UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Effects of cigarette smoke and smoke condensate on neutrophil extracellular trap formation.

by

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Honours

Submitted in fulfilment of the requirements for the degree of

Masters of Science

In

The Department of Immunology

Faculty of Health Sciences

University of Pretoria

September 2016

Acknowledgements

I would like sincerely thank the following people for their support through the duration of my studies, assistance in my research and accomplishing this dissertation.

- Professor GR Tintinger (supervisor)
- Professor AJ Theron (co-supervisor)
- Professor R Anderson (co-supervisor)
- Professor R Cockeran (head of department)
- Doctor PWA Peter (Scientist)
- My family (for the constant encouragement and support).

List of Publications

Bokaba, R, R. Anderson, A. Theron, GR. Tintinger (2016) “Cigarettes smoke condensate attenuates phorbol ester-mediated neutrophil extracellular trap formation” AJOL 1-19.

Abstract

Background: Neutrophil extracellular traps (NETs) constitute a network of chromatin fibres containing histone and antimicrobial peptides that are released by activated neutrophils. NETs protect the host against infection by trapping and facilitating phagocytosis of potentially harmful pathogens.

Objectives: The aim of the current study was to investigate the effects cigarette smoke condensate (CSC) on phorbol-ester (PMA)-mediated NETosis *in vitro*, as well as the effects of cigarette.

Methods: Isolated human blood neutrophils were exposed to PMA (6.25 ng/ml) in the presence or absence of CSC (40-80 µg/ml) for 90 min at 37ºC. Alternatively neutrophils of non-smokers and smokers were activated with PMA (6.25 ng/ml) for 90 min at 37ºC. NET formation was measured using a spectrofluorimetric procedure to detect extracellular DNA and fluorescence microscopy was used to visualize nets. Oxygen consumption by PMA-activated neutrophils was measured using an oxygen sensitive electrode. Cotinine levels were measured in smokers and non-smokers for objective confirmation of smoking status

Results: Activation of neutrophils with PMA was associated with induction of NETosis that was significantly attenuated in the presence of CSC (40 and 80 µg/ml), with mean fluorescence intensities of 65% and 66% of that observed with untreated cells, respectively, and confirmed by fluorescence microscopy. The rate and magnitude of oxygen consumption by activated neutrophils pre-treated with CSC (80 µg/ml) was significantly less than that observed with untreated cells (73% of the control system), indicative of decreased production of reactive oxidant species in the presence of CSC. When comparing smokers and non- smokers, neutrophils from smokers showed a decrease in both oxygen consumption and the number of NET-forming cells consistent with attenuation of NET formation due to inhalation of cigarette smoke.

Conclusion: The inhibition of NETosis observed in the presence of CSC and CS (in smokers) correlated with attenuation of oxygen consumption by PMA-activated neutrophils suggesting a mechanistic relationship between these events. Smoking-

related attenuation of NETosis may impair host immune responses and increase the risk of respiratory infections, *in vivo*.

KEYWORDS:

Neutrophils, reactive oxygen species, respiratory infection, smoking

CONTENT PAGE

LIST OF ABBREVIATIONS

Abbreviations	Full Scientific Names
AD/s	Autoimmune disease/s
AIDS	Acquired immune deficiency syndrome
CGD	Chronic granulomatous disease
CO	Carbon monoxide
CSC	Cigarette smoke condensate
DAPI	4', 6-Diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ILD	Interstitial lung disease
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NETs	Neutrophil extracellular traps
PAD	Peptidyl arginine deiminase
PBS	Phosphate- buffered saline
PFA	Paraformaldehyde
PKC	Protein kinase C
PI_3	Propidium iodide
PMA	Phorbol-12-myristate-13-acetate
RA	Rheumatoid arthritis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
sTREM-1	Soluble triggering receptor expressed on myeloid cells
TREM-1	Triggering receptor expressed on myeloid cells

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CHAPTER 1

1.1 Introduction

In this chapter the mechanisms of induction and the functions of neutrophil extracellular traps (NETs), as well as the constituents of cigarette smoke are described. This is followed by experimental sections (chapters 2 and 3) describing the effects of cigarette smoke condensate on the process of NETosis *in vitro,* as well as a comparison of NETosis by neutrophils isolated from smokers and non-smokers.

1.2 Literature Review

1.2.1 What are neutrophils

Neutrophils are essential immune cells that act in the innate immune system. They are part of the common myeloid progenitor lineage and are produced in the bone marrow (Futosi et al, 2013; Kolaparthy et al, 2014). From the bone marrow quiescent neutrophils travel to the blood where they become residents until they later migrate into infected tissues for activation and defence against infection. These immune cells make up about 60-70% of the total number of immune cells and are short lived after a cycle of phagocytosis (Metzler et al, 2014; Rodriguez-Espinosa et al, 2014). Structurally these cells contain a multi-lobed nucleus and three types of vesicles which are known as primary, secondary, and tertiary granules which are all found in the cytoplasm of the neutrophils. The primary function of neutrophils is to eliminate microbial and viral pathogens (Remijsen et al, 2011[b]; Kolaparthy et al, 2014). The neutrophils execute their protective activity by a coordinated process involving phagocytosis, the production of reactive nitrogen species (RNS), the production of reactive oxygen species (ROS), and the release of antimicrobial peptides and proteins, as well as by a more recently documented mechanism known as neutrophil extracellular trap formation (NET) which will further be discussed in detail, NET formation being the main focus of this study (Smith et al, 1994; Murphy. 2012, p80; Rodriguez-Espinosa et al, 2014).

Phagocytosis

Phagocytosis is the process whereby neutrophils internalise microbes and foreign matter forming a membrane-enclosed phagosome in the cytoplasm (Bjornsdottir, 2015). The phagosome rapidly acidifies creating an extremely hostile environment. The presence of the phagosome initiates the fusion of lysosomes (granules) with the phagosome which then forms a phagolysosome (Fuchs et al, 2007). The fusion of these primary, secondary and tertiary vesicles with the phagosome creates a favourable antimicrobial environment for the degradation of the contents in the phagosome. This process is initiated by the activation and triggering of receptors found on the surface membrane of neutrophils (Figure 1.1). Dectin-1 is a receptor mostly found on neutrophils and macrophages (another type of phagocytic cell found in the innate immune system) which binds to 1-3 glucans (fungal cell wall constituents) and activates the process of phagocytosis (Murphy. 2012, p77).

Production of antimicrobial peptides and proteins

Once phagocytosis has taken place the neutrophils need to produce toxic agents to eliminate the microbial constituents and therefore release antimicrobial peptides and proteins. The primary and secondary granules of the neutrophil release a number of antimicrobial peptides, with the most abundant being neutrophil elastase, α-defensins and cathelicidin. Neutrophil elastase is released from primary granules and is a proteolytic antimicrobial peptide that is activated in response to infection and the formation of a phagosome (Figure 1.1). Defensins are antimicrobial peptides released by the primary granules and are known to interfere with the cell membranes of microbes such as bacteria and fungi, by forming pores on the cell membrane causing the microbial cell to become leaky. Defensins can also interfere with the membrane envelope of some viruses. Cathelicidin is an antimicrobial peptide activated and released by the secondary granules. Its activation and release are induced when both the primary and secondary granules merge with the phagosome (Murphy. 2012, p45) (Figure 1.1).

Production of ROS

Receptors that trigger intracellular degradation are also found on the cell surface of neutrophils alongside receptors that activate other cellular pathways such as the phagocytosis activating receptors. The signalling and activation of the fMet-Leu-Phe receptor (fMLP receptor) and the C5a receptor of the complement system induces the production of ROS for the elimination of the ingested microbes (Murphy. 2012, p77). These toxic agents are produced by the activation of membrane-associated nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase). When the neutrophils are stimulated the detached constituents that make up the NADPH oxidase found in the different compartments of the cell (membrane of secondary granules, secretory vesicles and plasma membrane) translocate and assemble in the cell membrane (Figure 1.1). The constituents found in the cytosolic compartments translocate and bind to the membrane associated cytochrome b_{558} to make a fully functional NADPH oxidase (Borregaard, 1983; Anjos et al, 2014; Bjornsdottir, 2015). The activated NADPH oxidase induces a respiratory burst in the cell which then results in an increase in membrane-associated oxygen consumption, resulting in the production of superoxide anion (O_2^-) (Nishinaka et al, 2011). The enzyme, superoxide dismutase, transforms the O_2^- into hydrogen peroxide (H_2O_2), which is transformed by myeloperoxidase (MPO) into other toxic compounds such as hydroxyl radical (OH), hypobromite (OBr^-) and hypochlorite (OCl^-) (Nishinaka et al, 2011; Murphy. 2012, p80). The ROS produced are toxic and eliminate and degrade the phagocytosed microorganisms. The fMLP receptor stimulation also activates nitric oxide synthase (NOS) and inducible NOS2 (iNOS2) which also produces toxic agents collectively referred to as the reactive nitrogen species (RNS) such as nitric oxide (NO) which also helps in the intracellular elimination of microorganisms. ROS production plays a fundamental role in NET production and will be further discussed below (Murphy. 2012, p80).

1.2.2 NET production

An unusual defence mechanism directed against microbial pathogens was recently described by Brinkman et al. The basic concept of this defence mechanism is that after the neutrophil is activated the cell may then undergo a distinct type of cell death referred to as NETosis (Brinkmann et al, 2004). NETosis is characterised by the decondensation of chromatin, the appearance of intracellular vesicles, the formation of a meshwork of chromatin, the formation of citrullinated histones, NADPH oxidase activity and the release of antimicrobial neutrophil granule components such as elastase and myeloperoxidase (MPO) from the primary granules, lactoferrin from the secondary granules, and other bactericidal/permeability-increasing proteins from secretory vesicles of neutrophils (Anjos et al, 2014; Halverson et al, 2015)(Figure 1.1). All these changes occur in the intracellular space, terminating in the release of an extracellular network composed of a meshwork of chromatin fibres (Brinkmann et al, 2004; Brill et al, 2012). The main function of NETs is to immobilise, entrap, and kill microbes extracellularly, thereby preventing the spreading of the infection (Brinkmann et al, 2012; Kaplan et al, 2012).

Extracellular trap formation is not only restricted to neutrophils. Other granulocytes such as eosinophils and mast cells excluding basophils, are also able to extrude their DNA in the extracellular milieu to form extracellular traps (Brill et al, 2012; Anjos et al, 2014).

The formation and release of NETs can be stimulated by a number of physiological and non-physiological inducements. Physiologically, NETs can be induced by microorganisms such as *Pseudomonas aeruginosa* (which is a weak inducer), *Aspergillus fumigatus* and *Candida albicans*, as well as activators such as lipopolysaccharide, interferon gamma (IFN-γ), C5a complement and interleukin 8 (IL-8). Non-physiological stimulants include the phorbol ester, phorbol-12-myristate-13-acetate (PMA), and calcium ionophores (Guimaraes-Costa et al, 2012; Anjos et al, 2014; Halverson et al, 2015).

A number of studies have provided compelling evidence that the enzymatic processes involving NADPH oxidase and MPO, which mediate the production of ROS in activated neutrophils, play a fundamental role in NET production (Fuchs et al, 2007; Remijsen et al, 2011[a]; Arai et al, 2014). However, this does not appear to be a prerequisite for NET formation as NETosis induced by the pneumococcal pore-forming toxin, pneumolysin, the staphylococcal toxin, Panton-Valentine leukocidin, and the calcium ionophore, A23187, is not dependent on NADPH oxidase activity (Parker et al, 2012). This is an indication that there is a possibility of more than one pathway when producing NETs. Research studies have explained the possibility of two types of NETosis pathways, namely vital and suicidal NETosis (Parker et al, 2012; Yipp and Kubes, 2013; Kolapathy et al, 2014). The differences between these two pathways is the duration of the stimuli, the cellular changes leading to the release of NETs and the molecular pathways.

The duration of vital NETosis occurs rapidly with a time period of 5-60 minutes and does not involve cell death stimulation, furthermore this pathway involves the activation of a type of pattern recognition receptor mainly the toll like-receptors-4 (TLR-4) which recognize components of Gram-positive and Gram-negative bacteria (Semeraro et al, 2011; Yipp and Kubes, 2013; Kolapathy et al, 2014). When activated the neutrophils form vesicles comprising of decondensed chromatin and antimicrobial protein from secondary and tertiary granules, finally the vesicles are then released in the extracellular space where they form NETs (Parker et al, 2012; Kolapathy et al, 2014)

On the contrary, suicidal NETosis activates a cell death mechanism which takes 2-4 hours to occur (following prolonged exposure to activators) (Parker et al, 2012). This type of NETosis is observed during PMA-induced NET formation explained previously in the first paragraph of this section (section 1.2.2, page 4) and in the following paragraph.

Moreover, elucidation of the specific ROS and their involvement in the formation of NETs, requires further investigation (Park and Winterbourn, 2013; Anjos et al, 2014). It has been reported that neutrophils from patients with chronic granulomatous

disease (CGD), an inherited, primary immunodeficiency disease associated with the absence of NADPH oxidase activity in phagocytic cells, are unable to produce ROS and undergo NETosis when activated with PMA. (Fuchs et al, 2007; Nishinaka et al, 2011). The presence of MPO has also been shown to be an important factor in PMA-induced NET formation. In one study, neutrophils from individuals with a rare form of MPO deficiency were found to be unable to make NETs when activated by PMA (Metzler et al, 2014). Nishinaka et al reported that ROS (the specific active species have not been identified) and MPO are required for PMA-activated NETosis and that MPO converts hydrogen peroxide into singlet oxygen ($^1O_2$) (Nishinaka et al, 2011). They proposed that this may implicate the involvement of the singlet oxygen as being the putative ROS that is required for NET formation (Nishinaka et al, 2011; Arai et al; 2014). Given that MPO plays a fundamental role in ROS production, it is not surprising that this enzyme, like NADPH oxidase, is necessary for PMA-induced NET production (Bjornsdottir et al, 2015; Gupta et al, 2014; Metzler et al, 2014).

Calcium flux is also an important factor in NET production, with calcium ionophores having been shown to induce NET formation (Guimaraes-Costa et al, 2012; Gupta et al, 2014). In addition, thapsigargin, an inhibitor of the endomembrane Ca^{2+}-ATPase, which increases in cytosolic CA^{2+} due to leakage of the cation from intracellular stores can also induce NET formation. The key involvement of Ca^{2+} in the process of NETosis is linked to Ca^{2+}-dependent activation of peptidyl arginine deiminase 4 (PAD4), which mediates the citrullination of histones (Gupta et al, 2014).

Citrullination plays a key role in the early stages of NETosis. The histones extruded with nucleic acid during NET formation are citrullinated histones. Citrullination is the conversion or the post-translational modification of arginine into citrulline (Lugli et al, 2015). This process is a common biological process and is usually necessary for physiological functions such as skin formation, nerve growth and gene regulation. PADs are enzymes which mediate the conversion of arginine into citrulline (Anjos et al, 2014). This process is evident in a number of proteins such as histones, keratin, fibrinogen, collagen and myelin basic protein. The specific PAD produced by and responsible for the citrullination of the histones in neutrophils during NET formation is the PAD4 enzyme (Wang et al, 2009). Wang et al, who investigated the effects of hypercitrullination induced by the PAD4 on the decondensation of chromatin in

granulocytes, proposed that hypercitrullination may play a role in the decondensation of chromatin during NETosis (Wang et al, 2009).

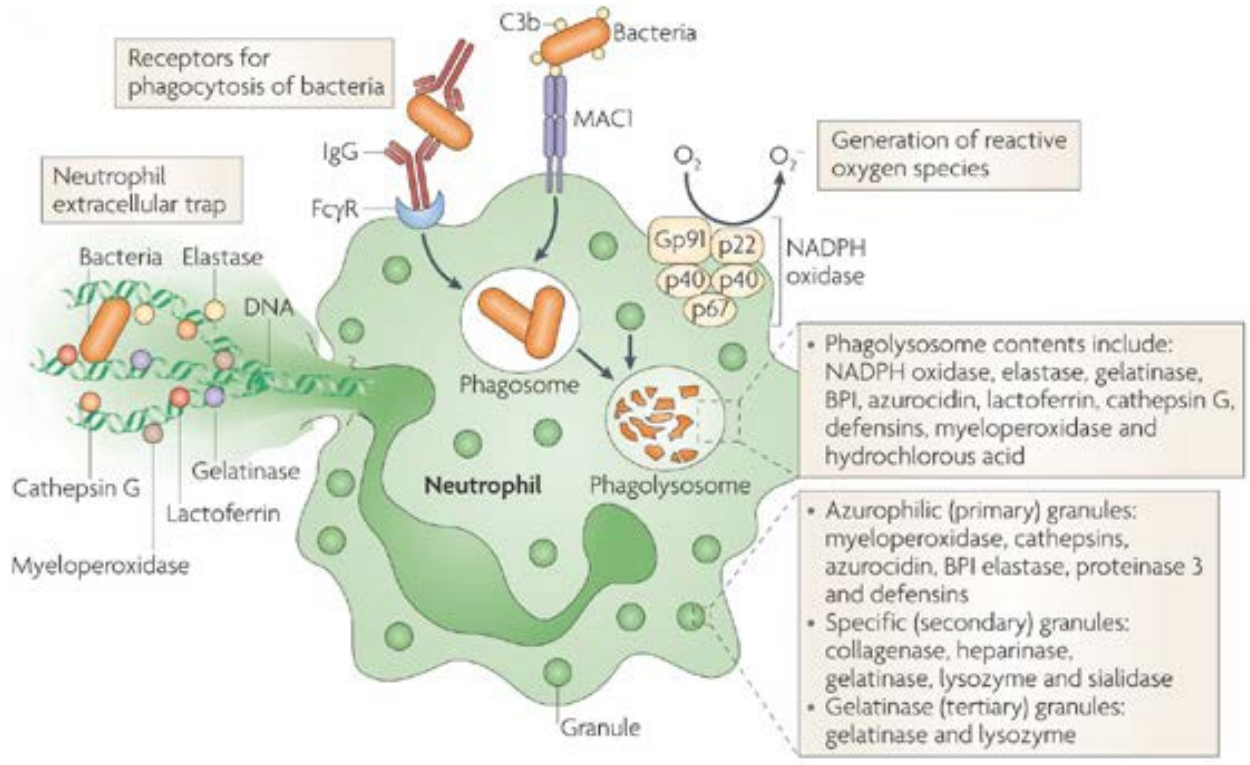


Figure 1.1. Summary of the processes of phagocytosis, neutrophil extracellular trap formation and NADPH oxidase activity. Microbial components that activate NET formation, found on the plasma membrane of the cells bind to phagocytosis activating receptors which initiate the phagocytosis of microbial components leading to the formation of a phagosome. This, in turn, activates NADPH oxidase and ROS production. The phagosome fuses primary and secondary granules which discharge their antimicrobial contents into the vacuole which is now known as the phagolysozome. The activated neutrophil also undergoes NETosis resulting in the formation of a network composed of decondensed chromatin, citrullinated histones, and antimicrobial proteins and peptides, which are collectively released extracellularly to capture and eliminate microbes (Hikey and Kubes, 2009).

As mentioned above, inappropriate, excessive NETosis has also been implicated in the pathogenesis of various disorders such as cancer via the sequestering of tumor cells and promotion of metastasis, thrombosis, autoimmune diseases such as SLE and RA, small vessel vasculitis and psoriasis (Kessenbrock et al, 2009; Lande et al, 2011; Knight et al, 2012; Park and Winterbourne, 2013; Cools-Lartigue et al, 2013; Lugli et al, 2015).

Sequestering of tumor cells and promotion of metastasis

Cancer is one of the most common causes of death worldwide with metastasis a major contributor. Neutrophils have been implicated in the promotion of metastatic disease (Cools-Lartigue et al, 2013). Some studies have even indicated that increased numbers of neutrophils in cancer are an independent marker of a poor prognosis, including malignancies such as lung and gastric cancer (Demers and Wagner, 2013).

The mechanisms by which neutrophils possibly contribute to progression to metastatic diseases are as follows. The first type is a contact-dependent-mechanism which occurs when neutrophils form a bridge between the terminal endothelial region of an organ and the circulating tumor cells, enabling the attachment of the circulating tumor cells to organs and promoting metastasis (Demers and Wagner, 2013; Cools-Lartigue et al, 2013). The attachment is executed by the interaction between the β_2 integrins found on the neutrophils and intracellular adhesion molecule-1 (ICAM-1) found on tumor cells (Cools-Lartigue et al, 2013). The second type of mechanism is also contact-dependent and involves adherence of the circulating tumor cells to organs and tissues, resulting from the release of soluble factors from neutrophils that activate endothelium and parenchyma cells, increasing adhesion between the circulating tumor cells and the tissue sites (Cools-Lartigue et al, 2013).

NET formation is also implicated in the progression of metastasis. In the study reported by Cools-Lartigue et al, the authors hypothesised that the occurrence of excessive NETosis in cancer patients is due to a severe post-operative infection. Prolonged systemic NETosis, in turn, would pose the risk of capture of the circulating tumor cells, initiating adhesion to tissues of the body at the sites capture (Cools-Lartigue et al, 2015).

Thrombosis

Haemostasis prevents the leakage of blood from ruptured or injured blood vessels in order for them to efficiently heal. This process consists of three phases, namely vasoconstriction, temporary blockage and blood coagulation (Silverthorn. 2010, p559). Following formation of platelet plugs as a temporary seal, studies have revealed the occurrence of concomitant NETosis. The NETs produced in this setting are able to bind to von Willebrand factor (vWF) and further increase platelet recruitment (Fuchs et al, 2010; Kolaczkowaska et al, 2015). The citrullinated histones produced in NET production, Histones 3 and 4 (H3 and H4 respectively), are also able to activate the platelets and further potentiate platelet activation and aggregation (Hahn et al, 2013). In the process of coagulation, NETs can also directly activate Factor XII, initiating a cascade reaction leading to clot formation (Silverthorn. 2010, p559) (Hahn et al, 2013). Further studies have also found that the extrinsic pathway can also be activated when NETs bind to tissue factor (TF) which also promotes clot formation (Zawrotniak and Rapala-Kozik, 2013). It is now believed that NET formation contributes to the pathogenesis of cardiovascular disease via pro-coagulant activity (Bjornsdottir et al, 2015).

Autoimmune diseases

Autoimmune diseases are caused by the failure of self-tolerance mechanisms (central and peripheral tolerance mechanisms) in the immune system which affects the entire immune system (Costenbader and Karlson, 2006; Lugli et al, 2015). NETosis has been implicated in the pathogenesis of a number of autoimmune diseases (AD). Autoimmune conditions result from the production of autoantibodies against host autoantigens. The basic concept of their involvement in triggering an autoimmune response is that NETs through the release of their constituent, autoantigenic cytosolic and nuclear constituents into the extracellular space pose the risk for development of AD (Chuahan et al, 2015; Lugli et al, 2015). If the NETs are efficiently degraded by the body after their release and their purpose is served, then the risk of recognition of NET-associated autoantigens is significantly increased.

Constituents such as citrullinated proteins and dsDNA have definite autoantigenic potential.

1.2.3 Cigarette smoking and neutrophils

Cigarette smoking (tobacco smoking) is one of the major public health problems that countries face worldwide. In 2012, 21% of the population worldwide, aged 15 years and onwards, smoked tobacco (WHO 2016, para. 1[a]). In that same year, the six most leading causes of death were ischaemic heart disease, stroke, chronic obstructive pulmonary disease (COPD), lower respiratory tract infections, lung cancers (trachea and bronchus) and human immunodeficiency virus/ acquired immune deficiency syndrome (HIV/AIDS) (WHO 2014, para 1.). The first five of these conditions are all associated with smoking, while those infected with HIV appear to be particularly vulnerable to the adverse health effects of smoking. Most of the above mentioned diseases are non-communicable diseases and in 2012, 68% of deaths globally were due to non-communicable diseases (WHO 2016, para 1[b]). Cigarette smoking does not only directly affect the respiratory system, but also indirectly affects various other organs in the human body (Yanbaeva et al, 2007).

Constituents of Cigarette smoke

Cigarette smoke consists of various chemical constituents and contains approximately 10^{10} particles/ml, containing an abundance of toxic chemicals (Valavanidis et al, 2009). A single puff of cigarette smoke contains a blend of various toxic and carcinogenic compounds. After combustion, over seven-thousand (7000) chemical compounds are generated, including nicotine, carbon monoxide, polycyclic aromatic hydrocarbons (PAH), aza-arenes and N-nitrosamines and high concentrations of oxidants and free radicals are present in both the gaseous phase and the tar phase of cigarette smoke (Rahman and MacNee, 1996; Rodgman et al, 2000). At least 40 of these compounds are known to be tumor promoters (carcinogenic) (Valavanidis et al, 2009). A majority of these compounds are highly pernicious and carcinogenic such as benzene, 2-napthylamine, cadmium, nickel and a variety of other carcinogens yet to be specified (Valavanidis et al, 2009). Various

studies have indicated that the carcinogenic particles contained in cigarettes smoke result in oxidative damage to cellular DNA (Church and Pryor, 1985). The induction of ROS production by cigarette smoke, also promotes depletion of cellular anti-oxidative vitamins and enzymes, predisposing the lungs to oxidative stress (Cross et al, 1999).

Effects of cigarette smoking on humans

Direct inhalation of cigarette smoking has been implicated as a risk factor for a number of conditions such as pulmonary emphysema, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis and lung cancer which are conditions that involve the impairment of the alveolar epithelial cells found in the lungs. Cigarette smoke damages the cells by increasing the permeability of the cells, decreasing the production of pulmonary surfactants which increase surface tension of the cells, promoting cell death either through necrosis or apoptosis, as well as triggering inflammatory responses by activating the production of pro-inflammatory cytokines and finally promoting tumorigenesis through inhalation of smoke-derived carcinogens and the production of growth factors by various types of pulmonary cells which may lead to lung cancer (Aoshiba and Nagai, 2003). The lungs of cigarette smokers are also populated by increased numbers of pulmonary neutrophils and aveolar macrophages when compared to non-smokers, which also contributes to the exacerbation of respiratory conditions (Rahman and MacNee, 1996). Cigarette smoking also indirectly affects peripheral organs in the body and is a recognised risk factor for conditions such as bladder cancer, hypertension and heart conditions. (Kalra et al, 1991; Plottner et al, 2012).

Cigarette smoking also increases oxidative processes such as lipid peroxidation, protein carbonylation, thiol peroxidation and DNA oxidation. As mentioned above, cigarette smoke increases the number of macrophages and neutrophils in the lungs which also contribute to oxidative stress through the production of ROS production (Valavanidis et al, 2009; Church and Pryor, 1985). Male students who smoked cigarettes relative to those who did not, exhibited increased oxidative stress as measured by increased circulating levels of lipid peroxides and lactate

dehydrogenase, as well as an impaired antioxidant system measured by decreased activities of the anti-oxidative enzymes catalase and superoxide dismutase, as well as low intracellular concentrations of the anti-oxidative tripeptide, glutathione (Mahaptra et al, 2008;).

1.2.4 Association between cigarette smoking and NETs

Associations with cigarette smoking have also been reported in the ADs rheumatoid arthritis (RA), SLE, multiple sclerosis and Graves' disease (Costenbader and Karlson, 2006). As mentioned above, the NET-derived constituents extruded in the extracellular space may activate autoantibody production and initiate an autoimmune response. Cigarette smoke also promotes the citrullination of proteins (Alsalahy, 2010; Lugli et al, 2015). A study done by Lugli et al in 2015 suggested that smoking could be a possible factor associated with the increased production of PAD2 and PAD4 in smokers (Lugli et al 2015).

1.2.5 Biomarker for smoking

Measurment of cotinine, a metabolite of nicotine, in urine or blood is considered to be a reliable, objective biomarker for smoking when determining the level of smoke exposure and the accuracy of self-proclaimed smoking histories (Benowitz et al, 2009; Avila-Tang et al, 2012). When nicotine enters the body it is extensively metabolised in the liver and an average of 75% of nicotine is converted into cotinine (Avila-Tang et al, 2012). A number of studies indicate that when it comes to interpretation of cotinine levels, 15ng/ml is considered to be a good cut-off point when distinguishing between active and a passive smokers (Jarvis et al, 2008).

1.3 The Aim of research study

The primary objectives of this study were to investigate the effects of cigarette smoke condensate on NET formation when using PMA as an inducer, and, in addition to compare the NET-forming activities of neutrophils from smokers and non-smokers. Secondary objectives included:

- Investigation of the role of NADPH oxidase in PMA-induced NET formation by measuring oxygen consumption by neutrophils treated with CSC.
- To measure the viability of the neutrophils when treated with CSC and PMA individually and in combination.
- To measure serum cotinine levels as a biomarker for smoking in order to effectively distinguish between the smokers and the non-smokers.

CHAPTER 2

Cigarette smoke condensate attenuates phorbol ester-mediated neutrophil extracellular trap formation

This chapter has been submitted for publication to the African Journal of Health Sciences.

2.1 Introduction

Neutrophil extracellular trap formation (NETosis) is a recently described host defence mechanism characterised by the decondensation of chromatin and nuclear segmentation resulting in the extracellular release of net-like structures (Brink et al, 2004; Zawrotniak et al, 2013; Anjos et al, 2014). NET formation can be triggered by a number of physiological and non-physiological activators such as interleukin-8 (IL-8), lipopolysaccharide (LPS), phorbol esters, interferon-γ (IFN-γ), various types of bacteria and their products, viruses, fungi and the complement cleavage component, C5a. The phorbol ester, phorbol-12-myristate-13-acetate (PMA) activates NETosis *in vitro* (Remijsen et al, 2011[a]; Guimaraes-Costa et al, 2012). PMA-induced NET formation requires protein kinase C (PKC)-mediated activation of the membrane-associated electron-transport, superoxide-generating complex, NADPH oxidase, as well as the activation of the enzyme peptidyl arginine deiminase 4 (PAD4), which leads to the citrullination of histones (Brill et al, 2012). This is followed by the release of decondensed chromatin web-like fibres entangled with citrullinated histones and impregnated with antimicrobial peptides and proteins including neutrophil elastase and myeloperoxidase (Park and Winterbourn, 2013; Anjos et al, 2014; Kolaparthy et al, 2014; Metzler et al, 2014; Rodriguez-Espinosa et al, 2014). Activation of NADPH oxidase is apparently a critical requirement for NETosis since patients with chronic granulomatous disease (CGD) who are unable to generate reactive oxidants, do not produce NETs when activated with PMA. Furthermore, diphenyleneiodonium chloride, an inhibitor of NADPH-oxidase activity, when added to neutrophils

effectively attenuates PMA-mediated NET formation (Remijsen et al, 2011[a]; Bjornsdottir et al, 2015).

NET formation protects the host against infection which is achieved via the capture of microbes and viruses in the net-like structures of decondensed chromatin fibres which are impregnated with antimicrobial agents resulting in the immobilisation, localisation and possibly elimination of the infection (Rodriguez-Espinosa et al, 2014; Bjornsdottir et al, 2015). In this setting, NET formation is potentially an advantageous host defence mechanism.

Cigarette smoking is a common lifestyle habit worldwide, with 21% of the population older than 15 years, smoking tobacco on a regular basis [13]. Cigarette smoke is composed of chemical compounds that include tar, nicotine, carbon monoxide, polycyclic aromatic hydrocarbons and high concentrations of oxidants and free radicals (Rahman and MacNee, 1995). Cigarette smoke has been shown to increase oxidant production by neutrophils (Kalra et al, 1995; Rahman and MacNee, 1995), and to promote oxidative processes such as lipid peroxidation, protein carbonylation, thiol peroxidation and DNA oxidation. Cigarette smoking also recruits macrophages and neutrophils to the lungs and increases the citrullination of proteins (Church and Pryor, 1985; Valavanidis et al, 2009; Alsalahy et al, 2010; Fuchs et al, 2010; Damgaard et al, 2015).

To date there are apparently no publications evaluating the effects of cigarette smoke and cigarette smoke condensate on NETosis. In the current study, the effects of exposure of isolated human blood neutrophils to cigarette smoke condensate (a surrogate for cigarette smoke) on PMA-induced NET formation *in vitro* have been investigated.

2.2 Methods and Materials

Ethics approval

The study was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Approval No. 2015/358).

Participants

The participants were healthy non-smoking individuals who were not taking any medication. They were aged 19-49 years old and consisted of 5 males and 7 females (general characteristics displayed in Table 2.1). Measurement of phorbol 12-myristate 13-acetate activated NETosis in the absence and presence of CSC (40-80 µg/ml) using spectrofluorimetry was performed on neutrophils from all 12 donors, while confirmatory microscopy, oxygen consumption and viability were performed on cells from 5, 6 and 9 donors respectively.

Reagents and Chemicals

Alexa Fluor 488-labeled goat anti-rabbit secondary antibody, 5 mM Sytox orange and 4',6-diamidino-2-phenylindole (DAPI) were all purchased from Life Technologies (Pty) Ltd (USA). Histopaque-1077 was purchased from Sigma Aldrich (Pty) Ltd (Johannesburg, South Africa) and the polyclonal rabbit anti-histone H4 (citrulline 3) was purchased from Merck Millipore (Pty) Ltd (Johannesburg, South Africa). Propidium iodide (50 µg/ml DNA Prep-Stain) was purchased from Beckman Coulter Pty (Ltd) (Johannesburg, South Africa) and the cigarette smoke condensate (CSC) from Murty Pharmaceuticals Inc, Lexington (KY) and dissolved in dimethyl sulphoxide to give a stock concentration of 40mg/ml. The total amount of condensate generated during the combustion of one cigarette is about 26.3 mg; therefore the concentrations of the condensate used in this study were relevant in the context of the smoking habit (Davis and Day, 1969). The CSC was prepared by burning University of Kentucky's 1R3E standard cigarettes and extracting the total particulate matter generated into DMSO using a smoking machine (Kulkarni et al, 2010; Lyn-Cook et al, 2014). The constituents of CSC include a complex mixture of phenolic compounds such as phenols, cresols and dihydroxybenzenes, as well as hydrogen

cyanide, acrolein and formaldehyde (Nikolic et al, 1997; Richter et al, 2010). Unless indicated the remaining chemicals and reagents mentioned in the methods were purchased form Sigma-Aldrich.

Methods

Neutrophil isolation

Following to obtaining informed consent, neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood and separated from mononuclear leukocytes by centrifugation on Histopaque-1077 cushions at 754 x g for 25 minutes at room temperature. The resultant cell pellet was suspended in phosphate-buffered saline (PBS, 0.15M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4^{o} C for 10 minutes. Following centrifugation (355 x g for 10min), the supernatant fluid was removed and the cells were washed in PBS and the neutrophils were then re-suspended to 1x10^{7}/ml in PBS and held on ice until used. Purity and viability were assessed using flow cytometry.

Exposure of neutrophils to PMA and CSC

A total of 4x10^{6} of cells (0.4 ml) was added to 3.6 ml of Hanks' balanced salt solution (HBSS, pH 7.4, indicator-free) and incubated for 5 minutes at 37^{o}C. Following incubation the cells were exposed to either DMSO (solvent control, 2µl/ml) or CSC (40-80 µg/ml, final). Following incubation for 10 minutes at 37^{o}C, the cells were activated by the addition of PMA (6.25 ng/ml), a potent activator of NETosis, and incubated for 90 minutes at 37^{o}C. Thereafter, the tubes were vortexed and centrifuged for 5 minutes, 4^{o}C at 355 x g to pellet the neutrophils. Three ml of the supernatant was then pipetted into new 5 ml tubes and the pellet resuspended and the cells retained for analysis of viability.

Spectrofluorimetry

Three microlitres (3 µl) of 5 mM Sytox orange, a DNA-reactive, fluorescent dye, was added to the harvested supernatant fluids in reaction cuvettes and transferred to the cuvette holder of a Hitachi 650-10S fluorimeter with the excitation and emission wavelengths set at 530 nm and 590 nm respectively. Fluorescence intensity as an index of NETosis was recorded as metered fluorescence units (MFUs).

Fluorescence Microscopy

Microscopy was performed to confirm that the extracellular DNA demonstrated by other techniques was the product of NETosis. Neutrophil suspension ($1.25x10^5$ cells in 250 µl) was allowed to adhere to glass cover slips for 30min. PMA (6.25 ng/ml) or an equal volume of HBSS in the presence and absence of CSC (80µg/ml) was added to the adherent cells and the coverslips were then incubated for 120min at 37ºC 5% CO_2. The cells were then fixed with 4% paraformaldehyde for 10min, washed 3 times in PBS and blocked with HBSS containing 5% goat serum and 5% bovine serum albumin for 30min at 37ºC. Neutrophils were incubated overnight with polyclonal rabbit anti-histone H4 (citrulline 3, Merck Millipore) and washed three times in PBS. Following addition of Alexa Fluor 488-labeled goat anti-rabbit secondary antibody, DNA present in the specimen was stained with the DNA-binding dye, DAPI, for 2 minutes and analysed for NET formation by fluorescence microscopy using a Zeiss Axio Vert. A1 fluorescence microscope and Axion Vision Software (Zeiss, Johannesburg, South Africa). Representative photomicrographs were taken, and used for the generation of counts of cells undergoing NETosis by a single non-blinded observer. Results are expressed as percentage of NET forming cells.

Cell Viability

This was measured after a 90 min incubation following the addition of PMA to neutrophils using a flow cytometric propidium idodide-based dye exclusion assay. The cells ($4x10^6$/ml) were incubated for 5 min with propidium iodide (50 µg/ml) DNA Prep-Stain, and cell viability assessed flow cytometrically with the results expressed as percentage of viable cells.

Oxygen consumption

A total of 4 x10^6 of cells (0.4 ml) were added to 1.6 ml of HBSS and incubated for 10 minutes at 37^{o}C in the absence or presence of CSC (80 µg/ml). The cells were then transferred to the thermoregulated compartment of an Oxygen Electrode (Hansatech, Instruments Ltd., Norfolk, UK). After a stable baseline was reached (in about 1 minute) the cells were stimulated with PMA (6.25 ng/ml) and oxygen consumption measured over a 10 min period and recorded as relative units.

Statistical analysis

Statistical significance was calculated from raw data collected from each series of experiments using a Mann-Whitney non-parametric test for analysis of the experiments measuring the effects of CSC on PMA-induced NET formation, oxygen consumption and cell viability. A computer-based software system was used (Graph Pad Instat 3®)) and the results are expressed as the median absolute values with 25th and 75th percentile values for each series of experiments.

2.3 Results

Effects of CSC on NET formation

Spectrofluorimetric analysis

These are shown in Figure 2.1 which depict the effects of activation of neutrophils with PMA (6.25 ng/ml) on NET formation following 90 min of incubation at 37^{o}C which resulted in a statistically significant increase in NET formation ($P<0.0002$). Figure 2.1 also depicts the effects of CSC (40 and 80 µg/ml) on PMA-activated NETosis. Exposure of PMA-activated cells to CSC resulted in attenuation of NETosis, the values for systems treated with 40 and 80 µg/ml being 65% and 66% of the corresponding untreated control system respectively ($P<0.0001$ and $P<0.0005$).

Microscopic analysis

These results are shown in Figures 2.2 and 2.3, which show representative fluorescence micrographs depicting PMA-activated NETosis in the absence and

presence of CSC at a concentration of 80 µg/ml (Figure 2.2). Averaged data from a larger number of experiments (Figure 2.3) resulted in statistical significance ($P<0.0001$) on the effects of neutrophils activated with PMA (6.25 ng/ml) on NET formation and further confirming the inhibitory effects of exposure of neutrophils to CSC on PMA-activated NETosis ($P<0.0001$).

Viability analysis

These results are presented in Figure 2.4 which show the viabilities of unstimulated and PMA-activated neutrophils in the absence and presence of CSC at 40 and 80 µg/ml following 90 min incubation at 37 ºC. Exposure of the cells to CSC alone did not affect viability, while activation with PMA resulted in statistically significant ($P<0.0003$) loss of viability consistent with lytic NETosis, with no additive effects of CSC.

Oxygen consumption

The results presented in Figure 2.5 are those for the averaged total amounts of oxygen consumed over the 10 min incubation period by resting, unstimulated neutrophils, as well as by PMA (6.25 ng/ml)-activated cells in the absence and presence of CSC (80 µg/ml). They demonstrate firstly, that exposure of neutrophils to PMA (6.25 ng/ml) results in marked consumption of oxygen by the cells ($P<0.0001$), and, secondly, that exposure of PMA-activated, but not resting cells, to CSC, results in statistically significant attenuation of oxygen consumption (73% of the CSC-free control system, $P<0.0001$). The time course of these experiments as oxygen depletion in the cell-suspending medium, averaged for each experimental system (resting and PMA-activated neutrophils in the absence and presence of CSC) are shown in Figure 2.6.

Table 2.1 Demographic data of the 12 healthy non-smoking donors involved in the study.

Characteristics	Current Non-smokers (n=12)
Age (Mean, year range):	28(19-49)
Gender:	
Female	7
Male	5
Race:	
Black	2
White	10

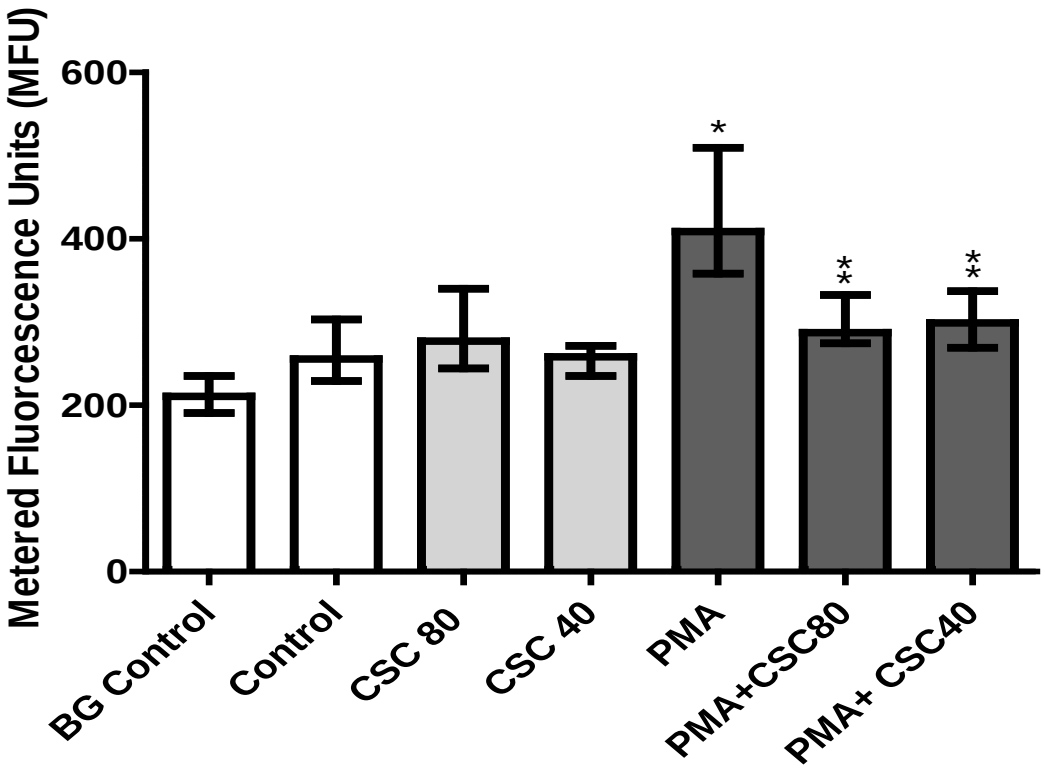
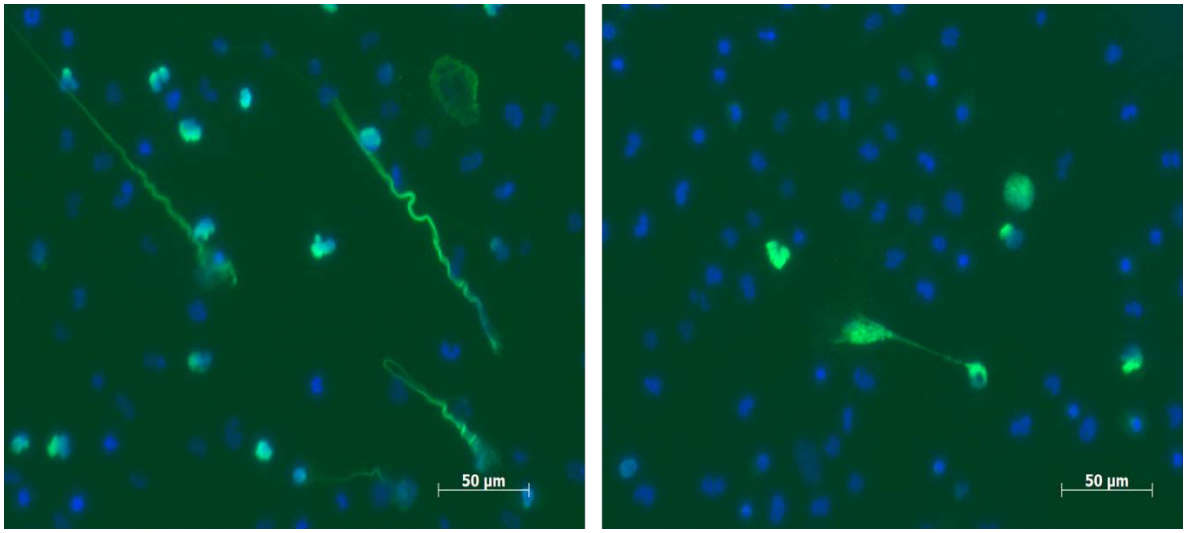


Figure 2.1

The effects of CSC (40 and 80 µg/ml) on PMA (6.25 ng/ml)-activated NETosis using spectrofluorimetric analysis for DNA measurement with results expressed as the median values in metered fluorescence units (MFUs) and with 25th and 75th percentiles. NETosis was significantly increased in PMA-activated neutrophils (*P<0.0002), while in the presence of CSC (40 and 80 µg/ml), the magnitude of PMA-induced NETosis was significantly attenuated (**P<0.0005 and **P<0.0001), respectively.

A) Control **B) CSC**



C) PMA **D) PMA+CSC 80**

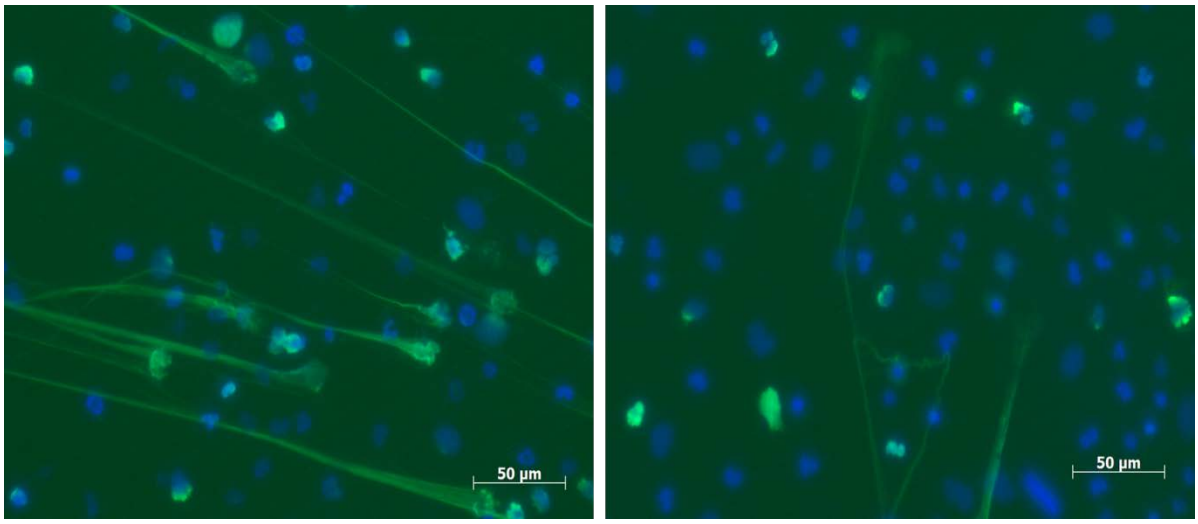


Figure 2.2

Fluorescence microscopic images from a single representative experiment (5 in the series) showing NETosis of resting neutrophils in the absence (A) and presence (B) of CSC (80 µg/ml) following 120 min incubation, as well as NETosis following activation of the cells with PMA (6.25 ng/ml) in the absence (C) and presence (D) of CSC.

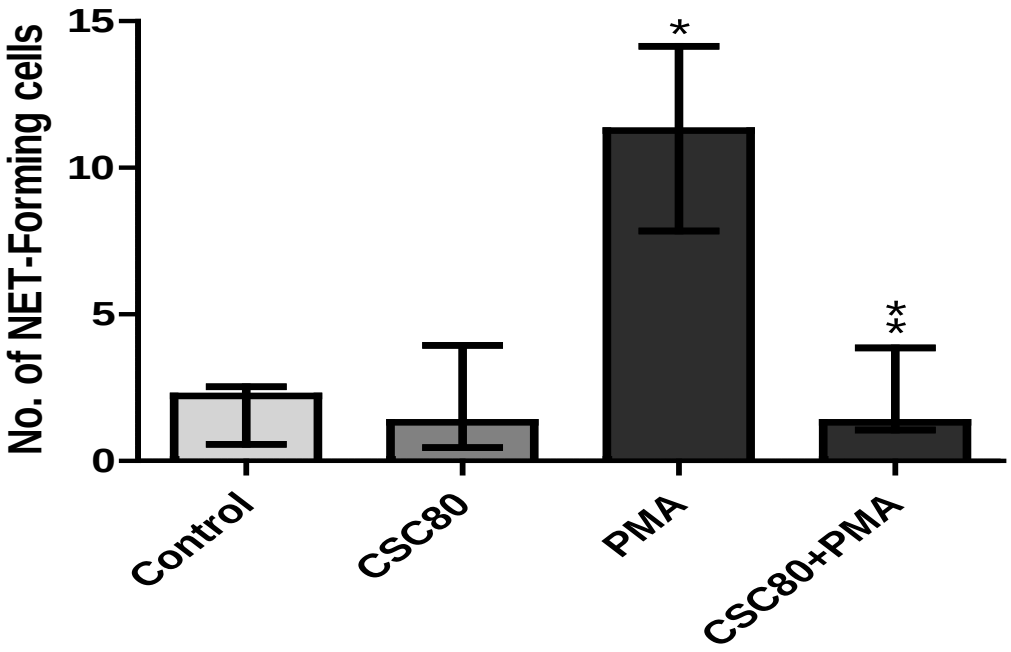


Figure 2.3

NET formation by resting and PMA (6.25 ng/ml)-activated neutrophils in the absence and presence of CSC (80 µg/ml) measured microscopically according to the numbers of NET-forming cells. The results of 5 separate experiments are expressed as the numbers of NET-forming cells for each system as median values and percentile values of 25% and 75%. PMA induced NETosis was significant (*P<0.0001) and attenuation of NETosis in PMA-activated systems treated with CSC (80 µg/ml) obtained statistical significance (**P<0.0001) as indicated.

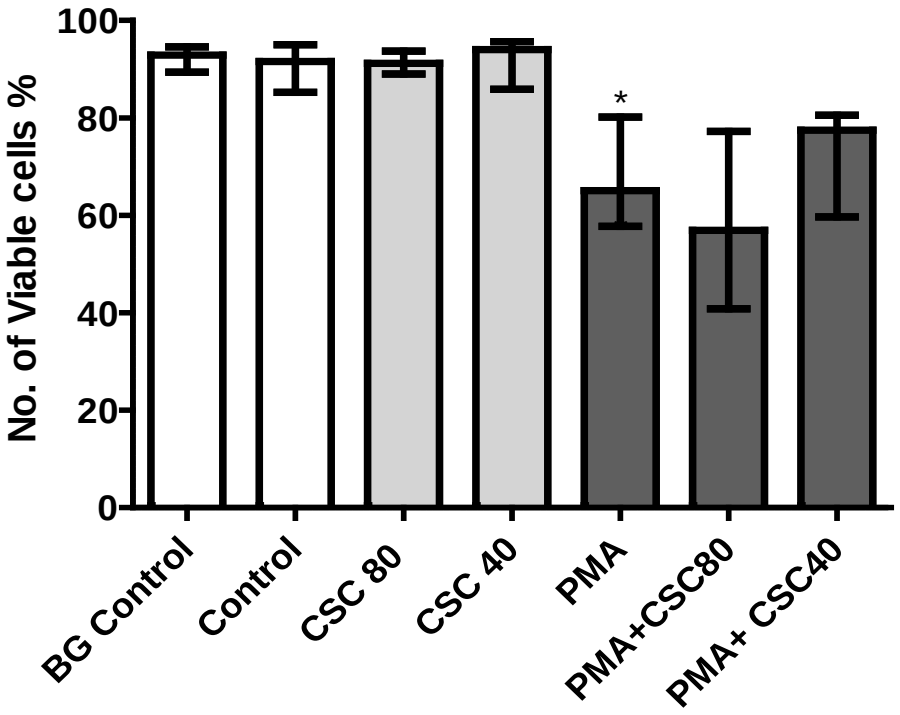


Figure 2.4

The effects of exposure of unstimulated and PMA (6.25ng/ml)-activated neutrophils to CSC (40-80 µg/ml) following 90 min incubation on neutrophil viability. The results of 9 separate experiments are expressed as the median values with percentile values of 25% and 75%. PMA activation resulted in (*P<0.0003) loss of viability.

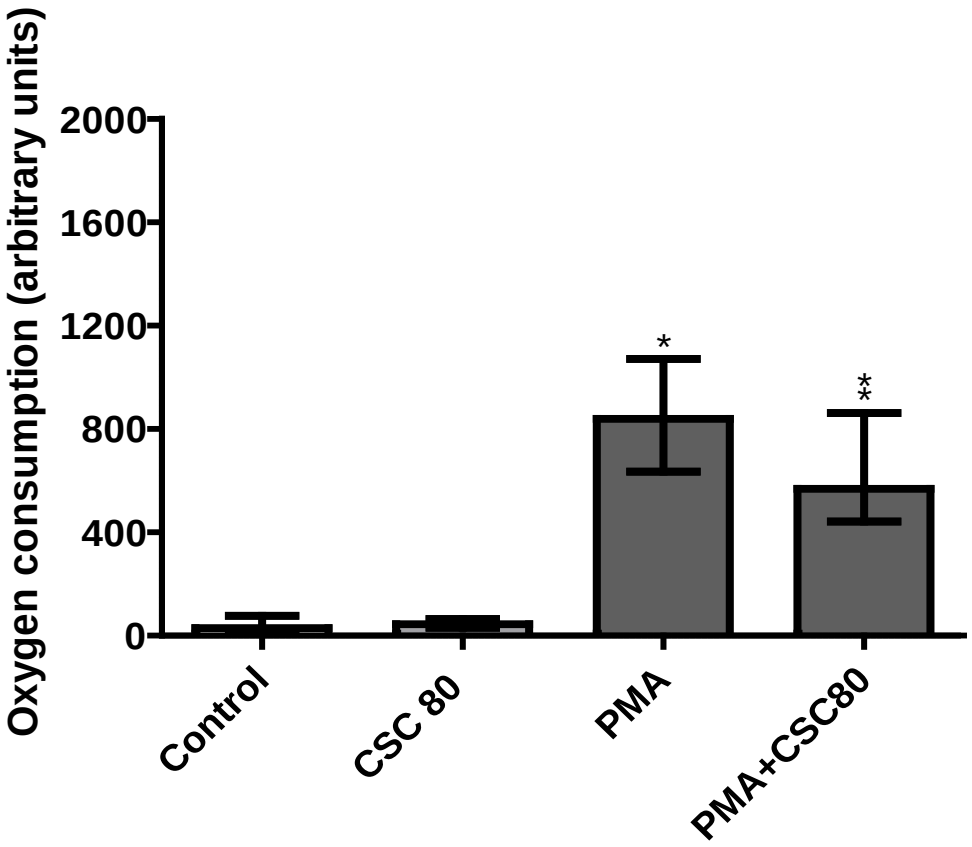


Figure 2.5

The effects of CSC (80 µg/ml) on oxygen consumption measured over a 10 min period by unstimulated and PMA (6.25 ng/ml)-activated neutrophils. The results of 6 separate experiments are expressed as median arbitrary units with percentile values of 25% and 75%. PMA-induced NET formation of neutrophils obtained statistical significance as indicated (*P<0.0001). **P<0.0001 for comparison of the PMA-activated systems in the presence and absence of CSC.

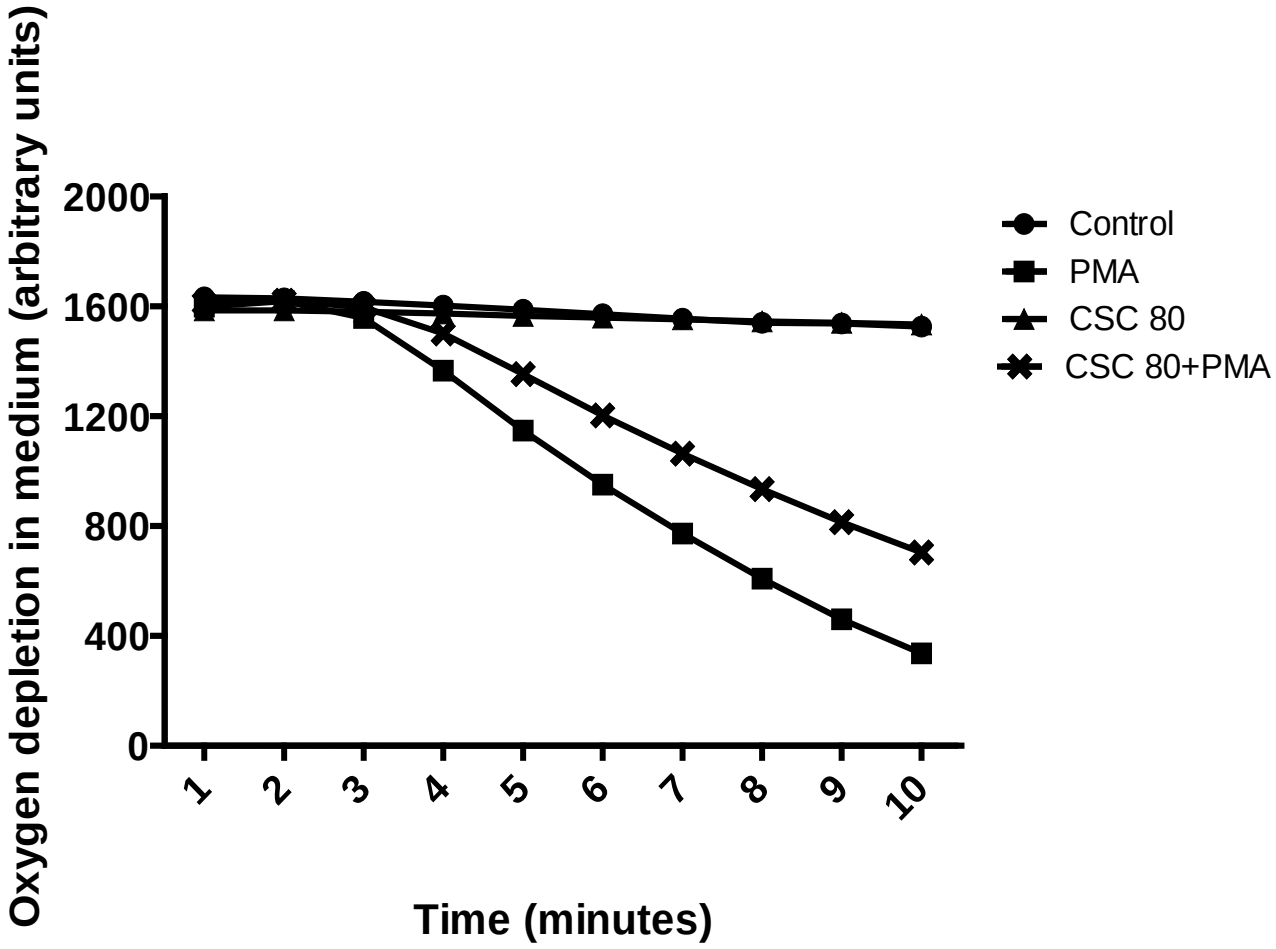


Figure 2.6

Time course of oxygen depletion in the cell-suspending medium by unstimulated and PMA (6.25 ng/ml)-activated neutrophils in the absence and presence of CSC (80 µg/ml). The data of 6 separate experiments are averaged for each time point.

2.4 Discussion and Conclusion

The current study was designed to investigate the effects of cigarette smoke condensate (CSC) on phorbol ester-mediated neutrophil extracellular trap formation (NETosis) *in vitro*. The phorbol ester, PMA, activates the neutrophil membrane-associated electron-transporting enzyme, NADPH-oxidase, which generates reactive oxidant species (ROS) from molecular oxygen (Fuchs et al 2007). ROS, in turn, activate intracellular signaling pathways which induce NETosis (Remijsen et al, 2011[a]). During NETosis, decondensation of nuclear chromatin precedes the disintegration of nuclear membranes and mixing of nucleic acids with cytosolic granules to form vacuoles (Brinkmann et al, 2004). The contents of these vacuoles are released into the extracellular environment forming web-like structures that trap microbial pathogens and promote their destruction by proteases and ROS (Brinkmann et al, 2004; Kolaparthy et al, 2014).

In the current study, PMA-mediated NETosis was detected 90 min and 2 hours following addition of the stimulant using a spectrofluorimetric procedure and fluorescence microscopy, respectively. In the presence of CSC, significant dose-dependent attenuation of NETosis was observed and this was associated with a reduction in the rate and magnitude of oxygen consumption by PMA-activated neutrophils. In this setting, the rate and magnitude of oxygen consumption by activated neutrophils reflects the activity of the ROS-generating NADPH oxidase. CSC has been reported to inhibit the activity of NAPDH oxidase (Dunn et al, 2005). Importantly, CSC did not affect the viability of neutrophils. As ROS have been shown to play an important role in inducing NETosis, it is likely that the inhibitory effects of CSC on NETosis may be attributed to attenuation of ROS production by neutrophils. Interestingly, nicotine, a constituent of CSC has recently been reported to activate NETosis (Hosseinzadeh et al, 2016). A possible explanation for this discrepancy is that CSC and cigarette smoke contain many other substances in addition to nicotine which may exert differential effects on NADPH oxidase and NETosis. The inhibitory effects of CSC on neutrophil NADPH oxidase activity and NETosis appears to contradict the well-recognized pro-oxidative potential of cigarette smoke in the lungs of smokers. However, additional mechanisms may contribute to smoking-induced oxidative stress in the setting of decreased neutrophil NADPH oxidase activity.

These include recruitment of macrophages and T-cells to the lungs, release of pro-inflammatory cytokines by lymphocytes and airway epithelial cells, enhanced release of matrix metalloproteinases (MMPs) and other proteases by neutrophils, as well as priming of neutrophils in the lungs with increased spontaneous release of O_2^- and H_2O_2 (van der Vaart et al, 2004; Mehta et al, 2008).

Although the experimental design of the current study focused primarily on the effects of CSC on neutrophils from non-smokers, a limited series of experiments was performed to evaluate the effects of cigarette smoking (CS) on NETosis by comparing individuals who smoke with those who do not smoke (chapter 3). The magnitude of PMA-mediated NETosis was significantly decreased in smokers compared to non-smokers with a similar reduction in the rate and magnitude of oxygen consumption by PMA-activated neutrophils.

The inhibitory effects of CSC on NETosis observed in the current study may be clinically relevant as NETosis promotes the destruction of microbial pathogens by facilitating phagocytosis and proteolytic degradation of microbes ensnared by extracellular NETs. Therefore, exposure to cigarette smoke may impair the immune response of the host and predispose these individuals to infections such as those caused by *Streptococcus pneumoniae, Mycobacterium tuberculosis* and the influenza virus (Bagaitkar et al, 2008). Indeed, it is well recognized that smokers have an increased risk of colonization by *Streptococcus pneumoniae* and are more likely to develop invasive pneumococcal disease (Mutepe et al, 2013). Cigarette smoking has also been reported to alter the immunological response to pulmonary tuberculosis (PTB) increasing susceptibility to PTB (Feldman et al, 2013; Yen et al, 2014) and delaying the clearance of acid-fast bacilli from the sputum of patients started on anti-tuberculous chemotherapy (Shang et al, 2013). In addition, smoking may be associated with treatment failure and higher recurrence rates compared to non-smokers treated for PTB (Bagaitkar et al, 2008). PTB and pneumococcal pneumonia are highly prevalent conditions in Africa accounting for significant morbidity and mortality.

In conclusion, the results of the current study suggest that CSC attenuates PMA-mediated NETosis *in vitro*. If operative in the clinical setting, this effect may at least

in part explain the exaggerated risk of infection, including pneumococcal disease, observed in individuals who smoke.

CHAPTER 3

Cigarette smoking attenuates phorbol ester-mediated neutrophil extracellular trap formation *ex vivo*

3.1 Introduction

As an extension to the previous chapter (chapter 2), the experiments described in the current chapter were designed to compare the pro-NETotic activities of neutrophils isolated from the blood of smokers and non-smokers.

3.2 Methodology

Ethics approval

The study was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Approval No. 2015/358).

Participants

The participants consisted of 8 healthy, non-smoking (average age, 37 years) and 8 apparently healthy smoking (average age, 34 years) individuals who by admission were not taking any medications. The age range was 28-50 years and consisted of 5 males (2 of whom were smokers) and 11 females (6 of whom were smokers). The smokers smoked a daily average of 8 cigarettes per day. PMA-activated NETosis of neutrophils from the smokers and non-smokers was measured using spectrofluorimetry and confirmatory microscopy. Additional investigations included measurement of oxygen consumption and viability (flow cytometry), while serum cotinine levels were also measured on all participants.

Reagents and Chemicals

The same reagents used in chapter 2 (page 18) were used in this part of the study excluding the CSC.

Methods

Neutrophil isolation and preparation were performed exactly as described in chapter 2 (Methods, page18). The spectrofluorimetry, fluorescence microscopy and oxygen consumption techniques were also described in chapter 2 (Methods, page 18-20), but exclude treatment with CSC. Instead these methods where applied to investigate differences between smokers and non-smokers.

Cotinine

This was measured using a commercial solid phase competitive ELISA procedure (Calbiotech, Spring Valley, California, USA). Cotinine in samples competes with a horseradish peroxidase (HRP)-labelled anti-cotinine enzyme conjugate for binding sites on a bound cotinine antibody. Upon the addition of the enzyme substrate, the intensity of colour is inversely proportional to the concentration of cotinine in the samples. For the assay procedure frozen serum was brought to room temperature and then 10µl of standards, controls and specimens (serum) were pipetted into selected wells. An enzyme conjugate (100 µl) was added to each well and subsequently the plate was shaken for 10-30 seconds to ensure proper mixing. Following the mixing step, the microwell plate was then incubated for 60 minutes and washed 6 times with 300 µl of distilled water using a suitable plate washer. The microwell plate was then inversely slapped down on absorbent paper to ensure that all the residual enzyme conjugate was removed. Subsequently, a 100 µl of substrate reagent was added to each well and incubated in the dark for 30 minutes, then a 100 µl of stop solution was also added to each well and shaken to mix and then finally after 15 minutes the absorbance was read with a plate spectrophotometer at 450nm. The concentration of the cotinine was calculated using a standard curve that was generated via serial dilutions of the standard with concentration ranges of 0-100 ng/ml.

3.3 Results

Spectrofluorimetric analysis

These results are shown in Figure 3.1 and depict the amounts of extracellular DNA released by PMA (6.25 ng/ml)-activated neutrophils from smokers and non-smokers following 90 min incubation at 37^{o}C. PMA-induced activation of the neutrophils from both smokers and non-smokers resulted in significant release of DNA ($P<0.02$ and $P<0.0002$, respectively). However, there was no difference in the magnitude of DNA release between cells from smokers and non-smokers ($P<0.2$).

Microscopic analysis

The results are presented in Figures 3.2 and 3.3. Figure 3.2 show representative fluorescence micrographs depicting PMA (6.25 ng/ml)-activated NET formation by neutrophils from a smoker and non-smoker. Data obtained from a larger number of experiments is shown in Figure 3.3. PMA-induced NETosis was found to be reduced in smokers compared to non-smokers ($P<0.0002$).

Viability analysis

The viabilities of unstimulated and PMA (6.25 ng/ml)-activated neutrophils from smokers and non-smokers following 90min incubation at 37^{o}C, are shown in Figure 3.4. PMA activation of neutrophils from smokers and non-smokers resulted in a significant loss of viability ($P<0.0003$ and $P<0.0001$), respectively. However, there were no significant differences between smokers and non-smokers ($P<0.8$).

Oxygen consumption

The results shown in figure 3.5 are the averaged total amounts of oxygen consumed over a 10 min incubation period by resting, unstimulated neutrophils, as well as by PMA (6.25 ng/ml)-activated neutrophils from smokers and non-smokers. Exposure of cells from both smokers and non-smokers to PMA resulted in increased consumption of oxygen ($P<0.0003$ and $P<0.0003$, respectively). The magnitude of oxygen consumption by PMA-activated neutrophils from smokers was significantly less than that observed with cells from non-smokers ($P<0.04$). Figure 3.6 shows the time course of oxygen depletion in the cell-suspending medium (as an indirect indication

of oxygen consumption) for unstimulated and PMA-activated cells from smokers and non-smokers.

Continine Levels

The cotinine results verify that all the smokers had high levels of cotinine (>100 ng/ml) while non-smokers only showed almost undetectable levels of the smoke metabolite of 0 ng/ml ($P<0.0002$). As mentioned before studies have indicated that the interpretation of cotinine levels, 15ng/ml is considered to be a good cut-off point when distinguishing between active and passive smokers (Jarvis et al, 2008)

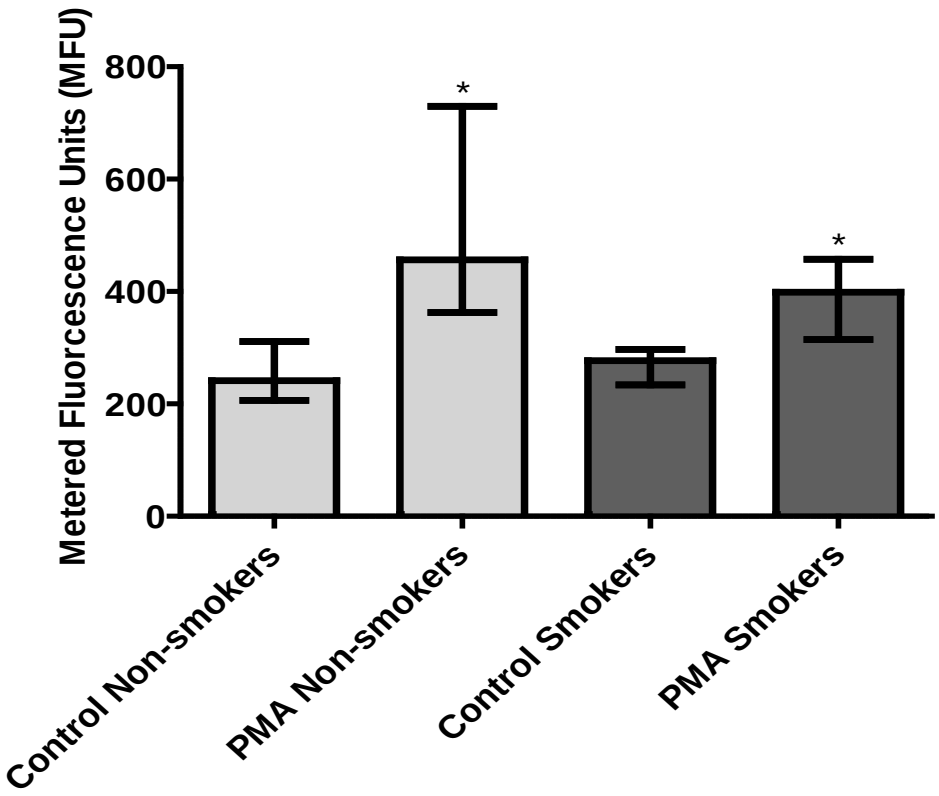
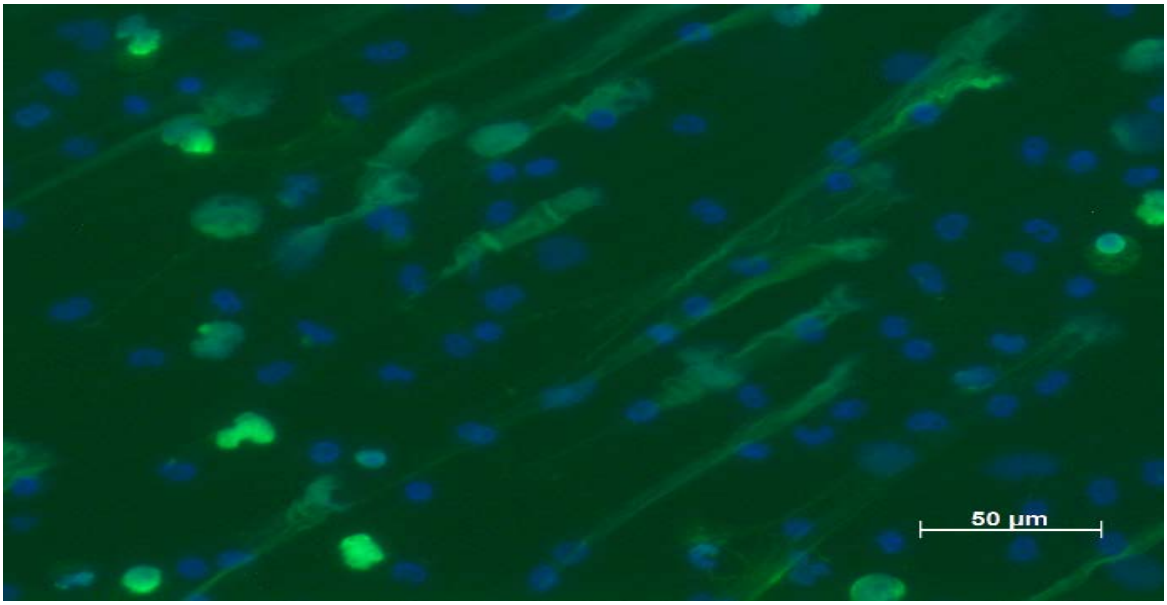


Figure 3.1

Extracellular DNA concentrations following activation of neutrophils from smokers and non-smokers with PMA (6.25 ng/ml). The results are expressed as the median absolute values in metered fluorescence units (MFU) with 25th and 75th percentiles. NETosis was significantly increased in PMA-activated neutrophils in both smokers and non-smokers (*P<0.02 and P<0.0002), respectively. The slight attenuation of PMA-induced NETosis in smokers when compared to non-smokers was not considered significant (P<0.2).

B) PMA-induced NETosis in a non-smoker



B) PMA-induced NETosis in a smoker

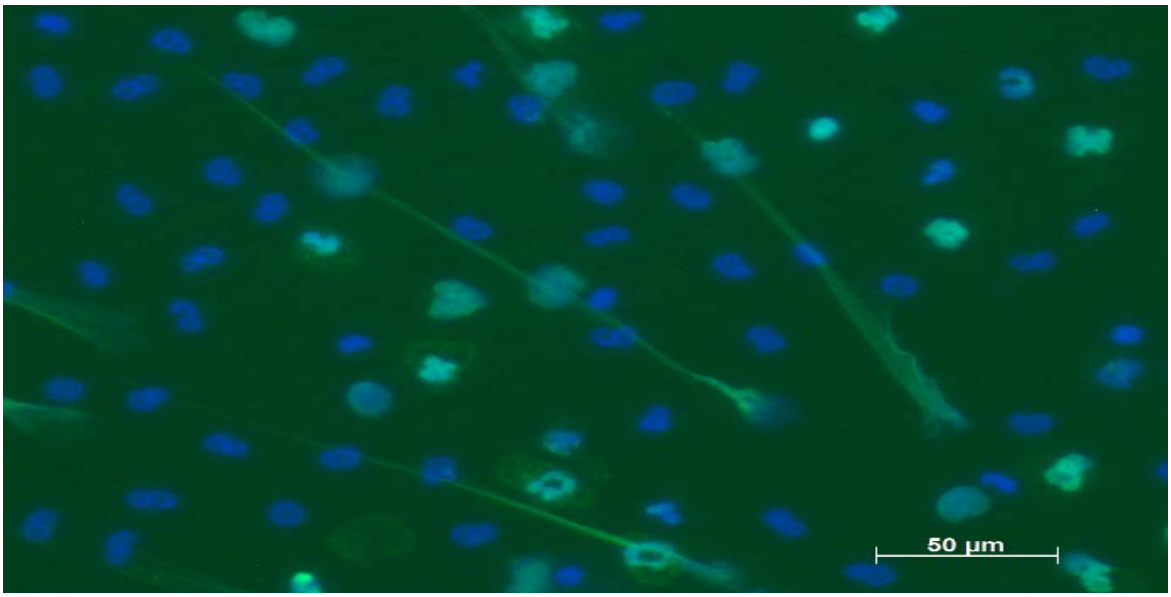


Figure 3.2

Representative fluorescence microscopic images of neutrophils following incubation for 2 hours with the stimulant PMA: A) PMA-induced (6.25 ng/ml) NETosis in a non-smoker and B) PMA-induced (6.25 ng/ml) NETosis in a smoker.

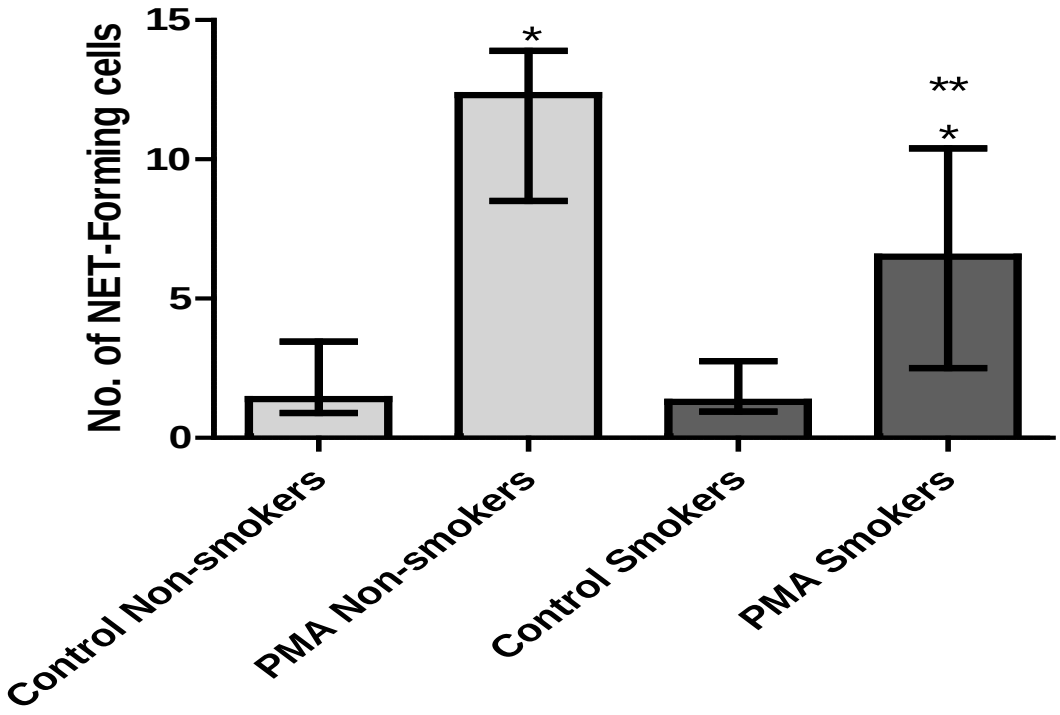


Figure 3.3

NET formation by resting and PMA (6.25 ng/ml)-activated neutrophils in cigarette smokers and non-smokers measured microscopically according to the numbers of NET-forming cells. The results of 5 separate experiments are expressed as the numbers of NET-forming cells for each system with median and percentile values of 25% and 75%. Addition of PMA to neutrophils from both smokers and non-smokers resulted in statistically significant activation of NETosis (*P<0.0001 and *P<0.0001 respectively), which was significantly greater in cells from non-smokers (**P<0.0002).

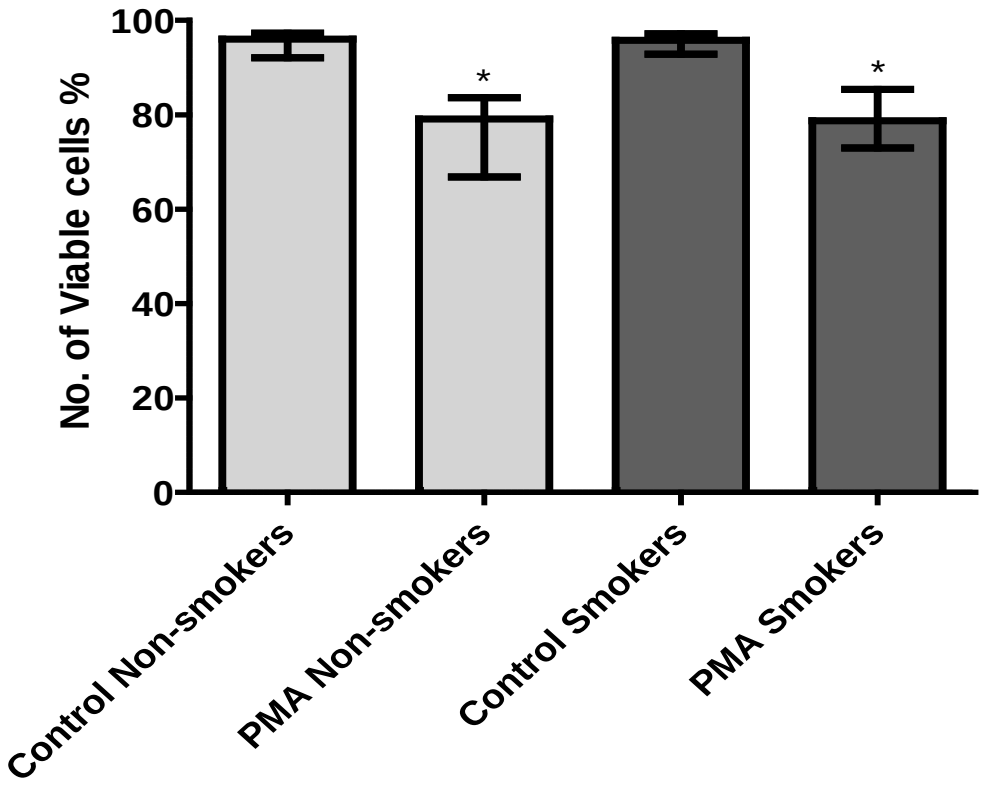


Figure 3.4

The effects of PMA (6.25 ng/ml) on neutrophil viability in non-smokers and smokers, measured using flow cytometry. The results are expressed as median percentages and 25th and 75th percentile values. PMA activation of neutrophils from smokers and non-smokers resulted in a loss of viability when compared to control, unstimulated cells (*P<0.0003 and *P<0.0001, respectively).

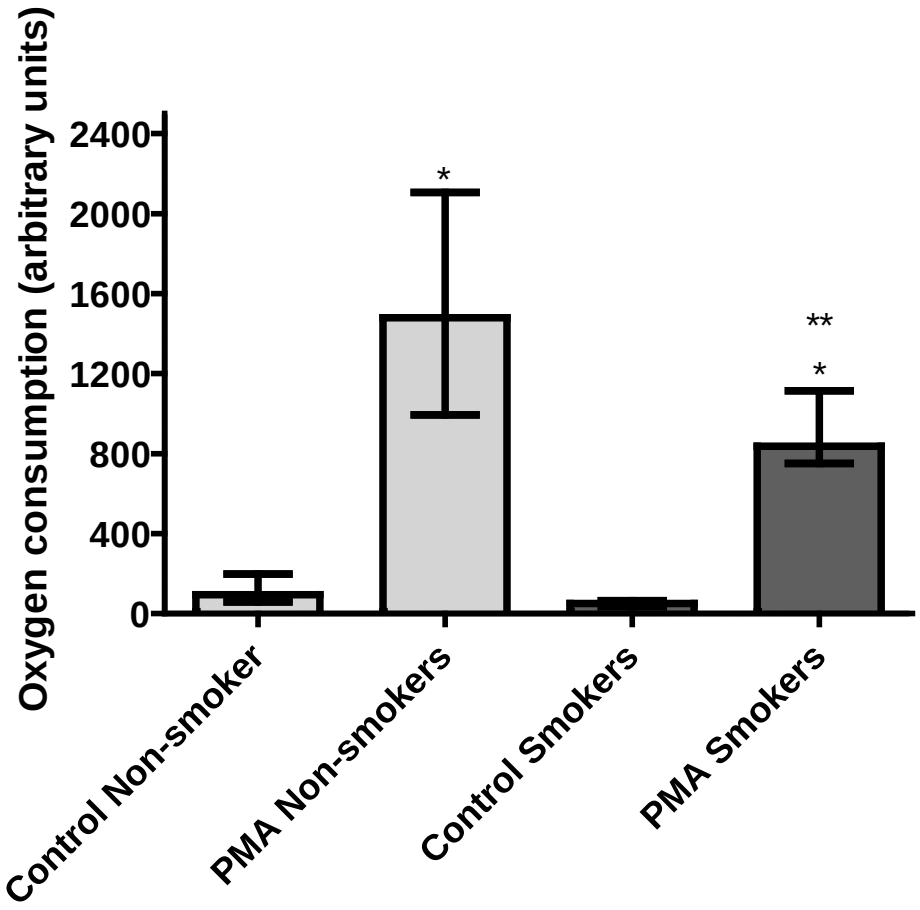


Figure 3.5

Oxygen consumption by control, unstimulated cells and PMA (6.25 ng/ml) – stimulated neutrophils from smokers and non-smokers measured over a 10 min period using an oxygen sensitive electrode as an index of the generation of reactive oxygen species. Data of 8 separate experiments are expressed as median absolute arbitrary units with 25th and 75th percentiles. Addition of PMA to neutrophils resulted in activation of oxygen consumption which achieved statistical significance with cells from both smokers and non-smokers (*P< 0.0003 and *P<0.0003, respectively). The magnitude of oxygen consumption by PMA-activated neutrophils from smokers was significantly less than that observed with cells from non-smokers (**P<0.04).

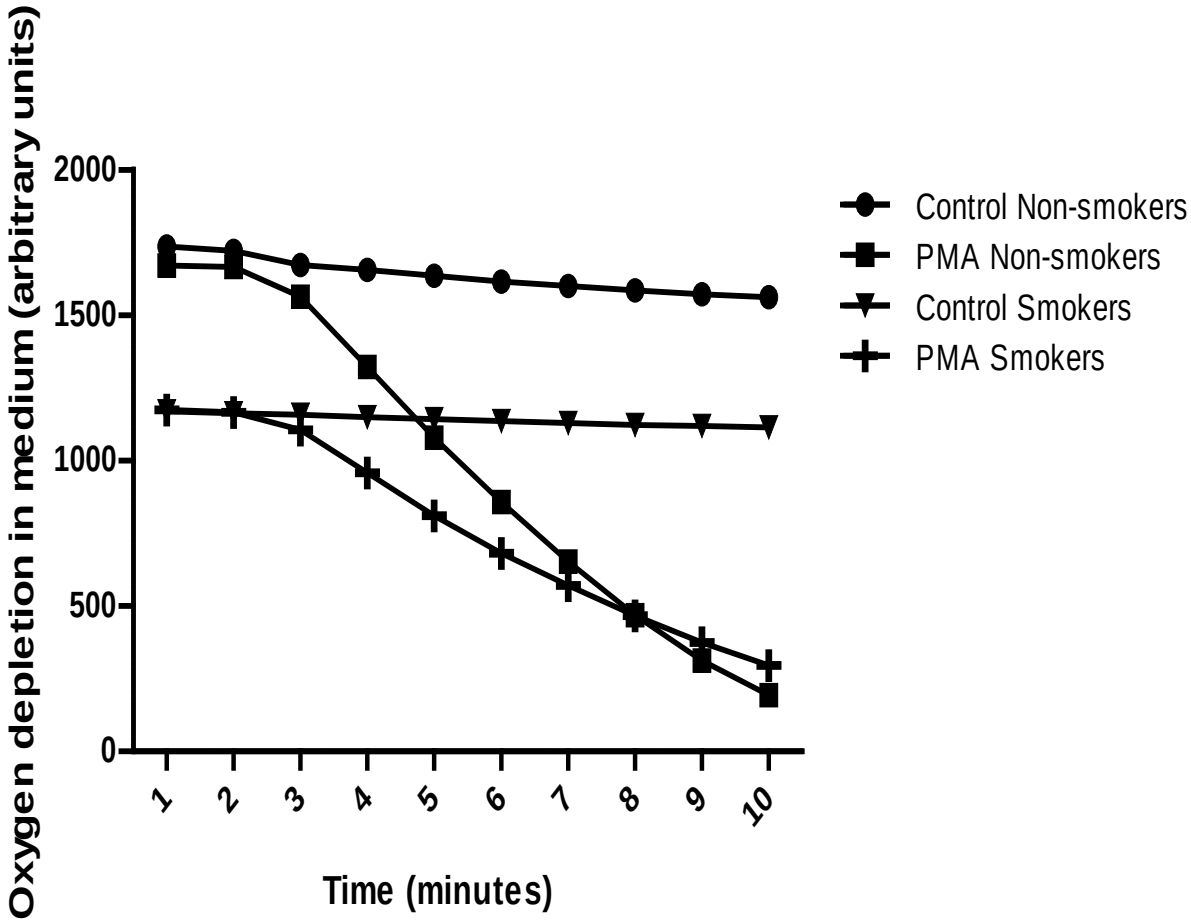


Figure 3.6

Time course of oxygen depletion in the cell-suspending medium as an indirect indication of production of reactive oxygen species using an oxygen sensitive electrode. The data of 8 separate experiments are averaged for each time point. The PMA (6.25ng/ml) -activated responses were slower in neutrophils from smokers compared to non-smokers.

3.4 Discussion and Conclusion

Studies have shown that compared to non-smokers, active and passive smokers have an increased susceptibility, not only to respiratory infections, but also to systemic bacterial infections (Feldman and Anderson, 2013). Cigarette smokers are at greater risk of developing respiratory infections (such as tuberculosis and pneumonia), sexually transmitted diseases (such as chlamydia and gonorrhoea) and post-surgical infections (Bagaitkar et al, 2008; Feldman and Anderson, 2013). Three possible mechanisms which may lead to the increased susceptibility for infections in smokers have been described namely, tobacco-induced physiological and structural changes in humans, tobacco-induced increase in bacterial virulence, and tobacco-induced dysregulation of immune function (Bagaitkar et al, 2008).

A number of studies have also shown the importance of NADPH oxidase activity against infection, and its involvement in PMA-induced NET formation (Fuchs et al 2007; Remijsen et al, 2011[a]). Therefore in this setting, oxygen consumption measurements serve as an indirect measure of oxidant activity and in the current study, a significant decrease in PMA-induced oxidant activity in neutrophils of smokers compared to non-smokers was observed. This suggests the possibility that cigarette smoke may dysregulate the immune responses of neutrophils by inhibiting NADPH oxidase activity. This mechanism may underpin the decrease in PMA-activated NETosis observed with neutrophils from smokers when using the microscopic procedure. The lack of statistical significance when using the spectrofluorimetric procedure may be related to the small number of participants in the study and clearly future studies will require recruitment of larger numbers of smokers and non-smokers.

In conclusion the current, albeit preliminary, study has shown possible attenuation of NET formation in smokers which may be related to smoking-associated inhibition of NADPH oxidase, but should further be investigated with a larger sample size.

CHAPTER 4

Final conclusion

Final conclusion

4.2 Concluding Comments

In conclusion both of the studies presented are consistent with suppressive effects of exposure of neutrophils to smoke condensate, as well as active smoking, on NET formation. Both studies indicated the existence of a possible relationship between decreased PMA-activated NETosis and attenuation of NADPH oxidase activity following exposure of neutrophils to smoke condensate, as well as to active smoking. This apparent relationship, however, requires further investigation in a larger sample size in order firstly to verify its existence, and, secondly to establish its possible association with the increased susceptibility of smokers for development of invasive bacterial infections.

CHAPTER 5

References

5.1 References

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