Lung (2019) 197:199–207
https://doi.org/10.1007/s00408-019-00200-z

AIRWAY BIOLOGY

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

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Received: 13 June 2018 / Accepted: 6 February 2019 / Published online: 14 February 2019

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,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00408-019-00200-z) contains supplementary material, which is available to authorized users.

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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

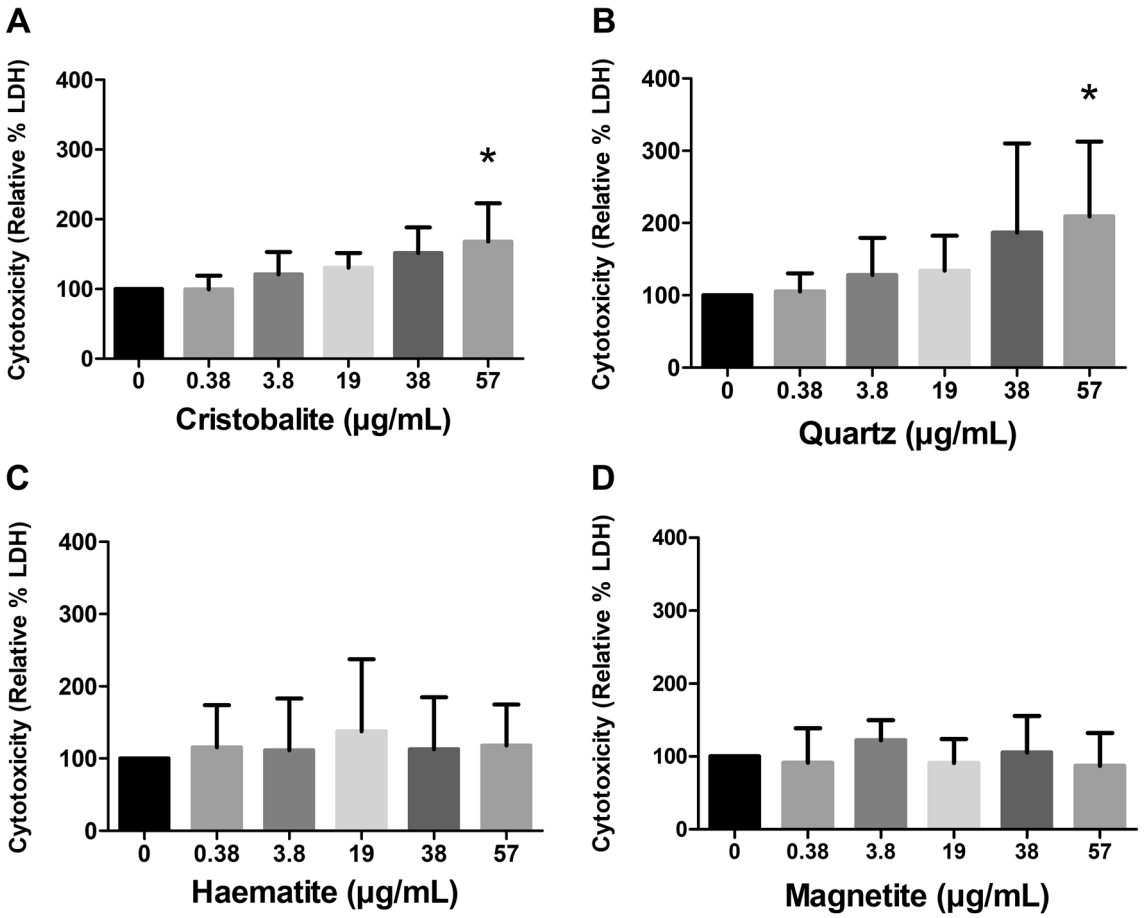

Fig. 1 LDH levels in the supernatant of BEAS-2B cells exposed to cristobalite (**a**), quartz (**b**), hematite (**c**) or magnetite (**d**) for 24 h. Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented as mean (SD) from 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

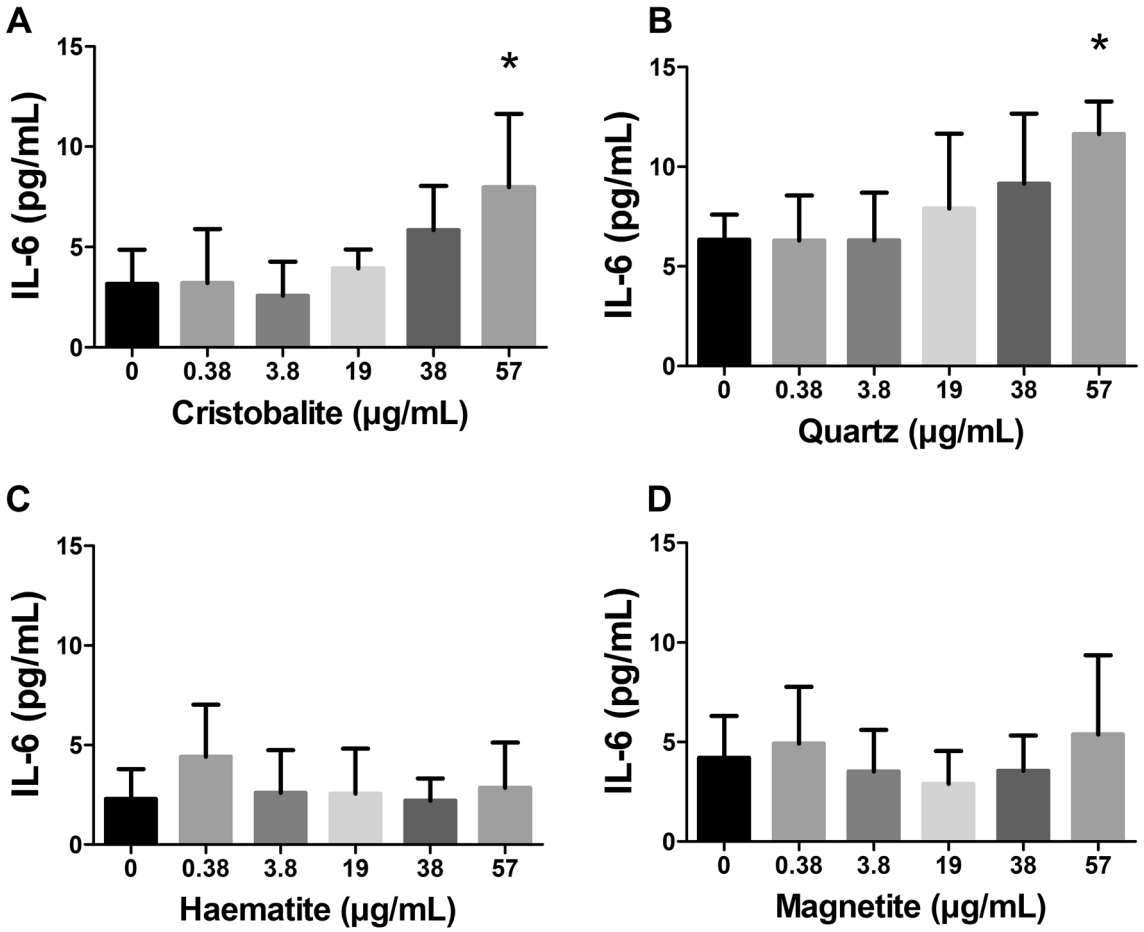

Fig. 2 Interleukin-6 (IL-6) levels in the supernatant of BEAS-2B cells exposed to cristobalite (**a**), quartz (**b**), hematite (**c**) or magnetite (**d**) for 24 h. Data are presented as mean (SD) from six independent replicates with asterisk indicating $p < 0.05$ versus control. Both cristobalite (**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

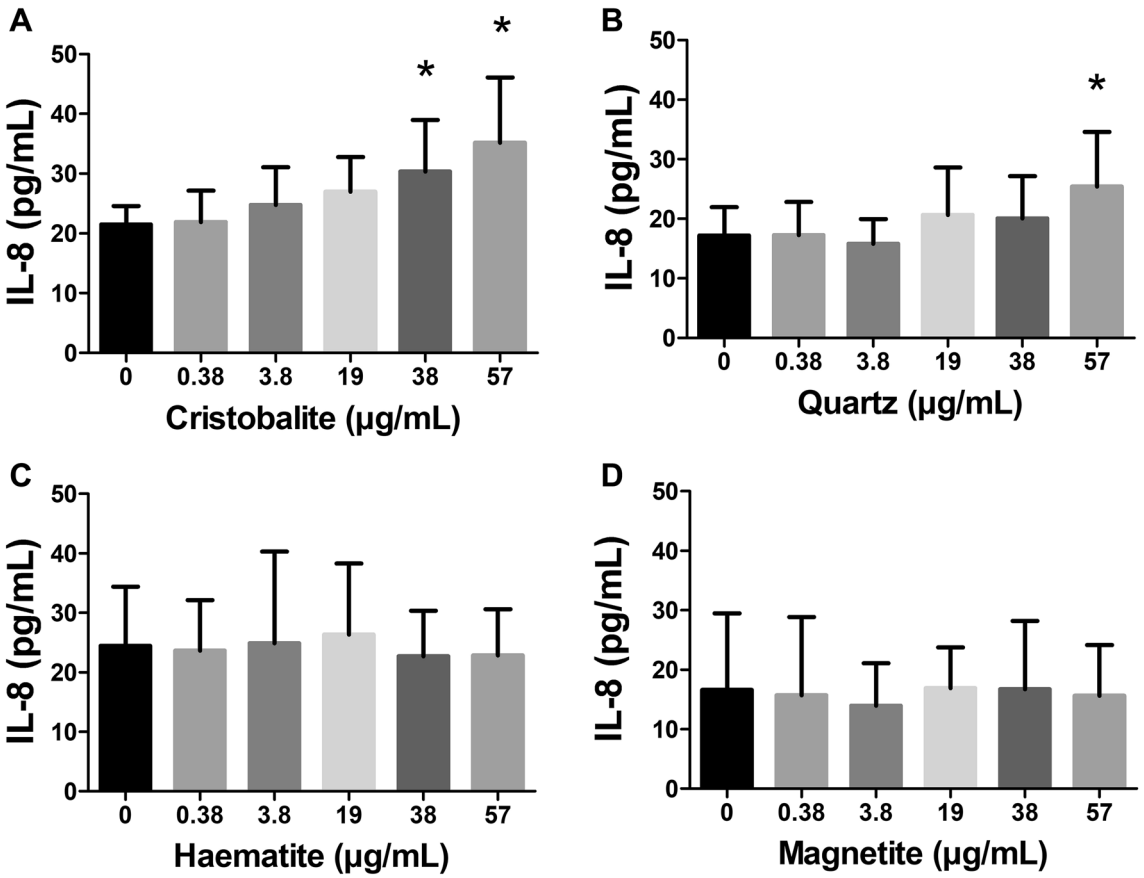

Fig. 3 Interleukin-8 (IL-8) levels in the supernatant of BEAS-2B cells exposed to cristobalite (**a**), quartz (**b**), hematite (**c**) or magnetite (**d**) for 24 h. Data are presented as mean(SD) from 6 independent replicates with asterisk indicating $p<0.05$ versus control. Cristobalite (**a**) caused 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.

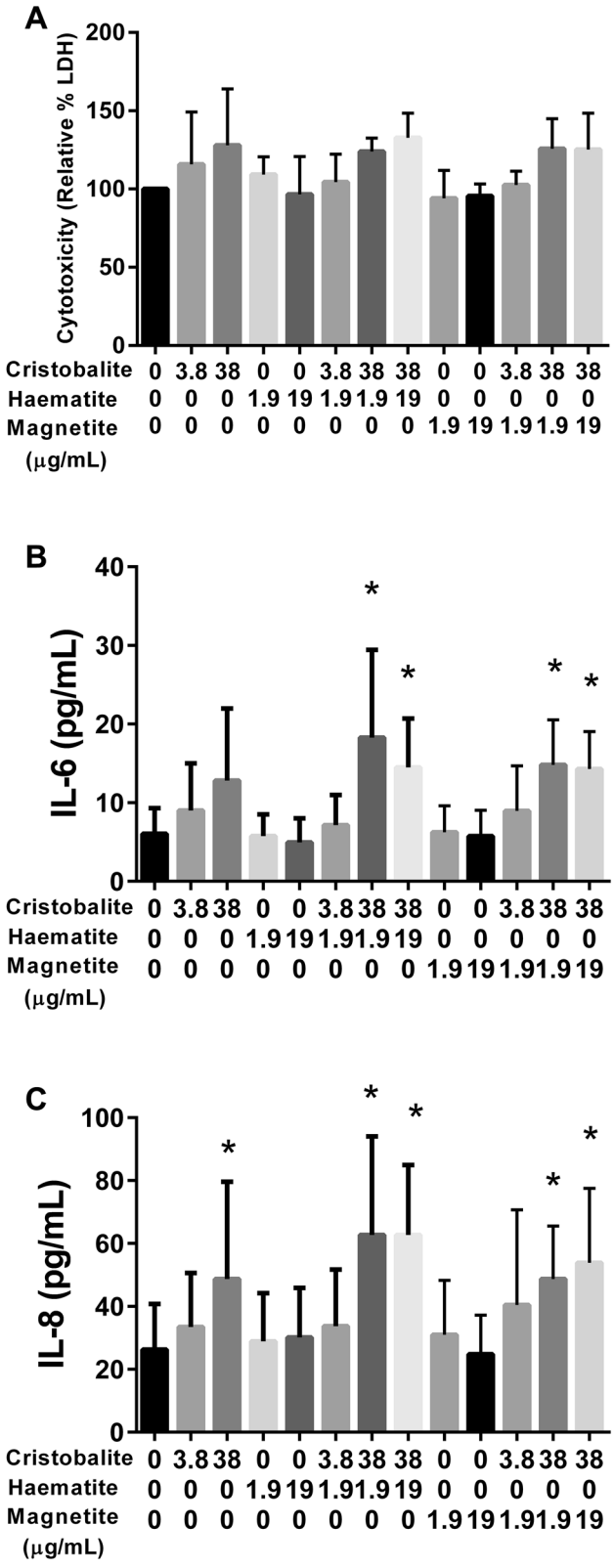

Fig. 4 Supernatant of BEAS-2B cells exposed to cristobalite–hematite or cristobalite-magnetite combinations for 24 h were assessed for relative LDH (**a**), IL-6 (**b**) and IL-8 (**c**). Data are presented as mean(SD) from six independent replicates with asterisk indicating $p<0.05$ versus control. 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

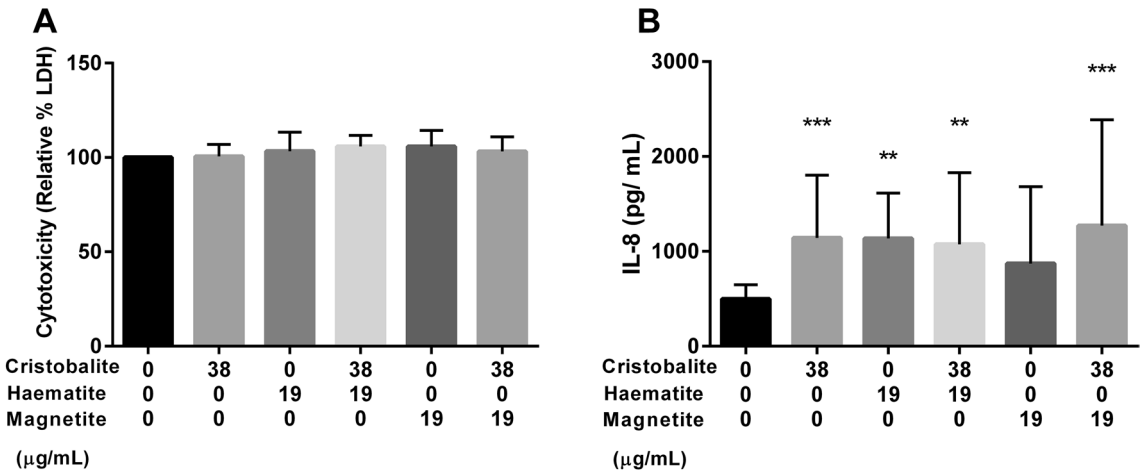

Fig. 5 Supernatant of A549 cells exposed to cristobalite–hematite or cristobalite–magnetite combinations for 24 h were assessed for relative LDH (**a**) and IL-8 (**b**). Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented 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.

Acknowledgements This work was funded by a Harry Windsor Grant from the Australian Respiratory Council.

Compliance with Ethical Standards

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

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