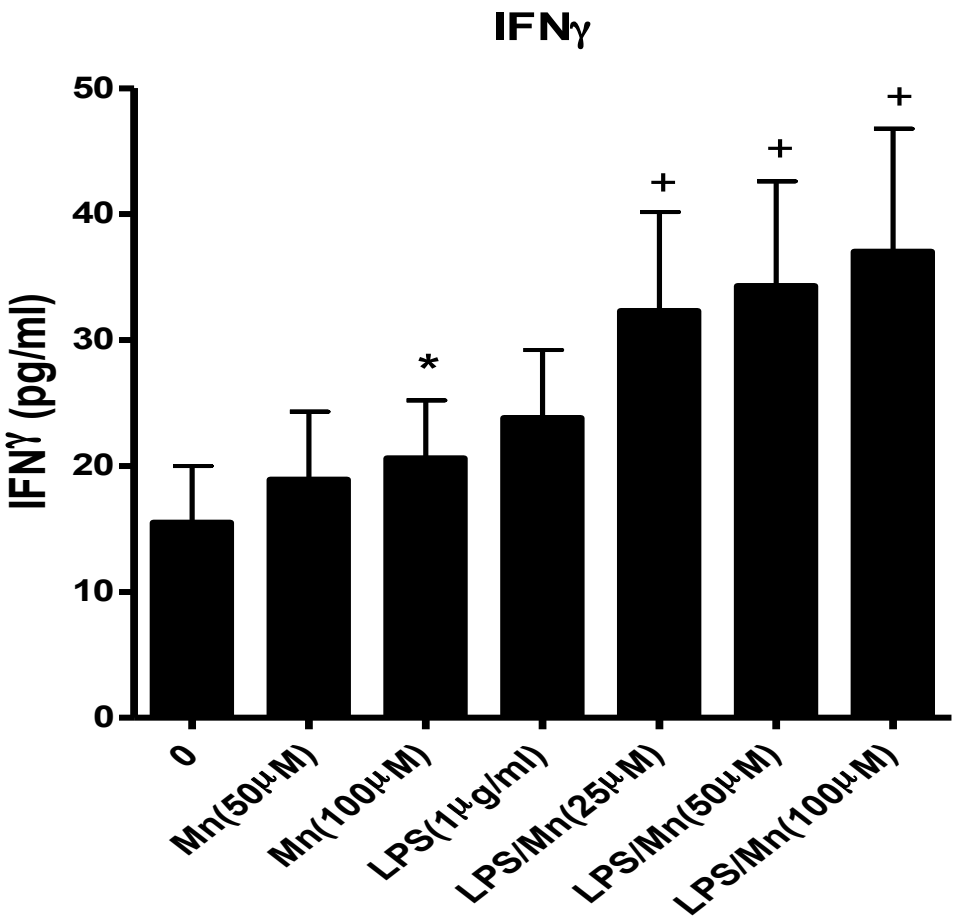


Figure 3.5

Effects of exposure to $MnCl_2$ (25-100μM) on the spontaneous production of IFNɣ as well as on LPS-stimulated production of this cytokine by monocyte-derived macrophages. The results are expressed as the mean ± SEM for 6 experiments (n=6).

*P<0.05 for comparison with the manganese-and LPS-free control systems.

+ P<0.05 for comparison with the manganese-free and LPS treated control systems.

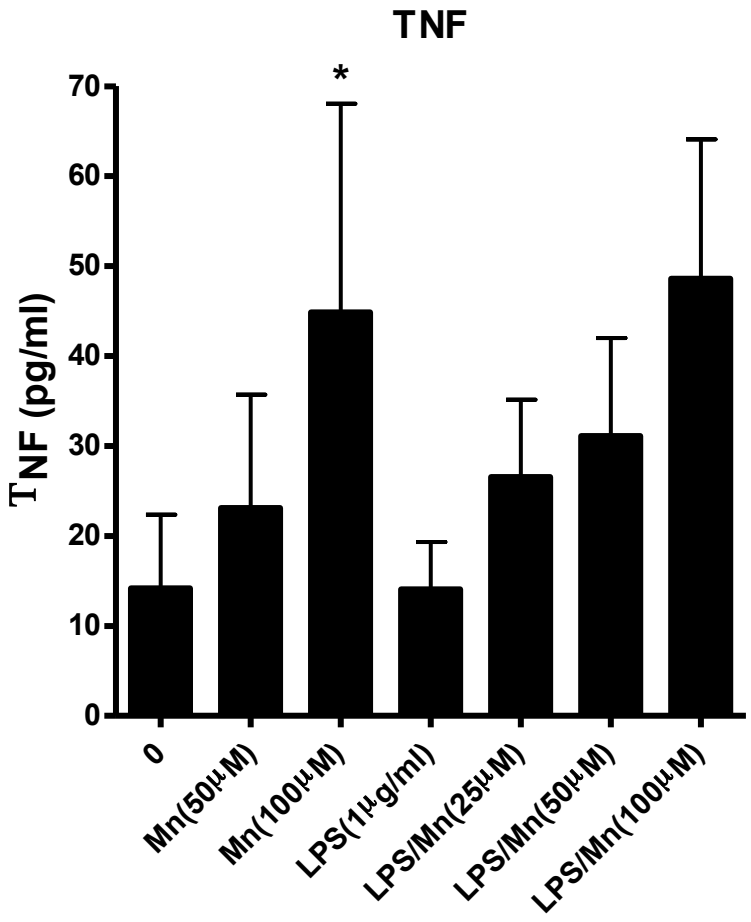


Figure 3.6

Effects of exposure to $MnCl_2$ (25-100µM) on the spontaneous production of TNF as well as on LPS-stimulated production of this cytokine by monocyte-derived macrophages. The results are expressed as the mean ± SEM for 6 experiments (n=6).

* $P<0.05$ for comparison with the manganese-and LPS-free control systems.

3.3.2 Effects of inhibition of signal transduction pathways on $MnCl_2$-mediated activation of IL-6 and IL-8 production by monocyte-derived macrophages

The effects of the NFκB activation inhibitor (1μM), SB202190 (20μM), and dithiothreitol (DTT, 5mM) on the $MnCl_2$-mediated increasesin IL-6 and IL-8 production by LPS-treated macrophages are shown in Figures 3.7 and 3.8, Pages 94-95,with the results expressed either as the absolute values or mean percentages of the stimulated control systems, respectively.

As observed in the previous series of experiments, exposure of macrophages to the combination of LPS and Mn^{2+} resulted in increased production of both IL-6 and IL-8. The absolute concentrations of IL-6 were, however, somewhat lower in this series of experiments, which in all probability reflects differences in the batches of reagents used; importantly, however, the trends were comparable. Exposure of the cells to DTT completely abolished the augmentative effects of $MnCl_2$ on the generation of both cytokines by LPS-activated cells, while in 4 of the 5 experiments the NFκB activation inhibitor and SB202190 caused partial inhibition ranging from 21-45% and 41-85% respectively for IL-6. The corresponding values for IL-8 ranged from 0-41% and 0-65%, respectively. Only those results for DTT-treated systems achieved statistical significance. These effectsof the inhibitors on cytokine production by Mn^{2+}-treated cells were not due to cytotoxicity as measured by the propidium iodide exclusion assay. The mean percentages viability for control cells and those treated with SB202190, NFκB activation inhibitor orDTT at the above mentioned concentrations, were 97.5 ± 0.4%, 97.2 ± 0.7%, 97.2 ± 0.4% and 97.2 ± 0.5%, respectively (3-5 different experiments).

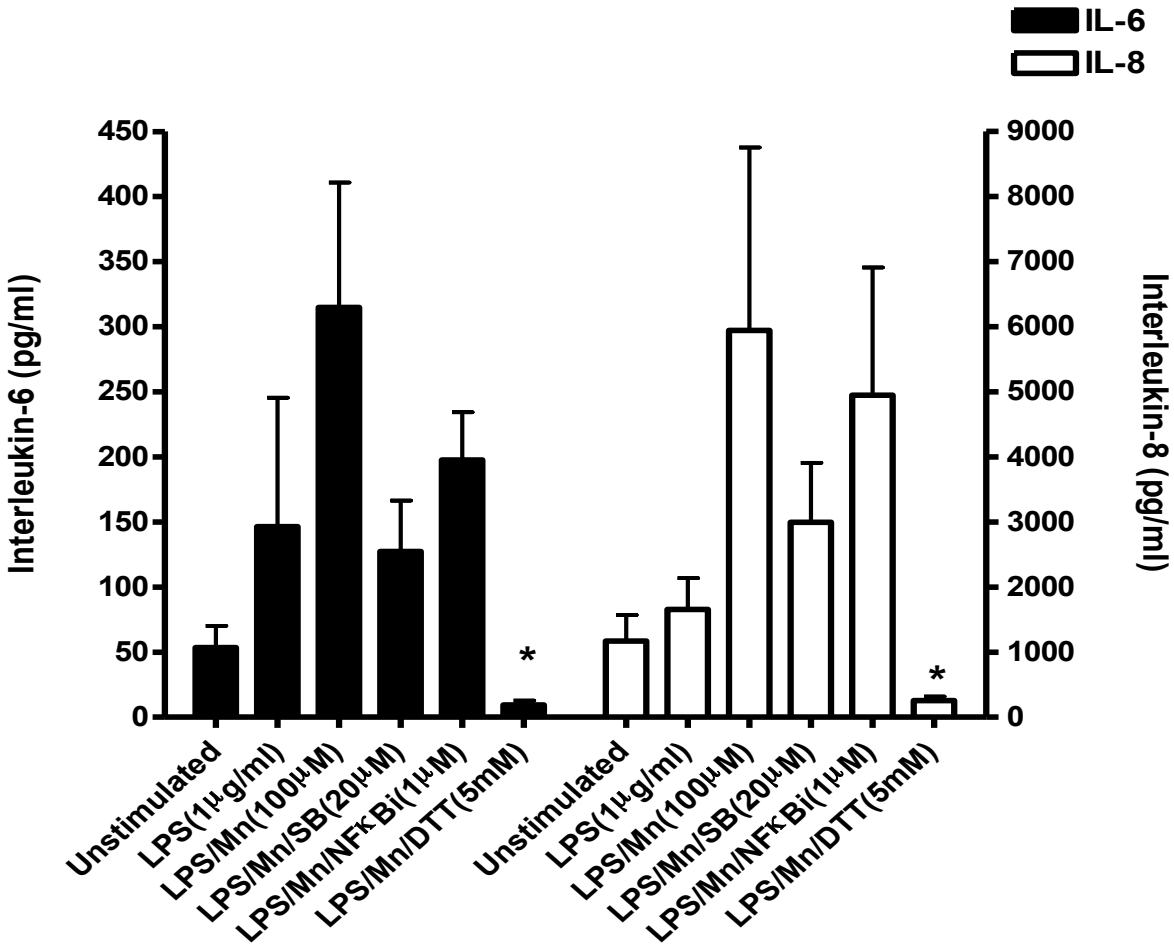


Figure 3.7

The effects of the NFκB activation inhibitor (1μM), SB202190 (20μM),and dithiothreitol DTT, (5mM) on the manganese-mediated increases inIL-6 and IL-8. The results are expressed as the mean ±SEM for 5 different experiments (n=5).

* $P<0.05$ for comparison with the LPS/Mn^{2+}-treated, inhibitor –free, control systems.

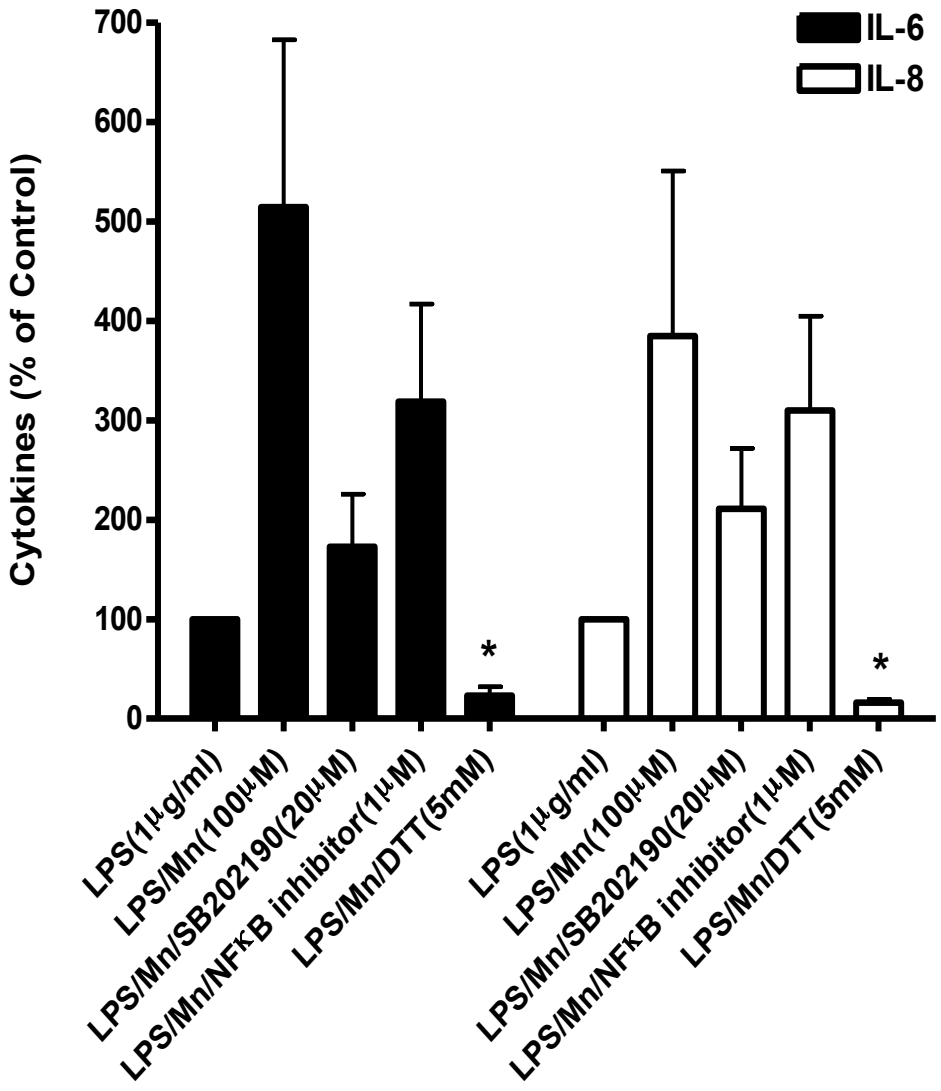


Figure 3.8

The effects of the NFκB activation inhibitor (1μM), SB202190 (20μM), and dithiothreitol DTT, (5mM) on the manganese-mediated increases in IL-6 and IL-8. The results are expressed as the % of Mn^{2+}free, LPS-stimulated, inhibitor-free-control system (n=5). The actual values for LPS control systems and inhibitor free Mn-treated systems for IL-6 and IL-8 were 147±99 and 315±96 for IL-6 and 1657±481 and 5945±2810 for IL-8, respectively.

* $P<0.05$ for comparison with the LPS/Mn^{2+}-treated, inhibitor-free control systems.

3.4 Discussion

Inflammation is the process by which the human body attempts to counteract potentially injurious agents such as invading bacteria, viruses, and other pathogens. Although it is essential, inflammation can be harmful to the host and, therefore, it is subject to multiple levels of biochemical, pharmacological, and molecular controls involving a diverse and potentially huge array of cell types and soluble mediators, including cytokines.[86] Good indicators of proinflammatory effects include upregulation of the production of cytokines, resulting from activation of transcription factors such as NFκB.[135]

The current study provides insights into the mechanisms by which $MnCl_2$ causes inflammation. The results demonstrate that Mn^{2+} potentiates the production of various pro-inflammatory cytokines. Exposure of the monocyte-derived macrophages to the metal alone resulted in significant increases in IL-1β, IL-6, IL-8, IFNγ and TNF but not G-CSF. When monocyte-derived macrophages were treated with the combination of $MnCl_2$ and LPS, the observed increases in the generation of IL-1β, IL-6, IL-8, IFNγ, and G-CSF were greater than those of cells exposed to the individual agents. Although there were only modest, albeit insignificant, increases in production of pro-inflammatory cytokines in LPS-treated systems in the absence of $MnCl_2$, this may be due to disagreement in literature in terms of the optimal LPS concentration to be used as well as duration of incubation with LPS. Macrophages release a number of proinflammatory cytokines with pathogenic potential. Most notable are TNFα, IL-1 and IL-6 that have been shown to be critical mediators of macrophage-induced tissue injury.[143] These cytokines cause the recruitment of inflammatory cells into tissues, stimulate the production of ROS and RNS, and release of other proinflammatory mediators, while being directly cytotoxic (TNF).[143]These findings are also consistent with those reported by Antonini *et al.*, who found that intratracheal exposure to stainless steel welding fumes in rats increased the levels of TNF and IL-1β in BALF.[141] These cytokines, produced predominantly by

alveolar macrophages, are involved in numerous inflammatory processes such as neutrophil recruitment and increased oxygen radical production.[141] IL-1 and TNF induce adhesion molecules, such as intercellular adhesion molecule-1, that promote leukocyte infiltration from blood into tissues. TNF and IL-1 together promote the proliferation of premalignant cells and thus serve as cofactors in wound-related carcinogenesis. It is also known that both pro-inflammatory cytokines (IL-1 and TNF) stimulate their own and each other's production, and this represents an important amplification loop of the inflammatory response.[144]

Proinflammatory cytokines (IL-6, IL-8 and TNF) were found elevated in, and may contribute to the pathogenesis of, pulmonary inflammatory responses such as tracheobronchitis, asthma, chemical pneumonitis, and alveolitis (metal fume fever), induced by human exposure to airborne metals.[145-147]It was also reported that transition metals vanadium, chromium and zinc induced cytokine expression in various cell types including monocyte/ macrophages.[145,148-150] Reports of dose-dependent increases in pro-inflammatory cytokines and neutrophilia in the human bronchoalveolar lavage fluid 20-22h following zinc oxide inhalation provide further evidence supporting the role of a cytokine-mediated mechanism causing the symptoms of metal fume fever.[151]

Occupational exposure to manganese has been linked to a specific neuropathology, manganism, that is characterized by clinical signs and lesions similar to Parkinson's Diesease.[9] Cytokines are increasingly implicated in acute and chronic neuronal demise with clinical studies having demonstrated increased levels of pro-inflammatory cytokines (TNF, IL-6, IL-1β, IFNγ) in postmortem brain, as well as blood or cerebrospinal fluid of patients with stroke, head injury, Alzheimer's disease and Parkinson's Disease.[152-156]Moreover, the levels of IFNγ, IL-1β and TNF, as well as those of the chemokines MCP-1, RANTES, MIP-1α and IL-8, were significantly correlated with the severity of Parkinsonism.[157] These reports underscore the involvement of pro-inflammatory cytokines and chemokines in the development of neurotoxicity in manganese-exposed workers.

In addition manganese has been implicated as one of the heavy metals found in high levels in soil, vegetable and fruit samples in an endemic region of upper gastrointestinal cancers in Eastern Turkey.[158]

The combination of cytokines generated by manganese-treated macrophages described in the current study appears to be consistent with predominantly neutrophil inflammatory response. The chemokine, IL-8 and the cytokines IL-1β, IL-6 and TNF interact to promote neutrophil extravasation, accumulation and activation while G-CSF decreases the process of apoptosis.[159]Interleukin-8 has also been implicated in tumour promotion/ progression. Neutrophils are attracted to sites of tissue injury/ infection by the subfamily of CXC/ELR-motif-positive chemokines and the predominant member of this sub-family is IL-8.[159]CXC/ELR^+ and CXCR2 are expressed by a diverse range of human cancers, including breast, bladder, cervix, colon, liver, lymphatics, oesophagus, ovary, prostate and skin. In this setting, these chemokines drive tumour expansion via the pro-proliferative interactions, both autocrine and paracrine with CXCR2-expressing tumour cells.[159]In addition, tumour neovascularisation is mediated via the pro-angiogenic activities of these chemokines, especially IL-8,[159]while the chronic influx of inflammatory cells exacerbates ROS-mediated oxidative damage to DNA and immunosuppression.

In the previous chapter the conclusion was drawn that $MnCl_2$ potentiates the production of H_2O_2 by neutrophils and macrophages, by acting as a superoxide dismutase mimetic. The fact that the metal potentiates the production of H_2O_2 clearly underpins its ability to increase the production of pro-inflammatory cytokines by monocyte-derived macrophages. This contention is based on the suggestion that H_2O_2 may act as an intracellular second messenger, whereby it modulates a number of major intracellular signalling cascades including ERK, JNK, MAPK, PI3K/Akt as well as transcription factors, by targeting the cysteine and methionine residues of these proteins.[141]Haddad reported that ROS can

induce the production of IL-6 and IL-8 in bronchial and epithelial cells, respectively. Furthermore, ROS can be released by many cell types in response to a variety of stimuli, such as TNF-α and LPS, serving as intracellular signals for the activation and regulation of redox-sensitive transcription factors.[160] According to Strassheim *et al.*, of the ROS commonly produced in tissues, H_2O_2 currently appears to be the most important in terms of signaling.[142] More recently, a role for H_2O_2 has been demonstrated in the activation of intracellular kinases, including MAPKs, PKC, and PKB, induced by interaction of growth factors and cytokines with their receptors. One of the principle means by which H_2O_2 affects signal transduction is through oxidation of susceptible cysteine residues to cysteine sulfenic acid or disulfides, a step reversed by cellular reductants.[141]

The Mn^{2+}-mediated increases in IL-6 and IL-8 reported in the current study were significantly inhibited by DTT, a cell-penetrating, sulphydryl antioxidant that effectively scavenges H_2O_2 and protects cellular sulphydryl groups. Partial, albeit insignificant, inhibition was also observed with the p38 MAPK inhibitor (SB202190) and a NFκB activation inhibitor. These inhibitors were used because NFκB and p38 MAPK are key regulators of pro-inflammatory cytokine gene expression. The approach of using inhibitors as opposed to direct monitoring of activation was used because of limitations on available cell numbers, as well as disagreement in previously published literature on the optimal concentration of LPS and duration of exposure of monocyte-derived macrophages to the activator required to cause maximal, detectable activation of NFκB. The efficacy of DTT in abolishing Mn^{2+}-mediated production of pro-inflammatory cytokines by monocyte-derived macrophages clearly underscores the actual involvement of this ROS in the activation of gene expression. The more limited efficacy of the pharmacological inhibitors of NFκB and p38 MAPK probably reflects the multiple redox signaling mechanisms activated by H_2O_2, rendering selective inhibition of a single pathway relatively ineffective. In addition, optimal inflammatory gene expression is accomplished by complementary interactions of several different transcription factors which

converge on large nuclear proteins known as co-activator molecules of which cAMP response element-binding protein (CBP) is a prototype. Following binding of transcription factors to CBP, the co-activator molecule acquires histone acetyltransferase (HAT) activity facilitating interaction of transcription factors with their response elements on target genes.[161]

In conclusion, the results of the current study are compatible with a pro-oxidative mechanism whereby $MnCl_2$, as a consequence of its superoxide dismutase mimetic activities, potentiates the generation of H_2O_2 by monocyte-derived macrophages. This ROS, in turn, via its REDOX intracellular signaling activity promotes the activation of genes encoding a range of pro-inflammatory cytokines. A summary of the proposed mechanism by which Mn^{2+} interacts pro-oxidatively with human neutrophils and macrophages is shown in Figure 3.9 (page 101). Although remaining to be conclusively proven, this pro-oxidative, pro-inflammatory mechanism is likely to be implicated in the (immuno) pathogenesis of Mn-associated neurological and respiratory dysfunction and may enable the formulation of preventive and therapeutic strategies, particularly in the setting of occupational exposure to this metal.

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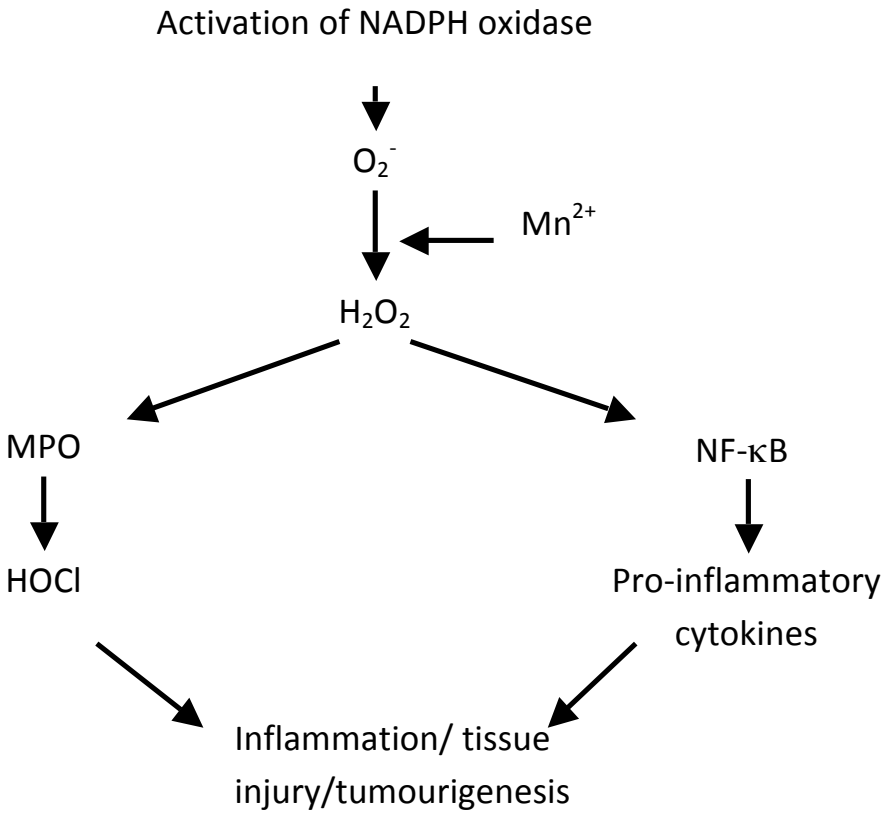

Figure 3.9

Summary of the mechanism by which Mn^{2+} interacts pro-oxidatively with human neutrophils and macrophages. Neutrophil and macrophage membrane-associated NADPH oxidase generates superoxide anions (O_2^-) which in the presence of Mn^{2+}, an SOD mimetic, is converted to hydrogen peroxide (H_2O_2). H_2O_2 activates NF-κB and promotes the synthesis of pro-inflammatory cytokines and is also transformed to HOCl by myeloperoxidase (MPO) released from neutrophil primary granules. Both HOCl and pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α contribute to neutrophil-mediated inflammation, tissue injury and tumourigenesis

CHAPTER 4

CONCLUDING COMMENTS

4.1 Concluding comments

Heavy metals possess a range of potentially toxic, pro-oxidative, pro-inflammatory activities. These include:

- Direct binding to protein sulphydryls leading to intense oxidative stress and activation of redox-based intracellular signaling mechanisms.
- Catalysis of the conversion of one type of ROS to another with increased reactivity, such as the conversion of H_2O_2 to hydroxyl radical.

The current study has documented an unusual mechanism of heavy metal-mediated toxicity in which Mn, via its well-documented superoxide dismutase mimetic activity, catalyses the conversion of superoxide to H_2O_2. Although counterintuitive (because elimination of superoxide is a potentially useful anti-oxidative strategy), excessive production of H_2O_2 clearly results in abnormal anti-oxidative homeostasis with the attendant risks of H_2O_2-mediated toxicity. $H_2O_2$is a particularly ominous type of ROS, this is because of its relative stability, enabling it to traverse membranes and activate intracellular redox signaling mechanisms via its interactions with protein sulphydryls. In addition, as a consequence of its interaction with intracellular heavy metals, particularly Fe^{2+}, H_2O_2 participates in the formation of hydroxyl radical, the most potent and damaging ROS in biological systems.

The limitations of the study include: i) it is an exclusively, pre-clinical, *in vitro* study, the results of which may not be entirely extrapolatable to the setting of Mn toxicity in humans; nonetheless such studies are necessary to provide an informed and discerning basis for future clinical/ epidemiological studies; ii) the numbers of experiments in the intracellular signaling component of the study were relatively small (n=5); however, these are lengthy multi-step and expensive experiments, which have, despite their limitations, provided novel mechanistic insights; and iii) in the occupational and environmental health settings, exposure

to a single metal toxicant is improbable; future studies should therefore focus on the pro-oxidative, proinflammatory interactions of combinations of heavy metals of occupational and environmental health significance with the major ROS-producing cells of the innate immune system.

Finally, the relevance of the current study should be viewed against the backdrop of: i) South Africa hosts 80% of the world's Mn reserves and mining and smelting of this mineral is due for major expansion; and ii) in keeping with this, the South African government and mining industry's drive to open up the manganese fields of the Kalahari, with its accompanying potential effects on occupational and environmental health in this country.

CHAPTER 5

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