Chapter 4

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

Hypothesis

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

Aim

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

4.1 Introduction

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

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

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

4.2 Materials and Methods

4.2.1 Chemicals and reagents

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

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

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

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

4.2.3 Neutrophils

Purified human neutrophils were prepared as described in 2.2.2.

4.2.4 Spectrofluorimetric measurement of cytosolic Ca^{2+}

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

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

4.2.5 Assay of neutrophil migration

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

4.2.6 NF-κB activation

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

4.2.7 Expression and statistical analysis of results

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

4.3 Results

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

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

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

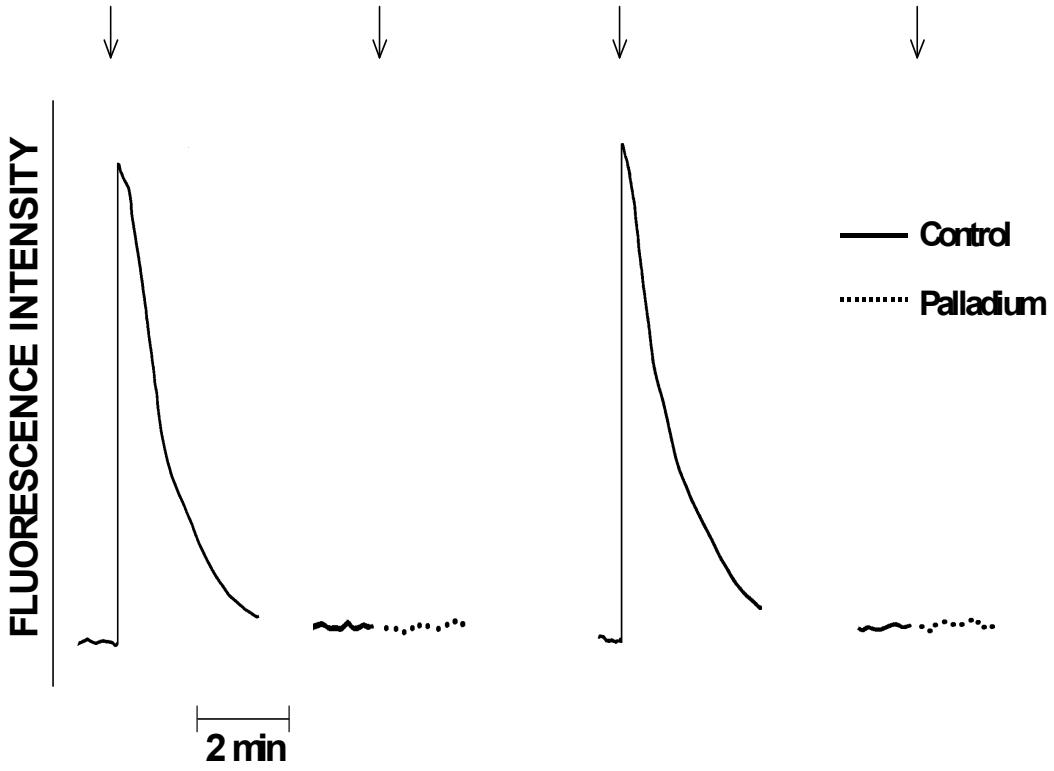

Figure 4.1

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

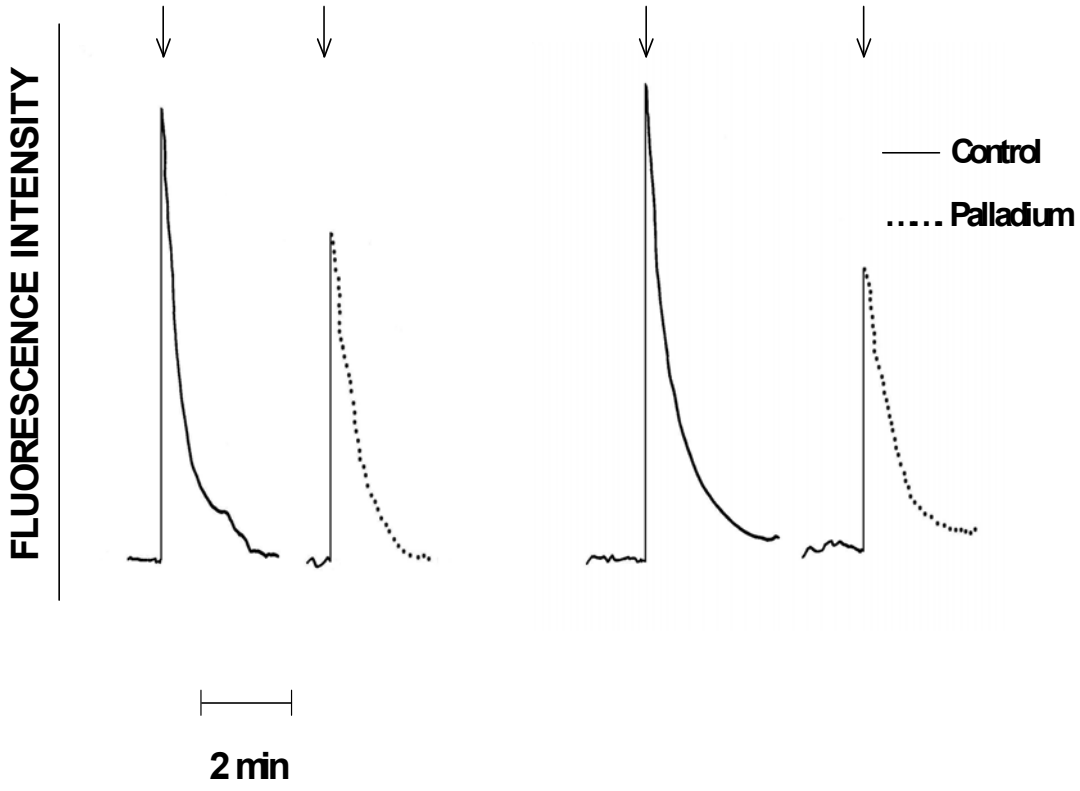

Figure 4.2

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

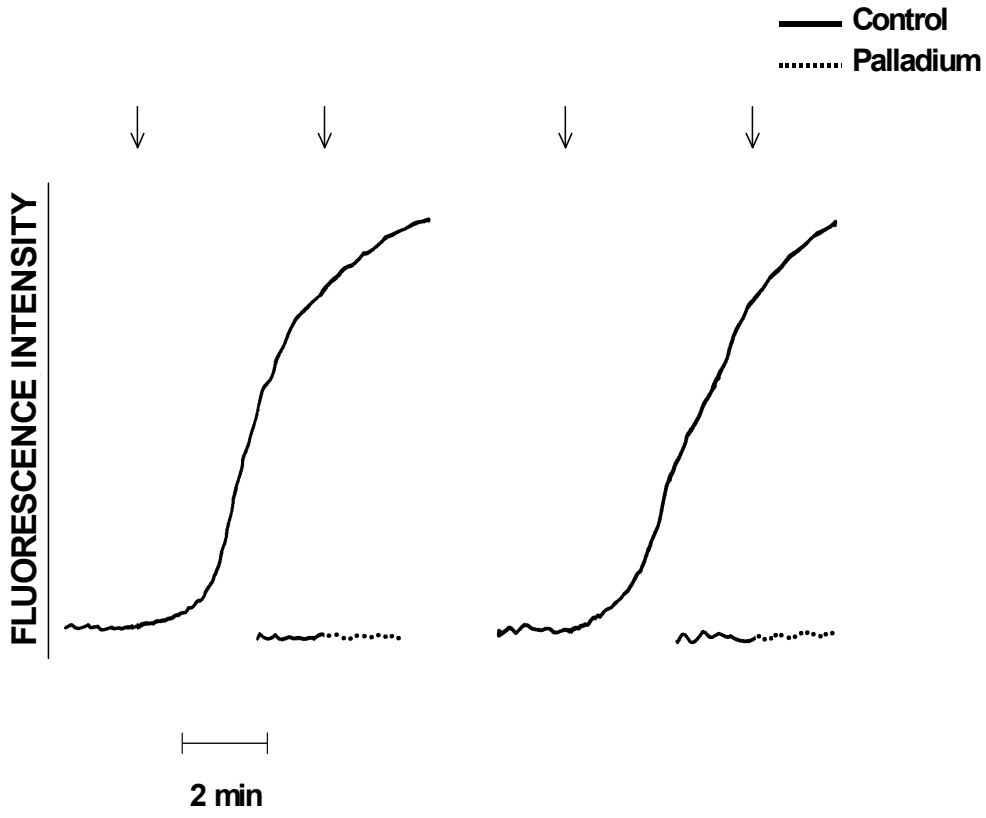

Figure 4.3

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

Table 4.1

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

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

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

* $p < 0.05$

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

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

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

4.3.3 Activation of NF-κB

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

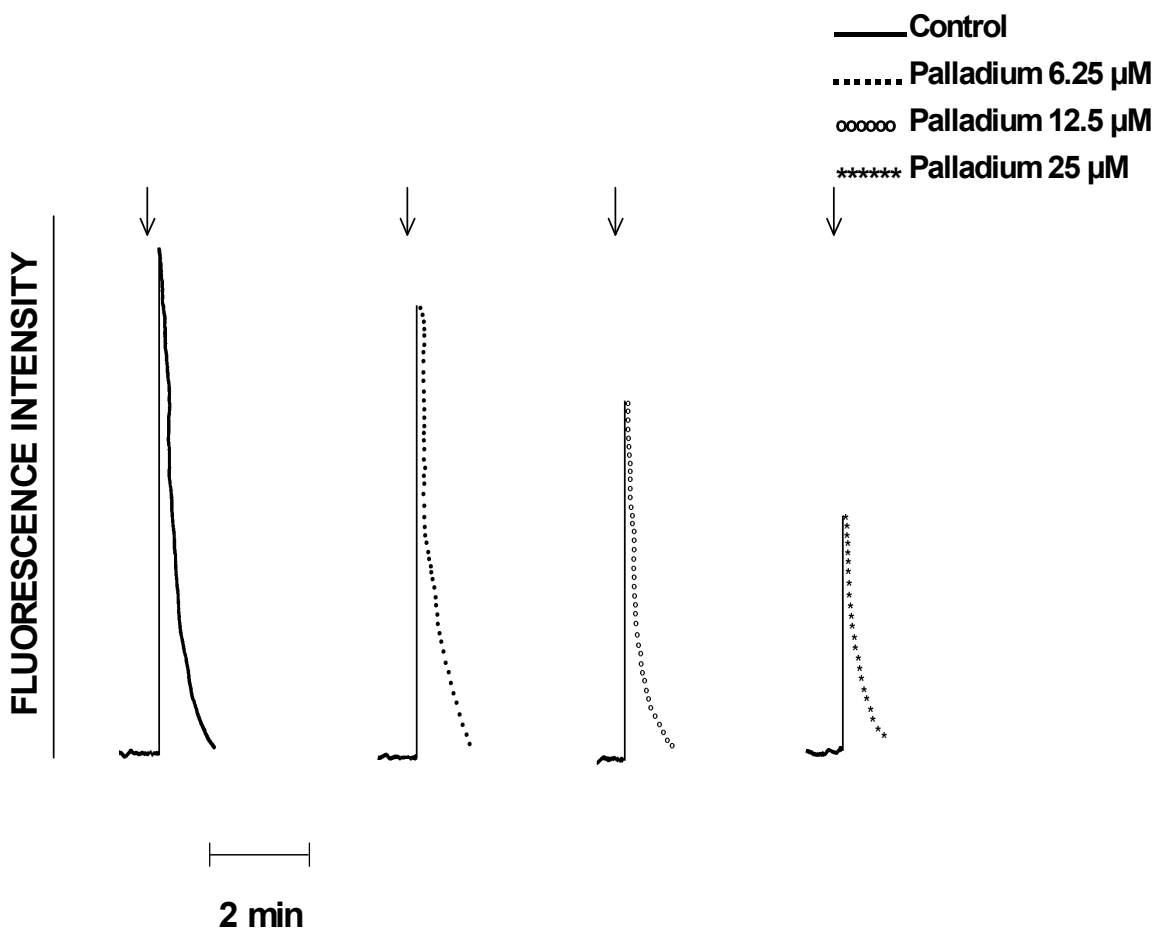

Figure 4.4

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

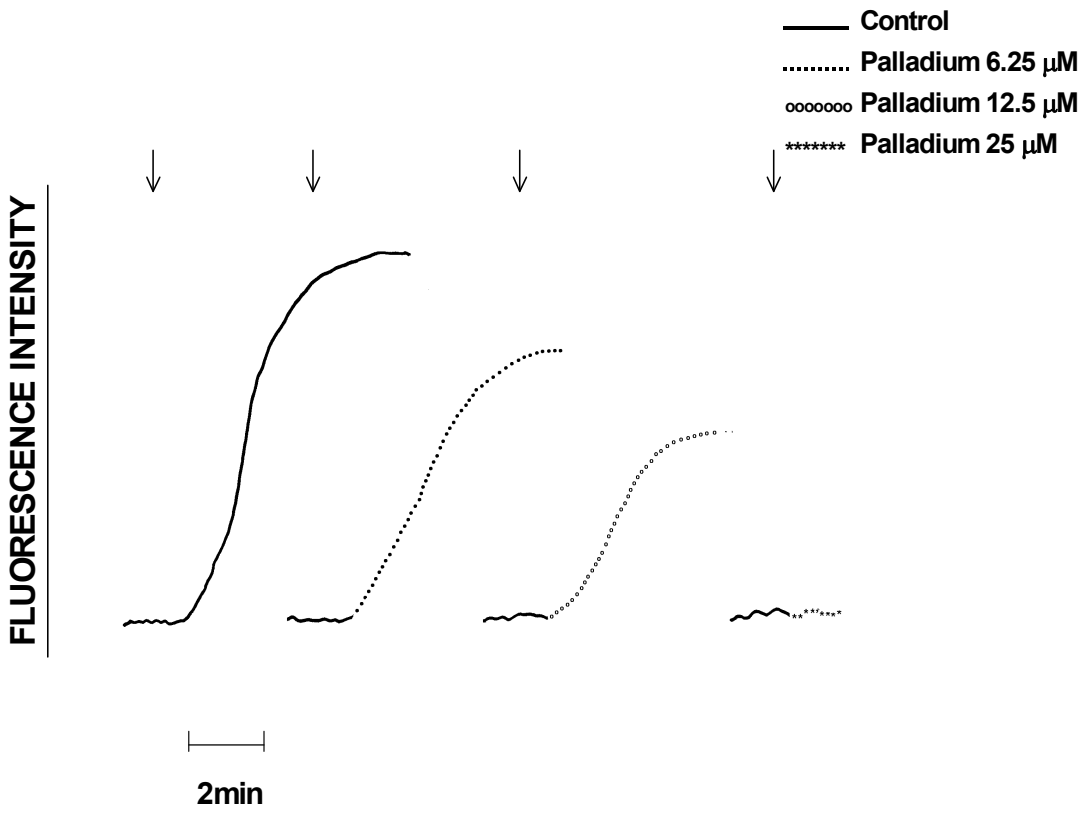

Figure 4.5

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

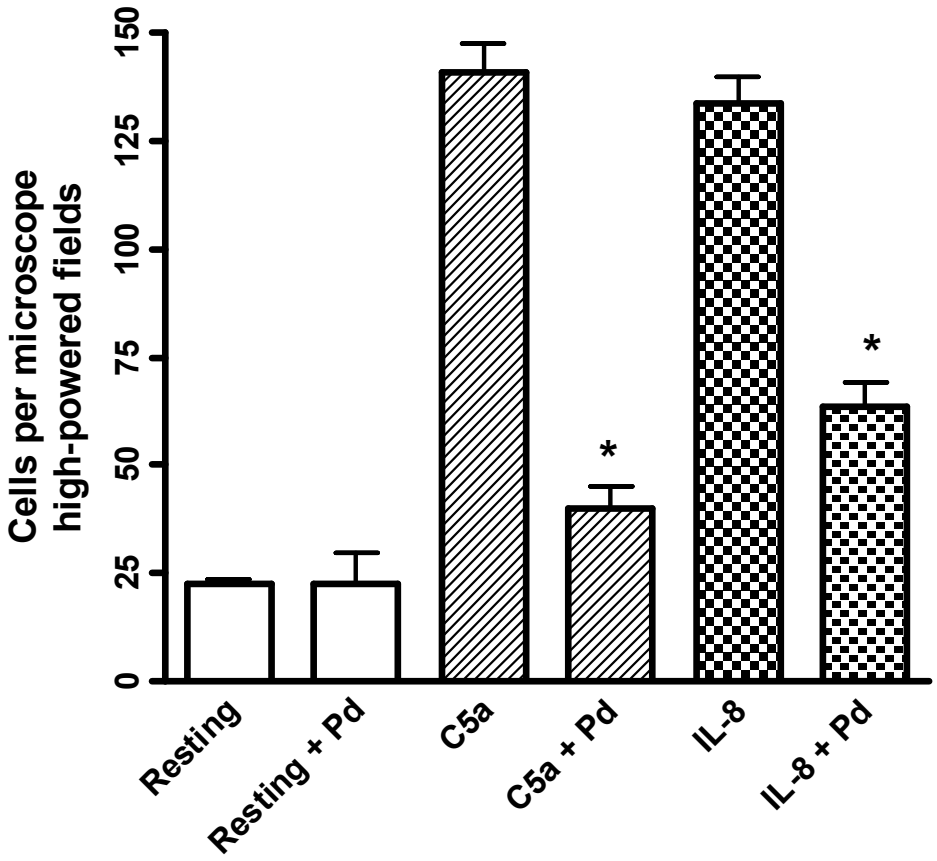

Figure 4.6

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

* $p < 0.05$

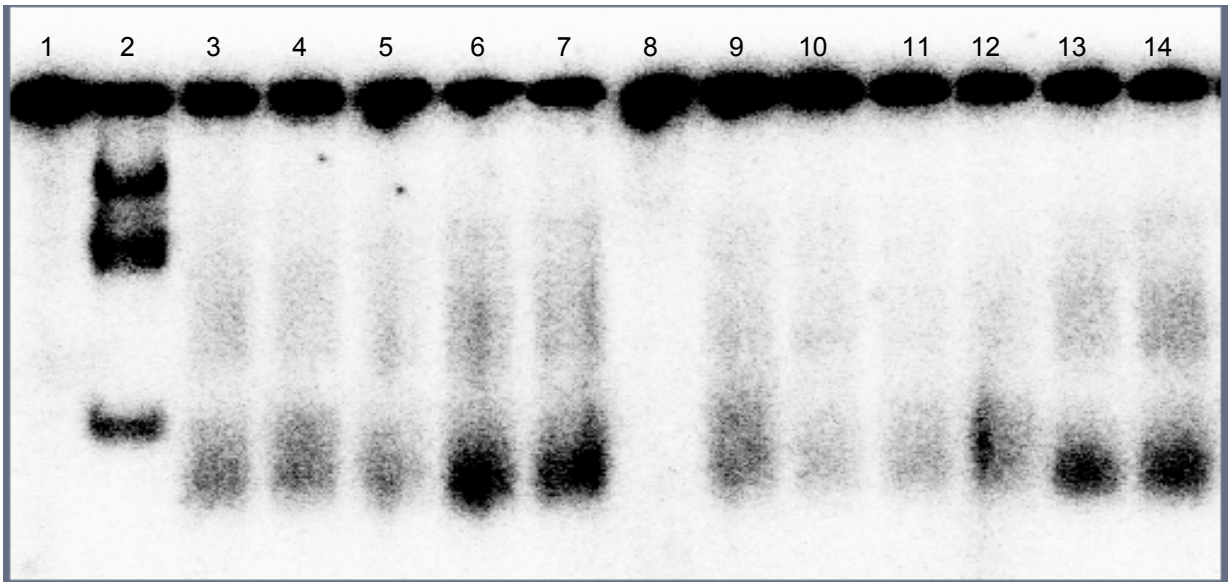

Figure 4.7
Phosphor-image showing the effect of untreated and Pd^{2+}-treated pneumolysin (Ply 20 ng/ml, Pd^{2+} 25 μM) on nuclear translocation of NF-κB in neutrophils.

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

4.4 Discussion

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

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

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

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

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

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

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

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

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

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