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Exposure to Iron-Laden Particulate Matter Impacts on Respiratory Health

By

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Statements and declarations

Declaration of originality

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Paper 2: Williams LJ, Zosky GR. The Inflammatory Effect of Iron Oxide and Silica Particles on Lung Epithelial Cells. Lung. 2019;197(2):199-207.

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Paper 3: Williams LJ, Tristram SG, Zosky GR. Iron Oxide and Silica Enhance the LPS-induced Inflammatory Response in Macrophages (currently under review with *Scientific Reports*).

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Statement of ethical conduct

The research associated with this Thesis abides by the International and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University of Tasmania.

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Abstract

Introduction

Ambient air pollution causes more than 4 million premature deaths globally every year. This excess mortality is primarily driven by inhalation of particulate matter (PM). The physicochemical properties of PM are important in determining the respiratory health outcomes but are typically not considered when setting air quality guidelines which are primarily based on PM size. The physicochemical properties of PM vary depending on the source. For example, urban PM is dominated by combustion-derived PM; particularly diesel exhaust particles. PM from these sources has been extensively studied in terms of its negative impact(s) on health. In contrast, geogenic (earth-derived) particles often affect populations in rural and arid areas, and our understanding of the respiratory health impacts of PM from these sources is far more limited. Exposure to geogenic PM is correlated with increased mortality and hospital admissions. Geogenic PM may be particularly relevant to the health of disadvantaged populations such as Australian Aboriginal children living in remote communities where geogenic PM levels are high and there is a disproportionate prevalence of chronic respiratory disease. Several studies suggest that inhalation of geogenic PM_{10} has the capacity to cause inflammation and exacerbate existing lung disease. It is also clear that the physicochemical characteristics of the particles have a significant impact on the response. However, research on the respiratory impacts of Fe, an important constituent of geogenic PM, is lacking.

Bronchiectasis, which is prevalent in some communities exposed to high levels of geogenic PM, is predominantly driven by respiratory infection. Bronchiectasis requires an impaired host response and/or increased pathogenicity of the bacteria. Geogenic PM has been shown to

increase the severity of viral infection *in vivo* and NTHi adhesion and invasion *in vitro*. This suggests that inorganic PM can reduce the host's ability to defend against infection. However, there are no data on the effect of these particles on bacterial growth or any comparative analysis of the effect of NTHi strain on respiratory cell invasion and how this is modified by silica and iron oxide. Thus, there is a need for further studies on the environmental contributions to susceptibility to bacterial infection as a potential explanation for the increased prevalence of bronchiectasis in Aboriginal Australian communities.

Methodology

To assess the impact of silica and iron oxide on inflammation and cytotoxicity in lung epithelial cells (Chapter 2), bronchial epithelial (BEAS-2B) and alveolar epithelial (A549) cell lines were exposed to increasing concentrations of silica (quartz; SiO_2) and/or iron oxide (haematite; Fe_2O_3 or magnetite; Fe_3O_4). Cytotoxicity was assessed by lactate dehydrogenase (LDH) assay and the production of the inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α (TNF-α) were measured by enzyme-linked immunosorbent assay (ELISA). To assess the effects of particles on inflammation and the response to a bacterial stimulus in macrophages (Chapter 3), THP-1 monocyte-derived macrophages were exposed to increasing concentrations of silica and/or iron oxide with or without lipopolysaccharide (LPS). The response was assessed by LDH assay and ELISA. To evaluate whether the PM could directly impact bacterial growth (Chapter 4), 9 clinical isolates of the iron-dependent bacteria *Non-typeable Haemophilus influenzae* (NTHi) were exposed to silica or iron oxide under a range of free iron growth conditions. Growth cycles of each isolate were evaluated for growth rate and maximal growth using spectral absorbance and agar plate

counts. Finally, to evaluate the impacts of these particles on the invasion of NTHi in lung cells and on phagocytosis by macrophages (Chapter 5), epithelial and macrophage cell lines and primary human peripheral blood mononuclear cell (PBMC)-derived macrophages were exposed to silica and/or iron oxide. Invasion of cells by NTHi was quantified by flow cytometry. Cellular and bacterial responses associated with bacterial invasion were assessed by flow cytometry, qPCR and whole genome sequencing.

Results

Silica caused mild cytotoxicity and a robust pro-inflammatory response in epithelial cells. Comparatively, iron oxide was not cytotoxic and had no effect on cytokine production in BEAS-2B cells but did increase IL-8 production in A549 cells (Chapter 2). In contrast, both silica and magnetite caused cytotoxicity in THP-1 cells. All particles caused an inflammatory cytokine response; although the silica response was more potent. Interestingly, haematite and magnetite augmented the IL-1β, IL-6 and IL-8 response to LPS to a similar extent as silica (Chapter 3). In the absence of cells, exposure to magnetite increased maximal growth in several isolates of NTHi. Comparatively, the response to silica and haematite was variable with some isolates exhibiting enhanced growth and others reduced growth (Chapter 4). The effect of particles on bacterial invasion (Chapter 5) varied depending on the PM source, cell type and NTHi isolate. Surprisingly, silica either had no effect on, or reduced, the invasiveness of NTHi in epithelial cells. In contrast, haematite significantly increased the invasion of most NTHi isolates in BEAS-2B cells. Conversely, both haematite and magnetite, but not silica, significantly reduced the phagocytotic capacity of macrophages in response to most NTHi isolates.

Discussion

Previous studies have shown a link between exposure to iron-laden geogenic PM and prevalence of bronchiectasis. In this Thesis, it has been shown that inflammatory response of epithelial cells and macrophages to iron oxide alone is unlikely to be making a major contributor to this association. However, iron oxide PM clearly enhanced the macrophage response to bacterial stimulation (LPS) and modified the growth of NTHi in a chemical (magnetite vs haematite) and isolate dependent manner. What was most striking, was the capacity of iron oxides to promote NTHi invasion in epithelial cells and impair macrophage phagocytosis of NTHi. The presented data provides insight into one of the mechanisms that may be contributing to excessively high burdens of chronic respiratory disease in regional communities, particularly bronchiectasis in Australian Aboriginals, and those in arid environments exposed to high levels of geogenic PM. In particular, this Thesis highlights that iron oxide PM may have profound impacts on the respiratory susceptibility to the severe bacterial infections.

Thesis structure

This Thesis consists of six Chapters:

- Chapter 1 is the Thesis introduction and includes the literature review, aims and hypotheses and an outline of the study approach utilised to address each specific aim. It includes a modified version of the manuscript published in *Inhalation Toxicology*.
- Chapter 2 is a results chapter that addresses the first aim of the study; To assess whether environmentally relevant forms of iron oxide are sufficient to induce inflammation in lung epithelial cells and whether they augment the silica-induced response. This Chapter is presented as published in the journal *Lung*.
- Chapter 3 is a results chapter that addresses the second aim of the study; To assess whether environmentally relevant forms of iron oxide cause a pro-inflammatory response in macrophages and whether they modify the response to a bacterial stimulus. This chapter is formatted as submitted to *Scientific Reports*.
- Chapter 4 is a results chapter that addresses the third aim of the study; To determine whether environmentally relevant forms of iron oxide promote NTHi growth. This chapter is formatted as submitted to *Environmental Geochemistry and Health*.
- Chapter 5 is a results chapter that addresses the fourth aim of the study; To assess whether environmentally relevant forms of iron oxide can alter NTHi invasion of lung cells and or phagocytosis by macrophages.
- Chapter 6 is the General Discussion which includes a consideration of limitations and outlines the conclusions of the Thesis.

Table of contents

List of Figures

List of Tables

Abbreviations

ABC: ATP-binding cassette

ADP: Adenosine diphosphate

ANOVA: Analysis of variance

AP: Activator Protein

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

BEGM: Bronchial Epithelial Growth Media

BHI: Brain heart infusion

HTM: Haemophilus test media

CCA: Base pair sequence CCA

CEBP: CCAAT-enhancer-binding proteins

CF: Cystic fibrosis

CFTR: Cystic fibrosis transmembrane conductance regulator

COPD: Chronic obstructive pulmonary disease

CTP: Cytidine triphosphate

DAMP: Damage-associated molecular patterns

DEP: Diesel exhaust particle

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked immunosorbent assay

ERK: Extracellular signal–regulated kinases

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

FMO: Fluorescent minus one

GAG: glycosaminoglycan

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GTP: Guanosine-5'-triphosphate

NADH: Nicotinamide adenine dinucleotide hydrogen

HMW: High molecular weight

HTH: Helix-turn-helix

IL: Interleukin

KDO: Lipopolysaccharide kinase

LDH: Lactate dehydrogenase

LOS: Lipooligosaccharide

LPS: Lipopolysaccharide

MMAD: Mass mean aerodynamic diameter

NCBI: National Center for Biotechnology Information

NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

NIST: National Institute of Science and Technology

NLRP: NACHT, LRR and PYD domain-containing protein

NTHi: Non-typeable *Haemophilus influenzae*

OD: Optical density

CFU: Colony forming units

OMP: Outer membrane protein

OR: Odds ratio

PAFr: Platelet activating factor receptor

PAMP: Pathogen-associated molecular patterns

PBMC: Primary blood-derived mononuclear cells

PBS: Phosphate buffered saline

PCA: Principal component analysis

PCR: Polymerase chain reaction

PM: Particulate matter

PMA: Phorbol 12-myristate 13-acetate

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute

SD: Standard deviation

SEM: Scanning electron microscope

USA: United States of America

TLR: Toll-like receptor

TNF: Tumour necrosis factor

UK: United Kingdom

TPS: Two partner secretion

UDP: Uridine phosphorylase

UV: Ultraviolet

Chapter 1: Literature Review

A modified version of this chapter has been accepted for publication in a peer-reviewed journal:

Williams LJ, Chen L, Zosky GR. The respiratory health effects of geogenic (earth derived) PM_{10}. Inhalation toxicology. 2017;29(8):342-55 (see Appendix).

This Chapter includes the literature review, aims and hypotheses and an outline of the study approach utilised to address each specific aim. It includes a large portion of text that appears in the manuscript published in *Inhalation Toxicology*.

1.1 Overview

The average human breathes 10,000 L of air every day [1]. The lungs are the key interface between the human body and the environment and play a critical role in gas exchange. Air pollution is a global health concern that poses a significant threat to lung health [2]. One of the important elements of air pollution that impacts on health is the suspended solids and liquids; also known as particulate matter (PM) [3]. PM consists of a particles of different size fractions, with finer particles (< 10 μm) being small enough to bypass the upper airways and deposit in the lung upon inhalation [4]. This can result in acute and chronic inflammation of the bronchial and alveolar epithelial lining and cause activation of resident immune cells in the lung [5]. This can have profound impacts on health, particularly the host response to an infection.

Bronchiectasis is a chronic respiratory disease characterised by irreversible dilation of the airways and mucous-induced airway obstruction [6,7]. Bronchiectasis occurs as a result of persistent chronic infection and inflammation [6]. One of the most common pathogens isolated from individuals with bronchiectasis is Non-typeable Haemophilus influenzae (NTHi) [8,6,9,7,10]. This pathogen is also associated with disease severity in Aboriginal children [11]. NTHi is a highly heterogenous bacteria and its success as a respiratory pathogen is attributed to its ability to adapt and evade host immunity [12,13].

Aboriginal Australians have the highest burden of non-cystic fibrosis (CF) bronchiectasis in the world [11,8]. While this could be linked to factors related to social disadvantage, Aboriginal Australians living in regional and remote are also exposed to high levels of earth-derived

(geogenic) PM [14,15]. Geogenic PM is a heterogenous mixture of inorganic compounds primarily consisting of silica, iron oxide and aluminium [16]. Experimental studies suggest that silica is the most damaging component of geogenic PM, however, recent evidence points towards a role for iron oxide in the respiratory response [16]. In line with this, recent studies have linked exposure to these particles to NTHi respiratory infections in Aboriginal children [14] and it is possible that the inhalation of these dusts may cause a response in the lung that predisposes to the development of bronchiectasis.

This literature review focuses on the possible link between exposure to geogenic PM and the risk of bronchiectasis. Firstly, an outline of our understanding of bronchiectasis and NTHi is provided. This is followed by an examination of the effect of silica and iron oxide particles on respiratory health. Finally, the plausibility of the link between exposure to geogenic PM and the response to infection is assessed with a summary of the gaps in the literature to inform the aims and hypotheses of this Thesis.

1.2 Bronchiectasis

Bronchiectasis is a chronic lung disease characterised by irreversible dilation of the airways as a result of progressive airflow obstruction due to mucous production [17,7]. Bronchiectasis is characterised by severe coughing, sputum production and recurrent respiratory infections resulting in decreased quality of life and reduced life span [18]. The disease is not fully understood however, to date, the most accepted model of bronchiectasis is the “vicious cycle hypothesis” [19]. This hypothesis proposes that the disease develops in individuals that are

subjected to both an impaired host immune response and a bacterial infection. This results in persistent bacterial infections [19,6] in which causes chronic inflammation including the release of tissue-damaging agents such as reactive oxygen species (ROS) and proteases. This leads to host tissue degradation, which impairs mucociliary clearance and further increases susceptibility to microbial infection [19]; hence the term "vicious cycle".

For example, bronchiectasis is a common feature of the lung in individuals with CF [20]. In cystic fibrosis, individuals have altered CFTR function which increases the viscosity of the airway surface liquid and promotes the formation of bacterial biofilms and persistent infection [21]. However, the origins of non-CF bronchiectasis are less clear and are likely to be diverse, as outlined below.

1.2.1 Pathogenesis

One of the early studies using lung samples from bronchiectasis patients showed inflammation of the bronchial walls in the smaller airways [22]. Inflammation-induced bronchial dilation was accompanied by an elastin deficiency, with persistent disease resulting in progressive degradation of the smooth muscle [6]. Inflammation in bronchiectasis is driven predominately by the recruitment of neutrophils, lymphocytes, and macrophages [23,24]. Neutrophils migrate to the bronchial lumen in response to the release of chemokines by resident innate immune cells such as macrophages and epithelial cells [23]. They target and phagocytose bacteria, using proteases to degrade internalised material [25]. However, excess migration and activation of these cells results in uncontrolled production and release of these proteases which can non-

specifically degrade host tissue resulting in elastin destruction [26,27] and changes to airway structure. In addition, airway wall inflammation in bronchiectasis is characterised by excess numbers of macrophages [23,28], suggesting that dysfunction in macrophage homeostasis is linked to neutrophil recruitment.

Bronchiectasis is associated with a variety of bacterial species, however, the most prevalent is Non-typeable *Haemophilus influenzae* (NTHi) which is isolated in 30-70% of patients [29-32]. This is followed by *Pseudomonas aeruginosa* (*P. aeruginosa*) which is found in 12-31% of patients [29-32]. Interestingly, the microbial flora shifts with the progression of disease. Typically, individuals with stable lung function have minimal bacterial infection [10] whereas a moderate decline in lung function is linked to the presence of NTHi, while *P. aeruginosa* is present in patients with severe loss of lung function [10]. It is unclear why different pathogens are linked to the progression of bronchiectasis and it is difficult to separate correlation and causation in these circumstances.

While the development of bronchiectasis is thought to require a pre-existing impairment in the host response to infection, the bacteria themselves can alter the lung environment to promote chronic inflammation and tissue damage. For example, lipopolysaccharide (LPS) and glycoproteins, potent inflammatory molecules on Gram-negative bacteria, induce macrophage and neutrophil activation, which can result in tissue damage [33]. Mediators produced by NTHi can damage ciliary function, the epithelium and mucous transport [34,35]. NTHi can also directly invade the bronchial epithelium and form resilient biofilms [36,13] which allows the pathogen to evade the host immune system and persist in the lung. Thus, when considering the

mechanisms of risk factors that predispose to the development of bronchiectasis it is important to consider the host and the bacterial response.

1.2.2 Prevalence and risk factors

Estimates of the prevalence of bronchiectasis vary geographically. For example, in the UK, the prevalence of bronchiectasis in 2013 equated to 485-566 cases per 100,000 people [37]. In contrast, in the USA, the prevalence in 2013 was lower, with 95 and 180 individuals per 100,000 people for women and men respectively, with an annual increase of 8% since 2001 [38]. The overall prevalence of bronchiectasis in Australia is unclear. However, in 2016/17 there were 36 and 19 hospitalisations for bronchiectasis per 100,000 for women and men respectively [39]. The prevalence of both, children of Pacific ethnicity have rates of bronchiectasis 12 times higher than those of European descent, with even higher rates amongst children from central Australia [40].

In many instances, the presence of bronchiectasis is linked to an existing underlying condition such as autoimmune disease, immune deficiency and inflammatory disease [41]. However, data on other risk factors for acquiring non-CF bronchiectasis are limited. There is some evidence to suggested that socioeconomic disadvantage is associated with bronchiectasis [11,8]. This is linked to a range of factors that may predispose to disease including smoking, poor nutrition and lower access to routine and specialist medical services [11,8]. Most of these factors negatively impact the host response to infection and thus increase the risk of chronic respiratory infection leading to the altered airway structure that is characteristic of bronchiectasis.

1.3 Bronchiectasis in Indigenous communities

Australian Aboriginal people suffer from rates of bronchiectasis almost three times higher than non-Aboriginal Australians [8]. Aboriginal children also have the highest incidence of non-CF bronchiectasis globally [11]. As mentioned, the aetiology of non-CF bronchiectasis usually occurs as a result of chronic pulmonary bacterial infection [6], typically NTHi [10,32]. Consistent with this, almost 90% of Aboriginal people with bronchiectasis are diagnosed with post-infective bronchiectasis as a result of pathogenic NTHi [8,42-44].

A range of socioeconomic and lifestyle factors likely contribute to this unacceptably high burden of disease [45-49]. These include high rates of smoking, poor living conditions, poor nutrition and lower access to medical services. However, recent evidence suggests environmental factors may also contribute [15] to the burden of respiratory disease in these communities. In particular, remote Aboriginal communities are also exposed to high levels of geogenic PM which may be linked to the severity of respiratory infections [47,15]. For example, Shepherd et al. (2019) showed a correlation between increasing geogenic dust exposure and hospitalisation for upper and lower respiratory tract infections in Aboriginal children [14]. Further experiments showed that community sampled dust increased the adherence and invasion of NTHi *in vitro*, with some evidence that these dusts may alter the inflammatory response of epithelial cells to NTHi [14]. These data suggest that geogenic PM may be directly responsible for the high prevalence of bronchiectasis in these communities. As discussed earlier (see Section 2.1), when considering the underlying mechanisms linking specific risk factors to the development of bronchiectasis it is important to consider the

bacterial response. The following sections will focus on the key factors that impact on NTHi pathogenesis.

1.4 Non-typeable Haemophilus influenzae (NTHi)

As discussed above, NTHi is one of the dominant pathogens associated with bronchiectasis [6] and is the predominant pathogen linked to the burden of bronchiectasis experienced by Australian Aboriginal people [11,8]. NTHi is an opportunistic Gram-negative human pathogen that is a major cause of morbidity and mortality around the world [13]. The ability of NTHi to cause chronic infections is attributed to its capacity to rapidly evolve and its use of multiple pathways for attachment and subsequent intracellular invasion [13]. The speed at which NTHi can respond to and evade host immunity enables it to remain incredibly resilient once it becomes established in the lung [12,13]. NTHi has a multitude of mechanisms for colonisation and immune evasion. The following sections will detail the initial stages of infection and subsequent survival within the host.

1.4.1 Cell adherence

The ability of a pathogen to infect the lung typically starts with the colonisation of the airways by attachment to host epithelial cells [50]. As a commensal organism, NTHi often colonises the airways of humans during childhood but remains non-pathogenic unless the immune system is compromised [13]. There are multiple pathways by which NTHi can attach to host cells to increase its virulence as discussed below.

1.4.2 Membrane proteins

The ability of NTHi to colonise regions of the airway is often dependent on the presence of underlying disease or pre-existing tissue damage. *In vitro* studies suggest NTHi preferentially adheres to cells that lack functioning cilia or have damaged cell membranes [51-53]. For example, PM can inhibit cilia function, and is cytotoxic, leading to the production of regions of necrosis in the airway epithelium [54] which may facilitate infection.

Attachment of the bacteria to the airway wall is the first phase of colonisation. Outer membrane proteins (OMPs), which vary in number and structure between NTHi strains, are a conserved amino acid cluster on the bacterial surface and are pivotal in the initial stages of NTHi attachment to host cells [13]. For example, OMP-P2 and P5 allow enhanced binding to mucin-coated nasopharyngeal epithelial cells [55]. Other NTHi related adhesion-related proteins include the Hap protein and High-molecular-weight (HMW) proteins-1 and 2 [56-58]. These proteins bind cells, epithelial cells and collagen in the extracellular matrix. Strains of NTHi that do not express these proteins have alternative HMW proteins coded by the *hia* gene [58]. The presence of one or more of these proteins may contribute to the regional pathogenicity of a given NTHi strain.

Some strains of NTHi are also able to produce the pilin protein [59-61]. Pilin-mediated binding occurs to lactosylceramide structures that contain sialic acid [61]. These structures are abundant on oropharyngeal epithelial cells and erythrocytes [61]. However, it is likely that these

structures are also utilised by the normal microbiome in the respiratory tract and are only involved in pathogenesis when NTHi dominates over resident non-pathogenic bacteria. To overcome the existing microbiome, NTHi is able to bind to the asialo-glycosphingolipids which are surface molecules that are found throughout the respiratory tract [62,63]. These act as additional anchorage sites allowing colonisation to occur. Similarly, fimbrin is an adhesin protein very similar to OMP-P5 which has been observed in all clinical isolates to date [13]. Deletion of the fimbrial gene reduces, but does not eliminate, the *in vitro* ability of the bacteria to adhere to oropharyngeal cells [64] resulting in a reduction in virulence *in vivo*.

Protein D is a surface lipoprotein located on the outer membrane protein of most NTHi strains [65]. Protein D, while not essential for bacterial survival, has been shown in animal models to increase the severity of respiratory disease by reducing the number of bacteria required to establish infection [65]. It is suggested that Protein D aids invasion by enhancing functional and morphological damage to ciliated epithelial cells [65]. It has been hypothesised that its glycerophosphodiesterase activity induces the release of phosphorylcholine from host epithelial cells [65]. This leads to an increased expression of platelet-activating factor receptor (PAF-r), a primary attachment point for NTHi on epithelial cells [66]. Adherence is the first stage of pathogenesis, however the subsequent host inflammatory response and capacity of NTHi to invade respiratory cells is driven by other factors. Endotoxins are potent inflammatory molecules released from Gram-negative bacteria which have a large impact on the host immune response [67-70].

1.4.3 Endotoxin

Endotoxin, or LPS, is a central component of the outer membrane in Gram-negative bacteria and frequently plays a key role in bacterial pathogenesis [71]. LPS coats the outer membrane and provides a level of protection against the host response [72]. An LPS molecule has a three-part structure; lipid A, which attaches the molecule to the outer membrane, an oligosaccharide core which, together with lipid A contributes to maintaining the integrity of the outer membrane, and an O antigen polysaccharide [73]. However, LPS produced by *Haemophilus* and many other human respiratory pathogens often do not contain the O antigen [67]; while still a functional form of LPS, these molecules are also known as Lipooligosaccharide (LOS). NTHi-produced LOS acts as a major surface antigen, inhibiting cilia function and inducing pro-inflammatory and cytotoxic effects in host cells [74-77]. LPS and LOS are recognised by the host Toll-like receptor (TLR)-4. Activation of these pattern recognition receptors stimulates a defensive response resulting in the activation of NF-κB and the release of cytokines such as IL-1, IL-6, IL-8, TNF-α and interferons [78].

The resulting migration of activated neutrophils in response to LOS increases inflammation in the pulmonary space [79]. This increases levels of proteins such as transferrin and lactoferrin. The binding of LOS to these host proteins downregulates the neutrophil response and is directly implicated in chronic low-grade inflammation on mucosal surfaces [80-83]. LOS can remain after pathogenic clearance by macrophages and neutrophils and may be involved in long-term disruption of clearance of transient bacteria [84-86]. Endotoxins, present on NTHi, induce profound pro-inflammatory effects on the host. This can alter the ability of cells to respond to other DAMPs/ PAMPs. Additionally, pro-inflammatory signalling upregulates host receptors

used as attachment points for NTHi during pathogenesis. Once colonised, NTHi uses different pathways to maintain infection.

1.4.4 Intracellular invasion

Once the lung has been colonised, the next phase of pathogenesis is the successful survival of the bacteria within the host. The human body has developed a range of innate and adaptive defence mechanisms to prevent long-term bacterial colonisation [87,88]. To combat this, NTHi has evolved several systems to evade host immune defences, predominantly through invasion of lung epithelial cells [89,90]..*In vitro*, NTHi adheres to, invades and replicate in human epithelial cells [91]. *In vivo*, NTHi penetrates the mucosal lining at points of necrosis and within cell junctions [91]. However, due to the heterogeneity of NTHi, there is no one mechanism or factor that can be globally attributed to cell invasion.

The invasiveness of a given NTHi isolate is highly correlated with its capacity for cellular adherence [9]. This appears to be independent of starting bacterial quantity, motility or serotype but is dependent on generation time, with rapidly replicating isolates penetrating the mucosal wall in 10-18 h compared to 30 h for slower isolates [92]. Once the intracellular space has been invaded, NTHi is able to propagate for extended periods of time [93]. Despite accumulating data on the invasiveness of NTHi, no single mechanism has been identified. Studies have suggested that cellular invasion is associated with the Haemophilus adherence and penetration (Hap), phosphocholine (PCho), Protein D, Protein E, glycosaminoglycan (GAG) and HMW proteins on the bacterial surface [53,94,57,95,70,96,97]. To date, there is no definitive list of genes or proteins that will determine if a bacteria will be invasive or not [12]. Nonetheless,

once the cell is invaded, the ability of the bacteria to persist in the intracellular space is linked to its ability to avoid immune recognition.

NTHi is the most prevalent pathogen amongst individuals with bronchiectasis in Aboriginal Australian communities [11,8]. This pathogen utilises multiple mechanisms during infection to adhere and survive within the host [13]; particularly its capacity to invade epithelial cells. In response, the innate immune system, driven in the first instance by epithelial cells and macrophages, generates an anti-microbial response. Understanding the capacity to invade cells, and the host response to the bacterial infection is important, however, infection itself is not sufficient to induce bronchiectasis [6]. The development of disease also requires a stimulus for impairment in the immune response that allows the persistence of the bacteria [6]. In the case of bronchiectasis in Aboriginal Australians, exposure to geogenic PM may provide this stimulus. For example, Shepherd et al (2019) showed that exposure to geogenic PM is correlated with the risk of hospitalisation for respiratory tract infections [14]. The following sections will focus on geogenic PM, its general effect(s) on respiratory health effects and how this may contribute to the severity of respiratory infection.

1.5 Health effects of geogenic PM

The deleterious health effects of inhaled particulate matter <10 μm in diameter (PM_{10}) are well established [2]. PM_{10}, which is small enough to bypass the upper airway defences and enter the lung, facilitates the progression of asthma and bronchitis, increases susceptibility to pneumonia and increases hospital admissions for respiratory conditions [98-103]. However, these

associations have primarily been demonstrated for PM_{10} in urban settings with limited data on the health effects of PM_{10} from non-urban regions. This is important because the physicochemical characteristics of PM_{10} vary geographically which will impact the potential health implications. For example, urban particles typically have a high combustion component, whereas nonurban particles often have a significant geogenic, or crustal, component [104].

Many of the conditions associated with exposure to PM_{10} have been directly linked to the pro-inflammatory potential of diesel exhaust particles (DEPs) that are a major component of urban PM_{10} [105]. This is supported by data from human, animal and cell culture studies [106-109]. However, less is known about the impact of PM_{10} with a geogenic origin on the lung. Occupational health data suggest a link between silica inhalation, a major constituent of geogenic PM_{10}, and the development of silicosis and chronic obstructive pulmonary disease (COPD) [110-113]. However, the respiratory impacts of other geogenic PM_{10} constituents are less certain. The limited data on the health effects of geogenic PM_{10} is concerning given the millions of people around the world living in remote and regional communities where exposure to geogenic particles is likely to be high.

1.5.1 Exposure to geogenic PM

While guidelines for PM exposure vary around the world, exposure limits (averaged over a 24-h period) are typically set at 50 $\mu g/m^3$ for particles less than 10 μm in diameter (PM_{10}) and 25 $\mu g/m^3$ for particles less than 2.5 μm in diameter ($PM_{2.5}$)[3]. Notwithstanding the fact that these limits are set on the basis of urban PM, acute geogenic exposure events (e.g. dust storms)

often vastly exceed these limits. For example, PM_{10} concentrations during dust storms have been measured at 11,000–15,366 μg/m^3 in Sydney, Australia [114,115] and 12,000 μg/m3 in Beijing, China [116] and concentrations of 710 μg/m^3 were detected in a more recent Saharan dust storm over Cape Verde [117]. In general, maximum PM_{10} levels during dust storms are approximately 200–3000 μg/m^3 [118], lasting up to 24 hours, which is orders of magnitude higher than what is typically considered to be "safe" based on current guidelines [119].

While eolian erosion is generally the largest cause of nontraffic-derived airborne particulates, anthropogenic activity can generate geogenic PM_{10} by disturbing surfaces that are otherwise stable enough to withstand natural erosion. This can occur on a large scale as a result of mining activities [120], or more locally as a result of off-road traffic [121]. While these are typically acute geogenic PM_{10} exposure events, it is the remote and regional communities that are chronically exposed to relatively high ambient levels of geogenic PM_{10} that are of greatest concern. For example, Australian Aboriginal communities exposed to high levels of geogenic dusts are located in the arid regions of the country that are closely associated with open cut mining operations and are regularly exposed to geogenic PM_{10} levels above 50 μg/m^3 [122]. Thus, there are a number of communities around the world that are exposed to very high levels of PM that is dominated by particles with a geogenic origin.

1.5.2 Non-communicable lung disease and geogenic PM

There are no direct human exposure trials using community sampled geogenic PM_{10}. Epidemiological studies that have examined the association between geogenic PM_{10} and

respiratory health of individuals without pre-existing respiratory disease have primarily come from health data in military personnel with some supplementary data from communities exposed to high levels of geogenic particles.

The majority of the Middle East region is an arid environment that is prone to dust storms and high levels of ambient dust of geogenic origin [123]. While there are limited data on the impact of geogenic particles on the >200 million inhabitants of the Middle East *per se*, some studies have followed the respiratory health of military personnel deployed to these regions. For example, military personnel and civilian workers in these areas, and those sent to southwest Asia, may be at increased risk of developing chronic lung diseases, including asthma and constrictive bronchiolitis [124-126]. It should be acknowledged, however, that it is often not possible to separate the specific effects of geogenic PM_{10} among other possible contributing factors such as burn pits, industrial fires and cigarette smoking that are also prevalent in this setting.

Notwithstanding this limitation, higher rates of newly reported respiratory symptoms have been observed in soldiers deployed to Iraq and Afghanistan (14.5% versus 1.8% in soldiers deployed elsewhere) [126] where deployment length was linearly associated with respiratory symptoms [127]. Additionally, land-based deployment correlated with higher rates of respiratory symptoms (OR [95% CI]; 1.79[1.63, 1.97]) in comparison to sea-based deployment (OR [95% CI]; 1.16[0.95, 1.43]) [127]. While this study showed no significant increases in the prevalence of chronic emphysema, bronchitis or asthma [127], deployment in Iraq, compared to non-deployment, has been associated with an increased the risk of new-onset asthma [125]. While

rates of clinical chronic bronchitis and asthma were not increased in all studies [128,127], enough studies suggest an increased risk of asthma, constrictive bronchiolitis [124] and allergic rhinitis [129] in soldiers deployed in arid regions to suggest further investigation is required. Of the soldiers deployed to Iraq, 69.1% reported experiencing respiratory illnesses [130] highlighting the potential scale of the issue. As previously stated, most epidemiology studies on the respiratory effects of PM_{10} have been conducted in the urban setting. Unfortunately, due to the low population sizes, the epidemiological studies that do exist in regional areas are largely cross-sectional and based on hospitalization data.

Perez et al. (2008) conducted a case-crossover study on the health effects of Saharan dust on daily mortality in Barcelona and found that, on days when Saharan dust levels were high, a 10 $\mu g/m^3$ increase in $PM_{10–2.5}$ was associated with an 8.4% increase in mortality [131]. A similar study in Trinidad found an association between Saharan dust exposure and hospital admissions for paediatric asthma with the authors suggesting that the response was driven by dust carriage of allergens and respiratory irritants rather than a direct effect of geogenic PM_{10} per se [132]. While exposure of remote sites to Saharan-derived PM_{10} may be considered an acute event, the regularity with which these exposure events occur may result in chronic health effects in these urban communities. This is supported by studies from the United States in urban centres including the Coachella Valley and Phoenix, where the contribution of geogenic particles to the ambient PM_{10} is relatively high, showing that increases in PM_{10} concentrations are associated with increases in daily mortality [133,134]. However, it should be acknowledged that these associations have not been shown in all studies [135,136]. Collectively, these epidemiological studies show that populations who are exposed to geogenic PM are at higher

risk to develop non-communicable respiratory diseases such as asthma suggesting that geogenic PM can promote inflammation in the lung.

1.5.3 Communicable lung disease and geogenic PM

There are very limited epidemiological studies on the association between geogenic PM and respiratory infection. Port Hedland is a mining port in Western Australia which stockpiles large quantities of uncontained iron ore. PM from this stockpile is frequently blown over the community [137-139]. Epidemiological studies in this region have shown that hospital admissions for respiratory diseases are significantly higher than the rest of the state [140]. In line with this, an epidemiological study based on hospital records from Whyalla, a South Australian community exposed to excessive iron oxide dust from a steel plant, found a higher prevalence of respiratory morbidity. In particular, there was an increased risk of hospitalization for respiratory infections in children, when compared to non-exposed, neighbouring towns [141]. In line with this, as discussed previously, Shepherd et al. (2019) showed a correlation between increasing geogenic dust exposure and hospitalisation for upper and lower respiratory tract infections in Aboriginal children [14].

Taken together, these studies point to a strong association between exposure to geogenic PM and the risk of respiratory infection and, as discussed in the previous section (Section 5.2), inflammatory lung diseases. When considering the mechanisms of these associations an important consideration is how the characteristics of the geogenic PM, which vary geographically [142], impact the lung response.

1.6 Physico-chemical properties of geogenic PM and their impact on respiratory health

As mentioned in Section 5, the health effects of respirable PM are dictated heavily by particle size and chemical composition. The size of the particle will not only determine its surface area but also how deep it penetrates the lungs, its method of deposition and to a certain extent, its bioreactivity. The chemical composition will largely determine the particles bioreactivity, but also secondary characteristics that influence health outcomes, such as solubility.

1.6.1 Particle size

PM is defined according to mass mean aerodynamic diameter (MMAD). PM_{10} is classified as PM under 10 μm in MMAD. PM_{10} can be further subdivided into the coarse, fine and ultrafine fractions. The coarse fraction consists of particulate matter between 2.5 and 10 μm in MMAD and generally constitutes the size range that includes geogenic particles. For example, the MMAD in community sampled geogenic PM_{10} from Western Australia is 3.15 μm [143,142,16], while dust collected from the Middle East has an MMAD of 2.5-5 μm depending on the location [144]. In line with this, PM_{10} collected from urban dust storms typically contains particles in the coarse size fraction.

While the MMADs typically fall within the coarse PM range, individual samples can have complex size distributions. For example, samples taken from an Australian dust storm had several size distribution modes with peaks at 0.6, 4.5, 9.3 and 20 μm (PM_{10} comprised 50% of

total PM in these samples) [115] while samples taken during Saharan dust storms had a bimodal size distribution (modes at 1–2 and 5–6 μm) [117,145]. Similarly, a Kosa (Asian dust) storm that travelled across several locations in China and Japan, had a bimodal distribution in MMAD with peaks at 0.43–0.65 μm and 3.3–4.7 μm [146].

The particle size has been shown to alter its bioreactivity. In general, the smaller the particle, the larger its reactivity. However, this is dependent on the size range of particles and the cell type. For example, within the ultrafine fraction, larger PM is more efficiently taken up by macrophages [147]. Accordingly, fine silica particles are more inflammatory than ultrafine silica particles in macrophages [148]. This is most likely due to the increased uptake of the larger particles. This is not surprising given the anti-microbial function of macrophages. Studies have shown that macrophages preferentially target foreign material in the 2-3 μm size range [149,150]. Within airway epithelial cells, the inflammatory response increases with decreasing particle size. Ultrafine silica induces a higher inflammatory response when compared to fine and coarse silica [151]. Interestingly, another study, using less bioactive particles, found less evidence for an effect of particle size on the response [152]. It has been suggested that the effects seen with epithelial cells and smaller particles may be more closely associated with the total surface area of the particle suspension. When the effects take into account surface area, the differences between large and small particles are reduced [151].

There are limited data on the effect of size for geogenic PM_{10}, although one study showed no correlation between particle size and the respiratory response to geogenic PM_{10} [16]. However, there was an association with the variation in particle sizes within the sample suggesting that

geogenic PM with a larger range of particle sizes had a bigger impact on respiratory health compared to geogenic PM with a more uniform size distribution [16]. The link between particle size variation and biological health impacts is not clear. However, a larger body of evidence has considered the importance of particle chemical composition on the response which will be detailed in the next section.

1.6.2 Chemical composition

The chemical composition of PM_{10} is entirely dependent on the source. In general, geogenic PM_{10} contains large amounts of amphiboles (predominantly silicates) alongside elemental metals depending on local geological and/or anthropogenic processes. For example, dust samples taken from a 2001 Beijing dust storm showed a predominance of Fe and Al with trace contributions from As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, U, Zn, Na, P, Ca, Sc, Ti, V, Sr, Y, B and La [146]. While dust samples collected in the rural city of Chuncheon, South Korea during a dust storm showed large concentrations of Si and Al with lesser quantities of other metals [153]. Aerosol samples taken from Saharan dust storms demonstrated large concentrations of Fe, Si, and Al and a range of trace elements [154]. Interestingly, samples from the Middle East collectively show lower proportions of Si, Fe and Mn and higher proportions of Ca and Mg when compared to dust samples from the Sahara, China and Australia [144]. Geogenic particles from Western Australia primarily consist of Si, Fe, Al with smaller amounts of As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, U and Zn [142,16]. Samples taken during a 2009 Sydney dust storm also showed large amounts of Si and Al, with moderate amounts of Fe and smaller amounts of C, Mg, S, K, Ca and Ti [115]. While Si, Fe and Al seem to dominate these particles, local geology can influence the contribution of other metals. For

example, in Nevada, arsenic concentrations as high as 7058 μg/g (bedrock and top-soil) and 0.03 μg/m3 (airborne) have been reported [155]. Further analysis found concentrations of As in soil and parking lot samples from varying locations within Nevada ranged from 3.49 to 83.02 μg/g and from 16.13 to 312 μg/g, respectively [156].

There are profound differences in tissue response to PM which is highly dependent on its chemical composition. The following sections consider the health effects of the primary constituents of geogenic PM; silica, iron oxide and aluminum.

1.6.2.1 Silicon

Typically, the major geological element of geogenic PM_{10} is silicon, in the form of silicon dioxide ("silica", SiO_2). Eurasian dust is predominantly quartz whereby the proportion of quartz in major dust storms (60.95% in North Africa; 60.26% in China) is close to the mean value for the Earth's crust (58.98%) [157]. The identification of the respiratory response to silicon is complicated by variation in the physical characteristics of silicon-containing particles and the impact this variation has on the respiratory response [158]. Amorphous and weathered silica is thought to be less detrimental to lung health [159,160] than crystalline silica; although it is not completely harmless [161]. Approximately 20% of silica in geogenic PM_{10} is crystalline [162]; however, this value may vary. The two most abundant polymorphs of crystalline silica are quartz and cristobalite; other less abundant polymorphs include tridymite, keatite, moganite, coesite, stishovite, seifertite and melanophlogite [163]. Exposure to crystalline silica can lead to silicosis, an irreversible fibrotic condition of the lung [164,158,110]

and has also been linked to pulmonary fibrosis, lung cancer, auto-immune dysfunction, COPD and tuberculosis [165].

Experimental studies have clearly demonstrated a causal association between exposure to silica and lung damage. Exposure of rats to a dose of silica, below that which causes cumulative apoptosis of alveolar macrophages (15 mg/m^3 for 6 h), elicits a dramatic increase in inflammatory cytokines, markers of tissue damage [166] and fibrotic lesions 71 days after inhalation [167,166]. In addition to the increased pulmonary inflammation and fibrosis, it was noted that the silica particles had cytotoxic properties, eliciting some level of pulmonary oedema and further promoting pulmonary fibrosis, which may contribute to the development of silicosis and COPD [167,166].

In vitro, crystalline silica causes the production of pro-inflammatory cytokines, including IL-6, IL-8, IL-12 and IL-13, in BEAS-2B and NHBE cells [168]. Furthermore, A549 cells show signs of acute cytotoxicity after 2–4 h of exposure to 25–100 µg/mL of silica including oxidation-induced DNA damage in the cells [169]. Alveolar macrophages facilitate the removal of silica particles via phagocytosis; however, excessive or repeated inhalation of crystalline silica can compromise macrophage function leading to apoptosis [170]. Apoptosis of macrophages causes the release of inflammatory mediators and other signalling molecules [171]. Which can cause fibroblast activation and augment inflammatory cascades, leading to the release of free oxygen radicals and nitrogen species [170,171]. Excessive fibroblast activation can lead to the accumulation of collagenous tissue and thus, fibrosis (silicosis) [171]. In addition, it is thought that excessive release of pro-inflammatory molecules under these

conditions can cause DNA damage which may be sufficient to disrupt normal cell function [170,110,172,171].

The chemical reactivity and toxic properties of crystalline silica appears to relate to the presence of silanol groups (SiOH) protruding from the crystal surface. Cristobalite and quartz possess a similar density of surface silanol groups and the weight of evidence from experimental studies *in vitro* and *in vivo* shows no differences in the cytotoxic, inflammatory or fibrogenic properties between these two polymorphs [173]. Meldrum & Howden (2002) provide a comprehensive review on this topic with a focus on the fibrogenic potential and toxicity of the crystalline silica sub-types [173].

On a molecular level, silica-associated tissue damage activates the NACHT, LRR and PYD domain-containing protein (NLRP)-3 inflammasome, a complex of proteins, which cleaves pro-IL-1β into its active analogue [174]. The NRLP-3 inflammasome is thought to be one of the most important particle-induced damage response pathways [174,175] and responds to changes in mitochondrial activity in response to exogenous stimuli [176]. NLRP-3 is stimulated directly by silica and molecules released from injured cells (e.g. damage-associated molecular patterns; DAMPs) that are produced in response to silica inhalation.

There is no doubt that crystalline silica has a high level of cytotoxicity and causes tissue damage in the lung which can lead to the development of chronic lung disease. This may have important implications for the respiratory health of communities exposed to high levels of geogenic

particles which typically contain a high proportion of silica. However, studies suggest there are potentially important implications from the inhalation of iron oxide.

1.6.2.2 Iron

Iron oxides are an abundant element in many geogenic PM_{10} samples [142,16]. There are 16 known iron oxides and oxyhydroxides. However, haematite (Fe_2O_3), magnetite (Fe_3O_4) and goethite (FeO(OH)) are three of the most abundant and important ferric compounds. Iron is usually inhaled as hematite or magnetite and can be found in respirable particulate form as a result of iron ore mining operations and natural erosion [177]. Evidence regarding the potential respiratory health impacts of iron oxide inhalation is conflicting. The literature on iron nanoparticles is more extensive, however these particles are not directly comparable to the micron-sized particles more commonly found in communities exposed to geogenic PM.

Lay et al. (1999) demonstrated that bronchoscopic instillation of 5 mg of sterilized haematite, at relatively high concentrations (5 mg/ person) resulted in transient, subclinical inflammation with markers indicative of alveolar damage including increased numbers of neutrophils and alveolar macrophages as well as increased protein, lactate dehydrogenase and interleukin (IL)-8 in the bronchoalveolar lavage [178]. In contrast, a follow-up study from the same group demonstrated that acute inhalation of iron oxide in humans, at a relatively high concentration (12.7 mg/m^3 for 30 min), left no evidence of significant alveolar or other lung damage [179]. However, these studies are based on acute exposure and it is likely that exposure at a community level is chronic. Goethite is composed of about 80–90% haematite and

approximately 10% water. When dehydrated, goethite forms pure haematite; upon hydration, goethite becomes limonite ($Fe_2O_3(H2O)$). There is limited data on the effect of limonite and other oxyhydroxides on lung health, however one study suggested that ROS production induced by ferric oxyhydroxides is minimal [180].

There are only a limited number of epidemiological studies based on the chronic health effects of iron oxide inhalation that are not confounded by the coexistence of silica in the particles. Port Hedland, a mining port in Western Australia, has PM_{10} that is 93% haematite [137-139]. Epidemiological studies in this region have shown that hospital admissions for respiratory diseases are significantly higher than the rest of the state [140]. In line with this, an epidemiological study based on hospital records from Whyalla, a South Australian community exposed to excessive iron oxide dust from a steel plant, found a significantly higher prevalence of COPD and asthma and an increased risk of hospitalization for respiratory infections in children, when compared to non-exposed, neighbouring towns [141]. However, it must be acknowledged that these studies are limited by their cross-sectional nature and inability to control for potential confounders.

One study, using relatively low concentrations (4.0 mg/kg) of small iron oxide particles (1.5 μm MMAD) in a rat model, demonstrated neutrophilia and IL-6 production by alveolar macrophages [181]. Similarly, while rats exposed sub-chronically to magnetite had no evidence of pathology or alterations in neutrophil function, it was observed that iron oxide inhalation (up to ~50 mg/m^3, 6 h per day for 13 weeks) resulted in an increased number of neutrophils and inflammatory markers in the bronchioalveolar lavage, as well as increased septal collagen

[182]. Likewise, sub-chronic haematite exposure resulted in immediate proliferation and thickening of the lung epithelium and bronchitis in guinea pigs [183]. However, the particles were rapidly cleared from the lung with only limited development of lung fibrosis. One study has demonstrated that intratracheal instillation of hematite (10 μg per mouse) caused an increase in mortality in mice from a *Streptococcus pneumoniae* infection suggesting that iron oxide may have the capacity to exacerbate existing respiratory disease [184].

The mechanism(s) by which iron oxide may elicit inflammation and cause lung damage is not clear. It is thought that iron is capable of driving redox reactions via a modified Haber–Weiss or Fenton reaction through the oxidization of Fe^{2+} to produce hydroxide (OH^-) and radical hydroxyl species (OH*) [185]. Experimental evidence suggests that iron oxide by itself may not be sufficient to elicit an inflammatory response, but it may have some synergistic potential and add to the response initiated by other compounds in particle mixtures. For example, in rats exposed to soot and/or iron for 3 days (250 $\mu g/m^3$, 6 h per day for 3 days), while there was no effect of either alone, the combination of these particles caused oxidative stress resulting in increased expression of IL-1β and nuclear factor-κB (NF-κB) [186]. This suggests that elements in a particle mixture may cause a response that is beyond the sum of responses induced by the individual elements alone. In line with this, recent *in vivo* evidence suggests that iron plays an important role in enhancing the respiratory health impacts of other constituents of geogenic PM_{10} [187,142,16].

Collectively, the evidence for inhalation of iron oxide suggests a low level of cytotoxicity in the lung, although there is some evidence to suggest reversible lung tissue damage and low

levels of inflammatory cytokines in response to iron oxide inhalation. There is also some evidence to suggest that iron may exacerbate the response induced by other elements and/or existing respiratory infection. More research is required to clarify this and the role of iron oxide in susceptibility to bacterial infection.

1.6.2.3 Aluminium

Aluminium inhalation at an occupational level has been, in some circumstances, correlated with lung diseases including asthma and fibrosis [188-195]. However, it is thought that the respiratory effects may be due to co-exposures and "dust overload" rather than the direct effect of aluminium itself [196]. Dust overload occurs when the volume of dust in the lungs markedly exceeds the capacity of pulmonary clearance mechanisms. Lung overload is not dependent on the inherent toxicity of the compound, and dust overloading has been shown to modify both the dosimetry and toxicological effects of an inhaled compound. There is mixed evidence to suggest that individuals exposed to aluminium oxide at an environmental level may be at increased risk of developing respiratory disease.

In line with this, animal experimentation has yielded inconsistent results. In one early study, rabbits exposed to aluminium dust daily (for 1–2 h) for 20 to 40 days had increased connective tissue in their lungs [197]. However, in other studies, inhalation of aluminium dust caused minor changes in lung health and no evidence of structural damage [198]. Interestingly, the addition of a pneumococcal infection resulted in diffuse sclerosis, collagen formation and rapid death demonstrating the importance of studying the effect of these particles when combined

with other respiratory insults. Lindenschmidt et al. (1990) also demonstrated, in rats, that intratracheal instillation of aluminium oxide (1–5 mg/100 g body weight) resulted in a dose-dependent, although minimal and generally transient, increase in inflammatory markers (total cells, neutrophils and lymphocytes) [199] with no evidence of fibrosis [200].

In vitro experimentation suggests that aluminium oxide does not significantly affect lung cell function. Rabbit alveolar macrophages and human granulocytes do not produce radical oxygen species in response to aluminium oxide at high concentrations (>5 mg per 1×10^6 cells) [201]. Similarly, Warshawsky et al. (1994) demonstrated that cell viability in hamster and rat alveolar macrophages is not altered in response to aluminium oxide exposure (0.5 mg per 1×10^6 cells) [202]. These data suggest that aluminium has limited potential to directly cause a response in healthy respiratory cells.

Collectively, the evidence for inhalation of aluminium oxide suggests a low level of cytotoxicity in the lung. Environmentally, aluminium oxide is considered a 'nuisance dust' that may cause airway irritation at high levels of exposure [196]. There is some evidence that aluminium oxide may accumulate in the lungs over prolonged periods of time and contribute to "dust overload" which may cause pathological changes in the lung but is not capable of directly causing lung injury [196]. Thus, when considering the health effects of geogenic PM, silica and iron oxide are likely to be the most critical determinants of outcome.

1.7 Geogenic PM and infection

The innate immune system is the first line of defence against foreign bodies, including PM. In contrast, the adaptive, or antigen-mediated, immune system has evolved to adapt and protect against pathogens by targeting specific antigens [87]. The health effects of PM and resulting infection primarily impact on the innate response; although it should be acknowledged that modulation of the adaptive immune system has important effects in some contexts [203].

The upper airways and bronchi house the mucociliary escalator which consists of a layer of ciliated epithelial and mucous-producing goblet cells [204,205]. Particles that deposit in the mucus are expelled by the upward beating of the cilia and the cough reflex. Particles that bypass the mucociliary escalator deposit into the lower airways and are cleared by alveolar macrophages [206,207]. Dysfunction in the mucociliary escalator, or epithelial lining, is often implicated in chronic respiratory diseases, such as bronchiectasis [6]. Additionally, excessive particle deposition in the alveolar space can alter the homeostasis and functional capacity of innate immune cells such as macrophages [208]. Both epithelial cells and alveolar macrophages are primary mediators of the initial immune response. As a result, dysfunction in these cells can have serious implications on the response to infection, tissue injury and stimulation of other immune cells.

1.7.1 Epithelial cells

The respiratory epithelial cell population consists of bronchial and alveolar epithelial cells, with the latter consisting of type 1 and 2 alveolar cells [209,210]. These cells differ in their morphology, function and sensitivity to stimuli [211].

As mentioned in Section 4.1, bacteria such as NTHi preferentially adhere to necrotic tissue [13,12]. This may be due to one of two key mechanisms. Firstly, non-viable cells produce no anti-microbial responses [13] and, secondly, bacteria prefer attachment to cells with damaged cilia which are unlikely to be present on dead cells [13]. Broadly, PM has been shown to induce acute cytotoxicity in both bronchial and alveolar epithelial cells [212]. This occurs through the induction of apoptosis as a result of excessive mitochondrial stress [213].

For example, silica particles activates the NLRP-3 inflammasome [214,215] following particle endocytosis which results in rupture of the lysosomal membrane [216]. The subsequent release of cathepsin B directly stimulates the formation of the inflammasome which elicits the release of IL-1β [216]. Inflammation of the airway epithelium is a hallmark characteristic of bronchiectasis [6]. The airway epithelium is one of the primary initiators of immune cell recruitment, particularly neutrophils [27] as a result of the release of the neutrophil chemoattractant, IL-8 [27,33]. Inherently, neutrophils release destructive proteases [27,33] which, in controlled quantities, act to degrade pathogens. However, in excess these proteases can result in the degradation of host tissues [27,26,33]. IL-8 release is induced by activation of NF-κB [217]. Upstream activators of NF-κB can vary, for example during silica exposure it is

most likely through NLRP-3 activation [174,214,215,218] while bacterial stimuli activate this pathway via TLR-4 [219]. Less is known regarding iron oxide, however, iron oxide nanoparticles have can activate TLR-4 [220,221], but these particles are smaller than those found with environmental exposures so more work is required. To date, the effect of these particle-induced TLR fluctuations is unclear.

1.7.2 Alveolar macrophages

Alveolar macrophages are the primary phagocyte in the lung [207]. They function to clear the airways of foreign bodies that are not cleared by the mucociliary escalator. Macrophages secrete anti-microbial proteins, such as lysozyme, but can also phagocytose and eradicate pathogens intracellularly [207]. Macrophages engulf PM which is then transported to the mucociliary escalator or lymph nodes if it is soluble [222,223]. However, unlike pathogens, inorganic PM is not as easily degraded. Insoluble PM deposited below the escalator relies heavily on removal by alveolar macrophages compared to any other mechanism [224]. At a minimum, non-toxic PM will be removed over the course of several weeks [224], however cytotoxic PM often induces macrophage apoptosis before it is successfully expelled from the lungs. As a result, during repeated exposures, these particles can build up in the lungs which can induce chronic inflammation.

Alveolar macrophages play a critical role in initiating, mediating and resolving pulmonary inflammation [207]. Alveolar macrophage activation occurs via two key pathways. The first is via damage-associated molecular patterns (DAMPs), such as cellular debris generated by

apoptotic cells. Macrophages also recognise pathogen-associated molecular patterns (PAMPs) [225]. PAMPs are expressed by bacteria, virus and their associated surface molecules such as LPS [226]. As discussed in Section 4.1.2, TLRs are a family of cell surface immune-modulatory receptor proteins that are the primary responders to PAMPs [78]. While they are present on epithelial cells, they are predominantly expressed on macrophages in the lung [227]. Upon binding with a ligand, TLRs are internalised and initiate an NF-κB-associated inflammatory cascade to resolve airway infection. Macrophages express a range of TLR sub-types that are involved in the recognition of a variety of PAMPs [226]. As mentioned, the impact of particle-induced changes in TLR expression on infection is unclear.

For example, TLR2 and TLR4 respond to bacteria. TLR2 is expressed in human alveolar epithelial type II cells and alveolar macrophages and recognises peptidoglycan and lipoprotein which are products of Gram-positive bacteria and mycobacterium [227,78]. TLR4 resides on the outer membrane and is responsible for the recognition of LPS [227,78]. A highly pro-inflammatory molecule produced and present on the outer membrane of Gram-negative bacteria. Dysfunction of TLR signalling has been associated with the pathogenesis of several airway diseases, including acute lung injury, asthma, and COPD [228].

PM has been shown to directly stimulate the expression of TLR2 and TLR4, even in the absence of known PAMPs such as LPS [229-234]. This suggests that PM may cause dysfunction in the capacity of macrophages to respond appropriately to bacterial infection. However, there are no studies on the effects of geogenic PM on TLRs and how this may impact on phagocytosis.

1.8 Summary

Several studies suggest that inhalation of geogenic PM_{10} has the capacity to cause inflammation and exacerbate existing lung disease. It is also clear that the physicochemical characteristics of the particles have a significant impact on the response. However, there are many gaps in the existing literature. Firstly, there is a large imbalance with a plethora of research studies on silica while research on the respiratory impacts of Fe, an important constituent of geogenic PM, is lacking.

Bronchiectasis, which is prevalent in some communities exposed to high levels of geogenic PM, is predominantly driven by respiratory infection. Bronchiectasis requires an impaired host response and/or increased pathogenicity of the bacteria. Geogenic PM has been shown to increase the severity of viral infection *in vivo* [235] and NTHi adhesion and invasion *in vitro* [14]. This suggests that inorganic PM can reduce the host's ability to defend against infection. However, there are no data on the effect of these particles on bacterial growth or any comparative analysis of the effect of NTHi strain on respiratory cell invasion and how this is modified by silica and iron oxide. Thus, there is a need for further studies on how geogenic PM can contribute to susceptibility to bacterial infection as a potential explanation for the increased prevalence of bronchiectasis in Aboriginal Australian communities.

1.9 Aims and hypotheses

The overall aim of this thesis was to assess the impact of geogenic PM, with a focus on iron oxide, on respiratory health and susceptibility to bacterial invasion.

1.9.1 Specific aims

Aim 1: To assess whether environmentally relevant forms of iron oxide are sufficient to induce inflammation in lung epithelial cells and whether they augment the silica-induced response.

Hypothesis: Iron oxide will induce an inflammatory response, but at a lower level than silica, and will augment silica-induced inflammation.

Study approach: Bronchial epithelial (BEAS-2B) and alveolar (A549) cells were exposed to increasing concentrations of two structural homologues of silica (quartz & cristobalite), or two chemical homologues of iron oxide (haematite & magnetite), for 4 or 24 hours. Subsequent experiments combined silica and iron oxide in a 2:1 ratio, reflecting the proportions found in real-world geogenic particles. The cytotoxic response was measured using lactate dehydrogenase (LDH), while inflammatory cytokines were measured using enzyme-linked immunosorbent assays (ELISAs), specific for interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α (TNF-α).

Aim 2: To assess whether environmentally relevant forms of iron oxide cause a pro-inflammatory response in macrophages and whether they modify the response to a bacterial stimulus.

Hypothesis: Iron oxide will induce an inflammatory response, but at a lower level than silica, while both exposures will induce significant inflammation in the presence of LPS.

Study approach: THP-1 monocyte-derived macrophages were exposed to increasing concentrations of silica and/or iron oxide (haematite or magnetite). Given macrophages are pivotal in the recognition and elimination of bacteria, the response to PM in the presence of LPS exposure was also assessed. The cytotoxic response was measured using LDH, while inflammatory cytokines were measured using ELISAs specific for IL-1β, IL-6, IL-8 and TNF-α.

Aim 3: To determine whether environmentally relevant forms of iron oxide promote NTHi growth.

Hypothesis: Iron oxide, but not quartz, will alter the growth of NTHi.

Study approach: Nine clinical isolates of pathogenic NTHi were used. Each isolate varied in invasiveness and genome. Bacteria were grown in deplete, limited or replete iron (heme) levels. Under each of these conditions, each isolate was exposed to either haematite, magnetite or silica. Alterations in the rate of growth and maximal growth were assessed by spectral absorbance and agar plate counts.

Aim 4: To assess whether environmentally relevant forms of iron oxide can alter NTHi invasion of lung cells and on phagocytosis by macrophages.

Hypothesis: Both silica and iron oxide will the increase invasion of lung epithelial cells and decrease phagocytosis of NTHi.

Study approach: BEAS-2B, A549, THP-1 and primary human peripheral blood mononuclear cell (PBMC)-derived macrophages were exposed to silica and/or iron oxide for 24 hours. Six NTHi isolates were cultured and fluorescently stained prior to cell exposure for 3 hours. NTHi invasion was quantified by flow cytometry. Differences in NTHi and invasion were assessed by genome comparison. Subsequent experiments quantified particle uptake and assessed expression of anti-microbial genes, beta-defensin-1 and -2 and bacterial recognition proteins; Toll-like receptors (TLRs)-2 and -4.

Chapter 2: The Inflammatory Effect of Iron Oxide and Silica on Lung Epithelial Cells

A modified version of this chapter has been accepted for publication in a peer-reviewed journal:

Williams LJ, Zosky GR. The Inflammatory Effect of Iron Oxide and Silica Particles on Lung Epithelial Cells. Lung. 2019;197(2):199-207 (see Appendix).

This Chapter addresses the first aim of the study; To assess whether environmentally relevant forms of iron oxide are sufficient to induce inflammation in lung epithelial cells and whether they augment the silica-induced response. This chapter is presented as published in the Journal *Lung*.

2.1 Abstract

Our understanding of the respiratory health consequences of geogenic (earth-derived) particulate matter (PM) is limited. Geogenic PM typically contains large concentrations of silica and iron oxide. While it is clear that silica particles induce chronic, irreversible lung damage, recent evidence suggests that iron may augment this response. We investigated the effect the inflammatory and cytotoxic potential of silica and iron oxide particles alone, and in combination, on lung epithelial cells. Bronchial epithelial cells (BEAS-2B) were exposed to 0-57 μg/mL silica or iron oxide. 4- and 24-hours post-exposure, cytotoxicity and cytokine production (IL-6, IL-8, IL-1β and TNF-α) by LDH assay and ELISA respectively. Subsequently, BEAS-2B and alveolar epithelial cells (A549) were exposed to combinations of silica and iron oxide to assess the effect of combined exposure to the particles. 24 hours post-exposure, cytotoxicity and cytokine production (IL-6, IL-8, IL-1β and TNF-α) by LDH assay and ELISA respectively. After 24 hours exposure, silica induced dose-dependent cytotoxicity and secretion of IL-6 and IL-8 in BEAS-2B cells. Iron oxide did not cause significant cytotoxicity or secretion of cytokines at any concentration, nor did it augment the response of silica in the BEAS2-B cells. In contrast, whilst the silica response was not augmented in the A549 cells by the addition of iron oxide, iron oxide particles alone were sufficient to induce IL-8 production in these cells. There was no response detected for any of the outcomes at the 4-hour time point, nor was there any evidence of IL-1β or TNF-α production. While previous studies have suggested that iron may augment silica induced inflammation, we saw no evidence of this in human epithelial cells. The response to iron oxide, which is generally thought to be biologically inert, was cell dependent with alveolar epithelial cells generating an inflammatory response in response to these particles.

2.2 Introduction

Particulate matter (PM) inhalation is strongly associated with an increased risk of respiratory disease, cardiovascular disease and overall mortality [236-239,2]. The sources of PM vary considerably between locations. For example, urban populations are typically exposed to PM derived from combustion sources; in particular, diesel exhaust particles (DEP) which have been extensively studied due to their impact on the pathogenesis of respiratory disease [240,241]. In contrast, crustal, or geogenic (earth-derived) particles often affect populations in arid areas. Our understanding of the respiratory health impacts from these sources of PM is much more limited [242].

Inhalation of geogenic PM is associated with increased mortality [131,134,133] and hospital admissions [132]. In experimental models, inhalation of geogenic PM results in oxidative stress, release of pro-inflammatory mediators, reduced lung mechanics and exacerbation of viral infections [243,244,16,142,235]. *In vitro*, geogenic PM increases interleukin (IL)-6 and IL-8 production bronchial epithelial cells [245] and increased tumour necrosis factor-α (TNF-α) and reactive oxygen species (ROS) in alveolar macrophages [246].

Oxides of silicon, aluminium and iron typically dominate geogenic PM. Silica (SiO_2) is the dominant constituent of geogenic PM and is well-known in the occupational setting for causing chronic lung disease [110] due its capacity to cause inflammation [166,168], cytotoxicity [247], DNA damage [248] and oxidative stress [170]. The effect of aluminium oxides on respiratory health is less well studied but the general consensus is that these particles are relatively biologically inert when inhaled [199,200]. In contrast, data on the effect of iron oxides are

contradictory. Epidemiologically, there is some evidence to suggest that exposure to iron oxide causes respiratory morbidity and *in vivo* studies have shown strong associations between the iron concentration in geogenic PM, inflammation, deficits in lung mechanics and the capacity of the particles to exacerbate viral infection [16,142,235][17,16,15]. However, this is not always the case with some studies suggesting that insoluble iron oxides are biologically inert [179]. In contrast, some studies have suggested that the presence of particulate iron may synergistically enhance the silica induced respiratory response [187].

In light of the controversy regarding the effect of iron oxide laden particles on respiratory health *in vivo*, we investigated the inflammatory and cytotoxic potential of iron oxide (Fe_2O_3 and Fe_3O_4) particles, alone and in combination with silica, on lung epithelial cells to provide further insight into the potential health implications of inhalation of these particles.

2.3 Methods

2.3.1 Particle preparation

Commercially available standard preparations of dry magnetite (Fe_3O_4; Sigma-Aldrich 310069), haematite (Fe_2O_3; Sigma-Aldrich 310050), α-quartz (SiO_2; NIST 1878B) and cristobalite (SiO_2; NIST 1879A) were used for cell exposure experiments. We assessed the effect of haematite (Fe^{2+}) and magnetite (Fe^{3+}) as the predominant forms of geogenic iron oxide. Particle samples were exposed to UV light for two hours and washed prior to experimentation to remove any viable bacteria and endotoxin contamination. Particle samples were dispersed in their respective media (BEGM for BEAS-2B and Ham's F-12K for A549 cells), sonicated and vortexed thoroughly for 30 sec to ensure even distribution of particles within the solution before application to the cells.

2.3.2 Particle characteristics

In order to confirm the particles were < 10 μm, samples of each particle type were attached to 12 mm diameter aluminium Scanning Electron Microscopy (SEM) mounts using conductive carbon double sided sticky tabs (Ted Pella, Redding, USA), coated with approximately 4nm platinum in a Bal-Tec SCD 050 sputter coater, and imaged with a Hitachi SU-70 field emission SEM at 1.5 kV accelerating voltage, at 1000-100,000x magnification.

2.3.3 Cell culture

The transformed human bronchial epithelial cell line, BEAS-2B (human bronchial epithelium, ATCC CRL-9609), was cultured in 75 cm^2 flasks (Corning CLS3290), using serum-free

bronchial epithelial growth medium (BEGM; Lonza CC-33170) at 37 °C in a humidified atmosphere of 5% CO_2. The human lung alveolar epithelial cell line (A549; lung adenocarcinoma, ATCC CCL-185) was cultured in 75 cm^2 flasks (Corning CLS3290) with Ham's F-12K medium (Gibco 21127022), supplemented with 10% fetal bovine serum and 1% glutamine and antibiotics. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

2.3.4 Cell exposure trials

Cells were suspended in BEGM solution and seeded into 12- and 96-well plates (Corning, CLS3512 & CLS3300) at a concentration of 1.9 x 10^5 cells/mL. To investigate the dose-dependent effects of iron oxide and silica individually, cells were exposed to 0 μg/mL, 0.38 μg/mL, 3.8 μg/mL, 19 μg/mL, 38 μg/mL or 57 μg/mL (0-15 μg/cm^2) of each particle type. Concentrations were chosen to be consistent with similar PM toxicology studies [168,249-252]. Cells were exposed for 4 or 24 hours. Having established the dose dependent effects of the individual particle types, we then assessed the impact of silica and iron in combination on the response in BEAS-2B cells. Cells were exposed to either a 2:1 silica: iron ratio, which reflects the proportion of these elements in real-world particles [16], or a 20:1 ratio to replicate a situation where iron particles are present in trace amounts [167]. Having established the response in BEAS-2B cells, we then determined whether the responses were consistent between lung cell types by repeating a sub-set of experiments in the A549 alveolar epithelial cell line. We assessed a range of outcomes including cytotoxicity and cytokine production (see below for details). All experiments were replicated in six independent trials conducted using fresh preparations of particle solutions and independently cultured cell batches between passages 8-12 to allow valid statistical comparisons between exposure groups.

2.3.5 Cytotoxicity

The lactate dehydrogenase (LDH) assay (Promega G1780) was used as a marker of cytotoxicity. LDH levels were measured after 24 hours of exposure according to the manufacturer's instructions. Briefly, 50 µL of LDH buffer was added to 50 µL of supernatant in a 96-well plate, incubated at room temperature and removed from light for 30 minutes. The absorbance was then read at 490 nm using the Spectra Max M2 plate-reader (Molecular Devices, USA).

2.3.6 Inflammatory cytokines

Inflammatory cytokines were assessed by enzyme-linked immunosorbent assay (ELISAs). We assessed levels of human interleukin-1β (IL-1β; R&D Systems DY201), interleukin-6 (IL-6; R&D Systems DY206), interleukin-8 (IL-8; R&D Systems DY208) and tumour necrosis factor-α (TNF-α; R&D Systems DY210) in the cell supernatant 4 or 24 hours post-exposure to the particles according to the manufacturer's instructions. The minimum detection limits for IL-1β, IL-6, IL-8 and TNF-α were 7.81, 9.38, 31.3 and 15.6 pg/mL respectively. Plates were read using a Spectra Max M2 plate-reader (Molecular Devices, USA) at 450/570 nm absorbance.

2.3.7 Statistical analysis

Comparisons between groups were made using repeated measures one-way ANOVA. When significance was determined for the main factors by ANOVA, the Holm-Sidak post-hoc test was used to examine individual between group differences. Where necessary, the data were

log transformed to satisfy the assumptions of normal distribution of the error terms and homoscedasticity of the variance. All data are presented as mean (SD) and values of $P < 0.05$ were considered statistically significant. All statistical analyses were conducted using SigmaPlot (v12.5).

2.4 Results

2.4.1 Assessment of particle structure

Cristobalite (Fig 2.1A) and quartz (Fig 2.1B) particle size ranged from 2 - 6 μm in diameter. The structure of both quartz and cristobalite consisted of flat surfaces with sharp edges. Particles were asymmetrical, often with jagged projections. Haematite (Fig 2.1C) and magnetite (Fig 2.1D) particle size ranged from 0.2-0.8 μm aerodynamic diameter. These particles sizes are within the range of those observed in real-world samples [15]. The structure of both haematite and magnetite consisted of flat surfaces with straight line edges. Particles seemed to be asymmetrical, often with projections that were not sharp, possibly representing agregates of smaller iron oxide particles. These particles sizes are within the range of those observed in real-world samples [15].

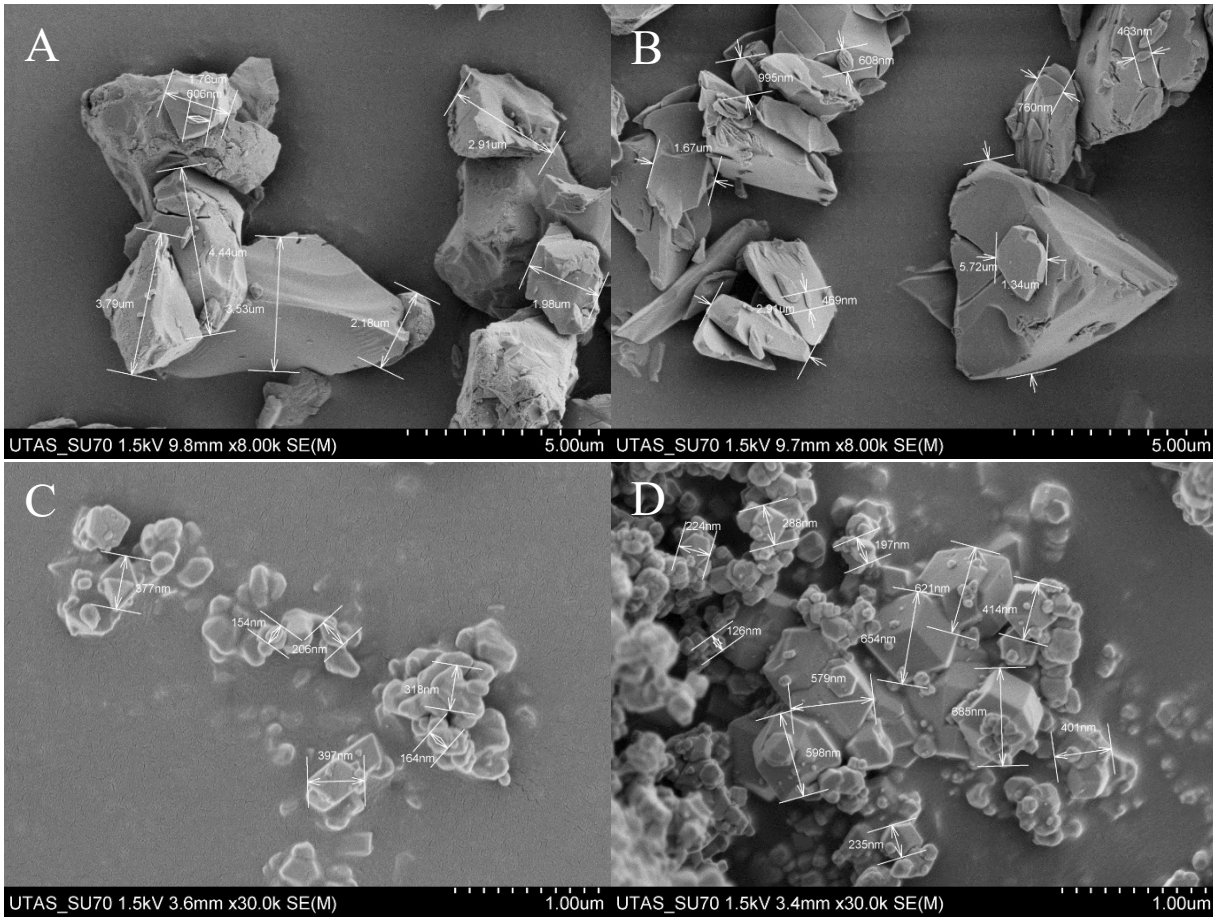


Figure 2.1: Particle samples were imaged with a Hitachi SU-70 field emission SEM at 1.5 kV accelerating voltage at 8000 times magnification. The level of magnification and scale are depicted at the bottom of each image. Images suggest size ranges of 2 - 6 μm in diameter for cristobalite (A) and quartz (B), 0.2 – 0.5 μm in diameter for haematite (C) and 0.2 – 0.7 μm in diameter for magnetite (D).

2.4.2 Response to individual particles types (BEAS-2B)

Cytotoxicity

LDH levels in the supernatant varied depending on particle type and concentration (Fig 2.2). Exposure of BEAS-2B cells for 24 hours to cristobalite (Fig 2.2A, p= 0.017) or quartz (Fig

2.2B, p= 0.009) elicited an increase in LDH levels at 57 µg/mL compared to control. Haematite (Fig 2.2C, p = 0.392) and magnetite (Fig 2.2D, p = 0.708) had no effect on LDH levels following 24 hours of exposure. There was no change in LDH levels in response to any particle type 4 hours post exposure (p > 0.05) (*data not shown*).

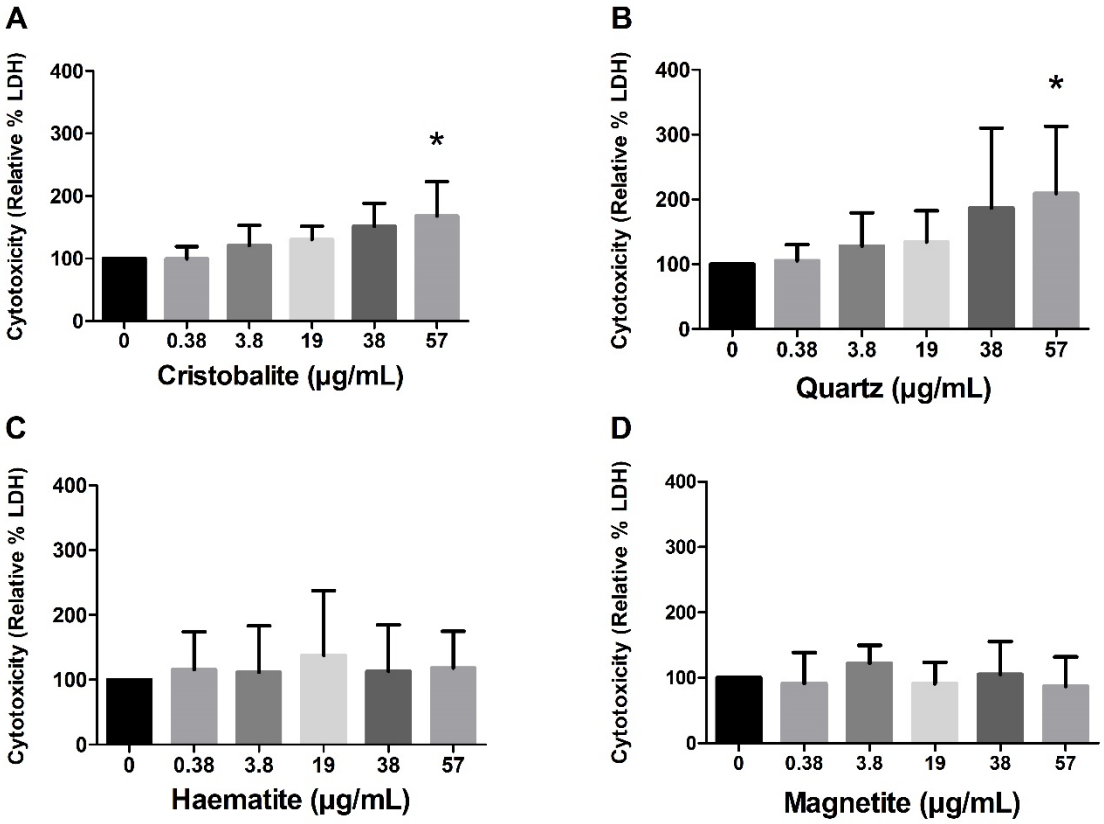


Figure 2.3: LDH levels in the supernatant of BEAS-2B cells exposed to cristobalite (A), quartz (B), haematite (C) or magnetite (D) for 24 hours. Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented as mean(SD) from 6 independent replicates with * indicating p < 0.05 versus control. Both cristobalite and quartz caused a significant increase in LDH, but only at a dose of 57 µg/mL (p

= 0.017 & p = 0.009). Haematite (p = 0.392) and magnetite (p = 0.708) had no effect on LDH levels.

Cytokines

Interleukin-6 (IL-6) levels in the supernatant varied depending on particle type and concentration (Fig 2.3). Exposure of cells for 24 hours to cristobalite (Fig 2.3A, p= 0.045) or quartz (Fig 2.3B, p= 0.009) elicited an increase in IL-6 levels at 57 μg/mL. Haematite (Fig 2.3C, p = 0.133) and magnetite (Fig 2.3D, p > 0.250) had no effect on IL-6 levels. There was no change in IL-6 levels in response to any particle type 4 hours post exposure (p > 0.05) (*data not shown*).

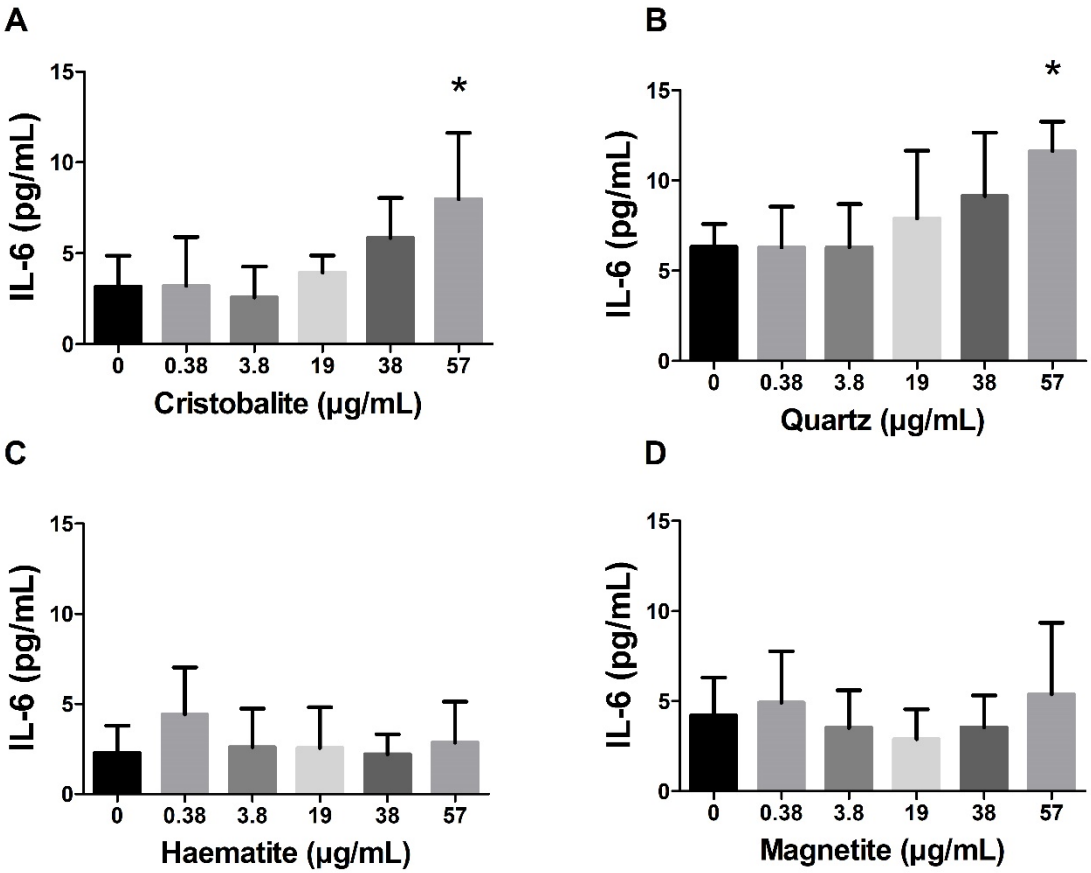


Figure 2.4: Interleukin-6 (IL-6) levels in the supernatant of BEAS-2B cells exposed to cristobalite (A), quartz (B), haematite (C) or magnetite (D) for 24 hours. Data are presented as mean(SD) from 6 independent replicates with * indicating $p < 0.05$ versus control. Both cristobalite and quartz caused a significant increase in IL-6, but only at a dose of 57 µg/mL ($p = 0.045$ & $p = 0.009$). Haematite ($p = 0.133$) or magnetite ($p = 0.250$) had no effect on IL-6 levels.

Interleukin-8 (IL-8) levels in the supernatant varied depending on particle type and concentration (Fig 2.4). Exposure of cells for 24 hours caused increased IL-8 for cristobalite at 38 µg/mL (Fig 2.4A, $p= 0.031$) and 57 µg/mL ($p < 0.001$). Quartz elicited an increase in IL-8

levels at 57 µg/mL (Fig 2.4B, p= 0.011). Haematite (Fig 2.4C, p = 0.857) and magnetite (Fig 2.4D, p = 0.775) had no effect on IL-8 levels following 24 hours of exposure. There was no change in IL-8 levels in response to any particle type 4 hours post exposure (p > 0.05) (*data not shown*). Tumour necrosis factor-α and interleukin-1β were measured, however all results were under the detection threshold (*data not shown*).

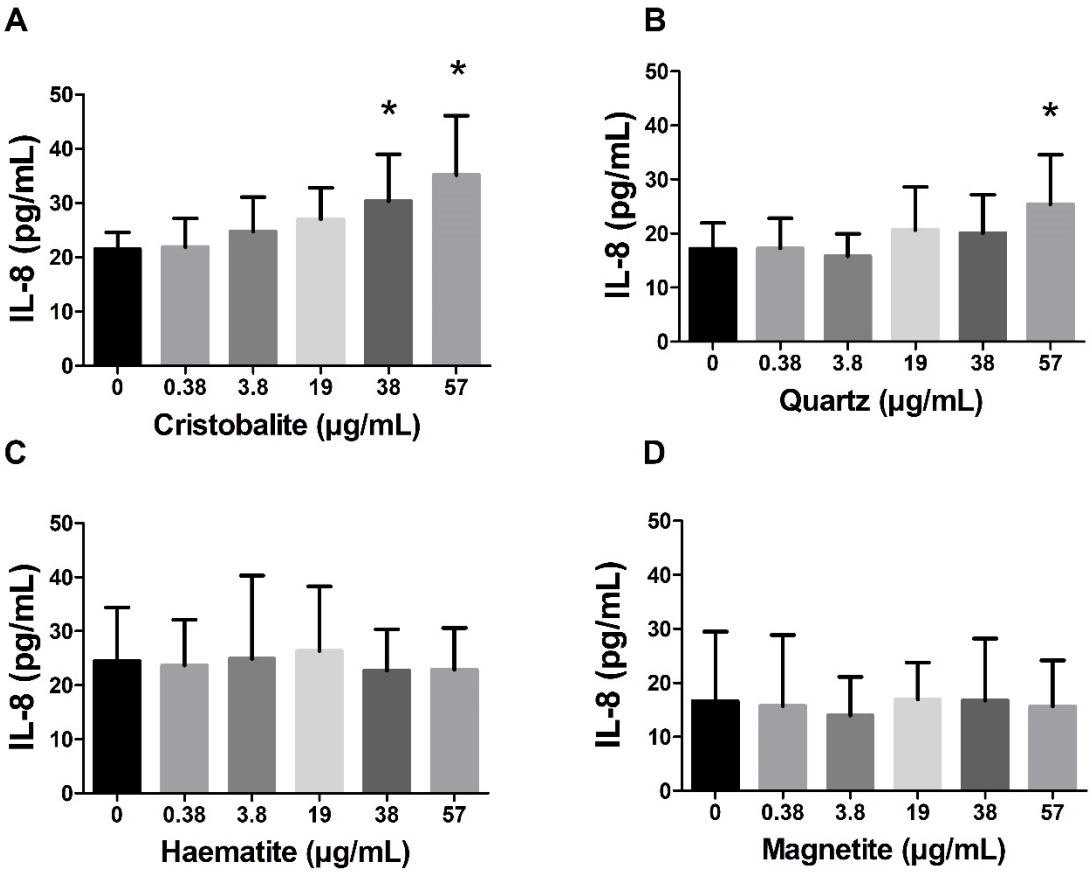


Figure 2.5: Interleukin-8 (IL-8) levels in the supernatant of BEAS-2B cells exposed to cristobalite (A), quartz (B), haematite (C) or magnetite (D) for 24 hours. Data are presented as mean(SD) from 6 independent replicates with * indicating p < 0.05 versus control. Cristobalite caused a significant increase in IL-8 at doses of 38 µg/mL (p = 0.031) and 57 µg/mL (p < 0.001). Quartz caused a significant increase in IL-8 but only at 57 µg/mL (p = 0.011). Both haematite (p = 0.857) and magnetite (p = 0.775) had no effect on IL-8 levels.

2.4.3 Combined effect of silica and iron oxide (BEAS-2B)

In initial experiments, described above, we determined the dose dependent cytotoxicity, cell metabolism and cytokine response to individual particle types. Subsequently, cells were exposed to combinations of particles to determine if the silica induced response was altered by the presence of iron oxide. For the combined exposure experiments, we chose to focus on the modifying effect of magnetite and hematite on the cristobalite induced response.

Cytotoxicity

When exposed for 24 hours, neither cristobalite-haematite (Fig 2.5A, $p = 0.096$) nor cristobalite-magnatite ($p = 0.253$) combinations elicited an increase in LDH levels in BEAS-2B cells above the cristobalite induced response.

Cytokines

38 µg/mL of cristobalite in combination with haematite (Fig 2.5B, 1.9 µg/mL; $p= 0.005$ & 19 µg/mL; $p = 0.04$) or magnetite (1.9 µg/mL; $p = 0.011$ & 19 µg/mL; $p = 0.012$) caused increased levels of IL-6 compared to controls when cells were exposed for 24 hours (Fig 2.5B). However, neither the addition of haematite (1.9 µg/mL; $p = 0.207$ & 19 µg/mL; $p = 0.649$) nor magnetite (1.9 µg/mL; $p = 0.933$ & 19 µg/mL; $p = 0.890$) significantly increased the IL-6 response compared to 38 µg/mL of cristobalite alone.

38 μg/mL of cristobalite alone (Fig 2.5C, p=0.021) and in combination with either concentration of haematite (1.9 μg/mL; $p < 0.001$ & 19 μg/mL; $p = 0.001$) or of magnetite (1.9 μg/mL; p= 0.035 & 19 μg/mL; p= 0.037) caused increased levels of IL-8 when cells were exposed for 24 hours (Fig 2.5C). However, neither the addition of haematite (1.9 μg/mL; $p = 0.207$ & 19 μg/mL; $p = 0.246$) nor magnetite (1.9 μg/mL; $p = 0.920$ & 19 μg/mL; $p = 0.913$) significantly increased the IL-8 response compared to 38 μg/mL of cristobalite alone. Tumour necrosis factor-α and interleukin-1β were measured, however all results were under the detection threshold (*data not shown*).

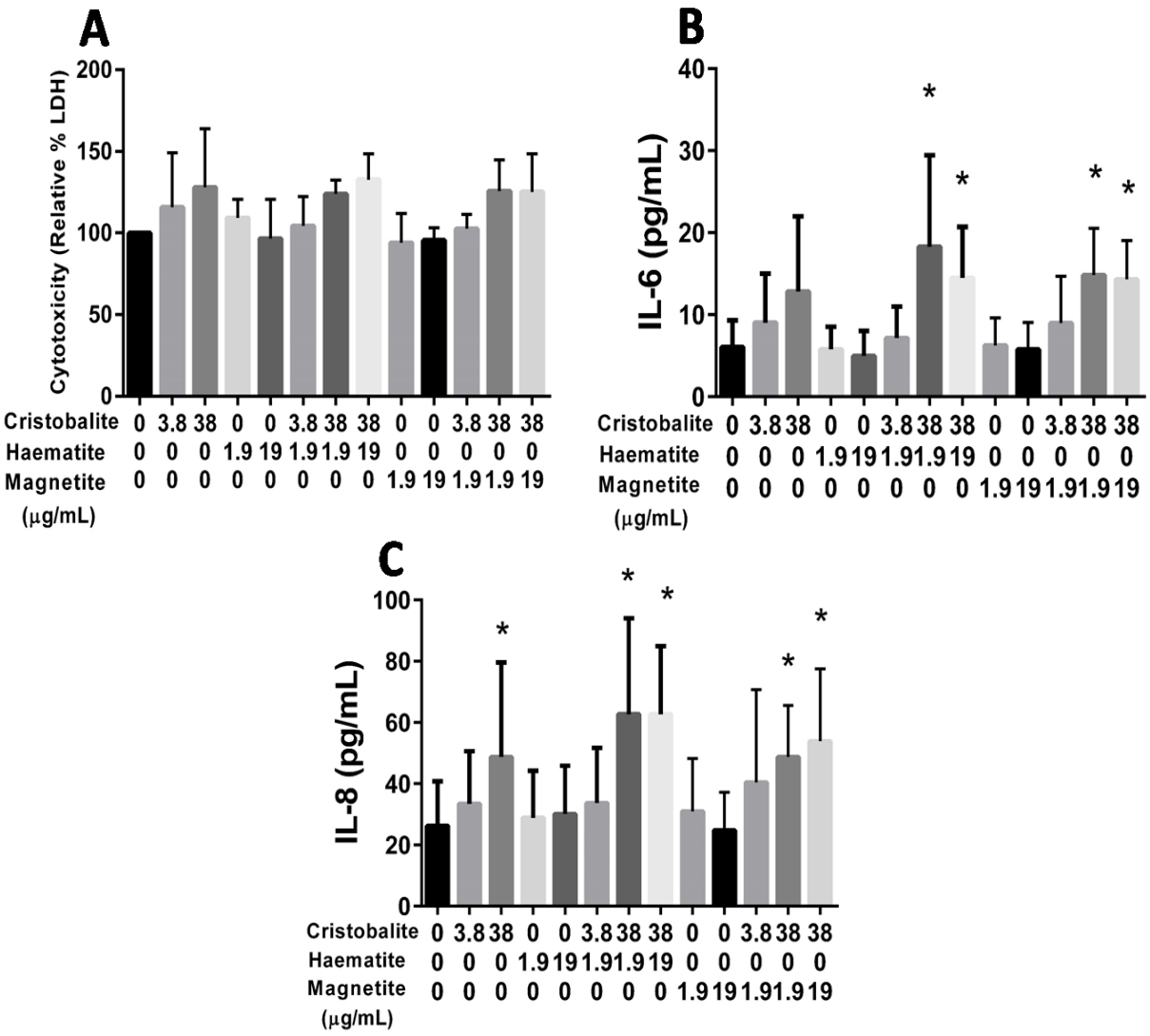


Figure 2.6: Supernatant of BEAS-2B cells exposed to cristobalite-haematite or cristobalite-magnetite combinations for 24 hours were assessed for relative LDH (A), IL-6 (B) and IL-8 (C). Data are presented as mean(SD) from 6 independent replicates with * indicating $p < 0.05$ versus control. Both cristobalite-haematite ($p = 0.096$) and cristobalite-magnetite ($p = 0.253$) had no effect on LDH levels compared to cristobalite treatment. The addition of haematite or magnetite to 38 µg/mL of cristobalite caused an increase in IL-6. However, the addition of haematite (1.9 µg/mL $p = 0.207$ & 19 µg/mL $p = 0.649$) or magnetite (1.9 µg/mL $p = 0.933$ & 19 µg/mL $p = 0.890$) was not significantly greater than the response induced by 38 µg/mL of

cristobalite alone. Likewise, the addition of haematite or magnetite to 38 μg/mL of cristobalite caused an increase in IL-8, however, the addition of haematite (1.9 μg/mL $p = 0.207$ & 19 μg/mL $p = 0.246$) or magnetite (1.9 μg/mL $p = 0.920$ & 19 μg/mL $p = 0.913$) was not significantly greater than the response induced by 38 μg/mL of cristobalite alone.

2.4.4 Combined effect of silica and iron oxide: the effect of cell type (A549)

Initial BEAS-2B experiments determined that both haematite and magnetite did not modify the silica-induced response. In order to test whether this observation is consistent in other cell types we also assessed the response in A549 cells, an alveolar type II epithelial cell line.

Cytotoxicity

There was no evidence of cytotoxicity in A549 cells in response to cristobalite and/or haematite (Fig 2.6A, $p = 0.157$) or magnetite ($p = 0.106$).

Cytokines

In contrast to the BEAS-2B cells, exposure to cristobalite (Fig 2.6B, $p < 0.001$) and haematite ($p = 0.008$), but not magnetite ($p = 0.060$), alone were sufficient to increase IL-8 levels (Fig 2.6B). The combined effect of cristobalite and haematite was equivalent to the effect of the individual exposures ($p = 0.740$). TNF-α, IL-1β and IL-6 were measured in the A549 cells, however all results were under the detection threshold.

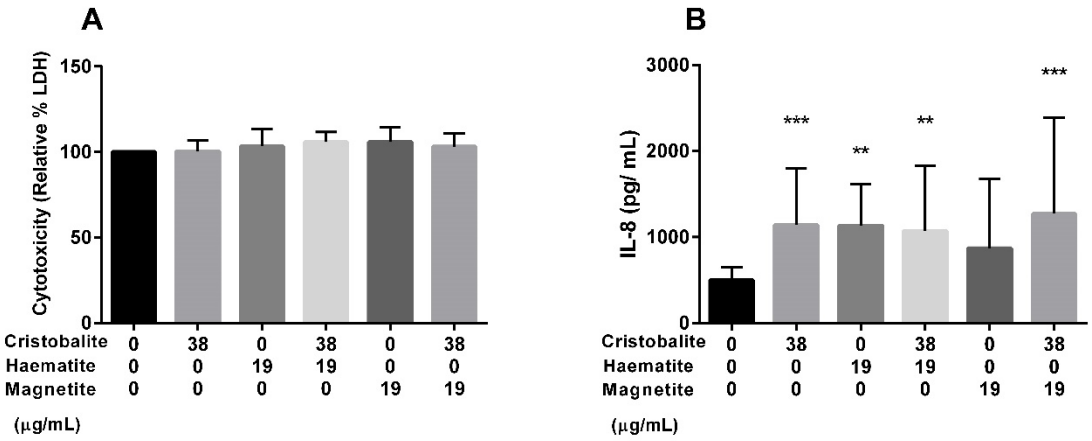


Figure 2.7: Supernatant of A549 cells exposed to cristobalite-haematite or cristobalite-magnetite combinations for 24 hours were assessed for relative LDH (A) and IL-8 (B). Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented as mean(SD) from 6 independent replicates with * indicating $p < 0.05$ versus control. Both cristobalite-haematite (Fig 5A; $p = 0.157$) and cristobalite-magnetite ($p = 0.106$) had no effect on LDH levels. Cristobalite (Fig 5B; $p < 0.001$), haematite ($p = 0.008$), cristobalite-haematite ($p = 0.001$) and cristobalite-magnetite ($p < 0.001$) had significant effects on IL-8 levels.

2.5 Discussion

The present study aimed to investigate the effect of iron oxide, alone and in combination with silica, on the inflammatory response in respiratory epithelial cells to determine whether these cells are responsible for the observed association between iron content and the inflammatory response induced by geogenic particles observed *in vivo* [142,16]. Collectively, our data from BEAS-2B cells, a bronchial epithelial cell line, suggest that iron oxide has no effect on inflammatory cytokine production, nor do these particles exacerbate the silica-induced response. In contrast to the lack of response observed in the BEAS-2B cells, iron oxide particles induced IL-8 production in A549 cells; although they did not enhance the response induced by silica. Collectively, these data suggest that alveolar but not bronchial, epithelial cells may be partly responsible for the association between the iron content and the inflammatory response to geogenic PM observed *in vivo* [16].

Using relatively low doses of particles compared to similar toxicological studies [215,152,151], we found that silica caused mild cytotoxicity and induced the production of IL-6 and IL-8 in BEAS-2B cells and IL-8 release in A549 cells. This is largely consistent with the wealth of literature on the known pro-inflammatory effect of silica [110] on BEAS-2B [168] and A549 cells [151]. There was no difference in the response between cristobalite and quartz, which is perhaps not surprising given the similarities in particle structure we observed under SEM. IL-1β and TNF-α release have long been associated with silica exposure in animal models [253,254]. Thus, secretion of these cytokines is most likely attributable to another cell type, such as macrophages [254,170,255].

In contrast, iron oxide, in the form of both haematite (Fe^{2+}) and magnetite (Fe^{3+}), was not cytotoxic at the doses used nor did it have any impact on the production of IL-6 and IL-8 by BEAS-2B cells or the silica induced IL-6 and IL-8 response. However, while neither were cytotoxic in A549 cells, both iron oxides elicited IL-8 release. This is consistent with previous epidemiological studies showing a positive correlation between exposure to iron oxide laden PM and adverse health outcomes [137,141] but is inconsistent with previous studies suggesting that iron oxide PM may be relatively inert [179].

It is generally thought that any cellular damage induced by iron is driven by the Fenton redox reaction whereby Fe^{2+} is converted into Fe^{3+} and a hydroxyl radical is produced [256]. Theoretically, with prolonged exposure to Fe^{2+}, this results in excessive production of radical oxygen species. This requires the presence of free Fe^{2+} which is dependent on the solubility of the iron compound. However, free iron rarely exists in nature [257] and the common forms used in this study, haematite and magnetite are largely insoluble at physiological pH. This implies that without a catalyst, there is no dissociated Fe^{2+} and no potential for a Fenton-like reaction to occur. While it has not been determined whether the previously studied geogenic samples contained dissociated Fe^{2+}, Lay *et al.* (1999) suggest only small amounts of iron (0.036% dissociation) are necessary to produce significant amounts of radical oxygen species [178]. It is unlikely that there was sufficient free iron in our system to induce this response. However, the fact that we were able to induce a response in the A549 cells suggests that the absence of a response to iron oxide is cell specific. Given that it is unlikely that high enough concentrations of free iron were liberated in our cell culture system, the increase in cytokine production in the A549 cells suggests that this is a direct effect of the particles on the cells.

In accordance with our data, silica has previously been demonstrated to elicit IL-8 release in A549 cells [151]. There is some evidence to suggest magnetite can induce genotoxicity and cytokine release [258]. Interestingly, Konczol *et al.* (2011) saw no cytotoxicity or genotoxicity [258], which is consistent with our data. Of note is the fact that the combined effect of silica and iron oxide on cytokine production was not greater than the effects of the individual particle types. It is likely that this is a threshold effect whereby the maximum production of IL-8 by these cells was reached.

IL-8 is a neutrophil chemoattractant and is key in recruiting neutrophils to a site of infection [259]. Recruitment of neutrophils results in endocytosis of invading pathogens and subsequent release of proteases and oxidant products [260]. Neutrophils naturally undergo autophagy, however, excessive or chronic IL-8 may lead to a disruption in the equilibrium of neutrophilic processes leading to excess and prolonged release of proteases and ROS and reduced anti-microbial function, which may result in damage to the lung tissue [261-263]. Our data suggests that exposure of alveolar cells to iron oxide containing particles may lead to tissue damage as a result of IL-8 production; an observation which is consistent with the long-term deficits in lung function that are observed *in vivo* [16].

In summary, we found that iron oxide particles can induce an inflammatory response in alveolar epithelial but appear to have no effect on bronchial epithelial cells. The iron oxide particles had no effect on the inflammatory response induced by silica, suggesting that the association between iron levels in geogenic particles and the inflammatory response *in vivo* is a direct

effect of iron oxide rather than an effect modifier. Collectively, these data highlight the importance of the iron oxide, as a key constituent of geogenic PM, when considering the health implication of PM dominated by wind-blown dust.

Chapter 3: Iron Oxide and Silica Enhance the LPS-induced Inflammatory Response in Macrophages.

A modified version of this chapter has been submitted for publication in *Scientific Reviews*:

Williams, L. J., Tristram, S. & Zosky, G. R. (**submitted**) Iron Oxide and Silica Enhance the LPS-induced Inflammatory Response in Macrophages.

In the previous Chapter (Chapter 2), the impact of silica and iron oxide on the inflammatory response in lung epithelial cells was assessed. This Chapter aimed to assess the impact of these particles on the response in macrophages and whether they modify the response to a bacterial stimulus. The text of this Chapter is presented as submitted to the Journal for publication.

3.1 Abstract

Exposure to geogenic (earth-derived) particulate matter (PM) is common in many communities around the world and is linked to respiratory morbidity and mortality. Experimental studies have shown that the concentration of iron in geogenic PM is associated with the magnitude of the health effects, however, the role of macrophages in this response is unclear. We investigated the inflammatory and cytotoxic potential of silica and iron oxide particles alone, and in combination with LPS, in macrophages. THP-1 monocyte derived macrophages were exposed to 0-50 μg/mL silica or iron oxide (hematite or magnetite) with or without 4 hours of prior incubation with LPS. After 24 hours of exposure to the particles, we assessed cytotoxicity and cytokine production (IL-6, IL-8, IL-1β and TNF-α) by LDH assay and ELISA respectively. In a subset of experiments, we assessed whether iron oxide modified the silica induced inflammatory response. Iron oxide increased IL-8 production while silica also induced significant production of IL-1β. Both iron oxide and silica enhanced LPS induced production of TNF-α, IL-1β, IL-6 and IL-8 by macrophages. Iron oxide did not modify the silica induced response. The macrophage response to PM varies depending on the particles. While silica produced a more potent response, iron oxide had the capacity to enhance LPS induced cytokine production in macrophages. These observations have health implications, particularly respiratory infections, for communities exposed to high levels of geogenic PM.

3.2 Introduction

In 2016, ambient air pollution was estimated to have caused 4.25 million premature deaths globally [264]. This excess mortality is primarily driven by respiratory and cardiovascular disease as a result of inhalation of particulate matter (PM) [264]. Importantly, the source of PM dictates its physical and chemical composition. For example, combustion products are the primary source of PM affecting urban populations [265]. PM in these areas is often dominated by diesel exhaust particles (DEP) which have well characterised effects on health [240,241]. However, populations in rural and arid environments, are primarily exposed to geogenic (earth-derived) PM [266,16,15,47] which is poorly understood.

Exposure to geogenic PM is associated with respiratory morbidity and mortality [131,133]. These epidemiological observations are supported by laboratory studies showing that community sampled geogenic PM causes inflammation *in vivo* [142,16], exacerbates respiratory infections [235] and causes the production of pro-inflammatory cytokines in airway epithelial cells and [245] and oxidative stress in alveolar macrophages [267,268].

Geogenic PM contains large concentrations of silicon, aluminium and iron [16]. Silica (SiO_2) is well-known for its contribution to pulmonary inflammation, cytotoxicity, the release of free radicals and progression of irreversible fibrotic lung diseases such as silicosis [110,269,166,170]. While less extensively studied, aluminium oxides are considered biologically inert [199,200]. In contrast, evidence regarding the health implications of iron oxide particles are conflicting. Epidemiological studies suggest that communities exposed to

high levels of ambient iron oxide dust [141,270,137,140,138,139] are at greater risk of respiratory morbidity. Consistent with this observation, experimental animal studies have shown that the concentration of iron in community sampled geogenic PM is associated with the magnitude of the detrimental lung response including inflammation, lung function deficits and impaired viral clearance [235,142,16]. In contrast, other studies suggest that insoluble iron oxides are relatively biologically inert [179]; although there is some evidence to suggest that iron oxide enhances silica induced inflammation [187]. We have recently shown that iron oxide particles generate minimal inflammation in airway epithelial cells [271] suggesting that other cell types may be involved in the response that is observed *in vivo*.

Given the critical role of macrophages the clearance of, and inflammatory response to, inhaled PM [272-274], we assessed the production of inflammatory cytokines in macrophages in response to iron oxide particles alone or in combination with silica. Based on the previously described modifying effect of geogenic PM on respiratory infection, we also investigated the effect of the particles on the macrophage response following prior exposure of the cells to bacterial endotoxin.

3.3 Methods

3.3.1 Particle preparation

Haematite (Fe_2O_3; Sigma-Aldrich 310050), magnetite (Fe_3O_4; Sigma-Aldrich 310069) and α-quartz (SiO_2; NIST 1878B) particles (<5 μm) were used for cell exposure experiments. Haematite and magnetite were chosen as common forms of naturally occurring iron oxide with different redox potentials (Fe II and Fe III) which may impact on the cellular response [271]. Particles were exposed to UV light for two hours prior to experimentation to remove any bacterial contamination. Particle samples were dispersed in RPMI-1640 media and vortexed thoroughly for 30 sec to ensure even distribution of particles within the suspension before application to the cells.

3.3.2 Cell culture

The human leukemic monocyte cell line, THP-1 (human leukemic monocyte, Sigma-Aldrich), was cultured in 25 cm^2 flasks (Corning CLS430639), using RPMI-1640 (ATCC 30-2001) supplemented with 10% Fetal Bovine Serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2.

3.3.3 Cell exposure trials

Cells were suspended in RPMI-1640 with 10% FBS and seeded into 12-well plates (Corning, CLS3512) at a concentration of 2 x 10^5 cells/well. THP-1 cells were differentiated into macrophages by exposure to 25 μM of Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich P1585) for 48 hours, followed by recovery in PMA-free growth media for 24 hours. Cells were

then either exposed to 10 ng/mL *E. Coli*-derived LPS (Sigma-Aldrich L4391) or LPS-free media for 4 hours before particle exposure. To investigate the dose-dependent effects of haematite, magnetite and silica individually, cells were exposed to 0 μg/mL, 10 μg/mL, 25 μg/mL or 50 μg/mL of each particle type. Concentrations were chosen to be consistent with similar PM toxicology studies [168,249-251,275,276]. Cytotoxicity and the production of interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α (TNF-α) were assessed after 24 hours of particulate exposure. The individual particle effects were established before assessing the impact of silica and iron, in combination, on the response. Cells were exposed to a 2:1, silica: iron ratio, which reflects the proportion of these elements in real-world particles [16]. The total dose used (50 μg/mL) was chosen based on the initial experiments, to reflect a concentration that would not cause large levels of cytotoxicity but was sufficient to produce a cytokine response. Cytotoxicity and cytokine production were assessed after 24 hours of exposure to particulates as described previously. All experiments were repeated over six independent experiments. Each experiment was conducted on a different day using fresh cell cultures and reagents including particles and LPS preparations to allow valid statistical comparisons between exposure groups.

3.3.4 Cytotoxicity

The LDH assay (Promega G1780) was used as a marker of cytotoxicity. LDH levels were measured according to the manufacturer's instructions. Absorbance was read with the Spectra Max M2 plate-reader (Molecular Devices, USA).

3.3.5 Inflammatory cytokine production

Levels of human interleukin-1β (IL-1β; R&D Systems DY201), interleukin-6 (IL-6; R&D Systems DY206), interleukin-8 (IL-8; R&D Systems DY208) and tumour necrosis factor-α (TNF-α; R&D Systems DY210) in the cell supernatant 24 hours post-exposure to the particles were assessed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The minimum detection limits for IL-1β, IL-6, IL-8 and TNF-α were 7.81, 9.38, 31.3 and 15.6 pg/mL respectively. Absorbance was read with the Spectra Max M2 plate-reader (Molecular Devices, USA).

3.3.6 Statistical analysis

Statistical analyses were conducted using two-way repeated measures ANOVAs to compare differences between groups. The Holm-Sidak post-hoc test was used to examine individual between group differences. Where necessary, the data were log transformed to satisfy the assumptions of normal distribution of the error terms and homoscedasticity of the variance. All data are presented as mean (SD) and values of $p < 0.05$ were considered statistically significant. All statistical analyses were conducted using SigmaPlot (v12.5).

3.4 Results

3.4.1 Response to individual particles types

Cytotoxicity

Quartz caused a dose-dependent increase in LDH production, with an increase observed at the 25 μg/mL concentration ($p < 0.001$) and a further increase at 50 μg/mL (Fig 3.1A, $p < 0.001$). Exposure to LPS reduced the quartz-induced LDH production, such that an increase in LDH was only detectable in response to the 50 μg/mL dose ($p < 0.001$), and the LDH production at this dose was significantly lower than the cells exposed to the same dose of quartz without LPS (Fig 3.1A, $p < 0.001$). Exposure to haematite had no effect on LDH levels (Fig 3.1B; $p > 0.05$ for all doses). In the haematite trials, LPS caused a small but statistically significant decrease in LDH production without particles (Fig 3.1B, $p = 0.02$). In contrast, when cells were exposed to LPS in combination with haematite there was a small increase in LDH production at the 25 μg/mL dose ($p = 0.04$) that did not increase further with exposure to 50 μg/mL (Fig 3.1B, $p = 0.74$). Exposure to magnetite also increased LDH production at the 50 μg/mL dose (Fig 3.1C, $p < 0.001$). Exposure to LPS changed the LDH-magnetite dose response relationship such that the increase in LDH production was detectable at 25 μg/mL ($p = 0.04$) but did not reach the same level at 50 μg/mL as that seen in cells exposed to magnetite without LPS (Fig 3.1C, $p < 0.001$) .

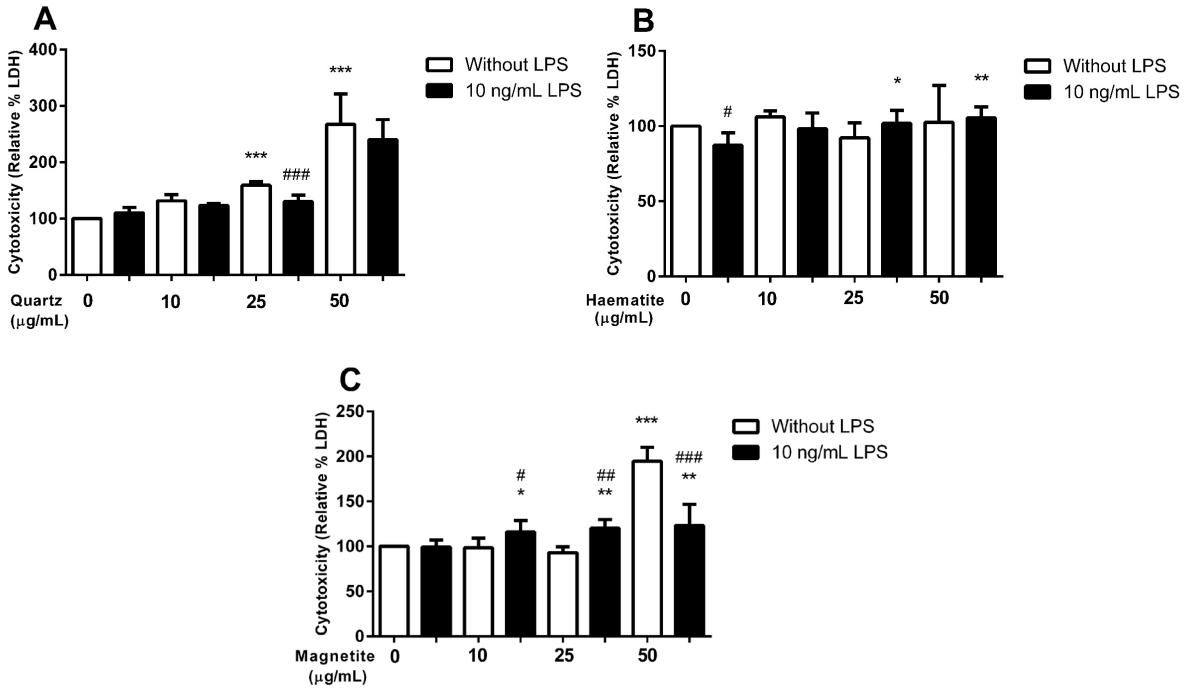


Figure 3.1: Lactate dehydrogenase (LDH) levels in the supernatant of THP-1 derived macrophages exposed to quartz (A), haematite (B) or magnetite (C) for 24 hours, with or without 4 hours of prior LPS exposure. Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented as mean(SD) from 6 independent experiments. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively versus control.. #, ## and ### indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively for LPS vs no LPS at the given dose.

Cytokines:

Interleukin-1β

Quartz induced a dose-dependent increase in interleukin (IL)-1β levels at 50 µg/mL (Fig 3.2A, $p = 0.03$). LPS significantly augmented this response ($p < 0.001$ for all doses). In contrast, haematite exposure did not alter IL-1β levels (Fig 3.2B, $p > 0.05$ for all doses). However, LPS resulted in significant increases in IL-1β expression at all concentrations ($p < 0.003$). This response was particle dose-dependent with the highest IL-1β observed at 50 µg/mL (Fig 3.2B, $p < 0.001$). Magnetite elicited a similar response when compared to haematite. There was no significant increase in IL-1β to any concentration of magnetite (Fig 3.2C, $p > 0.05$ for all doses) whereas LPS exposure elicited significantly increased IL-1β expression ($p < 0.001$ for all doses) with the highest expression observed in combination with 50 µg/mL of magnetite (Fig 3.2C, $p < 0.001$).

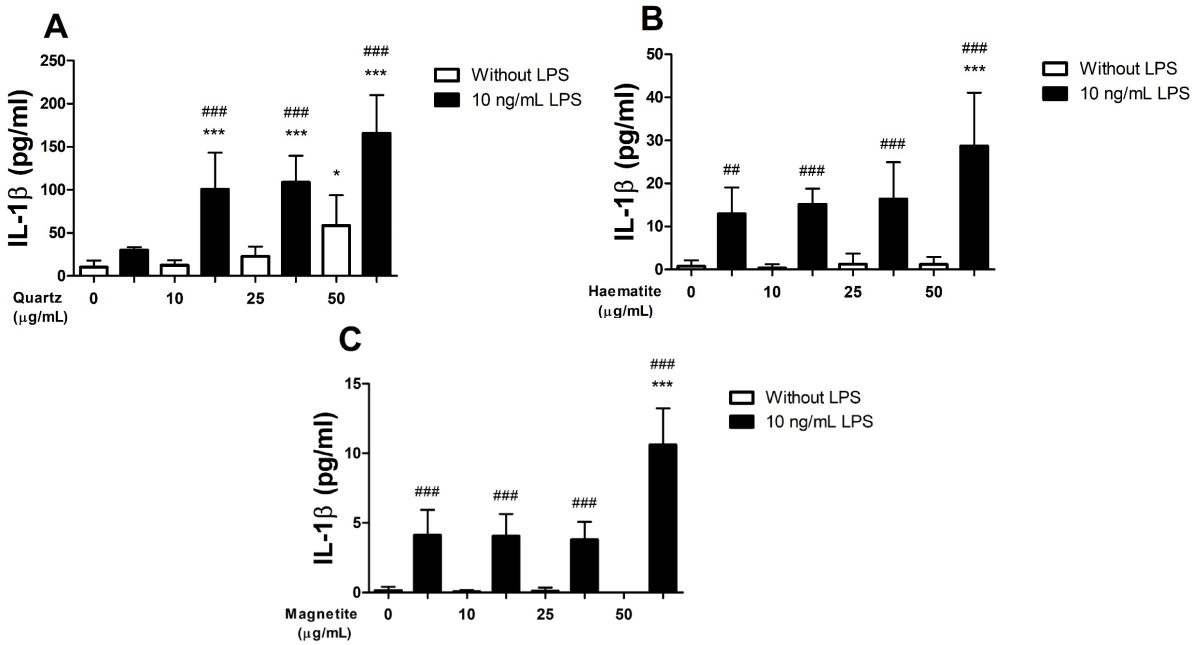


Figure 3.2: Interleukin-1β (IL-1β) levels in the supernatant of THP-1 derived macrophages exposed to quartz (A), haematite (B) or magnetite (C) for 24 hours and after 4 hours of LPS exposure. Data are presented as mean(SD) from 6 independent experiments. * and *** indicate

$p < 0.05$ and $p < 0.001$ respectively versus control. ## and ### indicate $p < 0.01$ and $p < 0.001$ respectively for LPS vs no LPS at the given dose.

Interleukin-6

Neither quartz (Fig 3.3A), haematite (Fig 3.3B) nor magnetite (Fig 3.3C) exposure caused any significant change in IL-6 ($p > 0.05$ for all doses). However, LPS elicited significantly increased the expression of IL-6 (Fig 3.3, $p < 0.001$ for all doses). When combined with 50 μg/mL of quartz and haematite, the IL-6 production was enhanced (quartz; $p = 0.006$ & haematite; $p < 0.001$). In contrast, magnetite did not enhance the IL-6 response induced by LPS, rather, IL-6 levels were decreased with exposure to 10 and 25 μg/mL of magnetite ($p < 0.001$).

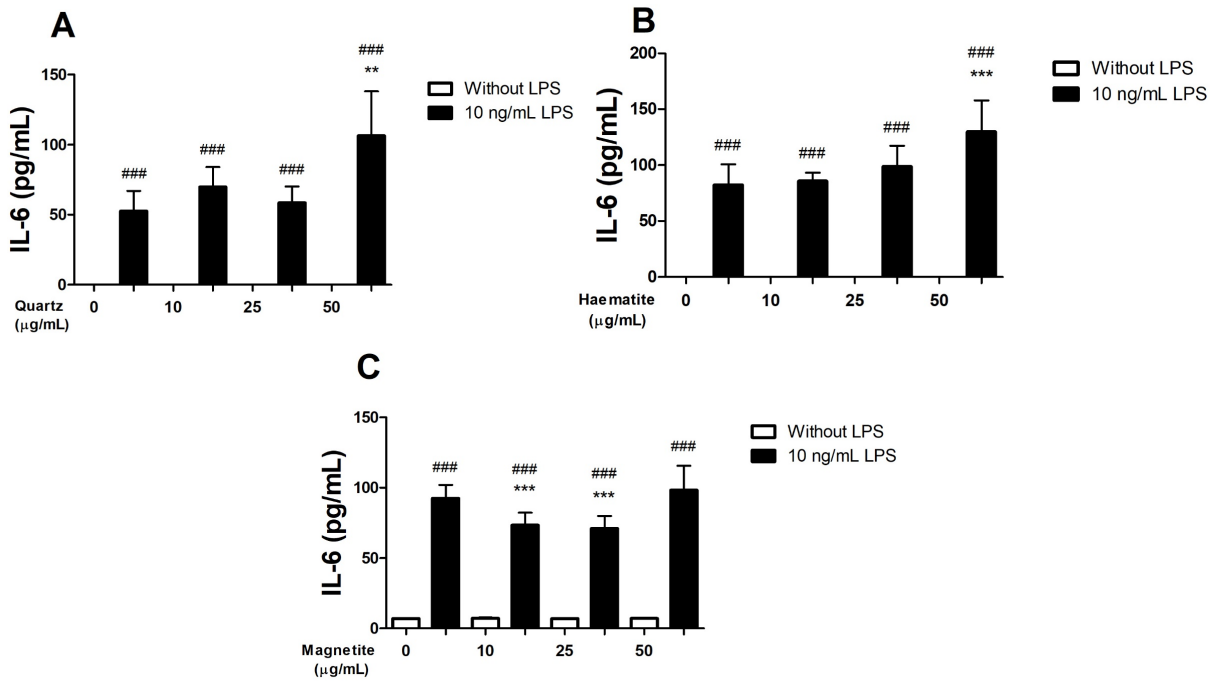


Figure 3.3: Interleukin-6 (IL-6) levels in the supernatant of THP-1 derived macrophages exposed to quartz (A), haematite (B) or magnetite (C) for 24 hours and after 4 hours of LPS exposure. Data are presented as mean(SD) from 6 independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$ respectively versus control. ### indicates $p < 0.001$ for LPS vs no LPS at the given dose.

Interleukin-8

Quartz induced a non-dose dependent increase in IL-8 protein expression (Fig 3.4A), with increased IL-8 production at 10 μg/mL ($p = 0.004$) and no further increase at higher particle doses ($p > 0.05$). LPS caused a profound effect on IL-8 production (Fig 3.4A, $p < 0.001$ for all doses) that was further enhanced by exposure to 10 μg/mL of quartz ($p = 0.004$) but did not increase further with higher doses of particles ($p > 0.75$). In contrast, haematite induced a dose-dependent increase in IL-8 with an increase in response to 25 μg/mL (Fig 3.4B, $p = 0.002$), but

no further increase at 50 µg/mL (p = 0.562). Exposure to haematite particles enhanced LPS induced IL-8 production resulting in a similar dose-dependent response to that observed without LPS; albeit at a higher overall level of IL-8 (Fig 3.4B). The same pattern was observed in response to magnetite, although the increase in IL-8 with (p = 0.002) and without (p = 0.002) was only observed at the 50 µg/mL particle dose (Fig 3.4C).

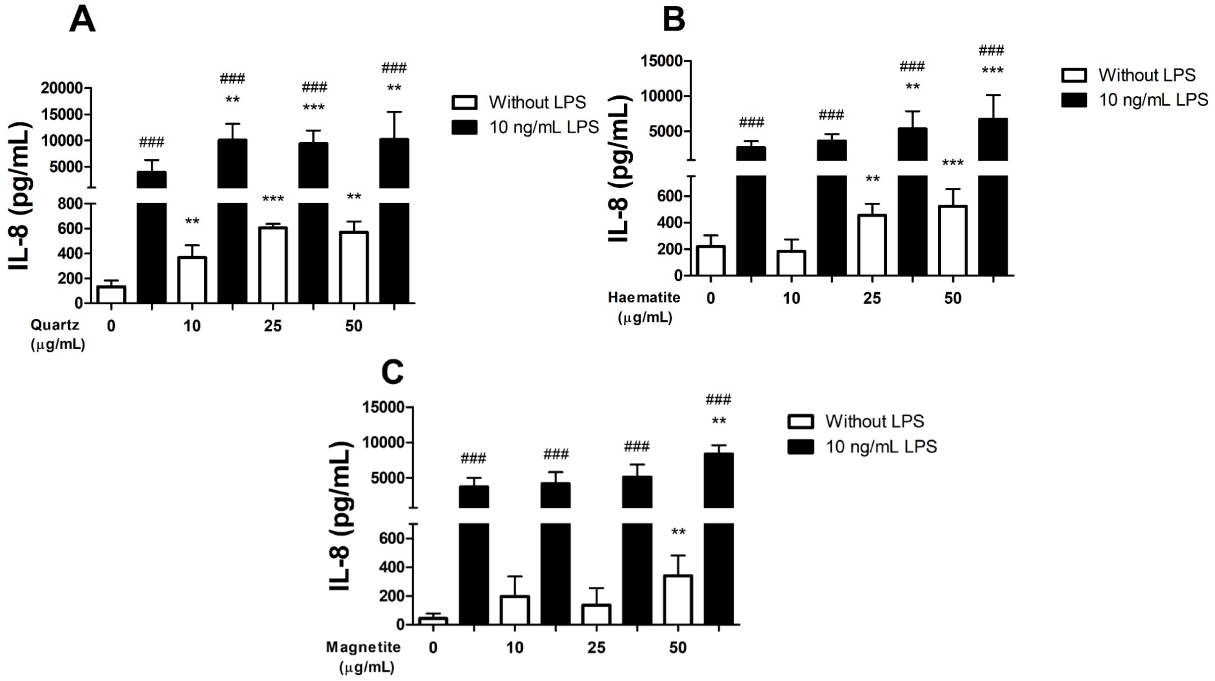


Figure 3.4: Interleukin-8 (IL-8) levels in the supernatant of THP-1 derived macrophages exposed to quartz (A), haematite (B) or magnetite (C) for 24 hours and after 4 hours of LPS exposure. Data are presented as mean(SD) from 6 independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$ respectively versus control. ### indicates $p < 0.001$ respectively for LPS vs no LPS at the given dose.

Tumour Necrosis Factor-α

Tumour Necrosis Factor-α (TNF-α) expression was not altered in response to quartz (Fig 3.5A), haematite (Fig 3.5B) or magnetite (Fig 3.5C) ($p > 0.05$ for all doses). LPS exposure increased the production of TNF-α which was enhanced further by exposure to 25 ($p = 0.010$) and 50 μg/mL ($p < 0.001$) of quartz particles (Fig 3.5A). In contrast, haematite ($p > 0.09$) and magnetite ($p = 0.146$) did not modify the LPS induced increase in TNF-α production (Fig 3.5B & 3.5C).

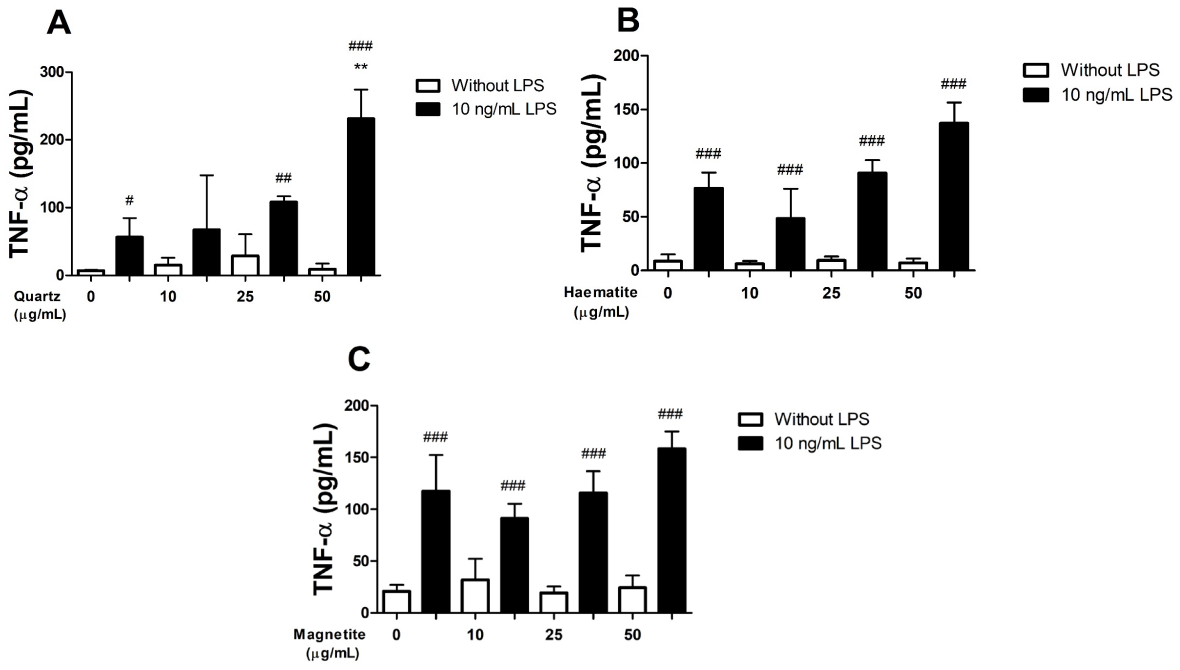


Figure 3.5: Tumour necrosis factor-α (TNF-α) levels in the supernatant of THP-1 derived macrophages exposed to quartz (A), haematite (B) or magnetite (C) for 24 hours and after 4 hours of LPS exposure. #, ## and ### indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively for LPS vs no LPS at the given dose.

3.4.2 Combined effect of silica and iron oxide

Having established the effect of iron oxide, and silica, particles alone on the response, we also determined whether iron oxide modifies the silica response. The outcomes of these experiments are detailed below.

Cytotoxicity

Regardless of prior LPS stimulation, neither the quartz-haematite ($p > 0.05$) nor quartz-magnetite ($p > 0.05$) combinations elicited a detectable increase in LDH levels (Fig 3.6).

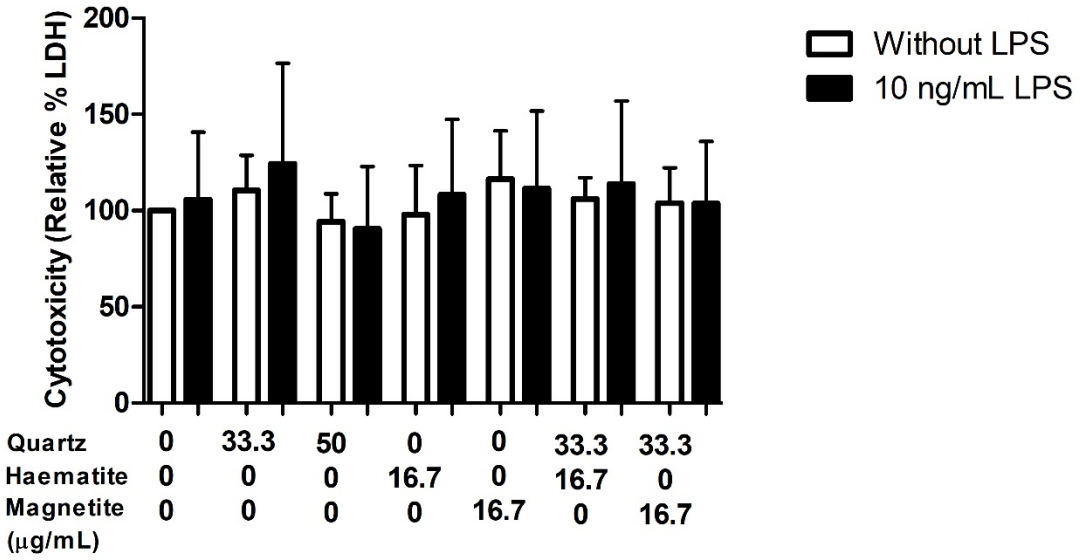


Figure 3.6: Lactate dehydrogenase (LDH) levels in the supernatant of THP-1 derived macrophages with or without prior 4-hour exposure to LPS. Cells were exposed to quartz,

haematite, magnetite or a combination of quartz and iron oxide for 24 hours. Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented as mean(SD) from 6 independent experiments.

Cytokines

The addition of haematite (IL-1β, p = 0.910; IL-8, p = 0.998) and magnetite (IL-1β, p = 0.367; IL-8, p = 0.926) had no effect on the quartz-LPS response (Fig 3.7A & Fig 3.7B).

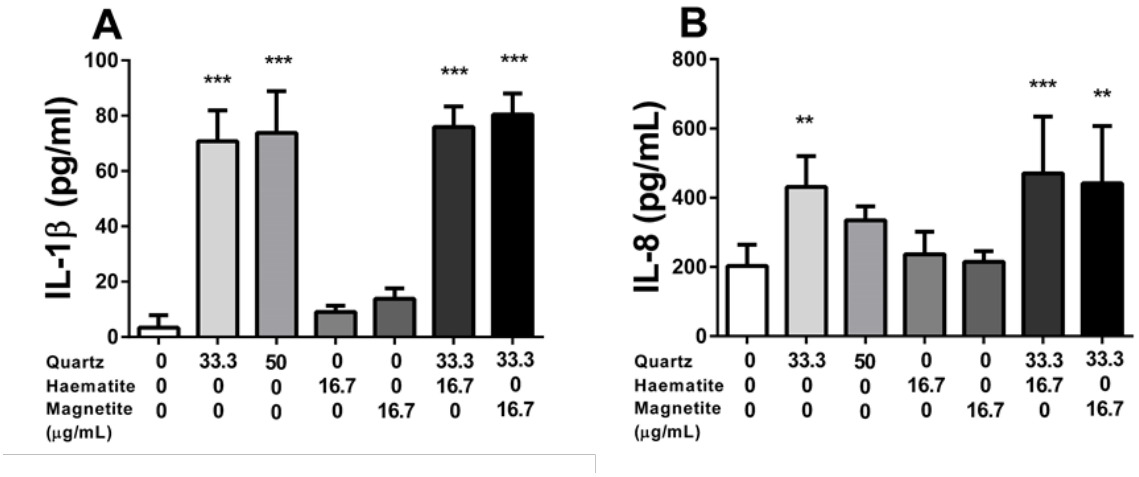


Figure 3.7: Interleukin (IL)- 1β (A) and IL-8 (B) with prior 4 hours L{S exposure, levels in the supernatant of THP-1 derived macrophages. Cells were exposed to quartz, haematite, magnetite or a combination of quartz and iron oxide for 24 hours. Data are presented as mean(SD) from 6 independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$ respectively versus control.

3.5 Discussion

The present study aimed to investigate the effect of iron oxide, with or without prior LPS exposure, on inflammation in macrophages, as a potential contributor to the inflammatory response induced by geogenic particles *in vivo* and the detrimental health outcomes observed in individuals exposed to iron-laden geogenic PM [16,142]. Iron oxide particles clearly modified the effect of LPS on the macrophages which varied between particle types and across cytokines. In particular, while haematite and magnetite had no effect on IL-1β production, they both enhanced LPS induced IL-1β production. Similarly, quartz and haematite, but not magnetite, enhanced LPS induced IL-6 production while all three particle types enhance LPS induced IL-8. Thus, the health implications of inhaled geogenic particles will vary depending on the chemical composition (e.g. quartz vs haematite vs magnetite) and whether there is a prior inflammatory stimulus present (e.g. infection).

LPS is a component of the outer membrane of Gram-negative bacteria and is central to mediating the inflammatory response via binding to specific host receptors [276]. LPS binds to Toll-like Receptor (TLR)-4 [277], leading to the upregulation of NF-κB, and enhanced transcription of a range of pro-inflammatory genes including *IL-1β*, *IL-6*, *TNF-α* and *IL-8* [278-280]. Accordingly, we found that LPS upregulated the expression of all the inflammatory cytokines we measured. The most overt difference between the effect of silica and iron oxide exposures we observed, was the release of IL-1β by quartz in absence of LPS. This effect of silica is consistent with previous studies and is likely to be driven by NF-κB-independent NOD-like receptor pyrin domain-containing 3 (NLRP-3) inflammasome activation [281,214,216,215]. The absence of an effect of iron oxide on the IL-1β production suggests

that iron oxide is unable to activate this pathway. While iron oxide nanoparticles can induce some level of IL-1β, due to partial NLRP-3 activation, this is highly dependent on the shape of the particles [282], whereby spherical iron oxide particles, which have a similar morphology to the particles used in the present study [271], caused a minimal response in this pathway. The particles used in the present study were all in the < 5μm size range [271] which is consistent with the particle size observed in community samples geogenic PM [142].

IL-8 is a neutrophil chemoattractant protein which can also be upregulated via NF-κB in response to LPS [283]. Our data showed that, in the absence of LPS, IL-8 was produced in response to all particle types. In epithelial cells, silica induces IL-8 through endocytosis-independent p38/AP-1 and ERK1/2/CEBP upregulation [284,285,217,286,151]. While the mechanism is unclear from our experiments, given the similarities between the IL-8 responses, it is possible that iron oxide particles may also activate the AP-1/CEBP pathway. This warrants further investigation.

IL-6 differs from the other cytokines discussed as it acts as both as an anti-inflammatory mediator and macrophage activator [278]. IL-6 synthesis is initiated via multiple pathways including upregulation of NF-κB, CEBP and IL-1β [287-294]. As expected, we observe a strong LPS induced response. We also observe a modifying effect of the particles. Interestingly, unlike both quartz and haematite, magnetite had a moderate suppressive effect on LPS-induced IL-6 at lower doses. As previously mentioned, IL-6 plays an anti-inflammatory role in the lung, supressing over-expression of TLRs, including TLR-4 [68] and that magnetite may have the capacity to prevent this response. We saw no effect of any particle on IL-6 production in the

absence of LPS, which is surprising given the increase in IL-1β production. Taken together, these observations suggest the regulation of IL-6 production in response to particles, and the modifying effect of these particles on the LPS response, is complex [295].

TNF-α induces a wide range of effects on macrophages and is proposed as a central player in inflammatory cell activation and recruitment with a critical role in the development of many chronic inflammatory diseases [296]. Consistent with previous studies [297,298], LPS increases TNF-α production, however, we observed no effect of any particles, without LPS, on TNF-α production. Unlike silica, the iron oxide particles did not modify the production of TNF-α.

Existing literature suggests silica modifies TLR-4 receptor function [219]. In our study, in the absence of LPS, quartz induced an IL-1β and IL-8 response, but not IL-6 or TNF-α. Iron oxide did not induce any cytokine except for IL-8. Thus, we hypothesise that quartz, haematite and magnetite induce NF-κB independent cytokine release, with only quartz able to induce NLRP-3 activation. The particle modifying effects on LPS exposure aligns with our suggestion that LPS induces aggressive NF-κB activation, with particles stimulating alternative pathways, resulting in a synergistic effect on cytokine production.

A secondary aim of this study was to determine whether iron oxide modifies the silica induced response. Silica and iron oxide were combined in a 2:1 ratio, representing the ratio commonly found in “real-world” particle samples [142,16]. Our data demonstrates that the addition of

either haematite, or magnetite, did not significantly augment the level of cytotoxicity or cytokine production induced by quartz. Prior stimulation of macrophages with LPS did not alter this response. Collectively, these data suggest that iron oxide increases IL-8 production in macrophages, which is consistent with the observed association between the iron content of geogenic PM and the magnitude of the neutrophil driven inflammatory response *in vivo* [16].

Limitations of the present study must be acknowledged. While we have investigated the effects and interactions of the two major components of geogenic PM on the cell response, silica and iron oxide do not fully represent geogenic PM in its entirety, nor do they fully represent the vast heterogeneity of naturally occurring geogenic PM. However, we chose to focus on silica and iron oxide as these are the two constituents that have shown the most robust correlation with negative health outcomes in previous studies [142,16]. Secondly, it is difficult to draw mechanistic conclusions from the present study as we focussed on the production of cytokines rather than cell signalling. For example, we did not quantify the phagocytic response to the particles by the macrophages. Given the potential impact of LPS on macrophage activation, this limits our capacity to comment on one of the key mechanisms that may underpin the enhanced inflammatory response to the particles with prior LPS exposure. Future work may focus on the macrophage response to other constituents of geogenic PM and the cellular mechanisms driving the variable response we observed.

The production of inflammatory mediators such as TNF-α, IL-1β and IL-8 are associated with the early progression of chronic diseases [185]. Lay *et al.* (2001) suggested that inhalation of iron oxide particles by healthy subjects led to no appreciable change in alveolar epithelial

permeability, diffusion capacity or pulmonary function [179]. However, inflammation was not measured, and it is unlikely a clinical response will become evident over the 24-hour period measured. We conclude that iron oxide contributes to the inflammatory response seen *in* vivo [142,16], partly by causing increased production of IL-8 production and cytotoxicity in macrophages. While iron oxide did not augment the inflammatory response induced by silica, it had a substantive impact on the response to LPS. Thus, iron oxide is likely to play a role in the association between exposure to geogenic PM and increase hospitalisations for respiratory infection. This has implications for the health of communities that are primarily exposed to non-combustion derived particulate matter.

Chapter 4: Inorganic Particulate Matter Modulates Non-Typeable Haemophilus Influenzae Growth: A Link Between Chronic Bacterial Infection and Geogenic Particles.

A modified version of this chapter has been submitted for publication in *Environmental Geochemistry and Health*:

Williams, L. J., Tristram, S. & Zosky, G. R. (**submitted**) Inorganic Particulate Matter Modulates Non-Typeable Haemophilus Influenzae Growth.

The previous Chapter (Chapter 3) assessed the inflammatory potential of silica and iron oxide on macrophages and whether these particles altered the response to a bacterial stimulus. This Chapter aimed to assess whether environmentally relevant forms of iron oxide promote NTHi growth. The text of this Chapter is presented as submitted to the Journal for publication.

4.1 Abstract

Australian Aboriginal populations have unacceptably high rates of bronchiectasis. This disease burden is associated with high rates of detection of pathogenic bacteria; particularly Non-typeable *Haemophilus influenzae* (NTHi). While there is evidence to suggest that exposure to inhalable inorganic particulate matter (less than 10 μm in diameter; PM_{10}) is associated with worse respiratory infections, no studies have considered the direct effect of this PM on bacterial growth. Nine clinical isolates of pathogenic NTHi were used for this study. Isolates were exposed to two common iron oxides, haematite (Fe_2O_3) or magnetite (Fe_3O_4), or quartz (SiO_2), as the main constituents of environmental inorganic PM. NTHi isolates were exposed to PM with varying levels of heme to identify whether the response to PM was altered by iron availability. The maximal rate of growth and maximum supported growth were assessed. We observed that inorganic PM was able to modify the maximal growth of selected NTHi isolates. Magnetite and quartz were able to increase maximal growth while haematite could both increase and suppress the maximal growth. However, these effects varied depending on iron availability and on the bacterial isolate. We found that inorganic PM may directly alter the growth of pathogenic NTHi. This observation may partly explain the link between exposure to high levels of crustal PM and chronic bacterial infection in Australian Aboriginals.

4.2 Introduction

Bronchiectasis is a chronic lung disease characterised by an irreversible dilation of the small airways which causes significant morbidity and reduced life-expectancy [18]. Urban air pollution is known to increase the risk of bronchiectasis exacerbations [299], with additional evidence supporting the negative outcomes of traffic-derived pollutants on patient mortality [300]. Interestingly, Australian Aboriginal people, often located in rural communities, experience a disproportionate burden of bronchiectasis, almost three times higher than the non-Aboriginal population [8]. Furthermore, Aboriginal children suffer from one of the highest prevalence's of non-cystic fibrosis bronchiectasis in the world [11,301]. While there are a range of factors associated with social disadvantage that may be contributing to this unacceptably high burden of disease [48,49,39,47,46,45,301], emerging evidence suggests environmental factors also contribute [15]. For example, geogenic (earth-derived) particulates are abundant in remote Aboriginal communities [15,47] and are now correlated with the prevalence of bronchiectasis in these communities [14].

Bronchiectasis typically develops as a result of uncontrolled, chronic bacterial infection in the airways [6]. Almost 90% of Aboriginal people with bronchiectasis return a positive result for the presence of pathogenic bacteria in sputum samples, predominately Non-typeable *Haemophilus influenzae* (NTHi) [8,42-44]. There is a growing body of evidence that inorganic particulates can contribute to the risk and severity of bacterial infections in the lung. For example, epidemiological studies demonstrate that occupational exposure to mineral dusts increases the risk of bacterial pneumonia [302,303]. In line with this, populations exposed to wind-blown dust from iron ore stockpiles have greater rates of hospitalisation for respiratory

conditions [270,140], particularly respiratory infections [141]. Inhalable particulate matter (PM) from a range of sources has been shown to modify the response to NTHi *in vitro* and *in vivo* [304-306]. However, these studies have primarily focussed on the host response to NTHi and have not considered the direct effect of PM on bacterial growth.

There is some evidence to suggest that iron nanoparticles can directly influence bacterial growth, however, the response varies considerably depending on the type of bacteria and physico-chemical characteristics of the particles used [307,308]. To date, no studies have characterised the response of NTHi to particulates in the coarse fraction respirable PM (1-10µm in diameter). As a result, the contribution of the direct effects of inorganic PM inhalation to NTHi infection are yet to be determined. The geogenic PM affecting Aboriginal children with high rates of NTHi infections dominated by high levels of silica (quartz; SiO_2) and iron oxides (haematite; Fe_2O_3 & magnetite; Fe_2O_3) [142,16]. Therefore, the aim of this study was to assess the effect of these particles on NTHi growth.

4.3 Methods

4.3.1 Particle preparation

Commercially available standard preparations of dry magnetite (Fe_3O_4; Sigma-Aldrich 310069), haematite (Fe_2O_3; Sigma-Aldrich 310050) and α-quartz (SiO_2; NIST 1878B) were used for bacterial growth experiments. NTHi responses to these particles were compared to particle-free bacterial growth. Particle samples were exposed to UV light for two hours prior to experimentation to remove any existing bacterial or endotoxin contamination.

4.3.2 Bacterial isolates

We used 4 clinical NTHi isolates from the lower respiratory tract, 3 from ear swabs or effusions and 2 from upper respiratory swabs of the oropharynx. The isolates were identified by the original laboratories where the isolates were collected as *H. influenzae*, without knowledge of this study, based on colonial morphology and X + V factor dependence, and assigned clinical significance based on criteria that involved relevant clinical history, dominant growth on primary culture and supporting microscopy of the clinical specimens. All isolates were retrospectively identified in this study as *H. influenzae* using a polymerase chain reaction (PCR) algorithm for key species marker genes including *fucK*, *hpd*, and sodC as previously described [309]. Isolates were chosen to span secondary characteristics including invasion rate of bronchial epithelial cells (Table 4.1).

Table 4.1: *Non-typeable Haemophilus influenzae* characteristics, including site of isolation and invasion rate of bronchial epithelial cells.

Identifier	Site of Isolation	% Invasion of bronchial epithelial cells (BEAS-2B) Mean (SD)
Ci8	Sputum	Moderate- 14.79 (9.11)
Ci16	Sputum	Low- 0.47 (0.15)
Ci34	Sputum	Low- 0.01 (0.01)
Ci43	Sputum	Low- 1.84 (0.17)
Ci37	Ear	Low- 0.01 (0.00)
L267	Ear	High- 36.69 (17.61)
L341	Ear	Moderate- 15.25 (6.25)
NF3	Oropharynx	Low- 2.59 (1.28)
L227	Oropharynx	High- 33.90 (6.31)

4.3.3 Conditions, exposures & growth curves

To quantitatively analyse bacterial growth, growth curves of each isolate were compared in two ways. Firstly, we assessed differences in maximum rate of growth. These data were generated from the highest rate of exponential growth observed for each isolate under each heme condition and particle exposure. Secondly, we assessed for the maximum supported

population, represented as the highest density of bacteria at any timepoint across the assessment period.

NTHi has a dependence on heme availability [310]. However, the strength of this dependency may vary between isolates. Initially, the effect of heme status on rate of growth was compared between replete (15 μg/mL heme), limited (2 μg/mL heme) and deplete (0 μg/mL heme) heme conditions in the absence of particles.

During routine culture, NTHi was grown under optimal conditions on Chocolate agar or in Brain Heart Infusion (BHI; Oxoid, UK) broth supplemented with Vitox and Haemophilus Test Media (HTM; Oxoid, UK). Under each heme condition, isolates were exposed to 0 or 50 μg/mL of particles (SiO_2, Fe_2O_3 or Fe_3O_4). Exit cultures were utilised to confirm the absence of bacterial contamination.

Bacteria were grown at 37ºC on an orbital shaker (220 rpm) in atmospheric CO_2. Absorbance (optical density; OD) of the cultures was measured at 600 nm on the BioPhotomer spectrometer (Eppendorf, Germany), hourly for 14 hours (Fig 4.1). This was sufficient to observe a complete growth curve for each isolate. To account for the colour pigment of the particles, standard curves were formulated for each isolate under each particulate exposure condition. The optical density measured by absorbance was then transformed into colony forming units (CFU)/mL by removing the colorimetric effects of the particles by comparison of OD_{600} to CFU/mL standard curves determined for each particulate and bacterial isolate.

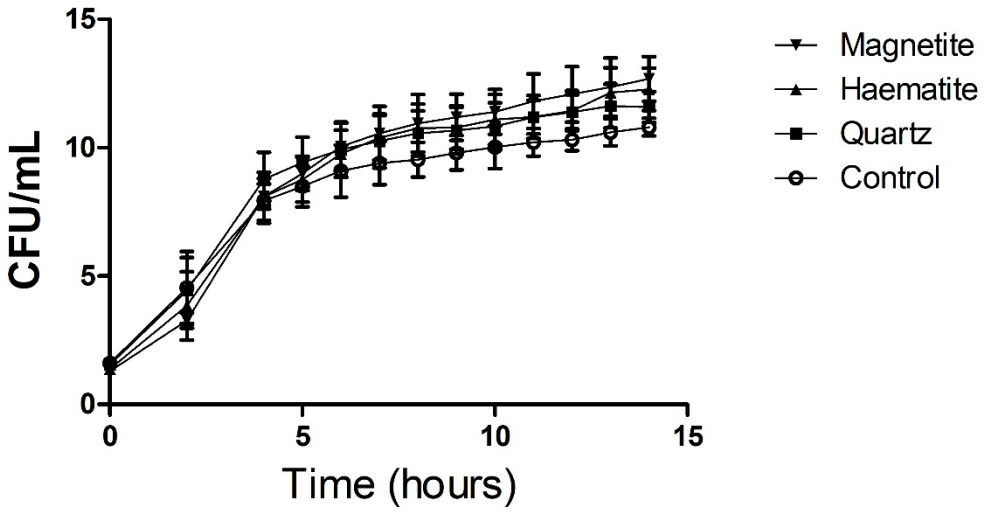


Figure 4.1: Example growth curve of isolate Ci34 under limited heme and in response to particulates. Growth is displayed as colony forming units/mL (CFU/mL) over 14 hours of measured growth. Magnetite and haematite both increased maximal growth compared to control ($p < 0.001$). Data are presented as mean(SD) from 6 independent experiments with * indicating $p < 0.05$.

4.3.4 Statistical analysis

Comparisons between groups were made using two-way repeated measures ANOVA. When significance was determined for the main factors by ANOVA, a Holm-Sidak post-hoc test was used to examine individual between group differences. Where necessary, the data were log transformed to satisfy the assumptions of normal distribution of the error terms and homoscedasticity of the variance. All data are presented as mean (SD) and values of $p < 0.05$ were considered statistically significant. All statistical analyses were conducted using SigmaPlot (v12.5).

4.4 Results

4.4.1 The effect of heme status

Rate of growth (generation time)

When compared to replete heme conditions, isolates Ci8 ($p = 0.010$), Ci43 ($p < 0.001$) and L227 ($p < 0.001$) grew slower under deplete heme. Interestingly, heme availability did not alter rate of growth in isolates NF3 ($p = 0.067$), Ci16 ($p = 0.148$), Ci34 ($p = 0.282$), Ci37 ($p = 0.161$), L267 ($p = 0.271$) or L341 ($p = 0.236$).

Of the heme-dependent strains, isolate Ci43 exhibited an intermediate level of growth under limited heme conditions when compared to growth under replete ($p < 0.001$) and deplete heme conditions ($p = 0.011$). In contrast, while there was a difference between replete and deplete conditions in the Ci8 and L227 isolates, the rate of growth was not modified by limited heme (Ci8, $p = 0.239$; L227, $p = 0.142$).

Maximum growth

When compared to replete heme conditions, heme depletion reduced maximal growth in the Ci8 ($p = 0.010$), Ci16 (Fig 4.2A; $p = 0.007$), Ci43 ($p < 0.001$), L227 ($p < 0.001$) and L267 (Fig 4.2B; $p = 0.007$) isolates. We observed no difference between replete and heme deplete conditions in maximal growth for the Ci34 ($p = 0.121$), Ci37 ($p = 0.053$), NF3 ($p = 0.067$) and L341 ($p = 0.297$) isolates. Limited heme resulted in significantly reduced growth compared to replete conditions for the Ci37 ($p = 0.046$), Ci43 ($p < 0.001$), L227 ($p < 0.001$) and L267 (Fig

4.2B; p = 0.008) isolated, but not for Ci8 (p = 0.138), Ci16 (Fig 4.2A; p = 0.150), Ci34 (p = 0.430), NF3 (p = 0.069) or L341 (p = 0.297). The main effects of heme availability on the rate of and maximum NTHi growth are summarised in Table 4.2.

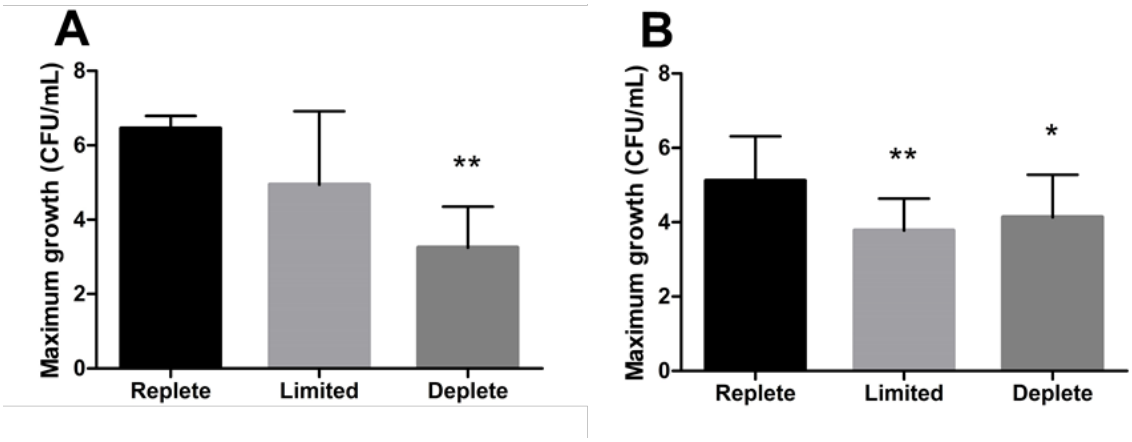


Figure 4.2: Maximum growth of isolates Ci16 (A) and L267 (B) in response to varying levels of heme. Deplete heme resulted in significantly decreased maximum growth (CFU/mL) when compared to replete heme for isolate Ci16 (A), but not isolate L267 (B). Data are shown as absolute CFU/ml values. Data are presented as mean(SD) from 6 independent experiments with * and ** indicating $p < 0.05$ and $p < 0.01$ respectively.

Table 4.2: The response of NTHi isolates to limited and deplete heme availability when compared to the response of NTHi in replete heme conditions.

Bacterial Characteristics			**Rate of Growth**		**Maximum Growth**	
Isolate	**Origin**	**Invasiveness**	**Limited**	**Deplete**	**Limited**	**Deplete**
NF3	Oropharynx	Low	-	-	-	-
Ci8	Sputum	Moderate	-	Decrease	-	Decrease
Ci16	Sputum	Low	-	-	-	Decrease
Ci34	Sputum	Low	-	-	-	-
Ci37	Ear	Low	-	-	Decrease	-
Ci43	Sputum	Low	Decrease	Decrease	Decrease	Decrease
L227	Oropharynx	High	-	Decrease	Decrease	Decrease
L267	Ear	High	-	-	Decrease	Decrease
L341	Ear	Moderate	-	-	-	-

4.4.2 The effects of particle exposure

Rate of Growth (generation time)

Rate of growth was not altered by particle exposure for the NF3 ($p = 0.227$), Ci8 ($p = 0.400$), Ci16 ($p = 0.420$), Ci43 ($p = 0.511$), L227 ($p = 0.132$), L267 ($p = 0.336$) or L341 ($p = 0.163$) isolates. Iron oxide altered the rate of growth for isolates Ci34 and Ci37. Magnetite exposure increased rate of growth of Ci34 ($p = 0.011$). Under replete heme, the Ci37 isolate demonstrated significantly slower growth when exposed to magnetite ($p = 0.004$) or haematite ($p = 0.003$).

In contrast, under deplete conditions magnetite increased rate of growth for the same isolate ($p < 0.001$). Quartz had no effect on rate of growth of any isolate ($p > 0.05$ for all comparisons).

Maximum Growth

There was no effect of particulates on maximum growth of the NF3 ($p = 0.227$), Ci8 ($p = 0.471$), Ci16 ($p = 0.064$), L227 ($p = 0.052$) or L267 ($p = 0.164$) isolates. Quartz increased the maximal growth of L341, regardless of heme status ($p < 0.001$). Magnetite increased growth of isolate Ci34 under both the limited (Fig 4.3; $p < 0.001$) and deplete heme conditions (Fig 4.3; $p = 0.001$) and the Ci37 isolate with deplete heme ($p = 0.046$). Regardless of heme, magnetite increased the maximum growth of Ci43 ($p < 0.001$). In comparison, haematite increased maximal growth of the Ci34 isolate under limited (Fig 4.3; $p < 0.001$) and deplete heme (Fig 4.3; $p = 0.004$) but suppressed the growth of L341 ($p = 0.014$). The main effects of particle exposures on the rate of and maximum NTHi growth are summarised in Table 4.3.

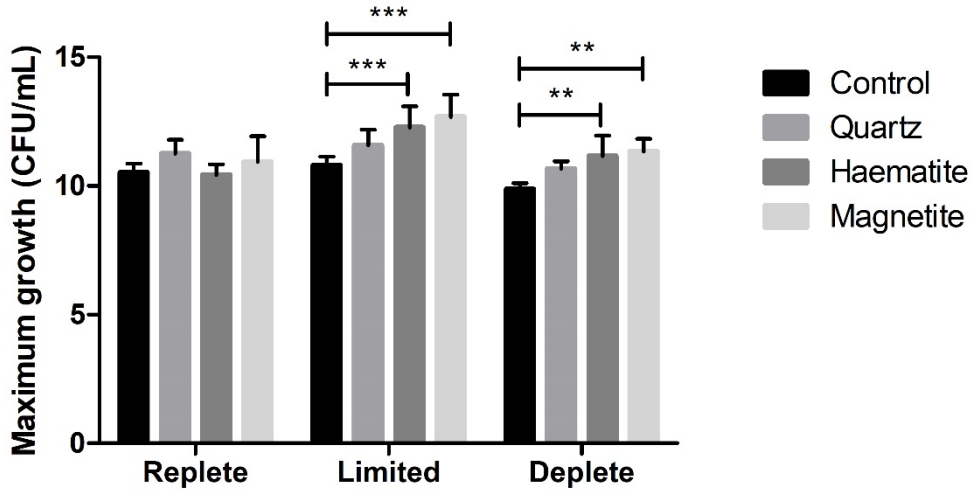


Figure 4.3: Maximum growth of isolate Ci34 in response to replete, limited and deplete heme availability as well as particulate exposure. Magnetite significantly increased growth compared to control under limited ($p < 0.001$) and deplete ($p = 0.001$) heme. Haematite increased growth under limited ($p < 0.001$) and deplete ($p = 0.004$) heme. Data are presented as mean(SD) from 6 independent experiments with *, ** and *** indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$ respevtively.

Table 4.3: The response of NTHi isolates to quartz, haematite of magnetite particulates. The responses listed are within replete heme availability unless otherwise specified.

Bacterial Characteristics			**Rate of Growth**			**Maximum Growth**		
Isolate	**Origin**	**Invasiveness**	**Quartz**	**Haematite**	**Magnetite**	**Quartz**	**Haematite**	**Magnetite**
NF3	Oropharynx	Low	-	-	-	-	-	-
Ci8	Sputum	Moderate	-	-	-	-	-	-
Ci16	Sputum	Low	-	-	-	-	-	-
Ci34	Sputum	Low	-	-	Increase	-	Increase (limited & deplete heme)	Increase (limited & deplete heme)
Ci37	Ear	Low	-	Decrease	Decrease (replete heme) and increase (limited heme)	-	-	Increase (deplete heme)
Ci43	Sputum	Low	-	-	-			Increase
L227	Oropharynx	High	-	-	-	-	-	-
L267	Ear	High	-	-	-	-	-	-
L341	Ear	Moderate	-	-	-	Increase	Decrease	-

4.5 Discussion

Remote Australian Aboriginal communities are exposed to high levels of inorganic particulate matter [14], dominated by silica and iron oxides [16], and have a disproportionate burden of chronic respiratory infections [8]. The present study aimed to identify whether silica or iron oxide PM can directly modify the growth of NTHi as one of the dominant forms of pathogenic

respiratory bacteria in these communities. We observed that inorganic PM was able to modify the maximal growth of selected NTHi isolates with the effect varying depending on iron availability. Interestingly, the effects were dependent on the bacterial isolate.

Our study highlights the heterogeneous nature of the bacterial response, even within a single species. Initial testing sought to identify the sensitivity of the isolate to heme. Two isolates were unaffected by heme availability, demonstrating uninhibited growth in the absence of heme. Given the complete dependence of NTHi on heme, this observation is most likely due to the ability of the organism to store heme during pre-exposure growth. While most of the isolates demonstrated diminished growth in response to lowering heme concentrations as expected [311,312], it clearly demonstrates the adaptive ability of human pathogens to operate under extremely low iron conditions [313-315].

The effects of PM on NTHi growth were heterogenous. While the mechanism is unclear, it is possible that bacterial sources of iron can be altered by environmental factors. Importantly, magnetite retains surface reactivity and protein binding affinity [316,317]. In this context, magnetite reduction is optimal at pH 5-6 and 22-37°C, which is consistent with our bacterial culture parameters and most biological enzymatic processes [318]. Magnetite ($Fe^{2+}Fe_2^{3+}O_4^-$) can undergo reduction to form Fe^{2+}, H_2O and HCO_3^- molecules [318]. Interestingly, this reduction process is suppressed in the presence of oxygen meaning this particulate would be relatively inert in the lung. We propose that partial reduction of magnetite under the conditions we used led to a moderate increase in Fe^{2+} which promoted bacterial growth. NTHi can utilise a range of heme containing proteins [319]. However, none of these include free Fe^{2+}, with even

relatively low levels of free iron being highly toxic. Thus, it is possible that the small increase in available Fe^{2+} from partial magnetite dissociation resulted in advantageous bacterial growth conditions by saturation of iron-containing proteins such as transferrin or ferritin.

In comparison, quartz is coated in oxygen-containing surface functional groups. Given their chemical nature, these silanol functional groups retain a high affinity for Fe^{3+} [320-322]. Despite varying chemically, haematite also possesses the ability for Fe^{2+} sorption [323,324]. It is unclear what effect this may have on the bioavailable iron. However, this may explain why we observe less consistent, and sometimes reduced growth, in the presence of these particulates, in contrast to the effect of magnetite. Thus, the effects of haematite are somewhat unexpected as the particle can undergo reduction and is often used for iron fertilization in water ecosystems to boost microbial growth [325]. However, this may reflect the specific condition of the *in vitro* environment we used.

We must acknowledge that the present study has some limitations. In particular, we have not established a mechanism by which the PM altered growth. Secondly, while we have attempted to cover a range of different isolates, we were not able to attribute variation in response to any specific characteristic of bacterial behaviour. Despite these limitations the present study demonstrates that pathogenic strains of NTHi can be directly influenced by inorganic PM. Future research should focus on linking the responses we observed with the host cell response and probe the possible mechanisms linked to the variable growth as a result of PM exposure.

NTHi infection leads to several diseases of the ear, upper and lower airways [13] and is associated with irreversible chronic respiratory diseases such as bronchiectasis [8] in vulnerable populations. Our data are the first to provide evidence that inorganic, environmentally relevant, PM matter can aid bacterial growth. Our work implies that reducing exposure to PM from geogenic sources may improve respiratory health in at risk communities.

Chapter 5: NTHi Invasion of Lung Cells Exposed to Inorganic PM

In Chapters 2 and 3 the pro-inflammatory potential of iron oxide and silica particles on epithelial cells and macrophages was determined. In these Chapters it was demonstrated that iron oxide caused inflammation in epithelial cells and macrophages and that the particles exacerbated the effect of LPS on macrophages. Furthermore, as outlined in Chapter 4, these particles can directly promote bacterial growth. Together, these observations suggest that there may be a simultaneous negative impact on the host response and a positive impact on the bacteria which may result in more severe infection. This Chapter aimed to assess whether environmentally relevant forms of iron oxide can alter NTHi invasion of lung cells and or phagocytosis by macrophages. This work is presented as a traditional Thesis Chapter.

5.1 Abstract

Post-infective bronchiectasis is particularly prevalent in communities exposed to high levels of geogenic (earth-derived) particulate matter (PM). In these communities, persistent Non-typeable *Haemophilus influenzae* (NTHi) infection is shown to be the predominant pathogen linked to disease. Experimental studies have shown that geogenic PM increases the invasiveness of NTHi, however the effects of silica, haematite and magnetite are not clear. The aim of this Chapter was to assess the effect of these particles on NTHi cell invasion in bronchial and epithelial cells and phagocytosis by macrophages. Alveolar (A549), bronchial epithelial cells (BEAS-2B) and macrophages (THP-1 & primary) were exposed to increasing concentrations of particles and assessed for the level of invasion/phagocytosis by multiple strains of NTHi. BEAS-2B cells were further assessed for particle uptake, beta-defensin-1 and -2 expression, TLR-2 and -4 expression, and production of inflammatory cytokines (tumour necrosis factor-α, interleukin (IL)-1β, IL-6 and IL-8). Genomic analysis of the NTHi strains was conducted to identify pathways that may be involved in cell invasion. Haematite significantly increased the rates of NTHi invasion in bronchial epithelial cells while both iron oxides decrease phagocytosis of NTHi by macrophages. While all particles influenced beta-defensin expression and cytokine production, haematite had the highest levels of endocytosis in bronchial epithelial cells and increased the expression TLR-4 in response to LPS. Genomic association analysis highlighted the complex association between bacterial genomics and particle-induced cell invasion, however, no firm conclusion could be drawn from this analysis. Taken together, these data suggest that haematite can increase bronchial epithelial cell invasion by NTHi which may be linked to the high levels of endocytosis of these particles. This suggests that one explanation for the high levels of bronchiectasis in remote Australian Aboriginal

communities is enhanced bacterial cell invasion leading to persistent infection as a result of inhalation of iron laden particulate matter.

5.2 Introduction

In Chapters 2 and 3 the pro-inflammatory potential of iron oxide and silica particles on epithelial cells and macrophages was determined. In these Chapters it was demonstrated that iron oxide caused inflammation in epithelial cells and macrophages and that the particles exacerbated the effect of LPS on macrophages. Furthermore, as outlined in Chapter 4, these particles can directly promote bacterial growth. Together, these observations suggest that there may be a simultaneous negative impact on the host response and a positive impact on the bacteria which may result in more severe infection. However, it remains unclear how quartz and iron oxide influence the response of these cells to bacterial invasion.

Geogenic PM has previously been shown to increase the invasiveness of Non-typeable *Haemophilus influenzae* (NTHi) in bronchial epithelial cells [304]. However, this study did not directly assess the effect of silica or different forms of iron oxide on the response, nor did it assess variability in the response between NTHi strains. The ability of NTHi to invade allows the bacteria to evade the host immune response [326,327,96,328]. Thus, it is a pivotal route of virulence for pathogenic NTHi and needs to be comprehensively assessed in the context of the effect of geogenic PM on bacterial infection.

In the lung, airway epithelial cells and alveolar macrophages are key components of the host innate immune response [329,330,206,226]. As the initial point of contact for inhaled particles, epithelial cells mediate cell signalling and inflammatory cascades induced by innate immune cells such as macrophages [88]. These functions are often tailored to recognition and

elimination of pathogenic bacteria. A primary anti-microbial response in the epithelium is through the expression of beta-defensins. These peptides act to strengthen epithelial integrity against bacteria by exerting a broad spectrum bactericidal and chemokine effect [331]. The expression of beta-defensins is often upregulated during chronic disease [332,333] and in response to inhaled pollutants such as cigarette smoke [334,335] and diesel exhaust particles [336].

Recognition of Gram-negative bacteria, including NTHi, typically occurs through endotoxin-induced activation of Toll-like Receptors (TLRs)-2 and TLR-4 [337-339]. Activation of TLRs results in the upregulation of NF-κB [340] and subsequent release of cytokines such as interleukin (IL)-1β, -6, -8 and tumour necrosis factor (TNF)-α [340]. Dysfunction of TLRs is often linked to respiratory disease [341] and these pattern recognitions receptors have been implicated in the response to air pollution [227,228,342]. Evidence suggests that silica, a primary constituent of geogenic PM, may be able to modulate the expression of TLR-2 [343] and -4 in macrophages [219]. However, there are no published data on the effects of silica on the expression of these cell surface receptors in epithelial cells or the effect of iron oxide particles on any cell type.

The overall aim of this Chapter was to investigate the effect of quartz, haematite and magnetite on the response of epithelial cells and macrophages to NTHi. Specifically, whether these particles enhance NTHi epithelial invasion or inhibit macrophage phagocytosis, and whether this differs between pathogenic strains of NTHi. Genomic analysis of NTHi was performed to determine the associations between differences in invasion between exposures and bacteria.

Having established these responses, their relationship to particle endocytosis, expression of TLRs, beta-defensins and inflammatory cytokines was assessed.

5.3 Methods

5.3.1 Particle preparation

Commercially available standard preparations of dry α-quartz (SiO_2; NIST 1878B), haematite (Fe_2O_3; Sigma-Aldrich 310050) and magnetite (Fe_3O_4; Sigma-Aldrich 310069) were prepared for cell exposure experiments as outlined in Chapter 2.

5.3.2 Cell line culture

The transformed human bronchial epithelial cell line (BEAS-2B; ATCC CRL-9609) and the human lung alveolar epithelial cell line (A549; lung adenocarcinoma, ATCC CCL-185) were cultured as outlined in Chapter 2. The leukemic peripheral blood monocyte (THP-1; ATCC TIB-202) cell line was cultured and differentiated into macrophages as outlined in Chapter 3.

5.3.3 Peripheral blood mononuclear (PBMC) cell isolation and culture

This study was approved by the Tasmanian health and Medical Human Research Ethics Committee (H0016505). Peripheral blood mononuclear cells (PMBCs) were isolated from six healthy adults aged between 20 and 50 years of age. Briefly, 32 mL of blood was collected from each individual using EDTA- BD Vacutainers (BD 366643). Samples were incubated at room temperature for 20 minutes with gentle mixing. Blood was aseptically layered onto 4 mL of Histopaque (Sigma-Aldrich 10771) in a 1:1 ratio before being centrifuged at 100 g for 30 min. Density gradient separation allowed the isolation of mononuclear cells which were then washed twice by centrifugation at 300 g. Cells were resuspended in serum-free RPMI-1640 and seeded at a density of 3 x 10^6 cells/mL. Cells were incubated for 1 hour at 37 °C in a

humidified atmosphere of 5% CO_2 and then washed twice to remove non-adherent cells, providing pure monocyte cultures. RPMI-1640 media containing 10% FBS was supplemented with 2 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech 300-03) for 12 days and refreshed every 3 days.

5.3.4 Cell exposure trials

Epithelial cells lines were seeded at a concentration of 2 x 10^5 cells/mL and THP-1 cells at 5 x 10^5 cells/mL. To investigate the dose-dependent effects of particulates, cells were exposed to 0 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL or 100 µg/mL of each individual particle type for 24 hours. Concentrations were chosen to be consistent with Chapter 2 and 3. In subsequent experiments comparing the repsonse for different strains of bacteria, exposures of 75 µg/mL and 50 µg/mL of each particle type were used for epithelial cells and macrophages respectively. All experiments were replicated independently six times using fresh preparations of particle solutions and cell cultures to allow valid statistical comparisons between exposure groups.

5.3.5 Bacterial isolates and culture

Eight clinical pathogenic strains of NTHi were utilised as described in Chapter 4. All isolates were cultured as outlined in Chapter 4.

5.3.6 Bacterial exposure

Bacterial broth suspensions were standardised to an optical density at 600 nm (OD_{600}) of 0.6. From this, 1 mL of each bacterial suspension was centrifuged at 6,000 g for 10 min before three washes in sterile-filtered phosphate buffered saline (PBS). Bacteria were resuspended in a total of 1 mL of PBS each and fluorescently tagged with 1 µL CellTrace Far Red (Thermofischer, C34564) for 20 min at room temperature in the absence of light. Bacteria were diluted to a 1:30 concentration before 30 µL of bacterial suspension was applied per well in a 96-well plate and incubated for 3 hours at 37 °C and 5% CO_2.

After bacterial exposures, samples were washed using PBS and exposed to their respective growth media supplemented with 200 µg/mL of gentamicin to remove remaining extracellular bacteria. Samples were incubated for 4 hours at 37 °C and 5% CO_2. Following PBS washing, trypsin detached cell samples were made to a final volume of 200 µL/ well for flow cytometry.

5.3.7 Cell surface markers

BEAS-2B cells were assessed for TLR-2 and -4 expression after 24 hours of particle exposure and a further 4 hours of 1 µg/mL E. Coli-derived LPS (Sigma-Aldrich L4391). TLR-2 (CD282 Alexaflour 488; Biolegend 309712) and TLR-4 (CD284 APC; Biolegend 312816) antibodies were diluted to 1:10 in FACS buffer (1% bovine serum albumin in PBS) and incubated with samples on ice for 30 min removed from light. Samples were then washed twice with FACS buffer, trypsinised for 20 min and resuspended in growth media for a total volume of 200 µL/sample prior to flow cytometry.

5.3.8 Flow cytometry

Fluorescent staining was analysed using a BD FACSCANTO II flow cytometer (BD Biosciences, USA). Data were analysed using FCS Express 6 (DeNovo software, USA), using fluorescence minus one (FMO) controls to determine gating strategies. Compensation was performed using single colour stained cells, and compensation matrices were calculated and applied. Cell populations were distinguished based on forward scatter (FSC) height and width. Gating strategies mitigated the presence of any remaining extracellular bacteria, while intracellular bacteria were quantified using the median fluorescent intensity.

5.3.9 Particle endocytosis

BEAS-2B cells were cultured on collagen-coated (Sigma-Aldrich C3867) 8-chamber slides (Thermo Scientific 154534) using DMEM media (Gibco 11965084) supplemented with 10% FBS. Cells were cultured to confluence and exposed to 75 μg/mL of quartz, haematite or magnetite for 24 hours. Cells were fixed in 10% formalin and stained with eosin for 2 min. Slides were imaged using the VS 120 automated slide scanner (Olympus, USA). ImageJ was used to quantify the endocytosed particle surface area and corrected for cell surface area to give a relative particle/ cell quantity.

5.3.10 Gene expression

RNA was isolated from BEAS-2B cells using the RNeasy Mini Kit (Qiagen 74106), followed by reverse transcription with the QuantiTect Reverse Transcription Kit (Qiagen 205311). Real-

time PCR was conducted using 100ng of cDNA, SYBR Select Master Mix (AppliedBiosystems 4472908) and primers for beta-defensin 1, beta-defensin 2/4A and housekeeper R18S. Primers used were beta-defensin 1; 5'- AGGTGGTAACTTTCTCACAG and beta-defensin 2/4A; 5' – GGTGTTTTTGGTGGTATAGG. Samples were normalised to R18S expression and assessed as relative fold change compared to control.

5.3.11 Cytokine production

Supernatant from BEAS-2B cells was collected after 24 hours of particle exposure and 4 hours exposure to 1 μg/mL of LPS. Inflammatory cytokines interleukin (IL)-1β (R&D DY201), IL-6 (R&D DY206), IL-8 (R&D DY208) and tumour necrosis factor-α (TNF-α; R&D DY210) were assessed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The minimum detection limits for IL-1β, IL-6, IL-8 and TNF-α were 7.81, 9.38, 31.3 and 15.6 pg/mL respectively. Absorbance was read at 450/570 nm with the Spectra Max M2 plate-reader (Molecular Devices, USA).

5.3.12 Whole genome sequencing (WGS)

Next generation sequencing was conducted using the Miseq (Illumina, USA) on NTHi strains used in the cell invasion assays. Galaxy Australia was used for all genomic preparation and annotation. Briefly, reads were trimmed using Trimmomatic [344] and assembled using SPAdes [345]. Assembled genomes were annotated using Prokka [346] and quality checked with Quast [347]. Roary was used to generate core gene alignments [348] and compared to the SwissProt protein database using NCBI BLAST + blastx [349-351]. RStudio 1.2.5 was then used to categorise genomes by principal component (PC) analysis which were used to assess

the relationship between gene expression and NTHi invasion. Unique genes and their respective proteins were then isolated by cross comparison of bacterial isolates and grouped functionally using the STRING functional protein network [352].

5.3.13 Statistical analysis

Comparisons between groups were made using repeated measures one-, and two-way ANOVA. When significance was determined for the main factors by ANOVA, the Holm-Sidak post-hoc test was used to examine individual between group differences. Where necessary, the data were log transformed to satisfy the assumptions of normal distribution of the error terms and homoscedasticity of the variance. Associations between continuous variables were analysed using linear regression. All data are presented as mean (SD) and values of $P < 0.05$ were considered statistically significant. All statistical analyses were conducted using SigmaPlot (v12.5).

5.4 Results

5.4.1 The effect of particle dose and type on NTHi invasion

To determine the effect of particles on epithelial cell invasion and macrophage phagocytosis cells were exposed to a highly invasive NTHi strain (L227) or a less invasive strain (Ci43) [353], with or without increasing concentrations of either silica, haematite or magnetite. Epithelial cells were exposed to 0-100 µg/mL, while macrophages were exposed to 0-50 µg/mL as concentrations above this were cytotoxic (see Chapter 3).

A549 – alveolar epithelial cells

There was a decrease in the invasiveness of L227 in response to 10 µg/mL of quartz (Fig 5.1A; $p < 0.001$), which did not decrease further at higher concentrations ($p = 0.262$). Haematite decreased the invasiveness of L227 (Fig 5.1B) at 10 ($p = 0.012$) and 100 µg/mL ($p = 0.008$). Magnetite induced a dose-dependent decrease in the invasiveness of L227 (Fig 5.1C) starting at 10 µg/mL ($p = 0.005$) which decreased further at 100 µg/mL ($p = 0.004$). There was no effect of any particle on the invasiveness of Ci43 on A549 cells (*data not shown*).

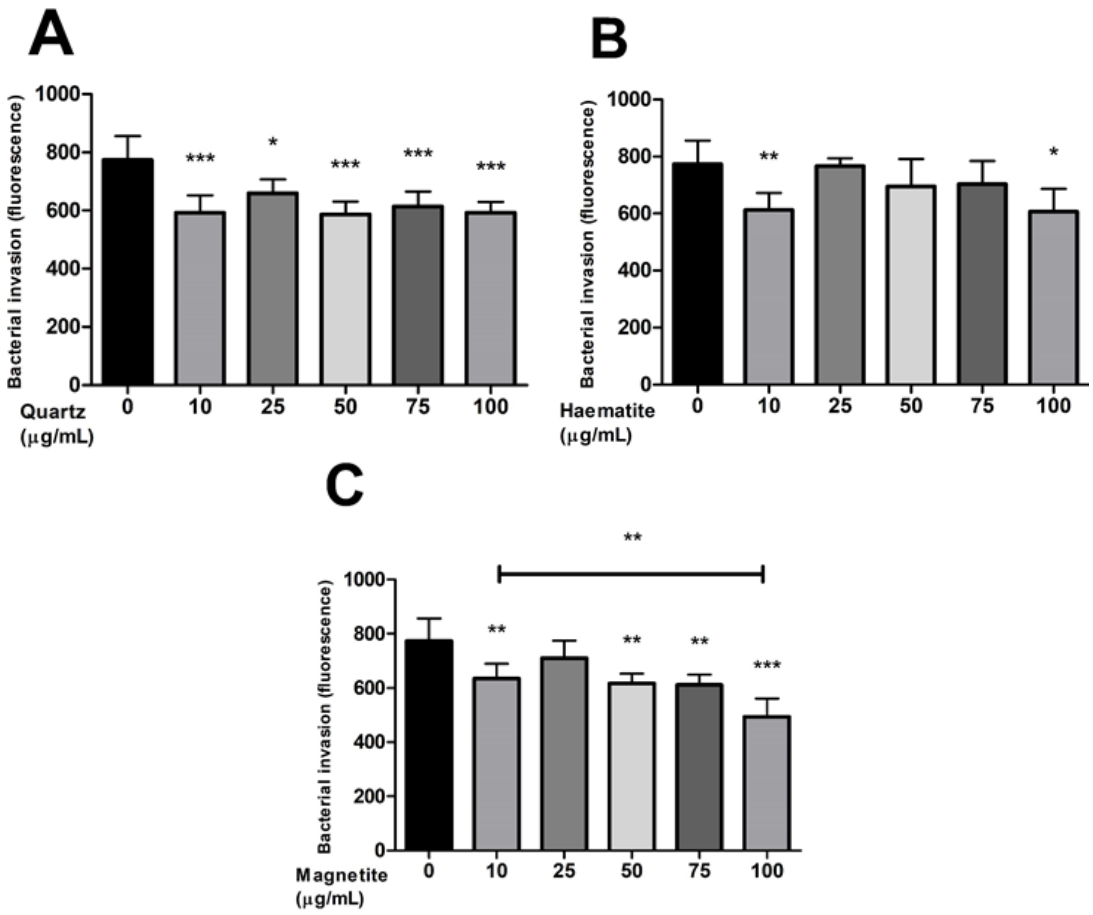


Figure 5.1: Bacterial invasion of a highly invasive NTHi bacterial isolate (L227) in A549 alveolar epithelial cells exposed to 0-100 µg/mL of quartz (A), haematite (B) or magnetite (C) for 24 hours. Data are represented as a relative percentage increase in median fluorescent intensity. Data are presented as mean(SD) from 6 independent experiments. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively versus control.

BEAS-2B – bronchial epithelial cell

Both quartz and haematite decreased the invasiveness of isolate L227 (Fig 5.2A & 5.2B) at 10 µg/mL ($p < 0.001$), which did not worsen with increasing concentrations of particles ($p > 0.353$). Magnetite (Fig 5.2C) induced a dose-dependent decrease in invasiveness, starting at 10 µg/mL

($p < 0.001$) which decreased further at 100 µg/mL ($p < 0.001$). In contrast, the invasiveness of Ci43 was not altered by quartz (Fig 5.3A; $p = 0.924$) or magnetite (Fig 5.3C; $p = 0.066$). However, haematite (Fig 5.3B) induced a substantial dose-dependent increase in the invasiveness of Ci43, starting at 25 µg/mL ($p < 0.001$) and increasing further at 50 µg/mL ($p < 0.001$).

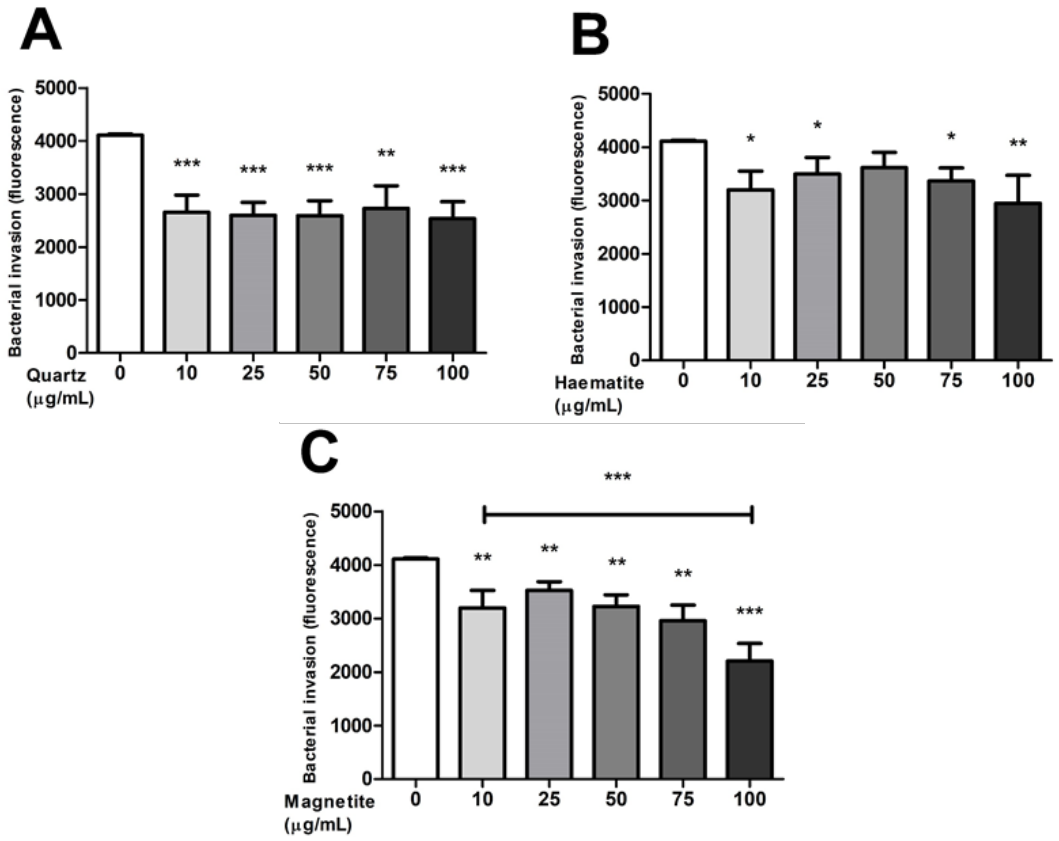


Figure 5.2: Bacterial invasion of a highly invasive NTHi bacterial isolate (L227) in BEAS-2B bronchial epithelial cells exposed to 0-100 µg/mL of quartz (A), haematite (B) or magnetite (C) for 24 hours. Data are represented as a relative percentage increase in median fluorescent intensity. Data are presented as mean(SD) from 6 independent experiments. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively versus control.

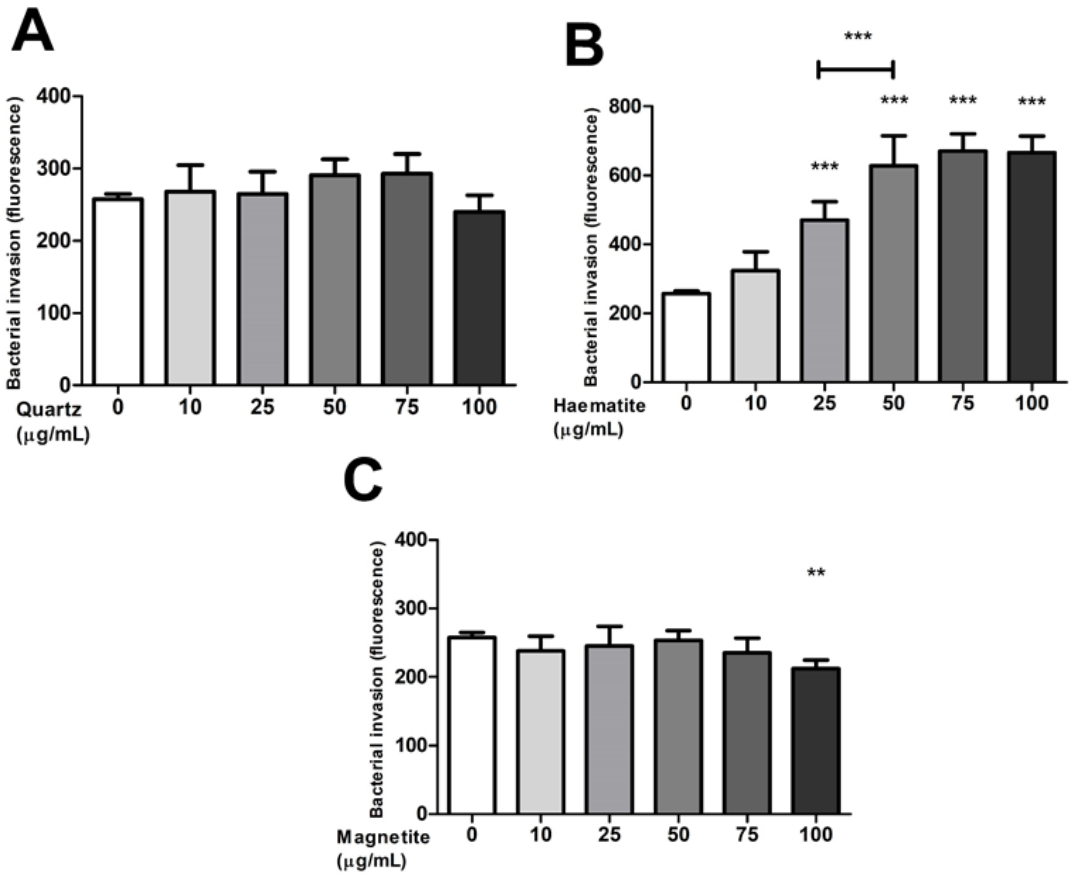


Figure 5.3: Bacterial invasion of a low level invasive NTHi bacterial isolate (Ci43) in BEAS-2B bronchial epithelial cells exposed to 0-100 μg/mL of quartz (A), haematite (B) or magnetite (C) for 24 hours. Data are represented as a relative percentage increase in median fluorescent intensity. Data are presented as mean(SD) from 6 independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$ respectively versus control.

THP-1– monocyte-derived macrophages

Quartz increased the phagocytosis of L227 at 25 μg/mL (Fig 5.4A; $p = 0.034$). In contrast, there was a significant decrease in the phagocytosis of L227 in response to all doses of haematite (Fig 5.4B; $p < 0.001$). Interestingly, there was no effect of magnetite on the

phagocytosis of L227 (Fig 5.4C; p = 0.361). Quartz also increased the phagocytosis of Ci43 at 10 µg/mL (Fig 5.5A; p = 0.005), but not at higher concentrations (p > 0.083). In contrast, haematite significantly decreased the phagocytosis of Ci43 at all concentrations (Fig 5.5B; p < 0.034) while magnetite had no effect on the phagocytosis of Ci43 (Fig 5.5C; p = 0.207).

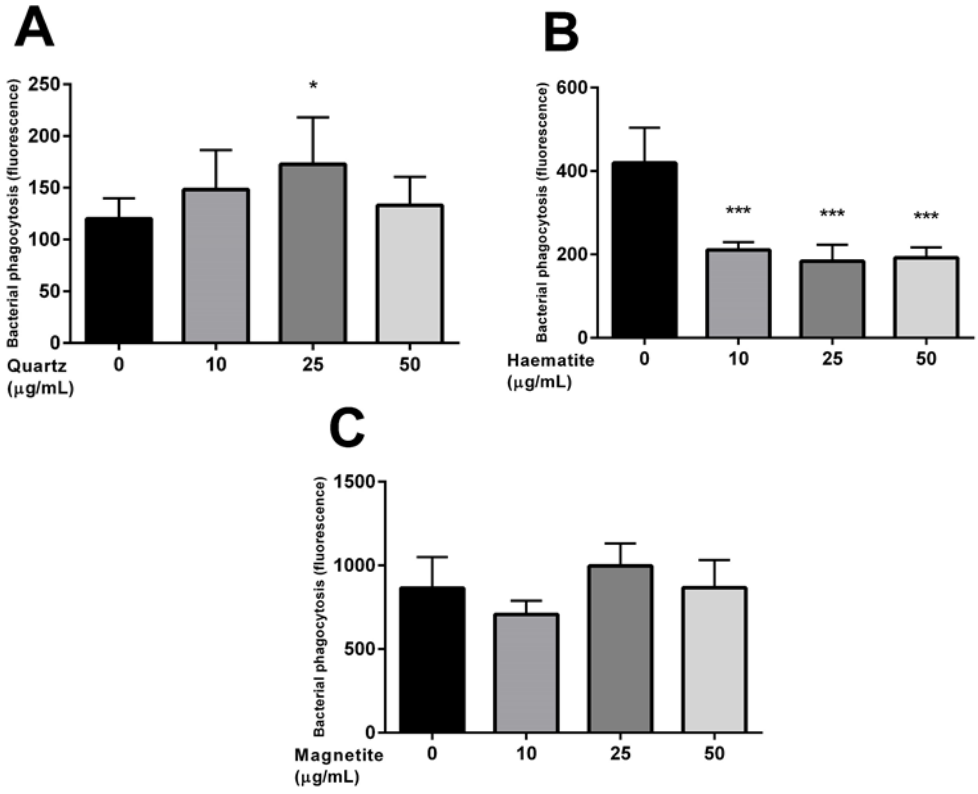


Figure 5.4: Bacterial phagocytosis of a highly invasive NTHi bacterial isolate (L227) in THP-1 derived macrophages exposed to 0-50 µg/mL of quartz (A), haematite (B) or magnetite (C) for 24 hours. Data are represented as a relative percentage increase in median fluorescent intensity. Data are presented as mean(SD) from 6 independent experiments. * and *** indicate p < 0.05 and p < 0.001 respectively versus control.

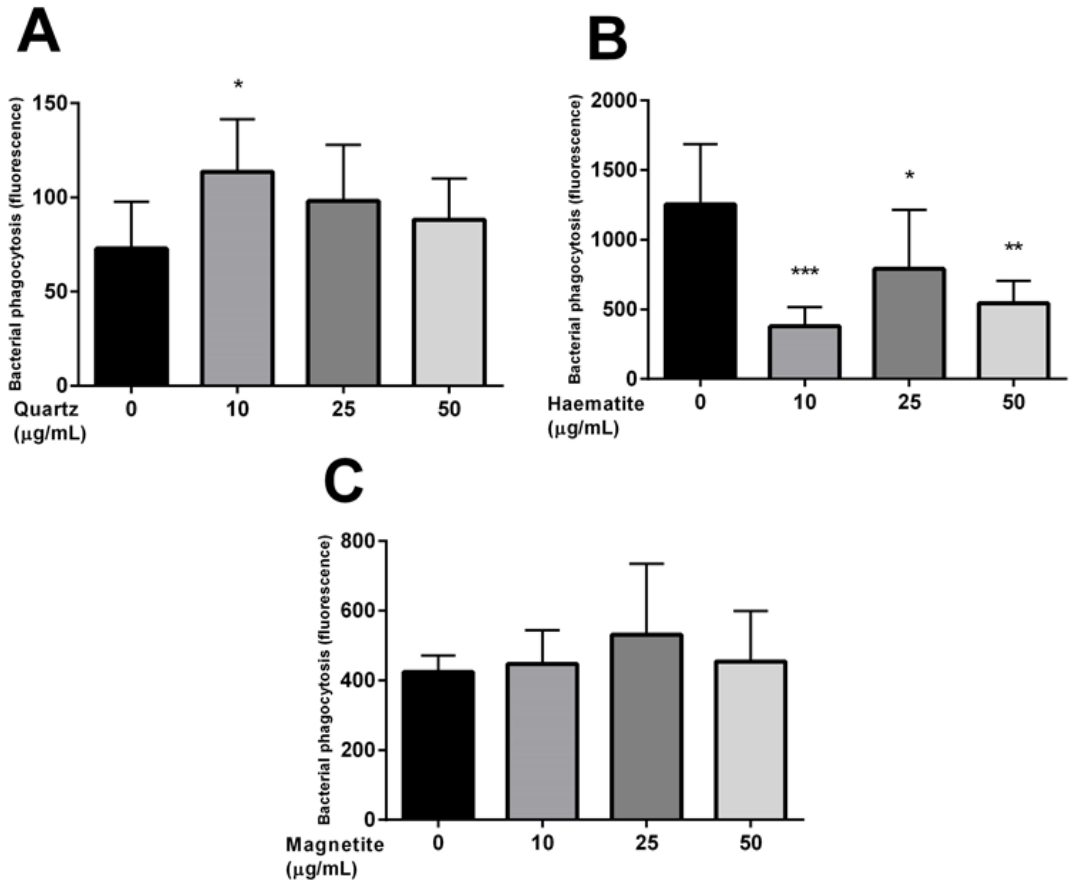


Figure 5.5: Bacterial phagocytosis of a low level invasive NTHi bacterial isolate (Ci43) in THP-1 derived macrophages exposed to 0-50 µg/mL of quartz (A), haematite (B) or magnetite (C) for 24 hours. Data are represented as a relative percentage increase in median fluorescent intensity. Data are presented as mean(SD) from 6 independent experiments. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively versus control.

5.4.2 The effect of NTHi strain on invasion in response to particle exposure

Given the differences displayed between the two NTHi isolates in the previous experiments, a subsequent set of trials were run to assess how NTHi strain influenced the effect of particle exposure on cell invasion and phagocytosis using 6 strains of NTHi.

A549– alveolar epithelial cells

At baseline, isolates L267 (Fig 5.6; $p < 0.001$) and L341 ($p < 0.001$) were more invasive than isolate NF3. In contrast, Ci37 ($p < 0.001$) and Ci34 ($p < 0.001$), but not Ci8 ($p = 0.417$), were less invasive compared to NF3. Quartz significantly increased the invasiveness of isolate Ci37 ($p > 0.001$) but had no effect on the other isolates ($p > 0.219$). In contrast, haematite significantly increased the invasiveness of isolate Ci8 ($p = 0.009$), while reducing the invasiveness of L267 ($p = 0.004$). Magnetite had no effect on the invasiveness of any isolate ($p > 0.222$).

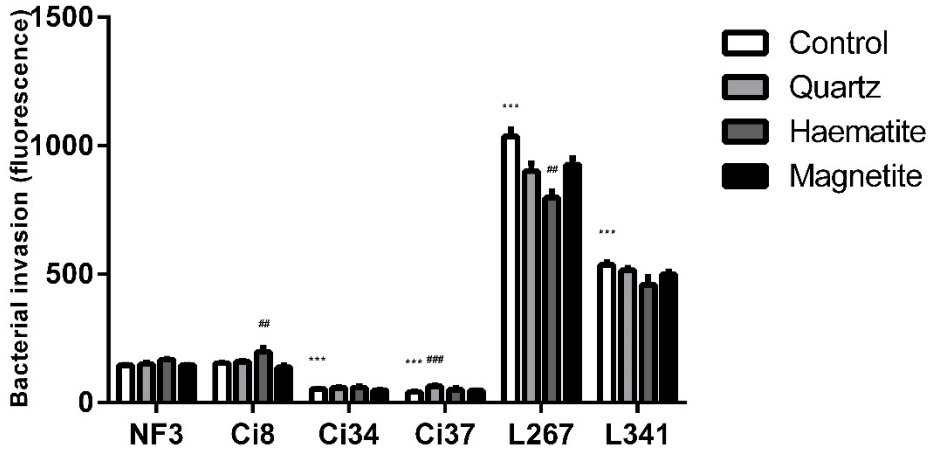


Figure 5.6: A549 alveolar epithelial cell invasion of six NTHi isolates in response to 75 µg/mL of quartz, haematite or magnetite exposure for 24 hours. Data are presented as mean(SD) from 6 independent experiments. *** indicates $p < 0.001$ of particle-free invasion versus isolate NF3. ## and ### indicate $p < 0.01$ and $p < 0.001$ respectively versus particle control.

BEAS-2B– bronchial epithelial cells

At baseline, the invasion of all isolates was lower than NF3 (Fig 5.7; $p < 0.001$). Quartz decreased the invasion of isolates NF3, Ci8 ($p < 0.001$) and Ci34 ($p = 0.046$) but increased the invasiveness of isolates Ci37 ($p = 0.031$), L267 and L341 ($p < 0.001$). Haematite increased the invasiveness of all isolates ($p < 0.001$) while magnetite decreased the invasiveness of isolate NF3 and Ci8 ($p < 0.001$) but increased invasiveness of L267 and L341 ($p < 0.001$).

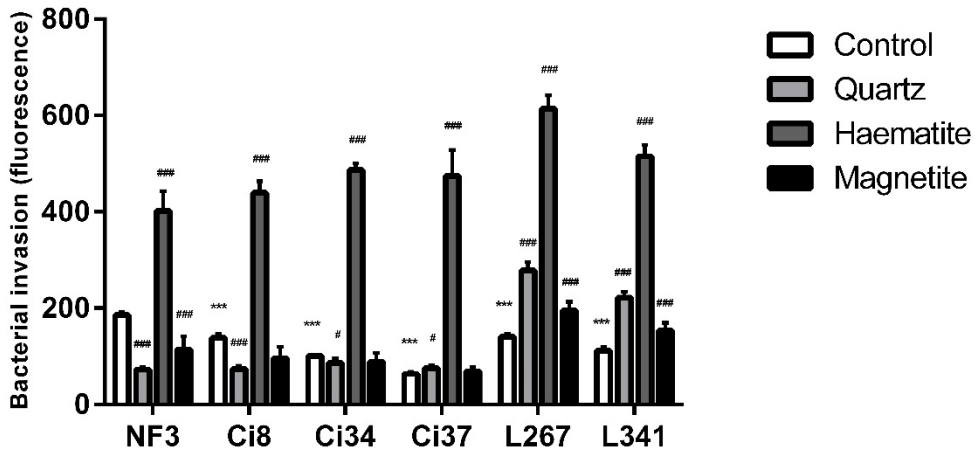


Figure 5.7: BEAS-2B bronchial epithelial cell invasion of six NTHi isolates in response to 75 µg/mL of quartz, haematite or magnetite exposure for 24 hours. Data are presented as mean(SD) from 6 independent experiments. *** indicates $p < 0.001$ of particle-free invasion versus isolate NF3. # and ### indicate $p < 0.05$ and $p < 0.001$ respectively versus particle control. δ δ indicates $p = 0.01$ of magnetite versus haematite.

THP-1– monocyte-derived macrophages

At baseline, isolate Ci34 (Fig 5.8; $p < 0.001$) was phagocytosed more than NF3, while Ci37, Ci8 ($p < 0.001$) and L267 ($p = 0.003$) were phagocytosed less. Haematite ($p = 0.013$) and magnetite ($p < 0.001$) decreased the phagocytosis of all bacteria while quartz had no effect ($p = 0.989$) Comparatively, magnetite reduced phagocytosis more than haematite ($p = 0.010$).

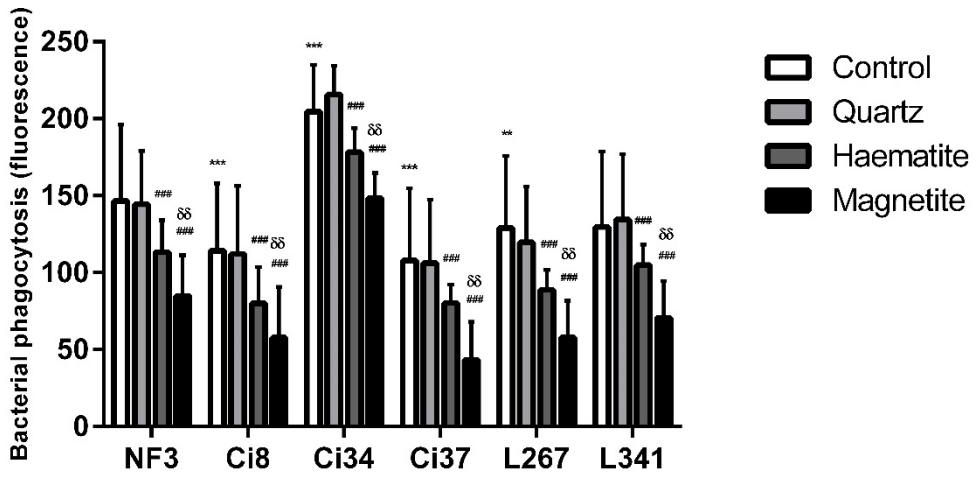


Figure 5.8: THP-1 derived macrophage phagocytosis of six NTHi isolates in response to 50 µg/mL of quartz, haematite or magnetite exposure for 24 hours. Data are presented as mean(SD) from 6 independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$ respectively of particle-free invasion versus isolate NF3. ### indicates $p < 0.001$ versus particle control.

PBMC– primary monocyte-derived macrophages

In order to assess the influence of macrophage source on the effect of particles on NTHi phagocytosis, a series of experiments using primary human macrophages were conducted.

Similar to the THP-1 response, there were phagocytic differences between NTHi (Fig 5.9; $p < 0.05$), however the effect of particles was, largely, not replicated. Overall, there was minimal effect of any particle exposure on PBMC-derived macrophage phagocytosis of NTHi. The exceptions were isolates L267 and L341 which were phagocytosed less after exposure to haematite ($p < 0.001$ & $p = 0.028$ respectively).

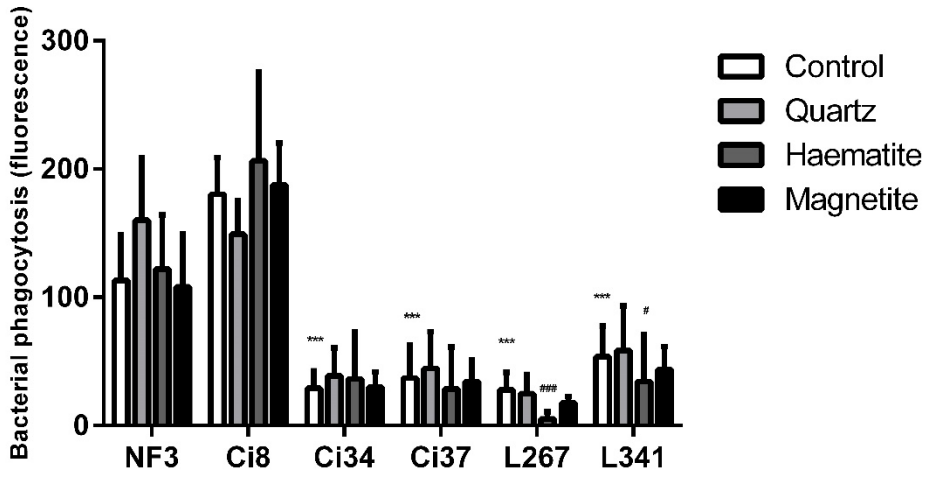


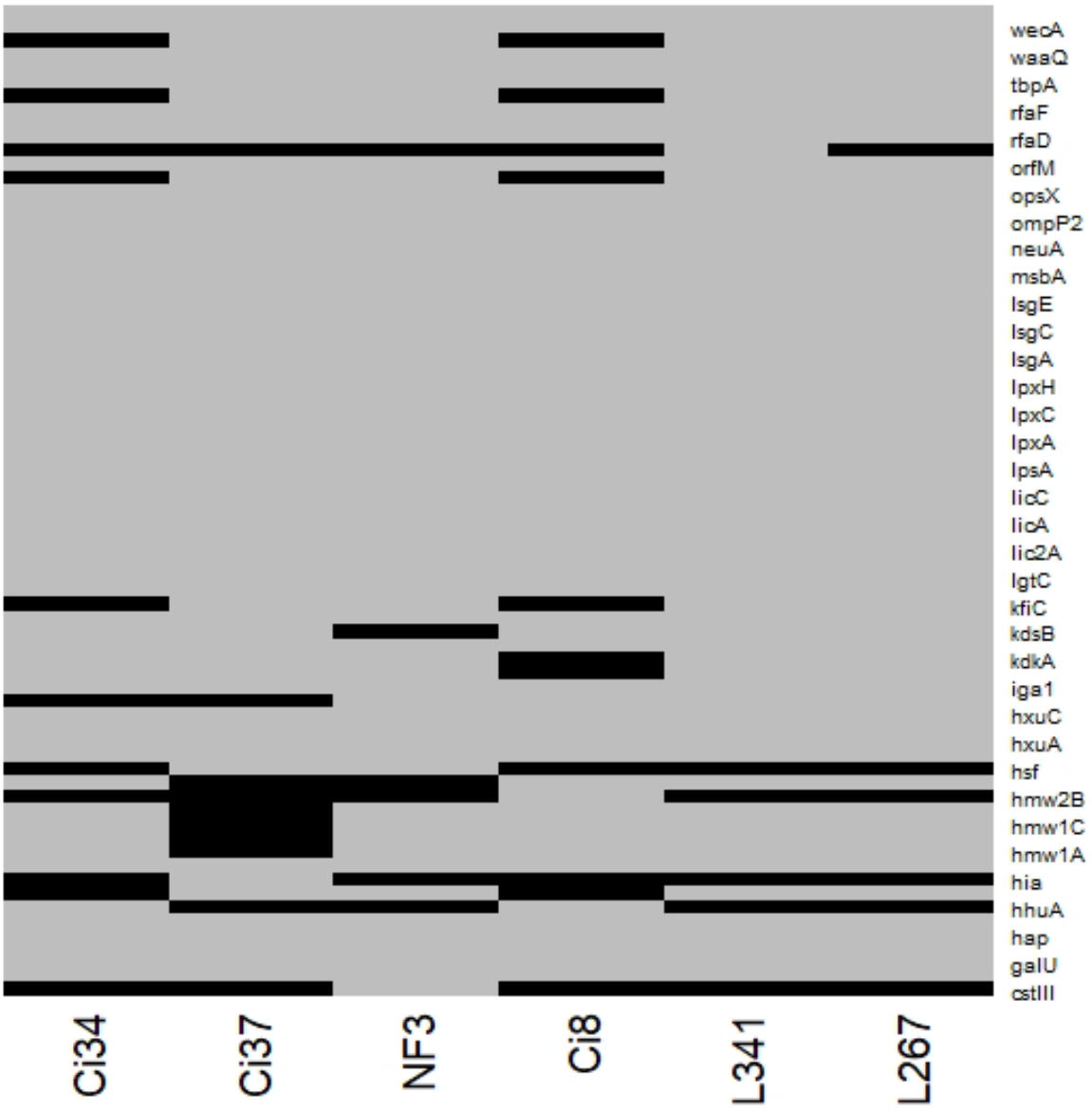
Figure 5.9: Primary blood-derived mononuclear cell (PBMC) derived macrophage phagocytosis of six NTHi isolates in response to 50 µg/mL of quartz, haematite or magnetite exposure for 24 hours. Data are presented as mean(SD) from 6 independent experiments. *** indicate $p < 0.001$ for particle-free invasion versus isolate NF3. # and ### indicates $p < 0.05$ and $p < 0.001$ respectively versus particle control.

5.4.3 Whole genome sequencing

Presence and absence of virulence genes

Given the significant differences in invasion at baseline in BEAS-2B cells (Fig 5.7), NTHi isolates were screened for known virulence genes (Table 5.1) to determine whether this could explain variations in invasion capacity. However, all NTHi strain expressed a large number of known virulence genes and there were no consistent differences between strains that could be related to differences in the invasion observed.

Table 5.1: Virulence genes screening of NTHi isolates with grey representing the presence of a gene and the absence indicated in black.



Principal component analysis (PCA)

Given there was limited effect of particle exposure on NTHi invasion in the A549 cells, the correlation between genomic data and the NTHi invasion response was assessed in BEAS-2B

and THP-1 cells. In order to achieve this, PCA was used as a data reduction strategy. Two principal components (PC1 and PC2) were identified that accounted for over 80% of genomic variance. Significant associations were found between both PC1 and PC2 and the effect of quartz, haematite ($p < 0.001$) and magnetite ($p = 0.001$) on NTHi invasion in BEAS-2B cells. In contrast, there were no associations between PC1 or PC2 and NTHi invasion in THP-1 macrophages ($p > 0.05$).

Given the lack of correlation between the genome and NTHi invasion in the THP-1 cells based on the PCA, further exploratory analysis was conducted focussing on the BEAS-2B cells. In order to identify the groups of genes that explained the observed associations in this cell type, a filtering process was applied whereby clusters of isolates were qualitatively identified when the regression analysis (described above and in Table 5.2) were significant and the relationship was monotonic (Fig 5.10). This filtration process isolated clusters of NTHi strains that were associated with invasion. The presence/absence of genome-related proteins between these clusters were compared to identify pathways involved in particle induced modulation of cell invasion. Based on this process isolates NF3, Ci8, Ci34 and Ci43 were compared to isolates L267 and L341 (based on the effect of quartz and haematite on NTHi invasion in BEAS-2B cells) and isolates L267, L341 and NF3 were compared to Ci8, Ci34 and Ci43 (based on the effect of magnetite on NTHi invasion in BEAS-2B cells).

Table 5.2: Linear regression analysis for the association between genomic principal component analysis and NTHi invasion.

Cell type		**Exposure**	**PC1**	**PC2**
BEAS-2B		Control	r^2: -0.0006031 p: 0.329	r^2: 0.2383 p: 0.001 **
		Quartz	r^2: 0.3012 p: < 0.001 ***	r^2: 0.3349 p: < 0.001 ***
		Haematite	r^2: 0.5228 p: < 0.001 ***	r^2: 0.7153 p: < 0.001 ***
		Magnetite	r^2: 0.2406 p: 0.001 **	r^2: 0.6486 p: < 0.001 ***
A549		Control	r^2: 0.4581 p: < 0.001 ***	r^2: 0.6316 p: < 0.001 ***
		Quartz	r^2: 0.4624 p: < 0.001 ***	r^2: 0.6501 p: < 0.001 ***

		Haematite	r^2: 0.4741 p: < 0.001 ***	r^2: 0.6481 p: < 0.001 ***
		Magnetite	r^2: 0.4761 p: < 0.001 ***	r^2: 0.6492 p: < 0.001 ***
THP-1		Control	r^2: -0.01012 p: 0.4259	r^2: -0.02918 p: 0.9311
		Quartz	r^2: -0.005048 p: 0.3703	r^2: -0.0268 p: 0.7703
		Haematite	r^2: -0.00112 p: 0.3339	r^2: -0.02503 p: 0.7053
		Magnetite	r^2: 0.001716 p: 0.3104	r^2: -0.0243 p: 0.683

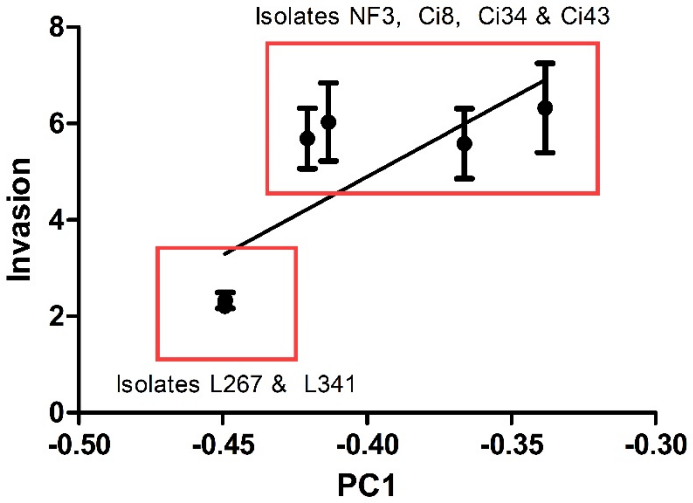


Figure 5.10: An example of a monotic association beteen the bacterial genome (on the basis of principal component 1) and invasion in BEAS-2B cells after exposure to 75 µg/mL of haematite for 24 hours. The upper box seperates isolates NF3, Ci8, Ci34 and Ci43, as more invasive, for further genomic comparison to the less invasive strains L267 and L341.

Protein presence associated with particle-dependent invasion

L267 and L341 were associated with higher invasion in BEAS-2B cells exposed to quartz based on the PCA. Forty-one proteins were found to be conserved between L267 and L341 but not in the remaining isolates (Table 5.3). Of these, 31 had known function including as transporters for nitrogen, organic substances, peptides and oligopeptides as well as monosaccharide metabolism while some were identified as cellular components implicated in outer membrane function. The expression of these proteins was significantly linked ($p = 0.003$).

Table 5.3: Proteins conserved within isolates L267 and L341.

Protein	**Gene**	**Function**
ABGA	abgA	p-aminobenzoyl-glutamate hydrolase subunit A homolog
CPDB	cpdB	2',3'-cyclic-nucleotide 2'-phosphodiesterase/3'-nucleotidase
CYDC	cydC	ATP-binding/permease protein CydC
CYDD	cydD	ATP-binding/permease protein CydD
CYOA	HI_1076	Probable cytochrome oxidase subunit 1
DDC	ddc	L-2,4-diaminobutyrate decarboxylase
FRDA	frdA	Fumarate reductase flavoprotein subunit
HBPA	hbpA	Heme-binding protein A
LEU1	leuA	2-isopropylmalate synthase
LEUC	leuC	3-isopropylmalate dehydratase large subunit

MNME	mnmE	tRNA modification GTPase MnmE
MURE	murE	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase
MURJ	murJ	Probable lipid II flippase MurJ
NANE	nanE	Putative N-acetylmannosamine-6-phosphate 2-epimerase
NQRC	nqrC	Na(+)-translocating NADH-quinone reductase subunit C
NQRD	nqrD	Na(+)-translocating NADH-quinone reductase subunit D
NRFA	nrfA	Cytochrome c-552
NRFE	nrfE	Cytochrome c-type biogenesis protein NrfE
OPPA	oppA	Periplasmic oligopeptide-binding protein
PCKA	pckA	Phosphoenolpyruvate carboxykinase (ATP)
PFLA	pflA	Pyruvate formate-lyase 1-activating enzyme
PRTH	prtH	Protease PrtH

PUR1	purF	Amidophosphoribosyltransferase
PUR9	purH	Bifunctional purine biosynthesis protein PurH
RECB	recB	RecBCD enzyme subunit RecB
RIBA	ribA	GTP cyclohydrolase-2
SAPA	sapA	Peptide transport periplasmic protein SapA
SYK	lysS	Lysine--tRNA ligase
TATC	tatC	Sec-independent protein translocase protein TatC
XYLA	xylA	Xylose isomerase
XYLB	xylB	Xylulose kinase
Y1005	HI_1005	Putative phosphoethanolamine transferase HI
Y1218	HI_1218	Putative L-lactate permease
Y152	HI_0152	Putative 4'-phosphopantetheinyl transferase HI

Y1637	HI_1637	Uncharacterized protein HI
Y220B	HI_220B	Uncharacterized protein HI
Y561	HI_561	Putative oligopeptide transporter HI
Y568	HI_568	Uncharacterized protein HI
Y594	HI_594	Uncharacterized protein HI
Y665	HI_665	Putative kinase HI
YIDC	YidC	Membrane protein insertase YidC

NF3, Ci8, Ci34 and Ci37 were associated with higher invasion in BEAS-2B cells exposed to haematite. Seventy proteins were conserved within isolates NF3, Ci8, Ci34, Ci37 but not L267 or L341 (Table 5.4). From these, 67 were verified proteins with known functions. A significant association was found ($p = 0.011$) between the expression of these proteins which are involved in catalytic activity, anion, ion and small molecule binding as well as binding of heterocyclic and organic cyclic compounds, purine and purine-free ribonucleotides, nucleotides, ATP and drug binding.

Table 5.4: Proteins conserved within isolates NF3, Ci8, Ci34 and Ci37.

Protein	**Gene**	**Function**
AAT	aspC	Aspartate aminotransferase
ACCA	accA	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha
AMIB	amiB	Probable N-acetylmuramoyl-L-alanine amidase AmiB
APAH	apaH	Bis(5'-nucleosyl)-tetraphosphatase, symmetrical
ARCC	arcC	Carbamate kinase
ARLY	argH	Argininosuccinate lyase
ASNA	asnA	Aspartate-ammonia ligase
DLD	dld	Quinone-dependent D-lactate dehydrogenase
KATA	KatA	Catalase-1
CCA	cca	Multifunctional CCA protein

COAA	coaA	Pantothenate kinase
COME	comE	Competence protein E
DGTL1	NTHI1825	Deoxyguanosinetriphosphate triphosphohydrolase-like protein
DLDH	lpdA	Dihydrolipoyl dehydrogenase
EX1	sbcB	Exodeoxyribonuclease I
EX7L	xseA	Exodeoxyribonuclease 7 large subunit
FTSH	ftsH	ATP-dependent zinc metalloprotease FtsH
FUCI	fucI	L-fucose isomerase
FUMC	fumC	Fumarate hydratase class II
GLGA	glgA	Glycogen synthase
GLMS	glmS	Glutamine-fructose-6-phosphate aminotransferase [isomerizing]
GLPG	glpG	Rhomboid protease GlpG

HGPA	hgpA	Hemoglobin and hemoglobin-haptoglobin-binding protein A
HLDE	hldE	Bifunctional protein HldE
HSCA	hscA	Chaperone protein HscA homolog
HI_1259	HI_1259	Probable periplasmic serine protease do/HhoA-like
ISCS	iscS	Cysteine desulfurase IscS
ISPG	ispG	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)
K1PF	fruK	1-phosphofructokinase
LCFA	fadD	Long-chain-fatty-acid-CoA ligase
LICA1	licA	Protein LicA
LLDD	lldD	L-lactate dehydrogenase
METK	metK	S-adenosylmethionine synthase
MUKF	mukF	Chromosome partition protein MukF

MURB	murB	UDP-N-acetylenolpyruvoylglucosamine reductase
NHAB	nhaB	Na(+)/H(+) antiporter NhaB
OTC	argF	Ornithine carbamoyltransferase
OTCC	arcB	Ornithine carbamoyltransferase, catabolic
PNTA	pntA	NAD(P) transhydrogenase subunit alpha
PRMC	prmC	Release factor glutamine methyltransferase
PSS	pssA	CDP-diacylglycerol--serine-o-phosphatidyltransferase
PTFAH	fruB	Multiphosphoryl transfer protein
PTFBC	fruA	PTS system fructose-specific EIIB'BC component
RECA	recA	Protein RecA
RLMN	rlmN	Dual-specificity RNA methyltransferase RlmN
RNG	rng	Ribonuclease G

RNPH	rph	Ribonuclease PH
RPOE	rpoE	ECF RNA polymerase sigma-E factor
RPOH	rpoH	RNA polymerase sigma factor RpoH
SECD	secD	Protein translocase subunit SecD
SRMB	srmB	ATP-dependent RNA helicase SrmB
SYH	hisS	Histidine--tRNA ligase
SYN	asnS	Asparagine--tRNA ligase
TILS	tilS	tRNA(Ile)-lysidine synthase
TLDD	tldD	Metalloprotease TldD homolog
TNAA	tnaA	Tryptophanase
TOLB	tolB	Tol-Pal system protein TolB
TOP3	topB	DNA topoisomerase 3

TRPC	trpC	Tryptophan biosynthesis protein TrpCF
TYPA	typA	GTP-binding protein TypA/BipA homolog
HI_1369	HI_1369	Uncharacterized protein HI
HI_1415	HI_1415	Uncharacterized protein HI
HI_1416	HI_1416	Uncharacterized protein HI
HI_1558	HI_1558	UPF0162 protein HI
HI_1667	HI_1667	Putative L,D-transpeptidase HI
HI_0183	HI_183	Uncharacterized transporter HI
HI_0361	HI_361	Probable iron transport system ATP-binding protein HI
HI_0367	HI_367	Uncharacterized protein HI
HI_0387	HI_0387	Probable ATP-dependent DNA helicase HI_0387
HI_0409	HI_409	Uncharacterized metalloprotease HI

HI_0419	HI_419	Uncharacterized protease HI
HI_0597	HI_597	Putative phosphatase HI

L267, L341 and NF3 were associated with higher invasion in BEAS-2B cells exposed to magnetite. There were 122 proteins conserved between isolates L267, L341 and NF3 that were not found in the other bacteria (Table 5.5). From this, 117 proteins had verified identity and function. There was a significant linkage found between these proteins ($p = 0.002$). Broadly, these proteins were associated with anion, ion, small molecule, nucleotide and ribonucleotide binding. There were also significant associations with heterocyclic compound binding, organic cyclic compound binding and purine ribonucleoside, with and without triphosphate, binding. As well as drug binding, ATP binding, macromolecule transmembrane transporter activity and catalytic and carboxy-lyase activity.

Table 5.5: Proteins conserved between isolates L267, L341 and NF3.

Protein	**Gene**	**Function**
3PASE	HI_1598	Inorganic triphosphatase
AAT	aspC	Aspartate aminotransferase

ABGA	abgA	p-aminobenzoyl-glutamate hydrolase subunit A homolog
ALR	alr	Alanine racemase
AMPP	pepP	Xaa-Pro aminopeptidase
APBE	apbE	FAD:protein FMN transferase
ARLY	argH	Argininosuccinate lyase
ATOE	atoE	Putative short-chain fatty acid transporter
CATA	KatA	Catalase
CCA	cca	Multifunctional CCA protein
COMM	comM	Competence protein ComM
CPDB	cpdB	2',3'-cyclic-nucleotide 2'-phosphodiesterase/3'-nucleotidase
CYDC	cydC	ATP-binding/permease protein CydC
CYDD	cydD	ATP-binding/permease protein CydD

CYOA	HI_1076	Probable cytochrome oxidase subunit 1
DCDA	lysA	Diaminopimelate decarboxylase ornithine decarboxylase
DDC	ddc	L-2,4-diaminobutyrate decarboxylase
DHNA	ndh	NADH dehydrogenase
DNAB	dnaB	Replicative DNA helicase
DSBD	dsbD	Thiol:disulfide interchange protein DsbD
FRDA	frdA	Fumarate reductase flavoprotein subunit
FTSK	ftsK	DNA translocase FtsK
FTSP	ftsP	Cell division protein FtsP
GLN1B	glnA	Glutamine synthetase
HBPA	hbpA	Heme-binding protein A
HMRM	hmrM	Multidrug resistance protein HmrM

HSRA	hsrA	Probable transport protein HsrA
ILVA	ilvA	L-threonine dehydratase biosynthetic IlvA
ILVI	ilvI	Acetolactate synthase large subunit
ISPE	ispE	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
KEFX	kefBC	Glutathione-regulated potassium-efflux system protein
KPRS	prs	Ribose-phosphate pyrophosphokinase
KPYK	pykA	Pyruvate kinase
LCFH	HI_0002	Putative long-chain-fatty-acid-CoA ligase
LEU1	leuA	2-isopropylmalate synthase
LEUC	leuC	3-isopropylmalate dehydratase large subunit
LSG1	HI_1700	Lsg locus putative protein 1
MGLA	mglA	Galactose/methyl galactoside import ATP-binding protein MglA

MNME	mnmE	tRNA modification GTPase MnmE
MURB	murB	UDP-N-acetylenolpyruvoylglucosamine reductase
MURC	murC	UDP-N-acetylmuramate-L-alanine ligase
MURE	murE	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase
MURJ	murJ	Probable lipid II flippase MurJ
NANE	nanE	Putative N-acetylmannosamine-6-phosphate 2-epimerase
NQRC	nqrC	Na(+)-translocating NADH-quinone reductase subunit C
NQRD	nqrD	Na(+)-translocating NADH-quinone reductase subunit D
NRFA	nrfA	Cytochrome c-552
NRFE	nrfE	Cytochrome c-type biogenesis protein NrfE
OPPA	oppA	Periplasmic oligopeptide-binding protein
PCKA	pckA	Phosphoenolpyruvate carboxykinase (ATP)

PFLA	pflA	Pyruvate formate-lyase 1-activating enzyme
PLDB	pldB	Probable lysophospholipase L2
PPID	ppiD	Peptidyl-prolyl cis-trans isomerase D
PRTH	prtH	Protease PrtH
PSD	psd	Phosphatidylserine decarboxylase proenzyme
PTFBC	fruA	PTS system fructose-specific EIIB'BC component
PUR1	purF	Amidophosphoribosyltransferase
PUR9	purH	Bifunctional purine biosynthesis protein PurH
PURK	purK	N5-carboxyaminoimidazole ribonucleotide synthase
PURR	purR	HTH-type transcriptional repressor PurR
PURU	purU	Formyltetrahydrofolate deformylase
PYRG	pyrG	CTP synthase

QSEC	qseC	Sensor protein QseC
RADA	radA	DNA repair protein RadA
RARA	rarA	Replication-associated recombination protein A
RECB	recB	RecBCD enzyme subunit RecB
RECJ	recJ	Single-stranded-DNA-specific exonuclease RecJ
RIBA	ribA	GTP cyclohydrolase-2
RISA	ribE	Riboflavin synthase
SAPA	sapA	Peptide transport periplasmic protein SapA
SECD	secD	Protein translocase subunit SecD
SECY	secY	Protein translocase subunit SecY
SFSA	sfsA	Sugar fermentation stimulation protein homolog
SYK	lysS	Lysine--tRNA ligase

SYP	proS	Proline--tRNA ligase
SYW	trpS	Tryptophan--tRNA ligase
SYY	tyrS	Tyrosine--tRNA ligase
TAMA	tama	Translocation and assembly module subunit TamA
TATC	tatC	Sec-independent protein translocase protein TatC
TKT	tktA	Transketolase
TORY	torY	Cytochrome c-type protein TorY
TORZ	torZ	Trimethylamine-N-oxide reductase
TRUD	truD	tRNA pseudouridine synthase D
TYPA	typA	GTP-binding protein TypA/BipA homolog
XYLA	xylA	Xylose isomerase
XYLB	xylB	Xylulose kinase

5.4.4 Particle uptake in BEAS-2B bronchial epithelial cells

It has previously been shown that internalization of particles PM disrupts the intracellular homeostasis [354]. Furthermore, uptake of particles is associated with altered cell membrane properties [355]. Together, this suggests that particle uptake may have implications for cellular integrity and the capacity of bacteria to invade the cell. Given the association between haematite and increased invasion of NTHi in BEAS-2B cells, the endocytosis of the PM was assessed in this cell type.

BEAS-2B cells endocytosed more haematite than quartz or magnetite (Fig 5.11; $p < 0.001$). While less than haematite, magnetite was also endocytosed more than quartz ($p < 0.001$). The magnitude of invasion across all NTHi isolates in BEAS-2B cells was significantly associated with the magnitude of particle uptake ($p < 0.001$).

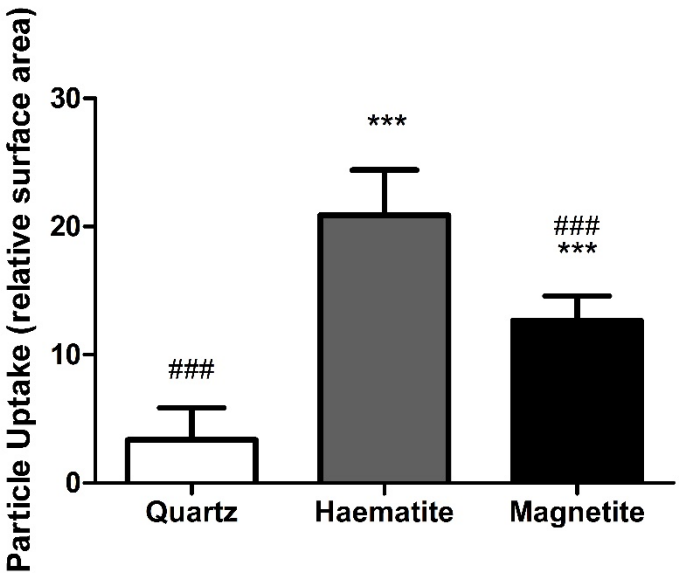


Figure 5.11: BEAS-2B bronchial epithelial cell particle uptake in response to 24 hours of exposure to 75 µg/mL of quartz, haematite or magnetite. Data are presented as mean(SD) from 6 independent experiments.*** indicates $p < 0.001$ versus quartz and ### indicates $p < 0.001$ versus haematite.

5.4.5 BEAS-2B bronchial epithelial cell response to particles

The data outlined above suggest that bronchial epithelial cells are the most susceptible to the effect of particles on NTHi invasion (see Figure 5.7). To further explore the effect of particles on the response to bacterial infection, BEAS-2B cells were exposed to 75 µg/mL of quartz, haematite or magnetite for 24 hours. Several pathways were assessed. Firstly, the gene expression of beta-defensin 1 and 2 these are a critical part of the epithelial anti-microbial response [356]. Secondly, particles have been shown to alter the expression of cell surface receptors [357,219] and thus the ability to respond to bacteria. The expression of TLR-2 and -4 as the primary responders to NTHi endotoxin, LPS [69,277,229], was measured to investigate

the role of particles in altered bacterial recognition. Additionally, given activation of TLRs drives NF-κB-associated inflammation [277], a panel of inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-8 were measured.

Beta-defensins

Quartz, haematite ($p = 0.001$) and magnetite ($p < 0.001$) all reduced the expression of beta-defensin 1 when compared to control (Fig 5.12A), with no differences between exposures ($p > 0.280$). Beta-defensin 2 expression was also suppressed by quartz (Fig 5.12B; $p = 0.005$), haematite ($p = 0.007$) and magnetite ($p = 0.019$) relative to control, with no differences between exposures ($p > 0.736$).

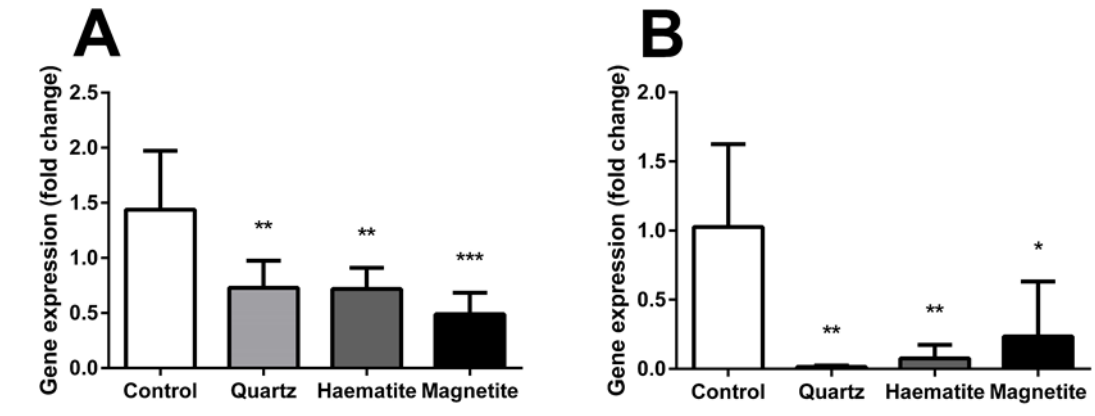


Figure 5.12: Fold change gene expression of beta-defensin-1 (A) and -2 (B) when BEAS-2B bronchial epithelial cells were exposed to 75 μg/mL of quartz, haematite or magnetite for 24 hours. Data are presented as mean(SD) from 6 independent experiments. *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ versus control.

Toll-like receptor-2 and -4

In absence of LPS, magnetite increased TLR-2 expression (Fig 5.13A; $p = 0.01$), while quartz and haematite had no effect ($p > 0.556$). When compared to LPS-free exposures, quartz ($p = 0.021$), haematite ($p = 0.020$) and magnetite ($p < 0.001$) decreased TLR-2 expression. with no difference between exposures ($p > 0.841$). In contrast, there was no particle effect on TLR-4 expression in the absence of LPS exposure (Fig 5.13B; $p > 0.958$). However, in the presence of LPS, while quartz and magnetite had no effect ($p > 0.701$), haematite exposure significantly increased the expression of TLR-4 ($p = 0.013$).

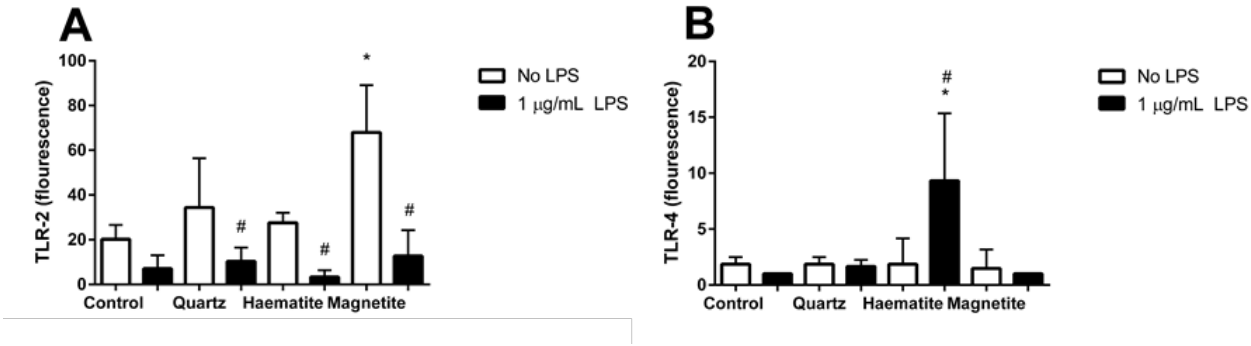


Figure 5.13: Toll-like receptor (TLR)-2 (A) and -4 (B) expression on BEAS-2B bronchial epithelial cells when exposed for 24 hours to 75 µg/mL of quartz, haematite or magnetite and either an additional 4 hours of 1 µg/mL of endotoxin (LPS) or LPS-free media. Data are presented as mean(SD) from 3 independent experiments. * indicates $p < 0.05$ versus control, # represents $p < 0.05$ versus respective LPS-free exposure.

Inflammatory cytokines

Magnetite supressed IL-6 (Fig 5.14A; $p = 0.001$) and TNF-α expression (Fig 5.14C; $p = 0.007$), however, there was no effect of either quartz or haematite ($p > 0.059$) on the inflammatory response to LPS. In contrast, the LPS-induced expression of IL-8 increased in BEAS-2B cells exposed to quartz and haematite (Fig 5.14B; $p < 0.001$), but not magnetite ($p = 0.222$).

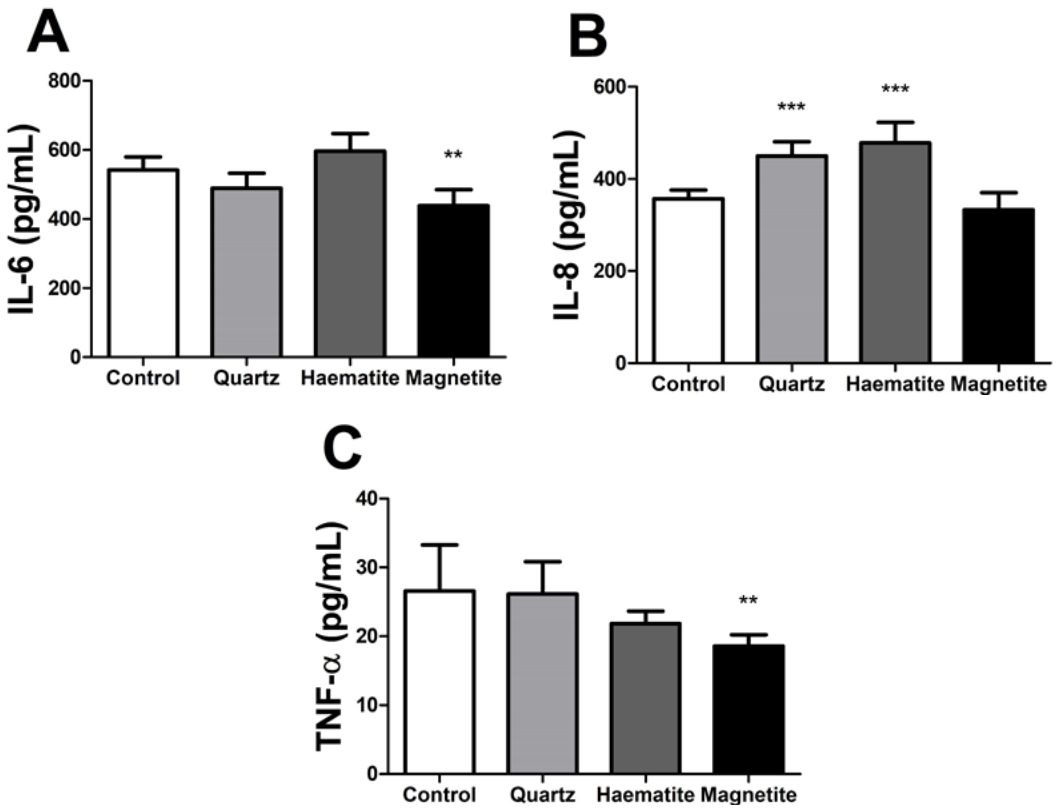


Figure 5.14: BEAS-2B bronchial epithelial cell supernatant analysed for inflammatory cytokines interleukin (IL)-6 (A), -8 (B) and tumour necrosis factor-α (C; TNF-α) after 24 hours of exposure to 75 μg/mL of quartz, haematite or magnetite. IL-1β levels were below detection

limits. Data are presented as mean(SD) from 6 independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$ respectively versus control.

5.5 Discussion

Regional Aboriginal Australian Aboriginal communities are exposed to high levels of geogenic inorganic particulate matter (Shepherd et al. 2019), dominated by silica and iron oxides (Zosky et al. 2014). These communities also have a disproportionate burden of chronic respiratory infections resulting in bronchiectasis (Blackall et al. 2018). The aim of this Chapter was to identify whether silica or iron oxide PM negatively impacted the respiratory cell response to NTHi infection. We observed that haematite substantially increased the rates of bacterial invasion in bronchial epithelial cells, while there was almost no response in alveolar epithelial cells. Similarly, both haematite and magnetite reduced macrophage phagocytosis of NTHi. There was a significant difference in these responses between NTHi strains, with protein binding capacity and metabolism linked to higher invasion in particle-exposed bronchial epithelial cells. While haematite was internalised more than the other particle types, all particles suppressed the expression of beta-defensins in bronchial cells and altered the inflammatory response to LPS. However, haematite induced significant upregulation of TLR-4 in response to LPS in bronchial epithelial cells.

Previous studies have shown that iron oxide can increase adhesion of NTHi to epithelial cells [14]. In this Chapter, the data suggest that iron oxide, a constituent of the red dust commonly found in areas where rates of bronchiectasis are high, increases bacterial invasion in epithelial cells. Cell invasion is a strategy used many pathogenic bacteria to evade the host immune response [358]. Interestingly, this response was only observed in haematite and was almost entirely absent in alveolar epithelial cells suggesting that particle chemistry and cell type influence this response. Both haematite and magnetite drastically reduced the ability of THP-

1-derived macrophages to phagocytose NTHi. However, this was not associated with bacterial genetics and was largely not replicated in primary macrophages suggesting that the effect of iron laden PM on the risk of bronchiectasis may be driven by their effect on bacterial invasion of bronchial epithelial cells.

In initial experiments it was found that low concentrations of haematite were sufficient to alter the bacterial response of bronchial epithelial cells. Currently there is little understanding on how haematite may impact the cell to facilitate this enhanced invasion by NTHi. Interestingly, bacterial invasion was significantly associated with particle endocytosis suggesting that this may explain the enhanced bacterial invasion. Both iron oxides would be expected to internalise at a higher rate given their smaller size (Chapter 2) than quartz [359]. However, an explanation for the significant difference in endocytosis by bronchial epithelial cells between haematite and magnetite is unclear. Both particle types are the same size but differ in valence, and while literature shows that a range of chemically different particles can be endocytosed by epithelial cells [359-361], there is currently no suggestion as to why they are internalised at different capacities.

"This Chapter is the first to demonstrate that silica and iron oxide PM suppresses the expression of beta-defensin 1 and 2. However, it must be acknowledged that gene and protein expression do not always equate, and gene expression is not indicative of protein function. However, the presence of such proteins should be examined with caution. Given their pH sensitivity, the presence of beta-defensins in neutral culture pH would be less effective against bacterial infection that when in the lung. While indicative of epithelial immune suppression, it does not

completely explain the invasive response observed in response to haematite. However, haematite significantly upregulated expression of TLR 4 in response to LPS. Previous literature shows that macrophage TLR-4 expression is altered in response to quartz [219]. While quartz did not alter TLR-4 expression in these epithelial cells, the increase expression of these surface receptors is consistent with the high levels of endocytosis of haematite and may be an adaptive response which may lead to an enhanced inflammatory response. This was observed in the case of the enhanced IL-8 production induced by haematite in response to LPS, however, this was also observed in response to quartz suggesting that it is not unique to haematite. The combined increase in bacterial invasion, reduced antimicrobial response, increase in TLR expression and inflammation induced by haematite suggests that this particle type, compared to quartz and magnetite, may pose the greatest risk to the severity of respiratory infection when inhaled.

NTHi are a unique opportunistic pathogen with large genetic heterogeneity [13]. In this Chapter, genomic analysis showed that all NTHi isolates had a large number of known virulence genes in their genome. Interestingly, there were no differences that could be associated with varying levels of invasion in the absence of particle exposure. Further association analysis suggested that presence of genes associated with transport of protein and organic metabolites were associated with higher invasion in cells exposed to quartz. Conversely, invasion by NTHi of epithelial cells exposed to haematite was associated with expression of molecular and compound binding genes, overall suggesting metabolic advantages in more invasive strains of NTHi, a paradigm apparent in haematite-exposed, but not exposure-free bronchial cells. Whilst these observations suggest fundamental differences in how bacteria can take advantage of the cell response to particle exposure to facilitate invasion, it is difficult to draw further conclusions

about the pathway the bacteria exploit without additional resolution. This analysis would greatly benefit from additional bacterial screening to identify pathways that could be tested in subsequent knockdown experiments

In summary, post-infective bronchiectasis can result from chronic inflammation linked to persistent respiratory tract infections. Exposure of individuals living in regional Australian Aboriginal communities to geogenic PM have been linked to the high prevalence of respiratory infections in these areas. Data from this Chapter suggest that exposure to iron laden geogenic PM can increase the capacity of NTHi to invade bronchial epithelial cells and that the effect is most pronounced for haematite. Invasion allows pathogenic bacteria to persist and avoid immune detection. This may lead to a persistent respiratory infection which is required for the development of post-infective bronchiectasis. This study provides evidence that iron oxide particles pose a significant threat to respiratory health.

Chapter 6: General Discussion

6.1 Discussion of findings

This thesis aimed to assess the impact of geogenic PM, in particular the iron oxide component, on respiratory health and susceptibility to bacterial infection as an explanation for the high burden of post-infective bronchiectasis in Australian Aboriginal communities. Overall, iron oxide induced no cytotoxicity and low levels of inflammation in epithelial cells (Chapter 2) and macrophages (Chapter 3) but clearly exacerbated the LPS induced inflammatory response in macrophages (Chapter 3). This suggests that iron oxide can modify the respiratory bacterial response. In addition, in some instances, iron oxide promoted the growth of the respiratory pathogen non-typeable NTHi (Chapter 4). Subsequent experiments showed that iron oxide, particularly haematite, enhanced NTHi invasion in epithelial cells and impaired macrophage phagocytosis of NTHi (Chapter 5). The increased invasion was linked to increased particle uptake and expression of Toll-like receptor (TLR)-4. Collectively these data suggest that haematite increases rates of bacterial infection in the lung by directly increasing the growth of NTHi and also increasing the susceptibility of bronchial epithelial cells to bacterial infection; most likely by suppressing the ability of the cell to respond appropriately to NTHi. Taken together, these data suggest that the haematite in geogenic PM is most likely the primary driver of the increased prevalence of respiratory infections, and possibly bronchiectasis, in remote Australian Aboriginal communities.

Initial experiments confirmed the cytotoxic and pro-inflammatory effects of silica on epithelial cells (Chapter 2) which is consistent with the existing literature [167,170,362,215]. In contrast, iron oxide was not cytotoxic and only caused a minimal inflammatory response; an increase in IL-8 production that was only observed in alveolar epithelial cells. This suggests that inhalation

of iron-laden PM alone is unlikely to induce severe epithelial inflammation, compared to other sources of PM such as DEP or silica [336,363,305,110,151].

In subsequent experiments, conducted using similar testing of the pro-inflammatory potential of these particles on another important lung cell, macrophages, silica was both cytotoxic and pro-inflammatory as expected [170,295,269]. This effect, while also present, was not as prominent in response to iron oxide (Chapter 3). Interestingly, when these cells were exposed to LPS in combination with particles, the iron oxide response was comparable to the silica response. With prior bacterial stimulation (LPS), macrophages showed an enhanced inflammatory response to both haematite and magnetite. In particular, there was upregulation of a range of NF-κB-activated cytokines including IL-1β, IL-8 and IL-6, suggesting a role for increased activation of TLR-4.

While there is evidence to suggest that exposure to inhalable inorganic PM is associated with worse respiratory infections [302,14], no studies have considered the direct effect of this PM on bacterial growth. While silica had little influence on the growth of NTHi, both iron oxides increased the growth of some NTHi strains (Chapter 4). This novel observation suggests that geogenic PM may have the capacity to directly influence bacterial growth, however, this response was only observed in some strains, so the biological relevance and potential health implications of these data are unclear. As a result, while iron oxide can modify the growth of NTHi, it is unlikely to be the main driver of the association between exposure to iron oxide

laden PM and respiratory infections. Nonetheless, these data highlight the importance of assessing between strain differences in bacterial responses to environmental stimuli.

The aim of the final series of experiments was to determine the effects of iron oxide particles on the ability of epithelial cells and macrophages to respond to NTHi (Chapter 5) with a particular focus on bacterial invasion in epithelial cells and macrophage phagocytosis. While silica caused a pro-inflammatory response in these cells (Chapters 2 & 3), it had little effect the internalization of NTHi. In direct contrast, while iron oxide causes a minimal inflammatory response, haematite caused a substantial increase in NTHi invasion of epithelial cells while both haematite and magnetite decreased NTHi phagocytosis. However, this result was, largely, not replicated in primary macrophages and, given the association between iron oxide induced alterations in NTHi invasion of BEAS-2B cells and the bacterial genome, further investigations into the cellular response by these cells were conducted.

It was found that bronchial epithelial cells had signification suppression of anti-microbial genes, beta-defensin-1 and -2, (Chapter 5) suggesting that geogenic PM may suppress the anti-microbial response. However, haematite was the only particle type that altered (increased) TLR-4 expression and had the highest level of endocytosis. The mechanisms of this observation are unclear, and it is difficult to determine cause and effect, however there is clearly a haematite specific effect on the bronchial epithelial cell response to NTHi that leads to increased invasion of the bacteria. It is possible that the increased endocytosis of haematite can act as a vehicle for NTHi entry into the cell; an effect that is likely to significantly increase

bacterial pathogenicity. The increase in TLR-4 expression may be an adaptive response to the increased bacterial invasion. This increase in NTHi invasion in response to haematite is both unsurprising, given the much smaller size when compared to quartz, and surprising given the similar physical characteristics compared to magnetite. This highlights the importance of chemistry when considering the health effects of PM. Similarly, while it was not possible to identify factors in the NTHi genome that could explain the strain variations in the response to PM, the variability that was observed also highlights the importance of considering multiple strains when assessing the effect of environmental stimuli on bacterial infection.

The disproportionate burden of bronchiectasis in Indigenous Australian communities has been recognized for several years [8,11]. However, new evidence has emerged suggesting that exposure to geogenic dust may be contributing to this disease burden [14,15]. Our understanding of health effects of geogenic PM exposure is limited. Shepherd *et al* (2019) showed that geogenic PM increases the invasion of NTHi in epithelial cells [14]. However, the effects of specific geogenic PM constituents such as silica and iron oxide are largely unknown. The data that does exist suggests that the presence and concentration of iron oxide in geogenic PM is correlated with poor lung function and inflammation [16] and impairs viral clearance [304]. Further evidence suggests that the addition of iron oxide to silica may augment the response when compared to silica alone [187]. The data presented in this Thesis are the first to demonstrate a role for iron oxide in increasing the severity of the response to bacterial infection by augmenting the bacterial inflammatory response in macrophages and increasing bacterial pathogenicity by promoting bacterial invasion in epithelial cells and damping the anti-microbial response. This is a significant step in our understanding of the health effects of

geogenic PM. Collectively, these observations provide a plausible biological mechanism by which exposure to iron laden geogenic PM, such as the situation that often occurs in remote Australian Aboriginal communities, may contribute to the development of bronchiectasis.It is important to note that this Thesis refutes the existing dogma that iron oxide is biologically inert [270]. The initial experiments presented (e.g. Chapter 2 and 3) are consistent with the limited pro-inflammatory effect of iron oxide which has been used to argue that iron oxide PM is a low health risk. However, later experiments (Chapter 3, 4 and 5) clearly demonstrate this is not the case which has important implications for community health. It is also interesting to note that the effect was dependent on iron oxide chemistry suggesting that areas where the haematite content of geogenic PM is highest may be most at risk.

Future studies should assess the effect of iron oxide on other pathogens. For example, *Pseudomonas aeruginosa*, which is the second most prevalent pathogen in bronchiectasis [17,31,6]. Furthermore, the *in vitro* work would be greatly complemented by translation into *in vivo* models to determine their effect on bacterial responses in the setting of a complete immune system. Next, further studies are needed to identify the key cellular, molecular and bacterial mechanisms that explain the increased invasion of NTHi observed in bronchial epithelial cells exposed to haematite. Our data suggests this could be a mechanism within the cell membrane and how iron oxide interacts with the membrane to leave it more susceptible to bacteria. An important aspect missing from these studies is the presence of bronchiectasis samples. The addition of primary bronchiectasis epithelial cells would allow a cross comparison of healthy cells and cells exposed to iron oxide and the relative effects of NTHi invasion. Neutrophil function is critical in the pathogenesis of excessive inflammation and, *in vivo*, geogenic particles increase neutrophil recruitment. However, this was an acute exposure

and the long-term influences on cytokine dysfunction are unknown. Given the effect of iron oxide on macrophages, it is plausible that similar effects may be seen in neutrophils and is an area that warrants further investigation. Lastly, we observed several responses that included a lack of inflammation in response to iron oxide (Chapter 2), yet a pro-bacterial response (Chapter 5). This Thesis focused on proinflammatory cytokines. However, it is possible that the results in from Chapter 2 and 5 are linked through a suppression of protective cytokines. Future research should investigate the influence of iron-laden particulates on cytokine such as IL-10, IL-27 or other anti-bacterial proteins.

6.2 Limitations

This Thesis had several limitations which should be acknowledged. Firstly, the experiments were conducted entirely in vitro. While it incorporated the use of human cell lines and primary human cells, which is a strength, it was not conducted in the context of a fully functioning lung or immune system. This Thesis also utilized submerged monolayer cell cultures. Various cell culture models such as air-liquid interface culture with primary cells from healthy or disease-state patients would have represented more advanced in vitro approaches. These models would be especially important in investigating the effects of these exposures on unwell individuals. Co-cultures would have also provided further insight into the interactions between airway cells. One of the larger downsides of submerged cultures is the lack of airway interface, including secretions of airway cells resulting is peptides and structures such as airway surface liquid. This lining holds importance in host defence against pathogens and particles through endogenous anti-bacterial peptides and mucous traps which. The interaction of iron-laden particles and these structures is of importance, especially in Cystic Fibrosis patients. However,

working within time constraints we chose to focus on the fundamental effects of these particles across a large number of variables to advance our knowledge before addressing more advanced and specific questions.

6.3 Conclusions

In conclusion, this Thesis has demonstrated the potential of iron oxide particles to enhance respiratory bacterial infection. This provides further evidence for a causal link between exposure to geogenic PM and bronchiectasis in Australian Aboriginal communities. The findings in this Thesis contribute to a greater understanding of the environmental influences on lung health. This work has public health implications and provides evidence to advocate for geogenic PM suppression, control and avoidance. By controlling these exposures, it may be possible to reduce the burden of chronic respiratory disease in susceptible communities. By controlling these exposures, it may be possible to reduce the burden of chronic respiratory disease in susceptible communities that extend to all regional communities around Australia.

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Appendix: Published Manuscripts

It is the following published article:
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AIRWAY BIOLOGY

The Inflammatory Effect of Iron Oxide and Silica Particles on Lung Epithelial Cells

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Abstract

Purpose Our understanding of the respiratory health consequences of geogenic (earth-derived) particulate matter (PM) is limited. Recent in vivo evidence suggests that the concentration of iron is associated with the magnitude of the respiratory response to geogenic PM. We investigated the inflammatory and cytotoxic potential of silica and iron oxide particles alone, and in combination, on lung epithelial cells.

Methods Bronchial epithelial cells (BEAS-2B) were exposed to silica (quartz, cristobalite) and/or iron oxide (hematite, magnetite) particles. Cytotoxicity and cytokine production (IL-6, IL-8, IL-1β and TNF-α) were assessed by LDH assay and ELISA, respectively. In subsequent experiments, the cytotoxic and inflammatory potential of the particles was assessed using alveolar epithelial cells (A549).

Results After 24 h of exposure, iron oxide did not cause significant cytotoxicity or production of cytokines, nor did it augment the response of silica in the BEAS2-B cells. In contrast, while the silica response was not augmented in the A549 cells by the addition of iron oxide, iron oxide particles alone were sufficient to induce IL-8 production in these cells. There was no response detected for any of the outcomes at the 4 h time point, nor was there any evidence of IL-1β or TNF-α production.

Conclusions While previous studies have suggested that iron may augment silica-induced inflammation, we saw no evidence of this in human epithelial cells. We found that alveolar epithelial cells produce pro-inflammatory cytokines in response to iron oxide particles, suggesting that previous in vivo observations are due to the alveolar response to these particles.

Keywords Iron oxide particles · Silica particles · Epithelial cells · Inflammation

Introduction

Particulate matter (PM) inhalation is strongly associated with an increased risk of respiratory disease, cardiovascular disease and overall mortality [1–5]. The sources of PM vary considerably between locations. For example, urban populations are typically exposed to PM derived from combustion sources; in particular, diesel exhaust particles (DEP) which have been extensively studied due to their impact on the pathogenesis of respiratory disease [6, 7]. In contrast, crustal, or geogenic (earth-derived) particles often affect populations in arid areas. Our understanding of the respiratory health impacts from these sources of PM is much more limited [8].

Inhalation of geogenic PM is associated with increased mortality [9–11] and hospital admissions [12]. In experimental models, inhalation of geogenic PM results in oxidative stress, release of pro-inflammatory mediators, reduced lung mechanics and exacerbation of viral infections [13–17]. In vitro, geogenic PM increases interleukin (IL)-6 and IL-8 production in bronchial epithelial cells [18] and tumor necrosis factor-α (TNF-α) and reactive oxygen species (ROS) in alveolar macrophages [19].

Oxides of silicon, aluminum and iron typically dominate geogenic PM. Silica (SiO_2) is well known in the occupational setting for causing chronic lung disease [20] due to its capacity to cause inflammation [21, 22], cytotoxicity [23], DNA damage [24] and oxidative stress [25]. The effect of aluminum oxides on respiratory health is less well studied,

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but the general consensus is that these particles are biologically inert when inhaled [26, 27]. In contrast, data on the effect of iron oxides are contradictory. Epidemiologically, there is some evidence to suggest that exposure to iron oxide causes respiratory morbidity and in vivo studies have shown strong associations between the iron concentration in geogenic PM, inflammation, deficits in lung mechanics and the capacity of the particles to exacerbate viral infection [15–17]. However, this is not always the case with some studies suggesting that insoluble iron oxides are biologically inert [28]. In contrast, some studies have suggested that the presence of particulate iron may synergistically enhance the silica-induced respiratory response [29].

In light of the controversy regarding the effect of iron oxide laden particles on respiratory health in vivo, we investigated the inflammatory and cytotoxic potential of iron oxide (Fe_2O_3 and Fe_3O_4) particles, alone and in combination with silica, on lung epithelial cells to provide further insight into the potential health implications of inhalation of these particles.

Methods

Particle Preparation

Commercially available standard preparations of dry magnetite (Fe_3O_4; Sigma-Aldrich 310069), hematite (Fe_2O_3; Sigma-Aldrich 310050), α-quartz (SiO_2; NIST 1878B) and cristobalite (SiO_2; NIST 1879A) were used. We assessed the effect of hematite (Fe^{2+}) and magnetite (Fe^{3+}) as the predominant forms of geogenic iron oxide. Particle samples were exposed to UV light for 2 h to remove any endotoxin contamination.

Particle Characteristics

See the online Supplement for details of the particle characterization.

Cell Culture

The transformed human bronchial epithelial cell line, BEAS-2B (ATCC CRL-9609), was cultured in 75 cm^2 flasks (Corning CLS3290), using serum-free bronchial epithelial growth medium (BEGM; Lonza CC-33170). The human lung alveolar epithelial cell line (A549; lung adenocarcinoma, ATCC CCL-185) was cultured in 75 cm^2 flasks (Corning CLS3290) with Ham's F-12K medium (Gibco 21127022), supplemented with 10% fetal bovine serum and 1% glutamine and antibiotics. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

Cell Exposure Trials

Cells were seeded onto 12- and 96-well plates (Corning, CLS3512 & CLS3300) at a concentration of 1.9×10^5 cells/mL. To investigate the dose-dependent effects of iron oxide and silica individually, cells were exposed to 0 µg/mL, 0.38 µg/mL, 3.8 µg/mL, 19 µg/mL, 38 µg/mL or 57 µg/mL (0–15 µg/cm^2) of each particle type. Concentrations were chosen to be consistent with similar PM toxicology studies [30–34]. Cells were exposed for 4 or 24 h. Having established the dose-dependent effects of the individual particle types, we then assessed the impact of silica and iron in combination on the response. Cells were exposed to either a 2:1 silica/iron ratio, which reflects the proportion of these elements in real-world particles [15], or a 20:1 ratio to replicate a situation where iron particles are present in trace amounts [35]. Having established the response in BEAS-2B cells, we then repeated a subset of experiments in the A549 alveolar epithelial cell line. We assessed a range of outcomes including cytotoxicity and cytokine production. All experiments were replicated in six independent trials conducted using fresh preparations of particle solutions and cell cultures to allow valid statistical comparisons between exposure groups.

Cytotoxicity

The lactate dehydrogenase (LDH) assay (Promega G1780) was used as a marker of cytotoxicity. LDH levels were measured after 24 h of exposure according to the manufacturer's instructions. Briefly, 50 µL of LDH buffer was added to 50 µL of supernatant in a 96-well plate, incubated at room temperature and removed from light for 30 min. The absorbance was then read at 490 nm using the Spectra Max M2 plate-reader (Molecular Devices, USA).

Inflammatory Cytokines

Inflammatory cytokines were assessed by enzyme-linked immunosorbent assay (ELISA). We assessed levels of human interleukin-1β (IL-1β; R&D Systems DY201), interleukin-6 (IL-6; R&D Systems DY206), interleukin-8 (IL-8; R&D Systems DY208) and tumor necrosis factor-α (TNF-α; R&D Systems DY210) in the cell supernatant according to the manufacturer's instructions. The minimum detection limits for IL-1β, IL-6, IL-8 and TNF-α were 7.81, 9.38, 31.3 and 15.6 pg/mL, respectively. Plates were read using a Spectra Max M2 plate-reader (Molecular Devices, USA) at 450/570 nm absorbance.

Statistical Analysis

Comparisons between groups were made using repeated measures one-way ANOVA. When significance was

determined for the main factors by ANOVA, the Holm–Sidak post hoc test was used to examine individual between group differences. Where necessary, the data were log transformed to satisfy the assumptions of normal distribution of the error terms and homoscedasticity of the variance. All data are presented as mean (SD), and values of $p < 0.05$ were considered statistically significant. All statistical analyses were conducted using SigmaPlot (v12.5).

Results

Assessment of Particle Structure

Cristobalite (Fig. S1A) and quartz (Fig. S1B) particle size ranged from 2 to 6 µm in diameter while hematite (Fig. S1C) and magnetite (Fig. S1D) particle size ranged from 0.2 to 0.8 µm aerodynamic diameter. See online Supplement for further details.

Response to Individual Particles Types (BEAS-2B)

Cytotoxicity

Exposure of BEAS-2B cells for 24 h to cristobalite (Fig. 1a, $p = 0.017$) or quartz (Fig. 1b, $p = 0.009$) elicited an increase in LDH levels at 57 µg/mL compared to control. Hematite (Fig. 1c, $p = 0.392$) and magnetite (Fig. 1d, $p = 0.708$) had no effect on LDH levels following 24 h of exposure. There was no change in LDH levels in response to any particle type 4 h post-exposure ($p > 0.05$) (*data not shown*).

Cytokines

Exposure for 24 h to cristobalite (Fig. 2a, $p = 0.045$) or quartz (Fig. 2b, $p = 0.009$) elicited an increase in IL-6 levels at 57 µg/mL. Hematite (Fig. 2c, $p = 0.133$) and magnetite (Fig. 2d, $p > 0.250$) had no effect on IL-6 levels. There was

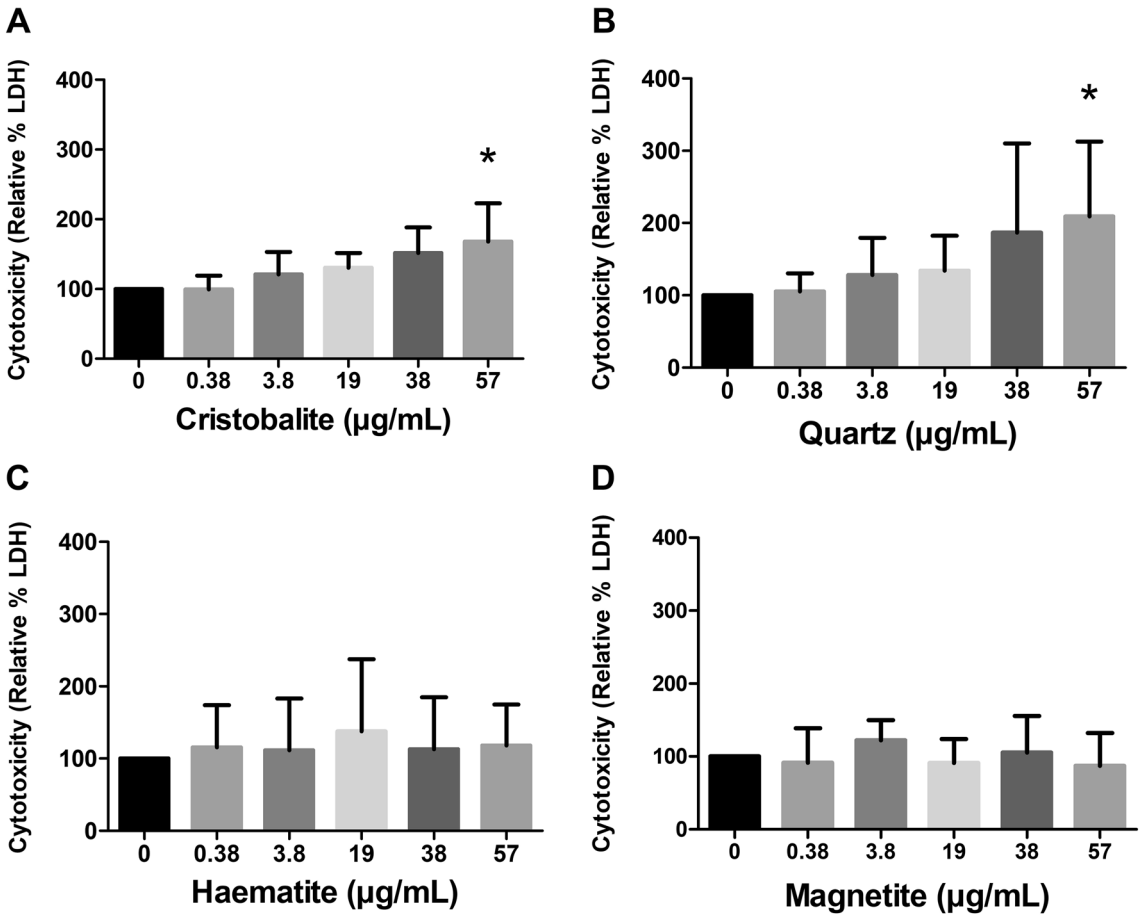

Fig. 1 LDH levels in the supernatant of BEAS-2B cells exposed to cristobalite (**a**), quartz (**b**), hematite (**c**) or magnetite (**d**) for 24 h. Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented as mean (SD) from six independent replicates with asterisk indicating $p < 0.05$ versus control. Both cristobalite (**a**) and quartz (**b**) caused a significant increase in LDH, but only at a dose of 57 µg/mL ($p = 0.017$ and $p = 0.009$). Hematite (**c**; $p = 0.392$) and magnetite (**d**; $p = 0.708$) had no effect on LDH levels

no change in IL-6 levels in response to any particle type 4 h post-exposure ($p > 0.05$) (*data not shown*).

Exposure for 24 h caused increased IL-8 for cristobalite at 38 µg/mL (Fig. 3a, $p = 0.031$) and 57 µg/mL (Fig. 3a, $p < 0.001$). Quartz elicited an increase in IL-8 levels at 57 µg/mL (Fig. 3b, $p = 0.011$). Hematite (Fig. 3c, $p = 0.857$) and magnetite (Fig. 3d, $p = 0.775$) had no effect on IL-8 levels following 24 h of exposure. There was no change in IL-8 levels in response to any particle type 4 h post-exposure ($p > 0.05$) (*data not shown*). Tumor necrosis factor-α and interleukin-1β were measured, however all results were under the detection threshold (*data not shown*).

Combined Effect of Silica and Iron Oxide (BEAS-2B)

In initial experiments, described above, we determined the dose-dependent cytotoxicity, cell metabolism and cytokine response to individual particle types. Subsequently, cells were exposed to combinations of particles to determine whether the silica-induced response was altered by the presence of iron oxide. For the combined exposure experiments, we chose to focus on the modifying effect of magnetite and hematite on the cristobalite-induced response.

Cytotoxicity

When exposed for 24 h, neither cristobalite–hematite (Fig. 4a, $p = 0.096$) nor cristobalite–magnetite ($p = 0.253$) combinations elicited an increase in LDH levels in BEAS-2B cells above the cristobalite-induced response.

Cytokines

38 µg/mL of cristobalite in combination with hematite (Fig. 4b, 1.9 µg/mL $p = 0.005$ and 19 µg/mL $p = 0.04$) or magnetite (1.9 µg/mL $p = 0.011$ and 19 µg/mL $p = 0.012$) caused increased levels of IL-6 compared to controls when cells were exposed for 24 h. However, neither the addition of hematite

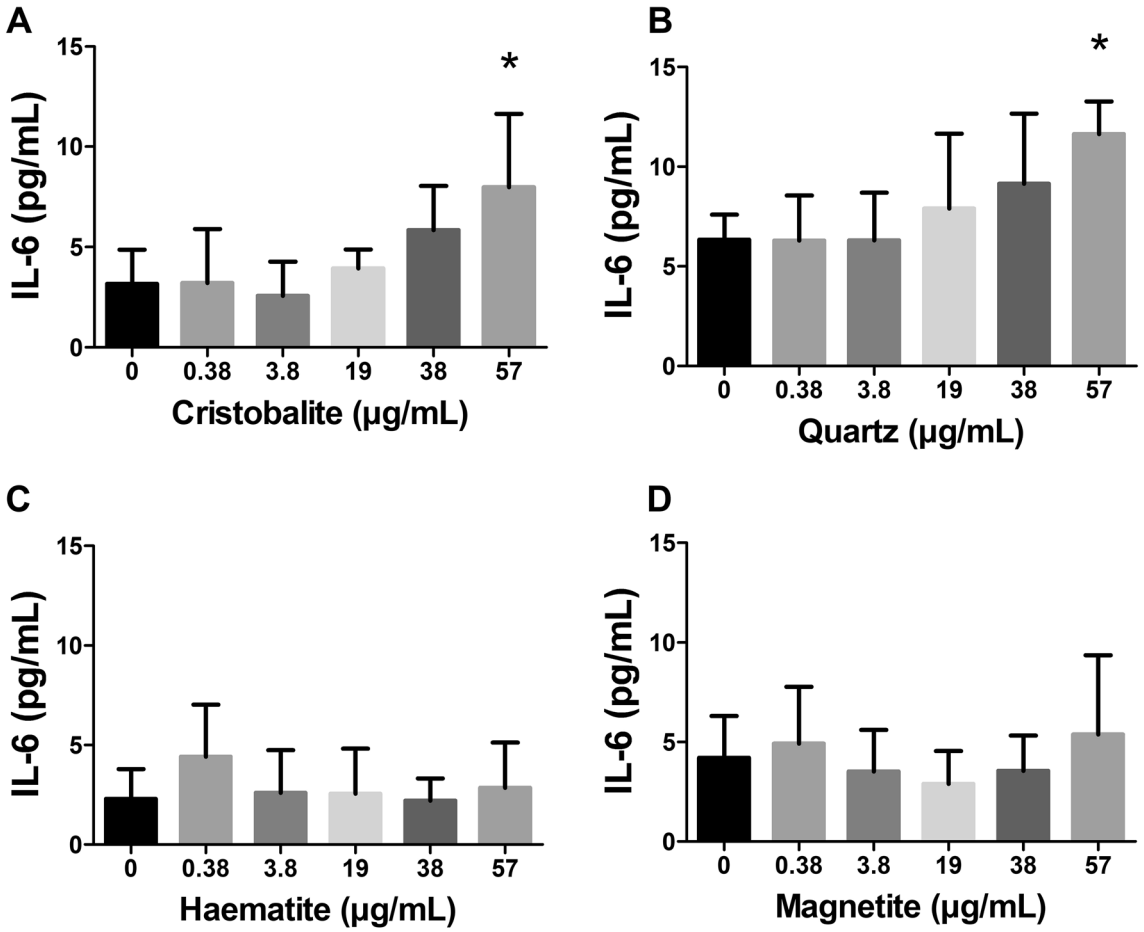

Fig. 2 Interleukin-6 (IL-6) levels in the supernatant of BEAS-2B cells exposed to cristobalite (**a**), quartz (**b**), hematite (**c**) or magnetite (**d**) for 24 h. Data are presented as mean (SD) from six independent replicates with asterisk indicating $p < 0.05$ versus control. Both cristobalite (**a**) or quartz (**b**) caused a significant increase in IL-6, but only at a dose of 57 µg/mL ($p = 0.045$ & $p = 0.009$). Hematite (**c**; $p = 0.133$) or magnetite (**d**; $p = 0.250$) had no effect on IL-6 levels

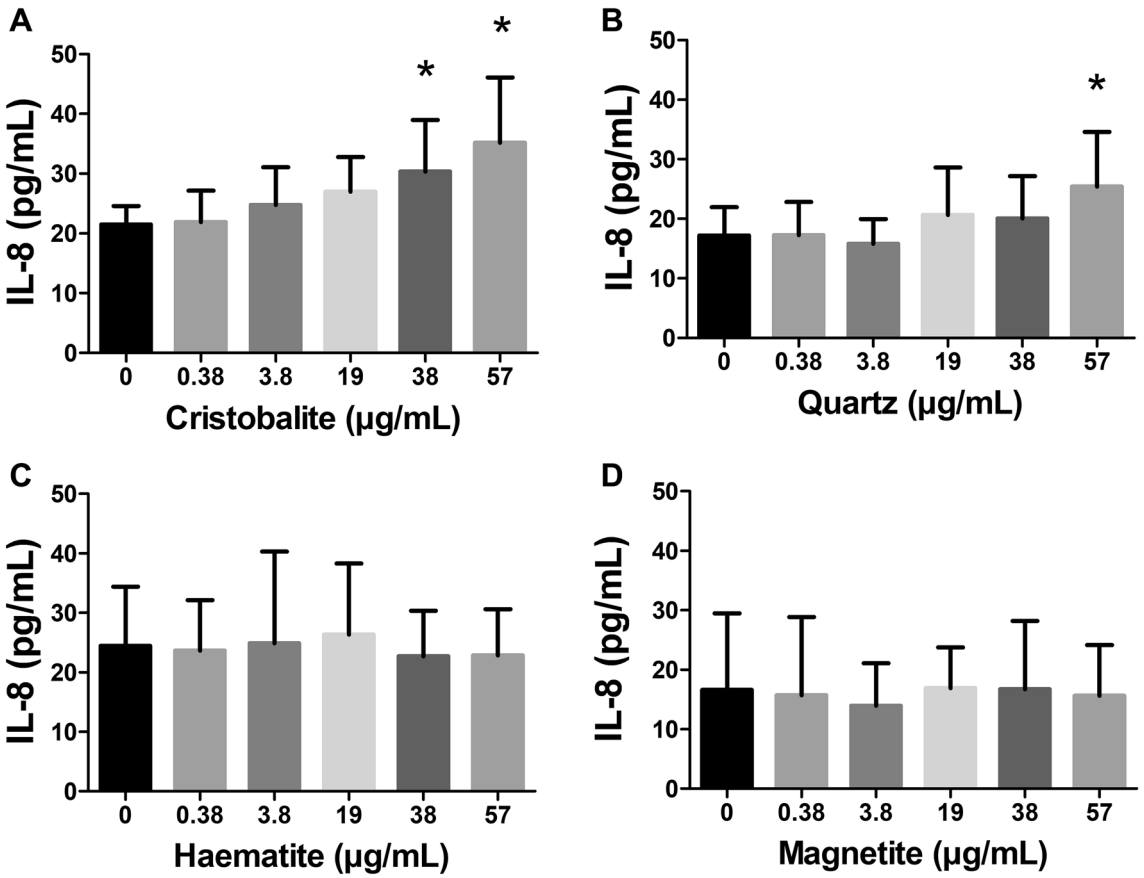

Fig. 3 Interleukin-8 (IL-8) levels in the supernatant of BEAS-2B cells exposed to cristobalite (**a**), quartz (**b**), hematite (**c**) or magnetite (**d**) for 24 h. Data are presented as mean(SD) from 6 independent replicates with asterisk indicating $p<0.05$ versus control. Cristobalite (**a**) caused a significant increase in IL-8 at doses of 38 µg/mL ($p=0.031$) and 57 µg/mL ($p<0.001$). Quartz (**b**) caused a significant increase in IL-8 but only at 57 µg/mL ($p=0.011$). Both hematite (**c**; $p=0.857$) and magnetite (**d**; $p=0.775$) had no effect on IL-8 levels

(Fig. 4b, 1.9 µg/mL $p=0.207$ and 19 µg/mL $p=0.649$) nor magnetite (1.9 µg/mL $p=0.933$ and 19 µg/mL $p=0.890$) significantly increased the IL-6 response compared to 38 µg/mL of cristobalite alone.

38 µg/mL of cristobalite alone (Fig. 4c, $p=0.021$) and in combination with either concentration of hematite (1.9 µg/mL $p<0.001$ and 19 µg/mL $p=0.001$) or of magnetite (1.9 µg/mL $p=0.035$ and 19 µg/mL $p=0.037$) caused increased levels of IL-8 when cells were exposed for 24 h. However, neither the addition of hematite (Fig. 4c, 1.9 µg/mL $p=0.207$ and 19 µg/mL $p=0.246$) nor magnetite (1.9 µg/mL $p=0.920$ and 19 µg/mL $p=0.913$) significantly increased the IL-8 response compared to 38 µg/mL of cristobalite alone. Tumor necrosis factor-α and interleukin-1β were measured; however, all results were under the detection threshold (*data not shown*).

Combined Effect of Silica and Iron Oxide: The Effect of Cell Type (A549)

Initial BEAS-2B experiments determined that both hematite and magnetite did not modify the silica-induced response. In order to test whether this observation is consistent in other cell types we also assessed the response in A549 cells, an alveolar type II epithelial cell line.

Cytotoxicity

There was no evidence of cytotoxicity in A549 cells in response to cristobalite and/or hematite (Fig. 5a, $p=0.157$) or magnetite ($p=0.106$).

Cytokines

In contrast to the BEAS-2B cells, exposure to cristobalite (Fig. 5b, $p<0.001$) and hematite ($p=0.008$), but not magnetite ($p=0.06$), alone were sufficient to increase IL-8 levels. The combined effect of cristobalite and hematite was equivalent to the effect of the individual exposures (Fig. 5b, $p=0.74$). TNF-α, IL-1β and IL-6 were measured in the A549 cells; however, all results were under the detection threshold.

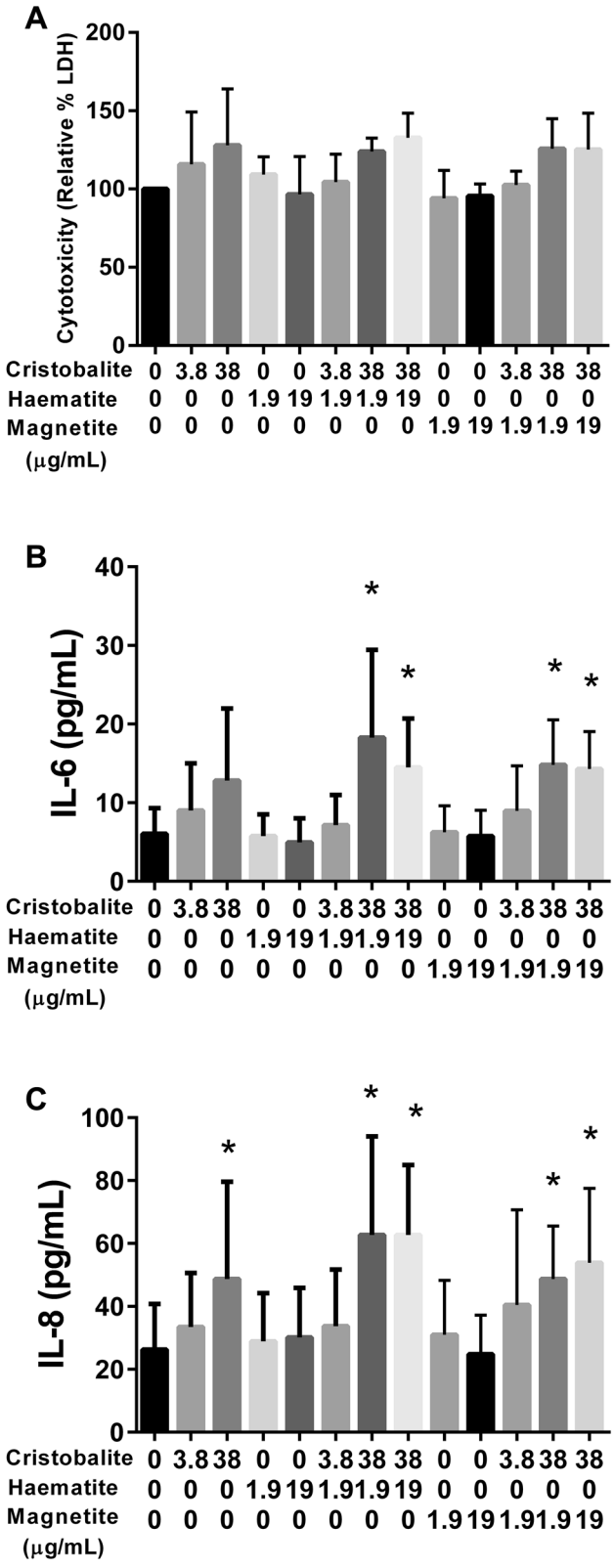

Fig. 4 Supernatant of BEAS-2B cells exposed to cristobalite–hematite or cristobalite-magnetite combinations for 24 h were assessed for relative LDH (**a**), IL-6 (**b**) and IL-8 (**c**). Data are presented as mean(SD) from six independent replicates with asterisk indicating $p<0.05$ versus control. Both cristobalite–hematite (Fig. 4a, $p=0.096$) and cristobalite–magnetite ($p=0.253$) had no effect on LDH levels compared to cristobalite treatment. The addition of hematite or magnetite to 38 μg/mL of cristobalite caused an increase in IL-6. However, the addition of hematite (Fig. 4b; 1.9 μg/mL $p=0.207$ & 19 μg/mL $p=0.649$) or magnetite (1.9 μg/mL $p=0.933$ and 19 μg/mL $p=0.890$) was not significantly greater than the response induced by 38 μg/mL of cristobalite alone. Likewise, the addition of hematite or magnetite to 38 μg/mL of cristobalite caused an increase in IL-8, however, the addition of hematite (Fig. 4c; 1.9 μg/mL $p=0.207$ and 19 μg/mL $p=0.246$) or magnetite (1.9 μg/mL $p=0.920$ and 19 μg/mL $p=0.913$) was not significantly greater than the response induced by 38 μg/mL of cristobalite alone

Discussion

The present study aimed to investigate the effect of iron oxide, alone and in combination with silica, on the inflammatory response in respiratory epithelial cells to determine whether these cells are responsible for the observed association between iron content and the inflammatory response induced by geogenic particles observed in vivo [15, 16]. Collectively, our data from BEAS-2B cells, a bronchial epithelial cell line, suggest that iron oxide has no effect on inflammatory cytokine production, nor do these particles exacerbate the silica-induced response. In contrast to the lack of response observed in the BEAS-2B cells, iron oxide particles induced IL-8 production in A549 cells, although they did not enhance the response induced by silica. These data suggest that alveolar, but not bronchial, epithelial cells may be partly responsible for the association between the iron content and the inflammatory response to geogenic PM observed in vivo [15].

Using relatively low doses of particles compared to similar toxicological studies [36–38], we found that silica caused mild cytotoxicity and induced the production of IL-6 and IL-8 in BEAS-2B cells and IL-8 release in A549 cells. This is largely consistent with the wealth of literature on the known pro-inflammatory effect of silica [20] on BEAS-2B [22] and A549 cells [38]. There was no difference in the response between cristobalite and quartz, which is perhaps not surprising given the similarities in particle structure we observed. IL-1β and TNF-α release have long been associated with silica exposure in animal models [39, 40]. Based on our data, secretion of these cytokines in vivo is most likely attributable to another cell type, such as macrophages [25, 40, 41].

In contrast, iron oxide, in the form of both hematite (Fe^{2+}) and magnetite (Fe^{3+}), was not cytotoxic at the doses used nor did it have any impact on the production of IL-6 and IL-8 by BEAS-2B cells or the silica-induced IL-6 and IL-8 response. However, while neither were cytotoxic in A549 cells, both iron oxides elicited IL-8 release. This is consistent with previous epidemiological studies showing a positive correlation between exposure to iron oxide laden

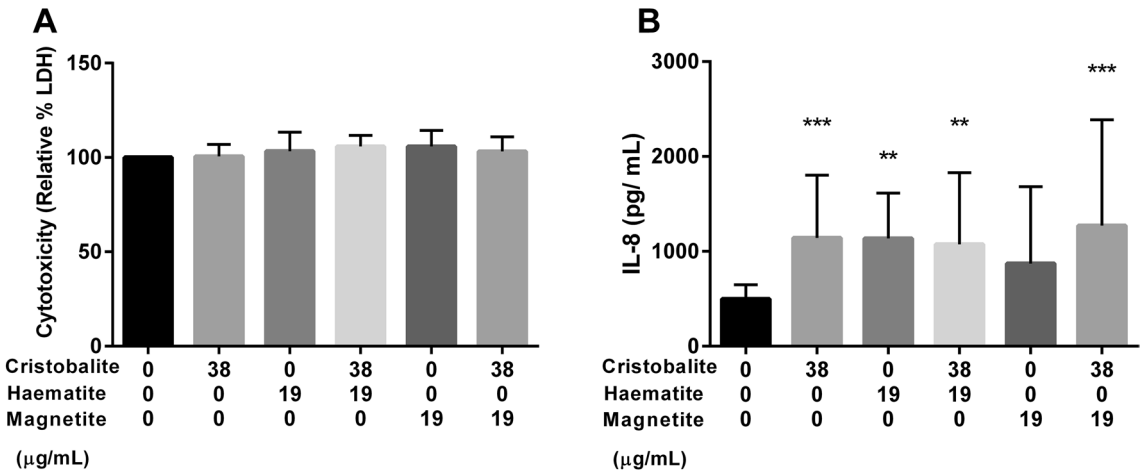

Fig. 5 Supernatant of A549 cells exposed to cristobalite–hematite or cristobalite–magnetite combinations for 24 h were assessed for relative LDH (**a**) and IL-8 (**b**). Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented as mean(SD) from six independent replicates with * indicating $p<0.05$ versus control. Both cristobalite–hematite (Fig. 5a, $p=0.157$)and cristobalite–magnetite ($p=0.106$) had no effect on LDH levels. Cristobalite (Fig. 5b, $p<0.001$), hematite ($p=0.008$), cristobalite–hematite ($p=0.001$) and cristobalite–magnetite ($p<0.001$) had significant effects on IL-8 levels

PM and adverse health outcomes [42, 43] but is inconsistent with previous studies suggesting that iron oxide PM may be relatively inert [28].

It is generally thought that any cellular damage induced by iron is driven by the Fenton redox reaction whereby Fe^{2+} is converted into Fe^{3+} and a hydroxyl radical is produced [44]. Theoretically, with prolonged exposure to Fe^{2+}, this results in excessive production of radical oxygen species. This requires the presence of free Fe^{2+} which is dependent on the solubility of the iron compound. However, free iron rarely exists in nature [45] and the common forms used in this study, hematite and magnetite are largely insoluble at physiological pH. This implies that without a catalyst, there is no dissociated Fe^{2+} and no potential for a Fenton-like reaction to occur. While it has not been determined whether the previously studied geogenic samples contained dissociated Fe^{2+}, Lay et al. [46] suggest only small amounts of iron (0.036% dissociation) are necessary to produce significant amounts of radical oxygen species. It is unlikely that there was sufficient free iron in our system to induce this response. Given that it is unlikely that high enough concentrations of free iron were liberated in our cell culture system, the increase in cytokine production in the A549 cells suggests that this is a direct effect of the particles on the cells.

In accordance with our data, silica has previously been demonstrated to elicit IL-8 release in A549 cells [47]. There is some evidence to suggest magnetite can induce genotoxicity and cytokine release [48]. Interestingly, Konczol et al. [48] saw no cytotoxicity or genotoxicity, which is consistent with our data. Of note is the fact that the combined effect of silica and iron oxide on cytokine production was not greater than the effects of the individual particle types. It is likely that this is a threshold effect whereby the maximum production of IL-8 by these cells was reached.

IL-8 is a neutrophil chemoattractant and is key in recruiting neutrophils to a site of infection [49]. Recruitment of neutrophils results in endocytosis of invading pathogens and subsequent release of proteases and oxidant products [50]. Neutrophils naturally undergo autophagy; however, excessive or chronic IL-8 may lead to a disruption in the equilibrium of neutrophilic processes leading to excess and prolonged release of proteases and ROS and reduced anti-microbial function, which may result in damage to the lung tissue [51–53]. Our data suggest that exposure of alveolar cells to iron oxide containing particles may lead to tissue damage as a result of IL-8 production, an observation which is consistent with the long-term deficits in lung function that are observed in vivo [15].

In summary, we found that iron oxide particles can induce an inflammatory response in alveolar epithelial cells, but appear to have no effect on bronchial cells. The iron oxide particles had no effect on the inflammatory response induced by silica, suggesting that the association between iron levels in geogenic particles and the inflammatory response in vivo is a direct effect of iron oxide. Collectively, these data highlight the importance of the iron oxide when considering the health implications of geogenic PM.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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