FINAL REPORT

ALPHA FOUNDATION FOR THE IMPROVEMENT OF MINE SAFETY AND HEALTH

Final Technical Report

Project Title: Asthma- and COPD-associated inflammasome activation induced by rock dust
Grant Number: AFC719-12
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Period of Performance: July 1, 2018 to December 31, 2019

Acknowledgement/Disclaimer: This study was sponsored by the Alpha Foundation for the Improvement of Mine Safety and Health, Inc. (ALPHA FOUNDATION). The views, opinions and recommendations expressed herein are solely those of the authors and do not imply any endorsement by the ALPHA FOUNDATION, its Directors and staff.

Brief Lay Summary:

Coal miners suffer from many respiratory diseases. Their disease vulnerability is believed to be due to rock particles produced in the mining environment, which stimulate an inflammation in the lungs.
It is not known how rock dust particles produced in mines can affect cells in the lungs. We therefore studied how cells from lung epithelium and macrophages react when they are exposed to different types of particles, which represent the array of particles that may be found in rock dust.
We found that the particles stimulate a protein complex within the cells that is called an inflammasome. This complex is sensitive to many kinds of stress. After the inflammasome is stimulated, the lung macrophages secrete a protein called interleukin-1β (abbreviated IL-1β) which is a strong inducer of inflammation in the lungs.
Our goal is to understand better how rock dust stimulates the inflammasome. This should help us to develop treatment and prevention of lung inflammation in the mining environment.

1.0 Executive Summary:

Coal miners are known to suffer from various respiratory disease including asthma and chronic obstructive pulmonary disease (COPD). It has been proposed that that the disease vulnerability is due to the unique occupational environment exposures faced by coal miners.

Asthma has three characteristics: intermittent and reversible airway obstruction; increases in airway responsiveness to contractile stimuli; and airway inflammation. Inflammation plays a

key role for asthma development and is directly related to asthma severity. Airway inflammation can be increased due to inhalation of airborne particles.

Occupational exposure, such as the working environment of the coal mining industrial, is considered a major risk factor for both smokers and non-smokers. However, the research for these airborne particles and airway inflammation has been very limited. It is known that people in coal mining communities have a 64% increased risk for developing COPD. However, the mechanistic link between coal mining and COPD has not been thoroughly investigated.

The objective of our research was to test the hypothesis that airborne explosion suppression rock dust induces lung inflammation by activating a complex of proteins called the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome. Our experiments showed in fact that rock dust particles activate the inflammasome in both lung epithelial cells and lung alveolar macrophages. When the macrophages are activated, they secrete pro-inflammatory proteins such as interleukin-1β (IL-1β). Our long-term goal is to characterize the mechanisms whereby the particles induce inflammation, with the goal of identifying therapeutic targets that could inhibit inflammation in the airway. In addition, understanding the interactive mechanisms between dust particles and inflammasome activation will facilitate the development of improved practice/use protocols or updated selection criteria for explosion suppression rock dust commonly found in the mining industry.

2.0 Problem Statement and Objective:

The focus area of our project was: "injury and disease exposure & risk factors". Chronic exposure to airborne particles (e.g. various types of rock dusts) can predispose humans to lung inflammation and increase the risk of COPD and asthma. However, whether mineral/rock dusts can stimulate inflammation by triggering inflammasome activation was no known. There was no research addressing the relationship between airborne dusts (especially in the case of rock dust in mining environments) and their links with inflammasome activation. Our study investigated whether dust particles trigger NLRP3 inflammasome activation in airway epithelial cells and alveolar macrophages, and characterized some of the mechanisms involved in inflammasome activation. We also identified some of the mechanisms that allowed the rock particles to activate the inflammasome. Many of the inhibitors used in our study in cells that block inflammasome activation could also be adapted in the future in humans.

Our SPECIFIC AIMS were twofold:

1. Determine whether rock dust particles activate the NLRP3 inflammasome in lung cells.
2. Identify pathways that are involved in inflammasome activation in the cells.

3.0 Research Approach:

We measured the ability of rock dust particles to activate the NLRP3 inflammasome in lung epithelial cells and alveolar macrophages grown in culture.

We used three types of rock particles. Mine BriteTM Rock Dust (UCRD; median size, 25 μm) was obtained from Huber Carbonates (Atlanta, GA) serving as the primary uncoated (hydrophilic) rock dust sample. ImerCoalTM Moisture-Tolerant Rock Dust (MT; median size, 19.5 μm) was obtained from Imery's Carbonates (Roswell, GA) serving as the primary

coated (hydrophobic) rock dust sample. MIN-U-SIL® Fine Ground Silica (median size, 3.4 μm) was obtained from U.S. Silica Company (Frederick, MD) (Silica) serving as additional control samples for impurities inherent in commercial dust products.

The human epithelial cell line, A549 cells, and alveolar macrophages were purchased from American Type Culture Collection (ATCC). Cells were grown and maintained in Dulbecco's Minimal Essential Medium (DMEM)/F12 supplemented with L-glutamine (5 mM), 1% Penicillin, 1% Streptomycin, and 10% Fetal Bovine Serum (FBS). Cells were passed every 3 days or when confluence reached 70%. Culture plates were pre-coated with pure bovine collagen 6 h before cell seeding. Cell counts were determined by trypan blue (Sigma Aldrich) exclusion and a Bright-Line hemocytometer.

Assessing caspase-1 activation and cell death by Western blot and IL-1β release by enzyme-linked immunosorbent assay (ELISA) have been used as standard indicators for NLRP3 inflammasome activation and cytotoxicity. We have utilized this approach to monitor NLRP3 inflammasome activation in previous studies on macrophages and epithelial cells, as previously described [1-8].

4.0 Research Findings and Accomplishments:

We initially performed experiments to evaluate whether treatment with different concentrations of rock dust would induce cell death in lung epithelial cells (A549 cell line – cat# ATCC CCL-185). As shown in Figures 1A, B and C, all particles induced cell death in a dose-dependent manner after lipopolysaccharide (LPS) treatment, and to a lower extent, without LPS treatment. Additionally, silica was the most toxic among all tested particles. Our data agree with other studies showing that silica induces cell death [9].

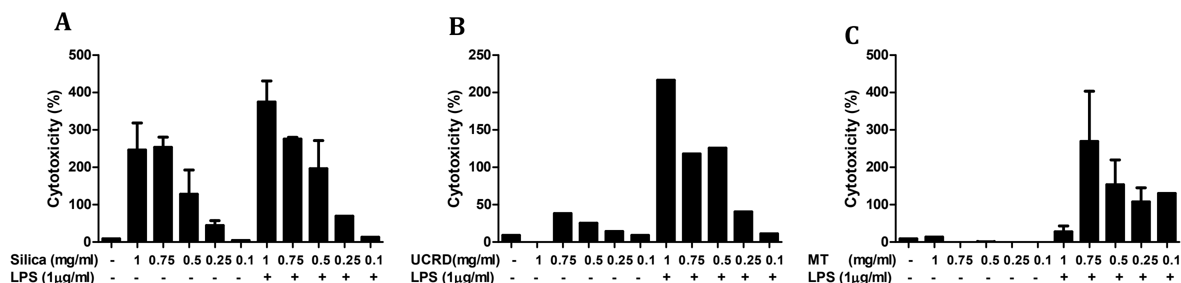

Figure 1. Rock dust particles induce cell death in lung epithelial cells in a dose-dependent manner. A549 cells were treated with or without LPS for 9h and with or without Silica (Fine Ground Silica), UCRD (Mine Brite Rock Dust – primary uncoated hydrophilic), or MT (ImerCoal Moisture-Tolerant Rock Dust – primary coated hydrophobic) for the last 6h. Supernatants were collected and LDH was analyzed using Pierce LDH Cytotoxicity Assay Kit, cat# 88954 – Thermo Scientific). Graphs show cell death relative to lysis buffer. The Y axis shows cell death (in percentage) related to treatment with a detergent as positive control during the last 30 minutes (condition not shown in the graph).

Next, we evaluated intracellular IL-1β production by lung epithelial cells after stimulation with different rock dust particles. As shown by western blot (Figure 2), all rock dust particles were able to induce intracellular IL-1β production in A549 cells. The presence of LPS did not seem to increase the intracellular cytokine production.

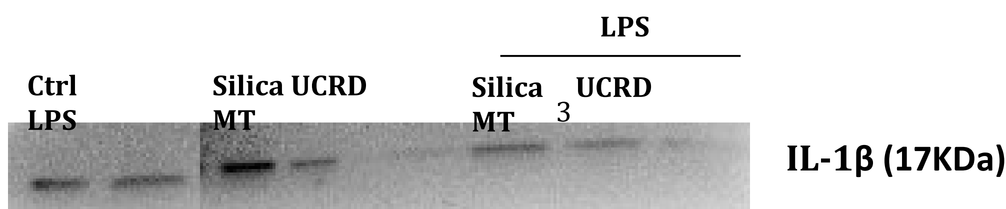

Figure 2. Different rock dusts induce intracellular IL-1β production in lung epithelial cells. A549 cells were treated with or without LPS for 9h and with or without 0.5mg/ml of Silica, UCRD, or MT for the last 6h. After 9h cell lysates were collected, and a Western Blot was done to evaluate IL-1β production. Actin was used as loading control.

Despite our numerous efforts using different techniques (ELISA, Western Blot, protein concentration), we could not detect IL-1β secretion in the supernatant of A549 cells treated with or without LPS and rock dust particles. Similar data were shown on A549 cells infected with *Mycoplasma pneumoniae* in which infection induced IL-1β production (detected by PCR and fluorescent microscopy) but could not be detected in the supernatants [10]. We believe that, in our experiments using rock particles, IL-1β is also produced by A549 cells but cannot be secreted. To overcome this issue, we performed experiments using lung alveolar macrophages (MH-S cell line - cat# ATCC CRL2019).

Using the MH-S cell line, we showed that different rock dust particles led to low levels of cell death (Figures 3A, B and C). LPS and silica-treated cells showed higher levels of cell death (approximately 20%) when compared with LPS and UCRD/ MT-treated cells (approximately 10%). Based on these data, we chose the three lower concentrations to work on additional experiments because they were considered relatively safe and nontoxic (at least in the case of UCRD and MT).

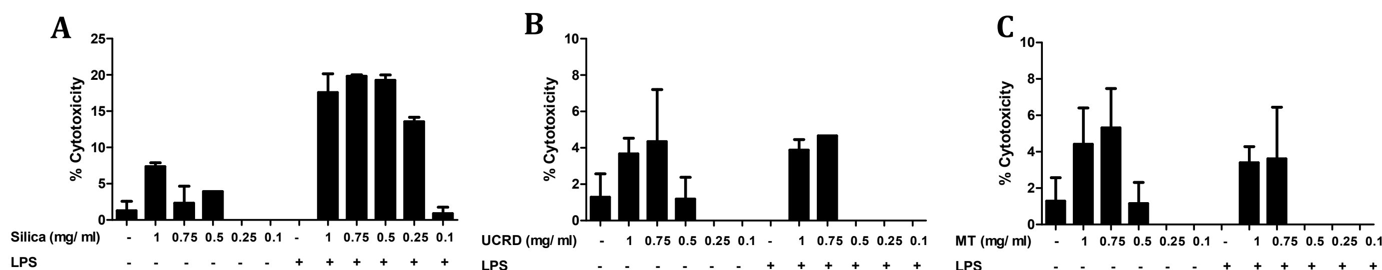

Figure 3. Rock dust particles induce low levels of cell death in alveolar macrophages. MH-S cells were treated with or without LPS from *Pseudomonas aeruginosa* for 9h and with or without Silica, UCRD, or MT for the last 6h. Supernatants were collected and LDH was analyzed using Pierce LDH Cytotoxicity Assay Kit, cat# 88954 – Thermo Scientific). Graphs show cell death relative to lysis buffer. Graphs are representative of 3 independent experiments. The Y axis shows cell death (in percentage) related to treatment with a detergent as positive control during the last 30 minutes (condition not shown in the graph).

Next, we determined whether treatment with rock dust particles would induce IL-1β secretion in LPS-treated MH-S cells. As shown in Figures 4A, B and C, rock dust particles treatment by itself could not induce IL-1β secretion. However, silica treatment promoted IL-1β secretion in LPS-treated MH-S cells in a dose-dependent manner, with higher levels of cytokine secretion compared to the other particles. UCRD and MT treatments induced IL-1β secretion in LPS-treated MH-S cells, but only when 0.5 mg/ml were used for treatments. In Figures 4B and C, even though concentrations of 0.25 and 0.1 mg/ml of UCRD and MT appeared to increase IL-1β secretion in LPS-treated cells, the data did not reach statistical significance. Our data are consistent with other studies showing that silica nanoparticles induce IL-1β secretion [11].

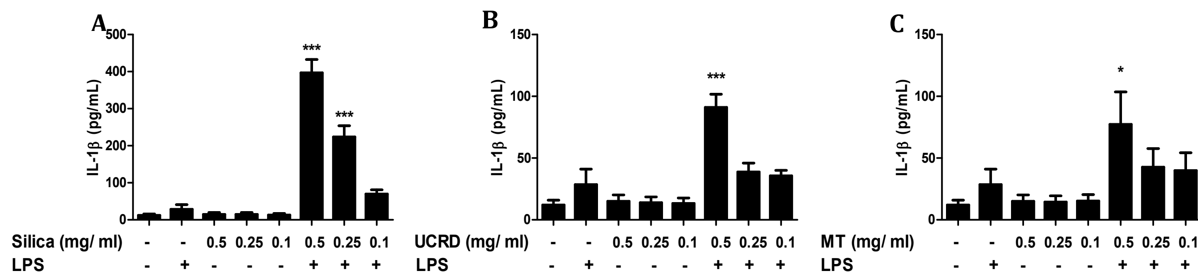

Figure 4. Rock dust particles treatment promotes IL-1β secretion in LPS-treated alveolar macrophages. MH-S cells were treated with or without LPS from *Pseudomonas aeruginosa* for 9h and with or without different concentrations of Silica, UCRD, or MT for the last 6h. Supernatants were collected and IL-1β was analyzed by ELISA (R&D Systems, cat# DY401). Graphs show IL-1β secreted in the supernatants in pg/ml. Rock dust particles concentrations are indicated in each graph. Graphs are mean of 3 experiments. Asterisks show statistically significant differences. ***$p \leq 0.001$; *$p \leq 0.05$. The Y axis shows levels (pg/ml) of the pro-inflammatory cytokine, IL-1β.

To understand better the mechanism of IL-1β secretion induced by LPS and rock dust particles treatment in MH-S cells, inhibitors for the NLRP3 inflammasome and caspase-1 were applied in the experiments. Figures 5A, B and C show that the NLRP3 inflammasome is required for IL-1β secretion in MH-S cells treated with LPS and Silica/ UCRD/ MT because the cytokine secretion was blocked in the presence of the inhibitors. During silica treatments, the caspase-1 inhibitor almost completely blocked IL-1β secretion, suggesting inflammasome activation was necessary for cytokine secretion. In this case, because the effect of the NLRP3 inhibitor was partial, we believe that other inflammasome platforms may also activate caspase-1 and could be involved in IL-1β secretion. Since inhibitors for NLRP3 and caspase-1 were able to completely block IL-1β secretion in UCRD- (except for a partial inhibition using the NLRP3 inhibitor) and MT-treated cells (Figures 5B and C), we believe that the NLRP3 inflammasome may be the main molecular mediator involved in this cytokine secretion. Thus, alveolar macrophages secrete IL-1β in response to LPS and rock dust particles through a mechanism dependent on the NLRP3 inflammasome.

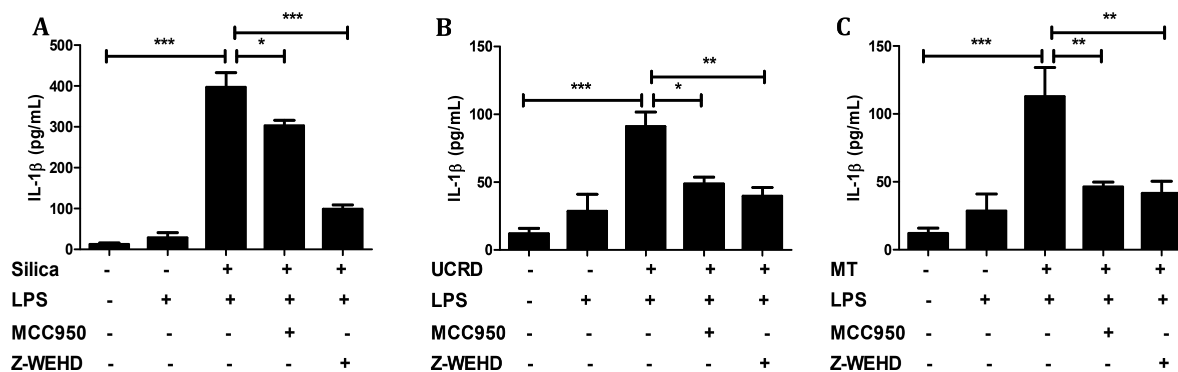

Figure 5. LPS and rock dust-induced IL-1β secretion is dependent on the NLRP3 inflammasome activation. MH-S cells were treated with or without LPS from *Pseudomonas aeruginosa* for 9h; treated with or without the NLRP3 inhibitor (MCC950 - 100μM) or the caspase-1 inhibitor (Z-WEHD-FMK - 100μM) during the last 7h; and with or without different concentrations of Silica, UCRD, or MT for the last 6h. Supernatants were collected and IL-1β was analyzed by ELISA (R&D Systems, cat# DY401). Graphs show IL-1β secreted in the supernatants in pg/ml. Rock dust particles concentrations are indicated in each graph. Graphs are mean of 3 experiments. Asterisks show statistically significant differences. ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$.

5.0 Publication Record and Dissemination Efforts:

We are in the process of writing two manuscripts. Dr. Cassio Almeida-da-Silva performed most of the experiments and will be first author on both publications. Dr. David Ojcius will be senior and corresponding author in both articles.

1. Almeida-da-Silva *et al*, Rock dust particles are cytotoxic and activate the NLRP3 inflammasome in lung epithelial cells. (Manuscript under preparation.)
2. Almeida-da-Silva *et al*, Rock dust particles activate the NLRP3 inflammasome and stimulate secretion of pro-inflammatory IL-1β in human alveolar macrophages. (Manuscript under preparation.)

The Alpha Foundation will be acknowledged for funding of both publications.

6.0 Conclusions and Impact Assessment:

The results from our study show convincingly that the NLRP3 inflammasome is activated in both lung epithelial cells and alveolar macrophages exposed to rock dust particles.

Based on the understanding regarding the interaction between dust particles and airway epithelial cells and alveolar macrophages obtained in this project, we propose that strategies could be developed to reduce airway inflammation to improve airway health for coal mine workers and to lower COPD/asthma cases within the coal mining community. Many of the inhibitors used in this study are being modified by pharmaceutical companies for the use of treating cancer and inflammatory diseases and could be adapted in the future in humans exposed to dust particles.

7.0 Recommendations for Future Work:

In the future, our initial project to characterize NLRP3 inflammasome activation in cells should be confirmed in animal models, by studying the role of NLRP3 in an animal model of nanotoxicity. Toxicity of nanoparticles has previously been shown in pulmonary and gastrointestinal models of toxicity in mice [12-14]. We would like to study these models of toxicity using wildtype and NLRP3-deficient mice, which has never been done for rock dust particles. The NLRP3-deficient mice are available commercially.

Furthermore, biopsies from diseased lungs of long-term members of the mining community should be used to confirm in humans that the NLRP3 inflammasome is activated in lung epithelium or alveolar macrophages, using standard techniques for histological characterization of the inflammasome [15, 16].

8.0 References:

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9.0 Appendices: None.

Glossary of Terms:

A549 cells: human epithelial cells

ATCC: American Type Culture Collection, a repository for many cell lines

COPD: chronic obstructive pulmonary disease

ELISA: enzyme-linked immunosorbent assay, an assay for measuring proteins secreted into supernatant

IL-1β: interleukin-1β, a key pro-inflammatory cytokine

LPS: lipopolysaccharide, a pro-inflammatory molecule present on the surface of Gram-negative bacteria

MH-S cells: lung alveolar macrophages

MT: a coated (hydrophobic) rock dust sample.

NLRP3 inflammasome: The NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome is a pro-inflammatory intracellular complex that is activated by environmental stress or infection.

UCRD: an uncoated (hydrophilic) rock dust sample