In Vitro Toxicity Studies of Rock Dust Samples
(*Assessments using Macrophage & Epithelial Cells)*

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NIOSH / HELD

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What Do We Know about Health Outcomes Elicited by Calcium Carbonate Dust ?

- Eight cases of suspected pneumoconiosis following inhalation of limestone dust with low silica content were described by Doig et al. (1953).
- Granulomatous lesions containing limestone particulates were reported in lungs of quarry worker (Crummy et al. 2004).
- Limestone quarry workers had increased prevalence of respiratory symptoms, e.g. various coughs, wheezing and shortness of breath (Bwayla et al. 2011).
- Pulmonary alveolar proteinosis observed in marble-cutter in Turkey (Case study: Yildirim et al. 2015).
- Erasmus syndrome (pneumoconiosis) in marble worker, most likely exposed to high silica concentrations (Bello et al. 2015).

Need for Developing Anti-caking Rock Dust

Coating with hydrophobic Stearate

- Limestone-based rock dusts are used to prevent explosions caused by high coal dust content in the air.
- Treating limestone will provide better dispersion of the materials.
  - Under humid conditions, limestone-based rock dusts have a tendency to cake.
- Treated limestone particles can fill the empty spaces between the larger untreated limestone particles, preventing or inhibiting the migration of water throughout the blend.

Need to assess the toxicity of anti-caking rock dust

NIOSH 2014 Study

- **Objective:** To test modified limestone based rock dust blend(s) that are developed for:
  - Effective dispersion (NIOSH dust dispersion chamber after being wetted, then dried)
  - Increasing the inertness of coal dust (NIOSH 20-L explosibility chamber).

- **Recommendations:** 20 – 25 μm untreated rock dust blended with 10% + 2.5% of a 3 μm treated component (e.g., stearate).

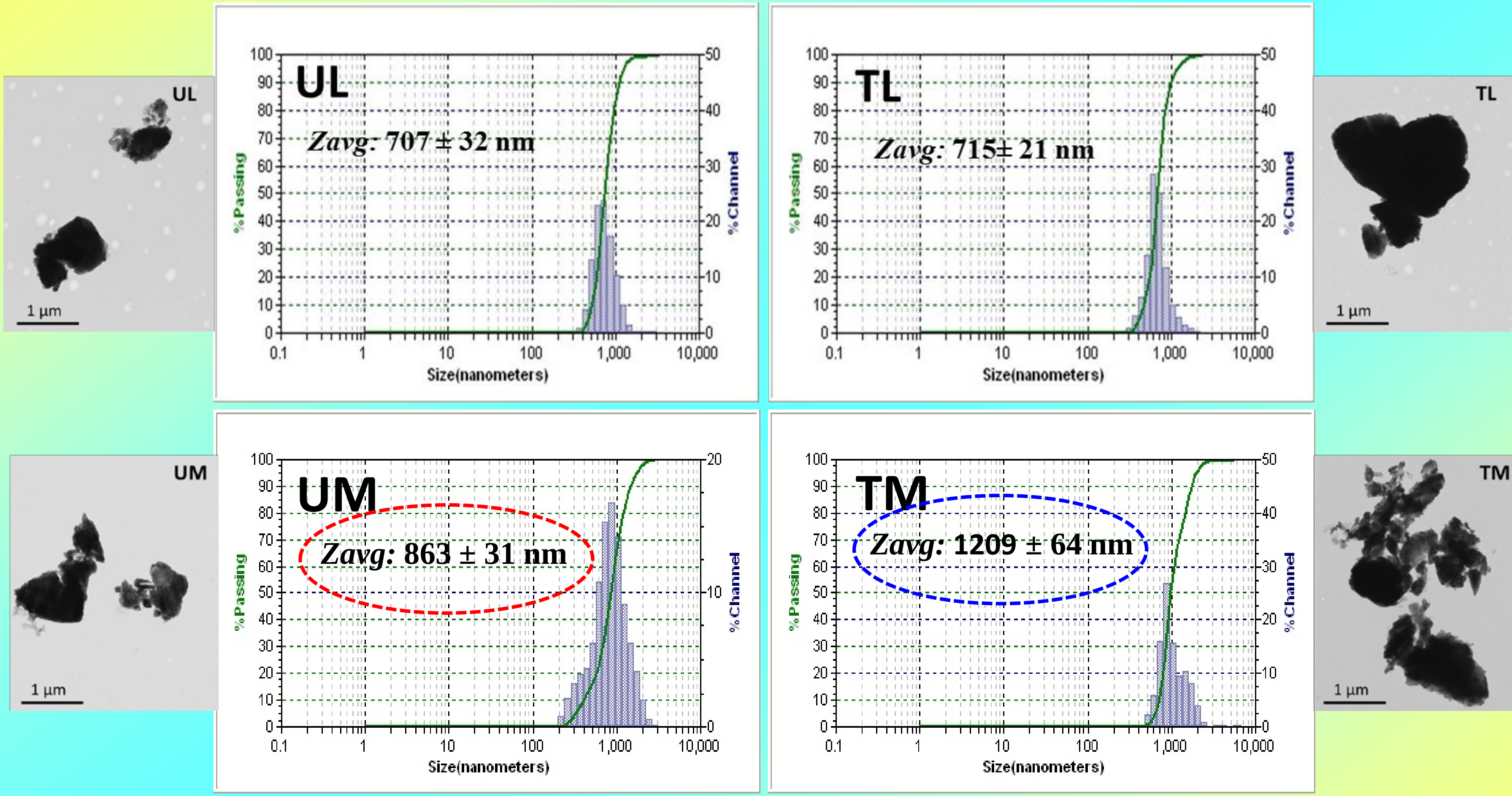
Calcium Carbonate Rock Dust Materials Investigated

- Untreated Limestone (UL), Allegheny Mining Corporation;
- Treated Limestone (TL), private enterprise;
- Untreated Marble (UM), Micro-White™ 100, Imerys Carbonates
- Treated Marble (TM) – a blend of Micro-White™ 100 (87.5%) and stearate coated Kotamite™ (12.5%), Imerys Carbonates
- Crystalline silica (Min-U-Sil®5, US Silica Corp)

- RD samples supplied by PMRD, respirable fractions were collected using FSP10 cyclone samplers, washed in 50% isopropyl alcohol, centrifuged and dried.

Particle Characterization

Average hydrodynamic diameter of respirable fraction of rock dust

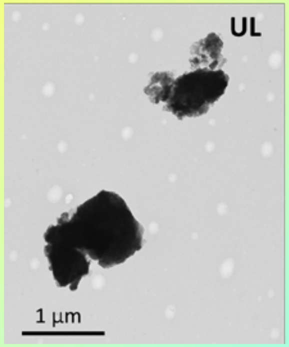


The average size/distribution of rock dust samples were determined using DLS measurements. The hydrodynamic diameter (Zavg) from DLS were represented as mean ± SD. The reported Zavg values correspond to a mean of six different measurements.

The average size of treated marble (TM) rock dust sample was higher compared to other rock dust samples investigated.

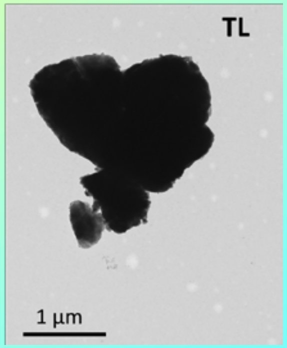
In vitro Toxicity Assessments of different Respirable Rock Dust

Experimental Design



Untreated Limestone

(or)



Treated Limestone

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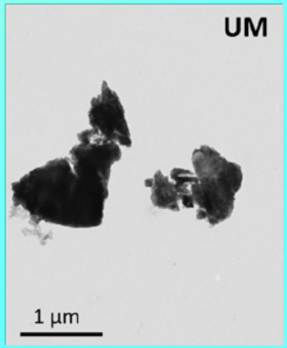

Untreated Marble

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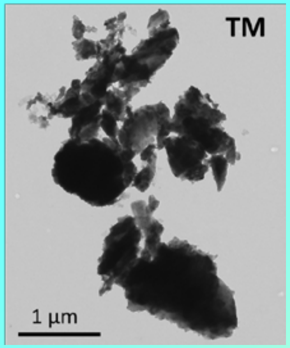
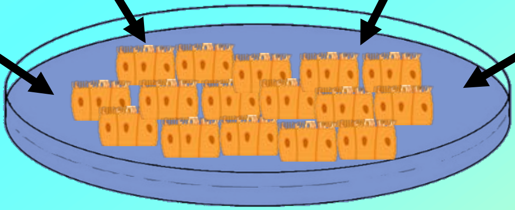


Treated Marble



A549
(Human pulmonary alveolar epithelial cells)

Particle Concentration's:

- 0 mg/ml
- 0.025 mg/ml
- 0.050 mg/ml
- 0.100 mg/ml
- 0.200 mg/ml
- 0.500 mg/ml
- 1.0 mg/ml

24h/72h

Cellular Viability/Damage
Inflammatory cytokines/chemokines

(human 27-plex kit from Bio-RAD)

Conclusions

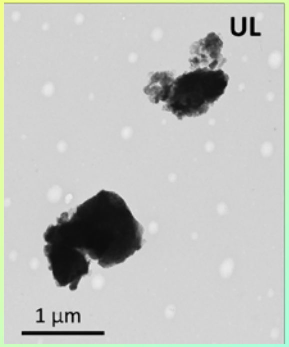
(A549 Cells)

- The results showed some dose-dependent cytotoxicity and cell damage at 72 h in A549 cells, with the least effect upon exposure to treated limestone (TL).
- Clustering analysis of the inflammatory cytokines/chemokines revealed an overall stronger effect of marble (i.e., UM,TM) compared to limestone samples (i.e., UL,TL).
- Furthermore, untreated rock dust induced an overall greater inflammatory response as compared to treated samples.
- Treatment related differences between limestone (TL) and marble (TM) samples were observed.

Overall, these results unveiled treatment related differences as well as material dependent changes in biological responses.

In vitro Toxicity Assessments of different Respirable Rock Dust

Experimental Design



Untreated Limestone

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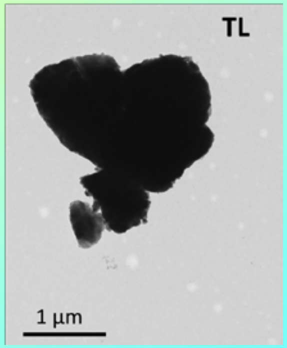

Treated Limestone

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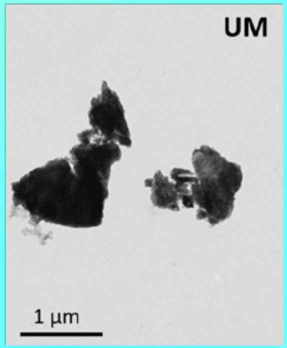
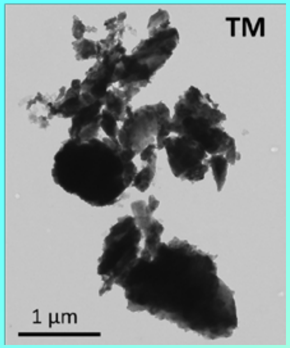


Untreated Marble

(or)

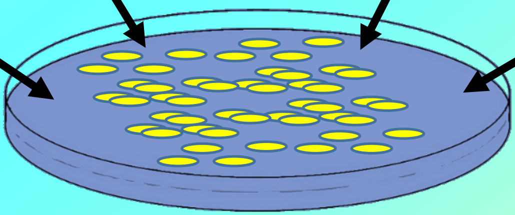


Treated Marble

<u>**Particle Concentration's:**</u>

- 0 mg/ml
- 0.005 mg/ml
- 0.01 mg/ml
- 0.025 mg/ml
- 0.05 mg/ml
- 0.1 mg/ml
- 0.200 mg/ml

Min-u-sil crystalline silica as positive control



THP-1
(Human peripheral blood monocytes)

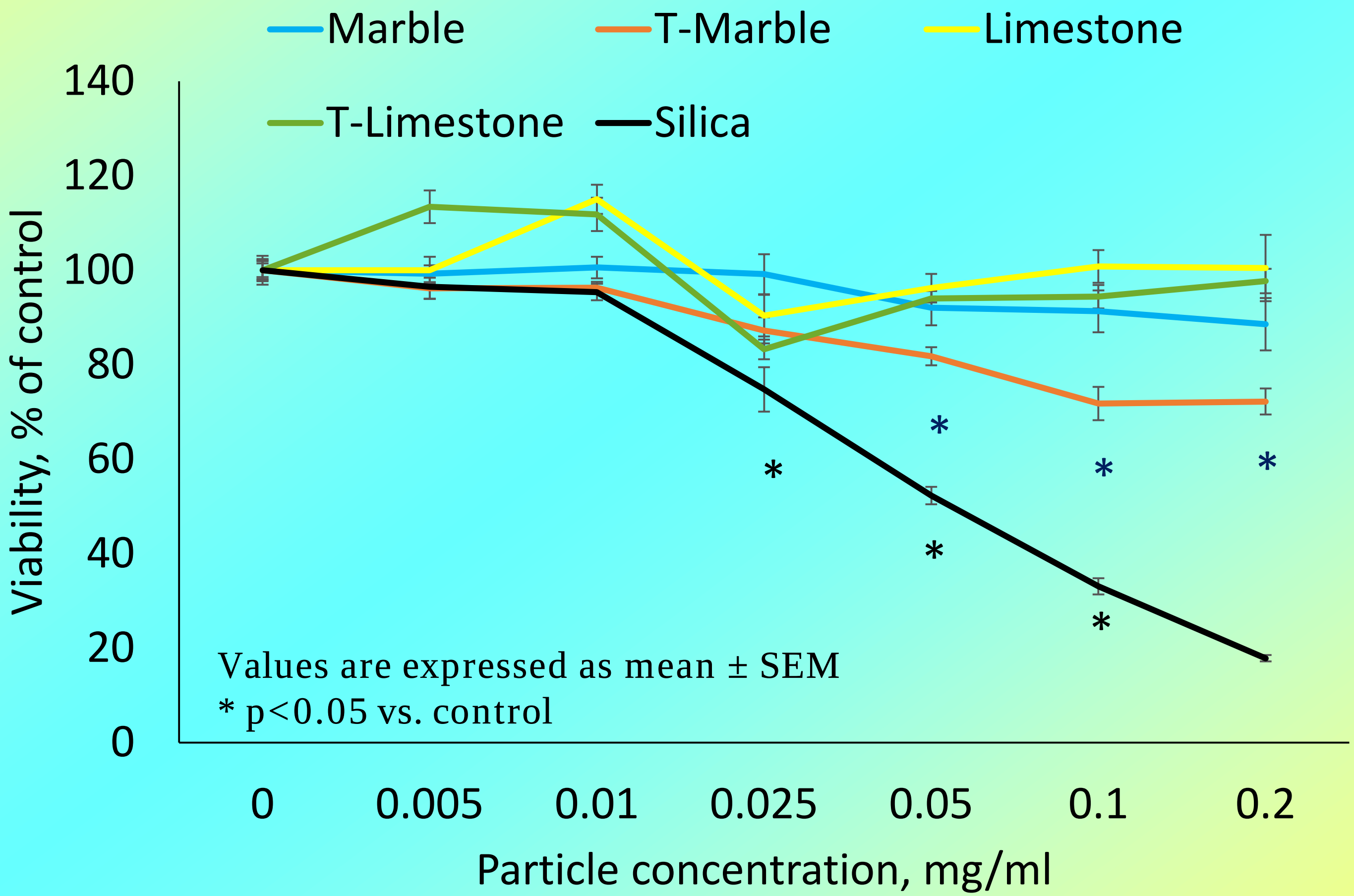
24h

Cellular Viability/Damage
Caspase-1 Activity
Inflammatory cytokines/chemokines
(human 27-plex kit from Bio-RAD)

In vitro Toxicity Assessments of different Respirable Rock Dust

THP-1 cells

Cytotoxicity (viability) of various respirable rock dust samples & silica

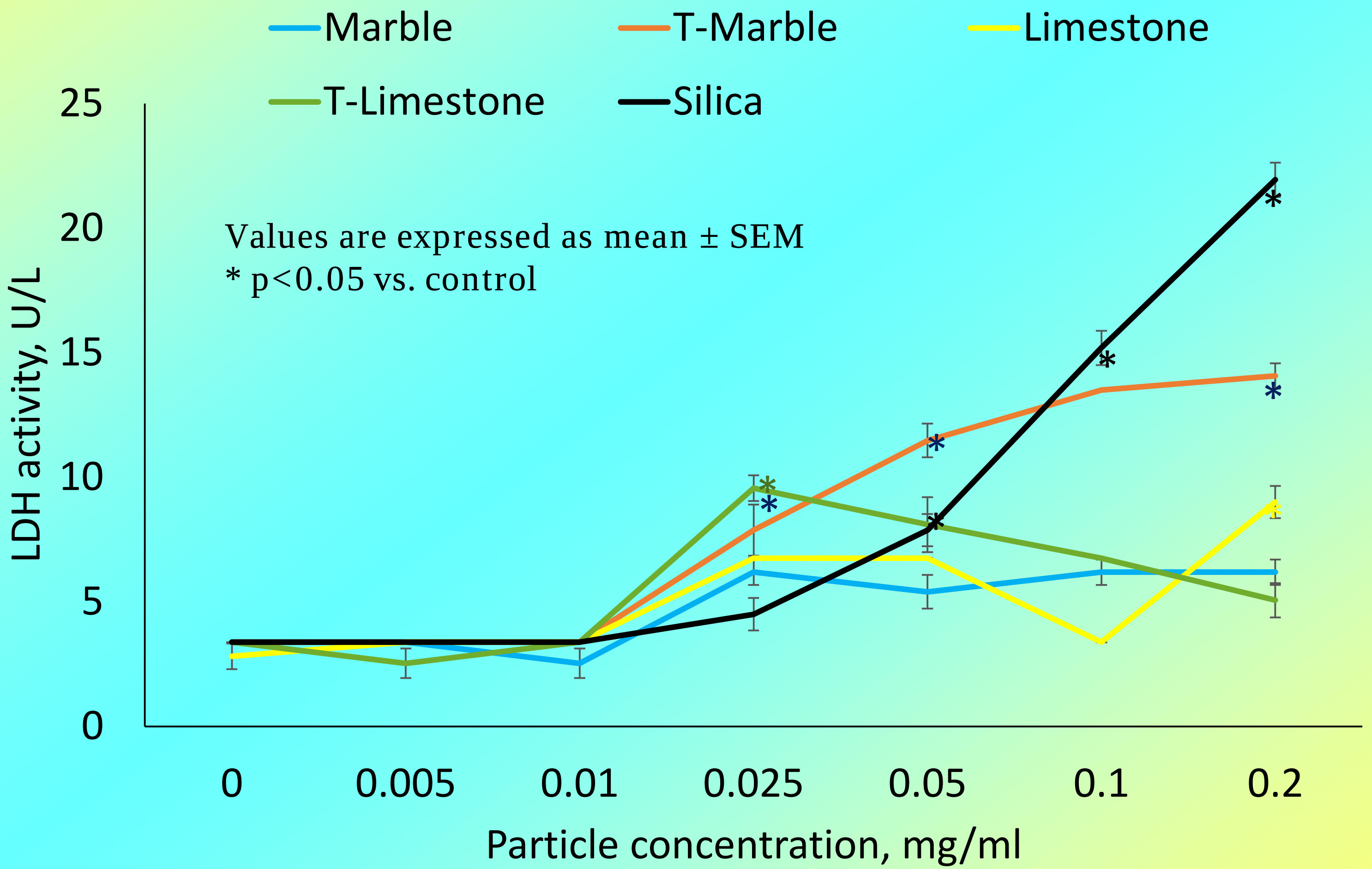


24 h after exposure, we observed a significant decrease in viability of the differentiated THP-1 cells treated with TM at 0.05, 0.1 and 0.2 mg/ml doses, while silica caused dose-dependent reduction in viability starting at 0.025 mg/ml.

In vitro Toxicity Assessments of different Respirable Rock Dust

THP-1 cells

Cellular Damage of various respirable rock dust samples & silica

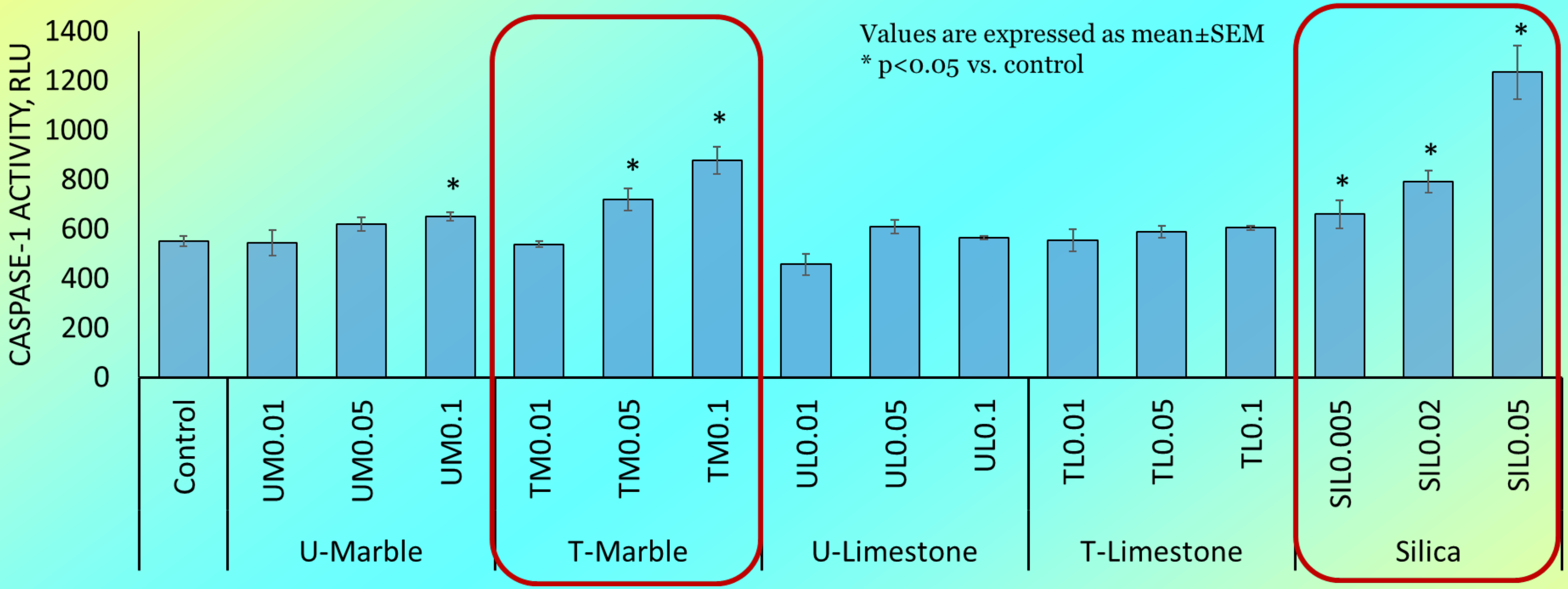


24 h after exposure LDH activity in supernatants increased for Silica and TM in dose-dependent manner

In vitro Toxicity Assessments of different Respirable Rock Dust

THP-1 cells

Caspase-1 Activity after exposure to rock dust & Silica



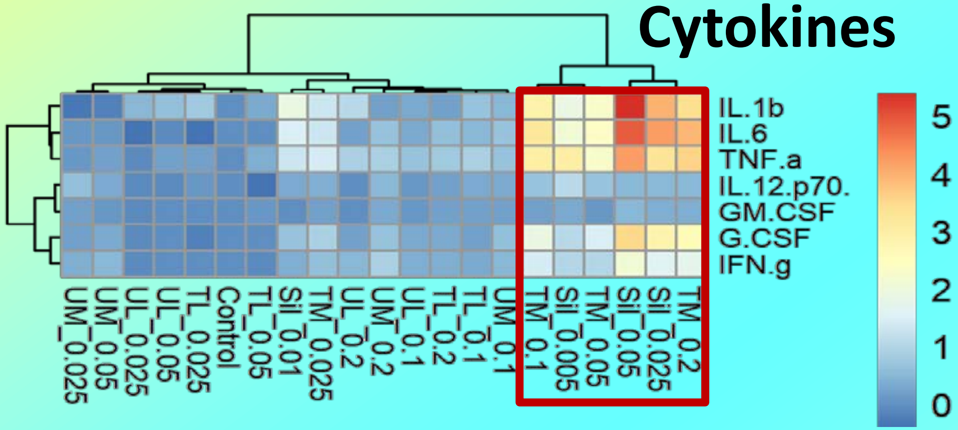
Macrophages, exposed to SiO_2, TM (0.05 and 0.1 mg/ml) and M (0.1 mg/ml) but not to any other rock dust displayed a dose-dependent increase in caspase-1 activity, indicative of the NLRP3 inflammasome activation.

In vitro Toxicity Assessments of different Respirable Rock Dust

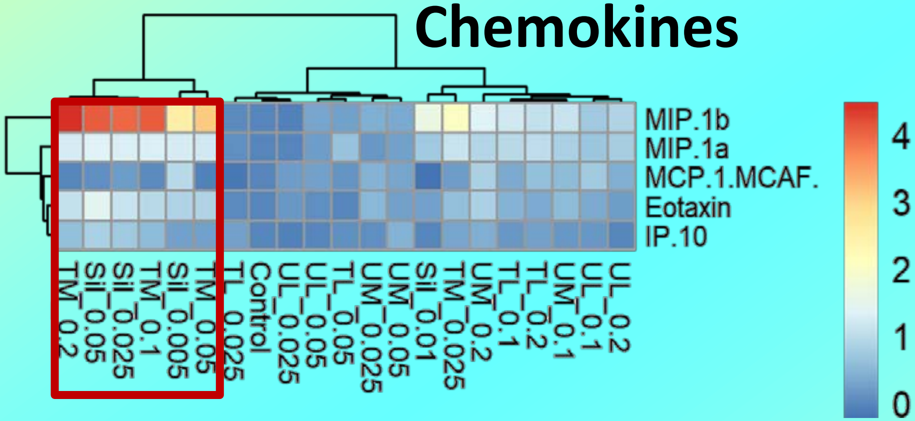
THP-1 cells

Hierarchical cluster analysis of cytokine profiles

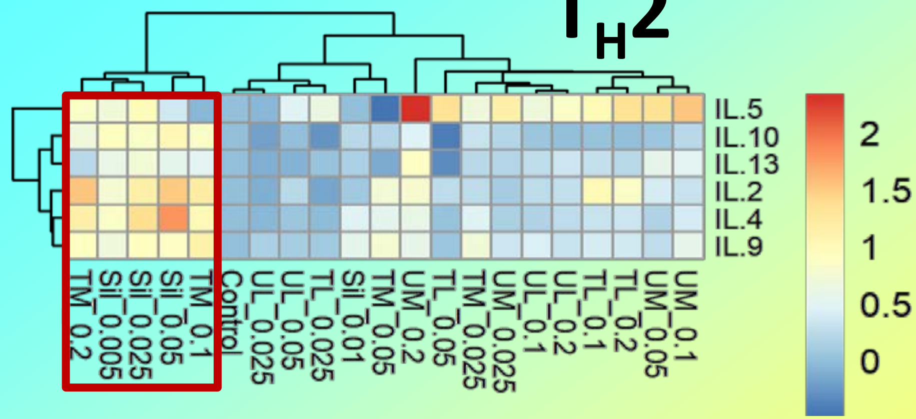
Cytokines



Chemokines



T_H2



Clustering analysis of the inflammatory cytokines/chemokines revealed an overall stronger effect of TM (*resembling silica*) compared to other rock dust samples.

Mechanistic Investigation of Similarities in Responses Between TM and Silica

Role of Particle Uptake

Inhibition of Uptake:

- **Cytochalasin D (Cyt D)**: inhibits the actin polymerization, blocking the particle uptake.
- **Bafilomycin A (Baf A):** inhibits phagolysosome acidification. Baf A is a vacuolar proton pump blocker, that prevents the acidification of lysosomes. Acidification of lysosomes is implicated in silica toxicity.

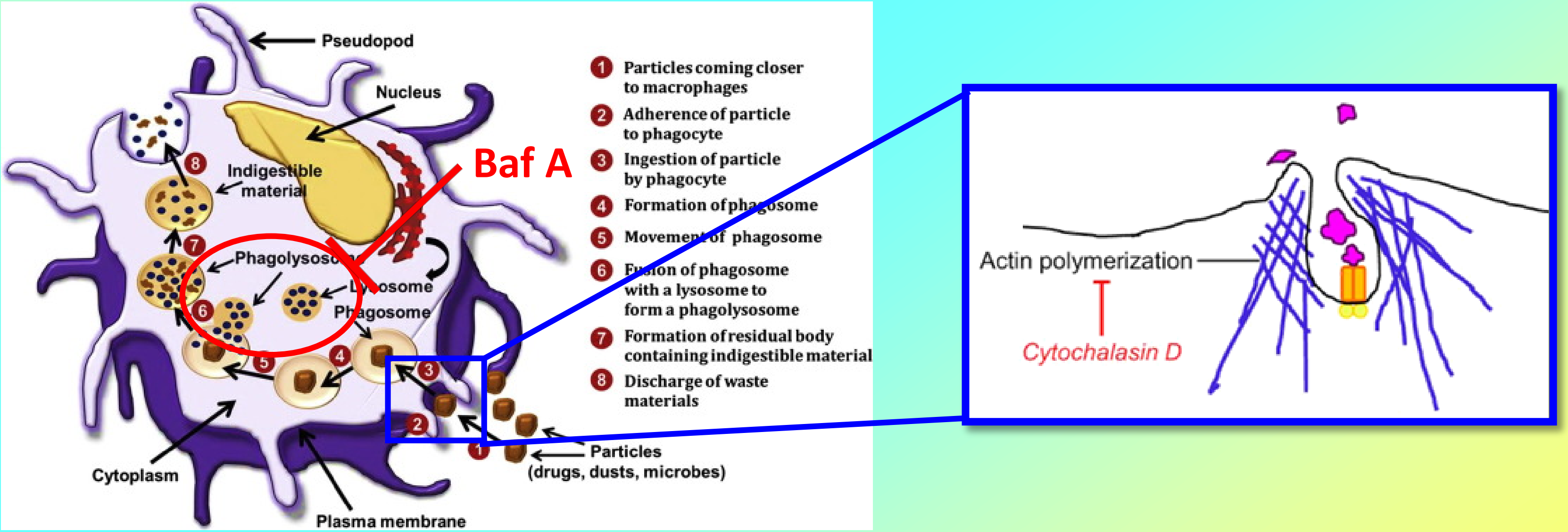
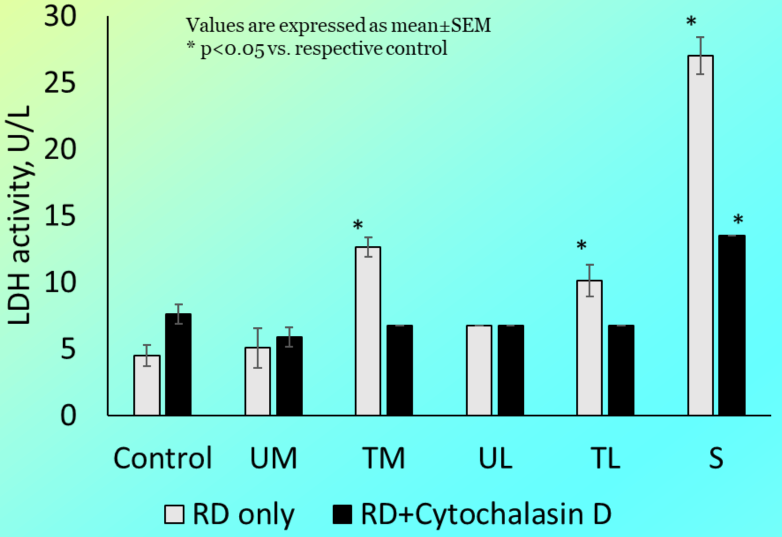
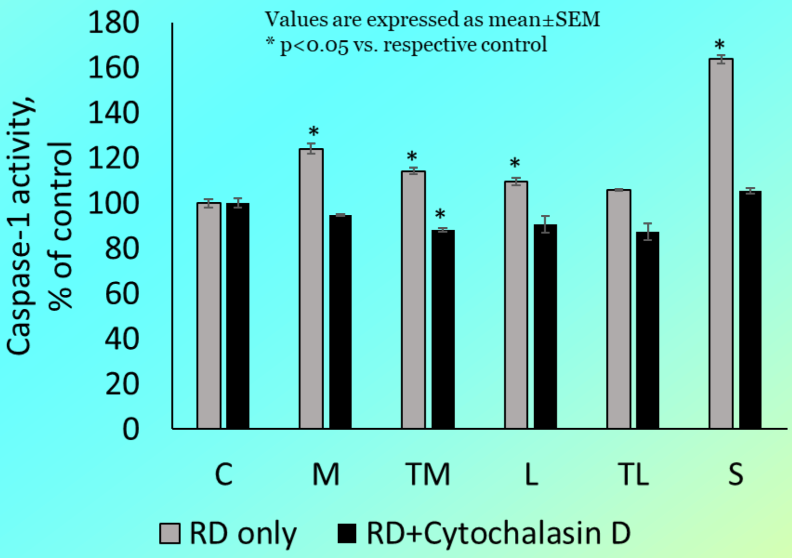


Image taken from : https://goo.gl/images/iaRGZ2

Mechanism of Uptake : Particle Toxicity

Pretreatment with Cytochalasin D and Exposed to rock dust or Silica

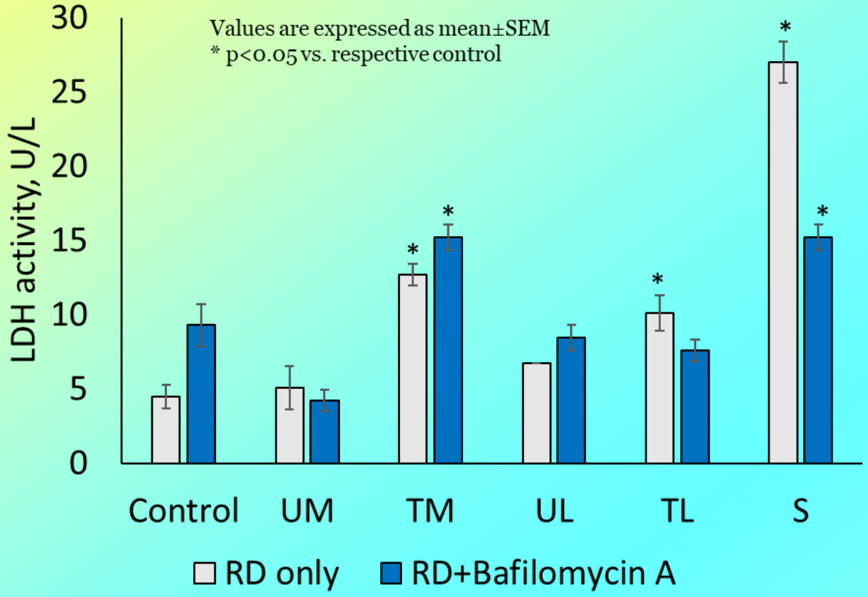


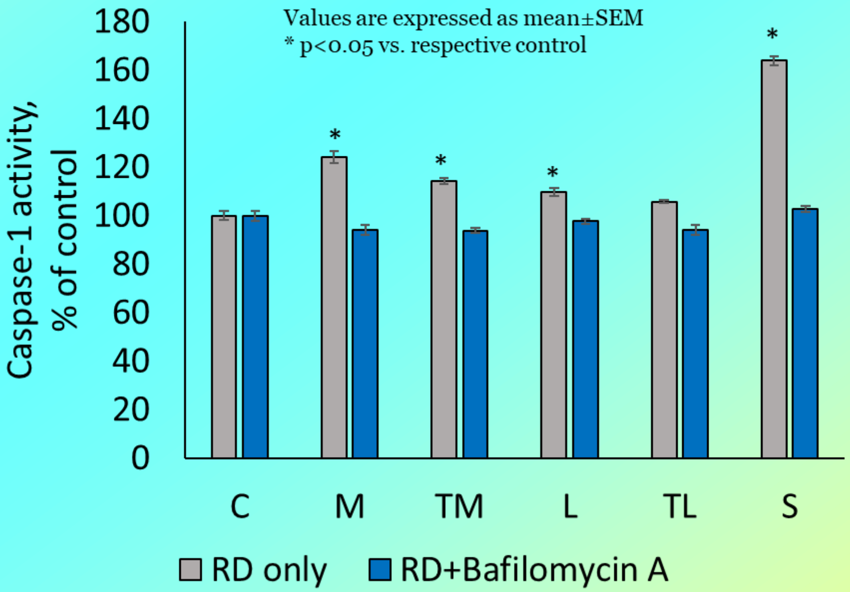


Cyt D treatment abrogated or significantly decreased LDH production and Caspase-1 activity in RD- and silica-treated cells compared to respective controls.

Mechanism of Uptake : Particle Toxicity

Pretreatment with Bafilomycin A and Exposed to rock dust and Silica





Baf A treatment prevented the activation of caspase-1 in both rock dust and silica exposed cells and only decreased the LDH leakage in silica.

Conclusions

Pretreating Cells with Cytochalasin D & Bafilomycin A

- Inhibition of particle uptake by Cytochalasin D reduced the cell damage and prevented the caspase-1 activation by both TM and silica.

- The use of Bafilomycin A (H^+V-ATPase blocker), decreased the cytotoxic effects of silica, but not TM.

Summary

(THP-1 Cells)

- Untreated rock dusts and Treated Limestone (TL) particles were readily internalized by macrophages, while causing very little or no toxicity even at high doses.
- Exposure of THP-1 cells to Treated Marble (TM) led to significant dose-dependent reduction in viability and LDH increase, however the observed effects were substantially less prominent compared to silica within doses used. Both TM and silica treatments led to elevation in caspase-1 activity.
- Cytokine profiling and hierarchical clustering revealed significant dose-dependent increase in the production of inflammatory and T_H2-type cytokines/chemokines for silica and TM, that formed a cluster, separated from other rock dust particles.
- Rock dust samples did not induced significant inflammatory responses in lung epithelial cells but stimulated some inflammatory responses in macrophage-like cells. TM was the most stimulatory of the RD, however, the response was much less pronounced compared to silica.

This study is published

- **Khaliullin TO, Kisin ER, Yanamala N, Guppi S, Harper M, Lee T and Shvedova AA (2019) *Comparative cytotoxicity of respirable surface-treated/untreated calcium carbonate rock dust particles in vitro.* Toxicol Appl Pharmacol. 362: 67-76. (available on line 25 Oct 2018)**

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